Floral evolution of long-tubed *Erica* species

Thesis submitted for the degree of Doctor of Philosophy at the University of Cape Town

December 2022

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Statement

The ideas and writing of this thesis were entirely my own, except in the instances mentioned below and in the acknowledgements at the end of every chapter. Chapter 5 was developed from an idea by my co-supervisor Steve Johnson, but I carried out all field work and analyses. For chapter 3 I collaborated with Ross Turner who collected data for it in 2012 and Genevieve Theron who identified the flies and provided helpful discussions on fly biology.

Three of my chapters were adapted from papers which were co-authored by my supervisor Jeremy Midgley. My co-supervisor Anina Coetzee also co-authored chapters 1 and 2, while my other co-supervisor Steve Johnson also co-authored chapter 4. All co-authors helped to improve existing manuscripts through a process of discussion and suggestion.

I confirm that I have been granted permission by the University of Cape Town's Doctoral Degrees Board to include the following publications in my PhD thesis, and where co-authorships are involved, my co-authors have agreed that I may include the publications:

1. McCarren, S., Midgley, J.J. & Coetzee, A. 2021. Sending Private Messages: Floral Ultraviolet Signals Are Associated with Pollination Syndromes in *Erica*. *Journal of Pollination Ecology*. 29:289–298. DOI: 10.26786/1920-7603(2021)648.
2. McCarren, S., Coetzee, A. & Midgley, J. 2021. Corolla stickiness prevents nectar robbing in *Erica*. *Journal of Plant Research*. 134(5):963–970. DOI: 10.1007/s10265-021-01299-z.
3. McCarren, S., Midgley, J.J. & Johnson, S.D. 2022. Biomechanics of nectar feeding explain flower orientation in plants pollinated by long-proboscid flies. *Science of Nature*. 109(47). DOI: 10.1007/s00114-022-01817-6.

16/12/2022

Acknowledgements

I want to thank my supervisor, Jeremy Midgley, who has been the best supervisor I could have asked for. He sparked my passion for plants and kindled it to the point of converting me into a botanist. It has been a pleasure to bounce ideas off him and he has taught me to think around the corner.
I am also grateful to my co-supervisors Anina Coetzee and Steve Johnson who have helped me immensely with my field work, the analysis of my data and pushing the quality of my work to international standards.
I also want to thank Betty Ann Illing for her hospitality, letting me stay with her on my numerous field trips to Hermanus and looking after my dogs while I am on top of some mountain.
Many landowners have allowed me access to their land, and I am particularly grateful to Vogelgat and Giorgio Lombardi, Thys de Villiers, Anthony van Hoogstraaten, Cape Nature and SANPARKS.
I am also very lucky to have a mother who has encouraged my love of nature since I was a little child and who is my biggest fan to this day.
Financially, this project was supported by funding from the National Research Foundation.

Abstract

The genus *Erica* has undergone an extreme radiation in the Cape and exhibits a diversity of pollination syndromes and floral traits. This makes *Erica* well-suited to study the evolution of floral traits and how they impact speciation.

The first chapter explored the role of ultraviolet colouration by recording its prevalence across *Erica* pollination syndromes. Ultraviolet was rare in wind-, rodent and small insect-pollinated species, but it was common in bird-pollinated species and ubiquitous in long-proboscid fly (LPF)-pollinated species. Testing their preference revealed that sunbirds can see ultraviolet, but they have no innate preference. LPFs on the other hand were not attracted to flowers where ultraviolet reflectance was removed, thus displaying a strong preference.

Chapter 2 focused on the role of stickiness for nectar robbers. I experimentally added stickiness to *Erica* flowers of one species and further compared stickiness to nectar robbing across several communities. Stickiness appears to reduce damage due to nectar robbing within and between species. Further, I found that stickiness is strongly correlated with pollination by birds and LPFs which might be due to their large nectar rewards.

Chapter 3 investigated how the sister species *Erica shannonea* and *Erica ampullacea* co-occur despite sharing a pollination syndrome. Pollination experiments and observations showed that they are pollinated by LPFs from two families. The horizontal flowers of *E. shannonea* are pollinated by a tabanid which has a fixed forward-pointing proboscis, while the vertical flowers of *E. ampullacea* are pollinated by a nemestrinid which can swivel its proboscis downwards. The nemestrinid in turn has a shorter proboscis which prevents it from accessing nectar in the long-tubed *E. shannonea*. Due to their different biomechanics, each fly can only access the flower it pollinates resulting in effective reproductive isolation between these species.

Chapter 4 compared flower orientation in relation to the two LPF families across all LPF-pollinated species. Using a phylogenetically corrected analysis, I found that flowers pollinated by Tabanidae tend to be horizontal, while nemestrinid flowers are more variable in orientation and more often vertical. This confirms the importance of pollinator biomechanics for the evolution of floral traits.

The last chapter investigated how pollen transfer efficiency differs between *Erica* pollination syndromes. I found that LPF- and bird-pollinated species have higher pollen transfer efficiency in comparison to bee-pollinated species which might have facilitated the shifts from ancestral bee pollination.

Introduction

The Cape Floristic Region in South Africa is one of the six floral kingdoms. With its over 9,000 species of vascular plants, it is an area of extraordinarily high diversity and endemism. About 69% of the plants are endemic and many genera show high rates of speciation. The genus *Erica* originated in Europe and colonized the Cape of South Africa about 15 million years ago (Pirie et al., 2016). While there are only 19 extant species in Europe, the genus has undergone an extreme radiation in the Cape leading to ca. 700 extant Cape *Erica* species that share a single ancestor (Pirie et al., 2016). The species diversity might even still be underestimated as there are numerous taxonomic challenges and species complexes that require in-depth phylogenetic assessment (Pirie et al., 2017). This makes *Erica* one of the most species rich plant genera not only in the Cape but globally and raises the question of how and why it radiated so spectacularly.

Erica flowers have a superior ovary, are radially symmetrical and have four petals which are fused into a corolla tube. They typically have eight anthers which are fused into an anther ring and a single style which protrudes beyond the anthers (Palser and Murty, 1967). It has been hypothesized that the radiation of the genus *Erica* has mainly been pollinator-driven due to the diversity of pollination syndromes that have been recorded. Their pollinators include bees, small flies, wind, rodents, moths, birds and long-proboscid flies (LPFs) (Rebelo, Siegfried & Oliver, 1985; Turner, Midgley & Johnson, 2011; Turner et al., 2012; van der Niet et al., 2014; Bouman, Steenhuisen & van der Niet, 2017; Lombardi et al., 2017, 2021; van der Niet & Cozien, 2022). Concurrently, a multitude of differences in floral traits has evolved such as corolla length and shape, anther exsertion, colour, orientation, and stickiness, most of which have occurred in multiple lineages independently (Pirie, Oliver & Bellstedt, 2011). These different pollinators and floral traits are expected to confer different costs and benefits and trade-offs between them might have facilitated the pollinator shifts. Measuring the costs and benefits, e.g., in form of pollen transfer efficiency could thus shed light on why there is such a big diversity in the genus *Erica*.

Many pollinators for *Erica* species have not been confirmed by observations, but have only been inferred based on flower traits (Rebelo et al., 1985) according to the concept of pollination syndromes (Faegri and van der Pijl, 1966). While this approach has been successful at predicting the pollinators of most species, it has been criticised as it is not always accurate (van der Niet, 2021). Due to a lack of data on many species, it is still widely used in the study of plant-pollinator interactions and also in this thesis, however where possible, additional observations were done to confirm the syndrome prediction.

Long-tubed *Erica* species are typically pollinated by birds or LPFs. There are at least 67 bird- and 30 LPF-pollinated species found in the Cape (Rebelo, Siegfried & Oliver, 1985; Turner et al., 2012; Lombardi et al., 2021; Newman & Johnson, 2021; Pauw, 2022). The bird-pollinated species almost all share the same pollinator, the Orange-breasted sunbird (*Anthobaphes violacea*) (Rebelo & Siegfried, 1985), which has led to research on how those species coexist (Heystek & Pauw, 2014; Coetzee, Spottiswoode & Seymour, 2020). On the other hand, there has been very little research on LPF pollination in *Erica* (Lombardi et al., 2021).

Pollinators can select for different flower shapes and accordingly, corolla length in long-tubed *Erica* species is associated with the beak or proboscis length of the pollinator (Rebelo & Siegfried, 1985; Newman & Johnson, 2021). Further, flower shape modularity in *Erica* differs between pollinators. In species pollinated by bees, small flies and wind selection for flower shape depends on developmental modules, while in bird- and LPF-pollinated species the modules are linked to function: pollen deposition and receipt for bird-pollinated species, and access-restriction to the floral reward in LPF-pollinated species (Reich et al., 2020). Additionally to corolla shape, *Erica* flowers also differ in shape by having exserted or included anthers and it is expected that anther exsertion confers a benefit in order for it to have evolved.

Floral colour plays an important role in pollinator specificity and changes in colour can result in pollinator shifts (Bradshaw & Schemske, 2003). The ancestral flower colour in the genus *Erica* is pink, but a huge variety of colours including white, red, orange, yellow, green, and even black have evolved in the Cape, with some species combining several flower colours or displaying colour polymorphisms (Oliver, 1975). There are multiple independent genetic mutations that have led to colour shifts in *Erica* flowers allowing for rapid adaptation (Le Maitre, Pirie & Bellstedt, 2019), and thus potentially facilitating pollinator shifts and species divergence. Ultraviolet (UV) colouration has not been studied in *Erica* yet, but it has been hypothesised that UV might play a role for some of the species (Rebelo & Siegfried, 1985).

Erica species differ in many further traits that are associated with pollination syndrome. These include scent (van der Niet et al., 2014; Bouman, Steenhuisen & van der Niet, 2017; van der Niet & Cozien, 2022), nectar volume and concentration (Nicolson, 2002; Heystek et al., 2014; Bouman, Steenhuisen & van der Niet, 2017; Newman & Johnson, 2021), and stem thickness. Scent and nectar properties are likely to have been selected for due to pollinator attraction, while thicker stems were selected for in bird-pollinated species since they can support the increased body weight (Siegfried, Rebelo & Prŷs-Jones, 1985). Further, it has been observed that *Erica* species differ in flower

orientation and that different pollinators are associated with certain flower angles (van der Niet & Cozien, 2022). Differences in this trait are expected to match pollinator biomechanics.

Floral traits are thought to evolve primarily in response to selection by pollinators, but selection can also be mediated by other environmental factors (Caruso et al., 2019). These can include biotic interactions with herbivores and nectar robbers. Especially in bird-pollinated *Erica* species, nectar robbing appears to be a frequent occurrence (Heystek et al., 2014) and it is expected that nectar robbers exsert selection on some flower traits. These could include traits like certain colourations that are less visible to nectar robbers or physical barriers to prevent them from accessing the nectar, like e.g., floral stickiness that some species produce in arrays of sessile glands located in the adaxial surface of sepals (Oliver & Oliver, 2002).

Abiotic factors like fire regime or water limitation might also play a role in shaping floral traits. *Erica* species employ different post-fire strategies, resprouting and seeding, which are linked to differences in flower colour, flowering phenology, and anther exsertion (Ojeda et al., 2019). Differences in water limitation between sites and seasons might also cause changes in flower traits like phenology (Rebelo, Siegfried & Crowe, 1984) or corolla stickiness as a way to limit transpiration (Vlok & Schutte-Vlok, 2003). While these traits appear to be selected for by abiotic factors, any changes in flower traits can also affect pollination and thereby impact speciation or co-existence.

The aim of this thesis is to further the understanding of floral evolution focusing on long-tubed *Erica* species that are typically pollinated by birds and LPFs by studying (1) the role of UV colouration for pollination, (2) the role of flower stickiness for antagonistic nectar robbers, (3) flower orientation as a reproductive barrier, (4) the role of pollinator biomechanics for flower orientation, (5) and differences in pollen transfer efficiency between pollination syndromes.

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Chapter 1: Sending private messages: Floral ultraviolet signals are associated with pollination syndromes in *Erica*

Abstract

The presence of ultraviolet (UV, wavelengths between 300-400 nm) reflectance in insect-pollinated flowers has been linked to pollination efficiency and pollination shifts, but little is known about its prevalence and function in other pollination systems and African species. We chose the genus *Erica* for studying the prevalence of UV because of its extreme radiation (c. 680 species) in the Cape, South Africa, with a diversity of pollination syndromes. This study quantified the prevalence and brightness of UV reflectance for five *Erica* pollination syndromes and tested pollinator preferences for UV reflectance in the two groups with the highest prevalence: sunbirds and long-proboscid flies. Our results show that UV colouration is absent or rare in *Erica* species pollinated by unclassified insects, rodents, or wind. About 17 % of bird-pollinated species reflected UV but choice experiments revealed that free-ranging sunbirds showed no preference for UV signals. All sampled long-proboscid fly-pollinated species reflected UV and its experimental removal decreased seed set drastically, suggesting that long-proboscid flies in the Cape strongly prefer or depend on UV and thereby contributed to selecting for the evolution of this signal.

Introduction

Colour is one of the most important flower advertisements for pollinators. Consequently, pollinators have contributed greatly to the large diversity of flower colours in angiosperms, which evoke specific behavioural responses in different flower visitors due to the differences in their colour vision systems and neural processing (Junker et al., 2013). Although many pollinators are able to see ultra-violet reflectance (UV, wavelengths between 300-400 nm; Shrestha, Dyer & Burd, 2016), it has only been studied for a few species in the Cape Floristic Region (e.g., Peter et al. 2004; Peter & Johnson 2008; Welsford & Johnson 2012). Plants in the Cape might benefit from using UV reflectance to be more conspicuous to their preferred pollinators (Chen et al., 2020). On the other hand, UV absorbing compounds protect plants against damage through UV-B radiation, thus UV reflecting flowers are left vulnerable (Llorens et al., 2015) and this signal should only evolve if increased pollination services offset the fitness costs incurred.

The mega-diverse genus *Erica* (c. 680 species in the Cape, South Africa) is highly suitable for studying this because of its diversity of flower colours and pollinators (Rebelo, Siegfried & Oliver, 1985). The species can be grouped into five pollination syndromes: insect-, bird-, long-proboscid fly- (LPF), rodent- and wind-pollinated (Rebelo, Siegfried & Oliver, 1985; Turner, Midgley & Johnson, 2011; Lombardi et al., 2017). Since these species share an evolutionary history, a comparison of the prevalence of UV reflectance between pollination syndromes could indicate the role of pollinators in selecting for UV signals. Differences in colour vision and behavioural responses between the pollinators might have selected for different signals amongst the pollination syndromes and thereby contributed to reproductive isolation and species divergence (e.g., Streisfeld, Young & Sobel, 2013).

There has been little research on the pollination of specific *Erica* species grouped into the insect-pollinated syndrome, thus there might be a variety of functional groups pollinating them (van der Niet, 2021). However, there seems to be a large number of bee-pollinated species (pers. obs., Bouman, Steenhuisen & van der Niet, 2017). Hymenopterans in general are able to perceive UV but mostly have lower discrimination abilities in the UV area of the spectrum (Peitsch et al., 1992). Thus, we expect little UV reflection for species with this pollination syndrome.

LPF-pollinated *Erica* flowers can be distinguished based on their long ampullaceous corollas with narrow openings and spreading lobes and therefore are the most discrete functional group within the insect-pollinated species (Rebelo et al. 1985; Lombardi et al. 2021; Newman & Johnson 2021). There are no studies on UV vision in LPFs, specifically, but other flies have been recorded to see UV (Troje, 1993). Fly-pollinated flowers have been shown to differ significantly in their colouration from bee-pollinated flowers (Shrestha et al., 2019) but different species of flower-visiting flies also differ greatly in colour preferences amongst each other (Lunau, 2014), thus making it difficult to predict the prevalence of UV in LPF-pollinated flowers.

It has been shown that African nectarivorous birds (sunbirds and sugarbirds, Nectariniidae and Promeropidae, respectively) have genes for the receptors that enable them to perceive UV reflectance (Ödeen & Håstad, 2010). Their behavioural response to UV, however, has not been tested yet. Bird-pollinated *Erica* species can be recognised by their long corollas, absence of floral scent and large nectar volume (van der Niet et al., 2014). Many bird-pollinated flowers around the world are red, which has been attributed to this colour being less conspicuous to bees that lack a photoreceptor for long wavelengths (Rodríguez-Gironés & Santamaría, 2004). By being less conspicuous to bees, bird-pollinated flowers can avoid being visited by these less efficient pollinators or nectar robbers (de Camargo et al., 2019). Due to bees having less discrimination ability in the UV range, bird-pollinated species may also make use of short-wavelength cues to attract their

pollinators and to be less conspicuous to bees (Lunau et al., 2011; Shrestha et al., 2013). Thus, we expect a higher prevalence of UV for bird-pollinated species than for bee-pollinated species.

Rodents possess photoreceptors that are sensitive to UV light (Jacobs, Neitz & Deegan, 1991), but since the rodents pollinating *Erica* species are nocturnal (Turner, Midgley & Johnson, 2011; Lombardi et al., 2017) we do not expect them to be attracted by visual cues. Thus, we do not predict rodent-pollinated *Erica* species to reflect UV. Wind-pollinated species, too, are not expected to experience any selection for UV reflectance.

This study aims to (a) quantify the prevalence and brightness of UV reflectance in *Erica* across different pollination syndromes, and (b) test pollinator preference in the groups with the highest prevalence since this may have been the mechanism that selected for the signal. We expect higher prevalence of UV reflectance in flowers pollinated by UV perceiving animals. Additionally, if certain pollinators have driven the evolution of UV signals, we expect them to show a preference for UV colouration.

Methods

UV prevalence across pollination syndromes

To quantify the prevalence of UV reflectance, flower reflectance was measured in 125 *Erica* species collected in the Cape Floristic Region, South Africa (Table S1, Table S2). For each species, the reflectance of three to five flowers was measured using a spectrophotometer (Jazz model with PX-2 Pulsed Xenon light source, Ocean Optics, Dunedin, FL) and then averaged. For the species with two-coloured flowers, both colours were measured separately and for 11 of the species, different colour morphotypes were measured. For each reflectance spectrum, the lowest reflectance value was added to the reflectance at all wavelengths to correct negative values in the spectra, whereafter the spectra were smoothed (smoothing parameter = 0.2), averaged and analysed using the R package pavo (Maia et al., 2019). The contribution of UV (300-400 nm) to total brightness (the sum of all reflectance values between 300-700 nm) was recorded as a percentage of the total reflectance between 300-700 nm. Additionally, it was recorded to which pollination syndrome the species belongs (bird, LPF, unclassified insect, wind, rodent; (Rebelo, Siegfried & Oliver, 1985; Turner, Midgley & Johnson, 2011; Lombardi et al., 2017). Variation in UV reflectance between pollination syndromes was analysed using a generalised linear model with quasipoisson error structure followed by a Tukey post-hoc test to identify the differences between the groups.

Table S1 *Erica* species sampled with contribution of UV reflectance to total brightness (> 10 % in bold), visible colour according to human vision, pollination syndrome and collection site. LPF = Long-proboscid fly. Location abbreviations match those in Table S2.

Erica species	Contribution of UV to total brightness	Visible colour	Pollinator	Location
abietina abietina	**0.15**	**red**	**bird**	**KP**
abietina atrorosea	**0.11**	**pink**	**bird**	**CP**
abietina aurantiaca	0.09	red	bird	SG
abietina perfoliosa	**0.10**	**yellow**	**bird**	SG
amoena	0.02	pink	insect	AB
ampullacea	**0.17**	**light pink**	**LPF**	**GR**
aneimena	0.05	pink	insect	KB
aristata	**0.25**	**light pink**	**LPF**	**VG**
articularis	0.03	pink	insect	VG
axillaris	0.01	green	wind	AB
azaleifolia	0.01	white	insect	AB
baccans	0.04	pink	insect	KB
barbigeroides	0.06	pink	insect	GR
baueri	0.06	white	bird	KB
baueri	0.02	pink	bird	KB
bergiana	0.04	pink	insect	BK
blandfordii	0.05	yellow	insect	KB
blenna	**0.11**	**orange**	**bird**	**KB**
brachialis	0.08	green	bird	KB
brevifolia	0.04	pink	insect	AB
bruniades	0.01	pink	insect	BB
caffra	0.04	white	insect	SM
calycina	0.05	pink	insect	KB
capensis	0.04	white	insect	KB
caterviflora	0.04	pink	insect	AB
ceraria	0.08	green	bird	KB
coccinea	0.02	yellow	bird	VG
coccinea	0.02	red	bird	CP
collina	0.01	pink	insect	HA
copiosa	0.08	light pink	wind	KB
corifolia	0.07	pink	insect	SB
cristata	**0.11**	**pink**	**LPF**	**VG**
croceovirens	0.02	orange	bird	KB
croceovirens	0.07	green	bird	KB
cruenta	**0.11**	**red**	**bird**	**KB**
cumuliflora	0.02	white	insect	JH
curviflora	0.04	pink	bird	SM
curviflora	0.04	orange	bird	GB
curviflora	0.02	yellow	bird	GB
curvirostris	0.07	white	insect	KP
curvistyla	0.07	white	insect	CB
denticulata	**0.12**	**pink**	**insect**	**BK**

Table S1 continued *Erica* species sampled with contribution of UV reflectance to total brightness (> 10 % in bold), visible colour according to human vision, pollination syndrome and collection site. LPF = Long-proboscid fly. Location abbreviations match those in Table S2.

Erica species	Contribution of UV to total brightness	Visible colour	Pollinator	Location
diaphana	0.07	purple	bird	KB
discolor	0.03	red	bird	SM
discolor	0.05	green	bird	SM
distorta	0.06	pink	insect	BK
elimensis	0.07	pink	insect	HA
ericoides	0.03	pink	insect	CP
exleeana	0.02	white	wind	AB
fascicularis	0.07	pink	bird	PB
fascicularis	0.06	green	bird	PB
fastigiata coventryi	**0.16**	**light pink**	**LPF**	**VG**
fastigiata fastigiata	**0.15**	**light pink**	**LPF**	**JH**
fontana	0.06	pink	bird	VG
formosa	0.06	white	insect	KB
fuscescens	0.02	white	insect	KB
gibbosa	0.06	light pink	insect	KB
glabella laevis	0.02	pink	insect	VG
glandulosa glandulosa	**0.15**	**pink**	**bird**	**SM**
glandulosa fourcadei	0.09	red	bird	KB
glauca	0.09	red	bird	KB
glomiflora	0.06	white	insect	KB
glomiflora	0.07	pink	insect	KB
grandiflora	0.07	yellow	bird	JH
grandiflora	**0.11**	**orange**	**bird**	**PM**
haematocodon	**0.12**	**red**	**insect**	**KB**
halicacaba	0.07	green	bird	KB
heleophila	0.02	white	insect	KB
hirtiflora	0.07	pink	insect	SM
hispidula	0.06	light pink	wind	GB
holoserica	0.03	pink	insect	VG
imbricata	0.03	white	insect	CP
intermedia	0.03	yellow	insect	KB
intervallaris	0.03	pink	insect	JH
irbyana	**0.13**	**pink**	**LPF**	**GR**
irregularis	0.06	pink	insect	GR
jasminiflora	**0.12**	**light pink**	**LPF**	**HA**
laeta	0.02	pink	insect	SB
lanuginosa	0.02	red	rodent	VG
lanuginosa	0.03	white	rodent	VG
lateralis	0.05	pink	insect	BK
leptopus	0.05	white	insect	KB
leucotrachela	**0.12**	**white**	**bird**	KB
leucotrachela	0.06	red	bird	KB

Table S1 continued *Erica* species sampled with contribution of UV reflectance to total brightness (> 10 % in bold), visible colour according to human vision, pollination syndrome and collection site. LPF = Long-proboscid fly. Location abbreviations match those in Table S2.

Erica species	Contribution of UV to total brightness	Visible colour	Pollinator	Location
limosa	0.05	red	insect	CB
lutea	0.05	white	insect	KB
macowanii lanceolata	0.07	white	bird	VG
macowanii lanceolata	0.01	purple	bird	VG
mammosa	0.03	white	bird	SB
mammosa	0.03	pink	bird	KB
mammosa	0.02	light pink	bird	SB
margaritacea	0.03	white	insect	KB
mauritanica	0.04	pink	insect	KB
melanthera	0.06	pink	insect	KB
melastoma minor	0.01	green	bird	VG
melastoma minor	0.04	black	bird	VG
mollis	0.06	pink	insect	CB
monsoniana	0.03	white	insect	KB
multumbellifera	0.07	purple	insect	SB
muscosa	0.06	white	wind	KP
nana	0.09	yellow	bird	KB
nudiflora	0.06	pink	insect	KP
obliqua	0.03	pink	insect	FK
obtusata	0.06	pink	insect	AB
palliiflora	0.02	pink	insect	CB
parviflora	0.03	pink	insect	VG
patersonii	**0.10**	**yellow**	**bird**	**BB**
pauciovulata	0.06	pink	insect	AB
penicilliformis	0.05	white	insect	KB
perspicua	0.04	white	bird	HH
perspicua	0.03	purple	bird	HH
physodes	0.07	white	insect	KB
pinea	**0.11**	**yellow**	**bird**	**BK**
placentiflora	0.02	purple	insect	VG
plena	0.04	white	insect	AB
plukenetii lineata	0.02	red	bird	PO
plukenetii plukenetii	0.07	pink	bird	PO
plukenetii plukenetii	0.02	red	bird	BK
pulchella	0.07	pink	insect	SB
recurvata	0.04	white	bird	KB
regia mariae	0.09	red	bird	PO
salax	0.06	white	wind	JH
sessiliflora	0.06	green	bird	VG
shannonea	**0.12**	**light pink**	**LPF**	**AB**
sitiens	0.05	pink	insect	KB
sparmannii	0.08	green	bird	KB

Table S1 continued *Erica* species sampled with contribution of UV reflectance to total brightness (> 10 % in bold), visible colour according to human vision, pollination syndrome and collection site. LPF = Long-proboscid fly. Location abbreviations match those in Table S2.

Erica species	Contribution of UV to total brightness	Visible colour	Pollinator	Location
sparsa	0.06	pink	insect	KB
spumosa	0.03	pink	insect	AB
tenella	0.03	white	insect	AB
tenella	0.08	pink	insect	AB
totta	0.07	white	insect	KB
triflora	0.04	white	insect	JH
triste	0.00	white	wind	SB
umbrosa	0.05	pink	insect	JH
unicolor	0.02	green	bird	KB
verecunda	0.02	light pink	insect	GB
verticillata	0.06	pink	bird	KB
vestita	**0.13**	**pink**	**bird**	**GR**
vestita	**0.13**	**red**	**bird**	**GR**
villosa	0.03	white	insect	VG
viridiflora	0.02	green	bird	KB
viscaria longiflora	**0.13**	**orange**	**bird**	**JH**
viscaria longiflora	**0.11**	**red**	**bird**	**JH**
viscaria longiflora	0.09	purple	bird	JH
viscaria longiflora	0.05	pink	bird	JH
viscaria macrosepala	0.05	green	bird	VG
viscaria pendula	0.02	white	bird	HH

Table S2 Locations and GPS coordinates at which *Erica* species were sampled

Location	Abbreviation	Coordinates
Akkedisberg	AB	34.417°S, 19.668°E
Bainskloof Pass	BK	33.580°S, 19.135°E
Betty's Bay	BB	34.354°S, 18.920°E
Cape Point	CP	34.266°S, 18.463°E
Cedarberg	CB	32.350°S, 19.150°E
Fernkloof Nature Reserve	FK	34.394°S, 19.265°E
Gifberg	GB	30.890°S, 18.676°E
Grootbos Private Nature Reserve	GR	34.545°S, 19.425°E
Hemel and Aarde Valley	HA	34.363°S, 19.351°E
Hottentots-Holland Mountain Catchment Area	HH	33.971°S, 18.899°E
Jonkershoek Nature Reserve	JH	33.971°S, 18.997°E
Kasteelspoort Hiking Trail	KP	33.965°S, 18.389°E
Kirstenbosch National Botanical Garden	KB	33.990°S, 18.428°E
Paarl Mountain Nature Reserve	PM	33.737°S, 18.936°E
Perdeberg	PB	34.307°S, 18.990°E
Potberg	PO	34.372°S, 20.564°E
Scarborough	SB	34.179°S, 18.390°E
Silvermine	SM	34.074°S, 18.398°E
Stellenbosch University Botanical Garden	SG	33.936°S, 18.866°E
Vogelgat Private Nature Reserve	VG	34.391°S, 19.315°E

UV preference in sunbirds

To test if sunbirds exhibit a preference for flowers which reflect UV, choice experiments were conducted with free-ranging sunbirds and model flowers. Each model inflorescence consisted of a wooden stick to which five 1.5 ml Eppendorf tubes (model flowers, opening width 9.8 mm, length 38.9 mm) were attached at the upper end approximately 1 m above the ground to imitate an inflorescence. For UV-reflecting model flowers, the Eppendorf tubes were covered with white UV-reflecting bleached printing paper and transparent tape (Sellotape). For non-UV-reflecting model flowers the Eppendorf tubes were additionally painted with a white non-UV-reflecting paint (Tipp-Ex) on top of the paper before covering them in transparent tape. Comparing the colours through bird vision models indicated that birds should easily be able to discriminate between them (6.8 JND, Figure S1). The model flowers were filled with 50 µl of 10% (weight/weight) sucrose solution and set up among natural fynbos vegetation at the Cape of Good Hope section of Table Mountain National Park (34.266^{o} S, 18,463^{o} E). Four pairs of UV-reflecting and non-UV reflecting inflorescences (50 cm apart) were set up at least 2 m apart.

Subsequently, visits by unmarked, free-ranging sunbirds were recorded through focal observations for 37 hours spread over six days. Consecutive visits to different inflorescences by the same

individual were classified as one foraging bout. After each foraging bout, the sugar water was refilled in visited flowers. To reduce the effect of inflorescence location and social learning on birds' foraging choices (Jackson & Nicolson, 1998; Kaczorowski et al., 2014), the inflorescences were switched around every hour. A two-sided t-test was used to compare the number of visits to UV reflecting and non-UV reflecting flowers.

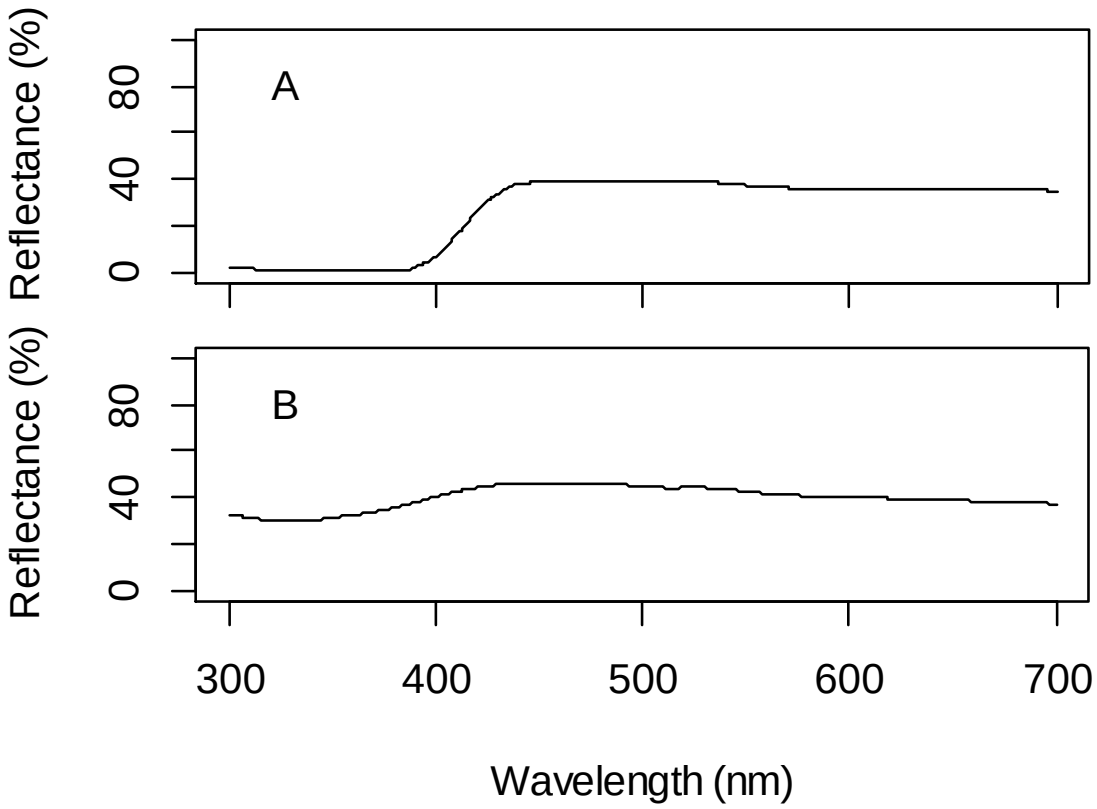

Figure S1 Spectral reflectance of A) White non-UV model flower (contribution of UV to total brightness: 9 %) and B) White UV model flower (contribution of UV to total brightness: 23 %). Bird vision was modelled in the R package pavo using the average avian ultra-violet-sensitive visual system, a D65 illuminant and a green foliage background. A receptor-noise limited model, with a Weber fraction of 0.1 for the long-wavelength sensitive photoreceptor type, indicated a chromatic contrast of 6.79 Just Noticeable Differences (JND)

UV learning in sunbirds

Sunbirds have been shown to learn from colour cues to increase their foraging success (Whitfield, Köhler & Nicolson, 2014) and thus, even if they do not exhibit a preference for UV-reflecting flowers, their ability to see UV and use this flower signal as a cue can be demonstrated with learning experiments. A learning experiment was set up at the same study site but in a different season and

with differently coloured model flowes to ensure the experiments did not influence each other. We used model inflorescences that were constructed with three 0.5 ml Eppendorf tubes (opening width 6.8 mm, length 30.0 mm) attached. Model flowers were painted with pink UV-reflective paint (UV Purple Fish Vision Paint, Reel Wings Decoy) and a UV-transparent polish (UV Fish Vision Gloss Coating, Reel Wings Decoy) for UV-reflecting model flowers or a UV-absorbent polish (Dulux Woodgard Timbavarnish clear) for non-UV-reflecting model flowers. The reflectance spectra of these two treatments were very similar except for the difference in the UV range (6.77 JND, Figure S2). The UV reflective model flowers were filled with 20 µl of 15 % (weight/weight) sucrose solution and non-UV flowers remained empty. Each model inflorescence with UV reflection was set up 50 cm apart from another one without UV reflection.

Subsequently, sunbird visits were recorded through focal observations for 18 hours spread over three days. After each visit the sugar water was refilled. To avoid any bias for positions, every 30 minutes the flowers were switched around. Visits within the same foraging bout were averaged and a linear model was fitted to explore the relationship between the proportion of rewarding choices (= UV-reflective flowers) per foraging bout and elapsed time, since we expected the birds to increasingly visit rewarding flowers over time if they were learning.

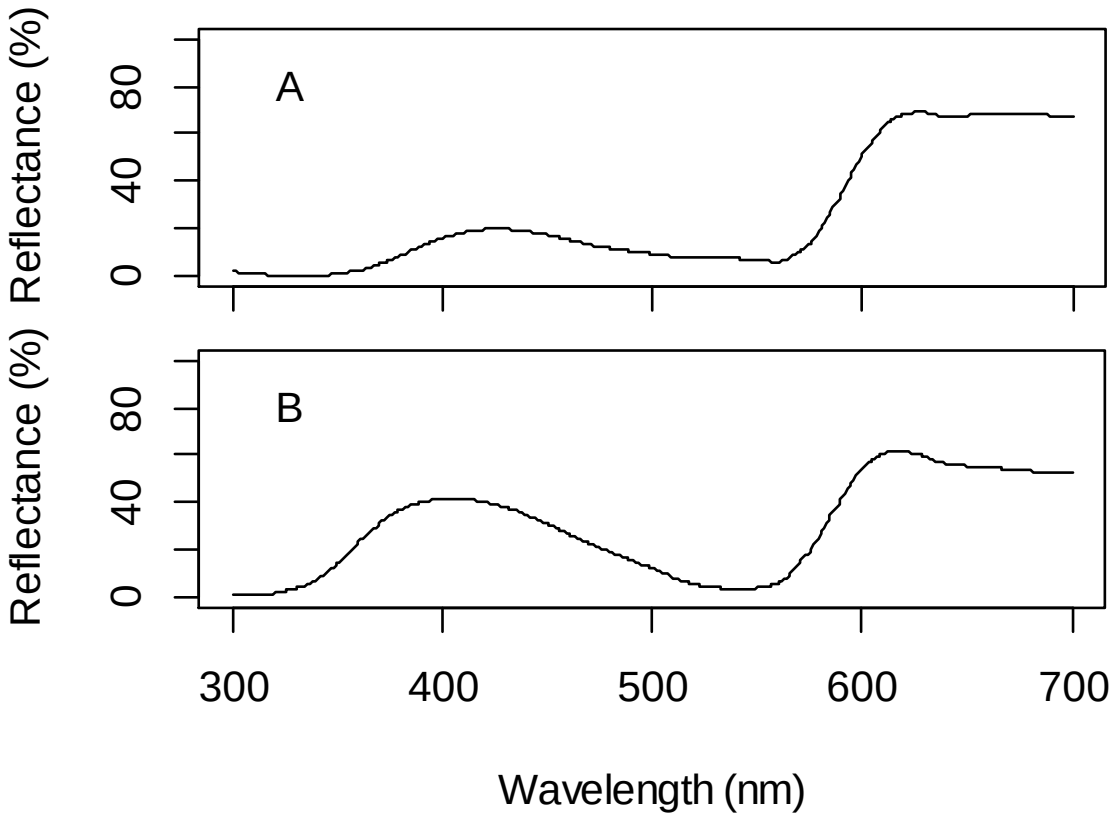

Figure S2 Spectral reflectance of A) Pink non-UV model flower (contribution of UV to total brightness: 4 %) and B) Pink UV model flower (contribution of UV to total brightness: 15 %), chromatic contrast = 6.77 JND (same method as described in Fig S1)

UV preference in long-proboscid flies

It has been shown that LPFs have colour preferences when visiting flowers (Valentin, Lunau & Johnson, 2006; Whitehead, Gaskett & Johnson, 2019). To test if LPFs in the Cape exhibit a preference for flowers that reflect UV, the seed set of flowers with UV, without UV and a scent control were compared. A population of *E. aristata,* which reflects UV and is pollinated by LPFs (Lombardi et al., 2021), occurring at Vogelgat Nature Reserve (34.391°S, 19.315°E) was used. For each treatment we randomly chose 15 plants that were about 1m apart from the next treated plant and had at least two unvisited flowers (Geerts & Pauw, 2011). For the treatment with UV reflection, the flowers were left unmanipulated. For the treatment without UV, the flowers were painted with sunscreen (Island Tribe SPF 50 Gel) to remove UV reflectance from their corolla sides and lobes without changing the rest of the reflectance spectrum (Figure S3). Modelling of fly vision shows that those two treatments are visually distinguishable to flies (Troje 1993; Figure S4). It has been shown in other systems that treatment with sunscreen itself does not deter pollinators (Johnson & Andersson, 2003).

Additionally, we do not expect scent to affect the pollinators, since LPF-pollinated flowers rarely produce scent (Goldblatt & Manning, 2000). Nevertheless, as a scent control we applied sunscreen only to the pedicels and bracts, so that the flowers remained UV-reflective and any changes in seed set would be due to altered scent. The three treatments were applied on separate plants to avoid pollinators choosing flowers based on their proximity to flowers with a different treatment. After flowering, the fruits were collected, and seed set was determined by extracting the seeds from the fruits and counting them under a dissection microscope. Seed set from the same plant was averaged and a generalised linear model with quasipoisson error structure was fitted to test for a difference in the average number of seeds in relation to treatment followed by a Kruskal-Wallis post-hoc test to identify the differences between the groups.

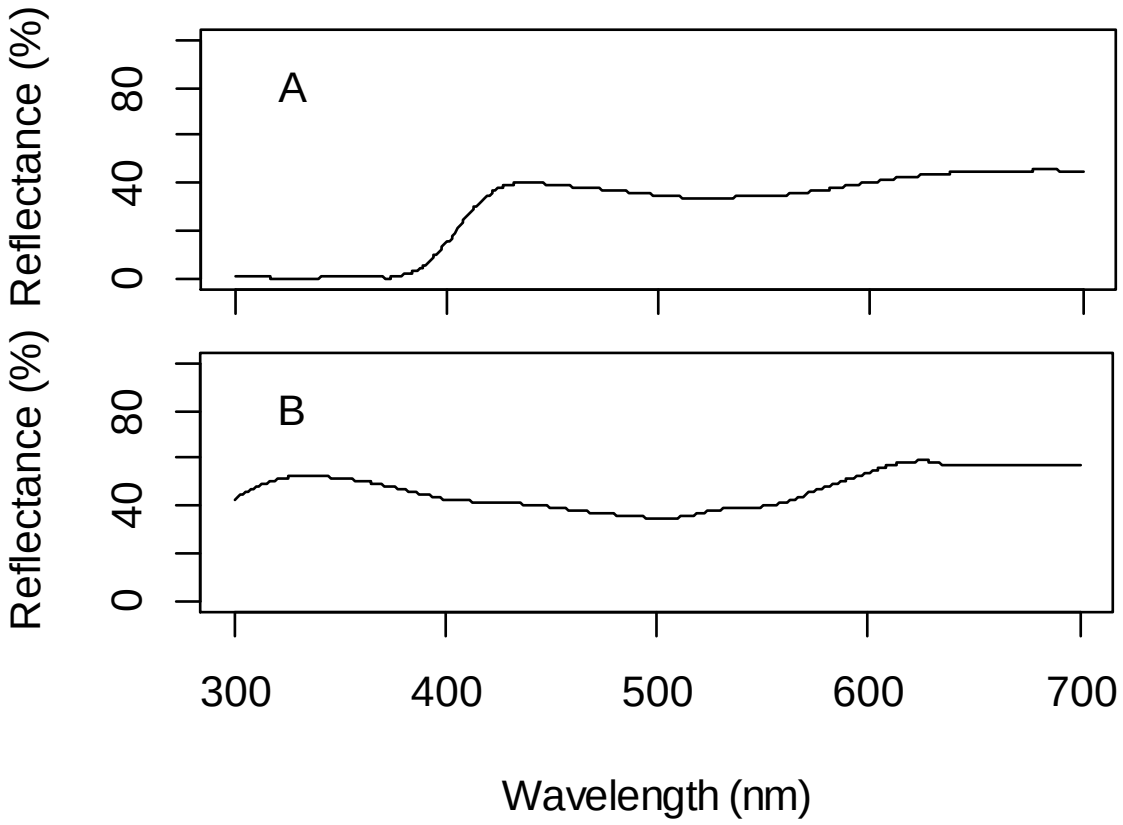

Figure S3 Spectral reflectance of A) *E. aristata* after treatment with sunscreen (contribution of UV to total brightness: 3 %) and B) untreated *E. aristata* flowers (contribution of UV to total brightness: 25 %)

Results

UV prevalence across pollination syndromes

To classify UV and non-UV reflecting species, a cut-off of 10 % contribution of UV to the total brightness was chosen based on the grouping in Figure 1. The overall prevalence of UV in the sampled *Erica* species according to this measure was 12.3 % with an average reflection maximum in the UV range of 6.0 %. For bird-pollinated species, the prevalence of UV was 16.9 % with an average reflection maximum in the UV range, for only the flowers with UV, of 12.1%. A 100 % of the LPF-pollinated species reflected UV (Table S1) with an average reflection maximum in the UV range of 41.6%, while only 1.4 % of unclassified insect-pollinated *Erica* reflected UV. We detected no UV reflection in wind- or rodent-pollinated species (Figure 1). The LPF-pollinated *Erica* species reflect significantly more UV than all other pollination syndromes and bird-pollinated *Erica* species reflect significantly more UV than unclassified insect-pollinated species (Table S3, Table S4).

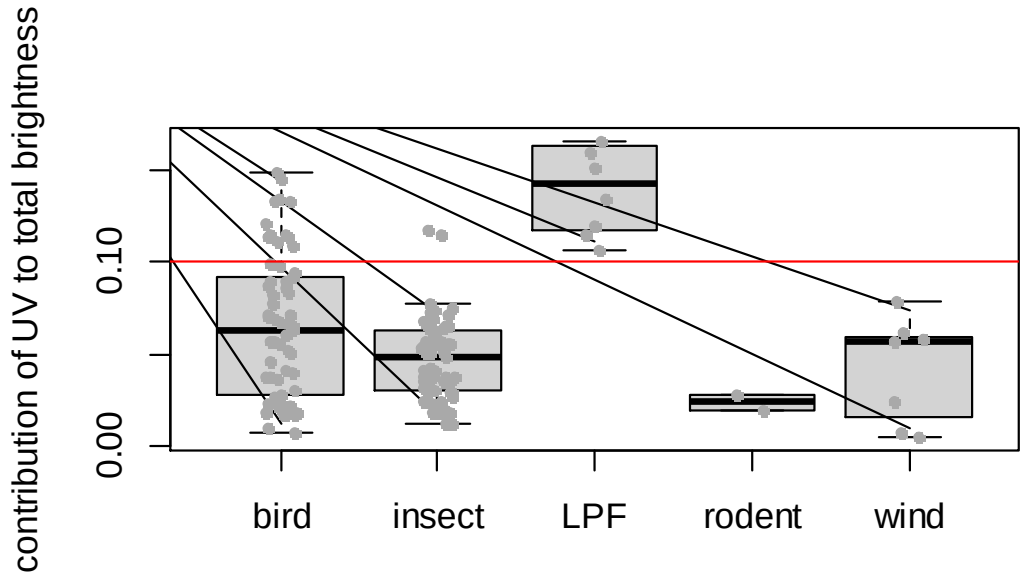

Figure 1 Contribution of UV reflectance (300-400 nm) to total brightness for *Erica* species in relation to their pollination syndrome (center line, median; box limits, upper and lower quartiles; whiskers, 1.5x interquartile range), red line indicating UV reflectance for samples above 0.1. Number of species: bird = 61, insect = 69, LPF = 8, rodent = 2, wind = 7.

Table S3 Contribution of UV to total brightness in 125 *Erica* species in relation to pollination syndrome. Output from generalised linear model; significant results in bold.

Variable	Estimate	SE	x^2	*P*-value
Intercept	-2.73	0.06	-	-
Pollinator (LPF)‡	**0.82**	**0.13**	**68.02**	**< 0.001**

‡Long-proboscid fly (LPF) pollination was used as a reference category.

Table S4 Contribution of UV to total brightness in 125 *Erica* species in relation to pollination syndrome. LPF = Long-proboscid fly. Output from Tukey post-hoc test; significant results in bold.

Contrast	Estimate	SE	*Z*-ratio	*P*-value
bird-insect	**0.34**	**0.09**	**3.55**	**0.004**
bird-LPF	**-0.83**	**0.13**	**-6.21**	**< 0.001**
bird-rodent	1.03	0.59	1.73	0.414
bird-wind	0.46	0.25	1.86	0.341
insect-LPF	**-1.17**	**0.14**	**-8.53**	**< 0.001**
insect-rodent	0.69	0.59	1.16	0.776
insect-wind	0.12	0.25	0.47	0.990
LPF-rodent	**1.85**	**0.60**	**3.09**	**0.017**
LPF-wind	**1.28**	**0.26**	**4.86**	**< 0.001**
rodent-wind	-0.57	0.63	-0.90	0.897

UV preference in sunbirds

Sunbirds showed no preference for flowers with or without UV reflectance (*P* = 0.939, t = 0.08, df = 170). A total of 171 visits to the model flowers during 74 separate feeding bouts were recorded, of which all, but one, were by malachite sunbirds *Nectarinia famosa*. Although none of the previously colour-ringed sunbirds visited the model flowers, male malachite sunbirds were moulting into their breeding plumage and exhibited unique moult patterns. A camera trap was set up by a feeder at the study site. From the pictures, the moult patterns were compared and at least ten different individual males could be identified. This way of identifying individuals is not possible for females, however at one point three female individuals were observed at the same time. Thus, the visitations recorded were from at least 13 different individuals.

UV learning in sunbirds

When presented with different rewards, sunbirds initially did not discriminate between UV and non-UV flowers, but with proceeding time their preference for the rewarding colour increased (Estimate = 0.02, SE < 0.01, $F = 14.02$, $P < 0.001$, Figure 3). A total of 587 visits to the model flowers during 201 separate feeding bouts were recorded, of which 45 were by southern double-collared sunbirds *Cinnyris chalybeus* and 156 were by orange-breasted sunbirds *Anthobaphes violacea*. Although only one of the previously colour-ringed sunbirds visited the model flowers, at least 9 different individuals could be identified based on their species, sex, age and number in a group.

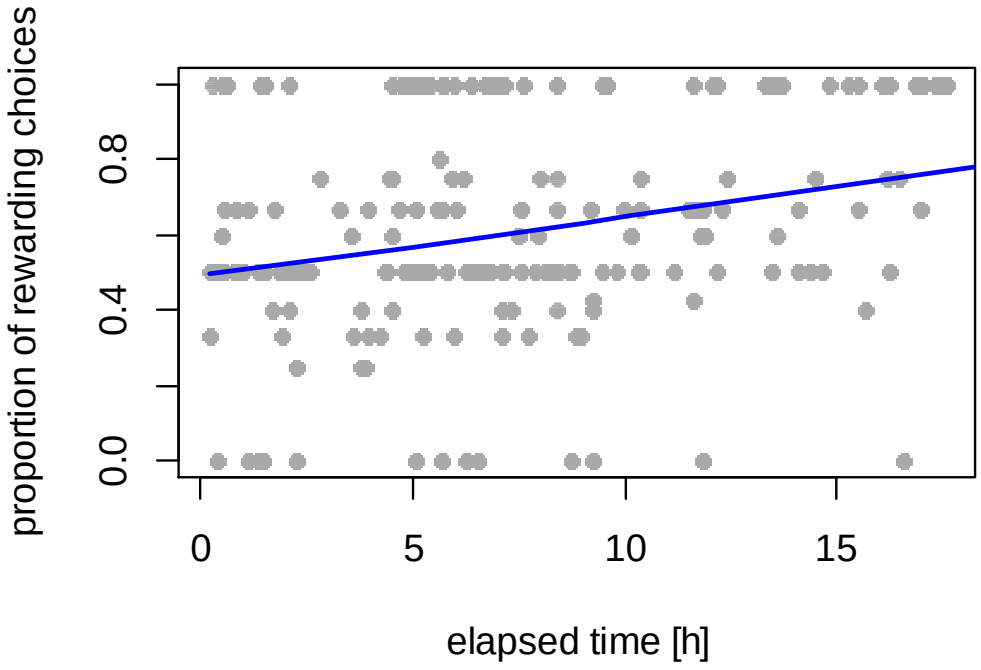

Figure 3 Proportion of rewarding choices (= UV-reflective flowers) made by sunbirds in response to elapsed time in hours, CI in light blue. Observations were conducted over three days (Day 1: 0- 2.5 hours, Day 2: 2.5-11 hours, Day 3: 11-16 hours).

UV preference in long-proboscid flies

There were significant differences between the treatments (Table S5). After experimentally removing UV reflection from the corollas in *Erica aristata* flowers (Figure S3, Figure S4), the treated plants without UV showed lower seed set than the flowers with UV and the scent control (Table S6, Figure 4). There was no difference between the scent control and the unmanipulated flowers with UV (Table S6, Figure 4). Looking at LPF-pollinated *Erica* flowers with a UV-sensitive camera, it is

noticeable that the corolla tube and lobes reflect UV strongly, but the centre around the corolla opening absorbs UV (Figure 2).

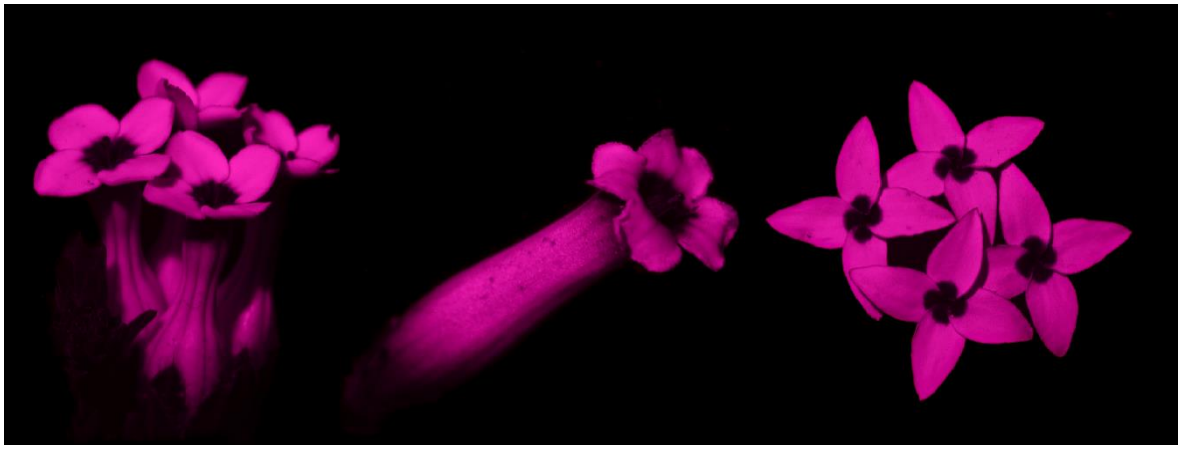

Figure 2 Long-proboscid fly-pollinated species *Erica ampullacea*, *Erica aristata* and *Erica fastigiata coventryi* (left to right) with ultraviolet reflectance (violet areas). Photos taken by S McCarren with a UV-sensitive camera.

Table S5 Average number of seeds per treatment (UV, non-UV, and scent control). Output from generalised linear model; significant results in bold.

Variable	Estimate	SE	x^2	*P*-value
Intercept	-16.30	3042.30	-	-
Treatment (UV)‡	**18.68**	**3042.30**	**8.79**	**0.012**

‡The treatment with UV reflection was used as a reference category.

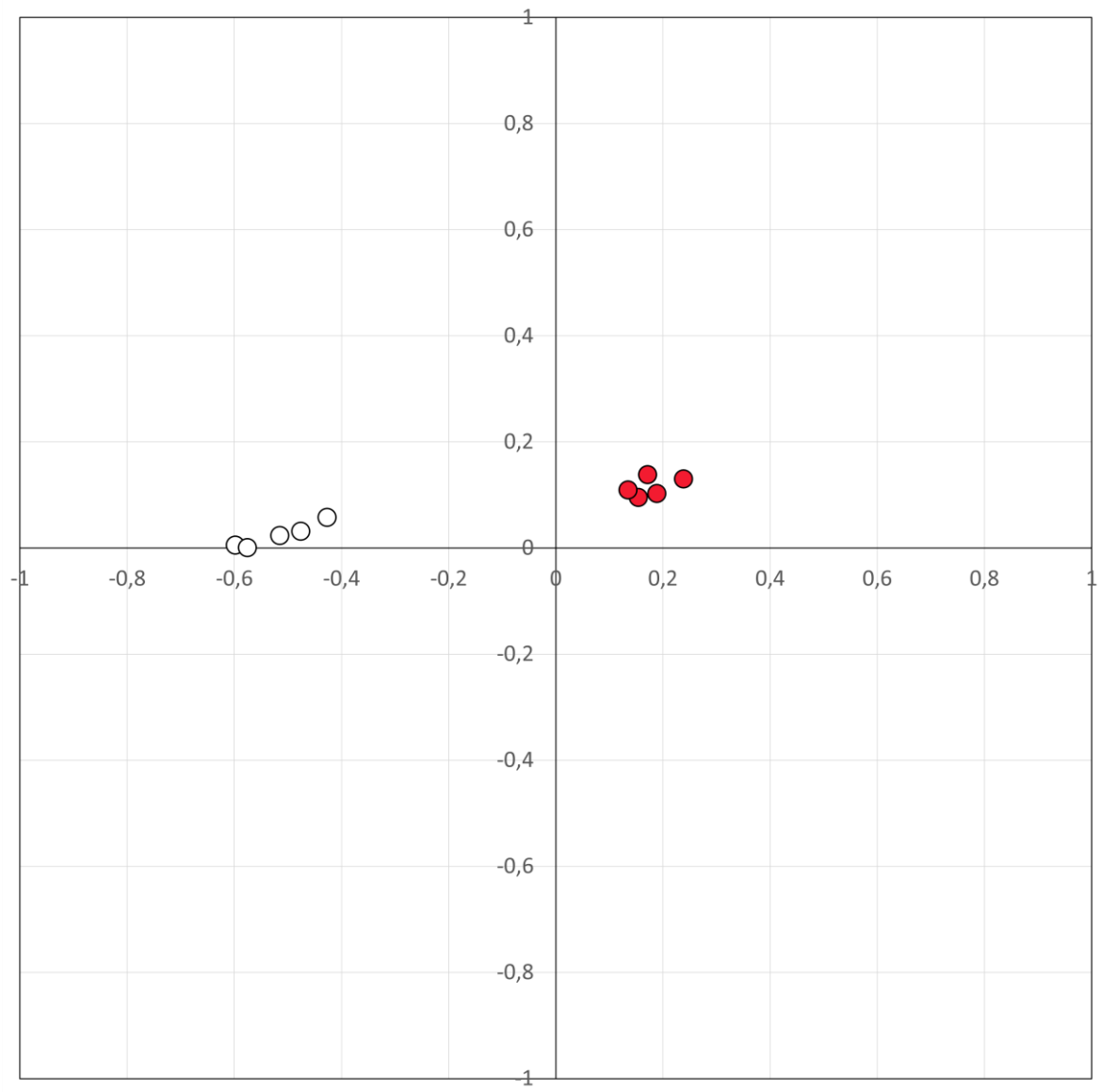

Figure S4 Reflectance spectra of untreated (red) *Erica aristata* flowers and flowers that were treated with sunscreen to remove UV reflectance (white). Treated and untreated flowers of *E. aristata* are different according to the Troje fly colour model as they are in different segments.

Table S6 Average number of seeds per treatment (UV, non-UV, and scent control). Output from Kruskal-Wallis post-hoc test; significant results in bold.

Contrast	x^2	Z-ratio	*P*-value
non-UV – scent control	**11.43**	**-2.83**	**0.002**
non-UV – UV	**11.43**	**-3.02**	**0.001**
scent control – UV	11.43	-0.19	0.423

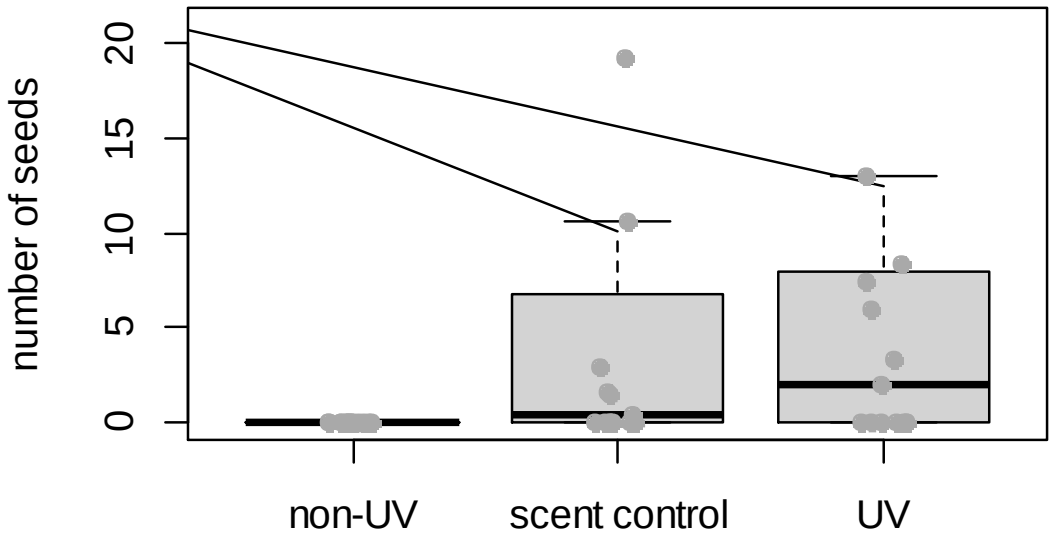

Figure 4 Average number of seeds per plant in *Erica aristata* without UV reflection, with UV-reflection and scent control (center line, median; box limits, upper and lower quartiles; whiskers, 1.5x interquartile range), n= 15.

Discussion

Our results show that UV colouration only plays a minor role for bird pollination of *Erica* but seems highly important for the evolution of the mutualism between LPFs in the Cape and the specialised *Erica* species they pollinate.

As expected, we found no UV reflection in the rodent- or wind-pollinated species. There was little UV reflection in the unclassified insect-pollinated, which might be due to lower discrimination abilities in hymenopterans (Peitsch et al., 1992). However, more ecological studies are necessary to identify the specific pollinators. The recorded UV reflection for the unclassified insect-pollinated species can be solely attributed to two species: *E. haematocodon* and *E. denticulata*. The pollinator of *E. haematocodon* has not been studied yet, but it has red flowers which is unusual for small-flowered *Erica* species (Rebelo & Siegfried, 1985). If it is pollinated by a hymenopteran, reflecting both red and UV light could increase the pollinator's ability to detect the flowers (Chen et al., 2020). *Erica denticulata*, on the other hand, is moth-pollinated (Rebelo, Siegfried & Oliver, 1985). Moths are able to see UV (Karalius & Būda, 2007) and use UV bullseye patterns for foraging (Hirota et al., 2019). In the moth-pollinated form of *E. plukenetii*, however, no UV was detected (van der Niet et al., 2014). Thus, further studies are necessary to understand the prevalence of UV patterns in moth-pollinated plants.

Some of the bird-pollinated species reflect UV but sunbirds do not show an innate preference for UV reflecting flowers and only learn to prefer them when the rewards differ. This suggests that UV plays only a small role for bird pollination in *Erica*. Sunbirds might primarily use their UV vision to perceive UV signals in plumage (Kevan, Chittka & Dyer, 2001) for sexual selection. Its role for bird pollination seems to be no bigger than for other floral colours (Kevan et al. 2001) but it might make it easier for sunbirds to discriminate between co-occurring species and therefore reinforce the pollinator's floral constancy (Lunau et al., 2011; Papiorek et al., 2016) and contribute to creating the geographic mosaic of flower colours in bird-pollinated *Erica* species (Coetzee A et al. 2021). Additionally, some bird-pollinated *Erica* species might have evolved UV reflectance as a mechanism to avoid being conspicuous to less effective pollinators and nectar robbers which might have lower discrimination abilities in the UV range (Lunau et al., 2011; Shrestha et al., 2013).

All LPF-pollinated species we tested reflect UV at high intensities, which suggests that it is important for their pollination. Additionally, Newman & Johnson (2021) found that *E. irrorata* and *E. junonia* which are both pollinated by LPFs also reflect UV. This is supported by the fact that untreated *Erica aristata* flowers were significantly more likely to produce seed than non-UV flowers. Colour preferences do not necessarily translate into observable fitness differences in *Erica* species (Heystek et al., 2014). Thus, the LPFs that pollinate these species seem to have an exceptional preference for UV. The absence of a difference between the scent control and the untreated flowers indicates that LPFs do not discriminate between flowers based on scent differences caused by the application of sunscreen.

The presence of a UV pattern on the LPF-pollinated *Erica* flowers with UV reflectance on the corolla tube and lobes and absorbance around the corolla opening suggests that UV might serve to establish a nectar guide. Floral UV patterns with central absorbance (UV bullseye) are common in nature (e.g., Koski & Ashman, 2016; Moyers et al., 2017; Hirota et al., 2019; Klomberg et al., 2019) and can increase flower conspicuousness (Koski & Ashman, 2014). It has been shown that the removal of nectar guides in LPF-pollinated flowers dramatically reduces the likelihood of proboscis insertion and consequently decreases plant fitness (Hansen, van der Niet & Johnson, 2012). Thus, the experimental removal of UV reflection in *Erica aristata* might cause blurring of the UV bullseye and thereby prevent LPFs from inserting their long proboscis.

All sampled LPF-pollinated *Erica* species are light pink with darker nectar guides, which aligns with the LPF pollination guild described for the Cape (Manning & Goldblatt, 1997). Flowers of the other genera in this guild also appear to reflect UV (e.g., *Adenandra villosa, Brachysiphon acutus, Gladiolus*

carneus, Pelargonium cuculatum, Tritonia cooperi quadrialata; McCarren S unpublished data) which indicates that LPFs in the Cape generally have a preference for UV.

There appear to have been several independent origins of UV reflectance in the genus *Erica* based on the current phylogeny (e.g., *E. viscaria, E. ampullacea, E. blenna, E. fastigiata, E. glandulosa* and *E. haematocodon* reflect UV and are on separate branches according to Pirie et al. 2016), however phylogenetic relationships were not included in this study, since not all the sampled species have been included in the phylogeny yet.

Our results found an association between UV reflectance and pollination syndromes, as well as between UV reflectance and pollinator behaviour. This, together with the patterns of phylogenetic independence of UV reflectance and pollination syndromes in *Erica*, suggests that UV reflectance may have contributed to driving shifts in pollination systems. Changes in UV reflection have also been associated with pollinator shifts in other genera (Martínez-Harms et al., 2020). However, more research is necessary to identify the changes in pigmentation that cause UV-reflection in some *Erica* species and to understand the visual system of LPFs.

Acknowledgements

We are grateful to Claire Spottiswoode for lending her UV-camera, to Ross Turner for help in identifying *Erica* species, to Seth Musker for assistance with the colour analysis and collecting *Erica* samples, to Craig Peter for help with the fly vision model, to Cape Point National Park, Kirstenbosch National Botanical Gardens, Vogelgat Private Nature Reserve, Giorgio Lombardi, Grootbos Private Nature Reserve, Thys de Villiers and Anthony van Hoogstraten for access and permission to sample on their land. We also wish to thank Betty Ann Illing for providing accommodation in Hermanus.

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Chapter 2: Corolla stickiness prevents nectar robbing in *Erica*

Abstract

Floral stickiness is a rare trait with unknown function, but it is common in the mega-diverse Cape genus *Erica* (Ericaceae). This study investigated the role of stickiness measured as adhesive strength in *Erica* as protection against nectar robbing and its correlation with floral traits. We compared the incidence of nectar robbing in flowers of the same species with or without experimentally added stickiness, and amongst communities of co-occurring species with flowers differing in stickiness. Additionally, we tested the relationship between stickiness and pollination syndrome, corolla shape, corolla length and sepal-corolla ratio across the whole genus. Stickiness was correlated with lower floral damage rates within and between species, indicating its functions as an anti-nectar robbing trait. Across the genus *Erica*, stickiness is most strongly correlated with bird and long-proboscid fly pollination, presumably because of their larger nectar rewards. Stickiness was also correlated with floral traits that are often associated with high risk of being damaged by nectar robbers: narrow-mouthed corollas, long corollas, and shorter sepals.

Introduction

Floral stickiness is a globally rare trait and thus its role has not yet been determined. Plant surface stickiness is typically caused by resin or mucin that is produced on glandular trichomes or from glands on glabrous plant surfaces (Dell & McComb, 1979). Floral stickiness has been suggested to limit water loss due to floral evapo-transpiration (Vlok & Schutte-Vlok, 2003), but no mechanism or analysis has yet been presented. Other studies have suggested (proto-) carnivory as the reason behind sticky flowers (Monteiro & Macedo, 2014), but there has been no evidence of nutrient uptake on those flowers. It has also been suggested that sticky flowers trap carrion, which attracts predators that defend the plants against herbivores in turn (Karban et al., 2019). While this might apply to other plant organs, predators on a plants' flowers also cause pollinators to avoid them (Huey & Nieh, 2017). General plant stickiness often serves as direct defence against herbivory (Mithöfer, Mithöfer & Boland, 2012). Likewise, it is more likely that floral stickiness reduces damage inflicted on flowers due to florivory and especially nectar robbing by impeding the movement of insects on the plant (Krimmel & Wheeler, 2015, Figure 1 D).

Nectar robbers can damage floral reproductive organs and thus render a flower infertile (Irwin et al., 2010). More importantly, nectar robbing can also alter pollinator behaviour, e.g., pollinators can be deterred by visible flower damage (Irwin & Brody, 1998; Carper, Adler & Irwin, 2016; Varma et al., 2020) or depletion of nectar (Irwin & Brody, 2000; Missagia & Alves, 2017). Thus, defence of the flowers against nectar robbing insects may carry a fitness benefit.

Erica is a highly suitable genus for such an analysis because it comprises many species (c. 680 in the Cape, South Africa) with a diversity of flower morphologies and pollinators (Rebelo & Siegfried, 1985). Many Cape *Erica* species have sticky flowers, some so remarkably that they are difficult to remove from one's finger. Nectar robbing by ants, carpenter bees, honeybees, monkey beetles and short-billed sunbirds can be observed frequently on non-sticky *Erica* flowers (Turner et al., 2012; Van Der Niet et al., 2014, Figure 1 A-C).

Studies have shown that long-tubed flowers are robbed more often than short-tubed flowers (Lara & Ornelas, 2001; Jogesh et al., 2017) and nectar robbing also inflicts higher fitness costs on bird-pollinated than insect-pollinated plants (Irwin, Brody & Waser, 2001). Additionally, bird-pollinated plants usually have relatively large amounts of nectar (Johnson & Nicolson, 2008), which might make them more attractive to nectar robbers. Thus, we expect to find more species that are bird-pollinated or have long corollas to have evolved stickiness, if it functions as an anti-robbing mechanism. Stickiness might have only evolved in species that lack other nectar robber deterrents, such as large sepals which protect the base of flowers from being punctured (Roubik, 1982).

We study the function and distribution of flower stickiness in *Erica*. We test whether stickiness reduces nectar robbing firstly, by experimentally adding stickiness to flowers and secondly, by analysing floral damage in relation to stickiness in natural communities. Then we investigate potential fitness implications of nectar robbing in *Erica* by looking at the correlation of nectar robbing and pollinator visitation. Finally, we determine in which functional groups stickiness has evolved by testing correlations between stickiness and morphological and pollination traits across the genus.

Figure 1 (A) *Erica coccinea* with damage close to the corolla base due to nectar robbing, (B) *Erica plukenetii* being robbed by an ant (C) *Erica plukenetii* being robbed by a carpenter bee (D) *Erica fascicularis* with a fly caught on the sticky corolla, photos taken by Sam McCarren.

Material and methods

Stickiness quantification

There are methods to determine stickiness known from the literature (Eisner & Aneshansley, 1983; Voigt, Gorb & Gorb, 2007), but for practical reasons we developed a relative field method. Thus, we measured adhesive strength by sticking 1 cm of cotton string along the lower side of a horizontally positioned corolla. We then attached small pieces of aluminium foil as weights until the string disconnected from the corolla. The mass of the string and the weights was multiplied with the gravitational constant g (~9.81 m/s^2) to obtain the force (in milli-Newton) required to break the connection between the corolla and the string.

Experimental test of the role of stickiness

To differentiate between nectar robbing by flying insects (e.g., honeybees and carpenter bees) and crawling insects (e.g., ants), we added stickiness experimentally to the corolla and the stem of the non-sticky *Erica plukenetii*. For this we applied a sticky, non-scented ointment used as insect barrier (Plantex, Chempack) to the corollas of immature inflorescences of 20 individual plants (19 individual flowers on average per plant) at Paarl Mountain Nature Reserve (-33.738^{o}, 18.937^{o}), causing an average stickiness of 2.35 mN. On each plant, we also applied Plantex to the stem just below another flowerhead, which deters only crawling insects, and an additional flowerhead was marked as control. Flower damage by robbing was visually assessed 17 days later. Flower damage was scored as nectar robbing (damage at the base or side of the corolla) and damage resulting in infertility (damage to male and/or female organs). A generalised linear mixed effects model with Poisson error structure was fitted to test the effect of treatment on the percentage of damaged flowers. Plant ID was chosen as a random effect to account for the paired design of the experiment. The differences between treatments were further explored using a Tukey post-hoc test.

Relationship between stickiness and flower damage in natural communities

Data for the community comparison of stickiness and flower damage was collected at seven sites in the Western Cape (Table S1). We concentrated on long-tubed species (>10mm), as other species did not appear to show much damage by nectar robbers. At each site, fifty random flowers per species were visually assessed for damage. From this, the percentage of damaged, robbed, and infertile flowers was calculated for each species at each site. Additionally, the degree of stickiness of each species was measured from five different flowers per species and then averaged. We modeled the percentage of damaged, robbed, and infertile flowers in relation to stickiness and site as fixed effects by fitting generalised linear models with negative binomial error structures. Site was included as a fixed effect to account for differences in the invertebrate community between the sites.

Table S1 Study sites in the Western Cape and the long-tubed *Erica* species used to assess the relationship between stickiness and nectar robbing.

Site name	GPS coordinates	*Erica* species
Cape Point A	-34.266° 18.463°	*E. cerinthoides, E. abietina atrorosea, E. plukenetii plukenetii*
Vogelgat Nature Reserve	-34.401° 19.321°	*E. melastoma, E. viscaria macrosepala, E. cerinthoides, E. macowanii, E. coccinea coccinea*
Kleinmond	-34.294° 19.118°	*E. coccinea coccinea, E. viscaria pendula, E. sessiliflora, E. plukenetii plukenetii, E. perspicua*
Betty's Bay	-34.327° 18.996°	*E. coccinea coccinea, E. viscaria macrosepala, E. plukenetii plukenetii, E. fascicularis*
Silvermine	-34.074° 18.397°	*E. plukenetii plukenetii, E. curviflora, E. discolour, E. glandulosa*
Paarl Mountain Reserve	-33.738° 18.937°	*E. plukenetii plukenetii, E. grandiflora grandiflora*
Akkedisberg	-34.416° 19.673°	*E. shannonii, E. ampullacea*

Relationship between nectar robbing and pollination rate in natural communities

To understand the impact of nectar robbing on pollination rates, we recorded these rates in 22 populations of 10 bird-pollinated *Erica* species at ten sites in the Western Cape (Table S2). At each site, at least 10 plants per species with at least 2 flowers each were visually assessed for damage caused by nectar robbing and for the presence of broken anther rings, as broken rings indicate pollinator visitation (Geerts & Pauw, 2011). Nectar robbing rates in this guild have been shown to decrease with patch floral density (Heystek & Pauw, 2014). We tested whether the proportion of flowers with broken anther rings is related to the proportion of robbed flowers, while accounting for individual plant floral abundance by including it as a fixed effect, by fitting a linear mixed effects model. Additionally, species and site were included as random effects to account for differences between species and for spatial effects.

Table S2 Study sites in the Western Cape and the bird-pollinated *Erica* species used to assess the relationship between the rates of pollinator visits and nectar robbing.

Site name	GPS coordinates	*Erica* species
Cape Point C	-34.29806° 18.42389°	*E. coccinea coccinea, E. mammosa*
Cape Point A	-34.2665° 18.46406°	*E. coccinea coccinea, E. abietina atrorosea,* *E. plukenetii plukenetii*
Suikerbossie	-34.00878° 18.35267°	*E. plukenetii plukenetii, E. cerinthoides*
Tafelberg road	-33.95433° 18.41494°	*E. plukenetii plukenetii, E. abietina abietina*
Paarl Mountain Reserve	-33.73725° 18.93703°	*E. cerinthoides, E. plukenetii plukenetii* *E. grandiflora grandiflora*
Du Toitskloof pass	-33.75325° 19.06508°	*E. plukenetii plukenetii,* *E. grandiflora grandiflora*
Lourensford	-34.00828° 18.93894°	*E. plukenetii plukenetii, E. curviflora*
Brodie Link	-34.35517° 18.83817°	*E. coccinea coccinea* *E. plukenetii plukenetii*
Perdeberg	-34.32783° 18.99647°	*E. fascicularis fascicularis* *E. sessiliflora, E. viscaria macrosepala*
Fernkloof	-34.39592° 19.27728°	*E. coccinea coccinea* *E. sessiliflora*

Relationship between stickiness and floral traits

For 427 *Erica* species flower stickiness, pollination syndrome, corolla shape, corolla length and sepal-corolla ratio were recorded from the literature (Baker & Oliver, 1967; Rebelo, Siegfried & Oliver, 1985; Schumann, Kirsten & Oliver, 1992; Volk et al., 2005; Turner, Midgley & Johnson, 2011; Turner et al., 2012; Lombardi et al., 2017). Baker & Oliver (1967) and Schumann, Kirsten & Oliver (1992) provide a brief description of *Erica* species, in which they also specify when the corolla is sticky. Species described as having any level of stickiness were designated as sticky and if stickiness is not mentioned then the species was assumed to be non-sticky. For flower stickiness, a binary variable was assigned (0 = not sticky; 1 = sticky). We explored the occurrence of sticky flowers in relation to pollinator, corolla shape (tubular, globular, open-mouthed, and ovoid), corolla length and sepal-corolla ratio each as fixed effect in four separate models, since pollination syndrome, corolla shape and length are correlated but still show overlap among pollination categories. Generalised linear models with binomial error structures were fitted. The differences between pollinators and corolla shapes were further explored using a Tukey post-hoc test. All statistical analyses were carried out in R (R Core Team, 2015).

Results

Experimental test of the role of stickiness

When experimentally adding stickiness to the bird-pollinated *E. plukenetii*, the incidence of nectar robbing in the treatment with sticky corollas was 3.4%. The treatment with sticky stems showed robbing of 36.4% of flowers and 38.6% of the control flowers were robbed (Figure 2A). There was a significant difference between the treatment with the sticky corolla and the other treatments (Tables S3 & S4). There was, however, no difference between the treatment with sticky stem and control (Table S4). At this site, we observed mainly flying nectar robbers such as honeybees (*Apis mellifera*) and carpenter bees (*Xylocopa caffra*).

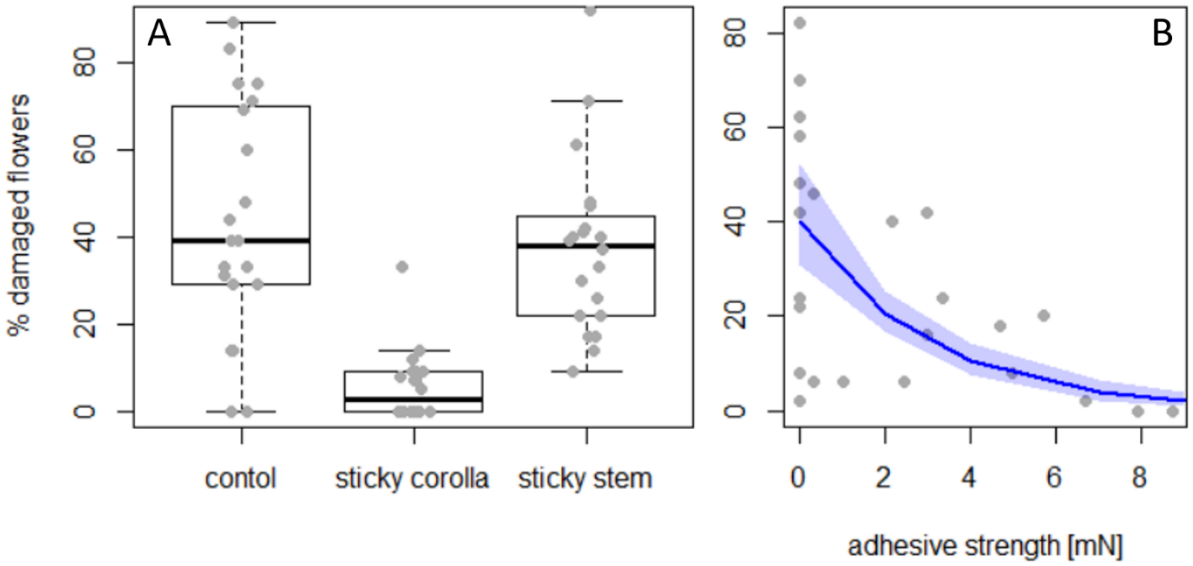

Figure 2 the percentage of damaged *Erica* flowers (A) declines for a non-sticky species when corollas are made sticky (center line, median; box limits, upper and lower quartiles; whiskers, 1.5x interquartile range) and (B) declines in response to increasing corolla stickiness measured in micro-Newton across communities at seven different sites in the Western Cape, South Africa (CI in light blue).

Table S3 Nectar robbing damage in *Erica plukenetii* in response to added stickiness at the base of the inflorescence, added stickiness on the corolla and a control treatment, measured 2019 at Paarl Mountain Reserve. Output from generalized linear model; significant results in bold.

Response	Variable	Estimate	se	x^2	P-value
Damage	Intercept	3.47	0.14	-	-
	Treatment‡	**-0.06**	**0.05**	**378.05**	**<0.001**

‡ the sticky stem treatment was used as a reference category

Table S4 Nectar robbing damage in *Erica plukenetii* in response to added stickiness at the base of the inflorescence, added stickiness on the corolla and a control treatment, measured 2019 at Paarl Mountain Reserve. Output from Tukey post-hoc test; significant results in bold.

Contrast	Estimate	se	Z-ratio	P-value
Control – sticky stem	0.06	0.05	1.14	0.489
Control – sticky corolla	**2.43**	**0.13**	**19.25**	**< 0.001**
Sticky stem – sticky corolla	**2.37**	**0.13**	**18.74**	**< 0.001**

Relationship between stickiness and flower damage in natural communities

Corolla stickiness across seven communities and 18 different long-tubed *Erica* (sub-) species ranged from not sticky at all (0 mN) to very sticky (up to 8.7 mN), such that these flowers were difficult to remove off one's finger. The percentage of nectar robbing holes ranged from 0% to 82% with a mean of 26% and flowers with damage causing infertility ranged from 0% to 50% with a mean of 5%. Total flower damage, as well as nectar robbing and damage causing loss of fertility, across communities were negatively associated with stickiness, and differed among sites (Table S5). With increasing stickiness of a species, the incidence of flower damage decreased (Figure 2B).

Table S5 Total corolla damage, corolla damage from only robbing and damage resulting in loss of fertility in response to corolla stickiness and location, measured in 2019 in seven communities of long-tubed *Erica* in the Western Cape. Output from generalized linear models; significant results in bold.

Response	Variable	Estimate	se	x^2	P-value
damaged	Intercept	4.19	0.27	-	-
	Stickiness	**- 3.28**	**0.44**	**52.96**	**<0.001**
	Location‡	**- 1.32**	**0.34**	**29.83**	**<0.001**
robbed	Intercept	4.14	0.29	-	-
	Stickiness	**- 3.26**	**0.47**	**43.94**	**<0.001**
	Location‡	**- 1.85**	**0.37**	**48.50**	**<0.001**
infertile	Intercept	4.19	0.27	-	-
	Stickiness	**- 3.28**	**0.44**	**52.96**	**<0.001**
	Location‡	**-1.32**	**0.34**	**29.83**	**<0.001**

‡Kleinmond was used as location reference category

Relationship between nectar robbing and pollination rate in natural communities

When studying the impact of nectar robbing on pollination, we found that across all measured *Erica* species, the incidence of broken anther rings and nectar robbing were positively correlated (Figure 3). Floral abundance, on the other hand, had no significant effect on pollinator visitation rates (Table S6).

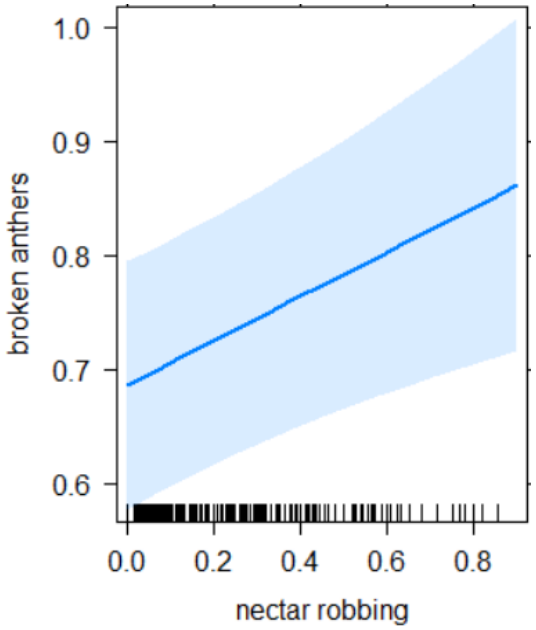

Figure 3 The number of *Erica* flowers with broken anther rings (indicating pollinator visits) increases with increased nectar robbing (CI in light blue).

Table S6 Proportion of broken anther rings in response to proportion of nectar robbing and floral abundance, measured in 2016 and 2017 at ten different sites with bird pollinated *Erica* in the Western Cape. Output from linear model with species and site as random effects; significant results in bold.

Response	Variable	Estimate	se	x^2	P-value
Anther rings broken	**Intercept**	**0.70**	**0.06**	**120.21**	**<0.001**
	Nectar robbing	**0.21**	**0.07**	**9.44**	**0.002**
	Floral abundance	-0.33	0.09	0.15	0.698

Relationship between stickiness and floral traits

Corolla stickiness was widespread in *Erica*, occurring in 82 (19%) of the sampled species (n = 427), and was significantly higher in species that are pollinated by birds or long-proboscid flies than in general insect-pollinated species (Figure 4A, Tables S7 & S8). Open-mouthed species had a significantly lower incidence of stickiness than tubular, globose or ovoid species (Figure 4B, Table S9). With increasing corolla length, the proportion of sticky species increased (Figure 4C) and with increasing sepal-corolla ratio the proportion of sticky species decreased (Figure .4D).

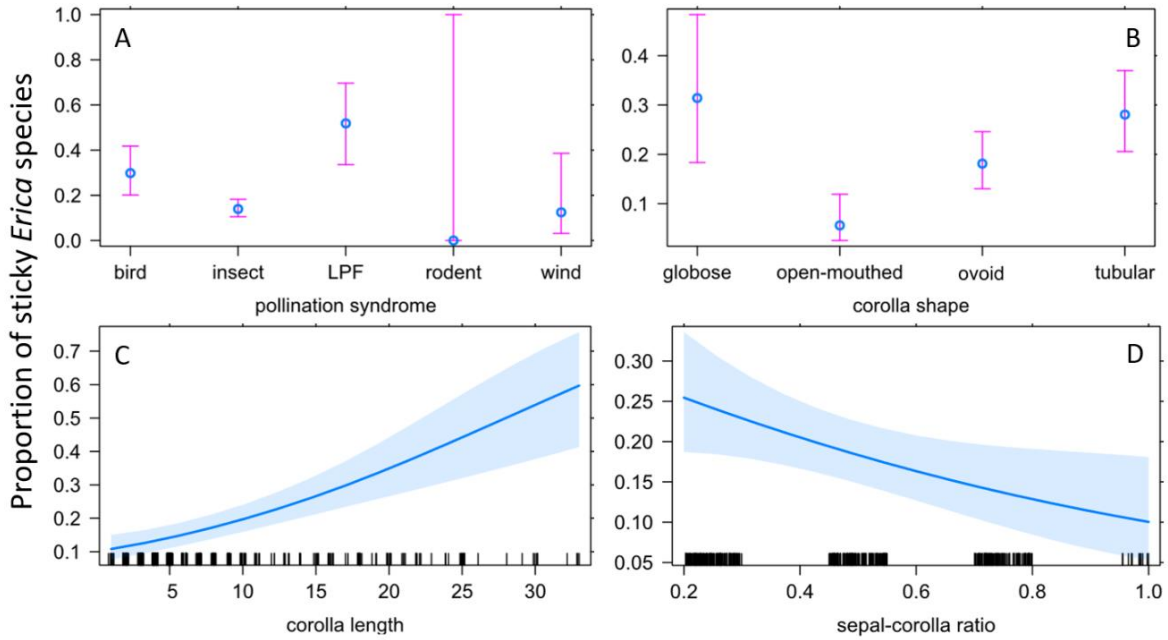

Figure 4 The proportion of stic*ky Erica* species, as described in the literature, grouped in (A) pollination syndromes (LPF = long-proboscid fly), (B) corolla shapes, (C) corolla length [mm] and (D) sepal-corolla ratio (CI in light blue).

Table S7 Corolla stickiness in response to pollination syndrome, corolla shape, corolla length and sepal-corolla ratio as described in the literature. Output from generalised linear models; significant results in bold.

Response	Variable	Estimate	se	x^2	P-value
Stickiness	Intercept	- 0.85	0.27	-	-
	Pollinator‡	**- 0.96**	**0.31**	**26.04**	**<0.001**
Stickiness	Intercept	- 0.78	0.36		
	Corolla shape*	**- 2.04**	**0.56**	**24.91**	**<0.001**
Stickiness	Intercept	- 2.19	0.21	-	-
	Corolla length	**0.08**	**0.02**	**24.43**	**<0.001**
Stickiness	Intercept	- 0.79	0.30	-	-
	Sepal-corolla ratio	**- 1.40**	**0.60**	**5.63**	**0.018**

‡ General insect was used as pollinator reference category
* Open-mouthed was used as corolla shape reference category

Table S8 Corolla stickiness in response to pollination syndrome as described in the literature. Output from Tukey post-hoc test; significant results in bold.

Contrast	Estimate	se	Z-ratio	P-value
bird - insect	**0.96**	**0.31**	**3.08**	**0.018**
bird - LPF	-0.93	0.47	-1.98	0.275
bird - rodent	13.71	624.19	0.02	1.000
bird -wind	1.09	0.80	1.36	0.652
insect - LPF	**-1.89**	**0.42**	**-4.53**	**<0.001**
insect - rodent	12.75	624.19	0.02	1.000
insect - wind	0.13	0.77	0.17	1.000
LPF - rodent	14.64	624.19	0.02	1.000
LPF - wind	2.02	0.85	2.38	0.121
rodent - wind	-12.62	624.19	-0.02	1.000

Table S9 Corolla shape in response to pollination syndrome as described in the literature. Output from Tukey post-hoc test; significant results in bold.

Contrast	Estimate	se	Z-ratio	P-value
globose - open-mouthed	**2.04**	**0.56**	**3.68**	**0.001**
globose – ovoid	0.73	0.42	1.75	0.296
globose – tubular	0.16	0.42	0.38	0.981
open-mouthed – ovoid	**-1.32**	**0.47**	**-2.83**	**0.024**
open-mouthed - tubular	**-1.88**	**0.47**	**-4.01**	**<0.001**
ovoid – tubular	-0.57	0.29	-1.97	0.200

Discussion

Adding stickiness to the corollas of non-sticky *Erica plukenetii* reduced nectar robbing significantly, which suggests that stickiness functions as an anti-robbing trait. When stickiness was only applied to the stem, the robbing rates remained similar to the control. This indicates that corolla stickiness can deter flying nectar robbers. Nectar robbing also differed between species, being inversely related to their stickiness, also supporting the hypothesis that stickiness acts an anti-robbing mechanism.

Contrary to our expectations, we found a positive relationship between broken anther rings (indicating pollinator visitation) and nectar robbing. This could be the result of nectar robbers triggering the anther rings while they are perched on the corolla and perhaps cause self-pollination. This, however, is unlikely for the long-tubed *Erica* species studied since the nectar robbers access the flower through a hole in the base of the corolla and do not make contact with the anthers (Figure 1). Additionally, (Geerts & Pauw, 2011) found that honeybees and solitary bees are incapable of breaking the anther ring. Instead, the observed pattern might be a result of bird-pollinated *Erica* species employing different strategies of counteracting fitness disadvantages caused by robbing, e.g., maintaining floral visitation by replenishing nectar (pers. obs.). Potentially, the advertising signals or rewards produced by flowers (such as flower colour or nectar concentration) that attract many pollinator visits are also attracting many visits by nectar robbers. Non-sticky corollas experience higher levels of nectar robbing but may still be selected for if moderate levels of nectar robbing are beneficial, e.g., by increasing the foraging distance of an individual pollinator which can ultimately increase cross-pollination (Irwin et al., 2010). In both surveys we found nectar robbing to be very variable within non-sticky species and between different sites. The relationship between nectar robbing and fitness is still unclear given that we don't know whether the flowers we assessed were pollinated or robbed first, therefore an assessment of the effect of nectar robbing on seed set is needed.

Corolla stickiness in *Erica* is correlated with pollination syndrome, corolla shape, corolla length and sepal-corolla ratio. As predicted, we observed higher prevalence of stickiness in bird-pollinated species, but also in species pollinated by long-proboscid flies. Both bird-pollinated species and long-proboscid fly-pollinated species have higher volumes of nectar than short-proboscid insect-pollinated species and the latter often also have higher sugar concentrations than bird-pollinated flowers (Heystek et al., 2014; Lombardi, 2014; van der Niet et al., 2014), thus offering a larger reward to nectar robbers and making defence mechanisms against nectar robbing more advantageous for the plants. It has also been shown that flowers with larger rewards are more likely to be robbed (Rojas-Nossa, Sánchez & Navarro, 2016). Furthermore, birds are less likely to get

caught on sticky corollas while feeding than smaller pollinators. Long-proboscid flies typically do not perch while feeding from a flower and instead hover in front of the flower, only grasping onto the non-sticky lobes (Goldblatt & Manning, 2000). Thus, plants pollinated by birds or long-proboscid flies are less likely to deter their own pollinator with a sticky corolla. Just like many other plant species with specialised pollinators have evolved mechanisms to deter ineffective pollinators from visiting their flowers (e.g., Vincentini & Fischer, 1999; Johnson, Hargreaves & Brown, 2006; Nicolson et al., 2015), *Erica* species that are specialised for bird or long-proboscid fly pollination are more likely to have evolved stickiness as a mechanism to deter nectar robbers as a form of preventing ineffective visits to their flowers. This is also supported by the high prevalence of stickiness among species with globose corollas (Fig 4B), which have small corolla openings that likely limit the type of insects that can access nectar in the corollas and are thus also relatively specialised.

Open-mouthed *Erica* species and those with shorter corollas are less likely to have evolved stickiness because it is not necessary for most visitors to damage the corolla in order to reach the nectar. Tubular, globose, and ovoid species, on the other hand, have smaller corolla openings and, like flowers with longer corollas, they are not accessible to generalist visitors unless they perforate the corolla. Also, *Erica* species with larger sepals were less likely to evolve stickiness, supporting its potential role as an alternative defence mechanism against nectar robbing, which has also been found in other systems (Roubik, 1982; Rojas-Nossa, Sánchez & Navarro, 2016).

Sticky flowers have evolved multiple times in the genus *Erica*, for example *E. cerinthoides, E. viridiflora, E. melastoma, E. shannonii, E. viscaria,* and *E. annectens* have sticky flowers and are widely distributed in the current *Erica* phylogeny (Pirie, Oliver & Bellstedt, 2011). However, flower stickiness is rare globally, having only been reported for some species of the New World genus *Vriesea* (Bromeliaceae; Monteiro & Macedo, 2014), some species of the family Solanaceae (Karban et al., 2019) and on the hairy calyx present in some species of the family Plumbaginaceae (Singh, Naidoo & Baijnath, 2018). We suggest that the high prevalence of stickiness in the Cape is due to it being a global hotspot for bird and long-proboscid fly pollination (Rebelo, 1987; Goldblatt & Manning, 2000). These results show that corolla stickiness defends *Erica* against nectar robbing, both in experimental and natural settings, and thereby potentially prevents infertility and decreased pollinator visits.

Acknowledgements

We are very grateful to all the landowners who permitted us to conduct fieldwork on their land, particularly to Vogelgat Private Nature Reserve and Giorgio Lombardi, Thys de Villiers, South African National Parks, and Cape Nature. We thank Felix Herzog for advice on how to measure stickiness. We also wish to thank Betty Ann Illing for providing accommodation in Hermanus.

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Chapter 3: Flower orientation and corolla length as reproductive barriers in the pollinator-driven divergence of *Erica shannonea* and *Erica ampullacea*

Abstract

A variety of reproductive barriers can enable reproductive isolation and stable coexistence of plant species. Differing floral traits might play an important role in reproductive isolation imposed by pollinators. Such shifts in pollinator use have been hypothesized to contribute to the radiation of *Erica* (Ericaceae) in the Cape Floristic Region, South Africa. The sister species *Erica shannonea* and *Erica ampullacea* co-occur and overlap in flowering phenology. Both have unscented long-tubed flowers consistent with adaptations for pollination by long-proboscid flies (LPFs), but differences in flower orientation and corolla tube length are indicative of a shift in pollinator species.

We conducted controlled pollination experiments and pollinator observations to determine the breeding system and pollinators of the two species.

Both species are self-incompatible and require pollinator visits for seed production, suggesting that pollinators could strongly influence flower evolution. The horizontally orientated flowers of *E. shannonea* were found to be pollinated by *Philoliche rostrata* (Tabanidae) which has a long, fixed forward-pointing proboscis, while the vertically orientated flowers of *E. ampullacea* were found to be pollinated by *Prosoeca westermanni* (Nemestrinidae) which has a shorter proboscis that can swivel downwards.

The nemestrinid's proboscis is too short to access the nectar in the relative long-tubed flowers of *E. shannonea* and the tabanid's proboscis cannot swivel down to access the upright flowers of *E. ampullacea*. These two *Erica* species thus have effective mechanical and ethological reproductive isolation barriers which may have contributed to speciation and enable stable coexistence.

Introduction

Population divergence in plants can involve relatively small changes in floral traits like corolla length, colour, or flowering time which can be critical for enabling subsequent sympatric coexistence (Johnson, 2010; Pauw, 2013). These floral trait changes are often driven by selection that favours use of a novel pollinator, resulting in a shift in pollination system. Such floral isolation can result

from mechanical (such as corolla length) and/or ethological mechanisms (such as floral scent preferences) that prevent a pollinator group or species from either visiting or legitimately pollinating a flower or both (Grant, 1994).

Corolla length is well-known to filter flower visitors (Geerts & Pauw, 2009). Long corollas can mechanically exclude flower visitors with short mouth parts as they cannot access rewards, but it is also common for flower-visiting animals to visit flowers with corollas shorter than their mouthparts (Johnson et al., 2017). In such cases, corolla length divergence could only be an effective isolating mechanism for co-occurring species if it is combined with another trait, such as increased nectar reward (Geerts & Pauw, 2009) that reinforces flower constancy. Flower orientation has been suggested to act as a visitation barrier for some pollinator species (e.g., hawkmoths and hummingbirds) and thus cause reproductive isolation based on both behavioural and mechanical isolation (Grant, 1992, 1994; Fulton & Hodges, 1999).

The Cape Floristic Region provides multiple opportunities to investigate the role of floral isolation in speciation and species coexistence due to its high diversity of plant species and pollinator functional types(van der Niet & Johnson, 2009). *Erica* (Ericaceae) has undergone a radiation that produced nearly 800 extant species which coexist in a small biome of only 90 000 km^2 (Pirie et al., 2016). Consequently, there has been much interest in the pollination systems for the genus which include pollination by long-proboscid flies (LPF; Lombardi et al., 2021; Newman & Johnson, 2021), other insects, wind, birds (Rebelo, Siegfried & Oliver, 1985) and rodents(Turner, Midgley & Johnson, 2011; Lombardi et al., 2017). Evidence suggests that pre-pollination reproductive barriers in bird-pollinated *Erica* species are relatively weak (Coetzee, Spottiswoode & Seymour, 2020), but stronger in bee-pollinated species (Bouman, Steenhuisen & van der Niet, 2017). Reproductive isolation has not been investigated in LPF-pollinated species yet.

LPF pollination is inferred for 9% of all insect-pollinated *Erica* species in the Cape (Rebelo, Siegfried & Oliver, 1985). Exceptionally long nectar-feeding proboscides (>15 mm) have evolved in two fly families in southern Africa: Nemestrinidae (Barraclough, 2006) and Tabanidae (Morita, 2008). In South Africa, LPFs have mainly been observed to pollinate species of Geraniaceae, Iridaceae, Orchidaceae (Goldblatt & Manning, 2000) and Lamiaceae (Potgieter & Edwards, 2005). These plants mostly have evolved elongated, tubular flowers, and a lack of scent (Goldblatt & Manning, 2000). Several guilds of LPF-pollinated plants have been identified (Manning & Goldblatt, 1996, 1997; Goldblatt & Manning, 2000; Potgieter & Edwards, 2005; Anderson & Johnson, 2009; Newman, Manning & Anderson, 2014) with examples of convergent evolution in flower colour among species pollinated by LPFs. For example, several species pollinated by *Prosoeca marinusi* (Nemestrinidae)

have purple colouration with white nectar guides (Hansen, van der Niet & Johnson, 2012), while different species pollinated by *Philoliche gulosa* (Tabanidae) tend to be cream coloured with reddish nectar guides (Combs & Pauw, 2009).

The *Erica* species inferred to be LPF-pollinated share traits with many other LPF-pollinated species, typically being odourless, and having long, ampullaceous corollas with flaring lobes, narrow openings, and a light pink colouration (Manning & Goldblatt, 1997). LPF-pollinated ericas also have been shown to reflect UV and some of them have a dark reddish nectar guide around the corolla opening (McCarren, Midgley & Coetzee, 2021). The sister species *Erica shannonea* and *Erica ampullacea* (Pirie et al., 2016) can both be found in the Kleinrivier Mountains and have been assumed to be pollinated by LPFs based on their corolla length and shape (Rebelo, Siegfried & Oliver, 1985). Flowers of both species are ampullaceous with a constricted corolla opening and flaring lobes, light pink in colour and do not emit a scent noticeable to humans. The flowering time of *E. shannonea* ranges from late October to mid-December while *E. ampullacea* flowers from late June to mid-December causing the two species both to co-occur and overlap in flowering phenology.

For sister species with the same pollination syndrome to co-occur and co-flower, there is a high risk of hybridisation, but no apparent hybrid plants have been reported and no phenotypically intermediate hybrids have been reported among other related *Erica* species found in this region. Therefore, it is expected that there is effective reproductive isolation between them which could be genetic, mechanical, ethological or any combination of these. Although, they are both believed to be LPF-pollinated, the corollas of *E. shannonea* are 22-33 mm long while the corollas of *E. ampullacea* are only 13-24 mm long (Baker & Oliver, 1967). Additionally, they differ in flower orientation, with *E. shannonea* facing sideways and *E. ampullacea* facing upwards. Upward orientation is rare in ericas and appears to be associated with pollination by moths and LPFs (van der Niet & Cozien, 2022). Therefore, we expect the two *Erica* species to be pollinated by distinct LPF species.

Methods

Study site

Field studies for *E. shannonea* and *E. ampullacea* were conducted in 2012, 2019 and 2021 at Boskloof in the Kleinrivier Mountains close to Hermanus in the south-west of the Cape Province, South Africa. At site 1 (-34.4008˚, 19.6800˚) the focal species was *E. shannonea* with about 60

mature flowering plants, which occur sympatrically with few non-focal plants of *E. ampullacea*, while at site 2 (-34.4168˚, 19.6768˚) the focal species was *E. ampullacea* with about 30 mature flowering plants, which co-occur with plants of the LPF-adapted plant *Gladiolus debilis* (Iridaceae) that flower at the same time. The two study sites are less than 2km apart and consist of fynbos vegetation on rocky northern slopes. While *E. shannonea* was only present at site 1, *E. ampullacea* was present at both sites. It was not a focal species at site 1 due to its low abundance there.

Controlled hand-pollination experiments

To determine whether pollinators are important for seed production in *E. shannonea* and *E. ampullacea*, and whether plants are capable of selfing, we carried out controlled hand-pollination experiments on 10 separate mature plants per species at its respective site. Each plant had two treatments applied: Exclusion with organza pollination bags and cross-pollination with toothpicks from a plant > 3m away, exclusion with pollination bags and self-pollination with toothpicks, and an open control. The flowers that were chosen for treatment were bagged before opening to prevent any chance of visitation and treated about a week later once the flowers had opened inside the pollination bags. For *E. shannonea* each treatment was applied to five flowers per plant at site 1, and plant was treated as a random factor in the further analysis. For *E. ampullacea,* due to fewer available flowers, each treatment was applied to one flower per plant at site 2. Treated flowers were examined one month later to assess seed set. For the seed set of both *E. shannonea* and *E. ampullacea* generalised linear models with negative binomial error structure were fitted in R (R Core Team, 2015) using the package MASS (Venables & Ripley, 2002). We explored seed set in relation to treatment as the fixed effect and a Tukey post-hoc test was performed to determine differences between the treatments. Additionally, we calculated pollen limitation index for both species (Larson & Barrett, 2000).

Pollinator observations

Flowers of *E. shannonea* were observed from 10am to 3pm for five days per year between October and November in 2012 and 2019 for a total of 50 hours. Additionally, four camera traps (Bushnell Natureview models 119740 and 119440) were set up during the flowering time in 2019 recording flower visitations for a total of 14 days and nights. *In situ* flower stems were tied to stakes to reduce false triggers of the camera traps through wind movement.

Flowers of *E. ampullacea* were observed from 10am to 3pm for three days per year between September and October both in 2019 and 2021 for a total of 30 hours, and the same four camera traps were set up on staked flowering stems in both years during the flowering time to record flower visitation for a total of ten days and nights.

We also measured the corolla lengths (from the corolla base to the point where the corolla tips start separating) and flower orientation (degree from vertical) of 20 randomly sampled flowers per *Erica* species.

The captured flower visitor on *E. ampullacea* was identified to species level, its proboscis was measured, and pollen on the proboscis and thorax was collected using fuchsin gel and identified using a compound microscope. Using ImageJ, we estimated the extended proboscis lengths of both visiting fly species that were photographed by the camera traps by using flower dimensions as a reference scale. For this, we chose pictures in which the flies were hovering in front of the flowers but had not inserted their proboscis yet. Additionally, we measured the proboscis length of museum specimens of both fly species using the collection in the Iziko South African Museum.

Due to its similar floral morphological characteristics, we also observed *Gladiolus debilis* for two hours, collected pollen from the anthers to compare it to the pollen found on the pollinator and measured the corolla length of five random flowers with digital callipers.

Nectar and colour properties

Nectar from 36 randomly sampled *E. shannonea* flowers and 10 randomly sampled *E. ampullacea* flowers from different plants was sampled with micro-syringes, accurate to 0.25 µl. Nectar concentrations were measured using a refractometer (Eclipse 0-50%). Both nectar volume and concentration were compared between the species using a t-test. Additionally, we recorded flower colour with a spectrophotometer in the laboratory (Jazz with PX-2 Pulsed Xenon light source, Ocean Optics, Dunedin, FL) for the lobes of three flowers per species that were collected from different individuals.

Results

Controlled hand-pollination experiments

Both *Erica* species appear to be dependent on pollinator visits for seed production. Seed set of cross-pollinated and open flowers was significantly higher than for self-pollinated flowers in both *E. shannonea* (χ^2 =355.07, df= 2, p <0.001, Figure 1) and *E. ampullacea* (χ^2 =38.48, df= 2, p <0.001, Figure 1). In *E. shannonea* cross-pollinated flowers also had a higher seed set than the open control (Figure 1). Autonomous selfing was not tested due to the limited number of flowers available, but since no seed set was observed for assisted selfing, the same result would have been observed for autonomous selfing. The pollen limitation index was 0.48 for *E. shannonea* and 0.60 for *E. ampullacea.*

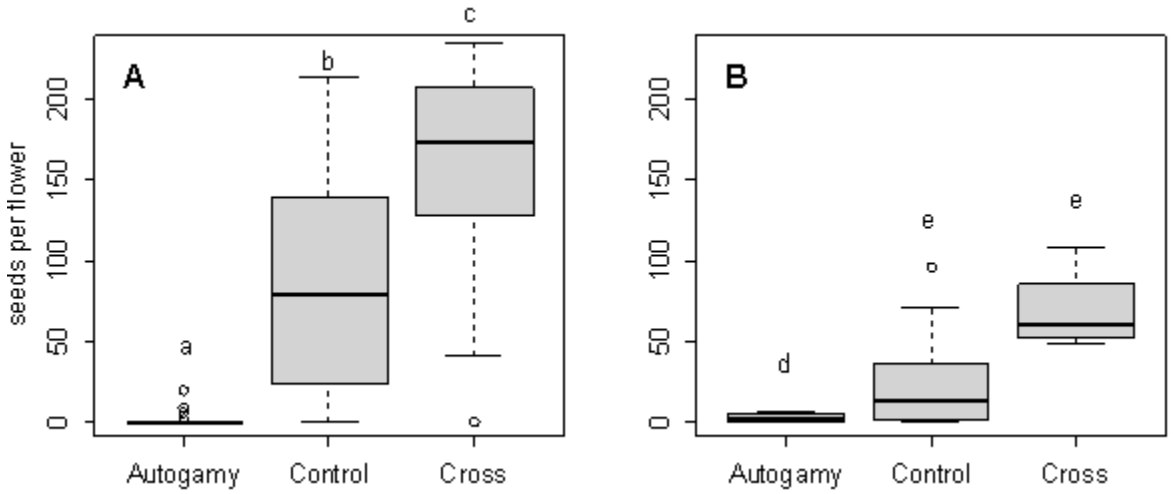

Figure 1 Seed set in controlled hand-pollinated experiments on A) *Erica shannonea* and B) *Erica ampullacea* (centre line: median; box limits: upper and lower quartiles; whiskers: 1.5x interquartile range; dots: outliers) Treatment groups that share letters are not significantly different.

Pollinator observations

Few visits were observed, but only *Philoliche rostrata* (Tabanidae) was observed legitimately visiting *E. shannonea*, while *Prosoeca westermanni* (Nemestrinidae) was the only visitor recorded to *E. ampullacea*.

The LPF *Ph. rostrata* (Tabanidae) visited the flowers of *E. shannonea* and had pollen deposited on its proboscis (Figure 2A). During all observations and recordings with camera traps on multiple plants, only six visits on separate plants from this species were recorded on two consecutive days in 2019 (Table 1). The flies appeared to visit all or multiple flowers on the plant, and in some cases visited several flowers repeatedly. One Malachite sunbird (*Nectarinia famosa*) was observed visiting the staked flowers of one plant and robbing nectar in 2019. In 2012, it was observed that 54% of *E. shannonea* flowers that were assessed had slits in the corolla, which is typically caused by sunbirds nectar robbing. No flower visits were recorded in 2012 or at night. Since the fly could be identified to species level from the observations and photographs, no individuals were caught.

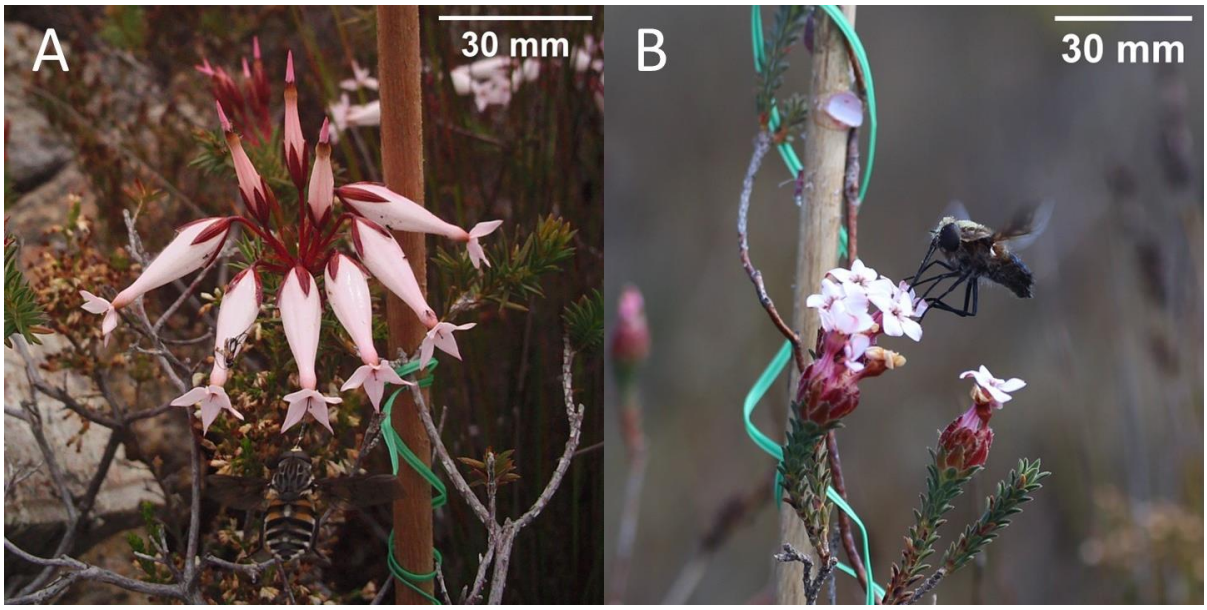

Figure 2 A) *Philoliche rostrata* visiting *Erica shannonea* B) *Prosoeca westermanni* visiting *Erica ampullacea*.

Table 1 Recorded flower visitations on *Erica shannonea*

Date	Focal observations	Camera traps
22/10/2012	0	0
23/10/2012	0	0
24/10/2012	0	0
25/10/2012	0	0
26/10/2012	0	0
23/10/2019	0	0
24/10/2019	0	1 Malachite sunbird
25/10/2019	0	0
26/10/2019	0	2 *Philoliche rostrata*
27/10/2019	2 *Philoliche rostrata*	2 *Philoliche rostrata*

The fly *Prosoeca westermanni* (Nemestrinidae, Figure 2B) was the only observed visitor to flowers of *E. ampullacea*. In 2019, two separate feeding bouts by this fly species were recorded by the camera traps, while in 2021, 15 feeding bouts were recorded (Table 2). The flies visited multiple flowers on each plant but rarely all of them. It was observed that the flies on the wing as well as the captured individual had pollen deposited on both their proboscis and dorsal part of the thorax. No flower visits were recorded at night. Since the fly could not be identified to species level from the observations or photographs, one individual was caught.

Table 2 Recorded flower visitations on *Erica ampullacea*

Date	Focal observations	Camera traps
17/09/2019	0	1 *Prosoeca westermanni*
18/09/2019	0	0
19/09/2019	0	1 *Prosoeca westermanni*
21/09/2021	6 *Prosoeca westermanni*	0
22/09/2021	6 *Prosoeca westermanni*	3 *Prosoeca westermanni*
23/09/2021	0	0

The mean corolla length of *E. shannonea* was 28.3 ± 1.8mm (n = 20) and its average flower angle 97 ° from vertical, while the mean corolla length of *E. ampullacea* was 18.8 ± 2.7mm (n = 20) and its

average flower angle was 11° from vertical. The corolla of *Gladiolus debilis* measured on average 15.6mm (n=5).

While the corollas of both *Erica* species are very sticky and often entrap and kill insects that land on them (Figure 2; McCarren, Coetzee & Midgley, 2021), we observed that the visiting flies grasped only onto the non-sticky corolla lobes during visits. *Ph. rostrata* hovered in front of *E. shannonea*, while *Pr. westermanni* hovered on top of *E. ampullacea*. All visits occurred at air temperatures between 26° and 31° C with little to no wind.

On the proboscis of the captured *Pr. westermanni* we found pollen tetrads that are typical for *Erica* (Wrońska-Pilarek, Szkudlarz & Bocianowski, 2018), and, on its thorax, we found pollen monads that are morphologically identical to the pollen collected from *Gladiolus debilis* anthers. No other pollen was found on the fly and no other LPF-adapted plants with similar pollen structure were flowering in the area. Only carpenter bees (*Xylocopa caffra*) were observed visiting the flowers of *Gladiolus debilis*.

The proboscis of the captured *Pr. westermanni* was 11.4mm long. From pictures we estimated the extended proboscis length to be approximately 28mm for *Ph. rostrata* and 13mm for *Pr. westermanni*. The mean (± se) proboscis length of the museum specimen was 23.65 ± 2.43mm for *Ph. rostrata* (n= 15) and 6.95 ± 1.00mm for *Pr. westermanni* (n = 8).

Nectar and colour properties

The mean nectar volume did not differ significantly among the *Erica* species ($t = 1.661$, $df = 44$, $p = 0.104$) and was 2.2 ± 0.2 µl for *E. shannonea* compared to *E. ampullacea* with 1.6 ± 0.5 µl. The mean nectar concentrations, however, differed between the species ($t = 5.926$, $df = 44$, $p < 0.01$) and were 41.4 ± 1.8% for *E. shannonea* and 20.5 ± 4.4% for *E. ampullacea*. Flowers of both species are light pink in human-vision colouration and reflect light in the UV range (Figure 3).

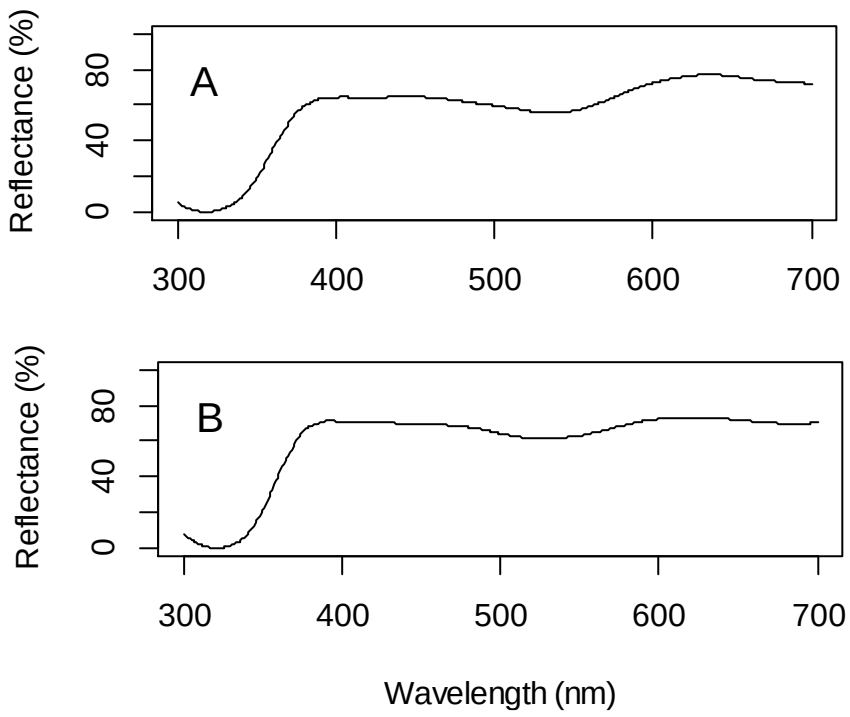

Figure 3 Average spectral reflectance of A) *Erica shannonea* and B) *E. ampullacea* flowers.

Discussion

The results of this study show that the sister species *E. shannonea* and *E. ampullacea* are pollinated by two different fly species from the families Tabanidae and Nemestrinidae. *Erica shannonea* is pollinated by the LPF *Ph. rostrata*, which made contact with anthers and stigmas, and carried pollen on its proboscis. Flowers that were self-pollinated produced few or no seeds relative to the open control and cross-pollination. Thus, *E. shannonea* is an obligate outcrosser and relies on *Ph. rostrata* for pollination, suggesting that the pollinators have a strong influence on the evolution of flower morphology. Birds were never seen foraging legitimately from the flowers, but only by puncturing the corolla side which might be due to the narrow corolla opening. The growth habit of *Erica shannonea* with very thin branches additionally impeded birds from visiting, since they require a perch (Siegfried, Rebelo & Prŷs-Jones, 1985; Anderson, Cole & Barrett, 2005); and birds only robbed nectar on very mature plants with thicker branches or when there was an artificial perch, e.g., stake (observed on camera trap). During focal observations, no birds were observed robbing nectar, thus we consider the impact of nectar robbing in this species as negligible.

Philoliche rostrata has also been observed to visit several other plant species apart from *E. shannonea* with similar floral traits such as species of *Gladiolus* (Iridaceae; Goldblatt & Manning,

1999), *Pelargonium* (Geraniaceae; Struck, 1997), *Geisshoriza, Ixia, Tritonia*, *Watsonia* (Irdidaceae; Goldblatt & Manning, 2000), *Leucospermum* (Proteaceae; Johnson, He & Pauw, 2014) and *Disa* (Orchidaceae; Johnson & Steiner, 1997). Many of those plants display a colouration similar to *E. shannonea*. The constricted corolla opening in LPF-pollinated plants, however, appears to be unique to the genus *Erica*.

Erica ampullacea is pollinated by the fly *Pr. westermanni*, which visited the flowers frequently making contact with both the anthers and the stigma while visiting and carried *Erica* pollen on its proboscis. The breeding system experiments confirmed that *E. ampullacea* cannot self-pollinate and thus relies on *Pr. westermanni* for pollination. Additionally, *Pr. westermanni* was carrying pollen of *Gladiolus debilis* on its thorax.

The lower seed set in the open control as compared to the facilitated outcrossing (Figure 1) observed in both species indicated that there might be pollen limitation in the study populations, which was confirmed by the high pollen limitation index in both species which may be caused by pollinator scarcity (Fernández et al., 2012) since there were few pollinator sightings. If pollen limitation is widespread in these species, this would increase the selective pressure to evolve floral traits that improve pollination efficiency (Ashman & Morgan, 2004).

In the literature, the term LPF is used widely and with varying definitions. There are studies that refer to flies with a proboscis as short as 7mm as LPFs (Devoto, Montaldo & Medan, 2006) and even instances where Bombyliidae (Johnson, 1992) or Acroceridae (Goldblatt, Manning & Bernhardt, 1997) are considered LPFs. Goldblatt & Manning (2000) defined a LPF as a fly with mouthparts of at least 15mm length. Thus, their proboscis is at least as long as their body, in many cases longer. This definition only applies to a few species of Tabanidae and Nemestrinidae in South Africa and explicitly excludes flies with medium length proboscis like *Pr. westermanni*. Thus, hereafter we will refer to *Pr. westermanni* and all fly species with a sucking proboscis shorter than 15mm as medium-proboscid flies (MPFs). They overlap in proboscis length with long-tongued bees and thus might also overlap in the plant species they visit, as for example *Gladiolus debilis* in this study and other *Gladiolus spp.* (Goldblatt, Manning & Bernhardt, 1998).

Prosoeca westermanni has also been observed as a visitor of other plant species in the Overberg region which have floral traits similar to those of *E. ampullacea*, including *E. fastigiata, Aristea spiralis* (Iridaceae)*, Adenandra villosa* (Rutaceae)*, Geissorhiza ovata* (Iridaceae)*, Pelargonium longicaule* (Geraniaceae)*, Gladiolus debilis* (Pauw, 2022), *E. vallis-gratiae* (https://www.inaturalist.org/observations/11124282), *Geisshoriza schinzii* (Iridaceae; Goldblatt,

Manning & Nänni, 2009) and *Disa fasciata* (Orchidaceae; Liltved & Johnson, 2012). In addition, *E. irbyana*, *E. cristata, E. gysbertii, E. hendricksei, E. ventricosa, E. curvifolia, E. intonsa, E. squarrosa, E. turrisbabylonica, E. coruscans, E. retorta* and *Brachysiphon acutus* (Penaeaceae) are also likely to be pollinated by *Pr. westermanni* or other similar MPFs such as *Pr. beckeri*, *Pr. handlirschi*, *Exoprosopa sp.* (pers. obs.), *Ph. arenoides* (Johnson, Alexandersson & Linder, 2003), *Psilodera valida* (Goldblatt, Manning & Bernhardt, 1997) or *Stuckenbergina africana* (Pauw, 2022), depending on the plant's flowering time and the time of year the flies are on the wing. As in (Johnson, Alexandersson & Linder, 2003)and (Pauw, 2022) we therefore propose to recognize the existence of one or more guilds of plants reliant on pollination by MPFs, which might have been the stepping-stone for the shift to long-proboscid fly pollination in some species. Those species share many traits with LPF-pollinated species, such as a straight floral tube with flaring petal lobes, white to pink colouration with darker nectar guides, and a lack of scent (Manning & Goldblatt, 1997). However, they differ in their tube length and have much shorter corollas adapted to the medium-length proboscis of their pollinators. There appear to be two peaks of flowering for MPF-pollinated plants which are during summer, like *E. ampullacea*, and in late autumn and winter, e.g., *E. gysbertii*. These differences in phenology might indicate another split within the MPF system depending on the season of fly activity.

Erica shannonea and *E. ampullacea* are extremely similar in colouration (Figure 3), which means their pollinators' visual preferences likely did not contribute to their divergence. The two species, however, differ in flower length and orientation. The corollas of *E. shannonea* match the proboscis of *Ph. rostrata* in length but are more than twice as long as the proboscis of *Pr. westermanni*, preventing it from accessing the nectar. Pollinator shifts have been identified as drivers for changes in flower tube length in other LPF-pollinated systems (e.g., (Johnson & Steiner, 1997; Anderson et al., 2014) and within the genus *Erica*, between *E. junonia* varieties (Newman & Johnson, 2021). In the case of *E. junonia*, both varieties have vertical flowers pollinated by Nemestrinidae. However, the varieties do not co-occur, and their pollinator shift might be linked to geographic variation in pollinator occurrence, while *E. shannonea* and *E. ampullacea* overlap in distribution and phenology. The LPF-pollinated *Tritoniopsis revoluta* (Iridaceae) has a bimodal distribution of tube lengths and there are co-occurring populations of long- and short-flowered individuals with divergent pollinators (Anderson et al., 2014). Thus, the diverging tube lengths in the focal *Erica* species might also have evolved in sympatry or allopatry with secondary contact as the result of a pollinator shift rather than a coevolutionary arms race between tube length and pollinator proboscis length (Whittall & Hodges, 2007).

Some LPFs, however, have been observed to visit flowers with short corollas (pers. obs.). Thus, divergence in tube length alone might not have been enough to exclude *Ph. rostrata* from *E. ampullacea* and the additional shift in flower orientation is necessary for complete reproductive isolation. The long-tubed *Erica shannonea* has a higher nectar sugar concentration than the shorter *E. ampullacea* which is a trend that has also been observed in other plants pollinated by long-proboscid insects where it promotes flower constancy for the long-tubed species (Johnson et al., 2017).

The difference in flower orientation between the two *Erica* species most likely ensures that only *Pr. westermanni* (Nemestrinidae) can visit *E. ampullacea*, since it has a swivelling proboscis (Karolyi et al., 2012) that can be inserted into the vertical flowers. *Ph. rostrata* (Tabanidae) on the other hand cannot swivel its proboscis (Karolyi et al., 2014) and therefore, it is expected to be restricted to plant species with horizontal flowers such as *E. shannonea*. There are some examples from other systems where changes in flower orientation coincide with pollinator shifts (e.g., (Campbell, Jürgens & Johnson, 2016; Xiang, Guo & Yang, 2021) but in the LPF pollination system, flower orientation as a driver for species divergence is a novel concept. Due to Nemestrinidae being able to swivel their proboscis, the difference in orientation alone would not suffice to exclude *Pr. westermanni* from visiting both the vertical and horizontal flowers. Thus, complete reproductive isolation is only realised in combination with different corolla lengths. It has been suggested that steep flower angles inaccessible to Tabanidae have evolved in almost half the plant species pollinated by Nemestrinidae (McCarren, Midgley & Johnson, 2022) which indicates that flower orientation might play an important role for divergence within the LPF pollination system and we expect to find more cases of pollinator shifts between tabanid and nemestrinid LPFs linked to flower orientation.

The combined differences in flower orientation and corolla length in *E. shannonea* and *E. ampullacea* are effective mechanical and ethological barriers preventing gene flow between the two species by excluding the other species' pollinator. The two *Erica* species therefore appear to have diverged from their common ancestor as a result of their pollinators' different biomechanical requirements, which may also facilitate the current sympatric coexistence of these species.

Acknowledgements

We wish to thank Thys de Villiers for granting permission to conduct research on his land and Betty Ann Illing for providing a base camp in Hermanus. Additionally, we thank Cape Nature (CN44-31-

2588) for permits. This study was funded by the South African National Research Foundation (Grant number: MND190724458797) and the UCT Science Faculty Research Committee.

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Chapter 4: Biomechanics of nectar feeding explain flower orientation in plants pollinated by long-proboscid flies

Abstract

The function of flower orientation is much-debated, with adaptation to pollinator mouthparts being a particularly compelling explanation, but also one that has lacked empirical support from broad-scale comparative studies. The two families of long-proboscid fly pollinators show similar hovering behavior while feeding on nectar but differ in the biomechanics of their proboscides which can be up to 80 mm in length: Tabanidae have a fixed forward-pointing proboscis while Nemestrinidae can swivel their proboscis downwards. We predicted that this difference has implications for the evolution of flower orientation. We established the flower angles of 156 South African plant species specialized for pollination by long-proboscid flies. Using a phylogenetically corrected analysis, we found that flowers pollinated by Tabanidae tend to be horizontally orientated, while those pollinated by Nemestrinidae tend to be more variable in orientation and more often vertically orientated. These results confirm the importance of pollinator biomechanics for the evolution of floral traits and highlight a potential mechanism of reproductive isolation between sympatric plant species pollinated by different long-proboscid fly families.

Introduction

Flower orientation is a key floral trait (Fenster, Armbruster & Dudash, 2009), but its function is not always readily apparent. It has been suggested, for example, that downward orientation (angle from vertical >90°) can protect nectar and pollen from rain (Aizen, 2003), but this may apply primarily to flowers with wide corolla openings (Tadey & Aizen, 2001; Huang, Takahashi & Dafni, 2002; Lin & Forrest, 2017; Yu et al., 2021). For some species downward orientation has been posited as a protection mechanism against adverse temperatures (Haverkamp et al., 2019; Xiang, Guo & Yang, 2021), while in others there is no apparent effect on temperature (Yu et al., 2021).

The most compelling explanations for flower orientation relate to its potential importance for accommodating the feeding behaviour and mouthparts of pollinators. Studies have shown effects of flower orientation on pollinator diversity (Wang, Xiao, et al., 2014) and behaviour (Ushimaru & Hyodo, 2005; Wang, Tie, et al., 2014; Johnson, Kiepiel & Robertson, 2020). In bilaterally symmetrical flowers, horizontal orientation (angle from vertical ≈90°) can enhance pollination accuracy (Fenster,

Armbruster & Dudash, 2009; Ushimaru et al., 2009), and such species even have the capacity to reorient after being damaged (Armbruster & Muchhala, 2020). Flower orientations that enhance accessibility for the main pollinator can also deter visitors that do not aid pollination (Fulton & Hodges, 1999; Gegear, Burns & Swoboda-Bhattarai, 2017) and some pollinators are even restricted to visiting flowers from certain angles; hawkmoths, for example, readily feed from horizontal and vertical (angle from vertical ≈0°) flowers but struggle to visit downward-facing flowers (Haverkamp et al., 2019). Thus, floral orientation might act as a visitation barrier for some pollinator species and result in behavioural and mechanical reproductive isolation (Grant, 1992, 1994). So far, most studies of floral orientation have focused on a single species or a few species from the same genus (Johnson et al., 2002; Xiang, Guo & Yang, 2021). Other floral traits, such as scent or colour, can influence pollinators by acting synergistically with orientation (Campbell, Jürgens & Johnson, 2016; Gegear, Burns & Swoboda-Bhattarai, 2017; del Carmen Salas-Arcos et al., 2019). Therefore, a broad-scale and phylogenetically corrected study of plants pollinated by animals that differ in proboscis biomechanics is needed to confirm the functional importance of flower orientation.

Long-proboscid flies (LPFs) are key pollinators of the southern African flora and occur in two families: Tabanidae and Nemestrinidae (Goldblatt & Manning, 2000). While Tabanidae have fixed horizontal proboscides (Karolyi et al., 2014), Nemestrinidae normally trail their proboscis beneath the thorax, and during foraging the membranous labial base allows them to swivel the proboscis forward into a vertical or horizontal position (Figure 1a, Karolyi et al., 2012). This suggests that Tabanidae might be restricted to feeding efficiently from flowers with angles close to horizontal (Figure 1b), while Nemestrinidae might be able to feed efficiently from flowers with a wider range of angles, including vertical. Thus, we expected LPF-pollinated plants to differ in their floral orientation depending on the pollinator family.

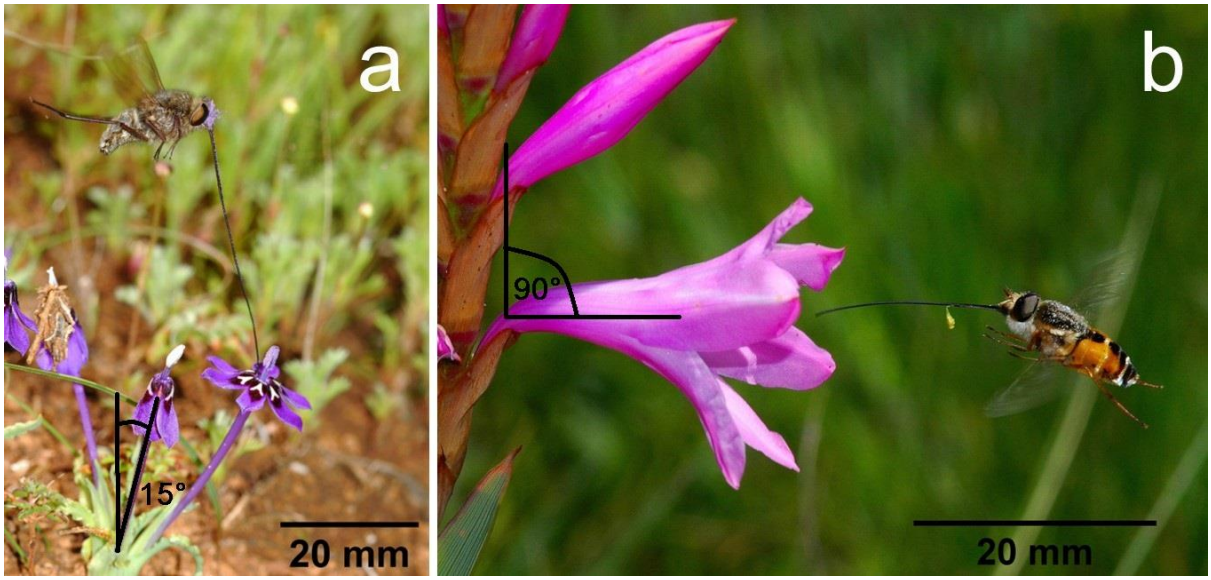

Figure 1 a) The nemestrinid *Prosoeca marinusi* swivels its proboscis downwards to feed on the near vertical flowers of *Lapeirousia oreogena*. b) The tabanid *Philoliche aethiopica* has a fixed forward-facing proboscis with which it feeds from the horizontal flowers of *Watsonia lepida.* Photos: SD Johnson.

Methods

We compiled pollinator data from available scientific literature for 156 plant species (Table S1) that are pollinated by LPFs, including 122 pollinated by Nemestrinidae, and 40 pollinated by Tabanidae. Six plant species are pollinated by both families and were excluded from statistical tests. For each plant species, a person who did not know the applicable pollinator family measured the flower angles from vertical (divergence of the floral tube from the vertical axis, see Fig 1) using a protractor on photographs and drawings included in the original articles or, in cases where this was not possible, from photographs available at iNaturalist (inaturalist.org). We selected photographs where the flowers could be seen from the side, so that the flower angle was not distorted by the angle of view, and the most typical angle was recorded when there were several pictures available. Any measurement errors would not be biased towards one or the other group of flowers. The data were then analysed using R (R Core Team, 2015). We compared the flower angles between species visited by Nemestrinidae and those visited by Tabanidae using a Mann-Whitney U test. Further, we compared the variability of flower angles between the fly families using an F-test. Bimodality was tested for both families using Hartigan's dip test (Hartigan & Hartigan, 1985). For 11 genera that had visitors from both families, differences in flower angles from vertical for tabanid- versus

nemestrinid-visited species were compared using a paired t-test (van Kleunen et al., 2008). We also created a phylogenetic tree for 79 of the plant species from OneTwoTree (Drori et al., 2018) and used it to run a phylogenetic ANOVA (Pennell et al., 2014).

Table S1 Plant species visited by nemestrinid and tabanid flies, and their flower angles in degrees from vertical (n=155). Abbreviations: *Philoliche* (Ph.), *Prosoeca* (Pr.), *Moegistorhynchus* (M.), *Stenobasipteron* (S.)

Plant species	Fly visitor	Fly family	Angle	Reference
Aristea spiralis	*Ph. gulosa*	Tabanidae	90	[1]
Babiana curviscapa	*Pr. peringuey*	Nemestrinidae	40	[1–3]
Babiana dregei	*Pr. peringuey*	Nemestrinidae	50	[2]
Babiana ecklonii	*Pr. peringueyi*	Nemestrinidae	30	[1,3]
Babiana engysiphon	*Pr. peringueyi*	Nemestrinidae	20	[3]
Babiana flabellifolia	*Pr. marinusi*	Nemestrinidae	0	[2,4]
Babiana framesii	*Pr. peringuey + marinusi*	Nemestrinidae	40	[2,3]
Babiana geniculata	*Pr. peringueyi*	Nemestrinidae	0	[3]
Babiana latifolia	*Pr. peringueyi*	Nemestrinidae	0	[3]
Babiana pubescens	*Pr. peringuey*	Nemestrinidae	0	[2,3]
Babiana rigidifolia	*Pr. peringueyi*	Nemestrinidae	45	[3]
Babiana tubiflora	*M. longirostris*	Nemestrinidae	30	[3]
Babiana tubulosa	*M. longirostris*	Nemestrinidae	20	[5]
Babiana vanzyliae	*Pr. marinusi + Pr. sp. nov. 2*	Nemestrinidae	20	[3]
Barleria obtusa	*S. wiedemanni*	Nemestrinidae	45	[6]
Brownleea coerulea	*S. wiedemanni*	Nemestrinidae	110	[1]
Brownleea macroceras	*Pr. ganglbaueri*	Nemestrinidae	90	[7]
Brunsvigia grandiflora	*Pr. ganglbaueri*	Nemestrinidae	90	[1]
Brunsvigia gregaria	*Pr. ganglbaueri*	Nemestrinidae	90	[1]
Brunsvigia radulosa	*Ph. aethiopica*	Tabanidae	90	[8]
Brunsvigia striata	*Pr. ganglbaueri*	Nemestrinidae	70	[1]
Cephalaria galpiniana	*Pr. ganglbaueri*	Nemestrinidae	20	[7]
Cyrtanthus leptosiphon	*Pr. longipennis*	Nemestrinidae	30	[9]
Delphinium caeruleum	*Nemetrinus sp.*	Nemestrinidae	90	[10]

Table S1 continued Plant species visited by nemestrinid and tabanid flies, and their flower angles in degrees from vertical (n=155). Abbreviations: *Philoliche* (Ph.), *Prosoeca* (Pr.), *Moegistorhynchus* (M.), *Stenobasipteron* (S.)

Plant species	Fly visitor	Fly family	Angle	Reference
Disa harveyana harveyana	*Ph. rostrata*	Tabanidae	90	[11]
Disa harveyana longicalcarata	*Ph. rostrata*	Tabanidae	80	[11]
Disa karooica	*Ph. gulosa*	Tabanidae	85	[12]
Disa nervosa	*Ph. aethiopica*	Tabanidae	110	[13]
Disa nivea	*Pr. ganglbaueri*	Nemestrinidae	90	[14]
Disa oreophila	*Pr. ganglbaueri*	Nemestrinidae	90	[7]
Disa pulchra	*Ph. aethiopica*	Tabanidae	90	[15]
Disa scullyi	*Pr. ganglbaueri*	Nemestrinidae	130	[16]
Embothrium coccineum	*Trichophthalma niveibarbis + philippi*	Nemestrinidae	110	[17]
Erica ampullacea	*Pr. westermanni*	Nemestrinidae	0	[18]
Erica aristata	*Pr. rubicunda*	Nemestrinidae	90	[19]
Erica irrorata	*M. sp. nov.*	Nemestrinidae	90	[20]
Erica jasminiflora	*Ph. rostrata*	Tabanidae	90	[21]
Erica junonia junonia	*M. perplexus*	Nemestrinidae	20	[20]
Erica junonia minor	*M. sp. nov.*	Nemestrinidae	20	[20]
Erica shannonea	*Ph. rostrata*	Tabanidae	90	[18]
Geissorhiza bonaspei	*Ph. rostrata + Pr. nitida*	Nemestrinidae + Tabanidae	90	[1]
Geissorhiza confusa	*Ph. rostrata*	Tabanidae	55	[1,11]
Geissorhiza exscapa	*M. longirostris*	Nemestrinidae	45	[5]
Geissorhiza fourcadei	*Pr. longipennis*	Nemestrinidae	40	[9]
Gladiolus angustus	*M. longirostris*	Nemestrinidae	70	[5,22]

Table S1 continued Plant species visited by nemestrinid and tabanid flies, and their flower angles in degrees from vertical (n=155). Abbreviations: *Philoliche* (Ph.), *Prosoeca* (Pr.), *Moegistorhynchus* (M.), *Stenobasipteron* (S.)

Plant species	Fly visitor	Fly family	Angle	Reference
Gladiolus bilineatus	*Pr. longipennis*	Nemestrinidae	90	[9,22]
Gladiolus calcaratus	*Pr. robusta*	Nemestrinidae	70	[22]
Gladiolus carneus	*Ph. rostrata + Pr. nitidula*	Nemestrinidae + Tabanidae	90	[22]
Gladiolus cylindraceus	*M. perplexus*	Nemestrinidae	30	[20]
Gladiolus debilis	*Pr. westermanni*	Nemestrinidae	50	[23]
Gladiolus engysiphon	*Pr. longipennis*	Nemestrinidae	30	[9,22]
Gladiolus floribundus	*Ph. rostrata + gulosa*	Tabanidae	70	[22]
Gladiolus inflatus	*M. sp. nov.*	Nemestrinidae	90	[20]
Gladiolus macneilii	*S. wiedmanni*	Nemestrinidae	45	[6,22]
Gladiolus martleyi	*Pr. longipennis*	Nemestrinidae	40	[9]
Gladiolus microcarpus	*Pr. ganglbaueri*	Nemestrinidae	90	[22]
Gladiolus monticola	*Ph. rostrata + Pr. nitidula*	Nemestrinidae + Tabanidae	90	[22]
Gladiolus mortonius	*Pr. ganglbaueri*	Nemestrinidae	90	[22]
Gladiolus oppositiflorus	*Pr. ganglbaueri*	Nemestrinidae	90	[9,22]
Gladiolus rhodantus	*M. sp. nov.*	Nemestrinidae	90	[22,24]
Gladiolus undulatus	*Ph. rostrata*	Tabanidae	70	[22]
Gladiolus varius	*Pr. ganglbaueri + robusta*	Nemestrinidae	60	[1,22]
Gladiolus vigilans	*Ph. rostrata*	Tabanidae	90	[22]
Gladiolus virgatus	*Ph. rostrata*	Tabanidae	90	[22]
Hesperantha brevicaulis	*S. wiedemanni*	Nemestrinidae	45	[1,6]
Hesperantha grandiflora	*Pr. ganglbaueri*	Nemestrinidae	90	[1,25]
Hesperantha huttonii	*S. wiedemanni*	Nemestrinidae	45	[6,25]
Hesperantha latifolia	*Pr. peringuey*	Nemestrinidae	0	[2,25]

Table S1 continued Plant species visited by nemestrinid and tabanid flies, and their flower angles in degrees from vertical (n=155). Abbreviations: *Philoliche* (Ph.), *Prosoeca* (Pr.), *Moegistorhynchus* (M.), *Stenobasipteron* (S.)

Plant species	Fly visitor	Fly family	Angle	Reference
Hesperantha pauciflora	*Pr. sp. nov. 2*	Nemestrinidae	10	[25]
Hesperantha scopulosa	*Pr. ganglbaueri*	Nemestrinidae	60	[2,25]
Hesperantha woodii	*Pr. ganglbaueri*	Nemestrinidae	50	[2,25]
Hypoestes arista	*S. wiedemanni*	Nemestrinidae	90	[6]
Impatiens hochstetteri hochstetteri	*S. wiedemanni*	Nemestrinidae	45	[6]
Isoglossa hypoestiflora	*S. wiedemanni*	Nemestrinidae	90	[6]
Ixia longituba	*Ph. gulosa*	Tabanidae	90	[1]
Ixia paniculata	*M. longirostris*	Nemestrinidae	25	[1,5,11]
Ixia paucifolia	*Ph. gulosa*	Tabanidae	60	[1]
Lapeirousia anceps	*M. longirostris*	Nemestrinidae	30	[11,26,27]
Lapeirousia dolomitica	*Pr. peringuey*	Nemestrinidae	30	[2,26]
Lapeirousia fabricii	*M. longirostris*	Nemestrinidae	45	[5,26]
Lapeirousia jacquinii	*Pr. peringuey + marinusi*	Nemestrinidae	30	[2,26]
Lapeirousia oreogena	*Pr. marinusi*	Nemestrinidae	0	[2,26]
Lapeirousia pyramidalis	*Pr. peringuey*	Nemestrinidae	35	[2,26]
Lapeirousia silenoides	*Pr. peringuey*	Nemestrinidae	20	[2,26]
Lapeirousia violacea	*Pr. peringuey*	Nemestrinidae	30	[2,26]
Leucospermum tottum tottum	*Ph. rostrata*	Tabanidae	90	[28]
Lobelia coronopifolia	*Ph. gulosa*	Tabanidae	90	[1]
Lobelia preslii	*Pr. ganglbaueri*	Nemestrinidae	90	[7]
Nerine angustifolia	*Pr. robusta*	Nemestrinidae	70	[1]
Nerine bowdenii	*Pr. ganglbaueri*	Nemestrinidae	60	[1]
Nerine humilis	*Pr. longipennis*	Nemestrinidae	40	[9]

Table S1 continued Plant species visited by nemestrinid and tabanid flies, and their flower angles in degrees from vertical (n=155). Abbreviations: *Philoliche* (Ph.), *Prosoeca* (Pr.), *Moegistorhynchus* (M.), *Stenobasipteron* (S.)

Plant species	Fly visitor	Fly family	Angle	Reference
Nivenia binata	*Pr. westmannii + sp.*	Nemestrinidae	10	[29]
Nivenia stenosiphon	*Pr. ganglbaueri*	Nemestrinidae	20	[29]
Ocimun tubiformis	*S. wiedemanni*	Nemestrinidae	100	[1,6]
Pelargonium alpinum	*M. perplexus*	Nemestrinidae	30	[20]
Pelargonium articulatum	*Ph. rostrata*	Tabanidae	70	[30]
Pelargonium barklyi	*Ph. rostrata*	Tabanidae	50	[30]
Pelargonium betulinum	*Ph. lateralis*	Tabanidae	50	[30]
Pelargonium capitatum	*Ph. lateralis*	Tabanidae	60	[30]
Pelargonium carneum	*Pr. ganglbaueri + longipennis*	Nemestrinidae	30	[1,9]
Pelargonium cucullatum	*Ph. rostrata*	Tabanidae	60	[11]
Pelargonium dipetalum	*Pr. longipennis*	Nemestrinidae	60	[9]
Pelargonium echinatum	*Pr. peringuey*	Nemestrinidae	70	[30]
Pelargonium gracillimum	*Pr. ganglbaueri*	Nemestrinidae	30	[1]
Pelargonium incrassatum	*Pr. peringuey*	Nemestrinidae	35	[2,26]
Pelargonium laevigatum	*Ph. rostrata + gulosa + Pr. longipennis*	Tabanidae + Nemestrinidae	70	[1,30]
Pelargonium longicaule	*M. longirostris*	Nemestrinidae	40	[5,26]
Pelargonium magenteum	*Pr. peringuey*	Nemestrinidae	40	[2,26]
Pelargonium myrrhifolium	*Ph. rostrata*	Tabanidae	55	[11]
Pelargonium peltatum	*Ph. rostrata + gulosa*	Tabanidae	70	[1]
Pelargonium pinnatum	*Pr. longipennis*	Nemestrinidae	40	[9]
Pelargonium reniforme	*Pr. longipennis*	Nemestrinidae	70	[9]
Pelargonium sericifolium	*Pr. peringuey*	Nemestrinidae	35	[2,26]
Pelargonium stipulaceum	*Ph. gulosa*	Tabanidae	70	[12]

Table S1 continued Plant species visited by nemestrinid and tabanid flies, and their flower angles in degrees from vertical (n=155). Abbreviations: *Philoliche* (Ph.), *Prosoeca* (Pr.), *Moegistorhynchus* (M.), *Stenobasipteron* (S.)

Plant species	Fly visitor	Fly family	Angle	Reference
Pelargonium suburbanum	*M. longirostris*	Nemestrinidae	30	[11]
Pelargonium tetragonum	*Pr. longipennis*	Nemestrinidae	40	[9]
Pelargonium zonale	*M. longirostris + Pr. longipennis*	Nemestrinidae	40	[9,26]
Plectranthus ambiguus	*S. wiedemanni*	Nemestrinidae	100	[6,31]
Plectranthus ciliatus	*S. wiedemanni*	Nemestrinidae	120	[6]
Plectranthus ecklonii	*S. wiedemanni + Ph. sp. + Pr. sp.*	Nemestrinidae + Tabanidae	110	[31]
Plectranthus fruticosus	*S. wiedemanni*	Nemestrinidae	90	[6]
Plectranthus hilliardiae	*S. wiedemanni*	Nemestrinidae	90	[6,31]
Plectranthus praetermissus	*S. wiedemanni*	Nemestrinidae	90	[6]
Plectranthus reflexus	*S. wiedemanni*	Nemestrinidae	110	[6]
Plectranthus saccatus	*S. wiedemanni*	Nemestrinidae	90	[6]
Plectranthus zuluensis	*S. wiedemanni*	Nemestrinidae	90	[6]
Plumbago auriculata	*Ph. aethiopica*	Tabanidae	45	[32]
Protea punctata	*Pr. longipennis*	Nemestrinidae	60	[33]
Pteronia incana	*Pr. sp. nov. 2*	Nemestrinidae	0	[34]
Romulea hantamensis	*Pr. marinusi*	Nemestrinidae	0	[2,4]
Romulea kamisensis	*Pr. peringueyi*	Nemestrinidae	0	[4]
Romulea syringodeoflora	*Pr. sp. nov. 2*	Nemestrinidae	0	[34]
Roscoea purpurea	*Ph. longirostris*	Tabanidae	45	[35]
Scabiosa columbaria	*Pr. ganglbaueri*	Nemestrinidae	10	[7]
Sparaxis metelerkampiae	*Pr. peringueyi*	Nemestrinidae	30	[36]
Sparaxis variegata	*Pr. peringuey*	Nemestrinidae	20	[2]
Streptocarpus formosus	*S. wiedemanni*	Nemestrinidae	90	[6]

Table S1 continued Plant species visited by nemestrinid and tabanid flies, and their flower angles in degrees from vertical (n=155). Abbreviations: *Philoliche* (Ph.), *Prosoeca* (Pr.), *Moegistorhynchus* (M.), *Stenobasipteron* (S.)

Plant species	Fly visitor	Fly family	Angle	Reference
Syncolostemon densiflorus	*Ph. aethiopica*	Tabanidae	90	[37]
Syncolostemon macranthus	*Pr. sp.*	Nemestrinidae	90	[37]
Syncolostemon rotundifolius	*Ph. aethiopica + Pr. sp.*	Nemestrinidae + Tabanidae	90	[37]
Theilera guthriei	*Pr. longipennis*	Nemestrinidae	10	[9]
Tritonia flabellifolia	*Ph. rostrata + gulosa*	Tabanidae	90	[1]
Tritonia lancea	*M. longirostris*	Nemestrinidae	30	[5]
Tritonia pallida	*Ph. rostrata*	Tabanidae	90	[1]
Tritoniopsis antholyza	*Pr. longipennis*	Nemestrinidae	80	[9]
Tritoniopsis revoluta	*Pr. ganglbaueri + longipennis*	Nemestrinidae	30	[1,9]
Watsonia borbonica	*Ph. rostrata + Pr. nitidula*	Nemestrinidae + Tabanidae	90	[1]
Watsonia densiflora	*Ph. aethiopica*	Tabanidae	90	[13]
Watsonia lepida	*Ph. aethiopica*	Tabanidae	90	[15]
Watsonia paucifolia	*M. sp. nov.*	Nemestrinidae	90	[1]
Watsonia wilmsii	*Pr. ganglbaueri + robusta*	Nemestrinidae	60	[1]
Zaluzianskya microsiphon	*Pr. ganglbaueri*	Nemestrinidae	60	[1,38]

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Results

Flower angles, measured from the vertical, for species pollinated by Nemestrinidae range from 0°-130° with a median of 45°, and for species pollinated by Tabanidae from 45°-110° with a median of 90°. The flower angles differ significantly between fly families ($Z = 3.835$, $p < 0.001$). The flower angles for Nemestrinidae have a bimodal distribution ($Dip = 0.087$, $p < 0.001$, Figure 2) with modes at 34° and 90°, while the angles of flowers visited by Tabanidae have a unimodal distribution with the mode at 89° ($Dip = 0.064$, $p = 0.326$, Figure 2). The F-test comparing variances between the fly families shows that the spread of flower angles is significantly larger in species pollinated by Nemestrinidae (coefficient of variation = 0.63), than in those pollinated by Tabanidae (coefficient of

variation = 0.22, F =3.850, p< 0.001). The pairwise comparison between the difference of the flower angles from vertical within plant genera shows a significant difference between the two fly families (t=2.904, df= 10, p=0.016) with a mean difference in flower angles of 19.6° between them. The phylogenetic ANOVA indicates that flower angles are related to fly families even after accounting for phylogenetic signal (F = 7.834, p = 0.0065, p given phylogeny = 0.0494, Table S2).

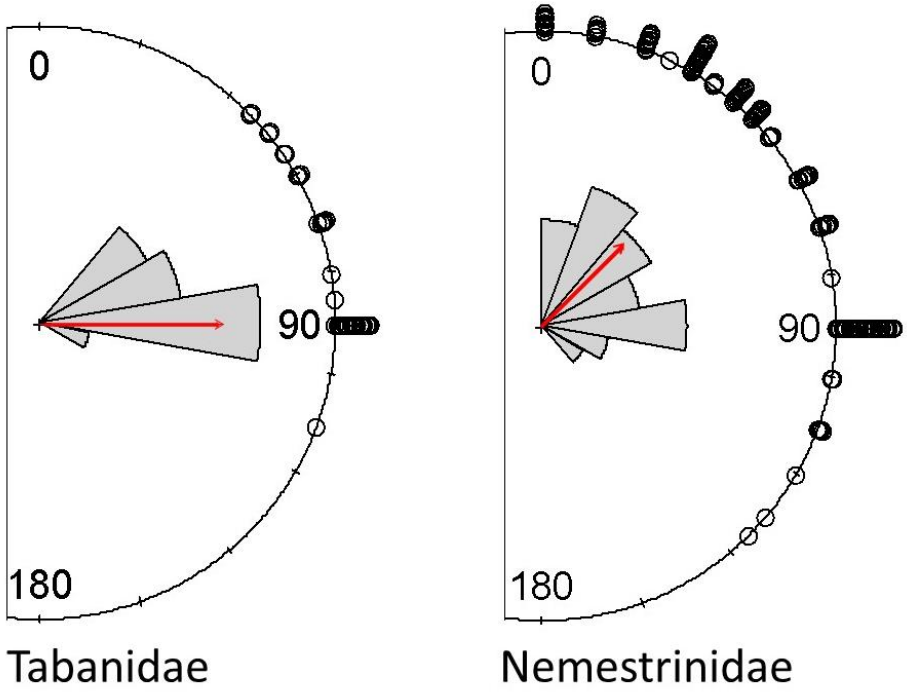

Figure 2 Circular plots of flower angles from vertical for plant species pollinated by Nemestrinidae (n= 121) and Tabanidae (n= 40), 0° being vertical and 90° being horizontal. In the rose diagram, the radius of a segment is the square root of relative frequency. The red arrows indicate the median.

Table S2 Analysis of variance table from a phylogenetic ANOVA for the flower angle from vertical of 79 plant species pollinated by long-proboscid flies in response to family of the pollinator (Tabanidae or Nemestrinidae)

	Degrees of freedom	Sum of Squares	Mean Squares	F	p	p given phylogeny
Fly family	1	7370	7369.6	7.834	0.0065	0.0494
Residuals	77	72438	940.8			

Discussion

Flower morphology can reflect the biomechanics of pollinators, including flight abilities, suction ability, and morphology of the mouthparts (e.g., Karolyi et al., 2013; Rico-Guevara et al., 2021). Here we specifically considered the relation between flower orientation and the ability of LPF pollinators to swivel their proboscis. Tabanidae mostly visit flowers with angles close to horizontal which is what we expected given that their proboscis is fixed in a horizontal position. They do sometimes visit flowers with steeper angles of up to 45° from vertical (Ferrero et al., 2009), but to do so they must bend their entire head or flex their proboscis while inserting it into curved floral tube while perched. Some tabanid-pollinated species with steeper flower angles have evolved a landing platform for the fly to sit on while bending its head and pollinating them (e.g., *Roscoea purpurea*; Paudel, 2015). However, the angle of proboscis insertion into flowers by hovering Tabanidae is always close to horizontal (Figure 1b).

The flower angles of plants visited by Nemestrinidae exhibit a much wider spread with a bimodal distribution. Nemestrinidae can swivel their proboscis into any position between vertical and horizontal which explains the wide range of flower angles they visit. The bimodal distribution of flower angles might be the result of two opposing strategies applied by the plants: Flowers with angles closer to vertical may be considered more specialised for Nemestrinidae as they exclude Tabanidae as pollinators, while flowers with angles closer to horizontal may be more generalized as they can make use of both fly families as pollinators. The six species visited by both families that we excluded from the statistical analysis all have horizontally orientated flowers.

We have shown that across different plant genera, tabanid-pollinated flowers exhibit a more horizontal flower orientation than do nemestrinid-pollinated flowers of the same genus, which indicates that this is a general trend across the whole LPF pollination syndrome. This is further supported by the observation that flower angle and fly family are still related after accounting for phylogenetic relations among the plant species. Flowers with orientation close to vertical can experience particular costs. They flood with rainwater more easily, which can dilute the nectar and wash away the pollen (Aizen, 2003). However, due to the surface tension of the raindrops, flowers with small openings, as is the case with most LPF-pollinated flowers, are unlikely to be affected by this phenomenon. Flowers with angles closer to vertical can also heat up more during hot days causing lower pollen germination success (Haverkamp et al., 2019). Additionally, vertical orientation might decrease pollination accuracy specifically in bilaterally symmetrical flowers due to the pollinator being able to visit from any direction (Fenster, Armbruster & Dudash, 2009; Ushimaru et

al., 2009), which could be offset by evolving radial symmetry and elaborate nectar guides (e.g., *Lapeirousia oreogena*, Figure 1a).

This study confirms the importance of pollinator biomechanics for floral evolution (Xiang, Guo & Yang, 2021) and suggests that flower orientation could be an important factor in pollination system specialization, reproductive isolation, and reduction of reproductive interference among plant species. Further work could include experimental manipulation of flower angles of plants pollinated by LPFs to assess the effect on pollination success and reproductive isolation.

Acknowledgements

We wish to thank Thaabiet Parker for assistance with the phylogenetic analysis.

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Chapter 5: Pollen transfer efficiency in *Erica* depends on pollinator type

Abstract

Pollen transfer efficiency (PTE) (the proportion of pollen removed from flowers that reaches stigmas) is expected to vary with type of pollinator. The ancestral pollinators of the genus *Erica* are bees (which consume pollen and should thus lower PTE of plants,) but during its radiation in the Cape, several independent shifts to both sunbird and long-proboscid fly (LPF) pollinators which do not consume pollen have taken place. Differences in PTE could be one of the selection factors driving morphological changes associated with these pollinator shifts. We collected PTE for five *Erica* species from each of the three pollination syndromes and compared their values in relation to pollination syndrome and anther exsertion. LPF- and bird-pollinated species had higher PTE in comparison to bee-pollinated species. Species with inserted anthers had higher PTE than those with exserted anthers. This confirms that sunbirds and LPFs are more efficient pollinators than bees. Additionally, the study suggests that anther exsertion is associated with lower PTE and thus costly.

Introduction

Pollen transfer efficiency (PTE; the proportion of pollen removed from flowers that reaches stigmas) is expected to vary with pollinator type (Laverty & Plowright, 1988; Shuttleworth & Johnson, 2008; Willmer, Cunnold & Ballantyne, 2017). For example, it was shown that PTE was higher in a hummingbird-adapted flower than in a bee-adapted flower, though it was unclear if this was related to an effect of pollinators as hummingbirds and bees did not differ in their per visit efficiencies in terms of pollen transfer (Castellanos, Wilson & Thomson, 2003). In general, increased PTE could explain why shifts to pollinators that require larger and more costly flowers like birds and potentially long-proboscid flies (LPFs) have occurred (Castellanos, Wilson & Thomson, 2003).

The genus *Erica* is highly suitable for studying differences in PTE between pollinator groups because of its species diversity (ca. 700 in South Africa) and diversity of pollinators (Rebelo, Siegfried & Oliver, 1985). The ancestral pollinators of *Erica* are bees (Pirie, Oliver & Bellstedt, 2011) but during

its radiation in the Cape, several independent shifts to both sunbird and LPF pollination have taken place in the genus (Pirie, Oliver & Bellstedt, 2011). Nevertheless, it is not understood what mechanisms have facilitated these shifts and differences in PTE could be one of the selection factors driving morphological changes associated with pollinator shifts (Kobayashi, Inoue & Kato, 1997). While it has been suggested that pollen transferred by bees is lower in quality than by generalist birds (Diller, Castañeda-Zárate & Johnson, 2022), there have been no studies comparing pollen transfer efficiencies of bees with African nectarivorous birds or long-proboscid flies (LPFs), which are important pollinator groups in southern Africa (Goldblatt & Manning, 2000).

Erica species pollinated by bees or other small insects are the largest group in the genus (Rebelo, Siegfried & Oliver, 1985). They typically produce many small flowers with low volumes of nectar (Bouman, Steenhuisen & van der Niet, 2017). Bees as pollinators have lower pollen carryover between plants compared to other pollinators which is most likely due to their pollen grooming behaviour (Castellanos et al., 2003; Holmquist et al., 2012). Thus, we expect the bee-pollinated species to have lower PTE than ericas with other pollinators.

Adaptations to sunbird pollination seen in *Erica* species include long corollas in a variety of colours (Rebelo & Siegfried, 1985), a higher volume (Rebelo, Siegfried & Crowe, 1984) of more dilute nectar (Nicolson, 2002), the lack of scent or nectar guides (Cronk & Ojeda, 2008) and the provision of a perch (Siegfried, Rebelo & Prŷs-Jones, 1985). These adaptations likely make bird pollination more energetically expensive for the plants and thus, it is expected that bird pollination should confer other fitness benefits (Stiles, 1978). These could include birds moving greater distances between plants while foraging, higher pollen carryover and limited pollen grooming (Krauss et al., 2017). Therefore, we expect higher PTE in *Erica* species pollinated by sunbirds.

Ericas pollinated by LPFs have long sticky corollas (McCarren, Coetzee & Midgley, 2021)with a narrow opening and flaring lobes (Rebelo, Siegfried & Oliver, 1985). They are unscented, typically produce nectar high in volume and concentration (Goldblatt & Manning, 2000; McCarren et al., 2022), and are white to pink in colour with reflection in the ultraviolet range (McCarren, Midgley & Coetzee, 2021). Reflecting ultraviolet light can make flowers more vulnerable to damage by UV-B radiation due to the lack of protection by ultraviolet-absorbing compounds (Llorens et al., 2015). These traits might confer higher costs to LPF-pollinated plant species since they need to allocate more resources to produce sticky, large flowers, and higher amounts of nectar. Additionally, LPFs visit *Erica* flowers infrequently, resulting in low pollination rates (McCarren et al., 2022). Investing in fewer, more costly flowers in both bird- and LPF-pollinated *Erica* species might incur the same costs as investing in many small flowers in bee-pollinated *Erica* species (Arendse, 2014). Or there could be

other fitness benefits to offset the costs. Few benefits of being specialised for pollination by LPFs have been recorded. They could be mediated by the relative local abundance of different pollinator groups. Further, LPFs are also not known to groom pollen off their bodies. In a different plant genus growing in forested areas, limited between-population foraging was observed for LPFs leading to lower gene flow compared to bird pollination (Hughes et al., 2007). The more open landscape of the Fynbos and small population sizes of LPF-pollinated *Erica* species might, however, lead to LPFs moving larger distances to find enough nectar. Additionally, *Erica* pollen is deposited on a small area at the base of the LPF proboscis which might increase pollination accuracy (Armbruster et al., 2009). Thus, we also expect a higher PTE for *Erica* species pollinated by LPFs compared to bee-pollinated species.

Further, many *Erica* species have exserted anthers, which appears to be a trait that evolved independently in multiple lineages (Pirie, Oliver & Bellstedt, 2011). *Erica* species with exserted anthers should gain a benefit from this trait in order for it to evolve. The function of exserted anthers, however, is yet to be uncovered. Nevertheless, it has been hypothesized that more exserted anthers in *Erica* might enhance male function by placing pollen further on the pollinator's body which could increase the chance of placing pollen on a stigma during subsequent visits (Ojeda et al., 2016). Thus, we expect higher PTE for *Erica* species with exserted anthers compared to species with included anthers.

This study aims to (a) compare PTE between bee-, bird- and LPF-pollinated *Erica* species, and (b) compare PTE between *Erica* species with exserted and included anthers. We expect higher PTE in species pollinated by sunbirds and LPFs compared to bees, and that exserted anthers convey higher PTE.

Methods

A total of 15 *Erica* species were sampled in the Cape Floristic Region of South Africa with five species from the three pollination syndromes: bird, LPF and bee (Table 1). Syndrome classification was based on flower morphology and confirmed by literature (Rebelo, Siegfried & Oliver, 1985; Lombardi, 2014; van der Niet et al., 2014; Bouman, Steenhuisen & van der Niet, 2017; Lombardi et al., 2021; McCarren et al., 2022) and pollinator observations. Six of these species have exserted anthers, with three of them being bee-pollinated and three bird-pollinated. For each species, ten unvisited flowers which can be recognised by their intact anther ring (Geerts & Pauw, 2011) and 20

flowers in late anthesis, whose corollas had begun to wilt (and had therefore had ample opportunity to be pollinated), were randomly collected from different individuals. We separated the anthers of the undisturbed flowers and the anthers and stigma of flowers in late anthesis and kept them individually in Eppendorf tubes. In the laboratory, the anthers were suspended in 1 ml ethanol and stained with fuchsin. The pollen suspension was homogenised with a vortex and then immediately four sample drops of each 20 µl were placed on a slide to count the pollen grains under a Leica DM500 compound microscope at 100 ✕ magnification.

The stigmas were mixed with molten fuchsin gel and mounted on a microscope slide using a cover slip. Then the deposited pollen was counted under a Leica DM500 compound microscope at 100 ✕ magnification. The flowers were mostly collected at sites where no other *Erica* species with the same pollinator were in flower at the time, except for some bee-pollinated species which co-flowered with no more than one other bee-pollinated *Erica*. However, even when sharing pollinators, the high levels of flower constancy exhibited by bees cause high pollen purity (i.e., pollen from only one species) on the stigmas of co-occurring *Erica* species (van der Niet, Pires & Steenhuisen, 2020). Thus, we assumed that the pollen we counted on the stigmas was monospecific.

Since most *Erica* species produce pollen in tetrads (Wrońska-Pilarek, Szkudlarz & Bocianowski, 2018), the number of pollen in the anthers and on the stigmas was further multiplied by four to calculate the total number of pollen grains, except for *E. cristata*, *E. ericoides* and *E. fastigiata* since those species produce pollen monads. Pollen removal was calculated per species by subtracting the mean pollen remaining in disturbed anthers from the mean pollen produced in unvisited anthers. Pollen transfer efficiency (PTE) was calculated for each species following the formula PTE= pollen deposition/pollen removal (Johnson, Neal & Harder, 2005). Statistical analyses were carried out in R (R Core Team, 2015) by fitting generalised linear models with negative binomial error structure to the data and testing for significance with a χ^2 test. We first explored pollen deposition (the response variable) in relation to pollination syndrome as explanatory variable with pollen removal as a covariate and no random effects. A Tukey's post hoc test was used to identify the differences between the pollination syndromes. We then repeated the same model with anther exsertion as the explanatory variable and both pollen removal and pollination syndrome as covariates. Since no LPF-pollinated flowers had exserted anthers, those species were excluded from this model and anther exsertion was only modelled for the bird- and bee-pollinated species.

Results

Almost all sampled flowers had at least some pollen deposited on their stigma (98.3%) and some pollen remaining in their anthers in late anthesis (85.0%). The recorded PTE values (Table 1) ranged from 0.1% to 7.5%. The mean number of pollen grains deposited after adjusting for removal was 101±26 (±standard deviation) for bird-pollinated species, 135±34 for LPF-pollinated species and 30±8 for bee-pollinated species. Species with exserted anthers had adjusted means for pollen deposition of 34±8 pollen grains, and species with included anthers 98±28 pollen grains. The model exploring pollen deposition in response to pollination syndrome with adjustment for pollen removal showed a significant association of pollen deposition with pollination syndrome (χ^2 = 7.75, p= 0.021, Figure 1). The post-hoc test showed that this difference was caused by the bee-pollinated species having less pollen deposited compared to both bird- (Z= 2.98, p= 0.008) and LPF-pollinated species (Z= -2.56, p= 0.029). There was no difference in pollen deposition between bird- and LPF-pollinated species (Z= -0.49, p= 0.878). In the model with pollen deposition in response to anther exsertion with adjustment for both pollen removal and pollination syndrome, pollen deposition was lower for species with exserted anthers than species with included anthers (χ^2 = 4.97, p= 0.026) (Figure 2).

Table 1: Raw data for number of pollen grains deposited and removed ± standard deviation in 15 *Erica* species, the calculated PTE, their pollination syndrome (long-proboscid fly = LPF), anther exsertion and sample location.

Species	Pollen deposition	Pollen removal	PTE (%)	pollinator	Anther exsertion	Sample location
E. aristata	199 ± 136	45130 ± 12752	0.4	LPF	included	Vogelgat
E. cristata	91 ± 56	3541 ± 1681	2.6	LPF	included	Vogelgat
E. retorta	362 ± 236	18873 ± 17444	1.9	LPF	included	Kogelberg
E. ampullacea	830 ± 296	38800 ± 29292	2.1	LPF	included	Boskloof
E. fastigiata	222 ± 149	2969 ± 2084	7.5	LPF	included	Vogelgat
E. sessiliflora	224 ± 208	15549 ± 9200	1.4	bird	included	Vogelgat
E. viscaria	790 ± 272	14205 ± 8768	5.6	bird	included	Vogelgat
E. plukenetii	206 ± 72	35935 ± 12152	0.6	bird	exserted	Vogelgat
E. monadelpha	246 ± 188	14003 ± 8972	1.8	bird	exserted	Fernkloof
E. melastoma	548 ± 248	36023 ± 15724	1.5	bird	exserted	Vogelgat
E. imbricata	49 ± 36	5480 ± 3132	0.9	bee	exserted	Vogelgat
E. laeta	168 ± 120	3488 ± 1596	4.8	bee	included	Vogelgat
E. labialis	33 ± 12	29810 ± 7492	0.1	bee	exserted	Vogelgat
E. ericoides	44 ± 21	9880 ± 3008	0.4	bee	exserted	Table Mountain
E. quadrangularis	198 ± 108	4785 ± 4868	4.1	bee	included	Hottentot Hollands

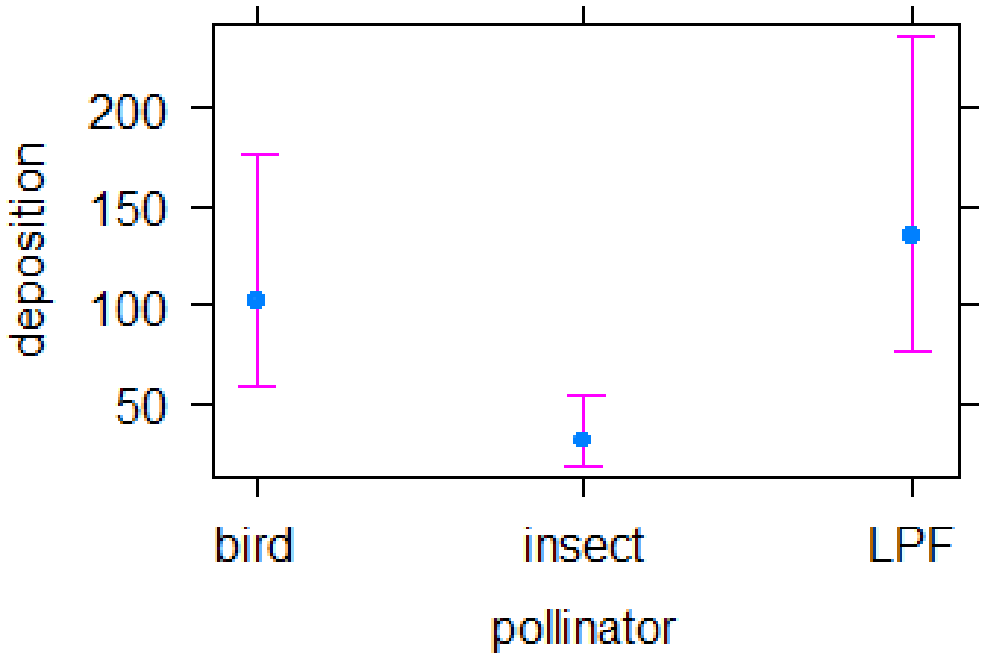

Figure 1 Pollen deposition for *Erica* species in relation to their pollination syndrome after adjusting for pollen removal, n = 15.

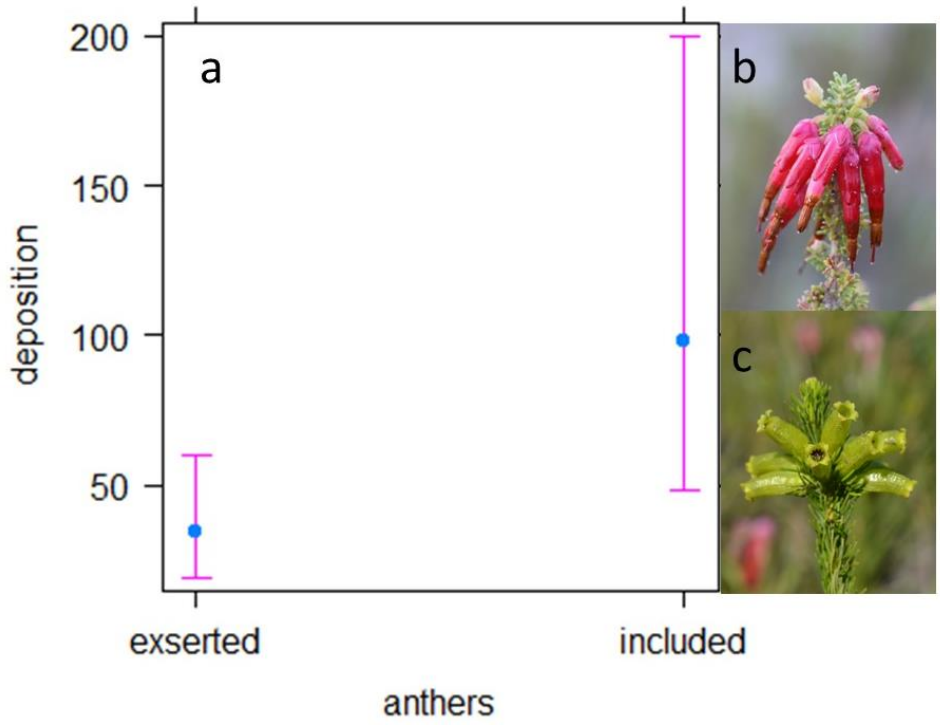

Figure 2 a) Pollen deposition for *Erica* species in relation to their anther exsertion after adjusting for pollen removal and pollination syndrome, n: exserted = 6, included = 4, b) exserted anthers in *E. monadelpha*, c) included anthers in *E. viscaria*

Discussion

PTE in the sampled *Erica* species is mostly higher than in other plants with granular pollen, where PTE is typically <1% (Harder & Johnson, 2008), which might be related to pollen aggregation in the form of tetrads in most *Erica* species. Similarly, in other families like orchids (Johnson, Neal & Harder, 2005; Hobbhahn & Harder, 2016) and asclepiads (Shuttleworth & Johnson, 2008) that produce aggregated pollen in the form of pollinia, high PTE values (2-16%) have been recorded. An inverse relationship of PTE and pollen-ovule ratios has been suggested (Gong & Huang, 2014), thus aggregated pollen is commonly associated with low pollen-ovule ratios (Harder & Johnson, 2008). This has been confirmed for *Erica* in a recent study despite pollen-ovule ratios in *Erica* not being associated with pollinator syndrome (Arendse et al., 2021)

As expected, we found relatively low PTE in bee-pollinated *Erica* species and higher PTE in both bird- and LPF-pollinated species. This confirms that specialists are more efficient pollinators than generalists (Laverty & Plowright, 1988) and nectarivorous birds are more efficient pollinators than bees (Castellanos, Wilson & Thomson, 2003). This study is the first to compare PTE between LPFs and other pollinators, and the observation that their PTE is higher than in bee-pollinated species, but does not differ from bird-pollinated species, could indicate that LPFs in the Fynbos have similar foraging behaviour to sunbirds.

Nectarivorous birds tend to avoid energetically unprofitable flower patches (Johnson, 2022). This might explain the low PTE in *E. plukenetii* in comparison to the other bird-pollinated species, since *E. plukenetii* occurred in a much lower density that the other bird-pollinated species. Thus, population size and other factors not included in this study, like reward (Hobbhahn & Harder, 2016) or ethological variation within a single pollination syndrome (Koski et al., 2018), might have also impacted PTE and caused e.g., the large variation of PTE in the bee-pollinated species.

Erica species pollinated by LPFs are often pollen limited (McCarren et al., 2022), but, nevertheless, their PTE is high in this study and their stigmas appear to be saturated with pollen grains. This is an indication that geitonogamy, as a result of the LPF visiting several flowers per plant, might play a role in clogging stigmas with self-pollen and reducing the number of seeds produced (Coetzee, Spottiswoode & Seymour, 2020) by LPF-pollinated *Erica* species through late-acting self-incompatibility (Arendse et al., 2021). Increased geitonogamy could also be a cost of producing more flowers per plant (de Jong et al., 1992). The inability to discriminate between outcrossed and geitonogamous pollen is a weakness of the PTE technique used here in general and more sophisticated methods like labelling pollen grains (Minnaar & Anderson, 2018) would be necessary

to better understand the importance of geitonogamy with different pollinators, however these methods are not feasible for *Erica* due to their poricidal anthers.

Previously, it has often been assumed that, due to the fused morphology of the *Erica* anther ring, all pollen is removed upon the first visit. This is clearly incorrect. It has been predicted that increased pollen removal by one pollinator causes diminishing returns in pollen deposition (Harder & Wilson, 1997) which would make it inefficient to place all pollen on the first visitor. This study shows that in most cases pollen still can be found in *Erica* anthers in late anthesis. The first visit to a flower causes the anther ring to break and release an explosive puff of pollen (Geerts & Pauw, 2011) which might cause the largest amount of pollen to be removed, but successive visits could still place some pollen on the pollinator. Further studies are required to understand what proportion of the pollen is removed by the first visit. The exploding anther ring might also serve to efficiently place pollen in hard-to-reach sites on the pollinator bodies and avoid being groomed off.

Surprisingly, we found that *Erica* species with exserted anthers have lower PTE than species with included anthers. Pollen removal typically increases with anther exsertion (Conner, Davis & Rush, 1995) but in *Erica* species with exserted anthers this increase in removal does not coincide with an increase in deposition, which indicates that more removed pollen is lost to the environment. It is not clear how the pollen is lost, but once the anther ring has been broken, it could more easily be blown away by wind and washed away by rain. Further, it might be easier for bees to collect and rob pollen from exserted anthers. Having exserted anthers imposes a cost since the plants produce more pollen while less of it ends up on conspecific stigmas. This could also be an indication of increased male-male competition, e.g., due to the physical size of the stigma. Therefore, we expect that future studies will reveal benefits conveyed by exserted anthers, e.g., they could result in better pollen placement or greater outcrossing. Additionally, having exserted anthers can cause more pollen to be removed during the first pollinator visit (Harder & Barrett, 1993) which could be beneficial when pollinators are rare or unpredictable.

This study has shown that PTE differs between pollination syndromes, as well as between anther morphologies. These differences in PTE might have driven pollinator shifts and consequently speciation in the genus *Erica*. However, more research is necessary to identify what causes the observed differences in PTE.

Acknowledgements

We are grateful to Ross Turner for help in identifying *Erica* species, to Cape Nature, SANPARK, Thys de Villiers, Vogelgat Private Nature Reserve and Giorgio Lombardi for access and permission to sample on their land. We also wish to thank Betty Ann Illing for providing accommodation in Hermanus.

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Synthesis

My research has added to our understanding of the role pollination syndrome plays for floral evolution both in the genus *Erica* and in general. The primary aim of this thesis was to explore previously neglected flower traits in long-tubed *Erica* species, namely ultraviolet colouration, corolla stickiness, flower orientation and pollen transfer efficiency. I also investigated how these traits correlate with other flower traits, especially pollination syndrome. I showed that long-tubed *Erica* species experience distinct selection pressures that have led to the evolution of specialised traits which set them apart from their short-flowered relatives. While there are shortcomings of the pollination syndrome concept, it has been useful for my research, and I have added to the knowledge of syndrome traits.

Nevertheless, there are many remaining questions about floral evolution in the genus *Erica* which would unfortunately exceed the scope of this thesis. The five chapters of this thesis focus mainly on the benefits of the studied floral traits, but it would add greatly to the understanding of the diversity in this genus, and the evolution of specialist versus generalist species to study the costs of these traits as well. Every trait is expected to be a trade-off between benefits and costs. Therefore, it would be worth testing if more resources are allocated to flowers with these traits, e.g., to produce pigments or sticky substances. Different flower visitors might also exert different selection pressures on *Erica* plants. Thus, measuring the strength and direction of selection exerted by different visitors on a trait in relation to the relative frequency of visits might help to explain the presence or absence of that trait in different species.

In chapter 1, I showed that UV reflection is uncommon or absent in the short-flowered wind-, rodent- or small insect-pollinated *Erica* species. On the other hand, the pollinators of long-tubed *Erica* species, birds and long-proboscid flies (LPFs), have selected for a higher prevalence of UV as a flower colour. Both sunbirds, as well as LPFs can see UV and react to it. When the reward is equal, sunbirds have no preference regarding UV but when UV flowers have a better reward, the birds learn to prefer them. This indicates that UV is no more important than other flower colours in bird-pollinated species, but the birds can use it to inform their foraging decisions. When UV was removed in LPF-pollinated species, seed set was suppressed which indicates that its presence is paramount for successful reproduction in those species.

The contrast in both hue and luminance between an object and its background plays an important role in vision (Chen et. al., 2020). A visually contrasting background could therefore make a flower more conspicuous to its pollinator. Thus, it would be of interest to further investigate how much contrast different flower visitors perceive between flowers of different pollination syndromes and their background. This could also help to understand the costs of this trait by indicating if UV-reflective flowers are more likely to be detected by nectar robbers.

When I started my thesis, I had little information on moth-pollinated *Erica* species, but recent studies have now revealed that UV-reflections are key to attract nocturnal pollinators, such as the hawkmoth which pollinates the UV-reflecting *Erica cylindrica* (van der Niet & Cozien, 2022) and I expect that future studies will reveal similar results for other *Erica* species pollinated by hawkmoths.

In chapter 2, I investigated the role of non-pollinator agents of selection in the form of biotic interactions with nectar robbers. Bees and other small insects typically act as pollinators for small-flowered species but are antagonistic nectar robbers for many long-tubed *Erica* species. My research has shown that floral stickiness can serve as a protection mechanism against said nectar robbers and as a result this trait has evolved more frequently in long-tubed species. This indicates that nectar robbers, too, can select for floral traits and thereby affect evolution in the genus *Erica*. In some cases, nectar robbers have been reported to act as pollinators (Navarro, 2000; Rojas-Nossa, Sánchez & Navarro, 2021) causing a positive effect on plant fitness and this could even lead to adaptations that favour nectar robbing.

While stickiness can repel nectar robbers, it is possible that this trait could also serve other functions like limiting water-loss in summer-flowering species or making the flowers look glossy and thereby increasing pollinator attraction. On the other hand, stickiness could also affect pollinators negatively if it decreases attraction e.g., because the pollinators themselves are at risk of getting stuck. Lastly, there are limitations to stickiness as an anti-robbing trait since larger animals like sunbirds or baboons also act as nectar robbers occasionally, but do not appear to be affected by stickiness.

While most bird-pollinated *Erica* species are pollinated by just one species, the Orange-breasted sunbird (*Anthobaphes violacea*), LPF-pollinated species are visited by a variety of different LPF species which has led to significant variation of flower traits within the pollination syndrome. In chapter 3, I used the example of *E. shannonea* and *E. ampullacea* to investigate what traits can lead to within-syndrome divergence. I showed that they are pollinated by flies with different morphological features and their differences in tube length as well as flower orientation serve as reproductive barriers between the two *Erica* species. Thus, differences in both tube length and

orientation can potentially facilitate speciation and maintain the coexistence of closely related species from the same pollination syndrome.

More research is needed to confirm the pollinators of the proposed MPF-pollinated species and put them into phylogenetic context with closely related LPF-pollinated species. This could shed light on the evolution of pollination by LPFs.

Further, I explored flower orientation for all LPF-pollinated species. I demonstrated that the floral orientation of LPF-pollinated species depends on the biomechanics of their fly pollinator. Nemestrinid flies can swivel their proboscis into any position between horizontal and vertical and consequently flowers pollinated by them have evolved a wide range of angles. Tabanid flies, on the other hand, have fixed forward pointing proboscis which has restricted plants that are pollinated by them to horizontal flower angles. This adds flower orientation as a trait indicative of pollination syndrome and confirms that flower orientation can potentially serve as a mechanism of reproductive isolation.

The plant species measured in this chapter mostly had LPFs as their main pollinator, but some of the species were more frequently visited by other insects, e.g., *Plumbago auriculata* which is sometimes visited by a tabanid fly despite having an angle of 45° (Ferrero et. al., 2009). In this case, the selection pressure exerted by the fly is likely to be small in comparison to its main pollinators and this also explains why the flowers are not horizontal.

Additionally, behavioural experiments with the two different LPF families will be necessary to confirm that their biomechanics lead to different preferences in flower orientation. This could be achieved by offering two artificial flowers, one with horizontal and one with vertical orientation, to LPFs from both families and comparing their first choice. Further, the impact of flower orientation on other pollination syndromes has not been investigated yet.

While we understand numerous floral traits that are mediated by pollinator selection, they alone don't explain what causes plant species to shift between pollinators. Different pollinators are expected to differ in the costs and benefits they confer, e.g., the resource investment required from the plant, the degree of flower constancy or pollen transfer efficiency. Therefore, I investigated pollen transfer efficiency for different *Erica* pollination syndromes in the last chapter. Birds and LPFs as pollinators confer higher pollen transfer efficiency to the long-tubed *Erica* species they visit compared to bees. This might have facilitated the shifts from ancestral bee pollination and ultimately the evolution of long-tubed *Erica* flowers.

Although the total cost of floral allocation is expected to be independent of flower size (many small versus few large flowers), many small flowers that open simultaneously, as in many bee-pollinated species, may cause higher levels of geitonogamy. I noted that I could not differentiate self-pollen versus crossed pollen on stigmas in determining pollen transfer efficiency. Ericas have a late-acting incompatibility mechanism (Arendse et. al., 2021) which makes it impossible to distinguish between pollen from conspecifics, selfing or other species through analysis of pollen tube growth. Thus, the impact of flower size and number, as well as pollination syndrome on geitonogamy should be further investigated using a different study system.

While I found differences in pollen transfer efficiency between species with exserted and included anthers, the sample size was small and future research should study anther exsertion in different pollination syndromes separately. Further, the function of having exserted anthers still needs to be determined. Approximately one quarter of *Erica* species have exserted anthers and they occur both in insect- and bird-pollinated species. I hypothesise that in bird-pollinated species this trait can serve to place pollen further on the pollinators body and thereby facilitate pollinator partitioning and coexistence of species that share a pollinator. This could be tested by comparing pollen placement of bird-pollinated *Erica* species with differences in anther exsertion.

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