

The reproductive biology of four geoflorous *Protea* species (Proteaceae)

Kim Christie Zoeller



Supervised by J. J. Midgley, S-L. Steenhuisen and S. D. Johnson

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.



University of Cape Town

Faculty of Science

Department of Biological Sciences

THESIS

Submitted in fulfillment of the requirements for the degree of

Master of Sciences (Biological Science)

August 2014

**The reproductive biology of four geoflorous *Protea*
species (Proteaceae)**

Kim Christie Zoeller

Supervisor: Prof Jeremy J. Midgley (Department of Biological Sciences, UCT)

Co-supervisors: Dr Sandy-Lynn Steenhuisen (Department of Biological
Sciences, UCT) and Prof Steven D. Johnson (School of Life Sciences,
University of KwaZulu-Natal)

Declaration 1-Plagiarism

1. I know that plagiarism is wrong. Plagiarism is to use another's work and pretend that it is one's own.
2. Each contribution to, and quotation in this research report from the work(s) of other people has been contributed, and has been cited and referenced.
3. This research report is my own work and has not previously in its entirety or in part been submitted at any other university for another degree.
4. I have not allowed, and will not allow, anyone to copy my work with the intention of passing it off as his or her own words

Signature: _____

Date: _____

Declaration 2-Publication

Publication 1.

K.C. Zoeller, S. D. Johnson, S-L. Steenhuisen, J. J. Midgley. New evidence for mammal pollination of *Protea* species based on remote camera analysis. Unpublished, to be submitted to South African Journal of Botany.

Author contributions:

KCZ, SDJ, JJM and SLS conceived paper. KCZ collected and analyzed the data and wrote the paper. JJM, SLS and SDJ contributed comments.

Publication 2.

K.C. Zoeller, S. D. Johnson, S-L. Steenhuisen, J. J. Midgley. Evolutionary specialization and ecological generalization in the pollination of geoflorous *Protea* species (Proteaceae). Unpublished, to be submitted to South African Journal of Botany

Author contributions:

KCZ, SDJ, JJM and SLS conceived paper. KCZ collected and analyzed the data and wrote the paper. JJM, SLS and SDJ contributed comments.

Publication 3.

S-L.Steenhuisen, A. Balmer, K. C. Zoeller, N. Kuhn, J.J. Midgley, S. D. Johnson. Carnivorous visitors to non-flying mammal pollinated *Protea* species (Proteaceae) in South Africa. Unpublished, to be submitted as a short communication to African Zoology.

Author contributions:

SLS and JJM conceived paper. SLS, AB, KCZ and NK collected and analyzed data. SLS wrote the paper. AB, KCZ, NK, JJM and SDJ contributed comments

Acknowledgements

I am hugely grateful to my three supervisors; Dr Sandy-Lynn Steenhuisen, Prof Jeremy Midgley and Prof Steve Johnson. In particular, to Sandy for your help with data analysis, constructive comments on previous drafts, field support, access to your photographs and instructions on experimental techniques. I am so thankful for your unwavering love, support and patience. To Jeremy for conceiving this project when I requested it, for all the valuable contributions you have made to every aspect of this thesis, and for leaving me at each meeting feeling happy and hopeful. To Steve for your thoughtful and inspiring edits of previous drafts of this thesis, and for providing me with a reliable 4x4 vehicle (with petrol!) that allowed me to access to remote *Protea* populations.

I would like to thank Cape Nature for allowing this project to proceed (permit numbers 0028-AAA008-00064 and 0056-AAA008-00004) and the University of Cape Town's Animal Ethics Committee (2012\46\SS). I am grateful to the Overstrand Municipality for allowing me to work at Fernkloof Nature Reserve, and The Lower Breede River Conservancy for permission to work in the Lower Breede River. Additionally, I would like to thank Di McKenzie for allowing us to access her farm on the Lower Breede River and Connie Peterse for permitting us to drive through his property on Houw Hoek Pass. I am very grateful to all those who supported me in the field and in the lab. Dr Adam Shuttleworth for use of his bee hexagon model, and Dr Timo van der Niet and Frank Greer for explaining bird colour vision on R. I would also like to thank Kirsten Packer for her tireless efforts in the field.

Finally, I owe a huge debt of gratitude to my friends and family. Michael Spreckley thank you not only for your help in the field, but also for your support every step of the way. To Lee Zoeller, thank you for keeping me laughing even in the most stressful of times. To my mother, Mandi, you have maintained an encouraging and positive attitude when I needed it

the most. I am in awe of your strength. And to my father, Chris. I'm sorry you didn't get to see me submit my MSc, but I know that I couldn't have reached this point without the drive, dedication and passion you instilled in me. I dedicate this thesis to you.

Abstract

Pollination systems of previously unstudied plant species are often inferred from floral traits that are shared among unrelated plant taxa. However, these ‘pollination syndromes’ are often not reliable predictors of the primary pollinator of a plant species, and may in fact represent adaptations to multiple pollinator groups or even minor pollinators. As such, conducting comprehensive field-based research into the pollination and breeding system of unstudied plant species is important for furthering our understanding of evolutionary processes underlying shifts in pollination systems within plant lineages. *Protea cordata*, *P. decurrens*, *P. scabra* and *P. subulifolia* are cryptic, geoflorous shrubs that are predicted to be pollinated by non-flying mammals based on their floral traits, although there has been no experimental evidence in support of this. The aims of this study were to quantify key functional traits that mediate interactions between these four *Protea* species and their pollinators, as well as to experimentally determine their pollination and breeding systems. We further aim to establish the main vertebrate pollinators of the study species, their relative rates of visitation and their temporal patterns of activity using remote camera footage. Rodents were found to be the most frequent visitors to the flower heads of the study species. Floral morphology, nectar volume and sugar concentration of the study species conform to the general syndrome of non-flying mammal pollination system in this genus. In particular, the short (14-20 mm) distance between pollen and nectar ensures pollen deposition on the body of rodent foragers. Excluding small mammals from flower heads did not significantly reduced seed set, suggesting that insects can play an important role in cross-pollination in the study species. Breeding system experiments revealed that *Protea cordata* and *P. scabra* are self-

incompatible, and are reliant on animal vectors for successful seed set. Supplemental hand pollination on *P. cordata* and *P. scabra* indicated that resource limitation is prevalent among these plant species. This study adds to the growing knowledge of the reproductive biology of *Protea* and will improve our ability to reconstruct the history of pollinator shifts in the genus.

Table of contents

Title page.....	ii
Plagiarism declaration.....	iii
Acknowledgments.....	v
Abstract.....	vii
1. Introduction	
1.1 Floral adaptations for pollination.....	1
1.2 Cape Proteaceae.....	2
1.3 Non-flying mammal pollination (NMP) in Cape <i>Protea</i>	3
1.4 <i>Protea</i> breeding systems.....	7
1.5 Specific Aims.....	8
1.6 References.....	9
2. New evidence for mammal pollination of <i>Protea</i> species based on remote camera analysis	
2.1 Abstract.....	16
2.2 Introduction.....	17
2.3 Methods.....	18
2.3.1 Study species.....	18
2.3.2 Remote cameras.....	21
2.3.3 Data analysis.....	22
2.4 Results.....	23
2.4.1 Pollinator identification and rates of visitation.....	23
2.4.2 Temporal patterns of visitation.....	25
2.4.3 Vertebrate visitors.....	27
2.5 Discussion.....	27
2.5.1 Non-species specific specialization for rodents.....	28
2.5.2 Temporal activity of mammals.....	28
2.5.3 Future uses of camera traps.....	29
2.6 References.....	30

3. Evolutionary specialization and ecological generalization in the pollination of geoflorous *Protea* species (Proteaceae)

3.1	Abstract.....	34
3.2	Introduction.....	35
3.3	Methods.....	37
	3.3.1 Study species and sites.....	37
	3.3.2 Morphology.....	39
	3.3.3 Controlled hand-pollination and selective exclusion...	43
	3.3.4 Statistical analyses.....	45
3.4	Results.....	46
	3.4.1 Morphology.....	46
	3.4.2 Controlled hand-pollination and selective exclusion.....	51
3.5	Discussion.....	53
	3.5.1 Floral specialization and ecological generalization.....	53
	3.5.2 Resource allocation to reproduction.....	58
	3.5.3 Ecological implications.....	59
3.6	References.....	62

4. Carnivorous visitors to non-flying mammal pollinated *Protea* species (Proteaceae) in South Africa

4.1	Abstract.....	68
4.2	Introduction.....	69
4.3	Methods.....	71
4.4	Results and discussion.....	72
4.5	References.....	75

5. Synthesis

5.1	Conclusion.....	77
5.2	Issues and future research.....	79
5.3	Implications.....	80
5.4	References.....	81

6. Appendix.....85

1. Introduction

1.1 Floral adaptations for pollination

Understanding the pollination biology of a plant species is important for gaining insights into the process of sexual reproduction through seeds (Johnson *et al.* 2009). Pollination occurs when pollen is released by the anthers (male), transferred to the stigma (female) and successfully placed on its receptive surface (Faegri & Van Der Pijl, 1979). Pollination systems of plants are inherently complex because plants are sessile and most rely on vectors to transport pollen from anther to stigma (Cheptou, 2011). These vectors are either abiotic (wind or water) or biotic (insects, birds and mammals). Utilization of biotic vectors requires numerous floral adaptations, ranging from suitable flower shape, through provision of rewards, to deployment of advertising signals. These generally correspond to the morphology, energetic requirements and sensory abilities of the “most effective” pollen vector in order to facilitate pollen receipt (Stebbins, 1970; Baker *et al.* 1998; Johnson & Steiner, 2000; Johnson *et al.* 2001; Knight *et al.* 2005; Steenhuisen and Johnson, 2012a; Steenhuisen *et al.* 2013).

Floral traits function as cues that are perceived by the pollinator which responds either innately or through associative conditioning with rewards, that, in turn, promotes repeat visitation (Schiestl and Johnson, 2013). Floral rewards can be functional (nest construction sites, heat sources and mating sites) or nutritional (nectar and pollen) (Simpson and Neff, 1981). When pollinators become conditioned to the plants specific traits and associated rewards, it promotes successful pollen placement and a reduction in stigma clogging (Steenhuisen *et al.* 2012b).

Plants pollinated by particular pollinator functional groups tend to exhibit convergent suites of floral traits or ‘pollination syndromes’ (de Merxem *et al.* 2009; Hargreaves *et al.* 2004;

Ollerton *et al.* 2009; Johnson & Steiner, 2000). These syndromes often provide a good basis for making initial hypotheses about the pollinators of a plant, to be tested using observations and field experiments (Johnson *et al.* 2001; Ollerton *et al.* 2009). However, research has suggested that pollinator-mediated selection for floral traits are often not based on a “lock and key” relationship (Grant and Grant, 1965), as floral traits may represent adaptations to pollinators from multiple functional groups or even ‘minor’ pollinators, (Spears, 1983; Waser *et al.* 1996; Johnson and Steiner, 2000; de Merxmen *et al.* 2009; Ollerton *et al.* 2009; Aigner, 2001). Consequently, most pollination systems exist along a continuum from many potential pollinators, to just one specialized pollinator species (Johnson & Steiner, 2000), but even plant species that possess specialized floral traits, such as long tubes and distasteful nectar that act as filters, may not entirely restrict the assemblages of animals that visit the plant (Johnson & Steiner, 2000).

1.2 Cape Proteaceae

The Cape Floristic Region (CFR) of South Africa is a biodiversity hotspot that is characterized by the unique vegetation of the fynbos biome (Valente *et al.* 2010). Fynbos is renowned for its exceptional species richness (around 9000 species), high endemism (68-69%), and adaptations for periodic burning (Manning and Goldblatt, 2012; Wiens *et al.* 1983). *Protea* (Proteaceae) is a prominent genus within fynbos. In southern African, *Protea* consists of 115 species, around 60% of which are endemic to the CFR (Schnitzler *et al.* 2011). All species are woody shrubs or trees, and are adapted for the region’s frequent fires in two ways; resprouting and reseedling (Bond and Midgley, 2003). *Protea* species have important ecological and economic significance, yet research on the evolutionary processes behind their diversification is still lacking.

Morphological diversity in *Protea* species is often associated with pollinator shifts (Steenhuisen *et al.* 2012a; but see Schnitzler *et al.* 2011). This occurs when a plant is exposed

to a novel environment wherein a new, pervasive pollinator exerts selective pressure on the functional traits of the plant (Stebbins, 1970; Hargreaves et al, 2004; Johnson *et al.* 2011). Consequently, pollination systems often shift rapidly, particularly when shifts are mediated by changes in just a few floral traits (Ollerton, 1996; Johnson *et al.* 2011). The extensive morphological and geographical diversity of *Protea* species implies that this genus may have undergone high levels of pollination shifts throughout its 140 million year evolutionary history (Valente *et al.* 2010; Rourke 1980). Currently, *Protea* species are known to be adapted to the unique foraging behavior and sensory preferences of birds (e.g. *Protea roupelliae*) (Hargreaves et al, 2004), insects (e.g. *Protea caffra*) ((Steenhuisen *et al.* 2012a) and non-flying mammals (e.g. *Protea humiflora*) (Wiens *et al.* 1983).

1.3 Non-flying mammal pollination in South African Protea

Research on non-flying mammal pollination (NMP) on South African *Protea* species has rapidly intensified since the 1970's (Rourke and Wiens, 1977), and has subsequently been identified in other key South African genera, including *Massonia* (Hyacinthaceae) (Johnson *et al.* 2001), *Whiteheadia* (Hyacinthaceae) (Wester *et al.* 2009), *Liparia* (Fabaceae) (Letten & Midgley, 2009), *Colchicum* (Colchicaceae) (Kleizen *et al.* 2008), *Cytinus* (Cytinaceae) (Johnson *et al.* 2011), and *Leucospermum* (Proteaceae) (Johnson and Pauw, 2014). While around 35 *Protea* species are inferred from their floral traits to be pollinated by small mammals, very few comprehensive studies on their pollination biology and reproductive ecology have been conducted (Rourke, 1980; Wiens *et al.* 1983; Biccard and Midgley, 2009). The pollination system of the basal *Protea* species has not yet been described, but current phylogenetic reconstructions place bird pollination as the ancestral condition, with some lineages showing shifts to insect and mammal pollination (Valente *et al.* 2010).

The shift from bird to small mammal pollination is thought to be a consequence of specialized plant species facing ecological restrictions associated with changes in habitat

(Wiens *et al.* 1983). This ‘restricted population hypothesis’, based on the well-known Allee effect (Ward & Johnson, 2005), suggests that the movement of *Protea* species into localized habitats over evolutionary time would diminish their attractiveness to birds, which tend to forage exclusively on large populations (Turner *et al.* 2011). Conversely, non-flying mammals within the CFR are generalist feeders and may be more attracted to ephemeral resources than specialist feeders (Wiens *et al.* 1983). The brief flowering period of NMP *Protea*, their restricted distribution, and the dichotomy between specialist floral traits and pollination by generalist foragers implies that NMP has evolved unilaterally (i.e. not through coevolution) in the Cape *Protea* (Wiens *et al.* 1983; Fleming and Nicolson, 2002; Kleizen *et al.* 2008).

1.3.1 NMP functional traits

The basic floral features indicative of NMP in *Protea* include geoflorous growth with “yeasty” scented, cryptic inflorescences that produce copious amounts of concentrated nectar (Wiens and Rourke, 1978; Johnson *et al.* 2001). Each inflorescence contains a large number of florets, often resulting in consistently low proportion of seeds set per inflorescence (Rourke and Wiens, 1977). The florets possess an effective 10 mm distance between pollen presenter and nectar (i.e. pollen-reward) (Wiens *et al.* 1983). The bracts of NMP *Protea* are generally dull red to brown (Wiens *et al.* 1983). While convergence in colour has been attributed to pollinator selection, there is no evidence to suggest that pollinators are the sole selective agent for flower colour (Carlson and Holsinger, 2010; Johnson and Steiner, 2000). NMP plants flower over early winter to late spring, providing a nectar reward that entices mammal foragers during winter months when alternative resources are low (Fleming & Nicolson, 2002). NMP *Protea* species that occur in sympatry tend to flower sequentially (Wiens *et al.* 1983). This non-overlapping phenology implies that pollinators are supplied

with resources over an extended period, and that the probability of inter-species reproductive interference is reduced (Biccard and Midgley, 2009; Botes *et al.* 2008).

The primary reward for pollinators is nectar (Johnson *et al.* 1999). The properties of nectar are variable over evolutionary time, and differ among angiosperms according to the dietary requirements of the major pollinator (Steenhuisen and Johnson, 2012a). Therophilous *Protea* species produce copious, viscous nectar reserves that provide foraging mammals with “junk food” rather than a sustainable resource on which small mammals can rely (Fleming & Nicolson, 2002). Nevertheless, nectar from NMP *Protea* is easily metabolized by most rodent foragers and may play an important role in female gestation, lactation and juvenile survival (Fleming and Nicolson, 2002; Rebelo and Breytenbach, 1987).

In addition to offering rewards, many plant species utilize distinct scents in order to attract pollinators. Olfactory cues are an important component in enticing pollinators as animals rely on scent to inform them on foraging sites, potential mates and defining territories (Steenhuisen *et al.* 2012b), and furthermore, enables plants to attract a large number of pollinators without relying on direct visual cues (Steenhuisen *et al.* 2012b). This is crucial for plants that are pollinated nocturnally, as visual cues are likely to be less effective (Johnson *et al.* 2011). For example, Wiens *et al.* (1983) found that small mammals were immediately attracted by the scent of *Protea amplexicaulis* and *Protea humiflora* even though the floral heads were concealed, and *Rhodomys pumilio* was innately attracted to the 3-hexanone compound in the floral scent of *Cytinus visseri*; (Johnson *et al.* 2011) and preferred the “sour-milk” scent of *Protea recondita* over sugar water in a choice experiment (Balmer, 2012).

1.3.2 Evidence for rodent pollination

Rodent pollination has been described frequently in the literature. Wiens et al (1983) provided evidence for NMP of several therophilous *Protea* species through *Protea* pollen deposits found on field-caught rodents, preference for therophilous *Protea* inflorescence in captive choice tests, as well as reduced seed set in rodent excluded inflorescences. However, in order to successfully demonstrate that rodents are the primary pollen vectors of suspected NMP plants, several important aspects need to be demonstrated; (1) that the proposed pollinator regularly visits the inflorescences in a non-destructive manner, (2) there is a substantial pollen load picked up by the visitor and transported to conspecific floral heads, (3) these pollination events produce viable seeds, (4) few visits by alternative pollinators (birds and insects), (5) seed set is reduced when small mammals are excluded from the inflorescence and (6) nectar secretion, scent production and floral anthesis correspond with activity times of the rodent pollinators (Goldingay *et al.* 1991; Johnson *et al.* 2001).

Various integrated techniques have been implemented in order to address these questions, allowing biologists to not only identify effective pollinators (Fleming and Nicolson, 2002; Biccard and Midgley, 2009; Spears, 1983), but also quantify floral advertisement and rewards (Johnson *et al.* 1999; Steenhuisen *et al.* 2012a), clarify reproductive biology using selective enclosure experiments (Johnson *et al.* 2001), determine population dynamics and genetic structure (Whitehead and Peakall, 2009), and evaluate these characteristics according to phylogenetic relationships (Johnson *et al.* 2009; Micheneau *et al.* 2009). One of the more novel, but highly informative approaches to pollination studies includes the development of remote video surveillance (Carthew and Slater, 1991). This technology gives new insight into natural behavior by providing information on pollinator assemblage, pollinator behavior and frequency of visitation.

1.4 Breeding Systems

Early papers on the breeding systems of *Protea* inferred that genetic self-incompatibility was ubiquitous in the genus (Horn, 1962), but is previously undetermined for NMP species (Steenhuisen and Johnson, 2012a; Wiens *et al.* 1983). Selection for self-compatibility is often linked to the colonization of pioneer environments on the margin of the species habitat range where the conditions are unfavorable for pollination (Stebbins, 1970). Pollen-limitation is less likely to occur in self compatible plants because the plants own pollen increases the pool of pollen available for fertilization (Larson and Barrett, 1999). In extreme pollen limited environments, autogamy – the ability of a plant to produce progeny in the absence of a pollination agent – can be selected (Cheptou, 2012; Knight *et al.* 2005). However, by accepting self-pollen, the plant is effectively reducing the gene pool of the species, thereby decreasing genetic diversity, viability and diversification (Busch and Delph, 2012). Plants that require facilitated self-pollen transfer usually have a mixed mating system (seed fertilization by both cross and self-pollen) (Lloyd and Schoen, 1992; Ollerton, 1996).

On the other end of the spectrum, species that outcross are introduced to novel genes via conspecific pollen expression, resulting in greater genetic variability and seedling establishment success, although this strategy necessitates interactions with pollen vectors (Faegri and van der Pijl, 1979). Investigating plant-pollinator relationships requires an in-depth examination of the potential for the pollinator to impact the plant's reproductive system (Spears, 1983). This entails not only study of the breeding system (such as the potential to self), but also measurement of pollinator effectiveness. These factors in the reproductive biology of many *Protea* species are mostly unknown, or based on circumstantial, anecdotal evidence (Steenhuisen and Johnson, 2012b).

Breeding systems can be studied by controlled pollinations, where seed set acts as a measure of plant fertility (Spears, 1983). This usually involves supplementing flowers with either

cross or self pollen to test for genetic incompatibility and excluding all visitors from unmanipulated control flowers to test for autogamy (Richards, 1997). In *Protea*, Biccard and Midgley (2009) utilized exclusion experiments to determine that self-pollination rates in *Protea nana* were low relative to control treatments, whereas Wiens et al (1983) suggested that self-pollination was partially successful in *Protea humiflora*. This result, however, was later amended by Fleming and Nicolson (2002), who suggested that viable seeds were not a consequence of self-pollen, but rather pollen supplementation by a variety of insects.

1.5 Specific aims

While pollination by non-flying mammals has been demonstrated in several South African *Protea* species (Wiens *et al.* 1983; Biccard and Midgley), most of the *Protea* species assumed to have this pollination system have not been studied in the field or have been looked at very superficially. To gain a deeper understanding of the evolution of pollination systems in the genus, it is therefore necessary to conduct research on the pollination biology of a wide set *Protea* species that have been inferred to be pollination by non-flying mammals.

The aim of this study was to conduct a comprehensive pollination and breeding system study on four species of *Protea* that conform to the therophilous pollination syndrome; *Protea cordata* Thunb., *P. decurrens* E.Phillips, *P. scabra* R.Br., and *P. subulifolia* (Salisb. ex Knight) Rourke. These four species represent two groups of sugarbush; rodent sugarbush (*P. cordata*, *P. decurrens* and *P. subulifolia*) and dwarf-tufted sugarbush (*P. scabra*). These species were selected due to their close proximity to one another, adequate population sizes, ease of access, and the lack of experimental evidence confirming their pollination systems. Rourke and Wiens (1977) and Wiens *et al.* (1983) observed captive rodent pollination of *Protea scabra* and *P. subulifolia*, while pollinator activity on *P. cordata* and *P. decurrens* has never been tested. My specific objectives were 1) to determine the identity of the main visitor species, their relative rates of visitation and temporal patterns of activity, using remote sensor

camera technology. 2) To describe potential functional floral traits of these species, and determine whether these traits facilitate effective pollination by vertebrates (principally rodents). 3) To investigate the breeding systems of these plant species, in order to evaluate the extent to which pollinators are required for successful seed set and whether the species are genetically self-compatible.

1.6 Reference

Aigner, P. A., 2001. Optimality modeling and fitness trade-offs: when should plants become pollinator specialists? *Oikos* 95,177–184.

Baker, H. G., Baker, I., Hodges, S. A., 1998. Sugar composition of nectar and fruits consumed by birds and bats in the tropics and subtropics. *Biotropica* 30, 559–586.

Balmer, A., 2012. A comprehensive ecological pollination study of non-flying mammal pollinated *Proteas*. MSc thesis, University of Zurich.

Biccard, A., Midgley, J. J. 2009. Rodent pollination in *Protea nana*. *South African Journal of Botany*. 75, 720-725.

Bond, W. J., Midgley, J. J., 2003. The evolutionary ecology of sprouting in woody plants. *International Journal of Plant Sciences* 164, 103-114.

Botes, C., Johnson, S. D., Cowling, R. M., 2008. Coexistence of succulent tree aloes: partitioning of bird. *Oikos* 117,875-882.

Busch, J. W., Delph, L. F., 2012. The relative importance of reproductive assurance and automatic selection as hypotheses for the evolution of self-fertilization. *Annals of Botany* 109, 553–562.

- Carlson, J. E., Holsinger, K. E., 2010. Natural selection on inflorescence color polymorphisms in wild *Protea* populations: the role of pollinators, seed predators, and intertrait correlations. *American Journal of Botany* 97, 934–944.
- Carthew, S.M., Slater, E., 1991. Monitoring animal activity with automated photography. *The Journal of Wildlife Management* 554, 689-692.
- Cheptou, P.O., 2011. Clarifying Baker's Law. *Annals of Botany* 109: 633–641.
- de Merxem, D.G., Borremans, B., De Jäger, M. L., Johnson, T., Jooste, M., Ros, P., Zenni, R.D., Ellis, A.G., Anderson, B., 2009. The importance of flower visitors not predicted by floral syndromes. *South African Journal of Botany* 75, 660-667
- Faegri K., van der Pijl L., 1979. *The Principles of Pollination Ecology*. Third revision. Pergamon Press, Oxford.
- Fleming, P., Nicolson, S., 2002. How important is the relationship between *Protea humiflora* (Proteaceae) and its non-flying mammal pollinators? *Oecologia* 132, 361-368.
- Goldingay, R.L., Carthew, S.M., Whelan, R.J., 1991. The importance of non-flying mammals in pollination. *Oikos* 61, 79–87.
- Grant, V. and Grant, K.A., 1965. *Flower Pollination in the Phlox Family*. Columbia University Press.
- Hargreaves, A. L., Johnson S. D., Nol E., 2004. Do floral syndromes predict specialization in plant pollination systems? An experimental test in an 'ornithophilous' African *Protea*. *Oecologia* 140: 295–301.
- Horn, W., 1962. Breeding research on South African plants: II. Fertility of Proteaceae. *Journal of South African Botany* 73, 259–268.

- Johnson, C. M., Pauw, A., 2014. Adaptation for rodent pollination in *Leucospermum arenarium* (Proteaceae) despite rapid pollen loss during grooming. *Annals of Botany* 113, 931-8.
- Johnson, S. A., van Tets, I. G., Nicolson, S. W., 1999. Sugar Preferences and Xylose Metabolism of a Mammal Pollinator, the Namaqua Rock Mouse (*Aethomys namaquensis*). *Physiological and Biochemical Zoology* 72, 438–444.
- Johnson, S. D., Steiner, K. E., 2000. Generalization versus specialization in plant pollination systems. *Trends in Ecology and Evolution* 15, 140-143
- Johnson, S. D., Pauw, A., Midgley, J. J., 2001. Rodent Pollination in the African Lily *Massonia Depressa* (Hyacinthaceae). *American Journal of Botany* 88, 1768–1773.
- Johnson, S. D., Manning, J. C., Pauw, A., 2009. Advances in the pollination biology of South African plants. *South African Journal Of Botany* 75, 625–629.
- Johnson, S. D., Burgoyne, P. M., Harder, L. D., Dötterl, S., 2011. Mammal pollinators lured by the scent of a parasitic plant. *Proceedings of the Royal Society, Biological Sciences* 278, 2303–2310.
- Kleizen, C., Midgley, J. J., Johnson, S. D., 2008. Pollination systems of *Colchicum* (Colchicaceae) in southern Africa: evidence for rodent pollination. *Annals of Botany* 102, 747-755.
- Knight, T. M., Steets, J. A., Vamosi, S. J., Campbell, D. R., Dudash, M. R., Johnston, M. O., Mitchell, R. J., 2005. Pollen limitation of plant reproduction: pattern and process. *Annual Review of Ecology Evolution and Systematics* 36, 467–497.
- Larson, B. M. H., Barrett, S. C. H., 1999. A comparative analysis of pollen limitation in flowering plants. *Biological Journal of the Linnean Society* 69, 503–520.

- Letten, A. D., Midgley, J. J., 2009. Rodent pollination in the Cape legume *Liparia parva*. *Austral Ecology* 34, 233–236.
- Lloyd, D. G., Schoen, D. J., 1992. Self- and Cross-Fertilization in Plants. I. Functional Dimensions. *International Journal of Plant Sciences* 153, 358-369.
- Goldblatt, P., Manning, P., 2012. Plants of the Greater Cape Floristic Region. 1: The Core Cape flora. *Strelitzia* 29. South African National Biodiversity Institute
- Micheneau, C., Johnson, S. D., Fay, M. F., 2009. Orchid pollination: from Darwin to the present day. *Botanical Journal of the Linnean Society* 161, 1–19.
- Ollerton, J., 1996. Reconciling ecological processes with phylogenetic patterns: the apparent paradox of plant-- pollinator systems. *Journal of Ecology* 84, 767-769.
- Ollerton, J., Alarcon, R., Waser, N. M., Price, M. V., Watts, S. Cranmer, L., Hingston, A., Peter, C. I., Rotenberry, J., 2009. A global test of the pollination syndrome hypothesis. *Annals of Botany* 103, 1471–1480.
- Rebelo, T. G., Breytenbach, G. J., 1987. Mammal Pollination in the Cape Flora, in: Rebelo, A. G. (Ed.), Preliminary synthesis of pollination biology in the Cape flora. South African National Science Program Report 141, 109–124.
- Richards AJ. 1997. Plant breeding systems. Chapman & Hall, London.
- Rourke, J. P., 1980. The Proteas of Southern Africa. Purnell, Cape Town.
- Rourke, J. P., Wiens, D., 1977. Convergent Floral Evolution in South African and Australian Proteaceae and Its Possible Bearing on Pollination by Nonflying Mammals. *Annals of the Missouri Botanical Garden* 64, 1–17.
- Schiestl, F. P., Johnson, S. D., 2013. Pollinator-mediated evolution of floral signals. *Trends in Ecology & Evolution* 28, 307-315.

- Schnitzler, J., Barraclough, T. G., Boatwright, J. S., Goldblatt, P., Manning, J. C., Powell, M. P., Rebelo, T., Savolainen, V., 2011. Causes of plant diversification in the Cape biodiversity hotspot of South Africa. *Systematic Biology* 60, 343–357.
- Simpson, B. B., Neff, J. L., 1981. Floral Rewards: Alternatives to Pollen and Nectar. *Annals of the Missouri Botanical Garden* 68, 301-322.
- Spears, E. E., 1983. A direct measure of pollinator effectiveness. *Oecologia* 57, 196-199.
- Stebbins, G. L., 1970. Adaptive radiation of reproductive characteristics in angiosperms. I: pollination mechanisms. *Annual Review of Ecology and Systematics*, 307–326.
- Steenhuisen, S.-L., Van der Bank, H., Johnson, S. D., 2012a. The relative contributions of insect and bird pollinators to outcrossing in an African *Protea* (Proteaceae). *American Journal of Botany* 99, 1104–1111.
- Steenhuisen, S.-L., Raguso R. A., Johnson, S. D., 2012b. Floral scent in bird- and beetle-pollinated *Protea* species (Proteaceae): Chemistry, emission rates and function. *Phytochemistry* 84, 78–87.
- Steenhuisen, S.-L., Johnson. S. D., 2012a. Evidence for Beetle Pollination in the African Grassland Sugarbushes (*Protea*: Proteaceae). *Plant Systematics and Evolution* 298, 857–869.
- Steenhuisen, S.-L., Johnson. S. D., 2012b. The influence of pollinators and seed predation on seed production in dwarf grassland *Protea* “sugarbushes” (Proteaceae). *South African Journal of Botany* 79, 77–83.

- Steenhuisen, S-L., Jürgens, A., Johnson, S. D., 2013. Effects of Volatile Compounds Emitted by *Protea* Species (Proteaceae) on Antennal Electrophysiological Responses and Attraction of Cetoniine Beetles. *Journal of chemical ecology*.
- Turner, R. C., Midgley J. J., Johnson, S. D., 2011. Evidence for Rodent Pollination in *Erica hanekomii* (Ericaceae). *Botanical Journal of the Linnean Society* 166,163–170.
- Valente, L. M., Reeves, G., Schnitzler, J., Mason, I. P., Fay, M. F., Rebelo, T. G., Chase, M. W., Barraclough, T. G., 2010. Diversification of the African genus *Protea* (Proteaceae) in the Cape biodiversity hotspot and beyond: equal rates in different biomes. *Evolution* 64, 745–759.
- Ward, M., Johnson, S. D., 2005. Pollen limitation and demographic structure in small fragmented populations of *Brunsvigia radulosa* (Amaryllidaceae). *Oikos* 108, 253–262.
- Waser, N. M., Chittka, L., Price, M. V., Williams, N. M., Ollerton, J., 1996. Generalization in pollination systems, and why it matters. *Ecology* 77, 1043-1060.
- Wester, P., Stanway, R., Pauw, A., 2009. Mice pollinate the Pagoda Lily, *Whiteheadia bifolia* (Hyacinthaceae)—First field observations with photographic documentation of rodent pollination in South Africa. *South African Journal of Botany* 75, 713-719.
- Whitehead, M. R., Peakall, R., 2009. Integrating floral scent, pollination ecology and population genetics. *Functional Ecology* 23, 863–874.
- Wiens, D., J. P., Rourke., 1978. Rodent pollination in southern African *Protea* spp. *Nature* 276:71-73.

Wiens, D., Rourke, J. P., Casper, B. B., Rickart, E. A., LaPine, T. R., Peterson, C. J., Channing, A., 1983. Nonflying mammal pollination of southern African *Proteas*: a non-coevolved system. *Annals of the Missouri Botanical Garden* 70, 1-31.

2. New evidence for mammal pollination of *Protea* species based on remote camera analysis

2.1 Abstract

Pollination of *Protea* species by small mammals has mostly been inferred from analysis of fur and faecal pollen loads of field-trapped animals. Actual observations of mammal pollination are rarely reported because of the sensitivity of small mammals to human presence. Behaviour of small mammals in the presence of humans may be altered and thus non-representative of natural activity. The development of heat- and movement-triggered motion sensor cameras overcomes these problems and allows for unprecedented insights into natural behaviours. We used this technology to test the hypothesis that mammals are the main floral visitors for four *Protea* species (*P. scabra*, *P. cordata*, *P. subulifolia* and *P. decurrens*). Camera footage revealed that small mammals are frequent visitors to flower heads, making up 100% of visits to *P. cordata*, 97.77% of visits to *P. decurrens*, 93.25% of visits to *P. scabra*, and 100% of visits to *P. subulifolia*, and while birds are relatively infrequent visitors. The *Protea* species are visited by a broad assemblage of rodent species. Although there was a clear separation in activity of diurnal and nocturnal species, there were no temporal differences in activity among the nocturnal species. The resolution of the cameras allowed us to determine that rodents made contact with reproductive parts of the flowers, but further work would be required to demonstrate unequivocally that rodents are the main pollen vectors of these species. These findings highlight the utility of employing camera technology in determining flower-visitation behaviour by small mammals and can provide new insights into plant pollination systems.

2.2 Introduction

Pollination by small, ground-dwelling mammals has evolved in several plant lineages, notably the Proteaceae in both Australia and South Africa. In South Africa, pollination by rodents was documented in a number *Protea* species in the 1970s and 1980s (Wiens and Rourke, 1978; Wiens *et al.* 1983) and, altogether, about 35 *Protea* species with cryptic, strong smelling geoflorous inflorescences are inferred from their floral traits to be pollinated by small mammals (Rourke, 1980; Rebelo and Breytenbach, 1987). The evidence supporting rodent pollination in *Protea* includes live rodent trapping and fur and faecal analysis of pollen loads (e.g. Biccard and Midgley, 2009; Carthew and Slater, 1991; Kleizen *et al.* 2008) as well as captive rodent behaviour (Fleming and Nicolson, 2002). These methods are potentially unreliable because the assemblage of rodents caught in traps often represents only a small subset of the rodent community as it may exclude trap-shy species (Carthew and Slater, 1991). Similarly, while choice trials run on captive rodents may provide evidence for rodent pollination of certain species (Fleming and Nicolson, 2002), it offers very little insight into important aspects of their behaviour in the wild, such as the overall frequency of visitation by different species and the incidence of visits between flowers on the same plant that can lead to geitonogamous self-pollen transfer.

Direct field observations of rodents, particularly nocturnal species, is difficult to achieve as they easily detect human presence, and are also potentially misleading if human presence alters their behaviour and activity patterns (Wester *et al.* 2009; Bridges and Noss, 2011). In contrast, the development of heat- and movement-triggered motion sensor cameras with infrared capacity allows for recording of natural mammal behaviour, and thus could give new scientific insights into mammal pollination systems (Bridges and Noss, 2011). For example, Wester *et al.* (2009) identified the visitation frequency of the dominant pollinator (*Aethomys*

namaquensis) on *Whiteheadia bifolia* (Hyacinthaceae), using remote camera technology.

While there are several weaknesses associated with evaluating pollination systems on camera footage, including difficulty distinguishing visiting species (Carthew and Slater, 1991) and assertion that rodent visitors are carrying pollen, remote cameras are useful in determining (1) the identity of vertebrate visitors, (2) rates of visitation by different vertebrate species, (3) contact between visitors and reproductive parts of flowers, (4) the incidence of visits to multiple flowers on the same plant and (5) temporal patterns of visitor activity, both across the flowering period and throughout the day and night. This latter aspect is particularly useful, as nocturnal activity by cryptic pollinators on inconspicuous floral heads was considered unobservable in the past (Wiens *et al.* 1983), and have thus often been underestimated due to the difficulty associated with monitoring their behaviour (Carthew and Slater, 1991; Goldingay *et al.* 1991).

In this study, we used remote video cameras to record vertebrate (mammal and bird) visitors to inflorescences of *Protea scabra*, *P. cordata*, *P. subulifolia* and *P. decurrens*. Based on their floral traits, which are consistent with the floral syndrome of rodent pollination, we hypothesized that rodents would be the most frequent visitors. Rourke and Wiens (1977) and Wiens *et al.* (1983) observed captive rodent pollination of *Protea scabra* and *P. subulifolia*, while pollinator activity on *P. cordata* and *P. decurrens* has never been tested. We aimed to determine the identity of the main visitor species, their relative rates of visitation and their temporal patterns of activity.

2.3. Methods

2.3.1 The study species

Protea cordata, *P. decurrens*, *P. subulifolia* (Rodent sugarbushes) and *P.scabra* (Dwarf-tufted sugarbush) are distributed in localised populations across the Cape Floristic Region.

All four species possessed traits indicative of the therophilous pollination syndrome, including small inflorescences that emit a strong “yeasty” scent, and short distances (10-20mm) between nectar presentation and stigma (Chapter 3). These species were selected due to their close proximity to one another, adequate population sizes, ease of access, and the lack of experimental evidence confirming their pollination systems. Inflorescences were produced axillary and clustered at the base of the stem (*P. cordata*), axillary and attached to the basal branches (*P. decurrens* and *P. subulifolia*), or borne close to the ground (*P. scabra*) (Figure 1) (Rebelo and Paterson-Jones, 2001). *Protea cordata* and *P. scabra* generally co-occurred, but differed in peak flowering period (Table 1).



Figure 1: Gross morphology of the non-flying mammal pollinated *Protea* study species: *P. cordata*, *P. decurrens*, *P. scabra* and *P. subulifolia*, with Bushnell cameras on *P. cordata* in Fernkloof Nature Reserve.

Table 1: Details of the locations and duration of camera activity on *Protea scabra*, *P. cordata*, *P. decurrens* and *P. subulifolia*.

Species	Dates of filming	Peak flowering period	Location	No. of cameras	Total duration of filming	Total no. of inflorescences observed	Total no. days	Total no. nights
			Houw Hoek Pass 34°11'48.49"S					
<i>Protea scabra</i>	26 July-12 Sep	July-Oct	19°10'29.50"E	4	862hrs44min	11	38	38
			Fernkloof Nature Reserve 34°23'33.74"S					
<i>Protea cordata</i>	25 June-8 July; 18 July-25 July	June-July	19°15'57.95"E	5	746hrs28min	6	38	32
			Lower Breede River 34°23'37.39"S					
<i>Protea decurrens</i>	2 Aug-26 Aug	July-Aug	20°43'01.43"E	4	998hrs5min	26	36	42
			Houw Hoek Pass 34°12'01.97"S					
<i>Protea subulifolia</i>	24 July-30 July	July-Sep	19°09'20.93"E	5	533hrs8min	28	26	23

2.3.2 Remote cameras

Bushnell (Trophy Cam HD Max-Colour LCD, 119577C) cameras were erected at four sites containing the study species within the Western Cape, during the winter of 2013 (June to September) (Figure 1). The placement of cameras was determined after careful examination of each *Protea* study species' population.

The cameras were positioned on tripods facing healthy (uneaten) inflorescences with bracts fully open and at least one outer ring of florets with dehisced anthers. A single layer of masking tape was wrapped around the LED screen to avoid over-exposure during nocturnal visitation. The overall duration of filming varied from 533 hours to 998 hours, and was dependent on the size of the digital memory card fitted in each camera (16 or 32 GB), the length of the flowering period, and the accessibility of each site. The cameras were removed when the inflorescences had senesced (passed the receptive stage as indicated by browning of stigma tips and anther lobes).

The number of inflorescences filmed by each camera was not standardised due to the close proximity of the plants and the differences in number and sizes of inflorescences typically borne on a branch for each *Protea* species (e.g. from only one to four inflorescences in the case of *Protea scabra* and *Protea cordata* and up to 10 visible inflorescences for *Protea decurrens* and *Protea subulifolia*).

Once triggered by movement and/or heat, the camera would film pollinator activity for 60 seconds. If there was successive triggering, the camera would resume filming after 10 seconds.

2.3.3 Data analysis

The footage from each camera was used to determine the diversity of pollinator species and the frequency of pollinator visitation. If there was successive triggering from each camera, we assumed that the individual from the previous footage was the same as the one 10 seconds

later. A visit was accepted as a pollination event when the individual's head probed the inflorescence ensuring contact with pollen presenters and stigmas. If an individual was not actively engaging with the flower, it was removed from the pollination analysis, but their behaviour was noted. The proportion that an animal visitor was observed at each *Protea* species relative to the total number of visits by all species enabled us to determine the total percentage of visits per pollinator for all study *Protea* species. Additionally, we determined the hourly visitation rates of each visitor per inflorescence. This was achieved by calculating the proportion of visits per inflorescence over the number of hours that each visitor species was active.

The time of day that each visit occurred was noted in order to determine if there was temporal partitioning of resource use among the small mammal community. From these data, we analysed the relative frequency of visits for each pollinator to the respective *Protea* species during successive one hour time intervals. This was determined using the following equation:

$$\text{relative frequency of species 1 at time } x = \frac{\text{frequency of visits for species 1 at time interval } x}{\text{total no. of visits at time interval } x}$$

2.4. Results

2.4.1 Pollinator identification and rates of visitation

The camera traps recorded foraging events by seven different vertebrate visitors across all *Protea* species under study. Visitors comprised five rodents (*Mus minutoides* A Smith, *Aethomys namaquensis* A. Smith (synonymous with *Micaelamys namaquensis*), *Acomys subspinosus* Waterhouse, *Rhabdomys pumilio* Sparrman, *Dendromus* species), one carnivore, the genet (*Genetta tigrina* Schreber) and one nectarivorous bird species, orange-breasted sunbird (*Anthobaphes violacea* Linnaeus) (Table 2) (Appendix, Video 1).

All *Protea* species received visits from at least three small mammal species. *Protea scabra* had the largest range of pollinator species, including the previously unobserved *G. tigrina* (Chapter 4) (Appendix, Video 2).

The rodents *R. pumilio* and *M. minutoides* made up the clear majority of visitors to flower-heads in total and per inflorescence per hour (Table 2; Fig. 2). *Protea scabra* and *P. decurrens* received the highest proportion of visits from *R. pumilio*, while *P. subulifolia* was foraged on almost exclusively by *M. minutoides* (Table 2; Fig. 2). *A. namaquensis* was the dominant visitor to *P. cordata* (Table 2). Only two foraging events were recorded by *Dendromus* sp.; one on *P. decurrens* and one on *P. scabra*. Orange breasted sunbirds (*Anthobaphes violacea*) visited both *P. scabra* and *P. decurrens*, but the percentage of visits by this species was minimal relative to visits by rodents (Table 2).

Table 2: The total percentage of visits by small mammal species (*Rhabdomys pumilio*, *Acomys subspinosus*, *Aethomys namaquensis*, *Dendromus* sp. and *Mus minutoides*) and bird species (*Anthobaphes violacea*) to *Protea scabra*, *P. subulifolia*, *P. decurrens* and *P. cordata*. The total number of visitation events per *Protea* species is indicated in parenthesis. Data on visits by the carnivorous Genet was omitted (but see Chapter 4).

<i>Protea</i> species	Total % (and number) of visitors					
	Small mammal species					Bird species
	<i>R. pumilio</i>	<i>A. subspinosus</i>	<i>A. namaquensis</i>	<i>M. minutoides</i>	<i>Dendromus</i> sp.	<i>A. violacea</i>
<i>P. scabra</i>	42.33 (69)	7.40 (12)	28.83 (47)	14.11 (23)	0.62 (1)	5.52 (9)
<i>P. subulifolia</i>	7.69 (29)	2.12 (8)	0	90.19 (340)	0	0
<i>P. decurrens</i>	91.95 (537)	0.34 (2)	0	5.30 (31)	0.17 (1)	2.28 (13)
<i>P. cordata</i>	0	7.41 (2)	55.56 (15)	37.04 (10)	0	0

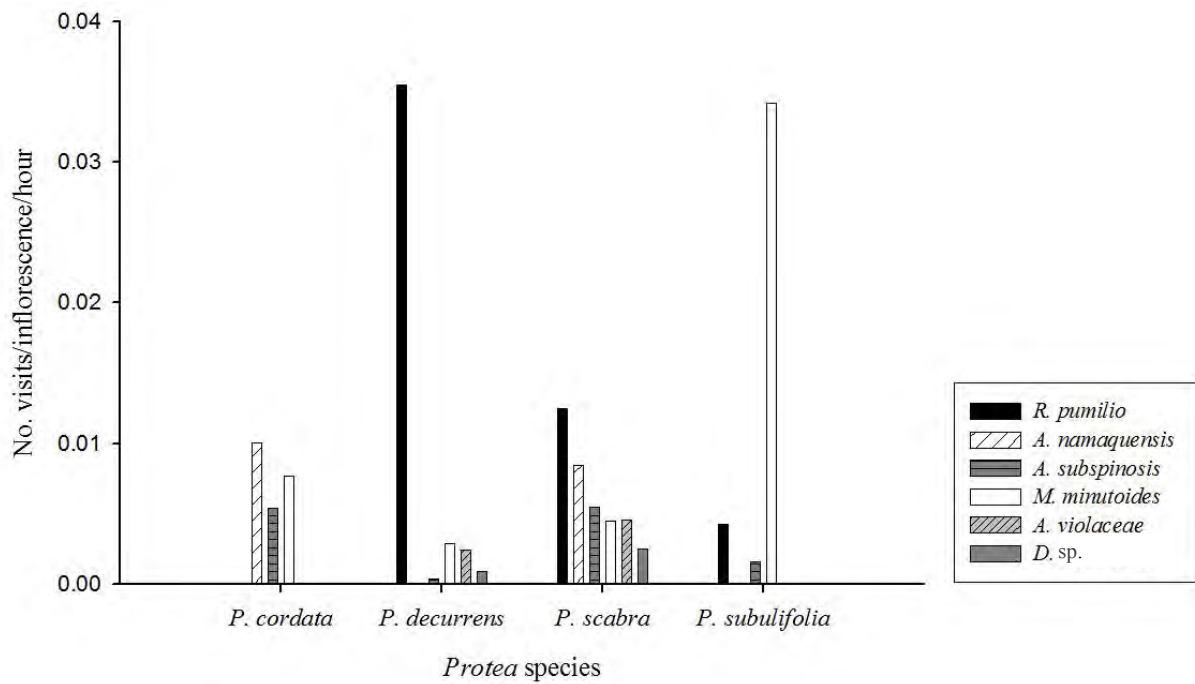


Figure 2: The visitation frequency of observed small mammal species (*Rhabdomys pumilio*, *Acomys subspinosus*, *Aethomys namaquensis*, *Dendromus. sp.* and *Mus minutoides*) and bird species (*Anthobaphes violaceae*) to *Protea scabra*, *P. subulifolia*, *P. decurrens* and *P. cordata* per hour per inflorescence

2.4.2 Temporal patterns of visitation

Diurnal pollination by *R. pumilio* was observed for three of the four study species, but activity patterns differed among *Protea* species (Fig. 3). While the frequency of *R. pumilio* visits to *P. scabra* was lowest at 11am, peak visitation frequency was observed between 09:00-13:00 for *P. subulifolia* and between 8:00-12:00 for *P. decurrens* (Fig. 3).

Additionally, sporadic visits were recorded during the day from two of the three ostensibly nocturnal rodent species. *Protea subulifolia* received occasional visits from *A. subspinosus* between 12:00-15:00. This corresponded with low activity by *R. pumilio* (Fig. 3c). *Mus minutoides* became active from 16:00 and 17:00 on *P. subulifolia* and *P. scabra* respectively (Fig 3).

There was no evidence for temporal segregation of nocturnal pollinators on *P. scabra*, as activity by *M. minutoides*, *A. namaquensis* and *A. subspinosus* occurred concurrently (Fig. 2d). Similarly, we observed nocturnal activity on *P. decurrens* and *P. subulifolia* at overlapping time intervals. However, peak activity by one pollinator species was often associated with a decline in another. Peaks in *A. subspinosus* activity on *P. decurrens* were generally corresponded with a decline in *A. namaquensis* activity. Additionally, *M. minutoides* was the only observed visitor to *P. subulifolia* between 0:00-06:30 (Figure 3c). *Protea cordata* only received visitors nocturnally, and these visits appeared to occur concurrently (Fig. 3a).

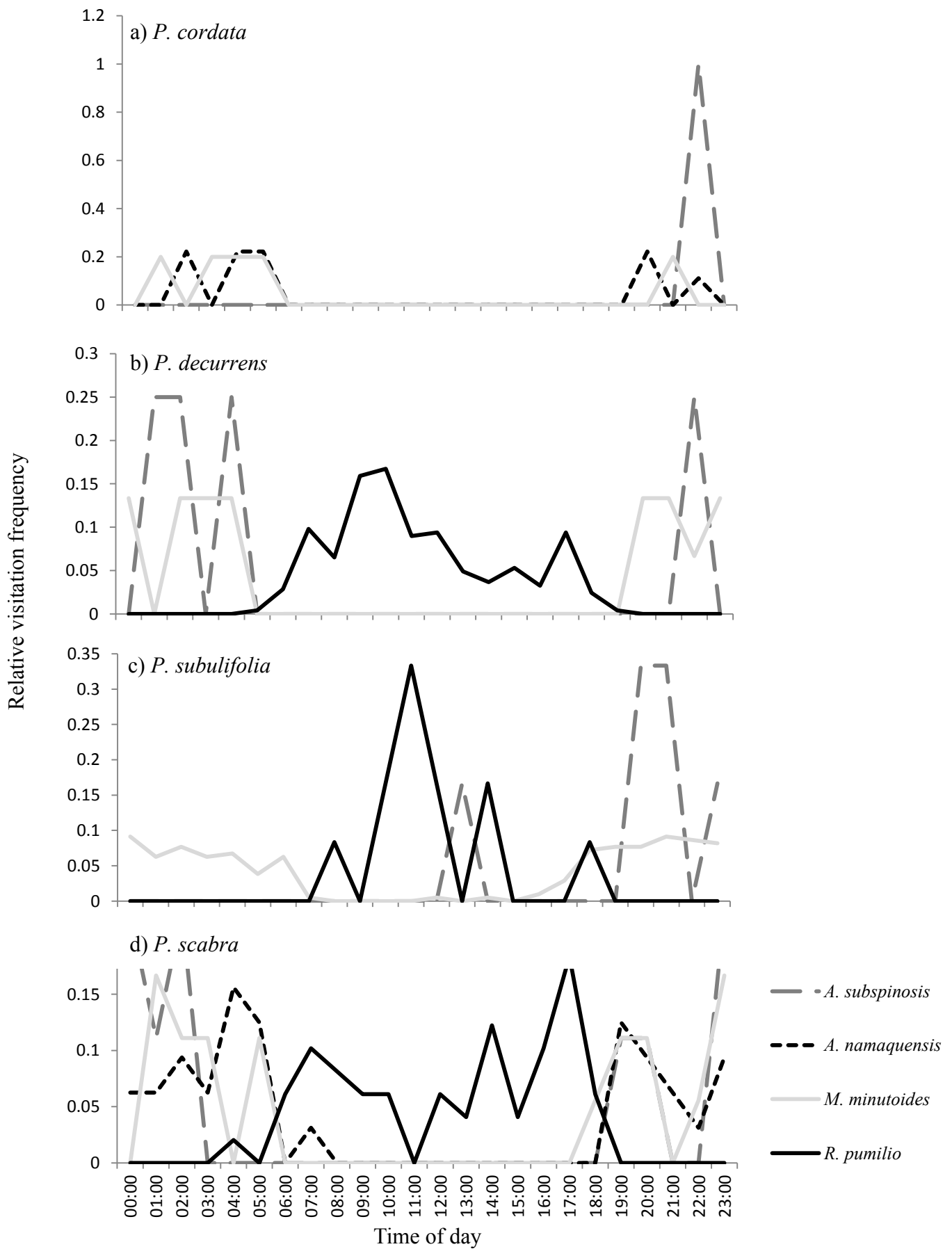


Figure 3: The relative visitation frequency of small mammals to a) *Protea cordata*, b) *Protea decurrens*, c) *Protea subulifolia* and d) *Protea scabra* combined over one hour time intervals. Due to low visitation rates, data on *Dendromus* species was omitted

2.4.3 Vertebrate visitors

Visitors generally foraged non-destructively, with the exception of one *R. pumilio* individual visiting a *P. decurrens* plant which bit into a bud and proceeded to remove and eat the florets contained inside the enclosed bracts (Appendix, Video 1). It is likely that all of the small mammal species in this study picked up pollen on their body and paws during foraging, as most individuals were observed to crawl onto or into inflorescences, probe florets with their snouts and lap nectar from the perianth and base of the florets. Individuals were often observed visiting more than one inflorescence on the same plant. In addition to the small mammals mentioned in this paper, *Otomys irrorata* was observed in four recordings of *P. decurrens*, but never engaged with the inflorescence, and was thus not counted in analyses as a pollinator.

2.5. Discussion

This paper has highlighted the utility of employing camera technology to help resolve the pollination systems of plant species. By using camera footage, we have shown that it is possible to detect patterns of pollinator activity, visitation frequency of different pollinators, as well as temporal variation in pollinator visitations with no impact on natural pollinator behaviour and allowing us to avoid sampling bias and constraints on direct observations. This technology has additionally enabled us to distinguish between rodent foragers and species that are merely caught in the vicinity of the inflorescences (such as *Otomys irrorata*).

The evidence presented in this paper has provided strong support for small mammal pollination of *P. scabra*, *P. cordata*, *P. decurrens* and *P. subulifolia*. Rodent species were the most common visitors to these plants and made consistent contact with the reproductive parts of flowers. Only two of the four plant species were visited by bird (Fig. 2). While birds are capable of carrying pollen over greater distances than rodents, and were observed at similar frequency to *Acomys subspinosus* on *P. decurrens* and *P. scabra* (Table 2, Fig. 2), the total

number of visits by all rodent species far exceeded visits by birds. Nectar removal from inflorescences occurred non-destructively with the exception of one *R. pumilio* individual, and the mechanism of accessing nectar ensured contact with pollen.

Discrepancies with camera traps are known from the literature and it is argued that not all species may be recorded by the cameras (Bridges and Noss, 2011). The great size range of recorded visitors from a relatively large genet to the tiny *M. minutoides* is evidence that the cameras were effective at detecting a diversity of animal visitors. As the remote cameras used in this study are designed for recording vertebrate behaviour, we were unable to quantify rates of visitation by insects (but see Steen, 2012 and Micheneau *et al.* 2009).

2.5.1 Non-species specific specialization for rodents

The *Protea* species received consistent visits from at least three rodent species (Table 2). This suggests that the floral traits of the plants promote visitation by a diverse range of rodent species. Non-specialisation by therophilous *Protea* species is likely to occur when the assemblage of rodent species are functionally equivalent (Johnson and Steiner, 2000), and promote successful pollination with no selective cost to the plant (Fenster *et al.* 2004). Considering that all pollinators contribute toward the overall fitness of the plant, it is possible for the plant to adapt to an array of pollinators within a specific guild (de Merxem *et al.* 2009). Increasing the pool of potential pollinators is likely to intensify the frequency of visitation to inflorescences, which impacts the breeding success of the plant (Welsford and Johnson, 2012).

2.5.2 Temporal activity by mammals

Nectar from therophilous *Protea* species rewards pollination services, and provides local small mammals with an energy supply during the otherwise resource-limited winter season (Fleming and Nicolson, 2002). As such, co-existing, closely-related rodent species may exhibit contrasting characteristics that enable shared resources to be partitioned in order to

prevent competitive exclusion (Brown *et al.* 1975). This is often expressed in differences in foraging time among rodent species with overlapping spatial distribution.

Daytime activity on floral heads was dominated by *R. pumilio* (Fig. 3). This result was expected as *R. pumilio* was the only extensive diurnal murid rodent species that was observed foraging on all the study species (Schradin and Pillay, 2004). In addition, we observed periodic daytime foraging events by *A. subspinosus* and *M. minutoides*. Daytime activity is highly unusual for these nocturnal species (Chimimba and Bennet, 2005), and occurred during periods of low activity by *R. pumilio*. This suggests that these animals are foraging outside of their usual activity hours.

In contrast, there was no subdivision of resources nocturnally (Fig. 3). All four study species received visits by different pollinators with overlapping activity patterns. However, the peak in activity time for one species usually corresponded with a slight depression in another for *P. decurrens* and *P. subulifolia*.

2.5.3 Future uses of camera traps

In the past, pollination by non-flying mammals in South African *Protea* species was inferred from traits that were convergent with Australian NFM pollinated Proteaceae (Rourke and Wiens, 1977). Since then, research on NFM pollination in South Africa has rapidly intensified, evoking movement away from the preceding descriptive approach to this research. The development of innovative principles and methods allow for a more integrated approach to research on pollination systems (Micheneau *et al.* 2009). Consequently, biologists are able not only to identify effective pollinators (Fleming and Nicolson, 2002; Biccard and Midgley, 2009; Spears, 1983), but also quantify floral advertisement and rewards (Johnson *et al.* 1999; Steenhuisen *et al.* 2012), evaluate the effect of pollinator grooming on pollen loss (Johnson and Pauw, 2014), clarify reproductive biology (Johnson *et al.* 2001), determine population dynamics and genetic structure (Whitehead and Peakall, 2009), and

evaluate these characteristics according to phylogenetic relationships (Johnson *et al.* 2009; Micheneau *et al.* 2009).

While the use of camera traps in validating pollination systems is a novel approach, we have shown here that they can be a useful tool for providing non-lethal, non-invasive and non-obstructive observations into who, how and when pollination of selected plant species occurs. This is an important component in enriching pollination studies and continuing the rapid progression of pollination research.

2.6. References

- Biccard, A., Midgley, J. J., 2009. Rodent pollination in *Protea nana*. South African Journal of Botany 75, 720-725.
- Brown, J. H., Grover, J. J., Davidson, D. W., Lieberman, G. A., 1975. A preliminary study of seed predation in desert and montane habitats. Ecology 556, 987-992.
- Bridges, A. S., Noss, A. J., 2011. Behavior and Activity Patterns, in: O'Connell, A. F., Nichols, J. D., Karanth, K. U. (Eds.), Camera Traps in Animal Ecology: Methods and Analyses. Springer, New York, pp. 57-65.
- Carthew, S. M., Slater, E., 1991. Monitoring animal activity with automated photography. The Journal of Wildlife Management 554, 689-692.
- Chimimba, C. T., Bennet, N. C., 2005. Order Rodentia, in: Skinner, J. D., Chimimba, C. T. (Eds.), The Mammals of the Southern African Subregion. Cambridge University Press, Cambridge, pp. 77-209.

- de Merxem, D. G., Borremans, B., de Jäger, M. L., Johnson, T., Jooste, M., Ros, P., Zenni, R. D., Ellis A. G., Anderson, B., 2009. The importance of flower visitors not predicted by floral syndromes. *South African Journal of Botany* 75, 660–667.
- Fenster, C. B., Armbruster, W. S., Wilson, P., Dudash, M. R., Thomson, J. D., 2004. Pollination Syndromes and Floral Specialization. *Annual Review of Ecology, Evolution, and Systematics* 35, 375–403.
- Fleming, P., Nicolson, S., 2002. How important is the relationship between *Protea humiflora* (Proteaceae) and its non-flying mammal pollinators? *Oecologia* 132, 361-368.
- Goldingay, R. L., Carthew, S. M., Whelan, R. J., 1991. The importance of non-flying mammals in pollination. *Oikos* 61, 79–87.
- Johnson, S. A., van Tets, I. G., Nicolson, S. W., 1999. Sugar preferences and xylose metabolism of a mammal pollinator, the Namaqua Rock Mouse (*Aethomys namaquensis*). *Physiological and Biochemical Zoology* 72, 438-44.
- Johnson, S. D., Steiner, K. E., 2000. Generalization versus specialization in plant pollination systems. *Trends in Ecology and Evolution* 15, 140-143.
- Johnson, S. D., Pauw, A., Midgley, J. J., 2001. Rodent pollination in the African Lily *Massonia depressa* (Hyacinthaceae). *American Journal of Botany* 88, 1768–1773.
- Johnson, S. D., Manning, J. C., Pauw, A., 2009. Advances in the pollination biology of South African plants. *South African Journal of Botany* 75, 625–629.
- Johnson, C. M., Pauw, A., 2014. Adaptation for rodent pollination in *Leucospermum arenarium* (Proteaceae) despite rapid pollen loss during grooming. *Annals of Botany* 113, 931-8.

- Kleizen, C., Midgley, J. J., Johnson, S. D., 2008. Pollination systems of *Colchicum* (Colchicaceae) in southern Africa: evidence for rodent pollination. *Annals of botany* 102, 747-755.
- Micheneau, C., Johnson, S. D., Fay, M. F., 2009. Orchid pollination: from Darwin to the present day. *Botanical Journal of the Linnean Society* 161, 1–19.
- Rebelo, T. G., Breytenbach, G. J., 1987. Mammal Pollination in the Cape Flora, in: Rebelo, A. G. (Ed.), Preliminary synthesis of pollination biology in the Cape flora. South African National Science Program Report 141, 109–124.
- Rebelo, T. G., Paterson-Jones, C., 2001. A Field Guide to the Proteas of Southern Africa. Fernwood Press in association with the National Botanical Institute.
- Rourke, J. P., 1980. The Proteas of Southern Africa. Purnell, Cape Town.
- Rourke, J. P., Wiens, D., 1977. Convergent Floral Evolution in South African and Australian Proteaceae and Its Possible Bearing on Pollination by Nonflying Mammals. *Annals of the Missouri Botanical Garden* 64, 1–17.
- Schradin, C., Pillay, N., 2004. The Striped Mouse (*Rhabdomys pumilio*) from the Succulent Karoo, South Africa: a territorial group-living solitary forager with communal breeding and helpers at the nest. *Journal of Comparative* 118, 37–47.
- Spears, E. E., 1983. A direct measure of pollinator effectiveness. *Oecologia* 57, 196-199.
- Steen, R., 2012. Pollination of *Platanthera chlorantha* (Orchidaceae): new video registration of a hawkmoth (Sphingidae). *Nordic Journal of Botany* 30, 623–626.
- Steenhuisen, S.-L., Raguso, R. A., Johnson, S. D., 2012. Floral scent in bird- and beetle-pollinated *Protea* species (Proteaceae): Chemistry, emission rates and function. *Phytochemistry* 84, 78–87.

- Welsford, M. R., Johnson, S. D., 2012. Solitary and social bees as pollinators of *Wahlenbergia* (Campanulaceae): single-visit effectiveness, overnight sheltering and responses to flower colour. *Arthropod-Plant Interactions* 6, 1–14
- Wester, P., Stanway, R., Pauw, A., 2009. Mice pollinate the Pagoda Lily, *Whiteheadia bifolia* (Hyacinthaceae)—First field observations with photographic documentation of rodent pollination in South Africa. *South African Journal of Botany* 75, 713-719.
- Whitehead, M. R., Peakall, R., 2009. Integrating floral scent, pollination ecology and population genetics. *Functional Ecology* 23, 863–874
- Wiens, D., Rourke, J. P., Casper, B. B., Rickart, E. A., LaPine, T. R., Peterson, C. J., Channing, A. 1983. Nonflying mammal pollination of southern African *Proteas*: a non-coevolved system. *Annals of the Missouri Botanical Garden* 70, 1-31.

3. Evolutionary specialization and ecological generalization in the pollination of geoflorous *Protea* species (Proteaceae)

3.1 Abstract

The ‘most effective pollinator’ principle formalized the idea of floral specialization for a single pollinator group. However, flowers that are seemingly evolutionarily specialized for a specific pollinator are often involved in generalized interactions with pollinators, resulting in an apparent paradox between floral adaptations to past pollinators and current ecological context. The first aim of this study was to determine whether vertebrates are the most effective pollinators of four species of *Protea* that conform to a non-flying mammal pollination (NMP) system: *P. cordata*, *P. decurrens*, *P. scabra*, and *P. subulifolia*. This was achieved by erecting enclosures over inflorescences in order to determine the relative contribution of insects and vertebrates to seed set. Secondly, we described their key functional floral traits and documented their breeding systems in order to evaluate the degree of self-compatibility and the extent to which they rely on animal pollinators for seed set. Investigations revealed that the *Protea* species possess floral traits that are specialized for pollination by non-flying mammals. Results from breeding system experiments indicated that *Protea cordata* and *P. scabra* are self-incompatible, and thus rely on external vectors for seed production, but *Protea decurrens* is self-compatible, meaning that the role of different groups pollinators cannot be determined by selective exclusion in this species. The enclosure experiments indicated that insects contribute to pollination of *Protea cordata* and *P. scabra* since exclusion of vertebrates did not significantly reduce seed set. However, seed set was lower in caged *P. scabra* plants and seed set was not increased by hand pollen supplementation in either *P. cordata* or *P. scabra*. This confirms that mammals are capable of pollination and that plants may be resource limited. These results further imply that these

species have evolved specialized morphological traits to attract mammals but that effective pollination can be carried out by multiple functional groups. Research on non-flying mammal pollination systems need to be revisited as we have shown here that ranking pollinators in terms of their functional fit to the floral phenotype often overlooks the contribution of generalist pollinators from other functional groups.

3.2 Introduction

Floral diversification in response to selection imposed by pollinators is considered the most outstanding example of adaptive evolution in angiosperms (Herrera, 1996). Much research has been concentrated on identifying and studying interactions of plants with their animal pollinators in order to determine how different traits function to attract efficient pollinators and filter out unwanted floral visitors (Shuttleworth and Johnson, 2009). Analyzing functional traits in a phylogenetic context further advances our understanding of evolutionary processes underlying shifts in pollination systems within plant lineages (van der Niet and Johnson, 2012).

On the assumption that unrelated plant taxa with analogous floral traits are likely to share a common pollinator functional group (Faegri and van der Pijl, 1979; Johnson *et al.* 2001; Fenster *et al.* 2004), pollination systems can be hypothesized for plant species that have not been studied in the field (Ollerton *et al.* 2009). However, traditional pollination syndromes are often not reliable predictors of the primary pollinator, as plants exhibiting highly specialized floral traits may have generalized visitation patterns from multiple functional groups (Aigner, 2001; Ollerton *et al.* 2009). Consequently, there is an apparent paradox between past floral traits and current ecological context (Ollerton, 1996; Aigner, 2001). For this reason, defining a pollination system of a plant species without experimental evidence has been widely criticized (Ollerton *et al.* 2009; Waser *et al.* 1996; Herrera, 1996; Johnson and Steiner, 2000).

The African genus *Protea*, centered in the Cape Floristic Region (CFR) of South Africa, is known for its spectacular morphological diversity (Valente *et al.* 2010; Rourke, 1980). The distinct floral morphological traits of species within this genus are thought to represent adaptations for pollination by different animals with unique foraging behaviour and sensory preferences (Valente *et al.* 2010; Knight *et al.* 2005), such as birds (e.g. *P. roupelliae*) (Hargreaves *et al.*, 2004), insects (e.g. *P. caffra*) (Steenhuisen *et al.* 2012) and non-flying mammals (e.g. *P. humiflora*) (Wiens *et al.* 1983).

Non-flying mammal pollination (NMP) has been inferred to occur in several *Protea* clades, but very few species have actually been investigated (Biccard and Midgley, 2009). The basis for inference is the possession by some species of traits that are consistent with a “therophilous” pollination syndrome (Rebelo and Breytenbach, 1987; Fleming and Nicolson, 2002). These traits include geoflorous growth with “yeasty” scented, cryptic inflorescences that produce copious amounts of concentrated nectar (Rourke and Wiens, 1977; Johnson *et al.* 2001). In addition, florets usually have an effective 10 mm distance between pollen presenter and nectar (Wiens *et al.* 1983). NMP plants typically flower over early winter to late spring, providing a nectar reward that entices mammal foragers during winter months when alternative resources are low (Fleming & Nicolson, 2002). While these traits are considered to represent a specialized system, inflorescences of NMP Proteaceae are frequently visited by other groups of diurnal pollinators, such as insects and birds (Rebelo and Breytenbach, 1987). This emphasizes the necessity for comprehensive experimental research to determine the relative importance of different animals for pollination of *Protea* species with the NMP floral syndrome.

Protea cordata, *P. decurrens*, *P. scabra*, and *P. subulifolia* have been classified as NMP species (Rebelo and Breytenbach, 1987; Protea Atlas Project, unpublished data). This is according to their convergent NMP floral traits (Rourke and Wiens, 1977). There have also

been video-based observations of visits by rodents (*Mus minutoides*, *Aethomys namaquensis*, *Acomys subspinosus*, *Rhabdomys pumilio*) at a much higher frequency than those of birds (Chapter 2). The breeding systems of these species have not been investigated previously. Early studies suggested that *Protea* species are self-incompatible (Horn, 1962), but self-compatibility has been demonstrated for some species (Steenhuisen and Johnson, 2012a; Wiens *et al.* 1983).

3.2.1 Aims

The aims of this study were 1) to describe key functional floral traits of *Protea cordata*, *P. decurrens*, *P. scabra*, and *P. subulifolia*, 2) to determine whether vertebrates are more effective pollinators of these species than insects, and 3) to document their breeding systems in order to evaluate the degree of self-compatibility and the extent to which they rely on animal pollinators for seed set.

3.3 Methods

3.3.1 Study species and sites

Protea cordata, *P. decurrens*, *P. scabra*, and *P. subulifolia* are geoflorous species distributed in localized populations across the Cape Floristic Region (Table 1). These species were selected due to their close proximity to one another, adequate population sizes, ease of access, and the lack of experimental evidence confirming their pollination systems. The four *Protea* study species are low growing and produce large inflorescences with tightly-packed, relatively small florets, resulting in a bowl-like structure. The inflorescences of *Protea cordata*, *P. decurrens* and *P. subulifolia* emit a weak, “yeasty” scent, and *P. scabra* releases a strong, cheesy scent. While remote camera analyses have identified rodents as the primary visitors to these species (Chapter 2), other important aspects of their pollination biology have not been studied in an experimental context. These species belong to two groupings based on their morphology, namely the “rodent sugarbushes” (*P. cordata*, *P. decurrens*, *P. subulifolia*)

and the “dwarf-tufted sugarbushess” (*P. scabra*) (Rebello and Paterson-Jones, 2001). The study species differ in fire survival strategy: *Protea scabra* is a resprouter, whereas *P. cordata*, *P. decurrens* and *P. subulifolia* regenerate from seeds (Protea Atlas Project, unpublished data). All four species flower during the winter rainfall period of the Western Cape (June-August, Table 1). Population size and geographic range varied among the study species, but estimati

Table 1: Description of study species and study sites. The GPS coordinates refer to the centroid of the study population

Species	Peak flowering period	Location	GPS coordinates	Estimated population size (number of plants)
<i>Protea scabra</i>	August-October	Houw Hoek Pass	34°11'48.49"S 19°10'29.50"E	150 over 125000 m ²
<i>Protea cordata</i>	June-July	Fernkloof Nature Reserve	34°23'33.74"S 19°15'57.95"E	100 over 2100 m ²
<i>Protea decurrens</i>	July-August	Lower Breede River	34°24'20.57"S 20°45'46.15"E	50 over 900 m ²
<i>Protea subulifolia</i>	July-September	Houw Hoek Pass	34°12'01.97"S 19°09'20.93"E	30 over 100 m ²

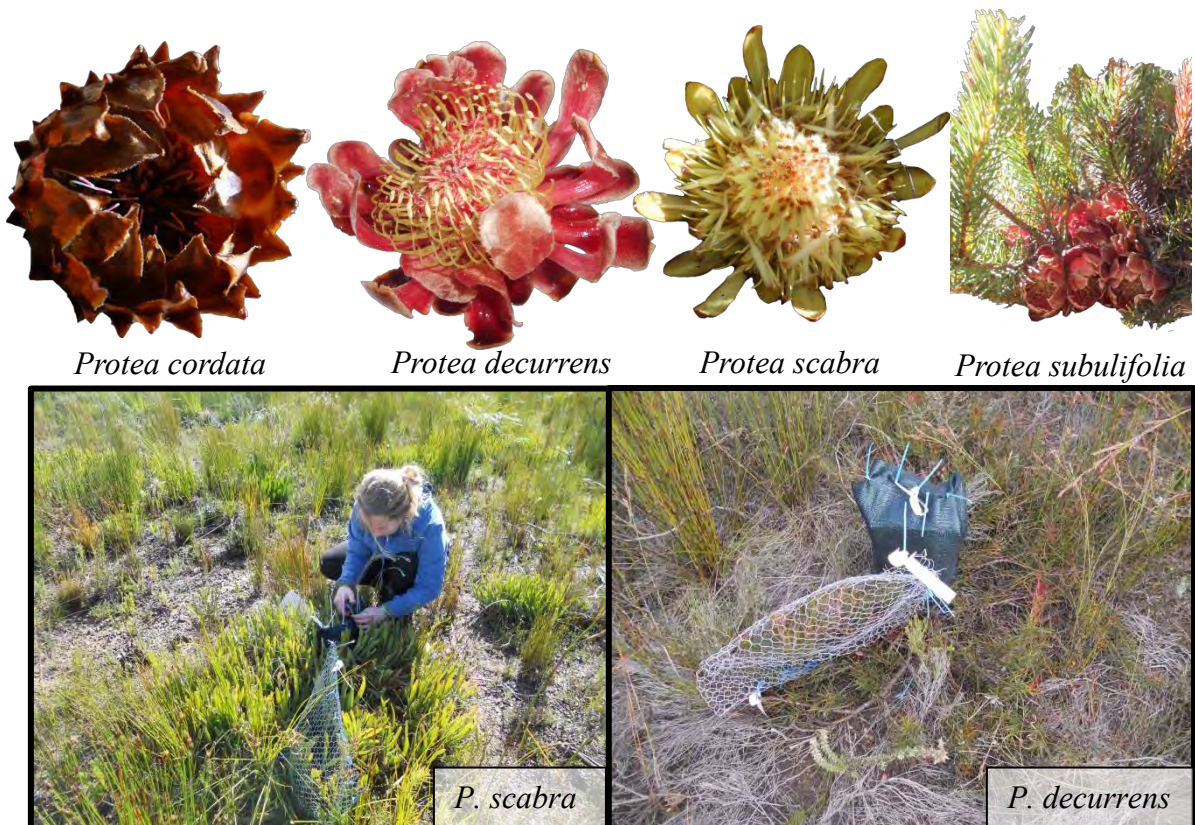


Figure 1: The gross morphology of the non-flying mammal pollinated *Protea* study species: *P. cordata*, *P. decurrens*, *P. scabra* and *P. subulifolia*, as well as caged and bagged enclosures erected around *P. scabra* and *P. decurrens*.

3.3.2 Morphology

3.3.2.1 Floral dimensions

Several measures of floral morphology were taken to determine their functional fit for small mammal pollination. These included inflorescence height, diameter, the number of florets contained in each inflorescence, and a number of floret traits that mediate pollinator interactions (floret height, style length, pollen presenter length, and the distance between: nectar presentation to nectar production; nectar presentation to the stigma; and nectar presentation to the base of the floret) for ten inflorescences of each species. For floret dimensions, we evaluated two florets per inflorescence: one occurring on the outer ring of the inflorescence, and one on the inner ring. As there was no statistical difference between inner

and outer floret measurements, the data were averaged to produce a mean measurement of floret dimensions.

These mean floral dimensions (\pm SE) were compared to “typical” floral traits of a NMP plant that were quantified in comparative literature.

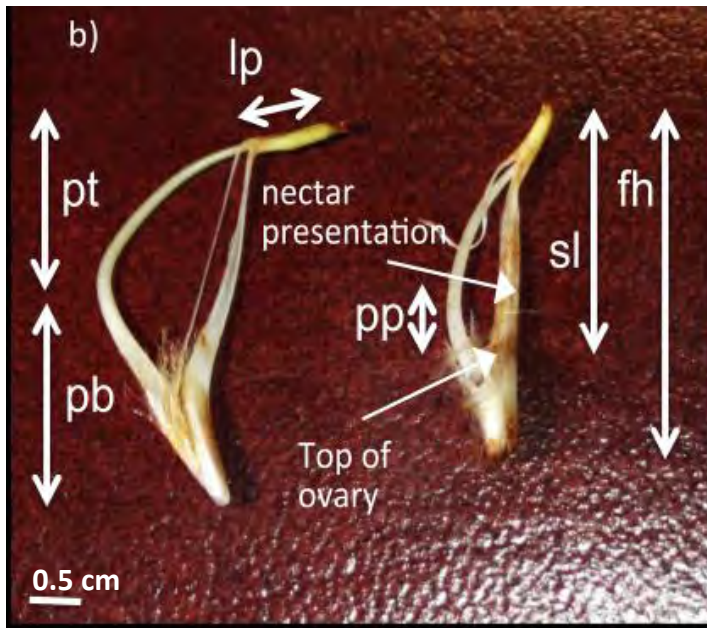


Figure 2: Dimensions of outer (left) and inner (right) florets of *P. sulphurea* from Balmer (2012). fh=floret height, sl=style length, lp=length of pollen presenter, pp=distance between nectar production and nectar presentation, pb=distance between nectar presentation and the base of the floret, pt=distance between nectar presentation and stigma.

3.3.2.2 Spectral reflectance

Protea species pollinated by rodents have been reported to have flowers which are dull in colour relative to their bird-pollinated congeners (Jacobs, 1992). To quantify flower colour, spectral reflectance was determined using a portable spectrophotometer (Ocean Optics S2000 spectrometer, Ocean Optics Inc., Dunedin, FL, USA). A fiber optic reflection probe was attached to the spectrometer and light source (Ocean Optics DT-mini deuterium tungsten halogen light source with an approximately 200-1100 nm spectral range) to create an optical-

sensing system for measuring reflection and fluorescence from the plant samples. The probe was aimed at the sample at a 45° angle. To calibrate the spectrometer, an Ocean Optics WS-1 diffuse reflectance standard was used (Johnson and Anderson, 2002).

For each of ten inflorescences of each species, reflectance measurements were taken for the top and bottom inner and outer surfaces of a bract, the top of the outer perianth, pollen, pollen presenters with pollen removed, bare styles, and a leaf. Spectral data were averaged over the ten measurements of each floral part, graphed, and described in terms of any UV-patterns or striking colour variations that exist.

Bees were observed visiting at least one of the *Protea* species (*P. cordata*). As colour models do not exist for insects other than hymenoptera, (developed by Chittka *et al.* 1993), bee hexagon models were used in order to determine how the trichromatic colour vision of bees affect their perception of floral colours of the *Protea* species. These data were included in the Appendix.

3.3.2.3 Nectar

The primary reward for pollination is nectar (Simpson and Neff, 1981). NMP *Protea* species produce large quantities of highly viscous nectar that provides small mammals with a reliable energy source during the resource-scarce winter months (Johnson *et al.* 2001; Fleming and Nicolson). Mean volume nectar production and mean percentage sugar concentration was determined from five florets from each of ten inflorescences. These measurements were made over two time frames: spot sampling and accumulated nectar measurements. Spot sampling of nectar was carried out at each site between 9:00-11:00, giving us an indication of standing crop nectar available to diurnal foragers: *Rhabdomys pumilio* (rodent), insects and birds (Chapter 2). In order to determine the properties of accumulated nectar, ten newly opened inflorescences on freshly cut branches were placed in water overnight at the University of

Cape Town, and measurements were made after 16-24 hours had elapsed. This was indicative of the physiological capacity of the plant for nectar production.

Nectar volume (ul) was determined by inserting a calibrated micropipette (Fisherbrand 1-5 μ l) between the style and the perianth tube (site of nectar production and nectar presentation), and drawing the nectar up by gently sucking from the free end of the pipette. The mean percentage sugar concentration was measured with an Atago N1 0–50% pocket refractometer.

Average accumulated nectar properties per floret were also obtained for three bird-pollinated *Protea* species (*P. nitida*, *P. obtusifolia*, and *P. roupelliae*), four beetle-pollinated *Protea* species (*P. caffra*, *P. dracomontana*, *P. simplex* and *P. welwitschii*) and five non-flying mammal pollinated *Protea* species (*P. pendula*, *P. recondita*, *P. sulphurea*, *P. namaquana* and *P. humiflora*), as reported by Steenhuisen and Johnson (2012) and Balmer (2012) (Appendix Table 1). The nectar properties from NMP *Protea* species were combined with the nectar data from our study species. Average standing crop nectar properties per inflorescence were also obtained for 10 bird-pollinated *Protea* species (*Protea compacta*, *P. cynaroides*, *P. exima*, *P. laurifolia*, *P. longifolia*, *P. magnifica*, *P. neriifolia*, *P. nitida*, *P. punctata* and *P. repens*; Schmid, unpublished data). Data on the standing crop nectar of beetle-pollinated *Protea* species was not available. As Schmid used average nectar volume for the overall inflorescence; our measurements were converted to reflect theirs by multiplying the nectar volume per floret by the average number of florets per inflorescence. It is important to note that this method may result in an inflated estimate of nectar volume per inflorescence since we did not account for florets that had senesced or were still in bud, and therefore not producing nectar.

3.3.3 Controlled hand-pollination and selective exclusion

Breeding system experiments using controlled hand-pollinations were performed in order to determine if the study species were dependent upon pollinators for seed set. Due to low population sizes, breeding and pollination system experiments were only conducted on *P. cordata*, *P. scabra* and *P. decurrens*.

Each treatment described below (1-2; 1-3) was conducted on fifteen inflorescences of *P. cordata*, twenty inflorescences of *P. scabra* and thirteen inflorescences of *P. decurrens*. Due to differences in the number of inflorescences borne on each plant, experimental treatments were conducted on inflorescences on separate plants for *P. cordata* and *P. decurrens* and conducted on inflorescences on the same plant for *P. scabra*. For treatments requiring total exclusion of all pollinators over the flowering period, wire cages made from 13 mm chicken mesh and covered with shade cloth were erected around each inflorescence and secured with pegs while the plant was in bud.

Once the inflorescences had reached anthesis and become receptive (following morphological indicators described by Steenhuisen and Johnson, 2012a,) hand pollination treatments were separately applied to each inflorescence: (1) self-pollination, by excluding all pollinators and using a toothpick to move self-pollen from each floret over the stigmatic grooves, and (2) pollen supplementation, where inflorescences left open to natural pollinators were hand-pollinated when receptive using a paintbrush coated in a mixture of cross pollen from freshly dehisced florets collected from separate plants. Both the self and cross pollen supplemented inflorescences were pollinated a minimum of three times throughout the inflorescence's lifespan.

Pollinator effectiveness was investigated on *P. cordata*, *P. scabra* and *P. decurrens* inflorescences by experimentally manipulating pollinator accessibility and pollen supply to

stigmas. This was achieved by excluding certain groups of pollinators, using the aforementioned cage and shade cloth apparatus. These exclusion treatments included: (1) a control, where the inflorescence was left open and accessible to all pollinators, (2) cage, wherein large mesh cages (without shade cloth) were constructed around the inflorescences to exclude vertebrates, but allow access to insects, and (3) a test for autonomous self-pollination (auto), wherein inflorescences in bagged cages that also exclude insects were left without manipulation during the study period in order to determine the extent to which pollinators are needed for pollen transfer.

Due to population constraints and accessibility issues, *P. decurrens* only received three treatments: cage, auto and control.

Three months after all the inflorescences had senesced and the bracts were closed, all treated infructescences were collected (November 2013 for *Protea cordata* and December 2013 for *P. scabra* and *P. decurrens*). The infructescences were air-dried at 25°C for four days to trigger the release of seeds. The proportion of fertilized seeds per inflorescence were counted and recorded. Fertilized seeds are morphologically similar to aborted seeds, but we were able to identify viable seeds by a slightly swollen endosperm and soft, milky-white texture of the embryo (Biccard and Midgley, 2009).

Due to incidences of seed predation, damage to cages and buds that did not open, several replicates for treatments were removed from the data analysis. Subsequently, all treatments except the control lost a replicate for *P. cordata*, *P. decurrens* consisted of 11 replicates for the auto treatment and 9 replicates for the cage treatment, and *P. scabra* consisted of 12 auto treatments, 18 cage treatments, 19 pollen supplementation treatments, and 16 self treatments.

In order to ensure that rodents were unable to access caged inflorescences, Bushnell (Trophy Cam HD Max-Colour LCD, 119577C) cameras were erected at two *Protea cordata* plants in

Fernkloof Nature Reserve from the 3rd June 2014 to the 12th June 2014. Due to time constraints and differing phenology, the rodent activity on caged inflorescences of the remaining study species were not recorded. The assemblage of rodent visitors and their ability to access caged inflorescences was noted in order to verify that insects alone were accountable for seed set in caged inflorescences.

3.3.4 Statistical analyses

One-way ANOVA's followed by Tukey HSD post-hoc tests were conducted on accumulated nectar data using STATISTICA (StatsSoft Inc. 12) to determine whether there were significant differences in both nectar concentration and production between NMP *Protea* (including study species), and bird- and beetle-pollinated *Protea* species. In order to establish whether standing crop nectar properties differed significantly between the study species and bird-pollinated *Protea* species, an independent 2-tailed t-test was performed on nectar concentration data and a non-parametric Mann-Whitney U-test was performed on the nectar production data as these did not conform to a known distribution.

We analyzed the effects of treatment on the proportion of florets that set seed for *Protea cordata* and *P. decurrens* using generalized linear models (GLMs) in SPSS Statistics v19 (IBM, Amonk, New York, USA). Wald Chi-square statistics type III models were used with logit link functions, and binomial error distributions, corrected for overdispersion where appropriate, and pairwise contrasts were made of the means for linear predictor using the sequential Šidák method (Steenhuisen *et al.* 2012). In order to run the analysis for *Protea cordata*, we added one seed to a single autogamy treatment since there was no seed set in that treatment for the species.

In order to run analyses for *Protea scabra*, we used plant as subject (to account for correlated responses among flowerheads) in Generalized Estimating Equations (GEE), implemented in

SPSS Statistics v19 (IBM, Amonk, New York, USA). We analyzed the effects of treatment on the proportion of florets that set seed in models with an exchangeable correlation matrix, binomial error distribution where plant was the subject. Significance was tested using Wald chi-square statistics in type III models with pairwise contrasts of means for linear predictor tested using the sequential Šidák method.

3.4 Results

3.4.1 Morphology

Nectar was produced at the base of each floret but presented between 14-20mm from the stigma (Table 1). Each study species produced copious amounts of concentrated nectar (Table 1). While volume of nectar increased over time, the concentration of nectar was reduced (Table 1). The traits of the *Protea* species are generally similar to the typical NMP system outlined by Rourke and Wiens (1977), Wiens *et al.* (1983) and Rebelo and Breytenbach (1987).

Accumulated nectar production in NMP *Protea* species did not differ significantly from bird- and beetle-pollinated species (Fig. 3c). The concentration of accumulated nectar in NMP species was consistent with bird-pollinated species, and significantly more concentrated than in beetle-pollinated species (Fig. 3d).

Standing crop nectar properties differed significantly between bird-pollinated and NMP *Protea* species (Fig. 3). Bird-pollinated *Protea* species produced greater quantities (Fig. 3a) of less concentrated (Fig. 3b) nectar in comparison to our NMP study species.

Table 1: Morphological measurements and nectar characteristics (mean±SE) of four NMP *Protea* species. Morphological measurements for NMP species reported in the literature are included for comparison (^aBalmer, 2012; ^bRebello and Breytenbach, 1987; ^cWiens *et al.*, 1983;).

Plant trait	<i>P. cordata</i>	<i>P. decurrens</i>	<i>P. scabra</i>	<i>P. subulifolia</i>	NFP <i>Protea</i>
Floret (mm)					
Floret height	21.76 ± 0.59	24.14 ± 0.57	28.56 ± 0.68	35.85 ± 0.85	
Style length	19.11 ± 0.55	21.02 ± 0.59	20.07 ± 1.48	31.43 ± 0.56	30-40 ^c
Pollen presenter	2.78 ± 0.13	4.52 ± 0.25	5.08 ± 1.62	7.43 ± 0.33	
Nectar presentation-nectar production	5.23 ± 0.35	4.88 ± 0.22	3.91 ± 0.17	12.63 ± 0.87	
Nectar presentation-stigma	14.02 ± 0.41	16.55 ± 0.39	19.30 ± 0.77	19.38 ± 0.83	10 ^c
Nectar presentation-base	8.12 ± 0.87	7.82 ± 0.48	9.15 ± 0.61	16.96 ± 0.93	
Inflorescence (mm)					
Height	40.68 ± 2.39	38.95 ± 1.50	44.68 ± 0.92	58.41 ± 1.55	15 ^b
Diameter	37.69 ± 2.12	37.82 ± 1.61	39.46 ± 1.59	49.14 ± 2.35	40-60 ^b
Number of florets	64.60 ± 2.81	60.00 ± 2.31	85.60 ± 6.45	117.10 ± 4.89	100-200 ^b
Plant (cm)					
Height	36.89 ± 1.29	39.88 ± 2.58	21.81 ± 0.80	30.20 ± 1.41	30 ^b
Nectar production (ul)					
Accumulated nectar	4.90 ± 0.34	3.59 ± 0.28	6.20 ± 0.26	5.51 ± 0.36	5.6 ^a
Standing crop nectar	4.23 ± 0.39	1.56 ± 0.11	5.12 ± 0.29	2.49 ± 0.18	
Nectar concentration (%)					
Accumulated nectar	14.86 ± 1.43	16.31 ± 1.25	11.59 ± 0.58	15.73 ± 0.57	13.26 ^a
Standing crop nectar	13.05 ± 1.32	30.49 ± 2.10	23.75 ± 1.13	37.75 ± 2.70	

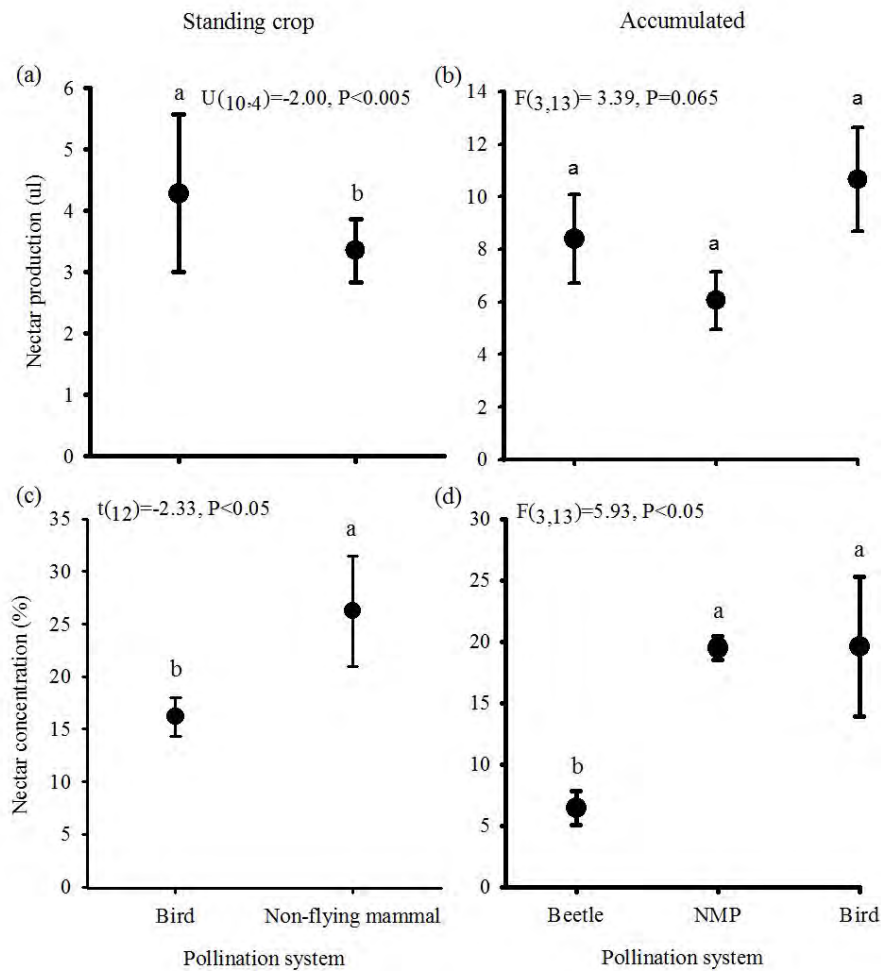


Figure 3: A comparison of (a) standing crop and (c) accumulated nectar production and (b) standing crop and (d) accumulated nectar concentration per floret for three typical pollination systems in *Protea*. Significant differences are indicated by the superscript. For accumulated nectar, NMP system refers to data from the study species and five confirmed NMP species, three bird-pollinated species and four beetle-pollinated species from the literature (Balmer, 2012; Steenhuisen and Johnson, 2012c). For standing crop nectar, data on 10 bird-pollinated species were obtained from Schmid *et al.* (unpublished data).

The study species did, to some degree, express convergence in bract colour. All of the bracts fell within the pink-red reflectance spectra, which appeared reddish brown to the human eye

(Fig. 4a-d), although the inner bracts of *P. scabra* appeared greenish to the human eye.

Protea decurrens and *P. scabra* showing the highest reflectance for the outer-bottom bract and the inner-bottom bract (Fig. 3b and c), a likely consequence of the tiny white hairs that cover the bracts. Additionally, pollen reflectance was consistently high for all study species, and appeared yellow-white to the human eye.

The remaining spectral reflectance of the floral traits were not constant among the study species (Fig. 4). Particularly, the perianth and style differed among the study species, as they were reflected in the white spectrum for *P. scabra* and *P. subulifolia*, and pink-red in *P. decurrens* and *P. cordata*. *Protea cordata* was measured at very low reflectance for all the remaining traits.

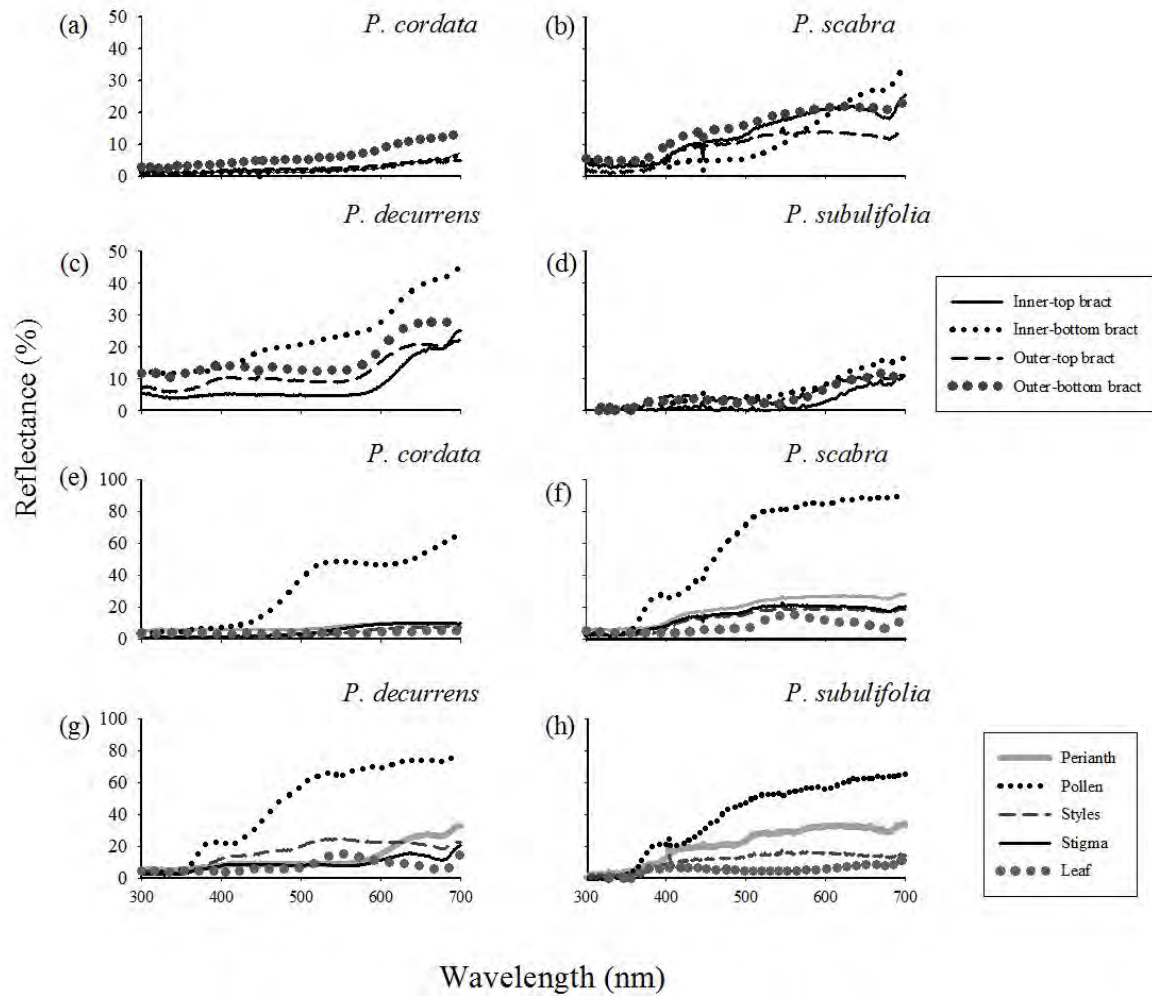


Figure 4: Mean spectral reflectance curves of four bract aspects (a-d) and five floral features (e-h) for 10 inflorescences of *Protea cordata*, *P. decurrens*, *P. scabra* and *P. subulifolia*.

3.4.2 Controlled hand-pollination and selective exclusion

Controlled hand-pollinated *P. cordata* plants that received pollen supplementation produced the highest percentage of fertilized seeds (13.5%); slightly greater than both the caged (12.8%) and open control (13.2%) treatments (Fig. 5a). Neither vertebrate exclusion nor pollen supplementation reduced seed set compared to the open controls (Fig. 5a). There was a significant reduction in seed set when inflorescences were left to set seed autonomously (0%) and treatments wherein self-pollen was experimentally added (2.1%), suggesting that this species is self-incompatible (Fig. 5a).

Seed set was consistently low in *P. scabra*, never exceeding 3% of florets, but varied significantly among treatments (Fig. 5b). Control plants open to all floral visitors produced the largest proportion of fertilized seeds, but not significantly more than caging inflorescences or pollen supplementation (Fig. 5b). *P. scabra* is evidently self-incompatible, as fewer seeds were produced when plants received self-pollen only (0.1%) or left to set seed autogamously (0%), Fig. 4b). While adding self-pollen to the inflorescences reduced the proportion of seeds set relative to adding pollen supplementation, this reduction was not significant (Fig. 5b).

Seed set was consistently high for all *P. decurrens* treatments, and did not differ significantly among treatments (Fig. 5c). Successful fertilization occurred in all three treatments, suggesting *P. decurrens* is capable of autogamous self-pollination (Fig 5c). Consequently, the role of pollinators cannot be determined by selective exclusion in this species.

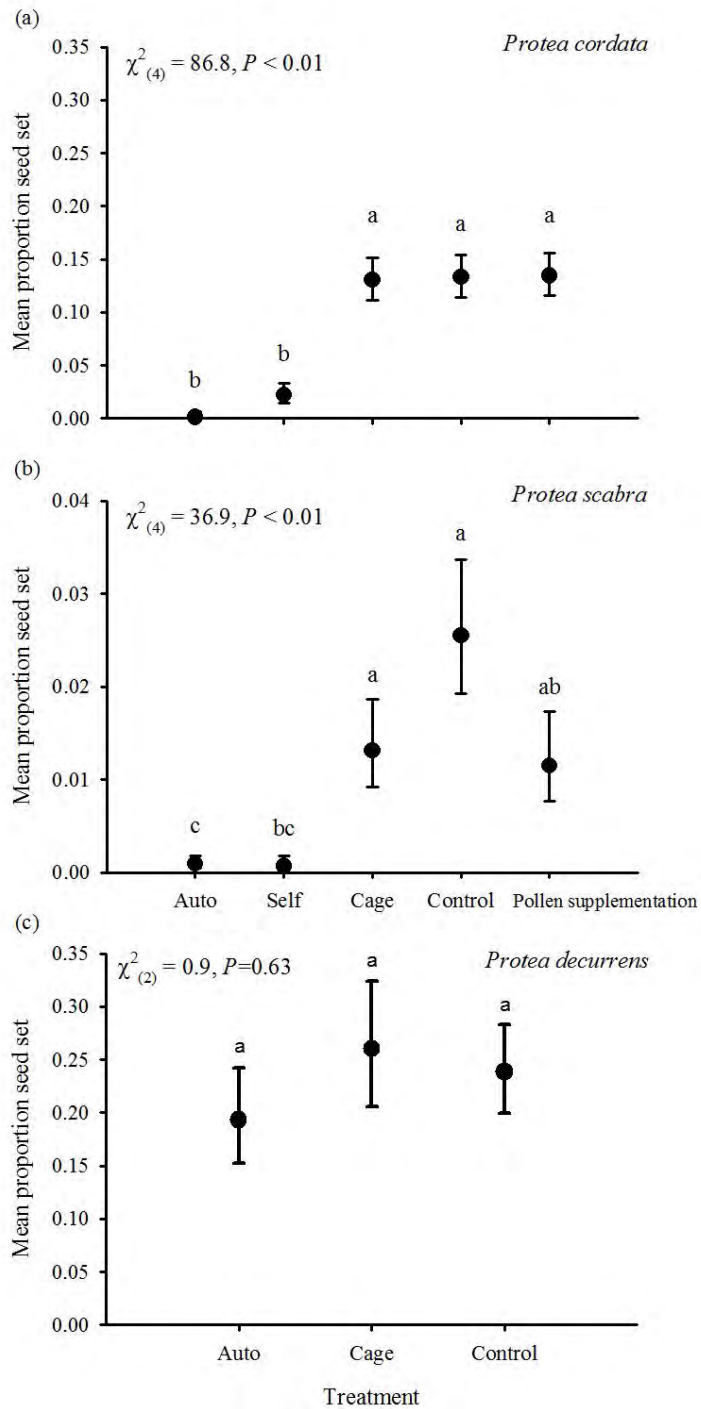


Figure 5: Mean (\pm SE) proportion of seed set per inflorescence for (a) *Protea cordata* (b) *Protea scabra* and (c) *Protea decurrens* under different treatment conditions.

The camera traps on cages picked up activity by four rodent species: *Mus minutoides*, *Aethomys namaquensis*, *Acomys subspinosus* and *Rhabdomys pumilio*. One *M. minutoides* and one *A. namaquensis* were observed near *P. cordata* but did not attempt to engage with the inflorescences. Three *A. subspinosus* and one *R. pumilio* were observed trying to access the inflorescences by climbing on the cages and pushing their rostrums against the bottom of the cages, but no individuals were able to breach the cages and subsequently no contact occurred between the rodents and the inflorescences. We further observed frequent movement of insects in the caged inflorescences, but as the remote cameras used in this study were not designed for recording invertebrate behaviour, insect identification and visitation rates could not be quantified.

3.5 Discussion

3.5.1 Floral specialization and ecological generalization

Remote camera analysis established rodents as the most frequent visitors to the four *Protea* species (Chapter 2). In order to evaluate these observations, this chapter has explored the NMP system further by describing the plant and floral traits that are important in attracting rodent foragers. The results suggested that although *Protea cordata*, *P. decurrens*, *P. scabra* and *P. subulifolia* exhibited floral morphologies that are specialized for pollination by non-flying mammals (Table 1) (Rourke and Wiens, 1977; Wiens *et al.* 1983; Rebelo and Breytenbach, 1987), they do not prohibit visitation by generalist pollinators as these species were shown to be dependent on insects for pollination in the absence of rodents.

The requirement of a plant for pollinator-mediated pollen transport determines the degree of floral adaptation for pollinators (Cheptou, 2011). For example, the strategy of autogamous self-pollination enables seed fertilization to occur without pollinator input (Cheptou, 2011). On the other hand, if a plant species is an obligate outcrosser, then the interaction between

plant and pollinator is a key determinant of the plant's persistence in the landscape (Spears, 1983). Both *Protea cordata* and *P. scabra* are self-incompatible as they were capable of neither facilitated nor autogamous self-fertilization (Fig. 5). Consequently, these species are dependent on external vectors for seed production.

There is a long tradition in pollination biology of explaining floral traits in terms of adaptations for pollinators (Aigner, 2001). The study species possess traits indicative of specialization for a non-flying mammal pollination system, including low growing, robust inflorescences that were concealed by dull coloured bracts (*Protea cordata*, *P. decurrens* and *P. subulifolia*) and rough, linear leaves (*P. scabra*) (Rebelo and Paterson-Jones, 2001). This increases the accessibility of inflorescences to small mammals while veiling the floral heads from opportunistic nectarivorous birds (Wiens *et al.* 1983), although the importance of geoflory in facilitating rodent pollination has not been established (Biccard and Midgley, 2009; Johnson and Pauw, 2014). The bracts of the inflorescences fell within the pink-red reflectance spectrum (Fig. 3), and were largely imperceptible to hymenoptera (Appendix, Fig. 1). Functional floral traits, such as small, wiry, tightly packed florets with a distance of 14-19 mm between the pollen presenter and nectar presentation further promote successful pollination by rodents (Table 1). Wiry florets prevent damage from aggressive foraging, and the short nectar-pollen distance ensures pollen deposition on the body of rodents (Wiens *et al.* 1983; Rebelo & Breytenbach, 1987). The foraging behavior of rodents (climbing into inflorescences and actively pushing their rostrum in amongst florets) provides additional support for floral adaptation to NMP, as pollen would be deposited on the fur of individuals as they brushes against several pollen presenters, which in turn will be deposited on the stigmas of neighboring plants in a similar way (Chapter 2).

Foraging animals are rewarded for their pollinating services with nectar (Simpson and Neff, 1981). Typically, *Protea* species produce copious amounts of nectar that is dilute in bird- and beetle-pollinated systems (Hargreaves *et al.* 2004; Steenhuisen and Johnson, 2012c), but viscous and concentrated in NMP systems (Wiens *et al.* 1983; Johnson and Pauw, 2014). Our investigation of nectar properties further suggested that these species are specialized for a NMP system, as the NMP *Protea* species yield significantly more concentrated accumulated nectar than beetle-pollinated *Protea* species, and significantly more concentrated standing crop nectar relative to bird-pollinated *Protea* species (Fig. 2). The requirement for small mammals of NMP *Protea* nectar has been demonstrated in the literature. For example, Fleming and Nicolson (2002) found that *Acomys* individuals were almost entirely reliant on *Protea humiflora* pollen and nectar during the resource-scarce winter months.

Nectar properties of NMP species differed according to time of sampling, suggesting that foragers with different activity patterns consume varying floral rewards (Table 1). The floral resources foraged on by nocturnal visitors (*M. minutoides*, *A. namaquensis*, *D. sp.* and *A. subspinosus*) consisted of larger quantities of less concentrated nectar than that accessed by diurnal species (*Rhabdomys pumilio*) (Table 1) (Chapter 2). A change in nectar concentration is thought to occur due to the evaporation of nectar water during the day, resulting in a more viscous solution (Nicolson and Thornburg, 2007). Patterns of nectar production are largely inconsistent in other NMP *Protea*, although much research has suggested that nectar is secreted nocturnally (Wiens *et al.* 1983). As such, the low volume of standing crop nectar we observed is likely due to nectar removal by nocturnal foragers with no nectar production during the day. The difference in nectar properties over time did not minimize visitation rates to these inflorescences, as rodent activity was consistently high throughout the day and night out (Chapter 2). In contrast to NMP *Protea* species, bird-

pollinated *Protea* nectar remains constant throughout the day, as the heavily furred bracts shields the nectar from dilution by rain and evaporation (Nicolson and Thornburg, 2007).

Subsequently, birds would be unlikely to be effective pollinators of the study species not only in terms of nectar properties, but also functional fit. Indirect evidence has suggested that pollination by birds is most effective when the pollen to nectar distance matches bill length (Hargreaves and Johnson, 2004). This ensures that pollen is deposited on the crown and throat of individuals during foraging events (Steenhuisen and Johnson, 2012b; Hargreaves and Johnson, 2004). The Orangebreasted sunbird (*Anthobaphes violacea*) was the only bird species observed engaging with the inflorescences of our study species (*P. decurrens* and *P. scabra*) (Chapter 2). However, it is unlikely that these specialist nectarivorous birds are effective pollinators. This is due to the small, bowl-like inflorescences of the study species, and the disparity between bill length (20-23 mm) and nectar-pollen distance (14-19 mm) (Maclean, 1993). These features suggest that Orangebreasted sunbirds are prevented from inserting their head into the inflorescence while foraging for nectar (Hargreaves and Johnson, 2004), implying that they do not pick up pollen and are consequently not contributing toward the reproductive effort of the study species.

While the *Protea* species under study possess traits consistent with a NMP system (Table 1) the pollinator exclusion experiments did not conclusively identify small mammals as the sole pollinator group. All four *Protea* species were shown to be visited by small mammals (Chapter 2), but the open pollinated treatment (Control) that allowed visitation by rodents and insects, did not produce a significantly greater seed set than the treatment that excluded rodents but allowed insects (Cage, Fig. 5). Based on camera recordings of *P. cordata*, it is evident that animals exceeding 13 mm were unable to access caged inflorescences, suggesting that insects were solely responsible for seed set in caged treatments of *P. cordata*

and *P. scabra*. Indeed, small insects were commonly observed on the inflorescences of the *P. cordata*, and were detected in abundance on some caged inflorescences.

The implication is that these species may employ a pollination system that is more generalized than their traits suggest (Fenster *et al.* 2004). This occurs because it is difficult for a plant to evolve morphological specializations that completely exclude any pollinators if they contribute toward the plants fitness (Aigner, 2001). Consequently, when there is efficient pollen transfer by two pollinator guilds with no selective cost to the plant (Johnson & Steiner, 2000; Fenster *et al.* 2004) species can then evolve specialized morphological traits in an environment that supports effective pollination by multiple functional groups (Aigner, 2001). Considering that the study species exist in localized, fragmented populations, increasing the pool of potential pollinators may be important in ensuring seed fertilization. Generalist insects are likely to be important pollinators to these plants as their preference for floral signals, such as colour, are not as innate as previously thought (Waser *et al.* 1996). This implies that floral specialization for non-flying mammal pollination may not necessarily deter insect visitors, as sensory preferences can be overcome by associative conditioning (Waser *et al.* 1996).

Indeed, insect activity has been observed on the inflorescences of species that are adapted for pollination by birds (e.g. *Aloe pluridens* and *A. lineate* var. *muirii*) (Botes *et al.* 2009), and non-flying mammals (e.g. *Protea nana* and *P. recondita*) (Biccard and Midgley, 2009; Balmer, 2012), and enclosure experiments have suggested that insects are effective pollinators of congeneric NMP species. Wiens *et al.* (1983) and Fleming and Nicolson (2002) found that small mammal exclusion reduced seed set by 50% in *Protea humiflora*, suggesting that insects contribute to the remaining seed fertilization (Fleming and Nicolson, 2002). While these findings are consistent with the evidence presented in this paper, they are impossible to

validate as no experimental design exists that allows small mammals to access inflorescences while simultaneously excluding insects (Wiens *et al.* 1983).

Nectar accumulates when rodents are prevented from foraging (Table 1), suggesting that the floral rewards available to insects in caged inflorescences were increased, while the probability of insectivorous predation is decreased (Hargreaves and Johnson, 2004).

Consequently, the non-significant difference in seed set between caged and control treatments for all study species may be due to increased insect activity following atypical volumes of nectar, and not indicative of insect activity under controlled conditions (Wiens *et al.* 1983).

Indeed, insects are unlikely to be reliable pollinators to these *Protea* species as their movement is restricted by persistent low temperatures (Wiens *et al.* 1983) The high incidences of seed set in caged treatments may therefore not represent the natural contribution of insects to the pollination of these species.

3.5.2 Resource allocation to reproduction

Considering that both *Protea cordata* and *P. scabra* were reliant on cross pollen for seed set (Fig. 5), we had expected that experimentally enhancing the number of cross-pollination events would increase seed set. However, supplementing pollen to *P. cordata* did not increase the proportion of fertilized seeds relative to the open control treatment (Fig. 5a), suggesting that pollen is not limited in the environment (Knight *et al.* 2005). Instead, it is likely that the plants are constraining the number of successful seed fertilization events due to the lack of extra resources available for the maturation of ovules that are artificially fertilized (Knight *et al.* 2005). The consistency in seed set between pollen supplementation and control treatments, and the probability that seed set is limited by resources rather than pollen, implies that the pollinators are transferring pollen at great efficiency.

Protea scabra produced an exceptionally small proportion of viable seeds in the control treatments relative to the other species (Fig. 5b) implying that although visitation frequency was high (Chapter 2), pollen deposition may not have been precise. Alternatively, the results may imply that resource allocation to reproduction is not consistent among NMP *Protea* species. Considering that *Protea scabra* was the only resprouting species in this study (Protea Atlas Project, unpublished data), this low seed set may be associated with fire survival strategies. Species that resprout allocate resources to storage rather than seed production, resulting in a trade-off between persistence and reproductive effort (Bond and Midgley, 2003). Consequently, seed set in resprouters is generally lower than congeneric, co-occurring nonsprouters (Bond and Midgley, 2003).

The greatest proportion of viable seeds was produced by *Protea decurrens*. Successful seed set occurred in all treatments, including those excluded from all pollinators (Fig. 5c), suggesting that *P. decurrens* is capable of autogamous self-pollination. While there was no significant difference between treatments, mean seed set through autogamy was slightly lower than the control treatment. The implication is that regeneration by seeds is greater when associated with outcross pollen, but viable seeds can be produced when pollinators are absent in the landscape. The strategy of self-compatibility is fairly common in reseederers (Lamont and Wiens, 2003). This is because unlike resprouters, reseederers are killed during fire events, and consequently rely on seeds for genetic persistence. Selection for self-compatibility assists in rapid post-fire recruitment by providing reproductive assurance irrespective of pollinator availability (Lamont and Wiens, 2003).

3.5.3 Ecological implications

While the evolution of animal pollination enhances pollen transfer efficiency, plants that depend on external pollen vectors for reproductive success are often more vulnerable to

fragmentation and disturbance due to the sensitivity of floral visitors to habitat changes (Aizen *et al.* 2002). As such, there is increasing global concern about the effect of climate and anthropogenic changes to ecosystems on plant-pollinator interactions (Mitchell *et al.* 2009).

The degree to which plants are affected by pollinator loss due to habitat degradation is determined by the plants breeding system and pollinator specialization (Bond, 1994), yet research on NMP *Protea* breeding systems is lacking in the literature. Around 35 *Protea* species have been proposed as non-flying mammal-pollinated (Rourke, 1980; Rebelo and Breytenbach, 1987), but the breeding systems of only two of these species have been published. Self-incompatibility was described for *Protea nana* (Biccard and Midgley, 2009), and autogamous self-fertilization was detected in *Protea humiflora* (Wiens *et al.* 1983). Additionally, Balmer (2012) established obligate outcrossing in *P. recondita* and *P. pendula*.

The results from comparative literature have suggested that although self-compatibility is common in this genus (Steenhuisen and Johnson, 2012a); there is no consistency in breeding systems among NMP *Protea* species. Indeed, the evidence presented in this paper has suggested that *Protea cordata* and *P. scbara* are self-incompatible, implying that the efficiency of their pollinators is an important component in ensuring their genetic persistence. In contrast, *P. decurrens* was capable of autogamous self-pollination. The strategy of autogamy is often facultative, suggesting pollinators may still promote seed production (Steenhuisen and Johnson, 2012b). Indeed, the high incidence of rodent visits to *P. decurrens* (Chapter 2) suggests that the floral traits of this species may function to attract potential rodent pollinators.

This study has indicated that although pollinator behavior and morphology may determine the direction of floral selection, traditional pollination syndromes often oversimplify the

complexity of plant-animal interactions (Ollerton *et al.* 2009). This is evident in this study, as while these *Protea* species have evolved traits that attract rodent pollinators (Chapter 2) (Table 1), insects may contribute to the reproductive effort of these plant species, since seed set was only marginally lowered when small mammals were excluded from the inflorescences (Fig.5). The implication is that the morphological traits of the study species do not filter insect visitors to floral heads.

Consequently, plants that display floral features for a certain pollination syndrome may rely on other pollen vectors for successful pollination (Spears, 1983). Floral adaptations to pollinators are then often not based on a “lock and key” relationship (Grant and Grant, 1965), as floral traits may represent adaptations to pollinators from multiple functional groups or even ‘minor’ pollinators, (Ollerton *et al.* 2009). This suggests that although these *Protea* species may be evolutionarily specialized for rodents, they are not ecologically dependent on them for pollination if insects are in abundance (Ollerton, 1996). Thus while selection for therophilous floral cues of the study species (Table 1) is likely to promote foraging constancy and prevent stigma clogging by rodents (Steenhuisen *et al.* 2010), generalist insects are likely to contribute toward the reproductive effort of these species (Fig. 5). However, it is important to note that these *Protea* species flower in winter when environmental conditions (snow, rain and low temperatures) do not favour a reliance on insect pollinators (Wiens *et al.* 1983).

While we have indicated that these species are more generalist in pollination systems than their traits suggest and therefore at lower risk of extinction than truly specialist species (Johnson and Steiner, 2000), their localized, fragmented distribution and the self-incompatibility of *Protea cordata* and *Protea scabra* implies that any environmental changes that exclude both insects and rodents from their landscape is likely to have dire consequences for these species (Aizen *et al.* 2002). This is important for the fire-dependent ecosystem of

fynbos, as research has suggested that fire dynamics are important in structuring vertebrate communities, with population numbers declining both immediately following a fire, and fourteen years thereafter (Willan and Bigalke, 1984). Consequently, these plant species that rely on animal pollinators for reproductive success are vulnerable to coextinction (Aslan *et al.* 2013), suggesting that the collapse of plant-animal mutualism would decrease the population viability of these species (Wiens *et al.* 1983, Aizen *et al.* 2002).

The unexpected nature of these results requires a reexamination of the NMP system in the Cape *Protea*. It is necessary for future research on non-flying mammal pollination systems to move beyond ranking pollinators in terms of their functional fit to the floral phenotype of the plant in order to better understand the underlying evolutionary processes that contribute toward pollination shifts (Aigner, 2001).

3.6 References

- Aigner, P. A., 2001. Optimality modeling and fitness trade-offs: when should plants become pollinator specialists? *Nordic Society Oikos* 95, 177-184.
- Aizen, M. A., Ashworth, L., Galetto, L., 2002. Reproductive success in fragmented habitats: do compatibility systems and pollination specialization matter? *Journal of Vegetation Science* 13, 885-892.
- Aslan, C. E., Zavaleta, E. S., Tershy, B., Croll, D., 2013. Mutualism disruption threatens global plant biodiversity: a systematic review. *PloS one* 8:e66993.
- Balmer, A., 2012. A comprehensive ecological pollination study of non-flying mammal pollinated *Proteas*. MSc thesis, University of Zurich.

- Biccard, A., and Midgley, J. J., 2009. Rodent pollination in *Protea nana*. South African Journal of Botany 75, 720-725.
- Bond, W. J., 1994. Do mutualisms matter? Assessing the impact of pollinator and disperser disruption on plant extinction. Philosophical Transactions: Biological Sciences 344, 83-90
- Bond, W. J., Midgley, J. J., 2003. The evolutionary ecology of sprouting in woody plants. International Journal of Plant Sciences 164, 103-114.
- Botes, C., Johnson, S. D., Cowling, R. M., 2009. The birds and the bees: using selective exclusion to identify effective pollinators of African Tree Aloes. International Journal of Plant Science 170, 151–156.
- Cheptou, P.O., 2011. Clarifying Baker's Law. Annals of Botany 109, 633–641.
- Chittka, L., Shmida, A., Troje, N., Menzel, R., 1993. Ultraviolet as a Component of Flower Reflections, and the Colour Perception of Hymenoptera. Vision Research 34, 1489-1508.
- Faegri K., van der Pijl L., 1979. The Principles of Pollination Ecology. Third revision. Pergamon Press, Oxford.
- Fenster, C. B., Armbruster, W. S., Wilson, P., Dudash, M. R., Thomson, J. D., 2004. Pollination syndromes and floral specialization. Annual Review of Ecology Evolution and Systematics 35, 375 – 403.
- Fleming, P., Nicolson, S., 2002. How important is the relationship between *Protea humiflora* (Proteaceae) and its non-flying mammal pollinators? Oecologia 132, 361-368.

- Grant, V. and Grant, K.A., 1965. Flower Pollination in the Phlox Family. Columbia University Press.
- Hargreaves, A. L., Johnson S. D., Nol E., 2004. Do floral syndromes predict specialization in plant pollination systems? An experimental test in an ‘ornithophilous’ African *Protea*. *Oecologia* 140: 295–301.
- Herrera, C. M., 1996. Floral traits and plant adaptation to insect pollinators: a Devil’s advocate approach, in: Lloyd, D. G., Barret, C. H. (Eds.), *Floral Biology: Studies on Floral Evolution in Animal-Pollinated Plants*. Chapman & Hall, New York, pp. 65-87.
- Horn, W., 1962. Breeding research on South African plants: II. Fertility of Proteaceae. *Journal of South African Botany* 73, 259–268.
- Jacobs, G. H., 1992. Ultraviolet Vision in Vertebrates. *American Zoologist* 32, 544-554.
- Johnson, S.D., Steiner, K.E., 2000. Generalization versus specialization in plant pollination systems. *Trends in Ecology and Evolution* 15, 140-143.
- Johnson, S. D., Pauw, A., Midgley, J. J., 2001. Rodent Pollination in the African Lily *Massonia Depressa* (Hyacinthaceae). *American Journal of Botany* 88, 1768–1773.
- Johnson, S. D., Andersson, S., 2002. A Simple Field Method for Manipulating Ultraviolet Reflectance of Flowers. *Canadian Journal of Botany*, 1325–1328.
- Johnson, C. M., Pauw, A., 2014. Adaptation for rodent pollination in *Leucospermum arenarium* (Proteaceae) despite rapid pollen loss during grooming. *Annals of Botany* 113, 931-8.

- Knight, T. M., Steets, J. A., Vamosi, S. J., Campbell, D. R., Dudash, M. R., Johnston, M. O., Mitchell, R. J., 2005. Pollen limitation of plant reproduction: pattern and process. *Annual Review of Ecology Evolution and Systematics* 36, 467–497.
- Lamont, B. B., Wiens, D., 2003. Are seed set and speciation rates always low among species that resprout after fire, and why? *Evolutionary Ecology* 17, 277–292.
- Maclean, G. L., 1993. Roberts' birds of southern Africa. John Voelcker Bird Book Fund. Cape Town.
- Mitchell, R. J., Irwin, R. E., Flanagan, R. J., Karron, J. D., 2009. Ecology and evolution of plant–pollinator interactions. *Annals of Botany* 103, 1355–1363.
- Nicolson, S. W., Thornburg, R. W., 2007. Nectar chemistry, in: Nicolson, Susan W., Nepi, Massimo, Pacini, Ettore (Eds.) *Nectar and Nectaries*. Springer.
- Ollerton, J., 1996. Reconciling ecological processes with phylogenetic patterns: the apparent paradox of plant-- pollinator systems. *Journal of Ecology* 84, 767-769.
- Ollerton, J., Alarcon, R., Waser, N. M., Price, M. V., Watts, S. Cranmer, L., Hingston, A., Peter, C. I., Rotenberry, J., 2009. A global test of the pollination syndrome hypothesis. *Annals of Botany* 103, 1471–1480.
- Rebelo, T. G., Breytenbach, G. J., 1987. Mammal Pollination in the Cape Flora, in: Rebelo, A. G. (Ed.), *Preliminary synthesis of pollination biology in the Cape flora*. South African National Science Program Report 141, 109–124.
- Rebelo, T. G., Paterson-Jones, C., 2001. *A Field Guide to the Proteas of Southern Africa*. Fernwood Press in association with the National Botanical Institute.

- Rourke, J. P., 1980. *The Proteas of Southern Africa*. Purnell, Cape Town.
- Rourke, J. P., Wiens, D., 1977. Convergent Floral Evolution in South African and Australian Proteaceae and Its Possible Bearing on Pollination by Nonflying Mammals. *Annals of the Missouri Botanical Garden* 64, 1–17.
- Shuttleworth, A., Johnson, S. D., 2009. The importance of scent and nectar filters in specialized wasp-pollination system. *Functional Ecology* 23, 931–940.
- Simpson, B. B., Neff, J. L., 1981. Floral Rewards: Alternatives to Pollen and Nectar. *Annals of the Missouri Botanical Garden* 68, 301-322.
- Spears, E. E., 1983. A direct measure of pollinator effectiveness. *Oecologia* 57, 196-199.
- Steenhuisen S.-L., Raguso, R. A., Jürgens A., Johnson, S. D., 2010. Variation in scent emission among floral parts and inflorescence developmental stages in beetle-pollinated *Protea* species (Proteaceae). *South African Journal of Botany* 76, 779–787.
- Steenhuisen, S.-L., Van der Bank, H., Johnson, S. D., 2012. The relative contributions of insect and bird pollinators to outcrossing in an African *Protea* (Proteaceae). *American Journal of Botany* 99, 1104–1111.
- Steenhuisen, S.-L., Johnson. S. D., 2012a. Evidence for autonomous selfing in grassland *Protea* species (Proteaceae). *Plant Systematics and Evolution* 298, 857–869.
- Steenhuisen, S.-L., Johnson. S. D., 2012b. The influence of pollinators and seed predation on seed production in dwarf grassland *Protea* “sugarbushes” (Proteaceae). *South African Journal of Botany* 79, 77–83.

- Steenhuisen, S.-L., Johnson, S. D., 2012c. Evidence for beetle pollination in the African grassland sugarbushes (*Protea*: Proteaceae). *Plant Systematics and Evolution* 298, 857-869.
- Valente, L. M., Reeves, G., Schnitzler, J., Mason, I. P., Fay, M. F., Rebelo, T. G., Chase, M. W., Barraclough, T. G., 2010. Diversification of the African genus *Protea* (Proteaceae) in the Cape biodiversity hotspot and beyond: equal rates in different biomes. *Evolution* 64, 745–759.
- Van der Niet, T., Johnson, S. D., 2012. Phylogenetic evidence for pollinator-driven diversification of angiosperms. *Trends in Ecology and Evolution* 27, 353-361.
- Waser, N. M., Chittka, L., Price, M. V., Williams, N. M., Ollerton, J., 1996. Generalization in pollination systems, and why it matters. *Ecology* 77, 1043-1060.
- Wiens, D., Rourke, J. P., Casper, B. B., Rickart, E. A., LaPine, T. R., Peterson, C. J., Channing, A. 1983. Nonflying mammal pollination of southern African *Proteas*: a non-coevolved system. *Annals of the Missouri Botanical Garden* 70, 1-31.
- Willan, K., Bigalke, R. C., 1984. Ecological Effects of Fire in South African Ecosystems
Ecological Studies 48, 255-271

4. Carnivorous visitors to non-flying mammal pollinated Protea species (Proteaceae) in South Africa

4.1 Abstract

Occasional visitors to flowers are often classified as inefficient or unreliable pollinators due to their rare occurrence and our inability to observe their behaviour. Recently, motion/heat-triggered camera traps have become instrumental in vertebrate pollination ecology studies. Our list of occasional nectar-feeders visiting several rodent-pollinated *Protea* species has expanded to include unexpected small carnivorous mammals. Nocturnal large-spotted genets (*Genetta tigrina* Schreber) were recorded licking nectar from inflorescences of *Protea scabra* and *Protea pendula*, and diurnal Cape gray mongoose (*Galerella pulverulenta* Wagner) foraged repeatedly on nectar from *Protea canaliculata* and *Protea recondita*. The foraging behaviour of these mammals was non-destructive and facilitated the transfer of pollen to other inflorescences, inferred by the heavy pollen loads observed on the snouts of the mongoose and visits by both mammals to inflorescences on the same and different plants during the observation period. These findings highlight the importance of the contribution to pollination by occasional flower visitors and promote the use of camera traps in observing animal behaviour.

4.2 Introduction

Difficulties in directly observing the behaviour of secretive and often nocturnal carnivorous mammals has sometimes lead to them being overlooked as contributors to pollination. Indeed carnivorous mammals are not considered specialist pollinators due to their diets consisting mostly of animal matter and assumptions that they may be destructive flower visitors. With the exception of nectarivorous bats, nectar usually cannot be the only source of nutrition for these mammals since due to their high energetic requirements and larger body mass than birds.

Tentative evidence of small carnivores visiting flowers is given in literature on mammal pollinated plants. Lack (1978) reported that besides bats and hawkmoths, genets (*Genetta tigrina* Schreber) foraged on nectar of *Maranthes polyandra* (Chrysobalanaceae), regularly visiting flowers over two days of observations at night when nectar production was at its highest. The genets foraged for nectar without damaging the flowers (Lack, 1977), but due to their low frequency of visitation, Lack concluded that they are probably only occasional pollinators, potentially also predated on the bats, the main pollinators (Lack, 1978).

Wiens *et al.* (1983) highlight the lack of information available on mammal pollinators and list the few studies that have provided evidence of non-rodent floral visitors (see also Rebelo and Breytenbach, 1987), before delving into a description of a non-flying mammal pollination system for South African Proteaceae. Besides rodents, various larger mammals documented as being involved in the pollination ranged from non-specialist nectarivores such as bush babies and genets in South Africa, mouse lemurs in Madagascar, sugar gliders and placental bushrats in Australia, and even monkeys in the New World, to specialist nectarivores such as the honey possums that are able to efficiently lap up nectar from *Banksia* inflorescences using a brush-like tongue (Collins and Rebelo, 1987; Hopper, 1980; Rebelo and Breytenbach, 1987; Wiens *et al.*, 1979; Wiens and Rourke, 1978).

As part of an investigation into the evolution of floral traits associated with pollinator shifts in African Proteaceae, our current research efforts have concentrated on evaluating mammals as pollinators and determining functional floral traits that may attract them to *Protea* species in the Cape Floristic Region. Many of the over 30 species of putatively mammal-pollinated *Protea* species have dull-coloured, geoflorous inflorescences that emit unpleasant yeasty/cheesy odours and provide principally rodent pollinators with copious amounts of sugar-rich nectar (0.1-2 ml, >30% Brix; Alice Balmer, unpubl. data; Rebelo and Breytenbach, 1987). During these investigations, however, we captured video evidence of larger carnivorous mammals visiting our study species to forage on nectar. Behavioural data for these mammal visitors obtained from video footage for the first time are presented here and discussed in terms of their ability to be occasional pollinators of these plant species.

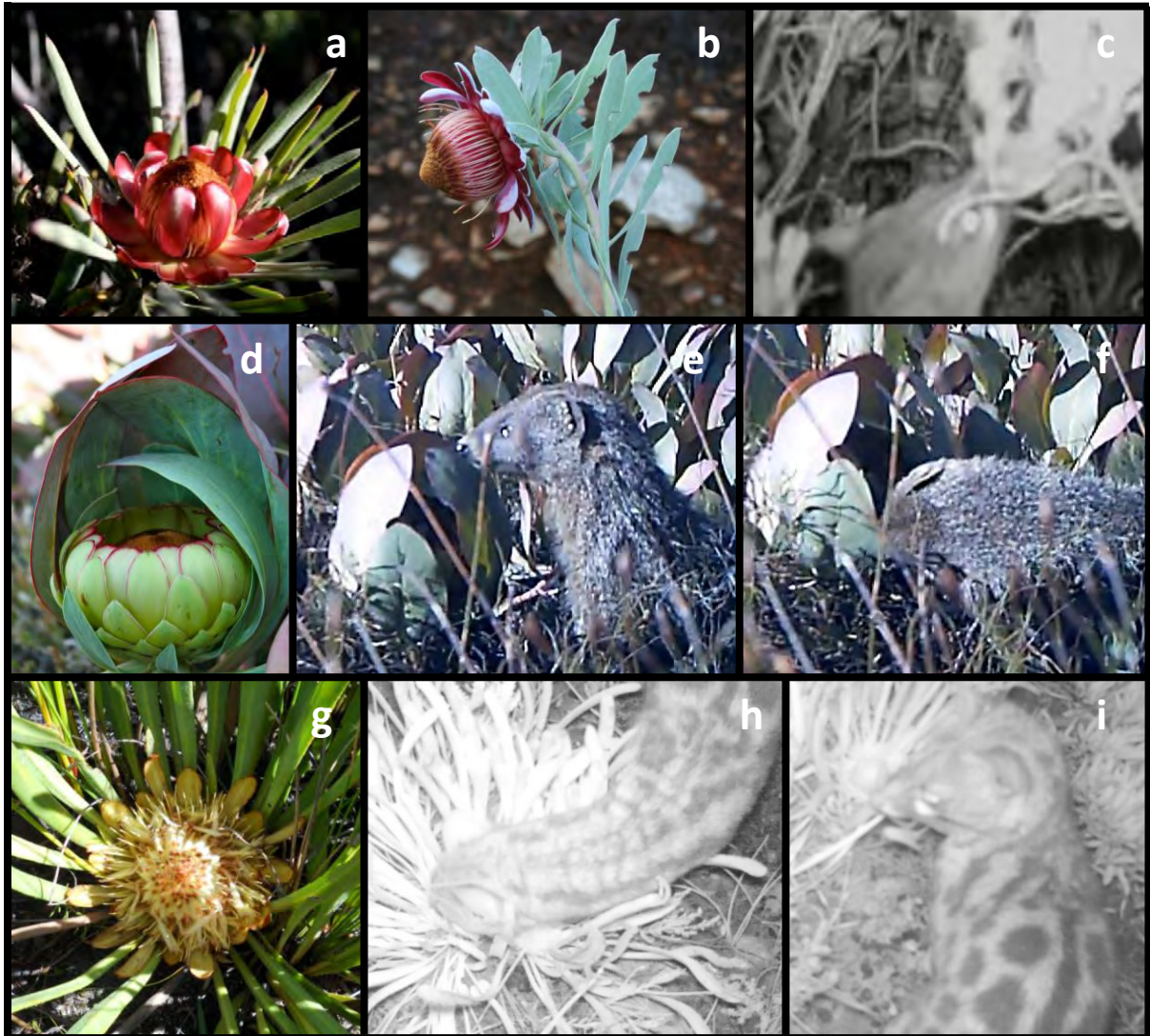


Figure 1. Gross morphology of inflorescences of (a) *Protea canaliculata*, (b) *Protea pendula*, (d) *Protea recondita* with leaves in front bent forward to reveal the inflorescence, and (g) *Protea scabra*. Still images from video footage are shown for large-spotted genet visiting (c) *P. pendula* and (h-i) *P. scabra*, and Cape gray mongoose visiting (e-f) *P. recondita*.

4.3 Methods

To determine the pollinators of a plant, invertebrate and vertebrate visitors are usually surveyed and their behaviour assessed as an indication of their efficiency as pollinators, ability to act as nectar/pollen thieves or occasional nectarivores. Vertebrate visitors to *Protea* inflorescences were monitored by motion and infrared sensor camera traps (Bushnell Trophy Cam HD Max-color LCD, 119577C and Bushnell Trophy Cam 119466) set to record a one

minute video with a ten second interval between successively triggered recordings. These cameras were aimed at flowering individuals of principally mammal pollinated *Protea* species of varying population size: *Protea canaliculata* Andrews (40 plants), *Protea pendula* R.Br. (25 plants), *Protea recondita* H.Buek ex Meisn. (200 plants), *Protea scabra* R.Br. (40 plants) (Fig. 1). At least three cameras, aimed at separate plants with one or two open inflorescences, were used to record vertebrate visitors during peak flowering periods for each species (Table 1). The cameras were frequently moved (about every 10 days) when their target inflorescences senesced, and aimed at other flowering plants. The number of visits that were deemed legitimate pollination events (i.e. involving contact between an animal and the pollen presenters/stigmas of an inflorescence whilst foraging) were determined for each visitor species. A “visit” was counted as new if the animal visited a different inflorescence in the field of view and returned to the first inflorescence or returned from being out of view of the camera for some time. The animal’s behaviour whilst foraging was also noted.

4.4 Results and discussion

With over 250 trap-nights/days worth of footage of vertebrate visitors to the *Protea* inflorescences, our cameras revealed two principally carnivorous large mammals foraging on nectar of these plant species (Table 1, Fig. 1, Appendix 2), in addition to confirming that the principal pollinators included rodents, elephant shrews, small generalist birds and more specialised nectarivorous sunbirds and sugarbirds. Only mammals larger than 100g are discussed in this study, other visitors together with pollinator exclusion and breeding system experiments will be described elsewhere (Alice Balmer, Nicola Kuhn and Kim Zoeller, unpubl. data).

Table 1. The number of video-recorded pollinating visits by genet and mongoose to each of four *Protea* species, the length of time the cameras were out in the field, and the localities of the *Protea* populations.

<i>Protea</i> species visited by:	Number of visits recorded (number of plants, number of inflorescences)	Recording period (No. of cameras, range of nights/days each was set out, total hours in field)	Site (coordinates)
Cape gray mongoose (<i>Galerella pulverulenta</i>)			
<i>P. canaliculata</i>	3 (2 plants, 3 infl)	3 cameras, 187 days/187 nights	Witteberge, Touws River (33°20'13.19"S, 20°23'35.74"E, 1191m)
<i>P. recondita</i>	8 (3 plants, 7 infl)	3 cameras, 69 days/60 nights	Koue Bokkeveld, Ceres (33°25'29.51"S, 19°28'32.01"E, 1566m)
Large-spotted Genet (<i>Genetta tigrina</i>)			
<i>P. pendula</i>	3 (1 plant, 3 infl)	3 cameras, 14 days/12 nights	Koue Bokkeveld, Ceres (33°15'2.38"S, 19°29'1.60"E, 1298m)
<i>P. scabra</i>	12 (3 plants, 4 infl)	4 cameras, 38 days/38 nights	Hottentots-Holland Mountain Range, Houw Hoek (34°11'54.73"S, 19°10'45.84"E, 571m)

At least three visits by either Cape gray mongoose (*Galerella pulverulenta* Wagner, family Herpestidae) or large-spotted genet (*Genetta tigrina* Schreber, family Viverridae) were recorded for each of the four *Protea* species investigated (Table 1, Fig. 1). We did not observe both carnivore species visiting the same *Protea* species. The greatest number of visits to inflorescences was by a genet that repeatedly visited four inflorescences from three different plants of *P. scabra* over three nights in a trapping area of 0.3 ha (Table 1). Of all the recorded foraging visits by various mammals and birds, genets were responsible for 0.4% and 10.3% of visits to *P. pendula* and *P. scabra* respectively, whilst mongoose made up 4.35% and 44% of visits to *P. canaliculata* and *P. recondita* respectively. The high percentage of mongoose visits to *P. recondita* may be overinflated by the fact that large leaves of this

Protea species characteristically curl over and shade inflorescences, thus obscuring rodent foraging from view of the camera. This is inferred by also seeing the rodents move in amongst inflorescences on the plants on the video footage, and captured rodents having had high pollen loads on their snouts and in their faeces (Alice Balmer, unpubl. data). Despite this uncertainty in actual numbers of rodent visits, enormous pollen loads were clearly visible on the snout of the mongoose and there is no doubt that these frequently visiting mammals would be contributing to pollination, especially given large size (91 mm in diameter, ~500 florets) and robustness of these inflorescences. The mongoose were observed to move leaves out of the way to gain access to nectar in the inflorescences. These visitors were diurnal compared to the nocturnal genets, and visited during snowfall at the Koue Bokkeveld site.

An additional two foraging visits, not included in Table 1, were recorded of mongoose lapping nectar off *P. recondita* involucre bracts without touching stigmas/pollen presenters. *Protea recondita* produces high amounts of sucrose-rich nectar (1.9 ml and 29.2% Brix; Alice Balmer, unpubl. data) that often pools in between the involucre bracts and leaves curled over the inflorescences, sometimes observed dripping down onto surrounding vegetation and rocks over which they sprawl. It is therefore very easy for a floral visitor to forage on nectar without being involved in pollinating inflorescences of this species.

With mongoose and genet both being of the order Carnivora, their diet is expected not to be reliant on flower resources. The fact that these *Protea* species are also visited by other families of mammals and birds, also makes us question the specialist nature of the non-flying mammal pollination system and how floral traits that attract pollinators with varied sensory abilities and preferences have evolved. These *Protea* species emit odours that resemble cheese, yeast or sour milk to the human nose (pers. obs.). Perhaps these animals all share preconditioning to detect compounds commonly found in carbohydrate and protein sources and that dietary specialisations are not involved in the selection of scent in these vertebrate

pollinated *Protea* species compared to fruity scents emitted by beetle-pollinated species. Our investigation into floral traits and pollinator shifts in the *Protea* genus will hopefully shed some light on this in future. Rather, these plants have opened themselves up to diverse group of generalist and specialist pollinators. Genets have a wide home range. Any pollen carried by them would potentially be carried further than the range of more common rodent visitors. Any pollen transfer events over these distances would be rare but still important for plant populations.

Speculative evidence of genet visiting mammal pollinated *Protea* species was given by Wiens *et al.* (1983) whilst this is the first record of mongoose visiting *Protea* inflorescences. Recently, Symes & Nicolson (2008) observed slender mongoose (*Galerella sanguinea* Rüppell) feeding on *Aloe marlothii* nectar. In the same way that Lack (1977) observed genet climbing branches of *Maranthes polyandra* to forage on nectar, our video recordings show a genet climbing up branches of *P. pendula* to reach inflorescences. Since our camera trap observations show this foraging to be non-destructive and potentially frequent, with regard to the mongoose, we are confident that these mammals are contributing to pollination of these *Protea* species and encourage the use of camera traps to observe the entire range of vertebrate visitors to a plant in future pollination studies.

4.5 References

- Collins, B.G., Rebelo, T., 1987. Pollination biology of the Proteaceae in Australia and southern Africa. *Australian Journal of Ecology* 12, 387-421.
- Hopper, S.D., 1980. Bird and mammal pollen vectors in *Banksia* communities at Cheyne Beach, western Australia. *Australian Journal of Botany* 28, 61-75.
- Lack, A., 1977. Genets feeding on nectar of *Maranthes polyandra* in northern Ghana. *East African Wildlife Journal* 15, 233-234.

- Lack, A., 1978. The ecology of the flowers of the savanna tree *Maranthes polyandra* and their visitors, with particular reference to bats. *Journal of Ecology* 66, 287-295.
- Rebelo, A.G., Breytenbach, G.J., 1987. Mammal pollination in the Cape Flora, in: Rebelo, A.G. (Ed.), *A Preliminary Synthesis of Pollination Biology in the Cape Flora*. South African National Scientific Programmes, Pretoria, pp. 109-125.
- Symes, C.T., Nicolson, S.W., 2008. Production of copious dilute nectar in the bird-pollinated African succulent *Aloe marlothii* (Asphodelaceae). *South African Journal of Botany* 74, 598-605.
- Wiens, D., Renfree, M., Wooller, R.O., 1979. Pollen loads of honey possums (*Tarsipes spenserae*) and nonflying mammal pollination in southwestern Australia. *Annals of the Missouri Botanical Garden* 66, 830-838.
- Wiens, D., Rourke, J.P., 1978. Rodent pollination in southern African *Protea* spp. *Nature* 276, 71-73.
- Wiens, D., Rourke, J.P., Casper, B.B., Rickart, E.A., Lapine, T.R., Peterson, C.J., 1983. Non-flying mammal pollination of southern African Proteas: a non-coevolved system. *Annals of the Missouri Botanical Garden* 70, 1-31.

5. Synthesis

5.1 Conclusion

Research on the reproductive biology and pollination ecology of *Protea* provides insights into the diversification of this genus and the ecological circumstances required for maintenance of the various species (Steenhuisen et al. 2012a). However, many suspected NMP *Protea* species have been inadequately studied (Steenhuisen and Johnson, 2012; Biccard and Midgley, 2009). In this thesis I conducted a comprehensive pollination and breeding system study on four species of *Protea* that conform to a non-flying mammal pollination system: *Protea cordata*, *P. decurrens*, *P. scabra*, and *P. subulifolia*. This involved determining pollinator effectiveness in terms of their influence on seed set, the functional traits that mediate plant-pollinator relations, and the requirement of these species for animal-facilitated pollen movement. In this concluding chapter, I will summarize the findings discussed in the previous chapters, and examine how camera technology, in conjunction with experimental evidence can further advance our understanding of the NMP system.

Pollination syndromes are generally evaluated on a subset of qualitative floral traits that represent pollinator-mediated selection (Waser et al. 1996). We have shown here that the *Protea* study species possessed traits that were indicative of a therophilous pollination syndrome, thereby enhancing the probability of pollen receipt by corresponding with foraging behavior and sensory preferences of rodent pollinators (Knight et al. 2005; Johnson & Steiner, 2000; Steenhuisen *et al.* 2013; Baker et al. 1998). One such trait was a consistently short distance between nectar and pollen (Chapter 3). This implies that pollen would be deposited on the head and fur of individuals, which would then be transferred to the stigmas of neighbouring plants during foraging events (Wiens et al. 1983). In contrast, the bird species observed foraging on *P. decurrens* and *P. scabra* (Orangebreasted sunbird) would be

ineffective pollinators as their beak length exceeds the distance between nectar and pollen, prohibiting pollen deposition on the body of individuals (Hargreaves and Johnson, 2004; Maclean, 1993).

While the use of motion sensor camera traps in validating pollination systems is a novel approach, they provided strong support for small mammal pollination of the study species, as rodent species were the most frequent visitors to flower heads (Chapter 2). While all of the study species received visits by at least three rodent pollinators, the frequency of these visits were not consistent among the study species (Chapter 2). For example, *Rhabdomys pumilio* was the dominant visitor to *P. decurrens* and *P. scabra*, *Mus minutoides* to *P. subulifolia*, and *Aethomys namaquensis* to *P. cordata* (Chapter 2). The frequent incidences of *R. pumilio* visits indicate that *P. decurrens* and *P. scabra* rely heavily on diurnal pollination.

Additionally, the data suggested that, although there was clear distinction between nocturnal and diurnal foraging events, there was no temporal subdivision of resources among nocturnal foragers.

The probability of rodent visits to the flower heads of the study species is a function of the floral traits that communicate potential rewards (Faegri and van der Pijl, 1979). However, signals produced by floral traits often represent a trade-off between being highly conspicuous to pollinators while remaining cryptic to herbivores and nectar thieves (Schaefer et al. 2004). Our results indicated that the study species produced large volumes of concentrated nectar that provided both nocturnal and diurnal small mammals with sugar-rich nectar supply (Chapter 3) (Fleming and Nicolson, 2002). While the qualitative plant traits were convergent with a NMP system, there were similarities in accumulated nectar properties between bird- and beetle-pollination systems (Chapter 3), implying that nectar traits of the study species may not deter foragers from different guilds.

My breeding system experiments indicated that while *Protea decurrens* was self-compatible neither *Protea cordata* nor *P. scabra* were capable of self-pollination (Chapter 3), yet rodent visitors to the study species were often observed probing several inflorescences on the same plant (Chapter 2). This can lead to geitonogamous self-pollen transfer, thereby negatively impacting plant mating success through reduced seed production, stigma clogging, and a reduction in siring success through pollen discounting (Mitchell et al. 2009). As such, the patterns of mammalian movement can ultimately affect the genetic structure of the population due to their impact on selfing rates (Goldingay et al. 1991; Mitchell et al. 2009).

The morphological traits of the study species and observations in the field indicated that rodents are the primary pollinators of *Protea cordata*, *P. decurrens* and *P. scabra* (Chapter 2). However, the results from the selective enclosure experiment suggested that insects contribute to the reproductive effort of these species since seed set was only marginally reduced when rodents were excluded (Chapter 3). The implication is that these species may have evolved a pollination system that is more generalized than their traits suggest (Fenster et al. 2004). This occurs because it is difficult for a plant to evolve morphological specializations that completely exclude any pollinators if they contribute toward the plants fitness (Aigner, 2001). Consequently, floral specialization for non-flying mammals does not restrict visitation by generalist insects.

5.2 Issues and future research

Several areas of concern were detected during the course of this study. While indirect evidence identified insects as pollinators of the study species, this could not be confirmed as we could not employ camera traps capable of directly recording invertebrate movement. Additionally, it is important to determine whether there is any insect-mediated selective pressure on the functional traits of the study species in order to resolve whether their

pollination systems are specialized for rodents or whether these species are ecologically generalized.

Additionally, while camera traps enabled us to quantify rates of visitation by observed visitors, we did not test the efficiency of this technique. Future studies would benefit from implementing measures to calibrate the findings of camera observations with real time observations. This can be achieved by comparing the number of times a camera picks up activity by captive rodents against the number of times captive rodents actually visit flower heads.

The floral scent and nectar of all four study species were sampled during the field season in order to determine the chemical profile of each floral scent and the sugar composition of nectar, but could not be quantified due to inadequate sampling methods and faulty equipment. These problems have been addressed and these data will be recollected in a subsequent field season and further investigations will be made into their functional significance for attracting pollinators. Insect preference for scent can also be determined by behavioral choice trials and manipulative field experiments in order to determine whether the floral traits of the study species attract insects (Steenhuisen et al. 2013).

5.3 Implications

This study has provided important insight into pollinator effectiveness, plant reproductive systems (Chapter 3) and pollinator behavior (Chapter 2) of four previously unstudied *Protea* species. Clarifying the pollination biology of species is an important component in informing conservation priorities of plant species that exist in small, localized populations, as they are more likely to be affected by anthropogenic activities and global change factors (Johnson and Steiner, 2000; Turner et al. 2011). Consequently, the dependence of plant species for pollen

vectors needs to be explored in order to assess the threat level that plant species face (Turner et al. 2011).

While *Protea decurrens* is self-compatible, both *P. cordata* and *P. scabra* are self-incompatible (Chapter 3), implying that the persistence of these species may be threatened by any habitat change that excludes pollinators from their landscape (Busch and Delph, 2011). However, these species have been shown to be successfully pollinated by both generalist rodent and insect foragers (Chapter 2; Chapter 3), which are less likely to be affected by habitat degradation than specialist pollinators (Wiens et al. 1983; Flemming and Nicolson, 2002; Chimimba and Bennet, 2005).

Analyzing functional traits in a phylogenetic context further advances our understanding of evolutionary processes underlying shifts in pollination systems within plant lineages (van der Niet and Johnson, 2012; Steenhuisen et al. 2012b). This study has provided data that can be mapped onto phylogenies in order to better understand evolutionary transitions in *Protea*, thereby adding to the growing database of knowledge for this genus.

5.4 References

- Aigner, P. A., 2001. Optimality modeling and fitness trade-offs: when should plants become pollinator specialists? *Nordic Society Oikos* 95, 177-184.
- Aizen, M. A., Ashworth, L., Galetto, L., 2002. Reproductive success in fragmented habitats: do compatibility systems and pollination specialization matter? *Journal of Vegetation Science* 13, 885-892.
- Baker, H. G., Baker, I., Hodges, S. A., 1998. Sugar composition of nectar and fruits consumed by birds and bats in the tropics and subtropics. *Biotropica* 30, 559–586.

- Biccard, A., Midgley, J. J., 2009. Rodent pollination in *Protea nana*. South African Journal of Botany, 75, 720-725.
- Busch, J. W., Delph, L. F., 2011. The relative importance of reproductive assurance and automatic selection as hypotheses for the evolution of self-fertilization. Annals of Botany 109, 553–562.
- Chimimba, C. T, Bennet, N. C, 2005. Order Rodentia, in: Skinner, J. D., Chimimba, C. T. (Eds.), The Mammals of the Southern African Subregion. Cambridge University Press, Cambridge, pp. 77-209.
- Faegri K., van der Pijl L., 1979. The Principles of Pollination Ecology. Third revision. Pergamon Press, Oxford.
- Fenster, C. B., Armbruster, W. S., Wilson, P., Dudash, M. R., Thomson, J. D., 2004. Pollination Syndromes and Floral Specialization. Annual Review of Ecology, Evolution, and Systematics 35, 375–403.
- Fleming, P., Nicolson, S., 2002. How important is the relationship between *Protea humiflora* (Proteaceae) and its non-flying mammal pollinators? Oecologia 132(3), 361-368.
- Goldingay, R. L., Carthew, S. M., Whelan, R. J., 1991. The importance of non-flying mammals in pollination. Oikos 61, 79–87.
- Hargreaves, A. L., Johnson S. D., Nol E., 2004. Do floral syndromes predict specialization in plant pollination systems? An experimental test in an ‘ornithophilous’ African *Protea*. Oecologia 140: 295–301.
- Johnson, S. D., Steiner, K. E., 2000. Generalization versus specialization in plant pollination systems. Trends in Ecology and Evolution 15, 140-143.

- Knight, T. M., Steets, J. A., Vamosi, S. J., Campbell, D. R., Dudash, M. R., Johnston, M. O., Mitchell, R. J., 2005. Pollen limitation of plant reproduction: pattern and process. *Annual Review of Ecology Evolution and Systematics* 36, 467–497.
- Maclean, G. L., 1993. Roberts' birds of southern Africa. John Voelcker Bird Book Fund. Cape Town.
- Mitchell, R. J., Flanagan, R. J., Brown, B. J., Waser, N. M., Karron, J. D., 2009. New frontiers in competition for pollination. *Annals of Botany* 103, 1403–1413.
- Schaefer, H. M., Schaefer, V., Levey, D. J., 2004. How plant–animal interactions signal new insights in communication. *Trends in Ecology and Evolution* 19, 577–584.
- Steenhuisen, S.-L., Johnson, S. D., 2012. Evidence for autonomous selfing in grassland *Protea* species (Proteaceae). *Plant Systematics and Evolution* 298, 857–869.
- Steenhuisen, S.-L., Van der Bank, H., Johnson, S. D., 2012a. The relative contributions of insect and bird pollinators to outcrossing in an African *Protea* (Proteaceae). *American Journal of Botany* 99, 1104–1111.
- Steenhuisen, S.-L., Raguso R. A., Johnson, S. D., 2012b. Floral scent in bird- and beetle-pollinated *Protea* species (Proteaceae): Chemistry, emission rates and function. *Phytochemistry* 84, 78–87.
- Steenhuisen, S.-L., Jürgens, A., Johnson, S. D., 2013. Effects of Volatile Compounds Emitted by *Protea* Species (Proteaceae) on Antennal Electrophysiological Responses and Attraction of Cetoniine Beetles. *Journal of chemical ecology*.

- Turner, R. C., Midgley, J. J., Johnson, S. D., 2011. Evidence for rodent pollination in *Erica hanekomii* (Ericaceae). *Botanical Journal of the Linnean Society* 166, 163–170.
- Waser, N. M., Chittka, L., Price, M. V., Williams, N. M., Ollerton, J., 1996. Generalization in pollination systems, and why it matters. *Ecology* 77, 1043-1060.
- Van der Niet, T., Johnson, S. D., 2012. Phylogenetic evidence for pollinator-driven diversification of angiosperms. *Trends in Ecology and Evolution* 27, 353-361.
- Wiens, D., Rourke, J. P., Casper, B. B., Rickart, E. A., LaPine, T. R., Peterson, C. J., Channing, A. 1983. Nonflying mammal pollination of southern African *Proteas*: a non-coevolved system. *Annals of the Missouri Botanical Garden* 70, 1-31.

Appendix

Table 1: accumulated nectar production and nectar concentration from the literature (Balmer, 2012; Steenhuisen and Johnson, 2012c) and my study species

Pollination system	<i>Protea</i> species	Nectar production (ul)	Nectar concentration (%)
NMP	<i>P. pendula</i>	3.53	25.18
NMP	<i>P. recondita</i>	6.5	24.16
NMP	<i>P. sulphurea</i>	6.44	21.2
NMP	<i>P. namaquana</i>	9.17	17.12
NMP	<i>P. humiflora</i>	9.62	34.1
BIRD	<i>P. nitida</i>	12.05	14.85
BIRD	<i>P. obtusifolia</i>	6.75	30.89
BIRD	<i>P. roupelliae</i>	13.15	13.06
Beetle	<i>P. caffra</i>	8.34	4.39
Beetle	<i>P. dracomontana</i>	8.69	4.66
Beetle	<i>P. simplex</i>	4.18	6.34
Study Species	<i>P. cordata</i>	4.9	14.86
Study Species	<i>P. scabra</i>	6.2	11.59
Study Species	<i>P. decurrens</i>	3.59	16.31
Study Species	<i>P. subulifolia</i>	5.51	15.73

Table 2: standing crop nectar production and concentration of ten bird-pollinated *Protea* species from Schmid (unpublished data) and my study species

Pollination system	<i>Protea</i> species	Nectar production (ul)	Nectar concentration (%)
Bird	<i>P. compacta</i>	19.1	0.57
Bird	<i>P. cynaroides</i>	7.53	14.01
Bird	<i>P. eximia</i>	23.22	3.11
Bird	<i>P. laurifolia</i>	19.1	3.45
Bird	<i>P. longifolia</i>	11.8	6.58
Bird	<i>P. magnifica</i>	24.02	2.89
Bird	<i>P. neriifolia</i>	15.94	5.39
Bird	<i>P. nitida</i>	18.44	0.76
Bird	<i>P. punctata</i>	7.33	0.35
Bird	<i>P. repens</i>	15.48	5.71
Study species	<i>P. cordata</i>	4.23	13.05
Study species	<i>P. scabra</i>	5.12	23.75
Study species	<i>P. decurrens</i>	1.56	30.49
Study species	<i>P. subulifolia</i>	2.49	37.75

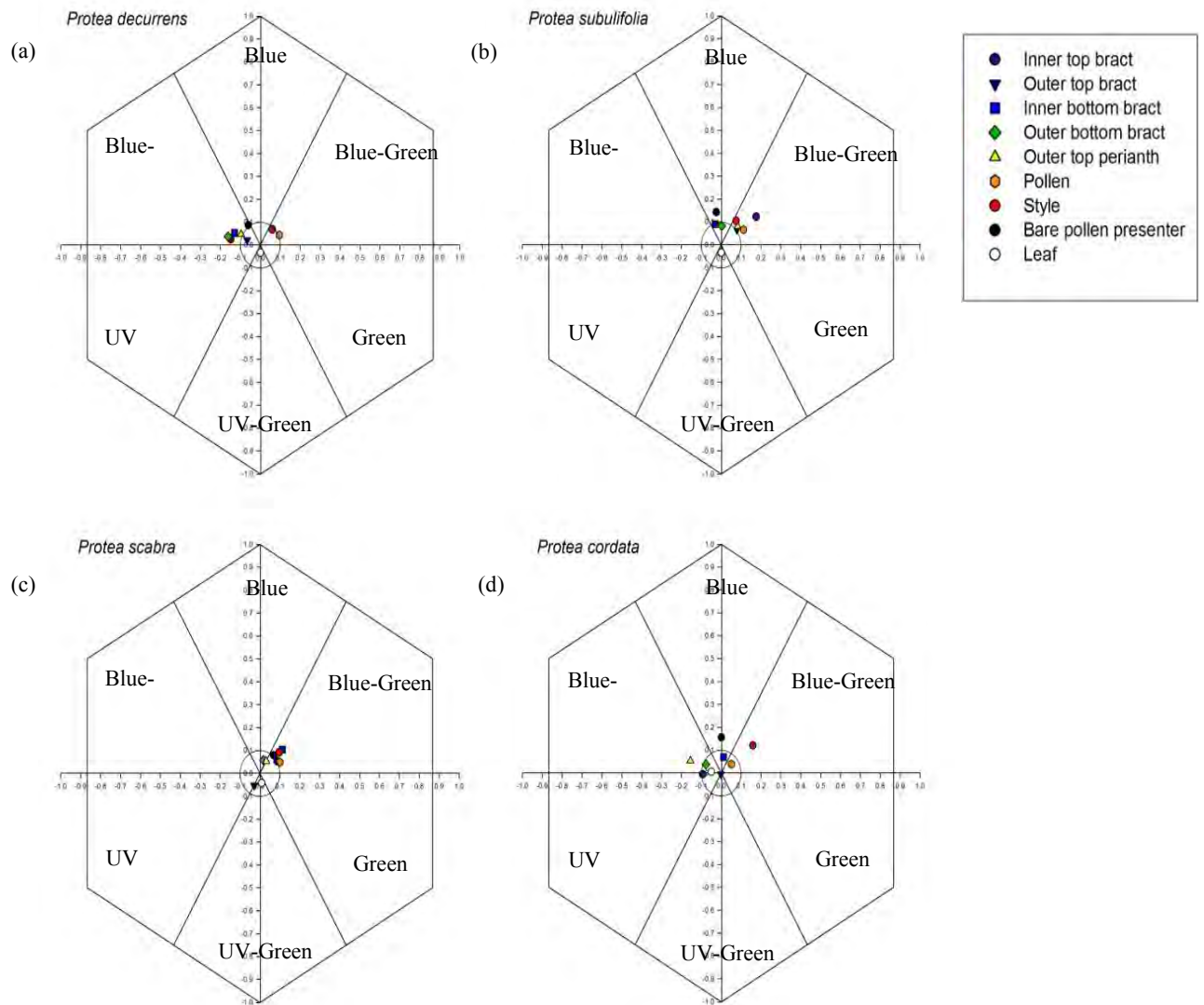


Figure 1: Bee hexagon indicating reflectance measurements of eight floral traits for (a) *Protea decurrens*, (b) *P. scabra*, (c) *P. subulifolia* and (d) *P. cordata*. Measurements that fall within the center circle of the hexagon are not distinguishable from the background for Hymenoptera, and those clustered around it are difficult to distinguish.

The bee hexagon models indicated that many of the floral traits, particularly leaves, are not perceptible to bees as they are indistinguishable from the background (Fig. 1). There was very little consistency in the reflectance of floral traits among the study species. For example, the style and perianth could only be perceived for *P. cordata* (Fig. 1). Additionally, bracts exhibited high variation in how they are perceived by bees. The inner and outer bracts of

Protea decurrens were visible in the blue-UV spectrum (Fig. 1), although the top of the bracts of *P. subulifolia* (Fig. 1c) and the inner bottom bract of *P. scabra* (Fig. 1c) were perceived in the blue-green spectrum.