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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 $\mathrm{flw}^{-1}\,\mathrm{h}^{-1}$ in these species compared with 0.09 ng $\mathrm{flw}^{-1}\,\mathrm{h}^{-1}$ 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 $\mathrm{flw}^{-1}\,\mathrm{h}^{-1}$ were recorded for *P. cynaroides* (L.)L., *Protea magnifica* Link, *P. nitida* and *P. repens*; 110–310 ng $\mathrm{flw}^{-1}\,\mathrm{h}^{-1}$ for the remaining four bird-pollinated species, and 685–6110 ng $\mathrm{flw}^{-1}\,\mathrm{h}^{-1}$ for beetle-pollinated species.

We found highly significant separation between species and pollination systems with respect to scent composition using emission rates (ng $\mathrm{flw}^{-1}\,\mathrm{h}^{-1}$; 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