

THE EVOLUTION OF FLOWER COLOUR VARIATION
IN THE ENDANGERED GRASSLAND DAISY
GERBERA AURANTIACA (ASTERACEAE)

by Isabel Marion Johnson

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Gerbera aurantiaca

It is a remarkably handsome plant, the rays mostly rich shades of red or orange, varying much in a single colony, and set off by the blackish disc.

Olive Hilliard – 1977



Plate 2220 *Gerbera aurantiaca*

Gerbera aurantiaca by Gillian Condy. Reproduced from *Flowering Plants of Africa* 59: t. 2220 with permission from the south African Biodiversity Institute, Pretoria.

ABSTRACT

Flower colour variation is widespread within and between populations and has long been a focus for research into natural selection. I investigated the potential factors influencing the capitulum colour variation exhibited by *Gerbera aurantiaca* (Asteraceae), an endangered grassland daisy endemic to the sub escarpment mistbelt grasslands of eastern South African. Widely scattered and fragmented flower colour polymorphic and monomorphic populations comprise long-lived clones with inflorescences ranging from yellow through orange to deep red. An earlier study investigated some aspects of pollination biology and population structure in the southern monomorphic, red-flowered *G. aurantiaca* populations, but little was previously known about the yellow and flower colour polymorphic communities in the central and northern parts of the distribution.

Geographical variation in flower colour of a plant species may reflect the outcome of selection by pollinators or may reflect abiotic factors such as soil chemistry or neutral processes such as genetic drift. I found no strong associations between morph colour and abiotic factors, including soil chemistry, and plants in a common garden retained the capitulum colour of the source population, even when grown from seed, suggesting that flower colour variation is not a result of phenotypic plasticity. Distribution of the various colour morphs in a flower colour polymorphic population further supported this lack of association. Hopliine beetles were the most abundant flower visitors in all populations and in a colour polymorphic population, all colour forms were equally attractive to these beetles and did not differ significantly in terms of fruit set. Native honeybees visited only yellow capitula in a monomorphic population. I found that *G. aurantiaca* is largely self-incompatible, completely dependent on pollinators for pollen transfer, and pollen quantity limited in some of the red coloured populations. This species is therefore highly susceptible to pollinator colour sensitivities for visits and fruit set. Hopliine beetle abundance appeared to be the most important factor in fecundity in all populations, but visited all colour forms equally, suggesting that it is maintaining the current colour pattern rather than driving change. The absence of this pollinator in two red-flowered populations has resulted in critically low fecundity. Pre-dispersal tephritid fly seed predators damaged similar numbers of capitula in all study populations making it unlikely that these biotic agents are driving colour variation.

Since community flower has been shown to influence the suite of pollinators and subsequently predominant flower colour, I compared the petal reflectance spectra of all

coflowering species across five representative *G. aurantiaca* populations but found no differences. Insect vision models showed that the yellow morphs were visible to honeybees, but the red forms were not different to the background, while all colour forms, in particular red, were conspicuous to the beetles. These results confirmed field pollinator observations.

Pigment distribution and petal structure may be important in pollinator attraction. In *G. aurantiaca* pigments are concentrated in the upper epidermal cells of the ray florets which have an epidermal cuticular surface micro structuring suggesting that pollinator attraction is important in this species. Flower colour inheritance may play a vital role in self-incompatible species in flower colour polymorphic populations. Here I crossed true breeding red and yellow colour forms from different populations which produced primarily yellow and orange F1 offspring, suggesting a polygenic inheritance of anthocyanins in *G. aurantiaca*.

The results presented here show that capitulum colour in *G. aurantiaca* is geographically structured, but the ultimate evolutionary basis of this colour variation remains elusive, and further research using genetic techniques should be pursued to answer this question. Some recommendations for the long-term conservation of this flagship endangered species are detailed.

PREFACE

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The work contained in this thesis was carried out in the Republic of South Africa at the University of KwaZulu-Natal under the supervision of Prof. Steven D. Johnson.



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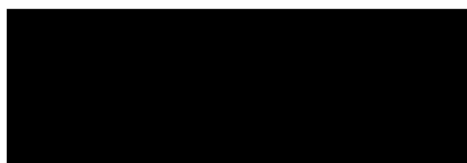
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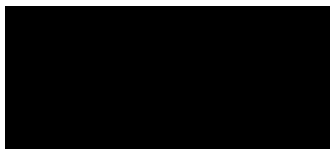
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Chapter 2 of this thesis has been published in *Frontiers in Ecology and Evolution* in 2021.

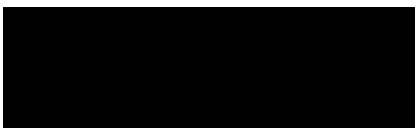
Author contributions are as follows: IMJ, SDJ, Trevor J Edwards conceived the study. IMJ collected and analysed the data with assistance from SDJ and TJE. IMJ wrote the manuscript with editorial inputs from SDJ and TJE.

Chapter 3 of this thesis has been prepared as a manuscript that is intended for submission to a relevant journal. Author contributions are as follows: IMJ, SDJ, Trevor J Edwards conceived the study. IMJ collected and analysed the data and wrote the manuscript with editorial inputs from SDJ, TJE and Carolina Diller.

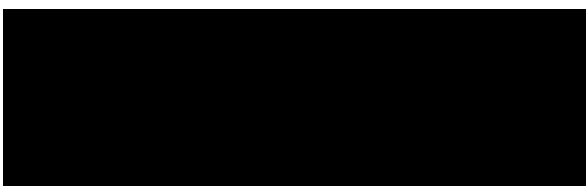
Chapter 4 of this thesis has been prepared as a manuscript that is intended for submission to a relevant journal. Author contributions are as follows: IMJ conceived the study, collected, and analysed the data and wrote the manuscript with editorial inputs from SDJ.

Chapter 5 of this thesis has been prepared as a manuscript that is intended for submission to a relevant journal. Author contributions are as follows: IMJ conceived the study, collected, and analysed the data and wrote the manuscript with editorial inputs from SDJ.

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Isabel Marion Johnson



Professor Steven D. Johnson (supervisor)

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CHAPTER 1: GENERAL INTRODUCTION

This introduction provides a general overview of flower colour variation and its determinants, followed by a detailed account of the study system and species, *Gerbera aurantiaca*, and then gives an outline of the specific objectives of this thesis.

Flower colour variation

Flower colour is among the most conspicuously variable traits in nature and angiosperms are diverse in flower colour, and traits that differ between closely related taxa often differing in petal colouring (Rausher, 2008). Most plant populations are monomorphic in floral colour, but a minority are polymorphic (Narbona et al., 2018; Sapir et al., 2021) and colour variation across a species range may show a complex mosaic, ranging from polymorphic to monomorphic populations of different colours (Dafni et al., 2020; Narbona et al., 2018). The factors influencing these colour differences, including selection by pollinators and predators, community effects, as well as abiotic drivers have been the source of many investigations over the last few decades.

The biotic determinants of flower colour variation: pollinators, predators, and community flower colour

The idea that animal pollinators play a critical role in shaping the diversity of flower colours through process of natural selection has received considerable support (Schiestl and Johnson, 2013; Trunschke et al., 2021; van der Niet and Johnson, 2012). Because different pollinator groups have different visual sensory capabilities and behavioural responses to colours, spatial variation in pollinator assemblages can lead to substantial selective pressure on flower colour (Chittka and Menzel, 1992; Chittka and Raine, 2006; Menzel and Shmida, 1993). Pollinator perception and discrimination of differently coloured flowers may therefore be instrumental in driving flower colour divergence among morphs, populations or species (Campbell et al., 1997). While the important role of flower colour for pollinator attraction has been widely acknowledged since the time of Darwin and his contemporaries (Fenster et al., 2004), over the past few decades an increasing number of studies have suggested that flower colour can also be influenced by a number of differing selective and often antagonistic, abiotic and biotic non-pollinator agents (Dalrymple et al., 2020; Strauss and Whittall, 2006). Some floral pigmentations can be signals to both pollinators and herbivores, in particular seed predators, that reduce plant fecundity by directly or indirectly damaging reproductive structures. In this way, the potential selection exerted by pollinators could be altered by herbivores, as in the case of *Protea aurea* where these inflorescence colour polymorphisms appear to be

maintained by the pleiotropy through a combination of seed predation differences in fecundity (Carlson and Holsinger, 2010; Carlson and Holsinger, 2013). Together these recent studies suggest that the evolution of flower colour polymorphism requires a broader focus that includes not only pollinators but also herbivores and pathogens (Strauss and Whittall, 2006).

Community flower colour signal is an additional biotic factor which has been implicated in influencing the evolution of flower colour. Clustered flower colour similarity in a community may increase visitation by shared pollinators, creating a ‘magnet effect’ (de Jager et al., 2011; Thomson, 1978). Rare plants may benefit from colour signals that mimic those of more common species in the community, for example, the rewardless orchid *Disa ferrugina* exhibits a geographic colour shift associated with mimicry of the flower colour of its pollinator’s locally abundant, primary nectar resource species (Newman et al., 2012). Conversely, plants might benefit more from having distinct signals from coflowering plants (Gumbert et al., 1999). The community colour signal of coflowering plants may be clustered, consistent with facilitation amongst species (Albor et al., 2022; Kemp et al., 2019), or may be more dissimilar in communities structured by competition for pollinators (Chittka, 1997; Makino and Yokoyama, 2015).

Insect vision models which take into account the specific wavelength sensitivities and processing systems of different pollinator groups have enabled quantification of colour differences as perceived by pollinators and had great utility in studies of these biotic determinants of flower colour variation (Paine et al., 2019). Possibly the best known and most widely used model of insect colour perception is the hymenopteran or bee colour hexagon (Chittka, 1992). Vision models have also been developed for flies (An et al., 2018; Troje, 1993), butterflies (Koshitaka et al., 2008), birds (Vorobyev and Osorio, 1998), and coleopterans (Martínez-Harms et al., 2012), although sensitivities and vision systems are recognised to vary within these groups. These models may allow the calculation of colour discrimination distances as for example, within the colour-hexagon model of bee vision, bees are unable to distinguish colours separated by a Euclidean distance of 0.11 units or less (Chittka, 1992), but more recently it has been established that this is context dependent and may be considerably less (< 0.05 hexagon units) with conditioning and under certain conditions (Dyer and Chittka, 2004; Dyer and Garcia, 2014; Garcia et al., 2017). This is important in establishing whether pollinators can distinguish between different flower species and different colour varieties of the same species.

The abiotic drivers of flower colour variation

Although it is now widely accepted that abiotic aspects can also impose selection on flower colour (Arista et al., 2013; Rausher, 2008; Strauss and Whittall, 2006), there have been fewer studies of such abiotic effects in influencing flower colour (Dalrymple et al., 2020). The most common floral pigments in angiosperms are anthocyanins, which may also play a role in abiotic stress tolerance, thereby also determining flower colour evolution through pleiotropy (Strauss and Whittall, 2006). In the classic study of *Linanthus parryae* it was found that the predominance of blue or white colour morphs was the result of environmental differences in soil types and moisture (Schemske and Bierzychudek, 2001; Schemske and Bierzychudek, 2007), while the maintenance of the orange form and blue forms of *Lysimachia arvensis* was found to be related to differences in the fitness of both morphs in differing moisture environments (Arista et al., 2013).

The relative importance of abiotic and biotic drivers of flower colour evolution is less well researched (Dalrymple et al., 2020; Strauss and Whittall, 2006). Multi-disciplinary studies that consider biotic and abiotic determinants as well as neutral processes such as genetic drift or historical events should be used in flower colour variation investigations (Narbona et al., 2018).

Reproductive systems as a factor maintaining flower colour variation

An aspect of flower colour variation and its maintenance that has been little studied so far is the role of breeding and reproductive systems (sexual and asexual) in influencing morph colour (Narbona et al., 2018). Both clonal reproduction and self-pollination may maintain colours in a population without pollinator intervention, thus it is important to establish the degree of dependence of a species on pollinating agents in studies of flower colour variation (Buide et al., 2021; Imbert et al., 2014). Maintenance of the orange morph of *Lysimachia arvensis* in the Mediterranean which is negatively selected for by both biotic and abiotic factors appears to be associated with a combination of low inbreeding depression and capacity for selfing (Arista et al., 2013; Jiménez-López et al., 2020). Overdominance (heterozygote advantage) has also been shown to maintain flower colour polymorphism in the orchid *Gymnadenia rhollicani* (Kellenberger et al., 2019). Vegetative reproduction or asexual reproduction by clonal growth may play a role in maintaining flower colour variants within a population for long periods (Wang et al., 2016). Lastly, although few examples of the role of random or neutral processes in maintaining colour variation in populations have been

reported it should not be ruled out as a factor maintaining flower colour polymorphism (Narbona et al., 2018; Wang et al., 2016).

The pigment and anatomical genetic basis of flower colour variation

The colour of a flower is determined by a combination of factors, including the absorption and reflectance of specific wavelengths by pigments, the backscattering of light within the interior of the petal, the thickness of the petal tissue (van der Kooi et al., 2016), and possibly the presence and arrangement of petal surface microstructures (Whitney et al., 2016) although the interaction of these components is not yet fully understood. Flower colours are primarily pigment-based, with betalains (in the Caryophyllales), carotenes and anthocyanins being the most important of these (Narbona et al., 2021) and most cases of flower colour polymorphism result from minor changes in pigment intensity, especially of anthocyanins (Warren and Mackenzie, 2001). It has been reported that almost 90% of the flower colour polymorphic species in the Mediterranean Basin have anthocyanin-based polymorphisms (Narbona et al., 2018). Structural modifications of the petal surface may play a role in flower colour with some types of modified epidermal cells believed to concentrate photons and to intensify pigment colour, and to produce iridescence which may enhance attractiveness to pollinators (Glover and Martin, 1998; Whitney et al., 2016). However, the latter has been debated since iridescence is rare in plants under natural light conditions (van der Kooi et al., 2014) and the significance of petal surface structure requires further investigation (Wilts et al., 2018).

The study of flower colour variation requires an approach that incorporates both abiotic and biotic agents of selection (Dalrymple et al., 2020; Strauss and Whittall, 2006), and several different areas of expertise ranging across botany, ecology and insect vision may be required since it is likely that a combination of these factors is often involved (Garcia et al., 2020).

The study system

The Genus *Gerbera*: Sect. *Lasiopus*

The genus *Gerbera* (Asteraceae: Mutisieae) has its centre of diversity in the southern part of Africa, seemingly originating in southern Gondwanaland (Hansen, 1985). About 14 species of *Gerbera* are found in southern Africa, with nine in eastern South Africa, where species delineations are often confusing as evidenced by the number of synonyms, with many specimens difficult to place. *Gerbera aurantiaca* is one of seven species in the Section

Lasiopus (*G. ambigua*, *G. aurantiaca*, *G. galpinii*, *G. jamesonii*, *G. natalensis*, *G. sylvicola*, *G. viridifolia*) (Conservatoire et Jardin Botaniques de la Ville de Genève and South African National Biodiversity Institute, 2024). Members of the section *Lasiopus* are stemless, perennial herbaceous plants with a rosette of basal leaves and with scapes (inflorescence stalks) bearing single heads or capitula. Distinguishing characteristics of this section are the ebracteate scapes not widened below the capitulum, showy outer ray florets which are considerably longer than the involucre bracts, and in various shades of pink, purple, red, white, or yellow (Hansen, 1985; Hilliard, 1977; Johnson et al., 2005). Some members of this section have dark centres in capitula in the female phase caused by the dark purple overarching pappus hairs and dark anther caps of the hermaphrodite disc florets (Johnson et al., 2005). Currently seven species, all occurring in KwaZulu-Natal and adjoining regions are placed in section *Lasiopus* (Conservatoire et Jardin Botaniques de la Ville de Genève and South African National Biodiversity Institute, 2024). These species exhibit a range of ray floret colours from white (often tinged pink below), in *G. ambigua*, *G. galpinii*, *G. natalensis* and *G. viridifolia*, yellow in *G. ambigua*, *G. aurantiaca* and *G. jamesonii*, and red in *G. aurantiaca* and *G. jamesonii* (Hansen, 1985; Hilliard, 1977; Johnson et al., 2005). Five of the seven species exhibit some floret colour variation of the ray floret upper surface (Fig 1.1, Table 1.1).

Table 1.1. Species and upper ray floret colour in *Gerbera*: Section *Lasiopus*

| <i>Gerbera</i>: Section <i>Lasiopus</i> | Upper ray floret colour |
|--|--------------------------------|
| <i>Gerbera ambigua</i> (Cass.) Sch. Bip. | White, yellow |
| <i>Gerbera aurantiaca</i> Sch. Bip. | Yellow, orange, red |
| <i>Gerbera galpinii</i> Klatt | White, yellow |
| <i>Gerbera jamesonii</i> Bolus ex Adlam | Yellow, orange, red |
| <i>Gerbera natalensis</i> Sch. Bip. | White |
| <i>Gerbera sylvicola</i> I.M. Johnson, N.R. Crouch & T.J. Edwards | White |
| <i>Gerbera viridifolia</i> (DC.) Sch. Bip. | White, pink, purple |

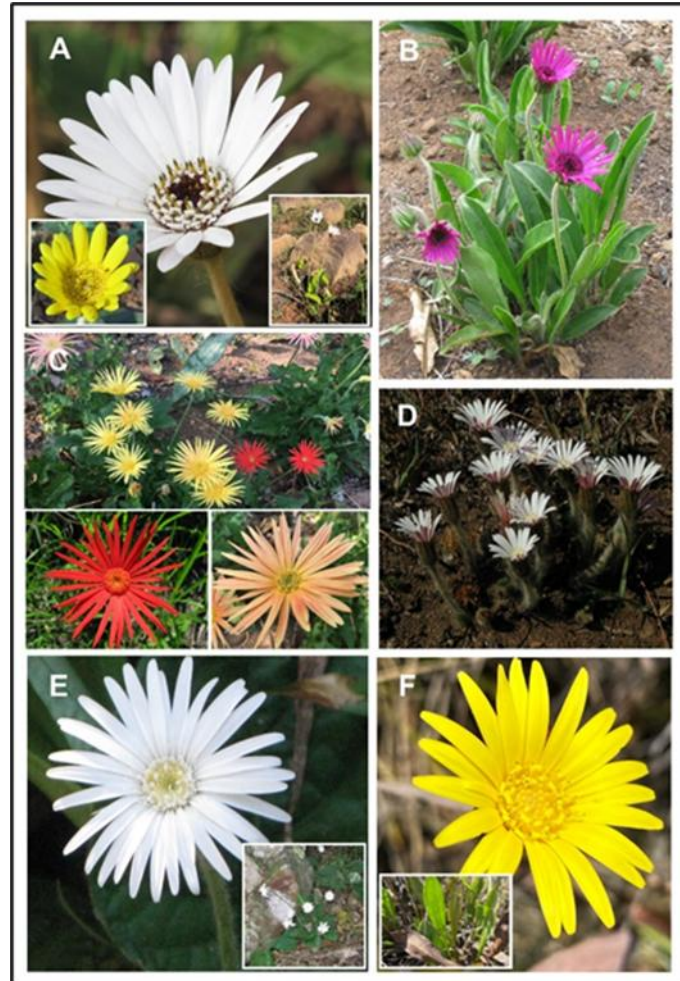


Figure 1.1. *Gerbera* : Sect. *Lasiopus* (excluding *Gerbera aurantiaca* which is depicted in Fig 1.3): **A.** *Gerbera ambigua*, **B.** *Gerbera viridifolia*, **C.** *Gerbera jamesonii*, **D.** *Gerbera natalensis*, **E.** *Gerbera sylvicola* and **F.** *Gerbera galpinii*. Photo (F) by S.Attwood. 2024. www.inaturalist.org/observations/240548108.

Phylogenetic studies have confirmed that *G. ambigua*, *G. aurantiaca*, *G. jamesonii* and *G. viridifolia* form a monophyletic clade (Pasini et al., 2016; Xu et al., 2018; Zheng et al., 2019), while *G. galpinii*, *G. natalensis* and *G. sylvicola* have not yet been included in a phylogenetic analysis (Fig 1.2).

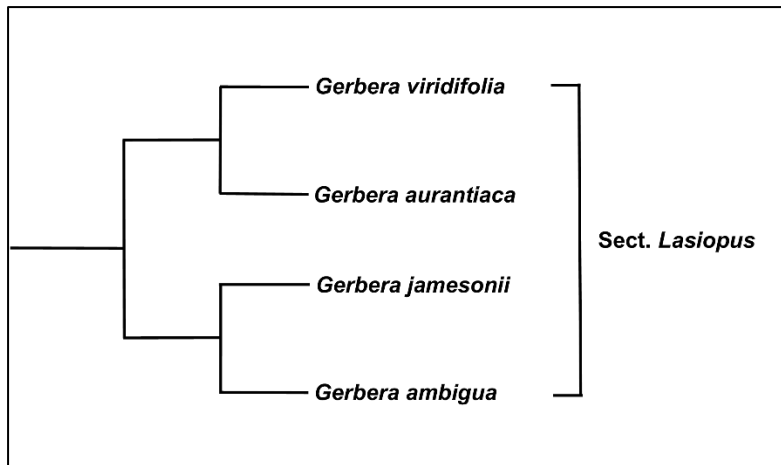


Figure 1.2. Cladogram of *Gerbera* Sect. *Lasiopus* (after Zheng et al 2019). *Gerbera natalensis*, and *G. galpinii* are not included

Gerbera has made an important contribution to international horticulture. The *Gerbera* commercial hybrids were developed in 1886 in the Cambridge Botanical Gardens in England when *G. viridifolia* and *G. jamesonii* when plants raised from seed sent from South Africa were hybridised (Codd, 1979; Johnson et al., 2005).

The study species: *Gerbera aurantiaca*



Figure 1.3. Capitulum colour variation in *Gerbera aurantiaca*.

Gerbera aurantiaca, commonly known as the Hilton daisy, is one of the more clearly defined species in the section *Gerbera* sect. *Lasiopus*, due to differences in leaf indumentum and venation from its sister species (Hansen, 1985). First collected between 1838 and 1840 by Ferdinand Krauss in “the hills near Pietermaritzburg” and given the specific epithet

“aurantiaca” by Schulz Bipontius due to its “beautiful orange” colour (Schulz, 1844), although most plants in these populations have capitula with deep red outer ray florets, (Fig 1.4 A&D), with some colour variation within populations (Hansen, 1985; Hilliard, 1977; Johnson et al., 2005) (Fig 1.3. In populations where *G. aurantiaca* occurs sympatrically with *G. ambigua* limited hybridisation takes place resulting in pink-flowered progeny (Hilliard, 1977; Johnson et al., 2004).



Figure 1.4. Typical landscape scenes of *Gerbera aurantiaca* habitat. **A.** Red colour form in the southern population, **B.** Northern colour polymorphic population at Rooihoochte, Mpumalanga, **C.** Monomorphic yellow population at Ngome, KwaZulu-Natal, and **D.** Monomorphic red population at Byrne, KwaZulu-Natal.

Until recently *G. aurantiaca* was thought to have primarily red capitula (Fig 1.4 A&D) and to be confined to the central KwaZulu-Natal mist belt grasslands, between Richmond in the south and Melmoth in the north. This initial view of the KwaZulu-Natal midlands extent of distribution for *G. aurantiaca* has more recently been extended much further north with the

collection in 1977 of a yellow flowered population near Ngome in northern KwaZulu-Natal (Fig 1.4 C), and with the discovery of another entirely yellow flowered population still further north in Mpumalanga at Lochiel near Barberton in 2002. In 2006 a further flower colour polymorphic population consisting of orange, red and yellow capitulum colour forms (Fig 1.4 B) was found by J.&S. Burrows near Badplaas in Mpumalanga (Fig 1.5).

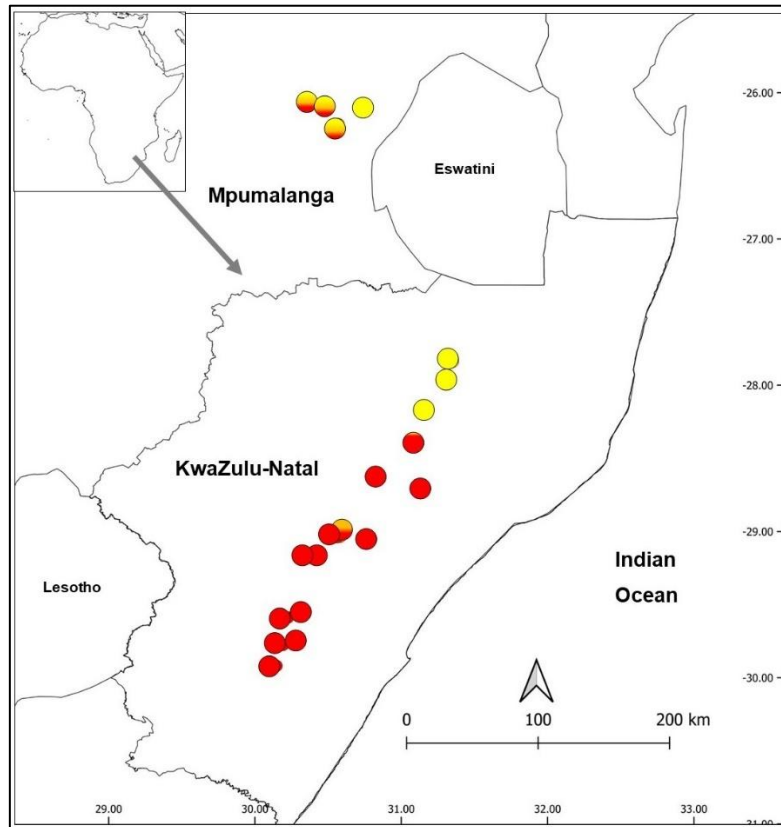


Figure 1.5. Map of eastern South Africa showing the positions and predominant colour forms of the *Gerbera aurantiaca* populations

Gerbera aurantiaca is found at elevations between 900 and 1700 masl in scattered fragments of summer rainfall mist belt grassland. The species grows clonally by underground stems with individual plants often extending to over a meter in diameter and is thought to live for extended periods of time (Scott Shaw, 1999). Flowering takes place in spring (September to November) and the plants become dormant during the dry winter months.

An earlier study of the variation in seed production of *G. aurantiaca* in southern monomorphic red populations (Johnson et al., 2004) found that *Eriesthis* beetles were the

primary pollinators, that seed production was low due to pollen limitation and seed predation, and that seed production was positively related to *G. aurantiaca* population size. Although, this study did not explore capitulum colour variation or pollination and breeding systems in the then unknown yellow and flower colour polymorphic northern populations it provides a sound basis for the investigations of this thesis.

Conservation status

Gerbera aurantiaca has been assessed as Endangered (A2ac) by the South African National Biodiversity Institute (SANBI) using the IUCN Red List categories and criteria and described as follows “A mistbelt grassland endemic, this species habitat has been over 90% transformed for commercial forestry plantations and crop and pasture cultivation over the past 120 years. There has also been extensive recent loss of habitat in the KwaZulu-Natal Midlands to urban development. *Gerbera aurantiaca* and we therefore extrapolate a 50% loss of subpopulations from the 90% loss of habitat.” (Scott-Shaw et al., 2005).

Greater Midlands Centre of Endemism in eastern southern Africa

Gerbera aurantiaca is confined to the greater Midlands in the summer rainfall area of eastern South Africa, a region of high plant endemism with many threatened species (Carbutt, 2023). The area consisting mainly of sub-escarpment (770-2200msl), mesic C₄ short, tufted grassland is highly fragmented due to agriculture and development. These grasslands and their highly threatened flora are in need of urgent conservation action and appropriate management The protection of critical habitats is essential to stem the loss of these endemic species and their evolutionary history (Carbutt, 2023; Jewitt, 2018).

Specific objectives of this thesis

In this thesis I use an integrated approach to investigate some abiotic, biotic and community factors which may influence flower colour variation in *G. aurantiaca*.

In Chapter 2 I evaluate associations between flower colour in *G. aurantiaca* and potential abiotic drivers of flower colour variation. I map the colour variation of *G. aurantiaca* across its distribution range, measure the spectral reflectance signals of five representative populations and investigate the relationship of population colour to climatic and edaphic factors. I use a common garden experiment to confirm the genetic basis for the colour variation and employ artificial colour traps to test colour preference of the most common

pollinators. In a colour polymorphic population, I map the distribution of the capitulum colour forms, record the colour preferences of the common beetle pollinator and compare the reproductive success of the *G. aurantiaca* colour forms.

In Chapter 3 I determine the breeding system and focus on the potential roles of pollinators and seed predators as drivers flower colour variation in *G. aurantiaca*, I use controlled pollination experiments to determine whether *G. aurantiaca* depends on pollinators, is self-compatible, and whether seed production is limited by pollinator activity. I further ask whether pollen limitation is caused by pollen quantity or pollen quality. I observe and identify insect visitors to capitula and measure fecundity under natural pollination conditions. Lastly, I measure seed predator abundance across the populations.

In Chapter 4 I investigate community flower colour as a potential driver of colour variation in *G. aurantiaca*. I measure community flower colour signal of five representative populations in relation to the colour forms of the local *G. aurantiaca* morph, compare spectral reflectance and inflection points of five populations independently of insect vision and then use insect colour vision models for the two most common pollinator groups, Hymenoptera and Coleoptera, to calculate the conspicuousness of the *G. aurantiaca* local colour forms to these insects in the different study populations.

In the fifth and final data chapter, Chapter 5, I focus on ray floret micromorphology. I use microscopy and spectrography to examine the petal surface microstructure and pigment distribution of the ray florets of red and yellow colour forms and discuss their possible roles in the colour signalling of *G. aurantiaca*. In this chapter I also report on a preliminary investigation into capitulum colour inheritance, where I cross true-breeding red and yellow colour forms of *G. aurantiaca* and record the colour of the F1 generation offspring to gain insight into the pattern of colour inheritance in this species.

In Chapter 6, I summarise the results of the four research chapters (Chapters 2-5) and discuss their relevance to current understanding of evolution of flower colour polymorphisms, with a particular focus on insights from the *G. aurantiaca* study system. I then speculate possible evolutionary history of colour variation in *G. aurantiaca*. I briefly discuss the relevance of my findings to the conservation of *G. aurantiaca* and make recommendations to contribute to its continued survival. Finally, I identify some areas for further research areas suggested from the findings of this thesis.

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**CHAPTER 2: GEOGRAPHICAL VARIATION IN FLOWER COLOR IN
THE GRASSLAND DAISY *GERBERA AURANTIACA*: TESTING
FOR ASSOCIATIONS WITH POLLINATORS AND ABIOTIC
FACTORS**



Geographical Variation in Flower Color in the Grassland Daisy *Gerbera aurantiaca*: Testing for Associations With Pollinators and Abiotic Factors

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Geographical variation in flower color of a plant species may reflect the outcome of selection by pollinators or may reflect abiotic factors such as soil chemistry or neutral processes such as genetic drift. Here we document striking geographical structure in the color of capitula of the endemic South African grassland daisy *Gerbera aurantiaca* and ask which of these competing explanations best explains this pattern. The color of capitula ranges from predominantly red in the southwest to yellow in the center, with some northern populations showing within-population polymorphism. Hopline scarab beetles were the most abundant flower visitors in all populations, apart from a yellow-flowered one where honeybees were frequent. In a mixed color population, yellow, orange and red morphs were equally attractive to hopline beetles and did not differ significantly in terms of fruit set. Beetles were attracted to both red and yellow pan traps, but preferred the latter even at sites dominated by the red morph. We found no strong associations between morph color and abiotic factors, including soil chemistry. Plants in a common garden retained the capitulum color of the source population, even when grown from seed, suggesting that flower color variation is not a result of phenotypic plasticity. These results show that flower color in *G. aurantiaca* is geographically structured, but the ultimate evolutionary basis of this color variation remains elusive.

Keywords: common garden, pollinator color preference, abiotic factors, beetle pollination, flower color polymorphism, honeybees

INTRODUCTION

Color is one of the most important cues used by pollinators to locate, recognize and discriminate between flowers (Menzel and Shmida, 1993; Schiestl and Johnson, 2013). There is good evidence for differences among pollinator functional groups in their perceptions of colors, their degree of innate attraction to certain colors and their abilities to learn to associate colors with rewards (Chittka and Menzel, 1992; Chittka and Raine, 2006). As a result, deployment of color signals is considered a key part of plant advertising strategies that influence pollination outcomes (Schiestl and Johnson, 2013; Reverté et al., 2016). This is reflected in the broad associations between various animal groups and the colors of flowers they visit (Fenster et al., 2004; Renoult et al., 2014). Some phylogenetic

studies have shown that macro-evolutionary transitions in flower color are statistically associated with pollinator shifts (Tripp and Manos, 2008) while others have found no relationship between pollination system and flower color (Smith et al., 2008). The role of selection in generating macro-evolutionary patterns in flower color has been highlighted by experimental studies using hybrid arrays with introgressed flower colors (Bradshaw and Schemske, 2003). These experiments, in which the effects of color and morphological signals on pollinator attraction can be uncoupled, have shown that pollinators frequently discriminate among plants according to flower colors (Bradshaw and Schemske, 2003; Hoballah et al., 2007). Similarly, experiments using arrays of model flowers that vary only in color have shown strong discrimination according to color by flower-visiting animals (Campbell et al., 2010).

Geographical variation in the availability of pollinators with different color preferences would be expected to lead to intraspecific divergence in flower color among plant populations (Stebbins, 1970). For example, the floral color polymorphism in *Drosera cistiflora* may be accounted for by the spatiotemporal variation in pollinator assemblages which display different color preferences (Johnson et al., 2020). This micro-evolutionary process could account for macro-evolutionary pattern if populations that differ in flower color were to diverge to the point of speciation (Johnson, 2006).

As an alternative to the hypothesis that changes in pollinator composition account for geographical variation in flower color, recent studies have identified geographical variation in color preferences of the same pollinator as a basis of selection (Newman et al., 2012; Whitehead et al., 2018). This can occur when color preferences of a pollinator vary as a result of conditioning due to associations between colors and rewards in local plant communities (Campbell et al., 2010; Whitehead et al., 2018). A much less likely possibility, given the conserved visual systems of insects, is that differences in color preferences among populations of a single insect species could be hard-wired (Raine and Chittka, 2007).

The relative importance of biotic and abiotic drivers of flower color polymorphisms within and between populations are not well understood (Strauss and Whittall, 2006; Dalrymple et al., 2020; Sapir et al., 2021) and there is considerable debate about the extent to which both within and between population flower color polymorphisms represent the outcome of pollinator-mediated selection, as opposed to other mechanisms (Hannan, 1981; Strauss and Whittall, 2006; Rauscher, 2008; Paine et al., 2019; Dafni et al., 2020). Flower color transitions have been attributed to pollinators (Fenster et al., 2004), herbivory (Carlson and Holsinger, 2013), rainfall and sunlight (Schemske and Bierzychudek, 2007; Arista et al., 2013; Vaidya et al., 2018) or neutral processes (Edh et al., 2007; Wang et al., 2016). Plant pigments responsible for flower color, in particular anthocyanins, have a number of functions other than pollinator attraction (Bohm and Stuessy, 2001), and may be associated with abiotic factors such as soil chemistry (Mogford, 1974; Schemske and Bierzychudek, 2007; Koski and Ashman, 2016) drought and heat stress (Strauss and Whittall, 2006; Arista et al., 2013), as well as interactions such as herbivory, seed parasitism and

fitness (Coberly and Rauscher, 2008; Carlson and Holsinger, 2013). Flower color may also evolve in response to pollen and nectar robbers that visit flowers and deplete their resources without effecting pollination and fertilization. Red color, for example, may reduce visits by pollen-depleting honeybees (Lunau et al., 2011; Santamaría and Rodríguez-Gironés, 2015). Since plant populations occur in intricate communities and habitats with complex interactions, traits may be the result of several interacting selective factors (Strauss and Whittall, 2006; Carlson and Holsinger, 2013). While flower color polymorphism between populations has been relatively well-researched, within-population variation, which may be either continuous or discrete, has been less well studied (Sapir et al., 2021).

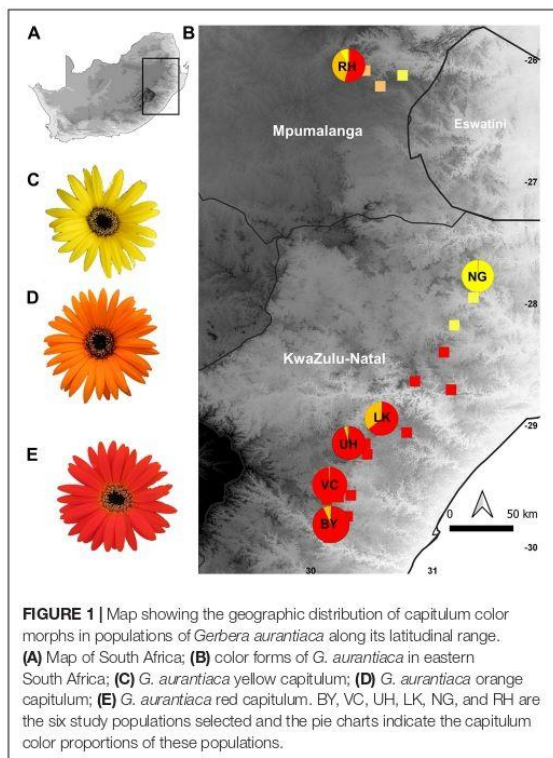
Here we investigated striking patterns of variation in capitulum color among and within populations of the daisy *Gerbera aurantiaca* and examined biotic and abiotic factors that could be correlated with this variation. Capitula of *G. aurantiaca* are typically bright red with a dark center, but several recently discovered populations are either entirely yellow-flowered (Johnson et al., 2005) or color polymorphic (ranging from yellow through orange to red). The combination of a red display and black center is typical of a guild of plants attractive to glaphyrid scarab beetles in the Mediterranean region (Dafni et al., 1990; Streinzer et al., 2019) and is also found in some members of various guilds of South African plants which are pollinated solely or primarily by hopliine scarab beetles (Bernhardt, 2000; Goldblatt and Manning, 2011; Johnson et al., 2020). The primary pollinators in red-flowered population of *G. aurantiaca* are hopliine scarab beetles, but honeybees are frequent visitors in yellow-flowered populations (Johnson et al., 2004; IM Johnson unpublished data). Hopliine scarab beetles use visual cues and are readily attracted to artificial flowers (Picker and Midgley, 1996; Johnson and Midgley, 2001; Van Kleunen et al., 2007), making them excellent experimental subjects for studies of pollinator-mediated selection on flower color.

Here we hypothesized that flower color polymorphism in *G. aurantiaca* is maintained by natural selection from pollinator preference or from differences in soil or climatic characteristics. We asked the following questions: (1) Does capitulum color variation in *G. aurantiaca* show a geographic distribution pattern? (2) Is morph color associated with abiotic factors? (3) What are the color preferences of the main pollinators? and (4) Do pollinators display a preference for color morphs in polymorphic populations of *G. aurantiaca*?

MATERIALS AND METHODS

Study Species and Sites

Gerbera aurantiaca Sch.Bip. (Asteraceae: Mutisieae) is a long-lived perennial herb endemic to the mistbelt grasslands of KwaZulu-Natal and Mpumalanga in eastern South Africa (Johnson et al., 2005). The species is known from approximately 25 scattered, isolated populations ranging in size from a few to hundreds of clones, and is listed as endangered due to habitat transformation resulting from commercial forestry, agriculture and urban development (Raimondo et al., 2009). The populations



occur in grasslands scattered along a roughly north-south axis between -26° and -30° S latitude along the eastern region of SA at elevations of between 900 and 1,700 m (Figure 1). *G. aurantiaca* populations are found on or near rocky outcrops in organic-rich topsoil overlying well-drained, acidic, nutrient-deficient sub-soils associated with a high degree of leaching typical of the high rainfall and cool temperatures of mistbelt grasslands (Fey, 2010).

Reproduction of *G. aurantiaca* is both clonal, by means of underground stems, and sexual with flowering taking place in austral spring (September to November). The radiate capitula contain about 200 florets, with approximately 100 female ray florets in three outer whorls, and with the central disc florets hermaphrodite but functionally male. Capitula are not nyctinastic and remain open throughout flowering. The species is largely self-incompatible and highly dependent on pollinators for fruit set (Johnson et al., 2004). Populations are typically predominantly red-flowered, but both completely yellow-flowered and color polymorphic populations with yellow, orange and red-flowered inflorescences also occur (Johnson et al., 2005). The chemical basis of capitulum coloration of *G. aurantiaca* has not been analyzed as far as we know but the flower color of *G. jamesonii* a sister species, and the related commercially important ornamental *Gerbera* hybrids, is derived from carotenoids which cause yellow and orange coloration, and the flavonoids, pelargonidin and cyanidin, which are responsible for the red coloration (Valadon and Mummary, 1967; Tyrach and Horn, 1997). A feature of *G. aurantiaca* capitula is the

dark center during the initial female stages of flowering when the disc florets are covered by the overarching dark purple pappus hairs and dark anther caps. Pollen is the primary floral reward as the florets produce little or no nectar and have no discernible scent.

The hairy monkey beetle *Eriesthis vulpina* Burmeister (Coleoptera: Scarabaeidae: Hopliini), appears to be the most important pollinator of *G. aurantiaca* (Johnson et al., 2004). Its use of color as a cue for locating flowers was evidenced by experiments in which large numbers of these beetles were captured in red plastic dishes placed in populations (Johnson et al., 2004). *Eriesthis* beetles almost completely ignored blue plastic dishes placed alongside red dishes in these populations (c. 100 beetles attracted to red dishes vs. one beetle attracted to blue dishes; SD Johnson, unpublished data), suggesting that they strongly prefer red over blue colors. The honeybee, *Apis mellifera scutellata* Lepeletier (Hymenoptera: Apidae) is also a common visitor in the yellow-flowered population.

Geographical Distribution of Color Morphs

We recorded the proportions of capitulum color morphs (as apparent in human vision) along transects across each of 23 populations ranging from southern KwaZulu-Natal to Mpumalanga (Figure 1 and Supplementary Table 1). At least one hundred clones were counted in larger populations and all clones were counted in the smaller populations. To minimize subjective bias in color allocation, one person (IMJ) carried out all counts. Ray floret samples were matched to a flower color chart (RHS, 2007) and broadly assigned to orange, red or yellow. Geographic position (south latitude and east longitude, WGS 84) and elevation (m.a.s.l) were measured using a handheld Garmin Etrex GPS. A generalized linear model (GLM) with a binomial error distribution, logit link function, and correction for overdispersion was applied to model the proportion of clones with red capitula in each population against latitude, longitude and elevation and we used likelihood ratios to test significance of fixed effects. All GLMs in this study were implemented in SPSS ver. 27 (IBM corp.).

Spectral Reflectance and Floral Morphological Trait Measurements

We selected six populations representative of the color variation across the distribution range (Figure 1) to investigate pollinator color preferences; of these three (BY, VC and UH) were predominantly red-flowered, one (NG) almost entirely yellow-flowered and two (RH and LK) polymorphic with a mixture of red, orange and yellow-flowered plants (Table 1). Here we measured spectral reflectance (300–700 nm) of the ray florets using an Ocean Optics S2,000 spectrometer (Ocean Optics Inc., Dunedin, Fla.), Ocean Optics DT-mini deuterium tungsten halogen light source and fibre optic reflection probe (QR-400-7-UUVVIS; 400 lm) held at 45° to the object surface in a probe holder (RPH-1). An Ocean Optics WS-1 diffuse reflectance standard was used to calibrate the spectrometer (Johnson and Andersson, 2002). Spectral reflectance readings were averaged from the mid-adaxial surface of three ray florets

TABLE 1 | Site name, abbreviation (abbr), geographical co-ordinates (dd), area (ha), approximate number of clones and percentage of clones with red orange and yellow capitula at the six *Gerbera aurantiaca* study sites.

| Site name | Abbr | Lat S; Long E | Area (ha) | No. of clones (approx.) | Capitulum color (%) | | |
|----------------|------|---------------|-----------|-------------------------|---------------------|--------|--------|
| | | | | | Red | Orange | Yellow |
| Byrne | BY | -29.81; 30.19 | 6.5 | 250 | 93 | 6 | 1 |
| Victoria Club | VC | -29.57; 30.33 | 2 | 100 | 99 | 1 | 0 |
| Umvoti Heights | UH | -29.18; 30.38 | 1.6 | 150 | 95 | 4 | 1 |
| Lookout | LK | -29.04; 30.58 | 6.2 | 250 | 64 | 34 | 2 |
| Ngome | NG | -27.84; 31.35 | 100 | > 1,000 | 0 | 1 | 99 |
| Rooihogte | RH | -26.05; 30.37 | 4 | 250 | 54 | 37 | 9 |

from at least 20 different plants from each population. All spectral reflectance curves are available from the Floral Reflectance Database (Arnold et al., 2010). Since we were interested in color as a floral signal and insect flower visitors most readily perceive rapid changes in spectral reflectance (Chittka and Menzel, 1992; Dyer et al., 2012) we used inflection or marker points which identify the wavelengths where change in spectral reflectance is maximal. We used the online *Spectral MP* (Dorin et al., 2020) to class capitulum color as yellow (inflection points from 520 to 530 nm), orange (inflection points from 530 to 600 nm) or red (inflection points from 600 to 625 nm). We recorded the positions of the different color morphs in the polymorphic RH population with a handheld Garmin Etrex GPS to investigate spatial structuring.

To test whether other floral traits that might influence pollinator attraction were associated with ray floret color we measured the capitulum diameter and ray floret length and ray floret inflection points for inflorescences from 50 clones collected randomly in the polymorphic RH population and tested for trait correlations using Pearson tests. We used finite mixture analysis allowing for unequal variance and with BIC criteria implemented in the *r* package *mclust* 5.4.6 (Scrucca et al., 2016) to assess whether the distribution of inflection points in populations were best explained by a single Gaussian distribution or by two or more Gaussian distributions. Both equal variance and unequal variance models were tested. We also tested for significant deviations from unimodality of inflection points in each population using Hartigan's diptest implemented in the *r* package *diptest* (Maechler, 2016).

The Influence of Abiotic Factors

We sourced the bioclimatic variables temperature (MAT: mean annual temperature °C) and precipitation (MAP: mean annual precipitation mm.) from www.worldclim.org/bioclim (Hijmans et al., 2005) for 23 populations across the distribution range of *G. aurantiaca* (Supplementary Table 1). A generalized linear model (GLM) with a binomial error distribution, logit link function, and correction for overdispersion was applied to model the proportion of clones with red capitula in each population against temperature and precipitation.

Since flower color in polymorphic species may be associated with differences in edaphic factors (soil characteristics)

(Horovitz, 1976; Rajakaruna and Bohm, 1999) we collected soil samples from 14 population locations of *G. aurantiaca*. For each population 15 augered subsamples of the top 15 cm layer taken randomly across the population patch were combined, air dried, stored at room temperature and analyzed for pH (KCL), exchange acidity, total cations, acid saturation, organic carbon (C), calcium (Ca), copper (Cu), potassium (K), magnesium (Mg), manganese (Mn), nitrogen (N₂), phosphorus (P), zinc (Zn), and clay content (Supplementary Table 2) by the Soil Fertility and Analytical Services Laboratory, KwaZulu-Natal Dept. of Agriculture (Manson and Roberts, 2000). We used Principal Components Analysis (Braak and Smilauer, 2002) to assess the relationship between the 14 edaphic variables (standardized to avoid using different scales for comparative purposes). Population scores were plotted onto the principal components axes and correlation vectors were used to examine relationships between the population samples and the first two PCA axes of the soil ordination. Linear regression was carried out using the first four regression factor scores and the proportion of red clones in the 14 populations.

Common Garden Experiment

To test whether the capitulum color of *G. aurantiaca* plants from different color morph populations changes when they are grown together under identical climatic and edaphic conditions, we cultivated both ramets and seeds collected from red and yellow flowered populations in a common garden at the KwaZulu-Natal National Botanical Garden in Pietermaritzburg where conditions (soil characteristics, temperature and precipitation) were identical. We did not include orange morphs since we were not aware of their extent in the polymorphic populations at the start of this experiment. Sample sizes were limited due to permit restrictions relating to the threatened status of the species. We collected flowering ramets from each of five widely spaced clones growing in red (BY) and yellow (NG) source populations and transplanted these into the common garden nursery bed. Ramets were used as they provided a baseline spectral reflectance and because they take less time to flower in cultivation than seed raised plants. In addition, seeds were collected from clones at least five meters apart in the red-flowered LM and the yellow-flowered NG populations and marked seedlings from each population were transplanted randomly into the common garden nursery bed. Reflectance spectra of ray florets from each individual were

measured at collection (in the case of ramets) and at intervals once flowering had occurred for both groups over a 5 year period. We compared mean inflection points of the spectral reflectance curves (Dorin et al., 2020) of ray florets for both ramets and seed grown plants using a GLM with normal distribution with source population and year as independent variables.

Color Choice Behavior of Insect Visitors

Pan traps have been widely used for passive sampling of flower-visiting insects and may give an indication of their color preferences as well as their abundance (Picker and Midgley, 1996; Leong and Thorp, 1999; Shrestha et al., 2019). Here we tested color discrimination of potential pollinators using colored pan trap arrays at the six study populations. Red and yellow traps were chosen as they represent the extremes of capitulum colors that occur naturally in the range of *G. aurantiaca*. We did not test for orange due to the unavailability of traps with suitable reflectance spectra. Spectral reflection curves were used to calculate the inflection points (Dorin et al., 2020) of the traps and compare these to those of *G. aurantiaca* capitula. We used twenty sets of red and yellow plastic traps (11 cm in diameter and 8.5 cm deep) filled with 150 ml of water and placed randomly in pairs 15 cm apart amongst flowering *G. aurantiaca* plants. We carried out these experiments during peak flowering on sunny days between 07:00 and 16:00 h in late October when insects were most active. Captured insects were identified at least to family level and counted. Beetles appeared to be unaffected by being trapped and most were released after recording on account of their role as important pollinators of this threatened study species. Pan trap catches of the dominant insect visitors, *E. vulpina* beetles and honeybees, were analyzed using a GLM with negative binomial distribution and log link with trap color and population site as independent variables. For the analysis of catches, one value at sites where no insects were caught in traps had to be adjusted from a choice for yellow to one for red in order for the model to converge (Zuur et al., 2009).

Since hymenopteran vision is well-researched we used the bee color hexagon (Chittka and Menzel, 1992; Peitsch et al., 1992) to assess how honeybees would perceive the color of capitula of *G. aurantiaca* and the pan traps. The mean reflectance spectrum calculated from three ray florets from each of 20 individuals of each color form and those from the red and yellow pan traps was plotted in the bee color hexagon. Background color was calculated from the spectra of *G. aurantiaca* leaves. Coleopteran vision has been less well studied and due to this we did not assess their perception of capitulum color.

Capitulum Visitation and Fruit Set in the Color Polymorphic Population

To determine if *E. vulpina* hopliine scarab beetles, the dominant insect visitors to *G. aurantiaca*, exhibit color preferences in a polymorphic population, we recorded the frequency of these beetles visiting capitula of different color morphs at the RH population along three 200 m transects during peak flowering in three separate years. As the intensity of flowering varied from year to year, we recorded different total inflorescence numbers

per transect (2008 $N = 155$, 2011 $N = 471$, 2013 $N = 291$). The proportion of capitula of different colors (yellow, orange, and red) that were occupied by beetles was compared using a logistic GLM with binomial distribution and logit link function.

We measured fecundity (mean fruit set per capitulum) in relation to morph color in the polymorphic RH population. In 2011 ($N = 61$: yellow = 19, orange = 28, red = 24), and 2013 ($N = 56$: yellow = 9, orange = 13, red = 34) capitula were bagged and labeled at the end of the male phase and harvested after 3 weeks. Filled fruit, easily distinguished by their larger size, darker color and firmness were counted for each bagged capitulum. Fruit set per capitulum for each year was analyzed using a GLM with a negative binomial distribution and log link function. Color morph, year and the interaction of color morph and year were fixed factors in this model.

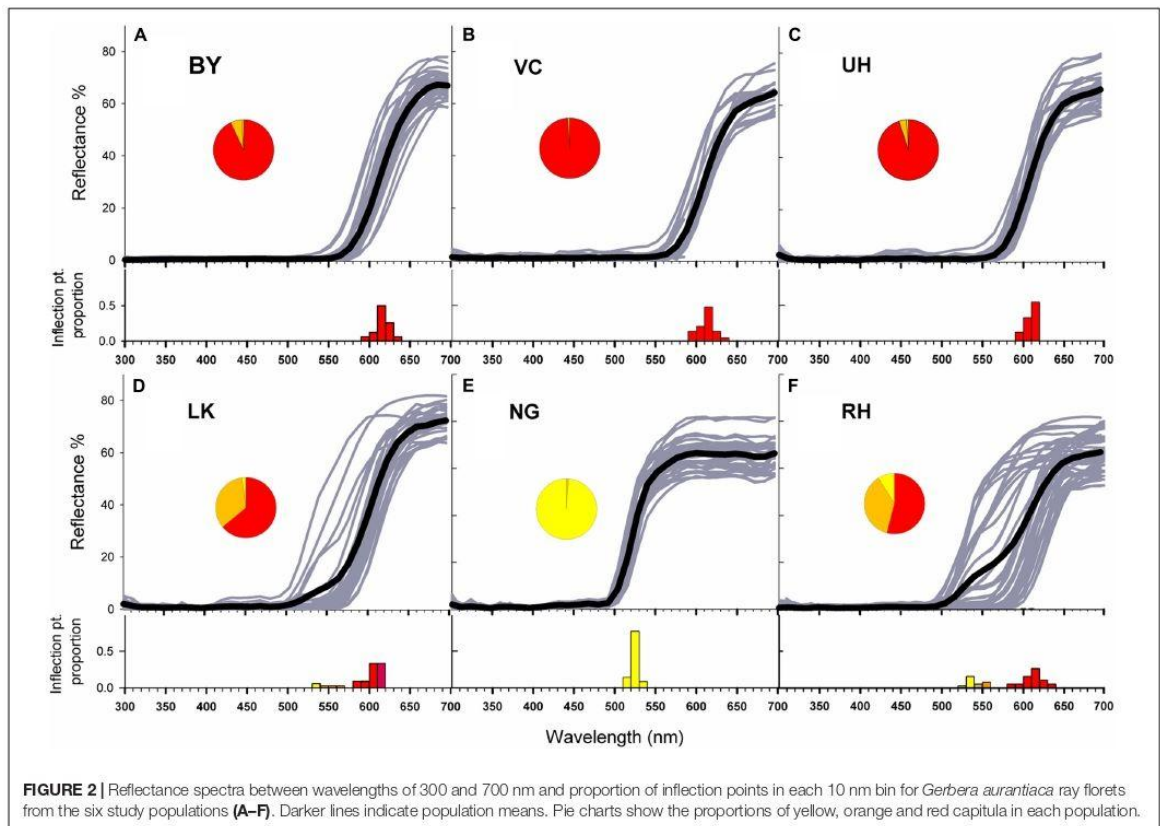
RESULTS

Geographical Distribution of Color Morphs

In human color vision 15 of the 23 *G. aurantiaca* populations measured had predominantly red-flowered clones, 5 were predominantly yellow-flowered and 3 were polymorphic with mixed yellow, orange and red-flowered clones. The red flowered populations occur mainly in the southern part of the distribution range with one polymorphic population (LK), the yellow cluster in the center, and northern populations are mainly color polymorphic with varying proportions of red, orange and yellow capitula (Figure 1 and Supplementary Table 1). The proportion of clones with red capitula in the 23 populations was significantly correlated with latitude ($\chi^2 = 9.460$, $df = 22$, $P = 0.002$), and longitude ($\chi^2 = 6.791$, $df = 22$, $P = 0.009$) but not with elevation ($\chi^2 = 1.805$, $df = 22$, $P = 0.179$).

Spectral Reflectance and Floral Morphological Trait Measurements

Mean inflection points of the reflectance spectra of the six representative populations were between 609 and 615 nm for the red-flowered populations (BY 615 nm, VC 611 nm, UH 609 nm, LK 600 nm) populations, 524 nm for the yellow-flowered NG population and 597 nm for the polymorphic RH population (Figures 2A–F and Supplementary Table 2). No UV reflectance (300–400 nm) was recorded (Figure 2). Although the northern color polymorphic RH and central LK populations showed a wide range of color variation we were able to assign plants to particular color classes as the distribution of inflection points in this population was broad (Figures 2D,F), with most inflection points clustered around 620 nm with a smaller peak around 540 nm for RH and 600–620 with a smaller cluster at 530–570 nm for LK. The frequency of inflection points in the NG, BY, VC and UH populations fitted a single Gaussian distribution better than two (ΔBIC range = 2.24–7.80), but the distribution of inflection points fitted two Gaussian distributions much better than one Gaussian distribution in the RH population ($\Delta BIC = 39.8$) and LK population ($\Delta BIC = 23.5$). Fits to three



or more Gaussian distributions were not supported for any population, but in the RH and LK populations, models with fits to three Gaussian distributions fitted better than models with fits to a single distribution ($\Delta\text{BIC} = 16.4\text{--}33.6$) but were still supported less than were models fitted to two Gaussian distributions ($\Delta\text{BIC} = 6.2\text{--}7.1$). However, we found no significant deviations from unimodality for the distributions of inflection points in any of the populations ($P > 0.095$). We also found no significant correlations between inflection points and capitulum diameter ($r = -0.15$, $P = 0.289$) or ray length ($r = -0.029$, $P = 0.845$) (Supplementary Table 3).

The Influence of Abiotic Factors

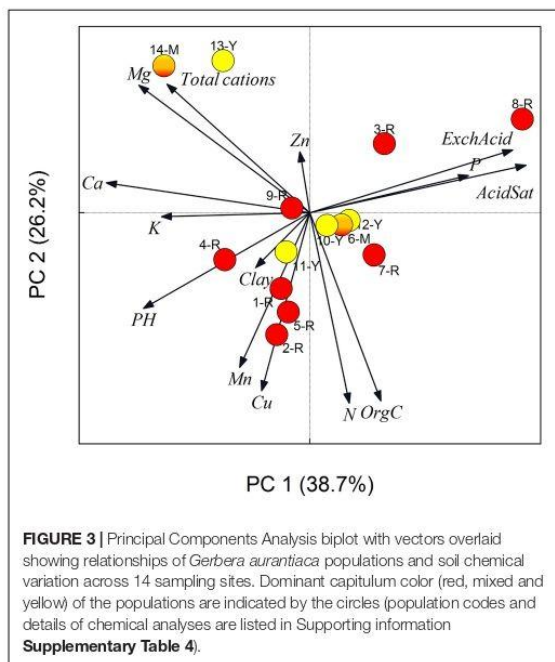
Environmental conditions were similar across the distribution range of *G. aurantiaca* with climatic variables for the 23 population localities ranging from 14.0 to 17.6°C for mean annual temperature (MAT), 878–1057 mm for mean annual precipitation (MAP) (Supplementary Table 1). The proportion of clones with red capitula in populations was not significantly correlated with temperature ($\chi^2 = 0.662$, $\text{df} = 22$, $P = 0.416$) or precipitation ($\chi^2 = 2.702$, $\text{df} = 22$, $P = 0.1$).

Differences in soil chemistry between sites were small (pH ranged from 3.94 to 4.69, N from 0.12 to 0.6% and organic C content 2.5–6%) (Supplementary Table 4). Principal

components of the edaphic variables were summarized into the first and second axes of the principal component analysis (PCA), which accounted for 64.9% of the total variation and were mainly explained by pH-acidity gradient (axis 1) and Org C/N gradient (axis 2) (Supplementary Figure 1 and Supplementary Table 5). The PCA biplot (Figure 3) shows an apparent pH-acidity gradient, and the two northernmost sites on the top left stand out because of their more weathered soils with higher Mg and lower than average organic carbon content. pH and Organic C can be used as largely independent surrogate variables to represent variation of other associated variables along these two typical soil chemistry gradients. We found no significant relationship between the proportion of red clones in a population and PCA Factor 1 ($r^2 = 0.037$, $P = 0.512$), Factor 2 ($r^2 = 0.24$, $P = 0.596$), Factor 3 ($r^2 = 0.008$, $P = 0.977$) or Factor 4 ($r^2 = 0.05$, $P = 0.441$) using linear regression. We further observed no spatial structure in the polymorphic RH population with different color morphs scattered randomly (Figure 4A) and often growing less than one meter from each other (Figure 4E).

Common Garden Experiment

Both ramets and seed-raised plants retained the capitulum color of the parent populations when grown in a common garden



over a 5-year period. Flowering ramets collected in the red-flowered BY (mean inflection point 616.0 ± 1.7 nm, $N = 5$) and yellow-flowered NG (mean inflection point 525.6 ± 1.8 , $N = 5$) populations showed no change from their original ray floret morph color ($\chi^2 = 3397.82$, $df = 1$, $P < 0.0001$) with no significant change in morph color recorded over the 5-year observation period ($\chi^2 = 1.319$, $df = 1$, $P = 0.517$) and no significant interaction between population and year ($\chi^2 = 1.530$, $df = 3$, $P = 0.465$) (**Figure 5A**).

Seed-raised plants flowered 4 years after germination and displayed the predominant capitulum color of the source populations: LM (red with mean inflection point 606.80 ± 1.8 nm, $N = 18$) and NG (yellow with mean inflection point 523.4 ± 1.9 nm, $N = 18$). There was a significant difference between the mean inflection points of the two seed populations ($\chi^2 = 2012.457$, $df = 1$, $P < 0.0001$) but there was no significant effect of time ($\chi^2 = 0.51$, $df = 1$, $P = 0.821$) or interaction between seed population and year ($\chi^2 = 0.003$, $df = 2$, $P = 0.957$) (**Figure 5B**).

Color Choice Behavior of Insect Visitors

A total of 682 *E. vulpina* beetles, and 39 honeybees, as well as some insects from other taxonomic groups were caught in the red and yellow pan traps. At all populations where beetles were present (UH, LK, NG, and RH) significantly more individuals of *E. vulpina* were caught in yellow than red traps (**Figure 6A** and **Supplementary Table 6**). The highest number of beetles overall was trapped at the red-flowered UH population, followed by RH (flower color polymorphic), NG (yellow), and LK (predominantly

red), while no individuals of *E. vulpina* were trapped at the two southernmost sites, BY and VC (red). We recorded significant effects of population ($\chi^2 = 97.951$, $df = 5$, $P = 0.001$), and of trap color ($\chi^2 = 14.904$, $df = 1$, $P = 0.001$) on the number of *E. vulpina* beetles caught, but the interaction between population and trap color was not significant ($\chi^2 = 7.182$, $df = 6$, $P = 0.207$) suggesting that there were no marked differences in beetle color preference between populations.

Very few honeybees were captured in pan traps, but despite this low statistical power we detected a slight significant effect of trap color ($\chi^2 = 5.172$, $df = 1$, $P = 0.023$), but not of population ($\chi^2 = 9.464$, $df = 5$, $P = 0.092$). There was a significant interaction between population and trap color ($\chi^2 = 15.352$, $df = 6$, $P = 0.009$) with more bees trapped in yellow than red traps at four of the populations (BY, VC, UH, and LK), equal numbers at NG and none trapped at RH although they were frequently observed on other plants at the site (**Figure 6B** and **Supplementary Table 6**).

When plotted in the model of hymenopteran color perception, loci of *G. aurantiaca* ray floret reflectance spectra were positioned primarily in the green and UV green segments of the hexagon with the florets of the red color forms clustered more closely to the origin of the hexagon than the yellow forms, suggesting that the yellow capitula are more visible to bees than the red. The loci of orange forms were intermediate between red and yellow in bee color space (**Figure 6C**). The spectral reflectances of the pan traps (**Supplementary Figure 2**) were similar to those of the red and yellow ray florets in terms of hymenopteran color perception (**Figure 6C**) and inflection points (red trap = 601 nm, floret = 608 nm; yellow trap = 519 nm, floret = 524).

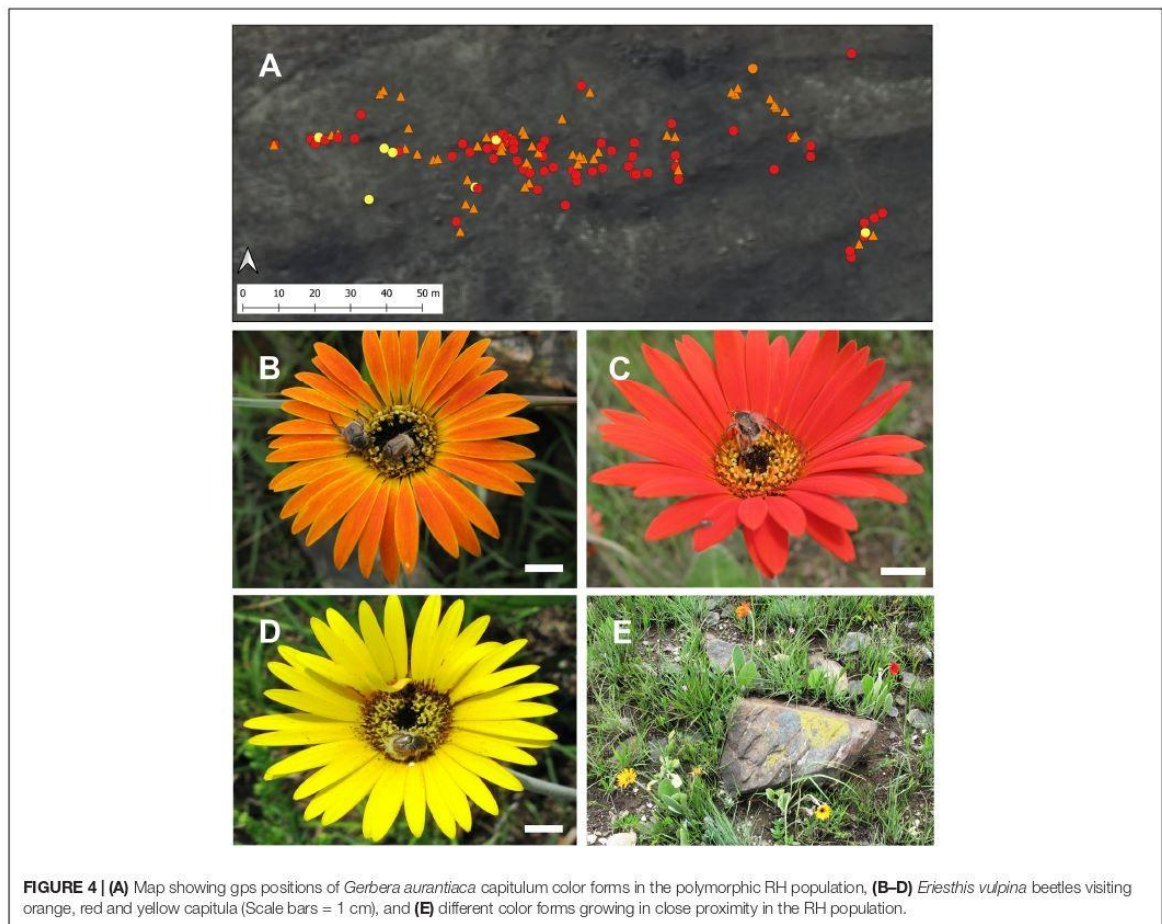
Capitulum Visitation and Fruit Set in the Color Polymorphic Population

Analysis of the incidence of *E. vulpina* beetles present on orange, red and yellow colored capitula (**Figures 4B–D**) at the mixed color site (RH) during transect surveys in three separate years indicated that the beetles did not discriminate among color morphs, with no significant differences found between the proportions of orange, red and yellow capitula that were occupied by beetles ($\chi^2 = 0.410$, $df = 2$, $P = 0.814$). The overall proportion of capitula occupied by beetles varied significantly among years ($\chi^2 = 24.90$, $df = 2$, $P < 0.0001$) with a higher incidence of beetles on capitula in 2013 than in 2008 or 2011 (**Figure 7A**). There was no interaction between year and color morph ($\chi^2 = 0.643$, $df = 4$, $P = 0.958$), implying that responses to colors by beetles did not vary among years.

We did not detect any significant differences in mean fruit set among color morphs in 2011 or 2013 ($\chi^2 = 0.349$, $df = 2$, $P = 0.840$) or year ($\chi^2 = 292$, $df = 1$, $P = 0.589$). There was no significant interaction between year and color morph ($\chi^2 = 0.216$, $df = 3$, $P = 0.898$) (**Figure 7B**).

DISCUSSION

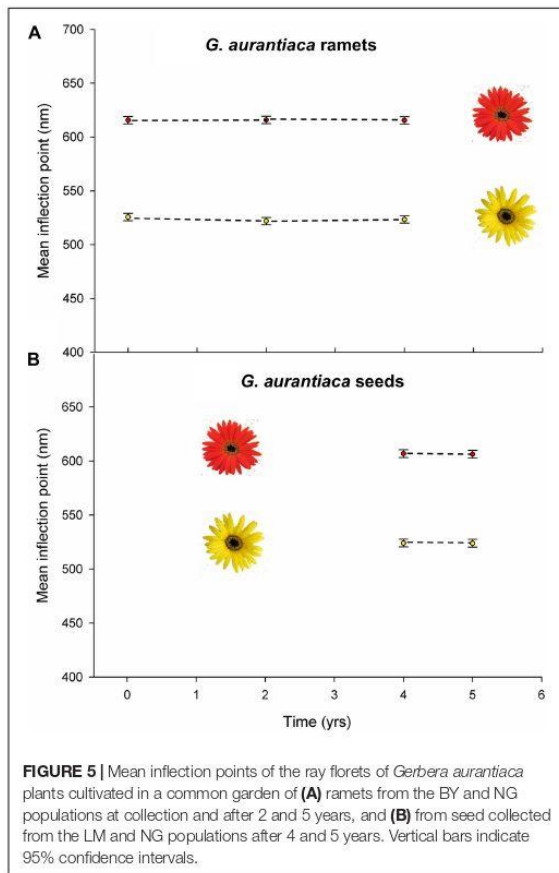
This study reveals a pattern of geographical structure of variation in capitulum color of *G. aurantiaca* along the north-south axis of its range with most southern populations being predominantly



red, the central cluster being predominantly yellow, and four populations, mainly in the north displaying a pattern of within population color polymorphism. Color variation in two polymorphic populations was continuous, but with inflection points clustering in two groups, as supported by the finite mixture analysis. We found no clear association between capitulum color and various ecogeographic and edaphic factors although a more detailed analysis of nitrogen in the forms utilized by plants should be carried out in future studies to confirm this. We recorded no spatial segregation of color morphs in the polymorphic RH population with plants of different inflorescence color growing intermixed in close proximity, suggesting that climate and soils do not influence floral color. Floral color was maintained over a 5-year period in a common garden experiment, both in ramet- and seed-grown plants, further indicating that the color polymorphism is not a response to soil or climatic variation (de Villemereuil et al., 2016). While the lack of a clear association with abiotic factors points to a biotic explanation for the evolutionary divergence in flower color among populations, the role of pollinators as drivers of the color change in *G. aurantiaca* is

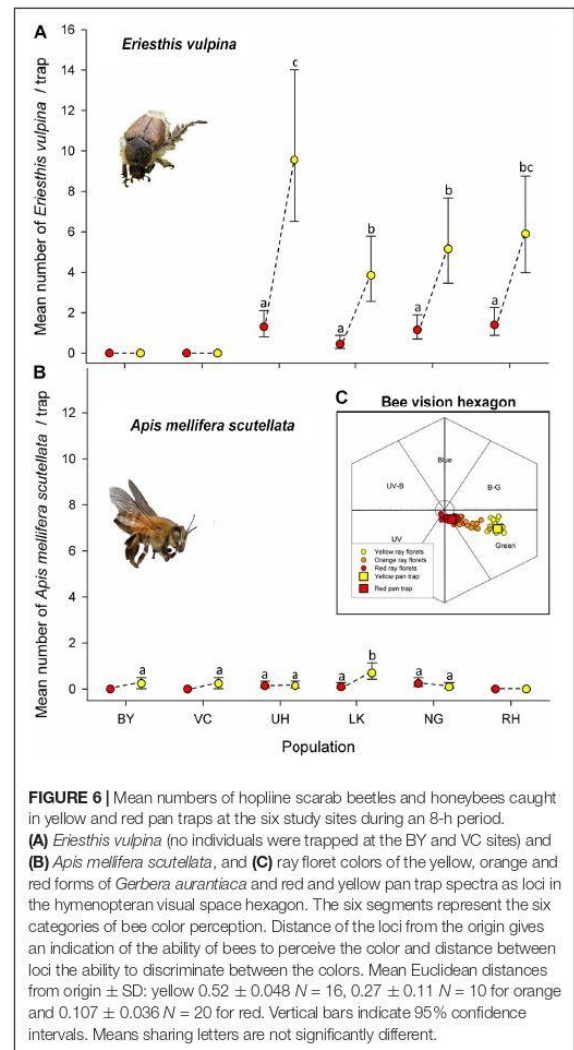
not entirely clear. Pollination studies have shown that a single hopliine scarab beetle species, *E. vulpina*, is responsible for the majority of all floral visits to *G. aurantiaca* (Johnson et al., 2004), unlike the color polymorphic *Drosera cistiflora* in the western Cape region of South Africa, where several different species of hopliine scarab beetles discriminate between different color forms in the same population (Johnson et al., 2020). Our study did not provide clear evidence that *E. vulpina* beetles prefer any particular capitulum color within a population as they were recorded visiting capitula in both the red and yellow-flowered populations, and they did not appear to discriminate between *G. aurantiaca* color morphs at the northern color polymorphic RH site. This was further supported by the lack of significant differences in fecundity of the three color morphs at the RH population, where capitula were almost exclusively visited by *E. vulpina* beetles.

Native honeybees are frequent visitors to the flowers of many plant species of the eastern South African grasslands (Hepburn and Radloff, 1995; Stanley et al., 2020) but there is some debate regarding their ability to easily detect red

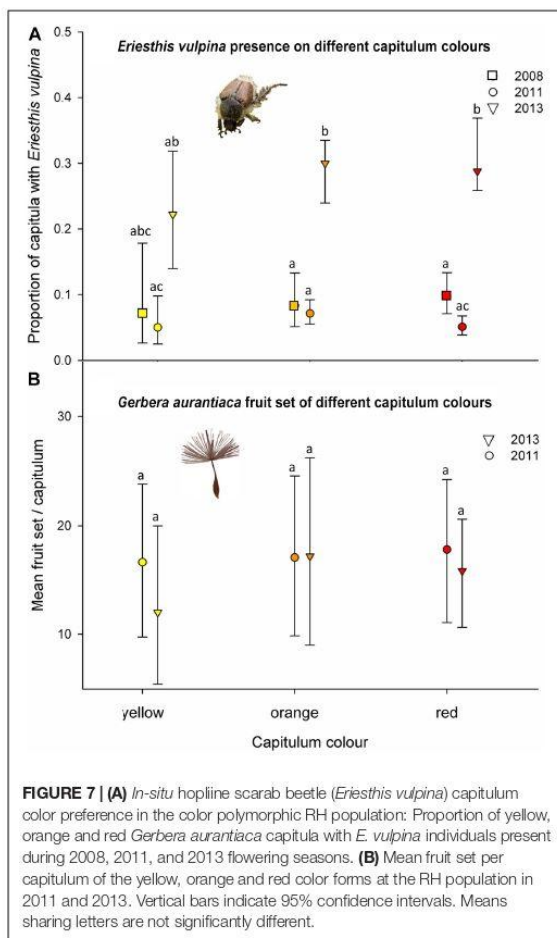


flowers (Chittka and Waser, 1997; Lunau et al., 2011). Although honeybees were observed visiting other flower species in the color polymorphic RH population and were frequently seen on yellow capitula in the monomorphic NG population, none were recorded on red *G. aurantiaca* capitula during the transect surveys. The relative importance of monkey beetles and honeybees as pollinators of yellow populations of *G. aurantiaca* is difficult to determine without further research into pollen deposition efficiency (King et al., 2013).

While *E. vulpina* preferred yellow over red in the color choice experiments using pan traps at all populations where it was present (including the predominantly red-flowered), approximately 25% of the total catch was from red traps. Based on this and the frequent presence of *E. vulpina* on red capitula it appears that red-colored flowers are easily detected by this beetle. Although beetles preferred yellow over red plastic bowls, this may reflect the particular spectral properties of the plastic bowls in this experiment. Attraction of hopline scarab beetles in the Western Cape to red bowl-shaped flowers has been recorded in previous studies (Johnson and Midgley, 2001; Johnson et al., 2004; Van Kleunen et al., 2007), but these studies indicate that



trap color choices do not necessarily reflect the flower colors favored by these beetles (Picker and Midgley, 1996; Mayer et al., 2006; Van Kleunen et al., 2007). We hesitate to conclude that beetles generally prefer yellow over red flower color as such a preference was not evident in our survey of beetles on capitula in polymorphic populations. This survey suggests that in natural polymorphic populations, red, orange and yellow capitula have equal probabilities of being visited by *E. vulpina*. This could also indicate that additional cues other than color, such as suitability of larger capitula for socializing, may be used by these beetles for recognition and choice of floral host plants (Dafni, 1997). We found no association between capitulum and ray floret size and flower color inflection points in the RH polymorphic population suggesting that capitulum color is not linked to morphological



traits and thus unlikely to evolve through pleiotropic effects of morphological evolution. We did not use a vision model to determine how *E. vulpina* beetles may perceive the ray floret and pan trap colors as the spectral sensitivity of photoreceptors of this species is not known. Coleopteran vision has been less well studied than hymenopteran vision but recent studies suggest that their visual capability can extend into longer wavelengths than that of Hymenoptera (Martínez-Harms et al., 2012; Lehardt and Desplan, 2017; Streinzer et al., 2019).

We caught relatively fewer honeybees than hopliine scarab beetles in the pan traps, but even our small sample suggested that bees showed a marked preference for yellow over red at most sites. When plotted in the bee color hexagon, the loci for red ray floret limbs of *G. aurantiaca* capitula and for the red pan traps used here are close to those for the vegetative background, and are therefore likely to be indistinct to honeybees, while the loci for yellow ray florets and for the yellow pan traps place further away from that of the background providing a heightened contrast and are therefore more visibly distinct to hymenopterans. This may

explain why bees seldom visit the red color forms of *G. aurantiaca* but were frequently observed visiting capitula in the yellow NG population, although, curiously, not in the polymorphic RH population. The yellow-flowered population of *G. aurantiaca* may thus have a bimodal pollination system involving both beetles and honeybees.

Red flowers that are not bird pollinated are uncommon in these mistbelt grasslands and as far as we are aware there are no insect pollinators that specialize on visiting red flowers in the region other than the butterfly *Aeropetes tulbaghia* (Johnson et al., 2009) which is a nectar feeder and unlikely to visit *G. aurantiaca*. The hopliine scarab beetles which pollinate the *G. aurantiaca* flowers appear to be attracted to all color forms equally and in red-flowered populations where they are absent fruit set is extremely low (Johnson et al., 2004) making the dominance of red capitula in these southernmost populations enigmatic. One possible scenario is that the red form of *G. aurantiaca* was historically associated with a pollinator which selected for the red color, but is no longer present (Cooley et al., 2008; Hopkins and Rausher, 2012) and that the long-lived clonal growth form of *G. aurantiaca* would allow the red color form to persist long after the selective preference of the now absent specialist pollinator had disappeared. A second possibility, yet to be tested, is that pollen robbers such as honeybees have a net negative effect on plant fitness because of their efficient collection and transfer of pollen to their corbiculae without depositing it on appropriate stigmas and therefore causing pollen limitation (Hargreaves et al., 2010). It has been proposed that red flower color could be a strategy to avoid pollen robbing by bees (Lunau et al., 2011; Santamaría and Rodríguez-Gironés, 2015). In this case, the red color of some morphs may represent a compromise between attraction and defense, in that red capitula are clearly attractive to beetle pollinators while at the same time are relatively inconspicuous to pollen-robbing insects such as honeybees. While this strategy appears effective in the case of *G. aurantiaca* since honeybees were seldom observed visiting red flowers, the high fruit set in the yellow NG population where honeybees were frequent recorded suggests that they may contribute to fruit set. Experimental observations of the relative success of single visits on fruit set by honeybees and comparisons of the efficiency of pollen transfer by beetles vs. honeybees at this site may help to clarify this.

A further possibility to consider is that the color polymorphic populations in the northern part of the range may be the result of red and yellow forms meeting and interbreeding. Preliminary crosses indicate that the red and yellow color forms of *G. aurantiaca* breed true, with inter-red and inter-yellow crosses producing entirely red and yellow F1 offspring, respectively. Crosses between red and yellow forms (both yellow pollen onto red mothers and red pollen onto yellow mothers) produce almost entirely orange and yellow F1 offspring (IM Johnson, unpublished data). However, we are not aware of red-flowered populations in the northern part of the distribution range of *G. aurantiaca*, which casts doubt on the idea that within-population color polymorphism reflects hybridization.

Since the capitulum color variation across the distribution range of *G. aurantiaca* described here does not appear to be influenced by pollinator color preference or climatic and

soil characteristics it provides an ideal system for testing whether there is a pattern of neutral evolution that reflects simple isolation by distance (Wright, 1943; Schemske and Bierzychudek, 2001, 2007). We are currently analyzing the population genetic structure across the geographic and color variation range using molecular markers to gain a better understanding of the genetic relationships among populations and color morphs.

In conclusion, we found that the geographic pattern of capitulum color distribution in *G. aurantiaca* populations does not appear to be associated with abiotic factors or pollinator color preferences. The idea that the divergence between red- and yellow-flowered population reflects a shift between beetle and bee populations is appealing, but we did not obtain clear evidence that hopliine scarab beetles favor red over orange or yellow inflorescences in the color polymorphic population. Further work needs to be conducted to explore the effects of capitulum color on interactions between *G. aurantiaca* and various antagonists, such as pollen thieves, herbivores and seed predators, as well as detailed genetic studies of within and between population structure in order to better understand the mechanisms driving color variation in this species.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

This manuscript is based on a Ph.D. thesis by IMJ who collected the data and wrote up the results under the supervision of SDJ and TJE. All authors contributed to the article and approved the submitted version and participated in the design of the study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2021.676520/full#supplementary-material>

Supplementary Figure 1 | Scree plot showing eigenvalues of principal components for edaphic variables of 14 *G. aurantiaca* population.

Supplementary Figure 2 | Reflectance spectra of red and yellow pan traps.

Supplementary Table 1 | Location, geographical and climatic conditions of *G. aurantiaca* populations across their distributional range.

Supplementary Table 2 | Mean inflection points of *Gerbera aurantiaca* ray florets from the six study populations.

Supplementary Table 3 | Floral morphological traits measurements (inflection points, capitulum diameter and ray floret length) from the color polymorphic RH population of *Gerbera aurantiaca*.

Supplementary Table 4 | Soil chemical properties for 14 sites across the geographical range of *Gerbera aurantiaca*.

Supplementary Table 5a | Total variance of explained soil variable components.

Supplementary Table 5b | Component matrix for *G. aurantiaca* soil variables.

Supplementary Table 5c | Regression factor scores for soils PCA of 14 *G. aurantiaca* populations.

Supplementary Table 6 | Pan trap catches: numbers of *Eriesthis vulpina* and *Apis mellifera* individuals caught in red and yellow pan traps.

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**CHAPTER 3: A BEETLE POLLINATOR REDUCES POLLEN-
LIMITATION OF FECUNDITY IN POPULATIONS OF A SELF-
INCOMPATIBLE COLOUR-POLYMORPHIC DAISY**

Abstract

Plants with specialized pollination systems may have a relatively high risk of reproductive failure if they are fully dependent on pollinators for seed production. Here we studied the breeding systems and the effects of pollinator and seed predator abundance on seed production of an endangered daisy, *Gerbera aurantiaca* which has a polymorphism in flower colour which ranges from red in most populations to yellow or a blend of these colours in a few populations.

We used controlled pollination experiments to investigate levels of self-incompatibility and pollen limitation of seed production in populations across the geographical range of *G. aurantiaca* and also studied pollinator abundance in these populations.

We found that *G. aurantiaca* is effectively genetically self-incompatible throughout its range. Seed production is severely pollen limited in some populations. Experiments involving inter-population crossing revealed that pollen limitation is determined by the quantity and not the quality of pollen on stigmas. The most numerous flower visitor was a hopliine scarab beetle *Eriesthis vulpina* that visited capitula of all colour morphs. Native honeybees were frequent visitors to *G. aurantiaca* only in a monomorphic, yellow-flowered population. Both fruit set and an index of pollen limitation were strongly correlated with the abundance of *E. vulpina* beetles in populations. The proportion of capitula impacted by ovule predation did not differ significantly between populations, or between colour morphs in a flower colour polymorphic population.

We conclude that the widespread red forms of *G. aurantiaca* have a specialised pollination system involving a single beetle pollinator species which increases its vulnerability, while the rarer yellow forms are possibly co-pollinated by other insect taxa. Because of its role in determining seed production of *G. aurantiaca*, the hopliine beetle *E. vulpina* can be considered critical for the pollination and survival of most populations of this endangered daisy species.

Key words: flower colour, breeding system, pollinators, ovule predation, *Gerbera aurantiaca*

INTRODUCTION

The risk of reproductive failure in plant populations is strongly affected by the combination of availability of pollinators, ability of plants to self-fertilize, and demographic dependence

on seeds (Bond 1994). Plant with highly specialized pollination systems often have compensatory mechanisms such as facultative self-fertilization or being long-lived that allow them to persist when pollinators are temporarily not available. However, for many endangered plants, available information on their reproductive systems is not sufficient to make an informed decision about risks of reproductive failure. This problem is particularly acute for species that are highly variable across their distribution range, as this variation may include traits that determine the risk of reproductive failure.

Flower colour is one of the most striking floral traits in nature, and while most plant species exhibit floral colours that are uniform among individuals, a small minority display variation both within and among populations (Narbona et al., 2018; Rausher, 2008). Flower colour polymorphism has been defined “as the presence of two or more genetically determined colour morphs within a single interbreeding population, the rarest of which is too frequent to be solely the result of recurrent mutation” (Ford, 1945; Huxley, 1955). These polymorphisms are of evolutionary interest because both selection and genetic drift usually lead to trait homogeneity, thus polymorphisms can be revealing about the existence of additional processes such as negative frequency dependent selection and introgression among populations (Sapir et al., 2021). Species that show within-population polymorphism also often show geographical variation in these traits. This can be due to divergent selection or neutral processes that lead to fixation of different traits in different populations.

The role of pollinators in colour variation has probably been the most widely studied (Sapir et al., 2021; Schiestl and Johnson, 2013). Colour perception depends on the sensory abilities of different groups of pollinators, and visual systems vary widely among pollinator guilds, with different pollinator groups able to discern certain spectral wavelength regions more clearly than others (van der Kooi et al., 2021). Bees, for example, most often visit blue and pink flowers, but longer wavelength reflecting red flowers are more easily detected by birds and certain butterflies and coleopterans (León-Osper and Narbona, 2022; Martínez-Harms et al., 2020; Shrestha et al., 2013). Flower colour preferences of flowers visitors can also be influenced by associative conditioning (Schiestl and Johnson, 2013). Different colour morphs within a population may attract and be pollinated by different groups of pollinators with differing visual capabilities, sometimes leading to population divergence and, ultimately, speciation (Campbell et al., 2010; Trunschke et al., 2021). A striking example here is the divergence of the red corn poppy *Papaver rhoeas*, which in the Middle East has non-UV reflecting petals which attract glaphyrid beetles, while those in Europe, which are UV reflecting, are visited by bees (Dafni et al., 1990; Martínez-Harms et al., 2020).

Although flower colour is well-known for its role in pollinator attraction, selection on floral traits seldom results only from pollinator preference (Strauss and Whittall, 2006). A number of studies have identified other possible important drivers of flower colour variation, including both abiotic and biotic factors (Carlson and Holsinger, 2013; Caruso et al., 2018) as well as lack of drivers or neutral processes (Wang et al., 2016). Abiotic factors include temperature and moisture (Arista et al., 2013; Schemske and Bierzychudek, 2007) as well as soil chemistry (Koski and Ashman, 2016; Mogford, 1974; Schemske and Bierzychudek, 2007), while biotic factors embrace pollinator mediated selection through colour choice (Fenster et al., 2004; Whibley et al., 2006), as well as non-pollinator biotic agents such as herbivores and florivores, in particular seed and ovule predators (Buide et al., 2021; Strauss and Whittall, 2006).

Although genetic drift and selection are both considered the main factors that might explain flower colour polymorphisms, the sexual systems of plants also need to be considered (Narbona et al., 2018). Sexual systems, including breeding systems (traits that influence the potential for plants to self-fertilize) affect genetic composition and patterns of genetic diversity (Charlesworth, 2006). Variation in breeding systems may also impact pollinator-mediated selection on floral traits (Jiménez-López et al., 2020; Vaidya et al., 2018). The potential for a species to self-fertilize can play an important role in the maintenance of flower colour variation, and colour morphs not favoured by pollinators may be maintained in populations by autonomous selfing (Buide et al., 2021; Jiménez-López et al., 2020; Vaidya et al., 2018), but the extent to which flower colour variation may be associated with variation in breeding system is largely unknown (von Witt et al., 2020).

The use of controlled pollination experiments is essential to establish the degree of self-incompatibility and the overall dependence of the study species on pollinator visits for reproductive success (Rodger and Ellis, 2016). This information is especially important for threatened plant species since the degree of pollinator-dependence dictates their vulnerability to local extirpation of pollinators (Bond, 1994; Pauw and Bond, 2011). In particular, autonomous selfing may enable reproductive assurance when opportunities for outcrossing are limited (Holsinger, 2000; Pannell et al., 2015).

Gerbera aurantiaca (Asteraceae), a long-lived clonal daisy endemic to the mistbelt grassland of eastern South Africa exhibits a geographical pattern of flower colour variation, with monomorphic populations of both red and yellow, and with discrete flower colour polymorphisms in two populations with individuals with either red, orange or yellow capitula (Johnson et al., 2021). Abiotic factors including elevation, temperature, precipitation and soil

chemistry do not appear to be related to differences in flower colour across the distribution range, and colour morphs in the mixed populations exhibit a mosaic rather than clinal distribution, further indicating that abiotic factors are unlikely to be important in influencing capitulum colour variation (Johnson et al., 2021). Red and yellow forms maintained their source colour over several years when grown under identical conditions, suggesting a genetic basis for the colour variation (Johnson et al., 2021). Previous studies on *G. aurantiaca* have shown that the most common and widespread visitor to capitula is a hopliine beetle *Eriesthis vulpina* (Johnson et al., 2021; Johnson et al., 2004), which does not appear to discriminate between the colour forms and visits red, orange and yellow morphs equally in the polymorphic population (Johnson et al., 2021). However, the extent to which the abundance of this beetle contributes to variation in seed production among populations of *G. aurantiaca* has not previously been investigated.

The aims of this study were to determine (1) whether *G. aurantiaca* is pollinator dependant across its geographical range, (2) whether seed production is pollen-limited and, if so, whether this is due to pollen quality or quantity, and (3) whether variation in seed production and pollen-limitation among populations can be explained by the abundance of *E. vulpina*.

METHODS

The study system

Gerbera aurantiaca Sch.Bip. (Mutisieae: Asteraceae), commonly known as the Hilton daisy, is a long-lived species endemic to eastern South Africa, with 25 isolated populations recorded along a 400 km north-south axis in high rainfall, species-rich mistbelt grassland fragments (Fig 1). *Gerbera aurantiaca* is listed as Endangered using IUCN criteria due to extensive habitat loss of the mistbelt grassland vegetation type by commercial forestry, urban development and agriculture (SANBI, 2020). Flowering takes place over about one month in the early summer (October and November) which is characterised by alternating hot sunny and cool misty periods. The bowl-shaped, dark-centred capitula bear approximately 35 outer female ray florets that are typically red, but sometimes yellow or orange, with no discernible UV reflectance (Johnson et al., 2021), and a further 55 dark inner ray florets. The outer, female ray florets open first, followed by the central, functionally male disc florets (Johnson et al., 2005). There is limited overlap between stigma receptivity of female florets and pollen production by the inner hermaphrodite florets, which minimises the opportunity for self-

pollination. As in all daisies the fruit are single seeded (Marzinek et al., 2008). The fruits are attached to an umbrella-like pappus of silky hairs characteristic of wind dispersal although distances are not known. Vegetative reproduction by underground stems gives rise to large clones (Johnson et al., 2005) which live for many generations and may form interspersed clumps, making the visual identification of genets challenging.

The most frequent visitor to *G. aurantiaca* capitula in the populations is *Eriesthis vulpina* Burmeister (Coleoptera: Scarabaeidae: Hopliini), a small (6.5-8.6 mm) beetle, with long, loosely matted hairs on its legs and abdomen which facilitate pollen transfer (Fig 3.1) (Johnson et al., 2021; Johnson et al., 2004). Hopliine scarab beetles are an important group of insect pollinators in south-western South Africa that often use flowers as platforms for social behaviour such as mating, and as a pollen food source (Ahrens et al., 2011; Goldblatt and Manning, 2011; Steiner, 1998). Many species have densely hairy bodies and legs, and their active diurnal behaviour makes them effective pollen vectors (Mayer et al., 2006). *Eriesthis vulpina* is a member of the embedding guild of hopliine scarab pollinators which seldom cause damage to flowers (Ahrens et al., 2011; Goldblatt and Manning, 2011). *Eriesthis vulpina* was observed visiting the differently coloured flowers of many other plant species (Table 3S1)



Figure 3.1. *Eriesthis vulpina* beetles dusted with pollen using a red *Gerbera aurantiaca* capitulum for social behaviour. Scale bar represents 1cm.

The distribution of *E. vulpina* overlaps but is more extensive than that of *G. aurantiaca* (Fig 3.2).

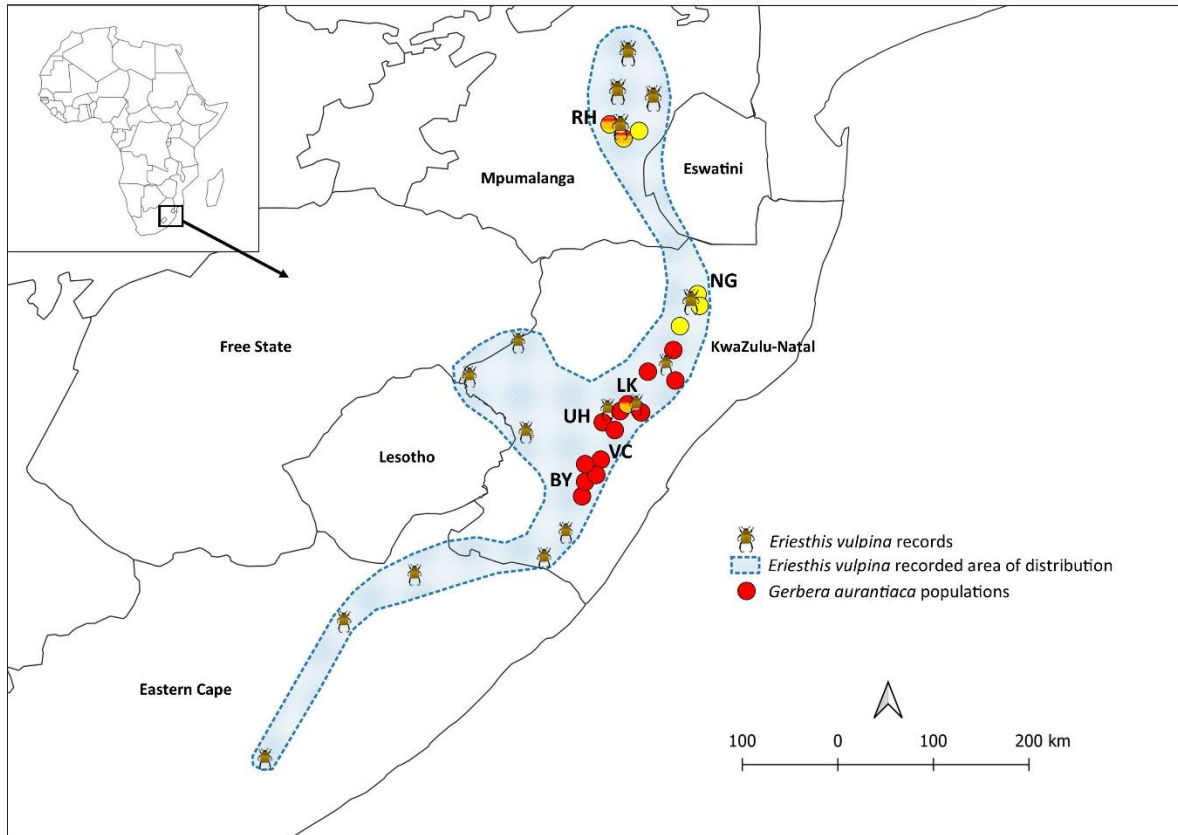


Figure 3.2. Map showing the geographic distribution of *Gerbera aurantiaca* in eastern South Africa and the recorded distribution of *Eriesthis vulpina* from museum records and this study. The localities of the six sites selected for breeding system experiments and pollinator and predation counts are labelled with their abbreviated names from Table 1.

Gerbera aurantiaca plants experience little leaf damage from herbivores, but pre-dispersal seed predation of developing ovules in inflorescences by the larvae of the African bollworm *Helicoverpa armigera* (Hübner) and more commonly the tephritid fly *Craspedoxantha marginalis* (Weideman, 1818) (Johnson et al., 2004) where the adult female typically inserts one or more eggs into the capitulum while still in bud phase and the larvae eat the developing fruit (Fenner et al., 2002).

We selected six representative populations of *G. aurantiaca*, varying in size and colour, along the north-south distribution range (Fig 3.2, Table 3.1). In 2009 we carried out pollinator observations and measured fruit set and predation in each of the six populations and performed controlled pollination experiments in five of these, Byrne (BY), Victoria Club (VC), Lookout (LK), Ngome (NG) and Rooihoogte (RH) (Fig 3.2, Table 3.1). We repeated the controlled pollination experiments in the BY population in 2010 and 2018.

Table 3.1. Site name, abbreviation, geographical co-ordinates (dd), area (ha), approximate number of clones, of the six *Gerbera aurantiaca* populations used for the breeding system experiments and pollinator observations. *Controlled breeding experiments were not carried out in the UH population.

| Site name | Abbreviation | Lat S; Long E | Area (ha) | No. of clones | Capitulum colour |
|-----------------|--------------|---------------|-----------|---------------|------------------|
| Byrne | BY | -29.81; 30.19 | 6.5 | 250 | red |
| Victoria Club | VC | -29.57; 30.33 | 2 | 100 | red |
| Lookout | LK | -29.04; 30.58 | 6.2 | 250 | polymorphic |
| Ngome | NG | -27.84; 31.35 | 100 | >1000 | yellow |
| Rooihooqte | RH | -26.05; 30.37 | 4 | 500 | polymorphic |
| *Umvoti Heights | UH | -29.18; 30.38 | 1.6 | 150 | red |

Controlled pollination experiments

We conducted controlled pollination experiments in the five populations mentioned above which extend along the entire north-south distribution axis of *G. aurantiaca* (Fig 3.2) to establish whether there are differences in breeding systems among populations. We individually enclosed 100 unopened capitula (five on each of 20 clones) within nylon insect exclusion bags in each population. We used clean fine paintbrushes to transfer pollen between florets. Hand pollination was performed under a magnifying lens to ensure that all stigmas received pollen and capitula were then rebagged. To avoid contaminations, paint brushes were replaced between treatments. Bagging prevented further pollination and ensured that all seeds were retained.

Four treatments were administered within each population: 1) bagging without manual pollination to test for autonomous fruit production; 2) hand-pollinated during the female phase with pollen from the same capitulum to test for self-compatibility, 3) hand-pollinated during the female phase with pollen from a different genet situated at least 10m away as a positive control for pollination technique and physiological ability of plants to produce seeds and 4) open pollinated capitula which were bagged after flowering to gauge natural fecundity. Since the tephritid fly ovule predators are introduced to capitula at the bud stage before bagging we assumed this would affect bagged and open pollinated capitula treatments equally.

To test for mate limitation such as may occur when there is inbreeding depression or a limited number of SI alleles in populations (Demauro, 1993; Pickup and Young, 2008), we used pollen from LM to manually pollinate flowers in the NG and BY populations (160 and 95km away respectively). The between-population crosses for BY were performed *in-situ* (N=23). Crosses for NG were performed *ex-situ* (N=14) at the KwaZulu-Natal National Botanical Garden due to logistic constraints. Pollen-bearing capitula were harvested and transported in cold boxes sealed in individual zip lock bags and used for inter-population crosses on the same day.

Bagged infructescences were harvested approximately three weeks after flowering when fruits were ready for dispersal. We measured fecundity as the number of filled fruits per capitulum. We recorded fruit set across all six study populations in 95 bagged, unmanipulated capitula; 105 self-pollinated capitula; 107 crossed pollinated capitula and 234 open-pollinated capitula. Some bagged capitula were damaged by strong winds resulting in slightly different treatment sizes. We compared number of fruits per capitulum (response variable) among treatments (explanatory variable) using generalized linear models (GLM) incorporating a Poisson distribution, log link function, and correction for overdispersion. GLM Models were implemented in SPSS 27 (IBM Corp.). Significance was assessed using likelihood ratios and post hoc testing was done using the Dunn-Šidák method.

Pollination indices: ISI, AFI, PC and PL

Using marginal (model-adjusted) means from the GLMs we calculated indices of self-incompatibility ($ISI = 1 - \text{fruit set after hand-self-pollination} / \text{fruit set after hand-cross-pollination}$) and auto-fertility ($AFI = \text{fruit set after autonomous-self-pollination} / \text{fruit set after hand-cross pollination}$) for each of the five populations (Rodger and Ellis, 2016). ISI values ≥ 0.8 indicate self-incompatibility, between 0.8 and 0.2 partial self-compatibility and ≤ 0.2 self-compatibility (Raduski et al., 2011). AFI values of less than 0.2 indicate pollinator dependence. We calculated a pollinator contribution metric (PC) by comparing fruit production when pollinators are artificially excluded from capitula (f_{excl}) with fruit set in naturally pollinated capitula (f_{nat}) as $PC = (f_{nat} - f_{excl}) / f_{nat}$ (Rodger et al., 2021). We further calculated an index of pollen limitation (PL = 1 - control open-pollinated/hand cross-pollinated) where 1 indicates complete pollen limitation and 0 indicates no pollen limitation (Larsen and Barrett, 2000).

Ovule predation

We examined a representative number of mature capitula *in-situ* in each of the five study populations (n = 48-155) and noted any showing evidence of ovule damage to obtain an estimate of the proportion affected.

To estimate the extent of predation of different coloured capitula in the flower colour polymorphic RH population we bagged 165 capitula at the completion of the male phase on separate plants with insect exclusion nets labelled with ray floret colour. After ripening, 159 intact bags were harvested and the any signs of predation of fruit were noted. We compared the number of predated versus intact capitula for each colour form (n = 39-66) (orange, red and yellow) using generalised linear models (GLM) incorporating a binomial distribution and logit link in SPSS 27 (IBM corp.) Significance was assessed using likelihood ratios and post hoc testing was done using the Dunn-Šidák method.

Fruit set

Fruit set per capitulum was determined by collecting samples of mature, intact infructescences from 15-79 ($\bar{x} = 43.7$) randomly selected plants in the six study populations. Capitula were covered with exclusion bags after the disc florets had finished flowering. Fruit production per capitulum is equivalent to seed production since the unilocular ovaries contain single ovules. Differences in the number of fertile fruits per capitulum (response variable) were compared among sites (explanatory variable) using a GLM with a Poisson distribution, log link function and correction for overdispersion.

Insect visitors

We carried out qualitative pollinator observations at the six populations (Fig 3.1, Table 3.1) between 2003 and 2018. Insect visitors were observed in the field and collected for identification. Taxonomic literature (Dombrow, 1997) was used to identify *Eriesthis* beetles to species level and voucher specimens were lodged with the University of KwaZulu-Natal, South Africa (Table 3S2).

Since beetles appeared to be the most common visitors to *G. aurantiaca* and may move slowly between capitula, we used line transects rather than fixed point observations to record presence or absence on capitula. We also recorded the presence or absence of other insect taxa, including honeybees, on *G. aurantiaca* capitula. Using visitor numbers on capitula (response variable) at each of the six study populations (fixed variable) we recorded their presence or absence on 100 inflorescences along each of three two meter wide transects at three hourly intervals. Insect visitors carrying visible pollen loads and contacting receptive

stigmas were recorded as potential pollinators. Insect visitor counts were carried out over a total of 108 hours on sunny days between 07:00 and 14:00 in 2009 and 2010. We followed and timed individual honeybees and beetles visiting capitula in the yellow NG population (the only population where honeybees visited capitula), to measure the relative times spent by these two visitors on and moving between capitula to assess their potential relative importance as pollinators in this population. We did these observations during the period when insects were most active, and when *G. aurantiaca* was presenting pollen, from 09.30 to 14.30.

Pollen loads

Pollen loads of insect visitors collected from *G. aurantiaca* at the colour polymorphic (RH), yellow (NG) and red-flowered (UH) sites were estimated by using blocks of fuchsin gel to pick up pollen grains from the surface of the insect's body and the killing bottle (Beattie, 1971). The gel was melted to form permanent slides, and *G. aurantiaca* pollen grains were counted. The mean number of pollen grains per insect individual was compared among sites and insect species using a generalized linear model (GLM) with negative binomial error distribution and log link function.

The relationship between beetle pollinator abundance, fruit set and pollen limitation index.

We used linear regression to establish the relationship between fruit set (response variable) and the proportion of *G. aurantiaca* capitula with *E. vulpina* beetles present per hour (explanatory variable) for six of the *G. aurantiaca* populations. To further investigate whether these beetles played an important part in the reproductive success of *G. aurantiaca* we used linear regression to determine the relationship between pollen limitation index (response variable) and the proportion of *G. aurantiaca* capitula with *E. vulpina* beetles present per hour (explanatory variable) for five of *G. aurantiaca* populations where we performed controlled breeding experiments.

RESULTS

Controlled pollination experiments

We found significant effects of treatments in the controlled pollination treatments in all populations that we tested. Relative to the cross-pollinated capitula, self-pollinated capitula in all populations produced significantly fewer fruits and the bagged unmanipulated capitula

seldom produced any fruits at all (Fig 3.3 A-E). In the BY population, where controlled hand-pollinations were performed in three separate years (Fig 3.3 A), we recorded remarkably consistent results (year by treatment interaction $\chi^2 = 2.5$, df = 2, P = 0.87).

Fruit set in open-pollinated capitula was significantly lower than that in cross-pollinated capitula in the two southernmost populations, BY and VC (Fig 3.3A-B), but not in other populations (Fig 3.3 C-E).

Inter-population crosses did not increase fruit set significantly compared to intra-population crosses (treatment effect $\chi^2 = 1.5$, P = 0.47) and this was consistent across populations (population by treatment interaction effect $\chi^2 = 1.2$, P = 0.28) (Fig 3.3F).

Pollination indices: ISI, AFI, PC and PL

The mean index of self-incompatibility (ISI) for the five populations was 0.7 (ranging from 0.6 to 0.79) across populations (Table 3.2) suggesting that *G. aurantiaca* is largely self-incompatible. Very low auto-fertility indices (AFI<0.02) in all populations indicate almost complete dependence on pollinators for fruit set. Pollinator contribution (PC) importance was high across all populations. The index of pollen limitation (PL) was high (0.59 - 0.79) in the BY and VC populations, intermediate (0.25) in the LK population and low (0.03 - 0.07) in the NG and RH populations (Table 3.2).

Table 3.2. Pollination indices for *Gerbera aurantiaca* populations calculated from the breeding system experiments.

| Population | Indices | | | |
|------------|----------------------------|----------------------|------------------------|------------------------------|
| | Self-incompatibility (ISI) | Auto-fertility (AFI) | Pollen limitation (PL) | Pollinator Contribution (PC) |
| BY | 0.60 | 0.02 | 0.59 | 1 |
| VC | 0.73 | 0.01 | 0.75 | 1 |
| LK | 0.79 | 0.01 | 0.25 | 1 |
| NG | 0.60 | 0.00 | 0.03 | 1 |
| RH | 0.79 | 0.01 | 0.07 | 1 |

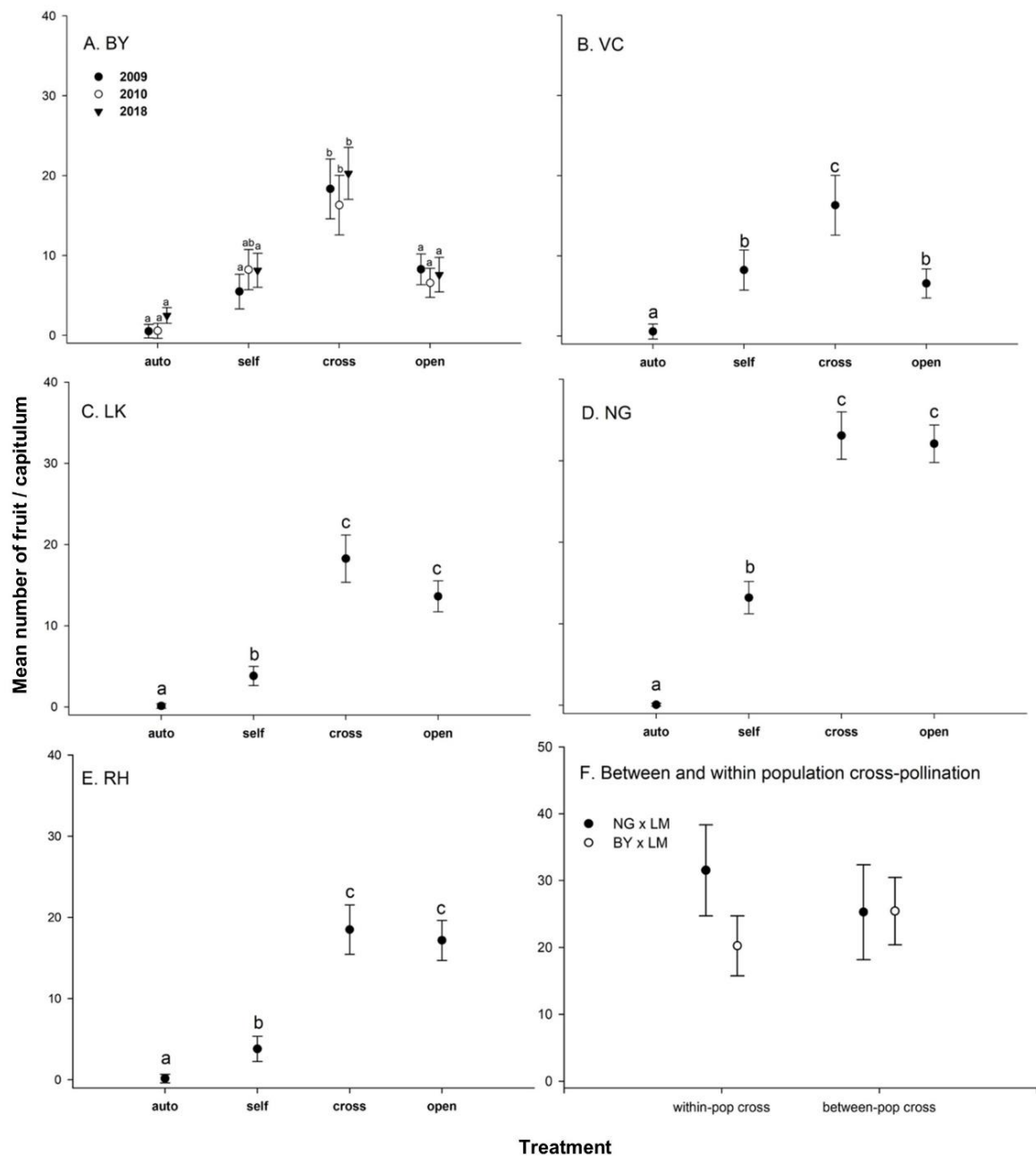


Figure 3.3. Mean fruit set per capitulum for bagged unmanipulated treatment (auton), controlled hand self-pollinated treatment (self), controlled hand cross-pollinated treatment (cross) and control unbagged open pollinated treatments of controlled breeding experiments in five populations of *Gerbera aurantiaca* (A to E) and (F) between and within population controlled hand pollination treatments for the NG (using LM pollen donor) and BY (using LM pollen donor) populations. Vertical bars represent standard errors and means that share letters are not significantly different.

Fruit set

The mean number of fruits produced by open-pollinated capitula differed significantly among the five populations ($\chi^2 = 117.3$, $df = 4$, $P = < 0.001$) (Fig 3.4). The red-flowered, southernmost populations (VC and BY) had extremely low mean fruit set (4.07 and 2.20 fruits per capitulum, respectively).

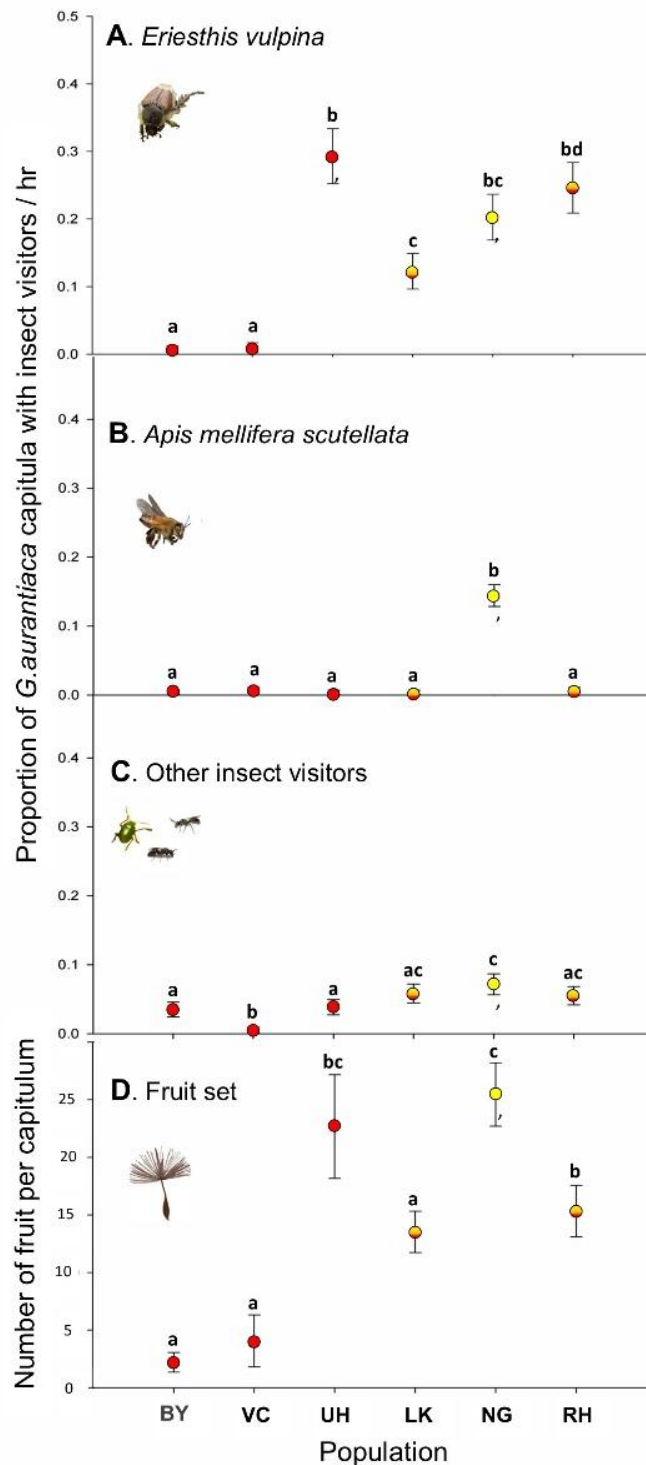


Figure 3.4. Mean numbers of: **A.** *Eriesthis vulpina*, **B.** *Apis mellifera scutellata* and **C.** other insect visitor on *Gerbera aurantiaca* capitula per hour along transects at the six study populations, and **D.** Fecundity (mean fruit set per capitulum) of six populations. Vertical bars represent standard errors and means that share letters are not significantly different. Population colour indicated by coloured circles.

Ovule predation

The mean number of capitula showing signs of predation did not differ significantly among the five populations $N=486$ ($\chi^2 = 7.072$, $df = 4$, $P=0.13$) (Fig 3.5A). In the FCP RH population the number of different coloured capitula with evidence of predation also did not differ significantly $N=159$ ($\chi^2 = 3.812$, $df=1$, $P = 0.15$) (Fig 3.5B).

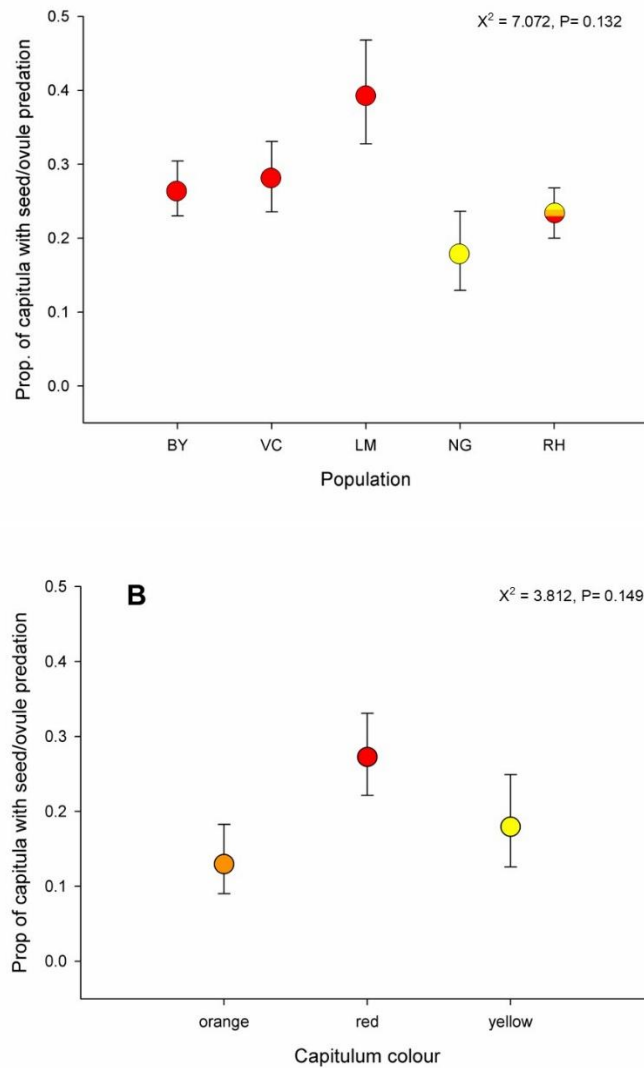


Figure 3.5. Proportion of *Gerbera aurantiaca* capitula with ovule predation **A.** Populations and **B.** Colour morphs in an FCP population. Vertical bars represent standard errors. Predominant population colour indicated by coloured markers.

Insect visitors

We recorded a total of 1077 individual insect visitors from four families across the six populations during transect surveys in 2012. The most common visitor to *G. aurantiaca* in all red populations where it was present, was the hopliine beetle *E. vulpina* (Fig 3.4A). Capitula at UH had the highest number of *E. vulpina* visits, followed by those at RH, NG, and LK, while these beetles were not seen on capitula at VC and BY. Although common on flowers of other plant species at all study localities, honeybees were rarely observed on *G. aurantiaca* capitula (Fig 3.4B) except in the yellow-flowered NG population where they accounted for 16% of all insect visitors. Insect species other than *E. vulpina* and *A. m. scutellata* made up less than 15% of all visitors and numbers were highest at NG, LK and RH followed by UH and BY with capitula at VC receiving very low numbers of insect visitors (Fig 3.4C).

We observed 178 visits to capitula of 80 clones by following 22 honeybees between 09.40 and 10.30. An average of four seconds (1-25) were spent per capitulum. Twelve beetles visited 31 capitula for an average of 19 secs (1-60) per inflorescence (Table 3S3).

Pollen loads

Pollen loads of *G. aurantiaca* on *E. vulpina* beetles were much higher than those on honeybees at the study site UH with red capitula (marginal means \pm SE adjusted for site: beetles 75.8 ± 34.8 , bees 0.82 ± 0.5 , $\chi^2 = 21.5$, $p < 0.001$). The highest *G. aurantiaca* pollen loads on beetles were recorded at the colour polymorphic RH site (248.5 ± 192 grains). There was a significant interaction of pollinator and site on pollen loads ($\chi^2 = 15.3$, $p < 0.001$). While honeybees carried very little *G. aurantiaca* pollen at the RH and UH sites (0.08 ± 0.09 and 0.17 ± 0.18 grains, respectively), at NG (the yellow-flowered population) they carried a mean of 43.3 ± 34.8 grains which was similar to that of beetles at this site (21.9 ± 17.6 grains).

The relationship between beetle pollinator abundance, fruit set and pollen limitation index

We found a significant positive linear relationship between *G. aurantiaca* fruit set and *E. vulpina* presence on capitula in six populations ($r^2 = 0.828$, $df = 5$, $F = 19.2$, $P = 0.012$) (Fig 3.6A) and a significant negative linear relationship between pollen limitation and *E. vulpina* presence on capitula in five populations (Fig 3.7B), $r^2 = 0.921$, $df = 4$, $F = 35.209$, $P = 0.010$). The two southernmost populations, BY and VC, where *E. vulpina* was rarely observed, had

low fruit set. In LK fruit set was intermediate, and at NG, UH and RH, where beetles were more abundant, fruit set was highest.

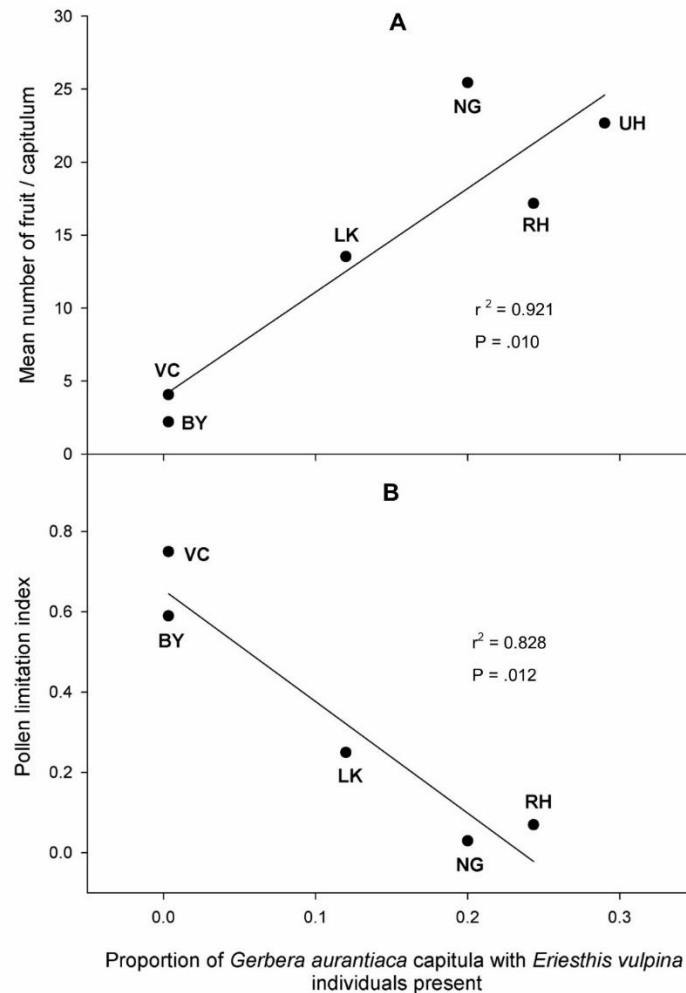


Figure 3.6. The relationship between **A.** Fecundity (mean number of fruit per capitulum) for six populations, and **B.** Pollen limitation index for five populations, and the proportion of *Gerbera aurantiaca* capitula occupied by the hopliine beetle *Eriesthis vulpina*.

DISCUSSION

Our study revealed that *G. aurantiaca* is largely self-incompatible and almost entirely dependent on pollinator visits for pollen transfer and seed production in all colour forms across its distribution range. A single hopliine beetle species, *E. vulpina*, was the most frequent visitor to capitula across all populations studied, with honeybees, and to a lesser

extent other insect taxa, being significant visitors only in the entirely yellow-flowered population. Fruit set varied among populations across the range, from very low in populations in which beetles were scarce or absent to much higher levels in populations which had a higher frequency of beetle visitors. Supplemental hand-pollinations indicated that low fruit set in populations was due to pollen-limitation. Results from both intra- and interpopulation cross-pollination treatments suggest that this pollen limitation is due to pollen quantity rather than quality, suggesting firstly, that there is no lack of compatible mates within populations, and secondly, that there is no genetic barrier to gene flow among populations. We found no evidence that seed/ovule predators of *G. aurantiaca* favour any colour morph, both across monomorphic and polymorphic populations, as well as across colour morphs in an FCP population, indicating that predator colour preference does not play a role in colour variation (Veiga et al., 2015). We established that pollen vectors are essential for seed production, as reflected by the extremely low (< 0.02) autofertility indices in all populations of *G. aurantiaca*.

We found that *G. aurantiaca* is largely self-incompatible across its distribution and therefore colour forms are unlikely to be retained in a population through autonomous selfing (Buide et al., 2021). Two of the five populations studied (LK and RH) had ISI (self-incompatibility index) values of 0.8, indicative of strong self-incompatibility, and three (BY, VC and NG) had lower ISI values (0.6, 0.7, 0.6), suggesting partial self-incompatibility (de Nettancourt, 1977). It is unlikely that these results arise from experimental technique because controlled pollination experiments conducted in the BY population over three separate, widely spaced years yielded similar ISI values of 0.7, 0.5 and 0.6. Approximately 25% of all plant species studied show partial SI (Raduski et al., 2011) and shifts in breeding system from self-incompatibility to self-compatibility have been recorded in several families, including the Asteraceae (Barrett et al., 2014).

Pollen limitation, which arises when pistils receive an inadequate quantity of compatible pollen, varied across the distribution range of *G. aurantiaca*, ranging from very low values (< 0.1), indicating the receipt of adequate suitable pollen in the central and northern populations, to much higher values (> 0.6), suggesting insufficient pollen receipt in the southern populations. Our between-population breeding system experiments in two of the study populations indicated that using pollen donors from distant ($> 100\text{km}$) populations did not significantly increase fruit set compared with that using pollen from different clones in the same population. These preliminary results indicate that pollen quantity rather than pollen quality is limiting fruit set in these populations.

By far the most abundant insect visitor species recorded on *G. aurantiaca* capitula across its geographic and colour range was the hopliine beetle *E. vulpina* which carries pollen on its hairy body and legs. This beetle visits the flowers of many different plant species (Table 3S1), does not discriminate between the different colour forms of *G. aurantiaca* (Johnson et al., 2021) and was recorded visiting all capitula during transects in both monomorphic red and yellow populations as well as all to colour forms in the colour polymorphic populations. The native honeybee *A. mellifera scutellata* was recorded visiting *G. aurantiaca* capitula in the monomorphic, yellow-flowered population, but we did not observe it on yellow capitula in other populations, even where its presence on other flower species was noted. The strong association between *E. vulpina* beetle abundance and fecundity of populations of *G. aurantiaca* (Fig 3.7) suggests that this beetle species is the most important pollinator of the species across its range and that the local frequency of this beetle is the main factor dictating the level of pollen-limitation of fecundity. The one possible exception may be the monomorphic, yellow-flowered population which may be co-pollinated by other insects and thus have a more generalized pollination system. Although pollen loads of honeybees were similar to beetles in this population, they tend to move more rapidly and spend much shorter periods on capitula than do beetles, and may thus contribute more to pollination (Streinzer et al., 2019). Conversely it is possible that beetles more thoroughly pollinate florets by spending longer periods on capitula than do bees. Our observations tracking honeybee visits between capitula in the yellow-flowered population showed that they spent an average of four seconds per inflorescence, while active beetles spent an average 19 seconds on a capitulum (Table 3S2). Factors such as within-clone capitulum visits due to flower constancy and return visits to the communal hive by honeybees may impact their pollination effectiveness, and further studies on pollen loads and single visit deposition (King et al., 2013) are needed to quantify the relative contribution and pollinator importance of these two insect visitors (Diller et al., 2019; Diller et al., 2022; Ne'eman et al., 2010).

Pollen limitation resulting from scarcity of *E. vulpina* beetles has serious consequences for the red-flowered southern populations of *G. aurantiaca*. Although the open bowl shape and short floret tube length of capitula are typical of generalist pollination, these red-flowered forms are visited almost solely by *E. vulpina* beetles, which do not exhibit a preference for any floral colour morph of *G. aurantiaca* (Johnson et al. 2021). The scarcity of other insect visitors to the red flowered morphs across the populations suggest that the red colour morph may exhibit ecological specialization for pollination, with ray floret colour functioning as an attraction filter (Armbruster, 2017). The red ray florets of *G. aurantiaca*, which display

little UV spectral reflectance (Johnson et al., 2021), may be at the edge of hymenopteran visual capabilities (Chittka and Waser, 1997; Lunau et al., 2011), and honeybees are likely to perceive these red flowers as achromatic (Martínez-Harms et al., 2010; Spaethe et al., 2001). Our results clearly indicate that in the absence of the pollinator contribution of *E. vulpina*, the red colour form of *G. aurantiaca* experiences severe pollen limitation and sets very little seed (Fig 3.7). The consequences of the loss of a generalist pollinator have been shown to cause linked declines in seed set of asymmetrically specialised, pollinator dependant plants in small populations of orchids in the Western Cape region of South Africa (Pauw, 2007). This confirms that this floral colour specialization strategy is risky as the long-term absence of *E. vulpina* from some red-flowered populations has resulted in severe pollen limitation and low seed set, and the persistence of these populations is currently dependent mainly on clonal proliferation.

The absence of *E. vulpina* from the southern, red-flowered populations is enigmatic. Long term observations of the BY population indicate that this is an enduring rather than recent or seasonal trend (Fig 3.3A) (Johnson et al. 2004). Reasons for this scarcity are unclear as edaphic and climatic conditions are similar to those of the central and northern *G. aurantiaca* populations where these beetles are more common (Johnson et al. 2021). The range of *E. vulpina*, inferred from museum records and personal observations, overlaps that of *G. aurantiaca* but is more extensive (Fig 3.1). While little is known about factors determining the distribution of hopliine scarab beetles (Colville, 2009), studies conducted in the Succulent Karoo Region of South Africa indicated that hopliine beetles were less sensitive to habitat size than to vegetation cover, grass dominance, and rockiness (Donaldson et al., 2012). A better understanding of the factors affecting the distribution of these beetles and the reasons for their absence may be very important in conservation plans for the endangered *G. aurantiaca*, in particular the red forms, which are highly dependent on hopliine beetles for pollination and sexual reproduction. Potential projects to reintroduce these insects to the populations where they are absent or scarce undertaken as part of a conservation plan require an understanding of what is influencing population fluctuations.

Since *G. aurantiaca* is an endangered species (SANBI, 2020) it is important to highlight some conservation issues. As both the total extent and fragment size decreases it is essential to consider plant breeding systems in conservation planning (Wagenius and Lyon, 2010). *Gerbera aurantiaca* is highly vulnerable to habitat fragmentation as it is largely self-incompatible and thus dependent on flower visitors for pollen transfer. While for long-lived species with multiple reproductive cycles such as *G. aurantiaca*, the short-term impact of

decreased seed set may be less important than adult survivorship (Bond, 1994), sexual reproduction through seed set determines the long-term persistence of a species through fitness, migration, and adaptation and is crucial to their survival, hence lack of recruitment will eventually lead to the demise of populations (Barrett and Harder, 2017; Levin, 2012). Securing formal protection and appropriate long-term conservation management for the remaining population habitats is clearly of foremost importance (Menz et al., 2011). Further conservation interventions in severely pollen limited plant populations resulting from pollinator demise could include within population artificial cross-pollination (Finger et al., 2011; Gemmil et al., 1998) or investigating pollinator reintroduction (Andrews et al., 2022; Phillips et al., 2015).

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**CHAPTER 4: THE COMMUNITY CONTEXT OF FLOWER COLOUR
VARIATION IN A GRASSLAND DAISY**

Abstract

Flower colour is one of the most variable traits of flowering plants, but the factors influencing colour variation are often difficult to identify. One possibility is that flower colour is shaped by co-flowering plants through processes such as competition and facilitation. Here I investigated a possible relationship between flower colour morphs of the daisy *Gerbera aurantiaca* and the colour signals of co-flowering plants. This was done using vision models for the two main pollinator groups, bees and scarab beetles. I obtained spectral reflectance data for flowers of plants growing sympatrically with either red, yellow, or mixed colour morphs *G. aurantiaca* at five sites. Principal components analysis (PCA) of spectra indicated that there were no overall differences in colour composition among species in the five communities. Analyses using vision models suggested that *G. aurantiaca* yellow colour morphs are distinct from the other species in the populations to bees, while red and orange forms are less so while a beetle vision model suggested that all the colour morphs are conspicuous to scarab beetles with red receptors. I conclude that flower colour variation is not explained by the community context of co-flowering plants and that the colours of *G. aurantiaca* are likely to act as a largely private signal for beetle pollinators. This is one of few studies that have used a community level approach investigate the signalling strategies of a flower colour polymorphic species that diverges in colour signal at different locations.

Keywords: spectra, inflection points, flower colour, intra-communal, *Gerbera aurantiaca*, bee vision, beetle vision

INTRODUCTION

One of the most widespread and conspicuous sources of variation in natural plant populations is flower colour. Plant phenotypic diversity may be influenced by several factors including biotic and abiotic influences, as well as evolutionary history, leading to varying functional patterns within plant communities (Dalrymple et al., 2020). However, the role of floral colour in community structure is still poorly understood despite its important role in plant-pollinator interactions (Torices et al., 2021). The importance of biotic factors, especially the part played by pollinators, and abiotic factors such as climate, in shaping community flower colour have been investigated in several studies over the past few decades. These studies have focused on the effects of abiotic factors (Dalrymple et al., 2020; Johnson et al., 2021), elevation (Arnold et al., 2009; Shrestha et al., 2014), phylogenetic relatedness (McEwen and Vamosi, 2010;

Torices et al., 2021), pollinator specialisation and generalisation (Renoult et al., 2014; van der Kooi et al., 2016) and alien plants (Makino and Yokoyama, 2015). The critical function of pollinator mediated selection in determining community flower colour has been the most widely investigated (de Jager et al., 2011; Dyer et al., 2012; Kemp et al., 2019; Lázaro et al., 2020; Menzel and Shmida, 1993; Reverté et al., 2016; Shrestha et al., 2019).

Flower colour plays a vital role in pollinator attraction, through foraging constancy and pollinator preference (Bradshaw and Schemske, 2003; Waser, 1986), and pollinators may influence the species composition of outcrossing plant communities by either promoting or inhibiting reproductive success through species preferences. Alternatively, pollinator preferences may allow coexistence through facilitation or character displacement (de Jager et al., 2011).

The role of bees in influencing community flower colour has probably been the most intensively studied (Dyer et al., 2012; Gumbert et al., 1999; Shrestha et al., 2019), while flies (Kemp et al., 2019; Shrestha et al., 2016), birds (Coetzee et al., 2021), and to a lesser extent butterflies (Dalrymple et al., 2015) have also been investigated. Although beetles are an important group of flower visitors in southern Africa (Arnold, 2010; Goldblatt and Manning, 2011; Kemp et al., 2019) their role in community flower colour determination has received the least attention, possibly because their visual capabilities are poorly known and are believed to be less constrained than that of hymenopterans (León-Osper and Narbona, 2022; Martínez-Harms et al., 2020; Martínez Harms, 2012; Sharkey et al., 2017).

Recent studies of flower colour distribution patterns in plant communities suggest that co-flowering species frequently display more similar floral phenotypes than expected by chance, and that this ‘clustering’ may be common (Bergamo et al., 2018; de Jager et al., 2011; Kemp et al., 2019). If this pattern of flower colour similarity produced competitive interactions between neighbouring species in communities, a more ‘overdispersed’ distribution would be expected (Bergamo et al., 2018). Ultimately patterns of trait clustering may thus be explained by a range of processes including facilitation, ecological filtering or evolutionary convergence (Anderson and Johnson, 2009; Kemp et al., 2019).

To ensure insect visitation and pollination within a community a plant species colour may be different to its co-flowering neighbours to ensure memorability and flower constancy by various pollinator groups with differing visual ranges, or be similar to one or more species of co-flowering plant leading to colour convergence and a “magnet effect” to potential pollinators (Arnold and Chittka, 2019). The neighbourhood colour environment of local populations may influence plant reproductive success of individual species or colour variants

by attracting different pollinating visitors, although this can also decrease reproductive success by increased heterospecific pollen deposition (de Jager et al., 2022). To my knowledge, the possible role of community flower colour in influencing intraspecific colour variation in a flower colour polymorphic species has not thus far been investigated (see Arnold and Chittka, 2019).

Here I combined a community level approach to flower colour (Gumbert et al., 1999; Shrestha et al., 2019) together with an investigation into the signalling strategies to its main insect visitor groups of a focal plant species which diverges in flower colour at different localities (Arnold and Chittka, 2019) to gain further understanding of the factors influencing capitulum colour variation in a South African daisy, *Gerbera aurantiaca*. Previous investigations of this species have indicated that the geographic colour variation of the species is genetically controlled, is not related to climatic and edaphic factors (Johnson et al., 2021) that native honeybees and a hopliine scarab beetle species are the most important pollinators, and that seed predation intensity is not associated with capitulum colour (see Chapters 2 and 3 of this thesis). It therefore seemed that investigation of co-flowering community colour patterns and their relationship to the resident *G. aurantiaca* colour variant may be informative in the current investigation into the drivers of flower colour variation in the species.

I asked whether the spectral characteristics of co-flowering neighbourhood plants differ between populations of *G. aurantiaca* across its colour and distribution range. Using two different vision models I then determined the conspicuousness of the different *G. aurantiaca* colour morphs within their intra-communal co-flowering populations to their two main insect visitor groups.

METHODS

The study system

Gerbera aurantiaca Sch. Bip. (Asteraceae: Mutisieae) is a flower colour polymorphic daisy endemic to the mistbelt grasslands of eastern South Africa (Johnson et al., 2021). The ray floret colour varies geographically from monomorphic red and yellow populations to polymorphic ranging from red through orange to yellow in a single population. Peak flowering in these grasslands, including that of *G. aurantiaca* takes place in the austral spring (late September to early November). *Gerbera aurantiaca* is self-incompatible and completely dependent on pollinators for pollen transfer (Chapter 4 of this thesis), and colour appears to

be the main attractant since no discernible nectar is present in the florets and a preliminary investigation of floral scent using dynamic headspace sorption methods (Shuttleworth and Johnson, 2009) revealed no floral volatile compounds.

In their natural state these endemic species-rich mistbelt grasslands (Carbutt, 2023) are composed of short perennial tufted grasses and a rich variety of long-lived forb species with subterranean tubers, corms or thickened roots which enable them to survive the dry winters and frequent fires typical of the region (Morris, 2021). Asteraceae, in particular the genera *Helichrysum*, *Senecio* and *Berkheya*, dominate these grasslands in both species richness and abundance (Hilliard, 1977) but a wide variety of species from the Fabaceae, Iridaceae, Orchidaceae and Apocynaceae are also present (Carbutt, 2023). Although alien invasive plant species are a major threat to mistbelt grassland fragments (Scott Shaw, 1999), in this study I found few alien plant species flowering concurrently with *G. aurantiaca*. Studies have shown that the dominant pollinators of the forbs in these grasslands are insects, primarily Hymenoptera, Diptera and Lepidoptera (Johnson, 2004; Johnson et al., 2009; Stanley et al., 2020), but Coleoptera are also frequent flower visitors (Ollerton et al., 2003; Peter and Johnson, 2009; Steenhuisen and Johnson, 2012) and see Chapter 4 of this thesis.

I selected five populations with varying capitulum colours from across the distribution range of *G. aurantiaca* (approx. 450km) to investigate whether the spectral signal of neighbourhood co-flowering plant species is related to the local *G. aurantiaca* morph. These five localities included two sites with dominantly red *G. aurantiaca* capitula (BY, UH), one entirely yellow-flowered population (NG), and two flower colour polymorphic populations (RH, LK) (Fig 4.1).

Sampling

I sampled all plant species in flower at each of five population localities in a 2ha (20000m²) area in the spring months during the approximately three-week peak flowering period of *G. aurantiaca*. I identified 119 species from 77 genera and 35 families from the five populations of *G. aurantiaca* localities (Table 4S1). The family Asteraceae was by far the most speciose (29%) followed by Fabaceae (9%). The number of species recorded per site ranged from 54 at BY to 19 at UH. Few species (2%) were recorded from all five populations while 64% occurred at only a single site.

Table 4.1. List of plant species sampled at five *Gerbera aurantiaca* localities (BY 54, UH 19, LK 30, NG 53, RH 29; a-abundant, c-common, o-occasional, n-number of species)

| Family | Species | Population | | | | |
|------------------|--|------------|----|----|----|----|
| | | BY | UH | LK | NG | RH |
| Euphorbiaceae | <i>Acalypha depressinerva</i> | | | | | c |
| Euphorbiaceae | <i>Acalypha peduncularis</i> | c | | | c | |
| Euphorbiaceae | <i>Acalypha punctata</i> | c | c | c | | |
| Asteraceae | <i>Adenanthemum osmitoides</i> | | | | | o |
| Asteraceae | <i>Afroaster hispida</i> | c | c | c | c | a |
| Lamiaceae | <i>Ajuga ophrydis</i> | o | | | o | |
| Hyacinthaceae | <i>Albuca setosa</i> | | | | o | o |
| Apiaceae | <i>Alepidia natalensis</i> | o | | | | |
| Ranunculaceae | <i>Anemone fanninae</i> | o | | | | |
| Fabaceae | <i>Argyrolobium stipulaceum</i> | o | | | | |
| Apocynaceae | <i>Aspidonepsis flava</i> | c | | | | |
| Asteraceae | <i>Berkheya insignis</i> | | | | | c |
| Asteraceae | <i>Berkheya setifera</i> | o | | o | o | c |
| Asteraceae | <i>Callilepis laureola</i> | | | o | | o |
| Agavaceae | <i>Chlorophytum cooperi</i> | | | | c | |
| Euphorbiaceae | <i>Clutia virgata</i> | | | | o | |
| Fabaceae | <i>Crotalaria globifera</i> | | | | o | |
| Commelinaceae | <i>Cyanotis speciosa</i> | o | | o | | |
| Orobanchaceae | <i>Cynium adonense</i> | | | | o | |
| Cyperaceae | <i>Cyperus obtusiflorus</i> var. <i>flavissimus</i> | | | o | | o |
| Lobeliaceae | <i>Cyphia elata</i> | o | o | o | | |
| Scrophulariaceae | <i>Diclis reptans</i> | | | | o | |
| Iridaceae | <i>Dierama gerstneri</i> | | | | r | |
| Iridaceae | <i>Dierama latifolia</i> | o | | | | |
| Iridaceae | <i>Dierama nixonianum</i> | o | | | | |
| Asteraceae | <i>Dimorphotheca jucunda</i> | o | | | | o |
| Acanthaceae | <i>Dyschoriste burchellii</i> | | | | o | |
| Fabaceae | <i>Eriosema distinctum</i> | c | c | c | o | |
| Fabaceae | <i>Eriosema ellipticifolium</i> | | | | o | |
| Fabaceae | <i>Eriosema kraussianum</i> | | c | o | | |
| Fabaceae | <i>Eriosema salignum</i> | | | | o | c |
| Ruscaceae | <i>Eriospermum abyssinica</i> | | | | o | |
| Orchidaceae | <i>Eulophia hians</i> var. <i>hians</i> | o | | | | |
| Orchidaceae | <i>Eulophia ovalis</i> | o | | | | |
| Asteraceae | <i>Euryops laxus</i> | o | c | c | c | |
| Asteraceae | <i>Euryops peduncularis</i> | | | | o | |
| Asteraceae | <i>Gazania krebsiana</i> | | | | o | |
| Asteraceae | <i>Gerbera ambigua</i> | o | o | | | |
| Asteraceae | <i>Gerbera aurantiaca</i> | c | c | c | c | c |
| Asteraceae | <i>Gerbera hybrid pink</i> | r | | | | |
| Asteraceae | <i>Gerbera piloselloides</i> | o | | o | | |
| Iridaceae | <i>Gladiolus longicollis</i> | | | | o | |
| Iridaceae | <i>Gladiolus parvulus</i> | c | | | | |
| Orobanchaceae | <i>Graderia scabra</i> | | | | | o |
| Asteraceae | <i>Haplocarpha scaposa</i> | | | | | c |
| Scrophulariaceae | <i>Hebenstretia dura</i> | | | | o | |
| Asteraceae | <i>Helichrysum</i> sp 1 | | | | | o |
| Asteraceae | <i>Helichrysum acutatum</i> | o | | o | o | |
| Asteraceae | <i>Helichrysum aureonitens</i> | c | c | | c | |
| Asteraceae | <i>Helichrysum aureum</i> | c | | | | |
| Asteraceae | <i>Helichrysum cephaloideum</i> | | | o | | |
| Asteraceae | <i>Helichrysum ecklonis</i> | o | | | | |
| Asteraceae | <i>Helichrysum pallidum</i> | | | | c | |
| Asteraceae | <i>Helichrysum nudifolium</i> var. <i>pilosellum</i> | | | c | | c |

| Family | Species | Population | | | | |
|------------------|--|------------|----|----|----|----|
| | | BY | UH | LK | NG | RH |
| Brassicaceae | <i>Heliophila rigidiuscula</i> | o | | | | |
| Malvaceae | <i>Hermannia cristata</i> | | | | | r |
| Asteraceae | <i>Hilliardiella elaeagnoides</i> | | | o | | |
| Asteraceae | <i>Hilliardiella hirsuta</i> | | o | | | o |
| Asteraceae | <i>Hilliardiella thodei</i> | | | | o | |
| Asteraceae | <i>Hirpicium armerioides</i> | o | | | | c |
| Hypericaceae | <i>Hypericum aethiopicum</i> | | | | o | c |
| Hypoxidaceae | <i>Hypoxis angustifolia</i> | | | | o | |
| Hypoxidaceae | <i>Hypoxis argentea</i> | | o | | | |
| Hypoxidaceae | <i>Hypoxis costata</i> | o | | | | |
| Hypoxidaceae | <i>Hypoxis filiformis</i> | r | | | | o |
| Hypoxidaceae | <i>Hypoxis rigidula</i> | | | o | o | |
| Fabaceae | <i>Indigofera hilaris</i> | | | o | | o |
| Fabaceae | <i>Indigofera velutina</i> | o | | | | |
| Convolvulaceae | <i>Ipomea obscura</i> | o | | | | |
| Ranunculaceae | <i>Knowltonia transvaalensis</i> | | | | | o |
| Rubiaceae | <i>Kouhoutia amatymbica</i> | | | c | c | |
| Thymeleaceae | <i>Lasiosiphon caffer</i> | o | | | | |
| Thymeleaceae | <i>Lasiosiphon kraussiana</i> | | | | o | |
| Thymeleaceae | <i>Lasiosiphon splendens</i> | | | | o | |
| Hyacinthaceae | <i>Ledebouria</i> sp 1 | o | | | | |
| Hyacinthaceae | <i>Ledebouria floribunda</i> | | | | o | |
| Hyacinthaceae | <i>Ledebouria saundersii</i> | c | | | o | |
| Fabaceae | <i>Leobordia corymbosa</i> | o | | o | o | o |
| Polygalaceae | <i>Muraltia lancifolia</i> | r | | | | |
| Scrophulariaceae | <i>Nemesia denticulata</i> | c | | | | |
| Lamiaceae | <i>Ocimum obovatum</i> | | c | c | | |
| Oxalidaceae | <i>Oxalis obliquifolia</i> | c | | | | |
| Oxalidaceae | <i>Oxalis semiloba</i> | | o | o | | |
| Oxalidaceae | <i>Oxalis smithiana</i> | | | | | o |
| Geraniaceae | <i>Pelargonium luridum</i> | | | | o | |
| Rubiaceae | <i>Pentania angustifolia</i> | | | o | | o |
| Rubiaceae | <i>Pentania prunelloides</i> | o | | | o | |
| Apiaceae | <i>Peucedanum caffrum</i> | | | | r | |
| Asteraceae | <i>Phymaspermum pinnatifidum</i> | | | | o | |
| Polygalaceae | <i>Polygala oldendorffiana</i> | | o | | | |
| Proteaceae | <i>Protea caffra</i> | o | | | | |
| Apocynaceae | <i>Raphionacme hirsuta</i> | o | | o | o | |
| Hypoxidaceae | <i>Rhodohypoxis baurii</i> | c | | | o | |
| Fabaceae | <i>Rhynchosia villosa</i> | | o | c | | |
| Lamiaceae | <i>Rothea hirsuta</i> | | o | | | |
| Rosaceae | <i>Rubus cuneiformis</i> | o | | | | |
| Dipsacaceae | <i>Scabiosa columbaria</i> | | | o | o | |
| Hyacinthaceae | <i>Schizocarphus nervosus</i> | | o | | | o |
| Apocynaceae | <i>Schizoglossum flavum</i> | c | | | | |
| Scrophulariaceae | <i>Selago longiflora</i> | o | | | | |
| Asteraceae | <i>Senecio broad leaf</i> | | | | o | |
| Asteraceae | <i>Senecio coronatus</i> | | | | c | |
| Asteraceae | <i>Senecio erubescens</i> | o | | | | |
| Asteraceae | <i>Senecio glaberrimus</i> | c | c | c | c | c |
| Asteraceae | <i>Senecio polyanthemoides</i> | o | | | | |
| Caryophyllaceae | <i>Silene burchellii</i> | | | | o | |
| Lamiaceae | <i>Stachys nigrescens</i> | | | | o | |
| Lamiaceae | <i>Syncolostemon teucrifolius</i> | | | | c | |
| Lamiaceae | <i>Syncolostemon transvaalensis</i> | | | | | o |
| Fabaceae | <i>Tephrosia natalensis</i> subsp. <i>pseudocapitata</i> | | | c | | |
| Acanthaceae | <i>Thunbergia atriplicifolia</i> | | | | o | o |
| Asteraceae | <i>Tolpis capensis</i> | o | | o | o | |

| Family | Species | Population | | | | |
|---------------|--|------------|----|----|----|----|
| | | BY | UH | LK | NG | RH |
| Asphodelaceae | <i>Trachyandra saltii</i> | o | | | c | |
| Alliaceae | <i>Tulbaghia leucantha</i> | o | | | | |
| Asteraceae | <i>Ursinea tenuiloba</i> | o | c | | | |
| Campanulaceae | <i>Wahlenburghia krebsii</i> subsp. <i>krebsii</i> | | | o | | |
| Apocynaceae | <i>Xysmalobium asperum</i> | | | | o | |
| Apocynaceae | <i>Xysmalobium involucreatum</i> | o | | | o | |
| Apocynaceae | <i>Xysmalobium parviflorum</i> | | | o | | |

Petal reflectance spectra and inflection points

Three replicate samples of the inflorescences of all plant species in bloom at each site were collected and placed into sealed ziplock bags in a cooler box and spectra were measured within 24 hours. Spectral reflectance across the 300–700 nm range was determined using an Ocean Optics S2000 spectrometer (Ocean Optics Inc., Dunedin, Fla.), Ocean Optics DT-mini light source and fibre optic reflection probe (QR-400-7-UV-VIS; 400 µm) held at 45° to the flower or leaf surface in a probe holder (RPH-1) (Shuttleworth and Johnson, 2010). The spectra of the three replicates were used to calculate a mean for each species. Spectral reflectance of background vegetation was measured from the upper surface of green leaves of several plant species from each population and averaged. Spectra of species occurring at more than one site were measured separately in case of colour differences. For species with multicoloured flowers the dominant colour area was selected for measurement. Petals of species with very small flowers were tiled onto clear adhesive tape to form a larger surface before measurement. Spectra from this study will be lodged with the FRED database (Arnold et al., 2010) to allow subsequent meta-analyses of flower reflectance data.

Inflection or marker points identify the region of wavelength where change in spectral reflectance is maximal (Chittka and Menzel, 1992; Dyer et al., 2012). Since I was interested in colour as a floral signal and since insect flower visitors most readily perceive rapid changes in spectra wavelengths, inflection points allow direct comparisons between flower colour signals that are independent of the assumptions used for the different pollinator group colour perception and are only a function of the reflectance curve (Dyer et al., 2012). To identify inflection points for each species spectral curve I used the automated online “Spectral-MP” (Dorin et al., 2020) which calculates the inflections points or regions of most rapid spectral change and checked these with manual calculations of spectra. The reflectance spectra and location of inflection points of four exemplary species from this investigation are shown in Figure 4.1. The number of inflection points per species varied from one (e.g. *G. aurantiaca*) to three (e.g. *Senecio glaberrimus*). Long-wavelength inflections (680–690 nm)

that occur in insect-pollinated flowers could be an artefact of the general reflectance properties of plant tissue (Chittka and Menzel, 1992; Dyer et al., 2012). Since they were present in between 90 and 100% of the species in the populations used in this study, they were disregarded here.

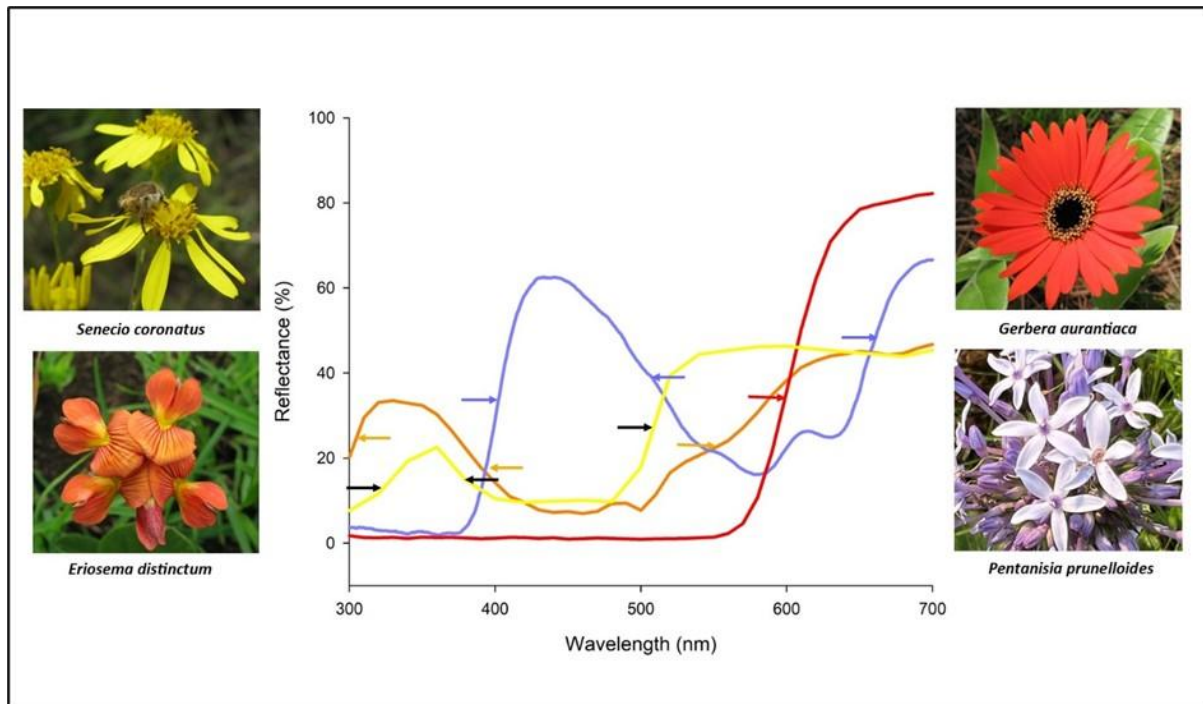


Figure 4.1. Reflectance spectra of four typical KZN mistbelt grassland species recorded in *Gerbera aurantiaca* populations. Inflection point positions are indicated by horizontal arrows. Note that some species such as *Senecio coronatus* and *Pentanisia prunelloides* may have two or more inflection points.

Data analysis

Spectra were first analysed independently of insect visual systems thereby considering the species flower colours without bias towards any particular pollinator group vision system. Spectra were simplified to 50nm wavelength interval values over the 300 to 700nm range measured as flowers typically have smooth reflectance functions with only two or three strong changes over this range (Arnold et al., 2009; Chittka and Menzel, 1992). A principal components analysis (PCA) was carried out on these spectra using PAST 4.14 to test whether the coordinates fell into distinct clusters according to population. A MANOVA was then carried out on the PCA scores to test whether the different populations were significantly

different in terms of reflectance spectra (Arnold et al., 2009; Chittka and Menzel, 1992) regardless of pollinator visual system.

Secondly, inflection points for all species in the five study populations were grouped into eight 50 nm wavelength bins across the UV–visible (300–700 nm) reflectance spectra (Kemp et al., 2019). A binomial logit GLM was carried out on proportions of inflection points in each 50nm bin at each site using SPSS 28 (IBM Corp) to examine the data for possible differences between the populations across the visible spectrum.

***Gerbera aurantiaca* capitulum colour and conspicuousness to pollinators**

Flower reflectance data was modelled in the visual systems of the two main functional groups of pollinators. I was interested in how the different coloured *G. aurantiaca* capitula and coflowering plants were perceived by bees and beetle visitors independently.

Firstly, for the native honey bees (*Apis mellifera scutellata*: Apidae), I used the widely accepted trichromatic visual model (bee hexagon) developed for hymenopterans (Chittka, 1992) since this model has been widely used and accepted in studies of hymenopteran colour perception.

In the case of modelling how beetles might perceive colour I used a receptor noise-limited (RNL) colour-opponent model (Vorobyev and Osorio, 1998). I did this because one of the only studies on coleopteran pollinator vision is the model developed for the trichromatic visual system of the red flower visitor *Pygopleurus israelitus* (Glaphyridae: Scarabaeoidea) (Martínez-Harms et al., 2012) which plots flower colours in a chromaticity diagram using the receptor noise-limited (RNL) colour-opponent model (Vorobyev and Osorio, 1998)

Although the visual sensitivities of the hopline scarab beetle *E. vulpina* are not yet known the coleopteran model for *P. israelitus* is used here since both beetle species belong to the superfamily Scarabaeoidea, and both frequently visit red flowers (Arnold, 2010; Martínez-Harms et al., 2020; Martínez-Harms et al., 2012). Euclidean distances between species loci in the different populations for the hymenopteran vision models were estimated using PAST 4.14 (Hammer et al., 2001).

Hymenoptera

To assess how the flower species in the five populations might be perceived by hymenopterans I employed the bee colour hexagon, a graphical representation of the perception of different colours to a bee, based on the relative excitations of the three types of hymenopteran photoreceptors (UV, blue and green) caused by the colours, and two

unspecified colour opponency mechanisms (Chittka, 1992; Gumbert et al., 1999). The colour of each species in bee vision can be categorised by the position of its locus in the colour which has six segments corresponding to bee-blue, blue–green, green, UV-green, UV or UV-blue. The hexagon allows the calculation of the perceptual similarity of different flower colours to a bee, with smaller Euclidean distances indicating similarity, and larger distances differences. I plotted the positions of all species from each of the five populations in the bee hexagon and then calculated the mean Euclidean distances for the various colour morphs of *G. aurantiaca* (orange, red and yellow) from all other co-flowering species (Gumbert et al., 1999). Loci which have Euclidean distances of less than 0.11 between them appear similar to honeybees, and those less than 0.05-0.04 to bumble bees and are likely to be confused, although this may be context dependent (Dyer and Chittka, 2004; Dyer and Garcia, 2014; Garcia et al., 2017; Wilmsen et al., 2021)

Coleoptera

To evaluate how beetles might perceive the flower colours here I used the model developed for the red-sensitive glaphyrid beetle pollinator *Pygopleurus israelitus* (León-Osper and Narbona, 2022; Martínez-Harms et al., 2020; Martínez-Harms et al., 2012). Spectral sensitivities for *P. israelitus* were kindly provided by J. Martinez-Harms. This model plots flower colours in a chromaticity diagram using the receptor noise-limited (RNL) colour-opponent model (Vorobyev and Osorio, 1998) where colours can be depicted as points in a chromaticity diagram and the distinction between colours is given by the Euclidean distance between points with the greater the distance, the more reliable the discrimination. Following Martinez-Harms et al (2020) a coordinate system (q_k) was used where quantum catches for stimuli were divided by those for a reference stimulus or background, Q^b_k , to give a receptor contrast space.

$$q_k = Q_k/Q^b_k$$

Assuming validity of Weber's law, the colour loci can be plotted with the coordinates

$$X_1 = A(f_L - f_M)$$

$$X_2 = B[f_S - (a f_L + b f_M)]$$

where the receptor signals f_k are

$$f_k = \ln(q_k)$$

with

$$A = 1/\sqrt{\omega_L^2 + \omega_M^2}$$

$$B = \sqrt{(\omega_L^2 + \omega_M^2) / (\omega_L^2 \omega_M^2 + \omega_S^2 \omega_L^2 + \omega_S^2 \omega_M^2)}$$

$$a = \omega_M^2 / \omega_L^2 + \omega_M^2$$

$$b = \omega_L^2 / \omega_L^2 + \omega_M^2$$

Note that the axes do not correspond to opponent mechanisms. The Weber fractions or noise values, ω_k , were set to $\omega_S = 0.13$, $\omega_M = 0.06$, and $\omega_L = 0.12$. The distance between the colour loci can be expressed as $\Delta S^2 = \Delta X_1^2 + \Delta X_2^2$.

RESULTS

Petal reflectance spectra and inflection points

The reflectance spectra (300-700nm) of all species from the five study populations with the *G. aurantiaca* spectra highlighted in colour are shown in Figure 4.2.

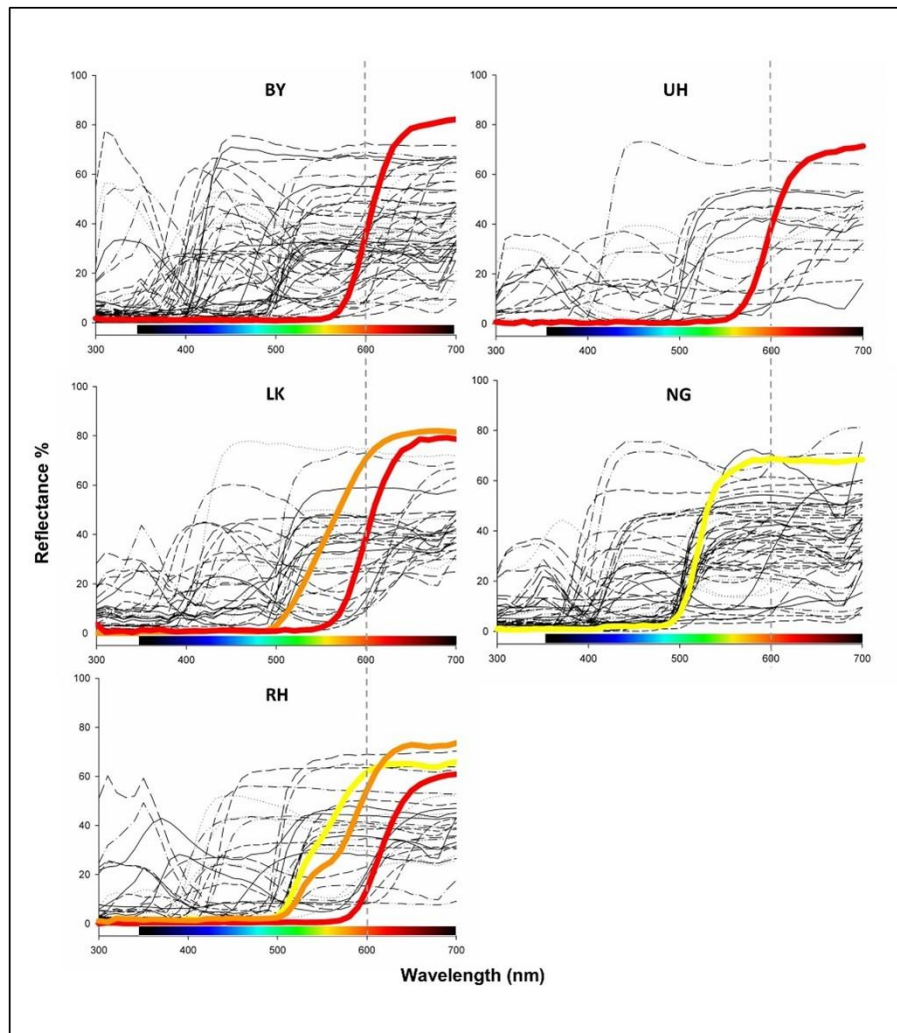


Figure 4.2. Spectral reflectance curves (300-700nm) of all flowering species sampled from the five study populations. *Gerbera aurantiaca* colour morph spectra are shown in bold, and the dashed vertical line marks 600nm.

The principal components analysis of all spectra (Fig 4.3) shows that the distribution of colours from all five populations appear to overlap strongly, and no distinct clusters or groups are evident. A MANOVA test of the PCA scores revealed no difference between the groups of points (Wilks' lambda, $F = 0.8119$, $P = 0.3912$, $df1 = 36$, $df2 = 661.3$) indicating that the distributions of reflectance spectra present in each population are statistically indistinguishable.

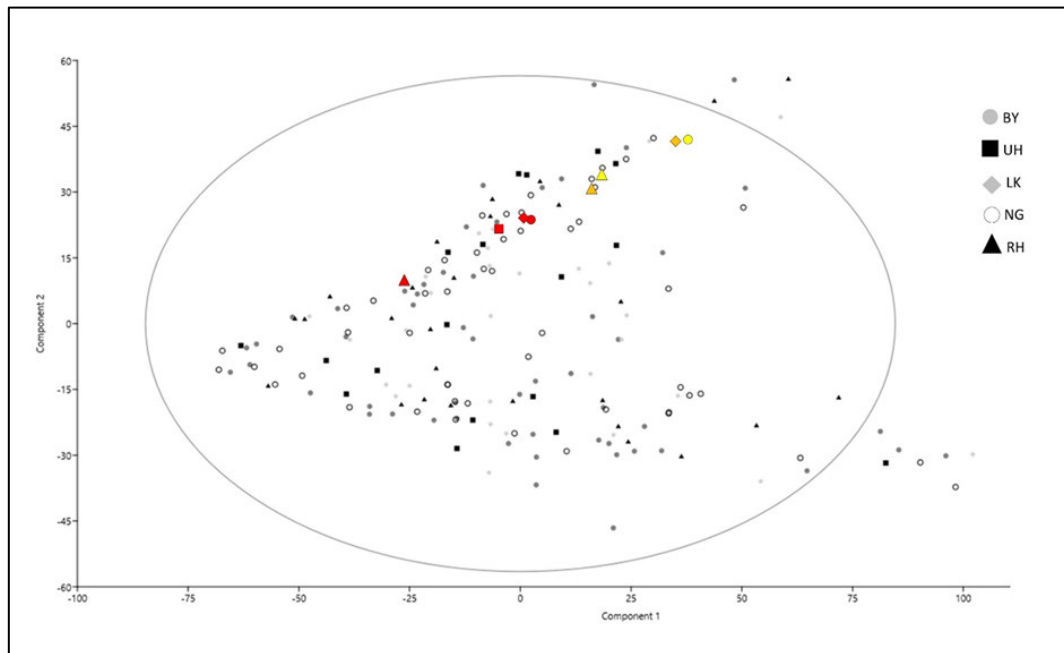


Figure 4.3. Principal components analysis and 95% confidence interval ellipse of the spectral reflectance data of all co-flowering species from five populations of *Gerbera aurantiaca* with monomorphic red and yellow and polymorphic colour morphs. *Gerbera aurantiaca* colour morphs are shown in colour in their population symbol. Variation accounted for by PC1: 53.75; PC2 = 23.98.

An analysis of inflection points from the five study populations indicated that there were no significant differences among sites in the proportion of inflection points in 50nm bins across the visible colour spectrum (300-700nm) (Fig 4.4). The highest proportion of inflection points in all five populations occurred in the 500-550nm (yellow) bin where the NG population was highest (although not significantly), followed by the 350-400nm (UV) bin.

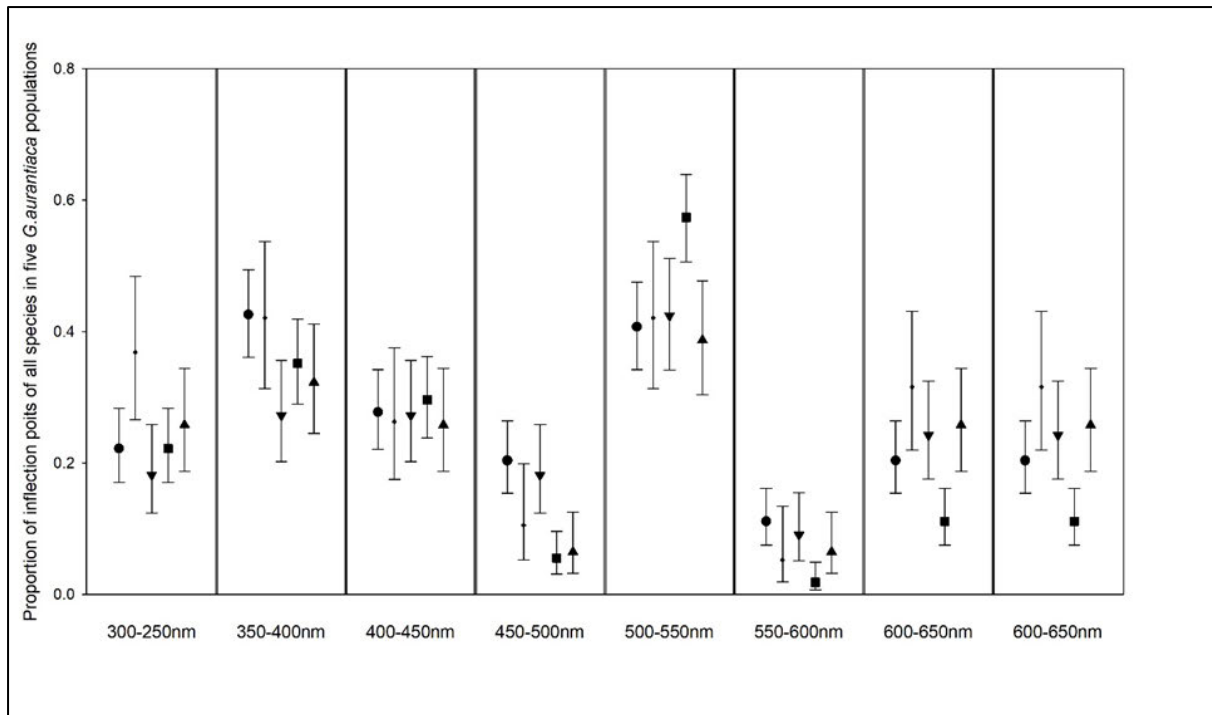


Figure 4.4. Proportions of inflection points (50nm bins) of the spectra of all co-flowering species from the five study populations. Vertical bars indicate standard error of the mean. (population symbols; black circle – BY, black dot – UH, inverted triangle – LK, black square – NG, black triangle –RH).

G. aurantiaca capitulum colour and conspicuousness to pollinators

Hymenoptera

The most frequent bee colour across the five populations was blue–green (25% of flowers overall), followed by bee UV–green (19%) and bee–green (18%) (Fig 4.5). A χ^2 test revealed no significant differences between the proportions of loci for each 60° bee colour segment ($\chi^2 = 2.9$, $df = 20$, $P = 0.293$).

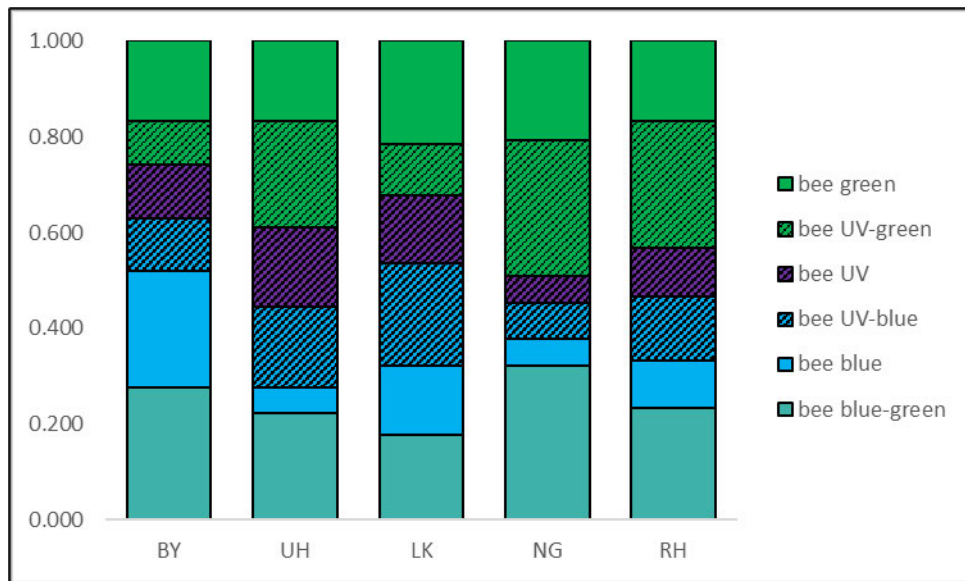


Figure 4.5. Relative proportions of different flower colours present at the study sites according to the bee visual system.

The loci of the *G. aurantiaca* colour forms are situated in the bee-green segment, with the loci of the red morphs falling close to the origin and leaf background, the yellow NG and RH morphs furthest away, while the orange forms in the polymorphic LK and RH populations lie in an intermediate position. (Fig 4.6).

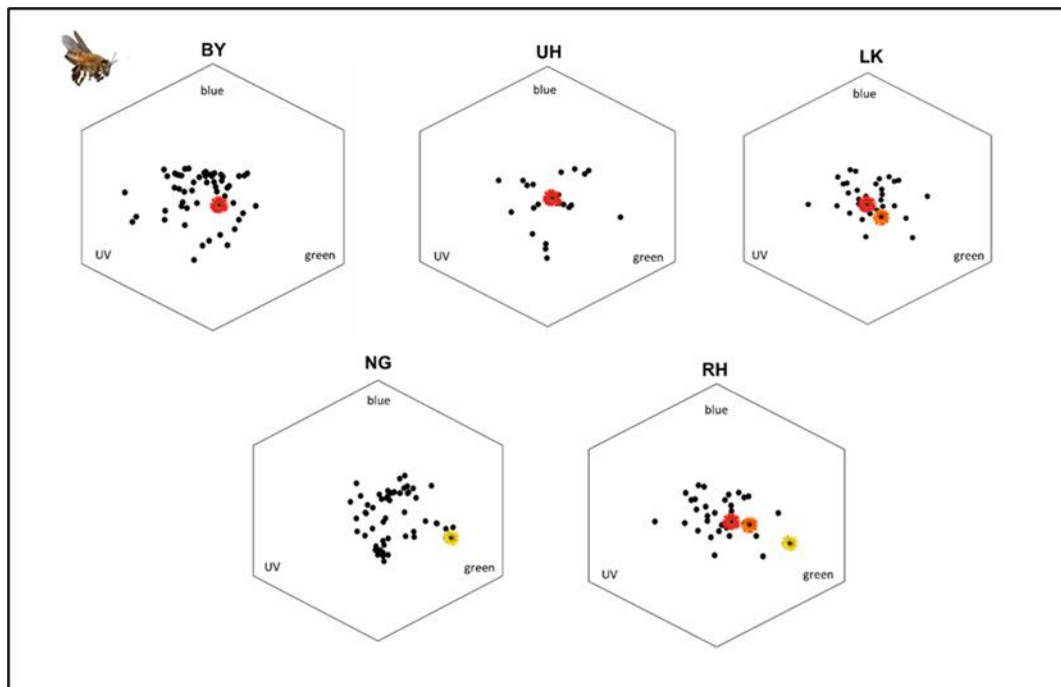


Figure 4.6. Reflection spectra loci of flowering species of the five study populations plotted into the bee vision hexagon (Chittka, 1992). The location of the *Gerbera aurantiaca* colour morphs in the hexagon are indicated with coloured capitula.

The mean Euclidean distances of *G. aurantiaca* colour morph loci from all other species in each population (Fig 4.7) indicate that the yellow morphs in both the monomorphic yellow NG population and the polymorphic RH population are a greater distance (>5) away from other species and from the origin and therefore probably highly distinct from the general flower population to hymenopterans. The red and orange colour morphs are a shorter mean distance away (0.2-0.3) and therefore less, but still easily distinguishable from most other flowers in the populations in hymenopteran vision, though the proximity of the spectra of the orange and red morph to the origin and leaf background indicates that these morphs are less conspicuous to bees than are the yellow morphs.

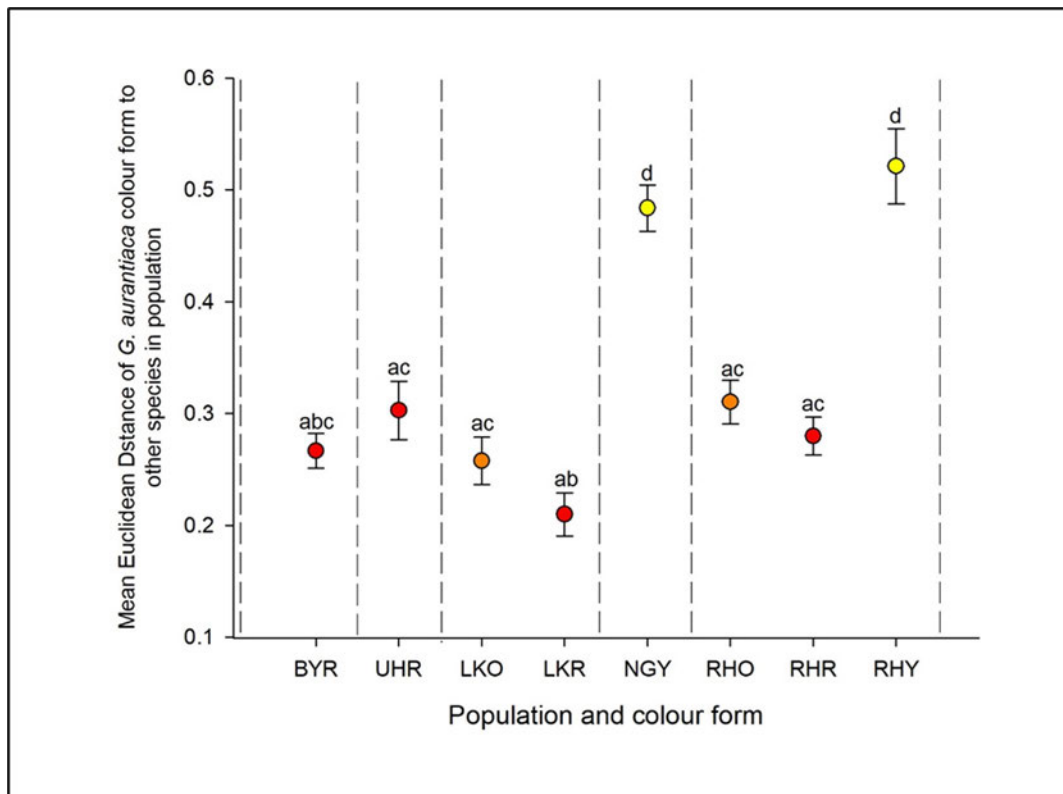


Figure 4.7. Graph showing the mean Euclidean distances of *Gerbera aurantiaca* colour variant loci in the bee hexagon from co-flowering species in each population. Vertical bars show the standard error of the mean and letters indicate significant differences between means.

Coleoptera

Chromaticity plots of the loci of all species at each of the five populations plotted using the model for *P. israelitus* (Martínez-Harms et al., 2020; Martínez-Harms et al., 2012) are shown in Figure 4.8. While it must again be emphasized that these findings should be viewed with some reservation since the visual sensitivities of *E. vulpina* are not yet known, it seems likely that these beetles possess red-sensitive receptors and this model may provide some information on the visibility of the different *G. aurantiaca* colour morphs to the hopliine beetle visitor which is fairly closely related to *P. israelitus*. The position of the *G. aurantiaca* morph loci in the chromaticity diagrams suggest that capitula of all colour morphs are situated at a greater than 1.5 unit mean distance away from the other flowering species in their respective populations and are therefore likely to be highly distinct from their neighbours to red-sensitive coleopterans.

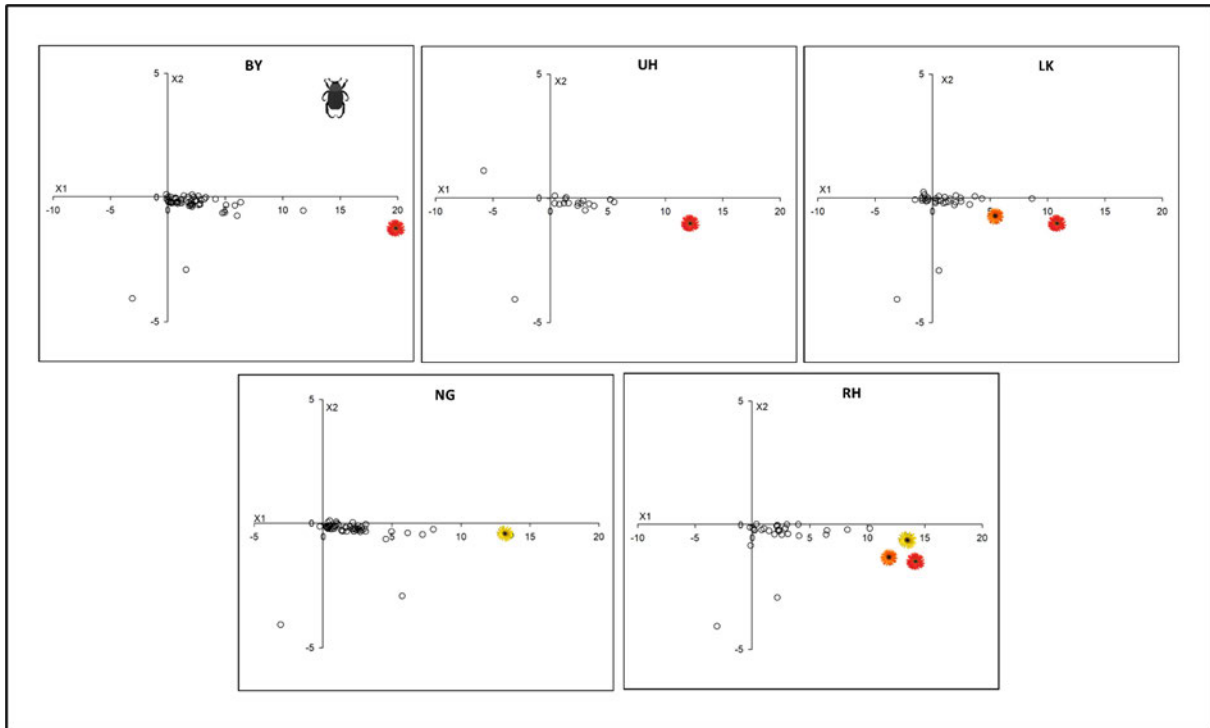


Figure 4.8. Loci of the flower colours of the plant species in five populations of *G. aurantiaca* represented in the chromaticity diagram of *Pygopleurus israelitus* according to the receptor noise-limited (RNL) colour-opponent model (after Martinez-Harms 2021). The loci positions of the orange, red, and yellow colour morphs of *G. aurantiaca* are indicated by coloured capitula.

DISCUSSION

Few studies have investigated the possible interactions between flower colour polymorphic species and their community colour environments. This investigation of the spectral properties of co-flowering plants within populations with differing capitulum colour morphs of *G. aurantiaca* indicated that there is little variation between the flower colour patterns of these communities when analysed independently of insect vision models. Analysis of the spectral reflectance curves (300-700nm) and inflection points (areas of spectral curves which show rapid change) of all co-flowering species in the five populations suggested that there are no distinct differences in the communal spectral signal between populations. Since the climatic and edaphic conditions were found to be similar among the *G. aurantiaca* populations (see Chapter 2 of this thesis) this further suggests that these abiotic factors are unlikely to play a role in colour variation, and that *G. aurantiaca* colour variation is more likely influenced by other factors. Since the *G. aurantiaca* colour morphs investigated here

are self-incompatible, pollinator dependent and often pollen-limited (see Chapter 3 of this thesis), reproductive output and consequently capitulum colour is likely to be strongly dependent on the attraction of pollinators.

I used insect vision models for the common pollinator groups to explore the possibility that community flower colour in these populations may be structured or influenced by plant-pollinator interactions for the two observed dominant insect visitor groups to *G. aurantiaca* (Hymenoptera and Coleoptera). Since very few of the herbaceous flowers in these grasslands are visited by birds, and few dipterans or lepidopterans were observed on *G. aurantiaca* capitula during this study I chose to not to include a quantitative analysis of flower colours as seen by these groups.

The hymenopteran colour hexagon model (Chittka, 1992) was used to categorise the flower species in bee vision by the position of their loci, and to calculate the Euclidean colour distance between loci of the *G. aurantiaca* colour variants in each population and that of their coflowering neighbours. The loci of the red colour morph of *G. aurantiaca* were positioned close to the origin suggesting that they are not easily distinguished by bees, while the loci of the yellow and the orange forms were located further from the origin in the bee-green segment of the hexagon, indicating that they are likely to be more easily discernible to hymenopteran visitors (Fig 4.7). These findings agree with the suggestion that pure red coloured flowers are not seen easily by bees since red is close to the limits of their visual abilities (Chittka and Waser, 1997; León-Osper and Narbona, 2022). Calculation of the mean Euclidean distance of the *G. aurantiaca* colour form loci to the loci of all coflowering species in each population supported these results and indicated clearly that the yellow colour morphs were distinct from most other flowering species in the population and highly conspicuous to hymenopterans. These results are supported by field observations made during this study when honeybees were frequently seen collecting pollen on the yellow capitula in the monomorphic NG population but were not observed on red capitula in other populations (see Chapter 3 of this thesis).

The potential visual capabilities of the hopliine beetle pollinator *E. vulpina* were explored using the model developed for the red sensitive beetle pollinator *P. israelitus* (Martínez-Harms et al., 2020; Martínez-Harms et al., 2012). The chromaticity plots from these calculations indicate that the orange, red and yellow morphs of *G. aurantiaca* are all clearly visible to *E. vulpina* and distinct from coflowering species in the populations. However, since the possibility exists that *E. vulpina* may be a tetrachromate with four visual sensitivity peaks rather than a trichromate like *P. israelitus* (Sharkey et al., 2017), these

results are tentative and must be viewed with caution until further information on *E. vulpina* vision is available. However, *E. vulpina* beetles visited orange, red and yellow colour forms equally in the polymorphic RH population (see Chapter 2 of this thesis) and were common visitors on both the red and yellow morphs in the monomorphic red and yellow populations which further supports these results. It must also be emphasized here that the role of *G. aurantiaca* capitula in providing a social platform the dark centre may be as or more important to beetle attraction as visual cues than colour (Johnson and Midgley, 2001).

This part of my study further raises the question of whether the monomorphic yellow population may have resulted from a temporary but prolonged absence or scarcity of a red-sensitive but colour unselective beetle, during which period honeybees actively selected for the highly visible yellow morphs and ignored the red less visible morphs.

Gerbera aurantiaca may have two strategies to ensure its long-term survival; firstly, clonal growth which allows it to survive long periods without insect pollinators or changing pollinator composition, and secondly, its flower colour polymorphism which enables it to attract hymenopteran pollinators (mainly honeybees) to yellow and possibly orange colour forms when coleopterans are scarce or absent. The high visibility of all colour forms to red-sensitive coleopteran pollinators may play a role in maintaining colour polymorphism in this species.

CONCLUSIONS

My study suggests that the colour morphs of *G. aurantiaca* are not correlated with differences in community patterns of flower colour signals. This is evident both using analyses that are independent of insect vision models, as well as vision models for the key insect pollinator groups. It seems likely that hymenopteran flower visitors can distinguish the yellow and orange capitula more clearly than the red, while preliminary data indicate that all colour forms of *G. aurantiaca* are easily detected by red-sensitive coleopterans. Further detailed research into the colour vision sensitivities of hopline scarab beetle pollinators, common flower visitors in southern Africa, is needed to obtain more definite results. These results may form the basis for further studies on the role of flower colour in structuring plant-pollinator networks.

Acknowledgements

I thank Adam Shuttleworth and Sandy-Lyn Steenhuisen for advice with spectral reflectance and scent analyses, J. Martinez-Harms for providing the spectral sensitivity curves for *P.*

israelitus, the Bews Herbarium (NU) for access to specimens for identification, Ezemvelo KZN Wildlife, Dept. of Environment, Forestry and Fisheries, and private landowners for access to the study sites.

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**CHAPTER 5: CAPITULUM COLOUR IN *GERBERA AURANTIACA*:
SURFACE MICROSTRUCTURE, PIGMENT DISTRIBUTION
AND INHERITANCE**

Abstract

Flower colour is influenced by the selective light absorption of different pigments, internal light scattering and the structure of epidermal cells. In plants with flower colour polymorphisms, the frequency of different colours in populations will also be determined by the genetic basis of flower colour (and associated patterns of inheritance) as well as pollinator-mediated mating patterns. I investigated the anatomical basis of flower colour and its inheritance in the flower colour polymorphic daisy *Gerbera aurantiaca*. Capitula are intensely red, orange, or yellow due to the presence of anthocyanin and carotenoid pigments, and the ray floret surfaces are reflective at certain angles to create a velvety effect in natural sunlight. Using spectrophotometry and microscopy to investigate the optical properties and ultrastructure of the ray florets, I found that in all colour morphs the pigments (anthocyanins and carotenes in the red and orange colour morphs, and carotenes only in the yellow morphs) are localised primarily in the upper epidermal layer of the ligule and that this surface is patterned with steeply sided longitudinal ridges which may have a structural effect on light reflectance and be important in visual signalling to pollinators. Preliminary *ex-situ* crossing of the red and yellow colour variants suggests polygenic inheritance of anthocyanin pigments with the mainly orange F₁ progeny colour array bearing a close resemblance to that in a naturally occurring polymorphic population.

Key words: *Gerbera aurantiaca*, petal surface structure, velvety effect, pigment, colour inheritance

INTRODUCTION

One of the most striking characteristics of flowers is their enormous range of colour variation. The colour of a flower is the result of complex biochemical processes influenced by the interplay of genetic, abiotic and biotic factors. The vast array of flower colours is primarily determined by spectral filtering that results from the presence of wavelength-selective absorbing pigments and light scattering structures inside the petals (van der Kooi et al., 2016). For example, flowers with blue-green absorbing anthocyanins are purple while flowers with the blue-absorbing carotenoids are yellow to humans (Grotewold, 2006). While carotenoids produce mainly yellow and orange colours, anthocyanins are a diverse group of pigments that produce colours ranging from white to dark purple (Kay et al., 1981). Flower colour is largely determined by pigments, but other factors by such as petal surface structure, soil or cell pH and water availability have also been considered as contributing to flower

colour (Glover and Whitney, 2010; Schemske and Bierzychudek, 2007; Vaidya et al., 2018; van der Kooi et al., 2014).

Approximately 80% of plant species investigated possess one or more forms of petal surface structuring, ranging from conical papillate cells to transverse striations, and some species such as *Hibiscus trionum* possess more than one type (Kay et al., 1981; van der Kooi et al., 2014). Dr Rudolph Marloth, in a study of South African flora, suggested that the petal colour in many species is influenced by epidermal structures, and more recent work suggests that it may also exhibit a particularly diverse array of petal surface microstructures (Marloth, 1915; Whitney et al., 2011). It has been proposed that the role of petal surface microstructures may be to enhance colour by focussing light into the pigment containing vacuole (Kay et al., 1981; Whitney et al., 2009b). A number of other suggestions include that the function of epidermal cell structure may be to influence flower temperature, hydrophobicity, petal strengthening to prevent buckling during development, provide a tactile pollinator guide when orientated towards the source of floral reward, and as a grip for insect pollinators (Antoniou Kourouniotti et al., 2013; Barthlott and Neinhuis, 1997; Garcia et al., 2020; Kevan and Lane, 1985; Wilts et al., 2018). However, the role of these petal epidermal microstructures is not fully understood and has been the subject of current discussion (van der Kooi et al., 2018). Several types of petal surface light reflection have been described using the following terms: ‘iridescence’ the phenomenon where the colour of an object appears angle-dependent with a prominent ‘rainbow effect’ as in a compact disc, and although found in many animals, this is thought to rarely be present in petals of wild species under natural light conditions (van der Kooi et al., 2014; Whitney et al., 2009b); ‘gloss’, a mirror-like surface reflection, as seen in *Ranunculus* spp. petals (van der Kooi et al., 2017); and a ‘velvety’ effect, caused by regularly arranged conical surface structures on the petal which reduce surface gloss e.g. *Mandevilla sanderi* (Stavenga et al., 2020).

One of the main purposes of flower colour is to attract insect pollinators, which perceive flower signals in different ways (Schiestl and Johnson, 2013). As the surface structure of petals may be an important trait that influences the type of flower visitor it should be considered in analyses of plant-pollinator and plant-antagonist interaction networks (Papiorek et al., 2014). Most flowering plant species are monomorphic within populations for colour, but a minority exhibit flower colour polymorphism (FCP) within populations (Sapir et al., 2021). Flower colour variation within a species may be caused by changes in pigment concentration that result in a gradient of flower colour intensity, or by differences in pigment types, e.g., in *Raphanus sativus*, petal colour is determined by the presence or absence of the

pigments carotene and anthocyanin (Narbona et al., 2021). As differences between pigment reflectance and localisation and petal surface microstructure among colour morphs in FCP populations may influence pollinator visitation and thus patterns of evolution arising from pollinator-mediated selection, these factors are worthy of further investigation.

Here I investigate some anatomical and structural aspects of flower colour in *Gerbera aurantiaca*, a brightly coloured, FCP daisy, as part of a broader investigation into the evolution of the species flower colour variation (Johnson et al., 2005; Johnson et al., 2021). I use field observations, spectrophotometry, and microscopy to investigate colour reflectance, pigment distribution and ray floret ligule surface microstructure of the three main colour morphs. In addition, I report on the results of a preliminary investigation into F₁ colour inheritance in *G. aurantiaca*. I ask the following questions: 1) What is the pigment distribution in the ray floret ligules of *G. aurantiaca* colour morphs? 2) Do *G. aurantiaca* ray floret ligules exhibit surface ornamentation and does this differ between different colour morphs? and 3) What is the colour of the ray floret ligules in F₁ progeny when red and yellow colour morphs are artificially crossed?

METHODS

The study species:

Gerbera aurantiaca (Mutisieae: Asteraceae) is a flower colour polymorphic daisy with capitulum ray florets ranging from yellow through orange to deep red, both within and between populations and which has dark centres in the female phase of flowering (Johnson et al., 2021). The species is endemic to mistbelt grasslands in the eastern regions of South Africa and is pollinated primarily by hopliine beetles and honeybees (Chapters 2 and 3 of this thesis). Although the pigments of *G. aurantiaca* have not yet been analysed chemically, the wide range of flower colour found in *Gerbera* hybrids is known to be based on the interaction of carotenoids and flavonoids: cyanic (with anthocyanin pigment) genotypes have both flavonoids and carotenoids and are red or orange coloured, while acyanic (lacking anthocyanin pigment) genotypes with carotenoids only are yellow or orange (Asen, 1984; Tyrach and Horn, 1997). The two main flavonoid classes in *Gerbera jamesonii* (a sister species to *G. aurantiaca*) hybrids are pelargonidin and cyanidin (Asen, 1984) and the following carotenoid pigments (phytofluene, β - and ξ -carotene, β -zeacarotene, 5,6-monoepoxy- β -carotene, 5,6-diepoxymethyl- β -carotene, cryptoxanthin, chrysanthemaxanthin, flavoxanthin and auroxanthin are present (Valadon and Mummery, 1967).

Plant material

Gerbera aurantiaca plants were observed in the field in natural light conditions and photographed using a Canon PowerShot G12 using automatic white balance. Capitula of *G. aurantiaca* were collected for further observations from both *in-situ* populations and from *ex-situ* plants growing in the KwaZulu-Natal National Botanical Garden in Pietermaritzburg, South Africa.

Spectral reflectance

The spectral reflectance of the ray floret ligule surfaces and the dark central disc area was measured using an Ocean Optics S2000 spectrophotometer (see Johnson et al 2021 for methods). The spectra of at least three ray floret ligules from 10 individuals of each colour morph were averaged. The spectral reflectance of both the adaxial (upper) and abaxial (lower) surfaces of the ray floret ligules, and the central disc area were measured. To measure possible effects of the orientation of the floret surface microstructure on spectral reflectance to the light source, both longitudinal and transverse orientations of the ray floret ligule sample adaxial surface were illuminated by the 45° light source and recorded.

Microscopy

To determine the location of pigments in fresh material of the ray floret ligules I hand-sectioned ray floret ligules transversely directly onto a microscope slide in a drop of distilled water and viewed them immediately with an Olympus Provis A×70 objective microscope. I examined the internal fine structure of the ray floret ligules after resin embedding of microtomed transverse sections with a Philips Biotwin C120 TEM. The surface structure of the florets was viewed with a Philips XL 30 /Zeiss EVO Ls15 ESEM scanning electron microscope. Since fresh petals contain water in their vacuoles and cannot be vacuumed for SEM, casts of the ray florets were sputtered with gold to prevent charging effects. At least 10 florets from each colour morph were viewed.

Colour crossing experiments

In a preliminary investigation of the inheritance of flower colour in *G. aurantiaca* I used *ex-situ* plants from red and yellow (i.e. pure breeding) populations raised from seed in the KwaZulu-Natal National Botanical Gardens. I used pollen from yellow capitula to fertilise receptive stigmas of red capitula (N=10) and pollen from red capitula to fertilise receptive stigmas of yellow capitula (N=7) using clean paintbrushes. Labelled capitula were bagged with exclusion nets prior to and following pollen transfer to minimise contamination.

Fertilised seeds were sown into labelled individual pots and planted out into nursery beds after twelve months. When flowering of the F₁ progeny commenced after approximately 36 months, capitulum colour was recorded and photographed over a three-year period. Further crosses between the F₁ progeny were made, but the offspring resulting from these crosses were inadvertently destroyed before flowering, thus precluding investigations of F₂ segregation.

RESULTS

Field observations

Field observations of *G. aurantiaca* populations *in-situ* revealed that the red morph frequently exhibits a “velvety” sheen on the ray floret ligule adaxial surface when viewed in sunlight, but this is less pronounced in the yellow form (Fig. 5.1A&B).



Figure 5.1. Capitula of *Gerbera aurantiaca* red (A) and yellow (B) colour morphs in natural light in *in-situ* populations. Arrows indicate the surface velvety sheen on the ray floret ligule adaxial surfaces of the red morph. The dark centre of disc florets overarched by purple pappus hairs is clearly visible.

Spectral reflectance

The reflectance spectra of the adaxial surface of the red, orange, and yellow ray floret ligules showed little reflectance in the shorter wavelength region of the spectrum for any of the colour forms. Reflectance of the yellow form rises sharply to approximately 70% between 500 and 520nm, that of the red form between 590 and 620nm with that of the orange form intermediate. The dark central disc area showed a slight increase in reflectance at very long wavelengths (between 700 and 800nm) (Fig 5.2).

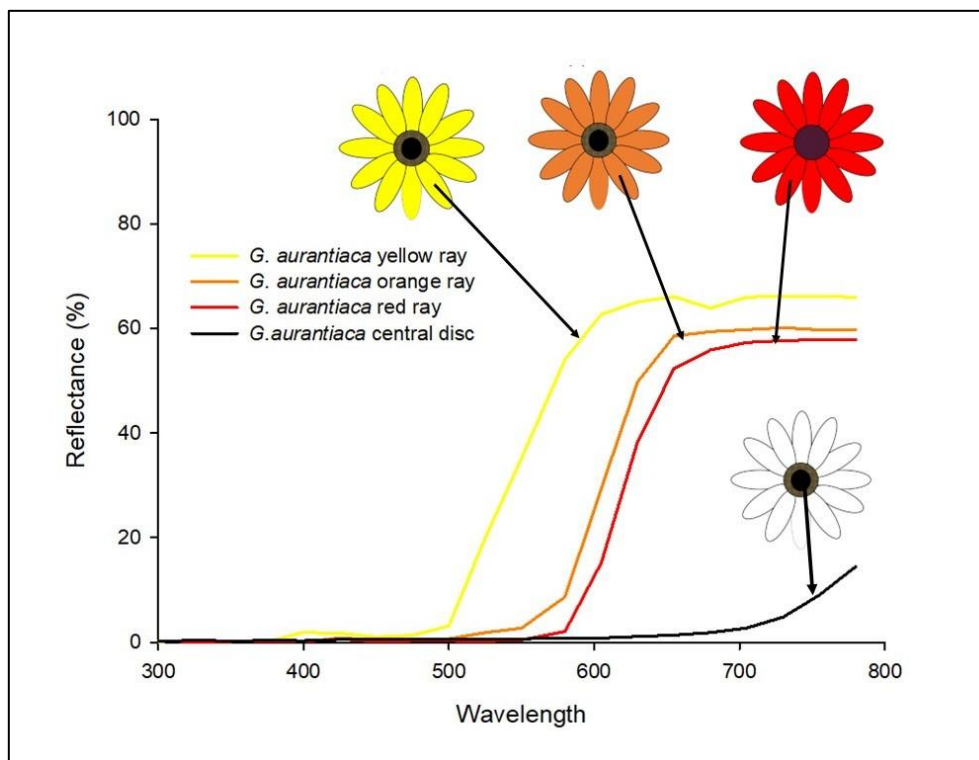


Figure 5.2. Reflectance spectra (300-800 nm) of the central surface of *Gerbera aurantiaca* adaxial ray floret ligule surface of red, orange, and yellow colour morphs, and of the disc floret central region of the capitulum.

In all three colour forms the adaxial ray floret ligule surface had a higher and more steeply curved reflectance than the duller coloured abaxial or lower surface (Fig. 5.3).

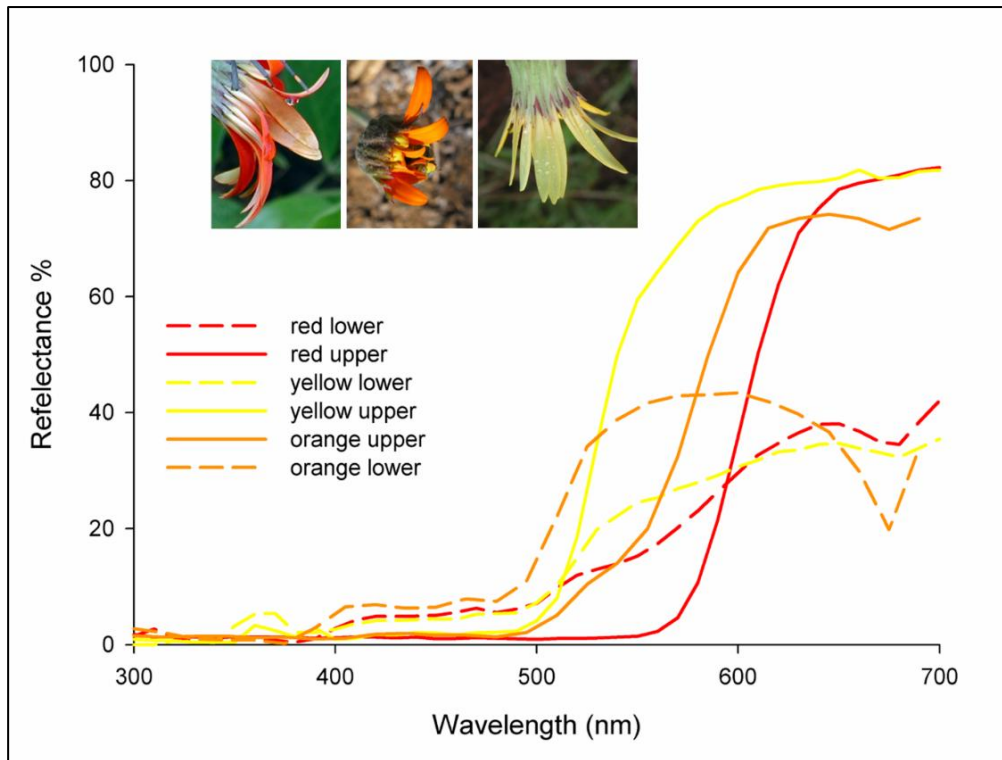


Figure 5.3. Reflectance spectra (300-700nm) of upper and lower ray floret ligule surfaces from red, orange and yellow colour morphs of *Gerbera aurantiaca*.

Reflectance spectra of the upper ligule surface showed lower reflectance across all visible wavelengths when the ligule was positioned so that the light source was directed at the longitudinal axis than when the ligule was positioned so that the light source was directed at the transverse axis of the ray floret (Fig. 5.4 B).

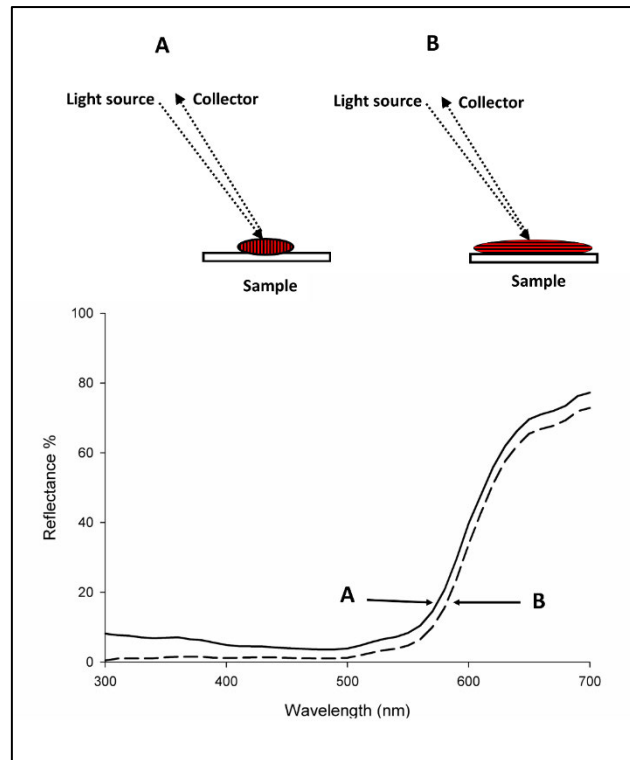


Figure 5.4. Reflectance spectra of *Gerbera aurantiaca* ray floret ligule adaxial surface when positioned at A: transverse axis orientation and, B: longitudinal axis orientation to the light source positioned at 45°.

Microscopy

Ray floret ligule structure

Viewed under the light microscope (40 × magnification) the upper surface of ray floret ligule of fresh material of all three colour morphs is comprised of narrow, elongated, pigment-filled cells aligned along the long axis of the ligule (Fig 5.5 A, D, G). Seen in cross section, the upper epidermal cells are capped by a sharply pointed epidermal structure which is much less pronounced in the lower epidermis.

Pigment distribution

Hand cut transverse sections of the ligules, which measured approx. 0.4 mm in thickness, clearly show that in all three colour forms the pigment is localized mostly in the upper adaxial epidermal cells with lesser amounts in the abaxial surface epidermal cells, and with very little in the mesophyll layer (Fig 5.5 B, E, H). The epidermal layers are separated by a mesophyll layer with small amounts of scattered carotene and chlorophyll pigment evident. (Fig 5.5 B, E, H). The brown-yellow hazy layer above the epidermal cells in 5.5 H is probably caused by the diffusion of pigment into the surrounding medium which may occur

with densely pigmented epidermal cells. In the red colour morph red anthocyanin pigment can be seen filling cell vacuole of the upper epidermal cells (Fig 5.4 C). In the yellow morph the carotene pigment appears to be confined to the cytoplasmic region below the epidermal cell vacuole (Fig 5.4 F) and in the orange morph the upper epidermal cell vacuoles are be filled with differing concentrations of flavonoid pigments (Fig 5.4 I).

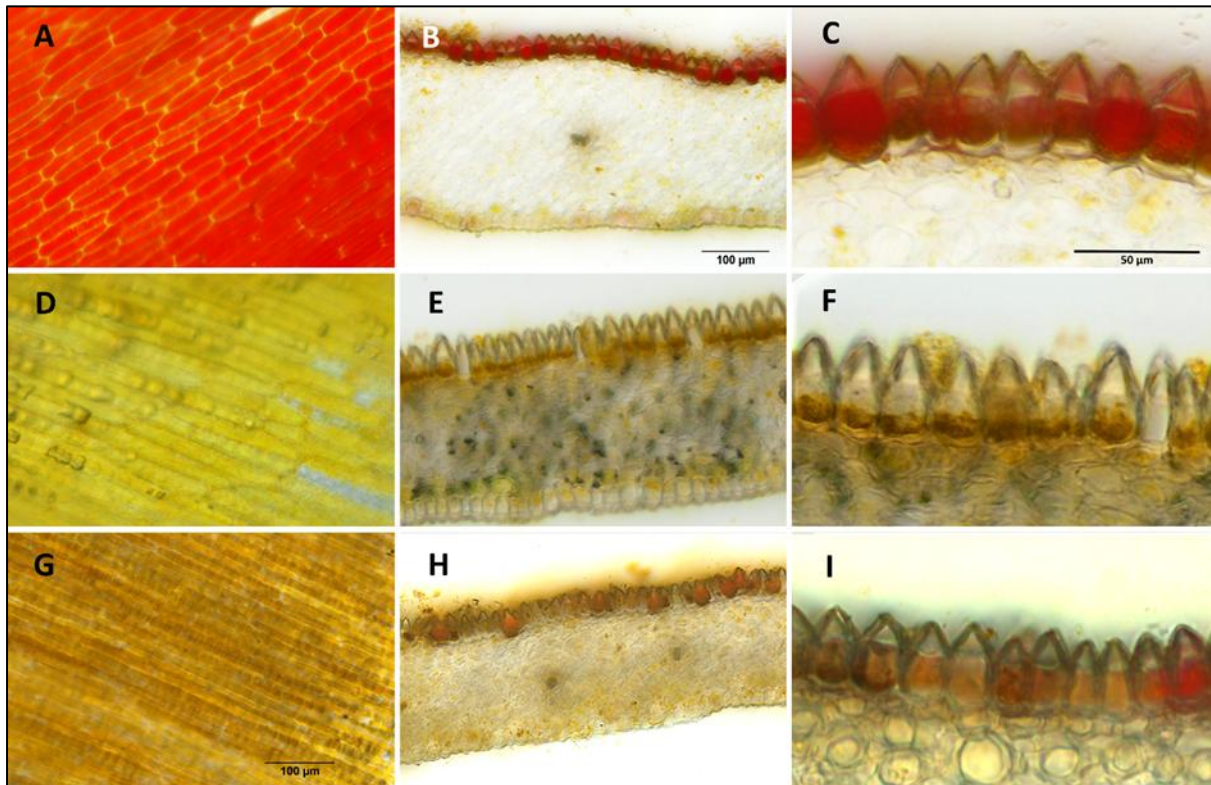


Figure 5.5. Ray floret ligules of red, yellow, and orange colour morphs of *Gerbera aurantiaca*. **A, D, G:** adaxial ligule surface structure (20 × magnification), **B, E, H:** hand-sectioned transverse sections (20 × magnification), and **C, F, I:** hand sectioned transverse sections (40 × magnification) showing structure of epidermal cells and pigment distribution.

At higher magnifications of 1500 × and 5000 ×, scanning electron microscope images show that the adaxial ray floret surface epidermal structure consists of narrow longitudinal ridges with sharply pointed, transversely patterned apices arranged in parallel rows (Figs 5.6 A&B). In transverse section, the structure of the upper epidermal cell ridges shows the sharply pointed cuticular apices capping a vacuole-filled cell with the cell contents situated mainly along the base of the cell (Fig 5.6 C&D).

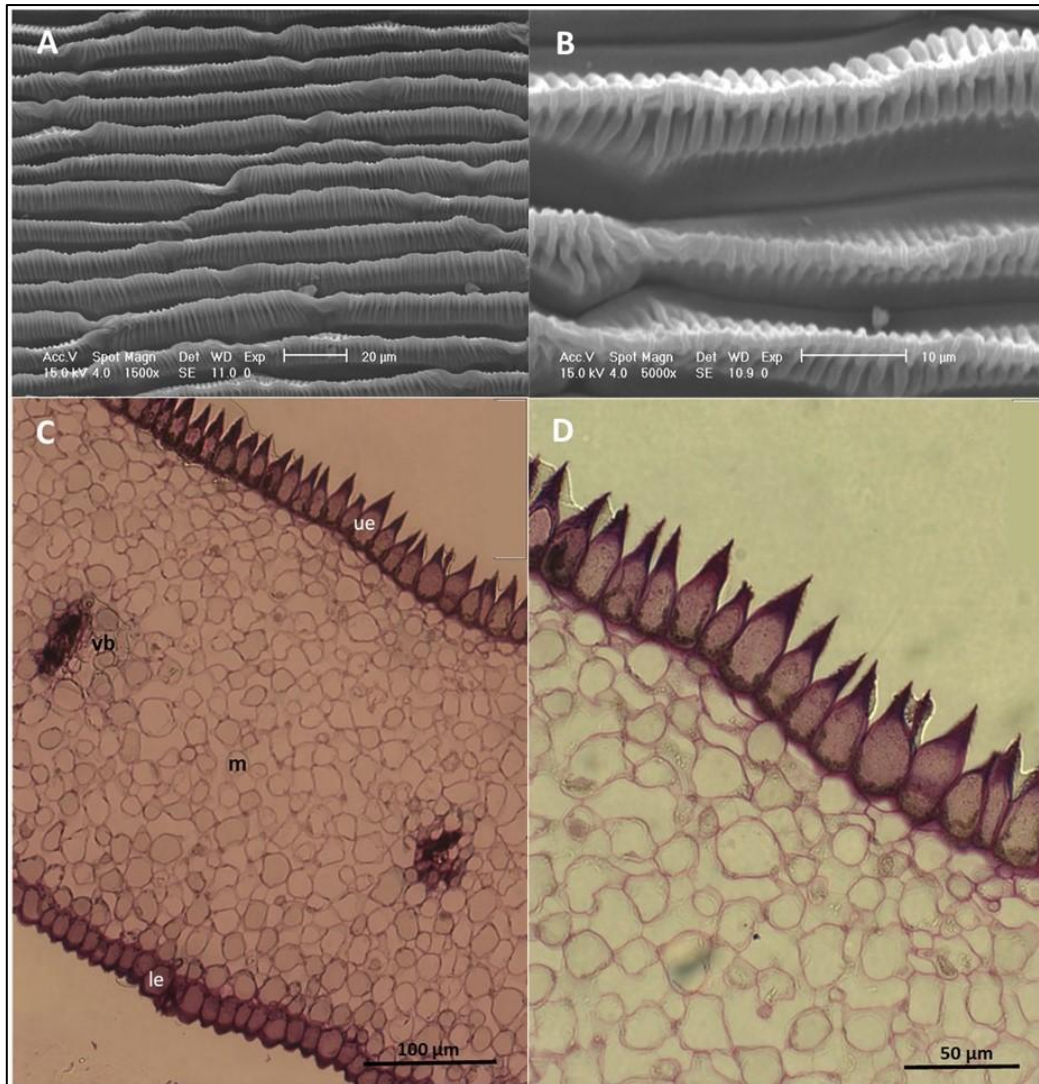


Figure 5.6. Scanning electron micrographs of the adaxial ray floret ligule surface of *Gerbera aurantiaca* **A**: 1500× magnification, **B**: 5000× magnification showing details of the parallel surface microstructures which run longitudinally along the long axis of the ligule surface; and light microscope images of a transverse section through a ray floret ligule showing **C**: the internal structure and details of the epidermal layers (20× magnification), and **D**: the adaxial surface showing epidermal cells with sharply pointed surface structures and concentration of cellular contents below the vacuole (40× magnification). (le-lower epidermis, ue-upper epidermis, m-mesophyll, vb-vascular bundle).

Colour inheritance crossing experiments

294 seedlings were raised from the ten crosses performed between red and yellow flowered *G. aurantiaca* individuals. A total of 121 plants flowered and capitulum colour was recorded. Since progeny ray floret colour was frequently not uniformly pigmented, but patchy, it was difficult to assign colours using an RHS colour chart category, or to measure reflectance

spectra. I recorded and photographed all progeny capitulum colours visually to reduce observer bias. Capitulum colours were allotted to one of the following the following categories: red, dark orange, medium orange, orange with yellow streaks, yellow with orange streaks pale orange and yellow (Fig 5.7 A). Images showing the colour range of the F₁ progeny of the experimental crosses ranging from red through various shades of orange to yellow are shown in Fig 5.7 B.

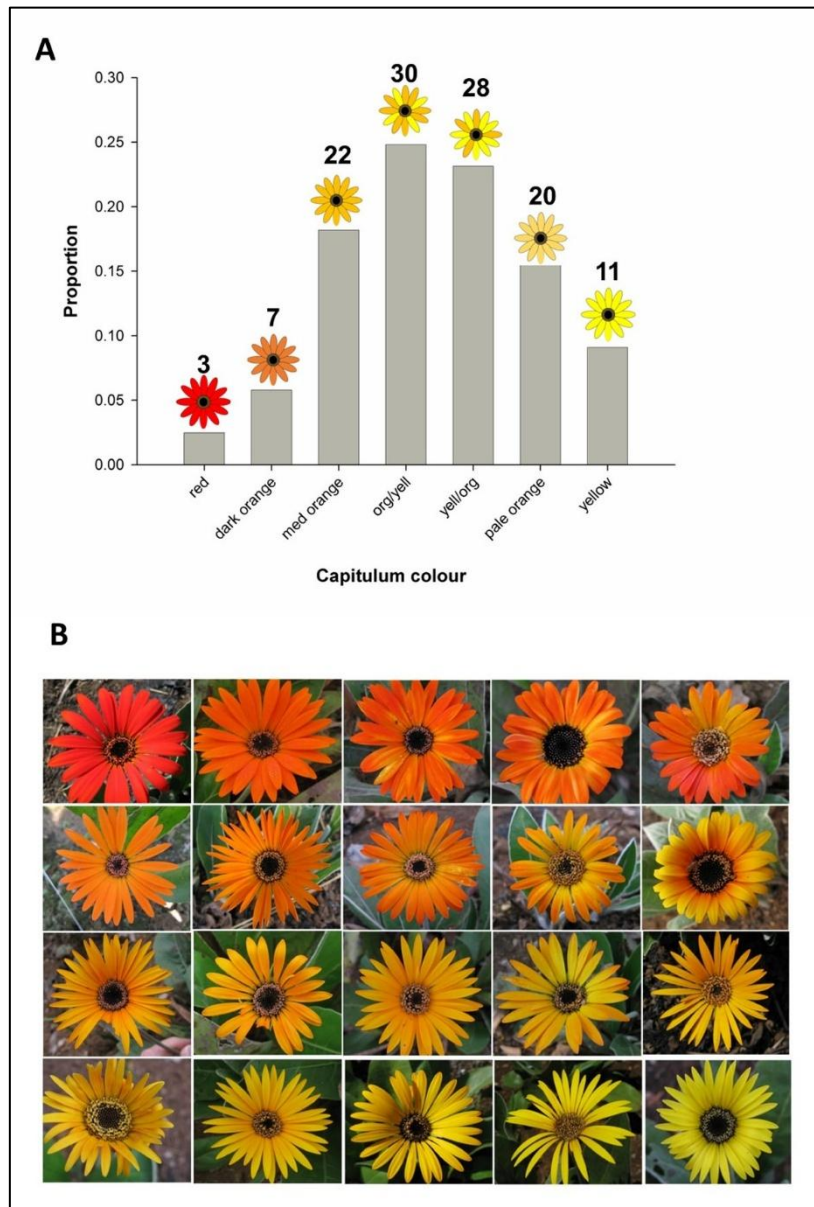


Figure 5.7. **A:** Bar graph showing distribution of F₁ colour forms of *Gerbera aurantiaca* red and yellow colour morph crosses (numbers above bars indicate sample size), and **B:** Images of the colour range of the *ex-situ* F₁ progeny.

DISCUSSION

These preliminary findings reveal that the vivid capitulum colour in *Gerbera aurantiaca* is the result of wavelength selective absorption of the carotenoid and anthocyanin pigments situated primarily in the epidermal layers of the adaxial ray floret ligules. Spectral analysis showed that the pigments of the ray floret ligules of all colour forms caused reflection of longer wavelengths between 500 and 600nm, with no ultraviolet reflection. Microscopic investigations revealed that the intense colouration may be influenced by the pigment localisation primarily in the adaxial epidermal cells of the ray floret ligules. Although localization of anthocyanins in epidermal cells is well known, the concentration of carotenoids in these cells was unexpected. The orange and red colour forms contain anthocyanin pigments in the upper epidermal vacuoles, with carotene plastids concentrated in the upper epidermal cell cytoplasm and to a lesser extent in the lower epidermal cells and scattered throughout the mesophyll, while the yellow forms contain carotene but no anthocyanin pigments. The surface microstructure of the adaxial ray floret ligule in all colour forms consists of steeply sided longitudinally aligned ridges with patterned apices.

The localisation of floral pigments in petals may vary among plant species in one of three ways: the pigment is evenly distributed throughout the floral tissue, the pigment is localised in the both the upper and lower epidermises while the mesophyll is unpigmented, or the pigment is localised in one of the two epidermises only (van der Kooi et al., 2016; Wilts et al., 2018). In *G. aurantiaca* the anthocyanin and carotenoid pigments are mostly concentrated in the adaxial epidermal cells, with small amounts of carotene scattered through the mesophyll layer and in the abaxial epidermal cells (Fig 5.5). In the adaxial epidermis the carotene pigments are situated in a cytoplasmic layer below the cell vacuole, and the epidermal cell vacuole is filled with anthocyanin pigment, from none in the yellow forms to varying amounts in the orange forms and a high concentration in the red forms. A similar pigment distribution of varying amounts of epidermal cell vacuole anthocyanin pigment to that found here in the orange morph of *G. aurantiaca* has been reported for orange *Gerbera* hybrids (Hatamzadeh et al., 2012). This asymmetrical pigment arrangement results in different coloured upper and lower petal surfaces and is to be expected in flowers that are only visited from one side (van der Kooi et al., 2016), as in the case of *G. aurantiaca* capitula. The presence of surface microstructures together with the concentration of the pigment in the upper, most clearly displayed, epidermis of the ray floret ligules of *G.*

aurantiaca suggested that these features may be involved with attraction of floral visitors to the capitulum.

Microstructure on the petal surface of the Compositae (Asteraceae) was reported in 1977 (Baagøe, 1977) and the presence of longitudinal ridges on the upper ray floret ligule surface of in *G. aurantiaca* was first noted in 2005 (Johnson et al., 2005). More recently this microstructure pattern has also been noted for *Gerbera* hybrids (Hatamzadeh et al., 2012), and it seems likely that it may be present throughout *Gerbera*: section *Lasiopus*. Similar microstructures were described for *Tulipa* sp. and *Hibiscus trionum*, where the ridged cuticle overlying elongated petal epidermal cells was thought to split the light reflecting from the surface into component wavelengths (Whitney et al., 2009b). The ray floret surface structure found in the current study shows a marked similarity to the morphology described for *Eschscholzia californica* which was suggested to enhance the strong colour intensity and increase angle dependent reflectivity (Wilts et al., 2018). *Antirrhinum majus* petals with conical cells, which were found to focus light into the anthocyanic vacuoles of the epidermal cells more strongly than was the case in mutants which lacked conical cells, were visited more frequently by foraging bees (Glover and Martin, 1998; Glover and Whitney, 2010; Whitney et al., 2009a). Conical cell colour enhancement of *A. majus* forms with conical cells was also found to be more visible to *Bombus terrestris* than forms lacking these cells (Dyer et al., 2007). However, the role of these focusing effects as biological cues remains controversial since many of the observations were not made under natural light conditions (reviewed by van der Kooi et al. 2019). While some researchers have suggested that flower colour signal and intensity may be enhanced and altered by microstructural epidermal elements on the petal surfaces (Whitney et al., 2011; Whitney et al., 2016), it has been argued that there is no evidence to suggest that structural coloration and its angle-dependence (i.e. iridescence) of flowers act as a signalling cue to pollinators under natural conditions (van der Kooi et al., 2015).

A recent study of deep red *Mandevilla sanderi* flowers concluded that the main function of conically shaped adaxial epidermis structures was to reduce surface gloss on the petal surface thereby creating an increased visual signal to pollinators (Stavenga et al., 2020). Similarly, the bright colour and distinct velvety effect of *G. aurantiaca* capitula in natural light in the red morphs and to a lesser extent in the orange and yellow colour morphs may be caused by a combination of the localisation of high concentrations of pigments in the upper epidermis and the reduction of gloss by the epidermal structures.

In the absence of a clear answer, this remains an intriguing avenue for further studies. A number of other possible functions of epidermal cell microstructure may be to influence flower temperature, hydrophobicity, petal strengthening to prevent buckling during development, to provide a tactile pollinator guide when orientated towards the source of floral reward, and as a grip for insect pollinators (Antoniou Kourounioli et al., 2013; Barthlott and Neinhuis, 1997; Garcia et al., 2020; Kevan and Lane, 1985; Wilts et al., 2018).

The dark capitulum centres of *G. aurantiaca* which are evident during the female phase of flowering are comprised of tightly packed purple black pappus hairs overarched over the developing anther caps of the hermaphrodite disc florets. The dark centres of brightly coloured, beetle pollinated flowers have been shown to increase visitation by male beetles (Dafni et al., 1990; Van Kleunen et al., 2007). If this is the case for *G. aurantiaca* it may be a strategy to attract pollen-dusted male beetles to visit capitula in the female phase before the hermaphrodite disc florets have emerged and thereby increase the chance of cross-pollination with pollen from other clones in the population.

The results of my preliminary investigation into colour inheritance in *G. aurantiaca*, where crosses between yellow and red colour morphs produce a broad spectrum of intermediate forms exhibiting various shades of oranges, often with uneven colour distribution across the individual ray floret ligules, suggest that colour inheritance may be polygenic, with the F₁ generation being intermediate in colour between the parents (Cantor, 1998). In one investigation into commercial *Gerbera* hybrid colour inheritance, F₁ progeny had the following proportions: 11.1% resembled the maternal genotype, 8.2% resembled the paternal genotype, 72.2% were intermediate and 8.5% did not resemble the parents (Cantor, 1998). These results are similar to my findings with *G. aurantiaca* where the F₁ progeny had the following proportions: 19.3% resembled the parental genotypes and 76% were intermediate (Fig 7 A), suggesting polygenic inheritance of the trait. It is worth noting that the range of capitulum colours in the polymorphic population of *G. aurantiaca* (Fig 5.8) bears a striking resemblance that of the F₁ generation obtained from artificial crossing of red and yellow morphs described here (Fig 5.7 B).



Figure 5.8. Capitulum colour range found in an *in-situ* flower colour polymorphic population of *Gerbera aurantiaca*.

CONCLUSIONS

This study provides an anatomical, structural and genetic context to my studies of the evolution of the flower colour polymorphism in *G. aurantiaca* (Chapters 2, 3 and 4 of this thesis). My examination of the ray floret ligules has revealed unusual features that may contribute to the vivid colouration of *G. aurantiaca* capitula; notably the concentration of pigments, including carotenoids, that are normally associated with mesophyll, located mainly in the upper epidermal cells and the potentially reflectance enhancing cuticular ridges. These longitudinal ridges may account for the velvety effect seen on the ray floret ligules, particularly of the red colour morph and to a lesser extent the yellow morph, when viewed from certain angles in natural light. Further investigations of the reflectance of *G. aurantiaca* ray floret ligules using an integrating sphere which captures most of the light from a sample and offers a more representative measurement may provide a more detailed understanding (van der Kooi et al., 2019). Despite the less intense velvety sheen in the yellow and orange forms, the structure and pigment localisation were similar to that of the red morph, with the colour forms differing only in the presence and amount of anthocyanin pigment in the epidermal cell vacuole. The F₁ generation of the preliminary experiment involving crossing between red and yellow morphs was mostly a spectrum of various combinations of orange, suggesting polygenic inheritance of anthocyanin in *G. aurantiaca*. This may be a situation

where random genetic recombinations are maintained in the absence of colour selection by the dominant pollinators (Johnson et al., 2021). Maintaining experimental crossing lines beyond the F1 generation are required to fully decipher the patterns of colour inheritance in this system.

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CHAPTER 6: CONCLUDING DISCUSSION

Together, results of this study show that despite flower colour in *Gerbera aurantiaca* being genetically based and geographically structured, no clear evidence supports that any of the abiotic or biotic factors investigated, including edaphic factors, climate, pollinators, seed predators and flower community colour context, are likely to underlie evolution of flower colour variation in this species. In sequence, the key findings of each chapter of this study can be summarised as follows: i) *G. aurantiaca* capitulum colour variation has a geographical structure, but abiotic factors do not play a significant role in determining this, and plants grown in a common garden experiment retain their source colours. A non-clinal, mosaic distribution of colour morphs in a flower colour polymorphic population further supports that abiotic factors do not play a role; ii) *G. aurantiaca* is largely self-incompatible, highly pollinator dependent and moderately to severely pollen limited across the populations, by pollen quantity not quality; iii) A single hopliine beetle species, *E. vulpina*, is responsible for most of the fruit set across all colour forms and populations. In populations where this beetle is scarce or absent fecundity is extremely low; iv) Although native honey bees are present in all populations they visit only yellow *G. aurantiaca* colour forms in the yellow monomorphic population; v) Seed predator damage to capitula did not differ between populations, vi) Community flower colour patterns do not differ between populations; vii) Insect vision models confirmed that red morphs are almost invisible to native honeybees but that yellow morphs are conspicuous to them, and that orange, red and yellow morphs are all highly visible to red-wavelength sensitive coleopterans; and viii) Pigments responsible for ray floret colour in *G. aurantiaca* are concentrated in the upper ray floret limb epidermal layer where they are most conspicuous to pollinators while a complex surface microstructure on the ray surface may be important in pollination but its function is currently unknown. In this concluding chapter, I summarize the results of this study and discuss them in the context of the roles of biotic and abiotic drivers of capitulum colour variation in *G. aurantiaca*, the interacting influences of insect pollinators, and suggest a possible hypothetical evolutionary scenario underlying the current geographical colour distribution in this species. I then highlight some implications of the findings of this study for conservation of this endangered daisy species. Finally, I conclude by suggesting areas for future research in the context of the findings of my study.

Summary of results

The primary aim of this study was to investigate the geographic distribution of capitulum colour forms in the endemic grassland daisy *G. aurantiaca* and to identify the factor or factors influencing this colour variation.

In Chapter 2 I documented the geographical structure of capitulum colour variation across the distribution range of *G. aurantiaca*. I found that capitula are predominantly red in the southernmost populations, populations in the central part are monomorphic with yellow capitula, and populations in the northern region show the greatest variation with colour polymorphic capitula in most northern populations. Analysis of the floret colour spectra in the colour polymorphic populations suggested that colour variation is continuous, not discrete, but analysis of inflection points showed cluster in two groups. I found no associations between capitulum colour and several abiotic factors including elevation, mean annual precipitation and temperature and soil chemistry for four monomorphic and two polymorphic coloured populations across the distribution range, suggesting that these abiotic factors they are unlikely to play a role in capitulum colour variation in *G. aurantiaca*. The lack of importance of abiotic drivers of colour variation was further supported by the mosaic, rather than clinal distribution, of different colour variants in a polymorphic population. A common garden experiment confirmed that capitulum colour remained constant when plants with orange, red and yellow capitula plants from different populations were grown under identical conditions, supporting the finding that capitulum colour is not influenced by environmental conditions and is likely to be genetically determined. In terms of biotic influences, a single hopliine beetle species was found to be the most common visitor to all colour forms of *G. aurantiaca* across the range, and did not show any preference for orange, red or yellow capitula in the northern polymorphic population, and fruit set was similar for the three colour groups in this population. The native honeybee *A. mellifera scutellata* was recorded visiting capitula only in the monomorphic yellow population.

Chapter 3: The breeding and reproductive systems of a colour polymorphic plant species may influence patterns of flower colour distribution (Buide et al., 2021; Jiménez-López et al., 2020). Here I report on the results of controlled pollination experiments which explored the levels of self-incompatibility, pollinator dependence and pollen limitation in populations of *G. aurantiaca* across its colour variation and geographical range. I found that *G. aurantiaca* is largely self-incompatible, completely dependent on pollinators for pollen

transport and has varying levels of pollen limitation in populations across its range. Using inter- and intrapopulation hand pollination experiments I established that this pollen limitation is a result of lack of pollen quantity rather than lack of compatible pollen suggesting that insufficient pollen transfer rather than the unavailability of compatible mates currently limits reproductive success in naturally pollinated populations of *G. aurantiaca*. I also confirmed through pollinator observations that the most widespread pollinator across all colour forms is the hopliine beetle *E. vulpina*. It is the only pollinator recorded in any numbers on *G. aurantiaca* red morphs suggesting that this colour form may only be visible to pollinators with long wavelength sensitivity photoreceptors. Honeybees were also important pollinators in the monomorphic yellow population but were not recorded visiting *G. aurantiaca* capitula elsewhere in this study, even though they were present in the local insect communities. The number of capitula damaged by a tephritid fly predispersal seed predator this investigation did not differ significantly between colour forms or populations. I found a strong positive relationship between the abundance of *E. vulpina* beetles and fruit set across all populations. Of concern was the extremely low fecundity in the two southernmost monomorphic red coloured populations where these beetles were scarce or absent, raising concern for long-term ongoing sexual reproduction in these populations.

Chapter 4 explored the possible influence of neighbourhood community flower on *G. aurantiaca* capitulum colour by measuring the spectral colour reflectance of co-flowering species in five populations of differing *G. aurantiaca* colour forms, both independently of insect vision using spectral reflectance curves and inflection points. Using principal components analysis, I found no significant differences between the distribution of spectra for the five study populations, and I found no significant differences in the distribution of inflection points across the visible spectrum for these communities. Using existing insect vision models for the two most important pollinators, the honeybee (*A. mellifera scutellata*, Hymenoptera), and the brown hairy monkey beetle (*E. vulpina*, Coleoptera) to investigate how conspicuous the different capitulum colour morphs are from the other co-flowering species in their respective populations I found that in bee vision, the loci of red colour morphs in all populations were positioned very close to the bee hexagon origin and leaf background and therefore probably not highly visible to bees, while the yellow morphs were positioned at much greater distances from the background and origin and other from the loci of co-flowering species indicating that this colour form is highly visible to bees, while orange forms were intermediate. These findings are supported by in situ observations reported in Chapters 1 and 2 where bees commonly visited the yellow forms in the monomorphic yellow

population but did not visit red colour morphs in any populations. Bees were not observed on yellow morphs in the polymorphic population, but this may reflect the smaller numbers and scattered mosaic distribution of this morph in this population. Conversely, using the coleopteran vision model developed for a red flower pollinating glaphyrid beetle, the red capitula, together with the orange and yellow, were very conspicuous to beetles both against the green background and the coflowering species loci. These results give further support to the field observations presented in Chapters 2 and 3, where I recorded beetles on all colour forms in the populations where they were present, and visiting equal proportions of red, orange and yellow capitula in the polymorphic population, and emphasizes their critical role in the fecundity of *G. aurantiaca* across its range.

Lastly, in Chapter 5, I investigated the basis of *G. aurantiaca* ray floret colour in terms of pigment distribution and petal surface structure using microscopy and anatomical techniques. Spectra showed that the adaxial ray floret limb lacked UV reflectance whether viewed longitudinally or transversely in all colour forms. In all three of the orange, red and yellow colour forms the pigment was concentrated in the upper adaxial epidermal cells. This is a documented phenomenon found in flowers which are only approached by pollinators from above. Anthocyanin pigments were situated in the epidermal cell vacuole for red and orange forms, carotenes appeared to be concentrated in the cytoplasmic layer at the base of these epidermal cells for all colour forms. This arrangement, which concentrates pigments in a focal part of the flower petal, may partly explain the brilliant red, orange and yellow hues of *G. aurantiaca* which is likely to play a role in signalling to potential pollinators. This investigation also revealed a distinct upper epidermal cuticular micro-structuring of regular, longitudinally arranged striations which are likely to be instrumental in creating the distinct velvety effect seen on the ray florets of the red, and to a lesser extent the yellow forms in natural sunlight. A combination of the pigment arrangement and the surface microstructure could be a factor explaining the rich hues exhibited by *G. aurantiaca* capitula. In this chapter I additionally report on the results of a preliminary breeding experiment where I crossed true red and yellow colour morphs and raised the resultant seedlings under nursery conditions. The resulting F1 progeny were predominantly yellow or orange suggesting a polygenic mode of anthocyanin inheritance in the species.

Collectively, the evidence presented in these chapters demonstrate that while extensive investigation has failed to definitively identify any specific abiotic or biotic factor influencing the colour variation in *G. aurantiaca*, pollinators do play an important role in

fecundity and lack of colour preference may contribute to maintenance of colour variation in polymorphic populations.

The role of abiotic and biotic factors in *G. aurantiaca* capitulum colour variation

Despite investigation of numerous candidate factors that have been implicated in driving floral colour evolution in other systems, my study did not identify any single abiotic factor determining the capitulum colour variation found in *G. aurantiaca*. The north-south geographical distribution of *G. aurantiaca* capitulum colour forms across approximately 450km, from predominantly red in the south, yellow in the central part and polymorphic in the north suggested that latitudinal environmental differences may influence colour, but I found no relationship between mean annual precipitation, mean annual temperature or edaphic factors and population colour (Arista et al., 2013; Dalrymple et al., 2020; Schemske and Bierzychudek, 2007; c.f. Strauss and Whittall, 2006). However, since *G. aurantiaca* grows along a fairly narrow altitudinal range (900-1700msl) of sub-escarpment hilly and low mountain terrain of predominantly C₄ grassland in mainly dolerite lithology, with a mesic rainfall (Carbutt, 2023) it was anticipated that environmental abiotic factors such as elevation, mean annual precipitation, and temperature and soil chemistry would be unlikely to influence flower colour on a broad spatial scale. The lack of detectable spatial gradient of capitulum colour in the polymorphic northern populations gave further support to the lack of influence by soil and or moisture factors on flower colouration (c.f. Arista et al., 2013; Schemske and Bierzychudek, 2007). The maintenance of flower colour over several years in common-garden experiments (using both translocated individual and plants raised from seed from monomorphic red and yellow populations) further supported a genetic basis rather than environmental heterogeneity influence for floral colour differences in *G. aurantiaca* (q.v. von Witt et al., 2020).

Interactions between the main pollinator groups and *G. aurantiaca* colour morphs

My investigations of the breeding and pollination systems of *G. aurantiaca* showed that the species is both largely self-incompatible, almost entirely dependent on pollen vectors for seed production, and critically pollen-limited in populations where beetle pollinators are absent. The vital role of *E. vulpina* as a pollinator is evident in the strong correlation between *G. aurantiaca* fecundity and abundance of the beetles in the populations, with seed set critically low in populations where they were scarce or absent. As a pollinator dependent, self-

incompatible and pollen limited species, *G. aurantiaca* is highly susceptible to selection through the pollinator activity and preferences (Ashman and Morgan, 2004). Since *G. aurantiaca* has no detectable nectar or scent, colour and pollen are the main potential attractants for insect visitors which may be under selection. In addition, the flat bowl-shaped capitulum surface may act as a platform for coleopteran social activities which could be an added attractant (Dafni et al., 1990; Goldblatt and Manning, 2011).

Pollinator observation revealed marked differences in the two main insect groups' capitulum colour preferences, with native honeybees only visiting yellow colour morphs in the monomorphic yellow population, while the hopliine beetle *E. vulpina* showed no preference for any colour morph of *G. aurantiaca*, and visited orange, red and yellow capitula equally in the colour polymorphic population.

Here I found that the different colour morphs of *G. aurantiaca* have a strong association with attraction of different insect groups which are their potential pollinators – likely mediated by differences in colour perception among insect groups. Using insect vision models (Chapters 2 and 4 of this thesis), I found that the yellow colour morphs are highly visible to both bees and hopliine beetles, while the red are conspicuous to beetles but not to bees. In the yellow monomorphic population honeybees move rapidly between capitula and collect pollen, while the beetles generally spend longer periods on the inflorescences feeding on pollen and using the capitula for social interactions and resting, where they burrow between the disc florets. Flies, other beetles and solitary bees were also recorded on the yellow capitula but in small numbers. However, the red form was visited exclusively by hopliine monkey beetles, in particular, *E. vulpina* which did not favour any colour form and visited all colours equally in a polymorphic population, and both red and yellow morphs in monomorphic populations. Honeybees did not visit red capitula of *G. aurantiaca* during this study in any populations presumably as the red form is almost indistinguishable from the background vegetation. It seems that the red colour forms are functionally specialist through using colour as a filter, possibly to deter pollen robbing bees. However, fruit set does not appear to be adversely affected by bee visitation in the monomorphic yellow population. Therefore, the yellow colour form appears to have a more generalist pollination system, and the red a more specialised pollination system.

A possible evolutionary scenario for the evolution of colour variation in *G. aurantiaca*

It is important to appreciate that this study has looked a ‘snapshot’ in the evolutionary history of *G. aurantiaca* and cannot evaluate for example historical factors such as climatic changes and associated pollinator and predator population fluctuations which may have influenced flower colour variation in the past (Jacquemin et al., 2020; Oellerton, 2017; Pauw and Hawkins, 2011). Despite the limitations, results of this study can be used to inform speculation on a hypothetical scenario of flower colour evolution in *G. aurantiaca*: one possible hypothesis is that the red capitulum colour form of *G. aurantiaca* was ancestral, since anthocyanins are more often lost than gained (Rausher, 2008), and was pollinated in the past by a now absent red wavelength sensitive insect which preferentially visited the predominantly red coloured capitula in the southern populations (see Cooley et al., 2008; Hopkins and Rausher, 2012). Most of the red populations have a small percentage of orange and yellow individuals suggesting that the potential for anthocyanin loss may be present. The cluster of monomorphic yellow populations in the Ngome Mountains in northern KwaZulu-Natal may have arisen by an event of seed dispersal from northernmost red population at Babanango (in which one yellow clone was recorded during this study) in southern Zululand which is situated only 25km away from of the southernmost monomorphic yellow population on Inhlazatshe Mountain. If the red selective pollinators were absent from this location, or alternatively, if a founder population of yellow individuals became established, bee pollinators could have preferentially visited these forms which subsequently migrated further north to the Ngome Mountains and further (Streisfield and Kohn, 2006).

A similar scenario to that proposed above for *G. aurantiaca*, has been investigated for the red poppy *Papaver rhoeas* in Europe. In *P. rhoeas* colour variation appears to reflect adaptation to a novel bee pollinator in regions into which the species has been introduced, but where the original beetle pollinator of its native regions does not occur. In the Mediterranean region, predominantly non-UV-reflecting red populations (with some red UV-reflecting individuals) are pollinated by glaphyrid beetles, whereas in populations introduced to Central Europe, UV-reflecting red flowered plants have been selectively pollinated by bees and are becoming the dominant form in the area in which the original pollinator does not occur (Martínez-Harms et al., 2020).

The construction of a phylogeography and historical range expansion (Hernández et al., 2022) and investigating the phylogeny of *G. aurantiaca* may help to solve the question of

whether red or yellow colour forms are ancestral to the current populations (Trunschke et al., 2021; Van der Niet et al., 2014).

Conservation:

Gerbera aurantiaca is currently considered Endangered (A2ac) by the South African National Biodiversity Institute (SANBI) using the IUCN Red List, primarily due to near complete transformation of its mistbelt grassland habitat for agricultural use (Scott-Shaw et al., 2005). Many of the remaining populations of *G. aurantiaca* are not in formally conserved areas. The possibility of protecting more of these populations, especially the polymorphic populations in the northern part of the range and providing specific management guidelines regarding grazing, fire frequency and intensity needs to be investigated and implemented. Several potential initiatives can be considered for implementation. Firstly, *G. aurantiaca* can be considered a flagship species for the recently proposed Greater Midlands Centre of Endemism (Carbutt, 2023). This region which consists of areas of endemic species rich old growth mesic grasslands has been heavily impacted by habitat modification and fragmentation and its endemic flora and fauna are currently relatively poorly known. A better understanding of the ecological requirements of the species could simultaneously contribute to improved conservation management of both *G. aurantiaca* in particular and the endemic flora of this region in general. Collection of seed from representative colour populations for *ex-situ* conservation with the Millennium Seedbank Partnership cryopreservation could also play a critical role and needs to be addressed urgently (White et al., 2023). A further, promising possibility is that reintroduction of *G. aurantiaca* into suitable sites may be used as a conservation measure: a pilot reintroduction study into a grassland where the species once occurred indicates that this is feasible, with re-introduced seedlings from a nearby population seed having survived and flowered for over ten years (I. Johnson, unpublished data). Lastly, in the light of critical importance of the pollinating beetle *E vulpina* for reproduction of *G. aurantiaca*, and the current absence of this pollinator in southern populations, both shown in this study, reintroduction of the pollinating beetle *E vulpina* to the southern *G. aurantiaca* populations should be investigated as a means to ensure long-term viability of populations in this region.

Conclusions

This study investigated multiple possible drivers of flower colour variation in a highly variable species yet found no clear evidence for a role of any single biotic or abiotic factor, nor for community context, in explaining colour variation. The evidence described in this study for identifying the factors responsible for flower colour variation in *G. aurantiaca* thus suggests that a complex interaction of influences rather than a single factor is probably at play. The findings presented here are important as they contribute to the increasing recognition that flower colour variation (in both species and communities) is seldom driven by a single influence, but is more often the result of a number of interacting biotic and abiotic factors, both current and historical (Arista et al., 2013; Dalrymple et al., 2020; Narbona et al., 2018; Ortiz et al., 2015), which may be accumulative or antagonistic (Strauss and Whittall, 2006). Studies attempting to disentangle the whole context of flower colour selection in polymorphic species should focus on a wide range of potential influences covering both abiotic and biotic factors rather than focusing solely on pollinator-mediated or abiotic selection (Arista et al., 2013; Dafni et al., 2020; Dalrymple et al., 2020; Narbona et al., 2018; Sapir et al., 2021).

In the light of the combined findings of this thesis, the main areas for further investigations of *G. aurantiaca* colour variation can be summarised as follows:

1. Firstly, application of molecular approaches holds great promise to both understand phylogeography and guide future conservation initiatives for this endangered species. Microsatellite markers have been developed for *G. aurantiaca*, and leaf material has been sampled to address several questions related to both flower colour evolution and aspects relevant for conservation. Analysis of *G. aurantiaca* specimens collected for microsatellite analysis during this study can be used to: i) investigate the historic phylogeography of *G. aurantiaca*, to elucidate whether and how founder effects contributed to the determination of flower colour in the red and yellow monomorphic populations and the flower colour variation in the polymorphic populations, ii) describe the population genetic structure and diversity of the remaining populations in association with flower colour, iii) estimate maximum clone size for an accurate evaluation of effective population size, iv) describe the mating system with molecular markers to confirm whether it is self-incompatible, v) assess whether there is assortative mating, specifically in relation to flower colour, and v) quantify phenotypic selection for several floral traits such as flower colour and capitulum size.

Answering these latter questions will assist conservation agencies to implement conservation evidence-based practices such as the identification of key populations needing protection based on their genetic health and demographics, reintroduction of the pollinator in populations that lack them, and genetic rescue (Basist et al., 2021).

3. Secondly, the study system is ideal to investigate anthocyanin inheritance patterns: For this, more extensive *ex-situ* crossing experiments of true-breeding red and yellow colour forms are required to provide a better understanding of the anthocyanin inheritance patterns in *G. aurantiaca* (Kirby, 2022; Rausher, 2008)
4. Addressing the *Gerbera aurantiaca* pollinator crisis: research into the life cycle, ecological requirements, and reasons for the apparent range contraction of the critically important monkey beetle pollinator *E vulpina* would provide vital information to guide a reintroduction programme to boost reproductive success in plant populations in which the pollinator no longer occurs.



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Plate 2220 *Gerbera aurantiaca*

Gerbera aurantiaca

Asteraceae: Mutisieae

South Africa

Gerbera aurantiaca Sch.Bip. in Flora 27: 781 (1844); Harv.: 523 (1865); J.M.Wood: t. 371 (1905); N.E.Br.: t. 8079 (1906); Dummer: 252 (1914); Hilliard: 590 (1977); H.V.Hansen: 24 (1985).

Gerbera aurantiaca, commonly known as the Hilton daisy, is a beautiful plant endemic to the mistbelt grasslands of KwaZulu-Natal and Mpumalanga. The species is threatened (Scott-Shaw 1999) primarily owing to habitat fragmentation and destruction by agriculture, commercial plantations and urban development. Because of its striking appearance, *G. aurantiaca* is regarded as a 'flagship' species for this threatened habitat type (Johnson 2004). The common name refers to a village near Pietermaritzburg, where it was once so abundant that in the 1900s the boys at Hilton College each wore one of these daisies as a buttonhole on 'Daisy Sunday' in spring (Nuttall 1971). Sadly, few plants remain.

The emergence of its green leathery leaves, initially coated with a web of white felt, in early to mid-September, is sometimes delayed if the spring rains are late. Flowering takes place in October and November when the plants form a dazzling display of brilliant red, dark-centred daisies among the grasses, especially after winter burning. The silky sheen on the ray florets is caused by the longitudinally arranged crested epidermal cells (Figure 1), which enhance absorbance and transmission of light (Baagøe 1977).

Considerable colour variation occurs within populations, ranging from deep red through orange to pink and yellow. This variability in the colour of the ray florets is common in the genus (Dyer 1944; Hansen 1985). *Gerbera aurantiaca* and *G. ambigua* occur sympatrically and form natural hybrids of intermediate colour and leaf morphology. Two of these hybrid clones were described as

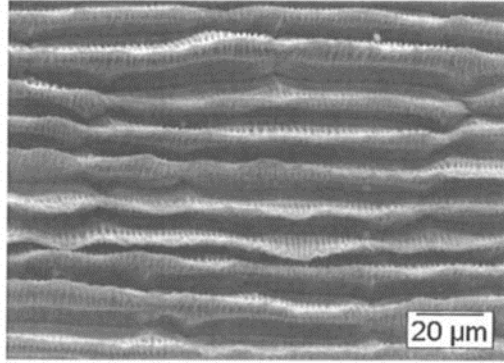


FIGURE 1.—Scanning electron micrograph of the upper surface of the ray floret lip of *Gerbera aurantiaca*.

PLATE 2220.—Habit, showing adult plant with flower heads in bud, in female phase and with mature seed head, $\times 1$. Voucher specimen: *Condy 138* in National Herbarium, Pretoria. Artist: Gillian Condy.

varieties, *G. aurantiaca* var. *alba* and var. *wyliei* (Dümmer 1914), but they are no longer recognised (Hilliard 1977).

Two exclusively yellow-flowered populations, which closely resemble *Gerbera aurantiaca*, were recently found in northern KwaZulu-Natal and southern Mpumalanga and may belong to an as yet undescribed subspecies. Hansen (1985) noted that northern KwaZulu-Natal and Mpumalanga are areas of high variability for the genus *Gerbera*.

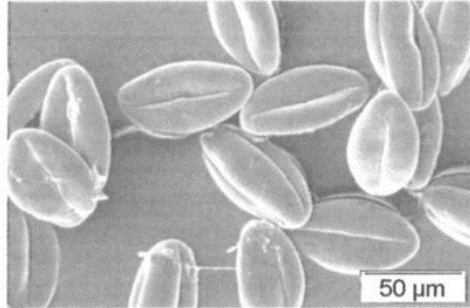


FIGURE 2.—Scanning electron micrograph of pollen grains of *Gerbera aurantiaca*.

Individual flower heads remain open for about three weeks, with the peripheral florets opening first, from the outer female ray florets towards the central hermaphrodite disc florets. This is accompanied by the gradual disappearance of the dark purple centre formed by protruding pappus hairs as the disc florets open and the yellow pollen is presented. When flowering is over, the involucre bracts recurve and the fluffy pappus-topped fruits are exposed for wind dispersal. The leaves turn brown and wither in April and May, and plants remain dormant throughout the dry winter months. Although the flower heads are scentless, at certain times the plants give off a noticeable odour very similar to allspice.

Little is known about the pollination of *Gerbera aurantiaca*, but one of the most frequent insect visitors to the plants is the brown hairy monkey beetle—*Eriesthis vulpina* Burmeister, 1844 (Scarabaeidae: Hopliini)—that feeds on the abundant, large pollen grains (Figure 2). Beetle pollination is common in red bowl-shaped flower heads. The flowers are damaged extensively by a lepidopteron caterpillar that devours the ripening fruits.

The Hilton daisy is known from 12 remaining, widely scattered populations in KwaZulu-Natal and Mpumalanga (Figure 3). The species is confined to Moist Mistbelt Grassland and occurs on warm slopes in shallow, well-drained doleritic soils (Scott-Shaw 1999), at altitudes between 800 and 2 000 m (Netnou 2003).

Southern Africa is the centre of diversity of the genus *Gerbera*, with four of the six sections represented. Section *Gerbera*, with five species, is confined to the Western Cape, while section *Piloselloides* contains one widespread

species, *G. piloselloides*, and *G. cordata* which occurs in the Western and Eastern Cape. Section *Parva*, with one species, is endemic to the Drakensberg in KwaZulu-Natal, and section *Lasiopus*, with species *G. ambigua*, *G. aurantiaca*, *G. galpinii*, *G. jamesonii* and *G. viridifolia*, occurs along the eastern seaboard.

Gerbera aurantiaca is closely related to the well-known Barberton daisy (*G. jamesonii*), and *G. viridifolia*, parents of the *Gerbera* hybrids used extensively in the cut-flower and nursery industries today.

Gerbera aurantiaca was first collected 'near Pietermaritzburg' by Christian Krauss, a prolific collector of plants in South Africa between 1838 and 1840. Brown (1906) wrote that it 'was remarkable that such a beautiful plant should not have been introduced from such a frequented locality (Pietermaritzburg) into cultivation long ago'. He comments on the fact that *G. aurantiaca* (*G. elsa* Hort.) was cultivated in Europe, and that specimens introduced from KwaZulu-Natal flowered at Kew in June 1905. Wood (1905) also reported that the plant was 'well worth cultivation', and that several specimens brought from near Pietermaritzburg were growing in the Durban Botanical Gardens and 'promising well so far'. In 1914, Dümmer stated that 'only two species have gained a hold on popular esteem, *G. jamesonii* and *G. aurantiaca*'. *G. jamesonii* has continued to be used extensively horticulturally, particularly as a parent of *Gerbera* hybrids, but *G. aurantiaca* has disappeared from horticulture. *G. aurantiaca* is notoriously difficult to grow in garden situations (Scott-Shaw 1999).

Plants removed from the wild usually disappear after a year or two in cultivation. Little water is required during the winter months and the plants dislike disturbance. Seeds germinate easily soon after ripening, but lose viability after a few months without refrigeration. Seedling mortality is high in the first year.

The genus *Gerbera* was named by Jan Gronovius in 1737 after Traugott Gerber, a German medical doctor and curator of the oldest botanical garden in Moscow. However, in spite of extensive investigations, no link or reason could

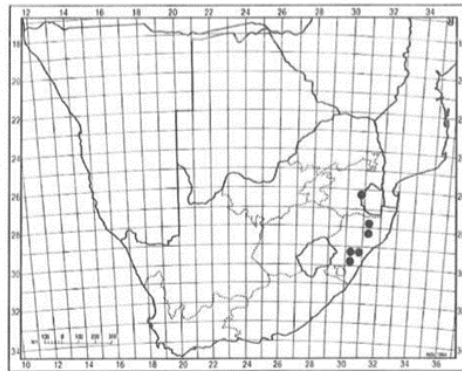


FIGURE 3.—Known geographical distribution of *Gerbera aurantiaca* in South Africa.

be found for the choice of the generic epithet (Ambrosius 2003). The specific epithet *aurantiaca* from the Latin for 'orange yellow' may seem somewhat puzzling, as the flowers are usually brilliant red. This can be explained by the fact that the type specimen collected by Krauss was an orange form of the species, as the original description states that the ray florets are 'pulchre aurantiacae' or 'beautiful orange' (Schultz 1844).

There is no record of ethnic uses of *Gerbera aurantiaca*, although the closely related *G. ambigua* is used by the Zulu people to treat tapeworm and coughs (Hutchings 1996; Pooley 1998).

Description.—Stemless, perennial herb up to 400 mm. *Roots* thick, thong-like, up to ± 3 mm in diameter. *Rhizome* stout, often horizontal, crown white silky-villous, up to 6-leaved. *Leaves* radical, leathery, erect, elliptical, 90–290 \times 20–65 mm; adaxial surface initially cobwebby, with persisting short bulbous-based hairs; abaxial surface villose, with prominent midvein, lateral veins scarcely raised; margin entire to obscurely denticulate, apex broadly apiculate, base cuneate; petiole winged. *Scapes* 1–3 from crown, ebracteate, cobwebby, 1-headed, 120–400 \times 3–4 mm. *Involucre* broadly campanulate, ± 30 mm across; bracts up to ± 55 in 3 or 4 series, lanceolate, 10–15 \times 1.5–2.5 mm, abaxial surface cobwebby. *Capitula* heterogamous, up to 90 mm across rays; receptacle convex, shallowly honeycombed. *Florets* bilabiate, lower lip 3-merous, upper lip 2-merous, corolla tube 6–8 \times 1 mm; pappus purple, setose-barbellate, 6–8 mm long; ovary 3.0–7.5 \times 0.5–1.0 mm, cylindrical, glabrous to strigose. *Female florets* peripheral, in 2 or 3 series; style 9–11 mm long, clavate, bilobed, lobes 0.5 mm long, stigma papillate; staminodes 5, filamentous; outer series radiate; lower limb linear-lanceolate, 15–30 \times 3–4 mm, yellow through orange to crimson above, paler often coppery below, apex minutely 2- or 3-toothed; upper limb 2-merous, linear, 2–3 \times 0.25 mm, tip curled, glandular outside; inner series discoid; lower lip truncate, 2.0–3.5 \times 2 mm, yellow to crimson, 3-toothed, recoiled, glandular; upper lip 2-lobed, linear, 1.5–2.5 \times 0.5 mm, curled, glandular outside. *Hermaphrodite florets* discoid; lower lip truncate, 2.0–3.5 \times 2 mm, yellow to crimson, 3-toothed, recoiled, glandular; upper lip 2-lobed, linear, 1.5–2.5 \times 0.5 mm, curled, glandular outside; anthers 5, syngeneicous, introrse, yellow, black-tipped, 5–6 mm long, caudate, tails ciliate at tip; style clavate, bilobed, lobes ciliate. *Pollen* elliptic, $\pm 50 \times 10 \mu\text{m}$, tricolpate, verrucate. *Cypselae* flask-shaped, 8–10 \times 1.5–2.0 mm, beaked, 5-ribbed, brown, pilose. Plate 2220.

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SUPPLEMENTARY MATERIAL:

CHAPTER 2: Geographical variation in flower colour in the grassland daisy *Gerbera aurantiaca*: testing for associations with pollinators and abiotic factors

The supplementary material for Chap 2 can be found online at

<https://www.frontiers.org/articles/10.3389/fevo.2021.676520/full#supplementarymaterial>

CHAPTER 3: A beetle pollinator reduces pollen-limitation of fecundity in populations of a self-incompatible colour-polymorphic daisy

Table 3S1: Flowers of plant species visited by *Eriesthis vulpina* at *Gerbera aurantiaca* populations

| Species | Family | Human colour | Bee colour | Inflection pts (nm) | Site |
|--|--------------|--------------|------------|---------------------|------------------------|
| <i>Afroaster hispida</i> | Asteraceae | mauve, white | blue-green | 415 | BY, VC, UH, LK, NG, RH |
| <i>Berkheya insignis</i> | Asteraceae | yellow | uv green | 525 | RH |
| <i>Berkheya setifera</i> | Asteraceae | yellow | uv green | 520 | BY, VC, UH, LK, NG, RH |
| <i>Cyperus obtusiflorus</i> var <i>flavissimus</i> | Cyperaceae | yellow | green | 500 | RH |
| <i>Dierama</i> sp. nov. | Iridaceae | pink | blue-green | 385 | NG |
| <i>Dimorphotheca jucunda</i> | Asteraceae | mauve | uv blue | 366 | LK, RH |
| <i>Euryops laxus</i> | Asteraceae | yellow | uv | 320,505 | BY, VC, UH, LK, NG, RH |
| <i>Gerbera aurantiaca</i> | Asteraceae | orange | green | 605 | LK, RH |
| <i>Gerbera aurantiaca</i> | Asteraceae | red | green | 615 | BY, VC, UH, LK, RH |
| <i>Gerbera aurantiaca</i> | Asteraceae | yellow | green | 545 | NG, RH |
| <i>Haplocarpha scaposa</i> | Asteraceae | yellow | uv green | 310 | RH |
| <i>Helichrysum</i> spp. | Asteraceae | yellow | green | 470 | BY, VC, UH, LK, NG, RH |
| <i>Hypoxis</i> spp. | Hypoxidaceae | yellow | uv | 310,520 | BY, VC, UH, LK, NG, RH |
| <i>Leobordea corymbosa</i> | Fabaceae | yellow | uv green | 515 | BY, VC, UH, LK, NG |
| <i>Ocimum obovatum</i> | Lamiaceae | white | uv blue | 400 | BY, VC, UH, LK, NG, RH |
| <i>Senecio glaberrimus</i> | Asteraceae | yellow | uv green | 310,505 | BY, VC, UH, LK, NG, RH |
| <i>S. coronatus</i> | Asteraceae | yellow | uv green | 320,510 | NG |
| <i>Ursinea tenuiloba</i> | Asteraceae | yellow | uv | 310,505 | BY, VC, UH, LK, NG, RH |

CHAPTER 3: A beetle pollinator reduces pollen-limitation of fecundity in populations of a self-incompatible colour-polymorphic daisy

Table 3S2: List of voucher specimens for insect visitors to *Gerbera aurantiaca*

| Species | Author | Family | Collector | Date | lat | long | Locality | Habitat | notes | Housed |
|----------------------------------|------------------|---------------|-------------|-----------|------------|-----------|-----------------------------------|--------------------|---------------------------------------|--------|
| <i>Eriesthis vulpina</i> | Burmeister, 1844 | Scarabaeoidea | I.M.Johnson | 31-Oct-13 | -29.03675 | 30.582486 | Lookout Hill, Lake Merthley, | Dolerite grassland | On <i>Gerbera aurantiaca</i> (red) | UKZN |
| <i>Eriesthis vulpina</i> | Burmeister, 1844 | Scarabaeoidea | I.M.Johnson | 19-Oct-12 | -27.840353 | 31.352402 | Ntendeka grassland, Ngome, | Dolerite grassland | On <i>Gerbera aurantiaca</i> (yellow) | UKZN |
| <i>Eriesthis vulpina</i> | Burmeister, 1844 | Scarabaeoidea | I.M.Johnson | 05-Nov-13 | -26.054611 | 30.371487 | Rooihooqte, Carolina-Badplaas Rd, | Rocky grassland | On <i>Gerbera aurantiaca</i> (red) | UKZN |
| <i>Eriesthis vulpina</i> | Burmeister, 1844 | Scarabaeoidea | I.M.Johnson | 03-Nov-11 | -29.17202 | 30.382780 | Umvoti Heights, Umvoti, | Dolerite grassland | On <i>Gerbera aurantiaca</i> (red) | UKZN |
| <i>Apis mellifera scutellata</i> | Lepeletier, 1836 | Apidae | I.M.Johnson | 20-Oct-11 | -27.840353 | 31.352402 | Ngome | Dolerite grassland | On <i>Gerbera aurantiaca</i> (yellow) | UKZN |
| <i>Apis mellifera scutellata</i> | Lepeletier, 1836 | Apidae | I.M.Johnson | 26-Oct-12 | -29.17202 | 30.382780 | Umvoti Heights | Dolerite grassland | On <i>Afroaster hispida</i> | UKZN |
| <i>Mecistes seriatus</i> | Lefevre, 1885 | Chrysomelidae | I.M.Johnson | 24-Oct-12 | -29.806724 | 30.192574 | Minerva NR, Byrne, KwaZulu- | Dolerite grassland | On <i>Gerbera aurantiaca</i> (red) | EKZNW |
| <i>Mecistes seriatus</i> | Lefevre, 1886 | Chrysomelidae | I.M.Johnson | 05-Nov-12 | -27.840353 | 31.352402 | Ntendeka grassland, Ngome, | Dolerite grassland | On <i>Gerbera aurantiaca</i> (yellow) | UKZN |

CHAPTER 3: A beetle pollinator reduces pollen-limitation of fecundity in populations of a self-incompatible colour-polymorphic daisy

Table 3S3: Honeybee and *Eriethes vulpina* visit times to yellow *Gerbera aurantiaca* capitula at Ngome

| Apis mellifera individual | Seconds per capitulum visit | | | | | | | | | | | | | | | | | | | | | | |
|------------------------------------|------------------------------------|----|----|----|---|----|---|---|---|---|----|----|---|----|---|---|---|---|---|---|---|---|---|
| Bee 1 | 4 | 1 | 3 | 1 | 2 | | | | | | | | | | | | | | | | | | |
| Bee 2 | 3 | 1 | 1 | 2 | 1 | | | | | | | | | | | | | | | | | | |
| Bee 3 | 4 | 1 | 4 | 1 | 1 | 1 | 2 | | | | | | | | | | | | | | | | |
| Bee 4 | 4 | 2 | 4 | 4 | 3 | 4 | 1 | 1 | 2 | 5 | 5 | | | | | | | | | | | | |
| Bee 5 | 2 | 1 | 1 | 2 | 6 | 3 | 3 | 1 | | | | | | | | | | | | | | | |
| Bee 6 | 14 | 2 | 4 | 1 | 3 | 2 | 4 | | | | | | | | | | | | | | | | |
| Bee 7 | 7 | 8 | 1 | 14 | 7 | 10 | 3 | 7 | 7 | 4 | | | | | | | | | | | | | |
| Bee 8 | 4 | 17 | 15 | 7 | 5 | 2 | 2 | 4 | | | | | | | | | | | | | | | |
| Bee 9 | 3 | 11 | 1 | 4 | 3 | | | | | | | | | | | | | | | | | | |
| Bee 10 | 9 | 1 | | | | | | | | | | | | | | | | | | | | | |
| Bee 11 | 8 | 15 | 6 | 15 | 4 | 4 | 4 | 8 | 4 | 7 | 2 | 5 | 7 | 9 | 2 | 7 | 4 | 4 | 2 | 6 | 5 | 8 | 8 |
| Bee 12 | 4 | 4 | 5 | 4 | 9 | 2 | 7 | 4 | 4 | 2 | 6 | 5 | 8 | 8 | | | | | | | | | |
| Bee 13 | 9 | 4 | 2 | 2 | 3 | 2 | | | | | | | | | | | | | | | | | |
| Bee 14 | 3 | 25 | 5 | 6 | 2 | 1 | 4 | 5 | 6 | 4 | 16 | 15 | 3 | 20 | | | | | | | | | |
| Bee 15 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | |
| Bee 16 | 4 | 1 | 3 | 1 | 2 | | | | | | | | | | | | | | | | | | |
| Bee 17 | 3 | 1 | 1 | 2 | 1 | | | | | | | | | | | | | | | | | | |
| Bee 18 | 4 | 1 | 4 | 1 | 1 | 1 | 2 | | | | | | | | | | | | | | | | |
| Bee 19 | 2 | 4 | 4 | 3 | 4 | 1 | 1 | 2 | 5 | 5 | | | | | | | | | | | | | |
| Bee 20 | 2 | 1 | 1 | 2 | 6 | 3 | 3 | 1 | | | | | | | | | | | | | | | |
| Bee 21 | 2 | 10 | 2 | 2 | 1 | 8 | 1 | 4 | 2 | 3 | | | | | | | | | | | | | |
| Bee 22 | 1 | 8 | 4 | 3 | 3 | 2 | | | | | | | | | | | | | | | | | |
| Mean | 4.3 | | | | | | | | | | | | | | | | | | | | | | |
| Eriethis vulpina individual | | | | | | | | | | | | | | | | | | | | | | | |
| Beetle 1 | 10 | 32 | | | | | | | | | | | | | | | | | | | | | |
| Beetle 2 | 60 | | | | | | | | | | | | | | | | | | | | | | |
| Beetle 3 | 5 | 33 | 1 | 1 | 2 | 1 | 8 | | | | | | | | | | | | | | | | |
| Beetle 4 | 13 | | | | | | | | | | | | | | | | | | | | | | |
| Beetle 5 | 2 | 17 | 2 | | | | | | | | | | | | | | | | | | | | |
| Beetle 6 | 20 | 42 | 10 | 4 | | | | | | | | | | | | | | | | | | | |
| Beetle 7 | 4 | 1 | | | | | | | | | | | | | | | | | | | | | |
| Beetle 8 | 15 | | | | | | | | | | | | | | | | | | | | | | |
| Beetle 9 | 15 | | | | | | | | | | | | | | | | | | | | | | |
| Beetle 10 | 72 | 20 | | | | | | | | | | | | | | | | | | | | | |
| Beetle 11 | 4 | 1 | | | | | | | | | | | | | | | | | | | | | |
| Beetle 12 | 28 | 3 | | | | | | | | | | | | | | | | | | | | | |
| Beetle 13 | 26 | 7 | 1 | | | | | | | | | | | | | | | | | | | | |
| Mean | 18.1 | | | | | | | | | | | | | | | | | | | | | | |