POLLINATION BY SHORT-TONGUED FLIES AND ASSOCIATED FLORAL TRAITS: A NOVEL POLLINATION GUILD IN THE HIGH-ELEVATION DRAKENSBERG OF SOUTH AFRICA

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PREFACE

The research described in this thesis was carried out in the Republic of South Africa under the auspices of the School of Life Sciences, College of Agriculture, Engineering and Sciences, University of KwaZulu-Natal, Pietermaritzburg campus, under the supervision of Prof. Timotheüs van der Niet and co-supervised by Drs Ruth J. Cozien, Adam Shuttleworth and Kurt Jordaens from April 2021 to February 2025.

This thesis represents the 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.

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I certify that the above statement is correct

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Professor Timotheüs van der Niet (supervisor)

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

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Dr Ruth J. Cozien (co-supervisor)

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Dr Adam Shuttleworth (co-supervisor)

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Dr Kurt Jordaens (co-supervisor)

DECLARATION 1 - PLAGIARISM

I, ……Nkitseng Oageng Modise……….……………………….., declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons’ data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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DECLARATION 2 - PUBLICATIONS

Chapters 2 has been submitted and accepted for publication to *South African Journal of Botany* (date accepted for publication: 12 May 2025). Chapter 3 has been submitted to *Botanical Journal of the Linnean Society*. Chapters 4 and 5 has been prepared as manuscripts and will be submitted to the relevant journals for publication.

Author contributions to manuscripts are as follows: NOM, RJC and TvdN contributed to field collections. NOM analyzed data and wrote the manuscripts with the assistance of RJC, AS, KJ and TvdN.

Signed:

[redacted]

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ABSTRACT

The diversity of angiosperm flowers is largely attributed to adaptations to pollinators with differing sensory modalities and morphology. Plant-pollinator interactions vary along elevation gradients and global trends suggest that true flies (Diptera) are often dominant pollinators at high elevations. Despite this, relatively few studies within sub-Saharan Africa have assessed the importance of short-tongued flies as pollinators at high elevations. In this thesis, I investigate the role of short-tongued flies as important pollinators and quantify floral traits of a putative pollination guild of plants that co-flower at multiple high-elevation communities in the Drakensberg Mountains of southern Africa.

For several species of the succulent genus *Crassula*, in its Drakensberg centre of diversity, pollinator-exclusion and pollen-supplementation experiments indicated strong dependence on pollinators and an absence of pollen limitation. The insect visitor assemblage for guild members, as assigned using morphological characters, was dominated by saprophilous, short-tongued flies (Diptera), except for one species, *C. vaginata*, in which beetles (Coleoptera) dominated the visitor assemblages. The saprophilous, short-tongued flies was predominated by species of the families Muscidae, Sarcophagidae, Calliphoridae and Tachinidae, amongst other species of various families. Assessment of pollen loads on these floral visitors further supported their importance as pollinators. All studied guild members produced an unpleasant scent. Scent chemistry was dominated aliphatic acids in some species, while other species were dominated by various aromatics, terpenoids and nitrogen-containing compounds. These scents likely mediate attraction of short-tongued fly pollinators. Flowers of all studied plants were relatively small in size, corresponding to flower visitor dimensions. Analysis of spectral reflectance using colour vision models revealed colour contrasts between different floral parts and background gravel from which species predominantly grow from. Fly behaviour on flowers was suggestive of the presence of a reward, but only trace amounts of nectar were recorded. Overall, studied plant species exhibit similarities in floral colour, but exhibit variation in the scent chemistry. Despite the diversity of fly species among the visitor assemblages of studied plant species, I argue that these plant species, with the exception of *C. vaginata*, are functionally specialized for pollination by a group of short-tongued flies and ecologically generalized for visitation and pollination by diverse species of short-tongued flies. Moreover, these plant species belong to a new pollination guild that relies on saprophilous, short-tongued flies for pollination in the high-elevation Drakensberg Mountains. These findings provide novel insights into the pollination ecology of a new saprophilous, short-tongued fly pollination guild that has remained largely unexplored from southern Africa and emphasize the importance of saprophilous, short-tongued flies as important pollinators in high-elevation ecosystems.

TABLE OF CONTENTS

PREFACE ii

DECLARATION 1 - PLAGIARISM iii

DECLARATION 2 - PUBLICATIONS iv

ACKNOWLEDGEMENTS v

ABSTRACT vi

TABLE OF CONTENTS vii

Chapter 1: General Introduction 1

Chapter 2: Short-tongued fly pollination of the vomit-scented *Crassula peploides* (Crassulaceae) in the southern African Drakensberg Mountains 26

Chapter 3: Absence of pollen-limitation in pollinator-dependent *Crassula* species in South Africa's Drakensberg mountains 66

Chapter 4: Diverse saprophilous, short-tongued Diptera pollinate multiple plant species in the Drakensberg Mountain Centre: evidence for a new pollination guild in the southern African flora 93

Chapter 5: Small, pale and stinky: floral traits associated with a fly pollination guild in the Drakensberg mountains of South Africa 147

Chapter 6: General discussion 205

Chapter 1:

General Introduction

Background

Interactions between species are among the important forces shaping ecological communities (Gilman et al., 2010). An example of such an interaction is between plants and pollinators, which is frequently perceived as a mutually beneficial relationship, in which both partners benefit (Olesen and Jordano, 2002; Proctor et al., 1996). Understanding of how interactions shape communities across space and time benefits from a combined evolutionary and ecological viewpoint. These viewpoints have largely influenced research focus on community ecology (Mitchell et al., 2009) by contributing to the understanding of various aspects of pollination biology, including competition for pollinators, pollen movement dynamics, patterns of specialization and generalization and biology of functional floral traits (Mitchell et al., 2009). Many plant-pollinator interactions are random (Chacoff et al., 2018), but some are less random and structured, with clear organization of interactions sometimes existing (see review by Bawa, 1990). This organization in plant-pollinator interactions is evident in the conceptual idea of guilds (Adams, 1985; Johnson, 2010) and the debate around specialization versus generalization in pollination systems (Johnson and Steiner, 2000; Waser et al., 1996). On the one end, plants can be specialized for pollination by a single pollinator type (Fenster et al., 2004; Johnson, 2010; Johnson and Steiner, 2000), whereas a generalized plant species relies on pollination by a diverse range of pollinators (Waser, 2006; Waser et al., 1996). Furthermore, specialization in plant-pollinator interactions is characterized by predictability in interacting members, both in space and time (Carstensen et al., 2014; Minckley et al., 1999), whereas generalized plant-pollinator interactions have the potential to be flexible and therefore vary both spatially and temporally (CaraDonna et al., 2017; CaraDonna and Waser, 2020; Gómez et al., 2008; Herrera, 1996). Specialized and generalized interactions can be better understood by studying functional traits that play a role in these interactions. These functional traits allow plant species to filter out the available pollinator assemblage and allow interaction with only a subset of these available pollinator community, which serves as a foundation for understanding the mechanisms that enable species to occupy diverse niches. (Junker et al., 2013; McGill et al., 2006). One way to view plant-pollinator interactions is through the concept of ecological niches.

Niches

The niche concept was initially defined by Joseph Grinnell as the distributional unit "occupied by just one species or subspecies" (Grinnell, 1924). Although the term was first used in 1924, the idea that species' geographical distributions can be described as a niche had already been developed in Grinnell's earlier work (see Grinnell, 1917). However, in 1957, the term 'niche' was defined differently by G. Evelyn Hutchinson (Hutchinson, 1957) and Amyan Macfadyen (Macfadyen, 1957), as a space within an environment that species can occupy and exploit successfully and independently (see Root, 1967).

In addition, Hutchinson (1957) also provided the theoretical framework for separating a niche into the fundamental and realized niche. Accordingly, Hutchinson (1957) defined the fundamental niche as the entire extent of the environment and all its conditions that a species can occupy and survive in, in the absence of biotic interactions such as competition and predation, whereas the realized niche is a subset of the fundamental niche that a species actually occupies and survives in, which is influenced by its interaction with other species through resource use in a competitive way or through predation (Drummond et al., 2006; Junker et al., 2013; McGill et al., 2006; Phillips et al., 2020). The conceptual terms of the niche and niche theory are now being applied to plant-pollinator interactions to understand their evolution and mechanisms that influence the coexistence of species (Johnson, 2010; Phillips et al., 2020; Valdovinos and Marsland, 2021) through the evolutionary and ecological viewpoints (see Johnson et al., 2017 and references herein). Within plant-pollinator interactions, pollinators represent ecological niches that can be exploited by plant species in a given community to maintain a positive population growth rate (Johnson, 2010; Phillips et al., 2020). In general, multiple species occupy similar niches, and as such, groups of species are considered guilds (Carvalheiro et al., 2014; Morris et al., 2004).

Pollination niches have been considered in the efforts to understand the evolution and coexistence of flowering plants, which are explained by competition for pollination resources (Pauw, 2013). Like any niche, pollination niches are also subdivided into fundamental and realized pollination niches (Hutchinson, 1957; Phillips et al., 2020). The fundamental pollination niche refers to the entire community of potential pollen vector that can potentially contribute to pollination of a given plant species in the absence of competition, whereas the realized pollination niche refers to the actual subset of pollen vectors used by plants amid competition with co-flowering plants for pollinator services (Phillips et al., 2020). Plant species minimize interspecific competition by exploiting diverse pollinator groups (Chesson, 2000; Johnson et al., 2017; Phillips et al., 2020) by partitioning a subset of these pollinator groups from the available pollinator assemblage (Johnson and Steiner, 2000), thereby occupying different realized pollination niches. These functional pollinator groups usually consist of one or more pollinator taxa that are functionally similar in terms of the selection pressures they exert (Johnson, 2010; Phillips et al., 2020). These functional pollinator groups usually function as part of pollination guilds, in which they are relied upon for pollination by multiple plant species within communities.

Guild concept and pollination guilds

Our understanding of the guild concept stems from the pioneering work of G. Evelyn Hutchinson (1959), who realized that the grouping of species is governed by competition for resources and the assumption that the utilization of the same resources by groups of species is common in nature. The term ‘guild’, however, was coined by Root (1967), who defined it as “a group of species that exploit the same environmental resources in a similar functional way”. Unlike Hutchinson’s pioneering work which recognized competition between species, Root’s (1967) definition, however, did not consider competition for resources, but instead focused solely on similar utilization of resources, in this instance food (Blondel, 2003). Although food has been the particular resource that defines guilds according to Root (1967), other resources, including nesting (Whittam and Siegel-Causey, 1981), habitat (Gorman, 1988) and pollinators, have also been utilized to distinguish guilds (Root, 1967; Simberloff and Dayan, 1991). Additionally, guild membership is not restricted by the degree of phylogenetic relationships among the species involved (Adams, 1985; Blondel, 2003; Findley, 1976; Root, 1967), meaning that species may belong to a particular guild if they utilize environmental resources in a similar function irrespective of them being closely related phylogenetically. Based on the definition and designations of guilds, guild membership is thus largely based on functional traits rather than taxonomic relationships.

Indeed, trait-based approaches have been employed in attempts to understand biotic interactions and species coexistence (Blondel, 2003; Calow, 1987; Dykman et al., 2021; Kraft et al., 2015; Pigot et al., 2016; Walker et al., 2022). Trait-based approaches are deeply embedded in the functional trait concept (Walker et al., 2022). Functional traits are defined as “a suite of physiological, morphological, phenological and behavioural characteristics of an organism that impact their fitness through their effects on survival, growth and reproduction”, usually through a link between an organism’s phenotype and exploitation of resources (Violle et al., 2007), and include diverse aspects such as body size, metabolic rates, morphology of the feeding apparatus, temperature tolerance, leaf mass per area, growth forms and lifespan, for example (McGill et al., 2006; Violle et al., 2007). The practicality of these functional traits in ecology hinges on the fact that their variation should be greater between species than within and should be measurable (McGill et al., 2006). As a result, functional traits play a crucial role in determining membership to guilds, since guild members are characterized by similarity in functional traits that enable them to exploit a common resource effectively (Adams, 1985; Blondel, 2003; Devictor et al., 2010; Simberloff and Dayan, 1991).

The concept of guild has gained widespread acceptance and has been incorporated in ecological studies (Adams, 1985; Brown, 1981; Jaksić, 1981; Simberloff and Dayan, 1991), including in pollination ecology. In pollination guilds, the species considered are a group of plant species who exploit pollinators as resource, as opposed to pollinator guilds, in which the focus is on animal species that visit plants to exploit floral rewards as a resource. Pollination guilds are defined as a group of plant species that rely on the same pollinator species or a few species of the same functional type and that share similar functional floral traits (Adams, 1985; Devictor et al., 2010; Johnson, 2010). Furthermore, pollination guilds are characterized by strong patterns of adaptation (and sometimes coevolution) between the guild members and associated pollinators within a community (see Johnson, 2010).

Floral traits play a crucial role in characterizing pollination guilds. By adapting to a common pollinator, plant species that constitute a guild typically display a suite of similar floral traits (i.e. pollination syndromes; Faegri and van der Pijl, 1979; Fenster et al., 2004; Johnson and Steiner, 2000) that function in the pollination process, including pollinator attraction and mediating contact between pollinator and plant reproductive organs. Functional floral traits include various aspects of the phenotype, including morphology, colour, scent and nectar characteristics. Functional traits not only mediate interactions with floral visitors, but can also determine access to rewards, which can lead to increased specialization. For example, plant species characterized by specialized pollination systems utilize ‘filters’ that typically narrow the spectrum of pollinators to one or a few species (Armbruster, 2017). Such ‘filters’ include flowers with long, narrow floral tubes to match the long proboscis of the pollinators and also facilitate precise pollen placement (Manning and Goldblatt, 1997), and unusual rewards in the form of oil and unpalatable nectar that are only appealing to specific pollinators (Armbruster, 2017; Johnson and Steiner, 2000). On the other hand, plant species that are characterized by generalized pollination systems lack such specialized ‘filters’ and are typically characterized by open flowers with easily accessible nectar (dos Santos et al., 2024; Faegri and van der Pijl, 1979; Waser et al., 1996), and pollen is generally deposited imprecisely on a diverse range of pollinators. However, plants with generalized floral morphologies may still be phenotypically specialized by using sensory cues and nectar as proximal mechanisms for specialization, which may include specific floral scent bouquets, to attract specific pollinators (*sensu* Niemirski and Zych, 2011; Shuttleworth and Johnson, 2009).

Nevertheless, plants characterized by typical generalist floral morphologies (i.e. small, open, bowl-shaped flowers with highly accessible nectar; Ollerton et al., 2009; Waser et al., 1996) are very seldom considered a part of a specialized pollination guild. Examples of such species may include representatives from Apiaceae (Niemirski and Zych, 2011; Proctor et al., 1996) and several members of Crassulaceae (Aigner, 2004; dos Santos et al., 2024). This is because these plant species lack specialized morphological traits, making them susceptible to visits and likely pollination by a diverse range of insects (Waser et al., 1996). Given that guilds are typically considered to comprise plant species with specialized pollination systems, it remains unclear whether plant species characterized by seemingly generalized phenotypes can be member of a pollination guild.

Pollination in high-elevation systems

Biological communities tend to vary along climatic gradients. Such gradients may be particularly steep (change over small scale) in montane systems. This makes such systems interesting arenas to test for the effect of climatic variables on biological communities and interactions therein since one effectively controls for other factors that may vary at broad spatial scales. In terms of plant-pollinator interactions, the diversity and abundance of pollinators tend to decrease with increasing elevation (McCabe et al., 2019; McCabe and Cobb, 2021). Furthermore, while bees are known to dominate low-elevation regions (McCabe et al., 2019), their diversity and abundance decrease as elevation increases, although some studies have shown that bumblebees and bees are common visitors in the subalpine regions in some ecosystems, such in the Qinghai-Tibet plateau of China (Zhao and Huang, 2013) and central and northern Japan (Kudo et al., 2024a). Nonetheless, the majority of evidence indicates that flies, particularly members of the families Muscidae and Tachinidae, constitute the dominant group of visitors in alpine regions. (Arroyo et al., 1982; Elberling and Olesen, 1999; Kudo et al., 2024a; Lefebvre et al., 2018; McCabe et al., 2019; McCabe and Cobb, 2021; Totland, 1993).

One particular reason why flies dominate in high-elevation regions is because these regions are characterized by cold temperatures and overall harsh weather conditions (Lefebvre et al., 2018; Ollerton, 2017), which flies appear to tolerate and thrive better in than bees. Also, similar to how insect activity is restricted by cold climatic conditions (Muñoz and Arroyo, 2006; Sinclair et al., 2015), flowering phenology of high-elevation plants is also restricted to a short period due to cold temperatures following snowmelt in regions that experience snow (Kudo et al., 2024a). In these ecosystems, the window for plant-pollinator interactions is relatively narrow and as such, both plants and pollinators are expected to exploit a wider range of resources in a short period. This ultimately alters plant-insect interaction dynamics in these regions and may lead to increased levels of generalization (Lara-Romero et al., 2019). It is unclear to what extent similar processes occur in high elevation regions in subtropical climates without substantial snow cover in winter, in which the flowering periods may be more extensive and similar to low elevation systems.

Although many plant species at high elevation are frequently visited by flies and other pollinator groups, the overall impact of climatic conditions on insect activity may reduce the possibilities of vector-mediated pollination and therefore affect plant reproductive success in pollinator-dependent high-elevation plants (Arroyo et al., 1982; Medan et al., 2002). As such, rates of pollen transfer may be reduced by low rates of visitation (Arroyo et al., 2017; García-Camacho and Totland, 2009). Failure to achieve reproductive success due to several factors, including inadequate receipt of conspecific pollen due to low pollinator activity, shortage of mates or high receipt of heterospecific or self-pollen, may result in plant species being pollen-limited (Arroyo et al., 2017; Ashman et al., 2004; Harder and Aizen, 2010; Knight et al., 2005). This phenomenon is widespread across angiosperms and is reported to be particularly prevalent in high-elevation ecosystems (García-Camacho and Totland, 2009; Jiang and Xie, 2020), although a recent study found no evidence for the effect of elevation on various measures of reproductive output, including pollen limitation, in high-elevation plants (Novaes et al., 2024). Pollen limitation has been reported in numerous high-elevation systems, such as in the Himalayas (Ai et al., 2013; Jiang and Xie, 2020), Chilean Andes (Muñoz and Arroyo, 2006) and in northern Europe (Elberling, 2001).

Substantial research has been conducted to understand pollination ecology in high-elevation ecosystems. However, high-elevation ecosystems vary geographically, thus potentially preventing extrapolation across regions. In the Northern Hemisphere, some regions are well-studies, including various systems in China (Zhao and Huang, 2013; Zhu and Lou, 2010), the Himalayas (Ai et al., 2013; Basnett et al., 2019; Jiang and Xie, 2020; Paudel et al., 2019; Zhao et al., 2016), mountains of Japan (Ishii et al., 2019; Kudo et al., 2024a; Matsubara et al., 2023; Toji et al., 2021), Taiwan (Kudo, 2022; Kudo et al., 2024a, 2024b), the alpine parts of Norway (Totland, 1993), Germany (Kohl and Steffan-Dewenter, 2022), the European Central Alps (Wagner et al., 2016), Hawaiian mountains (Aslan et al., 2019) and Colorado's Rocky mountains in the United States of America (Kearns, 1992; Kearns and Inouye, 1994). In the Southern Hemisphere, many case studies are present, but relatively few regions have been considered in a broader context. Regions for which some evidence is available include the Chilean Andes (Arroyo et al., 2017, 1982; Muñoz and Arroyo, 2006), the New Zealand Alps (Bischoff et al., 2013a, 2013b; Campbell et al., 2010; Campbell and Bischoff, 2013; Kudo et al., 2024a; Primack, 1983; Primack and Inouye, 1993) and southern Africa's Drakensberg Mountain (Black et al., 2019; Cozien, 2021; Cozien et al., 2019; Johnson and Hobbhahn, 2010; Steiner and Whitehead, 1988; Thupsie, 2023; van der Niet et al., 2010; Whitehead et al., 2019). Although multiple case studies focusing on plant reproductive ecology from southern Africa's Drakensberg Mountain region are available, a significant amount of research remains to be done and information about the pollination biology of most species is still unknown, despite the high level of plant endemism in this region. (Carbutt, 2019; Johnson, 2010).

Study system

The Drakensberg Mountain Range (DMR) in southern Africa is recognised as one of the seven regional mountain systems of the Afromontane region (White, 1978). This mountain range further forms part of the southern African Great Escarpment, which is a 5000 km-long mountain range that spans several countries, including Angola, Namibia, South Africa, Lesotho, Swaziland, Zimbabwe and Mozambique (Clark et al., 2011; Partridge and Maud, 1987). Furthermore, the Drakensberg Mountain Centre [DMC; using Carbutt's (2019) delineation], which is situated within the DMR, is recognised as the only alpine region and one of the centres of plant endemism in southern Africa (Carbutt, 2019; Carbutt and Edwards, 2006; Johnson, 2010). This summer-rainfall region is characterized by high levels of plant endemism per area, which is estimated at 9% at the species level (Carbutt, 2019).

Several plant lineages within the DMC have been the subject of pollination research. Some of these plant lineages include species that belong to pollination guilds and are specialized for pollination by a single species of long-proboscid fly of the families Nemestrinidae or Tabanidae, or oil-collecting bees of the genus *Rediviva* (Johnson, 2010). These plant species represent Orchidaceae, Scrophulariaceae, Iridaceae and Amaryllidaceae (Anderson and Johnson, 2009; Goldblatt and Manning, 2000; Steiner and Whitehead, 1988; Whitehead et al., 2008), however, they also include species that do not function as part of specialized pollination guilds, for instance some species of the genus *Schizochilus* (Orchidaceae; van der Niet et al., 2010). Furthermore, other plant lineages have also been studied to describe their pollination in general, and these include Colchicaceae (Johnson et al., 2020), Ericaceae (Thupsie, 2023), Asparagaceae (Shuttleworth and Johnson, 2009b) and Achariaceae (Cozien et al., 2019). Despite this, most other highly diverse families within the DMC remain to be studied.

Crassulaceae, a morphologically diverse angiosperm family, includes about 1500 described species worldwide, making it the most species-rich family in the order Saxifragales (Gontcharova and Gontcharov, 2007; Smith et al., 2019). Approximately 34 genera are represented in the family, which is distinguished primarily by the presence of leaf succulence in almost all members (Bruyns et al., 2019; Fradera-Soler et al., 2021; Gontcharova and Gontcharov, 2007; Thiede and Eggli, 2007). Members of Crassulaceae are widespread and are known to occur on almost all continents (Gontcharova and Gontcharov, 2007; Smith et al., 2019; Tölken, 1977), where they are mostly adapted to climatically harsh conditions across a diverse range of habitats including rocky outcrops, seasonally inundated aquatic pools (Smith et al., 2019), moist, rocky surfaces, in-between rock crevices and among grasses in open grasslands (Tölken, 1977).

Within southern Africa, Crassulaceae is the second largest group of leaf succulents, following Aizoaceae (Bruyns et al., 2019) and is represented by about 250 species across five of the 34 described genera, which include *Crassula* L., *Kalanchoe* Adans., *Tylecodon* Toelken, *Adromischus* Lem. and *Cotyledon* L. (Smith et al., 2019). Of these five genera, *Crassula* is the most species rich and phylogenetically placed in the subfamily Crassuloideae, separate from the other genera, and is the most speciose in southern Africa, with at least 150 species distributed in both the summer- and winter-rainfall regions (Goldblatt and Manning, 2002; Smith et al., 2019; Snijman, 2013). Members of *Crassula* are uniquely distinguishable from other members of Crassulaceae by the presence of hydathodes on the leaves, as well as flowers comprising stamens in equal number to the petals (Bruyns et al., 2019; Thiede and Eggli, 2007; Tölken, 1977). *Crassula* species vary in life-history strategies and growth forms, ranging from tree-like to perennial shrubs and soil-creepers and annual herbs (Smith et al., 2019; Tölken, 1985). Most species in *Crassula* are characterized by radially symmetrical flowers with varying floral traits, ranging from small, dull-coloured flowers to large flowers with bright colours (Smith et al., 2019); some are white-greenish or yellow, and some exhibit bicolouration with white floral petal tips and red petal base with red ovaries. In addition, most species are characterized by basal leaves arranged in rosettes (Tölken, 1977).

Despite their importance as one of the larger genera of the southern African flora, very little is known regarding the reproductive ecology of *Crassula*, or Crassulaceae in general, in this region. The majority of available research documenting pollination in Crassulaceae is concentrated in the Northern Hemisphere, where bumblebees and syrphid flies are the major pollinators in the genera *Rhodiola* (Zhu and Lou, 2010), bees of the genus *Apis* as pollinators of *Kalanchoe* (González de León et al., 2016) and various insects belonging to Diptera, Coleoptera and Hymenoptera in *Aeonium* species (Esfeld et al., 2009). Regionally in South Africa, pollination of *C. fascicularis* and *C. coccinea*, both unusual in the genus for their relatively long corolla tubes, has been documented, in which moths and butterflies were the respective pollinators (Johnson et al., 1993; Johnson and Bond, 1994). No other studies exist on understanding the pollination of this diverse family in southern Africa.

The angiosperm family Santalaceae comprise about 150 genera distributed worldwide (Der and Nickrent, 2008). Most members of Santalaceae are woody shrubs or perennial herbs and are characterized by simple leaves and varying flower sizes from small to larger flowers (Der and Nickrent, 2008). The monophyletic genus *Thesium* L. (Santalaceae) is the largest of the sandalwood family with over 350 described species worldwide (Mashego and le Roux, 2018; Moore et al., 2010; Nickrent and García, 2015; Visser et al., 2018) and over 150 of these described species are native to southern Africa (Moore et al., 2010). *Thesium* is one of the largest genera in the Cape Floristic Region (Goldblatt and Manning, 2002). The flowers of *Thesium* are usually small with white to a creamy-white colour, and bees and flies have been inferred as pollinators (Hendrych, 1972), but no actual pollination studies are hitherto available.

The family Apiaceae is well-represented and widely distributed in Africa, and is characterized by varying growth forms, ranging from herbs to trees (Kotina et al., 2012). A total of five genera within the subfamily Apioideae are present within the African continent (Downie et al., 2001). These are *Polemannia* Eckl. & Zeyh., *Anginon* Raf., *Dracosciadium* Hilliard & B.L.Burtt, *Glia* Sond. and *Heteromorpha* Cham. & Schltdl. (Kotina et al., 2012). Furthermore, these five genera are well-represented in southern Africa and are distinguishable from members of other subfamilies by the presence of compound umbels and well-developed vittae (Downie et al., 2001). Studies on the pollination biology of Apiaceae are quite limited in general, possibly because they are often considered archetypes of generalized pollination systems. Among the few available studies, flies are recorded as the dominant visitors and pollinators (Drabble and Drabble, 1927; Niemirski and Zych, 2011; Proctor et al., 1996; Zych, 2007), although a wide range of other pollinators such as bees, beetles and ants, have been recorded (Carvalheiro et al., 2008; Davila and Wardle, 2008; Niemirski and Zych, 2011). The genus *Polemannia* Eckl. & Zeyh comprises three species, namely *P. grossulariifolia* Eckl. & Zeyh, *P. simplicior* Hilliard & B.L.Burtt and *P. montana* Eckl. & Zeyh (Hllliard and Burtt, 1986; Van Wyk and Tilney, 2004), which are distributed in South Africa and Lesotho (Kotina et al., 2012), primarily along the Drakensberg Mountain range (Carbutt and Edwards, 2004). The only studies documenting visitors and pollinators of some Apiaceae species are from Europe (Niemirski and Zych, 2011; Zych, 2007) and Australia (Davila and Wardle, 2008). Apart from that, there are currently no available pollination studies conducted on representatives of the Apiaceae family, including any of the southern African *Polemannia* species.

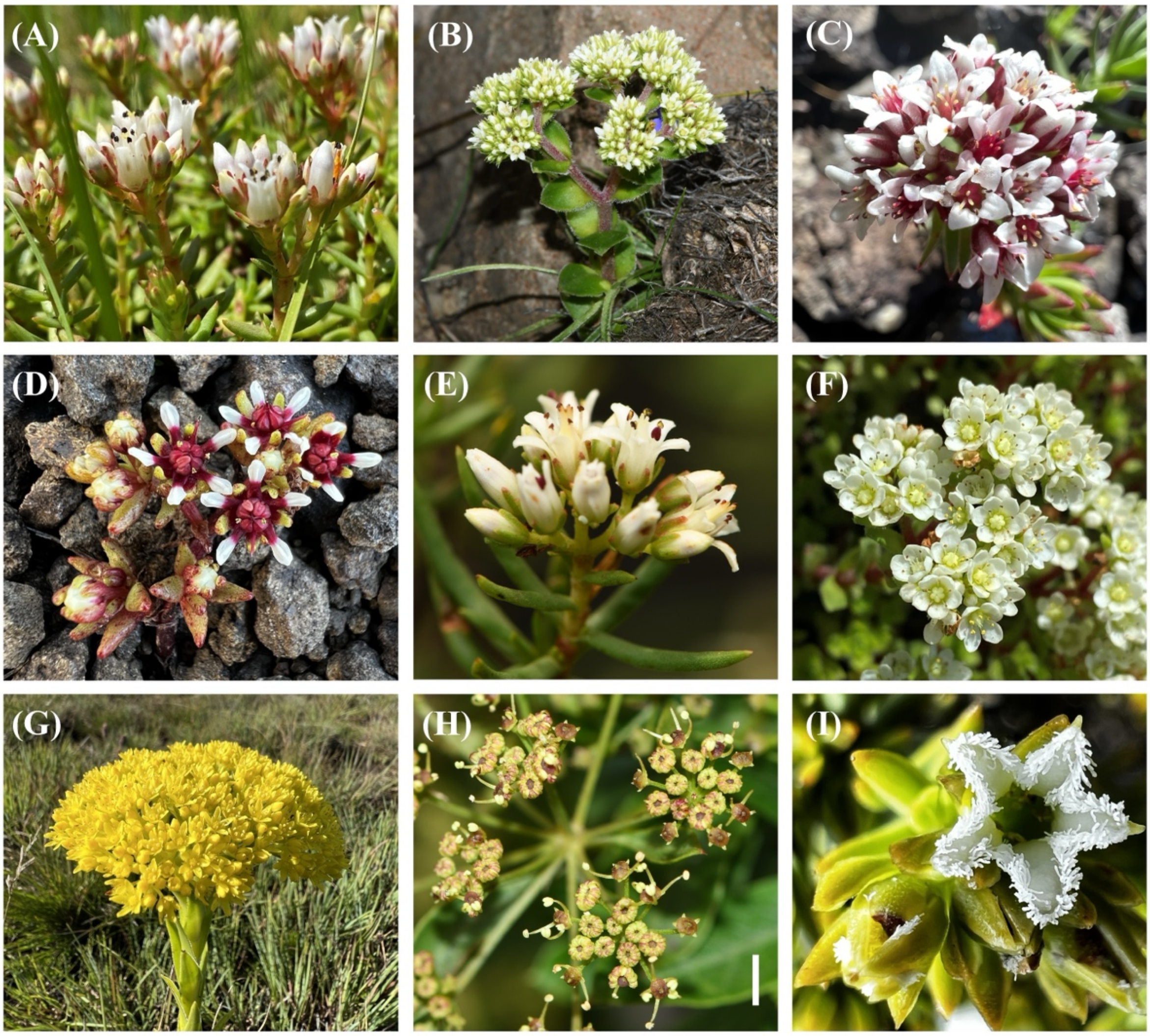

Figure 1.1: Studied plant species that were identified to belong to a putative pollination guild, *in situ*. (A) *Crassula dependens*, (B) *C. natalensis*, (C) *C. obovata* var. *obovata*, (D) *C. peploides*, (E) *C. sarcocaulis* subsp. *rupicola*, (F) *C. setulosa* var. *setulosa*, (G) *C. vaginata*, (H) *Polemannia montana* and (I) *Thesium imbricatum*. Photo credits: Nkitseng Modise (A, C, D, F, G, I) and Timotheus van der Niet (B, E, H). Scale bar: 2mm.

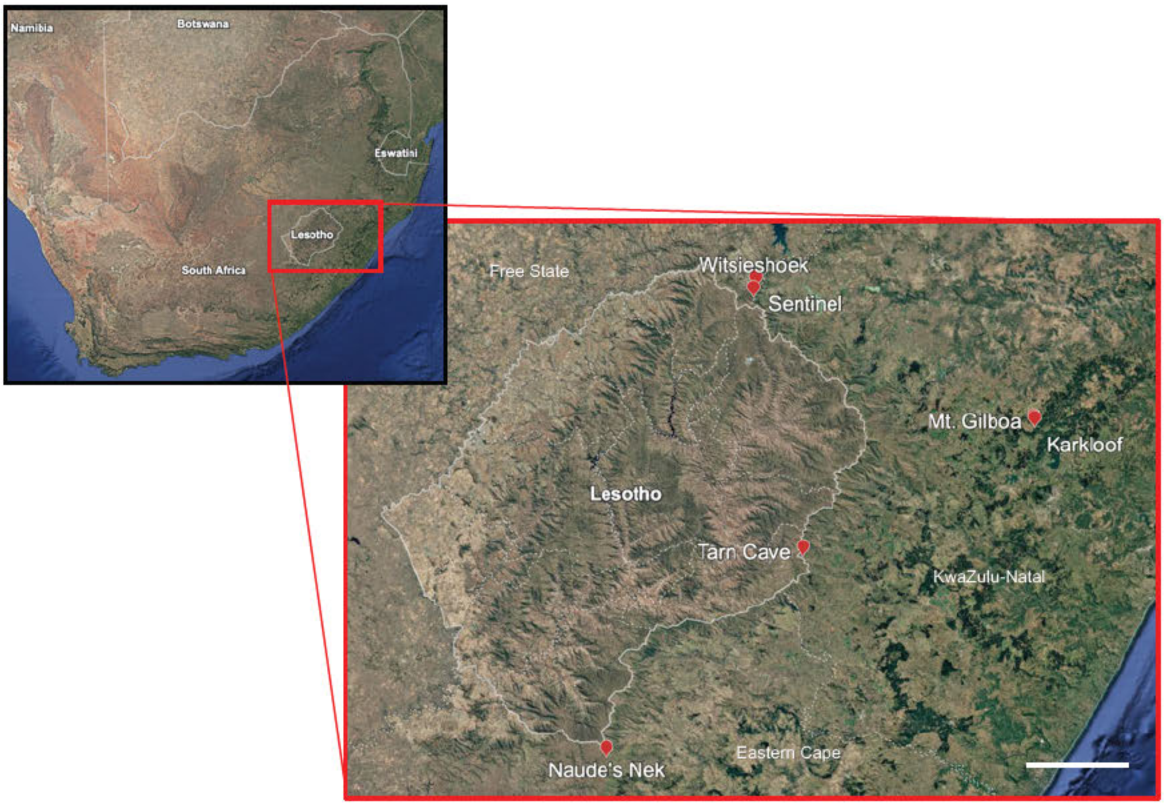

Figure 1.2: Annotated Google Earth image showing six high-elevation study sites within South Africa (red pins). The study sites were located in two provinces; KwaZulu-Natal (Witsieshoek, Sentinel, Mt. Gilboa, Karkloof and Tarn Cave) and Eastern Cape (Naude's Nek). Scale bar: 120km.

Subsets of several species of *Crassula*, *P. montana* and *T. imbricatum* (Figure 1.1) belonging to the above-mentioned plant families co-flower in high-elevation plant communities of southern Africa (Figure 1.2). These species display similar floral traits, such as the presence of small, white or yellowish flowers or a red gynoecium and petal bases, the production of a foetid smell to the human nose and all are visited primarily by seemingly functionally similar short-tongued flies and, in some species, various beetles (pers. observation). Yet their pollination biology is undocumented. The seeming overlap in visitor assemblages, coupled with the observation of similar floral traits further suggests that these plant species may form a guild of predominantly fly-pollinated flowers. Moreover, these plant species co-flower with several other fly-pollinated plant species that display similar floral traits. These plant species include *Wurmbea elatior* (Colchicaceae; Johnson et al., 2020), *Eucomis bicolor* (Asparagaceae; Shuttleworth and Johnson, 2010) and *Erica caffrorum* (Ericaceae; Thupsie, 2023). As such, the *Crassula* species, *P. montana* and *T. imbricatum* represent an ideal system for investigating the plant-pollinator interactions, their breeding systems, including their reliance on pollination vector for reproductive success and also for quantifying floral characteristics that are involved in mediating attraction of these insect visitors. Ultimately, studying the pollination system of these species may provide insights into the ecology of these co-flowering plant species, the significance of short-tongued flies as pollinators in this high-elevation region, and the potential existence of a previously undocumented pollination guild characterized by floral traits and pollinator similarities in the DMC.

Thesis outline

The main aim of this study was to evaluate evidence for a putative pollination guild, potentially including several co-flowering, plant species that are hypothesized to rely on short-tongued flies (Diptera) as pollinators. Based on field observations and available evidence of reliance on short-tongued flies for pollination, I hypothesize that short-tongued flies play an underappreciated role as pollinators of high-elevation flowers in the DMC and that the study species flowers represent a hitherto unrecognised pollination guild in the southern African flora.

In Chapter 2, I present a case study of the pollination ecology of *C. peploides* to identify floral visitors and potential pollinators and quantify floral colour, morphology, scent chemistry and rewards to determine evidence for functional specialization for pollination by short-tongued flies. This chapter introduces the methodology that was expanded to a broader group of species in subsequent chapters.

In Chapter 3, I present results from controlled pollination experiments in four of the putative guild members to investigate the breeding systems, reliance on pollinators, the contribution of pollinators to reproductive success, and evidence for pollen-limitation of reproductive success.

In Chapter 4, I report the identity of floral visitors and potential pollinators of nine putative guild members combined with assessment of pollen loads to determine whether visitors are potential pollinators. Using a plant-visitor network approach, I investigate patterns of spatial and temporal variation and local partitioning of the visitor assemblages at multiple sites and over two sampling seasons. By integrating these results, I assess whether plant species belong to a pollination guild utilizing a common functional pollinator type.

In Chapter 5, I report on the quantification of floral traits that may be associated with mediating interactions between pollinators and guild members. Specifically, I assess morphological differences and similarities among flowers of co-flowering plant species, compare their scent chemistry, and investigate flower colour characteristics to determine whether different floral parts are distinguishable from each other and the vegetation background using a model of fly colour vision, and I quantify the presence of nectar to distinguish a rewarding or deceptive pollination system.

In Chapter 6, I summarize the findings of the four research chapters, discuss the implications and contributions of these results to understanding of high-elevation pollination ecology in Southern Africa and identify avenues for future research.

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Chapter 2:

Short-tongued fly pollination of the vomit-scented *Crassula peploides* (Crassulaceae) in the southern African Drakensberg Mountains

Abstract

Saprophilous flies are frequent floral visitors, and growing evidence indicates that they play an important role as pollinators, especially at high elevation. Saprophilous fly pollination is often associated with foul-smelling flowers, but this has hitherto only been documented in a relatively limited number of plant families. We studied the pollination and floral traits of *Crassula peploides* (Crassulaceae), a species with vomit- and sweaty feet-scented flowers, at two sites in the Drakensberg Mountains of South Africa. Visitor observations and assessment of pollen loads revealed almost exclusive pollination by short-tongued flies (Diptera), primarily of the families Sarcophagidae, Tachinidae, and Muscidae, although pollinator assemblages varied between sites and seasons. Floral scent analysis using gas chromatography coupled with mass spectrometry showed that the odour of *C. peploides* is dominated by low molecular-weight acids, particularly butanoic and isovaleric acids, key compounds which characterize the smell of vomit and sweaty feet, respectively. Quantification of floral colour in a fly vision model indicated that petal tips, pollen and sepals contrast with petal bases, ovaries and the background gravel substrate, and may thus also function in pollinator attraction. Flies were consistently observed probing floral surfaces with their mouthparts when visiting flowers, and the presence of nectar, albeit in small quantities with low sugar concentration, was confirmed. These results provide the first evidence of functional specialization for pollination by short-tongued flies in Crassulaceae and suggest a role for the foul acid-dominated floral scent in pollinator attraction. Similarities between flowers of *C. peploides* and other fly-pollinated species in the Drakensberg Mountains suggest the presence of a guild of plants with relatively small, unpleasant-smelling, pale-coloured flowers and an important role for short-tongued flies as pollinators in this high-elevation region.

Keywords

Saprophilous flies, pollination guild, floral scent, spectral reflectance, floral morphology.

Introduction

Saprophilous flies (Diptera), typically of the families Muscidae, Sarcophagidae, and Calliphoridae, are important pollinators, especially at high elevations where bee (Hymenoptera) diversity is low (Arroyo et al., 1982; Bergamo et al., 2021; Elberling and Olesen, 1999; Fang and Huang, 2013). These short-tongued flies are commonly part of a broad assemblage of generalist visitors (Borkent and Harder, 2007), but are also involved in highly specialized pollination interactions, including brood- (Urru et al., 2011) and food-deceptive systems (Ackerman, 1986). In most fly pollination systems involving saprophilous flies, olfactory signalling, often coupled with visual traits, is thought to play a critical role in mediating pollination interactions (Chen et al., 2015a; du Plessis et al., 2018; Moré et al., 2013). However, documentation of saprophilous fly pollination systems, including a quantitative analysis of floral traits, is limited to a relatively small number of plant species and families.

Olfactory signalling has been shown to play a key role in mediating specialized interactions in certain saprophilous fly pollination systems. Existing case studies have demonstrated the functional significance of oligosulphides (Moré et al., 2013; Shuttleworth and Johnson, 2010; Stensmyr et al., 2002; Wee et al., 2018), *p*-cresol (Kite, 1995; Kite and Smith, 1997; Sayers et al., 2020), indole (Sayers et al., 2020; Zito et al., 2015a) and skatole (Johnson et al., 2020; Sayers et al., 2020) for the attraction of saprophilous flies as pollinators. However, the diversity of floral scents in flowers exhibiting these pollination systems is far more extensive than those that have been tested. Analysis of the scent chemistry of various foetid-smelling stapeliad flowers suggested that the scents of these flowers can be differentiated into multiple ‘chemotypes’, which may reflect mimicry of different substrates which are attractive to saprophilous fly species (Jürgens et al., 2006). Chemotypes were characterized by particular combinations of volatiles, including scents dominated by carboxylic acids (suggested to reflect mimicry of urine), scents dominated by *p*-cresol with small amounts of oligosulphides (suggested to be mimicking dung), scents dominated by oligosulphides with small amounts of *p*-cresol (suggested to be mimicking decaying meat or carnivore and omnivore faeces) and scents dominated by heptanal and octanal (also suggested to be mimicking decaying meat or carnivore and omnivore faeces) (Jürgens et al., 2006). The scents of flowers that exhibit a vomit-like odour to the human nose represent another possible chemotype, but the scent chemistry of such flowers has seldom been examined.

Crassulaceae (stonecrops), with c. 1500 described species, represent the third largest family of succulent plants after Aizoaceae and Cactaceae (Smith et al., 2019), and occur in all biogeographical regions except Antarctica. They are generally confined to semi-arid (Bruyns et al., 2019; Moteetee and Nagendran, 1997; Smith et al., 2019) or montane environments (DeChaine and Martin, 2005; Zhu and Lou, 2010), and grow in diverse habitats, ranging from aquatic pools to rocky outcrops to sun-exposed rocks (Bruyns et al., 2019; Smith et al., 2019). The family is particularly diverse in Mexico (Thiede,

1995) and in South Africa (Smith et al., 2019; Tölken, 1985), with high endemism in the Greater Cape Floristic Region (GCFR) (Bruyns et al., 2019) and the high elevation, summer-rainfall region of South Africa (Hilliard and Burtt, 1987). Despite the global diversity of Crassulaceae, very little is known about their reproductive biology, particularly pollination. Most of the few available studies are concentrated in the Northern Hemisphere (see González de León et al., 2016 and Zhu & Lou, 2010) and suggest that Crassulaceae in these regions are pollinated primarily by hover flies (Diptera: Syrphidae) and bumblebees (Hymenoptera: Apidae) in high-elevation regions (Zhu and Lou, 2010). *Crassula* is the second largest genus in the family after *Sedum* (Hassan et al., 2021) and a major component of the southern African flora, with the majority of species found in South Africa (Lu et al., 2022; Mort et al., 2010; Smith et al., 2019). Pollination of *Crassula* in southern Africa is poorly studied, although specialized pollination systems involving moths and the mountain pride butterfly (*Aeropetes tulbaghia*, Nymphalidae) have been documented in *C. fascicularis* and *C. coccinea*, respectively (Johnson et al., 1993; Johnson and Bond, 1994). However, these two species are exceptional in the genus due to their elongated, tubular flowers, which reach a length of several centimetres; the majority of other *Crassula* species are characterized by much smaller, open flowers.

Here, we focus on *Crassula peploides* Harv., flowers of which have a scent that is, to the human nose, reminiscent of vomit. This species grows at high elevations in seasonally inundated gravel beds on rocky outcrops in the summer-rainfall region of southern Africa, from the Eastern Cape province to the Drakensberg Mountain range in the province of KwaZulu-Natal (Tölken, 1977). The species shares floral characteristics with several other short-tongued fly-pollinated species with which it co-flowers, such as *Eucomis bicolor* and *E. humilis* (Shuttleworth and Johnson, 2010), *Wurmbea elatior* (Johnson et al., 2020) and *Erica caffrorum* (Thupsie, 2023). These species are all characterized by floral colours ranging from white to pale green, some with red markings on the ovaries and corolla and an unpleasant, albeit variable, foetid odour to the human nose. Based on these morphological similarities and foetid odour, we hypothesized that *C. peploides* is also pollinated by short-tongued flies.

This study aimed to document the pollination system and floral traits of *C. peploides.* Our specific objectives were to determine the visitors and pollinators and to objectively quantify floral traits, including scent, colour, floral morphology and reward, that are potentially involved in pollinator attraction.

Materials and Methods

Study species

Crassula peploides is a perennial, low-growing succulent plant species with open, star-shaped flowers (Figure 2.1; Tölken, 1977). Flowers have white petals with a red base and superior ovaries with five separate carpels, which is a distinguishing feature of Crassulaceae. *Crassula peploides* is distributed along most parts of the Drakensberg Mountain range in southern Africa, usually at elevations over 2000 m (Tölken, 1977). In this region, the species grows in flat gravel beds, on rocky, moist surfaces (Moteetee and Nagendran, 1997; Tölken, 1977) and flowers in summer between January and April (Tölken, 1977).

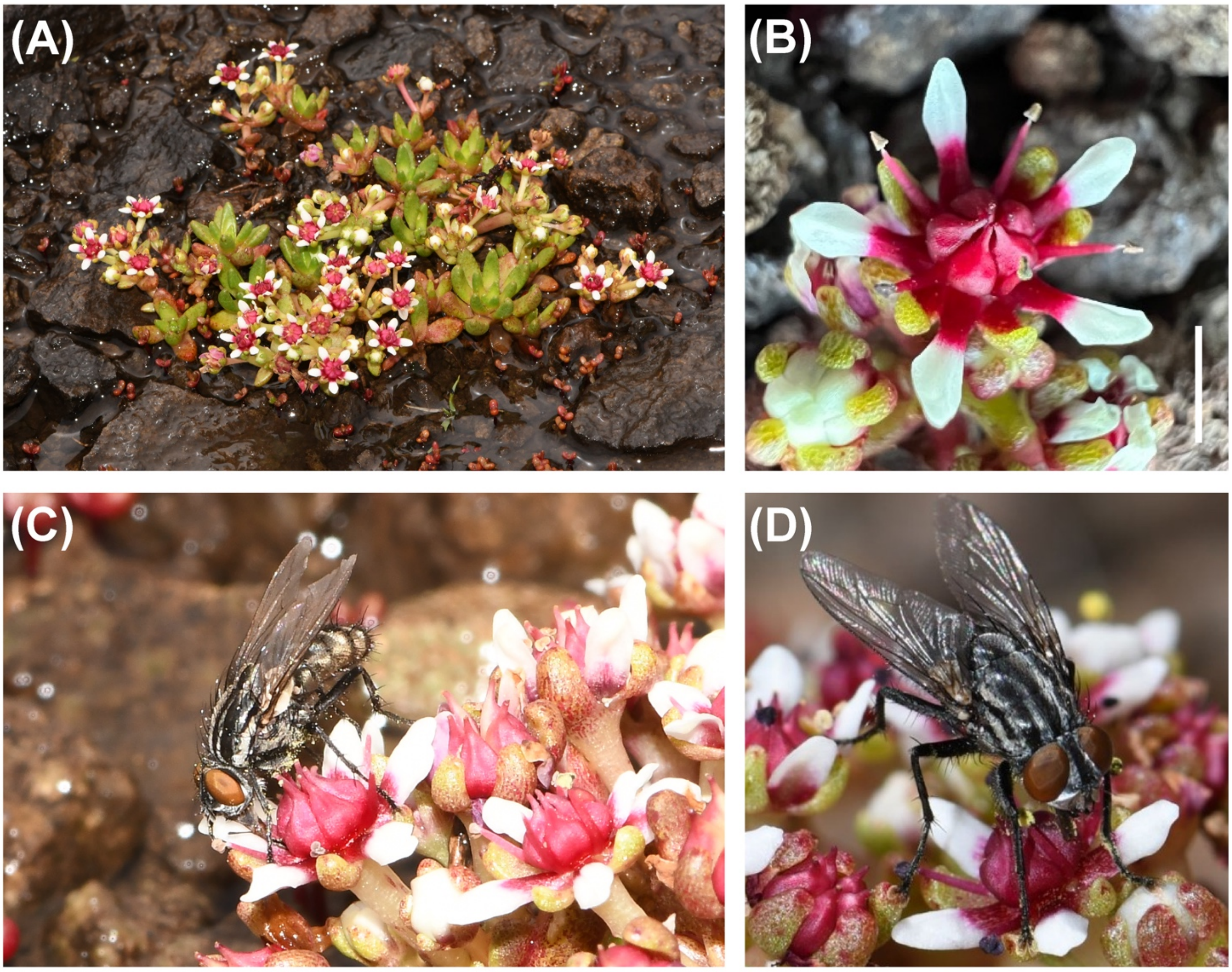

Figure 2.1: *Crassula peploides* in the Drakensberg Mountain Centre. **A** Gravel habitat of *C. peploides* inundated with water. **B** Close-up of *C. peploides* flower (Scale bar: 2.5 mm). **C** Flesh fly (Sarcophagidae) on *C. peploides* inflorescence, with pollen attached to its lower thorax contacting the

stigmas. **D** Flesh fly (Sarcophagidae) probing nectaries of *C. peploides*. Photographs taken by Timotheüs van der Niet (A, C, and D) and Nkitseng Modise (B).

Study sites

Fieldwork was conducted at four high-elevation sites in the Drakensberg Mountain range of South Africa. Two sites (30°40’55” S; 28°08’14” E, 2500 m.a.s.l. and 30°43’07” S; 28°08’23” E, 2511 m.a.s.l.) were situated in the vicinity of the Naude’s Nek Mountain Pass in the southern Drakensberg Mountains of the Eastern Cape province and two localities were in the Witsieshoek region (28°40’55” S; 28°54’19” E, 2180 m.a.s.l., 28°40’32” S; 28°55’38” E, 1937 m.a.s.l.), within the Royal Natal National Park in the northern Drakensberg Mountains in the KwaZulu-Natal province. Visitor assemblages were observed at all sites, and because the visitor assemblages were similar across the two southern sites and across the two northern sites, data from the individual southern sites and the individual northern sites were combined and are henceforth referred to as “Naude’s Nek” and “Witsieshoek” respectively.

Floral phenology

To quantify dichogamy, in particular to assess overlap in sexual phases and the associated potential for autonomous self-pollination (autogamy) in *C. peploides*, the sequence, duration and overlap of floral gender phases were monitored over five days during the peak flowering period at Witsieshoek in 2023. Ten randomly selected plants were photographed twice daily (in the morning and evening), and the gender phase of one to four open flowers on each plant (total $N = 31$) was recorded at each interval. Flowers with dehisced anthers and visible pollen were considered to be in male phase, whereas flowers were deemed to be in female phase (receptive for pollen) when the stigma lobes had mechanically separated and appeared visually wet and shiny (see Kalinganire et al., 2000 and references therein). Flowers in which pollen was present, and stigmas were receptive simultaneously, were scored as simultaneously male and female, i.e., with overlapping genders. The duration of each gender phase was determined by tracking individual flowers over the five days of observation. A minimum estimate of floral longevity of individual flowers was estimated from the sum of the recorded duration of male and female phases. To assess the potential for mechanical autonomous self-pollination, all flowers identified with overlapping genders were carefully inspected for physical contact between the anthers and stigma.

Pollinator observations

Floral visitors were observed and collected over ten days during the 2022 and 2023 sampling seasons (29 – 30 January 2022 and 27 – 28 January 2023 at Naude's Nek, 02 and 19 February 2022, 03 February 2023 and 20 and 22 – 23 February 2023 at Witsieshoek). Flower visitors were sampled during peak flowering on warm, sunny days and clear evenings when there was no rain. Flower visitors were captured haphazardly with an insect net and immediately placed in individual Eppendorff tubes and then frozen at -20 °C until they were sampled for pollen and/or pinned for identification. The method of capturing visitors haphazardly while patrolling, instead of recording visitation as insects visit individual flowers, was appropriate because the flowers received very few floral visits at a time, which some were difficult to identify to species-level during observations with the naked eye. To determine whether floral visitors came into contact with reproductive parts of flowers and carried visible pollen, interactions of visitors with flowers were photographed with an AF-S micro Nikkor 105 mm (1:2.8G) lens mounted on a Nikon D7500 camera. Dipteran visitors were identified to family level using the identification key of Marshall et al. (2017), and to the lowest possible taxonomic level (morphospecies) with help from taxonomic experts (see Acknowledgements). Specifically, visitors were identified to morphospecies level using morphological traits that are relevant for species separation in each group. To determine whether nocturnal insects visit flowers, evening observations were also done for approximately one hour after sunset during one evening at Naude's Nek and for three evenings at Witsieshoek in the 2023 sampling season. Plant voucher specimens and pinned insects were deposited in the Bews Herbarium (NU) and the Pollination Laboratory Collection, respectively, at the University of KwaZulu-Natal's Pietermaritzburg campus.

To establish whether floral visitors are likely pollinators, we quantified pollen loads for a representative subset of floral visitors collected in the 2022 flowering season. Pollen loads were assessed for 12 individuals from Naude's Nek and 17 from Witsieshoek, representing most insect morphospecies at each site. Pollen loads were sampled by dabbing the entire body with a 1 mm^3 cube of Fuchsin gel (Beattie, 1971). Cubes were subsequently melted on glass slides for pollen counting at 10× or 20× magnifications using a Zeiss compound microscope. *Crassula peploides* pollen was distinguished from heterospecific pollen based on pollen reference samples collected from co-flowering flowers at the study areas. However, it is possible that visitors carried pollen from co-flowering *Crassula* species, such as *C. dependens*, although this is unlikely as they had non-overlapping visitor assemblages during the study period (unpublished data).

To determine the most frequent interactions and to determine whether visitor assemblages were consistent across sampling seasons and sites, interactions with floral visitors were visualised in a modified bipartite visitation network and modularity analyses using the 'plotweb' and the 'Beckett' algorithm in the 'computeModules' functions in the 'bipartite' package (Dormann et al., 2009) in R statistical software (R Core Team, 2022). In the visitation network, the links represent the number of individuals per insect morphospecies that were caught visiting flowers (Dormann et al., 2009). We further performed a weighted modularity analysis of floral visitors of *C. peploides* to determine whether there is turnover of floral visitors at the two sites over two sampling seasons. Modules depict the grouping of interactions, with species within a module more strongly connected to each other at that site than at other sites (Olesen et al., 2007). Furthermore, significance of the modularity was determined by comparing the weighted modularity (Qw) values with null model values (Dormann and Strauss, 2014).

Scent

Floral scent samples were sampled *in situ* within two hours of midday to coincide with typical floral visitor activity in the 2022 sampling season from plants at both study sites using a dynamic headspace extraction method. Samples were taken by enclosing inflorescences of plants (three from Witsieshoek and four from Naude's Nek) in polyacetate bags and pumping air from the bag through a small cartridge containing 1.5 mg each of Carbotrap® B (20-40 mesh) (Sigma-Aldrich Co.; St Louis, MO, USA) and Tenax® TA (60/80) (Supelco^{TM}; Bellefonte, PA, USA). A control sample was taken for each sampling session from an empty polyacetate bag for the same duration. The sampling duration ranged between 30 to 50 min per sample. After sampling was completed, cartridges were removed and placed in separate glass vials and stored at -20 °C prior to analysis.

Floral scent was analysed using gas chromatography coupled with mass spectrometry (GC-MS) on a Scion 436 gas chromatograph coupled with a Scion single quadrupole mass spectrometer (Scion Instruments, Livingston, UK) (Johnson et al., 2020). Volatiles were separated on an SGE SolGel Wax standard polar capillary column (30 m X 0.25 mm ID, film thickness 0.25 µm). Helium was used as a carrier gas at a flow rate of 1 ml min^{-1}. Each sample was thermally desorbed in a Scion 1079 PTV injector port with a ChromatoProbe thermal desorption device. The injector port was held at 40 ºC for 2 min with a 20:1 split and then increased to 200 ºC at 200 ºC min^{-1} in splitless mode for thermal desorption and held for 2 min and then increased to 250 ºC for the remainder of the run. Following a 3 min hold at 40 ºC, the temperature of the GC oven was ramped up to 240 ºC at 10 ºC min^{-1} and then held at that temperature for 12 min (Shuttleworth et al., 2017). Compounds were identified using Scion MS Workstation v8.2.1 in combination with the NIST mass spectral library v2.4 (2020). Linear Retention Indices (LRI) relative to *n*-alkane standards (Van Den Dool and Kratz, 1963) were compared to published values to confirm library identifications. Library identifications were only accepted if they could be confirmed with comparison to published retention indices. Where possible, compounds identifications were further confirmed by comparison of retention times and mass spectra of authentic standards injected under identical conditions to samples. For compounds that could not be identified, the six most dominant mass fragments were reported. Compounds that were present in similar quantities between samples and the controls were considered contaminants and excluded from analysis.

To determine whether there is variation in the scent profiles between the two sites, we performed ANOSIM (Clarke and Warwick, 2001) with 10 000 random permutations. To down-weight the influence of a few dominant compounds in downstream analyses, proportions of each compound were square-root transformed. We quantified which compounds contributed the most to the variation between the two populations by conducting a similarity percentage (SIMPER) analysis based on Bray-Curtis dissimilarities. Both ANOSIM and SIMPER analyses were performed in PAST v4.03 software (Hammer et al., 2001).

Colour

To quantify the floral colour of sepals, petals, ovaries, pollen, and leaves, we measured their spectral reflectance across the 300 – 700 nm range. To quantify the potential contrast between flowers and the background against which they are likely perceived by insect pollinators, reflectance was also measured for the wet and dry gravel substrate in which plants grow. Spectral reflectance was obtained from one randomly chosen flower sampled from eight different plants per population with an Ocean Optics USB2000 spectrometer (Ocean Optics Inc., Dunedin, FL, USA) and fibre optic reflective probe (UV/VIS 400 µm) paired with an Ocean Optics DT-2-GS Deuterium-Tungsten-Halogen mini light source. We calibrated the spectrometer with a reflective standard (WS-1, Ocean Optics) before taking measurements. Reflectance was taken with the reflective probe held at a 45° angle to the surface being measured. All spectral reflectance measurements were taken in the Pollination Ecology laboratory at the University of KwaZulu-Natal's Pietermaritzburg campus.

To assess the chromatic contrast between the floral colours of the two populations and between different floral parts and the background on which they grow, as perceived by the fly visitors, we plotted all reflectance spectra as loci in the fly colour vision model by Troje (1993). The Troje (1993) fly colour vision model is based on behavioural experiments of colour discrimination and relative excitations of the two p- and y-type receptors of the blow fly *Lucilia* sp. (Calliphoridae) when stimulated by particular spectra in relation to the monochromatic background spectrum against which stimuli are viewed. This model has been used in other studies on fly pollination (see Arnold et al., 2009; Jersáková et al., 2012; Shuttleworth and Johnson, 2010). Plotting of spectral reflectance was done in R statistical software (R Core Team, 2022) using the default settings in the packages 'pavo' (Maia et al., 2019) and 'ggplot2' (Wickham, 2009).

Morphology

To quantify floral dimensions, we measured eight randomly selected flowers from each of the two study sites using digital callipers to the nearest 0.01 mm. Depending on availability, more than one flower was measured per plant. Measurements were taken for floral traits that are thought to be important for pollinator attraction and mechanical fit between pollinators and plant. Specifically, we measured the flower depth, defined as the distance from the receptacle to the highest terminal point when viewed from the side, flower diameter, pistil length, stamen length, petal length and sepal length for each of the eight flowers per population (see Fig. S1 for details of measured morphometrics).

To determine whether morphological traits vary among populations, we visualized the differences using a principal component analysis (PCA). In addition, we used a multivariate analysis of variance (MANOVA) using the raw morphological data to assess morphological differences between the two populations. Both PCA and MANOVA analyses was performed in PAST v4.03 software (Hammer et al., 2001). We further measured the body length and height of all visitors collected at Naude's Nek and Witsieshoek to compare floral dimensions with visitor dimensions and visitor dimensions between sites. A multivariate analysis of variance (MANOVA) was used to assess whether there are differences between the body lengths and heights of all visitors from the two populations.

Reward (nectar)

To determine the presence of a nectar reward, we quantified the volume and sugar content of nectar of plants from Witsieshoek during peak flowering in February 2024. The volume of nectar standing crop was drawn from nine randomly selected flowers by capillary action using 0.5 µl microcapillary tubes (microcaps®, Drummond Scientific Company, Pennsylvania). We estimated the percentage Brix sugar content in the resultant liquid using a Bellingham and Stanley sucrose pocket refractometer with a range of 0 – 50%.

Results

Floral phenology

Flowers of *C. peploides* are protandrous. The five anthers on each flower dehisce sequentially over the first four days of flower life, with one to three anthers presenting pollen simultaneously. Stigma lobes separate and become visually shiny (i.e., receptive) while the last one or two anthers still have pollen present, resulting in one to two days in which intra-floral self-pollination may be possible. After anthers have wilted, stigmas remain receptive for a further four to five days. Physical contact between anthers and stigmas was never observed in any of the monitored flowers in which pollen was available while stigmas were receptive ($N = 6$), rendering mechanical autonomous self-pollination highly unlikely.

Visitor assemblage and pollinators

Insect floral visitors (Figures 2.1C & D) were only observed during daylight hours, with a total of 63 individuals caught on flowers at Naude's Nek (comprising of 19 and 44 individuals in the 2022 and 2023 seasons, respectively) and 55 caught on flowers at Witsieshoek (comprising of 26 and 29 individuals caught in the 2022 and 2023 seasons, respectively; Figure 2.2, Table S2.1). At both sites, the vast majority of floral visitors were Diptera (97.44% of individuals), with only two Hymenoptera individuals and a single Lepidoptera individual found visiting flowers. During the 2022 season, Sarcophagidae dominated the visitor assemblage at Naude's Nek (a total of eight individuals from five morphospecies), whereas in the 2023 season, Muscidae dominated the visitor assemblage at that site (a total of 31 individuals from eight morphospecies; Fig. 2, Table S1). In contrast, at Witsieshoek, visitor assemblages in both sampling seasons were dominated by Tachinidae (14 individuals from five morphospecies in the 2022 season and 12 individuals from two morphospecies in the 2023 season; Figure 2.2, Table S2.1). The visitor assemblages from Naude's Nek and Witsieshoek comprised separate modules, which were further separated by seasons (Figure 2.3), indicating variation in floral visitor assemblages between sites and seasons. In addition, the network structure was significantly modular ($P < 0.05$, z-score = 7.90). The short-tongued flies visiting *C. peploides* were observed to move around the flower while lapping at minute quantities of nectar positioned at the nectary-like structure at the base of each carpel. During this process, they contact the anthers and stigma, usually with their ventral side, and photographs confirmed that pollen grains were visible on the flies' bodies (Figure 2.1C and D).

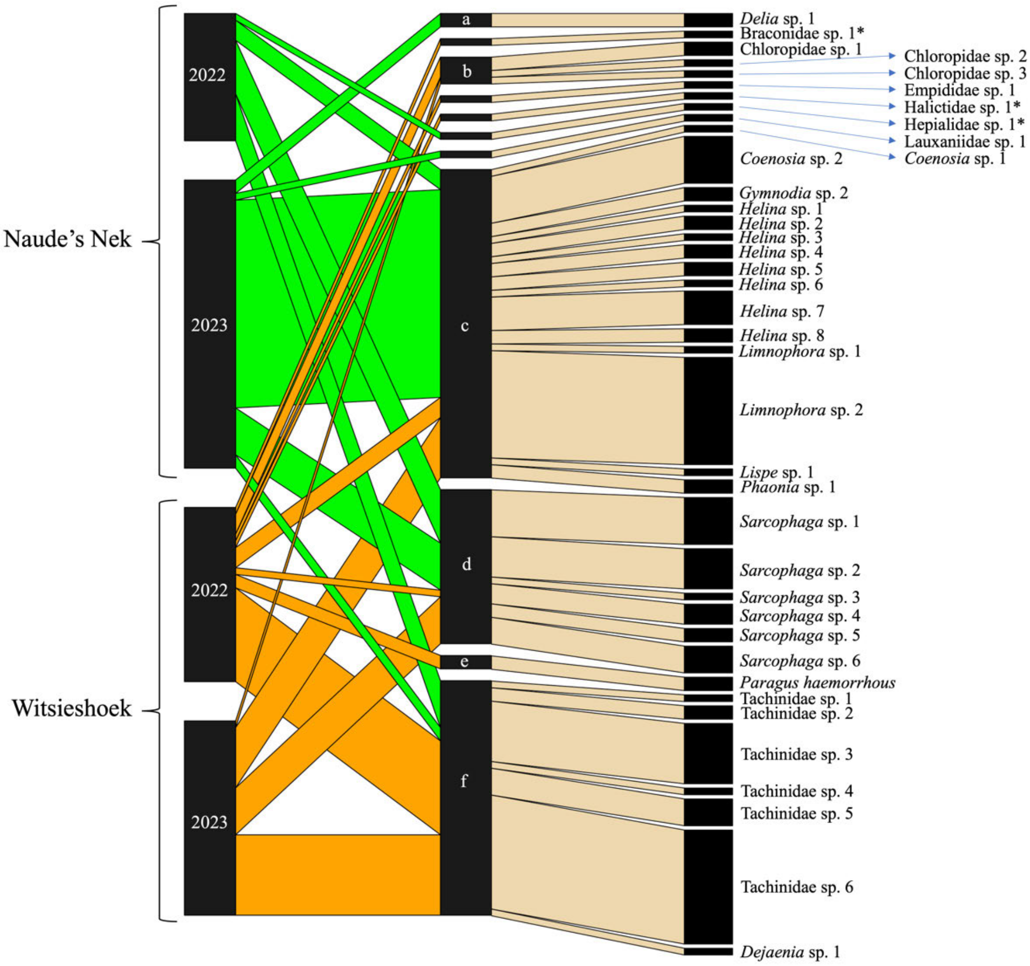

Figure 2.2: Bipartite interaction network of *C. peploides* with visitor species arranged by family at the two sites for the two sampling seasons, with an added interaction layer indicating visitor species. Fly family names are indicated by letters: (a) Anthomyiidae, (b) Chloropidae, (c) Muscidae, (d) Sarcophagidae, (e) Syrphidae and (f) Tachinidae. The network is based on 117 insects caught visiting flowers. The width of the green and orange interaction lines is proportional to the number of visitors per family, with the narrowest line representing one individual and the widest line representing 31 individuals. The narrowest line between fly families and fly species represents one individual and the widest line represents 17 individuals. Non-dipteran visitor families are marked with an asterisk (*) next to the species name. All individuals from the 2022 sampling season were assessed for pollen loads and all were found to be carrying *C. peploides* pollen.

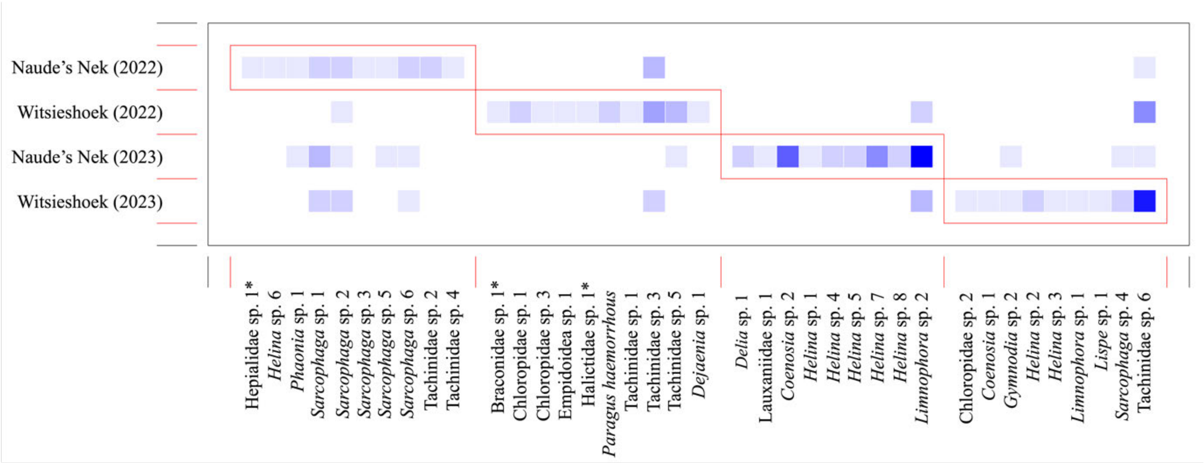

Figure 2.3: Modularity analysis for floral visitors interacting with *C. peploides* at the two sites. Floral visitors are listed on the *x*-axis and sites and sampling seasons are listed on the *y*-axis. Species within a module (enclosed with a red border) are more closely linked to each other than to species outside and in other modules. The darker the shade of blue, the higher the number of interactions observed with that species. Non-dipteran species are marked with an asterisk (*) next to the name.

A total of 3374 *Crassula* pollen grains were counted from a total of 27 fly individuals representing 20 species across six families and from one individual each of a moth (Hepialidae) and a bee (Halictidae) from both sites. Sarcophagidae carried the most pollen grains (mean ± SD: 178.29 ± 87.11; $N = 7$) at Naude's Nek, whereas Syrphidae (mean ± SD: 200.50 ± 235.47; $N = 2$), albeit only two individuals, carried the most pollen grains at Witsieshoek (Table S2.1). At both sites, pollinators mirrored the dominant floral visitors for that particular season: the most frequent floral visitors generally also carried the most *Crassula* pollen (Table S2.1). Pollen loads of only two individuals comprised pure *Crassula* pollen, whereas the rest of the individuals carried pollen from other plant genera too.

Scent analysis

The floral scent of *C. peploides* was dominated by butanoic acid (mean ± SD: 58.4 ± 13.0%; range: 38.9 – 77.5% in seven samples) and isovaleric acid (mean ± SD: 17.5 ± 12.4%; range: 2.6 – 32.5% in seven samples) (Table 1), with several additional aliphatic acids and a single C-5 branched chain acid occurring in smaller relative amounts. In addition to these acids, *p*-cresol was found in all samples, including in high relative amounts in some samples (mean ± SD: 5.3 ± 5.0%; range: 0.1 – 14.8% in seven samples), and octan-3-one was present in high relative amount in one sample (mean ± SD: 2.9 ± 4.5%; range: 0.1 – 11.6% in six samples; Table 2.1). Plants from the Naude's Nek site produced a larger number of compounds (a minimum of 20 compounds per sample), compared to the Witsieshoek site (maximum of 14 compounds found in any one sample) (Table 1). The Naude's Nek population contained 16 unique compounds, while the Witsieshoek population contained only two unique compounds (Table 2.1).

Scent profiles of the two populations were significantly different (ANOSIM, $R = 1$, $P < 0.05$). Based on the SIMPER analysis, differences in the relative amounts of several acids, octan-3-one and *p*-cresol contributed the first 50% of dissimilarity between the two populations (Table S2.2).

Table 2.1: Floral headspace compounds of *C. peploides* identified by GC-MS. Compounds are listed in order of Linear retention index (LRI) within each compound class[a]. The number of flowers in each sample is indicated in brackets.

			Naude's Nek				Witsieshoek		
	LRI	CAS[b]	1 (10)	2 (12)	3 (4)	4 (7)	1 (14)	2 (10)	3 (11)
Total number of compounds			23	26	28	22	5	13	14
Aliphatics									
Acids									
Isobutyric acid	1540	79-31-2	0.5	0.8	0.6	0.7	16.5	4.2	13.7
Butanoic acid[c,e]	1592	107-92-6	62.5	44.3	38.9	62.8	58.3	77.5	64.6
Pentanoic acid	1709	109-52-4	tr	tr	0.1	0.1	-	-	-
(E)-But-2-enoic acid	1750	107-93-7	1.6	15.3	16.1	2.1	-	1.6	3.5
4-Methylpentanoic acid	1774	646-07-1	tr	tr	tr	tr	-	-	-
Hexanoic acid[e]	1812	142-62-1	8.0	tr	0.6	0.6	-	-	-
Heptanoic acid	1920	111-14-8	0.1	tr	tr	tr	-	-	-
(E)-Hex-2-enoic acid	1941	13419-69-7	-	tr	tr	-	-	-	-
Octanoic acid	2026	124-07-2	0.1	0.2	0.1	0.2	-	-	-
Nonanoic acid	2129	112-05-0	1.1	1.2	0.5	0.8	-	-	-
Decanoic acid	2236	334-48-5	-	tr	0.1	tr	-	-	-
Alcohols									
(E)-Hex-3-en-1-ol	1357	928-97-2	0.2	0.1	tr	tr	-	-	-
Aldehydes									
(E)-Oct-2-enal	1411	2548-87-0	tr	tr	tr	tr	-	-	-
Alkanes									
Heptadecane[e]	1697	629-78-7	-	-	-	-	-	0.6	1.1
Octadecane[c,e]	1797	593-45-3	-	-	-	-	-	0.7	1.5
Ketones									
Heptane-2,3-dione[e]	1142	96-04-8	0.3	-	3.4	-	-	-	-
Octan-3-one	1234	106-68-3	0.1	0.2	0.1	-	11.6	2.4	3.1
Acetoin[e]	1262	513-86-0	-	tr	-	-	-	-	-
Ester									
Methyl palmitate	2192	112-39-0	-	-	-	-	-	0.5	0.7
Aromatics									
Benzyl acetate[e]	1699	140-11-4	-	tr	tr	tr	-	-	-
Benzyl alcohol[e]	1898	100-51-6	0.1	3.5	0.2	0.1	-	-	-
Phenylethyl alcohol[e]	1872	60-12-8	0.2	0.1	0.1	0.1	-	-	-

p-Cresol[e]	2036	106-44-5	0.9	14.8	4.3	0.1	5.2	8.3	3.8
C5-branched chain compounds									
Acids									
Isovaleric acid[e]	1637	503-74-2	23.5	18.5	32.5	31.9	8.4	2.6	5.4
3-Methylbut-2-enoic acid	1765	541-47-9	tr	0.4	1.1	0.2	-	-	-
Miscellaneous cyclic compounds									
γ-Caprolactone	1673	695-06-7	tr	tr	tr	tr	-	0.1	0.2
Nitrogen-containing compounds									
Succinimide	2404	123-56-8	tr	tr	tr	tr	-	-	-
Terpenoids									
Monoterpenes									
(Z)-Ocimene[e]	1243	3338-55-4	-	-	-	tr	-	-	-
Unknowns[d]									
m/z: 81, 43, 99, 71, 119, 142	1262		-	-	0.1	-	-	-	-
m/z: 69, 43, 87, 45, 41, 74	1448		tr	-	0.3	-	-	-	-
m/z: 46, 45, 82, 44, 95, 138	1575		-	tr	-	-	-	-	-
m/z: 57, 71, 43, 85, 113, 41	1663		-	-	-	-	-	0.2	0.8
m/z: 71, 57, 69, 43, 70, 84	1741		-	-	-	-	-	0.3	0.9
m/z: 117, 90, 116, 60, 42, 39	1893		tr	tr	tr	tr	-	-	-
m/z: 109, 81, 96, 124, 77, 53	2095		-	tr	0.2	-	-	1.2	-
m/z: 69, 55, 83, 41, 71, 97	2112		-	-	-	-	-	-	0.5
m/z: 91, 103, 121, 146, 77, 65	2219		0.3	-	0.1	-	-	-	-
m/z: 196, 69, 181, 93, 95, 110	2475		-	-	-	-	-	-	tr
Aliphatics			74.7	62.1	60.5	67.2	86.4	87.4	88.3
Aromatics			1.2	18.4	4.8	0.2	5.2	8.3	3.8
C5-branched chain compounds			23.5	18.9	33.6	32.1	8.4	2.6	5.4
Miscellaneous cyclic compounds			tr	tr	tr	tr	-	0.1	0.2
Nitrogen-containing compounds			tr	tr	tr	tr	-	-	-
Terpenoids			-	-	-	tr	-	-	-
Unknowns			0.3	tr	0.7	tr	-	1.6	2.2

[a] Compounds are indicated as relative amounts (%). tr = trace amount, referring to compounds amounting <0.1% of total sample.

[b] CAS = Chemical Abstracts Service Registry Number

[c] Compounds that are present in small amounts in some controls.

[d] Unknowns are listed with the base peak first, followed by other fragments with decreasing order of abundance.

[e] Compound identification confirmed with injection of authentic standard.

Colour

Crassula peploides floral parts from both sites exhibit similar spectral reflectance, with petal tips exhibiting a higher total reflectance compared to other floral parts (Figure S2.2). At both sites, the total reflectance of the petal tips did not exceed 60%. While all other floral parts exhibited less than 30% reflectance and are similar, the reflectance of pollen grains was slightly different, with pollen from Witsieshoek being brighter than that from Naude's Nek. In the fly colour vision model, *C. peploides* floral parts plotted in the fly-UV, fly-blue and fly-yellow quadrants, while the background substrate (wet and dry gravel) plotted in the fly-UV quadrant (Figure 2.4). Specifically, petal tips, pollen and some sepals plotted in the fly-blue and fly-yellow quadrants, suggesting that they are distinguishable from the background in fly visual systems, while petal bases, ovaries and the majority of the sepals plotted with the background substrate, suggesting that these parts are chromatically indistinguishable from each other and from the background to flies (Figure 2.4).

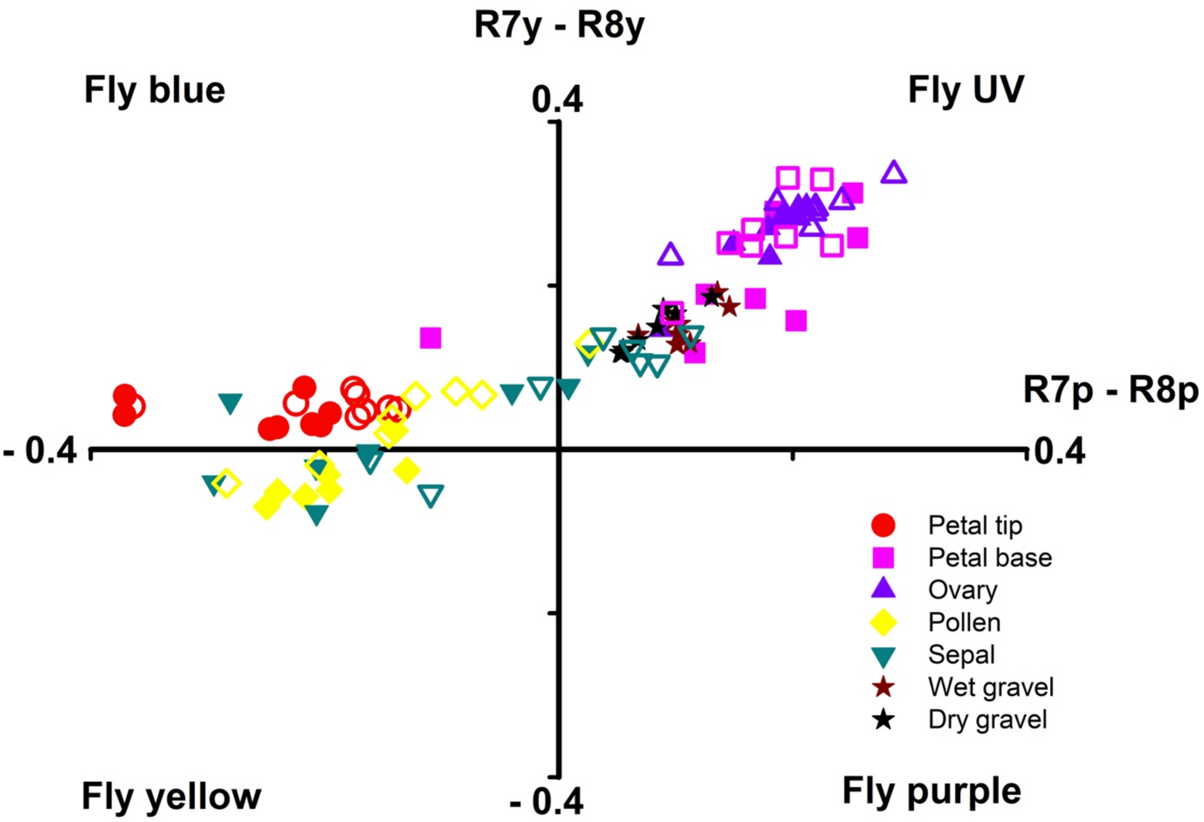

Figure 2.4: Spectral reflectance of different floral parts of *C. peploides* and the background substrate plotted in the fly colour vision model. Plots are based on individual colour loci of different floral parts. Open symbols represent flowers from Naude's Nek, and closed symbols represent flowers from Witsieshoek. Colours with loci that plot in the same quadrant are assumed to be indistinguishable to flies.

Morphology

Crassula peploides flowers from Naude's Nek were slightly larger for most measured traits compared with flowers from Witsieshoek (Table 2.2). PC1 and PC2 together accounted for 85.49% of the total variance (PC1: 56.33% and PC2: 29.16%; Figure 2.5). Flowers from the two sites were distinctly separated in the PCA (Figure 2.5), with stamen length (32.54%) and flower diameter (28.01%) contributing most to the variation. The two sites differed highly significantly in floral traits (MANOVA: $F = 41.28$, $P < 0.01$). Similarly, the body dimensions of visitors differed highly significantly between the two sites (MANOVA: $F = 6.58$, $P < 0.01$). The visitor assemblage varied in body length and height. Body length in most visitors, except for the moth, was less than 10.0 mm (Table S2.3). Overall, the mean body length and height of visitors were larger at Naude's Nek than at Witsieshoek.

Table 2.2: Mean ± SD of floral part measurements (in mm; $N = 8$) of two populations of *C. peploides*. Measured floral traits are indicated in Fig. S1.

Floral trait	**Naude's Nek**	**Witsieshoek**
Flower depth	2.78 ± 0.24	2.64 ± 0.15
Flower diameter	6.84 ± 0.71	7.16 ± 0.28
Pistil length	1.16 ± 0.11	1.14 ± 0.17
Stamen length	2.36 ± 0.09	1.59 ± 0.12
Petal length	2.69 ± 0.17	2.56 ± 0.19
Sepal length	1.76 ± 0.21	1.46 ± 0.09

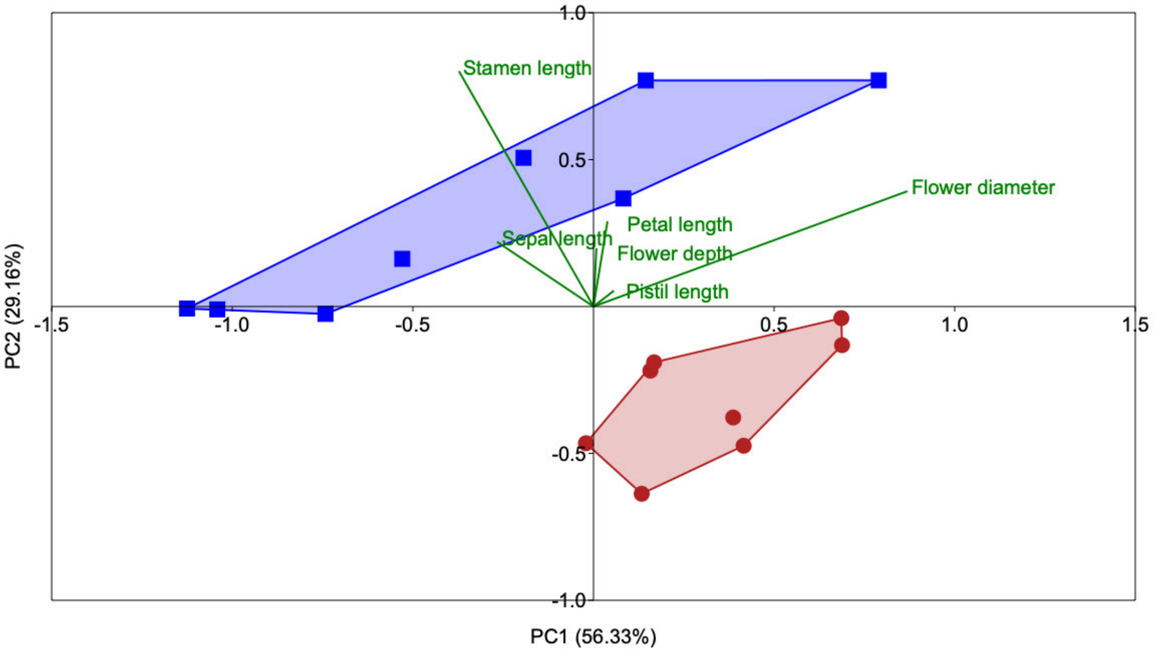

Figure 2.5: Principal Components Analysis (PCA) of *C. peploides* floral measurements showing PC1 and PC2. The different symbols and colours (Naude's Nek: square, blue; Witsieshoek: circle, red) represent the two populations. The labelled green lines indicate the influence of eigenvectors on the PCs. Percentages in parenthesis indicate variance explained by each PC.

Nectar

Crassula peploides flowers (*N* = 11) contained an average of 0.28 ± 0.25 µl (mean ± SD, range: 0.05 – 0.69 µl) of nectar with a sugar concentration of 10.0 ± 6.83% (mean ± SD, range: 1.50 – 18.0%).

Discussion

Results from this study show that *Crassula peploides* flowers are visited and pollinated almost exclusively by flies, particularly of the families Muscidae, Sarcophagidae and Tachinidae. Although substantial temporal and spatial variation in taxonomic composition at family and genus level was observed, floral visitor assemblages almost exclusively comprised saprophilous short-tongued flies and were therefore functionally similar across both study sites and sampling seasons. Observations of floral development indicate that individual flowers last for seven to ten days, and that protandry and herkogamy likely render the species reliant on pollinators for seed production. Analysis of floral traits showed that the unpleasant scent of the flowers is chemically dominated by acids, primarily butanoic and isovaleric acids, which are key components in the odour of vomit and sweaty feet, respectively. Analysis of flower colour identified chromatic contrasts among different floral parts from the perspective of the fly colour vision model. Similarities between *C. peploides* and other co-occurring fly-pollinated flowers suggest that this suite of floral traits represents adaptation for pollination by saprophilous short-tongued flies.

Observations of flower visitors and assessments of pollen loads showed that *C. peploides* flowers are pollinated almost exclusively by short-tongued flies, with variation in the relative dominance of Muscidae, Sarcophagidae and Tachinidae between the two study sites and sampling seasons. The consistent dominance of visitation by short-tongued flies suggests that *C. peploides* is functionally specialized for fly pollination, a finding that is common across high-elevation environments around the world (Arroyo et al., 1982; Larson et al., 2001; Lefebvre et al., 2018; McCabe and Cobb, 2021; Medan et al., 2002; Moré et al., 2019; Totland, 1993; Zhao and Huang, 2013). Given the presence of a range of other functional pollinator groups in the study area of the Drakensberg Mountains region (see Goldblatt and Manning, 2000; Johnson, 2005; Johnson et al., 2020, 2007; Johnson and Bond, 1994; van der Niet et al., 2010; Whitehead et al., 2008), specialization is not imposed by a paucity of pollinator diversity, but rather mediated by floral traits (see below). Although we found covariation between floral visitor dimensions and flower morphologies between the two sites, this is unlikely to represent functional ecotypic variation because the observed feeding behaviour does not result in particularly precise pollen placement, which was found to be placed haphazardly on the bodies of visitors. This is despite more pollen being placed on the dorsal side of the body, based on how visitors were observed to orientate themselves when contacting the nectaries (see Figure 2.1C and D).

Despite the functional specialization for short-tongued fly pollination in general, we observed temporal and spatial variation in visitor assemblages of *C. peploides*, with varying dominance of different visitor groups between the two sampling seasons. Many studies on plant-pollinator interactions of a single plant species have acknowledged the dynamic nature of interactions (Price et al., 2005). A pattern of floral visitor turnover, however, is somewhat unusual within many southern African pollination systems, which are characterized by high levels of specialization in which interactions are temporally and spatially consistent (Goldblatt and Manning, 2000; Johnson, 2005). There are several competing explanations for our findings. Firstly, the distribution of individual species making up local fly assemblages may vary geographically and this may result in spatial pollinator variation, consistent with the presence of multiple ecotypes. We consider this unlikely because the turnover in visitors did not only have a spatial component, but also a temporal component. Furthermore, comparison of floral visitors with other co-flowering *Crassula* species at the study sites, indicates that fly species that do not visit *C. peploides* at one site do visit co-flowering *Crassula* species (unpublished data). Another explanation for the observed spatio-temporal turnover is insufficient sampling effort (Rivera-Hutinel et al., 2012; Vizentin-Bugoni et al., 2016). Indeed, at Naude's Nek, the 2022 visitor assemblage is a nested subset of the 2023 visitor assemblage, possibly reflecting the lower sampling effort in 2022. Finally, the observed family-level turnover within the saprophilous fly assemblage could reflect generally broader ecological niches of short-tongued flies (Courtney et al., 2017; Skevington and Dang, 2002) which contrasts with the more specialized exclusively nectar-feeding pollinators such as long-tongued flies. Indeed, short-tongued flies explore diverse niches throughout their life cycles, including, amongst others, dung and carrion for oviposition and predating on other insects (Goff, 1993) and flowers for nectar and pollen feeding (Courtney et al., 2017; Doyle et al., 2020; Skevington and Dang, 2002).

Crassula peploides flowers appear white with a red base to the human eye. Although not identical, other studies have noted the dominance of white-coloured flowers in high elevations, such as in New Zealand (Newstrom and Robertson, 2005). Visitor assemblages to these white-coloured flowers in the New Zealand alpine habitats were also dominated by various flies, including Muscidae and Tachinidae (Ishii et al., 2019). Furthermore, our results suggest that colours of different floral parts of *C. peploides* contrast with the gravel substrate background against which flies see flowers and that there are clear chromatic contrasts between floral parts, when viewed by flies. The red colouring of the petal base and ovaries may appear to the flies as a dark spot surrounded by 'bright' white petals. Dark spots may mimic resting flies, which may potentially function to attract other flies seeking mates (Johnson and Midgley, 1997) and increase attraction of flies (see Dodson, 1962; Eisikowitch, 1980; Johnson et al., 2020), but support for this idea requires further work.

The scent of *C. peploides* was dominated by various aliphatic and C5-branched chain acids, particularly butanoic and isovaleric acid respectively, with *p*-cresol also present in small amounts in most samples. The dominance of butanoic acid and isovaleric acid, which characterize the odours of vomit and sweaty feet respectively, likely underlie the perception of the scent of *C. peploides* flowers as generally unpleasant and evocative of vomit in particular to the human nose. Acids have also been reported as part of a blend with other compounds in other foul-smelling, sapromyophilous flowers, such as in *Schizochilus bulbinella* (Orchidaceae; van der Niet et al., 2010) and *Leontopodium alpinum* (edelweiss, Asteraceae; Erhardt, 1993) and *Ceropegia pulchellior* (Apocynaceae; Heiduk et al., 2024). Jürgens et al. (2006) also reported the presence of acids (hexanoic and octanoic acids) and *p*-cresol at varying amounts in the foul-smelling stapeliads. The occurrence of isovaleric acid in particular, is rare in the scent of flowers but has been reported in a few sapromyophilous plant species, including *Senecio articulatus* (Asteraceae; Kite and Smith, 1997), *L. alpinum* (Asteraceae; Erhardt, 1993), *Sauromatum guttatum* (Araceae; Hadacek and Weber, 2002) and *Theophrasta americana* and *Deherainia smaragdina* (Theophrastaceae; Knudsen and Ståhl, 1994).

The combination of floral traits including floral colour and contrast, scent and a nectar reward and the visitor assemblages documented here for *C. peploides* are similar to other systems with pollination by short-tongued saprophilous flies, including *W. elatior* (Colchicaceae; Johnson et al., 2020), many stapeliads (Apocynaceae; du Plessis et al., 2018; Jürgens et al., 2006; Shuttleworth et al., 2017) and *Jaborosa rotacea* (Solanaceae; Moré et al., 2013). The scent of saprophilous plants which mimic dung and or carrion is typically characterized by the dominance of compounds including *p*-cresol, sulphides, heptanal and octanal and some acids (Jürgens et al., 2006), whereas the scent of *C. peploides* is dominated by acids, particularly butanoic acid and the less-common isovaleric acid (Kite and Smith, 1997; Knudsen et al., 1993), with *p*-cresol in lesser amounts. In addition, the attraction of flies to the acid-dominated *C. peploides* may reflect pre-existing bias to carboxylic acids, rather than *C. peploides* being a dung or oviposition site mimic. This is because both wild and domesticated animals roam around the sites where *C. peploides* grows, therefore flies could be attracted to the odours and dung from the animals, which comprise of carboxylic acids in their blend (see Marneweck et al., 2017 and refer). Further supporting that *C. peploides* is not a dung or oviposition site mimic, neither egg laying behaviour nor female bias in visitation was observed for *C. peploides*. Pollinating flies were observed lapping at the minute volumes of nectar, which was also observed for *W. elatior* (Johnson et al., 2020). Thus, it seems that only the attraction of flies to foetid scents and their associated feeding behaviour is exploited in the saprophilous fly-pollination system of *C. peploides*. Gas Chromatography coupled with Electroantennographic Detection (GC-EAD) and behavioural experiments could be used to further investigate the attractant cues involved in this pollination system, in particular, the functional aspect of the unusual acids underlying the vomit scent found in *C. peploides,* in attracting saprophilous flies.

Our study has shown that *C. peploides* in the high-elevation Drakensberg Mountains is functionally specialized for pollination by short-tongued saprophilous flies (Diptera), with olfactory and visual cues likely playing a role in mediating attraction. Several other studies from the Drakensberg Mountains have described similar fly-pollination systems involving specialized pollination by short-tongued flies, such as almost exclusive visitation by short-tongued flies belonging to the families Muscidae, Calliphoridae, Sarcophagidae and Tachinidae to the foetid-smelling *W. elatior* (Colchicaceae; Johnson et al., 2020), Calliphoridae on *Cineraria eroidioides* (Asteraceae; Black et al., 2019), Muscidae and Tachinidae on various *Schizochilus* species (Orchidaceae; van der Niet et al., 2010), Calliphoridae, Sarcophagidae and Muscidae on *Eucomis bicolor* and *E. humilis* (Asparagaceae; Shuttleworth and Johnson, 2009), Muscidae, Scathophagidae and Tachinidae on *Erica caffrorum* (Ericaceae; Thupsie, 2023) and on other co-flowering species of Crassulaceae, Apiaceae and Santalaceae (authors, unpublished data). Similarities in the scent profiles among these flowers, including dominance of strong, sweaty-smelling, foetid floral scents, as well as in the size and often colouration, suggest the presence of a guild of plants with small, pale-coloured and unpleasant-smelling flowers, all pollinated by short-tongued flies in the high-elevation regions of South Africa. Further research, including comparative analyses of the above-mentioned candidate species and non-fly pollinated congeners, could confirm whether convergent evolution has occurred, driven by floral adaptations for pollination by saprophilous flies whose importance as pollinators is currently underestimated.

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Supplementary materials

Figure S2.1: *Crassula peploides* flower with arrows illustrating measured floral traits. Scale bar: 1.0 mm.

Table S2.1: Visitor and pollinator assemblages of *C. peploides* from Naude's Nek (Eastern Cape) and Witsieshoek (KwaZulu-Natal) field sites. Individuals were sampled over two sampling seasons (2022 and 2023). Numbers in the years columns refer to number of individuals observed and caught in those years, and the number in brackets indicate the number of individuals sampled for pollen (pollinators). Conspecific (*C. peploides*) and heterospecific (non-*Crassula*) pollen are indicated as means.

	Naude's Nek			**Witsieshoek**		
Species	**2022 / 2023**	**Mean grains of conspecific pollen (range)**	**Mean grains of heterospecific pollen (range)**	**2022 / 2023**	**Mean grains of conspecific pollen (range)**	**Mean grains of heterospecific pollen (range)**
Diptera						
Anthomyiidae						
Delia sp. 1	0 / 2	-	-	-	-	-
Chloropidae						
Chloropidae sp. 1	-	-	-	2 (1) / 0	14	1
Chloropidae sp. 2	-	-	-	0 / 1		
Chloropidae sp. 3	-	-	-	1 (1) / 0	13	1
Empididae						
Empididae sp. 1	-	-	-	1 (1) / 0	14	0
Lauxaniidae						
Lauxaniidae sp. 1	0 / 1	-	-	-	-	-
Muscidae						
Coenosia sp. 1	-	-	-	0 / 1	-	-

Coenosia sp. 2	0 / 7					
Gymnodia sp. 1	1 (1) / 0	77	5	1 (1) / 0	31	6
Helina sp. 1	0 / 1	-	-	0 / 0	-	-
Helina sp. 2	0 / 0	-	-	0 / 2	-	-
Helina sp. 3	0 / 0	-	-	0 / 1	-	-
Helina sp. 4	0 / 2	-	-	0 / 0	-	-
Helina sp. 5	0 / 2	-	-	0 / 0	-	-
Helina sp. 6	1 / 0	-	-	0 / 0	-	-
Helina sp. 7	0 / 5	-	-	0 / 0	-	-
Helina sp. 8	0 / 2	-	-	0 / 0	-	-
Limnophora sp. 1	0 / 0	-	-	0 / 1	-	-
Limnophora sp. 2	0 / 11	-	-	2 (2) / 3	24.50 (7 – 42)	9.50 (7 – 12)
Lispe sp. 1	0 / 0	-	-	0 / 1	-	-
Phaonia sp. 1	1 (1) / 1	72	3	0 / 0	-	-
Sarcophagidae						
Sarcophaga sp. 1	2 (1) / 3	85	30	0 / 2	-	-
Sarcophaga sp. 2	2 (2) / 1	193.50 (159 – 228)	31.50 (29 – 34)	1 / 2	-	-
Sarcophaga sp. 3	1 (1) / 0	349	17	0 / 0	-	-
Sarcophaga sp. 4	0 / 1	-	-	0 / 2	-	-
Sarcophaga sp. 5	1 (1) / 1	154	20	0 / 0	-	-
Sarcophaga sp. 6	2 (2) / 1	137 (117 – 156)	17 (10 – 23)	0 / 1	-	-
Syrphidae						
Paragus haemorrhous	0 / 0	-	-	2 (2) / 0	200.50 (34 – 367)	50.50 (0 – 101)

Tachinidae						
Dejaenia sp. 1	0 / 0	-	-	1 (1) / 0	189	28
Tachinidae sp. 1	0 / 0	-	-	1 / 0	-	-
Tachinidae sp. 2	2 / 0	-	-	0 / 0	-	-
Tachinidae sp. 3	3 (1) / 0	27	14	4 (2) / 2	178 (68 – 288)	14 (11 – 17)
Tachinidae sp. 4	1 / 0	-	-	0 / 0	-	-
Tachinidae sp. 5	0 / 1	-	-	3 (2) / 0	132 (100 – 164)	5 (1 – 9)
Tachinidae sp. 6	1 (1) / 1	10	2	5 (2) / 10	186 (170 – 202)	16.50 (10 – 23)
Hymenoptera						
Braconidae						
Braconidae sp. 1	0 / 0	-	-	1 / 0	-	-
Halictidae						
Lasioglossum sp. 1	0 / 0	-	-	1 (1) / 0	46	165
Lepidoptera						
Hepialidae						
Hepialidae sp. 1	1 (1) / 1	10	2	0 / 0	-	-

Table S2.2: Compounds isolated from the scent of *C. peploides* contributing to the first 80% dissimilarity between the two sites following SIMPER analysis.

Taxon	Average dissimilarity	Contribution (%)
Isovaleric acid	5.88	12.72
Isobutyric acid	5.10	11.04
3-Octanone	4.15	8.98
(E)-2-Butenoic acid	3.62	7.82
p-Cresol	2.86	6.20
Hexanoic acid	2.41	5.21
Butanoic acid	2.14	4.63
Nonanoic acid	1.94	4.19
Benzyl alcohol	1.62	3.50
Octadecane	1.38	2.99
3-Methyl-2-butenoic acid	1.21	2.62
Heptadecane	1.20	2.59
2,3-Heptanedione	1.19	2.58
Methyl palmitate	1.01	2.19
m/z: 71, 57, 69, 43, 70, 84	0.97	2.10
m/z: 57,71,43,85,113,41	0.86	1.87

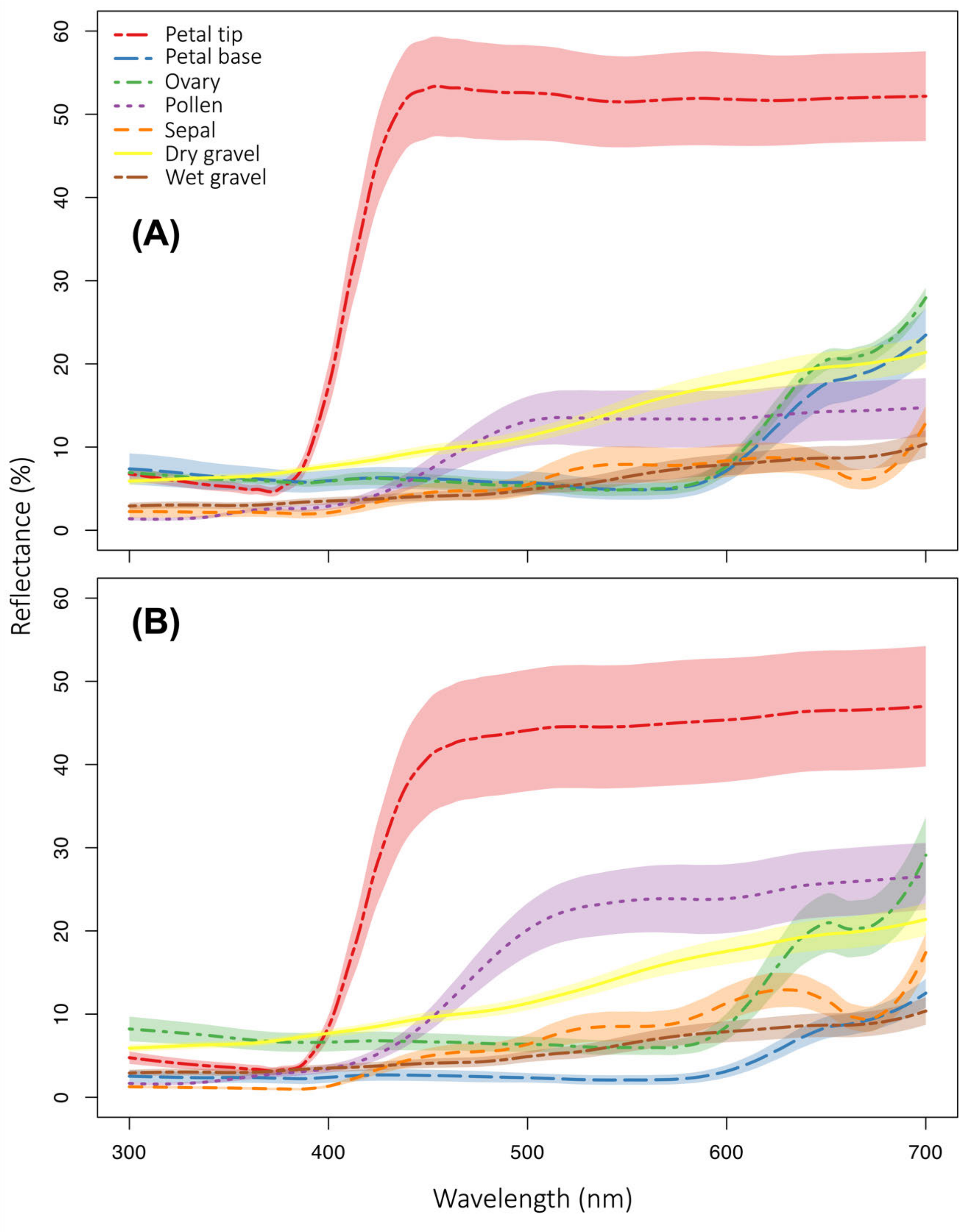


Figure S2.2: Spectral reflectance of the different floral parts of *C. peploides* and the substrate background (dry and wet gravel) from **A** Naude's Nek and **B** Witsieshoek. Lines represent the mean spectrum of eight replicates, while the standard deviation is indicated by the light colouring around the mean spectrum.

Table S2.3: Mean (± SD) of body dimensions (in mm) of *C. peploides* floral visitors. The number of measured individuals per species is indicated in brackets, with individuals from Naude's Nek first, followed by Witsieshoek where the same species was measured at both sites.

Order **Family**	**Naude's Nek**		**Witsieshoek**	
	Body length (mm)	**Body height (mm)**	**Body length (mm)**	**Body height (mm)**
Diptera				
Anthomyiidae				
Delia sp. 1 (1)	4.20	1.70	-	-
Chloropidae				
Chloropidae sp. 1 (2)	-	-	1.55 ± 0.35	0.55 ± 0.07
Chloropidae sp. 2 (1)	-	-	1.70	0.60
Chloropidae sp. 3 (1)	-	-	1.70	0.80
Empididae				
Empididae sp. 1 (1)	-	-	1.90	0.50
Lauxaniidae				
Lauxaniidae sp. 1 (1)	3.60	1.70	-	-
Muscidae				
Coenosia sp. 1 (1)	-	-	3.30	1.0
Gymnodia sp. 1(1;1)	6.30	2.30	4.50	1.60
Helina sp. 1 (1)	6.50	2.90	-	-
Helina sp. 2 (2)	-	-	4.55 ± 0.21	1.60 ± 0.14
Helina sp. 3 (1)	-	-	5.0	1.80
Helina sp. 4 (2)	5.55 ± 0.49	2.15 ± 0.07	-	-
Helina sp. 5 (2)	6.45 ± 0.07	2.80 ± 0.0	-	-

Helina sp. 6 (1)	5.10	1.60	-	-
Helina sp. 7 (2)	5.55 ± 1.91	2.45 ± 1.06	-	-
Limnophora sp. 1 (1)	-	-	3.80	1.30
Limnophora sp. 2 (4;5)	3.95 ± 0.30	1.33 ± 0.22	4.26 ± 0.31	1.58 ± 0.11
Lispe sp. 1 (1)	-	-	5.90	2.20
Phaonia sp. 1 (1)	7.20	2.70	-	-
Sarcophagidae				
Sarcophaga sp. 1 (5;2)	7.18 ± 1.30	2.48 ± 0.3	6.35 ± 0.07	2.25 ± 0.07
Sarcophaga sp. 2 (3;3)	6.93 ± 0.80	2.54 ± 0.06	8.35 ± 0.30	2.65 ± 0.17
Sarcophaga sp. 3 (1)	6.0	2.10	-	-
Sarcophaga sp. 4 (1;2)	10.0	3.0	10.50 ± 1.56	3.60 ± 0.71
Sarcophaga sp. 5 (2)	6.55 ± 0.35	2.40 ± 0.28	-	-
Sarcophaga sp. 6 (3;1)	7.57 ± 1.46	2.70 ± 0.56	6.90	2.80
Syrphidae				
Paragus haemorrhous (1)	-	-	4.20 ± 0.0	1.10 ± 0.10
Tachinidae				
Dejaenia sp. 1 (1)	-	-	10.70	3.40
Tachinidae sp. 1 (1)	-	-	4.10	1.80
Tachinidae sp. 2 (2)	6.45 ± 0.78	2.35 ± 0.49	-	-
Tachinidae sp. 3 (3;6)	6.33 ± 1.11	2.10 ± 0.53	6.30 ± 0.80	2.10 ± 0.30
Tachinidae sp. 4 (1)	7.60	2.50	-	-
Tachinidae sp. 5 (1;3)	4.20	1.70	6.60 ± 0.30	2.40 ± 0.20
Tachinidae sp. 6 (2;15)	5.70 ± 0.28	2.55 ± 0.35	6.20 ± 0.60	2.0 ± 0.30
Hymenoptera				

Braconidae				
Braconidae sp. 1 (1)	-	-	1.50	0.30
Halictidae				
Lasioglossum sp. 1 (1)	-	-	3.80	1.10
Lepidoptera				
Hepialidae				
Hepialidae sp. 1 (1)	18.60 (1)	6.10	-	-

Chapter 3:

Absence of pollen-limitation in pollinator-dependent *Crassula* species in South Africa's Drakensberg mountains

Abstract

Mountain regions are often characterized by highly variable pollination environments, in which self-compatibility and capacity for autonomous self-fertilization may buffer reproductive success of insect-pollinated plants. Within the biodiverse flora of southern Africa, the Drakensberg Mountain Centre (DMC) is characterised by high levels of floristic endemism, but very little is known about plants' dependence on pollinators and vulnerability to variation in pollinator activity in this region. We used controlled pollination experiments to investigate reliance on pollinators and the contribution of pollinators to reproductive success in four *Crassula* (Crassulaceae) species which are indigenous to the Montane elevation zone of the DMC. In all three species for which breeding systems were assessed, fecundity of flowers in the autonomous and hand self-pollination treatments was consistently lower than for flowers subjected to cross-pollination, natural pollination and pollen-supplementation treatments, indicating predominant reliance on receipt of cross-pollen for reproductive success. Indices of self-incompatibility (ISI) indicated strong self-incompatibility for *C. natalensis* and *C. sarcocaulis* subsp. *rupicola* (ISI = 0.84 and 0.94, respectively) and intermediate self-compatibility for *C. setulosa* var. *setulosa* (ISI = 0.54) and indices of autofertility (IAF) ranged from close to zero to a maximum of 0.2, indicating strong reliance on pollinators. Comparison of reproductive success of flowers which were naturally pollinated with that of flowers from which pollinators were excluded further confirmed the exclusive to near exclusive dependence of all studied *Crassula* species on pollinators for fecundity and revealed high levels of pollinator-mediated reproductive success under natural conditions: vector-mediated pollination contributes at least 80 to 90% of fecundity in all four studied species. Despite strong pollinator dependence, manual addition of cross pollen did not significantly increase fecundity in any of the three species, indicating that reproductive success is not limited by pollinator-mediated pollen-receipt (pollen limitation index values all < 0.15 for three species). Results show that reproductive success in the studied *Crassula* species is strongly pollinator dependent and yet may not be limited by pollinator activity in this high-elevation system.

Keywords

Drakensberg Mountain Centre, Crassulaceae, breeding system, autofertility, self-incompatibility, pollinator dependent.

Introduction

Mountain environments around the world are frequently characterised by relatively low insect diversity and abundance (Arroyo et al., 1985, 1982; Totland, 1994), potentially rendering reproductive success of insect-pollinated plants highly prone to pollen limitation (PL). However, unreliable pollinator visitation may also promote plant breeding systems which buffer reproductive success against variation in pollinator activity: self-compatibility (SC) facilitates reproduction in small populations and thereby reduces vulnerability to variation in pollinator service (Pannell and Barrett, 1998) and mechanisms of autonomous self-pollination (AF) enable reproduction independent of pollinators (Lloyd and Schoen 1992). Both SC and AF may thus be more common at high elevation and contribute to alleviating PL induced by low or variable insect activity (Billings, 1974; Mosquin, 1966), but evidence for this is fragmented and conflicting (Eriksen et al., 1993). Meta-analyses show that SC and AF are associated with reduced incidence and intensity of pollen limitation (PL) globally (Burd, 1994; Knight et al., 2005; Larson and Barrett, 2000) and SC and AF are overrepresented on island floras where they may have enabled colonisation and persistence in environments that are, similar to mountain systems, characterised by scarcity of pollinators or high variability in pollinator service (Grossenbacher et al., 2017; Razanajatovo et al., 2019). A few species-level studies in alpine species show that autonomous self-fertilization can assure reproductive success where pollinator service is low (e.g. Zhang and Tan, 2009; Zhang and Li, 2008). Some community-level studies show higher incidence of SC at higher elevation (the Andes; Medan et al. 2002), others find predominant self-incompatibility and reliance on pollinators (Japan; Kudo 2022). Few studies simultaneously consider SC, AF, and PL in the context of elevational gradients (Xu et al., 2023) and, in particular, estimates of pollinator contributions to fecundity are relatively scarce (Rodger et al., 2021, 2004). Because breeding systems which buffer against variation in pollination environments should also confer some resilience to reported global pollinator declines (Biesmeijer et al., 2006; Ollerton et al., 2014; Powney et al., 2019; Zattara and Aizen, 2021) which are of particular concern in mountain regions which are expected to be disproportionately affected by global change (Beniston, 2003; Knight, 2022; Pepin et al., 2015), data on breeding systems and pollinator service from less studied regions that are vulnerable to change may be especially valuable.

The southern African Drakensberg Mountain Centre (DMC) comprises high elevation hills and mountains along the interior of South Africa and into Lesotho, with an average lower elevation boundary around 1700 m above sea level (m.a.s.l.) and reaching up to almost 3500 m.a.s.l. (Carbutt, 2019). The region is recognised as one of southern Africa’s floristic centres of endemism with 227 endemic angiosperm species (9% of the flora; Carbutt, 2019). Pollinators are thought to have been a major driver of diversification in the southern African flora, including in the DMC (Johnson, 2010) however, comparatively few of the many pollination studies in the DMC (Anderson and Johnson, 2009; Brown et al., 2009; Goldblatt and Manning, 2000; Shuttleworth and Johnson, 2010; Steiner, 2010) have specifically investigated plant breeding systems to quantify autofertility and dependence on pollinators (but see Johnson et al. 2003; Springer 2019; Welsford and Johnson 2012). A few recent studies have used pollinator-exclusion experiments to quantify pollinator contributions to reproductive success in the DMC (e.g. Cozien et al. 2019) and determine dependence on pollinators for reproduction (Black et al., 2019; Kgaboesele et al., 2021). However, even studies such as these in which the focus is on a single or few species remain very limited and in the absence of any community-wide studies of pollinator importance and plant breeding systems (see Johnson et al. 2009) for the region, very little is known regarding the importance of pollinators for reproductive success and the vulnerability of DMC plant species to variation in pollinator service and to potential pollinator declines.

Crassulaceae is a cosmopolitan family that is represented on all continents except Antarctica (Mort et al., 2009). *Crassula* L., the second largest genus, is distributed mostly within southern Africa (Mort et al., 2009; Smith et al., 2019; Tölken, 1977), with at least 200 species in this region and over 150 species in South Africa alone (Bruyns et al., 2019; Smith and Crouch, 2021; Tölken, 1985, 1977). The majority of *Crassula* species within South Africa are found in the Greater Cape Floristic Region (Fradera-Soler et al., 2021) and Succulent Karoo Biome (Snijman, 2013), as well as along mountain slopes in the western part of the country (Tölken, 1977). In addition, 30 species occur above 1800 m.a.s.l. along the high-elevation DMC spanning the Eastern Cape, KwaZulu-Natal and the Free State (Carbutt and Edwards, 2004). Little is known, however, about the reproductive biology of *Crassula* in South Africa. Globally, self-compatibility has been reported in representatives of other genera of Crassulaceae, including *Echeveria gibbiflora* in Mexico (Parra et al., 1993), *Rhodiola dumulosa* in China (Zhu and Lou, 2010) and *Kalanchoe* species in Venezuela (Herrera and Nassar, 2009; Jaimes and Ramírez, 1999). Nonetheless, despite the high diversification of *Crassula* in southern Africa as a whole, no study has investigated the breeding system and importance of pollinators for reproduction in this genus.

In this study, we investigate the reproductive biology of four perennial *Crassula* species: *C. natalensis* Schonl., *C. obovata* Haw. var. *obovata*, *C. sarcocaulis* Eckl. & Zeyh. subsp. *rupicola* Toelken and *C. setulosa* Harv. var. *setulosa*, at two sites between 2200 and 2600 m.a.s.l., in their natural range in the Drakensberg mountains. We hypothesize that in this montane elevation habitat, self-compatibility and autonomous autofertility may boost fecundity which is limited by pollinator activity under natural pollination conditions. We used controlled pollination and pollinator-exclusion experiments to determine: (a) self-compatibility, (b) dependence on pollinators, (c) the contribution of pollinators to reproductive success and (d) pollen limitation.

Materials and Methods

Study species

We studied four *Crassula* (Crassulaceae) species: *C. natalensis*, *C. obovata* var. *obovata*, *C. sarcocaulis* subsp. *rupicola*, and *C. setulosa* var. *setulosa*, which all occur between 2248 and 2552 m.a.s.l. in the northern Drakensberg. All studied species are succulent perennials that bear small (diameter less than, or equal to, 1 cm), whitish flowers (Figure 3.1) over a period of four to six weeks between January and April. Flowers of these species are typical of the family Crassulaceae in that they are characterised by five separate carpels. *Crassula natalensis* is characterised by basal leaves arranged in rosettes, with inflorescences terminating in several flowers (Figure 3.1A; Tölken, 1977). *Crassula obovata* var. *obovata* is a freely branching species with flat-topped inflorescences that, similar to *C. natalensis*, terminate in several flowers (Figure 3.1B; Tölken, 1977). Both *C. natalensis* and *C. obovata* var. *obovata* were found growing in close proximity to each other among grasses on mountain slopes. Among the studied species, *C. sarcocaulis* subsp. *rupicola* is the most distinctive in terms of growth form, capable of forming a dense shrub and having fleshy stems with a peeling bark and growing up to 40 cm tall (Figure 3.1C; Tölken, 1977), and was found growing in-between wet boulders and from rock crevices on slopes. *Crassula setulosa* var. *setulosa* produces basal leaves which are arranged in rosettes, similar to *C. natalensis* (Figure 3.1D; Tölken, 1977) and was found growing in a very wet habitat, on steep basalt cliffs or from rock crevices on cliffs.

For three of the study species (*C. natalensis*, *C. sarcocaulis* subsp. *rupicola* and *C. setulosa* var. *obovata*), controlled pollination experiments were used to assess breeding systems, including dependence on pollinators (autofertility), self-compatibility, the contributions of pollinators to fecundity, and pollen limitation of reproductive success. For *C. obovata* var. *obovata*, scarcity of flowering plants precluded experiments to quantify breeding systems, and therefore only contributions of autonomous self-pollination and pollinators to reproduction were estimated.

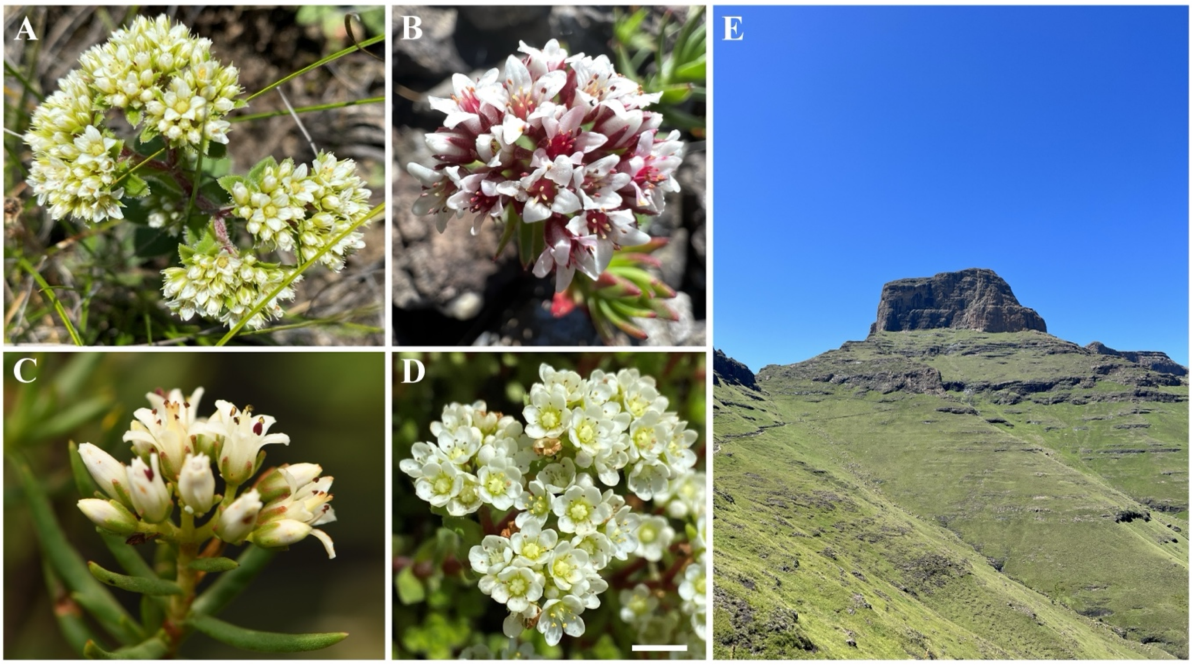


Figure 3.1: Illustrations of flowers and floral arrangement in the four studied *Crassula* species; (**A**) *C. natalensis*, (**B**) *C. obovata* var. *obovata*, (**C**) *C. sarcocaulis* subsp. *rupicola*, (**D**) *C. setulosa* var. *setulosa* and (**E**) The Drakensberg Basalt Grassland vegetation type between 2500 and 3000 m.a.s.l. characteristic of the habitat of the studied *Crassula* species in the DMC. Photographs were taken *in-situ* at the study sites in the northern Drakensberg Mountain range. Scale bar: 2.5 mm in D applies to panel A - D. Photographs: N.O. Modise (A, B, D, E) and T. van der Niet (C).

Study sites

Field work was conducted on the boundary of the Royal Natal National Park and the Orange Free State in the northern Drakensberg in the DMC, KwaZulu-Natal (Table 3.1) from January to March of 2022 (*C. natalensis*, *C. sarcocaulis* subsp. *rupicola* and *C. setulosa*) and 2023 (*C. obovata* var. *obovata*). Voucher specimens are deposited in the Bews Herbarium (NU) at the University of KwaZulu-Natal's Pietermaritzburg campus.

Table 3.1: GPS co-ordinates and elevation (m.a.s.l.) of sites within Royal Natal National Park and along the Kwa-Zulu Natal and Free State boundary (northern Drakensberg, South Africa) at which *Crassula* species were sampled and studied.

Coordinates		**Elevation**	**Habitat**	**Species**
S	E	m.a.s.l.		
28°40’48”	28°55’24”	2555	Rocky outcrops on mountain slopes, among grasses	*C. natalensis, C. obovata* var. *obovata*
28°42’40”	28°53’43”	2248	Between moist boulders and rocks and in rock crevices	*C. sarcocaulis* subsp. *rupicola*
			On very wet, steep basalt cliffs	*C. setulosa* var. *setulosa*

Breeding systems and pollinator contribution to reproduction

Controlled pollination experiments including five pollination treatments were used to assess 1) dependence on pollinators, by comparing relative reproductive success of pollinator-excluded and cross-pollinated flowers; 2) self-compatibility, by comparing relative reproductive success of self- and cross-pollinated flowers; 3) contribution of pollinators to reproductive success, by comparing relative reproductive success of pollinator-excluded and naturally-pollinated flowers, and 4) pollen limitation, by comparing relative reproductive success of naturally-pollinated and pollen-supplemented flowers.

According to population size, 10 – 20 individual plants were selected for pollination experiments in each study species and inflorescences were bagged. Multiple flowers were assigned to each treatment within each plant. Flowers assigned to self-pollination (“self-”) and cross-pollination (“cross-”) treatments were emasculated before anthers dehisced, to prevent within-flower contamination with self-pollen. Once stigmas were receptive (assessed by adherence of pollen to stigmatic surfaces), they were hand pollinated with pollen from the same plant (hand self-pollination), or from several flowers from different plants (hand cross-pollination). Flowers assigned to the autonomous autofertility (“auto”) treatment were bagged but otherwise not manipulated. Flowers assigned to self- and cross-pollination treatments to assess self-compatibility and autonomous autofertility were also enclosed in organza bags that excluded pollinators. Flowers assigned to open-pollination (“natural”) and pollen-supplementation (“pollen-supp”) treatments were not covered with pollinator-exclusion bags and left unmanipulated or supplemented with cross-pollen, respectively. An OptiVisor binocular headband magnifier (Donegan Optical Co., USA) was used to facilitate manipulation of reproductive parts of small *Crassula* flowers. Controlled-pollination experiments were conducted *in situ*, with the exception of *C. natalensis,* for which flowers were too small to manipulate in the field so breeding system experiments were conducted on plants transplanted to a shadehouse in the University of KwaZulu-Natal Pietermaritzburg campus' Botanical Gardens. "Bugdorm" insect exclusion tents (Bioquip) were used to prevent access by potential pollinators in the shadehouse. Plants were grown in a growing medium mixture of quartz sand, peat and vermiculite in a 2:1:1 ratio, and watered daily until fruits matured.

All pollination treatments were applied three to four weeks after buds were bagged. Experimental plants were marked with tags attached to the stem, while experimental flowers were marked with coloured wires corresponding to each of the five experiments. All tagged flowers and fruits were harvested once the fruits were sufficiently developed to count seeds, which was approximately four to six weeks after controlled pollination treatments were applied. The total numbers of flowers harvested for seed counting are indicated in Table 3.2. Fruits were placed in labelled seed envelopes and stored in a laboratory fridge at 4°C until further processing. To assess reproductive success, wilted flowers and fruits were dissected and the number of seeds formed was counted at magnifications of between 1× and 3× on 10× objective lens using a Zeiss Stemi 2000-C dissecting microscope. Fully developed seeds were distinguishable by their rough coating, greenish to brownish colour depending on species, and larger sizes compared to unfertilized seeds, which were smaller and white (see González de León et al., 2016).

Statistical analyses

Analysis of counts of seed set per flower obtained from pollination experiments utilised generalized linear mixed-effect models (GLMMs) implemented in SPSS v28 (IBM Inc.) and was modelled with a negative binomial distribution and log-link function (Bolker et al., 2009). To account for plant-specific effects, where multiple flowers were assigned to each treatment within each plant, plant identity was included as a random factor. Comparisons of seed set among five pollination treatments (auto, self, cross, natural and pollen-supp) for three studied species (*C. natalensis*, *C. sarcocaulis* subsp. *rupicola* and *C. setulosa* var. *setulosa*) considered the effect of treatment only in a separate analysis for each species. Comparisons of seed set between natural and pollen-supp treatments for four species (*C. natalensis*, *C. obovata* var. *obovata*, *C. sarcocaulis* subsp. *rupicola* and *C. setulosa* var. *setulosa*) assessed the effects of species, treatment and their interaction as fixed factors. Treatments were compared in post-hoc tests and significance was assessed using the sequential Šidák method for multiple comparisons. For graphical representation, adjusted means were back-transformed from the scale used for analysis before plotting.

Measures of pollinator dependence, self-incompatibility, pollinator contribution to fecundity and pollen limitation

Indices of autofertility (IAF), self-incompatibility (ISI), pollinator contribution (PC) and pollen limitation (PL) were calculated to quantify self-compatibility, capacity for autonomous self-fertilization, the contribution of pollinators to reproductive success and whether pollen receipt limits reproductive success. All indices were based on adjusted mean seed set per flower from the GLMMs, as follows:

The index of autofertility (IAF) was calculated following Lloyd and Schoen (1992), as:

$$\mathrm{IAF} = \frac{\text{fecundity when pollinators are excluded}}{\text{fecundity following cross-pollination}}.$$

IAF values range from 0 to above 1, with 0 indicating complete dependence on pollinators to set seeds and 1 indicating zero dependence on pollinators to set seeds. Autofertility was interpreted according to Rodger and Ellis's (2016) cut-off criterion values, which considers IAF < 0.2 pollinator-dependent and IAF ≥ 0.2 autofertile.

The index of self-incompatibility (ISI) was calculated following Lloyd (1965), as:

$$\mathrm{ISI} = 1 - \frac{\text{fecundity following self-pollination}}{\text{fecundity following cross-pollination}}.$$

ISI ranges between 0 and 1, with 0 indicating self-compatibility and 1 indicating total self-incompatibility. ISI was interpreted according to Bawa's (1974) widely used cut-off criterion of species with ISI ≥ 0.8 considered self-incompatible, ISI ≤ 0.2 considered self-compatible, and species with ISI between 0.2 and 0.8 considered intermediate.

The pollinator contribution (PC) was calculated following Rodger et al. (2021), as:

$$\mathrm{PC} = 1 - \frac{\text{fecundity when pollinators are excluded}}{\text{fecundity under natural pollination}}.$$

PC values range between 0 and 1, with 0 indicating all fecundity attributable to autonomous self-pollination with no contribution by pollinators, and 1 indicating that all reproductive success under natural pollination is attributable to pollinators.

The pollen limitation (PL) index was calculated following Larson and Barrett (2000), as:

$$\mathrm{PL} = 1 - \frac{\text{fecundity under natural pollination}}{\text{fecundity of flowers subjected to pollen-supplementation}}.$$

PL ranges from 0 to 1, with 0 indicating absence of pollen limitation and 1 indicating pollen limitation.

Results

Breeding systems: self-compatibility and autofertility

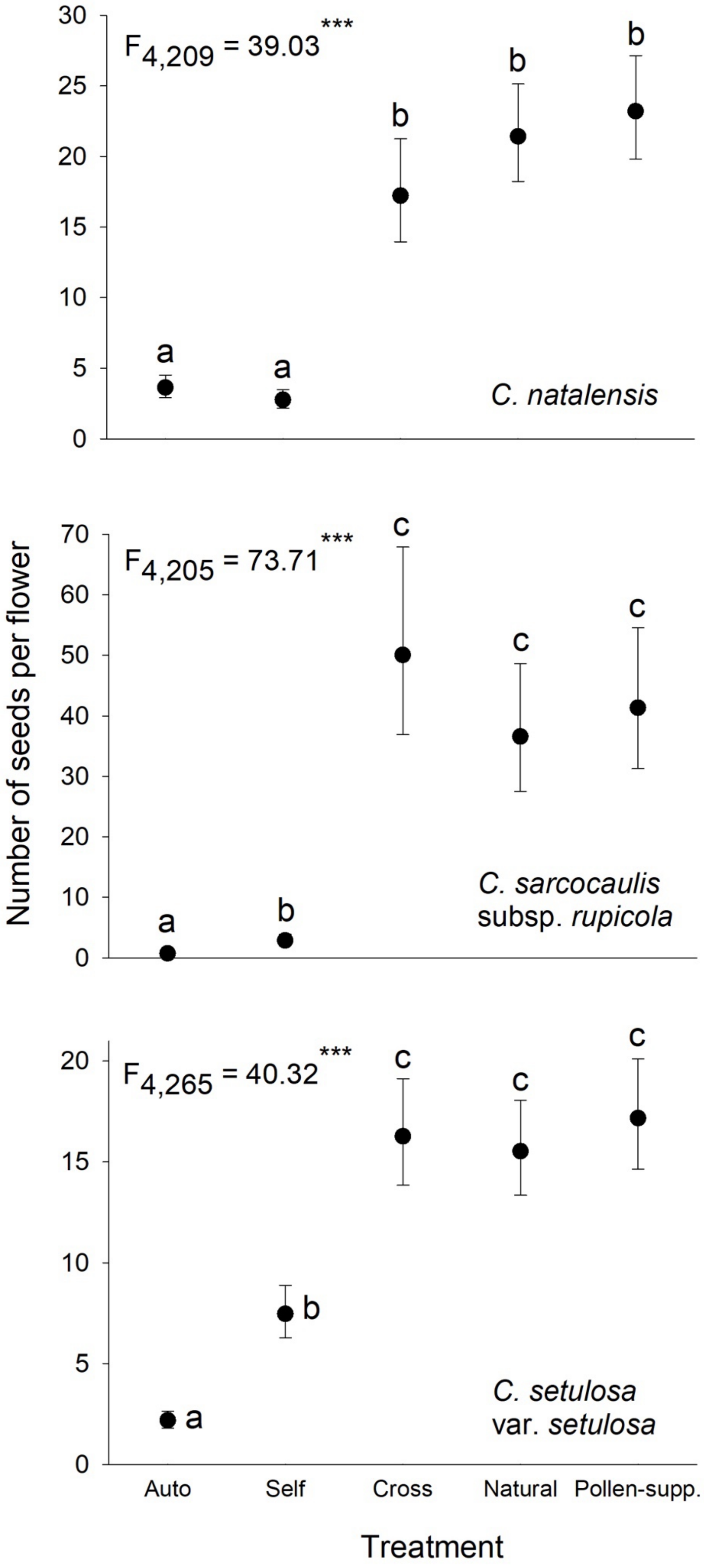
Across all three species, flowers in cross-pollination, natural-pollination and pollen-supplementation treatments set significantly more seeds than flowers in autonomous- and self-pollination treatments (Figure 3.2).

For *C. natalensis* and *C. sarcocaulis* subsp. *rupicola*, very few seeds were produced following autonomous- and hand self-pollination treatments (Figure 3.2). For both species, seed set in cross-, natural-pollination and pollen-supplementation treatments was similar and significantly higher (five-fold in *C. natalensis* and ten-fold in *C. sarcocaulis* subsp. *rupicola*) than seed set resulting from autonomous- and hand self-pollinated flowers (Figure 3.2). The associated indices indicate strong self-incompatibility in both species (ISI = 0.84 for *C. natalensis* and 0.94 for *C. sarcocaulis* subsp. *rupicola*) and low to no capacity for reproduction independent of pollinators (IAF = 0.21 for *C. natalensis* and 0.02 for *C. sarcocaulis* subsp. *rupicola*; Table 3.3).

The pattern for *C. setulosa* var. *setulosa* differed slightly from that found for *C. natalensis* and *C. sarcocaulis* subsp. *rupicola.* Although as for the other studied *Crassula* species, there was very low fecundity following the autonomous self-pollination treatment in *C. setulosa* var. *setulosa*, associated with very low capacity for reproduction independent of pollinators (IAF = 0.13), there was significantly higher seed set in hand self-pollinated flowers than in autonomously self-pollinated flowers as compared to both other species (Figure 3.2). Fecundity of hand self-pollinated flowers in *C. setulosa* var. *setulosa* was approximately half that of cross, natural and pollen-supplemented flowers (Figure 3.2). The observed higher fecundity of self-pollinated flowers is associated with intermediate self-compatibility (ISI = 0.54; Table 3.3).

Table 3.2: Samples size (number of plants; number of flowers) for five pollination treatments to determine breeding systems and assess pollen limitation in four high-elevation *Crassula* species in the northern Drakensberg, KwaZulu-Natal, South Africa.

	Pollinator-excluded			**Open-pollinated**	
***Crassula* species**	**Auto**	**Self-pollinated**	**Cross-pollinated**	**Natural**	**Pollen-supp**
C. natalensis	8; 29	9; 27	7; 27	14; 63	14; 68
C. obovata var. *obovata*	11; 34	-	-	11; 33	-
C. sarcocaulis subsp. *rupicola*	9; 33	9; 39	10; 33	12; 46	12; 59
C. setulosa var. *setulosa*	10; 44	10; 44	10; 51	10; 123	10; 58



30
25
20
15
10
5
0
F4,209 = 39.03***
a
a
b
b
b
C. natalensis
70
60
50
40
30
20
10
0
F4,205 = 73.71***
a
b
c
c
c
C. sarcocaulis subsp. rupicola
20
15
10
5
0
F4,265 = 40.32***
a
b
c
c
c
C. setulosa var. setulosa
Number of seeds per flower
Auto
Self
Cross
Natural
Pollen-supp.
Treatment

Figure 3.2: Number of developed seeds per flower following five experiments to determine autofertility, self-compatibility, pollen limitation and pollinator contribution to reproductive success in (a) *C. natalensis*, (b) *C. sarcocaulis* subsp. *rupicola*, and (c) *C. setulosa* var. *setulosa*. Treatments are abbreviated and refer to autonomous self-pollination (auto), hand self-pollination (self), hand cross-pollination (cross), natural, open pollination (natural) and pollen-supplementation (pollen-supp). Symbols indicate adjusted means ± SE which were back-transformed from the scale of analysis. Means that share a letter do not differ significantly. Reported statistics show results of generalized linear mixed models (GLMMs) to test the effect of pollination treatment, all overall $P < 0.001$, indicated by asterisks (Table 3.4).

Pollinator contribution to fecundity (PC) and pollen limitation (PL)

For flowers of all species, seed set was significantly higher under natural pollination conditions, compared to when pollinators were excluded from flowers (Table 3.2; Figure 3.3). The pollinator contribution (PC) index values for all studied species were high (Table 3.3), with a minimum of 80% of fecundity in all studied species attributable to pollinator-mediated pollen transfer.

Pollen-supplementation experiments showed no evidence of PL of reproductive success, i.e., manual addition of pollen did not significantly increase fecundity in any of the studied species (Table 3.3). Calculated indices of PL (< 0.2 for all species) confirmed that pollen receipt does not limit reproductive success (Table 3.3).

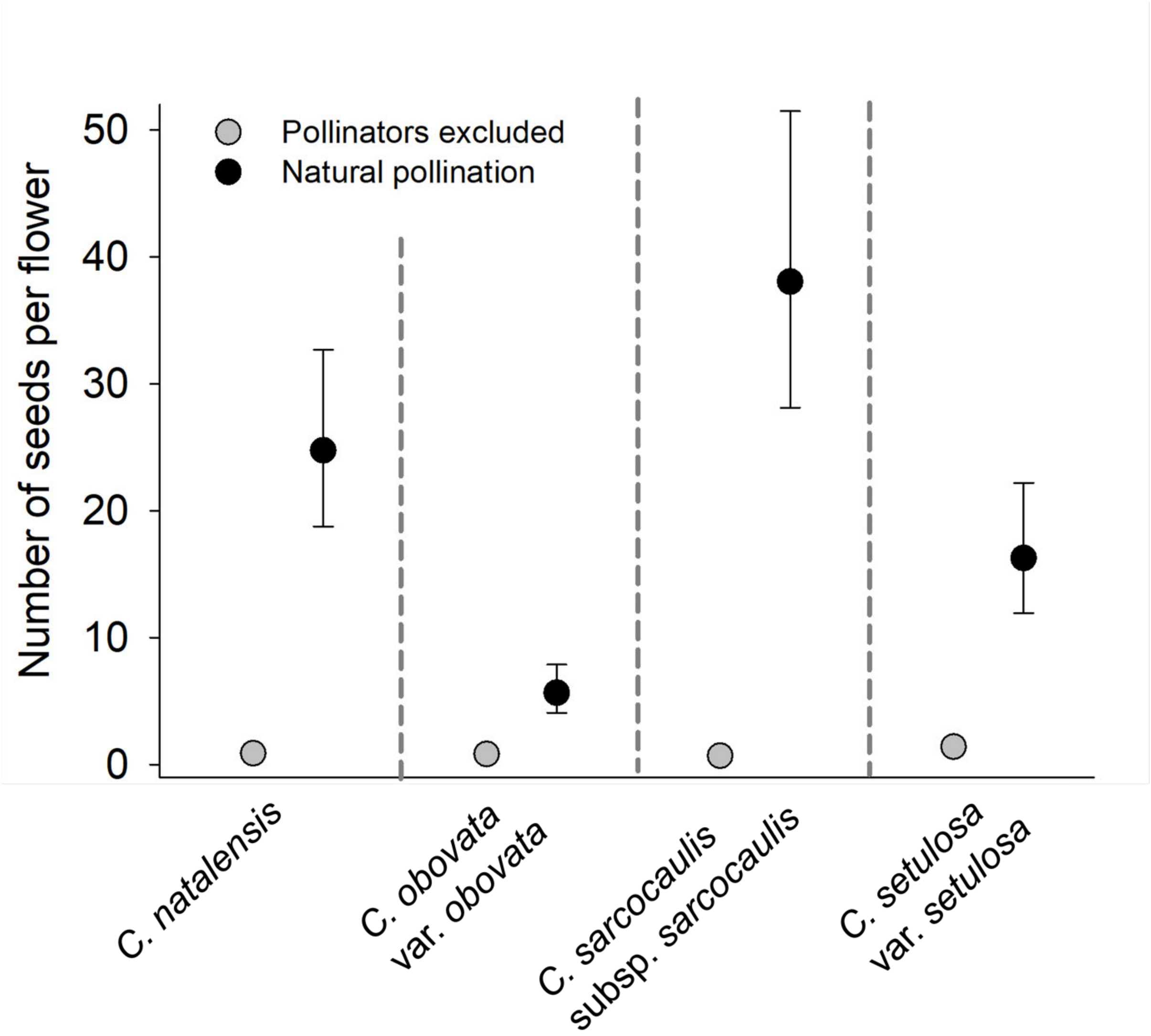


Figure 3.3: Number of developed seeds per flower of four *Crassula* species occurring in the northern Drakensberg in KwaZulu-Natal, South Africa, following treatments to determine pollinator contribution. There was significant difference between treatments ($P \leq 0.05$) within all species. Symbols show adjusted means ± SE which were back-transformed from the scale of analysis. All pairwise contrasts applied sequential Šidák correction for multiple contrasts. Sample sizes are reported in Table 3.2.

Table 3.3: Indices of autofertility (IAF), self-incompatibility (ISI), pollinator contribution (PC) and pollen limitation (PL) for four high-elevation *Crassula* species occurring in the northern Drakensberg in KwaZulu-Natal, South Africa. Indices were calculated from adjusted means.

Plant species	**IAF**	**ISI**	**PC**	**PL**
C. natalensis	0.21[a]	0.84[a]	0.83[a]	0.08
C. obovata var. *obovata*[b]	-	-	0.85[a]	-
C. sarcocaulis subsp. *rupicola*	0.02[a]	0.94[a]	0.98[a]	0.10
C. setulosa var. *setulosa*	0.13[a]	0.54[a]	0.86[a]	0.12

[a] Significant differences between treatments used to calculate the index.

[b] IAF, ISI and PL could not be calculated for *C. obovata* var. *obovata* because only autonomous- and natural-pollination treatments were performed on this species.

Table 3.4: Results of the generalized linear mixed models (GLMMs) testing the effects of pollination treatment to determine pollinator contribution to reproductive success for the four studied *Crassula* species occurring in the northern Drakensberg in KwaZulu-Natal, South Africa.

	Autonomous vs. natural pollination			
Source	**F**	**df1**	**df2**	***P***
Species	1.75	3	347	0.157
Treatment	359.42	1	347	< 0.001
Species × Treatment	8.31	3	347	<0.001

Discussion

In this study, we investigated the reproductive biology of four *Crassula* species from the summer-rainfall region in the Drakensberg Mountain Centre in South Africa. Results show that all studied species rely almost exclusively on pollinators to achieve reproductive success. Calculated indices of autofertility (IAF) ranged from 0.02 to a maximum of 0.21, indicating very low capacity to set seeds independent of pollinators. Results further indicate predominant reliance on receipt of outcross pollen: *C. natalensis* and *C. sarcocaulis* subsp. *rupicola* are self-incompatible, (ISI 0.84 and 0.94, respectively) whereas *C. setulosa* var. *setulosa* can be considered partly self-compatible (ISI = 0.54). Comparison of reproductive success of naturally pollinated flowers with that of flowers from which pollinators were excluded indicates that pollinator contribution to fecundity varies among species but for all species, at least 80% and up to almost 100% of seed set is attributable to vector-mediated pollination. Further, pollen supplementation experiments showed that reproductive success is not limited by pollen receipt under natural pollination conditions since manual addition of pollen did not significantly increase fecundity for any species. Although pollination services may vary between geographical areas and years, the absence of pollen limitation despite near-exclusive dependence on pollinators observed for all four studied *Crassula* species, all flowering at sites between 2000 and 2600 m.a.s.l. suggests these represent promising candidates to investigate pollination systems with capacity to assure high levels of pollinator-mediated reproductive success in a montane environment.

Despite the high diversity of *Crassula* (second-most speciose genus in the family Crassulaceae), particularly in southern Africa, nothing is known about the breeding systems of species in this genus. The uniform pollinator dependence and predominantly self-incompatible breeding systems of the *Crassula* species studied here (very weak self-compatibility in *C. natalensis* and *C. sarcocaulis* subsp. *rupicola* and intermediate self-compatibility *C. setulosa* var. *setulosa*) are consistent with pollinator dependence and self-incompatibility reported in *Kalanchoe* (González de León et al., 2016) and *Sedum* (Chapman, 1977; Wyatt and Stoneburner, 1981), although they contrast to the self-compatibility reported for representatives of several other genera of Crassulaceae, including pollinator-dependent *Echeveria gibbiflora* in Mexico (Parra et al., 1993), *Rhodiola* in China (Zhu and Lou, 2010) and partially autofertile species of *Kalanchoe* in Venezuela (Herrera and Nassar, 2009; Jaimes and Ramírez, 1999). Throughout angiosperms, self-compatibility is evolutionary labile (Igic et al., 2008), and indeed, greater variation in self-compatibility than found here for *Crassula* is suggested to occur even within other genera of Crassulaceae (e.g. *Sedum*; Chapman 1977; Denton 1979; Wyatt and Stoneburner 1981), with greater incidence of self-compatibility suggested to occur in taxa which lack capacity for asexual reproduction and rely more on sexual reproduction (Denton, 1979). However, several studies have found that for other Crassulaceae, estimates of self-compatibility based on fruit set rather than seed set or viability may not be reliable and even incorrectly infer self-compatibility for species that are self-incompatible (e.g. Rodríguez-Rojas et al., 2015; González de León et al., 2016, but see Wyatt 1983). Thus, while results indicating pollinator dependence are unambiguous for all the studied *Crassula* species, further research including analysis of mating systems and/or viability trials could be useful in *Crassula* species, such as *C. setulosa* var. *setulosa*, to confirm whether indices of self-compatibility are reliable indicators of the potential contribution of selfed progeny to reproductive success.

Although pollination services are expected to vary temporally and spatially, and this study quantified pollen limitation and pollinator activity in only a single season and at a single study area, the uniform absence of pollen limitation (PL) despite strong pollinator dependence observed for all species in this study is unusual, especially for species at high elevation (see García-Camacho and Totland 2009; Jiang and Xie 2020). Globally, pollinator dependence and self-incompatibility are more usually associated with higher levels of pollen limitation (Knight et al., 2005; Larson and Barrett, 2000). Autofertility is thought to provide a buffer in variable pollinator environments (Cruden and Lyon, 1989), such as those found at high elevations, where pollinator-dependent species are characterised by higher PL (Jiang and Xie 2020; Lázaro et al. 2015). Also, within the southern African diversity hotspots in particular, the levels of PL observed for the studied *Crassula* species are more typical for autofertile than pollinator-dependent taxa, whether SC or SI (see Rodger and Ellis 2016). Intriguingly, the combination of pollinator dependence and absence of pollen limitation found in this study for *Crassula* species appears not to be unusual among DMC species: although breeding system data for species from the summer rainfall region is scarce (Johnson and Steiner, 2003; Rodger and Ellis, 2016), the approximately 20 species for which breeding system data could be found are predominantly (>80%) pollinator dependent and commonly (c. 60%) show weak to no PL of reproductive success (Black et al., 2019; Kgaboesele et al., 2021; Rodger and Ellis, 2016; Springer, 2019). Additionally, the differences between the DMC and other regions globally may reflect relatively limited sampling, although studied taxa include representatives of several families including Crassulaceae (this study), Amaryllidaceae, Campanulaceae, Ericaceae, Iridaceae, Rubiaceae and Scrophulariaceae (Rodger and Ellis, 2016). Alternatively, high pollinator service and low PL may reflect that most of the DMC studies (but see Black et al. 2019) focus on species in the montane rather than alpine zone where harsher conditions are more likely to result in reduced pollinator service (Arroyo et al., 1985). However, many studies in the DMC document high pollination success (Brown et al., 2009; Johnson, 2006, 2005; Johnson and Hobbhahn, 2010). These studies support the idea that the climatic stability, absence of recent glaciation, and geological age of southern African systems including the Drakensberg Mountains (Mills et al., 2017), which are thought to have contributed to the high prevalence of specialized pollination here (Johnson and Steiner, 2003), may also underlie the apparently high pollinator service and dependence in the DMC. Community-wide studies (see Johnson et al. 2009), including assessments of breeding systems, pollinator service and pollen limitation, especially over multiple sites and years, would be valuable to determine whether the absence of pollen limitation despite dependence on pollinators found here for *Crassula* is common across the DMC flora, and whether the DMC is indeed characterised by breeding systems and pollinator service that are unusual among high elevation systems globally.

The absence of pollen limitation despite pollinator dependence observed in this study invites speculation on the potential pollination systems of these *Crassula* species. All the studied species appear to share similar floral traits (see Figure 3.1) and thus may have similar pollination systems, however, the only pollination studies in *Crassula* document specialized pollination systems involving moths and butterflies as pollinators of long-tubed flowers (Johnson et al., 1993; Johnson and Bond 1994), which are unlike the flowers of the species studied here. The small, white, strongly scented flowers of the studied species are consistent with biotic rather than wind pollination, but more suggestive of either generalised pollination by a variety of insects, or of pollination by short-tongued flies (Jimeno-Sevilla et al., 2014). Both of the latter pollination systems are frequent in pollinator-depauperate high-elevation floras (Arroyo et al., 1982; Lefebvre et al., 2018), and associated with lower incidence and strength of pollen limitation in montane environments (Johnson and Hobbhahn 2010; Lázaro et al., 2015; Jiang and Xie 2020). Generalist insects are common visitors to flowers with accessible pollen and/or nectar rewards (e.g. Brys et al., 2014; dos Santos et al., 2024), such as the bowl-shaped flowers of *C. setulosa* var. *setulosa* (Figure 3.1D). Pollination by short-tongued saprophilous flies, such as muscid and sarcophagid flies, has previously been recorded for other co-flowering, high-elevation Drakensberg mountain species including *Wurmbea elatior* (Colchicaceae; Johnson et al., 2020), *Eucomis bicolor* and *E. humilis* (Asparagaceae; Shuttleworth and Johnson 2010) and *Schizochilus angustifolius* (Orchidaceae; van der Niet et al., 2010), which all have strongly, often unpleasantly-scented, small pale to inconspicuously-coloured flowers. Studies to verify whether either hypothesis for generalized pollination or pollination by short-tongued, saprophilous flies is supported by observations would be useful to reveal the pollination systems that can provide such high pollination success in the DMC.

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Chapter 4:

Diverse saprophilous, short-tongued Diptera pollinate multiple plant species in the Drakensberg Mountain Centre: evidence for a new pollination guild in the southern African flora

Abstract

Pollination guilds include distantly related plant species that rely on a common pollinator. Here I investigate the assemblage of visitors and interaction patterns of nine high-elevation plant species in the Drakensberg Mountain Centre that are hypothesized, based on perceived similarities in floral traits, to represent a pollination guild. Across all studied plant species, a total of 859 floral visitors were caught. Based on morphospecies identifications the visitor assemblages were dominated (80.33% of the total visitor assemblage) by short-tongued flies (Diptera). In general, Muscidae, Tachinidae, Calliphoridae and Sarcophagidae dominated the visitor assemblages and were found visiting all studied plant species. The remaining visitors included Coleoptera, Hymenoptera, Hemiptera and Lepidoptera. Flies also carried large quantities of pollen from eight of the studied species, while beetles (and flies in almost equal quantities at one site) carried large quantities of pollen from *Crassula vaginata*, thereby indicating a role for flies and beetles as important pollinators of the respective studied plant species. Interaction network analyses at the visitor species- and family-level revealed near complete to complete spatial and temporal variation in visitor assemblages of selected guild members between sites and seasons at varying degrees, although these patterns were more pronounced at the species- than at the family-level. Partitioning of the local visitor community was evident at all three sampling sites at the insect species- and family-levels in varying degrees. Our findings suggest that the studied plant species, except *C. vaginata*, represent a pollination guild pollinated predominantly by saprophilous short-tongued flies. Spatial and temporal variation in visitation among sites and years and possible partitioning of the local visitor assemblage suggest not all species from the available fly assemblage interact with any plant species, which may be explained by fine scale heterogeneity in pollinator communities, although it can not be ruled out that the sampling strategy could have potentially resulted in insufficient quantification of visitor diversity, thus compromising the conclusions drawn regarding spatio-temporal turnover. The taxonomic diversity of saprophilous fly pollinators documented here suggests that an expansion of the pollination guild concept to include dependence on multiple, functionally similar pollinator species should be considered.

Keywords

High-elevation, Diptera, pollination, functional specialization, interaction turnover, local pollinator partitioning.

Introduction

An ecological guild is a group of species that similarly utilizes the same environmental resources (Root, 1967). Guilds are mostly considered in the context of sharing of resources such as food (Simberloff and Dayan, 1991), habitat (Gorman, 1988), microhabitat (Sedgwick and Knopf, 1987) and nesting (Whittam and Siegel-Causey, 1981), as well as other factors including similar mechanisms of reproduction (Berkman and Rabeni, 1987), but can also be applied to mutualistic interactions such as those between plants and pollinators (Manning and Goldblatt, 1997).

Distantly related plant species with similar floral traits such as colour, shape, size, scent, and rewards that exploit the same functional pollinator group (Fenster et al., 2004), belong to a pollination guild. Plants within a guild often reflect an ecologically specialized type of interaction between plants and one pollinator species or a small subset of functionally similar pollinators (Armbruster, 2017; Devictor et al., 2010; Johnson, 2010; Johnson and Steiner, 2000). This specialized interaction within a guild is achieved by 'filters' or 'private channels' that mediate reward access for specific pollinators, but exclude others (Armbruster, 2017; Nattero et al., 2010; Robert A Raguso, 2008; Stang et al., 2007). These 'filters' can include morphological adaptations, such as long floral tubes, that allows certain pollinators access to a reward but also restrict others. Filters may also include attraction by private communication channels such as chemical cues, which only specific pollinators can detect or are attracted to (Schiestl and Johnson, 2013; Shuttleworth and Johnson, 2010). On the other end of the spectrum, plant species with small and open flowers are seldom considered part of a specialized guild, as the accessibility of their rewards allows exploitation by a wide variety of pollinators, likely rendering them ecological generalists (Waser et al., 1996).

Potential plant-pollinator interactions depend on the geographical distribution of both interaction partners, and as such, spatial variation in these interactions can be expected to occur. However, this is seldom seen in specialized plant-pollinator interactions, which are usually characterized by highly consistent and spatially predictable plant-pollinator interactions (Carstensen et al., 2014; Johnson, 2010; Minckley et al., 1999; Poisot et al., 2013). This implies that the same pollinator species can be expected to consistently pollinate a plant across its distribution. One of the underlying reasons for the high levels of predictability may be that functional floral traits that are involved in specialized plant-pollinator interactions, usually evolved in response to natural selection imposed by a specific pollinator that can uniquely exploit floral rewards (Johnson, 2010). Typical examples of such systems in which species with particular functional traits are pollinated by the same pollinator species throughout their range include oil-producing plants with oil-collecting bees of the genera *Rediviva* (Melittidae) in South Africa (Steiner and Whitehead, 1988; Whitehead et al., 2008) and *Centris* and *Chalepogenus* (Apidae) in South America (Murúa and Espíndola, 2015), long-tubed plant species that rely on long-tongued pollinators such as flies of the family Nemestrinidae in South Africa (Goldblatt and Manning, 2000; Manning and Goldblatt, 1997), or *Agrius* (Sphingidae) hawkmoths (Johnson and Raguso, 2016), as well as the interactions of plants and hummingbirds of the family Trochilidae specifically in the Neotropical forests (Maglianesi et al., 2014; Stiles, 1981). On the other end of the spectrum, generalized interactions involving plants and a wide variety of taxonomically diverse pollinators (Herrera, 1996; Olesen, 2000; Waser et al., 1996) are highly flexible and spatially unpredictable. This may be because the traits that are required for pollinators to exploit floral resources are not particularly specialized and there is therefore ample flexibility in terms of the pollinator species that can successfully exploit a particular plant species (Gómez et al., 2008; Moeller, 2005; Ollerton et al., 2007; Price et al., 2005). Indeed, spatial variation (within the same year) in generalized interactions has previously been reported, including in the studies of the pollinator-generalist *Lavandula latifolia* (Labiatae) in Spain, with variable composition and abundance of pollinators between populations of the species (Herrera, 1988), varying abundances of pollinators between populations of *Phacelia linearis* (Hydrophyllaceae) in Utah, North America (Eckhart, 1992) and variations in pollinators of *Disa fragrans* subsp. *fragrans* (Orchidaceae) at multiple sites in the Drakensberg Mountains and KwaZulu-Natal Midlands, South Africa (Johnson and Hobbhahn, 2010).

In addition to spatial variation, plant-pollinator interactions are also characterized by temporal variation (Alarcón et al., 2008; Burkle and Alarcón, 2011; Olesen et al., 2011, 2008; Petanidou et al., 2008), mainly as a result of temporal variation in plant species and pollinator composition (Carstensen et al., 2014; Dupont et al., 2009). Temporal variation in floral visitor assemblages is very common in flexible and unpredictable interactions, i.e. in pollination systems characterized by generalized interaction patterns (Alarcón et al., 2008; CaraDonna et al., 2017). Studies evaluating temporal variation in floral visitors have indeed highlighted the dynamic nature of interactions at different timescales, ranging from intra-annually (daily, weekly and monthly; see CaraDonna et al., 2017; Fründ et al., 2011; Nagano, 2023; Olesen et al., 2008; Plein et al., 2013) to inter-annually (multiple years; Alarcón et al., 2008; Dupont et al., 2009; Price et al., 2005). With regards to the latter timescale, Alarcón et al. (2008) found temporal turnover in generalized pollination systems annually, with interactions involving more than half of the pollinator species being unique in each season over a 3-year period. Several studies have also reported similar temporal variations over multiple years (Dupont et al., 2009; Lázaro et al., 2010; Petanidou et al., 2008; Price et al., 2005), further echoing the dynamic temporal nature of plant-pollinator interactions.

Southern Africa is characterized by a high diversity of plant species, especially in the Greater Cape Floristic Region (GCFR; Johnson, 2010; Manning and Goldblatt, 2012) and the Drakensberg Mountain Centre (DMC; Carbutt, 2019) biodiversity hotspots. Diverse specialized pollination systems including several involving long-tongued nemestrinid and tabanid flies (Goldblatt and Manning, 2000; Manning and Goldblatt, 1997), oil-collecting bees (Steiner and Whitehead, 1988) and spider-hunting wasps

(Ollerton et al., 2003; Shuttleworth and Johnson, 2006), amongst others, have been recognized in these regions (see Johnson, 2010). Furthermore, a great diversity of fauna, including insects, birds and rodents, play important roles as pollinators in various systems in southern Africa (Johnson, 2010). As such, the presence of a diverse array of functional pollinator groups in the southern African region implies that the specialized interactions are not due to lack of potential pollinators in these environments (i.e. imposed specialization; *sensu* Armbruster, 2017), in contrast to pollinator-depauperate environments, which markedly include several high-elevation regions across the world (Jiang and Xie, 2020; Lefebvre et al., 2018; McCabe and Cobb, 2021). Despite availability of information on plant-pollinator interactions on a subset of plant species, the vast majority of plant species in the high-elevation regions of southern Africa, particularly the summer-rainfall region, remain to be studied in terms of pollinator identity and spatial-temporal dynamics.

Previous studies in high-elevation regions of South Africa suggest the possible existence of a pollination guild that relies on diverse saprophilous short-tongued flies. A previous study by Johnson et al. (2020) in the DMC reported the importance of diverse saprophilous short-tongued flies, including Muscidae, Sarcophagidae and Calliphoridae, as pollinators of *Wurmbea elatior* (Colchicaceae), which has white flowers and produces an unpleasant odour to the human nose. A study on *Crassula peploides* (Crassulaceae; see Chapter 2), which exhibits similar floral traits to *W. elatior* in that the floral petals have white-coloured tips and produce an unpleasant odour, showed that this species is also visited almost exclusively by a diverse assemblage of saprophilous short-tongued flies of the families Muscidae, Tachinidae and Sarcophagidae. Other co-flowering species in the DMC with similar floral characteristics, and that are visited by similar groups of short-tongued flies, include *Schizochilus angustifolius* (Orchidaceae; van der Niet et al., 2010), *Erica caffrorum* (Ericaceae; Thupsie, 2023), and *Eucomis bicolor* and *E. humilis* (Asparagaceae; Shuttleworth and Johnson, 2010). The similarity in floral characteristics and observed visitation by short-tongued flies of these aforementioned species suggest the existence of a hitherto undescribed pollination guild. However, evidence for this guild has not yet been systematically assessed, for instance, by identification of additional guild members based on perceived similarity in floral traits, and investigation of their pollination systems to determine if they share pollinators with other putative guild members.

The aim of this study was to evaluate evidence for a hypothesized short-tongued fly pollination guild, by surveying and identifying floral visitors and investigating their potential as pollinators. Nine plant species from three plant families were selected for this study. This was based on preliminary field observations that revealed frequent flower visitation by Diptera, as well as superficial similarity in floral traits with other species from the area known to be pollinated by saprophilous flies: all studied plant species, except the yellow-flowered *C. vaginata*, produce a putrid smell to the human nose and all have relatively small flowers which are white or greenish-white in colour, or pale with dark or red markings.

To evaluate the hypothesis that the study species represent a guild pollinated by short tongued saprophilous flies, I address the following questions:

1. What are the floral visitors of putative guild members and are these common across guild members?
2. Amongst the visitors, which are potentially important pollinators, as indicated by the presence of pollen from plant species on which visitors were caught on?
3. Is there spatial variation in the visitor assemblages among different study sites?
4. Is there temporal variation among the visitor assemblages between different sampling seasons?
5. Is there partitioning of the local visitor fauna among co-flowering plant species?

I then use the evidence provided to evaluate whether the studied plant species share a pollination system that is temporally and spatially consistent, and represents a pollination guild.

Materials and Methods

Study species

We studied nine plant species (Figure 4.1, Table S4.1) representing Crassulaceae, Apiaceae and Santalaceae, subsets of which co-flower during the months of December to March in high-elevation regions, mostly within the Drakensberg Mountains of South Africa. The studied Crassulaceae included seven *Crassula* L. species: *C. dependens* Bolus, *C. natalensis* Schonl., *C. obovata* Haw. var. *obovata*, *C. peploides* Harv., *C. sarcocaulis* Eckl. & Zeyh. subsp. *rupicola* Toelken, *C. setulosa* Harv. var. *setulosa*, and *C. vaginata* Eckl. & Zeyh.. We also studied *Polemannia montana* Schltr. & H.Wolff (Apiaceae) and *Thesium imbricatum* Thunb. (Santalaceae).

Crassula species are succulent, mostly herbaceous (apart from *C. sarcocaulis* subsp. *rupicola* that is woody and *C. dependens* that is woody at the base) perennials that are characterised by a short flowering season in summer (Tölken, 1977). *Crassula* species typically grow on moist, rocky surfaces, in-between rock crevices and in open grasslands. *Polemannia montana* is a woody small tree characterised by a slender, peeling bark surface (Kotina et al., 2012; Pooley, 2003). This species usually grows on rocky slopes associated with streams amongst large boulders (Pooley, 2003). *Thesium imbricatum* belongs to a large genus consisting mostly of perennial shrubs (Moore et al., 2010). The species grows on rocky surfaces and ridges (Pooley, 2003).

Study sites

The study was conducted at six high-elevation sites (mostly between 1490 and 3000 m.a.s.l.) across the provinces of KwaZulu-Natal and Eastern Cape in South Africa (Table S1). Five sites were located in KwaZulu-Natal. Two sites were in the KwaZulu-Natal Midlands (Karkloof Mountains and Mount (hereafter "Mt. Gilboa"), where *C. vaginata* was studied. Two other sites, Sentinel and Witsieshoek, were located in the Northern Drakensberg region within the Royal Natal National Park where large populations of all studied species were found. The fifth site was at Tarn Cave in the Bushman's Nek region, located in the Southern Drakensberg, where *C. sarcocaulis* subsp. *rupicola* was studied. In the Eastern Cape, the study site was on Naude's Nek mountain pass (hereafter Naude's Nek), where a total of five *Crassula* species and *P. montana* were studied.

All fieldwork was conducted in the 2021-2022 and the 2022-2023 flowering seasons. Voucher specimens for all studied plants were collected to confirm their identifications against herbarium collections at the start of the study and were subsequently deposited in the Bews Herbarium (NU, Pietermaritzburg campus) at the University of KwaZulu-Natal.

Floral visitor and pollinator sampling and identification

Floral visitor sampling was conducted at all sites over the two flowering seasons for a period of several hours over a few (typically two days) consecutive days. All fieldwork was conducted during warm, sunny days under conditions that were favourable for insect activity. The behaviour of visitors on flowers was observed. Visitors were considered to be potential pollinators if they made contact with the reproductive parts of the flowers, and based on presence of pollen on their bodies. Insect visitors seen visiting inflorescences were collected haphazardly using a hand net and immediately transferred to Eppendorf tubes individually. This non-standardized method of capturing visitors while patrolling, instead of recording visitation as insects visit individual flowers, was appropriate because 1) the flowers received relatively few floral visits at a time, and 2) most visitors cannot be identified to species-level during observations and therefore needed to be caught and killed prior to identifcation. All subsequent analyses are therefore based on caught visitors only. Visitors were killed in the laboratory by freezing and subsequently stored at -20 °C until further processing and identification. To determine the presence of nocturnal flower visitors, evening observations and sampling were done for *C. sarcocaulis* subsp. *rupicola*, *C. dependens* and *C. setulosa* var. *setulosa* at Sentinel and Naude’s Nek, *C. sarcocaulis* subsp. *rupicola* at Tarn Cave, *C. peploides* at Witsieshoek and Naude’s Nek, and *C. dependens* and *C. vaginata* at Witsieshoek. Sites for nocturnal sampling were limited by safety issues and accessibility under reduced visibility conditions. All collected insect specimens were pinned and stored in the Pollination Ecology laboratory at the University of KwaZulu-Natal’s Pietermaritzburg campus.

All collected floral visitors were identified to family level using a morphological identification guide (Marshall et al., 2017) and assigned morphospecies identity by Nkitseng Oageng Modise and Kurt Jordaens. Some morphological identifications were further confirmed by various fly experts.

Potential pollinators and pollen load analyses

Pollen loads collected from floral visitors of eight of the nine studied plant species were used as a proxy to determine whether visitors are effective pollinators. Pollen loads were assessed from a subset of floral visitors representing all morphospecies. Pollen loads were assessed from visitors caught in the 2021-2022 sampling season by dabbing the entire bodies of insect specimens with 1 mm^3 cubes of Fuchsin gel (Beattie, 1971; Kearns and Inouye, 1993). As some pollen attached to insects may have been lost during storage inside the Eppendorf tubes, the inside surface of each tube was also dabbed with a cube of Fuschin gel. Both Fuchsin cubes used to dab the individual insects and the Eppendorf tubes were subsequently melted on microscopic slides for pollen counting. Pollen loads were counted from all mounted slides under 10× or 20× magnifications using a Zeiss compound microscope. Pollen loads were distinguished into conspecific pollen, which referred to pollen grains that originated from the plant species on which visitors were caught and heterospecific pollen, which referred to any other pollen grain that did not originate from the plant species on which visitors were caught. To determine whether the pollen assessed belonged to the plant species on which visitors were caught, conspecific pollen was identified based on a pollen reference that was collected from the anthers of flowers of study plant species. Pollen grains from all *Crassula* species were visually identical to each other and therefore could not be distinguished between co-flowering species. At most, between one to three *Crassula* species were co-flowering at close proximity to each other and were all part of this study. Overall, pollen loads were used to determine where visitors carry pollen similar to that from the plants they were caught on, indicating their role as potential pollinators.

Visitor and pollinator interaction networks

To visualize interaction patterns between all studied plant species and insect visitors at all studied sites, I constructed bipartite interaction networks. To evaluate spatial and temporal variation in the interaction dynamics among the different plant species, I contructed bipartite interaction networks at the species-level. However, because of the sampling approach used in this study and the use of morphological identification technique, drawing conclusions from species-level analyses may be problematic. This is because there may be possible taxonomic oversplitting at the species-level. To partially control for this problem, I contructed the same bipartite interaction networks at the family-level. The disadvantages with analyses at the family-level over the species-level are that specific visitor details are lost and hyperspecialization on certain species that is otherwise visible at the species-level is not clear. As such, I argue that analysing at both the species- and family-level is optimal to investigate spatial and temporal variation in this study. The plant species and their respective study sites that were included in the evaluation of spatial variation were selected on the basis that the plant species were studied at at least two sampling sites, and insect visitors sampled at these sites across all sampling seasons. Furthermore, the difference in the total number of caught individuals for both sampling seasons per site was 20 or less. For temporal analyses, only plant species that were sampled at the same site for two consecutive seasons and had at least 15 individuals caught per season were selected. Additionally, the difference in the total number of caught individuals per sampling season was 20 or less, except for *C. peploides* at Naude’s Nek, where the difference was 24. The links between the interactions in the bipartite network represent the number of visitors per morphospecies or family caught visiting study flowers (Dormann et al., 2009).

Modularity analyses were performed to test for partitioning the local insect visitor assemblage within each year and site for the studied plant species. This was deemed appropriate because sampling was done within the same timeframe across sites and years and therefore better standardized within commuinities than it was among communities in different years or at different sites. Modules indicate the grouping of interacting species, with species within a module being more highly connected to each other than to those between modules (Olesen et al., 2007). To determine the significance of the modularity for each of the interaction networks indicating partitioning of the local insect visitor assemblages, I compared the weighted modularity (Qw) values of the networks with those generated by a null model (Dormann and Strauss, 2014). For each network, I generated 1000 random matrices of the same size using the 'r2d' method in R statistical software (R Core Team, 2022). Interaction networks were used for visual assessment of overlap in visitors, and are therefore used outside the realm for which they were developed (quantifying interactions within a community).

Results

Floral visitors and potential pollinators

A total of 859 individual insects were caught during the 2021-2022 and 2022-2023 sampling seasons (see Figures 4.1 and 4.2 for representatives, Table S4.2). The majority (80.21%) of all 859 visitors captured were Diptera. Muscidae (330 individuals; 47.90%) dominated, followed by Tachinidae (99 individuals; 14.38%), Calliphoridae (78 individuals; 11.32%) and Sarcophagidae (67 individuals; 9.72%, Figure 4.3, Table S4.2). Visitors in the family Muscidae exhibited the greatest within family-diversity, including a total of 44 morphospecies (see Figure 4.1 for some representatives and Figure S4.1). In addition, the family Tachinidae exhibited the second highest within family-diversity, with 17 morphospecies (see Figure S4.1). Among the non-Diptera insect visitors, individuals belonging to Coleoptera (94 individuals; 10.94%) were the most common, comprising of Scarabaeidae species and 16 other beetle morphospecies which could not be further identified to the family level (see Figure 4.2 for some representatives). Other non-Diptera visitors comprised mostly of moths and skipper butterflies (Lepidoptera; 46 individuals, 5.36%). which included nocturnal moths that were caught mostly visiting *C. sarcocaulis* subsp. *rupicola* (29 individuals; 3.38%; Table S4.2) during the evening, individuals belonging to Hymenoptera (23 individuals; 2.68%) and Hemiptera (seven individuals; 0.81%; Table S4.2).

A total of 132 321 pollen grains (100 271 (mean per individual: 256.05; range: 0 – 3247) conspecific and 31 950 (mean per individual: 82.51; range: 0 – 3015) heterospecific pollen grains) were counted on 392 caught individuals across eight plant species from five sites (Table S4.2). The majority of conspecific pollen from the studied plant species were carried by short-tongued flies (Diptera), which represent potential pollinators of all plant species except for *C. vaginata*. These short-tongued fly pollinators mirrored the most dominant visitors of these plant species. For *C. vaginata*, various beetle species (Coleoptera) carried the majority of conspecific pollen. Overall, all visitors to all plant species also carried varying amounts of heterospecific pollen loads from various plant species at the study sites (Table S4.2), as confirmed by contrasting morphological structures to pollen references.

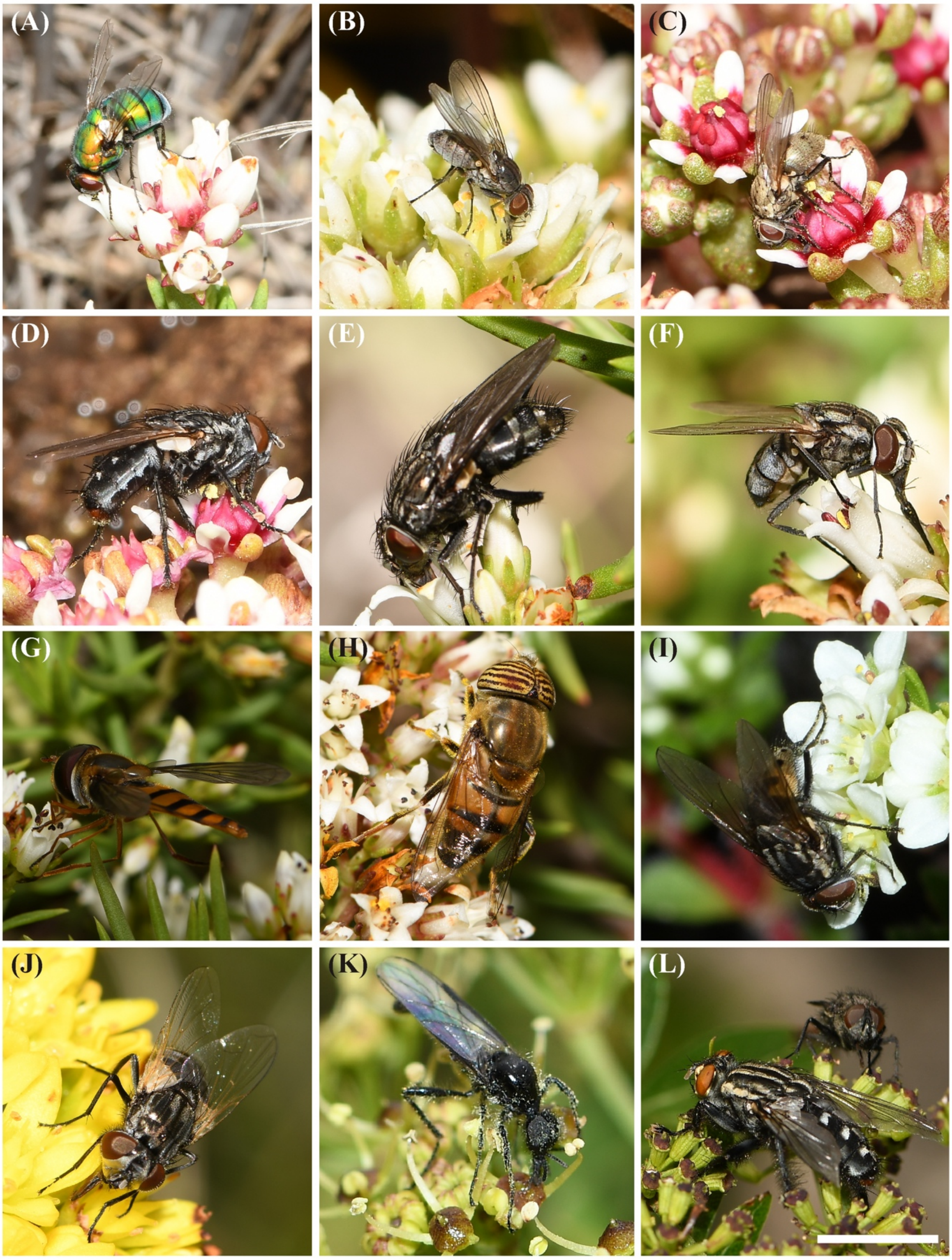

Figure 4.1: Examples of the diverse fly species (Diptera) observed and captured visiting studied plant species *in-situ*. All flies (with the except of *Sarcophaga* sp. in panel L) were captured contacting nectaries in search of a nectar reward, and simultaneously making contact with plant reproductive structures. (A) *Neomyia* sp. (Muscidae) on *C. dependens*; (B) muscid species (Muscidae) on *C. natalensis*; (C) *Coenosia* sp. (Muscidae) on *C. peploides*; (D) *Sarcophaga* sp. (Sarcophagidae) on *C.*

peploides; (E) tachinid species (Tachinidae) on *C. sarcocaulis* subsp. *rupicola*; (F) *Stomorhina* sp. (Calliphoridae) on *C. sarcocaulis* subsp. *rupicola* robbing the flowers of nectar; (G) *Asarkina* sp. (Syrphidae) on *C. sarcocaulis* subsp. *rupicola*; (H) *Eristalinus* sp. (Syrphidae) on *C. sarcocaulis* subsp. *rupicola*; (I) muscid species (Muscidae) on *C. setulosa* var. *setulosa*; (J) muscid species (Muscidae) on *C. vaginata*; (K) bibionid species (Bibionidae) on *P. montana* and (L) *Sarcophaga* sp. (Sarcophagidae) on *P. montana*. Scale bar: 4.1 mm. Photo credit: all photographs taken by Timo van der Niet.

Figure 4.2: Non-Diptera insects observed and caught *in situ* visiting the studied plant species, (A) beetles (Coleoptera: Merylidae) and (B) *Atrichelaphinis tigrina* (Coleoptera: Scarabaeidae) visiting *C. vaginata* at the Mt. Gilboa site, (C), (E) and (F) various moth species and (D) *Gegenes* sp. (Lepidoptera: Hesperiidae) that were observed on *C. sarcocaulis* subsp. *rupicola* during nocturnal observations. Scale bar: 9 mm. Photo credits: All photographs taken by Timo van der Niet.

Plant-visitor interaction network

A bipartite interaction network based on studied species at six sites with their interacting insect species and families revealed that various species of Muscidae dominated the overall visitor assemblage on all plant species at Naude's Nek and Sentinel (with the exception of *C. vaginata*), while Tachinidae dominated on the visitor assemblage on *C. peploides* at Witsieshoek (Figure 4.3, Table S4.2). At the species-level specifically, the genera *Helina* and *Coenosia* (Muscidae) were common across all studied plant species (Figure 4.3, Table S4.2). A similar pattern was also evident for the genus *Sarcophaga* (Sarcophagidae), with its representatives found visiting all plant species except *C. vaginata* at Witsieshoek (Figure 4.3, Table S4.2). Among the non-Diptera visitors, *Atrichelaphinis tigrina* (Coleoptera: Scarabaeidae) and beetle species belonging to Melyridae (Coleoptera) dominated the visitor assemblage of *C. vaginata* across sites in which this species was studied (Figure 4.3).

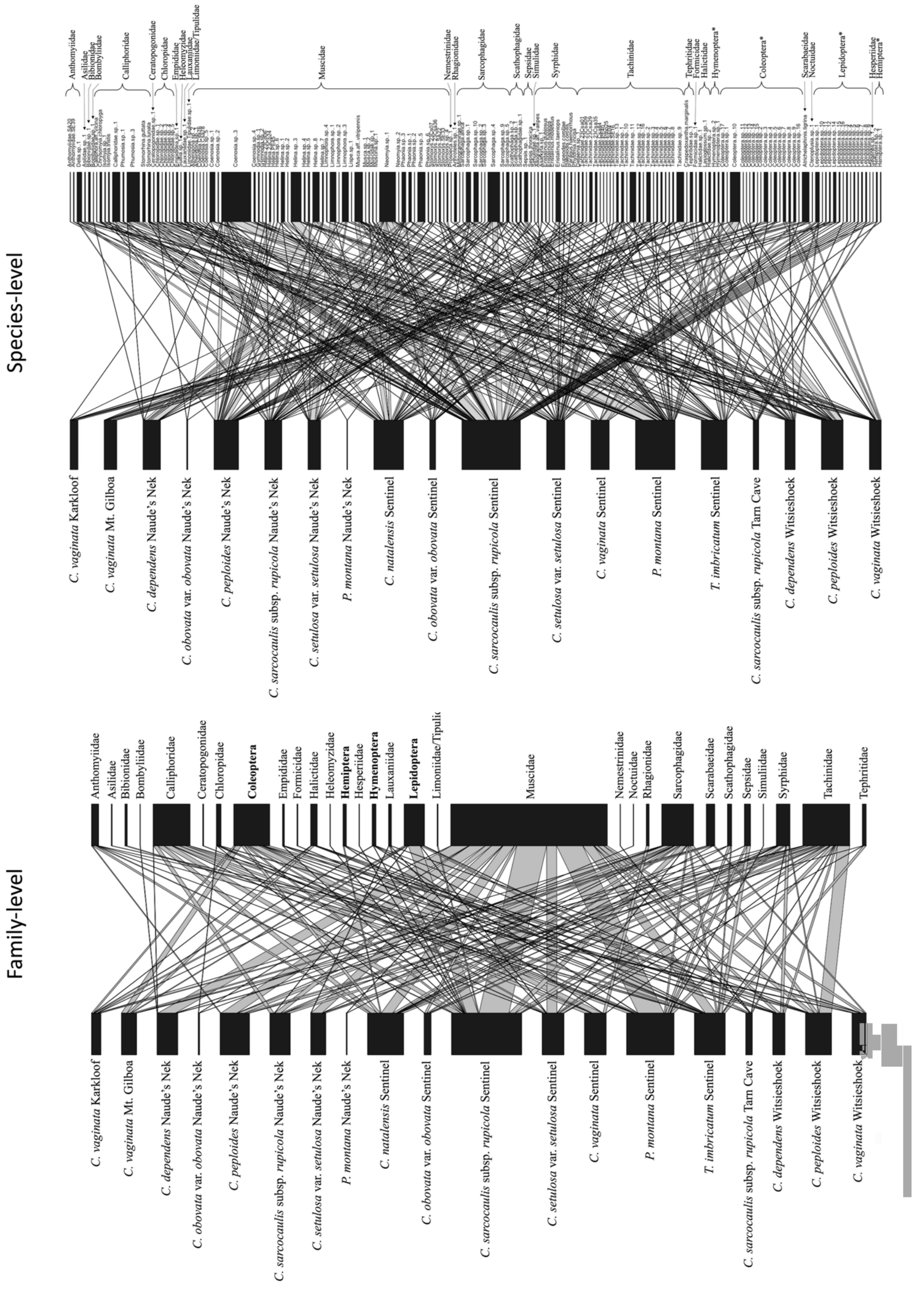

Figure 4.3: Bipartite interaction network of all studied plant species with insect visitor families at six study sites over two sampling seasons. The network analysis is based on a total of 859 individuals from several insect families caught visiting studied plant species. The width of the lines reflects the number

of individual visitors per plant species per family, with the narrowest line representing one individual and the thickest line representing 63 individuals. Bolded labels refer to order-level of non-Diptera individuals that could not be assigned to families.

Spatial variation among the visitor assemblages at different study sites

Spatial variation among visitor assemblages of three plant species that were studied at two sites in the same year each was evident at both the species- and family-level from bipartite interaction networks, albeit at varying degrees (Figure 4.4). At the species-level, *Crassula dependens* and *C. peploides* populations exhibited substantial spatial variation in visitor assemblages between sites, with three out of 25 insect species shared between *C. dependens* populations and nine out of 42 insect species shared between *C. peploides* populations. *Crassula vaginata* populations that were studied at Witsieshoek and Sentinel sites exhibited extreme spatial variation in insect visitor assemblages at the species-level, with no overlap in visitor assemblages at the two sites (Figure 4.4), despite both populations being commonly visited by beetles.

At the family-level, none of the species exhibited complete spatial variation and there was some overlap in families that visited at the two studied sites per species (Figure 4.4). Particularly for *C. peploides* and *C. vaginata*, families that comprise the majority of individuals are shared between sites, whereas those with a marginal number of individuals are unique to each species. In *C. dependens*, a similar pattern is also generally exhibited, although Calliphoridae, which comprised relatively few individuals, was also shared between sites (Figure 4.4).

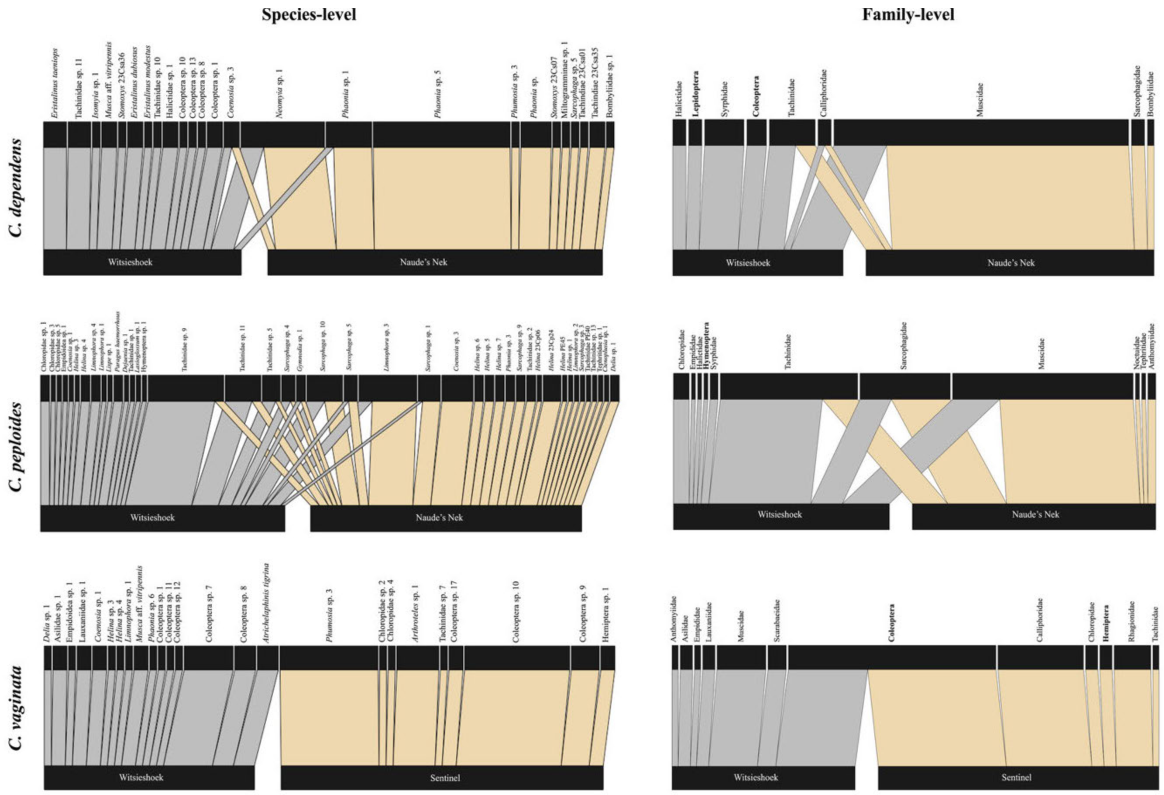

Figure 4.4: Bipartite interaction networks for selected plant species investigating spatial variation at the species- and family-levels between selected study sites. Interaction networks are based on caught individuals per insect family during the two sampling seasons. The studied species were selected on the basis that they were studied at two study sites, with both study sites comprising similar sampling effort, reflected by the total caught individuals in each season. The width of the lines is proportional to the number of individuals per morphospecies. Bolded labels refer to order-level of non-dipteran individuals that could not be assigned to families.

Temporal variation among the visitor assemblages between different sampling seasons

Temporal variation between two sampling seasons was evident in each of the six selected plant species for which data for two seasons from the same site was available at varying degrees (Figure 4.5), although none of the species exhibited complete spatial turnover at both the species- and family-levels. At the species-level, species that are frequent visitors were shared between sampling seasons in most species, except for *C. peploides* at Naude's Nek and *C. sarcocaulis* subsp. *rupicola* at Sentinel (Figure 4.5). At the family-level, the observed temporal turnover at the species-level diminishes considerably when analysed and is less pronounced at the family-level (Figure 4.5). In addition, for all plant species, the families comprising the most frequent visiting species were common between sampling seasons, except for *C. obovata* var. *obovata* at Naude's Nek, in which some families that comprised frequent visiting species were not common between the two seasons (Figure 4.5).

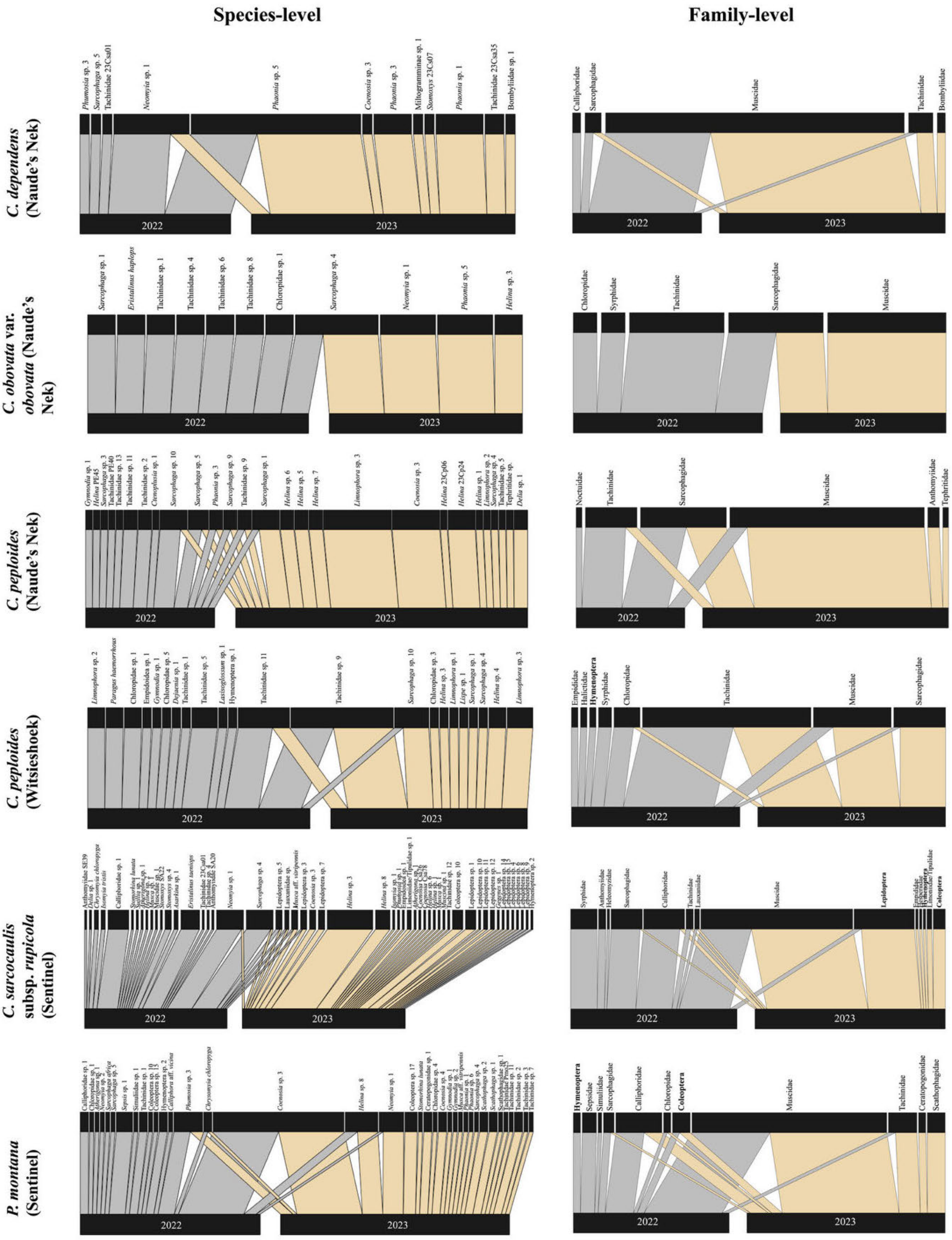

Figure 4.5: Bipartite interaction networks for selected plant species investigating temporal variation between the 2021-2022 and 2022-2023 sampling seasons at the insect- and family-levels. Interaction networks are based on caught individuals in each morphospecies per family, per sampling season. The studied species were selected on the basis that sampling effort was similar for both seasons, reflected by the total caught individuals in each season. The width of the lines is proportional to the number of

individuals per morphospecies. Bolded labels refer to order-level of non-dipteran individuals that could not be assigned to families.

Partitioning of the local visitor assemblages among co-flowering plant species

The pattern of partitioning of the local insect visitor assemblage was evident at the species-level (Figure 6 - 8). This partitioning of the local visitor assemblage was particularly extreme at the Witsieshoek and Sentinel sites in both sampling seasons and at Naude's Nek in the 2022 sampling season, at which each plant species occupied its own module, even though some species (such as *C. dependens* and *C. peploides* at Witsieshoek) flower side by side.

Extreme partitioning of the available local insect visitor community was also evident following modularity analyses at the family-level at Witsieshoek during both sampling seasons (Figure 4.6) and at Naude's Nek in the 2022 sampling season (Figure 4.8). The Sentinel site in both sampling seasons (Figure 4.7) and Naude's Nek site in the 2023 sampling season (Figure 4.8) exhibited substantial partitioning of the available local insect visitor community, although some plant species occupied the same module. Overall, at the species-level, all plant species occupy an individual module, whereas at the family-level, some plant species share a module. All modularity analyses at the species- and family-levels were statistically significant as determined by comparison with generated null models ($P < 0.05$, z-score > 2).

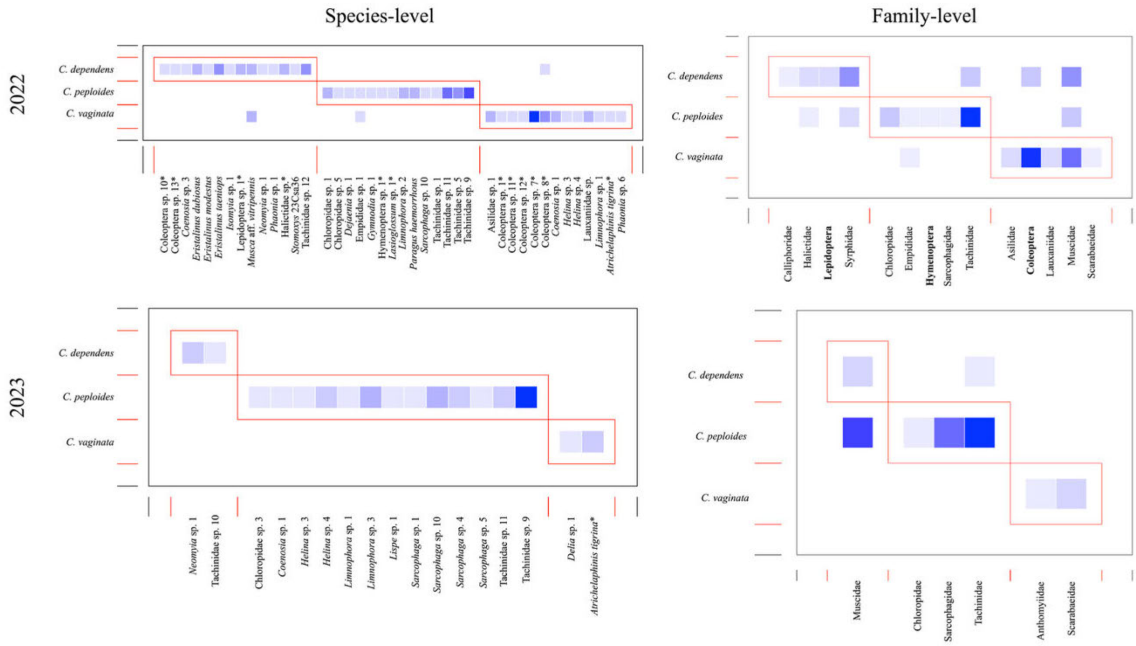

Figure 4.6: Modularity analysis of insect families and species interacting with studied plant species at Witsieshoek. The darker the colour of the blue square, the higher the number of interacting individuals within that family and species. Families and species within a module, enclosed within red borders, are

more closely connected than to those outside the module. Bolded labels refer to order-level of non-dipteran individuals that could not be assigned to families. The modules were overall statistically significant at the family-level (2022: $P < 0.05$, z-score = 7.47; 2023: $P < 0.05$, z-score = 2.19) and at the species-level (2022: $P < 0.05$, z-score = 7.71; 2023: $P < 0.05$, z-score = 3.59).

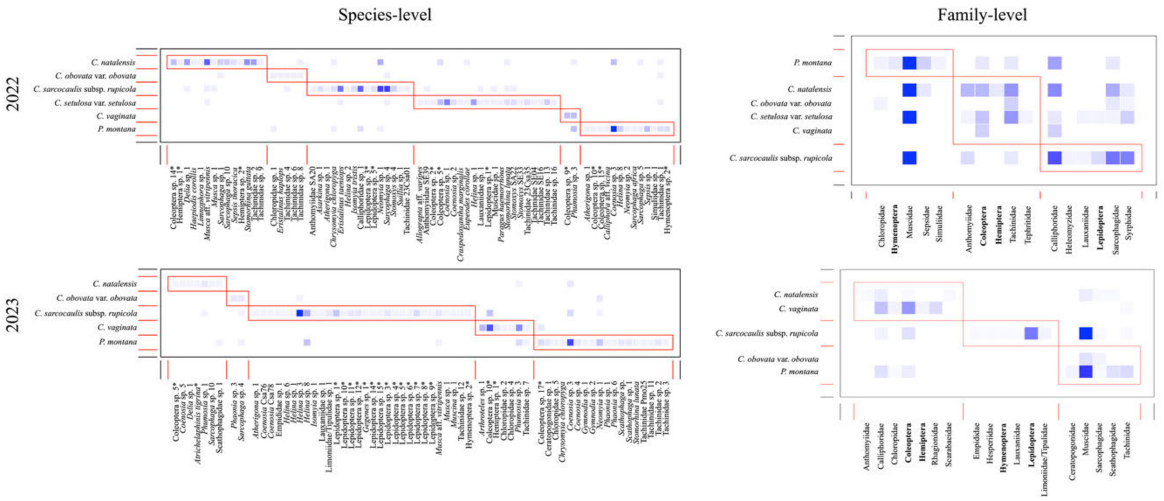

Figure 4.7: Modularity analysis of insect families and species interacting with studied plant species at Sentinel. The darker the colour of the blue square, the higher the number of interacting individuals that belong to that family and species. Families and species within a module, enclosed within red borders, are more closely connected than to those outside the module. Bolded labels refer to order-level of non-Diptera individuals that could not be assigned to families. The modules were overall statistically

significant at the family-level (2022: $P < 0.05$, z-score = 4.92; 2023: $P < 0.05$, z-score = 12.46) and species-level (2022: $P < 0.05$, z-score = 19.66; 2023: $P < 0.05$, z-score = 14.99).

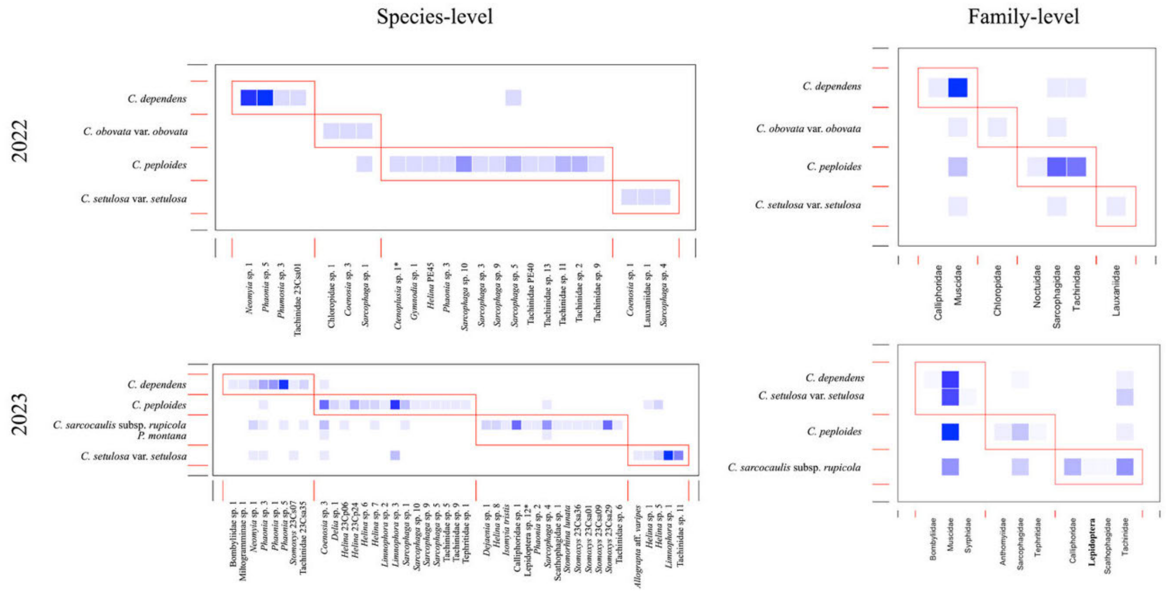

Figure 4.8: Modularity analysis of insect families and species interacting with studied plant species at Naude's Nek. The darker the colour of the blue square, the higher the number of interacting individuals that belong to that family and species. Families and species within a module, enclosed within red borders, are more closely connected than to those outside the module. Bolded labels refer to order-level of non-Diptera individuals that could not be assigned to families. The modules were overall statistically significant at the family-level (2022: $P < 0.05$, z-score = 5.00; 2023: $P < 0.05$, z-score = 5.92) and at the species-level (2022: $P < 0.05$, z-score = 5.34; 2023: $P < 0.05$, z-score = 13.30).

Discussion

The results from this study show that the studied plant species that occur at high elevation are visited predominantly by various saprophilous, short-tongued flies (Diptera), and to a lesser extent also by beetles (Coleoptera), bees (Hymenoptera) and moths (Lepidoptera). Flies of the families Muscidae, Tachinidae, Calliphoridae and Sarcophagidae generally dominated the visitor assemblages across all studied plant species. Specifically, many of the saprophilous, short-tongued flies (including species from the genera *Helina* and *Coenosia*, and *Sarcophaga*) visit most of the plant species. Pollen load analyses sampled from a subset of individuals encompassing all visitor taxa showed that all visitors carried pollen, with various short-tongued flies and beetles carrying the largest pollen loads. Results from interaction network analyses revealed that spatial and temporal turnover between sites and seasons was more pronounced when analysed at the species-level, than at the family-level. Lastly, the local visitor assemblages were partitioned between some species at the three studied sites when analysed both at the species- and family-levels, albeit at varying degrees. Overall, these results suggests that these plant species, except *C. vaginata*, are functionally specialized for pollination by saprophilous, short-tongued flies and represent a pollination guild that relies on short-tongued flies.

The results indicating dominance of short-tongued flies in the floral visitor assemblages of these studied high-elevation plant species highlight the widely-accepted idea that alpine plant visitor assemblages are dominated by flies (Kearns, 1992; Lefebvre et al., 2018; McCabe and Cobb, 2021; Zhao and Huang, 2013). Among the floral visitor assemblage, Muscidae were the most dominant group, followed by Tachinidae, Calliphoridae and Sarcophagidae, correlating with results from other high-elevations studies that reported similar trends (Arroyo et al., 1982; Elberling and Olesen, 1999; Lázaro et al., 2008; Lefebvre et al., 2014; Tiusanen et al., 2016). Particularly in southern Africa's Drakensberg Mountains, several other co-flowering plant species that display similar floral characteristics as the majority of the *Crassula* species, *P. montana* and *T. imbricatum* studied here have also been found to be visited primarily by short-tongued flies. These plant species include *Erica caffrorum* (Ericaceae; Thupsie, 2023), *Schizochilus angustifolius* (Orchidaceae; van der Niet et al., 2010), *Wurmbea elatior* (Colchicaceae; Johnson et al., 2020) and *Eucomis bicolor* and *E. humilis* (Asparagaceae; Shuttleworth and Johnson, 2009). Apart from saprophilous, short-tongued flies, various beetles species (Coleoptera) were the most common and dominant visitors on *C. vaginata* at all studied sites. A few beetles were also observed visiting *C. dependens*, *C. sarcocaulis* subsp. *rupicola* and *C. natalensis*, although in much smaller numbers than the flies. Visitation by beetles have also been reported on *Cineraria erodioides* (Asteraceae), which, like *C. vaginata*, is also characterized by yellow flowers in the Drakensberg region (Black et al., 2019).

Although a diverse assemblage of insect species visited the studied plant species, their functional contributions to pollination varied. Overall, short-tongued flies carried large quantities of *Crassula* pollen grains, except for *C. vaginata*. Indeed, flies were observed to be effective pollinators and their role has increasingly been highlighted, particularly in high-elevation regions (Lefebvre et al., 2018). Although pollen loads were counted from one sampling season, the overall composition of floral visitor assemblages and dominance of short-tongued flies in both sampling seasons suggest that our estimates of potential pollinators for each plant species would not have changed following pollen load assessment from an addition sampling season. This means that short-tongued flies are therefore likely the major pollinators of these guild members. Moreover, these guild members are functionally specialized for visitation by a functional group of saprophilous, short-tongued flies, but are also ecologically generalized in that they are visited by many different species of short-tongued flies.

Although short-tongued flies visited *C. vaginata* at all four sites where the species was studied, the visitor assemblage was dominated by beetles. Beetles also carried the highest amounts of *Crassula* pollen at the Karkloof and Mt. Gilboa sites. At the Sentinel and Witsieshoek sites, which are both higher in elevation than the other two study sites for *C. vaginata*, beetles also carried *Crassula* pollen, although in less quantities compared to saprophilous, short-tongued flies that were caught on the same flowers. Pollination by beetles has been observed within the southern African flora and particularly so in the summer rainfall region (Shuttleworth and Johnson, 2009c; Steenhuisen et al., 2010; Steenhuisen and Johnson, 2012). Based on the floral visitor assemblage, the pollen loads of floral visitors and lack of functional specialization for saprophilous short-tongued flies, *C. vaginata* is not considered to be a member of the guild that relies saprophilous short-tongued flies for pollination, although further research should be done to investigate the possibility of different pollination ecotypes of *C. vaginata* along an elevation gradient (cf. Sun et al., 2014), with greater abundance of flies at higher elevations.

The plant-visitor interaction networks analysed at the species- and family-level revealed both spatial and temporal variation in the visitor assemblages among the three study sites and between a selected number of plant species within study sites, respectively, to varying degrees. These results highlight a dynamic pattern of plant-pollinator interactions (see Burkle and Alarcón, 2011; Carstensen et al., 2014; Petanidou et al., 2008; Price et al., 2005). Such spatial and temporal dynamic interactions are unusual in southern Africa where many pollination studies are biased towards specialized pollination systems and very few generalized systems. In addition, these specialized pollination systems are characterized by interactions between guild members and pollinators that are rather predictable in time and space (Johnson, 2010), thus conforming to a traditional pollination guild concept. Indeed, the distribution of these specialized pollinators themselves is a strong predictor of the distribution of plants they pollinate (Duffy and Johnson, 2017). Nevertheless, spatial (Minachilis et al., 2023; Price et al., 2005) and temporal variation (Alarcón, 2010; Kudo et al., 2024b) in plant-pollinator interactions has been shown in high-elevation regions, but these studies focused on interactions within, and between, entire plant-pollinator communities as opposed to particular species (Alarcón et al., 2008; Kudo et al., 2024; Minachilis et al., 2023;but see Price et al., 2005).

Within pollination guilds, species often share pollinators (Goldblatt and Manning, 2000; Johnson, 2010; Manning and Goldblatt, 1997; Steiner and Whitehead, 1988; Whitehead et al., 2008) and are therefore subjected to interspecific competition (Phillips et al., 2020). However, our results indicated that the local visitor assemblage of functionally similar species was partitioned among co-flowering plant species at three studied sites when analysed at the species- and family-levels. Specifically, partitioning of the local insect assemblage was evident at both the species- and family-levels at the Witsieshoek and Naude's Nek sites during both seasons, although at Naude's Nek in the 2023 sampling season, two of the four plants species occupied the same module. Furthermore, the difference in results between analyses done at the level of visitor species versus visitor families was most pronounced at the Sentinel site: extreme partitioning of the local insect assemblage was evident in both sampling seasons at the species-level, but not at the family-level, in whch some plant species shared a module during both sampling seaons. Additionally, the *Crassula* pollinators also carried non-*Crassula* pollen which indicate some degree of pollinator sharing with other co-flowering, non-*Crassula* plants at these sites. Furthermore, pollen of *Crassula* species are similar, which means that the possibility of pollinator sharing among co-flowering *Crassula* species cannot be excluded, although this was not supported by visitor observations. In ecological guilds members are generally known to partition available resources, including pollinators, in space and time (Stone et al., 1998), and our results are consistent with this idea. Moreover, in other guilds where pollinators are being shared, there is character displacement where guild members deposit pollen on different parts of the insect's body. However, in this saprophilous, short-tongued fly guild, the actual fly pollinators are being partitioned.

In this study, the variation in visitor assemblages, both spatially and temporally, was more pronounced at the insect species- than at the family-level, as expected. In particular, spatial variation in visitor assemblages was extreme at the species-level in *C. vaginata*, but this pattern of extreme spatial variation was not evident in any of the three plant species at the family-level. Moreover, none of the species in which temporal turnover was investigated exhibited extreme temporal turnover at both species- and temporal-levels. However, this observed temporal variation pattern was less pronounced at the family-level. Several explanations for the perceived spatial and temporal variation and partitioning in the visitor assemblages at the species and family-level in this study could be considered. Firstly, variation may be an artefact of limited sampling. In order to survey multiple species across multiple seasons, sampling for each species in each season was restricted to observations for several hours, but not more than two consecutive days, and did not span the entire flowering season. Focus on several plant species across multiple sites may have compromised intensive within-site and within-season sampling. The latter strategy might have revealed a much broader assemblage of insect visitors with substantially more overlap between species. Alternatively, or in addition, variation and in particular, local partitioning of visitor assemblages between guild members, may reflect that guild members occupy different micro-habitats which may also be characterized by different assemblages of flies. For instance, *C. sarcocaulis* subsp. *rupicola* and *C. obovata* var. *obovata* at Sentinel occur along rock crevices on mountain slopes, while *C. natalensis* and *C. vaginata* occur in open grassland areas or rocky outcrops (as in *C. natalensis*) even though the latter two do not occur in close proximity. Lastly, insect visitor identifications using morphological characters may have resulted in possible taxonomic oversplitting at the species-level, since character differences may indicate variation within-species, rather than between species.

Overall, the contrasting explanations for the observed patterns at the insect species- and family-level highlight the need for integration of different spatial and temporal scales, as well as assessing variation at multiple taxonomic levels to address possible taxonomic oversplitting at one level, in understanding variations in plant-pollinator interactions. Future work could thus implement a standardized sampling approach in terms of equal sampling effort and keeping a record of which insects were caught when, to allow the possibility of calculating pollination rates, as opposed to the non-standardized approach used in this study. This standardized method would also allow calculation of rarefaction analyses to estimate whether sampling effort across the study sites was adequate. In addition, future work should involve an extensive, standardized within-season sampling approach that includes day-to-day and within-day sampling to determine whether temporal variations exist at these micro-scales.

The guild concept is essentially a way of structuring interaction patterns within communities. Here, I argue that the saprophilous, short-tongued guild described in this research is fundamentally different to other guilds, in that guild members rely on diverse pollinators that belong to a functionally similar group of saprophilous, short-tongued flies. Other described guilds are defined on the basis of sharing a single pollinator species, or few species from a similar functional group at the most (Goldblatt and Manning, 2000; Johnson, 2010; Manning and Goldblatt, 1997; Steiner and Whitehead, 1988; Whitehead et al., 2008). Moreover, following Fenster et al.'s (2004) argument that plant species that are visited by multiple species from a single functional group are specialized onto that functional group, I argue that this is the case with the studied guild members, which are specialized for visitation and pollination by the saprophilous, short-tongued fly functional group. Furthermore, the guild concept is rooted on similar use of a resource (Root, 1967; Simberloff and Dayan, 1991), which in this case are pollinators. Because of the role of pollinators as agents of natural selection on floral traits, adaptation to a common pollinator is expected to lead to convergence of floral traits, which can be considered as an additional defining criterion for guild membership (Manning and Goldblatt, 1997). Indeed, many of the described guilds are characterized by convergence in functional floral traits amongst guild members. Although trait convergence was not tested in this guild, I still argue that these plant species are functionally specialized for saprophilous, short-tongued flies and ecologically generalized for multiple species of these saprophilous, short-tongued flies based on the dominance of saprophilous, short-tongued flies visiting these plant species, and thus belong to a pollination guild based largely on utilization of a common resource. To further substantiate this idea, future research will investigate whether there is a pattern of trait similarities among guild members, and how this functional specialization for a single group of pollinators is achieved. Also, there is scope for future researchers to start viewing pollination systems as specialized, even if plant species are visited by many species of the same functional group, instead of concluding that such plant species are generalized. Studying these interactions at a community-level, rather than at the level of a single plant species, holds great promise for improving understanding of these concepts. Lastly, a wide variety of techniques, including camera traps and bottle traps using baits, to survey and characterize visitor assemblages to these guild members, should be considered to effectively sample the local visitor assemblages.

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Supplementary Information

Table S4.1: Species and site properties of the nine studied plant species (seven *Crassula*, one *Polemannia* and one *Thesium* species) each from the six study sites within South Africa.

Species	**Family**	**Site**	**Coordinates**	**Year sampled**	**Elevation (m.a.s.l.)**	**Characteristics**
C. dependens	Crassulaceae	Naude’s Nek	30°43’55” S; 28°08’14” E	2022; 2023	2499	Growing among grasses, on moist soils
		Witsieshoek	28°40’53” S; 28°54’18” E	2022; 2023	2171	Adjacent to a moist gravel bed among grasses, in close proximity to *C. peploides*
C. natalensis		Sentinel	28°40’48” S; 28°55’24” E	2022; 023	2552	Rocky outcrops on mountain slopes, among grasses
C. obovata var. *obovata*		Naude’s Nek	30°43’07” S; 28°08’22” E	2022	2511	Among grasses, close to moist gravel beds
		Sentinel	28°43’46” S; 28°53’32” E	2022; 2023	2552	Rocky outcrops on wet, moist mountain slopes,
C. peploides		Naude’s Nek	30°43’55” S; 28°08’14” E	2022; 2023	2499	Moist gravel beds
		Witsieshoek	28°40’53” S; 28°54’18” E	2022; 2023	2171	Moist gravel beds
C. sarcocaulis subsp. *rupicola*		Naude’s Nek (Pitseng Road)	30°45’22” S; 28°18’25” E	2023	1836	Between moist boulders and rocks and in rock crevices
		Sentinel	28°42’40” S; 28°53’43” E	2022; 2023	2248	Between moist boulders and rocks and in rock crevices
		Tarn Cave	29°51’29” S; 29°08’16” E	2023	2393	Between moist boulders and rocks and in rock crevices
C. setulosa var. *setulosa*		Naude’s Nek	30°43’07” S; 28°08’23” E	2022; 2023	2511	On gravel beds
		Sentinel	28°42’40” S; 28°53’43” E	2022	2248	Between boulders and rocks and in rock crevices
C. vaginata		Sentinel	28°44’08” S; 28°53’33” E	2022; 2023	2653	On grasslands along a mountain slope
		Witsieshoek	28°41’08” S; 28°54’05” E	2022; 2023	2194	On grasslands

		Mt. Gilboa	29°17’41” S; 30°17’52” E	2022	1758	On grasslands and along rocky outcrops
		Karkloof	29°17’39” S; 30°17’51” E	2022	1497	On grasslands
P. montana	Apiaceae	Sentinel	28°44’06” S; 28°53’36” E	2022; 2023	2627	Between crevices of big boulders, along a stream
T. imbricatum	Santalaceae	Sentinel	28°44’11” S; 28°53’41” E	2021	2733	On bare rocks, along a mountain slope

Table S4.2: Sampled insect visitors and mean grains of pollen loads quantified from individual morphospecies from seven *Crassula*, one *Polemannia* and one *Thesium* species from multiple high-elevation sites within South Africa over two sampling seasons. Visitor identifications were confirmed using morphological characters and molecular techniques for a subset of visitors that encompassed all caught visitor morphospecies. *N* = total number of collected individuals per morphospecies, * = number of individuals from which pollen loads were assessed.

					Species-level		**Family-level**	
Species **Site**	**Year**	**Insect order**	**Insect family**	**Insect species (*N*; *)**	**Mean grains of conspecific pollen (range)**	**Mean grains of heterospecific pollen (range)**	**Mean grains of conspecific pollen (range)**	**Mean grains of heterospecific pollen (range)**
C. dependens								
Naude’s Nek	2022	Diptera	Calliphoridae	*Phumosia* sp. 3 (1;1)	26	3	26	3
			Muscidae	*Neomyia* sp. 1 (6;6)	45.67 (11 – 102)	7.67 (1 – 24)	41.31 (5 – 102)	5.93 (0 – 24)
				Phaonia sp. 5 (7;7)	37.57 (5 – 76)	4.43 (1 – 17)		
			Sarcophagidae	*Sarcophaga* sp. 5 (1;1)	18	2	18	2
			Tachinidae	Tachinidae 23Csa01 (1;1)	4	2	4	2
	2023	Diptera	Bombyliidae	Bombyliidae sp. 1 (1)				
			Muscidae	*Coenosia* sp. 3 (1)				
				Neomyia sp. 1 (2)				
				Phaonia sp. 3 (4)				
				Phaonia sp. 1 (5)				
				Phaonia sp. 5 (11)				
				Stomoxys 23Cs07 (1)				
			Sarcophagidae	Miltogramminae sp. 1 (1)				
			Tachinidae	Tachinidae 23Csa35 (2)				
Witsieshoek	2022	Diptera	Calliphoridae	*Isomyia* sp. 1 (1;1)	50	36	50	36
			Muscidae	*Coenosia* sp. 3 (1;1)	2	4	22.83 (2 – 63)	14.67 (1 – 59)
				Musca aff. *vitripennis* (2;2)	13.50 (11 – 16)	10 (3 – 17)		
				Neomyia sp. 1 (1;1)	63	59		
				Phaonia sp. 1 (1;1)	43	1		

				Stomoxys 23Csa36 (1;1)	2	4		
			Syrphidae	*Eristalinus dubiosus* (2;2)	52.50 (18 – 87)	4.50 (4 – 5)	50.83 (14 – 124)	91.17 (4 – 270)
				Eristalinus modestus (1;1)	14	270		
				Eristalinus taeniops (3;3)	62 (23 – 124)	89.33 (36 – 189)		
			Tachinidae	Tachinidae sp. 12 (3;3)	17.67 (3 – 47)	16.33 (4 – 36)	17.67 (3 – 47)	16.33 (4 – 36)
		Coleoptera	Other	Coleoptera sp. 10 (1;1)	0	554	12 (0 – 34)	185.67 (1 – 554)
				Coleoptera sp. 13 (1;1)	2	1		
				Coleoptera sp. 8 (1;1)	34	2		
		Hymenoptera	Halictidae	Halictidae sp. 1 (2;2)	30 (4 – 56)	32 (5 – 59)	30 (4 – 56)	32 (5 – 59)
		Lepidoptera	Other	Lepidoptera sp. 1 (2;2)	5 (4 – 6)	16 (10 – 22)	5 (4 – 6)	16 (10 – 22)
	2023	Diptera	Muscidae	*Neomyia* sp. 1 (2)				
			Tachinidae	Tachinidae sp. 10 (1)				
C. natalensis								
Sentinel	2022	Diptera	Anthomyiidae	*Delia* sp. 1 (6;6)	114.67 (47 – 162)	22.83 (12 – 67)	114.67 (47 – 162)	22.83 (12 – 67)
			Calliphoridae	*Chrysomyia chloropyga* (1;1)	757	430	482 (120 – 1247)	140.10 (8 – 562)
				Phumosia sp. 3 (1;1)	120	20		
				Stomorhina guttata (7;7)	473.43 (191 – 1247)	134.71 (25 – 562)		
				Stomorhina lunata (1;1)	629	8		
			Muscidae	*Coenosia* sp. 3 (4;4)	173.50 (116 – 222)	32.25 (6 – 61)	244.52 (53 – 847)	39.86 (5 – 121)
				Limnophora sp. 1 (1;1)	80	22		
				Musca aff. *vitripennis* (10;10)	176. 70 (53 – 376)	32.60 (5 – 118)		
				Musca sp. 1 (1;1)	143	29		
				Neomyia sp. 1 (5;5)	490.20 (163 – 847)	66.20 (9 – 121)		
			Sarcophagidae	*Sarcophaga* sp. 1 (3;3)	914.33 (746 – 1064)	70.33 (51 – 107)	734 (206 – 1064)	55.71 (0 – 107)
				Sarcophaga sp. 10 (3;3)	729.67 (452 – 896)	59.67 (24 – 96)		
				Sarcophaga sp. 5 (1;1)	206	0		
			Sepsidae	*Sepsis thoracica* (2;2)	389 (263 – 515)	12 (4 – 20)	389 (263 – 515)	12 (4 – 20)
			Syrphidae	*Eupeodes corollae* (1;1)	234	191	174.50 (115 – 234)	170 (149 – 191)
				Haepiodis corollis (1;1)	115	149		
			Tachinidae	Tachinidae sp. 1 (1;1)	237	11	262.56 (45 – 877)	19.11 (6 – 54)

				Tachinidae sp. 2 (6;6)	179.83 (45 – 626)	15 (6 – 31)		
				Tachinidae sp. 8 (1;1)	877	17		
				Tachinidae sp. 9 (1;1)	170	54		
		Coleoptera	Other	Coleoptera sp. 14 (4;2)	761.50 (169 – 1427)	138.50 (41 – 208)	542.33 (17 – 1427)	119.33 (41 – 208)
				Coleoptera sp. 5 (2;2)	104 (17 – 191)	81 (55 – 107)		
		Hemiptera	Other	Hemiptera sp. 1 (1;1)	215	26	167.50 (120 – 215)	41.50 (26 – 57)
				Hemiptera sp. 2 (1;1)	120	57		
	2023	Diptera	Anthomyiidae	*Delia* sp. 1 (1)				
			Calliphoridae	*Phumosia* sp. 1 (2)				
				Phumosia sp. 3 (1)				
			Muscidae	*Coenosia* sp. 5 (1)				
				Coenosia sp. 3 (2)				
			Sarcophagidae	*Sarcophaga* sp. 10 (1)				
			Scathophagidae	Scathophagidae sp. (1)				
		Coleoptera	Other	Coleoptera sp. 5 (1)				
			Scarabaeidae	*Atrichelaphinis tigrina* (1)				
C. obovata* var. *obovata								
Naude's Nek	2022	Diptera	Chloropidae	Chloropidae sp. 1 (1;1)	43	0	43	0
			Muscidae	*Coenosia* sp. 3 (1;1)	16	0	16	0
			Sarcophagidae	*Sarcophaga* sp. 1 (1;1)	86	0	86	0
Sentinel	2022	Diptera	Chloropidae	Chloropidae sp. 1 (1;1)	25	1	25	1
			Sarcophagidae	*Sarcophaga* sp. 1 (1;1)	155	78	279.50 (155 – 404)	52.50 (27 – 78)
				Sarcophaga sp. 4 (1;1)	404	27		
			Syrphidae	*Eristalinus haplops* (1;1)	45	11	45	11
			Tachinidae	Tachinidae sp. 1 (1;1)	184	21	331.25 (117 – 604)	128.75 (21 – 352)
				Tachinidae sp. 4 (1;1)	117	352		
				Tachinidae sp. 6 (1;1)	420	68		
				Tachinidae sp. 8 (1;1)	604	74		
	2023	Diptera	Muscidae	*Helina* sp. 3 (1)				
				Neomyia sp. 1 (2)				

				Phaonia sp. (2)				
			Sarcophagidae	*Sarcophaga* sp. 4 (2)				
C. peploides								
Naude's Nek	2022	Diptera	Muscidae	*Gymnodia* sp. 1 (1;1)	77	5	74.50 (72 – 75)	4 (3 – 5)
				Helina PE45 (1)				
				Phaonia sp. 3 (1;1)	72	3		
			Sarcophagidae	*Sarcophaga* sp. 1 (1;1)	85	30	178.29 (85 – 349)	23.29 (10 – 34)
				Sarcophaga sp. 3 (1;1)	349	17		
				Sarcophaga sp. 9 (1;1)	154	20		
				Sarcophaga sp. 10 (3;2)	193.50 (159 – 228)	31.50 (29 – 34)		
				Sarcophaga sp. 5 (2)				
			Tachinidae	Tachinidae sp. 13 (1;1)	27	14	25 (23 – 27)	10.50 (7 – 14)
				Tachinidae sp. 2 (2)				
				Tachinidae sp. 9 (1;1)	23	7		
				Tachinidae sp. 11 (2)				
				Tachinidae PE40 (1)				
		Lepidoptera	Noctuidae	*Ctenoplusia* sp. 1 (1;1)	10	2	10	2
	2023	Diptera	Anthomyiidae	*Delia* sp. 1 (2)				
			Muscidae	*Coenosia* sp. 3 (7)				
				Helina sp. 6 (2)				
				Helina sp. 1 (1)				
				Helina sp. 5 (2)				
				Helina 23Cp06 (1)				
				Helina 23Cp24 (4)				
				Helina sp. 7 (2)				
				Limnophora sp. 2 (1)				
				Limnophora sp. 3 (10)				
				Phaonia sp. 3 (1)				
			Sarcophagidae	*Sarcophaga* sp. 1 (3)				
				Sarcophaga sp. 4 (1)				

				Sarcophaga sp. 9 (1)				
				Sarcophaga sp. 10 (1)				
				Sarcophaga sp. 5 (1)				
			Tachinidae	Tachinidae sp. 5 (1)				
				Tachinidae sp. 9 (1)				
			Tephritidae	Tephritidae sp. 1 (1)				
Witsieshoek	2022	Diptera	Chloropidae	Chloropidae sp. 1 (2;1)	14	1	13.50 (13 – 14)	1 (1 – 1)
				Chloropidae sp. 5 (1;1)	13	1		
			Empididae	Empididae sp. 1 (1;1)	14	0	14	0
			Muscidae	*Gymnodia* sp. 1 (1;1)	31	6	26.67 (7 – 42)	8.33 (6 – 12)
				Limnophora sp. 4 (2;2)	24.50 (7 – 42)	9.50 (7 – 12)		
			Sarcophagidae	*Sarcophaga* sp. 10 (1)				
			Syrphidae	*Paragus haemorrhous* (2;2)	200.50 (34 – 367)	50.50 (0 – 101)	200.50 (34 – 367)	50.50 (0 – 101)
			Tachinidae	*Dejaenia* sp. 1 (1;1)	189	28	170 (68 – 288)	13.75 (1 – 28)
				Tachinidae sp. 1 (1)				
				Tachinidae sp. 5 (3;2)	132 (100 – 164)	5 (1 – 9)		
				Tachinidae sp. 9 (5;2)	186 (170 – 202)	16.50 (10 – 23)		
				Tachinidae sp. 11 (4;2)	178 (68 – 288)	14 (11 – 17)		
		Hymenoptera	Halictidae	*Lasioglossum* sp. 1 (1;1)	46	165	46	165
			Other	Hymenoptera sp. 1 (1)				
	2023	Diptera	Chloropidae	Chloropidae sp. 3 (1)				
			Muscidae	*Coenosia* sp. 1 (1)				
				Helina sp. 3 (1)				
				Helina sp. 4 (2)				
				Limnophora sp. 1 (1)				
				Limnophora sp. 3 (3)				
				Lispe sp. 1 (1)				
			Sarcophagidae	*Sarcophaga* sp. 1 (1)				
				Sarcophaga sp. 4 (2)				
				Sarcophaga sp. 10 (3)				

				Sarcophaga sp. 5 (1)				
			Tachinidae	Tachinidae sp. 9 (10)				
				Tachinidae sp. 11 (2)				
C. sarcocaulis* subsp. *rupicola								
Naude’s Nek	2023	Diptera	Calliphoridae	*Isomyia tristis* (1)				
				Calliphoridae sp. 1 (7)				
				Stomorhina lunata (1)				
			Muscidae	*Coenosia* sp. 3 (3)				
				Helina 23Cp06 (1)				
				Helina sp. 8 (2)				
				Helina sp. 7 (1)				
				Neomyia sp. 1 (2)				
				Phaonia sp. 3 (1)				
				Phaonia sp. 2 (1)				
				Phaonia sp. 5 (1)				
				Stomoxys 23Csa36 (1)				
			Sarcophagidae	*Sarcophaga* sp. 1 (1)				
				Sarcophaga sp. 4 (5)				
			Scathophagidae	Scathophagidae sp. (1)				
			Tachinidae	*Dejaenia* sp. 1 (2)				
				Tachinidae sp. 6 (1)				
				Tachinidae 23Csa01 (1)				
				Tachinidae 23Csa09 (1)				
				Tachinidae 23Csa29 (7)				
				Tachinidae 23Csa35 (1)				
		Lepidoptera	Other	Lepidoptera sp. 11 (1)				
Sentinel	2022	Diptera	Anthomyiidae	Anthomyiidae SA20 (1;1)	33	18	32 (18 – 45)	19.67 (18 – 21)
				Anthomyiidae SE39 (1;1)	45	20		
				Delia sp. 1 (1;1)	18	21		
			Calliphoridae	*Chrysomyia chloropyga* (2;2)	869 (347 – 1391)	606 (20 – 1192)	851.87 (33 – 3052)	559.20 (0 – 1799)

			Isomyia tristis (2;2)	1420 (1055 – 1785)	1601 (1403 – 1799)		
			Calliphoridae sp. 1 (10;10)	815.90 (33 – 3052)	382.20 (0 – 1414)		
			Stomorhina lunata (1;1)	41	152		
		Heleomyzidae	*Suillia* sp. 1 (1;1)	11	4	11	4
		Lauxaniidae	Lauxaniidae sp. 1 (1;1)	6	1	6	1
		Muscidae	*Atherigona* sp. 1 (1;1)	18	1	194.81 (10 – 817)	29.14 (1 – 138)
			Helina sp. 2 (1;1)	66	7		
			Musca aff. *vitripennis* (1;1)	817	31		
			Musca sp. 2 (1;1)	84	3		
			Muscidae sp. 1 (1;1)	11	2		
			Neomyia sp. 1 (12;12)	225.92 (17 – 470)	45.58 (4 – 138)		
			Stomoxys sp. 4 (3;3)	101.67 (10 – 249)	5 (1 – 9)		
			Stomoxys SA22 (1;1)	79	6		
		Sarcophagidae	*Sarcophaga* sp. 4 (12;12)	402.33 (148 – 921)	47.25 (3 – 176)	402.33 (148 – 921)	47.25 (3 – 176)
		Syrphidae	*Asarkina* sp. 1 (2;2)	76 (68 – 84)	397.50 (44 – 751)	948.55 (53 – 3247)	672.36 (27 – 3027)
			Eristalinus taeniops (9;9)	1142.44 (53 – 3247)	733.44 (24 – 3015)		
		Tachinidae	Tachinidae sp. 4 (1;1)	137	32	99 (61 – 137)	127.50 (32 – 223)
			Tachinidae 23Csa01 (1;1)	61	223		
	Lepidoptera	Other	Lepidoptera sp. 2 (1;1)	241	128	122 (3 – 241)	64 (0 – 128)
			Lepidoptera sp. 4 (2;1)	3	0		
2023	Diptera	Calliphoridae	*Isomyia* sp. 1 (1)				
			Phumosia sp. 1 (1)				
		Empididae	Empididae sp. 1 (1)				
		Lauxaniidae	Lauxaniidae sp. 1 (1)				
		Limoniidae/Tipulidae	Limoniidae/Tipulidae sp. 1 (2)				
		Muscidae	*Atherigona* sp. 2 (1)				
			Coenosia sp. 3 (3)				
			Coenosia Csa76 (1)				
			Coenosia Csa78 (1)				
			Helina sp. 6 (1)				

				Helina sp. 1 (1)
				Helina sp. 3 (22)
				Helina sp. 8 (7)
				Musca aff. *vitripennis* (2)
				Musca sp. 1 (1)
				Muscina sp. 1 (1)
				Neomyia sp. 1 (1)
			Sarcophagidae	*Sarcophaga* sp. 4 (1)
			Tachinidae	Tachinidae sp. 12 (1)
		Coleoptera	Other	Coleoptera sp. 10 (5)
		Hymenoptera	Other	Hymenoptera sp. 1 (1)
		Lepidoptera	Hesperiidae	*Gegenes* sp. 1 (1)
			Noctuidae	Lepidoptera sp. 1 (5)
				Lepidoptera sp. 2 (2)
				Lepidoptera sp. 3 (1)
				Lepidoptera sp. 4 (1)
				Lepidoptera sp. 5 (1)
				Lepidoptera sp. 6 (3)
				Lepidoptera sp. 7 (1)
				Lepidoptera sp. 8 (1)
				Lepidoptera sp. 9 (1)
				Lepidoptera sp. 10 (2)
				Lepidoptera sp. 11 (2)
				Lepidoptera sp. 12 (1)
				Lepidoptera sp. 14 (1)
Tarn Cave	2023	Diptera	Muscidae	*Neomyia* sp. 1 (1)
			Nemestrinidae	*Prosoeca* sp. 1 (1)
			Syrphidae	*Asarkina* sp. 1 (1)
			Tachinidae	Tachinidae sp. 13 (1)
				Tachinidae Csa84 (1)

		Hymenoptera	Halictidae	Halictidae sp. 1 (3)				
		Lepidoptera	Other	Lepidoptera sp. 1 (2)				
				Lepidoptera sp. 5 (1)				
				Lepidoptera sp. 7 (2)				
				Lepidoptera sp. 15 (1)				
C. setulosa* var. *setulosa								
Naude's Nek	2022	Diptera	Lauxaniidae	Lauxaniidae sp. 1 (1;1)	43	0	43	0
			Muscidae	*Coenosia* sp. 1 (1;1)	16	0	16	0
			Sarcophagidae	*Sarcophaga* sp. 4 (1;1)	86	0	86	0
	2023	Diptera	Muscidae	*Coenosia* sp. 3 (1)				
				Helina sp. 1 (1)				
				Helina sp. 5 (2)				
				Limnophora sp. 1 (12)				
				Limnophora sp. 3 (3)				
				Neomyia sp. 1 (1)				
				Phaonia sp. (1)				
				Stomoxys 23Cs07 (1)				
			Syrphidae	*Allograpta* aff. *varipes* (1)				
			Tachinidae	Tachinidae sp. 11 (6)				
Sentinel	2022	Diptera	Anthomyiidae	Anthomyiidae SE39 (1;1)	12	2	12	2
			Calliphoridae	*Stomorhina lunata* (2;2)	485 (327 – 643)	92 (59 – 125)	485 (327 – 643)	92 (59 – 125)
			Lauxaniidae	Lauxaniidae sp. 1 (1;1)	10	3	10	3
			Muscidae	*Coenosia* sp. 1 (8;8)	46.86 (4 – 136)	9.13 (1 – 25)	145.38 (3 – 642)	18.43 (0 – 75)
				Coenosia sp. 2 (2;2)	99 (81 – 117)	17 (14 – 20)		
				Helina sp. 1 (6;6)	289 (28 – 642)	33.71 (0 – 75)		
				Musca aff. *vitripennis* (1;1)	492	18		
				Muscidae sp. 1 (1;1)	91	11		
				Stomoxys SA22 (2;2)	79 (3 – 155)	11.50 (2 – 21)		
				Stomoxys SE33 (1;1)	5	10		
			Sarcophagidae	*Sarcophaga* sp. 4 (1;1)	174	42	174	42

				Syrphidae	*Allograpta* aff. *varipes* (1;1)	18	55	80 (18 – 139)	179 (20 – 607)
					Eristalinus taeniops (1;1)	121	607		
					Eupeodes corollae (1;1)	139	34		
					Paragus haemorrhous (1;1)	42	20		
				Tachinidae	Tachinidae sp. 13 (3;2)	189 (18 – 279)	136.33 (11 – 342)	251.22 (18 – 483)	72.89 (4 – 342)
					Tachinidae sp. 1 (1;1)	319	56		
					Tachinidae sp. 16 (1;1)	55	4		
					Tachinidae SE04 (1;1)	536	110		
					Tachinidae SE16 (1;1)	69	5		
					Tachinidae 23Csa35 (1;1)	357.50 (232 – 483)	36 (17 – 55)		
				Tephritidae	*Craspedoxantha marginalis* (1)	77	4	77	4
			Coleoptera	Other	Coleoptera sp. 2 (1;1)	19	0	83.40 (19 – 204)	20 (0 – 47)
					Coleoptera sp. 5 (4;4)	99.50 (51 – 204)	25 (12 – 47)		
			Lepidoptera	Other	Lepidoptera sp. 1 (1;1)	6	4	6	4
C. vaginata									
	Sentinel	2022	Diptera	Calliphoridae	*Phumosia* sp. 3 (4;4)	275.75 (129 – 426)	34.75 (12 – 80)	275.75 (129 – 426)	34.75 (12 – 80)
			Coleoptera	Other	Coleoptera sp. 9 (4;4)	267.25 (163 – 341)	9 (3 – 19)	267.25 (163 – 341)	9 (3 – 19)
		2023	Diptera	Calliphoridae	*Phumosia* sp. 3 (10)				
				Chloropidae	Chloropidae sp. 2 (1)				
					Chloropidae sp. 4 (1)				
				Rhagionidae	*Arthroteles* sp. 1 (6)				
				Tachinidae	Tachinidae sp. 7 (1)				
			Coleoptera	Other	Coleoptera sp. (2)				
					Coleoptera sp. 10 (15)				
			Hemiptera	Other	Hemiptera sp. 1 (2)				
	Witsieshoek	2022	Diptera	Asilidae	Asilidae sp. 1 (2;2)	103.50 (5 – 202)	3.50 (0 – 7)	103.50 (5 – 202)	3.50 (0 – 7)
				Empididae	Empididae sp. 1 (1;1)	88	1	88	1
				Lauxaniidae	Lauxaniidae sp. 1 (2;2)	43 (5 – 81)	0	43 (5 – 81)	0
				Muscidae	*Coenosia* sp. 1 (2;2)	35 (28 – 42)	7 (0 – 14)	52.63 (20 – 169)	10.38 (0 – 29)
					Helina sp. 3 (1;1)	34	8		

				Helina sp. 4 (1;1)	34	4		
				Limnophora sp. 1 (1;1)	39	29		
				Musca aff. *vitripennis* (2;2)	112 (55 – 169)	13 (13 – 13)		
				Phaonia sp. 6 (1;1)	20	2		
		Coleoptera	Other	Coleoptera sp. 1 (1;1)	8	0	242.31 (8 – 846)	45.23 (0 – 262)
				Coleoptera sp. 7 (7;7)	347.48 (30 – 846)	66.43 (0 – 262)		
				Coleoptera sp. 8 (3;3)	171 (88 – 246)	40.67 (34 – 53)		
				Coleoptera sp. 11 (1;1)	169	1		
				Coleoptera sp. 12 (1;1)	28	0		
			Scarabaeidae	*Atrichelaphinis tigrina* (1;1)	421	0	421	0
	2023	Diptera	Anthomyiidae	Delia sp. 1 (1)				
			Scarabaeidae	*Atrichelaphinis tigrina* (2)				
Karkloof	2022	Diptera	Muscidae	*Coenosia* sp. 1 (1;1)	177	31	177	31
			Sarcophagidae	*Sarcophaga* sp. 2 (1;1)	176	35	176	35
		Coleoptera	Other	Coleoptera sp. 4 (1;1)	1582	314	540.25 (111 – 1582)	114.75 (7 – 314)
				Coleoptera sp. 6 (1;1)	111	35		
				Coleoptera sp. 9 (2;2)	234 (118 – 350)	55 (7 – 103)		
			Scarabaeidae	*Atrichelaphinis tigrina* (6;6)	883.50 (243 – 2163)	43.33 (0 – 106)	883.50 (243 – 2163)	43.33 (0 – 106)
		Hymenoptera	Apidae	Apidae sp. 1 (1;1)	326	73	326	73
			Other	Hymenoptera sp. 2 (1;1)	467	23	467	23
		Lepidoptera	Other	Lepidoptera sp. 14 (6;6)	80.17 (52 – 108)	5.5 (1 – 16)	80.17 (52 – 108)	5.5 (1 – 16)
Mt. Gilboa	2022	Diptera	Syrphidae	*Phytomia incisa* (1;1)	112	52	112	52
			Tachinidae	Tachinidae sp. 4 (1;1)	73	66	73	66
		Coleoptera	Other	Beetle sp. 3 (9;9)	160.22 (27 – 697)	170.67 (0 – 504)	285.46 (27 – 1197)	133.85 (0 – 504)
				Beetle sp. 8 (1;1)	184	0		
				Beetle sp. 10 (3;3)	695 (427 – 1197)	68 (1 – 202)		
			Scarabaeidae	*Atrichelaphinis tigrina* (8;8)	313.63 (27 – 1544)	57.13 (3 – 209)	313.63 (27 – 1544)	57.13 (3 – 209)
		Hymenoptera	Apidae	Apidae sp. 1 (4;4)	459 (62 – 1026)	286.75 (49 – 712)	459 (62 – 1026)	286.75 (49 – 712)
			Halictidae	Halictidae sp. 1 (3;3)	1477.67 (649 – 2251)	42.67 (7 – 104)	1477.67 (649 – 2251)	42.67 (7 – 104)
		Lepidoptera	Other	Lepidoptera sp. 14 (2;2)	40 (22 – 58)	7.50 (0 – 15)	40 (22 – 58)	7.50 (0 – 15)

P. montana								
Naude’s Nek	2023	Diptera	Muscidae	*Coenosia* sp. 3 (1)				
			Sarcophagidae	*Sarcophaga* sp. 4 (1)				
Sentinel	2022	Diptera	Calliphoridae	*Calliphora* aff. *vicina* (1;1)	132	0	89 (3 – 469)	3.63 (0 – 9)
				Chrysomyia chloropyga (1;1)	469	0		
				Calliphoridae sp. 1 (2;2)	30 (3 – 57)	5 (1 – 9)		
				Phumosia sp. 3 (4;4)	12.75 (7 – 24)	4.75 (1 – 9)		
			Chloropidae	Chloropidae sp. 1 (1;1)	22	0	22	0
			Muscidae	*Atherigona* sp. 1 (1;1)	29	1	27.86 (0 – 157)	4.36 (0 – 50)
				Coenosia sp. 3 (16;16)	13.63 (0 – 72)	1.06 (0 – 8)		
				Helina sp. 8 (3;3)	64 (9 – 157)	9 (0 – 15)		
				Neomyia sp. 1 (1;1)	83	1		
				Neomyia sp. 2 (1;1)	91	50		
			Sarcophagidae	*Sarcophaga africa* (1;1)	236	29	147 (58 – 236)	15.50 (2 – 29)
				Sarcophaga sp. 5 (1;1)	58	2		
			Sepsidae	*Sepsis* sp. 1 (4;4)	13.50 (2 – 25)	1 (0 – 2)	13.50 (2 – 25)	1 (0 – 2)
			Simuliidae	Simuliidae sp. 1 (1;1)	16	0	16	0
			Tachinidae	Tachinidae sp. 1 (2;2)	85 (46 – 126)	4 (0 – 8)	85 (46 – 126)	4 (0 – 8)
		Coleoptera	Other	Coleoptera sp. 10 (1;1)	8	106	8.5 (8 – 9)	57 (8 – 106)
				Coleoptera sp. 15 (1;1)	9	8		
		Hymenoptera	Other	Hymenoptera sp. 2 (2)	192.50 (5 – 380)	0.50 (0 – 1)	192.50 (5 – 380)	0.50 (0 – 1)
	2023	Diptera	Calliphoridae	*Chrysomyia chloropyga* (1)				
				Phumosia sp. 3 (3)				
				Stomorhina lunata (1)				
			Chloropidae	Chloropidae sp. 5 (1)				
			Muscidae	*Coenosia* sp. 3 (16)				
				Coenosia sp. 4 (2)				
				Gymnodia sp. 1 (1)				
				Gymnodia sp. 2 (1)				
				Helina sp. 8 (5)				

				Musca aff. *vitripennis* (1)
				Neomyia sp. 1 (5)
				Phaonia sp. 1 (1)
				Phaonia sp. 6 (1)
			Sarcophagidae	*Sarcophaga* sp. 4 (1)
			Scathophagidae	*Scathophaga* sp. (2)
				Scathophaga sp. 1 (2)
				Scathophagidae sp. (1)
			Tachinidae	Tachinidae sp. 2 (2)
				Tachinidae sp. 3 (1)
				Tachinidae sp. 7 (1)
				Tachinidae sp. 11 (1)
				Tachinidae Pmo25 (1)
		Coleoptera	Other	Coleoptera sp. 17 (3)
T. imbricatum				
Sentinel	2021	Diptera	Anthomyiidae	*Delia* sp. 1 (1)
			Bibionidae	Bibionidae sp. 1 (5)
			Calliphoridae	*Phumosia* sp. 3 (8)
			Chloropidae	Chloropidae sp. 3 (2)
			Empididae	Empididae sp. 1 (1)
			Muscidae	*Coenosia* sp. 3 (18)
				Coenosia sp. 4 (2)
				Helina sp. 3 (1)
				Musca aff. *vitripennis* (1)
				Stomoxys SA22 (1)
			Sarcophagidae	*Sarcophaga* sp. 1 (1)
				Sarcophaga sp. 4 (2)
			Scathophagidae	*Scathophaga* sp. 1 (1)
				Scathophagidae sp. 1 (1)
			Sepsidae	*Sepsis* sp. 1 (5)

	Tachinidae	Tachinidae sp. 8 (1)
	Tephritidae	Tephritidae sp. 1 (6)
Coleoptera	Other	Coleoptera sp. 3 (1)
		Coleoptera sp. 16 (1)
Hemiptera	Other	Hemiptera sp. 1 (2)
Hymenoptera	Formicidae	Formicidae sp. 1 (2)
	Other	Hymenoptera sp. 1 (2)
		Total: (859; 392)

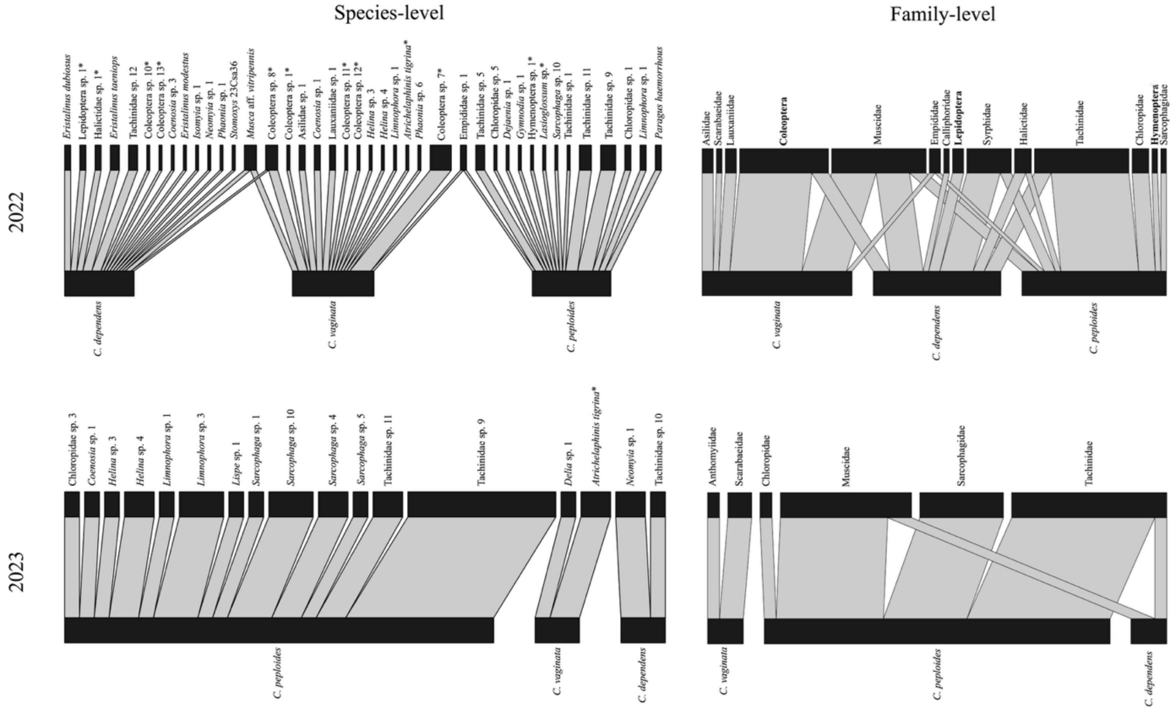

Figure S4.1: Bipartite interaction network of studied plant species and insect species and families at Witsieshoek over two seasons. Interactions are based on the number of individuals per species in each family that were caught interacting with a particular plant species, which is proportional to the width of the linking lines. Bolded labels refer to order-level of non-dipteran individuals that could not be assigned to families.

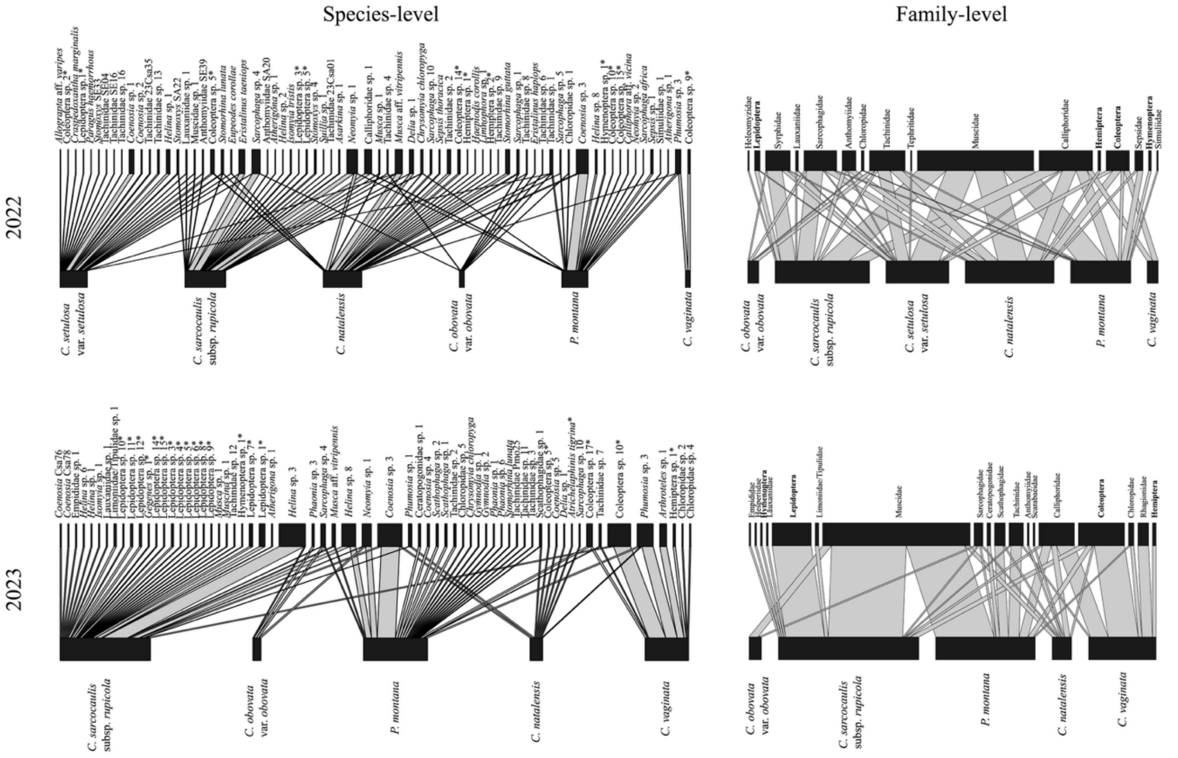

Figure S4.2: Bipartite interaction network of studied plant species and insect species and families at Sentinel over two seasons. Interactions are based on the number of individuals per species in each family that were caught interacting with a particular plant species, which is proportional to the width of the linking lines. Bolded labels refer to order-level of non-dipteran individuals that could not be assigned to families.

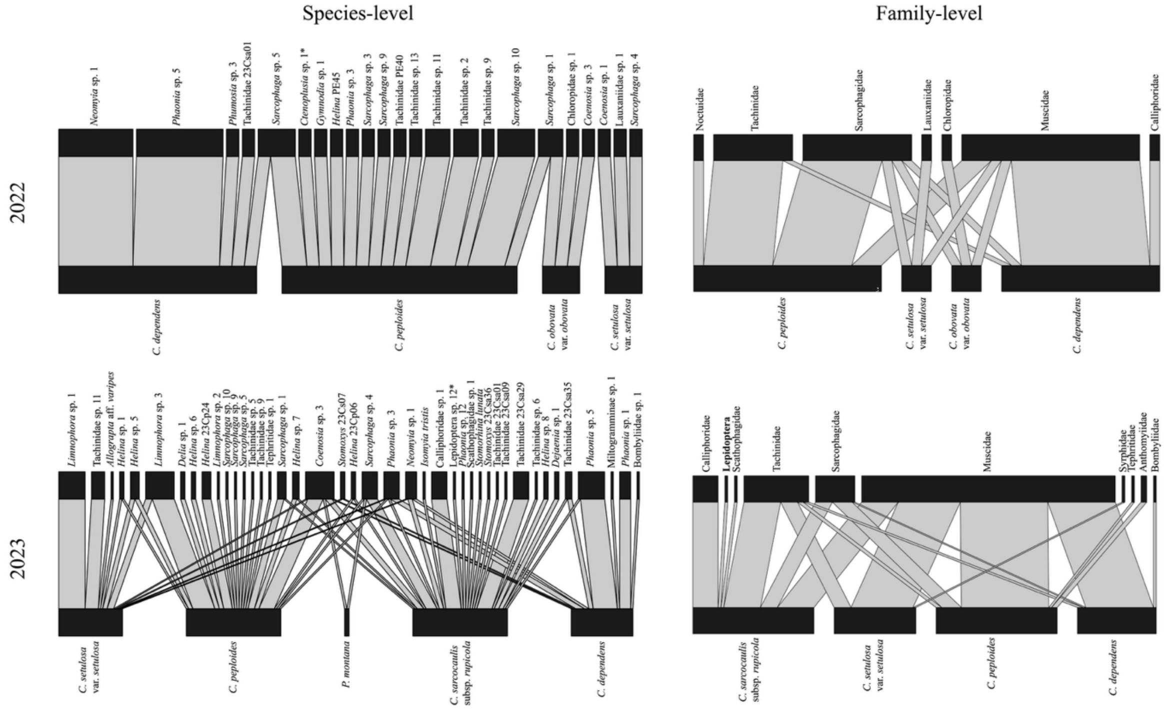

Figure S4.3: Bipartite interaction network of studied plant species and insect species and species at Naude's Nek over two seasons. Interactions are based on the number of individuals per species in each family that were caught interacting with a particular plant species, which is proportional to the width of the linking lines. Bolded labels refer to order-level of non-dipteran individuals that could not be assigned to families.

Chapter 5:

Small, pale and stinky: floral traits associated with a fly pollination guild in the Drakensberg mountains of South Africa

Abstract

Similarities in floral traits within pollination guilds of flowers that rely on one or a few functionally similar pollinator species provide some of the clearest demonstrations of adaptive trait convergence in flowering plants. The saprophilous, short-tongued fly pollination guild in the Drakensberg mountains in southern Africa is unusual in that the pollinators, although functionally similar, include multiple species from various families of flies. Despite this, to the human eye and nose, there still appears to be some degree of trait similarity among guild members that typically exhibit small white or greenish-white flowers and a foetid odour. However, evaluation of evidence for trait similarity among guild members requires objective quantification taking the pollinators' sensory modalities into account. Here, I quantified the floral traits of nine guild members to identify common traits that likely play a role in attracting fly pollinators and objectively assessed levels of variation and similarity among guild members. Morphometric analyses showed variation among the guild members. Spectral reflectance of petals was similar across the guild members. Moreover, the spectral reflectance of petals, pollen and ovaries varied among species, resulting in loci that plotted in different quadrants in the fly vision model, thus suggesting that pollinators would be able to distinguish between the different floral parts. Additionally, most floral parts plotted in different quadrants relative to the gravel background, implying that flowers are, to the fly pollinators, chromatically distinguishable from the background. Analysis of floral scent using coupled Gas Chromatography-Mass Spectrometry (GC-MS) showed that all guild members emitted scent volatiles that are normally associated with degradation of organic substrates, although the suites of compounds varied among species: some species were dominated by acids, specifically hexanoic, butanoic, isovaleric and butanoic acids, while some had very few or no acids, but were dominated by aromatics, including *p*-cresol, methyl benzoate and an isomer of methylanisole and others were dominated by terpenoids, including (E)-4,8-dimethylnona-1,3,7-triene and (Z)-ocimene. The variation in the scent profiles of the guild members was reflected by the clear separation of plant species in a multivariate analysis. The presence of nectar, albeit in minute quantities, confirmed that this is a rewarding pollination system. Similarities in colour and scent suggest that these floral traits may reflect adaptation to a common functional pollinator type within this pollination guild. In particular, the majority of scent compounds emitted by these guild members are known from degrading organic substrates to which the visitor assemblage would be drawn and this may explain the functional specialization of these guild members to saprophilous, short-tongued flies.

Keywords

Pollination guild, high-elevation, floral traits, scent chemotypes, myophily

Introduction

Angiosperms are the most diverse group of vascular plants worldwide, representing over 90% of extant species (Donoghue, 2019; Kearns et al., 1998; Sauquet et al., 2017). Presently, over 300 000 species of flowering plants have been described (Soltis et al., 2019). The diversification of major groups of insect pollinators and their interactions with flowering plants is often suggested as a major contributor to the evolutionary diversification of plants (Fenster et al., 2004; Johnson and Steiner, 2000; Stebbins, 1970; van der Niet et al., 2014). Through differences in their morphologies and sensory modalities, different pollinators exert divergent selective pressures on flowers and thus drive phenotypic divergence in flowering plants, resulting from adaptation to the locally most abundant and effective pollinators (Fenster et al., 2004; Stebbins, 1970; van der Niet and Johnson, 2012). Conversely, when a particular pollinator species or functional group of pollinators exert similar selective pressures on distantly related plant species within a community, this may result in the similarity of floral traits through convergent evolution. As such, plants adapted to pollination by a common pollinator species, or a group of functionally similar pollinators, will display a common suite of floral traits (i.e., pollination syndrome; Faegri and van der Pijl, 1979; Fenster et al., 2004). Patterns of floral trait similarity are often most clear when pollination systems are examined at the level of pollination guilds (Johnson, 2010; Manning and Goldblatt, 1997; Simberloff and Dayan, 1991), i.e. plant species (often unrelated species, but sometimes including related species) that share a common pollinator or functional pollinator group, as this is the unit within which selection occurs.

Functional floral traits can be divided into those that function to ensure optimal contact between pollinators and floral reproductive parts and others that attract pollinators. These functional floral traits can include morphology, colour, scent and nectar reward. Floral morphology plays an important role in plant-pollinator interactions by ensuring mechanical fit with pollinators, thereby achieving optimal pollen placement on pollinators and ensuring access to nectar (Gómez et al., 2015; Ibañez et al., 2023; Jürgens, 2006; Kaczorowski et al., 2012). Mechanical fit between flowers and pollinators may also result in filtering of floral visitors resulting in specialized pollination systems (Armbruster, 2017). For example, there is close matching between the dimensions of the long floral tubes of some flowering plants and the long proboscides of associated pollinators because potential visitors with proboscides substantially shorter than the floral tube cannot legitimately access the reward (i.e., Nemestrinidae; Manning and Goldblatt, 1997). In Manning and Goldblatt's (1997) system, guild members also vary regarding placing pollen on the pollinator's body, to minimise pollen contamination (Manning and Goldblatt, 1997). Another example is the close matching between the dimensions of *Ceropegia lutea* subsp. *lutea* (Apocynaceae) flowers and a single pollinating species of *Atherigona* (Muscidae; Shuttleworth et al., 2017). In the case of *C. lutea* subsp. *lutea* the floral dimensions only allow the head of this specific fly to fit between the inner corona lobes, which narrows the spectrum of visitors that can access nectar and contact the reproductive parts, resulting in a highly specialized pollination system (Shuttleworth et al., 2017). As such, quantifying floral morphometrics within guilds is important in understanding whether there are similarities or differences with regards to reward access, traits that mediate efficient pollen placement on pollinators and how the foraging behaviour of pollinators is influenced to ensure pollen transfer between individual flowers of the same plant species.

Of the traits that mediate pollinator attraction, visual signals related to flower colour and patterning likely play a functional role in attracting saprophilous flies. Many saprophilous fly-pollinated flowers have evolved unusual floral patterns (such as dark spots against a pale background) and colours (such as dull reddish pigmentation or purple flowers) which are suggested to resemble model substrates (Chen et al., 2015b; Johnson et al., 2020; Jürgens et al., 2013; Raguso, 2004; Urru et al., 2011). Other fly-pollinated flowers, especially those that are characterized by less specialized pollination systems, however, are white or yellow in colour and occur especially in high-elevation regions (Campbell et al., 2010; Newstrom and Robertson, 2005; Primack, 1983). Regardless of the variation, floral colour may represent an important cue used by flies to find flowers (Woodcock et al., 2014) and assessing similarity in colours is therefore necessary to understand how co-flowering plant species utilize a common pollinator and ensure efficient pollen transfer.

Further evidence in support for the ability of saprophilous flies to discriminate colour is only available for a few fly species through behavioural experiments (Lunau, 2014), but this includes a saprophilous blow fly species of the genus *Lucilia* (Calliphoridae; (Fukushi, 1990; Troje, 1993). In the blow fly, colour vision is thought to occur via two visual systems which are both facilitated by the rhabdomeres within the flies' compound eyes (Lunau, 2014; Yamaguchi et al., 2008) and are processed side by side (Strausfeld and Lee, 1991). The excitation of the peripheral rhabdomeres of the R1 – 6 photoreceptor cells activate the neural superposition system, which is used for motion detection but may also play a role in achromatic vision (Hardie and Kirschfeld, 1983). The other pathway, the tetravariant visual system, is based on the central rhabdomeres of the R7 and R8 photoreceptor cells, which comprise four photoreceptor subtypes: R7p, R8p, R7y and R8y, that are used for colour vision (Hardie and Kirschfeld, 1983; Troje, 1993). These four photoreceptors are specifically sensitive to different ranges of ultraviolet, green and blue wavelengths (Lunau, 2014). Troje (1993) proposed the fundamentals of how flies perceive colour, based on conditioning and discrimination experiments with a *Lucilia* species.

The role of visual cues, including colour contrasts, in influencing fly behaviours has previously been demonstrated. For instance, Prokopy (1968) demonstrated that small objects were more preferred by tephritid flies if they were a darker colour than a lighter colour, whereas larger objects were preferred when they were yellow in colour in the context of fruit-host searching. There is also evidence that flies exhibit a preference for darker colours against lighter colours, and the presence of dark spots on flowers has been shown to enhance overall fly attraction and visitation rates (Brodie et al., 2014; Eisikowitch, 1980; Johnson et al., 2020; Johnson and Dafni, 1998, but see du Plessis et al., 2018). Moreover, saprophilous flies are known to utilize bimodal cues (both visual and olfactory cues) to find foraging sites (Aak and Knudsen, 2011; Brodie et al., 2014). Indeed, some combinations, including foetid scents with dark colours resembling actual flowers, increased attraction compared to when either cue was tested alone (see Chen et al., 2015b; du Plessis et al., 2018).

Floral scent often plays a key role in attracting pollinators (Dötterl and Gershenzon, 2023; Robert A Raguso, 2008), but it is also highly variable. Indeed, distinct floral odours are often associated with different pollination systems, such as in sexual deceptive systems where specific sex pheromones are imitated (Johnson and Schiestl, 2016; Peakall, 2023), in sapromyiophilous systems, where, for instance, many fly-pollinated flowers mimic oviposition sites such as carrion and dung (Johnson and Schiestl, 2016; Jürgens et al., 2013) and in moth-pollinated systems where production of scent coincides with moth activity at night (Knudsen and Tollsten, 1993). Fly-pollinated flowers characterized by floral mimicry are known to produce odours that are reminiscent of the model substrate that is being mimicked, which includes both food and oviposition sites (Johnson and Schiestl, 2016; Jürgens et al., 2013; Stensmyr et al., 2002; Urru et al., 2011; Zito et al., 2013). The diversity of model substrates that are being mimicked are associated with distinct "chemotypes." For example, Jürgens et al. (2006) identified the following chemotypes in stapeliads and suggested they represent mimicry of different substrates: urine (characterized by carboxylic acids and pyrazine), herbivore dung (characterized by *p*-cresol, sometimes with polysulphides in low amounts), and rotting carcass or carnivore/omnivore dung (characterized by high amounts of octanal and heptanal, polysulphides and low amounts of *p*-cresol). Furthermore, the odours produced by these fly-pollinated flowers can be sweet to the human nose (although very seldom) or foetid, and include compounds that are associated with the degradation of organic substances, such as aliphatic acids, esters and alcohols, *p*-cresol, nitrogen-containing compounds like indole and skatole and sulphur-containing compounds like dimethyl disulphide (DMDS) and dimethyl trisulphide (DMTS; Johnson et al., 2020; Jürgens et al., 2013; Zito et al., 2015). Some of the compounds characterizing fly-pollinated flowers have been shown to elicit physiological and behavioural responses in saprophilous flies (see Johnson et al., 2020; Shuttleworth, 2016, 2017; Stensmyr et al., 2002; Zito et al., 2013 for examples). As such, the first step in understanding the attractiveness of floral cues to flies requires a qualitative analysis of scents in fly-pollinated plant species, which may also broaden understanding of how scent functions in mediating specialization.

Nectar is commonly the main reward offered by fly-pollinated plants (Baker and Baker, 1973; van der Kooi et al., 2021; Woodcock et al., 2014). However, some plant species that are pollinated by saprophilous flies do not provide nectar. For instance, some stapeliads do not offer a nectar reward, which is expected in carrion-mimicking plants (Ackerman, 1986; Dafni, 1984), whereas others do offer nectar rewards, albeit in very small quantities (Johnson, 2016; Jürgens, 2006; Meve and Liede, 1994).

Although the egg-laying behaviour of saprophilous flies on these stapeliad flowers that offer nectar suggests that the flowers are still deceptive and that the nectar functions to optimise the behaviour of the pollinator to contact the floral reproductive parts (Johnson, 2016). Overall, there is a general perception that fly-pollinated flowers are deceptive because they mimic oviposition sites that are attractive to saprophilous flies and are thus exploiting non-foraging behaviour by their pollinators. The presence of nectar in flowers that emit volatiles reminiscent of an oviposition site is somewhat paradoxical. Therefore, it is crucial to evaluate the presence of a reward, especially in guild members that have been observed to be visited by flies to confirm if nectar influences the behaviour of the flies on the flowers.

Because guilds are at times often defined by suites of floral traits, it is crucial to assess patterns of trait similarity or differences among guild members to understand the mechanisms of pollinator attraction. Here, I quantified floral traits of nine plant species in the genera *Crassula* (Crassulaceae), *Thesium* (Santalaceae) and *Polemannia* (Apiaceae) that, except for *C. vaginata*, belong to a saprophilous, short-tongued fly pollination guild in high-elevation regions of South Africa, to determine whether there is trait similarity among guild members. The specific objectives were to: (a) quantify the morphology of different floral parts of guild members, (b) compare floral colours of guild members and determine whether there is chromatic contrast between different floral parts and between flowers and the gravel substrate on which plants grow (and are viewed against by approaching pollinators), (c) quantify the chemical composition of floral scents emitted by guild members and lastly, (d) determine the presence of a nectar reward in guild members.

Materials and Methods

Study species

Floral traits that may be important for attracting pollinators to flowers and that may affect the functional fit of pollinators were quantified for *C. vaginata* and eight plant species that belong to the saprophilous, short-tongued fly pollination guild. Specifically, we investigated a total of seven plant species belonging to the genus *Crassula* (Crassulaceae) and one each from the genera *Polemannia* (Apiaceae) and *Thesium* (Santalaceae; see Figure 1.1 and Table S5.1 for photographs of species and study site details, respectively). Some floral traits were only measured for a subset of all the studied species (see Table 5.1). Studied species exhibited similarly sized small flowers, with most being white or greenish-white (some also have red petal tips and ovaries) and only *C. vaginata* being yellow in colour to the human eye. All species emitted a foetid odour to the human nose. In addition, quantification of floral visitors and pollinators (using pollen loads as a proxy) showed that these studied species, except for *C. vaginata*, were visited primarily by short-tongued, saprophilous flies (Diptera; see Chapter 4).

Study localities

Fieldwork was conducted in the 2021–2022 and 2022–2023 flowering seasons along Naude's Nek Mountain pass (hereafter Naude's Nek) in the southern Drakensberg of the Eastern Cape province, in the Witsieshoek and Sentinel regions of the northern Drakensberg in the KwaZulu-Natal province, and at Mount Gilboa (hereafter Mt. Gilboa) in the Karkloof mountains of KwaZulu-Natal province where only *C. vaginata* occurs (Figure 1.2 and Table S5.1). All studied species grow naturally at these sites.

Floral morphology

To assess the variation in floral morphology between flowers of the same species from different sites and between different species, measurements were taken for floral traits that are thought to be important for pollinator attraction and mechanical fit between pollinators and plant. Specifically, I measured flower depth (defined as the distance from the receptacle to the highest terminal point, which could include the petals, when viewed from the side), flower diameter (defined as the distance from petal tip to the tip of the opposite petal when viewed from the top) which allows pollinators to access nectar reward, pistil length (defined as the distance from the base of the ovary to the tip of the stigma), stamen length (defined as the distance from the base of the filament to the tip of the anther) that is relevant in the placement of pollen on visitors, and sepal length (defined as the distance from the base to the tip of the sepal; see Figure S2.1 in Chapter 2). In each population, eight randomly selected flowers per species were measured from eight different plants or multiple flowers from a single plant, depending on availability. Floral morphologies were measured from seven *Crassula* species as well as the *Thesium* species, but not for *P. montana*.

To determine which of the measured morphological traits contribute to the variation between species and populations, I performed a principal component analysis (PCA) with a correlation matrix using the PAST v4.03 software (Hammer et al., 2001). To investigate morphological differences between the studied species and populations, I performed a multivariate analysis of variance (MANOVA) using the raw, non-transformed morphological data, to preserve variance since all values were on a similar scale. A MANOVA was performed in PAST v4.03 software (Hammer et al., 2001).

Floral colour and perception of colour by pollinators

Colours from different flower parts of randomly selected flowers from all studied *Crassula* species (Table S5.1) were quantified by measuring spectral reflectance *ex-situ*. Flowers were collected in the field and placed in water for transport to the laboratory for measurements. Floral parts were mounted on clear sticky tape to keep them flat for spectral measurements. Spectral reflectance across the UV-visible range of 300–700 nm was measured for a total of eight flowers from different plants per studied plant species (see Table S5.1). Specifically, reflectance measurements were taken from the petal (separated into petal base and tip when a petal has two colours), sepal, ovary, pollen and leaf. It is worth noting that leaves were not measured for all plant species. In addition, the reflectance of the gravel substrate (eight replicates each of the wet and dry gravel) on which several of the plants typically grow was also measured *ex-situ*, by collecting gravel in the field and transporting it to the laboratory. Additionally, some of the gravel was kept wet by adding water to it to resemble growing conditions in the field.

Spectral reflectance was measured using an Ocean Optics USB2000 spectrometer (Ocean Optics Inc., Dunedin, FL, USA) with a fibre optic reflective probe (QR-200-7-UV-VIS; 200 μm) paired with an Ocean Optics DT-2-GS Deuterium-Tungsten-Halogen mini light source. The probe was held at a 45° angle to the surface being measured. Prior to the initial measurement and following every 8th measurement, the spectrometer was calibrated with a Labsphere USRS-99-010 white diffuse reflectance standard and a black standard. All reflectance measurements were taken using the Java-based SpectraSuite Spectroscopy software (Java v1.6.0_03, Oceans Optics Inc., Dunedin, FL, USA).

To assess chromatic contrast, as perceived by pollinators, between different parts of a flower and the background on which they grow, I plotted all reflectance spectra as loci in Troje's (1993) categorical colour vision model for each species. This colour vision model is based on the photoreceptor properties and spectral sensitivities of the house fly *Musca domestica* (Muscidae) (Hardie and Kirschfeld, 1983), and on behavioural experiments to determine wavelength discrimination in *Lucilia* sp. (Calliphoridae) (Troje, 1993). Troje (1993) proposed that flies have a categorical vision system in which colour is categorized into four quadrants, referred to as "ultraviolet", "blue", "green" and a "purple" category, which is mediated by the differences (whether positive or negative) between the excitations of the paired R7 and R8 cells. This model has been used for various pollinating fly families such as Syrphidae, Tabanidae, and Empididae (Jersáková et al., 2012; Ohashi et al., 2015; Shrestha et al., 2016). The basis of this categorical colour vision model relies on the four photoreceptor types used for colour vision in fly species (Troje, 1993) and calculates loci from the differences in relative excitations between the p-type (R7p and R8p) and the y-type (R7y and R87) receptors (Arnold et al., 2009). To compare the colours of floral parts to each other, I plotted the spectra of floral parts as loci in the fly colour vision model. Floral parts that plotted in different quadrants to the background substrate (wet and dry gravel in this instance) are suggested to be chromatically distinguishable from the gravel, according to the fly colour vision model, while those that plotted in the same quadrant as the background substrate are chromatically indistinguishable to the background substrate. Receptor excitations were calculated using standard daylight illumination (D65; Wyszecki and Stiles, 1982). I assumed that fly receptors would be adapted to the green leaf background of grasslands since these are grassland species. Therefore I used the spectrum of green foliage from a similar grassland at Midmar Nature Reserve in KwaZulu-Natal (Shuttleworth and Johnson, 2009b).

Scent chemistry

To identify the compounds that make up the scent bouquets of the study species (Table S5.1), including among populations (in the case of *C. peploides* and *C. vaginata*, which both grew abundantly at multiple sites and also received visitation by flies at high frequencies), floral volatiles were collected *in-situ* using dynamic headspace extraction methods during the 2021–2022 sampling seasons. Samples were taken by enclosing inflorescences of plants in Nalophan polyacetate bags (Kalle, Germany) and pumping air from the bags through a small glass cartridge (adsorbent trap) containing 1.5 mg each of Carbotrap® B (20-40 mesh) (Sigma-Aldrich Co.; St Louis, MO, USA) and Tenax® TA (60/80) (Supelco^{TM}; Bellefonte, PA, USA), which was attached to a mechanical portable pumping device (PAS500 Personal Air Sampler from Spectrex, Redwood City, CA, USA). A total of three to six inflorescences were sampled per species. Simultaneously, a control sample for each sampling session was taken from an empty polyacetate bag and used to identify background contaminants during analysis. The sampling duration for all samples and the corresponding controls was 50 minutes. Following sampling, the glass cartridges were detached from the pumping devices and placed separately in labelled glass vials, which were then stored at -20 °C prior to analysis.

Volatiles in samples were analysed by Gas Chromatography-Mass Spectrometry (GC-MS) following Shuttleworth and Johnson (2009b), with slight modifications. Saturated alkanes were also run on the GC-MS instrument, and were used to calculate linear *n*-alkane retention indices (Van Den Dool and Kratz, 1963). All analyses were performed on a Scion 436 gas chromatograph coupled with a Scion SQ single quadrupole mass spectrometer (Scion Instruments, Livingston, UK; Johnson et al., 2020). The GC was fitted with a Scion 1079 PTV injector port that was modified with a ChromatoProbe thermal desorption device. Volatiles were separated on an SGE SolGel Wax standard polar capillary column (Trajan Scientific and Medical, Melbourne, Australia) that was 30 m long and had an internal diameter of 0.25 mm and a film thickness of 0.25 µm, with helium as a carrier gas at a flow rate of 1 ml min^{-1}. The GC oven was programmed as follows: 40 ºC for 3 min, then raised to 240 ºC at 10 ºC min^{-1} and held at 240 ºC for 12 min. The injector was held at 40 ºC for 2 min with a 20:1 split and then increased to 200 ºC at 200 ºC min^{-1} and held in splitless mode for 2 min for thermal desorption, and then finally increased to 250 ºC at 200 ºC min^{-1} and held at 250 ºC with a 1:100 split for the rest of the run. Compounds were identified with the Scion MS Workstation v8.2.1 software in combination with the NIST mass spectral library (v2.4, 2020) and verified, where possible, using published Linear Retention Indices (LRIs) and by comparison with authentic standards. Two sets of LRIs are reported because samples were run on the GC-MS at different times, and because of column wear and change, retention times shifted. Relative amounts (%) of compounds, based on peak area, are reported according to compound classes following Knudsen et al. (2006) in the scent table. Compounds that could not be identified are listed with the base peak first, followed by remaining ion fragments in decreasing order of abundance. For some unknown compounds with typical ion fragments that are found in terpenoids, the mass spectrum was carefully analysed and compounds were either categorized under terpenoids or unknowns, based on a combination of typical ion fragments that define terpenoids. In addition, compounds that were present in the controls were considered to be background contaminants and were thus excluded from the analysis.

To compare scent profiles between species and study sites, I calculated Bray-Curtis similarity to accommodate the presence of a large number of zeros in the data (Clarke and Warwick, 2001). Prior to calculating Bray-Curtis similarity, I square-root transformed the proportion of each compound to down-weight the influence of dominant compounds (Clarke and Warwick, 2001). Differences in scent bouquets between species were visualised in a non-metric multidimensional scaling (NMDS) analysis that was based on Bray-Curtis similarity. Furthermore, to statistically compare differences in scent profiles among species, I implemented a one-way Analysis of Similarity (one-way ANOSIM) following 10 000 random permutations in PRIMER 6.1.6. This is a non-parametric permutational test based on the similarity matrix among samples (Somerfield et al., 2021) and generates the test statistic R values, with values closer to zero indicating minimal separation between species and values closer to unity indicating complete separation between species or groups (Clarke and Warwick, 2001). In addition, to determine which compounds characterize the scent of each species I performed a similarity percentage (SIMPER) analysis in PRIMER v6.1.6. This analysis calculates the contribution (in percentage) of each compound to average overall Bray-Curtis similarity between samples within a group (Clarke and Warwick, 2001). Compounds contributing 80% of Bray-Curtis similarity are reported for each species.

Floral rewards

To determine whether the studied plant species offer a reward in the form of nectar, the presence or absence of a nectar reward was investigated for six of the *Crassula* study species (see Table S5.1) in February 2022 and between January and February 2023, and repeated in February 2024 for *C. peploides* at Witsieshoek. Between 5 and 15 flowers from multiple inflorescences in each plant species were sampled for nectar and sugar quantification *ex-situ*. Where possible, we quantified the volume and sugar content of nectar (see Chapter 2 for a detailed explanation of the methods).

Inflorescences were excluded from visitors using fine mesh bags for a period of 24 hours, to allow accumulation of nectar. These pollinator-excluded inflorescences were then collected and maintained in water overnight. Because of the small sizes of the flowers, we could not use conventional methods for sampling nectar, and instead used the micro-rinse technique as outlined by Power et al. (2018). This was done by adding a 1 µl volume of water to the nectaries of individual flowers using a Gibson micropipette and allowing the water to sit for a maximum of 1 minute to dissolve the sugars. The resulting sugar solution was then carefully drawn up using a 1 µl microcapillary tube (microcaps®, Drummond Scientific Company, Pennsylvania, USA). The presence of sugars in the resultant sugar solution was determined using a Bellingham & Stanley 0–40% hand-held refractometer. Direct nectar sampling (without dilution) from flowers that were not pollinator-excluded was determined only for *C. peploides* flowers in the Witsieshoek population in February 2024 *in-situ* since the micro-rinse technique was not effective in this species (see Chapter 2). In addition, I noted the behaviour of flies on flowers to determine whether they consumed pollen or not.

Results

Floral morphology

Flowers of all species were characterized by relatively small size and shallow, open structure, and all had a floral diameter and functional floral depth of less than 8 mm (Table 5.1). Flowers of *C. peploides* had the largest diameter of up to 7 mm at both study sites, whereas flowers of *C. dependens* at Witsieshoek were the smallest, with an average diameter of 3 mm (Table 5.1). PC1 explained 51.59% of the observed variation, driven primarily by pistil length (loading = 0.59) and stamen length (loading = 0.57), whereas PC2 explained 24.24% of the variation, driven by sepal length (loading = 0.72) and flower diameter (loading = 0.67). Lastly, there was a highly significant difference in flower morphology between species (MANOVA: df = 55; $F = 48.35$, $P < 0.01$) and between populations (MANOVA: df = 10; $F = 5.16$, $P < 0.01$).

Table 5.1: Mean and standard deviation values of floral part measurements (in mm; $N = 8$) of studied plant species from one site in the southern Drakensberg (Naude's Nek) and two in the northern Drakensberg (Sentinel and Witsieshoek).

Species	**Site**	**Flower depth**	**Flower diameter**	**Pistil length**	**Stamen length**	**Sepal length**
C. dependens	Naude’s Nek	4.31 ± 0.24	3.60 ± 0.35	3.55 ± 0.09	3.59 ± 0.12	1.68 ± 0.13
C. dependens	Witsieshoek	4.86 ± 0.97	2.94 ± 0.57	3.81 ± 1.17	4.15 ± 1.03	2.19 ± 0.31
C. natalensis	Sentinel	3.58 ± 0.17	3.13 ± 0.15	3.14 ± 0.11	3.14 ± 0.07	1.04 ± 0.18
C. obovata var. *obovata*	Sentinel	3.33 ± 0.29	4.51 ± 0.39	1.85 ± 0.28	2.45 ± 0.33	2.03 ± 0.30
C. peploides	Naude’s Nek	2.78 ± 0.24	6.84 ± 0.71	1.16 ± 0.11	2.36 ± 0.09	1.76 ± 0.21
C. peploides	Witsieshoek	2.64 ± 0.15	7.16 ± 0.28	1.14 ± 0.17	1.59 ± 0.12	1.46 ± 0.09
C. sarcocaulis subsp. *rupicola*	Naude’s Nek	3.68 ± 0.16	4.21 ± 0.17	3.28 ± 0.15	3.28 ± 0.10	1.21 ± 0.12
C. sarcocaulis subsp. *rupicola*	Sentinel	3.76 ± 0.14	4.28 ± 0.23	3.13 ± 0.17	3.13 ± 0.13	1.13 ± 0.14
C. setulosa var. *setulosa*	Naude’s Nek	2.76 ± 0.09	3.05 ± 0.38	1.54 ± 0.13	1.68 ± 0.13	1.21 ± 0.12
C. setulosa var. *setulosa*	Sentinel	2.68 ± 0.13	2.50 ± 0.13	1.24 ± 0.09	1.45 ± 0.09	1.10 ± 0.13
C. vaginata	Sentinel	4.75 ± 0.12	4.55 ± 0.16	2.89 ± 0.15	3.15 ± 0.05	1.18 ± 0.09
T. imbricatum	Sentinel	4.14 ± 0.52	3.45 ± 0.34	1.28 ± 0.13	1.36 ± 0.09	1.59 ± 0.22

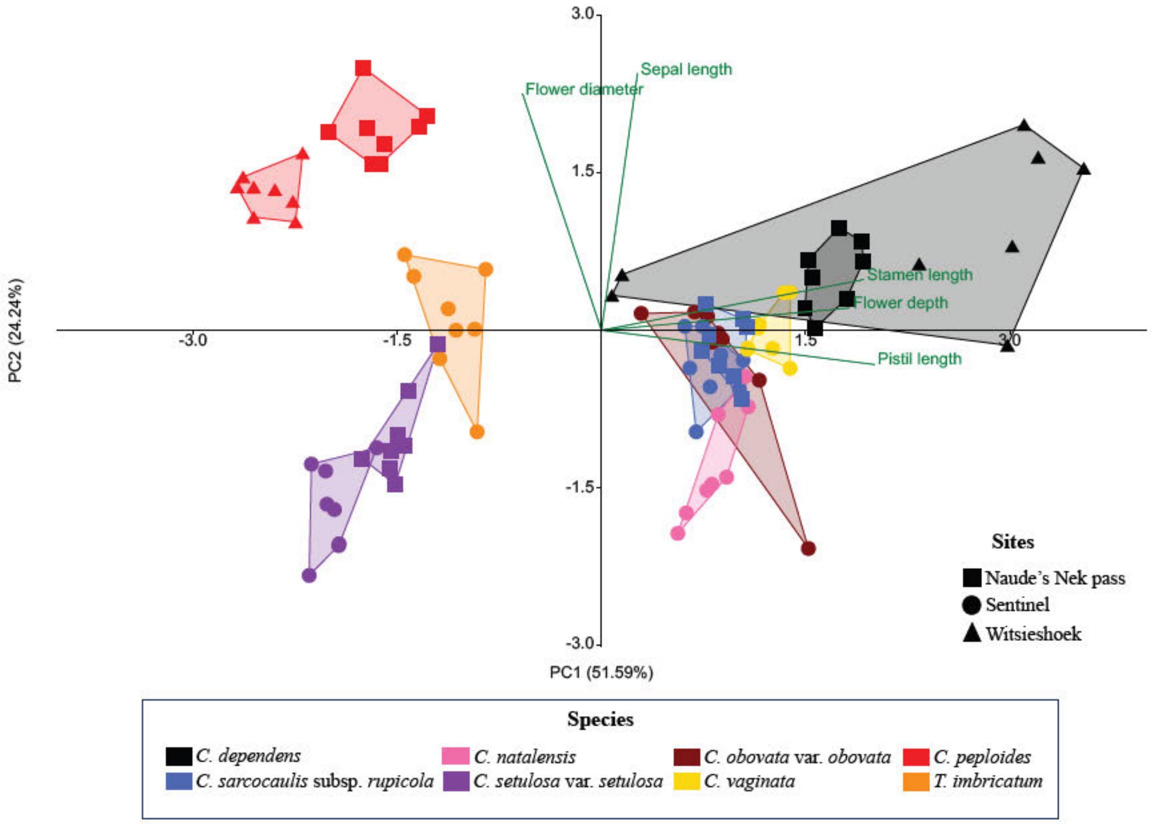

Figure 5.1: First two principal components (PC) of five floral morphological characters of seven studied guild members and *C. vaginata* across three sites. Individual plant species from each site are enclosed in convex hulls. The labelled green lines indicate the loading of eigenvectors on the two first PCs. Percentages in parentheses indicate variance explained per each PC. Symbol colours refer to species and symbol shapes refer to sites.

Floral colours and perception of colours by pollinators

The analysis of the spectral reflectance of studied species showed that the petals exhibited similar spectral reflectance curves (Figure S5.1). No species exhibited reflectance in the UV (300–400 nm) range (Figure S5.1). The spectral reflectance of petals was higher than all floral parts, increasing sharply around 400 nm (apart from *C. vaginata*, for which this increased at 500 nm), and remaining high throughout (Figure S5.1). Furthermore, the petals had a brighter reflectance compared to other floral parts, except in *C. natalensis*, *C. vaginata* and *C. sarcocaulis* subsp. *rupicola*, which did not exceed approximately 30% reflectance (Figures S5.1B, D and H). Overall, the petals had high chroma (pure chroma), while spectra of other floral parts were low in chroma (dull and impure; Figure S5.1).

Loci plotted within the fly-UV, fly-blue and fly-green quadrants of the fly colour vision model (Figure 5.2). Across all species, pollen, ovaries (except in *C. peploides*) and the white and yellow (in *C. vaginata*) petals plotted in a different quadrant to the gravel background loci (Figure 5.2), suggesting that they would be distinguishable from the background gravel. The red petal base and ovaries of *C. peploides* plotted with the background gravel, suggesting that these floral parts would not be chromatically distinguishable from the background gravel to flies (Figure 5.2E and F), but in this species, the petals, pollen and sepals are chromatically distinguishable from the background gravel. For all the species except *C. setulosa* var. *setulosa* and *C. vaginata*, the loci of the corolla, including the loci of the petal base of *C. peploides*, all plotted in the fly-blue quadrant (Figure S5.2), suggesting that these species will be perceived as the same in the eyes of the fly pollinators. Moreover, the loci of the corolla of *C. setulosa* var. *setulosa* and *C. vaginata* plotted in fly-green quadrant, suggesting these two species would be perceived as the same, but different from the other species, in the eyes of the fly pollinators (Figure S5.2). Overall, there are chromatic variations between floral parts and the background gravel for the different plant species, thus suggesting that fly visitors may be able to detect flowers against the background.

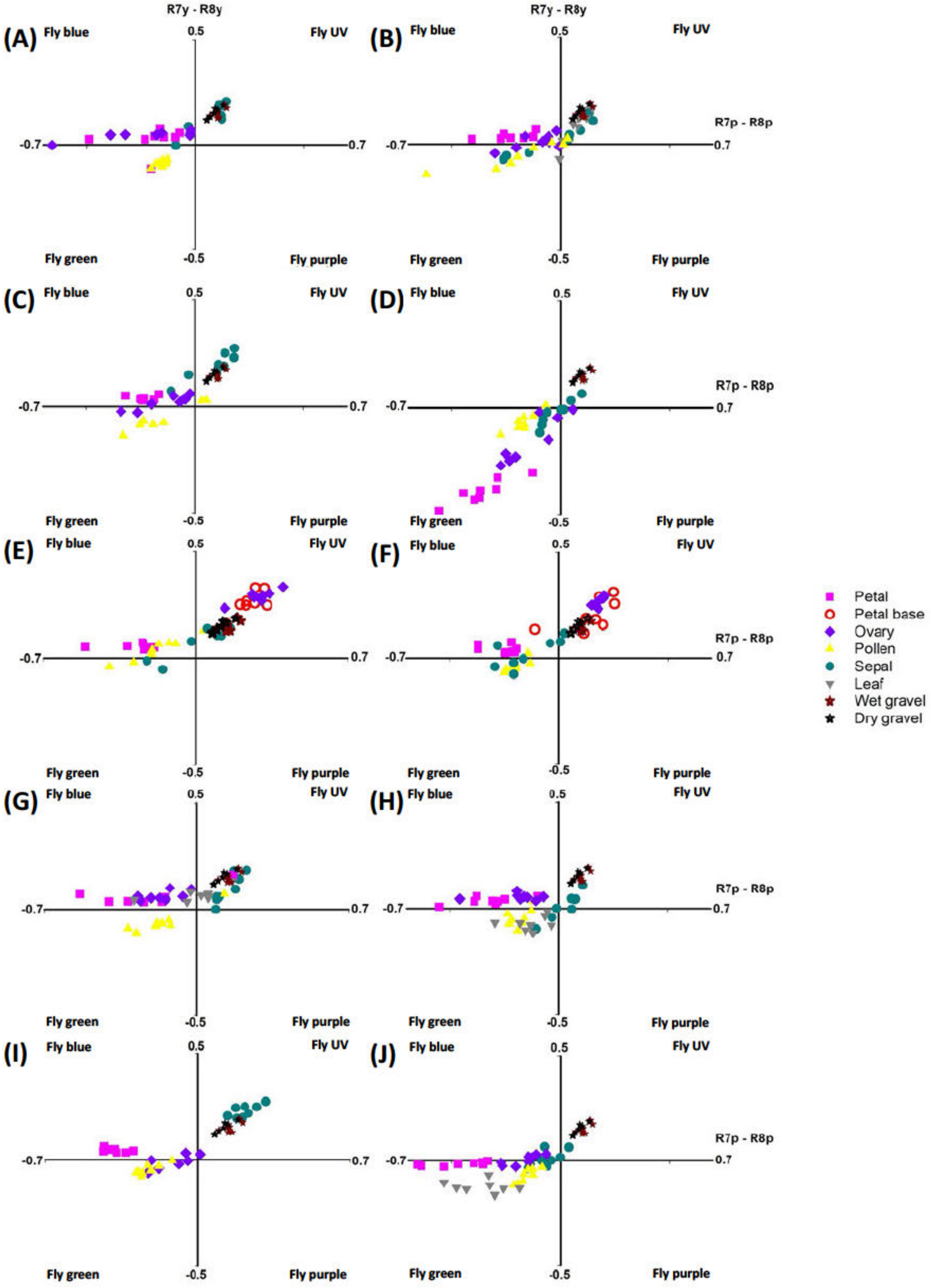

Figure 5.2: Floral colour loci of seven studied *Crassula* species, as perceived by flies according to the Troje (1993) categorical fly colour vision model. Loci within the same quadrant are not chromatically distinguishable from each other as perceived by flies. (A) *C. dependens* from Witsieshoek, (B) *C.*

natalensis from Sentinel, (C) *C. obovata* var. *obovata* from Sentinel, (D) *C. vaginata* from Witsieshoek, (E) *C. peploides* from Naude's Nek, (F) *C. peploides* from Witsieshoek and (G) *C. sarcocaulis* subsp. *rupicola* from Naude's Nek, (H) *C. sarcocaulis* subsp. *rupicola* from Sentinel, (I) *C. setulosa* var. *setulosa* from Naude's Nek and (J) *C. setulosa* var. *setulosa* from Sentinel. Assessed floral parts are distinguishable by the different colours.

Scent chemistry

The number of volatile compounds in floral headspace varied among species, ranging from two compounds in *C. dependens* to 43 compounds in *C. sarcocaulis* subsp. *rupicola* and *P. montana* (Tables 5.2, S5.2). Volatiles included 38 aliphatic, 22 aromatic, seven C5-branched chain, five miscellaneous cyclic, seven nitrogen-containing, and 36 terpenoid, as well as 95 unidentified compounds (Table S5.2).

Aliphatics dominated the scent profiles of *C. obovata* var. *obovata* (particularly hexanoic and butanoic acids, with a total relative amount of 50.88% ± 1.51), with a C5-branched chain compound also present in high amounts (2-methylbutyric acid; relative amount of 40.35% ± 6.98; Table S5.2). A total of 12 compounds, which includes these compounds plus other acids such as pentanoic, propanoic and isobutyric acids and *p*-cresol, accounted for the first 80% of Bray-Curtis similarity between samples of this species, with hexanoic acid characterizing this species the most (Table 5.2).

The scent of *C. peploides* was also dominated by aliphatics (particularly butanoic and isobutyric acids, totalling relative amount of 87.41% ± 3.94 at Witsieshoek and butanoic and (E)-2-butenoic acid, totalling relative amount of 66.37% ± 3.97 at Naude's Nek), with C5-branched chain compounds also present in higher amounts (particularly isovaleric acid in relative amounts of 5.47% ± 1.65 at Witsieshoek and 27.05% ± 10.77 at Naude's Nek; Table S5.2). At both populations of this species, these acids, plus *p*-cresol, accounted for the first 80% of Bray-Curtis similarity between samples of each population (Table 5.2).

The scent profile of *C. vaginata* varied between populations, with the scent of flowers from the Mt. Gilboa population dominated by aliphatics (relative amount: 35.4% ± 0.86), particularly octan-3-one (relative amount: 12.75% ± 4.81) and octan-1-ol (relative amount: 9.61% ± 3.02), while the Witsieshoek population was dominated by various aromatics (relative amount: 30.86% ± 1.33), particularly an isomer of methylanisole (relative amount: 12.74% ± 6.89; Table S5.2). At Mt. Gilboa, a total of 19 compounds comprising various aliphatics (including octan-3-one, octan-1-ol and hexanoic acid), aromatics (including benzyl and phenylethyl alcohols), miscellaneous cyclic compounds (δ-decalactone), terpenoids (including 4-oxoisophorone, linalool and (E)-4,8-dimethylnona-1,3,7-triene) and various unidentified compounds all accounted for the first 80% of Bray-Curtis similarity between samples of this population (Table 5.2). At Witsieshoek, 15 compounds of various aromatics (including m- or p-methylanisole and methyl benzoate), aliphatic (including acetic acid and octan-3-one), δ-nonalactone and various terpenoids (including α-terpineol, β-cubebene and (Z)-β-farnesene) and several unidentified compounds accounted for the first 80% of Bray-Curtis similarity between samples of this population (Table 5.2).

The scent profile of *C. natalensis* was dominated by aromatics (relative amount: 44.76% ± 1.72), particularly *p*-cresol (relative amount: 23.72% ± 8.21) and others in lesser amounts, an unidentified compound (unknown 10 in relative amount of 26.95% ± 7.75) and others in lesser amounts. These two compounds, along with nine other compounds including a C5-branched chain acid (2-methylbutyric acid), various other aromatics (including benzyl alcohol, benzyl acetate and salicylaldehyde) and an aliphatic (octan-3-one) accounted for the first 80% of Bray-Curtis similarity between samples of this species (Table 5.2).

The scent profile of *C. setulosa* var. *setulosa* was largely dominated by aromatics (relative amount: 75.69% ± 4.48), particularly *p*-cresol (relative amount: 47.23% ± 9.57), although this species also produced aliphatics (octan-3-one and methyl palmitate), one terpenoid (eucalyptol, which is unique to this species) and unidentified compounds (unknown 8 and 12) in lesser amounts (Table S5.2). Three of these compounds (*p*-cresol, eucalyptol and the unidentified compound, unknown 8), along with three further aromatics, accounted for the first 80% of Bray-Curtis similarity between samples of this species (Table 5.2).

The scent profiles of the remaining two species, *C. sarcocaulis* subsp. *rupicola* and *P. montana*, comprised the most compounds (43 compounds in each species) and were largely dominated by terpenoids (relative amount: 46.49% ± 1.30 and 89.14% ± 16.64, respectively). In *C. sarcocaulis* subsp. *rupicola*, the dominating terpenoid compounds included (E)-4,8-dimethylnona-1,3,7-triene (relative amount: 26.66% ± 3.63) and (Z)-4,8-dimethylnona-1,3,7-triene (relative amount: 7.16% ± 1.03), alongside aliphatics such as oct-1-en-3-ol (relative amount: 22.28% ± 4.14) and octan-3-one (relative amount: 4.54% ± 3.36). These compounds, including 11 other compounds of various aromatics (including methyl benzoate and phenylacetaldehyde), nitrogen-containing (including indole and benzyl nitrile), terpenoids (including β-pinene, β-caryophyllene and (-)-β-bourbonene) and an unidentified compound (unknown 9) accounted for the first 80% of Bray-Curtis similarity among samples of this species (Table 5.2). In the scent of *P. montana*, the terpenoid compound (Z)-ocimene dominated the scent bouquet (relative amount: 58.84% ± 15.3), in addition to other terpenoids (such as an unidentified sesquiterpene (unknown 3) in relative amounts of 21.08% ± 19.74, (E)-ocimene in relative amount of 6.5% ± 2.66 and linalool in relative amount of 1.56% ± 0.67) and nitrogen-containing compounds (such as skatole in relative amounts of 0.47% ± 0.28). These compounds, along with an additional 16 compounds of terpenoids and unidentified compounds, accounted for the first 80% of Bray-Curtis similarity between samples of this species (Table 5.2).

Species occupied distinct areas in the non-metric multidimensional scaling plot (NMDS; Figure 5.3), although some clustering can be seen between *C. peploides* and *C. obovata* var. *obovata*. In addition, the scent profiles were similar between the two studied populations (Witsieshoek and Naude's Nek) of *C. peploides* (Figure 5.3), which were both dominated by aliphatic acids (Table 5.1), whereas each studied *C. vaginata* population exhibited its own distinct profile (Figure 5.3), which were, respectively, dominated by aromatics (at Witsieshoek) and aliphatics (at Mt. Gilboa; Table S5.2). This clear distinction of scent profiles was further indicated by pairwise R values close to 1 between species, which were all significantly different (Table S5.3).

Table 5.2: Compounds characterizing the scent of seven studied *Crassula* species and *P. montana* based on SIMPER analysis. Only the compounds contributing to the first 80% of Bray-Curtis similarity are included in the table. Abbreviations are as follows: % = contribution to overall Bray-Curtis similarity between samples within each species or population in percentage, Sim/SD = % contribution divided by standard deviation. Compounds that best characterize a species' scent are indicated by high % contributions and sim/SD. MRA = Mean relative amount, indicates % of headspace based on peak area. Mass fragments for unknowns are presented with the base peak first, followed by remaining fragments in the decreasing order of abundance. Compounds whose values are not presented for a particular species may still have been produced but did not contribute to the first 80% similarity in the SIMPER analysis (see Table S5.2 for the complete scent profiles of species).

Compound	***C. dep* (Witsieshoek)**		***C. nat* (Sentinel)**		***C. obo* (Sentinel)**		***C. pep* (Witsieshoek)**		***C. pep* (Naude's Nek)**		***C. sar* (Sentinel)**		***C. set* (Sentinel)**		***C. vag* (Witsies)**		***C. vag* (Mt. Gilboa)**		***P. mon* (Sentinel)**	
	% (Sim/SD)	MNR (range)	% (Sim/SD)	MNR (range)	% (Sim/SD)	MNR (range)	% (Sim/SD)	MNR (range)	% (Sim/SD)	MNR (range)	% (Sim/SD)	MNR (range)	% (Sim/SD)	MNR (range)	% (Sim/SD)	MNR (range)	% (Sim/SD)	MNR (range)	% (Sim/SD)	MNR (range)
Aliphatic compounds																				
Acids																				
Acetic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5.8 (1.15)	3.3 (2.86-6.24)	-	-	-	-
Propanoic acid	-	-	-	-	2.1 (2.92)	0.6 (0.19-0.18)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Isobutyric acid	-	-	-	-	2.0 (14.17)	0.6 (0.21-1.47)	14.9 (2.73)	11.5 (4.16-16.54)	3.9 (10.31)	0.7 (0.53-0.81)	-	-	-	-	-	-	-	-	-	-
Butanoic acid	-	-	-	-	12.9 (5.51)	12.9 (6.81-16.60)	44.6 (23.70)	66.8 (58.27-77.49)	34.2 (6.06)	52.1 (38.91-62.77)	-	-	-	-	-	-	-	-	-	-
Pentanoic acid	-	-	-	-	3.2 (3.25)	1.1 (0.37-2.07)	-	-			-	-	-	-	-	-	-	-	-	-
(E)-2-Butenoic acid	-	-	-	-	-	-	-	-	8.9 (1.95)	8.8 (1.64-16.12)	-	-	-	-	-	-	-	-	-	-
Hexanoic acid	-	-	-	-	20.0 (11.81)	29.6 (20.17-38.99)	-	-	2.6 (1.66)	2.3 (0.05-7.98)	-	-	-	-	-	-	1.7 (2.33)	0.6 (0.12-1.03)	-	-
(E)-3-Hexenoic acid	-	-	-	-	5.5 (1.51)	5.2 (0.45-9.09)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(Z)-3-Hexenoic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.6 (5.28)	1.1 (0.19-3.47)	-	-
Nonanoic acid	-	-	-	-	-	-	-	-	4.2 (4.22)	0.9 (0.46-1.17)	-	-	-	-	-	-	-	-	-	-
Alcohols																				
Octan-3-ol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6.5 (2.33)	7.9 (2.05-12.23)	-	-

Oct-1-en-3-ol	-	-	-	-	-	-	-	-	-	-	12.7 (4.40)	22.3 (9.86-37.52)	-	-	-	-	-	-	-	-
(Z)-Oct-2-en-1-ol	-	-	-	-	-	-	-	-	-	-	3.0 (7.52)	1.2 (0.69-2.29)	-	-	-	-	-	-	-	-
3-Isopropylpentan-1-ol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5.4 (1.16)	2.9 (2.34-5.30)	-	-	-	-
Octan-1-ol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7.5 (4.72)	9.6 (4.40-17.71)	-	-
(E)-Oct-2-en-1-ol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.7 (6.89)	1.0 (0.52-1.30)	-	-
Esters																				
(Z)-Hex-3-en-1-yl acetate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.4 (0.86)	0.4 (0.02-0.89)
Ketones																				
Octan-3-one	-	-	5.6 (2.63)	6.3 (0.83-25.78)	-	-	-	-	-	-	4.5 (0.92)	7.5 (14.03-52.75)	-	-	6.0 (2.96)	2.7 (0.56-8.19)	8.7 (13.24)	12.7 (7.32-27.16)	-	-
Oct-3-en-2-one	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.9 (5.37)	2.1 (1.08-3.04)	-	-
Aromatics																				
o-Methylanisole											2.9 (3.18)	1.4 (0.41-2.58)								
m- or p-Methylanisole			4.1 (2.28)	56.0 (0.16-4.83)											8.5 (1.11)	12.7 (5.24-38.97)				
Methyl benzoate	62.4 (2.32)	66.2 (10.45-89.13)	-	-	-	-	-	-	-	-	4.2 (10.29)	2.2 (1.37-3.95)	-	-	6.8 (1.18)	5.3 (0.14-12.28)	-	-	-	-
Phenylacetaldehyde	-	-	-	-	-	-	-	-	-	-	6.3 (5.83)	4.9 (2.64-7.04)	-	-	-	-	-	-	-	-
Salicylaldehyde	-	-	5.1 (2.65)	2.1 (0.33-3.63)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Benzyl acetate	-	-	5.8 (1.96)	4.1 (0.71-10.20)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl phenylacetate	-	-	4.3 (3.75)	1.6 (0.38-3.61)	-	-	-	-	-	-	-	-	-	-	-	-	1.8 (13.77)	0.4 (0.30-0.64)	-	-
Benzyl alcohol	-	-	9.9 (3.05)	8.1 (1.65-18.76)	-	-	-	-	-	-	-	-	12.0 (5.34)	7.9 (3.55-17.83)	-	-	4.5 (2.05)	6.3 (0.61-15.28)	4.7 (1.08)	3.2 (0.95-8.38)
Phenylethyl alcohol	-	-	-	-	-	-	-	-	-	-	-	-			-	-	5.4 (16.92)	3.5 (2.74-4.56)		
p-Cresol	-	-	12.8 (1.58)	23.7 (0.90-55.68)	2.8 (1.82)	2.2 (0.28-5.73)	11.9 (6.38)	5.7 (3.78-8.25)	3.8 (1.15)	5.0 (0.05-14.76)	-	-	29.4 (2.32)	47.2 (6.95-74.01)	-	-	-	-	-	-
1,3,5-Trimethoxybenzene	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.3 (0.62)	2.6 (3.42-4.77)	-	-	-	-

4-Aminoacetophenone (= p-Aminoacetophenone)	-	-	3.6 (2.08)	1.7 (0.17-4.71)	-	-	-	-	-	-	-	-	11.0 (2.46)	9.6 (1.60-67.44)	5.7 (0.62)	8.7 (11.30-20.00)	-	-	-	-
2-Aminoacetophenone (= o-Aminoacetophenone)	-	-	-	-	-	-	-	-	-	-	-	-	12.3 (3.87)	7.7 (2.41-14.03)	-	-	-	-	-	-
C5-branched chain compounds																				
Acids																				
2-Methylbutan-1-ol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.7 (0.91)	4.3 (5.59-6.05)	-	-
2-Methylbutyric acid	-	-	7.5 (2.72)	9.3 (1.29-33.67)	22.8 (7.27)	40.4 (25.77-58.80)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Isovaleric acid	-	-	-	-	-	-	10.7 (4.53)	5.5 (2.62-8.35)	24.2 (6.73)	26.6 (18.51-32.53)	-	-	-	-	-	-	-	-	-	-
Miscellaneous cyclic compounds																				
γ-Caprolactone	-	-	-	-	2.3 (8.10)	1.1 (0.26-3.25)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
δ-Nonalactone	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5.8 (0.62)	14.0 (9.73-43.23)	-	-	-	-
δ-Decalactone	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5.8 (5.94)	8.1 (2.64-19.50)	-	-
Nitrogen-containing compounds																				
Benzyl nitrile	-	-	-	-	-	-	-	-	-	-	3.1 (4.57)	1.5 (0.51-2.88)	-	-	-	-	-	-	1.4 (1.10)	0.2 (0.08-0.43)
Indole	-	-	-	-	-	-	-	-	-	-	4.3 (5.22)	2.5 (0.92-4.54)	-	-	-	-	-	-	-	-
Skatole	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.7 (1.14)	0.5 (0.16-1.56)
Terpenoids																				
Irregular terpenes																				
(Z)-4,8-Dimethylnona-1,3,7-triene	-	-	-	-	-	-	-	-	-	-	7.5 (4.48)	7.2 (2.37-10.79)	-	-	-	-	-	-	-	-
(E)-4,8-Dimethylnona-1,3,7-triene	-	-	-	-	-	-	-	-	-	-	14.7 (5.98)	26.7 (11.57-93.95)	-	-	-	-	4.3 (2.04)	4.5 (1.07-8.54)	-	-

4-Oxoisophorone	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.6 (6.89)	2.3 (0.30-5.85)	-	-
2,2,6-Trimethylcyclohexane-1,4-dione	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.5 (2.88)	1.5 (0.28-3.47)	-	-
6,10-Dimethylundeca-5,9-dien-2-one	37.6 (4.47)	33.8 (10.87-89.55)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6,10,14-Trimethylpentadecan-2-one	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.2 (4.04)	1.2 (0.36-2.97)	-	-
Monoterpenes																				
β-Thujene	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.2 (0.83)	1.0 (0.08-2.67)
β-Pinene	-	-	-	-	-	-	-	-	-	-	1.8 (1.34)	0.8 (0.23-1.76)	-	-	-	-	-	-	2.5 (1.15)	0.5 (0.36-1.14)
Eucalyptol	-	-	-	-	-	-	-	-	-	-	-	-	9.6 (3.48)	8.7 (1.84-28.53)	-	-	-	-	-	-
(Z)-Ocimene[1]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	30.1 (1.15)	58.8 (63.61-88.93)
(E)-Ocimene[1]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6.4 (0.80)	6.5 (0.30-11.97)
γ-Terpinene	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.8 (1.15)	0.3 (0.19-0.89)
2,6-Dimethylocta-2,4,6-triene	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4.0 (1.15)	1.4 (0.88-2.87)
p-Mentha-1,5,8-triene	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.6 (1.15)	0.3 (0.06-0.61)
Linalool	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4.5 (1.11)	2.1 (1.35-3.56)	7.6 (3.91)	9.8 (3.37-16.06)	3.3 (0.96)	1.6 (0.27-3.43)
α-Terpineol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6.2 (1.26)	7.0 (0.35-23.63)	-	-	-	-
p-Mentha-1,5-dien-8-ol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.6 (1.15)	0.2 (0.16-0.38)
Sesquiterpenes																				
(-)-β-Bourbonene	-	-	-	-	-	-	-	-	-	-	3.0 (4.35)	1.3 (0.48-2.32)	-	-	-	-	-	-	-	-
β-Cubebene	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4.7 (1.06)	3.3 (1.07-10.24)	-	-	-	-
β-Caryophyllene	-	-	-	-	-	-	-	-	-	-	2.4 (3.81)	1.3 (0.30-4.02)	-	-	-	-	-	-	-	-
Humulene	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.7 (1.16)	0.2 (0.19-0.46)

(Z)-β-Farnesene	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.5 (1.96)	2.6 (0.07-11.28)	-	-	-	-
Unknown 3 (m/z: 186, 147, 204, 105, 119, 91)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6.9 (1.39)	1.1 (0.41-3.34)
Unknown																				
Unknown 1 (m/z: 43, 98, 71, 57, 69, 58)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5.7 (11.56)	4.6 (2.80-7.85)	-	-
Unknown 2 (m/z: 119, 93, 105, 91, 77, 92)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.8 (1.12)	2.0 (0.82-5.12)	-	-	-	-
Unknown 9 (m/z: 91, 92, 122, 65, 77, 51)	-	-	-	-	-	-	-	-	-	-	7.6 (2.79)	10.1 (2.20-20.69)	-	-	-	-	-	-	-	-
Unknown 10 (m/z: 91, 43, 162, 65, 119, 94)	-	-	17.1 (2.45)	27.0 (6.09-63.23)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Unknown 11 (m/z: 107, 105, 161, 93, 91, 109)	-	-	4.4 (1.17)	3.2 (8.28-22.29)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Unknown 12 (m/z: 107, 85, 57, 109, 108, 81)	-	-	-	-	-	-	-	-	-	-	-	-	11.6 (3.97)	6.7 (2.58-9.80)	-	-	-	-	-	-
Unknown 13 (m/z: 166, 135, 63, 165, 122, 137)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5.6 (0.85)	10.3 (0.23-36.37)	-	-	-	-
Unknown 14 (m/z: 196, 165, 122, 137, 63, 138)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5.2 (0.62)	8.7 (9.31-21.30)	-	-	-	-
Unknown 16 (m/z: 91, 161, 105, 93, 79, 94)*	-	-	-	-	-	-	-	-	-	-	2.2 (3.44)	0.8 (0.22-2.03)	-	-	-	-	-	-	-	-

Unknown 17 (m/z: 43, 41, 68, 39, 69, 40)*	-	-	-	-	2.6 (12.61)	0.5 (0.35-0.65)	-	-	-	-	-	-	-	-	-		-	-	-	-
Unknown 18 (m/z: 43, 55, 71, 41, 107, 149)*	-	-	-	-	2.5 (8.83)	0.5 (0.30-0.97)	-	-	-	-	-	-	-	-	-		-	-	-	-
Unknown 19 (m/z: 109, 135, 177, 41, 107, 149)*	-	-	-	-	2.3 (3.94)	0.7 (0.22-1.61)	-	-	-	-	-	-	-	-	-		-	-	-	-
Unknown 20 (m/z: 110, 81, 68, 67, 54, 82)*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.3 (2.41)	1.0 (0.21-1.27)	-	-
Unknown 21 (m/z: 109, 152, 91, 79, 137, 81)*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.5 (1.09)	0.6 (0.28-1.33)
Unknown 22 (m/z: 109, 152, 91, 79, 81, 77)*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.2 (1.10)	0.4 (0.21-0.65)
Unknown 23 (m/z: 82, 71, 67, 79, 81, 53)*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.7 (1.15)	0.22 (0.17-0.37)
Unknown 24 (m/z: 161, 105, 119, 91, 133, 120)*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.3 (0.94)	0.4 (0.04-1.31)
Unknown 25 (m/z: 192, 91, 19, 65, 77, 79)*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.2 (0.96)	0.3 (0.05-0.70)
Total	100		80.2		81		82.1		81.8		80.2		85.9		80.8		81		80.2	
Average similarity	78		65.5		77.6		75.8		76.5		76		73		47.2		70.8		49.21	
Number of compounds	2		11		12		4		7		15		6		15		19		20	

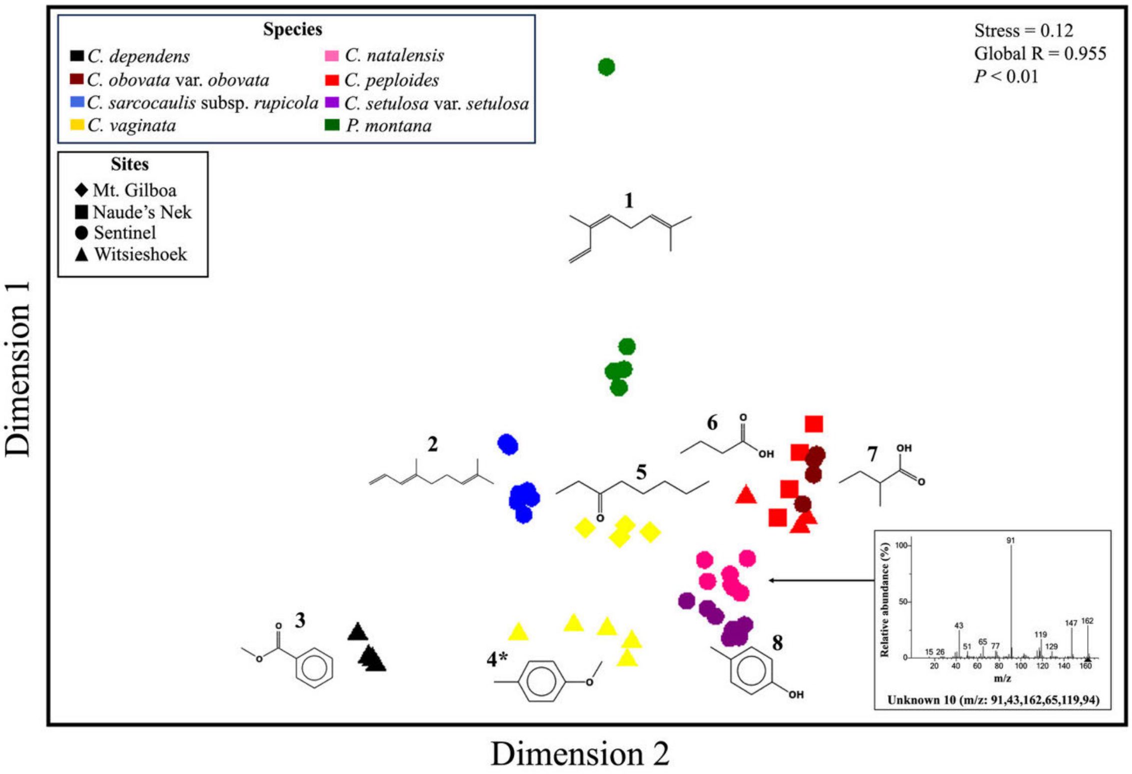

Figure 5.3: Non-metric multidimensional scaling (NMDS) ordination based on Bray-Curtis similarities of the scent profiles of seven *Crassula* and one *Polemannia* species from four different study sites. Note that symbol colours refer to species and symbol shapes refer to sites. Chemical structures refer to compounds that contributed most to the first 80% of Bray-Curtis similarity for each species following SIMPER analysis: 1, (Z)-ocimene; 2, (E)-4,8-dimethylnona-1,3,7-triene; 3, methyl benzoate; 4, p-methylanisole (the asterisk (*) denotes that this could also equally be m-methylanisole, but the compound with the best library match is indicated); 5, 3-octanone; 6, butanoic acid; 7, 2-methylbutyric acid; 8, *p*-cresol. See Table S5.3 for the pairwise ANOSIM contrasts between the species.

Nectar reward

The presence of sugar diluted in solutions obtained from rinsing flowers was confirmed for all seven of the studied plant species for which this was done (Table S5.1). During field observations, visitors were observed lapping at an apparent liquid substance on the shiny surface of the studied flowers, particularly in *C. peploides* and *P. montana*, thereby suggesting the potential presence of a reward. The presence of sugar content was further confirmed in *C. peploides* flowers in which nectar was directly sampled (mean sugar concentration: 6.92% ± 7.36%; range: 0.0 18.0%; $N = 18$), although this nectar was present in very small volumes (mean nectar volume: 0.19 µl ± 0.24 µl; range: 0.0–0.69 µl; $N = 18$; see Chapter 2). Visitors were never observed to collect or consume pollen as a reward.

Discussion

In this descriptive study, I quantified the floral traits of *C. vaginata* and several plant species which belong to the guild of species that are pollinated by saprophilous, short-tongued flies in high-elevation regions within South Africa. The results show that flowers of guild members are generally very small, but that floral morphologies are significantly different between species, with flower diameter and pistil length contributing most to the variation, and clustering of the species from multiple sites was visible in the PCA. Floral parts showed clear colour contrasts based on the fly colour vision model, but in general, floral colour was similar among the different guild species. Furthermore, some floral parts contrasted chromatically with the background gravel of their locality. The scent profiles of guild members were different, with the scent profiles of *C. obovata* var. *obovata* and *C. peploides* dominated by aliphatic acids, that of *C. dependens* and *C. vaginata* dominated by aromatics including *p*-cresol and an isomer of methylanisole. Surprisingly, scent profiles of *C. sarcocaulis* subsp. *rupicola* and *P. montana* were dominated by various terpenoids such as (E)-4,8-dimethylnona-1,3,7-triene and (Z)-ocimene, although they also included nitrogen-containing compounds such as indole and skatole. Overall, all species emitted scent compounds that are likely to be mimicking degrading substrates that are attractive to saprophilous, short-tongued flies. Lastly, the presence of nectar was confirmed in several guild members, confirming that flowers are rewarding.

Flowers of all guild members are characterised by a similarly small and open structure, with all species having flowers of less than 8mm diameter and 5mm functional floral depth, potentially facilitating interactions with small-proboscid pollinator species (Anderson and Johnson, 2009; Newman and Johnson, 2021). The studied guild members are overall characterized by open floral morphologies similar to those in other Crassulaceae members (for example, *Aeonium* species; dos Santos et al., 2024), but in contrast to the tubular *Crassula* species which are pollinated by moths and butterflies (Johnson et al., 1993; Johnson and Bond, 1994). The relatively small and open structure of flowers of the studied species suggests the lack of apparent morphological filters that would otherwise filter other animal pollinators from visiting these species. Despite the general similarity in size, however, multivariate analyses revealed significant differences among floral morphologies of the guild members, with three clusters evident in the PCA. Despite this variation, the flowers of guild members remain quite similar, and the inclusion of other closely related species adapted to different pollinators, such as the moth- and butterfly-pollinated *Crassula* species, in the analysis would likely reveal stronger similarities and a pattern of convergence among these guild members. All captured visitors to these plant species carried both congeneric and heterospecific pollen, which may suggest that there may be some specific pollen placement on the visitors, which may be consistent with the observed variation in floral morphologies of these guild members.

Floral colour is often one of the most important traits involved in attracting pollinators (Streinzer et al., 2021) and species that share pollinators often display similarities in floral colour. My results showed clear chromatic contrasts in the fly colour vision model between floral parts, such as petals and pollen and ovaries, and the gravel substrate on which several of these flowers grow (despite utilizing flies from a similar functional group, these guild members have different microhabitats; see Table S5.1). My results therefore suggest that visual signals play a role in the attraction of flies to guild members. Indeed, other studies have shown that flowers that exhibit clear contrast with the background on which they grow received more frequent visitation by pollinating insects than inconspicuous flowers (Rohde et al., 2013). The flower colour of the guild members deviates from those that are characteristic for sapromyiophilous flowers, which typically display dark brown colours with purple markings, such as in *Rafflesia* (Rafflesiaceae; Barkman et al., 2008) and stapeliads (Johnson and Jürgens, 2010; Jürgens et al., 2006). Our studied guild members, however, display white (with some red floral parts in some species) and greenish-white flowers (to the human eye), which do not conform to the typical sapromyiophilous syndrome (see Faegri and van der Pijl, 1979 and Vogel, 1954), despite being visited and pollinated by saprophilous, short-tongued flies. The species studied here rather show similarities to the flower colour of previously studied species that are pollinated by saprophilous, short-tongued flies (Johnson et al., 2020; Shuttleworth and Johnson, 2009b; Thupsie, 2023; van der Niet et al., 2010). Of particular interest is that in the *Crassula* species with white petals and red petal bases and ovaries, the overall inflorescence broadly resembles the typical pattern of a dark spot surrounding by a pale background that is seen in some fly-pollinated flowers. A similar colour pattern is found in some other fly-pollinated plants (du Plessis et al., 2018; Eisikowitch, 1980; Johnson et al., 2020; Johnson and Schiestl, 2016). The red petal bases and ovaries with white petals in some of the *Crassula* species, which may resemble a dark spot on a pale background, may mimic resting flies, which potentially functions to attract other flies seeking mates (Johnson and Midgley, 1997). Some studies have shown that dark spots increase attraction of flies, including Muscidae (Eisikowitch, 1980), Calliphoridae and Sarcophagidae (Johnson et al., 2020), Tachinidae (Dodson, 1962) and Bombyliidae (Johnson and Dafni, 1998), but in *Ceropegia mixta* (Apocynaceae) the black spots did not increase attraction of flies (du Plessis et al., 2018). Nevertheless, chromatic contrasts of some floral parts, such as petals and pollen to the background gravel, suggest that these floral parts may be important for saprophilous, short-tongued flies to differentiate between flowers at greater distances, while the combination of other cues may be of greater importance for detection at closer distances, although this effect is scope for future research.

Floral scent is thought to play a key role in pollinator attraction, particularly in saprophilous fly-pollinated plants (Chen et al., 2015a, 2015b; Cossé and Baker, 1996; du Plessis et al., 2018; Jürgens et al., 2006; Moré et al., 2019). In this study, the plant species produced highly diverse scent bouquets that varied in relative dominance of compounds among plant species (Table 5.2; Figure 5.3). Some species were dominated by aliphatic acids, particularly isovaleric, hexanoic and butanoic acids, while some, including *C. dependens*, *C. setulosa* var. *setulosa*, *P. montana* and *C. sarcocaulis* subsp. *rupicola*, did not produce any acids (except 2-methylbutyl isovalerate in trace amounts in *C. sarcocaulis* subsp. *rupicola*). Some species produced compounds such as *p*-cresol, indole and skatole; all these compounds are associated with degrading organic substrates and are attractive to flies (Erhardt, 1993; Shuttleworth, 2016; Urru et al., 2011; van der Niet et al., 2010). Contrastingly, some plant species were dominated by terpenoids, including (Z)-ocimene, (E)-4,8-dimethylnona-1,3,7-triene and linalool. The dominance of certain compounds, as indicated in this study by the area under the peak, does not necessarily equate to functional importance regarding attracting pollinators. These results, nonetheless, suggest that the studied plant species may have evolved diverse scent profiles, similar to other plant genera (see Dobson et al., 1997; Moré et al., 2021), to attract the observed a wide variety of saprophilous, short-tongued flies and beetles (see Chapter 4). Another alternative may be that majority of the studied species in this study are congenerics and closely related, therefore it is likely that these species have similar scent profiles, which could explain the common attraction of saprophilous, short-tongued flies that dominated the visitor assemblage.

One of the key findings from this study was the presence of carboxylic acid compounds in the scent profiles of five of the study species, including *C. natalensis*, *C. obovata* var. *obovata*, *C. peploides*, *C. sarcocaulis* subsp. *rupicola* and *C. vaginata*. These acids were primarily fatty-acid derivatives (with a few exceptions, such as isovaleric acid and 2-methylbutyl isovalerate: C5-branched chain compound). The presence of these compounds could explain the foetid and unpleasant odour (at least to the human nose) in these guild members (Jürgens et al., 2013, 2006). Specifically, carboxylic acids dominated the scent profiles of both populations of *C. peploides* (Witsieshoek and Naude's Nek; aliphatic-derived butanoic acid: 66.80% and 52.13%, respectively) and *C. obovata* var. *obovata* (2-methylbutyric acid: 40.35%). Butanoic acid has previously been found in pig manure, where it was determined to be one of the compounds emitted that elicited a behavioural response in *M. domestica* (Muscidae; Cossé and Baker, 1996). Similarly, butanoic acid, along with other acids including propanoic, acetic and hexanoic acid and aromatics such as *p*-cresol, has recently been found in high relative amounts in the fly-pollinated *Ceropegia pulchellior* (Apocynaceae; Heiduk et al., 2024). Isovaleric acid (C5-branched chain compound) was also found in higher amounts (26.61%) in *C. peploides* at Naude's Nek. The occurrence of isovaleric acid in floral scent is known from relatively few studies, where it constitutes the unpleasant scent produced by foul-smelling flowers (Kite, 1995; Shuttleworth, 2016, 2017; van der Niet et al., 2010), which are also pollinated by flies (Kite and Smith, 1997). These carboxylic acids likely represent relevant cues for flies to find degrading substrates and these compounds are likely to represent key signals for the attraction of these flies by flowers (see Cossé and Baker, 1996; Jürgens et al., 2006; Kite and Smith, 1997).

The presence of hexanoic acid in the scent profile of *C. obovata* var. *obovata*, *C. natalensis*, *C. peploides* (at Naude's Nek) and in relatively small amounts in *C. vaginata* (at Mt. Gilboa) is associated with the smell of urine (to the human nose). The and production of this acid by flowers has been suggested to be a way of mimicking urine (Jürgens et al., 2006), although this idea has been met with scepticism as it is not clear if this would be the only possible model that emits this acid (Urru et al., 2011). Whether the presence of carboxylic acids in these studied plants constitutes urine-mimicry is uncertain. The functional role of hexanoic and other acids, as well as other scent compounds, in this system warrants further research involving gas chromatography-electroantennographic detection (GC-EAD) experiments to determine the functional role of these carboxylic acids. This is particularly important because the observed visitor assemblages of the acid-dominated guild members are not unique to these species, but are also evident in other guild members, thereby potentially suggesting that acids may not play a significant role of attraction in this system. Nonetheless, this study presents a rare documentation of acid-dominated scents in fly-pollinated species in Crassulaceae.

The scent chemistry of the *C. vaginata* population from Mt. Gilboa is particularly intriguing, showing dominance of almost equal relative amounts of an aliphatic ester (methyl 2-methylbutyrate: 9.54%), aliphatic ketone (octan-2-one: 12.75%) and a monoterpene (linalool: 9.82%). This population of *C. vaginata* was visited predominantly by beetles (Coleoptera), in comparison to the Drakensberg populations (see Chapter 4). Similar patterns of the emission of monoterpenes, such as linalool, associated with beetle pollination at the same site (Mt. Gilboa) were reported by Steenhuisen et al. (2010) for *Protea* species (Proteaceae). Indeed, beetles are commonly attracted to linalool, amongst other compounds (Bengtsson et al., 2009; Dötterl and Gershenzon, 2023) and various other monoterpenes have been found in the scent profiles of beetle-pollinated flowers (Jürgens, 2009; Shuttleworth and Johnson, 2010). Ultimately, this correlation may explain the association between the presence of linalool and recorded visitors in this *C. vaginata* population and may also be influenced by the relative abundance of beetles at Mt. Gilboa.

Various aromatic compounds are commonly produced by plants (Rosenkranz and Schnitzler, 2016). In my results, aromatics dominated the scent profile of four species; in particular, the aromatic compounds that dominated were *p*-cresol in *C. natalensis* and *C. setulosa*, methyl benzoate in *C. dependens* and an isomer of methylanisole in *C. vaginata* at Witsieshoek. The aromatic *p*-cresol is a well-documented compound that is commonly associated with dung-like odours (Johnson and Jürgens, 2010; Jürgens et al., 2006; Kite, 1995; Shuttleworth, 2016; van der Niet et al., 2011) and flowers mimicking oviposition sites (Jürgens et al., 2013). Indeed, the attractiveness of *p*-cresol to muscid flies that are ecologically associated with dung (Johnson et al., 2020; Kite, 1995) has been shown previously (Jeanbourquin and Guerin, 2007). Based on the large relative amounts of *p*-cresol found compared to other compounds in *C. natalensis* and *C. setulosa* in this study, I infer that this compound may be functionally important in attracting flies to these species, although future research confirming its attractiveness needs to be explored (see Jeanbourquin and Guerin, 2007). On the other hand, many of the additional compounds in the scent of these studied plants occurred in relatively small amounts compared to the dominant compounds. This warrants further investigation to understand the role of these minor compounds. The aromatic methyl benzoate was one of two compounds, and the most dominant (relative amount of 66.23% ± 14.66) compound found in *C. dependens* and was also found in *C. sarcocaulis* subsp. *rupicola* (relative amounts: 2.21% ± 0.35). Methyl benzoate was also found in relatively high amounts in two *Schizochilus* species (Orchidaceae) that occur at high elevation in South Africa (van der Niet et al., 2010), and has been found in high amounts in moth-pollinated plants (Knudsen and Tollsten, 1993), where it has been shown to elicit a behavioural response (Plepys et al., 2002). I nevertheless did not observe any moths visiting *C. dependens* in this study. However, I did record two individual noctuid moths on this species during the daytime. On the other hand, I did record moths during nocturnal observations on *C. sarcocaulis* subsp. *rupicola*, which also produced the compound methyl benzoate.

Unlike many fly-pollinated flowers, especially deceptive flowers, the studied plant species do produce nectar, albeit in minute quantities. The presence of nectar supported observations of fly behaviour involving lapping at a shiny surface on the flowers. Based on this, I confirm that this particular fly pollination system is rewarding. The presence of nectar has previously been reported in some other fly-pollinated flowers, such as stapeliads (Jürgens and Shuttleworth, 2015; Meve and Liede, 1994). Furthermore, nectar is often offered to fly pollinators in high-elevation ecosystems, where it provides rewards such as energy and essential nutrients, including sugars, carbohydrates and amino acids (Larson et al., 2001; Woodcock et al., 2014). In this study, however, nectar constituents were not determined, and only the presence or absence of sugars as dissolved solids was determined using a refractometer. As such, a caveat to this method is that refractometer readings may be influenced by dissolved non-sugar constituents, which, although unlikely, cannot be discounted. The presence of a nectar reward in this pollination system presents a paradox to the concept of mimicry. This is because oviposition site mimicry systems are assumed to be exploiting the oviposition behaviour of flies, hence it is paradoxical for these flies to find food in such mimicry systems. This paradoxical aspect of a reward in plants that may be mimics of oviposition sites has been previously suggested for stapeliads that are carrion-scented (Heiduk et al., 2023; Jürgens et al., 2013; Meve and Liede, 1994). Considering that our results revealed the presence of a nectar reward, future studies should examine whether the nectar reward presented to flies may contain proteins (see Heiduk et al., 2023), especially because some stapeliads deceive flies, yet also exude protein-rich juices that flies feed on, so they are entirely not deceptive in that sense (Heiduk et al., 2023; Johnson and Schiestl, 2016). Moreover, the nectar produced in minute quantities in these plant species may function as a filter to prevent larger animal pollinators from visiting, as the this would not sustain the greater energy demands of these animal pollinators. Nonetheless, future studies will determine which filters are likely the proximal causes of the observed visitation by mostly short-tongued flies to these plant species.

Overall, the guild members display trait similarity, in that they all produce diverse scent volatiles that are characteristic of degrading organic substances. In addition, a similar pattern of floral colour reflectance among the studied plant species is evident in Troje's (1993) categorical fly colour vision model. Also, guild members produce minute nectar rewards to the fly visitor. These lines of evidence, including the specialization on saprophilous, short-tongued flies among the guild members (see Chapter 4), suggest trait similarity. Whether the observed trait similarity is due to convergence was not established in this study and requires a comparative phylogenetic perspective involving inclusion of non-fly pollinated congeners. Such an analysis can reveal whether guild membership involves *de novo* floral adaptations, or whether species were preadapted for the guild.

While all guild members display trait similarity in that they all produce volatiles that are reminiscent of degrading organic substances, there is variation in the specific compounds involved. This variation ultimately deviates from the expectation of the guild concept and argues against its existence. These guild members, however, are visited and pollinated by various functionally similar saprophilous, short-tongued flies, suggesting that the varied scent compounds involved may function to attract the diversity of saprophilous, short-tongued flies visiting these guild members and the partitioning of the local visitor assemblages (see Chapter 4). Moreover, the majority of the guild members are congeners and are therefore related, which could possibly mean that some observed similarity between guild members result from phylogenetic affinity. The emission of diverse degradation volatiles to attract flies suggests that these odours are not due to phylogenetic relatedness. Further research, however, will involve testing the functional aspects of these traits in attracting the diverse saprophilous, short-tongued flies. This will be achieved using GC-EADs and behavioural assays to provide additional evidence of the existence of this guild.

In this guild, scent and colour cues may be important in mediating specialization and may explain pollinator attraction. Furthermore, the studied plant species lack specialized floral morphologies, although may avoid heterospecific pollen transfer by potentially utilizing various pollen placement sites on the saprophilous, short-tongued flies. Nonetheless, these idea requires explicit tests, for example using dye as pollen analogues (Minnaar and Anderson, 2019) to determine whether specific pollen placement is indeed evident in this guild, and which plants are explicitly visited.

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Supplementary Information

Table S5.1: Study species covered in this study, along with localities and habitat characteristics at which they naturally grow, and quantification of floral traits measured. Sampling and quantification of floral traits on all species was done over two sampling seasons (2022 – 2023).

Species	**Locality**	**Coordinates**		**Elevation**	**Habitat**	**Floral colour**	**Scent**	**Floral**	**Nectar**
		S	**E**	**(m.a.s.l.)**	**characteristics**	**perception**	**chemistry**	**morphology**	**reward**
Crassula dependens	Witsieshoek (near Gudu stream)	28°40’48”	28°55’24”	2036	Among grasses near gravel beds	☐	☐	☐	X
C. natalensis	Sentinel	28°43’46”	28°53’32”	2552	Rocky outcrops on mountain slopes, among grasses	☐	☐	☐	☐
C. obovata var. *obovata*	Sentinel	28°43’46”	28°53’32”	2552	Rocky outcrops on mountain slopes, among grasses	☐	☐	☐	☐
C. peploides	Witsieshoek	28°40’53”	28°54’18”	2171	Moist gravel beds	☐	☐	☐	☐
	Naude’s Nek	30°43’55”	28°08’14”	2499	Moist gravel beds	☐	☐	☐	X
C. sarcocaulis subsp. *rupicola*	Sentinel	28°42’40”	28°53’43”	2248	Between boulders and	☐	☐	☐	X

					rocks and in rock crevices				
	Naude’s Nek (Pitseng Road)	30°45’22”	28°18’25”	1836	Between boulders and rocks and in rock crevices	☐	☐	☐	☐
C. setulosa var. *setulosa*	Sentinel	28°42’40”	28°53’43”	2248	Between boulders and rocks and in rock crevices	☐	☐	☐	☐
	Naude’s Nek	30°43’07”	28°08’23”	2511	On gravel beds	☐	☐	☐	☐
C. vaginata	Witsieshoek	28°41’08”	28°54’05”	2194	On grasslands along mountain slopes	☐	☐	☐	X
	Sentinel	28°44’08”	28°53’33”	2656	Along a mountain slope, on grasslands	X	X	X	☐
	Mount Gilboa	29°17’41”	30°17’52”	1758	On grasslands along mountain slopes	X	☐	X	X
Polemannia montana	Sentinel	28°44’06”	28°53’36”	2627	In-between crevices of big boulders	X	☐	X	X

Thesium imbricatum	Sentinel	28°44’11”	28°53’41”	2733	On bare rocks, along mountain slope	X	X	□	X

Floral spectral reflectance

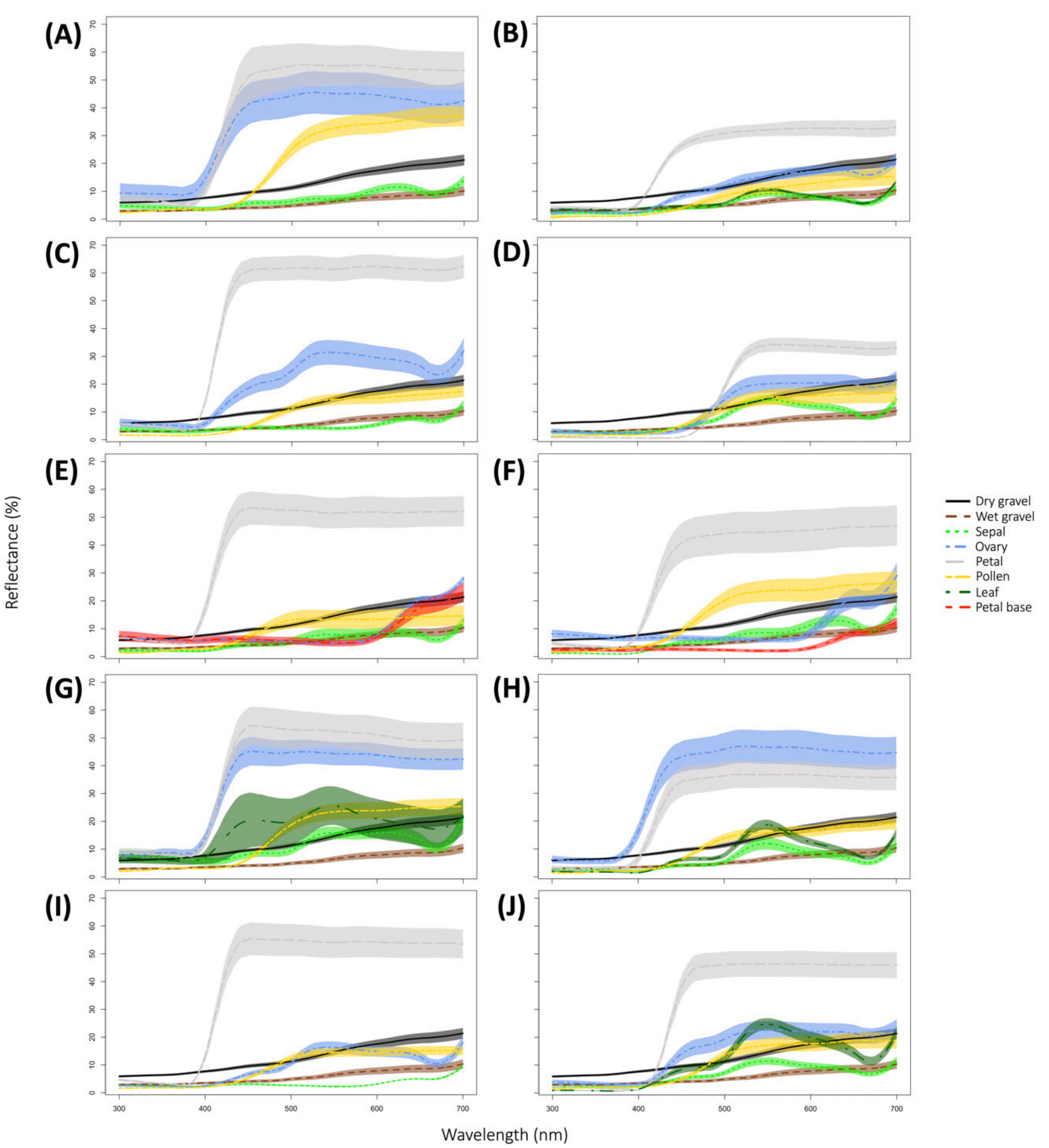

Figure S5.1: Spectral reflectance curves of different floral parts of (A) *Crassula dependens* from Witsieshoek, (B) *C. natalensis* from Sentinel, (C) *C. obovata* var. *obovata* from Sentinel, (D) *C. vaginata* from Witsieshoek, (E) *C. peploides* from Naude's Nek, (F) *C. peploides* from Witsieshoek and (G) *C. sarcocaulis* subsp. *rupicola* from Naude's Nek, (H) *C. sarcocaulis* subsp. *rupicola* from Sentinel, (I) *C. setulosa* var. *setulosa* from Naude's Nek and (J) *C. setulosa* var. *setulosa* from Sentinel in the Drakensberg, KwaZulu-Natal. Solid lines represent the mean spectrum of eight replicates ($N = 8$), shading indicates standard deviation.

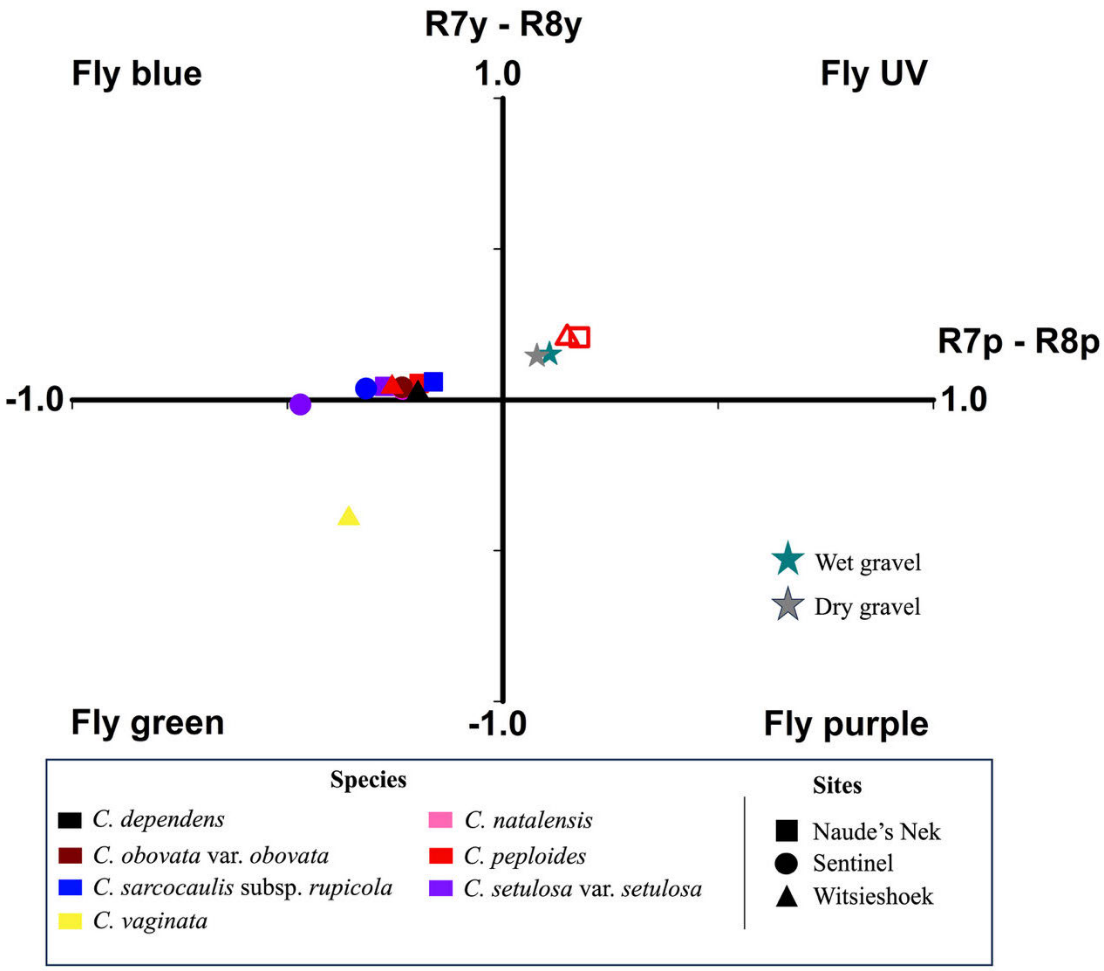

Figure S5.2: Colour loci of the corolla of seven studied *Crassula* species at the studied sites against the wet and dry gravel, as perceived by flies according to the Troje (1993) categorical fly colour vision model. Loci within the same quadrant are not chromatically distinguishable from each other, as perceived by flies. Note that symbol colours refer to the different species and symbols shapes refer to sites.

Scent chemistry

Table S5.2: Average relative amounts (%) of all scent compounds identified from flowers of seven *Crassula* and one *Polemannia* species. Average relative amounts are reported as mean ± SE (occurrence), where occurrence refers to the number of samples (out of total *N* = 8 per species) in which a compound was detected in each species. Compounds that were present in almost equal amounts in the control as in the samples were disregarded and not included in the scent table. Compounds are listed in order of increasing Linear Retention Indices (LRI) within each compound class[a]. Species and study site names are abbreviated as follows: *C. dep* = *C. dependens*, *C. nat* = *C. natalensis*, *C. obo* = *C. obovata* var. *obovata*, *C. pep* = *C. peploides*, *C. sar* = *C. sarcocaulis* subsp. *rupicola*, *C. set* = *C. setulosa* var. *setulosa*, *C. vag* = *C. vaginata*, *P. mon* = *P. montana*. CAS = Chemical Abstracts Service Registry number.

	LRI Mar 2022	**LRI Nov 2023**	**CAS**	**ID Criteria[b]**	***C. dep* (Witsieshoek)**	***C. nat* (Sentinel)**	***C. obo* (Sentinel)**	***C. pep* (Witsieshoek)**	***C. pep* (Naude's Nek)**	***C. sar* (Sentinel)**	***C. set* (Sentinel)**	***C. vag* (Witsies)**	***C. vag* (Mt. Gilboa)**	***P. mon* (Sentinel)**
Number of collected samples					5	7	4	3	4	7	7	5	4	5
Total number of compounds					2	25	33	15	29	43	11	28	42	43
Aliphatic compounds														
Acids														
Acetic acid		1472	64-19-7	A	-	-	-	-	-	-	-	3.28 ± 1.01 (4)	-	-
Propanoic acid	1508		79-09-4	A	-	-	0.6 ± 0.24 (4)	-	-	-	-	-	-	-
Isobutyric acid	1540		79-31-2	A	-	-	0.55 ± 0.31 (4)	11.47 ± 3.75 (3)	0.66 ± 0.06 (4)	-	-	-	-	-
Butanoic acid	1592		107-92-6	B	-	0.39 ± 0.23 (5)	12.87 ± 2.11 (4)	66.8 ± 5.65 (3)	52.13 ± 6.17 (4)	-	-	-	-	-
Pentanoic acid	1709		109-52-4	A	-	-	1.14 ± 0.37 (4)	-	0.07 ± 0.01 (4)	-	-	-	-	-
2-Methylhexanoic acid	1740		4536-23-6	A	-	-	tr ± tr (3)	-	-	-	-	-	-	-
(E)-2-Butenoic acid	1750		107-93-7	A	-	-	0.05 ± 0.03 (3)	1.71 ± 1.01 (2)	8.78 ± 4 (4)	-	-	-	-	-
4-Methylpentanoic acid	1774		646-07-1	A	-	-	-	-	tr ± tr (4)	-	-	-	-	-

Hexanoic acid	1812		142-62-1	A	-	1.75 ± 0.59 (7)	29.56 ± 3.89 (4)	-	2.31 ± 1.9 (4)	-	-	-	0.63 ± 0.23 (4)	-
Heptanoic acid	1920		111-14-8	A	-	-	0.28 ± 0.11 (3)	-	0.09 ± 0.02 (4)	-	-	-	-	-
(E)-3-Hexenoic acid	1921		13419-69-7	A	-	-	5.19 ± 2.16 (4)	-	-	-	-	-	0.21 ± 0.07 (4)	-
(Z)-3-Hexenoic acid	1933		1775-43-5	A	-	-	-	-	-	-	-	-	1.13 ± 0.78 (4)	-
(E)-2-Hexenoic acid	1941		13419-69-7	A	-	-	-	-	tr ± tr (2)	-	-	-	-	-
Octanoic acid	2026		124-07-2	A	-	-	0.15 ± 0.03 (4)	-	0.15 ± 0.02 (4)	-	-	-	-	-
Nonanoic acid	2129		112-05-0	A	-	-	0.36 ± 0.22 (3)	-	0.9 ± 0.16 (4)	-	-	-	-	-
Decanoic acid	2236		334-48-5	A	-	-	-	-	0.06 ± 0.02 (3)	-	-	-	-	-
Alcohols														
Octan-3-ol	1369	1391	589-98-0	A	-	-	-	-	-	-	-	0.38 ± 0.24 (2)	7.89 ± 2.48 (4)	-
(E)-Hex-3-en-1-ol	1357		928-97-2	A	-	-	-	-	0.12 ± 0.03 (4)	-	-	-	-	-
Oct-1-en-3-ol	1447		3391-86-4	B	-	-	-	-	-	22.28 ± 4.14 (7)	-	-	-	-
(Z)- Oct-2-en-1-ol	1489		26001-58-1	A	-	-	-	-	-	1.18 ± 0.21 (7)	-	-	-	-
3-Isopropylpentan-1-ol		1554	38514-13-5	A	-	-	-	-	-	-	-	2.89 ± 0.88 (4)	-	-
Octan-1-ol	1590		111-87-5	A	-	-	-	-	-	-	-	-	9.61 ± 3.02 (4)	-
6-Methylheptan-1-ol	1562		1653-40-3	A	-	-	0.13 ± 0.08 (4)	-	-	-	-	-	-	-
(E)-Oct-2-en-1-ol	1584		18409-17-1	A	-	-	-	-	-	-	-	-	0.96 ± 0.17 (4)	-
Aldehydes														
(E)-Hept-2-enal	1307		18829-55-5	A	-	0.5 ± 0.09 (7)	-	-	-	-	-	-	-	-

(E)-Oct-2-enal	1411		2548-87-0	A	-	-	-	-	0.05 ± 0.01 (4)	-	-	-	0.07 ± 0.03 (4)	-
Alkanes														
Heptadecane	1697		629-78-7	B	-	-	-	0.56 ± 0.32 (2)	-	-	-	-	-	-
Octadecane	1797		593-45-3	B	-	-	-	0.75 ± 0.45 (2)	-	-	-	-	-	-
Alkenes														
Octadec-1-ene	1842		112-88-9	A	-	-	-	-	-	0.05 ± 0.02 (6)	-	-	-	-
Esters														
(Z)-Hex-3-en-1-yl acetate		1322	72237-36-6	B	-	-	-	-	-	-	-	-	-	0.35 ± 0.16 (4)
Oct-1-en-3-yl acetate	1362		2442-10-6	A	-	-	-	-	-	0.07 ± 0.02 (6)	-	-	-	-
Octyl formate	1606		112-32-3	A	-	-	-	-	-	0.07 ± 0.02 (6)	-	-	-	-
Methyl palmitate	2192		112-39-0	A	-	-	-	0.39 ± 0.2 (2)	-	-	1.66 ± 1.48 (3)	-	-	-
Ketones														
Heptane-2,3-dione	1142		96-04-8	B	-	-	-	-	0.92 ± 0.82 (2)	-	-	-	-	-
Octan-3-one	1234	1263	106-68-3	A	-	6.29 ± 3.47 (7)	-	5.73 ± 2.96 (3)	0.11 ± 0.04 (3)	7.54 ± 2.2 (5)	4.54 ± 3.36 (3)	2.72 ± 1.41 (5)	12.75 ± 4.81 (4)	-
Acetoin	1262		513-86-0	B	-	-	-	-	tr ± tr (1)	-	-	-	-	-
6-Methylhept-5-en-2-one	1319		110-93-0	B	-	-	-	-	-	0.33 ± 0.1 (7)	-	-	-	-
Oct-3-en-2-one	1446	1506	1669-44-9	A	-	-	-	-	-	-	-	-	2.15 ± 0.41 (4)	-
Aromatics														
o-Methylanisole	1392		578-58-5	A	-	-	-	-	-	1.36 ± 0.31 (7)	-	-	-	-
m- or p-Methylanisole	1419	1445		A	-	1.9 ± 0.61 (7)	0.68 ± 0.44 (4)	-	-	-	3.17 ± 1.3 (7)	12.74 ± 6.89 (4)	-	-

Methyl benzoate	1596	1627	93-58-3	B	66.23 ± 14.66 (5)	-	-	-	-	2.21 ± 0.35 (7)	-	5.32 ± 2.49 (5)	-	-
Phenylacetaldehyde	1615		122-78-1	B	-	-	-	-	-	4.94 ± 0.72 (7)	-	-	-	-
Salicylaldehyde	1653		90-02-8	A	-	2.07 ± 0.49 (7)	-	-	-	-	-	-	-	-
1,2-Dimethoxybenzene		1723	91-16-7	A	-	-	-	-	-	-	-	0.22 ± 0.07 (4)	-	-
Benzyl acetate	1699		140-11-4	B	-	4.08 ± 1.6 (7)	-	-	tr ± tr (3)	-	-	-	0.08 ± 0.06 (3)	-
1,4-Dimethoxybenzene	1710		150-78-7	A	-	-	-	-	-	-	-	-	0.28 ± 0.19 (3)	-
Methyl phenylacetate	1730		101-41-7	A	-	1.57 ± 0.41 (7)	-	-	-	-	-	-	0.43 ± 0.07 (4)	-
Phenylethyl acetate	1785		103-45-7	B	-	-	-	-	-	0.54 ± 0.15 (7)	-	-	-	-
Guaiacol	1824		90-05-1	A	-	-	-	-	-	-	-	-	0.3 ± 0.07 (4)	-
Benzyl alcohol	1898	1878	100-51-6	B	-	8.11 ± 2.1 (7)	-	-	1.05 ± 0.83 (4)	-	7.92 ± 1.93 (7)	1.31 ± 0.54 (4)	6.35 ± 3.14 (4)	3.18 ± 1.51 (4)
(Z)-Cinnamaldehyde	1864		57194-69-1	A	-	-	-	-	-	-	-	-	0.44 ± 0.32 (4)	-
Phenylethyl alcohol	1872		60-12-8	B	-	-	-	-	0.12 ± 0.02 (4)	-	-	-	3.49 ± 0.39 (4)	-
Creosol	1918		93-51-6	A	-	0.07 ± 0.07 (1)	-	-	-	-	-	-	-	-
Benzenepropanol	2003		122-97-4	A	-	-	-	-	-	-	-	-	1.95 ± 1.59 (4)	-
p-Cresol	2036		106-44-5	B	-	23.72 ± 8.21 (7)	2.18 ± 1.28 (4)	5.75 ± 1.32 (3)	5 ± 3.38 (4)	-	47.23 ± 9.57 (7)	-	0.71 ± 0.45 (4)	-
Methyl 4-methoxybenzoate	2058		121-98-2	A	-	-	-	-	-	-	-	-	0.12 ± 0.06 (4)	-

1,3,5-Trimethoxybenzene		2151	621-23-8	A	-	-	-	-	-	-	-	2.56 ± 1.07 (3)	-	-
4-Aminoacetophenone (= p-Aminoacetophenone)	2174		99-92-3	A	-	1.73 ± 0.61 (7)	-	-	-	-	9.63 ± 3.08 (7)	8.71 ± 3.86 (3)	-	-
Cinnamyl alcohol	2235		104-54-1	A	-	-	-	-	-	-	-	-	0.36 ± 0.29 (4)	-
2-Aminoacetophenone (= o-Aminoacetophenone)	2527		551-93-9	A	-	1.51 ± 0.58 (7)	-	-	-	-	7.74 ± 1.47 (7)	-	-	-
C5-branched chain compounds														
Acids														
Methyl 2-methylbutyrate	1084		868-57-5	A	-	-	-	-	-	-	-	-	9.54 ± 8.28 (2)	-
2-Methylbutan-1-ol	1183		137-32-6	A	-	-	-	-	-	-	-	-	4.33 ± 1.45 (3)	-
2-Methylbutyl isovalerate	1286		2445-77-4	A	-	-	-	-	-	tr ± tr (1)	-	-	-	-
2-Methylbut-2-en-1-ol	1294		4675-87-0	A	-	-	-	-	-	-	-	-	0.34 ± 0.34 (1)	-
2-Methylbutyric acid	1633		116-53-0	A	-	9.28 ± 4.59 (7)	40.35 ± 6.98 (4)	-	-	-	-	-	-	-
Isovaleric acid	1637		503-74-2	B	-	-	-	5.47 ± 1.65 (3)	26.61 ± 3.4 (4)	-	-	-	-	-
3-Methyl-2-butenoic acid	1765		541-47-9	A	-	-	tr ± tr (2)	-	0.44 ± 0.23 (4)	-	-	-	-	-
Miscellaneous cyclic compounds														
γ-Caprolactone	1673		695-06-7	A	-	-	1.07 ± 0.73 (4)	0.1 ± 0.05 (2)	tr ± tr (4)	-	-	0.12 ± 0.04 (4)	-	-
Hex-2-en-4-olide	1726		112.1265	A	-	-	0.19 ± 0.11 (4)	-	-	-	-	-	-	-
δ-Caprolactone	1760		823-22-3	A	-	-	0.33 ± 0.25 (4)	-	-	-	-	-	0.12 ± 0.04 (4)	-
δ-Nonalactone		2200	3301-94-8	A	-	-	-	-	-	-	-	14.01 ± 7.98 (3)	-	-

δ-Decalactone	2160		705-86-2	A	-	-	-	-	-	-	-	-	8.1 ± 3.89 (4)	-
Nitrogen-containing compounds														
Benzyl nitrile	1891	1934	140-29-4	A	-	-	-	-	-	1.45 ± 0.3 (7)	-	-	-	0.2 ± 0.08 (4)
Indole	2395		120-72-9	B	-	-	-	-	-	2.46 ± 0.43 (7)	-	-	-	-
Skatole		2502	83-34-1	B	-	-	-	-	-	-	-	-	-	0.47 ± 0.28 (4)
3-Methoxy-2,5-dimethylpyrazine		1433	19846-22-1	A	-	-	-	-	-	-	-	-	-	0.06 ± 0.03 (4)
2-Methylbutanal oxime[e]	1462		53061-04-4	A	-	-	0.74 ± 0.31 (3)	-	-	-	-	-	-	-
3-Methybutanal oxime[e]	1467			A	-	-	0.37 ± 0.13 (3)	-	-	-	-	-	-	-
3-Methybutanal oxime[e]	1503			A	-	-	0.16 ± 0.08 (3)	-	-	-	-	-	-	-
Terpenoids														
Irregular terpenes														
(Z)-4,8-Dimethylnona-1,3,7-triene	1268			A	-	-	-	-	-	7.16 ± 1.03 (7)	-	-	-	-
(E)-4,8-Dimethylnona-1,3,7-triene	1300		19945-61-0	A	-	-	-	-	-	26.66 ± 3.63 (7)	-	-	4.49 ± 1.88 (4)	-
4-Oxoisophorone	1666		1125-21-9	B	-	1.76 ± 1.64 (7)	-	-	-	-	-	-	2.34 ± 1.24 (4)	-
2,2,6-Trimethylcyclohexane-1,4-dione	1748		20547-99-3	A	-	-	-	-	-	-	-	-	1.51 ± 0.69 (4)	-
6,10-Dimethylundeca-5,9-dien-2-one		1853	3879-26-3	A	33.77 ± 14.66 (5)	-	-	-	-	-	-	-	-	-
6,10,14-Trimethylpentadecan-2-one	2101		68607-88-5	A	-	-	-	-	-	-	-	-	1.21 ± 0.61 (4)	-
Monoterpenes														

β-Thujene	1122		28634-89-1	A	-	-	-	-	-	-	-	-	-	1.04 ± 0.53 (4)
β-Pinene	1229	1192	127-91-3	B	-	-	-	-	-	0.84 ± 0.24 (6)	-	-	-	0.55 ± 0.19 (4)
Eucalyptol	1203		470-82-6	B	-	-	-	-	-	-	8.68 ± 3.69 (7)	-	-	-
(Z)-Ocimene[f]	1243	1254	3338-55-4	B	-	-	-	-	tr ± tr (1)	2.95 ± 1.93 (2)	-	-	-	58.84 ± 15.3 (4)
(E)-Ocimene[f]		1269	3779-61-1	B	-	-	-	-	-	-	-	-	-	6.5 ± 2.66 (4)
γ-Terpinene		1297	99-85-4	A	-	-	-	-	-	-	-	-	-	0.33 ± 0.15 (4)
2,6-Dimethylocta-2,4,6-triene		1383	673-84-7	A	-	-	-	-	-	-	-	-	-	1.42 ± 0.48 (4)
Rose furan		1407	15186-51-3	A	-	-	-	-	-	-	-	-	-	0.08 ± 0.03 (4)
p-Mentha-1,5,8-triene		1441	21195-59-5	A	-	-	-	-	-	-	-	-	-	0.3 ± 0.12 (4)
Linalool	1517	1542	78-70-6	B	-	-	-	-	-	-	-	2.11 ± 0.67 (4)	9.82 ± 2.69 (4)	1.56 ± 0.67 (4)
α-Terpineol			98-55-5	A	-	-	-	-	-	-	-	6.99 ± 4.46 (5)	-	-
Isoborneol		1702	124-76-5	A	-	-	-	-	-	-	-	0.08 ± 0.07 (2)	-	-
Pinocarveol		1713	5947-36-4	A	-	-	-	-	-	-	-	-	-	0.1 ± 0.03 (4)
p-Mentha-1,5-dien-8-ol		1725	1686-20-0	A	-	-	-	-	-	-	-	-	-	0.22 ± 0.07 (4)
p-Cymen-2-ol		1847	499-75-2	A	-	-	-	-	-	-	-	-	-	tr ± tr (2)
Sesquiterpenes														
(-)-β-Bourbonene	1516	1523	5208-59-3	A	-	-	-	-	-	1.29 ± 0.25 (7)	-	-	-	0.07 ± 0.05 (4)
Unknown 3 (m/z: 186, 147, 204, 105, 119, 91)		1590			-	-	-	-	-	-	-	-	-	21.08 ± 19.74 (5)
β-Cubebene		1600	13744-15-5	A	-	-	-	-	-	-	-	3.25 ± 1.81 (4)	-	-
β-Caryophyllene	1592	1614	87-44-5	B	-	-	-	-	-	1.28 ± 0.5 (7)	-	2.53 ± 1.1 (3)	-	-
β-Guaiene		1696	88-84-6	A	-	-	-	-	-	-	-	-	-	tr ± tr (4)

Humulene	1663	1681	6753-98-6	A	-	-	-	-	-	0.08 ± 0.03 (7)	-	-	-	0.23 ± 0.07 (4)
(Z)-β-Farnesene		1730	28973-97-9	A	-	-	-	-	-	-	-	2.64 ± 2.16 (5)	-	-
(Z,Z)-α-Farnesene	1706		28973-99-1	A	-	-	-	-	-	0.18 ± 0.07 (7)	-	-	-	-
Germacrene D	1713		23986-74-5	A	-	-	-	-	-	tr ± tr (5)	-	-	-	-
α-Farnesene	1766		502-61-4	A	-	-	-	-	-	0.05 ± 0.01 (7)	-	-	0.12 ± 0.03 (4)	-
β-Caryophyllene oxide		1994	1139-30-6	A	-	-	-	-	-	-	-	-	-	tr ± tr (4)
Cedrol		2126	77-53-2	A	-	-	-	-	-	-	-	2.04 ± 1.29 (3)	-	-
Unknowns[c]														
Unknown 1 (m/z: 43, 98, 71, 57, 69, 58)		1465			-	-	-	-	-	-	-	-	4.64 ± 1.1 (4)	-
Unknown 2 (m/z: 119, 93, 105, 91, 77, 92)		1580			-	-	-	-	-	-	-	2.01 ± 0.87 (4)	-	-
Unknown 4 (m/z: 161, 93, 91, 79, 69, 41)	1585	1610			-	-	-	-	-	-	-	2.53 ± 1.1 (3)	-	-
Unknown 5 (m/z: 119, 93, 77, 121, 91, 41)		1687			-	-	-	-	-	-	-	1.09 ± 0.58 (3)	-	-
Unknown 6 (m/z: 161, 119, 105, 134, 91, 204)	1746				-	2.06 ± 0.85 (7)	-	-	-	-	-	-	-	-
Unknown 7 (m/z: 67, 80, 79, 81, 108, 109)	1810				-	1.2 ± 1.2 (1)	-	-	-	-	-	-	-	-
Unknown 8 (m/z: 137, 81, 95, 91, 108, 135)	1923				-	-	-	-	-	-	1.91 ± 0.5 (7)	-	-	-
Unknown 9 (m/z: 91, 92, 122, 65, 77, 51)	1947				-	-	-	-	-	10.11 ± 2.51 (7)	-	-	-	-
Unknown 10 (m/z: 91, 43, 162, 65, 119, 94)	1960				-	26.95 ± 7.75 (7)	-	-	-	-	-	-	-	-

Unknown 11 (m/z: 107, 105, 161, 93, 91, 109)	2064		-	3.18 ± 1.07 (6)	-	-	-	-	-	-	-	-
Unknown 12 (m/z: 107, 85, 57, 109, 108, 81)	2097		-	-	-	-	-	-	6.69 ± 1.16 (7)	-	-	-
Unknown 13 (m/z: 166, 135, 63, 165, 122, 137)		2289	-	-	-	-	-	-	-	10.26 ± 7.02 (5)	-	-
Unknown 14 (m/z: 196, 165, 122, 137, 63, 138)		2404	-	-	-	-	-	-	-	8.66 ± 4.04 (3)	-	-
Unknown 15 (m/z: 123, 105, 77, 57, 71, 69)	2699		-	-	-	-	-	-	-	-	1.24 ± 1.1 (3)	-
Unidentified compound[d]			-	1.87 ± 0.95^{6}	2.94 ± 1.06^{8}	1.26 ± 1.07^{4}	0.27 ± 0.2^{7}	4.69 ± 1.72^{23}	-	1.12 ± 0.08^{3}	2.28 ± 0.75^{9}	3.26 ± 1.42^{24}
Aliphatic compounds			-	8.93 ± 0.50	50.88 ± 1.51	87.41 ± 3.94	66.37 ± 3.47	31.52 ± 1.38	6.20 ± 0.52	9.27 ± 0.39	35.4 ± 0.86	0.35 ± 0.16
Aromatics			66.23 ± 14.66	44.76 ± 1.72	2.86 ± 0.33	5.75 ± 1.32	6.18 ± 0.52	9.05 ± 0.57	75.69 ± 4.48	30.86 ± 1.33	14.51 ± 0.45	3.18 ± 1.51
C5-branched chain compounds			-	-	40.38 ± 16.47	5.47 ± 1.65	27.05 ± 10.77	tr ± 0.01	-	-	14.21 ± 2.48	-
Miscellaneous cyclic compounds			-	-	1.59 ± 0.26	0.10 ± 0.05	tr ± 0.01	-	-	14.13 ± 4.42	8.22 ± 2.55	-
Nitrogen-containing compounds			-	-	1.31 ± 0.13	-	tr ± 0.01	3.91 ± 0.71	-	-	-	0.73 ± 0.10
Terpenoids			33.77 ± 14.66	1.76 ± 1.64	0.10 ± 0.17	-	tr ± 0.01	46.49 ± 1.30	8.68 ± 3.69	19.64 ± 0.50	19.49 ± 0.72	89.14 ± 16.64
Unknowns			-	35.26 ± 11.82	2.94 ± 1.06	1.26 ± 1.07	0.27 ± 0.2	14.80 ± 0.35	8.6 ± 0.33	25.67 ± 13.69	8.16 ± 2.95	3.26 ± 1.42

[a] tr = trace amount, referring to compounds amounting <0.05% of total sample. Compound classes follow Knudsen et al. (2006).

[b] Identification (ID) criteria: A = library match confirmed by comparing calculated Kovat retention indices (KRI) with published retention indices, B = identification confirmed with injection of an authentic standard.

[c] Unknowns are listed with the base peak first, followed by other fragments in decreasing order of abundance.

[d] Unknown compounds occurring in less than 1% relative amounts were pooled together. The number of compounds pooled are indicated as a superscript next to the relative amounts (% ± SE).

[e] Could not determine the stereoisomer.

[f] Identification was based on retention times and therefore the possibility that this is the E or Z isomer cannot be unequivocally discounted.

Table S5.3: Pairwise one-way ANOSIM test (*R*) results based on floral scent sampled from seven *Crassula* and one *Polemannia* species across four different study sites. Species are abbreviated as follows: *C. dep* = *C. dependens*, *C. nat* = *C. natalensis*, *C. obo* = *C. obovata* var. *obovata*, *C. pep* = *C. peploides*, *C. sar* = *C. sarcocaulis* subsp. *rupicola*, *C. set* = *C. setulosa* var. *setulosa*, *C. vag* = *C. vaginata* and *P. mon* = *P. montana*.

	C. pep **Witsieshoek**	***C. pep*** **Naude's Nek**	***C. sar*** **Sentinel**	***C. nat*** **Sentinel**	***C. set*** **Sentinel**	***C. obo*** **Sentinel**	***C. dep*** **Witsieshoek**	***C. vag*** **Witsieshoek**	***C. vag*** **Mt. Gilboa**	***P. mon*** **Sentinel**
C. pep **Witsieshoek**	-									
C. pep **Naude's Nek**	*R* = **1.0***	-								
C. sar **Sentinel**	*R* = 1.0**	*R* = 1.0**	-							
C. nat **Sentinel**	*R* = 1.0**	*R* = 1.0**	*R* = 1.0**	-						
C. set **Sentinel**	*R* = 1.0**	*R* = 1.0**	*R* = 1.0**	*R* = 0.999**	-					
C. obo **Sentinel**	*R* = **1.0***	*R* = **1.0***	*R* = 1.0**	*R* = 1.0**	*R* = 1.0**	-				
C. dep **Witsieshoek**	*R* = 1.0*	*R* = **1.0****	*R* = 1.0**	*R* = 1.0**	*R* = 1.0**	*R* = 1.0**	-			
C.vag **Witsieshoek**	*R* = 1.0*	*R* = **1.0****	*R* = 0.998**	*R* = 0.983**	*R* = 0.932**	*R* = 1.0**	*R* = 0.96**	-		
C. vag **Mt. Gilboa**	*R* = **1.0***	*R* = **1.0***	*R* = 1.0**	*R* = 1. 0**	*R* = 1.0**	*R* = **1.0***	*R* = 1.0**	*R* = 1.0**	-	
P. mon **Sentinel**	*R* = 1.0*	*R* = **0.931***	*R* = 0.853**	*R* = 0.876**	*R* = 0.858**	*R* = 1.0**	*R* = 1.0**	*R* = 0.82**	*R* = 0.719**	-

**$P<0.01$; *$P<0.05$

Chapter 6:

General discussion

In this study, I have described the reproductive and pollination ecology and quantified associated floral traits for nine plant species from three distantly related families, in the Drakensberg Mountain Centre (DMC) of southern Africa. I found that most of the study species are pollinated by saprophilous, short-tongued flies, emphasizing the importance of dipteran pollinators in this high-elevation region. Based on the common pollination system and similarities in floral traits, I suggest the recognition of a novel pollination guild, characterised by small, pale, foetid-smelling flowers, predominantly pollinated by a diversity of saprophilous, short-tongued flies, and occurring across high-elevation regions in the DMC. In this concluding chapter, I summarize the findings and discuss them in the context of ecological guilds in general, and pollination guilds in particular, with a specific focus on the unusual combination of functional specialization and ecological generalization found in the new guild which I describe. I then consider floral traits that likely mediate attraction and potentially facilitate specialisation in this pollination guild. Finally, I suggest future research to broaden understanding of this newly recognised pollination guild in the DMC.

Summary of results

The main aims of this study were to describe the breeding systems, pollination ecology and floral traits of several high-elevation plant species that were hypothesized to rely on short-tongued, saprophilous flies for pollination. A general introduction to the topic is presented in Chapter 1.

In Chapter 2, I focused on the pollination ecology of a single species: the vomit-scented *Crassula peploides* (Crassulaceae). I confirmed, using visitor observations and quantification of pollen loads, that this species is visited and pollinated primarily by saprophilous, short-tongued flies of the families Muscidae, Sarcophagidae and Tachinidae. Quantification of floral scent revealed an unusual acid-dominated odour bouquet that has not previously been documented for Crassulaceae. Analysis of floral colour revealed chromatic contrasts between petal tips, pollen and some sepals with the background gravel substrate in the fly colour vision model, indicating that flies can distinguish flowers from their *in-situ* background, as well as between different floral parts. Lastly, the presence of a nectar reward, albeit in minute quantities was confirmed.

In Chapter 3, I investigated characteristics of breeding systems including self-compatibility and reliance on pollinators (intrinsic factors) and quantified the contribution of pollinators (extrinsic factors) to reproductive success for four high-elevation *Crassula* species. The results from hand-pollination experiments revealed a reliance on receipt of cross-pollen for reproductive success for three of the four species for which breeding systems were assessed. The index of self-incompatibility revealed intermediate self-compatibility for *C. setulosa* var. *setulosa* and strong self-incompatibility for *C. natalensis* and *C. sarcocaulis* subsp. *rupicola*, and the index of autofertility revealed strong reliance on pollinators for these three species. For all four species, a comparison of the reproductive success of naturally pollinated flowers with flowers that were excluded from pollinators confirmed strong dependence on pollinators for fecundity, which is unusual in a potentially pollinator-depauperate high-elevation ecosystem. The lack of significant increase in fecundity following manual pollen supplementation indicated the absence of pollen limitation, in spite of the strong pollinator dependence for these three studied species.

The absence of evidence for pollen limitation despite strong dependence on pollinators found in the species studied in Chapter 3, motivated further research to identify pollinators of these, and of several other species which co-flower in the same high-elevation communities, which is reported in Chapter 4. I identified visitor assemblages of nine high-elevation plant species, at six different sites, and found that floral visitors of almost all were dominated by saprophilous, short-tongued flies. The only exception, *C. vaginata*, was predominantly visited and pollinated by various beetle species and is thus not considered a member of this guild. Overall, flies of the families Muscidae, Sarcophagidae, Tachinidae and Calliphoridae dominated the visitor assemblages of the eight putative guild members and carried large quantities of pollen of these plant species. Various other insects belonging to Coleoptera, Hymenoptera, Hemiptera and Lepidoptera also visited these nine high-elevation plant species but were less frequent visitors.

Chapter 5 objectively examined the floral traits of guild members and assessed similarities or differences among guild members. Specifically, I quantified floral colour, scent and nectar reward that likely mediate attraction and assessed the degree of trait similarity among these plant species. I also measured floral morphologies, which may influence which visitors can access nectar rewards and are important for pollen placement. Floral colour contrasts of pollen, ovaries (except in *C. peploides*) and petals against the wet and dry background gravel in a fly colour vision model suggest that flies can distinguish between the different floral parts and distinguish flowers from the background gravel. Analyses of floral scent revealed distinct scent profiles among the studied plant species. Three of the nine studied species exhibited an unusual dominance of hexanoic, butanoic and isovaleric acids in the floral scent. Three other plant species produced various acids in smaller relative amounts; another three plant species emitted no acids and the scent contained various terpenoids, aromatics (including *p*-cresol in five plant species) and nitrogen-containing compounds (skatole and indole, produced by one plant species each). Lastly, a nectar reward was confirmed for six of the nine studied plant species in which the presence of nectar was investigated, suggesting that these pollination systems are rewarding.

Overall, the combined evidence from Chapters 2, 3, 4 and 5 suggest that the studied plant species, with the exception of *C. vaginata*, are specialized for pollination by a group of functionally similar saprophilous, short-tongued flies. Although specialized for pollination by this functional group of pollinators, the diversity of flies involved makes these flowers quite ecologically generalized, in contrast to other known pollination guilds. This suggests ecological stability of these guild members as they do not depend on a single pollinating species, thus competition for pollinating services or local extinction would not have an impact on reproductive success of these guild members. This level of specialization is likely mediated by scent chemistry and visual signals that are particularly attractive to flies as proximal mechanisms. In addition, these results, considered in combination with previous studies of plant species with similar visitor and pollinator assemblages and floral characteristics, highlight the role of flies more generally as important pollinators in this high-elevation ecosystem and further support the presence of a previously undescribed pollination guild in the DMC that relies on saprophilous, short-tongued flies.

Pollinator dependence and visitor assemblage diversity

Seven out of the nine plant species on which I focussed in this study belong to the Crassulaceae. Generally, very limited information exists regarding the reproductive biology of Crassulaceae, including in southern Africa, despite this region being the centre of diversity for this family. For genera for which information is known, self-compatibility has been reported in *Kalanchoe* species from Madagascar (Smith et al., 2021) and Venezuela (Herrera and Nassar, 2009), *Echeveria gibbiflora* (Parra et al., 1993) and *E. rosea* (Jimeno-Sevilla et al., 2014) from Mexico, *Rhodiola dumulosa* in China (Zhu and Lou, 2010), as well as for *Sedum* species across North America (Denton, 1979; Wyatt, 1983). To the best of my knowledge, this particular research is the first to report breeding systems in the genus *Crassula* in southern Africa. The breeding systems of three high-elevation *Crassula* species that were examined in this thesis revealed that two of the three species were strongly self-incompatible (*C. natalensis* and *C. sarcocaulis* subsp. *rupicola*) and the remaining species was partly self-compatible (*C. setulosa* var. *setulosa*; Chapter 3). Moreover, vector-mediated pollination contributes over 80% to fecundity in these plant species, which are characterized by a lack of pollen limitation despite strong pollinator dependence.

Quantification of visitor assemblages of these pollinator-dependent plant species, and additional guild members (Chapter 4), revealed that saprophilous, short-tongued flies (Diptera) dominated the visitor assemblages. The remaining visitors observed in this study were Coleoptera, Hymenoptera, Hemiptera and Lepidoptera, although these visited in lower abundance than flies. Among the short-tongued flies, house flies (Muscidae), tachinids (Tachinidae), blow flies (Calliphoridae) and flesh flies (Sarcophagidae) dominated the visitor assemblages. Although not all visitors are pollinators, I have confirmed in Chapter 4 that flies from various families carried large quantities of pollen that is similar to that from the plant species on which they were caught and are therefore likely pollinators. Non-fly visitors caught on guild members also carried pollen, however, in relatively lower quantities compared to flies (Chapter 4). This is likely because flies, compared to non-fly visitors such as beetles, move more frequently and further distances between flowers and plant species, which would contribute to their quality-component as pollinators. Moreover, the large quantities of pollen observed on fly visitors, compared to non-fly visitors, may explain the observed lack of pollen limitation (i.e., guild members received adequate pollen from pollinators) within *C. natalensis*, *C. sarcocaulis* subsp. *rupicola* and *C. setulosa* var. *setulosa* guild members (Chapter 3). The lack of apparent pollen limitation has previously been reported in the DMC regions (Black et al., 2019; Kgaboesele et al., 2021; Rodger and Ellis, 2016; Springer, 2019), but is unusual in that many pollinator-dependent plant species in other high-elevation regions are strongly pollen limited (García-Camacho and Totland, 2009; Jiang and Xie, 2020; Lázaro et al., 2015). The combination of strong pollinator dependence and high pollinator-mediated fecundity outlined in Chapter 3 and the dominance of short-tongued fly pollinators revealed in Chapter 4, adds to the growing evidence that flies are particularly important pollinators of high-elevation ecosystems (Ishii et al., 2019; Kearns, 1992; Kudo et al., 2024b; Lefebvre et al., 2018, 2014; McCabe et al., 2019; Muñoz and Arroyo, 2006; Zhao and Huang, 2013).

One plant species, *C. vaginata*, was shown in this study not to be part of the guild of species pollinated by saprophilous, short-tongued flies. This species is exceptional in its flower colour (yellow instead of pale or white; Chapter 5) and is visited predominantly by various beetles, including cetoniid beetles (Chapter 4). Similar results of visitation and pollination by various beetles, including cetoniid beetles, in plant species that comprise similar scent compounds to the studied *C. vaginata*, are known in the Mt. Gilboa and Karkloof regions (Shuttleworth and Johnson, 2009c; Steenhuisen and Johnson, 2012). In the DMC, Johnson et al. (2007) reported visitation and pollination by cetoniid beetles of the yellow-flowered *Satyrium microrrhynchum* (Orchidaceae) and Black et al. (2019) reported beetles visiting *Cineraria eroidiodes* (Asteraceae) that is also yellow in colour. These species display trait similarities with *C. vaginata*. Moreover, similar visitation by beetles and flies has been observed on *Disa fragrans* subsp. *fragrans* (Orchidaceae) in the Drakensberg Mountain range (Johnson and Hobbhahn, 2010). The scent chemistry of this orchid shows similarity with that of *C. vaginata* and comprises various aromatic compounds, which are also produced by many other plants (Knudsen et al., 2006). The differences in the visitor assemblages (flies and beetles vs beetles, respectively) and scent profiles of *C. vaginata* at Sentinel and Karkloof suggest that these two populations may be ecotypes of the species, which have adapted to the visitor assemblage in their geographical locations. This adaptation may have occurred through pollinator shift from beetle-pollination at lower elevations (at Mt. Gilboa) to beetle- and fly-pollination at higher elevations (at Sentinel) within this species. Nonetheless, further research involving phylogenetics could shed light on the evolutionary history of this species and also indicate the direction of pollinator shift in this species.

Level of specialization and existence of a previously undescribed pollination guild

The southern African flora is characterized by many highly specialized pollination systems and multiple pollination guilds of up to 20 plant species which rely exclusively on a single pollinator species or few species from the same functional pollinator group, which are more species-rich and are ecologically-interchangeable, for pollination (Goldblatt and Manning, 2000; Johnson, 2010; Manning and Goldblatt, 1997; Pauw, 2006; Shuttleworth and Johnson, 2012; Steiner and Whitehead, 1988; Whitehead et al., 2008). Specialization in these pollination guilds is achieved with morphological or biochemical filtering, which only allows a small subset of potential visitors to access rewards or makes flowers differentially attractive to local insects (Armbruster, 2017; Johnson, 2010). Plant species with open-bowl flowers and highly accessible rewards (dos Santos et al., 2024; Fenster et al., 2004; Waser et al., 1996, but see Shuttleworth and Johnson, 2012) are seldom considered as part of a specialized pollination guild, yet, the plant species in the proposed guild described in this thesis are characterized by exactly these characteristics (Figure 1.1, Chapter 1). Sometimes, such floral characteristics are associated with the ability to self-pollinate (see Johnson and Hobbhahn, 2010), but I confirmed that three guild members are pollinator-dependent and depend on receipt of cross-pollen for reproductive success (Chapter 3) and were also visited primarily by saprophilous, short-tongued flies (Chapter 4). Although morphology suggests that these species could exhibit generalized pollination systems, I provide evidence that the studied plant species are pollinated predominantly by saprophilous, short-tongued flies. However, at the same time some evidence, specifically spatiotemporal variation in taxonomic identity within the broader group of saprophilous fly visitors, suggests some ecological generalization within the guild. Thus, the pollination guild in this study is defined following Root's (1967) definition of what constitutes a guild, which is based on similar utilization of a common resource, in this case, functionally similar saprophilous, short-tongued fly pollinators.

In this research, I describe this guild as functionally specialized for a group of functionally similar saprophilous, short-tongued flies. This specialisation could reflect imposed specialization (sensu Armbruster, 2017) since the flowers occur in a potentially pollinator-depauperate high-elevation environment where alternative pollinators may not be present. However, southern Africa in general, and the DMC in particular, are known to be characterised by a great variety of pollinating fauna, including a wide diversity of insects, birds, rodents and even reptiles, which are documented as important pollinators of DMC plants species (Black et al., 2019; Brown et al., 2009; Cozien et al., 2019; Johnson, 2010; Springer, 2019; Thupsie, 2023; van der Niet et al., 2015, 2010; Whitehead et al., 2019). Thus, there is abundant evidence for the greater diversity of potential pollinators than is observed visiting guild members locally in the DMC, countering imposed specialisation and supporting that the saprophilous guild filters a subset of pollinators via specialised floral attractant traits.

The saprophilous, short-tongued fly pollination guild documented in this research not only includes the studied plant species but also likely includes several other plant species in the DMC which are characterized by a similar pollination system. Previous studies in the DMC have documented pollination by saprophilous, short-tongued flies in several plant species including *Schizochilus angustifolius* (Orchidaceae; van der Niet et al., 2010), *Eucomis bicolour* and *E. humilis* (Asparagaceae; Shuttleworth and Johnson, 2010), *Wurmbea elatior* (Colchicaceae; Johnson et al., 2020) and *Erica caffrorum* (Ericaceae; Thupsie, 2023). Similar to the plant species in this research, these previously studied species are characterized by small, open flowers in which nectar is easily accessible. They emit a characteristic foul-smelling odour and are primarily visited and pollinated by saprophilous, short-tongued flies, and are thus potential candidates for this guild. A test of trait convergence should be done by performing comparative phylogenetic analysis between these fly-pollinated plant species, to determine whether there is evidence of convergence of traits to attract saprophilous, short-tongued flies.

Linking visitor and pollinator diversity to floral traits

Pollination guilds are often not only considered in the identity of species that utilize a shared resource but also in the context of the functional traits for exploiting the resource (Phillips et al., 2020). To the human eye, the members of the guild that was examined in this study all exhibit similar floral colours in that they display white to greenish-white flowers (except *C. vaginata*) with a foetid scent and nectar in minute quantities. With regards to floral morphology, the studied guild members all appear to have morphologically unspecialized flowers (Figure 1.1, Chapter 1) and the nectar is presented in shallow floral tubes and is highly accessible by flies with relatively short proboscides. The accessibility of floral rewards and the absence of morphological filters in a specialized pollination system is particularly intriguing given the apparent functional specialization for a group of saprophilous, short-tongued flies. The lack of morphological filters in this guild contrasts with some other specialized pollination systems found in the southern African flora in which some guild members are characterized by long floral tubes to only allow interaction with long-proboscid fly pollinators (Goldblatt and Manning, 2000; Johnson, 2010; Manning and Goldblatt, 1997; Pauw, 2022), and also with other pollination systems in which the plant species involved is characterized by elaborate floral morphologies to allow specific pollinators to access nectar and contact reproductive parts, as in some stapeliads (Shuttleworth et al., 2017).

The absence of distinct morphological filters coupled with the easy accessibility of nectar, raises the possibility that the proximal causes of the observed functional specialization within this guild might be sensory filters (scent and colour) and nectar quantity. Indeed, scent is thought to be an important communication channel in plant-pollinator interactions and has been attributed to driving specialization in some systems (Dötterl and Gershenzon, 2023; Knudsen et al., 2006; Robert A. Raguso, 2008; Shuttleworth and Johnson, 2012, 2009a, 2009b). Moreover, the volume and sugar concentration of a nectar reward mediates pollinator visitation, with different pollinators requiring certain concentrations of sugars to meet their energy requirements (Parachnowitsch et al., 2019; Woodcock et al., 2014). The guild members produced nectar in minute quantities, which is typical for fly-pollinated plant species

(Vandelook et al., 2019). Thus, scent, colour and minute amounts of nectar may all ultimately explain why other potential pollinators in the area where these guild members grow do not visit flowers of the saprophilous, short-tongued fly pollination guild.

In the absence of morphologically specialized flowers, it appears that floral scent may be important in this guild. Despite being visited predominantly by diverse saprophilous, short-tongued flies (Chapter 4), the guild members all emit a putrid odour with varying dominance of particular scent compounds (Chapter 5) that are associated with sapromyiophily. The dominance of carboxylic acids in this guild, including hexanoic and butanoic acids (aliphatics) and isovaleric and 2-methylbutyric acids (C5-branched chain compounds) in the scent profiles of *C. obovata* var. *obovata* and *C. peploides* (at both the Witsieshoek and Naude's Nek populations), is of particular interest, as it has only been reported in few sapromyiophilous plant species (Erhardt, 1993; Hadacek and Weber, 2002; Heiduk et al., 2024; Jürgens et al., 2006; Kite and Smith, 1997; Knudsen and Ståhl, 1994; Shuttleworth, 2016; van der Niet et al., 2010). In particular, isovaleric and butanoic acids characterize the scents of sweaty feet and vomit respectively and also occur in rotting organic matter that would be attractive to flies, which, in this research, may potentially explain the generally putrid scent (at least to the human nose) and the attraction of saprophilous, short-tongued flies to guild members that emit these compounds, as evident in Chapter 4.

Other particularly notable compounds found in the scent profiles of guild members are *p*-cresol (in five species) and indole and skatole (in one species each; in Chapter 5), which characterize the scent of dung (Jensen et al., 1995; Kite, 1995) and have been associated with possible dung mimicry in stapeliads (Jürgens et al., 2006), Araceae (Kite, 1995; Kite et al., 1998) and *Wurmbea elatior* (Colchicaceae; Johnson et al., 2020). Although the functional aspect of volatiles was not determined in this study (see below), these compounds have been demonstrated to elicit physiological and behavioural responses in flies (Johnson et al., 2020; Sayers et al., 2020; Zito et al., 2015b), including sarcophagids and muscids that were observed visiting guild members emitting these compounds (Chapter 4). Aside from foetid scents, the compound linalool, which is associated with plant species pollinated by moths, but also beetles (Johnson et al., 2007; Shuttleworth and Johnson, 2010; Steenhuisen and Johnson, 2012), was found in relatively larger amounts in the scent of *C. vaginata*, which could explain the dominance of various beetles (Chapter 4) visiting this species. Moreover, plants pollinated by cetoniid beetles, such as proteas, produce complex scents that are dominated by compounds such as linalool, benzaldehyde, methyl benzoate and benzyl alcohol (Steenhuisen et al., 2010). Interestingly, some of these compounds (methyl benzoate, benzyl alcohol and eucalyptol) were also produced, albeit in relatively lower amounts (except in *C. dependens* in which methyl benzoate dominated, although only two compounds were detected in this species; Chapter 5) in some of the guild members including *C. dependens*, *C. natalensis* and *C. setulosa* var. *setulosa*, which were also visited, albeit in relatively low quantities, by beetles.

One somewhat unexpected result from this research is that the interactions between guild members and the diverse floral visitors are highly dynamic, both spatially and temporally (Chapter 4). This observed spatial and temporal variation is somewhat unusual in the southern African region, in which most described guilds are characterized by specialized interactions that are predictable in space and time (Johnson, 2010). The dynamic nature of interactions, however, is common in some high-elevation systems (Alarcón, 2010; Kudo et al., 2024b; Minachilis et al., 2023; Price et al., 2005). Moreover, members of this saprophilous, short-tongued fly pollination guild appear to partition the local visitor assemblage (Chapter 4). This partitioning may result from the variable scent profiles of the guild members (Chapter 5) which may exploit subtle differences in the preferences of different flies to specialize to subsets of the local fly fauna. This pattern of partitioning the local visitor assemblage is unusual for co-flowering plant species that share a functional group of pollinators. While some co-flowering plant species that share pollinators limit the effects of interspecific competition by partitioning pollen on the insect's body (Goldblatt and Manning, 2000), the guild members in this research partition the actual fly assemblage. It would thus be interesting to understand how these guild members achieve this.

Plant-visitor interaction networks revealed spatial and temporal variation in the visitor assemblages for species that were sampled at multiple sites and/or seasons and showed that visitor assemblages were locally partitioned among co-flowering species. These results thus suggest a paradox: that the plant species are generalized for multiple species of short-tongued flies (indicated by variation space and time) but are also somewhat specialized in that not all locally available short-tongued flies will visit any plant species (indicated by local partitioning of visitors).

Future research avenues

The research in this thesis has presented reproductive and pollination ecology results of several high-elevation plant species with characteristic white and yellow flowers, putrid scents and minute nectar rewards, that were hitherto undocumented in the DMC. Furthermore, this research has revealed that these plant species belong to a previously undescribed pollination guild that is reliant on saprophilous, short-tongued flies for pollination (Chapter 4). The paradoxical guild described in this research merits further research as it deviates from the conventional pollination guilds that are known, which are characterized by the predictability of pollinators. Such research could investigate the plant-visitor interactions at multiple spatial and temporal scales (see CaraDonna et al., 2021; Trøjelsgaard and Olesen, 2016). Plant-pollinator interactions should be studied along the geographical extent of the plant species and on a weekly or monthly basis to encompass the entire flowering season which ranges between November and December for *T. imbricatum* and late January and March for *Crassula* species and *P. montana*, instead of inter-annually as in this research, which may either reveal more stable interactions between guild members and visitors, or rapid turnover.

In addition, a more extensive and standardized sampling protocol for floral visitors in which observation times and dates per flower are recorded and observation duration is similar across multiple sites, as well as for the complete local potential pollinator community, should be implemented in future studies to allow calculation of visitation rates. Such extensive sampling may provide contrasting results to the observed spatial and temporal scale and in fact, reveal stable interactions between these guild members and floral visitors. Furthermore, this standardized sampling approach would also allow for comparative analyses with other studies to identify patterns across regions. Future studies should also include quantification of pollen loads from multiple seasons and the incorporation of pollen tracking techniques (Minnaar and Anderson, 2019) to specifically distinguish pollen grains from different plant species since the guild visitors carry morphologically similar pollen that may originate from congeners. Furthermore, rarefaction curve analyses, which require a standardized sampling approach, could be performed for all guild members at all study sites to assess whether the sampling effort has been adequate. Moreover, incorporating molecular techniques, such as DNA barcoding, to identify the visitors and pollinators of these guild members could be beneficial in resolving the possible existence of taxonomic oversplitting and determine possible cryptic diversity, thereby highlighting the extent to which the DMC region may harbour a higher diversity of flies in the potential pollinator assemblage.

The results following quantification of floral traits of guild members revealed a carboxylic acid-dominated floral scent in some of the guild members, dominance of terpenoids and aromatics and the presence of nitrogen-containing compounds in other guild members, although all species attract saprophilous, short-tongued flies. The variation in scent compounds produced could indicate that scent cues are particularly important in attracting the diverse range of saprophilous, short-tongued flies, but could also mean that either these guild members partition the local pollinator assemblage using diverse floral scents, or that floral scent is not functionally important in determining guild membership. As such, future research should identify specific active compounds in the scents of different guild members that elicit physiological and behavioural responses in saprophilous, short-tongued flies from Muscidae, Sarcophagidae, Tachinidae and Calliphoridae. This could be achieved by implementing coupled gas chromatography-electroantennographic detection (GC-EAD) experiments (Shuttleworth and Johnson, 2020). Once identified, the attractiveness of these active compounds to different saprophilous, short-tongued flies could be demonstrated using behavioural assays in the field and a Y-maze in the laboratory. In addition, assays involving both active scent compounds and model flowers depicting varying colour contrasts, in isolation or combination, could provide more insight into whether saprophilous, short-tongued flies use either floral signal more than others or all equally to locate guild members. The combination of these methods could reveal whether the saprophilous, short-tongued flies are attracted by a single cue, such as a single scent that is made up of a combination of specific compounds or floral colour alone to locate guild members, or whether they use a blend of specific scent volatiles, colour cues and the presence of nectar as proxies to visit the guild members. In addition, these behavioural assays could reveal whether the dominance of carboxylic acids in the scent profiles of the guild members is particularly important in attracting the observed saprophilous, short-tongued flies or whether they also function to repel other potential visitors in these flowering communities. Particularly for the non-guild member *C. vaginata*, future research should investigate floral trait convergence at the population level between the different study sites to determine the possible existence of pollination ecotypes.

Flowers in pollination guilds often display convergence patterns of traits resulting from adaptation to a common pollinator (Bergamo et al., 2020; Garcia et al., 2022). Adding to this, guild members may also share this pollinator resource because shortage of available potential pollinators in pollinator-depauperate environments (i.e. imposed specialization; *sensu* Armbruster, 2017). In this research, the studied guild members exhibit similar white to greenish-white floral colours, emit a generally foul-smelling scent and all produce nectar in minute quantities. Testing for trait convergent evolution in this guild using comparative analyses (see Jürgens et al., 2013; Shuttleworth and Johnson, 2009c) would shed light on whether these guild members have evolved and adapted for a particular function. This testing could also be supplemented with a comprehensive phylogenetic analysis of the guild members to understand evolutionary relationships and phylogenetic dependence or independence of these guild members, especially in light of recent evidence of early radiation of *Crassula* in southern Africa (Bruyns et al., 2019).

Lastly, the guild described in this research is not limited to these guild members but also includes several other mentioned candidates which display similarities in traits and pollinators. Moreover, there are other candidates, including members of Apocynaceae, Molluginaceae and Asparagaceae, based on Pooley (2003), that may be potential candidates, and as such, future research should include studying the pollination ecology of these members to conclude whether they are part of this guild or not. Moreover, detailed plant-pollinator interaction studies will be very useful for long-term monitoring of the effects of climate change, putative pollinator shifts and the extinction of plant and pollinator species at these altitudes.

Conclusions

Results of this research have confirmed the existence of a novel pollination guild in the DMC region that is reliant on saprophilous, short-tongued flies as pollinators to achieve reproductive success and has also highlighted the importance of short-tongued flies in these high-elevation ecosystems. Moreover, the evidence provided in this research supporting the existence of this pollination guild deviates from the conventional criterion defining most pollination guilds, which is that guild members rely exclusively on one pollinating species or only a few species from the same functional group, and highlights the novelty of this guild in comparison to other known highly specialized guilds in the DMC (see Johnson, 2010). The deviation in the guild confirmed in this research is that the guild members in this research exhibit generalized floral morphologies and therefore lack distinct morphological filters, however, they are functionally specialized for a group of short-tongued flies and ecologically generalized for multiple species within this functional group. The functional specialization, but ecologically generalization indicates that olfactory and visual cues (scent, colour and a nectar reward) play a significant role as proximal mechanisms that facilitate the specialization within this guild. However, further research is necessary to explore the functional aspects of these traits to definitively conclude their effectiveness within this guild.

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