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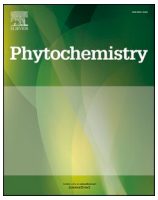

Unique chemistry associated with diversification in a tightly coupled cycad-thrips obligate pollination mutualism

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ABSTRACT

Cycad cone thermogenesis and its associated volatiles are intimately involved in mediating the behavior of their obligate specialist pollinators. In eastern Australia, thrips in the *Cycadothrips chadwicki* species complex are the sole pollinators of many *Macrozamia* cycads. Further, they feed and reproduce entirely in the pollen cones. *M. miquelii*, found only in the northern range of this genus, is pollinated only by a *C. chadwicki* cryptic species that is the most distantly related to others in the complex. We examined the volatile profile from *M. miquelii* pollen and ovulate (receptive and non-receptive) cones to determine how this mediates pollination mechanistically, using GC-MS (gas chromatography-mass spectrometry) and behavioral tests. Monoterpenes comprise the bulk of *M. miquelii* volatile emissions, as in other *Macrozamia* species, but we also identified compounds not reported previously in any cycad, including three aliphatic esters (prenyl acetate and two of uncertain identity) and two aliphatic alcohols. The two unknown esters were confirmed as prenyl (3-methylbut-2-enyl) esters of butyric and crotonic ((E))-but-2-enoic) acids after chemical synthesis. Prenyl crotonate is a major component in emissions from pollen and receptive ovulate cones, is essentially absent from non-receptive cones, and has not been reported from any other natural source. In field bioassays, *Cycadothrips* were attracted only to those volatile treatments containing prenyl crotonate. We discuss *M. miquelii* cone odorants relative to those of other cycads, especially with respect to prenyl crotonate being a species-specific signal to this northern *C. chadwicki* cryptic species, and how this system may have diversified.

1. Introduction

Animal pollination has been proposed as one of the drivers of angiosperm floral diversity and possibly speciation (Crepet, 1979; Grant, 1949; Johnson, 2006; Pellmyr and Thien, 1986; Stebbins, 1970; van der Niet and Johnson, 2012). Selection imposed by the behavior of different pollinators on particular floral traits may lead to changes in pollination mechanisms and consequently the independence (and diversification) of plant gene pools. Numerous examples suggest pollinator-driven diversification has occurred through changes in floral morphology and odor (van der Niet et al., 2014). This implies that a relatively small difference in the plant-emitted signals may represent a novel signal in a specialist pollinator-host mutualism and could, therefore, be associated with host specificity. Such differences thus define the independence of the gene pools or species involved, and explain their reproductive isolation as a consequence. This has been demonstrated in several highly specialized angiosperm pollination systems (Chen et al., 2009; Raguso, 2008; Schiestl and Peakall, 2005), and perhaps in a cycad (e.g., Suinyuy et al., 2018).

The degree of specificity in pollinators of more ancient plant and insect lineages and its influence on plant species diversification is particularly pertinent as most of these taxa are not particularly species rich. The cycads (Order Cycadales) are crucial to testing the importance of pollinator specificity in plant species diversification. Cycads are dioecious gymnosperms of ancient origin, at least into the Permian (Hermsen et al., 2006; Norstog and Nicholls, 1997), and their pollination systems involve some level of insect pollinator specialization (Toon et al., 2020). Some cycad species have only one specialist pollinator species, whereas others have a few, but usually fewer than three (Toon et al., 2020). Most cycad pollinators are beetles, with a major exception

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being species of thrips in the genus *Cycadothrips* Mound (Thysanoptera: Aeolothripidae). Species of *Cycadothrips* are the sole pollinators of some cycads in the Australian endemic genus *Macrozamia* Miq. (Zamiaceae) (Mound, 1991; Mound et al., 1998; Mound and Terry, 2001; Terry, 2001; Terry et al., 2005). *Cycadothrips* species use their specific host's pollen cones as mating and larval development sites, and they feed only on pollen from their host, in an obligate pollination mutualism sometimes referred to as a brood-site pollination system (Hossaert-McKey et al., 2010; Sakai, 2002). Other cycads are also involved in similar obligate mutualisms, mostly with specialist beetle pollinators (Toon et al., 2020).

Species of *Macrozamia* are found in three disjunct regions across Australia (Fig. 1, inset) (Jones, 2002). The genus *Cycadothrips* is associated with *Macrozamia* species in each of these regions, with a different species formally described from each region, and with only minor morphological characters separating them (Mound, 1991; Mound and Marullo, 1998; Mound and Terry, 2001). In eastern Australia, *Cycadothrips chadwicki* Mound pollinates many *Macrozamia* species that occur (coastally and subcoastally mainly) from as far south as the southeastern coast of New South Wales (NSW), near Bega, to just north of Rockhampton in central Queensland (Qld) (Fig. 1), over a span of ~1500 km, with species and populations discontinuous across that range (Jones, 2002). Compelling molecular and population genetics evidence has revealed up to five mostly allopatric cryptic species within *C. chadwicki*, despite the lack of distinguishing morphological characters (see Brookes et al. (2015) for their host associations and geographical distributions). Only one of these has been well studied along with the cone traits of their cycad hosts, *M. lucida* and *M. macleayi* in southeastern Qld (e.g., Terry et al., 2014). The thrips involved, *C. chadwicki* Species 4 (thereafter *C. chadwicki* 4) of Brookes et al. (2015) is the sole pollinator of these two cycad species (Terry et al., 2005).

During pollination, *Macrozamia* cones, like those of many other cycads, exhibit a daily thermogenic event and, at that time, emit volatile organic compounds that are critical in mediating pollinator behavior. These odors not only attract pollinators, but also with the help of thermogenic cones induce them to leave the cones in some cycads (Salzman et al., 2020; Suinyuy et al., 2010, 2012, 2013a, 2015; Terry et al., 2004b, 2007b, 2014). The cone volatiles of only eight of the 41 described *Macrozamia* species have been reported, and this includes five of the *Cycadothrips*-pollinated species from eastern Australia. Most of these

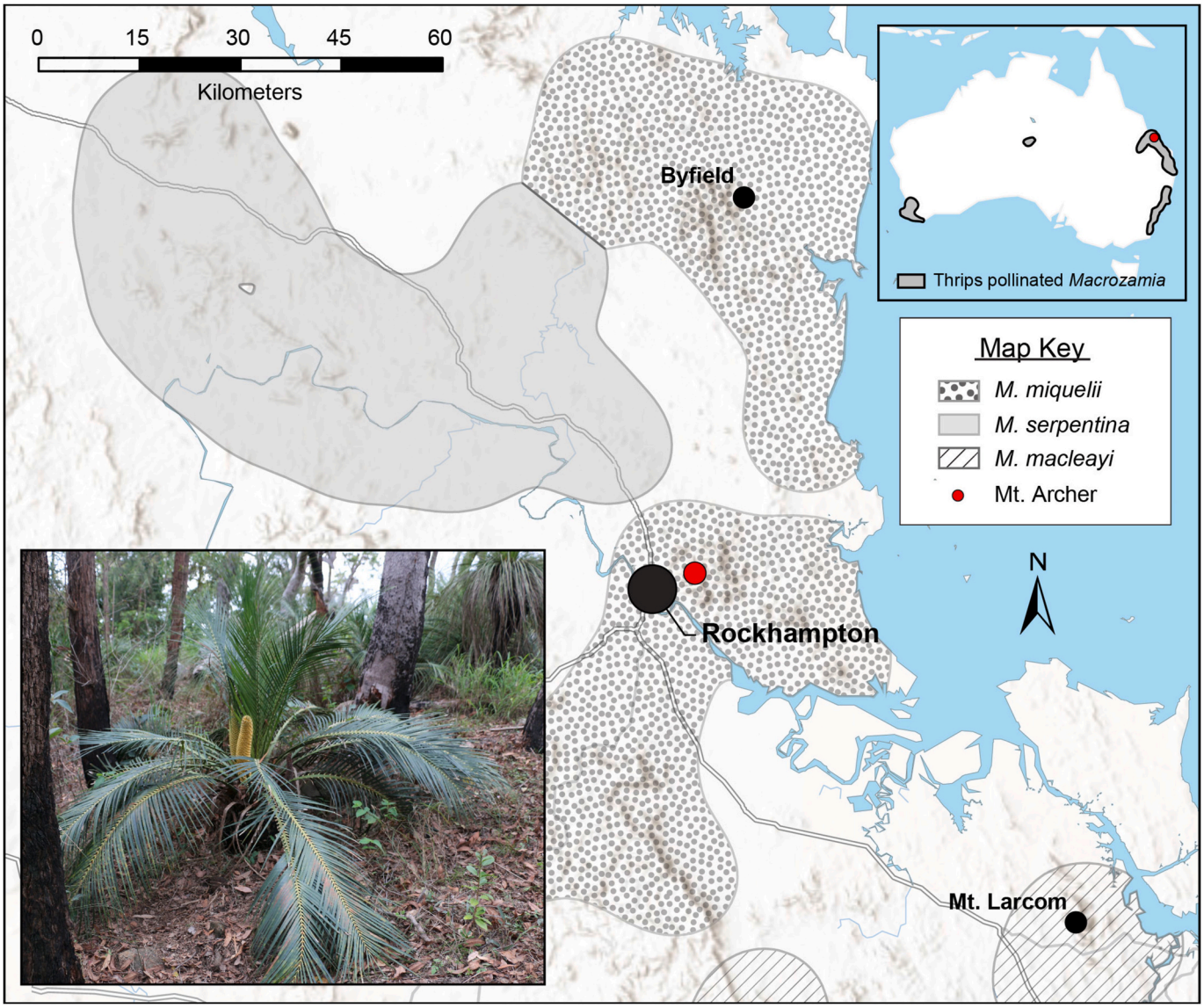

Fig. 1. Distribution of *Macrozamia miquelii* and two other *Macrozamia* species near the city of Rockhampton and the location of the Mount Archer National Park study site. The top inset figure shows the Australian distribution of *Cycadothrips*-pollinated cycads in the genus *Macrozamia*, and the bottom inset is a photograph of *M. miquelii* habitat at the study site, showing a cycad with maturing pollen cones.

eight have monoterpenes as the dominant (or only) odorant class, with β-myrcene present in all (Pellmyr et al., 1991; Terry et al., 2004a, 2004b, 2008; Wallenius et al., 2012). Some species also emit volatiles from a wider array of chemical classes, including sesquiterpenes and alcohols (3-octanol and 1-octen-3-ol, and octanol), some of which are dominant (Terry et al., 2008; Wallenius et al., 2012).

Investigation of *M. lucida* and *M. macleayi*, in southeastern Qld, has revealed an intricate set of daily interactions between specific cone chemicals (primarily β-myrcene), various thermogenic traits, and their shared sole pollinator, the thrips *C. chadwicki* 4 (Brookes et al., 2015; Terry et al., 2004a, 2005, 2007b, 2014). The thrips leave pollen cones en masse during the midday thermogenic peak, which also coincides with very high β-myrcene emission rates, and later in the afternoon return to cones as they cool down, with some thrips entering ovulate cones. Pollination of receptive ovules follows. We questioned whether each *C. chadwicki* cryptic species responds to something common across all *Macrozamia* species, such as β-myrcene or other common monoterpenes, or whether thrips respond to unique or alternative dominant volatiles emitted by their host cones. This issue is critical to understanding the possible role of these volatiles in the evolutionary divergence of the cycads and their pollinators.

We hypothesize that cryptic species in the *C. chadwicki* species complex, despite their morphological similarities to one another and their relatively recent divergences (from ~1–7.3 Mya, Brookes et al., 2015), will have a distinctly different basis from one another to their interactions with their host plants. The difference is likely to be in the chemistry that underpins the interaction, because previous studies show that cone chemistry is a key element in the behavioral interaction of specialist pollinators with their host plant (e.g., Salzman et al., 2020, Suinyuy et al., 2015; 2018; Terry et al., 2014). To test this hypothesis, we began an extensive study to determine the cone odorants of *Macrozamia* species associated with each of the *C. chadwicki* cryptic species in Qld. We report here our findings on *M. miquelii* (F.Muell.) F. DC., found in the northern range of this genus (Fig. 1) (Jones et al., 2001). The thrips on this cycad, *C. chadwicki* 1, diverged earliest (at an estimated 7.3 Mya) within the species complex, based on CO1 and 28S molecular analyses (Brookes et al., 2015). To this end, we characterized the cone volatiles of this cycad by gas chromatography-mass spectrometry (GC-MS) and compared the resulting profiles with those of the other *Macrozamia* species reported. We also compared the volatile profiles across the phenological stages of *M. miquelii* ovulate cones (pre--receptive, receptive and post-receptive, Fig. 2 a-d) to isolate potential compounds important in mediating pollinator behavior. Confirmation of the structures of several components of uncertain identities required the synthesis of candidate compounds. We tested several compounds, including one of novel chemistry, for their role in attracting *C. chadwicki* 1 in the field. The results are discussed in relation to the basis of the diversification in these cycads and their thrips pollinators.

2. Results

2.1. Overview of cone volatile chemistry

All compounds detected in *M. miquelii* cones and their mean emission rates are presented in Table 1. A total of 49 compounds was detected from 10 pollen cones (all dehiscing pollen and attracting thrips) and 23 ovulate cones, though not all compounds were present in all samples. Volatiles included the following chemical classes: nine aliphatics, nine benzenoids, 20 monoterpenes, five sesquiterpenes, one nitrogenous compound, and four unknowns. The maximum number of compounds detected in a single sample was 37 from a pollen cone and 33 from a receptive ovulate cone, and almost all compounds found in pollen cones

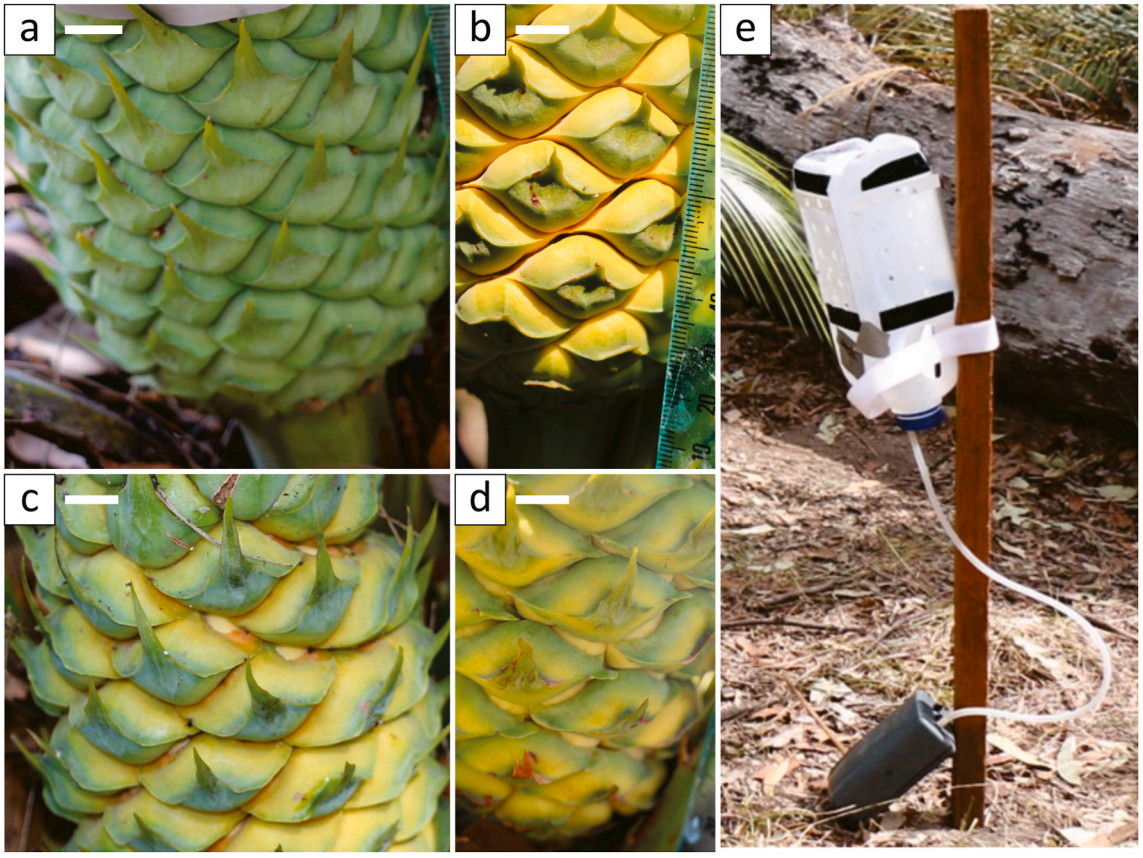

Fig. 2. *Macrozamia miquelii* ovulate cone sporophylls at (a) the pre-receptive stage, (b) receptive stage, and (c,d) post-receptive stage. Field bioassay device (e) to test chemical standards for their role in attracting pollinators. The white scale bar on cone photographs = 1 cm.

Table 1
The chemical compounds found on pollen and ovulate (receptive and non-receptive) cones of *Macrozamia miquelii* (n is given for the total number of volatile samples and the number taken at midday as indicated). For each compound is given: its Kovat's retention index (RI), and its average % contribution to total emissions, the number of samples in which it was detected (N), and measurements of emission rate (ng min^{-1}) within each cone type. In the average and minimum emission rate columns are the rates of all cone samples and, within parentheses, the rate for midday samples alone, if it changed. All maximum rates are from midday samples alone.

Compound[a]	CAS #	RI, column		Cone type, number of cones, n = total number of volatile samples (number of midday samples)[b]												
				Pollen, 10 cones, n = 38 (18)					Receptive ovulate, 14 cones, n = 19 (15)					Non-receptive ovulate, 9 cones, n = 11 (9)[c]			
				Avg. %	N	ng min^{-1}, all (midday only)			Avg. %	N	ng min^{-1}, all (midday only)			Avg. %	N	ng min^{-1}, all	
		Nonpolar	polar			Avg.	min	max			Avg.	min	max			Avg.	min-max
Aliphatics																	
2-methylbut-3-en-2-ol (MBO)*	115-18-4	592	1027	25.08	31	1165 (1632)	13 (88)	6382	15.07	8	317 (353)	10.0	1125	nd	1 pre	tr	nd-tr
3-methybut-2-en-1-ol (prenyl alcohol)*	556-82-1	799	1338	0.38	36	14 (18)	0.45	66	0.47	9	8	0.18	21	nd	nd	nd	nd
octanal *	124-13-0	1004	1306	0.12	6	1	0.50	1	0.24	7	1	0.50	1	0.46	5 pre	1	0.5–1
nonanal *	124-19-6	1108	1410	0.22	19	3	0.27	6	0.94	13	3	0.54	8	2.44	9	3	0.4–5.0
decanal	112-31-2	1208	1515	0.42	6	2	0.90	3	0.95	5	2 (3)	1.52	4	1.24	4 pre	2	1.2–3
prenyl acetate *	1191-16-8	925	1271	4.21	37	283 (436)	5 (34)	2275	5.19	13	84 (102)	0.41	508	9.26	1 pre	4	4
prenyl butyrate *	71820-56-9	1103	1411	0.06	11	2 (3)	1.20	6	0.24	4	3 (5)	1.04	7	nd	nd	nd	nd
prenyl crotonate *	211429-69-5	1161	1563	16.97	38	744 (1137)	1.35	2683	29.44	19	232 (386)	2(19)	1121	nd	3 post	tr	nd-tr
6-methyl-5-hepten-2-one*	110-93-0	988	1355	0.12	6	1	0.49	1	0.19	7	1	0.29	2	0.27	5 pre	0.3	0.3–0.5
Benzenoids																	
methyl benzoate *	93-58-3	1103	1636	0.08	5	1 (2)	1.00	3	nd	nd	nd	nd	nd	nd	nd	nd	nd
methyl salicylate *	119-36-8	1202	1796	0.13	23	9 (11)	0.53	66	0.04	4	0.3	0.03	1	nd	nd	nd	nd
cumic alcohol isomer	536-60-7	1259	2015	0.84	36	9	2.00	24	2.07	13	6	0.04	11	7.97	6	10	1.6–16
cumic alcohol isomer	536-60-7	1271	2029	0.76	36	9	2 (2.6)	27	1.81	14	5	0.13	10	7.51	6	10	1.6–14
4-ethylbenzaldehyde *	4748-78-1	1171	1725	0.81	25	15 (18)	0.10	40	1.45	14	6	0.58	13	4.19	5 pre	7	4.7–7
3-ethylbenzaldehyde	34246-54-3	1186	1756	0.26	10	2	1.10	4	0.54	11	1	0.19	3	1.56	5 pre	2	1.7–3
cinnmaldehyde *	104-55-2	1187	NA	0.14	6	1	1.00	1	0.26	6	1	0.26	1	0.50	5 pre	1	0.7–1
3-ethylacetophenone	22699-70-3	1282	1842	1.38	38	14 (16)	1.29	62	3.61	16	11	0.05	28	17.71	8	14	2.0–22
4-ethylacetophenone	937-30-4	1305	1852	1.00	38	12 (15)	1.3 (2.3)	77	3.20	14	10	0.03	19	11.78	8	11	1.6–19
Monoterpenes																	
bornylene	464-17-5	908	NA	0.25	26	71	13.61	165	tr	3	tr	tr	tr	nd	nd	nd	nd
α-pinene *	80-56-8	938	1011	23.32	34	5134 (7313)	0.93	21,514	9.76	19	830 (1124)	0.85	11,821	38.01	10	35	1.2–155
α-fenchene	471-84-1	951	1047	0.31	15	73 (97)	3 (10)	214	0.33	2	78	5.71	150	nd	nd	nd	nd
camphene *	79-92-5	951	1056	0.56	18	128 (147)	17.91	346	0.25	2	63	0.51	126	nd	nd	nd	nd
sabinene *	3387-41-5	977	1116	0.17	17	47 (64)	2.20	256	0.12	5	20	0.02	90	1.75	1 pre	3	3
β-pinene *	127-91-3	982	1100	0.79	27	211 (270)	0.14	1236	0.21	13	28 (40)	0.15	323	0.27	6	3	0.3–8
β-myrcene *	123-35-3	993	1174	6.63	38	624 (910)	1.6 (35)	4778	12.64	19	196 (247)	2.18	1748	24.90	9	37	0.9–73
α-phellandrene	99-83-2	1008	1170	0.35	25	37 (48)	2.10	191	1.10	11	11 (15)	1.06	53	2.63	5 pre	4	3.5–5
Δ-3-carene *	13466-78-9	1015	1148	0.17	19	50 (54)	0.36	256	0.08	4	11	1.05	37	nd	nd	nd	nd
α-terpinene *	99-86-5	1022	1190	19.63	30	4692 (5588)	1.70	29,979	7.13	14	668 (849)	0.57	7504	0.84	4	1	0.9–2
p-cymene *	99-87-6	1031	1285	3.10	32	788 (907)	0.82	5100	1.69	13	162 (232)	2.26	1979	1.93	10	2	0.7–5
limonene *	138-86-3	1035	1210	2.36	33	576 (731)	0.87	3836	1.55	14	81 (125)	0.95	912	2.24	8	3	0.4–13
β-phellandrene	555-10-2	1035	1221	0.91	18	233 (256)	0.32	1423	0.22	4	12	1.07	25	nd	nd	nd	nd
1,8-cineole	470-82-6	1035	1221	0.06	1	1	0.84	1	0.31	1	12	11.69	12	4.02	2 pre	5	3.3–7
(Z)-β-ocimene *	3338-55-4	1041	1250	0.20	34	23 (29)	0.4 (2.8)	169	0.12	11	3	0.42	18	nd	nd	nd	nd
(E)-β-ocimene *	3779-61-1	1052	1268	0.10	31	10 (14)	0.36	105	0.24	12	8 (10)	0.28	36	0.16	1	0.2	0.2
γ-terpinene *	99-85-5	1065	1259	0.60	22	159 (195)	1.75	784	0.40	12	24 (31)	0.40	236	1.21	2 pre	2	1.4–2
terpinolene *	586-62-9	1096	1298	0.35	22	95 (116)	0.89	413	0.18	6	33 (40)	0.08	179	nd	nd	nd	nd
allo ocimene *	7216-56-0	1134	1390	0.06	11	7 (10)	0.00	25	0.07	5	3	0.29	8	nd	nd	nd	nd

(*continued on next page*)

Table 1 (*continued*)

Compound[a]	CAS #	RI, column		Cone type, number of cones, n = total number of volatile samples (number of midday samples)[b]												
				Pollen, 10 cones, n = 38 (18)					Receptive ovulate, 14 cones, n = 19 (15)					Non-receptive ovulate, 9 cones, n = 11 (9)[c]			
				Avg. %	N	ng min^{-1}, all (midday only)			Avg. %	N	ng min^{-1}, all (midday only)			Avg. %	N	ng min^{-1}, all	
		Nonpolar	polar			Avg.	min	max			Avg.	min	max			Avg.	min-max
verbenone	80-57-9	1222	NA	0.05	4	4	1.71	5	0.02	1	0.5	0.54	1	nd	nd	nd	nd
Sesquiterpenes																	
(Z)-β-caryophyllene *	118-65-0	1420	1592	0.24	7	3	0.32	15	0.15	7	3	0.44	4	nd	nd	nd	nd
(E)-β-caryophyllene *	87-44-5	1448	1609	2.54	37	67 (94)	0.7 (12.8)	270	17.07	18	293 (394)	1.1 (3.6)	1171	1.52	6	2	0.6–3
α-humulene *	6753-98-6	1486	1675	0.25	34	5 (6)	0.60	21	1.59	15	30 (34)	0.30	95	nd	nd	nd	nd
(E,E)-α-farnesene *	502-61-4	1523	NA	0.02	6	2	0.63	5	0.08	7	0.9	0.16	2	nd	nd	nd	nd
caryophyllene oxide*	1139-30-6	1616	1996	0.16	10	2 (3)	0.40	9	0.46	13	5 (6)	0.43	14	0.22	6	0.3	0.2–0.5
N-containing																	
2,5 dimethyl pyrazine*	123-32-0	914	NA	0.04	1	38	38.00	38	nd	nd	nd	nd	nd	nd	nd	nd	nd
Unknowns (*m/z*)																	
154,111,85,69,68,67,41,39		1018	NA	0.05	1	2	1.61	2	nd	nd	nd	nd	nd	nd	nd	nd	nd
154,69,68,67,57,41		1079	NA	0.18	8	57	0.93	146	0.21	1	58	57.91	58	nd	nd	nd	nd
154,109,85,69,68,67,53,41		1126	NA	0.17	23	42	1.20	280	0.03	2	1	0.60	2	nd	nd	nd	nd
152,85,69,59,43,41		1228	NA	0.05	3	1 (2)	1.70	2	0.99	5	3	1.59	5	8.28	3	4	1.5–6

	Pollen cones	Avg.	Min	Max	Rec. Ovulate cones	Avg.	Min	Max	Non-Rec. Ovulate cones	Avg.	Min-max
All samples, ng min^{-1}		12,866	212	76,650		2635	152	27,017		110	4.9–187
Avg. No. compounds, all samples		25.66	10	37		21.89	8	33		12.64	3–22
Midday samples only, ng min^{-1}		17,028	1372	76,650		3454	204	27,017		110	4.9–187
Avg. No. compounds, midday only		27.4	16	37		21	8	33.0		12.64	4–22

[a] Names are derived from the mass spectral database best-fits. Those names marked with "*" can be considered certain identifications, with a strong match of mass spectrum, retention times and RIs to authentic standards. All others were not tested against a standard but can be considered highly probable identifications, with a very close RI and mass spectral match to published data. For 3-ethylacetophenone, the RI is ~40 units from the single data points published for both polar and nonpolar columns. For the cumic alcohol isomers, both are a close match for the nonpolar RI, but for the polar column, one isomer matched published data, but no data are available for the other isomer. For MBO, we include its published data on the nonpolar column. MBO was detected in all pollen cones and receptive pollen cones run on the polar column. All post-receptive and all but one pre-receptive cones were run on the polar column and it was only detected in one pre-receptive cone.

[b] Codes within table: NA = compound not found in samples run on polar column (either not found in samples, or compounds were identified from 2016 to 2017 samples that were not run on polar column); nd = not detected; tr = trace amounts.

[c] Non-receptive ovulate cones represent two stages of cone development, pre-receptive and post-receptive cones. Under the 'N' column, for compounds that include a notation of either a 'pre' or 'post' in addition to the number of samples, that means the compound was detected only in pre-receptive or post-receptive cones, respectively. For example, for MBO, there was only a trace amount detected in one pre-receptive cone (1 pre), and for prenyl crotonate a trace amount was detected in only three of the four post-receptive cones (3 post). If there is no 'pre' or 'post' notation, then that compound was detected in both pre- and post-receptive cones.

were also detected in receptive ovulate cones. Ovulate cones did not have any unique compounds. On average, the pollen cone emission rate was more than five times that of receptive ovulate cones, but some samples of the latter had a much higher emission rate than the pollen cone average (see totals for each cone group in Table 1). Receptive ovulate cones produced almost twice as many compounds and more than 20 times the emission rates of non-receptive ovulate cones.

Fig. 3 presents the average relative proportions of the different chemical classes to the total emissions (top row of pie charts) for each cone group (as listed across the top of the diagram) and the major contributors to the monoterpenes and the aliphatic esters (in the bottom two rows of the chart). The monoterpenes clearly contributed the highest proportion to the total emissions of all cones, and the aliphatic esters and alcohols the next highest proportions for the pollen cones (~7% each) and receptive ovulate cones (~9 and 14%, for the esters and alcohols, respectively). Sesquiterpenes contributed a substantial proportion in only the receptive ovulate cones (~12%), with (E)-β-caryophyllene as the most dominant (Table 1). This compound was present in all pollen and receptive ovulate cones and in some pre-receptive cones. Within the monoterpenes, α-pinene and α-terpinene were the dominant compounds, followed by β-myrcene, p-cymene and limonene. This dominance of monoterpenes was due to a number of pollen and receptive ovulate cones that emitted very high monoterpene levels, with the chromatogram shown in Fig. 4 being an example from a pollen cone (see section 2.3.2 for details on monoterpene emissions). The highest contributor to the aliphatic ester class proved to be prenyl crotonate, a compound previously unknown in nature (see section 2.2 for confirmation of its identity). It was detected in all pollen and receptive ovulate cones. Non-receptive ovulate cones were similar to the pollen and receptive ovulate cones in that monoterpenes represented a high proportion of the emissions, but were dissimilar in that the benzenoids were also dominant (Fig. 3, and see Table 1 for the specific benzenoids). Other differences in the non-receptive ovulate cones included the low level of emissions of α-terpinene, and prenyl crotonate was detected in only three of four post-receptive cones, at trace levels only, and it was not detected in any pre-receptive cones (Table 1).

Samples run on the Carbowax polar column revealed another compound not previously reported in cycad cone volatile samples, 2-methylbut-3-en-2-ol (MBO) (Table 1), a hemiterpene (Gray et al., 2011). This was not detected in samples run on the nonpolar columns because it elutes during the solvent delay. MBO was, however, detected in measureable levels in all pollen and receptive ovulate cone samples run on the polar column, but it was detected only at trace levels in one non-receptive (pre-receptive) ovulate cone (Table 1). Another hemiterpene, 3-methylbut-2-ene-1-ol (prenol, or prenyl alcohol) (Table 1), has also not been reported from any cycad to date. It was detected in

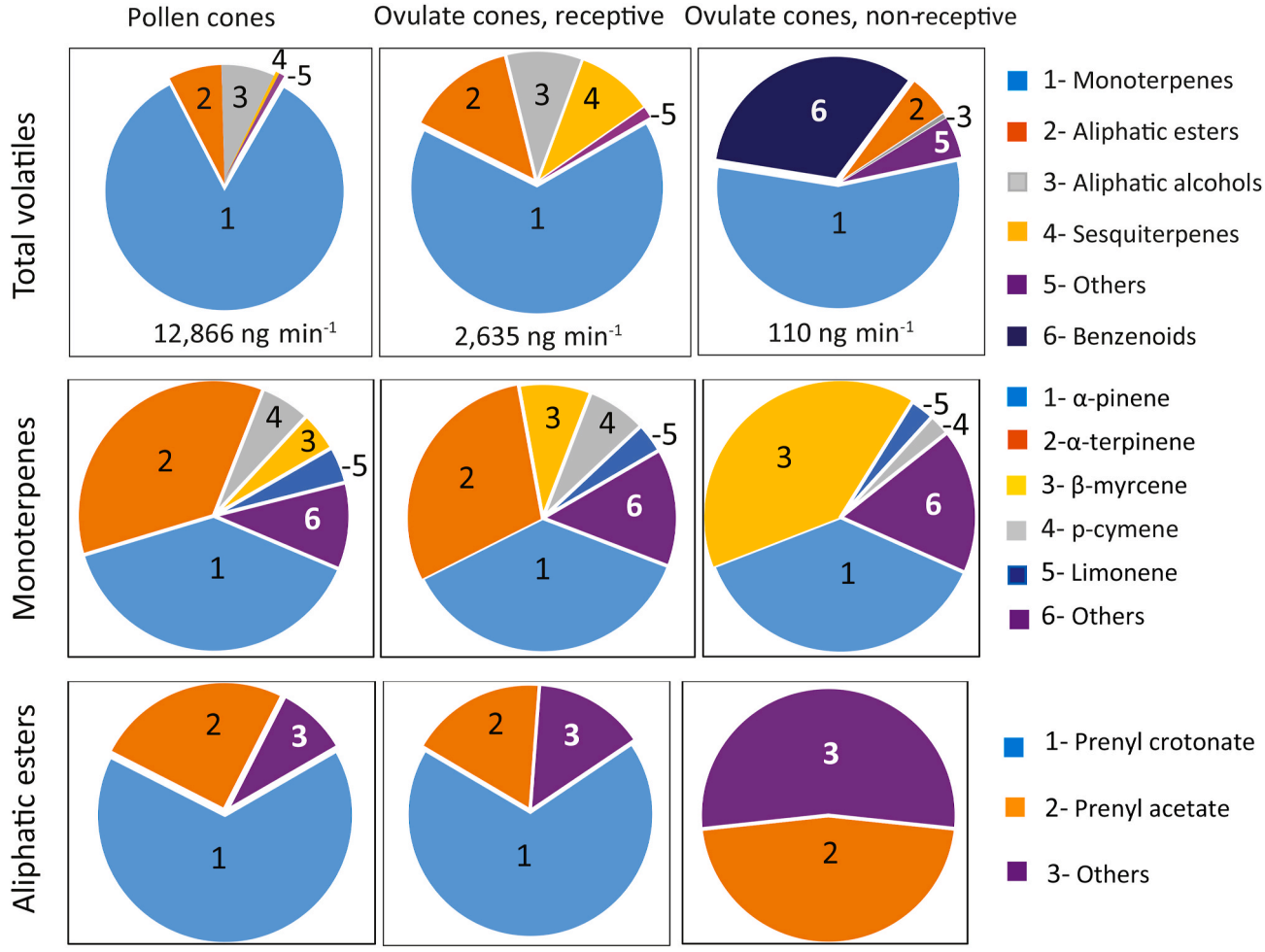

Fig. 3. Composition of volatiles emitted by *Macrozamia miquelii* cones. Pollen cones are presented in the left hand column of pie charts, receptive ovulate cones in the middle column, and non-receptive (pre-receptive and post-receptive) cones in the right hand column. The top row of pie charts presents the mean percentage volatile emission contributed by major chemical classes and the mean emission rate (ng min^{-1}) is presented below the chart for each cone type. The second row presents the mean percentage emissions of the different monoterpenes to the total monoterpene emissions, and the third row does the same for aliphatic esters. Each chemical or chemical class on the right hand side of the charts is identified by a matching number and color within each pie chart. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

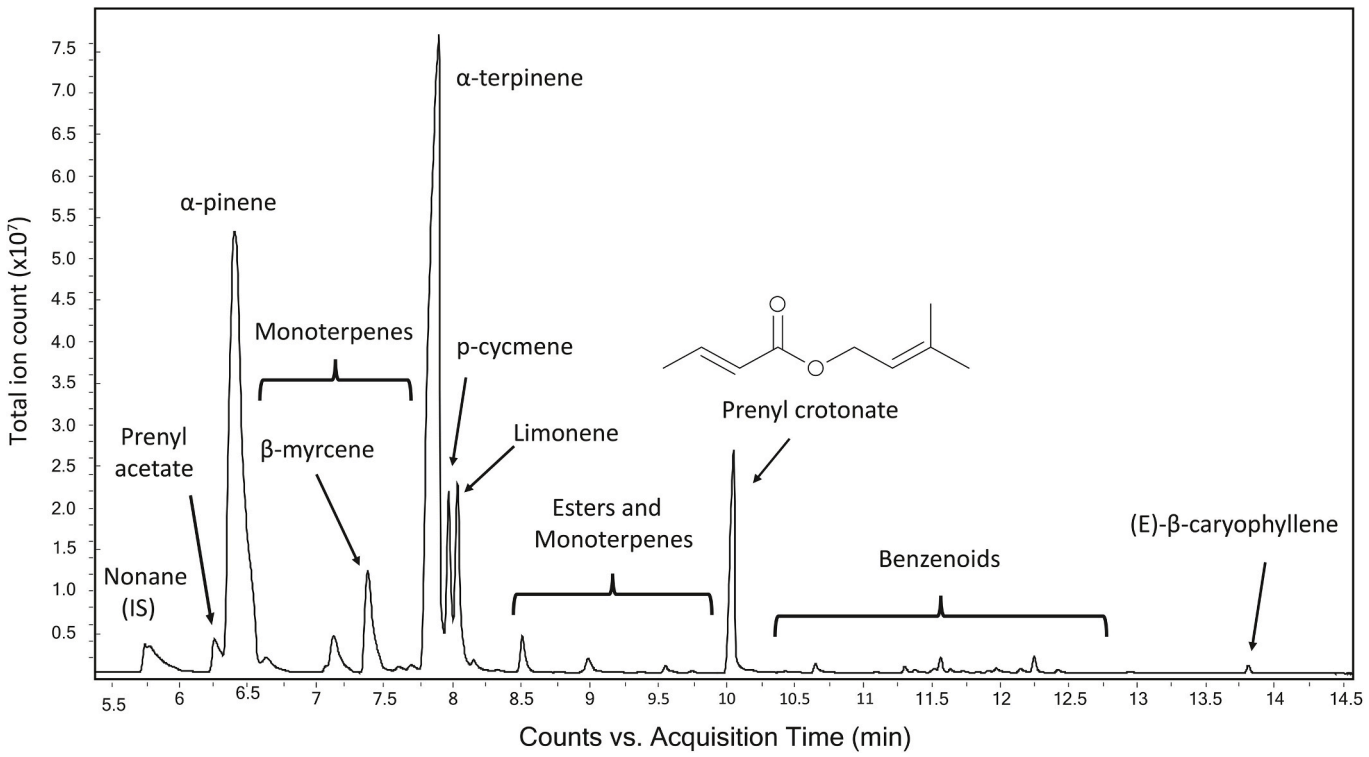

Fig. 4. A chromatogram of a *Macrozamia miquelii* pollen cone sample that exemplifies the emission of high levels of certain monoterpene components (run on the DB-5 column on Agilent QQQ GC-MS). The major confirmed components are indicated and include prenyl crotonate which was unknown until characterized in this study (see section 2.2). Nonane (IS) is the internal standard added for quantification.

some pollen and receptive ovulate cone samples but not in non-receptive ovulate cones.

2.2. Identification of unusual aliphatic esters

Fig. 5 shows a chromatogram of a sample from a pollen cone that emits relatively higher levels of aliphatic esters, but lower levels of certain monoterpenes, than the sample depicted in Fig. 4, but lower emissions of certain monoterpenes. This sample also shows a small peak of the prenyl alcohol. The process of elucidation of the structure of the major peak in Fig. 5a, prenyl crotonate (also present in Fig. 4), is described below in this section. Another minor ester component evident in the narrow segment depicted in Fig. 5b is shown to be prenyl butyrate. Other confirmed compounds are named on the chromatogram.

The mass spectrum of the major component in Fig. 5a, at 11.25 min (Fig. 6) showed a best-fit match (94%) to 3-methylbut-2-enyl cyclopropanecarboxylate, with matching base peak at *m/z* 69 and molecular ion at *m/z* 154. However, its Kovat's retention index (RI) of 903 (from Pubchem) differed significantly from the 1161 calculated value. The abundance of the m/z 69 fragment (100%, base peak) suggested that the acylium ion $[C_4H_5O]^+$ augments the $[C_5H_9]^+$ ion present in the prenyl (3-methylbut-2-enyl) moiety, as in prenyl acetate (Fig. 5a), where the relative abundance of *m/z* 69 is much less at 40–50%. Prenyl acetate is present in many samples (Table 1) as is prenyl alcohol (Table 1), though the relative abundance of *m/z* 69 in prenyl alcohol is less than 10%. Several alternatives to the cyclopropanecarboxylic acid moiety were considered and two compounds, prenyl crotonate and prenyl methacrylate, were synthesized (see chemical structures, Figs. 4,5 and Supplementary Fig. S1a,b). The database identified the peak at 10.36 min as prenyl butyrate ($[M]^+$ 156, base peak *m/z* 71) with a match of 95%. Both prenyl butyrate and its branched isomer, prenyl isobutyrate (Fig. 5a and Supplementary Fig. S1c,d), were also synthesized.

The retention time of the synthesized prenyl crotonate standard matches that of a cone sample containing the major peak in Fig. 5a on both nonpolar (Supplementary Fig. S2a) and polar columns, and the mass spectrum of the standard is a close match to that of the corresponding cone sample peak (Table 2, and comparable spectra in Supplementary Fig. S2b). Thus, the major peak is confirmed as prenyl crotonate. Its odor was described as 'anise-like' but sweeter by four biologists, independently, when given choices presented on a wine odor wheel. A fifth person described it as 'spicy-black pepper'. A similar compound, prenyl senecioate, has comparable organoleptics, described as 'chervil' which is anise-like (Good Scents Company, 2020a).

The peak "*" (Fig. 5b) mass spectrum also exhibits a base peak of *m/z* 69 and $[M]^+$ 154 and a clear similarity to prenyl crotonate (Table 2) and is likely therefore to be isomeric with it. One synthesized isomer, prenyl methacrylate, can be eliminated as a possibility since the RI of the synthetic standard on a nonpolar column (1096) is different from that of the sample peak (1126). Thus, this peak remains unknown (Table 1). Other isomers of both the acid and alcohol moieties of the ester are possible. The alcohol MBO, identified in pollen and ovulate cone samples (Table 1), is one candidate for the alcohol moiety.

Prenyl butyrate is confirmed since the synthetic standard and sample peaks had identical retention times and similar mass spectra (Table 2), and both mass spectra and RI show strong agreement to published data (RI = 1103, Table 1 and RI = 1101, González et al., 2012).

The mass spectra of several other peaks in Fig. 5b displayed abundant fragments characteristic of the prenyl moiety, (*m/z* 67, *m/z* 68, *m/z* 69) together with fragments at *m/z* 71 or enhancement of *m/z* 69, derived from the acid moiety of the esters whose identities have been established (see Table 1, unknowns).

In sum, five of the confirmed compounds have not been reported previously from cycad cone volatiles. These are two hemiterpenes, MBO and prenyl alcohol, together with three prenyl esters: acetate, crotonate and butyrate.

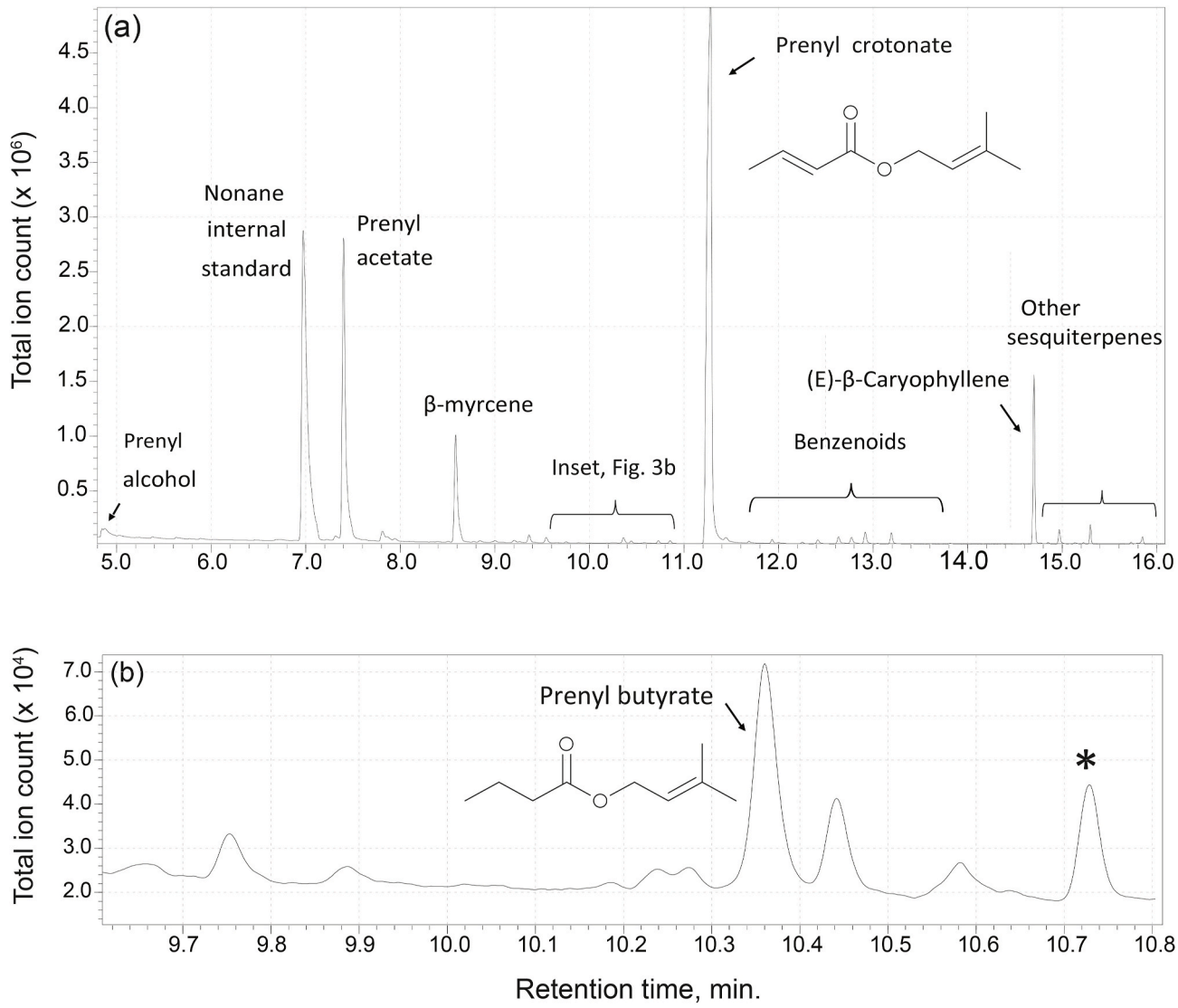

Fig. 5. A *Macrozamia miquelii* pollen cone sample chromatogram (Shimadzu GC-MS, ZB-5 column) representing samples in which aliphatic esters were more prominent than in Fig. 4 chromatogram. The relevant segment of the complete chromatogram is presented in (a), with confirmed components named, including prenyl crotonate and the nonane internal standard, and (b) shows an expansion of the 9.6–10.8 min retention time window as demarcated in (a) to reveal prenyl butyrate and a component marked (*), clearly related to prenyl crotonate.

2.3. Variation in volatile emissions of *M. miquelii* cones

2.3.1. Temporal patterns of cone thermogenesis and volatile emission rates

Both field and laboratory studies indicated that both pollen and receptive ovulate cones of *M. miquelii* exhibit a daily daytime thermogenic event, as in other-*Cycadothrips*-pollinated *Macrozamia* (Terry et al., 2004a). In the field, where ambient midday temperatures often exceeded 30 °C, pollen cone temperatures reached a maximum during 12:00–13:30 h, ranging from 0.7 to 8.5 °C above the ambient across all pollen cones, with an average of 3.7 °C (Fig. 7). The figure shows that thermogenic cones passed the ambient temperature around 11:30 h. By 17:00 h, cone temperatures cooled close to the ambient. Experiments on four excised pollen cones under controlled conditions in an environmental chamber also showed a midday thermogenic peak. Cones reached their temperature peak between 11:00 to 13:00 h exceeding the 25 °C ambient by 9–11 °C. On a different day, five receptive ovulate cones reached an average of 5.5 (±0.77 s.e.) °C above the ambient, measured between11:53 and 12:45 h, while five pre-receptive ovulate cones were not thermogenic with an average 0.16 (±0.37 s.e.) °C below the ambient temperature measured between 12:08 and 13:10 h.

The volatile emission rates of three pollen cones sampled on plants in the field were significantly higher (about five-fold) at midday (during peak cone temperatures) compared with morning or later afternoon rates (ANOVA, $F_{2,4} = 12.4$, $P = 0.019$) (Fig. 8). The number of compounds detected from each cone was numerically higher at midday, although the values were not statistically different from one another (Friedman test $\chi^2_2 = 4.7$, $P = 0.097$). Emission rates of major compounds of each cone also were the highest at midday (Supplementary Fig. S4). Only one receptive ovulate cone was sampled at these same periods in one day, and the midday sample emission rate was five-fold that of the morning and 4.4-fold that of the afternoon sample, with 20, 33, and 23 compounds detected in the early, midday and later afternoon samples, respectively. Another receptive ovulate cone had a midday sample with 2.1 higher emission rate over the morning sample, with 29 versus 24 detected compounds, respectively. In a third ovulate cone, the midday sample had a four-fold higher emission rate over the later afternoon sample, with 33 versus 31 compounds detected, respectively. All subsequent ovulate cone samples were taken during midday to synchronize the sample times near peak thermogenesis.

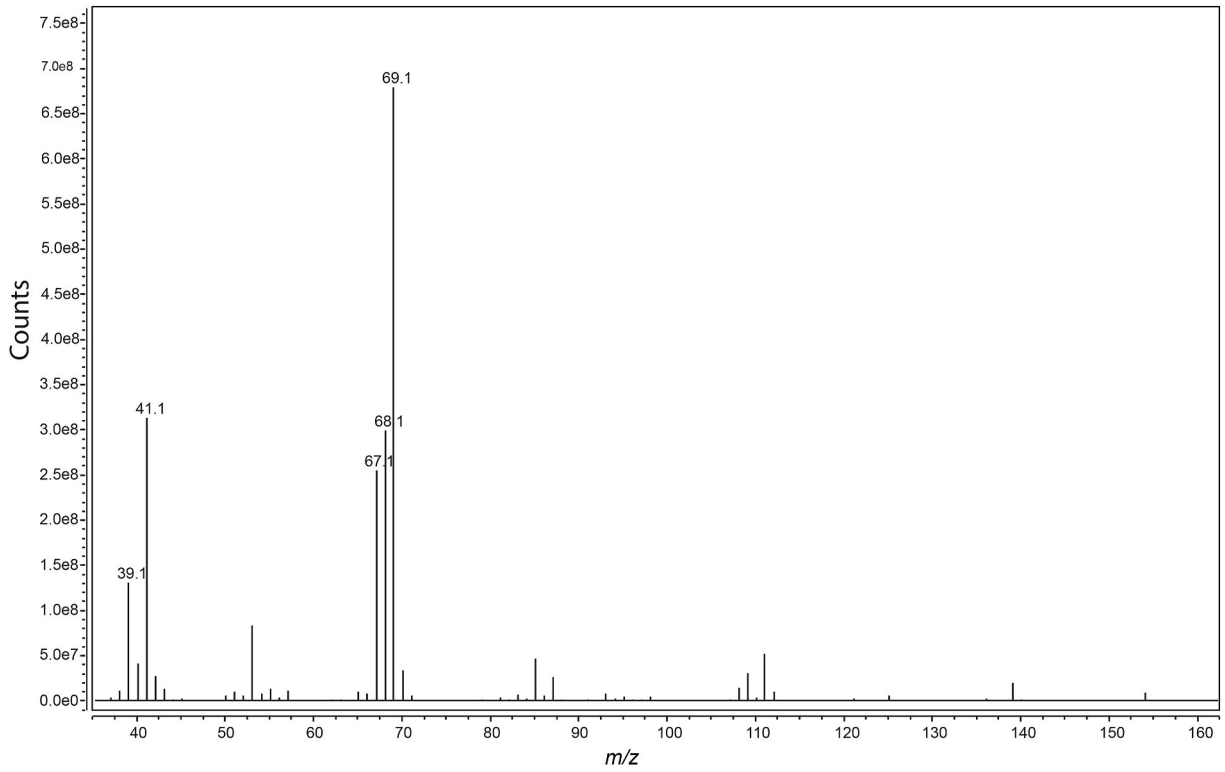

Fig. 6. Mass spectrum of the prenyl crotonate peak from a *Macrozamia miquelii* pollen cone sample (Thermo Scientific GC-MS run on TG-XLBMS nonpolar column). This has been reformatted for clarity. (See Supplementary Fig. S3 for the original mass spectrum.)

2.3.2. Variation in monoterpene emission rates among cones

The volatile emission rate across thermogenic events of the same cone (n = 4 pollen cones, each on a different plant, each sampled over six sequential thermogenic days in the field) revealed that the large differences in cone monoterpene emission rates were not the result of differences across cone stage or time of day, but rather to differences across individual cones in their emission rates of several monoterpene compounds. These were mainly α-pinene, α-terpinene and limonene (Fig. 9a), as is evident in the chromatograms of two cone samples that contrast one another in these monoterpene emissions (Figs. 4 and 5a). p-Cymene also varied in emission rate among cones but was highly correlated with limonene emission rate (Pearson $r = 0.91$, $P < 0.0001$), so it is not included in Fig. 9a for clarity. All other monoterpenes were minor components except for β-myrcene. Of the four cones tracked over six days, two were high emitters of these monoterpenes (e.g., maximum for α-terpinene, 29,979 and 9733 ng min^{-1}, Cones 1 and 2, respectively), and two barely emitted them (maximum for α-terpinene, 94 and 10 ng min^{-1}, Cones 3 and 4, respectively). These three compounds comprised >70% of the total volatile emissions from Cones 1 and 2 but ≤4% from Cones 3 and 4. Over the course of all the sample days, these percentages remained close to the same on each cone.

By contrast, emission rates of two other components, MBO and prenyl crotonate, were only about five-fold different across the four cones (Fig. 9b) during the same sample time period, and Cone 3 had the highest mean emission rate of both compounds. These two compounds comprised over 70% of the total emissions from Cones 3 and 4, but ≤7% from Cones 1 and 2. In addition, prenyl crotonate and MBO are present in all four cones at high levels (>800 ng min^{-1} at midday), and emission rates of both compounds increase between morning and midday samples.

Emission rates of β-myrcene, another monoterpene, varied less than ten-fold across the cones (data not shown). Together, these data demonstrate that plant-to-plant differences in emission rates of α-terpinene, α-pinene, limonene and p-cymene monoterpenes by cones is not simply a function of some cones being high emitters of all compounds.

Because two cones emit high levels and two cones very low levels of these four monoterpenes, the pattern could be interpreted as those monoterpenes being produced by the same terpene synthase (TPS). When we examined the emissions of all the pollen and receptive ovulate cones, six of 10 pollen and three of 14 receptive ovulate cones were high emitters of monoterpenes. However, three of the cones that were high emitters of α-pinene (>15,000 ng min^{-1} per sample) emitted very low α-terpinene levels (two cones emitted <115 ng min^{-1} and a third emitted ~ 2800 ng min^{-1}) (Supplementary Fig. S5a), demonstrating these two compounds' production are not always coupled. A similar pattern was found between limonene and α-pinene but there was a tight correlation between limonene and α-terpinene (Supplementary Fig. S5b, c).

2.3.3. Comparisons of volatiles across receptive and non-receptive ovulate cones

A significant difference was evident across receptive and non-receptive cones in the percentage that each compound contributed to the total volatile emissions. The compounds that contributed differentially across receptive and non-receptive ovulate cones were prenyl crotonate, α-pinene, prenyl acetate, β-caryophyllene, α-humulene, nonanal and limonene (Figs. 10 and 11), as well as the two ethyl-acetophenone compounds. Also, MBO was different across receptive and non-receptive cones. MBO was detected in all receptive ovulate cones at a mean emission rate of 250 ng min^{-1}, but was detected in only one non-receptive cone at trace level (Table 1). It could not be included in the ordination statistical test associated with Fig. 10 because all samples used in the test were not run on the polar column.

2.4. Attraction of pollinators

Field bioassays in 2018 indicated that thrips are not attracted to the two compounds tested, prenyl acetate and α-pinene, each one tested at

Table 2
Fragmentation data for two synthesized standards, prenyl crotonate and prenyl butyrate, each with data from a matching peak from a cone sample. The equivalent data for sample peak "*" (Fig. 5b) are also presented, to show the similarity to prenyl crotonate, especially, *m/z* 67, 68, 69, and likely molecular ion at 154. Dash (–) indicates 0 or <0.1% relative abundance.

m/z	Synthetic prenyl crotonate	Cone sample prenyl crotonate	Cone sample, peak "*"	Synthetic prenyl butyrate	Cone sample prenyl butyrate
	% Relative abundance				
39	17.2	19.3	8.8	18.1	8.9
40	5.5	6.1	2.2	7.8	4.4
41	42.0	46.2	24.1	64.0	46.1
42	3.6	4.0	–	8.7	6.7
43	1.8	2.0	–	72.1	65.3
44	0.2	0.2	–	2.8	4.0
45	0.3	0.4	–	0.8	2.4
53	10.9	12.3	7.4	18.9	13.4
54	1.1	1.2	–	2.0	1.3
55	1.8	2.0	0.7	4.2	5.8
56	0.4	0.5	0.6	1.3	1.3
57	1.4	1.6	0.6	2.9	5.4
65	1.3	1.5	0.8	0.1	1.2
66	0.9	1.1	0.6	1.6	1.3
67	33.2	37.5	22.2	67.4	50.2
68	39.5	44.1	39.8	89.1	89.7
69	100	100	100	64.3	54.2
70	4.8	5.0	4.1	4.8	3.3
71	0.7	0.8	–	100	100
72	–	–	–	4.4	4.5
85	6.2	7.0	0.3	7.5	6.3
86	0.8	0.9	–	1.5	2.6
87	3.5	3.8	–	0.2	–
89	–	–	–	27	2.1
108	1.9	2.1	1.6	–	–
109	4.1	4.4	1.5	–	–
111	6.9	7.7	2.2	–	–
112	1.3	1.5	1.3	–	–
114	–	–	–	1.8	1.9
128	–	–	–	0.3	0.1
139	2.6	2.9	1.0	–	–
154	1.2	1.3	1.2	–	–
156	–	–	–	1.7	2.2

10 μL per septum. Only two thrips were found on prenyl acetate traps, none on α-pinene traps, and one on the control traps (totals across four replicates and results from tests conducted on two days were combined). In 2019 assays, with a small volume of prenyl crotonate added to the standard bouquet (8 μL per septum), there was no significant difference among treatments in the number of trapped thrips at the end of the experiment (Fig. 12a, $F_{2,6} = 2.6$, $P = 0.108$)). When more prenyl crotonate was added to the standard mixture in a second experiment (at 40 μL per septum), significantly more thrips were captured on the prenyl crotonate treatment than on the non-prenyl crotonate treatment or the control (treatment effect, $F_{2,6} = 17.063$, $P = 0.0013$, Tukey's HSD, $\alpha < 0.05$; Fig. 12b). Many thrips were observed landing on the source pollen cones in the afternoon, so our treatments were competing with the host

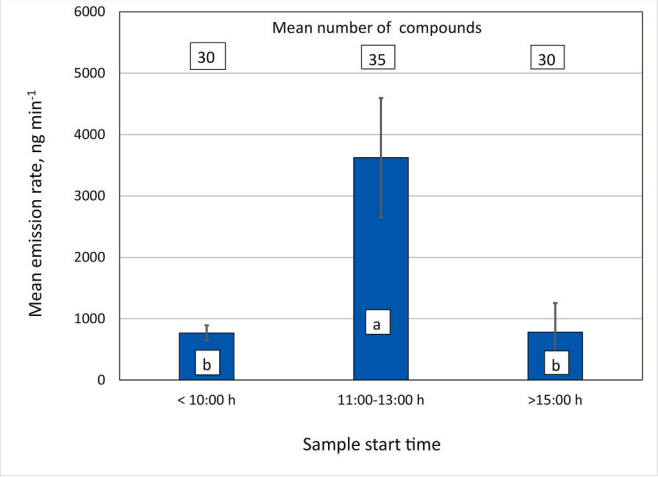

Fig. 8. Mean volatile emission rates (±1 s.e.) from three *Macrozamia miquelii* pollen cones, based on 30 min samples taken at one of three different times during one day: early thermogenesis in morning (before 10:00 h), peak thermogenesis (between 11:30–13:00 h), and late thermogenesis (>15:00 h). The mean number of compounds emitted during each period is presented above each. The midday sample start time was close to peak of thermogenesis. Bars with different letters are significantly different from one another (repeated measures test, and Bonferroni post hoc test of comparisons, $\alpha < 0.05$).

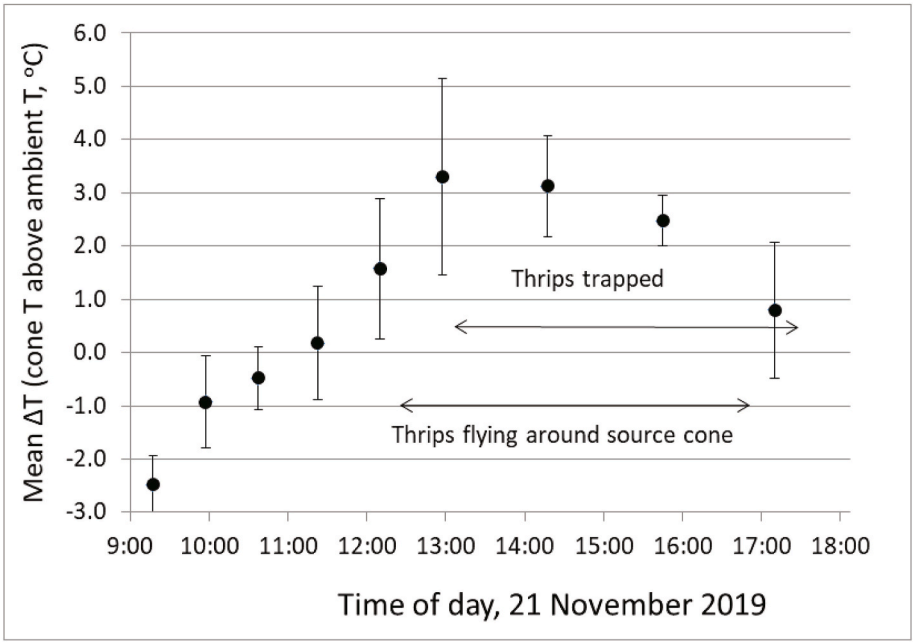

Fig. 7. Thermogenic temperature difference, ΔT (±1 s.e.), between pollen cones and their local ambient temperature (mean of four cones) in the field. The lower horizontal line indicates when the first thrips were observed leaving cones around noon and that they were observed flying around cones until about 16:00 h. The upper horizontal line indicates when the first thrips were captured on any treatment trap and the end time after which no more thrips were trapped that day. Ambient average was 26.3 °C at 09:15, 29.1 °C at 13:00 and 23.4 °C at 15:00 h.

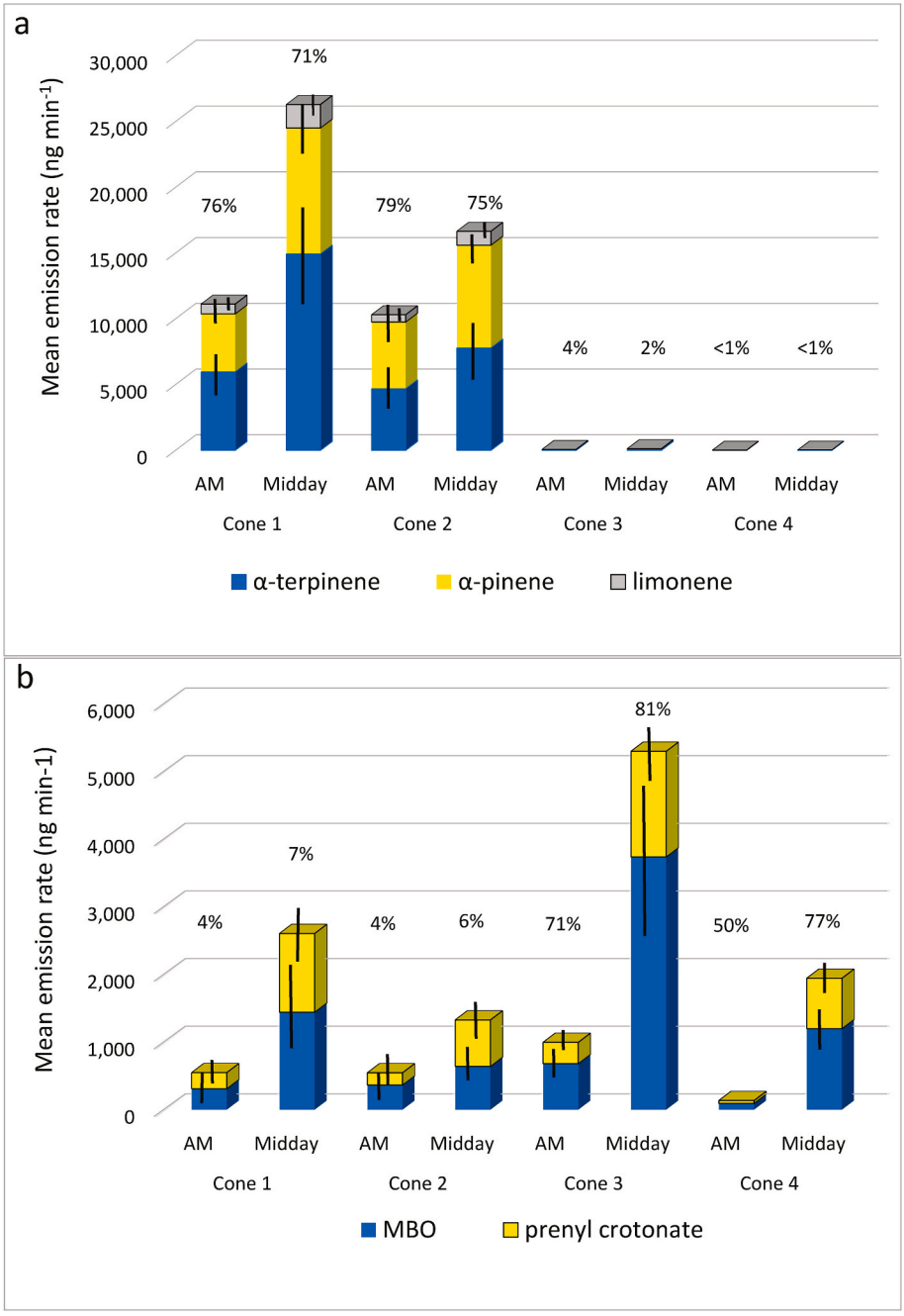

Fig. 9. Mean emission rates of major volatile components (±1 s.e., but not shown for those with very low emission rates) of four *Macrozamia miquelii* pollen cones. These were sampled in the field for 30 min over six days during pollination for both AM (morning sample start time, ~09:30 h) and midday samples (sample time started from ~11:30–13:30 h). The mean % contribution of these compounds to total emissions is reported above the bar graph of each cone and sample time. a) Emission rates of three monoterpenes, α-terpinene, α-pinene and limonene, which varied by > 5000 fold across cones (within the same sampling period). b) Emission rates of 2-methyl-but-3-en-2-ol (MBO), and an aliphatic ester, prenyl crotonate, with a five-fold difference or less across cones (within the same sampling period).

cones as well as other cones in the vicinity. The prenyl crotonate emission rate in the latter tests (463 ng min^{-1}) was slightly higher than its mean emission rate in the midday receptive ovulate cone samples (386 ng min^{-1}, Table 1) based on dynamic head space sampling of the bottle treatment apparatus.

In a Y-tube olfactometer experiment, both male and female thrips were attracted significantly to prenyl crotonate at the lower rate [binomial test, probability of going to prenyl crotonate vs control is equal to 0.5: 0.65 vs 0.35 (out of 143 thrips), $P < 0.002$; 0.67 vs 0.33 (out of 157 thrips), $P < 0.002$, $n = 14$ runs for both females and males, respectively with a 95% confidence interval of 0.66–0.998], but not to the higher rate [0.62 vs 0.38 (out of 103 thrips), $P = 0.064$; 0.55 vs 0.45 (out of 100 thrips) $P = 0.39$, $n = 9$ runs for both females and males, respectively), nor with males and female trials combined, $P = 0.14$, 95% confidence interval, 0.44 to 0.897]. There was no significant difference in response by sex ('prop test', $\chi^2 = 0.4$, $df = 1$, $P = 0.835$ for 1 μL test; $\chi^2 = 0.0.0023$, $df = 1$, $P = 0.96$ for the 5 μL test).

3. Discussion

In the following, we incorporate our major findings into the broader context of other research on cycad and floral volatiles. We deal with the timing of thermogenic events and its association with thrips behavior and volatile emissions, how *M. miquelii* cone volatiles differ from other cycad (even closely related ones) and plant volatiles, the attraction of *C. chadwicki* 1 to prenyl crotonate, and the implications for interpreting the evolution and divergence of *Cycadothrips chadwicki* cryptic species and their host cycads.

3.1. Thermogenesis and thrips pollinator movement

Macrozamia miquelii cones display a daily midday thermogenic event

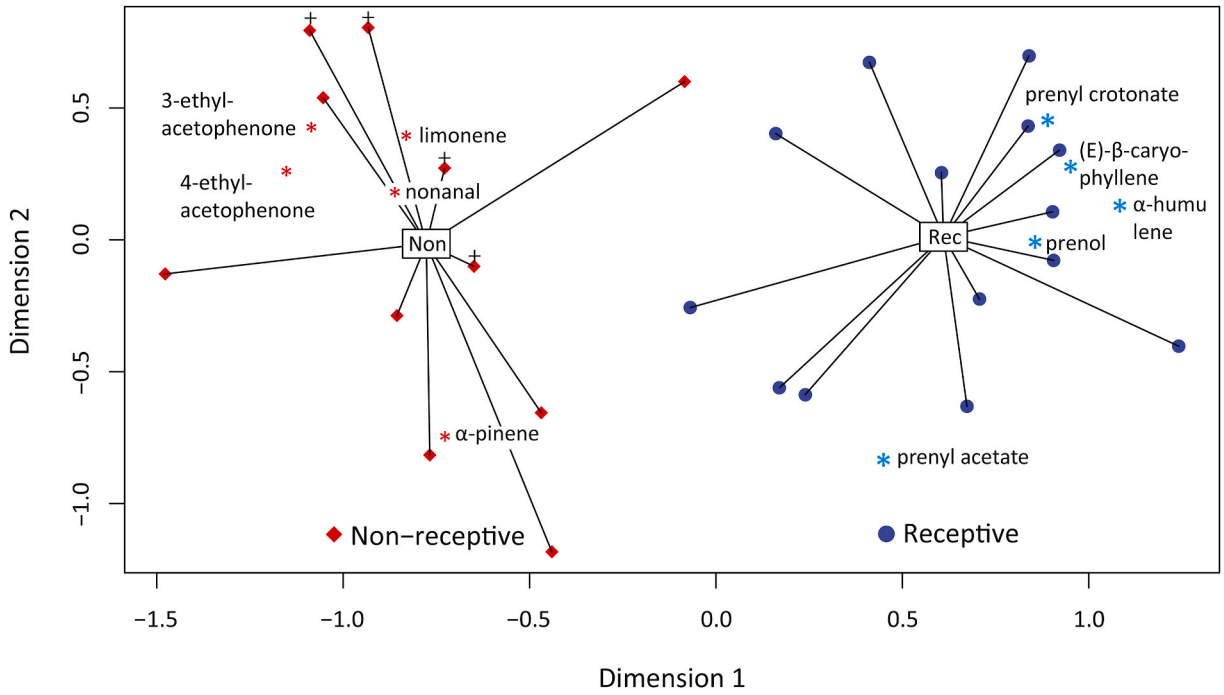

Fig. 10. Ordination of the volatile odor (% of each compound's contribution to the total emissions) across receptive (n = 14 samples) and non-receptive (n = 11 samples, 7 pre- and 4 post-receptive) ovulate cones of *Macrozamia miquelii*, by nonmetric multidimensional scaling (NMSD), Bray-Curtis similarity matrix. ANOSIM, Global R = 0.65, P < 0.0001, indicates a significant difference across groups, and the stress value = 0.11, K = 2, indicates a good fit of the data. There was no significant difference in the between-group multivariate dispersion test ('distance anova', P = 0.95). Each sample's position is indicated (by a dot for receptive (Rec.) or diamond for non-receptive (Non)) at the end of a spoke from the group's center. Post-receptive and pre-receptive cones are grouped together as non-receptive. The position of each post-receptive cone sample is indicated with a '+' over its diamond. The exact location of each compound that was significantly associated with each group (P < 0.04, Indicator Species Analysis in R 'indicspecies') is indicated by a '*' next to the compound's name.

(Fig. 7), with a concomitant increase in cone volatile emission rates of major compounds (Figs. 8 and 9). These results are similar to those of other *Cycadothrips*-pollinated *Macrozamia* species in having a daytime thermogenic peak compared with *Macrozamia* species pollinated by *Tranes* weevils, which have an evening thermogenic peak (Terry et al., 2004 a,b). Thus, thermogenic timing is not a feature that differs across the cycad species that host different cryptic species of *C. chadwicki*. Thrips begin to leave cone cones when thermogenesis begins to peak and continue their movement through the afternoon (Fig. 7). This pattern of emigration contrasts with that of the thrips pollinators of *M. lucida* and southern *M. macleayi*, which leave cones en masse over a 15–30 min period (Terry et al., 2004b) near the peak of thermogenesis.

This means that we cannot say that this *M. miquelii* pollination system operates in a 'push-pull' manner as in *M. lucida* and *M. macleayi* from southeastern Qld (Terry et al., 2004b, 2007b, 2014). In these southern Qld species, β-myrcene (together with thermogenic heating and associated high humidity) expels thrips from thermogenic cones, and emission rates of β-myrcene are more than 20 times higher than the rates of β-myrcene emission from *M. miquelii* cones (Terry et al., 2007a, 2014). Further, *M. miquelii* thrips were not attracted to β-myrcene at levels emitted by non-receptive ovulate cones (maximum of 73 ng min^{-1}, Table 1) nor were they attracted to the standard mix treatments that included β-myrcene (368 ng min^{-1} assessed from head space sampling of bottle trap apparatus). More significantly, in different bioassays, these thrips were not attracted to traps in the field that included *M. lucida* or *M. macleayi* cones emitting very high levels of β-myrcene (Terry et al. unpublished). Whether any of the compounds emitted by *M. miquelii* or the heating of the cones alone is involved with the 'push' aspect of pollination is unclear. Only a few cycad studies include sufficiently detailed behavioral observations to determine confidently whether push-pull is part of the pollination mechanism, but it clearly is in *Encephalartos ghellinckii* and *Zamia furfuracea* (Salzman et al., 2020; Suinyuy and Johnson, 2020), and certainly not so for *E. villosus* (Suinyuy et al., 2013a).

3.2. *Macrozamia miquelii* cone volatiles compared with those of other cycads

Only about 9% of the ~350 recognized species of cycads have had their cone volatile chemistry analyzed, so the published diversity of cycad chemistry is surely not representative. Nonetheless, several common elements in cone odors, as well as unusual findings, have been revealed in this study. Since so many more cycads await investigation of their cone volatiles, we may yet see other species with similar compounds.

1. Both pollen and receptive ovulate cones of *M. miquelii* emit a similar array of compounds to one another, the dominant compounds are the same across the sexes, and pollen cone emission rates are generally higher than those in ovulate cones (Table 1), as in other *Macrozamia* and even some species in other cycad genera (Azuma and Kono, 2006; Suinyuy et al., 2013a; Suinyuy and Johnson, 2018; Terry et al., 2004b; Wallenius et al., 2012). Some receptive ovulate cones of *M. miquelii* did, however, have a higher emission rate than some pollen cones (Table 1).
2. Almost all cycads, and all *Macrozamia* species including *M. miquelii*, produce monoterpenes, with the known exceptions being *Z. pumila* (now *Z. integrifolia* L. F.) (Pellmyr et al., 1991) and *Cycas revoluta* (Azuma and Kono, 2006). All *Macrozamia* species investigated produce some β-myrcene, but with considerable interspecies variation in the relative levels (Terry et al., 2004a, 2004b, 2008). *M. miquelii* also emits sesquiterpenes, as does *M. platyrhachis* among the *Macrozamia* species studied, and (E)-β-caryophyllene and α-humulene are also common to both species (Terry et al., 2008) suggesting they are produced by the same TPS enzyme, as has been reported in some angiosperms (Booth et al., 2017; Köllner et al., 2008.).

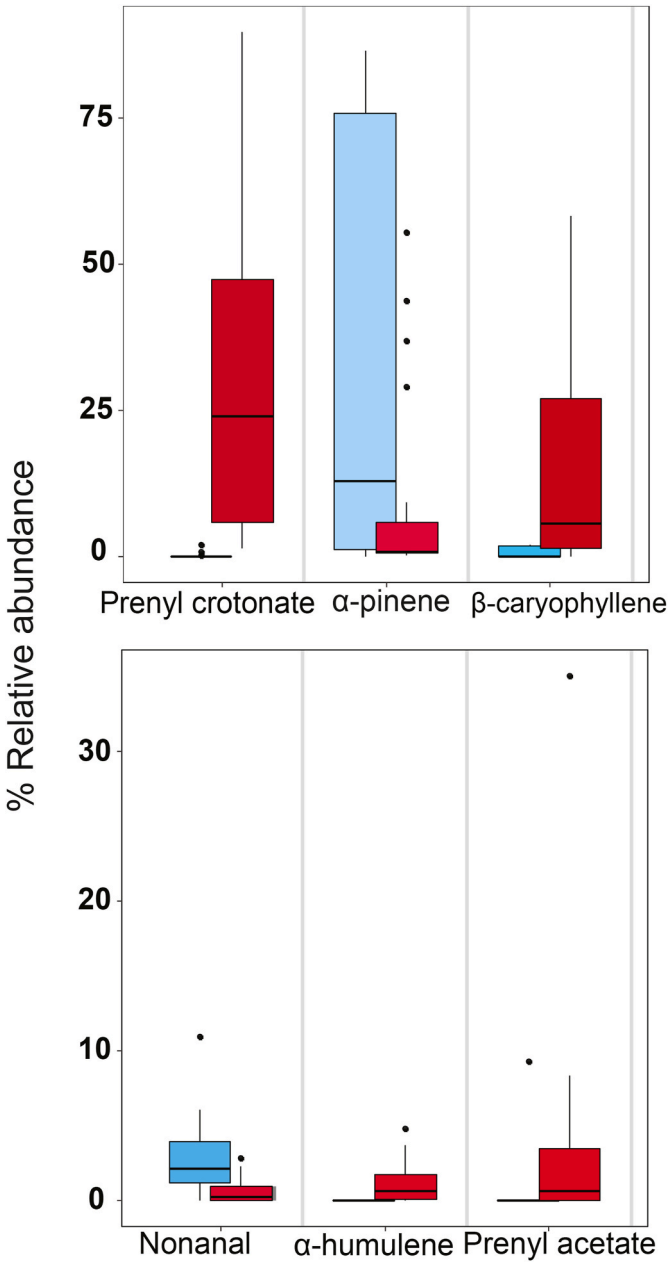

Fig. 11. The relative contribution (% of compound's emissions to the total volatile emissions of a sample) of six components that were statistically associated with either receptive (red, dark bar) or non-receptive (blue bar) ovulate cones, based on the results of the Indicator species analysis. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Sesquiterpenes, mainly (E)-β-caryophyllene, have been detected in some species of *Encephalartos, Zamia* and *Stangeria* (Pellmyr et al., 1991; Procheş and Johnson, 2009; Suinyuy et al., 2010, 2012, 2013b). The role of the sesquiterpene compounds remains unclear.

3. Clear plant-to-plant differences in the production of four monoterpenes, α-terpinene, α-pinene, p-cymene, and limonene, were evident, with some pollen cones emitting them at very high rates, and others only at low levels, or even just traces, regardless of cone stage (Fig. 9). Other major components did not show such extreme variation. Such large intra-population differences in production of compounds have not been reported in other cycads. Intraspecific variation in volatile emissions, both quantitative and qualitative, in plants across populations is common and may be the result of various influences including selection by pollinators, natural enemies, genetic drift, or physiological status, and such variation is not uncommon even within populations (see Delle-Vedove et al., 2017). Examples of intra-population variation have been reported in some orchid floral volatiles, with presence/absence of some compounds across different plants, perhaps because the odorants involved do not have a pollination, defensive or protective function, and are therefore not exposed to selection (Dormont et al., 2019). Pleiotropic or epigenetic changes could, under such circumstances, affect the biosynthetic enzymes or regulatory genes (Gang, 2005). This implies that the four monoterpenes involved are not functional in *M. miquelii* pollination, and results of field bioassays revealed that none these compounds attracted *Cycadothrips*, when tested alone (α-pinene) or as part of the standard mix.
4. Five of the *M. miquelii* cone volatile components have not been reported previously from any cycad. Two of them, prenyl alcohol and MBO (3-methylbut-2-en-1-ol and 2-methylbut-3-en-2-ol, respectively), are hemiterpenes. The three others are prenyl esters. Of these, prenyl acetate is found in most pollen and receptive ovulate cones at moderate levels, and prenyl butyrate is a minor component and relatively uncommon (Table 1).

Prenyl crotonate (3-methylbut-2-enyl (E)-but-2-enoate), by contrast, is a major component representing 70–84% of total emissions in many pollen cone volatile samples and up to 67% in some receptive ovulate cone volatile samples. It is one of the few compounds that was detected in all pollen and receptive ovulate cones (Table 1) at moderate to high emission levels in the midday samples (e.g., Fig. 9b), whereas this was not the case with the monoterpenes (Fig. 9a). That it was detected at trace levels in post-receptive cones, and then only three of the four, and not detected at all in the seven pre-receptive cone samples (Table 1), implies functionality. Indeed, prenyl crotonate has been confirmed as an attractant for the specific pollinator of *M. miquelii*, *C. chadwicki* 1 (Fig. 12). This information reinforces the interpretation that this volatile has been selected for its chemical mediation role with respect to the specific pollinator of this plant. These results not only substantiate the *M. miquelii* thrips pollinator being a different species to those associated with *M. lucida* and southern Qld *M. macleayi*, *C. chadwicki* 4, but also suggest that the host plant and pollinator specificity in these *Macrozamia* species is associated with major shifts in the compounds that mediate the interactions. Investigating these factors in the other species in relevant clades is crucial to determining the basis of their divergence, as elaborated in section 4.

3.3. The compounds newly reported from cycads

Prenyl alcohol and MBO, along with isoprene (another, and well known hemiterpene), are derived from dimethylallyl pyrophosphate (DMAPP) through the mevalonate (MVA) or the methylerythritol 4-phosphate (MEP) pathways (Zeidler and Lichtenthaler, 2001; Zhao et al., 2013; Zheng et al., 2013). Prenyl alcohol is found in a wide range of plants (Good Scents Company, 2020b). MBO has been found in insect gut fungal microbes and in insect sex and alarm pheromones (Wheeler et al., 1983; Zhang et al., 2012; Zhao et al., 2015). Also, it is produced by aspen, spruce, and pine trees (Gray et al., 2003, 2011; Zhao et al., 2015), where it has been proposed as having a similar protective function as isoprene. These compounds were included in the mixture of standards in our bioassay experiments and did not attract the thrips pollinator.

Prenyl butyrate is a male aggregation pheromone of the heteropteran *Thaumastocoris peregrinus,* a *Eucalyptus* feeder Calvo et al. (2018); González et al. (2012); Groba et al. (2019); Martins et al. (2012). It may be unusual in plants; it was not reported in the extensive review of floral scent compounds by Knudsen et al. (2006). It has, however, been reported since in the volatiles of the Chinese dwarf cherry (Ye et al., 2017). Prenyl acetate, by contrast, has been reported from many natural sources including plants (Pherobase, 2021a; Ye et al., 2017). Prenyl

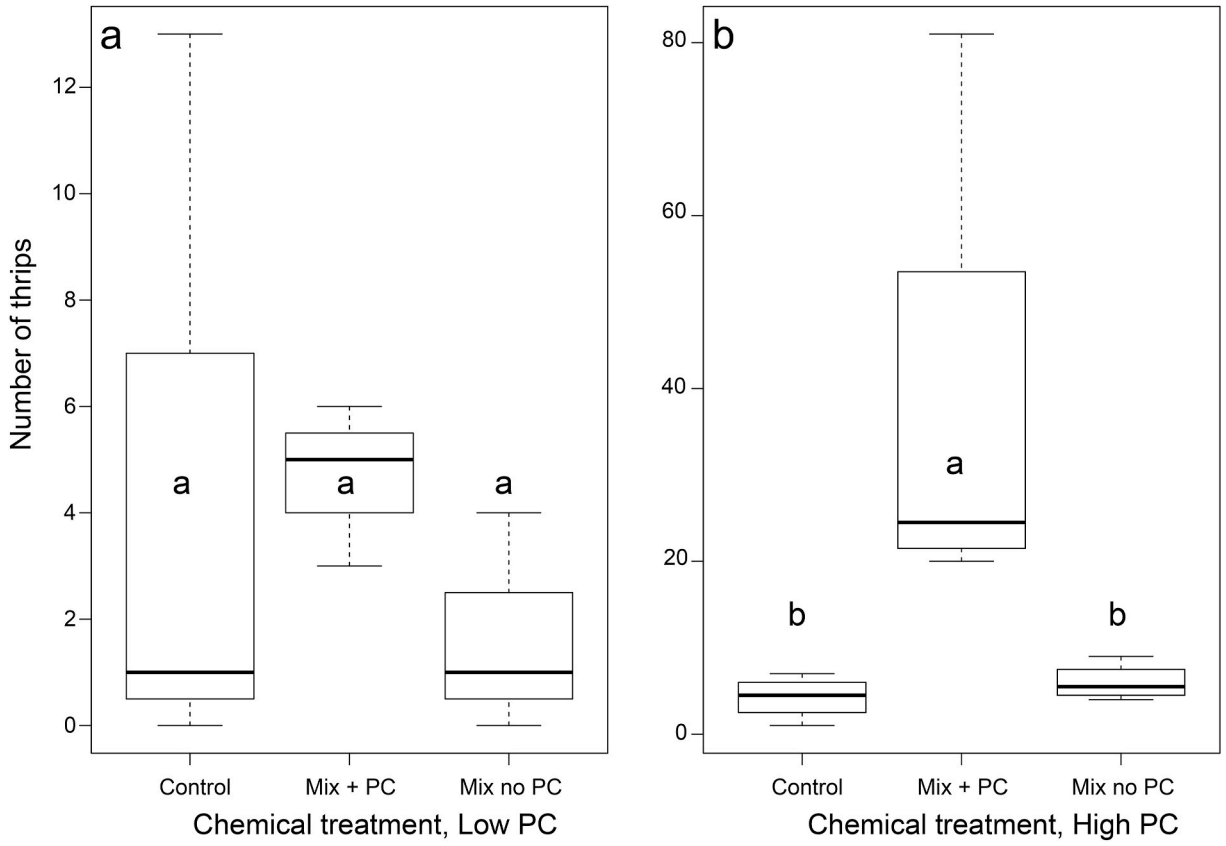

Fig. 12. Boxplots of the number of thrips caught per trap in field bioassays (black horizontal bar represents the median of four replicates). Each bioassay included rubber septa infused with: dichloromethane (DCM) evaporated solvent only (Control), a mixture of standards (see Supplementary Table S1) in DCM plus prenyl crotonate (Mix + PC), and the same mixture without prenyl crotonate (Mix no PC). a) Test with low rate of prenyl crotonate, 8 μL per septum, added to the standard mixture. b) Test with a much higher rate of prenyl crotonate, 40 μL per septum, added to the standard mixture, which attracted significantly more thrips in the Mix + PC treatment. Within each experiment's boxplot, different letters across treatments indicate significant differences from one another, $\alpha < 0.05$, Tukey's HSD).

acetate did not attract thrips in our field bioassay. The functional significance of these two prenyl esters in *M. miquelii* remains unknown.

Prenyl crotonate, the other confirmed prenyl ester, has not been reported from any natural source in all of our literature searches (Knudsen et al., 2006; SciFinder; Web of Science). This compound was reported in a laboratory study of a novel method of ester synthesis (Yadav et al., 1998), but no spectroscopic proof of structure has been published. A CAS number has been assigned to prenyl crotonate (SciFinder). Thus, our study is the first to report this ester's occurrence in nature, to characterize its structure spectroscopically, and to demonstrate a biological function.

Other esters of crotonic acid, the ethyl ester in particular, have been reported as possible attractants of Diptera (e.g., Jayanthi et al., 2012; George et al., 1986), and this ester also features in the volatile signature of more than 150 plant species (Pherobase, 2021b). In contrast, the prenyl ester of crotonic acid is unique and is one of very few examples of volatile prenyl esters found in nature. Two others are prenyl butyrate and the quite common prenyl acetate, both present in *M. miquelii*. In addition, we have established the presence of another compound (currently unidentified) very closely related to prenyl crotonate (Fig. 5b, compound marked '*', and Table 2) and strong indications that trace quantities represented by some of the peaks present in Fig. 5b are also related.

4. Conclusion

Our confirmation of a new chemical compound, prenyl crotonate, emitted by *M. miquelii* cycad cones to attract their thrips pollinator, raises questions relevant to understanding the evolutionary diversification of these thrips obligate pollinators and their plant hosts. How do the other cryptic species of the *C. chadwicki* complex respond to the cone odors of *M. miquelii* which contain many of the monoterpenes common to most other *Macrozamia* cycads? If the cryptic species *C. chadwicki* 1, alone, is attracted to *M. miquelii* cone volatiles, that is to prenyl crotonate, this pollination system would represent an example of a single compound species-specific cue involved in a specialist pollinator's attraction (Chen et al., 2009; Raguso, 2008). This cone odorant could, therefore, be involved in the divergence that occurred within the lineages of these two species. If so, is prenyl crotonate a novel compound recently added to the plant's genetic odor producing repertoire? Or, is this chemical a relictual volatile cone trait that has been lost in other cycads? If this is so, and prenyl crotonate had originally a non-pollination function, then it may have been subsequently co-opted for pollination (Armbruster, 1997). Knowledge of the molecular and genetic basis for production of the prenyl esters in these cycads, and an appreciation of any other role they may have in these plants, would be required to help answer these questions.

An understanding of whether there is congruence in the phylogenetic relationships of the pollinator and their *Macrozamia* hosts is also needed to answer broader questions concerning the diversification of these two taxa. In other obligate pollination mutualisms (fig-fig wasps, yucca-yucca moths, and leafflower), coevolution and co-speciation have been rejected as explanations in favor of changes after geographic isolation, or allopatric speciation, and usually underpinned by host shifts (Hembry and Althoff, 2016). This has also been demonstrated for the five *C. chadwicki* cryptic species, whose estimated divergence times across the different species range from 1.1 to 7.3 Mya (Brookes et al., 2015). Furthermore, if each of the cryptic species in the *C. chadwicki*

species complex is attracted to only its own regional host plants in response to species-specific signals, this would provide further evidence of the species status of each and explain the reproductive independence of their cycad hosts, as seen in some orchids (Dormont et al., 2019; Joffard et al., 2016; Vereecken et al., 2011). Similar odor-related diversification has been noted among *Encephalartos* cycad cones and their specialist pollinators (Suinyuy et al., 2012; Suinyuy and Johnson, 2018), suggesting that cycads on different continents have undergone diversification in similar ways to one another.

5. Experimental

5.1. Study species and site

Macrozamia miquelii is distributed across the Port Curtis District of Central Queensland, Australia, from near Mt. Larcom in the south to north of Byfield (Fig. 1) (Jones et al., 2001). The plants grow in open eucalypt forest and woodland, from elevations of 10 m–540 m a.s.l., primarily on stony soils, but also in deep sand near Byfield. *C. chadwicki* 1 is the only pollinator that has been found on *M. miquelii* (Brookes et al., 2015).

M. miquelii plants were investigated at Mt. Archer National Park (near Rockhampton) where hundreds of plants can be found in open eucalypt forest at the summit and on nearby slopes (Fig. 1 inset). Cones are produced for a pollination period that runs from October through November, and seeds mature in March and April (Jones et al., 2001). Voucher specimens of *M. miquelii* are available at the Queensland Herbarium, Brisbane Botanic Garden, PIF12252B (BRI), and C. *chadwicki* from *Macrozamia miquelii* have been deposited in the insect collection of the Queensland Department of Agriculture and Fisheries, Ecosciences Precinct, Dutton Park, Qld, catalogue # 0–168522, DJT152, and in the Australian National Insect Collection, CSIRO Black Mountain, ACT, Australia.

5.2. Cone volatile collection and measurement of cone thermogenesis

During the coning seasons of 2015–2019, we used dynamic headspace techniques developed previously to collect cone volatiles from other *Macrozamia* species (Terry et al., 2004a). A Pasteur pipette packed with 300 mg of Porapaq Q (50–80 mesh) was used as a volatile adsorbent device. This pipette was enclosed within an inert oven cooking bag (35 × 48 cm) placed over a cone. Air space was minimized, and the pipette then connected by tubing to a portable battery powered pump (Sensidyne Gilian LFS-113DC Low Flow Sampler, Florida, USA, or Ametek, alpha 2 model, Ametek, Inc. Berwyn, PA, USA). Pumps were calibrated to a flow rate of 100 mL min^{-1}, and cones were sampled for 30 min (up to 45 min for initial samples of ovulate cones). Adsorbed volatiles were desorbed with HPLC grade DCM, concentrated to 500 μL in GC-autosampler vials under a stream of high purity N_2, and then stored at −18 °C until analyzed. Control samples were taken of a Porapak tube and of the field bioassay bottle trapping device (including septa, see section 5.3), both were inside a volatile collecting bag, the same size as used on cones.

Initial temperature measurements indicated that pollen and receptive ovulate cones undergo a midday thermogenic event. To determine whether volatile emission rates are highest close to the peak of thermogenic temperatures, we sampled intact cones at three times during the day in the field. Three pollen cones (one in 2015 and two in 2016) were sampled with sampling start-times during the morning (~9:00 to 10:00 h), at midday (11:30 to 13:00 h) and the late afternoon (>15:00 h) (on the same day). Collecting bags were removed from cones between samples. Because ovulate cones follow the same volatile emission patterns as their conspecific pollen cones in other thrips-pollinated *Macrozamia* species (Terry et al., 2004a,b, and unpublished data), we tested only one ovulate cone at all three times, and two others were sampled either morning and midday, or midday and afternoon. We could then establish the best time of day to sample. The temperature of each cone was also monitored (see section 5.3).

Considerable variation was detected in cone emission rates across cones from different plants. We tested whether this was due to differences across cones in their total emissions of all compounds (or particular compounds) or to cone dehiscence stage (% of sporangia that have dehisced their pollen (Roemer et al., 2017)), as cones age through their pollination phase. In 2018, we selected four pollen cones, similar in size and phenology (% of sporangia dehisced) to one another, on different plants to monitor in the field. Cone samples started simultaneously, at <15% of pollen dehiscence on the first sample day, when large numbers of thrips were already present. We collected a morning sample (prior to or simultaneously with the beginning of a thermogenic event) on three days and a midday sample (peak thermogenesis) on four days over a six day period. Bags were removed between samples. All pollen sporangia were completely dehisced by the last sample day (some pollen and thrips were still present and cones remained thermogenic).

To compare volatile profiles and emission rates across receptive ovulate cones and non-receptive ones, we selected cones to sample based on our initial observations on their morphology and smell. Pre-receptive cones have no gaps between sporophylls (Fig. 2a), preventing insect entry, and on some cones a faint cinnamon-like odor is present. Receptive cones become flexible (they can be twisted or bent slightly), small gaps (<~ 3 mm) are visible between sporophylls (Fig. 2b), and at midday they are thermogenic and emit an odor similar to that of conspecific dehiscing pollen cones. In post-receptive ovulate cones, the expanding ovules become visible in widening gaps between sporophylls (Fig. 2c and d), and there is no detectable odor or thermogenic temperature. Cone receptivity was also assessed independently by testing for thrips attraction with a ring of clear sticky tape (~3 cm wide, facing outward) place around each cone's girth. Ovulate cones of each category (14 receptive, five pre-receptive, and four post-receptive cones) were sampled for volatiles around midday during the coning seasons of 2016, 2018 and 2019.

5.3. Behavioral bioassays (field and laboratory)

To determine whether a single volatile compound or a mixture of compounds from *M. miquelii* cones attracts *Cycadothrips*, we developed a device to simulate the emission of a cone's volatiles for field testing (Fig. 2e). We drilled a standard pattern of 10 holes (~2 mm diameter) on each of the four sides of a clean 2L plastic milk bottle. This pattern was used as a template to punch holes in clear double-sided sticky traps. A trap was attached to each side of a bottle with holes matching those of the bottle (to allow escape of volatiles). Thrips were trapped on the sticky trap and a few entered the holes of the bottle and remained there. A battery powered fish tank pump forced air (~1500 mL min^{-1}) through a tube inserted into a hole in the lid of the bottle so air then flowed out through the holes. We attached wires on the inside of the lid, and these extended into the milk bottle to hold a rubber septum (Suba-Seal R silicone rubber septa 9, 8 mm I.D., Sigma-Aldrich, Australia). A volatile treatment was prepared by adding 750 μL of DCM solvent alone (control) or DCM plus a single chemical standard (see below) or a mixture of standards (synthesized or purchased, see Supplementary Table S1 for amounts of each standard added to make this mixture) in DCM to each septum. Once the DCM had evaporated, two septa of the same treatment were suspended on a loop tip of wire and inserted into the bottle, which was attached to a stake. Each trap device was aligned to the height of a pollen cone growing nearby, and which provided a local source of thrips.

Several compounds identified as being different across receptive and non-receptive cones in section 2.3.3 were not tested individually in the field bioassay due to lack of sufficient quantity of standard or due to low emission rates in both cone groups (nonanal, α-humulene, limonene). In addition, (E)-β-caryophyllene was excluded because it was present in both groups but did not attract thrips in those non-receptive cones that had (E)-β-caryophyllene emission rates similar to those of some

receptive cones. In 2018, treatments involved either α-pinene (10 μL per septum) or prenyl acetate (10 μL per septum), and a solvent control. In 2019 the treatments were the standard mixture without prenyl crotonate, the standard mixture plus prenyl crotonate, and a solvent control. On the first test day, 8 μL of prenyl crotonate was added to the standard mixture for each septum. For the second day, 40 μL of prenyl crotonate was added per septum.

Each field bioassay was arranged as a randomized complete block of four blocks. Each block was associated with a plant that had a dehiscing pollen cone, ~30–50% dehisced, to provide a source of thrips. Each treatment and control was placed 1.5 m from the source cone, and each treatment was separated from other treatments and the control by at least 1.5 m. To calibrate emission rates of chemical standards from the test device we performed separate experiments that used the dynamic head space technique with a volatile collecting bag (same size as used for cones) placed over the bottle device. Air was supplied to the bottle device from the fish tank pump, and withdrawn from the bag into a Porapaq tube by a volatile sampling pump for 30 min. We also sampled the device without any chemical standards as a control.

During field bioassays, each of which lasted one day, we measured the temperature of each source pollen cone and the adjacent shaded ambient temperature hourly, beginning around 09:00 h until ~17:00 h, to determine the timing of peak thermogenesis. A type K thermocouple was inserted into each cone's core about mid-way along its length, and temperature was measured with a Fluke 500 data logger. We also visually observed each source cone, every 30 min, for the times when thrips first left pollen cones and when they were no longer flying around cones. In addition, at each treatment's sticky trap, we counted thrips to determine when thrips were first trapped and when no more thrips were trapped for the day.

To confirm the timing of thermogenesis under controlled conditions, we used the same thermocouple equipment as in the field to measure temperatures of excised pollen cones in an environmental chamber. (Excised cones continue to undergo daily thermogenic events in other species (Tang, 1987; Roemer et al., 2008)). The cones were kept at a regimen of 06:00/18:00 h, 25/15 °C, light/dark. Temperatures were measured continuously over two days from 08:00 to 17:00 h.

In the laboratory, we used a Y-tube olfactometer to test for *Cycadothrips* attraction to chemicals, as described previously (Terry et al., 2014). An excised pollen cone was kept in a mesh cage at 15 °C as a source of thrips until testing, when the cage was moved to a room at 22 °C. Thrips were captured and held separately by sex. They were tested in a dark windowless room, in nine replicates (for the high rate) and 14 replicates (at the low rate) of 10–14 males or females, for movement into arms of the Y-tube. The apparatus was illuminated evenly from above. Groups were given up to 5 min to respond. We tested a control (1 mL of castor oil) against prenyl crotonate (1 μL or 5 μL in castor oil) and reversed their positions after three experiments. Equipment was rinsed with acetone and dried when switching the Y-tube arms, or when changing a treatment.

5.4. Cone volatile analysis

Over the five years of this study, different GC-MS instruments were available for use. During the 2016 and 2017 coning seasons, a Shimadzu GCMS-QP2010 Ultra Gas Chromatograph Mass Spectrometer at the University of Queensland was used, equipped with a Zebron ZB-5 column from Phenomenex, Inc., Canada, with dimensions 30 m × 0.25 mm ID by 0.25 μm film thickness. Some samples from 2015, 2016 and 2017, as well as all samples from the 2018 and 2019 coning seasons, were analyzed on an Agilent QQQ Model 7000 GCMS at the University of Utah, Department of Chemistry Mass Spectrometry Laboratory fitted with an Agilent DB-5 column, and some samples were also analyzed using a Carbowax DB column, and both were of identical dimensions to that described above. All analyses were performed by splitless injections of 1 μL, with a helium flow rate of 1.5 mL. Oven temperature for the DB-5 column or ZB-5 equivalent was programmed from 40 to 260 °C at 10 °C min^{-1}, with a 2 min initial delay. The oven temperature for the Carbowax analyses was programmed from 40 to 67 °C at 10 °C min^{-1}, with an initial hold for 3 min and then at 5 °C min^{-1} to 190 °C. A third instrument, a Thermo Scientific ISQ 7000, fitted with a proprietary column (TG-XLBMS) of identical dimensions to the others, was used for analysis of synthesized standards and selected samples. The mass spectra used for confirmation of the structure of the major unknown compound were obtained on this instrument. Each instrument was operated at 70eV in EI mode and scanned from 35 to 350 mass units, following a 2 min solvent delay. A homologous series of alkanes was also analyzed to enable the calculation of Kovat's retention index (RI) for each compound.

Peak identities were tentatively determined using either Shimadzu software GCMS LabSolutions GCMSSolution 4.20 Shimadzu Corporation, with GCMS solutions library database NIST11 and NIST 11s or Agilent's MassHunter Workstation Software Qualitative Analysis vers. B.03.01 Agilent Technologies, Inc. 2009, with mass spectrum search NIST MS Program. Confirmation of a compound was based on its mass spectrum matching that of a compound identified in a database library and its calculated RI matching published data (Babushok et al., 2011, the NIST or PUBCHEM websites, or published work, as specified in the results). Further full confirmation of the identity of most peaks was based upon comparison of retention times and mass spectra with purchased standards or with compounds synthesized and characterized as part of this study. Nonane and in some samples, dodecane, were added to samples, each at 40 ng uL^{-1} for use as internal standards. Integrated peaks were quantified based on equivalence to ng of nonane or dodecane internal standard and then converted to ng min^{-1} emission rate based on the sample time. The emission rate of each compound and its percent contribution to the total volatile emissions in each sample were calculated.

5.5. Chemical synthesis

The four prenyl esters were synthesized at the Synthetic and Medicinal Chemical Core at the University of Utah.

Prenyl crotonate (3-methylbut-2-enyl (E)-but-2-enoate; Fig. 4; Supplementary Fig. S1a) was prepared as follows. 3-methyl-2-buten-1-ol (1.35 mL, 13.30 mmol) was added dropwise to a solution of (E)-crotonic acid (1.021 g, 11.86 mmol), *N,N′*-Dicyclohexylcarbodiimide (2.506 g, 12.15 mmol), and *N,N*-dimethylaminopyridine (72 mg, 0.59 mmol) in dry dichloromethane (DCM) (60 mL) at room temperature under N_2, and then stirred at room temperature for 24 h. The suspension was then filtered through Celite, and the filtrate was dry-loaded onto Celite (8 g) and purified by flash chromatography on silica gel eluting with DCM/hexanes to give the target compound as a colorless oil (910 mg, 5.90 mmol). RI (DB-5/Carbowax) 1161/1563, EI-MS, 70eV, *m/z* (rel. int.): 154 (1.2, M^+), 139 (2.6), 111(6.7), 109 (4.1), 87 (3.5), 85 (6.2), 70 (4.8), 69 (100), 68 (39.5), 67 (33.2), 53 (10.9), 41 (42), 39 (17.2) (Supplementary Fig. S2b; HRESIMS: $[M + Na]^+$ *m/z* 177.0892, calcd. for $[C9H14O2Na]^+$ 177.0892. (Δ = + 0.6 ppm) (Supplementary Fig. S6; ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm): 6.97 (dq, *J* = 15.4, 6.9 Hz, 1 H), 5.81–5.88 (m, 1 H), 5.33–5.40 (m, 1 H), 4.62 (d, *J* = 7.3 Hz, 2 H), 1.87 (dd, *J* = 6.8, 1.5 Hz, 3 H), 1.76 (d, *J* = 1.0 Hz, 3 H), 1.72 (s, 3 H) (Supplementary Fig. S7a). ^{13}C NMR (126 MHz, $CDCl_3$): δ (ppm): 18.05; 18.13; 25.88; 61.20; 111.88; 122.88; 139.01; 144.60; 166.71 (Supplementary Fig. S7b). The high resolution mass spectrum was obtained on a Waters Xevo G2-S Q-TOF with Acquity UPLC. A 0.05% methanolic solution was infused into the instrument at 5 μL/min, and spectra acquired over a 1 min period. ^{1}H NMR spectra were acquired on a Varian VXR 500 and ^{13}C NMR spectra on Bruker NEO500 MHz.

Prenyl methylacrylate (3-methylbut-2-enyl 2-methylprop-2-enoate; Supplementary Fig. S1b) was synthesized as follows. Methacrylic anhydride (2.0 mL, 13.42 mmol) was added dropwise to a mixture of 3-methyl-2-buten-1-ol (1.36 mL, 13.39 mmol), triethylamine (2.25 mL,

16.14 mmol), and *N,N*-dimethylaminopyridine (82 mg, 0.67 mmol) in dry DCM (60 mL) at room temperature under N_2, and then stirred at room temperature for 18 h. The reaction mixture was directly dry-loaded onto Celite (9 g) and purified by flash chromatography on silica gel eluting with DCM/hexanes to give the target compound as a colorless oil (1.268 g, 8.22 mmol). RI (DB-5/Carbowax 1096/1428. EI-MS, 70eV, *m/z* (rel. int.): 156 (1.5, M^+), 111 (13.9), 109 (16.5), 108 (8.7), 85 (5.9), 70 (7.7), 69 (100), 68 (46.3), 67 (50.1), 57 (3.6), 55 (2.8), 54 (1.7), 53 (15.6), 43 (3.4), 42 (6.1), 41 (83.5), 40 (8.2), 39 (24.2); ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm): 5.31–5.37 (m, 1 H), 4.57 (d, $J = 7.3$ Hz, 2 H), 2.28 (t, $J = 7.8$ Hz, 2 H), 1.76 (d, $J = 1.0$ Hz, 3 H), 1.71 (d, $J = 1.0$ Hz, 3 H), 1.61–1.69 (m, 2 H), 0.94 (t, $J = 7.3$ Hz, 3 H).

Prenyl butyrate (3-methylbut-2-enyl butanoate; Fig. 5b; Supplementary Fig. S1c) was prepared as for prenyl methacrylate except that butyryl chloride took the place of methylacrylic anhydride. RI (DB-5/Carbowax) 1103/1411. EI-MS, 70eV, *m/z* (rel. int.): 156 (1.7, M^+), 114 (1.8), 89 (2.7), 86 (1.5), 85 (7.5), 72 (4.4), 71 (100), 70 (4.8), 69 (64.3), 68 (89.1), 67 (67.4), 66 (1.6), 57 (2.9), 56 (1.3), 55 (4.2), 54 (2.0), 53 (18.7), 44 (2.8), 43 (72.1), 42 (8.7), 41 (64.0). Full fragmentation data are also presented in Table 2. These data and the NMR data agreed with that in the literature (Gonzales et al., 2012).

Prenyl isobutyrate (3-methylbut-2-enyl 2-methylpropanoate, Supplementary Fig. S1d) was prepared as for prenyl butyrate except that isobutyryl chloride replaced butyryl chloride. RI (DB-5/Carbowax) 1058/1315. EI-MS, 70eV, *m/z* (rel. int.): 156 (2.0, M^+), 114 (1.0), 89 (2.4), 85 (4.3), 72 (2.4), 71 (55.0), 70 (7.4), 69 (100), 68 (78.3), 67 (52.0), 66 (1.4), 65 (1.8), 57 (2.9), 56 (1.2), 55 (3.2), 54 (2.2), 53 (16.3), 52 (1.2), 44 (3.0), 43 (78.6), 42 (7.7), 41 (74.6), 40 (7.0). These data agreed with that in the literature (Seo et al., 2014). We were unable to source ^{1}H NMR data; ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm): 6.07–6.11 (m, 1 H), 5.52–5.56 (m, 1 H), 5.34–5.41 (m, 1 H), 4.65 (d, $J = 6.8$ Hz, 2 H), 1.92–1.96 (m, 3 H), 1.76 (d, $J = 1.0$ Hz, 3 H), 1.73 (s, 3 H).

5.6. Statistical methods

Statistical analyses and graphics were performed using R (version 3.6.3) except for some basic statistical tests and graphics with Excel (version 2013). Before performing the parametric statistical tests, we tested for assumptions of normality with the Shapiro-Wilk test and homogeneity of variance with Levene's test. If assumptions were not met, we tested them again after using a $\log_{10}$ transformation, or used a non-parametric test. We used Pearson correlation to examine the relationships between emission rates of pairs of some monoterpenes.

For the test examining changes in emission rates (ng min^{-1}) associated with time of day, we tested emission rates of each cone at three different start sample times: morning (before 10:00 h, near beginning of a thermogenic episode), midday (peak thermogenesis, 11:30 to 13:00 h), and afternoon (after 15:00 h, during the decline of thermogenesis). We used a one-way repeated measures ANOVA in R, ('anova_test') [rstatix package], as the data met the assumptions for this analysis, with post hoc pairwise t.tests, Bonferroni adjusted. Differences in the total number of compounds emitted by cones across these time periods were analyzed using the nonparametric Friedman test. These tests were performed to determine if emission rates and the number of compounds were highest during the peak of thermogenesis, and to determine the best time of day to take volatile samples.

In the field bioassay, which tested chemical standards for their attraction to *C. chadwicki* 1 (no other insect pollinators have been found on these cones), we used a randomized complete block design, with a thermogenic pollen cone (source cone) on plant growing in the field to define a block (n = 4 blocks or source cones) and provide a source of thrips. A single chemical standard or different mixtures of standards comprised the treatments to test as thrips attractants along with a control (DCM solvent only). The number of thrips trapped in each treatment was statistically tested using glm in R, with poisson or quasipoisson errors.

With the Y-tube olfactometer results, we tested for differences in responses across male and female thrips using a proportion test (χ^2, 'prop.test' in R). Each gender was then tested separately for its response to a chemical treatment against the control, using a binomial test ('binom.test' in R) with 95% confidence intervals.

A non-metric multidimensional scaling ordination (NMDS) in R, Bray-Curtis similarity matrix of the proportion of each odor component's emission relative to the total of all compounds in a sample (square root transformed), was used to separate and then graphically display the variation across non-receptive and receptive ovulate cone groups. The permanova test in R, ANOSIM ('adonis' in 9999 permutations), was used to determine the significance of the difference across the receptive and non-receptive ovulate cone groups, with the Global R value comparing the between-group ranked dissimilarities with that of the within-group dissimilarities (0 indicates no separation and 1 indicates complete separation). Because the pre-receptive and post-receptive ovulate cones overlapped in 2-diminsional space, and the Global R = 0.117, was low and non-significant (P = 0.17) indicating no separation when tested against each other, we grouped the pre-receptive and post-receptive cones together as a single 'non-receptive' group to compare with the receptive cones. We tested initially for the assumption of homogeneity of multivariate dispersion between-groups with a 'distance anova'. Finally, 'IndicatorSspecies' analysis (R 'indicspecies' package) determined which chemical compounds were significantly associated with either receptive or non-receptive ovulate cones. Cone samples were positioned visually in 2- dimensional space along with any chemical compounds that were significantly associated with cone stage group. These tests helped to identify significant compounds for further testing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.phytochem.2021.112715.

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