Chemoecology
https://doi.org/10.1007/s00049-024-00409-2

ORIGINAL ARTICLE

How to chew gum: the post-ingestion fate of foliar secondary compounds consumed by a eucalypt herbivore

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Received: 6 June 2024 / Accepted: 9 July 2024

Abstract

Herbivorous insects require mechanisms to deal with defence compounds produced by their host plants. Despite an array of secondary compounds associated with defence, eucalypts are hosts to many insect species that readily obtain nutrients also produced by these plants. *Gonipterus* weevils are foliage-feeding eucalypt specialists as larvae and adults, with a notable characteristic of protecting their eggs with a hardened frass-like substance. The aim of this study was to assess plant, weevil frass and egg capsule chemistry to determine how the weevil eliminates plant secondary metabolites. We hypothesised that noxious compounds would be metabolised prior to elimination and that egg capsules would be composed of frass and additional substances. Weevils were fed on *Eucalyptus globulus* plants for seven days, with their frass and egg capsules collected daily, and the damaged, first, fully-expanded leaves of the host collected at the end of the assay. Compounds present in each sample were extracted in hexane and analysed by gas chromatography-mass spectrometry. The most abundant compounds in each sample were waxes and terpenoids, and metabolism of 1,8-cineole was evident, with two metabolites that may have semiochemical activity. Comparative analysis revealed significant differences between all samples, with shared compounds varying in relative proportions and exclusive compounds in sample type. These findings contribute to the understanding of *Gonipterus* physiology and highlight the differences between frass and the cover of egg capsules.

Keywords Plant secondary metabolites · Egg capsule · Detoxification · 1,8-cineole · Aliphatic β-diketone

Introduction

Plants are the primary source of food for a large number of insect species that rely on this abundant resource to obtain essential nutrients, among them amino acids, lipids, carbohydrates and other growth factors (Chapman 1998). Countering this, plants have developed different strategies to reduce or avoid herbivory. For example, plants produce a variety of chemical compounds with repellent, antifeedant, antinutritive or toxic activity against target organisms, forming part of constitutive or induced defence (Furstenberg-Hagg et al. 2013).

Eucalypts are rich in a diversity of secondary metabolites many of which are used by the pharmaceutical, cosmetic, agricultural and food industries (Barbosa et al. 2016; Brophy and Southwell 2002). As well as these volatile and non-volatile metabolites, heavier compounds such as the waxes covering the epicuticular surface of leaves play a role in resistance against insect herbivores (Allen et al. 2004; Edwards 1982). Despite the complex chemical composition of eucalypts, an abundant vertebrate and invertebrate fauna is associated with these plants (Abbott et al. 1992; Bennett 2016). Many studies have addressed the mechanisms insects use to deal with eucalypt compounds including physical removal of leaf oil glands (Schmidt et al. 2010), detoxification pathways of terpenoids (Southwell et al. 2003), and

Communicated by Günther Raspotnig.

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Published online: 18 July 2024

storage and use for defence (Després et al. 2007; Morrow et al. 1976).

Gonipterus (Coleoptera: Curculionidae), is a genus of eucalypt-feeding weevils found along the east coast of mainland Australia and Tasmania on a diversity of *Eucalyptus* species (Clarke et al. 1998; Mapondera et al. 2012). Some species of *Gonipterus* are invasive in many parts of the world, causing economic impact in commercially grown plantations (Schröder et al. 2020), thus increasing the need for understanding its biology and relationship with different *Eucalyptus* species and natural enemies. An interesting characteristic of this genus is that females protect their eggs with a dark matter that hardens when dry, forming an egg capsule (egg case) that is laid on the surface of young eucalypt leaves. This egg capsule has been described as comprising a dark, gelatinous material, frass (excrement), or anal secretion by different authors (Oberprieler et al. 2014; Santolamazza-Carbone and Rivera 2003; Tooke 1955) but to date, its nature has not been elucidated.

Although some chemical ecology studies have investigated relationships between *Gonipterus* and their hosts, they have mostly addressed the attractiveness and repellency of plant- or insect-borne compounds to the weevils, in attempts to aid management strategies (Bouwer et al. 2014; Branco et al. 2018, 2020; Joubert et al. 2023; Mendes et al. 2022), while the metabolism and use, detoxification or discharge of plant compounds remains completely unexplored. In this study, we examined *Gonipterus* sp. n. 2 (*sensu* Mapondera et al. 2012), a species invasive in Western Australia, South Africa and parts of Europe to understand its relationship with *Eucalyptus globulus*, widely planted abroad and within Australia. We assessed host plant chemistry in comparison to two weevil-originated products - the frass and the cover of their egg capsule - to determine how these insects use plant compounds during herbivory. We hypothesised that noxious compounds would be metabolised prior to elimination and that egg capsules would be composed of frass added with other substances.

Methods and materials

Insect collection and rearing

Adult *Gonipterus* sp. n. 2 were collected in commercial plantations of *Eucalyptus globulus* and *Eucalyptus smithii* in southern Western Australia (WA) between Bunbury and East Nannup (approximately 33°19’12.0"S 115°38’24.0"E; 34°03’36.0"S 115°47’24.0"E), in October 2017. This species occurs throughout the east coast of Australia (Schröder et al. 2021) and is an invasive pest in WA (Loch 2006; Loch and Matsuki 2010; Mapondera et al. 2012) where populations are high enough to enable collection of large numbers of individuals. The weevils were taken to the Eco-Sciences Precinct, Dutton Park, Queensland (QLD, Australia) where they were kept in cages in rearing cabinets at 20 °C and 14 h photoperiod and fed branchlets of flushing *Eucalyptus dunnii* foliage for at least two weeks prior to the trial.

Plants

Seeds of *E. globulus* subsp. *globulus* “Geeveston trial F2 Bulk from orchard” were obtained from the Department of Agriculture and Fisheries, Forest Technologies Seed Store, Gympie, QLD, sown in small acrylic pots (70 cc), thinned after germination and transferred to 1.65 L pots once they reached approximately 15 cm. They were kept in a shade house throughout their development until they were 5 months old, when they reached adequate size to be used in the trials.

Experiment

Weevils used in the experiment were transferred from their *E. dunnii* rearing hosts into cages (60 cm height × 37.5 cm diameter) containing an *E. globulus* seedling and kept in a controlled environment room (CER) for one week at 20 °C, 70% RH and 14 h photoperiod. This first step was added to acclimatise the insects to the experimental conditions and to ensure that no *E. dunnii* residue was present in their digestive tract. For the experiment, foliage of new *E. globulus* seedlings was caged in a mesh bag of approximately 1 m height, the base consisting of a tray (38 × 29 cm) lined with white paper to facilitate frass collection (Fig. 1). Each caged plant received three male and seven female acclimatized adult weevils, with a total of six repeats conducted as two repeated sets of three under the same conditions. Egg capsules and frass produced in each cage were carefully collected daily for seven days and placed into pre-weighed glass vials (2 mL), each vial containing all eggs or all frass pellets from each plant. These were weighed (to nearest 0.1 mg) and extracted in 100 µL hexane per 10 mg of sample on each collection day. Samples were extracted for 30 min, shaken in vortex for 10 s at 10 min intervals. The extracts were collected into new glass vials closed with screw-caps with PTFE/RS septa and stored at -20 °C until analysis. At the end of the 7-day feeding period 5–6 damaged first fully-expanded leaves from each seedling were collected. Leaves were cut into small 5 × 10 mm sections and placed into Erlenmeyer flasks for extraction, at the proportion of 5 mL hexane per gram of sample and extracted and stored as above.

Fig. 1 Experimental set-up showing mesh cages used to contain *Gonipterus* weevils onto seedlings of *Eucalyptus globulus* allowing daily collection of frass and egg cases

Fifty to 60 µL aliquots of egg capsules and frass extracts from five collection days per repeat were combined to provide a single 250 to 300 µL sample for analysis. One repeat of egg capsule extract was excluded due to insufficient volume. The resulting blends and 30 µL aliquots of the leaf extracts were evaporated to dryness under a gentle nitrogen gas flow at 20 °C and re-suspended in hexane in one-tenth of the volume of the original extracts. A subsample of the original leaf extracts was kept prior to concentration (see Online resource 1 for work-flow diagram).

GC-MS analysis

Profiles of the compounds present in the extracts were obtained by analysing the samples (1 µL) using a gas chromatograph (GC) (Agilent 6890 Series) coupled to a mass spectrometer (MS) (Agilent 5975) and fitted with a silica capillary column (Agilent, model HP5-MS, 30 m × 320 µm ID × 0.25 µm film thickness). Data were acquired under the following GC conditions - inlet temperature: 250 °C, carrier gas: helium at 51 cm/s, split ratio 13:1, transfer-line temperature: 280 °C, initial temperature: 40 °C, initial time: 2 min, rate: 10 °C/min, final temperature: 300 °C, final time: 7 min. The MS was held at 230 °C in the ion source with a scan rate of 3.12 scans/s. Leaf extracts (original and concentrated) as well as the concentrated extracts from egg capsules and frass were all analysed by the same method. A blend of standard aliphatic hydrocarbons (C9 to C40, except C34) (Sigma®, St. Louis, Missouri, USA) was run in the same conditions for determination of Kovats retention indices (KI) of compounds in each sample using the following formula (van Den Dool and Kratz 1963):

$$KI = 100 \times \left[n + (N - n) \frac{t_{r(target\ compound)} - t_{r(n)}}{t_{r(N)} - t_{r(n)}} \right]$$

where: n = the number of carbon atoms in the smaller n-alkane;

N = the number of carbon atoms in the larger n-alkane;

t_r = the retention time.

Data analysis

To account for loss of more volatile compounds, such as monoterpenoids, during concentration, the average loss in area of the peaks from the leaf extract chromatograms pre and post concentration was calculated. These were plotted and the trend line equation was obtained for the first 15 min of the run (area = 0.2562 × ln(RT) + 0.2815; $R^2 = 0.69$), after which the areas had no appreciable loss (Online resource 1). The equation was applied to the areas of peaks of all concentrated samples up until the 15 min mark to adjust for loss. The presence of peaks was assessed and compounds present in at least 50% of the repeats of at least one sample were included in analysis. The relative corrected areas of remaining peaks were 4th root transformed and analysed by nonparametric methods (*Bray-Curtis cluster analysis* and *non-metric multidimensional scaling* (*nMDS*) ordination (Clarke 1993; Clarke et al. 2014) to ascertain whether differences could be detected among the samples. *Analysis of similarity* (*ANOSIM*) was used to determine difference among samples. *Similarity percentages* (*SIMPER*) analysis was performed to determine which compounds were the most important in contributing to any differences between samples, and to assess similarity between repeats within sample. The software used for multivariate analyses was Primer 7 (V 7.0.13, PRIMER-e).

Tentative identification of relevant compounds was carried out by comparison with NIST (2005) reference library (≥ 90%), analysis of their mass spectra, presence of

diagnostic ion fragments and comparison of their Kovats retention indices with literature (Adams 2007; Babushok et al. 2011; Branco et al. 2020; NIST 2005). Details of each tentative identification and spectra of undetermined compounds are presented in Online resource File 2 and 3, respectively.

Results

One hundred and twenty-eight organic compounds were present in at least 50% of samples per treatment and selected for analysis. Thirty-nine were detected in all three treatments while some compounds were treatment-specific (Fig. 2). Among leaves, frass and egg capsules, high molecular weight compounds such as waxes and sterols were present in high relative amounts (Table 1). Within those, tritriacontane-16,18-dione was abundant in all three treatments, representing 16.6 to 30% of the whole peak area. In leaf extracts 1,8-cineole was found in large amounts but was replaced by 2-α-hydroxy-1,8-cineole in weevil frass, a metabolite also found in egg capsule extracts. Although present in all treatments, α-pinene, was detected in high relative concentration in egg capsules, where only a few compounds were present. In each of the treatments, the five most abundant compounds represented over 50% of the total peak area of the chromatograms.

Analysis of similarity revealed that damaged leaves, frass and egg capsules were significantly different from each other (Global $R = 0.951$, $P = 0.001$) and the resulting nMDS shows clear separation of clusters according to these samples (Online resource 4). SIMPER analysis indicated that 59 compounds contributed to the differences between samples on a 50% cut off, those are presented (Table 1). In leaf samples, most of the exclusive compounds eluted late and are likely to be components of the cuticle (RT 25.66 to 31.60, KI > 3004).

Between samples of leaves and weevil frass, the remarkable shift in proportion of 1,8-cineole and its metabolite 2-α-hydroxy-1,8-cineole (Fig. 3) was reflected in the

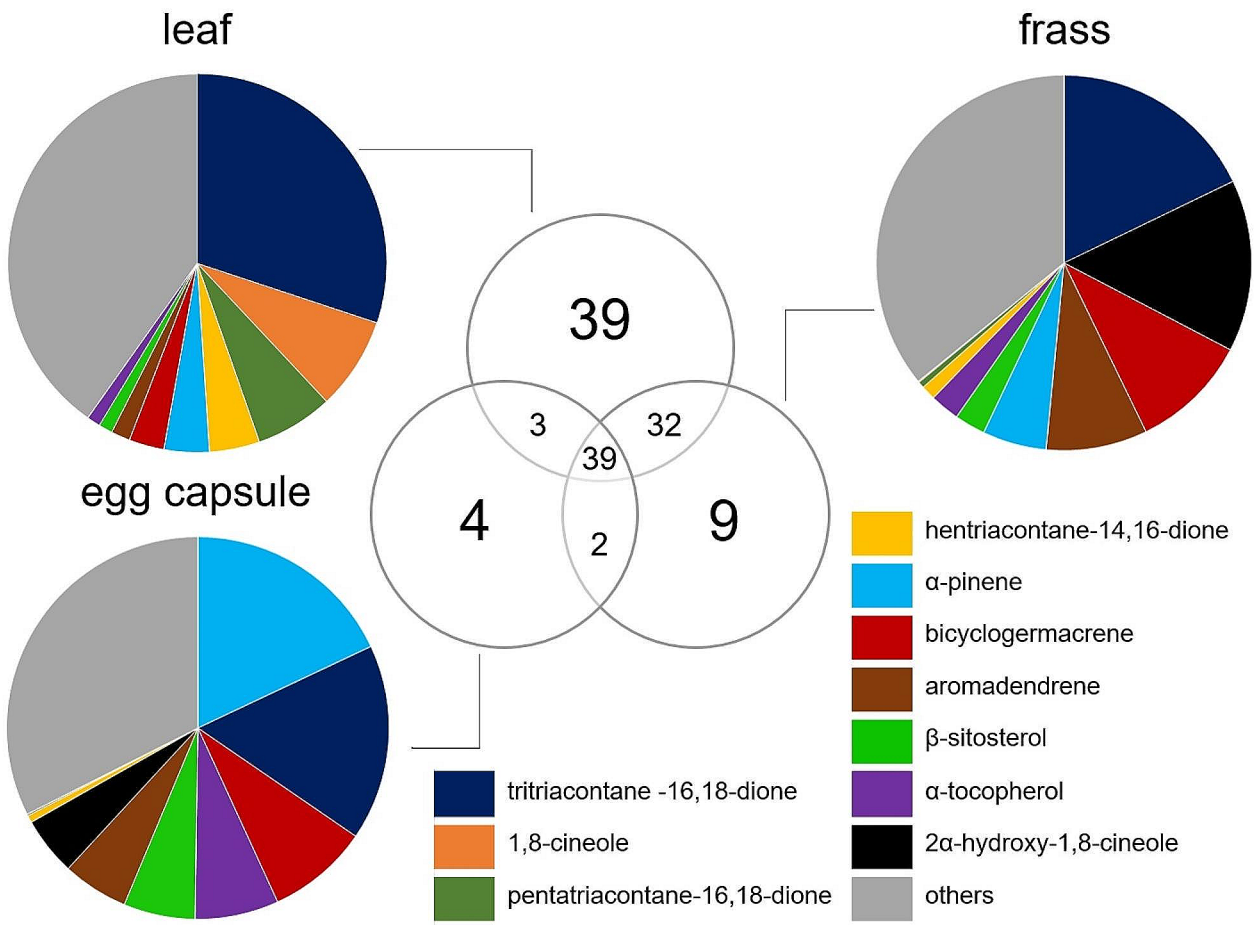

Fig. 2 Venn diagram of exclusive and shared compounds of *E. globulus* damaged leaves, *Gonipterus* sp. n. 2. frass and egg capsules and pie charts of main compounds presented in each sample

Table 1 Retention times (RT), Kovats retention index (KI), tentative identification, average abundance and contribution to pairwise differences of compounds contributing to the top 50% Of dissimilarities found on simper analysis (marked in bold) of *Eucalyptus globulus* and *Gonipterus* sp. n. 2 samples

			Average abundance (%)[a]			Contribution pairwise (%)		
RT	KI	Identification	Leaves ($n=6$)	Frass ($n=6$)	Egg capsules ($n=5$)	Leaves × Frass	Leaves × Egg capsules	Frass × Egg capsules
4.66	934	α-pinene	3.86 ± 0.69	5.51 ± 3.14	17.99 ± 3.26	0.70	0.99	**1.94**
5.37	977	β-pinene	0.48 ± 0.08	0.45 ± 0.29	2.18 ± 0.44	0.55	0.56	**1.47**
5.68	996	β-myrcene	0.20 ± 0.06	-	0.59 ± 0.61	**1.06**	0.84	1.15
5.86	1007	α-phellandrene	0.45 ± 0.17	0.25 ± 0.32	0.30 ± 0.67	**0.98**	**1.03**	1.06
6.27	1033	1,8-cineole	7.93 ± 1.95	0.09 ± 0.12	0.15 ± 0.35	**2.33**	**2.10**	0.88
9.31	1236	2α-hydroxy-1,8-cineole	0.02 ± 0.02	14.83 ± 5.45	4.94 ± 3.17	**2.79**	**1.54**	**2.20**
9.68	1263	7-hydroxy-1,8-cineole	-	1.40 ± 0.62	-	**1.48**	0.00	**2.36**
12.07	1447	aromadendrene	1.62 ± 0.42	8.72 ± 1.69	5.58 ± 1.57	**1.02**	0.65	0.84
12.22	1459	β-vatirenene isomer	0.15 ± 0.04	0.38 ± 0.16	-	0.66	0.88	**1.44**
12.47	1480	undetermined sesquiterpene A	0.03 ± 0.01	1.93 ± 0.86	-	**1.21**	0.51	**2.15**
12.51	1483	β-vatirenene isomer	0.07 ± 0.02	1.65 ± 1.96	-	**1.14**	0.72	**1.58**
12.68	1496	eremophilene	0.10 ± 0.02	0.77 ± 0.21	0.14 ± 0.13	0.76	0.63	**1.70**
12.99	1523	τ-cadinene	0.09 ± 0.02	0.23 ± 0.07	-	0.43	0.78	**1.53**
13.11	1533	δ-cadinene	0.21 ± 0.06	0.38 ± 0.13	0.03 ± 0.06	0.46	0.81	**1.54**
13.55	1571	epiglobulol	0.23 ± 0.06	0.44 ± 0.19	-	0.67	0.96	**1.49**
13.68	1583	β-copaen-4-α-ol	0.08 ± 0.03	0.34 ± 0.15	-	0.70	0.66	**1.40**
13.84	1597	globulol	1.13 ± 0.26	2.19 ± 0.75	0.69 ± 0.69	0.72	1.01	**2.04**
13.93	1605	viridiflorol	0.42 ± 0.10	1.27 ± 0.23	0.52 ± 0.28	0.45	0.64	**1.37**
14.05	1616	undetermined sesquiterpene hydrate A	0.17 ± 0.04	0.26 ± 0.09	0.02 ± 0.04	0.40	0.78	**1.38**
14.27	1636	undetermined A	0.19 ± 0.04	0.36 ± 0.11	-	0.45	0.94	**1.70**
14.62	1668	α-cadinol	0.48 ± 0.19	0.47 ± 0.28	-	0.50	**1.15**	**1.87**
16.05	1803	undetermined B	0.03 ± 0.01	0.46 ± 0.07	0.43 ± 0.28	**0.88**	0.71	1.11
16.24	1822	hexadecanal	0.22 ± 0.07	0.02 ± 0.03	0.03 ± 0.06	**0.84**	0.83	0.58
16.45	1843	undetermined C	-	0.14 ± 0.04	0.03 ± 0.06	**0.85**	0.16	1.19
17.09	1908	2-heptadecanone	0.33 ± 0.09	-	-	**1.24**	**1.09**	0.00
18.96	2111	2-nonadecanone	0.08 ± 0.02	-	-	**0.87**	0.77	0.00
19.08	2124	phytol	0.10 ± 0.12	0.19 ± 0.07	-	0.75	0.33	**1.44**
21.02	2355	undetermined D	-	-	0.43 ± 0.12	0.00	1.00	**1.83**
21.20	2377	dodecanoic acid, decyl ester	-	-	0.71 ± 0.12	0.00	**1.35**	**2.49**
21.86	2461	x-methyltetracosane	tr	0.90 ± 0.06	1.61 ± 0.09	**1.38**	**1.45**	0.42
22.14	2498	pentacosane	0.01 ± 0.01	0.51 ± 0.04	1.69 ± 0.27	**1.02**	**1.33**	0.78
22.89	2599	hexacosane	0.02 ± 0.01	0.50 ± 0.04	1.82 ± 0.32	**0.94**	**1.29**	0.85
23.33	2662	x-methylhexacosane	0.01 ± 0.00	1.91 ± 0.20	5.09 ± 0.27	**1.58**	**1.89**	0.91
23.60	2699	n-heptacosane	0.12 ± 0.02	1.16 ± 0.11	4.39 ± 0.60	0.77	**1.28**	1.10
24.13	2775	hexadecanoic acid, decyl ester	-	-	1.33 ± 0.46	0.00	**1.53**	**2.81**
24.50	2830	undetermined E	0.75 ± 0.21	0.53 ± 0.16	-	0.52	**1.30**	**1.88**
24.57	2840	undetermined F	1.98 ± 0.50	2.62 ± 0.78	0.06 ± 0.12	0.69	**1.49**	**2.61**
24.79	2873	undetermined G	1.04 ± 0.29	0.89 ± 0.29	-	0.57	**1.42**	**2.14**
25.46	2974	undetermined H	-	-	0.73 ± 0.18	0.00	**1.35**	**2.48**
25.66	3004	undetermined I	0.19 ± 0.04	-	-	**1.08**	0.95	0.00
26.27	3104	undetermined flavonoid derivative A	1.87 ± 0.33	-	-	**1.94**	**1.71**	0.00
26.88	3204	undetermined J	0.83 ± 0.26	-	-	**1.33**	**1.17**	0.00
26.94	3214	undetermined K	0.81 ± 0.12	-	-	**1.57**	**1.39**	0.00
27.15	3249	1,30-triacontanediol	0.25 ± 0.07	-	-	**1.03**	0.91	0.00
27.57	3318	undetermined L	0.80 ± 0.15	0.16 ± 0.07	0.25 ± 0.56	0.80	**1.14**	1.31
27.98	3385	hentriacontane-14,16-dione	4.30 ± 1.10	1.25 ± 0.41	0.61 ± 0.79	0.68	**1.51**	**1.98**
28.15	3413	undetermined M	2.86 ± 0.23	-	-	**2.19**	**1.93**	0.00
28.40	3455	undetermined N	0.89 ± 0.05	-	-	**1.63**	**1.44**	0.00

Table 1 (continued)

RT	KI	Identification	Average abundance (%)[a]			Contribution pairwise (%)		
			Leaves ($n=6$)	Frass ($n=6$)	Egg capsules ($n=5$)	Leaves × Frass	Leaves × Egg capsules	Frass × Egg capsules
28.59	3485	undetermined aliphatic β-diketone A	0.51 ± 0.04	-	-	**1.42**	**1.25**	0.00
28.80	3520	undetermined benzylester A	1.36 ± 0.12	0.03 ± 0.07	-	**1.64**	**1.59**	0.26
28.94	3543	undetermined O	1.68 ± 0.22	-	-	**1.90**	**1.68**	0.00
29.55	3631	undetermined P	1.84 ± 0.15	-	-	**1.96**	**1.73**	0.00
29.77	3656	undetermined aliphatic β-diketone B	1.85 ± 0.19	0.04 ± 0.04	-	**1.67**	**1.73**	0.46
30.08	3694	undetermined unsaturated aliphatic β-diketone A	1.82 ± 0.25	-	-	**1.95**	**1.72**	0.00
30.40	3731	undetermined benzylester B	0.42 ± 0.06	-	-	**1.34**	**1.18**	0.00
31.03	3803	pentatriacontane-16,18-dione	6.64 ± 0.75	0.55 ± 0.25	-	**1.70**	**2.38**	**1.56**
31.28	3824	undetermined unsaturated aliphatic β-diketone B	1.62 ± 0.57	-	-	**1.83**	**1.62**	0.00
31.39	3834	undetermined Q	0.47 ± 0.13	-	-	**1.19**	**1.04**	0.00
31.60	3851	undetermined R	0.83 ± 0.23	-	-	**1.36**	**1.20**	0.00

[a] from relative area, untransformed; tr = trace; - = not detected

large contribution these compounds had in the differences between samples, accounting for 5% in a total of 48.7% dissimilarity. Next, the high molecular weight compounds present exclusively or in much larger proportion in the leaves, with the exception of x-methylhexacosane, contributed 1.5 to 2.2% of the differences. Among the terpenoids, 7-hydroxy-1,8-cineole, another metabolite of 1,8-cineole was only detected in frass extracts.

Egg capsules were even more different to leaf extracts (63.28% dissimilarity). The biggest single compound differences between the two samples were given by pentatriacontane-16,18-dione and 1,8-cineole, each representing over 2% of the dissimilarities. These compounds were abundant in leaf extracts but appear in very low levels in egg capsules. Similar to frass, the metabolite 2-α-hydroxy-1,8-cineole was also present in egg capsules. X-methylhexacosane was also found in egg capsules, in the highest proportion among all samples.

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Seventeen compounds present exclusively in frass or in egg capsules played an important role in differentiation of these two samples, terpenoids and fatty acid esters among them. Interestingly, the metabolites 2-α-hydroxy-1,8-cineole and 7-hydroxy-1,8-cineole, were markedly reduced in egg capsules when compared to frass. In contrast, α- and β-pinene were proportionately higher in egg capsule extracts.

Discussion

The chemical profile of *Eucalyptus globulus* leaves is notoriously rich, having several terpenoids, particularly 1,8-cineole and α-pinene (Barbosa et al. 2016; Brophy et al. 1991), and an abundance of epicuticular waxes (Horn et al. 1964; Steinbauer et al. 2009). Our results corroborate the previously reported chemical composition of this species, presenting an abundance of terpenoids and heavier compounds, constituents of the plant waxes. Moreover, we found that not all the plant compounds are metabolized during feeding by *Gonipterus* sp. n. 2, and some are discharged unaltered. Regarding those compounds that were exclusive to leaves, we presume that they were metabolized by the weevils, since only trace amounts were detected in frass and egg capsules, if detected at all. Other compounds, present exclusively in frass or egg capsule extracts are likely produced by the weevils or are by-products of metabolism rather than derived directly from the host plant.

One of the most striking differences between extracts of leaves and weevil frass is the inverted proportion between 1,8-cineole and its metabolite 2-α-hydroxy-1,8-cineole present in those samples, respectively. 1,8-Cineole is a cyclic monoterpenoid commonly found in eucalypts, being the main component of the essential oils of several species within this group (Lassak et al. 1991), such that an alternative common name for the compound is “eucalyptol”. Organisms adapted to use *Eucalyptus* as food have mechanisms

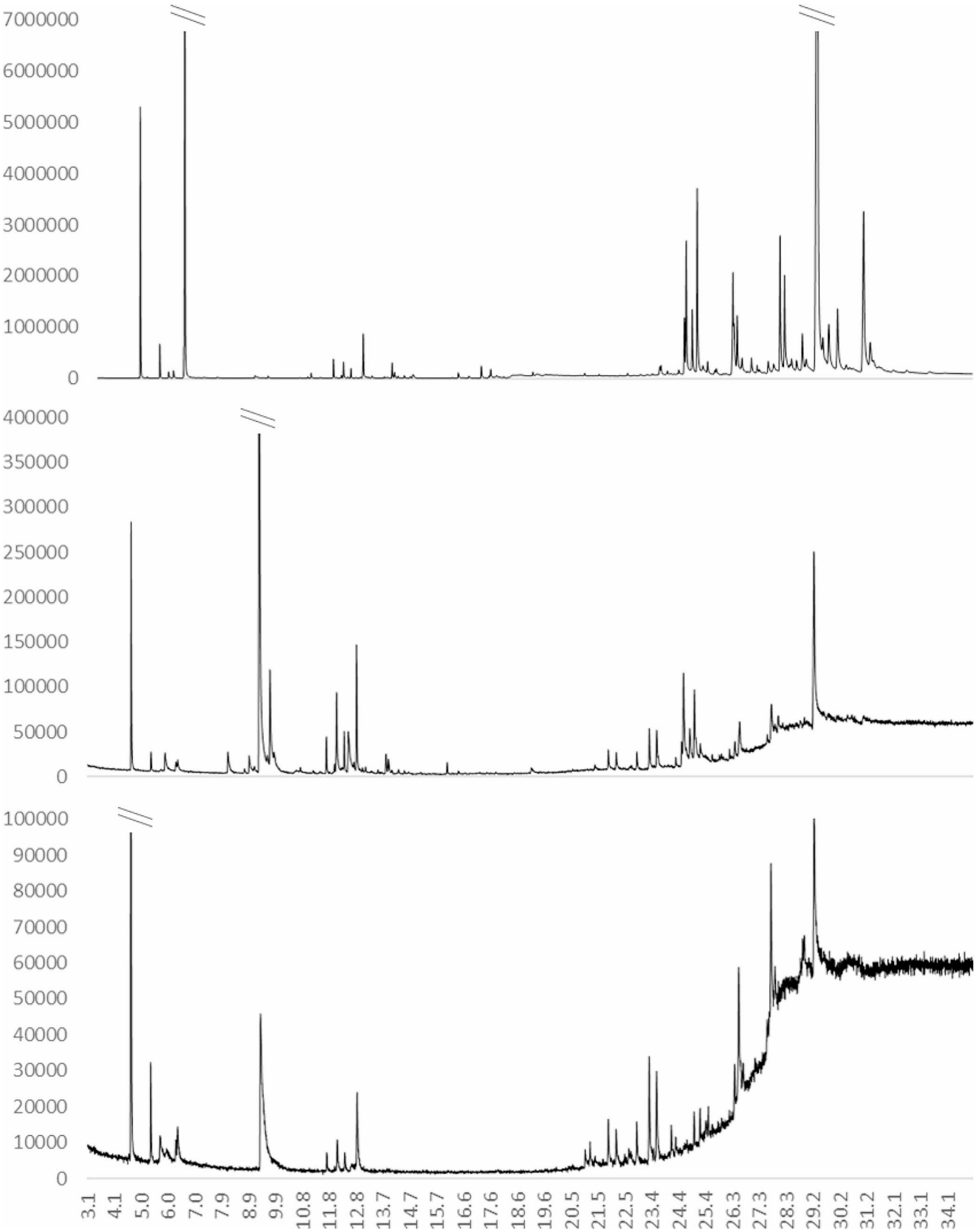

Fig. 3 Stackplot showing representative chromatograms from *E. globulus* leaf (top), *Gonipterus* sp. n. 2 frass (middle) and egg capsule (bottom) extracts

to detoxify this compound by hydroxylation or other processes (Azerad 2014; Cooper 2007; Schmidt et al. 2010), producing different metabolites (Azerad 2014; Southwell et al. 1995, 2003). In our study, we detected 2-α-hydroxy-1,8-cineole and 7-hydroxy-1,8-cineole, two of many possible metabolites of 1,8-cineole; both were present in frass, and the latter was not detected in egg capsules, demonstrating that *Gonipterus* sp. n. 2 has detoxification mechanism to deal with the noxious monoterpenoid. Both metabolites of 1,8-cineole were recently found to be released by adult weevils of the sibling species *G. platensis* (Branco et al. 2020; Mendes et al. 2022). They had behavioural effects on virgin males (attraction) and mated females (repellency) in olfactometer trials (Branco et al. 2020) and induced an electrophysiological response (Mendes et al. 2022). Presence of these compounds in frass suggests that this could be the source of this semiochemical and the mechanism used by these weevils to locate or avoid their conspecifics.

Among other terpenoids, α-pinene was present in high concentrations in all samples, and aromadendrene and bicyclogermacrene were abundant in *Gonipterus* sp. n. 2 frass. Metabolism of α-pinene from host plant results in production of *cis*- and *trans*- verbenol and verbenone, among others (Blomquist et al. 2010; Pierce et al. 1987). These compounds are utilized in chemical communication by some *Dendroctonus* and *Ips* bark beetles (Coleoptera: Curculionidae: Scolytinae) (Blomquist et al. 2010; Seybold et al. 2000; Symonds and Elgar 2004) and, the alcohols in particular, are female attractants of *G. platensis*, depending on the concentration (Branco et al. 2020). However, we were unable to detect the presence of such metabolites which, given their importance in other taxa, we expected in the analysis, especially considering the abundance of α-pinene present in the host to be metabolized. Branco et al. (2020) conducted their study comparing the headspace of *E. globulus* foliage with and without *G. platensis*, attributing to the weevil's activity the exclusive compounds found in the headspace of attacked plants with the weevils. We also collected headspace volatiles in our experiment (Souza, unpubl.) and did not detect the presence of the α-pinene metabolites.

The large representation of aromadendrene in frass extracts, less prominent in egg capsules, and bicyclogermacrene in both insect-derived extracts may be an indication that *Gonipterus* sp. 2 does not metabolise these sesquiterpenes. Although also present in *E. globulus* leaves, the higher proportion of both compounds in frass and egg capsules may be explained by the absence of several other plant compounds metabolized by the weevil. Aromadendrene has antimicrobial activity (Mulyaningsih et al. 2010), and its presence on the surface of egg capsules may have a protective function against bacteria and other pathogens.

Another group of compounds detected in this study, *Eucalyptus* epicuticular waxes, play an important role in interactions with other organisms: they are part of the resistance mechanisms used by these trees against herbivores (Allen et al. 2004; Gosney et al. 2017; Jones et al. 2002) and can influence the foraging and oviposition behaviour of insects (Steinbauer et al. 2004). Aliphatic β-diketones comprise a great part of these waxes (Horn et al. 1964; Steinbauer et al. 2009; Wirthensohn et al. 2000), forming tubular structures on the leaf surface (Huth et al. 2018) and several of them were detected in our *E. globulus* leaf extracts. Remarkably, an aliphatic β-diketone, tritriacontane-16,18-dione was the most or second most abundant compound in all samples indicating that this compound is most likely only partially metabolized by *Gonipterus* sp. 2. Most of the other leaf aliphatic β-diketones and high molecular weight compounds, part of the epicuticular wax or not, were not present in frass and egg capsules or were in substantially reduced proportions, indicating that these compounds were most likely digested.

Two of the most prominent compounds found in egg capsules were α-tocopherol and β-sitosterol, originating from the plant and also present in the frass. α-Tocopherol, also known as vitamin E, is an antioxidant found in green parts of plants involved in several different physiological processes (Munné-Bosch and Alegre 2002). Sterols such as β-sitosterol are essential part of the dietary requirements of the insects, being processed in the midgut and used as a source of energy and as part of fundamental physiological processes (Chapman 1998; Turunen and Crailsheim 1996); they are particularly important in an insect diet, given that insects are unable to synthetise these compounds by themselves, relying on external sources to obtain sufficient amounts to metabolize into the steroids needed (Chapman 1998). Both compounds were previously found in a blend collected from larval frass of the weevil *Sitophilus granarius* L. and stimulated drilling and drumming by its parasitoid *Lariophagus distinguendus* (Först.), in search for their hosts (Steidle and Ruther 2000); further studies into *Gonipterus* egg parasitoids could verify whether these compounds are also elicitors of host finding behaviours.

Comparison between frass and egg capsule extracts revealed that egg capsules are not comprised solely of compacted frass, and that other substances are present. Our hypothesis was that egg capsules were made of frass and additional compounds that bind the frass together to form a tough shield for the eggs, giving it hardness and resistance to field conditions such as solar radiation and rain. However, we detected fewer compounds in egg capsule extracts than in frass while also detecting exclusive compounds, among those two undetermined compounds and two fatty acid esters. These could have different functions including

participation in adhesive blends, even though egg anchorage blends are most commonly proteinaceous (Betz 2010; Souza et al. 2022). Further studies into the composition of the egg capsule cover and its nanostructure could help unravel which compounds are involved in this adhesive function and how they are organized to keep the egg capsule's structure.

Our study demonstrates the metabolism of several *Eucalyptus* secondary compounds, including those with toxic effect, by *Gonipterus* sp. n. 2, an important strategy to enable feeding on this host. This is the first time the use and metabolism of plant compounds is reported for this species and it helps elucidate the production of volatiles that are used as pheromones by a sibling species (Branco et al. 2020). Not all the resulting metabolites, however, are present in the hardened egg capsule that protects the eggs of this weevil while others are exclusive to this secreted matter, evidencing the difference in egg capsules and frass long speculated. This is a valuable insight into compounds that may be used by the weevil as adhesives to keep the structure of egg capsules or by the egg parasitoids as cues for host recognition. Further studies into the production of the egg capsule, including proteins and other substances involved are necessary for better understanding of this structure and its ecological role.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00049-024-00409-2.

Acknowledgements We thank Simon Lawson for research and travel funds through Forest and Wood Products Australia Limited (project number VNC 418–1617) (supported by Biological Control of Eucalypt Pests Alliance, Industry Plantation Management Group and University of the Sunshine Coast) towards a postgraduate research scholarship to NMS. Francisco Tovar and Industry Plantation Management Group for help with collection and access to plantations in southern Western Australia. Paulo Henrique M. da Silva (Instituto de Pesquisas e Estudos Florestais), Gustavo S. Martins (Central America and Mexico Coniferous Resources Cooperative - North Carolina State University), Jeremy Brawner (University of the Sunshine Coast - UniSC) and David Lee (UniSC, Department of Agriculture and Fisheries - DAF) for their advice on selection of species and seed provenances, and David Lee for providing access to seeds through Forest Technologies Seed Store, DAF and advice on sowing and seedling maintenance. We thank Flavia S. Bonora (UniSC) for her assistance with seedling sowing and maintenance. We would also like to thank Geoff Pegg (DAF) for access to the controlled environment rooms where the experiment was kept.

Author contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Natalia Medeiros de Souza, Helen F Nahrung and R Andrew Hayes. The first draft of the manuscript was written by Natalia Medeiros de Souza and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding Open Access funding enabled and organized by CAUL and its Member Institutions. This work was supported by Forest and Wood Products Australia Limited (project number VNC 418–1617) (supported by Biological Control of Eucalypt Pests Alliance, Industry Plantation Management Group and University of the Sunshine Coast).
Open Access funding enabled and organized by CAUL and its Member Institutions

Data availability The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request.

Declarations

Financial interests The authors have no relevant financial or non-financial interests to disclose.

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