MATING SYSTEMS, INSECT POLLINATION AND CHEMICAL ECOLOGY OF GRASSLAND *PROTEA* SPECIES (PROTEACEAE)

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FOR GRANDPA (ARNOLD) CARR

AND

MY FAMILY

ABSTRACT

Major transitions between vertebrate and insect pollination systems have occurred many times during the angiosperm radiation and are associated with evolutionary modifications in floral traits. In the large ancestrally bird-pollinated African genus *Protea* (Proteaceae), an evolutionary shift from bird to insect pollination in the genus is suggested by the fruity diurnal scent of flowers in a recently evolved clade of grassland species. In this study, I confirm that four of these grassland *Protea* species have mixed mating systems and are indeed insect pollinated, and furthermore demonstrate the functional significance of their floral presentation and scent chemistry for attraction of pollinators, specifically cetoniine beetles.

The study species, *Protea caffra, Protea dracomontana, Protea simplex* and *Protea welwitschii,* have colourful bowl-shaped inflorescences that produce copious amounts of pollen and dilute, xylose-rich nectar. Cetoniine beetles were found to be the most suitable pollinators due to their abundance, size, relatively pure *Protea* pollen loads, and their preference for the fruity scent and low growth form of these scented *Protea* species, as demonstrated by choice experiments in which inflorescences were offered at either end of a y-maze or at various heights above the ground, respectively.

Bagging and hand pollinations revealed that these *Protea* species are self-compatible and capable of autonomous selfing. Self progeny of *P. caffra* were as vigorous as cross progeny in terms of germinability and survivorship to two months. Vertebrate-excluded and open-pollinated inflorescences yielded similar seed numbers for all species. Supplemental hand-pollinations, however, failed to increase seed set substantially, an indication of resource limitation. Outcrossing rates estimated using polymorphisms at eight allozyme loci in progeny from vertebrate-excluded and open-pollinated treatments of *P. caffra* were no different (t=0.59), indicating outcrossing by insects and an equal or insubstantial contribution from bird pollinators.

The fruity-sweet scents of these species were more complex, with higher whole flower and mass-specific emission rates, than those in eight bird-pollinated congenerics. The overall floral scent is shown to be a blend of emissions from various plant parts, especially nectar. Electroantennography (EAG) revealed that the generalist pollinator *Atrichelaphinis tigrina* responds to a variety of volatile compounds found in fruity *Protea* scents. Field trapping confirmed that this cetoniine beetle is strongly attracted to *ß*-linalool (up to 60% of scent profile) and methyl benzoate.

In conclusion, this study demonstrates the evolution of beetle pollination and mixed mating systems in a grassland clade of *Protea.* Volatile compounds that make up the unique

(within *Protea*) fruity scent of the study species are shown to attract beetles, and the emission of large amounts of these compounds was probably a key step in the transition from bird to insect pollination in *Protea*.

PREFACE

The experimental work described in this thesis was carried out in the School of Biological and Conservation Sciences, University of KwaZulu-Natal, Pietermaritzburg, from January 2003 to January 2012, under the supervision of Professor Steven D. Johnson.

This thesis represents original work by the author and has not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

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Sandy-Lynn Steenhuisen
March 2012

I certify that the above statement is correct.

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Professor Steven D. Johnson (supervisor)
March 2012

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DECLARATION 1 – PLAGIARISM

I, Sandy-Lynn Steenhuisen declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.
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DECLARATION 2 – PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS THAT FORM PART OF AND/OR INCLUDE RESEARCH PRESENTED IN THIS THESIS

PUBLICATION 1.

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Author contributions:

SLS and SDJ conceived paper. SLS collected and analysed data, and wrote the paper. SDJ contributed comments.

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Author contributions:

SLS and SDJ conceived paper. SLS collected and analysed data, and wrote the paper. SDJ contributed comments.

PUBLICATION 3.

STEENHUISEN, S-L. AND S.D. JOHNSON. 2012. The influence of pollinators and seed predation on seed production in dwarf grassland *Protea* "sugarbushes" (Proteaceae). South African Journal of Botany. 79: 77-83.

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CONTENTS

"Amongst the legends of ancient Greece is a tale about Menelaus, King of Sparta, who tried to help Telemachus find his father Odysseus, who had been delayed on his way back from Troy. He told the young man that he had managed to capture the Greek god Proteus, also known as the old man of the sea, who would foretell the future if he were caught. Proteus changed into every imaginable shape in order to avoid being caught, but Menelaus struggled and managed to retain his grip while Proteus took on one shape after another. Finally he overcame him and forced him to reveal the destinies of the returning heroes. Proteus revealed that Odysseus was the captive of the nymph Calypso, who kept him prisoner on a lonely island.

The Proteus of many shapes was the mythological figure that sprang to the mind of Carl Linnaeus, the great Swedish naturalist, when he named the Protea."

Eliovson (1973, pg 3)

"*Insects could be more important than mammals or birds for plants whose inflorescences emit strong odours and produce little nectar*."

Collins and Rebelo (1987, pg 402) on the pollination of the Proteaceae

CHAPTER 1

INTRODUCTION

While animals usually move to find a mate, most flowering plants have to rely on wind, water or animal vectors to transfer pollen from the anthers of one flower to the stigma of another in order to reproduce. Most plants offer animal pollinators a reward for this service (Simpson and Neff, 1983). Through selective pressures for efficient transfer of gametes to maximise outcrossing, animal-pollinated plants have evolved traits attracting one or a group of pollinators, according to the vector's sensory abilities, morphology and energetic requirements (Chittka, 1996; Raguso, 2008; Barrett, 2010).

Visual and olfactory cues that have evolved to attract certain pollinators, can also act as filters of a general animal community of floral visitors, ensuring that rewards and pollen are not wasted on unsuitable pollinators and preventing potentially deleterious stigma clogging from foreign pollen sources. Scent is usually a long-distance attractant whereas colour cues are more important for creating contrast against the habitat background and orientating a pollinator on a flower for efficient pollen deposition and transfer (i.e. nectar guides; Raguso and Willis, 2002; Goyret and Raguso, 2006; Goyret et al., 2007; Raguso, 2008). To exploit the sensory abilities and motivation of different pollinators, colour and olfactory cues often mimic a pollinator's main food source, mate, brood site (Urru et al., 2011), or simply advertise a reward. Colour and olfactory cues have also been shown to change with flower ontogeny (Lamont, 1985) and pollination events. Changes in hue or scent emission of certain floral volatiles can signal whether or not a flower has been pollinated and/or advertise the presence/absence of a reward (Theis and Raguso, 2005). These floral signals can be learned by pollinators and used to guide floral visitors to un-pollinated flowers, thereby increasing foraging efficiency for the pollinator and potential outcrossing for the plant.

The most common floral rewards are nectar and pollen. Nectar rewards can either be exposed for short-tongued pollinators or hidden within modified floral spurs often corresponding to the shape and length of an animal's tongue. Sugar composition and concentration differ according to the energetic and physiological needs of the pollinator involved (Baker and Baker, 1983), whereas the presence of phenolics can repel unwanted nectar thieves (Johnson et al., 2006). Alternatively pollen-rewarding plants produce immense amounts of pollen or display heteranthy to ensure that some is transferred to stigmas of another flower before being consumed or carried off to brood sites (Vallejo-Marin et al., 2009).

Pollination syndromes — Adaptations of plants for pollination by particular functional groups of pollinators (e.g. bats, birds, bees, moths, beetles) result in convergent suites of floral traits (i.e. colour, scent, size, floral reward) across unrelated plant lineages known as pollination syndromes (Faegri and van der Pijl, 1979; Fenster et al., 2004). Convergence of these floral traits results from selection imposed by pollinators according to their size and shape, sensory abilities, dietary requirements and foraging behaviour. For example, plants adapted for beetle pollination often share the following floral traits: dull or white coloured perianth; fruity or aminoid scent; flat or bowl-shaped flowers with radial symmetry; large pollen rewards and, little or no nectar (Faegri et al., 1979; Howe and Westley, 1988; Bernhardt, 2000). There are, however, notable exceptions, including the brightly coloured, unscented flowers pollinated by scarab beetles in Southern Africa and Mediterranean Europe (Bernhardt, 2000). The concept of "pollination syndromes" implies some degree of specialization in pollination systems and has recently been challenged on the grounds that generalization is the rule in most pollination systems (Ollerton, 1996; Waser et al., 1996). In support of the concept of pollination syndromes, Johnson and Steiner (2000) and Fenster et al. (2004) highlight the fact that there is extensive evidence for specialization, at least at the level of the functional pollinator groups that are associated with syndromes.

Both generalist and specialist pollination systems can exist in closely related species and may represent shifts in the degree of specialization in pollination systems within a plant family or genus. For example, the orchid *Disa sankeyi* is specialized for wasp pollination (Johnson, 2005) whereas its co-occurring close relative, *Disa fragrans* (Johnson and Hobbhahn, 2010) is pollinated by generalist beetles, flies and bees. Despite the evidence for both generalisation and specialisation in plant pollination systems, pollination syndromes are particularly useful for developing testable predictions about pollination systems (Johnson et al., 2000).

Floral scents can play a key role in characterising pollination syndromes. Plants sharing a pollinator guild emit floral scents matching the animal's sensory abilities and "search image", such as food or a mate. Vertebrate pollination syndromes typically involve the yeasty-scented, fleshy flowers with wide flower openings and hexose-rich nectar rewards pollinated by bats (Fleming and Muchhala, 2008) and geoflorous, yeast-scented inflorescences pollinated by rodents (e.g. *Liparia parva* (Fabaceae); Letten and Midgley, 2009). The absence of scent is notable in the more derived bird pollination system. Convergent floral traits found in bird-pollinated plants include red-orange unscented flowers usually with narrow openings protecting an abundant and dilute nectar reward. Birds are thought to have poor olfactory senses (Knudsen et al., 2004) although there is growing

literature on odour signals playing an important role for nest recognition in passerine birds (Caspers and Krause, 2011) and petrels (Cunningham and Nevitt, 2011), orientation in doves, predator avoidance in Blue Tits (Amo et al., 2008) and foraging in penguins (Wright et al., 2011) and petrels (Nevitt, 2008). Fragrant signals in nectar have been shown to affect visitation by hummingbirds to *Nicotiana attenuata,* repelling pollinators with high levels of nicotine or increasing visitation rates and duration with increased levels of benzyl acetone (Kessler and Baldwin, 2007). In this case birds may be responding to taste as chemical volatiles can affect the taste of nectar.

With marked differences in the sensory abilities of different pollinators, changes in floral scent composition and timing of emission can be expected when plants shift pollinators. A shift from hawkmoth (19 species) to long-tongue fly pollination (1 species) in the genus *Zaluzianskya* involved a change to daytime-flowering and a loss of floral scent (Johnson et al., 2002). Shifts from wind to insect pollination in sedges involved the changes in colour and the production of floral scents to attract insect pollinators. Changes in scent chemistry occur even for pollinator shifts within insect orders. For example, beetle pollination systems vary from lilies that heat up their foul-smelling volatile scents to attract carrion beetles, cycads attracting weevils with one or two specific volatiles, to monkey beetles attracted to unscented red flowers in the Mediterranean and South Africa.

Shifts in pollination systems — As pollinators can differ in abundance and distribution over a plant's distribution, there have been frequent shifts between pollination systems in various plant clades, leading to speciation through divergence of phenotype and reproductive isolation (Johnson, 2006; Campbell, 2008). According to this pollinator-shift or "Grant-Stebbins" model (Grant, 1949; Stebbins, 1970, 1973, 1981), the immense diversification of floral form in angiosperms is considered a consequence of adaptations to different pollinators.

As predicted by Stebbins (1970), shifts usually occur where the main pollinator is less abundant. For example shifts from insect and bird pollination to wind pollination in *Espeletia* is associated with a decrease in animal pollinators with increased elevation in the Venezuelan Andes (Berry and Calvo, 1989). These shifts are usually associated with changes in floral traits, generally only one or two. More commonly we see shifts between different types of animal pollination systems, often clearly evident in highly diverse plant lineages (e.g. *Disa*; Johnson and Steiner, 1997; Johnson et al., 2000; Johnson and Steiner, 2003; Johnson et al., 2010). Shifts from wind to animal pollination are common but the reverse is much more rare (Berry et al., 1989; Wragg and Johnson, 2011). Reverse shifts are evident in *Espeletia* (Berry et al., 1989) and in sedges following changes in colour and scent (Wragg et al., 2011).

Recently researchers have begun clarifying the role of scent evolution in pollinator shifts. For example, a shift from moth pollination to bird pollination in the orchid sub-tribe *Hadrangis* was accompanied by the loss of scent and a change to shorter wider spurs (Micheneau et al., 2006). Shifts to bat pollination most often involve changes in pollen production (increase in pollen production per anther, greater number of anthers per flower, proportionally more male flowers per plant for bat-pollinated plants), visual (pale flowers with nocturnal opening), and scent cues (musty scents) (Muchhala, 2006; Muchhala and Thomson, 2010). A shift from bee- to hawkmoth-pollination in *Clarkia* represented by *Clarkia breweri* that evolved nocturnal anthesis, pale colouration and sweet floral fragrance, the latter being a unique floral trait for this genus (Raguso and Pichersky, 1995).

It may only take one trait shift for a plant to attract a different pollinator group. Meléndez-Ackerman and Campbell (1998) showed that red colour alone was enough to increase hummingbird visits and seed set in *Ipomopsis* species compared to pink or white-coloured hybrids. Similarly, a change in colour of F2 hybrids of *Mimulus lewisii* was sufficient to induce a shift in pollinators (Bradshaw and Schemske, 2003). The loss of floral oil as a reward in populations of *Pterandra pyroidea* (Malpighiaceae) may represent a reverse shift from pollination by oil-collecting bees that use the oil for reproduction or to line their nests, to pollination by bumblebees foraging for pollen (Cappellari et al., 2011).
Kessler et al. (2008) manipulated two volatile compounds in *Nicotiana attenuata* to show their singular affects moth and hummingbird pollination. Experimental addition of sulfur compounds to wasp-pollinated *Eucomis* flowers resulted in a shift to pollination by carrion flies (Shuttleworth and Johnson, 2010).

The functional significance of these traits can be tested using a variety of choice tests and electrophysiological techniques. For example, Fulton and Hodges (Fulton and Hodges, 1999) showed that hawkmoths were ten times more likely to visit an upright versus pendant flower of the hummingbird-pollinated species *Aquilegia pubescens*.

Such pollinator shifts may be evident in the large Gondwanaland family Proteaceae in which “retrograde” patterns of floral morphological development from brush-flowers to open-bowl shaped inflorescences prompted Faegri (1965) to suggest a “retrograde” shift in pollination systems from predominately ornithophily to the “assumed most primitive stage of cantharophily”. The fruity scents of four grassland *Protea* species in South Africa have recently been described (Steenhuisen et al., 2010), and this study addresses the prediction that changes in scent among other floral traits, are associated with this suggested shift from bird- to beetle-pollination in *Protea*.

The reproductive biology of the Proteaceae — The Proteaceae is one of the most prominent flowering plant families in terms of diversity and abundance in the southern hemisphere with ancient origins dating back to before the break-up of Gondwana. It is comprised of five subfamilies (major ones being Grevilleoideae and Proteoideae) that evolved prior to the separation of Gondwanaland into the Australian and African continents (Johnson and Briggs, 1975). The African clade has 14 genera (10 endemic), 317 species in the south-western Cape of southern Africa and 69 species in other parts of Africa. Except for *Brabejum* and *Malagasia* (Grevilleoideae), all African species are proteoids, whereas all five subfamilies are represented in Australia. No genus is common to both regions, with the exception of an invasive *Hakea* species in South Africa.

With many similarities between the Mediterranean climates and poor-quality soils of the habitats dominated by Proteaceae on both Australia and southern Africa, this plant family serves as a model group for evolutionary biologists to investigate convergence and divergence of morphological and functional traits that have occurred in genetically related groups. More practically, investigations into the reproductive biology of these plants help in their conservation and management. Their commercial value in the horticultural and wildflower industries is another good reason for studying this plant family, especially with regard to floral and seed predators, breeding systems and hybridisation. South Africa specifically takes pride in the King Protea (*Protea cynaroides*) as its national flower.

Early work on this family concentrated on seed biology (storage and dispersal) in a horticultural sense, not with regards to reproductive biology (e.g. Lamont, 1991). Later studies on the pollination ecology of the plants were concentrated in less diverse genera and biased to south-western regions of Australia and South Africa. Collins and Rebelo (1987) reviewed research conducted on this family and concluded that most work had been centred on *Banksia* and *Dryandra* in Australia, and *Leucospermum* and *Protea* in South Africa.

In the past two decades, research on the Proteaceae has grown rapidly. In addition to pollination and breeding systems, recent research questions on this family have explored phylogenetic trends of diversification (Schnitzler et al., 2011), maintenance of colour polymorphisms (Carlson and Holsinger, 2010) and even microbes in nectar (de Vega et al., 2009). The current study now adds to this by presenting a comprehensive investigation of functional floral traits, in particular scent, characterising a newly described pollination system in the largest genus in Africa, *Protea*.

Pollination systems — The Proteaceae is typically characterised by four pollination systems: wind, bird, non-flying mammal, and insect (Collins et al., 1987; Ayre and Whelan, 1989;

Goldingay and Carthew, 1998). Wind pollination is inferred for most *Leucadendron* species (Collins et al., 1987), and remaining species are insect-pollinated (e.g. Bond and Maze, 1999) and are speculated to attract pollinators with yeasty or fruity-sweet floral odours (described for some species by Rebelo, 2001). Similarly, the sweet odours of putatively insect-pollinated species in other genera of the Proteaceae, namely *Brabejum* and *Vexatorella* may represent adaptations for attracting insect pollinators (Collins et al., 1987).

There is a very high frequency of vertebrate pollination systems in the Proteaceae. Among Australian genera, bird- and mammal-pollination dominate in *Banksia* and *Grevillea* (Collins and Rebelo, 1987), while bird pollination dominates in the African genera *Leucospermum, Mimetes* and *Protea* (Faegri, 1965; Mostert et al., 1980; Rebelo, 2001; Hargreaves et al., 2004). Bird pollinated Proteaceae produce typically large, unscented red-coloured inflorescences, bearing long and directed pollen presenters, and copious amounts of nectar often only accessible with a long tongue/beak. Strong visual cues form the basis of attraction in this system (e.g. individual florets of the brush-type *Leucospermum* inflorescences; involucral bracts of bowl-shaped *Protea* inflorescences; red, tube-like flowers of *Lambertia* pollinated by honeyeaters; Pyke, 1982). The affect of bird pollinators on pollination has been shown typically through the use of exclusion experiments (e.g. Whelan and Burbidge, 1980), while the energetic value of *Protea* nectar for visiting sunbirds and sugarbirds has also received much attention (Mostert et al., 1980; Lotz and Nicholson, 1996; Downs, 1997)

Pollination systems involving non-avian vertebrates are evident in both Australian and South African Proteaceae flora. Marsupials and honey possums pollinate many *Banksia* and a few *Grevillea* species (Goldingay et al., 1987; Cunningham, 1991; Carthew, 1993; Carthew, 1994; Goldingay, 2000) in Australia. In Africa, rodent pollination has been recorded in geoflorous *Protea* species that smell yeasty (e.g. *Protea amplexicaulis* and *Protea humiflora*; Wiens and Rourke, 1978). They flower in winter rather than spring when insects are inactive and rodents would be attracted to nectar (Letten et al., 2009).

Pollination of the Proteaceae by invertebrates has received much less attention than pollination by vertebrates, and even less so for African species. For Australian Proteaceae, insect pollination has been recorded in *Banksia, Conospermum , Dryandra*, *Grevillea, Hakea, Macadamia*, *Persoonia, Petrophile* (Carolin, 1961; Lamont, 1982; Lamont and Collins, 1988; Bernhardt and Weston, 1996; Wallace et al., 1996; Ladd and Wooller, 1997; Lamont et al., 1998; Blanche et al., 2006). Birds and honeybees affect pollination of *Banksia ericifolia* but resource limitation made interpreting results from exclusion experiments difficult (Paton and Turner, 1985). *Stirlingia* species (endemic to south western Australia) exhibit a mix of wind

(*Stirlingia latifolia*) and insect (*Stirlingia tenuifolia*) pollination systems (Ladd et al., 1997). *Stirlingia simplex* is scented but evidence points to an intermediate pollination system of wind and/or insect pollination (Ladd et al., 1997).

The distribution and guild composition of insects inhabiting the inflorescences of South African Proteaceae has been documented mainly in the context of the marketability of cut *Protea* flowers (Gess, 1968; Myburgh et al., 1973; Myburgh and Rust, 1975; Coetzee, 1986; Coetzee and Latsky, 1986; Wright and Giliomee, 1990; Wright and Samways, 2000). There remains a controversy about whether insects found in *Protea* inflorescences are pollinators or just visitors scavenging for pollen and nectar (Collins et al., 1987). Coetzee & Giliomee (1985) performed exclusion experiments on *Protea repens*, whereby avian visitors were excluded from visiting inflorescences with wire mesh cages. They showed that insects were effective pollen vectors for this apparently bird-adapted species. Similar exclusion experiments conducted on six other ornithophilous protea species also showed that insects affected pollination to some degree, although birds contributed to pollination substantially more (Wright et al., 1991; Hargreaves et al., 2004). Apart from *P. roupelliae,* however, we don't know if seed set in plants excluded from bird pollinators was the result of autonomous selfing as it is uncertain if some of these species are capable of autonomous self-pollination.

Despite this, insects found in *Protea* inflorescences have been portrayed as just pests or pollen and nectar thieves without further investigation of their effectiveness as pollinators (e.g. Wright et al., 2000). There has been little investigation into pollen loads on insects, abundance, visiting frequencies, floral attractants, or energetic rewards for insects visiting *Protea* species (Collins et al., 1987). The honey bee (*Apis melifera*) and large scarab beetles (e.g. *Trichostetha fascicularis*) have been shown to carry heavy pollen loads from *Leucospermum* species (Collins & Rebelo 1987). Faegri (1965) has also observed beetles (*Genuchus hottentottus*) effectively pollinating *Leucadendron* species, and *Faurea* species exhibit traits conforming to an entomophilous pollination syndrome.

As mentioned above, insects have been shown to affect pollination in several *Protea* species. In general, the effectiveness of insects as pollinators was not investigated further upon assumption that, due to their foraging behaviour, birds should promote outcrossing more than insects. While a quality difference in seed from open-pollinated plants and those excluded from bird pollinators was shown for *P. laurifolia* (Wright, 1994), outcrossing by insect visitors to *Protea* warrants further investigation.

Breeding and mating systems in Proteaceae — Breeding system studies have revealed high rates of self-compatibility in major genera of the Proteaceae, namely *Banksia* and

Leucospermum (Collins et al., 1987; Goldingay et al., 1998). In contrast, most South African *Protea* species examined were reported self-incompatible (Horn, 1962; Collins et al., 1987; Coetzee and Littlejohn, 2001). With the exception of *P. roupelliae,* an ornithophilous and self-compatible species (Hargreaves et al., 2004), all other breeding system studies for *Protea* have been conducted on Cape species. This study incorporates breeding system studies for four other non-Cape *Protea* species.

Inflorescences of the Proteaceae are generally comprised of tightly packed hermaphroditic flowers. The pollen of individual flowers is commonly applied by four anther lobes to a specialised subapical region of the style known as a pollen presenter, exposed when the style elongates and/or straightens during anthesis (van der Walt and Littlejohn, 1996; Matthews et al., 1999). These pollen presenters typically exhibit terminal stigmatic grooves (van der Walt et al., 1996). Proteaceae flowers are generally protandrous. *Protea* flowers within an inflorescence mature centripetally, and, unless removed, self-pollen from central flowers is available to pollinate receptive stigmas of more mature outer flowers. Self pollination may be prevented in most cases by protandry or other mechanisms (e.g. cellular outgrowths blocking the path of self-pollen on the same flower from entering the stigmatic groove; Ladd et al., 1998) in Proteaceae. While some genetic studies of seed set imply strong selection for outcrossing among the Proteaceae (e.g. Carthew et al., 1988), geitonogamy has been shown to affect outcrossing rates in *Banksia brownii* (Sampson et al., 1994) and some populations of *Grevillea barklyana* (Ayre et al., 1994), suggestive that protandry may not be as effective for preventing self pollination as previously thought.

The Proteaceae are typically characterised by very low seed set, even in self-compatible species (Collins et al., 1987; Ayre et al., 1989). Ayre and Whelan (1989), in a review of factors affecting seed set, describe a number of hypotheses that attempt to explain very low fruit: flower ratios in potentially outcrossed hermaphroditic Proteaceae when no clear evolutionary benefits are evident. Two proximate hypotheses they suggest may be experimentally tested by pollen supplementation. These are (1) pollen limitation (insufficient pollen available to stigmas) and (2) resource limitation (insufficient availability of nutrients for the conversion of each pollinated flower into a fruit) (Ayre et al., 1989). There is a general trend toward resource limitation in the Proteaceae (Collins et al., 1987) but long-term studies have revealed mixtures of resource and pollen limitation for various species in different years (Copland and Whelan, 1989).

The study system: Protea, the largest genus of the Proteaceae, is comprised of 113 species with a range throughout southern Africa and north up to tropical Eritrea (Johnson et al., 1975).

Along with many other plant taxonomic groups, its center of endemism is in the Cape Floristic Region of South Africa (Schnitzler et al., 2011). Thus the majority of *Protea* species (72) are found in the Southern Cape, which receives winter-rainfall. Only 13 species have distributions in the summer rainfall region of South Africa (KwaZulu-Natal, Lesotho, Swaziland, Mpumulanga, Northern Province; Rourke, 1980; Rebelo, 2001). Of these 13, ten display floral traits characteristic of beetle pollinated plants (i.e. fruity scent, open bowl-shaped inflorescences, accessible nectar and large pollen rewards). Some grow from large underground rootstocks, and some form small trees that may reach several metres in height. Their habitats range from grassland and savanna to mountainous terrain. The inflorescences of most are recorded having a sweet, fruity scent and there is a wide range in the colour of the involucral bracts from pale green to carmine.

Based on floral and plant morphology, habitat and distribution, informal groupings have been used to describe the *Protea* genus since the late 1900's (e.g. grassland, spoonbract, and western ground sugarbushes) (Rourke, 1980; Rebelo, 2001). Some of these groupings do incorporate common pollination systems, such as the ground sugarbushes and putative rodent pollination. Recently, the majority of these groupings have been confirmed by molecular work on the genus (Valente et al., 2010).

Collins and Rebelo (Collins et al., 1987) summed up pollination systems within *Protea* by stating that only two species of *Protea* are putatively insect pollinated, the rest are bird- (c. 47 species) and mammal-pollinated (c. 24 species). They describe insect-pollinated sugarbush inflorescences as white/cream in colour, with 45-60 gullet-shaped flowers that emit sweet odours, have a nectar to stigma distance of 16-20 mm, and produce little or unknown amounts of low-energy nectar. Collins & Rebelo (1987) commented that "knowledge of invertebrate visitors is even more rudimentary than that for vertebrates." These authors also suggested that there is overlap of pollination syndromes for the Proteaceae, having observed a range of floral visitors to any one species. Even so, there are distinctive floral traits that have arisen to attract a few main pollinator-types that may help predict effective pollinators for a species. These include rodent-pollination of cryptic inflorescences that emit yeasty odours, conspicuously coloured inflorescences with hidden nectar rewards presented to bird pollinators, and, open inflorescences that emit strong sweet-fruity odours attracting insect pollinators.

Protea species of the section *Hypocephalae* are rodent pollinated (therophilous) with strongly scented cryptic inflorescences borne close to the ground. Opportunistic pollinators, such as elephant shrews, also pollinate these species while foraging for insects (Fleming and Nicolson, 2003). As mentioned previously the arthropod fauna in *Protea* flowers are seen as

pests, but also as an attractant and energy source for rodent and bird pollinators (Mostert et al., 1980; Fleming et al., 2003).

Besides the three major sugars (sucrose, fructose, and glucose) found in most floral nectars, xylose has been discovered as a major constituent (up to 39%) of nectar of *Protea* and *Faurea* species (van Wyk and Nicolson, 1995; Nicolson and Van Wyk, 1998). Insect and bird pollinators are averse to xylose, and although rodents will drink pure xylose, it is the least preferred sugar (e.g. Allsopp et al., 1998). The presence of this sugar in *Protea* nectar is puzzling as it does not seem to have an ecological significance and a rarity of studies on phloem sap composition makes it difficult to ascertain if it is a by-product from phloem tissue or enzymatic action on other nectar sugars (Jackson and Nicolson, 2002). Of the beetle visitors to *Protea* inflorescences, behavioural studies have been conducted with the cetoniine beetle *Trichostetha fascicularis* (S.W. Nicolson & S. Jackson, unpubl.data). This large species is mainly found in bird-pollinated *Protea* species with tree-like growth forms and little scent, and has shown a slight averse behaviour towards xylose (S.W. Nicolson & S. Jackson, unpubl.data).

Scarabs are unusual pollinators as they lack any unique morphology seen in other insect pollinators such as long-tongued flies, bees, butterflies or moths. They are thus thought to be unspecialist flower visitors (but see Gottsberger and Silberbauer-Gottsberger, 1991). Coleoptera have been found to be the most dominant and frequent invertebrate visitors of many *Protea* species because of the large pollen rewards offered, comprising, for example, 86.5 % (Coetzee et al., 1985) and 68 % (Mostert et al., 1980) of invertebrate visitors of *Protea repens*. The debate of "insect versus bird" pollination has been addressed several times and will be described in more detail in later chapters. However it should be noted that inconsistent methodology has made interpretation of past breeding system and pollinator exclusion experiments difficult and there is still considerable uncertainty about the contribution of insect visitors to seed set for most *Protea* species.

Protea farming is of great economic importance in South Africa in terms of the cut-flower industry. Besides seed predation, early work on this genus also centred on breeding systems and hybridisation, for use in creating new varieties for this industry. Following initial work by Horn (1962), *Protea* species were assumed to be self-incompatible. Later studies, however, have reported self-compatibility, contradicting Horn's results for at least one species (van der Walt, 1995; Hargreaves et al., 2004). While this is explored in more detail in later chapters, it is important to note that the breeding system of any plant species cannot be assumed from general trends and must be tested before results from any pollination study using seed set as a measure of pollinator effectiveness can be meaningful.

Objectives of the thesis — The majority of research on reproductive biology in *Protea* has been conducted on bird- and rodent-pollinated species in the Cape floral region (e.g. Wiens et al., 1983; Wright, 1994). My preliminary observations indicated that cetoniine beetles are regular visitors to the inflorescences of scented grassland and savanna species belonging to a clade centred outside the Cape region. Because birds were rarely seen on these *Protea* species and beetles make effective contact with the reproductive parts of their flowers, I hypothesized that insects, particularly beetles, are their most effective pollinators. Pollination by cetoniine (Scarabaeidae: Cetoniinae) beetles is common in the tropics, and is associated with open bowl-shaped flowers that emit strong odours (Bernhardt, 2000). Cetoniine beetles are known to be attracted to a variety of common floral volatiles. I therefore hypothesized that the fruity scent characteristic of these grassland *Protea* species is an adaptation for attracting cetoniine beetle pollinators.

Focusing on four common *Protea* species (*P. caffra, P. dracomontana, P. simplex* and *P. welwitschii,* Figs 1-4), I investigated the breeding systems and pollination systems of *Protea* species found in grasslands of eastern South Africa and tested the possibility that insects are attracted to their strong fruity-sweet floral scent. Specifically, my objectives were to (i) determine whether these species are self-incompatible and rely on pollinator visits for seed production (chapter 2), (ii) determine the effects of selective exclusion of vertebrates and supplemental hand-pollination on fecundity (chapter 4-5), (iii) determine the effectiveness of insect pollinators by measuring their contribution to outcrossing (chapter 5); and (iv), to describe floral traits (morphology, visual and scent cues) and assess their functional role in attracting beetle pollinators (chapters 3 & 6-8).

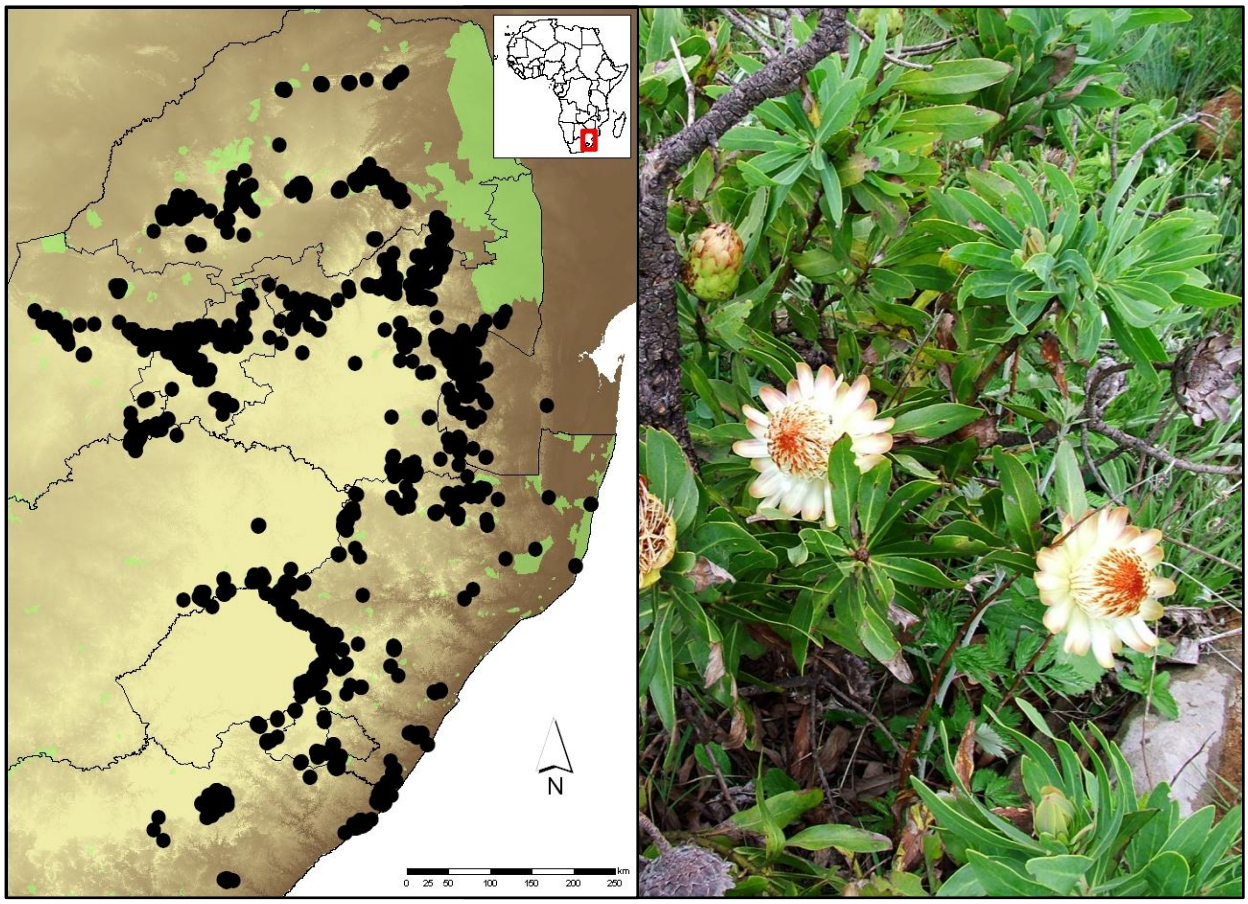

Fig. 1 The distribution (black dots) and growth form of *Protea caffra* in South Africa (Map: T. Rebelo, Photo: Bulwer Mountain).

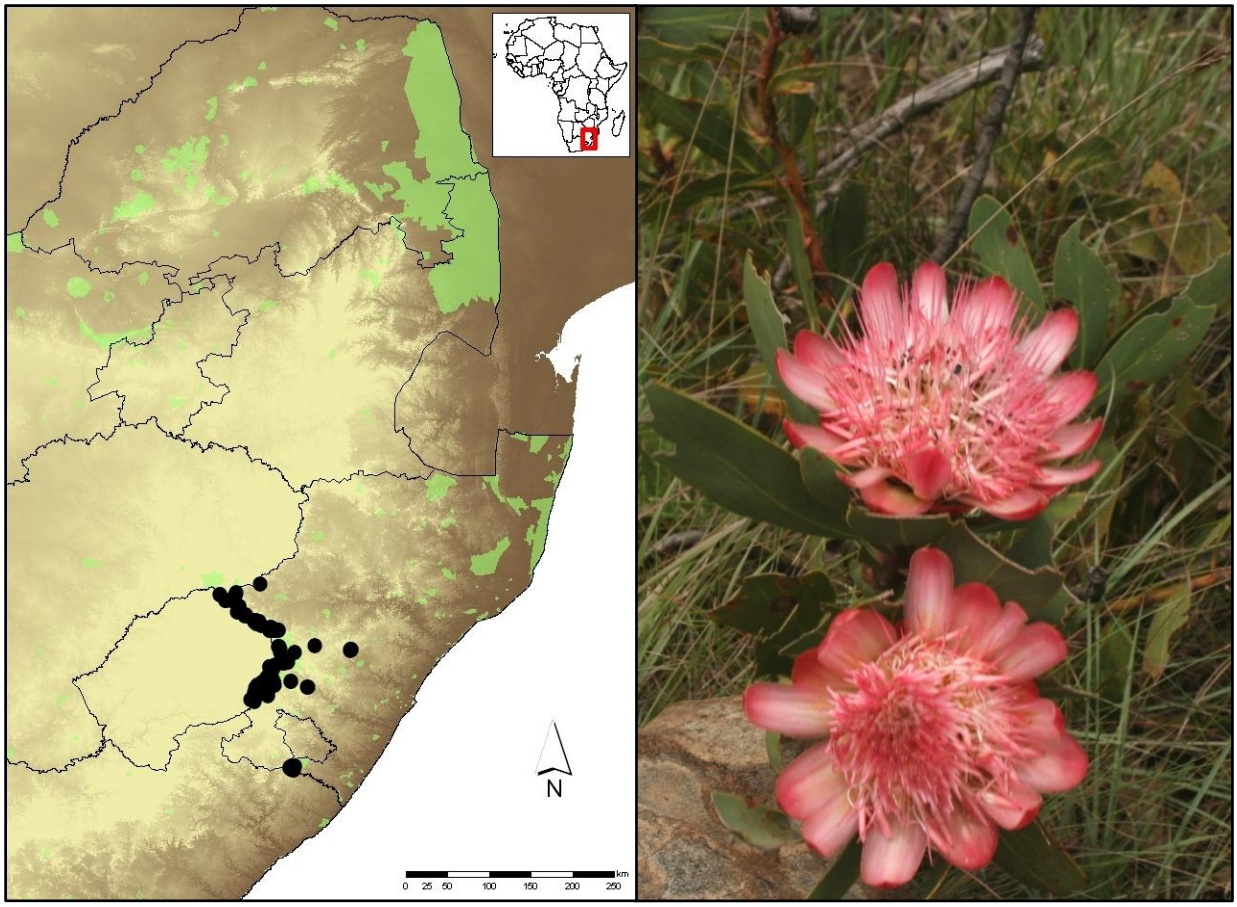

Fig. 2 The distribution (black dots) and growth form of *Protea dracomontana* in South Africa (Map: T. Rebelo, Photo: Garden Castle Nature Reserve).

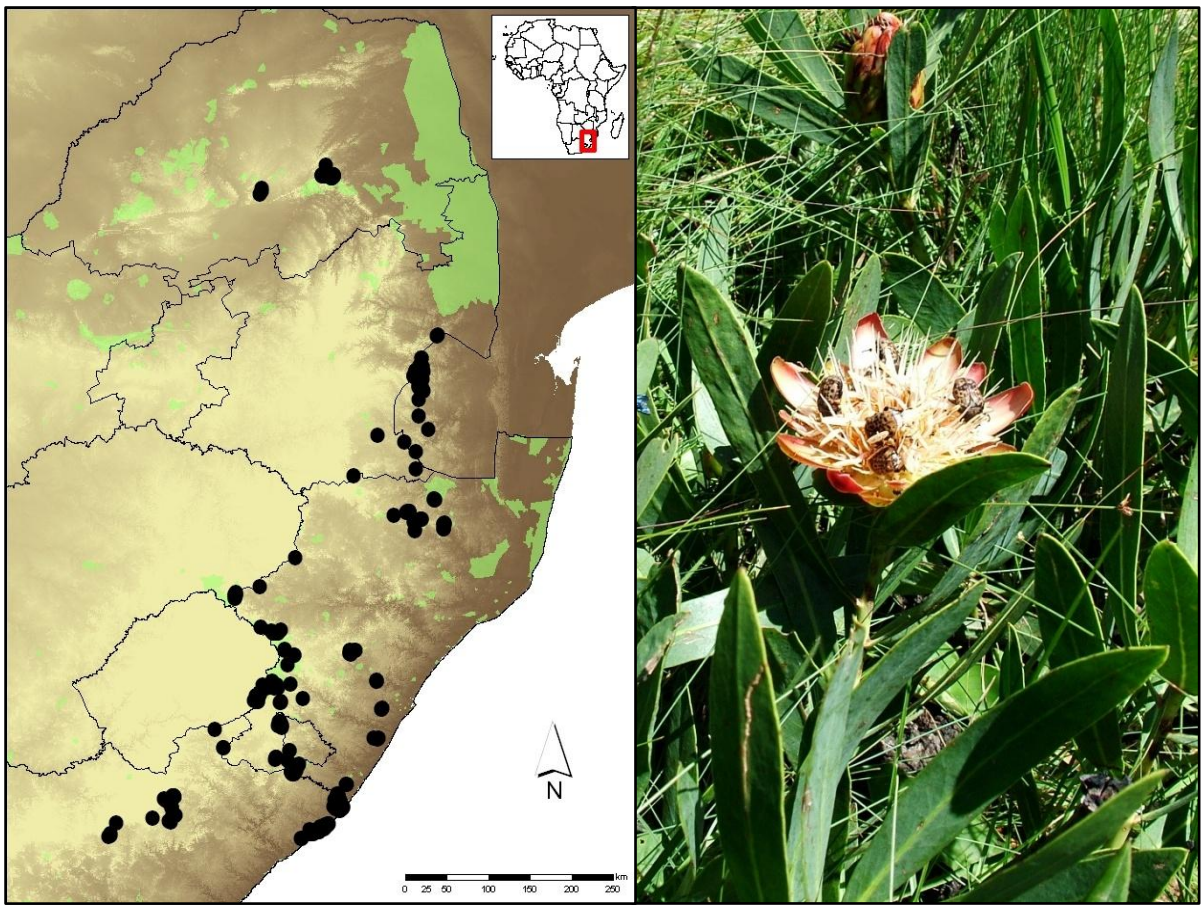

Fig. 3 The distribution (black dots) and growth form of *Protea simplex* in South Africa (Map: T. Rebelo, Photo: Mount Gilboa).

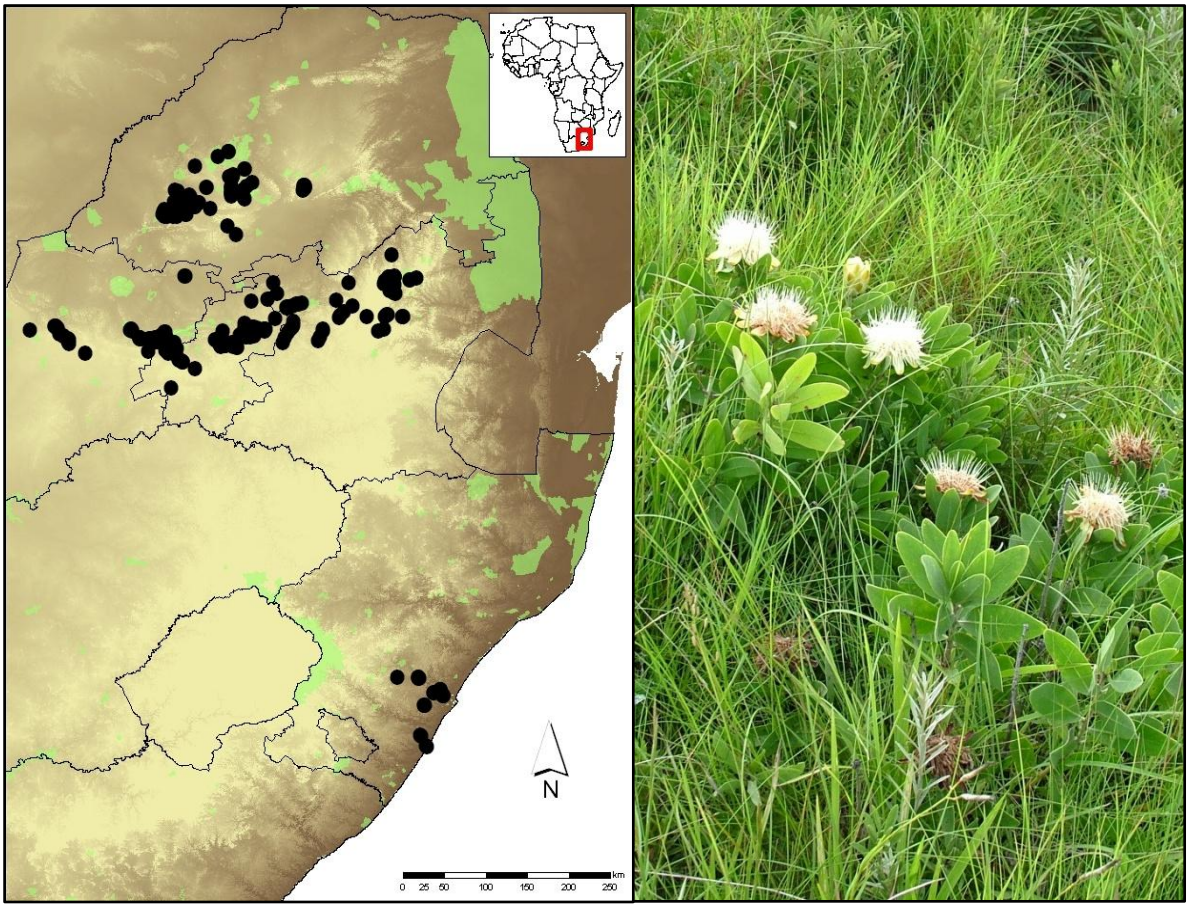

Fig. 4 The distribution (black dots) and growth form of *Protea welwitschii* in South Africa (Map: T. Rebelo, Photo: Winston Park).

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CHAPTER 2

EVIDENCE FOR AUTONOMOUS SELFING IN GRASSLAND *PROTEA* SPECIES (PROTEACEAE)

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Abstract

It has been assumed that species of the large African genus *Protea* have strong self-incompatibility systems. However, this assumption was based largely on studies conducted on a clade of bird-pollinated species that occur in the shrubby fynbos vegetation of the Cape region of southern Africa. To test whether self-incompatibility occurs in a grassland/savanna *Protea* clade, which is largely insect-pollinated, we performed controlled pollination experiments on four species - *Protea caffra*, *P. dracomontana, P. simplex* and *P. welwitschii*. Although pollen-ovule ratios of all four species fall within the range for outcrossers, all four species are self-compatible and capable of autonomous seed production. Using fluorescence microscopy, we found that self-pollen tubes had the same probability of reaching ovules as cross-pollen tubes. In the small tree *P. caffra*, selfed progeny had rates of germination and survivorship that were identical to those of crossed progeny. The grassland *Protea* species studied are likely to have mixed mating systems on account of being both visited by insects and capable of autonomous selfing. If one assumes previous reports of self-incompatibility in *Protea* to be reliable, there have been at least five losses of SI and two gains of autonomous selfing in this genus. However, earlier studies in the genus were often methodologically flawed and a thorough re-analysis of breeding systems in *Protea* is required.

Keywords: Autogamy – breeding system – genetic load –inbreeding depression–pollen-ovule ratios – pollen transfer efficiency– reproductive assurance – self-incompatibility – self-pollination

Introduction

Hermaphroditic flowers are prone to self-pollination. Traits of hermaphrodite flowers that have been interpreted as mechanisms to reduce self-pollination include temporal (dichogamy) and physical (herkogamy) separation of male and female parts (Barrett, 2002). Even if self-pollination does occur, about half of all angiosperms have genetic self-incompatibility mechanisms that prevent self-fertilization (Richards, 1997). The obvious benefit of the latter is to prevent uniparental inbreeding and thus reduce expression of deleterious alleles in progeny (Jarne & Charlesworth, 1993). However, self-compatibility can reduce mate limitation by allowing a plant to make use of its own pollen (Larson & Barrett, 2000) and provide reproductive assurance when associated with autonomous self-pollination (Eckert *et al.*, 2006).

Of the four larger genera of the Proteaceae, self-compatibility has been reported for 78% of 14 Australian *Banksia* L.f. species examined, 50% of six *Grevillea* R.Br. species examined , 75% of eight *Leucospermum* R.Br. species examined (Collins & Rebelo, 1987; Horn, 1962; Smith & Gross, 2002), and in only 21% of 14 South African winter-rainfall *Protea* L. species examined (Coetzee & Littlejohn, 2001; Collins & Rebelo, 1987; Horn, 1962). Breeding systems of *Protea* species outside the Cape region have not been investigated previously, with the exception of the ornithophilous *Protea roupelliae* Meisn. that was found to be self-compatible, but reliant on pollinator visits for seed production (Hargreaves *et al.*, 2004). Grassland and savanna *Protea* species mostly belong to an insect-pollinated clade that has been inferred to be ancestrally bird-pollinated (Valente *et al.*, 2010). This study focuses on the grassland/savanna clade of *Protea,* characterized by species that are mostly, but not always, small shrubs instead of trees, and have inflorescences that are bowl-shaped, scented (Steenhuisen *et al.*, 2010), and offer large pollen and nectar rewards (Steenhuisen & Johnson, 2012).

In the Proteaceae, inflorescences are comprised of numerous tightly packed hermaphroditic and protandrous flowers, in which pollen dehisces onto a specialised section of the style, the pollen presenter (i.e. secondary pollen presentation; Ladd, 1994). Self-pollen presented near the stigma needs to be removed before the stigmatic groove opens and becomes receptive to prevent self-pollination or allow cross-pollen to enter (e.g. *Lomatia;* Ladd *et al.*, 1998). However, the abundant flowers of a typical *Protea* inflorescence mature centripetally so that self-pollination is possible by two means: 1) pollen from central flowers contributes to pollinator-mediated geitonogamous pollen deposition on peripheral receptive flowers, or (2) autonomous self-pollination occurs spontaneously if pollen is situated near the stigmatic

groove when receptive. Protandry has long been thought of as the primary mechanism preventing self-pollination in this large Gondwanaland plant family, but this mechanism is often not effective for preventing geitonogamy within inflorescences. Researchers commonly report weak protandry in Proteaceae (see Offord, 2004) and a large proportion of self-pollen transfer to flowers within and between inflorescences of the same maternal plant. Self-pollination can also be due to inefficient pollinator behaviour promoting geitonogamy (e.g. *Banksia spinulosa* Sm.; Vaughton & Ramsey, 1991) supplemented by within-flower self-pollination due to stigma opening at or soon after anthesis before self-pollen has been removed (e.g. early receptivity in *Grevillea rhizomatosa* Olde & Marriott; Gross & Caddy, 2006). Other mechanisms that prevent autonomous self-pollination include narrowed stigmatic groove at anthesis (Matthews *et al.*, 1999), and complex stigmatic structures blocking the path for self-pollen to reach the stigmatic groove such as cellular outgrowths (Ladd *et al.*, 1998). In *Protea*, however, structural mechanisms to prevent self-pollination are absent (van der Walt & Littlejohn, 1996a)

In this study, we investigated the breeding systems of four grassland *Protea* species. Specifically, we 1) documented the timing of anthesis and stigma receptivity and quantified stigmatic pollen loads, 2) investigated whether the species are self-compatible and capable of autonomous self-fertilization; 3) compared the development of pollen tubes arising from self and cross pollen on stigmas, and 4) tested whether selfed progeny in one of the species exhibit inbreeding depression. Finally, we discuss the evidence for evolutionary shifts to autonomous self-pollination within *Protea*.

Materials and Methods

Study Species and Sites

The "sugarbushes" *Protea simplex* E.Phillips ex J.M.Wood, *Protea dracomontana* Beard, *Protea caffra* Meisn., and *Protea welwitschii* Engl. are common species inhabiting grassland vegetation, especially the escarpment, in the summer-rainfall region of South Africa (Rebelo, 2001). They are largely insect-pollinated (Steenhuisen *et al.*, 2010), but also visited by sunbirds and sugarbirds, especially *P. caffra* that is heavily utilised by bird pollinators in some populations (e.g. Potgieter *et al.*, 2008). These *Protea* species flower in summer between December and February, with some overlap in the flowering periods of *P. caffra* and the bird-pollinated *P. roupelliae* in sympatric populations. The current study was conducted on the following *Protea* populations occurring on grassland slopes in KwaZulu-Natal: sympatric populations of *P. caffra* (c. 200 plants), *P. simplex* (c. 550 plants) and *P. roupelliae* (c. 200 plants) located on the summit of Mount Gilboa (29.29°S, 30.29°E, 1770 m, January

2002 (*P. simplex* only) and 2005); *P. caffra* (c. 500 plants) in the Krantzkloof Nature Reserve (29.77°S, 30.84°E, 450 m, 2004); *P. welwitschii* (c. 500 plants) in a residential area in Winston Park (Giba Gorge) (28.75°S, 30.75°E, 550 m, summer 2003 and 2005); *P. caffra* (c. 50 plants) on Bulwer Mountain (29.75°S, 29.75°E, 1900 m, 2005); and, *P. dracomontana* (c. 500 plants) at Garden Castle (29.74°S, 29.20°E, 1900 m, 2006-2007) in the Drakensberg mountains. These sites receive summer rainfall, often accompanied by misty conditions. Voucher specimens have been deposited in the Bews Herbarium (UN) University of KwaZulu-Natal (voucher numbers 55, 57, 59, 60-62, collector: S.-L. Steenhuisen).

Floral biology, pollen-ovule ratios and pollen transfer efficiency

We measured the timing of anthesis, pollen dehiscence, opening of the stigmatic groove and its receptivity, and eventual wilting, for florets of up to twenty inflorescences of each species. The stage of receptivity (opening of the stigmatic groove) was determined using scanning electron microscopy. A stigma was harvested from each of five inflorescences at different stages of flowering from each species, and fixed using a method modified from Bozzola & Russell (1999), and the stigmatic grooves viewed with a Phillips XL30 environmental electron microscope. An open stigmatic groove indicated that the stigma was receptive to pollen and this corresponded to the loss of self-pollen from pollen presenters and the start of senescence of the perianth and dehisced anther lobes. These morphological changes were thereafter used to estimate the stage of receptivity in the field when conducting breeding system experiments.

The mean number of pollen grains produced by a floret was determined for *P. caffra, P. simplex* and *P. welwitschii* to assess pollen-ovule ratios (*Protea* florets have a single ovule). Five newly released pollen presenters were sampled from each of ten fully open inflorescences and vortexed individually in 1 ml of 70 % ethanol with dissolved fuchsin stain, to dislodge and visualise the pollen. All pollen grains in three sub samples of 1µl were counted microscopically, and mean estimates of whole pollen counts determined. *Protea dracomontana* also presents large amounts of pollen on pollen presenters but this was not quantified.

We determined stigmatic pollen loads of at least twenty naturally pollinated inflorescences to assess pollination (includes autogamous pollen). Five stigmas were taken from closing (*P. caffra, P. dracomontana, P. simplex*) or senescing (*P. welwitschii*) inflorescences and individually squashed on slides with fuchsin gel (Beattie, 1971). Using estimates of pollen production per floret (previous experiment) we determined the proportion of pollen produced that reached stigmas, a measure of pollen transfer efficiency (Harder, 2000).

Controlled pollination experiments

To isolate flowers of *P. simplex, P. caffra*, and *P. welwitschii*, inflorescences of 12-20 plants of each species were enclosed in fine nylon mesh bags from the bud stage to exclude all pollinators. A piece of wire was twisted around the stem and bent into a frame around each inflorescence to keep bags from touching pollen presenters and stigmas. We bagged sets of three inflorescences per plant for all species except *P. simplex* in which only one or two inflorescences per plant were bagged. These three inflorescences were randomly assigned to one of three treatments: 1) unmanipulated to test for autonomous selfing; 2) self-pollinated by brushing a toothpick over the pollen presenters of all flowers of the inflorescence at least twice to facilitate movement of self-pollen to neighbouring stigmas within an inflorescence; or 3) cross-pollinated at least twice by brushing five or more pollen presenters from inflorescences of a different plant (usually picked inflorescences allowed to dehisce indoors overnight) over the stigmatic grooves of all flowers of each experimental inflorescence during its receptive stage. As in experiments with *Grevillea repens* F.Muell. ex Meisn. (Holmes *et al.*, 2008) not all stigmas may have been receptive when self-pollinated at anthesis, but our results of pollen tube growth and seed set indicated that pollen applied at this stage was captured by the stigmas and still viable when stigmas became receptive. Unbagged naturally pollinated inflorescences on the same or a different plant (in the case of *P. simplex*) were used as open controls, together with a further 20-60 randomly chosen open-pollinated inflorescences in the population to increase sample sizes. For *P. dracomontana*, we conducted a simplified breeding system experiment involving a comparison between bagged and open-pollinated inflorescences, to test for autonomous selfing.

When the anthers had senesced and inflorescences were almost fully closed, five stigmas from each experimental inflorescence of *P. caffra, P. simplex* and *P. welwitschii* (2004 and 2005 seasons) were collected to determine the presence of pollen tubes in the upper style. Stigmas were fixed in glacial acetic acid/ethanol (1:3, v/v) for one hour, washed with distilled water, and thereafter stored in 70 % ethanol. These preserved stigmas were prepared for pollen tube analysis using a softening and staining procedure modified from Martin (1959). This procedure allowed for the examination of pollen tubes in the style through aniline blue UV-induced fluorescence of callose associated with the pollen tube wall. Preserved stigmas were rinsed in distilled water for ten minutes, softened in 4 N NaOH for 48 hours, rinsed in tap water for one hour, and stained with decolorized aniline blue-0.1 N K_2HPO_4 for four hours. The stained stigmas were stored in glycerin for no longer than three days before microscopic examination for which whole stigmas were mounted on slides in a drop of stain and glycerin, and squashed gently. The number of pollen tubes per stigma for each treatment was

determined by examining the stigmas with a Olympus Provis AX-70 light microscope equipped with a UV filter system consisting of a dichroic mirror (400 nm), an ultraviolet excitation filter (330-385 nm) and a barrier filter (420 nm). The proportions of styles per inflorescence with germinated pollen grains and mean counts of pollen tubes in the upper styles of each treatment group were determined. Insect-damaged stigmas were excluded from analyses.

All experimental infructescences were collected at the end of June in each year; none of these species are serotinous and annual winter fires make early collection of infructescences necessary. The number of fertile and infertile seeds was determined for each. Plump ovaries containing a large cream-coloured embryo with a spongy texture were scored as fertile seeds. All infructescences damaged by seed predators were excluded from analyses. Seed predation was extremely high, ranging from 29 % of experimental inflorescences in *P. caffra* populations to 80 % in the *P. dracomontana* population. The most common seed predator in these *Protea* populations is a tortricid moth that lays its eggs on buds (S-L. Steenhuisen, unpublished data), thus making it difficult to prevent predation, even through bagging. Bags were left on inflorescences until harvested. The heavy predation lowered sample sizes, resulting in the uncoupling of treated and control inflorescence pairs in many cases. Thus plant effects were not tested and all inflorescences were pooled for each treatment in analyses. Because resultant sample sizes were relatively low we confirmed the results of these experiments by repeating bagged (without manipulation) and open control treatments in 2005 for 20-80 inflorescences of *P. caffra, P. simplex and P. welwitschii* with additional populations of *P. caffra* at Bulwer Mountain and Mount Gilboa. Seeds from experimental plants of *P. welwitschii* in 2004 were not collected due to the site having been burnt.

Germination and inbreeding depression

Seeds from bagged (without manipulation), open, self- and cross-pollination treatments on each of twenty experimental *P. caffra* plants (Krantzkloof Nature Reserve, 2004) were germinated in late February 2005 to assess early fitness of selfed progeny. A maximum of thirty seeds per infructescence per treatment was soaked in Kirstenbosch Instant Smoke Plus Seed Primer overnight (germination cue for fireprone species, e.g. Brown, 1993). The treated seeds were planted individually in Growmor seedling mix (National Plant Food, Cato Ridge) in seedling trays treated with Plazdip rooting/pruning agent containing copper oxychloride (Natal Associated Chemicals), sprinkled with river sand, and watered for 30 second bouts twice a day for sixty days. The pollination treatments were alternated throughout the seedling trays and blocked by plant. The time to germinate (days), germination success and death rate

of seedlings were determined and averaged for seeds from each inflorescence over two months.

Statistical analysis

We analysed the effects of pollination treatment on the proportion of stigmas with pollen tubes in the upper style, the number of pollen tubes per style, the proportion of flowers that set seed, the proportion of seeds from each treatment that germinated, the number of days until germination, and the proportion of seedlings that died using generalized linear models (GZLMs) in PASW Statistics v18 (SPSS Inc, 2009, Chicago IL). Unless otherwise stated we used likelihood ratio Chi-square statistics, logit link functions, binomial error distributions and corrected for overdispersion where appropriate. We compared treatments using pairwise contrasts with sequential Sidâk adjustment for multiple comparisons (Field, 2009; Hosmer & Lemeshow, 2000; McCullagh & Nelder, 1989). Type I models were used to test the effect of year on the proportion of styles with pollen tubes and the number of pollen tubes per style for 2003 and 2004 for *P. welwitschii*, and plant effects for all germination and inbreeding depression measures for *P. caffra*. Treatment effects on the number of pollen tubes growing in styles were tested using means per inflorescence rounded to the nearest integer and fitted to models with a Poisson error distribution and log link functions. The number of days taken to germinate for *P. caffra* seeds fitted a normal distribution. When analysing the proportion of seedlings that died, we substituted one dead seedling for all treatments for four maternal plants that experienced zero progeny deaths, to provide a statistically conservative solution to the problem of undefined logits when there is no variance within a set of binomial data (Zuur *et al.*, 2009).

***Phylogenetic analysis of breeding systems in* Protea**

To determine the frequency of shifts in genetic self-incompatibility and autogamy in *Protea*, we reviewed the mating systems of 15 additional species from the literature. For each species we obtained data on the pollination system and natural seed set (percentage seeds per inflorescence averaged over populations within and between studies). We calculated indices of self-compatibility (ISC, percentage seed set from self-pollination divided by that from cross-pollination) and autonomous self-pollination (IAS, percentage seed set from unmanipulated bagged inflorescences divided by that of self-pollinated inflorescences). ISC values range from 0 (fully self-incompatible) to 1 (fully self-compatible), and IAS values range from 0 (flowers dependent on pollen vectors for seed set) to 1 (flowers capable of 100% seed set through autonomous selfing). We used an existing phylogenetic tree for *Protea* (Schnitzler *et al.*, 2011) and pruned unneeded taxa using Mesquite version 2.74 (Maddison & Maddison, 2010). After scoring each species as self-compatible or not and capable of

autonomous self-pollination (IAS>0.2) or not, we performed parsimony and maximum likelihood analyses to identify shifts in breeding system within the genus.

Results

Floral biology, pollen-ovule ratios and pollen transfer efficiency

The four *Protea* species flowered over a period of four months from December to March, with a peak period in January. The inflorescences were long-lived with individual flowers lasting for at least five days (Table 1). All flowers comprising an inflorescence opened fully, with anthers dehisced and pollen coating the pollen presenter, within approximately five days of the inflorescence bracts opening (Table 1). Anthers wilted 1-3 days after dehiscence, followed closely by the opening of the stigmatic groove and subsequent stigma receptivity (Table 1, Figure 1A-E). The progression of pollen presentation and receptivity was centripetal, allowing ample opportunity for facilitated self-pollination of outer flowers as inner flowers present pollen to floral visitors. The stigmas then remained receptive until the inflorescence bracts closed, either tightly or loosely, or when the bracts wilted and senesced in the case of *P. welwitschii*. Epidermal cells lining the stigmatic grooves were tightly interlocked at anthesis, separating soon after to become receptive to pollen grains that germinated along the full length of the groove, and along the curved surface of the style (Figure 1A-E). In contrast to the beetle-pollinated species, the stigmatic groove of *P. roupelliae* spanned one side of the style only (Figure 1F).

Table 1. Ontogeny of individual flowers in four *Protea* species. The number of inflorescences used to assess flowering stages is shown in parentheses.

Flowering stage	Days after opening of inflorescence bracts $\bar{x}$ ± SE			
	Protea simplex	*Protea caffra*	*Protea welwitschii*	*Protea dracomontana*
Anthesis of first flowers	4.8 ± 0.7 (16)	3.1 ± 0.2 (13)	3.6 ± 0.4 (17)	3.8 ± 0.4 (6)
Anthers of individual flowers begin to senesce	6.9 ± 0.9 (8)	5.4 ± 0.4 (8)	6.4 ± 0.5 (7)	4 ± 0.0 (2)
Receptivity of flower	7.8 ± 0.6 (17)	5.1 ± 0.5 (11)	5.8 ± 0.4 (16)	4 ± 0.0 (2)
Anthers of flower senesce	9.5 ± 0.9 (12)	6.2 ± 0.9 (6)	7.7 ± 0.3 (14)	-
Bracts of inflorescence close/senesce	17.3 ± 1.4 (17)	8.5 ± 0.5 (10)	11.4 ± 0.6 (13)	-

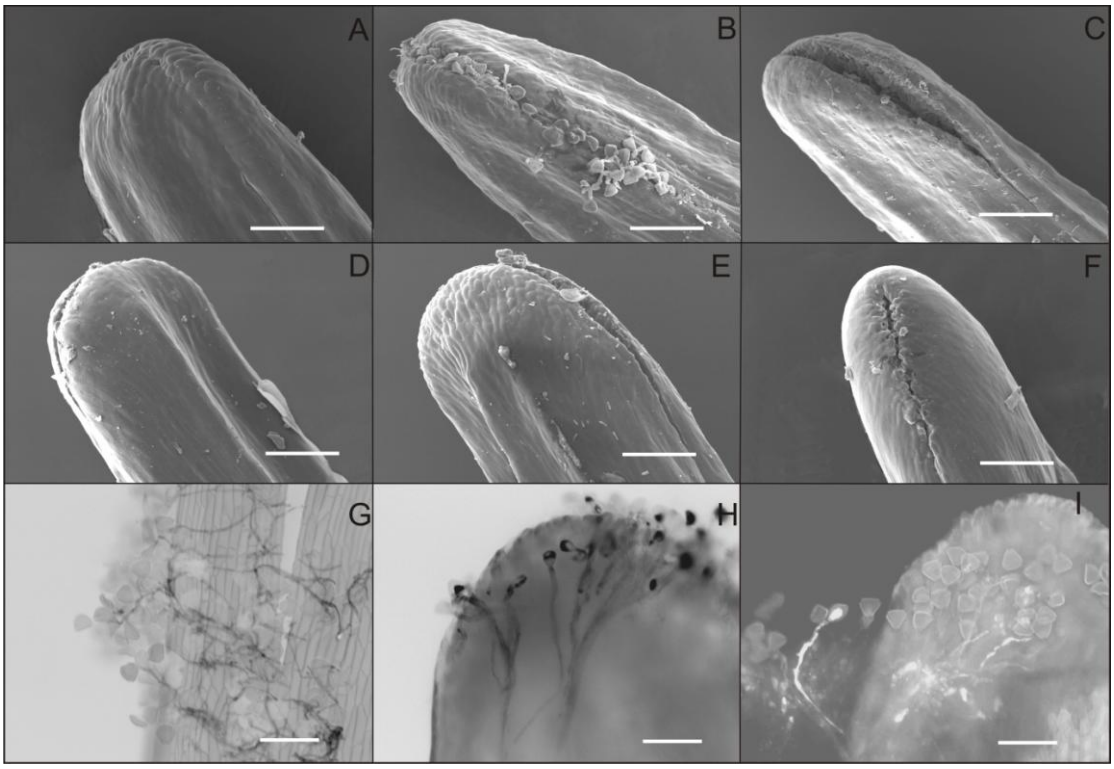


Figure 1. (A-F) Scanning electron micrographs of (A) non-receptive and (B) receptive (with germinating pollen grains) stigmatic grooves of *Protea simplex*; stigmatic grooves for (C) *Protea caffra*; (D) *Protea dracomontana*; (E) *Protea welwitschii*; (F) *Protea roupelliae*. (G-I) Light micrographs of pollen grains germinating on the stigmatic groove of (G) a bagged (pollinator-excluded) flower, (H) a self-pollinated flower, and (I) a cross-pollinated flower of *Protea welwitschii*. Scale bar = 100 μm.

Over 80 000 triporate pollen grains were produced by each flower of *P. caffra, P. simplex* and *P. welwitschii* (not quantified for *P. dracomontana,*Table 2). Abundant *Protea* pollen loads were found on more than 90% of stigmas sampled from open inflorescences of all species except *P. dracomontana* (Table 2). Collectively, minimal amounts of ten types of foreign pollen were present on some stigmas. Predation of stigma tips by lepidopteran larvae and possibly beetles foraging for pollen on pollen presenters was also minimal (less than 1 % of stigmas). The proportion of *Protea* pollen produced that reached stigmas was extremely low (0.005 %, 0.014 % and 0.010 % for *P. welwitschii, P. simplex* and *P. caffra* respectively; Table 2).

Table 2. Pollen production per flower and pollen load per stigma of naturally pollinated inflorescences of four *Protea* species. The number of inflorescences sampled per species and number of pollen presenters sampled per inflorescence are shown in parentheses.

Species (n)	Number of pollen grains per pollen presenter ($\overline{X}$ ± SE grains)	Pollen load per stigma ($\overline{X}$ ± SE grains)	Percentage of sampled stigmas with *Protea* pollen ($\overline{X}$ ± SE)
Protea welwitschii (57)	188484 ± 9841 (15)	12.1 ± 1.8	93.3 ± 0.2
Protea simplex (39)	83890 ± 6286 (11)	11.6 ± 2.3	99.6 ± 0.1
Protea caffra (60)	245455 ± 20744 (15)	18.0 ± 1.3	99.8 ± 0.03
Protea dracomontana (24)	-	2.6 ± 0.5	61.2 ± 1.0

Controlled pollination experiments

Most stigmas sampled from hand-pollinated inflorescences had pollen tubes present in the stigmatic groove and the upper style, sometimes in excess of 100 germinating grains (Figures 1G-I and 2A-C). Self pollen germinated readily in the upper style of all species (Figure 1H). A higher proportion of self- and cross-pollinated *P. welwitschii* flowers had pollen tubes penetrating the style than did unmanipulated bagged and open-pollinated inflorescences over two seasons (year: $\chi^2 < 0.1$, d.f. = 1, P=0.978;treatment: $\chi^2 = 17.62$, d.f. = 3, $P < 0.01$). Similarly for *P. caffra*, open-pollinated inflorescences had lower proportions of stigmas with pollen tubes than those of other treatments indicating that plants may be pollen-limited at Krantzkloof ($\chi^2 = 12.96$, d.f. = 3, $P < 0.01$), although this is not supported by the high seed set in open-pollinated plants in this population. *Protea simplex* showed the most variability between treatments with significantly higher proportions of styles with pollen tubes in self- and cross-pollinated inflorescences compared to unmanipulated bagged inflorescences ($\chi^2 = 31.19$, d.f. = 3, $P < 0.01$).

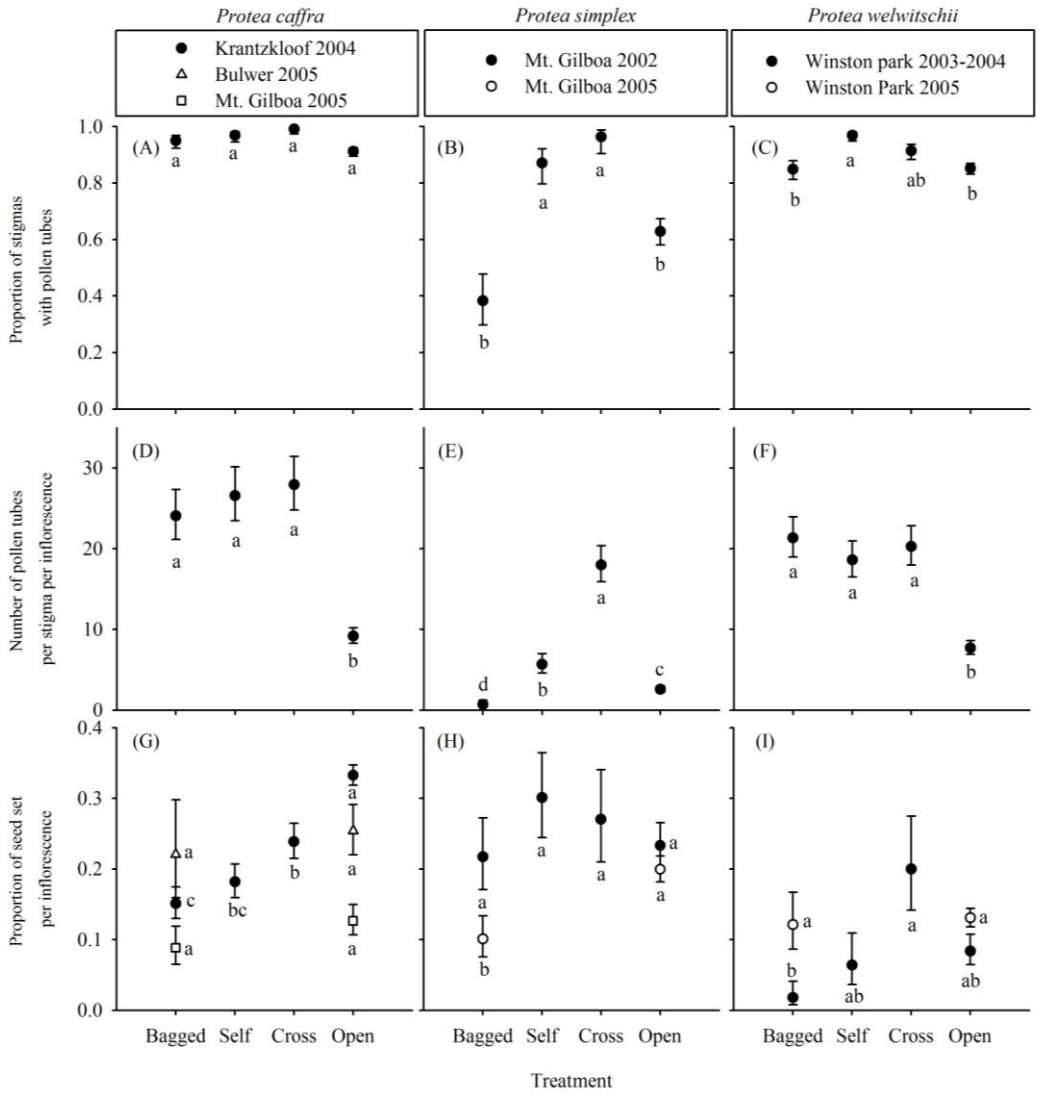


Figure 2. Measures of pollen tube growth and fertilization success in unmanipulated bagged, self-, cross-, and naturally (open) pollinated inflorescences of three *Protea* species in varying years and sites: (A-C) Adjusted mean proportions of stylar tips per inflorescence with pollen tubes; (D-F) adjusted mean numbers of pollen tubes in the upper style per inflorescence; and (G-I) adjusted mean seed set per inflorescence (excluding 2004 field season for *P. welwitschii* seed set). For each year of the experiments, mean symbols that share letters are not significantly different. Where similar treatments were repeated over two seasons, the data were analysed together taking year into account (i.e. 2003-2004 for *P. welwitschii*). Due to unbalanced experimental designs, data from unmanipulated bagged and open-pollinated plants for *P. welwitschii* and *P. simplex* were analysed separately to those from other seasons, and data from different populations of *P. caffra* were analysed separately from one another.

Hand pollinations resulted in significantly higher pollen tube loads on stigmas than in other treatments of *P. simplex* (unmanipulated bagged vs self vs cross vs open, $\chi^2 = 120.56$, d.f. = 3, $P < 0.01$; Figure 2D-F). In addition we found almost triple the number of pollen tubes in cross-pollinated stigmas compared to self-pollinated stigmas of *P. simplex* indicating that self pollen was less likely to germinate on a stigma on the same plant. Bagging and hand pollinations significantly inflated pollen tube loads compared to open pollinated controls for *P. welwitschii,* especially in 2004 (year: $\chi^2 = 7.78$, d.f. = 1, $P < 0.01$; treatment: $\chi^2 = 61.22$, d.f. = 3, $P < 0.01$). This trend was also evident in treated inflorescences of *Protea caffra* ($\chi^2 = 75.51$, d.f. = 3, $P < 0.01$).

In general, self-pollination in the study species resulted in levels of seed set that were comparable to those arising from cross-pollination (Figure 2G-I). Seed set in all species was low and at most 36% of flowers in an inflorescence set seed. While seed set for *P. simplex* was similar for all treatments in 2002 (unmanipulated bagged vs self vs cross vs open, $\chi^2 = 1.52$, d.f. = 3, $P = 0.68$), bagging halved seed set in 2005 (unmanipulated bagged vs open, $\chi^2 = 6.48$, d.f. = 1, $P = 0.011$). Cross pollination of *P. welwitschii* inflorescences resulted in significantly higher seed set than in unmanipulated bagged inflorescences in 2003 (unmanipulated bagged vs self vs cross vs open, $\chi^2 = 11.23$, d.f. = 3, $P = 0.011$), although seed set in both of these treatments were similar to selfed and open inflorescences. This trend was consistent in 2005 for *P. welwitschii* (unmanipulated bagged vs open, $\chi^2 < 0.1$, d.f. = 1, $P = 0.82$). For *P. caffra*, open and cross pollination increased seed set at Krantzloof (2004, unmanipulated bagged vs self vs cross vs open, $\chi^2 = 53.71$, d.f. = 3, $P < 0.01$) but no difference was found between unmanipulated bagged and open pollinated infructescences in other populations in a subsequent year (2005, Gilboa, $\chi^2 = 1.16$, d.f. = 1, $P = 0.28$; Bulwer, $\chi^2 = 0.17$, d.f. = 1, $P = 0.68$). Similar seed set (adjusted mean ± SE proportion of flowers that set seed per inflorescence) was found for both unmanipulated bagged and open-pollinated treatments of *P. dracomontana* (2007, unmanipulated bagged 0.19 (lower SE=0.07, upper SE=0.10) vs open 0.18 (lower SE=0.06, upper SE=0.08), $\chi^2 < 0.01$, d.f. = 1, $P = 0.97$).

Seeds from all treatment groups (unmanipulated bagged, hand self-pollinated, hand cross-pollinated, and open-pollinated) of *P. caffra* were found to have high germination success (> 80 %) and similar germination times (c. 36-37 days, Table 3). Seeds from unmanipulated bagged, self- and cross-pollinated inflorescences were 11% less likely to germinate than those from open pollinated inflorescences, but there was no significant difference in the germination success or survivorship of progeny arising from self- and cross-pollination (Table 3).

Table 3. Generalized linear models comparing germination and germination rate of seeds and death of seedlings from unmanipulated bagged, self-, cross- and open pollinated inflorescences of *Protea caffra* from Krantzkloof in 2004.

		Proportion of seeds that germinated (mean±SE)			Days to germinate (mean±SE)			Proportion of germinated seedlings that died (mean±SE)		
		Mean	Lower SE	Upper SE	Mean	Lower SE	Upper SE	Mean	Lower SE	Upper SE
Treatment	Bagged	0.80^{b}	0.04	0.04	36.29	0.81	0.81	0.04^{ab}	0.01	0.02
	Self	0.83^{ab}	0.04	0.03	36.94	0.80	0.80	0.05^{a}	0.01	0.02
	Cross	0.87^{ab}	0.03	0.03	37.08	0.81	0.81	0.03^{ab}	0.01	0.01
	Open	0.91^{a}	0.02	0.02	36.94	0.77	0.77	0.02^{b}	0.01	0.01
$\chi^2_{(19)}$	Plant	20.694			125.680**			63.209**		
$\chi^2_{(3)}$	Treatment	8.310*			0.586			9.271*		

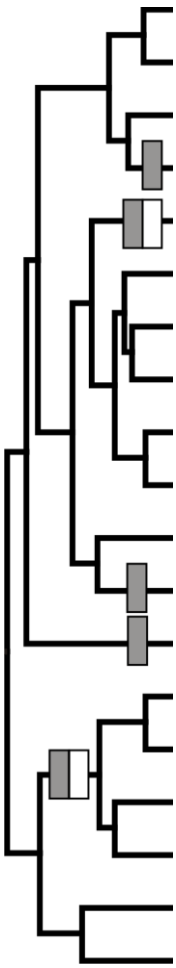
Means that share letters are not significantly different.

Significance level: *P<0.05; **P<0.01

***Phylogenetic analysis of breeding systems in* Protea**

Parsimony analysis, using available data, indicated at least five shifts from self-incompatibility to self-compatibility and at least two shifts from allogamy to autonomous self-fertilization in *Protea,* one of which involved the clade containing our four study species (Table 4). Optimization using maximum likelihood analysis did not alter these findings. Shifts to autogamy coincided with shifts to rodent (in the *P. humiflora* clade) and insect pollination (in the grassland clade), while shifts to self-compatibility included three bird-pollinated species (Table 4). Natural seed set was highly variable within the genus with no obvious associations with shifts in pollinators or self-compatibility, although seed set is comparatively high in the grassland taxa *P. caffra, P. dracomontana* and *P. simplex* (Table 4).

Table 4. Pollinators, seed set from natural pollination, and indices of autonomous self-pollination (IAS) and self-compatibility (ISC) f or 19 *Protea* species, w ith phy logenetic relatedness and shifts to autonomous self pollination shown in the first column. Bars on the phylogenetic tree indicate shifts to self-compatibility (grey bars) and autogamy (empty bars).

	Species	Pollinator[a]	Natural seed set %	IAS[b]	ISC[c]	References
	*lacticolor*	bird	8.6	0	0	Collins & Rebelo, 1987; Horn, 1962
	mundii	bird	5.3	0	0	Collins & Rebelo, 1987; Horn, 1962
	aurea	bird	7.7 (hybrid) 15.7 (sp.)	0	0	Collins & Rebelo, 1987; Horn, 1962
	roupelliae	bird	30	0.10	1	Hargreaves *et al*., 2004
	humiflora[d]	rodent	8.4	1.26	2.25	Collins & Rebelo, 1987; Wiens *et al*., 1983
	compacta	bird	3.3 (hybrid) 13.8 (sp.)	0	0	Collins & Rebelo, 1987; Horn, 1962
	neriiflora	bird	3.5	0	0	Collins & Rebelo, 1987; Horn, 1962; Wright *et al*., 1991
	longifolia	bird	0.7	0	0	Horn, 1962
	susannae	bird	10.3	0	0	Collins & Rebelo, 1987; Horn, 1962
	obtusiflora	bird	2.8	0	0	Collins & Rebelo, 1987; Horn, 1962
	laurifolia	bird	4.7	0	0	Collins & Rebelo, 1987; Horn, 1962; Wiens *et al*., 1983; Wright *et al*., 1991
	eximia[f]	bird	24.7	0	0.54	Collins & Rebelo, 1987; Horn, 1962; van der Walt, 1995
	repens[f]	bird	41.2	0.05	0.90	Coetzee & Giliomee, 1985; Collins & Rebelo, 1987; Horn, 1962; van der Walt, 1995
	caffra	insects	33.3	0.83	0.76	This study, 2004
	dracomontana	insects	18.1	1[e]	1	This study, 2006
	simplex	insects	23.3	0.72	1	This study, 2002
	welwitschii	insects	8.4	0.28	0.32	This study, 2003
	lanceolata	bird	12.1	0	0	Collins & Rebelo, 1987; Horn, 1962
	nana	insects and rodents	5	0	-	Biccard & Midgley, 2009; Collins & Rebelo, 1987

[a]Suggested pollinator by T. Rebelo (pers. comm.) and this study (for *P. caffra, P. dracomontana, P. simplex and P. welwitschii*).

[b]ISC = percentage seed set from self pollination / percentage seed set from cross pollination; for ratios using data from Horn (1962) seed set from cross hand-pollination was not available and was substituted by seed set from natural pollination

[c]IAS = percentage seed set from unmanipulated bagged treatment/ percentage seed set from self pollination; species with ratios above 0.25 were considered autonomous in the phylogenetic analysis.

[d]Indices for *Protea humiflora* based on number of seeds per head, not percentage seeds/head.

[e]IAS based on percentage seed set from autonomous self pollination/ percentage seed set from natural pollination.

[f]Includes measures of seed set from cultivars for *Protea repens* (L.) L. ‚Sneyd' and *Protea eximia* (Salisb. ex Knight) Fourcade ‚Fiery Duchess' (van der Walt, 1995).

Discussion

The results of this study indicate that grassland *Protea* species in South Africa are self-compatible and capable of producing viable seed through autonomous self-fertilisation to varying degrees. This is despite their extremely high pollen-ovule ratios (c. 80000:1) that are typical of animal pollinated, hermaphroditic xenogamous plants (Cruden, 2000). Self-pollen germinated readily along the entire stigmatic groove and was observed to enter the style without hindrance (Figure 1H). The masses of germinating pollen grains and prolific pollen tube growth observed in the upper styles (with the exception of *P. simplex*) of unmanipulated bagged plants was probably due to the bags preventing the loss of pollen from the pollen presenters via animals, wind or rain, and from possible contact with the bag (Figure1G). Open pollinated inflorescences had far fewer pollen tubes but similar seed set to those in the unmanipulated bagged and selfed treatments in most cases (Figure 2). *Protea welwitschii* showed the greatest increase in seed set after cross pollination and the lowest autonomous seed set despite being highly self-compatible (Figures 1H, 2F & I). However, a tenfold increase in autonomous seed set was observed in a second season. The opposite was found for *P. simplex* in which the numbers of self-pollen tubes evident in styles were lower compared to those in the outcrossed treatment, but still resulted in high seed set (Figure 2E & H). Low seed set (<35%) was observed across all species, as is typical of the Proteaceae (Ayre & Whelan, 1989; Collins & Rebelo, 1987). Cross pollination increased seed set in all cases, but seed germination experiments showed that self and cross progeny were equally viable for *P. caffra* (Figure 2G-I, Table 3). Vaughton & Ramsey (2006) also found similar germination of selfed and open-pollinated seeds in *Banksia marginata* R.Br. However, selfed seeds and seedlings were smaller and less likely to survive. Thus, even though *B. marginata* is self compatible, selfed seedlings suffer from inbreeding depression and were 62% less fit than open-pollinated progeny. A similar result was reported by Heliyanto *et al*. (2005) for *Banksia illicifolia*. By contrast, Wooller & Wooller (2004) found no difference in germination and seedling survival of selfed and open-pollinated progeny of *Banksia baxteri* R.Br., a result that is consistent with our findings for *P. caffra* (Table 3).

Protandry has been assumed to be the primary mechanism by which the Proteaceae avoid autonomous selfing, and self-pollen is removed before stigmas become receptive (Carolin, 1961). Our results show that protandry within inflorescences of these grassland species is not sufficient to prevent geitonogamy, with self-pollen and receptive stigmas present at the same time in an inflorescence, making autonomous and geitonogamous selfing likely. The stigmatic grooves of our study species were closed at the time of pollen presentation preventing immediate autonomous self-pollination and opened within 72 hours after anthesis (Table 1,

Figure 1A & B). These phenological trends have been reported for other Proteaceae (e.g. *Banksia* (Collins & Spice, 1986), *Telopea speciossima* R.Br. (Offord, 2004), *Protea repens* (L.) L. cv. Sneyd and *P. eximia* (Salis. ex Knight) Fourcade cv. Fiery Duchess (van der Walt & Littlejohn, 1996b). This is in contrast to *Grevillea rhizomatosa* stigmas for which receptivity is triggered by anthesis (Gross & Caddy, 2006), assisting in self-pollination. Studies of pollen viability have shown that Proteaceae pollen can be viable over the period of receptivity (e.g. 4 d for *T. speciossima*; Offord, 2004). During receptivity, the grassland proteas' stigmatic grooves were open over the entire curvature of the style tip increasing the probability of self pollen left on the pollen presenter to pollinate stigmas (evident from prolific growth of pollen tubes in grooves running lengthways down the styles, Figure 1G), but increasing the chances of scraping pollen off a pollinator's body as it brushes against the stigmas. In contrast, the stigmatic groove of *P. roupelliae* was much smaller and open only on one side of the style (that facing the centre of the inflorescence), which is more conducive to the precise transfer of pollen from a bird's head, beak or throat during foraging for nectar at an inflorescence, inadvertently brushing passed the stigmas (Figure 1F).

Self pollen remaining on a stigma may lead to stigma clogging preventing the entrance of cross-pollen that is deposited on top (Howell *et al.*, 1993). In self-incompatible species this would reduce seed set (Offord, 2004). As in *Banksia spinulosa* Sm., self-pollen covered the stigmas at anthesis in our study species (Vaughton, 1988; Vaughton & Ramsey, 1991). If self-pollen is not removed by the time the stigmatic groove opens, flowers would be pollinated autonomously. For our study species, the growth of self-pollen tubes was not hindered by physical barriers on the stigma (e.g. diameter or length of the stigmatic groove limiting pollen access) or within the style (e.g. narrowing of the transmitting tissue tract restricting growth to ovules) (Matthews *et al.*, 1999).

The proportion of pollen produced by a flower that was transferred to stigmas of open pollinated inflorescences of the study species was <0.02 %. This transfer efficiency is extremely low but similar to that of most wind and animal pollinated flowers reviewed by Harder (2000), and is expected in systems in which pollen is the main reward. *Atrichelaphinis tigrina* (Olivier, 1789), the most common Cetoniine visitor to inflorescences of our study species, was observed to consume substantial amounts of pollen indicating that pollen was a principal floral reward offered to these insect visitors. Studies in southern Africa have shown that pollen plays a major role in the diet of scarab beetles (Johnson & Nicolson, 2001). The presence of foreign pollen on stigmas, although minimal, does suggest that insect visitors are at least transferring pollen (Table 2).

Shifts to autonomous self pollination could have coincided with pollinator shifts (i.e. from bird to rodent or insect pollination, Table 4), but our preliminary phylogenetic analysis has highlighted several methodological problems and the need for more in depth studies of the breeding systems and pollination ecology of *Protea*. Wiens *et al*. (1983) performed self-pollinations by manipulating bags covering *P. humiflora* inflorescences to distribute pollen inside. The same method was used to cross pollinate inflorescences by placing pollen laden bags on inflorescences of another plant. However, Wiens *et al.* (1983) found higher seed set in open-pollinated control inflorescences than in their cross-pollination treatment and acknowledge that their pollination method may not have been effective. The majority of breeding system studies on *Protea* species were conducted by Horn (1962), who reported no seed set after autonomous and self pollination treatments on ten species (including *P. repens* and *P. eximia*) and three hybrids, but the method of self pollination was not explained. The *Protea* study by Horn (1962) evidently involved experimental hand self-pollination and pollinator exclusion experiments, but did not include a hand cross-pollination treatment to control for pollination technique. In contrast, Coetzee & Giliomee (1985) found partial autonomous self pollination (2.4-2.9% seed set) in a natural population of *P. repens*, and van der Walt (1995) found very high levels of self-compatibility in *P. repens*, contradicting Horn's (1962) results. Similarly, van der Walt (1995) found partial autonomous selfing and self-compatibility in *P. eximia*, previously reported as self-incompatible (Horn, 1962). Due to these differences in results, we recommend that further breeding system investigations be conducted for Horn's (1962) study species, as well as additional *Protea* species.

Our preliminary phylogenetic analysis indicates that shifts to autonomous self pollination in *Protea* may be accompanied with shifts from vertebrate to insect pollination systems in summer-rainfall species (Table 4). Most breeding system studies in South Africa have been conducted on bird pollinated *Protea* species, and, as discussed above, may have methodological flaws. Despite this, recent studies assume high levels of self-incompatibility in these species (e.g. four bird-pollinated *Protea* species in section *Exsertae* in Carlson & Holsinger (2010), based on the pollinator exclusion experiments of Horn (1962)). Fortunately, a few studies have conducted more thorough investigations of breeding systems and have indicated that some bird-pollinated *Protea* species may be partially autonomous and/or self-compatible (*P. repens* (Coetzee & Giliomee, 1985); *P. roupelliae* (Hargreaves *et al.*, 2004); *P. repens* and *P. eximia* (van der Walt, 1995)), although exposure to pollen vectors still yielded higher seed set. While Coetzee & Giliomee (1985) reported limited autonomous selfing in *P. repens* and evidence for insects contributing to seed set in inflorescences excluded from bird pollination. Wright *et al.* (1991) reported no difference in seed set between open pollinated inflorescences and those excluded from bird visitation by wire cages

for *P. nitida* and *P. cynaroides*, and concluded from this that the species experienced high levels of insect pollination, but the possibility of seed set through autogamy, which would yield similar results, was not investigated. However, in a more recent review of ornamental proteas, Coetzee & Littlejohn (2001) suggest that seed set in inflorescences exposed solely to insect visitation could be due to pollinator-mediated self-pollination.

Our review of *Protea* breeding system studies highlighted the need for standardising methods and the importance of controls for statistical comparisons. Seed set following hand self-pollinations is the appropriate control for autonomous seed set in unmanipulated inflorescences. In order to calculate an index of autonomous selfing, both treatments are needed. Similarly, both self and cross hand pollinations are needed to access self-compatibility of plants, while open pollinated treatments are needed to assess the effectiveness of these hand pollinations. Often investigations of plant breeding systems are missing the full spectrum of pollination treatments, making it difficult to interpret results. The growth of pollen tubes in the style and down to the ovary is also not a true indication of self-compatibility as many species show late-acting gametophytic self-incompatibility (e.g. *T. speciossima*; Offord, 2004). Vaughton and Carthew (1993) investigated low fruit:flower ratios in *B. spinulosa* and found support for the selective abortion hypothesis (post-zygote abortion) in which self-fertilised embryos developed in the absence of cross-fertilised embryos, but selectively aborted when cross progeny were present in the same inflorescence. The high incidence of self-incompatibility in *Banksia* presented by Collins and Rebelo (1987) may be an overestimate because they included species for which only data on pollen tube growth is available, rather than seed set. This further supports the need for documentation of fruit maturation and even seed germination after self-pollination treatments as species may be wrongly assumed to be self-compatible.

The apparent evolution of self-compatibility in *Protea* is puzzling when the plants produce large showy inflorescences with abundant rewards, typical of outcrossing species. Selfing can provide reproductive assurance in pollinator-limited environments (Eckert *et al.*, 2006). However, it has become increasingly evident that long-lived perennial species can carry a high genetic load of recessive deleterious alleles compared to short-lived annuals (Duminil *et al.*, 2009; Lande *et al.*, 1994). These alleles are then expressed in inbred progeny that are unlikely to reach reproductive maturity (Husband & Schemske, 1996; Morgan, 2001). This hypothesis has some support from an investigation of mating system using allozymes that revealed high inbreeding depression in the progeny of naturally pollinated plants of *P. caffra* (S-L. Steenhuisen, unpubl. data). This is contrary to our finding of an absence of inbreeding depression in germination rates or seedling survival within the first two months of growth for

selfed progeny in this species (Table 3). However, we were not able to observe long-term growth to reproductive maturity, and inbreeding depression in *P. caffra* may be more severe in its natural habitat. There is a paucity of literature on comparing inbreeding depression affects in a common-garden versus field environment for woody plants, but those few studies that do exist have shown higher inbreeding depression in the natural habitat over several years and flowering seasons for selfed plants compared to those grown under greenhouse conditions (e.g. *Eucalyptus regnans* F.Muell. (Hardner & Potts, 1997); *Fuchsia excorticata* L.f. and *Sophora microphylla* Meyen (Robertson *et al.*, 2011)). Thus our simple controlled pollination experiments and greenhouse trials for inbreeding depression may overestimate the extent to which selfed progeny contribute to future population demographics in grassland *Protea* species.

To investigate shifts to autonomous self pollination and their environmental basis, rigorous breeding system studies are required for many more *Protea* species. In addition, multilocus estimates of outcrossing rates are required to establish how particular breeding systems translate into mating patterns and to assess the contributions of selfed progeny to the demography of populations.

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CHAPTER 3

EVIDENCE FOR BEETLE POLLINATION IN THE AFRICAN GRASSLAND SUGARBUSHES (*PROTEA*: PROTEACEAE)

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Abstract

Most lineages in the African genus *Protea* consist of species with large unscented flowers pollinated principally by birds and several of these lineages also show evidence of shifts to rodent pollination, associated with concealed yeasty-scented flowerheads. In this study we investigated the hypothesis that brightly-coloured and fruity-scented flowerheads of four *Protea* species (*P. caffra*, *P. simplex*, *P. dracomontana* and *P. welwitschii*) represent a novel shift from bird to insect pollination in a grassland lineage in the genus. These species are visited by a wide range of insects, but cetoniine beetles were found to be the most important pollinators, due to their abundance, size, and relatively pure pollen loads. Three of the four putatively insect-pollinated *Protea* species have flowers presented at ground level and experiments showed that cetoniine beetles preferred inflorescences at ground level to those artificially elevated to the height of shrubs and small trees. Relative to insects, birds were infrequent visitors to all of the study species. The nectar of all the study species contained xylose, as documented previously in bird- and rodent-pollinated *Protea* species, suggesting that this is a phylogenetically conserved trait. However, the very low concentration of nectar (c. 8%), short nectar-stigma distance, and the fruity scent of florets, appear to be traits that are associated with specialization for pollination by cetoniine beetles.

Key words: *Atrichelaphinis tigrina*; Cetoniinae; nectar; specialized pollination systems.

Introduction

Beetle pollination is often regarded as a primitive condition in flowering plants (e.g. Willemstein 1987; Howe and Westley 1988). Because beetles can be destructive, eating petals and pollen, their contribution to pollination has sometimes been viewed as questionable (Gottsberger 1990). They can appear to be casual flower visitors lacking conspicuous morphological adaptations for collecting pollen or for exploring hidden nectar sources (Gottsberger 1990). However, beetle pollination has evolved in many plants characterised by bowl-shaped flowers (often serving as mating rendezvous sites) that present pollen as a primary reward. Beetles belong to the most diverse insect order and specialised pollination systems are becoming more apparent, in which plants exhibit floral traits associated with pollination by certain types of beetles (Armstrong and Irvine 1989). For example, pollination by cetoniine beetles (Scarabaeidae: Cetoniinae) is common in the tropics, and is associated with open bowl-shaped flowers that emit strong odours (Bernhardt 2000). Some groups of angiosperms have beetles as the most prominent or even exclusive pollinators (e.g. Magnoliaceae; Dieringer et al. 1999). Pollination systems involving hairy scarab beetles are also found in the Iridaceae (Goldblatt and Manning 2006) and Orchidaceae (Johnson et al. 2007), plant families that show high levels of pollination system specificity in southern Africa (Johnson and Steiner 2003).

Specialised pollination systems involving beetles have not previously been documented in the large Gondwanan family Proteaceae, although Collins and Rebelo (1987) noted beetles as co-visitors (along with bees) to several species of *Leucospermum*, *Banksia*, and *Grevillea* that are pollinated mainly by vertebrates. The distribution and guild composition of insects inhabiting the inflorescences of bird-pollinated South African Proteaceae have been extensively documented, mainly in the context of the marketability of cut *Protea* flowers (e.g. Gess 1968; Wright and Samways 2000). Their contribution to seed set, however, has been an issue of controversy. Some studies have used vertebrate exclusion to determine their contribution to seed set (e.g. *P. repens* in Coetzee and Giliomee (1985), *P. nitida* and *P. cynaroides* in Wright *et al.* (1991), *P. caffra, P. dracomontana, P. simplex,* and *P. welwitschii,* Steenhuisen and Johnson, in press, chapters 2 & 5). However, there is increasing evidence that many *Protea* species are self-compatible and capable of autogamous seed set (Steenhuisen and Johnson, in press; van der Walt 1995, chapter 2), making it difficult to

interpret the results of simple exclusion experiments without additional data on the abundance, behaviour and pollen loads of insect visitors.

Most Proteaceae are hermaphroditic with flowers arranged in capitula, racemes or spikes, often subtended by colourful and conspicuous involucral bracts (Collins and Rebelo 1987). The pollen of individual flowers is commonly applied by four anther lobes to a pollen presenter, a specialised subapical region of the style that is exposed when the style elongates and/or straightens during anthesis (van der Walt and Littlejohn 1996a; Matthews et al. 1999). Proteaceae are generally protandrous, with the stigmatic groove at the tip of the style being almost closed at the time of pollen presentation, and only opening after virtually all the pollen has been lost in some species (Collins and Rebelo 1987; van der Walt and Littlejohn 1996b) while weak protandry is reported in a few species for which receptivity is triggered by anthesis (Gross and Caddy 2006). Some species emit strong sweet or fruity odours (Steenhuisen et al. 2010) suggestive of insect pollination, and there is great variation in the amount and concentration of nectar produced by flowers (Collins and Rebelo 1987). The only examples of exclusive insect pollination in this family have come from studies of Australian genera (Carolin 1961; Lamont 1982; Bernhardt and Weston 1996; Phillips et al. 2010 and references within).

The present study investigates the pollination systems of four *Protea* species in KwaZulu-Natal, South Africa: *P. caffra* Meisn., *P. dracomontana* Beard, *P. simplex* E.Phillips ex J.M.Wood, and *P. welwitschii* Engl., known as the grassland or savanna sugarbushes (Rebelo 2001). The flowers of these species have an unusually strong fruity scent (Steenhuisen et al. 2010), and most possess an almost geoflorous growth form (when frequently burnt) with short stems growing from large underground rootstocks. In contrast, bird-pollinated *Protea* species are typically trees bearing large robust unscented inflorescences with hidden nectar sources exploited by sunbirds and sugarbirds with long slender bills (Hargreaves et al. 2004; Collins and Rebelo 1987). For example, *P. roupelliae* Meisn. shares a similar distribution with the grassland/savanna sugarbushes. It is self-compatible, but allogamous and inflorescences exposed to visits by malachite sunbirds show high seed set relative to those from which birds were selectively excluded (Hargreaves et al. 2004). The accessible nectar, large pollen rewards on exposed pollen presenters, fruity scent, and open bowl-shaped inflorescences of the grassland/savanna sugarbushes suggest that these plants may be pollinated by beetles, but there have been no previous investigations of the pollination

biology of these *Protea* species. Preliminary observations show that an array of insects, especially scarab beetles, visit these scented *Protea* species in the summer-rainfall areas of South Africa. This study investigates the pollination systems of these scented *Protea* species and presents information on floral biology, visitation frequency and pollen loads of potential insect pollinators, and determines the effect of inflorescence height above ground on insect attraction. We used the bird-pollinated *P. roupelliae* as a control species in comparisons of floral traits as it frequently co-occurs with the study species.

Materials and Methods

Study Species and sites

Protea caffra, *P. dracomontana, P. simplex* and *P. welwitschii* are common sugarbushes inhabiting grasslands and the escarpment in the Northern Province, Mpumulanga, Swaziland, and KwaZulu-Natal, through to the Eastern Cape of South Africa (Rebelo 2001) (Fig. 1). Flowering of these species coincides with summer rainfall and spans a period of four months from December to March, with a peak period in January. A population of approximately 550 *P. simplex* plants, located on the grassland slopes of the summit of Mount Gilboa (29.29°S, 30.29°E, 1770 m), KwaZulu-Natal, was studied in January 2002 and 2005 (Fig. 1A&B). Smaller sympatric populations (approximately 200 plants) of *P. caffra* and the bird-pollinated *P. roupelliae* were also studied here in 2005 (Fig. 1C&F). Populations of approximately 500 plants of *P. welwitschii* and *P. caffra* were studied in summer 2003/2005, and 2004 respectively, located on steep grassland slopes of a residential area in Winston Park (28.75°S, 30.75°E, 550m) and the Krantzkloof Nature Reserve (29.77°S, 30.84°E, 450m) respectively (Fig. 1C&D). A small population of 50 *P. caffra* plants on a hilltop slope of Bulwer Mountain (29.75°S, 29.75°E, 1900m) was used in 2005, sympatric with *P. roupelliae* (Fig. 1C&F). A large population (approximately 500 plants) of *P. dracomontana* on the lower slopes of Garden Castle (29.74°S, 29.20°E, 1900m) and a smaller population (300 plants) at Blind Man's Corner in Monk's Cowl Reserve (29.07°S, 29.38°E, 2016m) in the Drakensberg mountains were used in 2004 and 2005 respectively (Fig. 1E). Vouchers have been deposited in the Bews Herbarium (NU) University of KwaZulu-Natal (voucher numbers 55, 57, 59, 60-62, collector: S.-L. Steenhuisen).

Floral biology

For each of the study species, we measured floral dimensions that might influence pollinator-fit to flowers, spectral reflectance, and nectar and pollen production. We used the outermost and innermost ring of florets in each of 12 inflorescences of each species to measure floret height, style length, length of the pollen presenter, distance between the site of nectar production and presentation, the site of nectar presentation and tip of the stigma, the site of nectar presentation and base of the inflorescence, and also measured the height, diameter, and number of florets in each of these inflorescences (Fig 1B). The average plant height was also determined for these plants. Each trait was compared across species using an ANOVA with Tukey posthoc tests.

Spectral reflectance was measured for the inner and outer surfaces of the involucral bracts, perianth, pollen presenters bearing pollen (excluding *P. welwitschii*), bare styles, and stigma for each of five inflorescences of each species. Spectral reflectance across the 300-700 nm range was determined using an Ocean Optics S2000 spectrometer (Ocean Optics Inc., Dunedin, FL, USA) and fibre optic reflection probe (QR-400-7-UV-VIS; 400µm) held at 45º to the surface of the plant inflorescence part. The light source used was an Ocean Optics DT-mini deuterium tungsten halogen light source with an approx. 200 to 1100 nm spectral range. An Ocean Optics WS-1 diffuse reflectance standard was used to calibrate the spectrometer (Johnson and Andersson 2002).

Nectar properties were determined to investigate the quantity and quality of rewards offered to floral visitors. The average volume of nectar produced after a 24hr period was measured in two or more florets in each of six or more newly opened inflorescences on freshly cut branches by means of a calibrated micropipette (Fisherbrand 1-5 µl) inserted between the perianth and style of individual florets. The nectar of undisturbed florets often accumulated as a droplet midway up the style and perianth and was easily drawn into a micropipette by capillary action. The mean percentage sugar content was measured for the nectar samples using an Atago N1 0-50 % pocket refractometer. Nectar was also collected for high-pressure liquid chromatography (HPLC) to determine sugar composition. One to two samples were collected onto filter paper, air-dried, and analysed according to the methods of Nicolson and Van Wyk (1998) ("VW" in Table 2). A further 10-13 nectar samples were collected (stored frozen at -20 ºC until analysed), centrifuged at 10 000 rpm for 10 minutes and the supernatant

filtered with a 0.45 micron syringe filter. Filtered samples were analysed using a Shimadzu HPLC (LC-20AT) equipped with a differential refractometric detector (RID10A) and a Phenomenex column (Rezex RCM-Monosaccharide, 200 x 780 mm, 8 micron) ("UKZN" samples in Table 2). The elution was isocratic, using ultrapure water as the mobile phase. Species differences in the volume of nectar produced per floret and per inflorescence (volume per floret multiplied by number of florets per inflorescence), sugar concentration of nectar produced, and proportion of sucrose (for samples analysed at UKZN) were determined using ANOVA with arcsin-squareroot transformation of proportions and Tukey posthoc tests.

Field survey and pollen loads of floral visitors

To determine the spectrum of visitors to flowers, we surveyed at least twenty open inflorescences along 20 m line transects in single large populations of *P. simplex, P. caffra,* and *P. dracomontana* (Garden Castle), and random inflorescences in smaller populations for *P. dracomontana* (Monk's Cowl) and *P. welwitschii*. The number of individuals of each insect species found in each open inflorescence was recorded. The behaviour, especially with regard to feeding, of the insects was noted. Representative specimens of insect visitors and all of the medium-sized cetoniine beetles encountered were collected, and identified to family (or species) level. Fuchsin gel was used to pick up any pollen from the surface of the insect's body (excluding bee scopae for *Apis* species) and collecting bottle (Beattie 1971). The gel was then melted to form permanent slides and the pollen grains were counted using a compound microscope. Pollen loads carried on the upper and lower surfaces were determined separately for larger scarabaeid beetles and bees (≥10 mm in length) but not distinguished for the smaller insects. Mean estimates of the number of pollen grains (*Protea* and foreign pollen) carried by each insect species were determined. The placement of pollen on the body of one of the most frequent visitors, the cetoniine beetle *Atrichelaphinis tigrina* (Olivier), was also determined using scanning electron microscopy (Fig 1G). The mean body lengths of the captured insects were determined and compared with the dimensions of florets described above, namely the length of the style, pollen presenter, and the distance between the nectar and the stigmatic groove. All insects collected are stored in the entomological collection at the University of KwaZulu-Natal.

To test whether beetles had consumed *Protea* pollen, the contents of the faecal material from the cetoniine beetle species *Atrichelaphinis tigrina*, shown in this study to be a primary pollinator of some of the study species, was examined. Faecal material from these beetles

visiting *P. simplex* inflorescences was examined microscopically after softening with concentrated sulphuric acid and staining with fuchsin stain.

Height preference

By placing inflorescences at different heights and recording insect visitors, we determined the height preference of insect visitors to *P. simplex* on Mount Gilboa. We tested the prediction that insects prefer to visit inflorescences that are closer to the ground. Ten 2.5 m green-painted aluminium poles were erected in two parallel lines of five poles ten metres apart, with 2.5 m between adjacent poles. Plastic vases containing water and a newly opened *P. simplex* inflorescence (i.e. not containing insects) were wired on to each pole at heights of 0.5 m, 1.5 m, and 2.5 m (Fig. 1A). These heights correspond to the maximum height for *P. simplex*, average height for *P. caffra*, and minimum height of the *P. roupelliae* trees on Mount Gilboa respectively. The number of insects per inflorescence at each height was counted every half hour from 15:00-16:00 on February 28th, 9:00-12:30 on February 29th, and 10:00-14:00 on March 9th 2004. The total number of insects per survey at each height was compared using means per inflorescence per survey rounded to the nearest integer and fitted to generalized linear models with likelihood ratio Chi-square statistics, Poisson error distributions and log link functions (Field 2009; McCullagh and Nelder 1989; Hosmer and Lemeshow 2000). We also compared the number of Coleoptera, Diptera, and Hymenoptera, and the interaction between these three insect orders and height of inflorescence. We used pairwise contrasts with sequential Sidâk adjustment for multiple comparisons of height and insect order. To provide a statistically conservative solution to the problem of undefined log-link transformations when there is no variance within a set of count data (Zuur et al. 2009), we inserted one insect count in one inflorescence for counts of Diptera and Hymenoptera at heights of 1.5 and 2 m.

Results

Floral biology

Inflorescences of all the study species are extremely long-lived with individual florets lasting for at least five days. Open inflorescences provide large landing platforms for pollinators. The inflorescences of *P. caffra* are broader than those of the other species, while inflorescences of the bird-pollinated *P. roupelliae* are very tall with more tightly fitting and erect bracts and florets, making accessibility of nectar very difficult for medium-sized beetles

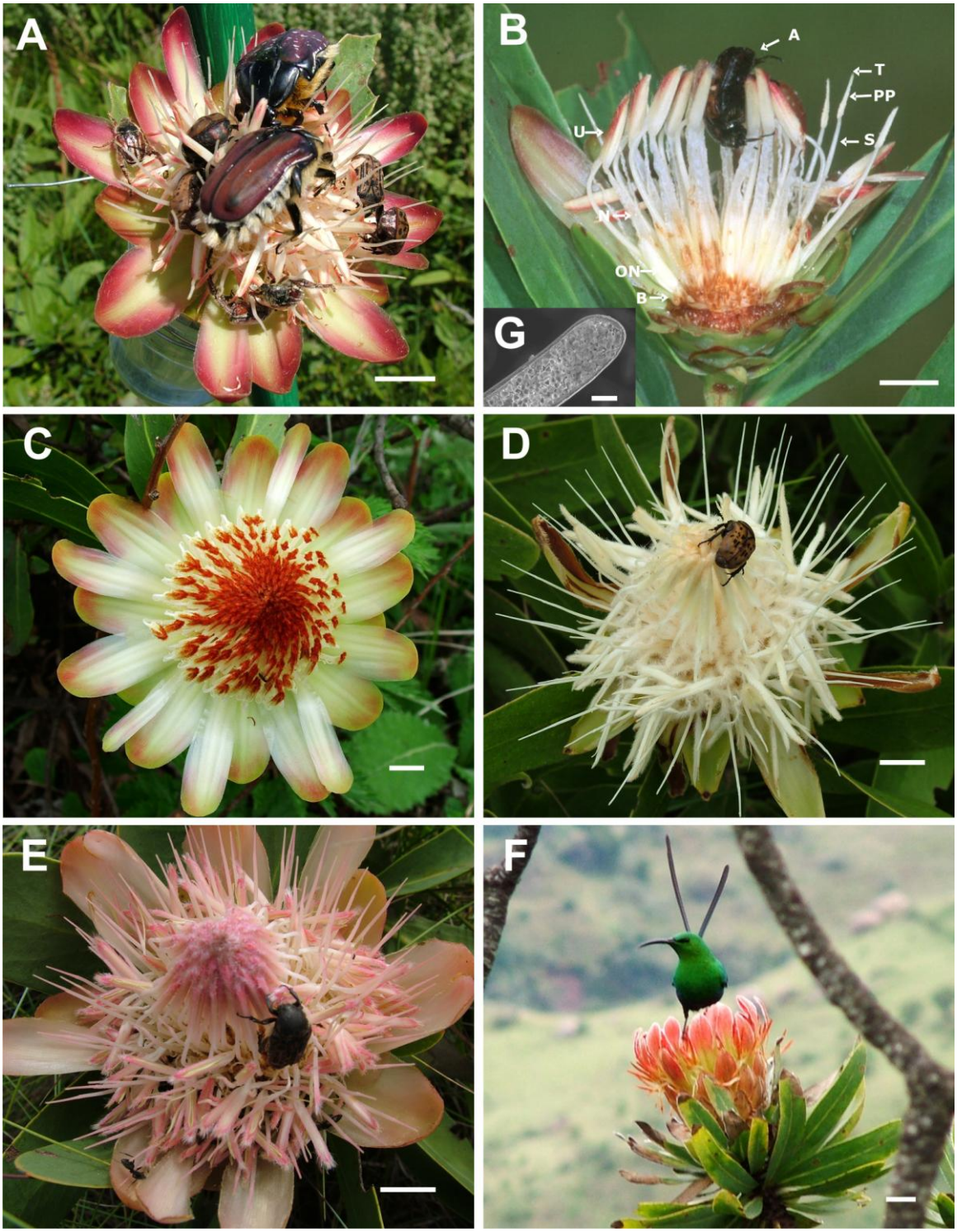

Fig.1 Inflorescences and pollinators of *Protea* species included in this study. (A) Inflorescence of *Protea simplex* visited by four *Atrichelaphinis tigrina*, two *Trichostetha fascicularis*, and three hopliine beetles. (B) Cross-section of a *Protea simplex* inflorescence showing nectar-feeding behaviour of a medium-sized cetoniine beetle (A- *Atrichelaphinis tigrina*, T-terminal stigmatic groove, PP-pollen presenter, S-style, B-base of inflorescence, ON-ovary and site of nectary, N-site of nectar presentation on perianth tube, U-undehisced

Fig. 1 *continued* — floret with perianth and anther lobes enclosing the pollen presenter). (C) *Protea caffra*. (D) *Protea welwitschii*. (E) *Protea dracomontana*. (F) *Protea roupelliae* inflorescence visited by a malachite sunbird, *Nectarinia famosa*. (G) Scanning electron micrograph of the underside of a hind tarsal claw of *Atrichelaphinis tigrina* carrying *Protea simplex* pollen. Scale bars for photos A-E are 10 mm, F is 20 mm and G is 100 µm.

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not strong enough to prise apart the woody florets (Fig. 1, Table 1). Individual florets ranged from 28.4 mm in height for *P. simplex* to 53.8 mm in *P. caffra,* and pollen presenters comprised about 13-21 % of the length of the style (Table 1, Fig 1B).

Spectral reflectance of involucral bracts and perianth for four of the five species was characterized by an overall red-pink hue (Fig. 1 and see Online resource 1). The greatest variation from the overall species pattern was the bracts of *P. welwitschii*, which appear as a green-cream colour in human vision. The gynoecia of all species were also cream. Pollen of all species and the silvery hairs on the bracts of *P. roupelliae* inflorescences showed a small amount of UV reflectance. Apart from browning during senescence, no distinct colour changes were observed at different stages of flowering (e.g. after anthesis).

Three of the four petals were fused forming a perianth sheath below the pollen presenter (Rourke 1980), drawing nectar from the site of secretion at the base of the floret up a distance of c. 1 cm by capillary action (Table 1). The nectar was very dilute and produced independently at the base of each floret (Table 1&2). Observations indicated that nectar production was usually at its highest just before and after anthesis (and in the morning), but this was not quantified. The nectar eventually drained to the base of the florets, as seen for example in inflorescences of *P. caffra*, which could have a pool of approximately 1 ml of 5 % sugar concentration at any one time if evaporation was kept to a minimum. Qualitative observations suggested that if removed this nectar can be replenished after only 6 hours. *P. roupelliae* produced larger volumes of more concentrated nectar than the other sugarbushes (Table 1&2). The nectars of all species contained xylose (4.0-42.1%) and were usually dominated by monosaccharide sugars (Table 2). *Protea caffra* and *P. dracomontana* nectars contained the highest proportion of xylose sugar (c. 24.3 % for each species, averaged over all sources, Table 2) and that of the bird-pollinated *P. roupelliae* the least (c. 4.3 %). For the grassland *Protea* species, the nectar of *P. caffra* contained a similar proportion of sucrose to

Table 1 Morphological and nectar characteristics of four insect-pollinated *Protea* species in comparison with those of the bird-pollinated species *P. roupelliae* with which they are often sympatric.

Floral character	*Protea caffra*	*Protea dracomontana*- Garden Castle	*Protea dracomontana*- Monk's Cowl	*Protea simplex*	*Protea welwitschii*	*Protea roupelliae*	
	$\bar{x}$ ±SE	$\bar{x}$ ±SE	$\bar{x}$ ±SE	$\bar{x}$ ±SE	$\bar{x}$ ±SE	$\bar{x}$ ±SE	*F*-value[d]
Floret height (mm)[a]	53.8 ± 0.7 (12)a	48.2 ± 0.8 (10)b	41.1 ± 0.7 (20)c	28.4 ± 0.5 (12)e	40.12 ± 0.9 (10)c	69.7 ± 0.6 (10)d	353.0*
Pollen presenter (mm)[a]	8.2 ± 0.2 (12)a	6.0 ± 0.2 (10)b	6.4 ± 0.1 (20)b	5.0 ± 0.1 (12)c	7.2 ± 0.2 (10)d	6.4 ± 0.1 (10)b	38.8*
Style (mm)[a]	50.0 ± 0.7 (12)a	45.2 ± 0.8 (10)b	36.5 ± 0.6 (20)c	22.8 ± 0.4 (12)e	34.8 ± 0.8 (10)c	64.8 ± 0.6 (10)d	415.9*
Stigma-nectar (mm)[a]	35.3 ± 0.9 (12)a	32.4 ± 0.9 (10)a	25.6 ± 0.5 (20)b	17.0 ± 0.6 (12)d	27.4 ± 0.7 (10)b	48.0 ± 0.6 (10)c	212.5*
Nectar-base (mm)[a]	18.8 ± 0.6 (12)a	15.8 ± 0.4 (10)b	15.3 ± 0.3 (20)b	10.2 ± 0.4 (12)d	12.0 ± 0.6 (10)d	21.7 ± 0.3 (10)c	79.6*
Nectar-nectary (mm)[a]	15.1 ± 0.7 (12)a	13.0 ± 0.4 (10)b	10.7 ± 0.3 (20)c	9.6 ± 0.5 (4)cd	7.3 ± 0.4 (10)d	16.7 ± 0.4 (10)a	50.7*
Inflorescence diameter (mm)[a]	101.1 ± 2.7 (12)a	84.3 ± 6.0 (10)bc	77.8 ± 1.7 (20)c	62.8 ± 1.9 (12)d	77.6 ± 3.2 (10)c	96.8 ± 4.5 (10)ab	18.5*
Inflorescence height (mm)[a]	78.7 ± 1.4 (12)a	66.4 ± 1.7 (10)b	57.7 ± 1.4 (20)c	41.0 ± 0.7 (12)e	48.3 ± 1.4 (10)f	98.6 ± 1.8 (10)d	181.8*
Number of florets per inflorescence[a]	158.3 ± 10.9 (12)a	130.7 ± 10.6 (10)ab	144.6 ± 9.6 (20)ab	78.2 ± 4.6 (12)c	80.2 ± 5.4 (10)c	114.5 ± 6.0 (10)bc	13.2*
Plant height (cm)[b]	147.3 ± 7.1 (30)a	44.5 ± 1.8 (40)b	30.6 ± 1.6 (20)bd	20.73 ± 0.66 (20)d	34.8 ± 1.7 (20)bd	295.1 ± 9.6 (20)c	417.7*
Volume per floret (µl)[c]	8.3 ± 0.9 (62)ab	8.7 ± 0.9 (52)ab		4.2 ± 0.3 (20)b	12.4 ± 1.6 (35)a	13.1 ± 1.0 (52)a	6.0*
Volume per inflorescence (ml)[c]	1.3 ± 0.1 (62)ab	1.1 ± 0.1 (52)bc		0.3 ± 0.02 (20)d	1.0 ± 0.1 (35)c	1.5 ± 0.1 (52)a	61.7*
Sugar concentration (%)[c]	4.4 ± 0.3 (37)a	4.7 ± 0.2 (52)a		6.3 ± 0.3 (20)c	10.4 ± 0.7 (35)d	13.1 ± 0.5 (52)b	54.3*

[a]Measurements from an inner and outer floret from each of (n) inflorescences

[b]Number of plants in parentheses

[c]Number of florets in parentheses

[d]df=5 for ANOVA with Tukey posthoc tests of species differences (for each trait, means that share letters are not significantly different) except for analyses of nectar properties for which df=4 (excludes *P. dracomontana* from Monk's Cowl). **P*<0.001

Table 2 Relative amount ($\bar{x}$ ±SE %) of nectar sugars for five species within the genus *Protea*. Source: NVW = from Nicolson and Van Wyk (Nicolson and Van Wyk 1998); VW = analysed by B.E. Van Wyk in 2004; UKZN = samples run at UKZN in 2009. Significant differences in the proportion of sucrose in UKZN samples of the grassland *Protea* species are indicated by different letters (P<0.05).

Species	Sample size	Sucrose (%)	Fructose (%)	Glucose (%)	Xylose (%)	Source
Protea caffra	6	2.0±1.4	33.2±4.1	49.3±3.07	15.8±4.5	NVW
	1	0.0	17.4	40.6	42.1	VW
	12	13.6±2.8 a	47.2±1.6	24.2±2.9	15.14±2.2	UKZN
Protea dracomontana	2	15.2±15.2	21.0±3.4	33.2±5.2	30.7±13.4	VW
	11	7.4±2.6 b	44.1±2.8	30.6±3.9	18.0±2.2	UKZN
Protea roupelliae	2	4.0±3.0	44.5±1.5	47.5±0.5	4.0±2.0	NVW
	2	0.0±0.0	46.2±1.1	49.2±1.3	4.6±2.4	VW
Protea simplex	2	0.0±0.0	31.2±7.4	54.0±1.4	14.8±6.0	VW
	13	6.7±1.1 ab	43.0±3.01	36.6±4.5	13.7±1.8	UKZN
Protea welwitschii	10	5.2±0.9 b	40.5±1.0	44.9±1.4	9.4±1.6	UKZN

P. simplex but a significantly higher proportion of sucrose than *P. dracomontana* and *P. welwitschii* ($F_{(3)} = 4.168$, $P = 0.011$; Table 2).

Pollinator survey and pollen loads

Insects, principally Coleoptera and Hymenoptera, were the sole animals observed to visit *P. simplex* and *P. welwitschii* inflorescences (Fig. 2, Table 3 and Online resource 2). Malachite sunbirds, *Nectarinia famosa*, were observed to visit *P. caffra* inflorescences at Bulwer Mountain and Mt Gilboa but not at Krantzkloof, and one sighting was made at Garden Castle of a malachite sunbird on *P. dracomontana*. Of the visiting insects, small (Chrysomelidae, Melyridae, Hopliinae) to medium-sized beetles (various Scarabaeidae, especially Cetoniinae) were the most frequent with the exception of a large number of flies (Chloropidae and Drosophilidae) and bees (Apidae: *Apis mellifera*) visiting *P. dracomontana* and *P. welwitschii* respectively (Fig. 2, Table 3). We recorded pollen grain loads of most insect visitors across 4 orders, 34 families and 77 species, 68 % of which were Coleoptera (Table 3). The majority of *Protea* pollen (up to 80 % for *Atrichelaphinis tigrina*) was carried on the

lower surface of the body for larger insects (≥10 mm, Online resource 2). Insect visitors carried low numbers of foreign pollen (usually < 20% of total pollen loads) although there was high variability between species (Online resource 2). Insect visitation was highest during full anthesis corresponding with the strongest scent emission (Steenhuisen et al. 2010).

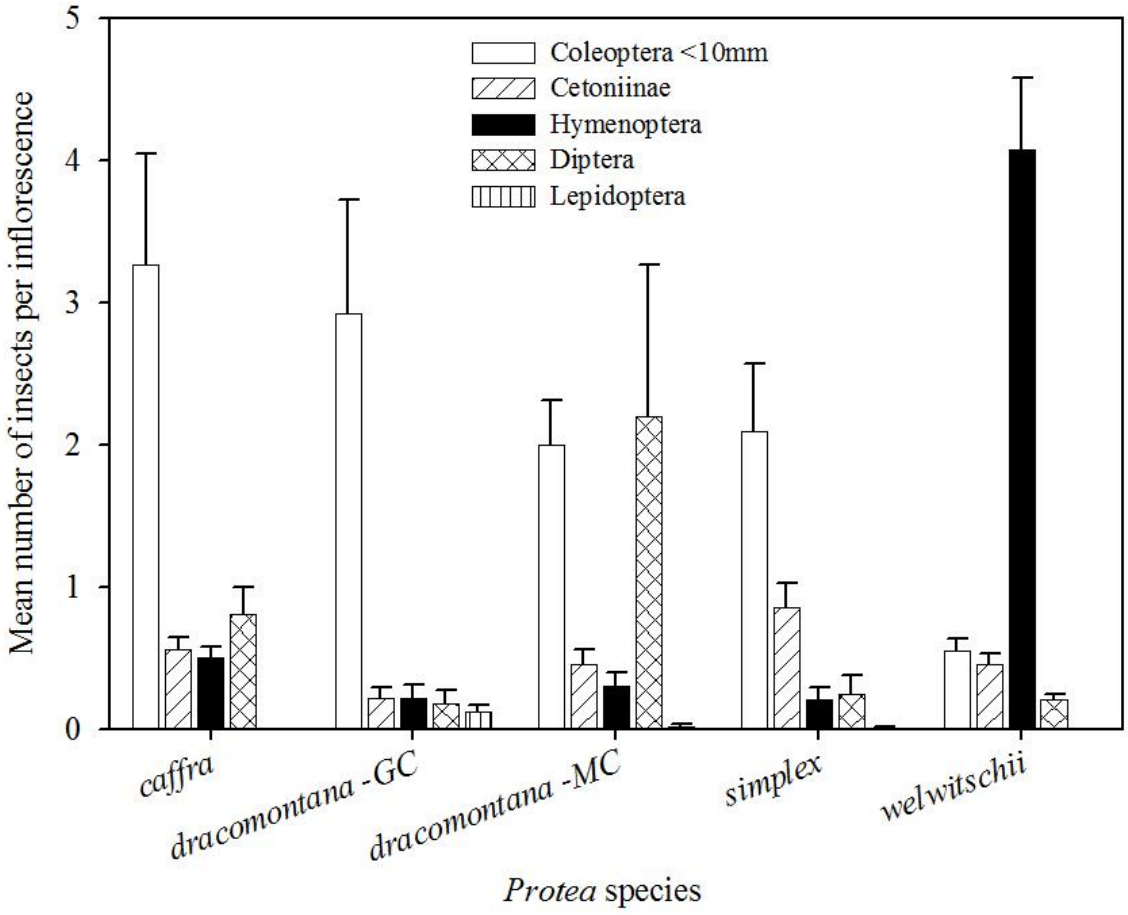

Fig. 2 The composition and frequency of insect visitors to open inflorescences of four grassland/savanna *Protea* species. Coleoptera are separated into beetles of <10 mm in body length and beetles of the sub-family Cetoniinae. For *P. dracomontana*: GC=Garden castle and MC=Monk's Cowl.

Cetoniine beetles carried large *Protea* pollen loads and most frequently contacted stigmas with the underside of their abdomens and legs whilst they consumed pollen from dehisced anthers, petals and pollen presenters (e.g. *Atrichelaphinis tigrina, Leucocelis haemorrhoidalis* and *Cyrtothryrea marginalis*) (Fig 1A, Table 3). Microscopic analysis of *A. tigrina* faeces revealed that these beetles consumed

extremely large amounts of *P. simplex* pollen (>10 000 grains present in faeces for a single beetle). These beetles also fed on nectar, drinking head-down from the accumulated nectar pool in the base or licking petals and styles (Fig 1A&B). Many of these beetles also used inflorescences as mating rendezvous sites and overnighted in both fresh and senescing inflorescences.

Smaller, more active beetles, such as Melyridae and Tenebrionidae, were observed scrambling over and in between perianth tubes and anthers, but very rarely brushing against stigmas (Table 3). Tiny Staphylinid beetles often swamped inflorescences of *P. dracomontana*. These beetles aggregated at sites of nectar production and presentation, but were also observed to crawl up and down styles. A diverse community of large (>20 mm) cetoniine beetles, including *Phoxomela umbrosa* and *Mecynorrhina passerinii* were observed as occasional visitors of *P. caffra* at Krantzkloof Gorge (see Table 3 for other species). Of the few large cetoniines, the most commonly recorded was the green protea beetle, *Trichostetha fascicularis*, particularly at Mt Gilboa and Garden Castle (Online resource 2). Flies (Diptera) were generally infrequent visitors, becoming more numerous as inflorescences aged and the nectar was characterised by a fruity wine-like scent, suggesting microbial fermentation (Steenhuisen et al. 2010). Ants (Hymenoptera, Formicidae) were usually present and observed to drink nectar. Small sweat bees (Halictinae) were more frequent visitors to *P. caffra, P. simplex* and *P. dracomontana* than larger Apidae (more frequent visitors to the sweeter-smelling *P. welwitschii*), and were observed to collect more pollen in their scopae than other bees. Pollen loads on halictid bees included pollen carried in their scopae, giving rise to markedly high pollen loads, most of which will not be available to pollination (Table 3, Online resource 2). All bee visitors probed the base of florets for nectar, especially honeybees (*Apis mellifera scutellata*). A few butterfly (Satyridae, Hesperiidae, Nymphalidae) and moth species were observed drinking nectar at the edge or under the bracts of inflorescences, especially on *P. dracomontana* (Fig. 2), but did not generally contact stigmas or pollen presenters. All species of insects recorded were consistently observed to visit inflorescences of the four scented *Protea* species over the duration of the study.

Table 3 The mean *Protea* pollen loads of representative insects collected from inflorescences of five *Protea* species.

Order	Family	Subfamily	Species	Mean *Protea* pollen load per insect found in inflorescences of 5 *Protea* species (location)				
				Protea caffra (Krantzkloof)	*Protea dracomontana* (Garden Castle)	*Protea dracomontana* (Monk's Cowl)	*Protea simplex* (Mount Gilboa)	*Protea welwitschii* (Winston Park)
Coleoptera	Carabidae		sp1	-	-	-	-	89.0
	Chrysomelidae		sp1	-	-	19.8	-	-
			sp2	-	26.4	4.0	-	-
			sp3	-	-	-	-	31.0
			sp4	-	-	-	-	52.0
			sp5	-	-	-	88.0	-
			sp6	-	-	-	1742.1	-
	Cleridae		sp1	-	-	26.1	-	-
			sp2	-	-	13.5	-	-
	Curculionidae		sp1	72.0	-	-	-	-
			sp2	104.0	-	-	-	-
			sp3	7.5	-	-	-	-
			sp4	unknown	-	-	-	-
			sp5	-	81.0	-	-	-
			sp6	-	-	-	-	15.0
			sp7	-	-	-	641.7	-
	Dermestidae		*Dermestes maculatus*	16.5	-	-	-	-
	Elateridae		sp1	-	69.0	-	13.0	33.8
			sp2	-	-	-	-	140.0
			sp3	-	-	-	-	10.0
	Endomychidae		sp1	-	-	-	89.0	-
	Eucnemidae		sp1	13.0	-	-	-	-
	Languriidae		sp1	-	-	-	66.0	-
	Meloidae		sp1	-	-	-	40.0	-
	Melyridae	Melyrinae	*Melyris sp.*	-	123.0	29.9	4561.5	14.7
	Nitidulidae	Carpophilinae	sp1	12.0	8.0	27.0	-	28.4
			sp2	13.5	24.0	60.0	17.9	23.1
		Epuraeinae	sp3	15.0	73.0	-	-	31.4
	Phalacridae		sp1	-	34.2	-	-	390.0
	Rhizophagidae		sp1	5.0	-	-	-	3.0
	Scarabaeidae	Cetoniinae	*Atrichelaphinis tigrina*	53.3	189.2	63.7	11198	199.5
			Cyrtothyrea marginalis	144.8	11.0	-	1627.4	137.1
			Leucocelis adspersa	71.8	-	-	-	-

			Leucocelis haemorrhoidalis	192.0	-	-	-	166.4
			Leucocelis rubra	695.7	-	-	-	-
			Pachnoda sinuata	113.5	-	-	-	-
			Porphyronota maculatissima	123.0	-	-	-	-
			Rhabdotis aulica	13.0	-	-	-	-
			Trichostetha fascicularis	-	70.4	-	1448	-
		Dynastinae	*Cyphonistes vallatus*	-	-	-	-	83.0
		Hopliinae	sp1	-	12.9	-	-	-
			sp2	-	12.3	-	-	-
			sp3	-	-	-	-	90.7
			sp4	-	-	10.0	-	-
			sp5	35.0±12.6	-	-	-	38.0
	Scirtidae		sp1	-	11.0	-	-	-
			sp2	-	10.0	-	-	11.0
	Staphylinidae		sp1	-	36.0	11.5	101.0	-
	Tenebrionidae		sp1	-	-	-	6222.3	-
			sp2	-	-	-	7.0	-
			sp3	-	7.0	-	-	-
Diptera	Calliphoridae		sp1	473.0	-	-	-	-
			sp2	-	-	-	417	-
	Chloropidae		sp1	473.8	-	8.5	30.0	-
	Drosophilidae		sp1	-	-	-	209.5	-
	Muscidae		sp1	-	-	-	54.0	-
			sp2	-	3.0	-	-	-
			sp3	-	62.0	-	-	-
			sp4	-	59.0	-	-	-
			sp5	-	85.0	-	-	-
	Platystomatidae		sp1	-	-	-	91.7	-
	Syrphidae		sp1	-	-	-	-	197.0
	Tephritidae		sp1	-	-	-	16.0	-
Hemiptera	Anthocoridae		sp1	-	1.0	-	-	-
	Lygaeidae		sp1	-	8.0	106.0	-	-
Hymenoptera	Apidae	(Xylocopini)	sp1	272.2	-	-	-	14.0
			Apis mellifera scutellata	63.8	13.5	-	578	30.6
	Braconidae		sp1	-	-	-	241.0	-
	Halictidae	Halictinae	sp1	-	24.0	-	-	-
			sp2	-	-	7.0	-	-
			sp3	-	4.0	84.7	>1000	208.0
	Pompilidae		sp1	20.0	-	-	-	-

Sphecidae	sp1	-	26.0	-	-	-	-	-
	sp2	-	-	-	-	-	62.0	-
Tiphiidae	sp1	-	1.0	-	-	-	-	-
	sp2	-	-	-	92.0	-	-	-

Height preference

Visiting insects showed a highly significant preference for *P. simplex* inflorescences at the low height of 0.5 m over those at heights of 1.5 and 2.5 m ($\chi^2_{(2)} = 64.3$, $P < 0.001$; Fig. 3). This pattern was also evident in analyses that included insect orders - Coleoptera, Diptera and Hymenoptera - as predictor variables (insect order: $\chi^2_{(2)} = 49.4$, $P < 0.001$; height: $\chi^2_{(2)} = 49.2$, $P < 0.001$; order*height: $\chi^2_{(4)} = 4.9$, $P = 0.30$). The coleopteran visitors mostly included Cetoniinae (*A. tigrina, T. fascicularis*), but also Hopliinae, Melyridae, Elateridae and other smaller beetles.

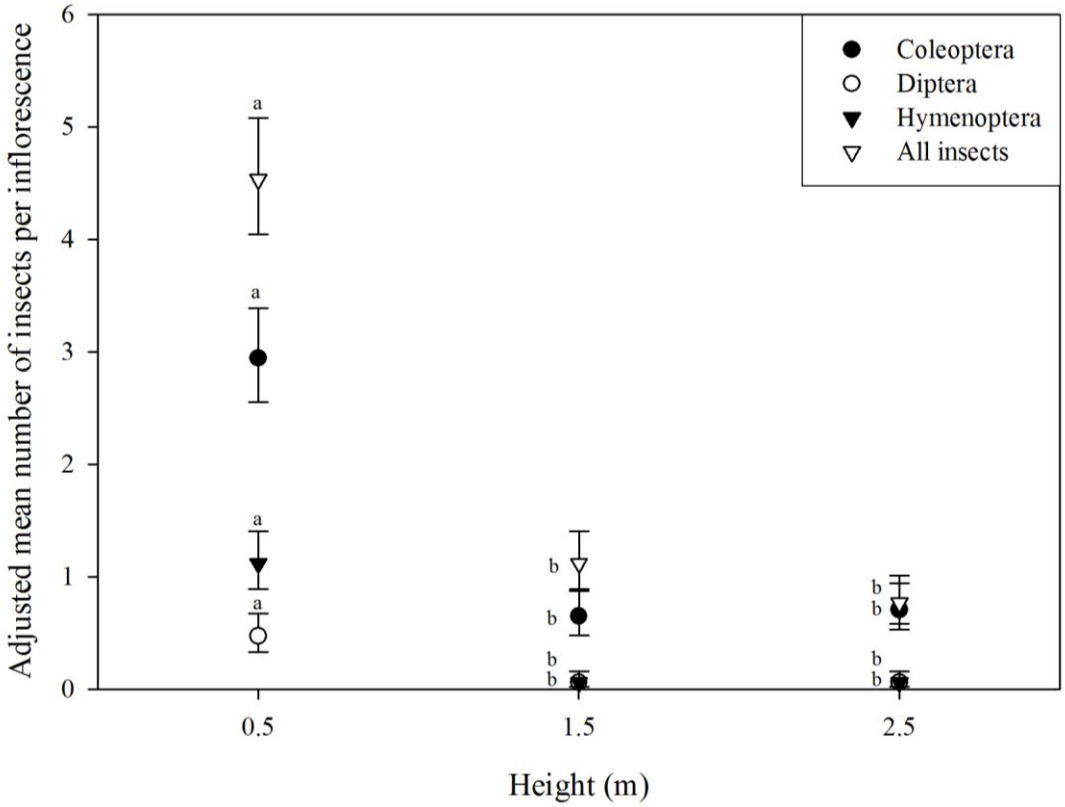

Fig. 3 The mean (± s.e) number of insects attracted to *Protea simplex* inflorescences presented at three different heights on aluminium poles. For each insect group, means that share letters are not significantly different.

Discussion

Dwarf grassland sugarbushes differ markedly from their bird-pollinated congeners (Fig. 1, Table 1). The latter, represented here by *P. roupelliae*, have tall erect bracts surrounding large woody florets producing hidden nectar that is available mainly to long-billed sunbirds and sugarbirds (Hargreaves et al. 2004). In addition, most bird-pollinated *Protea* species are large shrubs or trees above 3 m in height (Table 1,

Collins and Rebelo 1987). In contrast, the four species studied here were characterised by bowl-shaped inflorescences with open bracts, smaller and more flexible florets producing exposed nectar and pollen rewards (Table 1&2), a strong fruity scent (Steenhuisen et al. 2010), and were visited by a variety of insects (Table 3, Fig. 1). The low growth form of the plants was favoured by these floral visitors (Fig. 3), especially cetoniine beetles, which carried enormous pollen loads (Table 3), and were attracted to the fruity scent (S-L. Steenhuisen, unpubl. data, chapter 7).

Because of capillary action in the fused perianth, the site of nectar presentation was brought closer to the site of pollen presentation and stigma, potentially facilitating the deposition of pollen on insects feeding on nectar. This phenomenon has previously been observed for the Proteaceae (e.g. *Grevillea robusta*, Kalinganire et al. 2001). The ideal insect pollinator for the grassland sugarbushes may thus be predicted as exceeding 17-35 mm in size, corresponding to the distance between the stigma and site of nectar presentation across our four study species (Table 1). Collins and Rebelo (1987) reported stigma-nectar distances of 16-20 mm for two putatively insect pollinated *Protea* species, while those for bird-pollinated *Protea* species were 20-180 mm (c. 48 mm for *P. roupelliae*, Table 1, Collins and Rebelo 1987). This measure is highly variable across insect-pollinated species of the Proteaceae, for example, 28.6 mm for the beetle-pollinated *Dryandra lindleyana*, and < 13 mm for insect-pollinated *Hakea* species (Hanley et al. 2009; see also Collins and Rebelo 1987). The distance for rodent-pollinated proteas is c. 10 mm (Wiens et al. 1983). Our measurements of stigma-nectar distance in the dwarf grassland sugarbushes correspond to the body length of cetoniine visitors (8-23 mm for all species recorded). This, and the foraging behaviour and pollen loads of these insects, suggests that they are effective pollinators (Table 3 and Online resource 2). These beetles contact stigmas when landing on an inflorescence, promoting outcrossing (S-L. Steenhuisen unpublished data, chapter 5), and often crawl up and over pollen presenters and stigmas whilst foraging for nectar and pollen. Overnighting beetles would also encounter freshly dehisced pollen on presenters as they leave inflorescences the following morning.

The possibility that birds and rodents play a role in pollination of the study species was also considered. However, birds were seen visiting the inflorescences of *P. caffra* and *P. dracomontana* only on a handful of occasions over the months spent

observing in the field and they greatly preferred to visit the sympatric ornithophilous species *P. roupelliae*. Only one trapping night was used to assess visitation by rodents, using a hundred Sherman-type live traps at Mount Gilboa in February 2002. Only one individual rodent (*Mastomys natalensis*) was captured and it had just trace amounts of *Protea* pollen on its face and faeces. No other evidence of rodent visitation was found (i.e. scats, bites through nylon bags placed over flowers) that would warrant further investigation. In contrast to the winter flowering of most bird- and rodent-pollinated *Protea* species the dwarf sugarbushes flowered during the warm summer months when insects are most active.

The involucral bracts of these *Protea* species were diversely colourful and conspicuous and similar to those of bird-pollinated species, but were unlike the cryptic colouring of involucral bracts of rodent-pollinated proteas. No distinct colour changes followed anthesis and receptivity in the study species, although the contrast between dull pink-red perianth lobes and yellow pollen that has subtle UV-reflectance would possibly signal the presence of pollen to floral visitors (Online resource 1). Lamont (1985) showed a highly significant decrease in insect visitation to three species of *Grevillea, Petrophile* and *Isopogon* (Proteaceae) once their flowers had changed colour from yellow to red. There is some evidence that the evolution of inflorescence colour in Proteaceae is driven by pollinators (e.g. *Embothrium coccineum*, Chalcoff et al. 2008). Kalinganire et al. (2001) reported a six-fold increase in bird visitation to *Grevillea robusta* inflorescences that were bright glossy orange-yellow compared to duller colour variants. In contrast, Carlson and Holsinger (2010) suggest that colour polymorphisms (both pink and white colours) in a closely related group of white *Protea* species may be maintained by seed predators, rather than pollinators. Although not measured, there appeared to be no preference of insect visitors for any one particular colour among our study species, ranging from the white-green *P. welwitschii* to the carmine *P. caffra*. Cetoniine beetles pollinate a variety of cryptic coloured plants in South Africa e.g. *Satyrium microrrhynchum,* (Johnson et al. 2007) and various asclepiads ("human cream" is commonly the colour of beetle-pollinated asclepiads, Ollerton et al. (2003), Shuttleworth and Johnson (2009)), suggesting that they do not impose strong selection on flower colour.

The scented *Protea* study species produced large amounts of dilute nectar (Table 1). This contrasts to the smaller volumes typically produced by other Proteaceae with small insect-pollinated flowers, such as some *Leucospermum* and *Diastella* species (Collins and Rebelo 1987; Nicolson and Van Wyk 1998). Flowers of *S. microrrhynchum* which are adapted for pollination by the cetoniine beetle *A. tigrina* (the same insect that pollinates our *Protea* study species) also have highly dilute nectar (7.3-8.6 %) (Johnson et al. 2007). However, higher and more variable (12.5-30.0 %) nectar sugar concentrations were reported for four species of cetoniine-pollinated asclepiads (Ollerton et al. 2003; Shuttleworth and Johnson 2009). In contrast, the asclepiad *Xysmalobium undulatum* which has a bimodal wasp and beetle pollination system has extremely concentrated nectar (72.9%) (Shuttleworth and Johnson 2008). Nicolson and van Wyk (1998) extensively reviewed the nectar characteristics of *Protea* species in relation to other Proteaceae and found xylose to be a conserved nectar trait in this genus. This trend was confirmed by our study (Table 2).

A cetoniine beetle-pollination syndrome in South Africa?

While pollination of unscented flowers by hopliine beetles is common in the western winter rainfall regions of southern Africa (Goldblatt et al. 1998), pollination of fruity-scented flowers by cetoniine beetles appears to be more common in the eastern summer rainfall areas of South Africa (Shuttleworth and Johnson 2010; Steenhuisen et al. 2010). This raises the question of whether unrelated plants pollinated by cetoniine beetles in this region show convergent suites of floral traits that could be identified as a particular pollination syndrome (Faegri and van der Pijl 1979). In general, plants adapted for beetle pollination often share the following floral traits: dull or white coloured perianth; fruity or aminoid scent; flat or bowl-shaped flowers with radial symmetry; and, little or no nectar (Faegri and van der Pijl 1979; Bernhardt 2000; Howe and Westley 1988). A specific beetle "mess and soil" pollination system is well known from tropical regions (Gottsberger 1990; Englund 1993; Bernhardt 2000).

Pollination by cetoniine beetles has been recorded in Proteaceae (this study), various asclepiads (Shuttleworth and Johnson 2009; Ollerton et al. 2003) and orchids

(Johnson et al. 2007). Many of the asclepiads and orchids are also visited by pompilid wasps, suggesting that the two groups of insects use similar cues to locate flowers which have nectar which can be accessed by their short mouthparts. As is the case for our *Protea* study species, cetoniine-pollinated asclepiads have inflorescences which offer a large landing platform. Their inflorescences are often cream in colour to humans (Ollerton et al. 2003), like the gynoecia and parts of the perianth and bracts of our study species, and they also tend to reward chafer pollinators with dilute nectar, although there is considerable variation in this trait. A functional role for the fruity scent of species pollinated by cetoniine beetles is suggested by experiments which show that cetoniine beetles are attracted to the scent of *P. simplex* inflorescences, as well as individual compounds emitted by these inflorescences (S-L. Steenhuisen, unpublished data, chapters 7&8). Other studies have shown electrophysiological responses of the antennae of cetoniine beetles to some of the compounds which impart the fruity scent (Johnson et al. 2007). Cetoniine beetles such as *A. tigrina*, are generalist pollinators that are attracted to a broad spectrum of common floral volatiles, but may develop foraging constancy when blends of volatiles provide specific cues. In general, there is chemical convergence in the scents of cetoniine-pollinated asclepiad species (Shuttleworth and Johnson 2010), orchids (Johnson et al. 2007) and our four *Protea* study species (Steenhuisen et al. 2010). The dominant compound shared by these species is the monoterpenoid linalool, while some of the species share a high proportion of a variety of other monoterpenoids such as myrcene, (E)-ocimene, and α-pinene, and aromatics, particularly benzaldehyde. Available evidence thus supports the idea of convergent evolution in floral traits in species pollinated by cetoniine beetles.

Based on morphological similarities and preliminary observations of animal visitors to inflorescences of Proteaceae in South Africa, Faegri (1965) suggested that there is a "retrograde" development of pollination syndromes from the brush flower type (typical of bird-pollinated species) to the more primitive bowl-shaped one associated with beetle-pollination. Johnson and Briggs (1975) found these ideas untenable on the basis of a comparative study of inflorescence and flower morphology. However, recent phylogenetic data for *Protea* (Valente et al. 2010) revealed that the beetle-pollinated *P. simplex*, *P. welwitschii, P. caffra* and *P. dracomontana* occur in a clade in which bird-pollination is clearly ancestral, thus supporting Faegri's (1965)

speculations. Pollination systems in *Protea* will be investigated further, particularly with regard to our hypothesis that scent is a key functional floral trait that mediates shifts between birds, mammal and insect pollination systems in this genus.

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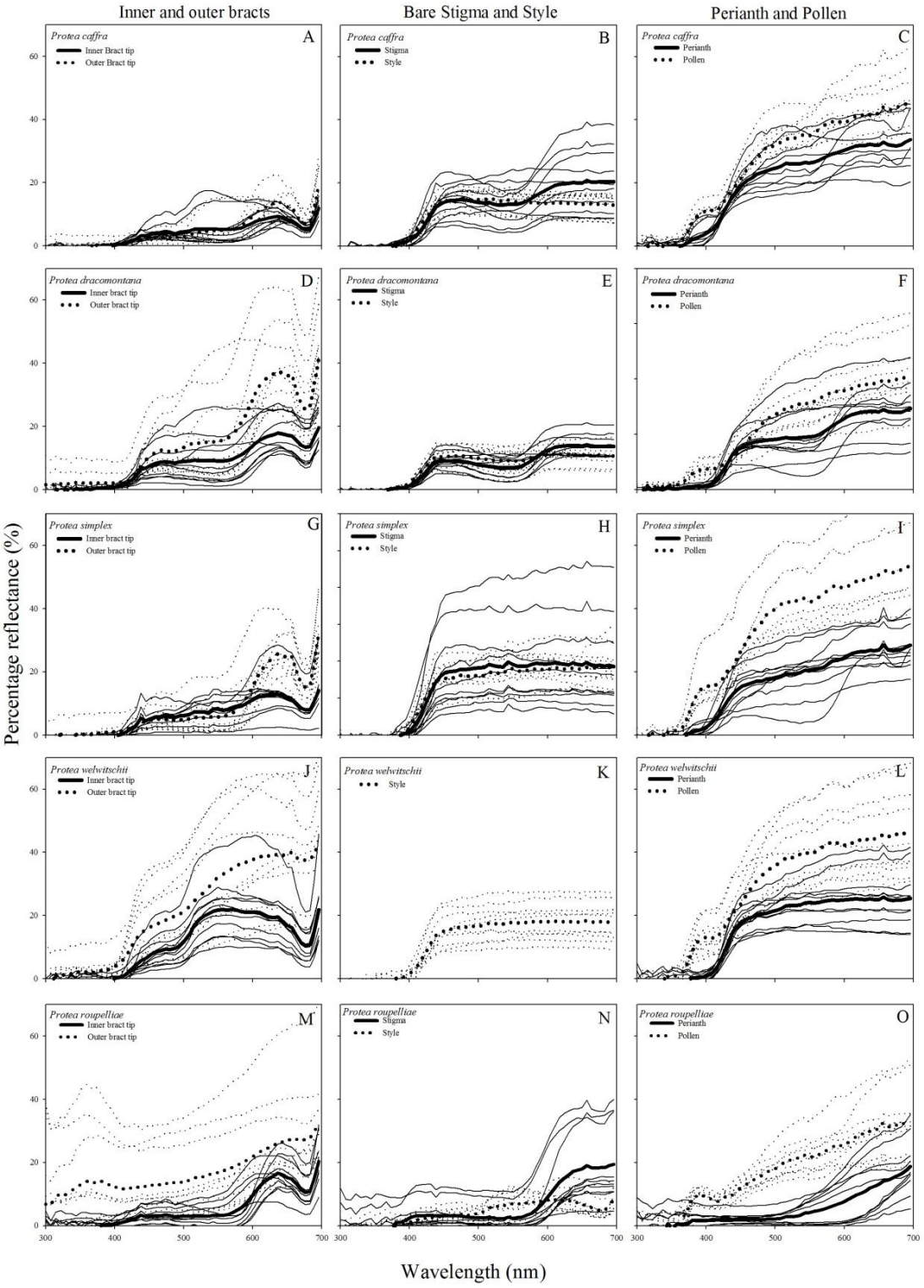
CHAPTER 3

SUPPLEMENTARY MATERIAL



Online resource 1 Comparison of colour spectra measured for six floral parts of five *Protea* species. Dotted and continuous lines represent individual measurements and means of these are shown in bold for each floral part.

Online resource 2 Body lengths, pollen loads, distribution on the body, and purity of pollen loads found on insects collected from inflorescences of five different *Protea* species.

Protea species (location)	Order	Family	Subfamily	Species	Sample size	Body length (mean ± SE mm)	Total *Protea* pollen (mean ± SE number of grains)	Percentage pollen on the upper surface of body (mean ± SE)	Percentage pollen on the lower surface of body (mean ± SE)	Percentage purity (mean ± SE)
Protea caffra (Krantzkloof)	Coleoptera	Curculionidae		sp1	1	5.1	72.0	-	-	96.0
	Coleoptera	Curculionidae		sp2	1	3.3	104.0	-	-	100.0
	Coleoptera	Curculionidae		sp3	2	4.1±0.6	7.5±2.5	-	-	66.7±16.7
	Coleoptera	Curculionidae		sp4	1	7.2	?	-	-	-
	Coleoptera	Dermestidae		*Dermestes maculatus*	2	8.7±0.3	16.5±10.5	-	-	69.6±26.8
	Coleoptera	Eucnemidae		sp1	5	3.4±0.4	13.0±7.4	-	-	51.0±20.9
	Coleoptera	Nitidulidae	Carpophilinae	sp1	2	3.4±0.3	12.0±9.0	-	-	100.0±0.0
	Coleoptera	Nitidulidae	Carpophilinae	sp2	4	2.7±0.2	13.5±6.5	-	-	63.7±10.8
	Coleoptera	Nitidulidae	Epuraeinae	sp3	66	2.8±0.1	15.0±3.4	-	-	66.0±3.8
	Coleoptera	Rhizophagidae		sp1	1	2.4	5.0	-	-	83.3
	Coleoptera	Scarabaeidae	Cetoniinae	*Atrichelaphinis tigrina*	13	12.6±0.2	53.3±10.2	43.5±9.0	56.5±9.0	88.5±4.6
	Coleoptera	Scarabaeidae	Cetoniinae	*Cyrtothrea marginalis*	60	9.6±0.1	144.8±42.6	37.1±6.6	62.9±6.6	91.1±1.5
	Coleoptera	Scarabaeidae	Cetoniinae	*Leucocelis adspersa*	10	10.2±0.3	71.8±40.6	30.0±12.2	70.0±12.2	84.1±5.6
	Coleoptera	Scarabaeidae	Cetoniinae	*Leucocelis haemorrhoidalis*	1	9.5	192.0	42.0	58.0	94.1
	Coleoptera	Scarabaeidae	Cetoniinae	*Leucocelis rubra*	3	13.4±0.2	695.7±687.2	26.7±26.7	73.3±36.7	77.6±11.6
	Coleoptera	Scarabaeidae	Cetoniinae	*Pachnoda sinuata*	4	21.7±0.6	113.5±54.5	67.5±12.7	43.4±9.2	98.8±1.2
	Coleoptera	Scarabaeidae	Cetoniinae	*Porphyronota maculatissima*	1	20.1	123.0	41.3	58.7	91.1
	Coleoptera	Scarabaeidae	Cetoniinae	*Rhabdotis aulica*	1	23.1	13.0	30.0	70.0	61.9
	Coleoptera	Scarabaeidae	Hopliinae	sp5	10	6.0±0.1	35.0±12.6	-	-	81.1±4.8
	Diptera	Calliphoridae		sp1	2	10.5±0.5	473.0±436	7.7	92.3	74.7±22.6
	Diptera	Chloropidae		sp1	4	2.2±0.4	473.8±426.8	-	-	81.9±16.2
	Hymenoptera	Apidae		*Apis mellifera*	25	10.1±0.3	63.8±27.2	43.9±7.6	59.6±7.1	80.2±3.0
	Hymenoptera	Apidae	Xylocopini	sp1	5	7.2±0.7	272.2±232.5	-	-	81.2±16.4
	Hymenoptera	Pompilidae		sp1	1	15.8	20.0	50.0	50.0	76.9
Protea dracomontana (Garden Castle)	Coleoptera	Chrysomelidae		sp2	12	3.8±0.1	26.4±7.1	-	-	87.8±6.1
	Coleoptera	Curculionidae		sp5	1	3.6	81.0	-	-	97.6
	Coleoptera	Elateridae		sp 1	2	6.8±0.6	69.0±66.0	-	-	99.3±0.7
	Coleoptera	Melyridae	Melyrinae	*Melyris*	34	5.3±0.1	123.0±85.4	-	-	94.1±1.9
	Coleoptera	Nitidulidae	Carpophilinae	sp1	1	2.9	8.0	-	-	72.7
	Coleoptera	Nitidulidae	Carpophilinae	sp2	10	2.1±0.1	24.0±7.0	-	-	82.7±6.0
	Coleoptera	Nitidulidae	Epuraeinae	sp3	2	2.7±0.2	73.0±54.0	-	-	92.8±6.4
	Coleoptera	Phalacridae		sp1	5	1.9±0.2	34.2±12.0	-	-	80.0±20.0
	Coleoptera	Scarabaeidae	Cetoniinae	*Atrichelaphinis tigrina*	22	12.2±0.4	189.2±32.6	39.0±5.2	61.0±5.2	98.1±0.7
	Coleoptera	Scarabaeidae	Cetoniinae	*Cyrtothrea marginalis*	1	9.1	11.0	-	-	78.6
	Coleoptera	Scarabaeidae	Cetoniinae	*Trichostetha fascicularis*	8	23.2±0.5	70.4±21.7	28.6±28.6	71.4±28.6	86.2±12.3
	Coleoptera	Scarabaeidae	Hopliinae	sp1	28	6.5±0.1	12.9±1.8	-	-	80.8±4.4
	Coleoptera	Scarabaeidae	Hopliinae	sp2	4	7.0±0.2	12.3±3.2	-	-	98.9±1.1
	Coleoptera	Scirtidae		sp1	1	2.3	11.0	-	-	84.6
	Coleoptera	Scirtidae		sp2	1	2.7	10.0	-	-	100.0
	Coleoptera	Staphylinidae		sp1	7	2.6±0.2	36.0±14.2	-	-	82.4±14.1
	Coleoptera	Tenebrionidae		sp3	1	5.7	7.0	-	-	87.5
	Diptera	Muscidae		sp2	1	6.7	3.0	-	-	100.0
	Diptera	Muscidae		sp3	1	12.0	62.0	-	-	100.0
	Diptera	Muscidae		sp4	2	5.6±0.1	59.0±20.0	-	-	96.3±1.2
	Diptera	Muscidae		sp5	1	3.9	85.0	-	-	96.6
	Hemiptera	Anthocoridae		sp1	1	4.2	1.0	-	-	100.0
	Hemiptera	Lygaeidae		sp1	1	12.2	8.0	100.0	0.0	80.0
	Hymenoptera	Halictidae	Halictinae	sp1	1	6.3	24.0	-	-	96.0

	Hymenoptera	Halictidae	Halictinae	sp3	1	4.7	4.0	-	-	80.0
	Hymenoptera	Apidae		*Apis mellifera*	2	10.2±0.0	13.5±2.5	-	-	100.0±0.0
	Hymenoptera	Sphecidae		sp1	1	15.3	26.0	-	-	92.9
	Hymenoptera	Tiphiidae		sp1	1	8.7	1.0	-	-	100.0
Protea dracomontana (Monk's Cowl)	Coleoptera	Chrysomelidae		sp1	5	5.1±0.2	19.8±11.7	-	-	87.4±7.3
	Coleoptera	Chrysomelidae		sp2	1	4.4	4.0	-	-	57.1
	Coleoptera	Cleridae		sp1	14	4.8±0.1	26.1±4.4	-	-	85.2±2.9
	Coleoptera	Cleridae		sp2	2	6.5±0.7	13.5±7.5	100.0	0.0	83.2±2.5
	Coleoptera	Melyridae	Melyrinae	*Melyris*	16	5.6±0.1	29.9±9.1	-	-	66.3±7.7
	Coleoptera	Nitidulidae	Carpophilinae	sp1	1	2.3	27.0	-	-	93.1
	Coleoptera	Nitidulidae	Carpophilinae	sp2	1	3.2	60.0	-	-	96.8
	Coleoptera	Scarabaeidae	Cetoniinae	*Atrichelaphinis tigrina*	23	12.7±0.2	63.7±12.9	51.7±5.8	48.3±5.8	82.3±2.5
	Coleoptera	Scarabaeidae	Hopliinae	sp4	1	7.1	10.0	-	-	55.6
	Coleoptera	Staphylinidae		sp1	2	2.2±0.3	11.5±1.5	-	-	82.1±10.7
	Diptera	Chloropidae		sp1	2	2.3±0.2	8.5±5.5	-	-	74.3±0.7
	Hemiptera	Lygaeidae		sp1	1	12.4	106.0	-	-	93.0
	Hymenoptera	Halictidae	Halictinae	sp2	2	7.7±0.3	7.0±1.0	-	-	61.4±11.4
	Hymenoptera	Halictidae	Halictinae	sp3	3	6.2±0.2	84.7±68.4	-	-	82.8±8.1
Protea simplex (Mount Gilboa)	Hymenoptera	Apidae		*Apis mellifera*	6	10.6±0.3	>100	41.0±7.0	59.0±7.0	98.0±1.0
	Hymenoptera	Halictidae	Halictinae	sp3	3	5.4±0.1	>1000	-	-	100.0±0.0
	Hymenoptera	Tiphiidae		sp2	1	12.4	92	-	-	100
	Hymenoptera	Braconidae		sp1	1	6.2	241	-	-	100
	Coleoptera	Scarabaeidae	Cetoniinae	*Atrichelaphinis tigrina*	80	12.1±0.1	11198±2802	19.5±2.1	80.5±2.1	89.9±2.2
	Coleoptera	Scarabaeidae	Cetoniinae	*Cyrtothrea marginalis*	5	8.8±0.1	1627.4±718.4	25.2±7.5	74.8±7.5	97.3±1.0
	Coleoptera	Scarabaeidae	Cetoniinae	*Trichostetha fascicularis*	2	23.4±0.6	1448.0±109.0	2.0±1.4	98.0±1.4	99.4±0.1
	Coleoptera	Chrysomelidae		sp5	1	4.2	88	-	-	98.9
	Coleoptera	Chrysomelidae		sp6	12	2.1±0.1	1742.1±989.0	-	-	97.0±2.5
	Coleoptera	Melyridae	Melyrinae	*Melyris*	24	5.0±0.1	4561.5±2112.4	-	-	96.6±2.5
	Coleoptera	Nitidulidae	Carpophilinae	sp2	11	2.0±0.1	17.9±6.1	-	-	80.2±7.8
	Coleoptera	Languriidae		sp1	1	1.7	66	-	-	91.7
	Coleoptera	Staphylinidae		sp1	1	2.2	101	-	-	99
	Coleoptera	Tenebrionidae		sp1	17	6.1±0.1	6222.3±3159.7	-	-	99.8±0.1
	Coleoptera	Tenebrionidae		sp2	1	2.5	7	-	-	100
	Coleoptera	Endomychidae		sp1	1	2.2	89	-	-	91.8
	Coleoptera	Curculionidae		sp7	3	3.6±0.1	641.7±601.2	-	-	93.9±2.8
	Coleoptera	Elateridae		sp1	1	5.7	13	-	-	92.9
	Coleoptera	Meloidae		sp1	1	7.8	40	-	-	97.6
	Diptera	Muscidae		sp1	1	4.7	54	-	-	80.6
	Diptera	Chloropidae		sp1	1	1.6	30	-	-	90.9
	Diptera	Calliphoridae		sp2	2	7.6±0.3	417.0±401.0	-	-	91.8±7.6
	Diptera	Platystomatidae		sp1	3	3.4±0.2	91.7±44.9	-	-	88.3±10.9
	Diptera	Drosophilidae		sp1	2	1.6±0.1	209.5±192.5	-	-	100.0±0.0
	Diptera	Tephritidae		sp1	1	5.4	16	-	-	94.1
Protea welwitschii (Winston Park)	Coleoptera	Carabidae		sp1	1	5.0	89.0	-	-	100.0
	Coleoptera	Chrysomelidae		sp3	2	3.7±0.5	31.0±6.0	-	-	98.7±1.3
	Coleoptera	Chrysomelidae		sp4	1	6.7	52.0	-	-	100.0
	Coleoptera	Curculionidae		sp6	1	5.0	15.0	-	-	100.0
	Coleoptera	Elateridae		sp1	19	6.0±0.1	33.8±9.4	-	-	93.2±2.2
	Coleoptera	Elateridae		sp2	1	10.7	140.0	28.6	71.4	94.6
	Coleoptera	Elateridae		sp3	2	3.6±0.5	10.0±8.0	-	-	89.1±10.9
	Coleoptera	Melyridae	Melyrinae	*Melyris*	3	5.7±0.1	14.7±0.3	-	-	100.0±0.0
	Coleoptera	Nitidulidae	Carpophilinae	sp1	7	2.8±0.1	28.4±9.1	-	-	86.3±8.1
	Coleoptera	Nitidulidae	Epuraeinae	sp3	5	2.3±0.1	31.4±13.1	-	-	87.4±12.2
	Coleoptera	Nitidulidae	Carpophilinae	sp2	7	2.4±0.3	23.1±8.0	-	-	94.6±3.4
	Coleoptera	Rhizophagidae		sp1	2	2.4±0.3	3.0±3.0	-	-	100.0
	Coleoptera	Scarabaeidae	Cetoniinae	*Atrichelaphinis tigrina*	28	11.9±0.1	199.5±42.5	28.2±4.2	71.8±4.2	97.9±0.7
	Coleoptera	Scarabaeidae	Cetoniinae	*Cyrtothrea marginalis*	8	9.7±0.2	137.1±41.9	19.1±5.6	80.9±5.6	72.5±11.8
	Coleoptera	Scarabaeidae	Cetoniinae	*Leucocelis haemorrhoidalis*	33	10.5±0.1	166.4±35.7	36.2±4.2	63.8±4.2	91.2±3.4
	Coleoptera	Scarabaeidae	Dynastinae	*Cyphonistes vallatus*	2	26.1±2.8	83.0±41.0	25.7±4.2	74.3±4.2	93.1±6.9
	Coleoptera	Scarabaeidae	Hopliinae	sp3	2	6.5±0.1	90.7±63.7	50.0±10.0	50.0±10.0	100.0±0.0

Coleoptera	Scarabaeidae	Hoplinae	sp5	1	7.1	38.0	-	-	100.0
Coleoptera	Scirtidae		sp2	1	2.6	11.0	-	-	100.0
Coleoptera	Phalacridae		sp1	1	2.0	390.0	-	-	95.6
Diptera	Syrphidae		sp1	2	9.6±0.4	197.0±88.0	57.1	42.9	94.7±3.9
Hymenoptera	Apidae	*Apis mellifera*		24	10.0±0.1	30.6±4.8	50.0±8.3	50.0±8.3	83.2±4.7
Hymenoptera	Apidae	Xylocopini	sp1	1	6.6	14.0	-	-	100.0
Hymenoptera	Halictidae	Halictinae	sp3	1	4.2	208.0	-	-	99.0
Hymenoptera	Sphecidae		sp2	1	14.8	62.0	68.4	31.6	96.9

CHAPTER 4

THE INFLUENCE OF POLLINATORS AND SEED PREDATION ON SEED PRODUCTION IN DWARF GRASSLAND *PROTEA* "SUGARBUSHES" (PROTEACEAE)

S-L. STEENHUISEN AND S.D. JOHNSON

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Chapter 4

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The influence of pollinators and seed predation on seed production in dwarf grassland *Protea* "sugarbushes" (Proteaceae)

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Abstract

Flowers of many plant species are visited by both birds and insects, making it necessary to establish their relative contributions to seed set. In *Protea*, available evidence points to an overwhelming preponderance of bird-pollination systems in the genus, but the scented flowers of several dwarf grassland "sugarbush" species suggest that some *Protea* species may be adapted for insect pollination. In this study, we used both selective exclusion of vertebrates and complete exclusion of all visitors to investigate whether the insects that visit the scented flowerheads of three *Protea* species (*Protea dracomontana*, *Protea simplex* and *Protea welwitschii*) in KwaZulu-Natal, South Africa contribute to seed production. We also performed supplemental hand pollinations to test for pollen limitation. Seed set was generally higher in inflorescences subjected to vertebrate exclusion than in those from which all visitors were excluded, suggesting that fertile cross-pollen was deposited by insects, but these differences were slight because of high levels of self-fertilization in the study species. Pollen deposition and pollen tube growth were similar for vertebrate-excluded and open-pollinated inflorescences. Supplemental hand-pollination treatments revealed that seed set in *P. simplex* and *P. welwitschii* was not pollen-limited. Overall seed set was low, typical of the family Proteaceae, and infructescences were highly predated by lepidopteran larvae. We conclude that insects are likely to contribute to seed set of the study species, but further studies using molecular markers are required to establish the actual level of insect-mediated outcrossing.

Keywords: Pollination; *Protea*; Resource limitation; Seed predation; Southern Africa

1. Introduction

The large Gondwanan plant family Proteaceae shows considerable diversity in reproductive systems (Ayre and Whelan, 1989; Collins and Rebelo, 1987; Goldingay and Carthew, 1998). Among Australian genera, bird- and mammal-pollination dominates in *Banksia* and *Grevillea* (Collins and Rebelo, 1987), while insect pollination has been recorded in *Banksia, Dryandra, Grevillea, Hakea, Macadamia* and *Persoonia* (Blanche et al., 2006; Carolin, 1961; Lamont, 1982; Lamont and Collins, 1988; Lamont et al., 1998; Wallace et al., 1996). Among the African genera, bird pollination dominates in *Leucospermum, Mimetes* and *Protea* (Faegri, 1965; Hargreaves et al., 2004; Mostert et al., 1980; Rebelo, 2001) although rodent pollination has been recorded for a few geoflorous *Protea* species (e.g. *Protea amplexicaulis* and *Protea humiflora*) (e.g. Wiens and Rourke, 1978), and insect and wind pollination is inferred for most *Leucadendron* species (Collins and Rebelo, 1987) and other genera. In *Protea*, the distribution and guild composition of insects inhabiting inflorescences have been documented over many years, mainly in the context of the marketability of cut *Protea* flowers (Coetzee and Latsky, 1986; Gess, 1968; Myburgh and Rust, 1975; Myburgh et al., 1973; Wright and Giliomee, 1990; Wright and Samways, 2000). For most *Protea* species, there is still considerable uncertainty whether the insects that frequent inflorescences contribute to seed production (Coetzee and Giliomee, 1985; Collins and Rebelo, 1987).

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Exclusion experiments, in which avian visitors were prevented from visiting inflorescences covered with wire mesh cages, have been conducted to ascertain the contribution of bird versus insect pollinators in a few *Protea* species. Caged inflorescences of *Protea laurifolia, Protea magnifica, Protea neriifolia* and *Protea roupelliae* set significantly less seed than open-pollinated treatments, supporting the idea that these species are predominantly bird-pollinated (Hargreaves et al., 2004; Wright et al., 1991). In contrast, caged and open-pollinated inflorescences of *Protea cynaroides*, *Protea nitida* and *Protea repens* set similar amount of seed (Coetzee and Giliomee, 1985; Wright et al., 1991). For five of these seven species, a treatment excluding all visitors was not included and it is thus unclear whether high seed set in vertebrate-excluded inflorescences was due to insect pollination or to autonomous self-fertilization. It is thus essential to establish the breeding system of each species in order to determine self-compatibility and, if so, whether they can set seed autonomously (without the use of pollen vectors). At least some *Protea* species are self-compatible, viz. *P. repens* (Van der Walt, 1995) and *P. roupelliae* (Hargreaves et al., 2004), and we have recently documented self-compatibility and autogamy in four grassland *Protea* species (unpublished results). Nevertheless, autogamy is most often facultative rather than obligate, and pollinators may therefore contribute to seed production in these *Protea* species.

While insects have been shown to affect pollination in ornithophilous *Protea* species, their contribution to pollination of *Protea* species with floral traits suggestive of insect-pollination has not been investigated. We excluded vertebrate visitors from three putatively insect-pollinated grassland *Protea* species (*Protea dracomontana, Protea simplex,* and *Protea welwitschii*) to investigate the contribution of insect visitors to seed set. These three species have floral traits similar to bird-pollinated *Protea* species, such as colorful involucral bracts and abundant nectar rewards, but they also have floral traits conforming to a beetle-pollination syndrome, notably a strong, fruity floral scent (Steenhuisen et al., 2010), smaller and more bowl-shaped inflorescences, immense pollen rewards and low plant growth form (Steenhuisen and Johnson, in press). The inflorescences of these species are visited by a variety of generalist insects, but most frequently by cetoniine beetles foraging on both nectar and pollen (Steenhuisen and Johnson, in press). Despite being self-compatible and partially autogamous (unpublished results) these species are characterized by low seed set (<40% florets set seed). We used supplemental hand-pollination (Bierzychudek, 1981) to test whether seed set in the study species was limited by either pollen or resource availability (Ayre and Whelan, 1989). We also quantified the rate of seed predation and identified insect seed predators (e.g. Myburgh et al., 1973).

2. Materials and methods

2.1. Study species

The role of insect pollinators in seed production in three grassland *Protea* species was investigated in KwaZulu-Natal, South Africa. A population of approximately 550 *P. simplex* E.Phillips ex J.M.Wood plants, located on the grassland slopes of the summit of Mount Gilboa (29.29°S, 30.29°E, 1770 m), KwaZulu-Natal, was used for experiments conducted in January 2002 and 2005. A population of about 300 plants of *P. welwitschii* Engl., located on steep grassland slopes of a residential area in Winston Park (SE-facing slope in 2003, NW-facing slope (Giba Gorge) in 2004) (28.75°S, 30.75°E, 550 m), was used in 2003 and 2005. A large population (approximately 500 plants) of *P. dracomontana* Beard on the lower slopes of Garden Castle (29.74°S, 29.20°E, 1900 m) in the Drakensberg mountains was used for this study in 2007. All three species have a flowering period ranging from December to March with a January peak. These sites receive summer rainfall. Voucher specimens have been deposited in the University of KwaZulu-Natal Herbarium (voucher numbers: Steenhuisen 55, 57, 59, 60, 62). The species were identified according to Rourke (1980).

2.2. Experimental design

To investigate the pollination effectiveness of beetles and other invertebrates, exclusion experiments were performed on the three *Protea* study species. Wire mesh cages painted green to reduce their conspicuousness were erected over single inflorescences to exclude vertebrate visitors to the flowers. Treatments applied to inflorescences consisted of (1) enclosure in a small diameter (15 mm) mesh cage that excludes vertebrates and larger insects, (2) enclosure in a larger diameter (30 mm) mesh cage that excludes vertebrates while allowing most insects to pass through, (3) enclosure in a fine mesh bag that excludes all visitors and thereby tests for autogamous seed production, (4) open inflorescences as a control to assess natural seed set, and (5) supplemental hand-pollination to test for pollen limitation. A wire support in (3) kept the bag from extensively touching the inflorescence and its pollen presenters. Inflorescences in (4) were cross-pollinated at least twice by brushing five or more freshly exposed pollen presenters from another inflorescence of a different plant over the stigmatic grooves of all florets of each inflorescence during its receptive stage, determined by previous observations (unpublished results). Bags and cages were applied at the bud stage before the inflorescences opened. From occasional observations of insects visiting caged inflorescences, although this was not formally quantified, it became apparent that similar-sized insects were found to visit inflorescences covered by different cage mesh sizes (e.g. Cetoniinae ranging from 10 to 25 mm in length; honeybees; flies; various Lepidoptera). We thus pooled data for all caged inflorescences. All treatments were applied to inflorescences on the same plant whenever possible or on adjacent plants in order to control for local habitat effects. In all experiments, 20–24 inflorescences were used for each treatment and corresponding open-pollinated controls. No pollen supplementation treatment was performed on *P. dracomontana*.

To investigate pollen deposition by insects on stigmas of *P. welwitschii*, five stigmas from each open, caged and pollen-supplemented inflorescence were collected from experimental plants in 2004 once the inflorescence bracts had withered and the anthers senesced. We did not collect stigmas from bagged

plants for this experiment as self-pollen coats pollen presenters and stigmas of these species and would not have been removed by pollinators or wind/rain. The stigma tips were squashed fresh in fuschin gel, which was melted to form permanent slides (Beattie, 1971) and the number of foreign (distinguishable from *Protea* pollen in shape and texture) and *Protea* pollen grains (using the unique smooth-edged triangular shape of *Protea* pollen as a reference) deposited on each stigma determined. A further five stigmas from each experimental inflorescence (including bagged ones) in *P. simplex* (2002) and *P. welwitschii* (2003/2004) were collected to determine pollen tube growth in the style. Each stigma was fixed in 2 ml 3:1 70% ethanol: acetic acid for 1 h, washed with distilled water, and stored in 2 ml 70% ethanol. Fixed stigmas were prepared for pollen tube analysis using a softening and staining procedure modified from Martin (1959), allowing for the examination of pollen tubes in the style through aniline blue UV-induced fluorescence of callose associated with the pollen tube wall. The stigmas were rinsed in distilled water for 10 min, softened and cleared by suspending them in 4 N NaOH for 48 h, rinsed in tap water for 1 h, and stained with aniline blue-0.1 N K_2HPO_4 for 4 h. The stained stigmas were stored in glycerin for no longer than 3 days before examination. Stigmas were mounted on slides in a drop of stain and glycerin, and flattened with a coverslip. The proportion of styles with pollen tubes in the upper first centimeter and the number of pollen tubes per stigma for each treatment were determined by examining the stigmas with an Olympus Provis, AX-70 equipped with a UV filter system consisting of a dichroic mirror (400 nm), an ultraviolet excitation filter (330–385 nm) and a barrier filter (420 nm). In all analyses, insect-damaged stigmas and the rare case of pollen/pollen tube loads of over 1000 grains were excluded from analyses.

Infructescences were collected 4 months after each flowering season, and the proportion of florets that set seed was determined for each treatment. Plump ovaries were treated as containing fertile seeds. Damaged infructescences were assessed for evidence of predation (dried frass, emergence holes in the base, damaged styles, eaten seeds) and excluded from analyses. The percentage of infructescences damaged by insect predators (partially or completely), lost to uncontrolled fires through the study sites or premature release of seeds was determined. Lepidopteran larvae found in damaged infructescences were collected into glass vials, and allowed to pupate and metamorphose. Emerged adults were identified by Dr M. Krüger (Transvaal Museum), and voucher specimens stored in the entomological collection at the University of KwaZulu-Natal.

2.3. Statistical analyses

We analyzed the effects of treatment on the number of pollen grains per stigma, proportion of stigmas that received pollen, proportion of deposited pollen that was *Protea* pollen, proportion of stigmas with pollen tubes in the upper style, the number of pollen tubes per style, and the proportion of florets that set seed using generalized linear models (GZLMs). Unless otherwise stated we used likelihood ratio Chi-square statistics, logit link functions, binomial error distributions corrected for overdispersion where appropriate, and compared treatments using pairwise contrasts with sequential sidak adjustment for multiple comparisons (Hosmer and Lemeshow, 2000). Type I models were used to account for the effect of year before treatment on the proportion of styles with pollen tubes, the number of pollen tubes per style (2003 and 2004), and seed set (2003 and 2005) for *P. welwitschii*. Treatment effects on the number of pollen grains deposited on stigmas and pollen tubes growing in styles were tested using means per inflorescence rounded to the nearest integer and fitted to models with a Poisson error distribution and log link functions.

3. Results

Very pure pollen loads (>90% *Protea* pollen) were deposited on all stigmas of unbagged *P. welwitschii* experimental inflorescences (Table 1). Stigmas of caged inflorescences received slightly lower pollen loads than open controls, while pollen supplementation increased pollen loads by about 60% (Table 1). Over 80% of *P. welwitschii* stigmas received pollen in all treatments. A small percentage (0–7%) of pollen loads found on *P. welwitschii* stigmas was comprised of pollen from up to eight other flowering plant species (see pollen purity measures in Table 1).

Seed set for all species was very low, never reaching above 40%, despite evidence of prolific pollen tube growth on most stigmas in pollen supplemented inflorescences in *P. simplex* and *P. welwitschii* (Figs. 1 and 2). For *P. simplex*, we observed lower proportions of stigmas with pollen tubes (2002, treatment: $\chi^2_{(3)}=23.302$, $P<0.001$) and lower numbers of pollen tubes per stigma (2002, treatment: $\chi^2_{(3)}=139.631$, $P<0.001$) for bagged, caged and open-pollinated inflorescences compared to pollen supplemented inflorescences (Fig. 1a,c). This pattern was not reflected by seed set for which we recorded higher seed set for caged and pollen supplemented inflorescences compared to open-pollinated and bagged inflorescences (year: $\chi^2_{(1)}=15.167$, $P<0.001$; treatment: $\chi^2_{(3)}=17.579$, $P=0.001$; Fig. 2a).

Pollen supplementation increased the proportion of stigmas with pollen tubes in *P. welwitschii* (2003–2004, year:

Table 1
The effect of natural pollination, caging and pollen supplementation on stigmatic pollen loads for *Protea welwitschii* in 2004.

		Treatment (sample size)			$\chi^2_{(2)}$
		Open control (57)	Cage (40)	Pollen supplemented (20)	
Pollen load per stigma	Mean	12.1[a]	10.5[a]	78.6[b]	184.914*
	Lower SE	1.5	1.7	6.8	
	Upper SE	1.8	2.0	7.4	
Proportion of sampled stigmas with *Protea* pollen	Mean	0.86[a]	0.83[a]	0.99[b]	22.258*
	Lower SE	0.02	0.03	0.02	
	Upper SE	0.02	0.02	0.01	
Protea proportion of pollen load	Mean	0.93[a]	0.96[a]	1.00[b]	45.082*
	Lower SE	0.01	0.02	0.003	
	Upper SE	0.01	0.01	0.002	

Significant differences between treatments are indicated by different letters next to the means (significance level: *$P<0.001$).

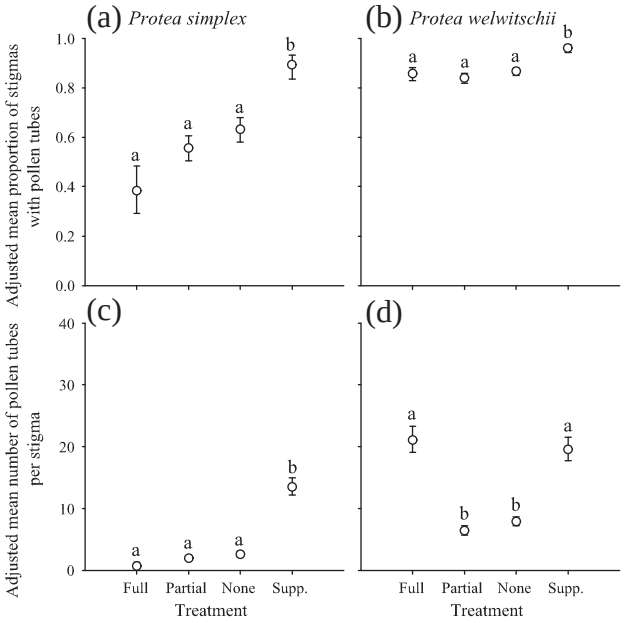

Fig. 1. The effect of "full" exclusion of pollinators by bagging, "partial" exclusion of pollinators by caging (allowing access to invertebrates only), natural ("none") and supplemental hand-pollination ("supp.") on the (a–b) proportion of stigmas with pollen tubes growing in the style, and (c–d) the pollen tube loads on stigmas for *P. simplex* (Mount Gilboa 2002) and *P. welwitschii* (Winston Park 2003 and 2004). Different letters indicate statistically significant differences between treatments ($P<0.05$).

$\chi^2_{(1)}=9.389$, $P=0.002$; treatment: $\chi^2_{(3)}=20.207$, $P<0.001$), and along with bagging, more than doubled the number of pollen tubes found on open or caged stigmas (2003–2004, year: $\chi^2_{(1)}=32.891$, $P<0.001$; treatment: $\chi^2_{(3)}=104.239$, $P<0.001$); Fig. 1b,d).

Seed set in *P. welwitschii* was very low (<15%), and with the exception of slightly higher seed set after pollen supplementation compared to caged inflorescences, seed set was similar for all other pairwise comparisons of treatments (year: $\chi^2_{(1)}=14.621$, $P<0.001$; treatment: $\chi^2_{(3)}=12.221$, $P=0.007$; Fig. 2b).

Seed set in *P. dracomontana* did not differ significantly between bagged, caged and open-pollinated treatments ($\chi^2_{(2)}=0.908$, $P=0.635$; Fig. 2c).

Insect predator species typically laid their eggs on the base of the inflorescence buds and the hatched larvae bored through the involucral bracts and devoured the receptacle, ovaries, or maturing seeds in the inflorescence. Lepidopteran larvae (Lycaenidae, Tortricidae) were solely responsible for seed and receptacle predation, while other lepidoteran larvae (Psychidae) and very large Coleoptera (Dynastinae, Cetoniinae, Scarabaeinae) predated on floral parts above the involucral bracts, damaging inflorescences and preventing them from setting seed. From pupation and subsequent emergence of lepidopteran predators from larvae found in experimental inflorescences, we determined that the majority of predation was due to the moth *Cydia* sp. cf. *ocnogramma* (Meyrick, 1910) (Tortricidae: Olethreutinae: Grapholitini). Minor predators were the orange banded protea butterfly, *Capys alpheus* (Cramer, 1777) (Lycaenidae: Theclinae: Deudorigini), and the small moth, *Epichorista* sp. cf. *galeata* Meyrick, 1921 (Tortricidae: Tortricinae: Archipini). Of all the larvae collected from experimental inflorescences (48 individuals) from Mt Gilboa, 50.0% were determined to be *C.* sp. cf. *ocnogramma*, 4.2% *E.* sp. cf. *galeata*, and the remaining 45.8% failed to metamorphose and were not identified. Very few infructescences opened prematurely or released dry, withered and aborted fruits without visible evidence of predation (Table 2). Missing infructescences were usually from the exclusion experiments and could be attributed to damage from caging and wires supporting them. Those missing from experimental plants of *P. dracomontana* were mostly due to removal by baboons.

4. Discussion

This study provides limited support for the hypothesis of insect pollination in *P. dracomontana, P. simplex* and *P. welwitschii*. Exclusion of vertebrates had little effect on the number of pollen tubes or seed set in all three species (Table 1; Figs. 1 and 2). With the exception of *P. dracomontana,* inflorescences from

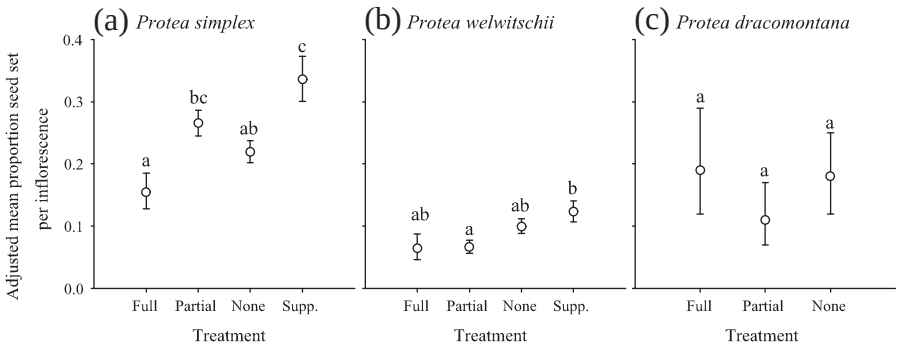

Fig. 2. The effect of "full" exclusion of pollinators by bagging, "partial" exclusion of pollinators by caging (allowing access to invertebrates only), natural ("none") and supplemental hand-pollination ("supp.") on the adjusted mean proportion of florets to set seed per inflorescence for (a) *P. simplex* (Mount Gilboa 2002 and 2005 combined), (b) *P. welwitschii* (Winston Park 2003 and Giba Gorge 2005 combined), and (c) *P. dracomontana* (Garden Castle 2007). Different letters indicate statistically significant differences between treatments ($P<0.05$).

Table 2
The proportion of experimental inflorescences lost to predation and other environmental factors for three *Protea* species spanning various years and sites.

Species	Undamaged (%)	Partially eaten (%)	Completely eaten (%)	Dry (%)	Burnt (%)	Missing (%)	Released seeds prematurely (%)	Total no. of inflorescences sampled
Protea simplex Gilboa 2002	58.33	27.27	12.12	2.27	0.00	0.00	0.00	132
Protea simplex Gilboa 2005	50.31	20.13	22.64	0.00	0.00	6.92	0.00	159
Protea welwitschii Winston Park 2003	25.42	41.53	32.20	0.85	0.00	0.00	0.00	118
Protea welwitschii Winston Park 2005	42.68	8.54	0.00	0.00	44.51	0.00	4.27	164
Protea dracomontana Garden Castle 2007	15.05	23.66	51.61	0.00	0.00	9.68	0.00	93

which vertebrates but not insects were excluded generally had slightly higher seed set than inflorescences bagged to exclude insects as well, indicating that insects transfer viable cross-pollen. These results do not, however, provide unambiguous evidence for the importance of insect pollination because there was also substantial autogamous seed production in bagged inflorescences. Supplemental pollination had little effect on natural seed set in two species, suggesting that seed production in the study species was not pollen-limited.

A more precise method for investigating the contribution of different pollinators in autogamous species is to use emasculated flowers, so that seed set reflects only transfer of cross-pollen. This was not feasible in inflorescences of these *Protea* species due to the gradual maturity of florets within an inflorescence and the immense amount of self-pollen covering pollen presenters and stigmas. Whelan et al. (2009) successfully washed self-pollen off pollen presenters of *Grevillea macleayana* to measure pollen deposition by pollinators. They were able to show that despite the high frequency of visits by honeybees to this species, that they deposited fewer pollen grains than birds. The presence of foreign pollen on stigmas of caged *P. welwitschii* inflorescences indicated that insects were transferring pollen from other flowering species in the community.

Seed set in autogamous plants is less likely to be pollen-limited than in allogamous plants but pollen-limitation is known to occur in some species in which autogamy is not efficient enough to result in all ovules setting seed (Rodger et al., 2010). In such plants, which are typically facultatively autogamous, pollinators can make significant contributions to seed production. In our study, inflorescences of *P. simplex* that were supplemented with pollen produced more seeds than those that were bagged but this was not evident in *P. welwitschii*, suggesting that autogamy in *P. simplex* does not reach the threshold that is physiologically possible.

Previous studies in the Proteaceae have identified a range of factors limiting seed production (Ayre and Whelan, 1989; Vaughton, 1991; Whelan and Denham, 2009). In some species, seed set can be increased with the addition of nutrients (e.g. *Banksia cunninghamii*; Vaughton, 1991), and Vaughton and Ramsey (1998) increased seed mass, but not seed set, from a redistribution of plant resources by removing inflorescences from *Banksia marginata.* Resource limitation was proposed as the major constraint on fruit and seed set in several *Grevillea* species (Hermanutz et al., 1998; Holmes et al., 2008). The lack of a significant increase in seed set following pollen supplementation in *P. simplex* and *P. welwitschii* suggests that seed set in these species is also limited by resources rather than pollen availability. A similar lack of response in seed set to pollen supplementation was reported in the bird-pollinated grassland species *P. roupelliae* (Hargreaves et al., 2004). In contrast, the effect of pollinator limitation was clearly demonstrated by extremely low natural fruit set in *Protea subvestita* studied by Carlson and Holsinger (only 18% of naturally pollinated infructescences investigated contained seed, 2010). Seed set may also vary from year to year (Vaughton, 1991) and pollen supplementation may affect seed set in subsequent flowering seasons by draining the plant's resources (Janzen et al., 1980). However, pollen supplementation failed to significantly increase seed set in *P. welwitschii* in two separate seasons (2003 and 2005).

The high levels of seed predation recorded in this study are typical for Proteaceae (Mustart et al., 1995; Wright, 1994; Wright and Samways, 2000). Insect predators halved seed set per plant in *Banksia spinulosa* var. *neoanglica* (Vaughton, 1990), and reduced seed set to zero for some Cape *Protea* species (Carlson and Holsinger, 2010). Coetzee and Giliomee (1987) found that more than 80% seeds of *P. repens* were destroyed by insect predators within 2 years after flowering. Like Wright and Samways (2000), we found that the major predators of seeds in *Protea* infructescences in KwaZulu-Natal were *Cydia* moths and other Olethreutinae species (Tortricidae), which are economically important pest species in South Africa (Timm et al., 2010). Other predators that we recorded included *C. alpheus* (Lycaenidae) and species of Curculionidae, Scarabaeidae and Psyllidae. Seed predators are undoubtedly a factor limiting seed production but their effect is hard to quantify. If we had applied insecticide (as done in Vaughton (1990) and Zammit and Hood's (1986) *Banksia* studies), then cross pollination by insects would have been compromised. We did attempt application of insecticide after pollination in some trials, but most lepidopteran predators had already laid eggs at the bud stage (mesh bags did not exclude predators from laying eggs on experimental inflorescences), and there was little effect on subsequent predation. Similarly, weevils laid eggs before experiments could commence on *Banksia grandis*, leading to predation of 79% of infructescences (Abbott, 1985). Wallace and O'Dowd (1989) were also able to increase seed set in *B. spinulosa* after applying insecticide to plants, but this increase was significant only with the addition of nutrients. However, as pointed out by Ayre and Whelan (1989), none of these manipulations (pollen-supplementation, resource addition, removal of seed predators) result in fruit:flower ratios near unity, indicating that there are other factors limiting seed set in this plant family.

Due to confounding factors of low seed set, self-compatibility and high seed predation, our exclusion experiments provided

limited evidence for the contribution of insect pollinators to seed production. One solution to this problem would be to compare outcrossing rates for seeds derived from bagged, vertebrate-excluded and open-pollinated inflorescences. Preliminary results from studies using multilocus outcrossing rates in *Protea caffra*, a grassland species with scented flowers, indicate that there is substantial outcrossing in seed derived from inflorescences from which vertebrates were excluded, lending support to the hypothesis of insect-pollination in this clade of *Protea*. This study underlines the problems of using seed production alone to estimate the contributions of different pollinators to seed production in self-fertilizing and resource-limited plant species.

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CHAPTER 5

THE CONTRIBUTIONS OF INSECT VERSUS BIRD FLORAL VISITORS TO OUTCROSSING IN AN AFRICAN *PROTEA* (PROTEACEAE)

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ABSTRACT

Premise of the study: A useful, but seldom applied, measure of the effectiveness of different pollinators is their contribution to the rate of outcrossing. This measure is particularly useful in facultatively autogamous plants for which seed set cannot be used as a direct measure of pollinator effectiveness. We used selective exclusion experiments to assess the importance of insects for outcrossing in *Protea caffra,* a facultatively autogamous shrub with scented flowers that are visited frequently by both birds and insects (mainly beetles).

Methods and results: Pollen loads on stigmas, pollen tube growth, seed set, seed mass, germination and early seedling survivorship were similar for vertebrate-excluded and open-pollinated inflorescences. Pollen-supplementation mostly did not increase seed set, revealing resource limitation. Mean multilocus outcrossing rates, estimated using eight polymorphic allozyme loci, were similar for progeny from inflorescences excluded from bird visitors (0.65) and for those visited by both birds and insects (0.59). Wright's fixation indices indicated that the adult population is near Hardy-Weinberg equilibrium but differed markedly for maternal plants (F_{IS} = –0.187) and their early stage progeny (F_{IS} = 0.258). Since seed from self and cross hand pollinations was equally viable in terms of germination, this discrepancy in F_{IS} could be explained by inbreeding depression that occurs between germination and reproductive maturity.

Conclusions: Since outcrossing rates were not reduced when birds were excluded, we infer that insects are effective agents of cross-pollination in *P. caffra*. This helps to explain the evolution of traits associated with insect-pollination, such as fruity floral scent, in this species.

Keywords: allozyme analysis; breeding system; cetoniine beetle pollination; inbreeding depression; mating system; self-compatibility

INTRODUCTION

Floral evolution in animal-pollinated plants typically results from selection imposed by their most effective and abundant pollinators, but they are usually also visited by many other animals that are of less importance for pollination (Johnson and Steiner, 2000; Fenster et al., 2004). A narrow range of important pollinators among a broad range of flower visitors can explain Ollerton's (1996) paradox of high levels of floral specialization in plants that are apparently ecologically generalized in their pollination systems. A classical example of this problem occurs in plants that show evolutionary specialization for bird-pollination, yet are also visited by insects. In such species, experimental exclusion of birds often results in substantially lowered seed production, indicating that birds are the most effective pollinators (e.g. Hargreaves et al., 2004; Botes et al., 2009). In response to variation in abundance and distribution of flower-visiting animals over plant geographical ranges, there have been frequent shifts between pollinators in various plant clades (Johnson, 2006; Campbell, 2008; van der Niet and Johnson, 2012). According to this pollinator-shift or ―Grant-Stebbins" model (Grant, 1949; Stebbins, 1970, 1973, 1981), the immense diversification of floral form in angiosperms is considered a consequence of adaptations to different pollinators with different morphologies and sensory abilities. Determining the most effective pollinator for a plant species therefore adds to our understanding of the evolution of floral traits associated with pollinator shifts.

Using seed set following experimental exclusion of pollinators to estimate pollinator effectiveness does not work well in species that are facultatively autogamous as seed set in these species occurs even in the absence of all pollinator visits. Self-pollination in such species can be prevented by emasculation, but this approach does not work when pollinators are attracted to pollen rewards or when emasculations are difficult to implement. An alternative measure of the effectiveness of different pollinators is their contribution to the rate of outcrossing. Multilocus estimates of outcrossing rates in plants can be estimated efficiently using co-dominant markers such as allozymes or microsatellites (e.g. Brown et al., 1989). Allozymes are still an ideal method for mating system studies as outcrossing rates in progeny can be estimated with a high degree of confidence from allelic variation at a small number of loci (May, 1998). In addition, studies of allozyme variation are simpler, cheaper, and faster to implement than those using microsatellite markers. Despite the ready availability of these methods, very few studies have attempted to partition the contributions of different pollinators to outcrossing rates. Brunet and Sweet (2006) showed that higher outcrossing

rates in *Aquilegia coerulea* James (Ranunculaceae) were associated with increased abundance of hawkmoth pollinators, whereas bees and flies did not affect outcrossing rates. Schmidt-Adam et al. (2009) found that open-pollinated plants of *Metrosideros excelsa* Gaertn. (Myrtaceae) had higher seed set and outcrossing rates than those from which vertebrates, but not bees, had been excluded, thus leading them to conclude that this species is predominantly pollinated by birds. Kudo et al. (2011) even used outcrossing rates to test differences in pollinator efficiency within a single pollinator group and found that differences in the foraging behaviour of early-emerging queens versus late-season foraging worker bumblebees accounted for seasonal fluctuations in mating patterns in *Rhododendron aureum* Georgi (Ericaceae).

The large African genus *Protea* L. (Proteaceae) exhibits pronounced variation in floral traits and breeding systems (Collins and Rebelo, 1987; Rebelo, 2001; Carlson et al., 2011). Most of the species are considered to be either bird- or rodent-pollinated and this has been supported by reduced seed set following experimental exclusion of vertebrates (Wiens and Rourke, 1978; Wiens et al., 1983; Wright et al., 1991; Hargreaves et al., 2004). However, it has been suggested that a small clade of grassland *Protea* species with fruity-scented, bowl-shaped inflorescences that produce copious pollen and nectar rewards are primarily insect-pollinated (Steenhuisen and Johnson, 2012a). Although these species are visited by both birds and insects, it has been suggested that they are pollinated mainly by fruit chafer beetles (Scarabaeidae: Cetoniinae) that carry large *Protea* pollen loads and are attracted to the papaya-like scent of the inflorescences (Steenhuisen et al., 2010; Steenhuisen and Johnson, 2012a; unpubl. data). These grassland *Protea* species are, however, also facultatively autogamous (Steenhuisen and Johnson, 2012b), making seed set an unreliable measure of the contribution of various pollinators to fitness. In this study, we use selective exclusion experiments coupled with estimates of outcrossing in the resultant progeny to assess the importance of insects for outcrossing in *Protea caffra* Meisn., a facultatively autogamous member of the grassland clade which has inflorescences that are visited regularly by both birds and insects (Steenhuisen and Johnson, 2012a&b).

Although cetoniine beetles are frequent visitors and carry large loads of *P. caffra* pollen (Steenhuisen and Johnson, 2012a), they also spend long periods of time within inflorescences, potentially increasing the likelihood of self-pollination within (autogamy) and between (geitonogamy) florets. Bird pollinators are expected to be better cross-pollinators than insects, as they disperse pollen longer distances between plants and have shorter within-plant foraging bouts (Mostert et al., 1980; Collins and Rebelo, 1987; Kalinganire et al., 2001;

Castellanos et al., 2003; Llorens et al.; 2012). In *Grevillea macleayana* (McGill.) Olde & Marriott (Proteaceae), for instance, birds were better outcrossers than bees, presumably because the latter seldom moved between plants (Whelan et al., 2009). However, in a study of *Grevillea beadleana* McGill., Smith and Gross (2002) found that birds made more within- than between-plant visits. The relative contributions of birds and insects to outcrossing are thus likely to be a result of their relative abundance, behaviour, and morphological suitability as agents of pollen transfer. Because flowers of *P. caffra* have traits such as a fruity scent that are associated with selection by beetles, we hypothesized that beetles are effective agents of cross-pollination in this species.

To test the hypothesis of effective insect pollination in *P. caffra* we carried out a series of experiments in which either vertebrates or all pollinators were excluded from flowerheads. We predicted that experimental exclusion of vertebrates would not affect pollen receipt, pollen tube growth, seed production and outcrossing rates, and that, on account of autogamy, exclusion of all pollinators would lead to only marginally lower levels of pollen receipt, pollen tube growth, and seed production, while outcrossing should drop to zero. To interpret whether seed set in *P. caffra* was naturally pollen-limited, we also performed supplemental hand-pollinations.

MATERIALS AND METHODS

The study species and study sites—*Protea caffra* is a common summer-rainfall sugarbush inhabiting upland grassland habitats in the eastern half of South Africa (Rebelo, 2001). Its growth form varies from dwarf shrubs in frequently burnt grasslands to trees of 2-5 m in height. Like other grassland *Protea* species, it produces large bowl-shaped, colourful inflorescences with copious nectar and pollen rewards (Steenhuisen and Johnson 2012a). Each inflorescence has c. 150 florets, each with over 80 000 pollen grains and c. 8 μL of dilute nectar. The fruity floral scent is very strong and has been shown to be attractive to cetoniine beetles (S-L. Steenhuisen, unpubl. data, chapter 7). A population of about 300 plants of *P. caffra* was used in December 2004, located on steep East-facing grassland slopes of a deep gorge consisting of scarp forest in the Krantzkloof Nature Reserve (29.77°S, 30.84°E, 450 m). A second population of approximately 220 *P. caffra* plants, located on the Northeast-facing grassland slopes of the summit of Mount Gilboa (29.29°S, 30.29°E, 1770 m), KwaZulu-Natal, was used in January 2005 and 2008. A third population of about 50

plants of *P. caffra* was studied on a hilltop slope of Bulwer Mountain (29.75°S, 29.75°E, 1900 m) in January 2005. A voucher specimen has been deposited in the Bews Herbarium (NU) at the University of KwaZulu-Natal (voucher number: S.-L. Steenhuisen 61). Inflorescences of *P. caffra* are visited by both birds and insects (e.g. Calf and Downs, 2002; Hargreaves et al., 2004). The most frequent floral visitors are beetles, particularly the cetoniine *Atrichelaphinis tigrina* (Olivier, 1789) (Steenhuisen and Johnson, 2012a). Bird visitors to *P. caffra* in the study population included Malachite sunbirds (*Nectarinia famosa* (Linnaeus, 1766)) and Gurney's sugarbirds (*Promerops gurneyi* (Verreaux, 1871)) (Hargreaves et al., 2004).

***Selective exclusion and controlled pollination experiment*—**For each experiment in a given year and location, we allocated inflorescences on 20-40 different *P. caffra* plants to three treatments with varying exclusion of different groups of pollinators, and a fourth treatment of supplemental hand-pollination to test for pollen limitation of seed set. Specifically, we either (1) bagged inflorescences with cloth mesh to exclude all pollinators (apertures 1 mm, N = 12-20); (2) caged inflorescences with wire mesh painted green to exclude vertebrates but allow access by insects (apertures 10-30 mm, N = 13-40); (3) left inflorescences unmanipulated and open allowing access to vertebrates and insects (N = 14-60); and (4) supplemented open-pollinated inflorescences with cross-pollen (N = 20). Inflorescences in treatment (4) were cross-pollinated at least twice by brushing five or more freshly exposed pollen presenters from inflorescences of different plants at least 20 m apart over the stigmatic grooves of all florets of each inflorescence during its receptive stage, as described by Steenhuisen and Johnson (2012c) for three other *Protea* species. It was difficult to experimentally exclude insect visitors without affecting all other pollinators. The cages used in this experiment have been shown to prevent bird visitation (Hargreaves et al., 2004). We observed similar mean numbers of insects (beetles and bees) visiting caged (mean ± SE = 2.23 ± 0.34 insects per inflorescence, N = 35 inflorescences) and uncaged inflorescences (mean ± SE = 2.50 ± 0.49 insects per inflorescence, N = 34 inflorescences; 2-tailed T-test $t_{(67)}$ = 0.454, P = 0.651), indicating that the cages did not prevent beetles from visiting the flowers.

Pollen receipt, seed set and progeny performance — To determine the abundance and purity of pollen loads, and to quantify pollen tube growth on stigmas, we collected ten stigmas from each of twenty experimental inflorescences for three treatments (vertebrate-excluded, open-pollinated and pollen-supplemented) at Krantzkloof once the inflorescence bracts were three quarters or more closed and the anthers had senesced. Five stigmas were

harvested from each of 20 bagged inflorescences and used to observe pollen germination (pollen loads on these stigmas were unnaturally inflated by pollen not having been removed from the pollen presenters and stigmas). We squashed the tips of five of the ten fresh stigmas collected from each inflorescence of vertebrate-excluded, open-pollinated and pollen-supplemented treatments in fuchsin gel that was melted to form permanent slides (Beattie, 1971) and counted the number of pollen grains deposited on each stigma. For pollen tube analysis, we fixed the remaining five stigmas from each inflorescence (including bagged inflorescences) in 2 ml of 3:1 70% ethanol: acetic acid for one hour, washed with distilled water, and stored in 2 ml of 70 % ethanol. These preserved stigmas were then subjected to a softening and staining procedure as described below and modified from Martin (1959). This procedure allowed for the examination of pollen tubes in the style through aniline blue UV-induced fluorescence of callose associated with the pollen tube wall. The stigmas were rinsed in distilled water for ten minutes, softened and cleared by suspending them in 4 N NaOH for 48 hours, rinsed in tap water for one hour, and then stained with aniline blue-0.1 N K_2HPO_4 for four hours. The stained stigmas were stored in glycerin for no longer than three days before use. The stigmas were mounted on slides in a drop of stain and glycerin, and flattened with a coverslip. The proportion of styles with pollen tubes in the upper first centimeter and number of pollen tubes per stigma for each treatment were determined by examining the stigmas with a microscope (Olympus Provis, AX-70) equipped with a UV filter system consisting of a dichroic mirror (400 nm), an ultraviolet excitation filter (330-385 nm) and a barrier filter (420 nm). Due to the thick stylar tissue obscuring pollen tubes and abundance of vasculature taking up the stain, it was difficult to distinguish pollen tube growth further down the style.

Infructescences from all populations and years were collected four months after flowering, seeds extracted and the proportion of seed set per inflorescence determined. In the case of open-pollinated treatments, the seeds were collated for each plant if more than one open-pollinated infructescence was collected from each experimental plant.

We investigated seed quality and possible inbreeding depression by weighing and germinating seed from vertebrate-excluded, open and pollen supplemented treatments in 2004 (Krantzkloof). A maximum of thirty seeds per twenty infructescences per treatment (total of 4582 seeds) was soaked in Kirstenbosch Instant Smoke Plus Seed Primer overnight in February 2005 and sown individually in Growmor seedling mix (National Plant Food, Cato Ridge) in seedling trays treated with Plazdip rooting/pruning agent containing copper oxychloride (Natal Associated Chemicals), and sprinkled with river sand. The three

pollination treatments were alternated throughout the seedling trays and blocked by maternal plant. The trays were checked for germination every third day over two months. After the trial, we calculated the mean number of days taken for seeds to germinate, proportion of seeds germinated and proportion of seedlings that died per maternal plant for each treatment.

We analysed the effects of treatment on the number of pollen grains per stigma, proportion of stigmas that received *Protea* and non-*Protea* pollen, proportion of deposited pollen that was *Protea* pollen, proportion of stigmas with pollen tubes in the upper style, the number of pollen tubes per style, the proportion of florets that set seed, the proportion of seeds from each treatment that germinated, the number of days until germination, and the proportion of seedlings that died using generalized linear models (GZLMs) in PASW Statistics v18. Unless otherwise stated we used likelihood ratio statistics, logit link functions, binomial error distributions and corrected for overdispersion where appropriate, and compared treatments using sequential Sidak adjustment for multiple comparisons (McCullagh and Nelder, 1989; Hosmer and Lemeshow, 2000; Field, 2009). To control for the effects of population, we entered this factor before treatment in Type I models. We also used Type I models to control for plant effects in analyses of the effects of treatments on seed mass, germination and other inbreeding depression measures. Treatment effects on the number of pollen grains deposited on stigmas and pollen tubes growing in styles were tested using means per inflorescence rounded to the nearest integer and fitted to models with a Poisson error distribution and log link function. The mass of individual seeds per treatment was also compared using analysis of covariance, with the number of seeds per inflorescence as a covariate. To determine if there is a trade-off between seed number and seed mass, mean mass per seed was regressed against the number of seeds per infructescence. The number of days taken for *P. caffra* seeds to germinate fit a normal distribution. When analyzing the proportion of seedlings that died, we substituted one dead seedling for all treatments for six maternal plants that experienced zero progeny deaths, to provide a statistically conservative solution to the problem of lack of binomial model convergence when there is no variance within a treatment group (Zuur et al., 2009).

***Outcrossing rates*—**We used starch gel electrophoresis to visualize allelic polymorphisms in mature seeds of *P. caffra* from bagged, vertebrate-excluded and open-pollinated plants from Mount Gilboa in 2008 (Wendel and Weeden, 1989). Seed families typically consisted of 10-40 seeds (median of 10 seeds, total of 479 seeds sampled over 39 seed families; bagged $N = 132$ seeds, caged $N = 192$ seeds, open $N = 155$ seeds). Seeds were stored at room temperature until lab work was completed in February 2009 (previous

germination tests demonstrated that seeds remain viable at room temperature for 18 months after harvesting). We did not count unfertilized ovules in 2008, but as in other years of the study (see Results), the number of seeds per inflorescence for vertebrate-excluded and open-pollinated treatments were similar to those in the fully bagged treatment, thus providing little information about pollinator effectiveness. The seed coat of each seed was slit before being soaked in water overnight. The seed coats were then removed and seeds placed in micro-centrifuge tubes on ice. The seeds were homogenized in a drop of cold sodium acetate extraction buffer (8.3 % (w/v) sodium acetate containing 16.7 % (w/v) sucrose, pH 7.38 adjusted with acetic acid) (Stuber et al., 1988), centrifuged at 4000 *g* for 3 min, and the supernatant absorbed onto paper wicks for starch gel electrophoresis. Twelve percent starch (SSEP Starch Products, Narayan & Company, India) gels were used.

Twenty-eight enzyme systems were screened for variability using four different electrophoretic buffers and a bulked sample of seed from open-pollinated plants (Wendel and Weeden, 1989; Murphy et al., 1996). Eleven enzyme systems gave interpretable banding patterns and eight of these were polymorphic (Table 1). Polymorphic loci were rather invariable, with Ldh showing the highest variability (Table 1). Fixation of alleles did not differ among treatments, and treatments were thus pooled to assess frequencies. Locus nomenclature and genetic interpretation of enzyme banding patterns based on the subunit structure of the enzymes followed Van der Bank (2002). Polymorphic enzymes and buffer system combinations are listed in Table 1.

***Outcrossing rates and inbreeding coefficients*—**Treatment-specific maximum likelihood estimates of single-locus (t_s) and multilocus (t_m) outcrossing rates were estimated using MLTR version 3.0 (Ritland and Jain, 1981; Ritland, 2002). Standard deviations for estimates of t_s, t_m, and $t_m - t_s$ were based on 1000 bootstraps with resampling. The difference $t_m - t_s$ represents a test for biparental inbreeding. These values would be the same in the absence of biparental inbreeding. The potential to detect outcrossing events increases with an increase in the number of loci sampled, and thus t_s will usually be lower than t_m in the presence of inbreeding. The difference in t_m between treatments (i.e. vertebrate-excluded vs. open, bagged vs. open, bagged vs. vertebrate-excluded) was assessed by a pair-wise comparison of 1000 bootstrap estimates generated from the maximum likelihood estimation of outcrossing rates (MLTR) analysis, following the method of Eckert and Barrett (1994). Outcrossing rates of two treatments were considered significantly different if 97.5 % (2-tailed test, $\alpha = 0.05$) of the differences between randomly paired bootstrap estimates of t_m were greater or less than zero (e.g. $t_{cage+k} - t_{open+k}$ for the k^{th} bootstrap estimates of t_m for two

treatments). This method was also used to test if all estimates of outcrossing and inbreeding were different to zero (1-tailed test, $\alpha = 0.05$).

Table 1. Nomenclature, allelic frequencies and sample sizes of polymorphic enzymes resolved from pooled progeny of *Protea caffra*. E.C. numbers, locus abbreviation, and optimal buffers used for electrophoresis are given for each locus.

Enzyme[a]	E.C number	Locus	Optimal buffer	Allele	Pollen/ ovule	Sample size
Alcohol dehydrogenase	1.1.1.1	Adh	MF	A	0.868	433
				B	0.132	
Glucose-6-phosphate isomerase	5.3.1.9	Gpi	A	A	0.147	479
				B	0.853	
L-Iditol 2-dehydrogenase	1.1.1.14	Iddh	TC	A	0.909	237
				B	0.091	
Isocitric dehydrogenase	1.1.1.42	Idhp-1	A	A	0.990	454
				B	0.010	
L-Lactate dehydrogenase	1.1.1.27	Ldh	MF	A	0.804	389
				B	0.196	
Malate dehydrogenase	1.1.1.37	Mdh	TC	A	0.854	251
				B	0.146	
Menadione reductase	1.6.99.2	Mnr	A	A	0.017	479
				B	0.983	
Phosphoglucomutase	5.4.2.2	Pgm	A	A	0.004	479
				B	0.996	

A: EDTA-Boric acid-Tris-Magnesium chloride continuous buffer (pH 8.6) system (Goncharenko et al., 1992)

MF: EDTA-Boric acid-Tris continuous buffer (pH 8.6) system (Markert and Faulhaber, 1965)

TC: Tris-citrate continuous buffer (pH 6.9) system (Whitt, 1970)

[a]Monomorphic loci included Idhp-2 (Isocitric dehydrogenase); Pgdh-1 and Pgdh-2 (Phosphogluconate dehydrogenase E.C. 1.1.1.44)

Inbreeding coefficients (Wright's (1978) fixation index F_{IS}) were estimated as: $F_{IS} = 1 - H_o/H_e$ for the maternal plants where mean observed (H_o) and expected (H_e) heterozygosity were estimated using POPGENE version 1.32 (Yeh et al., 1997), and as $F_{IS} = (1 - t_m)/(1 + t_m)$ (Hartl and Clark, 1989; Holsinger and Weir, 2009) for progeny from open-pollinated plants (using t_m estimate from MLTR). Maternal genotypes were inferred by the MLTR program by assessing individual progeny arrays. Standard deviation for maternal F_{IS} was calculated using single locus F_{IS} estimates for the maternal genotypes generated by POPGENE. Inbreeding depression (δ = 1 – fitness of selfed progeny/fitness of outcrossed progeny) was measured to

assess the survival of seed to reproductive maturity using $\delta = 1 - [(2t_mF)/((1 - t_m)(1 - F))]$ (Ritland, 1990), with an outcrossing rate (t_m) and parental inbreeding (F) estimated by MLTR.

RESULTS

***Pollen receipt, seed set and progeny performance*—**Very pure pollen loads (> 90 % *Protea* pollen) were recorded on stigmas of unbagged *P. caffra* experimental inflorescences (Table 2). Pollen loads on stigmas of vertebrate-excluded inflorescences did not differ significantly from those of open controls, while pollen supplementation generally inflated pollen loads by about 60 % (Table 2). Neither vertebrate-exclusion nor pollen supplementation significantly affected the proportion of stigmas receiving pollen (Table 2). Pollen from a maximum of six foreign plant species was found on stigmas. The proportion of stigmas that received non-*Protea* pollen was similar for open-pollinated and vertebrate-excluded florets (mean ± SE, 24.40 ± 0.02 % versus 21.17 ± 0.03 %; $\chi^2_{(1)} = 0.672$, $P = 0.412$).

Table 2. The effect of natural pollination, vertebrate-exclusion and pollen supplementation on stigmatic pollen loads for *Protea caffra* in 2004 (Krantzkloof Gorge). Sample sizes are shown in parentheses and significant differences between treatments are indicated by different letters next to the marginal means (significance level: **P* < 0.01; ***P* < 0.001).

Pollen measure		Treatment			$\chi^2_{(2)}$
		Open pollinated (60)	Vertebrate excluded (39)	Pollen supplemented (20)	
Pollen load per stigma	Mean	18.0[a]	14.7[a]	99.1[b]	185.108**
	Lower SE	2.0	2.2	8.2	
	Upper SE	2.2	2.5	9.0	
Proportion of sampled stigmas with *Protea* pollen	Mean	0.98[a]	0.93[b]	0.99[ab]	9.494*
	Lower SE	0.01	0.02	0.02	
	Upper SE	0.01	0.02	0.01	
Protea proportion of pollen load	Mean	0.97[a]	0.96[a]	1.00[b]	47.307**
	Lower SE	0.01	0.01	0.002	
	Upper SE	0.01	0.01	0.001	

The proportion of stigmas with pollen tubes was > 80% for all treatments with bagging and pollen supplementation inflating this measure significantly (2004, treatment: $\chi^2_{(3)}$ = 14.693, P = 0.002; Figs. 1A, B). Similarly, bagging doubled and pollen supplementation more than tripled the number of pollen tubes in styles compared to vertebrate-excluded and open-pollinated inflorescences (2004, treatment: $\chi^2_{(3)}$ = 165.071, P < 0.001; Figs. 1A, B).

Infructescences damaged by lepidopteran predators (as described in Steenhuisen and Johnson, 2012c for other *Protea* species) were excluded from analyses of seed set but unpredated seeds from damaged infructescences were used in germination trials. The population at Bulwer experienced the highest predation rate with sample sizes reduced to below 10 infructescences for three of the four treatments. Seed set was low over all populations, rarely exceeding 35 % of available ovules (Fig. 1C). Considering all populations together, pollen supplementation and vertebrate-exclusion (caging) resulted in seed set similar to that in open-pollinated inflorescences, while bagging significantly lowered seed set indicating that more viable cross pollen was deposited on vertebrate-excluded and open-pollinated inflorescences (population: $\chi^2_{(2)}$ = 97.374, P < 0.001; treatment: $\chi^2_{(3)}$ = 43.050, P < 0.001, Fig. 1C). These treatment differences were, however, only evident in the Krantzkloof population ($\chi^2_{(3)}$ = 46.442, P < 0.001; bagged N = 16, vertebrate-excluded N = 37, open N = 70, pollen supplemented N = 18; Fig. 1C). Seed set did not differ significantly among treatments in the Bulwer ($\chi^2_{(3)}$ = 1.591, P = 0.661; bagged N = 5, vertebrate-excluded N = 8, open N = 20, pollen supplemented N = 2) and Mount Gilboa ($\chi^2_{(3)}$ = 1.823, P = 0.610; bagged N = 11, vertebrate-excluded N = 11, open N = 23, pollen supplemented N = 4) populations, but power in these latter analyses was low because sample sizes were lowered substantially by seed predation (Fig. 1C).

The mean mass per seed for vertebrate-excluded inflorescences (mean ± SE g per seed; 0.035 ± 0.001 g) was similar to that in open-pollinated (0.036 ± 0.001 g), but significantly higher than that in pollen supplemented (0.033 ± 0.002 g) inflorescences (plant: F_{19} = 15.013, P < 0.001; treatment: $F_{2,86}$ = 3.772, P = 0.027). This effect, however, disappeared when seed number per inflorescence was included as a covariate (number of seed: F_1 = 7.449, P = 0.007; treatment: $F_{2,104}$ = 0.557, P = 0.574). A trade-off between seed mass and number is evident from a significant negative association between these variables (R^2 = 0.066, y = –0.0001x + 0.0378; $F_{1,106}$ = 7.511, P = 0.007; Fig. 2). Seeds from infructescences of vertebrate-excluded, open-pollination and pollen supplementation treatments of *P. caffra* from Krantzkloof were similar in terms of germination success (< 80

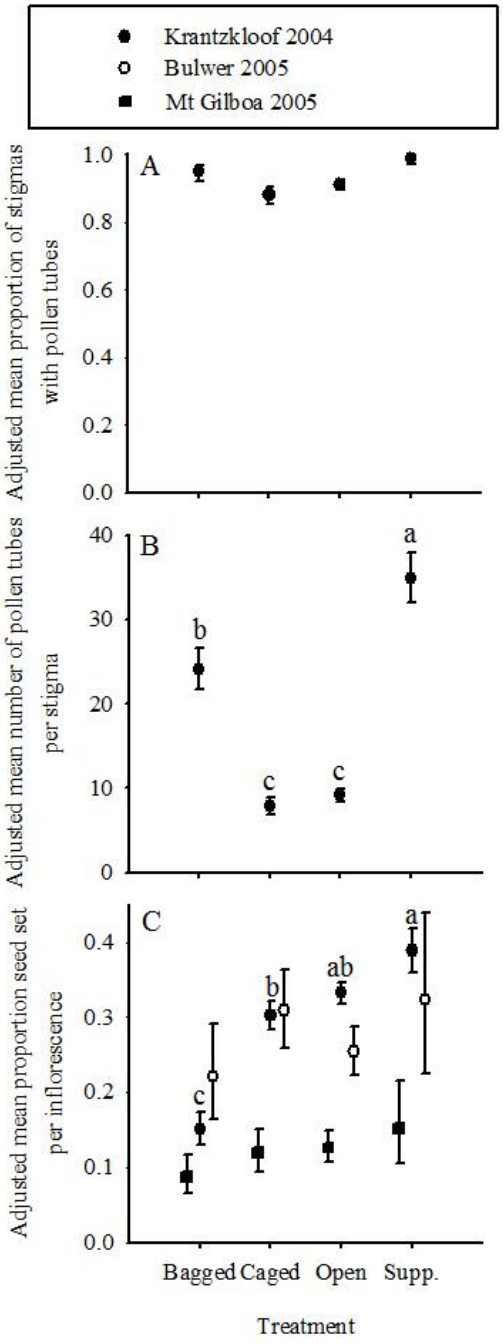

Figure 1. The effect of full exclusion of pollinators by bagging, experimental exclusion of bird pollinators by caging (allowing access to invertebrates only), open-pollination and supplemental hand-pollination (–supp.") on the marginal mean (A) proportion (± SE) of stigmas with pollen tubes growing in the style, (B) pollen tube loads (± SE) on stigmas, and (C) the proportion (± SE) of florets to set seed per inflorescence for three populations of *Protea caffra*. Different letters indicate statistically significant differences between treatments ($P < 0.05$). Means without letters are statistically similar for that population.

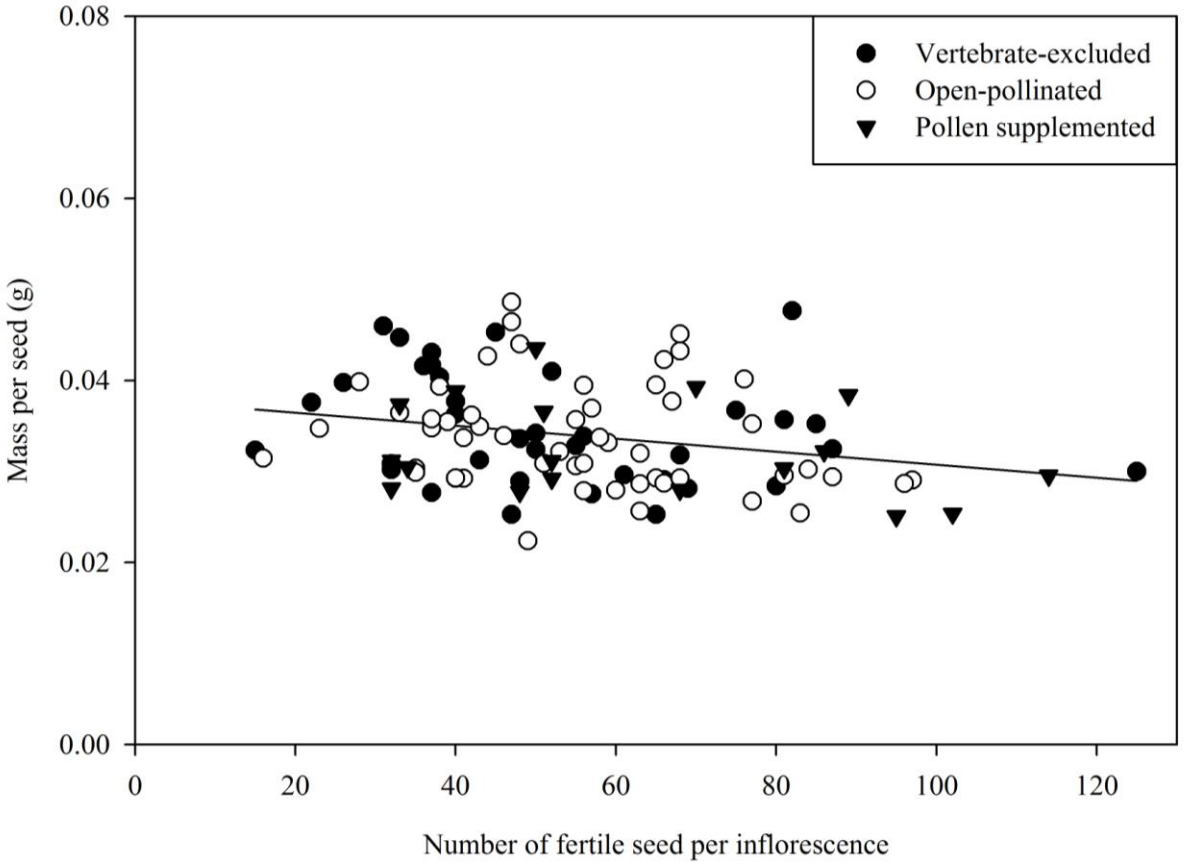

Figure 2. Regression of the number of fertile seeds and the mass per seed (g) for vertebrate-excluded, open-pollinated (no exclusion of pollinators), and open inflorescences supplemented with cross-pollen, for *Protea caffra* (Krantzkloof Nature reserve, 2004). Regression analysis: $R^2 = 0.066$, $y = -0.0001x + 0.0378$; $F_{1,106} = 7.511$, $P = 0.007$.

Table 3. Analyses comparing marginal means for germination success, germination rate and death toll of seeds and seedlings from vertebrate-excluded, open, and supplemental hand-pollinated inflorescences of *Protea caffra* from 2004. (The number of seeds sown from each treatment are shown in parentheses; Significance level: **P* < 0.01; ***P* < 0.001).

Fitness measure		Treatment			Plant: $\chi^2_{(19)}$	Treatment: $\chi^2_{(2)}$
		Open pollinated (1369 seeds)	Vertebrate excluded (882 seeds)	Pollen supplemented (451 seeds)		
Proportion of seeds that germinated	Mean	0.86	0.82	0.85	53.581**	1.462
	Lower SE	0.02	0.03	0.04		
	Upper SE	0.02	0.03	0.03		
Days to germinate	Mean	36.28	36.66	35.42	104.547**	1.545
	Lower SE	0.47	0.56	0.83		
	Upper SE	0.47	0.56	0.83		
Proportion of germinated seedlings that died	Mean	0.02	0.04	0.02	39.192*	5.359
	Lower SE	0.004	0.01	0.01		
	Upper SE	0.01	0.01	0.01		

%), germination rates (c. 35-37 d from sowing), and seedling survival after two months (1% deaths) (Table 3).

***Outcrossing rates*—**The outcrossing rates (t_m) of progeny from bagged plants were no different from zero, whereas t_m for open and vertebrate-excluded treatments was significantly greater than zero (Table 4). Pairwise comparison of bootstrap estimates for t_m indicated higher outcrossing rates in the progeny from vertebrate-excluded and open-pollinated plants compared with autonomously self-pollinated plants ($t_{cage+k} - t_{self+k}$, $P < 0.001$; $t_{open+k} - t_{self+k}$, $P < 0.001$; Table 4). There was no significant difference in t_m between vertebrate-excluded and open-pollinated plants ($t_{cage+k} - t_{open+k}$, $P = 0.41$; cage vs open: $\Delta\ t_m \pm SE = 0.061 \pm 0.182$; Table 4). The multilocus t estimates were only marginally higher than single locus estimates per treatment indicating little or no biparental inbreeding (Table 4).

The overall inbreeding coefficient ($F_{IS} \pm SE$) for the progeny of open-pollinated plants was indicative of inbreeding (0.258 ± 0.002). By contrast, F_{IS} for maternal plants (–0.187 ± 0. 065; CI = –0.315 – –0.059) indicated a lack of inbreeding and an excess of heterozygotes (F_{IS} maternal vs. F_{IS} progeny: $\Delta F_{IS} \pm SD$= –0.353 ± 0.094, $P < 0.001$). Our estimate of inbreeding depression for progeny exceeded one ($\delta = 1.281 \pm 0.308$), indicating that inbred progeny may not survive to reproductive age.

Table 4. Maximum likelihood (MLTR) estimates (± SD) of multilocus (t_m) and single locus (t_s) outcrossing rates and biparental inbreeding ($t_m - t_s$) for bagged (all pollinators excluded), vertebrate-excluded (access by insect pollinators only) and open pollinated (access by all pollinators) *Protea caffra* inflorescences (*denotes significantly different from zero; significance level: *$P < 0.05$; **$P < 0.001$).

MLTR estimates	Treatment		
	Bagged	Vertebrate-excluded	Open pollinated
t_m±SD	0.001±0.040	0.651±0.156**	0.591±0.084**
t_s±SD	0.001±0.033	0.638±0.153**	0.526±0.089**
t_m-t_s±SD	0.000±0.028	0.013±0.027	0.065±0.029*
Seeds (seed families)	132 (12)	192 (13)	155 (14)

DISCUSSION

Positive outcrossing rates in inflorescences excluded from birds (Table 4) show that insects cross-pollinate inflorescences of *P. caffra* at Mount Gilboa. In addition, these outcrossing rates did not differ from those of inflorescences exposed to vertebrates, further indicating that insects are effective agents of cross-pollination in *P. caffra*, and that any outcrossing by birds was similar or did not exceed that by insects. This could explain the evolution of entomophilous traits, such as a sweet-fruity floral scent, typical of many beetle-pollinated flowers (Bernhardt, 2000), in this species.

Environmental effects and suspected physical limits to the number of ovules that can set seed probably account for a low threshold seed set in this species (< 35 %), typical of the family Proteaceae (Collins and Rebelo, 1987; Ayre and Whelan, 1989). Pollen supplementation did not increase seed set or average seed mass, suggesting that *P. caffra* plants experience resource limitation (Fig. 1C & 2). Irrespective of treatment the average seed mass decreased slightly with an increase in the number of developing seeds in an infructescence (Fig. 2). This indicated that the threshold number of seed and the average seed mass were limited by physical restrictions of the size of an infructescence, rather than the quality of pollen.

The parental population at Mount Gilboa appears to be at Hardy-Weinberg equilibrium. In contrast, a significantly different and positive F_{IS} for progeny of open-pollinated plants indicates that the progeny contain a higher proportion of homozygote individuals than expected. Further investigation using progeny outcrossing rates and the maternal inbreeding coefficient to estimate inbreeding depression revealed that these progeny may suffer from complete inbreeding depression ($\delta = 1$). Since seed from self and cross hand pollinations in the Krantzkloof population were equally viable in terms of germination and early seedling survival (Steenhuisen and Johnson, 2012b), we suggest that this discrepancy in F_{IS} in the Mount Gilboa population could be explained by the death of inbred seedlings before they reach adulthood (3-4 years from seed; Eliovson, 1973), maintaining a low inbreeding coefficient for the parent population. In their study of the self-compatible species *Metrosideros excelsa*, Schmidt-Adam et al. (2000) detected inbreeding depression in seedlings after six months' growth, and F_{IS} was higher for progeny than for the maternal parents, similarly suggesting that there was selection against homozygotes before the plants reached reproductive maturity. It is possible, therefore, that the sheltered greenhouse conditions in which we germinated *P. caffra* seeds did not allow for the expression of deleterious genetic effects in the early life stages of inbred offspring. Inbred animals and plants often exhibit significant inbreeding depression only under stressful environmental conditions (e.g. Dudash, 1990; Fox and Reed, 2010). For example, Ramsey and Vaughton (1998) found that the effects of inbreeding depression in *Blandfordia grandiflora* R.Br. (Blandfordiaceae) were significantly greater under field conditions than under greenhouse conditions.

Mixed mating system — The only previous studies of mating systems in the family have been conducted on Australian species, particularly in *Banksia* L.f. In these Australian allozyme studies, only one to three polymorphic loci could be resolved sufficiently, even though 13-20 enzyme systems were screened for variability in each study (Scott, 1980; Carthew et al., 1988; Goldingay and Carthew, 1998). It was thus noteworthy that we found eight well resolved polymorphic loci in *P. caffra*, allowing very accurate estimation of the mating patterns in this species (Table 1). This was all the more surprising because *P. caffra* is facultatively autogamous and should therefore be expected to have relatively low levels of allelic diversity (Leimu et al., 2006).

Outcrossing rates for *P. caffra* are within the range reported for other self-compatible Proteaceae, although lower than for most woody plants reviewed by Goodwillie et al. (2005). Sampson et al. (1994) reported rates of outcrossing that were similar to ours, for the rare and

self-compatible *Banksia brownii* Baxter ex R.Br (t_m = 0.65 - 0.75 in two populations), while a large range of outcrossing rates from almost complete selfing (t_m = 0.07) to highly outcrossed (t_m = 0.85) were reported by Ayre et al. (1994) for *Grevillea barklyana* F.Muell. ex Benth. Vaughton and Carthew (1993) and Llorens et al. (2012) recorded almost complete outcrossing in the self-compatible *Banksia spinulosa* Sm. (t_m = 1) and *Banksia sphaerocarpa* (t_m = 0.86 - 0.99), respectively. Carthew et al. (1996) suggested preferential outcrossing as an explanation for high outcrossing rates in self-compatible *Banksia* species. This hypothesis was supported by selective abortion of self-fertilized ovules in *B. spinulosa* after experimental combinations of self and cross pollination treatments on inflorescences (Vaughton and Carthew, 1993). Selective abortion is also the proposed reason for low inbreeding in self-compatible yuccas (*Yucca filamentosa* L., Agavaceae; Pellmyr et al., 1996). In contrast, our finding of similar seed set and germination after self- and cross-pollination (Steenhuisen and Johnson, 2012b) indicates that selective abortion or inbreeding depression are not expressed at these early life stages. Vaughton (1995) similarly found no evidence of early inbreeding depression in the self-compatible *G. barklyana.*

Goldingay and Carthew (1998) argued that the reported high outcrossing rates for Proteaceae reflect post-zygotic processes rather the outcrossing efficiency of pollinators. Studies of pollinator behaviour indicate that pollinator-mediated selfing can occur frequently. For example, within-plant movements of honeyeaters promote geitonogamy in *B. spinulosa* in mid-seasonal flowering periods (Vaughton 1990). Cetoniine beetle pollinators of *P. caffra* are able to spread self-pollen from dehisced anthers from inner florets to receptive stigmas of the outer florets within an inflorescence. The inflorescences are long-lived and beetles spend long periods foraging in a single inflorescence. Upon observation, however, the beetles do not tend to visit several inflorescences on the same plant, but rather fly over several bushes before settling again, promoting outcrossing. Young (1988) and Englund (1993) found that scarab beetles were efficient pollinators and long-distance dispersal agents due to little grooming of pollen from their bodies, frequent inter-plant flights, and long flight distances between plants (average of 18 m for *Cetonia*, Englund, 1993; and sometimes > 300 m for *Cyclocephala* beetle species, Young, 1988).

***Beetle pollination in* Protea —** Overall, this study in conjunction with observations of pollinator and behavioural tests (Steenhuisen and Johnson, 2012a; unpubl. data) presents strong evidence for an effective beetle pollination system in *P. caffra.* This species belongs to a recently evolved clade of grassland *Protea* species which share a common suite of insect visitors and floral traits (Steenhuisen and Johnson, 2012a), including strong fruity scents

(Steenhuisen et al., 2010) which have been shown to be highly attractive to cetoniine beetles in laboratory and field conditions (S-L. Steenhuisen, unpubl. data). Recent phylogenetic analyses of *Protea* (Valente et al., 2010; Schnitzler et al., 2011) suggest that a shift took place from an ancestral condition of bird-pollination to insect pollination in the clade that includes *P. caffra*. It remains possible that birds contribute significantly to outcrossing in some populations of *P. caffra*, especially those in dense arboreal stands in the northern parts of its range (Calf and Downs, 2002; Nicolson, 2007), but the results of this study, together with the floral traits in *P. caffra* that are consistent with beetle pollination (Steenhuisen and Johnson, 2012a), support the idea of a shift from bird to beetle pollination in *Protea*. As *P. caffra* and most of the other species in its clade are facultative selfers (Steenhuisen and Johnson, 2012b), selection for this shift is likely to have occurred through increased male fitness arising from efficient pollen export by beetles.

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CHAPTER 6

VARIATION IN SCENT EMISSION AMONG FLORAL PARTS AND INFLORESCENCE DEVELOPMENTAL STAGES IN BEETLE-POLLINATED *PROTEA* SPECIES (PROTEACEAE)

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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

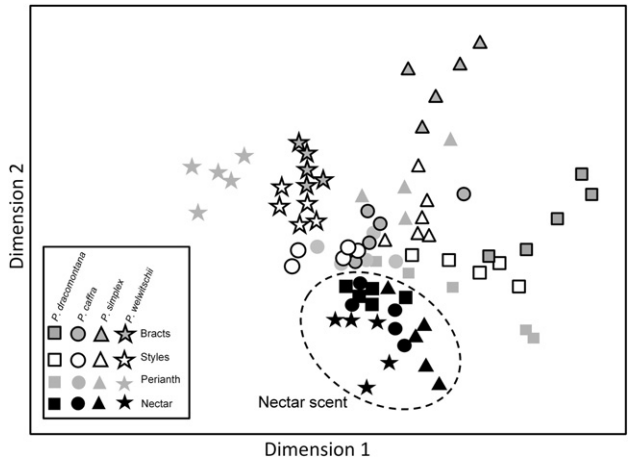

Fig. 1. Non-metric multidimensional scaling (NMDS) of the scent composition from different inflorescence parts (bracts, styles, and perianth) and nectar of four beetle-pollinated *Protea* species. NMDS is based on Bray Curtis similarities, samples are from five fully dehisced inflorescences for each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

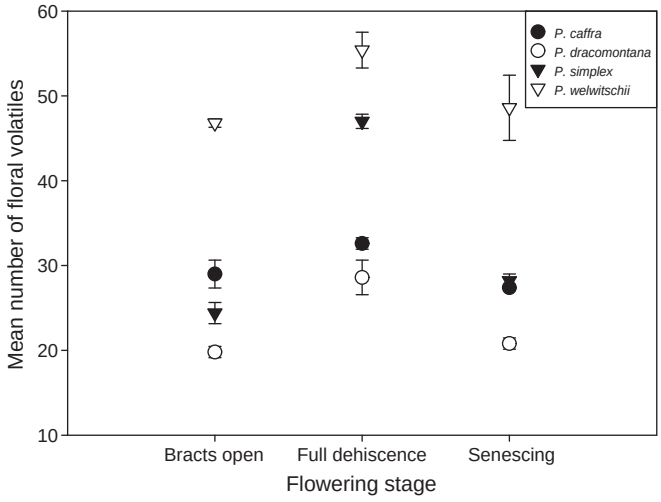

Fig. 2. The change in the number of floral volatiles emitted from four *Protea* species, each represented by 5 cut inflorescences from each of three different flowering stages (inflorescences with bracts open but no anther dehiscence; fully dehisced inflorescences; and, senescing inflorescences with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

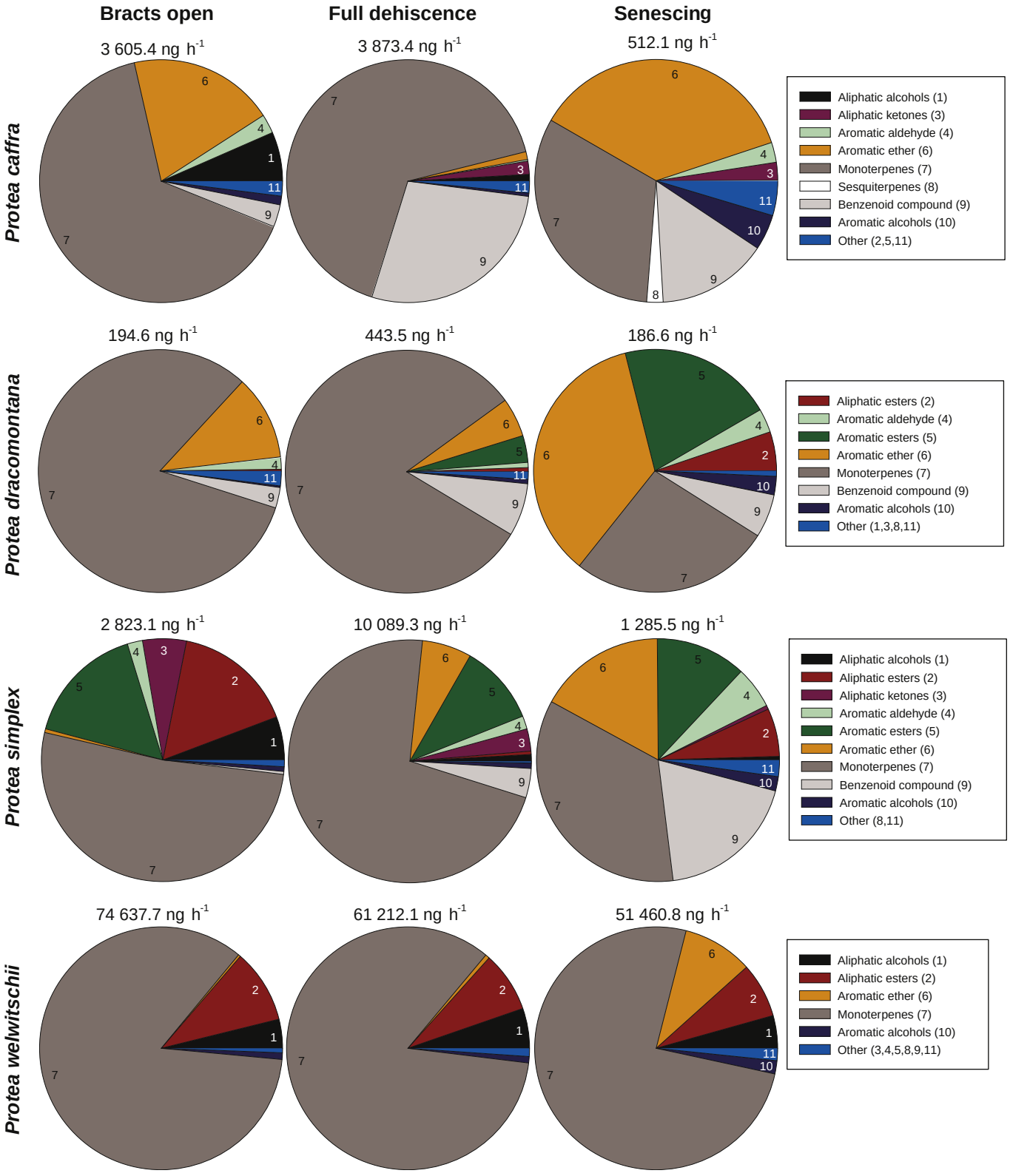

Fig. 3. The contribution of various compound classes to the scent of inflorescences of successive flowering stages for four *Protea* species. Total emission rates shown above each graph. Numbered pie slices refer to specific compound classes in legend. Data presented are average relative proportions 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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CHAPTER 6

SUPPLEMENTARY MATERIAL

Appendix 1. Compounds from inflorescence parts and nectar of four *Protea* species. Scent compounds are listed according to compound class and Kovats retention index (KRI). 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 for each species. Compound identification criteria and notes: 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.

Compound name			***Protea caffra***				***Protea dracomontana***				***Protea simplex***				***Protea welwitschii***			
Inflorescence part	**KRI**	**CAS**	**B**	**P**	**S**	**N**	**B**	**P**	**S**	**N**	**B**	**P**	**S**	**N**	**B**	**P**	**S**	**N**
Aliphatic compounds																		
Aliphatic acids																		
2-Methylpropanoic acid[b]	1518	79-31-2	-	-	-	-	-	-	-	-	-	-	-	tr	-	-	-	0.2
Pentanoic acid[c]	1687	109-52-4	-	-	-	-	-	tr	0.4	0.2	-	-	-	-	-	-	-	-
2-Methylhexanoic acid[b]	1612	4536-23-6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1
2-Methylbutanoic acid[b]	1630	116-53-0	-	-	-	0.3	-	0.1	0.2	0.1	-	-	-	-	-	-	-	-
Hexanoic acid[b]	1800	142-62-1	2.9	0.2	-	0.5	-	-	-	-	tr	-	-	-	-	-	-	-
Aliphatic alcohols																		
1-Butanol[c]	1121	71-36-3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.2
2-Methyl-1-butanol[a]	1178	137-32-6	-	-	-	-	-	-	-	-	-	0.8	-	-	-	0.1	-	0.9
1-Hexanol[c]	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[a]	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[a]	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
(E)-2-Hexen-1-ol[a]	1345	928-95-0	-	-	-	-	-	-	-	-	1.7	0.2	1.8	-	-	-	0.1	0.5
2-Heptanol[a]	1279	543-49-7	-	-	-	-	5.1	-	-	0.4	0.1	-	-	-	5.3	-	-	-
1-Octanol[a]	1512	111-87-5	-	-	0.3	0.9	-	-	-	-	-	-	-	-	-	-	tr	0.1
1-Nonanol[b]	1592	143-08-8	-	-	-	-	-	-	-	0.5	-	-	-	-	-	-	-	tr
Aliphatic aldehydes																		
trans-2-Methyl-2-butenal[a]	1088	497-03-0	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	-	-
(E)-2-Hexenal[b]	1183	6728-26-3	-	-	-	-	-	-	-	-	3.4	-	5.5	-	-	-	-	-
Aliphatic esters																		
Methyl tiglate[b]	1167	6622-76-0	-	-	-	-	-	3.5	-	-	-	-	-	-	-	-	-	-
Ethyl hexanoate[b]	1187	123-66-0	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	-
4-Hexen-1-yl-acetate[a]	1220	72237-36-6	-	-	-	-	-	-	-	-	8.1	-	-	-	2.7	-	-	-
Hexyl acetate[c]	1238	142-92-7	-	1.7	-	-	-	0.2	-	-	0.1	-	-	-	tr	0.8	tr	-
2-Methylbutyl 2-methylbutyrate[a]	1240	2445-78-5	-	-	-	-	-	-	-	-	-	-	-	-	tr	1.4	-	tr
Methyl 2-hexenoate [a]	1259	2396-77-2	-	-	-	-	-	-	-	-	-	0.1	0.1	-	-	-	-	-
Ethyl *(E)*-2-hexenoate[b]	1273	27829-72-7	-	-	-	-	-	-	11.6	-	-	-	-	-	-	-	-	-
Unidentified aliphatic ester[a]	1274		-	-	-	-	-	-	-	-	-	-	-	-	-	3.5	-	0.6
(Z)-3-Hexen-1-yl acetate[b]	1284	3681-71-8	8.9	6.5	-	tr	19.1	1.4	-	-	-	1.4	1.5	-	0.3	1.1	0.4	-
Butyl tiglate[a]	1386	7785-66-2	-	-	-	-	-	-	-	-	-	-	-	-	0.3	0.9	0.4	0.1

Unidentified aliphatic ester[a]	1391		-	-	-	-	-	-	-	-	-	-	-	-	0.2	1.3	-	-
Iso-amyl tiglate[a]	1420	66917-62-2	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	-	-
(3E)-3-Octenyl acetate [a]	1442	35602-33-6	-	-	-	-	-	0.8	0.1	-	-	-	-	-	-	-	-	-
(Z)-3-Hexenyl isovalerate[a]	1433	35154-45-1	-	-	-	-	6.3	-	-	-	0.5	-	-	-	2.7	2.2	-	tr
Unidentified aliphatic ester[a]	1473		-	-	-	-	-	-	-	-	-	-	-	-	-	tr	-	-
Unidentified aliphatic ester[a]	1526		-	-	-	-	-	-	-	-	-	-	-	-	tr	-	-	tr
Unidentified aliphatic ester[a]	1561		-	-	-	-	-	-	-	-	0.7	-	-	-	tr	-	-	-
Unidentified tiglate[a]	1581		-	-	-	-	-	-	-	-	-	-	-	-	0.5	1.8	1.1	-
Unidentified aliphatic ester[a]	1636		-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	tr	tr
Unidentified aliphatic ester[a]	1625		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1
Unidentified aliphatic ester[a]	1848		-	-	-	-	3.1	-	-	2.0	-	-	-	-	-	-	-	0.9
Unidentified aliphatic ester[a]	1788		-	-	-	-	0.8	0.4	1.5	0.4	-	-	-	-	-	-	-	-
Butyl butyrate[a]	1815	109-21-7	-	0.8	-	1.9	-	-	-	-	-	-	-	0.2	-	-	-	0.7
Unidentified aliphatic ester[a]	1826		-	-	-	-	3.1	-	-	3.2	-	-	-	-	-	-	-	-
Aliphatic ketones																		
2,3-Butanedione[a]	1019	431-03-8	-	-	-	-	-	-	-	-	-	2.4	-	-	-	-	-	7.1
2-Pentanone[a]	1023	107-87-9	-	-	-	-	-	-	-	-	6.5	0.2	1.0	2.1	-	tr	-	-
2,3-Heptanedione[a]	1138	96-04-8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.0
2-Heptanone[b]	1154	110-43-0	-	-	-	-	-	-	-	-	7.7	4.3	8.7	-	-	-	-	-
Acetoin[b] (3-hydroxy-2-butanone)	1257	513-86-0	-	-	-	6.8	-	7.1	-	4.4	0.2	-	1.2	7.1	-	-	0.9	5.5
5-Nonanone[a]	1297	502-56-7	-	-	-	-	-	-	-	0.2	-	-	-	-	-	-	-	-
2-Nonanone[b]	1355	821-55-6	-	-	-	-	-	13.3	-	-	-	-	-	-	-	-	-	-
2-Butanone[a]	1426	78-93-3	-	-	-	2.9	-	-	-	-	0.8	-	-	-	-	-	-	-
Aromatics																		
Styrene[b]	1225	100-42-5	1.3	1.3	0.4	0.8	-	0.6	-	1.0	1.4	0.2	0.3	1.4	0.4	-	0.6	0.2
p-Cymene[b]	1240	99-87-6	-	-	-	-	-	-	-	-	-	-	-	-	tr	-	-	tr
(E)-2-Hexenyl benzoate[a]	1284	76841-70-8	-	-	-	-	-	0.1	2.1	-	-	-	-	-	-	-	-	-
Anisole[b]	1311	100-66-3	-	1.1	2.3	tr	-	-	-	-	6.2	5.0	1.4	7.3	3.0	tr	3.3	-
Methoxymethylbenzene[a]	1350	538-86-3	-	-	-	-	-	-	-	-	-	0.1	-	-	0.1	-	tr	-
Benzaldehyde[c]	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
Phenylacetaldehyde[c]	1609	122-78-1	-	-	-	0.7	-	-	0.2	-	-	-	-	tr	-	-	-	-
Methyl benzoate[c]	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 acetate[c]	1691	140-11-4	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	tr
Methyl salicylate[c]	1738	119-36-8	-	-	-	-	-	0.6	0.6	-	tr	0.2	0.1	tr	-	-	-	-
Butyl benzoate[b]	1822	136-60-7	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-
Benzyl pentanoate[a]	1823	10361-39-4	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	tr	-
Benzyl alcohol[c]	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
3-Methyl-1-butyl benzoate [a]	1857	94-46-2	-	-	-	-	-	-	-	-	-	-	-	-	tr	-	-	-
Phenylethyl alcohol[c]	1863	60-12-8	0.6	0.2	0.4	2.3	1.2	0.7	0.6	1.3	0.1	0.3	0.5	1.5	0.2	0.1	0.4	0.7
(E)-Cinnamaldehyde[a]	1984	14371-10-9	-	-	-	-	-	-	-	-	-	tr	-	tr	-	-	-	-
3-Phenylpropanol[a]	1996	122-97-4	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-

Hexyl benzoate[a]	2025	6789-88-4	-	-	-	-	-	-	-	-	-	-	-	-	tr	tr	tr	-
Unidentified aromatic ester[a]	2057		-	-	-	-	-	-	-	-	-	-	-	-	tr	0.1	0.1	tr
(Z)-3-Hexenyl benzoate[b]	2062	72200-74-9	-	-	-	-	-	-	-	-	-	-	-	-	0.3	tr	tr	-
Monoterpenoids																		
alpha-Pinene[c]	1049	80-56-8	tr	-	0.9	-	-	-	-	-	8.4	7.1	0.4	7.9	1.3	0.3	4.3	0.5
Sabinene[b]	1099	3387-41-5	-	-	-	-	-	-	-	-	-	-	-	-	tr	2.0	0.3	tr
beta-Pinene[c]	1108	127-91-3	7.3	-	-	tr	-	-	-	-	1.7	0.4	0.2	0.2	0.3	1.3	0.3	tr
beta-Phellandrene[a]	1131	555-10-2	-	-	-	-	-	-	-	-	-	-	-	-	tr	-	-	-
beta-Myrcene[b]	1156	123-35-3	tr	-	-	tr	6.1	-	-	-	2.1	-	-	-	tr	0.8	0.1	tr
alpha-Terpinene[c]	1163	99-86-5	-	-	-	tr	-	-	-	-	-	-	-	-	-	-	-	-
Limonene[c]	1183	5989-27-5	-	-	-	0.1	-	-	-	-	-	-	-	0.3	0.1	tr	-	-
Eucalyptol[c]	1191	470-82-6	0.9	-	-	-	-	-	-	-	5.7	1.8	2.9	-	1.0	2.5	1.6	0.4
(Z)-Ocimene[c]	1195	3338-55-4	1.2	-	0.6	tr	-	-	-	-	-	-	-	-	1.1	2.0	0.4	tr
(E)-Ocimene[b]	1221	3779-61-1	tr	0.4	0.3	tr	-	-	-	tr	-	-	-	-	-	4.3	0.3	tr
Unidentified Monoterpene[a]	1255		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr
(E)-4,8-Dimethyl-1,3,7-nonatriene[a]	1277	51911-82-1	-	-	-	-	0.4	-	-	-	-	-	-	-	-	-	-	-
(E)-Linalool oxide (furanoid)[c]	1401	34995-77-2	2.2	1.5	4.5	1.8	tr	1.3	0.6	1.5	0.1	0.4	0.9	0.4	1.1	0.1	0.8	0.5
6-Methyl-5-hepten-2-ol[b]	1420	1569-60-4	-	-	-	-	-	-	-	-	-	-	0.2	-	-	-	-	-
(Z)-Linalool oxide (furanoid)[c]	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
Unidentified monoterpene[a]	1482		-	-	-	-	-	-	-	0.2	-	-	-	-	-	-	-	-
Linalool[c]	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
Unidentified monoterpene[a]	1501		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr
Lilac aldehyde (isomer 1)[a]	1439		-	tr	-	-	-	-	-	0.1	-	-	-	tr	-	-	-	-
Lilac aldehyde (isomer 2)[a]	1520		-	0.5	-	0.4	-	-	-	0.7	-	-	-	tr	-	-	-	-
Lilac aldehyde (isomer 3)[a]	1542		-	-	-	-	-	-	-	0.2	-	tr	-	tr	-	-	-	-
Hotrienol[b]	1563	29957-43-5	-	0.1	tr	0.7	-	-	-	1.1	tr	0.1	0.1	0.1	0.3	-	tr	tr
Unidentified monoterpene[a]	1597									tr								tr
gamma-Butyrolactone[a]	1604	96-48-0	-	-	-	-	0.9	0.2	0.5	0.2	-	-	-	-	-	-	-	-
Unidentified monoterpene[a]	1621		-	-	-	-	-	-	-	-	-	-	-	-	0.4	-	-	tr
alpha-Terpineol[b]	1648	10482-56-1	-	-	-	-	-	-	-	-	-	0.1	-	tr	0.1	0.1	0.1	0.3
(E)-Linalool oxide (pyranoid)[a]	1694	14009-71-3	tr	tr	0.3	0.2	0.1	0.8	-	-	tr	tr	tr	0.1	0.1	-	-	tr
(Z)-Linalool oxide (pyranoid)[a]	1715	14009-71-3	3.2	0.7	0.6	0.9	-	-	0.4	0.5	0.1	0.3	0.5	0.3	0.3	-	0.2	0.1
3,7-dimethyl-6-Octen-1-ol[a]	1721	106-22-9	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	-	-
(Z)-3,7-dimethyl-2,6-Octadien-1-ol[a]	1754	106-25-2	-	-	-	-	-	-	-	-	-	-	-	tr	-	tr	-	-
(E)-3,7-dimethyl-2,6-Octadien-1-ol[a]	1798	106-24-1	-	-	-	-	-	-	-	-	-	-	-	tr	tr	0.1	tr	-
Sesquiterpenoids																		
alpha-Bergamotene[a]	1522	17699-05-7	-	-	-	-	-	-	-	-	-	-	-	-	tr	-	-	0.1
beta-Caryophyllene[c]	1563	87-44-5	-	-	-	-	-	-	-	-	-	-	-	-	4.2	tr	tr	-

Unidentified sesquiterpene[a]	1597		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr
Unidentified sesquiterpene[a]	1601		-	-	-	-	-	-	-	-	tr	-	-	-	-	-	-	-
alpha-Farnesene[b]	1707	502-61-4	-	-	-	-	-	-	0.2	0.3	-	-	-	-	-	-	-	tr
Nitrogen Containing Compounds																		
Indole[c]	2376	120-72-9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr
Unknowns																		
Unknown	1391		-	-	-	-	-	-	-	0.5	-	-	-	-	-	-	-	-
Unknown	1541		-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	-
Unknown	1546		-	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-
Unknown	1559		-	tr	-	0.1	-	-	-	0.1	-	-	-	-	-	0.4	0.2	tr
Unknown	1590		-	-	-	-	0.9	-	0.8	0.2	-	-	-	-	-	-	-	-
Unknown	1603		-	-	-	-	-	-	-	-	-	-	-	-	-	1.0	2.0	tr
Unknown	1627		-	-	-	-	-	-	-	-	-	-	-	-	0.5	0.6	-	-
Unknown	1687		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr
Unknown	1654		-	-	0.1	-	-	0.1	0.7	-	-	-	-	tr	tr	-	-	-
Unknown	1887		0.5	0.2	0.2	0.2	1.0	0.3	0.7	0.2	0.2	0.1	0.4	0.1	tr	tr	tr	tr

Appendix 2. Key compounds and compound classes in the scent of inflorescences at three different flowering stages for four *Protea* species. Data presented are average relative proportions over 5 samples of each floral flowering stage.

Compound class and name		*Protea caffra*			*Protea dracomontana*			*Protea simplex*			*Protea welwitschii*		
Flowering stage	**CAS**	**New**	**Open full**	**Old**	**New**	**Open full**	**Old**	**New**	**Open full**	**Old**	**New**	**Open full**	**Old**
Number of compounds		37	34	32	28	38	25	39	53	35	51	61	59
Aliphatic acids													
2-Methylpropanoic acid[b]	79-31-2	-	-	-	-	-	-	-	-	-	-	-	-
Aliphatic alcohols													
1-Hexanol[c]	111-27-3	6.00	0.82	-	-	-	-	0.66	0.33	-	2.36	3.28	0.81
2-Heptanol[a]	543-49-7	-	-	-	0.68	-	-	1.26	0.40	-	-	-	-
(Z)-3-Hexen-1-ol[a]	928-96-1	0.25	0.07	0.09	-	0.05	0.10	3.85	0.12	0.49	1.48	1.61	3.49
(E)-2-Hexen-1-ol[a]	2305-21-7	0.29	-	-	-	-	-	-	-	-	0.02	-	-
Unidentified aliphatic alcohol[a]		-	-	-	-	0.15	-	-	-	-	-	-	-
Other		tr	tr	tr	0.19	tr	0.35	tr	0.05	tr	tr	0.43	0.04
Aliphatic aldehydes (All)		-	-	-	-	0.31	0.07	-	-	-	0.03	-	-
Aliphatic alkanes													
(E)-2-Hexenal[b]	6728-26-3	-	-	-	-	-	-	-	-	-	-	-	-
Aliphatic esters													
3-Methylbutyl acetate[a]	123-92-2	-	-	-	-	-	3.93	-	-	-	-	-	-
Unidentified aliphatic ester[a]		-	-	-	-	-	1.06	-	0.25	-	-	0.04	-
4-Hexen-1-yl acetate[a]	72237-36-6	-	-	-	-	-	-	-	-	-	2.02	-	-
(Z)-3-Hexen-1-yl acetate[b]	3681-71-8	-	-	0.30	-	-	-	14.37	-	-	-	0.39	0.28
(Z)-3-Hexenyl isovalerate[a]	35154-45-1	-	-	-	-	-	-	0.86	-	-	1.09	0.92	1.56
Unidentified aliphatic ester[a]		-	-	-	-	0.01	-	0.01	-	-	0.41	1.15	0.70
Hexyl acetate[a]	142-92-7	-	-	-	-	-	-	0.71	tr	-	1.36	0.26	-
Unidentified aliphatic tiglate[a]		-	-	-	-	-	-	-	-	-	1.93	2.06	1.28
Methyl 2-methylbutanoate[a]	868-57-5	0.24	-	-	-	-	-	-	0.15	5.84	-	-	-
Unidentified aliphatic ester[a]		-	-	-	-	-	-	-	-	-	2.04	1.35	2.63
Other		0.31	0.22	1.23	0.23	0.52	0.20	0.14	0.05	0.56	0.98	1.88	0.85
Aliphatic ketones													
2-Heptanone[b]	110-43-0	-	-	-	-	-	-	5.82	2.39	0.52	-	-	-
Acetoin (3-hydroxy-2-butanone)[b]	513-86-0	-	1.75	2.36	-	0.34	0.13	-	0.58	-	-	-	-
Other		-	-	-	0.34	tr	tr	-	-	-	-	-	-
Aromatic alcohols													
Benzyl alcohol[c]	100-51-6	1.08	0.37	4.17	0.18	0.30	1.76	0.53	0.68	1.56	0.65	0.74	1.28
Phenylethyl alcohol[c]	60-12-8	0.07	0.05	0.55	0.03	0.26	0.70	0.09	0.05	0.33	0.22	0.10	0.47
Aromatic aldehydes													
Benzaldehyde[c]	100-52-7	2.55	0.22	2.66	1.51	0.69	3.16	2.03	1.74	5.59	0.13	0.25	0.53

Aromatic esters													
Methyl benzoate[c]	93-58-3	0.09	1.11	0.88	0.12	3.56	20.23	15.65	10.41	11.81	-	-	0.02
Other		0.08	0.11	1.08	-	-	0.37	0.48	0.27	0.27	0.29	0.40	0.55
Aromatic ethers													
Anisole[c]	100-66-3	19.03	0.94	36.58	10.86	5.10	35.36	-	6.35	16.14	0.30	0.49	9.33
Other		0.40	0.09	-	0.40	0.11	-	-	0.25	0.80	-	0.08	0.05
Irregular terpenes													
6-Methyl-5-hepten-2-one[a]	110-93-0	1.02	0.16	1.01	0.59	-	-	0.70	0.11	0.53	0.01	0.04	0.02
Other		-	-	-	-	-	-	0.02	-	-	-	-	-
Monoterpenes													
(E)-Ocimene[b]	3779-61-1	0.99	0.24	0.52	1.90	1.44	1.85	0.34	1.13	0.22	2.09	1.18	-
(Z)-Ocimene[c]	3338-55-4	0.14	0.36	-	1.05	1.08	tr	0.02	0.70	-	1.00	0.72	3.53
Linalool[c]	78-70-6	31.71	57.15	6.75	49.66	66.66	22.05	48.54	57.94	26.21	66.06	61.25	47.59
3,7-dimethyl-*(E)*-2,6-Octadien-1-ol[a]	106-24-1	0.06	0.02	-	-	0.03	-	-	0.03	-	0.04	0.05	1.49
2,6-dimethyl-3,7-Octadiene-2,6-diol[a]	13741-21-4	0.03	0.04	1.79	-	0.05	-	0.05	0.06	-	0.07	0.12	0.22
alpha-Pinene[c]	80-56-8	9.16	0.27	tr	24.82	8.42	tr	0.36	3.55	4.08	0.78	1.55	1.03
beta-Myrcene[b]	127-91-3	16.67	4.64	17.70	1.90	1.13	-	0.33	0.71	0.08	0.80	0.73	1.18
beta-Pinene[c]	123-35-3	3.71	0.59	0.22	0.26	-	-	0.27	2.59	1.33	0.99	1.82	1.26
(E)-Linalool oxide (furanoid) [a]	34995-77-2	0.04	0.39	0.31	0.09	0.50	0.69	0.07	0.41	1.00	0.28	0.13	0.49
(Z)-Linalool oxide (furanoid)[a]	5989-33-3	0.12	1.28	0.94	0.47	0.93	1.07	0.21	1.01	0.92	0.16	0.01	0.23
p-Cymene[b]	99-87-6	-	-	-	-	-	-	-	0.24	-	tr	tr	0.73
Eucalyptol[c]	470-82-6	1.23	0.81	0.21	-	-	-	0.56	0.63	-	3.99	5.83	0.47
Limonene[c]	5989-27-5	1.19	tr	1.76	1.21	0.51	-	0.02	1.16	0.64	0.60	0.57	8.19
Sabinene[a]	3387-41-5	-	-	-	-	-	-	0.84	1.08	-	7.08	9.65	8.03
Other		0.21	0.42	1.87	0.60	0.62	1.06	0.15	0.56	0.51	0.61	0.53	1.14
Nitrogen containing compounds													
Indole[c]	120-72-9	0.25	-	0.09	-	-	-	-	-	-	tr	tr	tr
Other aromatic compounds													
Styrene[b]	100-42-5	2.97	27.79	14.74	2.72	7.05	5.77	0.39	3.89	18.89	-	-	-
Sesquiterpenes													
beta-Caryophyllene[c]	87-44-5	-	0.05	0.34	0.18	0.01	-	0.12	-	0.14	0.08	0.26	0.31
alpha-Bergamotene[a]	17699-05-7	-	0.03	1.73	-	-	-	0.01	0.04	0.01	-	-	-
Other		0.11	tr	0.10	tr	0.17	0.08	tr	0.08	1.53	0.05	0.04	0.17
Unknowns Total		-	-	0.04	-	-	-	0.02	tr	-	0.02	0.10	0.04

CHAPTER 7

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 for the floral scent chemistry in *Protea* species, and 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 ten-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, about two-fold greater than in bird-pollinated species. 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 *P. simplex* over those of the bird-pollinated sympatric congener, *P. roupelliae*. This study shows that shifts from passerine bird- to insect pollination may be caused by marked up-regulation and compositional changes in floral scent emissions.

KEY WORDS: Beetle pollination; Cetoniinae; gas chromatography-mass spectrometry; pollination shift; *Protea*

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. Both birds and beetles visit nearly all the *Protea* species presented in this study, but differences in plant and flower morphology, pollen and nectar rewards, in addition to stronger fruitier floral scents, result in a beetle pollination system in grassland and savanna *Protea* species.

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 (Knudsen and Tollsten, 1993, 1995; Jürgens et al., 2000; Stensmyr et al., 2002; Raguso et al., 2003).

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 experiments and manipulative field experiments. For example, Kessler *et al.* (2008) genetically manipulated the emission of two volatile compounds in *Nicotiana attenuata* 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 et al., 1979; Knudsen et al., 2004). 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* (Johnson et al., 2006)). Rodent-pollinated *Protea* species have a yeasty scent to humans, but chemical investigation of the scent of these species is in the preliminary stages and will be reported elsewhere.

Pollination by beetles has been documented in four grassland and savanna *Protea* species in South Africa (Steenhuisen and Johnson, 2012; chapter 3). 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 et al., 2010a). Olfactory signals to Cetoniinae have mostly received interest in terms of optimizing 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 (McGovern and Beroza, 1970; Ladd et al., 1976; Donaldson et al., 1986; Donaldson et al., 1990; Larsson et al., 2003; Johnson et al., 2007; Wolde-Hawariat et al., 2007).

The four beetle-pollinated *Protea* species included in this study belong to a clade (the "red, grassland, savanna and mountain sugarbushes") which includes eleven other species (Valente et al., 2010; Schnitzler et al., 2011). Floral scents of some of these species are also sweet or fruity and insects may play an important role in pollination for these related species. As the ancestors to this 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 evolutionary shifts from bird to insect pollination in this clade. The aims of this study were thus, 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 systems 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.

MATERIALS AND METHODS

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), some populations, especially of *P. caffra,* can be heavily visited by birds.

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*.

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 insects are important vectors of pollen in many of these species . Bird pollination of the white *Protea* species *P. punctata* and *P. subvestita* was predicted from observations by various researchers (De Swardt and Louw, 1994; Carlson and Holsinger, 2010). 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).

Gas chromatography-mass spectrometry (GCMS) analysis of floral scent — Floral scent was collected using dynamic headspace extraction methods 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 (3/4 - all florets fully dehisced) in a polyacetate bag (Toppits oven bags and Kalle Nalophan), allowing scent volatiles to equilibrate for 0-90 min, 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 realized 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 ionization mode (Amirav and Dagan, 1997; Gordin and Amirav, 2000;

Dötterl et al., 2005). 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 standards 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 standards and used to calculate the emission rate per compound and for whole inflorescences as $ng.^{-1}flw.^{-1}hr$. 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, 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* (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 generalized linear model with a Poisson distribution corrected for overdisperion, loglink function, and likelihood ratio Chi-square statistics in PASW SPSS version 18 (McCullagh and Nelder, 1989; Hosmer and Lemeshow, 2000; Field, 2009). 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 the similarity in scent composition between beetle- and bird-pollinated species in using mean whole flower emission rates for each compound ($ng.^{-1}flw.^{-1}hr$) and the proportion of each compound contributing to whole flower scents (percentage). 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. 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 beetle- and bird-pollinated species using ANOSIM (Analysis of Similarities). Significance of the test statistic *R* generated by ANOSIM was assessed by 10 000 random permutations of the grouping vector to obtain an empirical distribution of *R* under the null model. This was followed by a SIMPER analyses to determine which compounds were responsible for any differences between groups.

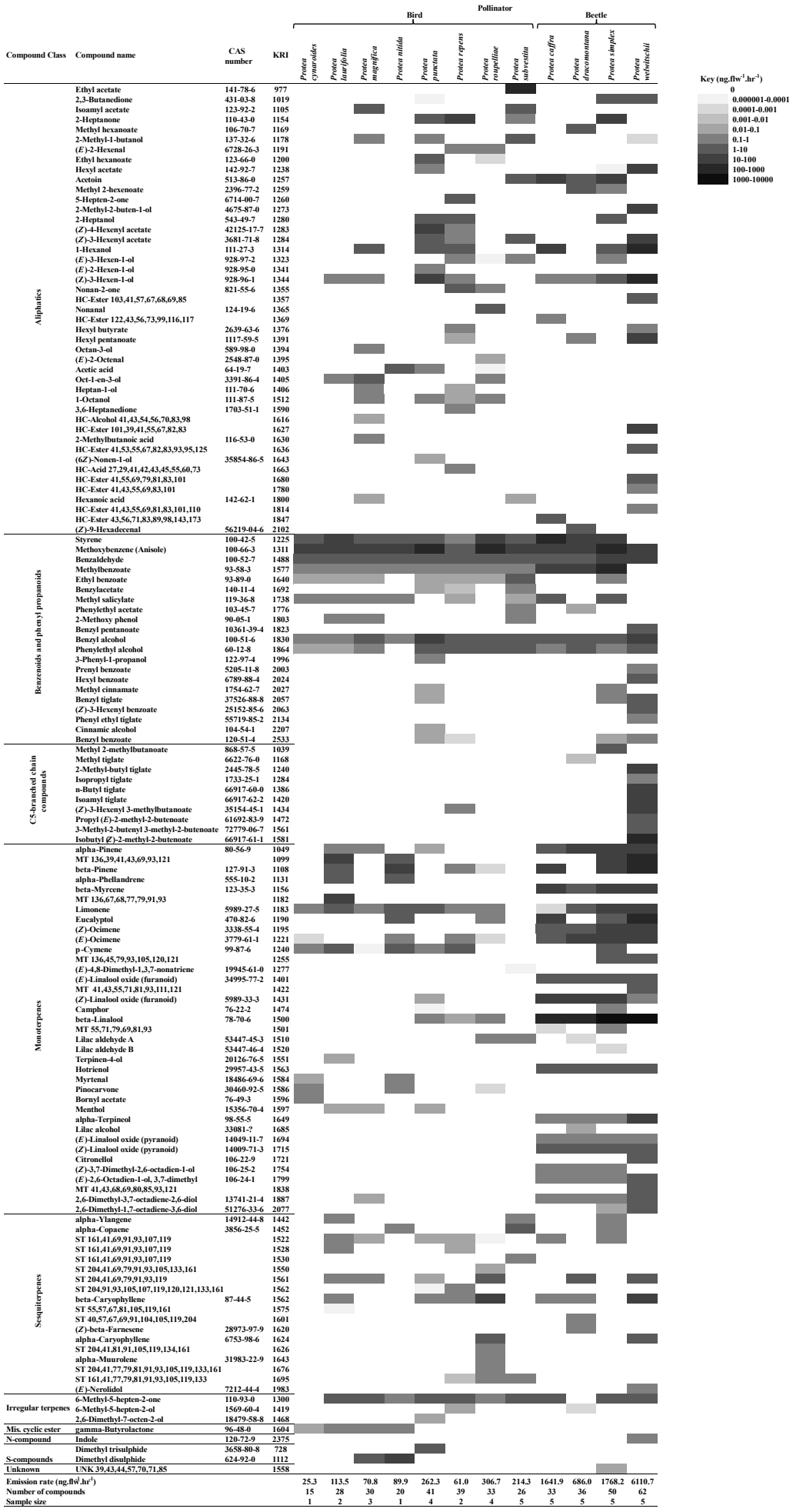
Beetle attraction to scent — Choice experiments were conducted to determine whether the cetoniine beetle, *Atrichelaphinis tigrina*, a common pollinator of the grassland *Protea* species preferred the fruity scent of flowers of *P. simplex* over that of the sympatric bird-pollinated *P. roupelliae*. 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 *Protea 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).

These same beetles were then used in choice experiments conducted with inflorescences of *P. simplex* and *P. roupelliae*. Two trials were conducted, each using thirty-five beetles. 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 beetles were stored frozen until they could be pinned and identified to sex (Holm and Marais, 1992).

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 were analysed using a binomial test.

RESULTS

Gas chromatography-mass spectrometry (GCMS) analysis of floral scent — We identified a total of 139 volatile compounds from the headspace of the twelve *Protea* species sampled. 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 *P. caffra, P. dracomontana, P. simplex* and *P. welwitschii* with an average emission rate of 1576 $ng.flw^{-1}.hr^{-1}$ in these species compared with 0.09 $ng.flw^{-1}.hr^{-1}$ for inflorescences of bird-pollinated species. Three benzenoid compounds (anisole, benzaldehyde, benzyl alcohol) were shared between all twelve *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 *P. nitida.* The fermentation volatile, acetoin, was evident in scent emissions of the three beetle-pollinated *Protea* species and the putatively bird-pollinated *P. subvestita*. 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, two groups were notable, one consisting of *P. laurifolia* and *P. nitida* that shared relatively higher emissions of the monoterpenes *beta*-myrcene, *beta*-pinene and *beta*-phellandrene, the other consisting of *P. punctata* and *P. repens*, which shared a variety of C6 aliphatics, or "green-leaf volatiles". The scent profile of *P. cynaroides* was the least diverse, with a total of only fifteen 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.



Compound Class	Compound name	CAS number	KRI
Aliphatics	Ethyl acetate	141-78-6	977
	2,3-Butanedione	431-03-8	1019
	Isoamyl acetate	123-92-2	1105
	2-Heptanone	110-43-0	1154
	Methyl hexanoate	106-70-7	1169
	2-Methyl-1-butanol	137-32-6	1178
	(*E*)-2-Hexenal	6728-26-3	1191
	Ethyl hexanoate	123-66-0	1200
	Hexyl acetate	142-92-7	1238
	Acetoin	513-86-0	1257
	Methyl 2-hexenoate	2396-77-2	1259
	5-Hepten-2-one	6714-00-7	1260
	2-Methyl-2-buten-1-ol	4675-87-0	1273
	2-Heptanol	543-49-7	1280
	(*Z*)-4-Hexenyl acetate	42125-17-7	1283
	(*Z*)-3-Hexenyl acetate	3681-71-8	1284
	1-Hexanol	111-27-3	1314
	(*E*)-3-Hexen-1-ol	928-97-2	1323
	(*E*)-2-Hexen-1-ol	928-95-0	1341
	(Z)-3-Hexen-1-ol	928-96-1	1344
	Nonan-2-one	821-55-6	1355
	HC-Ester 103,41,57,67,68,69,85		1357
	Nonanal	124-19-6	1365
	HC-Ester 122,43,56,73,99,116,117		1369
	Hexyl butyrate	2639-63-6	1376
	Hexyl pentanoate	1117-59-5	1391
	Octan-3-ol	589-98-0	1394
	(*E*)-2-Octenal	2548-87-0	1395
	Acetic acid	64-19-7	1403
	Oct-1-en-3-ol	3391-86-4	1405
	Heptan-1-ol	111-70-6	1406
	1-Octanol	111-87-5	1512
	3,6-Heptanedione	1703-51-1	1590
	HC-Alcohol 41,43,54,56,70,83,98		1616
	HC-Ester 101,39,41,55,67,82,83		1627
	2-Methylbutanoic acid	116-53-0	1630
	HC-Ester 41,53,55,67,82,83,93,95,125		1636
	(6*Z*)-Nonen-1-ol	35854-86-5	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	142-62-1	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	56219-04-6	2102
Benzenoids and phenyl propanoids	Styrene	100-42-5	1225
	Methoxybenzene (Anisole)	100-66-3	1311
	Benzaldehyde	100-52-7	1488
	Methylbenzoate	93-58-3	1577
	Ethyl benzoate	93-89-0	1640
	Benzylacetate	140-11-4	1692
	Methyl salicylate	119-36-8	1738
	Phenylethyl acetate	103-45-7	1776
	2-Methoxy phenol	90-05-1	1803
	Benzyl pentanoate	10361-39-4	1823
	Benzyl alcohol	100-51-6	1830
	Phenylethyl alcohol	60-12-8	1864
	3-Phenyl-1-propanol	122-97-4	1996
	Prenyl benzoate	5205-11-8	2003
	Hexyl benzoate	6789-88-4	2024
	Methyl cinnamate	1754-62-7	2027
	Benzyl tiglate	37526-88-8	2057
	(*Z*)-3-Hexenyl benzoate	25152-85-6	2063
	Phenyl ethyl tiglate	55719-85-2	2134
	Cinnamic alcohol	104-54-1	2207
	Benzyl benzoate	120-51-4	2533
C5-branched chain compounds	Methyl 2-methylbutanoate	868-57-5	1039
	Methyl tiglate	6622-76-0	1168
	2-Methyl-butyl tiglate	2445-78-5	1240
	Isopropyl tiglate	1733-25-1	1284
	n-Butyl tiglate	66917-60-0	1386
	Isoamyl tiglate	66917-62-2	1420
	(*Z*)-3-Hexenyl 3-methylbutanoate	35154-45-1	1434
	Propyl (*E*)-2-methyl-2-butenoate	61692-83-9	1472
	3-Methyl-2-butenyl 3-methyl-2-butenoate	72779-06-7	1561
	Isobutyl (*Z*)-2-methyl-2-butenoate	66917-61-1	1581
Monoterpenes	alpha-Pinene	80-56-9	1049
	MT 136,39,41,43,69,93,121		1099
	beta-Pinene	127-91-3	1108
	alpha-Phellandrene	555-10-2	1131
	beta-Myrcene	123-35-3	1156
	MT 136,67,68,77,79,91,93		1182
	Limonene	5989-27-5	1183
	Eucalyptol	470-82-6	1190
	(*Z*)-Ocimene	3338-55-4	1195
	(*E*)-Ocimene	3779-61-1	1221
	p-Cymene	99-87-6	1240
	MT 136,45,79,93,105,120,121		1255
	(*E*)-4,8-Dimethyl-1,3,7-nonatriene	19945-61-0	1277
	(*E*)-Linalool oxide (furanoid)	34995-77-2	1401
	MT 41,43,55,71,81,93,111,121		1422
	(*Z*)-Linalool oxide (furanoid)	5989-33-3	1431
	Camphor	76-22-2	1474
	beta-Linalool	78-70-6	1500
	MT 55,71,79,69,81,93		1501
	Lilac aldehyde A	53447-45-3	1510
	Lilac aldehyde B	53447-46-4	1520
	Terpinen-4-ol	20126-76-5	1551
	Hotrienol	29957-43-5	1563
	Myrtenal	18486-69-6	1584
	Pinocarvone	30460-92-5	1586
	Bornyl acetate	76-49-3	1596
	Menthol	15356-70-4	1597
	alpha-Terpineol	98-55-5	1649
	Lilac alcohol	33081-?	1685
	(*E*)-Linalool oxide (pyranoid)	14049-11-7	1694
	(*Z*)-Linalool oxide (pyranoid)	14009-71-3	1715
	Citronellol	106-22-9	1721
	(*Z*)-3,7-Dimethyl-2,6-octadien-1-ol	106-25-2	1754
	(*E*)-2,6-Octadien-1-ol, 3,7-dimethyl	106-24-1	1799
	MT 41,43,68,69,80,85,93,121		1838
	2,6-Dimethyl-3,7-octadiene-2,6-diol	13741-21-4	1887
	2,6-Dimethyl-1,7-octadiene-3,6-diol	51276-33-6	2077
Sesquiterpenes	alpha-Ylangene	14912-44-8	1442
	alpha-Copaene	3856-25-5	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	87-44-5	1562
	ST 55,57,67,81,105,119,161		1575
	ST 40,57,67,69,91,104,105,119,204		1601
	(*Z*)-beta-Farnesene	28973-97-9	1620
	alpha-Caryophyllene	6753-98-6	1624
	ST 204,41,81,91,105,119,134,161		1626
	alpha-Muurolene	31983-22-9	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	7212-44-4	1983
Irregular terpenes	6-Methyl-5-hepten-2-one	110-93-0	1300
	6-Methyl-5-hepten-2-ol	1569-60-4	1419
	2,6-Dimethyl-7-octen-2-ol	18479-58-8	1468
Mis. cyclic ester	gamma-Butyrolactone	96-48-0	1604
N-compound	Indole	120-72-9	2375
S-compounds	Dimethyl trisulphide	3658-80-8	728
	Dimethyl disulphide	624-92-0	1112
Unknown	UNK 39,43,44,57,70,71,85		1558
Emission rate (ng.flw^{-1}.hr^{-1})			
Number of compounds			
Sample size			

Protea cynaroides	*Protea laurifolia*	*Protea magnifica*	*Protea nitida*	*Protea punctata*	*Protea repens*	*Protea roupelliae*	*Protea subvestita*	*Protea caffra*	*Protea dracomontana*	*Protea simplex*	*Protea welwitschii*
25.3	113.5	70.8	89.9	262.3	61.0	306.7	214.3	1641.9	686.0	1768.2	6110.7
15	28	30	20	41	39	33	26	33	36	50	62
1	2	3	1	4	2	4	5	5	5	5	5

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 CAS numbers and Kovats indices are given for each. Grey shading is based on a log scale (the first shade of light grey spans two log increments instead of one). Abbreviations: HC = hydrocarbon; MT = monoterpene; ST = Sesquiterpene.

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

We found highly significant separation between beetle- and bird-pollinated species with respect to scent composition using emission rates (2D stress value=0.10; ANOSIM R=0.881, P<0.01) and proportional data (2D stress value=0.11; R=0.814, P<0.01) (Fig. 4). The higher emission and abundance of linalool contributed to the greatest difference between beetle- and bird-pollinated *Protea* scents (9.4 and 14.8 % 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 and an unknown; the 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 and benzaldehyde, 6-methyl-5-hepten-2-one and ethyl acetate in bird-pollinated species profiles.

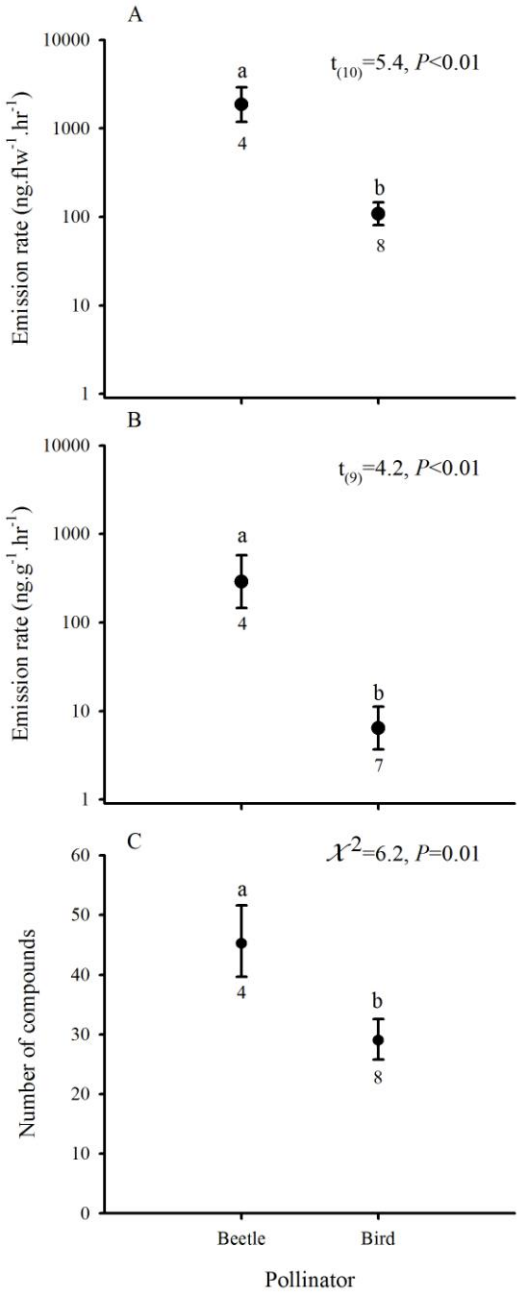


Fig. 3 Comparisons of mean floral scents of beetle- and bird-pollinated *Protea* species for (A) emission rates per inflorescence, (B) mass-specific emission rates (based on dry mass of inflorescence tissue), and (C) the mean adjusted number of compounds comprising the floral scents. Sample sizes are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

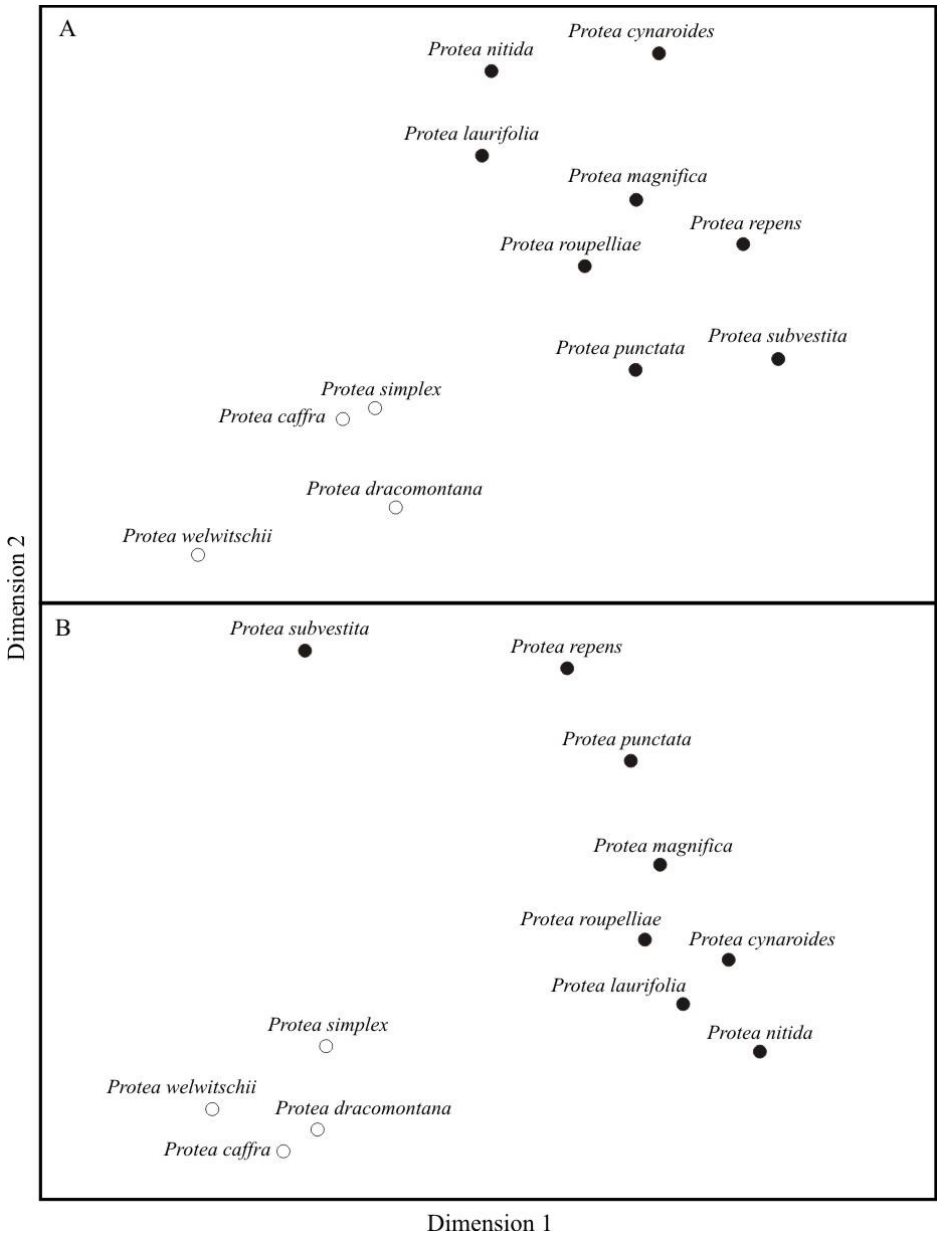


Fig. 4 Non-metric multidimensional scaling (NMDS) of (A) whole flower emission rates ($ng.flw^{-1}.hr^{-1}$) and (B) composition of scent from twelve *Protea* species. Open and closed circles depict beetle- and bird-pollinated species respectively. Both NMDS' are based on Bray-Curtis similarities (stress factor = 0.1 for both analyses).

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 *P. roupelliae,* for both the *Protea*-experienced beetles from Mount Gilboa (binomial test, $P<0.01$) and the naive beetles from Cobham Nature Reserve (binomial test, $P<0.01$; Fig. 5).

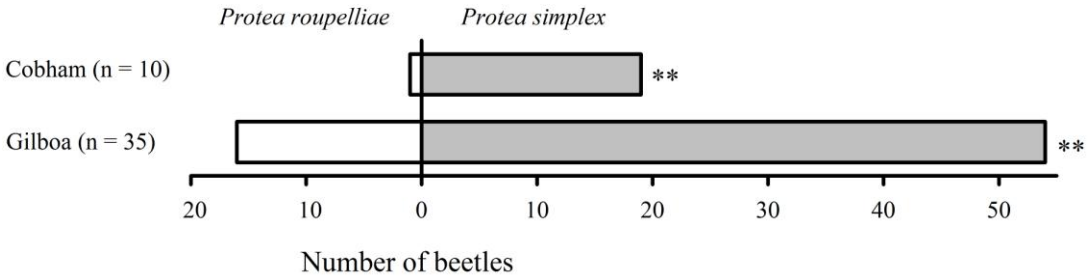


Fig. 5 The scent preference of *Atrichelaphinis tigrina* beetles from Cobham and Mount Gilboa, when offered the scents of whole inflorescences of sympatric *Protea simplex* and *Protea roupelliae* in a Y-tube olfactometer (binomial test: ** = $P < 0.01$). Each beetle was tested twice and with opposite orientation of stimuli to eliminate any environmental bias to either arm of the olfactometer.

DISCUSSION

This study confirms that the floral scents of beetle-pollinated *Protea* species are distinct from those of bird-pollinated congenerics 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.

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 twelve species, characterized by an absence of methyl benzoate and styrene, and the presence of over twenty unique aliphatic and benzenoid esters.

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 produced by plants (Cseke et al., 2007). Anisole is commonly reported in cockchafer (Melolonthinae) sex pheromones and used as an attractant to trap these pests. 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 pollinator shifts 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. 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 bird-pollinated *P. punctata.* Cinnamic alcohol along with other benzenoids and monterpenes 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 et al., 1985; Hargreaves et al., 2004) (e.g. Fig. 1D). We 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

stronger 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,* 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 numerous beetle-pollinated plant fragrances, (e.g. *Magnolia* species; Azuma et al., 2001), and also notably in most hawkmoth-pollinated plants worldwide (e.g. 56.8% linalool in sweet scent of *Coussarea papaya*; (Kaiser, 1993; Knudsen et al., 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* (0.2 %, S.D. Johnson & R.A. Raguso, unpubl. data), suggesting that 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 dominating compound in scents of beetle-pollinated *Protea* species, but it was emitted in 500-3500-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 it's 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 *C. breweri* is associated with the up-regulation of linalool and its oxides and a change to night-blooming (Raguso et al., 1995).

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 five-fold greater inflorescence mass. This was the case both for experienced beetles collected off *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 from trapping experiments conducted in the field with 69 different scent compounds, Donaldson *et al.* (1990) found linalool to be the seventh most attractive volatile to Cetoniinae and Rutelinae (family Scarabaeidae), although it isn't clear if this was a racemic mixture of the two chiral forms of linalool, each with its own biological roles and biosynthetic origin. This monoterpene is strongly attractive to several phytophagous cetoniine pests in olfactometer and field trapping experiments (Larsson et al., 2003; Bengtsson et al., 2009; Vuts et al., 2010).

In a study of the function of scent components of *Satyrium microrrhynchum* using gas chromatograph-electroantennographic detection (GCEAD), 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. 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, methyl2-methylbutanoate, found here only in the floral scent *P. simplex*, has recently been shown to be attractive to scarab beetles (Gottsberger et al., 2012).

Slight changes in chemical structure can affect the attractiveness of a compound to some cetoniines. For example, esterification of cinnamyl alcohol into cinnamyl 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). This trend could potentially be observed in *P. welwitschii* in which the esterification of alcohols lead to the production of over 20 volatile esters unique to this species. 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. The attractiveness of these compounds would need to be tested to determine if this trend is adaptive or just a consequence of particular enzymes found in this species.

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 (Gottsberger, 1990; Englund, 1993; Bernhardt, 2000). Our statistical analyses of emission rates and the number of compounds between beetle- and bird-pollinated species did not 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*, 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 complex scent chemistry 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.

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CHAPTER 7

SUPPLEMENTARY MATERIAL

Appendix Table 1. Sampling localities and dates for twelve *Protea* study species.

Species	Plant locality	Sampling date (d/m/y)
Protea caffra	Krantzkloof (29.77°S, 30.84°E, 450m)	1/2/2008
Protea cynaroides	Kirstenbosch Botanical Gardens (33.99°S, 18.43°E, 100m)	24/9/2006
Protea dracomontana	Garden Castle (29.74°S, 29.20°E, 1900m)	11/1/2008
Protea laurifolia	Franschhoek Pass (33.92°S, 19.16°E, 632m)	24/9/2006
	Bainskloof Pass (33.62°S, 19.10°E, 569m)	15/7/2008
Protea magnifica	Jonaskop (33.97°S, 19.50°E, 1027m)	24/9/2006, 15/7/2008
Protea nitida	Franschhoek Pass (33.92°S, 19.16°E, 632m)	24/9/2006
Protea repens	Franschhoek Pass (33.92°S, 19.16°E, 632m)	24/9/2006
	Bainskloof Pass (33.62°S, 19.10°E, 569m)	13/7/2008
Protea punctata	Jonaskop (33.97°S, 19.50°E, 1027m)	15-16/7/2008
Protea roupelliae	Mount Gilboa (29.29°S, 30.29°E, 1770 m)	15/2/2008, 30/1/2008
Protea simplex	Mount Gilboa (29.29°S, 30.29°E, 1770 m)	11/1/2008
Protea subvestita	Sani Pass (29.60°S, 29.34°E, 1963m)	20/2/2008
Protea welwitschii	Winston Park (28.75°S, 30.75°E, 550m)	1/1/2008

Appendix Table 2. Compounds (%) comprising the floral scents of twelve *Protea* species. KRI=Kovats retention index, tr = trace amount (<0.01%).

Species			*Protea cynaroides*	*Protea laurifolia*	*Protea magnifica*	*Protea nitida*	*Protea punctata*	*Protea repens*	*Protea roupelliae*	*Protea subvestita*	*Protea caffra*	*Protea dracomontana*	*Protea simplex*	*Protea welwitschii*
Pollinator			bird	bird	bird	bird	bird	bird	bird	bird	bird	bird	bird	bird
Number of samples			1	2	3	1	4	2	4	5	5	5	5	5
Number of compounds			15	28	30	20	41	39	33	26	33	36	50	62
Emission rate (ng/flw/hr)			25.32	113.53	70.79	89.88	262.26	61.00	306.65	214.27	1641.90	685.97	1768.18	6110.74
Compound class and name	CAS number	KRI												
Aliphatics														
Ethyl acetate	141-78-6	977	-	-	-	-	-	-	-	73.93	-	-	-	-
2,3-Butanedione	431-03-8	1019	-	-	-	-	tr	-	-	-	-	-	0.25	0.04
Isoamyl acetate	123-92-2	1105	-	-	9.37	-	-	-	-	2.09	-	-	-	-
2-Heptanone	110-43-0	1154	-	-	-	-	4.24	25.89	-	0.45	-	-	2.39	-
Methyl hexanoate	106-70-7	1169	-	-	-	-	-	-	-	-	-	0.22	-	-
2-Methyl-1-butanol	137-32-6	1178	-	-	1.72	-	1.69	-	-	1.94	-	-	-	tr
(E)-2-Hexenal	6728-26-3	1191	-	-	-	-	-	2.49	0.29	-	-	-	-	-
Ethyl hexanoate	123-66-0	1200	-	-	-	-	0.33	-	tr	-	-	-	-	-
Hexyl acetate	142-92-7	1238	-	-	-	-	0.28	-	-	-	-	-	tr	0.26
Acetoin	513-86-0	1257	-	-	-	-	-	-	-	1.03	1.76	0.34	0.58	-
Methyl 2-hexenoate	2396-77-2	1259	-	-	-	-	-	-	-	-	-	0.30	0.03	-
5-Hepten-2-one	6714-00-7	1260	-	-	-	-	-	2.82	-	-	-	-	-	-
2-Methyl-2-buten-1-ol	4675-87-0	1273	-	-	-	-	-	-	-	-	-	-	-	0.43
2-Heptanol	543-49-7	1280	-	-	-	-	1.22	1.89	-	-	-	-	0.40	-
(Z)-4-Hexenyl acetate	42125-17-7	1283	-	-	-	-	8.38	0.70	-	-	-	-	-	-
(Z)-3-Hexenyl acetate	3681-71-8	1284	-	-	-	-	11.05	1.64	-	0.63	-	-	-	0.39
1-Hexanol	111-27-3	1314	-	-	1.86	-	2.98	5.99	-	-	0.82	-	0.34	3.28
(E)-3-Hexen-1-ol	928-97-2	1323	-	-	-	-	-	0.29	0.00	0.08	-	-	0.05	-

(E)-2-Hexen-1-ol	928-95-0	1341	-	-	-	-	0.10	-	-	-	-	-	-	-
(Z)-3-Hexen-1-ol	928-96-1	1344	-	0.07	0.71	-	13.59	3.75	-	-	0.07	0.05	0.12	1.61
Nonan-2-one	821-55-6	1355	-	-	-	-	-	1.99	0.05	-	-	-	-	-
HC-Ester 103,41,57,67,68,69,85		1357	-	-	-	-	-	-	-	-	-	-	-	0.18
Nonanal	124-19-6	1365	-	-	-	-	-	-	3.00	-	-	-	-	-
HC-Ester 122,43,56,73,99,116,117		1369	-	-	-	-	-	-	-	-	0.01	-	-	-
Hexyl butyrate	2639-63-6	1376	-	-	-	-	-	0.45	-	-	-	-	-	0.01
Hexyl pentanoate	1117-59-5	1391	-	-	-	-	-	0.29	-	-	-	0.01	-	1.15
Octan-3-ol	589-98-0	1394	-	-	0.15	-	-	-	-	-	-	-	-	-
Acetic acid	64-19-7	1403	-	-	-	1.86	0.99	-	0.00	-	-	-	-	-
Oct-1-en-3-ol	3391-86-4	1405	-	0.08	6.39	-	-	-	0.22	-	-	-	-	-
Heptan-1-ol	111-70-6	1406	-	-	1.20	-	-	0.28	-	-	-	-	-	-
1-Octanol	111-87-5	1512	-	-	0.82	-	0.13	0.30	0.15	-	-	-	-	-
3,6-Heptanedione	1703-51-1	1590	-	-	-	-	-	0.24	-	-	-	-	-	-
Bornyl acetate	76-49-3	1596	1.92	-	-	-	-	-	-	-	-	-	-	-
HC-Alcohol 41,43,54,56,70,83,98		1616	-	-	0.04	-	-	-	-	-	-	-	-	-
HC-Ester 101,39,41,55,67,82,83		1627	-	-	-	-	-	-	-	-	-	-	-	1.29
2-Methylbutanoic acid	116-53-0	1630	-	-	0.39	-	-	-	-	-	-	-	-	-
HC-Ester 41,53,55,67,82,83,93,95,125		1636	-	-	-	-	-	-	-	-	-	-	-	0.04
(6Z)-Nonen-1-ol	35854-86-5	1643	-	-	-	-	0.01	-	-	-	-	-	-	-
HC-Acid 27,29,41,42,43,45,55,60,73		1663	-	-	-	-	-	0.31	-	-	-	-	-	-
HC-Ester 41,55,69,79,81,83,101		1680	-	-	-	-	-	-	-	-	-	-	-	0.02
HC-Ester 41,43,55,69,83,101		1780	-	-	-	-	-	-	-	-	-	-	-	0.01
Hexanoic acid	142-62-1	1800	-	-	0.11	-	-	-	-	0.04	-	-	-	-
HC-Ester 41,43,55,69,81,83,101,110		1814	-	-	-	-	-	-	-	-	-	-	-	tr
HC-Ester 43,56,71,83,89,98,143,173		1847	-	-	-	-	-	-	-	-	0.21	-	-	-
(Z)-9-Hexadecenal	56219-04-6	2102	-	-	-	-	-	-	-	-	-	0.32	-	-
Benzenoids and phenyl propanoids														
Styrene	100-42-5	1225	6.22	18.48	10.06	2.49	4.81	0.74	34.70	3.00	27.82	7.05	3.90	-
Methoxybenzene (Anisole)	100-66-3	1311	66.46	24.35	38.60	27.86	28.79	5.30	30.94	6.09	0.94	5.12	6.37	0.49
Benzaldehyde	100-52-7	1488	14.66	4.09	6.92	3.90	3.72	5.85	4.29	1.37	0.22	0.69	1.75	0.25
Methylbenzoate	93-58-3	1577	3.49	0.78	0.32	0.63	0.06	0.86	0.23	0.21	1.11	3.56	10.45	-
Ethyl benzoate	93-89-0	1640	0.25	0.03	0.02	-	0.03	0.11	0.01	1.05	-	-	0.01	-

Benzylacetate	140-11-4	1692	-	-	-	-	0.06	0.02	-	0.38	-	-	-	-
Methyl salicylate	119-36-8	1738	2.09	0.28	0.10	0.33	-	0.10	-	0.01	0.11	-	0.23	-
Phenylethyl acetate	103-45-7	1776	-	-	-	-	-	-	-	0.31	-	tr	-	-
2-Methoxy phenol	90-05-1	1803	-	0.51	0.49	-	-	-	-	0.11	-	-	-	-
Benzyl pentanoate	10361-39-4	1823	-	-	-	-	-	-	-	-	-	-	-	0.14
Benzyl alcohol	100-51-6	1830	0.45	0.26	4.98	0.38	7.17	2.06	4.08	1.67	0.38	0.30	0.68	0.74
Phenylethyl alcohol	60-12-8	1864	0.14	0.10	0.59	-	1.58	6.70	0.51	2.66	0.05	0.26	0.05	0.10
3-Phenyl-1-propanol	122-97-4	1996	-	-	-	-	0.52	-	-	-	-	-	-	-
Prenyl benzoate	5205-11-8	2003	-	-	-	-	-	-	-	-	-	-	-	tr
Hexyl benzoate	6789-88-4	2024	-	-	-	-	-	-	-	-	-	-	-	0.08
Methyl cinnamate	1754-62-7	2027	-	-	-	-	0.03	-	-	-	-	-	0.01	-
Benzyl tiglate	37526-88-8	2057	-	-	-	-	0.01	-	-	-	-	-	0.01	0.11
(Z)-3-Hexenyl benzoate	25152-85-6	2063	-	-	-	-	-	-	-	-	-	-	-	0.05
Phenyl ethyl tiglate	55719-85-2	2134	-	-	-	-	-	-	-	-	-	-	-	0.01
Cinnamic alcohol	104-54-1	2207	-	-	-	-	0.03	-	-	-	-	-	-	-
Benzyl benzoate	120-51-4	2533	-	-	-	-	0.04	tr	-	-	-	-	0.01	tr
C5-branched chain compounds														
Methyl 2-methylbutanoate	868-57-5	1039	-	-	-	-	-	-	-	-	-	-	0.15	-
Methyl tiglate	6622-76-0	1168	-	-	-	-	-	-	-	-	-	tr	-	-
2-Methyl-butyl tiglate	2445-78-5	1240	-	-	-	-	-	-	-	-	-	-	-	0.82
Isopropyl tiglate	1733-25-1	1284	-	-	-	-	-	-	-	-	-	-	-	0.01
n-Butyl tiglate	66917-60-0	1386	-	-	-	-	-	-	-	-	-	-	-	0.39
Isoamyl tiglate	66917-62-2	1420	-	-	-	-	-	-	-	-	-	-	-	0.36
(Z)-3-Hexenyl 3-methylbutanoate	35154-45-1	1434	-	-	-	-	-	0.42	-	-	-	-	-	0.92
Propyl (E)-2-methyl-2-butenoate	61692-83-9	1472	-	-	-	-	-	-	-	-	-	-	-	0.03
3-Methyl-2-butenyl 3-methyl-2-butenoate	72779-06-7	1561	-	-	-	-	-	-	-	-	-	-	-	0.07
Isobutyl (Z)-2-methyl-2-butenoate	66917-61-1	1581	-	-	-	-	-	-	-	-	-	-	-	2.06
Monoterpenes														
alpha-Pinene	80-56-9	1049	-	0.17	1.13		0.13	-	-	-	0.27	8.44	3.56	1.55
MT 136,39,41,43,69,93,121		1099	-	13.13	-	5.76	-	-	-	-	-	-	1.09	9.66
beta-Pinene	127-91-3	1108	-	5.49	-	12.27	-	0.59	tr	-	0.59	-	2.60	1.82
alpha-Phellandrene	555-10-2	1131	-	4.81	-	1.13	-	-	-	-	-	-	-	-
beta-Myrcene	123-35-3	1156	-	-	-	-	-	-	-	-	4.64	1.14	0.71	0.73

MT 136,67,68,77,79,91,93		1182	-	14.46	-	-	-	-	-	-	-	-	-	-
Limonene	5989-27-5	1183	1.51	4.82	1.35	1.56	1.71	2.30	1.18	-	tr	0.51	1.17	0.57
Eucalyptol	470-82-6	1190	-	-	-	5.91	-	-	1.01	-	0.81		0.63	5.84
(Z)-Ocimene	3338-55-4	1195	-	-	-	-	-	-	-	-	0.36	1.08	0.70	0.72
(E)-Ocimene	3779-61-1	1221	tr	-	-	0.52	-	2.02	tr	-	0.24	1.44	1.13	1.18
p-Cymene	99-87-6	1240	1.76	3.13	tr	6.09	1.19	2.19	-	-	-	-	0.24	-
MT 136,45,79,93,105,120,121		1255	-	-	-	-	-	-	-	-	-	-	0.22	0.04
(E)-4,8-Dimethyl-1,3,7-nonatriene	19945-61-0	1277	-	-	-	-	-	-	-	tr	-	-	-	-
(E)-2-Octenal	2548-87-0	1395	-	-	-	-	-	-	0.01	-	-	-	-	-
(E)-Linalool oxide (furanoid)	34995-77-2	1401	-	-	-	-	-	-	-	-	0.39	0.50	0.41	0.13
MT 41,43,55,71,81,93,111,121		1422	-	-	-	-	-	-	-	-	-	-	-	0.06
(Z)-Linalool oxide (furanoid)	5989-33-3	1431	-	-	-	-	0.01	-	-	-	1.28	0.93	1.01	0.01
Camphor	76-22-2	1474	-	-	-	-	tr	-	-	-	-	-	0.03	-
beta-Linalool	78-70-6	1500	-	-	-	-	1.02	0.18	0.02	-	57.20	66.84	58.07	61.30
MT 55,71,79,69,81,93		1501	-	-	-	-	-	-	-	-	tr	-	0.02	-
Lilac aldehyde A	53447-45-3	1510	-	-	-	-	-	-	0.20	0.43	-	tr	-	-
Lilac aldehyde B	53447-46-4	1520	-	-	-	-	-	-	-	-	-	-	tr	-
(-)-Terpinen-4-ol	20126-76-5	1551	-	0.03	-	-	-	-	-	-	-	-	-	-
Hotrienol	29957-43-5	1563	-	-	-	-	-	-	-	-	0.10	0.37	0.14	0.19
(1R)-(-)-Myrtenal	18486-69-6	1584	0.19	-	-	0.44	-	-	-	-	-	-	-	-
Pinocarvone	30460-92-5	1586	0.63	-	-	0.53	-	-	tr	-	-	-	-	-
Menthol	15356-70-4	1597	-	0.02	0.18	-	0.01	-	-	-	-	-	-	-
alpha-Terpineol	98-55-5	1649	-	-	-	-	-	-	-	-	0.01	0.04	0.03	0.16
Lilac alcohol	33081-?	1685	-	-	-	-	-	-	-	-	-	0.00	-	-
(E)-Linalool oxide (pyranoid)	14049-11-7	1694	-	-	-	-	-	-	-	-	0.05	0.01	0.02	0.01
(Z)-Linalool oxide (pyranoid)	14009-71-3	1715	-	-	-	-	-	-	-	-	0.22	0.18	0.10	0.02
Citronellol	106-22-9	1721	-	-	-	-	-	-	-	-	-	-	-	0.04
(Z)-3,7-Dimethyl-2,6-octadien-1-ol	106-25-2	1754	-	-	-	-	-	-	-	-	0.03	0.02	0.02	-
(E)-2,6-Octadien-1-ol, 3,7-dimethyl	106-24-1	1799	-	-	-	-	-	-	-	-	0.02	0.03	0.04	0.05
MT 41,43,68,69,80,85,93,121		1838	-	-	-	-	-	-	-	-	-	-	-	0.07
2,6-Dimethyl-3,7-octadiene-2,6-diol	13741-21-4	1887	-	-	0.05	-	-	-	-	-	0.04	0.05	0.06	0.12
2,6-Dimethyl-1,7-octadiene-3,6-diol	51276-33-6	2077	-	-	-	-	-	-	-	-	-	-	tr	0.02
Sesquiterpene														

alpha-Ylangene	14912-44-8	1442	-	0.18	-	-	-	-	-	0.44	-	-	0.05	-
alpha-Copaene	3856-25-5	1452	-	-	-	0.38	-	-	-	1.14	-	-	0.03	-
ST 161,41,69,91,93,107,119		1522	-	0.22	0.31	-	0.40	0.06	tr	-	0.03	-	0.04	-
ST 161,41,69,91,93,107,119		1528	-	0.06	-	-	-	0.18	-	-	-	-	-	-
ST 161,41,69,91,93,107,119		1530	-	-	-	-	-	-	-	0.09	-	-	-	-
ST 204,41,69,79,91,93,105,133,161		1550	-	-	-	-	-	-	0.01	-	-	-	-	-
ST 204,41,69,79,91,93,119		1561	-	0.11	0.09	-	tr	-	0.90	-	-	0.12	-	0.03
ST 204,91,93,105,107,119,120,121,133,161		1562	-	-	-	-	tr	2.00	-	-	-	-	-	-
beta-Caryophyllene	87-44-5	1562	-	0.19	-	-	0.05	0.60	14.62	-	0.05	0.01	-	0.26
ST 55,57,67,81,105,119,161		1575	-	tr	-	-	-	-	-	-	-	-	-	-
ST 40,57,67,69,91,104,105,119,204		1601	-	-	-	-	-	-	-	-	-	0.01	-	-
(Z)-beta-Farnesene	28973-97-9	1620	-	-	-	-	-	-	-	-	-	0.04	-	-
alpha-Caryophyllene	6753-98-6	1624	-	-	-	-	-	-	2.03	-	-	-	-	0.03
ST 204,41,81,91,105,119,134,161		1626	-	-	-	-	-	-	0.10	-	-	-	-	-
alpha-Muurolene	31983-22-9	1643	-	-	-	-	-	-	0.07	-	-	-	-	-
ST 204,41,77,79,81,91,93,105,119,133,161		1676	-	-	-	-	-	-	0.17	-	-	-	-	-
ST 161,41,77,79,81,91,93,105,119,133		1695	-	-	-	-	-	0.02	0.43	0.27	-	-	-	-
(E)-Nerolidol	7212-44-4	1983	-	-	-	-	-	-	-	-	-	-	-	0.01
Irregular terpene														
6-Methyl-5-hepten-2-one	110-93-0	1300	-	4.02	10.92	0.46	3.28	17.98	0.79	0.58	0.16	-	0.11	0.04
6-Methyl-5-hepten-2-ol	1569-60-4	1419	-	-	-	-	-	0.43	-	-	-	tr	-	-
2,6-Dimethyl-7-octen-2-ol	18479-58-8	1468	-	-	-	-	0.02	-	-	-	-	-	-	-
Miscellaneous cyclic ester														
gamma-Butyrolactone	96-48-0	1604	0.23	0.14	0.14	0.15	-	-	-	-	-	-	-	-
Nitrogen containing compound														
Indole	120-72-9	2375	-	-	-	-	-	-	-	-	-	-		0.00
Sulphur-containing compounds														
Dimethyl trisulphide	3658-80-8	728	-	-	-	-	0.38	-	-	-	-	-	-	-
Dimethyl disulfide	624-92-0	1112	-	-	1.00	27.34	-	-	-	-	-	-	-	-
Unknown														
UNK 39,43,44,57,70,71,85		1558	-	-	-	-	-	-	-	-	-	-	tr	-

CHAPTER 8

EFFECTS OF VOLATILE COMPOUNDS EMITTED BY *PROTEA* SPECIES (PROTEACEAE) ON ANTENNAL ELECTROPHYSIOLOGICAL RESPONSES AND ATTRACTION OF CETONIINE BEETLES

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ABSTRACT

Evolutionary shifts to beetle pollination are commonly associated with the use of scent as a primary floral attractant. The most common cetoniine beetle pollinator of grassland *Protea* species in South Africa, *Atrichelaphinis tigrina,* has previously been shown to have a strong preference for the fruity floral scent of these plants over the weak scent of their bird-pollinated congeners. However, it is not known which of the many compounds found in the scent of beetle pollinated *Protea* species play a role for pollinator attraction. Electroantennograms (EAG) from *A. tigrina* beetles were recorded in response to fifteen compounds emitted by *Protea* flower heads. EAG responses to all fifteen compounds were significantly greater than those to the paraffin solvent in which they were diluted. The greatest responses were observed for benzenoids (anisole, methyl benzoate, methyl salicylate, benzaldehyde) followed by the monoterpene β-linalool, which can comprise up to 66% of fruity *Protea* scents. Five compounds that elicited EAG responses (benzaldehyde, β-linalool, (*E/Z*)-linalool oxide (furanoid), methyl benzoate and methyl salicylate) were tested in commercially available yellow bucket traps in the field to test their attractiveness to beetles. Of these, methyl benzoate attracted the greatest number of insects overall, and *A. tigrina* beetles specifically, compared to paraffin baited controls. Traps baited with β-linalool, (*E/Z*)-linalool oxide (furanoid), benzaldehyde and methyl salicylate also caught higher insects numbers than paraffin controls. A second field experiment showed that trap colour had a significant effect on the number of trapped beetles. Yellow traps showed a ten-fold higher number of insect catches than green traps. However, the combination of yellow colour and a scent compound (β-linalool) yielded the highest number of catches. This study has shown that the cetoniine beetle *A. tigrina* can detect a variety of floral compounds and is attracted to compounds comprising a large proportion of the blend that makes up fruity *Protea* scents, adding support for the hypothesis that the shift from bird to cetoniine beetle pollination system in this genus may be associated with the evolution of a strong fruity floral scent.

KEY WORDS: Beetle pollination; Cetoniinae; colour and scent floral cues; electroantennographic detection (EAG); field trapping

INTRODUCTION

Plants from unrelated families that share the same functional group of pollinators tend to evolve similar floral traits that reflect the morphology, behavioural preferences and physiological characteristics of these pollinators. For example, beetle-pollinated plants typically produce bowl-shaped flowers that emit sweet, fruity or aminoid odours and some offer large pollen rewards (Gottsberger, 1999; Teichert, 2007; Thien et al., 2009). The great diversity of coleopteran flower visitors, from fruit chafers to carrion beetles, has resulted in a large variety of beetle pollination systems and associated floral traits (Jürgens, 2009). While many beetles are generalist flower visitors, there is evidence for specialist beetle pollination systems in several plant families (including gymnosperms and angiosperms) involving scent as a principal attractant (Gottsberger, 1999; Bernhardt, 2000; Goodrich et al., 2006).

Tropical beetle pollination systems typically involve olfactory cues to attract pollinators. These cues are usually yeasty or sweet-fruity odours attracting flower or fruit-eating beetles (Jürgens et al., 2000). A pantropical woody family, the Annonaceae, uses a fermented-fruit, yeast- or even fungus-like odour to attract beetle pollinators (Goodrich et al., 2006; Gottsberger et al., 2011). Thermogenesis has been reported for various beetle-pollinated *Arum* (Urru et al., 2011), *Asimina* (Goodrich et al., 2006), *Caladium* (Maia and Schlindwein, 2006), *Philodendron* (Dalia Maia et al., 2010) and cycad species (Terry et al., 2004) that use heat to volatize their odours, particularly notable at night when flowers/inflorescences/cones open to attract beetles. In Mediterranean systems, by contrast, some plants, such as the "Poppy guild" of orange-red flowers pollinated by *Amphicoma* beetles (Dafni et al., 1990), appear to rely solely on colour cues to attract beetle pollinators.

Studies decoupling the attractiveness and functional roles of colour versus scent cues have been conducted using insects from the orders Coleoptera, Diptera, Lepidoptera, Hymenoptera and Orthoptera (see Schmera et al., 2004 and references within; Milet-Pinheiro et al. 2012). Plants can use combinations of these cues that influence pollinator-specificity and learning (e.g. Gegear, 2005; Leonard et al., 2011). For example, the colour of various Australian Proteaceae flowers changes from yellow/white to red with age to encourage insect pollinators to visit unpollinated flowers (Lamont, 1985). Similarly the down-regulation of methyl benzoate in pollinated snapdragon and petunia flowers decreases their attractiveness to pollinators (see Negre et al., 2003 and references within).

In South Africa, pollination systems involving flower-visiting scarab beetles have been demonstrated in Asclepiadaceae, Proteaceae and Orchidaceae (Johnson et al., 2007;

Shuttleworth and Johnson, 2009; Steenhuisen and Johnson, in press). Two asclepiad species and the orchid *Satyrium microrrhynchum* use specific odour blends to attract generalist cetoniine beetles to cryptically coloured flowers in grassland habitats (Johnson et al., 2007; Shuttleworth and Johnson, 2010). In contrast the flowers of grassland *Protea* and *Senecio* species are usually brightly coloured with conspicuous hues of carmine-pink and yellow respectively, and emit sweet/fruity scents (Steenhuisen et al., 2010; Steenhuisen et al., in press). All of these species produce sweet scents, often with fruity undertones.

Studies of scent chemistry have contributed substantially to the understanding of how flowering plants attract and manipulate the behaviour of their pollinators. There is also growing knowledge of the sensory preferences of pollinators belonging to different pollination systems (Raguso, 2008). Traditionally these studies have involved simple choice experiments in wind tunnels, for example (e.g. Goyret et al., 2007). Since the odour blends of flowers can be complex (e.g. 62 volatile compounds in the scent of Protea welwitschii; Steenhuisen et al., 2010) only using choice experiments to determine which compounds can be detected or are important in attracting an insect can be laborious. Fortunately, new specific measures of pollinator scent preferences are being developed and refined. Although macro-experiments are advantageous in measuring behavioural responses of pollinators to various scents, methods such as gas-chromatography-electroantennographic detection (GC-EAD) and gas-chromatography single-cell recording (GC-SC) allow researchers to screen the plethora of compounds emitted by flowers for potential behavioural effectiveness (Dobson, 1994; Stensmyr et al., 2001; Schiestl and Marion-Poll, 2002; Johnson et al., 2007).

The strong sweet-fruity scents of *Protea* species in eastern South Africa have been shown to principally attract cetoniine beetles (S-L. Steenhuisen, unpublished data). These flowers present beetles with a large landing platform surrounded by colourful (cream to carmine) involucral bracts, and provide plenty of food reward in terms of pollen and nectar. The beetle-pollinated species emit very strong fruity floral scents, resembling that of ripe *Carica papaya* fruit, which are highly attractive to insect pollinators visiting the inflorescences for abundant pollen and nectar rewards. The fruity scents are comprised of up to 66% linalool, and a wide variety of aliphatic esters, benzenoids, and other monoterpenes (Steenhuisen et al., 2010). Bird-pollinated *Protea* species emit much weaker and less complex floral scents, although they do emit a number of compounds found in beetle-pollinated species scent profiles, an indication that emission of some volatiles may be a phylogenetically constrained trait. Cetoniine beetles, in particular *Atrichelaphinis tigrina*, are the most frequent floral visitors to scented grassland and savanna *Protea* species (Steenhuisen

et al., in press). The distribution of *A. tigrina* is largely limited to eastern South Africa (Holm and Marais, 1992). It is a common pollinator of flowering grassland plants ranging over many families (e.g. Orchidaceae, Asclepiadaceae, Proteaceae; Johnson et al., 2007; Shuttleworth et al., 2009; Steenhuisen et al., in press). Studies investigating host specificity, sex pheromones and the development of chemical lures for integrated pest management have found that cetoniine beetles are attracted to a wide variety of volatiles frequently found in fruity scents. In studies involving phytophagous pest cetoniine species strong EAG responses and attractiveness in field trapping experiments have been found for linalool and other odour compounds that are present in the scent of dwarf grassland *Protea* species (Stensmyr et al., 2001; Larsson et al., 2003; Steenhuisen et al., 2010).

The aims of this study were 1) to determine if volatile compounds comprising a large proportion of the scent of beetle-pollinated *Protea* species elicit electrophysical responses in their primary cetoniine pollinator, *A. tigrina*, 2) to establish whether or not these compounds are attractive in the field, and 3) to investigate possible synergistic effects of colour and scent on attraction of *A. tigrina.*

MATERIALS AND METHODS

Study site and species description — Field experiments were conducted on the summit of Mount Gilboa (29.29°S, 30.29°E, 1770 m, KwaZulu-Natal, South Africa) which has grassland vegetation dominated by three sympatric *Protea* species – the beetle-pollinated *P. caffra* Meisn.and *P. simplex* E.Phillips ex J.M.Wood, and the bird-pollinated *P. roupelliae* Meisn. subsp. *roupelliae.* These species flower from December to early March with a January peak at this site (Rebelo, 2001; pers. obs.). *Atrichelaphinis tigrina* (Olivier, 1789) used in choice experiments (described below) were collected from *Protea* inflorescences at this site. Voucher specimens of insect pollinators (previously surveyed) and plants have been deposited in the UKZN entomological collection and Bews herbarium (NU) respectively (plant vouchers: collector S.-L. Steenhuisen, 59 & 60).

Electroantennographic responses — The floral scents of beetle-pollinated *Protea* species are comprised of 30-60 volatile compounds (Steenhuisen et al., 2010), the most complex mixture being emitted by inflorescences of *Protea welwitschii* that includes unique esters, especially those of tiglate acid. Based on commonality (e.g. linalool oxides, myrcene), relatively high amount contributing to overall floral scent (e.g. linalool), uniqueness (tiglate acid esters) or

those most likely to contribute to the fruity aroma (e.g. methyl salicylate and methyl benzoate), we chose fifteen volatile compounds to test for electroantennographic (EAG) responses of the most common cetoniine beetle pollinator, *Atrichelaphinis tigrina*. Nine beetles were collected from *Protea* inflorescences at Mount Gilboa. A tri-lamellate antenna from each beetle was cut off and mounted between two glass micropipette electrodes filled with insect ringer's solution (8.0 g/l NaCl, 0.4 g/l KCl, 0.4 g/l $CaCl_2$). The micropipette electrodes were held with micromanipulators (Syntech MP15) and connected via silver wires to the EAG setup (high impedance input AC/DC amplifier model UN-06; Syntech, Hilversum, The Netherlands). The three lamellae were separated by tiny balls of dental wax to expose the sensilla between them (sensilla described for Pachnoda marginata by Stensmyr et al., 2001). The tip of the third lamella was cut and inserted into one micropipette while the pedicel of the antenna was inserted into the other micropipette. A drop (5 µl) of 1:19 test compound (Sigma or Fluka, >95%) in paraffin (alpha Pharm) was placed on filter paper in a glass Pasteur pipette connected to silicone tubing and glass capillary with it's opening aimed at the opened lamellae of the antenna. The antenna was kept in a constant flow of humidified air and each test mixture was puffed into this airstream and onto the antenna at a flow rate of 10 ml per second and a pulse duration of 0.5 seconds, regulated by a CS-05 Stimulus Controller (Syntech, Hilversum, The Netherlands). The response was recorded on the Syntech EAG software program. Responses to air (dry filter paper) and pure paraffin were recorded at the beginning and end of each series of test recordings for each of nine beetles and a benzaldehyde standard was puffed onto the antenna after every five test compounds to monitor the strength of the antenna over its lifespan in the electrodes. Non-normalized EAG responses of test compounds were compared to that of pure paraffin using pair wise contrasts and analysis of variance of logged absolute mVolt responses for each beetle in a Type I model to test the differences in overall responses to the various compounds (fixed factor) after accounting for the effects of individual beetles (random factor) (Zar, 1984).

Field experiments — Scent preference —We used five electrophysiologically active compounds to test their attractiveness in the field –benzaldehyde, β-linalool, (*E/Z*)-linalool oxide (furanoid), methyl benzoate, and methyl salicylate. Solutions of 2 ml 19:1 paraffin: test compound were placed in glass bottles with cotton wicks and used to lure insects to commercially available bucket funnel traps with yellow-coloured extensions (vanes, funnel and lid) and green collecting buckets (Insect Science™, Tzaneen, South Africa). A total of 18

traps (three of each of the five test compounds and three of pure paraffin) were presented a meter from the ground on metal poles in a randomized grid design (3 by 6 trap array) five meters apart from each other in any direction. The lures were placed within the bucket of each trap. The total number of insects caught in each trap was determined after two days. The positions of these traps were then re-randomized with fresh lures and left for two days for a total of eight trials of this experiment in January 2008. Total insect catches and the number of *A. tigrina* beetles per trap for each trial and test compound were compared using generalized estimating equations (GEE) with Poisson log-link function, trial as a subject effect, and scent compound as a within-subject effect. These analyses used an exchangeable correlation matrix structure, and employed sequential Sidâk comparisons to assess the significance of differences among means. Emission rates from the trap lures were monitored over four days by taking one minute headspace samples of two lures of each compound per day. Gas chromatography-mass spectrometry was used to compare the emission rates against injected amounts of known standards. Emission rates (approx. 40 $ng.hr^{-1}$) were found to be in the range of actual emission rates for these compounds from *Protea* inflorescences.

Colour preference — To test if colour in addition to scent is important for attracting insects to inflorescences of scented *Protea* species, we set out an array of 14 green and 14 yellow (green bucket) traps (see Fig. 1 for spectra), seven traps of each colour with or without linalool, a compound comprising 30-50% of the floral scent of beetle-pollinated species. The spectral reflectance across the 300-700 nm range was determined for green and yellow traps according to the methods of Johnson and Andersson (2002), and compared to that of various inflorescence parts (mean of 10 samples) and leaf samples mean of five samples) of *P.caffra* (Fig. 1). As in the previous experiment, 2 ml of 19:1 paraffin: *β*-linalool solution was used a lure in each of seven yellow and seven green beetle traps. Lures of pure paraffin were placed in a further seven yellow and seven green beetle traps. All 28 traps were placed a meter from the ground in a random grid design (3 traps wide). Two trials of 4-5 days each in January 2008 were conducted and the number of insects determined for each trap in each trial. The effects of scent and colour and their interaction on number of insects caught were analysed using GEE with Poisson log-link function, trial as subject effect, and scent, colour as fixed factors. These analyses used exchangeable correlation matrix structure, and employed sequential Sidâk comparisons to compare means.

RESULTS

Electroantennographic responses — In comparison with the paraffin and air controls, all fifteen volatile compounds elicited electrophysiological responses from the antennae of *A. tigrina* beetles (Fig. 2). The beetles responded most strongly to the aromatics anisole, methyl benzoate and methyl salicylate, and least strongly to two tiglate acid esters and Benzyl alcohol. Average responses were recorded for compounds such as myrcene, (*E/Z*)-linalool oxide (furanoid), limonene, β-pinene, and (*E/Z*)-ocimene.

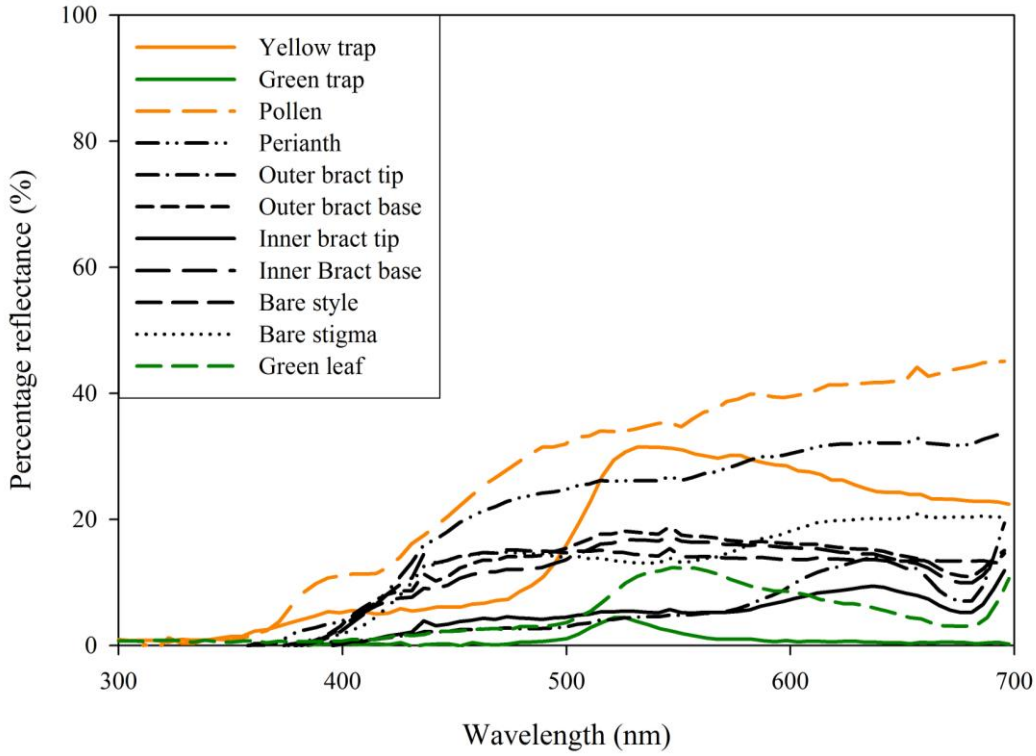

Fig. 1 The colour spectral reflectance for various inflorescence parts and leaves of *Protea caffra,* and green and yellow bucket traps used for field trapping experiments.

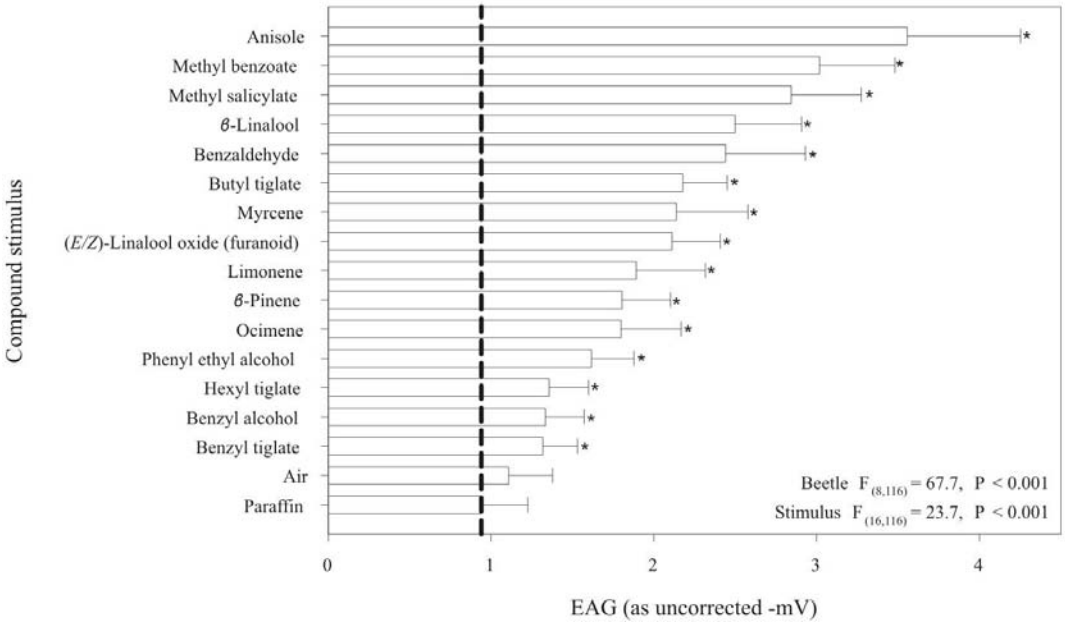

Fig. 2 Electroantennographic responses of *Atrichelaphinis tigrina* to a suite of 15 volatile compounds found in beetle-pollinated *Protea* species. Asterisks (*) indicate a significant difference to the mean antennal responses to paraffin with which the volatile compounds were mixed when presented to antennae.

Field experiments — Scent preference — There was a significant effect of compounds on insect catches ($\chi^2_{(5)}$ = 92.345, P<0.001; Fig. 3), with the highest catches recorded for traps with methyl benzoate standard lures (see Appendix 1 for by-catch). Traps with pure paraffin lures caught similar numbers of insects to those with benzaldehyde, but significantly lower numbers than those with β-linalool, (*E/Z*)-linalool oxide (furanoid) and methyl salicylate. Similar trends were recorded for the number of *A. tigrina* beetles, which comprised a high proportion of the insects in most traps, but only methyl benzoate lured a significantly higher number of *A. tigrina* beetles than paraffin controls (*A. tigrina*: $\chi^2_{(5)}$ =132.965, P<0.001).

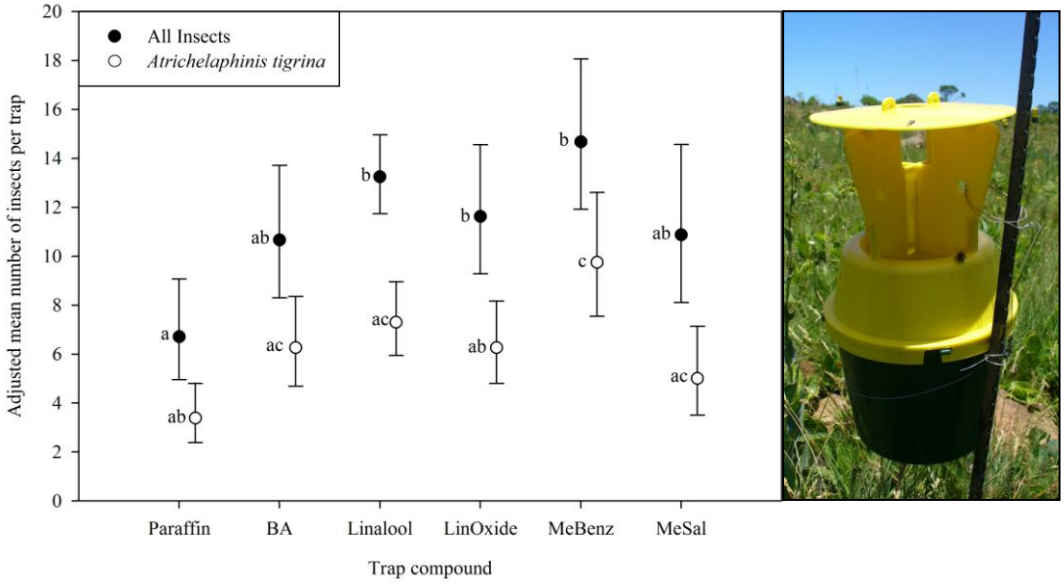

Fig. 3 The effect of five volatile compounds compared to a paraffin control on total insect numbers and specifically the cetoniine beetle, *Atrichelaphinis tigrina,* caught in bucket traps with yellow extensions on Mount Gilboa. BA = Benzaldehyde, LinOxide = (*E/Z*)-Linalool oxide (furanoid), MeBenz = Methyl benzoate, MeSal = Methyl salicylate. Different letters indicate significant differences between means ($P<0.05$).

Colour preference — Yellow coloured traps caught ten times more insects than green-coloured traps, regardless of whether they were baited with linalool or paraffin, but there was also a less marked, but significant, effect of linalool on insect catches (colour: $\chi^2_{(1)} = 91002.694$, $P<0.001$; scent: $\chi^2_{(1)} = 1345.991$, $P<0.001$; colour×scent: $\chi^2_{(1)} = 20.658$, $P<0.001$; Fig. 4).

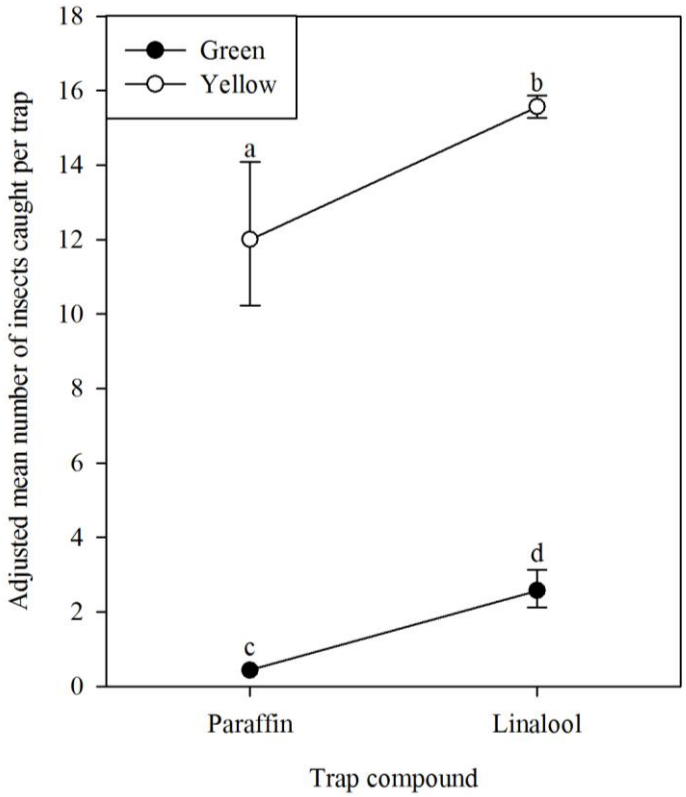

Fig. 4 The effect of colour (green versus yellow extensions) and scent (Paraffin versus β-Linalool) on the number of insects caught in bucket traps on Mount Gilboa. Different letters indicate significant differences between means (P<0.05).

DISCUSSION

Cetoniine chemical attractants and EAG — Our results concur with previous findings of attractiveness of fruity odours to cetoniine beetles (e.g. Donaldson et al., 1990). Although there was a strong colour effect, more than expected for a putatively scent-based pollination system, our results still showed that scent plays a role in attracting beetle pollinators (Figs 3-4).

The cetoniine *Atrichelaphinis tigrina* used in this study was able to detect all of the compounds tested for EAG responses. The highest EAG response was to anisole, a benzenoid compound comprising only 3% of the fruity scents of grassland *Protea* species but on average 28% of bird-pollinated *Protea* scents (S-L. Steenhuisen, unpublished data, chapter 7). The benzenoid methyl benzoate elicited the next highest EAG response and was most attractive in the field. Methyl salicylate elicited the third highest EAG response but was the

least attractive compound tested in the field. In contrast, (*E/Z*)-linalool oxides (furanoids) gave lower EAG responses compared to the other compounds used for trapping in the field but were responsible for the third highest insect catches. These findings show that field bioassays can sometimes give very different results to those expected from EAD responses.

The complex scent of the beetle-pollinated *Protea welwitschii* is comprised of many benzenoid esters and aliphatic esters, specifically those of tiglic acid (Steenhuisen et al., 2010). We were surprised then to have recorded mixed antennal responses to tiglic acid esters while other benzenoid esters elicited very high EAG responses. But, as mentioned above, a low EAG response does not necessarily mean low attractiveness in the field.

Much research that exists on the attractiveness of scent chemicals to cetoniines and other scarabs has been aimed at determining chemical lures for traps used in integrated pest management of phytophagous species (Donaldson et al., 1986; Donaldson et al., 1990; Cherry et al., 1996; Tóth et al., 2004; Robbins et al., 2006; Wolde-Hawariat et al., 2007; Bengtsson et al., 2009; Chen and Li, 2011). These and other researchers (Larsson et al., 2003; Johnson et al., 2007) concluded that Cetoniinae respond to a wide variety of scent compounds widely dispersed in nature and also attractive to other insects. This same trend was confirmed by significant EAG responses recorded here for *A. tigrina* to all compounds, and their attraction to methyl benzoate and linalool in separate field experiments. Despite this generalist behaviour, Stensmyr et al. (2001) suggest that even polyphagous cetoniines have very specific olfactory systems, as demonstrated by the specificity of olfactory receptor neurons (ORN's) in *Pachnoda marginata,* responding to only 48 out of over 200 volatile compounds extracted from a large variety of fruit, and even finer specificity demonstrated for single neurons. This specificity for fruit volatiles was confirmed for *P. marginata* and a congener *Pachnoda interrupta* by Bengtsson et al. (2011). Wolde-Hawariat et al. (2007) tested EAG responses and the attractiveness of five compounds in the field to *Pachnoda interrupta* in Japanese beetle traps. Methyl salicylate elicited significant dose-dependent responses in male and female *P. interrupta* beetles and traps baited with methyl salicylate were most attractive, catching over a thousand beetles per trap over 5 days. They also found a significant effect of the type of lure used in the traps on beetle catches. Isoamyl acetate, for example, evaporated very quickly from cotton wick lures, which did not attract more beetles than unbaited controls, but was among the most attractive compounds when presented on rubber septa that released the compound at a slower rate. None of the green leaf volatiles nor the lactones tested by Larsson et al. (2003) were attractive to *P. marginata* in laboratory choice tests.

The largest GC-EAD response recorded by Johnson et al. (2007) using antennae of *Atrichelaphinis tigrina* was to β-linalool in the floral scent of a beetle and wasp pollinated orchid *Satyrium microrrhynchum*. Johnson et al. (2007) also recorded GC-EAD responses to methyl salicylate, methyl eugenol, trans-β-caryophyllene, γ-amorphene, elemicin and an unknown compound. *Atrichelaphinis tigrina* males responded much more to methyl salicylate than did females (Johnson et al., 2007). These compounds are detected by several insect families, for example, EAG responses for *Hyles lineata* moths were strongest for β-linalool, benzyl acetate, methyl salicylate, and (*Z*)-linalool oxide (pyranoid) (Raguso et al., 1996). The polyphagous cetoniine *P. marginata* also responded to β-linalool, methyl salicylate and (*Z*)-linalool oxide (furanoid) in amongst a large variety of compounds tested, including a total of 17 compounds that are also found in *Protea* scents (Stensmyr et al., 2001). As in the present study, Vuts et al. (2010b) reported high EAG responses in *Epicometis hirta* males to methyl salicylate, followed by β-linalool and benzaldehyde. Female *E. hirta*, however, responded more strongly to methyl salicylate than any of the other 26 compounds tested, with β-linalool and benzaldehyde eliciting lower but similar responses. Methyl salicylate also elicited higher EAG responses than benzaldehyde and β-linalool in male and female *Cetonia aurata aurata* L. and *Potosia cuprea* Fabr. (Vuts et al., 2010a) although their attractiveness was not tested for these species. While methyl salicylate was highly attractive to the cetoniine *Protaetia brevitarsis,* methyl benzoate did not attract more beetles than an empty control in an olfactometer (Chen et al., 2011). These studies show that cetoniines differ in their responses to volatile compounds and thus we cannot infer the behaviour of our study species from studies involving other species.

Methodological considerations — The response to compounds tested in our field trapping experiments probably reflects both innate and learned responses in beetles. We didn't test responses of naïve beetles against innate responses but focussed on the role of scent in the natural environment, which would have been reinforced by a reward. This may account for the difference between the high EAG response and the low attractiveness of beetles to methyl salicylate as it is not a dominant compound in the chemical profiles of *Protea* inflorescences, although very attractive to other cetoniines. Instead, we found that *A. tigrina* was highly sensitive to methyl benzoate and the beetles responded positively to this compound in the field, perhaps because it reliably acts as a cue for the presence of rewards in *Protea* flowers in this particular plant community.

Donaldson, McGovern and Ladd (1990) used modified Japanese beetle traps to determine the attractiveness of 69 different scent compounds to cetoniine and ruteniline beetles in the field (McGovern et al., 1970b; Klein et al., 1973). They did not include blank control traps as experience had shown that beetle catches in such traps were very variable and low. In contrast, the number of insects trapped in our paraffin-baited controls were not much lower than in traps baited with floral compounds (Fig. 3). Given the highly significant difference in insect catches between traps with yellow or green extensions (Fig. 4), it appears that the colour of the traps played an important role in the attractiveness of control traps to insects. Studies have shown that cetoniines are attracted to various colour and scent cues and that the combination can be synergistic in their attractiveness. Schmera et al. (2004) found that the cetoniine pest *Epicometis* (*Tropinota*) *hirta* Poda is strongly attracted to yellow and blue and that there is a synergistic relationship between the blue colour of traps and a chemical cue (1:1 mixture of cinnamic alcohol and (*E*)-anethole). Tóth et al. (2005) found a significant effect of trap colour for catches of *Cetonia aurata aurata* L. and *Potosia cuprea* Fabr. (Cetoniinae) only in the presence of an olfactory cue (3-methyl eugenol, 1-phenylethanol and (E)-anethol in the ratio of 1 : 1 : 1). Our traps were also spaced closer together than the arrays used by Donaldson, McGovern and Ladd (1990). Their traps were set out 10m apart whereas ours were only five meters apart. Perhaps the close vicinity of different trap compounds resulted in a common mixed odour plume resulting in relatively high catches in the controls and almost uniform catches in the others.

Our field trapping experiments may have been further limited by the use of single compounds. The optimal lure for specifically attracting *Oxythyrea* beetles (Cetoniinae) was a mixture of cinnamic alcohol and eugenol (Donaldson et al., 1990). Similarly, the highest number of *Epicometis hirta* were caught in traps baited with a 1:1 blend of cinnamic alcohol and (*E*)-anethole (Tóth et al., 2004). A methodological challenge is, however, that different compounds also evaporate at different rates, and most studies do not report the emission rates of different chemicals used in their trap systems. Donaldson, McGovern and Ladd (1990) used amounts of volatile compounds in their trap lures similar to those described in McGovern and Beroza (1970a) which were ca. 19 g of undiluted compounds in mixes. Trap lures used for their field experiments had been optimized to evaporate slowly over a period of up to two months. We slowed evaporation by mixing our test compounds with paraffin and it's therefore difficult to compare emission rates to their lures. Even though there was a strong correlation between emission rates and vapour pressure (see Appendix 2), we did not find a correlation between emission rates/vapour pressure of the test compounds and

physiological/behavioural responses of the beetles, indicating that this factor did not play an important role in the physiological/behavioural responses of these beetles.

Furthermore it is difficult to compare the response of beetles to the bouquet of whole flowers with that to single compounds since some compounds may act as co-attractants. Therefore blends of compounds should be tested in future. For example, tiglate esters found in *P. welwitschii* might function as co-attractants and it would be interesting to test whether these esters are attractive in the field or only in combination with other attractants.

Insect numbers (14-16 per trap) attracted to traps in our field experiments for traps with yellow extensions and baited with linalool were comparable to the mean of 15.3 cetoniine beetles per trap recorded by Donaldson, McGovern and Ladd (1990). β-Linalool was the second most effective attractant (after methyl benzoate) we tested and the seventh most attractive out of the 69 compounds tested by Donaldson, McGovern and Ladd (1990). This compound makes up nearly 57-66% of the floral scents of beetle-pollinated *Protea* species. Larsson et al. (2003) also reported linalool as highly attractive to *P. marginata* in laboratory 2-way choice tests against a control and the only compound tested that attracted a significantly higher number of male beetles than females. The presence of β-linalool in a blend, however, with acetoin and (*E*)-2-hexenal, did not increase attractiveness beyond that of acetoin, the most attractive single compound in the blend.

Role of floral traits in the evolution of beetle-pollinated flowers — The shift from bird- to beetle- pollination was principally associated with the massive up-regulation of β-linalool, which, in general, is only weakly emitted from the inflorescences of bird-pollinated *Protea* species (S.-L. Steenhuisen, unpublished data, chapter 7). Since a bird-pollination system is ancestral in this genus, the up-regulation of β-linalool production may have a played an important role in the evolution of beetle-pollination systems in the recently evolved grassland clade. If stronger-smelling morphs of a bird-pollinated *Protea* ancestor attracted beetles in populations of low bird abundance, this floral trait may then have been selected, facilitating an evolutionary shift in pollinators. Cetoniine beetles are strongly attracted to β-linalool and the additional presence of certain benzenoids typically found in fruits, such as methyl benzoate, may be synergistic in its attraction. Furthermore it has been shown that beetles have multiple olfactory receptor neurons (ORNs) that can detect single compounds or general compound groups, although ORN more commonly respond to single compounds (Stensmyr et al., 2001; Bichao et al., 2005). There are basic structural similarities (benzene ring with

different side group) between anisole, methyl benzoate and methyl salicylate and it is possible that these compounds elicit responses from the same generic ORNs in our study beetles. However, this was not the case for ORNs tested in *P. marginata* (Stensmyr et al., 2001). For this species, methyl benzoate and methyl salicylate elicited responses in separate ORN classes.

The attraction of traps to cetoniine beetles in the field would have been influenced by innate and learned responses. We know little about what the beetles may have learnt in the natural environment but in addition to *Protea* we've observed these beetles visiting other species in the co-flowering community with similar floral traits, such as yellow-coloured Asteraceae and Asclepiadoideae (Shuttleworth and Johnson, 2008; Shuttleworth et al., 2010). These beetles may therefore be conditioned to a common colour cue, learnt in association with a food resource. Thus the higher attraction of insects to the yellow colour of the bucket traps may be due to a colour signal that has been learnt. At the community level, plant species should benefit from emitting similar scents to existing systems that attract these beetles. This is similar to a mimicry system, in which a plant mimics a model. Perhaps *Protea* scent and colour has therefore adapted to mimic (in the very general sense) a guild of plants that attract beetle pollinators. If colour is an easier cue to learn, naïve beetles may first use odour to locate the food resource, but once a colour signal has been learnt, beetles may use colour to locate food thereafter. This has been shown for bees (reviewed by Dötterl and Vereecken, 2010).

The broad olfactory response of our study cetoniine *A. tigrina* is likely adaptive for a generalist insect in a changing environment and it may be the particular blend of compounds that attracts these insects to a specific plant. By being able to detect a wide spectrum of compounds, these beetles keep the system of attraction open to responding to a variety of fruity odours. Fruity odours can be comprised of aromatics, aliphatics and/or monoterpenes that can represent different biosynthetic pathways in plants. Therefore, for plants, imitating a general fruit odour can be achieved in many ways. By having generalist olfactory senses, beetles can find many food resources, learn scent and colour signals associated with the resource, and show the necessary constancy to exploit it.

While this study has confirmed previous findings of beetle attractants it has also highlighted how shifts in pollination systems in *Protea* are associated with changes in floral traits that are both detectable and attractive to cetoniine beetle pollinators. Colour plays an important synergistic role, suggestive of a learnt signal associated with the abundant pollen

and nectar rewards in these plants. We predict that the scent of other closely related *Protea* species may play a similar role in attracting beetle pollinators.

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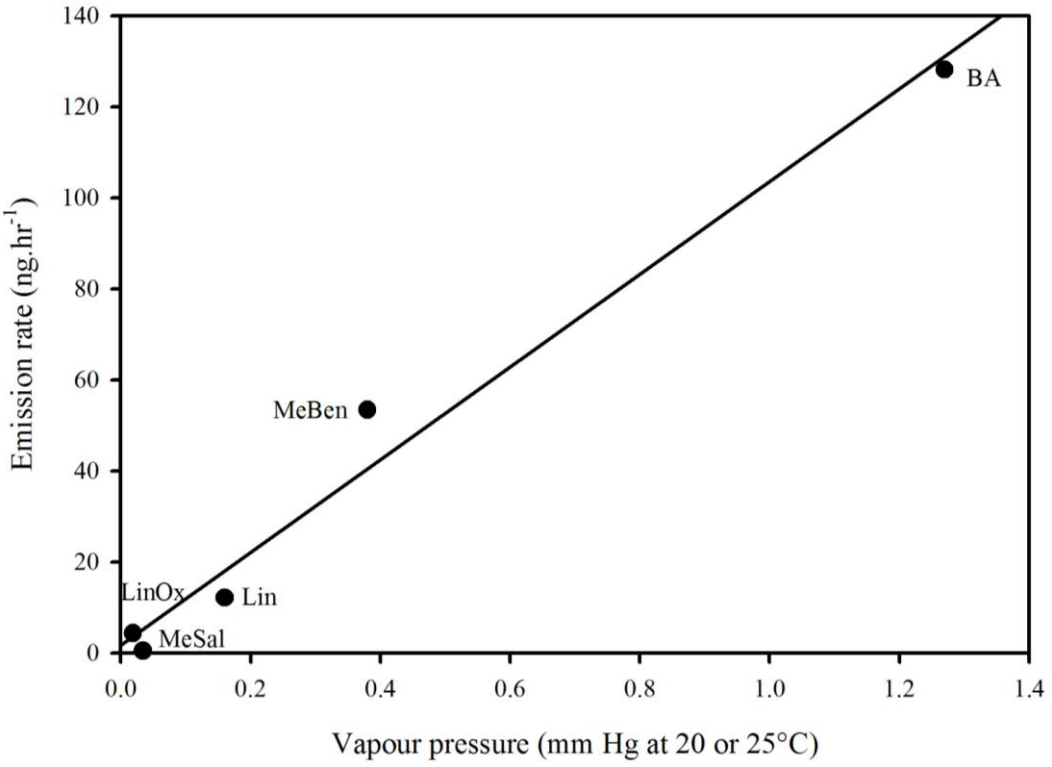
CHAPTER 8

SUPPLEMENTARY MATERIAL

Appendix 1. Total insect by-catch from bucket traps used to test the scent preference of insects in the field to five test compounds compared to paraffin controls.

Insect order	Test compound					
	Benzaldehyde	Linalool	Linalool oxide	Methyl benzoate	Methyl salicylate	Paraffin
Coleoptera	101	138	112	151	64	34
Dictyoptera	-	1	-	-	-	-
Diptera	-	-	3	1	4	4
Hemiptera	-	1	-	2	2	-
Hymenoptera	-	-	1	4	-	1
Lepidoptera	2	6	-	1	3	3



Appendix 2. A regression of emission rates and vapour pressure for 2 ml solutions of five test compounds (1:19 test compound: paraffin). BA = benzaldehyde, Lin = linalool, LinOx = linalool oxide, MeBen = methyl benzoate, MeSal = methyl salicylate. Vapour pressures were taken from the SRC PhysProp Database (http://www.syrres.com/esc/physdemo.htm). (Regression: $y = 1.71x + 101.92$, $R^2 = 0.97$)

CHAPTER 9

CONCLUDING DISCUSSION

In this concluding chapter I summarise the findings made in previous chapters, and discuss how they have advanced our current knowledge of the pollination ecology of Proteaceae and the more general issue of pollinator shifts associated with changes in floral scent chemistry. In addition, suggestions are made for future research on mating systems in *Protea* and the evolution of floral traits associated with pollinator shifts in the genus.

My findings were consistent with the hypotheses of beetle pollination in some grassland *Protea* species and attraction of beetles to the scent of these *Protea* species. Investigations of the breeding (chapter 2) and pollination systems (chapters 4 & 5) of four scented *Protea* species that occur in a clade in which bird-pollination is ancestral, as well as the functional roles of various floral traits (chapters 3 & 7), provide strong evidence for a shift from bird- to beetle-pollination in *Protea*. This conclusion is supported by experiments that showed that insects are effective agents of outcrossing in one of the study species (chapter 5) and that specific compounds in the scent of grassland *Protea* species are attractive to cetoniine beetles (chapters 8).

Background — There is a trend towards more integrated approaches to describing pollination systems that includes, for example, quantitative analyses of floral scent, colour, and pollinator effectiveness. Pollination studies have traditionally been focused on visual floral traits, while those investigating plant-herbivore interactions emphasized chemical traits (Raguso, 2008a; Raguso, 2008b). This is now changing as the expertise and means to study the role of chemical plant signals in pollination systems have become more accessible and widespread. This study attempted to explore many inter-connected aspects of the pollination ecology of a plant lineage, from pollinator effectiveness in terms of the genetic contribution to the next generation, the roles of traits, such as plant height, flower morphology, colour and scent, that could play a role in attraction of pollinators, quantification of floral rewards, factors that affect seed production and levels of seed predation.

Investigations of pollination systems help to identify convergent floral traits associated with pollination by certain functional groups, and thus allow predictions to be made about pollination systems of unstudied plant species (cf. Johnson et al., 2001; Pauw, 2006). Based on my findings for grassland *Protea* species, I suspect that other grassland plant species in South Africa that share floral traits such as large bowl-shaped inflorescences with fruity-sweet scents are also beetle-pollinated. Cetoniine beetles visit many flowering plant species within grassland communities (pers. obs.), including asclepiads and orchids (Ollerton et al., 2003; Johnson et al., 2007; Shuttleworth and Johnson, 2010), and I hypothesize that

they represent a specific functional group of short-tongued pollinators (Fenster et al., 2004) attracted to fruity scents. Cetoniines have generalist flower preferences, but the four *Protea* species studied here have traits such as low nectar concentration, short nectar-stigma distance and fruity scents (chapters 3 & 7-8) that appear to be associated with specialization to this specific functional group, as they are also found in asclepiads and orchids that share the same pollinators (Ollerton et al., 2003; Johnson et al., 2007; Peter and Johnson, 2009; Shuttleworth et al., 2010). Cetoniine beetles and pompilid wasps often visit the same flowers with exposed nectar (Shuttleworth and Johnson, 2008), but there is increasing evidence that plants can specialize on one or the other of these two groups of insects. Shuttleworth and Johnson (2012) have recently described the guild of grassland plant species that are specialized for pollination by pompilid wasps and I propose that a distinct cetoniine beetle pollination system also exists in these grassland communities. The cetoniine *Atrichelaphinis tigrina* that is reported to pollinate these plants has a distribution matching that of grasslands, and extending into savanna and thicket biomes in eastern South Africa (discussed further below, Fig. 1).

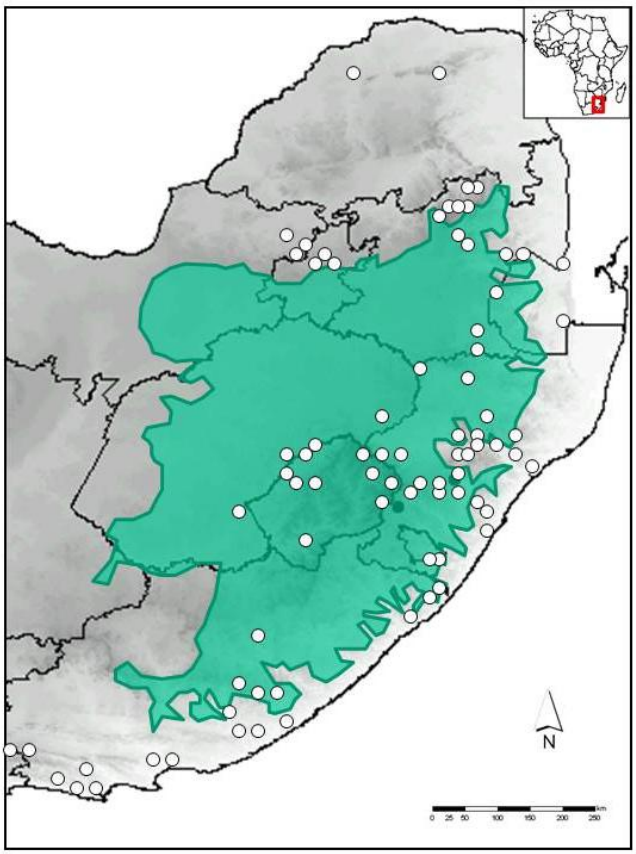


Fig. 1 The distributions of *Atrichelaphinis tigina* (Olivier, 1789) (white dots, Holm and Marais, 1992) and grassland biome (green shading, SANBI) in South Africa.

Pollinator shifts such as the one I have described in this thesis provide opportunities to investigate evolutionary mechanisms that cause speciation and/or extinction in plant lineages (Cappellari et al., 2011). Evolutionary reasons for shifts in pollination systems can include changes in the abundance of pollinators across a plant's distribution or altitudinal range (Johnson, 1997). A good case in point are shifts across the geographical range of columnar cacti in North America, from specialized bat pollination in the south to generalized pollination by several animal groups at more northern latitudes where bats are rarer (Valiente-Banuet et al., 2004). The reasons for pollinator shifts in *Protea* have not been explored here, but I propose that shifts have occurred due to differences in abundance of nectarivorous birds and cetoniine beetles across the plants' distribution. This raises some intriguing questions: are nectar-feeding bird species more abundant and diverse in the Cape fynbos, compared to grasslands of eastern South Africa, and, are beetle pollination systems involving cetoniines more common in grasslands than the fynbos?

Beetle pollination in Protea — Coetzee and Giliomee (1985) were first to provide evidence of insect pollination in *Protea* by describing pollen transfer on the bodies of small beetles (Halticidae, Nitidulidae and Staphylinidae) visiting the primarily ornithophilous *P. repens*. However, having only studied one *Protea* species they suggested that insect pollination in *Protea* could not yet be accepted as a "general rule". In this thesis I presented evidence that insects, particularly beetles, are the primary pollinators of some members of *Protea* (chapter 5) and that floral presentation and scent play a functional role in attracting these insects (chapters 3 & 8). My study species are visited by a variety of insects, but evidence presented in chapter 3 on visitation rates, body sizes, pollen loads and foraging behaviour of cetoniine beetles, as well as their preference for fruity *Protea* scents and dominant floral compounds in the field (chapters 7 & 8), suggest that these insects are the most important pollinators of these plant species.

Shifts from bird- to insect-pollination in the Proteaceae were suggested by Faegri (1965). This suggestion was based on changes in floral morphology from a "brush blossom" inflorescence associated with a more specialized ornithophilous pollination syndrome, to the "more primitive bowl-shaped" inflorescence associated with an "assumed most primitive stage of cantharophily", or beetle pollination (Faegri, 1965). This idea is generally supported by this thesis in that the cetoniine beetle-pollinated *Protea* species have bowl-shaped inflorescences and belong to a clade ancestrally derived from bird-pollinated species (Valente et al., 2010). However, the results of this thesis suggest that low nectar concentration, short

nectar-stigma distance, and fruity scents, chapters 3 & 7-8) are the key adaptations associated with the transition from bird pollination to cetoniine beetle pollination in this clade.

Breeding systems in Proteaceae — Following early studies by Horn (1962) and Collins and Rebelo (1987), the members of *Protea* were assumed to be almost entirely self-incompatible, a misconception that was perpetuated by further studies on *Protea* where breeding systems of species were not assessed (e.g. Carlson and Holsinger, 2010). In chapter 2, evidence is presented that suggests that self-compatibility may be more common in *Protea* than previously thought. Van der Walt (1995) was the first author to report results contradictory to Horn's (1962) claims for *P. repens* and my study presented a further opportunity to test the assumption of *Protea* being largely comprised of self-incompatible species. Experiments by Wiens et al. (1983) suggested autonomous selfing in the rodent-pollinated species *P. humiflora*. An investigation of breeding systems of the four study species in chapter 2 clearly demonstrated that self-compatibility and autonomous self-pollination occur in this genus. Furthermore, I have found that most of the earlier studies of breeding systems in *Protea* were methodologically flawed. It is thus not yet clear whether or not the self-compatibility and autonomous self-pollination that I found in grassland *Protea* species represent a recent shift from self-incompatibility in their immediate ancestors or a similar breeding system to that which occurred deeper in the lineage.

Pollinator shifts are sometimes associated with changes in breeding system. For example shifts to inbreeding are associated with some plants that occur on islands and experience different pollinator abundances compared to mainland populations (Inoue, 1993). A similar effect could also occur in isolated mainland populations; however, determining the relationship between autonomous selfing and pollinator shifts in *Protea* requires further work because of the uncertainty around the breeding systems of Cape species, as discussed above.

Using outcrossing rates to infer effective pollinators — Ever since Coetzee and Giliomee (1985) performed exclusion experiments with *P. repens* and revealed that insects contributed to seed set in *Protea*, the relative contribution of bird and insect visitors to outcrossing in *Protea* species have been in question (e.g. Wright et al., 1991). In an investigation of pollinator effectiveness using seed weight and germination for bird-excluded plants of *P. laurifolia* (suspected of wind-pollination), Wright (1994) proposed that a "better measure may be the extent of heterozygosity of seed arising after pollination by different vectors".

Pollinator effectiveness has rarely been measured using outcrossing rates of progeny from plants selectively excluded from different floral visitors (e.g. England et al., 2001), and my study was the first to attempt this in *Protea*. The approach is important in autogamous grassland *Protea* species because seed set is not affected strongly by pollinator exclusion. Using this approach, I found that insects contribute strongly to outcrossing in *P. caffra,* a species which can produce seeds facultatively through autonomous self-fertilization in the absence of pollinators.

By defining effective pollinators, hypotheses can be made about how selection by a specific pollinator group can drive the evolution of floral traits (Harder and Johnson, 2009). Ne'eman et al. (2010) reviewed methods of determining pollinator effectiveness for plants and the problems that can arise using these methods. The method of using codominant markers, such as allozymes or microsatellites, to determine outcrossing rates was not explored in great detail in their review, but presents a unique solution to assessing pollinator effectiveness in plants, especially facultative selfers in which seed production doesn't reflect pollinator visits. There is a wealth of comparative studies of outcrossing rates for plants, but very few studies that isolate the effects of particular pollinators on outcrossing rates. Using genetic variation in seed progeny circumvents the need for long-term germination studies of slow-growing plants and does not suffer from lowered sample sizes when inbred progeny fail to germinate. By using allozyme or microsatellite variation in whole seed families, a true measure of outcrossing can be obtained, unbiased by inbreeding depression on germinating seeds or plants that could cause underestimation of outcrossing in progeny. However, some enzymes may not be active in seed and the number of loci that are detected may be limited by this low activity (as seen in Weeden, 1984). This was not the case in *P. caffra* for which I detected eight polymorphic loci (chapter 5), a surprisingly high number compared to studies on the related genus *Banksia* (Scott, 1980). Maternal genotypes can be inferred from allele frequencies in progeny and used to estimate an inbreeding coefficient for the maternal population using free software such as MLTR (Ritland, 2002). This allows an estimate of inbreeding depression from the maternal inbreeding coefficient and progeny outcrossing rate. Using this approach (chapter 5) we inferred that a low inbreeding coefficient for *P. caffra* maternal plants was due to inbred progeny failing to reach reproductive maturity, as suggested, for example, by Schmidt-Adam et al. (2000). This study is thus consistent with many others that suggest that selfed progeny of woody plants may make limited demographic contributions because of high levels of inbreeding depression (Duminil et al., 2009; Robertson et al., 2011). Ideally, this approach should be supplemented by long-term growth

trials although potential problems with this method include the different expressions of inbreeding depression for plants grown in a greenhouse compared to the natural environment (Ramsey and Vaughton, 1998), and the length of time for long-lived plants to flower (i.e. 4 years in *Protea*). In this study, inbreeding depression of selfed progeny in a short greenhouse trial (chapters 2&5) was not detectable, which contradicted the independent estimates of inbreeding depression from the allozyme analysis, but this experiment will ultimately have to be repeated under field conditions.

Emission of scent from various flower parts — This study demonstrated that various floral parts contribute differently to the overall scent composition of *Protea* inflorescences (chapter 6). Headspace sampling indicated that, in comparison to other floral parts, nectar makes the most diverse contribution (33-55 compounds) to overall inflorescence scents. The nectar is also suspected to ferment as inflorescences senesce and this possibly affects pollinator attraction (see future directions below). During preliminary testing in the lab, the scent of fresh nectar was enough to elicit a feeding response from *Atrichelaphinis tigrina* beetles allowed to walk over nectar dotted on filter paper and hidden under porous cloth. The scent of nectar may therefore function as an honest floral signal advertising the presence of a reward to *Protea* pollinators as suggested by Raguso (2004). Additionally, the scent of nectar can be an important pollinator filter, especially with regards to bird-pollinated systems in which it can affect taste (Raguso, 2008a). However, linalool, the most abundant compound in *Protea* floral scents, was found to be emitted mainly by the perianth and stylar tissue. As linalool plays an important role in attracting pollinators (as demonstrated by bucket traps in chapter 8) and marks the most distinct change between bird- and beetle- pollinated *Protea* species (discussed in chapter 7), this is likely to be of functional significance. The high emission of linalool may act as a long-distance attractant and the other fruity-sweet emissions from nectar act as a pollinator filter or to elicit a feeding response.

The emission rates of overall inflorescence scent of the study species was shown to be highest at full anthesis. This probably coincides with maximum nectar production, pollen presentation and the start of receptivity for outer florets. Although the frequency of beetles visiting at different flowering stages needs more rigorous measurement, I tended to observe more beetles in fully dehisced inflorescences.

Emission of scent in insect- versus bird-pollinated congeners — Collins and Rebelo (1987) suggested that strong floral odours may be associated with insect- rather than bird- or

mammal-pollination systems in the Proteaceae. I characterised floral odours and emission rates for four beetle-pollinated and eight putatively bird-pollinated *Protea* species, and established that a marked increase in diversity and emission of fruity-sweet floral volatiles, namely linalool, is associated with a shift from bird- to beetle-pollination (chapter 7). Relating such evolutionary changes in floral traits to shifts between pollination systems adds to evidence linking adaptation and speciation as described by Johnson (2006). Studies documenting pollinator shifts are often used as a platform for describing evolutionary mechanisms leading to speciation, especially for shifts between pollinators with different sensory abilities (e.g. Bradshaw and Schemske, 2003; Campbell, 2008; Smith et al., 2008). Flowers of bird-pollinated plants are generally odour-less to humans, although it has been shown that some floral volatiles, such as sesquiterpenes, are not well detected by humans (see Knudsen et al., 2004) and thus human olfaction may not be the best judge of floral scent in some cases.

The floral scents of bird-pollinated *Protea* species studied here were surprisingly complex. To date, most documented bird-pollination systems involve odourless flowers (Knudsen et al., 2004) although some flowers with mixed bird and insect pollination systems are scented (e.g. *Iochroma*; Smith et al., 2008) Although Knudsen et al. (2004) suggest that bird-pollinated flowers are unscented their conclusions were based exclusively on studies of plants pollinated by hummingbirds. My study is the first to examine the floral scents of plants pollinated by passerine birds and suggests that bird pollinated flowers may not always be unscented. However, it's possible that the production of scent in bird-pollinated *Protea* species is driven by incipient transitions to other pollination systems.

Changes in floral scent have clearly played an important role in the shift from bird- to beetle-pollination in at least one clade of *Protea*. My results, in conjunction with reports of yeasty floral scents of rodent-pollinated *Protea* species in South Africa (Wiens and Rourke, 1978), suggest that shifts from bird to non-flying mammal-pollination in African and Australian Proteaceae will also be associated with a change in floral scent. Shifts such as these help to explain the immense floral radiation within genera of the Proteaceae. Pollinator shifts have often been used to explain adaptive radiations of plant lineages, as seen in *Disa* (Johnson et al., 1998). However, Schnitzler et al. (2011) found that species radiations for three Cape clades, specifically *Babiana, Moraea*, and *Protea,* were more strongly correlated with soil type. Pollinator shifts were only identified as the third out of five factors explored as causes of speciation in *Protea* (Schnitzler et al., 2011), but their findings have recently been

challenged on methodological grounds (Van der Niet et al., submitted). Nevertheless, pollination shifts should not be assumed to be the sole driver of speciation.

Responses of cetoniine beetles to flower volatiles — The floral odour blends of beetle-pollinated *Protea,* asclepiad and orchid species include linalool and a variety of other shared monoterpenes (Johnson et al., 2007; Shuttleworth et al., 2010; Steenhuisen et al., 2010). Whole *Protea* inflorescence scents and the single compounds linalool and methyl benzoate were shown to be attractive to cetoniines in chapters 7 and 8. The attraction of cetoniines by various other monoterpenes and benzenoids comprising *Protea* scents have been tested by other researchers working on different beetle species, particularly those that damage fruit crops (e.g. Donaldson et al., 1990). My results confirm that scent plays an important role in attracting cetoniine beetle pollinators. However, results from beetle trapping experiments in chapter 8 and in the literature (Schmera et al., 2004) have shown that colour cues are important in the attraction of cetoniines. I have observed cetoniines visiting many plant species comprising grassland communities, and which share floral traits such as yellow colour and sweet/fruity scents. One possibility is that scent may act as a long distance attractant while colour cues represent a conditioned response within particular communities. The scent of nectar, however, could advertise the presence of a reward at close range. The relative roles of colour and scent cues for attracting cetoniines therefore need further investigation.

Future research — This study demonstrates that beetle visitors to *Protea* inflorescences can be effective pollinators. Cetoniines aggregate on flowerheads of various plant families in grasslands of eastern South Africa. Medium sized cetoniines (*Atrichelaphinis, Cyrtothyrea, Leucocelis*) are principally pollen and nectar feeders in members of *Protea* that were investigated. I did not observe them eating floral parts, which contradicts the common perception that they are floral antagonists. They may actually be more important for the pollination of grassland species than previously realised. Cetoniine-pollinated plants, such as the *Protea* species in this study should be seen as moderately specialised, even though they have adapted to extremely generalist insects.

The shift from bird- to insect-pollination in *Protea* occurred in a non-Cape clade (Valente et al., 2010) with an additional eleven sister species to those used in this study. To determine if the evolution of strong fruity/sweet floral scents has occurred once only, the floral scents of the remaining members of this clade (i.e. *P. angolensis, P. comptonii, P.*

curvata, P. enervis, P. gaguedi, P. heckmanniana, P. laetans, P. nubigena, P. parvula, P. rubropilosa, and *P. wentzeliana*) need to be investigated. Furthermore, shifts to rodent pollination in *Protea* may be associated principally with the evolution of yeasty odours adapted to attracting mammal pollinators (S.D. Johnson, unpublished data). Evolutionary changes in floral odour may be associated with pollinator shifts in other Proteaceae in Africa (e.g. sweet scents of putatively insect-pollinated *Leucadendron*; M. Welsford, unpublished data) and Australia (e.g. sulphur compounds in scents of mammal-pollinated *Banksia*; S.D. Johnson, unpublished data). The ease with which floral odours can presently be quantitatively characterised has opened up new possibilities to explore earlier speculations that the evolution of floral scent has played a key role in pollinator shifts in the Proteaceae.

The four study species hybridise freely and hybrid zones including up to three of these species at any one time are evident in grasslands in KwaZulu-Natal. An interesting observation is the combination of floral traits arising from these hybridizations, such as plants producing inflorescences with hairy involucral bracts, a floral trait of *P. welwitschii*, coupled with the pink colour of *P. simplex*. These hybrid zones represent an exciting opportunity to investigate the inheritance of physiological pathways leading to the production of floral volatiles unique to each species, particularly the tiglic acid esters produced by *P. welwitschii.* In addition, hybrid zones provide an opportunity to further investigate scent-driven pollinator shifts if novel scents in the hybrids attract different pollinators compared to the parental plants, as seen in *Ophrys* hybrids (Vereecken et al., 2010). Hybrids zones are useful for selection studies because they can reintroduce phenotypic variation that has been removed by selection in the parent species (e.g. Meléndez-Ackerman and Campbell, 1998; Campbell et al., 2009).

Field tests determining the attractiveness of physiologically detected compounds found in beetle-pollinated flower odours should further help to understand the functional significance of compounds for attraction of cetoniines and, ultimately, to characterise the adaptive component of floral scents of plants conforming to a cetoniine beetle pollination system. It would be especially interesting to test the attractiveness of anisole, which gave the highest EAG response for *A. tigrina* (chapter 8), and the unique esters found in *P. welwitschii* floral odours (chapter 6-7). These beetles responded more strongly to butyl tiglate than to benzyl and hexyl tiglate (chapter 8). This represents an opportunity to test the attractiveness of different esters with the same basal group but differing in their attached side group. The production of tiglic acid esters by *P. welwitschii* may be autapomorphic and headspace samples of floral odours should be taken of its most closely related species (*P. angolensis, P*

gaguedi, P. heckmanniana) to track the evolution of this trait. In addition to tests of different compounds, EAG and field-trapping dose-dependent responses could also be determined, especially for compounds dominating floral scents of *Protea*, such as linalool. Synergistic relationships may also exist between volatile attractants and the attractiveness of combinations of compounds could be tested in the field. All of these tests will improve our understanding of how the evolution of one or many compounds has resulted in a shift to beetle pollination in this genus and in other plant groups.

The scent of nectar in this system needs more investigation to determine if it is a result of passive absorption of volatiles emitted from other floral parts or from active secretion of compounds into the nectar itself. To the human nose, the nectar of senescing flowers smells acidic and preliminary investigations have revealed that the nectars of these scented *Protea* species harbour an abundance of fermentative yeast and bacteria. A few typical fermentation volatiles (e.g. acetoin) were found in the nectar scents of these species and the question remains: is fermentation of *Protea* nectar contributing to the scent of these *Protea* inflorescences and if so, are scents from nectar fermentation attractive or repellent to pollinators? Interestingly, large numbers of beetles can be observed in older inflorescences. The attractiveness of fermentation compounds would be important to insects exploiting immense nectar sources in bird-pollinated *Protea*. However, preliminary results also suggest that the abundance and diversity of microbes is greater in beetle-pollinated species compared to bird-pollinated species. In addition, yeasts, including a new species (de Vega et al., submitted) were identified from the study species and were shown to be vectored by Cetoniinae and Hopliini visiting scented *Protea* species by allowing the beetles to walk over agar plates, followed by sequencing of the yeast cultures that grew. In addition, scent may affect the taste of nectar. To humans, the nectar of the study species tastes almost exactly how it smells —papaya-like. If scented nectar is acting as a pollinator filter based on taste, perhaps bird pollinators prefer the taste of non-scented *Protea* nectars although the concentration and composition of nectar sugars would also need to be taken into account in any comparison of nectars from beetle- and bird-pollinated *Protea* species.

Another floral trait that needs to be explored in more detail is colour. Our field-trapping experiments indicated that yellow played an important attractive role but our interpretation is limited by the use of only one colour comparison (green and yellow) and limited knowledge of beetle colour vision. The colour sense of some scarab beetles is currently under investigation by Lars Chittka's research group at Queen Mary University, London (Arnold, 2010), and it is hoped that a model similar to the bee-colour hexagon or bird

tetrahedron models will become available in the near future (Chittka, 1992; Endler and Mielke, 2005). This will allow spectral reflectance data such as that measured for *Protea* in chapter 3 and for the traps used in chapter 8 to be interpreted in terms of the sensory capabilities of beetles. Many grassland species visited by cetoniine beetles have yellow flowers that emit sweet scents from petals or foliage. The relative importance of colour cues should be investigated further, especially to determine if colour attraction is an innate or learnt response involving floral rewards (Kelber and Osorio, 2010). Since cetoniines visit cryptically coloured asclepiads and orchid species in grasslands (Johnson et al., 2007; Shuttleworth et al., 2008; Peter et al., 2009), I suspect that colour attraction is a consequence of learning, and not essential for initial visitation.

My review of *Protea* breeding systems uncovered fundamental methodological problems and contradictions in the literature (chapter 2). It is recommended that any further research on the pollination ecology of *Protea* species should be accompanied by a thorough investigation of the breeding system, including controls for hand pollination methods. The breeding systems of species used in Horn's (1962) study need to be verified, and hand-pollination methods explained and tested against cross-pollination treatments. Pollen and resource limitation and high seed predation has been shown for several Proteaceae (Steenhuisen and Johnson, in press; chapters 3&5), further complicating the interpretation of resulting seed set data from exclusion experiments. The estimation of outcrossing rates represents a unique approach for defining pollinator effectiveness in autogamous plant species. Outcrossing rates have been investigated for some Australian Proteaceae, using allozyme and microsatellite analyses (Scott, 1980; Ayre et al., 1994; England et al., 2001), but this is the first study to do so for an African member of the family. Microsatellite primers are now available for white proteas (Prunier and Latimer, 2010). These should yield more variable loci than the allozymes that were used in this study and thus create future opportunities to explore outcrossing rates in South African *Protea* populations. It would be particularly interesting to compare outcrossing rates and the distance of pollen-mediated gene flow in bird versus insect pollinated *Protea* species to answer the question of whether birds are generally better outcrossing agents than insects, and carry pollen longer distances (cf. Price and Waser, 1982; Waser, 1982; Schulke and Waser, 2001). Beetles can spend a few hours foraging in one inflorescence before moving onto another on the same or a different plant. Birds are much more active and fly between plants more frequently. Because many species are also rodent-pollinated, *Protea* offers ideal opportunities to understand the evolutionary consequences of shifts between different pollination systems.

Pollinator shifts have been shown to correspond to biogeographical patterns in pollinator distributions (e.g. Johnson, 1997; Johnson, 2010). In *Protea*, the shift from bird- to beetle-pollination may be associated with the distribution of different pollinator groups according to rainfall patterns and associated vegetation. Whilst the majority of *Protea* species in the winter-rainfall areas of the Cape which has largely shrubby vegetation are bird-pollinated, scented beetle-pollinated species are found in eastern South Africa, which receives summer-rainfall and has largely grassland vegetation. The distribution of *A. tigrina* matches that of these summer-rainfall species and thus there also may be a close correlation between the distributions of scented *Protea* species and their cetoniine beetle visitors (Fig. 1 and Figs 1-4 in chapter 1). Since *A. tigrina,* along with several other cetoniines, have been shown to pollinate scented *Protea,* asclepiad and orchid species in eastern South Africa, it would be interesting to investigate correlations between distributions of cetoniine pollinators and the plants they visit (Ollerton et al., 2003; Johnson et al., 2007; Shuttleworth et al., 2010). I predict that these beetles pollinate many more fruity-sweet scented grassland species across their range and that the guild of cetoniine-pollinated plants in southern Africa is concentrated in the eastern region.

The results of this thesis have added to evidence for a cetoniine beetle pollination system in grasslands of South Africa. Through headspace sampling of floral odours and choice tests with a common cetoniine pollinator, grassland *Protea* species were shown to employ fruity-sweet scents to attract pollinators to large colourful inflorescences. More specifically, a shift from bird- to beetle-pollination in *Protea* is associated with the up-regulation of linalool and a suite of other benzenoid and monoterpene compounds commonly found in floral scents, which elicit significant EAG responses and are attractive in the field to cetoniine beetles. Future research in this genus should be aimed at verifying the results of past breeding system studies, determining the evolutionary pattern of scent emission correlated with pollinator shifts in a phylogenetic context, and clarifying the role of microbial fermentation of nectar in the emission of volatiles that attract beetle pollinators.

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