ECOLOGY AND EVOLUTION OF THE SPECIALIZED *HEMIPEPSIS*-WASP (HYMENOPTERA: POMPILIDAE) POLLINATION GUILD IN SOUTH AFRICA

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FOR MY PARENTS

THE WASP

The wasp and all his numerous family
I look upon as a major calamity.
He throws open his nest with prodigality,
But I distrust his waspitality.

OGDEN NASH

ABSTRACT

Pollinators are believed to have played a key role in the radiation of flowering plants. The Grant-Stebbins model of pollinator-mediated speciation, in which evolutionary shifts between pollinators result in phenotypic diversification and enforce reproductive isolation, is one of the most compelling hypotheses for the rapid diversification of angiosperms. A key principle in this model is that plant pollination systems tend towards specialization, resulting in convergent suites of floral traits (syndromes) associated with particular types of pollinators. However, the expectation of pollination system specialization is not always supported by ecological data and has also been questioned on theoretical grounds. In this thesis, I examine pollination by *Hemipepsis* spider-hunting wasps (Hymenoptera, Pompilidae, Pepsinae) and use this system to address questions about levels and proximal mechanisms of floral specialization, floral shifts and convergent evolution of floral traits.

Specialized pollination by *Hemipepsis* wasps is a newly described pollination system within the angiosperms. I document pollination by these wasps for the first time in 15 South African grassland plant species, including two species of *Eucomis* (Hyacinthaceae) and 13 asclepiads (Apocynaceae: Asclepiadoideae). In one of the asclepiads, *Xysmalobium undulatum,* I describe a bimodal pollination system involving both *Hemipepsis* wasps and a cetoniine beetle. I also describe an unusual and potentially antagonistic pollination mechanism whereby wasps are systematically dismembered during the insertion of pollinia in the two asclepiads *Pachycarpus asperifolius* and *P. appendiculatus*. I have used these and previous case studies to establish the existence of a new pollination guild, consisting of at least 21 plant species (across 10 genera and three families), that are reliant on four functionally similar species of *Hemipepsis* wasp for pollination. Plants in the guild are distributed throughout the moist grasslands of eastern South Africa and flower from September through until early May, peaking in December/January.

The *Hemipepsis*-wasp pollination guild is characterized by high levels of functional specialization (17 of the 21 known guild members are pollinated exclusively by *Hemipepsis* wasps), despite the absence of morphological adaptations to prevent non-pollinating insects from accessing nectar. I used field and laboratory based experiments to explore the function of floral traits in enforcing specialization. These showed that *Hemipepsis* wasps primarily use scent, rather than visual cues, to locate flowers, but I was unable to firmly identify specific compounds responsible for the attraction of these wasps (compounds that elicited antennal responses in preliminary GC-EAD experiments did not attract wasps in bioassays). The chemical composition of the floral scents of guild members was examined for 71 individuals representing 14 species in addition to previous studies, and found to comprise complex blends of volatiles (usually containing between 30 and 50 compounds), typically dominated by aliphatics and monoterpenes with small amounts of aromatics. I also showed that the floral colours of guild members are similar to background vegetation, suggesting that floral colours are adapted for crypsis to avoid detection by non-pollinating insects. Palatability choice

experiments with honeybees showed that non-pollinating insects find the nectars of at least three of the asclepiad guild members distasteful. Plants in this guild thus appear to achieve specialization through biochemical filters (scent as an attractant and differentially palatable nectar) and cryptic coloration.

Pollinator-mediated convergence in floral traits is the fundamental basis for pollination syndromes, but has seldom been rigorously analyzed. Flowers in the *Hemipepsis*-wasp pollination guild share several qualitative traits, including dull greenish- or brownish-white colour, often with purple blotches, exposed sucrose dominant nectar with a relatively high sugar concentration (typically over 50% sugar by weight) and a sweet/spicy fragrance to the human nose. To test for convergent evolution in guild members, I compared scent, nectar and colour traits of guild members to those of congeners with different pollinators. Although traits often differed between guild members and their congeners, I found little evidence for overall convergence in floral scent profiles and nectar properties, but floral colours in the guild were significantly closer to the colour of background vegetation than those of congeners. At this stage, the lack of knowledge about specific floral volatiles that influence *Hemipepsis*-wasp behaviour and secondary nectar constituents that limit non-pollinator visits makes it difficult to identify the extent of biochemical convergent evolution within the guild.

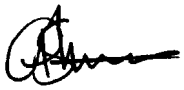
The directions and functional traits involved in evolutionary transitions between pollination by *Hemipepsis* wasps and other vectors are currently difficult to ascertain as there is limited phylogenetic data for the plant families concerned. In the genus *Eucomis*, fly and *Hemipepsis*-wasp pollinated species are very similar in floral morphology and colour, but differ strongly in floral scent. Using manipulative field experiments in conjunction with detailed analyses of colour, scent and morphology, I was able to show that a shift between wasp and fly pollination could be induced simply by manipulating oligosulphides in the scent emission from inflorescences. When considered in combination with other experiments highlighting the importance of scent as a pollinator attractant for all guild members, this suggests that scent properties may have played a key role in the evolutionary transitions between pollination by *Hemipepsis* wasps and other vectors.

This research has established that pollination by *Hemipepsis* spider-hunting wasps is more geographically and phylogenetically widespread than was previously known, and has confirmed that these wasps are important and consistent pollinators in southern African grassland ecosystems. I have shown that a distinct guild of plants is specialized for pollination by these wasps. The high levels of specialization within this guild highlight the effectiveness of biochemical filters and cryptic coloration in limiting the spectrum of flower visitors. The major challenge ahead will be to identify the floral volatiles that attract *Hemipepsis* wasps and the non-sugar constituents that make the nectars of some guild members differentially palatable. These would both contribute greatly to our understanding of floral specialization and the mechanisms involved in the radiation of the angiosperms.

PREFACE

The data described in this thesis were collected in the Republic of South Africa from January 2005 to November 2010. Experimental work was carried out while registered at the School of Biological and Conservation Sciences, University of KwaZulu-Natal, Pietermaritzburg, under the supervision of Professor Steven D. Johnson and co-supervision of Professor Denis J. Brothers.

This thesis, submitted for the degree of Doctor of Philosophy in the Faculty of Science and Agriculture, University of KwaZulu-Natal, Pietermaritzburg, represents original work by the author and has not otherwise been submitted in any form for any degree or diploma to any university. Where use has been made of the work of others, it is duly acknowledged in the text.



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Adam Shuttleworth

February 2011

I certify that the above statement is correct.

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Professor Steven D. Johnson (supervisor)

February 2011

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Professor Denis J. Brothers (co-supervisor)

February 2011

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DECLARATION 1 - PLAGIARISM

I, Adam Shuttleworth, declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

    a. Their words have been re-written but the general information attributed to them has been referenced.

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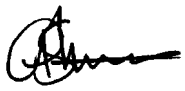

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Adam Shuttleworth

February 2011

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DECLARATION 2 - PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS THAT FORM PART OF AND/OR INCLUDE RESEARCH PRESENTED IN THIS THESIS.

PUBLICATION 1.

SHUTTLEWORTH, A. & JOHNSON, S.D. 2006. Specialized pollination by large spider-hunting wasps and self-incompatibility in the African milkweed *Pachycarpus asperifolius*. *International Journal of Plant Sciences* 167: 1177-1186.

Author contributions:

AS and SDJ conceived paper. AS collected and analyzed data, and wrote the paper. SDJ contributed comments.

PUBLICATION 2.

SHUTTLEWORTH, A. & JOHNSON, S.D. 2008. Bimodal pollination by wasps and beetles in the African milkweed *Xysmalobium undulatum*. *Biotropica* 40: 568-574.

Author contributions:

AS and SDJ conceived paper. SDJ contributed visitor observations and pollinium insertion rates for the Villiers study site. AS collected the remaining data, analyzed the data and wrote the paper. SDJ contributed comments.

PUBLICATION 3.

SHUTTLEWORTH, A. & JOHNSON, S.D. 2009. Palp-faction: an African milkweed dismembers its wasp pollinators. *Environmental Entomology* 38: 741-737.

Author contributions:

AS and SDJ conceived paper. AS collected and analyzed data, and wrote the paper. SDJ contributed comments.

PUBLICATION 4.

SHUTTLEWORTH, A. & JOHNSON, S.D. 2009. A key role for floral scent in a wasp-pollination system in *Eucomis* (Hyacinthaceae). *Annals of Botany* 103: 715-725.

Author contributions:

AS and SDJ conceived paper. AS collected and analyzed data, and wrote the paper. SDJ contributed comments.

PUBLICATION 5.

SHUTTLEWORTH, A. & JOHNSON, S.D. 2009. New records of insect pollinators for South African asclepiads (Apocynaceae: Asclepiadoideae). *South African Journal of Botany* 75: 689-698.

Author contributions:

AS and SDJ conceived paper. AS collected and analyzed data, and wrote the paper. SDJ contributed additional visitor observations and comments.

PUBLICATION 6.

SHUTTLEWORTH, A. & JOHNSON, S.D. 2009. The importance of scent and nectar filters in a specialized wasp-pollination system. *Functional Ecology* 23: 931-940.

Author contributions:

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PUBLICATION 7.

SHUTTLEWORTH, A. & JOHNSON, S.D. 2009. Specialized pollination in the African milkweed *Xysmalobium orbiculare*: a key role for floral scent in the attraction of spider-hunting wasps. *Plant Systematics and Evolution* 280: 37-44.

Author contributions:

AS and SDJ conceived paper. AS collected and analyzed data, and wrote the paper. SDJ contributed comments.

PUBLICATION 8.

SHUTTLEWORTH, A. & JOHNSON, S.D. 2010. The missing stink: sulphur compounds can mediate a shift between fly and wasp pollination systems. *Proceedings of the Royal Society B: Biological Sciences* 277: 2811-2819.

Author contributions:

AS and SDJ conceived paper. AS collected and analyzed data, and wrote the paper. SDJ collected solvent scent samples and additional visitor observations, and contributed comments.

PUBLICATION 9.

SHUTTLEWORTH, A. & JOHNSON, S.D. 2010. Floral scents of chafer-pollinated asclepiads and a potential hybrid. South African Journal of Botany 76: 770-778.

Author contributions:

AS and SDJ conceived paper. AS collected and analyzed data, and wrote the paper. SDJ contributed comments.

PUBLICATION 10.

SHUTTLEWORTH, A. & JOHNSON, S.D. The *Hemipepsis*-wasp pollination system in South Africa: a comparative analysis of trait convergence in a highly specialized plant guild. Unpublished, to be submitted to Botanical Journal of the Linnean Society.

Author contributions:

AS and SDJ conceived paper. AS collected and analyzed data, and wrote the paper. SDJ contributed comments.

PUBLICATION 11.

GLEN, M., NICHOLAS, A., LAMB, J. & SHUTTLEWORTH, A. In Review. A new species of *Pachycarpus* E. Meyer (Apocynaceae: Asclepiadoideae) from southern Africa. Submitted to Novon.

Author contributions:

MG, AN, JL and AS conceived paper. AS collected type specimens. MG collected the remaining data, analyzed the data and wrote the paper. AN, JL and AS contributed comments.

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“The attentions of this insect are paid to several other Asclepiads, such as *Periglossum*, as also to a *Cissus* and a *Eucomis*. These flowers are, most of them, dull-coloured and of very different size, but afford, apparently, a quality of nectar peculiarly pleasing to this wasp; for there were in blossom at the same time Asclepiads quite as conspicuous and more so than *Periglossum*, affording, too, an abundance of nectar, but which I have never seen it visit, although they appeared attractive to some other Hymenoptera.”

James Mansel Weale describing flower visitation by *Hemipepsis* wasps to flowers in the Eastern Cape Province, South Africa.

Taken from:

WEALE, J.P.M. 1873. Observations on the mode in which certain species of Asclepiadeæ are fertilized. *Linnean Journal - Botany* 13: 48-58.

Paper communicated by Charles Darwin on November 3, 1870.

CHAPTER 1

INTRODUCTION

BACKGROUND

Understanding the radiation of flowering plants has been one of the central aims of evolutionary biology. One of the most compelling hypotheses to explain the accelerated diversification of angiosperms is that animal pollinators, particularly insects, have imposed strong selection on floral traits. This idea was first suggested by Darwin (1859, 1862, 1877) and has been supported by more recent studies that have revealed patterns of repeated evolutionary shifts between different pollinators in many angiosperm lineages (Grant 1949; Grant & Grant 1965; Stebbins 1970; Johnson 2006). The diverse floral forms in the angiosperms are thus thought to have originated through a process of pollinator-mediated selection resulting from differences in the morphologies, behaviours and sensory modalities of different types of pollinators (Johnson 2006; Harder & Johnson 2009). This model of pollinator-driven speciation was originally developed by Grant & Grant (1965) and Stebbins (1970). The Grants used evidence from their studies of pollination systems in the phlox family (Polemoniaceae) to suggest that plants are exposed to different types of pollinators throughout their ranges (what they termed the "pollinator-climate") resulting in floral adaptations to the local pollinator fauna. Speciation, in their view, was an extension of this process to a point where specialization to different pollinators was sufficiently advanced to result in reproductive isolation. Building on this concept, Stebbins (1970) suggested five key principles: (1) the most effective pollinator principle, (2) the significance of character syndromes, (3) selection along lines of least resistance, (4) transfer of function via an intermediate stage of double function and, (5) reversals of evolutionary trends. Most aspects of the "Grant-Stebbins model" are well supported by macro- and micro-evolutionary studies (Hodges & Arnold 1994; Schemske & Bradshaw 1999; Bradshaw & Schemske 2003; Ramsey *et al.* 2003; Johnson 2006; Harder & Johnson 2009; Anderson & Johnson 2009).

An important implication of the Grant-Stebbins model of pollinator-mediated speciation is that plants tend to evolve specialization for pollination by particular types of pollinators, although the model can also encompass reversals to generalized pollination (Stebbins 1970; Armbruster & Baldwin 1998). This view is manifest in the development of pollination syndromes – suites of convergent floral traits that are associated with a particular type of pollinator (Faegri & van der Pijl 1979). Despite the widespread utility of pollination syndromes as a framework for studies of pollination ecology, several authors remained sceptical of the existence of universal specialization in plant-pollinator interactions, citing empirical evidence that flowers are often visited by a broad spectrum of visitors (Ollerton 1996; Waser *et al.* 1996). This has resulted in considerable discussion regarding the existence and mechanisms of specialization in pollination systems and provides the backdrop for this thesis.

Southern Africa has over 21 000 species of flowering plants (Germishuizen & Meyer 2003) and contains a diversity of highly specialized plant-pollinator interactions (Johnson & Steiner 2000, 2003; Goldblatt & Manning 2006; Johnson 2010). The region thus represents an ideal place to explore the

ecology of specialized plant-pollinator interactions and floral evolution, in the context of contemporary debates about floral specialization. In this thesis, I examine pollination by *Hemipepsis* spider-hunting wasps (Hymenoptera, Pompilidae, Pepsinae), a newly discovered pollination system within the angiosperms. This system is characterized by high levels of functional floral specialization in morphologically unspecialized, nectar-rewarding flowers, and I use the system to address questions about floral specialization (particularly the mechanisms of achieving specialization in the absence of morphological filters), floral evolution and pollination syndromes. To place these studies in context, it is necessary to review the development of the theory of floral syndromes from early studies of plant-pollinator interactions through to the contemporary debates that have surrounded the idea of specialized pollination systems in angiosperms.

POLLINATION SYNDROMES

Studies of interactions between plants and their pollinators have a long history and can be traced to the pre-Darwin studies of, amongst others, Joseph Gottlieb Kölreuter and Christian Konrad Sprengel. Kölreuter (1761) was the first to fully appreciate that plants often require the services of insects as pollen vectors in order to set seed. Building on the observations of Kölreuter, Sprengel's (1793) "*Das entdeckte Geheimnifs der Natur im Bau und in der Befruchtung der Blumen* (*The Secret of Nature in the Form and Fertilization of Flowers discovered)*" (published in German) marked the beginning of detailed studies of the interactions between flowers and their insect pollinators. In this book, Sprengel summarized the structural adaptations of hundreds of flowers and interpreted these as evidence of intentional design for insect pollination (Lloyd & Barrett 1996; Waser 2006). Charles Darwin was the first to suggest that flowers were shaped through natural selection imposed by their pollinators (Darwin 1859, 1862, 1877). Darwin's studies marked a change in the interpretation of floral forms and provided a new evolutionary framework for future studies.

Early pollination studies tended to be encyclopedic descriptions of floral morphologies and floral visitors with little attempt to explain the observed variety of floral types (Sprengel 1793; Müller 1883; Knuth 1898). Although Darwin introduced a new means to interpret the diversity of floral forms, the sheer volume of information about visitors to different plants made it difficult to identify trends and obtain a meaningful adaptive analysis of plant-pollinator interactions. The theory of pollination syndromes was originally developed, in this context, as a means to classify and make sense of the bewildering diversity of floral forms and to summarize the interactions between different flowers and their animal pollinators.

The classification of floral forms on the basis of a plant's mode of pollination was first suggested by the Italian botanist Federico Delpino in the late 1800s. Delpino (1868-1875) suggested two schemes for the classification of flowers, based on either the convergent morphological traits that represented the adaptations of flowers for visits by particular animals or purely on the agent of pollination (Waser 2006). Delpino's schemes had several shortcomings but represented a useful

starting point for subsequent workers. Building on the suggestions of Delpino, the German botanist Stefan Vogel (1954) used his studies of southern African pollination systems to develop a description of six floral "styles" in his classic "*Blütenbiologische Typen als Elemente der Sippengliederung: dargestellt Anhand der Flora Südafrikas (Floral biological types as elements for classification, demonstrated by the flora of South Africa)*" (published in German). Vogel's "styles" incorporated details of the floral reward, morphology, colour and scent that could be related to particular types of pollinators (summarized on pages 38-39 of Vogel (1954), see Fig. 1.2 in Waser (2006) for translation into English) and represent the basis for the development of traditional pollination syndromes: suites of floral traits (including aspects of morphology, colour, odour and reward) that are associated with different types of pollinators (Faegri & van der Pijl 1979; Proctor *et al*. 1996).

A pervasive feature of early methods of classification was the impression of a natural order or harmony between flowers and their pollinators (Sprengel 1793). Vogel's (1954) "floral styles" contain hints of the Victorian era belief in this fundamental nature or essence of plant-pollinator interactions. Indeed, hints of this are even suggested by Faegri & van der Pijl's (1979) "harmonic relations between pollinators and blossoms" (p. 97) in their development of the idea that particular floral traits can be associated with each pollinator type (Waser 2006). However, the contemporary development of pollination syndromes represents an intuitive adaptive interpretation of the diversity of floral forms, and floral syndromes have been widely used as a framework for pollination studies throughout the modern era (Proctor *et al*. 1996). Despite widespread use, however, the traditional concept of pollination syndromes has recently been criticized (Ollerton 1996; Herrera 1996; Waser *et al*. 1996), resulting in rigorous discussions of floral specialization in the literature (Johnson & Steiner 2000; Fenster *et al*. 2004). In this thesis, I have adopted the view that floral syndromes are simply patterns of convergent evolution that can be tested through the appropriate use of comparative biological methods.

SPECIALIZATION IN POLLINATION SYSTEMS

Floral specialization is implicit in both pollination syndrome theory and the prevailing Grant-Stebbins model of pollinator-driven speciation (Grant & Grant 1965; Stebbins 1970; Johnson 2006). However, empirical evidence does not always support a pattern of widespread specialization and many pollination systems are considerably more generalized (in terms of the spectrum of visitors) than would be expected if floral traits are shaped primarily through consistent selection by particular pollinators (Ollerton 1996; Waser *et al*. 1996). This contradiction between apparent evolutionary specialization in floral forms contrasted with diverse floral visitors became known as "Ollerton's Paradox" and has precipitated much discussion in the pollination biology literature.

In an influential paper, Waser *et al*. (1996) questioned the assumption that plants tend towards specialization in their interactions with pollinators. They pointed out that many plant-pollinator interactions are ecologically generalized (i.e. plants are visited by multiple species of potential

pollinators and pollinators, in turn, visit multiple plant species) and that generalization is predictable from a simple model which includes temporal variation in pollinator numbers and identity – patterns which had been identified in several studies (Herrera 1988, 1996; Horvitz & Schemske 1990; Fishbein & Venable 1996). The suggestion that generalization, rather than specialization, may be the rule in pollination systems was difficult to reconcile with the idea of pollinator-mediated speciation and the well established existence of suites of convergent floral traits (syndromes) traditionally associated with different types of pollinators (Faegri & van der Pijl 1979). Ollerton (1996) offered several possible explanations for this paradox, including recognizing that floral visitors are not equal in their pollinating abilities (relating back to Stebbins' (1970) "most effective pollinator" principle); considering the relative importance of micro- versus macro-evolutionary processes in promoting floral diversification; examining the possibility that contemporary floral traits may represent periods of pollinator specialization in the past or at the edge of a species range; or, finally, considering that processes such as hybridization and ploidy changes may be more important sources of novel floral traits than mutation and pollinator-mediated selection. The study by Waser *et al*. (1996) sparked renewed interest in the levels of specialization in plant pollination systems, and a number of workers began to critically examine the applicability of pollination syndromes as a conceptual framework for floral evolution.

Johnson & Steiner (2000, 2003) examined the levels of floral specialization in southern African pollination systems. They took issue with the fact that Waser *et al*.'s (1996) conclusions were based on studies conducted in cool temperate northern hemisphere ecosystems which may not be representative of ecosystems in other geographical regions. By comparing levels of specialization in southern African Orchidaceae and Iridaceae to European and North American Orchidaceae and Polemoniaceae, Johnson & Steiner (2000, 2003) revealed that southern African pollination systems often *are* remarkably specialized – a strikingly different pattern to that suggested by Waser *et al*. (1996) for temperate northern hemisphere ecosystems. A similar study by Ollerton *et al*. (2006) confirmed the trend, and illustrated higher levels of specialization in southern African members of the Apocynaceae compared to temperate northern hemisphere members of the same family. In a review of the pollination systems of southern African Iridaceae, Goldblatt & Manning (2006) concluded that 95% of the region's Iridaceae have highly specialized pollination systems. These studies provided strong support for floral syndrome theory and the existence of pollinator-mediated speciation, and suggested that Ollerton's (1996) Paradox may not hold for much of the southern African flora. Further supporting evidence was provided by studies in which authors used established syndromes or locally convergent floral traits to predict pollinators and then confirmed these with observations and experiments (Johnson *et al*. 2001; Hargreaves *et al*. 2004; Pauw 2006; Wolfe & Sowell 2006; Kleizen *et al*. 2008) and studies which tested the functional significance of convergent traits to confirm that they result from pollinator-mediated selection (Johnson & Bond 1994; Johnson & Midgley 1997; Van Kleunen *et al*. 2007).

However, support for syndromes was not universal. Using multivariate analyses to examine the match between pollinators and floral traits, Wilson *et al*. (2004) found that penstemons conform well to the broad categories of hummingbird versus hymenopteran pollination syndromes, but found that syndromes were less effective at predicting the differences between plants pollinated by different types of bees. Similarly, Hingston & McQuillan (2000) found that although floral visitor profiles in Tasmanian sclerophyllous communities were sometimes consistent with classic pollination syndromes, syndromes were mostly ineffective at predicting floral visitors. More recently, Ollerton *et al*. (2009) examined binary floral traits and floral visitors for communities from three continents and found that very few plant species fall within the classical syndrome clusters (based on qualitative floral traits described in Faegri & van der Pijl 1979 and Proctor *et al*. 1996) in multivariate phenotype space. They did, however, find partial support for the predictive utility of classic syndromes as the pollinators for about 30% of the plant species they studied could be predicted from their proximity to a particular syndrome in phenotype space.

An important development to emerge from this debate was the realization that specialization needs to be examined in terms of functional pollinator types rather than individual species (Johnson & Steiner 2000, 2003; Fenster *et al*. 2004). A “functional group” of pollinators is a group of species which exhibit similar morphology (and physiology) and behaviour, and consequently exert similar selection pressures. For example, a plant which is pollinated by 10 similar species of hawkmoths might traditionally be considered a generalist but is better thought of as functionally specialized for pollination by hawkmoths (Johnson & Steiner 2000; Fenster *et al*. 2004). Using this approach, Fenster *et al*. (2004) re-examined some of the data analyzed by Waser *et al*. (1996) and showed that most plant species were, indeed, specialized for pollination by a particular functional group. Studies of pollinator-mediated selection therefore need to group pollinator species into functionally similar types before trying to identify adaptive floral traits.

Southern African studies have consistently revealed high levels of floral specialization and provide support for the predictive utility of pollination syndromes. Several explanations for the high levels of specialization in southern African pollination systems have been suggested. These include high levels of phenotypic specialization (e.g. long spurs or oil rewards) which results in rewards which can only be accessed or utilized by a subset of the pollinator community (Johnson & Steiner 2003); and a pollinator fauna which is relatively depauperate at the species level but contains diverse functional types (Johnson & Steiner 2003). It has also been noted that generalist pollination systems appear to be associated with the post-glacial landscapes of Europe and the eastern and northern parts of North America (Johnson & Steiner 2003; Ollerton *et al*. 2006). This suggests that generalization may confer an ecological advantage for colonists of these post-glacial landscapes and that there has been insufficient time for specialized interactions to develop in these regions.

High levels of specialization may also relate to increased pollen limitation in areas of high species richness (Vamosi *et al*. 2006; Armbruster & Muchhala 2009). Recent studies have suggested

that pollen limitation increases with number of plant species (relating to increased competition for pollinators; Knight *et al*. 2005; Vamosi *et al*. 2006; Alonso *et al*. 2010). This would result in strong selection for mechanisms, such as pollinator specialization and phenological diversification, which increase the amount and quality of pollen transfer. Southern Africa is a floral biodiversity hotspot (Germishuizen & Meyer 2003; Raimondo *et al*. 2009) and pollen limitation may partly explain the trend towards floral specialization in this region. However, it is difficult to confirm whether pollen limitation is a cause rather than an effect of increased specialization. The pollen limitation theory could also explain higher levels of specialization in tropical regions compared to higher latitudes (Oleson & Jordano 2002; Armbruster 2006), although this trend remains uncertain (Ollerton & Cranmer 2002; see discussion of these studies in Ollerton *et al*. 2006). Understanding the differences in levels of specialization around the world ultimately requires more research in diverse but understudied regions such as South America and western Australia.

The proximal mechanisms of specialization in pollination systems are also not always clearly understood. Johnson & Steiner (2000, 2003) suggested that specialization can be achieved through particular floral filters (such as long spurs or specific rewards) or through a "private channel" of communication (such as a specific scent compound) which selectively attracts some insects but not others. Although filters are obvious in many systems (such as flowers with specific rewards or long spurs), specialization is more difficult to explain in morphologically unspecialized flowers which produce exposed nectar as a reward. Specialization in these flowers can possibly be explained by a private channel of communication in combination with cryptic colouring and differentially palatable nectar (Johnson *et al*. 2006; Chapter 7). However, the proximal mechanisms of achieving specialization remain poorly studied for many plant species.

POLLINATION GUILDS

The concept of a pollination guild is an extension of floral syndrome theory and refers to a group of plants, irrespective of taxonomy, which are ecologically reliant on a common pollinator (Manning & Goldblatt 1996). Pollination guilds represent the smallest unit of a pollination system and are the most relevant level at which to consider pollinator-mediated selection on floral traits. The southern African flora hosts a remarkable diversity of different pollination systems and Johnson (2010) recently summarized 24 specialized pollination guilds now known from the region. Many of these guilds comprise whole suites of plants that are reliant on a single pollinator species or type and these extreme levels of floral specialization are typically associated with remarkable convergence in the floral traits of the often unrelated guild members. These pollination guilds thus represent a fine scale test of pollination syndrome theory (Faegri & van der Pijl 1979; Manning and Goldblatt 1996; Fenster *et al*. 2004; Johnson 2010).

The large number of specialized pollination guilds in southern Africa and the identifiable patterns of convergent floral traits associated with the pollinators operating these guilds suggest that

studies of pollinator-mediated convergence (the central idea behind pollination syndromes) could be conducted at the guild level. This avoids making generalizations about the similarity of selection pressures exerted by potentially distantly related pollinators that might traditionally be included under a common functional group. In other words, the main problem with classical pollination syndromes may be the scale at which they are applied, rather than the concept itself.

Ollerton *et al*.'s (2009) multivariate analysis of pollination syndromes was based on floral traits established for classical pollination syndromes (Faegri & van der Pijl 1979; Proctor *et al*. 1996). As a result, traits associated with, for example, wasp-pollination were based primarily on observations of mostly generalist flowers visited by a variety of different wasps (mainly vespid and spheciform wasps; Faegri & van der Pijl 1979; Proctor *et al*. 1996). The wasp-pollinated flowers from eastern South Africa included in Ollerton *et al*.'s (2009) analysis, however, are highly specialized and are pollinated exclusively by pompilid wasps, but are considered under the broad category of "wasp-pollinated". In reality, different types of wasps represent functionally divergent pollinators. For example, pompilids typically have long legs and hairier bodies than vespids, and fall in separate clades within the aculeate Hymenoptera (Grimaldi & Engel 2005). While classical pollination syndromes would include both pompilid and vespid pollinated flowers under the broad functional type of "wasp", these two types of wasp would exert divergent selection pressures (relating to both their morphology and physiology) on the flowers that they pollinate (Fenster *et al*. 2004). Similarly, orders such as Diptera with pollinators as divergent as long-tongued nemestrinid or tabanid flies and carrion-seeking calliphorid or sarcophagid flies were collectively considered under the broad functional type of "fly" in Ollerton *et al*.'s (2009) analysis. Indeed, the pollinator types used in Ollerton *et al*.'s (2009) analysis (bee, beetle, bird, butterfly, fly, moth and wasp) can all, with the possible exception of "butterfly", be divided into two or more functionally divergent pollinator types which are unlikely to exert similar selection pressures.

While Ollerton *et al*.'s (2009) study achieves its aim of testing the validity of classical pollination syndromes, it also highlights the need to realistically consider which groups of pollinators are functionally similar before searching for pollinator-mediated convergence. It is thus more meaningful to examine the individual guilds within an overall classical pollination syndrome in order to detect patterns of convergence and pollinator mediated selection.

INCORPORATING OBJECTIVE MEASURES OF SCENT AND COLOUR INTO FLORAL SYNDROMES

Classical floral syndromes are based on qualitative descriptions of floral traits from a human perspective. However, a modern analysis of floral syndromes needs to incorporate objective assessments of traits such as colour and scent (both important traits for pollinator attraction) which are perceived very differently by humans and different types of pollinators. Colour vision is a function of both an animal's spectral receptors and their sensitivities as well as the neural opponency mechanism with which spectral stimuli are interpreted in the brain (Chittka 1992; Kelber *et al*. 2003; Chittka &

Raine 2006; Osorio & Vorobyev 2008). An objective comparison of floral colours from a pollinator perspective thus requires detailed sensitivity curves for spectral receptors as well as usable models of colour opponency for different animals. The development of the Chittka colour hexagon as a model of bee colour vision has provided an objective means to compare the colours of bee (and some other Hymenoptera) pollinated flowers (Chittka 1992; Chittka *et al*. 1992, 1994). Although the receptors and receptor sensitivities of other pollinator groups, such as flies, butterflies and birds, are relatively well studied, we still do not have useful models of opponency mechanisms for many animal pollinators (with the possible exception of carrion flies, see Troje 1993; Chittka & Menzel 1992; Kelber 1999; Osorio & Vorobyev 2008; Arnold *et al*. 2009; see reviews by Kelber *et al*. 2003 and Kelber & Osorio 2010). Nonetheless, the effects of changes in floral colours on pollinators have received much attention and colour mutations can clearly have profound effects on visitation rates to flowers (Bradshaw & Schemske 2003; Irwin & Strauss 2005; Hoballah *et al*. 2007; Cooley *et al*. 2008; Thomson & Wilson 2008). This suggests that floral colours should, depending on the degree to which they are phylogenetically constrained, be adapted to different pollinator types (Chittka & Menzel 1992).

In a first attempt to objectively test whether floral colours conform to syndromes, Lars Chittka examined the colours of 154 flower species in a German community using the Chittka colour hexagon (study published in Waser *et al*. 1996). He found that flowers do form clusters in bee colour space, but that these did not necessarily correspond to different pollinator types. It could be argued that the hexagon does not reflect colour perception by insects other than bees and trends relating to other pollinator types may therefore not be clear from this analysis. However, all clusters of flowers also contained some species that were visited by bees, although this could relate back to the dominance of "bee" functional types in the generalist pollination systems apparent from cool temperate northern hemisphere ecosystems (Waser *et al*. 1996; Johnson & Steiner 2003). An earlier study by Chittka & Menzel (1992) showed a good correlation between the reflectance spectra of 180 Israeli flowers and the spectral sensitivities of Hymenopteran pollinators, but again was hampered by the lack of knowledge of the neural processes underlying colour perception in other insect pollinators. Similar studies in species rich areas with high levels of floral specialization, such as southern Africa, may well reveal colour distributions that do correlate to particular pollinator types. A truly objective assessment of floral colour ultimately depends on how well we understand colour perception in different types of animal pollinators and will require the development of usable models of colour opponency for a wider array of animal pollinators.

Scent is a functional pollinator attractant in many systems (Raguso 2001, 2006; Dudareva & Pichersky 2006) but also remains to be objectively examined in the context of floral syndromes. Classical syndrome literature included descriptions of scent from a very basic human perspective (e.g. "yeasty" scent in rodent-pollinated flowers, "musty" scent in bat-pollinated flowers and "sweet" scent in moth-pollinated flowers; Faegri & van der Pijl 1979). However, these descriptions are not adequate

to describe the complexity and diversity of volatiles produced by flowers and advances in analytical techniques such as coupled gas chromatography-mass spectrometry (GC-MS) have now allowed pollination biologists to obtain a far greater understanding of floral fragrance (Knudsen *et al.* 2006). In a first attempt to include this wealth of new information into floral syndromes, Knudsen and colleagues (Knudsen & Tolsten 1993, 1995; Knudsen *et al*. 2004) summarized the volatiles collected from flowers involved in different pollination systems and identified broad trends in the composition of floral scents associated with different pollinators. For example, the scents of moth-pollinated flowers are often dominated by sweet-smelling benzenoid compounds (such as benzaldehyde, phenylacetaldehyde and benzyl alcohol) with small amounts of nitrogen-containing compounds such as indole (Knudsen & Tollsten 1993). A more strikingly convergent example is the production of oligosulphides by bat-pollinated species (Knudsen & Tollsten 1995; Bestman *et al*. 1997), although oligosulphides are now also known to be characteristic of carrion-fly pollinated flowers (Stensmyr 2002; Jürgens *et al*. 2006; Johnson & Jürgens 2010; see Chapter 9). Although these studies have provided a useful starting point, they are hampered by the large number of volatiles produced by flowers and the lack of outgroup comparisons to give some indication of convergence in the overall scent bouquets associated with different pollinators.

The difficulty with relating odour bouquets to particular pollinators is the diversity of volatiles that are produced by different species. A first requirement for many studies has thus been to try and identify physiologically active compounds using methods such as coupled gas chromatography-electroantennographic detection (GC-EAD) and electroantennography (EAG). These methods allow for the identification of compounds that elicit a physiological response from an insect antenna and can be used to exclude compounds which a particular insect cannot detect (Schiestl & Marion-Poll 2002). In combination with field bioassays, these methods have been used very effectively to identify compounds or blends of compounds that are attractive to particular pollinators (Schiestl *et al*. 1999, 2003; Ayasse *et al*. 2000; Schiestl & Ayasse 2002; Andersson & Dobson 2003; Dötterl *et al*. 2005). It is, however, still difficult to incorporate these analyses into a syndrome concept since they do not illustrate convergence unless specific physiologically active compounds are found across multiple species with a common pollinator.

An alternative method is to use multivariate analyses to examine the scents of plants within a particular guild alongside the scents of related plants outside the guild (outgroups) to identify levels of convergence in overall scent properties. This approach was used by Johnson & Jürgens (2010) to examine the scents of sapromyiophilous flowers and a stinkhorn fungus. These flowers (and the fungus) mimic carrion and faecal scents to attract pollinators or spore dispersers, and analysis with non-metric multidimensional scaling (NMDS) revealed clear convergence in the scents of the angiosperm flowers and the fungus in relation to different models and to the outgroup species (Johnson & Jürgens 2010). A similar study by Jürgens *et al*. (2006) showed clusters of scent profiles from various stapeliads with foetid odours in two dimensional scent space. This study demonstrated

convergence of scent profiles towards different models, but did not include non-sapromyiophilous outgroups to allow for an assessment of convergence towards an overall sapromyiophilous syndrome. Although these studies showed very clear convergent trends in floral scent properties, these may result from the mimicry of pollinator food and brood site odours in the sapromyiophilous syndrome. Similar studies comparing the scents of flowers within particular guilds to the scents of related non-guild member flowers would go a long way towards assessing levels of pollinator-mediated convergence in floral scents.

A further difficulty with incorporating floral scent into syndromes is that the volatiles for many plant species have not been analyzed in detail and the role of scent in many systems remains poorly examined. For example, the paucity of studies examining the role of floral scents in bee-flower interactions was recently highlighted (Dötterl & Vereecken 2010). However, bee pollination systems are some of the most intensely studied (reviewed by Dötterl & Vereecken 2010) and the visual systems of bees have received tremendous attention (Chittka 1992; Chittka *et al*. 1997; Dyer & Chittka 2004a,d,c,d; Dyer *et al*. 2008a,b). Floral scent has only become a quantifiable floral trait relatively recently and the traditional bias towards other floral traits probably stems largely from the difficulties of analyzing volatiles (Raguso 2008). In order to critically examine the levels of convergence in floral scent, future studies need to combine multivariate analyses such as NMDS with community (and guild) level studies of scent in relation to pollination systems.

WASP POLLINATION

> "… most of the 'wasps' are … unreliable and unsteady pollinators [and] the instinctive apparatus to build up a systematic utilization of one or very few suitable blossoms is not particularly well developed in these animals."
> Faegri & van der Pijl (1979, p. 107)

Aside from the pollination of deceptive orchids and the obligate brood-site pollination mutualisms between small chalcidoid wasps and figs (Moraceae), wasps have not traditionally been considered important pollinators. Faegri & van der Pijl (1979) were clearly uncertain of the value of wasps as pollinators. They did, however, acknowledge that wasps, mostly in the families Vespidae and Sphecidae (the latter now divided into several families within the Apoidea; Grimaldi & Engel 2005) were common visitors to some plants. In a similar vein, Proctor *et al*. (1996) do not highlight wasps as consistent pollinators although they do list several plants that are frequently visited by wasps and suggest the possibility that some of these may be adapted to pollination primarily by vespids, but include the caveat that these are also frequently visited by honeybees which may well effect pollination. Despite the general impression that wasps are usually visitors only to generalist plants,

both Faegri & van der Pijl (1979) and Proctor *et al*. (1996) identify some floral traits that are associated with flowers visited by wasps (summarized by Ollerton & Watts 2000). These include open or bell-shaped zygomorphic flowers sometimes with a short but wide tube, strong sour scent, drab brown or purplish colouring and moderate to small amounts of sucrose dominant nectar. Nonetheless, wasp pollination in rewarding plants has usually been associated with highly generalized pollination systems where wasps are part of a broad spectrum of floral visitors.

Published studies of wasp-pollination are not abundant in the literature (fig and fig-wasp systems excluded), although the highly specialized associations between sexually deceptive orchids and their wasp pollinators have received considerable attention recently (Wong & Schiestl 2002; Ayasse *et al*. 2003; Schiestl *et al*. 2003; Schiestl 2004, 2005; Mant *et al*. 2005; Schiestl & Peakall 2005; Ciotek *et al*. 2006; Hopper & Brown 2006, 2007; Gaskett *et al.* 2008; Gaskett & Herberstein 2010). In South Africa, Steiner *et al*. (1994) described sexual deception of a sphecid and pompilid wasp by two species of *Disa* orchids. Vespid and sphecid wasps are also involved in various food-mimicry deceptive orchid systems (Nilsson *et al*. 1986; Nazarov 1995). Aside from these deceptive systems, wasps have also been occasionally recorded as part of the pollinator fauna of plants with generalized insect pollination systems. These include the orchid *Listera ovata* which is sometimes pollinated by ichneumonid wasps (Nilsson 1981); and several species of North American *Asclepias* and *Gonolobus*, and South American *Oxypetalum* (Apocynaceae: Asclepiadoideae) which are occasionally pollinated by vespid and sphecid wasps (Vieria & Shepherd 1999; Kephart 1983; Kunze 1999; Kephart & Theiss 2004). Locally, pompilid wasps have been recorded as pollinators of the generalist *Xysmalobium gerrardii* (Apocynaceae: Asclepiadoideae; Ollerton *et al*. 2003) and have been observed visiting several other species with generalized pollination systems, including *Cyphostemma cirrhosum* and *C. natalitium* (Vitaceae), *Cissus* spp. (Vitaceae), *Sium repandrum* (Apiaceae), *Heteromorpha arborescens* var. *abyssinica* (Apiaceae) and *Peucedanum capense* (Apiaceae) (Weale 1873; A. Shuttleworth pers. obs; P. Wragg pers. comm.). In addition, long-tongued masarine wasps (Vespidae: Masarinae) are common floral visitors throughout the more arid regions of southern Africa, although the levels of specialization in these systems have not been assessed from the plants' perspective (Gess & Gess 1989, 2003, 2004; but see Goldblatt *et al*. 2009). Despite the paucity of published studies, however, most workers recognize that wasps commonly forage for nectar and occasionally pollinate generalist flowers throughout the temperate regions of the world (Gess & Gess 1989, 2003, 2004; Proctor *et al*. 1996).

Specialized wasp-pollination systems in rewarding plants, however, have seldom been reported. European *Epipactis* orchids are pollinated exclusively by vespid wasps (Vespidae; Judd 1971, 1979; Ehlers *et al*. 2002) and pollinator attraction in this system has been shown to be based on the production of green leaf volatiles by the orchids (Brodmann *et al*. 2008). Specialized vespid pollination systems have also been reported for some asclepiads (Apocynaceae: Asclepiadoideae), including four South American *Oxypetalum* species (Vieria & Shepherd 1999) and the South African

Gomphocarpus physocarpus (Coombs *et al*. 2009). In the Iridaceae, two South African species of *Ferraria* have recently been found to be pollinated exclusively by vespid (eumenine and masarine) wasps (Goldblatt *et al*. 2009). Recent studies have also revealed that *Hedera helix* (common ivy; Araliaceae) and *Croton suberosus* (Euphorbiaceae) are vespid wasp specialists (Jacobs *et al*. 2010 but see Vezza *et al*. 2006; Narbona & Dirzo 2010). These species are both visited by an array of different insects, but appear to be pollinated primarily by vespid wasps and are thus functionally specialized (Johnson & Steiner 2000; Fenster *et al*. 2004) but ecologically generalized (Ollerton *et al*. 2007). Until recently, specialized pollination systems operated by spider-hunting wasps (Pompilidae) were completely unknown in the angiosperms.

THE STUDY SYSTEM AND AIMS

This thesis examines the ecology and evolution of a guild of South African grassland plants pollinated exclusively by spider-hunting wasps in the genus *Hemipepsis* (Hymenoptera: Pompilidae: Pepsinae; Figs 1-17, see Fig. 18 for images of asclepiad pollinaria attached to wasps and Fig. 19 for map of field sites used in this study). *Hemipepsis* species represent some of the largest wasps in southern Africa and are reported to prey on rain spiders (Sparassidae: *Palystes*) or baboon spiders (Theraphosidae: *Harpactira*) (Skaife 1953; Brothers 1985). Further details of the biology of *Hemipepsis* wasps remain unknown, although pompilid wasps, in general, use a single spider for the development of each wasp larva (Evans 1953) and the adults of some species appear to feed chiefly on floral nectar.

Flower visitation by *Hemipepsis* wasps in South Africa was first noted by James Mansel Weale in the Eastern Cape grasslands around Bedford, Koonap and Port Elizabeth. Weale (1873) described extensive visitation by "a large black and yellow wasp … *Pallosoma*, one of the Pepsidae" (the genus *Pallosoma* has been synonymized with *Hemipepsis*) to several asclepiad species as well as to *Eucomis* (probably *E. autumnalis* subsp. *clavata*, see Fig. 15) and a *Cissus* (Vitaceae). Weale provided detailed descriptions of the behaviour of these wasps on *Woodia mucronata* (then called *Xysmalobium linguaeforme*) and *Periglossum* (probably *P. angustifolium*, see Fig. 10) inflorescences. He also noted the presence of numerous *Woodia* pollinaria attached to the tarsi and sternal hairs of wasps, but concluded that these were incidental and the elaborately shaped corona lobes could only be explained if pollination was effected by pollinia attached to the wasps' mouthparts – a view which is most likely correct for this species (c.f. Fig. 18; see Chapter 4). Although Weale's detailed observations suggested specialized pollination and some degree of adaptation by the asclepiad flowers, they were not noticed by subsequent workers. Importantly, Vogel (1954) in developing his "floral styles" based on pollination systems in the South African flora did not include any mention of pollination by pompilid wasps and failed to cite Weale (1873).

Specialized pollination systems operated by spider-hunting wasps thus remained obscure and essentially unknown in the angiosperms until the description of a sexually deceptive pollination system operated by the pompilid wasps *Hemipepsis hilaris* and *H. capensis* in the Cape orchid *Disa*

bivalvata (Fig. 17; Steiner *et al*. 1994). Specialized pollination by *Hemipepsis* wasps in rewarding plants was only recently re-discovered when a study by Ollerton *et al*. (2003) in the KwaZulu-Natal midlands revealed that three asclepiads, *Miraglossum pilosum*, *M. verticillare* and *Pachycarpus natalensis*, appear to be pollinated exclusively by *H. hilaris* and a few congeneric wasp species (see Figs 3, 4 & 9). This was followed by descriptions of specialized pollination systems operated by these wasps in the rewarding Drakensberg orchids *Disa sankeyi* (Johnson 2005) and *Satyrium microrrhynchum* (the latter also pollinated partly by cetoniine beetles; Fig. 17; Johnson *et al*. 2007). Specialized pollination by pompilid wasps is thus a recently discovered pollination system within the angiosperms and is currently unique to South Africa.

When I embarked on this research, specialized pollination systems operated by *Hemipepsis* wasps had been suggested by Weale's (1873) observations but were confirmed only in the three asclepiads described by Ollerton *et al*. (2003) and the sexually deceptive *D. bivalvata* (Steiner *et al*. 1994; Figs 3, 4, 9 & 17). However, reports of *Hemipepsis* wasps visiting *Pachycarpus grandiflorus* (Fig. 8) and *E. comosa* var. *striata* (Fig. 16) by Field (2002) combined with anecdotal observations by Steve Johnson and myself of wasps visiting *Pa. asperifolius*, *Pa. appendiculatus*, *E. autumnalis* subsp. *clavata* and two rewarding orchids (Figs 5, 6, 15 & 17) led me to hypothesize that there were many more *Hemipepsis*-pollinated species to be discovered. Some obvious convergent traits between the limited plant species that were known to be wasp-pollinated (dull colouring with purple blotches and exposed nectar in the asclepiads) and the highly specialized nature of their interactions with the wasps also led me to hypothesize that there would be patterns of convergent traits in these wasp-pollinated plants, as had been described for other South African pollination guilds (Johnson & Bond 1994; Manning & Goldblatt 1996, 1997; Goldblatt & Manning 2000; reviewed by Johnson 2010). Finally, the absence of any morphological adaptations to limit access to floral rewards (the deceptive *D. bivalvata* aside) led me to hypothesize that plants must be relying on cryptic colouring and biochemical (i.e. scent and nectar) adaptations to achieve specialization.

The aims of my research, broadly stated, were thus (i) to investigate the extent of specialized pollination by *Hemipepsis* pompilid wasps in South African grassland plants (Chapters 2-8), (ii) to investigate the means by which plants achieve this specialization (Chapters 5-8), (iii) to investigate floral evolution in relation to pollinator shifts (Chapter 9), and, (iv) to use comparative analyses with congeneric non-wasp-pollinated species to look for patterns of convergent floral traits (including scent) that can be associated with pollination by *Hemipepsis* wasps (Chapter 10). As with any ecological research, additional questions regarding the fine-scale ecology and evolution of particular systems emerged as different plant species were examined, and the specific hypotheses and aims for these individual studies are given in the relevant chapters. Two studies which stemmed from data collected during this thesis are included as Appendices as they are not directly related to the aims of the thesis. Appendix 1 represents scent data for chafer-pollinated asclepiads which were collected for the purposes of the congeneric comparisons presented in Chapter 10. Appendix 2 represents the

description of a previously undescribed species of *Pachycarpus* that I discovered during the collection of data on the pollination systems and floral traits of congeneric species used for the comparative analyses presented in Chapter 10.

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FIGURE 1. *Asclepias macropus* (Apocynaceae), Wahroonga Farm. Inset, close up of flower (left) and female *Hemipepsis capensis* visiting flowers (right). Flower diameter = 8 mm.

FIGURE 2. *Aspidoglossum glanduliferum* (Apocynaceae), Wahroonga Farm. Inset, close up of flower. Flower diameter = 10 mm.

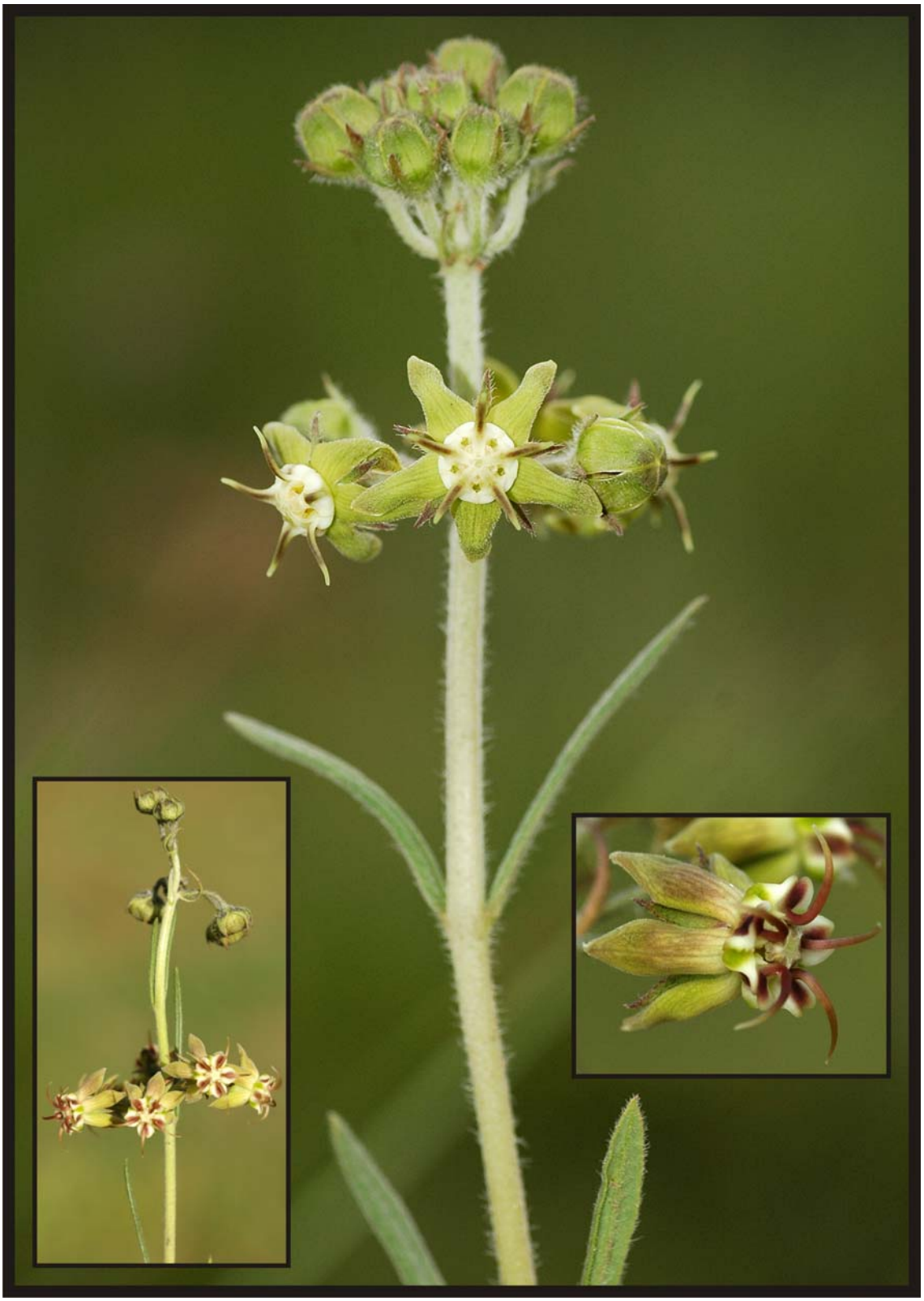

FIGURE 3. Two *Hemipepsis*-wasp pollinated *Miraglossum* species (Apocynaceae). *Miraglossum pilosum* (main spread), roadside near Gilboa Estate, flower diameter = 10 mm; and *M. pulchellum* (insets), Garden Castle, flower diameter = 12 mm.

FIGURE 4. *Miraglossum verticillare* (Apocynaceae), Wahroonga Farm. Inset, close up of flower. Flower diameter = 6 mm.

FIGURE 5. *Pachycarpus appendiculatus* (Apocynaceae), Sinangwana. Inset, close up of flower (top) and, male (left) and female (right) *Hemipepsis dedjas* visiting flowers. Flower diameter = 25 mm.

FIGURE 6. *Pachycarpus asperifolius* (Apocynaceae), Vernon Crookes Nature Reserve. Inset (all), female *Hemipepsis capensis* visiting flowers. Flower diameter = 20 mm.

FIGURE 7. *Pachycarpus campanulatus* var. *campanulatus* (Apocynaceae), Wahroonga Farm. Inset, close up of flower (top) and male *Hemipepsis capensis* visiting flowers (bottom). Flower diameter = 45 mm.

FIGURE 8. *Pachycarpus grandiflorus* subsp. *grandiflorus* (Apocynaceae), Gilboa Estate. Whole plant (top) and male *Hemipepsis hilaris* approaching flowers (bottom). Flower diameter = 45 mm.

FIGURE 9. *Pachycarpus natalensis* (Apocynaceae), Wahroonga Farm. Inset (both), female *Hemipepsis capensis* visiting flowers. Flower diameter = 40 mm.

FIGURE 10. *Periglossum angustifolium* (Apocynaceae), Midmar Nature Reserve. Inset, close up of flower. Flower diameter = 12 mm.

FIGURE 11. *Woodia verruculosa* (Apocynaceae), Midmar Nature Reserve. Inset (both), close ups of flowers. Flower diameter = 12 mm.

FIGURE 12. *Xysmalobium orbiculare* (Apocynaceae), Wodwo Farm and Wahroonga Farm (insets). Inset, close up of flower (top left), inflorescence (bottom) and female *Hemipepsis capensis* visiting flowers (top right). Flower diameter = 8 mm.

FIGURE 13. *Xysmalobium stockenstromense* (Apocynaceae), Royal Natal National Park, near Witsieshoek Resort. Inset, close up of flower. Flower diameter = 16 mm.

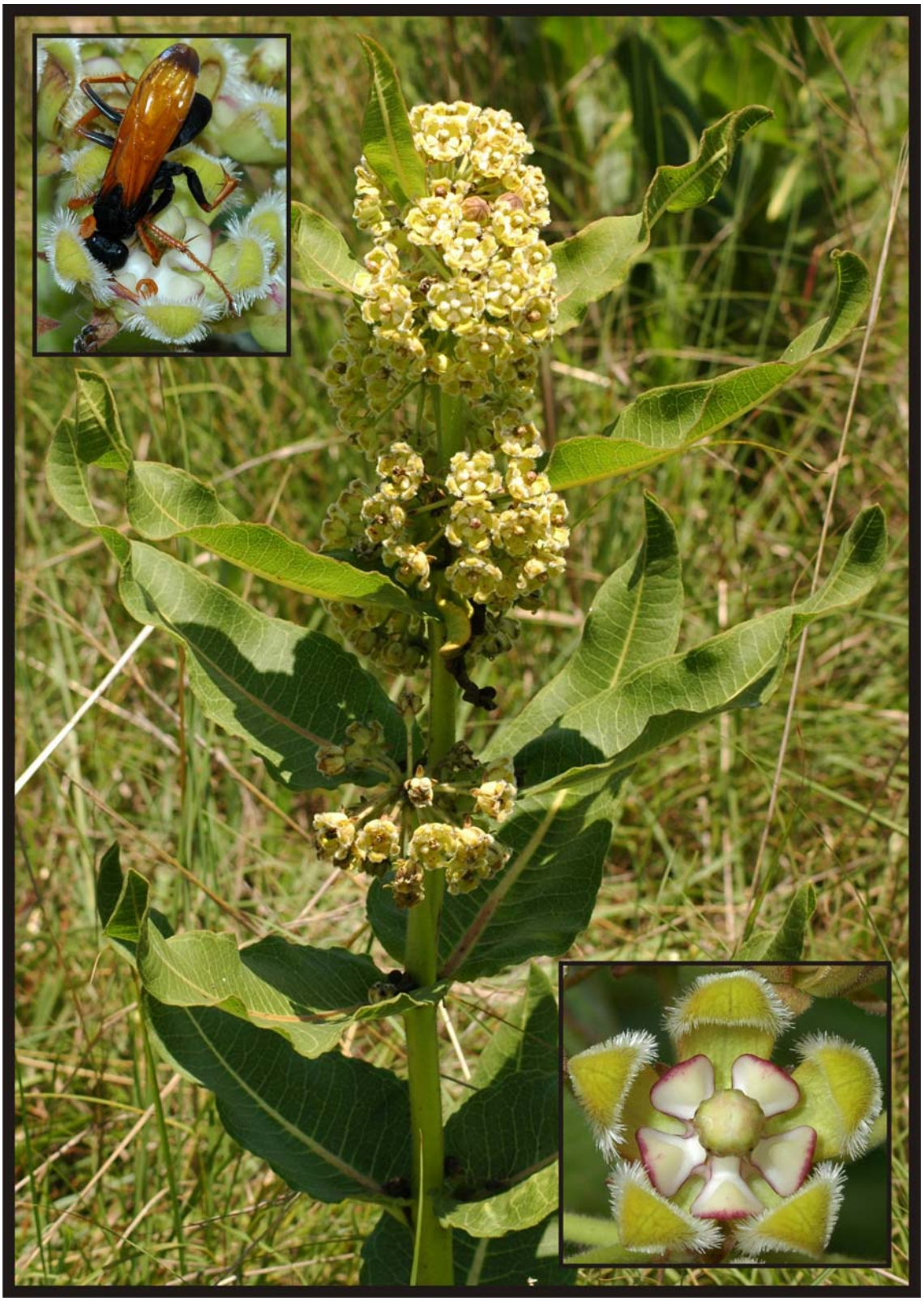

FIGURE 14. *Xysmalobium undulatum* var. *undulatum* (Apocynaceae), Midmar Nature Reserve. Inset, female *Hemipepsis capensis* visiting flowers (top) and close up of flower (bottom). Flower diameter = 16 mm. Inset photographs: S.D. Johnson.

FIGURE 15. *Eucomis autumnalis* subsp. *clavata* (Hyacinthaceae), Vernon Crookes Nature Reserve and Midmar Nature Reserve (inset). Inset, close up of flower. Flower diameter = 25 mm.

FIGURE 16. *Eucomis comosa* var. *striata* (Hyacinthaceae), Gilboa Estate. Inset, close up of flower. Flower diameter = 25 mm.

FIGURE 17. *Hemipepsis*-wasp pollinated orchids. *Satyrium microrrhynchum*, Monk's Cowl (main spread and bottom inset), flower diameter = 9 mm; *Disa sankeyi* being visited by *Hemipepsis capensis*, Sehlabathebe (inset, top left), flower diameter = 13 mm; and *Disa bivalvata*, Bainskloof (inset, top right), flower diameter = 45 mm. Photographs: S.D. Johnson.

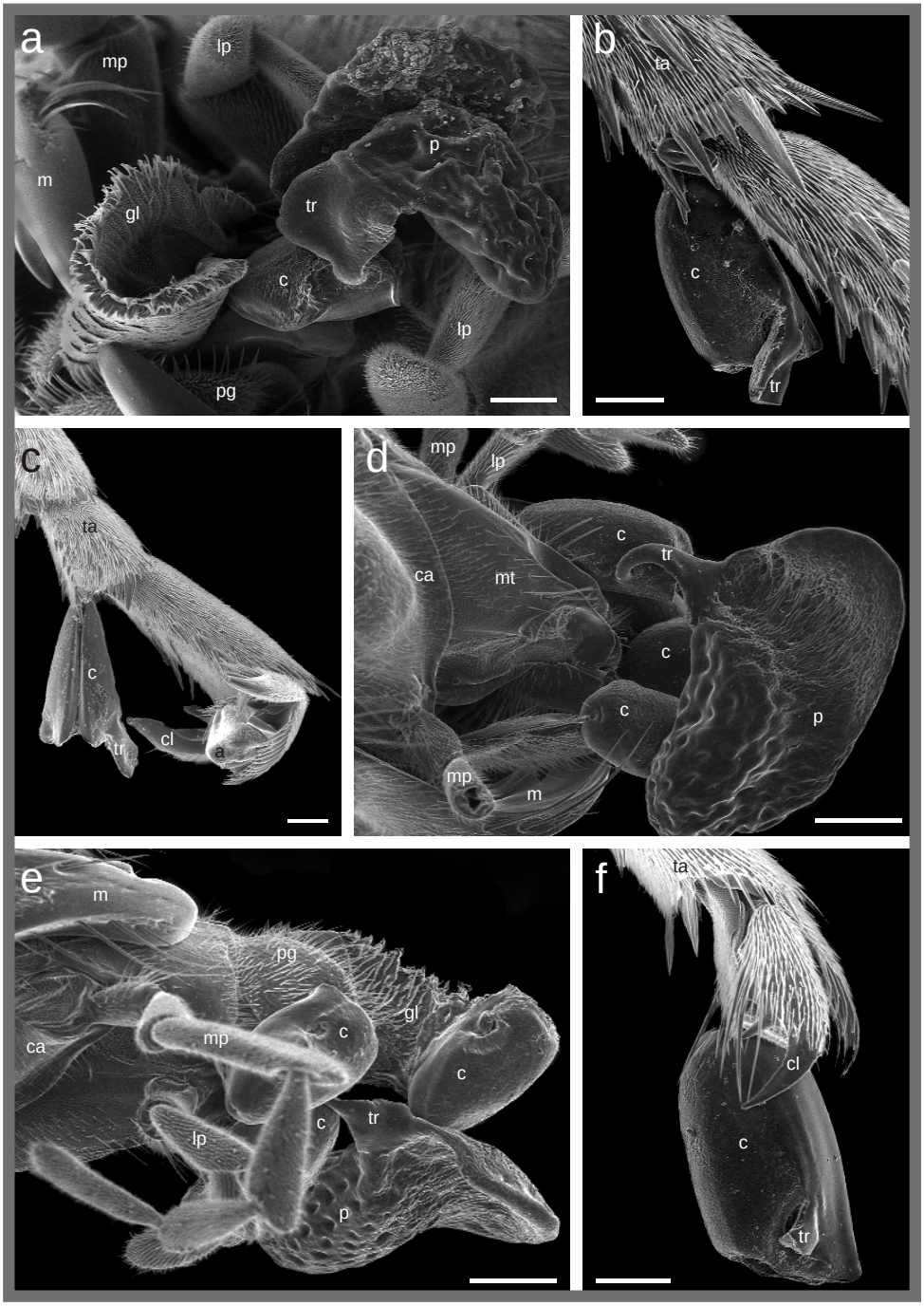

FIGURE 18. Scanning electron micrographs of asclepiad pollinaria (or just corpuscula) on *Hemipepsis* wasps. **a**, Ventral view of *H. errabunda* mouthparts with *Xysmalobium orbiculare* pollinarium, Wahroonga Farm; **b** & **c**, *Pachycarpus grandiflorus* corpuscula attached to *H. errabunda* tarsal spines, Fort Commonage; **d** & **e**, *H. hilaris* mouthparts with unidentified pollinaria (possibly *Miraglossum pilosum* or *M. verticillare*), Gilboa Estate; **f**, *P. grandiflorus* corpusculum attached to *H. capensis* arolium, Gilboa Estate. *a*, arolium; *c*, corpusculum; *ca*, cardo; *cl*, claw; *gl*, glossa; *lp*, labial palp; *m*, mandible; *mp*, maxillary palp; *mt*, mentum; *p*, pollinium; *pg*, paraglossa; *ta*, tarsus; *tr*, translator arm. Scale bar = 200 µm.

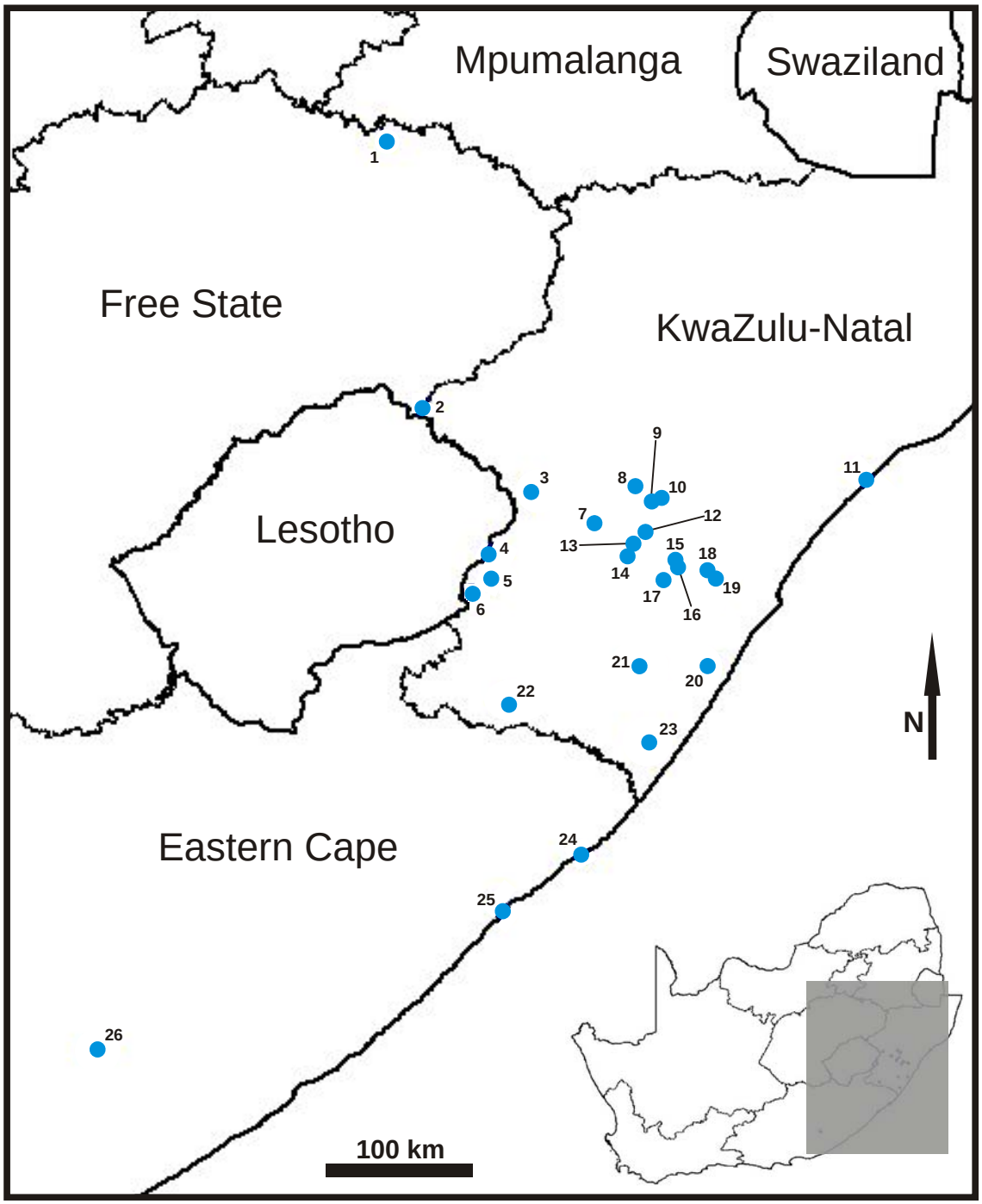

FIGURE 19. Field sites in South Africa. **1**, Roadside near Villiers; **2**, Royal Natal National Park; **3**, Giant's Castle; **4**, Sani Pass; **5**, Garden Castle; **6**, Bushman's Nek; **7**, Wodwo Farm/Fort Nottingham Commonage; **8**, Roadside between Mooi River and Greytown; **9**, Gilboa Estate; **10**, Roadside near Gilboa Estate; **11**, Amatikulu Nature Reserve; **12**, Howick; **13**, Midmar Nature Reserve; **14**, Wahroonga Farm; **15**, Hesketh Conservancy; **16**, Ashburton; **17**, Baynesfield; **18**, Cato Ridge Airfield; **19**, Monteseel; **20**, Vernon Crookes Nature Reserve; **21**, Highflats; **22**, Mount Currie Nature Reserve; **23**, Oribi Gorge Nature Reserve; **24**, Sinangwana; **25**, Lupatana; **26**, Hogsback.

CHAPTER 2

PALP-FACTION: AN AFRICAN MILKWEED DISMEMBERS ITS WASP POLLINATORS

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PLANT–INSECT INTERACTIONS

Palp-Faction: An African Milkweed Dismembers Its Wasp Pollinators

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ABSTRACT Interactions between pollinators and nectar-producing flowers are usually assumed to be mutualistic, but the exploitative basis of these relationships can lead to antagonistic interactions. Flowers of the African milkweed, *Pachycarpus appendiculatus* E. Mey, produce concentrated nectar that is consumed primarily by the large spider-hunting wasp *Hemipepsis dedjas* Guerin (Hymenoptera: Pompilidae). Pollinaria of this milkweed become attached to the palps of these wasps during nectar feeding. Broken wasp palps were found between guide rails, attached to corpuscula that were trapped behind the guide rails, and attached to pollinia that were inserted into the stigmatic chambers of the flowers. Approximately 85% of wasps captured on flowers of *P. appendiculatus* were missing one or more palps, whereas only 9% of wasps captured on flowers of another asclepiad species were missing any palps. It thus seems that wasps face a high risk of losing their palps when foraging on these flowers. The interaction may thus be antagonistic for the wasps if the cost of losing their sensory palps (not yet established) is greater than the benefits of the nectar reward. The plants, however, gain clear benefit from the interaction, as verified by the removal and insertion of pollinia in flowers exposed solely to visits by pompilid wasps.

KEY WORDS Apocynaceae, antagonism, mutualism, nectar, pollination syndromes

Plant–pollinator interactions have provided text-book examples of mutualisms in which both parties benefit (Proctor et al. 1996). However, in instances where the cost to either the plant or pollinator is greater than the benefit, these systems are better described as antagonistic. Examples include flowers that are damaged by nectar-robbing flower visitors (Irwin and Brody 1998, 1999, 2000, Maloof 2001) and insects that are deceived by rewardless orchids and arums (Steiner et al. 1994, Stensmyr et al. 2002, Wong and Schiestl 2002, Schiestl 2005, Diaz and Kite 2006, Jersakova et al. 2006). A different form of antagonism may occur when the pollinator is physically damaged, leading to loss of foraging efficiency (Morse 1981). Several examples of loss of body parts have been reported for milkweed pollinators, but these seem to be incidental, rather than a systematic feature of the pollination system (Morse 1981, Shuttleworth and Johnson 2006). This study was conducted after preliminary observations suggested that the South African milkweed *Pachycarpus appendiculatus* E. Mey invariably removes the palps of its pompilid wasp pollinators, and thus dismembers its pollinators to an extent that has not been reported previously in pollination systems.

The majority of literature regarding pollination by wasps is concerned with figs and fig-wasps or sexually deceptive orchids (Steiner et al. 1994, Herre and West 1997, Ayasse et al. 2000, Schiestl 2005). Aside from these types of studies, examples of pollination by wasps are remarkably scarce. Specialized pollination by nectar seeking wasps has until recently been considered a rare occurrence, with the only known examples being the pollination of asclepiads by vespids (Vieira and Shepherd 1999, J. Ollerton, unpublished data cited in Ollerton et al. 2003; S.D.J., unpublished data). However, recent studies have shown that a number of South African grassland plant species are specialized for pollination by pompilid wasps (Hymenoptera: Pompilidae) in the genus *Hemipepsis* (Ollerton et al. 2003, Johnson 2005, Shuttleworth and Johnson 2006, Johnson et al. 2007; unpublished data). Furthermore, specialization for pollination by these pompilids is not only found in asclepiads but has been described in the Orchidaceae (*Disa sankeyi* and *Satyrium microrrhynchum;* Johnson 2005, Johnson et al. 2007) and Hyacinthaceae (*Eucomis autumnalis* and *E. comosa;* unpublished data). In addition, Punzo (2006) described flower visiting by *Pepsis grossa* (Hymenoptera: Pompilidae) in North America, suggesting that pollination by pompilid wasps may be more widespread than it seems from the literature.

The asclepiads (Apocynaceae subfamily Asclepiadoideae *sensu* Endress and Bruyns 2000) and orchids (Orchidaceae) are unique among angiosperms in that they have pollen gathered into waxy masses known as pollinia. In asclepiads (unlike orchids), each pair of pollinia is attached by two translator arms to a mechanical clip known as the corpusculum. This corpus-

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culum attaches the pollinia to the pollinator. When a pollinium is inserted into the stigmatic groove of a subsequent flower, the translator arm usually breaks as the pollinator withdraws, separating the pollinium from the corpusculum (Wyatt and Broyles 1994). However, in the case of *P. appendiculatus*, preliminary observations showed that inserted pollinia were frequently still attached to a corpusculum, which itself was attached to a broken off insect palp.

The aims of the study were to document the interaction between *P. appendiculatus* and pompilid wasps, and specifically, to establish the degree of dismemberment incurred by the wasp pollinators during foraging on this plant species.

Materials and Methods

Study Site. This study was conducted in a population of ~60 plants extending for ~1.5 km north along the coastline from the mouth of the Sinangwana River (31°44′41.9″ S; 29°22′22.9″ E) in the Eastern Cape Province of South Africa. Plants were flowering in burnt patches of short grassland above coastal forest at altitudes of between 80 and 120 m.a.s.l.

Study Species. The genus *Pachycarpus* E. Mey. is endemic to Africa and contains ~40 species occurring in grasslands south of the Sahara (Smith 1988). *P. appendiculatus* is a robust erect perennial herb found in rocky grasslands in the eastern parts of South Africa (Smith 1988, Pooley 1998). Plants range from 22 to 50 cm in height and, at our study site, had a mean of 12.6 ± 0.90 (SE) flowers ($n = 50$). Flowers are large (14–26 mm diameter) with reflexed corolla lobes and large, distally flattened corona lobes, which fold over the staminal column (Fig. 1B). Both corolla and corona lobes are dull greenish-white with occasional purple spots or purple along the edges (Fig. 1B). A voucher specimen from the study site is lodged in the NU Herbarium, University of KwaZulu-Natal, Pietermaritzburg campus (Collectors No. Shuttleworth 5).

Nectar Properties. Nectar is secreted at the base of the corona lobes and gathers on either side of the guide rails. The volume (μl) and concentration (percentage sucrose equivalent by weight) of nectar produced over a 24-h period were measured for 21 and 19 flowers, respectively, from five plants on 12 December 2006. Plants were bagged in the field with fine mesh (1 by 1 mm) cloth pollinator exclusion bags for 24 h before measuring. Nectar present on these flowers at the start of the 24-h period was removed with capillary tubes. After 24 h, nectar volume and concentration were measured using 5-μl capillary tubes and a Bellingham and Stanley (0–50 or 45–80%) hand-held refractometer, respectively. Volume and concentration readings were averaged for all flowers measured on a plant, and these values used to calculate a grand mean ± SE per flower per plant.

Pollinator Composition. Visitor observations were conducted from 18 to 19 December 2005 and from 10 to 13 December 2006. Total observation time was ~50 h spread over these 6 d. All floral visitors were recorded, and representative individuals were captured for later identification. Floral visitors were checked (in the laboratory or in the field) for number and location of pollinaria. All insects were identified to family level using Scholtz and Holm (1996). Pompilid wasps were identified to species level using keys given in Arnold (1932), Day (1979), and Goulet and Huber (1993). Voucher specimens are kept in the university collection of S.D.J.

Pollinator Effectiveness: Cage Experiment. After preliminary observations suggested that the pompilid species *Hemipepsis dedjas* Guerin and *H. capensis* L. (both effectively a single functional group) were the primary visitors, a cage experiment was conducted to confirm the effectiveness of these wasps in removing and inserting pollinaria. Pompilid wasps, unlike bees, are remarkably unaffected by laboratory cage conditions. Wasps placed in a flight cage with *P. appendiculatus* flowers will immediately start feeding and exhibit behavior that is apparently identical to that exhibited in the field.

For this experiment, two *P. appendiculatus* plants were placed in a 1-m^3 fine mesh (1 by 1-mm cloth gauze) flight cage with wasps. The experiment was run from 1400 hours on 11 December, until 2100 hours on 14 December 2006. Two wasps (one *H. dedjas* and one *H. capensis*) were placed in the cage for the full duration of the experiment. A further two individuals of *H. dedjas* were introduced to the cage at 1830 hours on 11 December. One of the *H. dedjas* wasps had a single pollinium attached to a maxillary palp, while another had a single corpusculum attached to a maxillary palp when they were placed in the cage. At the start of the experiment, the *H. capensis* individual had all four palps intact, two of the three *H. dedjas* had both maxillary palps intact, and the third *H. dedjas* had a single maxillary palp intact. Labial palps are smaller and are difficult to see on a live wasp, and thus their presence could not be established at the start of the experiment (the state of the labial palps on the *H. capensis* individual was established at the end of the experiment).

The flowers of *P. appendiculatus* are large, and it is possible to see inserted pollinia in guide rails with a hand lens. Before starting the experiment, each flower was inspected, and the number of removed pollinia was written on one of the corolla lobes with a permanent marker. Any pollinia that were already inserted were removed with a fine pair of forceps or, if the pollinium had started growing pollen tubes, that flower was removed from the plant. A total of 22 flowers remained after this had been done. Several flowers were still in bud at the start of the experiment and opened during the course of the experiment.

At the end of the experiment, all flowers were inspected, and the number of pollinia that had been removed and inserted was recorded. The wasps were killed with ethyl acetate, and the number and location of pollinia on them was noted. The plants were kept in water-filled containers in the laboratory until 22 December to check for early stages of fruit development on pollinated flowers.

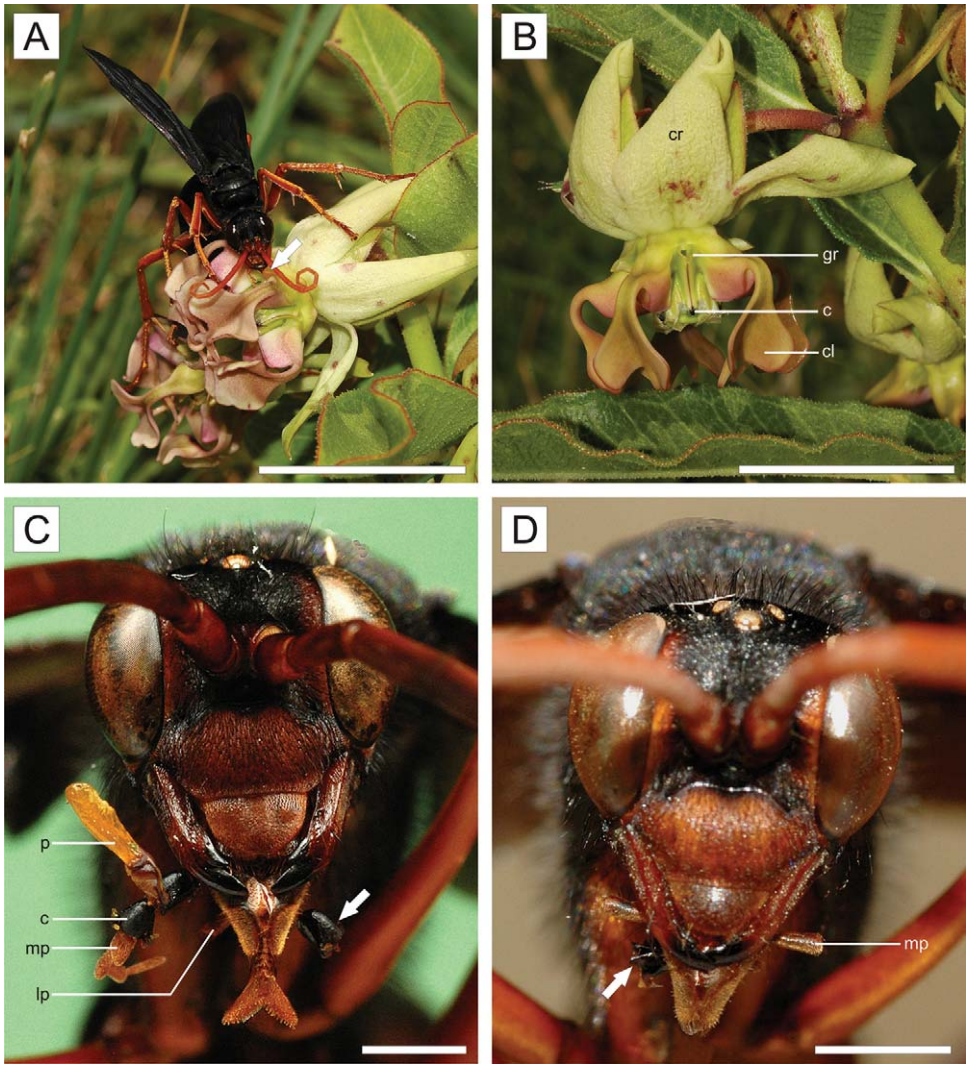

Fig. 1. *Pachycarpus appendiculatus* flowers and pompilid wasp visitors. (A) Female *H. dedjas* visiting a flower in the field. Note pollinia attached to palps (indicated by arrow). Scale bar = 20 mm. (B) Close-up of flower showing floral morphology. Scale bar = 20 mm. (C) Male *H. dedjas* with two corpuscula (one with a single pollinium) attached to a maxillary palp and a single corpusculum (indicated by arrow) attached to a labial palp. Note that one maxillary palp is missing, and both labial palps are reduced to a single segment each. Scale bar = 2 mm. (D) Male *H. dedjas* with all palps partially removed and a corpusculum attached to the remains of a labial palp (indicated by arrow). Scale bar = 2 mm. c, corpusculum; cl, corona lobe; cr, corolla lobe; gr, guide rail; lp, labial palp; mp, maxillary palp; p, pollinium. (Online figure in color.)

Pollinia Removal and Insertion Rates in the Field. The frequency of pollinia removal and insertion was determined for 189 flowers on 24 plants on 18 December 2005. Flowers were examined in the field. The average number of pollinia removed and inserted per flower was calculated for each plant, and a mean ± SE of these values obtained to represent the population mean. The percentage of flowers pollinated (containing at least one inserted pollinium) was calculated for each plant (n = 24), and a mean ± SE was obtained from these values to represent the percentage of flowers pollinated in the population. Pollen transfer efficiency (PTE) in each population was calculated as the percentage of removed pollinia that were inserted between guide rails (Johnson et al. 2004).

Fruiting Success. To calculate fruit set (as the percentage of flowers that set fruit), the number of unpollinated flowers and fruits present on 26 plants was counted on 18 December 2005. Unpollinated flowers could be easily counted because they leave a noticeable scar when they fall off the stem. Fruit set was calculated as the percentage of flowers (each flower on a plant represented by either a fruit or a scar) that formed a fruit. The total number of flowers (represented by either a fruit or a scar) was 214. The average percentage fruit set per flower was calculated for each

Table 1. *Pachycarpus appendiculatus* visitors and their respective pollen loads

Visitor	Number observed	Number captured	Number carrying pollinia (captured and observed)	Location of pollinia on the body
Hymenoptera				
Hemipepsis dedjas (Pompilidae)	45	29	10	Maxillary and labial palps
Hemipepsis capensis (Pompilidae)	4	1	0	
Tiphia sp. (Tiphiidae)	8	2	0	
Coleoptera				
Atrichelaphinis tigrina (Scarabaeidae: Cetoniinae)	1	0	0	
Hemiptera				
Lygaeidae sp.	8	3	0	
Diptera				
Calliphoridae sp.	1	0	0	
Sarcophagidae sp.	2	0	0	

plant, and the mean ± SE of these values used to represent fruit set in the population.

Dismemberment of Wasps. To test whether *P. appendiculatus* removes the palps of visiting wasps, we used three approaches. The first was to compare the number of intact palps on wasps before and after the cage experiment described above. The second was to compare the percentage of individuals with at least one palp broken or missing between wasps caught visiting *P. appendiculatus* at the study site and those caught on other flowering plants. Wasps used in this comparison consisted of 26 *H. dedjas* individuals caught on *P. appendiculatus* and 23 *H. capensis* individuals caught on another asclepiad species (*Xysmalobium undulatum*) at Midmar Nature Reserve (29°31′34.5″ S; 30°10′12.7″ E, altitude 1,090 m.a.s.l.) and Villiers (27°06′11.9″ S; 28°41′01.9″ E, altitude 1,565 m.a.s.l.) where *P. appendiculatus* does not occur. Although this comparison was weakened by the different wasp species caught on *P. appendiculatus* and *X. undulatum*, they are very similar morphologically and thus likely to have a similar probability of losing palps when foraging on a particular plant species. The third approach used was to inspect flowers in the field (n = 189) and in the cage experiment (n = 22) for the presence of broken-off pieces of wasp palp. Flowers inspected in the field were the same as those inspected for pollinia removal and insertion rates described above.

Results

Nectar Properties. Pachycarpus appendiculatus flowers produced 18.3 ± 6.09 (SE) μl (n = 21) of nectar over a 24-h period. The concentration of this nectar was 58.0 ± 1.99 (SE) % (n = 19) sucrose equivalents by weight.

Pollinator Composition. *Pachycarpus appendiculatus* flowers were visited almost exclusively by *H. dedjas* (Hymenoptera: Pompilidae) (Table 1). Note: female individuals key out to *Hemipepsis dedjas* Guerin variety *spinosior* Arnold, whereas male individuals key out to *H. gestroi* Gribodo (Arnold 1932). However, we are confident that these are the same species (A.S., unpublished data), and we therefore maintain the earlier name "dedjas" to refer to these individuals. A similar species, *H. capensis*, was also observed, but it was considerably less abundant (Table 1). Aside from the pompilid wasps, several other insect species were observed visiting flowers (Table 1). However, these visitors were not abundant and, with the exception of the cetoniin beetle, were all too small to remove pollinaria.

Pollinia were found only on *H. dedjas* individuals and were attached to both the maxillary and labial palps (Table 1). Of the 10 individuals carrying pollinia (9 males and 1 female), 6 were carrying a single pollinarium and 4 were carrying two pollinaria each. Of the 14 pollinaria that were attached to wasps, 10 had been reduced to just the corpusculum and 2 had only a single pollinium (indicating that either one or both pollinia had been successfully inserted between the guide rails of a flower). Note that in several instances these pollinaria (or just corpuscula) were attached to the first or second palp segment, with the remainder of the palp having been removed (Fig. 1, C and D).

Pollinator Effectiveness: Cage Experiments. In the cage experiment, 40 pollinia (on 20 pollinaria) were removed and 17 pollinia were inserted (see Table 2 for means per flower). Eight of the 22 flowers (36.4%) were effectively pollinated (having at least one pollinium inserted between guide rails; Table 2). Two

Table 2. Pollinia removal and insertion rates, PTE, and pollination success (fruit set) of *P. appendiculatus* flowers in the field and in the cage experiments (flowers exposed only to wasp visits) given as mean ± SE and percentage PTE

Experiment	Percentage of flowers pollinated	No. pollinia removed per flower	No. pollinia inserted per flower	PTE	Percentage fruit set
Field (n)	39.9 ± 5.59 (24)	2.4 ± 0.29 (24)	0.6 ± 0.08 (24)	28.1	24.1 ± 4.08 (26)
Cage (n)	36.4 ± 10.50 (22)	1.8 ± 0.47 (22)	0.8 ± 0.28 (22)	42.5	9.1 ± 0.06 (22)

Sample sizes for measurements in the field represent no. of plants (see text for no. of flowers). Sample sizes for the cage exp represent no. of flowers.

flowers showed distinctly swollen ovaries by 22 December 2007, indicating early stages of fruit development. One of these flowers was still in bud at the start of the experiment and opened during the course of the experiment, indicating that fruit development was definitely the result of pollination during the experiment. Corpuscula were still present on the palps of two of the three *H. dedjas* individuals at the end of the experiment. One wasp had a single corpusculum attached to the remains of a maxillary palp, and the other wasp had a corpusculum attached to the remains of one labial palp and two corpuscula (one with a single pollinium) attached an intact maxillary palp. The single *H. capensis* individual used carried no pollinia at the end of the experiment.

Pollinia Removal and Insertion Rates in the Field. Approximately 40% of *P. appendiculatus* flowers were pollinated, and PTE was ~28% (Table 2).

Fruiting Success. Approximately 24% of *P. appendiculatus* flowers set fruit (Table 2).

Dismemberment of Wasps. Of the three *H. dedjas* individuals used in the cage experiment, two had all four palps removed, and the third had both labial and a single maxillary palp removed. Thus, all five of the intact maxillary palps on *H. dedjas* individuals that were present at the start of the experiment were completely removed during the experiment. In contrast, the single *H. capensis* individual used in the cage experiment still had all four palps intact at the end of the experiment.

A significantly higher proportion (85%) of wasps visiting *P. appendiculatus* flowers in the field had one or more palps broken or missing than wasps visiting *Xysmalobium undulatum* flowers (9%; $G = 31.04$, $P < 0.001$). Furthermore, of the 26 *H. dedjas* individuals caught visiting *P. appendiculatus*, 16 had all four palps completely missing.

Flowers that were inspected in the field were found to contain 17 broken off wasp palps. Of these, five were trapped between guide rails, nine were attached to a corpusculum that had become trapped behind the guide rails and three were attached to a corpusculum that was itself still attached to an inserted pollinium (with the corpusculum outside the stigmatic groove). In the cage experiment, eight wasp palps were found broken off in flowers: seven were still attached to corpuscula (four still attached to a pollinium) that had been inserted and trapped behind the guide rails, and one was attached to a corpusculum that was still attached to an inserted pollinium.

Discussion

The results of this study show that *P. appendiculatus* is pollinated exclusively by pompilid wasps (specifically *H. dedjas*) and invariably removes the palps of these wasps during this process. Large pompilid wasps were by far the most abundant floral visitors and were the only insects found to carry pollinia at the study site (Table 1). The cage experiment showed that *H. dedjas* is capable of transferring pollinia between flowers of *P. appendiculatus*. We conclude that *P. appendiculatus* has a highly specialized pollination system that is operated only by large *Hemipepsis* pompilid wasps.

The pollinia of *P. appendiculatus* are attached to the palps of visiting wasps, and during this process, these palps are frequently broken off, as shown by the broken palps found in flowers in both the field and the cage experiments. Furthermore, a significantly higher proportion of the wasps visiting *P. appendiculatus* had one or more palps broken than did a similar sample of wasps caught visiting another asclepiad (*Xysmalobium undulatum*) at sites where *P. appendiculatus* does not occur. A number of the *H. dedjas* individuals visiting *P. appendiculatus* had all four of their palps completely removed. This study shows that palps are broken or removed in one of three ways: (1) when the palp gets trapped behind the guide rails and the wasp pulls away (without removing a pollinarium) breaking the palp, (2) when a corpusculum (with or without its pollinia) that is attached to a palp gets trapped behind the guide rails, breaking off the palp, or (3) when a pollinium is successfully inserted and the palp, rather than the translator arm of the pollinarium, is broken off.

Palps have a sensory function in insects and are used to locate and test the quality of food before ingestion (Chapman 1971, Gullan and Cranston 2005). In this study, wasps without palps seemed to be able to locate and feed on nectar in flowers. However, if damaged or missing palps seriously reduce the efficiency of feeding or cause the ingestion of unsuitable or low-quality nectar, the interaction between *P. appendiculatus* and pompilids is possibly antagonistic rather than mutualistic. Loss of palps was also observed for pompilid wasp pollinators of a related species, *P. asperifolius*, which also attaches pollinia to the wasps' palps (Shuttleworth and Johnson 2006). However, unlike the case in *P. appendiculatus*, pollinia of *P. asperifolius* are also placed on tibial spines, which are not damaged by the interaction. Physical damage has been reported for bumblebees that pollinate *Asclepias syriaca*, where ~30–40% of bumblebees were found to lose claws and tarsal segments when they became entangled in the guide rails during foraging on this milkweed (Morse 1981). The loss of these appendages was shown to reduce the foraging ability of the bumblebees by ~25% (Morse 1981). The cost of palp loss to wasps is difficult to quantify, but it is likely that wasps without palps are less efficient at foraging. However, evidence of this was not gathered during this study, and further research into the foraging abilities of wasps with and without palps is needed to establish whether palp loss does indeed constitute a cost to the wasps.

The balance between antagonism and mutualism has been explored in more detail for interactions in which plants incur loss of parts, such as ovules, during pollination (e.g. figs and fig-wasps, and yuccas and yucca-moths; Pellmyr and Huth 1994, Herre and West 1997, Holland and DeAngelis 2001). The cost of this damage to the plant, however, is counteracted by a benefit in terms of pollination. A number of studies have used these types of interaction to model the costs and benefits of mutualisms to obtain a greater understanding of the evolution of mutualisms in general

(Doebeli and Knowlton 1998, Holland and DeAngelis 2001, Holland et al. 2002). Further research to establish the cost of broken palps to *Hemipepsis* wasps would contribute to our general understanding of the roles of antagonism versus mutualism in plant-pollinator interactions.

The levels of pollinia removal and insertion and PTE were remarkably high for *P. appendiculatus* (Table 2), suggesting that attaching pollinia to palps is an effective mechanism for pollen transfer (cf. Johnson et al. 2004 for a discussion of PTE values and a comparative measure of PTE in an orchid species). Overall, almost 40% of flowers in the population were pollinated, and 24% of these set fruit (Table 2). The difference between the percentage of flowers that were pollinated and the percentage that set fruit suggests comparatively high levels of geitonogamous self-insertions. Although the breeding system of *P. appendiculatus* was not determined, two congeneric species (*P. asperifolius* and *P. grandiflorus*) are known to be genetically self-incompatible (Shuttleworth and Johnson 2006; A.S., unpublished data). The pollen transfer efficiency for *P. appendiculatus* (28.1%) is comparable to that found for another species, *P. asperifolius* (42.7, 19.0, and 15.0%, respectively, at different sites), which is also pollinated exclusively by pompilid wasps (Shuttleworth and Johnson 2006). However, *P. appendiculatus* had a higher number of removals and insertions per flower and experienced considerably higher levels of natural fruit set (only 1.1% of *P. asperifolius* flowers set fruit, see Shuttleworth and Johnson 2006). The 24% fruit set recorded in this study is remarkable for a milkweed as this group of plants typically experience low levels of fruit set (Queller 1985, Kephart 1987, Wyatt and Broyles 1994).

Pachycarpus appendiculatus is pollinated exclusively by pompilid wasps in the genus *Hemipepsis*, and the palps of these wasps are removed during this process. Further research into the costs of palp loss to the wasps would contribute to our understanding of the balance between antagonism and mutualism in plant–pollinator interactions.

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CHAPTER 3

BIMODAL POLLINATION BY WASPS AND BEETLES IN THE AFRICAN MILKWEED *XYSMALOBIUM UNDULATUM*

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Bimodal Pollination by Wasps and Beetles in the African Milkweed *Xysmalobium undulatum*

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ABSTRACT

It has been suggested that flowers of some plants are specialized for pollination by two unrelated species (or functional groups) of pollinators. However, evidence for 'bimodal pollination systems' has been extremely limited. Studies of the milkweed *Xysmalobium undulatum* in South Africa showed that its flowers are visited by a range of different insects (representing 18 families), but only two groups, represented by the chafer beetle *Atrichelaphinis tigrina* and pompilid wasps in the genus *Hemipepsis*, effect pollination. Experiments showed that both these pollinator groups are effective in removing and inserting pollinia. Pollinia are attached to clypeal hairs and mouthparts on the wasps and tarsal hairs and spines on the beetles. Although considerably less abundant than the beetles, *Hemipepsis* spp. wasps move more quickly among flowers and appeared to be more effective pollinators overall. Experimental hand-pollinations conducted in the field showed that *X. undulatum* is genetically self-incompatible and thus completely reliant on pollinators for reproduction. We conclude that *X. undulatum* has a bimodal pollination system, specialized for pollination by *Hemipepsis* pompilid wasps and the chafer beetle *A. tigrina*.

Key words: Apocynaceae; breeding system; pollination syndromes; South Africa; specialized pollination.

SOUTH AFRICAN POLLINATION SYSTEMS ARE CHARACTERIZED BY A HIGH PROPORTION OF SPECIALIZED plant–pollinator interactions (Johnson & Steiner 2000, Johnson & Steiner 2003; but see Waser *et al.* 1996). An interesting phenomenon within these highly specialized systems is the occurrence of bimodal pollination in some plant species (Manning & Goldblatt 2005, Johnson *et al.* 2007). These species are pollinated by two different pollinator types specialized to different pollination systems and exhibit morphological characteristics intermediate between different syndromes (Manning & Goldblatt 2005). Examples of bimodal pollination systems described in the Iridaceae include hopliine beetles/tabanid flies, bees/noctuid moths, bees/nemestrinid flies, bees/hopliine beetles, and sunbirds/butterflies (Goldblatt *et al.* 2000a,b, 2002, 2004; Manning & Goldblatt 2005). Johnson *et al.* (2007) described a bimodal wasp/beetle system in the orchid *Satyrium microrrhynchum* Schltr. Here we present evidence for a similar bimodal wasp/beetle pollination system in the African milkweed *Xysmalobium undulatum* (L.) Ait. f.

The asclepiads (Apocynaceae subfamily Asclepiadoideae *sensu* Endress & Bruyns 2000) and orchids (Orchidaceae) are unique among angiosperms in that they have pollen gathered into waxy masses known as pollinia (Harder & Johnson 2008). In asclepiads (unlike orchids) each pair of pollinia is attached via two translator arms to a mechanical clip known as the corpusculum, which attaches the pollinia to the pollinator. This whole structure is known as a pollinarium. Pollinia inserted in a stigmatic groove usually break away from the corpusculum when a pollinator withdraws (Wyatt & Broyles 1994).

Detailed studies of pollination in African milkweeds are scarce, with only a few published examples (Liede & Whitehead 1991; Pauw 1998; Ollerton *et al.* 2003; Shuttleworth & Johnson 2006, 2008). Pollination studies within the genus *Xysmalobium* are restricted to a single study by Ollerton *et al.* (2003), which found that chafer beetles (Scarabaeidae: Cetoniinae) visit and carry pollinaria of *Xysmalobium involucratum* (E. Mey.) Decne. However, they did not carry out experiments to show that beetles are effective pollinators of this species. Breeding systems in *Xysmalobium* have not been investigated previously.

The aims of this study were to describe the pollination and breeding system of the milkweed *X. undulatum* and to explore the relative importance of pompilid wasps and chafer beetles for the pollination of this species.

METHODS

STUDY SPECIES AND STUDY SITES.—*Xysmalobium undulatum* is an erect, multistemmed (mean ± SE number of stems per plant: 3.8 ± 0.42, $N = 35$) herb, which is widespread in South African grasslands. Plants have large leaves and are robust, attaining a height of up to 1.8 m (Pooley 1998). Flowers are small (*ca* 15 mm diameter) and are arranged in a dense umbel inflorescence with a diameter of approximately 15 cm (Fig. 1A). The dull green corolla lobes are erect with recurved tips, and form a cup around the central column and corona lobes (Fig. 1). Plants have 119.9 ± 12.86 (mean ± SE) flowers per stem ($N = 17$). Plants flower from October to January (Pooley 1998). This study was conducted during the three flowering seasons between 2004 and 2007 at nine field sites in South Africa (see Table S1 for details). All field sites are situated in subtropical grassland. Voucher specimens of plants studied are deposited in the NU Herbarium (University of KwaZulu-Natal, Pietermaritzburg).

FLORAL VISITORS AND POLLINATOR EFFECTIVENESS.—Floral visitors were observed at all field sites during the three flowering seasons (see Table S1 for observation times per site). Representative

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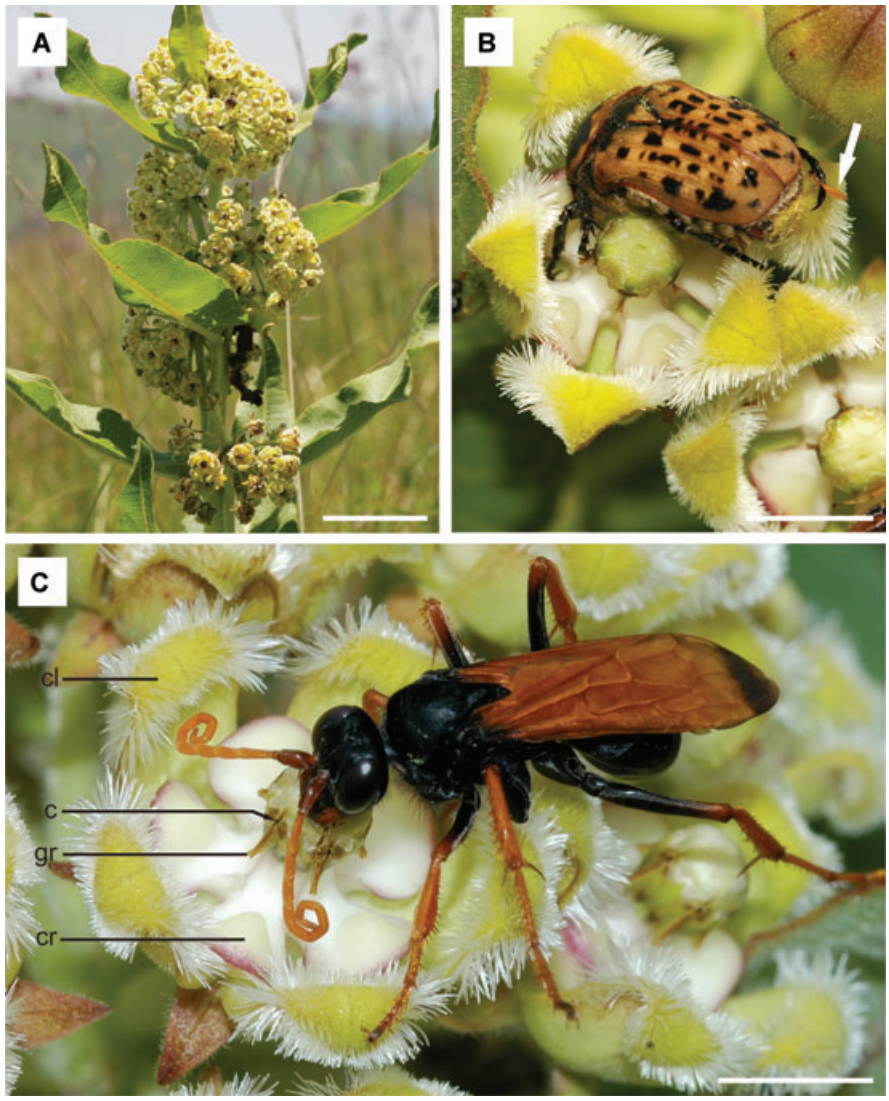

FIGURE 1. *Xysmalobium undulatum* and its pollinators. (A) single stem showing several umbel inflorescences. Scale bar = 50 mm. (B) *Atrichelaphinis tigrina* (Scarabaeidae: Cetoniinae) visiting a flower. Note pollinarium attached to metatarsal spine (indicated by arrow). Scale bar = 5 mm. (C) Female *Hemipepsis capensis* (Hymenoptera: Pompilidae) visiting a flower. Scale bar = 5 mm. c, corpusculum; cl, corolla lobe; cr, corona lobe; gr, guide rails.

individuals of all visitor species were captured for subsequent identification in the laboratory. Insects were identified by the authors, with the assistance of Denis Brothers (University of KwaZulu-Natal). Floral visitors were checked (either in the laboratory or in the field) for number and location of pollinaria. Representative insect specimens are deposited in the collection at the University of KwaZulu-Natal and the Natal Museum (Pietermaritzburg, South Africa). The median time spent per flower was determined for *Hemipepsis* spp. pompilid wasps and the chafer beetle *Atrichelaphinis tigrina*, after preliminary observations suggested that these were the only insects that consistently carried pollinia of *X. undulatum*. In total, we recorded times of visits by 12 wasps to 178 flowers and 31 chafer beetles to 54 flowers.

Cage experiments were used to establish the functional effectiveness of *Hemipepsis* spp. wasps and *A. tigrina* in removing and inserting pollinia of *X. undulatum*. Nectar feeding behavior in these insects is seemingly unaffected by laboratory cage conditions. For these experiments, virgin flowers (which had been previously bagged at the bud stage with fine mesh pollinator exclusion bags) were placed in 1-m^3 fine mesh flight cages with individuals of either the chafer *A. tigrina* or *Hemipepsis* spp. wasps. None of the insects carried pollinia at the start of the experiments. In total, three experiments were conducted with wasps and two with chafer beetles (see Table 1 for duration and sample sizes). At the end of each experiment, the insects were killed and the number and location of pollinia on them was noted. All flowers from the experiments

TABLE 1. *Pollen fates in five cage experiments conducted with* Hemipepsis *spp. wasps and the chafer beetle* Atrichelaphinis tigrina.

Pollinators in each experiment (*N*)	Experiment duration (h)	No. of flowers (no. of plants)	No. of pollinia removed	No. of pollinia inserted	No. of fruits
H. capensis (12) and *H. errabunda* (1)	139.5 [a]	74 (4)	102	6	2
Hemipepsis spp.[b] (2)	52	65 (7)	4	3	1
H. hilaris (3) and *Hemipepsis* spp. (7)[c]	119	389 (5)	2	0	Not checked
A. tigrina (19)	92	73 (4)	18	1	0
A. tigrina (10)[d]	119	346 (5)	12	1	Not checked

[a]Five of the wasps were removed after 12 h.

[b]Both these individuals went missing before they were identified.

[c]These individuals went missing before they were identified.

[d]Nine of these individuals escaped during the course of the experiment.

were inspected and the number of pollinia that were removed and inserted was noted. In the first two wasp experiments and the first beetle experiment, flowers were kept in vases in the laboratory for a period of two weeks after the end of experiment in order to allow for early fruit development.

NECTAR PROPERTIES.—Nectar is secreted on the sides of the corona lobes and gathers between the corona lobes. The standing crop volume (μl) and concentration (percentage sucrose equivalent by weight) of nectar produced by flowers was measured for 40 and 20 flowers, respectively on five plants. This was done at the Midmar Nature Reserve site between 0730 h and 0930 h on 14 December 2005. Nectar volume was measured using 20 μl capillary tubes. Nectar concentration was measured using a Bellingham and Stanley (0–50% or 45–80%) handheld refractometer. Volume and concentration readings were averaged for all flowers measured on a plant, and these values were used to calculate a grand mean $\pm$ SE per flower per plant.

POLLINATION SUCCESS.—The frequency of pollinia removal and insertion was determined for 55 flowers on nine plants at Villiers in December 2004 and 60 flowers on 10 plants at both Vernon Crooks and Midmar Nature Reserves in December 2005. Flowers were examined using a dissecting microscope in the laboratory or a 10$\times$ hand lens in the field. The mean number of pollinia removed and the mean number of pollinia inserted per flower was calculated for each plant sampled at a site, and a mean of these values was obtained to represent the population mean for each site. The percentage of flowers pollinated (containing at least one inserted pollinium) was calculated for each plant and a mean was obtained from these values to represent the population mean for each site. The pollen transfer efficiency (PTE) in each population was calculated as the percentage of removed pollinia that were inserted between guide rails (*cf.* Johnson *et al.* 2004).

BREEDING SYSTEM AND NATURAL FRUIT SET.—The degree of self-compatibility and capacity for autogamy in *X. undulatum* was determined using controlled hand–pollination experiments at Midmar Nature Reserve. These experiments were conducted in December 2005 and January 2006.

Plants in bud were bagged with fine mesh pollinator exclusion bags and left for approximately one week to allow for all or most of the flowers to open. Randomly selected flowers on an inflorescence were then assigned to one of three treatments: (1) cross-pollinated; (2) self-pollinated; and (3) control. Cross-pollinated flowers were pollinated with pollinia from flowers on a different plant, self-pollinated flowers were pollinated with pollinia from flowers on the same plant, and control flowers were left unmanipulated. Pollinia used for cross-pollinations were obtained from plants that were at least 5 m from the plant being pollinated to minimize inbreeding effects. Where possible, the number of flowers in each treatment on an inflorescence was kept equal. Hand-pollinations were performed using a fine (No. 2) pair of forceps. The corpusculum of a pollinarium was grasped with the forceps and the pollinarium gently removed from the flower. Each pollinium was then inserted with the convex surface innermost into the stigmatic chamber of a recipient flower (*cf.* Wyatt 1976). Pollinia were inserted into only one of the available stigmatic chambers of flowers being pollinated. In total, 141 flowers (47 per treatment) on 16 plants were used for these experiments.

Once an inflorescence had been pollinated, the mesh pollinator exclusion bag was replaced and the flowers were left to develop fruit. Once fruits were fully developed (*ca* 5 weeks after pollination), the bags were removed and the number of fruits from each treatment on an inflorescence was recorded. The number of seeds per fruit for each treatment on a plant was counted.

Natural levels of fruit and seed set were estimated for the Midmar Nature Reserve population. Fruit set per plant was estimated in January 2006. The number of fruits present on 34 randomly selected plants was counted at the end of the flowering season. Since plants are frequently multistemmed, the number of stems on each plant was noted and the number of fruits present on each plant was divided by the number of stems. A mean $\pm$ SE of these values was then obtained to represent the number of fruits per stem per plant for the population. Fruit set per flower were estimated from nine and eight randomly selected plants in the 2005–2006 and 2006–2007 flowering seasons, respectively. These plants were labeled and the number of flowers on each plant recorded early in the flowering season. Once all the flowers on a plant had either fallen off or developed into fruits, the fruit set for each plant was recorded.

A mean percentage of flowers that set fruit was calculated for each plant, and these means were used to calculate a grand mean ± SE percentage of flowers that set fruit for the population. Seed set per fruit was measured in January 2006. Eight mature fruits from separate randomly selected plants were dissected to count the number of seeds and a mean ± SE number of seeds per fruit was calculated. This was compared to the number of seeds per fruit present in fruits resulting from the hand pollinations. For this, all fruits obtained on a plant were averaged and the mean for each plant was used to represent seed set per fruit for that plant.

RESULTS

FLORAL VISITORS AND POLLINATOR EFFECTIVENESS.—Flowers of *X. undulatum* were visited by a large number of insects (comprising five different orders), which all obtained nectar. (Table S2). Of these, only the chafer *A. tigrina* (Fig. 1B) and the *Hemipepsis* spp. pompilid wasps (four morphologically similar species effectively representing a single functional group; see Fig. 1C) consistently carried pollinia (Table S2). Three other insect species were found to carry pollinia, but these insects were considerably smaller than both *A. tigrina* and the pompilid wasps and we believe they are unlikely to consistently effect pollinia removal and insertion in *X. undulatum*. A number of other insects were equally or more abundant than *A. tigrina* and the pompilid wasps, but never carried pollinia (Table S2). *Atrichelaphinis tigrina* and *Hemipepsis* spp. wasps were also the most widespread of the visitors, being found at six and seven of the study sites, respectively (Table S2). *Hemipepsis* spp. wasps were considerably more mobile than *A. tigrina* beetles and were often observed to be the only insects flying between plants.

Nectar is secreted at the base of the corona lobes and gathers between the corona lobes. *Hemipepsis* spp. wasps lap nectar from between the corona lobes and pollinia become attached to the hairs on the clypeus. Individuals of *H. hilaris* are typically smaller than the other *Hemipepsis* spp. and the pollinia are also attached to the mouthparts of this species. In contrast, *A. tigrina* beetles, being smaller, crawl inside individual flowers to reach the nectar (Fig. 1B). Pollinia are attached to tarsal hairs as the beetles crawl around in the flowers.

Atrichelaphinis tigrina individuals visited individual flowers for significantly longer periods of time than *Hemipepsis* spp. wasps (Mann–Whitney $Z = 10.2$, $P < 0.001$; Fig. 2). *Hemipepsis* spp. wasp visits were typically less than 30 sec long, in contrast to *A. tigrina* visits that were frequently longer than 300 sec (Fig. 2).

The cage experiments revealed that both *A. tigrina* and *Hemipepsis* spp. wasps are effective in removing and inserting pollinia (Table 1). Three flowers pollinated by *Hemipepsis* spp. wasps in the cage experiments developed fruits (Table 1). Three wasps from the first cage experiment carried pollinia at the end of the experiment (one had a whole pollinarium attached to a protarsal spine and two individuals each had a single corpusculum attached to clypeal hairs). None of the wasps involved in the third cage experiment were carrying pollinia at the end of the experiment. Both wasps from the second cage experiment went missing before they could be checked for pollinia. One *A. tigrina* from each of the beetle cage experiments had a whole pollinarium attached to a tarsal hair at the end of the experiment. Nine of the beetles from the second beetle cage experiment escaped during the course of the experiment and could thus not be checked for pollinia at the end of the experiment.

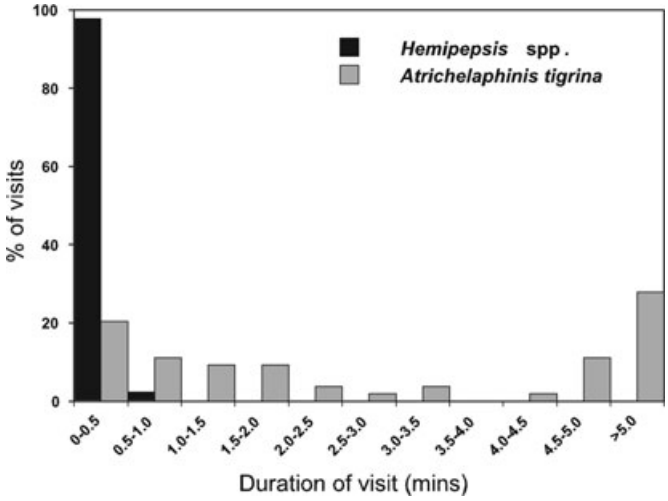

FIGURE 2. Duration of visits by *Hemipepsis* spp. wasps (Pompilidae) and *Atrichelaphinis tigrina* beetles (Scarabaeidae: Cetoniinae) to flowers of *Xysmalobium undulatum* at Midmar Nature Reserve. See text for statistical analysis.

NECTAR PROPERTIES.—The standing crop volume of nectar per flower was 8.5 ± 1.02 μl (mean ± SE) with a concentration of 72.9 ± 4.8 percent.

POLLINATION SUCCESS.—Plants at Vernon Crooks Nature Reserve and Villiers experienced similar pollination success with 21.7 (16.7) ± 7.47 percent and 21.7 (33.3) ± 5.45 percent (means [medians] ± SE) of flowers pollinated, respectively (Table 2). Pollination success was considerably lower for plants at Midmar Nature Reserve with only 1.7 (0) ± 1.67 percent of flowers pollinated. Differences between sites in the percentage of flowers pollinated were significant (Kruskal Wallis $\chi^2 = 7.99$, df = 2, $P = 0.018$; Table 2). PTE values showed similar trends, with Midmar (1.9%) being lower than both Vernon Crooks (16.4%) and Villiers (13.4%; Table 2). The number of pollinia removed and the number of pollinia inserted were both noticeably lower at Midmar than at the other two sites. These differences between sites for pollinia removals and insertions were significant ($\chi^2 = 10.0$, df = 2 , $P = 0.007$ and $\chi^2 = 8.42$, df = 2, $P = 0.015$, respectively).

BREEDING SYSTEM AND NATURAL FRUIT SET.—In the hand-pollination experiments, only outcrossed flowers developed fruits (Table 3). Percentage fruit set in naturally pollinated flowers was slightly higher in the 2006–2007 flowering season (Table 3), but these differences were not significant (Mann–Whitney $Z = 0.259$, $P = 0.80$). Fruits from naturally pollinated flowers

TABLE 2. *Pollination success and pollen transfer efficiency (PTE) in* Xysmalobium undulatum *at three different field sites. See text for statistical analysis.*

Site	Mean (median) ± SE percentage of flowers pollinated (/flower/plant)	Mean (median) ± SE pollinia removed (/flower/plant)	Mean (median) ± SE pollinia inserted (/flower/plant)	PTE (%)
Midmar Nature Reserve	1.7 (0) ± 1.7	0.87 (0.7) ± 0.22	0.02 (0) ± 0.17	1.9
Vernon Crooks Nature Reserve	21.7 (16.7) ± 7.5	2.13 (1.5) ± 0.66	0.35 (0.3) ± 0.13	16.4
Villiers	21.7 (33.3) ± 5.5	2.99 (2.7) ± 0.45	0.40 (0.5) ± 0.11	13.4

TABLE 3. *Fruit set in hand-pollinated and naturally pollinated* Xysmalobium undulatum *flowers at Midmar Nature Reserve.*

	Hand-pollinated % fruit set (*N*)			Naturally pollinated (mean ± SE)	
Flowering season	Control	Self	Cross	% fruit set (/flower/plant)	No. of fruits/plant (/stem/plant)
2005–2006	0 (47)	0 (47)	46.8 (47) [a]	0.2 ± 0.15	0.4 ± 0.09
2006–2007				0.7 ± 0.53	

[a] $\chi^2 = 52.1$, $P < 0.001$.

contained 326.1 ± 31.3 (mean ± SE, $N = 8$) seeds. This was not significantly different to the 376.7 ± 13.0 ($N = 9$) seeds per fruit obtained from flowers that had been cross-pollinated by hand ($t = 2.26$, $P = 0.17$).

DISCUSSION

The results of this study indicate that *X. undulatum* is genetically self-incompatible and, although visited by a range of different insects, is pollinated only by the chafer beetle *A. tigrina* and several morphologically similar pompilid wasps in the genus *Hemipepsis* (Table S2). *Atrichelaphinis tigrina* and the *Hemipepsis* spp. wasps were the only abundant insects that regularly carried pollinia and were both found at a wide range of study sites (Table S2). The effectiveness of both *A. tigrina* and the pompilid wasps as pollinators was confirmed by the successful removal and insertion of pollinia in controlled cage experiments with both these pollinator types (Table 1). We thus conclude that *X. undulatum* is an obligate outcrosser and exhibits a bimodal pollination system operated by the chafer beetle *A. tigrina* and pompilid wasps in the genus *Hemipepsis*.

Bimodal pollination systems have largely been described in the Iridaceae, but none of these examples involve chafer beetles or pompilid wasps (Manning & Goldblatt 2005). However, the orchid *S. microrrhynchum* is pollinated exclusively by chafer beetles and pompilid wasps (Johnson *et al.* 2007). It is interesting to note that the insects involved in the bimodal pollination system of *S. microrrhynchum* are precisely the same species which are involved in the *X. undulatum* system (Johnson *et al.* 2007). However, a wider range of insect species were recorded on flowers of *X. undulatum* (Table S2), which suggests that the mechanics of pollinarium attachment may play a larger role in the specificity of its pollination system than it does in *S. microrrhynchum*, which does not appear to attract insects other than chafer beetles and pompilid wasps (Johnson *et al.* 2007).

Our current understanding of specialized pollination by pompilid wasps and chafer beetles suggests a continuum between pompilid wasp and chafer beetle characteristics, with plant species that have bimodal systems, such as *S. microrrhynchum* and *X. undulatum*, lying somewhere in between. The chafer *A. tigrina* is known to be the specialist pollinator of several asclepiad and *Protea* species in South Africa (Ollerton *et al.* 2003; S.-L. Steenhuisen, pers. comm.). Nectar concentration in these specialist chafer-pollinated species is consistently low (*ca* 8–30% sugar; Ollerton *et al.* 2003; S.-L. Steenhuisen, pers. comm.). Likewise, *Hemipepsis* pompilid wasps are known to be specialist pollinators of a number of different asclepiads, three orchids, and two species of *Eucomis* (Hyacinthaceae) in South Africa (Steiner *et al.* 1994; Ollerton *et al.* 2003; Johnson 2005; Shuttleworth & Johnson 2006, 2008; A. Shuttleworth & S. D. Johnson, pers. obs.). However, specialist pompilid-pollinated species typically have highly concentrated nectar (*ca* 50–80% sugar), in contrast to the low concentrations found in the nectars of chafer-pollinated plants (Ollerton *et al.* 2003; Shuttleworth & Johnson 2006, 2008; A. Shuttleworth & S. D. Johnson, pers. obs.; but see Johnson 2005). The high nectar concentration in *X. undulatum* (*ca* 73% sugar) suggests that the evolution of nectar characteristics in this species has been driven by pompilid wasps rather than chafer beetles.

It is difficult to quantify the precise relative contribution of pompilid wasps and chafer beetles to fruit set in *X. undulatum*. The chafer *A. tigrina* was considerably more abundant (Table S2) and individuals frequently carried large numbers of pollinia. However, these beetles tend to spend large amounts of time visiting a single flower and were not as mobile between plants as the *Hemipepsis* spp. wasps (Fig. 2). *Hemipepsis* spp. wasps, by contrast, spent less than 30 sec visiting an individual flower and were frequently the only insects observed flying between plants. We believe the pompilids are more effective per unit time as pollinators. This idea is supported by the higher number of insertions (and subsequent fruit set) obtained

in the cage experiments with *Hemipepsis* spp. wasps (Table 1), although we cannot exclude the possibility that this reflects different responses by beetles and wasps to cage conditions. Furthermore, pollinia insertions and fruit set in *X. undulatum* appear to correlate better with wasp than with beetle abundance. Both fruit set and abundance of wasps (but not beetles) were low at Midmar Nature Reserve in the 2005–2006 season, while good pollination success was recorded at the Villiers site where wasps were common and beetles were absent (Table 2).

The PTE for *X. undulatum* flowers at Villiers and Vernon Crooks Nature Reserve was similar to PTE values recorded for other milkweed species (Ollerton *et al*. 2003; Shuttleworth & Johnson 2006, 2008; A. Shuttleworth & S. D. Johnson, pers. obs.). The remarkably low PTE value for Midmar Nature Reserve may have been the result of the scarcity of pompilid wasps at this site in that particular season (A. Shuttleworth, pers. obs.). *Atrichelaphinis tigrina* beetles were frequently observed to accumulate large pollen loads, suggesting that they may be less effective at depositing pollinia in comparison to the wasps.

Natural fruit set for *X. undulatum* was remarkably low. Although low fruit set is typical of milkweeds (Queller 1985), less than one percent of *X. undulatum* flowers set fruit in either of the two flowering seasons when fruit set was measured (Table 3). This is unlikely to be the result of resource limitation since *ca* 47 percent of flowers cross-pollinated by hand for the breeding system experiment developed fruit. The percentage fruit set was also considerably lower than the percentage of flowers that were pollinated, suggesting that most insertions were pollinia originating from the same plant. The breeding system results indicate that *X. undulatum* has a genetic self-incompatibility system that prevents fruits arising from self-pollination. Self-incompatibility is typical of milkweeds in the genus *Asclepias* (Wyatt & Broyles 1994; but see Lumer & Yost 1995, St Denis & Cappuccino 2004) and has also been recorded in the milkweeds *Gonolobus suberosus* (L.) R. Br. (Lipow & Wyatt 1998) and *Pachycarpus asperifolius* Meisn. (Shuttleworth & Johnson 2006).

We conclude that *X. undulatum* is an obligately outcrossing species that is pollinated only by pompilid wasps in the genus *Hemipepsis* and the chafer beetle *A. tigrina*. Further research is required to improve our understanding of the functional significance of floral scent, nectar, and morphology in bimodal pollination systems.

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SUPPLEMENTARY MATERIAL

The following supplementary material for this article is available online at: www.blackwell-synergy.com/loi/btp

Table S1. *Details of study sites and estimated observation times for each site*.

Table S2. *Visitors to* Xysmalobium undulatum *and their pollen loads*.

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SUPPLEMENTARY MATERIAL FOR CHAPTER 3

TABLE S1. *Details of study sites and estimated observation times for each site.*

Site Name (Province)[a]	Coordinates	Altitude (m asl)	Habitat	No. of plants	Estimated observation time (hours)
Amatikulu Nature Reserve (KZN)	29º08'58.0" S; 31º34'32.0" E	49	Moist grassland	*ca* 10	1
Hesketh Conservancy, Pietermaritzburg (KZN)	29º37'31.9" S; 30º25'31.2" E	850	Grassland surrounded by city	*ca* 15	10
Midmar Nature Reserve (KZN)	29º32'15.8" S; 30º10'13.1" E	1088	Moist montane grassland	*ca* 300	50
20 km from MooiRiver on R622 to Greytown (KZN)	29º10'48.5" S; 30º10'47.2" E	1432	Roadside grassland surrounded by farm lands	*ca* 10	1
Sinangwana (EC)	31º44'41.9" S; 29º22'50.8" E.	132	Coastal grassland	*ca* 30	10
10 km South of Villiers on R103 (FS)	27º06'11.9" S; 28º41'01.9" E	1565	Roadside grassland surrounded by farm lands	*ca* 50	1
Vernon Crooks Nature Reserve (KZN)	30º16'06.5" S; 30º37'14.5' E	447	Coastal grassland	*ca* 50	5
Wahroonga Farm (KZN)	29º36'35.9" S; 30º07'59.4" E	1350	Moist montane grassland	*ca* 10	1
Wodwo Farm (KZN)	29º24'08.1" S; 29º55'53.2" E.	1595	Montane grassland	*ca* 10	1

[a]KZN = KwaZulu-Natal Province, EC = Eastern Cape Province, FS = Free State Province

TABLE S2. *Visitors to* Xysmalobium undulatum *and their pollen loads.*

Visitor	Functional Group	No. observed (No. caught)	No. carrying pollinia (No. checked)	Placement of pollinia	Locality [a]
Hemiptera					
Lygaeidae					
Lygaeidae sp. 1	Lygaeid bug	195 (16)	0 (63)	None	M, H
Coleoptera					
Scarabaeidae					
Atrichelaphinis tigrina (Olivier, 1789)	Chafer beetle	308 (77)	47 (132)	Tarsal hairs	A, M, VC, S, Wa, Wo.
Cyrtothyrea marginalis (Swartz, 1817)	Chafer beetle	1 (1)	0 (1)	None	Mo
Plaesiorhinella plana (Wiedeman, 1821)	Chafer beetle	2 (2)	0 (2)	None	M, Mo.
Leucocelis haemorrhoidalis (Fabricius, 1775)	Chafer beetle	3 (2)	0 (2)	None	M
Porphyronota hebreae (Olivier, 1789)	Chafer beetle	1 (1)	0 (1)	None	M
Lycidae					
Lycidae sp. 1	Lycid beetle	1 (0)	Not checked	NA	M
Melyridae					
Astylus atromaculatus (Blanchard, 1843)	Melyrid beetle	2 (1)	0 (1)	None	M
Coccinellidae					
Coccinellidae sp. 1	Ladybird	1 (0)	0 (1)	None	M
Chrysomelidae					
Corynodes dejeani Bertoloni, 1849	Leaf beetle	8 (8)	0 (8)	None	M, VC.

Curculionidae					
Curculionidae sp. 1	Weevil	3 (2)	0 (3)	None	M
Curculionidae sp. 2	Weevil	1 (1)	0 (1)	None	M
Diptera					
Tachinidae					
Tachinidae sp. 1	Short-tongued fly	2 (2)	0 (2)	None	H
Calliphoridae					
Calliphoridae sp. 1	Short-tongued fly	11 (11)	1 (11)	Wing	M
Calliphoridae sp. 2	Short-tongued fly	1 (1)	0 (1)	None	M
Unidentified Calliphoridae	Short-tongued fly	169 (0)	0 (18)	None	M
Sarcophagidae					
Sarcophagidae sp. 1	Short-tongued fly	57 (0)	0 (6)	None	M
Tephritidae					
Tephritidae sp. 1	Short-tongued fly	9 (0)	0 (3)	None	M
Tephritidae sp. 2	Short-tongued fly	1 (0)	0 (1)	None	M
Muscidae					
Muscidae sp. 1	Short-tongued fly	1 (1)	0 (1)	None	M
Lepidoptera					
Noctuidae					
Noctuidae sp. 1	Moth	1 (0)	Not checked	NA	M
Nymphalidae					
Catacroptera cloanthe (Stoll, 1781)	Butterfly	30 (1)	0 (9)	None	M
Hymenoptera					

Argidae					
Argidae sp. 1	Sawfly	179 (15)	1 (55)	Rostrum	M
Tiphiidae					
Tiphia sp. 1	Tiphiid wasp	153 (18)	2 (55)	Tibia	H, M, S, VC.
Pompilidae					
Hemipepsis capensis (Linnaeus, 1764)	Pompilid wasp	23 (21)	4 (21)	Clypeal hairs	H, M, S, V, VC.
H. dedjas Guerin, 1848	Pompilid wasp	12 (4)	5 (6)	Clypeal hairs	M, S.
H. errabunda (Dalla Torre, 1897)	Pompilid wasp	14 (14)	0 (14)		M, V.
H. hilaris (Smith, 1879)	Pompilid wasp	15 (15)	1 (15)	Mouthparts	M
Hemipepsis spp. (not captured).[b]	Pompilid wasp	190 (0)	None checked	NA	H, M, S, V, VC, Wa, Wo.
Pompilinae sp. 1	Pompilid wasp	4 (1)	0 (1)	None	M
Pompilinae sp. 2	Pompilid wasp	1 (1)	0 (1)	None	H
Vespidae					
Polistes sp. 1	Vespid wasp	24 (5)	0 (5)	None	M
Apidae					
Apis mellifera Linnaeus, 1758	Bee	3 (3)	1[c] (3)	Claw	M

[a] A = Amatikulu, H = Hesketh Conservancy, M = Midmar Nature Reserve, Mo = Mooi River, S = Sinangwana, V = Villiers, VC = Vernon Crookes Nature Reserve, Wa = Wahroonga Farm, Wo = Wodwo Farm.

[b] These were all individuals of one of the four *Hemipepsis* species observed, but could not be identified to species level as they were not captured.

[c] This individual was found dead on a plant.

CHAPTER 4

NEW RECORDS OF INSECT POLLINATORS FOR SOUTH AFRICAN ASCLEPIADS (APOCYNACEAE: ASCLEPIADOIDEAE)

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New records of insect pollinators for South African asclepiads (Apocynaceae: Asclepiadoideae)

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Abstract

Studies of pollination in southern African asclepiads (aside from the stapeliads and members of the genus *Ceropegia*) are remarkably scarce given the diversity of asclepiad species in the region. In this study, we report new observations of insect flower visitors and their pollen loads for 15 species of South African asclepiads in the genera *Asclepias*, *Aspidoglossum*, *Miraglossum*, *Pachycarpus*, *Periglossum*, *Woodia* and *Xysmalobium*. Nectar properties are also presented for some species. Four specialized pollination systems are suggested by these observations: (1) pollination by wasps in the genus *Hemipepsis* (Hymenoptera: Pompilidae) in eight species, (2) pollination by chafer beetles (Scarabaeidae: Cetoniinae) in three species, (3) pollination by honeybees, *Apis mellifera* (Hymenoptera: Apidae) in two species, and (4) pollination by flies from various families in one species. The pollination system of *Asclepias crispa* remains unclear but appears to be one of generalized insect pollination. Future research is likely to confirm the preponderance of specialized pollination systems within this group of plants in southern Africa.

Keywords: Apocynaceae; Asclepiadoideae; Milkweed; Myophily; Pollination syndrome; Reproductive biology; Southern Africa; Spider-hunting wasp

1. Introduction

The pollination biology of southern African asclepiads (Apocynaceae subfamily Asclepiadoideae *sensu* Endress and Bruyns, 2000) is remarkably poorly studied compared with other regions, especially North America (see reviews by Wyatt and Broyles, 1994; Ollerton and Liede, 1997). The asclepiads have diversified tremendously in southern African grasslands, and South Africa is considered a centre of diversity and endemism for this group of plants (Victor et al., 2000). Approximately 600 species are currently described for southern Africa with 87% of these endemic to the region (Cowling and Hilton-Taylor, 1997; Victor et al., 2000). Knowledge of pollinator requirements is essential for conservation planning, especially given the high rates of habitat transformation in many of South Africa's grasslands.

Our current knowledge of the diversity of pollination systems in southern African asclepiads, apart from the succulent carrion-flower stapeliads and the genus *Ceropegia* (tribe Ceropegieae, see review by Meve and Liede, 1994; Ollerton et al., 2009), is somewhat limited. The earliest documented studies of asclepiad pollination in South Africa include descriptions of floral visitors to *Gomphocarpus*, *Periglossum* and *Woodia mucronata* (then known as *Xysmalobium linguaeforme*) in the Eastern Cape and on Table Mountain (Weale, 1873; Scott-Elliot, 1891). More recent studies have revealed a diversity of often specialized pollination systems within southern African asclepiads. These include specialized pollination by birds (Pauw, 1998), chafer beetles (Ollerton et al., 2003; Shuttleworth and Johnson, 2008), pompilid wasps (Shuttleworth and Johnson, 2006, 2008, 2009a,b,c), vespid wasps (Coombs et al., 2009) and possibly bees (Ollerton et al., 2003). Generalist insect pollination has also been described for several species (Liede and Whitehead, 1991; Ollerton et al., 2003). Nonetheless, pollination systems are known for a total of only 18 southern African asclepiad species excluding stapeliads and members of the genus *Ceropegia*.

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This study documents floral visitors (and likely pollinators) to a further 15 species of South African asclepiads in the genera *Asclepias*, *Aspidoglossum*, *Miraglossum*, *Pachycarpus*, *Periglossum*, *Woodia* and *Xysmalobium*. Rates of visitation to many of these species are typically low and visitor observations are consequently limited for some species. However, these have been included as they provide a valuable starting point for subsequent research. Furthermore, both *Woodia* species are listed as rare in the Red Data List of southern African plants (Hilton-Taylor, 1996) and knowledge of their pollinator requirements is essential for their conservation.

2. Methods

2.1. Study species and field sites

This study examined the pollination ecology of 15 perennial species of grassland asclepiad (Apocynaceae subfamily Asclepiadoideae *sensu* Endress and Bruyns, 2000) in the genera *Asclepias*, *Aspidoglossum*, *Miraglossum*, *Pachycarpus*, *Periglossum*, *Woodia* and *Xysmalobium* (Table 1; Figs. 1 and 2). Plant identifications were carried out with the assistance of Ashley Nicholas (University of KwaZulu-Natal, Westville) and were based on Langley (1980), Kupicha (1984), Smith (1988), Goyder (1998) and Nicholas (1999). Two of these species, *Woodia mucronata* and *W. verruculosa*, are listed as rare in the Red Data List of southern African plants (Hilton-Taylor, 1996). Voucher specimens of the species studied are deposited in the NU Herbarium (University of KwaZulu-Natal, Pietermaritzburg). This study was conducted over the course of five flowering seasons (between 2004 and 2009; see Table 1) at 12 sites in South Africa (Table 2).

2.2. Floral visitors, pollen loads and visitor behaviour

Floral visitors were recorded for all species and, where possible, representative specimens were collected for subsequent identification (Table 1). Pollen loads were determined for all collected individuals using a dissecting microscope. In some instances individual insects were inspected for pollinaria in the field and released. Representative insect specimens are deposited in the Natal Museum (Pietermaritzburg). The behaviour of pollinators and mechanism of pollinarium attachment was noted for species where sufficient visits were observed. Pompilid wasps were identified using keys given in Arnold (1932), Day (1979) and Goulet and Huber (1993). Chafer beetles were identified using Holm and Marais (1992). Visits by the beetle *Atrichelaphinis tigrina* to *Pachycarpus concolor* were inferred from the presence of the highly distinctive *Pa. concolor* pollinaria on individual beetles that were collected on the sympatrically occurring asclepiad *Xysmalobium undulatum* as part of a separate study (see Shuttleworth and Johnson, 2008). Visitors identified as *Hemipepsis* spp. (Table 1) are all individuals of one of the following species: *H. capensis*, *H. errabunda* or *H. hilaris*. These wasps are familiar to the authors from previous fieldwork but can usually only be identified to species where the individuals were collected or photographed.

2.3. Nectar properties

Total nectar production over a 24 h period was measured for five of the study species (Table 3). Flowers were bagged for 24 h prior to nectar sampling except for *Pachycarpus campanulatus* where plants were collected and kept in vases in the laboratory overnight (nectar present at the beginning of the 24 h period was removed with capillary tubes). The volume and the concentration (percentage sucrose equivalent by weight) of nectar were measured with 20 µl capillary tubes and a Bellingham and Stanley (0–50%) hand-held refractometer. Means were calculated per flower for each plant and these values used to calculate a grand mean for the species (see Table 3 for sample sizes).

3. Results

3.1. Floral visitors, pollen loads and visitor behaviour

Floral visitors suggest four distinct pollination systems in the species studied (Table 1): (1) pollination by *Hemipepsis* wasps (Hymenoptera: Pompilidae) in *Asclepias macropus*, *Aspidoglossum glanduliferum*, *Miraglossum pulchellum*, *Pachycarpus campanulatus*, *Periglossum angustifolium*, *Woodia verruculosa*, *W. mucronata* and *Xysmalobium stockenstromense* (Fig. 1); (2) pollination by chafer beetles (Scarabaeidae: Cetoniinae) in *Pa. concolor*, *Pa. scaber* and *Pachycarpus* sp. nov. (Fig. 2); (3) pollination by honeybees (*Apis mellifera*, Hymenoptera: Apidae) in *Asc. dregeana* and *Asc. gibba* (Fig. 2); and, (4) pollination by flies in *X. parviflorum* (Fig. 2). The pollination system of *Asc. crispa* is unclear, but this species appears to be a generalist insect-pollinated species.

Pollinaria were found on representative visitors to 11 of the 15 (73%) plant species studied (see Table 1 for summary and placement of pollinaria on insects). For eight of these plant species, pollinaria were carried by visitors belonging to a single functional group.

Hemipepsis wasps approach flowers with a zigzag flight path typical of insects tracking an odour plume (Raguso, 2006). In *Pa. campanulatus*, the flowers face down and wasps land on the outside of the corolla and crawl inside the large flowers. Once inside, the shape of the corona lobes forces the wasps to hang from the central column in order to access nectar (Fig. 1b) and in so doing, pollinaria are attached to their claws. In *Asc. macropus*, nectar gathers in the upward facing cup formed by the corona lobes. The small size of the flowers means that wasps accessing nectar from a particular flower cling to adjacent flowers and get pollinaria from these flowers attached to their claws (Fig. 1a). In *Asp. glanduliferum*, wasps land on the small flowers and hang below them whilst lapping the nectar (Fig. 1c). Pollinia are presumably attached to their mouthparts, although this was not actually observed. Weale (1873) provides detailed descriptions of wasp behaviour on *W. mucronata* (Fig. 1e).

Table 1
Insect visitors and their pollen loads for fifteen species of asclepiad.

Species and flowering times [a] (Estimated observation time/no. of seasons in which observed)	Visitors	No. observed (No. collected)	No carrying pollinaria (No. checked)	Pollinarium placement	Localities [b]	Flowering season
Asclepias crispa P.J.Bergius var. *plana* N.E.Br.	Coleoptera					
December–January	Scarabaeidae: Cetoniinae					
(1 h/1)	*Atrichelaphinis tigrina* (Olivier, 1789)	1 (0)	1 (1)	Hairs	S	2006/2007
	Diptera					
	Tabanidae					
	Tabanus sp. 1	4 (1)	1 (1)	Claws	S	2006/2007
	Calliphoridae					
	Calliphoridae spp.	10 (0)	None checked		S	2006/2007
	Sarcophagidae					
	Sarcophagidae spp.	5 (0)	None checked		S	2006/2007
Asclepias dregeana Schltr. var. *calceolus* (S.Moore) N.E.Br.	Coleoptera					
October–December	Scarabaeidae: Cetoniinae					
(4 h/2)	*Cyrtothyrea marginalis* (Swartz, 1817)	4 (3)	0 (3)		W	2008/2009
	Diptera					
	Calliphoridae					
	Calliphoridae sp. 1	1 (0)	0 (1)		W	2007/2008
	Hymenoptera					
	Apidae					
	Apis mellifera Linnaeus, 1758	17 (2)	1 (1)	Claws, proboscis	W	2007/2008; 2008/2009
Asclepias gibba (E.Mey.) Schltr. var. *gibba*	Coleoptera					
July–February	Scarabaeidae: Cetoniinae					
(3 h/2)	*Atrichelaphinis tigrina*	0 (1 [c])	1 (1)	Claws	M	2006/2007
	Diptera					
	Calliphoridae					
	Calliphoridae sp. 2	1 (1)	0 (1)		M	2006/2007
	Hymenoptera					
	Apidae					
	Apis mellifera	4 (2)	3 (3)	Claws	M	2006/2007; 2007/2008
Asclepias macropus (Schltr.) Schltr.	Coleoptera					
January–February	Scarabaeidae: Cetoniinae					
(20 h/3)	*Atrichelaphinis tigrina*	33 (13)	0 (13)		W, LB	2005/2006; 2007/2008
	Diptera					
	Sarcophagidae					
	Sarcophagidae spp.	2 (0)	None checked		W	2007/2008
	Hymenoptera					
	Tenthredinidae					
	Tenthredinidae sp. 1	1 (0)	None checked		W	2005/2006
	Pompilidae					
	Hemipepsis errabunda (Dalla Torre, 1897)	3 (3)	0 (3)		GC	2004/2005
	H. capensis (Linnaeus, 1764)	14 (14)	3 (14)	Claws	W, SP, GC	2004/2005; 2005/2006; 2007/2008
	H. hilaris (Smith, 1879)	14 (9)	2 (9)	Claws	W	2004/2005; 2005/2006; 2007/2008
	Hemipepsis spp.	53 (0)	None checked		W	2004/2005; 2005/2006; 2007/2008;
Aspidoglossum glanduliferum (Schltr.) Kupicha	Hymenoptera					

(continued on next page)

Table 1 (*continued*)

Species and flowering times [a] (Estimated observation time/no. of seasons in which observed)	Visitors	No. observed (No. collected)	No carrying pollinaria (No. checked)	Pollinarium placement	Localities [b]	Flowering season
September–January	Pompilidae					
(2 h/2)	*Hemipepsis capensis* [d]	2 (0)	None checked		W	2008/2009
	Hemipepsis sp.	1 (0)	Not checked		W	2007/2008
Miraglossum pulchellum (Schltr.) Kupicha	Hymenoptera					
October–January	Pompilidae					
(30 min/1)	*Hemipepsis capensis*	1 (1)	0 (1)		BN	2005/2006
Pachycarpus campanulatus (Harv.) N.E.Br. var. *campanulatus*	Hymenoptera					
November–February	Pompilidae					
(4 h/2)	*Hemipepsis capensis*	8 (4)	4 (5)	Claws	BN, W	2005/2006; 2007/2008
	H. hilaris	2 (2)	0 (2)		BN, W	2005/2006
	Halictidae					
	Halictidae sp. 1	1 (1)	0 (1)		W	2005/2006
Pachycarpus concolor E.Mey.	Coleoptera					
October–April	Scarabaeidae: Cetoniinae					
(20 h/3)	*Atrichelaphinis tigrina*	1 (25 [e])	26 (26)	Tibiae, tarsi	M	2005/2006; 2006/2007; 2007/2008
Pachycarpus scaber (Harv.) N.E.Br.	Hemiptera					
October–January	Lygaeidae					
(8 h/2)	Lygaeidae sp. 2	2 (0)	None checked		B	2008/2009
	Coleoptera					
	Scarabaeidae: Cetoniinae					
	Cyrtothyrea marginalis	131 (26)	4 (32)	Palps	B	2007/2008; 2008/2009
	Leucocelis adspersa (Fabricius, 1801)	8 (2)	0 (2)		B	2008/2009
	L. amethystina (MacLeay, 1838)	8 (2)	0 (2)		B	2007/2008
	L. haemorrhoidalis (Fabricius, 1775)	1 (1)	0 (1)		B	2007/2008
	L. rubra (Gory and Percheron, 1833)	2 (2)	0 (2)		B	2008/2009
	Scarabaeidae: Rutelinae					
	Rutelinae sp. 1	18 (7)	1 (7)	Mouthparts	B	2007/2008; 2008/2009
	Elateridae					
	Elateridae sp. 1	2 (0)	None checked		B	2007/2008
	Curculeonidae					
	Curculeonidae sp. 1	12 (2)	0 (2)		B	2007/2008
	Diptera					
	Diptera spp.	30 (0)	None checked		B	2007/2008
	Calliphoridae					
	Chrysomya chloropyga (Wiedemann, 1818)	1 (1)	0 (1)		B	2008/2009
	Calliphoridae sp. 3	2 (2)	0 (2)		B	2007/2008
	Muscidae					
	Orthellia sp. 1	10 (2)	0 (2)		B	2007/2008; 2008/2009
	Muscidae spp.	2 (0)	None checked		B	2007/2008
	Sarcophagidae					
	Sarcophaginae sp. 1	8 (3)	0 (3)		B	2007/2008; 2008/2009
	Hymenoptera					
	Tiphiidae					
	Tiphia sp. 1	1 (1)	0 (1)		B	2007/2008
	Pompilidae					
	Pepsinae sp. 1	1 (1)	0 (1)		B	2008/2009
	Apidae					
	Apis mellifera	1 (0)	None checked		B	2007/2008

Table 1 (*continued*)

Species and flowering times [a] (Estimated observation time/no. of seasons in which observed)	Visitors	No. observed (No. collected)	No carrying pollinaria (No. checked)	Pollinarium placement	Localities [b]	Flowering season
Pachycarpus sp. nov. [f]	Hemiptera					
November–December	Lygaeidae					
(3 h/2)	Lygaeidae sp. 1	1 (1)	0 (1)		Hi	2008/2009
	Coleoptera					
	Scarabaeidae: Cetoniinae					
	Atrichelaphinis tigrina	5 (3)	1 (3)	Tarsus	Hi	2008/2009
	Diptera					
	Diptera spp.	20 (0)	None checked		Hi	2007/2008
Periglossum angustifolium Decne.	Hymenoptera					
January–March	Pompilidae					
(6 h/1)	*Hemipepsis errabunda*	2 (2)	2 (2)	Mouthparts	M	2005/2006
	Hemipepsis spp.	9 (0)	7 (9)	Mouthparts	M, LB	2005/2006
Woodia mucronata (Thunb.) N.E.Br.	Coleoptera					
December–January	Scarabaeidae: Cetoniinae					
(1 h/1)	*Atrichelaphinis tigrina*	1 (0)	None checked		Ho	2007/2008
Red Data: Rare	Hymenoptera					
	Pompilidae					
	Hemipepsis spp.	5 (0)	None checked		Ho	2007/2008
Woodia verruculosa Schltr.	Hymenoptera					
October–February	Pompilidae					
(3 h/1)	*Hemipepsis capensis*	1 (1)	1 (1)	Mouthparts	M	2007/2008
Red Data: Rare	*Hemipepsis* sp.	1 (0)	Not checked		M	2007/2008
Xysmalobium parviflorum Harv. ex Scott-Elliot	Diptera					
October–April	Calliphoridae					
(3 h/1)	Calliphoridae sp. 4	3 (1)	2 (2)	Mouthparts	G	2007/2008
	Muscidae					
	Orthellia sp. 1	50 (3)	0 (3)		G	2007/2008
	Scathophagidae					
	Scathophaga sp. 1	1 (4)	1 (1)	Mouthparts	G	2007/2008
Xysmalobium stockenstromense Scott-Elliot	Hymenoptera					
November–January	Pompilidae					
(2 h/2)	*Hemipepsis capensis*	1 (1)	0 (1)		Se	2008/2009
	Hemipepsis spp.	5 (0)	None checked		Ho	2007/2008

[a] Flowering times taken from Pooley (1998) and Nicholas (1999), except for *Asclepias crispa* and *Woodia mucronata* which were inferred from our observations.
[b] B=Baynesfield, BN=Bushman's Nek, G=Gilboa Estate, GC=Giant's Castle, Hi=Highflats, Ho=Hogsback, LB=Lion's Bush Farm, M=Midmar Nature Reserve, S=Sinangwana, SP=Sani Pass, Se=Sentinel, W=Wahroonga Farm.
[c] This individual was captured carrying *Asclepias gibba* pollinaria, but was not directly observed visiting flowers.
[d] These individuals were identified from photographs.
[e] These individuals were collected on the sympatrically occurring asclepiad, *Xysmalobium undulatum*.
[f] This is a recently discovered species (M. Glenn, J. Lamb, A. Nicholas and A. Shuttleworth, unpubl. data).

Visits to *Pachycarpus concolor* and *Pachycarpus* sp. nov. were too limited to enable visitor behaviour to be described. In *Pa. concolor*, the widely spaced anther wings (forming the guide rails; Fig. 1f) combined with the placement of pollinaria on the tibiae and tarsi of visiting beetles suggests that the entire leg of the insect is trapped whilst accessing nectar. In *Pa. scaber*, the corona lobes curve back over the central column (Fig. 2b) and nectar gathers between ridges at the base of the corona lobe. Visiting beetles are thus forced to access the nectar from between the corona lobes (Fig. 2b). In doing so the palps are trapped between the guide rails and pick up the pollinaria.

In *Asc. dregeana*, flowers are suspended in an umbelliform inflorescence which faces down. Honeybees fly into the flowers from below and hang from the central column whilst probing for nectar. During this process, pollinaria are attached to the

Fig. 1. Plants pollinated by *Hemipepsis* wasps. (a) Female *H. capensis* visiting *Asclepias macropus*, Wahroonga Farm; (b) Male *H. capensis* visiting *Pachycarpus campanulatus*, Bushman's Nek. Note the pollinaria attached to tarsal claws (indicated by arrows); (c) Female *H. capensis* visiting *Aspidoglossum glanduliferum*, Wahroonga Farm; (d) Male *H. errabunda* visiting *Periglossum angustifolium*, Midmar Nature Reserve. Note the pollinaria attached to the mouthparts (indicated by arrow); (e) *Hemipepsis* sp. visiting *Woodia mucronata*, Hogsback; (f) *Xysmalobium stockenstromense* inflorescence. All scale bars = 10 mm.

claws as these get trapped between the guide rails (Fig. 2a). Observations of visits to *Asc. gibba* were insufficient to describe pollinator behaviour.

In *X. parviflorum*, the flowers are small and nectar appears to gather in the bottom of the cup formed by the corolla lobes (Fig. 2e). Flies visiting the flowers probe the base of the corolla, during which pollinaria are attached to their mouthparts and proboscides (Fig. 2e).

3.2. Nectar properties

Pachycarpus concolor and *Pa. scaber* (both chafer-pollinated) produced large amounts (3.7 and 6.6 µl respectively) of nectar with a concentration of 37% and 28% respectively (Table 3). *Pa. campanulatus* (*Hemipepsis* wasp pollinated) produced less nectar with a lower concentration (Table 3). The two honeybee-pollinated species (*Asc. dregeana* and

Fig. 2. Plants pollinated by honeybees, chafer beetles and flies. (a) *Apis mellifera* visiting *Asclepias dregeana*, Wahroonga Farm. Note the pollinaria attached to the tarsal claws and proboscis (indicated by arrows); (b) *Cyrtothyrea marginalis* visiting *Pachycarpus scaber*, Baynesfield; (c) *Asclepias gibba* flowers, Midmar Nature Reserve; (d) *Pachycarpus* sp. nov. flower, Highflats; (e) Calliphoridae sp. 4 visiting *Xysmalobium parviflorum*, Gilboa Estate. Note pollinarium attached to the proboscis (indicated by arrow); (f) *Pachycarpus concolor* flowers, Midmar Nature Reserve. All scale bars = 10 mm.

Asc. gibba) produced smaller amounts of nectar, but the concentration of *Asc. dregeana* nectar (54%) was considerably higher than that of *Asc. gibba* (36%; Table 3).

4. Discussion

The results of this study suggest that most of the asclepiad species studied have pollination systems which are highly specialized at the level of functional group. These groups are *Hemipepsis* pompilid wasps (for eight species of asclepiads), chafer beetles (for three species), honeybees (for two species) and flies (for one species) (Table 1). However, confirmation of the pollinator spectrum for some of the study species requires a larger sample of flower visitors and these results should thus be considered to be preliminary.

Table 2
Details of study sites and estimated population sizes for plants species.

Site name (province)[a]	Coordinates	Altitude (masl)	Habitat	Species (no. of plants)
Baynesfield (KZN)	29°45′13.3″ S; 30°21′29.9″ E	810	Rocky grassland	*Pachycarpus scaber* (c. 40)
Bushman's Nek, Drakensberg (KZN)	29°50′28.1″ S; 29°12′07.2″ E	1861	Montane grassland	*Miraglossum pulchellum* (c. 30) *Pachycarpus campanulatus* (c. 20)
Giant's Castle, Drakensberg (KZN)	29°13′06.0″ S; 29°33′18.0″ E	1600	Montane grassland	*Asclepias macropus* (c. 5)
Gilboa Estate (KZN)	29°16′30.7″ S; 30°16′45.0″ E	1607	Montane grassland	*Xysmalobium parviflorum* (c. 10)
Highflats (KZN)	30°16′10.3″ S; 30°12′09.3″ E	976	Annually mown grassland	*Pachycarpus* sp nov. (c. 30)
Hogsback (EC)	38°28′0.4″ S; 26°55′20.9″ E	1418	Montane grassland	*Woodia mucronata* (c. 15) *Xysmalobium stockenstromense* (c. 20)
Lion's Bush Farm (KZN)	29°24′25.1″ S; 29°56′19.6″ E	1476	Montane grassland	*Asclepias macropus* (c. 5) *Periglossum angustifolium* (c. 5)
Midmar Nature Reserve (KZN)	29°32′15.8″ S; 30°10′13.1″ E	1088	Moist montane grassland	*Asclepias gibba* (c. 30) *Pachycarpus concolor* (c. 45) *Periglossum angustifolium* (c. 10) *Woodia verruculosa* (c. 40)
Sani Pass, Drakensberg (KZN)	29°37′16.0″ S; 29°23′15.0″ E	1900	Montane grassland	*Asclepias macropus* (c. 5)
Sentinel, Drakensberg (FS)	28°42′54.0″ S; 28°53′43.0″ E	2400	Montane grassland	*Xysmalobium stockenstromense* (c. 15)
Sinangwana (EC)	31°44′41.9″ S; 29°22′50.8″ E.	132	Coastal grassland	*Asclepias crispa* var *plana* (c. 30)
Wahroonga Farm (KZN)	29°36′35.9″ S; 30°07′59.4″ E	1350	Moist montane grassland	*Asclepias dregeana* (c. 20) *Asclepias macropus* (c. 10) *Pachycarpus campanulatus* (c. 10)

[a] KZN = KwaZulu-Natal province, EC = Eastern Cape province, FS = Free State province.

Pollination by *Hemipepsis* pompilid wasps is now known for several South African asclepiads and these insects appear to be especially important as asclepiad pollinators within the region (Ollerton et al, 2003; Shuttleworth and Johnson, 2006, 2008, 2009a,b,c). Visitor observations and pollen load data are reasonably comprehensive for *Asclepias macropus* (Fig. 1a), *Pachycarpus campanulatus* (Fig. 1b) and *Periglossum angustifolium* (Fig. 1d), and these three species are clearly pollinated exclusively by these wasps (Table 1). Pollination by *Hemipepsis* wasps has not previously been described in the genus *Asclepias* but specialized pollination by these wasps is known in four other *Pachycarpus* species (*Pa. appendiculatus*, *Pa. asperifolius*, *Pa. grandiflorus* and *Pa. natalensis*; see Ollerton et al, 2003; Shuttleworth and Johnson, 2006, 2009a,c). The role of *Hemipepsis* wasps as the pollinators of *Pe. angustifolium* is consistent with early observations by Weale (1873) of visits by "a large black and yellow wasp … *Pallosoma*, one of the Pepsidae" to *Periglossum* in the Eastern Cape. The genus *Pallosoma* Lepeletier 1845 referred to by Weale (1873) is a synonym for *Hemipepsis* Dahlbom 1844 (Arnold, 1932). In addition, *Pe. angustifolium* pollinia have been found inserted between the guide rails of another exclusively *Hemipepsis* wasp pollinated species (*Xysmalobium orbiculare*) at a site near Midmar Nature Reserve (Shuttleworth and Johnson, 2009b; A. Shuttleworth, unpubl. data).

Table 3
Nectar properties for five of the asclepiad species studied.

Plant	Volume (μl)	Concentration (%)	Locality[a]
	Mean ± SE per flower per plant (no. of flowers/ no of plants)	Mean ± SE per flower per plant (no. of flowers/ no of plants)	
Asclepias dregeana	0.7 ± 0.46 (28/3)	54 ± 0.3 (12/2)	W
Asc. gibba	0.2 ± 0.04 (50/16)	36 ± 1.8 (20/10)	M
Pachycarpus campanulatus	0.9 ± 0.21 (12/5)	21 ± 2.2 (7/5)	BN
Pa. concolor	3.7 ± 1.23 (35/20)	37 ± 4.4 (25/15)	M
Pa. scaber	6.6 ± 1.63 (30/6)	28 ± 1.9 (30/6)	M

[a] BN = Bushman's Nek, M = Midmar Nature Reserve, W = Wahroonga Farm.

Observations of visitors to flowers of *Aspidoglossum glanduliferum*, *Miraglossum pulchellum*, *Woodia verruculosa*, *W. mucronata* and *Xysmalobium stockenstromense* were more limited (Table 1). However, *Hemipepsis* wasps were the only insects observed to visit these plants (except for a single chafer beetle on *W. mucronata*) and the floral characteristics of these species (see Fig. 1) appear to be consistent with a guild of cryptic flowers that are pollinated by *Hemipepsis* wasps (Ollerton et al., 2003; Johnson, 2005; Johnson et al., 2007; Shuttleworth and Johnson, 2006, 2008, 2009a,b,c,d). Pollination by *Hemipepsis* wasps is known for two other *Miraglossum* species (*M. verticillare* and *M. pilosum*) and two other *Xysmalobium* species (*X. orbiculare* and *X. undulatum*; Ollerton et al, 2003; Shuttleworth and Johnson, 2008, 2009b). *Hemipepsis* wasps have also been observed visiting *M. anomalum* (S.D. Johnson, unpubl. data). Weale (1873) provides detailed descriptions of the behaviour of "*Pallosoma*" (now *Hemipepsis*) wasps on "? *Xyomalobium* [sic.] *linguaeforme* ?" in the Eastern Cape. "*Xyomalobium linguaeforme*" presumably refers to *Xysmalobium linguaeforme* Harv ex. Weale which has subsequently been classified as *Woodia mucronata* (Victor et al., 2000, 2003). The results of our study, supplemented with Weale's (1873) observations, suggest that *Woodia mucronata* is indeed a *Hemipepsis* wasp specialist and it seems likely that *W. verruculosa* is similarly reliant on *Hemipepsis* wasps.

Specialized pollination by chafer beetles (Scarabaeidae: Cetoniinae) has been described in three South African

asclepiads (*Asclepias woodii*, *Sisyranthus trichostomus* and *Xysmalobium involucratum*; Ollerton et al., 2003). We suggest that specialized chafer pollination systems also occur in the genus *Pachycarpus*. Our observations for *Pa. scaber* are relatively comprehensive and this species appears to be pollinated almost exclusively by the chafer *Cyrtothyrea marginalis* (Fig. 2b) although a single monkey beetle (Scarabaeidae: Rutelinae) was also found to be carrying pollinia (Table 1). Visitor observations to *Pa. concolor* and *Pachycarpus* sp. nov. (Fig. 2d, f) were more limited, but suggest that these species are pollinated primarily by the chafer beetle *Atrichelaphinis tigrina*.

Apart from chafer beetles, *Pachycarpus* sp. nov. was also visited by a large number of flies (Table 1). However, we believe the delicate nature of the flies' legs makes them unlikely to systematically remove and insert pollinaria on the large and relatively robust flowers (Fig. 2d), although we cannot rule out the possibility that flies may contribute to the pollination of this species. *Pachycarpus* sp. nov. is currently known from only a single site near the village of Highflats in KwaZulu-Natal (M. Glenn, J. Lamb, A. Nicholas and A. Shuttleworth, unpubl. data) and its pollinator requirements should be assessed for its conservation.

In the case of *Pa. concolor*, only a single visit by *A. tigrina* was observed. However, a large number of these beetles were collected on the sympatric *X. undulatum* and found to be carrying *Pa. concolor* pollinaria (Table 1). Furthermore, the pollinaria on these beetles were frequently reduced to just the corpusculum suggesting successful insertion of individual pollinia in *Pa. concolor* flowers. *Pachycarpus concolor* pollinia are considerably larger than the stigmatic grooves on *X. undulatum* flowers and were thus unlikely to have been inserted into the grooves of these flowers. Furthermore, *Pa. concolor* pollinia were never discovered inserted in *X. undulatum* flowers from Midmar Nature Reserve when these were being inspected for removal and insertion rates of pollinia in a separate study (see Shuttleworth and Johnson, 2008). The flowers of *X. undulatum* are very attractive to *A. tigrina* beetles (Shuttleworth and Johnson, 2008) and the presence of a large *X. undulatum* population alongside the population of *Pa. concolor* at Midmar Nature Reserve may partly explain the low visitation rates of *A. tigrina* to *Pa. concolor* at this site (Table 1).

Pollination by bees (honeybees and halictids) has been suggested for *Aspidonepsis diploglossa* and *Asc. cucullata* (Ollerton et al., 2003). Our observations suggest that *Asc. dregeana* (Fig. 2a) and *Asc. gibba* (Fig. 2b) are pollinated primarily by honeybees (Table 1). Honeybees were the most abundant of the visitors to both species and appeared to be the most important pollen vectors (Table 1). However, a single chafer beetle (*A. tigrina*) was collected carrying *Asc. gibba* pollinia and these beetles may also contribute to the pollination of this species.

Myophily in asclepiads has typically been associated with the succulent stapeliads and members of the genus *Ceropegia* (Asclepiadoideae: Ceropegieae *sensu*; Endress and Bruyns, 2000; see review by Meve and Liede, 1994; Ollerton et al., 2009). Our observations suggest that *X. parviflorum* is pollinated exclusively by flies (Fig. 2e). This is consistent with the results of pollinator observations reported for this species by Johnson et al. (in press). This therefore represents the first record of myophily outside of the stapeliads and the genus *Ceropegia* in a southern African asclepiad. *Xysmalobium parviflorum* has a very powerful faecal odour (resulting from the production of high levels of *p*-Cresol by the flowers; A. Shuttleworth, unpubl. data) and flies are undoubtedly attracted by the strong odour of these flowers.

The pollination system of *Asclepias crispa* remains unclear. Pollinaria were found on a chafer beetle (*A. tigrina*) and on a tabanid fly suggesting that this species may have a more generalized pollination system than other species examined in this study (Table 1). However, visitor observations were limited and further research is required to determine the pollinator profile of this species.

The chafer-pollinated species reported here (*Pa. scaber* and *Pa. concolor*) produce large amounts of nectar (3–7 µl per flower) with a concentration of ±30% sugar (Table 3). These volumes are greater than those recorded for chafer-pollinated species by Ollerton et al. (2003) although the concentrations are comparable. The *Hemipepsis* wasp pollinated *Pa. campanulatus* produced a relatively low amount (less than 1 µl per flower) of dilute (21%) nectar (Table 3), in contrast with other *Hemipepsis* pollinated species which typically (but not always, see Ollerton et al., 2003; Johnson, 2005) produce copious amounts of very concentrated nectar (Ollerton et al., 2003; Shuttleworth and Johnson, 2006, 2008, 2009a,b,c,in press). The honeybee-pollinated *Asc. dregeana* and *Asc. gibba* produced low amounts of moderately concentrated nectar (Table 3), comparable with the putatively bee-pollinated species suggested by Ollerton et al. (2003).

The majority of the pollination systems documented in this study appear to be highly specialized. This contrasts with studies of North American asclepiads which typically have generalized pollination systems (cf. Fishbein and Venable, 1996). This study also adds several new species to a growing list of South African plants that are pollinated exclusively by *Hemipepsis* wasps. The high morphological diversity that has developed among southern African asclepiads suggests that future research is likely to reveal further interesting pollination systems within this diverse group of plants.

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CHAPTER 5

SPECIALIZED POLLINATION BY LARGE SPIDER-HUNTING WASPS AND SELF-INCOMPATIBILITY IN THE AFRICAN MILKWEED *PACHYCARPUS ASPERIFOLIUS*

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SPECIALIZED POLLINATION BY LARGE SPIDER-HUNTING WASPS AND SELF-INCOMPATIBILITY IN THE AFRICAN MILKWEED *PACHYCARPUS ASPERIFOLIUS*

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Specialized pollination systems in flowers with exposed nectar are difficult to explain because there are usually no morphological traits, such as long spurs, that could function to exclude particular flower visitors. Observations of the milkweed *Pachycarpus asperifolius* in KwaZulu-Natal, South Africa, showed that its flowers are visited mainly by large spider-hunting wasps belonging to the genus *Hemipepsis* (Hymenoptera: Pompilidae), despite producing copious amounts of nectar in an exposed position. Cage experiments showed that these wasps are effective in removing and depositing *P. asperifolius* pollinaria. Pollinaria become attached to the palps (and, to a lesser extent, the legs) of wasps. Palps are frequently broken, either when they become jammed in the guide rails or when pollinia are inserted. To try to understand why *P. asperifolius* flowers are visited almost exclusively by wasps, we presented droplets of nectar and control sugar solutions of the same concentration (ca. 70%) to honeybees (*Apis mellifera scutellata*). Honeybees readily consumed the sugar solutions but rejected *P. asperifolius* nectar, suggesting that secondary compounds in *P. asperifolius* nectar may deter insects other than pompilid wasps. Experimental hand pollinations conducted in the field showed that *P. asperifolius* is genetically self-incompatible and thus completely reliant on pollinators for seed production. We conclude that *P. asperifolius* is specialized for pollination by large pompilid wasps and that its nectar functions as the primary filter of flower visitors.

Keywords: Apocynaceae, antagonism, nectar, pollination syndromes, self-incompatibility, breeding system.

Introduction

The milkweeds (Apocynaceae subfamily Asclepiadoideae *sensu* Endress and Bruyns 2000) have long been considered a model system for the study of plant pollination (cf. Willson and Price 1977; Ollerton and Liede 1997; Kephart and Theiss 2004). In particular, the aggregation of milkweed pollen into pollinia (also found in orchids) makes it easy to estimate male and female components of pollination success and also to identify pollen-carrying insects. Studies of North American milkweeds have provided textbook examples of plants with generalized pollination systems (cf. Fishbein and Venable 1996). However, the few studies that have been conducted on African milkweeds suggest that the generalized pollination systems of North American milkweeds may not be typical of other regions (Ollerton et al. 2003).

Milkweeds have diversified tremendously in Africa, especially in southern Africa, where there are ca. 600 species (Victor et al. 2000). The milkweed genus *Pachycarpus* E. Mey. is endemic to Africa and contains some 40 species occurring in grasslands south of the Sahara (Smith 1988). Twenty species are found in southern Africa, with 15 of these occurring in KwaZulu-Natal (Smith 1988; Pooley 1998, 2003; Nicholas 1999). Apart from a single record for *Pachycarpus natalensis* (Ollerton et al. 2003), there have been no published studies of pollination within the genus, and nothing is known about breeding systems in the genus. In this study, we investigated the reproductive biology of the southern African milkweed *Pachycarpus asperifolius* Meisn., after preliminary observations indicated that its flowers are visited mainly by pompilid wasps.

Because flower-feeding wasps generally have short mouthparts, they tend to feed from generalist flowers with exposed nectar (Nilsson 1981; Kephart 1983; Proctor et al. 1996; but see Gess and Gess 1989). Floral specialization for pollination by wasps is well known in figs and deceptive orchids but appears to be rare among nectar-rewarding plants. Flowers pollinated by nectar-seeking wasps tend to exhibit dull perianth colors and unusual fragrances (Nilsson 1981; Johnson 2005). Wasps are generally less hairy than bees and are thus not considered efficient vectors of granular pollen; however, orchids and asclepiads pollinated by wasps are capable of either gluing or clipping pollinia onto hairless parts of the bodies of wasps (Nilsson 1981; Ollerton et al. 2003; Johnson 2005).

Among asclepiads (Apocyncaceae: Asclepiadoideae), specialization for pollination by vespid wasps (Hymenoptera: Vespidae) is known in South American *Oxypetalum* (Vieira and Shepherd 1999; J. Ollerton, unpublished data) and *Blepharodon* species (J. Ollerton, unpublished data). *Gomphocarpus physocarpus* is pollinated mainly by vespid wasps in South Africa (S. D. Johnson, unpublished data). This same plant species has been introduced to Australia, where it is pollinated by a variety of Vespidae, Pompilidae, and Ichneumonidae (Forster 1994).

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Pompilid wasps (Hymenoptera: Pompilidae) have been recorded among the visitors to flowers of a number of generalist North American *Asclepias* (Robertson 1928; Kephart 1979), South American *Oxypetalum* (Vieira and Shepherd 1999), and South African *Xysmalobium* species (Ollerton et al. 2003). Specialized pollination by pompilid wasps has been recorded in three South African asclepiads: *Miraglossum pilosum*, *Miraglossum verticillare*, and *P. natalensis* (Ollerton et al. 2003).

Breeding system data are generally scarce for asclepiads, probably because it is difficult to hand pollinate the flowers, which often have small pollinaria. Wyatt and Broyles (1994) reviewed studies on compatibility systems in the genus *Asclepias* (the only genus in the subfamily that had been studied at that stage) and found that most are self-incompatible. However, there appears to be considerable variation in the breeding systems of asclepiads. Self-compatibility and even autogamy have been reported in *Vincetoxicum rossicum* (Lumer and Yost 1995; St. Denis and Cappuccino 2004), while *Gonolobus suberosus* was found to be completely self-incompatible (Lipow and Wyatt 1998). Levels of self-compatibility may vary even among and within populations of asclepiads (Ivey et al. 1999; Lipow et al. 1999; Lipow and Wyatt 2000; Leimu 2004). The aims of this study of *P. asperifolius* were to describe the mechanism and level of specialization in the pollination system, to quantify the efficiency of pollen transfer in populations, and to determine the breeding system.

Material and Methods

Study Species

Pachycarpus asperifolius is a robust, erect herb ranging in size from 300 to 1000 mm. It is found in South African grasslands on rocky slopes from the Eastern Cape Province through KwaZulu-Natal Province to the Northern Province (Pooley 1998). Plants have a mean ± SE of 25.5 ± 1.54 ($n = 50$) flowers per plant. Flowers are a dull greenish white or yellowish green color, have strongly reflexed corolla lobes, and measure 11–28 mm in diameter (fig. 1*A*; Pooley 1998). Corona lobes are small and saccate and dark purplish red in color. A faint but discernible scent is emitted by the flowers. The flowering season is from October to March (Pooley 1998).

Study Sites

This study was conducted at three sites in the KwaZulu-Natal Province of South Africa. The first site was situated in Vernon Crooks Nature Reserve (lat. 30°16′S, long. 30°37′E; altitude 550 m). This site has a population of ca. 150 plants occurring in grassland on rocky slopes near the eastern boundary of the reserve. The second site was at Hesketh Nature Reserve (lat. 29°37′S, long. 30°25′E; altitude 850 m) in Pietermaritzburg. This site has a small population of ca. 20 plants growing in open grassland. The last site was situated on the edge of the Old Main Road (R103) between Pietermaritzburg and Durban, ca. 2 km before Monteseel (lat. 29°44′12″S, long. 30°39′58″E; altitude 750 m). This site has a small roadside population of ca. 15 plants. The Monteseel site was used only to measure pollen transfer efficiency (PTE) and pollination success.

Floral Visitors

Floral visitors were observed at Vernon Crooks and Hesketh throughout the flowering seasons from October 2004 to January 2005 and from October 2005 to January 2006. The total observation time was ca. 120 h spread over these 8 mo. All insects observed visiting flowers were recorded, and at least one individual of each species was captured and pinned. Insects that were observed carrying pollinaria in the field were noted, and all pinned insects were examined for the presence and position of pollinaria. At the end of the two flowering seasons, the number of individuals that were observed visiting flowers was estimated for each species of visiting insect. The length of the pompilid wasps (these were the only insects found to carry pollinaria, with the exception of a single lygaeid bug) was measured from the vertex to the tip of the abdomen with digital calipers. All insects were identified to family level following Scholtz and Holm (1996). Pompilid wasps were identified to species level using keys given by Arnold (1932), Day (1979), and Goulet and Huber (1993).

The number of flowers visited per plant and the length of time spent on each flower was measured for pompilid wasps with a digital dictaphone. Pompilids were observed at Hesketh and Vernon Crooks for these measurements. The mean ± SE number of flowers visited per plant and the mean ± SE time spent per flower were calculated for each pompilid species and for the total data set with all three species combined. Data for an additional two pompilid individuals that were not identified (but were one of the three species observed) were included in the analysis of the combined data set.

Some of the pollinia inserted in flowers of *P. asperifolius* examined in the cage experiments (see below) were observed to be attached to broken-off sections of insect palps. Broken-off insect palps were also observed between the guide rails of some flowers examined in the field and from the cage experiments. This suggested that *P. asperifolius* pollinaria might be carried on the palps of pompilids and that these palps are sometimes broken off when they become trapped between the guide rails or when the pollinia are inserted. This idea was tested by using a dissecting microscope to inspect the palps of all pompilids caught visiting *P. asperifolius* in the 2004–2005 flowering season. The percentage of wasps with at least one palp broken or missing was compared between individuals captured at the study sites and those captured on several other asclepiad species and *Eucomis autumnalis* (Hyacinthaceae) at Howick (lat. 29°26′S, long. 30°14′E; altitude 1300 m) and Gilboa Estate (lat. 29°17′S, long. 30°17′E; altitude 1650 m).

Nectar Production and Palatability Experiments

Nectar production over a 24-h period was measured for 31 flowers from six randomly selected plants at Vernon Crooks Nature Reserve in the 2005–2006 flowering season. Any nectar present on these flowers was rinsed off with water, and the flowers were allowed to dry. Once dry, the flowers were bagged with fine-mesh pollinator exclusion bags and left for 24 h. The volume and concentration (sucrose equivalent percentages by weight) of nectar were then determined for five

flowers on five of the plants and six flowers on one of the plants. Nectar volume was measured using 20-μL capillary tubes. Nectar concentration was measured using a Bellingham and Stanley handheld refractometer. To bring the nectar concentration within the range of the refractometer (0%–50%), each nectar sample was diluted with an equal volume of water directly on the glass plate of the refractometer. The resulting measured concentration was then doubled to obtain the actual nectar concentration. A mean volume and concentration were calculated per flower for each plant, and each of these mean values was then used to calculate a grand mean ± SE per flower.

The nectar of *P. asperifolius* is exposed and often produced in copious amounts. However, this nectar is not utilized by common nectar-feeding insects, such as honeybees (*Apis mellifera scutellata*), which are common at the study sites. The nectar of this species has an unpleasant bitter taste to humans, suggesting that the nectar may be unpalatable to nectar-feeding insects other than pompilids. To test whether the nectar of *P. asperifolius* is unpalatable to honeybees, individual bees were captured and placed in small glass vials for between 10 and 20 min. They were then offered a choice between ca. 1–5-μL droplets of *P. asperifolius* nectar and sugar solutions of identical volumes and concentrations made up of either sucrose or hexose sugars (a 1 : 1 mixture of glucose and fructose). Nectar was obtained directly from plants, using a 20-μL capillary tube, and was used immediately. Sugars were dissolved in water, and the solutions were diluted to match the sugar concentration of the nectar. The droplets of nectar and the two sugar solutions were placed on a petri dish in a triangular configuration. A vial containing a bee was then placed upside down over the three solutions so that the bee could crawl down and consume the solutions on the petri dish. The solutions that were either selected or rejected by each bee were noted. A solution was considered to have been selected if the bee consumed all (or nearly all) of the solution on the petri dish. A solution was considered to have been rejected if the bee probed but did not consume the solution. In total, 20 bees were tested, and each bee was used only once. Bees did not always sample all three solutions, and in instances where a bee did not probe a particular solution, that bee was not counted in the data for that solution. The percentages of droplets of the nectar and each of the sugar solutions that were rejected by bees were compared.

Pollinator Effectiveness

Pompilid wasps, unlike bees, are remarkably unaffected by laboratory cage conditions. Wasps placed in a flight cage with *P. asperifolius* flowers will immediately commence feeding and show behavior that is apparently identical to that exhibited in the field. Laboratory cage experiments were conducted to test the effectiveness of various pompilid species as pollinators. Plants in bud were bagged with fine-mesh pollinator exclusion bags in the field and left for ca. 2 wk to allow the flowers to open. Plants bearing these virgin flowers were then cut at ground level and taken back to the laboratory, where they were placed for at least 24 h in a 1-m^3 fine-mesh cage with newly caught pompilid wasps that did not carry pollinaria at the start of the experiment. After the experiment, wasps were killed with ethyl acetate, pinned, and examined under a dissecting microscope for the presence of pollinaria and to determine whether they had intact palps. Before the experiment it was established that all wasps had at least two intact palps. The number of removed and inserted pollinia in flowers was also determined using a dissecting microscope.

Three cage experiments were conducted. The first two were aimed at investigating the pollen transfer effectiveness of different-sized wasps, while the third was aimed at establishing whether fruit set results from visits by pompilid wasps. Since *P. asperifolius* plants grow in very rocky habitats, it was not possible to use rooted plants in pots in the cage experiments. However, cut stems placed in water remained turgid for 4–5 wk, which is long enough to observe initial fruit development.

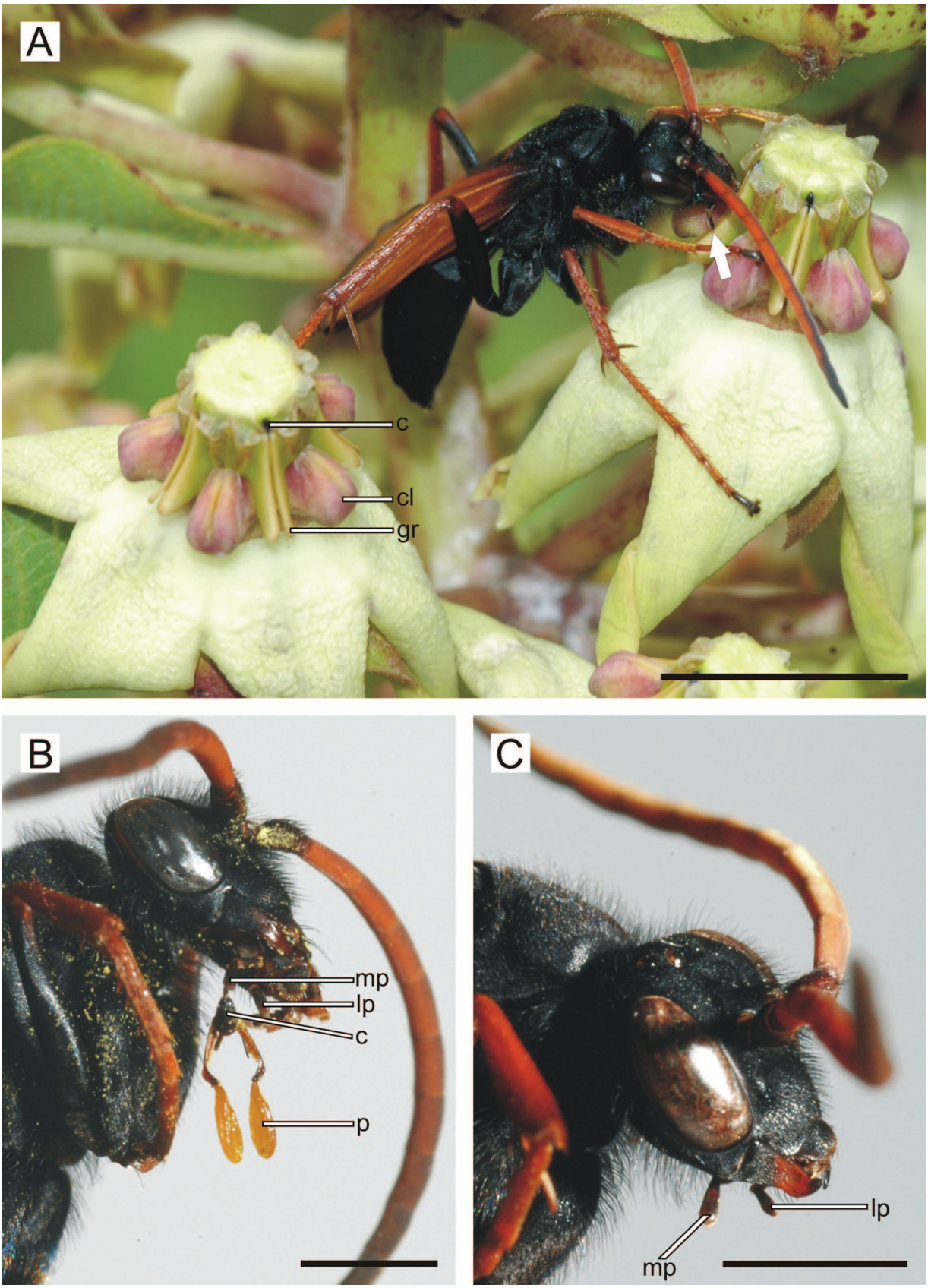
The first and second cage experiments were conducted from December 14 to 16, 2004, and from December 21 to 22, 2004, respectively. In the first cage experiment, two virgin plants, one with eight and one with 12 open flowers, were placed in the cage with four individuals of *Hemipepsis gestroi* Gribodo and a single individual of *Hemipepsis capensis* (Linnaeus) captured at Wahroonga Farm (lat. 29°37′S, long. 30°08′E; altitude 1350 m). These wasps were naive, since *P. asperifolius* does not occur at the site where they were captured. In the second cage experiment, two virgin plants, one with eight and one with 11 open flowers, were placed in the cage with six individuals of *Hemipepsis hilaris* (Smith) captured in Vernon Crooks Nature Reserve. These wasps were captured visiting *P. asperifolius* and were thus not naive. The *H. gestroi* and *H. capensis* individuals used in the first cage experiment were all >25 mm long (from the vertex to the tip of the abdomen), while the *H. hilaris* individuals used in the second cage experiment were all <15 mm long.

The third cage experiment was conducted from December 22 to 27, 2005. For this experiment, five virgin plants with a total of 76 open flowers were placed in the cage with five individuals of *H. capensis* (all >25 mm long) captured at Midmar Nature Reserve (lat. 29°31′S, long. 30°10′E; altitude 1090 m), where *P. asperifolius* does not occur naturally. The wasps in this experiment all had at least both maxillary palps intact. Plants were maintained in water for ca. 5 wk after the experiment had been terminated, to allow for fruit development. After 5 wk, all flowers were removed and inspected for pollinia removals and insertions and for swollen ovaries indicative of early fruit development.

Individuals of *H. gestroi* used in the cage experiments are almost certainly the same species as the "*Hemipepsis dedjas*" wasps observed visiting *P. asperifolius* at Hesketh (see "Results"). The *H. gestroi* individuals used were all males, while the individual *H. dedjas* Guerin was female. Using Arnold's (1932) key, the males keyed out to *H. gestroi*, while the female keyed out to *H. dedjas* var. *spinosior*. However, both have been observed in association with one another in the field. It appears that the species *H. dedjas* Guerin var. *spinosior* Arnold is in fact the same species as *H. gestroi* Gribodo (A. Shuttleworth, unpublished data). For the purposes of this article, this species will henceforth be referred to as *H. dedjas* (*H. gestroi*).

Pollination Success

The frequency of pollinia removal and insertion was determined for 82 flowers on 10 plants at Vernon Crooks Nature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Reserve, 59 flowers from seven plants at Hesketh Nature Reserve, and 70 flowers from nine plants at Monteseel in the 2004–2005 flowering season. Flowers were examined using a dissecting microscope in the laboratory or a ×10 hand lens in the field. The mean number of pollinia removed per flower and mean number of stigmatic chambers with a pollinium inserted per flower were calculated for each plant from a site, and a mean of these values was obtained to represent the population mean for each site. The percentage of flowers pollinated (containing at least one inserted pollinium) was calculated for each plant, and a mean was obtained from these values to represent the percentage of flowers pollinated at each site. Data for number of pollinia removed, number of stigmatic chambers with a pollinium inserted, and percentage of flowers pollinated were arcsine–square root transformed and analyzed using a one-way ANOVA. The PTE in each population was calculated as the percentage of removed pollinia that were inserted between guide rails (cf. Johnson et al. 2004).

Breeding System and Natural Fruit Set

The degree of self-compatibility and capacity for autogamy in *P. asperifolius* was determined using controlled hand pollinations at the Vernon Crooks and Hesketh Nature Reserve sites. These experiments were conducted in December 2004 and January 2005.

Plants in bud were bagged with fine-mesh pollinator exclusion bags and left for ca. 2 wk to allow all or most of the flowers to open. Individual flowers on an inflorescence were then assigned to one of three treatments: (i) cross-pollinated, (ii) self-pollinated, and (iii) control. Cross-pollinated flowers were pollinated with pollinia from flowers on a different plant, self-pollinated flowers were pollinated with pollinia from flowers on the same plant, and control flowers were left unmanipulated. Pollinia used for cross-pollinations were obtained from plants that were at least 5 m from the plant being pollinated in order to minimize inbreeding effects. Where possible, the number of flowers in each treatment on an inflorescence was kept equal. Hand pollinations were performed using fine forceps. The corpusculum of a pollinarium was grasped with the forceps and the pollinarium gently removed from the flower. Each pollinium was then inserted with the convex surface innermost into the stigmatic chamber of a recipient flower (cf. Wyatt 1976). Pollinia were inserted into all five of the available stigmatic chambers of flowers being pollinated. In total, 182 flowers on 18 plants were used for these experiments. Of these plants, four were in Hesketh Nature Reserve and 14 were in Vernon Crooks Nature Reserve.

Once an inflorescence had been pollinated, the mesh pollinator exclusion bag was replaced, and the flowers were left to develop fruit. Once fruits were fully developed (ca. 12–14 wk after pollination), the bags were removed, and the number of fruits from each treatment on an inflorescence was recorded. The number of seeds per fruit for each treatment on a plant was counted.

Natural levels of fruit and seed set were estimated for the Vernon Crooks Nature Reserve population. Fruit set per plant was estimated in both the 2004–2005 and the 2005–2006 flowering seasons. Fruits were counted on 68 randomly selected plants in January 2005 and from 14 plants on which the number of flowers had been previously recorded during December 2005. Ten fruits from the 2004–2005 flowering season were collected and dissected in the laboratory to determine seed set. The mean number of seeds per fruit was calculated from these 10 fruits. In the 2005–2006 season, the percentage of flowers that set fruit was calculated by dividing the number of fruits observed by the original number of flowers recorded for each of the 14 plants mentioned above. We then obtained a mean of these values to represent fruit set in the population.

Results

Visitor Observations and Nectar Measurements

Pompilid wasps (ca. 265 individuals observed) were by far the most abundant visitors to flowers of *Pachycarpus asperifolius* at the study sites, and, apart from a single lygaeid bug, they were the only insects found to carry pollinaria (table 1). Three pompilid species were identified from the two sites: *Hemipepsis capensis* and *Hemipepsis dedjas* (*Hemipepsis gestroi*) were found at both Vernon Crooks and Hesketh, while *Hemipepsis hilaris* was found only at Vernon Crooks. Although only six individuals of *H. dedjas* (*H. gestroi*) were captured, ca. 40 individuals were observed visiting flowers in the field. Individual plants were frequently observed being visited by large numbers (ca. 10) of pompilid wasps at a given time. *Hemipepsis hilaris*, the smallest pompilid species (ca. 12–25 mm in length), was generally more abundant than the larger pompilids, *H. capensis* (15–30 mm) and *H. dedjas* (*H. gestroi*) (25–35 mm). Female wasps tended to be larger than males, except in the case of *H. dedjas* (*H. gestroi*), in which the sexes are similar in size.

Overall, pompilids ($n = 18$) visited a mean ± SE of 10.0 ± 1.75 flowers per plant and spent a mean ± SE of 9.4 ± 0.63 s probing each flower. *Hemipepsis dedjas* (*H. gestroi*) individuals tended to visit the fewest number of flowers per plant, while *H. hilaris* individuals visited the most (table 1).

Pollinaria were found attached to 12 (four males and eight females) of the 29 individuals of *H. capensis* and *H. dedjas* (*H. gestroi*) that were captured. In addition, a single female *H. capensis* was observed removing a pollinarium from a *P. asperifolius* flower at Hesketh but was not captured. None of the 25 captured individuals of *H. hilaris* carried pollinaria.

Pollinaria were found to be attached to the palps (either maxillary or labial) (fig. 1*B*) and tarsal spines. In total, nine

Fig. 1 *Pachycarpus asperifolius* and its pollinators. *A*, Close-up of flowers showing floral structure and a male *Hemipepsis capensis* lapping nectar from around the corona lobe and alongside the guide rails. Note the maxillary palp inserted between the guide rails (indicated by arrow). Scale bar = 10 mm. *B*, Pollinarium attached to the maxillary palp of *H. capensis*. Scale bar = 2 mm. *C*, Individual of *H. capensis* with broken maxillary and labial palps. Scale bar = 2 mm. *c* = corpusculum; *cl* = corona lobe; *gr* = guide rail; *lp* = labial palp; *mp* = maxillary palp; *p* = pollinium.

Table 1

Insects Recorded as Visitors to Flowers of *Pachycarpus asperifolius* at Vernon Crooks and Hesketh Nature Reserves

Insect visitor	Number observed on *P. asperifolius*	Individuals captured[a]	Pollinaria placement	Mean ± SE flowers visited per plant[b]	Mean ± SE time per flower (s)[b]	Study sites
Hemipepsis capensis (Hymenoptera: Pompilidae)	ca. 75	23 (8)	Palps, tarsus	7.8 ± 6.75 (2)	10.9 ± 1.55 (2)	VC and H
Hemipepsis dedjas (*Hemipepsis gestroi*) (Hymenoptera: Pompilidae)	ca. 40	6 (4)	Palps, tarsi	10.6 ± 2.47 (10)	5.3 ± 0.33 (10)	VC and H
Hemipepsis hilaris (Hymenoptera: Pompilidae)	ca. 150	25 (0)	...	13.0 ± 3.56 (4)	16.3 ± 1.54 (4)	VC
Sphecidae sp. 1 (Hymenoptera)	ca. 50	12 (0)	...	...	...	VC
Tiphiidae sp. 1 (Hymenoptera)	ca. 50	4 (0)	...	...	...	VC and H
Vespidae sp. 1 (Hymenoptera)	4	4 (0)	...	...	...	VC and H
Cetoniinae sp. 1 (Coleoptera: Scarabaeidae)	6	6 (0)	...	...	...	VC
Melyridae sp. 1 (Coleoptera)	1	1 (0)	...	...	...	VC
Lygaeidae sp. 1 (Hemiptera)	1	1 (1)	Rostrum	...	...	H
Lygaeidae sp. 2 (Hemiptera)	1	1 (0)	...	...	...	VC
Reduviidae sp. 1 (Hemiptera)	1	1 (0)	...	...	...	H

Note. Pollen loads, number of flowers visited per plant, and time spent per flower are given for the principal pollinators. VC = Vernon Crooks; H = Hesketh.

[a] Number in parentheses is the number of individuals carrying pollinaria.

[b] Number in parentheses is the number of wasps used for that measurement.

pollinaria were found attached to palps, and seven pollinaria were attached to tarsal spines. The frequency of *H. capensis* and *H. hilaris* wasps with broken or missing palps was significantly greater for individuals caught on *P. asperifolius* than for individuals caught on other plant species ($G = 23.98$, $P < 0.001$; table 2), leading us to suspect that palps are frequently broken off during the pollination process. The single *H. dedjas* (*H. gestroi*) individual caught visiting *P. asperifolius* at Hesketh was missing both maxillary palps.

Several other wasps (unidentified Vespidae, Sphecidae, and Tiphiidae) were observed on the flowers, but, except for Sphecidae sp. 1, these were mostly infrequent (table 1). Of the insects other than pompilids caught or observed visiting flowers, only a single lygaeid (Hemiptera) was found to be carrying a pollinarium (attached to the rostrum).

Nectar Production and Palatability Experiments

Pachycarpus asperifolius secretes copious amounts of relatively concentrated nectar. The mean ± SE volume of nectar produced during 24 h was 18.4 ± 2.8 μL per flower. The mean ± SE nectar concentration was 72.7% ± 3.6% sucrose equivalent by weight. This nectar gathers at the sides of the corona and around the base of the guide rails (see Kunze 1997). Visiting wasps lap nectar from the sides of the corona and base of the guide rails (fig. 1*A*). Pollinaria are removed if one of the wasp's palps slides up through the guide rails during feeding (fig. 1*A*, arrow). However, palps caught between the guide rails do not always remove pollinaria. Wasps were occasionally observed to pull away from the flower before the palp reached the corpusculum, either pulling the palp out from between the guide rails or snapping the palp off in the stigmatic chamber.

The nectar of *P. asperifolius* appears to be unpalatable to honeybees. Nectar droplets that were probed by bees were rejected on 94% of occasions, while only 13% of the sucrose and 6% of the hexose (glucose and fructose) droplets were rejected after being probed ($G = 34.24$, $P < 0.001$; fig. 2).

Pollinator Effectiveness

In the first cage experiment (involving the large pompilids *H. dedjas* [*H. gestroi*] and *H. capensis*), 15 out of 20 (75%) of the flowers were effectively pollinated (having at least one pollinium properly inserted). In total, 32 pollinia (on 16 pollinaria) were removed, and 28 were inserted. Five corpuscula with both pollinia removed were also found inserted in stigmatic chambers. Four wasp palps were found broken off

Table 2

Percentage of Wasps with Broken or Missing Palps in a Sample Caught While Visiting *Pachycarpus asperifolius* and a Sample Caught While Visiting *Eucomis autumnalis* (Hyacinthaceae) and Several Other Asclepiad Species

Wasp species	Wasps with broken or missing palps (%): Visiting *P. asperifolius*	Wasps with broken or missing palps (%): Visiting other asclepiad species and *E. autumnalis*	*G*	*P*
Hemipepsis capensis	80 (20)	20 (15)	12.67	<0.001
Hemipepsis hilaris	82 (17)	29 (21)	11.15	<0.001
Both species combined	81 (37)	25 (36)	23.98	<0.001

Note. Wasps visiting *P. asperifolius* were caught at Vernon Crooks and Hesketh Nature Reserves. Wasps visiting *E. autumnalis* and other asclepiad species were caught at Howick and Gilboa Estate, where *P. asperifolius* does not occur. Numbers in parentheses are the total numbers of wasps caught.

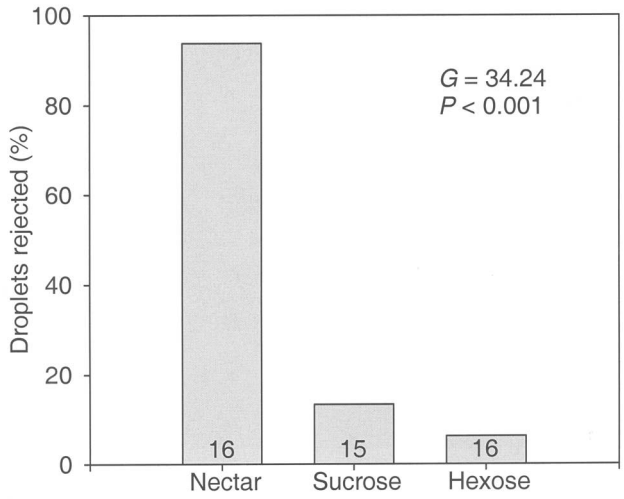

Fig. 2 Percentage of trials in which honeybees rejected a droplet of *Pachycarpus asperifolius* nectar, sucrose, or hexose (glucose and fructose mixed 1 : 1) solution in a choice test to determine the palatability of *P. asperifolius* nectar to honeybees. Sample sizes are shown at the base of the bar and represent the total number of droplets that were tasted by a bee.

between guide rails, while a further six palps with attached corpuscula were found broken off, some attached to pollinia that had been inserted in the stigmatic chambers. All wasps used in the first experiment had broken palps. Three of the five wasps had all four palps broken off, while the remaining two had three palps broken off. Thus, 18 of the 20 palps (four per wasp) were broken. A single pollinium was still attached to a palp on one of the *H. dedjas* (*H. gestroi*) individuals, while a corpusculum with both pollinia removed was attached to the palp of another. A corpusculum with both pollinia removed was attached to a spine on the right metatarsus of the *H. capensis* individual.

In the second cage experiment (involving the small pompilid *H. hilaris*), none of the flowers were pollinated, and no pollinaria were removed from any of the flowers, despite frequent visits by the wasps. In addition, none of the flowers contained broken-off palps. *Hemipepsis hilaris* individuals did not lose palps during this experiment.

In the third cage experiment, 28 out of 76 (37%) of the flowers had at least one pollinium inserted. In total, 134 pollinia (on 67 pollinaria) were removed and 43 were inserted. Three flowers developed fruits.

Three of the five wasps in this experiment each had two palps broken off. Four (40%) of the 10 maxillary palps that were present at the start of the experiment had been removed. Two pollinaria were still attached, one to a tarsal spine and one to a claw. Two corpuscula, each with one pollinium removed, were attached to claws, while a third was attached to a tibial spine. One corpusculum with both pollinia removed was attached to a tibial spine, one to a tarsal spine, and one to a claw.

Pollination Success

The mean number of pollinia removed per flower did not differ significantly between the three sites ($F_{2,23} = 0.19$, $P = 0.828$). The percentage of flowers pollinated was highest at Vernon Crooks (25.5 ± 8.37) and lowest at Monteseel (7.7 ± 3.35; table 3), but these differences were not significant ($F_{2,23} = 2.23$, $P = 0.130$). The number of pollinia inserted per flower was not significantly different between populations ($F_{2,23} = 2.51$, $P = 0.130$) but showed trends similar to those of the percentage of flowers pollinated. The PTE was considerably higher at Vernon Crooks (42.7%) than at Hesketh (19.0%) and Monteseel (15.0%; table 3).

Breeding System and Natural Fruit Set

Fruits formed only from outcrossed flowers at both sites (table 4). Two of the fruits resulting from hand pollinations consisted of two follicles, while the remainder all consisted of only a single follicle. Overall, 32% of outcrossed flowers set fruit, as opposed to the zero fruit set obtained in both the self-pollinated and control treatments ($\chi^2 = 43.49$, $P < 0.001$; table 4).

Of 68 plants checked for natural fruit set in January 2005, 52 (76.5%) had no fruits, 12 (17.6%) had a single fruit, three (4.4%) had two fruits, and one (1.5%) had four fruits. The mean ± SE number of fruits per plant in this flowering season was 0.3 ± 0.08 ($n = 68$). Of 14 plants checked for natural fruit set in December 2005, 11 (79%) had no fruits and three (21%) had a single fruit. The mean ± SE number of fruits per plant in this flowering season was 0.2 ± 0.11 ($n = 14$). Fruits from naturally pollinated flowers always consisted of a single follicle. The proportion of plants that set fruit was not significantly different between the two flowering seasons ($G = 0.03$, $P = 0.868$). Of the 14 plants checked for natural fruit set in December 2005, the mean ± SE percentage of flowers that set fruit was 1.1% ± 0.6%. Naturally pollinated fruits contained a mean ± SE of 219.4 ± 13.60 seeds in the 2004–2005 season, a similar value to the 211.6 ± 17.69 seeds in fruits that had been cross-pollinated by hand ($t = 2.131$, $P = 0.730$).

Discussion

The results of this study indicate that *Pachycarpus asperifolius* is genetically self-incompatible, and thus an obligate outcrosser, and that its flowers are pollinated almost exclusively by large pompilid wasps, such as *Hemipepsis capensis* and *Hemipepsis dedjas* (*Hemipepsis gestroi*). Field observations and the cage experiments show that these larger pompilids (>20 mm in length) are abundant plant visitors and are effective at removing and inserting *P. asperifolius* pollinia. Although flowers of *P. asperifolius* are also visited by large numbers of a smaller pompilid (*Hemipepsis hilaris*), this species does not appear to be effective as a pollinator. Aside from the pompilid wasps, flowers are also visited by several other insect species, but none of these appear to be capable of removing and inserting pollinia (table 1).

The utilization of only two pompilid species (effectively a single functional group) for pollination suggests that the pollination system of *P. asperifolius* is highly specialized (Johnson and Steiner 2000). This is despite the presence of a number of common flower visitors, such as bees, butterflies, and flies, on other plant species flowering in the same habitat as *P. asperifolius*.

Table 3

Pollination Success and Pollen Transfer Efficiency (PTE) of *Pachycarpus asperifolius* Flowers in Three Populations

Study site	Population size	No. plants sampled	Mean ± SE percentage of flowers pollinated	Mean ± SE pollinia removed (per flower per plant)	Mean ± SE pollinia inserted (per flower per plant)	PTE (%)
Vernon Crooks	ca. 150	10	25.5 ± 8.37	0.57 ± 0.217	0.32 ± 0.084	42.7
Hesketh	ca. 20	7	9.1 ± 8.06	0.66 ± 0.324	0.15 ± 0.142	19.0
Monteseel	ca. 15	9	7.7 ± 3.35	0.56 ± 0.153	0.08 ± 0.033	15.0

Note. There were no significant differences between populations for the measures of pollination success (see text for details).

One of the possible reasons why other insect species seldom visit flowers of *P. asperifolius* is that its nectar is very concentrated and appears to contain secondary compounds (A. Shuttleworth, unpublished data) that deter insects such as honeybees (fig. 2). Although honeybees prefer nectars with a sugar concentration of 30%–50% by weight (Butler 1945; Waller 1972; Baker 1975; Baker and Baker 1983) and show a distinct aversion to solutions containing a sucrose concentration of more than 50% by weight (Waller 1972), this cannot be the sole reason for their rejection of *P. asperifolius* nectar because hungry bees readily consumed sugar solutions of equal concentration to *P. asperifolius* nectar (fig. 2). It is also unlikely that the actual sugar composition (not yet known for *P. asperifolius*) plays a role because bees consumed both hexose and sucrose solutions. Preliminary investigations indicate that nectar of *P. asperifolius* has a relatively high phenolic content (A. Shuttleworth, unpublished data). These results, taken together, suggest that *P. asperifolius* has a specialized pollination system, partly because its nectar acts as a filter of flower visitors (cf. Johnson et al. 2006). Three other asclepiad species known to be specialized for pollination by pompilid wasps (*Miraglossum pilosum*, *Miraglossum verticillare*, and *Pachycarpus natalensis*) also have highly concentrated nectar (50%–70% by weight; Ollerton et al. 2003) but have not been investigated in terms of secondary compounds.

Aside from nectar properties, *P. asperifolius* flowers display further characteristics of specialization in that they are cryptic and dull colored (fig. 1*A*), with spectra similar to the background foliage and lacking in ultraviolet reflectance (A. Shuttleworth and S. D. Johnson, unpublished data). Pompilids often approach plants from downwind (A. Shuttleworth and S. D. Johnson, personal observation) and exhibit a typical zigzag flight pattern (see Johnson 2005). This suggests that wasps rely primarily on a scent rather than a visual cue to find plants. Further research is thus also required to determine the role of scent as a selective floral attractant in *P. asperifolius*.

The pollinaria of *P. asperifolius* are attached to the palps and legs of the larger pompilids during feeding. It is likely that the smaller size of most of the other wasp species allows them to access nectar without getting their body parts trapped between the guide rails. It is clear that the palps of pompilid wasps visiting *P. asperifolius* flowers are often removed (table 2). This was shown by the finding that wasps with broken palps were more common among individuals caught on flowers of *P. asperifolius* than on flowers of other species (table 2) and by the correspondence between pollinia transfer and loss of palps by wasps in the cage experiments. Thus, the loss of palps is a direct consequence of foraging on these particular flowers.

The palps of larger pompilids appear to break off in a number of situations. Palps can be broken when they become trapped between guide rails and the wasp pulls away before the palp reaches the corpusculum. This is evidenced by the remains of palps in between the guide rails of flowers examined in the field (A. Shuttleworth and S. D. Johnson, personal observation) and from the cage experiments. Alternatively, palps may be broken off if they carry a corpusculum with both pollinia removed and the corpusculum gets trapped between the guide rails of a flower. This was observed in several of the flowers in the cage experiments. Finally, palps to which corpuscula of inserted pollinia are attached may be broken off, as was observed in flowers examined in the field (A. Shuttleworth and S. D. Johnson, personal observation) as well as in the cage experiments. Further evidence for the pollination of *P. asperifolius* by large pompilids is provided by observations of *H. dedjas* visiting this species at a site between White River and Sabie in Mpumalanga Province, South Africa (lat. 25°03′35″S, long. 30°55′31″E) (C. Peter and J. Ollerton, personal communication). One of two males collected at this site was missing all four palps. The high number of broken palps in *H. hilaris* individuals captured while visiting *P. asperifolius* in the field suggests that these wasps also get their palps caught between the guide rails but are not effective at removing the pollinaria (table 2). This could be for two reasons. First, the pollinaria of *P. asperifolius* are relatively large and may require that the corpusculum be attached to an appendage that can withstand a certain amount of force in order to effect removal. The palps of smaller wasps may not be strong enough to do this and thus tend to get broken off in the corpusculum. Alternatively, the palps may just be broken off between the guide rails, before they actually reach the corpusculum. The latter explanation is more likely because no palps were observed in unremoved corpuscula. Interestingly, another *Pachycarpus* species, *P. natalensis*, with smaller guide rails and stigmatic chamber, is pollinated exclusively by

Table 4

Percentage Fruit Set Obtained in Each of the Hand-Pollination Treatments

Study site	Fruit set (%)			χ^2	P
	Control	Self	Outcrossed		
Vernon Crooks	0 (43)	0 (45)	30 (46)	29.91	<0.001
Hesketh	0 (16)	0 (16)	38 (16)	13.71	<0.001
Both sites combined	0 (59)	0 (61)	32 (62)	43.49	<0.001

Note. Numbers in parentheses are numbers of flowers.

H. hilaris individuals that carry the pollinaria on their mouthparts (Ollerton et al. 2003).

The fitness consequences for wasps of the loss of their palps is not easy to quantify. Palps have a sensory function in insects and are used to locate and test the quality of food before ingestion (Chapman 1971; Gullan and Cranston 2005). Wasps without palps appeared to be able to locate and feed on nectar in flowers. However, if damaged or missing palps seriously reduce the efficiency of feeding or cause the ingestion of unsuitable or low-quality nectar, then the interaction between *P. asperifolius* and pompilids may be antagonistic rather than mutualistic. Morse (1981) found that 30%–40% of the bumblebees that pollinate the milkweed *Asclepias syriaca* lose claws and tarsal segments when they become entangled in the guide rails of this species during foraging. The loss of these appendages was shown to reduce the foraging ability of the bumblebees by about 25% (Morse 1981). Further research to establish the cost of broken palps to *Hemipepsis* wasps would contribute to our general understanding of the roles of antagonism versus mutualism in plant-pollinator interactions.

The PTE was relatively high (15%–42%) in all of the populations (table 3). It was markedly higher in the Vernon Crooks population, where ca. 43% of all removed pollinia were successfully inserted into stigmas. The relatively low PTE for the smaller populations may be due to wasps showing less constancy and departing from the population with attached pollinia. There was some evidence of this at Hesketh, where two individuals of *H. capensis* captured while visiting another asclepiad species (*Xysmalobium undulatum*) were found to be carrying *P. asperifolius* pollinaria. Further research is, however, required to explore the possibility of a general relationship between PTE and population size in plants.

Pollinia removal from flowers did not vary significantly among the three populations (suggesting similar rates of visitation by wasps), but the greater PTE in the Vernon Crooks population translated into a relatively higher percentage (27%) of flowers pollinated at this site (table 3). However, fruits were set in <1% of all flowers in this population and in <24% of plants. Although low fruit set is often typical of milkweeds (Queller 1985; Kephart 1987), in the case of *P. asperifolius*, this is unlikely to be due to resource limitation because we found that ca. 32% of all cross-pollinated flowers in the breeding system experiment developed fruits. The most likely explanation for the low fruit set is that most of the inserted pollinia originated from the same plant, and, according to the breeding system results (table 4), they would not result in fruit set because of a self-incompatibility system. This is supported by our data on the foraging behavior of pompilids, which showed that wasps usually visit at least 10 flowers on a plant and spend ca. 9 s probing each flower (table 1). In plants with fewer flowers, wasps were occasionally observed to visit all the flowers on the plant and even to return to some of the flowers that they had already probed. In future studies, we plan to quantify the incidence of geitonogamous pollinium insertions in this species.

There appears to be a guild of southern African plant species that are specialized for pollination by *Hemipepsis* wasps (cf. Ollerton et al. 2003; Johnson 2005). Further research is required to better understand the role of pompilids as pollinators, as well as the extent of convergent evolution in the morphology, color, scent, and nectar of flowers they pollinate.

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CHAPTER 6

A KEY ROLE FOR FLORAL SCENT IN A WASP-POLLINATION SYSTEM IN *EUCOMIS* (HYACINTHACEAE)

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A key role for floral scent in a wasp-pollination system in *Eucomis* (Hyacinthaceae)

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• *Background and Aims* Floral scent may play a key role as a selective attractant in plants with specialized pollination systems, particularly in cases where floral morphology does not function as a filter of flower visitors. The pollination systems of two African *Eucomis* species (*E. autumnalis* and *E. comosa*) were investigated and a test was made of the importance of scent and visual cues as floral attractants.
• *Methods and Key Results* Visitor observations showed that *E. autumnalis* and *E. comosa* are visited primarily by pompilid wasps belonging to the genus *Hemipepsis*. These wasps carry considerably more *Eucomis* pollen and are more active on flowers than other visiting insects. Furthermore, experiments involving virgin flowers showed that these insects are capable of depositing pollen on the stigmas of *E. autumnalis*, and, in the case of *E. comosa*, pollen deposited during a single visit is sufficient to result in seed set. Experimental hand-pollinations showed that both species are genetically self-incompatible and thus reliant on pollinators for seed set. Choice experiments conducted in the field and laboratory with *E. autumnalis* demonstrated that pompilid wasps are attracted to flowers primarily by scent and not visual cues. Measurement of spectral reflectance by flower petals showed that flowers are cryptically coloured and are similar to the background vegetation. Analysis of headspace scent samples using coupled gas chromatography–mass spectrometry revealed that *E. autumnalis* and *E. comosa* scents are dominated by aromatic and monoterpene compounds. One hundred and four volatile compounds were identified in the floral scent of *E. autumnalis* and 83 in the floral scent of *E. comosa*, of which 57 were common to the scents of both species.
• *Conclusions* This study showed that *E. autumnalis* and *E. comosa* are specialized for pollination by pompilid wasps in the genus *Hemipepsis* and achieve specialization through cryptic colouring and the use of scent as a selective floral attractant.

Key words: *Eucomis*, Pompilidae, wasp pollination, breeding system, pollination syndrome, pollinator shift, floral volatile, floral filter.

INTRODUCTION

Plants with specialized pollination systems typically have complex morphology, such as floral spurs, that limits access by certain animals to rewards (Johnson and Steiner, 2000). Specialized pollination systems in plants with open and morphologically unspecialized flowers are more difficult to explain as they usually have rewards which are readily accessible to a range of different potential visitors. Some of these plants have toxic nectar which filters out certain flower visitors (Stephenson, 1981, 1982; Adler 2000; Johnson *et al.*, 2006; Shuttleworth and Johnson, 2006). Another possibility, still poorly documented, is that a combination of cryptic flower colour and a particular scent blend may allow morphologically unspecialized flowers to selectively attract specific pollinators (cf. Johnson, 2005; Johnson *et al.*, 2007; Brodmann *et al.*, 2008).

The genus *Eucomis* (Hyacinthaceae), commonly known in Africa as 'pineapple flowers', contains 11 species that occur in forest, grassland and wetland areas of southern Africa (Williams, 2000). *Eucomis* flowers are structurally unspecialized (see Fig. 1) and typically produce large amounts of exposed nectar. While these traits would normally be associated with generalist pollination systems, preliminary observations suggest that pollination systems in the genus are usually specialized and remarkably variable among species, ranging from rodent pollination in *E. regia* (S. D. Johnson, unpubl. res.) to pollination by spider-hunting wasps (Hymenoptera: Pompilidae) in *E. autumnalis* and *E. comosa* (this study). We hypothesized that *Eucomis autumnalis* and *E. comosa* have specialized pollination systems and achieve specialization through the use of cryptic colouring and floral scent as an attractant.

The specific aims of this study were: (*a*) to identify the most effective pollinators of *E. autumnalis* and *E. comosa*; (*b*) to determine the nectar properties of these plants' flowers; (*c*) to determine whether these plant species have breeding systems that make them reliant on pollinators for reproduction; (*d*) to determine if pollinators are attracted by floral scent or visual cues; (*e*) to determine the chemical composition of the floral fragrance; and (f) to determine if the spectral reflectance of the flower petals is similar to that of the background vegetation.

MATERIALS AND METHODS

Study species and field sites

Eucomis autumnalis (Mill.) Chittenden [subsp. *clavata* (Bak.) Reyneke used in this study] and *E. comosa* Houtt. ex. Wehrh. (Hyacinthaceae) occur throughout the eastern half of South

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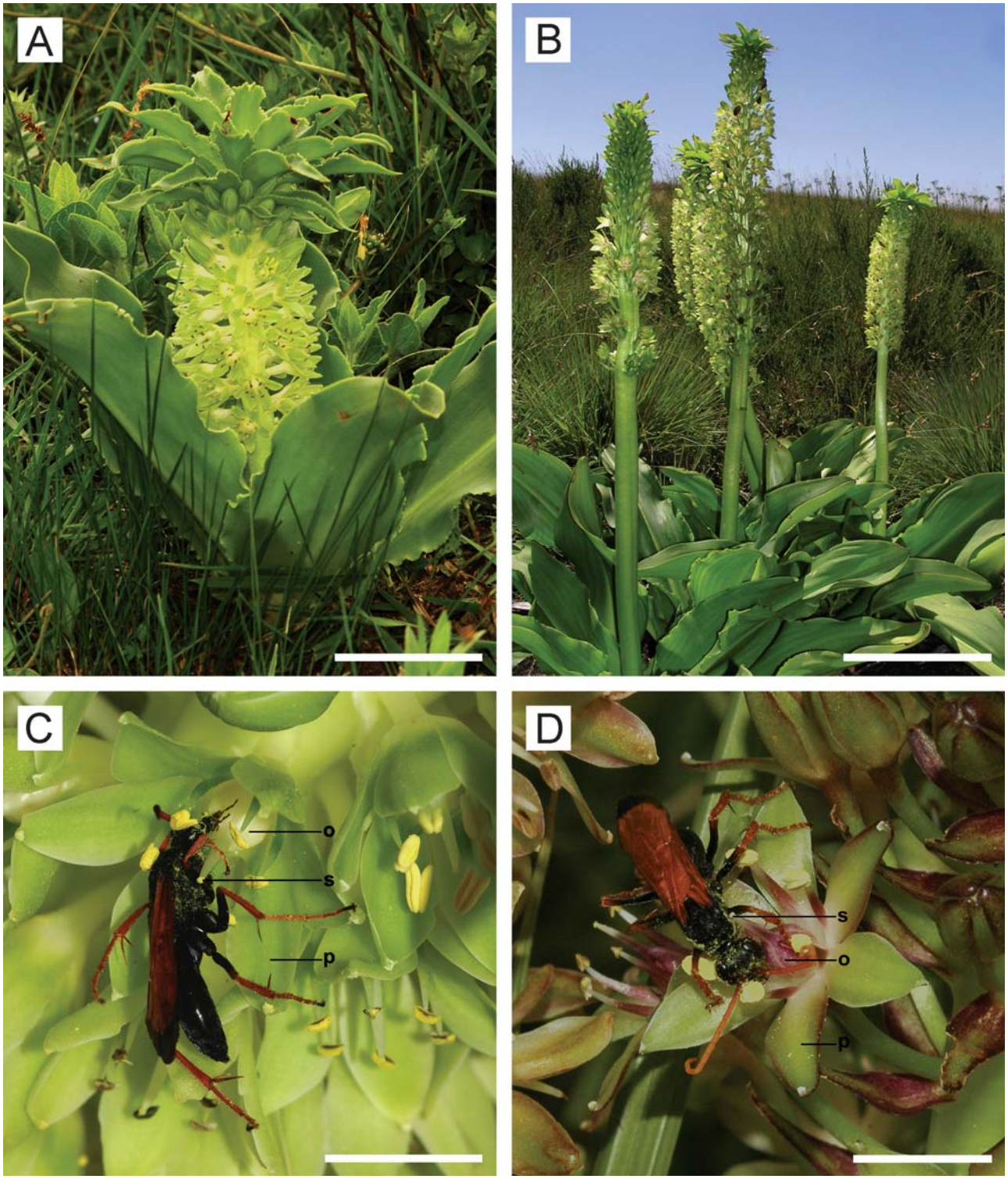

FIG. 1. *Eucomis* species and their visitors: (A) *Eucomis autumnalis* plant, Vernon Crooks Nature Reserve; (B) *Eucomis comosa* plants, Gilboa Estate (picture: Jana Jersakova); (C) male *Hemipepsis hilaris* lapping nectar from *E. autumnalis* flower, Vernon Crooks Nature Reserve; (D) female *H. capensis* lapping nectar from *Eucomis comosa* flower, Gilboa Estate. Note the pollen-covered thorax in contact with the stigma in (C) and (D). Abbreviations: o, ovary; p, petal; s, stigma. Scale bars: (A) = 60 mm; (B) = 200 mm; (C, D) = 10 mm.

Africa. Both species are found in damp grasslands or wetlands with dense vegetation (Pooley, 1998). The inflorescences of both species are similar and consist of pale yellow-green flowers arranged around a thick central axis and terminating in leafy bracts (Fig. 1). *Eucomis comosa* flowers occasionally have purple markings on the ovary and along the edges of the

petals. *Eucomis autumnalis* plants at Vernon Crooks Nature Reserve had 72 ± 8·4 (mean ± s.d., $n = 6$) flowers per plant and *E. comosa* plants at Gilboa Estate had 88 ± 12·5 (mean ± s.d., $n = 19$) flowers per plant. *Eucomis autumnalis* flowers earlier (October to November) than *E. comosa* (December to March) (Pooley, 1998). This study was conducted during the flowering seasons between 1999 and 2008 at seven field sites in South Africa (see Table 1).

Floral visitors and nectar rewards

Floral visitors were recorded at all study sites and representative individuals were collected for later identification and quantification of pollen loads. In some instances, visitors were noted but not collected. Pompilid wasps were identified to species level using keys given in Arnold (1932), Day (1979) and Goulet and Huber (1993). Floral visitors to *E. comosa* recorded as part of a separate study by Field (2002) are included and presented here. [Note that Field (2002) misidentified *E. comosa* and referred to it as *E. autumnalis* in her study.]

Nectar volume and concentration (% sucrose equivalents by weight) were measured using 5-μL capillary tubes and a Bellingham and Stanley 0–50 % or 45–80 % sugar concentration by weight, hand-held refractometer. The 24-h nectar production was measured for *E. autumnalis* and *E. comosa* at Vernon Crooks Nature Reserve in October 2006 and at Gilboa Estate in December 2006, respectively. Nectar present at the beginning of the 24-h period was removed and then plants were bagged for 24 h (for *E. autumnalis*) or cut and kept in water in the laboratory for 24 h (for *E. comosa*). The standing crop of nectar was measured for *E. autumnalis* only, at Midmar Nature Reserve at 0730 h in November 2006. Means were calculated per plant and these values used to calculate the population means (presented as the mean per flower per plant). Nectar measurements were taken from relatively few individuals in order to conserve plants for other experiments at the field sites.

Reproductive biology and reliance on pollinators

The breeding systems of *E. comosa* and *E. autumnalis* were determined in the 2005–2006 flowering season. For *E. autumnalis*, three plants were used at Vernon Crooks Nature Reserve and nine at Midmar Nature Reserve and, for *E. comosa*, ten plants were used at the Howick site. Flowers which had been bagged at the bud stage were randomly assigned to one of three hand-pollination treatments: (1) cross-pollinated, (2) self-pollinated, or (3) unmanipulated control. Pollen used for cross-pollinations was obtained from plants at least 5 m away to minimize bi-parental inbreeding effects. After pollination, flowers were rebagged and left to develop seeds. Once seeds had developed, plants were harvested and the number of seeds per flower in each treatment was counted. Twelve flowers (four per treatment) were used per plant, although some flowers were subsequently destroyed by caterpillars. Differences between treatments in the number of seeds produced per flower were analysed using a one-way ANOVA in conjunction with a Tukey test.

Seed set in naturally pollinated plants was measured as the number of seeds per flower per plant for both *E. autumnalis* and *E. comosa* (fruit set was not used as a measure of reproductive success as fruit-like swelling occurs in both unfertilized and fertilized ovaries). For *E. autumnalis*, natural seed set was measured at Highflats, Vernon Crooks Nature Reserve and Midmar Nature Reserve in the 2005–2006 flowering season. A second measurement was taken at Midmar Nature Reserve as a result of a large number of seed-containing fruits having been eaten by caterpillars. In the second sample, only undamaged fruits were selected. For *E. comosa*, natural seed set was measured at Gilboa Estate in the 2004–2005 flowering season and Howick in the 2005–2006 flowering season. Seed set was measured by dissecting individual fruits and counting the number of seeds present. Differences in seed set were compared using *t*-tests assuming unequal variance.

The number of ovules per flower was counted using a dissecting microscope (seeds present were counted as one ovule). This was measured on five *E. comosa* plants and six *E. autumnalis* plants.

Pollinator effectiveness

Pollen loads and placement were determined for all insect visitors (except where no individuals were collected) to *E. autumnalis* and *E. comosa*. Pollen was removed from each insect's body with fuchsin gel (Beattie, 1971) and a light microscope used to estimate the total *Eucomis* pollen loads per individual. In instances where individuals were carrying low amounts of pollen, the number of pollen grains was estimated directly on the insect using a dissecting microscope.

TABLE 1. *Details of the seven field sites at which* Eucomis autumnalis *and* E. comosa *were studied*

Site name	Co-ordinates and altitude	Habitat	*Eucomis* species	Approx. no. of plants
Gilboa Estate	29°15′01·6″S, 30°15′21·6″E; 1532 m	Wetland surrounded by pine plantation	*E. comosa*	100
Highflats	30°16′10·3″S, 30°12′09·3″E; 976 m	Cultivated grassland	*E. autumnalis*	100
Howick	29°27′34·8″S, 30°14′47·7″E; 1051 m	Roadside marsh	*E. comosa*	30
Midmar Nature Reserve	29°32′15·8″S, 30°10′13·1″E; 1088 m	Moist montane grassland	*E. autumnalis*	200
Vernon Crooks Nature Reserve	30°16′06·5″S, 30°37′14·5″E; 447 m	Coastal grassland	*E. autumnalis*	50
Wahroonga Farm	29°36′35·9″S, 30°07′59·4″E; 1350 m	Moist montane grassland	*E. autumnalis*	10
Wodwo Farm	29°24′08·1″S, 29°55′53·2″E; 1595 m	Montane grassland	*E. autumnalis* and *E. comosa*	10 of each

Pollen loads for *E. comosa* visitors measured by Field (2002) are included here.

As *Hemipepsis* wasps appeared to be the most important pollinators of both *Eucomis* species (they were the most abundant visitors, carried the highest pollen loads and were the only insects that moved frequently between inflorescences; Tables 2 and 6), further experiments were conducted to establish their effectiveness in transferring pollen to stigmas. For *E. comosa*, this was done by conducting an experiment to determine the effectiveness of single visits by *Hemipepsis* wasps for seed set in the 2003–2004 flowering season. Flowers on 14 inflorescences which had been bagged at the bud stage at Gilboa Estate were exposed in the field to visits by *Hemipepsis* wasps. After being visited by at least one wasp, the flowers were rebagged. A further 13 inflorescences were bagged at the bud stage but were not exposed, and served as controls. After 15 d, all inflorescences were removed to the laboratory and the number of seeds per exposed flower was counted. Seed set in exposed and control flowers was compared using a Mann–Whitney *U*-test as data were not normally distributed.

Logistical constraints prevented the use of a similar field-based single visit experiment with *E. autumnalis*. However, an experiment was conducted to determine the effectiveness of *Hemipepsis* wasps in depositing pollen on stigmas of *E. autumnalis* in the 2003–2004 flowering season. Flowers on seven inflorescences which had been bagged at the bud stage in Vernon Crooks Nature Reserve were placed in an 80-cm^3 wood and mesh cage in the laboratory. Stigmas of all flowers were examined with a dissecting microscope to ensure that they contained no pollen and flowers in which the stigmas contained pollen grains were removed. Six pompilids (five *H. capensis* and one *H. hilaris*), collected on *Pachycarpus asperifolius* (Apocynaceae) at Vernon Crooks Nature Reserve, were then placed in the cage with the virgin inflorescences. After 24 h, all flowers were removed and their stigmas inspected for pollen deposition using a dissecting microscope. The number of pollen grains on each flower was estimated to one of four categories: 0; 1–10; 10–100 and >100 pollen grains.

Responses of wasps to scent and visual cues

The flowers of *E. autumnalis* and *E. comosa* are morphologically similar (Figs 1 and 3) and the inflorescences of these two species are functionally identical. Experiments investigating the functional importance of scent and visual cues as attractants were thus conducted only with *E. autumnalis*. To do this, two field-based choice experiments (in October 2004) and a laboratory-based Y-maze choice experiment (in October 2007) were conducted. In all these experiments, *Hemipepsis* wasps and plants from Vernon Crooks Nature Reserve were used.

In the first field-based choice experiment, two plants were uprooted and placed in vases 2 m apart at right angles to the oncoming breeze. Flowers were removed from one inflorescence until both inflorescences had equal numbers of flowers. One of the inflorescences was then covered with the plant's own leaves as well as with leaves of a common sympatric plant species, *Gerbera ambigua* (Asteraceae), so that the inflorescence was completely concealed from view. The second inflorescence was left unmanipulated. Both the upper- and under-side of *E. autumnalis* leaves exhibit similar spectral reflectance properties to the flower petals, and also appear dull green in colour (see Fig. S1 in Supplementary Data, available online). *Gerbera ambigua* leaves are dull green on the upper side, with a similar spectral reflectance curve to the *E. autumnalis* flower petals and leaves (see Fig. S1). The underside of the *G. ambigua* leaves appears as a dull white colour with a maximum reflectance of approx. 50 % (see Fig. S1). The number of inspections (approached to within at least 15 cm but did not alight) or visits (actually landed) by *Hemipepsis* wasps to covered and unmanipulated inflorescences was recorded over a period of approx. 3 h. In addition, the direction of approach with respect to the prevailing breeze was recorded for 45 of the wasps observed in these experiments.

In the second field-based choice experiment, one of the inflorescences in a pair was enclosed in a transparent plastic ziplock bag to prevent it from emitting scent. Silica gel was added to the ziplock bag to prevent condensation as the plant transpired. A ziplock bag was placed in front of the second inflorescence such that it still emitted scent but approaching wasps would see both inflorescences through plastic. Covering flower petals with plastic reduced overall reflectance, but did not alter the hue of the petals (see Fig. S1 in Supplementary Data, available online).

Each of the above experiments was replicated and differences between the number of visits to each inflorescence in the two experiments were tested using a goodness-of-fit test. To quantify visual cues of flowers and leaves used in these experiments, the spectral reflectance of leaves of *G. ambigua*, and leaves, flowers and plastic-enclosed flowers of *E. autumnalis* were measured across the 300–700 nm range using the methods described below (see Fig. S1).

In the laboratory-based choice experiment, a 20-mm-diameter glass Y-maze placed on a light table was used. Each arm of the Y-maze was 90 mm long and the main arm was 170 mm long. The main arm of the Y-maze was connected to a suction pump such that air was drawn along each arm of the Y. One arm of the Y was then attached to a polyacetate bag containing an *E. autumnalis* inflorescence and the other arm attached to an empty polyacetate bag. A small hole was made in each bag to allow airflow through the bag. Wasps were inserted at the entrance to the Y-maze and allowed to walk down the Y-maze and select one of the arms. In total, 20 runs were made with four *Hemipepsis* wasps (five runs per wasp). The side containing the inflorescence was selected randomly for each run. As responses were identical for all wasps, individual choices were pooled and the number of choices made in favour of the arm with flowers was compared with the number of choices in favour of the arm without flowers using a binomial test to establish if wasps showed overall preference.

Spectral reflectance analysis of flowers and background vegetation

Spectral reflectance across the 300–700 nm range was determined using an Ocean Optics S2000 spectrometer (Ocean

TABLE 2. *Insects observed visiting flowers of* Eucomis autumnalis *and* E. comosa *at the study sites*

Visitors	Number observed: Total	Number observed: Captured	Functional group	Field site*	Reference
Eucomis autumnalis					
Hymenoptera					
Hemipepsis capensis (Pompilidae)	11	6	Pompilid	VC, W	This study
H. errabunda	1	1	Pompilid	M	This study
H. hilaris	35	11	Pompilid	Hi, M, VC	This study
H. capensis/*H. errabunda*/*H. hilaris*†	29	0	Pompilid	VC	This study
Hemipepsis sp. 1	1	1	Pompilid	VC	This study
Cryptochilus sp.1 (Pompilidae)	7	7	Pompilid	VC	This study
Priocnemis sp. 1 (Pompilidae)	1	1	Pompilid	M	This study
Pompilidae sp. 1	3	0	Pompilid	Hi	This study
Tiphia sp. 1 (Tiphiidae)	7	2	Tiphiid	Hi, VC	This study
Apis mellifera (Apidae)	1	0	Bee	Hi	This study
Halictidae sp. 1	1	0	Bee	VC	This study
Tenthredinidae sp. 1	2	0	Tenthredinid	VC	This study
Coleoptera					
Atrichelaphinis tigrina (Scarabaeidae: Cetoniinae)	31	15	Cetoniin	M, W, VC	This study
Cyrtothyrea marginalis (Scarabaeidae: Cetoniinae)	10	8	Cetoniin	M, VC	This study
Leucocelis haemorrhoidalis (Scarabaeidae: Cetoniinae)	1	1	Cetoniin	M	This study
Elateridae sp. 1	175	8	Elaterid	VC	This study
Chrysomelidae sp. 2	1	1	Chrysomelid	M	This study
Diptera					
Sarcophagidae sp. 2	4	0	Short-tongued fly	Hi	This study
Sarcophagidae sp. 3	1	0	Short-tongued fly	M	This study
Calliphoridae sp. 1	2	0	Short-tongued fly	Hi	This study
Calliphoridae sp. 2	3	0	Short-tongued fly	M	This study
Calliphoridae sp. 3	3	3	Short-tongued fly	M	This study
Muscidae sp. 1	1	0	Short-tongued fly	Hi	This study
Muscidae sp. 2	1	0	Short-tongued fly	M	This study
Hemiptera					
Reduviidae sp. 1	1	0	Hemiptera	Hi	This study
Lygaeidae sp. 1	1	1	Hemiptera	M	This study
Eucomis comosa					
Hymenoptera					
Hemipepsis capensis (Pompilidae)	34	34	Pompilid	G, Ho, Wo	Field (2002), This study
H. dedjas	3	1	Pompilid	Ho	This study
H. errabunda	7	7	Pompilid	G, Ho	Field (2002), This study
H. hilaris	27	27	Pompilid	G, Ho, Wo	Field (2002), This study
H. capensis/*H. errabunda*/*H. hilaris*†	86	0	Pompilid	G, Ho, Wo	Field (2002), This study
Cyphononyx sp. 1 (Pompilidae)	3	1	Pompilid	Ho	This study
Pepsinae sp.1 (Pompilidae)	8	5	Pompilid	G	This study
Pompilinae sp. 1 (Pompilidae)	1	1	Pompilid	G	This study
Pompilidae sp. 2	1	1	Pompilid	Ho	This study
Polistes sp. 1 (Vespidae)	1	1	Vespid	G	This study
Polistes sp. 2 (Vespidae)	1	1	Vespid	G	This study
Eumenidae sp. 1	1	0	Eumenid	Ho	This study
Halictus sp. 1 (Halictidae)	1	1	Bee	G	Field (2002)
Apidae sp. 1	1	1	Bee	G	This study
Apis mellifera (Apidae)	49	7	Bee	Ho	This study
Tiphia sp. 1 (Tiphiidae)	8	0	Tiphiid	G	This study
Tiphiidae sp. 1	1	1	Tiphiid	Ho	This study
Tenthredinidae sp. 1	1	1	Tenthredinid	G	This study
Formicidae sp. 1	2	0	Ant	G	This study
Coleoptera					
Lycidae sp. 1	2	2	Lycid	G	Field (2002); this study
Lycidae sp. 2	7	7	Lycid	Ho	This study
Lycidae sp. 3	3	3	Lycid	G, Ho	This study
Lycidae sp. 4	5	5	Lycid	G	Field (2002); this study
Lycidae sp. 5	7	7	Lycid	G, Ho	Field (2002); this study
Unidentified Lycidae	17	0	Lycid	G	This study
Atrichelaphinis tigrina (Scarabaeidae: Cetoniinae)	15	9	Cetoniin	G, Ho	Field (2002); this study
Cyrtothyrea marginalis (Scarabaeidae: Cetoniinae)	2	2	Cetoniin	G	This study
Cetoniinae sp. 1 (Scarabaeidae)	1	1	Cetoniin	G	This study
Cetoniinae sp. 2 (Scarabaeidae)	1	1	Cetoniin	G	Field (2002)
Chrysomelidae sp. 1	2	2	Chrysomelid	Ho	This study

Continued

TABLE 2. *Continued*

Visitors	Number observed: Total	Number observed: Captured	Functional group	Field site*	Reference
Cerambycidae sp. 1	1	1	Cerambycid	G	This study
Cantharidae sp. 1	1	1	Cantharid	Ho	This study
Elateridae sp. 1	1	1	Elaterid	G	This study
Coccinelidae sp. 1	1	1	Coccinelid	Ho	This study
Diptera					
Calliphoridae sp. 4	1	1	Short-tongued fly	Ho	This study
Tabanocella denticornis (Tabanidae)	8	8	Short-tongued fly	G	Field (2002); this study
Tabanus taeniatus (Tabanidae)	2	2	Short-tongued fly	G	Field (2002)
Sarcophagidae sp. 1	1	1	Short-tongued fly	G	Field (2002)
Hemiptera					
Lygaeidae sp. 2	1	1	Hemiptera	Ho	This study
Lepidoptera					
Catacroptera cloanthe (Nymphalidae)	1	0	Butterfly	Ho	This study
Danaus chrysippus (Danaidae)	1	0	Butterfly	Ho	This study
Stygionympha vigilans (Nymphalidae)	1	1	Butterfly	G	Field (2002)
Vanessa cardui (Nymphalidae)	1	0	Butterfly	Ho	This study
Nymphalidae sp. 1	1	1	Butterfly	G	Field (2002)
Nymphalidae sp. 2	1	1	Butterfly	Ho	This study
Nymphalidae sp. 3	1	0	Butterfly	Ho	This study

*G, Gilboa Estate; Hi, Highflats; Ho, Howick; M, Midmar Nature Reserve; VC, Vernon Crooks Nature Reserve; W, Wahroonga Farm; Wo, Wodwo Farm.
† These were individuals of these three *Hemipepsis* species but could not be identified to species as they were observed but not captured.

Optics Inc., Dunedin, FL, USA) and fibre optic reflection probe (QR-400-7-UV-VIS; 400 μm) held at 45° to the petal surface. The light source used was an Ocean Optics DT-mini deuterium tungsten halogen light source with an approx. 200- to 1100-nm spectral range. An Ocean Optics WS-1 diffuse reflectance standard was used to calibrate the spectrometer (Johnson and Andersson, 2002). Spectral reflectance was measured for petals of *E. autumnalis* from Vernon Crooks Nature Reserve in October 2004, and *E. comosa* from Gilboa Estate in November 2006. Spectral reflectance of background vegetation was measured from the upper surface of green leaves of 16 different plant species (various grasses, forbs and herbs) from Vernon Crooks Nature Reserve in November 2006. Four replicates were taken for each of the *Eucomis* species and three replicates for each of the background species. A mean spectrum was calculated for each plant species.

Gas chromatography–mass spectrometry (GC-MS) analysis of floral scent

Floral scent was collected using dynamic headspace extraction methods and analysed by coupled GC-MS. In total, eight samples for *E. autumnalis* and six samples for *E. comosa* were obtained. Two of the *E. autumnalis* samples and one of the *E. comosa* samples were analysed by Dr Roman Kaiser (Givaudan, Switzerland) as per the methods described in Kaiser and Tollsten (1995). For these three samples, inflorescences were cut and removed to the laboratory where they were enclosed in a glass vessel (excluding damaged plant tissue) and the air from the vessel pumped through a filter containing 3 mg Porapak™ Q for 6–7 h at a realized flow rate of 50 mL min^{-1}. One of the two *E. autumnalis* samples was taken from a single inflorescence from Vernon Crooks Nature Reserve in the 2004–2005 season (sample S1 in Table S1 in Supplementary Data available at *AoB* online), and the second *E. autumnalis* sample was taken from three inflorescences from Midmar Nature Reserve in the 2005–2006 season (sample S2 in Table S1). The *E. comosa* sample was taken from three inflorescences from Gilboa Estate in the 2005–2006 season. These samples were then eluted with approx. 30 μL of 9 : 1 hexane : acetone solvent and analysed by GC-MS using a DB-WAX column (J & W Scientific) and the instrumentation and temperature programmes as described in Kaiser and Tollsten (1995).

The remaining six *E. autumnalis* and five *E. comosa* samples were taken by enclosing inflorescences in polyacetate bags. Air from these bags was then pumped through small cartridges filled with 1 mg of Tenax® and 1 mg of Carbotrap™ activated charcoal at a realized flow rate of 50 mL min^{-1}. Controls were taken from an empty polyacetate bag sampled for the same duration. The *E. autumnalis* samples were taken in the field at Vernon Crooks Nature Reserve on 24 October 2007. Each sample was taken from a single inflorescence for a duration of 20 min. The *E. comosa* samples were taken from cut inflorescences collected at Gilboa Estate on 10 January 2008. Both these *Eucomis* species have thick fleshy stems and cut inflorescences survive for several weeks in vases without wilting. Each sample was taken from four inflorescences for a duration of 2 h. To minimize contamination by green leaf volatiles (as a result of using cut inflorescences), care was taken to bag only undamaged plant tissue. As previous attempts to sample this species had produced weak samples, the scent was accumulated in the bags for 3 h before the sample was taken. This served to minimize contamination from continuous pumping of background air. GC-MS analysis of these samples was carried out using a Varian CP-3800 GC (Varian, Palo Alto, CA, USA) with a 30 m × 0·25 mm internal diameter (film thickness 0·25 μm) Alltech EC-WAX column coupled to a Varian 1200 quadrupole mass spectrometer in electron-impact ionization mode. Cartridges were placed in a Varian 1079 injector

equipped with a 'Chromatoprobe' thermal desorbtion device. The flow of helium carrier gas was 1 ml min^{-1}. The injector was held at 40 °C for 2 min with a 20 : 1 split and then increased to 200 °C at 200 °C min^{-1} in splitless mode for thermal desorbtion. After a 3 min hold at 40 °C, the temperature of the GC oven was ramped up to 240 °C at 10 °C min^{-1} and held there for 12 min. Compounds were identified using the Varian Workstation software with the NIST05 mass spectral library and verified, where possible, using retention times of authentic standards and published Kovats indices. Compounds present at similar abundance in the controls were considered to be contaminants and excluded from analyses. For quantification, 68 different standards (comprising representatives from all compound classes) were injected into cartridges (200 ng of each) and thermally desorbed under identical conditions to the samples. *Eucomis* inflorescences are racemose with flowers maturing acropetally from the base. Individual inflorescences consequently contain a range of different aged flowers. We considered individual inflorescences to be complete functional units. The age and number of flowers per inflorescence in each sample were thus not recorded and emission rates were calculated per inflorescence.

RESULTS

Floral visitors and nectar rewards

Pompilid wasps (of both sexes) were the most abundant visitors to flowers of both *Eucomis autumnalis* and *E. comosa* (Table 2 and Fig. 1C, D). The most abundant pompilid species were identified as *Hemipepsis capensis*, *H. errabunda* and *H. hilaris. Eucomis autumnalis* was also visited by relatively large numbers of two cetoniin beetle species (*Atrichelaphinis tigrina* and *Cyrtothyrea marginalis*) and a single unidentified elaterid beetle species. *Eucomis comosa* was also visited by relatively large numbers of honeybees (*Apis mellifera*), lycid beetles (five species) and a single cetoniin beetle species (*A. tigrina*; Table 2). All other visitors to both plant species were observed infrequently and in relatively low numbers (Table 2). Of the visitors to *E. autumnalis*, *Hemipepsis* wasps were the only insects that were observed to move frequently between separate plants. This was in contrast to the cetoniin beetle species and the elaterid beetle species which spent long periods of time on each inflorescence and were seldom observed moving between plants. Likewise, *Hemipepsis* wasps were the only insects that moved frequently between *E. comosa* plants, although honeybees were also occasionally observed to move between plants. Floral visitors were active throughout the day on inflorescences of both plant species.

Eucomis autumnalis flowers produced a greater volume of more dilute nectar over a 24-h period, when compared with *E. comosa* flowers (Table 3). The standing crop of nectar on *E. autumnalis* flowers was considerably more concentrated than nectar that accumulated over 24 h (Table 3).

Reproductive biology and reliance on pollinators

Seed set was significantly higher in outcrossed flowers than in selfed or unmanipulated controls for both *E. autumnalis* and *E. comosa* (Table 4). Natural seed set was higher for *E. comosa* than for *E. autumnalis* (Table 5). For *E. autumnalis*, seed set was not significantly different between sites ($F_{2,13} = 2{\cdot}72$, $P = 0{\cdot}10$; for the Midmar Nature Reserve site, this analysis only included the seed set obtained by measuring all flowers on a plant). The two sampling methods used for measuring natural seed set in *E. autumnalis* at Midmar Nature Reserve yielded results which were not significantly different ($t = 0{\cdot}75$, $P = 0{\cdot}48$). For *E. comosa* there was no significant difference between seed set at Gilboa Estate in 2004–2005 and Howick in 2005–2006 ($t = 0{\cdot}55$, $P = 0{\cdot}59$).

Pollinator effectiveness

Pompilid wasps as a functional group carried considerably higher loads of *E. autumnalis* (mean = 964 grains per wasp) and *E. comosa* (mean = 1362 grains per wasp) pollen than all other visitors (Table 6). Pollen was located on the head

TABLE 3. *Nectar properties for* Eucomis autumnalis *and* E. comosa

Species	Sampling method	*n**	Volume (μL): mean ± s.d.	*n**	Concentration (%): mean ± s.d.
E. autumnalis	24 h production	16 (3)	15·8 ± 5·44	15 (3)	19 ± 4·8
	Standing crop	20 (4)	2·0 ± 1·04	20 (4)	71 ± 4·9
E. comosa	24 h production	25 (5)	2·8 ± 0·98	25 (5)	62 ± 3·8

*The numbers in parentheses refer to the number of plants.

TABLE 4. *Results of* Eucomis autumnalis *and* E. comosa *breeding system experiments*

	Seed set (mean ± s.d.)		
Species	Cross	Self	Control
E. autumnalis	5·8 ± 4·89 ($n = 46$)[a]	0·1 ± 0·25 ($n = 45$)[b]	0·02 ± 0·07 ($n = 48$)[b]
E. comosa	5·6 ± 4·23 ($n = 38$)[a]	0·1 ± 0·12 ($n = 40$)[b]	0·4 ± 0·39 ($n = 40$)[b]

Sample sizes refer to the number of flowers in each treatment.
Treatments with different letters are significantly different at the 5 % level.

TABLE 5. *Natural seed set for* Eucomis autumnalis *and* E. comosa *measured at different sites*

Field site	*n**	Percentage of flowers that set seed	No. of ovules: mean ± s.d. per flower (*n*)	Seed set: mean ± s.d. per flower per plant
Eucomis autumnalis				
Highflats	439 (6)†	62	29·0 ± 1·62 (20)	1·6 ± 0·73
Midmar	239 (4)†	54	Not measured	3·7 ± 2·83
Midmar‡	90 (9)	77	Not measured	5·0 ± 3·02
Vernon Crooks	429 (6)†	48	31·1 ± 4·46 (40)	1·4 ± 1·15
Eucomis comosa				
Gilboa	47 (5)	98	27·2 ± 2·91 (28)	7·4 ± 2·13
Howick	72 (9)	96	Not measured	8·3 ± 4·28

* The numbers in parentheses refer to the number of plants.
† Sampled all flowers on each plant.
‡ This sample excluded fruits damaged by caterpillars (see Materials and Methods).

TABLE 6. *Pollen loads for insects visiting* Eucomis autumnalis *and* E. comosa*, measured per functional group*

Functional group	No. examined for pollen	Pollen load: mean (range)	Pollen placement on body
E. autumnalis			
Hymenoptera			
Pompilid	13	964 (0–5000)	Head, thorax
Tiphiid	2	55 (10–100)	Head, thorax
Coleoptera			
Cetoniin	13	208 (0–800)	Head, thorax, legs
Elaterid	8	0 (0)	
Chrysomelid	1	0 (0)	
Diptera			
Short-tongued fly	3	4 (0–12)	Thorax
Hemiptera			
Lygaeid bug	1	0 (0)	
E. comosa			
Hymenoptera			
Pompilid	13	1362 (100–5000)	Head, thorax
Vespid	2	20 (0–40)	Thorax
Bee	9	154 (0–700)	Head, thorax, abdomen, legs, wings
Tiphiid	1	0 (0)	
Tenthredinid	1	80 (80)	Head, thorax
Coleoptera			
Lycid	18	367 (10–1500)	Head, thorax, legs
Cetoniin	13	43 (0–200)	Thorax, abdomen
Chrysomelid	2	0 (0)	
Cerambycid	1	50 (50)	Head, thorax, elytra
Cantharid	1	0 (0)	
Elaterid	1	0 (0)	
Diptera			
Short-tongued fly	7	68 (0–150)	Thorax
Lepidoptera			
Butterfly	3	7 (0–10)	Thorax

and thorax of pompilid wasps (see Fig. 1C, D). The only other insects which carried appreciable amounts of pollen were cetoniin beetles (mean = 208 grains per beetle) in the case of *E. autumnalis* and lycid beetles (mean = 367 grains per beetle) in the case of *E. comosa* (Table 6).

Eucomis comosa flowers which were exposed to visits by pompilid wasps ($n = 332$ flowers on 14 inflorescences) set significantly more seeds (mean ± s.d. = 0·14 ± 0·21 seeds per flower per plant, range = 0–5) than flowers that were not exposed ($n = 130$ flowers on 13 inflorescences; no seeds were produced; Mann–Whitney U, $Z = 3{\cdot}38$, $P = 0{\cdot}003$).

Pollen grains were deposited on 42 % of virgin *E. autumnalis* stigmas exposed to visits by *Hemipepsis* wasps in a cage (Table 7). Of the flowers which received pollen, most received between one and ten grains (Table 7).

Responses of wasps to scent and visual cues

There was no difference between the number of visits by pompilid wasps to *E. autumnalis* inflorescences concealed from view using leaves and to exposed inflorescences (Fig. 2). Of the 45 wasps that approached plants, 41 (91 %) approached from downwind, four (9 %) approached from 90° to the wind, and no wasps approached plants from upwind. Pompilid wasps visited the *E. autumnalis* inflorescence situated behind a plastic bag significantly more than they visited the inflorescence contained within a plastic bag to prevent emission of scent (Fig. 2). No other insects (aside from the pompilid wasps) were attracted to *E. autumnalis* flowers during these experiments.

In the Y-maze experiment, the *Hemipepsis* wasps selected the arm with the flowers in all 20 runs (Fig. 2).

Spectral reflectance analysis of flowers and background vegetation

Eucomis autumnalis petals are typically dull green in colour with maximum reflectance of approx. 20 % at 550 nm and low overall reflectance (Fig. 3). *Eucomis comosa* petals have a similar colouring to *E. autumnalis*, but had slightly lower overall reflectance (Fig. 3). The spectral reflectance of both *Eucomis* species was very similar to that of the background vegetation, and, although slightly brighter than the average background, fell within the range of green background reflectance (Fig. 3).

GC-MS analysis of floral scent

To the human nose the scents of both *E. autumnalis* and *E. comosa* are similar and have a sweet-spicy fragrance. The floral scent of *E. autumnalis* was dominated by monoterpenes

TABLE 7. *Pollen deposition on the stigmas of virgin* Eucomis autumnalis *flowers placed in a cage with* Hemipepsis *wasps*

			Number of flowers in each pollen load category			
	Flowers exposed	Stigmas with pollen deposited	0 pollen grains	1–10 pollen grains*	10–100 pollen grains	>100 pollen grains
Number	155	65	90	48	14	3
Percentage	100	42	58	30	9	2

*Category containing the median value.

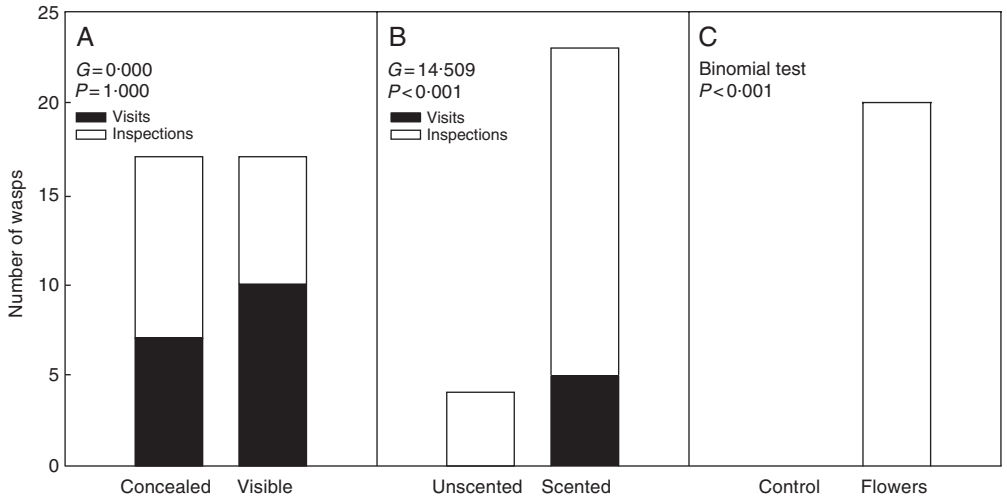

FIG. 2. Field-based and laboratory (Y-maze) choice experiments with *E. autumnalis* and *Hemipepsis* wasps: (A) number of visits and inspections by *Hemipepsis* wasps to inflorescences concealed from view (covered with leaves) compared with visible inflorescences; (B) number of visits and inspections by *Hemipepsis* wasps to unscented inflorescences (covered with a plastic ziplock bag) compared with scented inflorescences (placed behind a plastic ziplock bag, but not covered); (C) number of choices by wasps in favour of the arm containing flowers compared with the control (empty) arm in a Y-maze ($n = 20$ runs with four wasps).

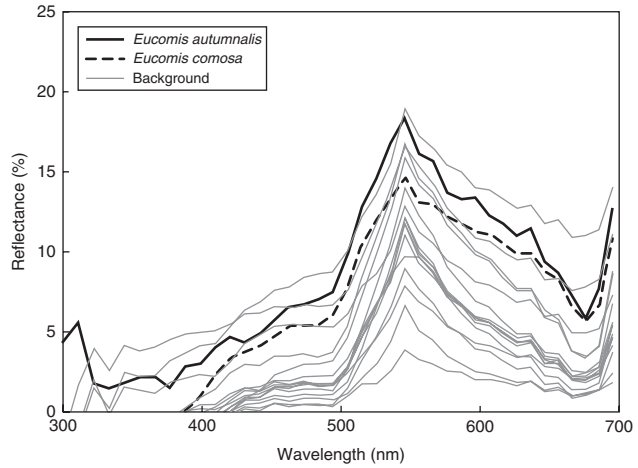

FIG. 3. Reflectance spectra for *Eucomis autumnalis* and *E. comosa* floral petals and background vegetation. Each curve represents the mean spectrum calculated from individual replicates.

and, to a lesser extent, benzenoids (see Table S1 in Supplementary Data, available online). The scent of *E. comosa* was dominated by monoterpenes and benzenoid compounds, with this species producing a comparatively larger proportion of benzenoids than *E. autumnalis* (see Table S1). Linalool and 3,5-dimethoxy toluene were the predominant monoterpene and benzenoid compounds in *E. comosa* and *E. autumnalis*, respectively, with 3,5-dimethoxy toluene forming a relatively higher proportion of the scent of *E. comosa*. Both species produced a large number of compounds (ranging from 41 to 61 among individual samples; see Table S1). Overall, a total of 104 compounds were identified in *E. autumnalis* samples and 83 compounds in *E. comosa* samples with 57 compounds occurring in samples from both species (see Table S1). *Eucomis autumnalis* samples contained 47 unique compounds (not found in any *E. comosa* samples) while *E. comosa* samples contained 26 unique compounds (see Table S1).

DISCUSSION

The results of this study indicate that both *Eucomis autumnalis* and *E. comosa* are pollinated primarily by *Hemipepsis* pompilid wasps and that scent is the key floral attractant. Field observations indicate that these wasps are the most abundant visitors to both plant species (Table 2) and, as a functional group, carry considerably higher pollen loads than any other visitors (Table 6). Exposure of virgin flowers of both species to visits from only *Hemipepsis* wasps showed that these wasps are capable of depositing pollen on the stigmas of *E. autumnalis* (Table 7), and, in the case of *E. comosa*, pollen deposited during a single visit is sufficient to result in seed set (although the low deposition rates and seed set

compared with pollen loads suggest that these wasps are not very efficient in this respect). Experimental hand-pollinations showed that both *E. autumnalis* and *E. comosa* are genetically self-incompatible and thus reliant on pollinators for seed set. GC-MS analysis showed that both plant species produce a large number of floral volatiles and behavioural experiments showed that the *Hemipepsis* wasps are attracted by scent rather than visual cues. Measurement of spectral reflectance showed that both *E. autumnalis* and *E. comosa* flowers have similar colouring to background vegetation (Fig. 3).

Aside from pompilid wasps, both *Eucomis* species were visited by several insects which may also contribute to pollination. In the case of *E. autumnalis*, the only other moderately abundant visitors were cetoniin beetles and a single species of elaterid beetle (Table 2). The cetoniin beetles, however, tend to spend long periods of time on a single plant and were seldom observed moving between plants [pers. obs.; see fig. 2 in Shuttleworth and Johnson (2008) for a comparison between floral visiting times of *Hemipepsis* wasps and *Atrichelaphinis tigrina*, one of the beetles observed here, on milkweed flowers]. In addition, the cetoniin beetles carried considerably less pollen than the pompilid wasps (Table 6). Individuals of the elaterid species, although abundant, were small enough to access nectar without contacting anthers or stigmas and thus did not carry pollen. In the case of *E. comosa*, flowers were also visited by relatively large numbers of honeybees, lycid beetles and cetoniin beetles (Table 2). Although these visitors did carry some pollen (Table 6), their presence on flowers was inconsistent between flowering seasons and field sites (pers. obs.; Table 2). The cetoniin beetles were also seldom observed moving between plants. These insects are thus unlikely to make significant contributions to outcross pollination in either *E. autumnalis* or *E. comosa*, suggesting that both *Eucomis* species are specialized for pollination by pompilid wasps.

Natural seed set in both *E. autumnalis* and *E. comosa* was notably low (Table 5). This may, to some extent, reflect pollen limitation. The single visit and pollen deposition experiments suggest that *Hemipepsis* wasps, although capable of depositing pollen on stigmas, are not very efficient in this respect (Table 7). The low seed set recorded could thus reflect the inefficiency of these wasps in terms of transferring pollen to stigmas. However, seed set of outcrossed flowers in the hand-pollination experiments (Table 4) was similar to natural seed set (Table 5), suggesting that natural seed set might be limited by physiological resources or genetic factors.

It appears that *E. autumnalis* and *E. comosa* achieve some degree of specialization through the combination of cryptic colouring and scent as a selective floral attractant. Choice experiments clearly demonstrated that wasps can find inflorescences by fragrance alone and do not require visual cues (Fig. 2). Indeed, flowers of the two study species are cryptic because of their dull yellow-green colour which reflects $<20\,\%$ of visible light and is similar to the background vegetation (Fig. 3). Furthermore, the scents of *E. autumnalis* and *E. comosa* are different to the scents of two morphologically similar congeners (*E. bicolor* and *E. humilis*) which are also cryptically coloured but pollinated by carrion flies and have very different scent composition (A. Shuttleworth and S. D. Johnson, unpubl. res.). This suggests some degree of adaptation to different pollinators in terms of the volatile compounds produced.

The absence of direct floral filters in either of these *Eucomis* species is intriguing. The flowers of both species produce relatively large amounts of concentrated nectar (Table 3). Without morphological filters this nectar is freely available to a wide variety of flower visitors, including nectar robbers. In some plants with exposed nectar, specialization can be achieved by distasteful compounds in nectar which renders it unpalatable to non-pollinating insects. This has been demonstrated in a milkweed pollinated by the same *Hemipepsis* wasps (Shuttleworth and Johnson, 2006) and in a bird-pollinated *Aloe* (Johnson *et al.*, 2006), both of which have nectar which is distasteful to bees. Although nectar palatability was not investigated in this study, we believe the production of toxic nectar is unlikely in these *Eucomis* species since the flowers were also visited by generalist nectar-feeding insects. Indeed, the relatively high number of non-pollinating visitors to the *E. autumnalis* and *E. comosa* flowers (Table 2) compared with other pompilid-pollinated flowers (see Ollerton *et al.*, 2003; Johnson, 2005; Shuttleworth and Johnson, 2006, 2008, 2009; Johnson *et al.*, 2007) suggests that the nectar of *Eucomis* flowers is at least partially palatable to other insects. The high concentration of nectar in *E. autumnalis* and *E. comosa* flowers is consistent with the nectars of other pompilid-pollinated flowers (Ollerton *et al.*, 2003; Shuttleworth and Johnson, 2006, 2008, 2009; Johnson *et al.*, 2007; but see Johnson, 2005), suggesting that *Hemipepsis* wasps have driven the evolution of at least some of the nectar characteristics in these species.

The high number of monoterpene and aromatic volatile compounds common to the scents of both species (see Table S1 in Supplementary Data available at *AoB* online) may be a result of common descent (the phylogenetic relatedness of the two species is unknown) or it could indicate that a blend of these compounds plays a key function for the attraction of pompilid wasps. Two orchid species, *Disa sankeyi* and *Satyrium microrrhyncum*, specialized for pollination by these same pompilid wasps also emit a large number of different floral volatile compounds (Johnson, 2005; Johnson *et al.*, 2007). The scent of *Disa sankeyi* is dominated by monoterpene and aromatic compounds, while that of *S. microrrhynchum* is composed almost entirely of monoterpenes, sesquiterpenes and a few aromatics (Johnson, 2005; Johnson *et al.*, 2007). Overall, only five compounds (elemicin, α-pinene, β-pinene, limonene and α-terpineol) were common to the scents of these two orchids and both *Eucomis* species. However, most of these are common floral volatiles and are unlikely to be a specific signal for wasps (see Knudsen *et al.*, 2006). It is possible that broad suites of compounds within particular classes will be found to characterize pompilid-pollinated plants, as in the example of scents of moth-pollinated flowers which often contain high proportions of terpenoid and aromatic alcohols with small amounts of nitrogen-containing compounds (Knudsen and Tollsten, 1993).

Alternatively, pompilid-pollinated plants may rely on specific prey-related compounds to attract spider-hunting wasps. A recent study by Brodmann *et al.* (2008) found that two European orchids (*Epipactis helleborine* and *E. purpurata*) have

developed a chemical-mimicry system in which they utilize green-leaf volatiles to attract their vespid pollinators. Green-leaf volatiles are produced by damaged plant tissues and the vespid wasps typically use these volatiles as a cue to find their herbivorous caterpillar prey. The key difference between the *Epipactis*–vespid system and this system is that pompilid wasps hunt spiders and not caterpillars. However, it is likely that pompilid wasps use chemical cues to locate their spider prey, as has been demonstrated for other prey-hunting wasps (Hendrichs *et al.*, 1994; Brodmann *et al.*, 2008). Pompilid-pollinated plants may thus be mimicking compounds produced by spiders which are attractive to these spider-hunting wasps. Unfortunately, the specific spiders used by these *Hemipepsis* wasps are not yet established, making it difficult to explore this idea further. Furthermore, pompilid-pollinated plants are visited by both male and female wasps, while only females hunt prey, suggesting that these wasps are not attracted solely by prey-related volatiles. Further research exploring the floral scents of other non-pompilid pollinated *Eucomis* species in conjunction with gas chromatography–electro-antennogram detection and bioassay experiments are ultimately required to fully understand the scent cues used by *E. autumnalis* and *E. comosa* to attract *Hemipepsis* wasps.

This study shows a clear role for floral scent in the specialized pollination of two *Eucomis* species. In future, we intend to use GC-EAD and bioassays to determine which of the dozens of floral volatiles produced by these flowers are attractive to pompilid wasps. Further research on other members of the genus may provide interesting insights into the role of floral scent in pollinator shifts by morphologically similar plant species.

SUPPLEMENTARY DATA

Supplementary data is available online at www.aob.oxfordjournals.org and consists of the following. Fig. S1: Reflectance spectra measured in the field-based choice experiments. Table S1: The floral volatiles identified by GC-MS.

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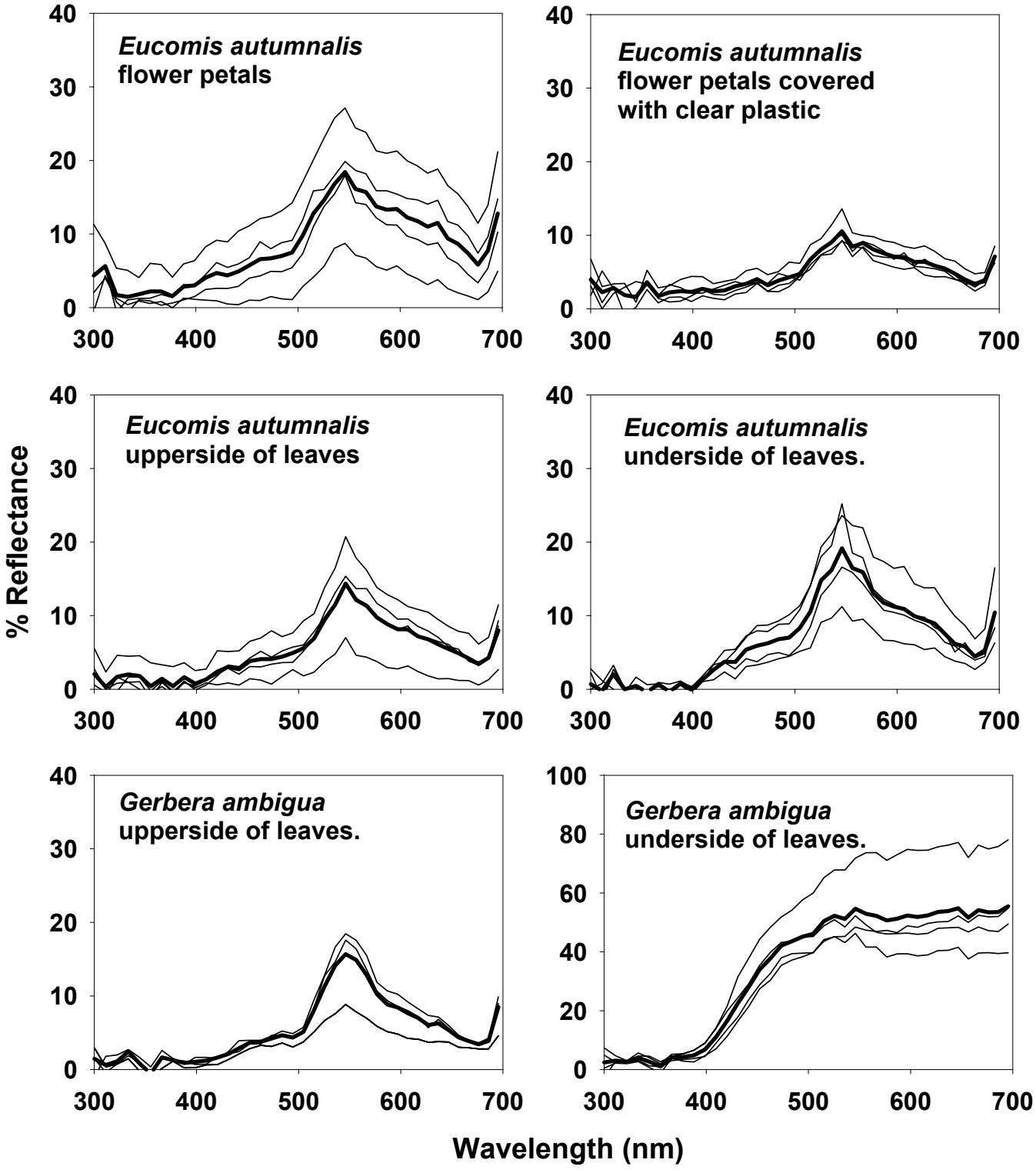
SUPPLEMENTARY MATERIAL FOR CHAPTER 6

SUPPLEMENTARY DATA

FIG. S1. Reflectance spectra for *Eucomis autumnalis* and the leaves used in the field choice experiments. In all graphs, the light curves represent individual replicates while the bold lines represents the mean reflectance.



SUPPLEMENTARY DATA

TABLE S1. Compounds identified by GC-MS from headspace samples of *Eucomis autumnalis* and *E. comosa*. Compounds are listed in order of increasing retention time (using an Alltech EC-WAX column) within each compound class[a]. T = thermal desorbtion, S = solvent extraction.

		Eucomis autumnalis								*Eucomis comosa*					
Compound	**Criteria** [b]	T1	T2	T3	T4	T5	T6	S1	S2	T1	T2	T3	T4	T5	S1
Aliphatics															
Alkanes															
Tridecane	D	-	-	-	-	-	-	-	tr	-	-	-	-	-	-
Tetradecane	D	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-
Pentadecane	D	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2
Ketones															
But-3-en-2-one	A	0.5	0.4	0.8	0.5	0.4	0.4	-	-	-	-	-	-	-	-
4-Methylpentan-2-one	A	1.8	1.5	2.9	1.7	1.1	0.9	-	-	0.8	1.0	2.8	0.8	1.7	-
Nonan-2-one	A	2.6	0.2	1.3	0.9	0.5	1.3	tr	tr	tr	tr	tr	tr	0.2	tr
2-Hydroxy-5-methylhexan-3-one	D	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2
Alcohols															
Heptan-2-ol	A	1.7	-	-	0.6	-	-	-	-	-	-	-	-	-	-
Hexan-1-ol	C	0.1	tr	0.6	tr	tr	tr	-	-	tr	0.1	tr	tr	tr	tr
Octan-1-ol	D	-	-	-	-	-	-	tr	0.1	-	-	-	-	-	tr
(Z)-Hex-3-en-1-ol	A	0.3	0.2	1.2	0.3	0.2	0.2	-	-	-	-	-	-	-	-
Oct-1-en-3-ol	C	3.8	1.7	3.4	6.7	4.1	1.7	0.2	tr	-	-	-	-	-	tr
Nonan-2-ol	A	3.4	-	-	1.9	0.1	0.6	-	-	tr	tr	-	tr	tr	0.1
(E)-Oct-2-en-1-ol	A	tr	tr	tr	tr	tr	tr	-	-	-	-	-	-	-	-
Aldehydes															
Octanal [c]	A	-	-	-	-	-	-	0.1	tr	-	-	-	-	-	tr
Nonanal [c]	A	-	-	-	-	-	-	0.2	0.2	-	-	-	-	-	0.1
Decanal [c]	A	-	-	-	-	-	-	-	0.2	-	-	-	-	-	0.2
Esters															
Isoamyl isovalerate	B	-	-	-	-	-	-	-	tr	0.2	-	-	-	-	0.1
(Z)-Hex-3-en-1-yl acetate	A	-	0.3	0.2	0.9	0.4	0.1	-	-	-	-	-	-	-	-

Isopropyl myristate	A	tr	tr	tr	tr	-	-	-	-	-	-	-	-	-	-
Benzenoids															
p-Cymene	A	-	-	-	-	-	-	-	0.4	0.1	0.5	0.1	0.3	0.4	-
Anisole	D	-	-	-	-	-	-	tr	-	-	-	-	-	-	-
α,*p*-Dimethylstyrene	D	-	-	-	-	-	-	-	0.5	-	-	-	-	-	-
1-Methoxy-4-methylbenzene	A	-	-	-	-	-	-	0.3	-	0.6	0.3	0.4	0.8	0.2	-
Benzaldehyde [c]	C	-	-	-	-	-	-	0.2	-	-	-	-	-	-	0.1
Estragole	A	-	tr	tr	tr	-	tr	tr	-	0.2	0.3	0.4	0.1	0.3	
1,2-Dimthoxybenzene	D	-	-	-	-	-	-	tr	-	-	-	-	-	-	-
3,4-Dimethoxytoluene	B	0.2	0.4	tr	0.2	0.2	0.4	0.3	-	0.4	0.5	0.3	1.2	0.7	0.5
Phenylethyl acetate	A	-	-	-	-	0.3	-	-	0.2	tr	0.1	tr	0.1	0.2	-
p-Cymen-8-ol	A	-	-	-	-	-	-	-	0.2	tr	tr	tr	tr	tr	-
3,5-Dimethoxytoluene	A	1.7	14.1	7.9	4.4	tr	0.1	7.9	tr	30.8	31.4	34.3	67.6	17.2	43.4
Benzyl alcohol	C	-	-	-	-	-	-	0.2	-	0.3	tr	tr	0.2	tr	tr
Phenylethyl alcohol	A	tr	tr	0.1	tr	0.3	tr	-	0.2	0.6	0.5	tr	0.4	1.0	0.2
Phenylethyl butyrate	A	-	-	tr	-	tr	-	-	tr	tr	tr	-	tr	tr	-
2-Methoxy-4-methylphenol	A	tr	tr	tr	tr	tr	tr	-	-	-	tr	-	tr	tr	-
Phenylethyl 2-methylbutyrate	D	-	-	-	-	-	-	-	tr	-	-	-	-	-	tr
Phenylethyl isovalerate	A	-	-	-	-	-	-	-	-	tr	tr	-	tr	tr	0.2
Methyleugenol	B	tr	tr	-	tr	-	tr	tr	-	0.1	tr	tr	0.2	tr	0.5
(E)-Cinnamaldehyde	A	-	-	-	-	-	-	-	-	tr	tr	tr	0.2	tr	-
3-Phenylpropanol	A	-	-	-	-	-	-	-	-	0.2	0.1	0.0	0.1	0.1	-
p-Cresol	A	tr	tr	tr	tr	tr	tr	-	-	-	-	-	-	-	-
Phenylethyl tiglate	D	-	-	-	-	-	-	-	tr	-	-	-	-	-	-
(E)-Cinnamyl acetate	A	-	-	-	-	-	-	-	-	-	0.3	-	tr	tr	-
Eugenol	A	tr	0.3	tr	tr	tr	0.4	-	-	-	-	-	-	-	-
Elemicin	B	tr	0.1	tr	tr	tr	tr	1.0	-	1.3	0.4	0.1	0.7	0.5	12.7
Anisyl alcohol	A	-	-	-	-	-	-	-	-	tr	tr	tr	tr	tr	-
(E)-Cinnamic alcohol	A	-	-	-	-	-	-	-	-	0.1	0.2	-	0.1	tr	-
2-(4-Methoxyphenyl)ethanol	B	-	-	-	-	-	-	-	-	tr	tr	tr	tr	tr	tr
3,4,5-Trimethoxy toluene	D	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-
5-Methoxyeugenol	A	-	-	-	-	-	-	-	-	tr	tr	-	tr	-	tr
Benzyl benzoate	C	-	-	-	-	-	-	-	-	tr	-	-	-	-	-
1-Isopropenyl-2-methoxy-4-methylbenzene	D	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-
Isoprenoids															
Monoterpenes															

α-Pinene	C	-	-	-	-	-	-	0.4	0.2	-	-	-	-	-	0.4
Camphene	D	-	-	-	-	-	-	tr	-	-	-	-	-	-	-
β-Pinene	C	-	-	-	-	-	-	0.1	-	-	-	-	-	-	0.2
Sabinene	D	-	-	-	-	-	-	tr	-	-	-	-	-	-	-
δ-3-Carene	D	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1
Myrcene	A	0.3	0.2	-	tr	1.3	0.3	0.6	5.1	-	-	-	-	-	0.3
Limonene	A	0.4	tr	tr	tr	7.5	0.9	5.0	26.9	0.5	0.7	0.4	tr	1.8	0.2
Eucalyptol	A	0.3	0.1	0.6	tr	0.8	0.1	0.6	0.1	-	-	-	-	-	0.1
β-Phellandrene	D	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-
(Z)-Ocimene	C	2.3	0.2	tr	0.3	0.2	1.1	0.6	0.3	0.3	0.8	0.5	1.2	4.5	0.7
(E)-Ocimene	A	16.8	0.7	0.8	1.5	1.3	2.5	0.8	1.0	2.3	5.0	1.2	9.7	14.9	9.4
Terpinolene	A	-	-	-	-	-	-	-	-	0.2	0.2	0.3	0.5	0.5	-
(E)-Limonene epoxide	D	-	-	-	-	-	-	-	tr	-	-	-	-	-	-
Perillene	B	-	-	-	-	-	-	tr	-	tr	tr	tr	tr	tr	tr
(E)-Linalool oxide (furanoid)	C	2.1	1.8	0.8	3.3	3.2	2.7	1.7	1.5	1.6	1.8	3.1	0.5	1.5	1.8
(E,E)-2,6-Dimethylocta-1,3,5,7-tetraene	B	-	-	-	-	-	-	-	-	-	0.2	tr	0.3	0.4	-
2,6,6-Trimethyl-2-vinyltetrahydropyran-5-one	D	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2
(Z)-Linalool oxide (furanoid)	C	-	-	-	-	-	-	0.2	0.1	0.3	0.2	0.3	0.5	0.2	0.2
Linalool	C	46.6	46.7	49.9	57.9	57.7	59.1	57.0	24.0	45.7	40.4	39.5	5.3	38.5	8.9
(Z)-*p*-Menth-2-en-1-ol	B	tr	tr	tr	tr	tr	tr	-	0.5	tr	tr	tr	tr	tr	-
Terpinen-4-ol	C	-	-	-	-	-	-	tr	0.2	tr	tr	tr	0.1	tr	tr
Hotrienol	A	7.8	15.7	7.6	10.6	9.1	10.4	9.9	9.7	8.3	6.7	14.2	2.2	9.2	4.8
β-Cyclocitral	A	0.3	tr	0.2	0.4	0.2	0.5	0.1	-	-	-	-	-	-	-
(Z)-*p*-Mentha-2,8-dien-1-ol	B	tr	-	-	-	tr	-	tr	0.5	-	-	-	-	-	-
6-Hydroxy-2,6-dimethyloct-7-en-4-one	D	-	-	-	-	-	-	1.4	2.8	-	-	-	-	-	2.1
Citronellyl formate	D	-	-	-	-	-	-	-	-	-	-	-	-	-	tr
(E)-*p*-Mentha-2,8-dien-1-ol	D	-	-	-	-	-	-	tr	0.2	-	-	-	-	-	-
(E)-Piperitol	D	-	-	-	-	-	-	-	tr	-	-	-	-	-	-
(E)-Isopiperitenol	D	-	-	-	-	-	-	-	5.5	-	-	-	-	-	-
Ipsdienol	B	tr	tr	-	tr	tr	tr	-	0.2	-	-	-	-	-	-
α-Terpineol	C	tr	-	tr	tr	0.3	0.3	0.7	3.3	0.4	0.2	0.1	0.8	0.4	0.1
Piperitone	A	tr	-	-	-	tr	tr	0.2	0.6	tr	tr	tr	tr	tr	-
Carvone	D	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-
β-Cyclogeraniol	B	tr	tr	tr	0.1	tr	0.1	tr	-	-	-	-	-	-	-
(E)-Linalool oxide (pyranoid)	A	0.2	0.6	0.3	0.3	0.3	0.4	0.3	0.5	0.2	0.1	0.2	0.1	0.1	0.3
(Z)-*p*-Mentha-6,8-dien-2-ol	A	tr	-	-	-	tr	-	0.7	0.2	-	-	-	-	-	-

(Z)-Linalool oxide (pyranoid)	A	-	-	-	-	-	-	-	tr	tr	tr	tr	tr	tr	0.1
Citronellol	D	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2
6-Hydroxy-2,6-dimethyl-2,7-octadien-4-one	D	-	-	-	-	-	-	tr	0.4	-	-	-	-	-	0.3
Geranyl acetate	A	-	-	-	-	-	-	-	-	-	tr	-	-	-	-
Nerol	A	tr	tr	tr	-	tr	-	-	-	-	-	-	-	-	-
p-Mentha-1,8-dien-3-one	A	0.1	-	-	-	0.6	0.5	0.7	2.3	-	-	-	-	-	-
Geraniol	C	-	-	-	-	-	-	-	-	tr	tr	tr	tr	tr	tr
2,6-Dimethyl-3,7-octadiene-2,6-diol [c]	A	-	-	-	-	-	-	-	0.2	-	-	-	-	-	0.2
(E)-Eldanolide	D	-	-	-	-	-	-	-	0.4	-	-	-	-	-	1.7
3,7-Dimethyl-1,6-octadien-3,5-diol	D	-	-	-	-	-	-	-	1.6	-	-	-	-	-	0.4
2,6-Dimethyl-1,7-octadiene-3,6-diol	B	tr	-	-	-	tr	tr	-	tr	-	-	-	-	-	-
Sesquiterpenes															
α-Cubebene	D	-	-	-	-	-	-	tr	-	-	-	-	-	-	-
α-Copaene	D	-	-	-	-	-	-	-	tr	-	-	-	-	-	-
Caryophyllene	C	tr	tr	tr	tr	tr	tr	1.4	1.8	-	-	-	-	-	0.3
Humulene	A	tr	tr	tr	-	tr	tr	tr	tr	-	-	-	-	-	-
Germacrene D	A	-	-	-	-	-	-	0.2	-	-	-	-	-	-	-
α-Farnesene	B	-	-	-	-	-	-	0.7	-	-	-	-	-	-	-
Caryophyllene epoxide	D	-	-	-	-	-	-	tr	0.4	-	-	-	-	-	-
Terpene derived compounds															
6-Methyl-5-hepten-2-one [c]	D	-	-	-	-	-	-	tr	0.1	-	-	-	-	-	tr
(E)-4,8-Dimethylnona-1,3,7-triene	D	-	-	-	-	-	-	-	0.2	-	-	-	-	-	-
(E)-Geranylacetone	D	-	-	-	-	-	-	0.1	tr	-	-	-	-	-	-
(E,E)-4,8,12-Trimethyltrideca-1,3,7,11-tetraene	D	-	-	-	-	-	-	0.2	tr	-	-	-	-	-	0.4
6,10,14-Trimethylpentadecan-2-one	D	-	-	-	-	-	-	-	tr	-	-	-	-	-	tr
Nitrogen-containing compounds															
(E)-2-Methylbutyraldoxime-O-methyl ether	D	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-
(Z)-2-Methylbutyraldoxime-O-methyl ether	D	-	-	-	-	-	-	tr	-	-	-	-	-	-	-
Phenylacetaldoxime-O-methyl ether	D	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2
Benzylnitrile	C	-	-	-	-	-	-	-	-	0.7	0.6	tr	0.3	0.8	0.4
1-Propyl-2-methyl-5-vinylpyrrole	B	0.8	3.0	4.9	1.2	2.3	2.4	-	-	-	-	-	-	-	-
1-Nitro-2-phenylethane	D	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2
Indole	C	tr	0.1	2.1	0.3	0.4	0.2	tr	-	tr	tr	tr	tr	tr	-
Miscellaneous cyclic compounds															

2,2,9-Trimethyl-1,6-dioxaspiro[4.4]nonane (Isomer A)	D	-	-	-	-	-	-	-	-	-	-	-	-	-	0.6
2,2,9-Trimethyl-1,6-dioxaspiro[4.4]nonane (Isomer B)	D	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2
2-Methylcyclopent-2-en-1-one	A	tr	tr	tr	tr	tr	-	-	-	-	-	-	-	-	-
δ-Octalactone	A	tr	tr	tr	tr	tr	0.1	-	-	tr	tr	-	tr	tr	-
δ-Decalactone	A	tr	3.9	4.2	0.6	1.2	5.9	2.2	1.5	tr	2.5	0.2	2.3	0.7	1.8
5-(Hydroxymethyl)-2-furancarboxaldehyde	D	-	-	-	-	-	-	-	-	tr	tr	-	tr	-	-
Unknowns [d]															
m/z: 114*, 43, 58, 71		1.4	tr	tr	0.3	0.3	0.4	-	-	-	-	-	-	-	-
m/z: 150*, 69, 41, 81,79,82,53		-	-	-	-	-	-	-	-	1.2	1.5	0.4	0.7	1.4	-
m/z: 121*, 106, 80, 79, 120		1.3	5.5	8.0	3.1	1.7	2.9	-	-	-	-	-	-	-	-
m/z: 71,43,82,67,41,55,39,53		1.0	0.7	0.6	0.8	0.7	0.9	-	-	tr	tr	0.2	tr	0.1	-
m/z: 152*, 84, 83, 41, 56, 69, 108		0.1	-	-	-	1.9	0.4	-	-	-	-	-	-	-	-
m/z: 168*, 99, 71, 43, 41, 69		0.3	0.4	tr	tr	0.2	0.3	-	-	0.2	1.3	0.1	0.5	0.3	-
Aliphatics		14.3	4.2	10.4	13.6	6.7	5.3	0.5	0.6	0.9	1.1	2.8	0.8	1.8	0.9
Benzenoids		1.9	14.8	8.0	4.6	0.8	0.8	10.0	1.6	34.7	34.6	35.7	72.0	20.5	57.6
Isoprenoids		77.5	66.0	60.2	74.3	82.5	78.9	83.6	91.0	59.8	56.4	59.9	21.3	71.9	33.4
Nitrogen-containing compounds		0.8	3.2	7.0	1.5	2.7	2.6	0.1	0.0	0.7	0.6	0.0	0.3	0.8	0.8
Miscellaneous cyclic compounds		0.0	3.9	4.2	0.6	1.2	6.0	2.2	1.5	0.0	2.5	0.2	2.3	0.7	2.6
Unknowns		4.1	6.7	8.6	4.2	4.8	4.9	0.0	0.0	1.4	2.8	0.7	1.1	1.8	0.0
Total of all compounds		98.6	98.8	98.5	98.7	98.7	98.6	96.4	94.7	97.5	98.1	99.3	97.8	97.5	95.3
Total number of compounds		50	43	42	44	50	46	54	61	49	51	41	50	48	58
Total volatiles (ng) emitted (per inflorescence per hour)		142250	41046	28288	58704	143729	50384			322	648	333	236	463	

[a] Relative amounts (%). tr = trace amount (< 0.1 % of total sample).

[b] Compound identification criteria: 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; D = compound identified by Dr Roman Kaiser.

[c] Compounds which are possible background contaminants as they were present in some control samples.

[d] Unknowns comprising <0.1% in all samples were excluded. Mass fragments for unknowns are listed with the molecular ion first (if known) marked with *, followed by the base peak and other fragments in decreasing order of abundance.

CHAPTER 7

THE IMPORTANCE OF SCENT AND NECTAR FILTERS IN A SPECIALIZED WASP-POLLINATION SYSTEM

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The importance of scent and nectar filters in a specialized wasp-pollination system

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Summary

1. Plants with open flowers and exposed nectar should attract a wide diversity of flower visitors, yet, for reasons that are not yet well understood, some plants with these 'generalist' floral traits have highly specialized pollination systems.

2. We investigated this problem in the African milkweed *Pachycarpus grandiflorus* which has open flowers that produce copious amounts of exposed and concentrated nectar, yet is visited almost exclusively by spider-hunting wasps in the genus *Hemipepsis*.

3. These wasps were the only visitors found to consistently carry pollinaria and a cage experiment showed that they are capable of successfully pollinating this plant. Furthermore, experimental hand-pollinations showed that *P. grandiflorus* is genetically self-incompatible and thus reliant on pollinators for seed set.

4. We investigated the roles of chemical (nectar and floral scent) and spectral properties in the selective attraction of wasps and the filtering out of other potential flower visitors. Nectar palatability experiments showed that the nectar is unpalatable to honeybees but palatable to the wasps. Choice experiments conducted in the field and using a Y-maze in the laboratory showed that wasps are attracted primarily by scent rather than visual cues. Analysis of scent using Gas Chromatography-Mass Spectrometry showed that these inflorescences produce 36 different compounds, mostly monoterpenes and aliphatics. Analysis of spectral reflectance showed that flowers have similar colouring to the background vegetation.

5. We conclude that *P. grandiflorus* is specialized for pollination by *Hemipepsis* wasps, and in the absence of morphological filters, achieves specialization through unpalatable nectar, cryptic colouring and scent as a selective pollinator attractant.

6. This study demonstrates that plants whose flowers are not morphologically adapted to exclude particular floral visitors can achieve specialization through non-morphological filters.

Key-words: Asclepiadoideae, breeding system, floral filter, *Pachycarpus grandiflorus*, pollination syndrome, Pompilidae, self-incompatibility, spider-hunting wasp

Introduction

Specialized pollination in plants is typically achieved through morphological adaptations (such as long spurs) which function to exclude particular floral visitors (Johnson & Steiner 2000). However, specialized pollination is also apparent in a number of plants with open, morphologically unspecialized flowers and the mechanisms through which these plants filter their visitors are still poorly understood (Johnson & Steiner 2000). In the absence of specialized morphology, these flowers appear to rely on chemical (nectar and scent) and spectral reflectance properties to selectively attract pollinators (Brodmann *et al.* 2008) and deter nectar thieves (Johnson, Hargreaves & Brown 2006). However, most studies of specialization in morphologically generalized flowers have focused only on single traits. For example, several studies have demonstrated a role for unpalatable nectar as a potential floral filter but have not explored the roles of floral scent and colouring (Stephenson 1981, 1982; Adler 2000;

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Johnson *et al.* 2006; Shuttleworth & Johnson 2006). In this study, we explore the combined roles of nectar, scent and cryptic colouring in a milkweed that has morphologically generalized flowers but exhibits a highly specialized pollination system.

Specialized interactions between plants and prey-hunting wasps are typically associated with sexually deceptive (Steiner, Whitehead & Johnson 1994; Schiestl *et al.* 1999; Schiestl 2005) and food-based mimicry systems (Nilsson *et al.* 1986; Nazarov 1995) but appear to be uncommon in rewarding plants. Documented examples in rewarding plants include pollination by vespids in *Oxypetalum* spp. and *Blepharodon nitidum* (both milkweeds) in South America (Vieira & Shepherd 1999; J. Ollerton *et al.*, unpublished data) and pollination by social vespids in the European orchids *Epipactis helleborine* and *E. purpurata* (Ehlers, Olesen & Ågren 2002; Brodmann *et al.* 2008). Another specialized system operated by pompilid wasps in the genus *Hemipepsis* has recently become apparent from studies of a number of rewarding South African grassland flowers. Plants involved in this system include orchids (Johnson 2005; Johnson, Ellis & Dötterl 2007), milkweeds (Ollerton *et al.* 2003; Shuttleworth & Johnson 2006, 2008, 2009a, 2009c) and pineapple flowers (Hyacinthaceae: *Eucomis*; Shuttleworth & Johnson 2009b). A general feature of these pompilid-pollinated flowers is the production of exposed nectar with no morphological means to prevent non-pollinator visits.

The genus *Pachycarpus* E. Mey. (Apocynaceae: Asclepiadoideae) is endemic to Africa and contains 37 species occurring in grasslands south of the Sahara (Goyder 1998; Smith 1988). Several South African members of the genus have flowers that produce copious amounts of exposed nectar and are known to be pollinated exclusively by pompilid wasps in the genus *Hemipepsis* (Ollerton *et al.* 2003; Shuttleworth & Johnson 2006, 2009a, unpublished data). Preliminary observations of *Pachycarpus grandiflorus* suggested that this species is also visited and pollinated almost exclusively by *Hemipepsis* pompilid wasps making it a suitable model to explore the roles of non-morphological traits in achieving floral specialization. We hypothesized that *P. grandiflorus* has a specialized pollination system (operated by *Hemipepsis* wasps) and achieves specialization through a combination of cryptic colouring, unpalatable nectar and specific floral scent.

The broad aims of this study were thus to determine whether *P. grandiflorus* has a specialized pollination system and, if so, to explore how these flowers achieve specialization in the absence of typical morphological filters. Our specific objectives were: (i) to identify the effective pollinators of *P. grandiflorus*, (ii) to determine whether *P. grandiflorus* has a breeding system that makes it reliant on pollinators for reproduction, (iii) to determine if *P. grandiflorus* nectar is unpalatable to non-pollinating insects but palatable to pollinating insects, (iv) to determine if pollinators are attracted by scent or visual cues, (v) to determine the chemical composition of the floral fragrance, and (vi) to determine if the spectral reflectance of the flower corolla is similar to that of the background vegetation.

Fig. 1. *Pachycarpus grandiflorus* and its pollinators, Gilboa Estate. (a) Whole plant. Note male *Hemipepsis capensis* (left) and male *H. hilaris* (right) approaching the plant. (b) Female *H. capensis* visiting an individual flower. Note the leg clinging to the central column with a tarsal claw trapped between the guide rails (arrow). c, corpusculum; cl, corona lobe; cr, corolla lobe; gr, guide rail.

Materials and methods

STUDY SPECIES AND STUDY SITES

Pachycarpus grandiflorus (L. f.) E. Mey. is a perennial herb found in grasslands and rocky slopes from the Eastern Cape through KwaZulu-Natal to Mpumalanga province, South Africa (Smith 1988; Pooley 1998). Plants are semi-decumbent with large inflated flowers (Fig. 1) which are dull green in colour with purple spots of varying density. The corona lobes extend horizontally from the central column and are folded over distally (Fig. 1b). Plants at Gilboa Estate had 16·1 ± 1·22 flowers per plant (Mean ± SE, n = 54). Flowering occurs from November to April (Pooley 1998). Voucher specimens from Gilboa Estate are deposited in the NU Herbarium, University of KwaZulu-Natal, Pietermaritzburg campus (Collectors Numbers: Shuttleworth 36, 37 and 50).

This study was conducted at three sites in KwaZulu-Natal province. At the first site, Gilboa Estate in the Karkloof mountain range, we located two populations of *c.* 60 plants each *c.* 1 km apart (29°16′30·7″ S; 30°16′45·0″ E. 1607 m and 29°16′56·9″ S; 30°17′33·8″ E. 1727 m, respectively). The second site, Wahroonga farm (29°36′22″ S; 30°07′42″ E. 1350 m), had a small population of approximately five plants growing in annually burnt montane grassland. At the third site, Fort Nottingham village commonage (29°23′55·0″ S; 29°55′30·0″ E. 1707 m), we located two plants growing in montane grassland. These

populations were all situated on rocky slopes in montane grassland. The study was conducted primarily in the Gilboa Estate populations, with additional visitor observations being conducted at the other two sites. This study was conducted during the five flowering seasons between 2003/2004 and 2007/2008.

FLORAL VISITORS

Floral visitors were observed at all field sites (total observation time *c.* 120 h spread over the five flowering seasons). Insect visitors were noted and representative individuals of each species were collected for subsequent identification. The *Hemipepsis* (Pompilidae) wasps were familiar to the authors and individual wasps could confidently be identified as belonging to one of the three *Hemipepsis* species identified (see Results) without collecting the individuals. Representative insect specimens are deposited in the university collection of SDJ and in the Natal Museum (Pietermaritzburg, South Africa).

Rates of visitation by insects to flowers of *P. grandiflorus* were measured at both the Gilboa Estate populations in the 2006/2007 flowering season. Seventeen plants were observed for a period of 25 min each and the number and identity of insects arriving during that period was recorded.

POLLINATOR EFFECTIVENESS AND MECHANISM OF POLLINATION

Pollen loads were determined for all species of insect visitor. Presence and placement of pollinaria (or just corpuscula) was assessed on collected individuals and, in instances where individuals could be confidently identified, on individuals which were captured and released. In some cases, pollinia were also observed on individual insects that were not captured.

Pompilid wasps, unlike bees, are seemingly unaffected by laboratory cage conditions. Wasps placed in a flight cage with *P. grandiflorus* flowers will immediately commence feeding and show behaviour which is apparently identical to that exhibited in the field. A laboratory cage experiment was conducted with plants from Gilboa Estate to test the effectiveness of *Hemipepsis* wasps as pollinators. Three plants bearing virgin flowers (previously bagged at the bud stage) were cut at ground level and placed in a 1-m^3 fine mesh cage in the laboratory with seven newly-caught *Hemipepsis* wasps (three *H. capensis*, one *H. errabunda* and three *H. hilaris*) from 7 to 20 February 2007. Wasps did not carry pollinia at the start of the experiment. After the experiment, wasps were killed and examined under a dissecting microscope for the presence and placement of pollinia. The number of removed and inserted pollinia in flowers was also determined using a dissecting microscope.

POLLINATION SUCCESS

The frequency of pollinia removal and insertion was determined for 65 flowers on 10 plants from Gilboa Estate in February 2004. Flowers were examined using a dissecting microscope in the laboratory. The mean number of pollinia removed per flower and mean number of pollinia inserted per flower were calculated for each plant. These mean values were then used to obtain a grand mean for the population. The percentage of flowers pollinated (containing at least one inserted pollinium) was calculated for each plant and a mean obtained from these values to represent the percentage of flowers pollinated in the population. The frequencies of removed and inserted pollinia in flowers was used to calculate the pollen transfer efficiency (PTE) as the percentage of removed pollinia which were inserted between guide rails (cf. Johnson, Peter & Ågren 2004).

FRUIT SET AND BREEDING SYSTEM

Percentage fruit set in naturally pollinated plants was measured at Gilboa Estate at the end of the 2007/2008 flowering season from 29 previously labelled plants. Percentage fruit set was calculated per flower for each plant and these values used to calculate a mean for the population. Seed set was measured as the number of seeds per fruit from 10 randomly selected fruits.

The degree of self-compatibility and capacity for autogamy in *P. grandiflorus* was determined using controlled hand-pollinations on five plants at Gilboa Estate in January 2006. Virgin flowers (previously bagged at the bud stage with fine mesh pollinator exclusion bags) were assigned to one of three treatments (three flowers per treatment on each plant): (i) cross-pollinated (pollinated with pollinia from flowers on a different plant), (ii) self-pollinated (pollinated with pollinia from flowers on the same plant), and (iii) control (unmanipulated). Hand-pollinations were performed using fine forceps. The corpusculum of a pollinarium was grasped with the forceps and the pollinarium gently removed from the flower. Each pollinium was then inserted with the convex surface innermost into the stigmatic chamber of a recipient flower (cf. Wyatt 1976). Pollinia were inserted into two of the five available stigmatic chambers of individual flowers. Once pollinated, flowers were rebagged and left for *c.* 8 weeks to develop fruits. Once fruits were fully developed, fruit set in flowers from each treatment was recorded. Fruit set in each treatment was compared using a χ^2 test. The number of seeds per fruit from each treatment was counted.

NECTAR PRODUCTION AND PALATABILITY EXPERIMENTS

Nectar properties were measured at Gilboa Estate. Nectar volume and concentration (percentage sucrose equivalent by weight) were measured using 5 μL capillary tubes and a Bellingham and Stanley (0–50% or 45–80%) hand-held refractometer. Means were calculated per flower. The standing crop volume and concentration of nectar was measured from 25 and 19 flowers, respectively, on four plants in February 2004. Nectar production over a 24-h period was measured from 25 flowers on five plants in February 2008. These flowers were bagged for 24 h prior to nectar sampling and nectar was measured once at the end of the 24 h period (nectar present on these flowers at the start of the 24 h period was removed with capillary tubes).

Pachycarpus grandiflorus nectar is secreted in an exposed position but is not utilized by common nectar-feeding insects, such as honeybees (*Apis mellifera scutellata*), which are common at the study sites. The nectar of *P. grandiflorus* has an unpleasant bitter taste to humans, suggesting that it may be unpalatable to nectar-feeding insects other than *Hemipepsis* wasps. We tested the palatability of *P. grandiflorus* nectar to honeybees and *Hemipepsis* wasps by offering individuals a three way choice between *c.* 1-2 μL droplets of *P. grandiflorus* nectar, and sucrose or hexose (a 1 : 1 mixture of glucose and fructose) sugar solutions of identical volume and concentration. To do this, individual bees and *Hemipepsis* wasps were placed in small glass vials. The droplets of nectar and the two sugar solutions were then placed 20 mm apart in a triangular configuration on a plastic petri dish and a vial containing a bee or a wasp was placed upside down over the three solutions such that the individual could crawl down and consume the solutions on the petri dish. The vials used were large

enough that the bees and wasps were able to crawl onto the petri dish while still covered by the vial. Nectar for these experiments was obtained from six plants (using capillary tubes) at Gilboa Estate. Sugars were dissolved in water and the solutions were diluted to match the sugar concentration of the nectar (45% in these experiments). Honeybees were collected in the University of KwaZulu-Natal botanical garden and *Hemipepsis* wasps were collected at Gilboa Estate. We noted which solutions were selected or rejected by each bee or wasp. A solution was considered to have been selected if the individual consumed all (or nearly all) of the solution on the petri dish. A solution was considered to have been rejected if the individual probed but did not consume the solution. In total, 18 bees and 18 wasps were tested and each individual was used only once.

POLLINATOR ATTRACTION

Pompilid wasps approaching *P. grandiflorus* plants exhibit a typical zig-zag flight pattern (see Johnson 2005) suggesting that wasps are attracted primarily by a scent cue. To test the importance of floral scent as an attractant, we conducted Y-maze choice experiments in the laboratory and choice experiments in the field.

Y-maze choice experiments were conducted in February 2008 with *Hemipepsis* wasps and *P. grandiflorus* flowers collected at Gilboa Estate. We used a 20-mm diameter glass Y-maze placed on a light table. Each arm of the Y-maze was 90 mm long and the main arm was 170 mm long. The main arm of the Y-maze was connected to a suction pump (flow rate = 6000 mL min^{-1}), such that air was drawn along each arm of the Y. One arm of the Y was then attached to a polyacetate bag containing a *P. grandiflorus* inflorescence and the other arm attached to an empty polyacetate bag. A hole was made in each bag to allow airflow through the bag. Wasps were inserted at the entrance to the Y-maze (by briefly disconnecting the pump) and allowed to walk down the Y-maze and select one of the arms. In total, 31 runs were made with 16 *Hemipepsis* wasps. Each wasp was used until it became visibly distressed resulting in a varied number of runs per wasp (range 1–5 per wasp). The side containing the flowers was selected randomly for each run. To establish if wasps show preference, the number of choices made in favour of the arm with flowers was compared to the number of choices in favour of the control arm using a binomial test (with individual runs for each wasp pooled). As a second analysis to control for potential individualistic behaviour of wasps, the percentage of choices in favour of the arm with flowers was also calculated for each individual wasp and these compared to 50% using a one sample *t*-test.

Field based experiments were conducted at Gilboa Estate in February 2007. Two *P. grandiflorus* inflorescences with a similar number of flowers were cut and placed in vases *c.* 1 m apart and at 90° to the prevailing breeze. One inflorescence was then covered with nearby vegetation [*Eriosema* sp. (Fabaceae) leaves and grass] such that the inflorescence was completely concealed from view. The other inflorescence was left exposed. The two inflorescences were then observed for a period of 25 min during which the number of *Hemipepsis* wasps visiting each of the covered and exposed plants was recorded. The covered and exposed plants were switched (i.e. the covered plant was exposed and the exposed plant covered) approximately half way through an observation period and both plants were moved to different positions between observation periods. This was repeated five times and two different pairs of plants were used. Wasp responses were classified as either visits (where the wasp actually alighted on the flowers) or inspections (where a wasp approached to within 5 cm of a plant and then flew off without actually landing on the flowers). The total number of visits and inspections to the covered and exposed inflorescences were compared.

SCENT SAMPLING AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS) ANALYSIS OF VOLATILES

The floral scent of *P. grandiflorus* was collected using dynamic headspace extraction methods and analyzed by coupled GC-MS. We sampled the scent of five plants in the field at Gilboa Estate in January 2008 by enclosing the inflorescence in a 25 × 20 cm polyacetate bag and pumping air from the bag through a small cartridge filled with 1 mg of tenax® and 1 mg of carbotrap® at a flow rate of 50 mL min^{-1} for a duration of 30 min. A control was taken from an empty polyacetate bag sampled for the same duration. GC-MS analysis of the samples was carried out using a Varian CP-3800 GC (Varian, Palo Alto, CA) with a 30 m × 0·25 mm internal diameter (film thickness 0·25 μm) Alltech EC-WAX column coupled to a Varian 1200 quadrupole mass spectrometer in electron-impact ionization mode at 70 eV. Cartridges were placed in a Varian 1079 injector equipped with a 'Chromatoprobe' thermal desorbtion device (Amirav & Dagan 1997; Dötterl, Wolfe & Jürgens 2005). The flow of helium carrier gas was 1 mL min^{-1}. The injector was held at 40 °C for 2 min with a 20 : 1 split and then increased to 200 °C at 200 °C min^{-1} in splitless mode for thermal desorbtion. Meanwhile, the GC oven was held at 40 °C for 3 min and then ramped up to 240 °C at 10 °C min^{-1} and held there for 12 min. Compounds were identified using the Varian Workstation software with the NIST05 mass spectral library and verified, where possible, using retention times of authentic standards and published Kovats indices. Compounds present at similar abundance in the control were considered to be contaminants and excluded from analysis. To ensure accuracy with quantification of emission rates, known amounts of standards were injected into cartridges and thermally desorbed under identical conditions to the samples.

SPECTRAL REFLECTANCE ANALYSIS OF FLOWERS AND BACKGROUND

Spectral reflectance across the 300–700 nm range was determined using an Ocean Optics S2000 spectrometer (Ocean Optics Inc., Dunedin, Fla.), Ocean Optics DT-mini light source and fibre optic reflection probe (QR-400-7-UV-VIS; 400 μm) held at 45° to the flower or leaf surface in a probe holder (RPH-1). Spectral reflectance was measured from the corolla lobes (including measurements from both the green background and the purple spots) of flowers from eight *P. grandiflorus* plants from Gilboa Estate. Spectral reflectance of background vegetation was measured from the upper surface of green leaves of nine different plant species (various grasses, forbs and herbs). Three replicates were taken for each of the background species and a mean spectrum was calculated for each plant species.

Results

FLORAL VISITORS

Pachycarpus grandiflorus flowers at all of the study sites were visited mostly by pompilid wasps in the genus *Hemipepsis,* with *H. capensis* being the most abundant (Table 1). *Hemipepsis* wasps obtained a nectar reward from plants and flowers were visited by both sexes (68% male for collected

Table 1. Insect visitors to *P. grandiflorus* and their respective pollen loads

Insect visitor	No. observed (No. collected)	No. carrying pollinaria (No. inspected)	Pollinaria placement	Locality*
Hymenoptera				
Pompilidae				
Hemipepsis capensis (Linnaeus, 1764)	116 (114)	30 (114)	Claws, tibial and tarsal spines	G, W
H. errabunda (Dalla Torre, 1897)	12 (12)	5 (12)	Claws, tarsal spines	FC, G
H. hilaris (Smith, 1879)	54 (43)	3 (47)	Claws, tibial and tarsal spines	G, W
Hemipepsis spp.†	542 (0)	3 (3)	Claws, tibial and tarsal spines	FC, G, W
Sphecidae				
Sphecidae sp. 1	2 (2)	0 (2)		G
Tiphiidae				
Tiphia sp. 1	51 (10)	0 (17)		G
Coleoptera				
Scarabaeidae (Cetoniinae)				
Atrichelaphinis tigrina (Olivier, 1789)	248 (8)	1 (116)	Claw	G, W
Cyrtothyrea marginalis (Swartz, 1817)	2 (1)	0 (1)		G
Diptera				
Sarcophagidae				
Sarcophagidae sp. 1	1 (0)	0 (1)		W
Sarcophagidae sp. 2	2 (1)	0 (1)		G
Sarcophagidae sp. 3	1 (1)	0 (1)		G

*FC, Fort Commonage; G, Gilboa Estate; W, Wahroonga Farm.
†These were all individuals of one of the three *Hemipepsis* species observed, but could not be firmly identified to species level as they were not captured (see Methods).

Table 2. Measures of pollination success for *P. grandiflorus* at Gilboa Estate

No. of pollinia removed Mean ± SE per flower per plant	No. of pollinia inserted Mean ± SE per flower per plant	% of flowers pollinated Mean ± SE per flower per plant	PTE%	*n* flowers (plants)
2·5 ± 0·52	0·4 ± 0·11	31·7 ± 10·01	19·8	65 (10)

individuals). Aside from *Hemipepsis* wasps, flowers were also commonly visited by the cetoniine beetle *Atrichelaphinis tigrina* and a single tiphiid wasp species (*Tiphia* sp. 1; Table 1). Individual *P. grandiflorus* plants were visited by 13·1 ± 3·25 *Hemipepsis* wasps per hour and 0·4 ± 0·31 *Tiphia* sp. 1 per hour (both Means ± SE, $n = 17$). No *A. tigrina* beetles arrived at *P. grandiflorus* flowers during the visitation rate observation periods.

POLLINATOR EFFECTIVENESS AND MECHANISM OF POLLINATION

Hemipepsis wasps were the only insects which consistently carried *P. grandiflorus* pollinia (23% of individuals inspected carried pollinia; Table 1). Nectar is secreted at the distal end of the corona lobe, forcing wasps to cling to the central column during foraging. During this process, the wasps' claws and spines on the tibiae and tarsi were trapped between guide rails and pollinaria were picked up when the wasp pulled away (Fig. 1b).

In the cage experiment, a total of 58 pollinia (on 29 pollinaria) were removed and 19 pollinia were subsequently inserted between guide rails. Of the 42 flowers (on three plants) used in this experiment, 12 (29%) were pollinated (having at least one pollinium inserted) during the experiment. Four of the *Hemipepsis* wasps had pollinaria (or just corpuscula indicating that pollinia had been inserted) attached to tarsal spines at the end of the experiment.

POLLINATION SUCCESS

Pachycarpus grandiflorus flowers experienced a PTE of 19·8% in the Gilboa Estate population (Table 2). The proportion of flowers pollinated was 31·7 ± 10·01% Mean ± SE; Table 2).

FRUIT SET AND BREEDING SYSTEM

Fruit set (Mean ± SE) occurred in 13·8 ± 2·0% of naturally-pollinated *P. grandiflorus* flowers (Table 3). In the controlled pollination experiment, fruits developed in 47% of cross-pollinated flowers while none of the self-pollinated or unmanipulated flowers set fruit ($\chi^2 = 16·6$, $P < 0·001$; Table 3). Seed set (measured as seeds per fruit) was higher in hand-pollinated fruits than in naturally-pollinated fruits (Table 3).

Table 3. Fruit and seed set in hand-pollinated and naturally pollinated *P. grandiflorus* flowers

	Hand-pollinated*			
	Crossed	Selfed	Control	Naturally pollinated
% Fruit set (*n*)	46·7 (15)	0 (15)	0 (15)	13·8 ± 2·00 (29)†
Seed set (*n*)‡	269·0 ± 26·85 (3)	–	–	139·4 ± 10·53 (10)

**n* = number of flowers not plants.
†Mean ± SE/flower/plant, SE not presented for hand-pollination means as these were calculated per flower.
‡Mean ± SE seeds per fruit.

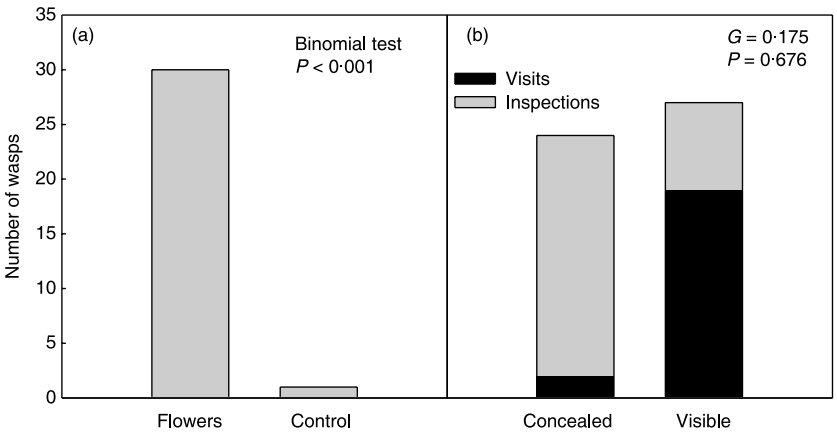

Fig. 2. Y-maze and field choice experiments with *Hemipepsis* wasps and *P. grandiflorus* flowers. (a) Number of choices by wasps in a Y-maze in favour of the arm containing flowers compared to the control (empty) arm. $n = 31$ runs with 16 wasps. (b) Number of visits and inspections by *Hemipepsis* wasps to inflorescences concealed from view (covered with leaves) compared to visible inflorescences. See text for statistical analysis of differences between visits and inspections.

NECTAR PRODUCTION AND PALATABILITY EXPERIMENTS

The standing crop volume of nectar available in *P. grandiflorus* flowers was 5·5 ± 1·90 µL (Mean ± SE, $n = 25$) with a concentration of 32·1 ± 1·12% (Mean ± SE, $n = 19$). Bagged flowers produced 16·4 ± 2·63 µL (Mean ± SE, $n = 25$) of nectar with a concentration of 44·8 ± 2·95% (Mean ± SE, $n = 25$) over a 24-h period.

In the palatability experiments, honeybees ($n = 18$) consumed all droplets of the two sugar solutions, but rejected all droplets of nectar (Friedman Test, $\chi^2 = 36$, $P < 0{\cdot}001$). In contrast, *Hemipepsis* wasps ($n = 18$) consumed all droplets of both the sugar solutions and the nectar. The number of nectar droplets consumed by *Hemipepsis* wasps was significantly greater than the number of nectar droplets consumed by the honeybees ($\chi^2 = 15$, $P < 0{\cdot}001$).

POLLINATOR ATTRACTION

In the laboratory Y-maze experiments, *Hemipepsis* wasps significantly favoured the arm of the Y-maze which contained *P. grandiflorus* flowers (Fig. 2). The percentage of choices made by individual wasps in favour of the arm containing flowers (Mean ± SE = 98·4 ± 1·56) was significantly greater than 50% ($t = 31$, df = 15, $P < 0{\cdot}001$).

In the field-based choice experiments, there was no difference between the number of visits and inspections (pooled) by *Hemipepsis* wasps to the concealed and the visible *P. grandiflorus* inflorescences ($G = 0{\cdot}175$, $P = 0{\cdot}676$; Fig. 2). However, the visible inflorescence experienced a significantly higher proportion of actual visits ($G = 21{\cdot}8$, $P < 0{\cdot}001$; Fig. 2). Apart from the pompilid wasps, no other insects approached the inflorescences during these experiments.

SCENT SAMPLING AND GC–MS ANALYSIS OF VOLATILES

To the human nose, *P. grandiflorus* flowers have a faint sweet spicy scent. A total of 36 compounds were identified in *P. grandiflorus* samples. Of these, 17 compounds were present in all samples and six were found in only a single sample (Table 4). The number of compounds in each individual sample ranged from 22 to 32 (Table 4). Overall, the scent of *P. grandiflorus* was dominated by aliphatic and isoprenoid compounds, with small amounts of benzenoids (Table 4). Four compounds [(Z)-hex-3-en-1-ol, (Z)-hex-3-en-1-ol acetate, (E)-ocimene and linalool] particularly dominated the scents in all samples, although the proportions of these compounds varied between samples (Table 4).

SPECTRAL REFLECTANCE ANALYSIS OF FLOWERS AND BACKGROUND

Pachycarpus grandiflorus flowers are typically dull green with maximum reflectance in the 500–650 nm range (Fig. 3). Overall brightness varied greatly between replicates, but maximum reflectance did not exceed 35% in any of the flowers sampled

Table 4. Compounds isolated by GC-MS from headspace samples of *P. grandiflorus*†

Compound	R_t	Criteria‡	Sample No. 1	2	3	4	5
Aliphatics							
Alcohols							
Hexan-1-ol	10·518	c	0·9	0·4	1·8	0·3	0·1
(E)-Hex-3-en-1-ol	10·551	a	0·2	0·1	0·4	–	tr
(Z)-Hex-3-en-1-ol	10·848	a	37·8	15·0	63·8	1·9	5·3
Aldehydes							
(E)-Hex-2-en-1-al	8·680	a	0·6	0·4	9·0	–	tr
Tetradecanal	21·204	b	0·1	0·3	0·2	0·5	0·2
Esters							
(Z)-Hex-3-en-1-yl acetate	10·009	a	0·5	53·4	1·0	1·3	34·2
(Z)-Hex-3-en-1-yl butyrate	11·850	a	0·9	3·3	1·5	0·5	5·6
(Z)-Hex-3-en-1-yl isovalerate	12·018	a	0·1	0·6	0·6	0·2	1·0
Aromatics							
Benzyl acetate	15·104	c	–	0·1	–	–	tr
Benzyl alcohol	16·708	c	0·9	1·4	1·1	1·5	0·4
Phenylethyl alcohol	17·118	a	tr	tr	tr	0·6	tr
(E)-Cinnamaldehyde	18·415	a	tr	0·6	0·4	1·3	tr
(Z)-Hex-3-en-1-yl benzoate	19·266	c	0·1	0·1	0·2	0·2	tr
Isoprenoids							
Monoterpenes							
Myrcene	8·136	a	–	–	–	–	0·3
(Z)-Ocimene	8·934	c	1·4	0·5	0·8	2·7	1·7
(E)-Ocimene	9·196	a	48·5	15·7	0·3	25·7	26·3
Linalool	12·847	c	0·2	5·1	15·9	46·6	14·3
2,6-Dimethyl-3,7-octadiene-2,6-diol	17·268	a	–	–	–	0·2	tr
Sesquiterpenes							
β-Caryophyllene	13·630	c	2·2	tr	0·1	8·4	7·6
Humulene	14·501	a	–	–	–	–	0·4
Germacrene D	14·938	a	–	0·2	–	–	0·7
α-Farnesene	15·299	b	0·7	0·1	0·2	0·3	0·1
Terpene derived compounds							
2,6,6-Trimethylcyclohex-2-ene-1,4-dione	14·682	a	–	0·3	–	–	–
Nitrogen-containing compounds							
Indole	22·090	c	–	0·1	–	0·2	tr
Miscellaneous cyclic compounds							
2-Methylcyclopent-2-en-1-one¶	10·755	a	2·9	tr	0·1	–	–
Unknowns§							
m/z: 204*, 55, 119, 161, 83	15·460		0·1	0·1	0·1	0·3	0·1
m/z: 120*, 105, 45, 57, 44	9·645		–	–	–	0·2	–
m/z: 150*, 69, 41, 79, 81	9·873		1·1	2·0	0·4	5·7	1·2
m/z: 96*, 81, 39, 41, 53	11·122		0·3	0·2	1·5	–	tr
m/z: 43, 80, 79, 39, 41	11·488		–	0·1	–	–	0·1
m/z: 204*, 41, 91, 79, 120	13·372		–	–	–	0·1	–
m/z: 71, 43, 82, 67, 41	13·376		–	–	–	–	0·1
m/z: 96, 71, 43, 32, 95	15·182		–	–	0·2	–	0·1
m/z: 204*, 121, 93, 41, 107	15·229		–	–	0·3	–	tr
m/z: 79, 131, 94, 103, 77	16·945		–	0·1	0·2	0·7	tr
Aliphatics			41·1	73·4	78·2	4·6	46·4
Aromatics			1·0	2·2	1·6	3·7	0·4
Isoprenoids			53·1	21·7	17·3	83·8	51·3
Nitrogen-containing compounds			–	0·1	–	0·2	tr
Miscellaneous cyclic compounds			2·9	tr	0·1	–	–
Unknowns			1·5	2·4	2·7	7·0	1·5
Total number of compounds			22	27	24	23	32
Total volatiles (ng) emitted (per inflorescence per hour)			1154	4435	2172	2073	15 157

†Relative amounts (in %) based on peak area with compounds arranged by retention time within compound class. tr, trace amount (< 0·1% of total sample). Totals are calculated from unrounded values and may differ slightly from totals of rounded values given in the table.
‡Compound identification criteria: 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.
¶This compound has not previously been described as a floral volatile (see Knudsen *et al*. 2006) and may be an artefact of our sampling materials.
§Mass fragments for unknowns are listed with the molecular ion first (if known) marked with *, followed by the base peak and other fragments in decreasing order of abundance.

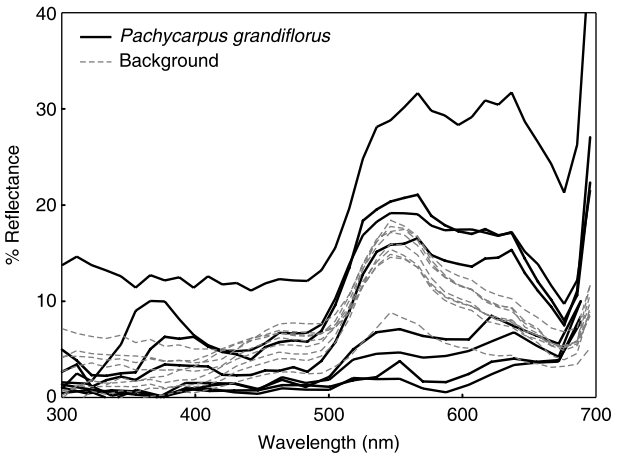

Fig. 3. Reflectance spectra for *P. grandiflorus* flowers (n = 8) and background vegetation (green leaves; n = 9). Background curves represent mean spectra calculated from individual replicates.

(Fig. 3). The reflectance of *P. grandiflorus* flowers was similar to that of the background vegetation, but flowers exhibit an additional peak (corresponding to the purple spots) in the 600–650 nm range (Fig. 3).

Discussion

The results of this study indicate that *P. grandiflorus* is pollinated exclusively by pompilid wasps in the genus *Hemipepsis*. Visitor observations and laboratory cage experiments showed that these wasps are abundant floral visitors which consistently carried pollinia and were capable of effectively pollinating plants (Table 1). Hand-pollinations showed that *P. grandiflorus* is genetically self-incompatible and thus reliant on pollinators for reproduction (Table 3). Palatability experiments with *P. grandiflorus* nectar and honeybees showed that the nectar is distasteful to non-pollinating insects. Choice experiments in the field and the laboratory showed that *Hemipepsis* wasps are attracted primarily by floral scent (Fig. 2, Table 4). Analysis of the spectral reflectance of *P. grandiflorus* flowers in comparison to typical background vegetation revealed that flowers are not brightly coloured and do not stand out from the background vegetation (Fig. 3). We conclude that *P. grandiflorus* is specialized for pollination by *Hemipepsis* pompilid wasps and achieves specialization through a combination of distasteful nectar that deters non-pollinating visitors and the production of specific scent in conjunction with cryptic colouring to selectively attract pollinators.

Although specialized pollination has been described in several plants which have open flowers and lack morphological filters, this is the first study to explore the combined roles of nectar, spectra and scent in achieving specialization. The role of distasteful nectar as a floral filter (deterring nectar thieves) has been demonstrated in several unrelated plants (Stephenson 1981, 1982; Adler 2000; Johnson *et al.* 2006; Shuttleworth & Johnson 2006). Unpalatability of nectar is typically attributed to secondary compounds (Stephenson 1981, 1982; Adler & Irwin 2005; Johnson *et al.* 2006; Gegear *et al.* 2007; Irwin & Adler 2008), but may also be due to specific sugar concentrations (Butler 1945; Waller 1972; Baker 1975) or a combination of secondary compounds and specific sugar concentrations (Liu *et al.* 2007). In the case of *P. grandiflorus*, it seems unlikely that nectar concentration (*c.* 30–45%) was a factor since honeybees readily consumed sugar solutions of the same concentration. A similar response has been shown by honeybees to the nectar of the congeneric *P. asperifolius* (Shuttleworth & Johnson 2006) which appears to have a high phenolic content (A. Shuttleworth, unpublished data). The distasteful qualities of *P. grandiflorus* nectar may, thus, be due to high levels of secondary compounds in the nectar, although the specific compounds responsible remain to be identified. Interestingly, the nectars of two other milkweeds (*Xysmalobium undulatum* and *X. orbiculare*), which are pollinated by the same *Hemipepsis* wasps (see Shuttleworth & Johnson 2008, 2009c), were more readily consumed by honeybees in similar experiments (Shuttleworth & Johnson 2009c, unpublished data). The *Xysmalobium* species, however, were visited by a much broader spectrum of non-pollinating insects (especially *X. undulatum*), suggesting that distasteful nectar in the two *Pachycarpus* species may indeed play an important functional role in reducing visits by nectar robbers (Irwin & Brody 1999; Maloof 2001).

The basis for the exclusion of other insects, such as honeybees, by nectar filters in *P. grandiflorus* could relate to their ineffectiveness as pollinators. Honeybees collected in the vicinity of the study population measure 9·4 ± 0·32 mm (Mean ± SE, n = 10) between their mouthparts and tarsi of their extended hind legs. Thus, because the nectar of *P. grandiflorus* is presented *c.* 15 mm from the column, honeybees, unlike the long-legged pompilid wasps (Fig. 1), would not remove or insert pollinaria while feeding on nectar. However, it is difficult to assess the adaptive significance of unpalatable nectar in pompilid-pollinated *Pachycarpus* species in the absence of a phylogeny for the genus (not yet available). Specialized pollination by pompilid wasps is known in five *Pachycarpus* species, two of which (*P. grandiflorus* and *P. asperifolius*) are known to have unpalatable nectar (Shuttleworth & Johnson 2006). However, nectar palatability in non-pompilid pollinated *Pachycarpus* species has not been explored. It is unclear whether unpalatable nectar is a characteristic of pompilid-pollinated species or a general property of this genus. Milkweeds are known to contain high levels of anti-herbivory compounds and it would not be surprising for some of these to be found in the nectar.

The proximal mechanisms of differential nectar palatability are difficult to determine as taste perception in insects is poorly understood. Honeybees are known to contain only 10 gustatory receptor genes (compared to 68 and 76 in fruitflies and mosquitoes, respectively) suggesting a limited capacity for taste (Robertson & Wanner 2006). However, despite using a limited number of gustatory receptors, honeybees may still be able to distinguish between a large number of compounds (de Brito Sanchez *et al.* 2007). At this stage, almost nothing is

known about the gustatory receptors of wasps. This makes it difficult to assess why wasps consume nectar which honeybees find aversive.

The results of this study show that *P. grandiflorus* flowers use floral scent as a long distance pollinator attractant (Fig. 2). It is interesting to note that in the field choice experiments, the proportion of actual visits (where the wasps landed on flowers) to visible plants was greater than the proportion of inspections (where the wasp only approached the plant, but did not land; Fig. 2). This suggests that while wasps are initially attracted only by floral scent, they rely on a visual cue to orient landing on flowers. At this stage, the specific compounds or blends of compounds in the scent of *P. grandiflorus* that are attractive to *Hemipepsis* wasps remain unknown. Other studies have shown that attraction of wasps to flowers can be due to blends of common compounds in some systems and specific compounds in other systems (e.g. Schiestl *et al*. 2003; Schiestl 2005; Brodmann *et al*. 2008).

The scent of *P. grandiflorus* is dominated by aliphatic and isoprenoid compounds with small amounts of aromatics (Table 4). Although several typical green leaf volatiles, such as (Z)-hex-3-en-1-ol and (Z)-hex-3-en-1-ol acetate, were identified, these were most likely produced by leaves enclosed with flowers during sampling. The scent of *P. grandiflorus* is similar to the scent bouquets of other pompilid-pollinated plants; however, no single compound is common to the scents of all of these plants (Johnson 2005; Johnson *et al*. 2007; Shuttleworth & Johnson 2009b). Furthermore, many of the compounds produced by these plants are ubiquitous floral volatiles which are unlikely to be specifically attractive to pompilid wasps. One possibility is that these wasps are responding to broad suites of compounds within particular classes, rather than to specific individual compounds. Alternatively, our analytical methods are not sufficiently sensitive to identify key compounds that are attracting pompilids in this system. In future studies we intend to use gas chromatography coupled to electroantennographic detection (GC-EAD) and behavioural assays with artificial scent bouquets to identify compounds that attract *Hemipepsis* wasps to flowers.

The role of specific floral scent compounds as selective pollinator attractants is well established in sexually deceptive and food-based mimicry systems (e.g. Schiestl *et al.* 1999, 2003; Schiestl 2005; Brodmann *et al*. 2008), but is poorly explored in rewarding plants. In a recent study, Brodmann *et al*. (2008) demonstrated a role for green-leaf volatiles in the highly specific attraction of pollinating vespids to the orchids *E. helleborine* and *E. purpurata*. However, nectar palatability to non-pollinating insects was not explored in this study, and the lack of visits by other insects was attributed to a combination of the plants' habitat (dark forest understorey) and specific floral scent (which mimics injured leaves) as a selective attractant. Our study of *P. grandiflorus* shows a clear role for scent as a long distance pollinator attractant, but also suggests that the nectar properties may play a functional role in preventing visits by non-pollinating insects. Interestingly, the nectars of both *E. helleborine* and *E. purpurata* are known to be toxic (Ehlers & Olesen 1997; Jakubska *et al.* 2005) and this may well play a role in deterring non-pollinating visitors.

The cryptic colouring of *P. grandiflorus* flowers (Fig. 3) is consistent with our hypothesis that pollinators are attracted primarily by scent. Flowers of *P. grandiflorus* are inconspicuous in the landscape and thereby probably avoid visual detection by other foraging insects. The role of the purple spots on *P. grandiflorus* flowers is unclear. Purple spots are found, to a greater or lesser degree, on several other *Hemipepsis* pollinated flowers (see Ollerton *et al*. 2003; Johnson 2005; Shuttleworth & Johnson 2009a). However, a number of cryptically coloured *Hemipepsis* pollinated flowers do not have purple spots or only occasionally have them (Ollerton *et al*. 2003; Shuttleworth & Johnson 2006, 2009b; Johnson *et al*. 2007) which suggests that they are not a critical cue for the attraction of these wasps.

This study adds another example to the growing list of South African plants that are reliant on *Hemipepsis* pompilid wasps for pollination (Ollerton *et al*. 2003; Johnson 2005; Shuttleworth & Johnson 2006, 2008, 2009a, 2009b, 2009c, Johnson *et al*. 2007; unpublished data). It is interesting that *P. grandiflorus* was also visited by large numbers of the cetoniine beetle *Atrichelaphinis tigrina* (Table 1). Intriguingly, these beetles were able to consume *P. grandiflorus* nectar. However, the beetles accessed nectar without contacting the central column and thus seldom removed pollinia. Furthermore, individual beetles were occasionally observed to have died in flowers after their legs had become trapped between the guide rails, suggesting that the beetles are not physically capable of removing pollinia and must, in this instance, be considered nectar thieves. The presence of these beetles on *P. grandiflorus* flowers, however, supports the broad overlap between pompilid and cetoniine pollination syndromes that has been suggested by previous studies (Ollerton *et al*. 2003; Johnson *et al*. 2007; Shuttleworth & Johnson 2008).

The PTE for *P. grandiflorus* flowers was comparable to values recorded in other milkweed species (Ollerton *et al*. 2003; Shuttleworth & Johnson 2006, 2008, 2009a, 2009c, unpublished data). The percentage fruit set in *P. grandiflorus*, however, was remarkably high (*c.* 14%) given that milkweeds typically exhibit very low levels of fruit set (Queller 1985; Lipow & Wyatt 1998). Fruit set in two other *Pachycarpus* species, also pollinated by *Hemipepsis* wasps, was found to be *c.* 1% and 24% (Shuttleworth & Johnson 2006, 2009a), suggesting that fruit set is highly variable within the genus.

Pachycarpus grandiflorus exhibits a highly specialized interaction with *Hemipepsis* pompilid wasps. This specialization is achieved through the selective attraction of *Hemipepsis* wasps using floral scent in conjunction with dull cryptic colouring. Visits by non-pollinating insects are minimized by specific properties of floral nectar which make it distasteful to nectar robbers. Further research to determine the specific scent compounds which attract *Hemipepsis* wasps and nectar compounds which are distasteful to non-pollinating insects will greatly enhance our understanding of specialized pollination systems in plants with exposed nectar.

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CHAPTER 8

SPECIALIZED POLLINATION IN THE AFRICAN MILKWEED *XYSMALOBIUM ORBICULARE*: A KEY ROLE FOR FLORAL SCENT IN THE ATTRACTION OF SPIDER-HUNTING WASPS

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ORIGINAL ARTICLE

Specialized pollination in the African milkweed *Xysmalobium orbiculare*: a key role for floral scent in the attraction of spider-hunting wasps

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Abstract Specialized pollination by prey-hunting wasps is poorly documented in rewarding plants. Furthermore, the mechanisms of achieving specialization are not clear since flowers typically produce exposed nectar and have no morphological adaptations (such as long spurs) to exclude non-pollinating visitors. We investigated the pollination of *Xysmalobium orbiculare* and explored the functional roles of floral scent and nectar in attracting pollinators and deterring nectar robbers. Floral visitor observations showed that this milkweed is visited almost exclusively by pompilid wasps in the genus *Hemipepsis*. These wasps were the only insects to carry pollinia, and a cage experiment confirmed their effectiveness in removing and inserting pollinia on flowers. Hand-pollinations showed that plants are genetically self-incompatible and thus reliant on pollinators for seed set. Palatability experiments with honeybees showed that nectar is distasteful to non-pollinating insects and is therefore likely to play a functional role in deterring nectar thieves. Choice experiments in the field showed that the wasp pollinators are attracted primarily by floral scent rather than visual cues. Analysis of spectral reflectance of flowers revealed that flowers are dull colored and are unlikely to stand out from the background vegetation. We conclude that *X. orbiculare* is specialized for pollination by spider-hunting wasps in the genus *Hemipepsis* and utilizes floral scent to selectively attract its pollinators and unpalatable nectar to deter non-pollinating visitors.

Keywords Apocynaceae · Asclepiadoideae · Pompilidae · Pollination syndrome · Breeding system · Self-incompatibility · Floral filter

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Introduction

Specialized pollination by prey-hunting wasps is not well known in rewarding plants (but see Vieira and Shepherd 1999; Ehlers et al. 2002; Brodmann et al. 2008). However, recent studies in South African grasslands have revealed a diverse guild of plants which are pollinated exclusively by spider-hunting wasps in the genus *Hemipepsis* (Hymenoptera: Pompilidae). This system includes milkweeds (Ollerton et al. 2003; Shuttleworth and Johnson 2006, 2008, 2009a; A. Shuttleworth and S.D. Johnson unpubl. data), orchids (Johnson 2005; Johnson et al. 2007) and pineapple flowers (*Eucomis*: Hyacinthaceae; Shuttleworth and Johnson 2009b). A uniting feature of these plants is the production of copious amounts of exposed nectar, which would typically be associated with generalist pollination systems.

Specialized pollination systems in plants with exposed nectar are difficult to explain as flowers have no morphological adaptations (such as long spurs) which could function to filter out certain floral visitors. In the absence of morphological traits, it appears that specialization is achieved through the production of distasteful nectar (to deter nectar thieves) and a combination of dull, cryptic coloring and specific floral scent (to selectively attract pollinators). The role of nectar in this respect has already been demonstrated in two members of this guild:

Pachycarpus asperifolius and *P. grandiflorus* are both pollinated exclusively by *Hemipepsis* wasps and have been shown to produce nectar which is distasteful to honeybees (Shuttleworth and Johnson 2006, in preparation). However, it is not clear whether this is a characteristic of the entire guild or only to members of the genus *Pachycarpus*. Specific floral scent and cryptic coloring, however, appear to play an important role in all guild members as flowers are typically dull colored and approaching wasps exhibit a characteristic zigzag flight path highly suggestive of insects following an odor plume (Raguso 2001, 2006).

The genus *Xysmalobium* R. Br. is endemic to Africa and contains some 40 species (Victor et al. 2000). Pollination and breeding system studies within the genus are scarce. Ollerton et al. (2003) found that *X. involucratum* is pollinated by chafer beetles (Scarabaeidae: Cetoniinae) while *X. gerrardii* appears to have a more generalist pollination system operated by a range of different insects. However, these conclusions were based only on visitor observations and pollen load data, and the effective pollinators of these two species were not experimentally established. The breeding systems of these two species were also not determined. In a separate study, we described effective pollination by chafer beetles and *Hemipepsis* wasps and genetic self-incompatibility in *X. undulatum* (Shuttleworth and Johnson 2008). Preliminary observations of *X. orbiculare* suggested that this milkweed is also pollinated exclusively by *Hemipepsis* wasps. We hypothesized that *X. orbiculare* exhibits a specialized pollination system operated by *Hemipepsis* wasps and achieves specialization through the production of distasteful nectar (to deter nectar robbers) and specific floral scent in combination with cryptic coloring to selectively attract pollinators.

The specific aims of this study of *X. orbiculare* were: (1) to describe the pollination system, including pollinator effectiveness, (2) to determine the breeding system, and therefore the plant's overall reliance on pollinators for reproduction, (3) to determine if nectar is distasteful to non-pollinating insects, (4) to determine if floral scent plays a functional role in attracting pollinators, and, (5) to compare the spectral reflectance of flowers to the spectral reflectance of background vegetation.

Methods

Study species and study sites

Xysmalobium orbiculare E. Mey. is an erect milkweed endemic to southern Africa (Nicholas 1999; Pooley 1998). Plants are robust, with large leaves and flowers in dense umbels (Fig. 1a). Flowers are small with reflexed corolla lobes and exposed corona lobes (Fig. 1b). Plants grow in rocky grasslands from the Eastern Cape Province of South Africa in the south through to Swaziland in the north (Nicholas 1999). Flowering occurs from October to May (Pooley 1998).

This study was conducted on the farm Wahroonga (29°36′22″S; 30°07′42″E. 1,350 m; ca. 30 plants in dense rocky grassland) and the farm Wodwo (29°24′08.1″S; 29°55′53.2″E. 1,595 m; five plants in tall montane grassland) in the flowering seasons between 2004/2005 and 2007/2008.

Floral visitors and pollinator effectiveness

Floral visitors were recorded at both study sites. Representative individuals were collected for subsequent identification and to measure pollen loads. In some instances, species level identification was also possible without collecting the individuals. Insect specimens are housed in the university collection of SDJ and representative individuals have been deposited in the Natal Museum (Pietermaritzburg, South Africa).

After preliminary observations suggested that *Hemipepsis* wasps were the primary pollinators, we conducted a cage experiment in the laboratory to establish the effectiveness of these wasps in removing and inserting pollinia. *Hemipepsis* wasps are unaffected by cage conditions and will commence feeding when placed in a flight cage with *X. orbiculare* flowers. For this experiment, ten *Hemipepsis* wasps (seven *H. capensis*, one *H. errabunda* and two *H. hilaris*) were placed in a 1 m^3 fine mesh flight cage with four *X. orbiculare* plants containing 190 virgin flowers (previously bagged at the bud stage). Plants and wasps were collected at Wahroonga. The experiment was run from 1 March to 5 March 2005 (90 h in total). At the end of the experiment, wasps were examined for presence of pollinia and the number of pollinia removed and inserted in flowers was recorded.

Pollination success and dependence on pollinators

Pollination success was measured at Wahroonga in Feb 2005. The frequency of pollinia removal and insertion was determined from 144 flowers (off 14 plants) which were examined using a dissecting microscope. The mean number of pollinia removed and inserted was calculated per flower for each plant, and these values were used to calculate a grand mean for the population. The percentage of flowers pollinated (containing at least one pollinium inserted) was calculated for each plant and these values were used to calculate a mean for the population. Pollen transfer efficiency (PTE) was calculated as the percentage of removed pollinia that were inserted between guide rails (cf. Johnson et al. 2004).

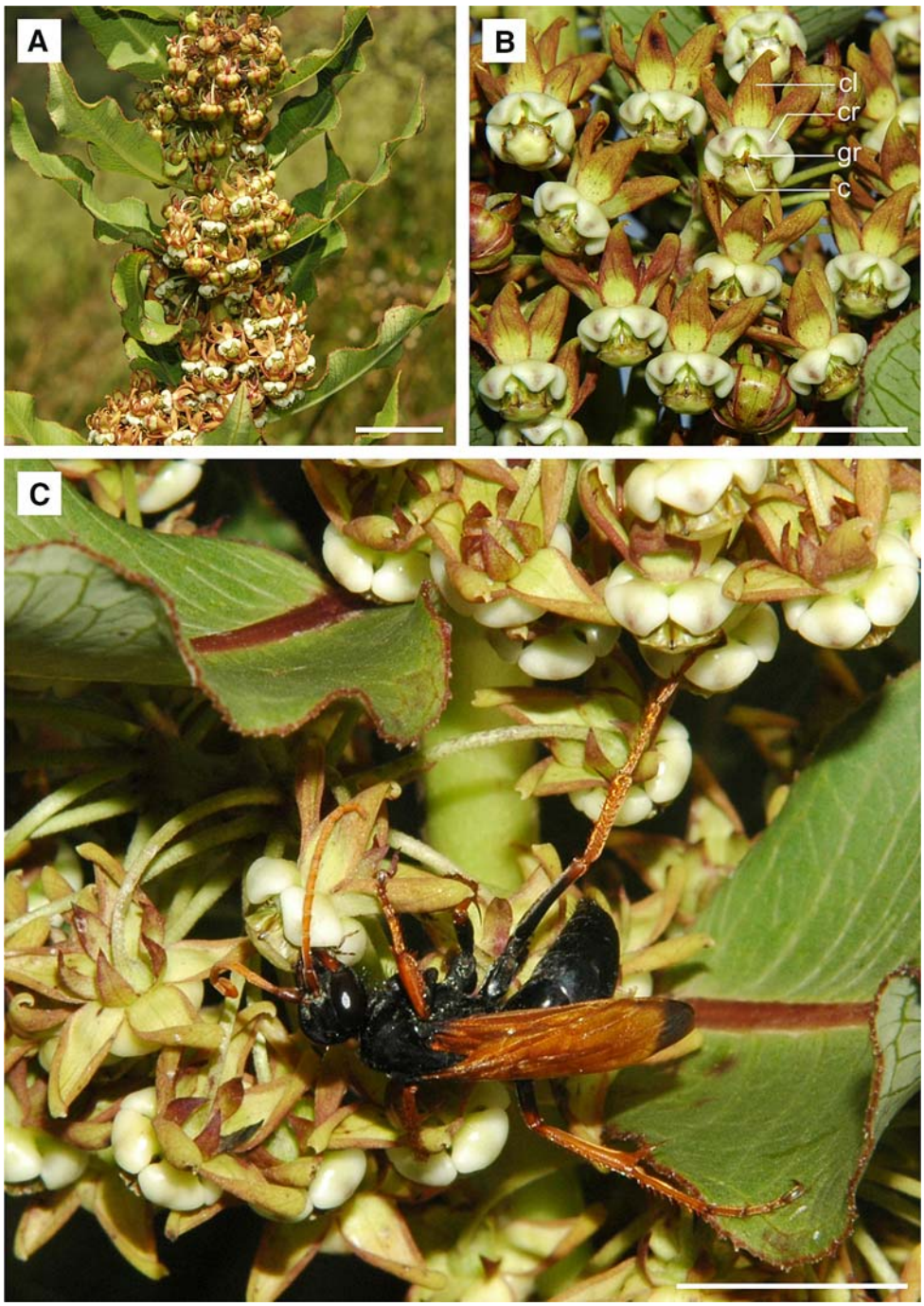

Fig. 1 *Xysmalobium orbiculare* and its pollinators. **a** Close-up of stem showing several inflorescences (the uppermost still in bud), Wahroonga farm. *Scale bar* = 50 mm. **b** Close-up of a single inflorescence showing floral morphology, Wodwo farm. *Scale bar* = 20 mm. **c** Female *Hemipepsis capensis* visiting flowers, Wodwo farm. Note the mouthparts pressed tightly against the guide rails between adjacent corona lobes. *Scale bar* = 20 mm. *c* corpusculum, *cl* corolla lobe, *cr* corona lobe, *gr* guide rails

To determine the dependence of *X. orbiculare* on pollinators, we explored the breeding system by conducting controlled hand-pollinations at Wahroonga in Mar 2005. Fifty-seven virgin flowers (previously bagged at the bud stage) on five plants were assigned to one of three treatments (19 flowers per treatment): (1) cross (pollinated with pollinia from a separate plant), (2) self (pollinated with pollinia from the same plant) or, (3) control (unmanipulated). The number of flowers per treatment on each plant was kept equal to control for any possible plant effects. To perform hand-pollinations, a fine pair of forceps (No. 2) was used to grasp the corpusculum and remove a pollinarium. A single pollinium was then inserted with the convex surface innermost into one of the five stigmatic chambers of a recipient flower (cf. Wyatt 1976). After pollination, flowers were rebagged and left for approximately 5 weeks to develop fruit. Fruit and seed set from flowers in the different treatments were compared.

Functional role of nectar

The volume and the concentration of nectar produced by *X. orbiculare* flowers over a 24 h period were measured from 15 flowers on five plants (three flowers per plant) at Wahroonga in March 2005. Flowers were bagged for 24 h prior to nectar sampling (nectar present at the beginning of this period was removed with capillary tubes). The volume and the concentration (percentage sucrose equivalent by weight) were measured with 20 µl capillary tubes and a Bellingham and Stanley (0–50%) hand-held refractometer.

Xysmalobium orbiculare nectar is secreted in an exposed position and has an unpleasant bitter taste to humans suggesting that nectar may play a role in deterring non-pollinating visitors. To test this, we conducted palatability experiments in Feb 2007 with honeybees (*Apis mellifera scutellata*) and *Hemipepsis* wasps (to control for possible changes in the nectar during experiments). Honeybees are common at the study sites but never visit *X. orbiculare* flowers. Individuals were offered a three-way choice between nectar (collected from flowers at Wahroonga and stored in an Eppendorf vial), and sucrose or hexose (a 1:1 mixture of glucose and fructose) sugar solutions. Sugar solutions were diluted with water to match the concentration of nectar (43% in these experiments). A 1.5 µl droplet each of the nectar and the two sugar solutions were placed in a triangular configuration on a petri dish and a vial containing either a honeybee or a *Hemipepsis* wasp placed upside over them such that the insect could crawl down and consume the droplets. The solutions which were either selected (if the individual consumed all of the solution on the petri dish) or rejected (if the individual probed but did not consume the solution) by each bee or wasp were noted. Honeybees were collected in the University of KwaZulu-Natal botanical garden and *Hemipepsis* wasps were collected at Wahroonga. In total, 15 bees and 13 wasps were tested and each individual was used only once. The percentage of droplets of the nectar and each of the sugar solutions which were rejected by bees and *Hemipepsis* wasps were compared.

Functional role of scent

Hemipepsis wasps approach *X. orbiculare* flowers from downwind (99%, $N = 68$) and exhibit a typical zigzag flight path (see Johnson 2005) suggesting that they are attracted primarily by a scent. To explore the importance of scent as an attractant, we conducted choice experiments in the field at Wahroonga in Feb 2007. Two inflorescences with a similar number of flowers were cut and placed in vases ca. 1 m apart and at 90° to the prevailing breeze. One inflorescence was then covered with vegetation [*Gunnera perpensa* (Gunneraceae) leaves and grass] such that the inflorescence was completely concealed from view. The other inflorescence was left exposed. A pile of grass and leaves was also placed ca 1 m from one of the plants to control for possible attraction to cut leaves (see Brodmann et al. 2008). The two inflorescences and the control pile of leaves were then observed for a period of 90 min during which the number of *Hemipepsis* wasps visiting or approaching to within 5 cm of each of the covered and exposed plants and the leaves was recorded. The covered and exposed plants were switched (i.e. the covered plant was exposed and the exposed plant covered) and both plants were moved to different positions every 15–20 min. The number of wasps visiting or approaching each of the inflorescences and the pile of leaves was compared.

Spectral reflectance analysis

Spectral reflectance across the 300–700 nm range was determined using an Ocean Optics S2000 spectrometer (Ocean Optics Inc., Dunedin, Fla.), Ocean Optics DT-mini light source and fiberoptic reflection probe (QR-400-7-UV-VIS; 400 µm) held at 45° to the leaf surface in a probe holder (RPH-1). We measured the spectral reflectance of the corolla lobes, corona lobes and the tip of the column of *X. orbiculare* flowers and the adaxial surface of *X. orbiculare* leaves from Wahroonga in March 2005. Four replicates (from separate plants) were taken for each, and a mean spectrum calculated. Spectral reflectance of background vegetation was measured from the upper surface of green leaves of nine different plant species (various grasses, forbs and herbs). Three replicates were taken for each of the background species and a mean spectrum was calculated for each plant species.

Results

Floral visitors and pollinator effectiveness

Xysmalobium orbiculare flowers were visited almost exclusively by wasps in the genus *Hemipepsis* (Hymenoptera: Pompilidae; Table 1). Of the four species of *Hemipepsis* recorded, *H. capensis* and *H. hilaris* were the most abundant (Table 1). Flowers were visited by both male and female wasps (65:35% male:female for collected individuals). These wasps were also the only insects which were found to be carrying pollinia, which were attached to the mouthparts and occasionally the feet (Table 1).

In the cage experiment, 76 pollinia (on 38 pollinaria) were removed and 27 pollinia were successfully inserted between guide rails. Of the 190 flowers used in the experiment, 23 (12%) were pollinated (having at least one pollinium inserted). After the experiment, one *H. hilaris* and two *H. capensis* individuals had pollinaria attached to

Table 1 Insect visitors to flowers of *Xysmalobium orbiculare* and their pollen loads

Insect visitor	No. observed (no. captured)	No. carrying pollinia (no. inspected)	Pollinia placement	Locality[a]
Hymenoptera				
Pompilidae				
Hemipepsis capensis (Linnaeus, 1764)	47 (29)	5 (29)	Mouthparts, tibial spine	Wa, Wo
H. dedjas Guerin, 1848	5 (3)	1 (3)	Mouthparts, arolium	Wa
H. errabunda (Dalla Torre, 1897)	13 (13)	7 (13)	Mouthparts	Wa, Wo
H. hilaris (Smith, 1879)	40 (40)	5 (40)	Mouthparts	Wa, Wo
Hemipepsis sp.[b]	249 (0)	2 (2)	Mouthparts	Wa, Wo
Vespidae				
Vespidae sp. 1	1 (0)	Not inspected		Wa
Vespidae sp. 2	1 (0)	Not inspected		Wa
Tiphiidae				
Tiphia sp. 1	4 (1)	0 (1)		Wa
Halictidae				
Halictidae sp. 1	13 (2)	0 (2)		Wa
Formicidae				
Formicidae sp. 1	2 (0)	Not inspected		Wa
Hemiptera				
Pyrrhocoridae				
Pyrrhocoridae sp. 1	1 (0)	Not inspected		Wa
Coleoptera				
Scarabaeidae (Cetoniinae)				
Atrichelaphinis tigrina (Olivier, 1789)	1 (0)	Not inspected		Wa
Chrysomelidae				
Chrysomelidae sp. 1	1 (1)	0 (1)		Wa
Carabidae				
Carabidae sp. 1	2 (0)	Not inspected		Wa
Unidentified Coleoptera				
Coleoptera sp. 1	5 (0)	Not inspected		Wa
Diptera				
Sarcophagidae				
Sarcophagidae sp. 1	1 (0)	Not inspected		Wa
Muscidae				
Muscidae sp. 1	1 (0)	Not inspected		Wa

[a] *Wa* Wahroonga Farm, *Wo* Wodwo Farm

[b] These individuals could not be firmly identified to species level (as they were not collected) but belonged to one of the four *Hemipepsis* species recorded at the study sites

their mouth parts (on two of these wasps, the pollinarium had been reduced to a corpusculum indicating that the pollinia had been successfully inserted in flowers).

Pollination success and dependence on pollinators

Xysmalobium orbiculare flowers typically had 1.1 ± 0.20 pollinia removed and 0.4 ± 0.07 pollinia inserted (both means ± SE per flower per plant). The proportion of flowers pollinated was 32.6 ± 6.25% (mean ± SE) and the PTE was 37.5%.

In the breeding system experiment, 11 out of 19 (57.9%) crossed flowers set fruit, while none of the selfed or control flowers set fruit ($\chi^2 = 27.2$, $P < 0.001$). Fruits resulting from cross-pollinations contained 239 ± 26.2 seeds (mean ± SE per fruit).

Functional role of nectar

Xysmalobium orbiculare flowers produced 174.8 ± 34.78 µl of nectar at a concentration of 29.1 ± 1.83% (both means ± SE per flower) over a 24 h period.

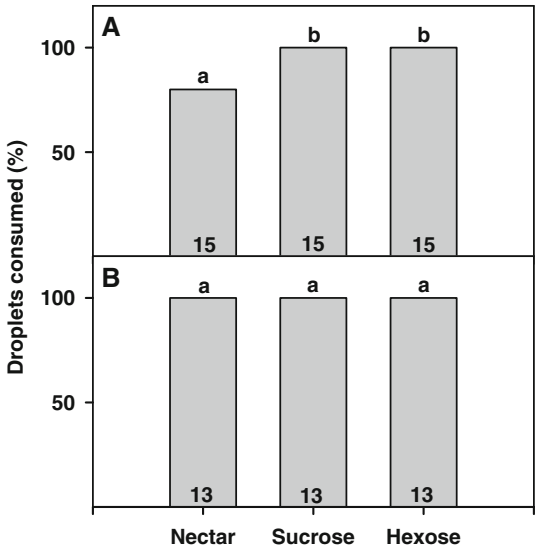

Fig. 2 Proportion of droplets of *Xysmalobium orbiculare* nectar, hexose and sucrose solutions consumed by honeybees (**a**) and *Hemipepsis* wasps (**b**). Sample sizes are shown at the base of the bar. Treatments with different letters are significantly different at the 5% level (see text for statistical analysis)

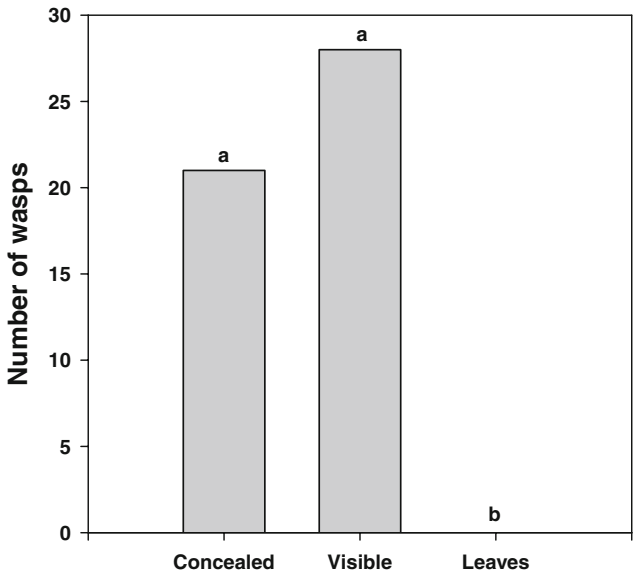

Fig. 3 Number of *Hemipepsis* wasps that approached or visited *X. orbiculare* flowers (either concealed from view or visible) or a control pile of leaves. Treatments with different letters are significantly different at the 5% level (see text for statistical analysis)

In the palatability experiments, honeybees rejected nectar droplets significantly more than droplets of the two sugar solutions ($\chi^2 = 6.43$, $P = 0.04$; Fig. 2). However, there was no significant difference between the proportion of nectar droplets consumed by *Hemipepsis* wasps and the proportion of nectar droplets consumed by the honeybees ($\chi^2 = 0.23$, $P = 0.89$).

Functional role of scent

There was no significant difference between the number of *Hemipepsis* wasps that approached or visited the concealed and the visible *X. orbiculare* inflorescence ($G = 0.99$, $P = 0.32$; Fig. 3). No wasps were attracted to the control pile of cut *Gunnera perpensa* leaves and grass (Fig. 3). The concealed and visible inflorescences were each visited (where the wasp landed on the flowers) by a single wasp while the remainder of wasps observed only approached the flowers without actually landing.

Spectral reflectance analysis

Xysmalobium orbiculare flowers exhibit low overall spectral reflectance (<40%) with no rapid changes in the gradients of individual curves (Fig. 4). Corolla lobes have similar spectral reflectance to the plant's own leaves and the background vegetation, but with a higher reflectance in the 600–700 nm range giving them purplish coloring (Fig. 4). The corona lobes and the tip of the column are greenish white, with higher overall reflectance than the leaves and background vegetation (Fig. 4). No floral parts had significant ultraviolet reflectance (Fig. 4).

Discussion

The results of this study indicate that *Xysmalobium orbiculare* is visited and pollinated exclusively by four functionally identical pompilid wasps in the genus *Hemipepsis* (Table 1). These wasps were the only abundant visitors and were the only insects found to carry pollinia (Table 1). The effectiveness of these wasps in removing

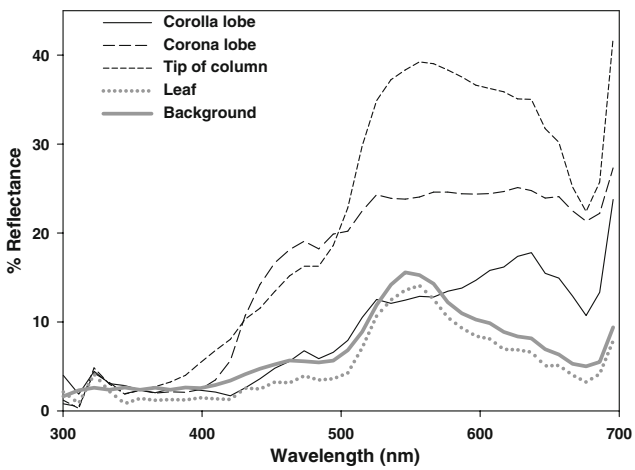

Fig. 4 Spectral reflectance of *X. orbiculare* flowers and leaves, and background vegetation. All curves represent mean spectra calculated from individual replicates

and inserting pollinia was also confirmed by a laboratory cage experiment. Hand-pollinations showed that *X. orbiculare* is genetically self-incompatible and thus reliant on pollinators for reproduction. Choice experiments in the field showed that *Hemipepsis* wasps are attracted primarily by floral scent rather than a visual cue (Fig. 3). This was backed up by the analysis of spectral reflectance which showed that flowers are typically dull greenish-white with similar coloring to the background (Fig. 4). The nectar of *X. orbiculare* appears to be distasteful to non-pollinating insects (Fig. 2). We conclude that *X. orbiculare* has a highly specialized pollination system operated by *Hemipepsis* wasps, and that specialization is achieved through the production of unpalatable nectar and specific floral scent in combination with cryptic coloring.

The exclusive use of four functionally identical wasp species for pollination suggests that *X. orbiculare* is a functional specialist (Johnson and Steiner 2000; Fenster et al. 2004). The presence of traits specific to pollination by pompilid wasps (such as concentrated nectar, specific floral scent and cryptic coloring) suggests that *X. orbiculare* is also a phenotypic specialist (Ollerton et al. 2007). The presence of these traits, which are likely adaptive to pompilid pollination, further suggests that *X. orbiculare* is an evolutionary specialist (Armbruster 2006). However, this is difficult to confirm in the absence of a detailed phylogeny as the genus is clearly polyphyletic (Goyder et al. 2007).

The apparent role of floral scent as a key attractant in this system is not surprising. Specific floral scent is a key component of the pollination systems of the rewarding orchids *Epipactis helleborine* and *E. purpurata* which are pollinated by vespid wasps (Ehlers et al. 2002; Brodmann et al. 2008). Furthermore, the role of floral scent has been suggested in other plants specialized for pollination by *Hemipepsis* wasps (Ollerton et al. 2003; Johnson 2005; Shuttleworth and Johnson 2006, 2008, 2009a, 2009b; Johnson et al. 2007). *Xysmalobium orbiculare* flowers have a weak but discernible sweet spicy scent to the human nose, but its chemical composition has not yet been determined. Prey-hunting wasps are typically attracted by highly specific compounds (rather than a complex floral bouquet, see Brodmann et al. 2008; Schiestl et al. 1999, 2003; Schiestl 2005) and we believe that flowers in the guild of plants pollinated by *Hemipepsis* wasps are likely to employ particular compounds that are highly attractive to *Hemipepsis* wasps. In future research we intend to use gas chromatography-electroantennogram detection (GC-EAD) and behavioral assays to identify compounds that are attractive to *Hemipepsis* wasps.

The nectar of *X. orbiculare* appears to be fairly unpalatable to honeybees and likely serves to reduce non-pollinating visitors (Fig. 2). Unpalatable, or even toxic, nectar is known to function as a floral filter to prevent or reduce non-pollinator visits in several unrelated plants (Stephenson 1981, 1982; Adler 2000; Johnson et al. 2006; Shuttleworth and Johnson 2006). Furthermore, distasteful nectar has been found in two species of *Pachycarpus* (Apocynaceae: Asclepiadoidea) also visited and pollinated exclusively by the same *Hemipepsis* wasps (Shuttleworth and Johnson 2006, in preparation). It is thus not surprising that *X. orbiculare* nectar, which is produced in an exposed position, should be unpalatable to non-pollinating insects. The similarity between the response of honeybees and the response of *Hemipepsis* wasps to nectar in this experiment, however, is intriguing and suggests that the nectar of *X. orbiculare* is not as distasteful to non-pollinating insects as is the nectar of the two *Pachycarpus* species tested in similar experiments (Shuttleworth and Johnson 2006, in preparation). An alternative possibility is that the honeybees used in these experiments were exceptionally hungry and therefore consumed nectar that they would not normally consume under natural conditions.

Specialized pollination by pompilid wasps in the genus *Hemipepsis* appears to be widespread in South African grassland plants and it is clear that these wasps are an important component of grassland ecosystems. Although specialized pollination by pompilid wasps is currently only known from South African systems, pompilid wasps are known to visit asclepiads in North America and South America suggesting that similar pollination systems operated by pompilid wasps may be more widespread (see discussion and references in Ollerton et al. 2003; Punzo 2006). Furthermore, species such as *Pepsis grossa* and *Hemipepsis ustulata* (Hymenoptera: Pompilidae) in North America are remarkably similar to the *Hemipepsis* species involved in the South African systems and are, in all likelihood, functionally identical to these South African *Hemipepsis* species. It would be interesting to confirm if specialized pollination by pompilid wasps is indeed more widespread and, if found to be, to globally compare floral traits of pompilid-pollinated flowers. Evidence from South African systems suggests that it may be possible to identify a syndrome of characteristics exhibited by pompilid-pollinated flowers.

The pollination success and PTE of *X. orbiculare* were comparable to other values obtained for milkweeds (Ollerton et al. 2003; Shuttleworth and Johnson 2006, 2008, 2009a). The high proportion of flowers pollinated (ca. 30%), however, is unlikely to translate into high fruit set (not measured in this study) as percentage fruit set in the congeneric *X. undulatum* was <1% even though ca. 20% of flowers were pollinated (Shuttleworth and Johnson 2008). Causes of this reduced fruit set are not fully understood, but high levels of geitonogamy in conjunction with genetic self-incompatibility are likely responsible.

Genetic self-incompatibility is typical of milkweeds (Wyatt and Broyles 1994, but see Lipow and Wyatt 1998, 2000; Lipow et al. 1999; Leimu 2004), and has also been found in *X. undulatum* (Shuttleworth and Johnson 2008).

The results of this study confirm that *X. orbiculare* is a member of a guild of South African grassland plants that are pollinated exclusively by pompilid wasps in the genus *Hemipepsis*. It appears that members of this guild are reliant primarily on floral scent for pollinator attraction. In future studies, we intend to identify specific floral volatiles that are attractive to *Hemipepsis* wasps and to explore the non-sugar components in the nectars of pompilid-pollinated flowers.

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CHAPTER 9

THE MISSING STINK: SULPHUR COMPOUNDS CAN MEDIATE A SHIFT BETWEEN FLY AND WASP POLLINATION SYSTEMS

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The missing stink: sulphur compounds can mediate a shift between fly and wasp pollination systems

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The radiation of the angiosperms is often attributed to repeated evolutionary shifts between different pollinators, as this process drives diversification of floral forms and can lead to reproductive isolation. Floral scent is an important functional trait in many pollination systems but has seldom been implicated as a key mechanism in pollinator transitions. In this study, we suggest a role for sulphur compounds in mediating a shift between specialized carrion-fly and pompilid-wasp pollination systems in *Eucomis* (Hyacinthaceae). Flowers of closely related *Eucomis* species pollinated by carrion flies or pompilid wasps have very similar greenish-white flowers, but differ markedly in floral scent chemistry (determined by GC–MS analysis of headspace extracts). Comparison of the floral colours of the four *Eucomis* species in the visual systems of flies and wasps suggests that colour plays little role in pollinator discrimination. Nectar properties and morphology also do not differ strongly between fly- and wasp-pollinated flowers. By comparing floral scent bouquets and experimentally manipulating the scent of plants in the field, we demonstrate that shifts between wasp and fly pollination in these four congeners can depend on the production or suppression of sulphur compounds (dimethyl disulphide and dimethyl trisulphide) in the fragrance bouquet. This suggests that mutations affecting the production of particular scent compounds could precipitate shifts between pollinators, independently of floral morphology, colour or nectar properties.

Keywords: pollinator transition; floral evolution; pollination syndrome; myophily; oligosulphide

1. INTRODUCTION

Pollinator-mediated selection on floral traits typically arises from the morphology, behaviour and sensory modalities of particular pollinator types (Harder & Johnson 2009). Evolutionary shifts between pollinators can therefore diversify floral forms and may result in reproductive isolation (Grant 1949; Stebbins 1970; Johnson 2006). Understanding the mechanisms responsible for precipitating a shift from one pollen vector to another is thus of particular interest, as it may often represent the initial stage of plant speciation. To this end, a number of studies have investigated the functional significance of morphology or floral colour for pollinator attraction in closely related species (Bradshaw & Schemske 2003; Irwin & Strauss 2005; Hoballah *et al.* 2007; Cooley *et al.* 2008; Thomson & Wilson 2008). However, the role of floral scent in these transitions has generally been ignored.

Floral scent functions as a pollinator attractant in many pollination systems (Raguso 2001; Dudareva & Pichersky 2006) and often forms the basis for highly specialized plant–pollinator interactions (Schiestl *et al.* 1999, 2003; Brodmann *et al.* 2008; Shuttleworth & Johnson 2009*a*,*b*,*c*). The adaptive significance of floral scent has also been suggested by studies that demonstrate associations between scent composition and various pollinator types (e.g. Knudsen & Tollsten 1993, 1995; Raguso & Pichersky 1995; Jürgens *et al.* 2002, 2003; Knudsen *et al.* 2004; Terry *et al.* 2004). The functional role of floral scent in many plant–pollinator interactions (e.g. Raguso & Pichersky 1995; Schiestl *et al.* 1999, 2003; Raguso 2001; Brodmann *et al.* 2008; Chen *et al.* 2009; Shuttleworth & Johnson 2009*a*,*b*,*c*) suggests that changes in the production of particular volatiles by flowers may, in some systems, alter their attractiveness and initiate shifts between different pollinator types (Kessler *et al.* 2008).

Groups of closely related species that show divergence in pollinators and floral scent, rather than morphology or colour, are ideal for exploring the role of fragrance evolution in pollinator transitions. The African genus *Eucomis* L'Hér. (Hyacinthaceae) represents a promising study system, as the 10 species have morphologically unspecialized flowers with exposed nectar and yet apparently have highly specialized and divergent pollination systems (Shuttleworth & Johnson 2009*a*; this study). In a previous study, we showed that pollinator attraction in the specialized pompilid-wasp pollination systems of *Eucomis autumnalis* and *E. comosa* is based primarily on floral fragrance (Shuttleworth & Johnson 2009*a*). Preliminary observations of several closely related *Eucomis* species (*E. bicolor*, *E. humilis*, *E. schijffii*, *E. montana* and *E. vandermerwei*) indicated that they differ dramatically from the wasp-pollinated species in scent chemistry and are pollinated by carrion flies. We thus hypothesized that the divergent pollination systems in *Eucomis* are mediated by differences in floral scent chemistry rather than morphology, floral colour or nectar properties.

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The aims of this study were (i) to establish whether some *Eucomis* species depend on carrion flies for pollination, (ii) to compare the floral fragrance, colour, morphology and nectar properties of these fly-pollinated species with wasp-pollinated *Eucomis* species to establish which traits are associated with divergent evolution, and (iii) to determine whether a pollinator shift could be induced by experimental manipulation of floral scent.

2. MATERIAL AND METHODS

(a) *Pollination systems and dependence on pollinators*

This study focused on the putatively fly-pollinated taxa *E. bicolor* Baker and *E. humilis* Baker, and was conducted in the montane grasslands of Royal Natal National Park (RNNP) in the South African Drakensberg mountains (28°42′54″ S, 28°53′43″ E, altitude 2400 m). Additional visitor observations for *E. bicolor* were obtained in a small population on Gilboa Estate in the Karkloof mountains of KwaZulu-Natal (29°15′01.6″ S, 30°15′21.6″ E, altitude 1532 m). Floral visitors to both species were recorded between 1999 and 2009 (electronic supplementary material, table S1). Representative insects were collected for quantification of pollen loads in the laboratory.

The dependence of *E. bicolor* and *E. humilis* on pollinator visits was established by comparison of seed set from naturally pollinated and bagged (for autonomous seed set) flowers at RNNP in the 2007/2008 flowering season. Plants were bagged at the bud stage and left for *ca* six weeks to allow fruit development (*E. bicolor*: $n = 793$ flowers on nine plants; *E. humilis*: $n = 610$ flowers on 10 plants). Natural seed set was measured from plants adjacent to the bagged plants and at the same stage of development (*E. bicolor*: $n = 689$ flowers on nine plants; *E. humilis*: $n = 537$ flowers on eight plants). Means were calculated per plant and used as replicates for obtaining a grand mean for each treatment group.

(b) *Morphometrics and spectral reflectance analysis of flowers*

Morphometrics were measured from specimens housed in the Bews Herbarium (University of KwaZulu-Natal, Pietermaritzburg). Lengths and widths of the inflorescence (cm) and of individual tepals (mm) were measured for *E. autumnalis* subsp. *clavata* ($n = 22$), *E. bicolor* ($n = 18$), *E. comosa* ($n = 17$) and *E. humilis* ($n = 20$). Plant means for tepal dimensions were calculated from measurements of three individual flowers. Morphometric measurements were plotted in two dimensions using non-metric multidimensional scaling (NMDS) and analysed as described for the fragrance analysis (see below).

Eucomis bicolor flowers are dull greenish-white with purple along the edges, whereas *E. humilis* flowers are more variably coloured, ranging from plain yellow-green to entirely purple (figure 1). Spectral reflectance (%) across the 300–700 nm range was measured using methods described in Shuttleworth & Johnson (2009*a*). The tepals (each from a separate plant) of four *E. bicolor* and eight *E. humilis* flowers (four green and four purple, to test whether the plants' pollinators perceived them differently) from RNNP were measured in December 2007 and December 2006, respectively. Spectra for *E. autumnalis* and *E. comosa* are presented in Shuttleworth & Johnson (2009*a*).

The similarity of these flowers as perceived by their pollinators was determined by plotting the reflectance spectra of the four *Eucomis* species as loci in the bee colour hexagon (Chittka 1992) and the blowfly colour vision model (Troje 1993). Although the visual abilities of pompilid wasps are not known, the colour hexagon is a suitable model for most higher Hymenoptera (Chittka *et al*. 1992). Pompilid wasps probably have a similar trichromatic visual system to the honeybee (*Apis mellifera*), although the peak sensitivities (λ_{max}) of the wasp photoreceptors may differ slightly. Colour distance in the hexagon was calculated as the Euclidean distance between loci. The blowfly model is based on behavioural experiments with *Lucilia* sp. calliphorids and should thus be well suited to modelling the visual capabilities of the carrion- and blowfly pollinators of *E. bicolor* and *E. humilis* (Troje 1993). According to this model, flies exhibit categorical colour vision, such that spectral stimuli are discriminated only when they fall within separate categories with boundaries at 400 and 515 nm (Troje 1993; Arnold *et al*. 2009). The opponent system is based on the relative excitations of the two p-type and two y-type receptors, so the perceived colour depends on the receptor of each pair that is stimulated most strongly. Thus, the four possible colour categories (p+y+, p+y−, p−y+ and p−y−) correspond to the difference in excitation between the receptors of each type (Troje 1993; Arnold *et al*. 2009). All stimuli within each category are considered indistinguishable to flies (Troje 1993).

(c) *Nectar properties*

Nectar production during 24 h was measured for *E. bicolor* and *E. humilis* in December 2007. Plants were cut and kept in vases overnight. Nectar present at the beginning of the period was removed. Nectar volume (μl) and concentration (percentage sucrose equivalents by mass) were measured using 5 μl capillary tubes and a Bellingham & Stanley 0–50% or 45–80% sugar concentration hand-held refractometer. Means were calculated per plant and these used to calculate a grand mean for the population. Differences in nectar properties between pollination systems were compared using an ANOVA with species nested within pollination system. Mean nectar volumes were log transformed to achieve homogeneity of variance.

Nectar sugar composition was determined by high-performance liquid chromatography using methods described in Brown *et al*. (2009). Nectar was collected from plants at RNNP in December 2007 (*E. bicolor* and *E. humilis*), Midmar Nature Reserve in November 2007 (*E. autumnalis* subsp. *clavata*) and Gilboa Estate in January 2008 (*E. comosa*; see Shuttleworth & Johnson (2009*a*) for details of the latter two study sites).

(d) *Fragrance analysis and behavioural assays*

Floral scent for *E. bicolor* and *E. humilis* was collected using dynamic headspace sorption methods (described in Shuttleworth & Johnson 2009*a*) and analysed by coupled gas chromatography–mass spectrometry (GC–MS). Solvent samples (*E. bicolor* S1 and *E. humilis* S1, see electronic supplementary material, table S2, for sampling details) were analysed by Dr Roman Kaiser (Givaudan, Switzerland) using methods described in Kaiser & Tollsten (1995). Thermal desorption samples (five for each species; see electronic supplementary material, table S2, for sampling details) were analysed on a Varian CP-3800 GC (Varian, Palo Alto, CA, USA) coupled to a Varian 1200 quadrupole mass spectrometer with a Varian 1079 injector equipped with a 'ChromatoProbe' thermal desorbtion device using methods

Figure 1. Flowers of the four *Eucomis* species with their pollinators. (*a*) *E. humilis* being visited by Sarcophaginae sp. 1, Witsieshoek Resort. (*b*) *E. bicolor* being visited by *Phaonia* sp. 1, Sentinel Peak. (*c*) *E. autumnalis* being visited by *Hemipepsis capensis*, Vernon Crookes Nature Reserve. (*d*) *E. comosa* being visited by *H. capensis*, Gilboa Estate. Scale bars, 10 mm.

described in Shuttleworth & Johnson (2009*a*). Quantification was based on 68 synthetic standards injected and thermally desorbed under identical conditions to the samples (Shuttleworth & Johnson 2009*a*).

To determine whether differences in the floral scents are related to the pollination system, we plotted fragrance data for the four *Eucomis* species (data for the wasp-pollinated species are presented in Shuttleworth & Johnson (2009*a*)) in two dimensions with NMDS using Primer 6.1.6 (2006) (Clarke & Warwick 2001; Clarke & Gorley 2006). NMDS was based on Bray–Curtis similarity and data were square-root transformed prior to analysis. Differences in the fragrance profiles between species and pollination systems were tested using ANOSIM (Clarke & Gorley 2006), a non-parametric permutation procedure based on the similarity matrix underlying the ordination (Clarke & Warwick 2001). The test statistic R compares average rank similarities within and among groups. R close to unity indicates complete separation of groups, whereas R close to zero indicates minimal separation among groups. Observed Rs were compared with the distribution of R generated by up to 10 000 random permutations of the sample labels to assess statistical significance, which depends on replicate sample size and must be interpreted accordingly (Clarke & Warwick 2001). Volatiles characterizing the fragrance of each species and each pollination system were explored using the similarity percentages (SIMPER) function in Primer (Clarke & Gorley 2006). SIMPER identifies compounds that contribute most to the average similarity within a particular group (a species or pollination system).

To establish the effects of sulphur-compound production by non fly-pollinated *Eucomis* flowers on pollinator preference, we added a 1 : 1 blend of dimethyl disulphide (DMDS) and dimethyl trisulphide (DMTS) to inflorescences of the wasp-pollinated *Eucomis* species and then recorded floral visitor behaviour. Two similar-sized inflorescences were cut and placed in vases. A small (10 ml) vial with a cotton dispenser wick and containing either 100 µl DMDS, 100 µl DMTS and 800 µl white mineral oil (to slow evaporation) or 1000 µl of white mineral oil only (for the control) was then attached to the base of each inflorescence and concealed from view with leaves. Emission rates of oligosulphides (quantified by injection and thermal desorption of synthetic standards under identical conditions to the flower samples) from vials identical to those used for the experimental inflorescences, quantified at 30 min

intervals during 3.5 h, ranged from 31.5 to 8.8 $\mu g\ min^{-1}$ for DMDS and from 35.9 to 19.0 $\mu g\ min^{-1}$ for DMTS. Experimental inflorescences were placed *ca* 1 m apart and the insects visiting flowers on each inflorescence were noted. The absence of sulphur compounds from the natural scents of the two inflorescences were confirmed by GC–MS after the experiments were completed (sufficient time was allowed for any residual sulphur compounds to evaporate prior to analysis). Flowers on both experimental and control inflorescences were emasculated to avoid contaminating gene pools.

Bioassays with *E. autumnalis* subsp. *clavata* were conducted near a patch of *E. humilis* at RNNP during December 2008. The inflorescences were moved to new positions three times during the 3.5 h of this experiment, but control and scent treatments were not switched between plants. Bioassays with *E. comosa* were conducted at Gilboa Estate (see Shuttleworth and Johnson (2009*a*) for details of the study site) during January 2009. The sulphur and the control vials were switched between inflorescences once and the inflorescences were moved to new positions once during the 2 h of this experiment.

3. RESULTS

Eucomis bicolor and *E. humilis* are both visited almost exclusively by flies in the families Calliphoridae, Muscidae and Sarcophagidae (electronic supplementary material, table S1; figure 1*a*,*b*). Pollen is deposited haphazardly on the flies' bodies as they lap nectar, which is secreted by the septal nectaries and gathers at the ovary base. Pollen transfer occurs as flies move around on flowers and brush against the erect stigmas (figure 1). Naturally pollinated flowers of both species produced significantly more seeds than bagged flowers (*E. bicolor*: bagged $= 0.003 \pm 0.002$, naturally pollinated $= 1.7 \pm 0.28$, $t = 5.87$, $p < 0.001$; *E. humilis*: bagged $= 0.1 \pm 0.04$, naturally pollinated $= 2.1 \pm 0.36$, $t = 5.30$, $p = 0.001$; means $\pm$ s.e. seeds per flower per plant).

Floral morphologies of fly- and wasp-pollinated *Eucomis* species are not strongly divergent (figure 1 and electronic supplementary material, figure S1). Tepals and individual flowers of the four *Eucomis* species have similar dimensions, although the inflorescences of *E. comosa* are larger than those of the other species (figure 1 and electronic supplementary material, figure S1). Morphometric data did not separate into discrete clusters in the NMDS analysis, although some species differed significantly (global $R = 0.43$, $p < 0.001$; figure 2*b*) because of the differences between *E. comosa* and the other species (figure 2*b*). Floral morphology differed significantly between pollination systems ($R = 0.168$, $p < 0.001$), although R was much smaller than for scent differences.

Colours of fly- and wasp-pollinated *Eucomis* flowers are also similar (electronic supplementary material, figure S2; Shuttleworth & Johnson 2009*a*). *Eucomis bicolor* flowers have a dirty white spectrum with low overall reflectance (electronic supplementary material, figure S2). *Eucomis humilis* flowers have low overall reflectance that peaks between 500 and 600 nm (green; electronic supplementary material, figure S2) with the purple flowers exhibiting an additional peak between 600 and 700 nm (red; electronic supplementary material, figure S2). Neither species reflects ultraviolet light (electronic supplementary material, figure S2). These reflectance spectra (especially for *E. humilis* and the two wasp-pollinated species) are similar to the spectra of background vegetation (Shuttleworth & Johnson 2009*a*).

Floral-colour loci for the four *Eucomis* species all fall in the blue–green to green region of the colour hexagon (figure 3*a*) and in the green quadrant of the blowfly colour vision model (with the exception of *E. bicolor*, which is marginally in the blue quadrant; figure 3*b*). These floral colours are also similar to the colour of green foliage background (represented by the centre of the hexagon; figure 3*a*). Loci for green and purple *E. humilis* flowers are close together in the colour hexagon and fall within the same category in the fly model (as a result of the limited sensitivity of Hymenoptera and fly photoreceptors in the red end of the spectrum (Troje 1993; Chittka & Waser 1997; figure 3). Using 0.1 hexagon units as a practical threshold for colour discrimination (Chittka *et al.* 1997, but see Dyer & Chittka 2004*a*,*b*; Dyer *et al.* 2008), wasps would not distinguish *E. comosa* (wasp-pollinated) from *E. humilis* (fly-pollinated), but would be able to discriminate between the other species (figure 3*a*). Flies would be unable to distinguish *E. humilis* from the two wasp-pollinated species (figure 3*b*).

Nectar properties were not clearly associated with pollination systems in the four *Eucomis* species (electronic supplementary material, table S3). Nectar volume was not significantly different between pollination systems ($F_{1,14} = 0.697$, $p = 0.418$). Nectar concentration varied considerably among species (electronic supplementary material, table S3) and differed significantly between pollination systems ($F_{1,14} = 76.4$, $p < 0.001$; see electronic supplementary material, table S3, for species comparisons). All four *Eucomis* species exhibit hexose-dominant nectar (electronic supplementary material, table S3).

All four *Eucomis* species produce many floral volatiles encompassing various compound classes (electronic supplementary material, table S2; Shuttleworth & Johnson 2009*a*). Compounds characterizing the fragrance of each species (accounting for 50% of the similarity between conspecific samples) were similar for all four species, aside from the sulphur-containing compounds, especially DMDS and DMTS, which were only produced by the fly-pollinated species (table 1 and electronic supplementary material, table S2; Shuttleworth & Johnson 2009*a*). Linalool contributed a large percentage to conspecific similarity for all species (table 1). When species were grouped by the pollination system, linalool, 3,5-dimethoxytoluene and hotrienol contributed to the similarity of the scents of both fly- and wasp-pollinated species. However, (*E*)-ocimene contributed only to the wasp-pollinated species while DMDS, DMTS and (*E*)-linalool oxide (furanoid) contributed only to the fly-pollinated species (table 1).

Fragrance data for the four species separate into discrete clusters in the NMDS analysis (global $R = 0.816$, $p < 0.001$; figure 2*a*). The relatively large R values for pairwise comparisons (greater than 0.6) indicate distinct separation between the fragrance profiles of all species (figure 2*a*). The greatest separation was between the fly-pollinated *E. bicolor* and both wasp-pollinated species (*E. comosa* and *E. autumnalis*), as well as between the fly-pollinated *E. humilis* and the wasp-pollinated

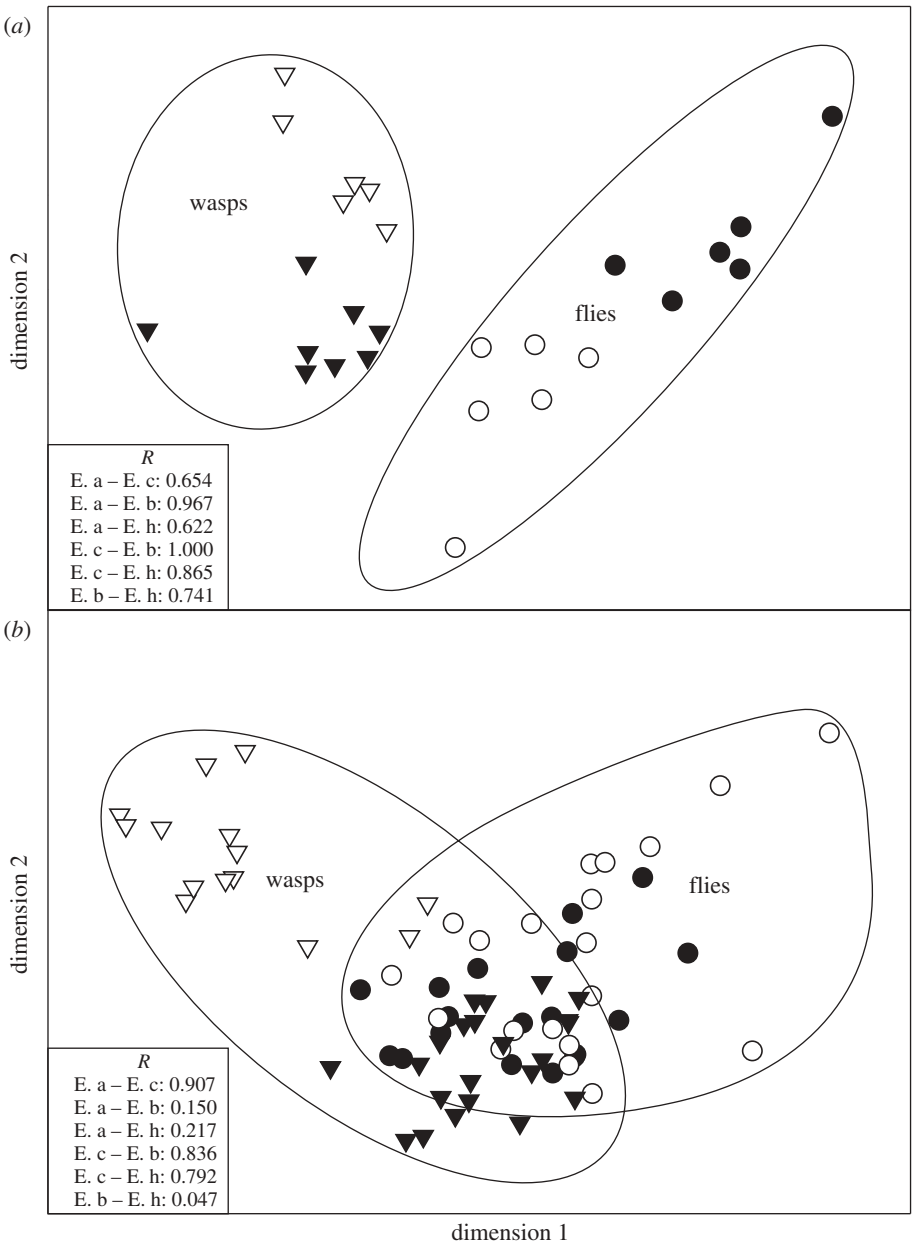

Figure 2. (*a*) Floral scent profiles (stress = 0.12) and (*b*) morphometric measurements (stress = 0.05) of fly- and wasp-pollinated *Eucomis* species plotted in two dimensions based on non-metric multi-dimensional scaling (NMDS). E. a, *E. autumnalis*; E. b, *E. bicolor*; E. c, *E. comosa*; E. h, *E. humilis*. Filled circles, *E. bicolor*; open circles, *E. humilis*; filled triangles, *E. autumnalis*; open triangles, *E. comosa*.

E. comosa (figure 2*a*). Fragrance profiles differed significantly between pollination systems (global $R = 0.678$, $p < 0.001$).

In the bioassays, inflorescences of the normally wasp-pollinated species with sulphur compounds experimentally added attracted large numbers of flies (mostly calliphorids and sarcophagids) that would not typically visit these flowers (Binomial test comparing fly visits to experimental and control inflorescences, $p < 0.001$ for both *Eucomis* species; figure 4 and electronic supplementary material, table S4). Addition of sulphur compounds did not deter pompilid wasps (binomial test, $p =$ n.s. for both species). Flies landing on experimental flowers exhibited differing behaviours: some perched on the inflorescences and then periodically explored flowers and drank nectar, whereas others (especially sarcophagids) immediately lapped nectar after arriving at flowers. Visiting flies frequently contacted stigmas. Very few flies crawled down to the base of the inflorescence to explore the vials from which sulphides were emitted.

4. DISCUSSION

Sulphur compounds in floral scent primarily differentiate fly and wasp pollination systems in *Eucomis*. Differences in floral colour (figure 3), nectar properties (electronic supplementary material, table S3) and morphologies (figures 1 and 2*b*; electronic supplementary material, figure S1) among the four species were minor and not clearly associated with the two pollination systems. Indeed, inflorescences of the fly-pollinated *E. humilis* are often difficult to distinguish from those of the wasp-pollinated *E. autumnalis* in the field (figure 1; Shuttleworth & Johnson 2009*a*). In contrast, floral scent chemistry

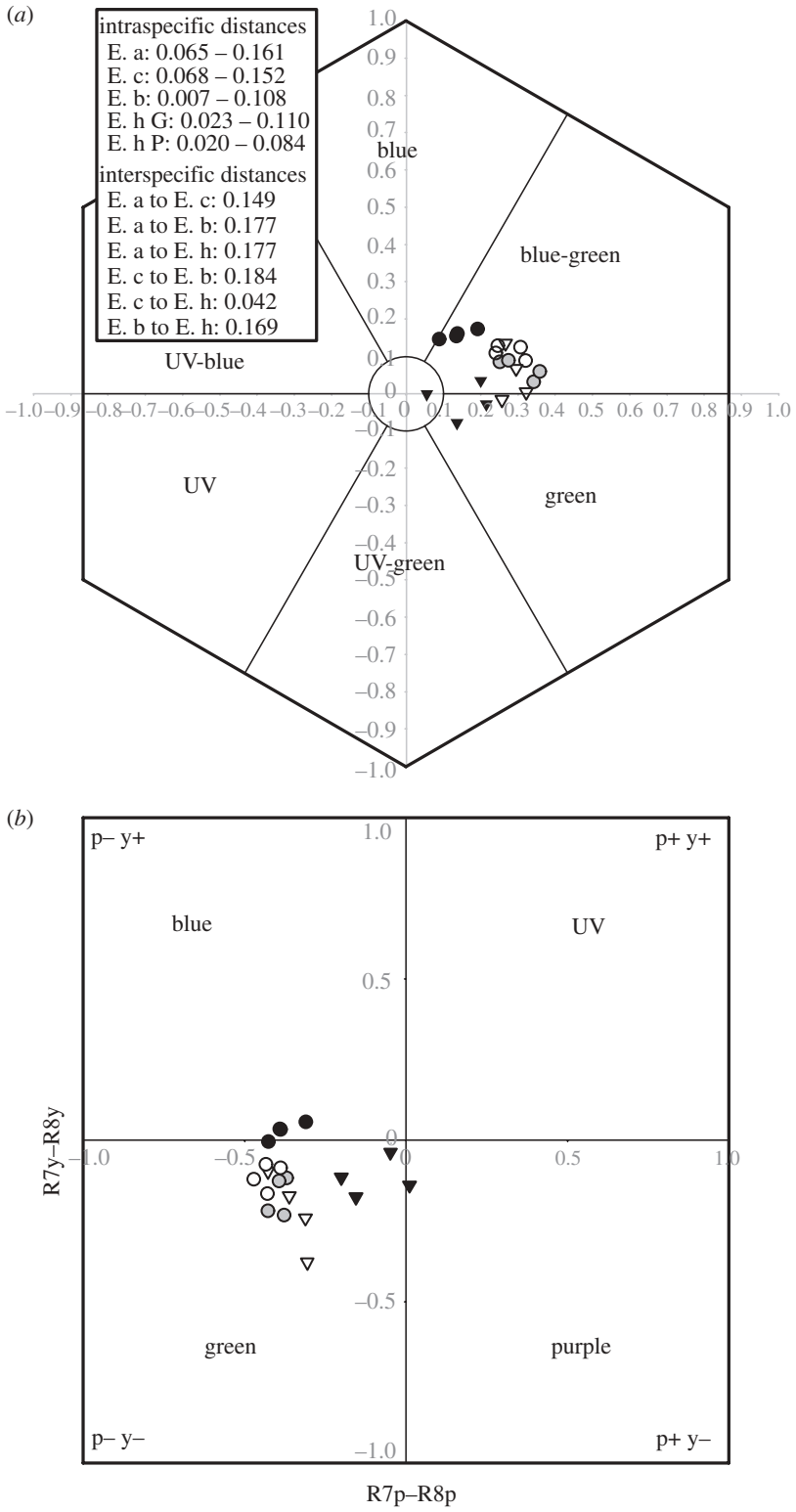

Figure 3. Floral colours of the four *Eucomis* species in bee and fly colour space. (*a*) Loci in the bee colour hexagon (in this case used as a model of wasp vision; Chittka 1992). The six segments represent the six categories of bee colour vision. Loci are calculated from the relative stimulation of the three receptor types (UV, blue and green) by the spectral reflectance of the flowers. Distance between loci in the hexagon correlates to discriminability of the colours by bees (or in this case wasps). Intraspecific distances (hexagon units) represent the range of distances between conspecific samples. Interspecific distances (hexagon units) represent the distances between the mean loci for each species (human green and human purple *E. humilis* pooled). E. a, *E. autumnalis*; E. b, *E. bicolor*; E. c, *E. comosa*; E. h, *E. humilis* (green and purple flowers indicated by G and P, respectively). (*b*) Loci in the blowfly colour vision model suggested by Troje (1993). Flies exhibit a categorical colour vision system such that spectral stimuli with loci in the same quadrant would not be discriminated (Troje 1993; Arnold *et al.* 2009). Filled triangles, *E. autumnalis*; open triangles, *E. comosa*; filled black circles, *E. bicolor*; filled grey circles, *E. humilis* human green; open circles, *E. humilis* human purple.

Table 1. Percentage contributions of particular scent compounds to the average similarity (based on Bray–Curtis similarity coefficient) between intraspecific fragrance samples for each *Eucomis* species (compounds listed here account for the first 50% of overall intraspecific similarity).

	wasp-pollinated		fly-pollinated			
compound	*E. autumnalis*	*E. comosa*	*E. bicolor*	*E. humilis*	fly	wasp
linalool	26.4	15.1	16.3	17.7	21.7	24.8
3,5-dimethoxytoluene	—	19.3	7.1	3.2	5.8	9.5
hotrienol	11.9	8.1	—	10.3	4.3	12.1
(*E*)-linalool oxide (furanoid)	5.0	4.0	—	7.3	4.4	—
(*E*)-ocimene	3.9	6.7	—	—	—	5.7
1-octen-3-ol	4.0	—	—	—	—	—
dimethyl disulphide	—	—	9.9	4.9	9.1	—
dimethyl trisulphide	—	—	6.4	—	5.2	—
(*Z*)-linalool oxide (furanoid)	—	—	6.4	—	—	—
indole	—	—	5.7	—	—	—
4-methylpentan-2-one	—	—	—	5.0	—	—
4-methylpent-3-en-2-one	—	—	—	3.4	—	—
total	51.2	53.1	51.8	51.9	50.5	52.1
average similarity	61.3	68.3	64.5	61.5	49.7	53.9

differed qualitatively between wasp- and fly-pollinated species, with the latter producing large amounts of sulphur-containing compounds that are absent from the scents of the two wasp-pollinated species (table 1 and electronic supplementary material, table S2; Shuttleworth & Johnson 2009*a*). Experimental addition of sulphur compounds to wasp-pollinated flowers induced a clear ecological shift in that experimental flowers immediately attracted flies, thus confirming the functional significance of sulphur compounds for fly-pollinated *Eucomis* plants (figure 4 and electronic supplementary material, table S4).

The overlap in the colours of wasp- and fly-pollinated *Eucomis* species in the colour hexagon and the blowfly model suggests that colour is not used by pollinators to discriminate these species (figure 3; Chittka 1992; Troje 1993). Floral colour in *Eucomis* species probably evolved as a form of crypsis to minimize visits by non-pollinating insects (Shuttleworth & Johnson 2009*a*) rather than as a pollinator attractant. The open, morphologically unspecialized, flowers of *Eucomis* species (figure 1) allow for pollen removal by a diverse spectrum of insects, and the costs of pollen loss to unfaithful floral visitors (Hargreaves *et al.* 2009) may be the basis for the evolution of this cryptic colouring.

Unlike other floral traits, odours clearly differ between pollination systems in these four *Eucomis* species (table 1 and figure 2*a*). Specialization in the wasp-pollinated *Eucomis* species is based on floral scent (Shuttleworth & Johnson 2009*a*), and this also appears to be the case in the fly-pollinated species. The sulphur compounds that characterize the scents of the two fly-pollinated species (table 1, figure 2*a* and electronic supplementary material, table S2) are commonly produced during protein decomposition and are known blowfly attractants, presumably guiding flies to feeding and oviposition sites (Stensmyr *et al.* 2002; Jürgens *et al.* 2006). Oligosulphides are also important floral volatiles of sapromyophilous stapeliads that mimic carrion odours (Jürgens *et al.* 2006). Our results suggest that sulphur compounds are a key functional trait that determines whether carrion- and blowflies visit (and potentially pollinate) *Eucomis* flowers

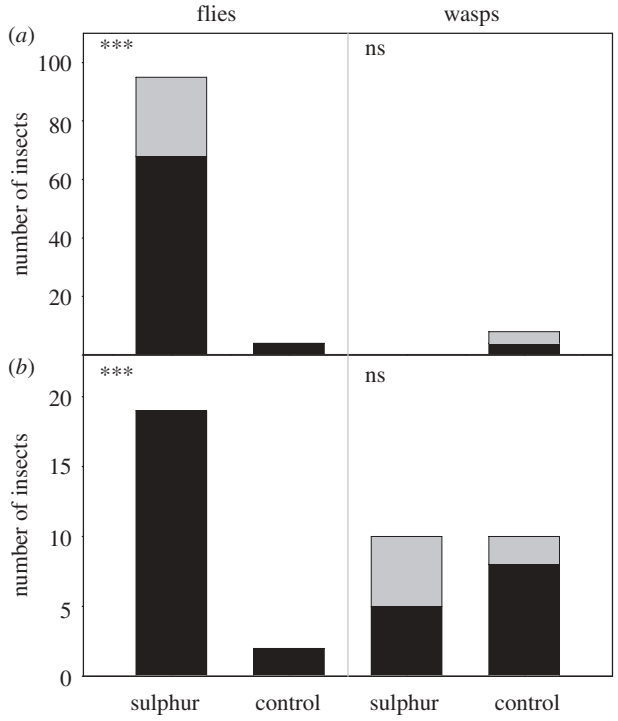

Figure 4. Attraction of flies and wasps to flowers of the wasp-pollinated (*a*) *E. autumnalis* subsp. *clavata* and (*b*) *E. comosa* with a 1 : 1 blend of DMDS and DMTS experimentally added to the inflorescences. The number of individual visits (excluding approaches) by different pollinator types to the inflorescences was compared with binomial tests. Approaches represent instances in which an insect flew up to the inflorescence, but did not actually land on the flowers. Grey bars, approaches; black bars, visits. ***, $p < 0.001$; ns, p not significant.

(figure 4 and electronic supplementary material, table S4). However, flies seldom sought the actual oligosulphide-emitting vial in these experiments which suggests that visual cues within a scent context may play a role at short distances.

Attraction of insects by scent cues alone has traditionally been associated with sexually deceptive systems (Schiestl *et al*. 1999, 2003), brood site mimicry (Stensmyr *et al*. 2002) and the highly coevolved obligate mutualisms of figs and fig wasps (Chen *et al*. 2009) and yuccas and yucca moths (Svensson *et al*. 2005, 2006). However, our results suggest that this phenomenon also occurs in typical food-rewarding plants. Although showy to the human observer, *Eucomis* flowers are cryptically coloured and likely do not stand out from the background vegetation in the eyes of their pollinators (figure 3; Shuttleworth & Johnson 2009*a*). Furthermore, *E. autumnalis* flowers attract their wasp pollinators even when completely concealed from view, suggesting that floral scent is the primary attractant (Shuttleworth & Johnson 2009*a*). This study of *Eucomis* shows that in such systems, simple changes in the production of particular compounds could dramatically alter the assemblage of visitors attracted, which could, in turn, lead to shifts between different pollinators.

Most *Eucomis* species appear to be either wasp- or fly-pollinated, suggesting one or more shifts between these pollination systems. One scenario is that a mutant wasp-pollinated *Eucomis* began producing sulphur compounds, thereby attracting two different pollinator types (*sensu* Stebbins' (1970) 'intermediate stage of double function principle'). Differences in effectiveness of the two pollinator types could then drive specialization to one or the other, ultimately resulting in a complete transition between pollinators (Stebbins 1970). In such a situation, differences in effectiveness may depend on the abundance of the different pollinator types in particular regions or habitats. Both fly-pollinated *Eucomis* species are more common in high-altitude grasslands than are the wasp-pollinated species, possibly correlating with higher fly abundance and lower wasp abundance in this habitat (Arnold *et al*. 2009).

Pollinator transitions have generated considerable interest, as they may often represent the beginning of plant speciation (Grant 1949; Stebbins 1970; Johnson 2006). We have shown that changes in the production of sulphur compounds have the potential to precipitate a shift from wasp to fly pollination in *Eucomis* without associated changes in the morphology, colour or nectar properties of flowers. This study is one of the first to show convincingly that simple changes in the floral scents of plants can profoundly influence the assemblage of floral visitors that they attract. Although the sulphur compounds involved in the *Eucomis* systems are attractive to carrion flies, the principle applies broadly to any shifts involving pollinators that are attracted exclusively by particular floral scents, and so deserves more focused study.

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SUPPLEMENTARY MATERIAL FOR CHAPTER 9

Electronic Supplementary Material Table 1. Floral visitors to *Eucomis bicolor* (c. 50 h observation time) and *E. humilis* (c. 40 h observation time) and their respective pollen loads.

Visitors	Number observed		Pollen load Mean (Range)	No examined for pollen	Pollen placement [a]	Field season	Field site [b]
	Total	Captured					
Eucomis bicolor							
Diptera							
Calliphoridae							
Phumosia sp. 1	5	4	5 (0-20)	4	T, L.	2005/2006; 2006/2007; 2007/2008	RNNP
Calliphoridae sp. 1	1	0	-	0	-	2006/2007	RNNP
Calliphoridae sp. 2	1	0	-	0	-	2008/2009	G
Muscidae							
Helina sp. 1	1	1	130 (130)	1	A, L, W.	2006/2007; 2007/2008; 2008/2009	RNNP
Orthellia sp. 1	1	0	-	0	-		RNNP
Phaonia sp. 1	8	8	9 (0-20)	8	A, T.	1999/2000	RNNP
Muscidae sp. 1	13	13	20 (0-80)	13	A, H, L, T	2006/2007; 2007/2008	RNNP
Muscidae sp. 4	2	2	10 (0-20)	2	H, L, T.	2008/2009	G
Sarcophagidae							
Sarcophaginae sp. 1	1	1	20 (20)	1	T.	2005/2006	RNNP
Scathophagidae							RNNP
Scathophaga sp. 1	3	1	0 (0)	1	-	2006/2007	RNNP
Scathophaga sp. 2	11	3	32 (0-80)	3	H, L, T.	2008/2009	G
Eucomis humilis							
Coleoptera							
Scarabaeidae (Cetoniinae)							
Atrichelaphinis tigrina (Olivier, 1789)	3	3	160 (0-400)	3	A, L, T.	2007/2008; 2008/2009	RNNP
Diptera							
Calliphoridae							
Phumosia sp. 1	20	15	4 (0-10)	16	A, L, T, W.	2006/2007; 2007/2008; 2008/2009	RNNP
Chrysomya marginalis (Wiedemann 1830)	3	0	-	0	-	2008/2009	RNNP
Muscidae							
Helina sp. 1	37	21	6 (0-30)	20	A, H, L, T, W.	2006/2007; 2007/2008; 2008/2009	RNNP
Orthellia sp. 1	42	23	52 (0-400)	20	A, H, L, T, W.	2006/2007; 2007/2008; 2008/2009	RNNP
Musca (*Byomya*) sp. 1	1	1	0 (0)	1	-	2007/2008	RNNP

Gymnodia sp. 1	1	1	0 (0)	1	-	2007/2008	RNNP
Muscidae sp. 1	1	1	2 (2)	1	T.	2008/2009	RNNP
Muscidae sp. 2	1	1	0 (0)	1	-	2007/2008	RNNP
Muscidae sp. 3	1	1	0 (0)	1	-	2007/2008	RNNP
Sarcophagidae							
Sarcophaginae sp. 1	15	9	60 (0-140)	9	L, T, W.	2006/2007; 2007/2008; 2008/2009	RNNP
Sarcophagidae sp. 1	2	0	-	0	-	2008/2009	RNNP
Syrphidae							
Syrphidae sp. 1	2	0	-	0	-	2006/2007; 2008/2009	RNNP
Tachinidae							
Goniinae sp. 1	1	1	-	0	-	2007/2008	RNNP
Unidentified Diptera	21	0	-	0	-	2006/2007; 2007/2008	RNNP
Hymenoptera							
Tiphiidae							
Tiphia sp. 1	2	0	-	0	-	2008/2009	RNNP
Apidae							
Apis mellifera Linnaeus, 1758	1	1	120 (120)	1	T, L.	2006/2007	RNNP

[a] A = abdomen, H = head, L = legs, T= thorax, W = wings.

[b] RNNP = Royal Natal National Park, G = Gilboa Estate.

Electronic Supplementary Material Table 2. Relative amounts (%) of compounds identified by GC-MS from headspace samples of *Eucomis bicolor* and *E. humilis* (see end of table for absolute amounts of sulfides and total scent emission). Compounds are listed in order of increasing retention time (using an Alltech EC-WAX column) within each compound class [a]. T = thermal desorbtion (each sample taken from a single inflorescence sampled in the field for 15 mins at RNNP during December 2006), S = solvent desorption [both samples taken for 6-7 h from cut inflorescences (one for *E. bicolor* and three for *E. humilis* in December 1999 and December 2005 respectively) collected at RNNP].

			E. bicolor						*E. humilis*					
Compound	R_t	Criteria [b]	T1	T2	T3	T4	T5	S1	T1	T2	T3	T4	T5	S1
Aliphatics														
Alkanes														
Tetradecane		D	-	-	-	-	-	3.0	-	-	-	-	-	0.1
Ketones														
But-3-en-2-one	5.781	A	-	-	-	-	-	-	1.3	1.3	1.4	tr	tr	-
4-Methylpentan-2-one	6.320	A	-	-	-	-	-	-	3.6	6.7	6.2	9.9	7.0	-
4-Methylpent-3-en-2-one	7.647	A	-	-	-	-	-	-	1.0	1.1	1.1	1.6	4.5	0.9
5-Methylhexan-2,3-dione		D	-	-	-	-	-	-	-	-	-	-	-	2.0
Heptane-2,3-dione	7.876	A	-	-	-	-	-	-	0.4	3.6	0.6	4.6	13.1	-
Heptan-2-one	8.252	B	-	-	-	-	-	-	1.0	tr	2.2	0.3	0.6	-
Octan-3-one	9.187	B	0.2	0.6	1.4	1.2	0.4	-	-	-	tr	-	tr	-
Acetoine	9.616	A	0.7	1.7	0.8	0.9	1.8	1.4	0.5	0.8	0.1	2.6	0.7	1.3
Nonan-2-one	10.987	A	-	-	-	-	-	-	0.4	-	10.7	-	0.5	-
2-Hydroxy-5-methyl-hexan-3-one		D	-	-	-	-	-	-	-	-	-	-	-	5.3
3-Hydroxy-5-methyl-hexan-2-one		D	-	-	-	-	-	-	-	-	-	-	-	4.9
Undecan-2-one	13.596	A	-	-	-	-	-	-	tr	-	0.7	-	-	-
Alcohols														
Ethanol	2.586	D	-	-	-	-	-	-	-	-	-	-	-	0.9
Isobutanol	6.929	D	-	-	-	-	-	-	-	-	-	-	-	2.5
Isoamyl alcohol	8.428	D	-	-	-	-	-	-	-	-	-	-	-	0.2
4-Methylpentan-1-ol	9.913	A	-	-	-	-	-	-	tr	0.4	tr	-	tr	0.5

Heptan-2-ol	9.952	A	-	-	-	0.3	-	-	0.4	0.1	0.3	0.1	0.1	-
Hexan-1-ol	10.398	C	0.8	2.1	2.9	1.4	0.5	-	0.2	0.4	0.1	0.6	1.3	-
(Z)-Hex-3-en-1-ol	10.848	A	0.2	1.7	1.0	0.4	0.1	-	0.1	0.8	0.3	0.5	2.6	0.1
Octan-3-ol	10.920	B	0.1	0.1	0.3	0.4	0.1	-	-	-	-	-	-	-
(E)-Hex-2-en-1-ol	11.098	A	0.7	5.0	5.2	2.1	0.6	-	0.5	0.4	0.3	1.7	2.0	0.1
1-Octen-3-ol	11.654	D	-	-	-	-	-	-	-	-	-	-	-	0.1
Nonan-2-ol	12.545	A	-	-	-	-	-	-	0.9	0.2	1.8	0.1	0.4	-
Nonanol		D	-	-	-	-	-	0.1	-	-	-	-	-	-
Undecan-2-ol	14.908	B	-	-	-	-	-	-	-	-	0.1	-	-	-
2,3-Butandiol		D	-	-	-	-	-	0.6	-	-	-	-	-	0.2
5-Methylhexan-2,3-diol		D	-	-	-	-	-	-	-	-	-	-	-	0.4
Aldehydes														
(E)-Hex-2-enal	8.680	A	0.4	3.2	3.5	1.2	0.2	-	0.1	0.1	tr	2.1	0.2	-
Octanal [c]	9.654	D	-	-	-	-	-	0.3	-	-	-	-	-	-
Nonanal [c]	11.013	D	-	-	-	-	-	1.0	-	-	-	-	-	0.1
Decanal [c]	12.357	D	-	-	-	-	-	1.5	-	-	-	-	-	0.1
(E)-2-Nonenal		D	-	-	-	-	-	0.1	-	-	-	-	-	-
Esters														
Propyl acetate		D	-	-	-	-	-	-	-	-	-	-	-	0.4
Isobutyl acetate		D	-	-	-	-	-	-	-	-	-	-	-	12.0
Butyl isobutyrate		D	-	-	-	-	-	-	-	-	-	-	-	1.6
Isoamyl acetate	7.520	A	-	-	-	-	-	-	-	0.1	tr	-	tr	-
Isobutyl 2-methylbutyrate		D	-	-	-	-	-	-	-	-	-	-	-	0.7
Isobutyl isovalerate		D	-	-	-	-	-	-	-	-	-	-	-	0.2
Hexyl acetate	9.413	C	-	-	-	-	-	-	0.1	0.4	0.1	0.5	0.2	0.1
Isohexyl acetate		D	-	-	-	-	-	-	-	-	-	-	-	0.3
(Z)-Hex-3-en-1-yl acetate	10.009	A	-	-	-	-	-	-	0.3	1.2	0.7	0.1	2.4	0.1
(E)-Hex-2-en-1-yl acetate	10.245	A	0.4	5.2	0.6	1.4	tr	-	0.2	0.3	1.2	2.2	1.6	-
Ethyl 3-hydroxybutanoate	12.630	A	-	-	-	-	-	-	-	tr	-	-	-	-
Diethyl propanedioate	13.334	B	-	-	-	-	-	-	tr	-	-	tr	-	-
Isovaleric acid		D	-	-	-	-	-	-	-	-	-	-	-	0.3
Aromatics														
p-Cymene [c]	9.421	D	-	-	-	-	-	-	-	-	-	-	-	0.1
1-Methoxy-4-methylbenzene	11.636	A	-	-	-	-	-	-	0.2	0.9	tr	tr	tr	-
Benzaldehyde	12.709	C	3.0	4.9	3.1	5.7	1.2	0.8	0.3	1.6	0.6	0.8	1.3	0.3
Phenylacetaldehyde	14.158	C	0.3	10.3	2.1	7.8	0.8	4.8	-	-	-	-	-	-

Estragole	14.452	A	-	-	-	-	-	-	tr	tr	tr	tr	tr	-
1,2-Dimthoxybenzene	15.072	B	tr	0.1	0.1	tr	tr	-	0.1	tr	tr	0.1	tr	-
Benzyl acetate	15.104	C	-	-	-	-	-	-	tr	0.5	tr	0.2	tr	-
2-Phenyl-2-propenal		D	-	-	-	-	-	0.1	-	-	-	-	-	-
Benzyl isobutyrate	15.747	A	-	-	-	-	-	-	tr	0.1	tr	tr	tr	-
3,4-Dimethoxytoluene	15.925	B	-	-	-	-	-	-	0.3	0.5	0.1	0.2	0.3	-
Phenylethyl acetate	16.073	A	-	-	-	-	-	-	tr	tr	0.2	tr	tr	-
3,5-Dimethoxytoluene	16.481	B	1.0	11.7	5.3	4.2	4.1	10.5	1.2	2.8	0.8	0.7	1.1	2.0
Benzyl alcohol	16.708	C	-	-	-	-	-	0.1	0.1	3.2	0.1	1.8	0.2	-
Phenylethyl alcohol	17.118	A	0.3	0.6	0.5	1.7	0.4	3.4	tr	0.1	0.1	tr	0.1	-
2-Methoxy-4-methylphenol	17.510	A	-	-	-	-	-	-	0.1	0.5	1.7	0.1	0.8	-
Methyleugenol	18.065	A	-	-	-	-	-	-	tr	tr	-	tr	tr	-
3,4,5-Trimethoxytoluene	18.478	A	-	-	-	-	-	-	tr	tr	tr	tr	0.1	-
p-Cresol	18.760	A	-	-	-	-	-	-	0.1	tr	tr	tr	0.1	-
Eugenol	19.605	A	-	-	-	-	-	-	0.1	0.1	0.1	0.1	0.9	0.1
3-Hydroxy-4-phenyl-2-butanone		D	-	-	-	-	-	-	-	-	-	-	-	0.2
Isoprenoids														
Monoterpenes														
a-Pinene	6.458	D	-	-	-	-	-	-	-	-	-	-	-	0.2
ß-Pinene	7.420	D	-	-	-	-	-	-	-	-	-	-	-	0.1
Sabinene	7.510	A	-	-	-	-	-	0.2	0.2	tr	tr	tr	tr	-
Myrcene	8.136	A	-	-	-	-	-	-	tr	-	tr	-	-	0.2
Limonene	8.522	C	0.2	0.8	0.7	0.6	0.1	0.7	-	-	-	-	-	0.1
Eucalyptol	8.686	A	-	-	-	-	-	-	-	-	0.1	-	-	-
(Z)-Ocimene	8.934	C	-	-	-	-	-	-	0.1	0.3	0.2	-	0.1	0.1
(E)-Ocimene	9.196	A	tr	tr	tr	tr	tr	-	0.9	0.4	0.5	0.2	0.5	1.6
(E)-Linalool oxide (furanoid)	11.600	C	4.6	tr	0.1	0.1	1.9	0.1	7.1	4.8	7.4	4.2	4.2	7.1
(Z)-Linalool oxide (furanoid)	11.962	C	2.1	4.2	6.4	7.1	1.0	17.0	0.2	0.1	0.1	0.1	0.1	-
2,6,6-Trimethyl-2-vinyl-tetrahydropyran-5-one	12.066	B	0.6	0.3	1.3	0.8	0.5	0.2	-	-	-	-	-	-
Linalool	12.847	C	48.6	21.0	30.8	30.9	76.4	3.5	57.0	42.5	37.4	32.4	31.0	19.0
(Z)-*p*-Menth-2-en-1-ol	13.137	A	-	-	-	-	-	-	tr	-	tr	tr	-	-
Pinocarvone	13.340	A	tr	0.1	-	tr	-	-	-	-	-	-	-	-
Hotrienol	13.651	A	0.1	0.1	0.2	0.5	0.5	-	13.3	9.6	16.4	7.6	12.7	10.5
ß-Cyclocitral	13.917	A	-	-	-	-	-	-	tr	tr	tr	-	tr	-
6-Hydroxy-2,6-dimethyl-oct-7-en-4-one		D	-	-	-	-	-	-	-	-	-	-	-	5.2
a-Terpineol	14.697	C	tr	tr	tr	tr	tr	-	tr	tr	tr	0.1	tr	-

(E)-Linalool oxide (pyranoid)	15.203	A	0.3	0.2	0.4	0.3	0.1	-	0.8	0.3	0.6	0.1	0.8	0.7
(Z)-Linalool oxide (pyranoid)	15.437	A	0.3	0.6	1.3	0.7	0.1	2.0	0.2	0.1	0.1	tr	0.1	-
6-Hydroxy-2,6-dimethyl-2,7-octadien-4-one		D	-	-	-	-	-	-	-	-	-	-	-	0.6
(E,E)-2,6-Dimethylocta-3,5,7-triene-2-ol	15.880	B	-	-	-	-	-	-	tr	tr	0.1	-	-	-
Geraniol	16.357	C	-	-	-	-	-	-	0.1	tr	tr	tr	tr	-
p-Mentha-1,8-dien-3-one	16.390	A	-	-	-	-	-	-	tr	tr	tr	tr	tr	-
2,6-Dimethyl-3,7-octadiene-2,6-diol	17.298	A	tr	tr	0.1	0.1	0.1	-	0.2	0.1	0.2	tr	0.1	0.2
3,7-Dimethyl-1,6-octadien-3,5-diol		D	-	-	-	-	-	0.3	-	-	-	-	-	3.1
2,6-Dimethyl-1,7-octadiene-3,6-diol	19.282	B	-	-	-	-	-	-	tr	tr	tr	-	-	-
Sesquiterpenes														
a-Cubebene		D	-	-	-	-	-	0.1	-	-	-	-	-	-
Caryophyllene	13.630	D	-	-	-	-	-	-	-	-	-	-	-	0.2
Humulene	14.501	A	-	-	-	-	-	-	tr	tr	tr	tr	tr	-
(E)-Geranylacetone		D	-	-	-	-	-	0.1	-	-	-	-	-	-
Caryophyllene epoxide	17.958	C	-	-	-	-	-	-	tr	tr	tr	tr	tr	-
Terpene derived compounds														
(E)-4,8-Dimethylnona-1,3,7-triene		D	-	-	-	-	-	-	-	-	-	-	-	0.1
Nitrogen-containing compounds														
1-Nitro-2-methylbutane		D	-	-	-	-	-	0.2	-	-	-	-	-	-
2-Methylbutyraldoxime		D	-	-	-	-	-	0.4	-	-	-	-	-	-
Acetyl pyridine	13.717	C	tr	tr		tr	0.1	-	-	-	-	-	-	-
Benzyl nitrile	17.282	C	tr	tr	tr	0.1	tr	-	tr	tr	tr	tr	tr	-
Indole	22.090	C	6.7	7.0	2.6	7.9	0.3	3.0	tr	tr	tr	tr	0.1	-
Sulfur-containing compounds														
Dimethyl disulfide	6.963	A	13.8	10.3	18.9	4.8	6.8	5.0	1.7	9.1	2.2	15.9	3.6	1.4
2-Methyl-1,3-thiazole	9.024	B	2.5	0.7	2.0	2.1	0.3	-	0.5	0.1	tr	0.2	0.1	-
2-Methyl-4,5-dihydro-1,3-thiazole	9.799	A	-	-	-	-	-	-	-	-	tr	-	-	-
Dimethyl trisulfide	10.981	A	10.3	4.2	4.8	1.9	1.1	18.0	1.5	1.2	tr	5.4	0.6	1.2
Methional	11.892	A	0.1	0.4	0.3	0.9	0.1	-	-	0.2	-	0.1	-	-
2-Methylthio-2,3-dimethylbutane	12.684	B	-	-	-	-	-	-	-	-	-	-	0.9	-
2,3,5-Trithiahexane	14.450	A	-	-	-	-	-	0.2	tr	tr	tr	tr	tr	-
Methionol	14.968	A	tr	tr	0.1	0.1	tr	-	tr	0.1	0.1	0.4	0.2	-
Miscellaneous cyclic compounds														
d-Decalactone	19.895	A	-	-	-	-	-	-	tr	-	tr	tr	0.1	-
Unknowns [d]														
m/z: 43, 73, 41, 87, 61, 56	5.940		0.8	-	-	8.3	-	-	-	-	-	-	-	-

m/z: 43,56,69,61,41,42,84,101	8.924	-	-	-	-	-	-	-	0.7	tr	-	-	-
m/z: 123*,80,81,93,53,68,57,67	9.321	-	-	-	-	-	-	-	-	-	-	0.4	-
m/z: 101,86,59,68,55,74,42,58	10.625	0.6	1.1	3.2	2.0	0.2	-	0.7	0.4	0.2	0.5	0.1	-
m/z: 137,94,95,80,82,53,41	10.692	-	-	-	-	-	-	0.1	tr	0.1		0.6	-
m/z: 102,43,88,55,41,60,84,67,73	10.852	0.2	-	-	1.2	0.2	-	-	-	-	-	-	-
m/z: 154*,43,71,93,81,55,41,111,69 [e]	11.857	-	-	-	-	-	-	0.1	-	tr	tr	tr	-
m/z: 43,87,55,41,56,69,70,71	11.871	-	-	-	-	-	-	-	-	0.6	-	-	-
m/z: 43,69,45,87,41,57,72,74	12.073	-	-	-	-	-	-	0.1	1.2	0.3	0.7	1.0	-
m/z: 95,41,69,67,55,43,68,85,82	12.716	tr	1.5	0.1	tr	tr	-	-	-	-	-	-	-
m/z: 71,43,82,67,41,55,39,53	13.376	-	-	-	-	-	-	1.3	0.4	1.0	0.2	0.3	-
m/z: 73,43,45,60,72,31,42,88	13.567	tr	tr	tr	0.1	tr	-	tr	0.1	tr	0.3	tr	-
m/z: 148*,43,88,73,61,41,74,45 [f]	14.007	-	-	-	tr	-	-	tr	0.1	tr	0.3	0.1	-
m/z: 71,43,85,57,58,42,68,55 [g]	14.043	-	-	-	-	-	-	tr	0.1	0.4	tr	0.1	-
m/z: 134,91,119,65,39,43,82,63	14.591	tr	0.1	tr	0.1	tr	-	-	-	-	-	-	-
m/z: 98*,41,69,39,40,53,43	15.080	-	-	-	0.4	-	-	-	-	-	-	-	-
m/z: 96,71,95,43,67,41,68,81	15.247	-	-	-	-	-	-	0.1	tr	tr	tr	0.1	-
m/z: 112*,83,55,57,84,44	15.545	-	-	-	-	-	-	tr	tr	tr	tr	0.1	-
m/z: 72,114,44,30,73,57,58	15.557	-	-	-	-	-	-	tr	tr	tr	-	0.1	-
m/z: 43,71,59,68,72,67,41,55	15.819	-	-	-	-	-	-	tr	tr	0.1	-	-	-
m/z: 152*,43,109,81,79,67,91,55 [e]	16.072	-	-	-	-	tr	-	tr	0.1	0.2	tr	tr	-
Aliphatics		3.5	19.6	15.7	9.2	3.6	8.0	11.0	17.6	27.8	27.0	37.2	35.2
Aromatics		4.5	27.6	11.1	19.4	6.4	19.7	2.5	10.3	3.6	4.0	4.8	2.6
Isoprenoids		56.8	27.3	41.1	41.3	80.7	24.2	80.0	58.2	63.0	44.6	49.6	49.0
Nitrogen-containing compounds		6.7	7.0	2.6	8.0	0.4	3.6	0.0	0.0	0.0	0.0	0.1	0.0
Sulfur-containing compounds		26.7	15.7	26.2	9.8	8.2	23.2	3.7	10.7	2.2	22.1	5.3	2.6
Miscellaneous cyclic compounds		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0
Unknowns		1.6	2.7	3.3	12.1	0.4	0.0	2.3	2.8	2.9	2.0	2.8	0.0
Total of all compounds		99.8	99.8	99.9	99.9	99.8	78.7	99.5	99.6	99.5	99.7	99.8	89.4
Dimethyl disulfide emitted (ng per inflorescence per minute)		135	30	92	41	158		65	263	86	164	13	
Dimethyl trisulfide emitted (ng per inflorescence per minute)		110	13	26	18	28		65	39	4	62	2.4	
Total volatiles emitted (ng per inflorescence per minute)		1760	528	881	1550	4217		7020	5250	7183	1872	985	
Total number of compounds		39	37	35	42	38	30	76	73	81	66	73	49

[a] Relative amounts (%). tr = trace amount (< 0.05 % of total sample). Retention times for compounds found only in solvent extractions are not presented as these samples were separately analyzed by Dr Roman Kaiser.

[b] Compound identification criteria: 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; D = compound identified by Dr Roman Kaiser.

[c] Compounds which are possible background contaminants as they were present in some control samples.

[d] Unknowns comprising <0.05% in all samples were excluded. Mass fragments for unknowns are listed with the molecular ion first (if known) marked with *, followed by the base peak and other fragments in decreasing order of abundance.

[e] Unidentified monoterpenes.

[f] Unidentified sulfur-containing compound.

[g] Unidentified ketone.

Electronic Supplementary Material Table 3. Nectar properties for fly- and wasp-pollinated *Eucomis* species. Data for wasp-pollinated species taken from Shuttleworth & Johnson (2009*a*). Species with different letters are significantly different at the 5% level[1].

Species	Volume (*n*) [2]	Concentration (*n*) [2]	Sugar composition (%)			
			Sucrose	Glucose	Fructose	*n* [3]
E. bicolor	1.4 ± 0.71 (26/5) [a]	50 ± 2.9 (24/5) [a]	34.8	32.5	32.7	4
E. humilis	16.0 ± 7.8 (25/5) [b]	8 ± 4.5 (25/5) [b]	7.8	39.5	52.7	12
E. autumnalis	15.8 ± 5.44 (16/3) [b]	19 ± 4.8 (15/3) [c]	5.0	45.9	49.2	12
E. comosa	2.8 ± 0.98 (25/5) [a]	62 ± 3.8 (25/5) [d]	2.6	42.2	55.2	12

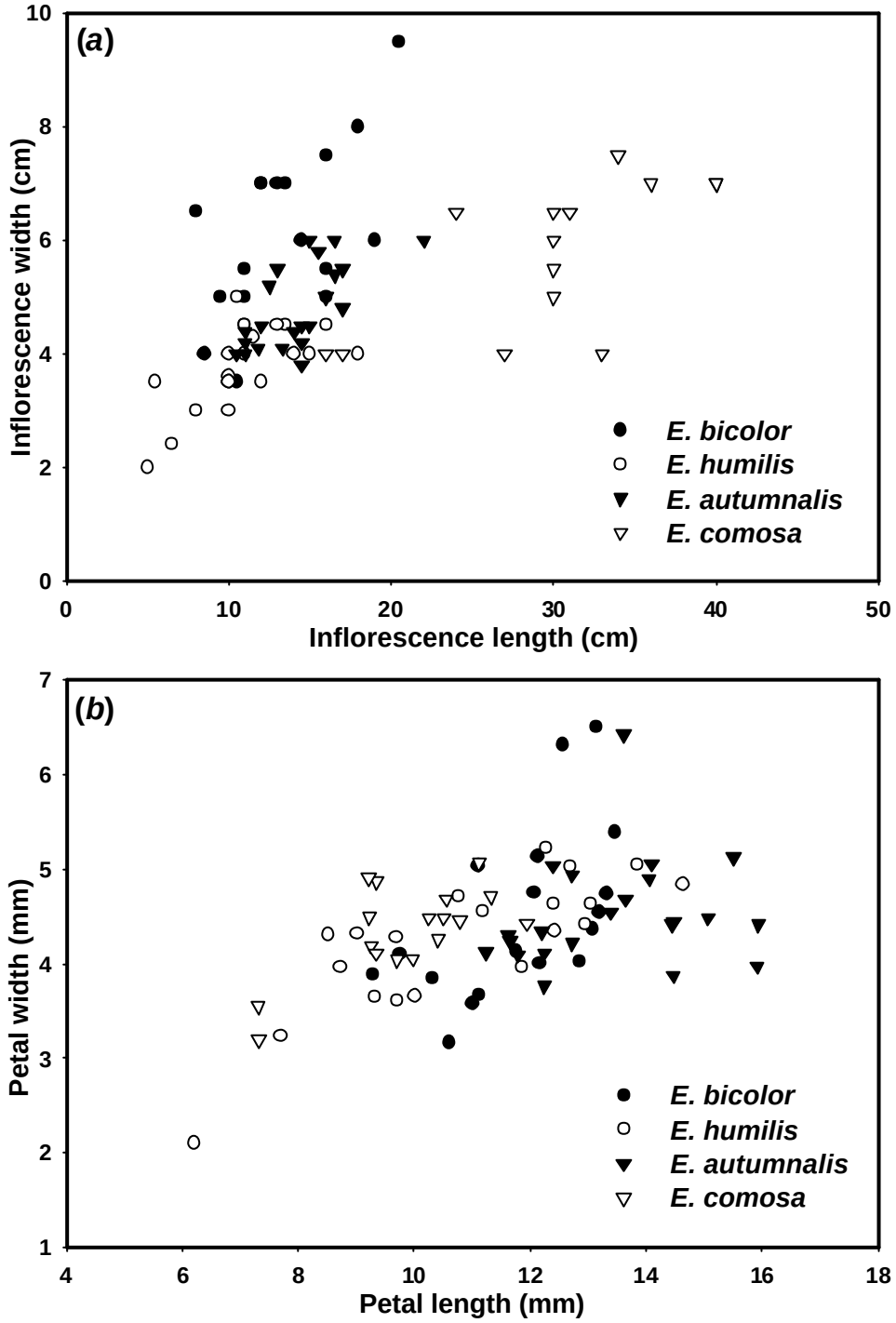
[1] compared with a single factor ANOVA and Tukey's test

[2] Volume in µl and concentration as % sugar. Means ± s.d. per flower per plant. Sample sizes = number of flowers/number of plants.

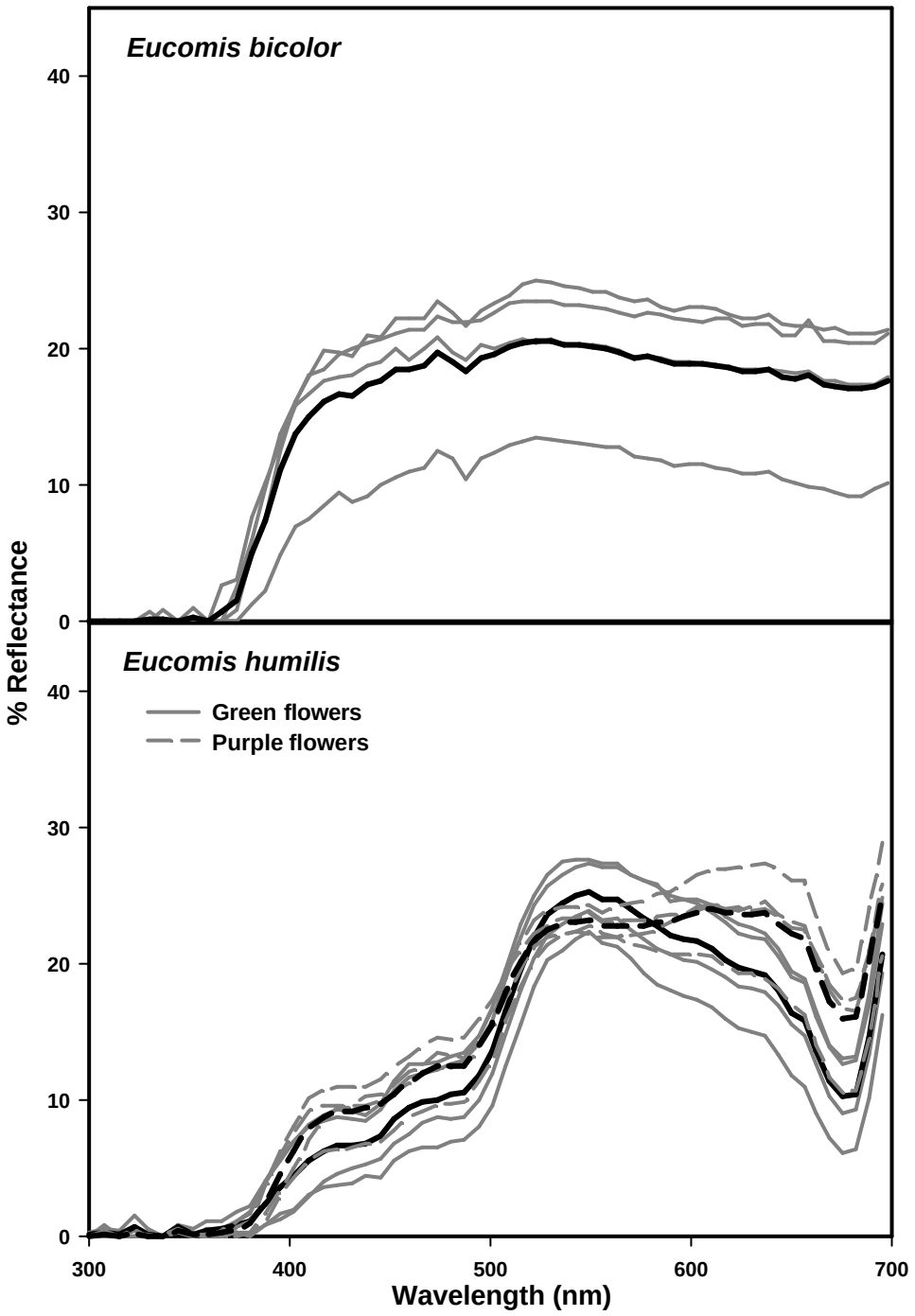
[3] Number of plants.

Electronic Supplementary Material Table 4. Number and identity of floral visitors to inflorescences of wasp-pollinated *Eucomis* plants with dimethyl disulfide and dimethyl trisulfide experimentally added. Numbers in parentheses represent instances where a visitor approached the inflorescence but did not actually land on the flowers.

Insect visitors	*E. autumnalis* plus sulfur compounds	*E. autumnalis* control	*E. comosa* plus sulfur compounds	*E. comosa* control
Diptera				
Calliphoridae				
Chrysomya chloropyga (Wiedemann 1818)	45 (26)	-	-	-
C. marginalis	4	-	-	-
Calliphoridae sp. 3	-	-	18	2
Calliphoridae sp. 4	-	-	1	-
Muscidae				
Orthellia sp. 1	1	1	-	-
Helina sp. 1	1	-	-	-
Sarcophagidae				
Sarcophaginae sp. 1	16 (1)	3	-	-
Sarcophagidae sp. 1	1	-	-	-
Hymenoptera				
Pompilidae				
Hemipepsis sp.	-	4 (4)	5 (5)	8 (2)
Total Diptera	68 (27)	4 (0)	19 (0)	2 (0)
Total Hymenoptera	0 (0)	4 (4)	5 (5)	8 (2)



Electronic Supplementary Material Figure 1. Relative sizes of inflorescences (*a*) and floral petals (*b*) of four species of *Eucomis* measured from all individuals lodged in the Bews Herbarium.



Electronic Supplementary Material Figure 2. Reflectance spectra for flower petals of the two fly-pollinated *Eucomis* species. Light curves represent individual replicates and bold curves represent the mean reflectance.

CHAPTER 10

THE *HEMIPEPSIS*-WASP POLLINATION SYSTEM IN SOUTH AFRICA: A COMPARATIVE ANALYSIS OF TRAIT CONVERGENCE IN A HIGHLY SPECIALIZED PLANT GUILD

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ABSTRACT

Pollinator-mediated convergence in floral traits is the fundamental basis for pollination syndromes, but has seldom been rigorously analyzed. Here we investigate the extent of trait convergence in a guild of South African plant species, representing three families (Apocynaceae, Orchidaceae and Hyacinthaceae), that are pollinated by functionally similar pompilid wasps in the genus *Hemipepsis*. This guild contains remarkably high levels of functional specialization with 17 of the 21 known guild members being pollinated exclusively by these wasps. The distribution of the guild is centered in the moist upland grasslands of eastern South Africa. Qualitative similarities among guild members include dull greenish- or brownish-white flowers, often with purple blotches, mid-summer flowering, sweet spicy scent and exposed nectar. To assess the extent of convergent evolution within the guild, we compared floral traits of guild members to those of congeneric non-wasp-pollinated species. Guild members typically produce moderate volumes (over 4 µl per flower per day) of concentrated (over 50% sugar by weight) sucrose dominant nectar. The nectar properties of guild members did not, however, differ significantly from those of congeneric species pollinated by other vectors. Non-metric multidimensional scaling (NMDS) of scent data for 15 guild members and 16 congeners (obtained through GC-MS analysis of headspace samples and supplemented with published data) yielded little evidence for convergent evolution in the overall scent composition of guild members. However, differences in scent between guild members and their congeners were apparent within particular genera. Convergence in floral spectral reflectance was evident in the guild members; in particular, spectra of guild members clustered in the blue to blue-green region of the hymenopteran colour hexagon and were significantly closer to the colour of background vegetation than those of congeneric species. These results confirm the existence of convergent floral traits, such as flower colour, in plants that are pollinated by *Hemipepsis* spider-hunting wasps, but also suggest that other traits, such as nectar properties, do not necessarily evolve during shifts between pollination systems. Identification of particular scent compounds that influence wasp behaviour and non-sugar nectar constituents will be essential for illuminating the extent of biochemical convergence in the guild members.

KEYWORDS: pollination syndrome; prey-hunting wasp; floral volatile; floral filter; toxic nectar.

> "This species [*Woodia mucronata*] is constantly visited by a large black and yellow wasp … *Pallosoma* [now *Hemipepsis*], one of the Pepsidae."
> Weale (1873, p. 50).

> "However, the adaptive problem is rather obscure and it is hardly possible to establish a syndrome of wasp blossoms, even if it should exist for blossoms adapted to visits by higher wasps."
> Faegri & van der Pijl (1979, p. 109).

INTRODUCTION

Convergence in floral traits of unrelated plants that share a common pollinator forms the basis for floral syndromes (Faegri & van der Pijl 1979) and is an expected outcome if shifts between different pollinators are a driver of floral evolution in plants (Grant & Grant 1965; Stebbins 1970; Johnson 2006). A pollination guild is an extension of floral syndrome theory and refers to a group of plants, irrespective of taxonomy, which are ecologically reliant on a common pollinator (Manning & Goldblatt 1996). Although floral syndromes are often linked with broad insect groups (such as bees or moths), there is an increasing realization that there are also fine-scale syndromes associated with adaptations to smaller sets of functionally similar species, and even particular insect species and their local geographical ecotypes (Faegri & van der Pijl 1979; Brown & Kodric-Brown 1979; Manning & Goldblatt 1996; Fenster *et al*. 2004; Anderson & Johnson 2009; Johnson 2010). These pollination guilds are thus the most relevant level at which to consider pollinator-mediated selection on floral traits. In this study, we synthesize information on a highly specialized guild of plants pollinated by spider-hunting wasps in the genus *Hemipepsis* (Hymenoptera: Pompilidae: Pepsinae) and test for convergent evolution by comparing traits of *Hemipepsis*-wasp pollinated species to those of congeners that are pollinated by other vectors. Specialized pollination by pompilid wasps has only recently been documented in rewarding plants, and the existence of a syndrome of floral traits associated with wasp-pollination has traditionally been considered unlikely (Faegri & van der Pijl 1979, see quote above; Proctor *et al*. 1996).

Pollination by wasps is usually associated with deceptive orchids (Nilsson *et al*. 1986; Nazarov 1995; Schiestl *et al*. 2003) and figs (Proctor *et al*. 1996), although vespid and spheciform (several families in the Apoidea; Grimaldi & Engel 2005) wasps commonly forage for nectar and occasionally pollinate generalist flowers (Gess & Gess 1989, 2003, 2004; Proctor *et al*. 1996). Records of specialized pollination by vespid wasps are scattered in the angiosperms, but include *Epipactis*

orchids (Judd 1971, 1979; Brodmann *et al*. 2008), four species of *Oxypetalum* and *Gomphocarpus physocarpus* (both Apocynaceae: Asclepiadoideae; Vieira & Shepherd 1999; Coombs *et al*. 2009), *Hedera helix* (common ivy, Araliaceae; Jacobs *et al*. 2010, but see Vezza *et al*. 2006), *Croton suberosus* (Euphorbiaceae; Narbona & Dirzo 2010) and two species of *Ferraria* (Iridaceae; Goldblatt *et al*. 2009).

Until recently, pompilid wasps had only occasionally been considered pollinators, and then only in generalist systems (Kephart 1983; Forster 1994; Ollerton *et al*. 2003). However, early observations by Weale (1873) suggested the existence of specialized pompilid pollination systems in South African grassland plants (see quote above). This has recently been confirmed by studies which have revealed a diverse assemblage of southern African plants that are pollinated exclusively by wasps in the genus *Hemipepsis* (Steiner *et al*. 1994; Ollerton *et al*. 2003; Johnson 2005; Johnson *et al*. 2007; Shuttleworth & Johnson 2006, 2008, 2009a,b,c,d,e). The existence of a guild of plants involved in specialized pollination interactions with pompilid wasps contradicts early assumptions that wasps are "unreliable and unsteady pollinators … [in which] the instinctive apparatus to build up a systematic utilization of one or very few suitable blossoms is not particularly well developed" (Faegri & van der Pijl 1979, p. 107), and suggests that it may now be possible to detect patterns of convergent floral traits that can be associated with pollination by pompilid wasps.

The specific aims of this study were (1) to review the evidence for specialized pollination by *Hemipepsis* wasps, (2) to summarize the floral traits of flowers pollinated by *Hemipepsis* wasps, (3) to assess levels of specialization within the guild, and, (4) to analyze the extent of evolutionary convergence in floral scent, nectar and colour properties of guild members through comparison to their non-wasp-pollinated congeners.

MATERIALS AND METHODS

SPECIES COMPOSITION, LEVELS OF SPECIALIZATION, PHENOLOGY AND DISTRIBUTION OF THE GUILD

Guild members were considered to be plant species for which *Hemipepsis* wasps are the primary pollinator, as established by pollen loads, consistent visitation across sites and seasons and, in some cases, single visit studies of pollinator effectiveness (Shuttleworth & Johnson 2006, 2008, 2009a,b,c,e). Levels of specialization within the guild were assessed in terms of the number of pollinator species and the number of pollinator functional groups (Johnson & Steiner 2000; Fenster *et al*. 2004). For species with granular pollen (*Eucomis* species), all insects which carried *Eucomis* pollen were considered to be potential pollinators. For the asclepiads and orchids, all insects carrying pollinaria were considered to be pollinators except where pollinaria were carried in a very low

frequency (less than 5% of individuals) or where attached pollinaria were obviously incidental (e.g. attached to an insect's wing). Species that carried no pollinaria but belonged to the same functional group as pollen carrying species were included as potential pollinators.

Flowering times for guild members were summarized from Pooley (1998, 2003), Nicholas (1999), McMurtry *et al*. (2008) and our own observations. The distribution of guild members was assessed from records in the South African National Biodiversity Institute's (SANBI) online SIBIS Database (http://sibis.sanbi.org; accessed August 2010) supplemented with records presented in Reyneke (1980), Hall (1982), Kupicha (1984), Smith (1988), Steiner *et al*. (1994), Pooley (1998, 2003), Nicholas (1999), McMurtry *et al*. (2008) and our own observations.

NECTAR ANALYSES

The nectar properties (volume, concentration and sugar composition) of guild members were summarized from previous studies (see Table 1 for references) and these data were supplemented with new analyses of nectar sugar compositions for a further seven guild members (see SM Table 2 for species and sampling details). To look for convergence in the nectars of guild members, we compared these data with the nectar properties of congeners with different pollination systems. Congener nectar properties were obtained from previous studies (see SM Table 3 for species and references) and supplemented with new analyses of the nectar sugar compositions for a further nine species (see SM Table 2). Nectar sugar composition was analyzed by high-performance liquid chromatography (HPLC) using methods described in Brown *et al*. (2009).

VOLATILE ANALYSES

Previous studies have shown that floral scent is an important functional trait in this system (Shuttleworth & Johnson 2009a,b,c). Floral scent profiles for five guild members were obtained from previous studies (Johnson 2005; Johnson *et al*. 2007; Shuttleworth & Johnson 2009a,b). We collected floral scents for an additional 11 guild members (3-6 replicates per species; see SM Table 2 for species and sampling details) using dynamic headspace sorption methods. These samples were analyzed by coupled gas chromatography-mass spectrometry (GC-MS) using a Varian CP-3800 GC (Varian, Palo Alto, CA, USA) coupled to a Varian 1200 quadrupole mass spectrometer with a Varian 1079 injector equipped with a 'ChromatoProbe' thermal desorbtion device (detailed methods described in Shuttleworth & Johnson 2009b). Where possible, samples were taken in the field, but some samples were taken from cut inflorescences in the laboratory, in which case care was taken to bag only undamaged plant tissue in order to minimize contamination by green leaf volatiles. Volatiles characterizing the fragrance of each species were identified using the similarity percentages (SIMPER) function in Primer 6.1.6 (2006) (Clarke & Warwick 2001; Clarke & Gorley 2006).

SIMPER calculates the percentage contributions of each compound to average overall similarity between samples (Clarke and Warwick 2001).

To look for convergence, we compared the floral scent profiles of 15 *Hemipepsis*-wasp pollinated flowers to those of 16 congeneric species with different pollination systems using non-metric multidimensional scaling (NMDS) implemented with Primer 6.1.6. Floral scent profiles for nine congeneric species were obtained from previous studies (Shuttleworth & Johnson 2010a,b) and we analyzed the floral scents for an additional seven species using the methods described above (see SM Table 2 for species and sampling details). NMDS was based on Bray-Curtis similarity and data were square-root transformed prior to analysis. NMDS analyses were also conducted for particular genera where sufficient congeners had been sampled.

Differences in the fragrance profiles between pollination systems, genera and plant families were each tested using a two-way ANOSIM with species nested within either pollination system, genus or plant family (Clarke & Gorley 2006). Differences between pollination systems within individual genera were tested using a one-way ANOSIM, as there was insufficient replication of species within pollination systems for the two-way nested design. ANOSIM is a non-parametric permutation procedure based on the similarity matrix underlying the ordination and generates the test statistic *R* through comparison of average rank similarities within and between groups (Clarke & Warwick 2001). High *R* values (close to unity) indicate complete separation of groups while low *R* values (close to zero) indicate minimal separation between groups (Clarke & Warwick 2001). Significance of differences is determined by comparison of the calculated *R* to values of *R* resulting from up to 10 000 random permutations of the sample labels (Clarke & Warwick 2001).

FLORAL COLOUR ANALYSIS

Reflectance spectra across the 300-700 nm range were measured for 13 *Hemipepsis*-wasp pollinated species and 15 congeneric species (see SM Table 2 for species and sampling details) using methods described in Shuttleworth & Johnson (2009a). Spectra were measured from the exposed surface of the corolla and, where possible, replicate measurements were taken from separate plants. Spectra for a further five wasp-pollinated and two congeneric species were obtained from previous studies (Shuttleworth & Johnson 2009a,b,c, 2010a; Johnson *et al*. 2007).

We used the bee colour hexagon (Chittka 1992) to objectively compare the flower colours of guild members to those of background vegetation and flowers of congeneric non-wasp pollinated species. The visual abilities of pompilid wasps are not known, but it is likely that pompilid wasps have a similar trichromatic visual system to the honeybee, although the peak sensitivities (λ_{max}) of the wasp photoreceptors may vary slightly from those of the honeybee (Chittka *et al*. 1992). Furthermore, the colour hexagon has been shown to be a suitable model for most higher Hymenoptera (Chittka *et*

al. 1992). The mean reflectance spectrum (calculated from individual replicates) for each species was plotted in the bee colour hexagon (methods described in Chittka & Kevan 2005; Chittka 1992). Colour distances in the hexagon were calculated as the Euclidean distance between loci. To test the idea that guild members are adapted for cryptic coloration (see Shuttleworth & Johnson 2009a,b,c), we compared the distances from the centre of the hexagon (representing green background vegetation) to loci of guild members and congeneric species.

RESULTS

SPECIES COMPOSITION, LEVELS OF SPECIALIZATION, PHENOLOGY AND DISTRIBUTION OF THE GUILD

The guild of plants pollinated by *Hemipepsis* wasps comprises at least 21 plant species in 10 genera and three families (Fig. 1; Table 1). The majority are asclepiads (Apocynaceae: Asclepiadoideae; 16 species in 7 genera). Plants in the guild are pollinated by four functionally similar wasp species (*H. capensis* (Linnaeus, 1764), *H. errabunda* (Dalla Torre, 1897), *H. dedjas* Guerin, 1848 and *H. hilaris* (Smith, 1879); Pompilidae: Pepsinae; Fig. 1; see references in Table 1). Within the guild, 17 species are pollinated exclusively by *Hemipepsis* wasps (median number of pollinator species = 2, range = 1–4; Table 1). Two species, *Satyrium microrhhynchum* and *Xysmalobium undulatum*, have bimodal pollination systems and are pollinated by *Hemipepsis* wasps and cetoniine beetles (Johnson *et al*. 2007; Shuttleworth & Johnson 2008). The two species of *Eucomis* are more generalized (*E. autumnalis*: 19 pollinator species in four functional groups; *E. comosa*: 35 pollinator species in five functional groups), although *Hemipepsis* wasps carried approximately four times as many pollen grains as other functional groups and were more consistent visitors across sites and seasons for both species (Shuttleworth & Johnson 2009a). Most guild members are seldom visited by non-pollinating insects, although the two *Eucomis* species and *X. undulatum* are visited by a broad spectrum of insects which contribute little to pollination (Shuttleworth & Johnson 2008; 2009a). The orchid *Disa bivalvata* has been reported to be sexually deceptive (Steiner *et al*. 1994).

Flowering of guild members occurs from late September through until early May, peaking in December/January (Table 2). The guild is distributed throughout the summer rainfall grasslands of Eastern South Africa and is particularly well represented in the high altitude grasslands from southern KwaZulu-Natal along the Drakensberg escarpment into Mpumalanga (Fig. 2). A single species, the orchid *D. bivalvata*, is endemic to the fynbos. *Xysmalobium undulatum* is the most widespread guild member and extends south into the fynbos in the Western Cape Province and west into the Karoo in the Northern Cape Province (Fig. 2).

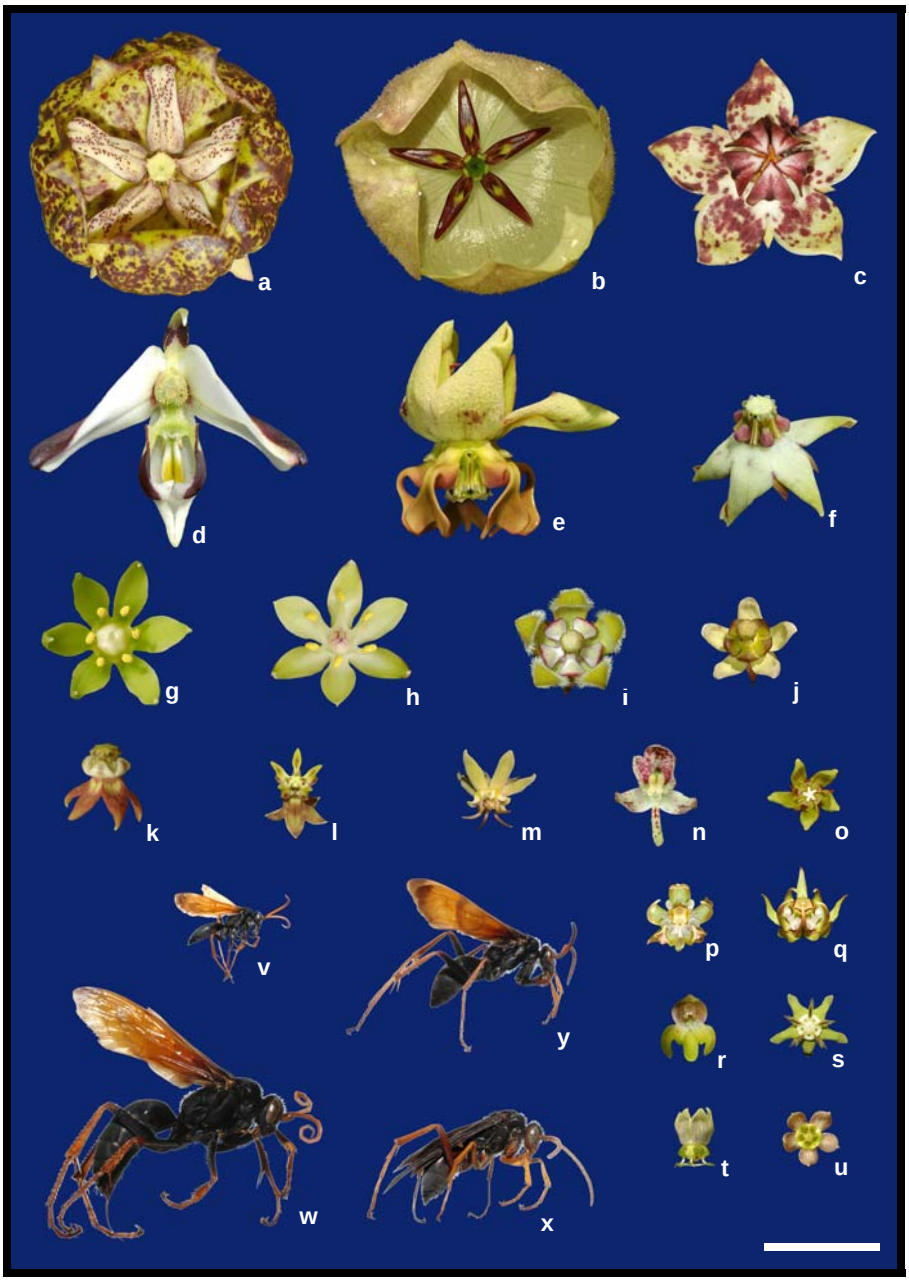

FIGURE 1. Flowers of guild members and their *Hemipepsis*-wasp pollinators. **Plants: a**, *Pachycarpus grandiflorus*; **b**, *Pa. campanulatus*; **c**, *Pa. natalensis*; **d**, *Disa bivalvata*; **e**, *Pa. appendiculatus*; **f**, *Pa. asperifolius*; **g**, *Eucomis autumnalis*; **h**, *E. comosa*; **i**, *Xysmalobium undulatum*; **j**, *X. stockenstromense*; **k**, *X. orbiculare*; **l**, *Asclepias macropus*; **m**, *Miraglossum pulchellum* (photo: Peter Wragg); **n**, *D. sankeyi*; **o**, *Periglossum angustifolium*; **p**, *Woodia mucronata*; **q**, *W. verruculosa*; **r**, *Satyrium microrrhynchum*; **s**, *M. pilosum*; **t**, *M. verticillare*; **u**, *Aspidoglossum glanduliferum*. **Wasps: v**, male *H. hilaris*; **w**, female *H. capensis*; **x**, male *H. dedjas*; **y**, male *H. errabunda*. Scale bar = 20mm.

TABLE 1. Floral traits, location of pollen placement on pollinators and pollen transfer efficiency (PTE) for members of the *Hemipepsis*-wasp pollination guild.

				Nectar									
				24h prod.		Stand crop		Sugars (%)					
Species	No. of pollinator spp. (functional groups)	Scent	Colour	µl	%	µl	%	Suc	Gluc	Fruct	Pollen placement	PTE (%)	Source[a]
Apocynaceae: Asclepiadoideae													
Asclepias macropus (Schltr.) Schltr.	3 (1)	faint spicy	greenish-brown	-	-	-	-	-	-	-	claws	18 [b]	1, 7
Aspidoglossum glanduliferum (Schltr.) Kupicha	2 (1)	sweet	greenish-brown	-	-	-	-	-	-	-	mouthparts (?)	-	7
Miraglossum pilosum (Schltr.) Kupicha	1 (1)	chemical	greenish-white	0.2	60	-	-	-	-	-	mouthparts (?)	17	10
M. pulchellum (Schltr.) Kupicha	1 (1)	faint	greenish-brown	-	-	-	-	-	-	-	mouthparts (?)	-	7
M. verticillare (Schltr.) Kupicha	2 (1)	faint spicy	greenish-white	0.3	68	-	-	0.3	28.4	71.3	mouthparts	80	1, 10
Pachycarpus appendiculatus E.Mey.	2 (1)	spicy	greenish-white with purple blotches	18.3	58	-	-	-	-	-	palps	28	8
Pa. asperifolius Meisn.	2 (1)	spicy	greenish-white with purple blotches	18.4	73	-	-	98.6	0.0	1.4	palps, tarsal spines	15 – 43 [c]	1, 2
Pa. campanulatus (Harv.) N.E.Br. var. *campanulatus*	2 (1)	spicy	greenish-white	0.9	21	-	-	97.6	0.0	2.4	claws	24 [d]	1, 7
Pa. grandiflorus (L.f.) E.Mey. subsp. *grandiflorus*	3 (1)	spicy	greenish-white with purple blotches	16.4	45	5.5	32	99.0	0.0	1.0	claws, tibial and tarsal spines	20	1, 5
Pa. natalensis N.E.Br.	2 (1)	spicy	greenish-white with purple blotches	4.2	50	-	-	96.7	0.0	3.3	mouthparts	6	1, 10
Periglossum angustifolium Decne.	2 (1)	faint spicy	green and reddish-brown	-	-	-	-	-	-	-	mouthparts	-	7
Xysmalobium orbiculare (E.Mey.) D. Dietr.	4 (1)	faint spicy	greenish-brown	174.8	29	-	-	98.1	0.0	1.9	mouthparts, tibial spines	38	1, 6
X. stockenstromense Scott-Elliot	2 (1)	faint spicy	greenish-white	-	-	-	-	-	-	-	mouthparts (?)	-	7
X. undulatum (L.) Aiton f. var. *undulatum*	12 (2)	faint spicy	greenish-white	-	-	8.5	73	79.4	2.3	18.3	mouthparts and clypeal hairs	2 – 16 [c]	1, 3
Woodia mucronata (Thunb.) N.E.Br.	1 (1)	faint	greenish-brown	-	-	-	-	-	-	-	mouthparts (?)	-	7
W. verruculosa Schltr.	2 (1)	faint	greenish-brown	-	-	-	-	-	-	-	mouthparts	-	7
Hyacinthaceae													
Eucomis autumnalis (Mill.) Chitt. subsp. *clavata* (Bak.) Reyneke	19 (4)	sweet spicy	greenish-white	15.8	19	2.0	71	5.0	45.9	49.2	head, thorax	-	4, 9
E. comosa (Houtt.) Wehrh. var. *striata* (Don) Willd.	35 (9)	sweet spicy	greenish-white with purple edges	2.8	62	-	-	2.6	42.2	55.2	head, thorax	-	4, 9
Orchidaceae													
Disa bivalvata (L.f.) T.Durand and Schinz	2 (1)	strong perfumy	greenish-white with purple edges	deceptive		-	-	-	-	-	sternum	-	13

D. sankeyi Rolfe	2 (1)	sweet spicy	yellow-green with purple spots	1.3	-	0.2	18	-	-	-	fore tarsi	-	11
Satyrium microrrhynchum Schltr.	2 (2)	sweet	greenish-white	-	-	0.3	8	-	-	-	frons	-	12

[a] 1, This study; 2, Shuttleworth & Johnson (2006); 3, Shuttleworth & Johnson (2008); 4, Shuttleworth & Johnson (2009a); 5, Shuttleworth & Johnson (2009b); 6, Shuttleworth & Johnson (2009c); 7, Shuttleworth & Johnson (2009d); 8, Shuttleworth & Johnson (2009e); 9, Shuttleworth & Johnson (2010a); 10, Ollerton *et al*. (2003); 11, Johnson (2005); 12, Johnson *et al*. (2007); 13, Steiner *et al*. (1994).

[b] Calculated from 73 flowers on three plants collected at Site 20 (see SM Table 1) in Feb 2004.

[c] Range of values across different sites.

[d] Calculated from 14 flowers on five plants collected at Site 4 in Dec 2005.

TABLE 2. Flowering times for members of the *Hemipepsis*-wasp pollination guild.

Guild member	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun
Asclepias macropus												
Aspidoglossum glanduliferum												
Miraglossum pilosum												
Miraglossum pulchellum												
Miraglossum verticillare												
Pachycarpus appendiculatus												
Pachycarpus asperifolius												
Pachycarpus campanulatus												
Pachycarpus grandiflorus												
Pachycarpus natalensis												
Periglossum angustifolium												
Woodia mucronata												
Woodia verruculosa												
Xysmalobium stockenstromense												
Xysmalobium orbiculare												
Xysmalobium undulatum												
Eucomis autumnalis												
Eucomis comosa												
Disa bivalvata												
Disa sankeyi												
Satyrium microrrhynchum												
Total no. of species flowering	0	0	1	12	16	18	19	15	8	3	1	0

NECTAR ANALYSES

With the exception of the deceptive orchid *Disa bivalvata*, all guild members provide a nectar reward for pollinators. Nectar volumes within the guild ranged from as low as 0.2 µl flower^{-1} day^{-1} in *Miraglossum pilosum* up to 175 µl flower^{-1} day^{-1} in *Xysmalobium orbiculare* (mean ± sd = 23.0 ± 50.9 µl, median = 4.2 µl, n = 11; Table 1). Nectar concentration is usually high (mean ± sd = 49 ± 19.5%, median = 54% sucrose equivalents by weight, n = 10) with several species producing nectar that is more than 60% sugar by weight (Table 1). Most guild members produce sucrose dominant nectar, although the nectar of *M. verticillare* and the two *Eucomis* species is dominated by hexose sugars (Table 1).

Differences between guild members and congeners in the mean (± sd) volumes and concentrations of nectars were not significant (volume for congeners = 3.3 ± 4.8 µl flower^{-1} day^{-1}, n = 11, t = 1.3, p = 0.23; concentration for congeners = 35 ± 15.3% sucrose equivalents by weight, n = 11, t = 1.7, p = 0.10; means for guild members presented above), although congeners produced lower

volumes of nectar (median = 0.9 µl flower^{-1} day^{-1}, n = 11) with a lower sugar concentration (median = 36%, n = 11) than guild members (Table 1, SM Table 3). Differences between guild members and congeners in the mean (± sd) proportions of sucrose in nectars were also not significant (guild members = 64 ± 46.6% sucrose, n = 9; congeners = 59 ± 43.0% sucrose, n = 9; t = 0.3, p = 0.80; Table 1, SM Table 3).

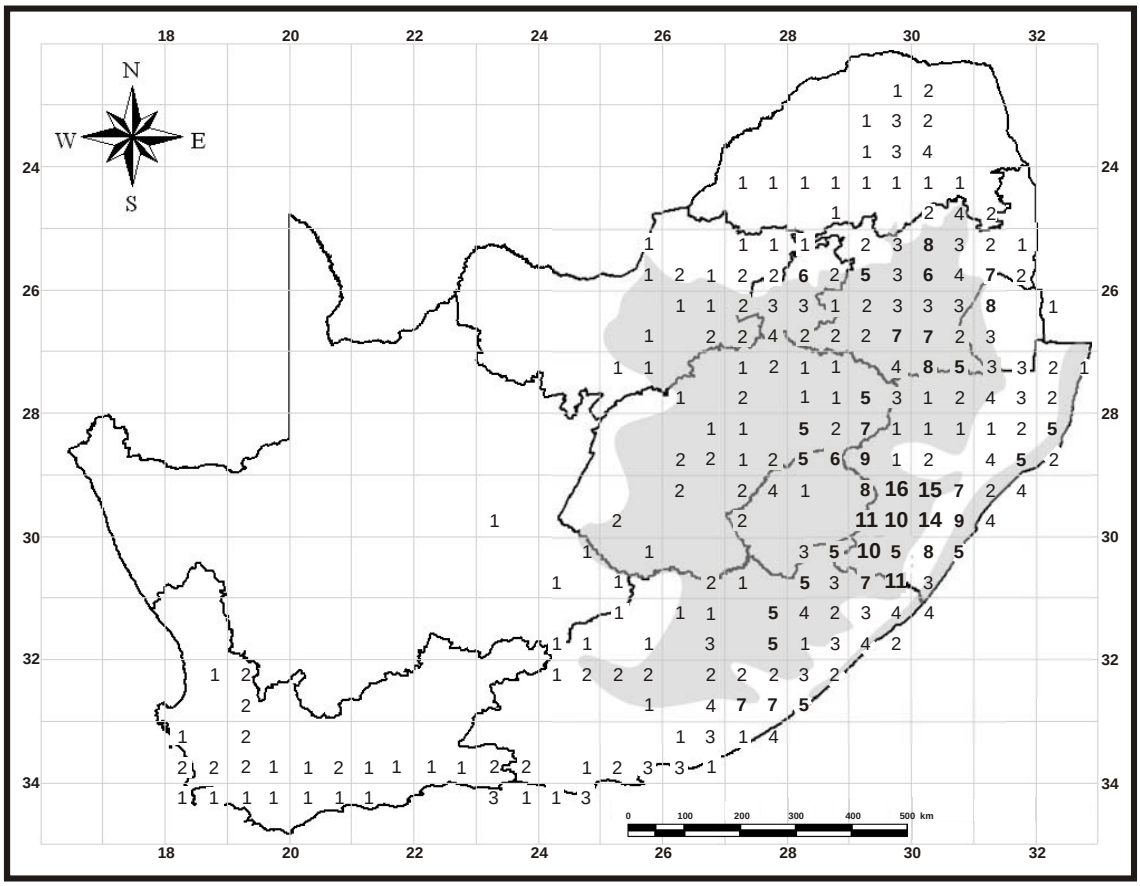

FIGURE 2. Distribution map for the *Hemipepsis*-wasp pollination guild in South Africa. Figures refer to the total number of plant species recorded per quarter degree square. Values between 5 and 9 in bold, values greater than 9 in bold and larger font. Shaded area represents the grassland biome.

SCENT ANALYSES

Members of the *Hemipepsis*-wasp pollination guild usually have a weak spicy fragrance to the human nose, although some species (especially the two *Eucomis* species and *Disa sankeyi*) are very strongly scented. Guild members typically produced a large number of floral volatiles with most scents being dominated by aliphatics and isoprenoids with small amounts of aromatics (SM Tables 4-6; Johnson 2005; Johnson *et al*. 2007; Shuttleworth & Johnson 2009a,b). The total number of volatiles produced

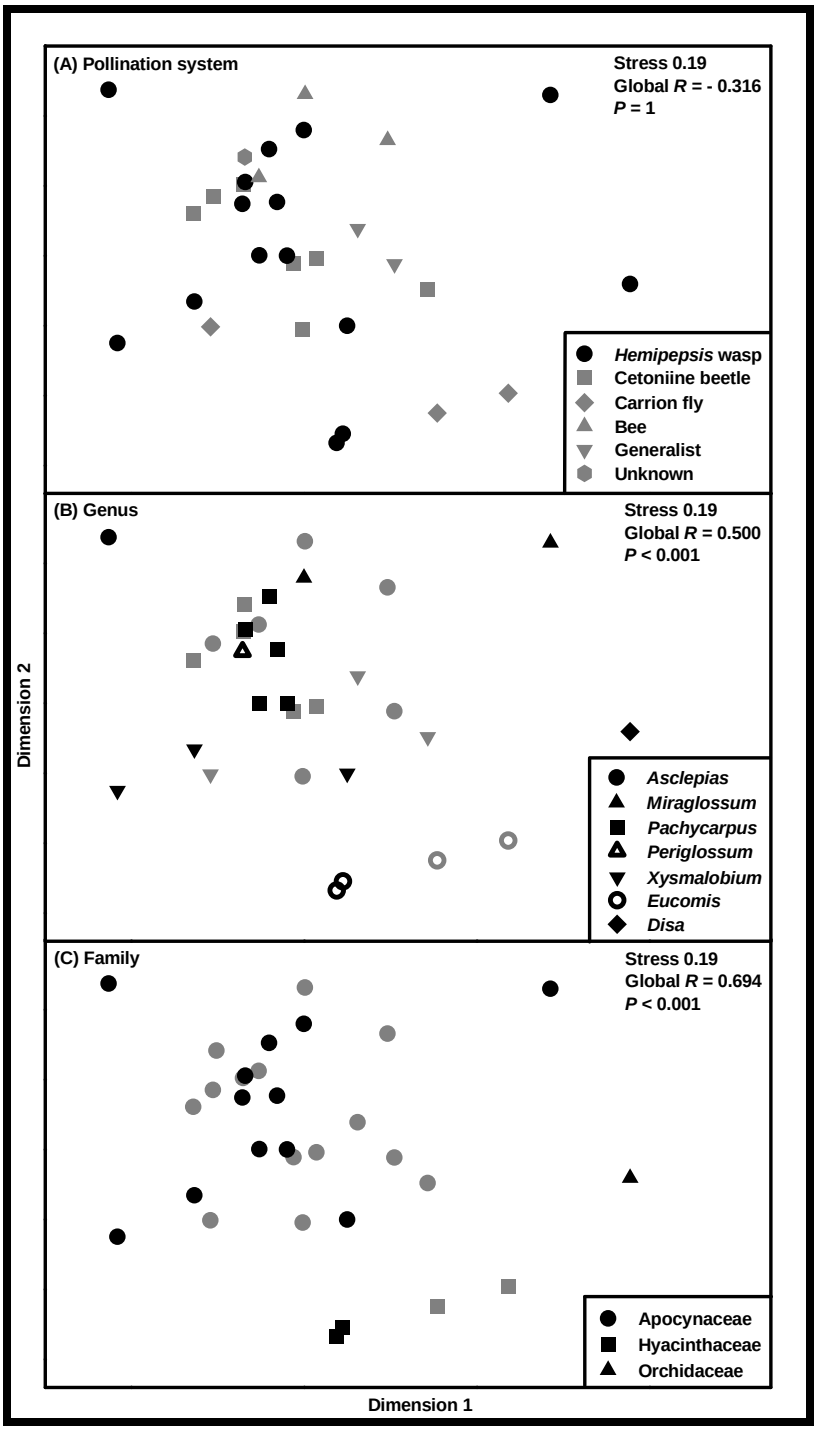

FIGURE 3. Non-metric multidimensional scaling (NMDS) ordination of floral scent profiles for members of the *Hemipepsis*-wasp pollination guild and congeneric species based on (A) pollination system, (B) plant genus and, (C) plant family. Loci represent species means. *P* values represent differences between pollination systems, plant genera or plant families respectively. In (B) and (C), bold symbols represent guild members within each genus or family.

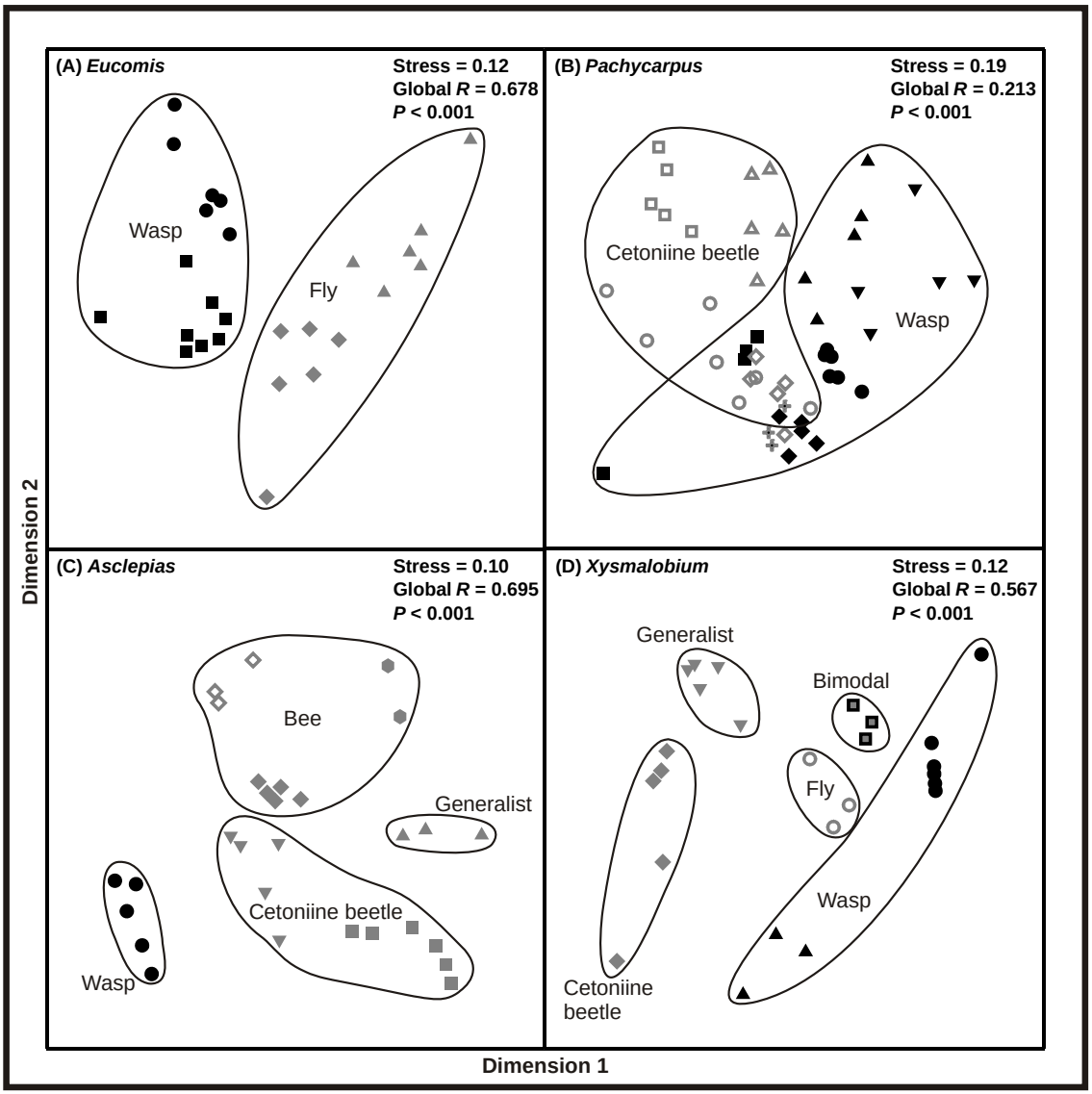

FIGURE 4. NMDS ordination of floral scent profiles based on pollination systems within particular genera for members of the *Hemipepsis*-wasp pollination guild and congeneric species. Different symbols represent different plant species. Crosses and open diamonds in (B) represent species with unknown pollination systems. Bimodal in (D) refers to *Xysmalobium undulatum* which is pollinated by both cetoniine beetles and *Hemipepsis* wasps. (A) reproduced from Shuttleworth & Johnson (2010).

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by each species varied from only 3 or 4 in *Asclepias macropus* up to c. 55 in the two *Eucomis* species and 62 in *D. sankeyi* (SM Tables 4-6; Johnson 2005; Shuttleworth & Johnson 2009a). Aside from *A. macropus*, asclepiad guild members typically produced c. 30 floral volatiles. Total emission rates (means ± sd) ranged from 0.04 ± 0.020 µg inflorescence $^{-1}$ hour $^{-1}$ in *Asclepias macropus* (n = 5; SM

Table 4) to 77.4 ± 51.8 µg inflorescence $^{-1}$ hour $^{-1}$ in *Eucomis autumnalis* (n = 6; Shuttleworth & Johnson 2009a). The mean (± sd) total emission rate (inflorescence $^{-1}$ hour $^{-1}$) across all guild members (calculated from species means) was 15.1 ± 20.8 µg (n = 15). No single compound was common to the scents of all species (SM Table 4-6). Compounds characterizing the scents of different species varied across the guild, although (Z)-hex-3-en-1-ol, (Z)-hex-3-en-1-yl acetate, (E)- and (Z)-ocimene, limonene, linalool and myrcene contributed large amounts to intraspecific similarity in most guild members (Tables 3 & 4).

Non-metric multidimensional scaling (NMDS) analysis of the floral fragrance profiles of guild members and congeneric (non-wasp pollinated) species revealed little evidence for overall convergence in the floral scents of wasp-pollinated species (Fig. 3). Floral scent profiles for the species sampled were not clearly associated with particular pollination systems (Fig. 3A), and loci for *Hemipepsis*-wasp pollinated species show considerable overlap with those of plants with other pollination systems (Fig. 3). Differences in fragrance profiles were more clearly associated with genera and plant family (Fig. 3B & C). However, fragrance profiles for species within particular genera were more clearly associated with different pollination systems (Fig. 4). Within the genus *Asclepias*, fragrance of the wasp-pollinated species was significantly different from other pollination systems (range of R = 0.665 – 1, $p \leq 0.018$; Fig. 4C). Similarly, the fragrances of wasp- and fly-pollinated *Eucomis* species were significantly different (R = 0.678, $p < 0.001$; Fig. 4A; see Shuttleworth & Johnson 2010a). In the genus *Xysmalobium*, the scents of wasp-pollinated species were similar to those of fly-pollinated species (R = 0.076, p = 0.286), but were distinct from other pollination systems (range of R = 0.638 – 1, $p \leq 0.018$; Fig. 4D). Finally, the scents of wasp-pollinated *Pachycarpus* species were similar to those of cetoniine beetle-pollinated species (R = 0.307, $p <$ 0.001; Fig. 4B).

TABLE 3. Compounds contributing to the first 80% of average similarity between conspecific scent samples (from SIMPER analysis) for *Asclepias*, *Miraglossum*, *Periglossum*, *Eucomis*, *Disa* and *Satyrium* members of the *Hemipepsis*-wasp pollination guild. % = percentage contribution of each compound to conspecific similarity, Sim/sd = percentage contribution/standard deviation (not presented for *D. sankeyi* as there were insufficient replicates to calculate sd). Compounds that characterize a species scent will exhibit high percentage contributions and high sim/sd values (Clarke and Warwick, 2001). Mass fragments for unknowns are listed with the molecular ion first (if known) marked with *, followed by the base peak and other fragments in decreasing order of abundance.

	A. macropus		*M. pilosum*		*M. verticillare*		*P. angustifolium*		*E. autumnalis*		*E. comosa*		*D. sankeyi*	*S. microrrhynchum*	
Compound	%	Sim/sd	%	Sim/sd	%	Sim/sd	%	Sim/sd	%	Sim/sd	%	Sim/sd	%	%	Sim/sd
Aliphatics															
Alcohols															
Hexan-1-ol	-	-	2.2	8.8	-	-	-	-	-	-	-	-	-	-	-
(Z)-Hex-3-en-1-ol	-	-	-	-	13.5	3.4	10.9	2.4	-	-	-	-	-	-	-
Oct-1-en-3-ol	-	-	-	-	-	-	-	-	4.1	1.5	-	-	-	-	-
(Z)-Dec-4-en-1-ol	-	-	3.1	6.1	-	-	-	-	-	-	-	-	-	-	-
Aldehydes															
Hexanal	-	-	4.0	3.7	-	-	-	-	-	-	-	-	-	-	-
(E)-Hex-2-enal	-	-	-	-	11.9	3.6	-	-	-	-	-	-	-	-	-
(Z)-Hex-3-enal	-	-	-	-	2.5	0.9	-	-	-	-	-	-	-	-	-
Octanal	-	-	19.4	6.8	-	-	-	-	-	-	-	-	-	-	-
Nonanal	-	-	-	-	-	-	-	-	-	-	-	-	2.0	-	-
(Z)-4-Decenal	-	-	22.5	5.9	-	-	-	-	-	-	-	-	-	-	-
Esters															
Hexyl acetate	-		7.6	4.0	-	-	4.9	4.1	-	-	-	-	-	-	-
(Z)-Hex-3-en-1-yl acetate	37.2	5.6	4.8	7.4	12.9	1.3	33.1	5.9	-	-	-	-	-	-	-
Octyl acetate	-	-	2.9	8.0	-	-	-	-	-	-	-	-	-	-	-
Ketones															
But-3-en-2-one	-	-	-	-	-	-	-	-	1.4	1.1	-	-	-	-	-
4-Methylpentan-2-one	-	-	-	-	-	-	-	-	2.4	1.0	2.4	1.3	-	-	-
Nonan-2-one	-	-	-	-	-	-	-	-	2.0	1.3	-	-	-	-	-
Aromatics															
Benzaldehyde	-	-	2.6	2.2	-	-	-	-	-	-	-	-	7.0	-	-
Benzyl (Z)-cinnamate	-	-	-	-	-	-	-	-	-	-	-	-	3.2	-	-
Benzyl alcohol	-	-	-	-	-	-	-	-	-	-	-	-	5.2	-	-

Compound	*A. macropus*		*M. pilosum*		*M. verticillare*		*P. angustifolium*		*E. autumnalis*		*E. comosa*		*D. sankeyi*	*S. microrrhynchum*	
	%	Sim/sd	%	Sim/sd	%	Sim/sd	%	Sim/sd	%	Sim/sd	%	Sim/sd	%	%	Sim/sd
(E)-Cinnamaldehyde	-	-	-	-	-	-	-	-	-	-	-	-	12.6	-	-
(Z)-Cinnamic aldehyde	-	-	-	-	-	-	-	-	-	-	-	-	2.8	-	-
Cinnamic alcohol	-	-	-	-	-	-	-	-	-	-	-	-	2.2	-	-
3,4-Dimethoxytoluene	-	-	-	-	-	-	-	-	-	-	2.4	15.5	-	-	-
3,5-Dimethoxytoluene	-	-	-	-	-	-	-	-	3.6	0.9	19.4	5.7	-	-	-
Eugenol	-	-	-	-	-	-	-	-	-	-	-	-	12.0	-	-
Elemicin	-	-	-	-	-	-	-	-	-	-	2.3	3.2	-	-	-
1-Methoxy-4-methylbenzene	-	-	-	-	-	-	-	-	-	-	1.4	1.3	-	-	-
Methyl (Z)-cinnamate	-	-	-	-	-	-	-	-	-	-	-	-	2.5	-	-
Methyl vanillate	-	-	-	-	-	-	-	-	-	-	-	-	1.7	-	-
Methylbenzoate	-	-	-	-	-	-	-	-	-	-	-	-	2.6	-	-
Methyleugenol	-	-	-	-	-	-	-	-	-	-	-	-	2.0	-	-
Phenylacetaldehyde	-	-	-	-	-	-	-	-	-	-	-	-	3.5	-	-
Phenylacetaldoxime	-	-	-	-	-	-	-	-	-	-	-	-	5.9	-	-
Phenylethyl alcohol	-	-	-	-	3.4	6.5	-	-	-	-	1.7	2.7	6.2	-	-
Isoprenoids															
Monoterpenes															
Terpinolene	-	-	-	-	-	-	-	-	-	-	1.3	1.3	-	-	-
(E)-Linalool oxide (furanoid)	-	-	-	-	-	-	-	-	5.0	4.6	4.0	3.6	-	-	-
(Z)-Linalool oxide (furanoid)	-	-	-	-	-	-	-	-	-	-	1.7	6.8	-	-	-
(E)-Linalool oxide (pyranoid)	-	-	-	-	-	-	-	-	2.2	9.8	1.2	4.4	-	-	-
(E)-Ocimene	-	-	-	-	-	-	7.9	5.1	3.9	5.6	6.7	2.7	-	-	-
(Z)-Ocimene	-	-	-	-	-	-	-	-	1.9	2.6	2.7	4.7	-	-	-
2,6-Dimethyl-1,5(E),7-octatrien-3-ol	-	-	-	-	-	-	-	-	-	-	-	-	-	5.5	1.9
Eucalyptol	-	-	-	-	-	-	-	-	1.4	1.6	-	-	-	11.5	2.5
Hotrienol	-	-	-	-	-	-	-	-	11.9	12.6	8.1	3.5	-	-	-
Lavandulol	-	-	-	-	-	-	-	-	-	-	-	-	2.0	-	-
Limonene	-	-	-	-	-	-	4.5	2.2	2.3	0.9	1.6	1.8	-	-	-
Linalool	-	-	-	-	2.7	6.2	4.6	3.6	26.4	5.4	15.3	2.1	-	65.9	5.3
Myrcene	-	-	-	-	3.6	3.4	15.8	2.0	1.4	1.2	-	-	-	-	-
α-Terpineol	-	-	-	-					-	-	1.6	3.1	-	-	-
Sesquiterpenes															
α-Copaene	-	-	-	-	2.3	2.8	-	-	-	-	-	-	-	-	-
α-Muurolene	-	-	-	-	2.0	4.9	-	-	-	-	-	-	-	-	-

Compound	*A. macropus*		*M. pilosum*		*M. verticillare*		*P. angustifolium*		*E. autumnalis*		*E. comosa*		*D. sankeyi*	*S. microrrhynchum*	
	%	Sim/sd	%	Sim/sd	%	Sim/sd	%	Sim/sd	%	Sim/sd	%	Sim/sd	%	%	Sim/sd
Miscellaneous cyclic compounds															
δ-Decalactone	-	-	-	-	-	-	-	-	3.7	1.5	2.3	1.4	-	-	-
2-Methylcyclopent-2-en-1-one	-	-	-	-	3.9	1.3	-	-	-	-	-	-	-	-	-
Nitrogen containing compounds															
1-Nitro-2-phenylethane	-	-	-	-	-	-	-	-	-	-	-	-	2.0	-	-
2-Phenylacetonitrile	-	-	-	-	-	-	-	-	-	-	1.8	2.3	5.7	-	-
Unknowns															
m/z: 112*,83,55,57,84	-	-	-	-	5.9	3.0	-	-	-	-	-	-	-	-	-
m/z: 112*,97,55,67,56,41,39	-	-	-	-	2.0	2.7	-	-	-	-	-	-	-	-	-
m/z: 121*,106,80,79,120	-	-	-	-	-	-	-	-	3.2	1.0	-	-	-	-	-
m/z: 150*,69,41,81,79,82,53	50.5	2.6	-	-	-	-	-	-	-	-	2.0	1.3	-	-	-
m/z: 55,56,41,69,70,84,43,42	-	-	6.4	16.9	-	-	-	-	-	-	-	-	-	-	-
m/z: 57,85,39,41,43,55,31	-	-	-	-	2.7	1.6	-	-	-	-	-	-	-	-	-
m/z: 57,85,86,43,55,42,41	-	-	-	-	10.7	3.4	-	-	-	-	-	-	-	-	-
m/z: 71,43,82,67,41,55,39,53	-	-	-	-	-	-	-	-	1.8	1.1	-	-	-	-	-
m/z: 79,67,41,108,55,39,81	-	-	5.7	7.4	-	-	-	-	-	-	-	-	-	-	-
m/z: 83,55,69,41,39,42,70,56	-	-	-	-	1.4	0.7	-	-	-	-	-	-	-	-	-
m/z: 117,91,90,65,89	-	-	-	-	-	-	-	-	-	-	1.2	1.2	-	-	-
m/z: 149,108,134,106,79	-	-	-	-	-	-	-	-	2.7	1.0	-	-	-	-	-
Total	87.7		81.1		81.3		81.7		81.2		81.1		81.2	82.9	
Average similarity (Bray-Curtis)	66.6		80.7		62.0		68.8		61.1		68.4		68.9	71.2	
Number of compounds	2		11		15		7		18		20		18	3	

TABLE 4. Compounds contributing to the first 80% of average similarity between conspecific scent samples (from SIMPER analysis) for *Pachycarpus* and *Xysmalobium* members of the *Hemipepsis*-wasp pollination guild. % = percentage contribution of each compound to conspecific similarity, Sim/sd = percentage contribution/standard deviation. Compounds that characterize a species scent will exhibit high percentage contributions and high sim/sd values (Clarke and Warwick, 2001). Mass fragments for unknowns are listed with the molecular ion first (if known) marked with *, followed by the base peak and other fragments in decreasing order of abundance.

	Pa. appendiculatus		*Pa. asperifolius*		*Pa. campanulatus*		*Pa. natalensis*		*Pa. grandiflorus*		*X. orbiculare*		*X. stockenstromense*		*X. undulatum*	
Compound	%	Sim/sd	%	Sim/sd	%	Sim/sd	%	Sim/sd	%	Sim/sd	%	Sim/sd	%	Sim/sd	%	Sim/sd
Aliphatics																
Alcohols																
Tetradecanol	-	-	-	-	4.6	1.4	-	-	-	-	-	-	-	-	-	-
Hexan-1-ol	2.8	8.0	-	-	-	-	1.9	3.3	3.0	2.2	-	-	-	-	2.4	4.3
(E)-Hex-2-en-1-ol	5.7	6.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(E)-Hex-3-en-1-ol	2.0	9.4	-	-	-	-	1.8	4.3	-	-	-	-	-	-	-	-
(Z)-Hex-3-en-1-ol	10.7	5.9	5.3	5.1	4.3	3.3	10.0	5.3	15.8	1.6	-	-	2.5	4.3	3.0	3.9
Aldehydes																
Propanal	-	-	-	-	-	-	2.6	1.2	-	-	-	-	-	-	-	-
(E)-Hex-2-enal	3.3	1.4	-	-	-	-	3.5	3.2	-	-	-	-	-	-	-	-
(Z)-Hex-3-enal	-	-	-	-	-	-	2.3	1.2	-	-	-	-	-	-	-	-
Hexadecanal	-	-	-	-	2.1	1.7	-	-	-	-	-	-	-	-	-	-
Esters																
Hexyl acetate	3.6	3.0	-	-	-	-	4.2	10.6	-	-	-	-	-	-	-	-
(Z)-Hex-3-en-1-yl acetate	26.4	10.8	24.2	1.0	15.0	2.3	32.3	9.1	8.2	0.9	-	-	-	-	-	-
(Z)-Hex-en-1-yl butyrate	-	-	-	-	1.7	1.1	-	-	5.9	3.1	-	-	-	-	-	-
Aromatics																
Benzaldehyde	-	-	-	-	7.7	3.1	-	-	-	-	-	-	15.9	6.2	-	-
Benzyl alcohol	3.1	8.4	-	-	1.9	2.6	-	-	5.1	3.6	-	-	20.8	4.0	-	-
Phenylethyl alcohol	-	-	-	-	-	-	-	-	-	-	-	-	5.7	3.2	-	-
1-Phenyl-1,2-propanedione	-	-	-	-	-	-	-	-	-	-	-	-	2.8	9.4	-	-
Isoprenoids																
Monoterpenes																
α-Pinene	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.7	4.4
(E)-Ocimene	5.7	3.2	7.8	0.9	17.2	7.6	3.4	1.9	17.2	1.4	10.6	2.1	-	-	16.5	10.7
(Z)-Ocimene	3.5	2.3	4.3	0.8	5.6	5.1	-	-	5.3	3.8	6.2	3.1	-	-	-	-

	Pa. appendiculatus		*Pa. asperifolius*		*Pa. campanulatus*		*Pa. natalensis*		*Pa. grandiflorus*		*X. orbiculare*		*X. stockenstromense*		*X. undulatum*	
Compound	%	Sim/sd	%	Sim/sd	%	Sim/sd	%	Sim/sd	%	Sim/sd	%	Sim/sd	%	Sim/sd	%	Sim/sd
Limonene	-	-	8.0	5.0	-	-	-	-	-	-	28.7	3.0	7.0	22.2	17.6	18.1
Linalool	2.1	4.6	-	-	6.6	2.6	-	-	11.7	1.4	2.9	3.9	-	-	3.3	4.1
Myrcene	-	-	14.4	3.5	-	-	2.9	1.7	-	-	21.2	2.0	11.5	4.9	23.9	9.8
Sesquiterpenes																
β-Bourbonene	-	-	-	-	-	-	-	-	-	-	2.2	1.3	-	-	-	-
Caryophyllene	3.8	2.7	3.8	1.8	4.7	4.7	-	-	4.0	0.8	3.8	2.9	-	-	2.4	4.5
α-Copaene	-	-	-	-	-	-	-	-	-	-	2.9	1.3	-	-	-	-
Germacrene D	-	-	-	-	1.7	2.0	-	-	-	-	2.3	1.2	-	-	-	-
Humulene	-	-	-	-	2.5	3.0	-	-	-	-	-	-	-	-	-	-
Terpene derived compound																
4-Oxoisophorone	-	-	-	-	-	-	-	-	-	-	-	-	2.2	1.3	-	-
Miscellaneous cyclic compounds																
2-Ethylfuran	-	-	-	-	-	-	4.4	6.5	-	-	-	-	-	-	-	-
δ-Decalactone	-	-	-	-	-	-	-	-	-	-	-	-	3.0	12.3	-	-
2-Methylcyclopent-2-en-1-one	-	-	3.0	0.8	-	-	1.8	1.2	-	-	-	-	-	-	-	-
Unknowns																
m/z: 112*,83,55,57,84	-	-	4.5	11.5	-	-	2.6	2.7	-	-	-	-	-	-	-	-
m/z: 150*,69,41,81,79,82,53	2.8	4.6	-	-	6.0	2.4	-	-	5.4	3.6	-	-	-	-	9.0	2.9
m/z: 168*,56,85,125,43,41,69,153,83	-	-	-	-	-	-	-	-	-	-	-	-	9.4	0.6	-	-
m/z: 43,80,79,39,41,77,81	2.5	5.9	-	-	-	-	1.5	3.7	-	-	-	-	-	-	-	-
m/z: 57,85,39,41,43,55,31	2.5	8.2	-	-	-	-	2.5	1.5	-	-	-	-	-	-	-	-
m/z: 57,85,86,43,55,42,41	-	-	7.4	2.6	-	-	5.1	3.9	-	-	-	-	-	-	-	-
Total	80.4		82.5		81.4		82.7		81.5		80.9		80.6		81.9	
Average similarity (Bray-Curtis)	77.1		53.8		70.2		75.6		57.3		68.6		57.2		78.9	
Number of compounds	15		10		14		16		10		9		10		9	

FLORAL COLOUR ANALYSES

Guild members typically exhibit dull greenish- or brownish-white flowers, often with purple or reddish-brown markings (Fig. 1; Table 1). Reflectance spectra were typically impure (low chroma) with maximum reflectance between 500 and 600 nm (green) and no ultraviolet (UV) reflectance (SM Fig. 1). Loci for these spectra cluster close to the background vegetation in the green to blue-green region of the colour hexagon (Fig. 5). Loci for guild members were significantly closer to the origin of the colour hexagon than loci for congenerics (mean ± s.d. distance for guild members: 0.15 ± 0.059 hexagon units, n = 17; mean ± s.d. distance for congenerics: 0.26 ± 0.019 hexagon units, n = 18; t = 4.7, $p < 0.001$; see SM Fig. 2 for reflectance spectra of congeneric species).

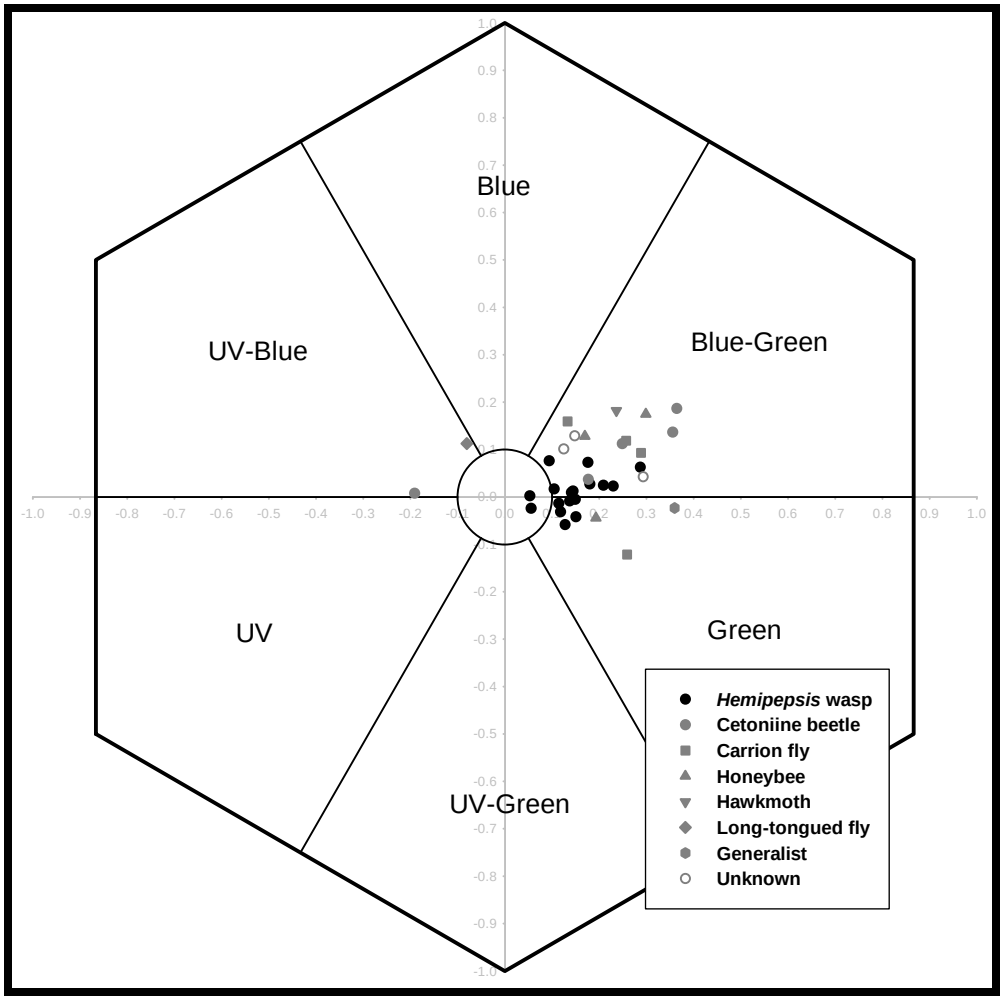

FIGURE 5. Floral colours of members of the *Hemipepsis*-wasp pollination guild and congeneric species as loci in bee colour space (Chittka 1992). The six segments represent the six categories of bee colour vision and loci are calculated from the relative stimulation of the three receptor types (UV, blue and green) by the spectral reflectance of the flowers.

DISCUSSION

The existence of a specialized system of pollination by *Hemipepsis* spider-hunting wasps in South African plants is firmly established by this synthesis of available data. The guild of plants pollinated by these wasps comprises at least 21 species, representing 10 genera and three families, which are pollinated exclusively or near exclusively by four functionally similar *Hemipepsis* wasp species (Table 1). The distribution of the guild is clearly centered in the moist grasslands of eastern South Africa (Fig. 2). Qualitative floral traits associated with the guild include dull greenish- or brownish-white flowers, often with purple blotches (Fig. 1), mid-summer flowering (Table 2), sweet spicy scent (SM Table 4-6) and exposed nectar (Table 1). Comparative analyses of floral traits for guild members and non-wasp pollinated congeneric species revealed limited evidence for convergence in nectar properties (volume, concentration and sugar composition) or floral scent profiles (although scent profiles for guild members were mostly distinct from congeners when examined within particular genera; Figs 3 & 4). However, floral colours of guild members clustered close to the background in the blue to blue-green region of the colour hexagon and were significantly closer to the colour of background vegetation than were those of congeneric species (Fig. 5).

The lack of convergence in the floral scent profiles of guild members (Fig. 3) is intriguing. Guild members produce a large number of volatiles in various compound classes and scent has been established as a key pollinator attractant in this system (Shuttleworth & Johnson 2009a,b,c; Tables 3 & 4, SM Tables 4-6). One reason for the lack of clear patterns of convergence in the scent phenotype space may be that we included both active and non-active compounds in the analysis, on account of being unable to distinguish between these two compound categories. Loci for *Ophrys* orchid species that share the same pollinator were tightly clustered in scent space when the analysis was limited to biologically active compounds but this overlap disappeared when the same analysis was applied to non-active compounds (Cortis *et al*. 2009). This suggests that patterns of convergence in the scent profiles of *Hemipepsis*-wasp pollinated flowers may be detected if we only examine biologically active compounds (not yet identified in this system). The congeneric comparisons of floral scent profiles may also be misleading if the current taxonomy does not reflect the true phylogeny. A recent molecular study suggests that the genera *Asclepias* and *Xysmalobium* are polyphyletic (Goyder *et al*. 2007) and congeneric comparisons may thus not be ideal for these particular genera.

The particular mechanisms of scent-based attraction in this system are not clear. One possibility is that wasps are attracted by broad classes of scents rather than specific compounds, in the same way that moth-pollinated flowers often produce high proportions of terpenoid and aromatic alcohols with small amounts of nitrogen-containing compounds (Knudsen & Tollsten 1993). The floral scents of most guild members are characterized by aliphatics and common monoterpenes (such as (E)- and (Z)-

ocimene, limonene, linalool and myrcene) with small amounts of aromatics (Tables 3 & 4). Physiological responses by wasps to several common monoterpene and aromatic compounds (such as linalool, myrcene, limonene, (Z)-ocimene, α-pinene and 3,5-dimethoxytoluene) were found in preliminary gas chromatography-electro antennogram detection (GC-EAD) experiments, but we did not get clear responses by wasps to these compounds in bioassays (F.P. Schiestl, S.D. Johnson & A. Shuttleworth, unpubl. data). Although these types of compounds often dominate the individual fragrance profiles of guild members (Tables 3 & 4; SM Tables 4-6; Johnson 2005; Johnson *et al*. 2007; Shuttleworth & Johnson 2009a,b), they also dominate the fragrance profiles of congeneric species (SM Tables 7 & 8; Shuttleworth & Johnson 2010b) and are ubiquitous floral volatiles (Knudsen *et al*. 2006), suggesting that they would be unlikely to mediate the exclusive attraction of *Hemipepsis* wasps.

Another possibility is that wasps are attracted by particular combinations of volatiles. The attraction of *Andrena* bees to sexually deceptive *Ophrys* orchids is based on a combination of common cuticular hydrocarbons in specific proportions (Schiestl *et al*. 1999). A synergistic effect on volatile attractiveness has also been shown for chafer beetles, where some compounds are only attractive in combination (Larsson *et al*. 2003; Toth *et al*. 2004; Vuts *et al*. 2010a,b). Guild members may thus be relying on combinations of common volatiles in specific proportions to attract *Hemipepsis* wasps. This could also explain the absence of any compound common to the scents of all guild members (SM Table 4-6), since different guild members may be utilizing different combinations of volatiles to attract the same wasps.

A final possibility is that *Hemipepsis* wasps may be attracted by particular compounds which are produced at levels below the threshold of detection by our analytical techniques. Wasps can be attracted by highly specific volatile compounds in some systems (Schiestl *et al*. 2003; Brodmann *et al*. 2008) and it is possible that these would be produced in very small amounts or have relatively low volatility such that they are present in only trace amounts in headspace samples (Schiestl *et al*. 1999). Indeed, wasps were found to respond physiologically to four pheromone-type hydrocarbons (heneicosane, tricosane, (Z)-9-tricosene and (Z)-9-pentacosene) in preliminary GC-EAD experiments using dichloromethane solvent extraction samples from flowers of guild members (F.P. Schiestl, S.D. Johnson & A. Shuttleworth, unpubl. data). These compounds, however, were not detected in headspace samples from the same flowers. The attraction of wasps may thus be mediated by highly specific compounds with low volatility which are consequently not detected in headspace samples. If this were the case, however, it would not explain why *Hemipepsis*-wasp pollinated flowers typically exhibit such complex fragrance profiles with high rates of emission (Johnson 2005; Johnson *et al*. 2007; Shuttleworth & Johnson 2009a,b; SM Tables 4-6). The production of "unnecessary" floral volatiles, while possibly costly for the plant, may also attract unwanted non-pollinating visitors. The

absence of floral scent in flowers pollinated by birds (Knudsen *et al*. 2004), which are generally assumed to use only colour and shape cues to locate flowers, suggests that volatiles are not produced by flowers unless they have a function. An understanding of the mechanisms of attracting *Hemipepsis* wasps will ultimately require more detailed GC-EAD and bioassay experiments.

Unlike floral scents, the floral colours of guild members do exhibit some level of convergence (Fig. 5). Floral colour in this guild appears to function primarily as a form of crypsis. Previous studies have shown that visual cues appear to play little or no role in the attraction of pollinators and the reflectance spectra of guild members are often similar to the spectra of green leaves (Ollerton *et al*. 2003; Shuttleworth & Johnson 2009a,b,c; Johnson *et al*. 2007; SM Fig. 1). Cryptic floral coloration within the guild is confirmed by the close proximity of the loci for guild members to background vegetation in the colour hexagon (Fig. 5). Differences between the colours of guild members and congeners (Fig. 5; SM Figs 1 & 2) suggest that the colours of guild members have evolved towards matching the background vegetation and selection pressures may, therefore, relate to the negative effects of non-pollinator visits on fitness (c.f. Hargreaves *et al*. 2009). The presence of purple blotches or colouring on the flowers of many guild members (Fig. 1) is another intriguing convergent trait. The wasps are unlikely to perceive these markings since purple, to humans, results from the reflectance of a combination of long (red) and short (blue) wavelengths, while wasps (like bees) have limited sensitivity to long (red) wavelengths (Chittka *et al*. 1992). It is thus unclear what role, if any, these markings play although one possibility is that the purple markings serve an additional cryptic function and result from selection by non-pollinating insects or even mammalian herbivores, rather than by the wasps themselves.

Differences between the nectar properties of guild members and congeners were not significant. However, clearer patterns of convergence may ultimately be found in the non-sugar constituents of nectars (not examined in this study). Bitter nectar is a characteristic of all the *Pachycarpus* guild members and a functional role has been demonstrated for two of these (Shuttleworth & Johnson 2006, 2009b). A functional role for nectar has also been found in *X. orbiculare*, although this was not as clear as for the *Pachycarpus* species (Shuttleworth & Johnson 2009c). Nectar palatability has not been tested in the orchids or *Eucomis* species. However, nectar of the *Eucomis* species is not as bitter to the human palate suggesting that differentially palatable nectar may be limited to certain genera within the guild. Nectar palatability has also not been explored in congeneric non-wasp-pollinated species, making it difficult to determine if the bitter nectar of some guild members represents an adaptation for wasp-pollination or is simply a phylogenetic property within some lineages. Nonetheless, the *Pachycarpus* species which have unpalatable nectar are visited by considerably fewer non-pollinating insects than some of the other guild members, suggesting that differentially palatable nectar may play an important role in reducing visits by nectar thieves. The compounds responsible for the

unpalatability of these nectars remain to be established, although preliminary studies with *Pa. asperifolius* nectar suggested a high phenolic content (A. Shuttleworth, unpubl. data). Future studies examining the non-sugar constituents of these unpalatable nectars would enhance our understanding of the role of nectar as a floral filter in some systems (Stephenson 1981; Adler & Irwin 2005; Johnson *et al*. 2006; Shuttleworth & Johnson 2009b).

The levels of specialization within this guild are remarkable given the presence of exposed nectar and the absence of any form of morphological filter (Table 1; Johnson & Steiner 2000, 2003). The proximate basis for specialization by plants in the guild appears to be biochemical (scent and nectar) and colour filters. Flowers of *Pa. grandiflorus*, for example, produce copious amounts of exposed nectar, yet are pollinated exclusively by *Hemipepsis* wasps. Experiments in which inflorescences were concealed from view, but still produced scent, showed that the wasps could locate flowers in the complete absence of visual cues (Shuttleworth & Johnson 2009b). The flowers have a very dull, impure reflectance spectrum which is similar to that of background vegetation. The nectar of this species is bitter to the human palate and proved unpalatable to honeybees (Shuttleworth & Johnson 2009b). This species thus uses floral scent as a specific pollinator attractant and appears to rely on cryptic colouring and unpalatable nectar to avoid detection and deter non-pollinating insects, respectively. A similar system was found for *X. orbiculare* and *E. autumnalis*, and it appears that a combination of specific floral scent and cryptic colouring are used by all guild members to filter floral visitors. Indeed, guild members are all inconspicuous in the landscape and many of the smaller asclepiads (such as *Aspidiglossum glanduliferum*, *Periglossum angustifolium* and the *Miraglossum* species) are best found by following foraging wasps, as was first noted by Weale (1873).

Guild members exhibit diverse floral morphologies and place pollen on various different parts of the wasps' bodies, including claws, tarsi, tibial and tarsal spines, palps, clypeal hairs, mouthparts, thorax and frons (Table 1). Pollen transfer efficiency (PTE; Johnson *et al*. 2004) was remarkably varied across the asclepiad guild members and ranged from 2% up to 80% (Table 1), possibly relating to both the placement of pollen on wasps and the morphologies of particular flowers. Placement of pollen on different parts of the same pollinator has been noted in other southern African pollination guilds (Manning & Goldblatt 1996, 1997; Potgieter & Edwards 2005) and may be the result of character displacement through reproductive interference between guild members (Armbruster *et al*. 1994). We found some evidence of reproductive interference in four species: a number of *Pe.angustifolium* pollinia were found inserted in *X. orbiculare* flowers (Shuttleworth & Johnson 2009c) and a number of *X. undulatum* pollinia were found inserted in *Pa. appendiculatus* flowers (Shuttleworth & Johnson 2009e). However, the costs of this interspecific pollen transfer are difficult to assess and a hypothesis of character displacement between guild members would require detailed studies of co-occurring species.

It is clear that *Hemipepsis* wasps are important components of the pollinator fauna in southern African grasslands and, as with other pollination guilds, should be recognized as keystone species within grassland ecosystems. Aside from the plants with specialized pollination systems examined in this study, *Hemipepsis* wasps also visit and may contribute to pollination in several plant species with generalist pollination systems, including *X. gerrardii* (Apocynaceae), *Cyphostemma cirrhosum* and *C. natalitium* (Vitaceae), *Cissus* spp. (Vitaceae), *Heteromorpha arborescens* var. *abyssinica* (Apiaceae), *Sium repandrum* (Apiaceae) and *Peucedanum capense* (Apiaceae) (Weale 1873; Ollerton *et al*. 2003; A. Shuttleworth pers. obs; P. Wragg pers. comm.). In addition, unidentified pollinaria have been found on several *Hemipepsis* wasps confirming that other guild members remain to be discovered. Asclepiad genera that are likely to contain additional guild members include *Asclepias*, *Miraglossum* and *Pachycarpus*.

The existence of floral traits that are associated with pollination by *Hemipepsis* wasps suggests that it may be possible to outline a syndrome of pompilid wasp pollination within the angiosperms. However, the difficulties associated with objectively assessing biochemical and colour traits means that patterns of convergence are not as clear as they are for other South African pollination guilds (Johnson 2010). The major challenge ahead will be to identify the floral volatiles that attract *Hemipepsis* wasps and the non-sugar constituents that make the nectars of some guild members differentially palatable. Having this information would contribute greatly to our general understanding of floral specialization in flowers with exposed nectar.

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SUPPLEMENTARY MATERIAL FOR CHAPTER 10

SM TABLE 1. Field sites used for the collection of floral scent, nectar sugars and reflectance spectra data.

Site No.	Locality (Province [a])	Habitat	Co-ordinates	Altitude (m.a.s.l.)
1	1 km North of Sinangwana River Mouth (EC)	rocky coastal grassland	31º44'41.9"S; 29º22'50.8"E.	132
2	Ashburton (KZN)	grassland	29°39'46.74"S; 30°26'27.65"E.	675
3	Baynesfield (KZN)	rocky grassland	29º45'13.27"S; 30º21'29.86"E.	810
4	Bushman's Nek, Drakensberg (KZN)	rocky montane grassland	29º50'28.1"S; 29º12'07.2"E.	1861
5	Cato Ridge Airfield (KZN)	rocky grassland	29°41'46.83"S; 30°37'0.39"E.	786
6	Garden Castle, 5km before resort, Drakensberg (KZN)	rocky montane grassland	29°44'20.41"S; 29°18'57.75"E.	1661
7	Gilboa Estate, Karkloof (KZN)	rocky montane grassland	29º16'30.7"S; 30º16'45.0"E.	1607
8	Hesketh Conservancy, Pietermaritzburg (KZN)	grassland	29º37'31.9"S; 30º25'31.2"E.	650
9	Highflats (KZN)	annually mown grassland	30º16'10.3"S; 30º12'09.3"E.	976
10	Midmar Nature Reserve 1 (KZN)	mistbelt grassland	29º32'15.8"S; 30º10'13.1"E.	1088
11	Midmar Nature Reserve 2 (KZN)	mistbelt grassland	29º31'34.5"S; 30º10'12.7"E.	1090
12	Mount Currie Nature Reserve, Kokstad (KZN)	montane grassland	30º29'58.2"S; 29º25'12.3"E.	1455
13	Roadside near Gilboa Estate (KZN)	montane grassland	29º15'29.0"S; 30º20'21.3"E.	1502
14	Royal Natal National Park, 2km before Sentinel car park, Drakensberg (FS)	rocky montane grassland	28º42'54"S; 28º53'43"E.	2400
15	Royal Natal National Park, below Witsieshoek Resort, Drakensberg (FS)	rocky montane grassland	28°41'20.57"S; 28°53'58.99"E.	2118
16	Royal Natal National Park, base of Sentinel Peak, Drakensberg (FS)	rocky montane grassland	28°44'30.04"S; 28°53'14.83"E.	2872
17	Sani Pass, Drakensberg (L)	rocky montane grassland	29°35'28.57"S; 29°17'45.91"E.	2570
18	Vernon Crookes Nature Reserve 1 (KZN)	coastal grassland	30º16'06.5"S; 30º37'14.5"E.	447
19	Vernon Crookes Nature Reserve 2 (KZN)	coastal grassland	30º15'53.8"S; 30º35'39.0"E.	479
20	Wahroonga Farm (KZN)	moist montane grassland	29º36'35.9"S; 30º07'59.4"E.	1350

[a] EC = Eastern Cape province; FS = Free State province; KZN = KwaZulu-Natal province.

SM TABLE 2. Sampling details for scent, nectar sugars and spectral reflectance of guild members and congeneric species.

	Scent					Nectar sugars			Spectral reflectance		
Species	*n*	Sample duration (mins) [a]	Sample type [a]	Date [a]	Site	*n*	Date	Site	*n*	Date	Site
Hemipepsis-pollinated											
Asclepias macropus	5	60	in situ	16 Jan 2008	20	-	-	-	-	-	-
Miraglossum pilosum	5	25	in situ	8 Nov 2008	13	-	-	-	4	29 Nov 2006	20
M. pulchellum	-	-	-	-	-	-	-	-	4	3 Dec 2007	6
M. verticillare	5	20	in situ	7 Nov 2008	20	4	12 Nov 2007	20	4	20 Nov 2006	20
Pachycarpus appendiculatus	6	20	in situ	11 Dec 2006	1	-	-	-	4	15 Dec 2006	1
Pa. asperifolius	4	30	in situ	21 Nov 2007	8	12	14 Nov 2007	18	4	7 Nov 2006	18
Pa. campanulatus	5	60	in situ	16 Jan 2008	20	3	16 Jan 2008	20	4	6 Jan 2006	20
Pa. grandiflorus	-	-	-	-		11	31 Jan 2008	7	-	-	-
Pa. natalensis	5	25	in situ	13 Nov 2007	20	7	19 Nov 2007	20	4	16 Nov 2006	20
Periglossum angustifolium	5	30	in situ	28 Jan 2008	10	-	-	-	4	28 Jan 2008	10
Xysmalobium orbiculare	6	20	in situ	15 Feb 2007	20	1	6 March 2008	20	-	-	-
X. stockenstromense	3	20	cut flowers	21 Dec 2006	14	-	-	-	4	21 Dec 2006	14
X. undulatum	3	20	cut flowers	4 Dec 2006	11	12	3 Dec 2007	19	4	6 Jan 2006	11
Woodia verruculosa	-	-	-	-	-	-	-	-	4	15 Nov 2005	10
Disa sankeyi	-	-	-	-	-	-	-	-	4	28 Oct 2004	14
Congeneric species											
Asclepias albens	-	-	-	-	-	-	-	-	4	7 Nov 2006	18
A. cucullata	3	25	in situ	7 Nov 2007	20	1	7 Nov 2007	20	4	9 Nov 2006	10

A. crispa var. *plana*	3	20	in situ	12 Dec 2006	1	-	-	-	-	-	-
A. dregeana	5	20	in situ	29 Oct 2007	20	12	31 Oct and 12 Nov 2007	20	4	29 Oct 2007	20
A. gibba	2	25	in situ	13 Nov 2007	10	14	19 and 21 Nov 2007	11	4	6 Nov 2006	2
A. humilis	-	-	-	-	-	-	-	-	4	21 Dec 2006	16
A. woodii	-	-	-	-	-	4	12 Nov 2007	11	4	16 Nov 2006	20
Pachycarpus concolor	-	-	-	-	-	7	19 and 21 Nov 2007	11	4	7 Nov 2006	18
Pa. coronarius	3	35	in situ	26 Nov 2007	5	-	-	-	-	-	-
Pa. plicatus	-	-	-	-	-	7	1 Dec 2007	12	4	3 Dec 2007	12
Pa. scaber	-	-	-	-	-	2	1 and 2 Nov 2007	3	4	29 Oct 2007	3
Pachycarpus sp. nov.	-	-	-	-	-	-	-	-	4	3 Dec 2007	9
Xysmalobium gerarrdii	5	20	in situ	29 Oct 2007	20	1	7 Nov 2007	20	4	16 Nov 2006	20
X. involucratum	-	-	-	-	-	-	-	-	4	16 Nov 2006	20
X. parviflorum	3	20	in situ	8 Nov 2007	7	-	-	-	4	9 Nov 2007	7
X. tysonianum									1	2002	7
Eucomis schijffii	-	-	-	-		4	15 Jan 2008	17	-	-	-
Satyrium longicauda	-	-	-	-	-	-	-	-	1	10 Nov 2009	20

[a] Number in parentheses refer to the sample number.

SM TABLE 3. Floral traits and pollination systems of plant species used for congeneric comparisons with members of the *Hemipepsis*-wasp pollination guild.

		Nectar						
		24h production		Sugars (%)				
Species	Colour	µl	%	Suc	Gluc	Fruct	Pollination system	Source
Apocynaceae: Asclepiadoideae								
Asclepias albens	pink and yellow	-	-	-	-	-	cetoniine beetle (?)	A. Shuttleworth unpubl. data
A. cucullata	brownish-white	0.4	44	100.0	0.0	0.0	bee (?)	Ollerton *et al*. 2003; This study
A. crispa var. *plana*	yellow-green	-	-	-	-	-	generalist insect	Shuttleworth and Johnson (2009d)
A. dregeana	green	0.7	54	97.1	0.0	2.9	honeybee	Shuttleworth and Johnson (2009d); This study
A. gibba	green and white	0.2	36	97.5	0.0	2.5	honeybee	Shuttleworth and Johnson (2009d); This study
A. humilis	greenish-white and purple	-	-	-	-	-	unknown	This study
A. woodii	yellowish-white	0.7	29	0.0	41.5	58.5	cetoniine beetle	Ollerton *et al*. (2003); This study
Pachycarpus concolor	wine red	3.7	37	96.2	0.0	3.8	cetoniine beetle	Shuttleworth and Johnson (2009d)
Pa. coronarius	greenish-white with purple blotches	-	-	-	-	-	unknown	
Pa. plicatus [a]	yellowish-green with purple blotches	5.9	54	94.6	0.8	4.7	unknown, possibly cetoniine beetle	This study
Pa. scaber	white	6.6	28	83.4	5.9	10.7	cetoniine beetle	Shuttleworth and Johnson (2009d); This study
Pachycarpus sp. nov.	white and yellow	-	-	-	-	-	cetoniine beetle	Shuttleworth and Johnson (2009d); This study
Xysmalobium gerarrdii	yellow	0.9	35	0.0	28.9	71.1	generalist insect	Ollerton *et al*. (2003); This study
X. involucratum	yellow-green	0.2	12.5	-	-	-	cetoniine beetle	Ollerton *et al*. (2003)
X. parviflorum	white, green and red	-	-	-	-	-	fly	Shuttleworth and Johnson (2009d); Johnson *et al*. (2009)
X. tysonianum	yellow	-	-	-	-	-	fly	S.D Johnson unpubl. data
Hyacinthaceae								
Eucomis bicolor	white with purple edges	1.4	50	34.8	32.5	32.7	fly	Shuttleworth and Johnson (2010)
E. humilis	greenish white and purple	16	8	7.8	39.5	52.7	fly	Shuttleworth and Johnson (2010)

E. schijffii	greenish white and purple	-	-	38.1	24.2	37.7	fly	A. Shuttleworth unpubl. data; This study
Orchidaceae								
Satyrium longicauda	white	-	-	-	-	-	hawkmoth	S.D. Johnson unpubl. data
Disa nervosa	pink	Deceptive		-	-	-	long-tongued fly	Johnson and Morita (2006)

[a] Nectar volume (n = 35 flowers) and concentration (n = 30 flowers) were measured using 5 µl capillary tubes and a Bellingham and Stanley 0–50 % or 45–80 % sugar concentration hand-held refractometer respectively, from eight plants at Site 12 (see SM Table 1) on 1 Dec 2007. Population means were calculated from individual plant means.

SM TABLE 4. Relative amounts (%) of compounds identified by GC-MS from headspace samples of *Asclepias*, *Miraglossum* and *Periglossum* members of the *Hemipepsis*-wasp pollination guild. Compounds are listed in order of increasing Kovats retention index (KRI, calculated from our retention times using an Alltech EC-WAX column) within each compound class [a].

			Asclepias macropus					*Miraglossum pilosum*					*Miraglossum verticillare*					*Periglossum angustifolium*				
Compound	KRI	Criteria[b]	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Aliphatics																						
Alcohols																						
Hexan-1-ol	1364	C	-	-	-	-	-	0.3	1.0	0.3	0.5	0.4	-	-	-	-	-	-	-	-	-	-
(Z)-Hex-3-en-1-ol	1398	A	-	-	-	-	-	0.1	0.1	0.2	2.6	0.5	12.4	5.4	24.2	23.4	10.6	4.2	21.1	7.0	1.4	16.5
(Z)-Dec-4-en-1-ol	1819	B	-	-	-	-	-	0.9	0.7	0.9	0.5	1.9	-	-	-	-	-	-	-	-	-	-
Aldehydes																						
Hexanal*	1131	A	-	-	-	-	-	1.3	4.9	0.6	3.2	1.1	-	-	-	-	-	-	-	-	-	-
(Z)-Hex-3-enal	1179	A	-	-	-	-	-	-	-	-	-	-	-	8.6	5.0	0.6	0.3	-	-	-	-	-
Heptanal	1212	A	-	-	-	-	-	0.1	0.7	0.6	1.1	0.2	-	-	-	-	-	-	-	-	-	-
(E)-Hex-2-enal	1240	A	-	-	-	-	-	tr	0.2	tr	-	0.1	33.8	10.7	23.8	7.6	4.0	0.7	2.8	2.5	-	4.6
Octanal*	1309	A	-	-	-	-	-	16.7	34.7	40.6	45.5	27.7	-	-	-	-	-	-	-	-	-	-
(E)-Non-4-enal	1457	B	-	-	-	-	-	0.1	0.2	0.1	0.1	0.1	-	-	-	-	-	-	-	-	-	-
(Z)-4-Decenal	1562	A	-	-	-	-	-	61.0	38.4	40.5	24.0	49.7	-	-	-	-	-	-	-	-	-	-
(E,E)-Deca-2,4-dienal	1835	A	-	-	-	-	-	0.1	0.1	tr	tr	tr	-	-	-	-	-	-	-	-	-	-
Esters																						
Isoamyl acetate	1161	A	-	-	-	-	-	0.1	0.1	tr	-	tr	-	-	-	-	-	-	-	-	-	-
Hexyl acetate	1292	C	-	-	-	-	-	8.2	8.2	2.2	4.0	5.4	tr	0.8	tr	0.2	0.5	3.1	1.3	1.9	0.5	0.8
(Z)-Hex-3-en-1-yl acetate [c]	1335	A	27.4	42.4	9.7	19.4	24.8	1.6	1.1	2.0	6.1	1.9	7.2	32.3	0.8	38.7	68.4	66.0	37.4	51.3	25.5	59.4
Heptyl acetate	1390	A	-	-	-	-	-	0.2	0.1	0.1	tr	0.1	-	-	-	-	-	-	-	-	-	-
Octyl acetate	1491	A	-	-	-	-	-	1.7	0.6	0.4	0.8	0.7	-	-	-	-	-	-	-	-	-	-
Aromatics																						
Benzaldehyde [c]	1546	C	-	-	-	-	-	0.1	2.3	1.2	1.0	0.6	-	-	-	-	-	-	-	-	-	-
2-Hydroxybenzaldehyde	1706	A	-	-	-	-	-	-	0.4	-	tr	tr	tr	0.3	-	tr	-	-	-	-	-	-
Benzyl acetate	1752	C	-	-	-	-	-	tr	0.1	0.3	0.1	0.2	-	-	-	-	-	-	-	-	-	-
Methyl phenylacetate	1788	A	-	-	-	-	-	-	-	-	-	-	0.9	tr	tr	1.1	-	-	-	-	-	-
Methyl salicylate	1794	C	-	-	-	-	-	-	-	-	-	-	tr	0.1	-	tr	-	-	-	-	-	-
3-Phenylpropanal	1806	B	-	-	-	-	-	tr	0.1	0.9	0.3	0.2	-	-	-	-	-	-	-	-	-	-
Phenylethyl acetate [c]	1841	A	-	-	-	-	-	0.2	tr	0.1	tr	tr	-	-	-	-	-	-	-	-	-	-
Methyl 3-phenylpropanoate	1872	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	3.4	0.1	tr	tr
Phenylethyl alcohol	1940	A	-	-	-	-	-	-	-	-	-	-	0.5	1.9	0.8	1.1	0.5	-	-	-	-	-
Phenylpropyl acetate	1968	B	-	-	-	-	-	0.1	0.3	1.1	0.7	0.6	-	-	-	-	-	-	-	-	-	-
2-Methoxybenzaldehyde	1997	B	-	-	-	-	-	-	-	-	-	-	-	0.7	-	-	-	-	-	-	-	-
(E)-Cinnamaldehyde	2067	A	-	-	-	-	-	tr	0.1	0.7	0.1	0.1	-	-	-	-	-	-	-	-	-	-
3-Phenylpropanol	2073	A	-	-	-	-	-	tr	tr	0.1	tr	tr	-	-	-	-	-	-	-	-	-	-
Methyl methoxybenzoate	2095	B	-	-	-	-	-	-	-	-	-	-	-	0.3	-	-	-	-	-	-	-	-
p-Cresol	2102	A	-	-	-	-	-	-	-	-	-	-	0.1	0.3	0.1	0.2	0.1	-	-	-	-	-
3-Phenylprop-2-en-1-yl acetate	2173	A	-	-	-	-	-	-	tr	tr	tr	tr	-	-	-	-	-	-	-	-	-	-

Elemicin	2241	B	-	-	-	16.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Isoprenoids																						
Monoterpenes																						
Sabinene	1160	A	-	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-
Myrcene	1202	A	-	-	-	-	-	-	-	-	-	-	0.5	8.0	2.3	0.5	0.9	12.9	2.1	28.4	62.6	10.8
Limonene	1229	C	-	-	-	-	-	-	-	-	-	-	-	0.3	-	tr	3.9	0.6	0.2	1.8	5.0	1.5
Eucalyptol	1240	A	-	-	-	-	-	-	-	-	3.7	-	-	-	-	-	-	-	-	-	-	-
(Z)-Ocimene	1257	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.8	-	0.3	0.3	-
(E)-Ocimene	1276	A	-	-	-	-	-	-	-	-	-	-	-	0.4	1.3	0.2	tr	9.3	4.0	2.6	2.4	1.3
Linalool	1557	C	-	-	-	-	-	-	-	-	-	-	0.4	0.5	0.3	2.2	0.6	1.0	14.9	1.6	0.9	0.3
Sesquiterpenes																						
α-Cubebene	1479	A	-	-	-	-	-	-	-	-	-	-	0.4	-	0.2	0.2	0.1	-	-	-	-	-
α-Copaene	1517	A	-	-	-	-	-	-	-	-	-	-	1.2	0.3	0.1	1.0	0.3	-	-	-	-	-
β-Bourbonene	1543	A	-	-	-	-	-	-	-	-	-	-	0.4	tr	-	tr	tr	-	-	-	-	-
β-Cubebene	1564	A	-	-	-	-	-	-	-	-	-	-	0.9	-	0.1	-	0.2	-	-	-	-	-
Caryophyllene	1623	C	-	tr	1.1	4.0	tr	-	-	-	-	-	1.7	1.9	-	-	0.4	0.5	-	0.3	tr	-
(E)-β-Farnesene	1685	A	-	-	-	-	-	0.1	0.2	tr	tr	tr	tr	0.1	0.1	tr	tr	-	-	-	-	-
Humulene	1698	A	-	-	-	-	-	-	-	-	-	-	0.1	0.2	tr	-	tr	-	-	-	-	-
Germacrene D	1739	A	-	-	-	-	-	-	-	-	-	-	0.8	0.2	0.1	-	0.2	-	-	-	-	-
α-Muurolene	1753	A	-	-	-	-	-	-	-	-	-	-	1.0	0.1	0.3	0.2	0.3	-	-	-	-	-
Caryophyllene epoxide	2022	C	-	-	-	-	-	-	-	-	-	-	0.1	0.2	0.1	-	-	-	-	-	-	-
(E)-Nerolidol	2055	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	tr	tr	0.1	0.2
δ-Cadinol	2222	A	-	-	-	-	-	-	-	-	-	-	1.5	-	0.2	0.2	0.5	-	-	-	-	-
Nitrogen-containing compounds																						
2-Phenylacetonitrile	1955	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	4.2	0.6	0.1	0.6
Miscellaneous cyclic compounds																						
2-Methylcyclopent-2-en-1-one	1391	A	-	-	-	-	-	-	-	-	-	-	7.0	0.5	6.3	0.1	2.4	0.3	1.8	0.6	0.1	0.3
Cyclohex-2-en-1,4-dione	1766	B	-	-	-	-	-	-	-	-	-	-	0.1	-	0.1	0.5	tr	-	-	-	-	-
5-Ethyl-2(5H)-furanone	1792	A	-	-	-	-	-	-	-	-	-	-	-	0.8	0.8	-	-	-	-	-	-	-
Unknowns [d]																						
Sesquiterpenes																						
m/z: 204*,105,161,57,119,91,94,93,120	1508		-	-	-	-	-	-	-	-	-	-	0.3	tr	-	tr	tr	-	-	-	-	-
m/z: 204*,161,105,119,81,41,91,93	1661		-	-	-	-	-	-	-	-	-	-	0.1	-	tr	-	0.1	-	-	-	-	-
m/z: 204*,57,161,41,71,105,85,93,96	1717		-	-	-	-	-	-	-	-	-	-	0.2	-	tr	tr	0.1	-	-	-	-	-
m/z: 204*,161,105,119,91,93,133	1747		-	-	-	-	-	-	-	-	-	-	0.4	tr	0.1	tr	0.1	-	-	-	-	-
m/z: 204*,161,119,105,134,91,41,55	1787		-	-	-	-	-	-	-	-	-	-	1.0	tr	tr	tr	0.5	-	-	-	-	-
m/z: 222*,43,71,161,207,105,41,56,83	1915		-	-	-	-	-	-	-	-	-	-	0.3	-	0.3	-	0.1	-	-	-	-	-
m/z: 222*,161,43,207,105,93,119,81,121,123	1967		-	-	-	-	-	-	-	-	-	-	2.7	-	0.1	tr	0.7	-	-	-	-	-
Unknown compound class																						
m/z: 83,55,69,41,39,42,70,56	1230		-	-	-	-	-	-	-	-	-	-	0.9	1.7	1.7	tr	-	-	-	-	-	-
m/z: 136*,41,44,57,93,121,91,56,45	1306		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.5	-
m/z: 150*,69,41,81,79,82,53	1325		10.6	38.4	89.2	60.5	60.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 43,80,79,39,41,77,81	1448		-	-	-	-	-	0.1	0.1	0.1	0.1	0.1	tr	0.1	-	tr	0.2	-	-	-	-	-
m/z: 112*,55,83,97,57,56	1541		-	-	-	-	-	-	-	-	-	-	tr	0.4	0.3	0.2	tr	-	-	-	-	-
m/z: 57,85,86,43,55,42,41*	1567		-	-	-	-	-	-	-	-	-	-	16.9	12.0	25.4	5.9	2.9	0.2	3.2	0.1	0.1	1.2
m/z: 55,56,41,69,70,84,43,42	1575		-	-	-	-	-	2.6	2.5	3.4	2.9	5.1	-	-	-	-	-	-	-	-	-	-

m/z: 112*,83,55,57,84	1619	-	-	-	-	-	-	-	tr	0.4	0.1	4.1	3.5	2.7	13.5	0.7	0.2	2.8	0.1	0.1	2.2
m/z: 69,43,41,93,68,67,121,39	1622	61.9	19.2	-	-	15.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 79,67,41,108,55,39,81	1626	-	-	-	-	-	3.2	2.2	2.9	1.8	2.7	-	-	-	-	-	-	-	-	-	-
m/z: 70,41,69,57,55,43	1647	-	-	-	-	-	tr	0.1	tr	tr	0.1	-	-	-	-	-	-	-	-	-	-
m/z: 168*,85,56,125,43,69,41,83,153	1693	-	-	-	-	-	0.1	0.1	0.1	0.1	-	-	-	-	-	-	-	-	-	-	-
m/z: 68,81,67,43,54,82,55,41	1721	-	-	-	-	-	1.1	0.3	tr	0.1	0.1	-	-	-	-	-	-	-	-	-	-
m/z: 93,69,57,41,68,121,55,80,85	1775	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	-
m/z: 79,67,41,68,93,55,81	1885	-	-	-	-	-	tr	tr	0.1	tr	0.1	-	-	-	-	-	-	-	-	-	-
m/z: 132*,103,78,77,51,104,50	1923	-	-	-	-	-	tr	tr	0.2	tr	tr	-	-	-	-	-	-	-	-	-	-
m/z: 131,103,162,77,161,51,57	1983	-	-	-	-	-	-	-	-	-	-	0.9	-	-	-	-	-	-	-	-	-
m/z: 57,85,39,41,43,55,31	1986	-	-	-	-	-	-	-	-	-	-	0.3	6.8	1.3	1.5	0.1	-	-	-	-	-
m/z: 112*,97,55,67,56,41,39	2111	-	-	-	-	-	-	-	-	-	-	0.4	0.3	0.6	0.5	0.1	tr	0.7	0.1	tr	0.3
m/z: 112*,97,55,67,56,57,41	2132	-	-	-	-	-	-	-	-	-	-	0.4	0.2	0.4	0.1	tr	-	-	-	-	-
Aliphatics		27.4	42.4	9.7	19.4	24.8	92.3	91.1	88.6	88.3	89.8	53.4	57.9	53.8	70.5	83.9	74.0	62.6	62.8	27.4	81.2
Aromatics		0	0	0	16.2	0	0.3	3.4	4.4	2.2	1.6	1.6	3.6	1.0	2.3	0.6	0	3.4	0.1	0	0
Isoprenoids (including unknown sesquiterpenes)		0	0	1.1	4.0	0	0.1	0.2	0	3.8	0	13.9	12.2	5.8	4.5	9.0	25.1	21.3	35.0	71.3	14.2
Nitrogen-containing compounds		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.1	4.2	0.6	0.1	0.6
Miscellaneous cyclic compounds		0	0	0	0	0	0	0	0	0	0	7.1	1.3	7.1	0.6	2.4	0.3	1.8	0.6	0.1	0.3
Unknowns (excluding those identified to compound class)		72.6	57.6	89.2	60.5	75.2	7.1	5.2	6.7	5.4	8.3	23.9	25.0	32.3	21.9	3.9	0.5	6.8	1.0	1.1	3.7
Total of all compounds		100.0	100.0	100.0	100.0	100.0	99.9	99.9	99.7	99.8	99.8	99.9	100.0	100.0	99.9	99.8	100.0	100.0	100.0	99.9	100.0
Total number of compounds		3	4	3	4	4	31	33	33	34	33	40	37	36	35	37	18	15	18	19	16
Total volatiles (ng) emitted (per inflorescence per hour)		66	46	58	26	21	6998	10660	9216	5079	9058	3510	8541	2626	4827	12953	1285	245	1082	4341	990

[a] Relative amounts (%). tr = trace amount (< 0.05 % of total sample).

[b] Compound identification criteria: 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.

[c] Compounds which are possible background contaminants as they were present in some control samples.

[d] Mass fragments for unknowns are listed with the molecular ion first (if known) marked with *, followed by the base peak and other fragments in decreasing order of abundance.

SM TABLE 5. Relative amounts (%) of compounds identified by GC-MS from headspace samples of four *Pachycarpus* members of the *Hemipepsis*-wasp pollination guild. Compounds are listed in order of increasing Kovats retention index (KRI, calculated from our retention times using an Alltech EC-WAX column) within each compound class [a]. Footnotes are as for SM Table 4.

			Pachycarpus appendiculatus						*Pachycarpus asperifolius*				*Pachycarpus campanulatus* var. *campanulatus*					*Pachycarpus natalensis*				
Compound	KRI	Criteria[b]	1	2	3	4	5	6	1	2	3	4	1	2	3	4	5	1	2	3	4	5
Aliphatics																						
Acids																						
2-Methylbutanoic acid	1690	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	-	0.1	-
Hexanoic acid	1864	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-
Alcohols																						
Hexan-1-ol	1364	C	0.5	1.3	0.5	0.6	1.5	0.6	-	-	-	-	-	-	-	-	-	0.4	0.4	0.1	0.2	1.1
(E)-Hex-3-en-1-ol	1375	A	0.2	0.4	0.3	0.3	0.6	0.4	-	-	-	-	-	-	-	-	-	0.3	0.4	0.1	0.2	0.4
(Z)-Hex-3-en-1-ol	1398	A	6.1	19.3	8.6	12.5	17.7	7.8	1.6	24.1	0.5	1.0	12.5	1.2	1.0	1.0	3.8	12.7	6.2	3.6	7.9	28.9
(E)-Hex-2-en-1-ol	1417	A	4.0	1.5	3.2	2.5	10.7	2.7	-	-	-	-	-	-	-	-	-	0.1	0.1	-	0.1	0.2
(E,Z)-Nona-3,6-dien-1-ol	1771	A	tr	0.5	-	tr	tr	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tetradecanol	2192	A	-	-	-	-	-	-	-	-	-	-	0.7	tr	13.8	4.4	0.3	-	-	-	-	-
Hexadecan-1-ol	2392	A	-	-	-	-	-	-	-	-	-	-	0.1	0.4	0.7	2.7	tr	-	-	-	-	-
Aldehydes																						
Propanal	1005	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.1	1.6	-	1.2	1.0
(Z)-Hex-3-enal	1179	A	-	0.5	0.7	0.7	5.9	0.7	-	-	-	-	-	-	-	-	-	0.8	2.2	-	1.5	1.0
(E)-Hex-2-enal	1240	A	-	1.8	1.9	2.3	5.4	1.5	-	-	-	-	-	-	-	-	-	1.5	0.9	0.3	1.4	3.2
(E,E)-Hexa-2,4-dienal	1419	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	0.1	0.1	0.2	0.2
Tetradecanal	1944	A	-	-	-	-	-	-	-	-	-	-	0.1	1.2	0.6	0.3	tr	-	-	-	-	-
Hexadecanal	2155	A	-	-	-	-	-	-	-	-	-	-	0.2	1.8	1.3	1.0	0.1	-	-	-	-	-
Octadecanal	2359	A	-	-	-	-	-	-	-	-	-	-	0.1	0.3	0.7	1.2	tr	-	-	-	-	-
Esters																						
Hexyl acetate	1292	C	0.3	1.7	1.6	1.5	1.3	1.8	0.1	-	0.5	1.6	-	-	-	-	-	1.4	1.5	1.0	1.0	0.9
(Z)-Hex-3-en-1-yl acetate [c]	1335	A	61.5	45.4	63.3	67.1	44.9	57.7	52.5	0.2	58.9	60.1	49.4	25.1	22.5	4.2	36.9	70.1	64.5	86.3	64.5	52.5
(Z)-Hex-en-1-yl butyrate	1479	A	-	-	-	-	-	-	-	-	-	0.8	0.4	0.7	-	0.8	0.8	0.1	1.0	0.1	0.1	0.1
(Z)-Hex-3-en-1-yl isovalerate	1490	A	-	-	-	-	-	-	-	-	-	-	tr	0.1	tr	0.2	0.1	0.2	0.6	0.1	tr	tr
Aromatics																						
Benzaldehyde [c]	1546	C	-	-	-	-	-	-	-	-	-	-	6.6	10.8	4.3	12.4	1.8	-	-	-	-	-
Ethylbenzaldehyde	1739	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	tr	tr	tr	tr
1,2-Dimthoxybenzene	1749	B	tr	tr	tr	tr	tr	tr	-	-	-	-	tr	0.3	-	-	-	tr	tr	tr	0.2	0.2
Methyl salicylate	1794	C	-	-	-	-	-	-	-	0.2	-	-	0.1	0.2	-	tr	tr	-	-	tr	tr	tr
p-Cymen-8-ol	1864	A	-	-	-	-	-	-	tr	-	-	tr	-	-	-	-	-	-	-	-	-	-
3,5-Dimethoxytoluene [c]	1879	B	0.1	tr	tr	tr	0.6	tr	-	-	-	-	tr	tr	tr	tr	0.2	-	-	-	-	-
Benzyl alcohol [c]	1900	C	0.8	0.7	1.2	1.4	0.7	0.6	-	-	-	-	0.4	1.0	0.2	1.1	0.1	-	-	-	-	-
Phenylethyl alcohol	1940	A	0.2	1.6	6.2	0.5	0.1	tr	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(E)-Cinnamaldehyde	2067	A	tr	tr	tr	tr	0.1	tr	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(Z)-Hex-3-en-1-yl benzoate	2154	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	tr	tr	tr	tr
Elemicin	2241	B	-	-	-	-	-	-	0.5	-	-	-	-	-	-	-	-	-	-	tr	-	-

Cinnamic alcohol	2301	A	-	tr	-	-	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Benzyl benzoate	2645	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	tr	tr	tr	tr
Isoprenoids																						
Monoterpenes																						
Sabinene	1160	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	0.2	tr	-
Myrcene	1202	A	0.7	4.1	0.3	1.0	-	0.4	7.0	3.7	21.6	10.8	-	-	-	-	13.9	1.5	4.1	0.1	2.0	0.6
Limonene	1229	C	-	-	-	-	-	-	1.3	3.3	2.0	10.8	-	-	-	-	-	tr	tr	0.2	tr	tr
(Z)-Ocimene	1257	C	0.4	0.7	4.9	1.6	0.8	8.3	15.1	-	1.1	4.3	8.7	2.0	3.2	3.5	1.5	0.9	-	0.2	0.8	tr
(E)-Ocimene	1276	A	2.3	7.3	1.0	2.9	4.5	8.3	9.5	-	7.6	5.8	13.5	26.8	31.2	39.3	22.7	0.4	0.4	3.3	3.6	1.0
(E)-Linalool oxide (furanoid)	1456	C	0.2	tr	0.1	tr	tr	0.1	0.2	-	0.1	-	-	-	-	-	-	tr	tr	-	-	tr
Linalool	1557	C	0.9	0.2	0.6	0.3	0.3	0.6	1.4	-	0.9	0.1	1.0	3.4	6.5	11.0	7.5	tr	0.1	0.1	0.1	0.1
α-Terpineol	1716	C	-	-	-	-	-	-	-	-	-	-	tr	0.2	0.1	tr	0.6	-	-	-	-	-
Eucarvone	1759	B	-	-	-	-	-	-	-	-	-	-	0.1	tr	0.1	0.3	0.1	-	-	-	-	-
Sesquiterpenes																						
α-Cubebene	1479	A	1.1	tr	0.3	0.1	0.1	0.3	0.4	-	0.3	-	-	-	-	-	-	-	-	-	-	-
α-Copaene	1517	A	3.8	0.2	0.6	0.3	0.2	0.5	0.8	-	0.4	1.0	-	-	-	-	-	-	-	-	-	-
β-Bourbonene	1543	A	0.1	tr	0.1	tr	tr	0.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Cubebene	1564	A	0.4	tr	0.1	tr	tr	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Elemene	1614	A	-	-	-	-	-	-	-	-	-	-	-	-	0.4	1.0	0.4	-	-	-	-	-
Caryophyllene	1623	C	9.5	5.3	1.2	0.7	0.6	1.7	1.8	2.1	0.1	0.5	0.8	2.9	2.1	4.9	1.9	0.1	0.1	0.2	0.1	tr
Humulene	1698	A	0.8	1.1	0.1	tr	0.1	0.1	0.2	0.8	tr	tr	0.3	0.2	0.9	2.5	1.1	0.1	0.1	0.1	tr	tr
Germacrene D	1739	A	0.2	0.1	0.1	tr	0.1	0.7	0.3	-	0.1	0.1	tr	0.3	0.5	0.6	1.0	tr	tr	tr	tr	tr
α-Farnesene	1772	B	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Caryophyllene epoxide	2022	C	tr	0.1	tr	tr	tr	tr	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(E)-Nerolidol	2055	C	-	-	-	-	-	-	-	-	-	-	-	0.3	tr	0.1	tr	-	-	-	-	-
Terpene derived compounds																						
4-Oxoisophorone	1721	A	2.7	1.1	0.5	0.2	tr	0.3	-	1.4	-	-	tr	tr	tr	0.1	tr	-	-	-	-	-
Nitrogen-containing compounds																						
Indole	2457	A	0.1	3.3	0.9	0.8	0.5	0.1	tr	tr	tr	0.1	tr	0.1	0.1	1.0	0.1	-	-	-	-	-
Miscellaneous cyclic compounds																						
2-Ethylfuran	1062	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.8	0.8	1.1	2.2	1.4
2-Methylcyclopent-2-en-1-one	1391	A	-	-	-	-	-	-	2.8	6.1	0.5	-	0.5	0.1	0.2	0.1	tr	0.9	tr	1.4	0.2	0.6
5-Ethyl-2(5H)-furanone	1792	A	-	-	-	-	-	-	tr	0.4	0.1	0.1	tr	-	-	-	-	0.2	0.5	tr	0.2	0.1
Unknowns [d]																						
Sesquiterpenes																						
m/z: 204*,105,161,57,119,91,94,93,120	1508		-	-	-	-	-	-	tr	-	-	0.1	-	-	-	-	-	-	-	-	-	-
m/z: 204*,93,69,41,91,133,55,79	1543		-	-	-	-	-	-	-	2.4	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 204*,120,161,105,93,91,79	1601		-	-	-	-	-	-	-	-	-	-	tr	tr	0.1	0.1	0.1	-	-	-	-	-
m/z: 204*,93,69,41,105,79,94,91	1754		-	-	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-	tr	-	-	-
m/z: 204*,121,93,41,107	1763		0.6	tr	0.4	0.1	tr	0.2	0.8	-	0.1	-	0.1	0.1	0.5	0.4	0.8	0.1	0.1	0.1	-	-
m/z: 204*, 55, 119, 161, 83	1784		-	-	-	-	-	-	-	-	-	-	-	-	tr	tr	tr	-	-	-	-	-
m/z: 204*,161,119,105,134,91,41,55	1787		0.2	tr	tr	tr	tr	0.1	0.2	-	0.1	tr	-	-	-	-	-	-	-	-	-	-
m/z: 204*,121,93,105,107,41,67,79,81,91	1862		-	-	-	-	-	-	-	-	-	-	-	-	tr	0.1	0.1	-	-	-	-	-
Unknown compound class																						
m/z: 83,55,69,41,39,42,70,56	1230		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.3	0.8	-	0.7	0.3

m/z: 93,55,69,41,83,79,80,57	1235	-	-	-	-	-	-	-	4.8	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 67,82,43,55,81,83	1295	-	-	-	-	-	-	-	24.7	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 150*,69,41,81,79,82,53	1325	0.5	0.8	0.4	1.0	1.0	2.8	-	-	-	-	0.8	9.4	8.7	4.6	2.9	-	-	tr	0.4	0.2
m/z: 43,80,79,39,41,77,81	1448	0.7	0.7	0.4	0.6	0.9	0.3	0.3	-	0.3	0.6	0.1	0.2	0.1	tr	0.1	0.1	0.1	0.2	0.2	0.3
m/z: 134*,91,119,77,79,92,105	1451	-	-	-	-	-	-	-	-	-	-	0.1	0.2	0.2	0.3	0.1	-	-	-	-	-
m/z: 112*,55,83,97,57,56	1541	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	0.1	tr	0.1	0.1
m/z: 57,85,86,43,55,42,41*	1567	-	-	-	-	-	-	2.5	15.7	3.7	0.7	2.8	0.4	tr	tr	0.5	2.6	1.7	0.7	6.1	3.8
m/z: 112*,83,55,57,84	1619	-	-	-	-	-	-	0.5	2.1	0.6	0.6	0.5	0.2	-	-	0.3	0.2	3.5	0.2	1.7	0.6
m/z: 82,54,43,68,41,53,69	1765	-	-	-	-	-	-	-	0.3	-	tr	-	-	-	-	-	-	-	-	-	-
m/z: 41,69,98,83,55,39	1781	0.7	tr	tr	0.2	tr	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 57,97,81,41,85,55,39,43	1783	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	0.2	-	0.1	0.1
m/z: 55,100,54,39,57,82,56,53	1809	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-
m/z: 112*,55,84,41,56,69,97,44	1847	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	-	-	-
m/z: 57,85,39,41,43,55,31	1986	0.4	0.4	0.5	0.5	0.9	0.8	0.1	5.9	0.4	0.6	0.2	tr	tr	tr	0.1	0.5	7.5	tr	2.3	0.9
m/z: 112*,97,55,67,56,41,39	2111	-	-	-	-	-	-	0.1	1.8	0.1	0.1	-	-	-	-	-	0.1	0.1	0.1	0.3	0.2
m/z: 112*,97,55,67,56,57,41	2132	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	tr	tr	0.1	0.1
m/z: 112*,84,69,41,43,42,39,55,56	2142	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	tr	tr	tr	tr
m/z: 82,57,96,68,43,83,55	2558	-	-	-	-	-	-	-	-	-	-	tr	0.1	0.1	0.4	tr	-	-	-	-	-
m/z: 57,97,83,55,69,43,110,71,71,41,70,56	2734	-	-	-	-	-	-	-	-	-	-	tr	0.1	tr	0.3	tr	-	-	-	-	-
Aliphatics		72.6	72.4	80.0	87.5	88.0	73.2	54.1	24.4	59.9	63.4	63.3	30.7	40.6	15.7	42.0	89.7	79.6	91.5	78.5	89.4
Aromatics		1.1	2.3	7.4	1.9	1.7	0.6	0.5	0.2	0	0	7.0	12.3	4.5	13.5	2.1	0.1	0	0	0.2	0.2
Isoprenoids (including unknown sesquiterpenes)		23.9	20.0	10.3	7.3	6.8	22.2	39.1	13.7	34.3	33.6	24.3	36.2	45.5	64.0	51.6	3.1	4.9	4.5	6.5	1.7
Nitrogen-containing compounds		0.1	3.3	0.9	0.8	0.5	0.1	0	0	0	0.1	0	0.1	0.1	1.0	0.1	0	0	0	0	0
Miscellaneous cyclic compounds		0	0	0	0	0	0	2.8	6.6	0.6	0.1	0.5	0.1	0.2	0.1	0	2.9	1.3	2.5	2.6	2.1
Unknowns (excluding those identified to compound class)		2.2	1.9	1.3	2.3	2.8	3.8	3.5	55.2	5.1	2.5	4.6	10.6	9.1	5.5	4.0	4.2	14.1	1.3	12.1	6.4
Total of all compounds		100.0	99.9	99.9	99.8	99.8	99.9	100.0	100.0	100.0	99.9	99.8	89.9	99.9	99.9	99.7	99.9	99.8	99.8	99.9	99.8
Total number of compounds		33	36	34	35	35	34	27	19	24	25	36	36	35	37	39	39	44	37	41	40
Total volatiles (ng) emitted (per inflorescence per hour)		3503	17131	19864	21305	14873	20663	4136	1681	9758	22721	2919	2593	1744	1799	6445	49397	59573	25576	43144	46495

SM TABLE 6. Relative amounts (%) of compounds identified by GC-MS from headspace samples of *Xysmalobium* members of the *Hemipepsis*-wasp pollination guild. Compounds are listed in order of increasing Kovats retention index (KRI, calculated from our retention times using an Alltech EC-WAX column) within each compound class [a]. Footnotes are as for SM Table 4.

			Xysmalobium orbiculare						*Xysmalobium stockenstromense*			*Xysmalobium undulatum*		
Compound	KRI	Criteria[b]	1	2	3	4	5	6	1	2	3	1	2	3
Aliphatics														
Alcohols														
Hexan-1-ol	1364	C	-	-	-	-	-	-	0.1	0.3	2.2	1.1	0.2	0.6
(Z)-Hex-3-en-1-ol	1398	A	-	-	-	-	-	-	0.2	0.4	5.4	1.0	0.9	0.4
(E)-Hex-2-en-1-ol	1417	A	-	-	-	-	-	-	-	-	-	1.1	0.2	0.5
Hexadecan-1-ol	2392	A	0.1	0.3	0.4	tr	0.2	tr	-	-	-	-	-	-
Aldehydes														
(E)-Hex-2-enal	1240	A	-	-	-	-	-	-	-	-	6.4	-	-	-
(Z)-Hept-2-enal [c]	1345	A	-	-	-	-	-	-	-	-	-	0.5	tr	tr
(E)-Non-2-enal	1557	A	-	-	-	-	-	-	-	-	-	0.1	tr	tr
Esters														
(Z)-Hex-3-en-1-yl acetate [c]	1335	A	-	-	-	-	-	-	-	-	-	tr	8.7	tr
Ketones														
Mesityloxide	1169	A	-	-	-	-	-	-	1.0	-	-	-	-	-
Hydroxyacetone	1323	B	-	-	-	-	-	-	tr	3.8	-	-	-	-
Nonan-2-one	1409	A	-	-	-	-	-	-	-	-	-	0.1	tr	tr
Aromatics														
Benzaldehyde [c]	1546	C	-	-	-	-	-	-	16.3	9.4	12.7	-	-	-
Methylbenzoate	1651	C	-	-	-	-	-	-	-	-	-	0.2	tr	tr
Benzyl formate	1716	A	-	-	-	-	-	-	0.1	0.2	0.1	-	-	-
Benzyl acetate	1752	C	-	-	-	-	-	-	0.1	0.1	0.3	-	-	-
Methyl salicylate	1794	C	-	-	-	-	-	-	-	-	-	0.5	0.1	tr
1-Phenyl-1,2-propanedione	1843	B	-	-	-	-	-	-	0.4	0.3	0.4	-	-	-
Guaiacol	1885	A	-	-	-	-	-	-	-	-	-	0.1	tr	0.2
Benzyl alcohol [c]	1900	C	-	-	-	-	-	-	25.7	14.3	28.6	6.1	0.1	0.9
Phenylethyl alcohol	1940	A	-	-	-	-	-	-	2.8	1.0	2.1	0.2	0.1	0.1
(E)-Cinnamaldehyde	2067	A	-	-	-	-	-	-	1.4	-	-	-	-	-
Elemicin	2241	B	-	-	-	-	-	-	tr	0.1	tr	-	-	-
Cinnamic alcohol	2301	A	-	-	-	-	-	-	-	0.4	-	-	-	-
Isoprenoids														
Monoterpenes														
α-Pinene	1093	C	-	-	-	-	-	-	-	-	-	1.3	1.6	0.6
Myrcene	1202	A	1.4	49.2	29.2	52.9	27.1	41.6	17.6	9.7	4.0	29.5	53.2	35.8
Limonene	1229	C	97.0	17.9	50.5	38.0	36.8	21.4	4.3	2.4	1.9	17.0	17.6	21.8
(Z)-Ocimene	1257	C	0.4	1.7	6.2	1.5	3.9	4.7	0.3	7.1	-	-	-	-
(E)-Ocimene	1276	A	0.5	13.2	7.2	4.6	19.5	20.8	0.2	1.9	-	28.7	13.0	19.6
(Z)-Linalool oxide (furanoid)	1485	C	-	-	-	-	-	-	0.6	tr	0.1	0.1	0.1	0.1

Linalool	1557	C	0.1	1.3	0.3	0.3	0.8	0.9	23.7	0.3	0.1	1.1	0.4	1.7
Hotrienol	1625	A	-	-	-	-	-	-	2.7	tr	-	-	-	-
(Z)-Linalool oxide (pyranoid)	1782	A	-	-	-	-	-	-	tr	-	tr	-	-	-
2,6-Dimethylocta-3,7-diene-2,6-diol [c]	1957	A	-	-	-	-	-	-	0.1	-	-	-	-	-
Sesquiterpenes														
α-Cubebene	1479	A	-	0.3	0.2	0.1	0.5	0.6	-	-	-	-	-	-
α-Copaene	1517	A	-	1.4	1.2	0.3	1.5	2.5	0.1	0.5	tr	-	-	-
β-Bourbonene	1543	A	-	0.5	0.5	0.3	2.2	0.9	-	-	-	-	-	-
Caryophyllene	1623	C	0.2	1.9	0.8	0.3	2.0	1.8	tr	0.1	tr	0.5	0.2	1.8
(E)-β-Farnesene	1685	A	-	0.1	tr	tr	tr	tr	-	-	-	-	-	-
Humulene	1698	A	-	tr	tr	0.1	0.1	0.1	-	-	-	0.1	tr	0.3
Germacrene D	1739	A	-	4.8	1.1	0.2	1.6	0.4	0.2	0.9	-	-	-	-
α-Muurolene	1753	A	-	0.3	0.1	tr	0.2	0.1	-	-	-	-	-	-
α-Farnesene	1772	B	-	-	-	-	-	-	-	-	-	0.4	tr	tr
Terpene derived compounds														
4-Oxoisophorone	1721	A	-	-	-	-	-	-	0.1	3.2	0.7	-	-	-
Nitrogen-containing compounds														
2-Phenylacetonitrile	1955	C	0.2	0.4	0.1	tr	0.1	1.1	-	-	-	-	-	-
Miscellaneous cyclic compounds														
2-Furanmethanol	1679	A	-	-	-	-	-	-	-	2.3	-	-	-	-
δ-Octalactone	1995	A	-	-	-	-	-	-	0.1	0.1	0.1	-	-	-
δ-Decalactone	2219	A	-	-	-	-	-	-	0.5	3.3	0.3	-	-	-
5-Hydrxoymethylfurfural	2500	B	tr	2.8	0.3	-	0.1	-	0.4	0.6	-	-	-	-
Unknowns [d]														
Sesquiterpenes														
m/z: 204*,161,105,82,91,119,81,93	1621		-	0.2	0.1	0.1	0.3	0.3	-	-	-	tr	tr	tr
m/z: 204*,108,91,93,105,107,119,133,79	1646		-	0.1	0.1	tr	0.1	0.1	-	-	-	-	-	-
m/z: 204*,91,161,105,92,120,119	1671		-	0.1	tr	tr	0.1	0.1	-	-	-	-	-	-
m/z: 204*,161,105,93,91,119,79,77,81,92,133	1718		-	0.2	0.2	tr	0.3	0.2	-	-	-	-	-	-
m/z: 204*,93,119,69,91,79,161,105,107	1748		-	0.2	0.1	tr	0.2	0.1	-	-	-	-	-	-
m/z: 204*,121,93,41,107	1763		-	2.2	0.6	0.3	0.7	0.5	-	-	-	-	-	-
m/z: 204*,161,119,105,134,91,41,55	1787		-	0.4	0.2	0.1	0.5	0.5	0.1	-	-	-	-	-
Unknown compound class														
m/z: 136*,41,44,57,93,121,91,56,45	1306		-	-	-	0.3	0.6	tr	-	-	-	-	-	-
m/z: 150*,69,41,81,79,82,53	1325		-	-	-	-	-	-	-	-	-	9.4	2.8	15.2
m/z: 86,41,123,73,43,91,67,45,55	1378		-	-	-	-	-	-	-	-	-	0.6	0.4	0.2
m/z: 134*,91,119,77,79,92,105	1451		-	0.1	0.3	0.1	0.3	0.3	0.5	0.1	tr	-	-	-
m/z: 69,41,39,79,57,53,81,93	1565		-	tr	0.1	0.2	tr	0.3	0.1	0.2	tr	-	-	-
m/z: 55,56,41,69,70,84,43,42	1575		0.1	0.2	-	0.1	0.1	0.2	-	-	-	-	-	-
m/z: 96*,68,42,54,39,40	1607		-	-	-	-	-	-	tr	0.4	tr	-	-	-
m/z: 112*,83,55,57,84	1619		-	-	-	-	-	-	-	-	-	0.1	0.2	tr
m/z: 168*,56,85,125,43,41,69,153,83	1697		-	-	-	-	-	-	-	33.7	34.2	-	-	-
m/z: 108,79,80,43,77,39	1699		-	0.3	0.1	0.1	0.1	0.4	-	-	-	-	-	-
m/z: 55,84,54,39,38,37	1785		-	-	-	-	-	-	tr	0.2	tr	-	-	-
m/z: 98,55,42,69,41,39,43,70	1791		-	-	-	-	-	-	tr	0.8	-	-	-	-
m/z: 152*,43,109,81,79,67,91,55	1841		-	-	-	-	-	-	-	-	-	0.2	0.1	0.1

m/z: 132*,103,78,77,51,104,50	1923	-	-	-	-	-	-	-	-	0.5	-	-	-	-	-	-	-	-	-
m/z: 43,144,101,44,73,72,55	2282	-	-	-	-	-	-	-	-	1.3	-	-	-	-	-	-	-	-	-
m/z: 102*,44,43,74,42,57	2593	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aliphatics		0.1	0.3	0.4	0	0.2	0	1.2	4.5	14.0	3.9	10.0	1.4						
Aromatics		0	0	0	0	0	0	46.8	25.8	44.3	7.1	0.3	1.2						
Isoprenoids (including unknown sesquiterpenes)		99.6	95.9	98.6	99.0	98.5	97.7	49.9	26.1	6.9	78.6	86.2	81.6						
Nitrogen-containing compounds		0.2	0.4	0.1	0	0.1	1.1	0	0	0	0	0	0						
Miscellaneous cyclic compounds		0	2.8	0.3	0	0.1	0	1.0	6.3	0.4	0	0	0						
Unknowns (excluding those identified to compound class)		0.1	0.5	0.5	0.8	1.1	1.1	0.8	37.2	34.2	10.4	3.4	15.6						
Total of all compounds		100.0	99.9	99.9	99.8	100.0	100.0	99.8	99.9	99.8	100.0	99.8	99.8						
Total number of compounds		10	27	26	27	28	27	35	34	25	26	26	26						
Total volatiles (ng) emitted (per inflorescence per hour)		4711	15227	3278	32027	14682	15077	20997	12117	17695	12142	27953	24773						

SM TABLE 7. Relative amounts (%) of compounds identified by GC-MS from headspace samples of four *Asclepias* species used for congeneric comparisons with members of the *Hemipepsis*-wasp pollination guild. Compounds are listed in order of increasing Kovats retention index (KRI, calculated from our retention times using an Alltech EC-WAX column) within each compound class [a]. Footnotes are as for SM Table 4.

			Asclepias cucullata			*Asclepias crispa* var *plana*			*Asclepias dregeana*					*Asclepias gibba*	
Compound	KRI	Criteria[b]	1	2	3	1	2	3	1	2	3	4	5	1	2
Aliphatics															
Acids															
Isovaleric acid	1690	A	-	-	-	tr	0.2	tr	-	-	-	-	-	-	-
Alcohols															
(Z)-Hex-3-en-1-ol	1398	A	16.3	30.5	20.6	1.3	1.0	0.2	5.3	6.0	2.1	4.5	9.5	1.5	8.0
(E)-Hex-2-en-1-ol	1417	A	2.1	2.6	4.4	-	-	-	-	-	-	-	-	-	-
Aldehydes															
(E)-Hex-2-enal	1240	A	5.5	2.2	7.8	0.9	0.3	tr	-	-	-	-	-	0.3	0.1
Esters															
Hexyl acetate	1292	C	-	0.9	5.2	-	-	-	0.1	0.5	0.4	0.2	0.4	-	-
(Z)-Hex-3-en-1-yl acetate [c]	1335	A	12.3	46.4	38.7	10.9	14.0	2.6	65.6	75.4	78.2	81.3	66.5	5.3	6.7
(Z)-Hex-en-1-yl butyrate	1479	A	-	-	-	-	-	-	1.3	1.6	0.2	1.1	4.9	-	-
Aromatics															
Benzaldehyde [c]	1546	C	-	-	-	43.4	34.6	59.5	-	-	-	-	-	6.0	18.0
Methylbenzoate	1651	C	-	-	-	-	-	-	-	-	-	-	-	0.7	1.3
Phenylacetaldehyde [c]	1668	C	-	-	-	0.6	0.3	12.1	0.3	tr	0.1	0.1	0.1	-	-
Benzyl acetate	1752	C	-	-	-	0.1	0.1	0.1	-	-	-	-	-	-	-
1,4-Dimthoxybenzene	1767	A	-	-	-	-	-	-	-	-	-	-	-	tr	0.2
Methyl phenylacetate	1788	A	-	-	-	-	-	-	-	-	-	-	-	tr	0.1
Methyl salicylate	1794	C	-	-	-	-	-	-	0.1	0.1	0.5	tr	0.2	0.1	0.1
1-Phenylbutan-2-one	1832	B	-	-	-	tr	0.1	tr	-	-	-	-	-	-	-
Phenylethyl acetate [c]	1841	A	-	-	-	-	-	-	0.4	0.1	0.2	0.1	0.1	-	-
1-Phenyl-1,2-propanedione	1843	B	-	-	-	1.5	0.9	0.8	-	-	-	-	-	-	-
Benzyl alcohol [c]	1900	C	0.2	0.1	0.3	1.1	3.0	1.8	-	-	-	-	-	0.3	0.6
Homoanisic acid	1935	B	-	-	-	-	-	-	0.1	0.1	0.1	0.1	0.2	-	-
Phenylethyl alcohol	1940	A	tr	0.1	0.2	1.2	5.4	5.3	2.9	5.5	8.9	0.8	1.3	-	-
Eugenol	2189	A	-	-	-	-	-	-	0.8	-	0.3	-	0.1	-	-
4-Methoxy-2-phenylethanol	2349	B	-	-	-	-	-	-	8.0	4.5	3.8	0.4	6.4	-	-
Isoprenoids															
Monoterpenes															
α-Pinene	1093	C	-	-	-	-	-	-	-	-	-	-	-	4.7	-
Myrcene	1202	A	-	-	-	0.2	0.2	0.7	1.7	4.3	1.9	2.5	2.2	0.4	-
Limonene	1229	C	-	-	-	tr	0.1	1.0	0.3	-	0.7	0.1	0.3	-	-
(Z)-Ocimene	1257	C	-	-	-	0.5	0.4	tr	0.6	-	-	-	-	-	0.3
(E)-Ocimene	1276	A	-	-	-	4.2	3.4	1.3	8.9	0.6	1.7	4.9	4.8	tr	1.5
(Z)-Linalool oxide (furanoid)	1485	C	-	-	-	2.4	1.2	0.7	-	-	-	-	-	-	-
Linalool	1557	C	0.1	tr	tr	9.2	3.6	2.1	0.3	0.1	0.1	0.1	0.3	0.1	0.2

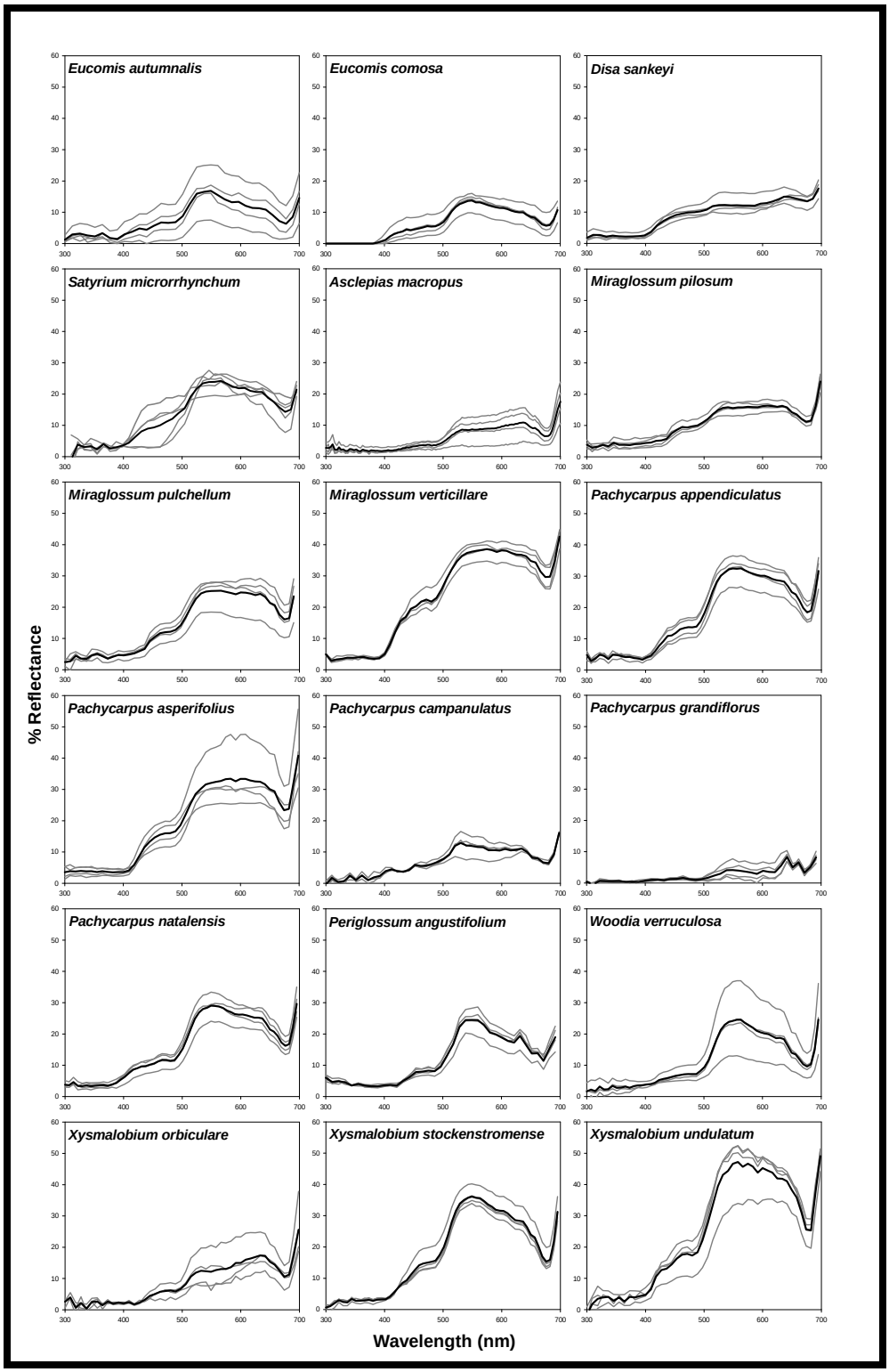
Hotrienol	1625	A	-	-	-	0.6	0.3	0.1	-	-	-	-	-	-	-
(Z)-Linalool oxide (pyranoid)	1782	A	-	-	-	5.7	2.4	0.8	-	-	-	-	-	-	-
Sesquiterpenes															
Caryophyllene	1623	C	-	-	-	-	-	-	0.7	tr	0.1	0.1	tr	0.1	3.2
Humulene	1698	A	-	-	-	-	-	-	0.1	-	-	-	-	-	0.2
Germacrene D	1739	A	-	-	-	-	-	-	tr	-	tr	-	0.6	-	0.3
Caryophyllene epoxide	2022	C	-	-	-	-	-	-	tr	-	-	-	-	-	-
Terpene derived compounds															
6-Methyl-5-hepten-2-one [c]	1357	A	-	-	-	-	-	-	-	-	-	-	-	53.2	43.4
6-Methyl-5-hepten-2-ol	1478	B	-	-	-	-	-	-	-	-	-	-	-	1.1	1.2
β-Cyclocitral	1648	A	-	-	-	-	-	-	1.3	-	-	0.4	0.2	-	-
4-Oxoisophorone	1721	A	1.4	0.9	0.7	0.2	0.2	0.3	-	-	-	-	-	-	0.3
(Z)-Geranyl acetone	1854	B	-	-	-	-	-	-	-	-	-	-	-	1.0	0.5
Geranyl acetone	1881	B	-	-	-	-	-	-	tr	tr	tr	tr	tr	13.5	7.4
6,10,14-Trimethylpentadecan-2-one	2147	B	-	-	-	-	-	-	-	-	-	-	-	0.1	0.5
Miscellaneous cyclic compounds															
2-Methylcyclopent-2-en-1-one	1391	A	39.1	0.2	-	-	-	-	0.1	0.1	-	2.1	0.5	1.6	3.2
5-Ethyl-2(5H)-furanone	1792	A	0.5	0.4	0.3	-	-	-	-	-	-	-	-	0.2	-
Unknowns															
Sesquiterpenes															
m/z: 204*,120,161,105,93,91,79	1601		-	-	-	-	-	-	tr	-	-	-	-	-	-
Unknown compound class															
m/z: 84,55,83,41,39,91	1227		-	-	-	-	-	-	-	-	-	-	-	1.1	0.3
m/z: 83,55,69,41,39,42,70,56	1230		-	0.3	0.4	-	-	-	-	-	-	-	-	-	-
m/z: 150*,69,41,81,79,107,119,79,82	1298		-	-	-	0.4	0.2	tr	-	-	-	-	-	-	-
m/z: 150*,69,41,81,79,82,53	1325		-	-	-	3.0	6.8	1.7	-	-	-	-	-	-	-
m/z: 43,80,79,39,41,77,81	1448		0.3	1.3	2.4	-	-	-	-	-	-	-	-	-	-
m/z: 57,85,45,41,43,74	1465		-	-	-	1.7	0.8	0.3	-	-	-	-	-	-	-
m/z: 112*,55,83,97,57,56	1541		0.3	0.4	0.3	-	-	-	-	-	-	-	-	-	-
m/z: 57,85,86,43,55,42,41*	1567		17.7	7.4	5.0	-	-	-	0.3	0.8	0.4	1.1	1.2	6.0	1.5
m/z: 112*,83,55,57,84	1619		2.2	3.0	7.9	1.0	0.3	0.1	0.3	0.1	0.1	tr	0.1	1.7	0.6
m/z: 152*,43,109,81,79,67,91,55	1841		-	-	-	-	-	-	tr	0.1	0.1	tr	0.1	-	-
m/z: 57,85,39,41,43,55,31	1986		0.2	2.6	5.5	0.6	0.2	0.1	0.1	0.1	0.2	tr	tr	0.4	tr
m/z: 162*,91,43,119,65,92	2022		-	-	-	5.7	16.9	6.0	-	-	-	-	-	-	-
m/z: 112*,97,55,67,56,41,39	2111		1.2	0.5	0.3	-	-	-	-	-	-	-	-	0.5	0.2
m/z: 112*,97,55,67,56,57,41	2132		0.4	0.3	0.1	-	-	-	-	-	-	-	-	0.2	0.1
m/z: 43,146,91,131,65,103	2235		-	-	-	0.1	0.4	tr	-	-	-	-	-	-	-
m/z: 91,43,115,87,65,92	2276		-	-	-	0.1	0.3	tr	-	-	-	-	-	-	-
m/z: 91,103,121,146,77,65,43,79	2287		-	-	-	0.4	1.4	0.9	-	-	-	-	-	-	-
m/z: 82,57,96,68,43,83,55	2558		-	-	-	0.2	0.1	0.1	-	-	-	-	-	-	-
m/z: 57,97,83,55,69,43,110,71,71,41,70,56	2734		-	-	-	0.5	0.3	0.4	-	-	-	-	-	-	-
m/z: 97,57,83,69,71,55,111,85	2879		-	-	-	0.3	0.1	0.1	-	-	-	-	-	-	-
m/z: 97,83,69,57,71,111,43	3059		-	-	-	1.3	0.5	0.3	-	-	-	-	-	-	-
m/z: 97,57,83,69,71,55,111,43,85	3605		-	-	-	0.8	0.3	0.2	-	-	-	-	-	-	-
Aliphatics			36.3	82.6	76.7	13.0	15.5	2.8	72.4	83.4	80.9	87.1	81.4	7.1	14.9
Aromatics			0.2	0.2	0.4	47.9	44.3	79.7	12.7	10.3	13.9	1.4	8.3	7.1	20.4

Isoprenoids (including unknown sesquiterpenes)	1.5	0.9	0.7	23.0	11.7	7.1	14.0	5.0	4.5	8.1	8.4	74.2	58.9
Nitrogen-containing compounds	0	0	0	0	0	0	0	0	0	0	0	0	0
Miscellaneous cyclic compounds	39.6	0.6	0.3	0	0	0	0.1	0.1	0	2.1	0.5	1.7	3.2
Unknowns (excluding those identified to compound class)	22.3	15.7	21.9	15.9	28.5	10.2	0.7	1.2	0.7	1.1	1.4	9.9	2.6
Total of all compounds	100.0	100.0	100.0	99.9	100.0	99.8	99.8	99.9	100.0	99.9	100.0	100.0	100.0
Total number of compounds	17	19	18	34	34	34	28	20	22	22	24	27	28
Total volatiles (ng) emitted (per inflorescence per hour)	799	12150	14667	13899	11100	27112	23050	5254	2251	1322	1100	3520	3712

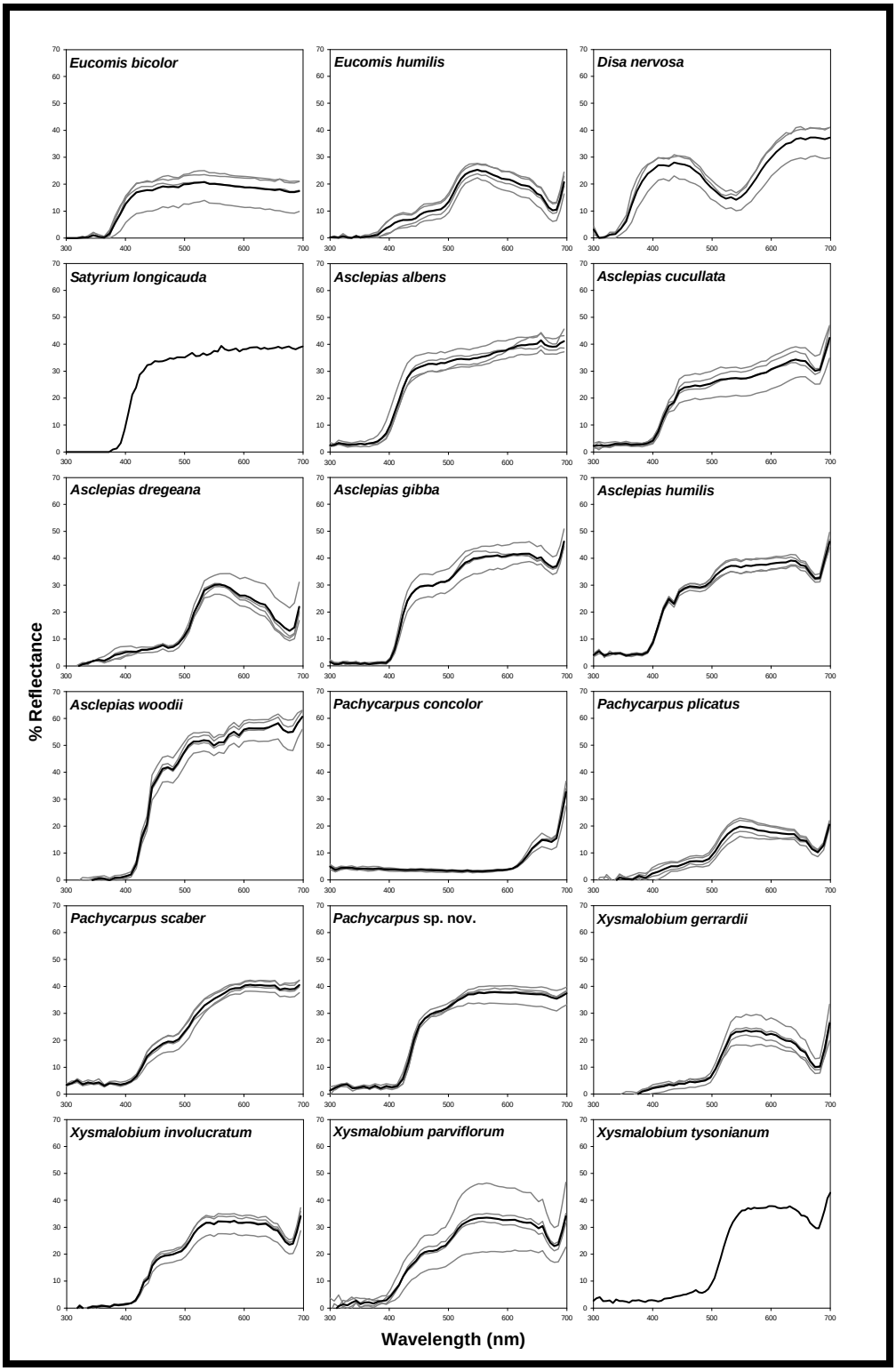
SM TABLE 8. Relative amounts (%) of compounds identified by GC-MS from headspace samples of one *Pachycarpus* and two *Xysmalobium* species used for congeneric comparisons with members of the *Hemipepsis*-wasp pollination guild. Compounds are listed in order of increasing Kovats retention index (KRI, calculated from our retention times using an Alltech EC-WAX column) within each compound class [a]. Footnotes are as for SM Table 4.

			Pachycarpus coronarius			*Xysmalobium gerrardii*					*Xysamlobium parviflorum*		
Compound	KRI	Criteria[b]	1	2	3	1	2	3	4	5	1	2	3
Aliphatics													
Alcohols													
Hexan-1-ol	1364	C	-	-	-	0.3	0.1	tr	0.1	0.1	-	-	-
(Z)-Hex-3-en-1-ol	1398	A	1.8	0.5	1.3	21.7	3.8	3.5	1.2	6.9	1.6	0.1	0.3
Aldehydes													
(Z)-Hex-3-en-1-yl formate	1288	A	-	-	-	0.1	-	-	-	0.1	-	-	-
Esters													
Hexyl acetate	1292	C	0.7	2.7	1.8	-	-	-	-	-	-	-	-
(Z)-Hex-3-en-1-yl acetate [c]	1335	A	79.6	89.5	86.8	14.4	6.1	8.4	5.5	24.4	5.5	0.4	0.3
(Z)-Hex-en-1-yl butyrate	1479	A	0.5	0.1	0.9	-	-	-	-	-	-	-	-
Aromatics													
Anisole [c]	1362	C	-	-	-	-	-	0.9	-	-	-	-	-
1-Methoxy-4-methylbenzene	1459	A	-	-	-	-	-	-	-	-	0.2	0.1	0.1
Benzaldehyde [c]	1546	C	-	-	-	20.3	7.3	25.3	32.5	29.2	17.3	13.2	9.6
Phenylacetaldehyde [c]	1668	C	-	-	-	10.8	33.3	25.1	14.4	2.2	-	-	-
2-Hydroxybenzaldehyde	1706	A	-	-	-	-	-	-	-	-	1.5	0.5	0.4
Benzyl acetate	1752	C	tr	tr	tr	-	-	-	-	-	-	-	-
2-Phenylethyl formate	1810	A	-	-	-	0.3	0.2	0.4	0.8	0.8	-	-	-
Phenylethyl acetate [c]	1841	A	-	-	-	0.2	0.4	0.3	0.3	0.2	-	-	-
Benzyl alcohol [c]	1900	C	-	-	-	0.4	0.5	1.0	tr	0.5	1.6	1.7	2.8
Phenylethyl alcohol	1940	A	0.4	0.1	tr	13.0	25.0	19.5	30.0	13.2	0.2	0.2	0.4
2-Methoxy-4-methyl-1-hydroxybenzene	1978	A	-	-	-	-	-	-	-	-	1.9	1.0	0.3
(E)-Cinnamaldehyde	2067	A	-	-	-	-	-	-	-	-	-	tr	-
3-Phenylpropanol	2073	A	-	-	-	-	-	-	-	-	-	0.1	-
p-Cresol	2102	A	-	-	-	-	-	-	-	-	28.6	11.3	13.6
(Z)-Hex-3-en-1-yl benzoate	2154	C	tr	0.1	tr	-	-	-	-	-	-	-	-
Elemicin	2241	B	-	-	-	0.1	0.1	0.1	0.1	0.1	-	-	-
Cinnamic alcohol	2301	A	-	-	-	-	-	-	-	-	-	tr	-
Isoprenoids													
Monoterpenes													
α-Pinene	1093	C	-	-	-	3.7	0.3	3.2	0.4	1.2	6.7	4.9	36.1
Myrcene	1202	A	-	-	-	0.5	0.8	tr	0.5	1.6	7.3	31.1	10.7
Limonene	1229	C	-	-	-	3.5	3.1	1.3	1.9	7.6	7.6	14.6	8.7
(Z)-Ocimene	1257	C	0.3	0.1	0.2	0.1	0.1	0.1	0.5	1.1	4.7	7.0	5.5
(E)-Ocimene	1276	A	2.5	0.6	3.6	0.9	3.2	0.7	1.5	3.7	13.0	8.8	9.7
Linalool	1557	C	0.3	0.8	1.5	0.1	0.1	0.1	tr	0.2	0.2	0.2	0.4
Sesquiterpenes													

α-Cubebene	1479	A	-	-	-	-	-	-	-	-	0.1	0.1	-
α-Copaene	1517	A	-	-	-	-	-	-	-	-	0.7	0.2	-
β-Bourbonene	1543	A	-	-	-	-	-	-	-	-	tr	0.1	tr
Caryophyllene	1623	C	0.3	1.6	1.3	0.1	0.3	0.1	tr	0.1	0.1	0.2	tr
(E)-β-Farnesene	1685	A	-	-	0.3	-	-	-	-	-	-	tr	-
Humulene	1698	A	tr	0.1	0.1	-	-	-	-	-	tr	0.1	-
Germacrene D	1739	A	tr	0.1	tr	0.2	0.4	0.1	tr	0.2	0.2	2.2	0.2
Terpene derived compounds													
4-Oxoisophorone	1721	A	-	-	-	4.7	10.7	7.2	7.3	4.0	-	-	-
Nitrogen-containing compounds													
Pyrrole	1536	A	-	-	-	-	-	-	-	-	0.1	0.1	0.7
Formanilide	2188	C	-	-	-	tr	tr	tr	tr	0.2	-	-	-
Indole	2457	A	tr	0.4	tr	-	-	-	-	-	-	-	-
Miscellaneous cyclic compounds													
2-Ethylfuran	1062	A	3.5	0.4	0.1	1.1	0.1	tr	tr	0.2	-	-	-
2-Methylcyclopent-2-en-1-one	1391	A	0.7	0.1	0.1	0.7	tr	tr	tr	0.1	-	-	-
δ-Decalactone	2219	A	-	-	-	-	-	0.1	-	-	-	-	-
Unknowns[d]													
Sesquiterpenes													
m/z: 204*,120,161,105,93,91,79	1601		-	-	-	-	-	-	-	-	tr	0.1	tr
m/z: 93,119,41,69,43,55,91,71,67,79,107	1608		-	-	0.2	-	-	-	-	-	-	-	-
m/z: 204*,161,105,93,91,119,79,77,81,92,133	1718		-	-	-	-	-	-	-	-	0.1	-	-
m/z: 204*,161,105,55,189,119,85,83,95,91,133	1734		-	-	-	-	-	-	-	-	0.1	-	-
m/z: 204*,93,69,41,105,79,94,91	1754		-	-	0.1	-	-	-	-	-	0.2	0.2	-
m/z: 204*,121,93,41,107	1763		-	-	-	tr	0.1	tr	tr	0.1	0.3	1.5	0.3
m/z: 204*,122,161,107,105,93,81,91	1798		-	-	-	-	-	-	-	-	0.2	tr	-
Unknown compound class													
m/z: 150*,69,41,81,79,107,119,79,82	1298		-	-	-	-	0.1	0.1	0.3	0.1	-	-	-
m/z: 136*,41,44,57,93,121,91,56,45	1306		-	-	-	-	0.2	tr	-	0.1	-	-	-
m/z: 150*,69,41,81,79,82,53	1325		-	-	-	1.2	2.7	1.6	2.3	1.1	-	-	-
m/z: 43,80,79,39,41,77,81	1448		0.2	0.9	0.4	-	-	-	-	-	-	-	-
m/z: 57,85,86,43,55,42,41*	1567		6.3	1.2	0.7	1.5	0.5	0.6	0.1	0.4	-	-	-
m/z: 112*,83,55,57,84	1619		1.8	0.5	0.4	-	-	-	-	-	-	-	-
m/z: 154*,56,42,41,69,70,139,120	1802		-	-	-	0.1	0.5	0.3	0.3	0.1	-	-	-
m/z: 57,85,39,41,43,55,31	1986		0.6	0.2	0.1	0.1	0.2	0.1	0.1	tr	-	-	-
m/z: 112*,97,55,67,56,41,39	2111		0.3	0.1	tr	-	-	-	-	-	-	-	-
m/z: 112*,97,55,67,56,57,41	2132		0.2	tr	tr	-	-	-	-	-	-	-	-
Aliphatics			82.7	92.8	90.7	36.6	10.1	11.9	6.8	31.6	7.1	0.5	0.5
Aromatics			0.4	0.2	0	44.9	66.7	72.7	78.0	46.2	51.3	28.1	27.2
Isoprenoids (including unknown sesquiterpenes)			3.4	3.2	7.3	13.8	19.0	12.7	11.9	19.9	41.5	71.2	71.5
Nitrogen-containing compounds			0	0.4	0	0	0	0	0	0.2	0.1	0.1	0.7
Miscellaneous cyclic compounds			4.2	0.5	0.2	1.8	0.1	0.1	0	0.3	0	0	0
Unknowns (excluding those identified to compound class)			9.4	2.9	1.6	2.9	4.2	2.6	3.0	1.8	0	0	0
Total of all compounds			100.0	99.9	99.8	100.0	99.9	100.0	99.8	100.0	100.0	99.9	100.0
Total number of compounds			22	22	25	28	29	31	28	30	28	30	21
Total volatiles (ng) emitted (per inflorescence per hour)			8508	10179	5346	4502	9813	3545	11012	5071	17380	15120	1840



SM FIGURE 1. Reflectance spectra for 18 members of the *Hemipepsis*-wasp pollination guild, measured from the exposed surface of the corolla. Bold curves represent the mean spectrum and light curves represent individual replicates.



SM FIGURE 2. Reflectance spectra for 18 species used in congeneric comparisons with members of the *Hemipepsis*-wasp pollination guild, measured from the exposed surface of the corolla. Bold curves represent the mean spectrum and light curves represent individual replicates.

CHAPTER 11

SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

In this thesis, I have established the existence of a guild of at least 21 grassland plant species (representing 10 genera and three families) that are specialized for pollination by *Hemipepsis* spider-hunting wasps (Chapters 2-8 & 10). Using this highly novel wasp-pollination system, I addressed questions about proximal mechanisms of floral specialization (Chapters 5-8), floral evolution in relation to pollinator shifts (Chapter 9), and pollination syndromes (Chapter 10). In this final section of the thesis, I examine some of the broader implications of the work and identify potential future studies that can build on aspects of this thesis to broaden our understanding of the ecology and evolution of specialized pollination systems.

PROXIMAL MECHANISMS OF SPECIALIZATION

Southern Africa is known for its large number of plants with highly specialized pollination systems (Johnson & Steiner 2000, 2003; Ollerton *et al*. 2006; Goldblatt & Manning 2006; Johnson 2010). Identifying the proximal mechanisms of specialization within particular guilds may help explain this trend. Levels of specialization in the *Hemipepsis*-wasp pollination guild are remarkably high. Of the 21 known guild members, 17 are pollinated exclusively by *Hemipepsis* wasps (median number of pollinator species = 2, range = 1–4; Chapter 10) while two species have bimodal pollination systems and are pollinated by *Hemipepsis* wasps and cetoniine beetles (Johnson *et al*. 2007; Chapter 3). Only two guild members, *Eucomis autumnalis* subsp. *clavata* and *E. comosa* var. *striata* have moderately generalized pollination systems (19 pollinator species in four functional groups and 35 pollinator species in five functional groups respectively), although *Hemipepsis* wasps carried four times as many pollen grains as other functional groups, moved more actively between plants and were consistent across sites and seasons suggesting that they are the primary pollinators of these species (Chapter 6). Thus, the *Hemipepsis*-wasp pollination system is characterized by levels of specialization that are consistent with other well-documented specialized pollination systems in southern Africa, such as those involving long-proboscid flies, oil-bees, monkey beetles, the mountain pride butterfly and small mammals (Johnson & Steiner 2000, 2003; Ollerton *et al*. 2006; Goldblatt & Manning 2006; Johnson 2010).

Floral specialization in the *Hemipepsis*-wasp pollination system is particularly intriguing in that the flowers of guild members are morphologically unspecialized with exposed nectar. This is in contrast to most specialized pollination systems in which specialization can be attributed to morphological adaptations (such as long spurs) or particular rewards (such as oils or particular fragrances) which can only be utilized by a subset of the pollinator community (Johnson & Steiner 2000, 2003). *Hemipepsis* wasps are short-tongued insects and plants pollinated by these wasps have no morphological adaptations that could prevent non-pollinating insects from accessing nectar. Despite this, most of the flowers pollinated by *Hemipepsis* wasps are visited by very few non-pollinating insects (Chapter 10). In the absence of typical morphological or reward-based floral filters,

the proximal basis for specialization in this guild appears to be biochemical filters (scent and nectar) and cryptic coloration (Chapters 5-8 & 10).

Floral scent

Floral scent has been suggested to contribute to specialization in systems where particular volatiles function as a "private channel" of communication between plants and specialist pollinators (Schiestl *et al*. 2003; Raguso 2008; Chen *et al*. 2009). In the specialized *Hemipepsis*-wasp pollination guild, the wasp pollinators appear to be attracted almost exclusively by scent cues (Chapters 6-8). This was empirically established for three guild members (*E. autumnalis*, *Pachycarpus grandiflorus* and *Xysmalobium orbiculare*; Chapters 6-8). In field experiments with these species, *Hemipepsis* wasps were consistently attracted to inflorescences which were concealed from view by being covered with leaves. In many instances, wasps would land on the leaves covering the inflorescences and then crawl inside and visit the flowers, confirming that wasps do not require a visual cue to find flowers. Foraging wasps also exhibit behaviour which suggests that they use scent as the primary cue to find host flowers (zig-zag flight typical of insects following an odour plume; Raguso 2006). The results of these field experiments were backed up by the attraction of wasps to flowers in laboratory Y-maze choice experiments (Chapters 6 & 7). The role of scent as the primary means of floral advertising is also suggested by the cryptic nature of many of the smaller asclepiad guild members such as *Aspidoglossum glanduliferum*, *Periglossum angustifolium* and the *Miraglossum* species. Indeed, Weale (1873) suggested that following foraging wasps was a good way to find some of the smaller asclepiads and this method has proven invaluable throughout this study.

Reliance on specific floral scent compounds to selectively attract pollinators is well established in mimicry systems (e.g. Schiestl *et al*. 1999, 2003; Schiestl 2005; Brodmann *et al*. 2008), but is poorly known in rewarding plants. However, a recent study by Brodmann *et al*. (2008) demonstrated the highly specific attraction of pollinating vespids by two orchids through the production of green-leaf volatiles normally associated with the wasps' herbivorous caterpillar prey. *Hemipepsis*-wasp pollinated flowers typically have complex scent profiles which are dominated by common monoterpenes and benzenoids but the particular compounds (or blends of compounds) responsible for the attraction of these wasps remain to be identified (Johnson 2005; Johnson *et al*. 2007; Chapter 6-8 & 10).

Identification of physiologically active compounds in this system requires coupled gas chromatography-electroantennographic detection (GC-EAD) experiments and behavioural assays. As a first step, I collaborated with Professor Florian Schiestl (University of Zürich) to conduct some preliminary GC-EAD experiments with *Hemipepsis* wasps between 2006 and 2008. These experiments were conducted in Professor Schiestl's lab in Zürich using *Hemipepsis* wasps (posted live from South Africa) and scent samples (both dichloromethane solvent extraction and headspace samples) collected by myself and Professor Schiestl. From these experiments, Professor Schiestl was

TABLE 1. Compounds that were found to elicit an electroantennographic response from *Hemipepsis* wasps and the frequency of occurrence of each compound in plant species pollinated by *Hemipepsis* wasps and congeneric plant species pollinated by other vectors. GC-EAD experiments were conducted by Professor Florian Schiestl (University of Zürich). Comparative data for headspace samples are based on the scent profiles of species (16 guild members and 16 congeners) used in Chapter 10. Comparative data for dichloromethane samples are based on samples from four guild members and two congeners.

Compound	Sample type for GC-EAD [a]	No. *Hemipepsis*-wasp pollinated plants that produce compound	No. non-wasp pollinated congeners that produce compound
Aliphatics			
Alkanes			
Heneicosane	Dichloromethane	4	2
Tricosane	Dichloromethane	4	2
Alkenes			
(Z)-9-Tricosene	Dichloromethane	4	2
(Z)-9-Pentacosene	Dichloromethane	4	2
Benzenoids			
p,α-Dimethyl styrene [b]	Headspace	1	0
3,5-Dimethoxytoluene	Headspace	5	3
Isoprenoids			
Monoterpenes			
α-Pinene	Headspace	5	6
Myrcene	Headspace	13	13
Limonene	Headspace	11	13
(Z)-Ocimene	Headspace	10	13
Linalool	Headspace	13	16
p-Mentha-2,8-dienol	Headspace	1	0
α-Terpineol	Headspace	4	3
(E)-Piperitol	Headspace	1	0
p-Mentha-1,8-dien-3-one	Headspace	1	0
2,6-Dimethylocta-3,7-diene-2,6-diol	Headspace	4	5
Sesquiterpenes			
Caryophyllene	Headspace	14	12
Miscellaneous cyclic compounds			
δ-Decalactone	Headspace	3	2

[a] Headspace samples were eluted with a 1:9 blend of hexane:acetone.

[b] This compound was likely a contaminant.

able to identify 18 floral volatile compounds which elicited a physiological response from wasp antennae (Table 1; Figs 1 & 2). Four of these active compounds were pheromone-type hydrocarbons with low volatility that were detected only in dichloromethane (DCM) solvent extractions (Table 1; Fig. 1). The remaining 14 active compounds, mainly monoterpenes, were identified from headspace

samples of *Eucomis autumnalis* and *Pachycarpus asperifolius* (Table 1; Fig. 2). However, preliminary bioassays with these compounds in the field and using a Y-maze in the laboratory failed to demonstrate attractiveness to the wasps (for the field trials, I used rubber septa, soaked overnight in a blend of hexane and active compounds, and attached with pins to the end of dowel rods placed in the ground; for the Y-maze experiments, I applied the hexane/active compound blend to filter paper placed at the entrance to the Y-maze).

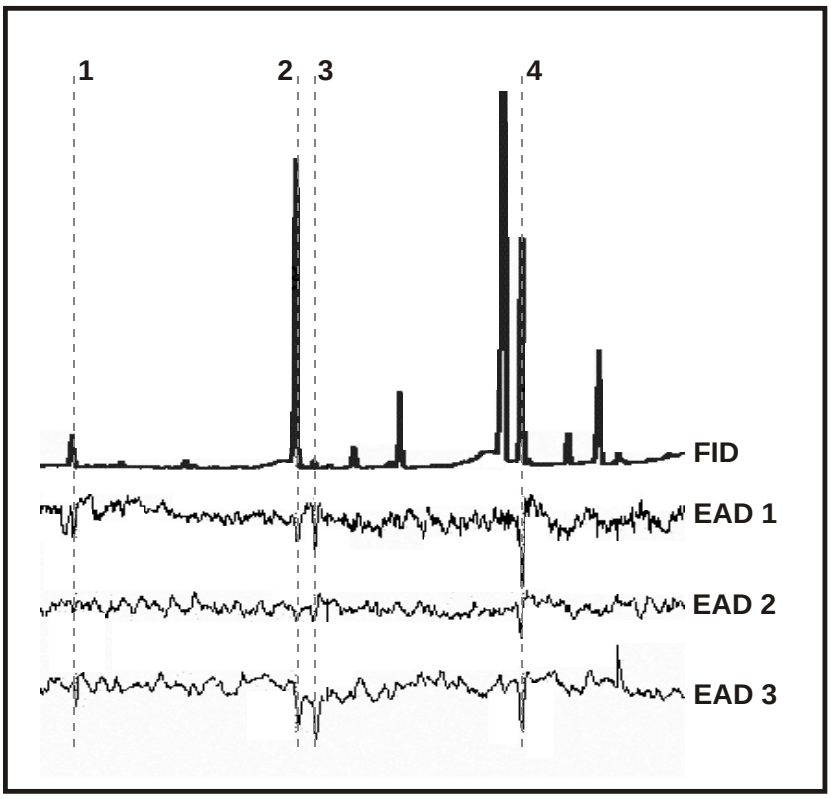


FIGURE 1. Coupled gas chromatography-electroantennographic detection (GC-EAD) responses of male *Hemipepsis capensis* antennae (from three individuals) to dichloromethane extraction samples from *Pachycarpus grandiflorus*. Active compounds: **1**, heneicosane; **2**, tricosane; **3**, (Z)-9-tricosene; **4**, (Z)-9-pentacosene.

The four pheromone-type hydrocarbons identified from the GC-EAD experiments with DCM samples (heneicosane, tricosane, (Z)-9-tricosene and (Z)-9-pentacosene; Table 1; Fig. 1) were subsequently also found in DCM samples from non-wasp-pollinated asclepiads and thus appear to be a general asclepiad signature that cannot by itself explain the specific attraction of spider-hunting wasps to some species (Fig. 3). The reason they elicit a response from wasp antennae is unclear, but dodecane present as a contaminant in some of the hexane solvent used in previous experiments also elicited a response, and it thus appears that these wasps may be sensitive to a range of simple aliphatic alkanes and alkenes. Indeed, blends of alkanes and alkenes characterize many hymenopteran

pheromones and are emitted by some sexually deceptive orchids that imitate certain female Hymenoptera (Schiestl *et al*. 1999). The remaining 14 active compounds are all common floral volatiles which are unlikely to function as specific *Hemipepsis* wasp attractants (Table 1; Knudsen *et al*. 2006). Furthermore, none of these 14 compounds are produced by all guild members and most of

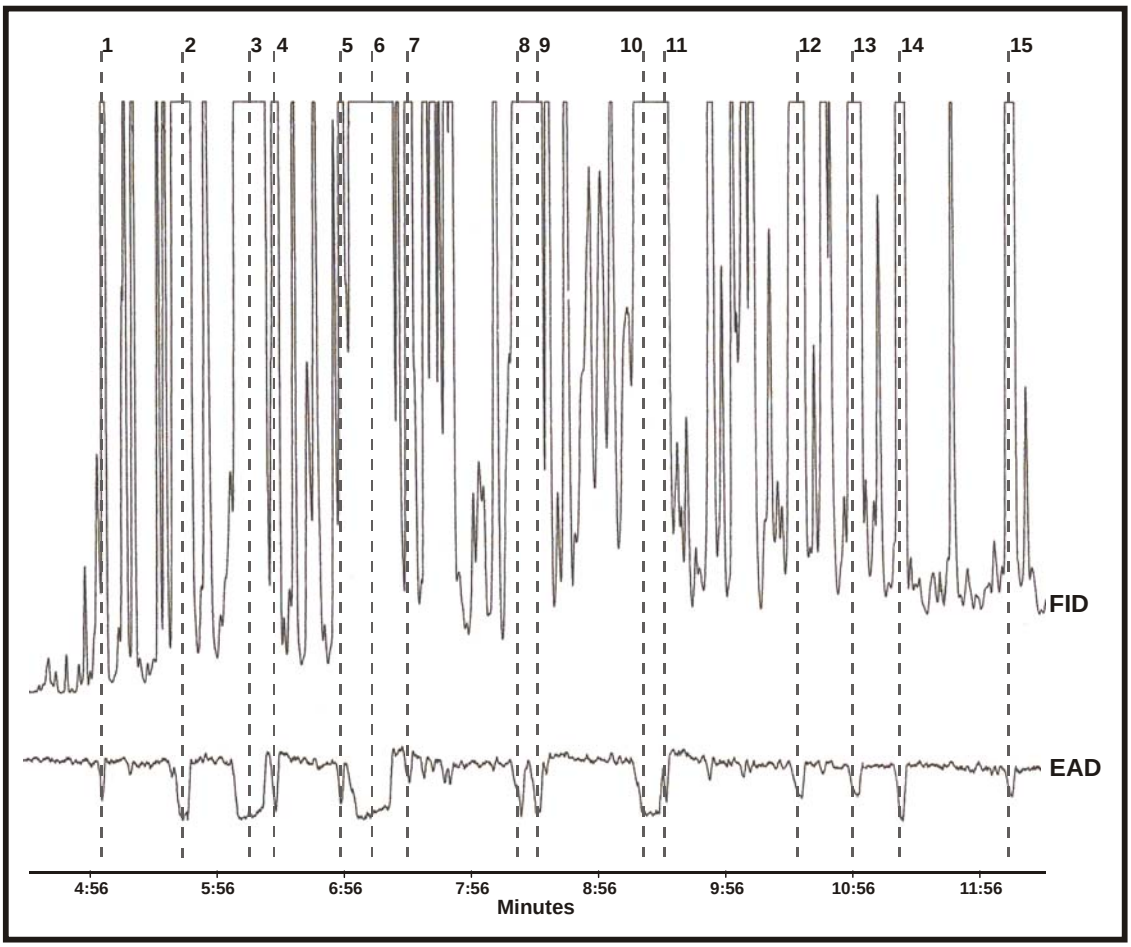


FIGURE 2. Coupled gas chromatography-electroantennographic detection (GC-EAD) response of a *Hemipepsis hilaris* antenna to headspace samples collected from *Eucomis autumnalis*. These responses were also obtained from an additional two wasps. Active compounds: **1**, α-pinene; **2**, myrcene; **3**, limonene; **4**, (Z)-ocimene; **5**, *p,α*-dimethyl styrene; **6**, linalool; **7**, *p*-mentha-2,8-dienol; **8**, *α*-terpineol; **9**, (E)-piperitol; **10**, 3,5-dimethoxytoluene; **11**, *p*-mentha-1,8-dien-3-one; **12**, 2,6-dimethylocta-3,7-diene-2,6-diol; **13**, unidentified compound; **14**, caryophyllene; **15**, δ-decalactone.

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them are produced equally by congeneric non-wasp pollinated flowers (Table 1). It is intriguing that several of the compounds which elicit a response in wasp antennae (such as 3,5-dimethoxytoluene, limonene, myrcene, linalool and (Z)-ocimene) are also produced in large amounts in the scents of particular individual guild members. However, it is difficult to understand how they could mediate a specific attraction when they are also produced by non-wasp-pollinated flowers and are not

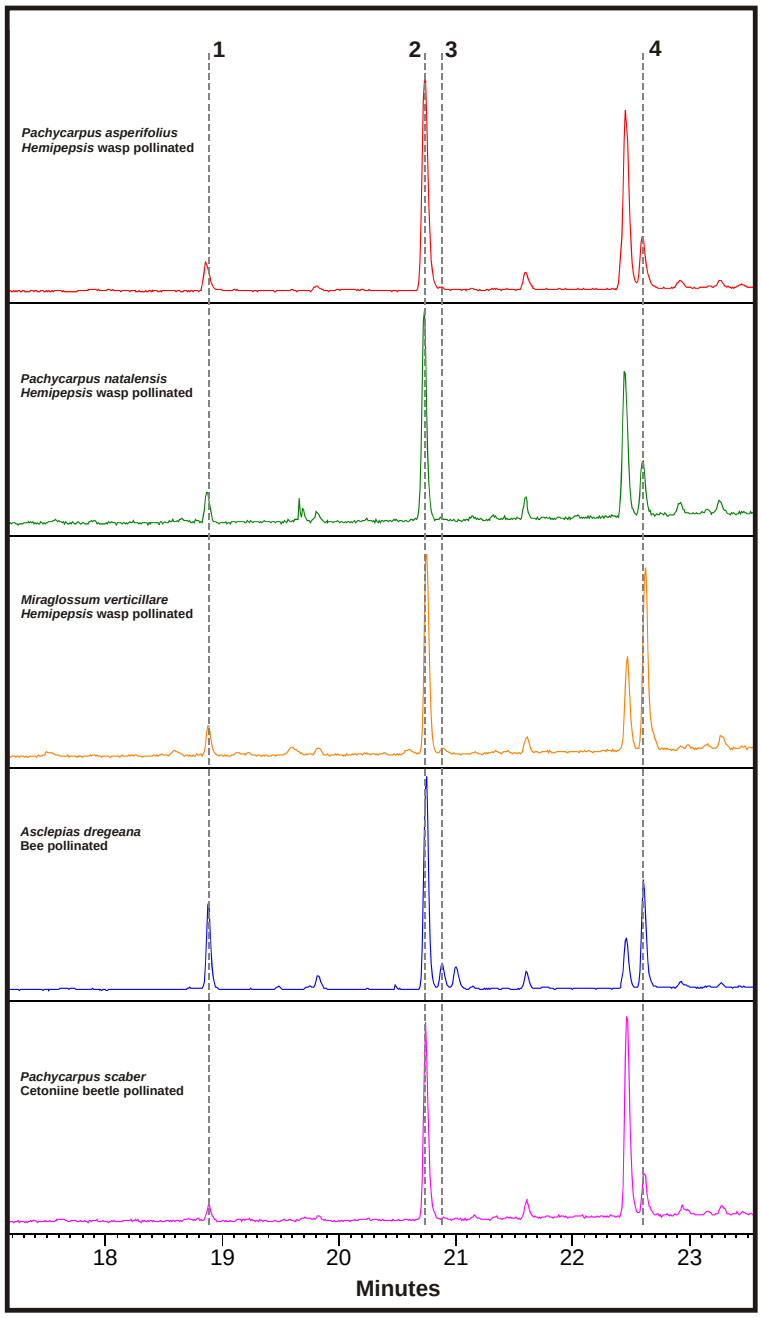

FIGURE 3. Chromatograms of dichloromethane (DCM) extraction solvent samples from three *Hemipepsis*-wasp pollinated species and two congeneric non-wasp-pollinated species. The four active compounds identified from GC-EAD experiments with male *H. capensis* wasps and DCM samples from *Pachycarpus grandiflorus* are labeled. **1**, heneicosane; **2**, tricosane; **3**, (Z)-9-tricosene; **4**, (Z)-9-pentacosene.

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universally produced within the guild. The particular scent-based mechanism for the attraction of these wasps thus remains unclear (see discussion in Chapter 10). I plan to conduct future studies which will include additional GC-EAD experiments and behavioural assays with samples from a

wider range of guild members, particularly some of the small, inconspicuous asclepiads (for example *A. glanduliferum* and the *Miraglossum* species) which are visited exclusively by *Hemipepsis* wasps.

Cryptic floral coloration

Floral colours can contribute to specialization if particular colours are attractive to specific pollinators (Grant 1966; Johnson & Bond 1994; Castellanos *et al*. 2004; Wilson *et al*. 2006). Alternatively, floral colours can contribute to specialization by serving a cryptic function in systems which do not rely on colour cues for floral advertising. From a human perspective, flowers pollinated by *Hemipepsis* wasps are usually inconspicuous and do not stand out from the background. Reflectance spectra for guild members are typically dull and impure, and similar to the spectra of green leaves (Ollerton *et al*. 2003; Johnson *et al*. 2007; Chapters 6-8 & 10). When plotted in the Chittka colour hexagon (as a model of hymenopteran colour vision; Chittka 1992; Chittka *et al*. 1992), reflectance spectra for guild members cluster close to the centre (representing the colour of typical green leaves) in the blue-green to green region of the hexagon (Chapter 10). This suggests that they are not easily distinguished from background vegetation by the wasps. Field experiments in which flowers were concealed from view showed that wasps can locate flowers in the complete absence of visual cues. Thus, floral colour appears to play little or no role in the attraction of *Hemipepsis* wasps.

The evolution of floral colour in this guild appears to have been driven primarily by the costs of visits by non-pollinating insects (c.f. Hargreaves *et al*. 2009). Colour is one of the best studied floral traits, but is usually examined in the context of floral advertising and pollinator attraction (e.g. Bradshaw & Schemske 2003; Irwin & Strauss 2005; Hoballah *et al*. 2007; Cooley *et al*. 2008). However, the evidence presented in this thesis suggests that floral colours may not always result from selection by pollinators. In this system, the reliance of *Hemipepsis* wasps purely on scent cues to find flowers appears to have freed the evolution of colour to proceed in a direction unrelated to the preferences of the wasp pollinators. The association of red colour with hummingbird pollination in penstemons has similarly been attributed to selection for reducing visits by less effective visitors such as bees, although, in this case, the red coloration could also result from selection for red by hummingbirds (Raven 1972; Grant 1966; Castellanos *et al*. 2004; Rodríguez-Gironés & Santamaría 2004; Wilson *et al*. 2006).

The costs of visits by non-pollinating insects are difficult to determine, but could be related to the loss of pollen to unfaithful visitors (Hargreaves *et al*. 2009) or to the reduction of available nectar reward making flowers less attractive for specialist *Hemipepsis*-wasp pollinators. Alternatively, the pigments required for bright colours may be costly to produce and are consequently lost from plants which do not require showy colours to attract their specific pollinators. It would be interesting to manipulate floral colours of highly specialized guild members and see if this alters the assemblage of insects that visit the flowers.

Differentially palatable nectar

Nectar properties can contribute to floral specialization if the nectar is differentially palatable (due to specific sugar concentrations or non-sugar constituents) to specialist pollinators and non-pollinating insects (Johnson *et al*. 2006). I conducted nectar palatability experiments with three *Hemipepsis*-wasp pollinated asclepiads (*Pachycarpus asperifolius*, *Pa. grandiflorus* and *Xysmalobium orbiculare*; Chapters 5, 7 & 8). Choice tests in which honeybees and *Hemipepsis*-wasps were offered a three way choice between nectar from each of these species and sucrose and hexose sugars at the same concentration showed that honeybees find these nectars distasteful while *Hemipepsis*-wasps appear unaffected (Chapters 5, 7 & 8). However, nectar palatability appears to be variable within the guild, with some nectars (particularly from *Pachycarpus* species) being especially bitter, while others (such as the *Eucomis* species) are not as unpleasant to the human palate.

Differentially palatable nectar has been demonstrated in several plants and is typically attributed to secondary compounds (Stephenson 1981, 1982; Johnson *et al.* 2006; Adler & Irwin 2005; Gegear *et al.* 2007; Irwin & Adler 2008), specific sugar concentrations (Butler 1945; Waller 1972; Baker 1975) or a combination of secondary compounds and specific sugar concentrations (Liu *et al*. 2007). In my studies with *Hemipepsis*-wasp pollinated flowers, nectar concentration was unlikely to be a factor since this was controlled for by matching the concentration of the sugars used in the choice experiments. Preliminary analyses of *Pa. asperifolius* and *X. undulatum* nectars using a colorimetric assay (Singleton *et al*. 1999) suggested that these nectars contain high levels of phenolic compounds and the distasteful qualities of the nectars are thus likely to be the result of non-sugar constituents (secondary compounds).

Nectar typically functions as a straightforward energy reward for pollinators (Baker & Baker 1983). However, floral nectars frequently contain non-sugar compounds which are unlikely to contribute to the energy value of the nectar (Baker & Baker 1983). The adaptive significance of these non-sugar compounds has only recently been recognized and a number of hypotheses have been proposed to explain their presence (see Adler 2000). The presence of secondary compounds in nectar is especially counter-intuitive since these are typically associated with plant defense and are generally unpalatable. However, several studies have shown that secondary compounds can function to deter non-pollinating visitors ("nectar-robbers"). In one of the first studies to test this empirically, Stephenson (1981, 1982) showed that iridoid glycosides present in the nectar of *Catalpa speciosa* (Bignoniaceae) are highly toxic to potential nectar robbers (non-pollinating ants and butterflies) but had no effect on the legitimate bumblebee and moth pollinators of this plant. Similar effects have subsequently been revealed in other plant species (Hagler & Buchmann 1993; Johnson *et al.* 2006). Toxic compounds present in nectars thus appear to serve as floral filters which prevent or reduce visitation by non-pollinating animals. This is supported by the results presented in this thesis, as species with unpalatable nectar were visited by very few non-pollinating insects (Chapters 5, 7 & 8).

Future studies should examine the nectars of a wider range of guild members and aim to identify particular secondary compounds present in the nectars of *Hemipepsis*-wasp pollinated flowers. In addition, palatability experiments need to be conducted with the nectars of non-wasp-pollinated congeners, to test the adaptive significance of distasteful nectar in this guild. A detailed analysis of the compounds responsible for the distasteful properties of some of these nectars would greatly enhance our understanding of some of the mechanisms by which plants with exposed nectar can filter floral visitors.

EVOLUTION OF THE GUILD

Specialized pollination by *Hemipepsis* wasps is currently known only from southern Africa, despite the fact that functionally similar *Hemipepsis* and *Pepsis* (Pompilidae) wasps are common in other regions and are known to visit flowers for nectar (Punzo 2006). Understanding the evolutionary transitions involved in the formation of the *Hemipepsis*-wasp pollination guild might explain why this pollination guild is particularly well represented in South African grasslands. This could also offer insights into the reasons for the high levels of specialization that have been suggested for southern African pollination systems compared with those in other regions (Johnson & Steiner 2000, 2003; Ollerton *et al*. 2006; Waser *et al*. 1996).

Macroevolutionary studies of the pollinator shifts that have led to the *Hemipepsis*-wasp pollination guild are confounded by the absence of phylogenetic data for the families and genera involved. Specialized pollination by *Hemipepsis* wasps is known for species from three families of angiosperms (Orchidaceae, Hyacinthaceae and Apocynaceae), with asclepiads (Apocynaceae: Asclepiadoideae *sensu* Endress & Bruyns 2000) being particularly well represented in the guild (16 out of 21 species). A molecular phylogeny for South African asclepiads would be especially useful, although optimizing pollination systems onto such a phylogeny would require more extensive studies of pollination systems within African asclepiads (but see Liede & Whitehead 1991; Pauw 1998; Ollerton *et al*. 2003). A detailed analysis of pollination systems and phylogenetic relationships in the genus *Eucomis* would also provide insights into the evolution of specialized pollination by *Hemipepsis* wasps. The genus *Eucomis* is small enough (12 species; Manning & Goldblatt 2003; Zonneveld & Duncan 2010) to provide a useful model study system for examining pollinator shifts. Aside from the two *Hemipepsis*-wasp pollinated taxa (*E. autumnalis* subsp. *clavata* and *E. comosa* var. *striata*), the majority of *Eucomis* species appear to be pollinated by carrion flies (Chapter 9; unpubl. data) while a single species, *E. regia*, is pollinated by rodents (P. Wester, A. Pauw & S.D. Johnson unpubl. data). In this study, I was able to show that the shift between wasp and fly pollination within the genus could be precipitated by relatively minor changes in the floral scents (particularly the production or suppression of oligosulphides; Chapter 9). However, it is difficult to speculate on the direction of these shifts. Floral scents are potentially labile (Dudareva & Pichersky 2006) and the close association between floral scents and pollination systems within this genus suggests that shifts

may have occurred on several occasions. In this respect, detailed comparison of the scents of the wasp- and fly-pollinated species to that of the rodent-pollinated *E. regia* would be particularly interesting. Quantitatively, *E. regia* has a nutty aliphatic-based odour very different to the sweet spicy scent of the wasp-pollinated species and the putrid rotting-carrion odour of the fly-pollinated species (P. Wester, A. Pauw & S.D. Johnson unpubl. data). Further studies examining the scents, pollination systems and phylogenetic relationships within this genus would enhance our understanding of the role of scent in mediating pollinator shifts.

It would also be interesting to examine the frequency of shifts between *Hemipepsis*-wasp pollination and cetoniine-beetle pollination. Ollerton & Watts (2000) suggested that wasp and beetle pollination syndromes are not widely separated in phenotype space, although their study was based on floral traits established for classic pollination syndromes (taken from Faegri & van der Pijl 1979 and Proctor *et al*. 1996) which may not be an ideal generalization for *Hemipepsis* wasps or cetoniine beetles (see discussion of pollination syndromes in Chapter 1). However, the similarity of these two syndromes is supported by several studies, including the results presented in this thesis (Ollerton *et al*. 2003; Johnson *et al*. 2007; Chapter 3). Two species, the asclepiad *Xysmalobium undulatum* and the orchid *Satyrium microrrhynchum* are specialized for pollination by both *Hemipepsis* wasps and the cetoniine beetle *Atrichelaphinis tigrina* (Johnson *et al*. 2007; Chapter 3). In addition, cetoniine beetles are frequent non-pollinating visitors to many of the specialized *Hemipepsis*-wasp pollinated flowers (Chapters 2 & 4-8). Similarly, *Hemipepsis* wasps occasionally visit specialized cetoniine-pollinated flowers (Ollerton *et al*. 2003; S-L. Steenhuisen pers. comm.). It would be interesting to examine the frequency and direction of shifts between these two pollination systems from a phylogenetic perspective. The genus *Pachycarpus* would be a particularly useful model system since all species for which pollination systems are known appear to be pollinated by either *Hemipepsis* wasps (Ollerton *et al*. 2003; Chapters 2, 4, 5 & 7) or cetoniine beetles (Chapter 4; Appendix 1). Intriguingly, cetoniine beetles often visit wasp-pollinated *Pachycarpus* species (Chapters 2, 4, 5 & 7) but *Hemipepsis* wasps have never been observed visiting cetoniine-pollinated *Pachycarpus* species, possibly relating to the beetles being more generalist floral visitors than the wasps. Comparison of scent profiles for wasp and beetle pollinated *Pachycarpus* species suggested a degree of separation between the syndromes in two-dimensional scent space, but with a broad overlap (Chapter 10). A detailed analysis of floral traits (including scents) and a phylogeny for the genus *Pachycarpus* would provide an opportunity to tease apart floral traits that are shared between these two syndromes and identify unique traits associated with wasps versus beetles.

ANTAGONISTIC PLANT-INSECT INTERACTIONS

The occurrence of a potentially antagonistic interaction between *Hemipepsis* wasps and two species of *Pachycarpus* was one of the interesting and unexpected results to emerge from my studies (Chapters 2 & 5). Plant-pollinator interactions have provided textbook examples of mutualisms and it is generally

assumed that both parties benefit. However, plants and their pollinators are involved in a process of mutual exploitation and there are several instances where this can progress to a point where one or the other is harmed by the interaction. Well known examples include systems in which the insect pollinators are deceived by rewardless orchids or arums (Steiner *et al*. 1994; Stensmyr *et al*. 2002; Wong & Schiestl 2002; Schiestl 2005; Diaz & Kite 2006; Jersakova *et al*. 2006) or are trapped and temporarily held captive by the flowers (Oelschlägel *et al*. 2009; Ollerton *et al*. 2009; Diaz & Kite 2006). However, a different form of antagonism occurs when the pollinator is physically damaged by the flowers. *Pachycarpus appendiculatus* and, to a lesser extent, *Pa. asperifolius* systematically remove the palps of their *Hemipepsis*-wasp pollinators. Pollinaria for these two species are attached to the palps of visiting wasps and the palps are broken off when the pollinia are subsequently inserted in flowers. Foraging on asclepiad flowers is well known to be hazardous for insects and there are several reports of insect body parts being removed by milkweed flowers (Weale 1873; Morse 1981). However, these are usually incidental occurrences rather than a systematic feature of the interaction. The two species of *Pachycarpus* mutilate their pollinators to a degree that has not previously been reported. For both species, over 80% of wasps collected on flowers were missing at least one palp, while 61% of the wasps collected on *Pa. appendiculatus* flowers were missing all four palps; Chapters 2 & 5).

The costs of palp loss to the wasps are difficult to assess. Palps serve a sensory function in insects and are involved in locating and testing the quality of food before ingestion (Chapman 1971; Gullan & Cranston 2005). If the removal of palps seriously reduced the foraging efficiency of wasps or caused them to ingest inferior quality nectar, this could reduce fitness of individuals foraging on these flowers. Morse (1981) showed that bumblebees lose claws and tarsal segments when foraging on *Asclepias syriaca*, and this loss reduced foraging efficiency by about 25%. Palps are more directly involved in foraging than claws and the loss of palps may thus be particularly detrimental to wasps. It would be interesting to examine the foraging efficiency of wasps with and without palps in more detail. *Hemipepsis* wasps behave well in laboratory cage conditions and it would be relatively simple to measure handling times for wasps with and without palps or to offer wasps choices between experimentally manipulated nectars of varying qualities. Examining the costs of palp loss would contribute to our understanding of the balance between antagonism and mutualism in plant-pollinator interactions.

Two types of potentially antagonistic interactions thus occur within the *Hemipepsis*-wasp pollination guild. In addition to the mutilation of pollinators by *Pachycarpus* species described above, the orchid *Disa bivalvata* is sexually deceptive and offers no reward for its *Hemipepsis*-wasp pollinators (Steiner *et al*. 1994). It is likely that the *Pachycarpus* species use the same cues to attract wasps as other, non-antagonistic guild members. It would thus be difficult for wasps to evolve mechanisms of avoiding these particular species as this would involve abandoning the specialized ecological interaction with their host plants. The chemical cues used by *D. bivalvata* to attract male

wasps have not been examined, but it is likely that these are related to the pheromones used by females to attract males (Schiestl *et al*. 1999, 2003). Male wasps are therefore unable to ignore the floral signals without ignoring signals from female wasps. These examples highlight the opportunity that exists for some plant species to exploit co-evolved ecologically specialized interactions which prevent selection for avoidance mechanisms in the pollinators.

ECOLOGICAL IMPLICATIONS

Knowledge of pollinator requirements and breeding systems are essential for plant conservation. Breeding systems dictate the reliance of a plant on pollinators for reproduction, and self-incompatible species with specialized pollination systems may be particularly sensitive to disturbances affecting pollinator abundance (Bond 1994). Of the 24 plant species whose pollination systems were described in this thesis, 21 are highly specialized and are pollinated by one or two functional types of pollinator (Chapter 10). In combination with genetic self-incompatibility, these highly specialized pollination systems may render these plants particularly vulnerable to disturbance (Bond 1994).

This is particularly important for southern African asclepiads as the region is considered a centre of diversity and endemism for this group, and contains approximately 600 species, many of which have limited geographical distributions and particular habitat requirements (Victor *et al*. 2003). The pattern emerging from the studies presented in this thesis and previous studies (Ollerton *et al*. 2003; Pauw 1998) is one of remarkably specialized pollination systems in southern African asclepiads. Generalized insect pollination has been suggested for three species, *Xysmalobium gerrardii* (Ollerton *et al*. 2003), *Sarcostemma viminale* (Liede & Whitehead 1991) and possibly *Asclepias crispa* var. *plana* (Chapter 4). However, the remaining southern African asclepiads for which detailed pollination studies have been conducted all exhibit high levels of floral specialization, including specialized pollination by birds (Pauw 1998), cetoniine beetles (Ollerton *et al*. 2003; Chapter 4), *Hemipepsis* wasps (Ollerton *et al*. 2003; Chapters 2-5, 7 & 8), vespid wasps (Coombs *et al*. 2009), bees (Ollerton *et al*. 2003; Chapter 4) and carrion flies (Johnson *et al*. 2009; Chapter 4).

At this stage, we have limited data on the breeding systems of southern African asclepiads. The four asclepiads for which breeding systems were examined in this thesis (*Pachcarpus asperifolius*, *P. grandiflorus*, *X. orbiculare and X. undulatum*; Chapters 3,5,7 & 8) were all genetically self-incompatible. Self-incompatibility has also been established for the African asclepiad *Gomphocarpus physocarpus* (Coombs *et al*. 2009). Furthermore, self-incompatibility is typical of North American *Asclepias* species (Wyatt & Broyles 1994). However, *G. fruticosus*, a species native to Africa which is introduced to tropical America and Australia, has been found to be self-compatible (Wyatt & Broyles 1997) and self-compatibility has been reported in several North American asclepiads, including *A. curassavica*, *Vincetoxicum rossicum* and *Gonolobus suberosus* (Lumer & Yost 1995; Wyatt & Broyles 1997; Lipow & Wyatt 1998; Ivey *et al*. 1999; Lipow & Wyatt 2000; Leimu 2004; St Denis & Cappuccino 2004). These studies suggest that southern African asclepiads are likely to be mostly self-

incompatible, although this trend needs to be confirmed with studies of breeding systems in a wider sample of the region's species and genera.

Pollination and breeding systems must be considered when formulating strategies for plant conservation. The plant species examined in this thesis are all important components of grassland ecosystems. Southern African grasslands are becoming increasingly fragmented through anthropogenic activities, and conservation planning needs to consider the effects of these disturbances on the specialist pollinators of grassland plants. To this end, it is essential that the insects operating pollination guilds, such as *Hemipepsis* wasps or cetoniine beetles, be recognized as keystone species and afforded appropriate protection. We still have very limited knowledge of asclepiad pollination and breeding systems and future studies are essential for the conservation of this group of plants.

CONCLUSION

The research presented in this thesis has confirmed the importance of *Hemipepsis* wasps as pollinators in southern African grassland ecosystems, and has revealed a diverse guild of plants that are reliant on these wasps for reproduction. The high levels of specialization within this guild highlight the effectiveness of biochemical traits, such as particular scent, cryptic coloration and distasteful nectar, as a proximal mechanism of filtering floral visitors. Future studies should be aimed at identifying the particular floral volatiles (or blends of volatiles) that attract *Hemipepsis* wasps and the secondary compounds in nectar that deter non-pollinating insects. Phylogenetic studies would also be useful for examining the direction and frequency of shifts involved in the formation of the guild.

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APPENDIX 1

FLORAL SCENTS OF CHAFER-POLLINATED ASCLEPIADS AND A POTENTIAL HYBRID

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Floral scents of chafer-pollinated asclepiads and a potential hybrid

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Abstract

Floral scent is a key functional trait for pollinator attraction to flowers, but is poorly documented in many plant lineages and pollination systems. In South African grasslands, chafer beetles (Scarabaeidae: Cetoniinae), particularly *Atrichelaphinis tigrina*, *Cyrtothyrea marginalis* and *Leucoscelis* spp., are common floral visitors and specialized pollination by these beetles has recently been established in several asclepiad, orchid and protea species. Chafer beetles are known to be attracted by a variety of floral volatile compounds and scent has been suggested to be an important signal in these chafer-operated pollination systems. In this study, we used dynamic headspace extraction methods and coupled gas chromatography–mass spectrometry (GC–MS) to examine the chemical composition of the floral scents of seven putatively chafer-pollinated asclepiad species in the genera *Asclepias*, *Pachycarpus* and *Xysmalobium*. We identified 15–57 compounds in the scents of these species, of which seven were common to all species examined. The scent profiles of each species separate into discrete clusters in two dimensional space based on non-metric multidimensional scaling (NMDS), indicating clear distinctions between species and suggesting that plants may use different combinations of volatiles to attract beetles. Two plants suspected to be intergeneric hybrids were also examined. Data on pollination systems, morphology and scent chemistry are consistent with the hypothesis that these plants are hybrids between the chafer-pollinated species *Asclepias woodii* and *Pachycarpus concolor*. The results of this study are discussed in relation to the role of chafer beetles as generalist pollinators of specialized asclepiads.

Keywords: Apocynaceae; Asclepiadoideae; Cantharophily; Floral volatiles; Introgression; Pollination guild

1. Introduction

Floral scent is increasingly being recognized as a functionally important trait in many plant–pollinator interactions, but remains poorly examined for many systems (Dudareva and Pichersky, 2006; Raguso, 2001). Recent studies in South African grasslands have revealed a guild of plants that are reliant on chafer beetles (Scarabaeidae: Cetoniinae), particularly *Atrichelaphinis tigrina* (Olivier, 1789), *Cyrtothyrea marginalis* (Swartz, 1817) and *Leucoscelis* spp. for pollination. These chafer beetles are ubiquitous and generalist floral visitors in South African grasslands (pers. obs.). Interestingly, while the beetles themselves are highly generalist, some of the plants they pollinate are highly specialized, in many cases being dependent almost entirely on just one beetle species for pollination. Chafer-pollinated species include asclepiads (Apocynaceae: Asclepiadoideae; Ollerton et al., 2003; Shuttleworth and Johnson, 2008, 2009a), orchids (Orchidaceae; Johnson et al., 2007; Peter and Johnson, 2009) and proteas (Proteaceae; Steenhuisen and Johnson, 2007).

Detailed studies of the mechanisms of chafer attraction by flowers have recently been conducted for proteas (Steenhuisen et al., 2008; Steenhuisen et al., 2010-in this issue) and have shown that floral scent is a key pollinator attractant. A role for scent in pollination of the chafer-pollinated orchid *S. microrrhynchum* was also suggested from antennal electrophysiological studies of *A. tigrina* (Johnson et al., 2007). Chafer-pollinated asclepiads are also often unusually fragrant in comparison to their congeners, further suggesting that volatiles may play an important role in the attraction of chafer beetles. The aims of this study were thus to examine the chemical composition of the floral scents of chafer-pollinated asclepiads and from the resultant patterns to evaluate the role of floral scent in the attraction of chafer beetles to asclepiad flowers.

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During the course of a previous study (Shuttleworth and Johnson, 2009a), we discovered two individuals of what appeared to be intergeneric hybrids between the two chafer-pollinated species *Asclepias woodii* and *Pachycarpus concolor*. These plants were growing at a site in Midmar Nature Reserve (Table 1) where both *A. woodii* and *P. concolor* co-occur and exhibited floral and vegetative traits intermediate between those of the suspected parent species (Fig. 1a,b,c). Hybridization is an important phenomenon which can result in novel traits being incorporated into parent species through backcrossing and introgression (Barton, 2001; Broyles, 2002; Lewontin and Birch, 1966; Rieseberg et al., 2003; Stebbins, 1959). Although hybridization in North American *Asclepias* species has frequently been examined (Kephart et al., 1988; Klips and Culley, 2004; Wyatt and Broyles, 1992; Wyatt and Hunt, 1991), it has seldom been reported in African asclepiads (but see Weale, 1873). We thus aimed to document these putative hybrids and examine the likelihood that they result from hybridization between *A. woodii* and *P. concolor* through comparison of pollination systems, morphologies and floral scent.

The specific aims of this study were thus (1) to determine the chemical composition of the floral scents of the five known chafer-pollinated asclepiads and an additional two species suspected to be chafer-pollinated, (2) to identify compounds in the floral scents of the seven species that may be attractive to chafer beetles and, (3) to compare floral and vegetative morphologies, and scent of the putative hybrids to those of the parent species.

2. Methods

2.1. Study species and their pollination systems

This study involved seven grassland asclepiads (Apocynaceae subfamily Asclepiadoideae *sensu* Endress and Bruyns, 2000) in the genera *Asclepias*, *Pachycarpus* and *Xysmalobium* (Fig. 1; Table 1). One of these species, *A. woodii*, is endemic to the KwaZulu-Natal midlands and is listed as vulnerable in the Red list for South African plants (Nicholas et al., 2009). *Pachycarpus* sp. nov. is a recently discovered species still in the process of formal description (M. Glenn, J. Lamb, A. Nicholas and A. Shuttleworth, unpubl. data), but is currently known from only a single locality and must also be considered threatened.

The pollination systems of five of these plant species have been examined in previous studies and shown to be operated primarily by the chafers *A. tigrina*, *C. marginalis* and *Leucoscelis* spp. (Scarabaeidae: Cetoniinae; Ollerton et al., 2003; Shuttleworth and Johnson, 2009a). The pollination system of *Asclepias albens* has not been examined in detail, but *A. tigrina* beetles have frequently been observed visiting these flowers and we have collected individuals of this beetle species carrying considerable numbers of *A. albens* pollinaria (unpubl. data). The pollination system of *Pachycarpus plicatus* is also unverified, but morphological similarities between this species and the beetle-pollinated *P. concolor* (such as a bowl shaped corolla and flattened gynostegial column with widely spaced anther wings) suggest that it is also adapted for pollination by chafer beetles. Voucher specimens of the study species are deposited in the Bews Herbarium (University of KwaZulu-Natal, Pietermaritzburg).

2.2. Floral scent collection, GC–MS analysis and comparison of fragrance data between species

Scent samples were collected between October and December 2007 at six sites in KwaZulu-Natal, South Africa (Table 1). Floral scent was collected using dynamic headspace sorption methods by enclosing individual inflorescences in polyacetate bags (Kalle, Germany) and pumping air from the bags through

Table 1
Pollinators and sampling details of floral scent collection for the study species.

Species	Pollinators		Scent sampling and plant localities					
	Principal pollinator	Source[a]	*n*	Sample duration min[b]	Sample date[b]	Locality[c]	Co-ordinates	Altitude (m.a.s.l.)
Asclepias albens (E.Mey.) Schltr.	*Atrichelaphinis tigrina* suspected	3	6	20	24 Oct 2007	VCNR	30°16′06.5″S; 30°37′14.5″E.	447
A. woodii (Schltr.) Schltr.	*Atrichelaphinis tigrina, Cyrtothyrea marginalis*	1	6	25 (1–3), 80 (4–5)	13 Nov 2007 (1–3), 26 Nov 2007 (4–5)	MNR	29°32′15.8″S; 30°10′13.1″E.	1088
Pachycarpus concolor E.Mey.	*Atrichelaphinis tigrina*	2	7	25 (1–3), 60 (4–6), 80 (7)	13 Nov 2007 (1–3), 23 Nov 2007 (4–6), 26 Nov 2007 (7)	MNR	29°32′15.8″S; 30°10′13.1″E.	1088
P. plicatus N.E.Br.	Unknown	–	5	60	1 Dec 2007	MCNR	30°29′58.2″S; 29°25′12.3″E.	1455
P. scaber (Harv.) N.E.Br.	*Cyrtothyrea marginalis, Leucoscelis* spp.	2	5	20	1 Nov 2007	B	29°45′13.3″S; 30°21′29.9″E.	810
Pachycarpus sp. nov.	*Atrichelaphinis tigrina*	2	5	30	3 Dec 2007	H	30°16′10.3″S; 30°12′09.3″E.	976
Xysmalobium involucratum (E.Mey.) Decne.	*Atrichelaphinis tigrina, C. marginalis*	1	5	20	29 Oct 2007	WF	29°36′35.9″S; 30°07′59.4″E.	1350
A. woodii X *P. concolor* hybrid	–	–	1	25	13 Nov 2007	MNR	29°32′15.8″S; 30°10′13.1″E.	1088

[a] 1 = Ollerton et al. (2003); 2 = Shuttleworth and Johnson (2009a); 3 = Pers. obs.
[b] Numbers in parentheses refer to the sample number.
[c] B = Baynesfield; H = Highflats; MCNR = Mount Currie Nature Reserve, Kokstad; MNR = Midmar Nature Reserve, Howick; VCNR = Vernon Crookes Nature Reserve, Umzinto; WF = Wahroonga Farm.

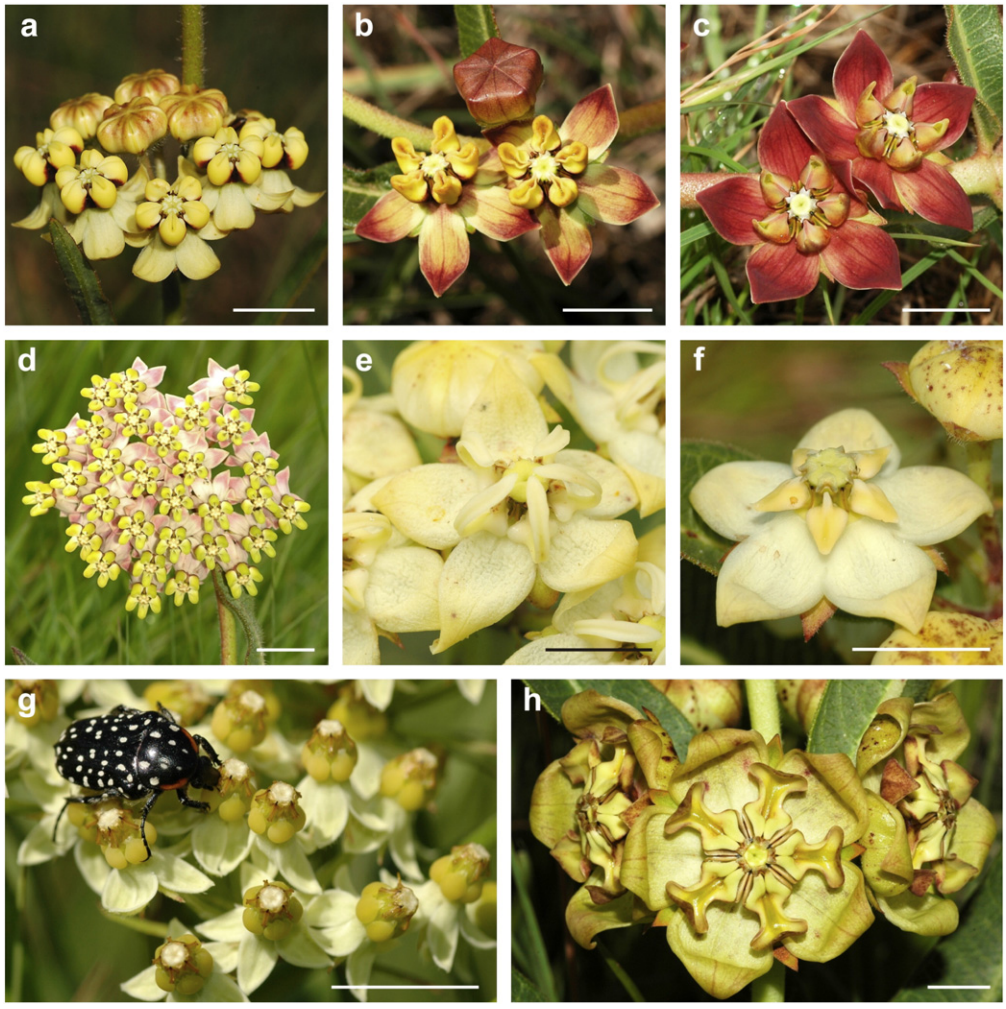

Fig. 1. Flowers of the seven study species and the suspected hybrid. (a) *Asclepias woodii* inflorescence, Wahroonga Farm; (b) flowers of the *A. woodii* X *P. concolor* hybrid, Midmar Nature Reserve; (c) flowers of *Pachycarpus concolor*, Midmar Nature Reserve; (d) *Asclepias albens* inflorescence, Wahroonga Farm; (e) flower of *P. scaber*, Baynesfield; (f) flower of *Pachycarpus* sp. nov., Highflats; (g) flowers of *Xysmalobium involucratum* being visited by *Cyrtothyrea marginalis* (Scarabaeidae: Cetoniinae), Wahroonga Farm; (h) flowers of *P. plicatus*, Mount Currie Nature Reserve. All scale bars = 10 mm.

small cartridges filled with 1 mg of Tenax® and 1 mg of Carbotrap™ activated charcoal. Pumps ran at a realized flow rate of 50 ml/min. Controls were taken from an empty polyacetate bag sampled for the same duration. Care was taken to exclude green leaves from the polyacetate bag, but control samples from green leaves were not collected and some non-floral plant volatiles may have been collected in the samples. All samples were collected in the field except for *P. concolor* samples 4–6 (Table 1) which were taken from recently cut flowering stems in the laboratory. Samples were analyzed by coupled gas chromatography–mass spectrometry (GC–MS) using a Varian CP-3800 gas chromatographer (Varian, Palo Alto, CA, USA) with an Alltech EC-WAX column (optimal for polar compounds) coupled to a Varian 1200 quadrupole mass spectrometer with a Varian 1079 injector equipped with a 'ChromatoProbe' thermal desorbtion device (see Shuttleworth and Johnson (2009b) for a detailed explanation of methods).

Fragrance profiles for the seven species were compared using non-metric multidimensional scaling (NMDS) implemented with Primer 6.1.6 (2006) (Clarke and Warwick, 2001; Clarke and Gorley, 2006). NMDS was based on Bray–Curtis similarity and data were square-root transformed prior to analysis. The stress value is a measure of how well the two dimensional configuration matches the similarity matrix, such that stress below 0.1 represents a good ordination while stress values between 0.1 and 0.2 represent a useful 2-dimensional picture but patterns should be interpreted in conjunction with the results of the ANOSIM analysis (Clarke and Warwick, 2001). ANOSIM tests the differences in the fragrance profiles between species using a non-parametric permutation procedure which generates the test statistic R based on the similarity matrix underlying the ordination (Clarke and Warwick, 2001). Values of R close to one indicate complete separation of groups while R values close to zero indicate minimal separation between groups. Significance of differences is determined by comparison of the calculated R to values of R resulting from up to 10 000 random permutations of the sample labels. It should be noted, however, that comparisons between groups with small numbers of replicates will yield insufficient permutations to produce meaningful levels of significance, although this problem was only encountered for

the hybrid comparisons (see subsequent discussion) and the *P* values for these were therefore not presented (Clarke and Warwick, 2001). The NMDS and ANOSIM analyses were repeated with typical green leaf volatiles (such as (Z)-hex-3-en-1-ol and (Z)-hex-3-en-1-yl acetate) excluded, in order to confirm that these compounds were not unduly affecting the results.

Volatiles characterizing the fragrance of each species were identified using the similarity percentages (SIMPER) function in Primer (Clarke and Gorley, 2006). SIMPER calculates the percentage contributions of each compound to average overall similarity between samples from a particular species (Clarke and Warwick, 2001).

2.3. Morphometrics and scent of putative hybrids

A single individual of the putative hybrid between *A. woodii* and *P. concolor* was discovered at Midmar Nature Reserve in each of two flowering seasons (November 2006 and November 2007). Both individuals were collected and deposited in the Bews Herbarium for subsequent analysis. The floral scent of one of these individuals was sampled prior to collection (using the methods described earlier; see Table 1 for details) and this fragrance data was included in the NMDS analysis described previously in order to compare it with the suspected parent species. Morphological measurements of the two hybrids and of the two parent species were taken using herbarium specimens. For the parent species, we used specimens that were collected by us in Midmar Nature Reserve in the same flowering seasons as the hybrids. We measured the diameter of the flower (measured across the corona) as well as the length and diameter of the corona lobes (taken from the base along the outermost edge), corolla lobes and leaves. Plant means were not calculated as it was not always possible to differentiate which flowers/leaves originated on separate plants in the herbarium collection (i.e. flowers/leaves from multiple plants were sometimes collected and preserved together as part of the same herbarium specimen).

3. Results

3.1. Floral scents

The seven study species are all scented to the human nose. Two species, *Pachycarpus scaber* and *Xysmalobium involucratum*, are particularly fragrant with *P. scaber* exhibiting a strong sweet scent (emission rate per inflorescence: 26.5±13.34 mean± sd μg/h) and *X. involucratum* exhibiting a powerful sweet spicy/cinnamon-like fragrance (emission rate per inflorescence: 18.2± 5.93 μg/h). The remaining species are relatively weakly scented both to the human nose and in terms of actual emission rates (emission rate per inflorescence ranging from 0.4±0.25 μg/h in *P. plicatus* to 8.6±12.53 μg/h in *P. concolor*) but have a similar sweet scent. A wide range of floral volatiles in various compound classes were identified from the headspace samples of these species (ESM Tables 1 and 2). The scents of *A. woodii*, *P. concolor*, *P. plicatus*, *Pachycarpus* sp. nov. and *Xysmalobium involucratum* were all similarly dominated by aliphatics and isoprenoids (ESM Tables 1 and 2). In contrast, the scent of *A. albens* was dominated by isoprenoids with small amounts of aliphatic and aromatic compounds; and the scent of *P. scaber* was dominated by aromatics and isoprenoids with small amounts of aliphatics (ESM Tables 1 and 2). Seven compounds (myrcene, limonene, (E)-ocimene, linalool, (Z)-hex-3-en-1-ol, (Z)-hex-3-en-1-yl acetate and 2-methylcyclopent-2-en-1-one) were common to the scents of all seven species (ESM Tables 1 and 2). *A. woodii* produced the fewest compounds (ranging from 9 to 15 between samples) while *P. scaber* (36–40 compounds) and *X. involucratum* (45–57 compounds) produced the highest number of compounds. A total of 32 compounds account for the first 80% of average Bray–Curtis similarity between conspecific samples across all species (Table 2). Between seven and 15 of these compounds accounted for the first 80% of average similarity within individual species (Table 2). Three compounds (myrcene, (E)-ocimene and (Z)-hex-3-en-1-yl acetate) contributed to average similarity in all seven species (Table 2).

Floral scent profiles for each of the seven species separate into discrete clusters in the non-metric multidimensional scaling (NMDS) ordination (Global $R=0.844$, $P<0.001$; Fig. 2). Exclusion of typical green leaf volatiles from this analysis did not dramatically alter the NMDS and ANOSIM analyses (results not shown). The scent profiles of *A. albens*, *P. scaber*, *Pachycarpus* sp. nov. and *X. involucratum* were all clearly distinct and well separated from each of the other species (range of *R* for pairwise contrasts between species=0.755–1; $P<0.01$). The scent profile of *P. plicatus* was poorly separated from that of *P. concolor* ($R=0.263$; $P=0.047$), but was well separated from the remaining six species ($R=0.768$–1; $P<0.01$). The scent profiles of *P. concolor* and *A. woodii* were moderately separated ($R=0.506$; $P<0.01$). Aside from *P. concolor*, the scent of *A. woodii* was well separated from all other species ($R=0.768$–1; $P<0.01$).

3.2. Morphometrics and scent of putative hybrids

Flowers and leaves of the putative hybrid plants were consistently intermediate between those of *A. woodii* and *P. concolor* in shape and size (Fig. 1a,b,c; Fig. 3).

Eighteen compounds were identified in the headspace sample taken from the putative hybrid (ESM Table 1). Of these, seven were common to the scents of both parent species, while seven compounds and one compound were also found in the scents of *P. concolor* or *A. woodii* respectively (ESM Table 1). Three compounds (oct-1-en-3-ol, (Z)-hex-3-en-1-yl isovalerate and cyclohex-2-en-1,4-dione) were unique to the hybrid and were not found in the scents of the parent species. The hybrid scent profile fell midway between the scents of *A. woodii* and *P. concolor* in the NMDS ordination (Fig. 2), although the ANOSIM analysis suggested that the hybrid was closer to *P. concolor* ($R=0.197$) than to *A. woodii* ($R=0.4$). The hybrid scent profile was also close to *P. plicatus* in the ordination (Fig. 2), although the ANOSIM analysis suggests that the hybrid scent was distinct from the scent of *P. plicatus* ($R=1$). Comparison of the hybrid sample to each of the other five asclepiad species yielded *R* values of 1 for all species.

Table 2
Compounds contributing to the first 80% of average similarity between conspecific scent samples (from SIMPER analysis). % = percentage contribution of each compound to conspecific similarity, Sim/sd = percentage contribution/standard deviation. Compounds that characterize a species scent will exhibit high percentage contributions and high sim/sd values (Clarke and Warwick, 2001). Compounds in bold are those that have been shown to elicit an electroantannagraphic response (superscript a) or to be attractive (superscript b) to cetoniine beetles (see footnote to ESM Table 1 for references). Mass fragments for unknowns are listed with the molecular ion first (if known) marked with *, followed by the base peak and other fragments in decreasing order of abundance. KRI = kovats retention index (calculated from retention times).

Compound		*Asclepias albens*		*Asclepias woodii*		*Pachycarpus concolor*		*Pachycarpus plicatus*		*Pachycarpus scaber*		*Pachycarpus* sp. nov.		*Xysmalobium involucratum*	
	KRI	%	Sim/sd	%	Sim/sd	%	Sim/sd	%	Sim/sd	%	Sim/sd	%	Sim/sd	%	Sim/sd
Aliphatics															
(Z)-Hex-3-en-1-yl acetate[a]	1335	3.6	1.0	39.7	2.4	27.5	1.8	36.5	18.5	4.8	3.8	11.5	4.2	2.0	0.6
Hexyl acetate[a]	1292	–	–	–	–	–	–	4.6	7.0	–	–	2.1	2.8	–	–
(Z)-Hex-3-en-1-ol[a]	1398	–	–	6.0	1.1	6.4	1.7	4.1	4.6	4.0	3.1	4.9	5.0	1.5	1.6
Hexan-1-ol[a]	1364	2.0	4.5	–	–	–	–	–	–	–	–	–	–	–	–
(E)-Hex-2-en-1-ol[a]	1417	–	–	–	–	–	–	–	–	–	–	2.7	4.2	–	–
(E)-Hex-2-enal[a]	1240	–	–	–	–	–	–	–	–	–	–	2.5	1.4	–	–
Aromatics															
Benzaldehyde[a]	1546	2.2	3.3	–	–	–	–	–	–	29.2	3.9	–	–	16.4	4.7
Benzyl alcohol[a]	1900	2.2	2.2	–	–	–	–	–	–	3.0	3.5	–	–	4.1	9.0
Methylbenzoate[a,b]	1651	2.1	1.2	–	–	–	–	–	–	–	–	–	–	11.6	4.5
Methyl salicylate[a,b]	1794	–	–	–	–	–	–	–	–	–	–	–	–	3.7	2.6
Phenylethyl alcohol[a]	1940	–	–	–	–	–	–	–	–	–	–	–	–	8.3	3.8
Phenylacetaldehyde[a,b]	1668	–	–	–	–	–	–	–	–	4.6	2.0	–	–	3.5	2.1
Isoprenoids															
α-Pinene	1093	–	–	–	–	–	–	–	–	2.8	2.1	–	–	–	–
(E)-Ocimene	1276	5.2	3.4	6.0	1.3	11.9	2.1	2.3	1.4	4.2	5.6	2.8	1.7	14.6	1.2
(Z)-Ocimene	1257	5.1	5.1	–	–	–	–	–	–	2.6	1.4	4.9	3.3	1.8	1.8
Myrcene	1202	8.6	3.7	5.0	0.9	14.0	1.5	3.0	0.8	9.9	2.9	11.8	4.0	2.0	1.6
Limonene	1229	4.4	3.4	–	–	7.6	1.1	2.4	0.7	6.7	3.4	3.0	4.1	–	–
Linalool[a,b]	1557	33.6	1.9	8.7	2.5	–	–	4.2	3.7	–	–	11.5	7.2	4.1	0.7
Hotrienol	1625	–	–	–	–	–	–	–	–	–	–	5.4	3.9	–	–
Caryophyllene[a,b]	1623	2.0	2.5	–	–	5.8	1.4	7.6	8.8	–	–	–	–	–	–
Germacrene D	1739	–	–	–	–	3.2	0.8	–	–	2.1	7.5	–	–	–	–
(E)-β-Farnesene	1685	–	–	4.9	0.6	–	–	–	–	–	–	–	–	–	–
4-Oxoisophorone	1721	–	–	–	–	–	–	–	–	1.8	1.0	11.1	5.0	–	–
Unknowns															
m/z: 150*,69,41,81,79,82,53	1325	7.4	4.0	13.5	2.8	1.9	0.8	4.9	6.9	–	–	–	–	–	–
m/z: 57,85,86,43,55,42,41	1567	–	–	–	–	3.4	1.0	7.0	4.4	2.1	2.2	2.2	0.9	–	–
m/z: 112*,83,55,57,84	1619	–	–	–	–	–	–	2.7	4.5	–	–	2.4	4.5	–	–
m/z: 57,85,39,41,43,55,31	1986	–	–	–	–	–	–	2.5	2.2	–	–	–	–	1.4	1.4
m/z: 150*,69,41,81,79,107,119,79,82	1298	1.9	2.8	–	–	–	–	–	–	–	–	–	–	–	–
m/z: 152*,43,109,81,79,67,91,55	1841	–	–	–	–	–	–	–	–	–	–	–	–	2.0	1.1
m/z: 79,81,77,41,72,53	1503	–	–	–	–	–	–	–	–	–	–	–	–	3.4	1.0
m/z: 168*,85,56,125,43,69,41,83,153	1693	–	–	–	–	–	–	–	–	3.4	1.6	–	–	–	–
m/z: 152*,43,69,109,55,67,41,95	1732	–	–	–	–	–	–	–	–	–	–	2.1	3.0	–	–
Total		80.2		83.8		81.7		81.6		81.2		80.9		80.3	
Average similarity (Bray–Curtis)		66.6		62.2		51.5		75.7		72.6		68.9		54.4	
Number of compounds		13		7		9		12		14		15		15	

4. Discussion

The results of this study are consistent with the suggestion that floral scent may play an important functional role in specialized chafer beetle (Scarabaiedae: Cetoniinae) pollination systems. Flowers of the seven asclepiads examined are all scented and produced between 15 and 57 floral volatiles (ESM Tables 1 and 2), many of which may play a role in the attraction of beetles. Floral scents of the seven species formed distinct and mostly well separated clusters in the non-metric multidimensional scaling (NMDS) ordination (Fig. 2) indicating that the scents of these species are distinguishable from one another and suggesting that these species may use different combinations of volatiles to attract beetles. The scent and morphometrics of the putative hybrids suggest that these plants were indeed derived from intergeneric hybridization between *A. woodii* and *P. concolor* (ESM Table 1; Figs. 2 and 3), although more comprehensive sampling of the parent species would be required to resolve this fully. The hybrid scent was also close to the scent of *P. plicatus* in the ordination (Fig. 2) although the ANOSIM analysis suggests that these scents are distinct. This counter-intuitive result may relate to the similarity between the scents of *P. plicatus* and *P. concolor* or, alternatively, this may simply reflect an imperfect representation of the similarity matrix in two dimensions. The latter possibility is supported by the relatively high stress value (0.16) for the 2-dimensional

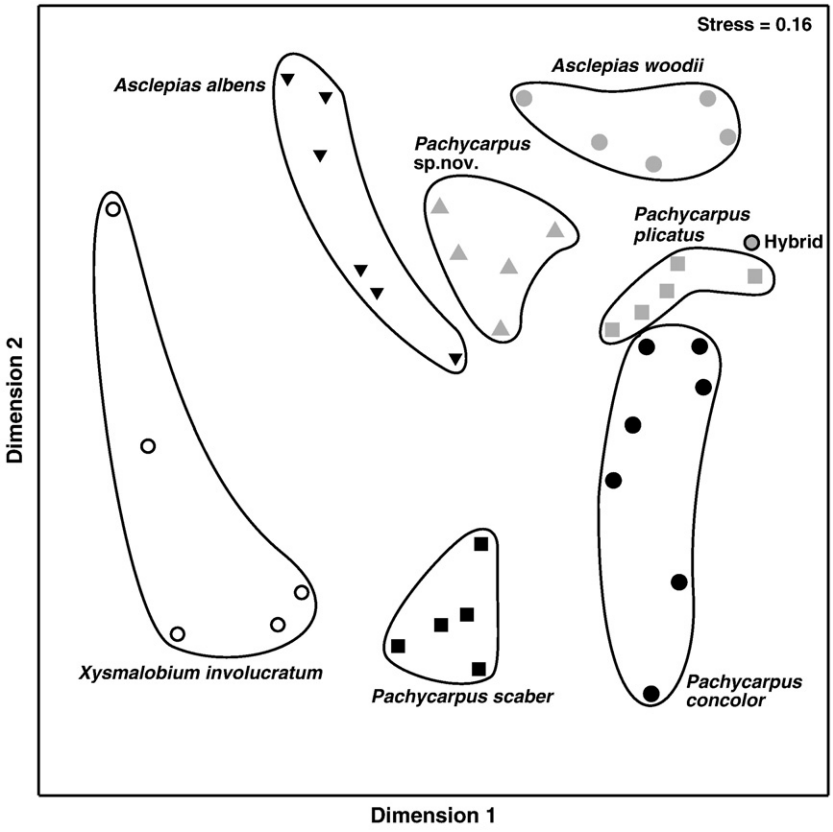

Fig. 2. Non-metric multidimensional scaling ordination of floral scent profiles for the seven asclepiad species and the hybrid.

ordination (Clarke and Warwick, 2001). It is unlikely that the putative hybrids are related to *P. plicatus* as the floral morphologies are not similar (Fig. 1; A. Shuttleworth pers. obs.), although we cannot exclude the possibility that *P. plicatus* may be a parent species.

Our results suggest that chafer beetles are attracted by broad classes of floral volatiles rather than by specific compounds. Only seven compounds (myrcene, limonene, (E)-ocimene, linalool, (Z)-hex-3-en-1-ol, (Z)-hex-3-en-1-yl acetate and 2-methylcyclopent-2-en-1-one) were common to the scents of all the asclepiad study species (ESM Tables 1 and 2), and only four of these (myrcene, linalool, (Z)-hex-3-en-1-ol and (Z)-hex-3-en-1-yl acetate) have also been found in the scents of chafer-pollinated proteas and the chafer-pollinated orchid *Satyrium microrrhynchum* (Johnson et al., 2007; Steenhuisen et al., 2010-in this issue). However, myrcene, limonene, (E)-ocimene and linalool are ubiquitous floral volatiles (Knudsen et al., 2006), while (Z)-hex-3-en-1-ol and (Z)-hex-3-en-1-yl acetate are typical green leaf volatiles. Although green leaf volatiles elicit an electrophysiological response in chafers, they do not appear to be attractive (Bengtsson et al., 2009; Larsson et al., 2001, 2003). The final compound common to the scents of the asclepiad study species, 2-methylcyclopent-2-en-1-one, is unusual but, aside from our studies (Shuttleworth and Johnson, 2009b,e), has not previously been described as a floral volatile (Knudsen et al., 2006) and was not found in the scents of non-asclepiad chafer-pollinated species (Johnson et al., 2007; Steenhuisen et al., 2010-in this issue).

A similar mechanism of attraction has been suggested by other studies of scent in chafer-pollinated flowers. The fragrance of *S. microrrhynchum* contains over 50 volatiles, but is dominated by common floral volatiles such as linalool, α- and β-pinene, myrcene, eucalyptol and methyl eugenol, several of which were shown to elicit an electrophysiological response in the antennae of *A. tigrina* beetles (Johnson et al., 2007). Likewise, chafer-pollinated proteas produce complex floral fragrances but are typically dominated by common volatiles such as linalool, benzaldehyde, methyl benzoate, benzyl alcohol, α-pinene and eucalyptol (Steenhuisen et al., 2010-in this issue). These studies are consistent with our results and suggest that chafer beetles utilize various blends of common floral volatiles as cues to identify potential food plants.

Chafer beetles are typically polyphagous nectar or fruit feeding insects and appear to be attracted primarily by volatiles that represent cues to food substrates (Larsson et al., 2003). Studies examining olfaction in pest chafer species have shown that these beetles can detect a wide range of volatiles in various compound classes and many of these (especially aromatics) are attractive to chafer beetles (e.g. Bengtsson et al., 2009; Donaldson et al., 1986, 1990; Johnson et al., 2007; Larsson et al., 2003; Toth et al., 2004; Vuts et al., 2010a,b; Wolde-Hawariat et al., 2007). Many of the compounds found in the scents of the seven study species are common floral volatiles (ESM Tables 1 and 2; Knudsen et al., 2006) and several of these are established chafer attractants (highlighted in Table 2 and ESM Tables 1 and 2). Although certain single compounds can be attractive to chafers (Donaldson

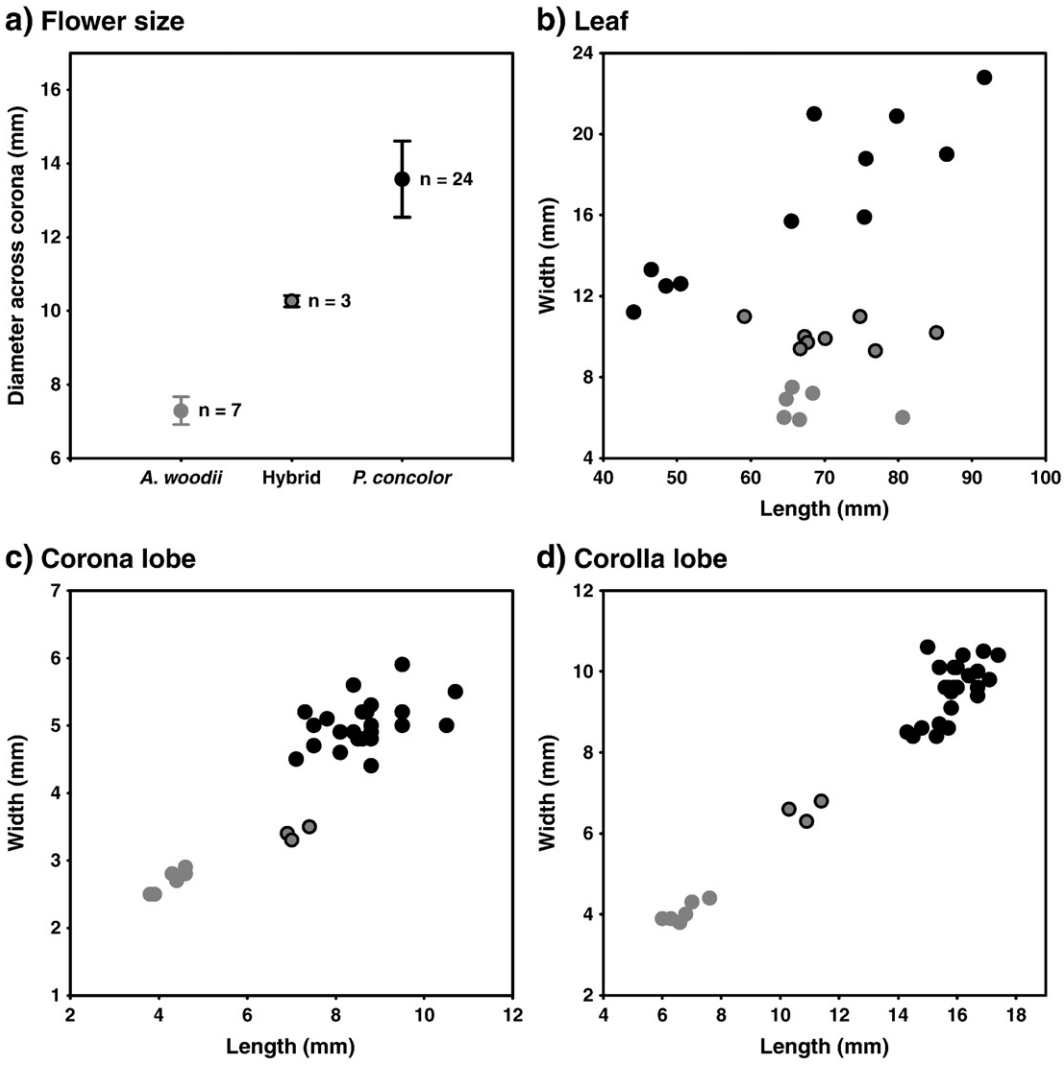

Fig. 3. Morphometrics of the flowers and leaves of the hybrid plants and of the putative parent species, *Pachycarpus concolor* and *Asclepias woodii* from Midmar Nature Reserve. Flower sizes represent means±s.d. Black circles = *P. concolor*; grey circles = *A. woodii*; black and grey circles = hybrid.

et al., 1986, 1990; Larsson et al., 2003; Wolde-Hawariat et al., 2007), there is also evidence that compounds can have synergistic effects, such that they are more attractive in combination than singly, or are only attractive in combination (Larsson et al., 2003; Toth et al., 2004; Vuts et al., 2010a,b). This, together with phylogenetic effects, could explain the lack of overlap between the scents of the seven chafer-pollinated asclepiads (Fig. 2), as different species may be utilizing different combinations of compounds to attract the same beetles.

Differences in the scents of the study species may also relate to the particular beetle species involved as their relative abundances varied between plant species (Ollerton et al., 2003; Shuttleworth and Johnson, 2009a). Thus, *P. concolor* and *Pachycarpus* sp. nov. appear to attract only *A. tigrina*; *A. woodii* and *Xysmalobium involucratum* attract both *A. tigrina* and *C. marginalis*; and, *P. scaber* attracts only *C. marginalis* and *Leucoscelis* spp. (Table 1). Differences in the rank order of attractiveness of particular compounds to different scarab species have been noted in previous studies (Larsson et al., 2003) and a similar mechanism may be responsible for the partitioning of beetle species between chafer-pollinated plants. This partitioning of beetles between plant species represents further specialization within the overall guild, and may be an adaptive response to reproductive interference resulting from the utilization of highly generalist pollinators.

The potential for reproductive interference between guild members is well illustrated by the suspected *A. woodii*× *P. concolor* hybrids discovered at Midmar Nature Reserve. This site contains a population of c. 40 plants of each species growing side by side, and evidence from morphometrics (Fig. 3) and scent (Fig. 2; ESM Table 1) suggests that the putative hybrids are the result of cross pollination between *A. woodii* and *P. concolor*. In addition, the putative hybrids exhibited a semi-decumbent growth habit intermediate between the erect habit of *A. woodii* and the decumbent habit of *P. concolor*. Movement of pollen between these two species is likely since both are pollinated by the beetle *A. tigrina* which is common at this site (Ollerton et al., 2003; Shuttleworth and Johnson, 2008, 2009a). Furthermore, the pollinaria of both plant species are attached to the beetles' legs (Ollerton et al., 2003; Shuttleworth and

Johnson, 2009a) and transfer between species could conceivably occur. If this were the case, *A. woodii* would most likely be the pollen donor since the relatively large pollinia of *P. concolor* would not be easily inserted in the small flowers of *A. woodii*. Alternatively, these plants may be the result of hybridization between either of these species and other sympatric asclepiads as several species of *Asclepias* and *Pachycarpus* are known to occur in the region. More comprehensive sampling of the parent species as well as molecular fingerprinting studies would be required to resolve this question fully.

Hybridization has seldom been documented in African asclepiads, although Weale (1873) described the occurrence of possible hybrids between *Gomphocarpus physocarpus* and *G. fruticosus* where their ranges overlap in the Eastern Cape. In contrast, hybridization has been reported in several North American *Asclepias* species (Broyles, 2002; Kephart et al., 1988; Klips and Culley, 2004; Wyatt and Broyles, 1992; Wyatt and Hunt, 1991). A possible explanation may be that the relatively specialized pollination systems found in South African asclepiads (Ollerton et al., 2003, 2006; Pauw, 1998; Shuttleworth and Johnson, 2006, 2008, 2009a,b,c,d; but see Liede and Whitehead, 1991) are a more effective isolating mechanism than the generalized pollination systems found in North American asclepiads (Fishbein and Venable, 1996; Ivey et al., 2003; Kephart and Theiss, 2004; Kephart, 1983; Theiss et al., 2007; Willson and Bertin, 1979).

Hybridization in plants is an interesting phenomenon as it has been suggested to result in novel traits which may contribute to adaptive variation through introgression (Barton, 2001; Broyles, 2002; Lewontin and Birch, 1966; Rieseberg et al., 2003; Stebbins, 1959). In some instances, hybridization may also result in novel recombinant species if the hybrid genotypes establish and maintain isolation from the parents (Rieseberg, 1997, 2006; Rieseberg et al., 1995, 2003). The putative hybrids reported in this study represent one of the few documented cases of hybridization in African asclepiads and warrant further study. Future research needs to examine pollination success in putative hybrids to assess the possibility of introgression between *A. woodii* and *P. concolor*. Interestingly, the floral scents of *A. woodii* and *P. concolor* were more similar to each other than to most of the other species analyzed (including congeneric species; Fig. 2), suggesting the possibility of some gene flow between these two species, although a plausible alternative explanation would be that the scents are similar because of convergence driven by adaptation to the same pollinators. *A. woodii* has a limited distribution in KwaZulu-Natal (Nicholas et al., 2009) where it overlaps with the widely distributed *P. concolor* (Smith, 1988). It would also be interesting to compare traits of *P. concolor* plants from regions where *A. woodii* does not occur to those of plants in the hybrid zone.

In conclusion, future studies need to examine the contributions of adaptation versus phylogenetic history to the blends of compounds that characterize chafer beetle-pollinated species. It would be interesting to distinguish between selection for attractiveness versus selection for blends that promote foraging constancy and thereby limit reproductive interference resulting from the common utilization of generalist pollinators. This study also demonstrates that floral volatile data can be useful as additional evidence for hybridization.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.sajb.2010.07.023.

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SUPPLEMENTARY MATERIAL FOR APPENDIX 1

Electronic supplementary material table 1. Relative amounts (%) of compounds identified by GC-MS from headspace samples of *Asclepias albens*, *A. woodii*, *Pachycarpus concolor* and the *A. woodii* X *P. concolor* putative hybrid. Compounds are listed in order of increasing Kovats retention index (KRI, calculated from our retention times using an Alltech EC-WAX column) within each compound class [a]. Compounds common to the hybrid and either or both parent species are shaded. Compounds unique to the hybrid in bold. Compounds in bold are those that have been shown to elicit an electroantannagraphic response (superscript e) or to be attractive (superscript f) to cetoniin beetles (see footnotes for references).

			Asclepias albens						*Asclepias woodii*					Hybrid	*Pachycarpus concolor*						
Compound	KRI	ID [b]	1	2	3	4	5	6	1	2	3	4	5	1	1	2	3	4	5	6	7
Aliphatics																					
Acids																					
2-Methylbutanoic acid	1690	A	-	-	-	-	-	-	1.6	tr	tr	1.6	tr	1.4	-	-	-	-	-	-	-
Alcohols																					
Hexan-1-ol [e]	1364	C	0.2	0.3	0.1	0.1	0.9	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-
(Z)-Hex-3-en-1-ol [e]	1398	A	0.3	0.1	0.5	tr	0.2	0.5	2.5	2.2	8.7	2.0	-	4.8	3.8	0.5	2.2	1.4	8.9	7.6	0.2
Oct-1-en-3-ol [c, e]	1461	C	tr	0.3	0.1	0.2	0.1	0.1	-	-	-	-	-	**0.1**	-	-	-	-	-	-	-
Hexadecan-1-ol	2392	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2
Aldehydes																					
(Z)-Hex-3-enal	1179	A	-	-	-	-	-	-	-	-	-	-	-	-	-	2.1	-	-	-	-	-
(E)-Hex-2-enal [e]	1240	A	-	-	-	-	-	-	-	-	1.2	-	-	-	-	1.4	-	-	-	-	-
Esters														-							
Hexyl acetate [e]	1292	C	-	-	-	-	-	-	1.2	0.8	4.7	0.1	tr	0.2	1.0	0.6	0.4	-	-	0.3	0.4
(Z)-Hex-3-en-1-yl acetate [e]	1335	A	3.1	10.2	5.9	-	1.8	0.2	83.4	85.7	58.6	41.2	14.3	87.0	73.5	51.6	73.9	3.7	25.9	9.9	42.8
(Z)-Hex-en-1-yl butyrate [e]	1479	A	-	-	-	-	-	-	-	-	-	-	-	0.6	-	0.3	0.5	-	-	-	0.1
(Z)-Hex-3-en-1-yl isovalerate	1490	A	-	-	-	-	-	-	-	-	-	-	-	**0.2**	-	-	-	-	-	-	-
Aromatics																					
Anisole [c]	1362	C	-	-	tr	-	0.3	tr	-	-	-	-	-	-	-	-	-	-	-	-	-
Benzaldehyde [e]	1546	C	0.3	0.4	0.6	0.1	0.5	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-
Methylbenzoate [e, f]	1651	C	1.4	15.5	tr	1.0	0.2	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl salicylate [e, f]	1794	C	0.2	0.8	-	tr	tr	tr	0.2	-	0.2	1.1	-	-	-	-	-	-	-	-	-
3,4-Dimethoxytoluene	1827	B	tr	tr	0.1	tr	0.1	tr	-	-	-	-	-	-	-	-	-	-	-	-	-
3,5-Dimethoxytoluene [e]	1879	B	-	0.1	0.1	0.1	tr	tr	-	-	-	-	-	-	-	-	-	-	-	-	-
Benzyl alcohol [c, e]	1900	C	0.9	1.7	0.1	0.2	0.7	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-
Phenylethyl alcohol [e]	1940	A	0.1	1.5	0.1	tr	-	tr	-	-	-	-	-	-	-	-	-	-	-	-	-
2-Methoxy-4-methyl-1-hydroxybenzene	1978	A	0.4	2.2	tr	tr	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3-Phenylpropanol	2073	A	0.1	0.1	-	tr	1.8	tr	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl methoxybenzoate	2095	B	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-

***p*-Cresol** [e]	2102	A	tr	0.8	tr	tr	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-
(Z)-Hex-3-en-1-yl benzoate	2154	C	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	0.1	-	-	-
Benzyl benzoate	2645	C	0.7	1.1	tr	tr	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-
2-Ethylhexyl-4-methoxycinnamate	3057	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	tr	-	tr	0.4
Isoprenoids																				
Monoterpenes																				
Myrcene	1202	A	11.6	5.5	57.0	1.6	2.1	2.9	-	0.4	3.8	6.8	15.4	1.2	9.8	0.6	3.0	33.1	20.1	6.2	34.0
Limonene	1229	C	1.6	3.5	4.9	0.8	0.7	0.3	-	-	0.7	2.7	4.2	0.1	0.7	tr	1.5	22.4	17.3	2.4	10.2
(Z)-Ocimene	1257	C	2.2	1.7	0.8	1.5	0.7	1.0	-	-	-	-	-	-	0.8	1.2	tr	-	tr	0.5	tr
(E)-Ocimene	1276	A	5.2	1.1	5.5	0.9	0.8	0.7	1.5	tr	2.6	5.5	8.5	0.5	2.0	33.3	1.1	5.3	18.9	8.2	5.9
(Z)-Linalool oxide (furanoid) [e]	1485	C	tr	tr	tr	tr	tr	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-
2,6,6-Trimethyl-2-vinyltetrahydropyran-5-one	1494	B	0.4	0.3	tr	0.2	0.2	tr	-	-	-	-	-	-	-	-	-	-	-	-	-
Linalool [e, f]	1557	C	66.5	40.0	9.0	89.2	85.7	91.9	0.7	5.1	1.7	3.9	12.2	0.4	tr	tr	tr	0.2	1.1	tr	0.1
Hotrienol	1625	A	0.2	0.2	tr	0.2	0.3	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-
(Z)-Linalool oxide (pyranoid) [e]	1782	A	0.1	0.1	-	tr	0.1	tr	-	-	-	-	-	-	-	-	-	-	-	-	-
2,6-Dimethylocta-3,7-diene-2,6-diol [c]	1957	A	0.1	tr	-	tr	tr	tr	-	-	-	-	-	-	-	-	-	-	-	-	-
Sesquiterpenes																				
α-Cubebene	1479	A	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	3.2	-	tr	0.1
α-Copaene	1517	A	0.3	tr	0.1	-	tr	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Bourbonene	1543	A	-	-	-	-	-	-	-	-	-	-	-	-	tr	-	0.1	0.6	-	tr	tr
β-Cubebene	1564	A	-	-	-	-	-	-	-	-	-	-	-	-	tr	-	tr	2.8	0.7	0.2	0.1
β-Elemene	1614	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.8	1.3	-	13.3	-
Caryophyllene [f, e]	1623	C	0.7	0.4	1.4	0.1	0.3	0.1	-	-	-	-	-	-	0.7	0.1	4.7	7.4	3.2	18.6	0.4
γ-Elemene	1667	A	-	-	-	-	-	-	-	1.1	0.3	0.4	0.8	-	-	-	0.6	-	-	4.2	-
(E)-β-Farnesene	1685	A	-	-	-	-	-	-	-	tr	12.4	16.1	5.4	-	-	-	tr	-	-	-	-
Humulene	1698	A	0.1	tr	0.2	tr	-	tr	-	-	-	-	-	-	tr	-	2.5	1.1	-	12.6	tr
Germacrene D	1739	A	0.5	0.4	3.0	0.1	tr	0.1	-	-	-	-	-	-	0.1	-	0.8	12.9	4.0	4.5	tr
α-Farnesene	1772	B	tr	0.3	tr	tr	tr	tr	-	-	-	-	-	-	-	-	-	-	-	-	-
Caryophyllene epoxide	2022	C	-	-	-	-	-	-	-	-	-	-	-	-	0.7	-	tr	-	-	0.4	-
(E)-Nerolidol	2055	C	0.2	0.2	tr	tr	0.1	tr	-	-	-	-	-	-	-	-	-	-	-	-	-
Terpene derived compounds																				
4-Oxoisophorone	1721	A	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	-	-	-	-	0.3
Miscellaneous cyclic compounds																				
2-Ethylfuran	1062	A	-	-	-	-	-	-	-	-	-	-	-	-	0.6	1.3	0.2	-	-	-	1.0
2-Methylcyclopent-2-en-1-one	1391	A	0.1	0.1	0.2	tr	0.1	tr	2.4	1.6	1.5	-	0.4	-	1.4	0.3	0.1	-	-	0.2	0.5
Cyclohex-2-en-1,4-dione	1766	B	-	-	-	-	-	-	-	-	-	-	-	**0.1**	-	-	-	-	-	-	-
5-Ethyl-2(5H)-furanone	1792	A	-	-	-	-	-	-	-	-	-	-	-	-	tr	0.2	tr	-	-	-	tr
Unknowns [d]																				
Sesquiterpenes																				
m/z: 204*,105,161,119,91,189,55,93,41	1559		-	-	-	-	-	-	-	-	-	-	-	-	tr	tr	tr	0.6	-	-	0.1
m/z: 93,119,41,69,43,55,91,71,67,79,107	1608		-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	-	-	0.1	tr

m/z: 204*,93,81,161,79,67,105,91	1606	-	-	-	-	-	-	-	-	-	-	-	-	tr	-	0.1	0.7	-	0.6	tr
m/z: 204*,93,69,41,105,79,94,91	1754	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	0.1	0.4	-	0.5	tr
m/z: 204*,121,93,41,107	1763	0.1	0.1	0.3	tr	-	tr	-	-	-	-	-	-	-	-	-	1.0	-	0.4	-
m/z: 204*,161,119,105,134,91,41,55	1787	-	-	tr	-	-	-	-	-	-	-	-	-	-	-	-	1.8	-	0.4	-
m/z: 204*,67,93,107,68,105,79,161,119	1797	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	1.7	tr
m/z: 204*,121,93,105,107,41,67,79,81,91	1862	-	-	-	-	-	-	-	0.6	0.8	0.6	0.4	-	-	-	0.8	-	-	6.3	-
Unknown compound class																				
m/z: 83,55,69,41,39,42,70,56	1230	-	-	-	-	-	-	-	-	-	-	-	-	-	0.6	-	-	-	-	-
m/z: 150*,69,41,81,79,107,119,79,82	1298	0.4	0.6	1.0	0.1	0.1	tr	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 150*,69,41,81,79,82,53	1325	2.1	10.2	8.7	3.3	1.5	1.3	6.6	2.5	2.8	17.9	38.5	-	2.3	0.6	1.3	-	-	0.2	0.4
m/z: 43,80,79,39,41,77,81	1448	-	-	-	-	-	-	-	-	-	-	-	0.1	0.2	0.1	0.3	-	-	-	0.3
m/z: 112*,55,83,97,57,56	1541	-	-	-	-	-	-	-	-	-	-	-	-	tr	0.1	tr	-	-	-	tr
m/z: 57,85,86,43,55,42,41 [c]	1567	-	-	-	-	-	-	-	-	-	-	-	1.1	1.5	3.5	1.9	tr	tr	0.4	1.7
m/z: 112*,83,55,57,84	1619	tr	tr	tr	-	0.1	tr	-	-	-	-	-	1.9	0.3	0.4	0.2	-	-	-	0.3
m/z: 152*,43,109,81,79,67,91,55	1841	0.1	tr	0.1	tr	tr	tr	-	-	-	-	-	-	-	0.1	-	-	-	-	-
m/z: 57,85,39,41,43,55,31	1986	-	-	-	-	-	-	-	-	-	-	-	0.5	-	0.9	0.2	-	-	-	0.3
m/z: 79,93,91,77,80,94,41,55,67,121	2055	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	-	-
m/z: 112*,97,55,67,56,41,39	2111	-	-	-	-	-	-	-	-	-	-	-	0.1	0.1	0.1	0.1	-	-	tr	0.1
m/z: 112*,97,55,67,56,57,41	2132	-	-	-	-	-	-	-	-	-	-	-	tr	tr	tr	tr	-	-	tr	tr
Aliphatics		3.6	10.9	6.7	0.2	2.9	1.1	88.7	88.6	73.1	45.0	14.3	94.3	78.4	56.6	77.0	5.1	34.8	17.9	43.6
Aromatics		4.0	24.2	0.9	1.4	4.0	0.2	0.2	0	0.2	1.1	0	0.0	0	0	0.1	0	0	0	0.4
Isoprenoids (including unknown sesquiterpenes)		89.6	53.9	82.2	94.6	91.1	97.1	2.2	7.2	22.4	36.0	46.8	2.2	14.9	35.1	18.4	94.9	65.2	81.1	51.1
Miscellaneous cyclic compounds		0.1	0.1	0.2	0	0.1	0	2.4	1.6	1.5	0	0.4	0.1	2.1	1.7	0.4	0	0	0.2	1.5
Unknowns (excluding sesquiterpenes)		2.6	10.8	9.7	3.5	1.7	1.3	6.6	2.5	2.8	17.9	38.5	3.7	4.3	6.5	3.9	0	0	0.6	3.1
Total of all compounds		99.8	99.9	99.7	99.7	99.9	99.7	100	100	100	100	100	100	99.7	99.9	99.7	100	100	99.9	99.8
Total number of compounds		36	37	35	34	36	34	9	12	15	13	12	18	27	30	37	20	11	30	34
Total volatiles (µg) emitted (per inflorescence per hour)		5.6	2.6	5.8	10.4	4.0	9.0	1.0	1.6	1.1	0.4	0.1	14.6	4.6	32.6	19.2	0.2	0.03	0.9	3.0

[a] Relative amounts (%). tr = trace amount (< 0.05 % of total sample).

[b] Compound identification criteria: 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.

[c] Compounds which are possible background contaminants as they were present in some control samples.

[d] Mass fragments for unknowns are listed with the molecular ion first (if known) marked with *, followed by the base peak and other fragments in decreasing order of abundance.

[e] Compounds that have been shown to elicit an electroantanngraphic (EAG) response from cetoniin beetles (Larsson et al., 2001; Johnson et al., 2007; Bengtsson et al., 2009; Vuts et al., 2010a,b).

[f] Compounds that have been shown to attract cetoniin beetles in traps or mazes (Donaldson et al., 1986; Larsson et al., 2003; Wolde-Hawariat et al., 2007).

Electronic supplementary material table 2. Relative amounts (%) of compounds identified by GC-MS from headspace samples of *Pachycarpus plicatus*, *P. scaber*, *Pachycarpus* sp. nov. and *Xysmalobium involucratum*. Compounds are listed in order of increasing Kovats retention index (KRI, calculated from our retention times using an Alltech EC-WAX column) within each compound class [a]. Compounds in bold are those that have been shown to elicit an electroantannagraphic (EAG) response (superscript e) or to be attractive (superscript f) to cetoniin beetles (see footnotes for references). Footnotes are as for ESM Table 1.

			Pachycarpus plicatus					*Pachycarpus scaber*					*Pachycarpus* sp. nov.					*Xysmalobium involucratum*				
Compound	RRI	ID [b]	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Aliphatics																						
Acids																						
2-Methylbutanoic acid	1690	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.2	-	-	-
Alcohols																						
Hexan-1-ol [e]	1364	C	-	-	-	-	-	-	-	-	-	-	0.7	0.2	0.2	0.2	0.2	tr	tr	0.1	tr	0.1
(E)-Hex-3-en-1-ol [e]	1375	A	-	-	-	-	-	-	-	-	-	-	0.3	-	tr	0.2	tr	tr	tr	tr	tr	tr
(Z)-Hex-3-en-1-ol [e]	1398	A	3.8	0.7	1.0	1.8	0.6	3.2	2.1	0.4	2.0	0.9	16.6	1.0	3.8	1.8	1.8	tr	0.2	0.6	tr	1.5
(E)-Hex-2-en-1-ol [e]	1417	A	-	-	-	-	-	-	-	-	-	-	1.0	0.8	0.6	0.7	0.3	-	-	-	-	-
Hexadecan-1-ol	2392	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	tr	tr	-	tr
Aldehydes																						
(Z)-Hex-3-enal	1179	A	-	2.6	0.7	0.1	0.1	-	-	-	-	-	-	-	-	-	-	-	0.1	tr	-	-
(E)-Hex-2-enal [e]	1240	A	0.3	1.8	1.2	1.1	-	-	-	-	-	-	3.7	5.3	0.6	0.2	0.2	-	-	tr	-	0.1
Esters																						
Methyl tiglate	1214	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	0.2	-	tr
Hexyl acetate [e]	1292	C	1.6	1.7	1.5	0.9	0.9	-	-	-	-	-	1.1	0.4	0.4	0.1	0.7	-	-	-	-	-
(Z)-Hex-3-en-1-yl acetate [e]	1335	A	69.7	64.5	76.4	70.8	66.9	1.1	4.8	1.0	9.7	1.1	28.0	5.0	20.9	8.2	14.1	0.3	4.1	tr	tr	7.7
(Z)-Hex-3-en-1-yl isovalerate	1490	A	0.7	0.2	0.6	tr	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ketones																						
But-3-en-2-one	1051	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	tr	-
Mesityloxide	1169	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.3	-	-	0.1	-
Aromatics																						
Anisole [e]	1362	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	tr	tr	-	-
Benzaldehyde [e]	1546	C	-	-	-	-	-	67.7	73.5	72.5	30.5	52.3	-	-	-	-	-	14.8	22.4	16.7	4.9	17.9
Methylbenzoate [e, f]	1651	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7.6	12.4	23.8	2.5	6.8
Phenylacetaldehyde [c, e, f]	1668	C	-	-	-	-	-	0.4	3.5	5.3	4.4	0.8	-	-	-	-	-	0.5	1.1	6.9	0.1	2.1
Ethyl benzoate	1689	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	0.1	0.1	tr	0.2
Benzyl formate	1716	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	tr	tr	-	tr
Benzyl acetate [f]	1752	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	tr	tr	tr	tr
Methyl phenylacetate	1788	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	-	-

Methyl salicylate [e, f]	1794	C	-	tr	tr	0.2	tr	tr	0.1	0.4	0.3	tr	-	-	-	-	-	1.2	1.3	1.3	0.1	0.9
3-Phenylpropanal	1806	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	tr
2-Phenylethyl formate	1810	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.4	tr	tr	tr	tr
Phenylethyl acetate [c, e]	1841	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.3	0.1	-	-	0.4
Methyl 3-phenylpropanoate	1872	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	-	-	-
Benzyl alcohol [c, e]	1900	C	-	-	-	-	-	0.5	1.0	1.2	1.1	0.3	-	-	-	-	-	1.1	0.9	2.1	0.4	0.8
Phenylethyl alcohol [e]	1940	A	-	-	-	-	-	0.1	0.3	0.6	0.6	0.1	-	-	-	-	-	11.9	1.6	7.7	3.8	2.5
(E)-Cinnamaldehyde [e]	2067	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.7	tr	2.6	tr	tr
3-Phenylpropanol	2073	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	tr	0.5	tr	tr
Methyl cinnamate [e, f]	2100	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	0.3	-	tr	0.2
***p*-Cresol** [e]	2102	A	-	-	-	-	-	tr	tr	tr	tr	tr	-	-	-	-	-	-	-	-	-	-
(Z)-Hex-3-en-1-yl benzoate	2154	C	-	-	-	-	-	tr	tr	-	1.2	tr	-	-	-	-	-	-	-	-	-	-
Cinnamic alcohol [e, f]	2301	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	-	0.2	-	-
Benzyl benzoate	2645	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	tr	-	tr	0.5
Isoprenoids																						
Monoterpenes																						
α-Pinene	1093	C	-	-	-	-	-	7.4	0.4	0.2	0.4	2.3	-	-	-	-	-	0.1	-	-	-	-
Myrcene	1202	A	-	1.0	0.5	7.7	7.5	5.5	2.7	7.6	21.8	24.0	8.2	52.2	24.0	11.6	6.1	0.9	0.4	tr	0.7	0.1
Limonene	1229	C	-	0.1	1.0	5.6	10.2	3.3	3.6	1.1	8.1	7.1	1.1	5.3	1.2	0.4	0.5	-	-	-	tr	-
Eucalyptol	1240	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.2	tr	-	-	tr
(Z)-Ocimene	1257	C	tr	5.2	tr	-	0.4	0.5	0.3	0.1	5.7	3.9	0.7	2.5	3.5	4.5	1.9	0.1	0.5	0.4	tr	0.8
(E)-Ocimene	1276	A	tr	2.8	0.7	0.5	1.0	0.8	0.8	1.4	4.6	2.0	0.1	1.1	0.7	1.7	48.7	19.3	46.7	22.6	0.1	53.1
Terpinolene	1307	A	-	-	-	-	-	0.1	0.1	0.1	0.6	0.6	-	-	-	-	-	-	-	-	-	-
(E)-Linalool oxide (furanoid) [e]	1456	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5.9	-	-	1.1	-
(Z)-Linalool oxide (furanoid) [e]	1485	C	-	-	-	-	-	-	-	-	-	-	0.1	0.3	1.3	0.4	tr	0.5	tr	tr	0.9	-
2,6,6-Trimethyl-2-vinyltetrahydropyran-5-one	1494	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	-	-	-	-
Linalool [e, f]	1557	C	1.9	0.9	1.3	0.5	3.0	-	0.2	0.4	0.8	0.1	9.4	12.0	11.7	27.5	6.2	16.9	0.1	0.5	83.6	0.1
Hotrienol	1625	A	-	-	-	-	-	-	-	-	-	-	1.3	2.6	5.2	7.2	1.5	0.1	-	-	0.2	-
α-Terpineol	1716	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.8	tr	-	tr	tr
Pinocarveol	1732	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	0.1	tr	tr	0.1
Geranial	1753	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	tr	tr	0.1	tr
(E)-Linalool oxide (pyranoid) [e]	1761	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7.0	tr	tr	0.9	tr
(Z)-Linalool oxide (pyranoid) [e]	1782	A	-	-	-	-	-	-	-	-	-	-	0.1	0.1	0.4	0.1	0.3	0.9	-	3.2	-	-
Nerol	1822	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	-	tr	tr	-
(E,E)-2,6-Dimethylocta-3,5,7-triene-2-ol	1823	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	0.1	0.1	tr	0.2
Geraniol [e, f]	1867	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	-	tr	0.1	-
2,6-Dimethylocta-3,7-diene-2,6-diol [c]	1957	A	-	-	-	-	-	-	-	-	-	-	tr	-	0.1	0.1	-	tr	-	-	0.2	-
2,6-Dimethylocta-1,7-diene-3,6-diol	2155	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-
Sesquiterpenes																						

α-Cubebene	1479	A	-	-	-	-	-	0.1	0.1	tr	0.3	tr	-	-	-	-	-	-	-	-	-	-
α-Copaene	1517	A	-	-	-	-	-	0.2	0.2	0.2	1.2	0.1	-	-	-	-	-	-	-	-	-	-
β-Cubebene	1564	A	-	-	-	-	-	tr	tr	tr	0.2	tr	-	-	-	-	-	-	-	-	-	-
Caryophyllene [f, e]	1623	C	4.4	3.2	3.4	2.2	3.7	0.1	0.1	0.1	0.7	0.1	-	tr	-	tr	tr	-	-	-	tr	-
Humulene	1698	A	0.2	0.3	0.4	0.4	0.3	tr	tr	tr	0.2	tr	-	-	-	-	-	-	-	-	-	-
Germacrene D	1739	A	0.3	0.1	0.1	0.1	0.5	0.3	0.5	0.2	1.2	0.2	0.1	0.1	0.1	0.3	tr	-	-	-	-	-
α-Farnesene	1772	B	-	-	-	-	-	0.1	tr	-	0.1	tr	-	-	-	-	-	-	-	-	-	-
(E)-Nerolidol	2055	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	-	-
Terpene derived compounds																						
4-Oxoisophorone	1721	A	-	-	-	-	-	0.1	0.1	6.1	0.1	2.2	5.2	8.6	11.8	28.5	12.2	-	-	-	-	-
Nitrogen-containing compounds																						
2-Phenylacetonitrile [e]	1955	C	1.0	-	0.4	0.4	0.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indole	2457	A	tr	tr	0.1	tr	0.1	0.1	tr	tr	tr	tr	-	-	-	-	-	-	-	-	-	-
Miscellaneous cyclic compounds																						
2-Ethylfuran	1062	A	-	-	-	-	-	1.0	0.4	tr	0.2	0.1	-	-	-	-	-	-	-	-	-	-
2-Methylcyclopent-2-en-1-one	1391	A	tr	1.3	0.5	3.6	tr	1.4	0.1	tr	0.1	0.1	0.3	tr	tr	tr	0.5	0.1	tr	tr	tr	0.5
3-Oxo-7,8-dihydro-α-ionone	2557	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	tr	0.3	tr	tr
Unknowns [d]																						
Sesquiterpenes																						
m/z: 204*,105,161,57,119,91,94,93,120	1508		-	-	-	-	-	tr	-	tr	0.2	-	-	-	-	-	-	-	-	-	-	-
m/z: 204*,120,161,105,93,91,79	1601		-	-	-	-	-	tr	tr	tr	0.1	tr	-	-	-	-	-	-	-	-	-	-
m/z: 204*,93,119,69,91,79,161,105,107	1748		-	-	-	-	-	0.1	0.1	tr	0.1	tr	-	-	-	-	-	-	-	-	-	-
m/z: 204*,93,69,41,105,79,94,91	1754		-	-	-	-	-	0.1	0.1	tr	0.1	tr	-	-	-	-	-	-	-	-	-	-
m/z: 204*,121,93,41,107	1763		0.1	0.1	0.3	tr	0.1	0.3	tr	0.6	1.4	0.1	0.2	0.5	0.2	1.4	tr	-	-	-	-	-
m/z: 204*,161,119,105,134,91,41,55	1787		-	-	-	-	-	tr	tr	0.1	0.2	tr	-	-	-	-	-	-	-	-	-	-
Unknown compound class																						
m/z: 83,55,69,41,39,42,70,56	1230		-	0.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 150*,69,41,81,79,82,53	1325		2.1	1.1	5.4	1.2	1.1	-	-	-	-	-	0.1	0.4	0.4	0.6	0.2	tr	0.1	0.4	tr	tr
m/z: 43,80,79,39,41,77,81	1448		0.1	0.4	0.3	0.1	0.1	-	tr	-	tr	-	-	-	-	-	-	-	-	-	-	-
m/z: 134*,91,119,77,79,92,105	1451		-	-	-	-	-	-	-	-	-	-	-	-	0.1	tr	0.2	tr	0.1	tr	tr	0.1
m/z: 154*,43,71,93,81,55,41,111,69	1477		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	-
m/z: 79,81,77,41,72,53	1503		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5.0	1.0	7.1	-	1.2
m/z: 112*,55,83,97,57,56	1541		0.2	0.2	tr	tr	tr	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 57,85,86,43,55,42,41 [c]	1567		5.5	8.5	3.6	2.2	1.6	1.4	1.1	0.2	0.4	0.1	8.6	-	2.3	0.3	1.8	-	0.3	0.2	-	0.3
m/z: 71,43,82,67,41,55,39,53	1601		-	-	-	-	-	-	-	-	-	-	tr	0.1	0.2	0.3	tr	-	-	-	tr	-
m/z: 112*,83,55,57,84	1619		2.7	0.9	0.4	0.2	0.4	0.4	0.4	tr	0.2	tr	3.1	0.3	1.0	0.5	0.3	tr	0.5	0.1	tr	tr
m/z: 168*,85,56,125,43,69,41,83,153	1693		-	-	-	-	-	3.3	3.1	0.1	1.0	1.2	5.7	0.1	6.7	0.2	0.2	-	-	-	-	-
m/z: 152*,43,69,109,55,67,41,95	1732		-	-	-	-	-	-	-	-	-	-	0.2	0.2	1.2	1.7	0.3	0.1	tr	tr	tr	tr

m/z: 59,94,79,77,91,93	1742	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	0.1	tr	-	0.1
m/z: 96,71,95,43,67,41,68,81	1765	-	-	-	-	-	-	-	-	-	-	tr	tr	0.1	0.1	0.2	-	-	-	-	-
m/z: 154*,56,42,41,69,70,139,120	1802	-	-	-	-	-	-	-	-	-	-	0.1	0.4	0.5	0.6	0.8	-	-	-	-	-
m/z: 152*,43,109,81,79,67,91,55	1841	-	-	-	-	-	-	-	-	-	-	0.1	0.1	0.3	0.4	0.6	0.7	1.3	0.3	-	0.8
m/z: 132*,103,78,77,51,104,50	1923	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	tr	0.5	-	-
m/z: 57,85,39,41,43,55,31	1986	5.1	1.4	0.2	0.1	0.6	tr	0.1	tr	tr	tr	3.1	0.1	0.3	0.1	0.1	0.1	2.2	0.5	tr	0.2
m/z: 72,97,43,41,68,57,67,95,79,91	2016	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	0.3	0.2	-	0.3
m/z: 72,97,43,41,68,57,55,79,95	2041	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	0.2	0.2	-	0.1
m/z: 112*,97,55,67,56,41,39	2111	0.4	0.2	0.1	0.1	0.1	0.1	0.1	tr	tr	tr	0.3	-	0.1	-	tr	-	-	-	-	-
m/z: 112*,97,55,67,56,57,41	2132	tr	0.1	tr	tr	tr	0.1	tr	-	tr	tr	0.2	-	0.1	-	tr	-	-	-	-	-
Aliphatics		76.1	71.5	81.3	74.7	68.6	4.3	6.9	1.4	11.6	2.1	51.5	12.8	26.4	11.4	17.2	0.7	5.7	0.9	0.1	9.4
Aromatics		0	0	0	0.2	0	68.6	78.3	80.0	38.2	53.5	0	0	0	0	0	38.6	40.1	62.1	11.8	32.4
Isoprenoids (including unknown sesquiterpenes)		6.9	13.7	7.7	17.0	26.8	19.1	9.3	18.1	48.1	42.8	26.6	85.5	60.2	83.8	77.3	54.2	47.9	26.8	87.8	54.3
Nitrogen-containing compounds		1.0	0	0.5	0.4	0.7	0.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Miscellaneous cyclic compounds		0	1.3	0.5	3.6	0	2.5	0.5	0	0.3	0.1	0.3	0	0	0	0.5	0.1	0	0.3	0	0.5
Unknowns (excluding sesquiterpenes)		16.0	13.4	10.0	4.0	3.8	5.3	4.8	0.3	1.6	1.3	21.5	1.7	13.4	4.8	4.7	6.0	6.0	9.5	0	3.1
Total of all compounds		100	99.9	99.9	99.9	99.9	99.9	99.8	99.8	99.9	99.8	100	100	100	100	99.8	99.5	99.8	99.5	99.7	99.8
Total number of compounds		23	27	27	26	26	38	39	36	40	38	32	28	33	32	33	57	50	51	45	46
Total volatiles (µg) emitted (per inflorescence per hour)		0.1	0.4	0.5	0.4	0.8	14.4	16.2	33.6	46.3	22.2	0.9	0.4	0.6	0.2	0.8	21.3	21.3	14.1	24.3	10.0

APPENDIX 2

A NEW SPECIES OF *PACHYCARPUS* E. MEYER (APOCYNACEAE: ASCLEPIADOIDEAE) FROM SOUTHERN AFRICA

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Submitted to Novon

A new species of *Pachycarpus* E. Meyer (Apocynaceae: Asclepiadoideae) from southern Africa

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ABSTRACT. *Pachycarpus acidostelma* Glen M & Nicholas is a new species described from Highflats in the KwaZulu-Natal Province of South Africa. It is differentiated from other closely-related *Pachycarpi*,(*P. scaber* and *P. asperifolius*) by its small stature, inflorescence type, catilliform corolla and corona lobe shape.

During recent fieldwork undertaken at Highflats one of the authors (Shuttleworth) collected a number of specimens of *Pachycarpus* E. Meyer that could not be placed into any previously-known species of this genus. Preliminary assessments of these collections suggested that they maybe allied to *P. asperifolius* C.D.F. Meisner or *P. scaber* (W.H. Harvey.) N.E. Brown, and it was originally thought that they may be of hybrid origin between these two species. Analyses of morphological data, however, suggest that the Highflats population is a distinct entity, most similar to *P. scaber*, and is not a hybrid between this species and *P. asperifolius* (Figures 1 & 2). Based on these findings it was decided that the Highflats *Pachycarpus* population, although of restricted distribution (Figure 3), is deserving of recognition at species level and here receives the name *P. acidostelma* in reference to its sharply-pointed corona lobes.

Pachycarpus acidostelma Glen M & Nicholas, *sp. nov*. **Type:** South Africa, KwaZulu-Natal Province, Ixopo District, 1km from Highflats, 03 Dec. 2007, *A. Shuttleworth 38* (holotype, NU; isotype, MO). Figure 4.

Herbae brevis, erectus, folio late oblongus, floribus albidus vel crememus, corollae ubi juvenis catilliformis, ubi maturus apices reduncus. Affinis Pachycarpus scaber *et* Pachycarpus asperifolius, *coronae brevis et patens, et inflorescentia subcorymbus differt respectus.*

Habit erect perennial geophytic herb. **Stems** mostly solitary, 370--400 mm tall. **Leaves** oval, oblong, 38--79 mm long, 20--34 mm wide; apex obtuse with slight mucronate point; base shortly attenuate, rounded; margin thickened, callose and sparsely ciliate, occasionally almost revolute; vestiture glabrous, with the abaxial midrib hispid; venation prominent on the underside; petiole 3.0--6.5 mm long. **Inflorescences** terminal and axillary, 4-per stem, corymbose or subcorymbose; 4--6 flowered; peduncle free, 11--29 mm long. **Flowers** white to cream, sweetly scented, 13--17 mm tall, 23--25 mm wide; pedicels pubescent, 15--21 mm long. **Sepals** reflexed, scabrid, 7. 5--9.0 mm long, 3.5--6.5 mm wide. **Corolla** cream, spreading and catilliform when young, tips reflexed when mature; lobes ovate, 9--12 mm long, ± 9 mm wide. **Gynostegial column** 3.9--4.8 mm tall. **Corona** cream, tinged yellow at base; lobes 1.4--3.2 mm long, 1.7--2.4 mm wide, 2.5--2.7 mm tall, distal appendage deltoid with sharp apex, spreading, 2.0--2.7 mm long, 0.7--1.9 mm wide. **Androecium:** anther-appendages oblong with acute apex, 1.7--2.0 mm wide, ± 2.0 mm long; anther-wings moderately prominent, 2.4--3.4 mm long, 1.0--1.4 mm wide. **Pollinarium**: corpusculum 0.75--0.85 mm long, ± 0.45 mm wide; translator arm winged, 0.9--1.4 mm long; pollinium dorso ventrally flattened, oblong, 1.35--1.8 mm long, 0.65--0.9 mm wide. **Gynoecium** with style-apex 3.3--3.7 mm in diameter. **Follicle** inflated, ovoid ±90 mm long, ± 34 mm wide; apex angular. **Seeds** not yet seen. IUCN Conservation Status: Endangered.

The genus *Pachycarpus* was formerly recognized by N.E. Brown (1902 & 1908) and currently comprises 44 taxa throughout Africa (Goyder 1998). Thirty of these taxa occur within in southern Africa, 90% of which are endemic to this region (Smith 1988). Excluding the taxa within section Trichocodon, described by D.M.N. Smith (1988), the genus is congruent and has many correlated diagnostic characters that seem to hold true throughout Africa (Nicholas 1990). This has been partially confirmed by molecular data (Goyder et al., 2007).

The corona lobe of *Pachycarpus acidostelma* is somewhat similar in structure to that of *P. asperifolius*, with the result that a specimen (*Shirley 328*) has previously been erroneously named and filed under this latter species. However *P. acidostelma* differs from *P. asperifolius* in having leaves that are oblong, with a mucronulate to rounded apex, corymbose or subcorymbose inflorescences, more uniformly-colored flowers, shorter gynostegial column, and a far more angular follicle. *P.acidostelma* more closely resembles *P. scaber* in flower color, inflorescence type, corolla morphology, and gynostegial column height, and especially in its corymbose inflorescences, but differs significantly from this species in its general, more dainty facie, and quite different corona lobe structure (Fig. 1). Being extremely restricted in distribution, and surrounded by extensive farming and increasing human populations the future conservation of this species relies on its official recognition and inclusion on Red Data lists.

Key to *P. acidostelma* and allies:

1a. Inflorescence a raceme; flowers at different heights along flowering stem; staminal column 5.0 to 13.5 mm tall ……...…….. *P. asperifolius*

1b. Inflorescence corymbose or subcorymbose, flowers held at almost the same level on the flowering stem; staminal column 2.3 to 7.5 mm tall ……...…..... 2

2a. Distal corona-lobe with filiform appendage 5.6 to 10.5 mm long, recurving and connivent over the style-stigma head …….……. *P. scaber*

2b. Distal corona lobe without an appendage, tapering horizontally to a sharp deltoid point ……..……..... *P. acidostelma*

Specimens Consulted:

SOUTH AFRICA. **KwaZulu-Natal:** Highflats, *Shirley 328* (paratype, NU). *Shuttleworth 39,* (paratypes, NU, PRE).

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Figure 1: A: *Pachycarpus acidostelma* flowering branch (Photo: A. Shuttleworth). B. *P. asperifolius* flower with tall staminal column, and small corona-lobes (Photo: P. Wragg). C: *P. acidostelma* flower with deltoid corona-lobes (Photo: A. Shuttleworth). D: *P. scaber* flower with long corona-lobe appendages (Photo: M. Glen).

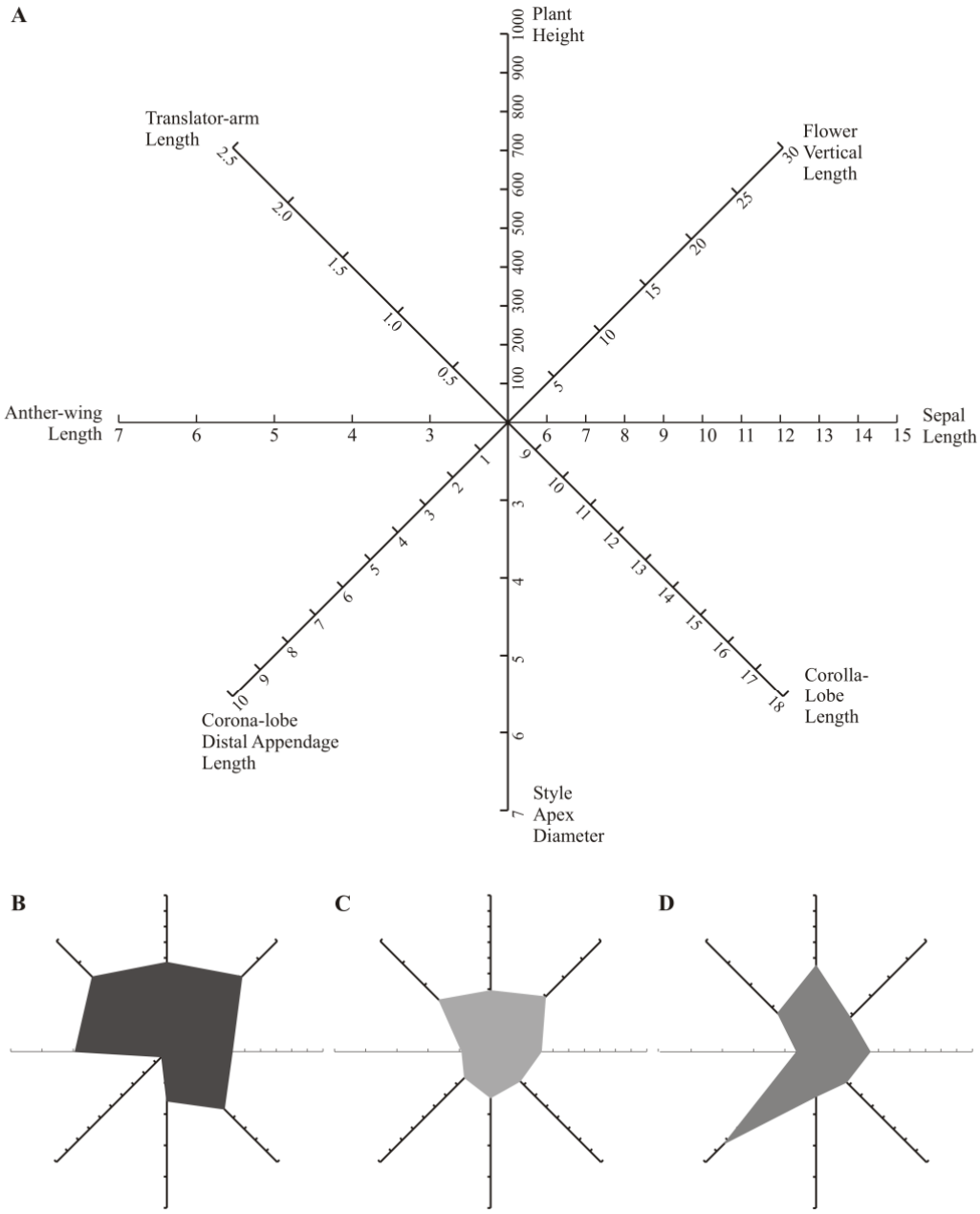

Figure 2: A: 8-Dimensional polygonal graph illustrating relationships between plant height, flower vertical length, sepal length, corolla lobe length, style-apex diameter, distal corona-lobe appendage length, anther-wing length, and translator-arm length B: *Pachycarpus asperifolius*. C: *P. acidostelma*. D: *P. scaber*.

Figure 3: Known distribution of *Pachycarpus acidostelma*.

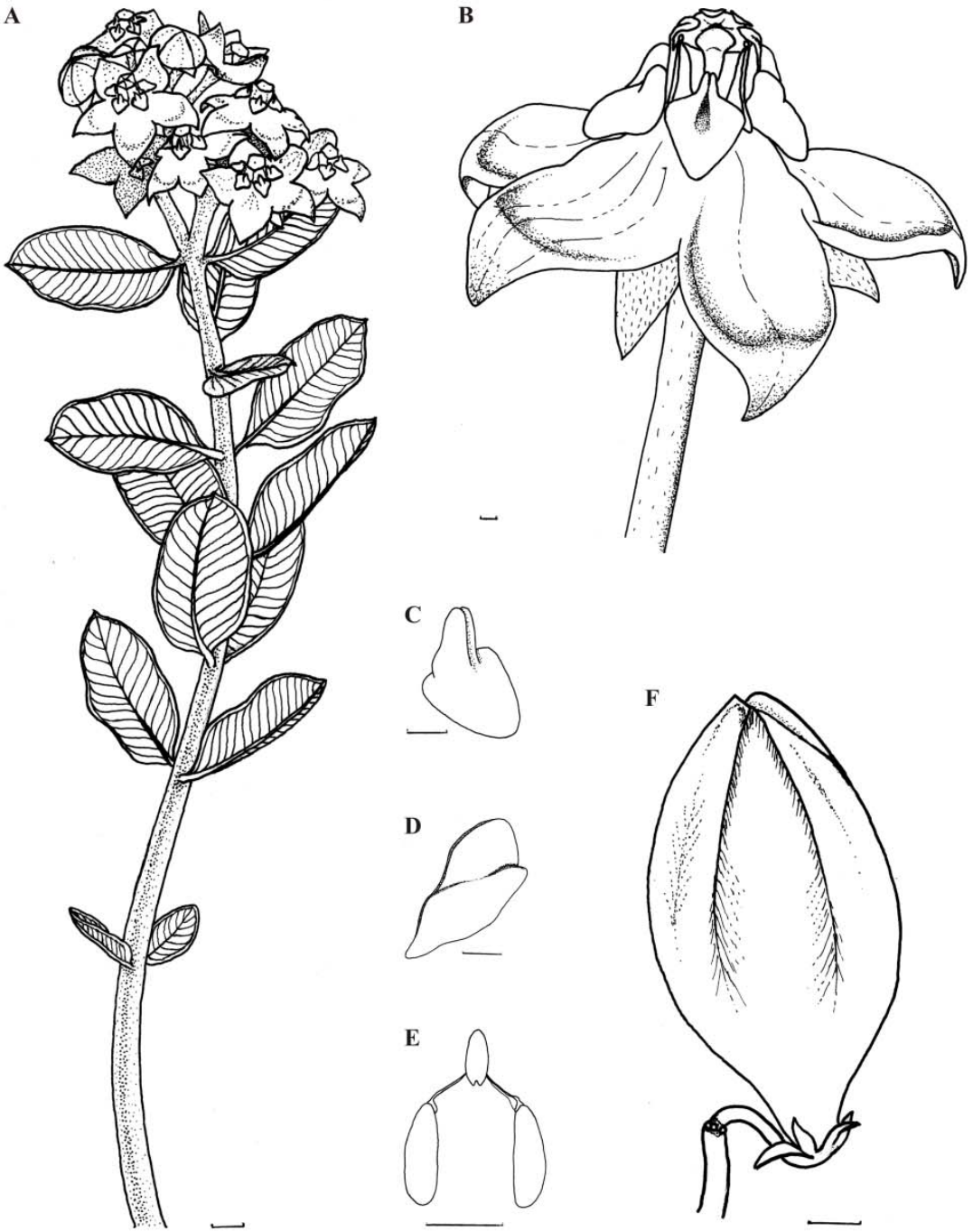

Figure 4: *Pachycarpus acidostelma*. A: Whole plant. B: Flower. C & D: Corona-lobes. E: Pollinarium. F: Fruit. (All scale bars represent 1mm, except A & F, which are 1cm.)