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Oviposition substrate location by the invasive woodwasp *Sirex noctilio*: The combined effect of chemical cues emitted by its obligate symbiont *Amylostereum areolatum* and different host-tree species.

Short title: Oviposition substrate localization by *Sirex noctilio*: symbiont and host chemical cues.

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Abstract

BACKGROUND: *Sirex noctilio* is an invasive forest wasp that affects pines and can result in severe economic losses. The use of semiochemicals offers an opportunity to develop sensitive and specific capturing systems to mitigate negative impacts. Previous research showed that female *S. noctilio* would use volatiles emitted by its fungal symbiont, *Amylostereum areolatum*, but little is known about how these modulate behaviour when combined with pine-wood emissions. Our aim was to understand the relevance of fungal volatiles grown on artificial media and wood from two hosts trees, *Pinus contorta* and *P. ponderosa*, on behavioural and electroantennographic responses of wasp females. Because background odors can modify an insect's response towards resource-indicating semiochemicals, we propose that the behavior towards the symbiont (resource) will be modulated by host pine emissions (background odors). RESULTS: Olfactometric assays showed that both host species with fungus were attractive when contrasted against air (*P. contorta* vs Air, $\chi^2=12.19$, $p<0.001$; *P. ponderosa* vs Air, $\chi^2=20.60$, $p<0.001$) and suggest a clear hierarchy in terms of female preferences towards the tested stimuli, with response highest towards the fungus grown on *P. contorta* (Olfactory Preference Index: 5,5). Electrophysiological analyses indicate that females detect 62 volatile compounds from the tested sources. CONCLUSION: Results indicate a strong synergy between symbiont and host semiochemicals, suggesting that the pine species could play a fundamental role in the interaction. Further understanding of the chemical basis of this, could guide the development of specific and attractive lures, in order to maximize attraction of wasps in surveillance programs.

Keywords

chemical ecology, pest management, semiochemicals, background odors, resource indicating odors (RIOs)

1 Introduction

Sirex noctilio Fabricius (Hymenoptera; Symphyta; Siricidae), is a hymenopteran endemic to Europe, Asia and North Africa. More than a century after its first detection outside its native range, the wood-boring wasp *Sirex noctilio* (Fabricius) (Hymenoptera: Siricidae) is present in most regions where *Pinus* spp. are cultivated for commercial purposes (1,2). Although it can develop in several conifer species, *S. noctilio* shows a marked preference for pines, being the only woodwasp that can kill living trees (3). Despite the fact that in its native range *S. noctilio* is not considered a threat as it attacks largely suppressed and dying trees, severe impacts have been reported in pine-growing regions around the world (4–7). In the invaded range, such as Patagonia (Argentina), populations can grow and spread fast, causing significant economic losses (3,8–10).

The flight season of *S. noctilio* spans from early summer to early autumn in temperate regions. Females oviposit below the bark using a relatively long ovipositor. The energy required for adult survival, movement and reproduction comes from the metabolic resources accumulated during the larval stages, which are strongly linked to the growth of the symbiotic fungus *Amylostereum areolatum* (Basidiomycota: Corticiaceae) (11). Symbiosis with this species is obligatory, being until recently the only known member of the Siricidae family that exclusively used *A. areolatum* (12). However, more recent studies indicate that other siricids native to North America, where *S. noctilio* has recently arrived, can carry *A. areolatum* in mycangia. This novel association between *A. areolatum* and a second Siricid species is thought to be due to the recent introduction of *S. noctilio* to the area. (13–15). The fungus is transported by the females in special structures called mycangia (16) and is essential for larval development due to its role in their nutrition (17,18). The fungus mycelia are initially inoculated into trees during oviposition together with a phytotoxic venom. Both promote the initial physiological deterioration of the tree and create suitable conditions for the development of the fungus and, therefore, the survival of the larvae (19–21).

Generally, healthy trees have more defences than stressed ones. In this sense, the latter are more susceptible for oviposition (22) and were shown to be the preferred substrate for wood wasp females (23). Spatial aggregation of attacked trees may contribute to the population dynamics of the species, as wasp outbreaks could be explained by a shift in attack from suppressed trees when wasp population levels are low, to healthy trees when outbreaks occur (24). At this stage, multiple ovipositions by females in a single tree are crucial to overcome tree defences (20). In Patagonia, *P. contorta* and *P. ponderosa* are among the most cultivated pine trees, and both are susceptible to *S. noctilio* attack, but *P. contorta* is frequently affected, while *P. ponderosa* rarely (25).

Surveillance and monitoring of *S. noctilio* populations has been typically implemented through the deployment of trap tree plots and/or by visual recognition of affected host trees through ground or aerial surveys (10,26). However, these methods have important limitations. On the one hand, the installation of trap trees (artificially stressed trees) involves elevated costs, logistics and the handling of toxic substances (herbicides) posing a negative impact on human health and the environment (27). On the other hand, given the invasive nature of the species, this approach does not allow for monitoring in pine-free areas that have a high risk of entry (e.g., ports and borders). Therefore, by increasing our knowledge of the chemical ecology and behaviour of the pest, there is potential to improve monitoring techniques to facilitate early detection and favour the efficacy of control practices (28).

Chemical cues are of paramount importance for almost every insect species to locate food, mates, hosts, etc. (29–33). For invasive insects, species-specific detection methods are critical to successful surveillance and monitoring programs. It is well known that *S. noctilio* females are attracted to volatile organic compounds (VOCs) released by stressed host trees (34–36). Some of these rather ubiquitous compounds (e.g., pinenes, α and β) are used to monitor and control female wasp populations in affected areas (37–41). Recently, female responses towards other sources have been investigated. For instance, Cooperband *et al.* (42) identified a close-range pheromone produced by

males that is behaviorally-active in both females and males in laboratory trials, but not in the field (43). It has also been suggested that successful host searching by *S. noctilio* may rely on VOCs release both by *A. areolatum* and the host plant (37,39,44). Under controlled laboratory conditions *S. noctilio* females have shown strong attraction to the fungus *A. areolatum* grown on artificial culture medium (45). Although major VOCs emitted by the effluvia of *A. areolatum* grown in artificial medium have been identified (46–48) and the full genome of this symbiotic fungus has been recently described (49), detailed information on how volatiles emitted by the symbiont growing in wood could regulate the behaviour of female wasps is lacking. Schröder & Hilker (2008) (50) proposed a framework that discerns the role of background odors with those released by a particular resource (resource indicating odors – RIOs), during the process of insect orientation. These authors suggest that the response towards a specific volatile stimulus leading to a resource could be modulated by the perception of other volatiles. These background odors, that include the volatiles of the habitat in which insects forage, can be either irrelevant, mask the RIOs or “sharpen the view” by enhancing the response towards a source.

The wasp *Sirex noctilio* evolved in Europe, Western Asia and Northern Africa in environments where susceptible pine trees are native to and are found in mixed forests, together with other woody species. In areas where it has been introduced, *S. noctilio* is usually found in healthy commercial pine forests, where the environment tends to be homogeneous. In this scenario, finding a suitable host can be a complex process. The detection of a previously attacked tree can result advantageous for larval development, since chances of overcoming tree defences are greater with increased fungal inoculation and phytotoxic venom. Thus, the volatiles emitted by a stressed pine with fungal growth (which itself represents a critical resource), may be result in high levels of attraction for a female seeking an optimal oviposition substrate. Therefore, we hypothesize that *S. noctilio* females use the volatiles of *A. areolatum* to detect previously attacked trees, which in a diverse landscape may be difficult to find, favouring the development of offspring. We further propose that in a scenario with emissions from a pine species as background odors combined with emissions from the symbiont

(RIOs), female attraction would be maximized. By means of behavioural assays in the laboratory, electroantennographic studies, and identification of volatile compounds, we investigated the relevance of VOCs released by the symbiotic fungus *A. areolatum* grown on wood of *Pinus ponderosa* and *Pinus contorta* (Pinus: Pinaceae). We predict that *S. noctilio* females will show a stronger behavioural response (preference) towards the symbiont growing on the wood of potential host species. This behavioural preference will correspond with antennal responses of females to particular combination of volatile compounds, dependent on the tested source.

2 Materials and methods

2.1 Insects

Adult *S. noctilio* females used in all the experiments were obtained from attacked *Pinus contorta* in Northwestern Patagonia, Argentina. Trees were selected and felled based on typical *S. noctilio* oviposition symptoms (i.e., chlorotic trees and resin beads on the main stem). Trees were then cut into 0.8 m billets, taken to the rearing facilities at GEPI – IFAB (Bariloche, Argentina) and kept under room temperature and natural light conditions in netted cages (0.8 x 0.8 x 0.8 m) until adult emergence. *Sirex noctilio* females were collected daily and kept in individual containers under controlled temperature and light (12 ± 1 ° C and L16: D8 photoperiod) for 3 ± 2 days, until used in experiments. The mating status of females was unknown since it is possible some females could have mated during the short period (<24h) they remained with males in the rearing cages. It's important to note that the species is parthenogenetic, therefore virgin females still have the capacity to oviposit.

2.2 Volatile sources

A total of seven volatile sources were evaluated: (i) clean air (Air), (ii) artificial culture medium (A-M), (iii) *A. areolatum* grown in artificial culture medium [Fungus (A-M)], (iv) *P. contorta* (Contorta), (v) *P. ponderosa* (Ponderosa), (vi) *A. areolatum* grown in *P. contorta* (Contorta+Fungus), (vii) *A. areolatum* grown in *P. ponderosa* (Ponderosa+Fungus). Source (i) consisted of empty olfactometer chambers in bioassays or clear oven cooking bags for volatile collections (30 x 40,5 cm, Frozens®, Argentina). Source (ii) consisted of sterile agar, malt and yeast culture medium (Martínez et al. 2006) left for 18 ± 3 days at 22 ± 1 ° C and 65 ± 5 % RH in the dark. Source (iii) was obtained from hyphal fragments extracted from female mycangia of *S. noctilio* females and cultured in A-M (51,52). Sources (iv) and (v) were obtained by felling pine trees of *P. contorta* and *P. ponderosa* (10 cm diameter at breast height). After felling the pine trees, a portion of the main stem was cut into discs (0,9 cm thick), placed in individual glass Petri dishes (1x1,5 cm, Anumbra®, Czech Republic), and sterilized in an autoclave (80 min at 120°C). Petri dishes with wooden discs were kept in the

incubator at 22 ± 1 °C and $65 \pm 5\%$ RH in the dark for 18 ± 3 days. Sources (vi) and (vii) were obtained by inoculating *A. areolatum* hyphal fragments from *S. noctilio* females (see source iii) onto discs of *P. contorta* and *P. ponderosa* (see sources iv and v), respectively. The fungus was allowed to grow at 22 ± 1 °C and $65 \pm 5\%$ RH in the dark until the entire surface of the wooden disc was covered with mycelium (18 ± 3 days).

2.3 Behavioural experiments

2.3.1 Olfactometer

To test the behavioural response of *S. noctilio* females towards olfactory stimuli, a four-way olfactometer was used (Figure 1). The device consisted of an experimental arena designed for this specific purpose in-house, made out of Grilon® (EMS Grivory, Switzerland) with a central area (13 cm²) and four symmetrical arms (odour areas). The length from the center to each of the four arm ends was 17.5 cm. Each arm was connected to a cylindrical glass chamber (odor chamber, 5.5 cm long; 2.5 cm in diameter) where the stimuli sources were placed. The whole arena was covered by a glass lid (37 × 37 cm, 4 mm thick) to achieve an airtight seal. To generate the airflow, a conventional commercial suction pump was connected to an orifice located in the centre of the arena with Teflon tubing (diameter: 12 mm, Swagelock®, USA). Air entering to the system through the odor chambers (2 l/min) was first filtered using activated charcoal (0.5 kg) and humidified by bubbling it in distilled water (1 l). All tubing connecting the system was with Teflon tubes (diameter: 6 mm, Swagelock®, USA). All system connections were made with brass fittings (Swagelock®, USA). 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 centred above the olfactometer.

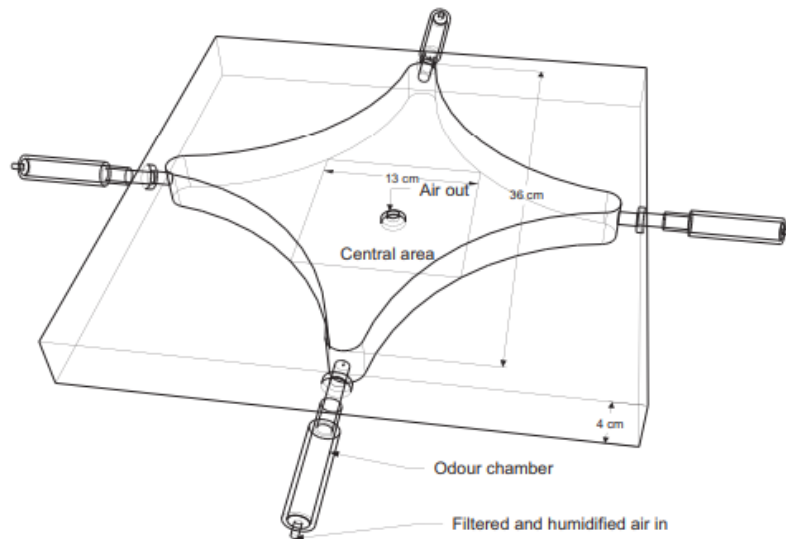


Figure 1. Four-way olfactometer used in bioassays with *Sirex noctilio* females. Odours were presented in pairwise combinations and 28 females were tested in each. Image credit: AS Martínez.

2.3.2 Behavioural assays

Two hours before the experiments, female wasps were moved from the incubator to an acclimation room with artificial light, 24 ± 1 °C and 65 ± 5 % RH. A single wasp was introduced in the central area of the olfactometer. Once released, the female was given 2 min for further acclimation inside the olfactometer. After this period, the wasp's movement was recorded with a webcam centred 150 cm above the olfactometer (Facecam 1000 Genius,) at 5 frames/s for 10 min. All bioassays were conducted under controlled conditions (24 ± 1 °C temperature and 65 ± 10 % RH). For each treatment, 28 *S. noctilio* adult females were tested. The olfactometer was used as a two-choice, since two sources of volatile stimuli were presented simultaneously to individual wasps in each replicate (Table 1). Thus, in each trial, two branches of the olfactometer contained the same source of volatiles and the other two contained a different source. The same volatile sources were always placed in adjacent arms and their positions were rotated according to a predefined scheme every two trials in order to minimize orientation biases. When wood or artificial culture were used as volatile sources (ii-vii), one rectangular (2.5 cm x 1 cm x 1 cm) portion (plug) of the source was introduced into each chamber. When contrasting the response towards air, two adjacent chambers

were left empty. After each replicate, volatile sources were renewed and the arena and odor chambers were thoroughly rinsed with water, alcohol and hexane. Additionally, two controls were performed: Air vs. Air (system control) and Artificial culture Medium (A-M) vs. Air.

Table 1. Pairwise combinations (grey cells) of 6 odor sources presented to a *Sirex noctilio* female in the olfactometer during a 10 min-long bioassay. Control assays are not shown on the table for clarity purposes (i.e., Air vs. Air and Artificial culture Medium (A-M) vs. Air).

Stimulus 1 Stimulus 2	Fungus (A-M)	Contorta	Ponderosa	Contorta+Fungus	Ponderosa+Fungus
Air					
Fungus (A-M)					
Contorta					
Ponderosa					
Contorta+Fungus					

2.3.3 Data analysis

The video files recorded for each individual wasp were used to determine the position (x and y coordinates in pixels) of the wasp at each frame using the software ImageJ (Rasband, 2016). The percentage of time spent in each odor area during the bioassay was calculated for each individual wasp. For this purpose, the time spent in the central area was excluded from the analysis. The percentage of time spent in an odor area for each volatile source was compared to each other with Wilcoxon signed-rank tests since data did not meet normality assumptions. To rule out possible effects due to positional biases (i.e., the position of the olfactometer), an additional Wilcoxon signed-rank test was performed considering the response of the wasps in relation to each of the olfactometer arms (1-4). In this case, non-significant results are not reported. All statistical analyses were performed with R (version 3.6-2) (R Core Development Team 2019).

2.3.3.1 Olfactory Preference Index (OPI)

An Olfactory Preference Index (OPI) was designed *ad-hoc* to rank the intensity of the preference in the overall responses towards the six odor sources: Air, Fungus (A-M), Contorta, Ponderosa, Contorta+Fungus and Ponderosa+Fungus. The OPI is based on the statistical significances (p-values

from Wilcoxon signed-rank tests) of a particular source vs. the other five tested sources. For each contrast from the Wilcoxon signed-rank test, the p-value was converted into a Partial Olfactory Preference Score (POPS) by assigning a score ranging between 2 and -2 (Table 2). The OPI for a specific odor source was then conformed by adding all the POPS that involved that same odor source: e.g., $-5,5$ (OPI_{Air}) = $-1,5$ ($POPS_{vs.Fungus(A-M)}$) + 0 ($POPS_{vs.Contorta}$) - 2 ($POPS_{vs.Contorta+Fungus}$) + 0 ($POPS_{vs.Ponderosa}$) - 2 ($POPS_{vs.Ponderosa+Fungus}$).

Table 2. Partial Olfactory Preference Score (POPS) given according to the result of the behavioural tests. The score considers the statistical significance of a particular contrast between two sources and assigning an arbitrary partial score between 2 and -2 to the response evoked towards each source evaluated in that contrast. For instance, in the case of the contrast between Air and Fungus, where Fungus was preferred over Air with a statistical significance of $p < 0.01$, a POPS of +1.5 was assigned to Fungus and a POPS of -1.5 to Air. The OPI was then conformed by adding all the POPS obtained from each contrast in which a particular odor source was tested.

p value	POPS if stimulus was preferred	POPS if the other stimulus was preferred
$p < 0.001$	2	-2
$p < 0.01$	1,5	-1,5
$p < 0.05$	1	-1
$p \geq 0.05$	0	

2.4 Volatile collection

Headspace volatiles were collected from the different sources: (i) Air, (ii) A-M, (iii) Fungus (A-M), (iv) Contorta, (v) Ponderosa, (vi) Contorta + Fungus and (vii) Ponderosa + Fungus. This was carried out using an 8-arm volatile collection system. Air was pumped at 0.55 l/min through activated charcoal to the oven cooking bags containing the odor sources. The air was extracted from the bags with a vacuum pump (0.5 l/min) passing through a porous polymer adsorbent matrix (30 mg of HayeSepQ®) where the volatiles were retained. Two samples of volatile emissions were collected per odor source (4 Petri dishes of each odor source per sample). These were performed in darkness

and at a constant temperature of 25 ± 1 °C and 65 ± 5 % RH. Volatiles were collected during 24 h. Afterwards, the volatile traps were eluted with 200 μ l of dichloromethane ($\geq 99.5\%$, Sintogran®) containing 5 ng of tridecane ($\geq 99\%$, Sigma-Aldrich®) as an internal standard. Each sample was then evaporated to 50% of total volume with a N₂ flow to further increase concentration. The different samples of the same odor source were pooled for EAD and GC-MS analyses.

2.5 Antennal response

Antennae from adult *S. noctilio* females (3 ± 2 days old) were cut with a dissection knife and exposed to the following headspace samples: (i) Air (N=4 antennae), (ii) Artificial culture medium (N=7 antennae), (iii) Fungus (A-M) (N=8 antennae), (iv) Contorta (N= 7 antennae), (v) Ponderosa (N=8 antennae), (vi) Contorta+Fungus (N=8 antennae) and (vii) Ponderosa+Fungus (N=8 antennae). All antennae used in each assay were extracted from different females and used once. To record the antennal response towards the volatile compounds we used an electroantennographic detection system (EAD Syntech, Germany), coupled to a gas chromatograph (GC, Agilent 7890B, Agilent Technologies, Inc., USA) with a flame ionization detector (FID) fitted with a DB5 capillary column (0.25 mm inner diameter, film thickness 0.25 μ m, Agilent Technologies, Inc.). The EAD consisted of 2 electrodes in the form of a fork for antenna mounting (with conductive gel), connected to a 10X high impedance pre-amplifier. This was connected to the signal acquisition system via an analog-to-digital converter (iDAC-2, with filters and amplifier) and finally to a PC. A constant stream of humid and filtered air bathed the antenna. The tube through which the air was transferred was connected to a solenoid valve that was activated by a “stimulus controller” (generic), which is activated from a manual switch. Once the antenna was mounted, this electrovalve was only used to apply the positive controls (2 μ l of α -pinene on a 0.75 x 0.75 cm filter paper), without using the GC input. After manually activating the solenoid valve, a clean air pulse (2 s) was delivered to the antenna and its response recorded. Two minutes after ensuring the antenna responded correctly to the α -pinene ($\geq 98\%$, Sigma-Aldrich®), 2 μ l of a sample was injected into the GC at 260 °C and nitrogen was used as carrier gas at 0.75 ml/min. The column temperature was maintained at 35 °C for 1 min, then

increased at a rate of 5 °C/min until it reached 100 °C, then 12 °C/min until 230 °C and maintained during 10 min. As the compounds emerged, they were detected by the FID and simultaneously exposed to the insect antenna and its electrical response recorded. Finally, the same positive controls with α -pinene were performed to evaluate the antennal response at the end of the trial. Antennae not responding to α -pinene before or after, were discarded. The parameters for logging and antenna recording were configured and analysed using GcEad 2014 v1.2.5 (Syntech) and in addition, Chemstation software (Agilent) was used to examine the GC profiles in detail. True responses were considered to be those observed in at least 50% of the antennae tested for each particular source.

2.5.1 Data analysis

The analysis of the antennal responses was carried out with five of the biologically relevant odor sources: Fungus (A-M), Contorta, Ponderosa, Contorta + Fungus and Ponderosa + Fungus, (air and artificial culture medium were performed as controls) through a non-metric multidimensional scaling (NMDS) using antennal response amplitude. For this purpose, a Bray-Curtis distance matrix (Vegan V2.4-6 package for R) was used. To test for differences in the antennal responses between sources a multivariate analysis of variance (perMANOVA) was also performed on the distance matrix based on 9999 permutations. The analysis was performed using the Adonis function in R (Vegan V2.4-6 package for R). Finally, to analyse the level of relevance of the detected compounds in the ordination and the results obtained, the corresponding vectors were analysed using the data.envfit function (Vegan V2.4-6 package).

Finally, from a qualitative analysis, a Venn diagram was created (53). Following the background odors-resource indicator odors (RIOs) conceptual framework, we grouped compounds that were produced by a single odor source and those that were shared by two or more.

2.6 Identification of volatile compounds

Volatile compounds collected and evaluated in electroantennography assays were tentatively identified (i.e. we did not confirm compounds by injection of synthetic standards) through a gas chromatograph (Agilent 7890A, Agilent Technologies, Inc.) fitted with a DB5MS capillary column (0.25 mm inner diameter, film thickness 0.25 μ m) and coupled with a mass spectrometer (Agilent 5977 selective mass detector, Agilent Technologies, Inc.). Samples (1 μ l) were injected at 240 °C in splitless mode. Helium was used as carrier gas at 0.75 ml/min. The temperature ramp used was the same as the one used for GC/FID-EAD.

2.6.1 Data analysis

Compounds were identified by computer matching (>95%) with our own mass spectral library and a comparison of Kovats retention indices and compound spectra with published data (Adams, 2007) and web databases, (<https://www.pherobase.com/>, <https://webbook.nist.gov/> and <https://pubchem.ncbi.nlm.nih.gov/>). Data acquisition was carried out with MassHunter Data Acquisition software (version B.06.00, Agilent Technologies, Inc.).

3 Results

3.1 Behavioural assays

Sirex noctilio female responses towards control sources were non-significant (Air vs. Air, $n=28$, $\chi^2 = 1.66$, $p>0.05$ and Air vs. A-M, $n=28$, $\chi^2 = 1.53$, $p>0.05$), confirming the correct system performance and that A-M is not attractive. A preference was detected towards the Fungus (A-M) when it was presented against Air ($n=28$, $\chi^2=7.87$, $p<0.01$) (Figure 2). Non-significant differences were observed in female response when presented Contorta vs. Air ($n=28$, $\chi^2=2.08$, $p>0.05$) or Ponderosa vs. Air ($n=28$, $\chi^2=0.99$, $p>0.05$). When the response towards Fungus (A-M) was contrasted with *P. contorta*, a behavioral preference was observed towards the latter ($n=28$, $\chi^2=3.87$, $p<0.05$), whereas non-significant differences were observed when the Fungus (A-M) was contrasted with *P. ponderosa* ($n=28$, $\chi^2=0.0003$, $p>0.05$). A preference in response towards *P. contorta* was observed when contrasted against *P. ponderosa* ($n=28$, $\chi^2=5.18$, $p<0.05$).

When contrasting Contorta+Fungus against Air we recorded a clear preference of *S. noctilio* females towards the stimulus containing the host wood with the symbiont ($n=28$, $\chi^2=12.19$, $p<0.001$). Non-significant differences were observed when Contorta+Fungus was confronted with Fungus (A-M) ($n=28$, $\chi^2=0.67$, $p>0.05$). A clear preference towards Fungus+Contorta was observed when contrasted with Contorta or Ponderosa ($n=28$, $\chi^2=8.24$, $p<0.01$, $\chi^2=21.36$, $p<0.001$). When contrasting Ponderosa+Fungus against Air we recorded a clear preference of *S. noctilio* females towards the stimulus containing the other potential host with the symbiont ($n=28$, $\chi^2=20.60$, $p<0.001$). No significant differences were observed in contrasts involving Ponderosa+Fungus or Fungus (A-M) ($n=28$, $\chi^2=0.67$, $p>0.05$), whereas when Ponderosa+Fungus was tested against Contorta or Ponderosa, the latter were preferred ($n=28$, $\chi^2=11.84$, $p<0.001$, $\chi^2=5.15$, $p<0.05$). Finally, both pine species with the fungus growing on them resulted in similar responses ($n=28$, $\chi^2=0.47$, $p>0.05$).

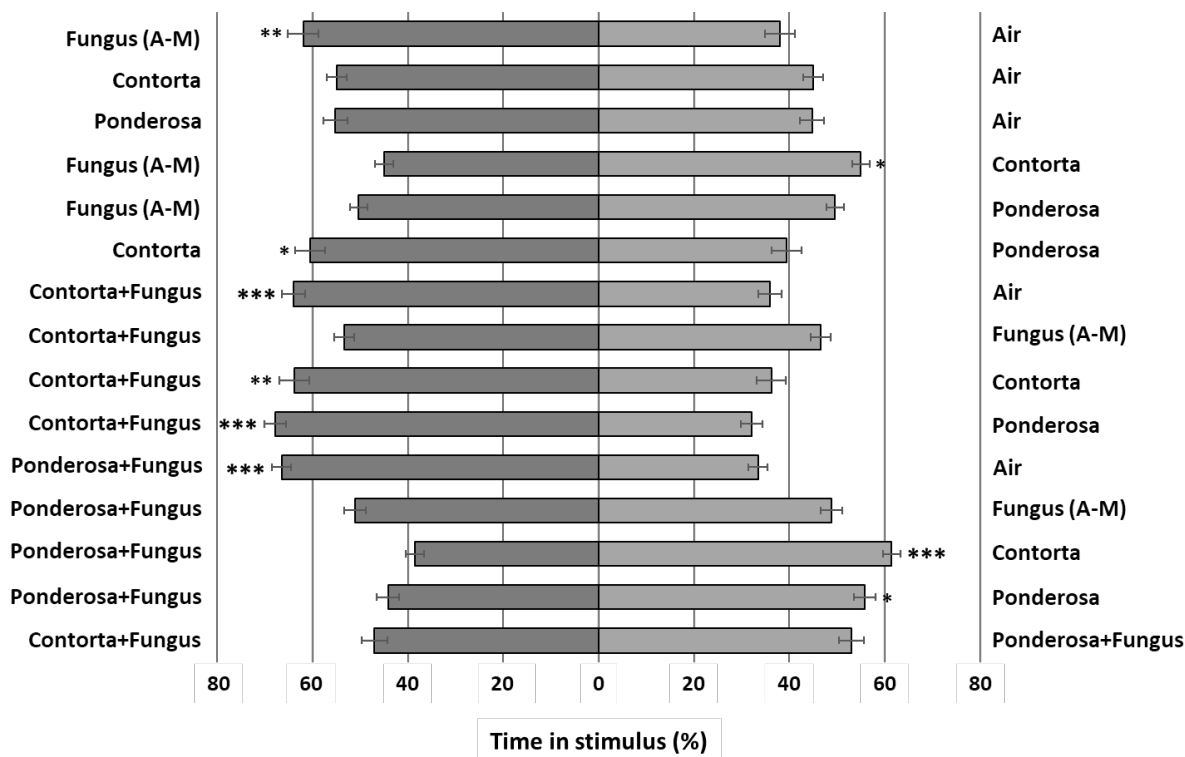


Figure 2. Behavioural responses of *Sirex noctilio* females in an olfactometer towards pairwise combinations of clean air (Air), fungus grown on artificial culture medium (Fungus (A-M)), *Pinus contorta* wood only (Contorta), *Pinus ponderosa* wood only (Ponderosa), fungus grown on *P. contorta* wood (Contorta+Fungus), and fungus grown on *P. ponderosa* wood (Ponderosa+Fungus) (n=28 females). Odor sources combinations are detailed at the right and left of the figure. Bars represent the mean (\pm SE) percentage time spent in the area corresponding to the stimulus. Significant results are indicated by asterisks (*:p<0.05, **:p<0.01, ***:p<0.001).

3.2 Olfactory Preference

The OPI indicates a hierarchy in terms of preference of female wasps towards the 6 sources presented (Figure 3, Table S1). The stimulus that was found to be the most preferred in the ranking according to the evaluation was Contorta+Fungus (OPI=5.5), followed by Contorta and Fungus (OPI=2.5 and 0.5, respectively). The stimuli that were relatively least preferred were Ponderosa+Fungus (OPI=-1), Ponderosa (OPI=-2) and finally Air (OPI=-5.5).

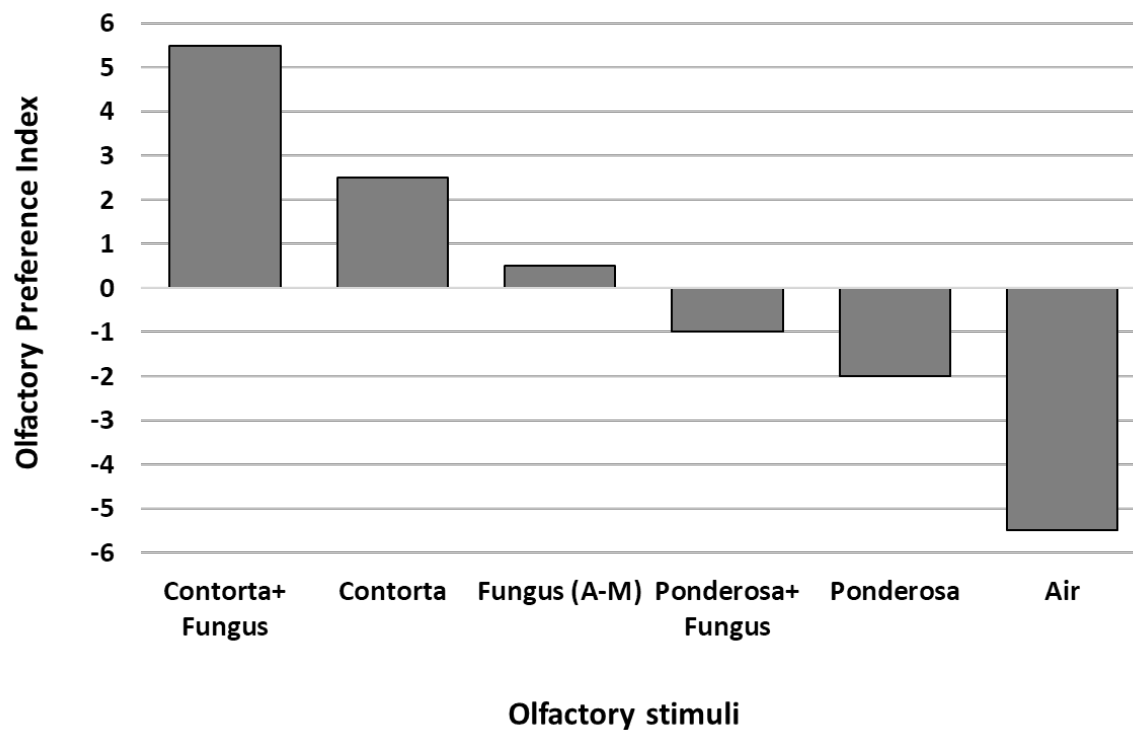


Figure 3. Olfactory Preference Index (OPI) obtained from olfactometer assays performed with *Sirex noctilio* females. The index considers the statistical significance of a particular contrast between two specific odors and assigns an arbitrary partial score between 2 and -2 to the response evoked towards each source evaluated in that contrast. For instance, for Air vs. Fungus, where Fungus was preferred over Air with a statistical significance of $p < 0.01$, a partial score of +1.5 was assigned to Fungus and a score of -1.5 to Air. The OPI was then conformed by adding all the partial scores obtained from each contrast in which an odor source was tested.

3.3 Antennal response

In GC analysis a total of 515 compounds were detected from all tested samples. According to our analysis criteria, GC-EAD showed antennal electrophysiological responses to 62 of these compounds (Figure 4 and Table S2). Entrainments of Fungus (A-M) resulted in 16 positive responses out of 62 compounds ; for Contorta, 15 positive responses out of 141 compounds; for Ponderosa, 21 positive responses out of 120 compounds; for Contorta+Fungus, 20 positive responses out of 93 compounds; for Ponderosa+Fungus, 23 positive responses out of 99 compounds. For Artificial culture Medium

(A-M) we observed a total of 24 compounds and 3 responses, which were discarded from the analyses since they do not represent, a priori, ecologically-relevant stimuli (also did not prove to be an attractive stimulus and the compounds only appear in this source). Additionally, one compound (Nonanal) was discarded as responses towards it were observed in all sources, including the blank system (Air, not shown) and as such considered a contaminant.

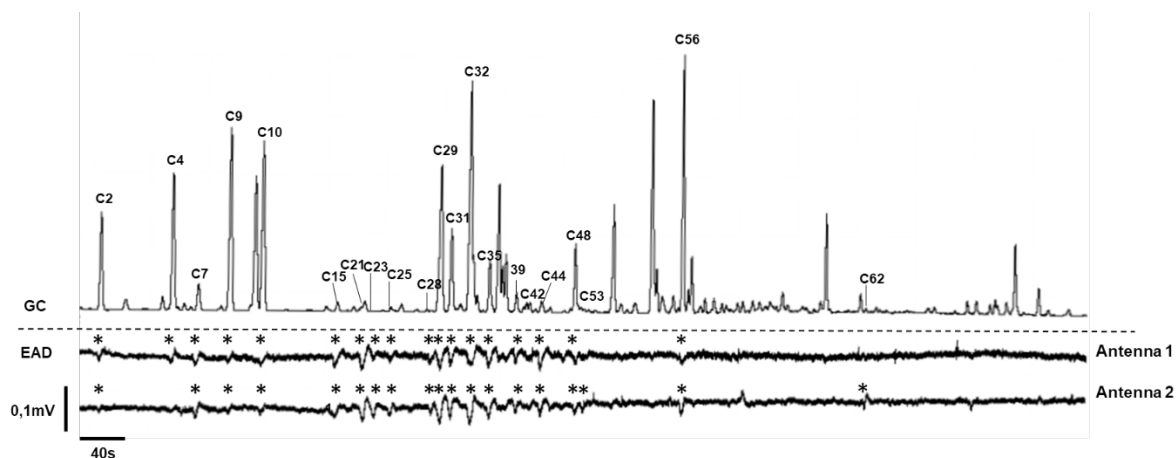


Figure 4. Example of a GC-EAD output. On the top (GC), the detected compounds emitted by Contorta+Fungus. Below, the coupled responses of 2 antenna (EAD) towards volatiles emitted by this odor source. The asterisks indicate the antennal responses.

The NMDS based on the antennal response amplitude showed a spatial arrangement revealing a separation between the sources (Figure 5). The analysis showed that there were significant differences in the antennal responses of *S. noctilio* females to VOCs emitted by these sources (perMANOVA: $F_{2, 30}=19.929$, $p<0.001$, permutations=9999). Furthermore, multivariate pairwise comparison showed significant differences between the VOCs of all evoked responses (Table S3).

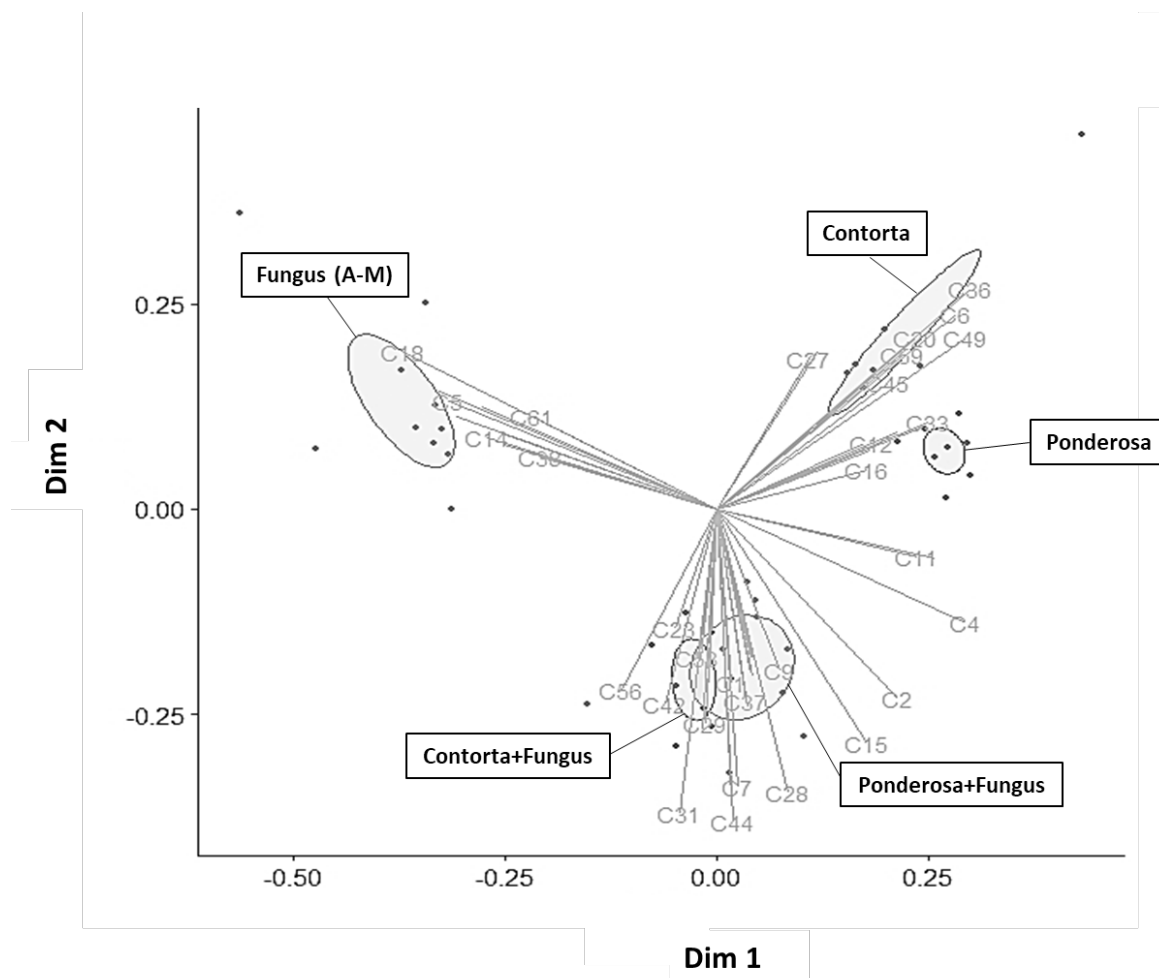


Figure 5. Non-metric multidimensional scaling (NMDS) ordination based on Bray-Curtis dissimilarities of antennal response amplitude towards volatile organic compounds from five different sources: Fungus (A-M) (N=10; Contorta (N=7), Ponderosa (N=8), Contorta+Fungus (N=8) and Ponderosa+Fungus (N=9). Stress value=0.09.

We also established the incidence of the active compounds in distinguishing the different sources of volatiles. The analysis showed that 53 out of the 58 compounds contribute to the separation of the sources (Table S2) and these were plotted in a Venn diagram created under the background odors-RIOs framework (Figure 6).

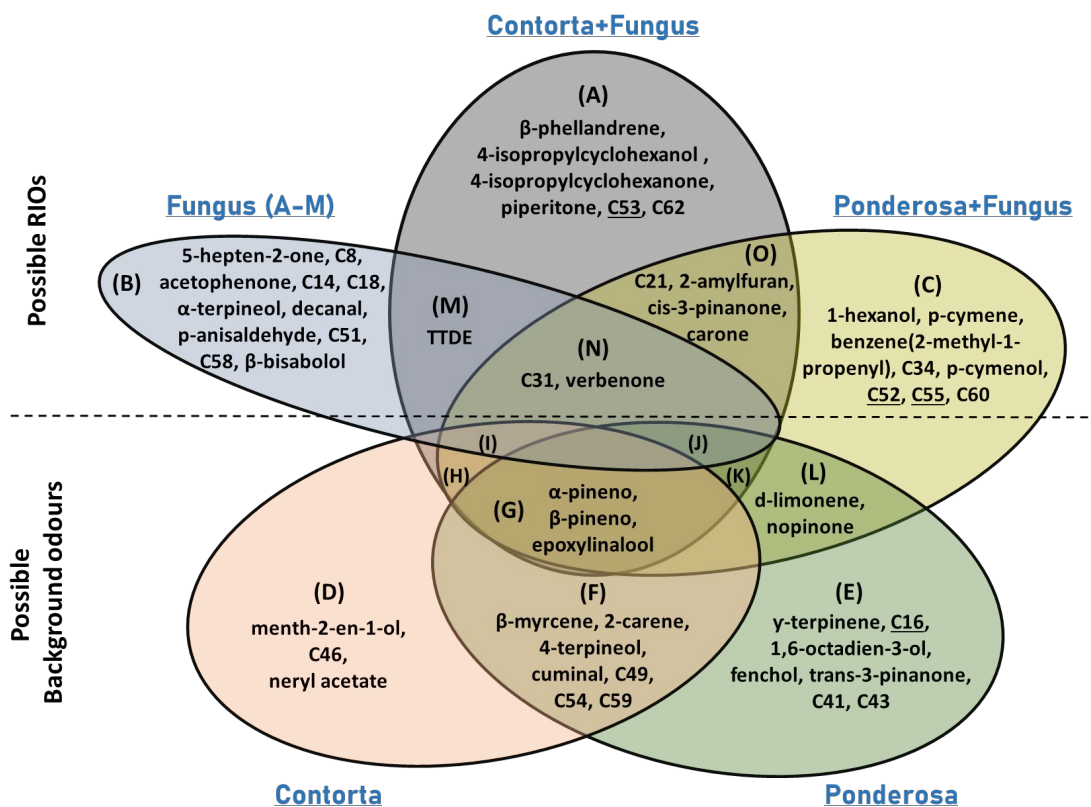


Figure 6. Volatile compounds emitted by five different sources that triggered electroantennographic responses in *Sirex noctilio* females. The detected compounds from each particular source (groups A-E) and those shared by 2 or more (groups F-O) are shown. Statistically significant and non-significant compounds (underlined) from the analysis of the vectors resulting from NMDS sorting are shown. For better visualization and understanding, certain compounds are not shown in the figure: group H: 3-carene; I: methylbenzoate; J: estragole; K: C28. Compounds below the dotted line suggests possible background odors, while those above, possible resource indicating odors (RIOs).

4 Discussion

Our study is the first to report the behaviour of *S. noctilio* females towards combinations of chemical cues emitted by its symbiont *A. areolatum* grown on different host-tree species. Both pine species inoculated with the growing fungus showed to be strong attractants on their own when contrasted against air. Interestingly, we found a clear hierarchy in preferences displayed by *S. noctilio* females, with the highest level evidenced towards the volatiles released by the symbiont *A. areolatum* growing on *P. contorta*, followed by *P. contorta* on its own, and then by the fungus growing on artificial culture medium. Notably, the preference level towards *P. contorta* was higher than the one observed for the alternative host species *P. ponderosa*, even when it presented symbiont growth. *Sirex noctilio* female's clear preference towards *P. contorta*, could be the result of early experiences with the natal host, since all females used in bioassays were obtained from *P. contorta* infested trees (it is challenging to find *P. ponderosa* with attack in the region (25). It is well known that pre-emergence and early adult exposure to a particular host species/cultivar, can affect the olfactory preferences in later stages of the life cycle in certain insect species (54–57). For instance, adults of the aphid-parasitic wasp *Aphidus ervi* preferred volatiles from aphid-infested host plants to volatiles from intact plants only when they had been exposed to volatiles from aphid-infested host plants during the developmental stages (58).

Alternatively, females could show a preference towards *P. contorta* because of its unique chemotype, regardless of the host species where individuals developed. It is important to note that both *P. contorta* and *P. ponderosa* are native to North America, while *S. noctilio* is of Eurasian origin. Innate responses may be due similarities in volatile profiles of North American pine species respect to those of Eurasian origin. Additionally, differences in preference could be related to bark structure (critical at the time of oviposition) or the resin production/composition that could be perceived through differences in volatiles composition (*P. ponderosa* have thicker bark and higher resin production than *P. contorta*) (59,60). Whether the observed hierarchy in the attraction levels

towards the tested sources is a result of the wasp's natal environment or a difference due to innate responses, remains to be confirmed in future studies. In this context, if larval or early adult experiences affect the behaviour in later stages of *S. noctilio* females, special attention needs to be given to the chemical signature of monoculture pine species, since integrating this particular signature into a lure used for the region, could maximize its attractiveness.

Resource and background odors

Previous studies have recorded increased behavioural responses when background odors and RIOs interact. For instance, Landolt et al. (61) showed that females of the cabbage moth, *Trichoplusia ni* (Hübner) were attracted more frequently in a flight tunnel to male pheromones when combined with cotton foliage. The woodwasp *Sirex noctilio* evolved in mixed-forest environments, where susceptible pine trees are located in a complex and unpredictable matrix that also contains other tree species. Fungal volatiles could signal previously infected (hence weakened) trees, in addition to specific locations within them that are most suitable for larval development, conveying the offspring a fitness advantage (45,62). This positive response to previously attacked trees results in aggregative attack, which has been suggested as a mechanism underlying the damaging outbreaks of *S. noctilio* in the invaded range (10,24,25). In this scenario, pine wood emissions (background odors) combined with symbiont emissions (RIOs) could signal the presence of an optimal oviposition substrate, thus increasing the female's response when perceived together.

Possible background odors

Antennal responses indicate that those compounds shared by all four sources (α pinene, β pinene and epoxylinalool) emitted by pine wood-containing sources, could be part of the background stimuli. This is in agreement with previous studies that suggest that pine trees emitting α and β pinene evoked positive responses from *S. noctilio* females (38). Moreover, these compounds are part of the kairomone-based attractants currently used in intercept panel trap or Lindgren multiple funnel trap in many parts of the world for monitoring purposes (10). It's important to note that in

our study, compounds found in groups D, E and F (Figure 6) could also be relevant as background stimuli, but not appearing in wood with fungal growth due to being masked during the chromatographic identification by some compounds released from wood with growing fungus. For instance, compounds such as β -myrcene and 4-terpineol, that have been shown to trigger antennal responses in *S. noctilio* (34,35,63), were not detected in our study in group G (Figure 6).

Resource indicating odors

Our results suggest that compounds produced by sources containing *A. areolatum* could conform the RIOs. For instance, β -phellandrene (Figure 6, A), is a known semiochemical to which female *S. noctilio* elicited antennal responses in earlier studies (34,35). A second compound of interest is 2,2,8-trimethyltricyclo[6.2.2.0^{1,6}]dodec-5-ene (TTDE), which was found in the present study within emissions of the symbiont growing on artificial medium and also on *P. contorta* (group M). This compound was also tentatively identified by Jofré et al. (46) obtained from volatile collections of the symbiont and thought to be relevant for the *S. noctilio* parasitoid, *Ibalia leucospoides* (Hymenoptera:Ibaliidae). It is worth noting that particular compounds emitted exclusively by the symbiont grown in artificial medium (Figure 6, B), such as p-anisaldehyde and β -bisabolol, could also be relevant in defining the RIOs. However, were not registered when the fungus was grown on wood, possibly due to being masked during chromatography by other compounds emitted by these sources (e.g., Contorta+Fungus). In this regard, β -bisabolol was previously detected in volatile entrainments of *A. areolatum* (48), while antennal electrophysiological activity has been detected in *S. noctilio* towards p-anisaldehyde (64).

Preference towards *P. contorta*

Based on our antennal response analysis, there are several compounds (Figure 6; Contorta: A, D and M; Ponderosa: C, E and L) that could be involved in the preference variations of the females towards *P. contorta*. In this sense, certain volatile compounds emitted by the sources could act as deterrents, resulting in *P. ponderosa* being less preferred than *P. contorta*. Interestingly, d-limonene is present

in both sources with *P. ponderosa* (with and without the fungus). This compound has been detected in previous studies involving terpenoid extractions from this particular pine species (61) and has also been shown to be toxic, repellent, and to have fungicidal properties (66–69). In line with this, Kile & Turnbull (70) showed that limonene is a growth inhibitor for *A. areolatum* mycelium in laboratory bioassays. Considering that limonene was only detected in odor sources from *P. ponderosa*, this compound could be signalling a possible "less suitable" host that restricts symbiont growth. More research is needed to determine which compounds are responsible for the preference between *P. contorta* vs. *P. ponderosa*.

Conclusions

Our study is indicative of the highly complex chemical world in which *S. noctilio* females navigate in order to locate suitable oviposition sites, with interactions between different compounds appearing to be important during the search and the host species being more important than initially assumed. Our results support the hypothesis that female behaviour is regulated by pine wood with signs of previous attack from conspecifics (i.e. fungal growth). Additionally, the pine species seems to be key, with the attraction maximized when the fungus grows on *P. contorta*, highlighting the synergy of both odor sources. Future research should be aimed at establishing the particular odor blend that maximized the response, with emphasis on establishing the relevance of learning processes during early life stages.

5 Conflicts of interest

The authors declare that they have no competing interests.

6 Authors' contributions

Conceived and designed the study: SM, JV, JC and AM. Collected the data: SM, PF, DS, FD. Analysed the data: SM, GDLV, PF, DS, FD and AM. Drafted the manuscript: SM and AM. All authors read and approved the final manuscript. Secured Funding: JV and AM.

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9 Supplementary material

Table S1. Olfactory Preference Indexes (OPI) obtained by adding the Partial Olfactory Preference Scores (POPS) of olfactometric behavioural tests of female *Sirex noctilio* with six different olfactory sources presented in pairwise combinations. The OPI was used to rank the preference towards the tested stimuli of the females.

Stimulus	Source preferred	POPS	No preference	POPS	Other source preferred	POPS	OPI
Contorta + Fungus vs.	Contorta, p<0,01	1,5	Fungus(A-M), p>0,05	0		0	5,5
	Ponderosa, p<0,001	2	Ponderosa+Fungus, p>0,05	0			
	Air, p<0,001	2					
Contorta vs.	Fungus(A-M), p<0,05	1	Air, p>0,05	0	Contorta+Fungus, p<0,01	-1,5	2,5
	Ponderosa+Fungus, p<0,001	2					
	Ponderosa, p<0,05	1					
Fungus vs.	Air, p<0,01	1,5	Contorta+Fungus, p>0,05	0	Contorta, p<0,05	-1	0,5
			Ponderosa+Fungus, p>0,05	0			
			Ponderosa, p>0,05	0			
Ponderosa + Fungus vs.	Air, p<0,001	2	Contorta+Fungus, p>0,05	0	Contorta, p<0,001	-2	-1
			Fungus(A-M), p>0,05	0	Ponderosa, p<0,05	-1	
Ponderosa vs.	Ponderosa+Fungus, p<0,05	1	Fungus(A-M), p>0,05	0	Contorta+Fungus, p<0,001	-2	-2
			Air, p>0,05	0	Contorta, p<0,05	-1	
Air vs.		0	Contorta, p>0,05	0	Fungus(A-M), p<0,01	-1,5	-5,5
			Ponderosa, p>0,05	0	Contorta+Fungus, p<0,001	-2	
					Ponderosa+Fungus, p<0,001	-2	

Table S2. Amplitude of antennal responses (mV) recorded in *Sirex noctilio* females when exposed to compounds emitted by the different volatile sources. Individual compounds are listed together with their respective Kovats indices and an identification used throughout the manuscript for ease of identification. The means (\bar{x}) of the responses are shown, with their respective standard deviations (Sd) and the number of antennas that showed a response (n). The p-values of the vector analysis resulting from the NMDS are also shown (*=p<0.05, **=p<0.01, ***=p<0.001). Empty cells indicate absence of compound on that specific treatment.

Table S3. Multivariate pairwise comparison of the permutational ANOVA ("adonis") showing comparisons of *Sirex noctilio* GC-EAD responses among the five different stimuli, calculated on Bray–Curtis dissimilarity measures with Bonferroni *p-value* adjust.

Pairs	F-Model	r^2	<i>p</i>-adjusted
P.contorta+Fungus vs P.contorta	21,48	0.62	0.003*
P.contorta+Fungus vs P.ponderosa+Fungus	9,11	0.38	0.001**
P.contorta+Fungus vs P.ponderosa	31,35	0.69	0.003*
P.contorta+Fungus vs Fungus	21,88	0.58	0.001**
P.contorta vs P.ponderosa+Fungus	17,1	0.55	0.001**
P.contorta vs P.ponderosa	9,88	0.43	0.002*
P.contorta vs Fungus	19,66	0.57	0.002*
P.ponderosa+Fungus vs P.ponderosa	20,74	0.58	0.002*
P.ponderosa+Fungus vs Fungus	19,87	0.54	0.001*
P.ponderosa vs Fungus	28,9	0.64	0.001*

Oviposition substrate location by the invasive woodwasp *Sirex noctilio*: The combined effect of chemical cues emitted by its obligate symbiont *Amylostereum areolatum* and different host-tree species.

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Our results suggest a hierarchy in terms of female preferences towards the tested stimuli and also provides information on candidate compounds for the design of a highly sensitive trapping mechanism.

Olfactory sources



Fungus in artificial culture medium (Fungus (A-M))



Pinus contorta



Pinus ponderosa

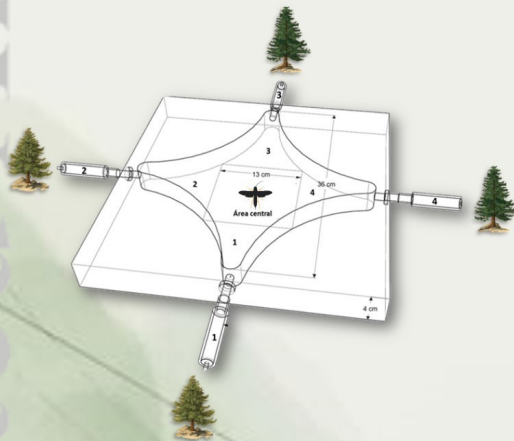


P. contorta + Fungus



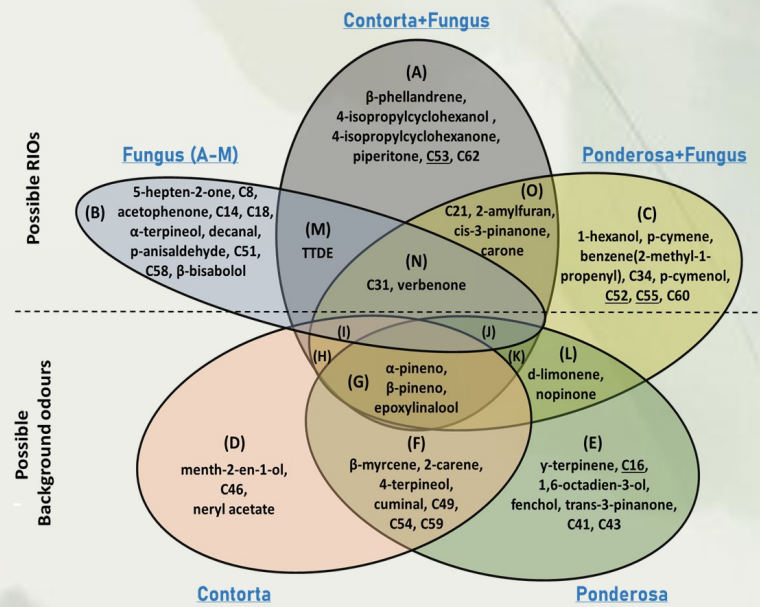
P. ponderosa + Fungus

Olfactometry



Antennal response

Compounds with antennal response



The detected compounds from each particular source (groups A-E) and those shared by 2 or more (groups F-O). Statistically significant and non-significant compounds (underlined) from the analysis of the vectors resulting from NMDS are shown. Unknown compounds are shown (C). Group H: 3-carene; I: methylbenzoate; J: estragole; K: C28.

Olfactory preference

