The importance of flies for pollination at high elevation: a case study of *Erica caffrorum*

By

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Preface

The research conducted in this dissertation was completed by the candidate while based in the Discipline of Biological Sciences, School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg, South Africa. The research was supported and funded by the Royal Museum of Central Africa (RMCA).

This dissertation represents original work by the author and has not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text, see the accompanying supervisor's report for a breakdown of data collection by student and supervisors respectively.

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Viren Thupsie

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19 July 2023

Professor Timo Van der Niet (supervisor)

As the candidate's supervisor I have approved this thesis for submission.

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Doctor Ruth J. Cozien (co-supervisor) Doctor Kurt Jordaens (co-supervisor)

Declaration-Plagiarism

I, Viren Thupsie declare that

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Abstract

Pollinator communities typically vary across elevation gradients. In particular, the contribution of bee pollination typically declines at higher elevations in favour of other pollinators, such as birds, butterflies and flies. *Erica caffrorum* occurs at high-elevation sites in the South African Drakensberg Mountains and has floral traits suggestive of pollination by short-tongued insects. Most *Erica* species with similar floral traits studied to date occur in the Cape Floristic Region at low elevations and are pollinated by honey bees. I present a case study of pollination in *Erica caffrorum*, including characterization of the breeding and pollination system, quantification of the associated floral traits, and experimental determination of the significance of flower colour and scent for pollinator attraction. Hand-pollination experiments confirmed that similar to most *Erica* species, *E. caffrorum* is self-incompatible. At five study sites, between 1800 and 2500 meters above sea level, pollinator observations revealed that most visitors were not honey bees but Diptera, which were identified into morphospecies and identifications verified using DNA barcoding. Assessment of pollinator importance based on pollen loads and visitation frequency identified Muscidae, Rhiniidae and Scatophagidae as the most important pollinators. Analysis of floral scent using GC-MS indicated that benzaldehyde and 2-methyl butanoic acid dominated the odour bouquet across all populations. In bioassays, a significant preference for white colour in the presence of scent was found for flies across all three experimental sites and for other visitors at one of two sites, but no other consistent preferences for colour or scent alone were detected. Across three sites, a combination of acid and benzaldehyde attracted significantly more flies than the unscented control. A similar pattern was observed for acid alone but not for benzaldehyde. This study provides novel evidence for short-tongued fly pollination in an *Erica* species, mediated through a combination of floral scent and colour. The similarity in traits between *E. caffrorum* and several other distantly related plant species in the high-elevation ecosystems of the Drakensberg mountains suggests that the pollination of scented, small, white flowers by short-tongued flies found here likely represents a more widespread pollination system whose importance in southern African mountain regions is currently underestimated.

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This thesis is dedicated to my grandmother.

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Chapter 1: Introduction

1.1 Introduction

Angiosperms are characterised by tremendous variation in how they reproduce; some species can reproduce asexually, independently of pollinators, whereas others depend completely on pollinators for sexual reproduction (Darwin, 1897). Plant species dependent on animals for pollination and effective reproduction have evolved highly diverse traits to attract a range of different pollinators (Schiestl and Johnson, 2013). These traits include brightly coloured flowers, specific scents, and rewards such as nectar to attract bees, birds, butterflies and many other animals to transfer pollen from one flower to another. Understanding plant-pollinator interactions is complex, but has been helped by the concept of the pollination niche (Phillips et al., 2020).

The pollination niche concept considers pollen vectors as ecological niches to which flowers adapt (Johnson, 2010; Phillips et al., 2020). As in the utilisation of any ecological niche, exploitation of the pollination niche is mediated by specific traits involved in attracting and interacting with pollinators. Such functional (floral) traits, such as colour, morphology and scent, can be considered adaptations to pollinators (Phillips et al., 2020). Whether a trait performs a particular function can be experimentally tested by manipulating a trait (e.g., removing the scent or experimentally changing flower morphology) and then evaluating whether this affects pollinator attraction or successful pollination (e.g., de Jager and Peakall, 2019; du Plessis et al., 2018; Johnson et al., 2020; Trunschke et al., 2020).

1.2 Pollination along elevation gradients

Pollination niches have a strong geographical component (e.g., Duffy and Johnson, 2017) because the distribution of pollinators and plants vary spatially (Gómez et al., 2015). This variation can occur at different scales: at the continental scale, major pollinator groups may be completely absent, as bumble bees are in sub-Saharan Africa, whereas hummingbirds only occur in the new world (Ollerton, 2017). At finer scales, there may be more subtle geographical turnover in species composition and relative abundance of particular pollinator groups. For instance, there appears to be a global trend of transitions of pollination systems along elevation gradients, most notably a trend of replacement of bees at low elevations by birds and other insects, such as flies, at higher elevations (Arnold et al., 2009; Cruden, 1972; Kearns, 1992; McCabe and Cobb, 2021; Warren et al., 1988). Areas at low elevations are often characterised by considerably higher temperatures compared to areas at high elevations. This creates an optimal temperature for bees to be active and forage for food resources (Herrera, 1995). An increase in elevation is directly proportional to a decrease in the temperature, so there may be thermal constraints for bees at these higher elevations (Corbet et al., 1993). Indeed, bees' abundance and species richness are shown to decrease with an increase in elevation (Hoiss et al., 2012).

Plants that occur in high-elevation environments differ compared to their congeners that occur at lower elevations (Arroyo et al., 2021) and are often characterised by floral traits that function as adaptions to the unique environmental conditions found at high elevations (Pellissier et al., 2010). Floral traits of plants at high elevations may vary depending on the geographic region and the type of pollinators present. Some plant species that depend on pollinators for reproduction may produce strong scent to attract pollinators (like flies and moths) that rely on such cues to locate flowers, especially when visual cues may be reduced due to frequent occurrence of fog or low light conditions at high elevations (Galen and Kevan, 1980). Flower colour shifts from bee-blue and UV-blue flowers (human-perceived blue/violet) to bee-blue-green and green (human-perceived white and yellow) with increasing elevation (Arnold et al., 2009). This observation may result from selection by pollinators that is associated with a shift from bee-pollination to fly-pollination (Lefebvre et al., 2018).

Shifts in the pollination systems between high and low elevations among species within a family are exemplified in Melastomataceae, in which *Meriania* species that occur at low and high elevations exhibit specific floral adaptations to bee pollinators at low elevations and vertebrate pollinators at high elevations, respectively (Dellinger et al., 2021). Also, at high elevations, bimodal pollination systems associated with distinct functional traits in other *Meriana* species may indicate relatively generalised pollination systems (Dellinger et al., 2019). Some Melastomataceae plant species have shifted pollination systems from specialised bee pollination through the buzzing of flowers to more generalist systems incorporating flies and wasps into their pollination systems (Brito et al., 2016). Importantly, this shift is mainly found in lineages that occur at high elevations, where buzzing bees may be absent (Brito et al., 2016). These examples show that within particular plant families differences in pollination systems and associated floral traits have evolved along elevation gradients.

1.3 Pollination Systems in southern Africa

Pollination systems have generally been well-studied in southern Africa and are characterised by an unusually high level of specialisation, particularly in the Orchidaceae and Iridaceae (Goldblatt and Manning, 2006; Johnson, 2010; Johnson and Steiner, 2000; Johnson and Steiner, 2003). Specialisation has multiple connotations in pollination (Armbruster, 2017). Ecological specialisation is the most common specialisation concept, where a plant species interacts or is pollinated by one or only a few pollinators (Armbruster, 2017). Phenotypic specialisation is another specialisation concept concerned with having specific or derived physical traits which are a characteristic of a plant or pollinator (Ollerton et al., 2007). Evolutionary specialisation in pollination is the process in which genes/traits of a plant or pollinator evolve to become more specialised, resulting in a specialised relationship between the plant and pollinator (Fenster et al., 2004). In some cases, a plant species can have a specialised pollination system at the functional group level but can remain an ecological generalist by having many closely related pollinator species (Armbruster, 2017). High levels of specialisation are typically associated with floral traits that attract specific functional pollinator species or groups [here defined as an assemblage of often taxonomically related species with similar behaviour, morphology and/or functional ecology in terms of attractants and rewards associated with pollination interactions (Fenster et al., 2004)] and other traits which may restrict particular potential pollinators (Johnson and Steiner, 2000).

High levels of specialisation are typically associated with floral traits that attract specific functional pollinator species or groups [here defined as an assemblage of often taxonomically related species with similar behaviour, morphology and/or functional ecology in terms of attractants and rewards associated with pollination interactions (Fenster et al., 2004)] and other traits which may restrict particular potential pollinators (Johnson and Steiner, 2000). The specialised relationships between plants and pollinators in southern Africa are particularly evident in the Cape Floristic Region (CFR) and other biodiverse regions such as the Drakensberg Mountain region (Johnson, 2010). Specialised pollination guilds documented in southern Africa include Cape sugarbird pollination in

Proteaceae in the Cape mountains and several guilds including species in the Iridaceae, Orchidaceae and Geraniaceae that are pollinated by different species of long-proboscid flies (Diptera, Nemestrinidae and Tabanidae) which occur in various habitats including fynbos as well as sub-Alpine grasslands and Namaqualand semi-desert (Johnson, 2010). Some of the most remarkable southern African biotic pollination systems include pollination by oil-collecting bees, long-tongued flies and moths, and vertebrates such as birds and non-flying mammals (Johnson, 2010).

1.4 Fly pollination in South Africa

Fly pollination in South Africa has been primarily studied in the context of the highly specialised pollination systems comprising long-proboscid fly pollination (Goldblatt and Manning, 2000; Johnson and Steiner, 1995; Johnson and Steiner, 1997; Lombardi et al., 2021; Newman and Johnson, 2021) and various forms of oviposition site mimicry (du Plessis et al., 2018; Heiduk et al., 2016; Heiduk et al., 2021; Johnson and Jürgens, 2010; Jürgens et al., 2015; Van der Niet et al., 2011).

The plant species that long-proboscid flies pollinate are usually highly specialised, whereby a single fly species is responsible for the pollination of multiple plant species (Johnson, 2010). These fly species are, therefore, sometimes considered keystone pollinators in the ecosystems they inhabit (Goldblatt and Manning, 2000). Long-proboscid fly pollinators in Southern Africa are represented mostly by the families Nemenstinidae and Tabanidae (Goldblatt and Manning, 2000), although it has been suggested that some Bombyliidae pollinators could also be considered long-proboscid flies (Goldblatt and Manning, 2013; Vogel, 1954). Long-tongued fly pollinators often have tongue lengths that are at least twice as long as their bodies and exceed 15 mm in length (Goldblatt and Manning, 2000; Manning and Goldblatt, 1996). Geraniaceae and Iridaceae are particularly rich in species that long-proboscid flies pollinate (Manning and Goldblatt, 1996). Plants pollinated by long-proboscid flies typically have flowers with a long, cylindrical floral tube 15-60 mm long, usually containing relatively dilute, sucrose-rich nectar (Goldblatt and Manning, 2000); the floral dimensions of fly-pollinated flowers correlate with the mouthpart lengths of the pollinators (Anderson et al., 2016; Newman et al., 2014). Flower colour is considered a particularly important feature of plants pollinated by long-proboscid flies (Jersáková et al., 2012; Whitehead et al., 2018). Flower colour varies across different pollination guilds pollinated by different fly species, but the presence of nectar guides that flies use to orientate the proboscis to probe the flower-opening is a common feature (Hansen et al., 2012). Most long-proboscid fly-pollinated flowers are unscented to the human nose (Campbell et al., 2016). Since long-proboscid flies generally visit multiple plant species that co-flower in communities, species have adapted to utilise different sites of pollen deposition on the body of the fly to avoid heterospecific pollen deposition and receipt (Goldblatt and Manning, 2000; Manning and Goldblatt, 1996). Long-proboscid flies also pollinate some Orchidaceae species that do not contain nectar through deception and floral mimicry of rewarding co-flowering model species (Goldblatt and Manning, 2000).

A second well-documented specialised fly pollination system in southern Africa is pollination through various kinds of oviposition site mimicry (du Plessis et al., 2018; Heiduk et al., 2016; Heiduk et al., 2021; Johnson and Jürgens, 2010; Jürgens et al., 2015; Van der Niet et al., 2011). The flowers are thought to mimic the scent of decaying animal carcasses or dung to attract flies to visit these flowers, mistaking them for brood or food sites and inducing pollination through deception or via a small nectar reward that may function as a mechanism for proper orientation rather than as a reward for pollinators, seen in many species in the Apocynaceae (du Plessis et al., 2018; Johnson and Jürgens, 2010; Stensmyr et al., 2002; Van der Niet et al., 2011). The insect species attracted to oviposition site mimics are primarily flies (e.g., Calliphoridae, Muscidae and Sarcophagidae). The plant families Apocynaceae and Orchidaceae have multiple species with oviposition site mimics (e.g., du Plessis et al., 2018; Van der Niet et al., 2011). The floral traits associated with most oviposition mimicry pollination systems are red-brown colour and specific scent to mimic the appearance of rotting flesh (Jürgens et al., 2015).

1.5 High elevation pollination in South Africa

Despite extensive studies of pollination in southern Africa, relatively little is known about high-elevation pollination systems and the identities of pollinators in the Alpine regions of southern Africa. A few recent studies are beginning to explore pollination systems in this region in general and fly pollination in particular (Johnson and Hobbhahn, 2010; Van der Niet et al., 2010). The Drakensberg range in southern Africa covers approximately 40 000 km^2 (Carbutt and Edwards, 2006) and is part of the great escarpment. The Drakensberg Mountain range can be categorised into floristic alpine regions. The North and South regions of the KwaZulu-Natal Drakensberg escarpment, the combined

KwaZulu-Natal and Lesotho region, and the southerly Eastern region of the Cape Drakensberg (Hilliard and Burtt, 1987). The Drakensberg region in southern Africa is recognised as the only true alpine region (Carbutt and Edwards, 2006; Linder, 1990); despite the known high levels of endemic plant species (Van Wyk and Smith, 2001), the extant endemic plant species and the accompanying floral ecology are not well understood (Carbutt and Edwards, 2006).

Studies from the Drakensberg Mountain region have suggested the importance of short-tongued flies as crucial pollinators of some plant species (Johnson et al., 2020; Shuttleworth and Johnson, 2010; Van der Niet et al., 2010). The flowers of *Wurmbea elatior*, for example, occur at high-elevation grassland sites in the Drakensberg and are pollinated by members of the fly families Calliphoridae, Muscidae, Sarcophagidae and Scathophagidae (Johnson et al., 2020). The flowers contain indole and skatole scent compounds that are thought to mimic the scent of animal dung, despite the appearance of the flowers not resembling dung to humans. *Eucomis* species like *E. bicolor* and *E. humilis* are equally visited by flies from the families Sarcophagidae, Calliphoridae, and Muscidae, which are attracted to the sulphur scent compounds that are found in rotting meat (Shuttleworth and Johnson, 2010). Despite differences in flower and inflorescence traits between *W. elatior* and *E. bicolor*, in both cases scent, and possibly flower colour, are important traits for attracting short-tongued flies for pollination (Johnson et al., 2020; Shuttleworth and Johnson, 2010). The presence of these plant species at high elevations in the Drakensberg region may indicate a guild of plant species found in this region that may share the same assemblage of pollinators, although potential guild members appear to have subtly varying functional traits primarily in terms of scent compounds (cf. Johnson 2010).

1.6 Knowledge Gaps in southern Africa for high-elevation pollination

There are considerable gaps in our knowledge of the pollination systems of plant species in the Drakensberg, pertaining to the extent of reliance on pollinators, levels of specialisation in pollination, the identities of important pollinators and floral traits associated with the attraction of these pollinators. The Ericaceae are a prime candidate for investigation as a well-studied plant family in the CFR, but with a large number of species for which little is known about pollination in other regions of southern Africa (Van der Niet, 2021). Some of these species produce a putrid scent reminiscent of fly pollination, but observations have not verified this trait-based hypothesis. Within other plant genera found in the Drakensberg region, fly-pollinated species, such as *Wurmbea* and *Eucomis*, appear to use different scent compounds to attract similar assemblages of fly pollinators (Johnson et al., 2020; Shuttleworth and Johnson, 2010).

The fly species that are found visiting *Wurmbea* and *Eucomis* species (Johnson et al., 2020; Shuttleworth and Johnson, 2010) can be classified as short-tongued flies because their proboscis length never exceeds 15 mm (cf. Goldblatt and Manning, 2000). Although long-tongued fly pollination systems are characterised by extreme specialisation from the plant's perspective, it remains unknown how specialised short-tongued fly pollination is at high elevations in southern Africa.

The evidence from studies in the Drakensberg region suggests that different fly families and fly species are associated with specific floral (scent) traits (Johnson et al., 2020; Shuttleworth and Johnson, 2010). This could represent a signature of hyper-specialisation associated with particular traits. However, perceived hyper-specialisation could also be reflect failure to sample fly communities across multiple sites resulting in overestimation of the specialisation in a pollination system. For example, if a plant species is studied over multiple years and at multiple sites, each site/year combination could return a unique pollinator assemblage. Taking this temporal and spatial variation together, a relatively broad pollinator assemblage would characterise the study species. If, however, this study species was only sampled at one site during one sampling year, then this undersampling would lead to a false conclusion of hyper-specialisation. Thus, when characterising pollination systems, pollinator communities and floral traits should ideally be sampled across multiple sites and years to understand the specialisation of pollination systems, but especially at high elevations, practical considerations such as access may restrict the spatiotemporal scope of pollination studies (e.g., Cozien et al., 2019; Ollerton et al., 2003).

1.7 The plant genus *Erica*

The plant genus *Erica* (Ericaceae) comprises a large number of species. Its roughly 800 southern African representatives are mainly found in the CFR of South Africa and the Drakensberg Mountain region (Bouman et al., 2017; Carbutt and Edwards, 2006; Hilliard and Burtt, 1987; Pirie et al., 2016). *Erica* species are often called "heaths" or "heathers" and have diverse floral traits that attract many different functional pollinator groups. Almost all *Erica* species studied to date are dependent on pollinators for reproduction (Arendse et al., 2021; Lombardi et al., 2021; Newman and Johnson, 2021; Van der Niet and Cozien, 2022; Turner et al., 2011). Rebelo et al. (1985) classified 426 Erica species into four pollination syndromes based on their floral traits, primarily the shape of the flowers in the CFR. These pollination syndromes comprise bird-pollination, insect-pollination, long-proboscid fly-pollination and wind-pollination syndromes, with the insect-pollinated syndrome accounting for 80% of these *Erica* species, and pollination by long-proboscid flies representing a distinctive subcategory within the insect-pollinated syndrome (Rebelo et al., 1985).

Specialisation in terms of pollination is highly evident in *Erica*, especially within the long-proboscid fly pollination systems (Van der Niet, 2021). Many *Erica* species depend on a single functional group for pollination and effective reproduction (Van der Niet, 2021). Studies of pollination systems of *Erica* species from the CFR have included quantification of pollinator assemblages and of functional floral traits such as colour, scent and floral morphology (Bouman et al., 2017; Heystek et al., 2014; Newman and Johnson, 2021; Ojeda et al., 2019; Turner et al., 2011; Van der Niet and Cozien, 2022; Van der Niet et al., 2014). Many kinds of pollination systems in *Erica* are being continually discovered and described. For example, over the last two years, empirical studies revealed evidence for hawkmoth (Van der Niet and Cozien, 2022), long-proboscid fly (Lombardi et al., 2021; Newman and Johnson, 2021) and rodent pollination (Turner et al., 2011). Some species appear to have ecotypes with different pollination systems (Van der Niet et al., 2014), suggesting that pollinators are important evolutionary drivers of species and functional diversity in *Erica*.

Despite the available studies, pollination systems of a vast number of *Erica* species remain unknown, and no work has been done on species that occur in southern Africa outside of the CFR, for instance, on those that occur in the Drakensberg Mountain region (see Carbutt and Edwards, 2006; Hilliard and Burt, 1987), where many of the pollinator species that are found in the CFR may not occur (e.g., Johnson 2010). Therefore, descriptive, and experimental work in the Drakensberg is merited for characterising the pollination system of *Erica* species in this region, including an assessment of functional floral traits that these *Erica* species may use for attracting pollinators. Such studies can be used to explain the role of pollinators driving the radiation and diversification of *Erica* species in this high-elevation region and can serve as a further example of pollination systems in high-elevation environments.

1.8 Drakensberg study sites and *Erica caffrorum* study species

The Drakensberg Mountain region of southern Africa comprises an alpine environment with many different angiosperm growth forms that occur in habitats such as rocky slopes, grasslands and basalt ridges (Carbutt and Edwards, 2006). An estimated 394 angiosperm species are endemic to this region (Hilliard and Burt, 1987). Although the percentage of species in the Drakensberg region represents a significantly lower proportion of the South African flora than occur in the CFR, the region is nevertheless characterised by a very high level of endemism (Carbutt and Edwards, 2006). The dominant plant families are Asteraceae, Scrophulariaceae, Iridaceae and Ericaceae (Carbutt and Edwards, 2006).

Erica caffrorum (Mountain heath) is distributed predominantly in the high-elevation Drakensberg region of KwaZulu-Natal (Guthrie and Bolus, 1905). The species is a shrub, usually found on rocky mountain slopes or near cliff edges (Guthrie and Bolus, 1905). The floral traits of *E. caffrorum* may indicate that flies might be important pollinators of the species. Yet, whether this small-flowered *Erica* species relies on pollinators is unknown. Neither the identities of the pollinators, nor the specific traits that are involved in pollinator attraction and functional pollination, have been studied. Based on an initial assessment of its floral traits, with a particular emphasis on the putrid scent, and its occurrence at high elevations, I hypothesise that short-tongued flies are the main pollinators.

1.9 Outline of thesis structure

In this thesis, I report results from a case study of the pollination system of *E. caffrorum* to determine if this *Erica* species is specialised at the functional group level for pollination by short-tongued flies, which would represent a novel pollination system in *Erica*. In Chapter 2, I provide an analysis of the reproductive ecology of *E. caffrorum*. I first used hand-pollination experiments to determine whether this plant species is self-incompatible and whether it is reliant on pollinators to reproduce effectively. I then identify the pollinator species (using DNA barcoding to verify their identity) that are responsible for the pollination of *E. caffrorum* across five study sites: Carter's Nek, Kamberg Reserve, Ntsikeni, Tarn Cave and Witzieshoek, including an evaluation of pollinator importance using the mean number of individuals as a proxy for visitation rates and pollen loads as a proxy for effectiveness. Finally, I determined whether pollinator service limits reproductive success in this species.

In Chapter 3, I present results of quantification of floral traits, including flower morphology, reflectance pattern of the flowers (sepals and petals) and background leaves, as well as floral scent at different sites. I plotted the loci of the reflectance pattern of sepals and petals, and background leaves into a categorical fly vision model to determine if these are distinguishable by the main pollinators. In order to determine the role that different scent compounds and colour may play in pollinator attraction, I performed a series of bioassay choice tests; the first aimed to test the role that colour and scent may play in attracting pollinators, whereas in a second experiment, I determined the specific *E. caffrorum* scent compounds that may function in pollinator attraction.

In the final chapter, I draw conclusions from the two data chapters to illustrate the importance of flies for pollination at high elevations, using my case study of *E. caffrorum.* Furthermore, I recommend avenues for additional studies in this chapter, suggesting a hitherto unidentified pollination guild of flowers in the high-elevation Drakensberg, similar to *E. caffrorum* in floral traits and pollination by short-tongued flies.

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Chapter 2: Pollination and breeding system of *Erica caffrorum* (Ericaceae)

Abstract:

Erica is among the most species-rich genera of the southern African flora, with diversity centred in the Cape Floristic Region (CFR) and Drakensberg Centres of Endemism. Adaptation to different pollinators has been suggested to play an important role in diversification in the genus, but pollination studies to date are limited to the CFR and focus primarily on relatively large-flowered species. I studied the reproductive ecology of the foul-smelling small-flowered *Erica caffrorum* across five high-elevation study sites in the Drakensberg mountains of South Africa. A breeding system experiment showed that *E. caffrorum* is self-incompatible and depends almost exclusively on pollinators for reproduction (indices of self-incompatibility and autonomous self-fertilisation 0.05 and 0.10, respectively). Pollinator observations augmented by DNA barcoding of visitors revealed that 88.3% of visitors were Diptera. Assessment of pollinator importance (PI) based on visitation frequency and pollen loads showed that the relative importance of dipteran taxa differed among sites and years and confirmed Muscidae (PI 12.28% to 75.70 %), Scathophagidae (PI 0.17% to 65.97%), and Rhiniidae (PI 0.30% to 43.94%), as the most important pollinators. The dominance of flies over other visitors in combination with variation in assemblages among sites and years suggest that the fly pollination system in *E. caffrorum* represents a relatively specialised pollination system in which potentially functionally similar flies of different families are presumably attracted by similar floral cues, potentially buffering reproductive success of *E. caffrorum* in a high elevation against a variable pollination environment.

Key Words: Diptera, DNA barcoding, Ericaceae, high-elevation, pollen limitation

2.1 Introduction

Florally diverse lineages are often considered to have evolved by a process of adaptation to an array of different pollinators (Van der Niet and Johnson, 2012; Van der Niet et al., 2014a). Indeed, there are many examples of genera in which floral diversity is associated with frequent shifts among pollination systems (Goldblatt and Manning, 2006; Johnson et al., 1998; Valente et al., 2012). Floral radiations often occur in regions with high biodiversity, such as tropical rainforests (Boyce and Lee, 2010), island ecosystems (Lunau, 2004) and montane regions (Medail and Quezel, 1997).

The southern African flora, including three hotspots of biodiversity (Myers, et al., 2000) has been a model system in which to explore adaptations to different pollination systems and its resultant macroevolutionary patterns (Johnson, 2010). Wind pollination is also common in South Africa, particularly in the Cape Floristic Region (CFR) (Welsford et al., 2014) plants with highly reduced floral features such as grasses, sedges, and Restionaceae rely on wind for pollination (Friedman and Barrett, 2009). Some plant species in South Africa are adapted to reproduce independently from pollen vectors through self-pollination (e.g., Van der Niet, 2018), a common strategy in arid environments where pollinators may be scarce (Harder and Johnson, 2005).

The South Africa flora is characterised by a high level of specialisation in pollination systems, including plant species that are pollinated by a single pollinator species (Johnson and Steiner, 2003; Johnson, 2010). High levels of pollinator specialisation in plant species are usually associated with a combination of floral traits including colour, scent and floral morphology that only attract particular functional groups or may restrict particular pollinator groups from visiting (Johnson and Steiner, 2000). Families in which high specialisation is particularly common include Orchidaceae and Iridaceae (Goldblatt and Manning, 2006; Johnson and Steiner, 2000; Johnson and Steiner, 2003).

A pollination syndrome is a set of associated features that exhibit floral phenotypic homogeneity, and represent adaptations for pollination by a particular group of animals (Faegri and van der Pijl, 1979; Fenster et al., 2004; Sprengel, 1793). Although specialisation in pollination systems is a key underlying assumption of the pollination syndrome concept (Johnson and Steiner, 2000), in which floral traits are interpreted as adaptations for particular pollinators, Waser et al. (1996) proposed that most plants rather display generalisation in their pollination systems, despite conforming to a particular pollination syndrome (Ollerton, 1996). Plant species with generalised pollination systems are frequently characterised by relatively small flowers (Zych et al., 2019), resulting from an absence of morphological specialisation (e.g., due to the accessibility of floral rewards in flowers that lack a long floral tube) (Bergamo et al., 2021; Johnson and Anderson, 2010; Klumpers et al., 2019). However, small-flowered species may be ecologically specialised due to signalling traits that filter species not involved in pollination (Johnson and Steiner, 2000; Martos et al., 2015). Specialisation observed in small-flowered species may also be due to their occurrence in particular environments with lower pollinator diversity (e.g., high elevation), representing “imposed specialisation” (cf. Armbruster, 2017).

Studies of plant species with small flowers that are candidates for representing generalised pollination systems are rare in South Africa (but see Johnson and Hobbhahn, 2010). Indeed, the data obtained by Waser et al. (1996) were limited to the northern hemisphere (Johnson and Steiner, 2003). The geographical bias in assessing specialisation and generalisation in pollination systems can underestimate the degree of generalisation or specialisation in a particular pollination system (Johnson and Steiner, 2000).

Pollinator assemblages vary spatially and temporally (Herrera, 1988; Price et al., 2005), and this variation may result in slight changes to pollination systems (Kearns and Inouye, 1994). Thus, the assessment of specialisation might change temporally or geographically, and limited sampling in time and space may result in an overestimation specialisation if studies are done at a single site and/or in a single year. For example, an overestimation of specialisation can occur if a particular plant species may appear specialised to a pollinator in one year at one site but may tend to be generalised to other pollinators, observed after a few years or multiple sites (Petanidou et al., 2008), although specialised pollination may also be underpinned by considerable spatiotemporal variation in the role of more than one pollinator group (Fenster and Dudash, 2001). Therefore, when attempting to characterise a potential pollination system, it is important to do so at multiple years and at multiple sites to gain a more accurate representation of that particular pollination system (Price et al., 2005; Dupont et al., 2009).

Specialisation in pollination systems is evident in the plant genus *Erica* (Ericaceae), (see Bouman et al., 2017; Rebelo et al., 1985), and many species of *Erica* appear relatively specialised for biotic pollination often relying on a single functional group (Rebelo et al., 1985; Van der Niet, 2021). The genus *Erica* occurs in the old world and is most diverse in southern Africa, with the centre of diversity in the CFR (Manning and Goldblatt, 2012; Pirie et al., 2011; Pirie et al., 2016), and with a lesser radiation in the summer rainfall region, where it nevertheless is one of the most species-rich genera (Carbutt and Edwards, 2006; Hilliard and Burtt, 1987). *Erica* comprises remarkable floral diversity (Baker and Oliver, 1967; Pirie et al., 2011; Pirie et al., 2016), which is associated with diverse biotic pollinators, including bees (Bouman et al., 2017; Van der Niet et al., 2020), long-proboscid flies (Lombardi et al., 2021; Newman and Johnson, 2021), moths and hawkmoths, (Van der Niet et al., 2014b; Van der Niet and Cozien, 2022), non-flying mammals such as rodents (Lombardi et al., 2013; Turner et al., 2011) and birds (Angoh et al., 2021; Heystek et al., 2014; Turner et al., 2012; Van der Niet et al., 2014b). However, all hitherto studied species occur in the CFR, rendering information on *Erica* pollination systems outside that region unknown.

Several plant species in the summer rainfall region of South Africa appear specialised for pollination by short-tongued Diptera (true flies) of the families Muscidae, Calliphoridae, Sarcophagidae, Tachinidae and Scathophagidae (Johnson et al., 2020; Shuttleworth and Johnson, 2010). Rebelo et al. (1985) classified 72% of *Erica* species as insect-pollinated, including pollination by bees and small flies in this category. Other *Erica* species that appear specialised for pollination by Diptera are pollinated exclusively by long-tongued Nemestrinid and Tabanid flies (Goldblatt and Manning, 2000; Lombardi et al., 2021; Newman and Johnson, 2021). Hitherto there is no study that shows specialisation for short-tongued fly pollination in *Erica*, even though many *Erica* species are characterised by small flowers that would provide a good morphological match with pollinators with particularly short proboscises. Although flies have been recorded as visitors for five small-flowered *Erica* species, these species were found to be pollinated primarily by honey bees (Bouman et al., 2017). An alternative explanation for small flowers in *Erica* is that these species may reproduce independently of pollinators. Capacity for autonomous self-pollination is often associated with small flowers, since investment into features for pollinator attraction are no longer maintained by selection (Primack, 1985).

Therefore, it is necessary to understand the reproductive ecology of small-flowered *Erica* species in terms of their reliance on pollinators for reproduction. Our current understanding of breeding systems in *Erica* suggests that many *Erica* species cannot successfully reproduce through autogamy and rely on pollinators for successful reproduction (Angoh et al., 2017; Arendse et al., 2021), but these studies were limited to representatives from the Cape clade of *Erica*.

The high-elevation Drakensberg region of South Africa is a centre of diversity for *Erica* (Carbutt and Edwards, 2006). In other areas of the world, there is a trend for increased reliance on flies at high elevations, but this has not been studied in South Africa. *Erica caffrorum* is a species that is mainly present at high-elevation sites of the Drakensberg region of southern Africa and blooms in early summer. It is characterised by small pale-white tubular flowers that emit a strong, somewhat unpleasant scent. The latter trait is particularly inconsistent with the bee pollination systems described by Bouman et al. (2017) for small-flowered *Erica* species. Given its floral traits and distribution, I hypothesise that *E. caffrorum* is primarily pollinated by short-tongued flies.

A particular challenge when identifying Afrotropical Diptera to genus or species level is that there is immense morphological variation in some families, and for many Diptera genera identification keys are unavailable (Jordaens et al., 2013; Jordaens et al., 2015; Kirk-Spriggs and Sinclair, 2017). Thus, the identification of morphologically similar flies is often very difficult and as such molecular techniques, such as DNA barcoding, could be used to complement identifications based on morphology (e.g., Jordaens et al., 2013; Jordaens et al., 2015).

The aim of this study is to characterise the pollination system of *E. caffrorum*. Specifically, I determine whether this species depends on pollinators for reproduction and whether it is self-compatible. Furthermore, I identify floral visitors and evaluate their importance for pollination based on visitation frequency and pollen loads, and verify their identification based on morphological characteristics using DNA barcoding. Finally, I quantify pollen limitation to evaluate the pollination service provided by the pollinators.

2.2 Methods

2.2.1 Species and study sites

Erica caffrorum (Ericaceae), commonly known as the "mountain heath", is endemic to southern Africa and occurs in high-elevation regions, including the Drakensberg mountain range in the Eastern Cape and KwaZulu-Natal provinces of South Africa and in full flower produces several thousand flowers. To determine the pollination system of *E. caffrorum*, five study sites were selected across the species' distribution (Table 2.1). Breeding system and pollen limitation experiments were conducted at Carter's Nek. Visitors were observed and visitor pollen loads were quantified at all sites.

Table 2.1: GPS coordinates and elevation (meters above sea level) for the five study sites of *E. caffrorum* with the years visitors were sampled.

Site	Elevation m.a.s.l.	Latitude	Longitude	Year sampled 2017-2018	2018-2019	2019-2020	2020-2021	2021-2022
Carter's Nek	1846	29°31'30.6"S	29°41'17.8"E				x	
Kamberg Reserve	2100	29°22'22.0"S	29°39'35.8"E				x	
Ntsikeni	2080	30°07'56.6"S	29°28'29.1"E	x		x		
Tarn Cave	2395	29°51'29.9"S	29°08'16.1"E	x	x		x	
Witzieshoek	2100	28°41'28.7"S	28°53'55.8"E			x		x

2.2.2 Quantification of breeding system

Controlled hand pollination experiments were conducted to determine whether *E. caffrorum* is self-compatible and can reproduce independently of pollen vectors (auto-fertility). Prior to the commencement of these experiments, buds were enclosed in breathable mesh bags to exclude flower visitors and ensure that the treatment pollen was the only pollen reaching the stigmas of these flowers. To assess self-compatibility, reproductive success was compared between flowers that were hand-pollinated with self-pollen of the same flower and flowers pollinated with cross-pollen from a flower of another plant approximately 20 meters away. Reproductive success was compared between flowers that were left unmanipulated to flowers also pollinated with cross-pollen from a flower of another plant, to assess auto-fertility. A minimum of 10 flowers were assigned to each treatment on each of 20 plants. Self- and cross-pollen were applied to flowers of which stigmas were receptive. Pollen grain adherence to stigmas was confirmed with a 10× hand-lens. Sufficient pollen grains were placed on the stigma to cover it entirely.

All treated flowers were marked with coloured wires and covered with fabric pollinator exclusion bags that allow passage of wind and water but exclude insect visitors. Flowers assigned to the autonomous self-pollination treatment were left unmanipulated inside the exclusion bags.

After two months, all treated flowers and the enclosed fruits were harvested. Harvested flowers were dissected under a dissecting microscope at a 40× magnification, and the presence or absence of developed fruits was recorded. For each fruit, the number of mature seeds and undeveloped ovules was recorded. Unfertilised ovules were distinguished from developed seeds on the basis of size: unfertilised ovules were 125 to 200 µm in diameter, whereas seeds were larger than 400 µm in diameter (Supplementary Fig. S2.1).

Indices of self-incompatibility and auto-fertility were calculated following Zapata and Arroyo (1978), based on the marginal means estimated from statistical analysis of the seed set (see below). The index of self-incompatibility (ISI) was calculated as the mean number of seeds per flower pollinated with self-pollen divided by the mean number of seeds per flower for cross-pollinated flowers; this gives a value ranging from zero to one, for which “zero” indicates complete self-incompatibility and “one” indicates full self-compatibility.

The index of autonomous self-fertilisation (IAS) indicates a species’ capacity to set fruit independently of pollinators (Schoen and Lloyd, 1992). This index was calculated as the mean of the number of seeds produced by flowers from which pollinators were excluded, divided by the mean number of seeds per flower for cross-pollinated flowers. The IAS ranges from zero to one, for which “zero” indicates a complete dependence on pollinators for fruit set, whereas “one” indicates complete independence of pollinators through autonomous autogamy.

2.2.3 Visitor observations and assessment of pollinator importance

Floral visitors were identified through direct observations on *E. caffrorum* on 22-23 November 2019 and on 28–30 November 2021 at Witzieshoek, on 5 January 2020 at Tarn Cave and on 29 December 2021 and 3-4 January 2022 at Carter's Nek; visitor species identity and their behaviour while visiting flowers were recorded. Observations were carried out typically from 07:00 to 17:00, but usually in the late morning when visitor activity was the highest and then in the evening from 18:00 to 19:00.

Flower visitors were collected using sweep nets at five sites over a total of 42 hours over seven days (Table 2.2). When an insect visitor landed on the flowers of *E. caffrorum* a sweep net was placed directly over the visitor. This was done randomly throughout the day when a visitor was seen visiting *E. caffrorum* flowers. All captured insects were placed in 1ml Eppendorf tubes and frozen at -20 °C before further processing. Although this approach should result in a representative sample of captured floral visitors, there is potential for bias due to different capture rates for different types of visitors.

For Diptera, which generally comprised the most common visitors, each visitor was identified to genus level. Other visitors were identified to family level. All identifications were based on matching morphological characteristics viewed under a 20× dissecting microscope with the identification keys described in Kirk-Spriggs and Sinclair, (2017) and Nihei and de Carvalho, (2009). DNA barcoding (see below for methods) was used on a subset of caught visitors to confirm the morphospecies identification of Diptera. All downstream analyses were based on the dataset that was obtained subsequent to DNA barcoding, incorporating any changes to initial morphospecies designations.

Pollen loads of all captured individuals were assessed by dabbing them with a two mm^3 cube of fuchsin gel that adheres to, and stains pollen grains (Beattie, 1971). The fuchsin gel was then melted on a microscope slide, and pollen grains were counted under a stereo microscope using 400× magnification. *Erica* pollen can be readily identified by their distinctive tetrad pollen from other plant species’ pollen grains (Sarwar and Takahashi, 2014). Pollen grains of other plant species, which are visibly different from the tetrad pollen of *Erica*, were also recorded on any visitor that did carry pollen of *E. caffrorum.* .

Pollinator Importance (PI) was calculated for each visitor taxon at each site, following a modification of the protocol of Bouman et al. (2017). The mean pollen load was calculated for each visitor taxon and then multiplied by the mean visitation frequency, as inferred from the proportion of the species among the total number of insects captured. The relative importance of each visitor group was calculated as the contribution of each group to the total contribution summed for all groups and expressed as a percentage.

2.2.4 Analysis of pollination networks and pollinator importance

To visualise geographical variation in pollinator assemblages and to assess whether and how pollinator assemblages vary among sites, visitor assemblages were analysed using pollination network tools, including an analysis of modularity (cf. Phillips et al., 2020). Modularity is mainly used here as a visualisation tool, and not interpreted in its mostly used form as a measure for interactions between plants and pollinators that all occur within a particular community. The flower-visitation network is a visual representation of the frequency of visitors at each site. The width of the connecting lines of sites to visitor taxa indicates the frequency of visitors at each site; the thicker the connecting line, the greater the visitor frequency. The pollinator importance network is a visual representation that includes information related to pollen loads that is used for calculating pollinator importance (see above). The width of the connecting lines of sites to pollinator taxa indicates the pollinator importance of pollinator taxa at each site; the thicker the connecting line, the greater the pollinator importance.

To determine the number of modules representing visitors and their pollinator importance, a weighted interaction matrix was created with sites in rows and visitors and pollinator importance, respectively in columns (Figs 2.4a, 2.4b). Modularity (Q value) is a measure of network structure calculated to measure the strength of grouping a network into modules (Newman and Girvan, 2004). A modularity analysis creates discrete compartments that group strong interactions together and indicate lesser interactions outside of each unit compartment (Olesen et al., 2007) and is visualised using a set of coloured squares, with the darker squares representing more frequent interactions and greater pollinator importance respectively. The observed Q value was compared against a null model derived from 1,000 randomised networks using the vaznull function (Vázquez et al., 2007). Modularity was calculated in R version 4.2.1 (R Core Team, 2022) with the bipartite package version 2.16 (Dormann et al., 2008; Dormann, 2020).

A similarity percentage (SIMPER) analysis was implemented to determine which visitor taxon contributes most to dissimilarity among sites (i.e., which specific visitor taxa are characteristic of a particular site). This analysis ranks visitor taxa based on their importance in explaining dissimilarity (Clarke, 1993).

Variation of visitor taxa across sites and different sampling years was also visualised in two dimensions using non-metric multidimensional scaling (NMDS) implemented in PAST Version 4.03 (Hammer et al., 2001), based on the Bray–Curtis similarity because the data matrix contained many zero values of taxa that were absent at a particular site (Bray and Curtis, 1957).

2.2.5 Procedure for DNA barcoding and PCR

Since most visitors were Diptera that are often difficult to identify at the species level based solely on morphological characters, DNA barcoding was used to verify the morphological identifications. Specimens were preserved in 100% ethanol and stored in a -20°C freezer until further analysis. The procedures for DNA barcoding followed Jordaens et al. (2015). The Genomic DNA was extracted from the tissue of a single leg of fly specimens using the NucleoSpin Tissue Kit (Macherey-Nagel, Düren), following the directions of the manufacturer. PCR reactions commenced in 25 µl reaction volumes comprising 1.5 mM $MgCl_2$ in 1× PCR buffer (Invitrogen), 0.2 mM of each dNTP, 0.2 µM of each primer and 0.5 units of Taq polymerase (Invitrogen). Using the primer pair LCO1490 and HCO2198, the DNA barcode fragment of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene was amplified (Folmer et al., 1994). The PCR program started with an early denaturation phase of 5 minutes at 95 °C, followed by 35 cycles of 45 seconds denaturation at 95 °C, 45 seconds at an annealing temperature of 45 °C and 1.5 minutes extension at 72 °C, and ending with a final extension stage of 5 minutes at 72 °C. PCR products were purified using the GFX PCR DNA Purification Kit (GE Healthcare, Machelen, Belgium) and diluted in 15 µl of sterile water or using the ExoSap protocol (Thermo Fisher Scientific, Merelbeke, Belgium) following the instructions of the manufacturer. The PCR products were bidirectionally sequenced using the ABI PRISM BigDye ® Terminator v3.1 Cycle Sequencing Kit and executed on an ABI3130xl Genetic Analyzer. Sequences were assembled in GeneiousR10 v.10.0.9 (https://www.geneious.com), and discrepancies were checked by eye on the chromatogram.

DNA sequences were analysed using a Neighbour-Joining analysis (Saitou and Nei, 1987) based on 81 DNA barcodes. A Neighbour-Joining (NJ) tree was constructed using the p-distance model in MEGA v.7 (Kumar et al., 2016) and pairwise p-distances (i.e., the proportion of sites at which two sequences differ) within and among species were calculated. Commonly, in DNA barcoding, a 2% threshold is used as an arbitrary cut-off value used to delimit species from another based on the distribution of inter and intra-specific genetic distance values of the DNA barcodes (Jordaens et al., 2015). In this case, the distance measure is the p-distance, which is the proportional distance between the DNA barcodes.

Each DNA barcode (query) was then blasted in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) using the Basic Local Alignment Search Tool (option Megablast), yielding a list of highly similar sequences of which the best match was retained (i.e., the DNA barcode with the lowest p-distance with the query). The percentage of query covered with the best match and the percentage identity (p-distance) between the query and the best match was noted. Best matches with a p-distance of < 2% were considered a conspecific match with the query (Sonet et al., 2013). The morphological ID of the best match was then compared to the morphological identification of the query. If the identification returned by GenBank and morphological identification did not correspond, I re-examined the specimen morphologically and inserted all the corrected identifications in the analyses outlined above. Best matches with a p-distance > 2% were considered heterospecific with the query and suggested that GenBank did not contain a reference DNA barcode for the query.

2.2.6 Assessment of pollen limitation

In order to assess whether pollinator-mediated pollen receipt limits the reproductive success of *E. caffrorum*, a pollen supplementation treatment was applied to a minimum of 13 flowers and a maximum of 24 flowers on 20 plants and reproductive success was compared with that of naturally pollinated flowers. The method of hand pollination was similar to that used in the breeding system experiment. However, to allow natural visitation and pollination, flowers in the pollen limitation experiment were not enclosed in bags prior to or after treatment. (cf. Burd, 1994).

The treatment and control flowers were marked with coloured wires. Reproductive success was assessed as described above. An index of pollen limitation was calculated as 1 – (the mean number of seeds following natural pollination) divided by the mean number of seeds per flower following pollen supplementation (Bierzychudek, 1981, Baskin and Baskin, 2018). Values range from "zero" indicating an absence of pollen limitation, to "one" indicating maximum pollen limitation. Pollen limitation occurs when pollinators deposit incompatible pollen or when pollinators are infrequent (Fernández et al., 2012) and is indicated by significantly greater seed set of pollen-supplemented pollinated flowers than that of the unmanipulated natural flowers (Bierzychudek, 1981).

2.2.7 Statistical analysis of breeding system and pollen limitation experiments

Generalised Estimating Equations (GEE) with a binomial distribution and a log link function were used to test the reproductive success among treatments in the breeding system and pollen limitation experiments. To account for non-independence among measurements from the same plant, individual models included plant identity as a subject variable, and multiple flowers measured on the same plant as within-subject effects with an "exchangeable" correlation matrix, and models were implemented using SPSS Version 27 (IBM Corp). To adjust significance for multiple comparisons among means, the Dunn-Šidák method was used (Šidák, 1967). For visual comparison of means on the original measurement scale, the data was back-transformed from the scale of analysis, resulting in marginal means and asymmetrical standard error bars, that were plotted using SigmaPlot version 8.0 (Systat Software, Inc. 2005).

2.3 Results

2.3.1 Breeding system

Comparison of seed set shows that the reproductive success of autonomously self-pollinated and hand self-pollinated flowers was similar and very low, with less than 10% of ovules developing into seeds, whereas cross-pollinated flowers showed significantly higher reproductive success, with approximately 75% of ovules developing into seeds (Fig. 2.1). The IAS was 0.05, indicating that *E. caffrorum* is dependent on pollinators for reproduction. The ISI was 0.09, indicating that *E. caffrorum* is strongly self-incompatible.

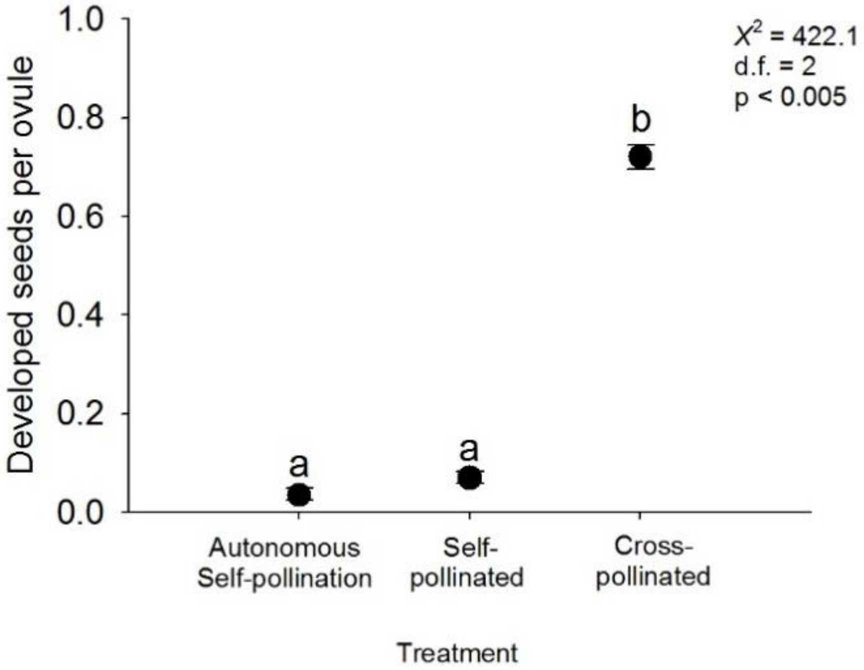

Figure 2.1: Mean ± SE seed production per ovule for unmanipulated flowers and flowers pollinated with self and cross pollen. Symbols that share letters indicate values that do not differ significantly in post-hoc comparisons ($p < 0.005$).

2.3.2 Identification of flower visitors and calculation of pollinator importance

Across all sites, a total of 618 insect visitors, including 546 Diptera (all consisting of the suborder Brachycera) visitors and 72 other visitors, were caught or observed visiting flowers of *E. caffrorum* (Table 2.2). In total, there were 17 fly morphospecies. Other visitors included bees, wasps, butterflies, moths, true bugs and thrips, together classified as “other pollinators” (six taxa recorded). Some Diptera visitor taxa, including *Neomyia* (Muscidae), *Sarcophaga* (Sarcophagidae), and *Scathophaga* (Scathophagidae) were present consistently regardless of site or sampling year. The most frequently occurring family among the dipteran visitors comprised the Muscidae, specifically the genera *Musca (Byomya* and *Musca)*, *Neomyia* and *Stomoxys,* which tallied a total of 309 flies. At Carter’s Nek and Kamberg Reserve, there were seven and nine fly morphospecies visitors, respectively, and one non-dipteran visitor morphospecies per site. At Ntsikeni, there were five fly visitor morphospecies with no non-dipteran visitors. At Tarn Cave there were 11 fly morphospecies and three non-dipteran morphospecies visitors. Witzieshoek had the most fly morphospecies, with 14 visitors and six non-dipteran morphospecies present (Table 2.2; Table 2.3).

The number of *E caffrorum* pollen grains on visitors ranged from 0–800 (Table 2.2). A visitor that carried pollen grains of *E. caffrorum* was generally also observed to carry non-*Erica* pollen grains. *Anthomyia* (Anthomyiidae), *Ceratitis* (Tephritidae) and *Lauxania* (Lauxaniidae) were the only dipteran visitor taxa that did not carry pollen grains of *E. caffrorum* or any other plant species pollen, but individuals of these groups were in any case represented rarely among caught visitors.

Table 2.2: Identities, number of individuals caught and mean pollen loads of visitors to flowers of *Erica caffrorum* at five high-elevation sites across four flowering seasons and five years studied. Values indicate mean ± SD pollen grains across sampled individuals; numbers in square brackets indicate individuals caught.

	Carter's Nek	Kamberg Reserve	Ntsikeni			Tarn Cave				Witzieshoek		
Family/(Sub)-Genus	2020–2021 Pollen	2020–2021 Pollen	2017–2018 Pollen	2019–2020 Pollen	All Ntsikeni Pollen	2017–2018 Pollen	2018–2019 Pollen	2020–2021 Pollen	All Tarn Cave Pollen	2019–2020 Pollen	2021–2022 Pollen	All Witzieshoek Pollen
Diptera												
Anthomyia	-	-	-	-	-	0 [1]	-	-	0 [1]	0 [1]	-	0 [1]
Ceratitis	-	-	-	-	-	0 [1]	-	-	0 [1]	0 [1]	-	0 [1]
Chrysomya	2 [1]	3 [1]	-	-	-	-	-	-	-	712 [1]	-	712 [1]
Eristalinus	-	-	-	-	-	8 [9]	-	-	8 [9]	76.7 ± 84.7 [5]	-	76.7 ± 84.7 [5]
Hilarempis	-	-	-	-	-	5.1 ± 9.2 [12]	-	9.8 ± 15.8 [4]	7.45 [16]	2 [1]	-	2 [1]
Isomyia	65.3 ± 38.7 [11]	-	-	-	-	9.0 ± 14.2 [10]	4.4 ± 11.5 [2]	1.6 ± 2.3 [7]	7 ± 11.90 [19]	-	-	-
Lauxania	-	-	-	-	-	-	-	-	-	0 [1]	-	0 [1]
Musca (Byomya)	-	14.2 ± 8.1 [5]	-	-	-	3.0 ± 4.8 [20]	-	1.3 ± 2.4 [12]	2.31 ± 4.15 [32]	1.5 ± 1.5 [2]	4.6 ± 10.0 [7]	3.89 ± 8.94 [9]
Musca (Musca)	-	14.2 ± 20.2 [5]	-	-	-	-	-	3.1 ± 7.2 [16]	3.1 ± 7.2 [16]	0 [1]	32.8 ± 47.3 [5]	27.33 ± 44.88 [6]
Muscoidea	-	1	-	-	-	-	-	2.3 ± 2.3	2.3 ± 2.3	-	27	27

		[2]						[4]	[4]		[1]	[1]
Neomyia	15.4 ± 15.1	13.0 ± 13.9	14.9 ± 22.3	0	14.1 ± 21.34	14.6 ± 38.1	16.0 ± 11.0	4.9 ± 13.4	11.39 ± 32.50	5.3 ± 9.3	19.5 ± 21.1	10.37 ± 16.17
	[23]	[25]	[18]	[1]	[19]	[69]	[13]	[24]	[106]	[34]	[19]	[53]
Sarcophaga	13.0 ± 13.1	103.0 ± 87.0	6	-	6	29.5 ± 68.4	-	8.9 ± 16.5	22.62 ± 57.45	16.6 ± 38.6	0.7 ± 1.1	10.2 ± 30.89
	[5]	[4]	[1]		[1]	[14]		[7]	[21]	[9]	[6]	[15]
Scathophaga	48	44.6 ± 58.8	10	0.2 ± 0.4	1.83 ± 3.67	3.0 ± 1.9	-	2.4 ± 2.7	2.67 ± 2.40	7	-	7
	[1]	[39]	[1]	[5]	[6]	[4]		[5]	[9]	[1]		[1]
Sepsis	-	-	-	-	-	-	-	-	-	1.3 ± 1.5		1.3 ± 1.5
										[22]		[22]
Stomorhina	24.7 ± 32.0	8	34.5 ± 6.5	-	34.5 ± 6.5	8	-	1.6 ± 1.5	2.33 ± 2.45	-	-	-
	[18]	[1]	[2]		[2]	[1]		[8]	[9]			
Stomoxys	2	3	-	-	-	-	-	-	-	-	-	-
	[1]	[1]										
Tachininae	-	-	-	0	0	-	-	-	-	4.5 ± 6.4	-	4.5 ± 6.42
				[1]	[1]					[14]		[14]
Other Visitors												
Apis	-	-	-	-	-	0	-	3.0 ± 3.7	1.5 ± 3.01	2.5 ± 2.5	-	2.5 ± 2.5
						[5]		[5]	[10]	[2]		[2]
Hesperiidae	-	0.8 ± 1.6	-	-	-	22.3 ± 16.0	-	-	22.3 ± 16.0	2.0 ± 2.8	-	2.0 ± 2.8
		[5]				[3]			[3]	[3]		[3]
Noctuidae	1	-	-	-	-	-	-	-	-	-	23.1 ± 46.6	23.1 ± 46.6
	[1]										[16]	[16]
Pyrrhocoridae	-	-	-	-	-	-	-	-	-	210.4 ± 198.9	35.3 ± 48.0	135.36 ± 176.33
										[8]	[6]	[14]
Thripidae	-	-	-	-	-	-	-	1.5 ± 1.5	1.5 ± 1.5	0.3 ± 0.7	1.0 ± 1.4	0.88 ± 1.41
								[2]	[2]	[7]	[8]	[15]
Vespidae	-	-	-	-	-	-	-	-	-	4	-	4
										[1]		[1]

Similar to the visitor assemblages, pollinator importance also varied at each site. Only *Neomyia*, *Sarcophaga* and *Scathophaga* were pollinators across all sites (Table 2.3). At Carter's Nek, *Isomyia* (43.94%) (Rhiniidae) had the greatest pollinator importance even though it was the second most frequently occurring visitor, behind *Stomorhina* (27.17%) (Rhiniidae) (Figs 2.2a, 2.2b). At Kamberg Reserve, *Scathophaga* (65.97%) was the most important pollinator and the most frequent visitor. Ntsikeni and Tarn Cave had *Neomyia* spp. (75.70% and 53.44%, respectively) as the most important pollinator and the most frequent visitor (Figs 2.2a, 2.2b). At Witzieshoek, the true bug family of Pyrrhocoridae (46.04%) was the most important pollinator, and *Chrysomya* (17.30%) (Calliphoridae) was the second most important pollinator (Fig.2.2b). This was mostly determined by the large amounts of pollen found on true bugs, even though they were not the most frequent visitors, which was *Neomyia* (Fig. 2.2a).

Table 2.3: Pollinator importance for different flower visitors to *E. caffrorum* across the five sites. Pollinator importance is expressed as a percentage, calculated as the product of mean visitation frequency, as inferred from the proportion of the species among the total number of insects captured, and mean pollen load for each visitor category, out of the total for all visitors at each site. Visitor taxa are arranged into Diptera and other visitors (each arranged alphabetically, with the dipteran families in parenthesis). Numbers in bold indicate the most important pollinator at each site.

	Carter's Nek	Kamberg Reserve	Ntsikeni	Tarn Cave	Witzieshoek
	Pollinator Importance				
Family/Genus					
Diptera					
Anthomyia (Anthomyiidae)	-	-	-	0.00	0.00
Ceratitis (Tephritidae)	-	-	-	-	0.00
Chrysomya (Calliphoridae)	0.12	0.11	-	-	17.30
Eristalinus (Syrphidae)	-	-	-	3.16	1.86
Hilarempis (Empididae)	-	-	-	5.23	0.05
Isomya (Rhiniidae)	**43.94**	-	-	5.83	-
Lauxania (Lauxanidae)	-	-	-	-	0.00
Musca (Byomya) (Muscidae)	-	2.69	-	3.24	0.85
Musca (Musca) (Muscidae)	-	2.69	-	2.17	3.98
Muscoidea (Muscidae)	-	0.08	-	0.40	0.66
Neomyia (Muscidae)	21.66	12.28	**75.70**	**53.44**	13.35
Sarcophaga (Sarcophagidae)	3.98	15.61	1.70	20.83	3.72
Scathophaga (Scathophagidae)	2.94	**65.97**	3.10	1.05	0.17
Sepsis (Sepsidae)	-	-	-	-	0.82
Stomorhina (Rhiniidae)	27.17	0.30	19.50	0.92	-
Stomoxys (Muscidae)	0.12	0.11	-	-	-
Tachininae (Tachinidae)	-	-	0.00	-	1.53
Other Visitors					
Apis	-	-	-	0.66	0.12
Hesperiidae	-	0.15	-	2.93	0.15
Noctuidae	0.06	-	-	-	8.98
Pyrrhocoridae	-	-	-	-	**46.04**
Thripidae	-	-	-	0.13	0.32
Vespidae	-	-	-	-	0.10

Modularity analysis derived from the visitation data (Fig. 2.3a) showed significant modularity (Q = 0.28; 95% CI, Q null-vaznull = 0.18-0.57; Fig. 2.3a). Four modules were detected for the modularity analysis of visitor frequency formed by two to nine visitor taxa. Tarn Cave, Witzieshoek and Carter's Nek represented independent modules. Seven visitor taxa characterise Tarn Cave, with *Neomyia* being the main visitor taxon. Five visitor taxa characterise Witzieshoek, and two visitor taxa (*Isomyia* and *Stomorhina*) characterise the visitors at Carter's Nek, while two visitor taxa (Hesperiidae and *Scathophaga*) characterise Ntsikeni and Kamberg Reserve (Fig. 2.3a).

Significant modularity was also detected for the pollinator importance data (Q = 0.53; 95% CI, Q null-vaznull = 0.14-0.44; Fig. 2.3b). Four modules were also detected for pollinator importance, formed by two to nine important pollinators. Tarn Cave, Witzieshoek and Kamberg Reserve Nek represented independent modules. Three taxa were important pollinators at Tarn Cave, with *Neomyia* being the most important pollinator. Six taxa were important pollinators at Witzieshoek, with Pyrrhocoridae the most important. Four taxa were important pollinators at Kamberg Reserve, with *Scathophaga* being the most important pollinator. Two taxa (*Isomyia* and *Stomorhina*) characterise important pollinators at Ntsikeni and Carter's Nek (Fig. 2.3b).

For both modularity analyses, the clearest module was found at Carter's Nek and comprised the Rhiniidae genera *Isomyia* and *Stomorhina* as the most frequent visitor and most important pollinator (Fig. 2.3).

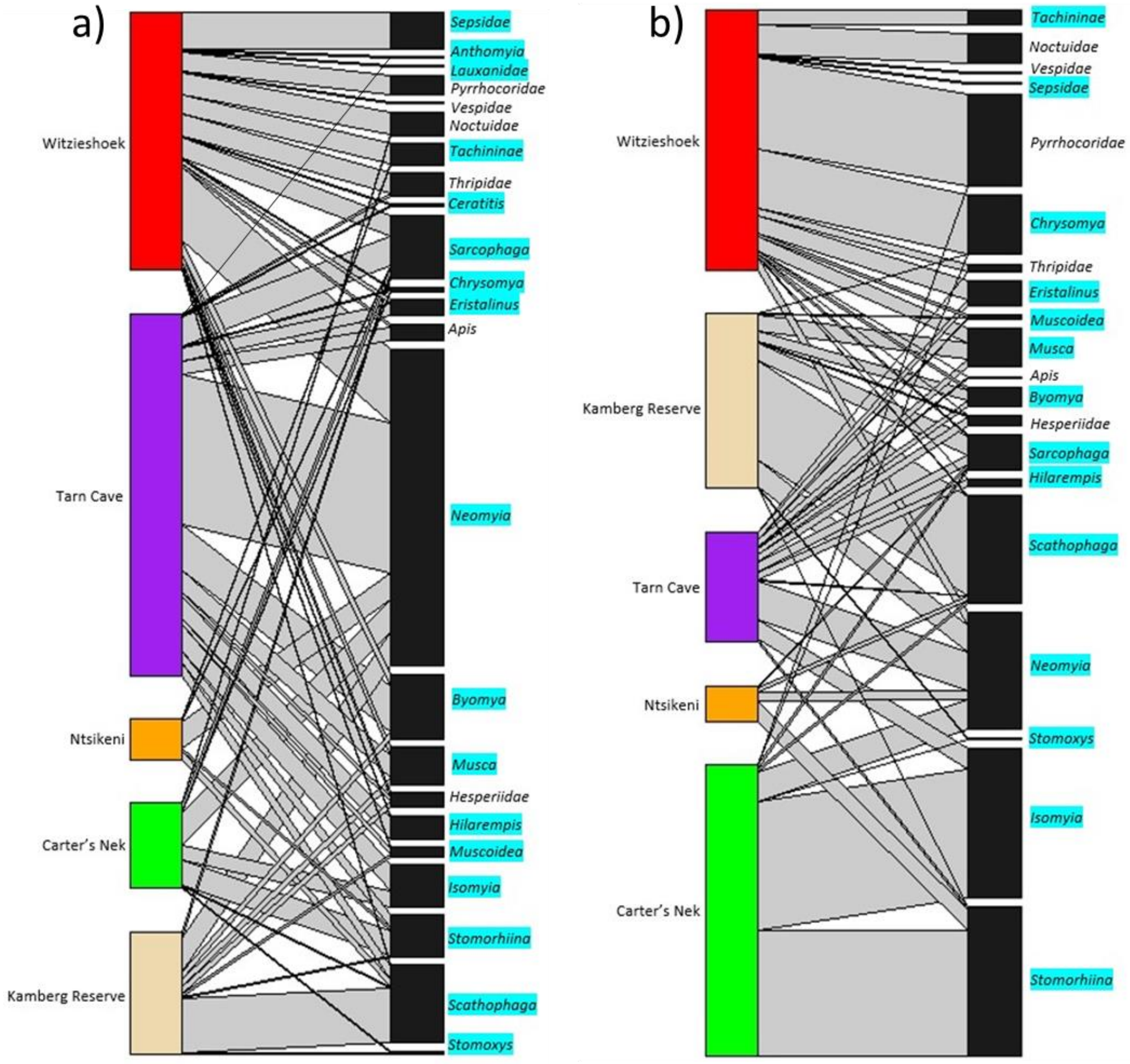

Figure 2.2: Bipartite network analyses of visitors to *Erica caffrorum* at five high-elevation sites based on visitation frequency only (a) and on pollinator importance calculated as the product of visitation frequency and pollen loads (see methods text for details) (b). Names of dipteran visitor taxa are highlighted in blue. The width of connecting lines indicates interaction frequency (a) and strength of pollinator importance (b).

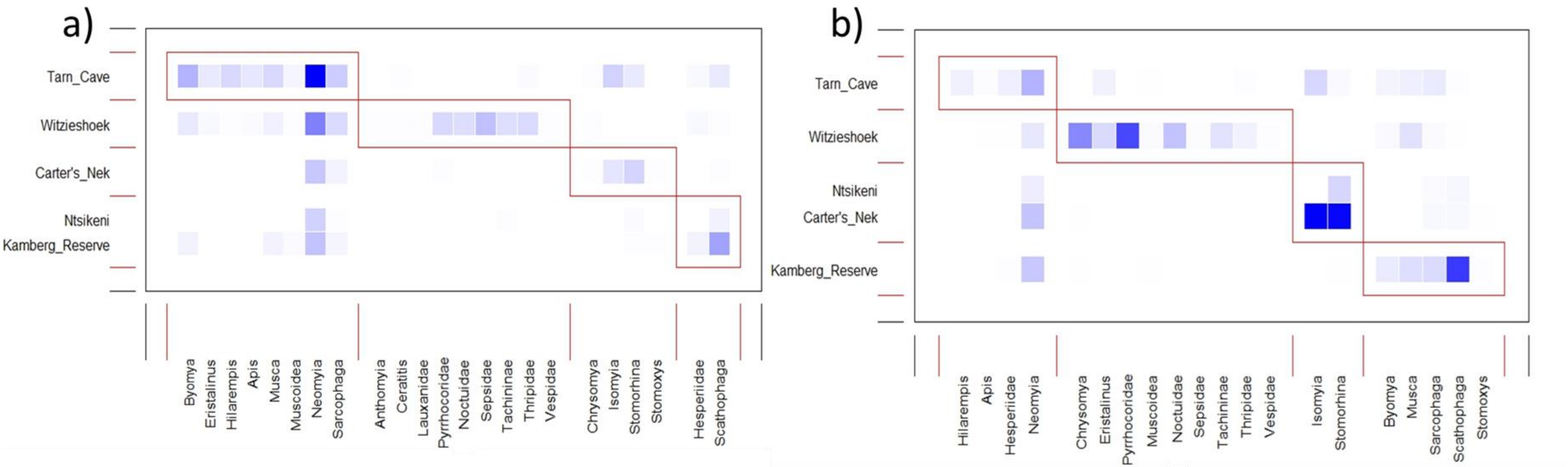

Figure 2.3: Modularity diagrams for *E. caffrorum* based on visitation frequency (a) and including pollinator importance based on visitation frequency and pollen loads (b). Each module, with sites as rows and pollinator genera as columns, is enclosed within a red box. Both analyses identify four modules, but the inclusion of pollen loads changes module composition in terms of both site similarity and pollinator composition for each module. Darker colours indicate greater interaction frequency (a) and pollinator importance (b). The red boxes separate the modules, and the cells inside the boxes are the interactions that are shared within the modules.

Visualisation of the visitor taxa among different sites at different sampling years using NMDS revealed an inconsistent temporal spread of the sites (Fig. 2.4). The SIMPER analysis identified *Neomyia*, *Scathophaga* and *Sarcophaga* as the main visitor taxa that are responsible for the dissimilarity of visitor taxa among different flowering seasons, *Neomyia* was the only visitor taxon that was present across all sites and flowering seasons (Table S2.1).

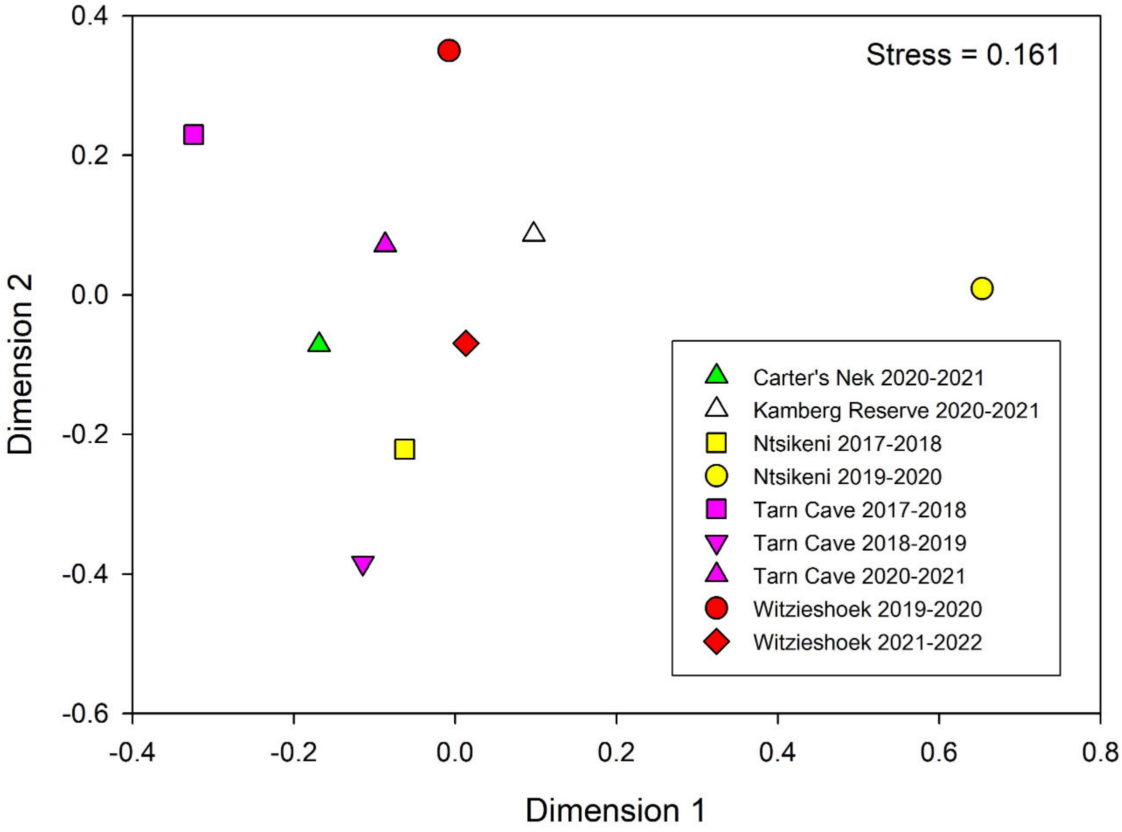

Figure 2.4: Non-metric multidimensional scaling of visitors assemblages of *E. caffrorum* at five sites and over multiple sampling years; sites are indicated by symbols with the same colour, different years are indicated by different symbols.

2.3.3 Verification of flies DNA barcoding

In total, 92 fly legs were used for DNA barcoding, of which 81 were successfully barcoded, which was a 88.04% success rate (Table S2.2). DNA barcoding largely confirmed family-level identifications: only two out of 81 were incorrectly identified at the family level of the barcodes that were successful. Eleven specimens showed a best match with a p-distance of < 2%, which was considered a supposed match to flies in the GenBank database that also matched their morphological identification. Seventy flies had best matches with a p-distance > 2%, suggesting that DNA barcoding corroborated the identification to the family level, but that GenBank does not yet contain a DNA barcode of these species. Six flies had a 100% match with their p-distance and the query cover, suggesting that these six flies DNA sequences matched those already deposited in the GenBank database.

The Neighbour-Joining (NJ) tree comprised 81 DNA barcodes, which created clusters that grouped morphospecies based on molecular similarity (Fig. 2.5). There was a great correspondence between the identification based on morphology and clustering using DNA barcoding suggesting that the morphological identifications were accurate. Most Syrphidae, Tachinidae, Sarcophagidae, and Scatophagidae specimens fit in their respective clusters at the family level. However, there are some considerable outliers. For example, the muscid TC 40 South Africa Tarn Cave 2017-2018 is grouped near the Rhiniidae clusters suggesting a potential contamination in the lab.

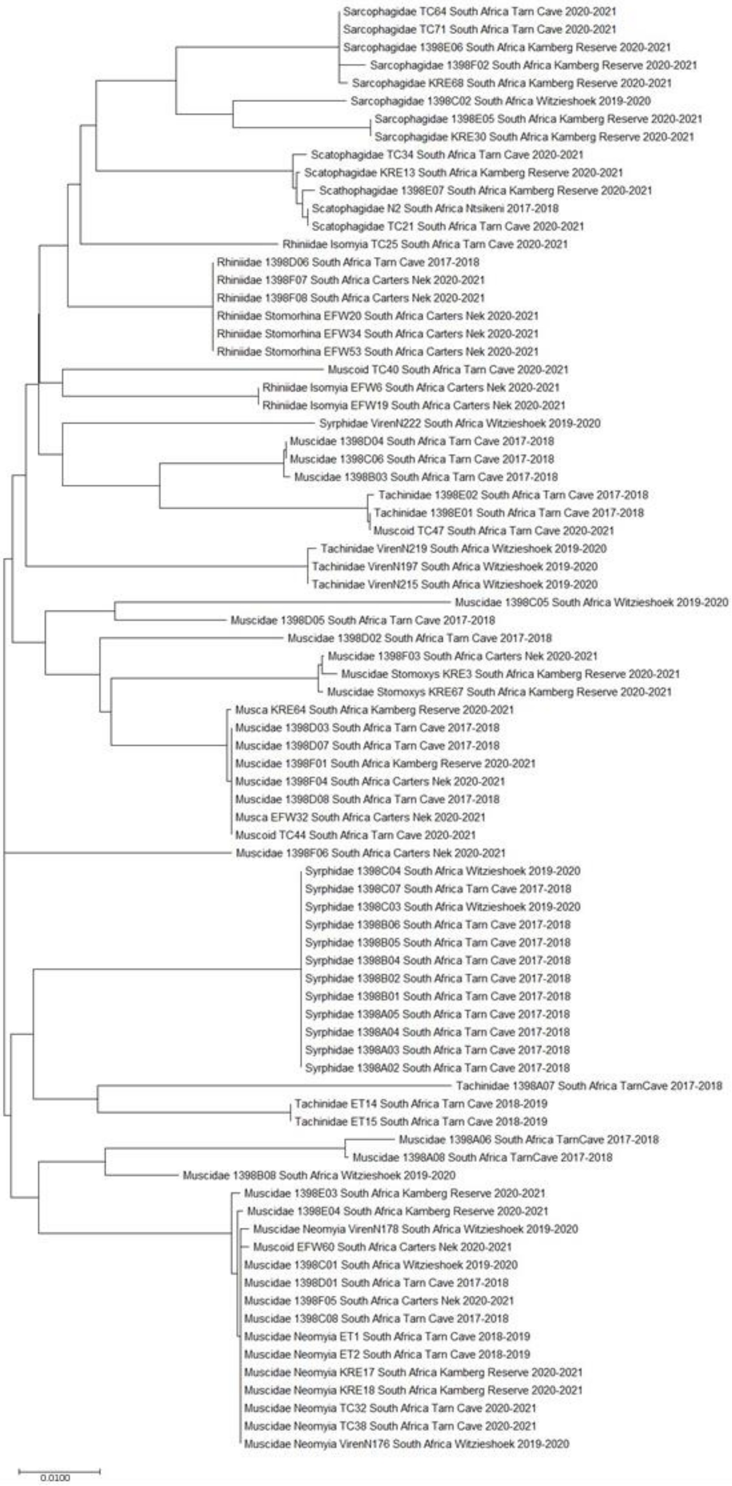

Figure 2.5: Neighbour-Joining tree of 81 DNA barcodes of 24 Diptera morphospecies that were collected from *Erica caffrorum*. 1398A01 to 1398F08 are flies that were identified at the family level. The other unique codes represent specimens identified to the genus level.

2.3.4 Pollen limitation

Reproductive success of naturally pollinated flowers is significantly less than that of pollen-supplemented flowers (Fig. 2.6). Despite the significant difference in fecundity between pollen-supplemented flowers and naturally pollinated flowers, the index of pollen limitation (PL) was 0.18, indicating weak pollen limitation.

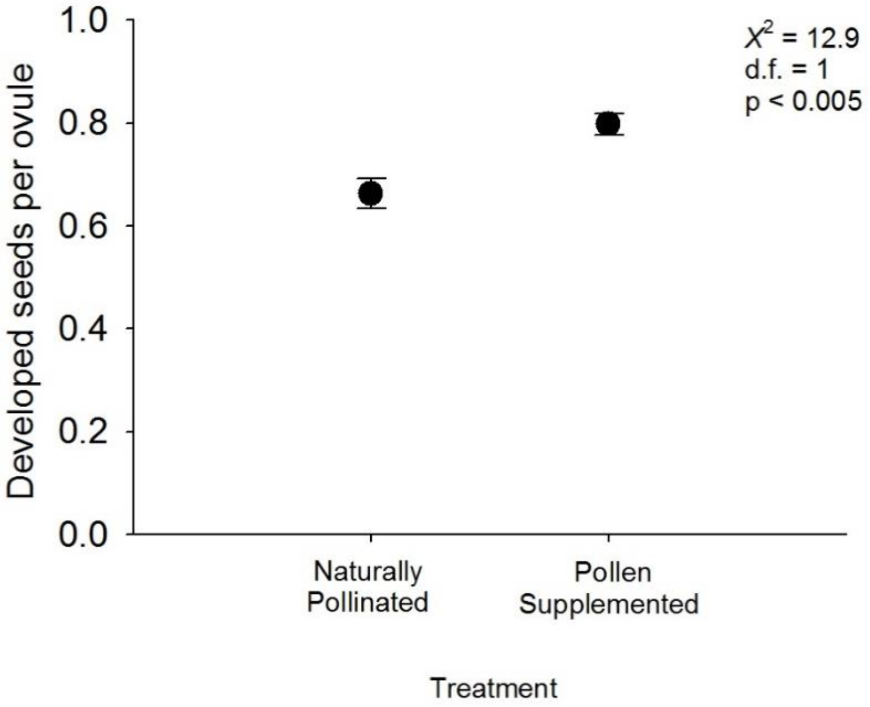

Figure 2.6: Mean ± SE seed production per ovule for naturally pollinated flowers and for flowers supplemented with pollen in *Erica caffrorum* ($p < 0.005$).

2.4 Discussion

In this study, I aimed to quantify and characterise the reproductive ecology of *E. caffrorum*. I found that this plant species relies on pollinators for reproduction and is self-incompatible and weakly pollen limited at Carter's Nek (Figs 2.2, 2.6). Of the collected flower visitors, 88.3% belonged to the insect order Diptera (Table 2.1), with each of the five sites having different species assemblages (Figs 2.2, 2.3). DNA barcoding used to verify identifications of the most abundant and taxonomically challenging order revealed that morphospecies grouped together in clusters in the Neighbour-Joining (NJ) tree (Fig. 2.5). The DNA barcoding results thus corroborate the identifications based on morphological characters. The difference in fly assemblages is also evident across years (Fig. 2.6). Different fly taxa were associated with varying levels of pollinator importance (Table 2.3).

2.4.1 Breeding system

The main results of the breeding system experiment at the Carter's Nek site show that *E. caffrorum* is self-incompatible (ISI = 0.05) and relies on pollinators to set seed (IAS = 0.09). In a previous breeding system study in *Erica,* which included six species, all species had an IAS value < 0.2, and five had an ISI value < 0.2 (Angoh et al., 2017), similar to *E. caffrorum.* In a subsequent study of a bigger sample of species, for 12 out of 13 *Erica* species studied almost no seed set resulted in the autonomously self-pollinated flowers, and had significantly lower reproductive success than cross-pollinated flowers (Arendse et al., 2021). My results on a species from a different *Erica* clade therefore conform with our current understanding of *Erica* breeding systems.

In certain high-elevation environments, like the habitat of *E caffrorum,* plants may show decreased dependence on pollinators and a higher selfing rate due to the lower visitation frequency of pollinators at higher elevations (e.g., Eriksen et al., 1993). Conversely, some plant species do not conform to this general trend of increasing self-fertilisation rates with increased elevation (Wirth et al., 2010), as was found here for *E. caffrorum*. These plant species' reproductive success may be unrelated to the variation of pollinators along an elevation gradient (cf. Arroyo et al., 2006). The high pollination success of *E. caffrorum* at high elevation despite complete pollinator dependence, may be due to its reliance on a group of pollinators that is particularly common at high elevation.

2.4.2 Flower visitors and their pollinator importance

Diptera was the most abundant order of visitors accounting for 88.3% of all visitors to *E. caffrorum*. Despite their general importance, there was ample variation in the composition of fly assemblages at the five different study sites (Figs. 2.2, 2.3), in association with differences in pollinator importance (Table 2.3). The fly families Muscidae, Rhiniidae and Scathophagidae were the dominant families that characterised each site. At Carter's Nek, the Rhiniidae (genera *Isomyia* and *Stomorhina*) were the most frequent and important pollinators, whereas, at Kamberg Reserve, Scathophagidae (genus *Scathophaga*) was the most frequent and important pollinator. Although at Witzieshoek, the true bug family Pyrrhocoridae was the most important pollinator, the Muscidae genus *Neomyia* was the most frequent visitor. Interestingly, *Neomyia* was the only genus present at each site and across sampling years (Figs. 2.2, 2.3; Table 2.2).

Flies were not the only visitors to *E. caffrorum*. Bees, butterflies, moths, thrips, true bugs, and wasps were all observed to visit plants and carried pollen grains of *E. caffrorum*. Yet, they only accounted for 11.7% of all visitors (Table 2.2), and at some sites they were virtually absent. Despite the relatively low abundance of non-fly visitors on *E. caffrorum*, some groups were regarded as the most important pollinator at one site (Figs. 2.2, 2.3; Table 2.3). This is evident for the true bug family Pyrrhocoridae at Witzieshoek, which was a less frequent visitor but carried copious amounts of *E. caffrorum* pollen grains, possibly leading to misleadingly high calculated pollinator importance values: True bugs were never observed to move from flower to flower as Diptera did, and may have been visiting for the consumption of multiple plant parts including nectar, pollen, and seeds (Ahmad, 1987). Based on the breeding system, their general lack of movement would mostly result in ineffective within-flower pollination, and not contribute to effective pollination. I therefore consider Pyrrhocoridae unlikely to be important pollinators, although further research including quantification of single visit effectiveness for seed set is ideally required to determine the pollination effectiveness of each visitor group.

Overall, the general dominance of flies as important pollinators of *E. caffrorum* could have resulted from a bias in observation time, especially a lack of observations during the evening when moths are particularly active. On the other hand, flies were visiting throughout the day, regardless of weather conditions, whereas other insects, such as bees and butterflies, were generally only active during sunny conditions when most observations were carried out. Furthermore, moths were only observed during particularly warm and dry nights, rare in the summer evenings of eastern South Africa. I, therefore, think that a biased observation time has not likely resulted in an overestimation of the importance of flies as pollinators.

Many of the most important fly families in this study, including Anthomyiidae, Calliphoridae, Muscidae, Sarcophagidae, Scathophagidae, Syrphidae, and Tachinidae, are often considered as pollinators in high-elevation regions (Kearns, 1992). These families seem well-adapted to high-elevation environments (Lefebvre et al., 2018) and this may correspond to their lower energy requirements and better thermoregulation ability compared to bees (Hocking and Sharplin, 1965). The scarcity of bees at higher elevations may be because of nesting constraints and lower flying ability at low temperatures (Kearns, 1992).

Flies from the families Calliphoridae, Muscidae and Sarcophagidae are found to be visiting high-elevation plant species found in the Drakensberg, such as *Eucomis bicolor* and *E. humilis* (Shuttleworth and Johnson, 2010). *Wurmbea elatior* is also pollinated by these fly families and other fly families, such as Tachinidae and Scathophagidae (Johnson et al., 2020). Lastly, *Schizochilus angustifolius* (Orchidaceae) is mainly visited by muscid flies (Van der Niet et al., 2010). All these fly families found in these studies also were regarded as important pollinators of *E. caffrorum.* Indeed, every visitor that carried pollen grains of *E. caffrorum* also carried pollen from other plant species (which was not further quantified in this study). Although they were not readily observed within an approximate 300-400 m range of *E. caffrorum* (V. Thupsie, personal observation), other *Erica* species may have been in the same habitat and due to flies ability to travel long distances it cannot be ruled out that the flies pollinating *E. caffrorum* only carry pollen from this plant species and not of other *Erica* species.

The sharing of similar fly pollinators among multiple species that occur in the high elevation environments of the Drakensberg mountains implies the existence of a pollination guild. All the flies found on these high-elevation plant species, including *E. caffrorum*, can be categorised as short-tongued flies as their proboscis length was shorter than 15 mm and never exceeded the length of the body of the fly (Goldblatt and Manning, 2000). Thus, *E. caffrorum* and the other plant species can be described as being specialised at the functional group level for short-tongued fly pollination.

Although flies were the most important pollinator at four out of five sites, multivariate analyses showed that visitor/pollinator assemblages varied across sites and years (Fig. 2.4). The results of the SIMPER analysis further reiterate the inconstancy of visitor taxa found at differing sampling years across each site. These results imply that it is critical for pollination studies to sample visitors at multiple sites across multiple years. Had this study been done at a single site in a single year, this would have suggested a hyper-specialised pollination system of *E. caffrorum* (see Van der Niet et al., 2011). Instead, it appears that *E. caffrorum* is specialised at the functional group level, but not at the pollinator species level.

2.4.3 Verification of visitor identification using DNA barcoding

In this study, the construction of a Neighbour-Joining (NJ) tree based on p-distances among DNA barcodes has proven to be a useful visual and diagnostic tool for the identification of Diptera species. Indeed, the clustering of DNA barcodes per morphospecies and the high interspecific p-distances among species affirms that DNA barcoding can be used to identify Diptera to species level.

Eleven flies showed a best match with a p-distance of < 2%, which was considered a putative match to GenBank entries. Thus, only a small proportion of the Diptera flower visitors of *E. caffrorum* can be identified using DNA barcodes. Indeed, 70 Diptera flower visitors had best matches with a p-distance > 2% suggesting that the best match and the flower visitor of *E. caffrorum* are heterospecific. However, this does not mean that my morphological identifications are incorrect. Rather, it shows that currently, GenBank does not contain DNA barcodes of these Afrotropical species. It also shows that my study will provide the first DNA barcodes to GenBank for a number of Diptera species. When based on the NJ tree (Fig. 2.5), the identities of morphologically similar visitors to genus level give an excellent visual representation of flies that were deemed to be morphologically similar by grouping the same genus clusters together with the exception of a few outliers which may prove to be of the same genus, but comprise distantly related species. The use of DNA barcoding for this study is currently a limited tool that supports identification based on morphology. Yet, it revealed the morphospecies that require further identification by taxonomic experts of the various Diptera families.

The short-tongued fly pollination system of *E. caffrorum* adds a novel pollination system for the plant genus *Erica* for which hitherto fly pollination was known for long-tongued species (Lombardi et al., 2021; Newman and Johnson, 2021). Pollination by short-tongued flies may be more widespread in *Erica* species from the Drakensberg, as other species with similar floral traits can be found there.

2.4.4 Pollen limitation

The pollen limitation experiment at Carter's Nek revealed that although *E. caffrorum* is significantly pollen limited, it is only weakly so (small effect size: pollen limitation index of 0.18). Naturally pollinated flowers, on average set more than 60% seed, whereas for flowers to which pollen was manually added, fecundity increased to 80%. The weak pollen limitation observed in *E. caffrorum* is surprising as it contrasts with general trends found for self-incompatible species, and in combination with a relatively specialised pollination system, both of which are typically associated with stronger pollen limitation (Larson and Barrett, 2000). For *E. caffrorum,* flies appear to be important and effective pollinators resulting in only weak pollen limitation in a specialised plant-pollinator relationship.

2.5 Conclusion and new questions raised by this study

The pollination systems of *E. caffrorum* is characterised by specialisation for pollination by short-tongued flies that vary locally and temporally in assemblage composition, self-incompatibility, and a significant but small amount of pollen limitation. This combination of features of the reproductive ecology of *E. caffrorum* brings about new questions: if short-tongued flies are the main pollinators, what traits does *E. caffrorum* use for attracting these flies? Is there variation in floral traits that attract different pollinators at particular sites, and could the variation in floral traits or variation in pollinator assemblages possibly be the driving force for the formation of ecotypes for this plant species? Could the short-tongued flies found in this study be more prevalent for other pollination systems of plant species at higher elevations? These questions require further research, in particular analyses of functional floral traits of *E. caffrorum* and an extension of pollination studies in other species that co-occur in the same communities.

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2.8 Supplementary Material

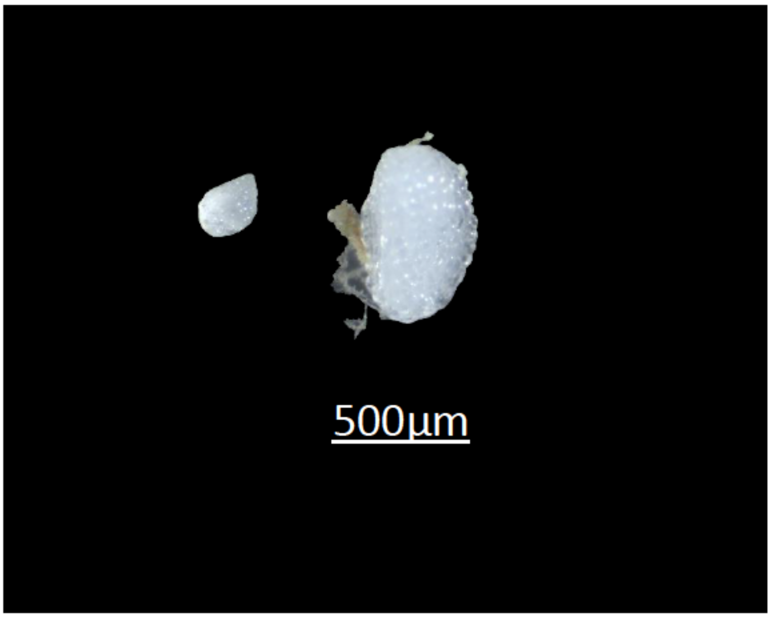

Figure S2.1: Size differentiation of immature ovule (left) vs a developing seed (right) that was used to distinguish ovules from seeds.

Figure S2.2: *Erica caffrorum* habitat and flower visitors: Typical *E. caffrorum* habitat at the Tarn Cave site (a), Sarcophagidae (*Sarcophaga*) (b), Muscidae (*Neomyia*) (c) and Lepidoptera (Noctuidae) (d) pollinators [Photographs T. Van der Niet].

Table S2.1: SIMPER analysis of visitor taxa to *E. caffrorum* that contribute to cumulative Bray–Curtis dissimilarity (Diss.) between flowering seasons among sites. Values represent number of insects captured at each site. The Average dissimilarity measures how visitor taxa differ from one another, with 0 indicating no difference and the higher the number, indicating a greater difference. The Contribution % shows the percentage dissimilarity explained by that visitor taxon, and the Cumulative % tallies the cumulative Bray Curtis dissimilarity metric for the visitor taxon represented in the table.

Taxon	Av. dissim	Contrib. %	Cumulative %	Carter's Nek 2020-2021	Kamberg Reserve 2020-2021	Ntsikeni 2017-2018	Ntsikeni 2019-2020	Tarn Cave 2017-2018	Tarn Cave 2018-2019	Tarn Cave 2020-2021	Witzieshoek 2019-2020	Witzieshoek 2021-2022
Neomyia	15.47	23.67	23.67	23	25	18	1	70	13	24	34	19
Scathophaga	7.933	12.14	35.8	1	39	1	5	4	0	5	1	0
Sarcophaga	5.886	9.004	44.81	5	4	1	14	0	0	7	9	6
Byomya	5.479	8.381	53.19	0	5	0	0	20	0	12	2	7
Stomorhina	4.971	7.604	60.79	18	1	2	0	1	0	8	0	0
Isomyia	4.106	6.281	67.07	11	0	0	0	10	2	7	0	0
Sepsidae	3.203	4.9	71.97	0	0	0	0	0	0	0	26	0
Thripidae	2.654	4.06	76.04	0	0	0	0	0	0	2	7	8
Musca	2.637	4.034	80.07	0	5	0	0	0	0	0	16	0
Pyrrhocoridae	2.16	3.305	83.37	0	0	0	0	0	0	0	8	6
Hilarempis	2.066	3.16	86.53	0	0	0	0	12	0	4	1	0
Tachininae	1.994	3.05	89.58	0	0	0	1	0	0	0	14	0
Apis	1.452	2.222	91.81	0	0	0	0	5	0	5	2	0
Hesperiidae	1.401	2.143	93.95	0	5	0	0	3	0	3	0	0
Eristalinus	1.375	2.104	96.05	0	0	0	0	9	0	0	3	0
Muscoidea	0.991	1.516	97.57	0	2	0	0	0	0	4	1	0
Chrysomya	0.3866	0.5913	98.16	1	1	0	0	0	0	0	1	0
Stomoxys	0.3182	0.4868	98.65	1	1	0	0	0	0	0	0	0

Ceratitis	0.221	0.338	98.98	0	0	0	0	1	0	0	1	0
Anthomyia	0.221	0.338	99.32	0	0	0	0	1	0	0	1	0
Noctuidae	0.1968	0.301	99.62	1	0	0	0	0	0	0	0	0
Vespidae	0.1232	0.1885	99.81	0	0	0	0	0	0	0	1	0
Lauxanidae	0.1232	0.1885	100	0	0	0	0	0	0	0	1	0

Table S2.2: DNA barcodes blasted in GenBank database along with morphological identifications of the dipteran visitors of *E. caffrorum*

code	success/failed	GenBank blast family	GenBank blast best match	query cover	% identity/ p distance	Best match	mophology ID	Site/season
1398A01	failed	failed	(Barcoding failed)	failed	failed	failed	Rhiniidae	Tarn_Cave_2017-2018
1398A02	success	Syrphidae	*Eristalinus vicarians*	99%	94.77	NC_042909.1	Syrphidae	Tarn_Cave_2017-2018
1398A03	success	Syrphidae	*Eristalinus vicarians*	99%	94.77	NC_042909.1	Syrphidae	Tarn_Cave_2017-2018
1398A04	success	Syrphidae	*Eristalinus vicarians*	99%	94.77	NC_042909.1	Syrphidae	Tarn_Cave_2017-2018
1398A05	success	Syrphidae	*Eristalinus quinquelineatus*	91%	95.09	KR831000.1	Syrphidae	Tarn_Cave_2017-2018
1398A06	success	Muscidae	*Helina annosa*	89%	89.82	MG090929.1	Muscidae	Tarn_Cave_2017-2018
1398A07	success	Tachinidae	*Mysceristops stolida*	69%	89.77	KX844256.1	Tachinidae	Witzieshoek_2019-2020
1398A08	success	Muscidae	*Helina annosa*	98%	91.19	JF877149.1	Muscidae	Tarn_Cave_2017-2018
1398B01	success	Syrphidae	*Eristalinus vicarians*	99%	94.77	NC_042909.1	Syrphidae	Tarn_Cave_2017-2018
1398B02	success	Syrphidae	*Eristalinus quinquelineatus*	89%	94.98	KR831000.1	Syrphidae	Tarn_Cave_2017-2018
1398B03	success	Muscidae	*Phaonia valida*	100%	91.49	MN555673.1	Muscidae	Tarn_Cave_2017-2018
1398B04	success	Syrphidae	*Eristalinus vicarians*	99%	94.77	NC_042909.1	Syrphidae	Tarn_Cave_2017-2018
1398B05	success	Syrphidae	*Eristalinus vicarians*	99%	94.77	NC_042909.1	Syrphidae	Tarn_Cave_2017-2018
1398B06	success	Syrphidae	*Eristalinus quinquelineatus*	91%	95.13	KR831000.1	Syrphidae	Tarn_Cave_2017-2018
1398B07	failed	failed	(Barcoding failed)	failed	failed	failed	Tephritidae	Tarn_Cave_2017-2018
1398B08	success	Muscidae	*Helina latitarsis*	100%	91.84	MT410783.1	Muscidae	Witzieshoek_2019-2020
1398C01	success	Muscidae	*Haematobia irritans*	100%	91.48	KM669714.1	Muscidae	Witzieshoek_2019-2020
1398C02	success	Sarcophagidae	*Sarcophaga burungae*	98%	100	KU746592.1	Sarcophagidae	Witzieshoek_2019-2020
1398C03	success	Syrphidae	*Eristalinus vicarians*	99%	94.77	NC_042909.1	Syrphidae	Witzieshoek_2019-2020
1398C04	success	Syrphidae	*Eristalinus vicarians*	99%	94.77	NC_042909.1	Syrphidae	Witzieshoek_2019-2020
1398C05	success	Anthomyiidae	*Delia sanctijacobi*	85%	89.06	MT808064.1	Muscidae	Witzieshoek_2019-2020
1398C06	success	Muscidae	*Phaonia valida*	100	91.64	MN555673.1	Muscidae	Tarn_Cave_2017-2018

1398C07	success	Syrphidae	*Eristalinus vicarians*	99%	94.77	NC_042909.1	Syrphidae	Tarn_Cave_2017-2018
1398C08	success	Muscidae	*Haematobia irritans*	100%	91.48	KM669714.1	Muscidae	Tarn_Cave_2017-2018
1398D01	success	Muscidae	*Haematobia irritans*	100%	91.63	KM669714.1	Muscidae	Tarn_Cave_2017-2018
1398D02	success	Muscidae	*Musca vitripennis*	99%	93.57	MW592366.1	Muscidae	Tarn_Cave_2017-2018
1398D03	success	Muscidae	*Musca vitripennis*	99%	97.01	MW592366.1	Muscidae	Tarn_Cave_2017-2018
1398D04	success	Muscidae	*Phaonia valida*	100%	91.64	MN555673.1	Muscidae	Tarn_Cave_2017-2018
1398D05	success	Anthomyiidae	*Delia florilega*	98%	95.59	MZ632781.1	Muscidae	Tarn_Cave_2017-2018
1398D06	success	Rhiniidae	*Stomorhina lunata*	100%	100	OW121746.1	Rhiniidae	Tarn_Cave_2017-2018
1398D07	success	Muscidae	*Musca vitripennis*	98%	95.91	MW592366.1	Muscidae	Tarn_Cave_2017-2018
1398D08	success	Muscidae	*Musca vitripennis*	99%	97.01	MW592366.1	Muscidae	Tarn_Cave_2017-2018
1398E01	success	Tachinidae	*Tachinidae sp. bold*	92%	89.68	KP043003.1	Tachinidae	Witzieshoek_2019-2020
1398E02	success	Tachinidae	*Tachinidae sp. bold*	93%	89.41	KP043003.1	Tachinidae	Witzieshoek_2019-2020
1398E03	success	Muscidae	*Hydrotaea sp.*	95%	91.82	KM903081.1	Muscidae	Kamberg_Reserve_2020-2021
1398E04	success	Muscidae	*Haematobia irritans*	100%	91.63	KM669714.1	Muscidae	Kamberg_Reserve_2020-2021
1398E05	success	Sarcophagidae	*Sarcophaga sushkini*	100%	95.07	KX094928.1	Sarcophagidae	Kamberg_Reserve_2020-2021
1398E06	success	Sarcophagidae	*Sarcophaga tuberosa*	99%	93.11	NC_047405.1	Sarcophagidae	Kamberg_Reserve_2020-2021
1398E07	success	Scathophagidae	*Scathophaga stercoraria*	93%	94.04	MT622510.1	Scathophagidae	Kamberg_Reserve_2020-2021
1398E08	failed	failed	(Barcoding failed)	failed	failed	failed	Calliphoridae	Kamberg_Reserve_2020-2021
1398F01	success	Muscidae	*Musca vitripennis*	99%	97.31	MW592366.1	Muscidae	Kamberg_Reserve_2020-2021
1398F02	success	Sarcophagidae	*Sarcophaga tibialis*	90%	91.64	MK679959.1	Sarcophagidae	Kamberg_Reserve_2020-2021
1398F03	success	Muscidae	*Stomoxys calcitrans*	100%	99.7	KU543641.1	Muscidae	Carters_Nek_2020-2021
1398F04	success	Muscidae	*Musca vitripennis*	99%	97.01	MW592366.1	Muscidae	Carters_Nek_2020-2021
1398F05	success	Muscidae	*Haematobia irritans*	100%	91.48	KM669714.1	Muscidae	Carters_Nek_2020-2021
1398F06	success	Muscidae	*Haematobia irritans*	100%	92.83	DQ029097.1	Muscidae	Carters_Nek_2020-2021
1398F07	success	Rhiniidae	*Stomorhina lunata*	100%	100	OW121764	Rhiniidae	Carters_Nek_2020-2021
1398F08	success	Rhiniidae	*Stomorhina lunata*	100%	100	OW121764	Rhiniidae	Carters_Nek_2020-2021
KRE3	success	Muscidae	*Stomoxys calcitrans*	100%	99.85	KU543641.1	Muscidae *stomoxys*	Kamberg_Reserve_2020-2021
KRE67	success	Muscidae	*Stomoxys calcitrans*	100%	99.55	KU543641.1	Muscidae *stomoxys*	Kamberg_Reserve_2020-2021
KRE10	failed	failed	(Barcoding failed)	failed	failed	failed	Muscidae *Byomya*	Kamberg_Reserve_2020-2021
KRE77	failed	failed	(Barcoding failed)	failed	failed	failed	Muscidae *Byomya*	Kamberg_Reserve_2020-2021
KRE8	failed	failed	(Barcoding failed)	failed	failed	failed	Muscidae *Musca*	Kamberg_Reserve_2020-2021
KRE64	success	Muscidae	*Musca vitripennis*	99%	96.86	MW592366.1	Muscidae *Musca*	Kamberg_Reserve_2020-2021
KRE9	failed	failed	(Barcoding failed)	failed	failed	failed	Scathophagidae	Kamberg_Reserve_2020-2021
KRE13	success	Scathophagidae	*Scathophaga stercoraria*	89%	96.18	MT622510.1	Scathophagidae	Kamberg_Reserve_2020-2021
KRE30	success	Sarcophagidae	*Sarcophaga sushkini*	100%	95.07	KX094928.1	Sarcophagidae	Kamberg_Reserve_2020-2021
KRE68	success	Sarcophagidae	*Sarcophaga brevicornis*	100%	92.68	NC_047404.1	Sarcophagidae	Kamberg_Reserve_2020-2021

KRE17	success	Muscidae	*Haematobia irritans*	100%	91.48	KM669714.1	Muscidae *Neomyia*	Kamberg_Reserve_2020-2021
KRE18	success	Muscidae	*Haematobia irritans*	100%	91.48	KM669714.1	Muscidae *Neomyia*	Kamberg_Reserve_2020-2021
EFW7	failed	failed	(Barcoding failed)	failed	failed	failed	Muscidae *Musca*	Carters_Nek_2020-2021
EFW60	success	Muscidae	*Neomyia yunnanensis*	100%	91.18	MF511762.1	Muscidae *Muscoid*	Carters_Nek_2020-2021
EFW6	success	Rhiniidae	*Isomyia electa*	98%	93.63	KY031768.1	Rhiniidae *Isomyia*	Carters_Nek_2020-2021
EFW19	success	Rhiniidae	*Isomyia electa*	98%	93.63	KY031768.1	Rhiniidae *Isomyia*	Carters_Nek_2020-2021
EFW20	success	Rhiniidae	*Stomorhina lunata*	100%	100	OW121746.1	Rhiniidae *Stomorhina*	Carters_Nek_2020-2021
EFW32	success	Muscidae	*Musca vitripennis*	99%	97.31	MW592366.1	Muscidae *Musca*	Carters_Nek_2020-2021
EFW34	success	Rhiniidae	*Stomorhina lunata*	100%	100	OW121746.1	Rhiniidae *Stomorhina*	Carters_Nek_2020-2021
EFW53	success	Rhiniidae	*Stomorhina lunata*	100%	100	OW121746.1	Rhiniidae *Stomorhina*	Carters_Nek_2020-2021
Viren-176	success	Muscidae	*Haematobia irritans*	100%	91.48	KM669714.1	Muscidae *Neomyia*	Witzieshoek_2019-2020
Viren-178	success	Muscidae	*Haematobia irritans*	100%	91.33	KM669714.1	Muscidae *Neomyia*	Witzieshoek_2019-2020
Viren-197	success	Tachinidae	*Siphona nigricans*	98%	94.38	KX843940.1	Tachinidae	Witzieshoek_2019-2020
Viren-219	success	Tachinidae	*Siphona nigricans*	98%	94.22	KX843940.1	Tachinidae	Witzieshoek_2019-2020
Viren-199	failed	failed	(Barcoding failed)	failed	failed	failed	Tachinidae	Witzieshoek_2019-2020
Viren-215	success	Tachinidae	*Siphona nigricans*	98%	94.38	KX843940.1	Tachinidae	Witzieshoek_2019-2020
Viren-222	Success	Syrphidae	*Eristalis tenax*	85%	98.26	OL981998.1	Syrphidae	Witzieshoek_2019-2020
Viren-210	failed	failed	(Barcoding failed)	failed	failed	failed	Syrphidae	Witzieshoek_2019-2020
TC25	success	Calliphoridae	*Calliphora vicina*	100%	97.76	KX893333.1	Rhiniidae *Isomyia*	Tarn_Cave_2020-2021
TC47	success	Muscidae	*Phaonia subfuscinervis*	98%	89.5	KM929221.1	Muscidae *Muscoid*	Tarn_Cave_2020-2021
TC32	success	Muscidae	*Haematobia irritans*	100%	91.48	KM669714.1	Muscidae *Neomyia*	Tarn_Cave_2020-2021
TC38	success	Muscidae	*Haematobia irritans*	100%	91.48	KM669714.1	Muscidae *Neomyia*	Tarn_Cave_2020-2021
TC40	success	Muscidae	*Limnophora sp*	98%	93.47	KX230675.1	Muscidae *Muscoid*	Tarn_Cave_2020-2021
TC44	success	Muscidae	*Musca vitripennis*	99%	97.16	MW592366.1	Muscidae *Muscoid*	Tarn_Cave_2020-2021
TC64	Success	Sarcophagidae	*Sarcophaga tuberosa*	99%	93.11	NC_047405.1	Sarcophagidae	Tarn_Cave_2020-2021
TC71	Success	Sarcophagidae	*Sarcophaga tuberosa*	99%	93.11	NC_047405.1	Sarcophagidae	Tarn_Cave_2020-2021
TC21	success	Scathophagidae	*Scathophaga stercoraria*	100%	95.67	MT622510.1	Scathophagidae	Tarn_Cave_2020-2021
TC34	success	Scathophagidae	*Scathophaga stercoraria*	100%	95.67	MT622510.1	Scathophagidae	Tarn_Cave_2020-2021
N1	failed	failed	(Barcoding failed)	failed	failed	failed	Scathophagidae	Ntsikeni_2017-2018
N2	success	Scathophagidae	*Scathophaga stercoraria*	100%	95.67	MT622510.1	Scathophagidae	Ntsikeni_2017-2018
ET1	success	Muscidae	*Haematobia irritans*	100%	91.48	KM669714.1	Muscidae *Neomyia*	Tarn_Cave_2018-2019
ET2	success	Muscidae	*Haematobia irritans*	100%	91.48	KM669714.1	Muscidae *Neomyia*	Tarn_Cave_2018-2019
ET14	success	Tachinidae	*Agaedioxenis succulentus*	100%	96.71	KP189255.1	Tachinidae	Witzieshoek_2019-2020
ET15	success	Tachinidae	*Agaedioxenis succulentus*	100%	96.71	KP189255.1	Tachinidae	Witzieshoek_2019-2020

Chapter 3: Quantification and functional significance of floral traits of *Erica caffrorum* (Ericaceae) in relation to pollinator attraction

Abstract:

In plant species dependent on pollinators for reproduction, floral traits are thought to reflect adaptations to optimise pollination, including colour and scent for pollinator attraction and flower dimensions that optimise the contact of reproductive parts with pollinators. However, for species pollinated by short-tongued flies, relatively few studies have quantified these traits and tested their function across multiple sites. Here, I focus on *Erica caffrorum*, for which pollinator observations have confirmed that it is predominantly pollinated by short-tongued flies (Insecta, Diptera) across five study sites., To quantify floral dimensions, colour, and scent and then use bioassays to test the functional significance of flower colour and scent components for attraction of flower visitors. Floral dimensions varied significantly among sites, including flower opening width and functional depth. The white to cream-coloured flowers show a steep increase in reflectance from 400 nm to approximately 450 nm, followed by constant reflectance to 700 nm. When plotted in the fly vision model, the spectral loci of the sepals, petals and leaves fell in the same quadrant, suggesting an absence of colour contrast between flowers and their immediate background. The floral scent, unpleasant to the human nose, comprised between 40 and 90 compounds per plant individual sampled and varied among sites. The dominant compounds comprised aromatics (benzaldehyde, phenylethyl alcohol), monoterpenes (linalool) and sesquiterpenes (caryophyllene), as well as various acids (including 2-methylbutanoic acid). In an experiment in which insects were offered choices among combinations of colour and scent, flies preferred white over green in the presence of scent at two out of three sites. Overall, other insects (including bees, butterflies, moths, grasshoppers and thrips) showed no preference for a particular colour or scent. In choice test experiments among different scent compound combinations at three sites, flies preferred a mixture of a 2-methylbutanoic acid and benzaldehyde over the acid or benzaldehyde alone or a hexane control. For other insects, including pollinators of *E. caffrorum*, no consistent preferences for particular scent compounds were detected. I conclude that both flower colour and the scent of *E. caffrorum* have functional significance for attracting pollinators. Further research is required to test

whether site-specific variation in scent blends reflects function in attraction of different fly families across the species' range.

Key Words: bioassay experiments, flower colour, flower morphometrics, flower scent, spectral reflectance.

3.1 Introduction

Flowering plant species that depend on pollinators for reproduction have developed numerous ways to attract diverse pollinators. Flowers are the primary organs involved in pollinator attraction and are characterised by great variation in shapes, sizes, orientation, colours, scents and rewards (Schiestl and Johnson, 2013). The tremendous variety of these floral traits is thought to have evolved as diverse adaptations to the many animal species that contribute to pollination (Phillips et al., 2020), because these pollinator species are characterised by variation in sensory modalities and morphologies (Schiestl and Johnson, 2013).

Floral scent is a key trait for pollinator attraction and typically comprises a blend of low molecular weight compounds (Knudsen and Tollsten, 1993). Indications for its function in pollination come from two lines of evidence. Firstly, bioassays have demonstrated a key role for scent in attracting pollinators (Stensmyr et al., 2002; Urru et al., 2011). Secondly, comparative studies show widespread similarities in scent compounds among plant species that share major pollinator groups (Knudsen and Tollsten, 1993). For instance, bird-pollinated flowers produce no scent, moth-pollinated flowers emit aromatic compounds (Knudsen and Tollsten, 1993), and distantly related carrion-mimics pollinated by flies produce sulphur or nitrogen-containing compounds (Jürgens et al., 2013). The scent compounds emitted by fly-pollinated flowers is particularly variable, especially in the context of scent-mimicry. It has been suggested that mimicry of a range of specific models, including carrion, dung, and urine, underlies this highly diverse scent chemistry (Jürgens et al., 2006).

Although one of the main functions of floral scent in angiosperms is to attract pollinators, supplementary functions include defence against herbivores and protection against abiotic stress (Pichersky and Gershenzon, 2002). These additional functions may help explain the substantial number and variety of scent compounds often detected in floral scent bouquets (Schiestl, 2010). Therefore, quantification for identification and experimental tests to verify function is required to understand the role of scent in pollinator attraction.

In addition to scent, flower colour is also considered key for pollinator attraction (Schiestl and Johnson, 2013) and is highly variable among angiosperms (Narbona et al., 2021). Like scent, distinct patterns of colour similarity are associated with different functional pollinator groups (Reverté et al., 2016). For instance, many bird-pollinated flowers are red or orange (Shrestha et al., 2013), whereas moth-pollinated flowers are typically pale to white (Oliveira et al., 2004). In many short-tongued fly-pollinated plant species, especially oviposition site mimics, the colour of the flowers is typically brown to purple (Jürgens et al., 2015), whereas hoverfly pollinated plant species are usually yellow and white (Lunau, 2014), and long-proboscid fly pollinated flowers are pale blue to pale pink with UV guides (Goldblatt and Manning, 2000).

Colour variation in flowers is thought to be associated with variation in visual systems among pollinating animal species based on the number of receptor types and their sensitivity to specific wavelengths (Reverté et al., 2016). For instance, the visual system of the honeybee, often used as a model for bees in general, is sensitive to wavelengths between 300 and 700 nm and is based on a trichromatic system. Many other insects also have trichromatic vision systems, but there are taxa with dichromatic (flies) and tetrachromatic (e.g., some butterflies) systems (Briscoe and Chittka, 2001; Van der Kooi et al., 2021).

Although flower colour is an important cue to attract pollinators, the pigments that produce colour, such as anthocyanins, carotenoids, and flavonoids (Tanaka et al., 2008), may also protect the plant from UV radiation (e.g., Mori et al., 2005) or herbivory (e.g., Kemp and Ellis, 2019) or help regulate plant metabolism or development (Trunschke et al., 2021). Therefore, it is important to analyse flower colour and evaluate its function in relation to pollination.

Morphological flower traits are important in the pollination process, as it mediates contact between the pollinator body and plant reproductive organs. The length of the nectargland, which frequently varies across flowering plant species pollinated by different functional pollinator groups, is among the most important of these (Whittall and Hodges, 2007). Nectar tube length varies with pollinator mouth-part length (e.g., Whittall and Hodges, 2007; Anderson and Johnson, 2009). In some cases, floral morphology is not considered an adaptation to match the shape of its pollinator but rather functions as a filter to exclude non-pollinating visitors (Castellanos et al., 2004).

Techniques for the objective characterisation of floral traits, including morphometrics for quantification of floral shape, spectrophotometry for quantifying flower colour and gas chromatography coupled with mass spectrometry (GC-MS) for quantifying floral scent enable patterns of association between traits and pollination systems to be identified. Such associations suggest the putative functional significance of traits in pollination; however, testing requires controlled manipulative experiments. For instance, the function of floral scent can be tested by experimentally manipulating the scent of plants in their environment, whereas for flower colour, field-based behavioural experiments can be conducted using artificial flowers (e.g., Shuttleworth and Johnson, 2010; Jersáková et al., 2012). Manipulative experiments are often performed at a single site. Although insightful, the limitation of this approach is that it does not consider the possibility of context-dependent outcomes (Jersáková et al., 2012; Whitehead et al., 2018). To generalise whether a particular trait serves a particular function in pollination requires testing at multiple sites (Sletvold, 2019).

The species-rich plant genus *Erica*, which comprises a diverse floral radiation (Rebelo et al., 1985; Pirie et al., 2016), has been extensively studied for associations between floral traits and functional pollinator groups. In a study of five *Erica* species that are all predominantly pollinated by honeybees, all species had floral tube lengths that matched honeybee mouth-parts despite considerable variation in scent and colour among species (Bouman et al., 2017). The hawkmoth-pollinated *Erica cylindrica* exhibits traits typical of a hawkmoth pollination syndrome, such as narrow and upright-oriented tubular flowers and emission of benzenoid scent compounds (Van der Niet and Cozien, 2022). Experiments have also established that foraging preferences of sunbirds in relation to flower colour in *Erica* indicate that they prefer colours that are typically associated with the ornithophilous syndrome (Heystek et al., 2014), such as reddish-pink (Rebelo and

Siegfried, 1985). Intraspecific variation in floral traits of *Erica plukenetii* has been shown to reflect the evolution of three different pollination ecotypes, each with characteristic traits: two long-tubed and weakly scented bird-pollinated ecotypes and a short-tubed, but strongly scented moth-pollinated ecotype (Van der Niet et al., 2014). *Erica aristata* has flowers that have long, narrow corolla tubes with narrow openings and contrasting white and pink petals, traits typically associated with pollination by long-proboscid flies and is exclusively pollinated by a tangle-veined fly species (Diptera, Nemestrinidae) (Lombardi et al., 2021). Similarly, different varieties of *Erica junonia* with different flower tube lengths are visited by different long-proboscid flies (Nemestrinidae, *Moegistorhynchus*) with different proboscis lengths (Newman and Johnson, 2021). Finally, *Erica hanekomii* has flowers that occur close to the ground and that are dull-coloured with large volumes of sucrose nectar and appears to be adapted to rodent pollinators (Turner et al., 2011). Together, these studies show convincingly that *Erica* species with different pollination systems are characterised by distinctive floral traits, including scent, colour, and morphology.

All studies to date that quantify colour, scent and other floral traits relevant for pollination in *Erica* focus on species that occur in the Cape Floristic Region (CFR) (Bouman et al., 2017; Heystek et al., 2014; Newman and Johnson, 2021; Ojeda et al., 2019; Turner et al., 2011; Van der Niet et al., 2014; Van der Niet and Cozien, 2022). However, a substantial proportion of *Erica* diversity occurs outside the CFR, including a secondary centre of diversity in the Drakensberg (Pirie et al., 2016), where many of the available pollinator species in the CFR may not occur (e.g., Johnson, 2010). Thus, studies from outside the region are required. Furthermore, relatively few available studies have implemented experimental work to verify the adaptive significance of the trait-pollinator associations, suggesting pollinators' role in driving diversification in *Erica* (but see Heystek et al., 2014, McCarren et al., 2001). More experiments, therefore, need to be conducted to understand the function of floral traits for pollination in *Erica* (Van der Niet, 2021).

Erica caffrorum, found in high-elevation habitats of the summer rainfall region of southern Africa, is dependent on pollinators for reproduction (Chapter 2). Pollinator observations at five sites showed that, overall, 88.3% of flower visitors were true flies (Insecta, Diptera) (Chapter 2), although an assessment of pollinator importance (PI) incorporating visitation frequency and pollen loads of all visitors showed that the relative importance of particular dipteran taxa differed among sites and years (Chapter 2). *Erica caffrorum* can thus be considered specialised at the functional group level for short-tongued fly pollination, representing a novel pollination system in *Erica*. Based on general associations between specific floral traits, including morphology, scent, and colour, and particular pollinator groups in *Erica*, it seems likely that in *E. caffrorum*, colour and scent also play a role in the attraction of short-tongued flies. Quantification and experiments are needed to characterise traits and test their function. Furthermore, given the site-specific dominance of particular fly groups, local adaptation may be associated with population-level trait variation. Therefore, it is important that floral traits are quantified and experimentally tested at multiple sites.

The aim of the study presented in this chapter is to objectively quantify floral traits that are likely relevant for pollination. The specific objectives were to 1) measure floral morphology, 2) quantify flower colour using spectrophotometry, 3) quantify floral scent using GC-MS, and 4) use bioassays to test the function of flower colour and scent for pollinator attraction in *E. caffrorum*. A first bioassay focused on determining the potential function of flower colour and the scent of *E. caffrorum* for pollinator attraction, and a second bioassay was used to assess whether dominant scent compounds found in the scent of flowers of *E. caffrorum* function to attract flower visitors. These experiments were conducted at multiple sites to establish whether floral traits, especially key scent compounds, function to attract the observed locally varying assemblages of pollinating short-tongued flies.

3.2 Methods

Erica caffrorum (Ericaceae) occurs in high-elevation regions of the Drakensberg mountains in the Eastern Cape and KwaZulu-Natal provinces of South Africa. *Erica caffrorum* is a dense creeping to erect shrub 0.2–3 m in height found on grassy mountain slopes, cliffs, or forest edges between 1,500–2,500 m in elevation. The main stem is densely branched, and the leaves are crowded in whorls of four along the entire branchlet, tightly clasping the stem. The small, dull white to pale pink flowers are enclosed for up to two-thirds of their length by large calyx lobes and droop in clusters of four near the end of the branchlets (Guthrie and Bolus, 1905). Populations at five study sites were included in this investigation; at Carter’s Nek, Kamberg Reserve, Ntsikeni, Witzieshoek and Tarn Cave (for details and GPS coordinates, see Chapter 2, Table 2.1).

3.2.1 Flower morphometrics

To quantify floral morphology and to determine whether there are differences in floral morphology among plants from different sites, floral morphology was measured from three flowers of 20 plants from Tarn Cave, three flowers of ten plants from Witzieshoek and five flowers of five plants from Carter’s Nek. All measurements were taken to the nearest 0.1 mm using a pair of digital callipers. Floral tube length was measured as the distance from the sepal base to the sepal tip. The flower opening width was measured as the gap between the sepal tips. Stamen length was measured from the base of the flower to the tip of the anther ring. Style length was measured from the base of the ovary to the stigma.

3.2.2 Flower colour measurement

To quantify the colour of *E. caffrorum* flowers, the spectral reflectance of a total of 15 flowers (sepals) and 15 leaves of *E. caffrorum* at Carter’s Nek, as well as 16 flowers at Witzieshoek and six flowers at Tarn Cave, all sampled from different individual plants, were recorded using the method described in Whitehead et al. (2018). The spectral reflectance of collected flowers and leaves from different individual plants and bioassay experiment discs (see below) was measured over the UV-visible range (300–700 nm) using an Ocean Optics USB2000 spectrometer with an Ocean Optics DT-mini light source (200–1,100 nm; Dunedin, FL, USA) and fibre optic reflection probe (UV/VIS 400 µm) taken at 45 ° angles.

To determine if the sepals and petals are perceived as indistinguishable from each other and from materials used for bioassay experiments, the loci representing the measurements were analysed using the Troje (1993) fly vision model, based on the chromatic receptors of Calliphoridae flies, since most visitors were flies. This model uses the photoreceptor types of flies to plot spectra as loci in four quadrants of a two-dimensional co-ordinate plane. Loci within the same quadrant represent colours that are thought to be indistinguishable. However, it is to be noted that this fly vision model may not apply to all visitors of *E. caffrorum*.

3.2.3 Flower scent

To quantify the scent bouquet of *E. caffrorum* flowers, headspace was sampled following a protocol modified from Van der Niet et al. (2014) at all five study sites during peak flowering. Scent was sampled from a single branch of *E. caffrorum* with circa 100–1,000 flowers from four plants at Carter's Nek and four plants Kamberg Reserve, three plants at Tarn Cave and three at Witzieshoek, and two plants at Ntsikeni. At each site, a sample of the surrounding air was taken simultaneously to control for any compounds present in the ambient air.

Scent traps comprised 1.5 mg of Tenax® and 1.5 mg of Carbotrap™ activated charcoal. Inflorescences were each enclosed in a poly acetate oven bag that was closed with plastic cable ties at both ends and in which a small incision was made to allow for airflow (Kalle Bratschlauch, Wiesbaden, Germany). Sampling was done at a flow rate of 50 mL per minute for 30 minutes with a portable blue vacuum suction pump (Spectrex PAS500; Spectrex Corp., Redwood City, CA, USA). Sampling started in the late morning, representing the peak activity time of many of the insects pollinating *E. caffrorum*. Traps were stored individually in 1.8 ml clear glass vials, which were kept at -20 °C until analysis.

3.2.3.1 Gas chromatography-mass spectrometry analysis of floral scent

Scent samples were analysed using a Varian CP-3800 SCION model gas chromatograph (Varian, Palo Alto, California) equipped with a polar Alltech EC-WAX column with a 30 m × 0.25 mm inner diameter (film width of 0.25 µm. This column was attached to a Varian 1200 quadrupole mass spectrometer in electron-impact ionisation mode.

The scent traps were inserted into a Varian 1079 injector equipped with a ‘Chromatoprobe’ thermal desorption device (Amirav and Dagan, 1997; Dötterl et al., 2005). The sample vapour was mixed with inert gas (helium) and moved through the column at a flow rate of 1 ml per minute, following the procedure by Van der Niet et al. (2014). The injector temperature started at 40 °C for two minutes with a split ratio of 20:1. After this, the temperature of the injector was ramped up to 200 °C at 200 °C per minute in the split-less mode for thermal desorption and held for ten minutes. Simultaneously the GC oven was maintained at 40 °C for a three-minute period. After this, the GC oven was ramped up to 240 °C before it was cooled down to 10 °C per minute for 12 minutes.

Each compound in plant samples was recorded and quantified by integrating the area under the peak of the chromatograms. Compounds were identified using Scion Workstation software (Version 8.2.1) with the NIST 05 mass spectral library. Scent compounds present in the controls of the ambient air were considered contaminants and omitted from further analysis if they were present in the same quantity as in the plant samples. Compound identification was based on comparing the mass spectra and the Kovats retention index of compounds present in the NIST library. The Kovats retention index of peaks in the chromatograms was obtained by comparison of retention times with those of a series of alkanes (Van den Dool and Kratz, 1963; Zenkevich, 2010) that were run separately on the GC-MS device under identical conditions to the samples. The compounds that did not match any mass spectra of compounds in the NIST library with appropriate Kovats retention indices were considered unknown, with the six most prominent mass fragments provided.

3.2.4 Bioassay choice tests: colour/scent experiment

To determine the potential function of flower colour and scent of *E. caffrorum* for pollinator attraction, a bioassay experiment (hereafter, “colour/scent experiment”); was used to test the role that the white flower colour has against the green leaf background in the presence or absence of a scent blend comprising two dominant *E. caffrorum* compounds.

The colour/scent experiment was carried out at Witzieshoek on 24 November 2019 over two days and at Carter’s Nek and Kamberg Reserve on 18 January 2022 and 2 April 2022, respectively. The experiment was set up during the peak flowering of *E. caffrorum*, except for Kamberg Reserve, where the flowers were approximately two months past their peak flowering phase. However, the most frequent flower visitors (Chapter 2) were still present and active at this site (V. Thupsie, personal observation). In the colour/scent experiment, each replicate included eight treatments arranged in four binary choices. Within each pair, treatments differed in a single factor, either in colour or in the presence of scent and were placed one meter apart. Each binary pair was then placed two meters apart from another binary pair and then each replicate was placed at least five meters apart from another replicate. This design assessed both preferences for individual colour or scent cues and compared the strength of the preference associated with each cue in isolation (scent alone, colour alone) or combination (alone versus both combined). Specifically, the paired treatments were as follows: the functional significance of white flower colour was tested in the presence and absence of scent by comparing the proportion of visitors to paired white and green discs for which in each case one disc contained the scent, and one did not. The functional significance of floral scent was assessed by comparing the proportion of visitors attracted to scented treatments in which both treatments in a pair were either white or green.

The scent standard used in this experiment comprised a mixture of 2-methylbutanoic acid and benzaldehyde, which were the two dominant compounds of two different classes (aromatics and acids) (Table S3.1; S3.2). At the Witzieshoek site, a ratio of 1 µl:9 µl of 2-methylbutanoic acid to benzaldehyde was used, whereas, at the Carter's Nek and Kamberg Reserve sites, a ratio of 3 µl:7 µl 2-methylbutanoic acid to benzaldehyde was used. The difference in ratios used in the experiments was due to variation in ratios that was detected in initial scent samples (that were not further used). Over the course of the experiments, it was decided that a consistent 3:7 ratio was, however, preferable for comparison of results across multiple experiments and ratios were not further modified. As a consequence, it cannot be excluded that differences in results may stem from different compound ratios. Hexane was added to make up a total of 1 ml. All treatments in which the role of colour was tested comprised a vial with 1 ml of hexane to control for a potential effect of this solvent on insect attraction. All scent standards were pipetted into 1.8 ml vials and stored in separate boxes to avoid cross-contamination until further use.

To provide a visual target for insects and facilitate the trapping of insects attracted to each treatment, vials containing scent standard mixtures were positioned in the middle of plastic discs with a diameter of 75 mm, of which the upper surface was covered with a layer of Tanglefoot® (The Tanglefoot Company, Grand Rapids, Michigan) (Fig. 3.1). A 4 mm wide hole was cut in the centre of the disc to place the scent compound vials. Discs were cut from clear plastic sheeting and spray-painted with two layers of "matt white" (Sprayon Products (PTY) LTD) for the white discs and two layers of "matt green" (Sprayon Products (PTY) LTD) (Fig. 3.1), to match the reflectance of the white flowers and sepal region of flowers of *E. caffrorum* and the green leaves (green was used to replicate the background vegetation)respectively, before they were covered with Tanglefoot®.

The scent standards in 1.8 ml vials were inserted in OASIS® Floral Foam at Witzieshoek and inside 5 ml vials at Carter's Nek and Kamberg Reserve (this subtle difference in the placement of the 1.8 ml vials was cosmetic and likely had negligible influence in providing a visual target for visitors). The combination of discs and scent vials were attached to a 30 cm long wooden skewer. The skewer was positioned in the soil to hold scent vials 20 cm above the ground, within the height range of small plants of *E. caffrorum* (Fig. 3.1).

After each bioassay experiment was completed, discs were placed individually in Ziplock™ bags for transport back to the laboratory. All insects on each disc were identified as Diptera versus "other pollinators." Damage to specimens due to contact with Tanglefoot® prevented species-level identification in many cases and precluded verification using DNA barcoding. However, the visiting Diptera trapped on the discs had similar morphological characteristics to the fly families caught on *E. caffrorum,* and, as such, there was likely substantial overlap in the visitors on the trapped discs and those visiting *E. caffrorum*.

3.2.5 Scent choice experiment

To assess whether dominant scent compounds found in the scent of flowers of *E. caffrorum* function to attract flower visitors, a four-way choice experiment (hereafter "scent choice experiment") was performed, among three scent blends; benzaldehyde only; 2-methylbutanoic acid (hereafter referred to as "acid") only; a mixture of benzaldehyde and acid and a hexane control. These compounds were chosen because they are known to be involved in the attraction of broad groups of pollinators [benzaldehyde for bees (e.g., Theis, 2006) and acids for flies (e.g., Shrestha and Lee, 2021)]. White discs were used for all scent choice treatments. For the scent composition in the benzaldehyde-only treatment, seven µl of benzaldehyde was used. For the acid-only choice, three µl of 2-methylbutanoic acid was used. The benzaldehyde and acid mixture choice comprised three µl of 2-methylbutanoic acid and seven µl of benzaldehyde. Hexane was added to all these solutions to make up 1 ml for each choice solution. As a control to account for the possible effect of hexane on insect attraction, a fourth treatment containing only 1 ml hexane was also included in the experiment.

The scent choice experiment was conducted at three populations of *E. caffrorum*: Carter's Nek, Kamberg Reserve and Witzieshoek. At each population, replicates of four treatments were set up among *E. caffrorum* plants, with one meter between each treatment and at least five meters between replicates. At Witzieshoek, 12 replicates were exposed from 09:00 until 15:00 on 16 and 17 November 2019, coinciding with peak flowering for *E. caffrorum* and run over two days. At the start of the second day, the inner glass vials containing scent solutions were replaced. At Carter's Nek and Kamberg Reserve, eight replicates were set up at each site on 18 January 2022 and 2 April 2022, respectively. The collection and processing of the discs mirrored that of the colour/scent experiment.

3.3 Data analysis

3.3.1 Flower morphometrics

To test whether floral dimensions differed across sites, Generalised Estimating Equations (GEE) with a gamma distribution and log link function were used. To account for non-independence among measurements from the same plant individual, models included plant identity as a subject variable, and multiple flowers measured on the same plant as within-subject effects with an "exchangeable" correlation matrix. Analyses were performed using SPSS Version 27 (IBM Corp). To adjust significance for multiple comparisons, the Dunn-Šidák method was used (Šidák, 1967). All means for this analysis, and those described below, were back-transformed to the original scale for visualisation, resulting in asymmetrical error bars. Marginal means and standard errors were plotted together using SigmaPlot version 8.0 (Systat Software, Inc., 2005).

3.3.2 Flower colour

Reflectance spectra of *E. caffrorum* and background leaves were analysed with the PAVO package (Maia et al., 2019) using R version 4.2.1 (R Core Team, 2022). Spectra were trimmed to the 300 and 700 nm range, the UVA and visible spectral range, and averaged at 1 nm intervals (resulting in 401 reflectance values). The loci of the spectra were plotted in the Troje (1993) fly vision model.

3.3.3 Floral scent

Prior to the analysis of scent compounds, the mean relative proportion of each scent compound was square-root transformed to down-weight the impact of the dominant compounds. A dissimilarity matrix for the square-root transformed scent data was then calculated using the Bray-Curtis method in PAST Version 4.03 (Hammer et al., 2001) because the data matrix contained many zero values (Bray and Curtis, 1957).

To test whether scent composition varies across sites a one-way ANOSIM (Analysis of similarity) test was done. ANOSIM provides an R-value, the ratio of the between-site variation to the within-site variation. The R-value varies between "zero" and "one", where a value close to "one" indicates a high separation between levels of the factor (in this case, sites), whereas R-values close to "zero" indicate no separation between levels. The significance of dissimilarity is then evaluated by comparing the R-value to those generated using 9999 permutations (Chapman and Underwood, 1998). Furthermore, because of the small number of samples for Ntsikeni, this site was excluded from the pairwise comparison in the ANOSIM.

A similarity percentage (SIMPER) analysis was implemented to determine which compounds contribute most to dissimilarity among sites (i.e., which specific compounds are characteristic of a particular site). This analysis ranks scent compounds based on their importance in explaining dissimilarity (Clarke, 1993).

The variation of scent compounds across sites was then visualised in two dimensions using non-metric multidimensional scaling (NMDS) implemented in PAST Version 4.03 (Hammer et al., 2001), using the Bray-Curtis similarity of square-transformed proportions of compounds.

3.3.4 Bioassay experiments

The colour/scent choice experiment was analysed as a choice test, using a binary logistic distribution to model the number of insects attracted to each treatment within a pair out of the number attracted to both treatments in that pair, with an “events out of trials” structure. To account for correlated responses within replicates, analyses implemented GEEs with “replicate” included as a subject and treatment as a within-subject effect and an exchangeable correlation matrix. The data from the Witzieshoek site were analysed separately from Carter’s Nek and Kamberg Reserve sites because the use of different scent standard mixtures precluded simultaneous analysis. Furthermore, at Witzieshoek, there were too few “other visitors” to compare their abundance, so analyses were only carried out for Diptera. A preference was considered significant if the confidence interval of the proportion of trapped insects for a particular treatment in the binary choice did not overlap 0.5 choice (representing random choice). The strength of preference was compared between treatments using pairwise comparisons of the proportion of insects attracted to each treatment.

For the scent choice experiment, the number of trapped insect visitors attracted to each of the four treatments was compared using GEE with a negative binomial distribution and log link function. To account for the non-independence of data within replicates, “replicate” was included as the subject variable and treatment as a within-subject effect, with an exchangeable correlation matrix (similar to the colour/scent experiment). For both bioassays, the Dunn-Šidák method was used to adjust the significance for multiple comparisons (Šidák, 1967).

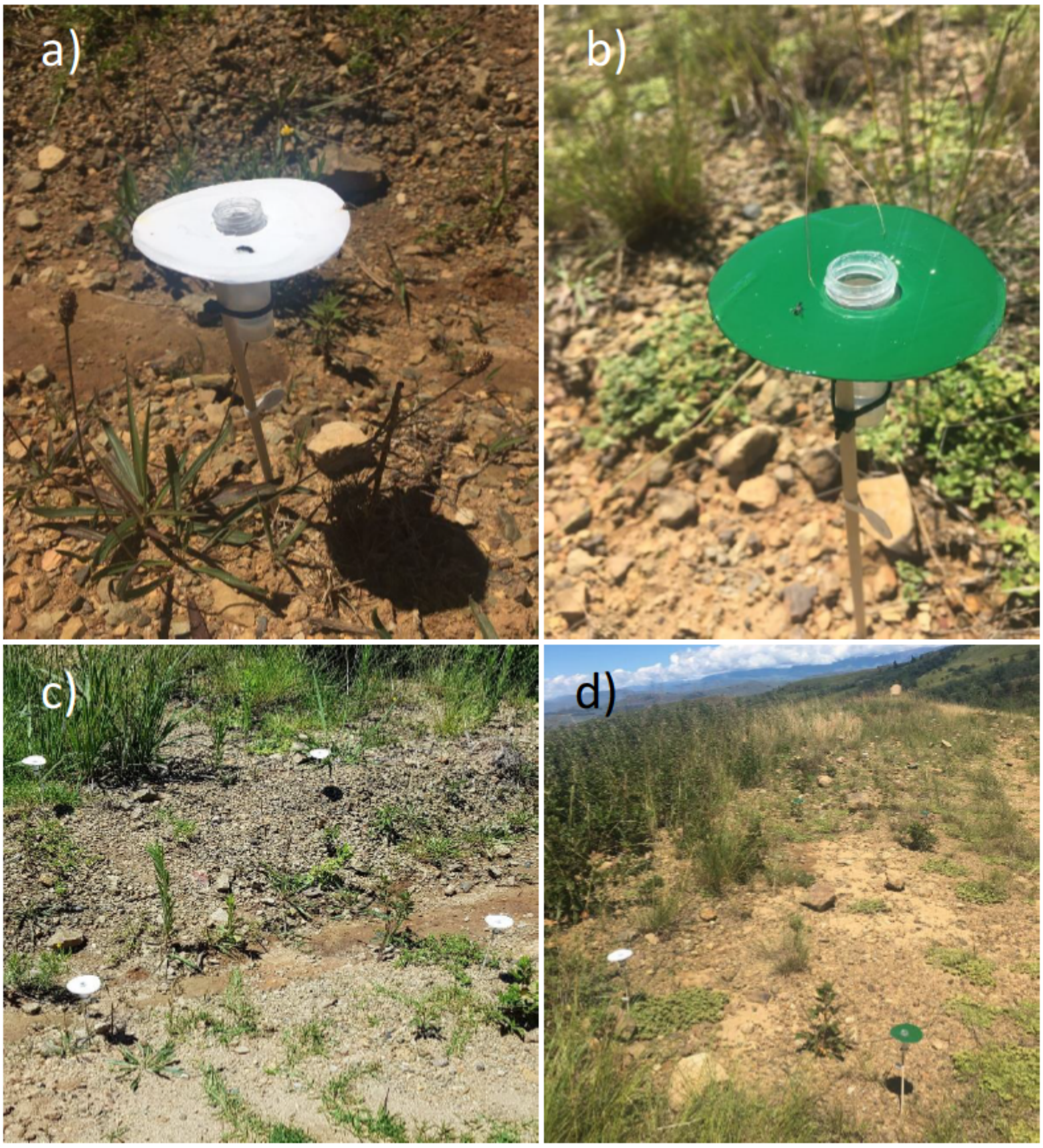

Figure 3.1: White (a) and green (b) discs used in the colour/scent experiment and scent choice experiments with the layouts and experimental sites for the scent composition experiment showing the four white discs used for the four treatments (c) and the paired white and green model discs used in the binary colour/scent experiment (d). [Photographs taken by T. Van der Niet (a–b) and V. Thupsie (c–d)].

3.4 Results

3.4.1 Flower morphometrics

Flower opening width (χ^2= 94.9, $p < 0.005$), stamen filament length (χ^2= 17.7, $p < 0.005$), and style length (χ^2= 33.4, $p < 0.005$) differed significantly across the three sites, whereas tube length was similar across populations (χ^2= 0.6, $p = 0.749$) (Fig. 3.2). Although the absolute magnitudes of the differences among populations are relatively small (<1 mm; Fig. 3.2) these differences represent variation of up to a third of the dimensions of floral parts (Fig. 3.2).

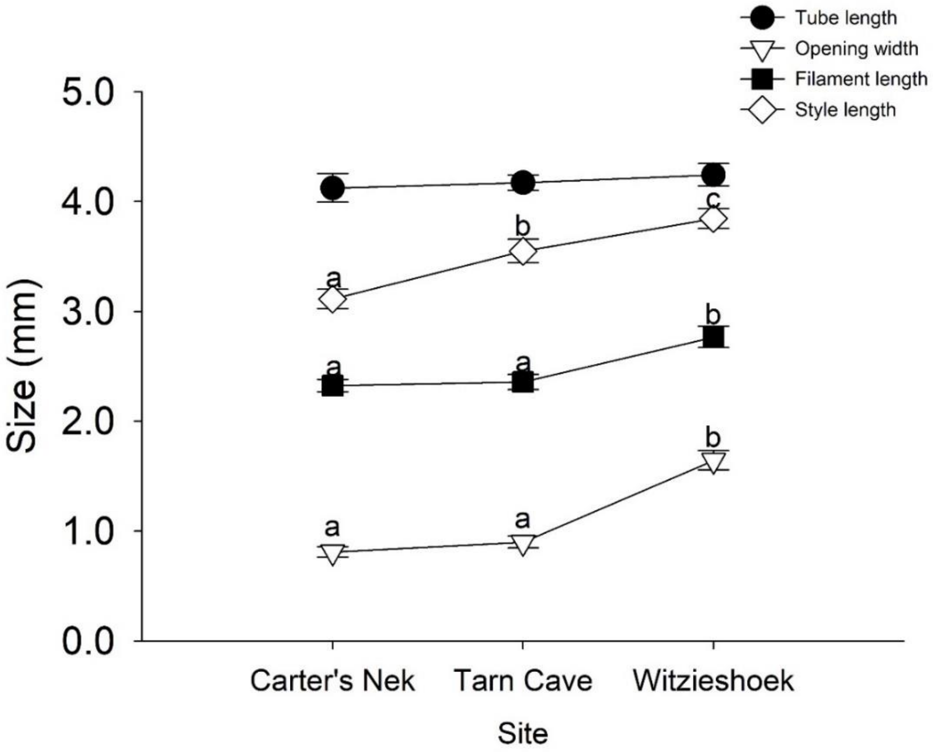

Figure 3.2: Mean ± SE of different flower parts. Symbols that share letters indicate values that do not differ significantly in post-hoc comparisons among sites ($p < 0.05$).

3.4.2 Flower colour

Sepals and petals of *E. caffrorum* showed a similar spectral reflectance, characterised by a steep increase in reflectance from 400 nm to approximately 450 nm, with a relatively constant reflectance between 450 nm and 700 nm (Figs 3.2a–c). None of the floral parts of *E. caffrorum* showed reflectance in the UVA range (300–399 nm). The white disc used for the bioassay experiments had a similar reflectance pattern to the sepals and petals (Fig. 3.2d). Leaves of *E. caffrorum* showed a reflectance pattern typical for green leaves, with an increase in reflectance at 500 nm and a peak at 550 nm (Fig. 3.2c). For spectra plotted in Troje's (1993) fly vision model, all points fell in the same quadrant, suggesting that no within-flower contrast between sepals and petals is detectable by flies (Fig. 3.4).

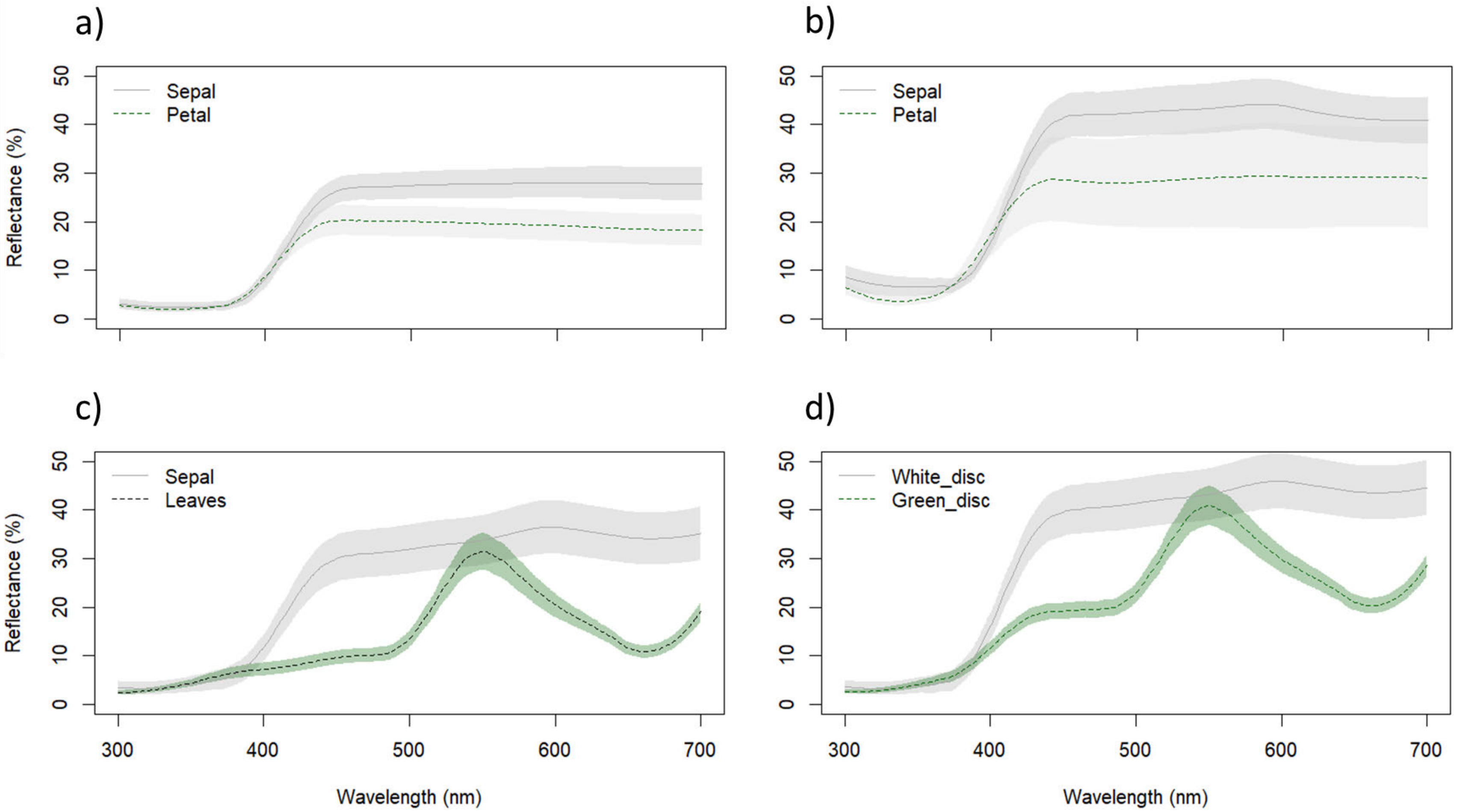

Figure 3.3: Mean reflectance spectra of flowers (sepals and petals) and leaves of *E. caffrorum* and of discs used in bioassay choice tests. Shading indicates 95% confidence intervals for reflectance measurements. Measurements are from Witzieshoek (a) (n=8 sepals; n=8 petals), Tarn Cave (b) (n=3 sepals; n=3 petals), Carter's Nek (c) (n=15 sepals; n=15 leaves), and discs used for the scent composition and colour/scent choice test experiment (d) (n=5 white discs; n=5 green discs).

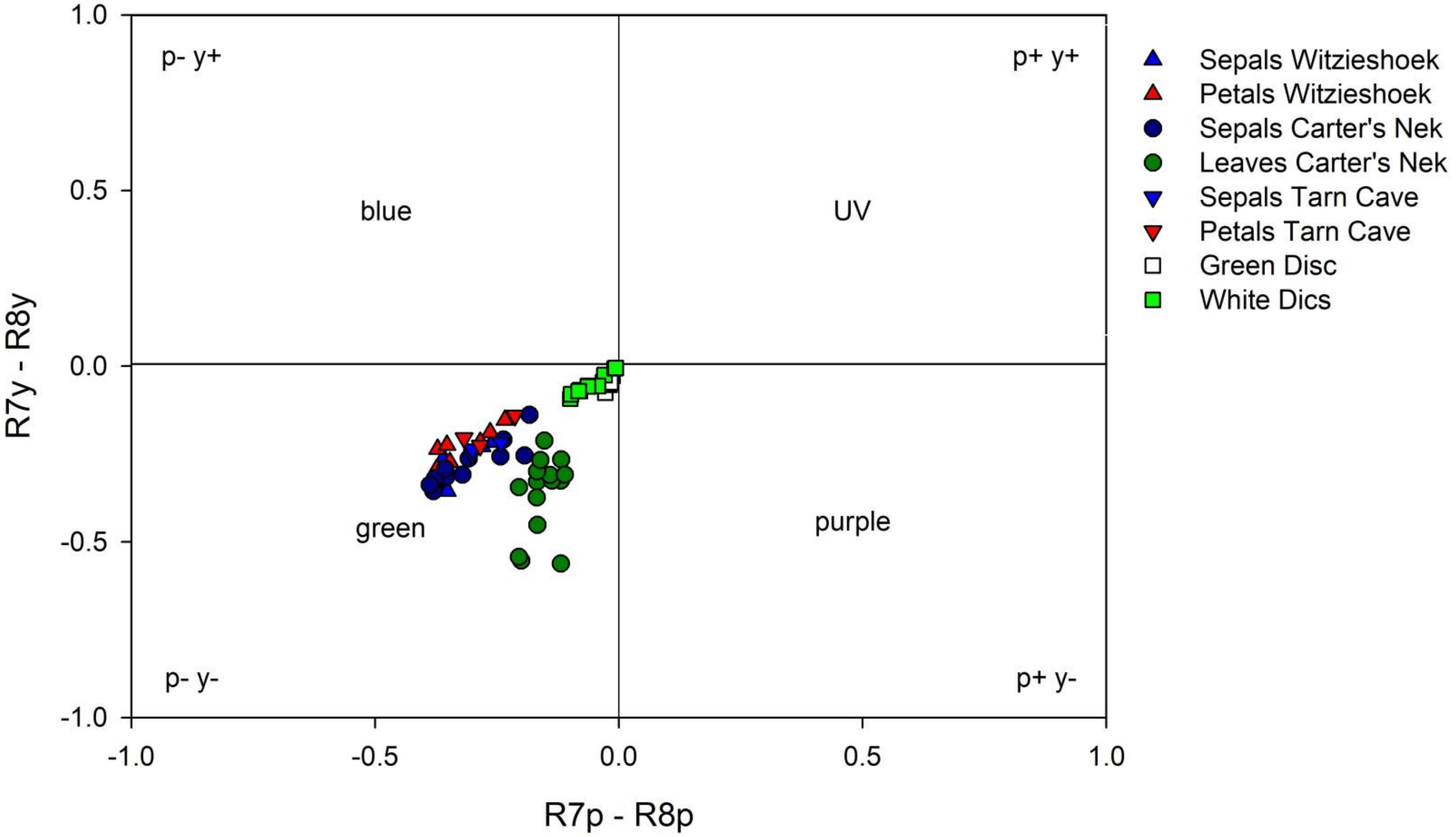

Figure 3.4: Loci of the spectra of sepals, petals and leaves of *E. caffrorum* sampled from three different sites plotted in two-dimensional space according to Troje's (1993) fly vision model, along with the loci of the spectra of the discs used for the scent composition and colour/scent choice test experiments.

3.4.3 Flower scent

A total of 140 scent compounds were detected in *E. caffrorum* flowers sampled across all five sites (mean ± SD: 64.4% ± 25.5% across sites). Of these, 58 compounds were identified and verified with known Kovats values. The main scent compounds consist of aromatics (benzaldehyde, phenylethyl alcohol), monoterpenes (linalool), sesquiterpenes (caryophyllene), and a carboxylic acid (2-methylbutanoic acid) (Table S3.1). A total of 82 compounds could not be identified based on the criteria implemented here and are listed as unknowns (Table S3.1). Although this comprises the majority of compounds, these compounds represented a minority (mean ± SD: 24.8% ± 16.4%) of compounds in each blend per site.

There was an overall significant difference in scent compounds among the five sites (one-way ANOSIM, factor site: Global $R = 1, p < 0.0001$). Pairwise comparisons suggested that profiles at Witzieshoek and Tarn Cave were similar to each other but indicated significant differences in scent profiles in all other possible pairwise contrasts between populations (Table 3.1). Sites that are geographically in closer proximity were not similar to each other: Although Witzieshoek and Tarn Cave are furthest apart in space, these were the only populations that did not differ significantly in scent profile, whereas the scent at the two geographically most proximate sites, Kamberg Reserve and Carters Nek, differed significantly from each other and from all other compared sites (Table 3.1). Visualisation of the samples using NMDS revealed that scent samples clustered by site (Fig. 3.5).

Table 3.1: ANOSIM Bonferroni-corrected *p*-values for multiple comparisons of floral scent data from four *E. caffrorum* sites. R-values for all comparisons was 1. Cells that show significant differences are shaded in grey ($p < 0.05$).

	Tarn Cave	Witzieshoek	Carter’s Nek	Kamberg Reserve
Tarn Cave	-	0.10	0.03	0.03
Witzieshoek		-	0.03	0.03
Carter’s Nek			-	0.03
Kamberg Reserve				-

The one-way SIMPER analysis identified benzenoid aromatics (benzaldehyde), monoterpenoid volatiles (linalool) and 4-methyl-3-penten-2-one as the main compounds that are responsible for the dissimilarity of compounds among sites, which is caused by variation in the relative contributions of these various compounds rather than presence/absence (Table S3.2). 2-methylbutanoic acid, the compound that gives *E. caffrorum* its distinctive odour, is present across all sites.

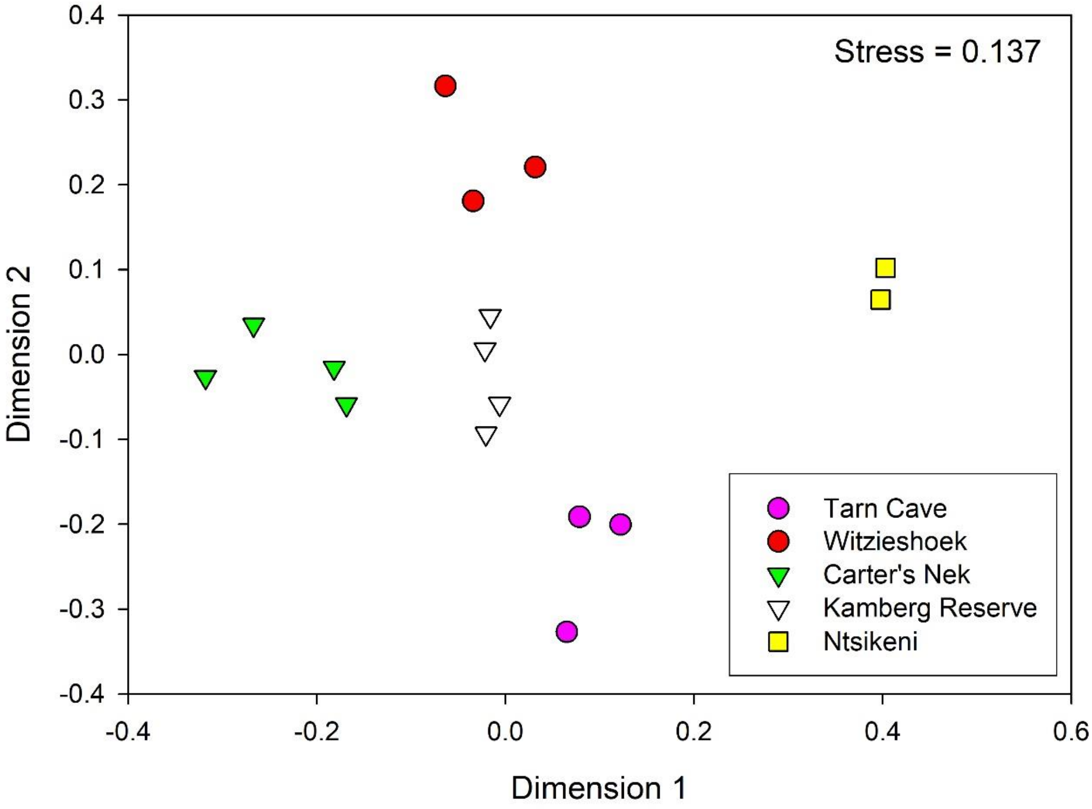

Figure 3.5: Two-dimensional ordination of scent samples of *E. caffrorum* from five localities based on non-metric multidimensional scaling using Bray-Curtis similarities. 2D stress value = 0.137. Each symbol represents one scent sample.

3.4.4 Colour/scent choice test experiment

The colour/scent choice experiment showed slightly different patterns among sites, although at all three sites, flies showed a significant preference for white over green in the presence of scent (Fig. 3.6a–c). In addition, at Kamberg Reserve (Fig. 3.6b) and Witzieshoek (Fig. 3.6c), the addition of scent resulted in a significantly greater attraction of flies in choices between green discs. At Witzieshoek, significant preferences were evident in all four binary choice tests involving fly visitors: flies showed significant preferences for both white colour and for the addition of scent, and these preferences were evident in choices for colour with or without scent present and for scent with or without differences in colour (Fig. 3.6c) indicating the independent contributions of both scent and colour for attraction of flies. At all three sites, preference strength differed among choice combinations (Carters Nek $\chi2= 11.38$, $p = 0.010$, Fig. 3.6a; Kamberg $\chi2= 13.59$, $p < 0.001$, Fig. 3.6b; Witzieshoek $\chi2= 22.87$, $p < 0.001$, Fig. 3.6c). At Kamberg Reserve and at Witzieshoek, choices in which only colour differed were associated with the strongest increase in visitor attraction, although scent addition consistently increased attraction at Witzieshoek (Fig. 3.6c).

For non-fly visitors, colour and scent had less effect on attraction: significant preference was found in only one of the eight choice experiments, and no difference in preference strength was found at either site (Carter's Nek $\chi2= 7.88$, $p = 0.050$, Fig. 3.6d; Kamberg Reserve $\chi2= 1.57$, $p = 0.210$, Fig. 3.6e). At Kamberg Reserve, differences in colour and scent among models did not affect the numbers of non-fly visitors attracted to either model in any of the four binary choices (Fig 3.6e). However, at Carter's Nek, in the choice between white and green discs, in which both were scented, white was associated with a greater number of non-fly visitors (Fig. 3.6d).

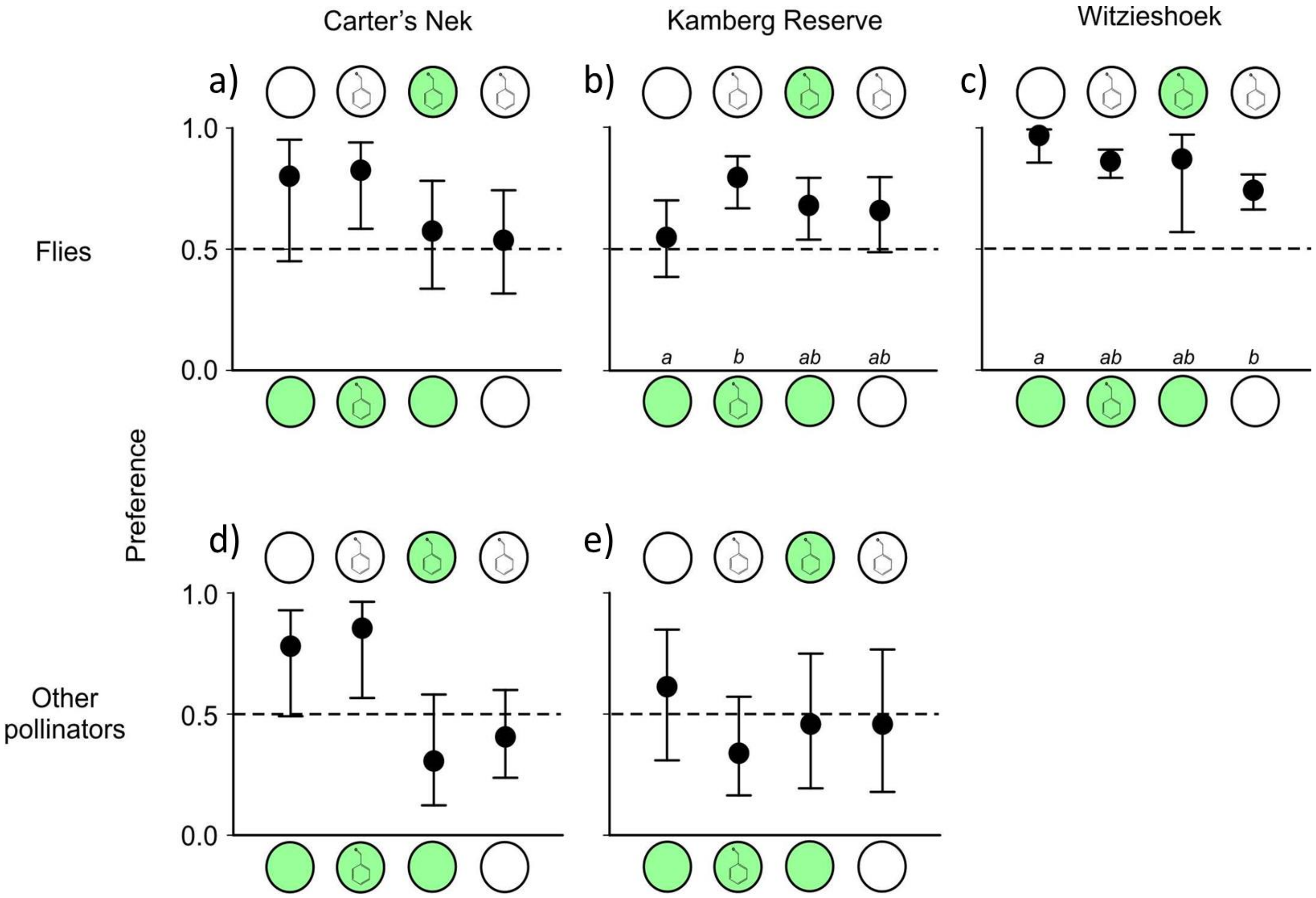

Figure 3.6: Mean ± CI of the preferences of flies (a-c) and other pollinators (d, e) in binary choice experiments to assess the significance of colour and scent for pollinator attraction at three sites. The circle colour indicates the disc colour. The presence or absence of the organic compound symbol within a circle indicates the presence or absence of the scent compound mixture (2-methyl butanoic acid and benzaldehyde). Choices that share letters indicate values that do not differ significantly in post-hoc comparisons. A preference was considered significant if the 95% confidence interval did not intersect the dashed line (0.5, random choice).

3.4.5 Scent choice experiment

The choice experiments with different scent compounds showed that compound type or combination had a significant effect on attraction of flies, with significant effects of compound type at all three sites (Carter's Nek $\chi^2 = 9.09$, $p = 0.028$, Fig. 3.7a; Kamberg

Reserve χ^2= 92.3, $p < 0.005$, Fig. 3.7b; Witzieshoek χ^2= 24.7, $p < 0.001$, Fig. 3.7c). In all cases, the mixture of benzaldehyde and acid attracted more flies than the hexane control (Figs 3.7a–c). The acid alone also attracted significantly more flies than the hexane control at Kamberg and Witzieshoek (Figs 3.7b–c). Benzaldehyde alone always attracted fewer flies than the benzaldehyde-acid mixture but did not consistently attract fewer flies than the acid alone (Figs 3.7a–c).

For the non-fly flower visitors, the differences in the number of individuals attracted by different scent types were less pronounced. There was an overall difference among treatments at Carter’s Nek (χ2= 35.8, $p < 0.001$, Fig. 3.7d) and Witzieshoek (χ2= 16.90, $p < 0.001$, Fig. 3.7f), but not at Kamberg Reserve (χ2= 6.25, $p = 0.100$, Fig. 3.7e). However, there were contrasting results between Carter’s Nek and Witzieshoek with regards to benzaldehyde: at Carter’s Nek, benzaldehyde attracted significantly fewer visitors than the other treatments, whereas, at Witzieshoek, benzaldehyde attracted significantly more non-fly visitors than the control or acid treatments did (Figs 3.7e–f).

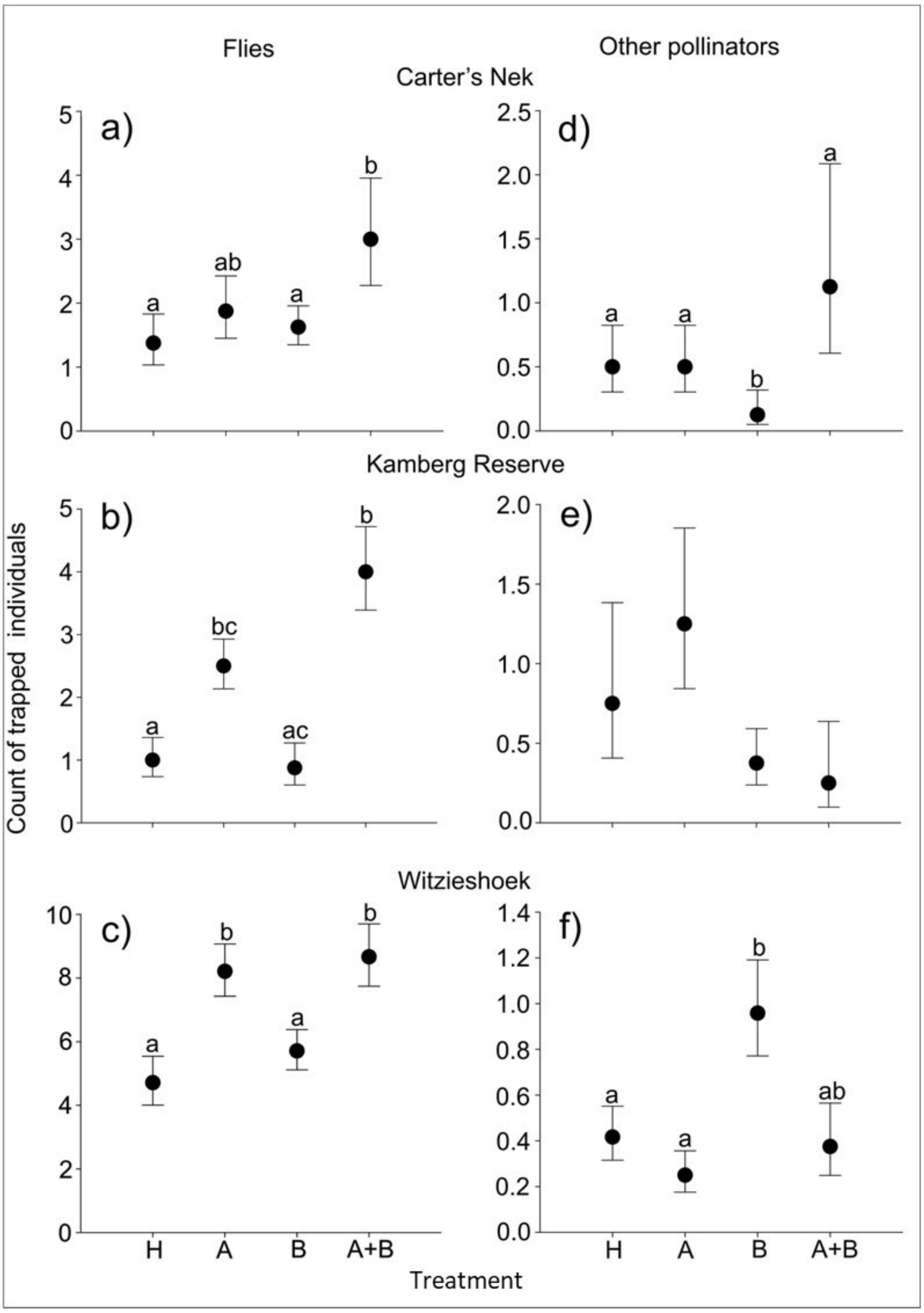

Figure 3.7: Mean ± SE numbers of trapped individuals attracted to different scent treatments with H = hexane, A = 2-methylbutanoic acid, B = benzaldehyde, A+B = 2-methyl butanoic acid and benzaldehyde mixture, for flies and other visitors at three sites. Within each panel, treatments that share letters indicate values that do not differ significantly in post-hoc comparisons (*p* < 0.05).

3.5 Discussion

In this chapter, I aimed to quantify and characterise the functional significance of floral traits of *E. caffrorum* in relation to pollinator attraction and plant-pollinator fit. Characterisation of flower colour and scent showed that flowers of *E. caffrorum* showed a typical white flower spectrum with no UVA reflectance (Fig. 3.3), and the scent profile comprised considerable amounts of aromatics, monoterpenoids and sesquiterpenes, but also 2-methylbutanoic acid, with significant differences in relative amounts of dominant compounds across the five sites (Table S3.1). Bioassay experiments showed that both scent and colour of *E. caffrorum* function in pollinator attraction. Further bioassay experiments with different scent compounds in isolation and as part of a mixture suggested that flies are generally attracted to acids (either as part of a mixture or alone), whereas numbers of other pollinators attracted to the different scent treatments, were lower overall and did not vary among scent treatments.

The typical diet of the fly families that are found pollinating *E. caffrorum* share many similarities, with the larvae of all families developing and feeding in or around decaying plant or animal matter and dung (Kirk-Spriggs and Sinclair, 2017; Picker et al., 2008). The adults of some species of Lauxanidae, Syrphidae, Tachinidae and Tephritidae typically feed on pollen, nectar, and decaying plant material (Cumming and Wood, 2009). In contrast, the adults of Sarcophagidae and Sepsidae occasionally feed on nectar but typically feed on decaying animal matter and dung (Hafez, 1948; Villet et al., 2017), along with Scathophagidae and Empididae, which are predators of other flies (Kirk-Spriggs and Sinclair, 2017). In addition to feeding on nectar sources, some adults of Anthomyiidae, Calliphoridae, Empididae, Muscidae, and Rhiniidae feed on various other substrates like decaying organic matter, usually of faecal or plant sources (Kirk-Spriggs and Sinclair, 2017; Picker et al., 2008). Thus, the fly families found on *E. caffrorum* can be described as opportunistic nectar feeders because they do not feed on nectar only, which is dissimilar from long-tongued flies, which are usually specialised to feed only on nectar sources.

3.5.1 Flower morphometrics

Erica caffrorum, like many other *Erica* species, has small flowers (e.g., Bouman et al., 2017). Even though there were significant differences in three of the four traits measured, these differences were relatively small but were large relative to the size of the flower parts (Fig. 3.2). Although this study did not specifically further explore the significance of the variation in flower dimensions found among populations of *E. caffrorum*, correlations between floral trait variation and pollinator mouth-part variation are thought to reflect adaptations for other pollination systems in *Erica*. For instance, flowers of species pollinated by hawkmoths and long-proboscid flies have much longer tubes than those pollinated by honeybees (Bouman et al., 2017; Lombardi et al., 2021; Van der Niet and Cozien, 2022), and the tube length in *E. junonia* correlates with pollinator mouth-part length (Newman and Johnson, 2021), similar to what was found in *E. plukenetii* in which ecotypes have different tube lengths that correlate to the mouth-parts of their pollinators (Van der Niet et al., 2014). For *E. caffrorum,* the functional tube length (in this case represented by the length of the sepals, while in other *Erica* species, this is comprised of the corolla length) is relatively short compared to other *Erica* species. Although the proboscis length of fly pollinators of *E. caffrorum* was not measured in this study, these are known to be relatively short, less than 10 mm and not exceeding the total length of the fly (Cumming and Wood, 2009). The fit of proboscis length to tube length in *Erica* flowers may also apply to *E. caffrorum.* The small size of the flowers of *E. caffrorum* may therefore likely represent an adaptation to the short tongues of the dominant pollinators.

The morphology of the fly-pollinated small flowers of *E. caffrorum* have similarities with other plant species pollinated by short-tongued flies: *Schizochilus angustifolius* is pollinated mostly by muscid flies and is characterized by very short nectar spurs (Van der Niet et al., 2010). In *Wurmbea elatior*, nectar is readily available and accessible to its short-tongued fly pollinators (Johnson et al., 2020). Finally, fly-pollinated species of *Eucomis* also have nectar that is readily accessible to short-tongued insects (Shuttleworth and Johnson, 2010).

3.5.2 Flower colour

Flowers of *E. caffrorum* are characterised by a typical white-flower spectrum with no UV reflectance and an absence of colour contrast within flowers (Fig. 3.3, 3.4). These colour characteristics differ from *Erica* species pollinated by long-proboscid flies (Nemestrinidae), which typically exhibit UV reflectance, bright colours and colour contrast within flowers that provide nectar guides (McCarren et al., 2021). Flowers of fly-pollinated *E. junonia* varieties show UV reflection (Newman and Johnson, 2021). Similarly, flowers of the fly-pollinated *Erica aristata* also reflect UV and different flower parts have different UV reflectance patterns, although the Troje (1993) fly vision model suggests that this is not perceived as colour contrast by flies (Lombardi et al., 2021). This difference in pollinator species may explain the variation in UV reflectance among the fly-pollinated *Erica* species. Interestingly, the white-flowered moth-pollinated ecotype of *E. plukenetii* shares a similar reflectance pattern to that of *E. caffrorum* but differs in the most effective pollinators (flies in *E. caffrorum*, moths in *E. plukenetii* subsp. *breviflora*); however, at some sites moths were observed visiting *E. caffrorum* flowers. Thus, the white colour of *E. caffrorum* may be a potential adaptation to short-tongued fly pollinators, that is still to some extent attractive to other functional pollinator groups.

The white-cream colour of *E. caffrorum* flowers follows the reflectance pattern typical of many plants pollinated by insects, especially Diptera (true flies) (Shrestha et al., 2019). Many short-tongued fly-pollinated plant species share a similar reflectance pattern to *E. caffrorum* extending to genera other than *Erica*, that include, but are not limited to southern African representatives of *Wurmbea*, *Eucomis* and *Schizochilus* (Johnson et al., 2020: Shuttleworth and Johnson, 2010: Van der Niet et al., 2010). Results from community-level analyses in other regions showed that the loci of spectra of primarily dipteran-visited nectar-rewarding plant species most frequently fall into the "fly-yellow" quadrant of Troje's (1993) fly vision model (Ishii et al., 2019), as was the case for the loci of spectra of *E. caffrorum* (Fig. 3.4). Despite the difference in flower colour, there was considerable overlap in flower-visiting fly families between *E. caffrorum* and the species studied by Ishii et al. (2019).

Within southern Africa, several other plant species pollinated by short-tongued flies are characterised by different flower colours, including yellow and maroon, and are often patterned (Jürgens et al., 2015). For example, the reflectance pattern of carrion mimicking fly-pollinated flowers like *Ceropegia mixta* (Apocynaceae) is pale yellow with UV reflectance and is characterised by varying degrees of dark red streaking on the corollas (du Plessis et al., 2018). One important difference between *E. caffrorum* and many carrion mimics is that the latter group usually offers no or very small amounts of nectar reward, whereas nectar is present in *E. caffrorum*, although this was not quantified in this study

(T. Van der Niet, unpublished data). This suggests a general difference in pollination mechanism related to fly behaviour in the context of foraging and other aspects of the life cycle between carrion-mimicking flowers and flowers that provide a food reward. Further research is required to understand whether and how nectar influences pollinator behaviour.

3.5.3 Flower scent

Erica caffrorum has a unique scent profile compared to other *Erica* species, especially due to the presence of the acidic compound 2-methylbutanoic acid. The classes of compounds that could be identified otherwise mostly comprised aromatics, monoterpenoids and sesquiterpenes (Table S3.2). Although there was some quantitative variation in relative abundance, these compounds are present across all five sites where *E. caffrorum* was studied (Table S3.1, S3.2).

Acid compounds, particularly organic acids, have a strong scent that may attract flies and other insects. Flies are attracted to the pungent smell of these acid compounds because they may perceive it as a potential food resource or breeding site (Jürgens et al., 2006). Gas chromatographic-electroantennographic detection (GC-EAD) and wind tunnel bioassays conducted by Cossé and Baker, (1996) show that acid compounds, and in particular butanoic acid and 3-methylbutanoic acid, which are found in pig manure, elicit antennal responses in female house flies (*Musca domestica*). The antennal response of these flies may mirror parallel responses to 2-methylbutanoic acid of similar flies found visiting *E. caffrorum*.

The carboxylic acid (2-methylbutanoic acid) class of compounds produced in *E. caffrorum* is often found in the excretory waste of animals as well as in decomposing plant material (Cossé and Baker, 1996; Schulz and Dickschat, 2007). However, it remains debatable whether the presence of acids in *E. caffrorum* means that it functions as a mimic of brood and food substrates. In plant species that rely on mimicry, specific acids are often the dominant floral volatiles. In contrast, the scent profile of *E. caffrorum* may not fit a mimicry system. Although *E. caffrorum* does share similar fly pollinators with those found in mimics, the presence of other fly pollinators like Rhiniidae and different functional pollinator groups, as well as the distinct nectar-seeking behaviour of floral visitors, suggest that this *Erica* species may not fit into a mimicry system and further research is needed to test a mimicry hypothesis for *E. caffrorum*. Furthermore, *E. caffrorum* produces many other scent compounds that may attract various insect pollinators (including flies, bees, and moths).

Although most *Erica* species were considered to be unscented (Rebelo et al., 1985), quantification using objective methods has revealed the presence of many scent compounds in most *Erica* species studied to date (Bouman et al., 2017; Van der Niet et al., 2014; Van der Niet and Cozien, 2022). Comparison of scent profiles across *Erica* species with different pollination systems reveals different scent profiles and, therefore, likely a key role for scent in pollinator attraction. For instance, the pollination ecotypes of *E. plukenetii* have different scent profiles; an unscented bird-pollinated ecotype and a scented moth-pollinated ecotype (Van der Niet et al., 2014). In particular, flowers of the moth-pollinated ecotype produce numerous, mostly aromatic, scent compounds likely to attract moth pollinators (Van der Niet et al., 2014). *Erica caffrorum* does not contain these scent compounds except for caryophyllene, which may therefore be a moth attractant since moths were occasionally observed on flowers of *E. caffrorum* too. However, similarities in scent chemistry between *E. caffrorum* and the moth-pollinated *E. cylindrica*, with an overlap in compounds such as benzaldehyde and benzyl alcohol (see Van der Niet and Cozien, 2022), suggest that these compounds may also be important for moth attraction. There is also evidence that flies of the family Muscidae, similar to the *Musca* genus observed visiting *E. caffrorum*, are attracted to aromatic compounds (Howse, 1997), and therefore these aromatics may also serve as a possible fly attractant (see below).

Compared to *E. caffrorum*, the scent of other short-tongued fly-pollinated plants for which this has been quantified is quite different. For instance, the scent of *Wurmbea elatior* is dominated by indole and skatole, which are also typically found in faecal bouquets that attract coprophagous (dung-feeding) flies (Johnson et al., 2020) and electroantennographic detection (EAD) experiments showed that these two compounds play a key role in fly attraction. In contrast, indole and skatole scent compounds are absent from *E. caffrorum* scent, even though there is some overlap in the fly visitor assemblage between *W. elatior* and *E. caffrorum* with Muscidae, Sarcophagidae and Scathophagidae visitors. Sulphur scent compounds characterise fly-pollinated *Eucomis* species, which play a key role in fly attraction (Shuttleworth and Johnson, 2010). Sulphur scent compounds are absent from *E. caffrorum.* These comparisons suggest that fly attraction may be achieved through many different pathways. At the level of compound classes, a mixture of different compound classes ('unpleasant' versus 'sweet' smelling) is not unique to *E. caffrorum*. *Periploca laevigata,* a fly-pollinated plant species in the Mediterranean region, has a scent profile that contains unpleasant scent compounds like sulphides and sweet compounds like terpenoids (Zito et al., 2015) and *P. laevigata* also shares similarities in fly pollinators with *E. caffrorum* including visitation by Anthomyiidae, Calliphoridae, Muscidae, Sarcophagidae and Scathophagidae as pollinators of both plant species (see Pisciotta et al., 2011).

The results of the ANOSIM showed a significant difference in scent compounds of *E. caffrorum* at five sites (Table 3.1). Furthermore, the results of the SIMPER analysis indicated that the main variation is quantitative; the same compounds are present but emitted in different relative amounts (Table S3.1). Variation among populations may stem from various processes. First, it may be non-adaptive and due to genetic drift or may be related to sampling artefacts such as different sampling times, variations in the abiotic factors at each site, including different soil types and other environmental factors or climatic factors like temperature and humidity that may have influenced the concentrations of compounds that were emitted at each site (Eisen et al., 2022; Majetic et al., 2009). However, it may also indicate adaptive differences related to the presence of different pollinator assemblages (see Chapter 2), resulting in the evolution of scent ecotypes (chemotypes) for *E. caffrorum.* Given the presence of different fly pollinators at each site (Chapter 2; Table 2.2), it is possible that different fly communities could drive the formation of chemotypes of *E. caffrorum.* This would require that local pollinator communities are stable over long periods of time and that the constituent species vary in preference for the relative abundance of particular compounds. Although this cannot be excluded, further work, including reciprocal translocation experiments, is needed to determine the cause and to evaluate the potential significance of population-level variation.

It was not possible to identify all compounds produced by *E. caffrorum*, and the number of unknown compounds was relatively large. In most cases, compounds could not be identified because of impure mass spectra and associated non-matching Kovats indices. These problems are most frequently associated with compounds present in very small quantities. Indeed, the contribution of unknown compounds was relatively small, (mean ± SD: 24.8% ± 16.4%) unknown compounds per site. Although it cannot be excluded that some unknown compounds play a role in pollinator attraction, I consider the presence of a relatively large number of unknown compounds as unproblematic in this study.

3.5.4 Bioassay experiments

3.5.4.1 Colour/scent experiment

Colour preferences have been established for many fly species (Lunau, 2014). In the colour/scent experiment performed in this study, flies consistently preferred white over green in the presence of scent (Figs 3.6a–c). The results are most apparent at the Witzieshoek site, potentially because the experiment there was done over multiple days resulting in higher numbers of trapped flies on the discs compared to the experiments that were only run over one day (Fig. 3.6c). Furthermore, the experiment conducted at the Kamberg Reserve site was done past the optimal flowering time of *E. caffrorum* (April); as such, fewer fly pollinator individuals may have been present, as a result of potential temporal variation of fly abundance.

At all three sites, there was a trend of preference for the white treatment over the green treatment (green was used to replicate the background vegetation) for flies, although only choices involving scented colour pairs were uniformly significant across all sites. This may indicate that both colour and scent are important for fly attraction and that the colour white is particularly preferred in the presence of scent; thus, colour may be an integral aspect of fly attraction, but its attractive function is enhanced in the presence of scent. A possible caveat to this result may be that white was the only colour used in these colour/scent experiments. As such, it may be unclear whether there was a preference for white specifically or whether a similar result would have been obtained for any colour that flies perceive as contrasting against the green background vegetation. The other flower visitors showed no significant preference among the colour and scent choices (Fig.

3.6d–e). This may be due to a lack of statistical power resulting from the generally small number of individuals attracted to the experiment. Alternatively, they may not prefer the scent mixture and colours used in the colour/scent choice experiment. Further experimental work is needed to distinguish between these explanations.

3.5.4.2 Scent choice experiment

In the scent choice experiment, flies preferred 2-methylbutanoic acid alone, or in conjunction with benzaldehyde over the hexane control and benzaldehyde alone (Figs 3.7a–c). Furthermore, benzaldehyde alone never trapped more flies than the hexane control. This suggests that the presence of an acid, either alone or in a mixture, serves an important function in attracting flies. The reason for this might be that the flies found on *E. caffrorum* prefer the acid scent compound due to the fact that they may respond to the acid compound as an indicator for a site for breeding or laying eggs because 2-methylbutanoic acid is found in the excretory waste of animals (Cossé and Baker, 1996; Schulz and Dickschat, 2007).

The results of the scent choice experiment further suggest that other pollinators have varied preferences for scent compounds at Carter’s Nek versus Witzieshoek (Fig. 3.7d, f). These results could reflect differences in local assemblages of other pollinators, although benzaldehyde is usually considered an attractant for a broad range of insects (Farré-Armengol et al., 2020). Further work is needed to understand the difference in choice between the two sites. What can be concluded from the scent choice experiment, however, is that both the acid and possibly benzaldehyde are crucial scent compounds for pollinator attraction in *E. caffrorum,* especially given the consistent choices across multiple sites (Figs 3.7a–c). The consistent choice of specific scent compounds could also be found in bioassay experiments done by Moré et al. 2013 using the oligosulphide compounds (dimethyl disulphide and dimethyl trisulphide) emitted by *Jaborosa rotacea* a plant species native to South America. Bioassay experiments conducted in North America on *J. rotacea,* showed that the same fly families of Calliphoridae, Muscidae, and Sarcophagidae (also found on *E. caffrorum*) were attracted to the oligosulphide bait on artificial flowers instead of the artificial flowers that did not contain the scent bait (Moré et al., 2013).

3.5.4.3 Opportunities for future research

For the scent composition experiment, only two scent compounds were used in the bioassays, and it is unclear whether these two scent compounds, instead of the many other scent compounds that are emitted by *E. caffrorum*, are critical for pollinator attraction. *Erica caffrorum* produces a large number of different scent compounds, and further studies, therefore, need to be performed to determine whether insect visitors respond to further *E. caffrorum* scent compounds that may serve a function in attraction. A particularly useful method for this purpose would be gas chromatography coupled with electroantennographic detection (GC-EAD).

Further, the use of Tanglefoot® and placement of discs with insects stuck to them in Ziplock™ bags precluded accurate identification of insects because of damage to key morphological characteristics. Identification based on DNA barcoding could also not be performed on these flies, as the Tanglefoot® may result in challenges for DNA extraction. In future, identification of trapped visitors could be done prior to placement in Ziplock™ bags to identify visitors more accurately.

A factor which may contribute to the response of pollinators to model flowers in the bioassays is that of their past foraging experience as they may have previously encountered other white flowers that provided a nectar reward, which would have influenced their behaviour towards the discs in the bioassays. A problem of the bioassay experiments is a potential bias due to the effect of aggregation of flies, as flies are known to be attracted to surfaces with other flies (Ives, 1991). When flies become trapped on the disc’s surface, other flies may be attracted to this accumulation of flies for mating or may be attracted to the stress pheromones emitted from the flies or insects instead of the colour or scent compounds in the experiment. A solution may be to catch visitors as they land on the disc’s surface. However, this may lead to a reduction in the number of replicates and an added expenditure of time catching visitors. Therefore, I consider the experimental design implemented as an optimal compromise, but further experiments need to be done to evaluate the potential bias due to the effects of fly aggregation on fly attraction.

3.6 Conclusions and avenues for further research

The results presented in this chapter shed light on the functional significance of floral scent and colour traits of *E. caffrorum* for pollinator attraction. The floral morphology of

E. caffrorum may correlate to the mouth-parts of the short-tongued pollinators, facilitating contact with stigmas and deposition of pollen released from anthers as insects probe flowers for nectar. The white-coloured flowers are similar to those of many short-tongued fly-pollinated plant species. The scent profile of *E. caffrorum* contains many different scent compounds, some of which are known attractants of multiple functional pollinator groups. The acidic compound 2-methylbutanoic acid is uncommon in many *Erica* species and may be an essential scent compound in attracting flies for pollination. Based on the two bioassay experiments, it appears that both colour and scent play an integral role in attracting the fly pollinators of this plant species. *Erica caffrorum* does have different pollinator assemblages at different sites associated with site-specific scent blends, indicating the potential presence of chemotypes. Genetical profiling of *E. caffrorum* at different sites could further explore this idea of chemotypes. *Erica caffrorum* is sister to a large clade, to which some species with similar traits and pollination systems belong (Pirie et al., 2016). This suggests that traits have evolved multiple times. However, given the topological position of *E. caffrorum*, it is likely impossible to reconstruct transitions in pollination system with certainty. Furthermore, work needs to be undertaken to explore the role of nectar and its function in pollinator attraction for *E. caffrorum*. Gas chromatography coupled with electroantennographic detection (GC/EAD) could be used to determine which specific scent compounds are detectable from insect visitors. Further bioassays could be conducted to confirm the role of individual scent compounds of *E. caffrorum* for pollinator attraction or if these scent compounds illicit a repulsive response by the insect visitors.

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3.6 Supplementary Material

Table S3.1: Mean relative amounts (%) ± SD of scent compounds of *E. caffrorum* across five sites, organised by compound class and Kovats Retention Index (Kovats RI).

Compounds	Kovats RI	Tarn Cave	Witzieshoek	Carter’s Nek	Kamberg Reserve	Ntsikeni
Acids						
Acetic acid	1441	-	0.39 ± 0.21	0.5 ± 0.3	-	-
Butanoic acid, 3-hexenyl ester, (Z)-	1446	0.29 ± 0.15	-	-	-	-
Benzoic acid, methyl ester	1621	0.09 ± 0.06	0.27 ± 0.13	0.28 ± 0.06	0.41 ± 0.25	-
Butanoic acid, 2-methyl-	1664	13.79 ± 1.65	3.65 ± 1.17	7.24 ± 3.85	6.85 ± 4.26	16.57 ± 3.6
Heptanoic acid	1942	0.05 ± 0.04	0.14 ± 0.11	-	0.02 ± 0.01	0.27 ± 0.05
Octanoic acid	2040	-	0.1 ± 0.05	0.04 ± 0.01	0.03 ± 0.01	-
Alcohols and Phenols (Aromatics*)						
3-Hexen-1-ol, acetate, (Z)-	1301	1.35 ± 0.5	-	-	-	-
1-Hexanol	1324	0.07 ± 0.02	-	-	-	-
3-Hexen-1-ol, (E)-	1357	0.24 ± 0.12	0.3 ± 0.09	0.31 ± 0.08	0.4 ± 0.41	5.78 ± 2.06
1-Octen-3-ol	1425	0.26 ± 0.15	0.05 ± 0.02	0.47 ± 0.1	0.69 ± 0.63	0.37 ± 0.01
1-Heptanol	1433	0.46 ± 0.19	0.03 ± 0.02	-	-	-
1-Hexanol, 2-ethyl-	1475	-	0.21 ± 0.03	-	-	-
1-Octanol	1546	1.78 ± 0.88	0.69 ± 0.13	1.49 ± 0.85	0.64 ± 0.58	0.41 ± 0.04
1,5,7-Octatrien-3-ol, 3,7-dimethyl-	1602	2.65 ± 1.26	0.53 ± 0.27	-	0.04 ± 0.03	0.37 ± 0.18
1-Nonanol	1651	-	-	0.2 ± 0.12	0.17 ± 0.08	-
Phenylethyl Alcohol*	1896	2.82 ± 2.19	1 ± 0.42	15.72 ± 3.7	0.82 ± 0.66	1.84 ± 0.3
Phenol*	1980	0.01 ± 0	0.12 ± 0.03	-	0.04 ± 0.02	0.71 ± 0.05

Aldehydes						
Hexanal	1056	0.18 ± 0.09	0.1 ± 0.07	-	-	-
Octanal	1268	1.63 ± 1.22	0.95 ± 0.29	2.41 ± 0.97	1.27 ± 0.83	2.65 ± 0.26
Nonanal	1382	2.49 ± 1.1	3 ± 0.41	4.21 ± 0.41	2 ± 1.26	5.91 ± 0.81
2-Octenal, (E)-	1417	0.18 ± 0.06	0.09 ± 0.01	0.14 ± 0.06	0.11 ± 0.06	-
Undecanal	1606	0.35 ± 0.27	0.15 ± 0.06	-	1.91 ± 1.63	1.1 ± 0.75
Dodecanal	1714	0.02 ± 0	0.01 ± 0	-	-	-
Alkenes						
1-Decene	1044	0.26 ± 0.08	0.08 ± 0.05	0.04 ± 0.01	-	-
1-Tridecene	1337	0.16 ± 0.06	-	-	0.14 ± 0.13	-
Benzenoids (Aromatics*)						
Benzaldehyde*	1506	37.8 ± 16.31	24.47 ± 7.71	18.83 ± 12.1	37.29 ± 25.7	4.25 ± 1.54
Benzeneacetaldehyde	1639	1.41 ± 0.05	1.66 ± 1.13	1.44 ± 0.46	0.18 ± 0.1	0.3 ± 0.07
Naphthalene	1743	-	0.19 ± 0.12	-	0.23 ± 0.11	0.55 ± 0
Benzyl alcohol*	1854	5.14 ± 0.99	4.68 ± 0.84	5.61 ± 1.11	4.32 ± 3.24	2.96 ± 0.1
Benzyl nitrile	1907	-	0.02 ± 0.01	-	0.01 ± 0.01	0.35 ± 0.11
E-2-Hexenyl benzoate	2083	0.03 ± 0	-	-	-	-
Esters						
cis-3-Hexenyl isovalerate	1469	-	-	0.17 ± 0.15	0.12 ± 0.07	-
2(3H)-Furanone, 5-ethyldihydro-	1698	-	0.06 ± 0.02	0.14 ± 0.08	0.03 ± 0.02	-
Methyl salicylate	1768	0.03 ± 0	0.04 ± 0.01	0.14 ± 0.03	0.06 ± 0.04	0.09 ± 0
2(3H)-Furanone, dihydro-5-propyl-	1799	0.05 ± 0.02	0.1 ± 0.01	0.12 ± 0.04	0.03 ± 0.02	-
Ketones						
3-Penten-2-one, 4-methyl	1120	0.56 ± 0.15	0.17 ± 0.1	-	-	15.82 ± 4.39
5-Hepten-2-one, 6-methyl-	1317	0.35 ± 0.25	0.79 ± 0.36	-	0.6 ± 0.33	6.86 ± 3.16

2-Nonanone	1370	0.14 ± 0.03	-	-	-	-
2-Decanone	1484	0.12 ± 0.03	0.09 ± 0.02	-	0.07 ± 0.04	-
6-Methyl-3,5-heptadiene-2-one	1577	0.05 ± 0.02	-	-	-	0.18 ± 0.01
Acetophenone	1647	-	0.1 ± 0.03	-	-	-
2-Tridecanone	1800	0.01 ± 0	-	-	-	-
Monoterpenes						
β-Myrcene	1159	6.4 ± 3.51	4.45 ± 0.75	0.87 ± 0.19	2.6 ± 1.71	4.13 ± 0.32
Limonene	1191	0.11 ± 0.07	0.49 ± 0.23	0.34 ± 0.03	0.34 ± 0.24	1.11 ± 0.27
(Z)-Ocimene	1221	0.71 ± 0.44	0.24 ± 0.06	0.36 ± 0.09	0.48 ± 0.26	0.22 ± 0.03
(E)-Ocimene	1235	1.49 ± 1.48	0.47 ± 0.51	1.72 ± 1.06	0.83 ± 0.59	1.69 ± 0.34
(E)-4,8-Dimethylnona-1,3,7-triene	1295	2.92 ± 1.58	9.37 ± 3.58	14.9 ± 7.18	1.52 ± 1.3	3.72 ± 1.15
Linalool	1534	3.61 ± 0.86	9.8 ± 6.47	12.41 ± 4.13	8.57 ± 10.25	1.2 ± 0.41
α-Terpineol	1691	0.13 ± 0.09	0.03 ± 0.03	0.07 ± 0.02	0.02 ± 0.01	-
Nitrogen containing compounds						
2H-Pyran-2-one, tetrahydro-6-methyl-	1781	0.02 ± 0.02	-	-	-	-
Sesquiterpenes						
Copaene	1501	0.14 ± 0.13	0.23 ± 0.11	-	0.31 ± 0.21	0.51 ± 0.3
Caryophyllene	1603	0.3 ± 0.17	4.57 ± 2.32	1.98 ± 2.52	0.46 ± 0.37	1.95 ± 1.13
Humulene	1688	0.08 ± 0.02	0.17 ± 0.05	0.04 ± 0.04	0.1 ± 0.05	-
β-Bisabolene	1726	0.21 ± 0.07	0.01 ± 0	-	0.24 ± 0.28	-
(1S,2E,6E,10R) -3,7,11,11-Tetramethylbicyclo[8.1.0]undeca-2	1738	0.06 ± 0.06	-	5.17 ± 3.1	-	-
α-Farnesene	1754	0.04 ± 0.01	0.2 ± 0.03	-	0.04 ± 0.02	0.04 ± 0
Caryophyllene oxide	1986	-	0.05 ± 0.01	-	0.01 ± 0.01	-
Toluenes						
3,5-Dimethoxytoluene	1843	0.05 ± 0.03	0.42 ± 0.08	0.37 ± 0.36	0.09 ± 0.1	0.29 ± 0.03
Unknowns						

m/z 67,44,68,53,39,41	891	1.93 ± 1.12	-	-	-	10.62 ± 0.07
m/z 45.43,31,46,86,70	901	-	1.17 ± 0.88	-	-	-
m/z 73,87,56,100,41,54	956	-	13.12 ± 10.4	-	-	-
m/z 55,43,207,70,67,82	988	0.31 ± 0.11	-	-	-	-
m/z 69,41,43,81,79,67	1260	0.29 ± 0.12	2.29 ± 0.09	-	-	-
m/z: 43,55,67,41,83,57	1302	0.33 ± 0.19	-	-	-	-
m/z 70,102,85,40,96,32	1345	-	0.03 ± 0.01	-	-	-
m/z: 55,83,69,41,57,97	1442	0.09 ± 0.12	-	-	-	-
m/z: 57,67,83,68,70,69	1460	0.03 ± 0.01	0.06 ± 0.03	-	-	0.78 ± 0.09
m/z: 55,41,69,83,43,57	1547	0.03 ± 0.05	-	-	-	-
m/z: 71,43,82,67,99,41	1567	0.17 ± 0.08	-	0.59 ± 0.31	0.07 ± 0.04	-
m/z: 93,119,41,69,43,55	1570	0.1 ± 0.08	-	-	-	-
m/z: 70,69,99,56,41,55	1584	-	0.05 ± 0.01	-	-	-
m/z: 93,91,133,79,82,107	1592	-	0.06 ± 0	-	0.04 ± 0.01	0.11 ± 0.01
m/z 60,73,41,67,68,43	1623	0.01 ± 0	-	-	-	-
m/z 56,55,70,41,69,43	1641	0.12 ± 0.09	-	-	-	-
m/z 43,55,69,41,57,83	1651	0.03 ± 0.01	0.07 ± 0.01	-	-	0.75 ± 0.08
m/z 69,43,41,112,39,91	1675	0.03 ± 0.02	-	-	-	-
m/z 117,90,91,89,65,118	1679	-	1.97 ± 1.76	0.68 ± 0.57	0.35 ± 0.35	0.12 ± 0.05
m/z 85,57,55,41,43,56	1692	0.03 ± 0.02	-	-	-	0.45 ± 0.08
m/z 55,41,67,69,68,43	1703	0.08 ± 0.04	0.01 ± 0	-	-	-
m/z 171,75,127,101,117,77	1708	-	0.01 ± 0	-	-	-
m/z 57,71,43,85,41,55	1710	0.02 ± 0.01	-	-	-	0.17 ± 0.01
m/z 69,79,105,71,67,77	1721	-	0.04 ± 0.01	-	-	-
m/z 60,73,55,67,41,43	1733	0.02 ± 0.01	-	-	0.04 ± 0.01	-

m/z 79,93,43,91,94,133	1760	-	0.03 ± 0.02	-	0.02 ± 0.02	0.05 ± 0
m/z: 93,83,71,43,119,91	1772	0.01 ± 0	-	-	-	-
m/z 70,42,55,71,43,41	1787	-	0.02 ± 0	0.03 ± 0.02	-	0.09 ± 0.05
m/z 104,41,55,69,68,43	1803	-	-	-	-	-
m/z 43,57,55,71,41,69	1812	0.02 ± 0	0.04 ± 0.01	-	-	-
m/z 43,57,85,72,71,100	1817	0.01 ± 0	0.04 ± 0.01	-	-	1.08 ± 0.75
m/z 69,41,79,67,93,57	1825	0.01 ± 0	-	-	-	-
m/z 43,71,59,55,70,69	1831	0.02 ± 0.02	-	-	-	-
m/z 59,69,55,68,97,71	1833	-	0.01 ± 0	-	-	-
m/z 107,136,69,151,93,85	1852	-	0.03 ± 0.03	-	-	-
m/z 43,126,193,55,41,69	1870	0.02 ± 0	-	-	-	-
m/z 71,43,69,159,98,111	1876	-	0.1 ± 0.07	-	-	1.38 ± 0.62
m/z 55,43,41,57,70,42	1878	0.03 ± 0.02	-	-	-	-
m/z 59,79,43,69,141,142	1888	-	0.07 ± 0.01	-	0.02 ± 0	-
m/z 85,60,55,41,39,68	1902	0.02 ± 0.01	-	-	-	-
m/z 82,71,67,43,55,41	1917	3.73 ± 1	-	0.35 ± 0.16	0.71 ± 0.59	-
m/z 42,54,98,43,55,39	1922	-	-	-	-	-
m/z 82,71,67,43,85,83	1929	0.01 ± 0	3.41 ± 1.12	-	-	-
m/z 85,57,69,41,43,55	1931	-	0.01 ± 0	-	-	-
m/z 71,97,55,43,83,70	1952	-	0.04 ± 0.01	-	-	-
m/z 55,41,69,43,71,83	1953	0.01 ± 0	-	-	-	0.11 ± 0
m/z 85,55,98,57,80,43	1958	-	-	-	-	-
m/z 69,43,57,85,141,55	1960	-	0.01 ± 0	-	-	-
m/z 43,58,55,71,57,41	1968	0.01 ± 0.01	-	-	-	-
m/z 68,67,85,43,83,55	1989	0.28 ± 0.25	-	-	-	-
m/z: 85,170,141,142,77,84	2002	-	0.05 ± 0.02	-	-	-
m/z 85,43,59,71,58,55	2013	-	0.07 ± 0.01	0.08 ± 0.05	0.02 ± 0.01	0.29 ± 0.07

m/z 107,108,79,77,71,84	2050	-	0.01 ± 0	-	-	0.64 ± 0.13
m/z 85,55,191,84,109,43	2056	-	0.06 ± 0.05	-	-	-
m/z 57,44,43,71,42,41	2060	-	0.1 ± 0.07	-	-	-
m/z: 43,105,55,67,77,109	2070	0.03 ± 0.01	-	-	-	0.31 ± 0.08
m/z 67,71,82,43,68,55	2084	-	0.05 ± 0.01	-	-	-
m/z 105,57,71,43,77,55	2102	0.05 ± 0.03	-	-	-	-
m/z 94,138,77,57,95,66	2106	-	0.07 ± 0.02	-	-	-
m/z 109,127,77,71,105,81	2124	-	-	-	-	-
m/z 69,55,83,43,57,70	2127	-	0.05 ± 0.02	-	-	-
m/z 73,60,115,57,129,55	2135	-	0.34 ± 0.24	-	-	-
m/z: 162,164,63,98,56,126	2149	0.01 ± 0	-	-	-	-
m/z 43,69,55,83,87,74	2191	0.02 ± 0	-	-	-	-
m/z 120,138,121,92,83,43	2260	-	0.08 ± 0.07	0.05 ± 0.07	0.02 ± 0	-
m/z 91,175,105,79,133,77	2267	-	-	0.07 ± 0.04	-	-
m/z 57,71,43,85,55,41	2288	0.05 ± 0.04	-	-	-	-
m/z 149,177,176,150,71,93	2306	-	0.11 ± 0.1	-	-	0.24 ± 0.03
m/z 85,69,84,57,83,82	2317	-	0.07 ± 0.03	-	-	-
m/z 57,71,43,85,56,41	2349	0.05 ± 0.03	-	-	-	-
m/z 117,90,89,57,70,85	2366	-	0.05 ± 0.02	-	-	-
m/z 99,56,71,100,89,59	2375	-	0.04 ± 0.02	-	-	-
m/z 138,109,120,69,121,83	2391	-	0.14 ± 0.11	-	0.57 ± 0.33	-
m/z 105,83,73,87,82,60	2405	-	0.04 ± 0.02	-	-	-
m/z 149,85,57,150,223,122	2450	-	0.17 ± 0.15	-	-	-
m/z 57,71,43,85,99,41	2467	0.11 ± 0.09	-	-	-	0.38 ± 0.16
m/z 57,71,85,43,55,99	2488	0.24 ± 0.22	-	-	-	-
m/z 57,43,71,85,55,56	2534	0.05 ± 0.04	-	-	-	-
m/z 57,71,85,43,55,56	2556	0.03 ± 0.02	-	-	-	-

m/z 105,77,91,212,51,57	2569	0.01 ± 0	-	-	-	-
m/z 149,91,92,71,150,73	2602	-	0.12 ± 0.08	0.22 ± 0.19	-	-
m/z 73,129,60,43,57,55	2915	-	0.47 ± 0.51	-	-	-

Table S3.2: SIMPER analysis *E. caffrorum* at five sites. Values represent mean percentages of the total peak area. These scent compounds contribute to 60% average Bray–Curtis dissimilarity (Diss.) among five sites of *E. caffrorum*. The average dissimilarity is a measure of how scent compounds differ from one another, with 0 indicating no difference and the higher the number, the more different they are. The Contribution % shows the percentage dissimilarity explained by that scent compound, and the Cumulative % tallies the cumulative Bray Curtis dissimilarity metric for the scent compound thus far represented in the table.

Scent compound	Average. Dissimilarity	Contribution %	Cumulative %	Mean Tarn Cave	Mean Witzieshoek	Mean Carter’s Nek	Mean Kamberg Reserve	Mean Ntsikeni
Benzaldehyde	2.427	5.498	5.498	0.602	0.488	0.407	0.651	0.203
Phenylethyl Alcohol	1.665	3.772	9.27	0.155	0.0975	0.394	0.116	0.135
Linalool	1.517	3.436	12.71	0.189	0.295	0.346	0.276	0.108
(E)-4,8-Dimethylnona-1,3,7-triene	1.452	3.288	15.99	0.165	0.301	0.375	0.157	0.191
3-Penten-2-one, 4-methyl	1.345	3.046	19.04	0.0742	0.0386	0	0	0.394
m/z 67,44,68,53,39,41	1.268	2.872	21.91	0.132	0	0	0	0.326
Caryophyllene	1.263	2.861	24.77	0.0515	0.206	0.115	0.162	0.133
m/z 73,87,56,100,41,54	1.205	2.729	27.5	0	0.318	0	0	0
Butanoic acid, 2-methyl-	1.144	2.59	30.09	0.371	0.189	0.261	0.269	0.405
(1S,2E,6E,10R) -3,7,11,11-Tetramethylbicyclo[8.1.0]undeca-2	1.138	2.578	32.67	0.023	0	0.216	0	0
5-Hepten-2-one, 6-methyl-	1.009	2.285	34.95	0.053	0.0872	0	0.0931	0.254
m/z 82,71,67,43,55,41	0.9366	2.121	37.08	0.191	0	0.0582	0.0922	0
β-Myrcene	0.8879	2.011	39.09	0.243	0.21	0.0931	0.198	0.203

Undecanal	0.8232	1.864	40.95	0.055	0.0386	0	0.14	0.0976
1,5,7-Octatrien-3-ol, 3,7-dimethyl-	0.7677	1.739	42.69	0.159	0.0695	0	0.0262	0.0593
m/z 82,71,67,43,85,83	0.7201	1.631	44.32	0.0126	0.182	0	0	0
m/z 69,41,43,81,79,67	0.6908	1.565	45.89	0.0531	0.151	0	0	0
m/z 117,90,91,89,65,118	0.6895	1.562	47.45	0	0.124	0.0721	0.0768	0.0339
3-Hexen-1-ol, (E)-	0.6562	1.486	48.93	0.0472	0.0543	0.0552	0.0682	0.236
(E)-Ocimene	0.5694	1.29	50.22	0.107	0.0555	0.125	0.105	0.13
Benzeneacetaldehyde	0.5388	1.22	51.44	0.119	0.122	0.118	0.0506	0.0548
Octanal	0.538	1.218	52.66	0.108	0.0962	0.151	0.135	0.163
Copaene	0.497	1.126	53.79	0.0318	0.0465	0	0.0789	0.0684
Nonanal	0.471	1.067	54.85	0.154	0.173	0.205	0.169	0.243
Benzyl alcohol	0.4643	1.052	55.91	0.226	0.216	0.236	0.221	0.172
m/z 138,109,120,69,121,83	0.4607	1.043	56.95	0	0.0351	0	0.0798	0
Acetic acid	0.4528	1.026	57.98	0	0.0608	0.0686	0	0
3-Hexen-1-ol, acetate, (Z)-	0.4385	0.9931	58.97	0.114	0	0	0	0
1-Octanol	0.4231	0.9582	59.93	0.13	0.0827	0.117	0.0884	0.0644
m/z: 71,43,82,67,99,41	0.41	0.9287	60.86	0.0406	0	0.074	0.0267	0

Chapter 4: Conclusion and recommendations for further research

4.1 Outline and Conclusions

Numerous studies have investigated the reproductive ecology of *Erica*, which is the most species-rich genus of the Cape Floristic Region (Van der Niet, 2021), and recent work continues to provide insight into the diversity of pollination systems in the genus (Lombardi et al., 2021, Newman and Johnson, 2021, Van der Niet and Cozien, 2022). However, few studies have been done on the pollination of *Erica* species outside of the Cape Floristic Region (CFR), even though it is also among the more species-rich lineages in the high-elevation region of the Drakensberg Mountains of southern Africa (Carbutt and Edwards, 2006). This study used a multidisciplinary approach to study the pollination system of *Erica caffrorum* and verify a syndrome-based hypothesis of fly pollination, using observations and experiments at five high-elevation study sites in the Drakensberg Mountains: Carter's Nek, Kamberg Reserve, Ntsikeni, Tarn Cave and Witzieshoek. Hand-pollination experiments were used to quantify the breeding system and pollen limitation, floral visitors were observed and identified based on morphology coupled with DNA barcoding for verification, and visitation rates were combined with the assessment of pollen loads to quantify pollinator importance. Spectrophotometry and chemical analysis of floral scent was combined with bioassay experiments to test the function of floral scent and colour traits for pollinator attraction. These techniques and experiments were used to determine the pollination niche of *E. caffrorum* and the relevant functional traits for exploiting this niche.

In Chapter 2, the results of an investigation of the reproductive biology of *Erica caffrorum* are reported. The hand-pollination experiments showed that *E. caffrorum* could not set seed through autonomous autogamy and is not self-compatible; it is therefore reliant on pollinators for reproduction. The pollinators of *E. caffrorum* comprise a range of insects, but across all five study sites, the most abundant insect order was Diptera, comprising 88.3% of the insects captured visiting flowers of *E. caffrorum*. *Erica caffrorum* can thus be regarded as specialised at the functional group level for pollination by short-tongued flies. Even though there was tremendous diversity among fly identity at the family, genus, and species level, individuals of all fly families found pollinating this plant species have tongue lengths that never exceed 15 mm, or the body length of the flies (Goldblatt and

Manning, 2000), and therefore comprise a single functional pollinator group that is attracted to similar scent cues for possible brood or feeding sites. Although some *Erica* species are specialised for pollination by flies (Lombardi et al., 2021: Newman and Johnson, 2021), these are long-tongued flies that comprise a different pollination niche with different functional floral traits. Specialisation for short-tongued fly pollination is, therefore, a novel occurrence for *Erica*.

Fly identification solely based on morphology has many drawbacks because some traits are notoriously difficult to differentiate (Lambkin et al., 2013), resulting in challenges associated with species-level identification with a high chance of cryptic species (e.g., Smith et al., 2006). Thus, DNA barcoding was used as a tool to confirm the identities of flies.

Flies were not only the most common visitors but also the most important pollinators in four out of the five study sites when pollen loads were taken into consideration. Interestingly, there were marked differences in fly assemblages at each site and year sampled. Regardless of the variation in fly communities, Muscidae (and especially the genus *Neomyia*) were consistently present at each site and each year sampled. Other dipteran families, such as the Scathophagidae and Rhiniidae, were also important pollinators at Kamberg Reserve and Carter's Nek, respectively. The results of the pollen limitation experiment at Carter's Nek showed that *E. caffrorum* is weakly pollen limited with a small effect size and a pollen limitation index of 0.18.

In Chapter 3, I quantified floral traits and performed experiments to test their function. One of the most studied floral traits in the context of pollination biology is the length of the nectar-bearing organ (Alexandersson and Johnson, 2002; Anderson and Johnson, 2009; Darwin, 1897; Johnson et al., 2017; Peng et al., 2018). I measured several floral traits using traditional morphometrics and found significant differences among the three sites where flower morphology was measured, with the exception of floral tube length. Floral tube length and functional floral depth (anther height and style length) never exceeded five mm; this may reflect morphological matching with the short tongues of the visitors, especially flies (e.g., Anderson and Johnson, 2009; Whittall and Hodges, 2007). The reflectance pattern of the flowers (sepals and petals) and background leaves at multiple sites showed that the flowers had increased reflectance at wavelengths of 400 nm onwards; this is characteristic of human “white-cream” flowers also found in other fly-pollinated plant species (Johnson et al., 2020; Klecka et al., 2018; Lunau, 2014). When the loci of the flowers were plotted in the Troje (1993) categorical model, all loci fell in the same quadrant, suggesting that flies cannot discriminate between different flower parts (also see Johnson et al., 2020).

I also investigated the floral scent chemistry of *E. caffrorum*. The main scent compounds are aromatics (benzaldehyde, phenylethyl alcohol), monoterpenoids (linalool) and sesquiterpenes (caryophyllene). A carboxylic acid (2-methylbutanoic acid) is likely the scent compound that gives *E. caffrorum* its characteristic scent that is unpleasant. These scent compounds are known to attract various insects, including moths and flies (Johnson and Jürgens, 2010; Johnson et al., 2020; Knudsen and Tollsten, 1993).

The bioassay experiments I performed tested the function of colour and scent for pollinator attraction. The colour/scent choice experiments showed that fly visitors preferred white over green with and without the presence of a scent mixture; flies always preferred the option with scent present when offered a choice between two treatments that did not differ in colour. The results of this experiment show that colour and scent both function for fly attraction in *E. caffrorum*. The results for the other flower visitor groups were less conclusive, partly due to a small sample size.

The scent composition choice experiment results showed that the flies were consistently attracted to the acid-benzaldehyde blend over the hexane control at two of the three experimental sites. The flies were also attracted to the acid by itself but did not show attraction to benzaldehyde alone. Other flower visitors were attracted in much lower numbers to the scent experiments, and neither clear nor strong trends in the attraction of non-fly visitors were detected: At one site, benzaldehyde appeared to deter non-fly visitors, but at another it functioned to attract. Although results of the scent bioassays for non-fly flower visitors to *E. caffrorum* were inconclusive, these experiments clearly demonstrate the function of the acid component of the scent bouquet, either alone or in conjunction with benzaldehyde, for attraction of fly pollinators to flowers of *E. caffrorum.*

Fly pollination in *Erica* has hitherto only been documented in the context of highly specialised long-proboscid fly pollination systems, which are specialist nectar feeders, in southern Africa (Lombardi et al., 2021; Newman and Johnson, 2021). For example, *Erica* *aristata* has flowers that have long, narrow corolla tubes with narrow openings and contrasting white and pink petals (Lombardi et al., 2021), which may function as nectar guides (Hansen et al., 2012). These traits are typically associated with long-proboscid fly pollination generally, and *E. aristata* is exclusively pollinated by a single tangle-veined fly species (Diptera, Nemestrinidae). In contrast, *E. caffrorum* has relatively small flowers with no contrasting reflectance patterns of sepals and petals, although flowers have a similar human-perceived white petal colour to *E. aristata* (Lombardi et al., 2021). Many other small-flowered *Erica* species share various degrees of overlap in reflectance patterns to *E. caffrorum,* and flies have also been recorded as visitors to some of these small-flowered *Erica* species; however, these species were pollinated mainly by honeybees (Bouman et al., 2017) and were characterised by different scent chemistry. The scent chemistry of *E. caffrorum* sets it apart from the long-proboscid fly-pollinated *Erica* species, that are usually unscented, and other small-flowered *Erica* species because of the scent compounds that are known to function for fly attraction such as carboxylic acids function in the attraction of *Drosophila* species (e.g., Shrestha and Lee, 2021).

Results of this study strongly suggest specialisation for short-tongued fly pollination in *E. caffrorum* with short-tongued flies, opportunistic nectar feeders, as the predominant visitors at all of the five sites at which pollination was studied. The short-tongued flies comprised 88.3% of pollinators of *E. caffrorum,* which is higher than the 75% threshold suggested by Fenster et al. (2004) to be characteristic of specialised pollination. However, an alternative explanation for the observations of predominantly flies as pollinating agents of *E. caffrorum* may be that the sites where *E. caffrorum* was studied have a depauperate insect fauna in which flies dominate. This would constitute a case of “imposed specialisation” (sensu Armbruster, 2017), in which specialisation is not necessarily due to functional traits that partition the local pollinator community but due to the absence of many common pollinator groups. Indeed, at the high-elevation sites where *E. caffrorum* was studied, flies are often one of the dominant pollinators of other plant species, too (see McCabe et al., 2019). In case of imposed specialisation, if other insects had been present, they might have also been found visiting *E. caffrorum*, as was the case for the CFR-based small-flowered *Erica* species that were pollinated primarily by honeybees but also visited by flies (Bouman et al., 2017). Under this scenario, the presence of benzaldehyde, which is a known attractant of bees (e.g., Theis, 2006), may be easier to explain compared to a scenario of functional specialisation for flies. Distinguishing between these two scenarios can be resolved by 1) reconstructing pollination networks for the entire community that *E. caffrorum* is part of and 2) characterising the pollinator community objectively by catching insects using techniques such as malaise traps and pitfall traps in order to objectively sample the community of insects present at these high-elevation study sites. Both of these techniques have advantages and disadvantages and should ideally be used complementarily to assess the insect community at high elevations.

Erica caffrorum shows similarities with several short-tongued fly-pollinated plant species in the Drakensberg Mountain range that do not have the floral appearance of known carrion or dung mimicry pollination systems: species of *Eucomis, Wurmbea* and *Schizochilus* are pollinated by species within Calliphoridae, Muscidae, Sarcophagidae and Scathophagidae (Johnson et al., 2020; Shuttleworth and Johnson, 2010; Van der Niet et al., 2010). *Erica caffrorum* produces small quantities of nectar, for which quantification was outside this project's scope. *Erica caffrorum* may conform to a similar Drakensberg-centred pollination guild, including *Eucomis bicolor* and *E. humilis,* which are also nectar-producing and exclusively fly-pollinated, although the functional scent compounds are oligosulphides rather than acids (Shuttleworth and Johnson, 2010). These compounds are also important in stapeliads that mimic carrion scent, but these species often produce minute amounts of nectar that may not serve as a food reward but as a "teaser" to help position the pollinator for contact with reproductive parts (Jürgens et al., 2006). Another member of the high-elevation fly pollination guild may be *Wurmbea elatior,* which uses indole and skatole, which are thought to mimic faeces (Johnson et al., 2020). The scent compounds produced by *E. caffrorum,* especially the carboxylic acid 2-methylbutanoic acid, are also found in the excretory waste of animals (Cossé and Baker, 1996). For this and other species of the putative guild, it remains unclear whether the flies attracted to these scent compounds are initially attracted through innate preferences, similar to the mechanism considered important for mimicry, or through other mechanisms (Johnson and Schiestl, 2016). Further behavioural experiments are required to fully understand whether mimicry plays a role in the pollination of these species. Experiments using gas-chromatography coupled with mass spectrometry (GC-MS) combined with electroantennographic detection (EAD) could test the role of specific candidate scent compounds of other plant species in similar high elevation environments that could attract a similar assemblage of short-tongued fly pollinators.

The similarity in traits between *E. caffrorum* and several other distantly related plant species in high-elevation ecosystems suggests that the pollination of scented, small, white flowers by short-tongued flies found here likely represents a more widespread pollination system whose importance in southern African mountain regions is currently underestimated. Further empirical studies of pollination and macroevolutionary research is needed to help us better understand the patterns of short-tongued fly pollinator-driven diversification of high-elevation plant species.

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