The role of pollinators in generating and maintaining floral polymorphism: phylogeographic and behavioural aspects

By

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Abstract

Pollinators play a fundamental role in floral evolution. They can exert selection on the flowers they visit in a plethora of different ways, ranging from innate floral preferences to differences in body size and shape and behavioural elements such as flower constancy and learning capacity. Since different pollinators exhibit differences in these characters, shifts between pollinating species are often considered the most likely drivers of floral diversification. While many lines of evidence support this claim, numerous angiosperms pollinated by a single species also exhibit floral variation. Throughout my thesis, I explore and investigate floral diversification in such species in the absence of pollinator shifts.

In Chapter 2, I investigate variation in the preference of conspecific male and female pollinators for the floral traits of a sexually deceptive daisy that comprises distinct floral forms. I show that its pollinator exhibits gender-specific variation in floral preferences, and that some floral forms have specialized on the male pollinator. This chapter thus illustrates the importance of intraspecific variation in pollinator preference for floral diversification, an underappreciated mechanism in this field of research.

The innate preferences of pollinators are likely to have a genetic basis, especially innate preferences that govern mate choice. Genetic structure within the pollinators of sexually deceptive plants, which mimic female insects to achieve pollination, may thus provide an important source of selection on the plants they pollinate. This depends on an association between genetic divergence and divergent mate preferences, and I explore this intriguing idea in Chapter 3. While pollinators associated with sexually deceptive floral forms did exhibit significant genetic structuring, male pollinators from different phylogeographic clades all exhibited preference for the same sexually deceptive floral form, thus rejecting this hypothesis.

Another behavioural attribute of pollinators that may affect floral evolution, particularly in deceptive plant species, is learning ability. Studies on sexually deceptive orchids often report that male pollinators tend to avoid sexually deceptive flowers with experience. In Chapter 4, I systematically investigate learning abilities within male pollinators and the costs they suffer on sexually deceptive floral forms that vary in deceptiveness. Results reveal a positive relationship between the level of floral deceptiveness and the

associated mating costs that deceived males suffer. Pollinator learning, however, appears to occur only on the most deceptive floral forms, suggesting a link between the costs suffered to the occurrence of learning.

In Chapter 5, I explore the importance of florivory damage in a polymorphic daisy. Studies on floral evolution often overlook the significance of florivorous visits and focus only on pollinator-mediated selection. I show that floral polymorphism is maintained by antagonistic selection exerted by pollinators and florivores on the same floral traits.

Lastly, I focus on evolutionary history to explore similarity in the patterns of South African angiosperm evolution and the pollinator species used throughout my thesis. Molecular dating shows this pollinator exhibits broadly congruent evolutionary patterns to these angiosperms, indicative of a shared biogeography. Taken together, my thesis demonstrates the vast impact of floral visitors, in particular pollinating insects, on the evolution of floral form.

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Chapter 1

Introduction

One of the primary aims of evolutionary biology has been to explore the processes that result in diversification of traits and species (Darwin 1859, Mayr 1942, Carson 1996, Schluter 2001, Johnson 2006, Hoskin and Higgie 2010). With millions of described species on earth, and countless undescribed ones, this remains a critical and active area of research. Angiosperms alone contain over 260 000 extant species (Takhtajan 1997) and often serve as models for the study of evolutionary processes. A defining feature of this group is the incredible variation of floral forms that it exhibits, both between and within species. Since pollinators exert selection on various floral traits like colour (Jones and Reithel 2001, Bradshaw and Schemske 2003), size (Galen 1989, Campbell et al. 1991) and scent (Raguso 2008) they are frequently implicated as potential drivers of this vast diversity (Grant and Grant 1965, Stebbins 1970, Johnson 2006). The preferences of pollinators for these traits varies between species (Schemske and Bradshaw 1999, Bradshaw and Schemske 2003, Ramsey et al 2003, Vereecken et al. 2010), but is assumed to be uniform at the intra-species level. Shifts between different pollinator species are consequently regarded as the main drivers of floral divergence (the pollinator shift model, or Grant-Stebbins model *sensu* Johnson 2006). This model has gained supported from many studies investigating floral variation between closely related species utilizing different pollinators (Steiner et al. 1994, Johnson et al. 1998, Whittall and Hodges 2007, Smith et al. 2008, Van der Niet and Johnson 2012).

Floral variation within species, however, may be a more fruitful area of research as the time since divergence between populations is less and differential selection can be observed directly (Johnson 2006). Although floral variation within species has also been linked to the use of different pollinators (Johnson 1997, Boyd 2004), few experiments explore the adaptive value of divergent traits within these species (Johnson and Steiner 1997), which make the contributions from pollinator shifts difficult to determine. An overlooked challenge to the prevalence of the pollinator shift model comes from floral variation in species that employ one predominant pollinator (Ellis and Anderson 2012). This phenomenon is far from rare (Herrera et al. 2006, Anderson and Johnson 2008, Anderson and Johnson 2009, Ellis and Johnson 2009, Pauw et al. 2009, Schlumpberger et al. 2009, De Jager et al. 2011), which suggests that preferences within pollinating species

may also vary and that pollinator-mediated selection likely operates within a geographic mosaic determined by more variables than simply pollinator type. The consequence of intraspecific variation in pollinator preferences and behaviour for floral divergence, however, remains largely unexplored (Ellis and Anderson 2012).

Throughout this thesis, I explore my overarching theme of floral diversification in the absence of pollinator shifts. I focus mostly on the pollination biology of the self-incompatible annual daisy, *Gorteria diffusa* Thund. (Arctotideae: Asteraceae). This species is one of three in the genus *Gorteria* L. (Roessler 1959). It is endemic to the Succulent Karoo biome of South Africa and flowers from August to September throughout its arid winter rainfall regions. In the Namaqualand region, it exhibits incredible floral variation comprising 14 distinct floral forms (Ellis and Johnson 2009; Figure 1.1). All of these floral forms occupy distinct geographical ranges and a given locality never contains more than a single floral form, except for a few narrow contact zones where hybrids can sometimes by found (Ellis and Johnson 2009). All these forms readily produce hybrids in the greenhouse and appear to be fully compatible with each other (Ellis unpubl. data). Twelve of the 14 floral forms are characterized by dark spots at the base of its ray florets and some forms exhibit remarkably complex spots that contain various specialized cell types (Thomas et al. 2009). In all forms, the entire receptacle becomes lignified after flowering and drops off to act as a single diaspore (Karis et al. 2009) with limited dispersal ability.

All floral forms of *G. diffusa* are pollinated predominantly by a single bee fly species *Megapalpus capensis* (Bombyliidae: Diptera - Ellis and Johnson 2009, Figure 1.2). *M. capensis* is the only species in its genus and is small (5-10mm) and easily distinguished from other bee flies in South Africa by its uniform black and hairless bodies. It appears to have a short lifespan of only a few days and is a very common visitor in Namaqualand during the austral spring where its range and flight times broadly overlaps with the flowering times of *G. diffusa*. It also occurs in reduced numbers in the Fynbos biome to the south where it has previously been implicated in the pollination of several *Pelargonium* spp. within the Geraniaceae family

(Struck 1997). Some of the floral forms of *G. diffusa* have been found to mimic *M. capensis* females as their complex ray floret spots elicit mating behaviour from *M. capensis* males (Ellis and Johnson 2010). This makes *G. diffusa* the first documented angiosperm outside of the Orchidaceae to use sexual deception for pollination.

Apart from *G. diffusa*, I also investigated pollinator and herbivore interactions in another annual daisy in Namaqualand, *Ursinia calenduliflora* (Anthemidea: Asteraceae). This species is part of a genus that is abundant in both the Succulent Karoo and the Fynbos biomes of South Africa. It consists of two floral forms that often co-occur in the arid winter rainfall area of Namaqualand: a plain orange form and a spotted form that contains small black spots inside a band of dark red at the base of its ray florets (Figure 1.3). It bears solitary inflorescences on the end of long peduncles during September to October and can grow in great abundance in favourable conditions. Winged seeds are produced after flowering and are dispersed from the capitulum by the wind. Like the sexually deceptive daisy, *G. diffusa*, it is often visited by the bee fly *M. capensis*, as well as a multitude of other insect species. In fact, *M. capensis* are frequent visitors to various spotted daisies in Namaqualand and may be an important pollinator for many daisies within this area.

Figure 1.1. The incredible diversity of floral forms exhibited by *Gorteria diffusa* in South Africa. Floral forms are a = Khubus; b = Rich; c = Okiep; d = Buffel; e = Oubees; f = Worc; g = Garies; h = Worc; I = Soeb; j = Spring; k = Nieuw; l = Koma; m =Kz; n = Naries; o = Nieuw; p = Cal. Scale bar = 1cm. Image from Ellis and Johnson 2009, *American Journal of Botany* 96: 793-801.

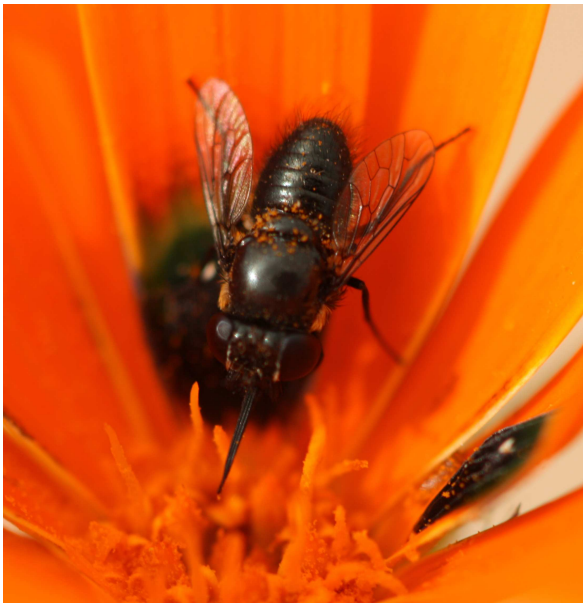

Figure 1.2. The bee fly, *Megapalpus capensis*, on one of the black petal spots of the daisy, *Gorteria diffusa*. Note the *G. diffusa* pollen on its back.

Figure 1.3. The annual daisy *Ursinia calenduliflora* with the two floral morphs in sympatry: the spotted morph contains a red ring at the base of the ray florets with pronounced dark spots while the plain morph only has plain orange ray florets.

Chapter background and objectives

Chapter 2

Since different pollinator species often exhibit different preferences and behaviours, shifts between alternative pollinator types is considered the main source of divergent selection driving floral diversification. However, considerable intraspecific variation in important morphological and behavioural traits has been reported within various pollinating species. This variation typically entails gender-specific preferences for floral colour (Kandori et al. 2003, Temeles and Kress 2003), nectar production (Temeles and Kress 2003, Ne'eman et al. 2006, Alarcón et al. 2010) and floral elaborations (Ellis and Johnson 2010a, De Jager and Ellis 2012). The consequence of such intraspecific pollinator variation on floral divergence has very seldom been considered (but see Temeles and Kress 2003). Here I explore the floral preferences of the bee fly *M. capensis* for floral elaborations in the sexually deceptive daisy, *G. diffusa*. Specifically I ask whether pollinators exhibit gender-specific variation in their preferences for visual, olfactory and tactile floral traits and whether this can act as a driver of floral diversification in species that employ a single predominant pollinator.

Chapter 3

Specialization of plants on their pollinators is a key component of floral divergence (Grant and Grant 1965, Stebbins 1970, Eriksson and Bremer 2002). Within the Greater Cape Floristic Region (GCFR) in South Africa, floral specialization is relatively common compared to the northern hemisphere (Johnson and Steiner 2000) where pollination systems are often generalized (Waser et al. 1996). With over 10000 flowering species in the GCFR, of which more that 70% are endemic (Born et al. 2006), ecological interactions with specific pollinators are often invoked as a potential cause of this diversity (Goldblatt and Manning 1996, Johnson 2006, Anderson and Johnson 2009, Pauw et al. 2009, Ellis and Johnson 2010a, De Jager and Ellis 2012). No studies, however, have explored the impact of the evolutionary history of important pollinators in the GCFR on the plants they pollinate. This may be of great importance for floral diversification in specialized systems if pollinator preferences have a genetic basis. I address the influence of genetic structure

in the widespread bee fly pollinator *M. capensis* on its interactions with *G. diffusa*. This daisy exhibits remarkable geographically structured floral variation and interactions between *M. capensis* and its different floral forms ranges from mutualistic to antagonistic across the landscape, making it an ideal system to study potential associations between pollinator genotype and floral preferences and interactions. Firstly, I determined if phylogeographically distinct flies are associated with different floral forms. I then investigated whether genetically distinct male flies exhibit more mating behaviour on their local sexually deceptive floral forms in order to determine if adaptation to local male preferences has driven floral diversification in sexually deceptive *G. diffusa*.

Chapter 4

Antagonistic interactions between plants and their pollinators often influence pollinator preferences and behaviours (Johnson et al. 2003, Anderson and Johnson 2006). Unrewarding plants that rely on deceptive pollination in particular represent potential costs to pollinators, which form the basis for pollinator learning (Ferdy et al. 1998). Sexually deceptive plants that mimic the mating signals of female insects in order to elicit mating behaviour from conspecific males could hold potentially severe costs to deceived males. This is because not only foraging success, but also mating success is likely affected. Consequently, many field-based studies on sexually deceptive pollination systems have reported evidence of male pollinator learning (Ayasse et al. 2000, Wong and Schiestl 2002, Gaskett et al. 2008). None, however, has investigated the costs that deceived males suffer, or its relationship to the observed rates of pollinator learning. I investigate the various costs suffered by the male pollinators of the sexually deceptive daisy *G. diffusa*. I also study the learning abilities of male pollinators and investigate the link between the costs suffered and the occurrence of learning in natural pollinator populations. I then explore the potential role of such antagonistic coevolutionary dynamics in driving floral diversification within deceptive systems.

Chapter 5

Although the importance of pollinator-mediated selection on floral evolution has been well established (Galen 1989, Campbell et al. 1991, Jones and Reithel 2001, Bradshaw and Schemske 2003), visits by non-pollinating animals may also be of great importance (Strauss and Whittall 2006). Herbivorous insects that damage flowers in particular can exert strong selection on floral phenotypes (Irwin et al. 2003, Cariveau et al. 2004). When pollinators and florivores exhibit preference for the same floral traits this will lead to antagonistic selection that may contribute to the maintenance of floral polymorphism (Strauss and Whittall 2006). Floral polymorphism within species is an intriguing phenomenon that often stimulates evolutionary studies, because directional selection by pollinators (Schemske and Bradshaw 1999, Jones and Reithel 2001) and genetic drift (Wright 1943) are expected to eliminate such polymorphisms (Frey 2004). The composition and abundance of floral visitors throughout a plant species' range, and the various selective pressures each represent may thus be important for the maintenance of floral polymorphism. In this chapter I address the influence of community context on floral phenotype and investigate the relative importance of both pollinator- and florivore-mediated selection on floral polymorphism in the South African daisy, *Ursinia calenduliflora*.

Chapter 6

The ecological interactions of plants with their animal visitors form the bulk of evidence for the important role that pollinators play in floral evolution. Comparative studies on the evolutionary history of pollinators and flowering plants, however, can also offer support for their significance (Dodd et al. 1999, Grimaldi 1999, Mant et al. 2005a, Schiestl and Dotterl 2012). The evolutionary history of flowering plants in the GCFR in South Africa has been extensively investigated (Goldblatt and Manning 1996, Richardson et al. 2001, Klak et al. 2004, Linder et al. 2006, McKenzie and Barker 2008, Verboom et al. 2009). However, no study to date has investigated the genetic structure of a pollinating species within this remarkable area that comprises two biodiversity hotspots: the mesic Fynbos biome and the semi-arid Succulent Karoo biome (Myers et al. 2000). A recurring pattern from the molecular investigations into GCFR flowering plants is ancient Fynbos lineages

with recent radiations in the Succulent Karoo (Verboom et al. 2009). Molecular studies on important pollinating species in the GCFR would be of great value to determine if plants and pollinators show congruent patterns. Such patterns have been observed in North American pollination systems and interpreted as a signal of shared biogeography between plants and pollinators (Smith et al. 2011, Althoff et al. 2012). Of prime importance in the GCFR would be the dates of divergence of pollinating species that are present in both biomes, relative to the divergence dates of flowering plants. Here I address this gap in our knowledge and produce a fossil calibrated phylogeography of the important GCFR pollinator, *M. capensis*, and I explore any congruence between the evolutionary histories of plants and pollinators in this area in the light of shared biogeography.

Chapter 2

Gender-specific pollinator preference for floral traits

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ABSTRACT

Shifts between alternative pollinator types are regarded as the main source of divergent selection underlying angiosperm floral diversification. However, pollinating species can exhibit substantial intraspecific variation, particularly between genders, in key morphological and behavioural traits determining their interactions with flowers. This potential mechanism of floral diversification remains largely unexplored. The bee fly, *Megapalpus capensis*, is the predominant pollinator of the remarkable array of floral forms of the sexually deceptive daisy *Gorteria diffusa.* Flies exhibit strong gender-specific interactions with the variable insect-like spots which characterize *G. diffusa* inflorescences. In order to explore variation in the preferences of male and female pollinators for the visual, tactile and olfactory components of these spots, and its implications for floral diversification, we used a sequence of binary choice tests where we manipulated individual spot components. Male and female flies exhibited contrasting preferences for spot components with females preferring simplistic spots and avoiding UV highlights, whilst males prefer any additional visual and tactile phenotypic complexity. Floral odour alone elicited significant preference in females only, indicating that, in contrast to orchids, sexual deception in *G. diffusa* is achieved largely through visual mimicry of female pollinators. Our results clearly show that elaboration of the insect-like spots has evolved in response to male preferences and suggest that a trade-off exists between the attraction of male and female flies, which may have contributed to the divergence in floral phenotype between morphotypes of *G. diffusa*. Pollinators exhibit gender differences in floral preferences and behaviour, which is another potential source of divergent selection contributing to angiosperm floral diversification.

INTRODUCTION

The use of different pollinator species, which can vary substantially in their floral preferences (Bradshaw and Schemske 2003) and sensory systems (Chittka 1992, Troje 1993), is regarded as the main source of divergent selection underlying the remarkable diversification of angiosperm flowers (Johnson et al. 1998, Whittall and Hodges 2007). However, pollinators also exhibit considerable intraspecific variation,

particularly between genders, in sensory, morphological and behavioural traits relevant to their interaction with flowers that could impose divergent selection on floral traits (reviewed in Ellis and Anderson 2012). Previous studies have reported differences between genders in colour preference and behaviour within Lepidopteran (Rusterholz and Erhardt 2000, Alarcón et al. 2010), Hymenopteran (Ne'eman et al. 2006) and Dipteran (Ellis and Johnson 2010a) pollinators, respectively. In one of the few studies investigating the role of gender-specific selection in floral diversification, Temeles and Kress (2003) revealed that differences in foraging preferences of male and female hummingbirds drive divergence in floral shape and nectar production within the *Heliconia* species they pollinate.

Sexually deceptive orchids that achieve pollination through the attraction and elicitation of copulation attempts from male pollinators only (Schiestl et al. 2003; Mant et al. 2005b), are well-known examples of flowers shaped by gender-specific pollinator-mediated selection. Diversification of sexually deceptive orchid lineages, however, does not result from gender-based differences in their pollinators, as only male pollinators are involved. In contrast, *Gorteria diffusa* Thund., a sexually deceptive African daisy (Ellis and Johnson 2010a), employs both male and female pollinators and therefore offers a unique opportunity to investigate the effect of gender-specific pollinator behaviour on floral divergence. *G. diffusa* exhibits geographically structured floral variation and comprises distinct floral morphotypes, despite being pollinated by a single species of bee fly (Ellis and Johnson 2009). Both male and female flies visit all the *G. diffusa* morphotypes, but only males exhibit mate searching and copulation behaviour on a subset of these morphotypes (Ellis and Johnson 2010a).

Male sexual responses are elicited by black spots on the ray florets of *G. diffusa*, which are required for the attraction of these flies (Johnson and Midgley 1997). These spots vary in complexity and show substantial differentiation between floral morphotypes. In this study, we investigate gender-specific responses to visual, olfactory and tactile components of the spot phenotype. Firstly, we ask which components of spot phenotype influence pollinator preference and behaviour, and secondly whether male and female pollinators exhibit differences in their preference for these components. Should variation in the preference for spot components be gender-specific and the importance of each gender as pollinator vary

between populations, this mechanism likely contributed to floral diversification within this system. If spot complexity is associated with the mimicry of females, we may also expect to find male, but not female, preference for increasing complexity within the spots.

MATERIALS AND METHODS

Study system*: Gorteria diffusa* is a self-incompatible spring flowering annual daisy from the arid winter-rainfall areas of South Africa. It has a prostrate growth form with branches up to 0.5 meters long that carry a profusion of inflorescences (10 - 60 per plant) on individual peduncles. Inflorescences vary in colour from orange through pale yellow and are characterized by black spots at the base of some, or all, of the ray florets. These spots vary in complexity from simple black pigment patches to three-dimensional structures containing specialized epidermal cells (Thomas et al. 2009). Allopatric populations vary substantially in spot phenotype as well as ray floret shape, number and colour, which has led to the description of 14 distinct floral morphotypes, all pollinated predominantly by the bee fly *Megapalpus capensis* Wiedemann (Ellis and Johnson 2009). Although all floral morphotypes induce feeding behaviour in *M. capensis* males and females, five morphotypes induce inspection / mate-searching behaviour predominantly in males whilst three sexually deceptive morphotypes elicit copulation attempts exclusively from males (Ellis and Johnson 2010a). These gender-specific differences in behaviour on *G. diffusa* morphotypes suggest that *M. capensis* males and females exhibit differential responses to the variation in floral traits observed across the range of *G. diffusa* (see Figure 2.1).

M. capensis, like many bombyliids, is a common flower visitor and is regularly seen visiting a range of spring flowering species in Namaqualand. Mating takes place on the open inflorescences of daisies such as *G. diffusa*. Females typically sit within inflorescences and feed whilst males exhibit mate-searching behaviour by flitting between inflorescences, landing on the petal spots and other flies when present. They often exhibit mating attempts on the spots of sexually deceptive *G. diffusa* identical to those exhibited on females (Ellis and Johnson 2010a). Mating attempts with female flies, however, seldom result in copulation; perhaps indicating that females only mate once during their lifetime or are only receptive for a limited

period. Males, however, will repeatedly land on and exhibit mating behaviour towards females along their flight path, as well as in captivity (M. de Jager, pers. obs), indicating that they are likely polygynous and that the first males to mate could experience paternity advantage.

Experimental set-up: In order to determine the influence of various components of spot phenotype on gender-specific pollinator preference we designed a sequential series of binary choice experiments, starting with simplistic spot models and gradually increasing complexity by adding visual, tactile and olfactory components. Spot models were attached with re-useable adhesive to model inflorescences (3 cm diameter orange paper discs) that had a similar reflectance spectrum to the ray florets of the Spring morphotype of *G. diffusa* (Figure 2.4). Each model inflorescence contained a single spot model and was presented 5 cm above ground level. Model inflorescences used in binary choice pairs were placed 3 cm apart and differed only in the specific spot component under investigation. The Spring morphotype of *G. diffusa* was selected as the basis for the complex spot models in our experiments since it has been shown to frequently elicit mating behaviour from *M. capensis* males (Ellis and Johnson 2010a). *M. capensis* individuals naïve to the floral spots under investigation were caught on daisy inflorescences at a site where no *G. diffusa* occurs (S 30, 12, 33.3; E 18, 2, 58.4). Sex was visually determined before releasing the flies individually into a $1m^3$ gauze cage that contained a binary choice of model inflorescences. Each fly was observed for 10 minutes and its preference for spot models in each choice experiment, as well as its behaviour on them, was recorded with a digital voice recorder (Bell Office 600D, Korea). We used flies on the same day we caught them and exposed them to the various binary choice experiments in a random order, generally using each fly for a given experiment only once in order to exclude potential learning. Our experiments were conducted between August and September in 2006, 2009, 2010 and 2011 on warm sunny days between 11am and 4pm when *M. capensis* are most active. Sample sizes for all experiments are reported in Table 2.1.

Visual signals: Black spots on a coloured ray floret have previously been suggested as pollinator attractants in *G. diffusa* (Johnson and Midgley 1997). To confirm this we offered flies a choice between a plain orange model inflorescence and one containing a centrally placed 1 cm diameter matt black paper

spot (Experiment 1). Because *G. diffusa* spots are often raised above the ray floret surface we also offered flies a choice between a plain orange model inflorescence and one containing a centrally placed 5 mm diameter odourless black plastic bead (Experiment 3). Flies potentially perceive two visual components of the raised spot, which may be important for pollinator attraction, the reflective highlight associated with a convex surface and the three-dimensionality itself. In order to tease apart these components we first offered flies a choice between black paper spots (1 cm diameter) painted with either gloss or matt transparent acrylic paint (Experiment 2) and then between a flat gloss spot and a raised odourless black plastic bead (Experiment 4). The gloss-matt choice tested the importance of reflectance off the black surface whereas the gloss-raised choice tested the importance of three-dimensionality, although the nature of reflectance from these two surfaces likely also differed.

Olfactory signals: Floral odour has often been shown to attract pollinators (reviewed in Raguso 2008), especially within sexually deceptive orchids that often employ surface hydrocarbons to attract mate-seeking visitors (Schiestl 2005, Mant et al. 2005b). In order to test its importance within *G. diffusa* we extracted cuticular compounds from the sexually deceptive Spring morphotype of *G. diffusa* by individually submerging spotted ray florets for 4 minutes in 8 mℓ glass vials containing 200µl hexane (C_6H_{14}). Using the odourless black plastic beads from experiment 4 we then offered flies a choice between model inflorescences with a black plastic bead covered in either spotted ray floret extract or pure hexane (50µl each – Experiment 5). To determine if the spots produce any unique compounds that males might be responding to we also offered males a choice between model inflorescences with a black plastic bead covered in either spotted ray floret extract or non-spotted ray floret extract (50µl each). As a control to test whether our extraction method was effective in capturing the putative hydrocarbon compounds that might affect pollinator preference we caught two females of *M. capensis* in copula in the field and extracted them separately in 400µl hexane for 2 minutes (Mant et al. 2005b). We then offered flies a choice between model inflorescences with a black plastic bead covered in receptive female extract or pure hexane (100µl each - Experiment 6). All extracts were applied with a 500 µl SGE LC glass syringe (Supelco, St. Louis, USA).

Tactile signals: The surface cell structure of ray floret spots within sexually deceptive *G. diffusa* morphotypes are complex and contain specialized multicellular papillae (Thomas et al. 2009) which may have a tactile effect on pollinators. To explore the importance of these potential tactile signals we used the protocol of Whitney et al. (2009) to create odourless epoxy casts of spotted and non-spotted ray florets of the sexually deceptive Spring morphotype. These casts captured all the minute details present on the surface of the ray florets at a µm scale, but were odourless and did not include visual differences as we used black pigment to colour all epoxy casts uniformly black. Using these casts we offered flies a choice between a black epoxy cast of a spotted ray floret and a black epoxy cast of a non-spotted ray floret (Experiment 7).

Combination of signals: Previous studies demonstrated that pollinators can use multiple sensory modalities to respond to flowers and that multiple floral traits can differentially affect pollinator choice and behaviour, compared to a single trait (Kunze and Gumbert 2001, Raguso and Willis 2002). We therefore tested the effect of different combinations of spot components on fly preference and behaviour, using the same protocols described above. Since the ray floret spots of many *G. diffusa* morphotypes contain white UV reflective highlights we used Titanium oxide pigment to strategically add white UV reflective highlights to the black epoxy casts of spotted ray florets from experiment 7. We then offered flies a choice between spotted ray floret epoxy casts with or without UV reflective highlights (Experiment 8). Finally, in order to test the combined effect of visual, olfactory and tactile spot components we used the black epoxy casts described in experiment 8 and added the floret odour extracts from experiment 5. Flies were thus offered a choice between a spotted ray floret epoxy cast with UV highlights and either spotted ray floret extract or pure hexane (50µl each - Experiment 9).

Statistical analyses: We ran separate Generalized Estimating Equations (GEE) analyses for each binary choice experiment in order to test for significant differences between male and female preference for the more complex model. We chose to use GEE's, which control for fly identity, since our data are correlated responses. The influence of gender on preference for the more complex spot model was modeled using an

exchangeable correlation structure, which assumes that observations within a subject are equally correlated. We used a binomial distribution with a logit link function to obtain the estimated marginal means and their 95% confidence intervals (CI's) which we back-transformed before plotting. Departure of preference from random choice was confirmed for each gender when the 95% CI's did not overlap with the random expectation of 50% preference for the more complex model. We also ran separate Generalized Linear Model (GLM) analyses for each experiment using only the first choice of each fly as dependant variable, but since these results were qualitatively similar to our GEE analyses we do not report results here. Next we ran GEE's with the same parameters to model the influence of the model type used on the behavioural responses of each gender during our binary choice experiments. We used preference for landing on the spot model attached to the orange model inflorescence, as opposed to landing on the model inflorescence itself, as our dependant variable and ran a single analysis for each gender, grouping all data by the model type used. Analyses were carried out in the SPSS 19 statistical package (SPSS Inc., Chicago, USA).

RESULTS

Visual signals: Although female flies significantly preferred model inflorescences with a simple black spot over those without (Figure 2.2, Experiment 1), they exhibited no preference for visual elaboration of the black spot, whether this involved three-dimensionality or glossiness. In contrast, males showed significant preference for these spot elaborations when contrasted with spotless models or those with a simple matt spot. Results suggest that both reflectance from the shiny black surface (gloss - Experiment 2) and three-dimensionality (raised spot - Experiment 3) contribute to male preference, although preference was stronger for three-dimensionality (Experiment 4). Despite these differences, the preferences of males and females were only significantly different for the choice between a raised spot and no spot at all.

Olfactory signals: In contrast to the visual components, males did not exhibit preference for the floral odour of spotted *G. diffusa* ray florets, whereas females did (Experiment 5). Male flies (n=14) also did not discriminate between spotted and non-spotted ray floret extracts (Goodness of fit Test; $G = 0.05$, *df*

= 13, $p = 0.82$) suggesting that spotted ray florets do not produce any unique compounds relevant to the attraction of male flies. Interestingly, the control for our extraction method revealed significant differences between genders with males, but not females, exhibiting strong preference for the odour extracts of receptive female flies (Experiment 6). This result confirms that our extraction protocol captured compounds involved in male sexual responses.

Tactile signals: When investigating further structural elaboration of the spots with regards to the specialized papillae present within the spot epidermis of some *G. diffusa* morphotypes we again found male, but not female, preference for the more complex models (Experiment 7). Male and female preferences also differed significantly.

Combination of signals: When we combined visual and tactile components by adding UV reflective highlights to the black epoxy casts of spotted ray florets bearing epidermal papillae, we again found significant preference exhibited by males only (Experiment 8). In contrast to the random choices exhibited by females in previous experiments, females significantly avoided models containing UV highlights, indicating a potential trade-off for *G. diffusa* when producing this floral trait. When we combined visual and tactile, as well as olfactory traits, only males showed significant preference for the more complex model bearing all three stimuli (Experiment 9). Again, there were significant differences between male and female preferences.

Although copulation behaviour was not observed on our spot models, male flies exhibited significantly different behaviour from females by landing on various spot models significantly more often than on the orange model inflorescences to which they were attached (Figure 2.3). Exceptions were the most simplistic of spot models (black Matt and Gloss spot), epoxy models of the non-spotted ray florets which do not bear papillae (Non-spotted floret cell structure) and the most complex spot models (Spot cell structure with UV and either hexane or floret odour extract). In contrast, females always significantly avoided landing on the spot models, except for the Raised spot containing female fly odour on which they exhibited no landing preference.

DISCUSSION

Our results show that male and female bee flies exhibit contrasting preferences for most of the spot components of *G. diffusa.* Such divergent selection (or opposing selection in the case of UV reflective highlights) imposed by different fly genders can generate floral diversification within this system without the need for pollinator shifts. Male flies select for any visual elaboration of the spots (including glossiness, raised three dimensionality and UV reflective highlights), whilst females only exhibit significant preference for the most simplistic visual spot models. This result strongly suggests that visual elaboration of spot complexity has evolved under selection exerted by male flies. The fact that most of *G. diffusa*'s morphotypes bear some reflective UV highlights (Ellis and Johnson 2009) suggests that selection through male flies in this system is widespread. Johnson and Midgley (1997) suggested that the UV highlights within *G. diffusa* spots mimic the reflective highlights on the convex thorax of *M. capensis* flies, implying that the attraction of male flies to these UV highlights may be related to mate searching. The presence of UV highlights in sexually deceptive orchids has also been attributed to the visual mimicry of females in order to attract males searching for mates (Gaskett and Herberstein 2010).

In contrast to studies on sexually deceptive orchids where olfaction is the key stimulus used to attract male Hymenopteran pollinators (Schiestl et al. 2003, Mant et al. 2005b) we found female, but not male, preference for the spotted ray floret odour extracts from sexually deceptive *G. diffusa* when used in isolation. Applying floral odour extract in our study also did not elicit copulation behaviour from male flies, suggesting that it does not contain gender-specific pheromonal signals, as is the case in sexually deceptive orchids (Schiestl et al. 2003). We did however find male, but not female, preference for the odour extract of sexually receptive females. This result indicates that there are indeed gender-specific compounds produced by receptive *M. capensis* females which males are responding to, but which *G. diffusa* has not employed in its mimicry of female flies. It should be noted that males did not exhibit mating behaviour in response to the receptive female extracts either. This suggests that male bee flies use signals besides olfaction to detect and react to receptive females, although it is possible that our extraction method did not capture all the relevant compounds dictating male mating behaviour. A previous study on Mediterranean bee flies,

however, found male mating behaviour in response to simple black ink spots (Johnson and Dafni 1998) which strongly suggests that odour signals are less important in governing mating behaviour than in Hymenoptera.

Our experiments investigating the importance of the specialized epidermal papillae structures present within sexually deceptive *G. diffusa* spots also revealed male, but not female preference, indicating that these potential tactile components also evolved under selection from male flies. Studies on sexually deceptive orchids bearing papillae have suggested that these features might help mimic the body textures of females (Blanco and Barboza 2005). Although males did not exhibit mating behaviour on our spot models bearing papillae, they did significantly prefer to land on them during our experiments and therefore must have been able to discriminate them prior to landing. It has been observed that the raised papillae in *G. diffusa* spots possess light reflecting tips that appear similar to the dorsal surface of *M. capensis* flies (Johnson and Midgley 1997), making it possible that this spot component is also involved in visual mimicry of *M. capensis* females.

Interestingly, our experiment combining visual, tactile and olfactory spot components contrasts with our experiment testing olfaction in isolation by finding male preference for the spotted ray floret odour extract of *G. diffusa.* This could be due to a hierarchal system used by flies in decision making, much like Hymenopteran pollinators confronted with the floral traits of sexually deceptive orchids. Streinzer et al. (2009) found that bees are firstly attracted to the flowers of sexually deceptive orchids in the genus *Ophrys* through olfactory signals, but once they are close enough they also use visual signals in decision making. Similarly, *G. diffusa* may also be using multimodal signals to attract and elicit responses from mate seeking *M. capensis* males. Multimodal signals can improve signal detection and performance by simultaneously stimulating different sensory systems in the receiver (Candolin 2003), and the interplay between visual, tactile and olfactory signals may therefore potentially be important for mate location in *M. capensis*. Male flies are likely under strong selection to find a mate quickly, as females often appear to be unreceptive to courting males, and they may therefore employ multiple sensory modalities in the detection of potential mates.

Potential cause and effects of gender-specific variation in pollinator preference

As the UV reflective highlights and the specialized papillate structures within raised *G. diffusa* spots are proposed bee fly mimics (Johnson and Midgley 1997), the preponderance of male preference for these elaborate traits might be linked to mate searching behaviour. Male insects are known to devote more time to mating behaviour than females, which spend more time collecting nutritional rewards related to brood care (Alcock et al. 1978). *G. diffusa* spots might therefore be exploiting various search images that males use to locate potential mates in its mimicry of female bee flies. This is further supported by the fact that males behaved significantly differently from females by consistently landing on elaborate spot models during our binary choice experiments. One benefit of exploiting mate seeking behaviour in males is that they move more between plants than females, which can increase pollen export (Ellis and Johnson 2010a). Increased outcrossing is one of the most compelling hypotheses invoked to explain the evolution of sexual deception in orchids (Scopece et al. 2010) as it can increase the quality of the seeds produced (Peakall and Beatie 1996). Within *G. diffusa*, however, only sexually deceptive morphotypes gain the benefit of increased pollen export from mate seeking male flies (Ellis and Johnson 2010a), which begs the question why non-sexually deceptive morphotypes also bear UV highlights, One explanation might be that most of the *G. diffusa* morphotypes experience selection by mate seeking male flies, which move more between plants than females (Ellis and Johnson 2010a), and may therefore relatively increase outcrossing rates even without the elicitation of copulation attempts. The observed female avoidance of black spot models bearing UV highlights may stem from an attempt to reduce competition, either for food or for potential mates. This result also suggests that females do not aggregate together to form leks, which is supported by the observation that solitary females are typically found within inflorescences.

Female pollinators have also been found to spend more time per flower and move less between plants than males (Ne'eman et al. 2005, Ellis and Johnson 2010a). This behaviour is probably related to differences in energy requirements. Within Hymenoptera it has been noted that females spend more time feeding than males (Alcock et al. 1978) and that they collect more pollen in order to provide for their young

(Michener 2000). Female butterflies have also been found to move less between flower patches than males and to target nectar sources high in amino acids that are probably linked to egg development (Rusterholz and Erhardt 2000). Whereas attraction of male insects can increase pollen export as a consequence of mate searching behaviour, attraction of females may contribute more to pollen import, relative to males, if spending more time on flowers results in greater loads of conspecific pollen. This has been found to be the case with female hawkmoths (Alarcón et al. 2010), as well as female bee pollinators (Ne'eman et al. 2005).

Variation in the effectiveness of male and female pollinators can produce allopatric divergence in floral phenotype when the relative importance of males and females as pollinators varies geographically, but is constant within populations (Ellis and Anderson 2011). One geographically variable factor that can produce this is pollen limitation. Since the importance of selection acting through pollen import has been shown to be highest when pollinators are limited (Ashman and Morgan 2004), selection through pollen export is likely to become disproportionately important under pollinator abundance. Muchhala et al. (2010) found similar results in a modelling study, but also showed that under high visitation rates specialization tends to evolve due to competition acted out through pollen export. Within a sexually deceptive *G. diffusa* morphotype the removal of ray floret spots did not result in decreased fruit set (Johnson and Midgley 1997), suggesting that sexually deceptive morphotypes are not pollinator limited. Under such conditions, selection exerted by mate seeking male flies will be strong as they can increase relative fitness through increased pollen export (Ellis and Johnson 2010a). The evolution of sexual deception will therefore be favoured in these populations, resulting in complex spots specialized on the attraction and elicitation of mating behaviour from male flies. Geographic variation in pollinator limitation might therefore affect the strength of selection exerted by male and female pollinators in different populations.

Another mechanism that could generate asymmetry in the importance of male and female pollinators is variation in the sex ratio of flies between populations. If ratios within a population remain stable over generations, selection exerted by the dominant gender may influence and determine floral phenotype. The presence and importance of alternative pollinators within each population may also have an impact on the effectiveness of a given *M. capensis* gender and the strength of selection it exerts. Some of

the *G. diffusa* morphotypes are visited by insects other than *M. capensis* (Ellis and Johnson 2009), although there is no obvious relationship between their presence and floral phenotype. Lastly, the strength of selection exerted by food seeking female pollinators will likely be affected by the availability and attractiveness of alternative rewarding plant species in the community and thus the degree of reliance of *M. capensis* on *G. diffusa* as a food source. These are some mechanisms by which male and female pollinators can exert differential selective pressures in different populations and highlight the importance of ecological context in the evolution of specialization. Since specializing on different pollinating genders will differentially affect plant fitness, floral diversification within plants pollinated by a single species is also likely to occur and may hitherto have been an overlooked mechanism of floral divergence.

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Table 2.1

Details of the choice experiments to determine the preference of male and female flies for the various spot components present within *G. diffusa*. Experiment numbers are listed with the binary choices flies were exposed to in each, increasing from top to bottom in spot complexity. Along each row the presence (X) or absence (0) of these spot components are indicated for each choice in every experiment. The relevant sensory system investigated in each experiment is also indicated, as well as the sample size of each fly gender we used and the total number of visits they made in each experiment.

Experiment	Spot	Gloss	Raised spot	Spot odour	Female odour	Spot cell structure	UV highlights	Sensory system investigated	*N* (flies) ♂ / ♀	*N* (visits) ♂ / ♀
1. Matt spot	**X**									
No spot	**0**							Visual	11 / 11	26 / 54
2. Gloss spot	**X**	**X**								
Matt spot	**X**	**0**						Visual	43 / 37	194 / 153
3. Raised spot	**X**	**X**	**X**							
No spot	**0**	**0**	**0**					Visual	17 / 15	40 / 30
4. Raised spot	**X**	**X**	**X**							
Gloss spot	**X**	**X**	**0**					Visual	29 / 5	104 / 28
5. Spot odour	**X**	**X**	**X**	**X**						
No odour	**X**	**X**	**X**	**0**				Olfactory	33 / 26	308 / 146
6. Female odour	**X**	**X**	**X**		**X**					
No odour	**X**	**X**	**X**		**0**			Olfactory	24 / 12	74 / 48
7. Spot cell structure	**X**	**X**	**X**			**X**				
Floret cell structure	**X**	**X**	**X**			**0**		Tactile / Visual	21 / 33	162 / 152
8. UV highlights	**X**	**X**	**X**			**X**	**X**			
No highlights	**X**	**X**	**X**			**X**	**0**	Tactile / Visual	30 / 25	170 / 191
9. UV highlights + Spot odour	**X**	**X**	**X**	**X**		**X**	**X**			
UV highlights	**X**	**X**	**X**	**0**		**X**	**X**	Tactile / Visual / Olfactory	12 / 11	78 / 96

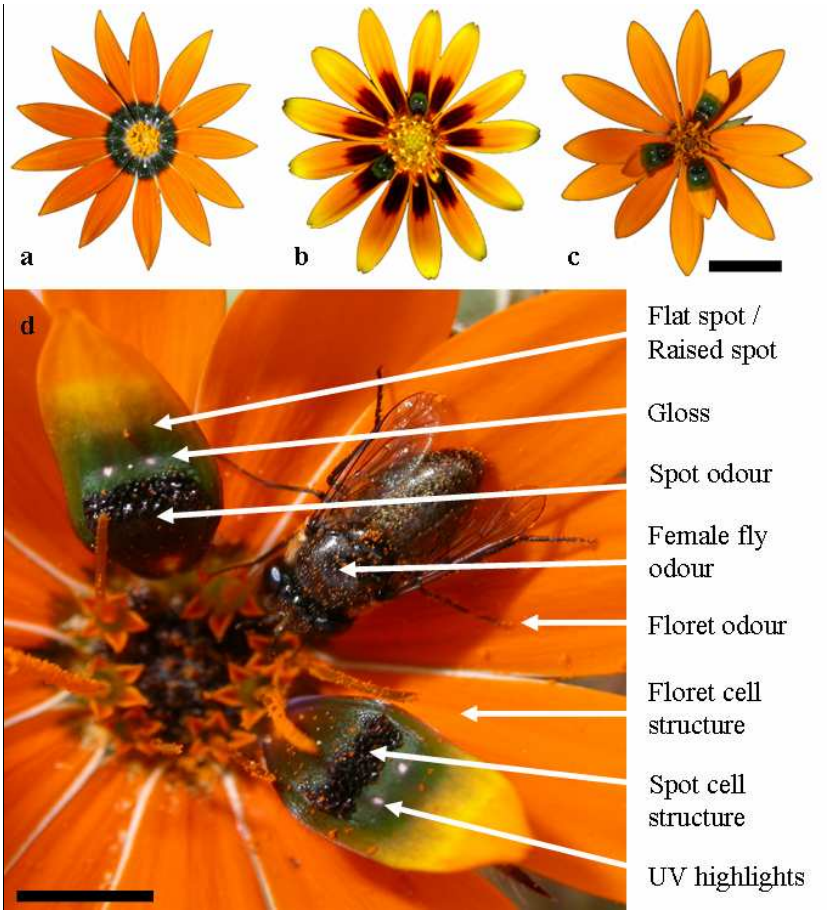

Figure 2.1. Photos *a – c* depict floral morphotypes representing the three functional categories found within *G. diffusa*: a) is a feeding morphotype (Garies) that induces only feeding behaviour in *M. capensis* males and females; b) represents an inspection morphotype (Okiep) which induces feeding behaviour in both sexes, as well as mate searching behaviour from *M. capensis* males and c) a sexually deceptive morphotype (Spring) which induces feeding behaviour in both sexes, but elicits copulation behaviour exclusively from *M. capensis* males. Note that all morphotypes exhibit dark spots at the base of some or all ray florets and that these spots increase in complexity from left to right. d) Shows a close up of the sexually deceptive Spring morphotype in (*c*) with its pollinator, the bee fly *M. capensis*. Arrows indicate the main ray floret spot components that we investigated in this study and includes visual, olfactory and potential tactile components. Scale bar in (c) = 1cm and (d) = 0.5 cm. Photos by Allan Ellis.

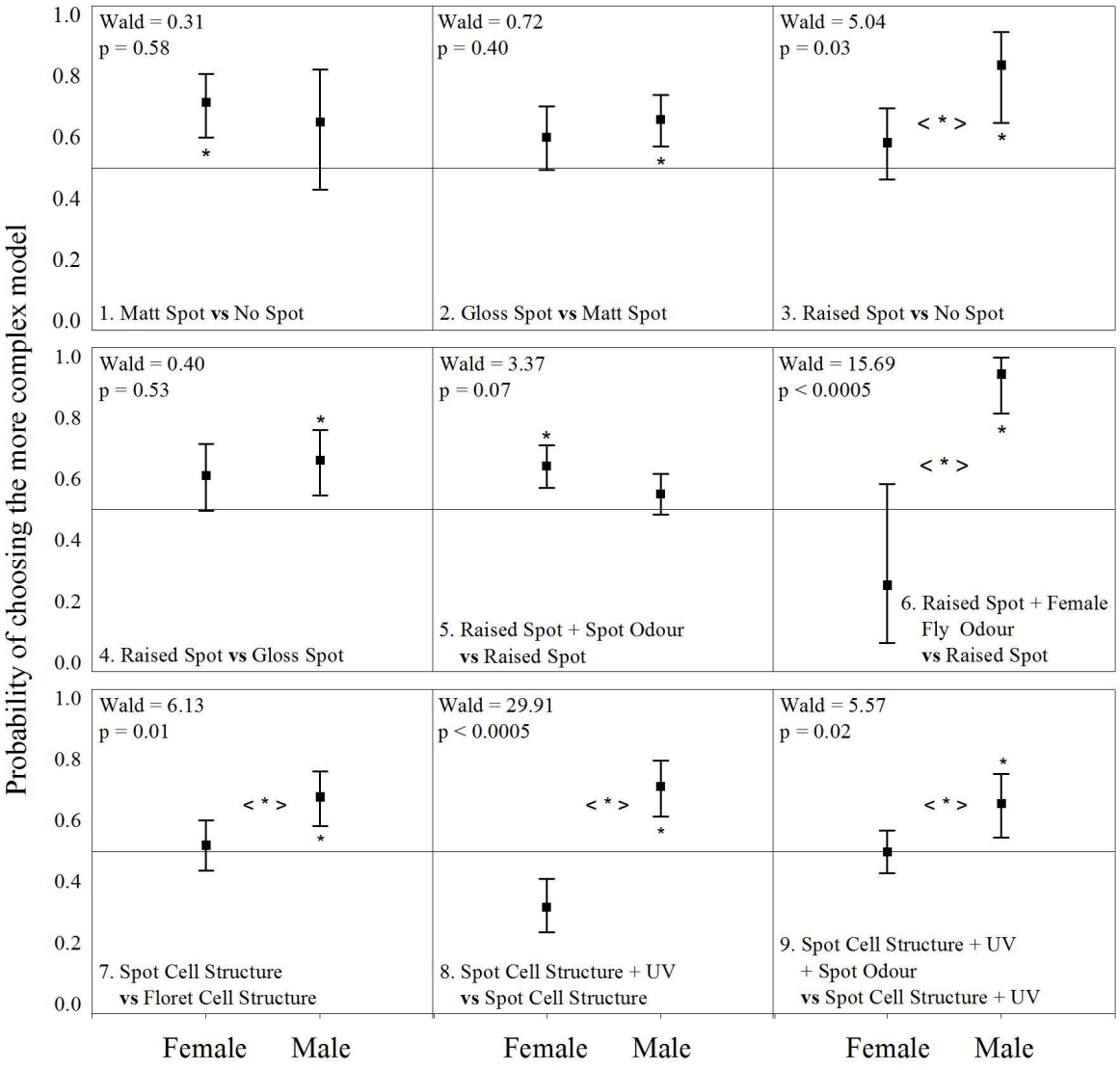

Figure 2.2. Probability that *M. capensis* females and males will choose the more complex spot model (first description in the explanation within each panel) in each choice experiment (experiments 1 – 9, details in methods). Backtransformed marginal means from GEE models with 95% Wald confidence intervals are shown. * indicates significant ($p < 0.05$) preference for one model over the other in male and female flies (i. e. confidence intervals do not overlap with the 0.5 expectation under random visitation). Wald Chi χ^2 and p values are reported for the differences between male and female preference and significance is indicated with < * >.

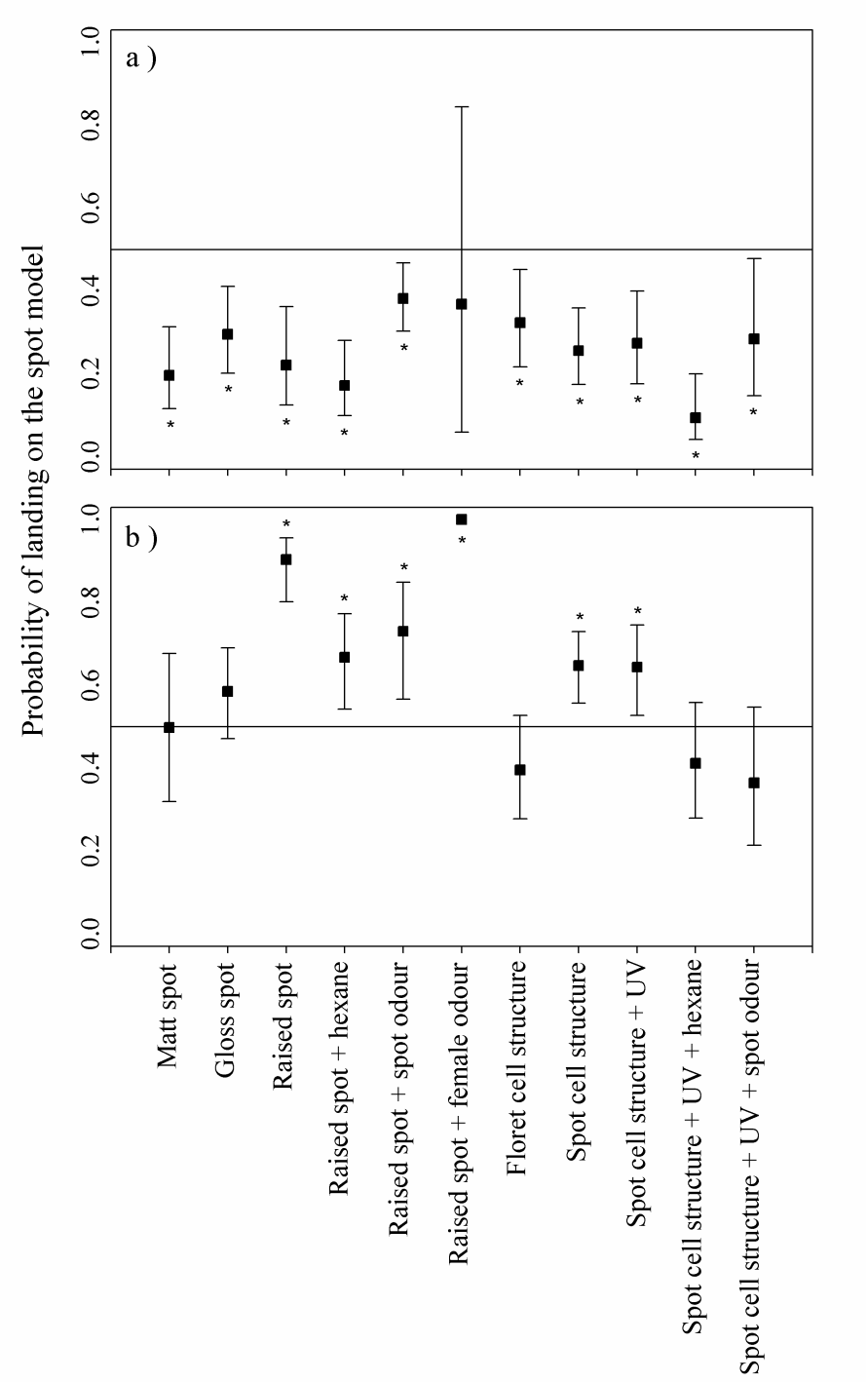

Figure 2.3. Different behavioural responses of a) female and b) male flies to the model inflorescences they visited during our choice experiments. The probability of landing on the spot model attached to model inflorescences, as opposed to landing on the model inflorescence itself,

is indicated. Female flies significantly avoided landing on the spots in most cases whereas males tended to land on the spots. Backtransformed estimated marginal means are plotted with the upper and lower 95% Wald confidence intervals for each model type used. * indicate significant ($p < 0.05$) deviation from the random expectation of equal expression of both behaviours.

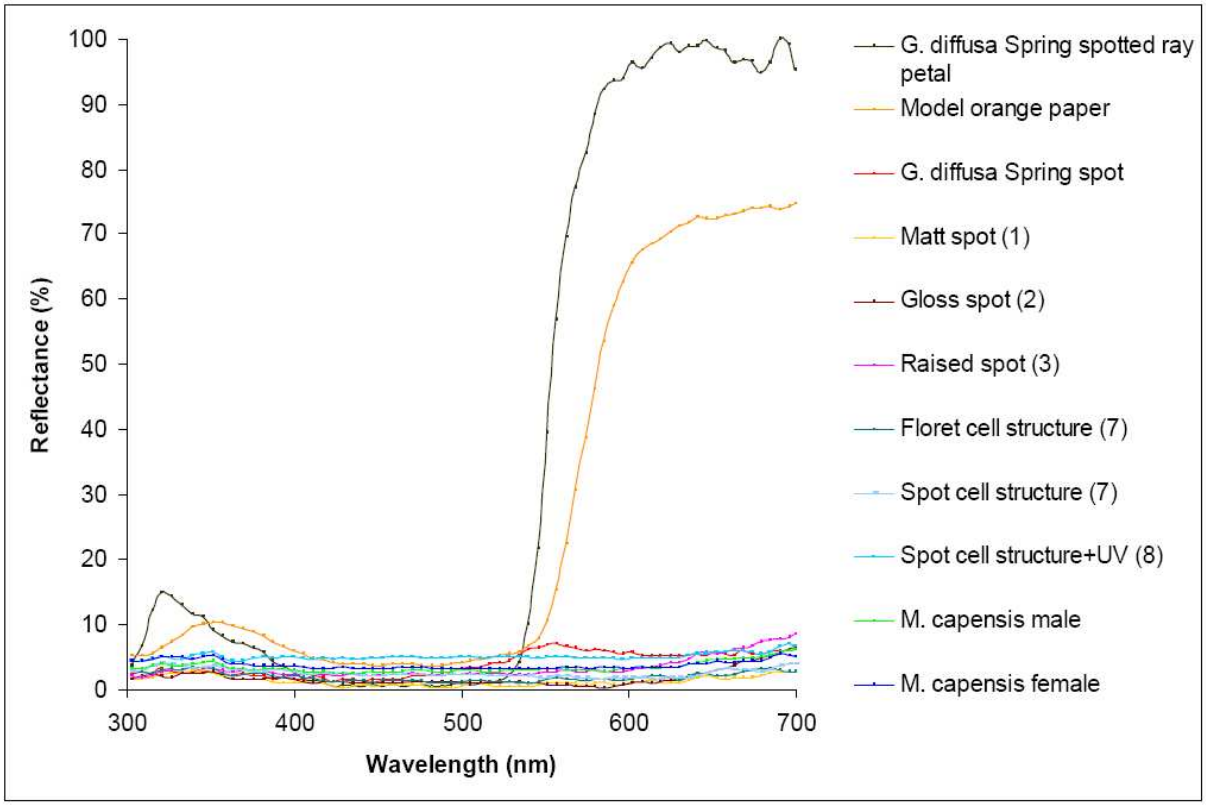

Figure 2.4. Spectral reflectance of the spotted ray petals of *G. diffusa*'s Spring morphotype and the orange paper used as model inflorescences in our experiments. Also shown are the spectral reflectance of the insect-mimicking petal spots of the Spring morphotype and the various spot models used in our experiments, with the experiment number they were first used in shown in parentheses. Male and female individuals of the bee fly pollinator, *M. capensis*, which the petal spots of *G. diffusa* mimics, are also shown.

Chapter 3

The influence of pollinator phylogeography and mate preference on floral divergence in a sexually deceptive daisy

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ABSTRACT

Divergent mate preferences and subsequent genetic differentiation between populations has been demonstrated, but its effects on interspecific interactions are unknown. Associated species exploiting these mate preferences, for example, may diverge to match local preferences. We explore this idea in the sexually deceptive, fly-mimicking daisy, *Gorteria diffusa*, by testing for association between genetic structure in the fly pollinator (a proxy for mate preference divergence) and geographic divergence in floral form. If genetic structure in flies influences interactions with *G. diffusa*, we expect phylogeographically distinct flies to be associated with different floral forms. Flies associated with forms exploiting only feeding behaviour often belonged to several phylogeographic clades, while flies associated with forms exploiting male mating behaviour always belonged to distinct clades, indicating the possibility of pollinator-mediated floral divergence through phylogeographic variation in mating preferences of male flies. We tested this hypothesis with reciprocal presentations using male flies from distinct clades associated with separate floral forms. Results show that males from all clades exhibit similar preferences, making pollinator driven divergence through geographic variation in mate preference unlikely. Males, however, showed evidence of learned resistance to deceptive traits, suggesting antagonistic interactions between plants and pollinators may drive deceptive floral trait evolution in *G. diffusa*.

INTRODUCTION

Adaptation to local environments often results in genetic divergence between populations, at least at selected loci (Galen et al. 1991, Carroll et al. 1997, Quinn et al. 2000). Such local adaptation can facilitate reproductive isolation when, for example, it influences the evolution of mating signals and the perception of signal receivers (e.g. sensory drive – Boughman 2002). Under this hypothesis, the effectiveness of a signal will likely depend on how well it matches the receiver's perceptive abilities and these elements of communication systems will co-diverge between populations experiencing different environments (Boughman 2001, Seehausen et al. 2008).

Genetic divergence in neutral markers typically follows (Seehausen et al. 2008) and may therefore potentially be indicative of divergent mate preferences. Some studies investigating this link within invertebrates have found that preference for local mates over foreign ones is stronger with increased genetic divergence between them (Sutherland et al. 2010). Local mate preference has also been reported in vertebrate taxa comprising genetically distinct groups (Wong et al. 2004, Knight and Turner 2004).

If phylogeographic variation does reflect divergent mate preferences, it could also have interspecific effects by driving phenotypic divergence in closely interacting species that exploit these mating preferences. Sexually deceptive plants in particular present a promising system for investigation of this idea. The flowers of these plants actively mimic female-specific olfactory (Schiestl et al. 2003) and visual (De Jager and Ellis 2012) signals which male insects respond to and achieve pollination through successful elicitation of mating behaviour from mate searching males. These flowers are therefore acting as a sensory trap, exploiting biases within males that evolved outside of a foraging context. Since pollination success is directly correlated with the intensity and frequency of mating behaviour that plants elicit from male pollinators (Ellis & Johnson 2010, Gaskett 2011), sexually deceptive flowers will be subject to sexual selection exerted by males through mate preference. Variation in mating preferences between geographically separated populations of their male pollinators may subsequently lead to divergence in the flowers that are under selection to deceive these males (Mant et al. 2005), explaining why floral phenotypes of some sexually deceptive plants vary across their ranges.

Gorteria diffusa Thund., a South African daisy comprising 14 geographically distinct floral forms (Ellis and Johnson 2009), elicits mating behaviour from its male pollinators with fly-mimicking spots on its ray florets (Ellis and Johnson 2010). The bee fly *Megapalpus capensis* Wiedemann pollinates all of the *G. diffusa* floral forms and is its main and often only visitor throughout the flowering season (Ellis and Johnson 2009). While feeding behaviour in both male and female flies is elicited by all floral forms, only three forms elicit mating behaviour from *M.*

capensis males (Ellis and Johnson 2010), which actively search for female flies and often interact with potential mates within inflorescences (De Jager and Ellis 2012). These sexually deceptive forms are separated geographically and differ significantly in floral phenotype, including the petal spot ornaments with which *M. capensis* males attempt to mate (Ellis and Johnson 2009).

One explanation for this pattern could be that phylogeographically distinct *M. capensis* males from different areas vary in mate preference and thus exert differential selective pressures on their local *G. diffusa* populations, thereby contributing to diversification of the sexually deceptive forms. In order to test this intriguing hypothesis systematically, we firstly investigated genetic structure within *M. capensis* and determined whether genetically similar flies are associated with the same floral forms of *G. diffusa*, and whether this association is stronger for sexually deceptive forms than for the less specialized feeding forms. We then conducted reciprocal presentation experiments with genetically distinct males, which may represent divergent mating preferences, to determine if males exhibit more mating behaviour on the fly-mimicking spots of their local sexually deceptive floral forms. Such a pattern would offer support for local adaptation to male mating preferences driving floral diversification in sexually deceptive *G. diffusa.*

MATERIAL AND METHODS

Genetic sampling & laboratory protocol

M. capensis individuals of both sexes were collected from 36 sites across Namaqualand in South Africa where its range coincides with *G. diffusa*'s (Figure 3.1). In addition, outgroup samples (*Corsomyza* sp. in the Bombyliid subfamily Mariobeziinae with *Megapalpus*) were collected at two locations. Fly vouchers are held in the AGE collection at Stellenbosch University, South Africa. For each fly sample we recorded the local floral form of *G. diffusa* that grows in the area it was caught. All samples were preserved in 95% ethanol and we extracted genomic DNA from each fly following the Promega Wizard Genomic DNA purification kit (Madison, USA) protocol.

Fragments of both mitochondrial (*cox1* and *cox2*) and nuclear genes (*EF1A*) were amplified. A total of 92 samples were amplified for *cox1* with a universal primer pair (LCO1490 5'-GGTCAACAAATCATAAAGATATTGG-3' & HCO2198 5'-TAAACTTCAGGGTGACCAAAAAATCA-3') yielding a 623bp fragment of the gene. We selected a subset of 31 representatives from this dataset (representing all the major *cox1* clades) for the amplification of additional genes, including *cox2* (C457B 5'-AACTAGTATCCTTTCATGAYCAYGC-3' & C457C 5'-GTGATTAGCACCGCARATYTC-3') which yielded a 478bp fragment and the intronless nuclear *EF1A* gene (EF-F05 5'-CCTGGACATCGTGATTTCAT-3' & EF-F06 5'-TTACCTTCAGCGTTACCTTC-3') which yielded a 303bp fragment of DNA.

Every 50µl PCR reaction contained 1.5mM $MgCl_2$, 1µM of the forward and reverse primers each, 0.2mM of each dNTP, 1 unit Taq polymerase (Super-Therm JMR-801; Southern Cross Biotechnologies), 5µl 10X Buffer (Southern Cross Biotechnologies), 25µl dH_2O and about 100ng (1µl) of DNA template. For the PCR amplifications we used the following thermal regimes (*cox1 / cox2 / EF1A*): a denaturation step of 94°C for 5 min / 4 min / 3 min followed by 30 cycles of 94°C for 1 min / 30 sec / 30 sec, 50°C for 30 sec/ 50°C for 1min / 45.9°C for 30 sec and 72°C for 1 min / 2 min / 90 sec. All were followed by a final elongation step at 72°C for 5 min / 10 min / 10min. Amplifications were performed on a Labnet Multigene gradient PCR thermal cycler (Sigma-Aldrich, St. Louis, USA). PCR products were stained with ethidium bromide and run on a 1% agarose gel for confirmation under UV- light. Products were purified using QIAquick®spin columns (Qiagen, Valencia, USA) and sequenced in the forward direction only with a BigDye terminator kit version 3.0 (Applied Biosystems, Foster City, USA) and analyzed on a ABI 3130XL Genetic Analyzer (Applied Biosystems, Foster City, USA) as preliminary sequencing in both directions yielded clean and unambiguous sequences. Any ambiguous sites in the nuclear dataset were coded using the IUPAC codes. Trace files were imported into MEGA v.4 (Tamura et al. 2007) and edited by hand before alignment with ClustalW (Thompson et al. 1994).

Phylogeographic analysis

We conducted two analyses, one using only the *cox1* dataset (*Cox1*) and the other using only the representatives that had been sequenced for both mitochondrial genes, as well as the nuclear gene (*Combined*). Using the Akaike Information Criterion (Akaike 1974) as implemented in jModelTest v0.1.1 (Posada 2008) the HKY+I+G model of sequence evolution was selected for the *Cox1* analysis. The dataset was partitioned into 3 codon positions with substitution rates, rate heterogeneity and base frequencies unlinked across codon positions and run with a strict molecular clock and an uncorrelated lognormal relaxed molecular clock using a mean substitution rate of 1.7% sequence divergence per million years for both clock runs (*Drosophila* mtDNA – Brower et al. 2004). Bayes factors were used to determine which model performed best (with 2ln $BF_{10} \geq 2$ indicating positive support for model 1 over model 0 - Nylander et al. 2004). We also ran the best model without partitioning by codon positions and compared it with the partitioned model using Bayes factors.

For the *Combined* analysis we partitioned the various genes and selected the HKY+I+G, HKY+G and HKY+G models for the mitochondrial (*cox1*, *cox2*) and nuclear (*EF1A*) genes, respectively, as determined by AIC. We partitioned each gene by the 1^{st}+2^{nd} and 3^{rd} codon positions and ran it with an uncorrelated lognormal relaxed molecular clock using a mean substitution rate of 1.7% for the mitochondrial genes and 1.1% for the nuclear gene (*Drosophila* nuclear DNA – Tamura et al. 2004). We also ran this model without partitioning by codon positions and used Bayes factors to determine which model performed the best given our data. MCMC were run for 20 million (*Cox1*) and 60 million (*Combined*) generations in BEAST v1.5.3 (Drummond and Rambaut 2007), sampling parameters every 2000 or 6000 states, respectively. Starting trees were randomly generated and a constant size coalescent prior was selected for all tree models. Results were checked in Tracer v1.4 (Rambaut and Drummond 2007) for reliable effective sample sizes and convergence of MCMC likelihoods. We ran the preferred models

identified with Bayes Factors twice and combined results with Log Combiner v1.5.3, discarding the first 10% of samples as the burnin phase in each case. Resulting trees were summarized with TreeAnnotater as part of the BEAST package and viewed and edited in FigTree v1.3.1 (Rambaut 2006).

Association between M. capensis phylogeographic clades and G. diffusa floral forms

In order to determine whether flies associated with the same *G. diffusa* forms grouped together genetically we employed randomization tests where we shuffled the floral forms associated with our fly samples across the tips of our *Cox1* tree (see Figure 3.2). Only flies associated with a *G. diffusa* floral form were used, and only floral forms associated with more than one fly sample were shuffled. We randomized this dataset for 1000 iterations in Excel. During each we calculated, for every floral form, the percentage of associated flies that fell within each of the clades (determined in our phylogeographic analysis as all samples sharing a common ancestor within each of the main Namaqualand groups), controlling for sample size differences in the number of flies caught within the range of every floral form. For every floral form we then collected during each iteration the maximum percent clade membership (MPCM - the percentage of flies that belonged to the fly clade most frequently associated with that floral form) to create a null distribution of the expected probability of each MPCM category in 10% increments. Significant deviation from random association was determined using one-tailed tests (i.e. flies within the range of a given floral form exhibit significantly higher MPCM than expected). We also calculated the mean of MPCM categories across all floral forms from randomizations and compared this to the observed average MPCM of flies associated with sexually deceptive floral forms that mimic female mating signals and feeding floral forms that do not.

In addition to our randomization tests, we ran an AMOVA (Excoffier et al. 1992) in Arlequin 3.10 (Excoffier and Schneider 2005) on our only dataset with sufficient sample size (*Cox1*), using sampling sites as populations to determine whether genetic variation of the flies

was structured by the local floral form present in each area (i.e. most genetic variation is found between flies associated with different floral forms). Fly samples that were not associated with *G. diffusa* were excluded and all samples were grouped by the floral form they were associated with.

Local adaptation experiments

To test if variation in pollinator mate preferences can drive the divergence of sexually deceptive floral phenotypes we investigated whether phylogeographically distinct male flies associated with different sexually deceptive floral forms exhibit more mating behaviour on the fly-mimicking spots of their local floral form. We employed reciprocal presentation experiments where we exposed male flies from two different clades to two floral arrays in random order; one containing a local floral form they are familiar with and the other a foreign form they had not encountered before. We used the Spring and Nieuw floral forms (Figure 3.1) which are widely separated geographically (over 150 km) and differ significantly in floral phenotype, including their fly-mimicking spots (Ellis and Johnson 2009). *M. capensis* males from both these areas regularly exhibit mating behaviour in response to these spots (Ellis and Johnson 2010a).

Male flies were caught close to the towns of Springbok (S 29 39 14.5; E 17 53 20.9, N = 16) and Nieuwoudtville (S 31 22 46.8; E 19 5 38.1, N = 15 - top and bottom stars in Figure 3.1). We released each male into a $1m^3$ pollinator cage containing a floral array composed of 12 fresh inflorescences (replaced as necessary) of a given floral form for 10 minutes, before resting it for at least 10 minutes and exposing it to the floral array of the second floral form for 10 minutes. We recorded how many visits each male made to the inflorescences within each array, as well as their behaviour during these visits. Behaviour was categorized as sitting (inactive or grooming), feeding (actively consuming pollen or nectar with an extended proboscis) or mating. Mating behaviour was only exhibited on the fly-mimicking spots and is composed of various motor responses including; *inspecting* (quick landings on spots < 1 second), *changing* (flitting between different spots within an inflorescence), *hopping* (repeatedly hopping and arching abdomen

downwards on the spot) and *turning* (rotating on the spot). We also recorded the amount of time males spent exhibiting the various behaviours during each visit, as well as whether they landed on the fly-mimicking spots when visiting the inflorescences of a given array. We compared all results of individual male fly behaviour between the two floral arrays using paired T-tests or Wilcoxon Matched Pairs Test, depending on the normality of the data.

In addition to these reciprocal presentation experiments with experienced males from within the ranges of the Spring and Nieuw floral forms, we also used the same experimental design to investigate males from an area between their respective ranges where no *G. diffusa* occurs (S 30, 12, 33.3; E 18, 2, 58.4, $N = 10$ – middle star in Figure 3.1). To these males, both the Spring and Nieuw forms are foreign and this experiment thus investigates the innate preference of naïve males for the female fly cues which *G. diffusa* mimics. For this experiment, we also employed paired T-tests / Wilcoxon Matched Pairs Tests. Using naïve males in an identical experimental design also offers us the opportunity to investigate any putative differences between experienced and naïve males regarding their response to sexually deceptive inflorescences. To do this we compared the pollination behaviour and time spent per visit exhibiting these behaviours between naïve and experienced males from different areas on both the Spring and Nieuw forms using T-tests for independent samples or Mann Whitney U tests where applicable. We used the SPSS 19 statistical package for all analyses (SPSS Inc., Chicago, USA) and conducted all experiments at the Succulent Karoo Knowledge Centre in Kamieskroon (S 30, 12, 20.6; E 17, 56, 12.1) and in Nieuwoudtville (S 31 22 46.8; E 19 5 38.1) during August and September 2009 and 2010 on warm sunny days.

RESULTS

Phylogeographic analysis

Within the *Cox1* dataset (containing 92 *M. capensis* sequences from 36 locations) 15.41% of characters were Parsimony-Informative. Bayes factors indicated that the uncorrelated lognormal

relaxed molecular clock analysis performed better than both the strict clock analysis ($2\log_e BF_{1-0}$ = 5.29) and the uncorrelated lognormal relaxed molecular clock without partitioning by codon position analysis ($2\log_e BF_{1-2}$ = 11.02). Based on our Bayesian analysis of the *Cox1* dataset *M. capensis* is monophyletic and separates into three main Namaqualand clades (Northern, Central and Southern), as well as a fourth basal group sister to these clades, which comprises samples from a single population along the Namaqualand coast (Figure 3.2). For the *Combined* dataset, (containing 31 *M. capensis* sequences from 25 locations) 9.13% of the *cox1* characters, 9.83% of the *cox2* characters and 5.94% of the *EF1A* characters were Parsimony-Informative. The uncorrelated lognormal relaxed molecular clock analysis with partitioning by 2 codon position also performed better than the same analysis without partitioning ($2\log_e BF_{1-0}$ = 9.24). From the *Combined* analysis we retrieved the same clades as with the *Cox1* analysis with better nodal support (Figure 3.3).

The Northern clade of *M. capensis* flies in our *Cox1* analysis was associated with the Rich, Spring, Garies, Naries, Okiep and Koma forms of *G. diffusa*, some of which occur in northern Namaqualand (see Figure 3.1). The Central clade was associated with the Soeb, Garies, Naries, Koma, Buffels, Cal and Okiep forms, which mostly occur in central Namaqualand, including the coastal plain to the west. The Southern clade was associated only with the Nieuw form, which occurs in a wide distribution within southern Namaqualand. The association between the phylogeographic pollinator clades from our *Cox1* analysis and the floral forms of *G. diffusa*, however, was clearly not absolute, as flies associated with four of the ten floral forms belonged to more than one clade of *M. capensis* (see Table 3.1 for the number of flies analysed for each floral form). Our randomization tests revealed that flies associated with two sexually deceptive floral forms and two feeding forms showed significant genetic structuring by exhibiting a higher observed maximum percent clade membership (MPCM) than expected under random association (Table 3.1). It is, however, important to note that for all sexually deceptive floral forms the flies caught within their respective ranges belonged to single clade (i.e. MPCM = 100%), while this

only occurred for less than half of the feeding forms. The average observed MPCM of flies associated with sexually deceptive *G. diffusa* also exhibited overall significant genetic structuring (Table 3.1). All flies associated with the sexually deceptive Spring form belonged to the Northern Namaqualand clade, while all flies associated with the sexually deceptive Buffels and Nieuw forms belonged to the Central and Southern Namaqualand clades, respectively (Figure 3). Male flies from these three clades were chosen to be used in our reciprocal presentation experiments and from here on we refer to them as "Spring", "Buffels" and "Nieuw" clade males, respectively.

The AMOVA analysis revealed that genetic variation was significantly structured by the floral form with which flies were associated, with 29.07% of the variation found among groups (F_{CT} = 0.291, p < 0.001). Other major sources of genetic variation were found among populations within groups at 28.39% (F_{SC} = 0.400, p < 0.001) and within populations at 42.53% (F_{ST} = 0.575, p < 0.001).

Local adaptation experiments

Results from our experiments investigating the preferences of genetically distinct *M. capensis* males for the fly-mimicking spots of *G. diffusa* revealed that there is no difference between phylogeographic clades. Experienced males from both the "Spring" (Wilcoxon Matched Pairs Test Z = 2.98, df = 15, p = 0.003) and "Nieuw" (Z = 2.47, df = 14, p = 0.013) clades exhibited significantly higher proportions of total visits including mating behaviour on the Spring floral array (Figure 3.4 a & b). In addition, naïve males from the "Buffels" clade also exhibited significantly higher proportions of mating visits on the Spring array (Z = 2.55, df = 9, p = 0.011 - Figure 3.4 c), indicating that the Spring floral form is more effective at exploiting innate mating preferences of *M. capensis* males and that these preferences are essentially the same throughout its range in Namaqualand. Experienced "Spring" and "Nieuw" clade males also spent significantly more time per visit exhibiting mating behaviour on the Spring array (Z = 2.90, df = 15, p = 0.004; Z = 2.12, df = 14, p = 0.034, respectively), despite making the same number of

visits to each array (Table 3.2). In line with the Spring form being a more successful fly-mimic, "Nieuw" clade males landed on the fly-mimicking spots of Spring more often ($Z = 2.50$, $df = 14$, $p = 0.012$).

"Nieuw" clade males, however, spent significantly more time per visit being active on, and feeding from the Nieuw form ($Z = 2.56$, $df = 14$, $p = 0.011$, $Z = 2.61$, $df = 14$, $p = 0.009$, respectively). The naïve males from the "Buffels" clade mirrored this pattern and spent more time per visit being active on and feeding from the Nieuw form ($Z = 1.99$, $df = 9$, $p = 0.047$, $Z = 2.09$, $df = 9$, $p = 0.037$, respectively), suggesting that Nieuw is relatively better at eliciting a feeding response from male flies and may be a better food source. In contrast to the similar number of visits made by experienced males from the "Spring" and "Nieuw" clades to the two floral forms, naïve "Buffels" clade males made almost four fold as many visits to the Spring array (Paired T-tests $t = -2.54$, $df = 9$, $p = 0.032$). During visits they also spent more than five times as much time exhibiting mating behaviour on the Spring array ($Z = 2.67$, $df = 9$, $p = 0.008$). This result confirms that the Spring form is clearly more effective at eliciting mating responses from *M. capensis* males in all clades.

Our results also revealed that there are significant differences between the behaviour of naïve males and males experienced with the fly-mimicking spots of *G. diffusa*. Experienced males landed on the deceptive spots of the Spring form significantly less often than naïve males (Mann Whitney U-test $U = 37$, $df = 25$, $p = 0.023$ – Figure 3.5). They also exhibited mating behaviour in much lower proportions of all visits ($U = 14$, $df = 25$, $p < 0.001$), and spent less time exhibiting mating behaviour per visit than naïve males ($U = 29.0$, $df = 25$, $p = 0.007$). This is despite the fact that they did not exhibit any differences in the total number of visits they made, or the amount of time they were active per visit on the two arrays (T-test $t = 1.45$, $df = 25$, $p = 0.159$, Mann Whitney U-test $U = 46$, $df = 25$, $p = 0.073$, respectively). In contrast, there was no difference between naïve and experienced males on the Nieuw form for any of these measures,

indicating that this form does not induce putative learned avoidance of mating behaviour within *M. capensis* males.

DISCUSSION

Our results show that *M. capensis* flies within Namaqualand fall into three well-supported phylogeographic clades. The association between genetically similar flies and *G. diffusa*'s floral forms was not absolute, as flies associated with four of the ten floral forms we investigated belonged to more than one clade. Such patterns of incomplete association between plants and their pollinators are often reported within the well-studied yucca-yucca moth system (Leebens-Mack and Pellmyr 2004, Smith et al. 2009). Flies associated with sexually deceptive floral forms that elicit mating behaviour from male flies, however, exhibited overall significant genetic structuring. This pattern may suggest a role for phylogeographic variation in male mating preferences as a driver of floral divergence between sexually deceptive floral forms.

Our behavioural experiments clearly rejected this hypothesis as male flies from three distinct clades all exhibited significantly more mating behaviour on the Spring form of *G. diffusa*. These results indicate either that there is no geographic variation in male mating preferences within *M. capensis*, or that *G. diffusa*'s mimicry of *M. capensis* females has not responded to this level of variation. This latter explanation seems somewhat unlikely, as sexually deceptive *G. diffusa* are under selection to elicit copulation attempts from male flies that consistently result in significantly higher levels of pollen export compared to non-mating male or female visits (Ellis and Johnson 2010a). Attempted copulation with fly-mimicking spots will only occur once males have selected the "female" (deceptive spot) on which to focus their efforts. Since the deceptive spots of the Spring form of *G. diffusa* elicits strong mating responses from males from all three of the *M. capensis* phylogeographic clades, any variation not mimicked by the Spring form is likely to contribute little to overall mate preference within *M. capensis* males.

Our results thus suggest that neutral genetic divergence within species does not necessarily indicate the potential for divergent mate preferences, and that mate preference within *M. capensis* males are uniform, although experiments with real females are required for confirmation. Some studies have reported that preference for local mates over foreign ones only occurs after considerable genetic divergence between them (Sutherland et al. 2010). Within *Drosophila*, however, females have also failed to exhibited preference for essential sexual traits in local males over those of foreign males that are phylogeographically highly divergent (Klappert et al. 2007). Such patterns might be the result of selection to promote outbreeding through reduced sibling mating, as has been suggested for solitary bees that exhibit preference for exotic mates over local, presumably genetically similar, mates (Vereecken et al. 2007). These bees pollinate sexually deceptive orchids in the genus *Ophrys* and their preference for exotic sexual signals may considerably affect floral divergence in these plants (Vereecken et al. 2007). This mechanism is unlikely to drive plant-pollinator interactions within our study system as all *M. capensis* males exhibit clear and uniform preference for the female mimicking spots of a single floral form. This preference is independent of geographic origin and genetic association of the males, indicating that phylogeographic structure does not play a large role in mating preferences within *M. capensis*. Rather, males throughout the landscape have similar preferences that appear to have driven the evolution of complex female-mimicking forms in allopatric populations of *G. diffusa.*

This is supported by the observation that only sexually deceptive forms of *G. diffusa* possess raised, multicellular papillate trichomes within their spots (Ellis and Johnson 2009), which have been shown to be crucial for the attraction of male, but not female flies (Chapter 2, De Jager and Ellis 2012). This may of course also be the result of sharing a recent common ancestor and the evolutionary history of these forms is currently under investigation. Although the fly-mimicking spots of sexually deceptive forms share such similarities, there are nonetheless significant differences in their overall spot phenotype (Ellis and Johnson 2009). This implies that

the same preference in a pollinator may result in similar, but not identical, phenotypes in different populations, determined by which mutations happen to occur in each. This phenomenon is also important in the sexually deceptive orchid genus *Ophrys*, where geographically separated species sometimes evolve similar phenotypes to exploit the mating preferences of a single pollinating species (Mant et al. 2005b, Paulus 2006).

While such one-sided evolution is a likely scenario, floral diversity may also be the result of more interactive relationships between plant and pollinator. If male pollinators suffer potential costs when deceived (Gaskett et al. 2008) they may learn to reduce the amount of mating behaviour they exhibit on sexually deceptive flowers with time. Since the reproductive success of these plants is determined by the intensity and frequency of mating behaviour they elicit from their male pollinators (Ellis & Johnson 2010a, Gaskett 2011), this might exert selective pressure on flowers to increase deceptiveness (Wong and Schiestl 2002). This scenario may be less likely within *Ophrys* where deceived males quickly decrease their mating behaviour on deceptive flowers with repeated exposure, but will renew their mating efforts if a new flower is introduced (Ayasse et al. 2000, Paulus 2006). This strongly suggests that they learn the identity of individual flowers, but not the actual signals used to mimic females and will therefore remain effective pollinators for other flowers in the population. In our study, however, experienced males were tested in a new location on inflorescences of the sexually deceptive Spring form they had not encountered before and still they exhibited significant reductions in mating behaviour relative to naïve males, which implies they have learned to discriminate the deceptive spots of this form as female mimics.

This will certainly pose a reproductive cost for sexually deceptive *G. diffusa*, which achieve increased levels of pollen export only when they elicit mating behaviour from male flies (Ellis and Johnson 2010a). The costs suffered by male pollinators due to sexual deception have rarely received investigation and little is known about their strength and frequency (Gaskett 2011), which will likely determine the scope for antagonistic coevolution to operate within these systems.

This process is important in pollination systems involving long-tubed flowers that place their pollen on the bodies of long-proboscid flies searching for nectar within their tubes. The fitness of both plant and pollinator is determined by the difference between their floral tube lengths and proboscid lengths and each benefit by outdistancing the other, resulting in a coevolutionary arms race (Pauw et al. 2009). Such arms races generate floral diversity between isolated populations of many long tubed angiosperms, each linked to the proboscid length of their local pollinators (Anderson and Johnson 2009).

Similar processes may also promote floral divergence between isolated populations of sexually deceptive flowers, dependent on male pollinators possessing the necessary learning abilities to avoid deceptive flowers. Pollinator learning appears to be common within sexually deceptive pollination systems (Gaskett 2011) and learning in insects is known to comprise genetically based and therefore heritable variation (reviewed in Dukas 2008). This, together with the fact that *M. capensis* males show substantial variation in their mating responses towards a given sexually deceptive *G. diffusa* form, and that both *G. diffusa* and *M. capensis* have short annual life cycles, may make antagonistic coevolution likely between these two species. Owing to the allopatric nature (Ellis and Johnson 2009) and very low dispersal ability (M. de Jager pers. obs.) of *G. diffusa* forms, putative arms races in different populations will run along different trajectories, which could promote floral diversification. Although coevolution is an established mechanism generating diversity within interacting species, its role in deceptive systems has not received much attention and its importance, as well as the potential cause and effect of pollinator learning requires much needed experimental investigation.

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Table 3.1

The observed maximum percentage clade membership (MPCM) of flies caught (*N*) within the range of every *G. diffusa* floral form and the expected probability of that percentage occurring under random association between flies and floral forms.

Flies (*N*) from	Functional type	Observed MPCM	Expected probability
Spring (6)	Sexual	100	**0.0140***
Nieuw (7)	Sexual	100	**0.0080 ***
Buffels (3)	Sexual	100	0.1980
Rich (6)	Feeding	100	**0.0120 ***
Okiep (9)	Feeding	56	0.3990
Naries (9)	Feeding	67	0.2460
Koma (8)	Feeding	75	0.1300
Soeb (9)	Feeding	100	**0.0040 ***
Cal (3)	Feeding	100	0.2010
Garies (6)	Feeding	83	0.1360
Average for	Sexual forms	100 ± 0	**0.0455 ***
Average for	Feeding forms	83 ± 18	0.0502

Table 3.2

The number of visits made to inflorescences in each *G. diffusa* array by males from different clades, the proportion of visits where they landed on the fly-mimicking spots and the amount of time (s) they were active per inflorescence visit, as well as the amount of time spent exhibiting feeding and mating behaviour. Medians and quartile ranges (75^{th} - 25^{th} percentile) are displayed.

Male clade	*G. diffusa* array	Total visits made	Prop visits landed on spot	Time active per visit	Time feeding per visit	Time mating per visit
Exp "Nieuw"	Nieuw	11 (12)	**0.4 (0.4) ***	**37.8 (29.7) ***	**37.5 (29.6) ****	**0 (0.1) ***
(*N* = 15)	Spring	11 (18)	**0.8 (0.3)**	**15.3 (19.5)**	**15.2 (21.2)**	**0.3 (0.4)**
Exp "Spring"	Nieuw	10.5 (10.5)	0.51 (0.2)	23.2 (27.6)	23.2 (27.8)	**0 (0.1) ****
(*N* = 16)	Spring	14 (6.5)	0.61 (0.2)	20.5 (21.3)	20.3 (22.4)	**0.2 (0.8)**
Naïve "Buffels"	Nieuw	**5.5 (6) ***	0.6 (0.6)	**19.1 (37.7) ***	**19.1 (37.8) ***	**0 (0.6) ****
(*N* = 10)	Spring	**20 (18)**	0.9 (0.4)	**8.3 (12.5)**	**2.3 (13.8)**	**2.3 (4)**

* indicates $p < 0.05$, ** $p < 0.01$

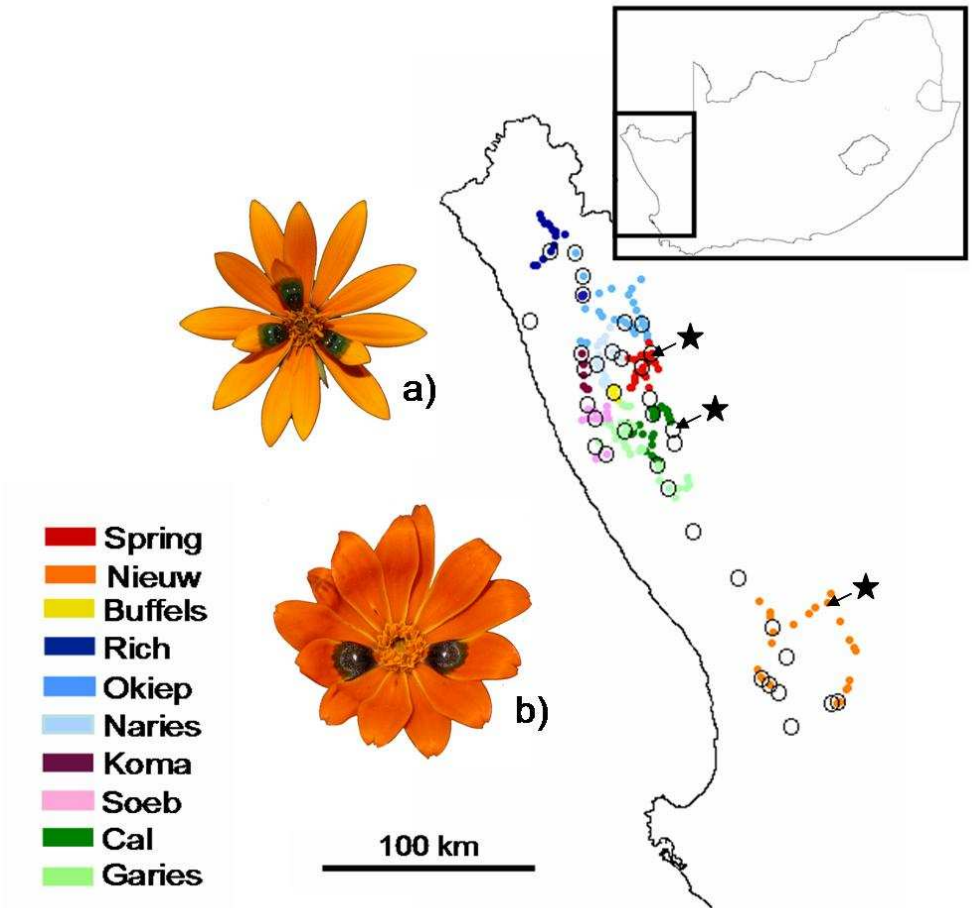

Figure 3.1. The range of *G. diffusa* within Namaqualand in South Africa, colour coded by floral form. Black rings indicate locations where we sampled and sequenced their pollinator, *M. capensis*. The two sexually deceptive forms investigated in our reciprocal pollinator behaviour experiments are shown in a) Spring, and b) Nieuw. Stars indicate localities where we caught *M. capensis* males for use in these experiments.

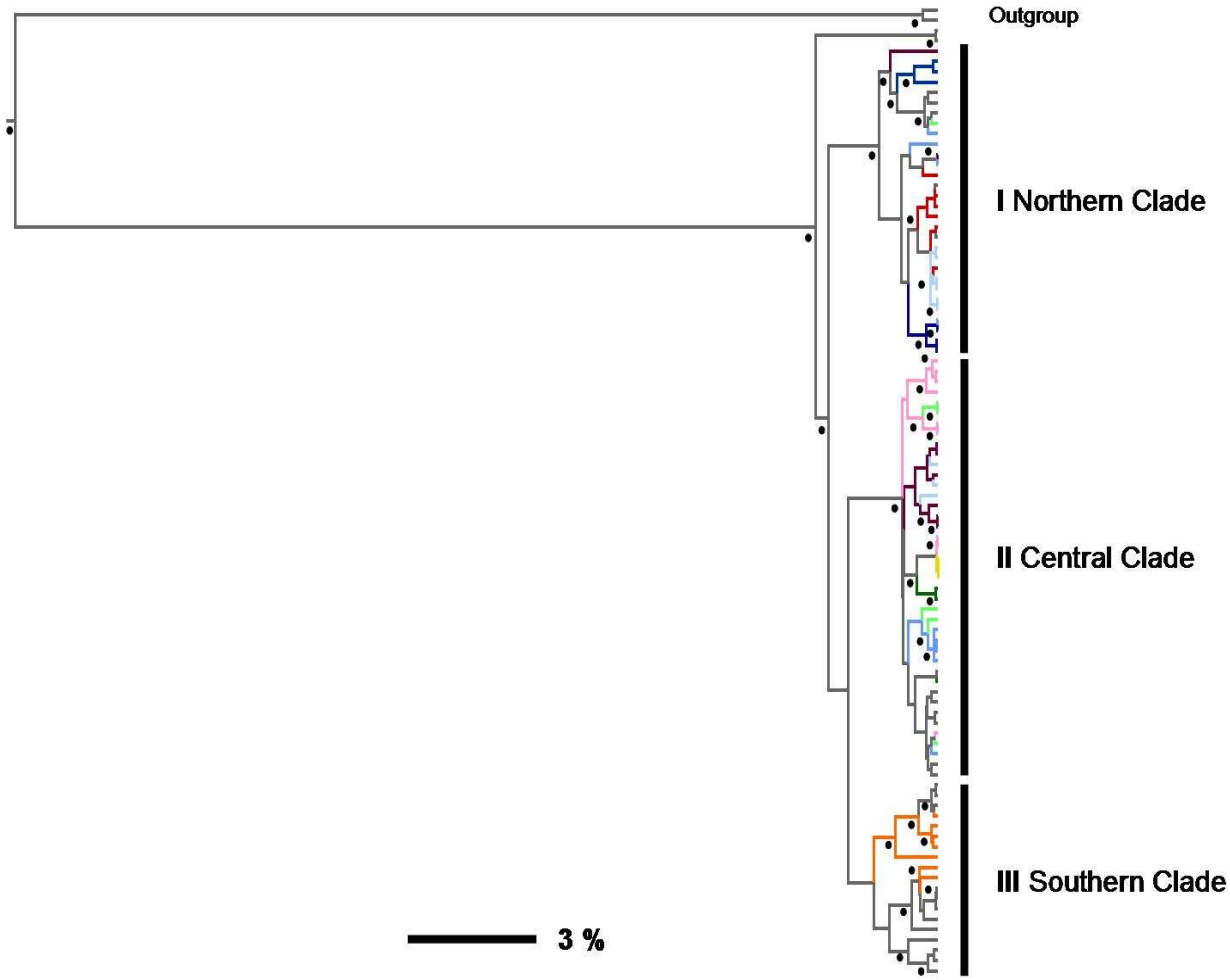

Figure 3.2. Maximum credibility tree based only on the *Cox1* dataset with posterior probabilities above 0.9 indicated by dots alongside nodes. Sampled *M. capensis* individuals resolve into three Namaqualand clades, as well as a basal coastal group comprising a single population. The floral form of *G. diffusa* associated with each fly sample is indicated using the colours from Figure 3.1. Tips in grey represent flies caught in areas where no *G. diffusa* grows. Scale bar represents percent divergence.

Figure 3.3. Maximum clade credibility tree based on the *Combined* dataset showing the same topology as the *Cox1* dataset. Nodes with higher than 0.9 posterior probability are indicated with dots. Namaqualand clades designated in the *Cox1* tree are indicated by Roman numerals. Flies caught within the ranges of the three sexually deceptive floral forms are indicated using the same colours as in Figure 3.1. The sexually deceptive floral forms associated with each of these fly samples are depicted to the right; with a close-up of the petal spot of each form that *M. capensis* males attempt to copulate with. Scale bar represents percent divergence.

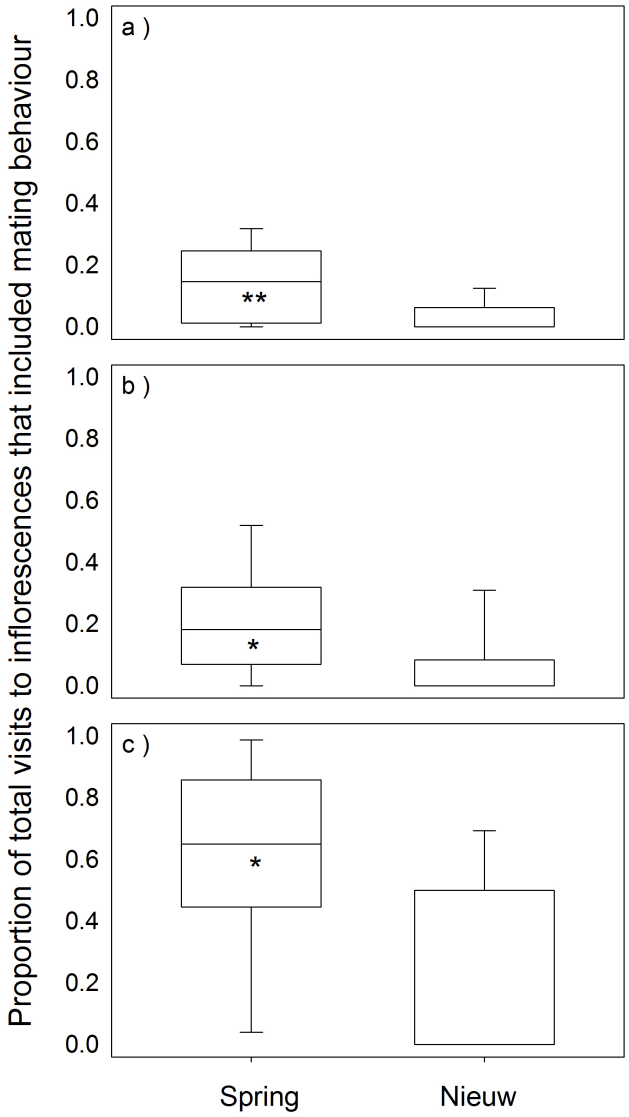

Figure 3.4. The proportion of total visits to inflorescences of each sexually deceptive *G. diffusa* floral form that included mating behaviour by a) experienced "Spring" clade males; b) experienced "Nieuw" clade males and c) naïve "Buffels" clade males. Medians with 5th and 95th percentiles are shown and significance is indicated by * $p < 0.05$ and ** $p < 0.005$

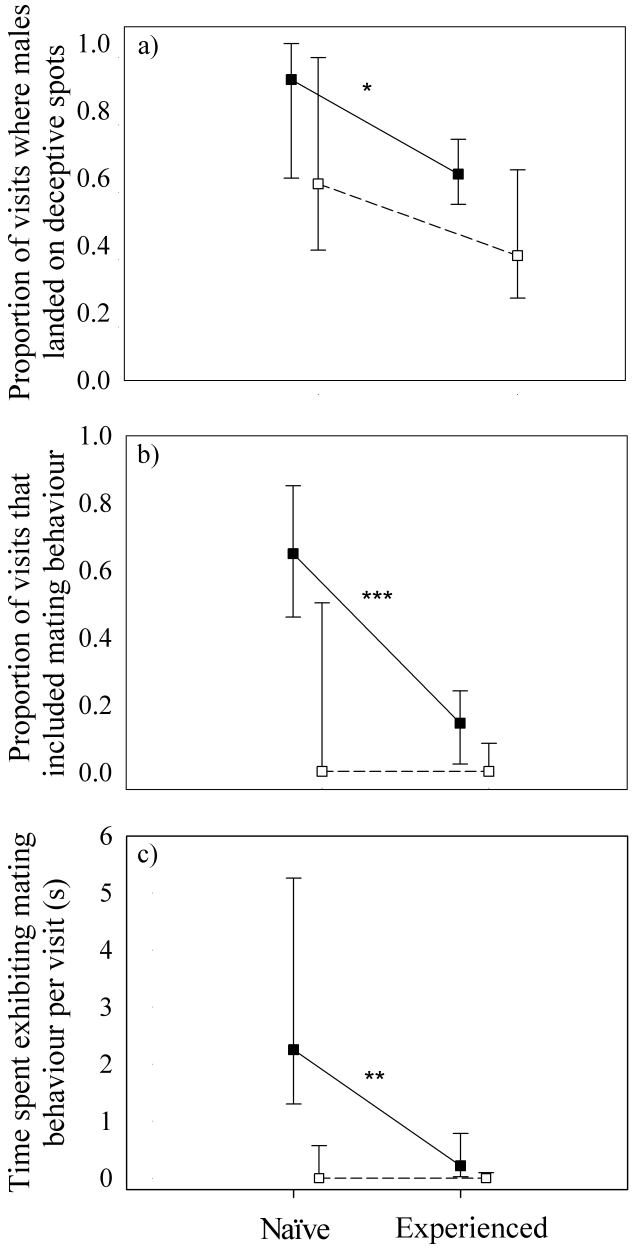

Figure 3.5. The difference between naïve and experienced male flies in a) the proportion of visits where they landed on the deceptive spots, b) the proportion of visits where they exhibited mating behaviour and c) the amount of time they spent exhibiting mating behaviour per visit on the sexually deceptive Spring (black squares) and Nieuw (open squares) forms. All plots show medians with their upper and lower quartiles. * indicates $p < 0.05$, ** $p \leq 0.01$, *** $p < 0.001$

Chapter 4

The costs of deception determines learned resistance in deceived pollinators

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ABSTRACT

The costs that species suffer when deceived are expected to drive learned resistance, although this link has seldom been demonstrated experimentally. Flowers that elicit mating behaviour from male insects by mimicking conspecific females provide an ideal system for investigating this link. Here we explore the interactions between a sexually deceptive daisy that exhibits multiple floral forms, which vary in deceptiveness, and the male flies that pollinate it. We show that male pollinators experience negative impacts via this interaction and suffer not only potential mating costs in terms of the ability and time taken to locate genuine females within deceptive inflorescences, but also foraging costs through reduced feeding activity. The severity of costs that males suffer correlates strongly with the level of floral deceptiveness. Male flies, however, exhibit the ability to learn to discriminate the most deceptive inflorescences as female mimics and subsequently reduce the amount of mating behaviour they exhibit on them with increased exposure. This demonstrates the important relationship between the costs suffered and the likelihood of learned avoidance within deceptive systems. It also offers support for antagonistic coevolution as an important process affecting floral evolution in sexually deceptive plants.

INTRODUCTION

Deceptive interactions between animal species often serve as models for the study of evolutionary processes (Jiggins et al. 2001, Kapan 2001). Plant-pollinator interactions, however, may have much to contribute to this field of research since floral mimicry and pollinator deception is widespread (Dafni 1984, Roy and Widmer 1999). However, for floral resemblance to be considered mimetic, the adaptive significance of a given phenotype needs to be demonstrated experimentally (Roy and Widmer 1999). This was recently done for food deceptive species, where non-rewarding flowers have higher reproductive success when occurring with the rewarding flowers they resemble (Johnson et al. 2003, Benitez-Vieyra et al. 2007, Peter and Johnson 2008). Sexually deceptive species also exhibit highly specialized flowers that elicit

mating behaviour from male insects by resembling the mating signals of pollinator females (Schiestl et al. 2003, De Jager and Ellis 2012). The adaptive advantage of mimicry in these plants lies in increased levels of outcrossing and pollen export (Peakall and Beattie 1996, Ellis and Johnson 2010a).

While the benefit, for the plants, of deceiving male insects to act as pollinators is well documented, almost nothing is known of the impact of these interactions on the insects. Recent reviews have suggested that any costs suffered by deceived males may be negligible, although experimental evidence is required (Schiestl 2005, Gaskett 2011). The only study investigating the negative effects of sexual deception on a pollinating species, however, has suggested it may result in reduced fitness for the mimicked female insect (Wong and Schiestl 2002). Studies exploring the costs to deceived male pollinators are completely lacking. This is surprising, as most studies report that male pollinators reduce the amount of mating behaviour they exhibit on sexually deceptive orchids with exposure (Ayasse et al. 2000, Wong and Schiestl 2002, Gaskett et al. 2008). This strongly suggests that pollinators can learn to avoid deceptive flowers, which is only likely to occur if some cost is involved in being deceived. The extent and nature of costs suffered by deceived males, as well as their influence on pollinator learning in deceptive pollination systems remain unknown.

The bee fly *Megapalpus capensis* is the predominant pollinator of the African sexually deceptive daisy, *Gorteria diffusa*. This species exhibits multiple floral forms that vary in their level of deceptiveness and elicit mating behaviour from male flies with fly-mimicking spots on their ray florets (Ellis and Johnson 2010a). The frequency with which *G. diffusa* elicits mating behaviour is much higher than that observed within the sexually deceptive orchids on which most research in this field has been conducted (Ellis and Johnson 2010a). It is also relatively common in the landscape and can grow in dense aggregations, and may thus pose significant costs to deceived males, both in terms of reduced mating and foraging success. Using this study system, we investigated the impact of sexual deception on male bee fly pollinators. Specifically we aimed

to determine: (1) whether deceived males suffer any costs and how these are influenced by floral deceptiveness, (2) whether deceived males can learn to reduce mating behaviour on deceptive inflorescences and (3) how the costs of deception influence the likelihood of male learning.

MATERIAL AND METHODS

Study system: *G. diffusa* comprises 14 geographically distinct floral forms within Namaqualand in South Africa (Ellis and Johnson 2009). These include feeding forms that induce only feeding behaviour in male and female flies, inspection forms that elicit inspection behaviour predominantly from mate seeking males and sexually deceptive forms that elicit mating behaviour exclusively from males (Ellis and Johnson 2010a). The ray floret spots of these sexually deceptive forms possess specialized papillate structures (Thomas et al. 2009) and well-defined UV highlights which likely mimic mate recognition cues that male flies are strongly attracted to (De Jager and Ellis 2012). Mating in *M. capensis* often takes place on open daisy inflorescences wherein females sit and feed. Males exhibit mate searching behaviour by moving repeatedly among inflorescences, landing on other flies and fly-mimicking spots alike (De Jager and Ellis 2012).

Costs to deceived males and the influence of deceptiveness: We investigated male behaviour on various deceptive forms of *G. diffusa*, which included two feeding (Soeb, $N = 14$; Garies, $N = 15$), two inspection (Cal, $N = 13$; Okiep, $N = 14$) and two sexually deceptive forms (Nieuw, $N = 13$; Spring, $N = 15$). These six forms represent the continuum of mating behaviour exhibited by male flies in response to *G. diffusa* (Ellis and Johnson 2010a). We caught wild *M. capensis* males near the town of Kamieskroon (S 30, 12, 20.6; E 17, 56, 12.1) and used them in experiments on the same day. For each floral form, we created arrays of 20 fresh inflorescences spaced six cm apart. Before releasing individual male flies into $1m^3$ pollinator cages containing one of these arrays, we attached a dead *M. capensis* female (killed by exposure to -18^oC for 30 minutes) next to a ray

floret spot on a single inflorescence on each array, selecting females of similar size for the various arrays. We categorised male behaviour on inflorescences as either sitting, feeding or mating behaviour, which comprises *inspecting* (quick landings on the ray floret spots), *changing* (flitting between different spots in an inflorescence), *hopping* (repeatedly hopping and arching abdomen downwards on the spot) and *turning* (rotating on the spot). We calculated the percentage of total active behaviour (excluding sitting) that included mating behaviour for every male, as well as the average percentage mating behaviour that males exhibited on every floral form to get an estimate of each form's level of deceptiveness.

We allowed males 20 minutes (maximum) to locate the female and exhibit mating behaviour towards her. When males landed on the same inflorescence as the dead female without discovering her, we scored it as a missed mating opportunity. When they discovered the female, we scored it as a successful mating opportunity, recorded the time elapsed (proportion of the maximum 20 minute period it took the male to locate the female) and stopped the experiment. We then created a compound index of mating costs on every floral form by dividing the mean proportion of maximum time that males took to find females on a particular form by the proportion of males that managed to find them on that form. This metric includes both the ability and time taken to locate females, which we reasoned reflects the capacity of male flies to discriminate true females from the deceptive spots of *G. diffusa*. We then regressed the mating costs males experienced against the deceptiveness of each floral form. We only used males on a particular form once and exposed them to different forms in a random order to avoid any potential effects of learning. Only males that were active on arrays for at least five minutes were used. We conducted all experiments in Kamieskroon during August and September 2011 on warm sunny days when flies are most active. During August 2009 we ran a smaller mating cost experiment with males using the same protocol described above on the two sexually deceptive floral forms (Nieuw and Spring, $N = 5$). Arrays in these experiments contained 12 fresh inflorescences and one live feeding female fly.

Male learning in response to deception: To determine whether male flies possess the necessary learning capabilities to alleviate the costs of deception we caught naïve males unfamiliar with sexually deceptive *G. diffusa* at two sites (Kamieskroon, $N = 10$; Englishman's Grave, $N = 10$, S 32 04 00.0, E 19 07 37.9). To test their putative learning abilities we exposed these males repeatedly to arrays of 20 fresh inflorescences of the Spring form of *G. diffusa*, as this form elicits the strongest mating response from male flies and is thus most likely to induce learning. Our protocol consisted of releasing captured naïve males into a pollinator cage containing an array and recording their pollination behaviour as described above for 10 minutes (**1st exposure**). We left males in the cage for an additional 10 minutes to ensure that they familiarize themselves with the deceptive spots and allow any putative learning to take place. Males were then caught and rested for 10 minutes to prevent fatigue from affecting subsequent behaviour, before being released back onto the same floral array and recording their mating behaviour for another 10 minutes (**2nd exposure**). We investigated differences in mating behaviour between the two exposures with Wilcoxon Matched Pairs Tests. For Kamieskroon males, we also investigated differences in time spent exhibiting behaviours between the exposures. Experiments were conducted in Kamieskroon in 2010 and the Biedouw Valley in 2011 during August and September on warm sunny days.

Influence of costs on learning in deceived males: To explore how costs can affect learning we firstly quantified the costs that were experienced by male flies exposed to each of the two sexually deceptive forms (Spring and Nieuw) during our mating costs experiments ($N = 15$). We recorded the proportion of these males that found females on each form, and the mean number of missed mating opportunities they experienced and analyzed this paired dataset with McNemar Chi-square test. To investigate how these costs may affect learning in males on these two forms we ran additional experiments during 2010 where we recorded the amount of time that

experienced and naïve males from separate populations spent exhibiting behaviours on arrays of the Spring and the Nieuw forms (12 inflorescences each). Experienced males were caught within the ranges of the respective forms (Spring, N = 14, S 29 39 14.5; E 17 53 20.9; Nieuw, N = 14, S 31 22 46.8; E 19 5 38.1), whilst naïve males unfamiliar with either floral form were caught at a site where no *G. diffusa* grows (N = 11 for each form, S 30, 12, 33.3; E 18, 2, 58.4). All males were moved to Kamieskroon where we released them individually into pollinator cages with an array and recorded their behaviour for 10 minutes. We used a factorial ANOVA to analyse differences between naïve and experienced males on either floral form. During all our experiments inflorescences in arrays were replaced as necessary. Statistical analyses were performed in the SPSS 19 statistical package (SPSS Inc., Chicago, USA).

RESULTS

Costs to deceived males and the influence of deceptiveness: We used 45 male flies in 94 experiments (mean number of floral forms males were exposed to = 2.1). Our results show that deceptive floral forms pose significantly greater mating costs to male flies, as measured by our compound index that included both ability and time to locate females (r^2 = 0.87, $F_{1,4}$ = 12.87, p < 0.01) and the mean number of missed mating opportunities (r^2 = 0.97, $F_{1,4}$ = 115.77, p < 0.0005 – Figure 4.1). The Spring floral form elicited considerably more mating behaviour (mean of 77% of total active behaviour) than the other forms and posed relatively greater costs, which may have strongly influenced this pattern. To determine its influence we removed this outlier and still found a significant relationship between deceptiveness and the mean number of missed mating opportunities (r^2 = 0.83, $F_{1,3}$ = 14.65, p < 0.05), but not between deceptiveness and our index of potential mating costs (r^2 = 0.34, $F_{1,3}$ = 1.57, p > 0.05). Costs suffered by males in the 2009 experiment using live females were qualitatively similar.

Male learning in response to deception: Our experiments to explore learning revealed that male flies drastically reduce the amount of mating behaviour they exhibit towards the fly-mimicking spots of *G. diffusa* with increased exposure. Males from both the Kamieskroon (Wilcoxon Matched Pairs Test: $Z = 2.367$, $N = 10$, $p = 0.018$) and Englishman's Grave ($Z = 2.521$, $N = 10$, $p = 0.012$) sites exhibited significantly less mating behaviour during their 2nd exposure to floral arrays of the Spring form (Figure 4.2). Males from Kamieskroon also showed significantly less mating behaviour in all the mating categories we observed except hopping (inspecting; $Z = 1.992$, $N = 10$, $p = 0.047$; changing $Z = 1.992$, $N = 10$, $p = 0.047$ and turning $Z = 2.547$, $N = 10$, $p = 0.011$), whilst males from Englishman's Grave only did so for changing ($Z = 2.023$, $N = 10$, $p = 0.043$). One possible explanation for this reduction in mating behaviour during the 2nd exposure could be reduced activity caused by fatigue. However, males from Kamieskroon that showed a significant reduction in time spent exhibiting mating behaviour during their 2nd exposure to Spring arrays ($Z = 2.599$, $N = 10$, $p = 0.009$), were in fact significantly more active during this exposure as a result of increased feeding activity ($Z = 2.497$, $N = 10$, $p = 0.012$ - Figure 4.3). In addition, experienced males caught within the range of the Spring form during 2010 spent the same amount of time exhibiting mating behaviour on Spring arrays (median = 2.5 seconds) as the naïve males mentioned above during their 2nd exposure (median = 3.5 seconds: Mann Whitney U test: $U = 66$, $p = 0.815$). This comparison strongly suggests that the reduction in mating behaviour we observed during our repeated exposure experiments is due to learning and not fatigue.

Influence of costs on learning in deceived males: Male flies that were exposed to arrays of both the sexually deceptive Spring and Nieuw forms found significantly fewer females on the more deceptive Spring form (Figure 4.4 A) than the Nieuw form (frequency of males that found females against those that did not; McNemar Chi-square test; $p = 0.009$). They also experienced significantly more missed mating opportunities on the Spring form (frequency of successful mating opportunities against missed mating opportunities; McNemar Chi-square test; $p = 0.027$),

confirming greater mating costs experienced by males on this form. When analysing the time spent exhibiting mating behaviour by experienced males from within the range of the Spring form we found significant reductions compared to naïve males from outside its range (Factorial ANOVA: MS = 472.6, df = 46, $p < 0.0005$ - Figure 4.4 B). For the Nieuw form, which poses less severe mating costs to males than the Spring form, there was no difference between experienced and naïve males (MS = 472.6, df = 46, $p > 0.05$). Whether males learn to reduce the amount of mating behaviour they exhibit toward deceptive spots is thus likely determined by the extent of mating costs they suffer when deceived. Experienced males on both the Spring (MS = 472.6, df = 46, $p < 0.005$) and Nieuw (MS = 472.6, df = 46, $p < 0.01$) forms, however, were significantly more active than naïve males, due to the greater amount of time they spend exhibiting feeding behaviour (Figure 4.4 B). This suggests that increased feeding behaviour is also a learned response and another potential selective force on male flies to reduce mating behaviour directed towards the deceptive spots of *G. diffusa*.

DISCUSSION

We found that male pollinators deceived by *G. diffusa*'s fly-mimicking spots suffer potential mating costs and that the severity of these costs depends on the deceptiveness of the floral form involved. Our learning experiments reveal that males learn to discriminate deceptive spots as female mimics through increased exposure and thus alleviate these costs by reducing their mating behaviour on these spots. This explains why experienced males from within populations of the very deceptive Spring form exhibit less mating behaviour on its spots than naïve males. Field studies on the sexually deceptive European *Ophrys* orchids have also documented that male bee pollinators quickly learn to avoid deceptive flowers (Ayasse et al. 2000). Males in these systems seem to learn to identify individual flowers, but not the signals involved in deception, as mating behaviour remains high when exposed to new flowers (Ayasse et al. 2000).

Male wasp pollinators of sexually deceptive orchids in Australia, however, reduce their mating behaviour with exposure, even if this is to new flowers (Ayasse et al. 2000, Wong and Schiestl 2002, Gaskett et al. 2008). In our study, experienced male flies from populations of the sexually deceptive Spring form also exhibited reduced mating behaviour compared to naïve males when tested on new inflorescences in a new locality. This suggests that male flies learn to recognize the deceptive signals of *G. diffusa*, and not just individual flowers or the location of deceptive plants. Whether male learning occurs appears to be influenced by the actual costs suffered when they are deceived, as only males experienced with the most deceptive form (Spring) that held the largest costs exhibited a reduction in mating behaviour with exposure. Such learning capacities may represent an evolved response to being deceived, or a convenient preadaptation originating in male-female interactions to avoid wasting time and energy on unsuccessful mating attempts. With experience, males from different insect orders have been found to reduce mating behaviour in response to heterospecific or unresponsive conspecific females (Wcislo 1987, Dukas 2004). This suggests that learned avoidance is widespread amongst insects and not limited to species involved in antagonistic or deceptive interactions. Even if this ability evolved in male-female interactions, experience with sexually deceptive flowers can still modify and shape the observed rates of learning since variation in the capacity to learn is heritable (Dukas 2008).

This suggests that antagonistic coevolution may potentially operate in these systems. Within *G. diffusa* (Ellis and Johnson 2010a) and sexually deceptive orchids (Gaskett et al. 2008), the forms/species that elicit the most intense mating behaviour in male pollinators experience the highest reproductive success. Unless they rely solely on newly emerged naïve males for pollination, learned avoidance could place them under strong selection to increase their deceptiveness and/or deter learning. Males, for their part, suffer reproductive costs when deceived and may therefore experience selection to increase learning capacity, dependant on the proportion of the male pollinator population that actually encounters sexually deceptive plants. Learning, however, may also largely be influenced by the ratio of deceptive flowers to their models (female

insects). This factor has been demonstrated to be important, both experimentally (Anderson and Johnson 2006) and theoretically (Ferdy et al. 1998), for pollinator learning in food deceptive species. Whatever the ultimate causes of learning, we illustrate that pollinators suffer potentially severe costs when deceived and subsequently learn to discriminate mimics with exposure. These results have important implications for evolutionary interactions in all deceptive systems as they show that responses to exploitation may depend on various factors, potentially including the severity and frequency of the costs suffered as well as preadaptations. Learning also seems to take place with relatively little exposure to deceptive flowers. Future studies may help elucidate the intriguing role of antagonistic coevolution within sexually deceptive pollination systems by comparing the learning abilities of pollinators inside and outside the ranges of sexually deceptive plants.

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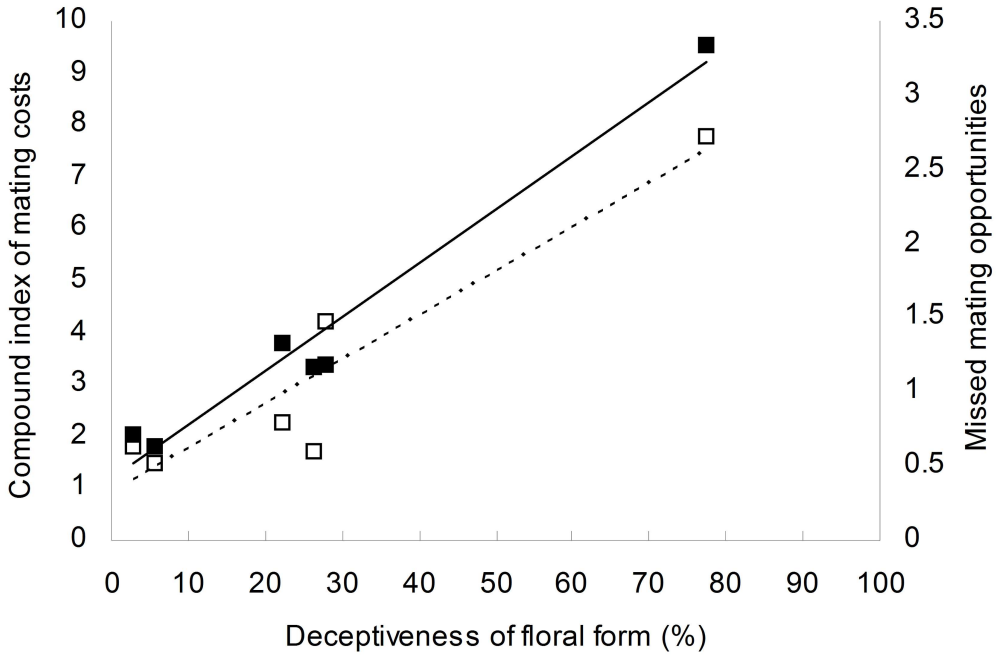

Figure 4.1. The deceptiveness (mean percentage mating behaviour of total active behaviour that was elicited from male flies) of the six *G. diffusa* floral forms investigated regressed against the compound index of mating costs (mean proportion of maximum time taken to locate female/the proportion of males that found females; dashed line □) and the mean number of missed mating opportunities they experienced on each form; solid line ■.

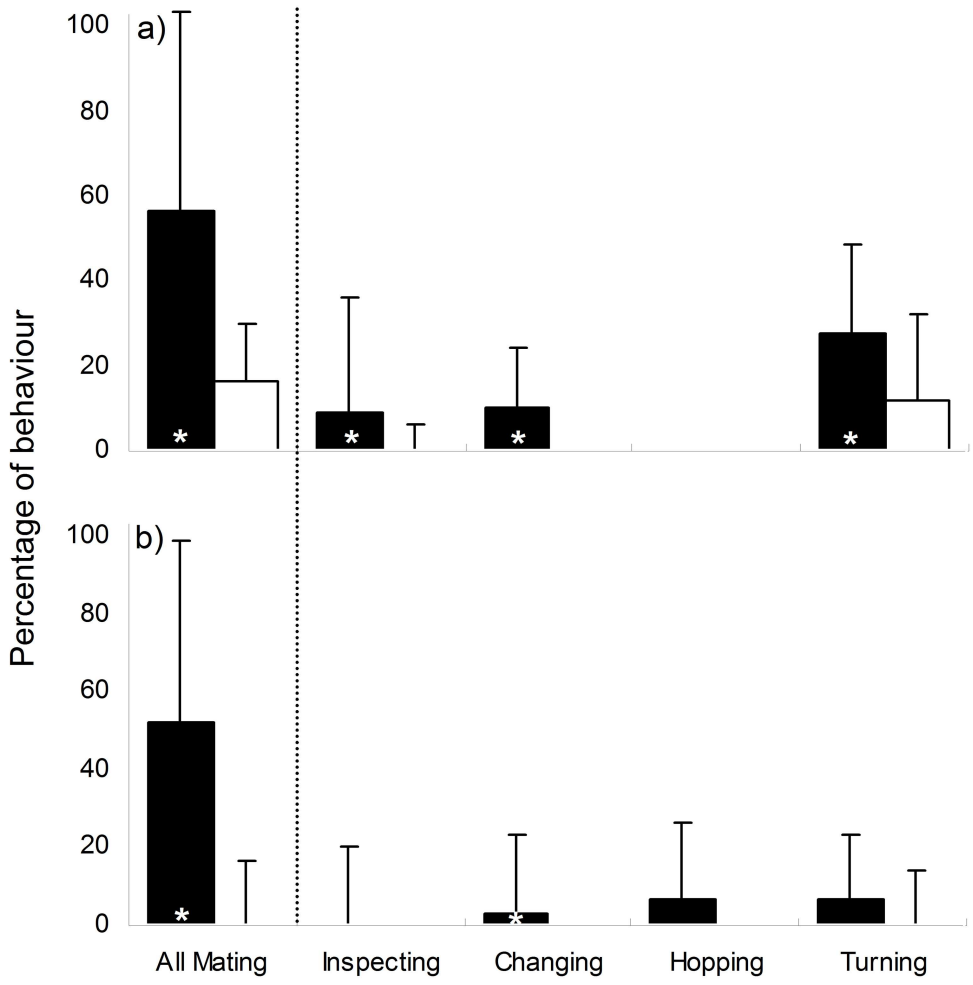

Figure 4.2. The median and quartile ranges for the percentage of mating behaviour of total activity exhibited by naïve males from a) Kamieskroon (*N* = 10) and b) Englishman's Grave (*N* = 10) during their 1st (black bars) and 2nd (white bars) exposure to the sexually deceptive Spring floral form of *G. diffusa*. * indicates significant difference between exposures at $p < 0.05$.

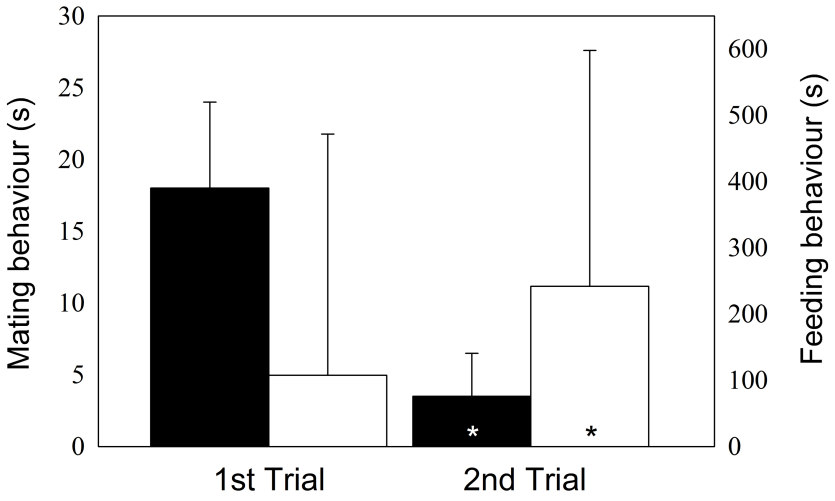

Figure 4.3. The median and quartile ranges of time spent exhibiting mating (black bars) and feeding behaviour (white bars) by naïve males from Kamieskroon (N = 10) during their 1st and 2nd exposure to the sexually deceptive Spring floral form of *G. diffusa*.

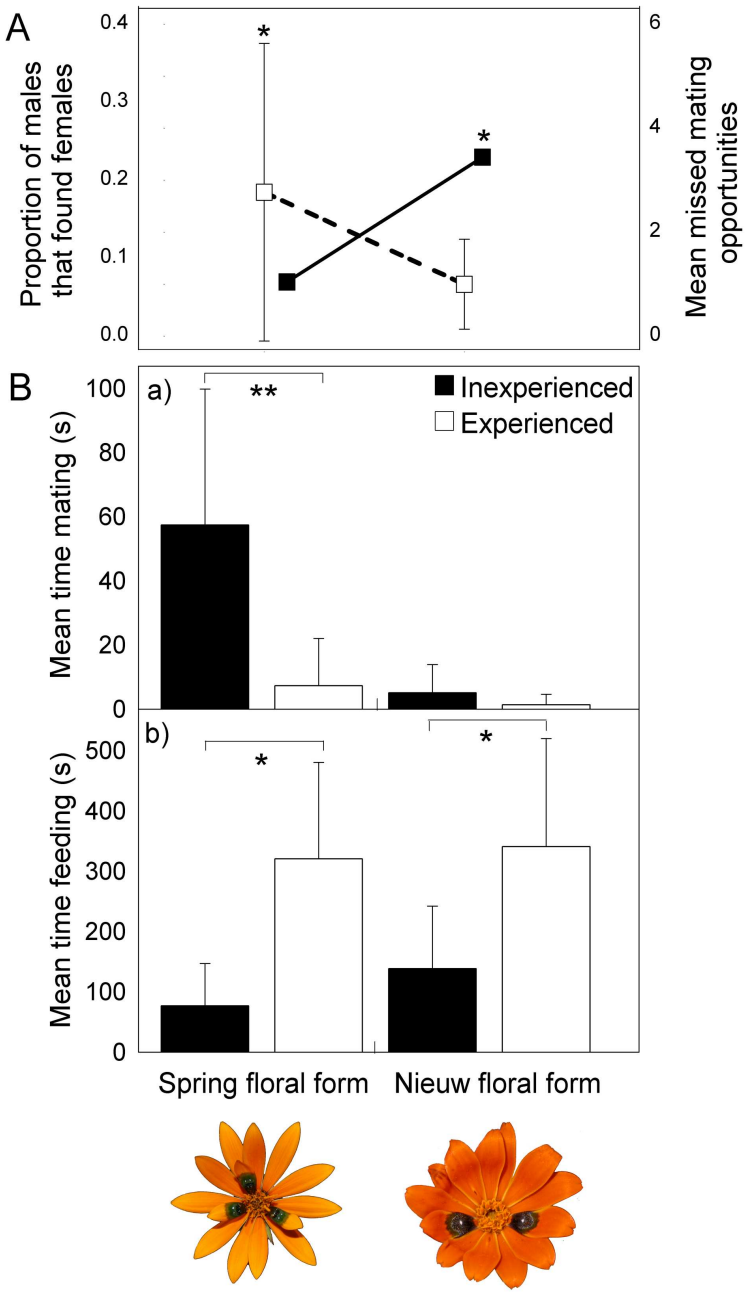

Figure 4.4. A) The proportion of male flies that found females (■) on arrays of the sexually deceptive Spring and Nieuw floral forms, and the mean number and standard deviations of missed mating opportunities (□) they experienced on each form. Significant differences between costs experienced on the two forms are indicated with * = $p < 0.05$

B) The average amount of time that inexperienced and experienced male flies spent exhibiting a) mating behaviour and b) feeding behaviour on arrays of either the sexually deceptive Spring or Nieuw floral forms. The Spring form poses greater potential mating costs to males flies and induces learned resistance in experienced males, while the less deceptive Nieuw form does not. Both forms, however, induce a significant increase in feeding behaviour in experienced males. Significant differences between inexperienced and experienced males on each floral form are indicated with * = $p < 0.05$, ** = $p < 0.0005$

Chapter 5

Floral polymorphism and the tradeoffs of attracting pollinating and florivorous insects

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ABSTRACT

Floral polymorphism is common in angiosperms and traditionally attributed to pollinator-mediated selection. While many studies support the role of pollinators in floral phenotype evolution, a growing number of studies also reveal the importance of non-pollinating visitors, like florivores. Using the polymorphic annual South African daisy, *Ursinia calenduliflora,* we investigated the importance of insect visitors and their potential effects on fitness in the maintenance of floral polymorphism. Firstly, we characterized the spatial and temporal structure of this floral polymorphism in *U. calenduliflora* over three years. We then conducted pollinator observations at polymorphic sites over three years and analysed differences in the assemblage of visitors to the two morphs. Controlled experiments were also conducted to investigate variation in the preference of male and female flies of the dominant visitors, *Megapalpus capensis,* for the two morphs, as well as experiments on the effectiveness of *M. capensis* as pollinators. Next, we surveyed floral damage by antagonistic florivores, as well as the reproductive success of the two morphs in multiple sites over three years. Results show that *M. capensis* males are effective pollinators and exhibit clear preference for the spotted morph. These morphs, however, also suffer significantly greater costs due to florivory than the plain morphs. Measures of both reproductive success and florivory damage were significant factors in determining the proportion of spotted inflorescences at a site the following year. These results reveal that pollinators, as well as florivores, are strong selective agents and exhibit antagonistic selection that contributes to the maintenance of floral polymorphism in *U. calenduliflora*. The relative strength of each selective agent is likely determined by the insect community patterns at each site and year, highlighting the importance of community context in the evolution of floral phenotypes.

INTRODUCTION

Floral polymorphism is a common phenomenon in angiosperm species (Galen 1999, Schemske and Bierzychudek 2007, Ellis and Anderson 2012). It includes variation in floral size (Galen 1989,

Schlumpberger et al. 2009, Geelhand de Merxem 2009), scent (Ayasse et al. 2000) and most commonly, floral colour (Warren and McKenzie 2001, Schemske and Bierzychudek 2001, Ellis and Johnson 2009, Carlson and Holsinger 2010, De Jager et al. 2011). Although intraspecific variation in floral colour has been associated with variation in plant size (Rausher & Fry 1993), flower production (Levin & Brack 1995) and survivorship (Coberly and Rausher 2008), its most researched role is in pollinator attraction (Schemske and Bradshaw 1999, Jones and Reithel 2001, Bradshaw and Schemske 2003, Newman et al. 2012). Since pollinators exhibit differential preference for floral colour morphotypes that can affect plant fitness in natural populations (Johnson 1997, Boyd 2004) they are often considered the drivers of floral polymorphism. Such pollinator-mediated selection is viewed as ubiquitous, giving rise to the floral syndrome concept (Fenster et al. 2004) that has been used to predict pollinator type based on floral phenotype alone (Armbruster et al. 2011).

Floral visits by non-pollinating species, however, can also have a strong influence on the evolution of floral form (reviewed in Strauss and Whittall et al. 2006). Both floral size (Galen 1999) and colour (Irwin et al. 2003), for example, have been shown to respond to selection exerted by florivores, which may be even stronger than selection exerted by pollinators for some floral traits (Cariveau et al. 2004). Pollinator-mediated selection may therefore only achieve its full potential in the absence of florivores (Herrera 2000). Pollinators and florivores also often exhibit preference for the same floral traits that can result in antagonistic selection (Strauss and Whittall 2006). In polymorphic wild radish, both pollinators and florivores prefer the floral morphs that do not contain anthocyanin (Irwin et al. 2003, Strauss et al. 2004). Variation in the presence of anthocyanin is one of the most common forms of floral polymorphism (Levin and Brack 1995, Warren and McKenzie 2001, Strauss and Whittall 2006), suggesting that antagonistic selection via pollinators and florivores may be widespread.

Species that exhibit anthocyanin based floral polymorphism in sympatry could thus provide an ideal system to explore these factors, as spatial and temporal variation in the

dominance of a given floral morph across its range will potentially be determined by the importance of pollinators versus florivores in each year and site. Using the polymorphic South African annual daisy *Ursinia calenduliflora* as a model, we investigate the importance of these factors and specifically ask: 1) is there spatial and temporal variation in the distribution of floral morphs; 2) are the morphs interfertile and self-compatible; 3) are there any differences in insect visitation patterns; 4) florivory damage and 5) reproductive success between these morphs and finally 6) how do all these factors influence the distributions of floral morphs in multiple sites over multiple years.

MATERIALS AND METHODS

Study system: *Ursinia calenduliflora* grows in the Succulent Karoo winter rainfall biome of South Africa. It bears solitary inflorescences on the end of long peduncles and exhibits two floral morphs: a spotted anthocyanin containing morph characterised by a red ring with black spots at the base of all ray petals and an anthocyanin-less plain morph without markings on its orange ray petals (Figure 5.1). Although different insect species visit *U. calenduliflora* the bee fly, *Megapalpus capensis*, is a very common visitor in all populations (Figure 5.1 c). These flies pollinate another annual daisy (*Gorteria diffusa*) in this area, which exhibits elaborate fly-mimicking spots on its ray florets (Ellis & Johnson 2009). *M. capensis* flies are attracted to these dark spots (Johnson & Midgley 1997), especially the males that exhibit mate-searching behaviour on them (Chapter 2, De Jager & Ellis 2012), suggesting the possibility that male *M. capensis* may also prefer the spotted morph of *U. calenduliflora*.

Spatial and temporal variation in the distribution of floral morphs: To explore spatial and temporal variation in the proportion of spotted inflorescences we repeatedly surveyed *U. calenduliflora* populations across Namaqualand during 2010-2012. We estimated the proportion of spotted inflorescences by walking random transects through sites scoring about 500

inflorescences per population as either spotted or plain in a 1m section along the transect. To determine if this ratio varies in time and space we used an arcsin transformation on the proportional data and a Generalized Linear Mixed Model (GLMM) to analyse these data. We used a Gaussian distribution with year, site and altitude as random factors. To determine the significance of each factor we used an analysis of deviance approach where we compare the likelihoods of models with and without the effect of interest with a Chi-square test.

Insect visitation patterns

a) Field observations: To determine whether the two morphs are attracting different insect visitors we conducted observations in two large polymorphic populations over three years (Nourivier 2010-2012, 34 hours; Bovlei 2012, 18hours). We observed patches roughly $1m^2$ in size for 30 minutes, recording the number of spotted and plain inflorescences in each patch as well as the identity of all insect visitors to each morph. From this dataset we calculated the mean number of visits per inflorescence for each floral morph per patch by all the dominant visitors (*M. capensis* flies; monkey beetles - Coleoptera: Hopliini and blister beetles - Coleoptera: Meloidea). We also separated *M. capensis* flies into females and males, which could be distinguished from females by their sex-specific mate searching behaviour (De Jager and Ellis 2012). To analyse these data we used visits per inflorescence per hour as dependant variable in a GLMM with a Gaussian distribution. We treated morph as a fixed factor, and year and observation patch nested within year as random factors. We ran a separate analysis for each of the dominant visitor groups and obtained F-statistics for the fixed factor (morph), as well as the estimated means and standard errors for the visitation rates to each of the two morphs. For the random factors, we calculated the Wald Z statistic to determine the significance of year and patch on visitation rates.

b) Preference of male and female M. capensis: Since *M. capensis* males are strongly attracted to dark petal spots in other daisies (Ellis and Johnson 2010a; De Jager and Ellis 2012, Chapters 3 & 4) we also explored the preferences of male and female flies for the two floral morphs of *U.*

calenduliflora during 2009. We caught flies in a large polymorphic population of *U. calenduliflora* (Bovlei) and transported them to the Succulent Karoo Knowledge Centre in Kamieskroon for experiments. We confirmed genders of the flies visually before releasing them into a $1m^3$ mesh cage containing an array composed of 10 fresh inflorescences each of the spotted and plain morphs in an alternating pattern (four by five inflorescences). We observed each fly for 10 minutes and recorded the number of landings it made on each floral morph. To model the influence of gender on fly preferences we employed Generalized Estimating Equations (GEE) using fly identity as our repeated subject variable. We coded all fly choices as binary responses and used a binomial distribution with an underlying logit link function. We selected an exchangeable correlation construct, which assumes that choices are equally correlated within each fly. From this analysis, we obtained the estimated marginal means and 95% Wald confidence intervals of each gender's preference.

c) M. capensis males as effective pollinators on the different morphs: To ensure that *M. capensis* are effective pollinators and to investigate whether there is any difference in the effectiveness of male flies as pollinators for the two floral morphs, we conducted pollen export and deposition experiments using fluorescent powder (Dayglo Color, Cleveland, OH, USA) as a pollen analogue in 2010. We applied powder to all exerted pollen presenters on two inflorescences in an array containing 24 fresh inflorescences of either the spotted or the plain morph before releasing individual male flies ($N = 9$) into cages containing one of the arrays. We left males for 20 minutes before catching them and releasing them on an array of the other morph. We always used different colour powders on the two floral arrays and randomized colours and floral arrays before experiments. We confirmed the export of fluorescent powder with UV light and replaced all inflorescences that received powder before starting a new experiment. We used T-tests for dependant samples to analyse these data since the same flies were used on both arrays.

Florivory damage

a) Damage to ray florets in natural populations: During our 2010-2012 transects mentioned above we estimated the incidence of damage to ray florets by florivores for all scored inflorescences. We coded all inflorescences as damaged or undamaged based on evidence of foraging by florivorous insects (Figure 5.1 e). We analysed these data with a GLMM using a binomial distribution with a logit link function and year and site as random factors and morph as a fixed factor. Significance was determined by analysis of deviance.

b) Damage to ovules inside maturing infructescences: To explore the extent of ovule predation, a potentially severe cost of florivory, we collected about 15 mature infructescences from multiple populations during the end of the flowering season in 2010-2012. We identified mature infructescences by their dried ray florets and nodding habit, which occurs after flowering, but before their seeds are released and dispersed by wind. We dissected each infructescence under a dissection microscope, cutting it at a perpendicular angle and investigating the disc florets for any evidence of ovule predation. This comprised the presence of larvae or pupae inside dissected infructescences and the remains of ovules consumed by florivorous insects. We also measured the diameter of the infructescences to control for variation in inflorescence size. We then analysed the incidence of ovule predation by florivores with a GLMM using an underlying binomial distribution and logit link function. We treated year, site and diameter as random factors and morph as a fixed factor.

Reproductive success of floral morphs

a) Proportion fertilized ovules across sites and years: To investigate reproductive success and pollen limitation of the two floral morphs we counted the total number of ovules in each dissected infructescence mentioned above. We then recorded the number of fertilized ovules, which we identified by their larger size and considerable swelling of the ovary walls, which were dark green compared to those of unfertilized ovules. By dividing fertilized ovules by the total ovules for each infructescence, we got a proportional measure of reproductive success that incorporates pollen

limitation and controls for individual variation in reproductive potential. We arcsin transformed these data and analysed it across multiple sites and years with a GLMM and a Gaussian distribution treating year, site and diameter as random factors and morph as a fixed factor.

b) Breeding system of U. calenduliflora: We collected seeds from natural populations in Namaqualand during 2010 and grew them in a greenhouse under ambient conditions and a set water cycle at Stellenbosch University, South Africa during 2011. We sowed seeds in pots containing a mixture of sand and compost (1:1). At 84 days after planting, we measured seedling heights (from the Bovlei population) and thinned seedlings to the strongest individual. For the remainder of the experiment we added additional water and nutrients (Nitrosol, Fleuron, South Africa) as required. Focal plants received some, or all of four treatments: a) outcross pollen from another individual of the same morph, b) outcross pollen from another individual of the other morph, c) self pollination, d) no pollination (inflorescences bagged before opening). We applied pollen with artist brushes that we dipped in alcohol between treatments to remove pollen grains. Kruskal Wallis ANOVA was used to analyse differences in seed production between the various pollination treatments.

Influence of ecological factors on the distribution of floral morphs: To investigate how these ecological factors influence the observed proportion of spotted inflorescences (dependent variable) in populations we built a model to include all the factors we measured. These comprised measurements of relative performance of the two morphs at each site, including the mean proportion fertilized ovules, proportion of inflorescences with damaged ray florets and proportions of infructescences that suffered ovule damage for each morph. We used these measures as factors in determining the proportion spotted inflorescences at a site the following year with a Generalized Linear Model (GLM) with a Gaussian distribution. Statistical analyses including the nested GLMM for insect visitation were carried out in the SPSS 19 package (SPSS Inc., Chicago, USA). All remaining GLMM analyses were conducted with the lme4 package in R

(R Development Core Team 2008). Interaction terms were investigated in all models and excluded from final analysis if they were non-significant and models without them had better likelihood scores.

RESULTS

Spatial and temporal variation in the distribution of floral morphs: During three years of sampling we found 21 *U. calenduliflora* populations (Figure 5.2). Only eight of these were polymorphic and the rest were monomorphic for the spotted morph and typically occurred at lower altitudes. We found no populations monomorphic for the plain morph. The proportion of spotted inflorescences did not change significantly between years or sites (Table 5.1). Altitude, however, had a significantly negative effect on this measure, because the plain morph occurred mostly at sites with elevations greater than 1000m above sea level.

Insect visitation patterns

Field observations: For all dominant flower-visiting groups, there was significant variation in visitation rates between floral patches (Table 5.2), indicating substantial spatial variation in insect visitation. *M. capensis* males, which were the most common visitor to both morphs, exhibited strong and consistent preference for the spotted morph (Figure 5.3). Blister beetles (Meloidea) exhibited preference for the plain morph, although their visitation rates were the lowest of all the visiting insect groups and were seldom observed (Figure 5.3).

b) Preference of male and female M. capensis: Male individuals of *M. capensis* exhibited significant preference for the spotted morph of *U. calenduliflora* during our cage experiments, while females showed no preference (Figure 5.4). This pattern mirrors that of our field observations. If *M. capensis* males act as effective pollinators, their preference for the spotted morph may represent a selective advantage through increased reproductive success.

c) M. capensis males as effective pollinators on the different morphs: Our experiments investigating the effectiveness of *M. capensis* males as pollinators revealed that they can successfully collect and deposit pollen on both floral morphs of *U. calenduliflora*. There were no significant differences ($t = 0.42$, $df = 8$, $p = 0.68$) between pollen analogues exported to other inflorescences on floral arrays of the plain (mean inflorescences receiving pollen analogue = 5.22) and the spotted morph (mean inflorescences receiving pollen analogue = 4.78), indicating they can effectively pollinate both morphs.

Florivory damage

a) Damage to ray florets in natural populations: Results from the GLMM analyses on our transect data revealed that there was no variation in the incidence of damage to ray florets by florivores between morphs (Table 5.3). There were, however, significant effects of site and year, indicating that there is significant spatial and temporal variation in the extent of this antagonistic interaction within natural populations of *U. calenduliflora*.

b) Damage to ovules inside maturing infructescences: During our dissections we found multiple, as yet unidentified, species of beetle larvae and pupae inside the infructescences of *U. calenduliflora*, all of which we are currently sequencing to confirm identification. The presence of larger larvae and pupae generally resulted in more damage, although this was not quantified. Results from our GLMM analyses showed that the only significant factor affecting ovule damage by florivores is morph, with most of the damage being suffered by the spotted morph (Table 5.4). There was little spatial or temporal variation suggesting that antagonistic selection exerted by florivores on this trait was consistent between sites and years.

Reproductive success of floral morphs

a) Proportion fertilized ovules across sites and years: Results revealed significant temporal variation in the proportion of fertilized ovules with year being a highly significant factor (Table

5.4). Reproductive success was also influenced by diameter with larger inflorescences exhibiting a higher proportion fertilized ovules than smaller inflorescences. Morph was a near significant factor with the spotted morph having higher overall reproductive success than the plain morph. Again, there was very little spatial variation in this measure.

b) *Breeding system of U. calenduliflora:* This species is largely self-incompatible and incapable of autogamy as most inflorescences in the self-pollination treatment produced very few seeds (Spotted mean = 1.53 ± 5.56 *N* = 10; Plain mean = 1.44 ± 1.97 *N* = 10) and those in the unmanipulated bagged treatment produced no seeds at all (*N* = 13). There were no significant differences between outcross treatments as analysed by Kruskal-Wallis ANOVA (Figure 5.5), except for the *Plain-Plain* cross that produced significantly less seeds that the *Spot-Spot* cross ($z = 2.97$; $p = 0.018$). The two morphs are thus interfertile and can freely outcross in natural populations. There was no difference in seedling height between the two morphs as measured at day 84 (T-test for independent groups: $t = -1.62$, $df = 42$, $p = 0.11$).

Influence of ecological factors on the distribution of floral morphs: Results from our GLM analysis revealed that the proportion of fertilized ovules for spotted morphs had a significantly positive effect on the proportion of spotted inflorescences at a site the following year (Table 5.5). Conversely, the proportion of fertilized ovules for plain morphs had a significant effect in the opposite direction. The proportion of spotted infructescences that experienced ovule damage by florivores had a significantly negative effect on the observed proportion spotted inflorescences the following year. Ovule damage to plain morphs, however, had a significantly positive effect. The proportion inflorescences that experienced ray floret damage showed similar patterns, although these were not significant, suggesting that the main cost of florivory is the loss of ovules.

Although these measures do not take into account possible dormancy in the seeds of *U. calenduliflora* it does shows how reproductive success and ovule damage may influence the observed proportion of spotted inflorescences in natural populations. This proportion itself, may

of course also affect pollination and florivory rates at sites, although we detected no significant spatial variation for either reproductive success or ovule predation across multiple sites (Table 5.4), even though sites exhibited some variation in their proportion of spotted inflorescences (Figure 5.2). Measures of the proportion fertilized ovules at a site may also be influenced by the proportion of inflorescences that experienced ovule predation, although we did not find a strong relationship between these measures for either spotted (Pearson r = -0.047, P > 0.05) or plain morphs (Spearman R = 0.286, P > 0.05). This is probably because the proportion of inflorescences that suffered predation were typically low (< 0.15), indicating that this factor did not confound our measures of reproductive success.

DISCUSSION

Our results reveal two consistent and opposing selective pressures on floral phenotype. Male *M. capensis* flies, the dominant visitors and effective pollinators of *U. calenduliflora*, always exhibit preference for the spotted morph of *U. calenduliflora*. This is probably a result of innate preferences in male flies for dark spots related to mate-searching behaviour, which the spotted morph is likely exploiting like other daisies in this region (*Gorteria diffusa* - De Jager and Ellis 2012, Chapter 4). Florivory damage due to larvae feeding on ovules inside infructescences, however, was also significantly more prevalent in the spotted morph. These two forces of selection may thus be maintaining floral polymorphism in *U. calenduliflora*, as both had a significant effect in determining the observed proportion of spotted inflorescences at a site the following year. Shared preference between mutualists and antagonists for the same floral traits is often reported (Irwin et al. 2003, Frey 2004, Strauss et al. 2004, Ashman et al. 2004) with either pollinators (Sanchez-Lafuente 2002) or herbivores (Cariveau et al. 2004, Parachnowitsch and Caruso 2008) exerting the strongest selection.

The greater occurrence of florivorous larvae on the anthocyanin containing spotted morph may be influenced by the fact that anthocyanins are the end products of the same

biochemical pathway that produces anti-herbivorous compounds such as flavones, flavonols and tannins (Fineblum and Rausher 1997). A relative increase in anthocyanin production may thus be associated with a relative decrease in the production of anti-herbivory compounds. If, for instance, mutations block the production of floral anthocyanin in one morph, it may accumulate more defensive compounds than morphs still producing anthocyanins, which may affect observed florivory rates. However, this will only be the case if the mutations blocking anthocyanin production does not also affect the production of intermediate, defensive compounds, (i.e. only end products are affected - Fineblum and Rausher 1997). Since pollinator attraction and herbivory defense can be linked by a common biosynthetic pathway, it provides a mechanism where both pollinators and florivores can exert selection on the same floral trait, although in different directions. The relative strength of selection exerted by each will thus probably influence the abundance of the two morphs in natural populations of *U. calenduliflora*.

While ovule predation by antagonistic florivores significantly affected the spotted morph, this morph only enjoyed a near significant increase in female reproductive success due to enhanced pollinator attraction. *M. capensis* males, which act as effective pollen exporters for *U. calenduliflora*, visited the spotted morph nearly five fold as many times as the plain morph in natural populations, suggesting that the benefit of increased pollinator attraction to spotted *U. calenduliflora* may rather lie in greater male reproductive success (pollen export).

Variation in the composition and abundance of pollinators versus florivores at each site and year will likely be of great importance in determining the impact of each on morph ratios in *U. calenduliflora*. Our insect observations revealed spatial variation in the visitation rates of all dominant visiting groups, including pollinators (*M. capensis*) and florivores (monkey beetles and blister beetles). This may strongly influence pollination rates, as well as the extent of florivory damage suffered at each site. In fact, florivory damage to the ray florets of *U. calenduliflora* showed significant spatial and temporal variation. Although this type of floral damage was not specifically directed at either morph, it could still indirectly affect pollinator-mediated selection

as multiple studies have reported that damaged flowers suffer reduced attractiveness to pollinators (Krupnick et al. 1999, McCall and Irwin 2006) and produce less anthers and nectar that serve as rewards for pollinators (Krupnick et al. 1999). Differences in ray floret damage between sites may thus also potentially affect the relative strength of pollinator- and florivore-mediated selection.

While biotic factors are clearly important, floral polymorphism can also be maintained by abiotic factors such as the presence of drought conditions. Floral morphs containing anthocyanin often exhibit higher reproductive fitness during drought than floral morphs without anthocyanin (Schemske and Bierzychudek 2001, Warren and McKenzie 2001). This is likely due to the role that anthocyanin plays in maintaining metabolic activity under stressed conditions (Tholakalabavi et al. 1997) and is actually a correlated response to selection on anthocyanin levels throughout the entire plant (Warren and McKenzie 2001). Although we did not investigate this in *U. calenduliflora,* we did find a significant effect of altitude on the presence and abundance of the plain morph. This observation may be important in light of the fact that floral morphs that do not contain anthocyanin often produce more seeds than anthocyanin containing morphs under well watered conditions (Warren and McKenzie 2001), which are more likely at higher elevations.

Our hand crosses between plain morphs without floral anthocyanin, however, produced significantly less seeds than crosses between spotted morphs and there was no difference in the seedling heights of plain and spotted morphs grown under well-watered conditions in the greenhouse, suggesting there may be no inherent fitness advantage to plain morphs at high elevation sites. Rather we argue that variation in the composition and abundance of pollinating versus ovule damaging florivorous insects across the landscape determines morph ratios in *U. calenduliflora*. Damage to ray florets by another group of indiscriminate florivores may also play a potential role by reducing the strength of pollinator-mediated selection. These results may offer some insight as to why many studies fail to detect pollinator-mediated selection (reviewed in Harder and Johnson 2009) or report substantial spatio-temporal variation in the strength of

selection exerted by pollinators (Thompson 2001, Ellis and Johnson 2010b, Lay et al. 2011). It also highlights the many players in the selective arena that affect floral phenotype and the value of a more inclusive approach to the study of floral evolution.

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Table 5.1
Results from GLMM analysis testing the effects of Altitude, Year and Site on the proportion of spotted inflorescences in *U. calenduliflora* populations.

Source	χ^2	*P*
Altitude	8.456	* (-)
Year	0	NS
Site	0	NS

χ^2*statistics calculated with analysis of deviance. * = $P < 0.05$. (-) indicates a negative effect

Table 5.2
Results from GLMM analyses investigating the effects of Morph, Year and Patch (nested within Year) on the visitation rates of the dominant insect visitors to natural patches of *U. calenduliflora*.

Source	*M. capensis* males		*M. capensis* females		Monkey beetles		Blister beetles	
	Test statistic	*P*	Test statistic	*P*	Test statistic	*P*	Test statistic	*P*
Morph	$F = 45.39$	**	$F = 2.414$		$F = 0.19$		$F = 4.90$	*
Year	$z = 0.84$		$z = 0.92$		$z = 0.84$		$z = 1.00$	
Patch	$z = 3.45$	**	$z = 4.64$	**	$z = 6.50$	**	$z = 2.00$	*

* = $p < 0.05$; ** = $p < 0.001$

Table 5.3
Results from GLMM analysis testing the effects of Morph, Year and Site on the incidence of ray floret damage due to florivores in *U. calenduliflora*.

Source	χ^2	*P*
Morph	0.131	NS
Year	52.827	**
Site	141.37	**

χ^2*statistics calculated with analysis of deviance. * = $P < 0.05$, ** = $P < 0.001$

Table 5.4
Results from our GLMM analyses testing the effects of Morph (fixed effect) and Diameter, Year and Site (random effects) on the proportion of fertilized ovules, and the incidence of ovule predation in *U. calenduliflora*.

	Fertilized ovules		Ovule predation	
Source	χ^2	*P*	χ^2	*P*
Morph	3.539	^	16.188	**
Diameter	11.816	** (+)	0	NS
Year	498.44	**	1.319	NS
Site	2.097	NS	1.039	NS

χ^2*statistics calculated with analysis of deviance.
* = $P < 0.05$, ** = $P < 0.001$, ^ = $P < 0.06$
(+) indicates a positive effect

Table 5.5
Results from GLM analysis investigating the effects of various ecological factors on the observed proportion of spotted inflorescences the following year in polymorphic *U. calenduliflora* populations.

Source	*B*	Wald Chi-χ^2	*df*	*P*
PropSpot Fertilized	0.059	8.011	1	*
PropPlain Fertilized	-0.119	5.284	1	*
PropSpot RayDamage	0.690	1.827	1	NS
PropPlain RayDamage	-8.504	3.090	1	NS
PropSpot OvuleDamage	-2.761	4.764	1	*
PropPlain OvuleDamage	2.690	5.157	1	*

Regression coefficients (*B*) indicate the direction of effect on the proportion spotted inflorescences the following year. * = $P < 0.05$

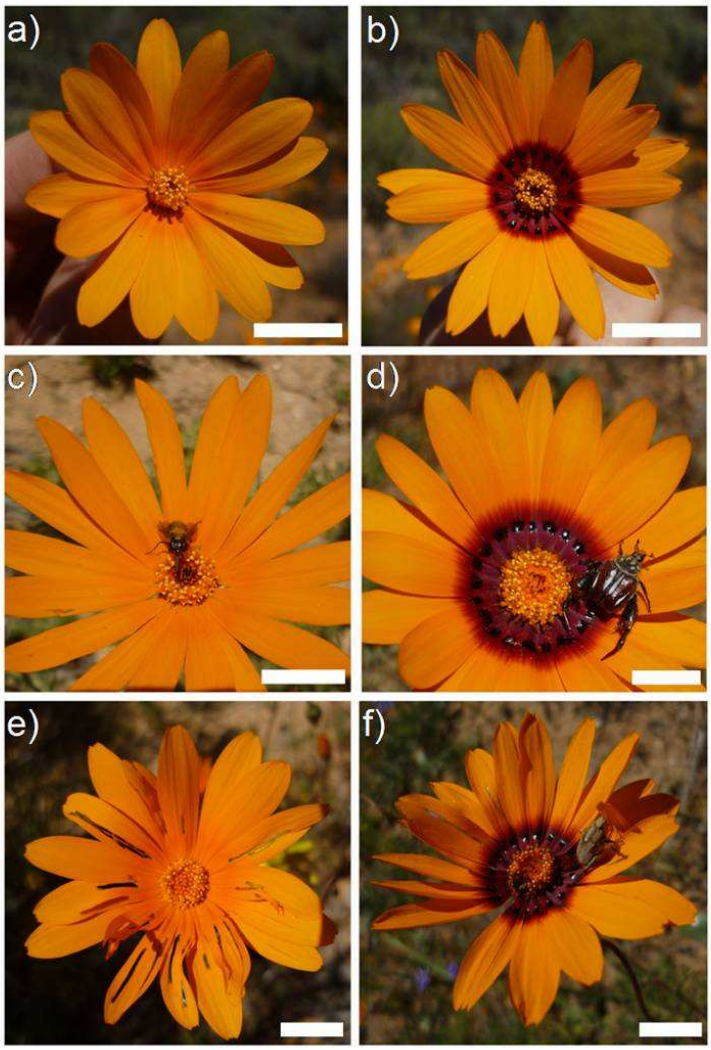

Figure 5.1. The a) plain and b) spotted floral morphs of *Ursinia calenduliflora*. c) shows the common bee fly visitor *M. capensis* drinking nectar from a plain inflorescence while d) shows a monkey beetle resting on the ray florets. These and blister beetles often cause damage to ray florets as seen in e) and f) where a monkey beetle is eating ray petals. Scale bar represents 1cm. All photos by Marinus de Jager.

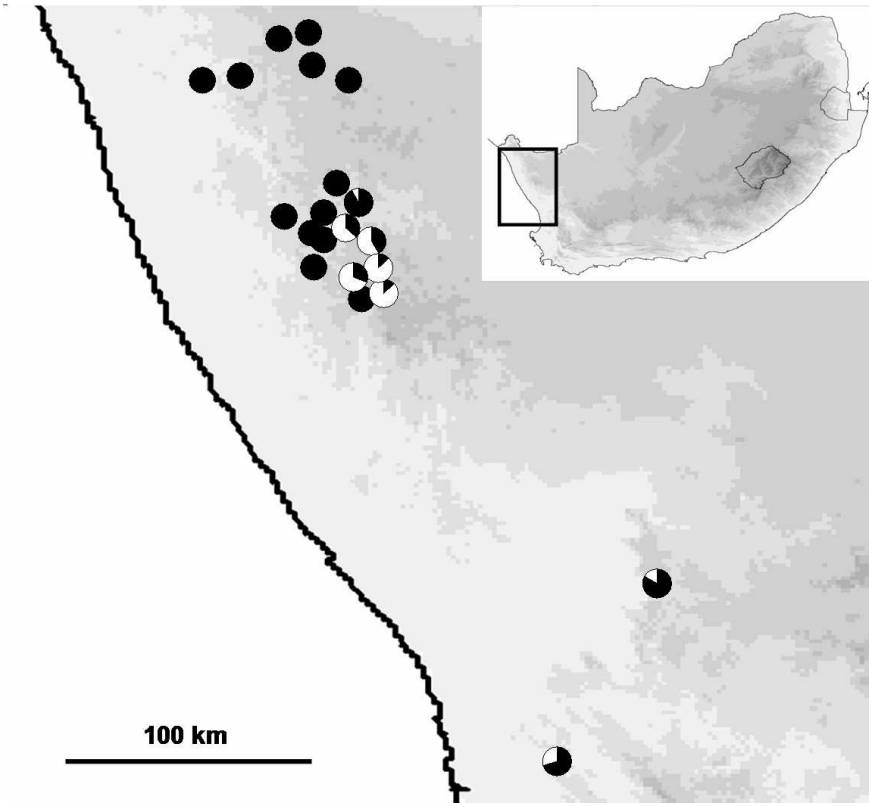

Figure 5.2. The spatial pattern of floral polymorphism in *U. calenduliflora* within Namaqualand, South Africa. Pie charts show the proportion of spotted (black) inflorescences within populations. The average proportion was used for polymorphic sites sampled over multiple years.

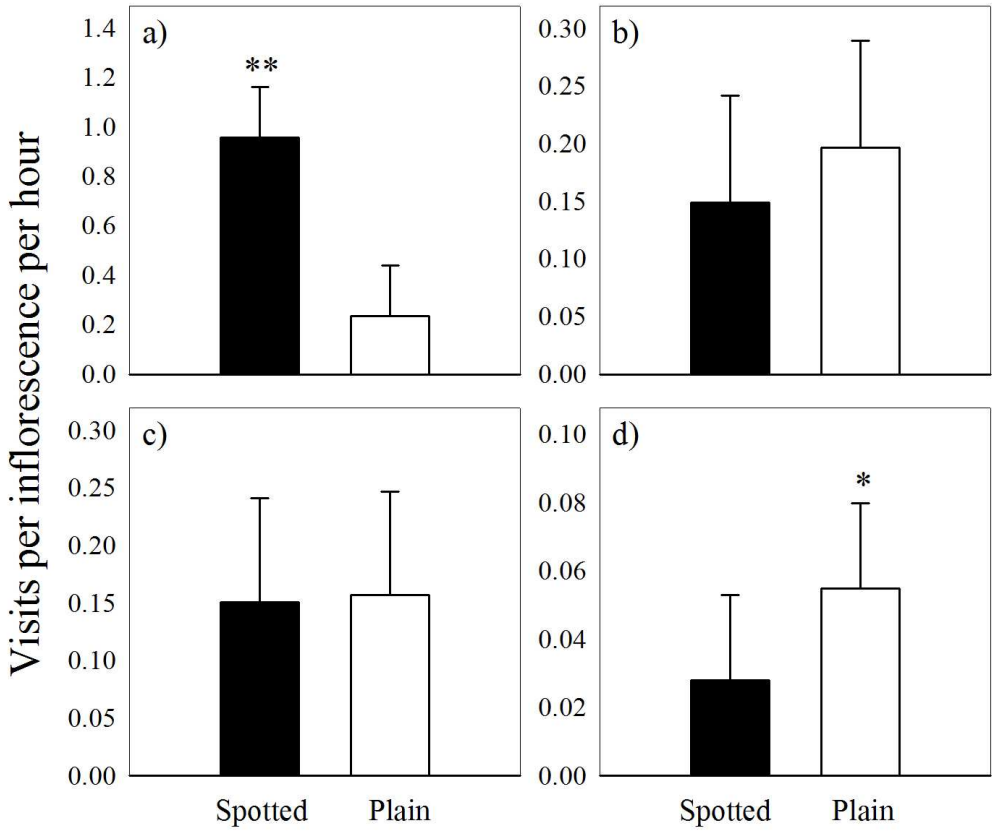

Figure 5.3. The means and standard errors for the number of visits per inflorescence per hour to spotted and plain floral inflorescences of *U. calenduliflora* made by a) *M. capensis* males, b) *M. capensis* females, c) various species of monkey beetles and d) various species of blister beetles. Significant differences between visits to plain and spotted floral morphs are indicated with * ($p < 0.05$) and ** ($p < 0.005$).

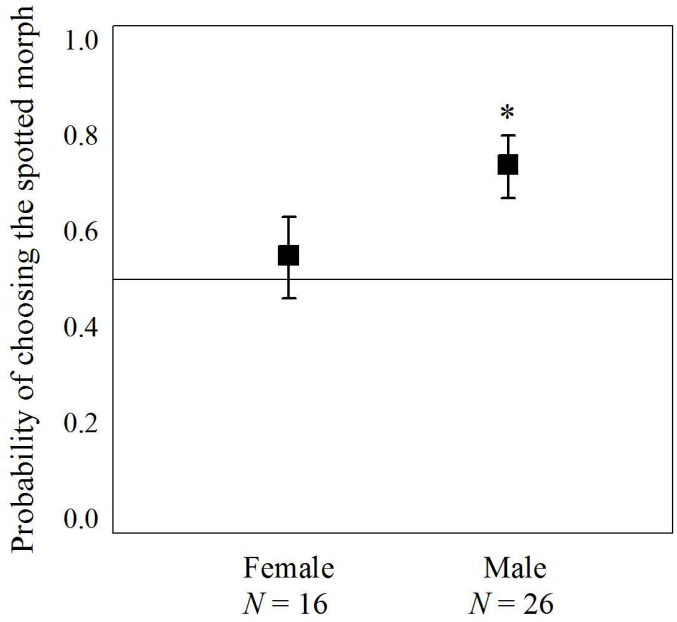

Figure 5.4. Estimated marginal means with their 95% Wald confidence intervals for male and female *M. capensis* flies are shown for their probability of choosing to land on the spotted morph during cage experiments with mixed arrays of both floral morphs of *U. calenduliflora*. * indicates $p < 0.05$

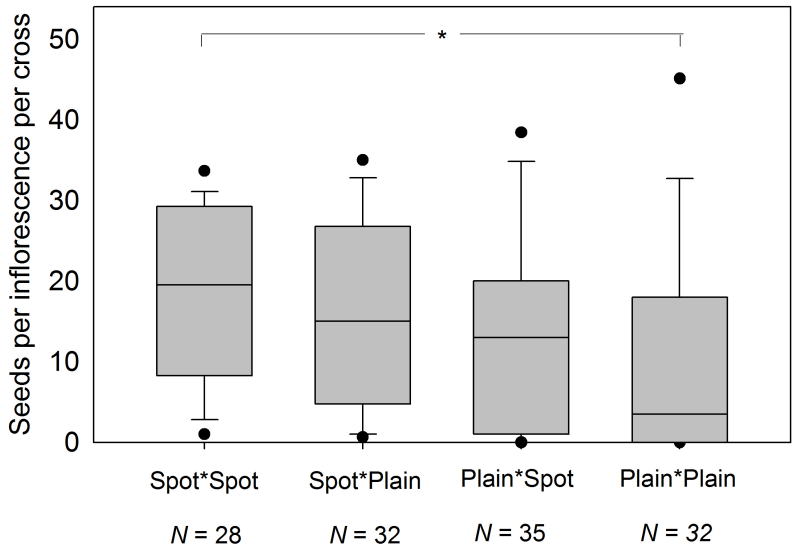

Figure 5.5. The median and 95th and 5th percentile of the number seeds produced per inflorescence per cross between the morphs of *U. calenduliflora*. The number of plants receiving each pollination treatment is indicated on the X-axis. The only significant difference was between the Plain-Plain and the Spot-Spot cross, * indicates $p < 0.05$

Chapter 6

Phylogeography of the pollinator *Megapalpus capensis* (Diptera: Bombyliidae) in the Greater Cape Floristic Region

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ABSTRACT

The Greater Cape Floristic Region (GCFR) in South Africa exhibits astounding plant species richness and comprises two biodiversity hotspots, the relatively mesic Fynbos biome and the semi-arid Succulent Karoo biome. Many studies investigating the evolutionary history of GCFR angiosperms report increased diversification associated with aridification beginning in the middle of the Miocene, as well as relatively older Fynbos biome lineages compared to Succulent Karoo lineages. No molecular studies are presently available for animals (such as pollinators) directly associated with the diverse GCFR flora. We present here the first study to investigate evolutionary relationships within an important pollinator whose range spans both these biomes. Fynbos lineages of the bee fly *M. capensis* (Diptera: Bombyliidae) are revealed to be relatively older than lineages from the Succulent Karoo, as determined by a mitochondrial *cox1* fossil calibrated BEAST analysis. Investigation into the demographic history of *M. capensis* from both mitochondrial (*cox1* and *cox2*) and nuclear (*EF1A*) genes reveal that Succulent Karoo lineages underwent a recent population expansion, while Fynbos lineages have remained demographically stable. Together, these results indicate that *M. capensis* has a longer evolutionary history in the Fynbos biome and has recently colonized the Succulent Karoo biome, a pattern matching that of many flowering plants from this area.

INTRODUCTION

The Greater Cape Floristic Region (GCFR) in South Africa boasts astounding biological diversity and comprises two of the world's biodiversity hotspots (the Fynbos and the Succulent Karoo biomes - Myers et al. 2000). Aridification beginning in the Miocene (Tyson and Partridge 2000, Diester-Haass et al. 2004) is believed to have contributed to it's high diversity of flowering plants (Linder 2003), which comprises over 10000 species, 71.6 % of which are endemic (Born et al. 2006). This remarkable diversity has prompted investigations into the evolutionary history of GCFR plants, many of which report increased diversification rates during the Miocene to

Pleistocene (Richardson et al. 2001, Klak et al. 2004, Linder et al. 2006, McKenzie and Barker 2008). Aridification during this period has also been invoked as a potential mechanism for radiation of reptiles (Tolley et al. 2006, Daniels et al. 2007, Swart et al. 2009) and small mammals (Russo et al. 2010) in the GCFR.

Molecular (Verboom et al. 2003, McKenzie and Barker 2008) and modelling studies (Midgley et al. 2005) suggest that the Succulent Karoo biome has recently expanded as aridification increased within the GCFR. In fact, a large comparative study investigating dated phylogenies for many angiosperm taxa revealed that Fynbos endemic lineages are significantly older than Succulent Karoo endemic lineages (Verboom et al. 2009). The Succulent Karoo is thus regarded as a relatively young biome comprising multiple recent radiations of angiosperms (Verboom et al. 2009). Succulent Karoo Aizoaceae, for example, contain more than 1500 species that have emerged in the last 3.8-8.7 million years, making this one of the fastest angiosperm radiations known globally (Klak et al. 2004).

Compared to floras of the northern hemisphere, angiosperms in the GCFR exhibit a relatively high degree of specialization on their pollinators (Johnson and Steiner 2000), which are often implicated as drivers of botanical diversity in this region (Johnson 1996, Johnson 2006, Van der Niet and Johnson 2009). Despite this, there is considerable paucity of knowledge regarding the evolutionary history of pollinators within this remarkable area, with no published studies to our knowledge. Some studies investigating comparative phylogeographies of plants specialized on their pollinators in North America have reported congruent demographic and evolutionary histories that have been interpreted as a potential signal of either coevolution (Smith et al. 2008) or shared biogeography (Smith et al. 2011, Althoff et al. 2012). Studies on plants and pollinators involved in specialized interactions in Europe, however, have found non-congruent evolutionary patterns (Espindola and Alvarez 2011), suggesting this is not always a likely outcome of specialization. Information on the evolutionary history of important GCFR pollinators would thus

be useful for investigating the roles of shared biogeographic history and potential coevolution in shaping molecular patterns of biota in this remarkable area.

Megapalpus capensis Wiedemann, a small beefly within the Mariobeziinae subfamily of the Bombyliidae (Diptera), is abundant in the Succulent Karoo biome where it visits and pollinates multiple annual daisies (Ellis and Johnson 2010a, Chapter 5). Its range, however, also covers the Fynbos biome to the south where it has been implicated in the pollination of several Geraniaceae species (Struck 1997). Within the Succulent Karoo, *M. capensis* is the predominant pollinator and most likely driver of floral diversification for the endemic daisy *Gorteria diffusa* (Ellis and Johnson 2010a, De Jager and Ellis 2012). Using this widespread pollinator as a model, we aim to a) explore the genetic structure and timing of *M. capensis* lineage diversification across the GCFR and b) to determine whether this pollinator exhibits similar patterns of ancestral biome affinity as angiosperms within the GCFR.

MATERIAL AND METHODS

Sampling & laboratory protocol: *M. capensis* individuals of both sexes were collected from sites (median flies per site = 3) across its known geographic range in South Africa (Figure 6.1). This included locations in both Succulent Karoo (36) and Fynbos (7) vegetation, where they are much less abundant. We collected outgroup species in the *Corsomyza* genus, which falls within the same subfamily as *Megapalpus*, from two different sites within the Fynbos biome. We caught all flies in the field and preserved them in 95% ethanol. Fly vouchers are held in the AGE collection at Stellenbosch University, South Africa. We extracted DNA with the Promega Wizard Genomic DNA purification kit (Madison, USA) protocol and sequenced both mitochondrial (*cox1* and *cox2*) and nuclear (*EF1A*) genes. We sequenced 109 samples for *cox1* with the universal primer pair (LCO1490 5'-GGTCAACAAATCATAAAGATATTGG-3' & HCO2198 5'-TAAACTTCAGGGTGACCAAAAAATCA-3') which yielded a 623bp fragment of the gene. From these samples we selected a subset of 39 representatives (representing all major *M. capensis*

cox1 clades) to be sequenced for additional genes, including *cox2* (C457B 5'-AACTAGTATCCTTTCATGAYCAYGC-3' & C457C 5'-GTGATTAGCACCGCARATYTC-3'), which yielded a 478bp fragment and the intronless nuclear *EF1A* gene (EF-F05 5'-CCTGGACATCGTGATTTCAT-3' & EF-F06 5'-TTACCTTCAGCGTTACCTTC-3'), which yielded a 303bp fragment.

PCR reactions (50 µl) contained 1.5mM $MgCl_2$, 1µM of the forward and reverse primers each, 0.2mM of each dNTP, 1 unit Taq polymerase (Super-Therm JMR-801; Southern Cross Biotechnologies), 5 µl 10X Buffer (Southern Cross Biotechnologies), 25 µl dH_2O and about 100ng (1 µl) of DNA template. Our thermal regimes for fragment amplifications were (*cox1 / cox2 / EF1A*): a denaturation step of 94°C for 5 min / 4 min / 3 min followed by 30 cycles of 94°C for 1 min / 30 sec / 30 sec, then 50°C for 30 sec/ 50°C for 1min / 45.9°C for 30 sec and lastly 72°C for 1 min / 2 min / 90 sec. All amplifications contained a final elongation step at 72°C for 5 min / 10 min / 10min. A Labnet Multigene gradient PCR thermal cycler (Sigma-Aldrich, St. Louis, USA) was used for amplification and PCR products were stained with ethidium bromide and run on a 1% agarose gel for confirmation under UV- light. All products were purified with QIAquick®spin columns (Qiagen, Valencia, USA) and sequenced with a BigDye terminator kit version 3.0 (Applied Biosystems, Foster City, USA) and analyzed on an ABI 3130XL Genetic Analyzer (Applied Biosystems, Foster City, USA). We only sequenced samples in the forward direction as preliminary sequencing in both directions yielded clean and unambiguous sequences. Ambiguous sites in the nuclear dataset were coded using the IUPAC codes. We viewed trace files in MEGA v.4 (Tamura et al. 2007) and edited them by hand before alignment with ClustalW (Thompson et al. 1994).

Phylogeographic analysis

Divergence dating: To investigate the evolutionary history and time of divergence of *M. capensis* we conducted a Bayesian analysis using the *cox1* dataset as this contained most samples and

produced a high resolution tree. Using the Akaike Information Criterion (Akaike 1974) as implemented in jModelTest v0.1.1 (Posada 2008) we selected the HKY+I+G model of sequence evolution and partitioned the dataset by the 1^{st}+2^{nd} and 3^{rd} codon positions. Substitution rates, rate heterogeneity and base frequencies were unlinked across codon positions. Starting trees were randomly generated and a constant size coalescent prior was selected for the tree models. We ran both a strict molecular clock and an uncorrelated lognormal relaxed molecular clock. To obtain an absolute date on divergence times we calibrated the root between *M. capensis* and *Corsomyza* to 44Mya and calculated the age of the time to most common recent ancestor (TMRCA) for internal nodes and their corresponding 95% highest posterior density (HPD) confidence intervals. This date is based on fossilized beeflies (*Corsomyza*) retrieved from Baltic Amber by Loew (1850) that have been dated to the mid Eocene through stratigraphic studies (Kosmowska-Ceranowicz and Muller 1985). A K-Ar radiometric study (Ritzkowski 1997) later confirmed this and placed the amber at 44.1±1.1 Mya.

Since fossil dates represents strong minimum age constraints, but weak maximum age constraints (Donoghue and Benton 2007) we used an exponential parametric distributions as prior for this root with an offset of 44Mya (mean 1 Mya) allowing us to impose a hard minimum (fossil age) with a soft maximum age bound, which clearly outperforms analyses employing only hard bounds (Yang and Ranala 2005). All models were run for 60 million generations in BEAST v1.5.3 (Drummond and Rambaut 2007), sampling parameters every 6000 states. We used Bayes factors to determine which clock performed best (with $2\ln BF_{10} \geq 2$ indicating positive support for model 1 over model 0 - Nylander et al. 2004). We also ran the best model without partitioning by codon positions and compared it with the partitioned model using Bayes factors. The model selected by Bayes Factors was run three times and we combined them with Log Combiner v1.5.3, discarding the first 10% of samples as the burnin phase in each case. We summarized resulting trees with TreeAnnotater (part of the BEAST package) and used FigTree v1.3.1 (Rambaut 2006) to view and edit it.

Ancestral state reconstruction: To determine whether ancestral lineages of *M. capensis* were associated with the Fynbos or the Succulent Karoo biome we scored each ingroup sample as either Fynbos or Succulent Karoo, based on the vegetation type where we caught the fly. Ancestor biome association was then reconstructed in Mesquite version 2.75 (Maddison and Maddison, 2011) using an unordered parsimony method.

Multi gene tree: We also ran an uncorrelated lognormal relaxed molecular clock analysis using both mitochondrial and nuclear genes for comparison to the topology obtained from our *cox1* analysis. Using jModelTest v0.1.1 (Posada 2008) we selected the HKY+I+G, HKY+G and GTR+G models of sequence evolution for the *cox1*, *cox2* and *EF1A* genes respectively. We partitioned all datasets by the 1^{st}+2^{nd} and 3^{rd} codon positions and unlinked substitution rates, rate heterogeneity and base frequencies across codon positions. This analysis was run for 60 million generations under the same parameters as our *cox1* analysis.

Network analyses: We conducted a separate analysis for each gene using the NeighborNet algorithm (Bryant and Moulton 2004) as implemented in SplitsTree 4.10 (Huson and Bryant 2006). For mitochondrial datasets, we used the DNA sequence alignments as input to generate networks. Due to potential heterozygosity within the nuclear dataset, however, we first resolved ambiguous positions within each sequence by determining the gametic phase of alleles with the program PHASE v2.1.1 (Stephans et al. 2001) as implemented in DNAsp v.5 (Librado and Rozas 2009). We ran the algorithm for 10000 generations with a burn-in period of 1000 and a thinning interval of one and considered phases resolved at the default probability threshold of 0.9. We then used POFAD v.1.03 (Joly and Bruneau 2006) to construct a standardized matrix of the pairwise distances among individuals from their pairwise allelic distances, which we used as input in our NeighborNet analyses for the nuclear *EF1A* gene.

Multi gene inference of demographic history: To investigate changes in potential population size in the past for the two biomes we calculated Tajima's D for each gene, which, when significantly negative, indicates population expansion or positive selection. We also calculated Fu's Fs values for detecting population growth. For comparison, we also calculated mismatch distributions for each gene in each biome. In these tests, observed pairwise differences are compared to data that has been simulated under an expansion model. Sum of square deviations (SSD) and Harpending's raggedness index were used determine the fit of observed data to the model, with significant values rejecting a hypothesis of population expansion. All test were carried out in Arlequin 3.10 (Excoffier and Schneider 2005), except those for the nuclear *EF1A* gene that we conducted in DNAsp v.5 after determining the gametic phase of all alleles.

Genetic variation between biomes: We used an AMOVA (Excoffier et al. 1992) analysis as implemented in Arlequin 3.10 (Excoffier and Schneider 2005) on the *cox1* dataset, which had sufficient sampling size, using sites as populations. We grouped all populations as either Fynbos or Succulent Karoo to investigate the partitioning of genetic variation between these two biomes. We included the sequences from flies caught in the Succulent Karoo on the mountainous Fynbos peaks within the Succulent Karoo group, as they clearly group there genetically (Figure 6.2).

RESULTS

Phylogeographic analysis

Divergence dating: For the *cox1* dataset, 24.4% of the ingroup sites were Parsimony-Informative. As determined by Bayes factors the uncorrelated lognormal relaxed molecular clock analysis outperformed both the strict clock analysis ($2\log_e$ BF = 2.66) and the uncorrelated lognormal relaxed molecular clock without partitioning by codon position analysis ($2\log_e$ BF = 6.88). Our analysis combining three runs of the preferred model revealed that *M. capensis* separates into

three Fynbos clades and five Succulent Karoo clades (Figure 6.2). Ancestral state reconstruction revealed that Fynbos biome lineages were basal, except for the samples from the three high elevation sites in Namaqualand that contain fynbos peaks in an otherwise entirely Succulent Karoo landscape. Two Succulent Karoo lineages, corresponding to the Olifants river valley situated between the two biomes and a northern coastal site, form the basal branches for this biome. Two samples from this coastal site, however, form part of the Northern Namaqualand clade, which suggest this site may contain ancestral polymorphisms.

The divergence date for the oldest Fynbos clade was 16.79 Mya (95% HPD: 31.13 – 8.68 Mya), whereas the oldest Succulent Karoo clade was 8.49 Mya (95% HPD: 14.83 – 3.88 Mya). *M. capensis* appears to have colonized the Succulent Karoo biome round this time from ancestral Fynbos biome lineages. Succulent Karoo lineages subsequently diversified with most lineages arising during the Pliocene. The three main Namaqualand clades in our sampling area diversified roughly during the last 4 million years; Northern Nama clade = 3.37 Mya (95% HPD: 4.91 – 1.23 Mya); Central Nama clade = 4.06 Mya (95% HPD: 7.08 – 1.95 Mya) and the Southern Nama clade = 3.35 Mya (95% HPD: 5.16 – 1.38 Mya).

Multi gene tree: Results from our Bayesian analysis using those samples sequenced for both mitochondrial (*cox1* and *cox2*) and nuclear genes (*EF1A*) revealed a similar topology to our more detailed *cox1* tree (Figure 6.3).

Network analyses: The network analysis we conducted for the mitochondrial genes investigated in this study show similar patterns as the *cox1* BEAST analysis, with well-resolved Namaqualand clusters in the Succulent Karoo biome and deeply divergent Fynbos clusters (Figure 6.4 a & b). The nuclear *EF1A* gene, however, failed to resolve the various clades in these biomes and exhibits no clear patterns (Figure 6.4 c), indicating limited utility of this gene to resolve genetic relationships in this species.

Multi gene inference of demographic history: Fu's Fs values that deviate significantly from zero and indicate population growth (Fu 1997) were only observed for the Succulent Karoo samples (Table 6.1), indicating widespread expansion within this region. We also found negative values for Tajima's D, which indicate population expansion or positive selection (Tajima 1989) for Succulent Karoo samples, although these were not significant (Table 6.1). From our mismatch distribution analyses, all three genes failed to reject a population expansion hypothesis for the Succulent Karoo samples.

Genetic variation between biomes: Our AMOVA analysis revealed that genetic variation is indeed strongly structured by biogeographical biome (Table 6.2).

DISCUSSION

This study is the first to investigate the evolutionary history of a pollinator within the biodiversity rich GCFR in South Africa. The bee fly pollinator, *M. capensis*, comprises several lineages in this area with a clear separation between lineages in the Fynbos biome to the south and the Succulent Karoo biome towards the north. Fynbos lineages are substantially older than the Succulent Karoo lineages, confirming the relatively older age of this biome as inferred from molecular studies on angiosperm taxa in the GCFR (Verboom et al. 2009). While the Succulent Karoo lineages show modest divergence, the ancestral Fynbos lineages exhibit deep divergence suggesting little geneflow between southern populations. *M. capensis* has a less continuous distribution in the Fynbos biome, which may strongly influence this pattern, although our small sample size for this biome likely also affects phylogenetic signal (Blomberg et al. 2003). We are currently in the process of increasing our sample size for flies in the Fynbos biome, which are considerably scarcer and harder to find than in the Succulent Karoo biome.

Results from our network analyses for mitochondrial genes confirmed the topology of our *cox1* tree and also found two of the four samples from the coastal Kleinzee population to group on their own in a basal Succulent Karoo clade. This was the only locality to show such disparity in terms of the placement of its samples and suggest that lineages at this site may be relatively old for this biome. This could be due to ancestral polymorphism and potentially indicates that Fynbos lineages colonized the Succulent Karoo via a coastal route. Unfortunately, we have relatively few coastal samples and are currently unable to statistically test this hypothesis. Network analysis of DNA fragments from the nuclear *EF1A* gene could not resolve the various clades confirmed by the mitochondrial genes. Investigation into the demographic history of *M. capensis* using all molecular datasets, however, offered some support for colonization of the Succulent Karoo. Both mitochondrial and nuclear datasets for only the Succulent Karoo biome exhibited significant values of Fu's Fs, which detects population growth (Fu 1997). These datasets also showed consistently negative, although non-significant, values for Tajima's D test that indicates population expansion or positive selection (Tajima 1989).

In addition to these results, neither of the mitochondrial datasets for the Succulent Karoo could successfully reject the hypothesis of population expansion as determined by the sum of squares deviations from mismatch distributions. Together, these results suggest that Succulent Karoo lineages have undergone recent population expansion. Combined with the relatively younger age of Succulent Karoo lineages this indicates that, like angiosperm taxa (Verboom et al. 2009), the pollinator *M. capensis* has a longer history in the Fynbos biome and have colonized the Succulent Karoo biome from the late Miocene onwards. Pollen cores from areas in the GCFR suggest that it was much more mesic in the distant past and contained forest and fynbos elements (Linder 2003, Udeze et al. 2005) and has steadily been experiencing aridification from about 6 Mya (Tyson and Partridge 2000). As the area aridified some lineages may have tracked the movement of the Fynbos biome, while other may have adapted to the new Succulent Karoo climate. This seems to be the dominant pattern for clades of GCFR angiosperms (*Moraea* -

Goldblatt et al. 2002, *Ehrharta* - Verboom et al. 2003, *Melianthus* – Linder et al. 2006, *Tribolium* – Verboom et al. 2006, *Muralitia* – Forest et al. 2007 and *Pentachistis* – Galley and Linder 2007), as well as the pollinator *M. capensis* in this study.

Such patterns of shared biogeography between plants and pollinators have been documented for North American yuccas and the yucca moths that pollinate them (Smith et al. 2011, Althoff et al. 2012). These taxa show contemporaneous signals of population expansion (Smith et al. 2011) as well as cospeciation (Althoff et al. 2012), which has been attributed to shared biogeography. Some studies in this system, however, have found asynchronous divergence dates between plants and pollinators that may cast doubt on the importance of shared biogeography (Smith et al. 2009). Within the GCFR, there are some taxa that show an opposite pattern of colonization from the more arid north into the less arid south, for instance, arid adapted reptiles (Portik et al. 2011). Another study on insects has also reported ancestral lineages from arid Namibia with the more mesic lineages in South Africa being derived (Damgaard et al. 2008).

For the GCFR, however, our results with *M. capensis* suggest that evolutionary patterns between plants and pollinators may be congruent, although further studies on important pollinators are required. Comparison of the phylogeographies of plants and pollinators involved in specialized and /or obligate interaction would be of special interest as these taxa are expected to exhibit the most similar evolutionary histories due to the potential role of coevolution on evolutionary history. While patterns of codivergence and cospeciation may be indicative of this process, they can also arise through shared biogeographical history (Althoff et al. 2012), indicating that ecological and evolutionary field studies of interactions between specialized species are needed to help interpret patterns of congruent evolutionary histories. The pollinator *M. capensis* is strongly implicated in the divergence of floral forms in the Succulent Karoo endemic *Gorteria diffusa* (Ellis and Johnson 2010a, De Jager and Ellis 2012, Chapters 3 and 4). This annual daisy exhibits great variation in its interactions with *M. capensis*, ranging from antagonistic sexual deception to mutualistic foraging – pollination interactions (Ellis and Johnson

2010a), and has responded to selection exerted by *M. capensis* (De Jager and Ellis 2012). *M. capensis* may also respond to selection exerted by *G. diffusa* (Chapter 4) and comparative molecular analyses of these two species may thus offer some insight into the potential role of coevolution in shaping the genetic histories of closely interacting species. While this is part of an ongoing project, our current study is the first to reveal the evolutionary history of a GCFR pollinator and the first to report congruent patterns to the plants that characterize this remarkable area.

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Table 6.1
The values for SSD, Raggedness Index, Tajima's D and Fu's Fs for each gene in each biogeographical area.

Biome	Gene	SSD	Ragged Index	Tajima's D	Fu's Fs
Succulent Karoo	*cox1*	0.003	0.002	-1.027	-24.030 **
	cox2	0.006	0.006	-1.039	-19.912 **
	EF1A	-	0.003	-1.375	-67.368**
Fynbos	*cox1*	0.031 *	0.028	1.516	-0.883
	cox2	0.054	0.070	0.814	0.379
	EF1A	-	0.017	-1.303	-4.005

Significant values for Fu's Fs and SSD are indicated with * = $p < 0.05$; ** = $p < 0.001$. Fu's Fs indicates population growth whereas significant values for SSD reject a population expansion hypothesis.

Table 6.2
Results from our hierarchical AMOVA that partitioned variance in the mitochondrial *cox1* dataset between the Succulent Karoo and Fynbos biomes.

Variation	df	Sum of squares	Variance component	% Variance	*P*
Among biomes	1	228.10	12.14	51.29	<0.001
Between populations	38	829.43	6.63	28.01	<0.001
Within populations	63	308.5	4.50	20.70	<0.001

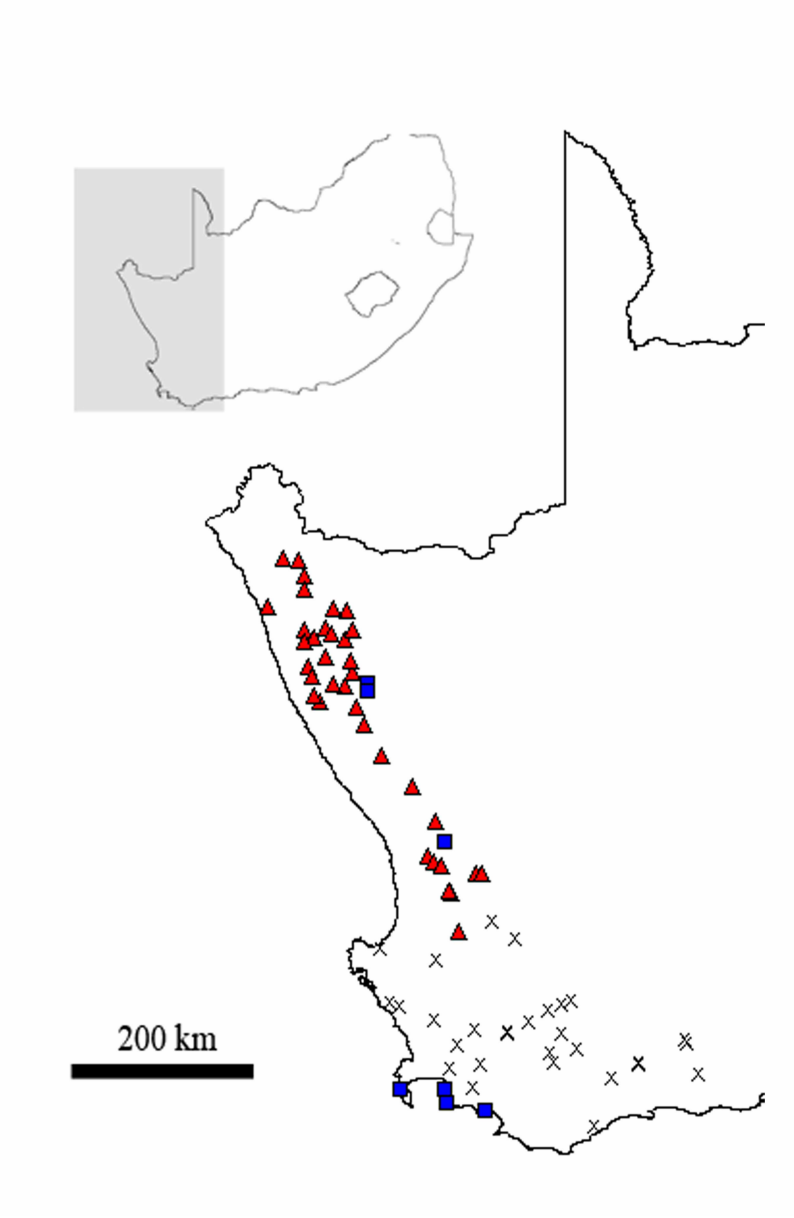

Figure 6.1. Sampled localities of *M. capensis* coded by the vegetation type where they were caught. Red triangles indicate the Succulent Karoo biome while blue squares indicate the Fynbos biome. Black crosses are locations predominantly in the Fynbos biome where we have searched for *M. capensis* over three consecutive years without finding them and thus indicate potential gaps in its range.

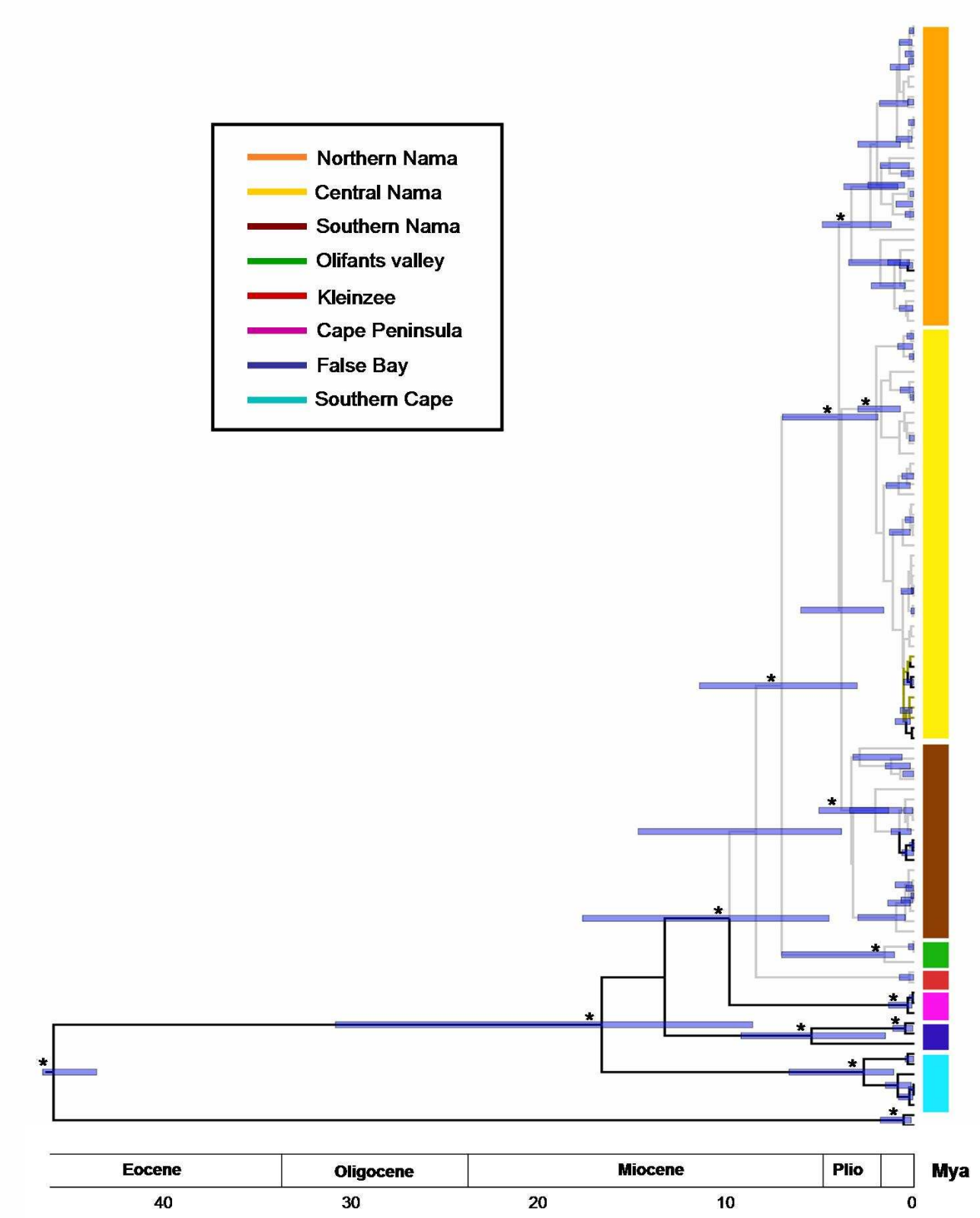

Figure 6.2. Divergence dated *cox1* Bayesian tree based on 107 sequences from 43 *M. capensis* sites across the GCFR. Biome affinity of ancestral nodes has been reconstructed and are shown by black (Fynbos biome), grey (Succulent Karoo biome) and green (unresolved) branches. Main clades are indicated with coloured bars described in the figure. Node bars show the TMRCA with the distribution of the 95% HPD for each estimate. The scale bars show time in millions of years and internal nodes with posterior probability higher than 95% are indicated with *.

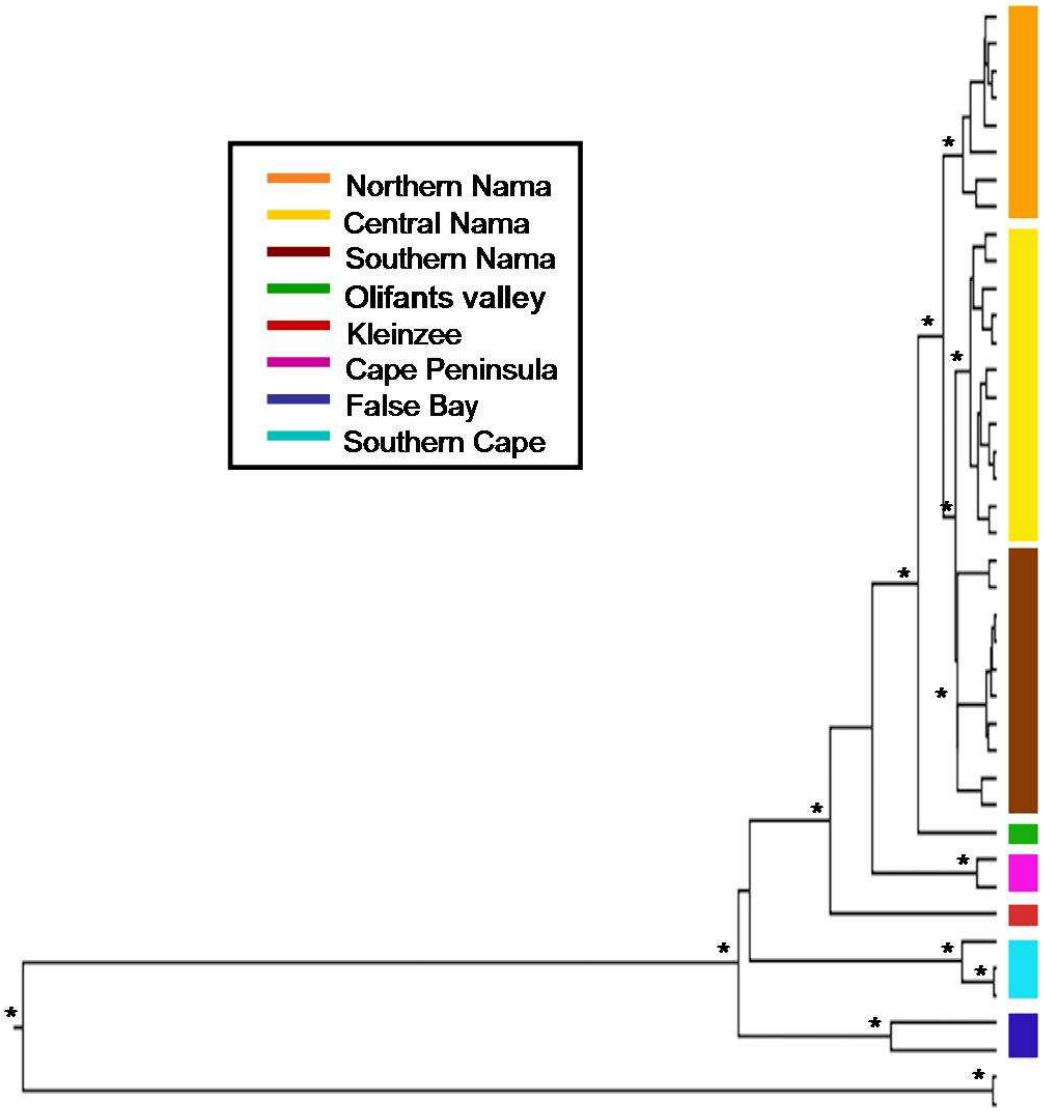

Figure 6.3. Multi gene Bayesian tree based on the subset of samples sequenced for both mitochondrial (*cox1* and *cox2*) and nuclear (*EF1A*) genes. Clades are colour coded and nodes with posterior probability higher than 95% are indicated with *.

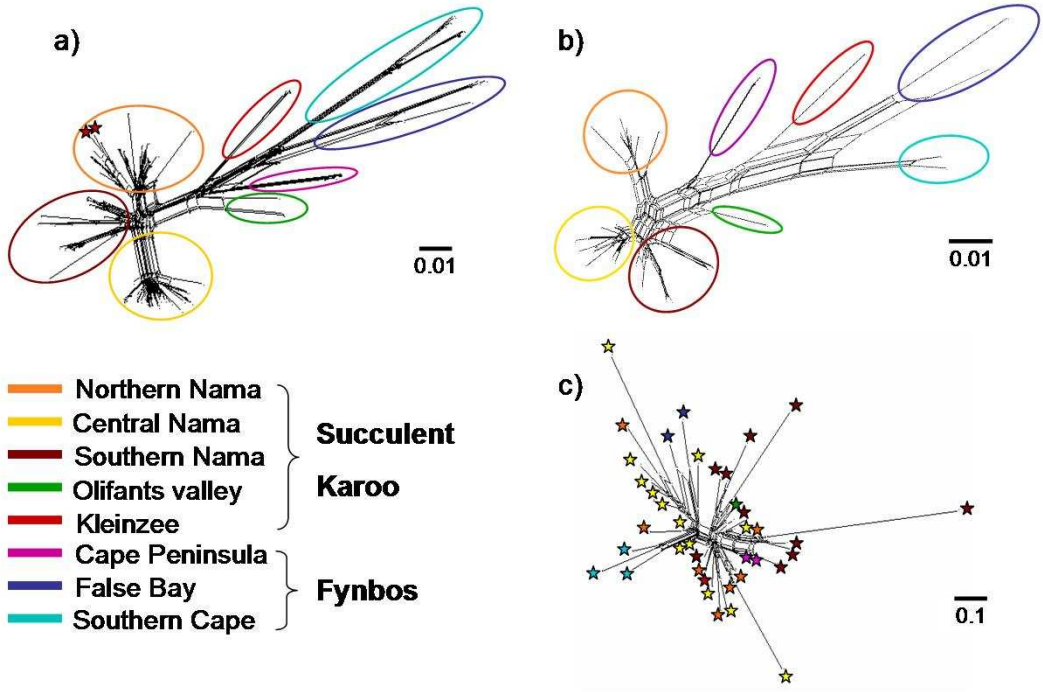

Figure 6.4. Network analyses conducted with NeighborNet in SplitsTree 4.10 for the a) *cox1*, b) *cox2* and c) *EF1A* genes. Both the mitochondrial genes recovered the main Fynbos and Succulent Karoo biome clades, although the nuclear *EF1A* failed to do so.

Chapter 7

Conclusions

The influence that animal visitors have on floral evolution is clearly complex and dynamic. While some aspects can be relatively straightforward, such as directional selection by a pollinator for a given floral trait, other factors also play important roles in the evolution of floral form and the realized phenotypes observed in nature. These can include layers of supplementary variation within the selective pressure exerted by a single pollinating species, such as gender-specific preferences and learning abilities that can affect the fitness of deceptive plant species. In addition to pollinator-mediated selection, other floral visitors also exert selection on floral form, which can either reinforce or counter the selection exerted by pollinators. Most of my research focused on the bee fly pollinator, *M. capensis*, which pollinates various polymorphic annual daisies in South Africa. I show that this pollinator exhibits strong gender-specific preferences for the floral traits of one of these daisy species, *G. diffusa*. Males and females exhibit opposing preference for some floral traits, which may result in diversifying selection across the range of *G. diffusa.* Some *G. diffusa* floral forms have clearly specialized on the attraction of *M. capensis* males, which can increase pollen export relative to females, while others have not. Whether specialization on a specific pollinating gender occurs will be determined by the importance of each gender as a pollinator. This is likely affected by the significance of pollen import and pollen export at each site, variation in pollinator sex ratios across the landscape and the presence of secondary pollinators within each population.

Pollinator preferences may also differ between genetically distinct lineages. I investigated this hypothesis, but found no evidence to support it as male *M. capensis* pollinators from different phylogeographic clades all exhibited preference for the same sexually deceptive floral form of *G. diffusa*. Rather, there were significant differences between naïve and experienced males indicating potential male pollinator learning in this system. Learned pollinator avoidance, which can affect the reproductive success of sexually deceptive plants, was confirmed for *M. capensis* males and found to be associated with the potential mating costs they suffer when deceived by *G. diffusa*. While plants may benefit from increased deceptiveness, pollinators can reduce the costs they suffer through learned avoidance, indicating that antagonistic coevolution may potentially operate within these

systems. Such interactions will also influence floral evolution, as plants may be under strong selection to increase floral deceptiveness in order to keep deceiving males for successful pollination.

Selection from other sources, like florivores, also has to be considered when investigating floral evolution. Previous studies have pointed to their importance in the maintenance of discrete floral polymorphisms. By exploring the selective landscape for floral phenotype in another polymorphic daisy, *U. calenduliflora*, I show that pollinators and florivores exert opposing selection on the same floral traits and thus produce the maintenance of floral polymorphisms. In this system, male *M. capensis* flies again exhibit preference for dark ray floret spots while females do not. This suggests that multiple species may be exploiting the preference of *M. capensis* males for dark spots, which could drive its repeated evolution in different plant lineages. This indicates the likely importance of *M. capensis* as a pollinator in the GCFR in South Africa.

As part of my thesis, I also investigated the evolutionary history of *M. capensis* in the GCFR, which contains two biodiversity hotspots: the Fynbos and Succulent Karoo biomes. Many studies have investigated the evolutionary history of flowering plants in this area, but no studies to date have considered its pollinators. Results from this chapter show that *M. capensis* mirrors certain genetic patterns observed in the angiosperms. In particular, this pollinator exhibits ancestral Fynbos biome lineages in the south with more derived lineages in the Succulent Karoo biome to the north. Most flowering plants in the GCFR exhibit similar patterns, suggesting shared biogeography between its plants and pollinators. *M. capensis* also exhibit signals of population expansion in the Succulent Karoo, but not the Fynbos biome, suggesting it has colonized the Succulent Karoo in the last 5 million years. This date matches well with the appearance of Succulent Karoo endemic plants, placing the likely origin of this biome around that time.

Taken together, my research illustrates the importance of pollinators, and florivores, for floral evolution. It confirms the fundamental role played by pollinators and contributes evidence for additional and often overlooked mechanisms whereby even a single pollinating species can exert diversifying selection on the plants it visits. This may help to explain the vast diversity of floral forms

found within the angiosperms. It also, however, cautions against a purely pollinator centred view of floral evolution, as florivores clearly exert their own selective pressures. This research contributes to our understanding of the complex interactions between species in natural populations and the adaptive landscape in which angiosperms must operate in order to produce their most familiar attribute, the flower.

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