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Volatiles from Eucalyptus Trunks and Forest Floor Humus Influence the Habitat Transfer, Host Selection, and Aggregation of *Endoclita signifer* Larvae

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Abstract: *Endoclita signifer* is a major wood-boring pest of eucalyptus trees in China, and its third instar larvae can accurately find and infest eucalyptus trees in mixed-species forests, although it can also feed on another 51 tree species in the same planted area. A total of 34 volatile compounds were identified from eucalyptus trunks, including non-infested and infested trunks with single or multiple (more than three) larval entrance cover packets, and forest floor humus. Of these, o-cymene showed a strong gas chromatography-electroantennographic detection (GC-EAD) activity and significant attraction of the third instar *E. signifer* larvae. Higher abundance of alpha-phellandrene, o-cymene, and the unique 2-phenyl-2-propanol in the volatile profile from infested eucalyptus trunks indicated that they were herbivore-induced plant volatiles (HIPVs). The larvae showed significantly higher attraction to volatile blends, especially those from infested eucalyptus trunks, than to single volatiles. A blend of the three HIPVs, α-pinene, D-limonene, and n-butyl ether may serve as an attractant for the control of *E. signifer* larvae in eucalyptus plantations. Further, exposure of third instar *E. signifer* larvae to some of these volatiles which also elicited electroantennogram and behavioral responses, influenced expressions of some olfactory proteins. Our results show that third instar *E. signifer* larvae can recognize o-cymene from host eucalyptus trunks and are attracted to the trunk by the three HIPVs when they shift their habitats from the forest floor humus to the tree trunks.

Keywords: volatile organic compounds; herbivore-induced plant volatiles; eucalyptus boring pest; conspecific larvae; attraction

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1. Introduction

The ghost moth, *Endoclita signifer* Walker (Lepidoptera, Hepialidae), infests a broad range of host plants spanning 30 families, 40 genera, and 51 species [1]. It is distributed widely from Japan and Korea in East Asia, through central, southern, and southwestern China, India, Thailand, and Myanmar in South Asia [2]. It is the major native polyphagous wood-boring pest of eucalyptus in China and is mainly distributed in Guangxi and Guangdong in China [3].The damage caused by *E. signifer* to eucalyptus was first recorded in China in 2007 after eucalyptus were introduced to large areas in Guangxi. Currently only 17.1% of counties in Guangxi are free from its infestation [1]. Notably, the larvae hatch and survive in forest floor humus following egg-laying by the adults, after which the third instar larvae translocate to standing trees, feed on bark, bore into stems, and weave larval entrance cover packets with fragments of wood and silk to cover larval entrance holes, causing damage to eucalyptus trunks (Figure 1A). Because the damage to the trunk is obvious only after the formation of the larval entrance cover packet, the control method usually applied is injection of pesticides into each packet, but this is inefficient and therefore requires the development of new methods.

Figure 1. Larval entrance cover packet and worm holes of 3rd instar larvae transferred to eucalyptus trunks (circle shows the caudal spine) (**A**), aggregation infestations of *E. signifer* (**B**), and collection of eucalyptus volatiles (**C**).

Hepialidae larvae are known to transfer host and also move from the forest floor humus to roots [2]. Uniquely, *E. signifer* larvae have the ability to move from forest floor humus to trunks. Additionally, third instar larvae can precisely identify eucalyptus trees in mixed forests (which can contain eight different species) [1] to form multiple infestations of a single tree, which is common in eucalyptus plantations (Figure 1B). Given the unique habitat transfer behavior, host-specific transfer, and precise host selection of the third instar larvae, they represent a unique model to understand insect habitat selection and adaption. Polyphagous herbivores have the capacity to detect common cues from multiple host species as well as specific cues from individual host species [4]. We therefore sought to find out how constitutive and larvae-induced volatiles from eucalyptus trunks, influence the third instar larvae of the polyphagous *E. signifer* to find and gather on eucalyptus trunks.

Chemosensation shapes insect behavior related to survival and reproduction, such as food searching, choice of oviposition substrate, mate seeking, and detecting dangers, including predators or parasitoids [5]. Insects do not attack all plant species with equal frequency or incidence. Thus, for many herbivorous species, host plant selection is mediated mainly through volatile organic compounds (VOCs) [6]. VOCs and their recognition by insects have received significant research attention, especially in adults. For example, in eucalyptus, the leaf VOCs emitted by *Eucalyptus globulus* [7] and *E. citriodora* [8] were studied. Additionally, *Xanthogaleruca luteola* females were reported to have responded to ten compounds such as α-pinene, eucalyptol, 4-terpineol, and so on, of a eucalyptus non-host plant [9].

Meanwhile, 12 volatile compounds identified from *E. globulus* leaves were reported to elicit electroantennographic responses by *Helicoverpa armigera* females, among which α-phellandrene, α-pinene, and α-farnesene acted as oviposition attractants, while 1-methyl-4-isop and 1,8-cineole showed repellency [10]. In the above work, although eucalyptus was not a host, its VOCs could attract adult insects. Moreover, an attractant blend of myrcene, ethyl butyrate, and p-mycene was developed, which was reported to have slightly improved field attraction of *Diaphorina citri* to yellow traps [11]. This indicated that host plant volatiles have great potential for use as attractants for *D. citri* [12]. Additionally, in holometabolous insects such as Lepidoptera, the larval stage causes the main damage to plants, as it is the stage for feeding and growth, whereas adults are dedicated to reproductive tasks. The evaluation of chemical cues from their ecological niches is therefore

different between larvae and adults, and likewise between the physiological and molecular equipment required for detecting odors [13]. For example, methyl salicylate and (E)-alpha-bergamotene served as oviposition attractants for *Spodoptera frugiperda* adult moths, but they did not impact larval behavior; however, (E)-4,8-dimethyl-1,3,7-nonatriene, an oviposition deterrent in adults, was attractive to larvae [14]. Since larvae are the most damaging stage to plants and can also recognize VOCs, the identification of VOCs to which they are attracted can serve as evidence for their host selection behavior.

Herbivore-induced plant volatiles (HIPVs) are released by plants in response to herbivore feeding [15,16]. HIPVs mainly include green leaf volatiles (GLVs-C6 aldehydes, alcohols, and their esters), terpenoids, aromatics, and amino acid volatile derivatives [17]. Importantly, there are quantitative and qualitative differences in HIPV blends between non-infested and herbivore-infested plants [17], as has been recorded from tree trunks of *Semanotus bifasciatus* [18] and *Platycladus orientalis* infested by *S. bifasciatus* and *Phloeosinus aubei* [19]; these differences are known to induce different behaviors in pests and parasitoids [4,20]. For instance, adult *Pandemis pyrusana* were more attracted to apple trees damaged by conspecific larvae than to undamaged trees [21]. On the other hand, *Ostrinia furnacalis* neonate larvae were attracted to extracts collected from self-damaged plants but were repelled by (*Z*)-3-hexen-1-ol [22]. Additionally, *Cydia pomonella* larvae were more attracted by codling moth larvae-infested apple and (*E*,*E*)-a-farnesene [23], and *S. frugiperda* larvae were strongly attracted to HIPVs from conspecific-infested cowpea plants [24]. All the above showed that HIPVs affect the behavior of pests and parasitoids but also attract conspecific larvae. They therefore serve as indicators for host selection of larvae. However, reports on the specific VOCs used by larvae for host selection are limited. The identification of such VOCs can be used as a new strategy to control larvae.

Recognition of volatiles by insects is based on olfactory proteins. For example, a combination of genome editing (CRISPR-Cas9), electrophysiological recordings, calcium imaging, and behavioral analyses demonstrated that ionotropic receptor 8a (IR8a) was involved in the acid-mediated feces avoidance behavior by ovipositing female *Manduca sexta* [25]. Furthermore, a heterologous expression study identified 2-methoxyphenol as a key ligand for the *S. littoralis* odorant receptor SlitOr59 [26]. Additionally, odorant binding proteins (OBPs) are involved in the discrimination of odorant molecules. They serve as vital factors for the selective solubilization and transportation of external hydrophobic odorant molecules across the lymph. They can therefore be used for screening potential active compounds involved in insect behaviors [27]. In *E. signifer,* based on the high and biased expressions of genes in young larval heads, we identified EsigOBP2 (odorant binding protein OBP), EsigOBP8, EsigGOBP2 (general odorant binding protein GOBP), EsigGOBP4, EsigGOBP5, EsigCSP1 (chemosensory protein CSP), EsigCSP5, EsigOR1 (odorant receptor OR), EsigGR1 (gustatory receptor GR) [28], and EsigGR3 (unpublished data) as key olfactory proteins that bind eucalyptus volatiles. Given that the third instar larvae can recognize constitutive VOCs and HIPVs of eucalyptus at the physiological level, unraveling the genetic basis of this response may show the link between these levels.

Although both adults and larvae are attracted to plant volatiles [4,15,20], adults and larvae behave differently, and detection of odors differ between these two life stages [13,29], as demonstrated in *S. frugiperda* [14]. For boring pests, the attraction to plant volatiles have been reported for adults, such as *Dendroctonus armandi* [30], but reports on larval attraction to plant volatiles have been rare except for third instar *E. signifer* larvae. Furthermore, first and second instar larvae of *Plagiodera versicolor* used volatiles from newly-infested willow leaves by conspecific larvae, as cues for re-aggregation [31], which supported the knowledge that volatiles are involved in the host selection and aggregation of insect larvae. In the present study, we identified volatiles emitted from eucalyptus trunks (non-infested trees, single packet, and multiple packets) and forest floor humus, assessed their attractiveness to third instar *E. signifer* larvae, and analyzed the responses of key olfactory proteins following exposure to these volatiles. Finally, we discuss our findings in the

context of developing volatile attractant-based lures to recruit *E. signifer* larvae for the protection of eucalyptus plantations.

2. Materials and Methods

2.1. Insect Rearing

Third instar larvae were collected from newly infested eucalyptus trees with a small amount of wood chips (Figure 1A) found in the Changke forestry station in the national Guangxi gaofeng forestry station in China (22.907° N, 108.266° E), from June to July 2021 in a *Eucalyptus grandis* × *Eucalyptus urophylla* plantation. After collection all larvae were fed on artificial diet prepared with eucalyptus bark.

2.2. Volatile Collections from Eucalyptus and Forest Floor Humus

Volatile compounds emitted from one-year-old trunks, including non-infested, single packet (one worm packets in one trunk), and multi packets (three worm packets in one trunk) *E. grandis* × *E. urophylla* trees, and forest floor humus were collected July to August 2021 at the Liuli forestry station, in the national Guangxi gaofeng forestry station in China (22.941° N, 108.336° E). Constitutive volatiles from non-infested eucalyptus trunks were collected at a height of 0.8–1.0 m (Figure 1B), which was the part primarily damaged by 3rd instar larvae (unpublished data). Single and multiple packets were selected based on the larval damage heights on the trunks. Three biological replicates (individual trees) were sampled at the same height. All eucalyptus trunk volatiles were collected in vivo and in situ (Figure 1C). The soil volatiles were collected from shallow layers of humus soils (50 mm) in year 1 eucalyptus plantations. Volatiles emitted during the night affect the behavior of nocturnal herbivores [32], since *E. signifer* is a nocturnal pest, sample collections were done from 7:00 p.m. to 7:00 a.m. and three biological replicates were conducted. VOCs from eucalyptus trunk and forest floor humus were collected using dynamic headspace adsorption (DHS). Two 55 × 60 cm jumbo® roasting Bags (Planit Products Ltd., Worcs, England) were used to cover eucalyptus trunks. Eucalyptus trunks were enclosed in the combined bags and sealed tightly with cotton inside, and rubber bands and plastic adhesive tape outside. A pump (QC-1S; Beijing Institute of Labor Instrument, Beijing, China) was used to circulate air through each bag at 300 mL/min. Charcoal-filtered air was introduced into the lower part of the bag, which exited the top via a collection filter that contained 1.0 g of Porapak-Q (200 mg, 80/100 mesh, CNW, Irelan) in a glass column (I.D. 5 mm). All parts of the system were connected by tetrafluoroethylene tubes. Prior to volatile collection, the collection filter was cleansed with high-pressure liquid chromatography (HPLC)-grade n-hexane (Aladdin, Shanghai, China) and conditioned under N_2 flow (ca. 10 mL/min) at 150 °C for 30 min in an oven. Immediately after collection, volatiles were eluted from the trap with 1 mL of n-hexane. Each extract was concentrated to 500 µL under a mild N_2 stream, analyzed by gas chromatography (GC), and stored at 4 °C for later use. Two kg of forest floor humus was put in a roasting bag and sampled using the same methodology as that carried out on eucalyptus trunks.

2.3. Chemical Identification

All volatile treatments were analyzed on an Agilent 7890B-7000D Gas chromatograph (GC) coupled to a 5975C Mass Selective Detector (Agilent MSD 5975, Agilent Technologies, Palo Alto, CA, USA. GC-MS) with electron impact (EI) ionization mode at 70 eV. The GC was equipped with an HP-5 (19091J-413-INT, J&W Scientific, Santa Clara, CA, USA) polyethylene glycol column (0.325 mm diameter, 0.25 µm film thickness, 30 m length). Two µL were injected splitless (250 °C) at an oven temperature of 40 °C (1 min) into the GC followed by heating to 250 °C at a rate of 5 °C/min. The ChemStation Enhanced Data Analysis program was used. Scanning was started after a 180 s solvent delay, and ranged from m/z 40 to 400. Compounds were tentatively identified by comparison of MS spectra obtained from the treatments, with those from the National Institute of Standards and Technology (NIST) mass spectral library (17 library, version B.07.00, Gaithersburg, MD,

USA). Comparison of compound spectra which yielded a match of above 90% quality, were judged as tentatively identified. The peak area was used for quantification of the volatile compounds. All chemical analyses were conducted at the state key laboratory for conservation and utilization of subtropical agro-bioresources.

2.4. Chemicals

Based on the identified chemicals by GC-MS, we purchased their synthetic standards. All chemical purity and supplier of synthetic standards were as follows: alpha-pinene (99%), β-pinene (98%), alpha-phellandrene (99%), d-limonene (99%), benzene, 1,2-diethyl- (97%), naphthalene (99%), eucalyptol (99%), 2-phenyl-2-propanol (97%), butyl acrylate (99%), and camphene (96%) were purchased from Macklin, China; o-cymene (98%), 4-ethylacetophenone (97%) were purchased from Aladdin, China; 3-carene (90%) was brought from Klamar, China; benzene, 1-ethyl-2-methyl- (99%), and n-butyl ether (99%) were purchased from Rhawn, China.

2.5. GC-EAD Electroantennal Detection of Volatile Compounds

GC-EAD analyses of volatile samples were performed using a gas chromatograph (Agilent 7890 B, Agilent, Santa Clara, CA, USA) equipped with a heated outlet for electroantennographic recordings (Syntech, Buchenbach, Germany). Antennal responses of the 3rd instar larvae were measured via electroantennographic detection (EAD). For EAD recordings, the tip of the excised antenna (three replicates) was abscised and the antenna was mounted on an antennal holder using a glass electrode (IDAC4, Syntech, Germany) with normal saline. The antennal holder was connected via a signal interface box to a computer. The temperature programming and carrier gas condition were the same as the GC-MS. The GC was equipped with an HP-5 column (as described above) and a flame ionization detector (FID). Helium was used as carrier gas at a flow of 1 mL/min. A GC effluent splitter was used to direct 50% of the effluent over a heated transfer line (200 °C) into a purified and humidified air stream (250 mL/min) in a stimulus delivery tube (10 mm diameter), which was directed over the excised antenna. EAD and FID signals were recorded simultaneously on a computer using GC-EAD software (GC/EAD, version 1.2.4 or 4.4, Syntech, Germany). Volatiles were assumed EAD-active if reproducible responses at the same retention time were seen in three larval antennae and each with three repetitions. Nine compounds suspected to be GC-EAD active volatile, were confirmed by comparison with a synthetic standard (0.1 g/L, n-hexane solvent) to determine whether their retention times and the response of larval antennae were the same.

2.6. Behavioral Experiments

To assess the attraction of the 3rd instar *E. signifer* larvae, behavioral bioassays were performed in a dynamic Y-tube olfactometer bioassay (glass tube: entrance arm length: 20 cm, test arm length: 15 cm, inner diameter: 2.5 cm, angle: 75 degrees) with specifications as follows. The method followed that used previously for spongy moth caterpillars [33]. The olfactometer experiments were conducted between 20:00 to 24:00 at room temperature (24–26 °C and 60–65% RH) since *E. signifer* larvae are nocturnal. For each formulation, six bioassays were performed using five 3rd instar *E. signifer* larvae, totaling 30 larvae tested for each treatment (N = 30). All tested compounds were prepared in hexane and diluted to 10 g/L. One glass bulb holding a filter paper (1 × 2 cm) loaded with 10 µL of the sample solution served as treatment, while the other glass bulb holding a filter paper disk of the same size and loaded with 10 µL of hexane served as the control. Single larva was placed in a glass Petri dish (6 cm) for ca. 30 min to adapt to the glass environment before the olfactometer tests and were kept in the dark. Each larva was released at the base of the entrance arm of the olfactometer. The number of larvae that entered one of the test arms (7.5 cm) and stayed there for at least 30 s was counted. Larvae that did not reach one of the test arms within 2 min were recorded as "no choice". After the bioassays all tubes and glass olfactometers were cleaned with ethanol (70%) and heated at 230 °C for 2 h.

2.7. Expression of Olfactory Proteins from Exposure to Volatiles

Eight volatiles, which included four GC-EAD active compounds, were selected for this experiment. Exposure of the 3rd instar larvae followed the protocol described by [34]. Twenty-four larvae were placed in a 50 mL jar covered with aluminum foil, in which a glass pipe containing a piece of Whatman filter paper soaked with 50 µL of the odorant diluted at 10 g/L in methanol was placed. Exposure of larvae to methanol only, served as the control. Total RNA from heads of the larvae (nine RNA treatments each with three larval heads) were extracted, the residual genomic DNA in the total RNA was removed using DNase I (Thermo Scientific, Waltham, MA, USA) then purified RNA used to synthesize cDNA following methods described by Zhang [28]. We selected the highly-expressed olfactory proteins (EsigGOBP2, EsigGOBP4, EsigGOBP5, EsigCSP1, EsigCSP5, EsigOR1, EsigGR1 reported by Zhang, [28] and EsigGR3), with their primers and reference genes the same as previously provided. PCR was performed on the Roche LIGHT CYCLE 480II (Salt Lake City, UT, USA). Genious 2X SYBR Green Fast qPCR Mix (No ROX) (No. RK21205; ABclonal, Wuhan, China) was used for the PCR reaction under a three-step amplification. Each PCR reaction was conducted in a 20 µL reaction mixture containing 10 µL of Genious 2X SYBR Green Fast qPCR Mix (No ROX), 0.8 µL of each primer (10 mM), 2 µL of sample cDNA (2.5 ng of RNA), and 7.2 µL of dH2O (sterile distilled water). The real-time fluorescence quantitative PCR (RT-qPCR) cycling parameters were as follows: 95 °C for 180 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s, and 65 °C to 95 °C in increments of 0.5 °C for 5 s to generate the melting curves. Each RT-qPCR reaction for each volatile exposure was performed in three biological replicates and three technical replicates. Negative controls without either template were included in each experiment. Roche LIGHT CYCLE 480II was used to normalize expression based on the ΔΔCq values, with EsigGR3 in alpha.-phellandrene as control samples. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative gene expressions between samples [35].

2.8. Statistical Analysis

The average relative content of the VOCs was calculated based on the percentage of the normalized peak area of the total ion current chromatogram. The comparative analyses for the amounts of each compound emitted by eucalyptus trunk (non-infested, single packet, and multiple packets) and forest floor humus and every gene exposure to volatiles were assessed by a one-way nested analysis of variance (ANOVA), followed by LSD tests implemented in SPSS Statistics 18.0. Before the comparative analyses of expression of olfactory proteins, the data was checked for normality and equal variances. If the data were not normally distributed and their variance not homogenous, a nonparametric Kruskal–Wallis ANOVA was performed to compare amounts of VOCs and expression of olfactory proteins, using the post hoc Dunn's test with Bonferroni's adjustment for mean separation [36]. Heatmap clustering was also performed to illustrate variation in the VOCs across non-infested, single packet, and multiple packet eucalyptus trunks and forest floor humus using ClustVis (https://biit.cs.ut.ee/clustvis/) (accessed on 25 March 2022) [37]. In addition, a principal component analysis (PCA) was performed to determine whether the four treatments could be separated based on the quantitative differences in their VOCs. PCA was performed with R software (Auckland, New Zealand) (Core Team, 2018), using the factoextra Packet (Kassambara and Mundt 2017). The positive or negative correlations between these two variables in the VOCs olfactometer test were determined and their significance was tested by the χ^2 test (Chi-squared) [38].

3. Results

VOCs and HIPVs collected by in vivo and in situ DHS from eucalyptus trunks (non-infested, single packet, and multiple packets) and forest floor humus volatiles were analyzed by GC-MS. Thirty-four compounds were identified, and quantitative differences between treatments were assessed.

3.1. Constitutive VOCs of Non-Infested Eucalyptus Trunks and Forest Floor Humus

Differences in the composition eucalyptus trunks and forest floor humus were observed (Table 1). For non-infested eucalyptus trunks, the most abundant compounds were alpha-pinene, alpha-phellandrene, and n-butyl ether in that order; alpha-pinene, butyl acrylate, and d-limonene were the most abundant compounds from forest floor humus treatments. The compounds common to both forest floor humus and eucalyptus trunks were butyl acrylate, alpha-pinene, d-limonene, o-cymene, 2,3-dimethyl-pentane, 2,4-dimethylhexane, eucalyptol, and nonanal, among which the level of d-limonene in forest floor humus was significantly higher than in non-infested eucalyptus trunks ($p < 0.05$, Table 1). Compounds specific to non-infested eucalyptus trunks were 3,3-dimethyl-6-methylenecyclohexene, n-butyl ether, β-thujene, β-pinene, alpha-phellandrene, 4-ethylacetophenone, and decanal. Benzene, 1-ethyl-2-methyl-, 1,3,5-trimethyl-benzene, naphthalene, toluene, and 1,4-xylene were unique to forest floor humus (Table 1).

Table 1. VOCs identified by dynamic headspace adsorption of the eucalyptus trunk (non-infested, single packet, and multiple packets) and forest floor humus.

Compound Types	Name	CAS	Retention Time	NO. of PCA and GC-EAD	Volatile Abundance (%)			
					Multi-Packets	Single-Packet	Non-Infested Eucalyptus	Forest Floor Humus
Olefin	3,3-Dimethyl-6-methylenecyclohexene	20185-16-4	5.432	1	0.042 ± 0.016	0.046 ± 0.005	0.107 ± 0.031	_
Esters	Isobutyl acetate	110-19-0	3.517	2	0.022 ± 0.022	_	_	_
Ethers	Butyl acrylate	141-32-2	5.978	3	0.025 ± 0.015	0.053 ± 0.008	0.061 ± 0.044	0.203 ± 0.072
	n-Butyl ether	142-96-1	5.703	4	0.046 ± 0.024	0.011 ± 0.011	0.118 ± 0.052	_
Terpene	β-Thujene	28634-89-1	6.718	5	0.022 ± 0.011	0.019 ± 0.111	0.008 ± 0.008	_
	alpha-Pinene	7785-70-8	6.873	6	0.155 ± 0.023 [b]	0.196 ± 0.029 [b]	0.325 ± 0.053	0.355 ± 0.05 [a]
	β-Pinene	18172-67-3	7.857	7	0.016 ± 0.004 [b]	0.021 ± 0.006 [b]	0.044 ± 0.008 [a]	_
	Myrcene	123-35-3	8.222	8	0.002 ± 0.002	_	_	_
	.alpha.-Phellandrene	99-83-2	8.529	9	0.385 ± 0.082 [a]	0.238 ± 0.029	0.133 ± 0.037 [b]	_
	Terpilene	99-86-5	8.833	10	0.001 ± 0.001	_	_	
	D-Limonene	5989-27-5	9.115	11	_	0.030 ± 0.03 [b]	0.023 ± 0.023 [b]	0.132 ± 0.03 [a]
	3-Carene	13466-78-9	9.551	12	_	0.005 ± 0.005	_	_
	γ-Pyronene	514-95-4	10.462	13	0.002 ± 0.002		_	_
	camphor	464-49-3	11.746	14	0.028 ± 0.024	0.027 ± 0.011	_	_
Phenolic compound	Benzene, 1-ethyl-2-methyl-	611-14-3	7.544	15	0.006 ± 0.006	0.020 ± 0.011	_	0.022 ± 0.002
	1,3,5-trimethyl-benzen	108-67-8	8.274	16	0.005 ± 0.005	0.023 ± 0.012	_	0.027 ± 0.003
	o-cymene	527-84-4	9.005	17	0.131 ± 0.061	0.089 ± 0.055	0.041 ± 0.022	0.038 ± 0.001
	Benzene, 1,2-diethyl-	135-01-3	9.596	18	_	0.039 ± 0.039	_	_
	Naphthalene	91-20-3	12.587	19	0.001 ± 0.001	0.002 ± 0.002	_	0.011 ± 0.007
	Toluene	108-88-3	3.443	20	_	_	_	0.013 ± 0.005
	1,4-Xylene	106-42-3	5.41	21	_	_	_	0.096 ± 0.09
	carvacrol	499-75-2	14.049	22	_	0.021 ± 0.021	_	_
Alkane	2,3-dimethyl-Pentane	565-59-3	3.136	23	0.019 ± 0.012	0.006 ± 0.003	0.030 ± 0.015	0.027 ± 0.009
	2,4-Dimethylhexane	589-43-5	3.998	24	_	_	0.042 ± 0.025	0.032 ± 0.007
	Heptane, 2,2,4,6,6-pentamethyl-	13475-82-6	8.505	25	0.007 ± 0.007	_	_	_
Ketone	Acetophenone	98-86-2	10.025	26	0.002 ± 0.002	0.002 ± 0.002	_	_
	4-Ethylacetophenone	937-30-4	14.23	27	0.024 ± 0.013	0.069 ± 0.069	0.024 ± 0.013	_
	5-methyl-5-hexen-3-ol	19780-40-6	3.944	28	_	0.021 ± 0.015	_	_
Alcohol	Eucalyptol	470-82-6	9.136	29	0.034 ± 0.034	0.008 ± 0.008	0.027 ± 0.014	0.041 ± 0.035
	2-Phenyl-2-propanol	617-94-7	10.418	30	0.012 ± 0.012	0.017 ± 0.017	_	_
	4-Isopropylbenzyl Alcohol	536-60-7	13.873	31	_	0.014 ± 0.014	_	_
Aldehyde	Nonanal	124-19-6	10.832	32	0.013 ± 0.009	0.019 ± 0.006	0.013 ± 0.006	0.003 ± 0.003
	Benzaldehyde, 4-ethyl-	4748-78-1	12.159	33	_	0.006 ± 0.006	_	_
	Decanal	112-31-2	13.016	34	_	_	0.004 ± 0.004	_

Note: eleven volatiles with red typeface refer to GC-EAD active compounds; underlined compounds were confirmed with synthetic standards (0.1 g/L, n-hexane solvent) by GC with retention times. _ means compound not detected. Different lowercase letters denote significant differences at $p < 0.05$.

3.2. Herbivore-Induced Plant Volatiles (HIPVs) of Eucalyptus Trunk

The most abundant compounds from injured eucalyptus trunks (both single and multi-packets) were alpha-pinene, alpha-phellandrene, and o-cymene (Figure 2 and Table 1), and alpha-phellandrene from multi-packet trunks was significantly higher than that from non-infested trunks ($p < 0.05$, Table 1). Comparison of non-infested and injured eucalyptus trunks showed that the common compounds included all specific volatiles identified from non-infested eucalyptus trunk as given above (except decanal), as well as all common

compounds of both forest floor humus and eucalyptus trunks (except 2,4-dimethylhexane). Of these, β-pinene from non-infested trunks was significantly higher than that from damaged eucalyptus trunks ($p < 0.05$, Table 1). Furthermore, camphor, 1,3,5-trimethyl-benzene, naphthalene, benzene, 1-ethyl-2-methyl-, acetophenone, and 2-phenyl-2-propanol were unique to injured eucalyptus trunks (both multi and single packet), and 2,4-dimethylhexane and decanal were exclusive to non-infested trunks (Table 1).

Multi-packet trees were common in the plantation; hence, we explored differences between single and multi-packet eucalyptus trunks. The most abundant compounds of both single and multi-packet eucalyptus trunks were also the most abundant compounds from injured eucalyptus trunks. The common compounds included all the above unique compounds of both injured eucalyptus trunks and all common compounds of both non-infested and injured eucalyptus trunks (except d-limonene). In addition, the compounds specific to multi-packet treatments were isobutyl acetate, myrcene, terpilene, γ-pyronene and heptane, 2,2,4,6,6-pentamethyl- (Figure 2 red compounds and Table 1). Compounds unique to single-packet trunks were D-limonene, 3-carene, benzene, 1,2-diethyl-, carvacrol, 5-methyl-5-hexen-3-ol, 4-isopropylbenzyl alcohol, and benzaldehyde, 4-ethyl- (Table 1). The levels of alpha-pinene from forest floor humus were significantly higher than from damaged eucalyptus trunks, and D-limonene was more abundant from forest floor humus than from single-packet and non-infested eucalyptus trunks ($p < 0.05$, Table 1).

3.3. Principal Component Analysis (PCA) of Volatile Treatments

Data for the 34 identified volatiles were evaluated by PCA to determine whether the volatile compounds could be used to discriminate the chemical environment of transfer and the degree of infestation of eucalyptus trunks. Results of PCAs showed the first (horizontal axis) and second (vertical axis) principal components (PCs) explained 40.39% and 18.87%, respectively, of the total variance across treatments in Figure S1 (Supplementary Material S1). PC1 separated the forest floor humus treatment from the eucalyptus tree treatment. The volatile compounds with high positive scores for PC1 included toluene, 1,4-xylene, butyl acrylate, and D-limonene, and were designated as group1 (red ellipse), which showed a high positive correlation with forest floor humus. Volatile compounds with high positive scores for PC2 included decanal and were designated as group2 (turq ellipse), which showed positive correlation with non-infested eucalyptus trunks, and 3-carene, o-cymene, acetophenone, 5-methyl-5-hexen-3-ol, and 2-phenyl-2-propanol were designated as group3 (black ellipse) and correlated positively with damaged eucalyptus (Figure S1).

3.4. Gas Chromatography-Electroantennographic Detection (GC-EAD)

At least 11 reproducible EAD responses in the antennae of third instar larvae of *E. signifer* were observed following exposure to volatiles from eucalyptus trunks (non-infested, single-packet, and multi-packet) and forest floor humus (Figure 2). GC-EAD-active compounds were identified as alpha-pinene, butyl acrylate, n-butyl ether, β-thujene, alpha-phellandrene, d-limonene, 1,3,5-trimethyl-benzene, o-cymene, 4-ethylacetophenone, 2-phenyl-2-propanol, and 3,3-dimethyl-6-methylenecyclohexene (Figure 2). Furthermore, synthetic standards (0.1 g/L for each compound) for all the above identified compounds were confirmed to have electrophysiological activity by GC-EAD (Figure 2), except β-thujene and 3,3-dimethyl-6-methylenecyclohexene, for which no synthetic standards were available.

3.5. Olfactometer Assay

We tested the choice of third instar *E. signifer* larvae to eight GC-EAD-active compounds, five identified volatiles in this study and camphene (new volatile substance of 5th instar larval period). Among the GC-EAD-active compounds, the behavior choice ratio in o-cymene was significantly higher than control ($\chi^2 = 6.76$, df = 1, $p = 0.009$) (Figure 3), and alpha-phellandrene ($\chi^2 = 3.24$, df = 1, $p = 0.072$), d-limonene ($\chi^2 = 1.20$, df = 1, $p = 0.273$), 2-phenyl-2-propanol ($\chi^2 = 1.20$, df = 1, $p = 0.273$), 4-ethylacetophenone ($\chi^2 = 0.571$, df = 1, $p = 0.450$), alpha-pinene ($\chi^2 = 0.037$, df = 1, $p = 0.847$), n-butyl ether ($\chi^2 = 0.00$, df = 1,

$p = 1.000$), butyl acrylate ($\chi^2 = 0.133$, df = 1, $p = 0.715$), naphthalene ($\chi^2 = 2.79$, df = 1, $p = 0.705$), benzene, 1-ethyl-2-methyl- ($\chi^2 = 0.615$, df = 1, $p = 0.433$), benzene, 1,2-diethyl- ($\chi^2 = 0.615$, df = 1, $p = 0.433$), 3-carene ($\chi^2 = 0.048$, df = 1, $p = 0.827$), β-pinene ($\chi^2 = 0.143$, df = 1, $p = 0.715$), and eucalyptol ($\chi^2 = 0.044$, df = 1, $p = 0.835$) were observed. Interestingly, the third instar larvae were significantly attracted to camphene ($\chi^2 = 5.54$, df = 1, $p = 0.019$) (Figure 3), and the behavior choice of crude extract of multi ($\chi^2 = 4.48$, df = 1, $p = 0.034$) and single packets ($\chi^2 = 4.17$, df = 1, $p = 0.041$) was significantly stronger than control, but those from non-infested trunks ($\chi^2 = 1.20$, df = 1, $p = 0.273$) and soil ($\chi^2 = 2.79$, df = 1, $p = 0.095$) were not (Figure 3).

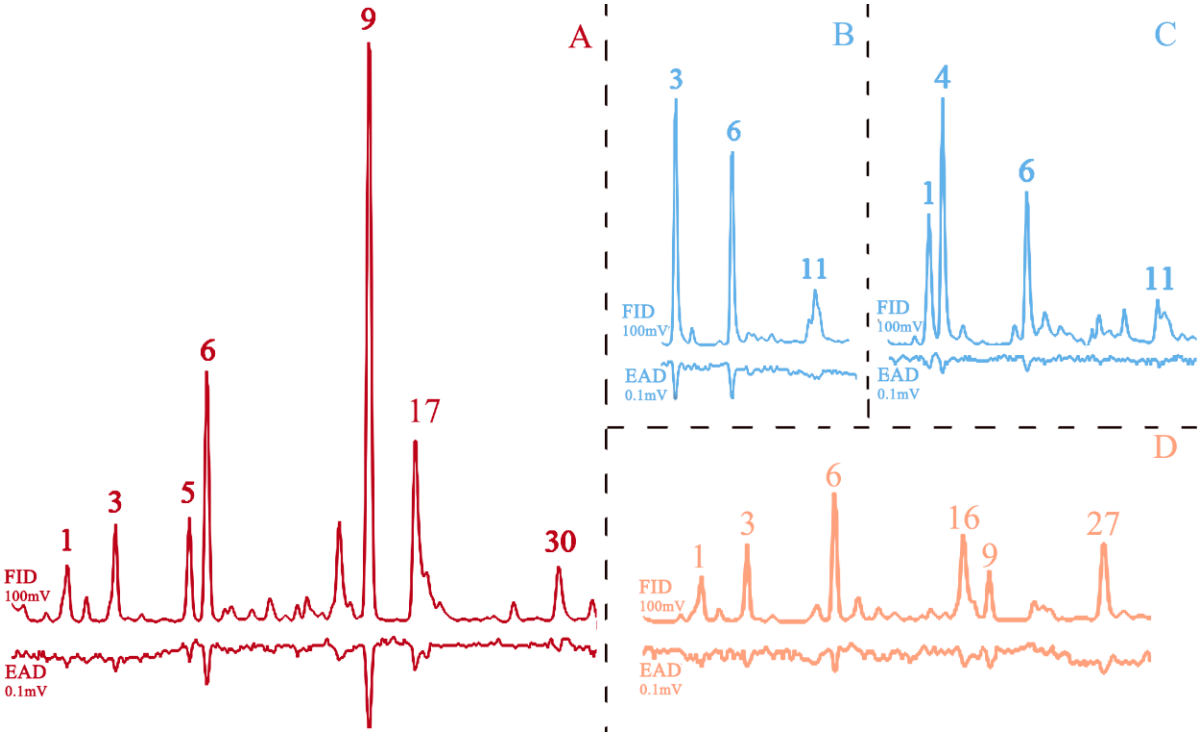

Figure 2. GC-EAD record of eucalyptus trunk and forest floor humus volatiles. **A**: multi packets; **B**: forest floor humus; **C**: non-infested trunk; **D**: single packet. Peak numbers are the same as Table 1.

3.6. Expression of Olfactory Proteins from Exposure to Important Volatile

The expression of EsigGOBP2 was up-regulated by exposure to camphene, naphthalene, and eucalyptol ($p < 0.05$, Figure 4). The expression of EsigGOBP4 was up-regulated by exposure to camphene and naphthalene, ($p < 0.05$, Figure 4), and the expression of EsigGOBP5 was also up-regulated following exposure to camphene and eucalyptol ($p < 0.05$, Figure 4). The expression of the chemosensory protein, EsigCSP1 was up-regulated by exposure to camphene. The expression of EsigCSP5 was up-regulated after exposure to five volatiles (camphene, naphthalene, eucalyptol, n-butyl ether, and 4-ethylacetophenone), ($p < 0.05$, Figure 4). Conversely, the expression of the olfactory receptor EsigOR1 was down-regulated following exposure to o-cymene and n-butyl ether, ($p < 0.05$, Figure 4). The expression of EsigGR3 was up-regulated by exposure to camphene ($p < 0.05$, Figure 4) but was down-regulated by exposure to o-cymene, α-phellandrene, n-butyl ether, and 4-ethylacetophenone ($p < 0.05$, Figure 4). The expression of EsigGR1 was not affected by exposure to all eight volatiles. Additionally, the expression of other olfactory proteins from exposure to other volatiles were not significantly different.

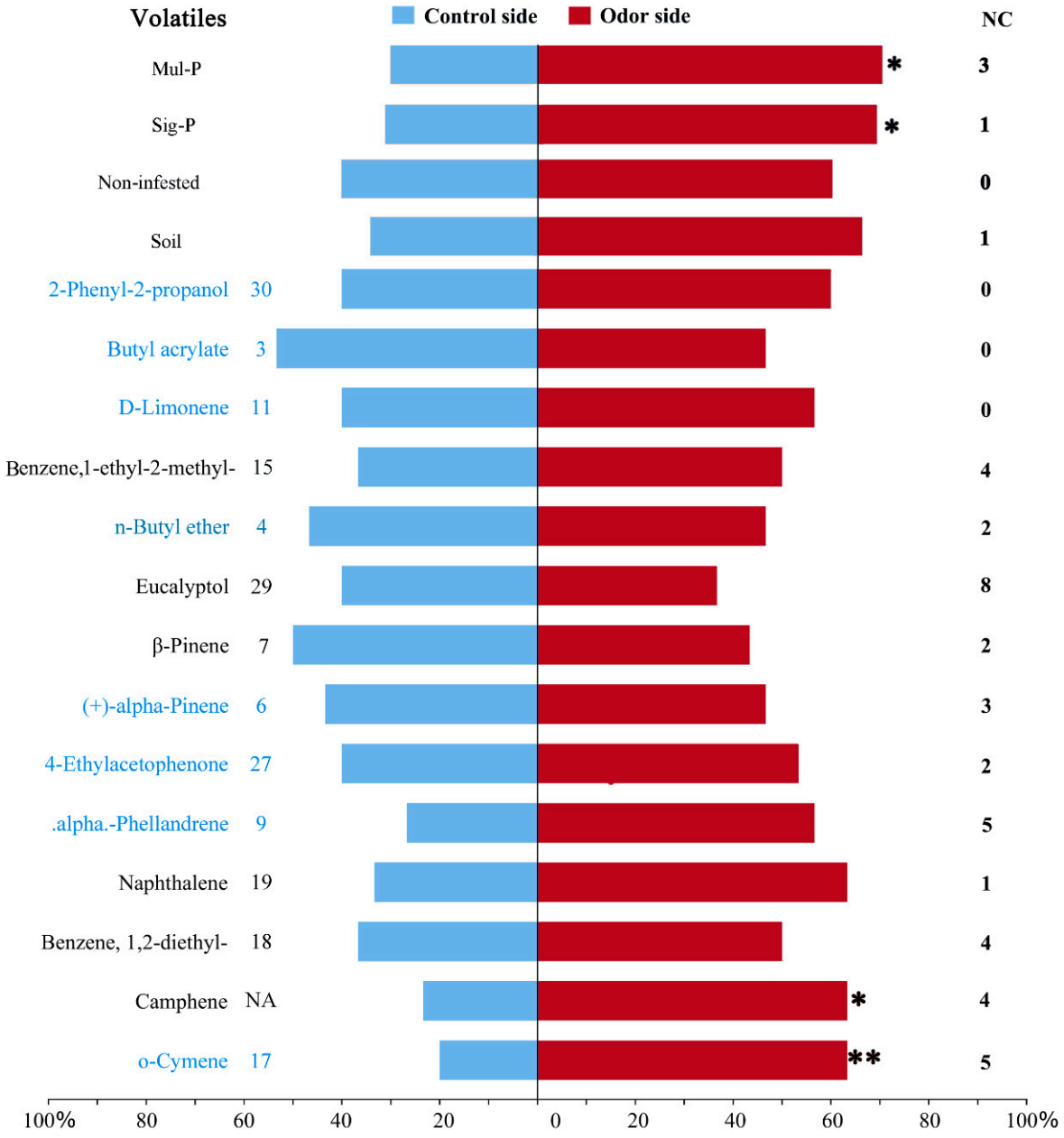

Figure 3. Y-tube olfactometer bioassay of eucalyptus trunk and forest floor humus volatiles. NC means no choice, blue volatiles were GC-EAD-active compounds, * denotes significant differences at $p < 0.05$, ** denotes significant differences at $p < 0.01$. Numbers beside compounds are the same as Table 1. NA means no compound in Table 1.

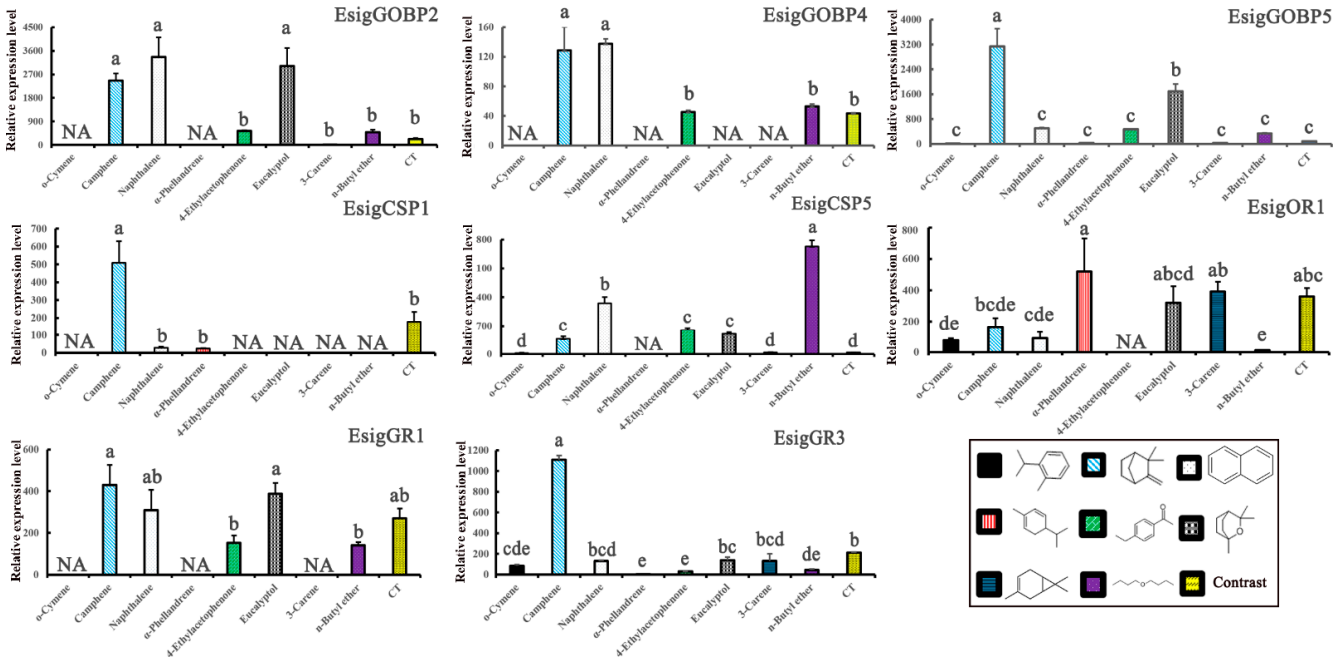

Figure 4. Expression profile (mean + SE) of *E. signifer* larvae after exposure to volatiles. NA: no expression. Different lowercase letters above the bars denote significant differences at $p < 0.05$.

4. Discussion

Third instar larvae of the polyphagous wood-boring pest *E. signifer* transfer from forest floor humus to eucalyptus in a precise manner and often in aggregation (Figure 1B). We explored their attraction to non-infested and larvae-damaged eucalyptus trunks and the chemical environment variation in forest floor humus from where they transfer. A total of 34 compounds were identified, among which we assayed 14 compounds, 2 of which (camphene and o-cymene) were attractive to third *E. signifier* larvae. This study supports the view that polyphagous larvae largely depend on plant olfactory cues to locate food [39,40].

4.1. Main VOCs and the Movement of third Instar E. signifer Larvae

A significant effect on the movementof larvae wasonly observed to one compound. Interestingly, third instar larvae did orient to the single and multiple packets treatments. This indicated that the selection behavior of the larvae largely depends on volatile blends, especially from infested trees. This in turn indicated the need to first identify, the specific compounds that form the blend. We first identified the constitutive volatiles of non-infested eucalyptus trunks and forest floor humus, which represents important components of the varied chemical environment experienced by third instar larvae of *E. signifer*. Our results show that alpha-pinene, alpha-phellandrene, n-butyl ether, butyl acrylate, and d-limonene are common volatiles in both non-infested eucalyptus trunks and forest floor humus. Alpha-pinene was one of the main volatiles identified from pine trees, olfactometer bioassays and field experiments, which showed an enhanced ability to lure pine beetles [41]. Therefore, it can form part of the attractant volatile blends for attracting the third instar larvae of *E. signifer*. Alpha-phellandrene was one of the three most abundant compounds detected in eucalyptus trunk treatments (but not forest floor humus), and its levels in multi-packet trunks were higher than in non-infested trunks ($p < 0.05$). GC-EAD results showed that it may be a main functional compound in eucalyptus, especially released from multiple packets to aggregate conspecific larvae. Alpha-phellandrene showed no significant effect in the behavior experiments; the addition of other volatiles may be necessary to attract third instar larvae of *E. signifer*. It has been used as the main compound of a food bait for *Zeugodacus cucurbitae* females [42], and elicited EAG responses (regardless of concentration)

in *D. armandi* females [30]. D-limonene was detected by GC-EAD and was significantly higher in the forest floor humus than from non-infested and one packet eucalyptus trunks ($p < 0.05$), indicating that it may be one of the main functional compounds in blends that determines larval orientation to already infested trees. It was also identified as a major volatile of citrus, which attracted pests and parasitoids. Moreover, field experiments suggested that it increased parasitism of *Aonidiella urantia* following a decrease in the amount of VOCs [43]. n-Butyl ether may also form part of the attractant volatile blends for the attraction of the third instar larvae of *E. signifer*, as it was identified as one of the main volatile compounds in non-infested eucalyptus trunks, was GC-EAD active. In addition, it was reported to attract *Adelphocoris suturalis* adults in field trial experiments and be EAG active [44].

Behavioral responses to VOCs from plants do not always mirror electrophysiology results in some insects, such as for (Z)-3-hexenyl acetate, (Z)-3-hexen-1-ol, and (Z)-β-ocimene [45]. For example, butyl acrylate elicited an electrophysiological response in third instar larvae of *E. signifer*, but showed no attraction in behavioral tests. Butyl acrylate was identified as an electro-physiologically active volatile of *A. lucorum* adult [46] but acted as an attractant to *Harmonia axyridis* adult in both behavior and field tests [47].

Among the six common compounds detected from forest floor humus and eucalyptus trunks, the larvae responded to eucalyptol in the GC-EAD experiment. Eucalyptol was found to strongly reduce pheromone attraction and inhibited the activation of the pheromone olfactory sensory neurons (OSN) through activation of a co-localized OSN [48] in bark beetles. It was also reported to have shown repellency against foraging ants under field conditions [49].

4.2. Volatiles from Non-Infested and Infested Eucalyptus Trunks

O-cymene, alpha-phellandrene, and alpha-pinene were highly abundant compounds identified from infested eucalyptus trunks. Interestingly, larvae were onlysignificantly attracted to o-cymene in olfactometer bioassays ($p < 0.01$) and o-cymene and was one of the three most abundant compounds emitted from injured eucalyptus trunks (single- and multi-packet). Moreover, PCA analysis revealed that it positively correlated with injured eucalyptus. These results suggested that o-cymene is a major attractant to the third instar *E. signifer* larvae, released by injured eucalyptus trunks. Similarly, o-cymene was an attractant to *Plagiodera versicolora* adults [50]; it was identified in conspecific-infested tomato plants [51] and was one of the most EAD-active components and one of the seven compounds constituting the behaviorally-active blend for both sexes of *Zeugodacus cucurbitate* [52]. In addition, o-cymene has been reported as a toxin to *Bemisia tabaci* [53] and showed antioxidant and antimicrobial properties in *Citrus acida* against bacteria [54].

Besides the common compounds identified from non-infested and infested eucalyptus trunks, 4-ethylacetophenone was also detected in the GC-EAD experiments. Previous reports showed that 4-ethylacetophenone served as an attractant for insects; for instance, it induced the expression of the ApisOR4/Orco in the *Xenopus* system and elicited EAG responses in *Acyrthosiphon pisum* [55] and, as an HIPV, attracted *Peristenus spretus* and improved the parasitism rate of *Apolygus lucorum* [56]. Although β-pinene was one of the common compounds identified from non-infested and infested eucalyptus trunks, it did not influence the selection behavior of *E. signifer* larvae. Further, its concentration decreased with increasing larval packets, with a significant difference between non-infested and infested eucalyptus trees ($p < 0.05$). It has been previously reported as repellent for *A. lucorum* adults [57]. The functions of β-thujene and 3,3-dimethyl-6-methylenecyclohexene were not evaluated because their standards were not available.

The characteristic compounds of damaged eucalyptus trunks were 1,3,5-trimethyl-benzene, 2-phenyl-2-propanol, acetophenone, camphor, naphthalene, benzene, and 1-ethyl-2-methyl- and acetophenone. Only 1,3,5-trimethyl-benzene and 2-phenyl-2-propanol elicited electrophysiological response in *E. signifer* larval antennae. There are currently no reports on the functions of 2-phenyl-2-propanol in other insects. However, it was

reported to be a primary metabolite of cumene, which is frequently used in industrial applications and also found in the environment, cigarette smoke, and food [58]. The effects of 2-phenyl-2-propanol on insects therefore requires further study.

4.3. HIPVs from Single and Multiple Infestation Trunks

In principle, infested plants emit HIPVs in a dose-dependent manner; the greater the number of herbivores, the more HIPVs are emitted [20,59]. We explored the difference in HIPVs in terms of degree of infestation of eucalyptus trunks, i.e., between single- and multi-packet-infested trunks. There was dynamic volatile emission from eucalyptus trunks due to the different levels of *E. signifer* infestation. A similar observation was also recorded from the trunks of *P. orientalis* [18,19]. Compared to single-packet-infested trunks, multi-packet-infested eucalyptus trunks emitted five specific compounds, but none elicited larval electrophysiological response. Among the seven unique compounds identified from single-packet trunks, benzene, 1,2-diethyl-, and 3-carene influenced the selection behavior of larvae but did not elicit electrophysiological responses in the antennae; therefore, the functions of these compounds need to be investigated further.

Notably, volatiles from single- and multi-packet-infested eucalyptus trunks may be HIPVs due to *E. signifer*, or from feces, wood parts, silk, and other symbiotic organisms. For example, acetophenone, p-cymene, m-cymene, and o-cymene were identified from the frass of *Opisina arenosella* [60]. A previous study showed that fourth instar larvae of *S. littoralis* were attracted to the frass volatile guaiacol (2-methoxyphenol) [26]. Additionally, feces from conspecific caterpillars were sufficient to deter female *M. sexta* from ovipositing; this deterrence was based on feces-emitted carboxylic acids 3-methylpentanoic acid and hexanoic acid [25]. Thus, further exploration of volatiles from different parts of packets is need to understand the associated relationships.

4.4. Response of Olfactory Proteins to Exposure to Volatiles

Insect OBPs can be used for screening potential active compounds which influence insect behavior [27]. Our results showed that the binding proteins (CSPs + OBPs) in *E. signifer* larvae were all up-regulated after exposure to volatiles. All five binding proteins showed up-regulation from exposure to camphene; EsigGOBP2, EsigGOBP4, and EsigCSP5 to naphthalene; EsigGOBP2, EsigGOBP5, and EsigCSP5 to eucalyptol; and EsigCSP5 to 4-ethylacetophenone and n-butyl ether. Similarly, several OBPs were reported to have been upregulated in *Holotrichia oblita,* from exposure to (E)-2-hexenol [61] and also of pheromone binding proteins involved in behavior modulation of moths [62], e.g., in *S. exigua* larvae [34] and *S. littoralis* [63]. Our results showed that the attraction of *E. signifer* larvae to 4-ethylacetophenone and n-butyl ether correlated with the up-regulation of EsigCSP5. However, o-cymene and α-phellandrene did not induce any significant difference in the expression of binding proteins. Notably, eucalyptol did not elicit GC-EAD recording nor influence avoidance behavior in choice bioassays but caused the up-regulation of three genes. Additionally, naphthalene and camphene, which showed attractive properties, up-regulated all genes. This indicated that the three compounds may be important compounds in the olfactory response of third instar *E. signifer* larvae. The olfactory receptors, EsigOR1 and EsigGR3 were down-regulated from exposure to o-cymene and n-Butyl ether; EsigGR3 was also down-regulated by exposure to α-phellandrene and 4-ethylacetophenone. It has been reported that odorants induce a fast and reversible concentration-dependent decrease in the transcription of genes corresponding to activated receptors in intact mice [64]. Interestingly, in some cases of *Drosophila,* the exposure of a chemosensory receptor to high concentration of its best ligand led to measurable alterations in mRNA levels, but this was not the same as that observed in mice [65]. The up-regulation of EsigGR3 from the exposure to camphene was similar to the functional characterization of the expression of SlitOR40 in first instar larvae from the exposure to β-caryophyllene and α-humulene [66]. The up- and down-regulation of these receptors suggested that they may be involved in the detection of semiochemical stimuli. Further, the expressions of the receptors after exposure

to these important volatiles were consistent with the physiological result that showed that o-cymene, α-phellandrene, n-butyl ether, and 4-ethylacetophenone were olfactory response-active compounds, and camphene influenced the choice behavior of larvae.

5. Conclusions

We studied the olfaction response of the third instar larvae of the polyphagous wood-boring pest *E. signifer* to volatiles emitted from eucalyptus trunks and forest floor humus. The key volatiles involved in olfactory functions in the larvae were identified among 34 compounds. O-cymene and camphene showed attraction. Alpha-pinene, alpha-phellandrene, 4-ethylacetophenone, d-limonene, 2-phenyl-2-propanol, and n-butyl ether were attractive candidates. These can be developed into attractant volatile blends for improved attraction of third instar *E. signifer* larvae. Future studies should evaluate the attraction of these seven compounds, using different mixture ratios, which can then be developed into field traps for the attraction of *E. signifer* larvae in eucalyptus plantations. Additionally, the functions of β-thujene, 1,3,5-trimethyl-benzene, and 3,3-dimethyl-6-methylenecycloh- exene should highlighted in future studies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f13122058/s1, Figure S1: Principal component analysis (PCA) of volatiles identified by dynamic headspace adsorption on the eucalyptus trunk and shallow soil.

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Institutional Review Board Statement: The ghost moth *E. signifer* is a forestry pest in China and is not included in the "List of Endangered and Protected Animals in China". All operations were performed according to ethical guidelines in order to minimize pain and discomfort to the insects.

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Conflicts of Interest: The authors declare no conflict of interest.

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