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Variation in scent emission among floral parts and inflorescence developmental stages in beetle-pollinated *Protea* species (Proteaceae)

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Abstract

Floral fragrances are an important component for pollinator attraction in beetle-pollinated flowers. Several genera in the Proteaceae contain beetle-pollinated species. However, there is no information on the floral scent chemistry of beetle-pollinated members of the family. In this paper we report on the spatial variation and differences between developmental stages in emission of inflorescence (flowerhead) volatiles of four South African *Protea* species (*P. caffra*, *P. dracomontana*, *P. simplex*, and *P. welwitschii*) that are pollinated by cetoniine beetles. The scents from different inflorescence parts (bracts, perianth, styles, and nectar) and from successive anthesis stages of whole inflorescences were sampled using dynamic headspace collection and identified using GC–MS. Although the four species shared many scent compounds, possibly reflecting their close phylogenetic relationships and common pollinators, they showed significant differences in overall scent composition due to various species-specific compounds, such as the unique tiglate esters found in the scent of *P. welwitschii*. The strongest emissions and largest number of volatiles, especially monoterpenes, were from inflorescences at full pollen dehiscence. Senescing inflorescences of two species and nectars of all species emitted proportionally high amounts of acetoin (3-hydroxy-2-butanone) and aromatic alcohols, typical fermentation products. As a consequence, the scent composition of nectar was much more similar among species than was the scent composition of other parts of the inflorescence. These results illustrate how the blends of compounds that make up the overall floral scent are a dynamic consequence of emissions from various plant parts.

Keywords: Anthesis; Beetle pollination; Fermentation volatiles; Flower scents; GC–MS; Scented nectar

1. Introduction

Pollinator attraction is mainly based on visual cues (flower colour and shape) and olfactory cues (floral scent) that guide insects to flowers. Olfactory cues seem to play a particularly important role in many beetle-pollinated plants that have been described as emitting strong and characteristic fragrances reminiscent of ripe or rotting fruits, sometimes with a spicy aroma (Gottsberger, 1999; Proches and Johnson, 2009; Proctor et al., 1996). It was hypothesized that floral fragrances of beetle-pollinated flowers mimic fruit odours, because aliphatic esters such as those emitted by fruits have been found as major components especially in flowers of families of the primitive subclass Magnoliidae (e.g. Magnoliaceae, Annonaceae) where beetle pollination is a common pollination system (Jürgens, 2009; Jürgens et al., 2000; Thien et al., 1975). Although magnoliid inflorescence morphology was thought of as unspecialised with many exposed anthers that cover the whole body of a beetle in pollen, it is possible that these beetle-pollinated species evolved specialist fruity scents to attract more generalist beetle visitors (Jürgens, 2009). There are several documented examples of floral scents based on fermenting fruit odours that attract saprophilous flies and beetles (e.g. Goodrich et al., 2006), and the current study investigates the change from a pleasant fruity scent to that of fermenting fruit odours emitted over flower development for four species of beetle-pollinated *Protea*.

Flower scent is a relatively difficult component of floral phenotype to investigate, because flowers can emit very complex blends, with up to 100 compounds from different biosynthetic pathways (Pichersky and Gershenzon, 2002).

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There are many different factors to be considered when investigating floral scent compounds, especially in efforts to identify their functional roles in plant–pollinator interactions. These include scent emission by different floral parts (perianth, pollen, style, nectar etc.) and how this varies according to flowering stages (see e.g. Schiestl and Ayasse, 2001), times of the day, and different ecological conditions.

The Proteaceae have a Gondwanan distribution and the ecology and biogeography of several species of this family have been well-documented (e.g. Collins and Rebelo, 1987). This study is, however, the first analysis of the floral scent of any species of *Protea*, the largest genus in the family Proteaceae, and forms part of a larger investigation of beetle pollination systems in this genus. Most *Protea* species are either bird- or rodent-pollinated and have been described as either unscented or having a yeasty scent, respectively (e.g. Hargreaves et al., 2004; Wiens and Rourke, 1978). Our field experiments have revealed that four *Protea* species (known as grassland and savanna sugarbushes) are insect-pollinated, with cetoniine beetles as their most frequent visitors. These *Protea* species belong to a non-Cape clade of 15 species (Valente et al., 2009) and have floral traits that conform to a beetle pollination syndrome, namely open bowl-shaped inflorescences emitting strong fruity scents, low growth form, and abundant pollen rewards (Rebelo, 2001). In addition, these species produce copious amounts of dilute nectar. *Protea* inflorescences are typically large capitula surrounded by colourful bracts and comprised of numerous tightly packed hermaphroditic florets with pollen presenters. In each floret, the anther lobes are fused to the reduced perianth and fall to the base of the inflorescence after dehiscence, leaving pollen on the surface of the presenter. Florets are protandrous and mature centripetally. Nectar is produced at the base of each floret and often presents as a droplet held by the fused perianth lobes before accumulating at the base of the inflorescences once the florets start dehiscing and the perianth lobes fall.

Preliminary GC–MS results using SPME (solid-phase micro-extraction) of various floral parts of *P. caffra* revealed that the nectar is scented, a phenomenon only recently described in several diverse angiosperm species by Raguso (2004). While inflorescences of *P. caffra, P. dracomontana, P. simplex* and *P. welwitschii* emit a sweet, fruity scent when the bracts open and during early flowering stages, older inflorescences, after all florets becoming receptive to pollen, often emit a more acidic wine-like fragrance, probably as a result of nectar fermentation.

In this study we describe the scent composition of inflorescences at various developmental stages and for different floral tissues and nectar. In addition, we consider the possible origin and role of the scented nectar in relation to the beetle pollinators. We also test the prediction that nectar of senescing flowers will be characterised by a relatively high proportion of fermentation volatiles.

2. Materials and methods

2.1. Study species

We sampled scent from four *Protea* species in KwaZulu-Natal between 2006 and 2008. The "sugarbushes" *P. caffra* Meisn., *P. dracomontana* Beard, *P. simplex* E. Phillips, and *P. welwitschii* Engl. are common species inhabiting grassland vegetation, especially in the vicinity of the escarpment, in the summer-rainfall region of South Africa (Rebelo, 2001). They are members of the same clade and are beetle-pollinated, but also visited by sunbirds, and sugarbirds in more northern populations of *P. caffra* (e.g. Calf and Downs, 2002; Hargreaves et al., 2004). Inflorescences were collected from separate plants from the following populations in KwaZulu-Natal: sympatric populations of *P. caffra* (c. 200 plants) and *P. simplex* (c. 550 plants) located on the grassland slopes of the summit of Mount Gilboa (29° 17′ 10″ S, 30° 17′ 33″ E, 1770 m); *P. welwitschii* (c. 500 plants) located on steep grassland slopes of a residential area in Winston Park (28° 45′ 00″ S, 30° 45′ 00″ E, 550 m); and, *P. dracomontana* (c. 500 plants) from the lower slopes of Garden Castle (29° 44″ 30′ S, 29° 12″ 08″ E, 1900 m) in the Drakensberg mountains.

2.2. Scent sampling — scent emitted from different parts of the inflorescence

For a spatial analysis of the floral scent emission we sampled scent from bracts, styles with freshly dehisced pollen on pollen presenters, perianth (with attached dehisced anthers), and nectar, for five fully dehisced inflorescences from five different individuals of each of the four beetle-pollinated *Protea* species (80 samples) in January 2006. Inflorescences with only the extreme outer ring of florets dehisced were taken from plants, placed in water-filled vases and allowed to dehisce fully over 24–48 h in laboratory conditions. Preliminary results of scent samples from morning versus evening surveys showed that the inflorescences were more strongly scented in the morning. Therefore scent sampling was conducted between 0900 and 1500 h. Pooled nectar (200 μl) at the base of the florets was removed from each inflorescence using calibrated microcapillaries and blotted onto a small disc of Whatman's No. 1 filter paper. All bracts, styles and perianth lobes were excised from each inflorescence and excess nectar or plant sap from cut surfaces dabbed with absorbent paper. The different floral parts from each inflorescence were then placed in separate 8 × 8 cm polyacetate bags (Kalle Nalo, Germany), sealed and left to equilibrate for 1 h. The air from each bag was then pumped through a small cartridge filled with 1.5 mg of Tenax® and 1.5 mg of carbotrap® at a flow rate of 200 mL/min for a duration of 2 min. An ambient control sample was taken from an empty polyacetate bag sampled for the same duration.

2.3. Scent sampling — scent emission from different developmental floral stages

For analysis of temporal changes in the scent composition of whole inflorescences, we sampled five cut inflorescences at three different stages for each of the four study species in January 2008, resulting in a total of 15 samples per species. The inflorescence stages from which scent was collected were: (1) inflorescence bracts fully open but all florets before anthesis, none or little nectar production in florets; (2) full anthesis, which includes pollen presentation in inner florets and the start

of receptivity in outermost florets, and highest nectar production; and (3) older inflorescences with all florets having senesced perianth and anther lobes (brown in colour), senescing non-receptive stigmas, little or no nectar production, and bracts enclosed half to three quarters inwards (except in *P. welwitschii* in which the bracts drop outwards). Inflorescences of all stages were open to pollinators before collection. Cut stems were placed in water while headspace samples were taken by placing each inflorescence in a polyacetate bag, allowing scent volatiles to equilibrate for 20 min, and pumping the air through a small cartridge for 5 min. A control was taken from an empty polyacetate bag sampled for the same duration. The *Protea* inflorescences are more strongly scented during the day, thus scent sampling was mostly conducted during 0900 to 1500 h. Preliminary tests in which we compared the scent of inflorescences of *P. simplex* sampled in the field and in the laboratory showed little difference between the two methods in terms of the quantity and diversity of floral volatiles.

2.4. Gas chromatography–mass spectrometry (GC–MS) analysis of floral scent

Scent sampling cartridges were placed in a Varian 1079 injector equipped with a Chromatoprobe thermal desorption device and processed using a Varian CP-3800 GC with a 30 m × 0.25 mm internal diameter (film thickness 0.25 μm) Alltech EC-WAX column coupled to a Varian 1200 quadrupole mass spectrometer in electron-impact ionization mode (Amirav and Dagan, 1997; Dötterl et al., 2005; Gordin and Amirav, 2000). Details of the pressure program and method of analysis were described by Shuttleworth and Johnson (2009).

2.5. Statistical analysis of scent data

Prior to statistical analysis all compounds considered potential artefacts were excluded. Multivariate analysis, implemented in the Primer 6 program (Clarke and Gorley, 2001), was used to assess the variability in the floral scent samples of different plant parts. Percentage data for compounds (relative amounts with respect to total peak areas) were used, because the total amount of emitted volatiles varied greatly among different individuals. The data were square root transformed before calculating Bray–Curtis similarities to detect similarities among samples. To obtain a two-dimensional representation of the data Non-Metric Multidimensional Scaling (NMDS) was used. The stress value is given to evaluate how well or poorly the particular configuration produces the observed distance matrix. The smaller the stress value, the better the fit of the reproduced ordination to the observed distance matrix (Clarke, 1993). The significance of differences in scent profiles between species and dissected floral parts was assessed by ANOSIM (Analysis of Similarities) in a 2-way crossed layout (factors: inflorescence parts and nectar; plant species) implemented in the Primer 6 program (Clarke and Gorley, 2001) with 10,000 random permutations. The ANOSIM test calculates the test statistic R as well as a level of significance. Statistical significance of R is assessed by random permutations of the grouping vector to obtain an empirical distribution of R under the null model. SIMPER (factor: species) was used in Primer to identify the compounds responsible for dissimilarities among species (Clarke and Warwick, 2001).

In addition to the mean relative proportions of compounds making up the scent of whole inflorescences of three different flowering stages, we report on the change of the average number of volatiles emitted and the median emission rate per hour. The number of volatiles emitted by all samples of each stage was compared using Analysis of Variance. For quantification of emission rates per hour, known amounts of methyl benzoate were injected into thermal desorption cartridges and desorbed in the same manner as the samples. For each species, compounds and cumulative compound classes comprising less than 2% of the averaged samples were combined under the heading "Other" in Fig. 3.

3. Results

3.1. Species-specificity and spatial patterns of scent emission

A total of 118 compounds were found in the scent of the different floral parts (for details see the complete list of compounds in Appendix 1 - Supplementary material). Marked differences in chemical composition were identified between the inflorescence parts of all *Protea* species studied here. In Table 1, we list the key compounds found in the different inflorescence parts. Fig. 1 shows that the four different species are distinct regarding the scent composition of their constituent inflorescence parts during full anther dehiscence, with little variation between individual samples of the different floral parts. Using a two-way cross design, we found highly significant separation between species and dissected floral parts (2D stress value = 0.21; ANOSIM R (species) = 0.924, $P < 0.01$; ANOSIM R (inflorescence parts and nectar) = 0.852, $P < 0.01$). All species differences were significant with the highest separation found between *P. dracomontana* and *P. welwitschii* ($R = 1.0$, $P < 0.01$), and the least separation between *P. caffra* and *P. simplex* ($R = 0.837$, $P < 0.01$). Similarly, significant differences were found between floral parts, the highest separation being between nectar and pollen-bearing styles ($R = 0.966$, $P < 0.01$), and the least separation between the perianth lobes and pollen-bearing styles ($R = 0.654$, $P < 0.01$). In contrast, nectar scents were much less distinct between species (Fig. 1).

During full anthesis, all four species were characterised by emission of high relative amounts of linalool, followed by benzaldehyde. We found the highest relative amounts of linalool in samples from *P. caffra* and *P. welwitschii* (Table 1). *Protea dracomontana* scents comprised the highest relative amount of methyl benzoate, while *P. welwitschii* emitted only trace amounts from the bracts and nectar. *Protea caffra* and *P. dracomontana* scent samples shared relatively high amounts of benzyl alcohol and (*Z*)-linalool oxide (furanoid), while *P. simplex* and *P. welwitschii* shared high amounts of monoterpenes such as *alpha*-pinene and eucalyptol.

Table 1
Key compounds and compound classes from inflorescence parts and nectar of four *Protea* species. Floral parts: B = bracts, P = perianth, S = styles, N = nectar. Data presented are average relative proportions over 5 samples of each floral part and nectar from fully dehisced inflorescences of each species (compounds were identified by comparing MS and retention time with published works (e.g. Linstrom and Mallard, 2010).

Key compound and compound class	*Kovats*	*CAS*	*P. caffra*				*P. dracomontana*				*P. simplex*				*P. welwitschii*			
Floral parts and nectar			B	P	S	N	B	P	S	N	B	P	S	N	B	P	S	N
Number of compounds (max)			19	24	21	33	20	25	23	35	34	31	28	36	49	43	38	55
Aliphatic compounds																		
2,3-Butanedione	1019	431-03-8	–	–	–	–	–	–	–	–	–	2.4	–	–	–	–	–	7.1
2-Pentanone	1023	107-87-9	–	–	–	–	–	–	–	–	6.5	0.2	1.0	2.1	–	tr	–	–
2-Heptanone	1154	110-43-0	–	–	–	–	–	–	–	–	7.7	4.3	8.7	–	–	–	–	–
Acetoin (3-hydroxy-2-butanone)	1257	513-86-0	–	–	–	6.8	–	7.1	–	4.4	0.2	–	1.2	7.1	–	–	0.9	5.5
2-Nonanone	1355	821-55-6	–	–	–	–	–	13.3	–	–	–	–	–	–	–	–	–	–
Other aliphatic ketones			–	–	–	2.9	–	–	–	0.2	0.8	–	–	–	–	–	–	2.0
2-Heptanol	1279	543-49-7	–	–	–	–	5.1	–	–	–	0.1	–	–	–	5.3	–	–	–
1-Hexanol	1314	111-27-3	–	1.0	3.3	1.6	–	1.5	4.5	0.8	3.5	1.2	4.8	1.9	1.2	1.5	3.5	22.7
(*E*)-3-Hexen-1-ol	1323	928-97-2	–	1.5	9.4	0.2	–	–	–	0.1	2.2	0.1	0.2	0.1	0.2	–	–	–
(*Z*)-3-Hexen-1-ol	1344	928-96-1	5.8	0.2	0.6	0.6	12.0	0.6	3.8	0.5	29.8	5.2	17.3	0.2	10.0	1.1	6.8	4.1
(*Z*)-4-Hexen-1-yl acetate	1220	42,125-17-7	–	–	–	–	–	–	–	–	8.1	–	–	–	2.7	–	–	–
Ethyl (*E*)-2-hexenoate	1273	72,237-36-6	–	–	–	–	–	–	11.6	–	–	–	–	–	–	–	–	–
(*Z*)-3-Hexen-1-yl acetate	1284	3681-71-8	8.9	6.5	–	tr	19.1	1.4	–	–	–	1.4	1.5	–	0.3	1.1	0.4	–
(*Z*)-3-Hexen-1-yl isovalerate	1434	35,154-45-1	–	–	–	–	6.3	–	–	–	0.5	–	–	–	–	–	–	–
Other aliphatic esters			tr	2.5	0.1	1.9	3.9	5.1	4.4	3.6	0.1	0.1	0.1	0.2	3.8	12.1	1.5	1.5
(*E*)-2-Hexenal	1183	6728-26-3	–	–	–	–	–	–	–	–	3.4	–	5.5	–	–	–	–	–
Aliphatic acids			2.9	0.2	–	0.8	–	0.1	0.6	0.3	0.7	–	–	tr	tr	–	–	0.3
Other aliphatic compounds			–	–	0.3	0.9	–	–	–	0.9	–	0.8	–	–	–	0.2	tr	2.2
Monoterpenoids																		
alpha-Pinene	1049	80-56-8	tr	–	0.9	–	–	–	–	–	8.4	7.1	0.4	7.9	1.3	0.3	4.3	0.5
beta-Pinene	1108	127-91-3	7.3	–	–	tr	–	–	–	–	1.7	0.4	0.2	0.2	0.3	1.3	0.3	tr
beta-Myrcene	1156	123-35-3	tr	–	–	tr	6.1	–	–	–	2.1	–	–	–	tr	0.8	0.1	tr
Eucalyptol	1191	470-82-6	0.9	–	–	–	–	–	–	–	5.7	1.8	2.9	–	1.0	2.5	1.6	0.4
cis-Linalool oxide (furanoid)	1430	5989-33-3	8.3	3.3	4.6	4.8	1.5	3.7	1.3	3.8	0.4	1.4	2.8	1.5	0.7	–	1.2	0.6
Linalool	1500	78-70-6	35.0	56.6	54.9	28.9	2.7	26.4	23.1	56.2	2.7	31.2	19.3	11.5	59.6	67.4	68.6	16.6
Other monoterpenes			7.1	3.3	6.5	3.6	1.9	2.4	1.6	3.4	0.4	0.9	1.9	1.3	3.7	8.6	2.2	1.1
Sesquiterpenoids			–	–	–	–	–	–	0.2	0.3	tr	–	–	–	4.2	tr	tr	0.1
Aromatic compounds																		
Anisole	1311	100-66-3	–	1.1	2.3	tr	–	–	–	–	6.2	5.0	1.4	7.3	3.0	tr	3.3	–
Benzaldehyde	1488	100-52-7	12.4	8.0	5.2	34.8	13.7	3.7	9.5	13.9	4.2	4.3	14.2	49.9	0.7	0.1	0.3	30.2
Methyl benzoate	1578	93-58-3	–	8.8	6.9	0.6	12.2	29.1	27.8	1.1	0.6	29.6	12.0	0.3	tr	–	–	0.1
Benzyl alcohol	1830	100-51-6	9.5	5.4	4.2	6.8	9.6	3.4	8.8	4.1	0.6	1.7	1.7	5.3	0.5	0.4	1.3	2.6
Other benzenoid compounds			1.8	1.5	0.8	4.0	4.3	1.9	1.4	4.3	1.6	0.7	0.9	3.1	1.0	0.3	1.1	1.8
Nitrogen containing compounds			–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	tr
Unknowns			–	tr	–	0.1	0.9	–	0.8	0.8	–	–	–	0.1	1.0	2.1	2.2	0.1

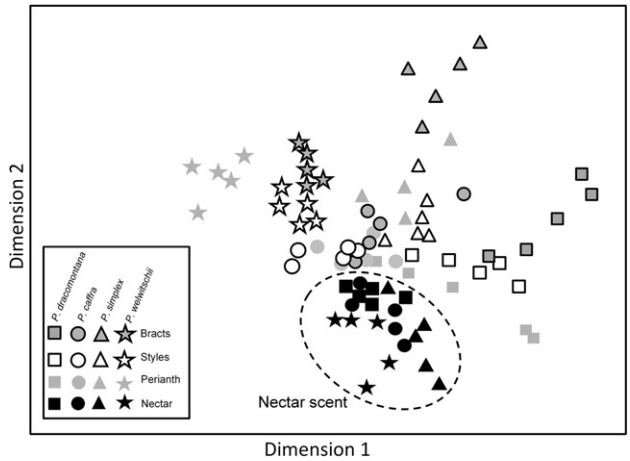

Fig. 1. Non-metric multidimensional scaling (NMDS) of the scent composition from different inflorescence parts (bracts, styles, and perianth) and nectar of four beetle-pollinated *Protea* species. NMDS is based on Bray–Curtis similarities, samples are from five fully dehisced inflorescences for each species.

Protea welwitschii scents were comprised of the highest number of volatiles emitted from all floral parts, including 34 unique compounds of which six were different tiglic acid esters. Compounds unique to *P. simplex* were mainly 2-heptanone, 2-pentanone and (*E*)-2-hexenal, and to *P. dracomontana* were 2-nonanone (perianth scent) and ethyl (*E*)-2-hexanoate (styles with pollen) (Table 1; Appendix 1 - Supplementary material).

Across all species, nectar scents contained the highest number of volatiles, especially for *P. welwitschii* (Table 1). The so-called "green leaf volatiles", such as (*Z*)- and (*E*)-3-hexen-1-ol and related esters were most commonly found in the scents of excised fleshy inflorescence and floral parts, especially bracts and styles across all species. Linalool and methyl benzoate were emitted mostly by the perianth and styles, while benzaldehyde dominated nectar scent. Acetoin (3-hydroxy-2-butanone) was found in higher amounts in the nectar scents, but was also present in perianth scent in *P. dracomontana*.

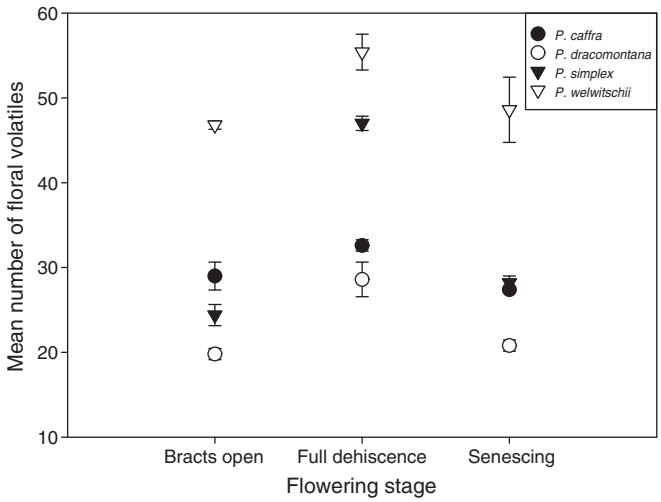

Fig. 2. The change in the number of floral volatiles emitted from four *Protea* species, each represented by 5 cut inflorescences from each of three different flowering stages (inflorescences with bracts open but no anther dehiscence; fully dehisced inflorescences; and, senescing inflorescences with bracts closing or wilting).

3.2. Changes in scent emission for various developmental floral stages

We found distinct changes in scent composition across flowering stages for all species. Fig. 2 shows that the fully dehisced inflorescences emit the most diverse floral scent (Species F = 158.2, $P < 0.01$; Flowering stage F = 55.8, $P < 0.01$; Interaction F = 7.2, $P < 0.01$; Fig. 2), corresponding with the strongest emission of scent as indicated in Fig. 3. *Protea welwitschii* emitted the strongest and biosynthetically most diverse scent, comprising from 10 to 15 more compounds than were emitted by the other species at any one stage, and contributing to a significant interaction between species and flowering stage in the analysis (Fig. 2).

Linalool dominated the samples from younger stage inflorescences (open bracts before anthesis, and full dehiscence, 31–66%) (Fig. 3; for a complete list of compounds see Appendix 2 - Supplementary material). Correlated with a marked decrease in linalool emissions in senescing inflorescences (e.g. down to 6% in *P. caffra*) we found a change in the proportion of a variety of monoterpenes such as *beta*-myrcene in *P. caffra*, *alpha*-pinene in *P. simplex* and limonene in *P. welwitschii*. Similarly, there was an increase through time in the proportion of the aromatic ether anisole for all species, although absolute amounts were similar across all stages of flowering. Of the aromatic esters, methyl benzoate dominated all three stages of *P. simplex* scent and showed a notable increase in senescing inflorescences of *P. dracomontana*. Aliphatic alcohols, mainly 1-hexenol and (*Z*)-3-hexen-1-ol were present in inflorescence scents of *P. caffra* and *P. simplex* before anther dehiscence, while they occurred in similar proportions in all three stages of *P. welwitschii*. Within the aliphatic esters, the green leaf volatile (*Z*)-3-hexen-1-yl acetate was prominent in *P. simplex* inflorescences before dehiscence, while methyl-2-methyl butanoate dominated this compound class in senescing inflorescences. Aliphatic esters were most diverse for the scent of *P. welwitschii,* for which (*Z*)-3-hexenyl isovalerate, isobutyl tiglate and an unknown tiglate dominated this compound class in all three stages. Styrene, a benzenoid compound, was found in high proportions in inflorescence scents of *P. caffra* and *P. simplex* after dehiscence and during senescence (Fig. 3).

4. Discussion

Spatiotemporal variation in floral scent has biological significance in mediating pollinator attractiveness over the life of a flower, and pollinator behaviour once they arrive at a flower (e.g. Dötterl and Jürgens, 2005). Differentiation in floral scent leads to efficient learning and flower handling in pollinators, and if associated with a reward, promotes constancy, efficient pollen placement and lowered stigma clogging (Wright and Schiestl, 2009). Limiting scent production to certain flowering times such as anthesis and receptivity also limits the unnecessary use of resources into producing scent after pollination. For example, *Clarkia breweri* flowers only emit linalool from when the flowers open until they are pollinated (Dudareva et al., 1996). In the case of beetle pollination systems, beetles often visit flowers for extended periods of time, slowing the movement of pollen between flowers and also increasing the frequency of geitonogamy in monoecious plants. Interestingly, Terry et al. (2007) found that in dioecious cycads, male cones control the movements of visiting thrips by up- or down-regulating the emission of certain monoterpenes, preventing pollinators from “lingering” for days on the same cone. In the same way, flowers of *Ophrys sphegodes* emit increased amounts of (*E*)-farnesyl hexanoate after pollination, becoming less attractive to bee pollinators, indirectly guiding them to unpollinated flowers (Schiestl and Ayasse, 2001). The current study found that scent emission from *Protea* inflorescences peaked during full anthesis of all the florets of an inflorescence (Figs. 2 and 3), and that nectar scent may be signalling the presence of nectar to a pollinator (Table 1; Fig. 1). Although total emission was lower in senescing inflorescences, and linalool production decreased, as seen in *C. breweri* flowers, a wide spectrum of volatiles were still emitted during this late flowering stage, together with the introduction of typical fermentation odours.

The scent samples of the investigated floral parts of the *Protea* species showed a wide range in the number of compounds per sample with 19 compounds found in the scent of bracts of *P. caffra* to 55 compounds found in the scent of *P. welwitschii* nectar (Table 1). Our investigation showed that inflorescence parts of *P. welwitschii* emitted a much more diverse and distinct scent compared to those of the other species. This was mostly due to its wider variety of monoterpenes and aromatic esters, and more specifically the tiglic acid esters (fruity/spicy odours), which were unique among the *Protea* species studied here, but found in other plants (e.g. Canada thistle, Japanese honeysuckle, gardenia, and Roman chamomile) (El-Sayed et al., 2008, 2009; Joulain, 2008; Omidbaigi et al., 2004). These compounds, together with the immense amount of linalool, result in an overall sweet

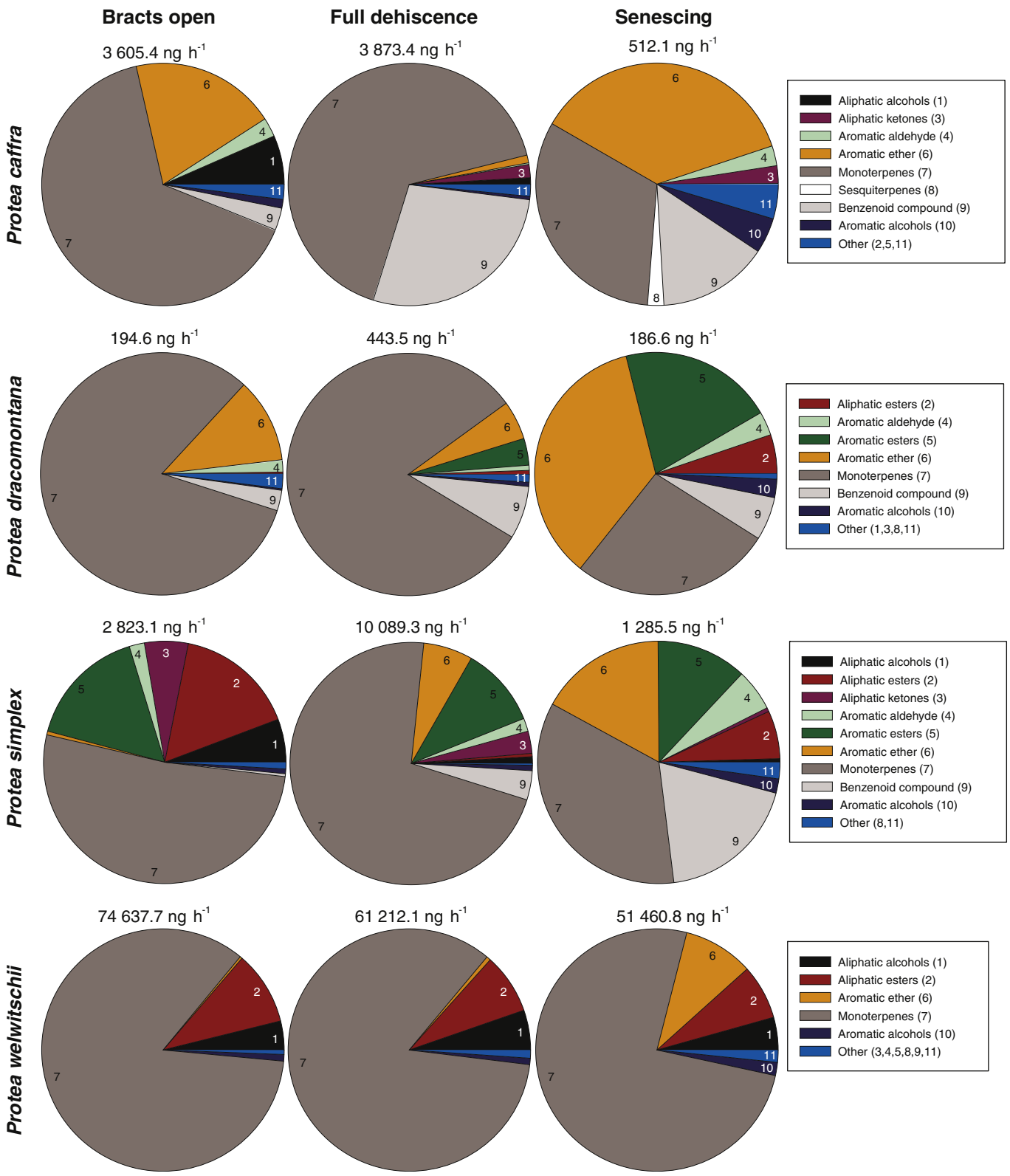

Fig. 3. The contribution of various compound classes to the scent of inflorescences of successive flowering stages for four *Protea* species. Total emission rates shown above each graph. Numbered pie slices refer to specific compound classes in legend. Data presented are average relative proportions from 5 samples. Compound class "Other" contains all compounds and compound classes that contribute under 2% each to the overall scent of the inflorescence.

honey-like scent in *P. welwitschii* compared to the papaya-like scent of the other *Protea* species. The scent of *P. dracomontana* was most similar to that of *P. caffra*, both comprised of high relative emissions of the fruity-smelling methyl benzoate, a compound almost absent from *P. welwitschii* scents. These patterns seem to reflect phylogenetic relationships, in that *P.*

dracomontana is more closely related to *P. caffra* than to *P. welwitschii*, the latter falling into a group that is sister to the other two species (Valente et al., 2009).

Temporal changes and spatial patterns in scent composition are likely to affect the attraction and behaviour of flower visitors (e.g. Theis and Raguso, 2005). Although there was an overall decrease in scent emission rates with senescence, the inflorescences of these *Protea* species appear to emit scent from pre-anther dehiscence until after stigma receptivity (Figs. 2 and 3). Cetoniine beetles were often found aggregated in older flowers, together with fruit flies, especially near the end of the flowering season when freshly opened flowers were scarce. Thus, senescent inflorescences still attracted insects, albeit with a much weaker scent emission as floral tissues die. Bracts and styles (and nectar, discussed below) may contribute to overall emissions at this late flowering stage, as these floral tissues last for much longer than the perianth. There is no further reason for the inflorescences to attract pollinators with scent near senescence, but it may be a consequence of the large mass of floral tissue that was emitting scent during flowering and the slow "shutting down" of pathways producing chemical volatiles, together with microbial action. The scent composition of these inflorescences changed over time, mostly due to a decrease in relative amounts of linalool. This accounts for the higher proportion of benzaldehyde and methyl benzoate in senescing inflorescences. Anisole was also curiously present in high proportions in senescing inflorescences. Few changes in the scent composition of *P. welwitschii* flowers were observed for different flowering stages and this may be the result of morphological differences in that the bracts do not enclose the florets during senescence, exposing nectar and florets to higher evaporation rates than the other species, and preventing nectar fermentation. However, scent emissions at the senescence stage of *P. welwitschii* were still very strong compared to the other species, suggesting that they may have not been collected at the same advanced stage of senescence.

Beetle visitors were most abundant during full anthesis (all florets dehisced and up to when all florets are receptive) stages of inflorescence flowering. They were found digging amongst fallen perianth lobes in the base of the inflorescences, licking nectar off floral tissues, drinking nectar collected at the base, eating pollen left in dehisced anther lobes or on the pollen presenters themselves, crawling over stigma tips in the process of moving around the inflorescence or when landing or taking off. In earlier stages before anther dehiscence, beetles can be found between perianth lobes and styles where nectar is secreted. The strong scent of *Protea* inflorescences may act as a long range attractant of pollinators, but the nectar scents may guide foraging insects to this resource once they have entered the inflorescence.

Most floral scent is emitted by petals but many studies show that distinct pollinator attractants can also be emitted by pollen (Dobson et al., 1999) and nectar (Raguso, 2004). Here we found that the perianth lobes of three of the *Protea* species, and styles to an extent, seemed to be responsible for emitting the fruity-smelling methyl benzoate, an aromatic ester occurring in fruits such as *Carica papaya*, which the scents of these *Protea* species strongly resemble (Pino et al., 2003). Methyl benzoate is also under investigation for use in lure-and-toxicant pest control systems as a cetoniine beetle attractant (Bengtsson et al., 2009). The variety of "green leaf volatiles" in the scents of the perianth, styles and especially bracts was probably due to the sampling method and exposed plant tissues at cut surfaces. But the most interesting result was that while differences between the scents of bracts, perianth and styles reflected species differences, the scent of nectar of all four species was similar, resulting in a common signal to pollinators. Prominent in the nectar was acetoin (3-hydroxy-2-butanone), a known product of sugar fermentation (see Goodrich et al., 2006) and a sign of nectar fermentation in the inflorescences (discussed below). In addition, nectar scents were dominated by benzaldehyde and linalool, common attractants of cetoniine beetles (Bengtsson et al., 2009; Donaldson et al., 1990). Other suites of volatiles found in these *Protea* scents may owe their presence to biosynthetic pathway flux, as benzoic acid, benzaldehyde, methyl benzoate and other oxygenated benzenoids have precursor–derivative relationships in the shikimate pathways (Moerkercke et al., 2009).

The potential proximate causes of scented nectar were extensively reviewed by Raguso (2004). It may be due to the high solubility of some of the more polar scent constituents in the aqueous medium of *Protea* nectar. In addition, volatile compounds could be secreted directly into the nectar, or conversely, some compounds may be metabolic products of microbial fermentation of nectar constituents. The absorption of some volatiles by nectar may occur since the perianth with fused anther lobes, bracts and the base of styles are often in contact with nectar before florets dehisce, and when nectar accumulates in the base of the *Protea* inflorescences. There is thus sufficient physical contact to allow nectar to absorb volatiles passively from floral tissues. However, this hypothesis is not well supported because the nectar scents were often stronger and always more diverse than those of other floral tissues. Curiously benzaldehyde is not readily soluble in water (Stephenson, 1993), yet dominated nectar volatile samples in these species (Table 1). Contrasting nectar and corolla scents were also found in *Oenothera primiveris*, where methyl benzoate and 1-pyrrholine are probably secreted into the hypanthium (Raguso, 2004; Raguso et al., 2007). Although we believe that there may be active secretion of some scent volatiles into the nectar, the bracts of the *Protea* inflorescences form a bowl allowing nectar to pool at the base, creating ideal conditions to house fermenting yeast and bacteria. This may also be the case for *Agave* flowers that produce large nectar pools open to microbial infestation for 4–6 days, and for which fermentation volatiles such as ethanol and ethyl sorbate, probably resulting from fermentation, were found in headspace samples (Raguso, 2004). We found few typical fermentation volatiles in the nectar scents, such as acetoin, which were probably due to fermentation processes that cannot be regulated by the plant but are mainly a result of the micro-organisms (Table 1). De Vega et al. (2009) reported that yeasts were present in 58% of *P. caffra* inflorescences sampled at the stage of full anthesis, and our preliminary investigation found that yeasts and bacteria were abundant in nectar of all

four *Protea* species at the senescence stage (S-L. Steenhuisen, unpublished results).

Scented floral nectar is an honest signal of a reward to a pollinator and ultimate causes of the evolution of scented nectar include the antimicrobial activity of certain scent compounds secreted into the nectar. Like many monoterpenes, linalool has antimicrobial properties (e.g. Queiroga et al., 2007), and although it does not prevent fermentation of nectar in these *Protea* species, future experiments should assess nectar volatiles retard the onset or rate of nectar fermentation. In the context of the foraging behaviour of cetoniine beetles, there may not be any selective forces for antimicrobial agents because most fruit chafer beetles feed on rotting fruit that may have already been inoculated with fermenting yeast and bacteria. These beetles are vectors of a variety of microbes (S-L. Steenhuisen, unpublished results) and are not deterred by fermenting odours, although these may be deterrents to other pollinators such as bees. Thus, for a *Protea,* the inability to prevent fermentation in such an open inflorescence appears not to have a negative effect on beetle visitation.

We found an increase in the relative amounts of only a few fermentation volatiles with senescence, such as phenylethyl alcohol and isoamyl acetate (3-methylbutyl acetate) (only in *P. dracomontana*). Thus, our data from the scent emission of different developmental stages did not fully support our expectations of a greater abundance and amount of fermentation volatiles with senescence. However, even fresh nectar in beetle-pollinated *Protea* species is very dilute (4–10% sugar refractometer reading; S-L. Steenhuisen, unpublished results) and may ferment quickly. Hence already fermented nectar from older flowers may mostly consist of rain or dew water, containing little or no sugar (0–1%), and hence little substrate for further microbial action. Similarly, in the case of *Asimina* flowers, although suitable domatia for floral yeasts and bacteria were provided, Goodrich et al. (2006) found that fermentation volatiles were emitted by various floral tissues, and so could not conclude that microbes were responsible for the fermented scents without more experimentation. Alternatively, the scents of inflorescences at senescence may be affected by evaporation of nectar and/or use by foraging insects. Older inflorescences that contain some moisture are much more strongly scented to the human nose, than those in which all moisture has evaporated. Additionally, the role of fallen *Protea* pollen and beetle faeces as a microbial substrate at the base of the inflorescences was not investigated in this study but should be considered in future investigations.

Aliphatic compounds such as acetoin, 3-methyl 1-butanol, ethanol, and isobutyl alcohol were present in the headspace of baker's yeast (Goodrich et al., 2006). Acetoin, a commonly encountered microbial metabolite (Schultz and Dickschat, 2007) with one chiral center, has been identified in very few flowers (see Knudsen et al., 2006) and is described as an aggregation signal for male summer chafers (*Amphimallon solstitiale*; Francke and Dettner, 2005). Acetoin (potentially two enantiomers) was mostly found in the nectar and is probably produced through its fermentation, rather than as a signal produced by the flowers. Lacking an appropriate enantioselective column, we could not establish the absolute configuration of acetoin in the present study.

In contrast to other floral parts the scent of nectar was very consistent across species, with few fermentation volatiles emerging in late flowering stages. This lack in variation could be attributed to the stable biochemical cycles by which microbes ferment nectar, but also to possible strong selection for physiologically active compounds, maintaining the attractiveness of these species to their beetle pollinators. Such a case was described for the orchid genus *Ophrys*, where pollinators exerted strong stabilising selection on active floral volatiles that elicit specific behavioural responses in their hymenopteran pollinators (Mant et al., 2005; Salzmann et al., 2007). Non-active compounds were found to be more variable among *Ophrys* species.

5. Conclusions

The four investigated beetle-pollinated *Protea* species showed different scent compositions, with *P. welwitschii* having the highest number of compounds and the highest emission rate. Inflorescences of all species showed variation in floral scent emissions from different floral parts and developmental stages. This study has also shown that the nectar of these *Protea* species emits a chemically complex scent blend, but more work needs to be done to establish its function and to determine if volatile compounds are present in nectar through passive absorption or active secretion of volatiles into the nectar.

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