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Floral scent in bird- and beetle-pollinated *Protea* species (Proteaceae): Chemistry, emission rates and function

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ABSTRACT

Evolutionary shifts between pollination systems are often accompanied by modifications of floral traits, including olfactory cues. We investigated the implications of a shift from passerine bird to beetle pollination in *Protea* for floral scent chemistry, and also explored the functional significance of *Protea* scent for pollinator attraction. Using headspace sampling and gas chromatography–mass spectrometry, we found distinct differences in the emission rates and chemical composition of floral scents between eight bird- and four beetle-pollinated species. The amount of scent emitted from inflorescences of beetle-pollinated species was, on average, about 10-fold greater than that of bird-pollinated species. Floral scent of bird-pollinated species consists mainly of small amounts of "green-leaf volatiles" and benzenoid compounds, including benzaldehyde, anisole and benzyl alcohol. The floral scent of beetle-pollinated species is dominated by emissions of linalool, a wide variety of other monoterpenes and the benzenoid methyl benzoate, which imparts a fruity odour to the human nose. The number of compounds recorded in the scent of beetle-pollinated species was, on average, greater than in bird-pollinated species (45 versus 29 compounds, respectively). Choice experiments using a Y-maze showed that a primary pollinator of *Protea* species, the cetoniine beetle *Atrichelaphinis tigrina*, strongly preferred the scent of inflorescences of the beetle-pollinated *Protea simplex* over those of the bird-pollinated sympatric congener, *Protea roupelliae*. This study shows that a shift from passerine bird- to insect-pollination can be associated with marked up-regulation and compositional changes in floral scent emissions.

1. Introduction

Through selection, flowers become adapted to the morphology and sensory physiology of their pollinators. This also produces patterns of convergent floral evolution—pollination syndromes (Faegri and van der Pijl, 1979)—when unrelated plants become adapted to the same functional group of pollinators. These syndromes can be used to generate hypotheses about the evolutionary modifications that take place during shifts between different pollinators. For example, since bird-pollinated flowers tend to emit very little scent (Knudsen et al., 2004) and flowers pollinated by cetoniine beetles are often highly scented (Johnson et al., 2007; Shuttleworth and Johnson, 2010a), it could be predicted that a shift between these two pollination systems in a particular lineage would be associated with marked changes in scent production, both in terms of emission rates and chemical composition. Here we confirm this particular prediction for a shift from bird- to beetle-pollination in *Protea* (Proteaceae) and show that beetles strongly prefer scented *Protea* flowers.

Most, but not all, animal pollinators have acute olfactory senses which aid them in finding food, mates and in defining territories. Chemical signals have the potential to act over long distances, attracting pollinators from a greater area than visual cues visible only at close range (Kite et al., 1998). Floral odours are thus subject to selection when they affect reproductive success. There is now good evidence for associations between chemical composition of scent and various pollination systems, such as those involving bats, moths, flies and beetles (Jürgens et al., 2000; Knudsen and Tollsten, 1993, 1995; Raguso et al., 2003; Stensmyr et al., 2002).

Fenster et al. (2004) found that 14 of 59 pollinator shifts analysed in their study involved a qualitative change in floral fragrance, with the majority of these cases involving shifts to nocturnal Lepidoptera as pollinators. Studies that link quantitative changes in scent composition and emission rate to pollinator shifts in specific clades are still relatively rare (e.g. Cyperaceae, Wragg and Johnson, 2011; *Eucomis*, Shuttleworth and Johnson 2010b; Nyctaginaceae, Levin et al., 2001). The functional significance of scent traits involved in pollinator shifts has been demonstrated using electrophysiological techniques, behavioural choice

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experiments and manipulative field experiments. For example, Kessler et al. (2008) genetically manipulated the emission of two volatile compounds in *Nicotiana attenuata* Torr. and showed that they affected moth and hummingbird pollination, and Shuttleworth and Johnson (2010b) added oligosulphides to flowers of wasp-pollinated pineapple lilies (*Eucomis*: Hyacinthaceae) and found that this scent modification resulted in pollination by carrion flies.

Protea (Proteaceae) is well-suited for investigations of floral scent evolution associated with pollinator shifts. Three pollination systems, involving beetles, birds and rodents have been established in the genus. A phylogeny for the genus indicates that bird-pollination is ancestral to both beetle- and rodent-pollination in *Protea* (Valente et al., 2010). Flower heads of bird-pollinated *Protea* species are weakly scented to the human nose. It is generally assumed that flowers pollinated by birds are usually unscented, presumably because birds tend to use visual rather than olfactory cues for finding flowers (Faegri and Van Der Pijl, 1979; Knudsen et al., 2004). However, However, existing studies of floral scent in bird-pollinated plants are confined to hummingbird-pollinated species (Knudsen et al., 2004). Olfactory signals are used by certain birds for foraging and nest recognition (e.g. petrels and penguins; Nevitt, 2008; Wright et al., 2011), and the possibility that passerine flower-visiting birds use olfactory signals therefore cannot be ruled out. In addition, the nectar of beetle-pollinated *Protea* species is generally scented (Steenhuisen et al., 2010), and would thus have a flavour as well as an odour. For many species of passerine birds, the flavour of nectar is an important determinant of food choice, as shown in repellant studies with lithium chloride, methyl anthranilate and sodium chloride with avian crop pests (Werner and Provenza, 2011) and bitter nectar repelling less effective sunbird pollinators of *Aloe vryheidensis* Groenew. (Johnson et al., 2006). Rodent-pollinated *Protea* species have a yeasty or "sour-milk" scent to humans.

Pollination by beetles has been documented in four grassland and savanna *Protea* species in South Africa (Steenhuisen and Johnson, 2012; Steenhuisen et al., 2012). These beetle-pollinated *Protea* species have scents which to humans are papaya- or honey-like. In a previous study of volatile emissions from various floral parts in these species, we found that the nectar emits a rich blend of volatiles that is very similar among the four species (Steenhuisen et al., 2010). Studies of other plants pollinated by the same cetoniine beetles have shown that floral scent is a major attractant of these insects (Johnson et al., 2007; Shuttleworth and Johnson, 2010a). Olfactory signals to Cetoniinae have mostly been studied in the context of optimising odour lures for use in traps and integrated pest management. Electroantennogram (EAD), olfactometer and field trapping experiments have more specifically shown that cetoniines are attracted to a wide variety of fruit and flower volatiles, in particular benzenoids such as cinnamic alcohol and methyl salicylate, and monoterpenes such as linalool and related compounds (Donaldson et al., 1986, 1990; Johnson et al., 2007; Ladd et al., 1976; Larsson et al., 2003; McGovern and Beroza, 1970; Wolde-Hawariat et al., 2007).

The four beetle-pollinated *Protea* species included in this study belong to a non-Cape clade (the "red, grassland, savanna and mountain sugarbushes" found outside of the Cape Floristic Region in South Africa) which includes eleven other species (Schnitzler et al., 2011; Valente et al., 2010). Floral scents of some of these related species are also sweet or fruity, suggesting that insect-pollination may be more widespread in this clade. The eight bird-pollinated species included in this study are representative of six different clades in the genus (Valente et al., 2010). As the ancestors to the beetle-pollinated clade have been inferred as being bird-pollinated (Schnitzler et al., 2011), we predict that a change in scent composition and up-regulation of emission of compounds attractive to cetoniine beetles may have facilitated the evolutionary shift from bird to insect pollination in this clade. The aims of this study were, firstly, to document the changes in floral scent (in terms of chemical composition and emission rates) associated with the shift from bird- to beetle-pollination in *Protea*, and secondly, to determine whether differences in scent between bird- and beetle-pollinated species have a functional significance for attraction of beetle pollinators.

2. Results

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

We detected a total of 139 volatile compounds in headspace samples taken from the 12 *Protea* species (Figs. 1 and 2). The majority of these were aliphatic alcohols, esters and ketones as well as monoterpene olefins and alcohols (Fig. 2, Appendix Table 2). Headspace sampling revealed that the monoterpene alcohol linalool (3,7-dimethyl-1,6-octadien-3-ol; enantiomeric configuration unknown) comprised approximately 57–66% of total scent emissions from *Protea caffra* Meisn, *Protea dracomontana* Beard, *Protea simplex* E.Phillips ex J.M.Wood and *Protea welwitschii* Engl. with an average emission rate of 1576 ng flw^{-1} h^{-1} in these species compared with 0.09 ng flw^{-1} h^{-1} for inflorescences of bird-pollinated species. Three benzenoid compounds (anisole, benzaldehyde, benzyl alcohol) were shared between all 12 *Protea* species sampled. In addition the benzenoids styrene and methyl benzoate were present in all species profiles except *P. welwitschii*, and phenylethyl alcohol was present for all species except *Protea nitida* Mill. The fermentation volatile, acetoin, was evident in scent emissions of the three beetle-pollinated *Protea* species and the putatively bird-pollinated *Protea subvestita* N.E.Br. Of all the species, the four beetle-pollinated species were most similar, sharing a wide range of floral volatiles (reported below). Of the bird-pollinated *Protea* species, although none were sister species, two groups were notable, one consisting of *Protea laurifolia* Thunb. and *P. nitida* that shared relatively higher emissions of the monoterpenes *beta*-myrcene, *beta*-pinene and *beta*-phellandrene, the other consisting of *Protea punctata* Meisn. and *Protea repens* (L.) L., which shared a variety of C6 aliphatics, or "green-leaf volatiles". The scent profile of *Protea cynaroides* was the least diverse, with a total of only 15 compounds. Notable also, are the benzenoids cinnamic alcohol and methyl cinnamate in the scent of *P. punctata*, and trace amounts of sulphur-containing compounds in three bird-pollinated species.

The mean rate of volatile emissions (both per flower head and per unit dry mass of flower head) was about 10-fold higher in the beetle-pollinated species than in the bird-pollinated species (Fig. 3A–C). Emission rates below 100 ng flw^{-1} h^{-1} were recorded for *P. cynaroides* (L.)L., *Protea magnifica* Link, *P. nitida* and *P. repens*; 110–310 ng flw^{-1} h^{-1} for the remaining four bird-pollinated species, and 685–6110 ng flw^{-1} h^{-1} for beetle-pollinated species.

We found highly significant separation between species and pollination systems with respect to scent composition using emission rates (ng flw^{-1} h^{-1}; 2D stress value = 0.15; ANOSIM R(species) = 0.75, $P < 0.01$; ANOSIM R(pollinator) = 0.836, $P < 0.01$) and percentage data (2D stress value = 0.17; ANOSIM R(species) = 0.813, $P < 0.01$; R(pollinator) = 0.840, $P < 0.01$) (Fig. 4). The higher emission and abundance of linalool contributed to the greatest difference between beetle- and bird-pollinated *Protea* scents (10.5% and 16.0% contribution for emission rates and percentage composition, respectively). When using emission rates, all other compounds contributing to the top 50% of the difference between the two pollinator groups were emitted in higher amounts from beetle-pollinated plants (e.g. monoterpenes *alpha*- and *beta*-pinene, *beta*-myrcene, eucalyptol, isomers of ocimene, furanoid linalool oxides, limonene and an unknown; the

Fig. 1. Inflorescences and animal visitors of *Protea* species included in this study: (A) *Protea caffra**; (B) *Protea cynaroides*; (C) *Protea dracomontana** and beetle pollinator, *Atrichelaphinis tigrina*; (D) *Protea laurifolia* visited by a protea beetle, *Trichostetha fascicularis*; (E) *Protea magnifica*; (F) *Protea nitida*; (G) *Protea punctata* (photo: Jane Carlson); (H) *Protea repens*; (I) *Protea roupelliae* visited by a bird pollinator, the malachite sunbird, *Nectarinia famosa*; (J) *Protea simplex** pollinated by *A. tigrina*; (K) *Protea subvestita* visited by melyrid beetles (photo: Michelle Tedder); and (L) *Protea welwitschii**. Photos by the authors unless otherwise indicated. An asterisk after the species name denotes that the species is beetle-pollinated.

benzenoids styrene and methyl benzoate; the aliphatics acetoin and 1-hexanol, all contributing 2–4% each to the difference). For percentage scent composition, large differences between the two groups were caused by higher relative abundance of the benzenoids anisole, styrene and benzaldehyde, 6-methyl-5-hepten-2-one and ethyl acetate in bird-pollinated species profiles.

2.2. Choice experiments

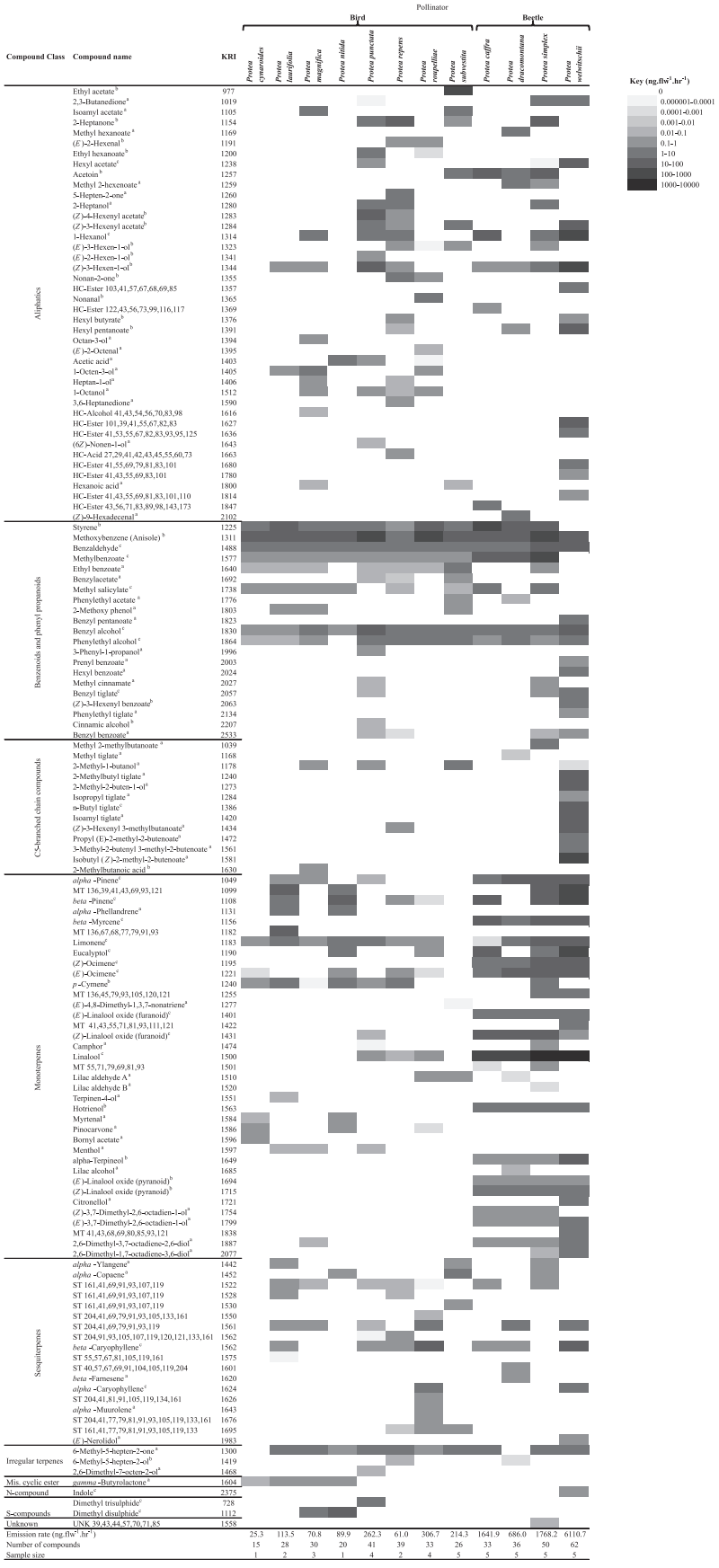
In experiments using the Y-shaped olfactometer, there was a highly significant preference for the scent of *P. simplex*, as opposed to that of *Protea roupelliae* Meisn. subsp. *roupelliae*, for both the *Protea*-experienced beetles from Mount Gilboa (binomial test, $P < 0.01$, Fig. 5) and the first trial involving naive beetles from Cobham Nature Reserve (binomial test, $P = 0.02$, Fig. 5). In a repeat of the latter trial, 100% of beetles chose the arm with the scent of *P. simplex* ($P = 0.02$).

3. Discussion

This study confirms that the floral scents of beetle-pollinated *Protea* species are distinct from those of bird-pollinated congeners in terms of chemical composition, whole flower and mass-specific emission rates (Figs. 2–4). Furthermore, choice experiments with *Atrichelaphinis tigrina* (Cetoniinae) using whole *Protea* inflorescences as an attractive unit revealed that these beetles show a significant preference for the strong fruity scent of *P. simplex* over the faint, nondescript odour of *P. roupelliae* (Fig. 5). Thus, there is chemical and biological justification for our human perception that beetle-pollinated species smell differently and more strongly than those of bird-pollinated species.

3.1. Scent composition and emission rates

Two patterns emerge from the compositional data on *Protea* scents that could represent strategies that have evolved to attract beetles. One involves a benzenoid and phenyl propanoid pathway with the up-regulation of methyl benzoate and anisole. The other involves the up-regulation of linalool and the production of other monoterpenoid compounds (e.g. *beta*-myrcene, eucalyptol, furanoid and pyranoid linalool oxides, hotrienol, (*E*) and (*Z*)-ocimene) giving these species a sweet scent with fruity notes. As reported by Steenhuisen et al. (2010) the scent profile of *P. welwitschii* is the most distinct and complex out of the 12



Compound Class	Compound name	KRI	25.3	113.5	70.8	89.9	262.3	61.0	306.7	214.3	1641.9	686.0	1768.2	6110.7
	Emission rate (ng.flw⁻¹.hr⁻¹)													
	Number of compounds		15	28	30	20	41	39	33	26	33	36	50	62
	Sample size		1	2	3	1	4	2	4	5	5	5	5	5
Aliphatics	Ethyl acetate [b]	977												
	2,3-Butanedione [a]	1019												
	Isoamyl acetate [a]	1105												
	2-Heptanone [b]	1154												
	Methyl hexanoate [a]	1169												
	(*E*)-2-Hexenal [b]	1191												
	Ethyl hexanoate [b]	1200												
	Hexyl acetate [c]	1238												
	Acetoin [a]	1257												
	Methyl 2-hexenoate [a]	1259												
	5-Hepten-2-one [a]	1260												
	2-Heptanol [a]	1280												
	(*Z*)-4-Hexenyl acetate [b]	1283												
	(*Z*)-3-Hexenyl acetate [b]	1284												
	1-Hexanol [a]	1314												
	(*E*)-3-Hexen-1-ol [b]	1323												
	(*E*)-2-Hexen-1-ol [b]	1341												
	(*Z*)-3-Hexen-1-ol [b]	1344												
	Nonan-2-one [b]	1355												
	HC-Ester 103,41,57,67,68,69,85	1357												
	Nonanal [b]	1365												
	HC-Ester 122,43,56,73,99,116,117	1369												
	Hexyl butyrate [b]	1376												
	Hexyl pentanoate [b]	1391												
	Octan-3-ol [a]	1394												
	(*E*)-2-Octenal [a]	1395												
	Acetic acid [a]	1403												
	1-Octen-3-ol [a]	1405												
	Heptan-1-ol [a]	1406												
	1-Octanol [a]	1512												
	3,6-Heptanedione [a]	1590												
	HC-Alcohol 41,43,54,56,70,83,98	1616												
	HC-Ester 101,39,41,55,67,82,83	1627												
	HC-Ester 41,53,55,67,82,83,93,95,125	1636												
	(6*Z*)-Nonen-1-ol [b]	1643												
	HC-Acid 27,29,41,42,43,45,55,60,73	1663												
	HC-Ester 41,55,69,79,81,83,101	1680												
	HC-Ester 41,43,55,69,83,101	1780												
	Hexanoic acid [a]	1800												
	HC-Ester 41,43,55,69,81,83,101,110	1814												
	HC-Ester 43,56,71,83,89,98,143,173	1847												
	(*Z*)-9-Hexadecenal [a]	2102												
Benzenoids and phenyl propanoids	Styrene [b]	1225												
	Methoxybenzene (Anisole) [b]	1311												
	Benzaldehyde [c]	1488												
	Methylbenzoate [c]	1577												
	Ethyl benzoate [a]	1640												
	Benzylacetate [a]	1692												
	Methyl salicylate [c]	1738												
	Phenylethyl acetate [a]	1776												
	2-Methoxy phenol [a]	1803												
	Benzyl pentanoate [a]	1823												
	Benzyl alcohol [c]	1830												
	Phenylethyl alcohol [c]	1864												
	3-Phenyl-1-propanol [a]	1996												
	Prenyl benzoate [a]	2003												
	Hexyl benzoate [a]	2024												
	Methyl cinnamate [a]	2027												
	Benzyl tiglate [c]	2057												
	(*Z*)-3-Hexenyl benzoate [b]	2063												
	Phenylethyl tiglate [a]	2134												
	Cinnamic alcohol [b]	2207												
	Benzyl benzoate [a]	2533												
C5-branched chain compounds	Methyl 2-methylbutanoate [a]	1039												
	Methyl tiglate [a]	1168												
	2-Methyl-1-butanol [a]	1178												
	2-Methylbutyl tiglate [a]	1240												
	2-Methyl-2-buten-1-ol [a]	1273												
	Isopropyl tiglate [a]	1284												
	n-Butyl tiglate [c]	1386												
	Isoamyl tiglate [a]	1420												
	(*Z*)-3-Hexenyl 3-methylbutanoate [a]	1434												
	Propyl (*E*)-2-methyl-2-butenoate [a]	1472												
	3-Methyl-2-butenyl 3-methyl-2-butenoate [a]	1561												
	Isobutyl (*Z*)-2-methyl-2-butenoate [a]	1581												
	2-Methylbutanoic acid [b]	1630												
Monoterpenes	*alpha* -Pinene [c]	1049												
	MT 136,39,41,43,69,93,121	1099												
	beta -Pinene [c]	1108												
	alpha -Phellandrene [a]	1131												
	beta -Myrcene [c]	1156												
	MT 136,67,68,77,79,91,93	1182												
	Limonene [c]	1183												
	Eucalyptol [c]	1190												
	(*Z*)-Ocimene [c]	1195												
	(*E*)-Ocimene [c]	1221												
	p -Cymene [b]	1240												
	MT 136,45,79,93,105,120,121	1255												
	(*E*)-4,8-Dimethyl-1,3,7-nonatriene [a]	1277												
	(*E*)-Linalool oxide (furanoid) [c]	1401												
	MT 41,43,55,71,81,93,111,121	1422												
	(*Z*)-Linalool oxide (furanoid) [c]	1431												
	Camphor [a]	1474												
	Linalool [c]	1500												
	MT 55,71,79,69,81,93	1501												
	Lilac aldehyde A [a]	1510												
	Lilac aldehyde B [a]	1520												
	Terpinen-4-ol [a]	1551												
	Hotrienol [b]	1563												
	Myrtenal [a]	1584												
	Pinocarvone [a]	1586												
	Bornyl acetate [a]	1596												
	Menthol [a]	1597												
	alpha-Terpineol [b]	1649												
	Lilac alcohol [a]	1685												
	(*E*)-Linalool oxide (pyranoid) [b]	1694												
	(*Z*)-Linalool oxide (pyranoid) [b]	1715												
	Citronellol [a]	1721												
	(*Z*)-3,7-Dimethyl-2,6-octadien-1-ol [a]	1754												
	(*E*)-3,7-Dimethyl-2,6-octadien-1-ol [c]	1799												
	MT 41,43,68,69,80,85,93,121	1838												
	2,6-Dimethyl-3,7-octadiene-2,6-diol [a]	1887												
	2,6-Dimethyl-1,7-octadiene-3,6-diol [a]	2077												
Sesquiterpenes	*alpha* -Ylangene [a]	1442												
	alpha -Copaene [a]	1452												
	ST 161,41,69,91,93,107,119	1522												
	ST 161,41,69,91,93,107,119	1528												
	ST 161,41,69,91,93,107,119	1530												
	ST 204,41,69,79,91,93,105,133,161	1550												
	ST 204,41,69,79,91,93,119	1561												
	ST 204,91,93,105,107,119,120,121,133,161	1562												
	beta -Caryophyllene [c]	1562												
	ST 55,57,67,81,105,119,161	1575												
	ST 40,57,67,69,91,104,105,119,204	1601												
	beta -Farnesene [a]	1620												
	alpha -Caryophyllene [c]	1624												
	ST 204,41,81,91,105,119,134,161	1626												
	alpha -Muurolene [a]	1643												
	ST 204,41,77,79,81,91,93,105,119,133,161	1676												
	ST 161,41,77,79,81,91,93,105,119,133	1695												
	(*E*)-Nerolidol [a]	1983												
Irregular terpenes	6-Methyl-5-hepten-2-one [a]	1300												
	6-Methyl-5-hepten-2-ol [b]	1419												
	2,6-Dimethyl-7-octen-2-ol [a]	1468												
Mis. cyclic ester	*gamma* -Butyrolactone [a]	1604												
N-compound	Indole [a]	2375												
S-compounds	Dimethyl trisulphide [c]	728												
	Dimethyl disulphide [c]	1112												
Unknown	UNK 39,43,44,57,70,71,85	1558												

species, characterised by the presence of over 20 unique aliphatic and benzenoid esters. The compounds methyl benzoate and styrene are also notably absent from the scent profile of this species.

Benzenoid compounds shared between all species included anisole, benzaldehyde and benzyl alcohol. These compounds are very common among plants, benzenoids being one of the largest classes of essential oils in terms of number of compounds produced by plants (Cseke et al., 2007; Levin et al., 2003). Anisole has been found in small amounts in scarabaeid sex pheromones (Bengtsson et al., 2010) and it and related compounds are used as attractants to trap scarab pest species (see Leal et al., 1996). It is unknown whether it is found in cetoniine sex pheromones, nor if it is also attractive to this subfamily. Styrene was found in all scent profiles, except that for *P. welwitschii*, and was absent in control samples. The presence of styrene is puzzling as it is seldom emitted by plants. One possibility is that it is an insect faecal artefact, although this needs to be confirmed. It seems therefore that the common benzenoids found in *Protea* scents are either symplesiomorphic or insect contaminants and have little to do with the pollinator shift in this clade.

Some compounds known to be attractive to cetoniines such as cinnamic alcohol and its relatives were unexpectedly absent from beetle-pollinated species profiles. Pure cinnamic alcohol was the most attractive compound to cetoniine beetles and second most attractive compound to ruteline beetles caught in field traps set out by Donaldson et al. (1990). Interestingly we found cinnamic alcohol only in the putatively bird-pollinated *P. punctata*. Cinnamic alcohol along with other benzenoids and monoterpenes found in bird-pollinated *Protea* scents are probably attractive to insects in the field, as researchers report beetles visiting these plants, especially the large cetoniine *Trichostetha fascicularis* (e.g. Coetzee and Giliomee, 1985; Hargreaves et al., 2004) (e.g. Fig. 1D). We however, also have preliminary observations of aggregations of up to 50 melyrid beetles per inflorescence of *P. subvestita* (Fig. 1K) and several families of beetles are proposed as co-pollinators of *P. nitida* (Lach, 2007). In these cases, there may be a strong affect of colour, a learned response reinforced by abundant pollen and nectar rewards, on the attractiveness of beetles to these species, which are potentially more generalist than previously thought.

Apart from cinnamic alcohol, linalool and its oxides have also been reported as cetoniine beetle attractants. Linalool oxides are potentially responsible for the distinctive papaya-like fragrance of beetle-pollinated *Protea* inflorescences since they are prominent as flavour components of papaya (*Carica papaya* L., Caricaceae), grapes and tea leaves (reviewed by Raguso and Pichersky, 1999). Overall, these *Protea* species share over 30 volatiles with the scent of papaya fruit (Pino et al., 2003). Linalool and its oxides are found in fragrances of numerous beetle-pollinated plants, (e.g. *Magnolia* species; Azuma et al., 2001), and also notably in most hawkmoth-pollinated plants worldwide (e.g. 35.3–51.5% and 56.8% linalool compounds in the sweet scents of two *Coussarea* species and *Carica papaya*, respectively (Knudsen and Tollsten, 1993). Linalool was found in small amounts in the scent of three bird-pollinated *Protea* species investigated here (<1.02%) and in the rodent-pollinated *Protea humiflora* Andrews (0.2%, S.D. Johnson & R.A. Raguso, unpublished data), suggesting that up-regulation of the biosynthesis of linalool and its oxides by fruity scented *Protea* species may be an important adaptation for beetle pollination.

Not only was linalool the dominant compound in scents of beetle-pollinated *Protea* species, but it was emitted in 500- to 3500-fold greater amounts compared to those of the three bird-pollinated species in which it was also found. While we have mentioned that linalool is emitted by many plant species in small amounts (possibly just metabolic noise in some species), it can function as a distance attractant when its production is ramped up, as it almost certainly does in sphingophilous flowers (Raguso and Pichersky, 1995). For example, the genus *Clarkia* is dominated by "scentless" bee-pollinated species. However, moth pollination in *Clarkia breweri* Greene is associated with the up-regulation of linalool and its oxides and a change to night-blooming (Raguso and Pichersky, 1995).

3.2. Functional significance of scent in beetle-pollinated Protea species

The scent of the smaller *P. simplex* inflorescences was significantly more attractive to cetoniine beetles than its sympatric congener, *P. roupelliae* which has a 5-fold greater inflorescence mass. This was the case both for experienced beetles collected from *P. simplex* inflorescences and "naive" beetles from the Cobham Nature reserve. The functional significance of individual compounds dominating the scent of these *Protea* species is beyond the scope of this study and will be addressed elsewhere, but olfactometer and field trapping experiments have shown linalool to be strongly attractive to several phytophagous cetoniine pests (Bengtsson et al., 2009; Donaldson et al., 1990; Larsson et al., 2003; Vuts et al., 2010).

In a study of the function of scent components of *Satyrium microrrhynchum* using gas chromatography–electroantennographic detection (GC–EAD), linalool, which comprised up to 70% of the floral scent of this orchid in one population, gave the strongest response in the antennae of the beetle *A. tigrina* (Johnson et al., 2007) which was used in the choice experiments in this study. This technique will be employed in future studies to determine detectable compounds in *Protea* by cetoniine beetle pollinators.

Many of the aliphatic compounds found in the bird-pollinated *Protea* scents were ubiquitous C6 "green-leaf" volatiles, emitted at similarly low levels to aliphatics emitted by "scentless" hummingbird-pollinated plants documented by Knudsen et al. (2004). Donaldson et al. (1990) found that (*E*)-2-hexenoic acid was completely unattractive to Cetoniinae and due to their ubiquity in plant tissues, we suspect these "green-leaf" volatiles do not play a specific role in the attraction of insect pollinators to *Protea* inflorescences. Of the C5-branched chain compounds, methyl 2-methylbutanoate, found here only in the floral scent *P. simplex*, has recently been shown to be attractive to scarab beetles (Gottsberger et al., 2012).

The esterification of alcohols lead to the production of over 20 volatile esters unique to the floral scent of *P. welwitschii*. Chromatographic data suggest that organic acids (butyric acid, isovaleric acid, tiglic acid, caproic acid) and alcohols (benzyl alcohol, phenylethyl alcohol, hexenol, 2-methyl-heptanol) are esterified to form a variety of acetates, tiglates, butyrates, valerates and benzoates. Further experiments are required to determine if the esterification of these alcohols plays a functional role in attraction of pollinators. Slight changes in chemical structure have been found to affect the attractiveness of a compound to some cetoniines. For example, esterification of cinnamyl alcohol into cinnamyl

Fig. 2. A heat map showing a visual representation of emission rates per inflorescence for all volatile compounds emitted from (reading left to right) eight bird-pollinated *Protea* species and four beetle-pollinated species. Compounds are grouped by compound class according to Knudsen et al. (2006) and Kovats indices are given for each. Grey shading is based on a log scale (note that the first shade of light-grey spans two log increments instead of one). Abbreviations: HC = hydrocarbon; MT = monoterpene; ST = Sesquiterpene. Compound identification criteria: a = comparison of MS with published data; b = comparison of MS and retention time with published data (e.g. http://webbook.nist.gov (Linstrom and Mallard, 2010) and references therein); c = comparison of MS and retention time with published data and authentic standard.

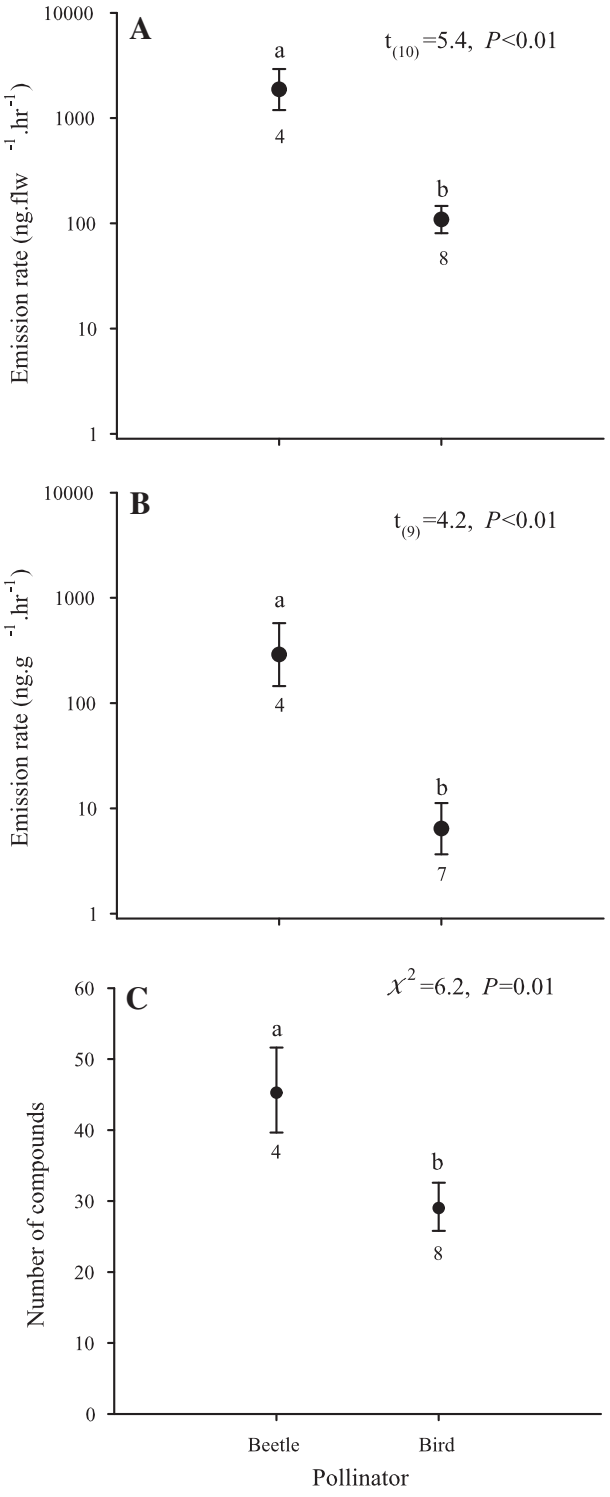


Fig. 3. Comparisons of floral scents of beetle- and bird-pollinated *Protea* species for (A) emission rates per inflorescence, (B) mass-specific emission rates (based on dry mass of inflorescence tissue), and (C) the mean number of compounds comprising the floral scents. Sample sizes are shown below each mean and different letters depict significant differences between means. Note the logarithmic scale for (A and B).

acetate changed the proportion of *Oxythyrea* species (Cetoniinae) caught in traps from 38% with the alcohol to 96% with cinnamyl acetate (Donaldson et al., 1990). Both birds and beetles visit nearly all the *Protea* species presented in this study (e.g. Coetzee and Giliomee, 1985; Hargreaves et al., 2004), but their relative frequency and importance as pollinators of particular *Protea* species appears to vary in relation to a suite of traits, including plant and flower morphology, pollen and nectar rewards, and the amount and composition of floral scent emissions (this study; Steenhuisen and Johnson, 2012; Steenhuisen et al., 2012; S.-L. Steenhuisen, personal observation).

3.3. Concluding remarks on trends in the floral scent of Protea

All except two of the bird-pollinated *Protea* species investigated in this study occur in south-western winter rainfall regions of South Africa. In contrast, the more strongly fruity scented and beetle-pollinated *Protea* species occur in the north-eastern summer rainfall areas, which is consistent with a trend for cetoniine beetle pollination systems involving scent cues to be more frequent at lower latitudes (Bernhardt, 2000; Englund, 1993; Gottsberger, 1990). Our statistical analyses of emission rates and the number of compounds between beetle- and bird-pollinated species did not explicitly control for phylogenetic relatedness, and thus should be viewed as simple tests of associations between scent patterns and pollination systems, and not statistical tests of adaptation (Felsenstein, 1985). Since beetle-pollination probably evolved only once in *Protea* (Schnitzler et al., 2011), sampling of other genera would be required to confirm the evolutionary generality of the changes in scent chemistry that we observed in *Protea*. More sampling is also needed to determine if the fruity scents are only found in the non-Cape clade and if the high diversity of scent compounds in *P. welwitschii* is autapomorphic.

Due to their relatedness, similar floral morphology and summer-rainfall distributions, we predict that taxa closely related to our four beetle-pollinated species would emit similarly fruity floral scents attractive to cetoniine beetles. In the same way that up-regulation of linalool may be the principal adaptation associated with a shift from bird- to beetle-pollination, up-regulation of other compounds may be associated with a shift from bird- to rodent-pollination in other clades of *Protea*. More intense sampling of pollinators and scent chemistry in *Protea*, together with bioassays that test the effects of individual compounds on attraction of birds, beetles and rodents, is required to fully reveal the role of pollinator-mediated selection in the evolution of volatile chemistry in this genus.

4. Experimental

4.1. Study species

The beetle-pollinated *Protea* species included in this study (*P. caffra* Meisn., *P. dracomontana* Beard, *P. simplex* E.Phillips ex J.M.Wood, *P. welwitschii* Engl.) are common in grassland vegetation in the summer-rainfall region of South Africa (Rebelo, 2001) (Fig. 1). While cetoniine beetles are the principal pollinators of these species (Steenhuisen and Johnson, 2012; Steenhuisen et al., 2012), some populations, especially of *P. caffra*, can be heavily visited by birds. The bird-pollinated species sampled for this study were *P. roupelliae* Meisn. subsp. *roupelliae* and *P. subvestita* N.E.Br, which are often sympatric with the beetle-pollinated species, and another six species (*P. cynaroides* (L.) L., *P. laurifolia* H.Beuk ex Meisn., *P. magnifica* Andrews, *P. nitida* Mill., *P. punctata* Meisn. and *P. repens* (L.) L.) which are restricted to fynbos vegetation in the winter-rainfall Cape region (Rebelo, 2001) (Fig. 1). Study sites and sampling dates for each species are given in Appendix Table 1. Through the use of exclusion experiments, birds have been shown to be the principal pollinators of *P. cynaroides*, *P. laurifolia*, *P. magnifica*, *P. nitida* (Wright et al., 1991), *P. repens* (Coetzee and Giliomee, 1985) and *P. roupelliae* (Hargreaves et al., 2004), although

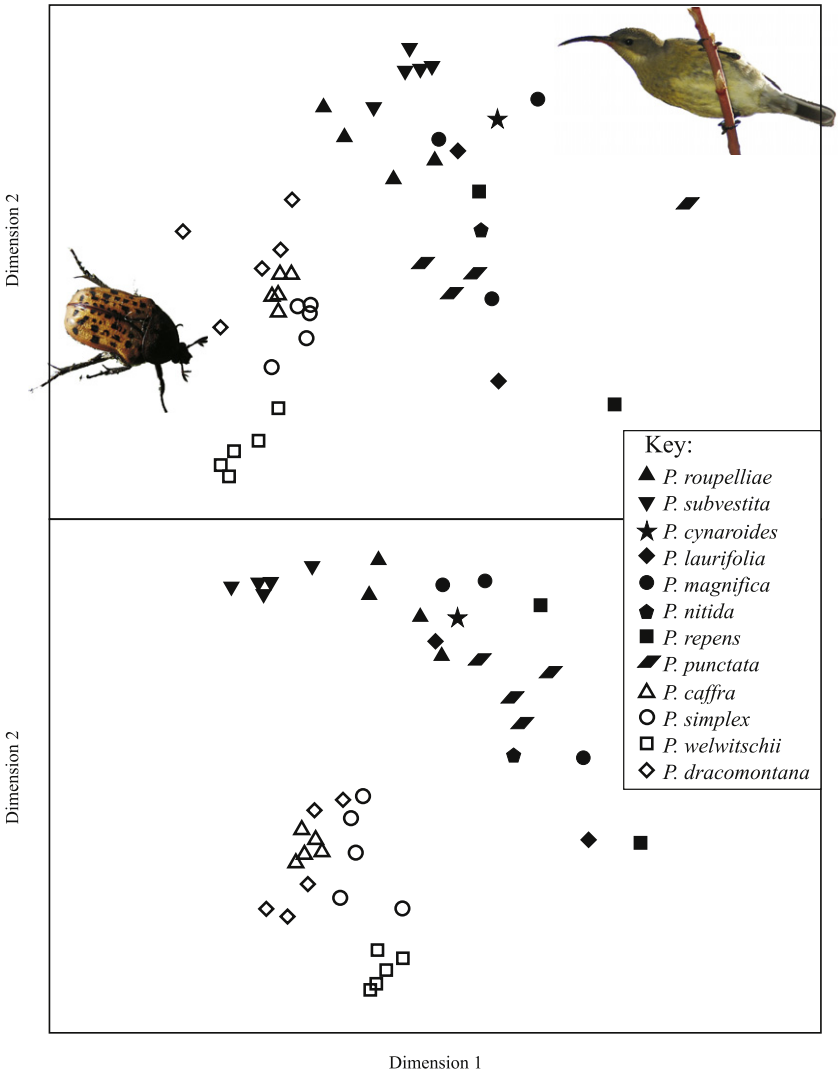


Fig. 4. Non-metric multidimensional scaling (NMDS) based on Bray–Curtis similarities of (A) whole flower emission rates (ng flw^{-1} h^{-1}; stress factor = 0.15) and (B) composition (stress factor = 0.17) of scent from twelve *Protea* species. Open and closed symbols depict beetle- and bird-pollinated species, respectively.

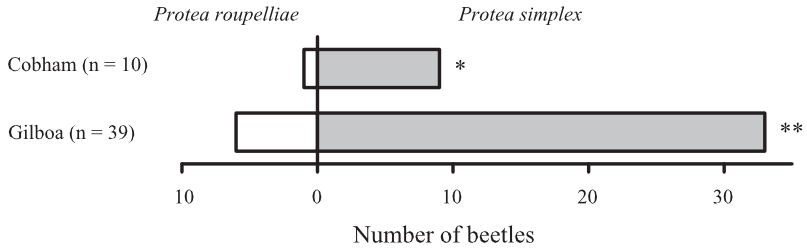


Fig. 5. The scent preference of *Atrichelaphinis tigrina* beetles from Cobham and Mount Gilboa, when offered the scents of whole inflorescences of sympatric *Protea simplex* and *Protea roupelliae* in a Y-tube olfactometer (binomial test: $^{*}P < 0.05$, $^{**}P < 0.01$).

insects are important vectors of pollen in many of these species (e.g. Coetzee and Giliomee, 1985). Bird pollination of *P. punctata* and *P. subvestita* was predicted from observations by various researchers (Carlson and Holsinger, 2010; De Swardt and Louw, 1994), although long-proboscid flies and butterflies have recently also been implicated as pollinators of *P. punctata* (Johnson et al., 2012). Plant vouchers are stored at the Bews Herbarium (NU) University of KwaZulu-Natal Herbarium (accessions R.A. Raguso RAR-ZA-01-05 and S.-L. Steenhuisen 54-66).

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

Floral scent was collected from the headspace of inflorescences and analysed by coupled GC–MS. Scent profiles of fully dehisced inflorescences of beetle-pollinated *Protea* species were taken from Steenhuisen et al. (2010). For the other species used in this study, cut stems were placed in water while headspace samples were taken by placing each inflorescence ($\frac{3}{4}$ – all florets fully dehisced) in a

polyacetate bag (Toppits oven bags and Kalle Nalophan), allowing scent volatiles to accumulate for 0–90 min (to reduce the background noise per volume of air pumped in relation to the scent emitted from the plant; modified from Heiduk et al., 2010; Andreas Jürgens, personal communication), and pumping the air for 5–180 min through a small cartridge filled with 1.5 mg of Tenax and 1.5 mg of Carbotrap™ activated charcoal at a realised flow rate of 50 mL min^{-1}. Controls were taken from an empty polyacetate bag sampled for the same duration. As pollinators were active during the day, 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 (data not shown). Scent sampling cartridges were placed in a Varian 1079 injector equipped with a Chromatoprobe™ thermal desorption device and stripped volatiles were separated using a Varian CP-3800 GC with a 30 m × 0.25 mm internal diameter (film thickness 0.25 μm) Alltech EC-WAX polar column coupled to a Varian 1200 quadrupole mass spectrometer in electron-impact ionisation 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).

Compounds were identified using the Varian Workstation software with the NIST05 mass spectral library and were verified, when possible, using retention times of authentic standards and published Kovats indices. Compounds present at similar abundance in the controls were considered to be contaminants and were excluded from analyses. Once volatile compound peaks were identified, manual integration of the peaks was performed. Known amounts of four standards (benzyl alcohol, *cis*-ocimene, linalool, phenylethyl alcohol) were injected into thermal desorption cartridges and desorbed in the same manner as the samples. The peak areas of compounds in the samples were compared to those of the mean peak area per ng of the standards and used to calculate the emission rate per compound and for whole inflorescences as ng flw^{-1} h^{-1}. Volatile emission rates for the four beetle-pollinated species were reported previously by Steenhuisen et al. (2010), but the rate for *P. dracomontana* was underestimated in that publication due to a failure to account for a change in baseline associated with a faulty MS filament and has been corrected here. Emission rates for the 12 study species were used to generate a heat-map (Wragg and Johnson, 2011), in which emission rates on a log scale are represented by different shades of grey (Fig. 2). The average emission rate and dry mass measured for one inflorescence of each species, except for *Protea punctata* (originally sampled herbarium specimen not available), was used to calculate a mass-specific emission rate for each species. These mass-specific emission rates, and whole flower emission rates of beetle- and bird-pollinated *Protea* species were separately compared using a 2-tailed *t*-test on log-transformed data assuming equal variances (Zar, 1984). The total number of compounds for beetle- versus bird-pollinated *Protea* species was compared using a generalised linear model with a Poisson distribution corrected for overdispersion, loglink function, and likelihood ratio Chi-square statistics in PASW SPSS version 18 (Field, 2009; Hosmer and Lemeshow, 2000; McCullagh and Nelder, 1989). For graphical representation of means and standard errors, we used values that were back-transformed from the log scale, resulting in asymmetric standard errors. We used multivariate analysis, implemented in the Primer 6 program, to further assess similarities between beetle- and bird-pollinated species. Two-dimensional non-metric multidimensional scaling (NMDS) was used to obtain visual representations of patterns of scent composition in beetle- and bird-pollinated species based on whole flower emission rates for each compound (ng flw^{-1} h^{-1}) and the percentage of each compound contributing to whole flower scents. The data were $\log(x+1)$ transformed for emission rates and square root transformed for proportional data before calculating Bray–Curtis similarities to detect similarities between species and pollination systems. The stress values are included to evaluate the fit of the particular configuration produced to the observed distance matrix (the smaller the value the better the fit; Clarke, 1993). The significance of differences in emission rates and proportions of scent compounds was compared between species and pollination systems (beetle- versus bird-pollinated species) using 2-way ANOSIM (Analysis of Similarities). Significance of the test statistic *R* generated by ANOSIM was assessed by 10 000 random permutations of both grouping vectors (species and pollination system) to obtain empirical distributions of *R* under the null model (*R* close to unity indicates complete separation of groups while *R* close to zero indicates minimal separation among groups). This was followed by SIMPER analyses to determine which compounds were responsible for any differences between pollinator groups.

4.3. Beetle attraction to scent

Choice experiments were conducted to determine whether the cetoniine beetle, *A. tigrina*, a common pollinator of the grassland *Protea* species preferred the fruity scent of flowers of *P. simplex* over that of the sympatric bird-pollinated *P. roupelliae*. Although flower heads of *P. roupelliae* are about five times greater in dry mass than *P. simplex* flowers, we used whole flower heads of these species in the choice tests in order to accurately represent the unit of attraction in the field. We used a Y-shaped olfactometer placed in a greenhouse. The run was composed of three sections of clear Perspex pipe, one central tube and two tubes forming the arms of the "Y" with metal box compartments and fans fitted to their ends. As the *Protea* inflorescences were too large to be held in the compartments, plastic bottles with cut ends were used to house the flowers on the outside of the fans, which drew air over the flowers and into the chamber from both ends. To ensure that a beetle's choice of scent was not influenced by other variables besides scent, an experiment testing for random choice was first conducted. In this experiment, no flowers were present in the bottles and the olfactometer was positioned precisely to face the direction of the sun by using the shadow cast by a vertical metal rod. The airflow from the fans was regulated to ensure equal flow down both arms of the olfactometer. Thirty-five cetoniine beetles collected from *P. simplex* inflorescences at Mount Gilboa were allowed to choose (individually) which arm of the olfactometer they would enter. A non-significant percentage ratio of 49:51 in the choice of direction was obtained (binomial test, $P = 1.0$).

Thirty-nine beetles (including the 35 beetles used in the random test) were then used in choice experiments conducted with inflorescences of *P. simplex* and *P. roupelliae*. They were placed consecutively in the chamber and each was considered to have made a choice once it had walked at least half way down one of the arms. The positions of the inflorescences were swapped periodically. The results were analysed using a binomial test in SPSS version 18.

The previous experiment was repeated using ten cetoniine beetles (*A. tigrina*) from Cobham Nature Reserve, Drakensberg (29.70°S, 29.41°E, 1640 m), where neither of the *Protea* species used in the choice experiments were flowering at the time. The beetles were thus considered to be naive toward the scent of either *Protea* species. Two trials were conducted using each beetle twice with opposite orientation of the inflorescences in the arms of the maze. The results of each trial were analysed separately using binomial tests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2012.08.012.

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