The pollination and scent ecology of selected Cape milkweeds (Apocynaceae: Asclepiadoideae)

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THESIS
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Dedicated to my mother

Abstract

Milkweeds (Asclepiadoideae, Apocynaceae) possess a complex floral morphology that has made them prime candidates for investigating the evolution of plant-pollinator relationships. In South Africa, the pollination ecology of this diverse group has largely been focused in the summer rainfall region. This study focused on Western Cape species in the winter and summer-winter rainfall transition zones. The aim was to determine the pollinators of the study species and assess, describe and quantify their floral attractants. Thus offering a basis of comparison with the previously studied summer rainfall species.

The pollination systems of seven milkweed species occurring in the Western Cape were investigated by determining floral visitors and several floral traits that may act as attractants of these visitors. For each study species an attempt at pollinator observations was carried out in several sites across the Cape; floral scent samples were collected through headspace sampling and analysed using GC-MS (gas chromatography-mass spectrometry); floral colour was analysed using spectral reflectance measurements; and nectar was measured to quantify floral rewards.

Some Cape milkweeds displayed a more generalized pollination system compared to their summer rainfall congeners. A diverse range of visitors were observed on two *Gomphocarpus* species, *Gomphocarpus cancellatus* and *G. filiformis* in the Fynbos and Succulent Karoo biomes respectively, in contrast to their summer rainfall congeners, which are exclusively wasp pollinated or much less generalized. These two *Gomphocarpus* species offered floral visitors nectar as a reward with a concentration of 53% in *G. cancellatus* and a lower 15% in *G. filiformis*. The species emitted very different scents, *G. cancellatus* produced a scent dominated by irregular terpenes while *G. filiformis* scent largely consisted of benzenoid compounds. Although visited by a variety of different insect families and lesser-double collared sunbirds, honeybees and Balbyter ants were found to be the most efficient at removing pollinaria from *G. cancellatus* and *G. filiformis* respectively.

Eustegia minuta, a Cape endemic, is almost exclusively pollinated by bibionid flies. This is the first record of the pollinators for this monotypic genus, as well as the first for the tribe Eustegieae. Additionally, this study is the first record of a milkweed-bibionid pollination system. The flowers produced very low nectar volumes, displayed minimal visual cues but produced a strong pungent odour dominated by an unidentified compound. This strong scent is thought to play an important role in attracting bibionids while also deterring other potential visitors.

The scent profiles of four other previously unstudied Cape milkweeds, namely *Cynanchum obtusifolium, C. zeyheri, Fockea capensis* and *Secamone alpinii* were also analysed and shown to be mainly dominated by monoterpenes and benzenoids. Their scent profiles differed markedly from each other as well as to their congeners. The presence of skatole in *S. alpinii* suggests that it may attract coprophagic flies, small Nematoceran flies were observed drinking nectar from its flowers. While *F. capensis* produced scent associated with moths. Further observations are needed to confirm true pollinators for both species. The two *Cynanchum* species produced scent that was very different to each other as well as their congeners. *Cynanchum obtusifolium* is known to be bee pollinated however its scent profile did not align with this. While *C. zeyheri* produced a benzenoid-dominated scent with compounds associated with moths.

Scent appears to be the most prominent pollinator attractant in the study species compared to floral colour. The concentration and volume of nectar rewards may also be significant. The structural complexity of the flowers is suggestive of a strong floral filter in some species (e.g. *Eustegia minuta*). Morphologically similar species were found to employ vastly different floral chemical strategies to attract but possibly also deter and filter out floral visitors. The Cape milkweeds therefore offer many opportunities for further pollination studies.

1. Introduction

1.1 Background

The large-scale diversification of plant lineages is closely linked to variation in plant reproductive biology (Stebbins 1974, Barrett et. al. 1996). About 80% of plant species depend on animal pollinators for their reproduction (Ollerton et al. 2011). Additionally, a quarter of documented angiosperm divergence events have been linked to pollinator shifts (van der Niet & Johnson 2012). Floral adaptations to pollinators have had a large impact on angiosperm diversity (Grant & Grant 1965, Stebbins 1974, Eriksson & Bremer 1992, Dodd et al. 1999, Vamosi & Vamosi 2010). Therefore the co-evolution of plants and their pollinators has received considerable attention, much of which has focused on understanding specialized and generalized pollination systems.

Plant-pollinator interactions fall on a continuum from narrow specialization, characterized by plants utilizing a single type of pollinator, to broad generalization, involving a wide range of pollinator types (Waser et al. 1996, Johnson & Steiner 2000). While it was previously thought that most pollination systems are specialized (Faegri & van der Pijl 1979), studies have revealed that generalized pollination systems may be more widespread (Waser et al. 1996, Herrera 1996, Ollerton 1996, Wilson & Thomson 1996).

The debate around generalization versus specialization has boiled down to differences between considering species versus functional types. Waser et al. (1996) considered specialization in terms of number of species. However, several studies (Johnson &

Steiner 2000, 2003, Fenster et al. 2004) argue that functional type is a much more realistic measure of specialization, i.e. plants specialize to functional (e.g. long-tongued flies or similar hawkmoths, or similar sized bees or carrion flies) rather than to individual species. Thus, a plant pollinated by ten similar hawkmoth species would have been classified as generalized by Waser et al. (1996), when it is in fact specialized for pollination by hawkmoths. The groups of pollinators are categorized into functional groups according to similarities in the selection pressure they exert (Fenster et al. 2004). Therefore a plant is considered to have a specialized pollination system, and occupies a pollination niche, if it is only successfully pollinated by a subset of functionally similar potential pollinators (Beattie 1971, Armbruster et al. 1994, Gomez & Zamora 1999). The functional types can include behaviour; for example a plant's structure could compel floral visitors to behave in a similar manner in order to reach the plant's reproductive organs, morphology; floral visitors may be taxonomically unrelated but possess a similar morphology, e.g. long tongue, elongated front legs, and sensory modes; the ability to detect similar colour signals or chemical compounds in a flower's scent. Functional groups of pollinators can therefore contain many species, or just one species, and a particular pollinator species can belong to multiple functional groups (Fenster 2004). When Fenster et al. (2004) applied this approach to the same data analyzed by Waser et al. (1996) most species were actually quite specialized.

Ollerton et al. (2007) reviewed how specialization and generalization mean different things to different people and how there are many ways for a flower to be generalized or specialised. Ecological generalization or specialization can refer to the number of effective pollinators that interact with a plant, while the functional generalization or specialization refers to a higher taxonomic level (Family or above, e.g. fly or bird pollinated) to describe the diversity of pollinators (Ollerton et al. 2007). Whereas phenotypic generalization and specialization are the adaptations displayed by a flower usually in relation to functional specialization (e.g. complex scent, specialist rewards, radial or zygomorphic symmetry) (Ollerton et al 2007). Additionally, some research areas have constructed indices of specialization incorporating phylogenetic information such as plants exploited by phytophagous insects or parasite host diversity (Symons & Beccaloni 1999, Poulin & Mouillot 2005). Ollerton et al. (2007) highlighted that while a plant may be considered phenotypically specialized, this may not necessarily equate to ecological specialization as a plant may be phenotypically specialized but ecologically generalized and vice versa. Therefore these terms mean different things to researchers in different botanical fields. The semantics around the terminology of specialization and generalization, therefore, present a further hindrance to a more unified approach. This may explain why the debate concerning the frequency of generalized versus specialized plant-pollinator relationships as well as the ecological and evolutionary importance of either system is still ongoing.

Flowers utilize a combination of floral traits such as colours, and scents to advertise rewards and attract potential pollinators. Selection on the variation in these features is responsible for the specificity in pollination systems (Johnson & Steiner 2000). Therefore pollination ecology studies often focus on determining the significance of these floral traits in shaping particular plant-pollinator relationships.

An example of this is the importance of floral scent in plant-pollinator communication (Raguso 2008, Schiestl 2005). Plants emit a wide range of scents to entice potential pollinators from a distance; additionally this can be instrumental in keeping particular visitors away (Shuttleworth & Johnson 2009a). The burgeoning field of plant chemical ecology has identified a significant amount of chemical compounds and scent combinations involved in this process. Revelations about this complex communication system are largely due to technological advancements such as the use of gas chromatography-mass spectrometry (GC-MS) (Raguso 2008). This has resulted in a more rapid identification of floral volatiles, with an impressive 1700 organic compounds found in 991 flowering plants across 90 families and 38 orders identified (Knudsen et al. 2006). This progress has also been witnessed in research focusing on floral scent signal production as well as the floral visitor's perception of these signals (Dudareva & Pichersky 2006, Gang 2005, Carlsson & Hansson 2006, Smith et al. 2006).

Such studies have revealed a wealth of knowledge not only on the variation (both subtle and elaborate) displayed in floral traits, but can potentially provide insights into broader concepts of the evolutionary mechanisms involved in pollinator-driven diversification, which is responsible for a vast majority of the plant diversity we see today (Stebbins 1970, van der Niet & Johnson 2012).

1.2 Asclepiadoideae: structural features and important pollination mechanisms

The subfamily Asclepiadoideae (family Apocynaceae *sensu* Endress & Bruyns 2000, Endress et al. 2014) commonly known as asclepiads or milkweeds have been used in several studies around the world to better understand pollination systems (e.g. Ramakrishan & Arekal 1979, Pant et al. 1982, Chaturvedi & Pant 1986, Ollerton et al. 2003). They are a diverse group of plants with highly derived floral traits (Endress & Bruyns 2000) and their complex floral morphology has made them ideal candidates for further understanding the evolutionary ecology of plant reproduction, particularly plant-animal interactions and host-plant co-evolution (Ollerton 1998, Van Zandt & Agrawal 2004).

Several of their unique morphological features are responsible for this. Their floral morphology consists of synorganized arrangements which are fairly consistent and exclusive to the Asclepiadoideae subfamily. These elements include: elaborate coronas, which are sterile whorls in addition to their corolla; a gynostegium formed by a fused androecium and gynostecium differentiated into five sectors arranged in a revolver-like system; and their unique mode of pollen presentation (described below) (Bookman 1981, Fallen 1986, Endress 1994, Kunze 1981, 1996, Verhoeven & Venter 2001).

Asclepiads are largely insect pollinated with some exceptional cases of bird pollination (Ollerton & Liede 1997, Ollerton 1998, Pauw 1998). They have aggregated pollen which is contained in sacs to form pollinia grouped into pairs (five per flower). Each pair is held in place by two translator arms (caudicles) connected to a central corpusculum known collectively as the translator apparatus which connects the pollinia of two adjacent anthers (Kunze 1991). This type of pollen presentation is shared with orchids (Orchidaceae), however orchids lack translator arms and rely on sticky viscidium rather than a mechanical clip to attach pollinia to pollinators (Endress 2016). During pollination an insect visitor's appendage is trapped between the guide rails formed by the anther margins and forced into the translator apparatus where the corpusculum clamps to it. As the insect attempts to free itself it dislodges the pollen pair which will stick to the appendage until the insect visits another flower where the pollinia will get dislodged into the stigmatic chamber between the anther slits (Kunze 1991, 1995, Liede 1994, Ollerton & Liede 1997, Pauw 1998). The corpusculae often stay attached to the pollinator's appendages after pollination and more pollinia may attach to it to form aggregates of pollinia, a process referred to as concatenation, resulting in long chains of corpusculae and pollinaria (Morse 1981, Coombs et al. 2009, 2012, Wiemer et al. 2012, Cocucci et al. 2014). A recent study of *Cynanchum ellipticum* found that concatenation does not interfere with foraging behaviour or efficiency and it was suggested they may even add the advantage of increasing the attachment points on an insect (Coombs et al. 2012). This feature makes it easy to distinguish between visitors and effective pollinators. However the attachment mode is sometimes detrimental as highlighted in the case of dismembered body parts of wasp pollinators left in the guide rails documented in *Pachycarpus appendiculatus* and *Asclepias syriaca* (Morse 1981, Shuttleworth & Johnson 2009b).

Species of the Asclepiadoideae are thought to be more generalized in the New World while displaying an affinity for specialization in the Old World (Ollerton & Liede 1997).

African milkweed studies support this finding with recent studies further establishing this trend (Ollerton et al. 2003, Shuttleworth & Johnson 2009a, 2009c, 2012). The group is well represented in Africa particularly in southern Africa where there is an estimated 600 species (Victor et al. 2000). Milkweeds are the eighth largest plant family in southern Africa and show an incredible 87% endemism (Cowling & Hilton-Taylor 1997), thus offering an excellent opportunity to study complex floral morphologies and their impact on plant-pollinator interactions in this region.

Studies of the pollination systems of South African species have highlighted several specialized pollination systems, with some species relying on a single functional group of pollinators e.g. *Asclepias woodii* and *Sisyranthus trichostomus* are pollinated by chafers beetles (Coleoptera, Scarabaeiae, Cetoniini) while *Miraglossum pilosum* and *Pachycarpus natalensis* by pompilid wasps (Ollerton et al. 2003), as well as specialized pollination by four functionally similar *Hemipepsis* wasps in *Pachycarpus grandiflorus, Xysmalobium orbiculare*, *Aspidoglossum glanduliferum*, *Periglossum angustifolium*, and a *Miraglossum* spp. (Shuttleworth & Johnson 2012). This may, however, result from a sampling bias towards more specialized species, as these typically offer more opportunities to explore evolutionary questions about the functional significance of floral traits (Shuttleworth & Johnson 2009c, Coombs et al. 2010). Moreover, generalist pollination systems have been suggested for some species, including *Xysmalobium gerrardii*, *Asclepias crispa* and *Cynanchum viminale* (Ollerton et al. 2003, Shuttleworth & Johnson 2009a, Liede & Whitehead 1991). Nonetheless, the species studied to date suggest that specialization is frequent within the species occurring in the summer-rainfall grasslands of KwaZulu-Natal and the dry regions of Western and Northern Cape. They have found specialized pollination by spider-hunting wasps (Ollerton et al. 2003, Shuttleworth & Johnson 2012), vespid wasps (Coombs et al. 2009), chafer beetles (Shuttleworth & Johnson 2009c), bees (Ollerton et al. 2003, Shuttleworth & Johnson 2009a, Coombs et al. 2012), saprophilous flies (Meve & Liede 1994, Meve et al. 2004, Shuttleworth & Johnson 2009a, Coombs 2010, 2011), as well as birds (Pauw 1998).

The functional floral trait studies on South African milkweeds have produced very interesting findings on the importance of different floral cues in attracting and filtering pollinators, and identified features involved in the extreme pollinator specificity observed. Examples include highlighting the role of unpalatable nectar in *Pachycarpus* and *Xysmalobium* as a deterrent of a wide range of floral visitors while also enticing a select few (Shuttleworth & Johnson 2008, 2009d, 2009e). Presenting evidence of convergent evolution in an assemblage of chafer-beetle pollinated milkweed species although they utilise different chemicals to lure the same pollinator (Shuttleworth & Johnson 2010). Apart from the summer rainfall milkweeds the group is still understudied in the country, particularly in the winter rainfall region.

1.3 Study aims

The aim of this study was to enhance our understanding of plant-pollinator interactions in the South African Asclepiadaceae, specifically selected Cape milkweeds for which no knowledge of the degree of specialization in pollination systems or the floral traits objectively quantified. The study includes a comparison of the pollination ecology of a genus that occurs in the summer and winter rainfall regions of South Africa, with focus on a Fynbos species and a Succulent Karoo/Desert species. Thus creating a basis of comparison of generalist and specialist pollination systems utilized by this genus in different geographical regions. I also present the first pollination study on the monotypic Cape endemic *Eustegia*, representing the first pollinator data for the tribe Eustegieae. Lastly I investigated the floral scent chemistry of four additional milkweed species, assess the floral scents' ability to accurately predict the species' pollination systems and conduct a species and genus level comparison with similar studies from around the world.

2. Pollination systems of two South African winter rainfall region milkweeds: *Gomphocarpus cancellatus* and *Gomphocarpus filiformis*

2.1 Abstract

The Asclepiadoideae (Apocynaceae) are well known for their floral complexity and widespread diversity. A large proportion of which is centered in southern Africa. South African studies on the group's pollination system have focused on species in the summer rainfall region where they have been shown to be specialized. To determine if this system is consistent throughout their range, a pollination study was carried out on winter rainfall species occurring in Fynbos and the Succulent Karoo. The study investigated the pollination ecology of two *Gomphocarpus* species through pollinator observations, qualitative and quantitative assessments of floral traits in the form of colour spectra reflectance analysis, gas chromatography mass spectrometry (GC-MS) to determine floral scent components and compared these to summer rainfall congeners. Both species attracted a wide range of floral visitors, *G. cancellatus* displayed a generalized pollination system while *G. filiformis* showed a more restricted pollination system with *Camponotus* ants as its most abundant pollinator. Both species had distinct floral scents, different from each other and from their congeners. Floral scent and nectar rewards appeared to be the most important floral attractants. The study highlighted that congeneric species can look similar in appearance but utilize completely different chemicals, which may result in different pollination systems.

2.2 Introduction

Complex flowers present evolutionary ecologists with ample opportunities to study functional traits and cue combinations shaping their relationship with plant visitors. Asclepiads or milkweeds (Apocynaceae; Asclepiadoideae) are playing a major role in illuminating the intricacies of specialized and generalized pollination systems. Their vast diversity and elaborate floral structures have made them ideal candidates for enhancing our understanding of such complex plant-animal interactions. The trap-flowers of *Ceropegia* L. (Apocynaceae: Asclepiadoideae-Ceropegieae) used to detain small flies overnight has offered insights to the extent that plants will specialize their features to achieve pollination (Masinde 2004, Ollerton et al. 2009, Heiduk et al., in press). Additionally, the deceptive mimicry of carrion plants that smell and in some cases look like dead animal flesh or fecal matter in the Stapeliinae has expanded our understanding of the wealth of chemical arsenals and extraordinary morphological extremes used to lure specific pollinators (Meve & Liede 1994, Jürgens et al. 2006, 2013, Shuttleworth et al., in press).

Asclepiads are abundant in Africa, with a large variety occurring in southern Africa, making up 90 genera and 600 species (Victor et al. 2000). South Africa in particular is one of the main centers of diversity and endemism for this group (Victor et al. 2000). Pollination studies carried out in South Africa have identified several specialized asclepiad pollination systems (Coombs et al. 2009, Shuttleworth & Johnson 2012).

Gomphocarpus offers an excellent opportunity for a comparative pollination study across a genus distributed in very different environments in South Africa. Additionally, its structure, and the existence of comprehensive pollination studies already carried out in the summer rainfall region of the country on two species makes it an ideal candidate (Coombs et al. 2009, Burger et al., in press). The genus is made up of 32 species of short-lived perennial shrubs with an average height of 1-1.5m, with larger species reaching heights of 2.5 - 3m. Each plant produces several umbels containing 20 or more individual, pendant, cream to yellow flowers. The genus is distributed in Africa and the Arabian Peninsula, with ten species occurring in southern Africa (Goyder & Nicholas 2001). They predominantly occur on rocky hills, with some found on sandy banks and disturbed areas (Bullock 1952).

Gomphocarpus physocarpus is mainly found in the grasslands on the southern and eastern parts of South Africa, overlapping its range with *G. fruticosus* along the east coast (Goyder & Nicholas 2001). The latter is more widespread throughout the country. Structurally, these summer rainfall species are soft shrubs with several cream flowers bunched into an inflorescence and occur on stony flats, roadsides or disturbed areas (Manning & Goldblatt 2012). *Gomphocarpus physocarpus* is exclusively pollinated by vespid wasps, namely *Belanogaster* and *Polistes* (Hymenoptera), which are thought to be attracted to the plant's floral scent (Coombs et al. 2009). It also offers abundant nectar rewards, which are avoided by other pollinators except wasps, suggesting that nectar may act as a repellent to other visitors much like for some *Pachycarpus* species and *Xysmalobium orbiculare* E. Mey. where the bitter taste of the nectar is avoided by bees (Burger et al., in press, Shuttleworth & Johnson 2006, 2009d, 2012). While it has been suggested that *G. fruticosus* is predominantly bee pollinated (Burger et al., in press), this species is visited by a large array of pollinator types.

This chapter investigates the pollination biology of two *Gomphocarpus* species from the winter rainfall regions for which preliminary observations suggested more generalist pollination systems than their summer rainfall congeners; *G. cancellatus* (Burm.f.) Bruyns and *G. filiformis* (E.Mey.) D.Dietr. The pollination systems of both species have not been previously studied, however there is a historical observation of 'small mosquito-like flies' on *G. cancellatus* (formerly known as *G. arborescens*) (Elliot 1891, Goyder & Nicholas 2001).

The overall plant structure of the two study species is very different (Figure 1a and b). *Gomphocarpus cancellatus* is an upright solid shrub and *G. filiformis* is a very tall and slender grass-like shrub. Despite this, their flowers look very similar to each other at a superficial level and to the previously studied specialist *G. physocarpus* and less specialized *G. fruticosus*. The flowers of *G. filiformis* are almost inconspicuous with spaced umbels that blend in with the leaves. The most significant structural floral difference in terms of pollination is the presence of teeth in the corona of *G. filiformis*, which are absent in *G. cancellatus*. These may act as a pollinator filtering mechanism as is the case for *G. physocarpus* (Burger et al., in press) and may point to a more specialized pollination system. However, characteristics such as small flower size, flowering times and preliminary observations of many different insect floral visitors suggest that both species have a generalized pollination system.

I hypothesized that despite the apparent similarities in floral structures, the summer and winter rainfall species will have different pollination systems due to their different habitats, climate, and flowering and pollinator emergence times. I expected the Cape plants to exhibit a more generalized pollination system, and this will be reflected in differences in their attractants and rewards. The goal of this chapter was to determine the pollinators of *G. cancellatus* and *G. filiformis*, quantify the efficiency of the pollen transfer, determine the breeding system of *G. cancellatus* and use this data to address the following questions:

1. Do the winter rainfall *Gomphocarpus* species have similar specialist pollination systems to their summer rainfall congeners?

2. Are the pollinator attractants (colour and scent) used by *Gomphocarpus* species in the Western Cape similar to those of summer rainfall species?

I hypothesized that both species will display generalized pollination systems, while *G. filiformis* will have a narrower range of effective pollinators due to the filtering function of the corona teeth. I also predicted that the attractants of the two species would differ from their summer rainfall congeners as well as from each other primarily due to the more cryptic appearance of *G. filiformis*; this species will have a stronger olfactory signal than *G. cancellatus*.

2.3 Methods and Materials

Study species

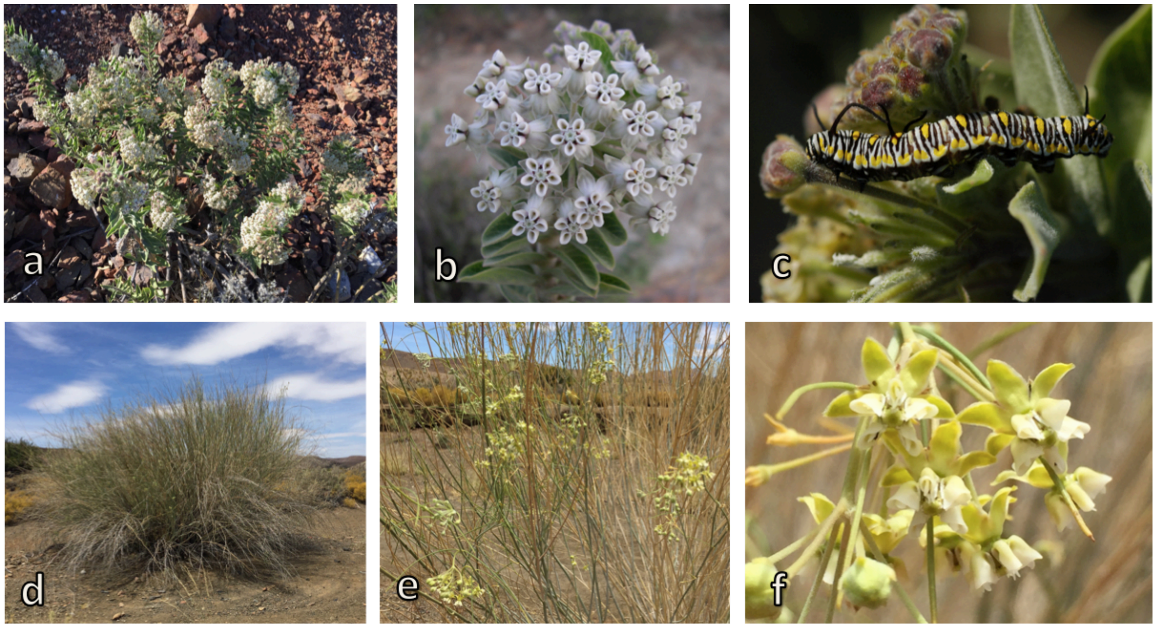

Figure 1. Gross morphologies of a *Gomphocarpus cancellatu*s plant (a), inflorescence (b) and an African Monarch butterfly (*Danaus chrysippus*) caterpillar feeding on the leaves (c); and a *Gomphocarpus filiformis* plant (d) and flowers (e and f).

Gomphocarpus cancellatus (Burm.f.) Bruyns is a rigid hairy shrub of 1-1.5m height. Its flowers are greyish-white to cream coloured extra-axillary inflorescences with reflexed corollas grouped in nodding umbels of 10-20 flowers (Figure1a). *Gomphocarpus cancellatus* occurs in rocky hillsides, crevices and disturbed slopes (Goldblatt & Manning 2012). Its primary habitats are Albany Thicket, Fynbos and the Succulent Karoo (Von Staden 2012). It flowers from March – December (Goldblatt & Manning 2012).

In contrast *G. filiformis* (E.Mey.) D.Dietr. is a slender 1-3m tall shrub that branches from the base (Figure 1d, Goyder & Nicholas 2001). It has very thin stems and filiform leaves. Its flowers are creamy white to yellow-green coloured extra-axillary inflorescences with 6-12 flowers and reflexed corollas in a nodding umbel (Figure 1e-f, Goyder & Nicholas 2001). The flowers have a flattened tooth in the corona cavity (seen in Figure 1f) (Goyder & Nicholas 2001). *Gomphocarpus filiformis* is found in deserts, arid shrublands and along dry river courses scattered in the Nama and Succulent Karoo, as well as the Namib

Desert (Goyder & Nicholas 2001, von Staden 2012) and flowers throughout the year (P. Bruyns per. comm).

Study sites

The study was carried out on the Cape Lime Plant; a quarry located in Langvlei, between Worcester and Robertson in the Western Cape (-33.738847, 19.763514) for *G. cancellatus* in April – May of 2015 and 2016. The site had a population of ca. 40-50 plants, all located on a rocky slope along the side of the road. A roadside population along the Rawsonville – Worcester south road (-33.674885, 19.386132) was used to supplement the scent samples. In this population both white and slightly pink morphs of *G. cancellatus* were scattered along the road. *Gomphocarpus filiformis* was studied in December 2015 on a farm in Laingsburg (-33.184531, 20.806581) also in the Western Cape. This site had a population of ca. 60 plants along a sandy river course with many others scattered in the surrounding farms and roadsides.

Floral visitors

Floral visitors were observed for a total of 23 hours over four days for *G. cancellatus* (17th and 23rd May 2015, 15th and 19th May 2016). Due to distance and other logistical reasons *G. filiformis* pollinator observations were only carried out for one day (9th December 2015). Morning and afternoon observations were conducted from 10am-3pm for *G. cancellatus* and from 12pm-3pm for *G. filiformis* and involved observing a randomly selected plant for five minutes and recording all its visitors. Visitors that were directly on the plants were noted and their behaviour (e.g. drinking nectar) was noted and, when possible, at least ten representative specimens of each insect species were collected and pinned. All insects were identified to family level (Picker et al., 2004), with further help from professional entomologists for identification. The insects were assessed for pollen load and pollinaria attachment zones using a dissecting microscope. The number of pollinaria and corpusculae was assessed for each specimen. As bees were the most prominent visitor for *G. cancellatus,* additional behavioural observations were carried out at midday. A total of 13 randomly selected bees were observed for two minutes each and the number of flowers and plants they visited was recorded.

Pollination success

Pollination success was assessed using the pollen transfer efficiency (PTE), which is the proportion of pollinaria removed from a flower and deposited on another flower of the same species. This measure of PTE has been utilized in many milkweed (Ivey et al., 2003, Shuttleworth & Johnson, 2006, 2008, Coombs et al., 2009) and orchid studies to measure pollination success (Peter & Johnson 2008a; Johnson et al., 2009; see review by Harder and Johnson 2008). The PTE in this study was determined by counting the number of pollinia removed and the number deposited in the guide rails of ten flowers from ten randomly selected plants for each *Gomphocarpus* species under a dissecting microscope.

Nectar

To determine floral rewards, nectar was sampled from flowers from each species. Open *G. cancellatus* flowers were bagged with fine-mesh pollinator exclusion bags overnight onsite to prevent nectar feeders from accessing the nectar. Nectar was sampled the next morning from 18 flowers from four plants. The volume was measured using 1μL capillary tubes. The nectar's sugar concentration in sucrose equivalents was determined using a Bellingham and Stanley handheld sugar refractometer. Sucrose concentrations for *G. cancellatus* flowers were too high for the refractometer's range of 0-50% therefore the nectar samples were diluted with a known volume of water. The measured concentrations were then adjusted accordingly to get the actual nectar concentration. *Gomphocarpus filiformis* had no nectar at the time of sampling most likely due to the activity of nectar feeders and high evaporation as sampling was conducted in the dry afternoon weather. Due to the travel distance to this site making multiple field trips a challenge, a flowering branch from ten randomly selected plants was collected, placed in water and the flowers bagged overnight in a lab. Nectar was sampled from a total of 58 flowers over all the branches the following morning. The average nectar volume and sugar concentration per flower was then calculated.

Colour

The colour of the flowers and leaves was determined using an Ocean Optics S2000 spectrophotometer (Ocean Optics Inc., Dunedin, FL. USA) with a fibre optic reflection probe (QR-200-7-UV-VIS; 200μm) at a 45° angle to the specimen's surface. The reflectance of the corona, corolla, stigmatic surface and the upper side of leaves was measured and the reflectance spectra recorded from 300-700nm. An Ocean Optics DT-mini deuterium tungsten halogen light was used as the light source. To calibrate the spectrophotometer an Ocean Optics WS-1 diffuse reflectance standard was used. A total of ten different plants were used for each species.

The reflectance values were plotted on a graph. They were also plotted on a colour hexagon model (Chittka 1996) following methods described in Chittka and Kevan (2005) which involves using reflectance data to give a visual representation of the three-dimensional photoreceptor signal space with each axis corresponding to a photoreceptor signal. This is representative of Hymenopteran optical space. When different floral structures are represented in the hexagon, the distances between them represent their perceptual colour difference. Therefore the more distant objects are from each other within the hexagon the greater the differences between the colours from an insect's perspective. The average reflectance of the leaves was used as the background for each species.

Scent collection and analysis

Floral scent was collected using dynamic headspace adsorption of volatiles by enclosing an individual inflorescence in a polyacetate bag (Kalle, Germany) and pumping the air in the bags through a small glass cartridge containing 1mg of Tenax ® and 1mg of Carbotrap™ activated charcoal using a battery operated membrane pump (PAS-500 personal air sampler, Spectrex, CA, USA) (Shuttleworth & Johnson 2010). Leaf and air controls were taken for comparison. Field and lab scent sampling was carried out for both species. Inflorescences from separate plants were sampled (each sample represents a different individual plant) in the morning and afternoon for *G. cancellatus* (9 samples from Worcester and 2 from Rawsonville), and midday for *G. filiformis* (5 samples).

Scent was analysed by coupled gas chromatography-mass spectrometry (GC-MS) with a Varian CP-3800 GC (Varian, Palo Alto, CA, USA) using Bruker BR-Swax column coupled with a Varian 1200 quadrupole mass spectrometer and a Varian 1079 injector which was equipped with a 'Chromatoprobe' thermal desorption device (see Dötterl et al. 2005, Shuttleworth & Johnson 2010 for further details). The scent profiles of the species were identified and quantified using Varian Workstation software with the NIST 11 V2.0 mass spectral library and verified in many cases by comparing to Kovats indices in published literature and retention times of a number of known standards. Compounds present in similar amounts in the control were considered contaminants and not included in the analyses unless stated otherwise. Emission rates were calculated by comparing peak areas to that from known amounts of methyl benzoate injected onto a trap and analysed using the same protocol as the plant samples.

To determine differences and similarities in the floral scent profiles, the percentages of volatile compounds were square root transformed and visualized using non-metric multidimensional scaling (NMDS) based on Bray-Curtis similarity using the PRIMER 6.1.6 software (Clarke & Warwick 2001, Clarke & Gorley 2006). Additional scent profiles from a *G. fruticosus* and *G. physocarpus* study (Burger et al., in press), which occur in the summer rainfall region of South Africa, were included in the PRIMER scent analysis for comparison. A one-way Analysis of Similarity (ANOSIM), which is a non-parametric permutation procedure that is based on the similarity matrix of the ordination, was also carried out in PRIMER 6.1.6 to test for differences in the scent profiles of the included species. ANOSIM produces a test statistic R which represents how separated the groups are. Values of R close to unity indicate complete separation of groups, whereas the R values closer to zero indicate a smaller separation of the groups (Clarke & Warwick 2001). The significance of the differences in the scent profiles was determined through a comparison of the samples' R values to the R values produced from 10 000 random permutations of the scent samples (Clarke & Warwick 2001). The compounds which best characterized the scent of each species were determined using SIMPER. This is a function in PRIMER that compares similarity percentages by calculating the percentage contribution of each compound to average overall Bray-Curtis similarity between samples within a group (Clarke & Warwick 2001), thus outlining the compounds that best characterize a group.

Dependence on pollinators for reproduction

The autogamous reproductive ability of *G. cancellatus* was investigated by covering fresh buds on each of 20 plants with mesh bags to exclude all floral visitors. The number of bagged buds and therefore flowers at maturity were counted at the beginning of the experiment, as the flowers tend to fall off easily. Unbagged buds on a different inflorescence on the same plant were also counted and tagged as open-pollinated controls. Additionally, single inflorescences on different plants were tagged in a similar fashion to determine the general pollination success of the population. The number of pods of the controls and bagged treatments were counted upon maturity (about 3 months later). A large number of plants later suffered from disease and the sample size was reduced by ca. 30%.

2.4 Results

Floral visitors and pollen transfer

A wide range of floral visitors was observed visiting both *G. cancellatus* (Figure 2) and *G. filiformis* (Table 2). A total of 16 different species were observed for *G. cancellatus* and 12 for *G. filiformis*. These ranged from small Melyrid beetles to several fly families, butterflies, ants and birds, all of which were drinking nectar (Figure 2, Table 2). The most abundant visitors and subsequent pollinators of *G. cancellatus* were bees, particularly Cape honeybees (*Apis mellifera capensis*). A total of 36 honeybees were collected and every bee had at least one pollinarium as well as several corpusculae attached to its tongue or tarsus. The presence of corpusculae represents successful pollination as it implies that a pollinaria sac was successfully picked up and deposited in the guide-rails of a flower (Bookman 1981, Johnson et al. 2004).

Balbyter ants (*Camponotus fulvopilosus*) were the most abundant visitor on *G. filiformis* and carried large loads of pollinaria, single pollinia and/or had several corpusculae attached. There were at least 30 Balbyter ants on each *G. filiformis* plant drinking nectar but only a representative sample was collected from several plants. There were fewer honeybees found visiting *G. filiformis* as only two were observed, one of which was collected and possessed 18 pollinia pairs and 34 corpusculae. A different species of *Camponotus* ants were also abundant on *G. cancellatus* flowers, but were not moving pollinaria around. A total of three *Camponotus fulvopilosus* ants were observed on *G. cancellatus* flowers, one was collected and had a pollinia pair attached to it. Lesser double-collared sunbirds were spotted drinking nectar in the morning from both species.

A much larger proportion of *G. cancellatus* visitors carried pollinaria and showed signs of successful pollination in the form of corpusculae compared to *G. filiformis* visitors. The most common pollinaria attachment zone was the arolium of the tarsi; this is a lobe-like structure between the tarsal claws (Table 2, Figure 3). For those insects lacking arolia, the corpusculae was attached directly to their tarsi. The main attachment points for bees were the tarsal claws and mouthparts, while in some cases the pollinia were directly attached to the bee's tongue.

Apis mellifera capensis observed on *G. cancellatus* during midday visited an average of 8.7 (±2) flowers and 0.9 (±0.3) plants per minute. This highlights that generally bees spent more time foraging on several flowers of the same plant but also moved between flowers of different plants.

Gomphocarpus cancellatus flowers showed high pollination levels (35%) compared to the meager levels in *G. filiformis* (1.33%) (Table 4). The overall pollen transfer efficiency was therefore higher in *G. cancellatus* (21.3%) compared to *G. filiformis* (9%).

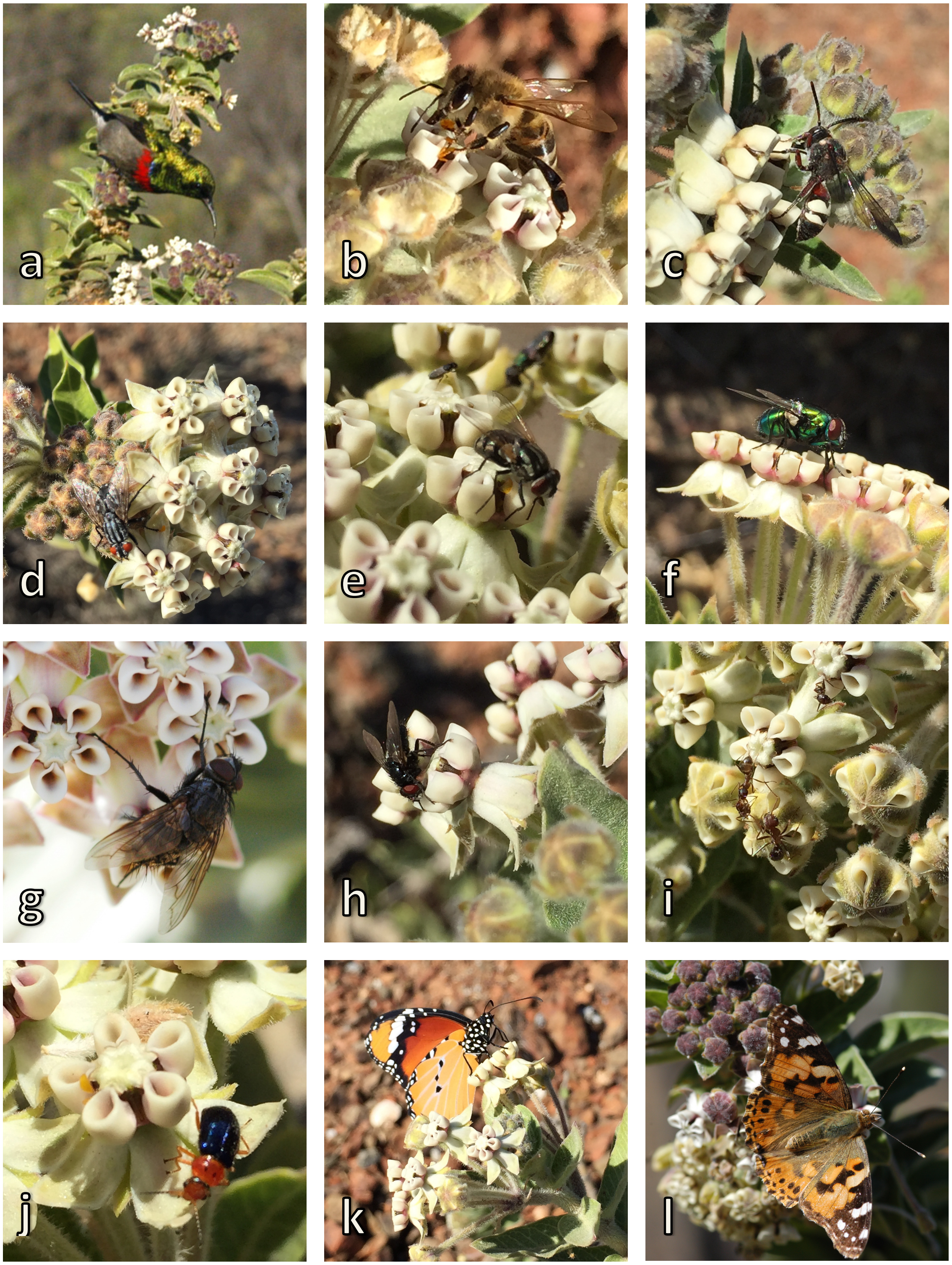

Figure 2. Observed floral visitors and pollinators on *G. cancellatus*. a) Lesser double-collared sunbird (*Cinnyris chalybeus*), b) honey bee (*Apis mellifera*), c) Vespid wasp, d) and e) Sarcophagid flies, f) green blowfly (*Lucilia* sp.), g) and h) Tachinid flies, i) *Camponotus* sugar ants, j) Melyrid beetle, k) African Monarch butterfly (*Danaus chrysippus*) and l) Painted Lady butterfly (*Cynthia cardui*).

Table 2. Floral visitors and pollinators of *G. cancellatus* and *G. filiformis*, both observed and analyzed *(in bold)*, with the average number of pollinia pairs, halves and corpusculae present on different body parts, along with the standard error (in parentheses).

Floral visitor	No. observed (**No. analyzed**)	No. carrying pollinaria	Average pollinaria (±SE) per visitor	Average half pollinaria[1] (±SE) per visitor	Average corpusculae[2] (±SE) per visitor	Pollinaria placement
Gomphocarpus cancellatus						
Diptera						
Tachinidae						
Tachinid sp. 1	9 (**5**)	4	0.6 (0.4)	0	0.2 (0.2)	Tarsal claw arolium
Caliphoridae						
Lucilia sp.	4 (**1**)	1	0	0	3 (0)	Tarsal claw arolium
Sarcophagidae						
Sarcophagid sp. 1	2 (**1**)	1	1	1	3	Tarsal claw arolium
Sarcophagid sp. 2	1 (**1**)	1	0	1	0	Tarsal claw arolium
Sarcophagid sp. 3	3					
Hymenoptera						
Apidae						
Apis mellifera capensis	36 (**14**)	14	1 (0.2)	0.9 (0.3)	0.9 (0.3)	Tarsal claw arolium, tarsal hair, mouthpart
Apis sp. 1	9 (**9**)	8	2.8 (0.8)	2.8 (0.8)	4.8 (1)	Tarsal claw, mouthpart
Apis sp. 2	1					
Formicidae						
Camponotus fulvopilosus	3 (**1**)	1	1	0	0	Tarsus
Camponotus sp. 1	20 (**6**)	0	0	0	0	
Braconidae						
Braconid sp. 1	4 (**1**)	1	1	1	0	Mouthpart
Vespidae						
Vespid sp. 1	2					
Coleoptera						
Chrysomelidae						
Sonchia sternalis	4 (**1**)	1	1	0	0	Tarsus
Melyridae						

Melyrid sp. 1	3 (**3**)	1	1	0	0	Tarsus
Lepidoptera						
Hesperiidae						
Danaus chrysippus	4 (**1**)	0	0	0	0	
Cynthia cardui	1					
Passeriformes						
Nectariniidae						
Cinnyris chalybeus	3					
Gomphocarpus filiformis						
Diptera						
Tachinidae						
Tachinid sp. 1	3					
Hymenoptera						
Formicidae						
Camponotus fulvopilosus	46 (**10**)	10	2.5 (0.8)	0.8 (0.3)	1.9 (0.6)	Tarsus
Formicid sp. 1	2					
Apidae						
Apis mellifera capensis	2 (**1**)	1	18	6	34	Tarsus, mouthparts
Apidae sp. *1*	1					
Anthophoridae						
Xylocopa caffra	3					
Vespidae						
Vespula germanica	3(**3**)	3	4.7 (1.8)	1.3 (0.3)	5.6 (3.7)	Tarsus
Pompilidae						
Pompilid sp.1	1 (**1**)	0	0	0	0	
Coleoptera						
Chrysomelidae						
Cryptocephalus decemnotatus	4					
Lepidoptera						
Hesperiidae						
Danaus chrysippus	4 (**2**)	0	0	0	0	
Passeriformes						

Nectariniidae	
Cinnyris chalybeus	4

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[1] Half a pollinaria refers to a pollinaria where one pollinium has been deposited.

[2] Corpusculae refers to the remaining clip after both pollinia have been removed/deposited.

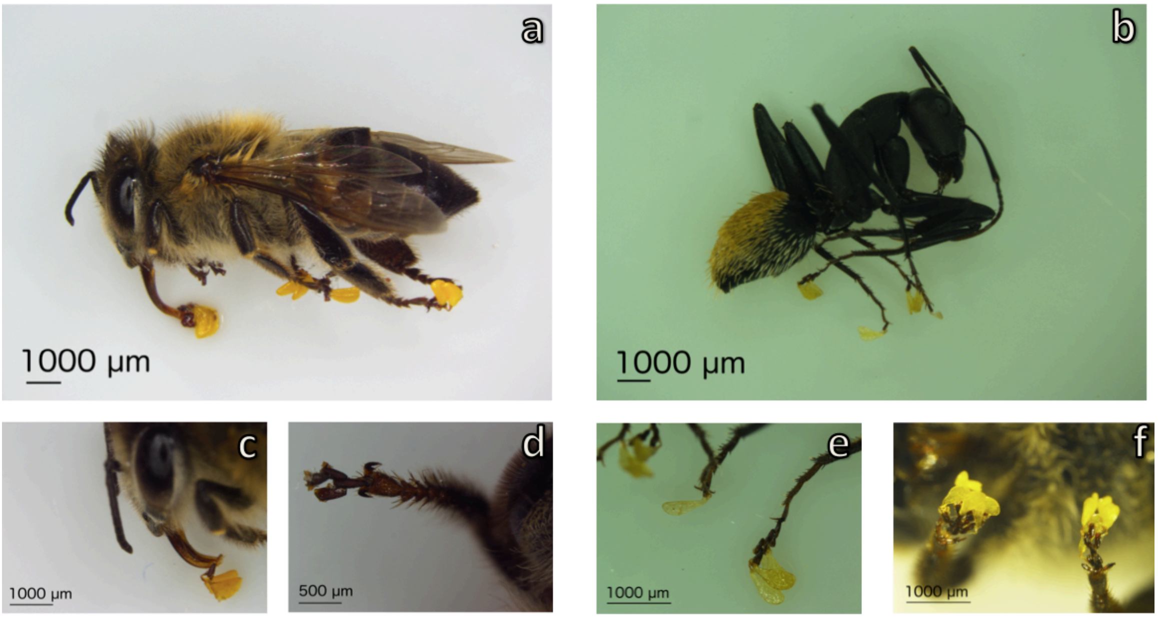


Figure 3. The most abundant visitor with pollinia for *Gomphocarpus cancellatus* (a) Cape honeybee (*Apis mellifera capensis*) with pollinia attached to its mouthparts and several tarsal claws and arolia. The most abundant insect visitor for *G. filiformis* (b) Balbyter ant (*Camponotus fulvopilosus*) with pollinaria attached to the tarsal claws. A close up of a different honeybee with a pollen pair on its mouthparts (c) and 4 pollinium clips (corpusculae) attached to the same bee's tarsal claw (d) found on *G. cancellatus*. Pollen pairs and single pollinium attached to a Balbyter ant's tarsi (e) and on a honeybee's tarsi found on *G. filiformis* (f).

Nectar

The average nectar volume produced by *G. cancellatus* was 16.1µl ± 1.7 (range 6-31µl) per flower while the average sucrose concentration was 53.7 ± 3.7 % with a range of 23-72 %. *Gomphocarpus filiformis* produced higher volumes (22.2µl ±1, range 5-40µl) of more dilute nectar per flower 15.6 ±1% and a range of 5-37µl.

Table 4. Mean (median) ± SE percentage of pollinia removed and inserted per flower per plant for ten flowers from each species. Pollen Transfer Efficiency (PTE) represents a measure of the success rate of pollination.

Species	Site	Pollinated flowers (%)	Pollinaria removed (no. /flower/plant)	Pollinaria inserted (no. /flower/plant)	PTE (%)
Gomphocarpus cancellatus	Worcester	35(30) ± 7.2	3.2(3.1) ± 0.4	0.7(0.6) ± 0.2	21.3
Gomphocarpus filiformis	Laingsburg	1.33(0.7) ± 0.7	1.3(0.7) ± 0.7	0.1(0) ± 0.05	9.2

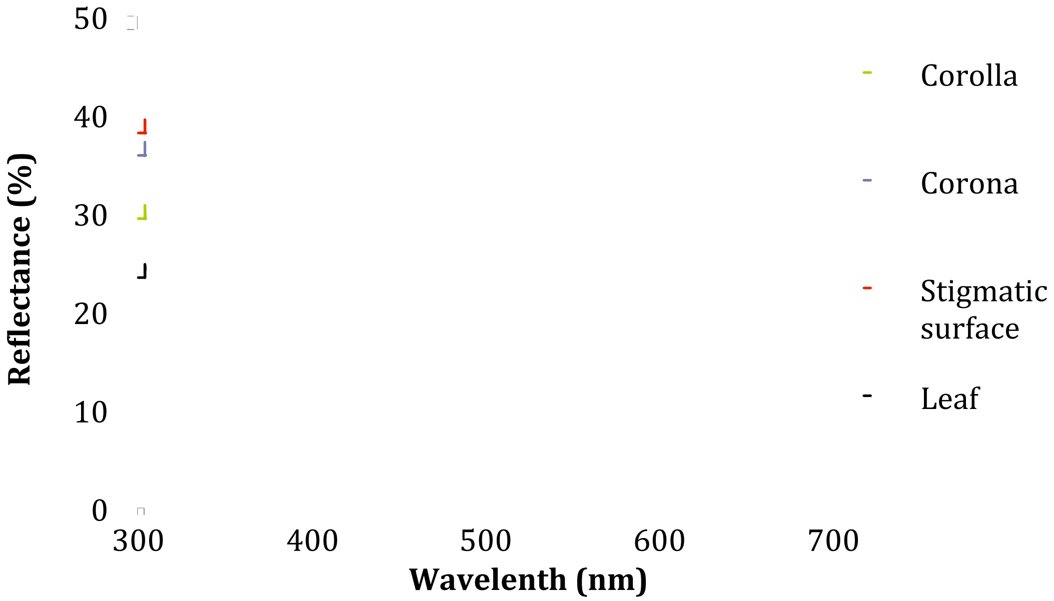
Colour

Gomphocarpus cancellatus flowers consist of dull white to cream coloured coronas, with white-light green stigmatic surfaces and similarly coloured reflexed corollas. The leaves are dark green. *Gomphocarpus cancellatus* floral structures show a marked difference in reflectance from the leaf (Figure 3). The stigmatic surface in particular shows the highest reflectance with peaks in the 550-650nm range, while the corolla and corona show similar peaks but at lower reflectance levels. In *G. filiformis* the reflectance of the corona and corolla start to stand out in the 450-500nm range with peaks in the 550-650 range. While the stigmatic surface shows very little difference in reflectance to the leaves. Colour analysis shows that the floral structures start to show differences in the blue light zone, and the stigmatic surface and corona show the highest reflectance. The colour hexagon shows that all the floral features fall in blue-green receptor zone for honeybees (Figure 4a). The colours are a slight contrast to the dark green leaf background at the center. Multiple measurements of the corona, corolla and stigmatic surfaces do cluster but also show slight separation. The stigmatic surface and corona show the most similarity.

In contrast, floral parts of *G. filiformis* show a much greater difference in colours (Figure 3b and 4b) highlighted by more scattered points. These flowers are made up of dull white-yellow coronas, with a dull light green stigmatic surface similar in colour to the reflexed corollas which display a brighter light green hue whilst the thin leaves are a slightly darker green. The floral structures also fall within the blue-green region with the stigmatic surface falling slightly into the green light receptor zone (Figure 4b).

Gomphocarpus filiformis shows a much more clear distinction between the different floral parts, particularly the pronounced separation of the corolla which is the most prominent colour of the flower. The corona and stigmatic surface, which are clustered close together, are much closer to the leaf background as in *G. cancellatus* but more spread out and also overlap in some areas. The colour differences in floral structures are therefore more prominent in *G. filiformis* than in *G. cancellatus*.

a) *G. cancellatus*



b) G. *filiformis*

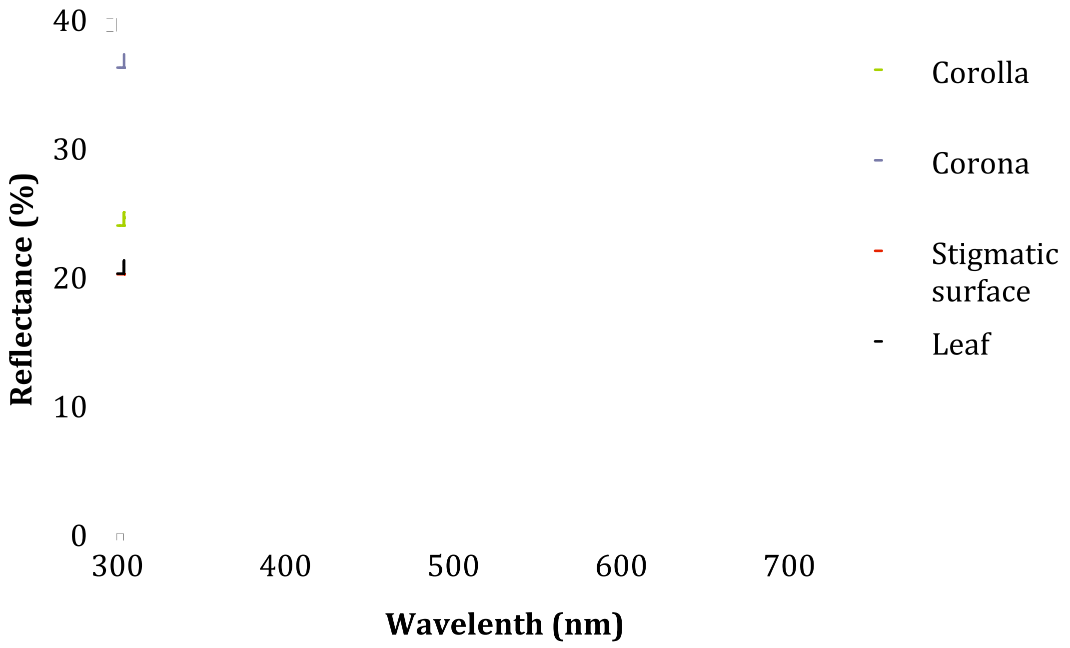
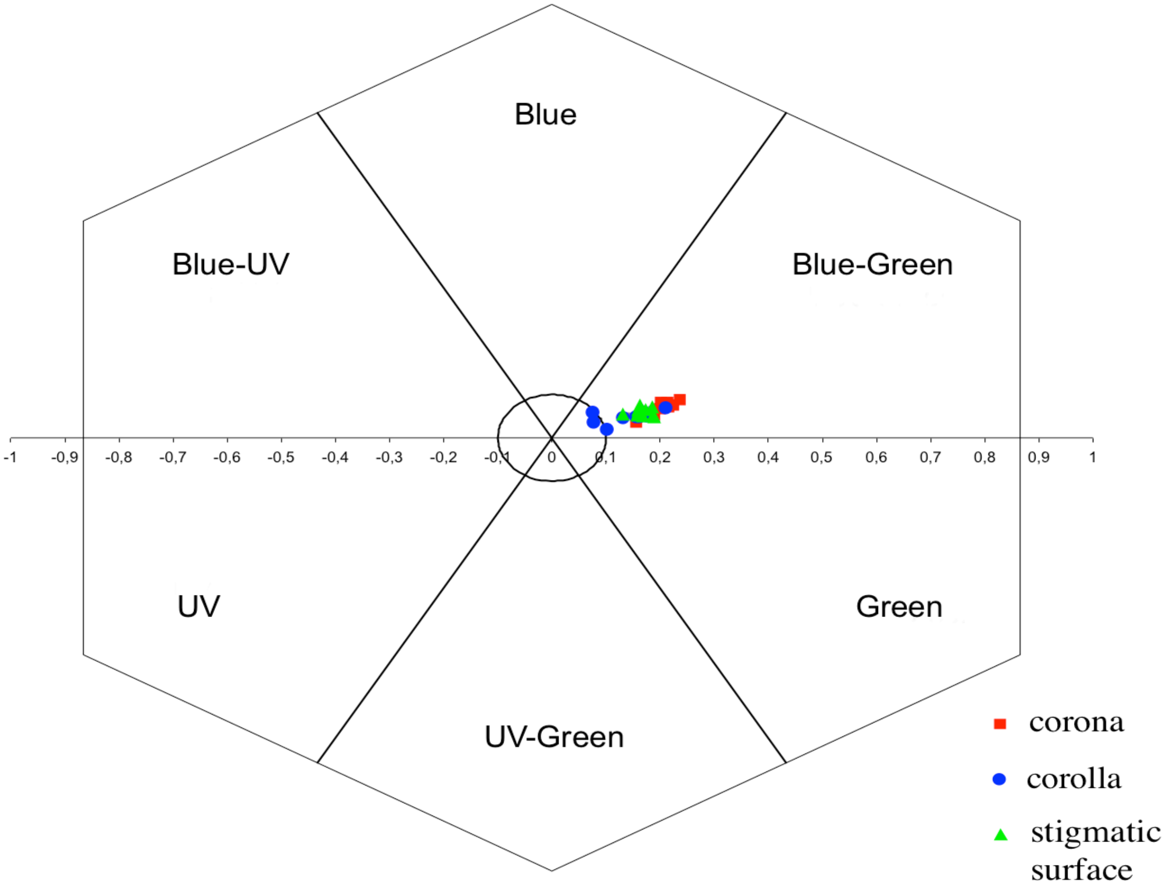


Figure 3. Reflectance spectra for a) *G. cancellatus* flowers and b) *G. filiformis* flowers separated into their floral components. (*n* = 10 for both species).

a) *Gomphocarpus cancellatus*



b) *Gomphocarpus filiformis*

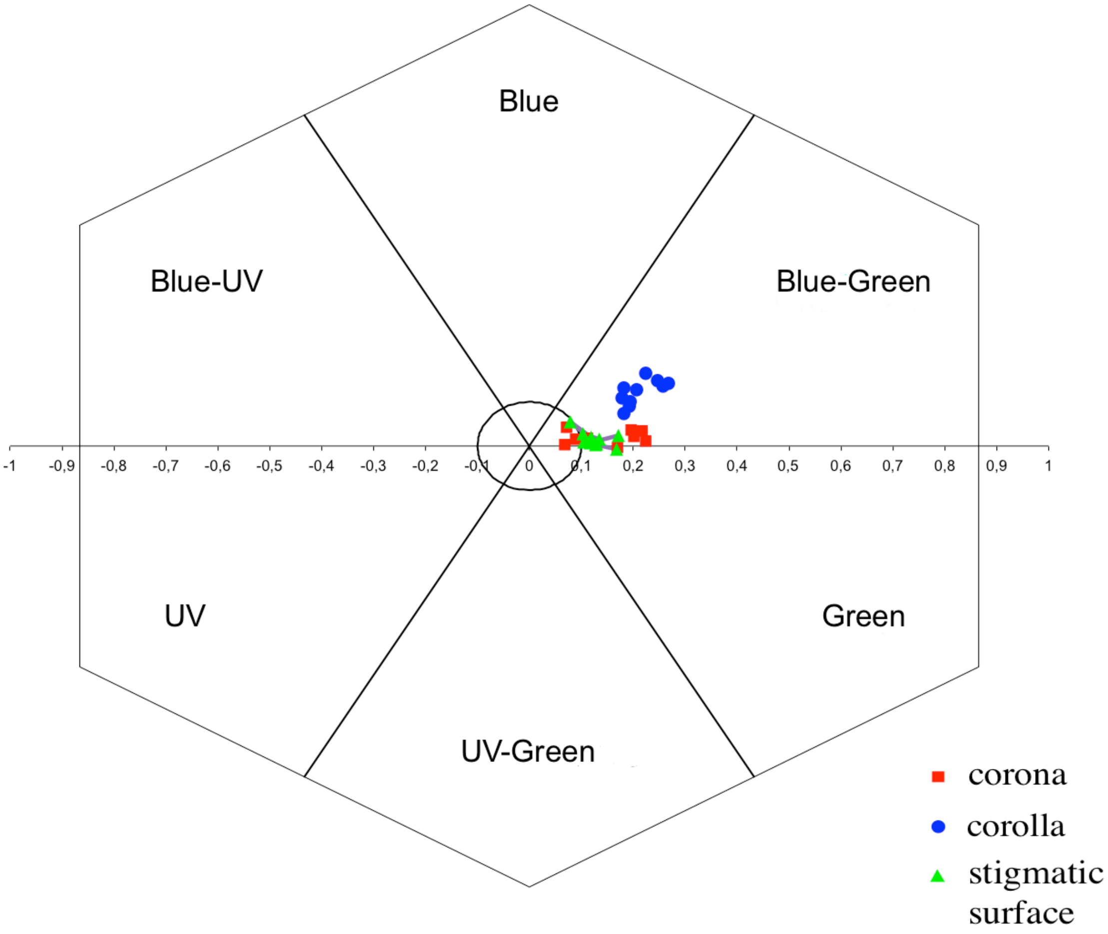


Figure 4. Colour hexagons showing a visual representation of the different parts of the floral display of *G. cancellatus* (a) and *G. filiformis* (b) as visual cues according to a honeybee's perception.

Scent

(Table 4 : Appendix 1)

Both species produced a faint sweet smell with *Gomphocarpus cancellatus* producing an average of 15.3 different scent compounds, compared to the slightly lower average of 13.2 in *G. filiformis* (Table 4). The two species show clear differences with 4-oxoisophorone dominating *G. cancellatus* scent (84.7%), while being completely absent in *G. filiformis*. The floral scent of *G. filiformis* is largely made up of benzyl acetate (33.3%) and benzyl alcohol (21.5%), which are both absent in *G. cancellatus*.

Benzaldehyde, also a prominent compound in *G. filiformis* (27%), is present in trace amounts in *G. cancellatus* (0.05%). *Gomphocarpus filiformis* therefore emitted large amounts of a variety of benzenoid compounds while *G. cancellatus* emitted a wider range of aliphatic compounds and monoterpenes, though in much smaller proportions. *Gomphocarpus cancellatus* plants from Rawsonville appear to be missing some of the compounds present in the Worcester population and only contain an average of 8.5 compounds. Additionally the flowers of these plants emitted a marked proportion of limonene (31.6%), which is produced in the other *Gomphocarpus* population as well as in *G. filiformis* but in very small percentages (1.1% and 0.2% respectively). *Gomphocarpus cancellatus* had a higher emission rate (747ng/min/flower) compared to *G. filiformis* (588ng/min/flower) but both were fairly high. Overall, their scent profiles showed marked differences with an average dissimilarity of 94%.

The NMDS shows that *G. cancellatus* and *G. filiformis* have distinct scents that are different from each other as well as from the summer rainfall species *G. fruticosus* and *G. physocarpus*. The summer rainfall species; *G. fruticosus* and *G. physocarpus* are stacked on top of each other and show much greater similarity. These results are statistically significant.

Reproduction

All *G. cancellatus* flowers excluded from pollinators did not produce fruit (two plants were diseased) while 27% (n=15) of the controls did (five control inflorescences were diseased). Surrounding plants with a single tagged inflorescence to determine the general reproductive success of the population displayed a higher fruit set with 53% (n=17) producing an average of two fruits each (three plants were diseased). Generally most plants produced fruit including some that were diseased. Single inflorescences were diseased but this did not impact the entire plant and other inflorescences produced fruit. The plants had several inflorescences, most with well over 50 flowers but produced very few fruits. Therefore relative to the number of flowers fruit set was very low.

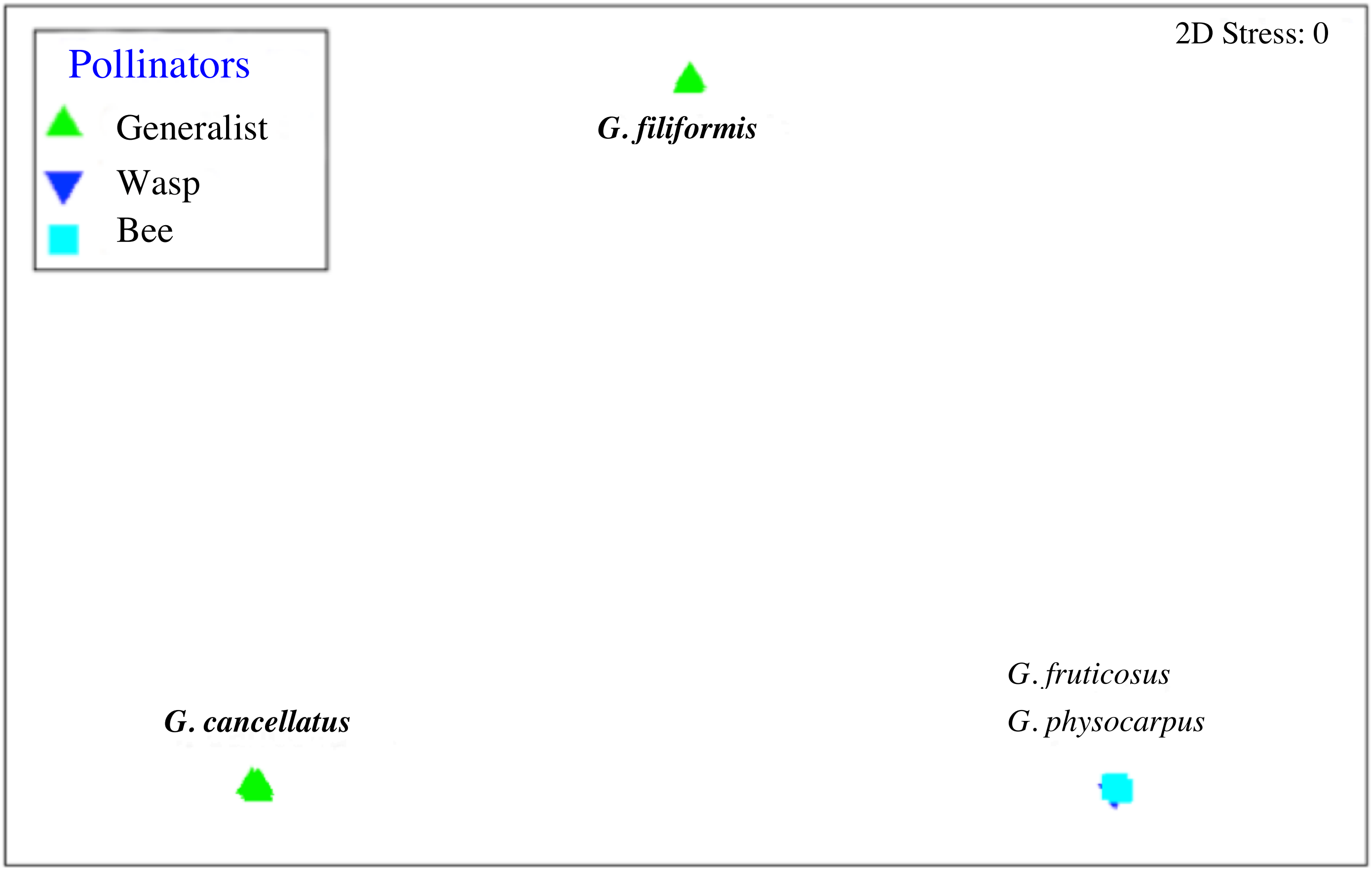


Figure 5. A visual representation of the floral fragrances based on non-metric multidimensional scaling using Bray-Curtis similarities of relative amounts of scent compounds found in the flower samples of *Gomphocarpus physocarpus* and *G. fruticosus* from the summer rainfall region (with data from Burger et al., in press) and *G. filiformis* and *G. cancellatus* from the winter rainfall region. Global R: 0.529, $p < 0.01$.

2.5 Discussion

The wide range of visitors in both *Gomphocarpus* species highlights that unlike the summer rainfall congeners, the winter rainfall species exhibit a more generalist pollination system (Table 2). While both species were similarly visited by sunbirds, based on the larger number and variety of insect visitors, *G. cancellatus* appears to be more generalized than *G. filiformis*. This was expected as *G. cancellatus*' floral display forms a starker contrast to its background (the large white umbels against the dark green leaves) (Figure 1a, b). Although the various components of the flower itself, i.e. the corolla, corona and stigmatic surface are very similar in terms of their colour and visibility (Figure 3a, 4a), the overall floral presentation seems to be effective in making the flowers more accessible to a large variety of pollinator types. However this may just be the case because more time was spent observing *G. cancellatus* compared to *G. filiformis* due to logistical reasons. Therefore a more unbiased comparison would be one comparing the number of different successful pollinators based on the presence of corpusculae. In this case *G. cancellatus* has five different pollinating species falling into two functional types; fly and bee pollinators, while *G. filiformis* has three different species falling into two groups, ants and bee pollinators. Evidently *G. cancellatus* still appears to be more generalist. Its ability to attract a wide range of pollinators may explain how it has successfully established itself in South Australia and Victoria where it is invasive (Forster et al. 1996).

Despite the cryptic appearance of *G. filiformis* flowers, colour analysis shows that there is a larger contrast between the different floral components (Figure 3b, 4b), which may explain why it too attracted a wide range of visitors. Several other factors could be contributing to this. For example, both plants produced large amounts of nectar therefore it is not surprising that most of their visitors were nectar-seekers. The fact that so many insects, and even birds were feeding on the nectar suggests that the nectar is palatable to a variety of groups (Table 3). This is unlike *G. physocarpus* which has bitter nectar that is only enticing to wasps but similar to *G. fruticosus* which attracts a wider range of pollinators with its nectar (Burger et al., in press).

The plants had some interesting visitors including the lesser double-collared sunbird which has also been observed visiting *Microloma sagittatum*, now known as a specialized bird pollinated species (Pauw 1998, Ollerton 1998). The birds in this case would be supplementary pollinators. Another fascinating observation was the overwhelming presence of Balbyter ants (*Camponotus fulvopilosus)* on *G. filiformis* (at least 30 on each plant). The presence of corpusculae and concatenations (a chain of corpusculae), which are indications of repeated successful pollinaria deposition (Coccuci et al. 2014, Peter & Shuttleworth 2014) suggests that they are in fact pollinators. This is highly unusual as ants are known nectar robbers (Wyatt 1980, Frits & Morse 1981) and in some cases they even repel pollinators (Willmer & Stone 1997, Tsuji et al. 2004, Ness 2006). However the ants observed on *G. filiformis* were successfully moving pollinaria around suggesting that this might not be the case. The dry environment with sparse vegetation as well as fewer potential pollinators due to the climatic region could have resulted in the plant adapting to a wide range of pollinators including ants. This means they are most likely passing around pollen from the same plant, thus potentially negatively impacting the plant if it is self- incompatible, which is common in most milkweeds (P. Bruyns pers. comm). The ants could be clogging the guide rails with pollen from the same plant therefore reducing the impact that other pollinators may have when they bring pollinaria from a different plant. *Gomphocarpus cancellatus* had a fair amount of sugar ants visiting as well as a few smaller ants which were feeding on nectar but not nearly as abundant as the Balbyter ants on *G. filiformis*. Furthermore, only one sugar ant had picked up pollinaria suggesting this may be a rare occurrence. Like the ants in *G. filiformis* the ants moved between several flowers of the same plant.

Ant pollination is controversial as there are many arguments against it. It tends to be rare and this has been attributed to the ants' aggressive and destructive nature, their inability to travel far enough between plants to cause cross pollination and their tendency to feed on floral organs like ovaries (Beattie 2006). Most notably it has been shown that ant metapleural antibiotic secretions interfere with pollen viability, and this may be a major contributor to the paucity of ant-pollination systems (Beattie et al. 1984, 1986, Hull & Beattie 1988). The few ants that have been observed pollinating plants produce very small amounts of this secretion or none at all such as the genus *Camponotus* (Hull & Beattie 1988, Gomez et al.1996) that are abundant on *G. filiformis* flowers. Additionally, the fact that milkweed pollinaria are attached via corpusculae and the pollen itself is not exposed to harmful secretions may have allowed these flowers to start utilizing ants. Some cases of ant pollination have not been attributed to evolutionary relationships but simply as a result of the ants being more abundant than flying insects in particular regions (Gomez & Zamora 1992). This may certainly be the case for *G. filiformis* as the ants greatly outnumbered all other visitors.

Ants have been observed on other asclepiads in the summer rainfall region on several occasions however they did not have any pollinaria (A. Shuttleworth pers. comm.). Invasive *G. physocarpus* in Australia are also visited by ants, they extracted pollinaria but did not manage to insert any and also remained on the same plant (Forster 1994). Ants have been reported on other asclepiads including *Asclepias curassavica, A. exaltata, A. syriaca, Ditassa banksii, D. rothundifolia, Leptadenia pyrotechnica, L. reticulate* and *Gomphocarpus physocarpus* within its natural range in South Africa, but they were not observed inserting pollinaria (Kephart 1979, Pant et al. 1982, Chaturvedi & Pant 1986, Ali 1994, Betz et al. 1994, Coombs et al. 2009, A. Domingos-Melo, unpublished). The only reported occurrence of an ant-asclepiad association where pollinia deposition and subsequent fertilization occurred is a very recent study of *Ditassa capillaris* and *D. hastata* in Brazil (Domingos-Melo et al. 2017). This interaction also occurred in a semi-arid environment. The plants had exposed nectar and attracted other Hymenopteran pollinators, however, much like in *G. filiformis*, the ants were the most abundant and frequent pollinator. Therefore if *G. filiformis* is self-compatible and the ant activity does in fact lead to successful fertilization then this study further supports that ants may be effective milkweed pollinators in some cases and may have been wrongfully dismissed as nectar robbers in this family.

The *Gomphocarpus* scent profiles also explain their ability to lure a large assortment of visitors, with common floral compounds like limonene, benzyl acetate, benzyl alcohol and benzaldehyde making up some of the dominant scents (Table 4) (Knudsen et al. 2006). *Gomphocarpus cancellatus* had the more unique 4-oxoisophorone as its most dominant compound. This is also found in Apiaceae, Asteraceae, Dipsacaceae, Iridaceae, Orchidaceae, Polemoniaceae, Scrophulariaceae and Theophrastaceae and is associated with Lepidopteran pollinators (Andersson et al. 2002, Andersson 2003, Andersson & Dobson 2003, Knudsen et al. 2006). Incidentally, oxoisophorone and its derivatives are also butterfly pheromones (Schlutz et al. 1988). Its overwhelming proportion in *G. cancellatus* would suggest a strong butterfly association. Butterflies were observed visiting the plants, however these were very few in number and the assessed butterflies did not carry any pollinaria (Table 2). The large proportion of limonene in the

Rawsonville *G. cancellatus* population which is not present in the larger *G. cancellatus* population from Worcester is also interesting because it suggests that the populations may be utilizing different scents to attract pollinators. *Gomphocarpus filiformis* and *G. cancellatus* exhibited a large difference in their scents. Octanal, limonene, and methyl salicylate were responsible for most of this difference supporting my prediction that there will be disparities between the two species based on the observation that *G. filiformis* appeared to be investing less in visual cues but was still managing to attract pollinators.

Based on the cryptic nature of *G. filiformis* flowers I suggested that the plant might invest in more non-visual attractants to lure pollinators unlike the more elaborate and densely packed *G. cancellatus* inflorescences. I hypothesized that this further investment may be an olfactory cue, resulting in *G. filiformis* having a more complex or a much stronger scent. However this was not particularly the case. Both species emitted fairly large amounts of scent, 747ng/min/flower in *G. cancellatus* and 588ng/min/flower in *G. filiformis*. The species show moderate differences in the overall scent compounds that they produce; *G. filiformis* has 13-14 compounds making up its scent profile while *G. cancellatus* has 8-17 with an average of 14. This is not a large difference and in terms of floral scent complexity both species tended to have fairly common floral compounds (Knudsen et al. 2006), with the exception of 4-oxoisophorone. The two Cape species showed more differences between each other compared to the two specialists (Figure 5). This is surprising, as I would have expected the two specialists to show a larger difference both from each other as well as the generalists. The study did highlight that nectar palatability played a major role in repelling certain insects therefore the scents produced may not have to be distinctly different if the nectar is sufficiently filtering pollinators (Burger et al., in press).

In the winter rainfall species, flowering time may play a key role in the plant's ability to attract more visitors. In the summer rainfall region the *Gomphocarpus* species flower from November to April, while *Gomphocarpus cancellatus* flowers between March and April and *G. filiformis* flowers at various times throughout the year (Manning & Goldblatt 2012, P. Bruyns personal communication). *Gomphocarpus cancellatus* flowers when the cold wet winter is starting and not much is flowering. The flowers also exhibited asynchronous blooming. Staggering the blooming process may increase the chances of successful pollination occurring in unpredictable conditions. For *G. cancellatus* this is most likely to make up for the days that are too cold for any pollinators to visit. By spacing out the blooming it ensures that fresh flowers will always be available under ideal pollinator conditions. Therefore harsh weather conditions and the consequential reduction in pollinators on particularly cold, rainy or windy days could have shaped this system.

Unlike *G. cancellatus*, *G. filiformis* flowers throughout the year. The study population mostly blooms in the summer, characterised by hot dry conditions. *Gomphocarpus filiformis* is especially adapted to surviving in harsh dry conditions, evidenced by its reduced filamentous leaves to reduce water loss and its photosynthetic stems (Goyder & Nicholas 2001). The location of *G. filiformis* was dry farmland with very few plants surrounding it. Therefore the presence of large numbers of pollinators and such a diverse range was unexpected. Despite the large variety and numbers of visitors *G. filiformis* displayed a very low pollen transfer efficiency (9%) compared to the higher 21.3% of *G. cancellatus*. This is unprecedented as both plant species occurred in fairly large populations. The assumption is that a large plant population will result in pollinators spending more time foraging, thus increasing the transfer of pollinaria (Coombs et al. 2009). This was not the case however, as the two species exhibited somewhat low PTE similar to *G. fruticosus* (Harder & Johnson 2008: 15.2%), *Asclepias verticillata* (Bertin & Willson 1980: 20%), *A. tuberosa* (Wyatt 1976: 22%), *A. curassavica* (Wyatt 1980: 29%) and *Cynanchum viminale* (Liede & Whitehead 1991: <20%). This may be due to both *Gomphocarpus* species attracting a wide variety of pollinator groups, some of which are more effective than others. Discrepancies in pollinator quality have been highlighted in other generalists like *Asclepias syriaca* and *A. exultata* (Broyles & Wyatt 1991, Morse & Fritz 1983, Morse & Jennerstern 1991). In more specialized systems a much higher PTE would be expected due to high pollinator fidelity i.e. the ideal pollinators are the ones consistently visiting the plant as exhibited in Coombs et al.'s study of *G. physocarpus* (2009).

The moderate to low PTE translated to low reproductive success in *G. cancellatus*, which displayed very low fruit set relative to the number of flowers. Asclepiads are generally characterised by low fruit set due to very few compatible pollinia reaching the stigmatic chambers and limited resources leading some asclepiads to abort fertilized fruits (Queller 1983, 1985, Wyatt & Broyles 1994, Willson & Price 1977, Wilson & Rathcke 1973). Like *G. physocarpus* (Coombs 2009), pollinator exclusion of *G. cancellatus* flowers showed that facilitated pollination is necessary for reproductive success. Further studies with hand pollinations are needed to confirm if this species is self-compatible. Attempts to self-pollinate flowers in this study were unsuccessful due to the flowers falling off within days of being handled and hand-pollinated. Self-incompatibility has been established in various South African *Pachycarpus* and *Xysmalobium* species (Shuttleworth & Johnson 2006, 2008, 2009d). Other self-incompatible asclepiads include the *Asclepias* genus and *Gonolobus suberosus* (Wyatt & Broyles 1994, Lipow & Wyatt 1998). Generally, in-depth studies of asclepiad breeding systems are scarce due to the extreme difficulty in hand pollination caused by their small pollinaria. The few studies that have been successful in this regard have shown that self-compatibility varies for asclepiads, with some variation even found within the same populations (Ivey et al. 1999, Lipow et al. 1999, Lipow & Wyatt 2000).

This study has offered a basis of comparison for the reproductive biology of a Fynbos and desert *Gomphocarpus* species with the grassland species. An additional study investigating the impact that adaptations to different environments have on the plant-pollinator relationship could include *G. rivularis* Schltr. which is a widespread rheophyte occurring along riverbanks and seasonal flood zones. This would provide an interesting contrast to the xerophytic *G. filiformis* and offer valuable insights into the different ways a single genus adapts and attracts pollinators in a wide range of climatic environments across the country.

2.6 Conclusion

Gomphocarpus cancellatus displayed a generalized pollination system. While *G. filiformis,* though visited by a wide range of visitors, appears to have a less generalized pollination system based on the limited visitors that were actually pollinators. Therefore *G. cancellatus* differs from its summer rainfall congeners but *G. filiformi*s exhibited a pollination system similar to *G. fruticosus*, where it is visited by various pollinator types but actually pollinated by a select few (Burger et al., in press). The most noteworthy trait in *G. cancellatus* seems to be its abundant and sugar rich nectar unlike in *G. filiformis* where nectar is not as concentrated. In terms of pollinator attractants, scent appears to be important in both species. Though they both attract a wide range of pollinators their scent profiles as well as their colour signals are distinct from each other as well as from their summer rainfall counterparts.

Both species appear to be generalized to different extents. Location as well as flowering time has likely played a role in shaping this kind of generalized pollination system. The lower number of pollinators for *G. filiformis* in the hot dry Laingsburg region may have contributed in creating a novel pollination system in the group predominantly involving *Camponotus* ants in addition to other pollinators. It is yet to be determined if lesser double-collared sunbirds, which frequent both species, are playing a role in pollination. A study where the birds are captured and their tongue and beaks assessed for pollinaria or pollinia clips would verify this. Overall these two species have offered great insights into the reproductive biology of *Gomphocarpus* in the Cape region and highlighted the potential for some very exciting future research.

3. Minute pollinator for a minute asclepiad: pollination of *Eustegia minuta* R. Br. (Apocynaceae -Asclepiadoideae) by bibionid flies

3.1 Abstract

An investigation was carried out on the monotypic *Eustegia minuta* (Apocynaceae, Asclepiadoideae), which exhibited a specialized pollination system involving microdipterans in the family Bibionidae. Such pollination systems are uncommon therefore a floral trait analysis was carried out to determine which floral cues could be shaping this system. This was conducted through analysis of the floral spectral properties as well as scent analysis using gas chromatography mass spectrometry (GC-MS) and pollinator observations. The flowers produced a strong pungent odour dominated by an unknown compound. The plant species is characterized by unique features such as a ring of corona lobes that limit access to its deep-set nectaries and its tiny size and cryptic appearance, are all thought to contribute in filtering plant visitors. Floral scent is thought to play the most important role in attracting bibionid flies. *Eustegia* also exhibited several features associated with bibionid-pollinated species. This is the first pollination study for the tribe Eustegieae and the first to describe a specialized bibionid-asclepiad pollination system.

3.2 Introduction

Pollinator-mediated speciation and extensive ecological niche partitioning have been credited as major contributors to plant diversification (Grant 1994, Waterman et al. 2011, Johnson 2010, Schiestl & Schlüter 2009, Chesson 2000, van der Niet & Johnson 2012, Sargent 2004). This is apparent in biodiversity hotspots like the Cape Floristic Region of South Africa with its disproportionately high plant diversity (Goldblatt & Manning 2002). Several other factors such as the presence of climatic and geological gradients have likely played a larger role in its origin (Cowling & Lombard 2002). Competition for pollinators can lead to diversification and specialization of plant-pollinator relations (Rathcke 2012). All of these combined factors have created the unique conditions that have led to high levels of endemism with a large number of notable specialised pollination systems in the region (Rebelo 1987, Johnson 1994, Manning and Goldblatt 1996, 1997, Pauw 1998, 2006). Studying such systems has greatly enhanced global understanding of evolutionary diversification and plant-pollinator relationships, particularly those involving plants with complex floral structures. This includes the study of specialized pollination relationships in highly derived plants and microdipterans (Coleman 1934, Meeuse 1978, Thien 1980).

One such example is the rare pollination system involving the family Bibionidae. This dipteran family is made up of small to medium-sized flies (2.5-15mm) known to be nectar and pollen feeders (Kevan & Baker 1983, D'Arcy-Burt & Blackshaw 1991, Skartveit 1996). Bibionid-pollination systems, while not common, have been observed in various parts of the world. Bibionid flies have been reported visiting the flowers of Mexican coffee (*Coffea Arabica* L.) (Vergara & Badano 2009), *Cyathodes parvifolia* R. Br. in Australia (Higham & McQuillan 2000), flowers in the Argentinian Andes (Medan et al. 2002), as well as *Earina mucronata,* an epiphytic orchid in New Zealand (Lehnebach & Robertson 2004). In Africa they are thought to be the exclusive pollinator of the Cape orchid *Disa obtusa* Lindl. and have been observed visiting *Disa obtusa hottentotica* (Johnson & Steiner 1994). They are also the only known pollinator of *Melasphaerula ramosa,* a Cape endemic Iridaceae (Goldblatt et al. 2005). But generally, very little is known about their pollinating activity.

Preliminary observations of visitors to *Eustegia minuta* R. Br. (Apocynaceae), an asclepiad endemic to the Cape suggested that bibionid flies might be the principal pollinators of this species. *Eustegia* is an unusual monotypic genus, which has recently been placed in the tribe Eustegieae (Endress et al. 2014) solely consisting of the genera *Eustegia* and *Emicocarpus* K.Schum. & Schltr., both of which exhibit unique features amongst the Asclepiadeae. *Eustegia* flowers have bright green corollas with relatively hairless leaves and stems, a clear sap instead of the characteristic asclepiad milky sap and the entire plant is covered in a thin wax (Bruyns 1999). However the most distinguishing feature of this genus is the unusual gynostegium. This consists of a large protruding style-head surrounded by three series of corona lobes instead of the usual one or two as in other asclepiads. *Eustegia*'s series of corona lobes consists of 25 lobules, the outer 20 are white while the five inner lobules tend to be green (Bruyns 1999). These unique features set *Eustegia* apart from the rest of the southern African asclepiads, with the exception of *Emicocarpus*, a closely related Mozambican endemic (Bruyns 1999). *Eustegia* corona lobes occur in white or pink morphs, previously thought to be separate species or subspecies, but the genus has been reduced to just one species, *Eustegia minuta* (L.f.) Schult (Bruyns 1999). The unusual floral morphology of this species could not immediately be related to known pollination systems within South African asclepiads (see Ollerton & Liede 1997; Shuttleworth & Johnson 2012 for reviews of these), and the observation of visitation by bibionid flies suggested the possibility of a novel pollination system for South African asclepiads. I hypothesized that *Eustegia minuta* was specialized for pollination by bibionid flies and that the unusual floral morphology of this species represented an adaptation to these flies.

The broad aims of this study were thus to describe the pollination system of *E. minuta* and explore levels of floral specialization and dependence on pollinators for reproduction. The specific aims were to determine the pollinators of *Eustegia minuta*, characterize the floral features used by *E. minuta* to attract pollinators, and determine if the species can reproduce autogamously.

3.3 Methods and Materials

Study species

Eustegia minuta is a small (150 – 300 mm) erect plant with very thin slender green leaves (sometimes inconspicuous). The flowers occur as inflorescences made up of several umbels (Figure 1a). The corolla is light green and the corona lobes are usually white but can also occur in pink or a mixture of the two (Figure 1b-d). At the center of the flower is the stylar head (Figure 1d), which contains the pollinaria.

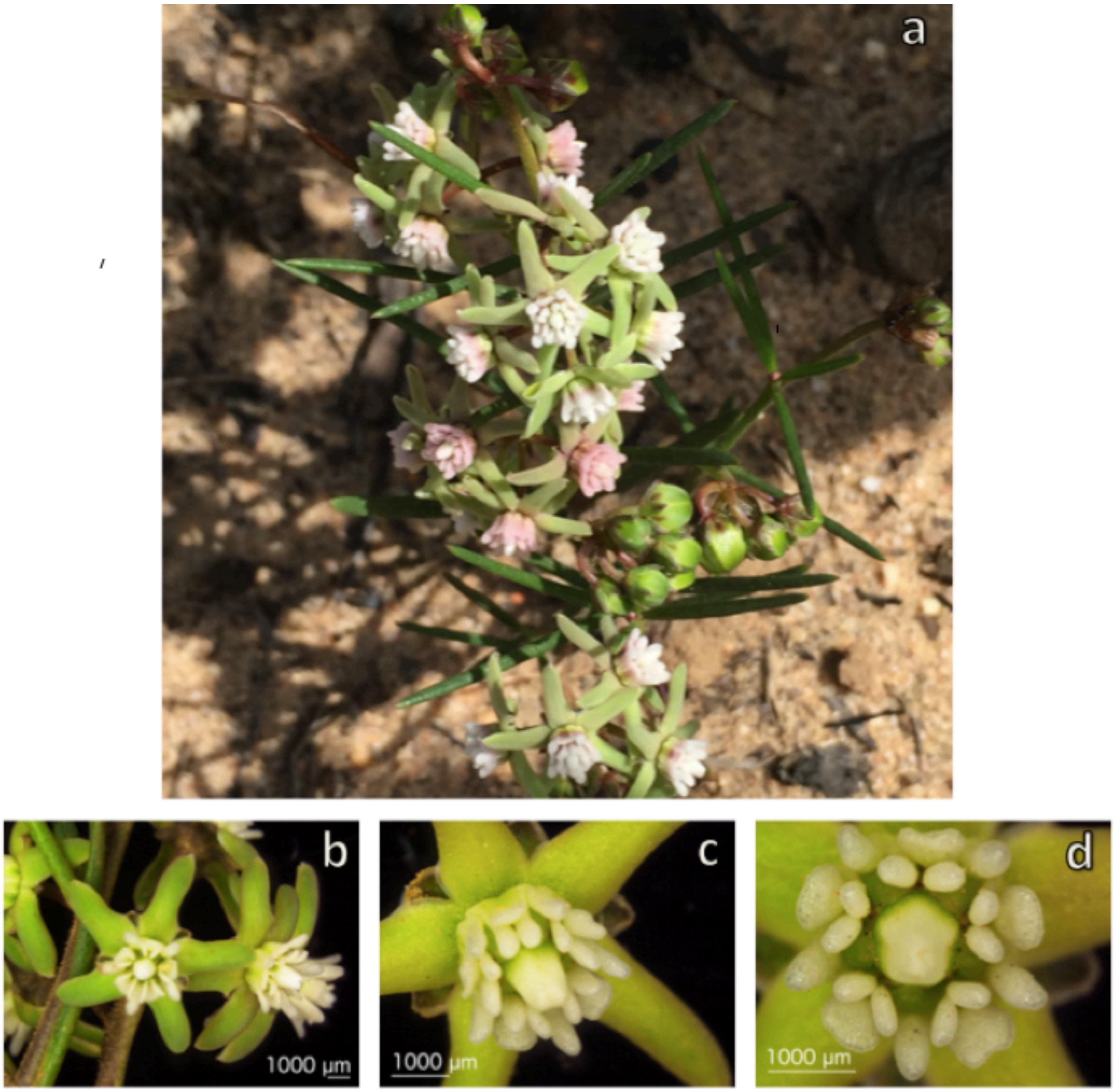


Figure 1. a) *Eustegia minuta* plant with a mixture of pink and white corona lobes and some buds. Gross morphology of the b) flowers, showing the characteristic series of corona lobes surrounding the central stigma (c and d).

Eustegia occurs exclusively in the Western Cape with a distribution from the Cape Town region to Namaqualand as well as isolated locations in the eastern parts of the Western Cape (Bruyns 1999). It is extinct in many historically recorded locations, particularly in the southwestern Cape due to agricultural expansion and urbanization. However flourishing populations exist in more isolated areas (P. Bruyns, personal communication, categorized as of “Least Concern”: Foden & Potter, 2005). It is restricted to the winter rainfall regions of the Cape with the peak flowering season occurring in September. The plants are found in Fynbos, particularly on sandstone soils and in Renosterveld on shale and granite soils (Bruyns 1999). They also occur in disturbed areas and seem to flourish in post-fire environments.

Study site

Plants from a large population of ca. 75 plants occurring in a valley in Jonkershoek Nature Reserve, Stellenbosch, Western Cape (GPS: -33.990662,18.967079) were used in this investigation. The population occurred along the main tourist road on a sandy bank with another population of ca. 30 plants occurring on the opposite side of the road. The area had experienced a fire 6 months prior to sampling in September of 2015. I revisited the site in September and November 2016.

Floral visitors

Field observations of floral visitors were carried out over five days from 10am until 3pm on September 16th, 25th, October 1st, 4th and 16th. Night observations were carried out on September 25th and October 1st from 6pm to 8pm. Generally, days were sunny, warm and windy with two calm days. The night observations were carried out in cold dry conditions. A total of 29 hours were spent observing and recording plant visitors. Insect visitation and general activity in the area was very low so insects in the vicinity of the flowers were also collected for inspection. Insects were identified to at least family level and inspected on site and microscopically for the presence and location of attachment of pollinaria and corpusculae (the clips that attach the pollinaria to pollinators).

Pollen analysis

A total of ten inflorescences (each from a separate plant) were collected and assessed under a dissecting microscope for pollinia insertions and removals. The number of flowers on each plant varied from three to nine. Pollination success was determined by calculating the pollen transfer efficiency, which is the proportion of removed pollinia that are subsequently inserted. It has been utilized in many milkweed and orchid studies to measure pollination success (Ivey et al., 2003, Shuttleworth and Johnson 2006, Coombs et al., 2009, Peter & Johnson 2008a, Johnson et al., 2009; see review by Harder & Johnson 2008).

Colour

The colour of the flowers and leaves was determined using the same protocols as Chapter 2. A total of ten different plants were used. A graph of the reflectance spectra produced by each floral component was constructed.

Scent collection and analysis

Floral scent was collected from six plants using the same protocols as Chapter 2. All scent sampling was carried out in the field from 2-4pm on the 19th of September and 2nd of October 2015.

Nectar

To determine rewards for pollinators, nectar was sampled from 11 plants. The volume produced per flower was low therefore an accumulated volume from 10 flowers was sampled from each plant. Fine mesh bags were placed on the plants to exclude all floral visitors and nectar was collected in the morning the day after bagging. The volume of nectar from each flower was determined by extracting it using 1μL capillary tubes, and the concentration in sucrose equivalents determined using a Bellingham and Stanley (0-50% Brix) handheld sugar refractometer.

Autogamy

To determine if *Eustegia minuta* needs pollen vectors for pollination to take place, pollinator exclusion experiments were carried out, and seed set determined. Fine mesh bags were placed over multiple fresh buds of ten plants and left unmanipulated until fruit set. Buds from a further ten plants were tagged as controls. All experimental buds were counted at the start of the experiment to determine fruit set (proportion of flowers that set fruit). The bags were removed when fruit (pods) were mature and before seeds were released 2 months later. The number of bagged and unbagged plants with and without fruit was noted. The fruit was collected and the number of seeds per pod recorded.

3.4 Results

Floral visitors

A few *Camponotus vestitus* ants from a colony located next to the plant population were seen on some of the flowers drinking nectar and several dead ants were collected on the flowers (Table 1). There were about two ants on each plant on ca. 15 plants closest to the ant colony most of which were dead. *Eustegia* was only directly visited by bibionid flies, which were observed visiting several flowers and moving between plants on two of the observation days. The flies appeared to be probing for nectar. They landed on the corona lobules and dipped their head in between the lobules for a few seconds and moved to another flower or plant. Attached pollinaria were visible on the mouthparts as the bibionids were flying from flower to flower. They were particularly difficult to catch and netting near the ground was not always successful. Therefore several observed to be carrying pollinaria escaped and a smaller total of 25 were caught and inspected. Of the 25 that were caught only three carried pollinaria, however five more were spotted flying with pollinaria attached to their mouthparts (Table 1). Both ants and bibionid flies had pollinaria attached to their mouthparts (Figure 2).

Table 1. Insects surveyed for pollinaria caught within the *Eustegia minuta* plant population. Species names in bold indicate insects collected on flowers. The rest were caught in the plants' general vicinity.

Insect	No. observed (No. collected)	No. carrying pollinaria (escaped)	Average pollinaria (±SE)	Average half pollinaria[1] (±SE)	Average Corpusculae[2] (±SE)	Pollinarium placement
Observed on *Eustegia* flower						
Diptera						
Bibionidae						
***Bibio* sp.**	ca.40 (25)	3[*](5)	2.7(1.7)	0	0	Mouthpart
Hymenoptera						
Formicidae						
Camponotus vestitus	17 (10)	3[*]	1	0	0	Mouthpart
Observed around *Eustegia* plants						
Diptera						
Bombyliidae						
Exoprosopa sp.	1 (1)	0				
Calliphoridae						
Calliphorid sp.	4 (4)	0				
Muscidae						
Muscid sp. 1	2 (2)	0				
Muscid sp. 2	1 (1)	0				
Sarcophagidae						
Sarcophagid sp.	1 (1)	0				
Tachinidae						
Tachinid sp. 1	2 (2)	0				
Tachinid sp. 2	2 (2)	0				
Hymenoptera						
Formicidae						
Camponotus sp. 2	20 (10)	0				
Mutillidae						
Mutillidae sp. 1	2 (2)	0				
Hymenopteran sp. 1	2	0				
Coleoptera						

Cocconellidae		
Henosepilachna sp.	1(1)	0
Elateridae		
Elaterid *sp*.	4(2)	0
Scarabaeidae		
Pachycnema marginella	4(4)	0
Meloidae	2(1)	0
Meloe sp.		
Synhoria sp.	1(1)	0
Lepidoptera		
Sphingidae		
Sphingid sp.[4]	2(2)	0
Lepidoptera sp. 1[5]	3	0

Notes:

[1] Half a pollinaria refers to a pollinaria where one pollinium has been deposited.

[2] Corpusculae refers to the remaining clip after both pollinia have been removed/deposited.

[3] Observed carrying pollinaria but not caught.

[4] Moths were seen flying in the plant vicinity but none observed landing on the plants during night observations.

[5] Lepidoptera sp.1 refers to a small white moth observed but not caught during night observations.

[*] Each *Bibio* fly was seen carrying 1-6 pollinaria while each ant was seen carrying a single pollinaria.

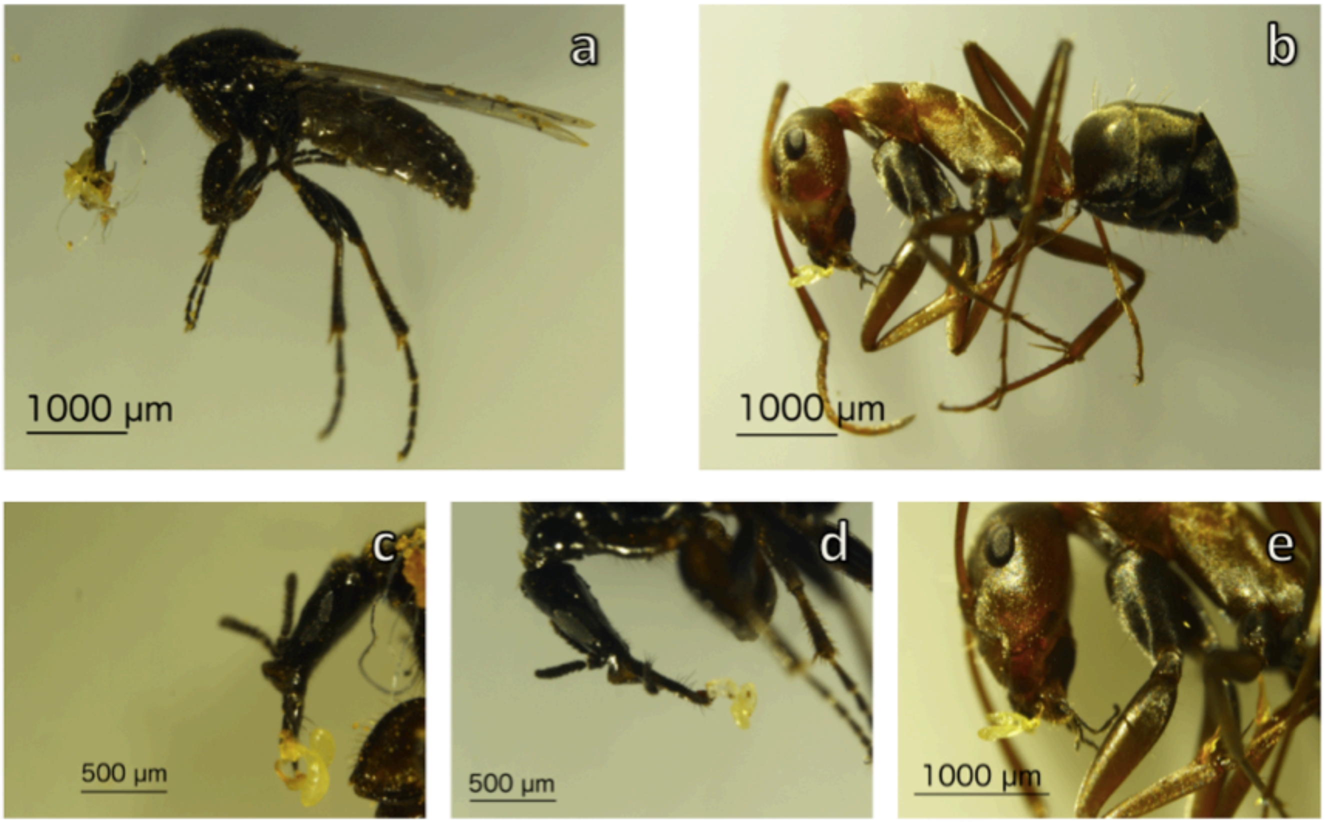


Figure 2. Insects with pollinaria from *Eustegia minuta*. (a) Bibionid midge with 6 pollinia pairs attached to its mouthparts. (b) *Camponotus vestitus* with a pollinia pair attached to its mouthparts with a close up of the same ant (e). (c and d) Close ups of two different Bibio nidae (*Bibio* sp.) midges, each with at least one pollinia pair attached to its mouthparts.

Pollination success

In terms of pollination success an average of 24.7% (±12.8) open-pollinated flowers (n= 24 flowers) from five different plants had received at least one pollinium (median of 16.7%). The pollen transfer efficiency was 6.4%.

Colour

The white corona lobes showed the highest albedo with up to 50% at a wavelength of 570nm (Figure 3). Generally the rest of the floral features displayed much lower reflectance with the corolla appearing to be very similar to the leaf, displaying their dull coloration. The stigmatic surface, which is also white, showed a marked difference to the corona lobes.

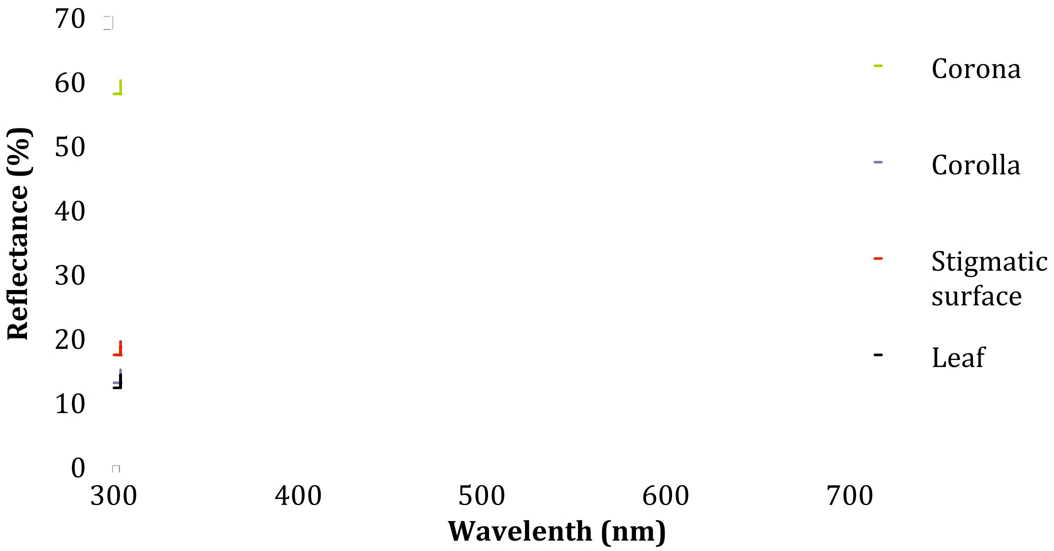


Figure 3. Mean spectral reflectance curves of *Eustegia minuta* floral components in the 300-700nm spectral range.

Scent

Eustegia flowers emitted a fairly strong unpleasant smell, which peaked in the middle of the day. The emission rate was 36ng/hr/flower (6 samples, 137 flowers). Scent analysis showed that this was produced by a wide range of scent compounds with an average of 24 different compounds dominated by aliphatics and benzenoids. The most abundant compounds were Unknown mz/ 57,85,45,41,43,74,86,87,69,39 (21.8%), 2.6-dimethyl-4-heptanol (21.2%), and to a lesser extent 2.3-heptanedione (15.8%)and benzaldehyde (12.6%). The scent profile was also characterised by low concentrations (<5%) of a diverse range of benzenoid compounds.

Table 2. Floral scent compound contributions (%) to the scent profiles of six *Eustegia minuta* plants. KRI = Kovats Retention Index. Contributions of more than 10% are in bold, trace amounts (tr) refers to compounds that make up less than 0.05% of the total sample.

Compounds	KRI	P1	P2	P3	P4	P5	P6
Aliphatics							
2,3-Heptanedione	1137	**28.3**	6.0	9.8	**12.2**	**22.6**	**16.1**
Nonanal	1346	0.5	1.6	1.7	0.3	**15.2**	**18.5**
2,6-Dimethyl-4-heptanol	1430	**22.3**	**14.2**	**16.5**	**22.3**	**14.2**	**16.5**
Decanal	1454	0.1	5.9	6.8	0.1	5.9	6.8
Hexanoic acid	1800	-	-	tr	tr	-	-
Irregular terpene							
6-Methyl-5-hepten-2-one	1300	0.1	0.1	0.2	0.1	tr	6.7
4-Oxoisophorone	1658	0.1	tr	0.2	tr	tr	0.3
Benzenoid & Phenyl Propanoids							
Benzyl isobutyl ketone	1141	0.0	0.0	0.0	0.0	**21.4**	**15.0**
Benzaldehyde	1482	1.2	**21.4**	**23.5**	**25.7**	1.7	2.3
Methylbenzoate	1577	0.1	3.0	4.0	4.4	1.1	1.3
Phenylacetaldehyde	1598	0.0	0.1	0.1	0.1	0.2	0.4
1,4-Dimethoxybenzene	1678	0.6	0.1	0.0	0.1	0.2	0.6
Methyl salicylate	1738	0.0	0.2	0.1	0.1	0.1	0.0
Acetylbenzoyl	1762	0.0	1.8	1.0	0.5	0.4	0.2
Benzyl alcohol	1830	0.0	0.2	0.2	0.1	0.2	0.9
Phenylethyl alcohol	1864	0.2	0.1	0.1	0.1	0.0	0.0
Benzoin	2025	0.0	0.3	0.2	0.2	0.0	0.0
Benzyl benzoate	2533	0.0	0.5	0.0	0.0	0.0	0.0
Miscellaneous cyclic compounds							
beta-Octalactone	1921	0.0	0.1	0.0	0.0	0.0	0.0
2,3-Dimethyl-cyclohexa-1,3-diene	2044	0.3	3.1	2.5	3.1	0.0	0.0
Cyclohexene, 4-ethyl-3-ethylidene	2054	0.0	0.7	0.7	0.8	0.0	0.0
δ-Decalactone	2136	0.3	5.3	2.8	2.8	0.0	0.0
Jasmine lactone	2194	0.1	0.9	0.3	0.2	0.0	0.0
Unknowns							
mz/ 69,79,41,81,67,107,82,135,77,53	1275	0.5	**15.1**	5.4	3.9	0.0	0.0
mz/ 57,85,45,41,43,74,86,87,69,39	1405	**38.1**	**16.7**	**22.8**	**22.6**	**16.5**	**14.3**
mz/: 41,43,56,57,67,68,77, 83,85,106	1495	0.3	0.1	0.0	0.1	0.4	0.2
mz/: 41,43,57,85,123	1528	0.1	0.2	0.2	0.2	0.0	0.0
mz/: 39,41,67,73,79,87,93,105,122,131,149	1891	0.0	0.1	0.1	0.0	0.0	0.0

Aliphatics	**51.2**	**27.7**	**34.9**	**34.9**	**57.9**	**57.9**
C5-Branched chain compound	**35.7**	**12.5**	**19.1**	**19.3**	**16.3**	**14.2**
Irregular terpene	0.2	0.1	0.4	0.1	0.0	7.0
Benzenoid & Phenyl Propanoids	2.1	**27.7**	**29.2**	**31.3**	**25.3**	**20.7**
Miscellaneous cyclic compounds	0.7	**10.1**	6.3	6.9	0.0	0.0
Unknowns	**39.0**	**32.2**	**28.5**	**26.8**	**16.9**	**14.5**

Nectar

Nectar production was very low with an average of 12.1µL (±1.4) accumulated from ten flowers, single flowers produced immeasurable nectar volumes. The concentration was also low with five accumulated volumes (each accumulated from ten flowers) not reflecting any sugar at all. The four samples that did contain sugar ranged from 26 – 42% with an average of 32.5% (±2.5) sucrose equivalents by weight.

Autogamy

None of the flowers excluded from floral visitors using mesh bags produced any fruit, and only three of the ten open pollinated plants produced fruit. Of the open pollinated plants that did produce fruit, one fruit was produced by plants that had 22 and 19 flowers each, while a plant with eight flowers produced two fruits. Of the 75 plants in the population a total of 15 fruits were produced on seven plants collectively therefore natural fruit set was a mere 9.3% in the population of ca. 75 plants. Most plants produced one fruit whilst four individuals produced two fruits each with an average of 12.5 (±1.5) seeds per fruit.

3.5 Discussion

Bibionid flies were the most prominent *Eustegia* visitors with about 40 observed flying directly on the flowers and moving pollinaria across plants (Table 1). Ants were the only other insects observed on the flowers and carried limited numbers of pollinaria. However the fact that an ant colony was located right next to the plants raises questions about their role as pollinators. They are clearly able to remove pollinaria (Figure 2), however it is yet to be determined if they are capable of depositing it, or if they move between plants to cause cross-pollination. Ants, particularly *Camponotus* ants are associated with milkweeds (Picker et al, 2004) however pollination by this vector is reported as rare (see Chapter 2, Beattie 2006, Domingos-Melo et al. 2017). Additionally the ants were only on the plants closest to the colony and there were considerably fewer ants on the plants compared to the surrounding area; most ant activity took place on the ground directly under the plants. The presence of dead ants on the flowers is puzzling. Perhaps it might be an indication that the nectar is toxic to the ants and possibly other insects, acting as a pollination filter. Further investigation would be needed to give support to this speculation. Bitter or toxic nectar is utilized as a filter in some plants (Adler 2000, Shuttleworth & Johnson 2009).

Based on *Eustegia's* floral structure, the bibionids appear to be a more suited potential pollinator than the ants. The pollinaria are deep seated in the lower sides of the stylar disc and access is somewhat obstructed by the rows of corona lobes. It was therefore predicted that a long tongued insect was the likely pollinator. Bibionid flies' morphological structure and foraging behaviour makes them ideal pollinators. Their tiny size as well as their elongated mouthparts are the right size and shape for pollinaria removal and deposition. This is the first record of bibionids pollinating an Apocynaceae species. The closest resemblance to such a pollination system within the Asclepiadoideae is the early record of "mosquitoe-like" flies visiting a *Gomphocarpus* species on Table Mountain (Elliot 1891). More recently, emphidid flies (which are morphologically similar to these bibionids, although with longer mouthparts) have been observed visiting asclepiads such as *Aspidonepsis reenensis* (subtribe Asclepiadinae) in the Drakensberg mountains (A Shuttleworth, pers. comm.) as well as *Orthosia ellemannii* (subtribe Orthosiinae) in the southern Ecuadorian mountains (Wolff et al. 2008). Emphidid flies are superficially similar in morphology to bibionids (Skartveit, unpublished), therefore the mechanism of the pollination process by bibionid midges could be similar.

Bibionids are known from the literature to pollinate orchids (Johnson & Steiner 1994, Lehnebach & Robertson 2004). This is significant because orchids share similarities with asclepiads not just in terms of their floral complexity but also because they share the distinct feature of producing aggregated pollen packaged in pollinia (Endress 2016). The orchids that are pollinated by bibionids also have small, pungent flowers much like *Eustegia minuta* (Lehnebach & Robertson 2004, Johnson & Steiner 1994). The Cape orchid *Disa obtusa*, which is pollinated by *Bibio turneri* is characterised as pungent smelling and produces no nectar (Johnson & Steiner 1994). Pollinaria placement on *Bibio turneri* differs from that on the *Bibio* species that pollinates *Eustegia* however. *Disa obtusa* pollinaria are attached to the swollen front legs of the females, and to the ventral surface of the male's thorax since males have slender forelegs. While in the bibionid flies that visited *Eustegia* the pollinaria were attached to the rostrum. Sexual dimorphism is pronounced in bibionids both behaviourally and morphologically however the mouthparts in *Bibio* species do not show any differences between the sexes (J Starkveit pers. comm.). Therefore if male and female nectar-seeking behaviour is the same in this particular species, the attachment zone should consistently be the mouthpart. Reported differences in behaviour have been found in Norwegian bibionids where females are the main floral visitors while males are rarely associated with flowers (J Starkveit pers. comm.). Therefore the sex ratio of *Eustegia*'s bibionid visitors may be expected to be skewed. What is evident in the comparison of *Disa obtusa* and *Eustegia* pollination is that attachment zones can differ within the same species and is additionally shaped by the floral structure which influences the behaviour of the nectar or pollen-seeking bibionid.

The influence of floral structure on pollinator behaviour may be prominent in *Eustegia* although the same cannot be said about its visual cues. The flowers do not display dull coloration like other Nematoceran-pollinated plants (Proctor & Yeo 1972, Kevan & Baker 1983, Johnson & Steiner 1994) as the erect white (and sometimes pink) corona lobes and stigmatic surface form some contrast to the light green corolla (Figure 1). However this is negated by the tiny size of the plants so the flowers do not stand out against the surrounding matrix. This is exacerbated by their proximity to the ground, which they tend to blend into quite considerably, making them almost completely camouflaged. Colour analysis showed that the different floral components display a lot of similarity to the green leaves (Figure 3) with the exception of the high reflectance shown by the corona lobes. The large difference shown in the reflectance of the stigmatic surface to the corona lobes is suspicious since the two are the same colour and should therefore be reflecting the same amount of light. This is most likely a result of the tiny flowers whose separate components are extremely difficult to work with therefore repetition is highly recommended. Despite this, it can still be asserted that the cryptic nature of the overall plant makes it highly unlikely that visual cues play a major role in attracting pollinators.

This tiny plant species makes up for what it lacks in visual splendor with its surprisingly strong smell. The unpleasant scent dominated by aliphatics and a diverse range of aromatic compounds (Table 2) is likely to have been unattractive or even repellent to other potential floral visitors. The unknown compound (*mz*/ 57,85,45,41,43,74,86,87,69,39) making up the largest proportion of the scent is most likely responsible for this. Bruyns (1999) stated that *Eustegia* flowers emit a strong sweet smell with some populations smelling like honey while others emitted a vanilla scent. This was not entirely the case for the Jonkershoek population, which mostly smelt unpleasant but also had a hint of vanilla undertones. Incidentally studies show that bibionids, along with other Nematocera are attracted to pungent smelling flowers (Kevan & Baker 1983, Johnson & Steiner 1994). Therefore the smell of *Eustegia minuta* is likely to play the most prominent role in attracting this particular pollinator while effectively excluding a vast majority of insects in the surrounding area.

Selective filtering of plant visitors is characteristic of specialized plant-pollinator systems. Many specialized asclepiads use filtering mechanisms to increase the chances of attracting their desired pollinator. *Pachycarpus grandiflorus* attracts *Hemipepsis* pompilid wasps with its distinct scent and filters out non-pollinating visitors with its nectar, which is exclusively palatable to the pollinating wasps (Shuttleworth and Johnson 2009c). The same strategy is utilized by *Xysmalobium orbiculare* to attract spider-hunting wasps, while deterring other potentially inefficient pollinators (Shuttleworth and Johnson 2009d). The *Pachycarpus grandiflorus* study suggested that the cryptic nature of its flowers also aids in this filtering process. The flowers blend in with their surroundings while emitting a distinct scent only attractive to the desired visitor. This may certainly be the case for *Eustegia*. Experiments on the bibionid flies to determine whether they respond to *Eustegia*'s visual cues would verify this.

Investigating the functional significance of floral traits such as colour and scent cues for pollinator attraction was planned for *Eustegia*'s second flowering season (September 2016), by performing choice tests using a Y-tube. Unfortunately this was not possible because only one plant with frail looking flowers was in bloom, making sampling impossible. There was also no visible midge activity in the vicinity. A follow up trip the next month to determine if flowers might have bloomed later than usual was made, but was also not fruitful. Bruyns (1999) postulates that after fires *Eustegia* plants flower in large numbers but their flowering becomes sporadic with time. The absence of a second flowering season certainly supports this. Therefore future studies to investigate pollinator responses to different floral traits should be planned in the first post-burn flowering season. Intriguingly, *Eustegia* also shares this trait with the midge-pollinated *Disa obtusa*, known to only flower during the first post-fire floral season (Johnson & Steiner 1994).

The final part of this investigation was to determine if *Eustegia* is autogamous. Pollinator exclusion experiments indicate that *Eustegia* appears to be incapable of autonomous selfing. The whole population had a very low fruit set (9%) despite its large size and mass flowering. This was expected based on the low pollinator activity and low PTE (6.4%). Additionally, Nematoceran flies are associated with pollination systems involving low fruit and seed set (Thien & Utech 1970, Bierzychudeck 1981). This is not always the case as studies have shown that high pollination success can be achieved when Nematoceran numbers are high or when they are pollinating a large plant population made up of mass flowering flowers to increase the chances of cross pollination (Mesler et al. 1980, Johnson & Steiner 1994). In the case of *Eustegia*, the latter was true but the bibionid numbers were low. We can therefore conclude that pollinators appear to play an important role in *Eustegia*'s propagation, as the plants are reliant on facilitated pollen transfer. Further studies with hand pollinations could be used to verify if this species is self-compatible.

3.6 Conclusion

This study suggests that *Eustegia minuta* has a specialised pollination system involving Bibionidae flies. The strong pungent floral scent most likely plays a significant role in the attraction of these flies as is common in other bibionid pollinated plants. Scent may also be utilized to repel other visitors. Similarly the overall floral features, particularly the minimal visual cues, low nectar production and even the plant's apparent low reproductive success are all characteristic of a plant that is specialised for bibionid pollination. This study represents the first investigation into the pollination system of this unique genus as well as the first for the early diverging tribe in the Asclepiadoideae subfamily. It is also the first record of a bibionid-asclepiad pollination system and therefore presents many opportunities for further research to better understand this novel interaction.

4. Floral scent chemistry of four Cape milkweeds (Apocynaceae: Asclepiadoideae and Secamonoideae)

4.1 Abstract

Floral scent plays an important role in plant-pollinator communication. The diverse and prolific Apocynaceae have been shown to utilize a wide range of chemical compounds to attract pollinators. Different plants within and across genera can use different combinations of volatiles to attract the same pollinator group or show distinctions to attract different pollinators while restricting others. Therefore further analyses, particularly comparative studies, are essential in enhancing our understanding of the intricate ways plants are utilizing scent components. This study aimed to supplement the available data on *Cynanchum*, *Fockea* and *Secamone* by analyzing the scent compounds of previously unstudied Cape species using dynamic headspace extraction and (GC-MS) gas chromatography-mass spectrometry and comparing it to existing data to investigate similarities and differences at both species and genus levels. *Fockea capensis* had a largest amount of monoterpenes, which made up 54.9% of its scent profile. *Secamone alpinii* also had large amounts of monoterpenes (41.6%) with a fair amount of aliphatics (26.7%) as well as unique compounds such as skatole. While benzaldehyde contributed a 11.9% to the scent of *C. obtusifolium* and 60% in *C. zeyheri*. All four species had distinct scents dominated by benzenoids and monoterpenes, and were distinct from each other as well as their congeners.

4.2 Introduction

Angiosperms employ many strategies to attract animal pollinators. This can be in the form of bright colouration, presenting rewards such as nectar or oils, or by emitting a diverse range of chemicals into the air to create distinct floral scents (Dobson et al. 1996, Galetto & Bernadello 2005, Peter & Johnson 2008b). Many studies have focused on the pivotal role floral scent plays in attracting pollinators to angiosperms (Raguso 2008, Jürgens 2009). These scents can vary from unique fragrances in closely related species, which attract different kinds of pollinators indicating divergent evolution (Shuttleworth & Johnson 2009e). Alternatively, similar fragrance patterns have been documented amongst distantly related species adapted to similar pollinators highlighting convergent evolution (Knudsen & Tollsten 1995, Jürgens et al. 2013). Thus floral odour chemistry is an important component of the floral phenotype and analyses of scent chemistry are as important as morphological studies in attempts to understand the evolution of diverse plant groups.

Members of the subfamily Asclepiadoideae (Apocynaceae *sensu* Endress et al. 2014) possess a complex floral morphology with equally distinct floral scents (Jürgens et al. 2010, Shuttleworth & Johnson 2009c, d, e). Although not as well represented in floral scent studies, the milkweed studies that have been conducted certainly highlight this. Within this subfamily, the stapeliads (tribe Ceropegia: subtribe Stapeliinnae) have been the focus of much of the floral scent and general plant volatile research in this group (Meve & Liede 1994, 2002). Their scent is characterized by oligosulphides, phenol and skatole (Jürgens et al. 2006, 2013).

Additionally, some interesting scent research has been carried out on the genus *Ceropegia* L., which have deceptive pitfall flowers mainly pollinated by microdipterans (Masinde 2004, Heiduk et al. 2010, 2015, in press, Coombs et al. 2011). It is thought that the remarkable scent and visual mimicry of dipteran food resources and oviposition sites plays a pivot role in attracting pollinators within this genus (Masinde 2004, Ollerton et al. 2009). However a recent extensive scent study (Heiduk et al., in press) of 14 *Ceropegia* species suggests that floral scent chemistry is not necessarily correlated with pollinator assemblages, instead they showed scent components and pollinators associated with those components were species specific. Furthermore the same functional groups of pollinators were attracted to *Ceropegia* species that were utilizing different scent components (Heiduk et al., in press).

A similar finding was obtained in a South African study on an assemblage of milkweeds from different genera; *Asclepias*, *Pachycarpus* and *Xysmalobium* all attract chafer-beetles (Scarabaiedae: Cetoniinae) despite producing a wide range of distinct scents, with minimal similarities between them (Shuttleworth & Johnson 2010). Shuttleworth and Johnson (2009c) also highlighted that scent plays a key role in the pollination of *Pachycarpus grandiflorus,* which has a specialized wasp pollination system. A floral scent study of some Asclepiadeae species characterized their floral scents as sweet and associated with Dipteran, Hymenopteran and Lepidopteran pollinators (Jürgens et al. 2008). Generally a vast array of plant volatiles has been documented for the family as a whole, representing a large variety of attractants and facilitating shifts between different pollinator guilds (Ollerton and Liede 1997). The scent profiles of seven milkweeds, namely *Cibirhiza, Fockea, Gymnema, Hoya, Marsdenia, Stephanotis* and *Telosma* (all primitive Asclepiadoideae taxa), along with the basal *Secamone* (tribe Secamoneae, subfamily Secamonoideae) were analysed by Jürgens et al. (2010). They found some commonly shared floral scent compounds but also observed a lot of diversity both within and across different genera. Even closely related species showed some distinction.

These studies highlight that different plants, either within the same genus or across genera, can use different combinations of volatiles to attract the same pollinator group. While others show that distinctions can occur even within closely related species in order to attract different pollinators while restricting others. Therefore further analysis of taxa is essential, particularly comparative analyses within genera to increase our understanding of the intricate and novel ways plants are utilizing different scent components to attract or repel floral visitors. Apocynaceae's floral and evidently chemical diversity presents an excellent avenue to explore this.

The present study aimed to investigate the floral scent chemistry of four milkweed species in the Western Cape. The study species were selected due to the fact that their floral scent has not been previously studied but comprehensive chemical analyses exist for different species within the same genera, offering an opportunity to investigate possible variation in chemical composition both within and across genera. Specific aims were to:

1) Determine the chemical composition of the floral scent of each species,

2) Compare the scent chemistry both within and across genera from previous studies, and

3) Present a preliminary record of floral visitors for some of these species.

4.3 Methods and Materials

Study species

Scent samples were collected from four Apocynaceae species: *Cynanchum obtusifolium, C. zeyheri, Fockea capensis* and *Secamone alpinii* (see Table 1 for sampling localities).

(Scents of the species they will be compared to are described below.)

Cynanchum obtusifolium L.f (subfamily Asclepiadoideae, tribe Asclepiadeae, subtribe Cynanchinae) is a common milkweed creeper that can twine 2-3m high. It is found along the southern and eastern South African coast as well as southern Mozambique (Liede 1993). It has tiny flowers (3-4mm) with light green corollas and white coronas occurring in small clusters along the twining stems in groups. *Cynanchum obtusifolium* occurs on dune scrubs and coastal vegetation, and is sometimes found in disturbed habitat.

In contrast, *C. zeyheri* Schltr. is a dwarf decumbent shrublet that is 150-200mm tall with small flowers (2-3mm) that have maroon-brown corollas and white coronas. It is endemic to the Cape and grows on shale and limestone in Fynbos, Renosterveld and Strandveld (Liede 1993). A previous study included *C. altiscandens* K. Schum. which was sweet and slightly musky, as well as *C. auriculatum* Blume. described as sweet smelling (Jürgens et al. 2008).

Fockea capensis Endl. (subfamily Asclepiadoideae, tribe Fockeeae), is a twining herb, sometimes erect, and characterized by its massive underground tuber (Endress & Bruyns 2000). Flowers are small (8-22mm), made up of green-yellow or green-brown corollas with tiny white coronas. It occurs in arid to semi-arid regions, mostly restricted to the Little Karoo and some drier mountainous regions of the Succulent

Karoo (Jürgens 1991). Scent analysis exists for *F. angustifolia* and *F. edulis,* which are described as sweet (benzenoid dominated) and slightly sweet (aliphatic compound dominated) respectively (Jürgens et al. 2010).

Secamone alpinii Schult. (subfamily Secamonoideae, tribe Secamoneae) is a forest creeper with small white flowers naturally occurring in afro-montane forests (Goyder 1992). It is predominantly found in eastern and southern Africa (Goyder 1992). The genus is made up of 80 species found in the tropics and subtropics, with 16 species occurring in mainland Africa with an additional 62 occurring in Madagascar, which is believed to be the main center of radiation (Goyder 1992). The species is known for its tiny flowers and minute pollinaria, which, unlike the Asclepiadoideae, have four pollinia attached to the corpusculum instead of the usual two (Goyder 1992). The scent of *S. afzelii* has been described as sweet and musky, while *S. parviflora* was described as sweet (Jürgens et al. 2010).

Table 1. Focal study species with descriptions of floral scent, populations, sampling locations and collection dates and sampling times.

Species and Description	Location	Population description	GPS Coordinates	Date sampled (scent sampling time)
Cynanchum obtusifolium **Sweet smell**	Silvermine Nature Reserve Cape Town	Small scattered population along road	-34.11012, 18.4061	28 July 2015 (11:30 – 12:30pm)
Cynanchum zeyheri **Strong musky, rotting kelp smell**	Signal Hill Cape Town	Two isolated plants along hiking trail	-33.9176, 18.4032	30-31 July 2015 (3 - 4:30pm, 4 - 5pm)
Fockea capensis **Slightly musky smell**	MinWater Oudtshoorn	>200 on mountain top	-33.7143, 22.0173	24-25 March 2016 (11am - 12:30pm)
Secamone alpinii **Very strong fetid smell**	Newlands Forest Cape Town	Three scattered plants along hiking trail	-33.9672, 18.4479	11-13 December 2015 (10 - 11am, 12 - 1pm)

Figure 1. Focal study species a) *Cynanchum zeyheri*, b) *Fockea capensis*, c) *Cynanchum obtusifolium*, and d) *Secamone alpinii*

Scent collection and analysis

Floral scent was collected and analysed using the methods described in Chapter 2 for three *Cynanchum obtusifolium*, six *C. zeyheri*, three *Secamone alpinii* and six *Fockea capensis* plants. All scent samples were taken in the afternoon in the field, with the exception of *C. zeyheri* where additional samples were taken in the lab. The chemical compounds making up the floral scents of the four study species were compared to each other and to previously studied congeners (data obtained from Jürgen et al. 2008, 2010) in Primer 6.1.6 software (Clarke & Warwick, 2001; Clarke and Gorley, 2006) with the previously mentioned protocols (Chapter 2).

Floral visitors

Floral visitors were observed for *Fockea capensis* from 8am until 5pm on each of 3 days; 23—26 of March 2015. A total of four motion sensor cameras (Bushnell Trophy Cam HD Max-Colour LCD, 119577C) were set up from 5pm to 8am on the 23rd, 24th and 25th of March 2016, and collected the next morning to detect any night floral visitors and possible pollinators for each of three consecutive nights. Each camera was directed at clusters of flowers on a different plant, and placed about a metre away from each plant to create the optimal camera focus. Once triggered by motion or heat the cameras would record a 60-second video with a 10 second interval between consecutively triggered videos. Footage was later analysed for the type of floral visitor species, time of day of the visit, time spent foraging, behaviour and number of flowers visited.

Secamone alpinii was observed for pollinators on the 11th and 13th of December 2015 from 10am -12pm and 1pm to 2pm respectively. Floral visitors to *F. capensis* and *S. alpinii* were caught and inspected for pollinaria, and the place of attachment and the number of pollinaria each insect carried determined.

The pollinators of *Cynanchum obtusifolium* have already been examined. Studies show that this species is pollinated by honeybees (*Apis mellifera*) (Coombs 2010 PhD Thesis, Coombs et al 2012) therefore floral visitors were not examined. Furthermore, pollinator observations were not carried out for *C. zeyheri* due to safety issues.

Autogamy and natural fruit set

The number of buds on ten different plants were counted and tagged to investigate natural fruit set in the study population of *F. capensis*. An additional ten plants with only buds were bagged with fine mesh cloth baths to exclude all floral visitors and determine if this species was capable of autogamous self-pollination. Fruits were collected four months later and the number of seeds within each pod determined.

4.4 Results

The scents are dominated by benzenoid compounds and monoterpenes (Figure 2). *Fockea capensis* had the largest relative amount of monoterpenes making up 54.9% of its total scent while 41.8% consists of benzenoid compounds. *Cynanchum zeyheri* emitted scent that was almost completely dominated by benzenoid compounds (86.3%). In contrast benzenoids only makes up 30.4% of *C. obtusifolium*'s scent, while monoterpenes and irregular terpenes made up the remaining majority (35.7% and 18.9% respectively). Irregular terpenes made up less than 1% of the scents of the rest of the species, as did nitrogen-containing compounds with the exception of *C. obtusifolium* where they made up 7.5%. *Secamone alpinii* also had large amounts of monoterpenes (41.6%) with a fair amount of aliphatics (26.7%). However 14% of *Secamone's* compounds are unknown. Emission rates varied with *F. capensis* (100.2 ng/hr/flower) and *C. obtusifolium* (102.4 ng/hr/flower) emitting large amounts of scent while *C. zeyheri* (29.3 ng/hr/flower) and *S. alpinii* (9.7 ng/hr/flower) had very low emission rates.

Table 2: Floral scent compounds in the four study species, showing relative amounts (%) of each compound present in headspace samples. Scent compounds are listed according to class and their Kovats Retention Index (KRI). Amounts larger than 10% are highlighted in bold and trace amounts (< 0.05%) are noted as tr.

		Cynanchum obtusifolium	*Cynanchum zeyheri*	*Fockea capensis*	*Secamone alpinii*
No. of samples collected		3	5	6	5
	KR1				
Aliphatics					
3-Methylhexane[b]	675	-	9.2	-	-
Hexyl acetate[b]	1238	-	-	-	0.9
(Z)-3-Hexen-1-yl, acetate[b]	1271	4.0	-	1.0	**17.5**
(Z)-2-Hexen-1-yl, acetate[b]	1283	-	-	-	0.5
Hexan-1-ol [a]	1314	0.1	-	-	-
(E)-2-Hexen-1-ol[b]	1341	0.7	-	-	-
(Z)-3-Hexen-1-ol[b]	1344	-	-	-	3.3
Nonanal[b]	1365	2.3	1.6	-	3.6
Acetic acid[b]	1403	-	-	-	0.4
Oct-1-en-3-ol[b]	1405	-	0.2	-	-
Decanal[b]	1454	-	-	-	0.5
Nitrogen containing compounds					
Phenyl formamide	2120	5.0	-	-	-
Methyl anthranilate[b]	2186	0.5	-	-	-
Indole	2375	8.9	0.1	0.5	-
Skatole[b]	2385	-	-	-	0.5
Benzenoid & phenyl propanoids					
Benzaldehyde [b,c]	1488	**14.1**	**64.7**	1.6	-
Methyl benzoate [b]	1577	0.1	-	**17.5**	-
Phenylacetaldehyde [b]	1511	-	**13.7**	1.5	-
Benzylacetate [b, c]	1692	tr	-	1.1	-
Methyl salicylate [a]	1738	2.8	-	**14.3**	-
Acetylbenzoyl	1762	-	0.1	-	-
Phenylethyl acetate [b]	1776	-	-	1.7	-
Benzyl alcohol	1830	**13.9**	0.4	0.6	-
3-Phenylhexane	1835	-	-	-	3.3
Phenylethyl alcohol	1864	-	7.2	3.4	6.4
Dimethyl salicylate	2008	0.3	-	0.2	-
Benzyl benzoate [b]	2533	-	-	tr	-

Monoterpenes					
Limonene	1122	-	1.6	-	-
β-Ocimene	1228	5.2	-	**32.6**	2.2
(E)-Linalool oxide (furanoid)	1401	0.2	-	-	-
(Z)-Linalool oxide (furanoid) [b]	1431	0.2	-	-	-
β-Linalool	1500	**11.6**	**-**	**22.1**	**33.6**
Hotrienol [b]	1563	0.3	-	0.1	-
α-Terpineol [b]	1649	-	0.1	-	3.8
(E)-Linalool oxide (pyranoid) [b]	1694	5.2	-	-	1.7
(Z)-Linalool oxide (pyranoid) [b]	1715	**19.7**	-	-	-
Sesquiterpenes					
α-Copaene	1452	-	-	-	0.5
ST 204,41,69,79,91,93,105,133,161	1550	-	-	-	0.9
Himachala-2, 4-diene	1560	-	-	-	6.4
Bourbonene [b]	1483	0.1	-	-	-
α-Caryophyllene	1624	-	-	-	tr
α-Farnesene [a]	1687	tr	-	tr	-
Nerolidol	1979	0.9	-	-	-
Irregular terpenes					
6-Methyl-5-hepten-2-one [a]	1300	3.6	0.7	0.2	-
Unknowns					
mz/: 59,69,40,55,43,60,126,44,57,71	1133	-	-	0.8	-
mz/: 119,43,85,57,105,84,92,64,131,71	1264	-	0.1	-	-
mz/: 91,119,134,77,92,79,105,117,93,41	1338	-	-	0.2	-
mz/: 57,71,55,43,85,145,82,67,73,147	1329	-	0.4	-	-
mz/: 79,81,77,93,91,72,42,53,80,41	1368	-	-	0.4	-
mz/: 59,94,93,68,67,111,55,81,83,79	1427	-	-	-	**11.6**
mz/:105,119,93,91,161,92,81,77,79,120	1437	-	-	-	0.5
mz/: 43,109,95,69,84,54,81,79,91,40	1572	-	-	tr	-
mz/: 91,161,105,81,119,79,93,40,77,55	1578	-	-	0.1	-
mz/: 93,80,78,91,92,121,77,107,94,105	1609	-	-	-	0.9
mz/: 39,50,65,75,77,89,91,108,122,136	1652	0.1	-	-	-
mz/: 81,57,67,68,82,53,43,41,95,71	1655	-	-	-	1.1
mz/: 93,69,41,42,107,81,71,136,79,54	1871	-	-	tr	-
Total percentage of identified compounds		99.9	99.5	98.5	85.9
Aliphatics		7.2	**11.0**	1.0	**26.7**
Nitrogen compounds		**14.3**	0.1	0.5	0.5
Benzenoids & phenyls		**31.2**	**86.0**	**41.8**	9.7
Monoterpenes		**42.5**	1.7	**54.9**	**41.3**
Sesquiterpenes		1.0	0.0	0.0	7.8
Irregular terpenes		3.6	0.7	0.2	0.0
Unknowns		0.1	0.5	1.5	**14.1**

Total no. of compounds	25	14	23	22
Emission rates (ng/hr/flower)	102.4	29.3	100.2	9.7

[a] Comparison of MS and retention time with published data.

[b] Comparison of MS with published data.

[c] Comparison of MS and retention time with authentic standard.

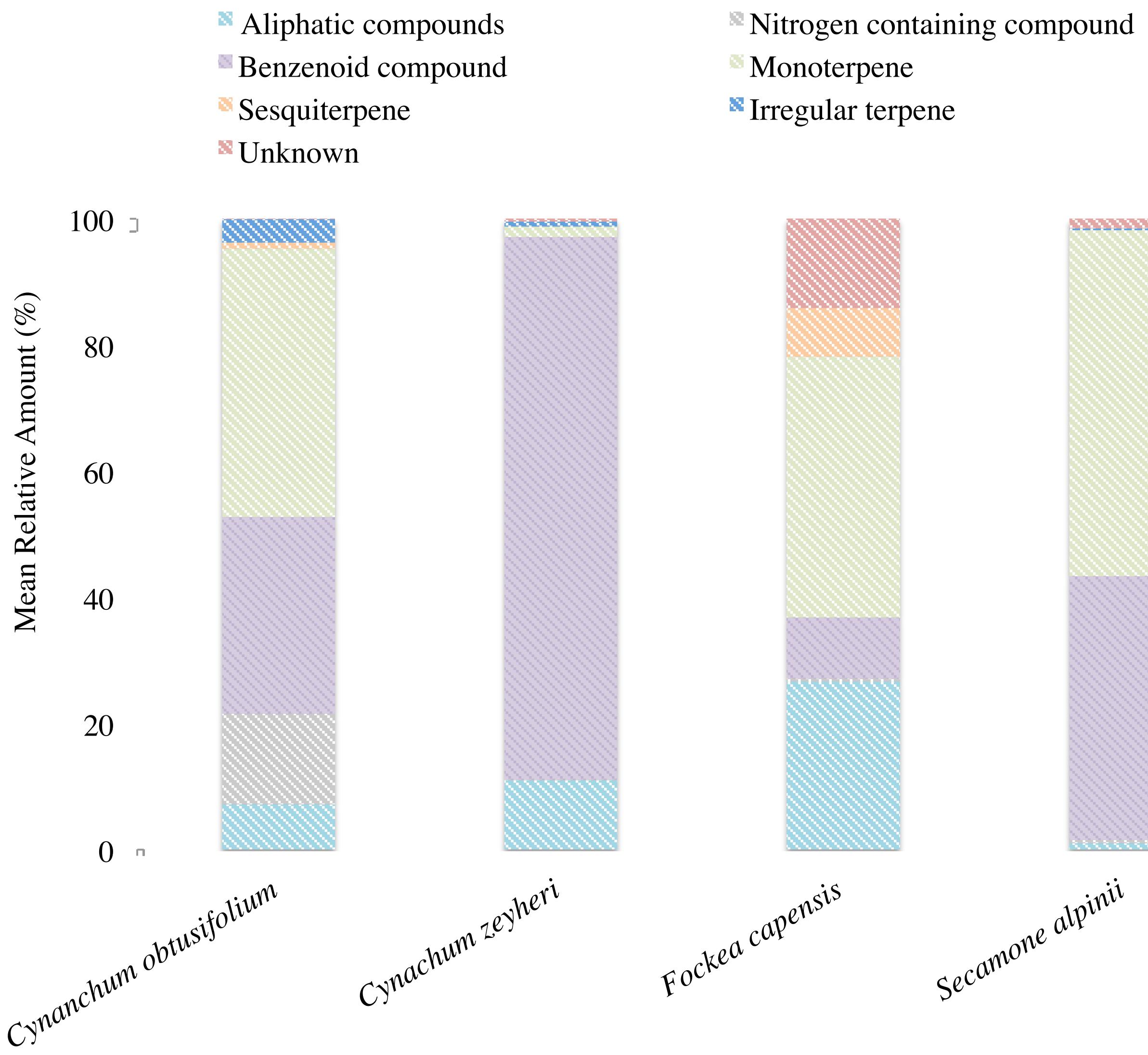


Figure 2. Floral scent composition of four Apocynaceae species displaying relative amounts of compound classes for each species.

The NMDS shows that there is large variation in scent compounds both within and across genera. The exceptions are *Fockea angustifolia* and *Secamone parviflora* whose floral scents show some similarity, however this is only at the 40% similarity level therefore all species had distinct scents. However the results were not statistically significant.

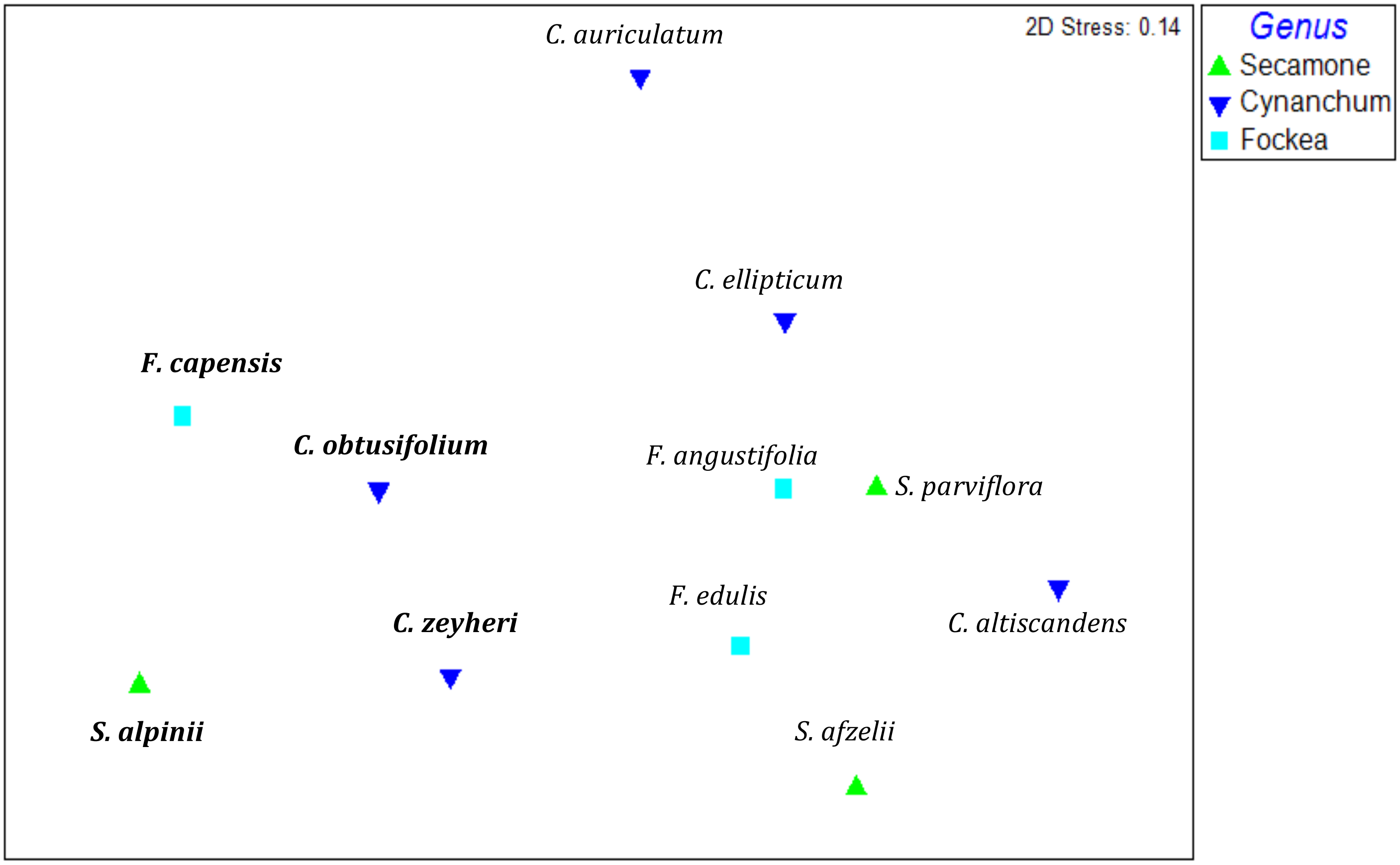


Figure 3. Non-metric multidimensional scaling (NMDS) based on Bray-Curtis similarities of the odour composition of *Cynanchum*, *Fockea* and *Secamone*. Focal study species are in bold. Scents of *C. auriculatum, C. altiscandens* and *C. ellipticum* were obtained from Jürgens et al. 2008, while *S. afzelii* and *S. parviflora*, as well as *F. angustifolia* and *F. edulis* were obtained from Jürgens et al. 2010). ANOSIM Global R_{genera} = -0.149; p > 0.01

Floral visitor observations

Pollinator activity was very low in the *Fockea capensis* population. Video footage did not show any nocturnal visitors. None of the collected daytime floral visitors had pollinaria, and no pollinaria were visible on the visitors that were observed but not collected. Of the observed *Fockea* visitors only the flies drank nectar and moved from flower to flower. The observed ants were present on several plants, not drinking nectar and not on the flowers themselves but were moving around the plants' stems. The rest of the visitors landed on the corollas and left without making contact with the nectaries or central part of the flower.

Secamone alpinii was visited by six tiny Nematoceran species (mosquito-like flies) which drank nectar for several seconds and moved from flower to flower on the same plant (separate plants were located very far away from each other). One of the small Dipteran species was collected and no pollinaria was found on it. It was also partially damaged during collection therefore identification was not possible. A single honeybee (*Apis mellifera)* was observed drinking nectar and moving from flower to flower, it was collected and no pollinaria was found on it.

Fruit set

In the population of ca. 200 *Fockea capensis* plants, none of the ten bagged plants or the controls produced fruit. The population in general had a very low fruit set with only two plants producing fruit (0.02%). The two plants only produced two fruits each. The collected fruit was taken back to the lab to mature so seeds could be counted. They matured after a few weeks but all the fruits were infested with newly hatched *Dacus ciliatus* (Tephritidae) and all the seeds had been eaten or were badly decayed.

Table 3. *Fockea capensis* and *Secamone alpinii* floral visitors.

Floral visitor	Observed (collected and analysed)
Secamone alpinii	
Nematoceran sp.	6(1)
Fockea capensis	
Diptera	
Drosophiliidae	
Drosophiliidae sp.	1 (1)
Sarcophagidae	
Sarcophagidae sp.	2
Tachinidae	
Tachinidae sp. 1	6 (2)
Tachinidae sp. 2	2 (1)
Hymenoptera	
Apidae	
Apis mellifera	2
Apidae sp.	
Formicidae	
Camponotus fulvopilosus	23 (10)
Lepidoptera	
Lepidopteran sp.	1
Coleoptera	
Coleopteran sp.	1

Note: All assessed visitors lacked pollinaria.

4.5 Discussion

All four species have very distinct scents, different from each other as well as their congeners (Table 2, Figure 3). Only benzaldehyde, phenylethyl alcohol, benzyl alcohol, β-ocimene and β –linalool were common amongst the focal species, present in three out of four of the species. These are all very common floral compounds, especially β –linalool and β-ocimene (Knudsen et al. 2006). The two monoterpenes were relatively high in *Secamone alpinii* and *Fockea capensis*.

Fockea capensis also had a large relative amount of methyl benzoate (17.5%). The combination of methyl benzoate and linool are commonly associated with moth-pollinated flowers (Dobson 2006, Knudsen & Tollsten 1993). The expectation would therefore be that *F. capensis* is moth-pollinated. However night pollination observations did not support this. *Fockea capensis* floral visitors were almost exclusively active in the early morning. The video recorders were set up on very cold nights and the fruit set was extremely low, therefore it is possible that pollinator activity was low during the observation periods and moths may still be observed in more favourable conditions. Some of the other abundant *F. capensis* compounds such as methyl salicylate (14.3%), β-ocimene (32.6%) and linalool (22.1%) are known to be detected by Andrenid bees (Dotterl et al. 2005), although only one bee was observed visiting *Fockea* (Table 3). The combination of all four dominant compounds in addition to the smaller compounds may produce a completely different smell that is attractive to a different pollinator group. Based on floral visitor observations, this combination may be attractive to flies.

The previous *Fockea* study (Jürgens et al. 2010) showed that *F. angustifolia* scent is mostly made up of (E,Z)-2,6-nonadienal (33%), while *F. edulis* is dominated by benzaldehyde (52%) and both these species have very small amounts of a range of sesquiterpenoids making up the rest of their scent profiles. Therefore all the *Fockea* species seem to be producing different scent components. It is possible that the different combinations could all be attracting flies, but the absence of pollinaria or corpusculae on any of the flies collected in my study makes it difficult to confirm if flies are in fact pollinators. It should be noted that *Fockea* species possess a weak corpusculum that is believed to easily disintegrate (Bruyns & Klak 2006) so evidence of it on pollinators is very difficult to obtain. Additionally *Fockea* is unique among milkweeds for its very restrictive gynostegium morphology, with elongated anther appendages blocking access to the corona tube (Bruyns & Klak 2006). This suggests a pollinator with long mouthparts and the flies observed were most likely incidental visitors. The low fruit set further suggests that the true pollinators were absent. Therefore, its pollinator remains elusive, as thus far no studies have successfully confirmed the pollinator of *Fockea* species.

The two *Cynanchum* species also showed little similarity. They only shared five compounds with benzaldehyde contributing a significant amount to both scents (11.9% in *C. obtusifolium* and 60% in *C. zeyheri*). The rest of the shared compounds made minimal contributions to either scent profiles with the exception of benzyl alcohol (11.6 % in *C. obtusifolium* and only 0.4 in *C. zeyheri*). *Cynanchum obtusifolium* is predominantly pollinated by honeybees (*Apis mellifera*) but also visited by other small hymenopteran families (Coombs 2010). However the presence of compounds known to be detectable to bees such as α-farnesene and indole, (though present in small amounts), in combination with its more dominant compounds, may contribute to its bee and overall Hymenopteran attracting abilities (Dotterl et al. 2005).

The large presence of aromatic compounds explains *C. zeyheri*'s strong scent. Jürgens et al. (2008) also found a large amount of benzaldehyde in *C. formosum* (87.6%) which was attractive to Vespidae and Apidae Hymenopterans. Other studies have also highlighted the potential importance of benzaldehyde in attracting Hymenopteran species (Han & Chen 2002, Theis 2006). *Cynanchum zeyheri* also contained some phenyacetaldehyde (12.9%), which is a strong moth attractant. While the sweet and slightly musky *C. altiscandens* had almost half its scent consisting of linalool and a large amount of α-terpineol and *C. auriculatum* was predominantly made up of benzyl acetate and lilac alcohol (Jürgens et al. 2008).

The scent of *Secamone alpinii* mostly consisted of β –linalool (33.6%) and (Z)-3-hexen-1-yl acetate (17.5%). While linalool is common in many floral scents (Knudsen et al. 2006), *Secamone's* does have some interesting compounds such a skatole, which is associated with flowers that smell like feacal matter (Jürgens et al. 2006). Some milkweeds associated with carrion flies produce pungent odours. Flowers that mimic the smell of feaces to attract such flies are characterized by the emission of oligosulphides, phenol and skatole (Jürgens et al. 2006, 2013). *Secamone alpinii* was visited by small mosquito-like flies, which moved from flower to flower. Morphologically their size would be suitable for pollinaria removal. A single honeybee was also observed visiting several flowers and drinking nectar however it did not have any pollinaria on it. Although based on the size of the tiny flowers a Nematocera sp. is the most likely pollinator.

It is thought that the basal Asclepiadoideae plants were fly pollinated with this system of pollination being lost and regained throughout the family's evolution (Ollerton & Liede 1997). *Secamone,* which belongs to the primitive Secamonoideae subfamily, appears to be fly pollinated; supporting the notion that fly pollination was most likely the initial pollination system within this family. A variety of fly pollinators encompassing many groups are associated with the Ceropegieae tribe within Asclepiadoideae, including Calliphoridae, Drosophiliidae, Empididae, Muscidae, Sarcophagidae, Sepsidae, Tachinidae as well as Tephritidae (Meve & Liede 1994). The large quantity of volatiles present in asclepiads is thought to be a reflection of the different scents used to attract different kinds of flies (Meve and Liede 1994).

The study species emitted a wide range of floral volatiles, dominated by benzenoids and monoterpenes. They showed a marked difference from their congeners, highlighting the complexity and diversity both within and across species in this group. Supplementary studies involving extended pollinator observations as well as lab experiments to determine which compounds different visitors respond to would further enhance the results of this study.

5. Synthesis

5.1 Summary

The Cape milkweeds covered in this study revealed some thought provoking results. Two *Gomphocarpus* species, one occurring in Fynbos (*G. cancellatus*) and the other in the Succulent Karoo (*G. filiformis*) had a wide range of floral visitors. Hymenopteran pollinators were the most abundant and frequent visitors for both plant species. *Gomphocarpus cancellatus* was predominantly pollinated by bees (*Apis mellifera*) while *G. filiformis* exhibited an unlikely plant-pollinator relationship with *Camponotus fulvopilosus*, which were the most abundant pollinator. Both species were also visited by lesser double-collared sunbirds (*Cinnyris chalybeus*). *Gomphocarpus cancellatus*' pollination system is a contrast to milkweeds in the summer rainfall region, which are mostly specialized (Ollerton et al. 2003, Coombs et al. 2009, Shuttleworth & Johnson 2010). However *G. filiformis* shows similarities in its somewhat filtered pollination system to *G. fruticosus*, which also had more visitors than pollinators (Burger et al., in press).

The large amounts of nectar rewards offered by both species may be responsible for the large amount of visitors. The sweet smelling flowers dominated by very different floral compounds may also contribute to the observed systems. *Gomphocarpus filiformis* emitted a benzenoid-dominated scent with relatively large amounts of commonly found floral compounds whose combination is associated with generalist plant species (Tollsten et al. 1994, Knudsen et al. 2006,). However *G. cancellatus* floral emissions were almost completely dominated by 4-oxoisophorone normally associated with the attraction of Lepidopterans (Andersson et al. 2002, Andersson 2003, Andersson & Dobson 2003). The abundance of other visitors, despite a strongly butterfly-biased smell, suggests that scent might not be a prominent factor in pollinator attraction in this species or the compound may be associated with a wider variety of insect groups than previously thought. The colours of both plants' floral components were shown to be very similar to their backgrounds in Hymenopteran optical space. Therefore nectar and scent appear to be the main pollinator attractants. Additionally, evidence of the importance of these pollinators was highlighted by the inability of *G. cancellatus* to produce fruit autogamously.

In contrast to the generalist system displayed by *Gomphocarpus,* the Cape endemic *Eustegia minuta* had a specialized pollination system. This species is characterised by its unusually small size, complex floral morphology and sporadic floral blooms stimulated by fire (Bruyns 1999). It showed an exclusive association with bibionid flies. The plant's complex floral structure, with its deep-set nectaries obstructed by two rings of protruding corona lobes, may act as a filtering mechanism to keep certain visitors away. Additionally it emits a large amount of pungent floral odour dominated by an unidentified compound, which may attract the tiny bibionid flies whilst also repelling other potential visitors. This unique scent is likely the most important floral feature in this system. The combination of these and other floral traits for *Eustegia*, such as minimal visual cues, low nectar production and low reproductive success are all characteristics associated with a Bibionidae specialised pollination system. This study is the first to observe such a pollination system in Asclepiadoideae and represents the first pollination study of the Eustegieae tribe.

The floral scent diversity observed in Apocynaceae was further highlighted by the assortment of floral compounds in *Cynanchum obtusifolium, C. zeyheri, Fockea capensis* and *Secamone alpinii.* These species mainly emitted benzenoid and monoterpenoid scents. The combinations of compounds found within each species however, were quite distinct. The floral scent of *Fockea capensis* consisted of compounds typically associated with moth pollination, i.e. methyl benzoate and linalool (Dobson 2006, Knudsen & Tollsten 1993). The scent was also suggestive of bee pollination due to the presence of β-ocimene, β-linalool and methyl salicylate (Dotterl et al. 2005). Pollinator observations did not confirm either prediction, as flies were found to be the most common floral visitor. However the *Fockea* population, though large, had very low pollinator activity and therefore observations were minimal. *Secamone alpinii*'s musty smell was most likely due to the presence of skatole, common in some *Stapelia* species that mimic feacal matter to attract coprophagic flies (Jürgens et al. 2006). The flowers attracted tiny

Nematoceran flies, whose size potentially makes them ideal pollinators of *Secamone alpinii*'s equally tiny flowers.

The floral scents of the two *Cynanchum* species studied were also quite dissimilar in their chemical composition. *Cynanchum obtusifolium*, a bee pollinated species (Coombs 2010), displayed the largest floral compound diversity with 27 different compounds, the largest proportions consisting of commonly encountered plant compounds such as linalool oxide, linalool, benzyl alcohol and benzaldehyde. However these are not exclusively associated with bees but attract a wide range of visitors (Dudareva & Pichersky 2006). *Cynanchum zeyheri* emitted fewer compounds, predominantly consisting of Hymenopteran and Lepidopteran volatile attractants (large proportion of benzaldehyde, with smaller amounts of phenyl acetaldehyde, and phenyl alcohol). The combination of benzaldehyde and phenyl acetaldehyde is known as a very powerful moth attractant (Heath et al. 1992). Therefore I predict that *C. zeyheri* may be moth pollinated and recommend that night observations be conducted in a population in which it is safer to do so.

The assortment of compounds not only made the scents of these species distinct from each other but also from their congeners. The emitted scents were complex and did not necessarily align with the scent associated with their predicted or observed pollinator. Additionally predicting a pollinator group based on scent may not be reliable as different plants may use completely different compound combinations to attract the same kind of pollinator (Shuttleworth & Johnson 2010).

5.2 Limitations of this study

This study suggests that floral scent and possibly nectar play an important role in pollinator attraction. However direct tests to determine their functional significance were not carried out due to time constraints, and in *Eustegia minuta*'s case, unpredictable flowering. Therefore behavioural observations under different controlled conditions would have greatly enhanced the study by elucidating how certain potential functional floral traits affect visitors. This is particularly evident in Chapter 4, which shows that floral scent alone does not necessarily predict the kind of pollinator attracted to certain plant species and can result in conflicting ideas. Therefore descriptive pollination ecology studies benefit from also including substantial behavioural manipulations and lab experiments to determine the function of various floral traits in attracting or filtering insect visitors. Another limitation is determining the pollinators of small flowered species such as *Secamone* and *Fockea* species, which have tiny pollinaria. In the case of *Fockea* there is no physical evidence on pollinators expected to show successful pollination due to the weak corpuscula.

5.3 Implications and future research

This study has highlighted how genera with similar floral morphology can have very different pollination systems. The *Gomphocarpus* species both in the Cape and in the summer rainfall regions look like they would attract similar visitors based on their similar floral structure, yet the chemicals emitted by those flowers were very different and most likely a reflection of their environment and the availability of pollinators. The species in harsher environments attract completely different pollinators from their congeners by producing chemical compounds that lure a wide range of potential pollinators. Or in the case of *Eustegia minuta* and possibly *Fockea capensis*, *Secamone alpinii* or *Cynanchum zeyheri*, their chemical arsenal may restrict certain visitors. While the focus of this study was to establish plant-pollinator relationships and qualitatively and quantitatively describe potential floral functional traits shaping these interactions in the Cape asclepiads, the ultimate goal would be to determine the function of these traits and the efficiency of these pollination systems. Other questions to explore would be, for example, why have the Cape *Gomphocarpus* species evolved more generalist pollination systems while their summer congeners are specialists? Do the unique scents of the other study species point to specialised systems? This certainly seems to be the case for *Eustegia*. Is this a response to their environment and availability of pollinators across the geographic range of the family? Are these plant traits pollinator driven and indicative of coevolution?

Some of these questions were answered by Grant’s Polemoniaceae studies (Grant 1949, Grant & Grant 1965). The studies analyzed the pollinators of the family in different geographical locations and noted how the modified flowers in the family utilized different pollinators and resulted in speciation. It was also posited that pollinators occur in a geographic mosaic creating a "pollination climate" thus explaining the difference in floral selection across a species' range (Grant & Grant 1965). Differences in the pollination climate across the milkweed's range could therefore explain some of the obtained results.

The present study also highlighted that discrepancies can occur between a plant's floral volatiles and its expected pollinators. This suggests that knowing the pollinators of a plant does not necessarily make it easier to predict what scent it is emitting and vice versa. Milkweeds display an incredible amount of scent diversity, both within and across genera (Jürgens et al. 2008, 2010). Different combinations could end up producing similar scent to attract specific pollinators. Shuttleworth and Johnson (2010) showed evidence of this in their study of specialized plants that lure chafer beetles with very different scent compound combinations. Therefore observations should always be supplemented with chemical analyses to make an accurate association between the plant's volatiles and what they are attracting or repelling.

With regards to pollinator efficiency, the effectiveness of ant pollinators observed in *G. filiformis* and *E. minuta* could be tested by isolating and harvesting plants that have been exclusively pollinated by ants to see if they develop fruit with viable seeds. A morphological analysis inferring if the corona teeth of *G. filiformis* flowers act as a filter could be conducted. This would involve morphometric measurements of the floral components and floral visitors and extensive observations on how different guilds interact with these structures. Furthermore, investigations to determine the potential impact of birds as pollinators in both *Gomphocarpus* species can be carried out. Birds can be caught with mist nets and analyzing their tongues and beaks (using similar protocols as Pauw 1998) will help to determine if they do in fact act as supplementary pollinators in these generalized pollination systems.

Pollinator choice tests would also be highly beneficial to determine what function scent and colour have. Y-tube tests could be used to determine if visitors are lured by smell. Additionally, electrophysiological investigations could be carried out to determine exactly which individual compounds or group of compounds can be detected by the floral visitors and their response to those compounds can be assessed through behaviour observations. This could potentially identify new compound combinations that are being utilized to attract or repel common or generalist pollinators as well as which compounds are keeping most visitors away from specialized plants such as *Eustegia minuta*. To clarify if *Eustegia* nectar is unpalatable to the common nectar-seeking insects in the area, nectar choice tests involving different insect groups' response could be carried out. This will determine if nectar is involved in the visitor filtering process.

Another interesting aspect would be a community level assessment of the insect activity present in the absence of *Eustegia* flowers several seasons after a fire. This could be in the form of a succession study keeping track of which plants move into the area. Do other flowering plants facilitate pollinator attraction to *Eustegia* or do they outcompete *Eustegia* when it comes to pollinators and could this be the reason behind *Eustegia's* post-fire blooming? Observing if bibionids still visit the area in *Eustegia*'s absence would also highlight weather they depend on it or are generalist pollinators as observed in the *Disa obtusa* study (Johnson & Steiner 1994). This kind of study would be possible as the entire *Eustegia* population occurs in a very small area. Moreover, pollinator observations carried out in one of the rare seasons when *Eustegia* flowers bloom before a fire removes the competition would also yield interesting results and provide further information about this unique specie's reproductive strategy.

Evidently milkweeds present many opportunities for interesting studies that can further inform us about the complex relationship between plants and their pollinators. Particularly how floral traits such as morphology, nectar rewards and scent and colour cues differ across genera.

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APPENDIX 1

Table 4. Relative amounts (%) of scent compounds of *G. filiformis*, and two *G. cancellatus* populations. Compounds are arranged according to Kovats retention index (KRI) and compound class. tr = trace amount (< 0.05% of total sample). Percentages greater than 10% are highlighted in bold.

		G. filiformis Laingsburg					*G. cancellatus* Worcester									*G. cancellatus* Rawsonville	
		P1	P2	P3	P4	P5	P1	P2	P3	P4	P5	P6	P7	P8	P9	P1	P2
Aliphatics		8.4	**13.6**	5.6	5.5	**21.5**	-	-	-	-	-	-	-	-	-	-	-
Octanal	1259	-	-	-	-	-	1.3	0.2	0.1	0.2	0.2	0.1	0.5	0.3	0.8	-	-
(*Z*)-3-Hexen -1-yl acetate	1271	8.4	13.6	5.6	5.5	21.5	tr	0.1	0.1	0.2	1.2	0.2	0.4	0.5	0.1	-	-
Hexyl acetate	1238	1.2	2.5	1.1	1.6	3.5	-	-	-	-	-	-	-	-	-	0.3	3.6
Nonanal	1365	-	-	-	-	-	3.3	0.4	0.3	0.5	0.5	0.4	0.9	0.7	1.0	-	-
(*Z*)-3-Hexen-1-ol	1328	-	-	-	-	-	-	-	-	-	-	tr	0.5	0.2	0.1	-	-
Heptyl acetate	1324	0.1	0.1	tr	0.1	0.3	-	-	-	-	-	-	-	-	-	-	-
Oct-1-en-3-ol	1405	-	-	-	-	-	0.2	tr	tr	tr	tr	-	-	-	-	-	-
Octyl acetate	1416	0.1	0.1	tr	0.1	0.2	-	-	-	-	-	-	-	-	-	-	-
Decanal	1454	-	-	-	-	-	1.0	0.1	0.1	0.2	0.2	0.1	0.9	0.5	0.5	-	-
Non-2-enal	1501	-	-	-	-	-	tr	-	-	-	tr	4.8	tr	tr	tr	-	-
Octan-1-ol	1514	-	-	-	-	-	0.3	0.1	tr	tr	0.1	tr	0.1	0.1	0.1		
Monoterpenes																-	-
β-Myrcene	1156	-	-	-	-	-	2.4	0.2	0.5	-	0.8	0.8	1.2	0.8	0.5		
Limonene	1183	tr	0.1	0.1	0.1	0.5	3.7	0.1	0.4	0.1	0.5	0.4	1.2	1.6	2.0	**11.4**	**52.5**
(Z)-Ocimene	1216	2.7	2.0	1.3	2.7	3.5	3.0	0.6	0.2	0.6	1.1	0.5	1.2	0.1	0.5	-	-
(*E*)-Ocimene	1221	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.8	6.7
p-Cymene	1247	-	-	-	-	-	-	-	-	-	-	0.1	0.1	-	-	-	-

3,7-Dimethyloctan-3-ol	1371	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	1.6
β-Linalool	1500	-	-	-	-	-	-	-	-	tr	-	-	-	-	-	-	1.1
(Z)-Geraniol	1772	0.3	0.4	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-
Irregular terpenes																	
4-Oxoisophorone	1658	-	-	-	-	-	**83.2**	**96.0**	**96.9**	**96.8**	**93.2**	**91.0**	**84.7**	**88.7**	**90.9**	**79.4**	**31.3**
2,2,6-Trimethyl-1,4-cyclohexanedione	1743	-	-	-	-	-	0.3	0.4	0.5	0.9	0.9	1.0	6.7	4.7	2.5	7.0	3.2
(Z)-Geranylacetone	1786	-	-	-	-	-	-	-	-	tr	tr	tr	-	-	-	-	-
Benzenoid & Phenyl propanoids																	
Benzaldehyde	1488	**30.2**	**28.1**	**39.0**	**23.1**	**15.1**	0.2	0.1	tr	tr	tr	tr	-	-	-	0.1	0.1
Methylbenzoate	1590	1.7	1.6	1.0	1.4	1.2	-	-	-	-	-	-	-	-	-	-	-
Benzylacetate	1692	**29.1**	**37.2**	**20.8**	**40.0**	**39.6**	-	-	-	-	-	-	-	-	-	-	-
Methyl salicyate	1718	1.3	0.3	0.8	0.4	-	-	-	-	-	-	-	-	-	-	-	-
Benzyl alcohol	1830	**24.6**	**13.9**	**29.9**	**24.8**	**14.2**	-	-	-	-	-	-	-	-	-	-	-
Phenylpropyl acetate	1871	0.3	0.1	0.1	0.2	0.2	-	-	-	-	-	-	-	-	-	-	-
C5-branched chain																	
Isovaleric acid	1636	-	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-
Miscellaneous cyclic compounds																	
Dihydro-5-methyl-2(3H)-furanone	1581	-	-	-	-	-	tr	-	-	-	tr	-	0.1	0.1	tr	-	-
																-	-
Unknowns																-	-
UNK: 41,69,79,105	1279	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	-	-
UNK:53,68,79, 91,107,136	1301	-	-	-	-	-	-	-	-	-	-	-	0.2	0.1	0.1	-	-
UNK:39,43,55,79,81,10	1305	-	-	-	-	-	-	-	-	-	-	-	0.2	0.2	0.2	-	-

8,136																	
UNK:43,59,67,81	1468	tr	tr	-	tr	0.2	-	-	-	-	-	-	-	-	-	-	-
UNK:39,68,96,138	1479	-	-	-	-	-	1.1	1.8	1.0	0.5	1.2	0.4	1.3	1.4	0.7		
Aliphatics		9.7	**16.3**	6.7	7.3	**25.5**	6.1	0.9	0.6	1.0	2.1	5.7	3.2	2.3	2.6	0.3	3.6
Monoterpenes		3.0	2.5	1.6	2.8	4.0	9.1	0.9	1.1	0.7	2.5	1.7	3.6	2.5	2.9	**13.3**	**61.9**
Irregular terpenes		-	-	-	-	-	**83.5**	**96.3**	**97.4**	**97.7**	**94.2**	**92.1**	**91.4**	**93.4**	**93.4**	**86.4**	**34.4**
Benzenoid & Phenyl propanoid		**87.3**	**81.2**	**91.6**	**89.9**	**70.4**	0.2	0.1	tr	tr	tr	tr	0.0	0.0	0.0	0.1	0.1
C5-Branched chain compounds		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Miscellaneous cyclic compounds		0.0	0.0	0.0	0.0	0.0	tr	0.0	0.0	0.0	tr	0.0	0.1	0.1	tr	0.0	0.0
Unknowns		tr	tr	-	tr	0.2	1.1	1.8	1.0	0.5	1.2	0.4	1.7	1.7	1.0	0.0	0.0
No. of compounds		14	14	13	13	12	15	13	13	15	16	17	17	16	16	7	8
No. of flowers		16	43	17	28	25	70	38	45	38	81	61	41	57	61	44	31

Emission rates: *G. cancellatus* (747ng/min/flower)
G. filiformis (588ng/min/flower)