ECOLOGY AND DEGREE OF SPECIALIZATION OF SOUTH AFRICAN MILKWEEDS WITH DIVERSE POLLINATION SYSTEMS

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THESIS

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Abstract

Like orchids, the complexity of flowers found in asclepiads (Asclepiadoideae, Apocynaceae) and the fact that pollen is presented as pollinaria, offers excellent opportunities to study various aspects of plant-pollinator interactions. In this thesis I investigated two broad themes: ecological aspects of the pollination biology of hymenopteran and fly-pollinated asclepiads as well as the degree of specialization to certain pollinators in these species.

Colonizing plants often reproduce through self-pollination, or have highly generalized pollination systems, or both. These characteristics facilitate establishment in small founding populations and generates the prediction that reproductive success should be independent of population size in these species. Chapter one examines the pollination biology of *Gomphocarpus physocarpus*, an indigenous, weedy species and investigates the relationship between reproductive success and population size. In this species, there is no evidence of an Allee effect and reproductive success is not correlated with population size. In addition *G. physocarpus* is not capable of self-pollination, suggesting it is completely reliant on pollinators for seed set. The lack of a relationship between pollination success and population size is therefore likely explained by the generalized wasp pollination system of this species.

Several milkweeds are invasive outside of their native ranges. Invasive species either need to co-opt native pollinators in order to reproduce or reduce their reliance on pollinators through having the ability to self-pollinate. Co-opting native pollinators is expected to be

easier in species that have generalized pollination systems, alternatively species with specialized flower morphologies need to rely on similar functional groups of pollinators to be present within the invaded range. Chapter two investigates the pollination biology and pollination success of the invasive milkweed, *Araujia sericifera*, and finds that in South Africa, this species is visited mainly by native honeybees and nocturnal moths. Moths however contribute little to pollen removal, and deposition. Based on the apparent morphological mismatch between the flower of *A. sericifera* and native honeybees, I propose that the native pollinators of this species are likely to be larger Hymenoptera (e.g. Bumblebees). Data from a breeding system study, indicated that this species is not capable of automatic self-pollination, but could set fruit from geitonogamous self-pollinations pointing to the importance of native pollinators for successful reproduction.

The pollinaria of milkweeds can accumulate on pollinators to form pollen masses large enough to physically interfere with the foraging behaviour of pollinating insects. In chapter three I describe the pollination biology of *Cynanchum ellipticum* and find that this species is mainly pollinated by honeybees although this species is visited by several other members of Hymenoptera, Lepidoptera and Diptera. Due to the structure of the pollinaria, these chain together relatively efficiently and frequently form large pollinarium loads on the mouthparts of honeybees. However there is little evidence that these pollinarium loads influence the foraging times of pollinators and only a few individual honeybees exhibited longer foraging times and most honeybees were unaffected by the presence of large pollinarium loads.

Within the genus *Cynanchum* there is large variation in the gynostegium structure that may influence the pattern of pollinarium loading on pollinators as well as pollen reception as shown in chapter three. In Chapter four, the pollination biology of *Cynanchum obtusifolium* is examined, and like that of *C. ellipticum*, this species is visited by a wide diversity of pollinators but honeybees appear to be the primary pollinators. More importantly this species is shown to be andromonoecious and produces two morphologically different flower types, that may be distinguished based on differences in the gynostegium structure. These two types of flower could mainly be distinguished by the length of the anther wings. I found that flowers with short anther wings function as male flowers by only exporting- and rarely receiving pollinia. Flowers with longer anther wings function as hermaphrodite flowers and can both export and receive pollinia. The ratio of male to hermaphrodite flowers varied at different times during the flowering season, but preliminary data suggested that this was not related to levels of pollination success.

The genera *Stapelia* and *Ceropegia* are well known for their intricate floral adaptations that mimic the brood and feeding substrates of pollinating flies. Despite several studies that have documented the various adaptations to fly pollination in different species, there is a lack of natural history studies documenting different flower visitors, pollen loads and long term levels of pollination success in these species. In Chapter six I document the pollination biology of *Ceropegia ampliata* by documenting different pollinators and quantifying average levels of pollination success and the nectar reward. I also experimentally manipulated the trapping hairs of this species to determine whether trapping hairs influence average levels of pollen export and receipt. I show that *Ceropegia ampliata* is pollinated by a generalist guild of flies (mainly Tachinidae, Sarcophagidae, Muscidae and Lauxaniidae) and produces minute quantities of relatively dilute nectar as a reward. Pollination success was generally low in this species and increases periodically suggesting that the abundance of pollinators is patchy. I found that flowers with trapping hairs that had already wilted had higher levels of pollinarium removal than flowers with erect hairs, however experimentally removing the hairs had no significant effect on pollen export and receipt.

In Chapter seven, I document the pollinators, pollen loads and long term levels of pollination success in *Stapelia hirsuta var. bayllissi*, a rare sapromyiophilous stapeliad. I find that, in contrast to *C. ampliata*, this species was specialized to pollination by small flies of the family Anthomyiidae. Similar to the results from Chapter seven, I find that long term levels of pollination success were typically low but could increase periodically, although such increases were generally unpredictable.

There are currently very few records documenting pollinator interactions in the Periplocoideae. Many species within this subfamily exhibit open-access flowers suggestive of pollination by short-tongued insects. I investigated the pollination biology *of Chlorocyathus lobulata*, a rare species with a highly localized distribution. I aimed to determine the pollinators, average levels of pollination success and demography of this species in order to determine whether this rare species is suffering from the collapse of a highly specialized pollinator mutualism. I also quantified the high incidence of flower herbivory caused by larvae of the moth, *Bocchoris onychinalis*. I find that *C. lobulata* has a highly generalized fly pollination system and average levels of pollination success suggested that a large proportion of flowers had pollen removed and deposited suggesting that this species is not experiencing pollination failure. The large numbers of juveniles present also indicated that recruitment is taking place.

ECOLOGY AND DEGREE OF SPECIALIZATION OF DIVERSE POLLINATION SYSTEMS IN SELECTED SPECIES OF MILKWEEDS

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Part A: Ecological studies on hymenopteran pollinated systems

Part B: Degree of specialization in fly-pollinated asclepiads

Declaration

I, Gareth Coombs declare that the work within this thesis is my own with the exception of data collected by Eleanor Brassine in Chapter 4 as part of her 3rd year project and Sarah Taylor who carried out the breeding systems between different populations of *Gomphocarpus physocarpus* (Chapter 2). In cases where I have made use of the work of others, this has been cited in text. This thesis has not been submitted for examination to any other University.

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G. Coombs

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List of Appendices

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Preface:

Papers from this thesis that have been published or submitted:

Chapter 2: Published in Austral Ecology: Coombs, G., Peter, C.I. and Johnson, S.D. 2009. A test for Allee effects in the self-incompatible wasp-pollinated milkweed *Gomphocarpus physocarpus. Austral Ecology* **34**: 688 – 697.

Chapter 3: Published in *AoB Plants*: Coombs, G. and Peter, C. I. 2010. The invasive "mothcatcher" (*Araujia sericifera* Brot.; Asclepiadoideae) co-opts native honeybees as its primary pollinator in South Africa. Published online doi:10.1093/aobpla/plq021

Appendix 1B: *Chlorocyathus lobulata* – Accepted in *Flowering plants of Africa*

Chapter 1

Introduction

Pollination systems in the Asclepiadoideae (Apocynaceae)

Milkweeds show adaptations to exploit nearly all groups of flower visiting insects, but the main groups of pollinators include the Hymenoptera, Diptera, Lepidoptera and Coleoptera in order of decreasing importance (Ollerton and Liede, 1997). The single report of pollination by sunbirds is considered exceptional (Pauw, 1998). Until recently, most of the detailed studies were limited to the genus *Asclepias* in North America (see review by Wyatt and Broyles, 1994; Ollerton and Liede, 1997), which may significantly bias our understanding of milkweed pollination systems in general (Ollerton and Liede, 1997). There has however been a recent increase in the interest from other regions such as South Africa (Liede and Whitehead, 1991; Ollerton *et al*., 2003; Shuttleworth and Johnson, 2006, 2008, 2009 a, b, c), Kenya (Masinde, 2004), South America (Viera and Shepherd, 1999), Japan (Tanaka *et al*., 2006; Yamashiro *et al*., 2008) and Ecuador (Wolff *et al*., 2008)

The flowers of milkweeds have diversified to exploit different pollinators through various mechanisms. These include floral isolation by scent, colour, reward chemistry and flower morphology or a combination of these traits (Ollerton *et al.,* 2003; Meve *et al*., 2004; Shuttleworth and Johnson, 2009a). Pollination systems vary in the degree of specialization ranging from those species that are exclusively pollinated by one or a few species of insects (*e.g*. Pompilid wasp pollination in *Pachycarpus species* - Shuttleworth and Johnson, 2006, Shuttleworth and Johnson, 2009a,b,c) to highly generalized pollination systems (*e.g. Asclepias verticillata*; Ollerton and Liede, 1997). Examples of generalized pollination systems occur in several genera. For instance extreme generalization is seen in *Asclepias verticillata* where up to 126 pollinators have been reported to date (Ollerton and Liede, 1997). Highly generalized pollination systems have also been reported in trap-flowers systems (*Ceropegia* species) where several different species from a wide range of fly families serve as pollinators of different subspecies of *Ceropegia aristolochioides* (Ollerton *et al.*, 2009).

The flowers of more generalized species presumably have flower fragrances that attract a greater diversity of insects and provide easier access to rewards and reproductive structures. This is in contrast to more specialized species where access to rewards may be physically restricted (i.e. long tubular corolla tubes in *Microloma sagittatum*; Pauw, 1998) or in the case where nectar is exposed the chemical composition of nectar may distasteful to non-target flower visitors (Shuttleworth and Johnson, 2006; 2009a).

Current evidence suggests that most species for which pollinator records have been made are relatively specialized (< 5 pollinators), but data are limited (Ollerton and Liede, 1997). Examples of highly specialized species include different species of *Pachycarpus* which are specialized towards pollination by Pompilid wasps (Shuttleworth and Johnson, 2006; Shuttleworth and Johnson, 2009 a, b, c). The flowers of these plants are visually cryptic but emit a scent to which these wasps are highly sensitive. In addition to the cryptic colouration and specialized scent, the unpalatable nectar produced by these flowers is also not readily consumed by other potential hymenopteran visitors (e.g. honeybees) which further serves to restrict the diversity of different flower visitors (Shuttleworth and Johnson, 2006, 2009a).

Pauw (1998) reports specialized sunbird pollination in *Microloma sagittatum*, where the flowers of this species display classic features of bird pollination (Faegri and van der Pijl, 1979) and are coloured red, have short tubular corollas and produce copious volumes of dilute nectar.

Hymenoptera have been reported as the most common pollinators of milkweeds (Ollerton and Liede, 1997). Much of the research into pollination by Hymenoptera has focussed on the members of the North American genus *Asclepias*, where pollination by bumblebees (*Bombus* species) and other Hymenoptera such as honeybees (*Apis mellifera*) is common (*e.g*. Fishbein and Venable, 1996; Ivey *et al*., 2003). Most North American members of the genus *Asclepias* have broadly similar flower morphologies (see Wyatt and Broyles, 1994; see Woodson, 1954 for details of different species) and are pollinated mainly by Hymenoptera and Lepidoptera (Ollerton and Liede, 1997). In *A. tuberosa,* bumblebees and honeybees are the most effective pollinators although the inflorescence displays characteristics of a butterfly-pollinated syndrome (i.e. red or orange flowers and nectar contained in deep corona cups; Fishbein and Venable, 1996). Other hymenopteran-pollinated milkweeds include *A. syriaca*, which is pollinated by bumblebees (Morse, 1981) and *Asclepias incarnata* which is pollinated by several hymenopteran pollinators, including carpenter bees (*Xylocopa virginica*), bumblebees (*Bombus* species) and other wasps (Ivey *et al.,* 2003). Despite the similarities in flower morphologies between different species, there is evidence that pollinators can discriminate between the flowers of *A. syriaca*, *A. verticillata* and *A. incarnata*, but may also regularly switch between the flowers of these different species (Kephart and Theiss, 2004). South African examples of bee and wasp-pollinated asclepiads include *Sarcostemma viminale*, which is pollinated by honeybees (Liede and Whitehead,

1991) and several indigenous species of *Asclepias* (Shuttleworth and Johnson, 2009c). Shuttleworth and Johnson (2006, 2009a,b,c) and Ollerton *et al.* (2003) have recently described specialized pollination by Pompilid wasps in several *Pachycarpus* species and other species within the genera *Asclepias*, *Aspidoglossum*, *Miroglossum*, *Periglossum*, *Woodia* and *Xysmalobium.*

The flower morphology of wasp - pollinated milkweeds may vary from cryptic flowers with nectar that is only palatable to certain types of wasps (Shuttleworth and Johnson, 2006, 2009a) to the well known members of North American genus *Asclepias* where the flowers have cup-like corona hoods within which large amounts of nectar accumulates and is consumed by a wide variety of diurnal and nocturnal insects (e.g. Jennerston and Morse, 1991; Ivey *et al.,* 2003). Flower colours of this genus are variable, even between species that are pollinated by the same pollinators. For example within the genus *Asclepias*, colours of those species that are visited by honeybees may vary from pink, orange and red (North American *Asclepias* species.: Fishbein and Venable, 1996) to various shades of green and yellow that are more common in wasp-pollinated milkweeds (Viera and Shepherd, 1999; Ollerton *et al.,* 2003; Shuttleworth and Johnson, 2006; Shuttleworth and Johnson, 2009). It has been suggested that the morphology of the corona of *Cynanchum adalinae subsp. adalinae* physically restricts access to nectar to pollinators with sufficiently long proboscides (Ollerton and Liede, 2003; see section on “Gynostegium structure and pollinarium loading”).

Many stapeliads (e.g. *Stapelia*, *Orbea, Huernea, Duvalia* etc.) and members of the genus *Ceropegia* are sapromyiophilous and have flowers that exhibit traits attractive to carrion flies (Vogel, 1961; Meve and Liede, 1994). These features are interpreted as adaptations that mimic the various morphological features such as the texture and scent of dipteran brood sites. Characteristic features include hairy petals or moveable hairs that mimic the surface texture of rotting animal (*e.g.* fur) or plant material (Meve and Liede, 1994; Meve *et al.,* 2004; Jurgens *et al.,* 2006). In particular, the flowers of stapeliads (Apocynaceae – Asclepiadoide – Ceropegieae) are known for their intricately textured petals, that through a combination of elongated hairs, heavily ridged petals and various colour combinations (red, dark-brown, yellow) further serve to mimic the external appearance of carrion (Meve and Liede, 1994; Meve *et al.,* 2004; Jurgens *et al.,* 2006). Other species have smoother petals with lighter colours (e.g. yellow in *Desmidorchis flava*, Jurgens *et al.*, 2006). The flowers of various species of *Stapelia* augment morphological deception by emitting foul odours that mimic the scent of carrion, rotting plants or urine and faeces (Meve and Liede, 1994; Jurgens *et al.,* 2006).

In some fly pollinated genera such as *Ceropegia*, pollinators are not only lured to the flower, but flowers are also morphologically shaped to trap pollinators (Vogel, 1961). Pollinating flies are lured to flowers that emit a pungent scent and are trapped inside a trapping bulb at the base of the flower (Vogel, 1961). In order to access the trapping bulb, flies have to navigate through a relatively long corolla tube that is lined on the inside with rigid hairs that are presumed to prolong the visit of the pollinating fly by preventing its escape (Vogel, 1961). Flies are then released after some time when hairs relax and become flaccid (Vogel, 1961). Several studies have investigated the morphology of different flowers structures associated in sapromyiophilous flowers in the genera *Stapelia* and *Ceropegia* (Vogel, 1961; Meve *et al.,* 2004; Poppinga *et al.,* 2010). However, with the exception of Oelschlagel *et al.*

(2009); the question as to how these structures interact with pollinators and the influence of different flower structures on pollen export and reception has not been investigated.

The degree of specialization in sapromyiophilous asclepiads is uncertain, largely owing to the lack of detailed studies documenting different flower visitors and pollen loads. Available data suggests that the diversity of elaborate flower structures and other traits such as colour and scent is not matched by the same diversity in terms of different pollinator families (Ollerton and Liede, 1997). For instance most records indicate that the main fly families pollinating stapeliads are Sarcophagidae, Calliphoridae and Muscidae with relatively little specialization towards specific pollinator families by different species of stapeliad (Meve and Liede, 1994; Ollerton and Liede, 1997). There has however been a recent study indicating that *Caralluma europaea* is specialized towards pollination by a single fly species (*Milichiella lacteipennis* – Milichiidae; *Raspi et al.,* 2010), suggesting that specialized pollination mutualisms may at least occur in stapeliads.

A large volume of literature has been generated on the morphological adaptations to sapromyiophily in milkweeds (Vogel, 1961; Meve and Liede, 1994; Bruyns, 2000; Meve *et al.,* 2004), however in the vast majority of these species, the identity of the pollinators remain unknown. In general the degree of specialization in trapping sapromyiophilous flowers varies between different species and is likely to be indicated or alluded to by the size of the flower and the nature of other floral attractants such as scent (Knuth, 1909 cited in Burgess *et al*., 2004). In trapping flowers such as *Aristolochia,* degrees of specialization range from highly generalized species (e.g. *Aristolochia grandiflora*, Burgess *et al.,* 2004) to relatively specialized (e.g. *Aristolochia inflata*; Sakai, 2002a). In sapromyiophilous flowers where pollinators use flowers as breeding sites and larvae consume floral parts (e.g. ovule parasites) pollinator specificity may be high (Sakai, 2002b). Trapping flowers such as *Ceropegia* species are generally considered to be mimetic (although flowers do produce nectar; Lock, Endress and Ollerton, unpublished data) and have been found to vary in the degree of specialization with some species being specialized with others being pollinated by a wide diversity of flies from several different families and genera of flies (Ollerton *et al.,* 2009).

I know of no experimental studies that investigate specific traits (*c.f* Coombs and Peter, 2009; Appendix 1A), in order to determine the role of trapping structures in sapromyiophilous flowers. For the most part, the role of certain flower structures is deduced from natural history studies documenting the interaction between these flowers and pollinators.

Gynostegium structure and pollinarium loading

Pollination in milkweeds is facilitated by both the external characteristics that serve to attract pollinators as well as the intricate mechanical mechanisms involved in pollen removal and deposition. A key component to the pollination system of the flowers of milkweeds is the gynostegium (Wyatt and Broyles, 1994), a central columnar structure formed by the fusion of the androecium and gynoecium (Liede, 1996). Pollen is presented as coalesced masses known as pollinia which are removed by pollinators as pairs, each pair forming a pollinarium. Each pollinarium consists of the two pollinia attached to clam-like mechanical clip (corpusculum) by a small section of elastic tissue known as the caudicle. The corpusculum is nested at the top of the alar fissure with each pollinium situated at either side of the alar fissure. Pollinaria are removed when the appendage of an insect is dragged through the alar fissure and the corpusculum clips onto an appendage of the pollinator. When the pollinator visits a subsequent flower and drags it's limb through the alar fissure the interior of which forms the stigmatic surface, a pollinium may be broken off within the alar fissure resulting in pollination (Wyatt, 1976; Wyatt and Broyles, 1994).

The morphology of the gynostegium is highly variable between different genera and even between different species within the same genus (e.g. *Cynanchum*, Liede, 1993; *Stapelia*, Meve *et al.,* 2004). Similarly the pollinaria are highly variable in shape and size (see Ollerton *et al.,* 2003) and this variation has been suggested to limit hybridization between co-occurring species through preventing the insertion of pollinia into incorrect stigmatic chambers of congeners (Kephart and Heiser, 1980). The isolation mechanism described above is known as the lock and key hypothesis (Kephart and Heiser, 1980). It is not known however to what extent the morphology of the gynostegium influences the pattern of pollinarium attachment to pollinators. The placement of pollinaria on pollinators is likely to be influenced by both the structure of the gynostegium and flower morphology. Ollerton *et al.* (2003) refers to flowers that place pollinaria non-specifically on various parts of the body as messy pollination systems in contrast to other systems where pollinarium placement may be more exact. Such exact placement of pollinaria on specific parts of the body is common place in orchids where there is close morphological fit between the pollinator and flowers (e.g. *Disa draconis*, Johnson and Steiner, 1997; *Disa scullyi*, Johnson, 2005). The precision of pollinarium placement is also influenced by whether plants have actinomorphic or zygomorphic flowers, as pollen placement is generally considered to more precise in the latter (Sargent, 2004). Similar precise placement of pollinaria on milkweed pollinators has also been documented in sunbirds that bear pollinaria on the tongue (Pauw, 1998) and in wasp-pollinated *Pachycarpus appendiculatus* where pollinaria attach almost exclusively to the labial palps of the pollinator (Shuttleworth and Johnson, 2009b). At least in different species of *Cynanchum*, placement of the pollinaria may be influenced by the morphology of the corona lobes (Ollerton and Liede, 2003). In species where the corona lobes obscure the gynostegium, only the mouthparts of pollinating insects are inserted into the flower and pollinaria are carried on these (Ollerton and Liede, 2003). In all documented cases of fly-pollinated species, pollinaria are attached to the proboscis of the pollinating fly (Meve and Liede, 1994; Ollerton *et al.,* 2009 and references therein).

In addition to influencing the placement of pollinaria on the proboscis of pollinators there may be large variation in the dimensions of different structures of the gynostegium (Morse and Fritz, 1985). For instance variability in the dimensions of the alar fissure may result in some flowers being morphologically incapable of receiving pollinaria and are thus rendered functionally male flowers (Beare and Perkins, 1982). In at least one species, *Metaplexis japonica*, this variation causes this species to be andromonoecious, where plants produce two distinct groups of flowers, one group being male flowers, the other being hermaphrodite flowers (Tanaka *et al.,* 2006).

An intriguing feature of the pollinaria of milkweeds is that pollinaria will frequently form chains with the corpusculum of one pollinarium attaching to the caudicle tissue of other pollinaria that have already attached to the pollinators (Frost, 1965; Morse, 1981). Such pollinarium chains may accumulate on the mouthparts of an insect throughout a foraging bout and increase in size to the point that these are large enough to negatively influence their foraging behaviour through slowing the foraging speed of pollinators (Morse, 1981). The effects of large pollinaria loads are however likely to be insignificant with some insects showing no attempts to remove pollinaria from the proboscis (pers. obs.). Pollinarium chaining does not occur in all species (Vieira and Shepherd, 1999) and the reasons why pollinaria form chains in some species and not in other related species are not known.

A consequence of the fact that limbs of pollinators have to be drawn through the stigmatic chamber to either pick up or deposit pollinia is that insects frequently become wedged between the anther wings. In some cases this may fatally restrain both pollinating and non-pollinating insects or result in limbs being broken off the insects (Robertson, 1887; Romeo, 1933; Coleman, 1935; Morse, 1981; Shuttleworth and Johnson, 2009b). Moths and bees often become stuck within the flowers of the invasive milkweed *Araujia sericifera* (Coleman, 1935; Romeo, 1933 and references therein) and the labial palps of large pompillid wasps are frequently broken off between the rigid anther wings of *Pachycarpus appendiculatus* (Shuttleworth and Johnson, 2009b). For this reason, pollination in milkweeds has been described as a “high-cost” system (Morse, 1981).

Pollination success

As is the case in orchids, the presence of pollinaria in milkweeds means that their pollination success can be relatively easily estimated (Wyatt and Broyles, 1994). Estimating pollination success in terms of pollen removal and deposition is difficult and laborious for most species with granular pollen. Quantifying pollination success is an important part of pollination system studies as it gives a measure of the pollinator services and is an indication of the efficacy of a given system. In milkweeds and orchids, levels of pollinarium removal and deposition have been used to calculate pollen transfer efficiency (PTE), which gives a measure of the proportion of removed pollinia that are deposited on conspecific stigmas at the population level (Johnson *et al.,* 2005). Pollen transfer efficiency has been increasingly used as a measure of pollination success in milkweeds (Ivey *et al.,* 2003; Shuttleworth and Johnson, 2006; Shuttleworth and Johnson, 2008; Coombs *et al*., 2009; Shuttleworth and Johnson, 2009a, b) and orchids (Peter and Johnson, 2008; Johnson *et al*., 2009; see review by Harder and Johnson (2008)).

Most estimates of pollination success in asclepiads have been for hymenopteran-pollinated species and have indicated that pollination success can reach relatively high levels (sometimes exceeding 40%) in such species, but is generally variable (Wyatt, 1980; Forster, 1994; Ivey *et al.,* 2003; Ollerton *et al*., 2003; Harder and Johnson, 2008; Shuttleworth and Johnson, 2006, 2008, 2009a, b). Estimates of pollen transfer efficiency in beetle pollinated species (*Asclepias woodii* and *Sisyranthus trichostomus*) indicated that PTE may be low in these species with PTE estimates of values of zero and 10% respectively (Ollerton *et al*., 2003). To my knowledge, no data is available on the average levels of pollination success in fly-pollinated genera such as *Stapelia* and *Ceropegia*. That is in spite of the large amount of literature that has been written about the intricate morphological adaptations of these species to lure fly pollinators (Vogel, 1961; Meve and Liede, 1994; Ollerton *et al*., 2009). There is not enough data available on the pollen transfer efficiency of various species in order to generalize about the pollination efficiency of taxa pollinated by specific pollinators.

Available data on the pollination success of hymenopteran pollinated species suggests that average levels of pollination success differs between different sampling dates (Ivey *et al.,* 2003) and between different populations (Shuttleworth and Johnson, 2008). Most estimates of pollinarium removal and deposition have however only been collected at a single date with few estimates of pollination success made at several sampling dates throughout the flowering season (e.g. Ivey *et al.,* 2003). Data by Ivey *et al*. (2003) indicates that pollinarium removal and deposition rates vary significantly between different sampling dates, while data by Shuttleworth and Johnson (2008) shows significant variation between different sites. Peter and Johnson (2008) documented the long term pollination success of *Acrolophia cochlearis* and demonstrated the pollen removal, deposition and PTE was variable throughout the flowering season but relatively predictable and peaked when flowering intensity was the highest. Harder and Johnson (2008) provide a review of pollen transfer efficiency in various orchids and milkweeds.

An alternative advantage of being able to easily quantify pollination success is that other ecological and evolutionary questions pertaining to pollination success may be more easily answered using milkweeds as model taxa (Wyatt and Broyles, 1994). For instance a number of studies have found evidence that reproductive success declines with population size or density in plants (Groom, 1998; Cappucino, 2004; Davis *et al.,* 2004). A negative relationship between reproductive success and the number or density of individuals in a population is referred to as the Allee effect (Allee, 1931). In plants, such reductions in reproductive success are frequently a consequence of a break-down of pollination services at low abundances (Groom, 1998; Courchamp *et al.,* 1999), although reduced genetic diversity in small populations and consequent inbreeding effects may also play a role in certain instances (Ellstrand and Elam, 1993; Leimu *et al.,* 2006). Not all studies have found these relationships (see review by Ghazoul, 2005) which suggests that compensatory mechanisms may exist in small populations. Mechanisms that may prevent small populations from having reduced fitness include reduced reliance on pollination services through automatic self-pollination (Baker, 1955; Cheptou, 2004), or in some species where single individuals may produce large flower displays that attract a sufficient number of pollinators (e.g. *Senna didymobotrya*; Van Kleunen and Johnson, 2005). The degree of specialization towards certain pollinators could conceivably influence whether species suffer from Allee effects with plants that have specialized pollinators being considered to be vulnerable to Allee effects (Ghazoul, 2005), however few studies have explored the likelihood of Allee effects in species with generalized pollination systems (Morgan *et al.,* 2005).

The ability to quantify pollination success relatively easily is also beneficial when investigating the levels of pollination success maintained by invasive milkweeds. Several species of milkweed have become invasive in other countries with Australia having 10 invasive species (Forster, 1994), while in North America two species of *Vincetoxicum* have become invasive (Daehler, 1998; Cappucino, 2004). Quantifying the average levels of pollination success in invasive milkweeds gives information on the success with which these species co-opt pollinators in the invasive range. Given the complex flower morphology of milkweeds it is expected that invasive milkweeds are either capable of automatic self-pollination or geitonogamy (pollinator facilitated self-pollination) or must rely on similar functional groups (*sensu* Fenster *et al.*, 2004) of pollinators to be present in the invaded range. Current evidence suggests that this is indeed the case with several invasive milkweeds successfully pollinated by native insects within the exotic range (Coleman, 1935; Forster, 1994; Herrera and Nassar, 2009).

Relatively few milkweeds are capable of autonomous self-pollination or geitonogamy (pollinator mediated self-pollination; Wyatt and Broyles, 1994; Lipow and Wyatt, 1998; Shuttleworth and Johnson, 2006; Shuttleworth and Johnson, 2008). As the number of studies on the reproductive ecology of milkweeds increases, this pattern is likely to change and there are some weedy North-American species of the genus *Asclepias* which are capable of pollinator mediated selfing (Wyatt and Broyles, 1997; Lipow *et al.,* 1999; Finer and Morgan, 2003). There have also been reports of autogermination of pollinia in *Vincetoxicum rossicum* (Asclepiadoideae; Cappuccino, 2004).

Aims and Hypothesis

This work was inspired by the relatively limited knowledge of the pollination biology of milkweeds in South Africa and more broadly, throughout Africa (Ollerton and Liede, 1997). Although studies on local milkweeds have been undertaken, these have largely been limited to a few species and are geographically restricted to the Western Cape and Kwazulu-Natal regions (Liede and Whitehead, 1991; Pauw, 1998; Ollerton *et al*., 2003; Shuttleworth and Johnson, 2006; Shuttleworth and Johnson, 2008, Shuttleworth and Johnson, 2009 a, c). Most of the work on pollinator interactions in South African Asclepiadoideae is also confined to these two areas (Rodger *et al*., 2004), with very little work having been done on milkweeds in the Eastern Cape region (but see Shuttleworth and Johnson, 2009b).

Part A: Ecological studies on hymenopteran pollinated systems

Pollination studies on South African milkweeds pollinated by wasps, have thus far mostly been limited to work done by Shuttleworth and Johnson (2006; 2009a,b,c) and Ollerton *et al.* (2003) who documented highly specialized pollination systems in the genera *Pachycarpus* and *Miraglossum* by pompilid wasps. This thesis aimed to document the pollination biology of *Gomphocarpus physocarpus*, a native weedy shrub that commonly grows alongside road edges and ploughed farm lands, where our preliminary observations suggested that this species is visited by several different wasp species and is presumably more generalized in its pollination system. Given that this species is a weedy colonizing species I set out to test other predictions posed by Baker (1955) who proposed that the establishment of colonizing species is facilitated through self-pollination by reducing their reliance on pollinators. I also used this system to test whether there is a positive relationship between pollination success and population size (i.e. Allee effect, Allee (1931)), and documented other characteristics of its reproductive biology (i.e. breeding system, diversity of pollinators and pollinarium reconfiguration) which could explain our results.

Several milkweeds have become invasive in various countries around the world. Examples of species where the pollination biology has been documented include *G. physocarpus* which is invasive in Australia (Forster, 1994) and *Stapelia gigantea* that is invasive in Venezuela (Herrera and Nassar, 2009). Despite milkweeds possessing flowers that are highly mechanically complex, some species (e.g. *Asclepias*) have highly generalized pollination systems (Ollerton and Liede, 1997). Invasion of species with specialized flower morphologies is thought to be facilitated if plants can co-opt native pollinators belonging to similar functional groups are present within the invaded range (Forster, 1994; Liu and Pemberton, 2010; Rodger *et al.,* 2010). For instance *Gomphocarpus physocarpus* is an invasive milkweed in Australia, and is pollinated within its exotic range by a similar diversity of wasps that pollinate this species in South Africa (Forster, 1994). Similarly, *Stapelia gigantea* has become a naturalised invasive in Venezuela, where it is pollinated by carrion flies (Herrera and Nassar, 2009). In this thesis, I investigate the pollination biology of *Araujia sericifera,* a common invasive milkweed in South Africa, in order to determine which pollinators pollinate this species and test the hypothesis that this species reproduces successfully in its exotic range through co-opting native pollinators of a similar functional group. In addition, to documenting the pollinators of *A. sericifera*, I quantified various other aspects of its pollination biology such as average levels of pollination success maintained by exotic pollinators, nectar rewards, breeding system and the relative contribution of diurnal and nocturnal pollinators to the pollination of this species.

The genus *Cynanchum* displays a wide diversity in the morphology of the gynostegium, however the functional significance of this diversity remains unexplained (Liede, 1996). My preliminary observations on the pollination biology of *Cynanchum ellipticum* indicated that honeybees foraging on the flowers of this species accumulate large pollinarium loads on their mouthparts. The presence of these large pollinarium loads may interfere with the foraging behaviour of these pollinators in much the same way as as that described by Morse (1981) who found that large pollinarium loads significantly slowed the foraging speed of bumblebees foraging on *Asclepias syriaca*. I documented the pollination biology of this species to determine the degree of specialization of the pollination system, average levels of pollination success and to test the hypothesis that large pollinarium loads accumulating on the proboscis of pollinators reduces their foraging speeds, causing increased foraging times per flower or resulting in bees visiting fewer flowers per inflorescence.

Andromonoecy occurs when plants bear both male and hermaphrodite flowers and has been documented in approximately 400 plant species within 33 families (Miller and Diggle, 2003), including at least one species in the Apocynaceae (Tanaka *et al.,* 2006). My preliminary observation on the pollination biology of *Cynanchum obtusifolium* suggested that this species produces two different types of flowers which could be distinguished by the size of the flowers that corresponded to differences in the size of the gynostegia. I documented various aspect of the pollination biology of *Cynanchum obtusifolium*, including the diversity of pollinators, average levels of pollination success and nectar rewards. I used morphometric measurements to test the hypothesis that this species is andromonoecious and produces two different flower types.

Part B: Degree of specialization in fly-pollinated asclepiads

Fly-pollinated flowers can be broadly classified into myophilous and sapromyiophilous syndromes (Faegri and van der Pijl, 1979). Sapromyiophilous genera display flowers that mimic the brood and food substrate of flies and most plants within the genera *Stapelia* and *Ceropegia* are assumed to be sapromyiophilous (Vogel, 1961; Meve and Liede, 1994).

Stapelia flowers are known for their highly “sculptured” petals caused by a combination of ridges and hairs that cover the petal surface and mimic the texture of decaying plant or animal material (Meve and Liede, 1994; Meve *et al*., 2004). Most studies on sapromyiophilous milkweeds to date have documented pollination by carrion flies (Sarcophagidae, Calliphoridae, Muscidae; Meve and Liede, 1994). There is uncertainty about the degree of specialization in different species, however some specialized relationships do exist (Raspi *et al.,* 2009; Jonkers, 2010). To my knowledge, there is no information on the average levels of pollination success and fruit set in *Stapelia* as well as other aspects such as the demographic profile of different species. In Chapter 7, I document the pollinators and pollination success of *Stapelia hirsuta var. baylissi*, a rare stapeliad with a very narrow geographic distribution, with the aim of determining whether this species has a relatively specialized or generalized pollination system and to documenting long term levels of pollination success. I also use these estimates of pollination success to determine whether such a small isolated population experiences Allee effects through low pollinator visitation, as has been documented in other plant species (Groom, 1998; Ward and Johnson, 2005).

The flowers of *Ceropegia* are shaped to form a lantern-shaped trap within which pollinators are imprisoned for a period of several days. Flies are trapped inside a bulbous base at the bottom of the flower, which is reached once they have crawled through an elongated corolla tube lined with stiff hairs. Flies are released after these hairs wilt (Vogel, 1961). Surprisingly, however there have been relatively few natural history studies that have documented the pollinators of these species (but see Masinde, 2004; Ollerton *et al.,* 2009), none that have quantified the average levels of pollination success and nectar rewards in these species. There have also been no studies that have empirically tested the function of these hairs to establish whether trapping pollinators increases levels of pollinarium removal or reception. In this thesis I documented the different pollinators, average levels of pollination success and nectar rewards of *Ceropegia ampliata*, in order to determine whether this species has a highly generalized or specialized pollination system and how efficient the system is in terms of pollen transfer. I also carried out manipulative experiments with the specific aim to determine the influence of trapping hairs on pollen export and receipt in this species.

Pollination studies on pollination biology of members of the subfamily Periplocoideae (Apocynaceae) are relatively scarce. Very little is known about the degree of specialization in different species as well as the average levels of pollination success in members of this subfamily. I studied the pollination biology of *Chlorocyathus lobulata*, a rare endemic milkweed that is only known from one site, the Kap River reserve (Venter *et al.,* 2006). I investigated which species of pollinators pollinate this species and attempted to determine whether its rarity could in part be ascribed to the collapse of a highly specialized mutualism, as has been reported before in other rare species (Steiner, 1993).

Specific Aims

1) Determine the natural pollinators of *Gomphocarpus physocarpus* and establish whether pollination success varies with population size.
2) Determine the role of honeybees in facilitating the invasion of *Araujia sericifera* as an invasive species in South Africa and what contribution nocturnal moths have to average levels of pollinarium deposition and removal.

3) Document the pollination biology and pollination success of *Cynanchum ellipticum* (Apocynaceae – Asclepiadoideae) and establish whether large pollinarium loads influence the foraging behaviour of one of its main pollinators, *Apis mellifera*.
4) Document the pollination biology and pollination success of *Cynanchum obtusifolium* and determine whether this species is andromonoecious.
5) Determine whether *Stapelia hirsuta* var. *baylissi* and *Ceropegia ampliata* are generalized or specialized pollinated taxa and whether the trapping hairs of *C. ampliata* influence pollen removal or deposition.
6) Document the pollination ecology of *Chlorocyathus lobulata* and determine whether its rarity is caused by the break-down of a highly specialized pollination mutualism.

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Part A: Ecological studies on hymenopteran pollinated systems

Chapter 2

A test for Allee effects in the self-incompatible wasp-pollinated milkweed *Gomphocarpus physocarpus*

Published in *Austral Ecology*: See Coombs, G., Peter, C.I. and Johnson, S.D. 2009. A test for Allee effects in the self-incompatible wasp-pollinated milkweed *Gomphocarpus physocarpus***.** *Austral Ecology* **34**: 688 - 697.

Abstract

It has been suggested that plants which are good colonizers will generally have either an ability to self-fertilize or a generalist pollination system. This prediction is based on the idea that these reproductive traits should confer resistance to Allee effects in founder populations and was tested using *Gomphocarpus physocarpus* (Asclepiadoideae; Apocynaceae), a species native to South Africa that is invasive in other parts of the world. There was no significant relationships between the size of *G. physocarpus* populations and various measures of pollination success (pollen deposition, pollen removal, and pollen transfer efficiency) and fruit set. A breeding system experiment showed that plants in a South African population are genetically self-incompatible and thus obligate outcrossers. The breeding system of this species did not vary between different populations. Outcrossing is further reinforced by mechanical reconfiguration of removed pollinaria being required for the pollinaria to be inserted and the time that pollinaria take to do so exceeding the average duration of pollinator visits to a plant. Observations suggest that a wide variety of wasp species in the genera *Belonogaster* and *Polistes* (Vespidae) are the primary pollinators. It is concluded that efficient pollination of plants in small founding populations, on account of their generalist wasp-pollination system, contributes in part to

the colonizing success of *G. physocarpus*. The presence of similar wasps in other parts of the world has evidently facilitated the expansion of the range of this milkweed.

Introduction

The fitness of individuals is frequently positively related to either the number or density of conspecifics (Allee 1931). This phenomenon has become known as the Allee effect (Groom, 1998; Stephens *et al.,* 1999; Courchamp *et al.,* 1999; Stephens and Sutherland, 1999). The basis for Allee effects may be genetic (Charlesworth and Charlesworth, 1987; Ellstrand and Elam, 1993; Courchamp *et al.,* 1999; Herlihy and Eckert, 2002) or ecological, as in reduced cooperative interactions between individuals in small or sparse populations (Groom, 1998; Courchamp *et al.,* 1999). There may also be interactions between ecological and genetic effects. For example, small plant populations may be less attractive to foraging insect pollinators because of reduced floral display and rewards (Schmid-Hempel and Speiser, 1988; Klinkhamer and de Jong, 1990; Goulson, 1999; Thompson, 2001). Pollinators that do visit small populations may, in turn, also increase their intrapatch foraging time culminating in increased self-pollination, which in self-compatible plants leads to inbreeding (de Jong *et al.,* 1993; Oostermeijer, 2003). Self-pollination can also compromise the export of pollen by wasting pollen that potentially could have been exported. This process is known as pollen discounting (de Jong *et al.,* 1993).

Most attention has been paid to Allee effects in rare native species (Ward and Johnson, 2005). The persistence of small populations is, however, undoubtedly also important for colonizing and invasive species (Liebhold and Bascompte, 2003; Van Kleunen and Johnson, 2005; Taylor and Hastings, 2005). Single founders, in particular, would be more likely to establish populations if they are able to self-fertilize (Baker, 1955), since they would be relatively immune from ecological Allee effects due to an absence of pollinators or mates. Available data suggest that there is a tendency for Allee effects to be weakened or absent in self-compatible plant species (Leimu *et al.,* 2006).

Pollination success is difficult to quantify directly in most plants, but several studies have demonstrated that plants in small populations often show markedly increased seed production following supplemental hand-pollinations using pollen from within the same population (Ågren, 1996; Ward and Johnson, 2005). This provides direct evidence for ecological Allee effects through decreased pollinator visitation in small populations. On the other hand, pollination success is seemingly unaffected by population size in other plant species (Van Treuren *et al.,* 1993; Kunin, 1993; Kunin, 1997; Van Kleunen and Johnson, 2005; Grindeland *et al.,* 2005).

Asclepiads and orchids are ideal subjects for studying factors that influence pollination success because their pollen is packaged in pollinia which makes it relatively easy to directly quantify rates of pollen removal and deposition in flowers. A further advantage of these plants is that it is relatively easy to calculate pollen transfer efficiency (PTE), a measure of the proportion of removed pollen that reaches stigmas (Johnson *et al.,* 2005). PTE has been used to investigate mating patterns in orchids (Johnson *et al.,* 2005), rates of selfing (Johnson *et al.,* 2004) as well as the evolution of pollen aggregation in the Angiosperms (Harder and Johnson, 2008). In general, PTE would be expected to decrease in small populations because of lower levels of pollinator foraging constancy, leading to higher levels of pollen transport losses.

In comparison to those species from North America, the pollination biology of African milkweeds has been poorly studied (but see Liede and Whitehead, 1991; Pauw, 1998; Ollerton *et al.,* 2003; Ollerton and Liede, 2003; Shuttleworth and Johnson, 2006). For this study we focused on the Asclepiad *Gomphocarpus physocarpus* E. Mey. which is an indigenous weedy species in South Africa where it rapidly colonizes roadsides and other disturbed habitats. It is also invasive in other regions, including Australia, China, Hawaii and other pacific islands (Orchard, 1994; Forster, 1994; Wagner *et al.,* 1999). Forster (1994) documented a wide range of wasp pollinators of this species in Australia, but its pollination biology has not been studied in its native range. In South Africa, isolated plants usually set fruit which led us to suspect that the species is genetically self-compatible. We also noticed that pollinaria withdrawn from flowers must undergo gradual reconfiguration before they can be inserted into stigmas. Available evidence suggests that this serves to reduce self-pollination among flowers of orchids and milkweeds (Peter and Johnson, 2006).

The aim of this study was to determine the relationship between population size and various measures of pollination success in *G. physocarpus*, including rates of pollinia removal and insertion, pollen transfer efficiency and fruit set. In order to interpret our results we also investigated basic aspects of the reproductive biology of the species, including its breeding system, pollen vectors and post-removal pollinarium reconfiguration.

Materials and Methods

Study species

Gomphocarpus physocarpus (Asclepiadoideae, Apocynaceae; Fig. 1A-C) is a common plant occurring in disturbed habitats such as ploughed farming lands and road verges. It is found throughout the southern and eastern parts of South Africa at lower altitudes (Fig. 2). Individual plants produce large numbers of flowers that are arranged in pendant umbels. *G. physocarpus* has a floral morphology similar to that found in members of the relatively well studied genus *Asclepias* (Bookman, 1981; Wyatt and Broyles, 1994) with actinomorphic flowers each bearing five prominent corona lobes which accumulate copious amount of nectar (Fig. 1C). Flowering occurs throughout the austral summer from September to late April, but peaks in December. Populations of *G. physocarpus* were studied at a number of sites in the Eastern Cape and KwaZulu-Natal provinces (Fig. 2).

Breeding systems

To enable the delicate hand-pollinations needed for the breeding system experiment to be done under observation with a microscope, potted plants needed to be brought into the laboratory (cf. Lipow and Wyatt, 2000). This was achieved by propagating plants in 10 litre pots from wild harvested seeds originating from one large population of plants occurring on Mountain Drive near Grahamstown. Plants were fertilised and well watered. With the aid of a dissecting microscope, three treatments were performed on at least one umbel on each of 7 plants. These flowers where then rebagged to exclude pollinators. Treatments included cross-pollination with pollen from another plant; self-pollination with pollinia from the same plant; and un-pollinated to test for auto-pollination. A total of 8 additional umbels were bagged on 4 of these plants and left untreated to test for auto-pollination. Cross and self-pollination treatments where done by inserting two pollinia into two stigmatic cavities. This procedure was based on suggestions by Moore (1946), Moore (1947) and Sparrow and Pearson (1948) who showed that two pollinaria, inserted into separate stigmatic chambers, are needed for effective pollination. The proportion of flowers in each treatment group that set fruit was analyzed using the non-parametric Friedman test which considered each plant as a "block".

In addition to the treatments on the potted plants, additional flowers were bagged on six plants growing in the gardens of the Department of Botany, Rhodes University. These were left untreated as a further test for auto-pollination.

Due to evidence that the degree of self-compatibility may vary between different populations of *Asclepias* (Lipow *et al.,* 1999), the breeding system of *G. physocarpus* was determined for three additional populations, these were from Port-Alfred, Kasouga and Grahamstown. In all three populations, wild plants were collected and grown in 20 litre buckets, until these had established and continued flowering. Plants were then kept in an enclosure made of 80% shade cloth to exclude all flower visitors. Breeding systems were performed using the same techniques and applying the same treatments as described in Coombs *et al*. 2009. Each treatment was replicated at least twice on each of 5 plants from for different populations. Differences in the proportion of flowers that set fruit in different treatments were tested for using a Chi-square test.

Pollinators

Insects visiting flowers of *G. physocarpus* were collected at a number of sites in KwaZulu-Natal and the Eastern Cape provinces of South Africa between 1997 and 2007 (Fig. 2). Insects were identified and the number of pollinaria and corpusculae attached to each insect was determined. The corpusculum is the mechanical clip that attaches the pollinarium to the pollinating insect (Fig 1D) and in most cases remains attached even after individual pollinia have been deposited (Brown, 1833; Frost, 1965; Bookman, 1981). The total duration that pollinators spent visiting flowers was observed on individual plants in the Mountain Drive population, a natural population near Grahamstown.

Nectar rewards

The standing crop of nectar was measured the standing crop of nectar from randomly selected flowers between 7:00 and 8:30 at the start of pollinator activity. A second measurement was made from 10:30 to 11:30 to determine nectar utilization by pollinators. Nectar volumes were determined using 5µl micropipettes. Nectar concentration was determined using an Atago refractometer. During the second interval of nectar measurements, the nectar quantities of individual corona lobes were very low, so the nectar volume and concentration was determined for the nectar pooled from all five corona lobes of flowers.

Pollinaria reconfiguration

Pollinaria were removed from freshly harvested flowers using an insect pin. The pollinaria were orientated so that the longitudinal axis could be viewed using a dissecting microscope. To monitor the rate of pollinaria reconfiguration, digital pictures were taken at intervals of approximately 30s. The angle between the pollinia was then measured from these images. Pollinaria reconfiguration is complete once the angle stops changing.

Cellular mechanisms for pollinaria reconfiguration

Pollinaria were removed from freshly harvested flowers, fixed in Acrilene and then dehydrated in an alcohol-butanol series before being embedded in paraplast wax. Whole flowers were also fixed and embedded using this technique to examine pollinaria prior to reconfiguration. Mounted sections of approximately 15 µm thickness were stained with safranin and fast green, and imaged.

Population surveys and test for the Allee effect

Nineteen populations between Grahamstown and Kenton-on-Sea were examined (between sites 1 and 2 in Fig. 2). These ranged from single isolated plants to a large population of approximately 1000 plants. All flowering plants were counted in each population.

In small populations the number of fruit on each plant was counted. In large populations a subset of twenty plants were randomly sampled and the number of fruit on each plant counted.

We also randomly sampled one inflorescence from each of twenty plants bearing open but not senescent flowers. In smaller populations, it was sometimes necessary to collect more than one inflorescence per plant to make up a sample of twenty inflorescences. One flower from each of these inflorescences was randomly selected and scored for pollinaria removal and pollinia deposition in stigmatic cavities. These data were also used to calculate pollen transfer efficiencies (PTE) in each population. PTE is the proportion of removed pollinia (removed pollinaria multiplied by two as there are two pollinia per pollinaria) that are deposited in stigmatic cavities.

The relationships between log-transformed population size (predictor variable) and various measures of pollination success (response variables) were determined using univariate regressions. The proportion of flowers pollinated, proportion of flowers with pollinaria removed and PTE were arcsine square-root transformed prior to these analyses.

Results

Breeding systems

Only cross-pollinated flowers set fruit indicating that plants in the study population are genetically self-incompatible (Table 1). This difference was statistically significant (Friedmans test, χ^2=10.0, p=0.007).

Table 1: Results of an experiment to determine the breeding system of *Gomphocarpus physocarpus*

Variable	Unmanipulated	Self-pollinated	Cross-pollinated
Number of plants	13	7	7
Total number of flowers	168	22	22
Number of fruits set	0	0	7
Overall proportion of flowers that set fruit	0	0	0.32
Median proportion of flowers that set fruit per plant	0	0	0.33**
Mean (± se) number of seeds per fruit.	n/a	n/a	110.3 ± 16.5

** P = 0.007 (Friedman test involving seven plants on which all three treatments were applied)

Results testing for differences in the breeding system between different populations showed that only outcross treatments set fruit, while none of the plants from different populations were capable of either automatic self-pollination or geitogamy (Table 2), indicating that *G. physocarpus* is likely to be largely genetically self-incompatible. Differences in the proportion of successfully fertilized flowers between different treatements were statistically significant (Pearson chi-square, χ^2= 96.05, p < 0.0001).

Table 2: Results of the breeding system carried out on three different populations of *G. physocarpus*.

Population	Unamanipulated control	Geitonogamy	Cross-pollinated
Port Alfred	0 (n=13)	0 (n=12)	0.636 (n=11)
Grahamstown	0 (n=5)	0 (n=5)	0.5 (n=4)
Kasouga	0 (n=11)	0 (n=10)	0.9 (n=10)

Pollinators

Flowers were visited by a wide diversity of Hymenoptera, as well as a few Diptera and Coleoptera (Table 3). The majority of the insects bearing pollinaria or corpusculae, however, belong to the wasp family Vespidae and the bulk of these to two genera, *Belonogaster* and *Polistes*. In most cases the corpusculum of the pollinarium is attached to the arolium (a fleshy pad between the claws of the insects' limbs). In many cases these insects had multiple corpusculae attached in chains (Fig. 1D), indicating that even when the initial attachment sites on the arolium are full, the insect can still remove further pollinaria from the flowers.

Numerous Pompilid wasps were collected at the Mountain Drive site, however these wasps carried few pollinaria. Honey bees bearing pollinaria were occasionally collected at a number of the sites and small *Lassioglossum* bees (Halictidae) were abundant at the Hesketh site, but only a few of these bees carried pollinaria.

In most of the cases we observed, the wasps approach the plant often from a down wind position before briefly hovering in front of an umbel of flowers and grappling them with the front legs to alight upside down. The insects then hang from the flowers while probing the shallow corona nectar cups for the abundant nectar (Fig. 1A-C). The wasps often clamber from one flower to another in an umbel. In windy conditions wasps may walk between umbels on a plant. With the insects hanging from the drooping flowers the tarsi of the wasps are drawn through the open proximal end of the stigmatic slit down towards the apex of the gynostegium where the corpusculae are positioned at the termination of the stigmatic slit. When a wider structure, such as an insect limb, is drawn through the stigmatic slit, not only is the stigmatic cavity opened but also the mechanical clip of the corpusculum allowing the arolium to be inserted. As the limb of the insect is drawn further the support of the stigmatic slit no longer holds the clip of the corpusculum open and so the corpusculum close onto the arolium or another corpusculum already attached to the insect, firmly attaching the pollinarium to the insect.

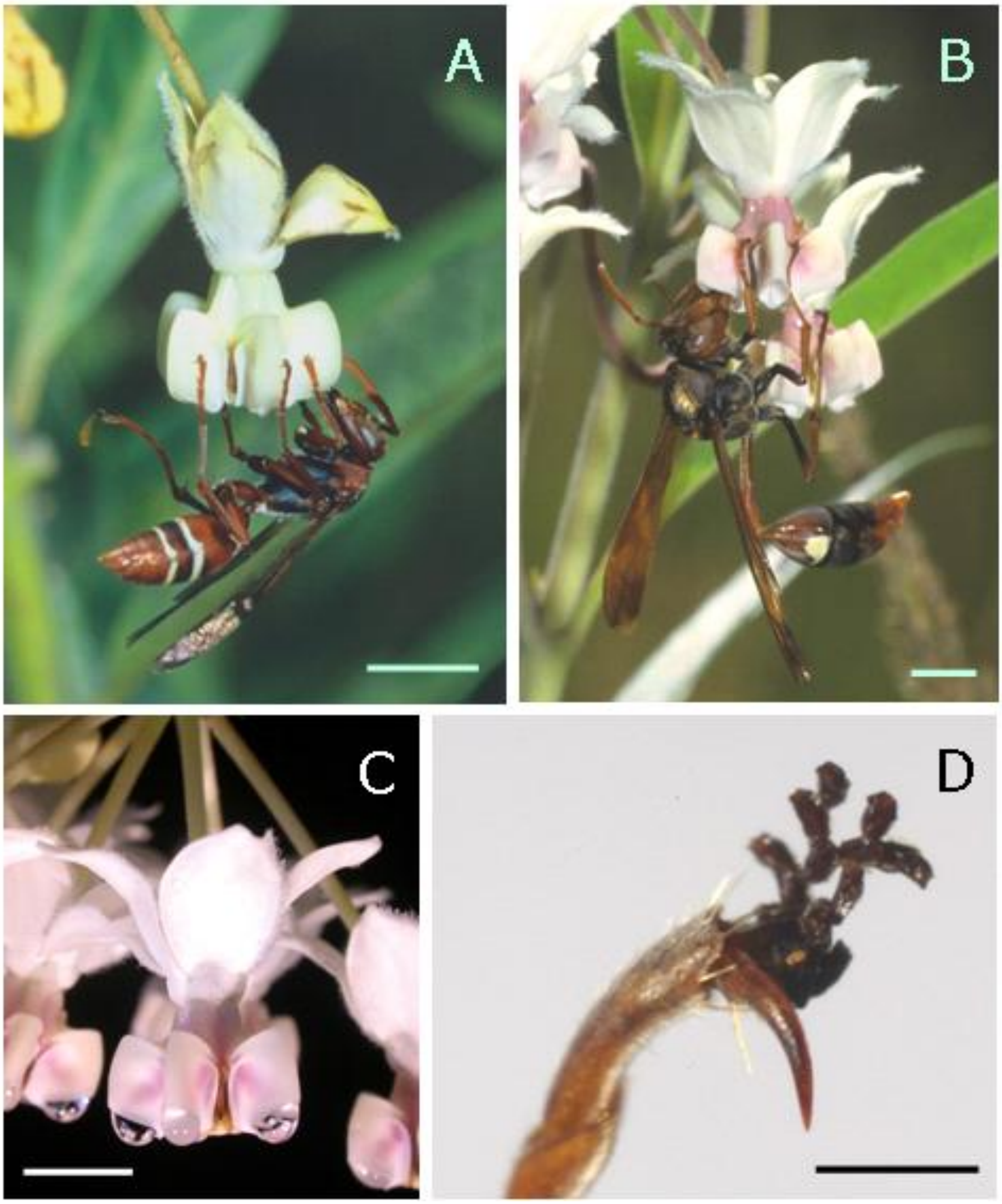

Figure 1: A) *Polistes* species showing typical position when visiting the flowers of *Gomphocarpus physocarpus*. B) *Belonogaster* wasps are equally important pollinators of this species. C) Nectaries formed by the corona lobes produce large quantities of accessible nectar. D) Multiple corpusculae attached in chains to the arolium (fleshy pad between claws of tarsi) of a species of *Belanogaster* wasp (Bars: A, B and C = 5 mm, D = 1 mm).

Table 3: Identity, gender, and mean (± standard error) pollinarium loads of insects collected visiting the flowers of *Gomphocarpus physocarpus*. Values in bold are means (± standard error) for individuals belonging to the respective insect families.

Taxon	*Study Sites*[1]	*Number captured and Sex*	*Average Corpus-culae*[2]	*Average Pollinaria*	*Average Half Pollinaria*[3]
Vespidae (Hymenoptera)		**39 F, 9 M**	**10.5 (1.4)**	**1.0 (0.2)**	**1.4 (0.3)**
Belonogaster (*B. dubia, B. lateritia, B. petiolata* and 2 unidentified species)	1, 2, 3, 4, 6	20 F, 3M	12.5 (3.5)	1.1 (0.4)	1.9 (0.4)
Polistes (*P. fastidiosus* and 7 unidentified species)	1, 3, 4, 5, 6	19 F, 3M	9.4 (2.9)	1.0 (0.4)	1.3 (0.7)
Ropalidia (three unidentified species)	3, 4, 5	3 F	9.3 (4.1)	1.0 (1.0)	0.7 (0.7)
Pompilidae (Hymenoptera)		**5 F, 11 M**	**1.2 (0.4)**	**0.5 (0.3)**	**0.4 (0.2)**
Hemipepsis (*H. capensis, H. hilaris* and 1 unidentified species)	1	4 F, 11 M	1.0 (0.9)	0.1 (0.1)	0
Batozonellus (unidentified species)	3	1 F	2	2	2
Apidae (Hymenoptera)					
Apis mellifera	1, 5	6 F	4.6 (1.6)	1.9 (0.7)	0.6 (0.4)
Halictidae (Hymenoptera)					
Lassioglossum (unidentified species)	4	16 F	0.2 (0.1)	0.3 (0.1)	0.1 (0.1)
Occasional visitors					
Cerceris sp (Sphecidae, Hymenoptera)	6	F	0	0	0
Scoliidae (Hymenoptera)	1	F	6	3	0
Ichneumonidae (Hymenoptera)	3	F	0	0	0
Formicidae (Hymenoptera)	1	2 F	0	1	0
Muscidae (Diptera)	1, 3	3?	0	0	0
Syrphidae (Diptera)	1	?	0	2	2
Chrysomelidae (Coleoptera)	5	?	0	0	0
Lycidae (Coleoptera)	1	?	1	0	0

1 Numbers refer to study sites given in Figure 2.

2 Corpusculae refers to pollinaria where both pollinia have been removed and only the mechanical clip remains.

3 Half polliniaria are defined as polliniaria where one pollinium has been deposited.

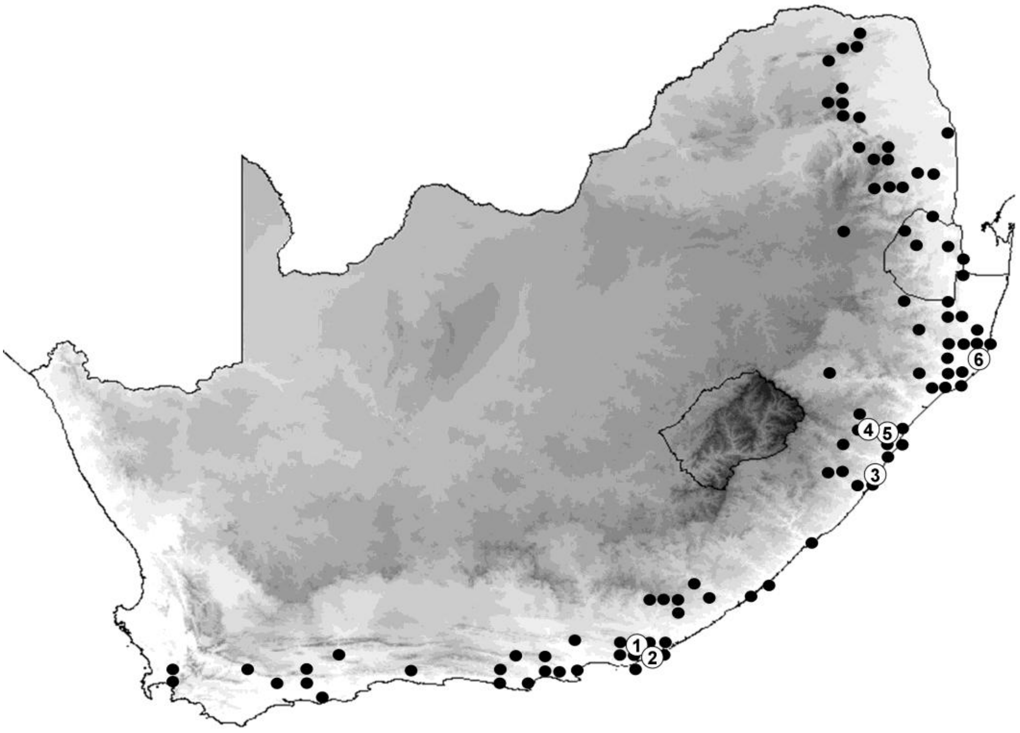

Figure 2: Distribution of *Gomphocarpus physcocarpus* in South Africa. Study sites in the Eastern Cape include **1**) Mountain Drive and Woest Hill on the Rietberg, Rhodes University, all in the vicinity of Grahamstown; **2**) Kasouga and Kenton-on-Sea. Sites in KwaZulu-Natal include **3**) Vernon Crookes nature reserve; **4**) Hesketh conservation area Pietermaritzberg; **5**) Ashburton and Thornville; and **6**) Cape Vidal and Lake St Lucia.

Nectar rewards

The nectar volume per coronal lobe decreased from 0.7 µl (SD = 0.44, n = 18, median = 0.63) between 7:00-8:30 am, to 0.04 µl (SD = 0.05, n = 14, median = 0.02) between 10:30-11:30am (Fig. 3A). This difference was statistically significant (Mann-Whitney U test, $p<0.0001$). At the same time nectar concentration increased from 18.9% (SD = 8.21, n = 17, median = 16.17%) to 43.2% (SD = 30.28, n = 8; Fig. 3B, median = 38.37%), but this difference was marginally non-significant (Mann Whitney U test, p = 0.071), probably as a result of the smaller sample size and greater variability. The increase in nectar concentration was insufficiently high to have been caused by evaporation alone as there was a near 18 fold decrease in the average volume of nectar. The increase in concentration was less and only increased to slightly more than double that of the initial concentration (although these differences were non-significant). These results suggest that both pollinators and evaporation played a role in reducing the nectar quantity; however our experimental protocol cannot separate these effects.

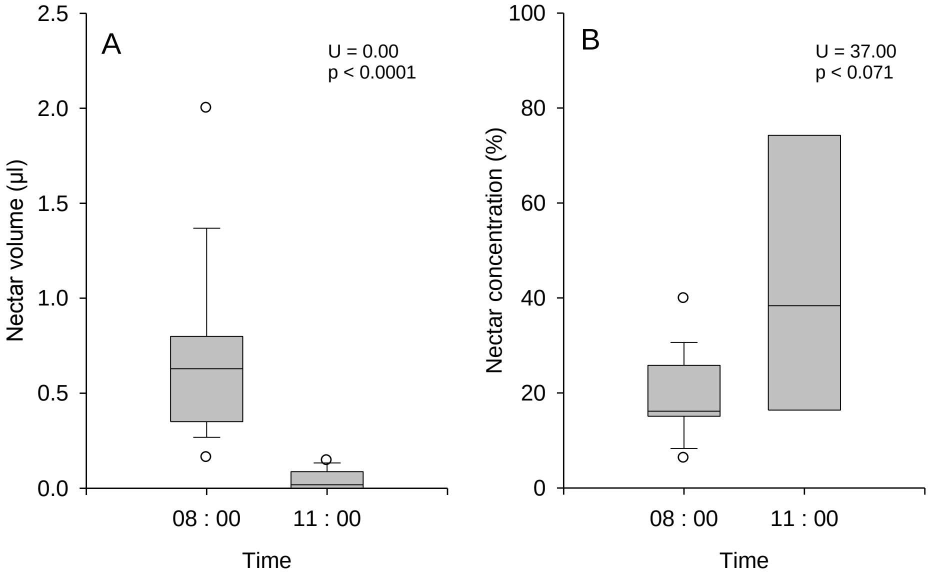

Figure 3: Boxplots indicating changes in **A**) nectar volume and **B**) nectar concentration between the start of insect visitation (7:30 to 8:30) and late morning (10:30 to 11:30) when insect activity decreased. Open circles indicate outliers, details of the statistics are given in the text.

Pollinaria reconfiguration

Pollinaria undergo marked reconfiguration. In a longitudinal plane, the two pollinia are at an angle of approximately 90 degrees to one another when removed from the flower (see Wyatt, 1976). Over the course of the reconfiguration this angle is reduced to nearly zero, with pollinaria coming to rest parallel to each other. This reconfiguration takes 224 seconds on average (SD = 77, n = 20) and is significantly longer than the average visit duration of 106s (SD = 62, n = 23) by pollinators to individual plants ($t_{(41)} = 5.56$, $p<0.0001$).

Cellular mechanisms for pollinaria reconfiguration

Transverse sections through the pollinarium indicated regions of large thin-walled cells located on the inside of the translator arms, next to the corpusculum (Fig. 4). The shape of this region of cell before and after reconfiguration suggests they may play a role in pollinarium reconfiguration (Fig. 4A and B).

In addition there is a layer of apparently turgid cells situated along the inner surface of the translator arm (Fig. 4A, 1 and 2). These cells also appear to lose water when pollinia are removed from the flower resulting in the bending of translator arms towards one another (Fig. 4B, 1 and 2).

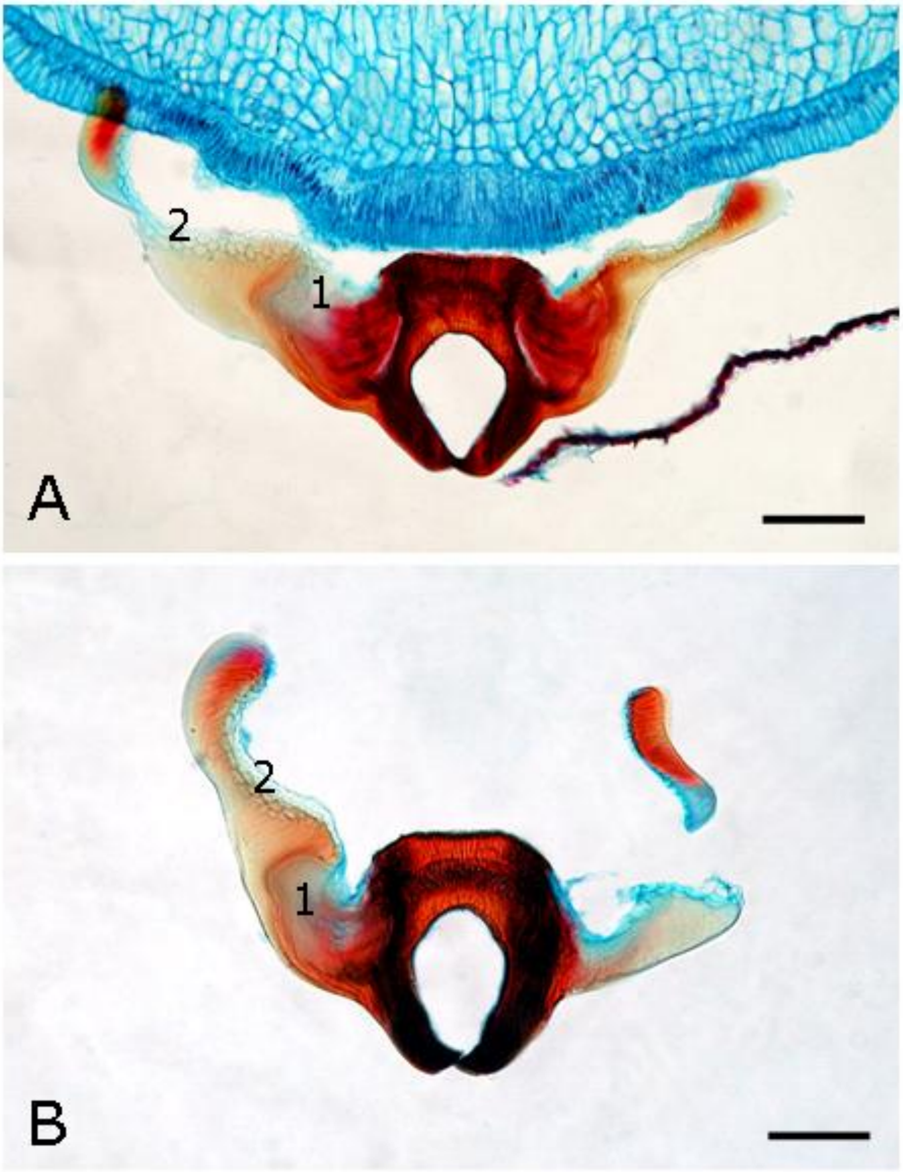

Figure 4: Anatomy of the corpusculum of *Gomphocarpus*. A) Corpusculum still attached to the gynostegium before reconfiguration. B) Corpusculum following removal from the flower and reconfiguration. Labels 1 and 2 are discussed in the text.

Population survey and test for the Allee effect

We found no significant relationships between population size and various measures of reproductive success in *G. physocarpus*, including the number of fruits per plant (Fig. 5a), the proportion of flowers with pollinaria removed (Fig. 5b), proportion of flowers with at least one pollinium inserted (Fig. 5c) and the proportion of removed pollinia inserted into stigmas (Fig. 5d). Population size and number of flowers per plant were also not significantly correlated ($p = 0.20$, $n = 23$).

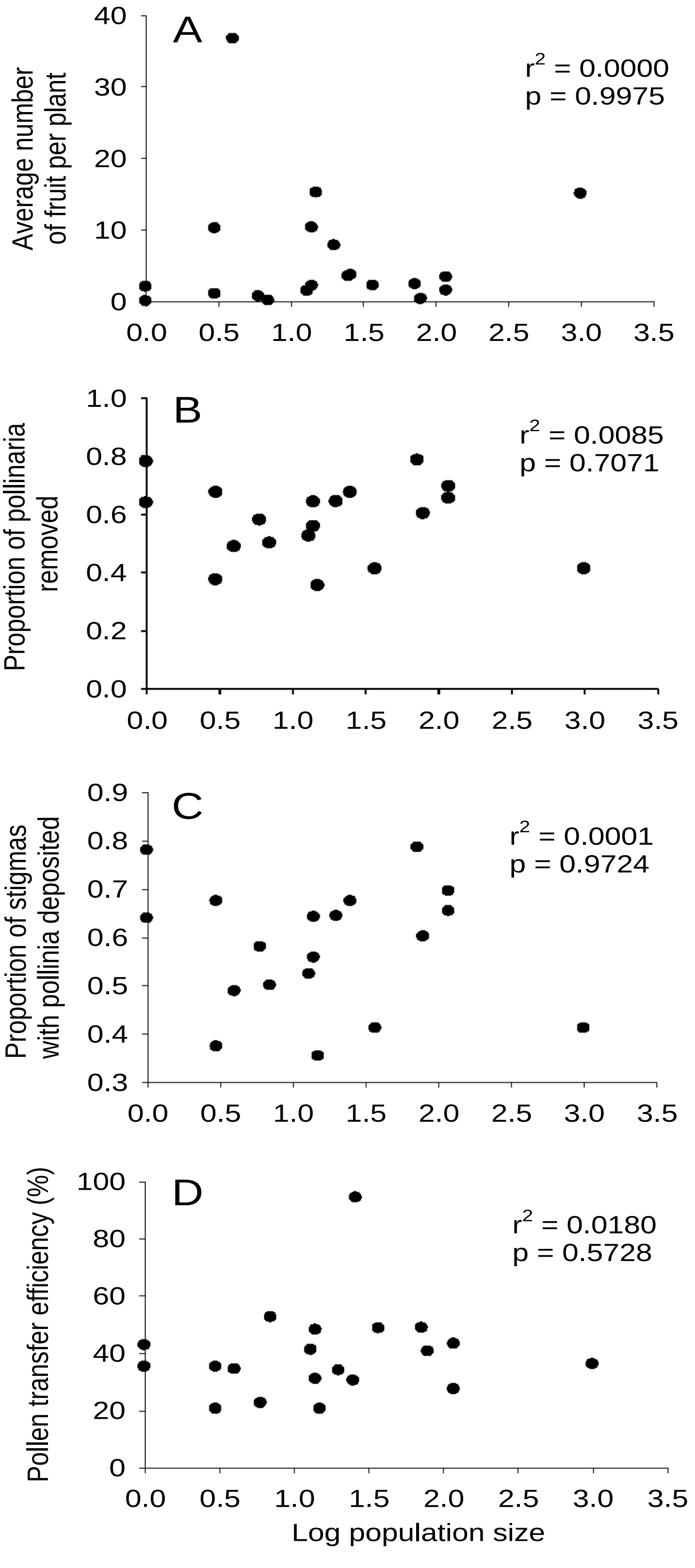

Figure 5: Measurements of reproductive fitness in relation to population size in *Gomphocarpus physocarpus*.

A) average fruit set, **B**) pollinaria removal, **C**) pollinia deposition and **D**) pollen transfer efficiency.

Discussion

Small populations of *Gomphocarpus physocarpus* can achieve high levels of pollination success (pollen removal and deposition), pollen transfer efficiency and fruit set. Contrary to our initial predictions, the breeding system experiment indicates that at least some populations are genetically self-incompatible and therefore completely reliant on insect flower visitors to transfer pollen among different plants. However, some populations in Australia, where this species is invasive, appear to be self-compatible (M. Ward, University of Queensland, personal communication). Compatibility in milkweeds may vary among individuals and populations (cf. Ivey *et al.,* 1999; Lipow and Wyatt, 2000). Even in self-compatible milkweeds, cross-pollination usually produces higher fruit set (Ivey *et al.,* 1999; Lipow and Wyatt, 2000).

Our results show that the pollination system is essentially specialized at the level of functional group (medium-sized vespid wasps), but generalist, and thus flexible, at the species level. This flexibility is also evident from the similarly wide range of wasp species, mainly vespids, which have been shown to pollinate the species in Australia (Forster, 1994). These results contrast with those recently obtained for another South African milkweed, *Pachycarpus asperifolius*, which is pollinated by just 2-3 species of pompillid wasps (Shuttleworth and Johnson, 2006). Pollination success and fruit set in *G. physocarpus* is considerably higher than in *P. asperifolius* which may reflect the broader spectrum of insects that can function as its pollinators.

The high levels of pollen transfer efficiency observed in *G. physocarpus* populations are comparable to other weedy milkweeds, such as *G. fruticosa* (15.2%, Harder and Johnson 2008), and *Asclepias curassavica* (2.2-17%, Wyatt 1980). PTE in *G. physocarpus* exceeds that of almost all the species studied by Ollerton *et al.* (2003). High levels of PTE in *G. physocarpus* must be due to a mechanically efficient pollen transfer system combined with high levels of fidelity by vespid wasps.

Floral specialization for pollination by wasps has been considered mainly in the context of brood site mutualisms, as in figs and fig wasps (Weiblen, 2002), and sexual deception systems in orchids (e.g. Steiner *et al.,* 1994; Mant *et al.,* 2002; Ciotek *et al.,* 2006). However, there is increasing evidence that flowers that provide nectar rewards can be specialized for pollination by wasps (Sahagun-Godinez and Lomeli-Sencion, 1997; Vieira and Shepherd, 1999; Ollerton *et al.,* 2003; Fenster *et al.,* 2004; Johnson, 2005). Other wasp-pollinated milkweeds include *Pachycarpus natalensis* and *Miraglossum verticillare* that are both pollinated by *Hemipepsis hilaris* (Ollerton *et al.,* 2003).

An important trait associated with many wasps seems to be the accessibility of nectar for these short-tongued insects. In *G. physocarpus* the abundant nectar supply accumulates in the shallow, cup-like corona lobes, accessible to the wasp and heavily utilised by these insects in the first half of the day. An exception to this pattern is the long-tongued masarid wasps which can access concealed nectar (Gess, 1996).

Although rates of self-pollination could not be quantified in this study, our data suggest that pollinarium reconfiguration times in *G. physocarpus* are generally longer than the duration of pollinator visits. As insertions are impeded mechanically until reconfiguration is completed, (C. Peter and G. Coombs, unpublished data), this would strongly promote cross-pollination (Peter and Johnson, 2006). Although the possible role of pollinarium reconfiguration in promoting cross-pollination in asclepiads has been discussed previously (Queller, 1985), the general association between reconfiguration and pollinator visit times in a range of orchids and asclepiads provides compelling support for the cross-pollination hypothesis (Peter and Johnson, 2006). In orchids, reconfiguration of pollinia after removal from the anther sac is thought to occur as a result of differential drying of cell layers of accessory tissue of pollinaria (Peter and Johnson, 2006). In *G. physocarpus*, two areas of pollinarium tissue have large, thin walled cells which appear to result in pollinarium reconfiguration when they desiccate.

Allee effects

The absence of Allee effects in populations of *G. physocarpus* is consistent with its weedy life history and relatively generalized wasp pollination system, but nevertheless surprising for a self-incompatible species. Our data suggest that efficient pollination in small populations, combined with a mechanism (pollinarium reconfiguration) that reduces self-pollination enables plants in small populations to achieve levels of fruit set comparable to those in larger populations. Contrary to the expectation of net pollen flow out of small populations, pollen transfer efficiency was unaffected by population size in *G. physocarpus*. This suggests either that pollinators show foraging constancy in small populations or that a net outflow of pollinia from small populations is balanced by an inflow from other populations. A more detailed analysis of pollen fates in this species would require direct labelling of pollinia, as has been done in orchid populations (e.g. Johnson *et al.,* 2005) and once in asclepiads (Pleasants, 1991).

There are still too few studies for general conclusions to be reached about whether colonizing species are relatively buffered against Allee effects and, importantly, whether Allee effects pose a significant ecological barrier to establishment and persistence (Liebhold and Bascompte, 2003; Davis *et al.,* 2004; Taylor and Hastings 2005, but see Groom, 1998). Pollen receipt and fruit set were not affected by population size in the self-compatible but allogamous invasive species *Senna didymobotrya* (Van Kleunen and Johnson, 2005). On the other hand, Allee effects have been detected in naturalized populations of the partially self-compatible invasive taxa *Spartina alterniflora* (Davis *et al.,* 2004) and in artificial populations of the self-incompatible invasive herb *Raphanus sativa* (Elam *et al.,* 2007).

Allee effects on seed production have been documented in the self-compatible colonizing milkweed *Vincetoxicum rossicum*, but the mechanism appeared to be through the inability of small populations to suppress competing vegetation, rather than through an effect of population size on pollination processes (Cappuccino, 2004).

Conclusion

Despite its reliance on cross-pollination for fruit set, *G. physocarpus* is able to reproduce efficiently in small populations. Even though the plant seems specialized for pollination by vespid wasps, these pollinators are common and diverse enough not to be a limiting factor for reproduction in small populations. It would be particularly interesting to study the successful naturalization of this species in Australia where it is considered a serious weed (Forster, 1994). One possibility is that substitute wasp pollinators are common enough in Australia to allow establishment of small populations. Another is that there has been evolutionary change in the compatibility system. Wyatt and Broyles (1994) document both self-incompatible and self-compatible breeding systems in asclepiads. In addition, several studies have found the breeding systems of milkweeds to be variable between different populations and different individuals within the same population (Lipow *et al.,* 1999; Lipow and Wyatt, 2000; Leimu, 2004).

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Chapter 3

The invasive "mothcatcher" (*Araujia sericifera*; Asclepiadoideae) co-opts native honeybees as its primary pollinator in South Africa

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Abstract

Background and aims

Successful invasive plants such as *Araujia sericifera* usually are either capable of automatic self-pollination or maintain pollinator services by having generalized pollination systems to make use of local pollinators in the invaded range. Alternatively plants must co-opt new pollinators with similar morphology to native pollinators or reproduce asexually. We aimed to document the pollination biology of *A. sericifera* in South Africa and given the success of this species as an invader predicted that sexual reproduction in this species either occurs through self-pollination or *A. sericifera* has successfully co-opted native insects as its pollinators.

Methodology

We examined the pollination biology of the South American *A. sercifera* in South Africa. We documented the effective pollinators including a comparison of the efficacy of nocturnal versus diurnal pollinators as well as the breeding system and long term natural levels of pollination success of this species.

Principal results

We found that native honeybees (*Apis mellifera*) were the main pollinators of *A. sericifera* in South Africa, while moths are unimportant pollinators despite pale flower colours and nocturnal scent production by the flowers. Plants from the Grahamstown population were incapable of autonomous self-pollination but pollinator mediated self-pollination does occur. However the highest fruit initiation resulted from out-crossed pollination treatments. The high pollen transfer efficiency of this species was comparable to other hymenopteran - pollinated exotic and native milkweeds, suggesting that *A. sericifera* maintains pollinator services at levels experienced by indigenous asclepiad species.

Conclusions

A. sericifera reproduces successfully in South Africa due to the combined ability of this species to successfully attract and exploit native honeybees as its pollinators as well as the ability of individual plants to set fruit from pollinator mediated self-pollination.

Introduction

Invasive species introduced into new environments in small numbers could experience pollen limitation or pollination failure if they cannot shift to new pollinators (Parker 1997, Larson *et al.* 2002, Parker and Haubensak 2002). However, pollination failure (lack of seed set due to pollinator absence) rarely occurs in invasive species and is more likely to prevent species with highly specialised pollination systems and intricate flower morphologies (*e.g.* figs and orchids) from becoming invasive (Richardson *et al.* 2000), although exceptions occur (e.g. *Ficus species*: Nadel *et al.* 1992, Gardner and Early 1996, Orchids: Liu and Pemberton 2010). Many invasive species typically either have generalized pollination systems and flowers with open accessible rewards (Richardson *et al.* 2000, Bjerknes *et al.* 2007), or overcome pollinator limitation through autonomous or pollinator mediated self-pollination (Baker 1974; van Kleunen *et al.* 2008).

The mechanism of pollination in milkweeds (Asclepiadoideae-Apocynaceae) is mechanically complex and requires the accurate re-insertion of pollinia (aggregated compact pollen masses) that are removed as pairs and deposited individually into a snugly fitting stigmatic groove (Wyatt and Broyles 1994, Ollerton *et al.* 2003; Fig. 1C). Two pollinia are suspended off a clam-like mechanical clip (the corpusculum) that attaches to the pollinator, and constitute a single structure, the pollinarium, that is removed by pollinators. Pollinia are deposited when the insect that is already bearing a pollinarium drags a pollinium through one of the five specialized stigmatic grooves where it may become lodged, breaking off to effect pollination (Wyatt 1976; Wyatt and Broyles 1994). This relatively specialised floral morphology translates into specialised interactions with pollinators in 70% of examined asclepiads which have less than five species of pollinators, while 38% have only a single pollinator (Ollerton and Liede 1997). Nevertheless, several milkweeds including the well known North American species in the genus *Asclepias*, have highly generalized pollination systems (Ollerton and Liede, 1997). Despite such generalization many of these are functionally specialized (*sensu* Fenster *et al.* 2004) to a group or family of pollinators with the right morphology and behaviour (Wolff *et al.* 2008).

Ten of the 94 species of milkweed occurring in Australia are naturalised invasive species (Forster 1994). In North America at least two species of *Vincetoxicum* are invasive (Cappucino 2004, Daehler 1998) while there are two naturalised Asclepiadoideae in South Africa (Victor *et al.* 2000). Invasive mikweeds are likely to depend largely on co-opting new pollinators as few species can set seed through autonomous self-pollination (Wyatt and Broyles, 1994).

Araujia sericifera (Brot.) is an invasive tropical vine that is famous for catching both diurnal and nocturnal Lepidopteran flower visitors. This results from the long proboscides of these insects becoming wedged between the rigid anther wings of its flowers – giving rise to common names of “mothcatcher” or “cruelplant”. Smaller insects may also be trapped in the corpusculum and are incapable of escaping as these insects are too small to remove pollinaria. *A. sericifera* is pollinated by honeybees in Australia (Coleman 1935) and bumble bees (*Bombus* species) and Scoliid wasps (*Scolia species* - Scoliidae) in Europe (Romeo 1933). Several notes and papers have enumerated insects that visit the flowers of *A. sericifera* in other countries (Romeo, 1933 and references therein; Coleman, 1935; Hicken, 1928) although records from the native range are limited to a single observation (Morong 1889).

Given the success of this species as an invader in South Africa and the rarity of autonomous self-pollination in the Asclepiadoideae, we hypothesised that *A. sericifera* successfully utilizes native pollinators to maintain pollination success. We therefore set out to (1) determine the reliance of *A. sericifera* on pollinators by documenting its breeding system; (2) determine the functional pollinators of *A. sericifera* in South Africa; (3) quantify the consistency of pollination success in this species for several consecutive flowering seasons; (4) determine the relative contribution of diurnal and nocturnal pollinators to pollination success; and (5) to compare whether the levels of pollination success in *A. sericifera* are similar to a native milkweed with similar growth form and pollination biology.

Methods

Study species

Araujia sericifera (Apocynaceae-Asclepiadoideae) is indigenous to tropical (including Peru, Argentina, Paraguay, Brazil) and temperate (Uruguay) regions of South America, and has become invasive in several countries in Europe (France, Greece, Italy, Portugal, and Spain), Australia, New Zealand, North America, Israel and South Africa (Forster and Bruyns 1992, EMPPO 2008). In South Africa it commonly grows in abandoned fields and on fences in urban environments (Fig. 1A; Henderson and Anderson 1966). Flowers are white, streaked with light purple and scented day and night. Flowers are borne on pedunculate axillary inflorescences (*sensu* Henderson and Anderson 1966). In South Africa, flowering begins in late November and ends in May with the mid-season peak occurring in December (pers. obs.).

Cynanchum ellipticum (Apocynaceae-Asclepiadoideae) is a common milkweed, endemic to southern Africa (Liede 1993). Both species share broad similarities including growth form and pollination biology. *Cynanchum ellipticum* also grows on fences in urban environments, forming large, dense floral displays. *Cynanchum ellipticum* flowers semi-continuously throughout the year whereas *A. sericifera* only flowers between November and March in Grahamstown.

Breeding systems

The breeding system of *A. sericifera* was determined using 20 wild plants growing around Grahamstown during 2007-2008 flowering season and again on ten plants during the 2008-2009 flowering season. During each year the duration of the breeding system study was 6 weeks.

We performed three treatments per plant and replicated each treatment between one to four times per plant throughout the study period. Treatments were (1) out-crossed flowers pollinated with pollen from another plant, (2) self-pollinated flowers pollinated with pollen from the same plant and (3) unmanipulated control, where no pollination was carried out to test for autonomous self-pollination. Only one of each treatment was made per umbel. Following Wyatt (1976) we inserted only one pollinium per flower using small forceps. Due to the tubular shape of the flower, we made a longitudinal slit down one side of the corolla to access the gynostegium. We only bagged flowers with light nylon mesh bags until buds opened. After treatments were performed, we prevented access to pollinators by wedging a cotton wool plug into the corolla. Because milkweeds often abort their fruit even after successful initial fertilization (Lipow and Wyatt 1998; Finer and Morgan 2003) we scored fruits as initial fertilizations and regularly inspected initiated fruits to record what proportion of initial fertilizations matured into fruit.

We tested for differences in the number of fruits that were initiated between different pollination treatments by using two sample *t*-tests based on different proportions of pollinations that initiated fruit (*e.g.* Lipow *et al*. 1999). In both years we only tested within year differences between the number of fruits that were initiated from either cross-pollinated flowers, self-pollinated flowers and unmanipulated controls. Results from the 2007-2008 season indicated that *A. sericifera* does not undergo autonomous self-pollination, so we did not repeat unmanipulated controls during the 2008-2009 season. All tests were at the 5% level of significance.

Pollinators and pollinator behaviour

Diurnal visitors were caught while visiting individuals of *A. sericifera* in Grahamstown. Bees were the most abundant diurnal visitors and we limited our sampling to a total of five days in 2007 and one day in 2008. Bees were normally caught between 0800 and 1030 h, with most sampling periods not exceeding one hour, for a total of approximately eight hours observation time. For all insects we counted the number of full pollinaria (pollinaria with no pollinia removed), half pollinaria (pollinaria with one pollinium removed) and corpusculae (pollinaria with both pollinia removed) present on the mouthparts.

Nocturnal visitors were collected during sampling periods ranging from 20 minutes to 2 hours. All observations where made between 1930 (sunset) and 2200 h. During each observation period we attempted to catch all observed moths and counted any additional visits where moths could not be caught. Moth visits were observed over 15 evenings (*ca.* 15 h observation time). Moths were only caught when visiting flowers, or collected after recently becoming stuck within a flower and were still alive.

Comparison between diurnal versus nocturnal pollination

We used seven large flowering individuals of which three were exposed to nocturnal pollinators, three to diurnal pollinators and a seventh plant was exposed to both (i.e. exposing part of the plant to nocturnal pollinators and another part to diurnal insects). Bagging consisted of either covering a large part of the plant with fine nylon mesh or by bagging entire inflorescences with large mesh bags. All open flowers were removed from plants prior to bagging and exclusion experiments were started once a sufficient number of flowers had opened per plant. Bags on plants exposed to nocturnal pollinators were removed at dusk (1900 - 1930 h) and replaced the next morning between 0440 - 0530 h before bees started visiting. Bags on plants that were only exposed to diurnal pollinators were removed at 0440 - 0530 h and then replaced again at dusk before moths started visiting. All plants were open to either nocturnal or diurnal pollinators for three to five days or nights. At the end of the bagging period we randomly picked up to 50 open flowers from each of the four plants and picked another 50 flowers from an unbagged section on the same plant to serve as control flowers being open day and night to all pollinators. This resulted in a sample size of each between 190 - 200 flowers for each of the four treatments. For statistical analysis we grouped flowers into those exposed to pollinators during the day or night only and the control flowers to either group.

We tested for differences between treatments by testing for differences in the percentage of flowers with pollinaria removed or deposited. Non-parametric analysis of variance was done using the program PERMANOVA (Anderson 2001; McArdle and Anderson 2001) as the small sample size for each category (*N* = 4) violated the assumptions of normality. Pairwise *post-hoc* differences were tested using this program. For both the overall model and *post-hoc* tests we used 999 permutations to obtain accurate *p*-values at the 5% level of significance (Anderson 2001; McArdle & Anderson 2001).

Pollinarium removal, deposition and pollen transfer efficiency

Flowers of *A. sericifera* were sampled once at the beginning of 2007 and at three different dates within each of the 2007-2008 and 2008-2009 flowering seasons. During these later two seasons, the sampling intervals were spaced approximately one month apart. At each sampling date we randomly picked three different flowers per plant from a subsample of plants (range: 9 – 28 for different sampling dates) growing on fences around Grahamstown. Due to the low number of flowering individuals during February 2007, we sampled up to 20 flowers per plant. For all flowers we scored pollination success by counting the number of pollinaria removed and the number of pollinia deposited per flower and used this to calculate the average percentage of flowers with at least one pollinarium removed, one pollinium deposited and the pollen transfer efficiency (PTE). PTE is the proportion of removed pollinia that are deposited on conspecific stigmas, calculated by dividing the number of deposited pollinia by the number of removed pollinia (removed pollinaria multiplied by two; Johnson *et al.* 2005). PTE can be considered a population level estimate of the efficiency with which pollinators move pollen between anthers and stigmas. It is a commonly used measure of pollination success in milkweeds (Coombs *et al*. 2009;

Shuttleworth and Johnson, 2008; Shuttleworth and Johnson, 2009) and Orchids (Peter and Johnson, 2008; Johnson *et al*. 2009; see Harder and Johnson (2008) for review).

Given the broad similarities in growth form and pollination biology we compared pollination success of *A. sericifera* and the native *C. ellipticum*. *C. ellipticum* flowers were sampled during the closest peak flowering period of this species to *A. sericifera* which, in Grahamstown, was from late February 2008 to May 2008. *C. ellipticum* flowers were sampled on three dates by picking three flowers per plant from between 22 - 31 plants. We then compared the percentage of flowers with pollinaria removed, flowers with pollinaria deposited and PTE between these two species using the program PERMANOVA due to the non-parametric nature of the data. We used 720 permutations and calculated *P*-values using the Monte - Carlo method which is advised for small samples (Anderson 2005).

Most pollinia of *A. sericifera* were deposited as whole pollinaria with one pollinium inserted into the stigmatic chamber while the other pollinium and connected corpusculum remained on the outside of the anther wings (Fig. 1C). We believe this to be an unusual pattern of pollinium deposition for an asclepiad as the deposited pollinium typically breaks away from the caudicle and is left behind. Therefore this pattern of pollinium deposition is likely the result of a morphological mismatch between *A. sericifera* and its co-opted pollinators. To document whether this pattern of pollinium deposition differs to that of *C. ellipticum*, we used the same flowers that were used to calculate pollen transfer efficiency and for three of the sampling dates of both species we counted the relative proportions of pollinaria that were deposited in this way and compared this to the proportion of pollinaria that were deposited “normally”, where a single pollinium is broken off of the corpusculum and seated within the stigmatic chamber.

Colours and Reward

Flowers colours of *A. sericifera* were measured on one flower selected randomly from each of ten different plants (*N* = 10 flowers). Colour spectra of *A. sericifera* were measured using a USB 2000 photo spectrometer (Ocean Optics, Dunedin Florida, see Peter and Johnson 2008a for details). Two measurements were made, the first on the white part of the petal and the second on the inner corolla where flowers are frequently dappled with purple spots and streaks.

To measure the standing nectar volume and concentration of *A. sericifera*, we bagged 2 inflorescences per plant on 10 plants at 1800 h using nylon mesh bags. Inflorescences were harvested on the following morning between 0800 h and 0900 h, and nectar extracted from one randomly selected flower per inflorescence using 10 µl micropipettes and the concentration measured as percentage sucrose equivalents using an Atago 0 - 50 percent sucrose refractometer.

Results

Breeding system

Only cross-pollinated and self-pollinated treatments initiated fruit, suggesting that autonomous self-pollination or agamospermy does not occur (Table 1). During 2007-2008 the percentage of successful fertilizations from cross-pollinated treatments was not significantly greater than that in self-pollinated treatments (P = 0.096, t_{76} = 1.66). The percentage of flowers that received out-cross pollen and initiated fruit was 39.0% (2007-2008) compared to 20.5% for self-pollinated flowers. The percentage of cross-pollinated and self-pollinated flowers that initiated fruit were both significantly greater than the unmanipulated control where none of the flowers initiated fruit (cross pollination vs. unmanipulated control: t_{88} = 4.82, P < 0.001; self-pollination vs. unmanipulated control: t_{86} = 3.32, P < 0.001). During 2008-2009 the percentage of flowers that initiated fruit from cross pollination treatments significantly exceeded that initiated by self-pollination treatments (52% vs. 21.4%, t_{51} = 2.32, P = 0.02). Fruit abortion was generally high in both outcross and self-pollination treatments and only a fraction (maximum = 30.8%) of successful pollinations matured into fruit. Only two cross-pollinated fruit matured in the 2007-2008 flowering season and four cross-pollinated and one self-pollination fruit matured in the 2008-2009 flowering season. The small sample size of matured fruit precluded any statistical analyses on these data.

Table 1: Results of a breeding system for two years on *Araujia sericifera*. *A. sericifera* was not capable of autonomous self-pollination but was capable of geitonogamy although outcross pollination treatments had the highest percentage successful fertilizations.

Flowering season (Year)	**Treatment**	**No. of flowers per treatment**	**Initiated fruit**	**%†**	**Initiated fruit that matured**	**%**
2007-2008	Cross-pollination	41	16	39.0^{a}	2	13.0
	Self-pollination	39	8	20.5^{a}	0	0
	Unmanipulated control	49	0	0^{b}	0	0
2008-2009	Cross-pollination	25	13	52.0^{*}	4	30.8
	Self-pollination	28	6	21.4^{**}	1	16.7

†Superscript letters and symbols indicate significant differences using two-sample t-tests based on proportions. Different symbols were used for different years to indicate that tests were not done between different years.

Pollinators and pollinator behaviour

Honey bees (*Apis mellifera*) were the main diurnal visitors to *A. sericifera* with 158 bees being caught in approximately 8 hours of sampling effort spread over six sampling dates (Fig. 1, B&D; Table 2). The majority (69.6%) of bees bore pollinaria which were carried exclusively on the proboscides. The average total number of pollinaria per bee ranged between 1.0 (SE = 0.2) and 1.30 (SE = 0.2) on different sampling dates. The mean number of full pollinaria always exceeded that of ½ pollinaria, which was in turn generally higher than the number of corpusculae carried (Fig. 2). Visits were initiated by first hovering in front of the flower before alighting on the dissected part of the petals and then crawling into the corolla tube as the proboscides of the bees were too short to access nectar merely by inserting the proboscis into the flower.

Other diurnal visitors collected include single individuals of the day flying *Cephanodes hylas* (Sphingidae) and two butterfly species*, Acraea horta* (Nymphalidae) and *Catopsilla florella* (Pieridae). Two other individuals of *C. hylas* were observed visiting *A. sericifera* during the day but not captured (Table 2).

Nocturnal visitors included at least 11 different species of moths visiting *A. sericifera*, most of which were small settling noctuids (Table 2). The most abundant noctuid species were *Tycomarptes inferior* (Fig 1 F), *Spodoptera cilium* and *Helicoverpa armigera*. Pollinarium loads borne by these moths ranged from a maximum of 0.8 (SE = 0.2) in *T. inferior* to 0.5 (SE = 0.50) in *H. armigera.* Larger Noctuids (*Ericeia congressa* or *E. sobria* and *Anomis subulifera*) and one hawkmoth (*Theretra capensis* - Sphingidae) were also caught visiting *A. sericifera*. Moths where less abundant than bees and in 15 hours we caught 17 moths and saw approximately 50 visits. Another 5 moth species were collected while stuck in flowers during the day and are listed as “additional collections” in Table 1. Moths carried pollinaria on the tip of the tongue with the corpusculum either surrounding the tip or clipping on to the side of the proboscis tip in larger Sphingids. The abundance of moths was typically low and very variable (range: 0 – 24 observations per evening). During five of the 15 evenings no moth visits were seen. The highest visitation rate was recorded on one large plant on the evenings of 29 January 2008, 30 January 2008 and on 1 February 2008, where we saw 6, 24 and 8 visits respectively. Moths visited the flowers of *A. sericifera* by making hovering approaches before alighting on the petals and extending their proboscides into the basal nectar cavities of the flower. Smaller sized moths also crawled into the short tubular corona in order to reach nectar. We inspected several moths that were caught within the flowers and found that either the tongue itself was wedged between the anther wings or moths carrying a pollinarium are caught when the entire pollinarium is dragged into the stigmatic chamber and wedged behind the anther wings (Fig. 1, E). Smaller moths may be too weak to break the caudicle when a pollinium is deposited correctly (Fig. 1, F).

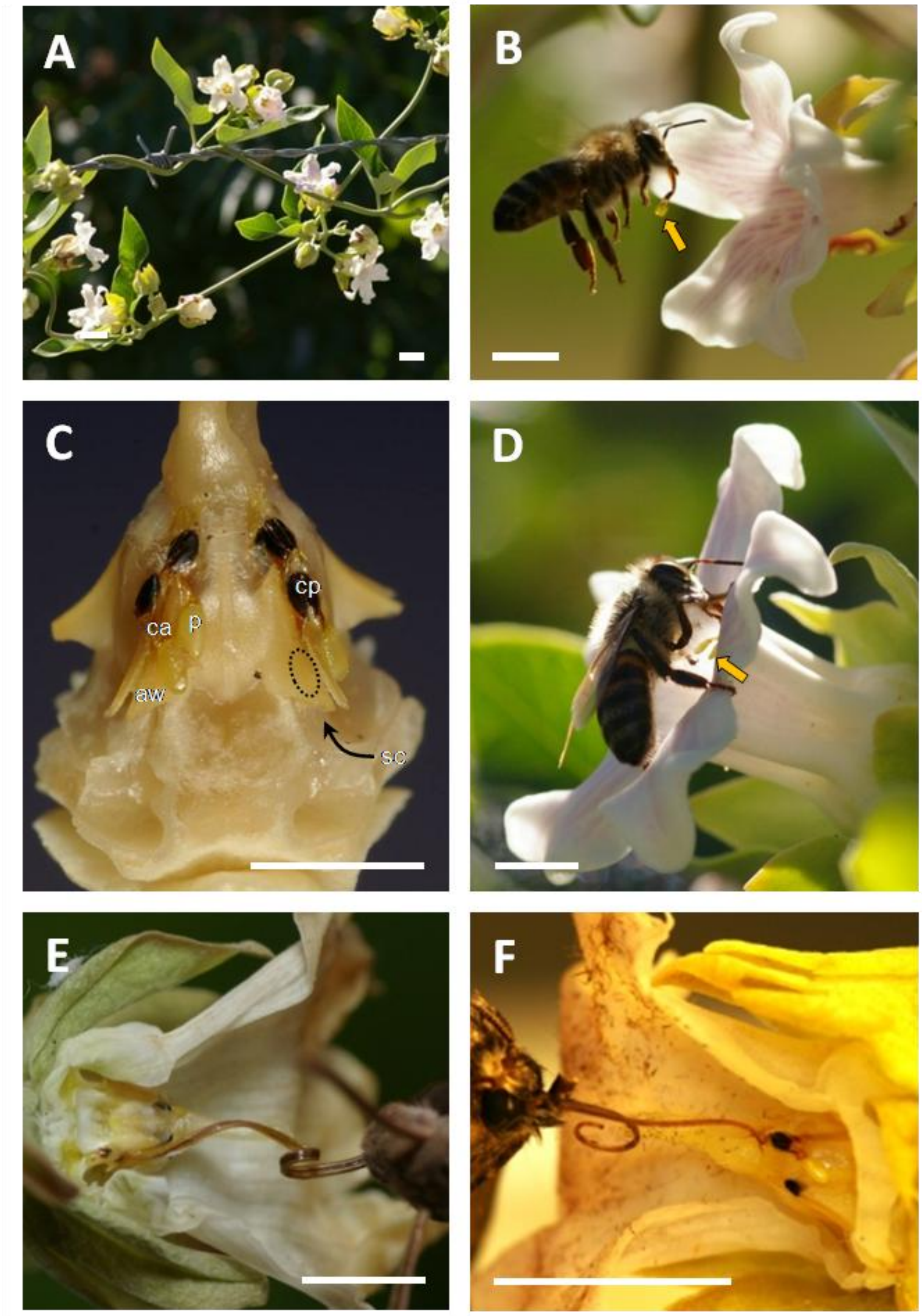

Figure 1. The invasive *Araujia sericifera* is commonly found growing on urban fence-lines (A). Honey bees (*Apis mellifera*) visit *A. sericifera* by initially hovering in front of the flower (B) and then landing on the petals (F). A flower of *A. sericifera* with the petals removed showing the gynostegium (C, aw = anther wing, ca = caudicle, cp = corpusculum, p = pollinium, sc = stigmatic chamber, dashed oval indicates position of a deposited pollinium). The photo shows 4 whole pollinaria that have been deposited in two stigmatic chambers (two per chamber). This way of pollinarium deposition is considered unusual as pollinaria are typically deposited individually with only one pollinium lodged inside the stigmatic chamber (dashed oval, C), and not with one pollinium inside the stigmatic chamber while the other pollinarium is on the outside (see text for further discussion). A sphingid moth, *Temnora plagiata* (E) and noctuid, *Tycomarptes inferior* (F, insert) found stuck inside the flower of *A. sericifera* (Scale bars: A = 10mm, D = 3mm; All others = 5mm).

Table 2: Summary of the total numbers of different species of insects caught visiting the flowers of *A. sericifera*, sampling effort (sampling days and hours) and the average numbers of whole, ½ pollinaria and corpusculae borne by each taxon. Honeybees were the most abundant flower visitors and the majority of these insects bore pollinaria. A wide diversity of moths visited *A. sericifera*, but moths were less abundant than honeybees and generally carried lower numbers of pollinaria.

Species	**Order**	**Family**	**No. of days sampled**	**Total number of hours sampled**	**Number of individuals caught**	**Number of individuals bearing pollinaria**	**Whole pollinaria (mean ±1SE)**	**½ Pollinaria (mean ±1SE)**	**Corpusculi (mean ±1SE)**	**Total pollinium load (mean ±1SE)**
Diurnal visitors										
Apis mellifera	Hymenoptera	Apidae	6	8	158	110	0.85±0.84	0.21±0.42	0.082±0.34	1.14±0.1
Xylocopa caffra	Hymenoptera	Apidae	6	8	1	0	0	0	0	0
Xylocopa flavicollis	Hymenoptera	Apidae	6	8	1	0	0	0	0	0
Nocturnal visitors										
Anomis sabulifera	Lepidoptera	Noctuidae	15	15	1	0	0	0	0	0
Athetis pigra	Lepidoptera	Noctuidae	15	15	1	0	0	0	0	0
Borolia spp.	Lepidoptera	Noctuinae	15	15	1	1	0	0	1	1
Ericeia congressa	Lepidoptera	Noctuidae	15	15	1	0	0	0	0	0
Ericeia congressa or E. sobria	Lepidoptera	Noctuidae	15	15	2	0	0	0	0	0
Helicoverpa armigera	Lepidoptera	Noctuidae	15	15	2	1	0.50 ± 0.50	0	0	0.50 ± 0.50
Spodoptera cilium	Lepidoptera	Noctuidae	15	15	3	2	0.67 ± 0.33	0	0	0.67 ± 0.33
Theretra capensis	Lepidoptera	Sphingidae	15	15	1	0	0	0	0	0
Tycomarptes inferior	Lepidoptera	Noctuinae	15	15	5	4	0.60 ± 0.24	0.2 ± 0.20	0	0.8 ± 0.20
Additional collections*										
Acraea horta	Lepidoptera	Nymphalidae	2		2	1	0.50 ± 0.50	0	0	0.50 ± 0.50
Borolia spp.	Lepidoptera	Noctuinae	2	-	2	1	0.50 ± 0.50	0	0	0.50 ± 0.50
Catopsilla florella	Lepidoptera	Pieridae	1	-	1	0	0	0	0	0
Cephanodes hylas#	Lepidoptera	Sphingidae	1	-	1	1	0	0	1	1
Temnora plagiata	Lepidoptera	Sphingidae	1	-	1	1	1	1	1	3
Temnora pylas	Lepidoptera	Sphingidae	1	-	1	1	0	0	0	0
Theretra capensis	Lepidoptera	Sphingidae	1	-	1	1	1	0	0	1

*Additional collections refer to insects not collected during sampling times.
Day-flying hawkmoth

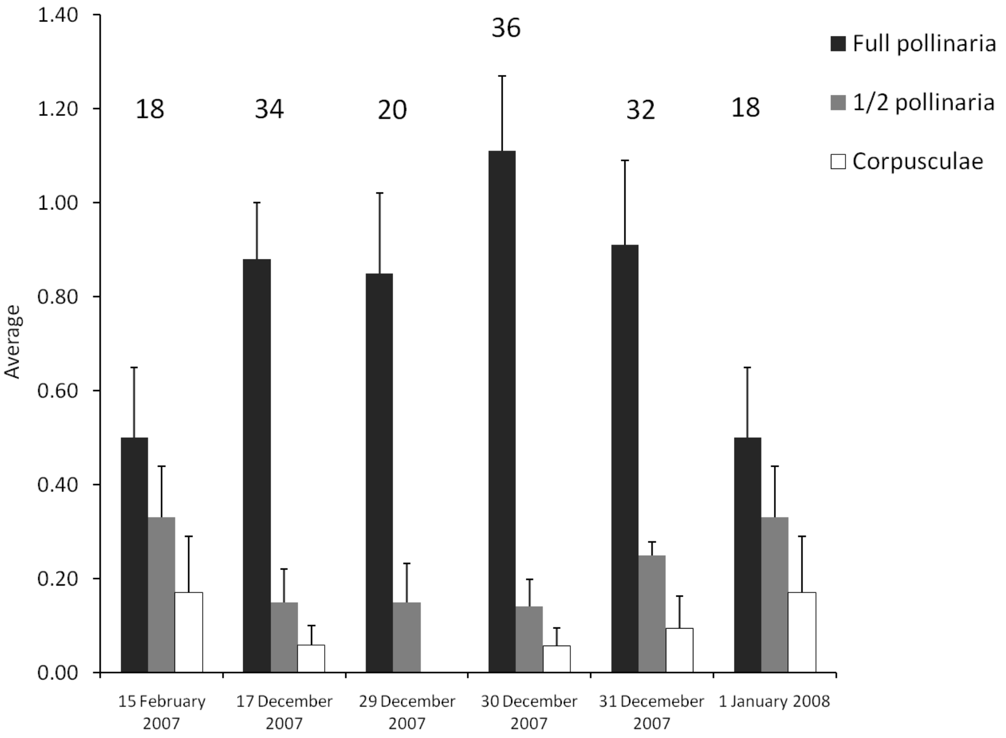

Figure 2. Changes in the mean number of full pollinaria, ½ pollinaria and corpuscula carried by honeybees visiting *A. sericifera*. Pollinarium loads on honeybees caught on different days indicated that honeybees mostly carried full pollinaria. The lower number of ½ pollinaria and corpusculae present on the mouthparts results from most pollinaria being deposited as full pollinaria due to the morphological mismatch between pollinaria and native honeybees (see text for details). Numbers appearing above bars indicate the number of bees caught at each sampling date (Bars = mean ± 1SE).

Comparison between diurnal and nocturnal pollination

The average percentage of flowers with pollinaria removed from plants that were only exposed to nocturnal pollination was 4.5% (SE = 1.9; Fig 3). This was significantly lower than the percentage of flowers with removals from plants exposed only to diurnal pollinators (average “Diurnal pollinators only” = 44.5%, SE = 8.4, *t (4)* = 4.65, *P* = 0.034) and the control of flowers exposed to nocturnal pollinators only (average control = 56.5 %, SE = 9.7, *t (4)* = 5.27, *P* = 0.030). The average percentage of flowers with pollinaria removed from plants exposed only to diurnal pollinators was 44.5% (SE = 8.4) and was not significantly different from its control (average control = 52.5%, SE = 5.7, *t (4)* = 0.79, *P* = 0.47).

The average percentage of flowers with pollinia deposited in plants exposed only to nocturnal pollination was 1% (SE = 1.0) and was significantly lower than the control flowers of this group (average control = 30.5%, SE = 10.5, *t (4)* = 2.79, *P* = 0.03) and to the percentage of pollinated flowers exposed to diurnal pollinators (*t (4)* = 3.97, *P* = 0.034). The average percentage of flowers that received at least one pollinium was 25.7% (SE = 6.1) for plants exposed only to diurnal pollinators and did not differ significantly from the control flowers of this group (average control = 28.1%, SE = 9.6, *t (4)* = 0.21, *P* = 0.94).

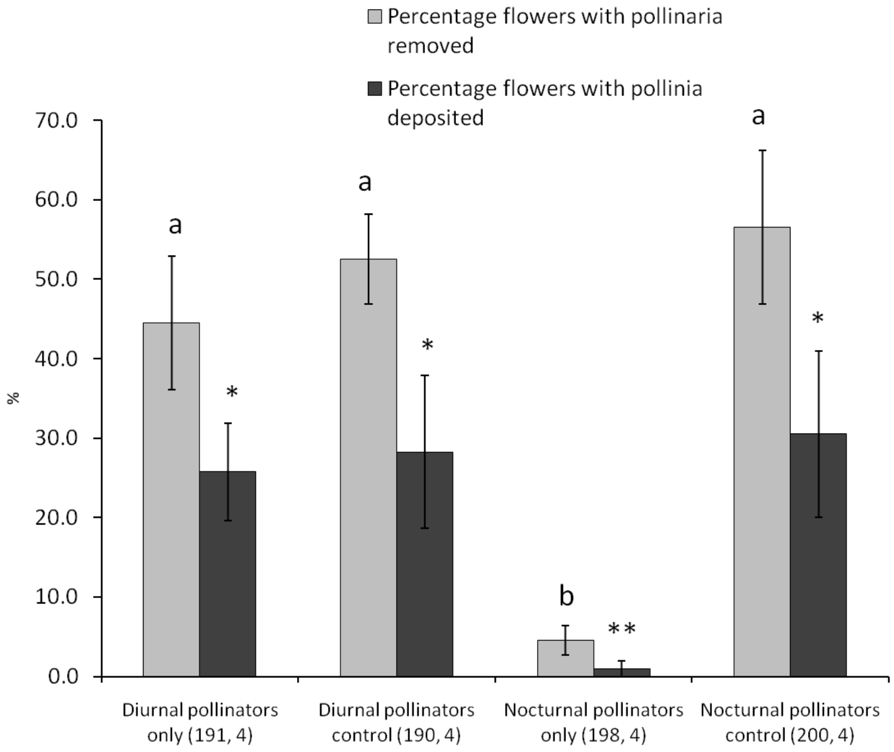

Figure 3. Differences in the percentage of flowers with pollinaria removed and the percentage of flowers with pollinia deposited in plants that have been exposed either to diurnal pollinators only, nocturnal pollinators only, or both (control). Plants exposed to diurnal pollinators only had a significantly higher percentage of flowers with pollinaria removed and deposited than plants exposed only to nocturnal pollinators. Asterisks above bars correspond to significant differences between treatments. Sample sizes (n flowers, n plants) for each treatment are included in parentheses below bars. All bars = mean ± 1SE.

Pollinarium removal, deposition and pollen transfer efficiency

In *A. sericifera*, the percentage of flowers with at least one pollinarium removed ranged between 30.5 to 54.2 % on different sampling dates. The percentage of pollinated flowers ranged from a minimum of 14.7% to a maximum of 23.9% (Fig. 4). PTE was generally high, and ranged from a minimum of 12.8% to a maximum of 36% across both years. The trend of PTE however, did not vary predictably across sampling dates.

The average percentage of flowers with at least one pollinarium removed was 77.4% (SE = 7.6) for *C. ellipticum* which was significantly higher than the 52.6% (SE = 1.0) obtained for *A. sericifera* (t_5= 3.25, P = 0.035; Fig. 5). The percentage of flowers that received pollinia was 65.5% (SE = 10) in *C. ellipticum* and was significantly higher than the 18.8% (SE = 2.4) received by *A. sericifera* (t_5 = 4.53, P = 0.007). The average PTE of *C. ellipticum* was 36.9 % (SE = 4.5) which was not significantly higher than that of *A. sericifera* (mean = 22.9.0%, SE = 6.9; t_5 = 1.71, P = 0.17).

In *A. sericifera* the majority (61.2%; 30 of 49) of pollinaria were deposited as whole pollinaria with one pollinium inserted in the stigmatic chamber while the other remaining pollinium and corpusculum was left outside (Fig 1, C). This was significantly lower than the 1.9% (8 of 427; *t*-test based on proportions, $P < 0.0001$) of *C. ellipticum* pollinia deposited in this manner, the majority being deposited as single pollinia. The remaining percentage of depositions in *A. sericifera* were either deposited normally (22%, 11 of 49) or were deposited as either the entire pollinarium (i.e. both pollinia and corpusculum) inside the stigmatic chamber or as a ½ pollinarium. The vast majority of *C. ellipticum* depositions (97.9%, 418 of a total of 427) were deposited “normally” as explained in the methods.

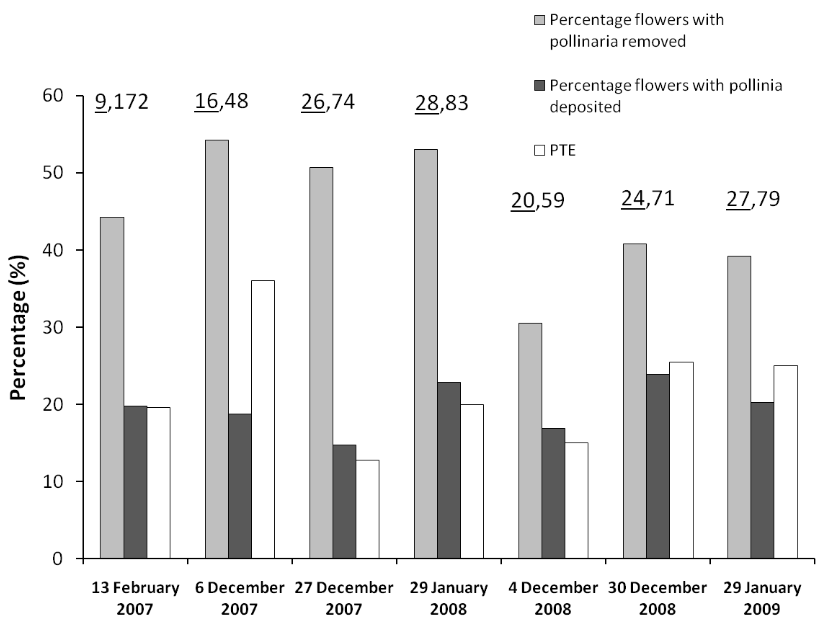

Figure 4. Changes in the percentage of flowers with at least one pollinarium removed, with at least one pollinium deposited and pollen transfer efficiency (percentage of removed pollinia that are deposited on conspecific stigmas) at different sampling dates between February 2007 and January 2009. The pollination success of *A. sericifera* was generally high suggesting that this species effectively maintains pollination service to its flowers outside its native range by attracting native diurnal pollinators (Numbers above each sampling date contain number of plants (underlined) followed by number of flowers for each sampling date).

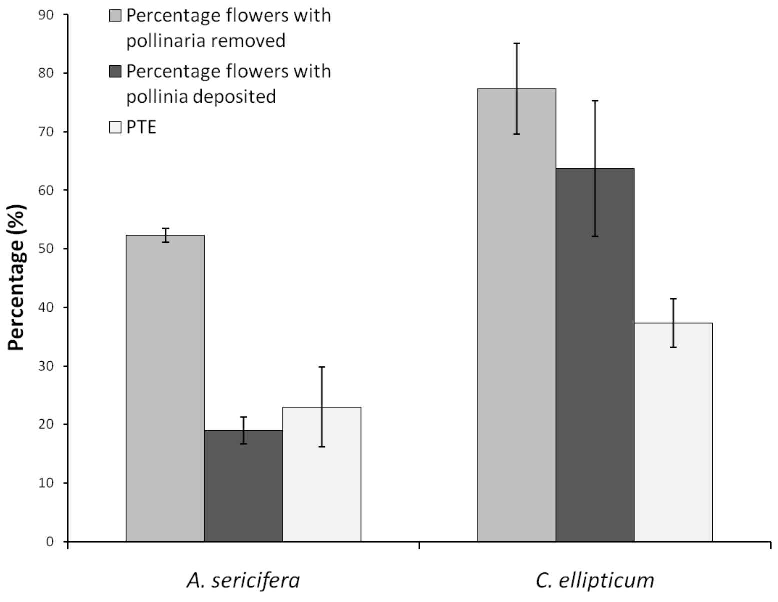

Figure 5. Comparison of the average percentage of flowers with pollinaria removed, - flowers with pollinia deposited and pollen transfer efficiency between the exotic *A. sericifera* and native *C. ellipticum*. Pollinarium removal and deposition was significantly higher in *C. ellipticum* but pollen transfer efficiency was similar and not significantly different between these two species (Bars = mean ± 1SE). Flowers of *A. sericifera* were sampled on (6 December 2007, *N* = 16 plants, 48 flowers; 27 December 2007, *N* = 26 plants, 74 flowers, 29 January 2008, *N* = 28 plants, 83 flowers). Flowers of *C. ellipticum* were sampled on 17 March 2008, (*N* = 22 plants, 64 flowers); 29 March 2008 (*N* = 31 plants, 87 flowers) and 14 April 2008 (*N* = 31 plants, 92 flowers).

Colours and reward

Colours for the tips of the corolla and the centre varied between purple and white. Both areas only reflected above 400nm indicating no UV reflectance from the petal. Nectar volumes were large (Average = 17.27µl; SE = 2.54, *N* = 19), but highly variable (range: 0.97 µl to 48.81 µl). The concentration per flower ranged from 5.90 to 50.75 sucrose equivalents with an average concentration of 22.0 % sucrose equivalents (SE = 2.66, *N* = 19).

Discussion

In South Africa, *Araujia sericifera* is pollinated primarily by native honeybees (*Apis mellifera*) while nocturnal moths are relatively ineffectual pollinators. Other diurnal flower visitors such as carpenter bees, day flying hawkmoths and butterflies were sometimes seen visiting this species but only did so infrequently and rarely carried pollinaria. Honeybees have learnt to access the nectar of the oversized flowers but like moths, bees were sometimes “caught” by the anther wings of the flower, but most freed themselves after a brief struggle. Working in Europe, Coleman (1935) and Romeo (1933) also observed that larger hymenoptera such as carpenter bees (*Xylocopa violacea*), bumblebees (*Bombus pascuorum* and *B. terrestris*), Scoliidae (*Scolia flavifrons* and *S. sexmaculata*) and honeybees manage to escape from the anther wings more often than not.

Moths visiting *A. sericifera* in Grahamstown removed and deposited only a fraction of pollinaria when compared to honeybees. Moths and butterflies have also been observed visiting this species in Europe (Romeo 1933, Hicken 1928), although these authors report more butterflies than were observed in the current study. Moths bearing pollinaria had the corpusculae attached around the tip of the tongue, similar to pollinaria of the moth-pollinated vine *Metaplexis japonica* (Asclepiadoideae; Sugiura and Yamazaki 2005). The efficacy of moths in pollinating *A. sericifera* is limited due to the tendency of these insects to get stuck and die within the flowers. This ineffectiveness of moths in depositing pollinia is further confirmed by the relatively few ½ pollinaria carried by these insects. Similarly Romeo (1933) found that several genera of Noctuidae (*e.g. Plusia* species, *Heliothis* species and *Caradrina* species) and Sphingidae (*Deilephila* species and *Macroglossa* species) visited the flowers of *A. sericifera* in Europe and supposedly also play a minor role in the pollination of this species. It is worth noting that the appendages of both pollinating and non-pollinating insects regularly become stuck between the anther wings or within the corpuscular groove of milkweed flowers and this does not only occur in invasive species (see Robertson 1887, Hicken 1928, Frost 1965, Morse 1981, Shuttleworth and Johnson 2009).

Understanding the pollination biology of *A. sericifera* requires examining pollinator records from its native range. Most of the records of insects pollinating *A. sericifera* are old (1825 - 1935) and are confined to areas where it is exotic (*e.g.* Romeo 1933, Hicken 1928, Coleman 1935). Honeybees that frequently pollinate *A. sericifera* in its invasive range are not native to its region of origin in South America (Ruttner 1988). Bumblebees are native to South America (Michener 2000), and were proposed by Coleman (1935) to be the pollinator in the native range. The only record of a potential pollinator in its natural range was a visit by a day flying hawkmoth in Paraguay (Morong 1889). The large nectar volume, white flowers and nocturnal scent is typical of moth pollinated flowers (Faegri and van der Pijl 1979) and may explain the attractiveness of these flowers to moths around the world. The nectar concentration is relatively low and typical of hawkmoth pollinated species (Cruden *et al*. 1983). White coloured flowers, bulbous nectar cavities and filaments emerging from the top of the pistil are also present in the moth-pollinated *Metaplexis japonica* (see Tanaka *et al.* 2006) suggesting that moths could be the natural pollinators. Pollination by Hymenoptera is equally likely - the large, sharply-pointed and rigid anther wings are also present in some *Pachycarpus species* pollinated by large Pompilid wasps (Shuttleworth and Johnson 2006).

The large flower size of *A. sericifera* suggests that it is not optimized for pollination by relatively small honeybees. Despite this, honeybees are efficient at removing and depositing pollen. The nectar volumes of this species were generally large but highly variable making it difficult to say whether these nectar volumes point to larger insects being the natural pollinators. Inferring the natural pollinator from the size of the nectar reward is also difficult as the standing crop of nectar is known to be variable (Keasar *et al*. 2008). The range of nectar concentrations recorded for flowers of this species is however well within the range of most bee-pollinated plants (Cruden *et al.* 1983).

One possibility is that *A. sericifera* is highly generalised in its native range which enables it to exploit diverse assemblages of pollinators in various parts of the world where it has become invasive. It seems likely from morphological evidence presented above (white, scented flowers, long corolla tube for an asclepiad, abundant nectar, large pollinaria), that native pollinators are either relatively large moths with relatively short tongues such as large noctuids or relatively large, long-tongued bees (*Bombus* or euglossine bees). As noted above, honey bees do not occur in South America where eusocial bees include only smaller *Meliponini* stingless bees or larger *Bombus* bees (Michener, 2000). Honey bees mismatch with morphological aspects of the flower such as the large corolla tube and large pollinaria which attach poorly to the bee resulting in messy deposition of whole pollinaria - all of these features point to *A. sericifera* being adapted to pollinators larger than honey bees.

The interaction of *A. sericifera* with native honeybees in South Africa and with honeybees and bumblebees in other invaded areas confirms that the intricate flower morphology of milkweeds is not a barrier to co-opting new pollinators, particularly in species that attract honeybees and other generalist Hymenoptera. For instance exotic honeybees are one of the most effective pollinators of *Asclepias incarnata* within its home range (Ivey *et al.* 2003). Similar groups of Hymenoptera (pompilids, vespids and ichneumonids) pollinate *Gomphocarpus physocarpus* in its invasive (Australia) and native (South Africa) ranges (Forster 1994, Coombs *et al*. 2009). Milkweeds that are pollinated by pollinators other than the Hymenoptera have also become invasive. One species, *Vincetoxicum nigrum*, is an invasive fly-pollinated vine occurring in the USA (Lumer and Yost 1995), while Herrera and Nassar (2009) have reported fly pollination (Muscidae, Calliphoridae and Sarcophagidae) in naturalised populations of *Stapelia gigantea* in Venezuela.

Despite having to co-opt native honeybees as pollinators, *A. sericifera* maintains relatively high levels of pollination success that are lower but still comparable to a native honeybee-pollinated milkweed. During some periods over half of all flowers of *A. sericifera* had pollinaria removed and more than third of all removed pollinia were subsequently deposited. Although the estimates of pollen removal and deposition were higher in *C. ellipticum*, this is to be expected as bees pollinating *C. ellipticum* carry some of the largest numbers of pollinaria recorded for any African milkweed (Chapter 4). The pollen transfer efficiency of *C. ellipticum* was not significantly higher than that of *A. sericifera* which is impressive considering that *A. sericifera* is exotic and has inherent pollination inefficiency introduced by honeybees which frequently deposit whole pollinaria with one of the paired pollinia positioned outside of the stigmatic groove thereby wasting half of the pollinia. Although it is tempting to conclude that this pattern of pollinium deposition is entirely due to a mismatch between honeybees and the pollinaria of *A. sericifera*, regular deposition of entire pollinaria (i.e. both pollinaria and the corpusculum) has been reported in wasps (*Polybia species*) pollinating *Oxypetalum appendiculatum* (Viera and Shepherd, 1999).

The seasonal variability in pollination success of *A. sericifera* is not uncommon in plants. Peter and Johnson (2008b) demonstrated that pollen transfer efficiency in *Acrolophia cochlearis* (Orchidaceae) ranged from 0% to 60% throughout the 5 month flowering period of this species. Similar results have been reported for milkweeds (Ivey *et al.* 2003). Estimates of pollen removal and deposition for other invasive milkweeds include those made by Coleman (1935) who indicated that on average 80% of the pollinaria had been removed and 40% deposited in flowers of *A. sericifera* that apparently showed signs of being fertilized. Forster (1994) reported that 38.9% of flowers had been pollinated in an Australian population of *G. physocarpus* and the average pollen transfer efficiency was 24.9% per plant. Although data is clearly limited our findings suggests that the measures of pollination success in *A. sericifera* are comparable to that experienced by other invasive milkweeds both in magnitude and variability.

Unlike the breeding systems of many other invasive species, *A. sericifera* is not capable of autonomous self-pollination, making this species entirely reliant on bees for pollination and fruit set. This type of breeding system is however expected within the Asclepiadoideae where automatic self-pollination is rare (Wyatt and Broyles, 1994). To our knowledge the only exotic milkweeds which have been reported to have this ability have been *Vincetoxicum nigrum* (Lumer and Yost 1995) and observations by Cappuccino (2004) that suggested automatic self-pollination to be present in *V. rossicum*. *Araujia sericifera* is however genetically self-compatible and capable of pollinator facilitated self-pollination (geitonogamy), a trait present in most invasive species (van Kleunen *et al*. 2008), but relatively rare in the Asclepiadoideae although this mode of reproduction is known from some weedy North American milkweeds (*e.g. Asclepias exaltata, A. speciosa*, *A. currassavica* and *A. fruticosa*; Finer and Morgan 2003, Lipow *et al.* 1999; Broyles and Wyatt, 1997). The ability of this species to self-pollinate could facilitate reproduction in the early stages of invasion, although the tendency for geitonogamous pollinations to initiate and mature less fruit, leads us to conclude that in larger, well established populations with relatively high and consistent pollen transfer, most fruit set is likely to come from cross-pollinations carried out by honeybees.

Conclusion

We have shown support for our hypothesis that *Araujia sericifera* has successfully co-opted a native generalist pollinator (honeybees) in its invaded range in South Africa. The high pollination success of *A. sericifera* suggests it does not suffer pollination failure in South Africa and consistently maintains relatively high levels of pollen transfer efficiency throughout several flowering seasons. The species is also capable of reproducing in small populations owing to the ability of single individuals to set fruit through geitonogamous pollinations. The results of this study combined with others (e.g. Liu and Pemberton 2010) represent mounting evidence that invasive plants are not necessarily prevented from invading new regions to specialized flower morphologies. Future studies should focus on documenting the natural pollinators, pollination success and breeding system of *A. sericifera* in its natural range. This data would reveal whether this species maintains equally high levels of pollination success in its native versus exotic ranges and whether geitonogamy is present in natural populations or is an acquired trait present only in exotic populations (e.g.van Kleunen *et al*. 2008). A further point of interest will be to examine the degree to which invasive asclepiads have generalist pollination systems as a preadaptation to exploiting novel pollinators when invading new areas.

Acknowledgments

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Chapter 4

Cynanchum ellipticum loads large numbers of pollinaria onto its principle pollinator *Apis mellifera* without interfering in the foraging behaviour of these insects.

Abstract

The pollen of most plants is granular and its presence on pollinators does not negatively influence the behaviour of these insects. Orchids and asclepiads however present pollen as large aggregated masses known as pollinia that have the potential to negatively affect the foraging behaviour of pollinators by causing increased flower handling times or interfering in the biology of the pollinator. The pollination biology of members of the genus *Cynanchum* (Apocynaceae – Asclepiadoideae) is poorly documented. In this study various aspects of the pollination biology of *C. ellipticum* were examined including the identity of pollinators, pollination success and nature of the floral rewards. This revealed that large masses of pollinaria accumulate on the mouthparts of the primary pollinators, honeybees. The influence of large pollinarium loads on the foraging behaviour of honeybees was therefore investigated and I found a positive relationship between pollinarium loads and flower visiting times, however longer flower handling times were restricted to only a few individuals. There was no other evidence to suggest that large pollinaria loads influence the foraging behaviour of honeybees as bees did not visit fewer flowers per umbel and showed no increase in wing wear with increasingly large pollinarium loads. *Cynanchum ellipticum* displayed consistently high levels of pollination success that may be attributed to a highly

efficient system of pollinarium loading with minimal impact on the foraging behaviour of pollinating insects.

Introduction

Flower visiting insects frequently accumulate large pollen loads during a foraging bout. The pollen of most plants is carried on the pollinator's body without influencing the behaviour of the animal. Excess pollen is typically groomed off by some insects (*e.g*. honeybees) and other animals such as birds and bats. The pollen of orchids and asclepiads may be more difficult to remove as the pollen of these families attaches to pollinators as large masses of pollen (termed pollinia) that is attached to a part of the insect either through a sticky pad (Orchidaceae and Periplocoideae; Johnson and Edwards, 2000; Verhoeven and Venter, 2001) or through a mechanical clip (asclepiads; Wyatt and Broyles, 1994). In some orchid species where the morphology of the flower and pollinator correspond closely, pollinarium placement is specific to one area of the pollinator's body and can accumulate to form large pollen masses (*e.g.* long-tongued flies, Johnson and Steiner, 1997; hawkmoths, Johnson and Litved, 1997; short-tongued bees, Peter and Johnson, 2008). In milkweeds pollinaria may continue to accumulate by linking to other pollinaria as the corpusculae of pollinaria can attach to the small remaining piece of tissue (caudicle) that is left attached to the corpusculum when a pollinarium is removed (Morse, 1981; Coombs *et al.,* 2009).

Pollinaria of some species of milkweed and orchids can accumulate on pollinators to the point where pollinarium loads become large enough to physically interfere with the foraging behaviour of the insect (Morse, 1981; Johnson and Liltved, 1997), or in some cases lead to the death of pollinators in both orchids (*Bonatea speciosa*, Johnson and Liltved, 1997) and asclepiads (Romeo, 1933; Coleman, 1935). Adaptations that promote pollinarium chaining are therefore only likely to evolve in species with small pollinaria as the advantage in terms of promoting male fitness and increasing pollen transfer efficiency (Johnson and Harder, 2008), may be eroded if the behaviour of pollinators is negatively influenced through carrying these structures. While larger pollinarium loads may increase chances of successful pollination, carrying large pollinarium loads may negatively influence the foraging behaviour of the pollinator (Morse, 1981). To date, the only evidence collected for this idea has shown that the main influence of pollinaria is to increase flower handling times, which is caused by the claws of bumblebees either breaking off between the relatively rigid anther wings or by pollinia on the mouthparts slowing foraging times (Morse, 1981). The influence of large pollinarium loads also depends on the size of the pollinaria relative to the pollinator. It is possible that large pollinarium loads may influence the number of flowers that pollinators visit per umbel by reducing the volume of nectar that bees may consume. The resultant behaviour may therefore be similar to that seen in pollinating bees that visit fewer flowers per inflorescence when they encounter reduced nectar rewards (Pleasants *et al.,* 1979; Jersakova and Johnson, 2006; Johnson and Nilsson, 1999).

The genus *Cynanchum* contains approximately 400 species of which about 100 are African (Liede, 1993; Ollerton and Liede, 2003). Despite the large number of species within *Cynanchum*, little is known about the pollination biology of species in this genus. Currently pollinator observations have only been made for 13 species (Ollerton and Liede, 2003; Ollerton *et al.,* 2010; Chapter 5) of approximately 400 species (Liede, 1997), which represents slightly more than 3% of the total number of species. In this study the pollination biology of *Cynanchum ellipticum* (Apocynaceae – Asclepiadoideae), a common milkweed vine endemic to southern Africa (Liede, 1993) was investigated. Initial observations suggested that honeybees visiting this species may accumulate very large pollinarium loads (>200) on their mouth parts, and is thus a good study species to test the hypothesis that large pollinarium loads influence the foraging behaviour of pollinators. In this study I investigated (1) the identity of the main pollinators of *C. ellipticum*, (2) the average levels of pollinarium removal, deposition and pollen transfer efficiency in populations of this species and 3) whether large pollinarium loads negatively influence the foraging behaviour of honeybees?

Methods

Study species and study site

Cynanchum ellipticum (Harv.) R.A.Dyer is a common perennial creeper found along the South African coast (Liede, 1993). The species flowers almost continuously throughout the year and produces flowers on umbels that bear between 4—10 open flowers per umbel (Liede, 1993). Flowering typically occurs throughout the year but peaks in April and September (Liede, 1993). During peak flowering periods large plants can produce large flower displays consisting of several hundred inflorescences, each inflorescence displaying several flowers simultaneously. Although the exact flowering phenology of this species was not determined, it appears that most individuals in a population flowered synchronously with several flowering events occurring at different times throughout the year. However there was a small fraction of individuals that would flower unpredictably at times when most other plants were not in flower. Flowers produce nectar as a reward.

This study was carried out at three different sites in the Eastern Cape, South Africa. These were Grahamstown (33^{o} 18’ 20”S, 26^{o} 31’ 28” E), Port Alfred (33^{o} 36’ 00”S, 26^{o} 53’ 00” E) and Kenton-on-Sea (33^{o} 40’ 50”S, 26^{o} 40’ 14”E). At each of these three locations *C. obtusifolium* is a common creeper growing on coastal vegetation and on garden fences. The study populations consisted of plants growing on fences and natural vegetation throughout these three towns.

Pollinator observations and pollinarium loads

Pollinators were collected at all three study sites during 2007. Sampling efforts were mostly centred on the morning peak period of insect activity (8:30 - 10:30) but sampling of pollinators was continued throughout the day at Kenton-on-Sea and Port Alfred. During these periods all flowers visitors were collected or only a subsample of more common species. During 2008 and 2010, I only collected flower visiting species other than honeybees such as flies and other smaller Hymenoptera. Nocturnal visitors were collected only in Grahamstown where I spent three evenings consisting of one hour of observation per evening from dusk (19:00 - 20:00). All insects were captured, pinned, identified and for each specimen, the number of full pollinaria (pollinaria with both pollinia attached), ½ pollinaria (pollinaria with one pollinium removed) and corpusculae (pollinaria with both pollinia removed) were counted.

Pollinarium removal, deposition and pollen transfer efficiency

To quantify average levels of pollen removal, deposition and pollen transfer efficiency I sampled flowers from all three sites during 2007. Flowers were sampled by randomly picking three flowers per plant from between 30—50 individuals. During 2008 I repeated sampling for only one date at Port Alfred and for three dates in Grahamstown. During the same year, in Kenton-on-Sea I tracked pollination success throughout the year by sampling flowers during peak flowering periods when most individuals were flowering synchronously. Sampling at Kenton-on-Sea started from May 2008 and ended during March 2009. For all flowers that were sampled I counted the numbers of pollinaria removed and deposited and used this to calculate the pollen transfer efficiency which is defined as the fraction of removed pollinaria that are deposited on conspecific stigmas (Johnson *et al.,* 2005).

Nectar rewards

The nectar volume produced by *C. ellipticum* is minute and is accumulated at the base of the corona cup that is very small (*ca*. 2mm wide). I therefore allowed bagged flowers to accumulate nectar over a period of one day between the afternoon when flowers were bagged and the morning that the volume and concentration of the nectar was measured. I bagged one to three umbels per plant with fine white nylon mesh bags. Depending on the volume of nectar that each flower accumulated I collected all the nectar from between two to five flowers per umbel and divided the final volume by the number of flowers to obtain an average nectar volume per flower. Nectar volumes and concentration for *C. ellipticum* were measured only in the Grahamstown population. Nectar measurements were made by sequentially probing flowers with on an umbel until a large enough volume of nectar was collected to accurately measure on the refractometer. All nectar concentration measurements were made using an Atago sucrose refractometer.

The effect of large pollinarium loads on the foraging efficiency of honeybees

To investigate the possible role of large pollinarium loads on the foraging efficiency of honeybees I first quantified whether pollinaria accumulate on bees through different times of the day and whether bees groom pollinaria off over night. I then quantified whether large pollinarium loads affected the foraging behaviour of honeybees. This was done by (1) correlating the average time spent by honeybees per flower against the pollinarium load carried by the bee, (2) determining whether honeybees visit fewer flowers as a result of large pollinium loads potentially limiting the volume of nectar consumed per flower.

Diurnal pollinarium accumulation and removal of pollinaria through grooming

Pollinarium accumulation throughout the day was monitored by collecting honeybees foraging on *C. ellipticum* at the following time intervals: 6:00 - 7:00 (Dawn); 7:00 - 8:00, 8:00 - 9:00, 10:00 - 11:00, 12:00 - 13:00, 14:00 - 15:00, 16:00 - 17:00. For each of these time intervals, between 3 - 8 plants were examined and up to three bees were collected per plant per day. Time intervals were stacked more closely from 7:00 - 11:00 in order to have scale resolution of how pollinaria may accumulate throughout the foraging period which typically peaks in mid-morning. Each sampling interval was replicated on three different days. The only exception was the dawn time interval which was only sampled on two days. Pollinarium accumulation of *C. ellipticum* was compared to that of *C. obtusifolium*, a co-occurring congener that is also bee-pollinated although bees carry fewer pollinaria of *C. obtusifolium* (Chapter 4). Using the same sampling strategy as was used to quantify pollinarium accumulation in *C. ellipticum*, I also quantified pollinarium accumulation in *C. obtusifolium* and sampled bees for a period of three days over the same time intervals. I also inspected whether bees carry pollinia overnight by catching the first bees arriving at plants at dawn.

To determine whether there is a pattern of pollinarium accumulation throughout the day, a one way ANOVA was used to test for differences in the mean pollinarium loads of *C. ellipticum* borne by bees at different times of the day. Data of total pollinarium loads for *C. ellipticum* at different times of the day was Box - Cox transformed to meet the assumptions of normality and homoscedascity.

To determine whether bees carry pollinaria overnight, the first bees arriving at the plants at dawn (i.e. “first arrivals”) were collected and examined for the presence of pollinaria. During these sampling intervals I caught up to nine first arrivals, killed and mounted these insects and counted the number of pollinaria. The time spent catching these first arrivals were limited to 10 minutes to ensure minimal pollinarium accumulation subsequent to the arrival of the bees.

Weight of pollinarium loads carried by bees

Bees carrying pollinaria were caught and immobilized by quickly cooling them in a freezer. They were then weighed on an electronic balance before and after the pollinaria were removed under a dissecting microscope using a fine pair of forceps. The difference between the weights before and after pollinaria were removed was assumed to be the weight of the total pollinarium load. The pollinarium load was thereafter counted. The relationship between the number of pollinaria making up the load and the weight of the pollinaria was examined to determine how accurately increases in the weight of the pollinarium load corresponds to increases in total pollinarium load and whether increasingly large pollinarium loads constitute a larger burden to the honeybee (expressed as a larger percentage of their body weight).

Influence of pollinarium loads on the foraging times and percentage of flowers that bees visit per umbel

To calculate the average time that bees spent foraging per flower, we tracked the foraging bouts of individual honeybees using an electronic data logger. Foraging bees were selected randomly and their foraging bout tracked until individuals had visited up to a maximum of 10 flowers. After the foraging bout was recorded, bees were caught, killed and mounted. For each bee the number of full, ½ pollinaria and corpusculae carried on the mouth parts was counted. Sampling periods were confined to two main sessions in the morning and afternoon and up to 10 individual bees where caught in any single sampling period. Morning and afternoon sessions started at 10:30 and 14:30 respectively with sampling intervals lasting approximately 1 hour.

On two days during March 2009 I collected data to see whether bees carrying large pollinarium loads visit fewer flowers per umbel. On each day I selected between one to three foraging bees per plant and followed each bee until it had visited between one to three umbels. Only visits to umbels with two or more flowers were included. While the bee was visiting an umbel the number of flowers that the bee visited and the number of flowers on the umbel were recorded. Thereafter the bee was caught and the total pollinarium load was counted. All data was collected on two mornings between 8:00 - 11:00am. The relationship between the number of flowers visited per umbel and the total number of pollinaria on the bee was investigated by fitting a 2nd degree polynomial function to this data.

Relationship between pollinarium loads and degree of wing wear in pollinating honeybees

Wing wear has been suggested to be an indication of the foraging effort of bees, with older individuals that have spent greater periods of time foraging expected to exhibit a greater degree of wing wear (Cartar, 1992). Thus I expected that honeybees bearing large pollinarium loads may exhibit higher levels of wing wear if pollinaria frustrate the feeding efforts of these insects. Alternatively honeybees bearing large pollinarium loads could be older and have accumulated more pollinaria purely as a result of having spent longer periods foraging. Similarly Morse (1981) used this approach when studying the influence of large pollinarium loads on bumblebees foraging on *Asclepias syriaca*. I inspected the wing wear of a subsample of bees that were caught visiting *C. ellipticum* and categorically classified bees according to a slightly adapted scheme suggested by Cartar (1992). Wings with no wear were given a score of 0; wings with minor indentations along the margin were given a score of 1 and any worse damage was given a score of 2. All four wings were scored individually and an average calculated across all four wings for each individual bee. Using non – parametric correlation I determined whether there was a positive correlation between the degree of wing wear and the total pollinarium load carried on the mouth parts.

Results

Pollinator observations and pollinarium loads

The flowers of *Cynanchum ellipticum* (Fig. 1, A) were visited by a wide variety of Hymenoptera, Diptera and Lepidoptera (Fig. 1, Table 1). However, honeybees (*Apis mellifera*) carried by far the most pollinaria and, particulary in Grahamstown, accumulated large amounts of pollinaria (maximum recorded = 224) on the mouth part parts (Table 1, Fig 1: D & E). Bees were the most common flower visitor caught in Grahamstown and remained the main flower visitors at this site during all study years. Although fewer bees were caught while visiting *C. ellipticum* in Kenton-on-Sea and Port Alfred, bees at these sites also characteristically accumulated large amounts of pollinaria on the mouth parts. One bee caught on 16 May 2008 in Port Alfred bore a total pollinarium load of 73 (25 full, 17 ½C and 31 corpusculae). Thus, while for the previous year only two bees were collected from Port Alfred, neither of which bore pollinaria, this is likely to be an artefact of the small sample size (Table 1).

The stigmatic chamber of *C. ellipticum* is quite small and flowers of *C. ellipticum* typically only received one pollinium per stigmatic chamber of *C. ellipticum* (Fig. 1, B). The mechanism of pollinaria attachment to pollinators in *C. ellipticum* is the same as other milkweeds but is particularly efficient in forming such long continuous chains (Fig. 1F). It is relatively easy to artificially construct pollinarium chains by hand using a small insect “minuten” pin mounted in a holder to simulate the proboscis of the pollinator. This can be done if the pollinium is seated within the alar fissure and dragged along this groove; as the pollinarium wedges the caudicle breaks and the remaining piece of caudicle tissue passes through the corspusculum of the pollinarium seated above this groove and removes this pollinarium (Fig 1, B). The caudicle of *C. ellipticum* is roughly triangularly shaped, narrower at the base where it connects to the corpusculum than at the end where it attaches to the pollinium (Fig. 1F, See also description by Liede, 1993). This likely creates a wedge that slides into the corpuscular groove of subsequent pollinaria where it lodges firmly and so removes the pollinarium which becomes the next in the chain. As the insect continues to forage, the pollinarium load is thought to be further held together by the pollinarium load becoming covered in nectar. Although some exact mechanical details are still unknown this arrangement combined with the relatively small size of the pollinaria appears to be the reason why such large continuous chains of pollinaria are formed.

Surprisingly, although the dimunitive size of the pollinaria and gynostegium suggests that smaller wasps and bees should also carry pollinaria, very few of these insects carried pollinaria. Smaller Hymenoptera visiting this species include female *Allodape pernix* and *Allodapula melanopus* and both sexes of *Allodapula variegata*. The number of pollinaria carried by these insects was generally much lower than that carried by honeybees (Table 1). Smaller Hymenoptera appear for the most part to be opportunistic nectar thieves. When grouped together a significantly greater proportion of Hymenoptera (56%) bore pollinara than Lepidoptera (16%: proportions based t-test, $t_{(124)} = 4.68$, $p < 0.0001$).

Butterflies appeared secondarily important as pollinators (Fig. 1G, Table 1). The most frequent visitors were *Dira clytus eurina* (Nymphalidae, Satyrinae) that visited for a short period during March following their brief emergence in long grass near patches of *C. ellipticum*. These butterflies also frequently bore the pollinaria of *C. obtusifolium* alongside pollinaria of *C. ellipticum,* indicating that they visit both species. Other less common butterflies included *Eronia cleodora cleodora* (Pieridae) and *Metisella metis* (Hesperidae). Most of the nocturnal moths that were collected were *Echaea lienardi* (Noctuidae) owing to a mass-emergence of these insects in Grahamstown and surrounding areas during 2008. These moths visited many other plant species during the day and night and were highly generalist visitors to flower and decaying fruit. Only one moth of a total of 31 collected bore two full pollinaria.

Figure 1: The small flowers (2-4mm) of *C. ellipticum* are arranged as umbels A). Gynostegium of *C. ellipticum* showing position of anther wings (aw) covering the stigmatic chamber ("sc", B). Cross section through the gynostegium of *C. ellipticum* showing a pollinarium deposited ("dp") within the chamber (C). D) The primary pollinators of *C. ellipticum* are honeybees that can accumulate large numbers of pollinaria on the mouth parts E), which occurs when pollinaria attach form long chains by attaching to other corpusculae via the caudicle (arrow, F). Other visitors to *C. ellipticum* that also bore pollinaria include *Dyra clytus eurina* (Nymphalidae, Satyrinae; G), and flies such as species of Tachinidae (H & I). Scale bars: A – C, F = 1mm; others = 3mm.

Flies were frequent visitors to *C. ellipticum* (Fig. 1 H, I; Table 1) and were generally present year round. As a group the proportions of flies that bore pollinaria (15%) was similar and not significantly greater than the proportion of Lepidoptera that bore pollinaria (16%;

proportions based t-test, $t_{(94)} = -0.13$, $p = 0.90$). The most common families were Tachinidae, Calliphoridae and more rarely Muscidae and Syrphidae. Most flies bore few or no pollinaria. The maximum amount of pollinaria carried by a fly was on a tachinid that bore a total of seven pollinaria. The presence of half pollinaria and corpusculae indicates that flies may effectively pollinate *C. ellipticum* but the lower proportion of individuals that bear pollinaria suggests that their overall contribution is likely to be insignificant compared to that of honeybees. Although I did not identify flies beyond the family level there were at least eight different species in total. The only other flower visitors that were collected were three Lycid beetles (Lycidae) that bore no pollinaria and probably only stole nectar (Table 1).

Table 1: Summary of the different insect flower visitors to *Cynanchum ellipticum* and the average amount of full pollinaria, ½ pollinaria and corspusculae borne by each species (asterisks above family names indicate significant differences in the proportion of individuals carrying pollinaria).

Order	**Family**	**Site**	**Species**	**No. of individuals sampled**	**No. individuals carrying pollinaria (percentage)**	**Full pollinaria (mean ± 1SD)**	**1/2 pollinaria (mean ± 1SD)**	**Corpusculae (mean ± 1SD)**	**Total (mean ± 1SD)**
Hymenoptera*	Apidae	Grahamstown	*Apis mellifera*	26	25 (96)	13.35 ± 9.33	19.96 ± 17.31	29.88 ± 21.57	63.19 ± 46.29
	Apidae	Kenton-on-Sea	*Apis mellifera*	11	8 (73)	4.45 ± 3.98	6.36 ± 6.47	9.18 ± 8.48	20 ± 17.37
	Apidae	Port Alfred	*Apis mellifera*	3	1 (33)	8.33 ± 14.43	4.0 ± 6.93	10.33 ± 17.90	22.67 ± 39.26
	Apidae	Kenton-on-Sea	*Allodape pernix*	14	1 (7)	0.71 ± 0.27	0	0	0.71 ± 0.27
	Apidae	Grahamstown	*Allodapula variegate*	5	0 (0)	0	0	0	0
	Apidae	Kenton-on-Sea	*Allodapula melanopus*	2	0 (0)	0	0	0	0
	Apidae	Grahamstown	*Allodapula melanopus*	1	0 (0)	0	0	0	0
	Vespidae	Kenton-on-Sea	*Belonogaster* spp.	1	0 (0)	0	0	0	0
	Hesperidae	Grahamstown	*Metisella metis*	1	0 (0)	0	0	0	0
Lepidoptera**	Nymphalidae	Kenton-on-Sea	*Dira clytus eurina*	24	7 (29)	0.71 ± 1.40	0.67 ± 1.71	0.96 ± 2.54	2.33 ± 5.33
	Nymphalidae	Grahamstown	*Dira clytus eurina*	5	2 (40)	1.20 ± 1.64	0.80± 1.10	0	2.0 ± 2.74
	Nymphalidae	Port Alfred	*Dira clytus eurina*	2	0 (0)	0	0	0	0
	Noctuideae	Grahamstown	*Achaea lienardi*	29	1 (3)	0.07 ± 0.37	0	0.07 ± 0.37	0.14 ± 0.74
	Hesperidae	Grahamstown	*Metisella metis*	1	0 (0)	0	0	0	0
	Nymphalidae	Grahamstown	*Bicyclus safitza*	1	0 (0)	0	0	0	0
	Nymphalidae	Grahamstown	*Eronia cleodara cleodore*	1	0 (0)	0	0	0	0
Diptera**	Tachinidae	Kenton-on-Sea	-	2	0 (0)	0	0	0	0
	Tachinidae	Grahamstown	-	24	4 (17)	0.42 ± 1.06	0.21 ± 0.66	0.08 ± 0.41	0.71 ± 1.81
	Muscidae	Grahamstown	-	1	0 (0)	0	0	0	0
	Syrphidae	Grahamstown	-	1	0 (0)	0	0	0	0
	Calliphoridae	Grahamstown	-	9	1 (11)	0.11 ± 0.33	0	0.11 ± 0.33	0.22 ± 0.41
Coleoptera‡	Lycidae	Grahamstown	-	3	0 (0)	0	0	0	0

‡Due to small number of Coleoptera the pollinarium loads of this family were not statistically compared to other families.

Pollinarium removal, pollinia deposition and pollen transfer efficiency

Data for pollinarium removal and deposition indicated that *C. ellipticum* generally has very high levels of pollination success at all three study sites. Data collected on single sampling dates during 2007 and 2008 indicated that the percentage of flowers with pollinaria removed exceeded 40% for most sampling dates, while pollen deposition was generally lower than pollinarium removal but nevertheless relatively high, exceeding 40% for most sampling dates (Table 2).

Pollination success varied at different times in the flowering season for the population at Kenton-on-Sea. Estimates of pollinarium removal ranged from a maximum of 79.6 % to a minimum of 22.6 % on different sampling dates. The percentage of flowers with pollinaria deposited was generally lower and ranged between 7.5% and 76.4%. PTE was typically high (range = 5% - 48%) and for most sampling dates PTE exceeded 30%. When data was averaged over all dates, more than half of all flowers had pollinaria removed (average = 53.4% SD = 20.9) and slightly lower than half of all flowers had pollinia deposited (average = 41.8%, SD = 28.8; Fig. 2). The average PTE across all dates was 35.7% (SD = 16.1), which is high considering that the theoretical maximum value that this species may achieve is 50%, a limitation imposed by the pollinarium chamber nearly always accommodating only one pollinium (i.e. flowers may export 10 pollinaria but typically may only receive five).

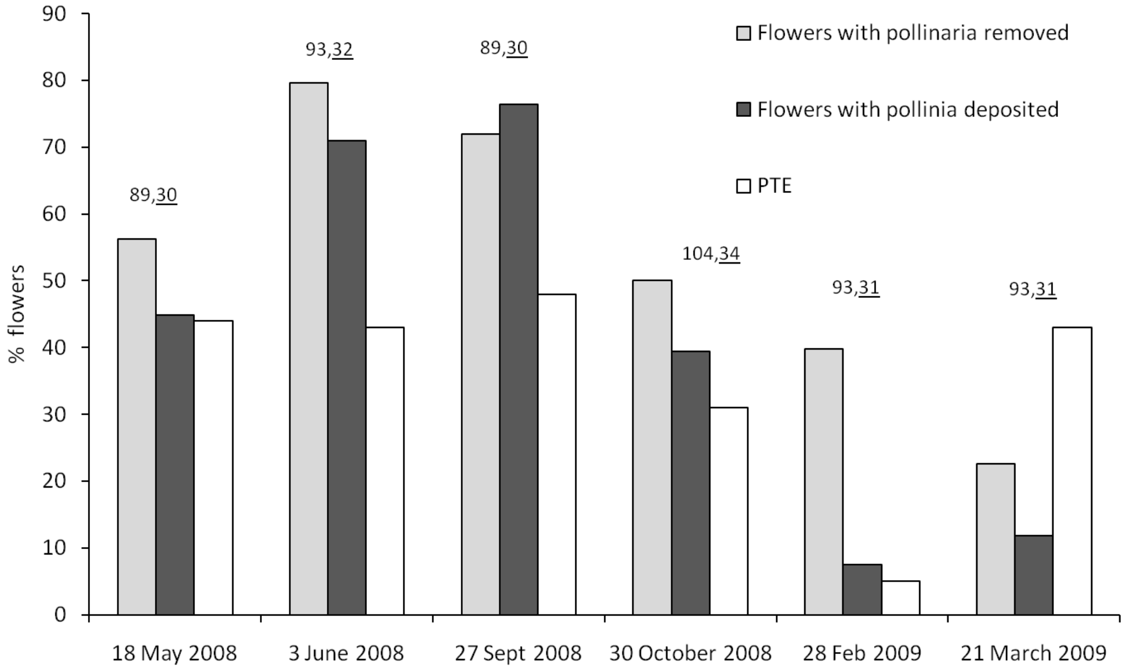

Figure 2: Changes in the percentage of flowers with pollinaria removed, flowers with pollinaria deposited and pollen transfer efficiency (PTE) in flowers of *C. ellipticum* sampled on different dates at Kenton-on-Sea (numbers above bars indicate sample size of flowers followed by number of plants (underlined)).

Table 2: Summary of the percentage of flowers with pollinaria removed, pollinia deposited and PTE at different sampling dates at three different sites of *C. ellipticum*.

Location	**Date**	**Number of plants (no. of flowers)**	**Percentage of flowers with pollinaria removed**	**Percentage of flowers with pollinia deposited**	**PTE (%)**
Grahamstown	02 April 2007	19 (57)	52.6	49.1	39.7
Grahamstown	17 March 2008	22 (64)	62.5	46.0	28.0
Grahamstown	29 March 2008	30 (87)	82.8	71.3	40.5
Grahamstown	14 April 2008	31 (92)	87.0	79.3	42.4
Port Alfred	11 April 2007	42 (126)	27.8	15.1	25.5
Port Alfred	16 May 2008	32 (96)	41.7	31.3	32.1
Kenton-on-Sea	04 April 2007	48 (144)	45.1	25.7	18.1
Kenton-on-Sea	Average all sampling dates	30 – 48 (561)	53.4 (SD = 20.9)	41.8 (SD = 28.8)	35.7 (SD = 16.1)

Nectar measurements and flower colours

The average nectar concentration of flowers from Grahamstown was 31.16 % sucrose equivalents (SE = 4.60, n = 19 flowers, 12 plants) and the average volume was 0.83 µl (SE = 0.50, n = 38 flowers, 5 plants). Colour measurements of *C. ellipticum* indicated that flower colours are similar to those observed by the human visual system with no reflectance in the ultraviolet part of the spectrum.

The effect of large pollinarium loads on the foraging efficiency of honeybees

Diurnal pollinarium accumulation and removal of pollinaria through grooming

In Grahamstown, the pollinarium loads on the proboscides of bees feeding on *C. ellipticum* ranged between 1 and 169 across all sampling intervals (Fig. 4). The average pollinarium load ranged between an average of 37 (SD = 29) to 62.9 (SD = 42) on different sampling intervals. There was no significant overall effect of the time of day on the total pollinarium load of that bees were carrying ($F_{(6,207)} = 1.95$, $p = 0.074$). There were no differences between particular sampling times using Scheffe post-hoc test for homogenous groups.

The pollinarium load carried by bees visiting *C. obtusifolium* ranged between 2.1 (SD = 1.5) and 2.8 (SD = 1.3) at different sampling intervals (Fig. 4). Similar to the case in *C. ellipticum* there was no pattern of pollinarium accumulation throughout the day in either *C. ellipticu*m or *C. obtusifolium*.

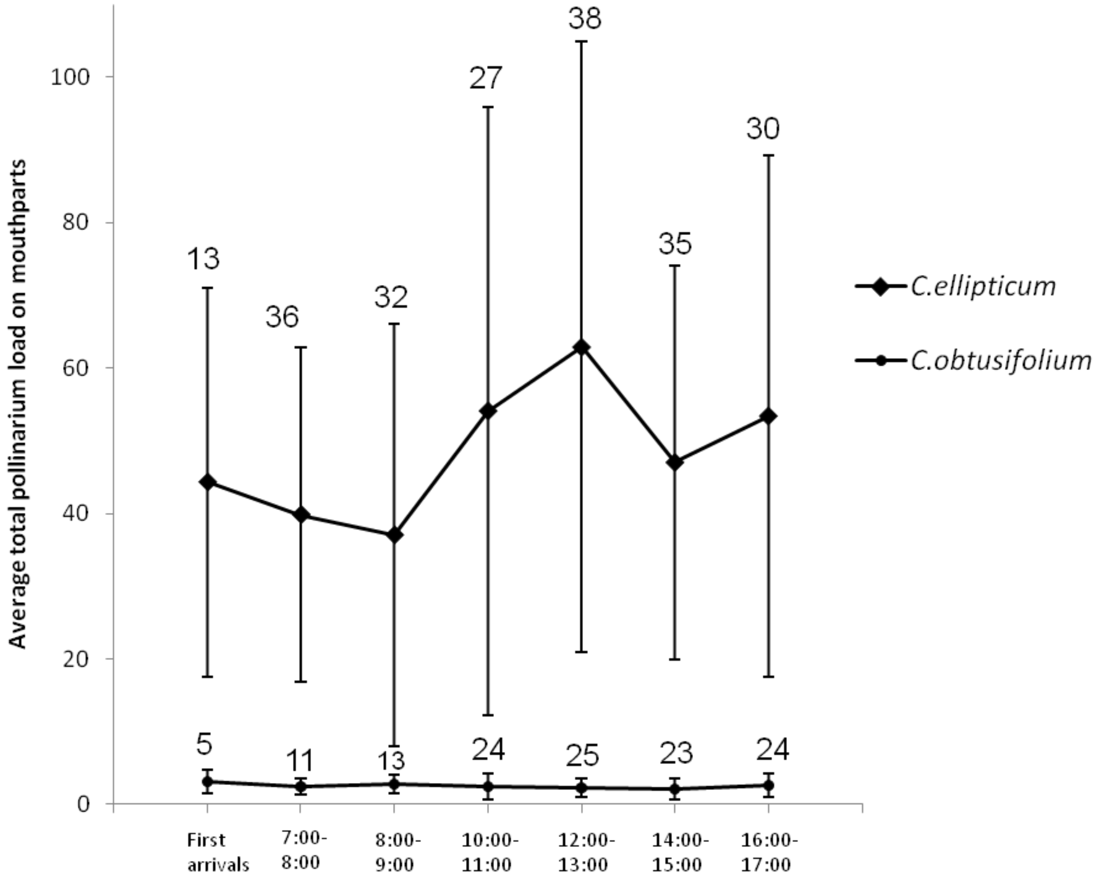

Figure 4: Changes in the average total number (± 1 SD) of pollinaria carried by bees visiting *C. ellipticum* at different times of the day (values above bars indicate number of bees in each sample.

In *C. ellipticum*, the average pollinarium load of bees caught at first light was 44.30 (SD = 26.7; n = 13 bees), indicating that bees do carry pollinaria overnight. Similarly bees arriving at dawn to feed on *C. obtusifolium* also carried pollinaria (average = 3.2, SD = 1.6). These results indicate that bees either struggle to remove pollinaria or the impact of pollinaria on bees is not sufficient to force the insects to groom pollinaria off.

Weight of pollinarium loads carried by bees

The weight of the pollinarium load was positively related to the total number of pollinaria ($r^2 = 0.74$, $n = 30$, $p < 0.0001$; Fig. 5). As pollinarium loads increased in size, these constituted a larger percentage of the bees' total weight ($r^2 = 0.72$, $n = 30$, $p < 0.0001$), suggesting that the weight of the pollinarium load is not negated by increases in the weight of the bee due to larger crop loads. Within the range of values recorded here (4 – 134), the total pollinarium load never exceeded more than 2.5% of the bee's body mass. Even large pollinarium loads therefore constitute a very small percentage of the bees' total body weight. Extrapolating beyond this range, it can be estimated that even at pollinarium loads as high as 250 (maximum recorded = 224), the total percentage mass of the bee pollinaria take up is unlikely to exceed 5%. This makes it unlikely that pollinarium loads are heavy enough to influence the foraging behaviour of honeybees as a consequence of weight.

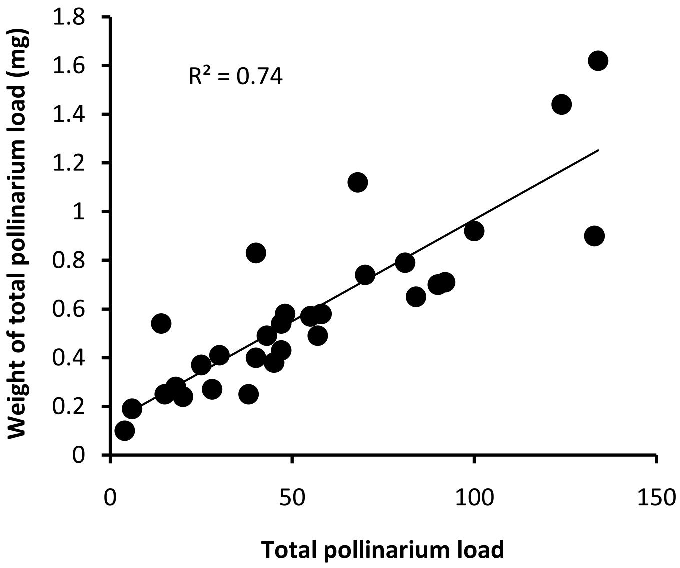

Figure 5: Relationship between the weight of the pollinarium load and the total number of pollinaria.

Influence of pollinarium loads on the foraging times and percentage of flowers that bees visit per umbel

Due to flower visiting times being non-normally distributed I performed non-parametric correlation analysis between the average flower visiting time of bees and the total pollinarium load borne on the mouthparts (Fig. 6). There was a positive (Spearman's rank correlation coefficient, $r_s = 0.39$, $n = 38$, $p < 0.05$) relationship between the time spent visiting flowers and the total pollinarium load. However the positive correlations was influenced by a few bees ($n = 3$) that bore large pollinarium loads and exhibited unusually long visiting times. However, only one of these bees carried a pollinarium load large enough to be a statistical outlier suggesting that other factors (e.g. age of bees) may have caused the increase in foraging times of these individuals. The correlation values became non-significant when these three values were excluded, suggesting that most bees carrying pollinarium loads not exceeding *ca*. 150 pollinaria do not spend longer times foraging on flowers. The strength of this relationship could also be influenced by the lower number of individuals that were sampled with large pollinarium loads. Bees that carry exceptionally large loads may however struggle to forage due to such loads interfering with the bee's ability to access the nectar in the corona tube and not due the weight of the pollinaria (See previous section).

I found no relationship between the numbers of pollinaria that bees carried on the mouth parts and the percentage of flowers that bees visited per umbel (Fig. 7), suggesting that bees are not likely to be frustrated in their feeding effort and leave an umbel more quickly. Bees typically visited between two to four open flowers per umbel (median = 2 flowers visited, n = 27) and rarely visited all open flowers on an umbel regardless of the number of pollinaria that bees were carrying. The relationship between the percentage of flowers that bees visited per umbel and the number of flowers per umbel fit a 2nd order polynomial (F (1, 25) = 4.83; r^2 = 0.13; p = 0.037, Fig. 8), indicating that bees visited a smaller percentage of flowers as the number of open flowers per umbel increases.

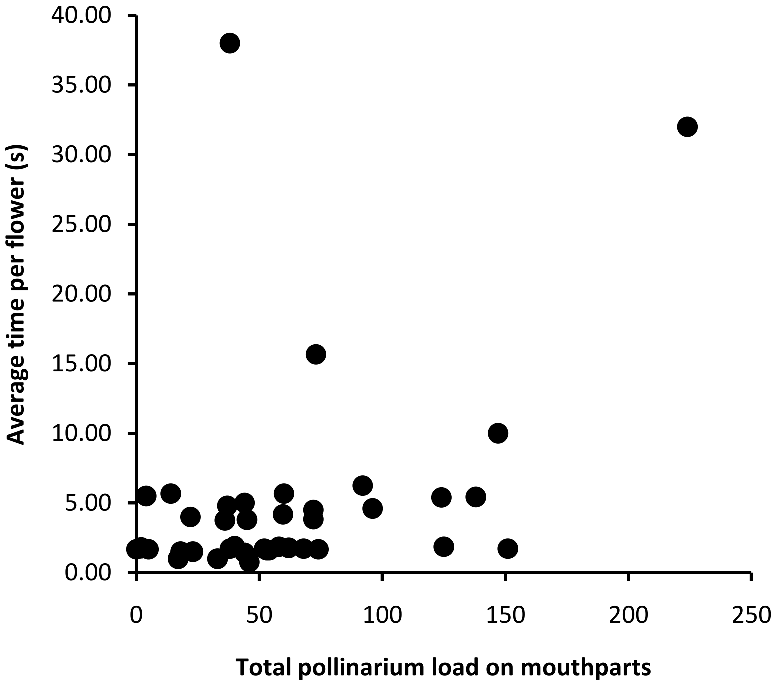

Figure 6: Relationship between the time that bees spent per flower and the total pollinarium load carried on the mouthparts.

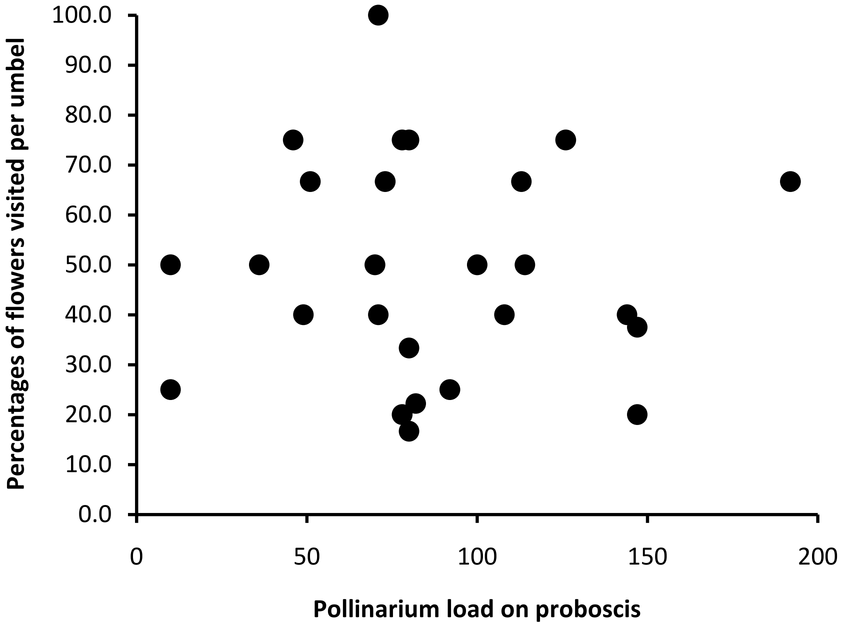

Figure 7: Relationship between the percentage of flowers that bees visited per umbel and the total pollinarium load carried on the mouth parts.

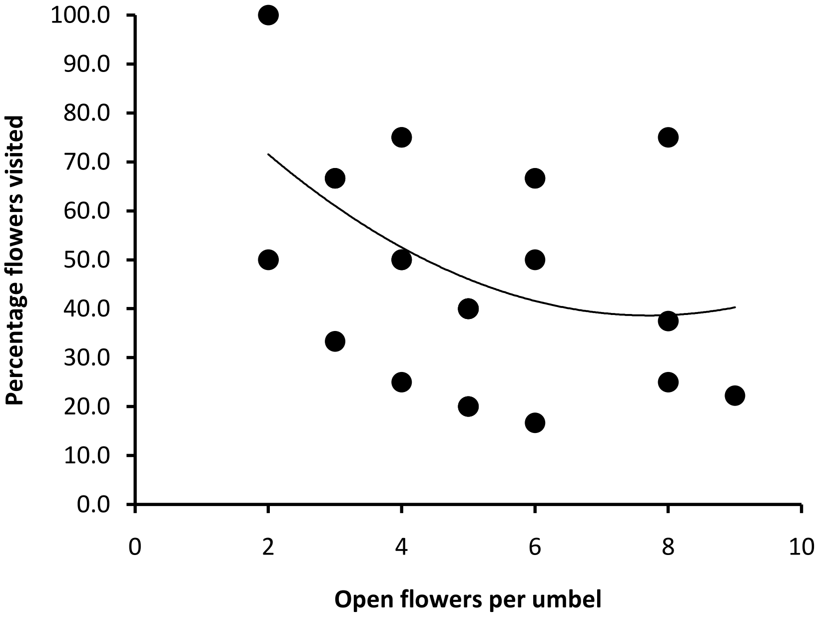

Figure 8: Relationship between the percentage of flowers that bees visited per umbel and the number of open flowers per umbel. Curve represents a 2nd order polynomial function fitted to data (see text for details).

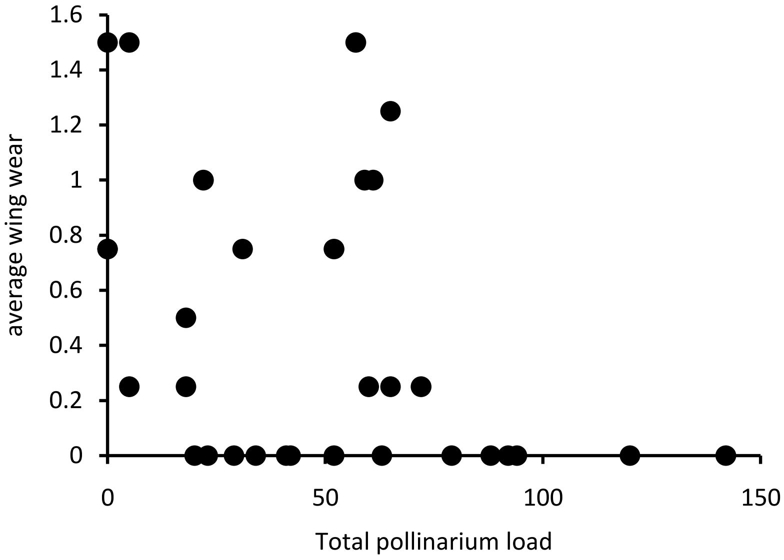

Figure 9: Scatterplot of average wing wear of honeybees against the number of pollinaria carried by each bee.

Relationship between pollinarium loads and degree of wing wear in pollinating honeybees

The amount of wing wear was significantly negatively related to the total pollinarium load (Spearman's rank correlation coefficient $r_s = -0.41$; $p < 0.05$; $n = 31$; Fig. 9), which was contrary to my expectation that bees with large pollinarium loads would be older bees with higher amounts of wing wear.

Discussion

Cynanchum ellipticum is visited by a large number of flower visitors but the abundance of honeybees and their large pollinarium loads, indicates that this species is relatively specialized for pollination by honeybees. Butterflies may be secondarily important as pollinators but the relatively short flight period and patchy occurrence due to specific habitat requirements necessarily constrains their contribution to a short period of the flowering season. Although *C. ellipticum* produces minute nectar volumes per flower, the large floral display collectively amplifies the total reward attracting many different nectar feeding insects. The relatively shallow corona tube allows a large number of these insects to access the nectar and clearly does not restrict the access to the flower to just honeybees and butterflies. Despite obvious differences in morphology, smaller flower visitors such as Hymenoptera and Diptera can also access the nectar of this species and undoubtedly occasionally effect legitimate pollination (*cf.* Maloof and Inouye, 2000). Geographical variation in pollinator abundance may also play a role in relative abundance and contribution of these different insects to the pollination of *C. ellipticum* (e.g. Herrera, 1988). In Grahamstown bees appeared to be the most abundant flower visitors and exceeded the number of other flower visitors, a pattern that could be influenced by the presence of numerous wild nests around the Rhodes University campus. At Kenton-on-Sea and Port Alfred visits by bees were less common, but the small number of bees collected at these sites still bore large pollinarium loads. Observations on all other species of *Cynanchum* where pollinators have been observed also indicate different bees (Ollerton and Liede, 2003; Yamashiro *et al.,* 2008) and flies (Wolff *et al.,* 2008) as pollinators, but field observations on *Cynanchum* pollinators are too scarce to generalize. Owing to the lower number of pollinaria borne by Lepidoptera in general, and the few moths that were caught visiting *C. ellipticum* at night, the importance of nocturnal lepidopteran pollinators is likely to be limited and such minute nectar volumes is not typical of hawkmoth or settling moth pollinated species (Faegri and van der Pijl, 1979).

Insects that visit *C. ellipticum*, particularly honeybees, accumulate large pollinarium loads on their mouth parts. Pollinarium chaining has been reported previously in the Asclepiadoideae (Frost, 1965; Morse, 1981; Coombs *et al.,* 2009) but may be particularly efficient in *Cynanchum ellipticum*. Chaining is caused by the positioning of the corpusculum at the distal end of the alar fissure, such that when a pollinarium is broken off inside the stigmatic chamber, the remaining piece of caudicle often passes through the corpuscular groove of the pollinarium seated above the chamber causing it to attach to the caudicle tissue and to be removed (Morse, 1981, Coombs *et al.,* 2009). This could explain the high correlation between pollinarium deposition and removal in this species. In *C. ellipticum*, 92.3% (394 of 427) of stigmatic chambers with pollinia depositions also had the associated pollinarium above the chamber removed. Data taken from samples collected from Grahamstown and Kenton-on-Sea showed a highly significant correlation between pollinium deposition and pollinarium removal (Kenton-on-Sea: Spearman's rank correlation coefficient, $r_s = 0.77$, $n = 705$; Grahamstown: Spearman's rank correlation coefficient $r_s = 0.84$, $n = 238$; all $p < 0.05$). This is similar to the positive correlation between pollinarium removal and deposition in *Asclepias tuberosa* (Wyatt, 1976). However, I found that the correlation between pollinarium removal and deposition does not necessarily mean that pollinarium removal and deposition co-occur. This was demonstrated by artificially dragging pollinaria through the stigmatic chamber, which caused pollinaria to be picked up in 42.7% (15 of 36) of cases while in 57.3 % of cases the associated pollinarium was left behind. Dragging only the caudicle through the pollinarium chamber caused the seated pollinarium to become attached in 81.4 % (22 of 27) of cases which indicates that pollinarium removal occurs preferentially if only a caudicle is dragged through the stigmatic cavity and pollinia can be deposited while not removing any pollinaria.

There may be several advantages to such an efficient chaining mechanism in *C. ellipticum*. Firstly, in species where there is a close morphological fit between the pollinator and flower, there may be a limited number of sites for pollinaria attachment to the pollinator's body. Adaptations that promote the linking of individual pollinaria to other pollinaria can conceivably increase the amount of pollen removed by pollinators by increasing the number of attachments sites. Larger pollen loads on insects may in turn increase the chances of successful pollen deposition, which could explain why the average levels of pollinarium removal, deposition and PTE are high in this species (see later).

The ultimate effect of this pollinating mechanism in *C. ellipticum*, is that honeybees carry large numbers of pollinaria with minimal detectable impact on their foraging behaviour. This was surprising as it was expected that flower handling times would increase due to large pollinarium loads frustrating the foraging behaviour of honeybees causing honeybees bees to spend longer periods consuming nectar from flowers. In this study, increased flower handling times were isolated to only a few individuals (one of which carried the largest pollinarium load) and may indicate that the effect of pollinaria on the foraging times is non linear with pollinaria having no effects on pollinators within a certain range while the effects may suddenly increase once this threshold is exceeded. Over the course of this study, relatively few pollinators were found to carry such large pollinarium loads. In addition, the pollinaria attach to the mouthparts of the honeybees and therefore do not affect the grip of the insect (bees were however occasionally observed falling from plants). This differs from the pollinaria of *Asclepias syriaca* where pollinaria attached to the tarsi caused the pollinating bumblebees to often fall off the flowers of *A. syriaca* (Morse, 1981).

The absence of significant variation in foraging times of bees with pollinarium loads up to *ca.* 150 is probably due to the fact that the tiny weight addition that pollinarium loads add to the honeybee is not sufficient to affect foraging behaviour either through weighing the insects down or physically interfering with the bee's foraging. This is particularly true considering the research by Schmid-Hempel (1986) that showed honeybees could forage while bearing several small weights, each weighing 7mg each. The maximum pollinarium load that was weighed in this study amounted to only 1.62mg. It is also not unusual for honeybees to carry average pollen loads of other plants equalling 15 mg (Fukuda *et al.,* 1969 cited in Wolf *et al.,* 1989; Winston, 1987 cited in Feuerbacher *et al.,* 2003) and nectar loads of up to 40 mg (von Frisch 1965, cited in Wolf *et al.,* 1989), whereas even exceptionally large pollinarium loads of *C. ellipticum* would weigh approximately 2 mg (value calculated by substituting maximum pollinarium load found on bees (224) into equation of regression line in Figure 5). The position where the weight is carried could also be important, as weight added to the mouth parts may physically interfere with foraging movements, while weights attached to the back (Schmid – Hempel, 1986) or pollen carried naturally in the corbiculae is likely to be less cumbersome. Larger pollinarium loads may either be groomed off, or excessively large chains may be limited by the pollinarium chain breaking under its own weight. Grooming behaviour was observed on several occasions although the frequency of this behaviour was not quantified. Such grooming may explain the regular occurrence of pollinaria on the tarsi of honeybees even when bees forage for nectar by gripping the flower on the outside. Honeybees could also pick up pollinaria on the tarsi when crawling over umbels while foraging between different flowers.

The potential negative effects that large pollinarium loads may have on the foraging behaviour of honeybees could also be inferred from the pollination success of the plant. Estimates of pollen removal, deposition and PTE in *C. ellipticum* were generally very high and frequently approached the maximum attainable values suggesting that this strategy of loading pollinators with long chains of pollinaria is unlikely to negatively influence pollination success and may be a strategy to increase it. The habit of the pollinaria of *C. ellipticum* to chain may thus be an adaptive strategy that allows this species to load large pollinarium loads on one part of the pollinator without negatively influencing its behaviour.

The hypothesis that the gynostegium structure of *C. ellipticum* has adapted to increase pollinarium chaining is further confirmed by the observation that the pollinarium loads on honeybees visiting *C. ellipticum* invariably exceeded that of the pollinarium loads of honeybees visiting C. *obtusifolium* by several orders of magnitude, suggesting that pollinarium loads in this species are not merely a function of visitation rate or pollinator behaviour.

Rates of pollinarium removal, deposition and PTE varied stochastically throughout flowering season similar to that seen in some other species (e.g. *Araujia sericifera*, Chapter 2) but not in some orchids (e.g. Peter and Johnson, 2008). Pollination success may also vary between different sited in some milkweed species (*Pachycarpus asperifolius*, Shuttleworth and Johnson, 2006). For instance PTE was found to vary three fold in the invasive milkweed *Araujia sericifera* where PTE ranged from 12.8 to 36 % on different sampling dates (Coombs and Peter, 2010). Similarly, PTE for the wasp-pollinated *Pachycarpus asperifolius* varied by the same magnitude (range: 15 – 42.7%) between different populations (Shuttleworth and

Johnson, 2006). High levels of pollination success have also been reported in other *Cynanchum* species, for instance Wolff *et al.* (2008) report PTE of 73.7 % in *Cynanchum harliingii*.

The pollinaria of *C. ellipticum* do not re-configure once removed from the flower as has been reported in other species of Asclepiad (Wyatt, 1976; Coombs *et al.,* 2009). Pollinarium re-configuration is considered to function to reduce self-pollination in milkweeds and Orchids (Peter and Johnson, 2006) and the absence of this trait in *C. ellipticum*, combined with the high levels of PTE, suggests that it must either have high levels of self-pollination or rely on other mechanisms to prevent self-pollination. Harder and Barret (1995) demonstrated that geitonogamous self-pollination increases with increased flower display sizes and highlighted the inherent tradeoffs between increased pollinator attraction and increased self-pollination in such species. The large flowering display of *C. ellipticum* attracts high numbers of insects resulting in high PTE ((nearly half of all PTE estimates exceeded 40% (6 of 13)). It is thought that the small stigmatic chamber that typically only accommodates one pollinium (two pollinia were very rarely found to be inserted) could function to limit the amount of pollen that may be received. Deposited pollinia also frequently protrude slightly from the stigmatic chamber thus preventing other pollinia from being inserted. This mechanism could reduce levels of geitonogamy (i.e. pollen discounting: Harder and Wilson, 1998), by reducing the amount of pollen a flower may receive. The inflorescence architecture of *C. ellipticum*, combined with the small rewards offered to pollinators per flower, may further contribute to minimizing geitonogamous self-pollinations through reducing the percentage of flowers that bees visit in larger umbels (See Harder *et al.,* 2001; Ohashi *et al.,* 2001 for reviews of this argument). Further research is needed to establish alternative mechanisms by which milkweeds that lack pollinarium re-configuration may prevent self-pollination.

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Chapter 5

Functional andromonoecy in a South African milkweed

Abstract

Very little is known about the underlying function of the wide variation in the structure of the gynostegium found in different species of *Cynanchum* (Asclepiadoideae - Apocynaceae). This results from few pollination studies that have documented the pollination biology of members of this genus leaving aspects such a description of the pollinator fauna, average levels of pollination success and flower rewards largely undescribed. I studied the pollination biology of *Cynanchum obtusifolium*, a common asclepiad vine in South Africa. I documented the pollinator fauna, average levels of pollination success and flower rewards of this species and also describe a flower polymorphism present in this species. *Cynanchum obtusifolium* is pollinated mainly by honeybees, but a wide variety of other Hymenoptera and Diptera were frequent visitors suggesting that the species has a more generalized pollination system. Flowers produce minute quantities of nectar as a reward. *C. obtusifolium* is andromonoecius and produces both functionally male and hermaphrodite flowers. These two flower types can be distinguished by the length of the anther wings. Flowers with long anther wings functioned as hermaphrodites and were capable of both exporting and receiving pollinia whereas flowers with short anther wings seldomly received pollinaria and mainly function as male flowers through exporting pollinaria.

Introduction

In the Asclepiadoideae (family Apocynaceae) the basic mechanism of pollination requires that pollinia, which are small coalesced packages of relatively large pollen grains, are removed in pairs (pollinaria) and subsequently deposited individually in a specialized groove known as a stigmatic chamber (Wyatt and Broyles, 1994). The stigmatic chamber is formed by two rigid anther wings from adjacent anthers that cover the stigmatic surface forming a slit, the alar fissure, through which a pollinating insect drags its appendage and removes or deposits pollinia (Fig 1; F; see also Wyatt, 1976). There is however large variation in the basic structure of this pollination mechanism. Structures such as the anther wings, pollinaria and stigmatic chamber vary considerably between different species and the functional significance of such variation is not yet fully understood (Ollerton and Liede, 2003), although studies of co-occurring assemblages of milkweeds indicate that flowers pollinated by different pollinator groups have divergent flower characteristics (Ollerton *et al.,* 2003; Wolff *et al.,* 2008). The role of structural variability in flower characters not directly related to pollinator attraction (i.e. parts of the gynostegium) is poorly understood, but recent evidence suggests that the dimensions of these structures are correlated with morphological aspects of pollinators (Yamashiro *et al.,* 2008). As pollinia need to have some degree of morphological matching to the stigmatic chamber within which these are deposited, differences in the size and shape of the pollinaria and stigmatic chambers have been suggested to act as mechanical barriers to hybridization (Kephart and Heiser, 1980). There may also be large variation in the size of structures within a species (e.g. *Asclepias syriaca*; Morse and Fritz, 1985), which can cause a percentage of flowers with large stigmatic chambers to function more as hermaphrodites while others function mainly as male flowers by only exporting pollen (Morse and Fritz, 1985; Tanaka *et al.,* 2006). This type of breeding system whereby plants bear both male and hermaphrodite flowers is known as andromonoecy and has been described in approximately 4000 plant species and 33 families (Miller and Diggle, 2003). To my knowledge it has only been reported once before in the Asclepiadoideae (Tanaka *et al.,* 2006).

The pollination biology of the genus *Cynanchum* (Apocynaceae – Asclepiadoideae) is poorly documented with pollinator observations having been made for approximately 13 species (Ollerton and Liede, 2003; Ollerton *et al.,* 2010) of approximately 400 species (Liede, 1997). Such a lack of data is unfortunate because the genus displays a wide diversity in the structure of the gynostegia, much of which is influenced by differences in the structure of the anther wings (Liede, 1996). Observations on the pollination biology of these species can give valuable data on the underlying functions of differences in morphology (Ollerton and Liede, 2003; Liede, 1996) and in at least one species, *C. ellipticum*, the structure of the pollinaria and gynostegium has been shown to be associated with forming exceptionally large pollinarium balls on the mouthparts of pollinating honeybees (Chapter 4).

In this study I set out to document the pollination biology of *Cynanchum obtusifolium*, a common milkweed with a creeping habit occurring along the coast of South Africa and southern Mozambique (Liede, 1993). Specifically, I aimed to identify the main pollinators of this species and quantify the average levels of pollination success in terms of pollinarium removal, - deposition and the efficiency with which pollinators move pollen between flowers.

During my work on *C. obtusifolium* I also noticed that this species produces two morphologically distinct flower morphs and therefore I tested the hypothesis that this species is andromonoecious. I further investigated other aspects of andromonoecy in this species including which morphological characteristics prevent pollinium deposition in male flowers and whether the proportion of male and hermaphrodite flowers changes at different times during the flowering season. I also investigated whether there is a relationship between pollen transfer efficiency and the proportion of hermaphrodite flowers within the population.

Methods

Study species

Cynanchum obtusifolium L.f is a common creeping milkweed endemic to the southern and eastern coastal regions of South Africa and southern Mozambique (Liede, 1993). Within its range, *C. obtusifolium* is very common and characteristically covers other plants growing in dune scrub and coastal vegetation (Liede, 1993). This species adapts well to urban environments and is also commonly found growing in gardens and on fences in coastal towns and cities. Flowering occurs continuously throughout the year (Liede, 1993) but occurs in distinct pulses when the majority of flowering individuals within a population are flowering and I therefore consider the species to flower episodically (*cf.* Bawa, 1983). The intensity of flowering peaks from March to June at these study sites. Flowers are produced in umbels of between 8 to 15 buds with several (typically three to seven) open flowers (Liede, 1993; pers. obs.). Flowers are sweetly scented with a scent reminiscent of coconuts.

Study sites

Work on this species was done at three sites: Grahamstown, Port Alfred and Kenton-on-Sea. Within these three towns I worked on plants growing on hedges and fences in suburban areas or plants in adjacent natural coastal vegetation. Thus the study population should be considered semi-natural.

Pollinators and pollinarium loads

Pollinators visiting both species were observed and collected at all three sites during 2007. Sampling intervals occurred primarily from 08:30 - 10:30 but continued throughout the day in Kenton-on-Sea and Port Alfred. A total of approximately 24 hrs of sampling was spent catching insects visiting this species. During 2008, I focussed on catching visitors other than bees such as smaller wasps, flies and butterflies that were regularly seen visiting the flowers. During each sampling period an effort was made to catch pollinators from several plants. For all insects that were captured, the numbers of whole pollinaria (pollinarium with both pollinia attached), ½ pollinaria (pollinarium with one pollinium removed) and corpusculae (pollinarium with both pollinia removed) per pollinator was counted and I noted to which part of the body the pollinaria were attached (i.e. average number of pollinaria on the mouthparts, -tarsi and body).

Nectar rewards and flower colours

The small flowers of *C. obtusifolium* produce minute quantities of nectar that accumulates at the bottom of the corona at the base of the white, pointed corona lobes. To measure the volume of nectar that is produced, I allowed bagged flowers to accumulate nectar over a period of one day between the afternoon that flowers were bagged and the morning when measurements were taken. Working on plants at Kenton-on-Sea and Grahamstown, I bagged two to three umbels per plant with white nylon net bags and due to the small volume of nectar, measured the volume and concentration separately. In flowers where the nectar volume was too small, I pooled nectar values from several flowers and calculated the average volume per flower by dividing the total volume by the total number of flowers sampled. All nectar measurements were made from 7:30 – 9:00am, except for measurements taken at Kenton-on-Sea where, due to the travelling distance, I continued measurements until 10:30 am. Nectar measurements were measured as percentage sucrose equivalents using a 0 – 50 percent Atago handheld refractometer. All colour measurements were made using a USB 2000 photospectrometer (Ocean Optics, Dunedin Florida). The white corona lobes of *C. obtusifolium* were too small to measure individually, therefore I took all five corona lobes per flower and stacked these together such that petals were tightly abutting or slightly overlapping (similar to “fish scales” *sensu* Chittka and Kevan, 2005).

Pollen removal, - deposition and pollen transfer efficiency

During 2007 I collected flowers at all three study sites on a single date during the peak flowering period. In order to measure pollination success throughout the year at different flowering intervals, I continued sampling flowers once a month at Kenton-on-Sea starting from February 2008 and continued through to March 2009. Sampling was conducted on a single date in 2008 at Port Alfred and Grahamstown. At each sampling date I collected flowers by picking three flowers per individual from between 30 - 50 individuals. For all flowers I recorded the number of pollinaria removed and pollinia deposited per flower. This data was then used to calculate the pollen transfer efficiency, a ratio of the proportion of removed pollinia that are deposited on conspecific stigmas (*sensu* Johnson *et al.,* 2005). At all sampling dates I attempted to control for variation in the number of flowering individuals by only sampling when at least 30 plants were in flower.

Flower dimorphism

Morphological evidence for the presence of two different flower types in C. obtusifolium

I noticed that certain flower characteristics of *C. obtusifolium* appeared to be dimorphic, suggesting that this species may display a flower polymorphism. One "type" of flower had short anther wings and seldomly had pollinia deposited within the stigmatic chamber. The other "type" of flowers had longer anther wings and a relatively larger opening of the alar fissure. The latter group of flowers regularly had pollinia deposited within the stigmatic chamber. Brown (1908), noted a similar dimorphism in the anther wing length and gynostegium height, but did not quantify these differences and made no comments on apparent differences in other characteristics such as the size of the alar fissure opening and the differences in pollen receipt between the two flower morphs. Based on my personal observations I aimed to determine whether flowers of *C. obtusifolium* with long anther wings (termed LW flowers) were morphologically distinct from flowers with short anther wings (termed SW flowers) and hence whether this species is functionally andromonoecious.

From a subsample of 10 flowers taken from each of 10 different plants collected at the Kenton-on-Sea population, I measured the length and width of petals and corona lobes;

height and width of gynostegium; length of anther wings and maximum width of base of the alar fissure, length, width and height of pollinaria. All flower measurements were made with a calibrated Olympus graticule on a stereo dissecting microscope (Olympus SZH). Due to the small size of flowers, handling of flowers was minimized by mounting flowers on an insect pin while measurements were made. Principal component analysis (PCA) based on these ten characters was used to determine whether flowers with long and short anther wings formed two distinct groups. Additional morphological measurements (minimum width of alar fissure, minimum height of alar fissure and maximum height of alar fissure) were made from samples of flowers collected from Kenton-on-Sea to estimate pollination success. These measurements could not be included in the PCA as they where not made from the same flowers.

In addition I investigated whether this dimorphism was present in other populations by measuring the anther wing lengths in samples of flowers collected on from Port Alfred (6 April 2008; n = 32 plants, 95 flowers) and Grahamstown (22 April 2008; n = 57 flowers, 19 plants).

Finally, I also measured the anther wing lengths of a sample of 93 flowers picked from 31 plants growing at Kenton-on-Sea, to investigate whether the same flower polymorphism exists in *Cynanchum ellipticum*, a common co-occurring vine that is also mainly pollinated by honeybees (Chapter 4).

Difference in pollen export and receipt between LW and SW flower

Due to the low numbers of depositions in most samples, I combined data from four PTE sampling dates and recorded the number of pollinaria removed and deposited as well as the length of the anther wings and calculated the average percentage of flowers with pollinaria removed and percentage of pollinated flowers in SW and LW flowers in these samples. Due to the small sample size (n = 4 dates) I performed a one way ANOVA using non-parametric analysis of variance (PERMANOVA; see Anderson, 2001 and McArdle and Anderson, 2001 for details of technique) to test whether there were differences in the number of flowers with pollinaria removed and deposited between LW and SW flowers. This technique is suitable for small samples sizes as it does not assume normally distributed response variables or homoscedasticity. To obtain p and t values I used 99 permutations (See Anderson, 2005).

I also sought to explain the apparent differences in pollen receiving ability between flowers with long and short anther wings. This was done by making measurements of the minimum and maximum height and minimum and maximum width of the alar fissure. I then compared the dimensions of these structures to the height and width of pollinaria in order to establish whether differences in these characters may explain why differences in pollinium deposition between the presumed different flower types.

Flowering phenology of andromonoecy in C. obtusifolium

I marked a random sample of 32 inflorescences on four plants and monitored whether inflorescences display both types of flowers simultaneously and if individual inflorescences continuously produced the same flower type on different flowering periods. This was done by sampling all flowers produced by an inflorescence once a week for four sampling dates. At every sampling date I picked all flowers per umbel and determined whether these were SW or LW flower types by measuring the anther wing length. Some inflorescences could either not be found or were not flowering on all sampling dates and I therefore calculated the percentage of inflorescences which produce both types of flowers only for those inflorescences that flowered on at least two sampling dates.

Temporal variation in the ratio of LW and SW flowers and the relationship between pollen transfer efficiency and the proportion of LW flowers

Flower samples collected previously for PTE measurements at four different sampling dates (30 October 2008, 16 January 2009; 28 February 2009; 21 March 2009) were used to determine whether the ratio of male to hermaphrodite changes at different flowering times throughout the season. I inspected whether there was a temporal change in the proportion of flowers by fitting a polynomial regression line between the sampling date and the proportion of SW and LW flowers present at each date. In addition, I pooled all data for which I had estimated PTE and the proportion of male to hermaphrodite flowers (all above dates, n = 6) and investigated the relationship between PTE and the proportion of hermaphrodite flowers.

Results

Pollinators and pollinarium loads

The most abundant and consistent visitors to *C. obtusifolium* at all sites were honeybees (*Apis mellifera*). Pollinaria carried by these insects were either carried on the proboscis, tarsi (Table 1; Fig. 1, C, D) or occasionally attached to hairs on the body (Table 1). The placement of pollinaria on the tarsi is the result of the dissected corona of *C. obtusifolium* which causes the tarsi of bees to pass through the corona and grip the gynostegium, resulting in the tarsal claws of the bees being dragged through the alar fissure and in so doing pick up pollinaria. Honeybees visited plants throughout the day with noticeable peaks in the morning between 09:00 and 11:00am. Bees visited plants by initially hovering in front of umbels before alighting. Once bees had landed on an umbel, nectar was consumed by probing at the bases of the corona lobe. Subsequent umbels were located by a combination of short flights and crawling. Although both *C. obtusifolium* and *C. ellipticum* are often found growing intertwined and flowering times overlap, honeybees specialize on one species during a foraging bout. Honeybees crossing over between the two species was rarely observed, however a few of the bees caught on *C. obtusifolium* bore the pollinaria of *C. ellipticum* showing that some individuals do switch between the two species.

Other smaller Hymenoptera that visited the flowers of *C. obtusifolium* and bore small numbers of pollinaria included members of the families Vespidae, Halictidae, Scoliidae, Argidae and Crabronidae (Table 1). Small bees of the genus *Allodapula* were also occasionally seen visiting the flowers of *C. obtusifolium* but none were collected (Fig. 1D). The abundance of these insects was generally sporadic, although they could at times be common visitors their presence during my sampling periods was not as constant as that of honeybees. Flies were common flower visitors and also carried small numbers of pollinaria on the proboscis. The main families were Tachinidae (*ca.* 8 species.), Calliphoridae and Sarcophagidae in order of decreasing abundance. Lepidoptera were uncommon visitors to this species and only two individuals of *Belenois gidica abyssinica* (Pieridae) were collected visiting this species. None of these bore any pollinaria and are considered opportunistic nectar thieves.

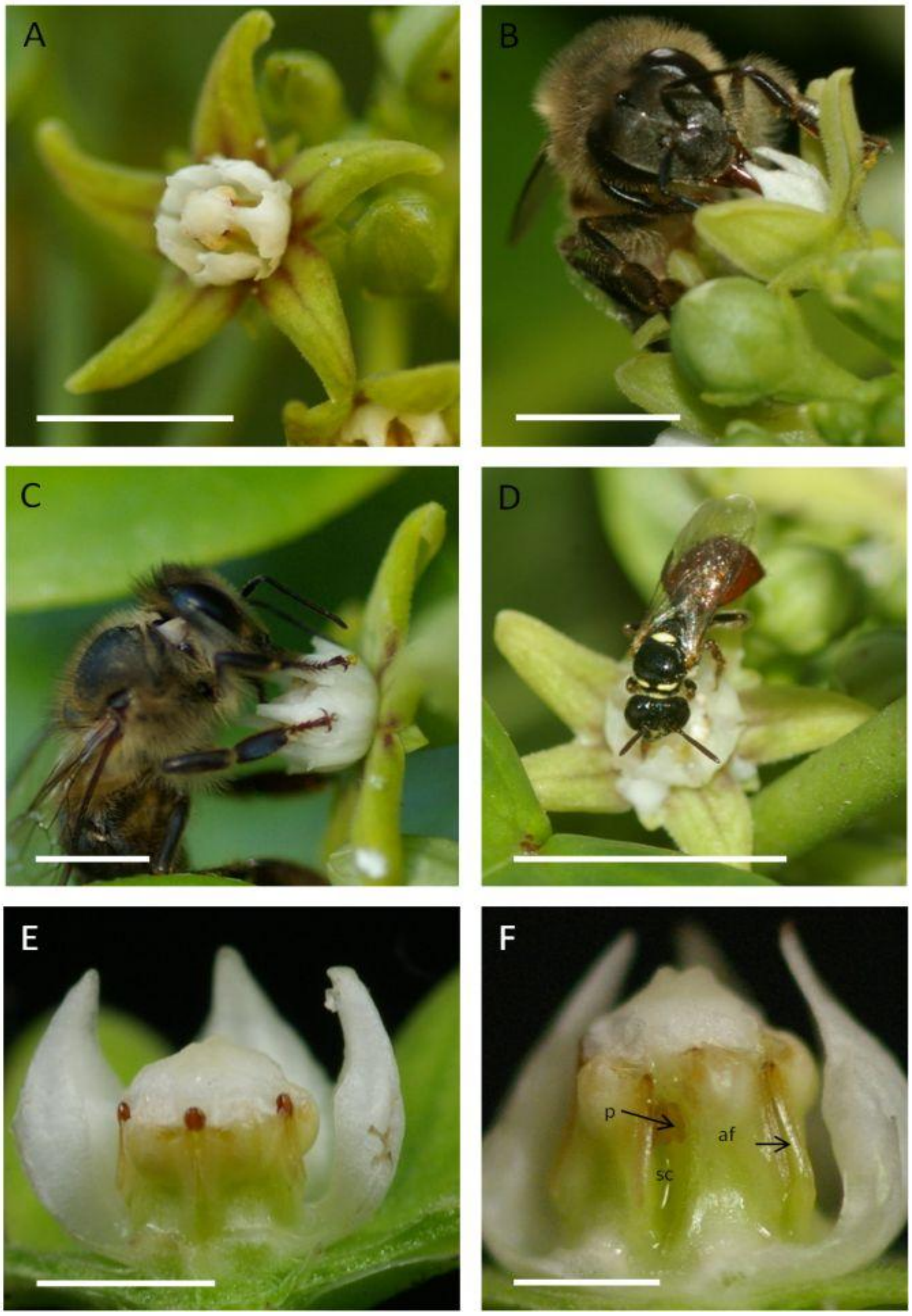


Figure 1: The flowers of *C. obtusifolium* (A) are mainly pollinated by honeybees (B, C). Other flower visitors included small Hymenoptera (*Allodape sp*. D). *C. obtusifolium* produces two distinct types of flower that are distinguishable from the length of the anther wing. Flowers with short anther wings, SW flowers (E), are functionally male and very seldomly have pollinia inserted in the stigmatic chamber, while flowers with long anther wings, LW flowers (F) are functional hermaphrodites and may receive several pollinia within a single stigmatic chamber (F; see text for further details). af = alar fissure, sc = stigmatic chamber, p = pollinia. Scale bars A – D = 5mm; E, F = 1mm.

Table 1: Summary of insect visitors collected while visiting *Cynanchum obtusifolium* and the pollen loads carried by each species. Full pollinaria = pollinaria with both pollinia attached; ½ pollinaria = pollinaria with one pollinium removed; corspusculae = pollinaria with both pollinia removed. Superscripts refer to position of pollinaria, m = mouthparts, t = tarsi, b = body.

Species	Family	Site	No. of individuals sampled	Number of individuals carrrying pollinaria	Full pollinaria (mean ± 1SD)	1/2 pollinaria (mean ± 1SD)	Corpusculae (mean ± 1SD)	Total (mean ± 1SD)
Hymenoptera								
Apis mellifera	**Apidae**	Grahamstown	37	31	0.78 ± 1.00^{m}	0.43 ± 0.69^{m}	0.32 ± 0.71^{m}	1.54 ± 1.52^{m}
					0.43 ± 0.60^{t}	0.11 ± 0.31^{t}	0^{t}	0.54 ± 0.73^{t}
					0.16 ± 0.55^{b}	0^{b}	0^{b}	0.16 ± 0.55^{b}
Apis mellifera	**Apidae**	Kenton-on-Sea	34	31	1.24 ± 1.10^{m}	0.79 ± 0.84^{m}	0.94 ± 1.07^{m}	2.97 ± 1.45^{m}
					0.12 ± 0.41^{t}	0.26 ± 0.57^{t}	0.09 ± 0.29^{t}	0.47 ± 0.90^{t}
					0^{b}	0^{b}	0^{b}	0^{b}
Apis mellifera	**Apidae**	Port Alfred	3	3	1.33 ± 0.58^{m}	0.67 ± 0.58^{m}	1.33 ± 0.53^{m}	3.33 ± 2.52^{m}
					0.67 ± 1.15^{t}	0^{t}	0^{t}	0.67 ± 1.15^{t}
					0^{b}	0^{b}	0^{b}	0^{b}
Xylocopa flavicaulis	**Apidae**	Kenton-on-Sea	1	1	0	1	1	2
	Halictidae	Kenton-on-Sea	6	0	0	0	0	0
		Port Alfred	1	0	0	0	0	0
Belonogaster sp.	**Vespidae**	Kenton-on-Sea	1	1	0	0	0	2

Table 1 (continued):

Species	**Family**	**Site**	**No. of individuals sampled**	**Number of individuals carrrying pollinaria**	**Full pollinaria (mean ± 1SD)**	**1/2 pollinaria (mean ± 1SD)**	**Corpuscula e (mean ± 1SD)**	**Total (mean ± 1SD)**
	Argidae	Kenton-on-Sea	1	1	1	0	0	1
?	**Scoliidae**	Kenton-on-Sea	2	2	2 ± 1.41	0	0	2 ± 1.41
Campsomeriella caelebs			1	1	1	1	0	2
Leomeris leonine			2	1	1±1.41	0	0	1±1.41
?		Grahamstown	1	1	3	0	0	3
Liris sp.	**Crabronidae**	Kenton-on-Sea	1	0	0	0	0	0
Bembix melanopa			2	0	0	0	0	0
Stizus fuscipennis			1	0	0	0	0	0
Liris sp.			1	1	0	1	1	2
Diptera								
Degenea	**Tachinidae**	Grahamstown	3	0	0	0	0	0
		Grahamstown	8	4	0.88 ± 1.13	0.25 ± 0.46	0	1.13 ± 1.36
		Kenton-on-Sea	2	1	0.5 ± 0.71	0	0	0.5 ± 0.71
	Sarcophagidae	Kenton-on-Sea	2	1	0	0.5 ± 0.71	0	0.5 ± 0.71
	Calliphoridae	Grahamstown	3	2	0.67 ± 0.58	0	0	0.67 ± 0.58
Lepidoptera								
Belenois gidica abyssinica	**Pieridae**	Kenton-on-Sea	2	0	0	0	0	0

Nectar rewards and flower colours

Cynanchum obtusifolium produces relatively minute amounts of nectar that is housed at the base of the corona. The average volume of nectar produced per flower in the Grahamstown population was 0.51 µl (SE = 0.11, n = 12 plants, 33 flowers). The average concentration of nectar produced by *C. obtusifolium* was 22.49 (SE = 1.54, n = 12 plants, 19 flowers) in Grahamstown and 30.53 (SE = 9.60, n = 16 plants, 25 flowers) in Kenton-on-Sea. Flower colours agreed with that observed by the human visual system, indicating that the corona lobes reflected relatively uniformly from 400 – 700 nm (i.e. white) and the petals showed a sharp peak in reflectance between 500 – 650nm (i.e. green).

Both LW and SW flowers both produce nectar although I did not quantify differences in the volume between these two flower types and nectar volumes and concentration presented here are averaged across both flower types.

Pollen removal, -deposition and pollen transfer efficiency

Pollination success measured at single sampling dates at all three sites showed that in all samples at least 25% or more of flowers had pollinaria removed, while pollinarium deposition was generally lower and had a minimum of 3.3% of flowers with depositions and a maximum of 19.3% pollinated flowers (Table 2). These data collectively indicate that the average level of pollinator visitation to *C. obtusifolium* is relatively high but variable across the different study areas.

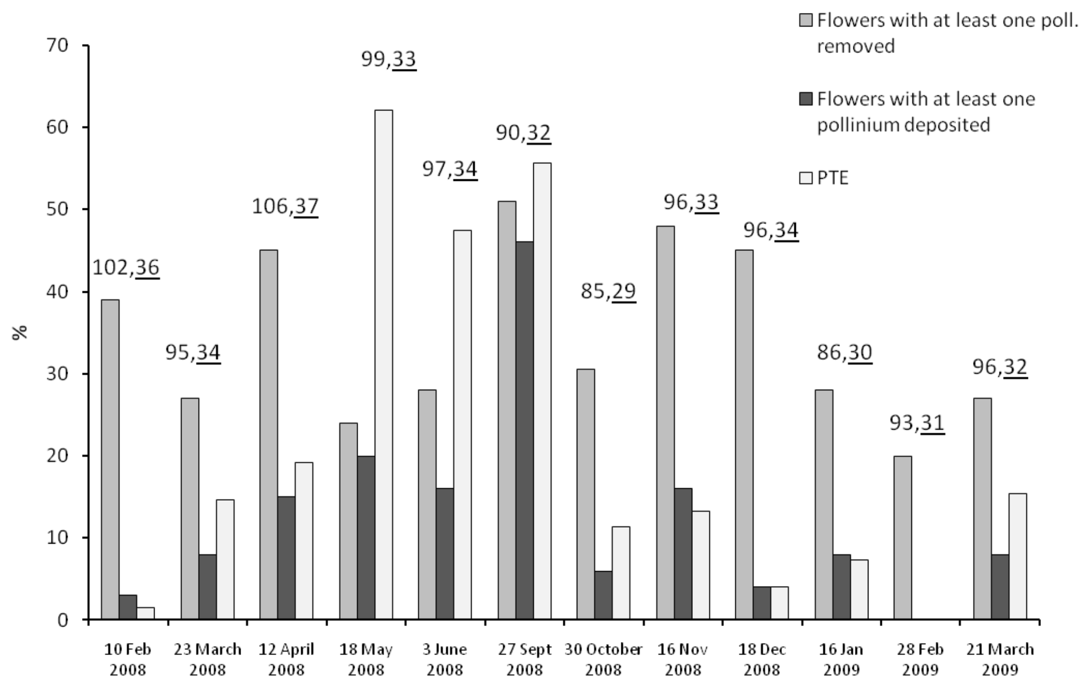


Figure 2: Changes in the percentages of flowers with pollinaria removed, - pollinia deposited and pollen transfer efficiency measured over year long period in a population of *Cynanchum obtusifolium* at Kenton-on-Sea (Figures above bars indicate number of flowers and number of plants (underlined) sampled at each sampling date).

Table 2: Summary of estimates of pollinarium removal, deposition and pollen transfer efficiency collected at different dates from three locations during 2007 and 2008.

Location	Date	Number of plants (no. of flowers)	Percentage of flowers with pollinaria removed	Percentage of flowers with pollinia deposited	PTE (%)
Grahamstown	02 April 2007	13 (39)	28.2	12.8	35.3
Grahamstown	22 April 2008	19 (57)	31.6	8.8	8.9
Port Alfred	11 April 2007	38 (114)	39.5	12.3	16.5
Port Alfred	06 April 2008	31 (91)	25.3	3.3	10.5
Kenton-on-Sea	03 April 2007	39 (117)	29.1	19.3	36.5
Kenton-on-Sea*	2008 - 2009	29 - 36 plants (1141)	34.4 (SD = 10.6)	12.5 (SD = 12.2)	21 (SD = 21.5)

*Average values calculated for year-long sampling interval at Kenton-on-Sea (Fig. 2).

Data for pollinarium removal, deposition and PTE collected over a year long period at Kenton-on-Sea indicated large variation in the proportion of flowers with pollinaria removed at different flowering times throughout the flowering season (Fig. 2). The percentage of flowers with pollinaria deposited was always lower than the percentage of flowers with removals. During this continuous sampling period, pollinium deposition did not vary as unpredictably as pollinarium removal and the percentage of pollinated flowers increased steadily from its lowest value during February 2008 to its highest during September 2008. PTE followed the same pattern as pollinarium deposition and peaked from May to September where it reached its highest value of 62.1% recorded during May 2008.

Interestingly there is a decoupling of pollinarium removal and deposition in *C. obtusifolium*. The correlation between the percentage of flowers with pollinaria removed and percentage of flowers with pollinia deposited was relatively weak and not significant (Spearman's rank = 0.29, $p > 0.05$, $n = 12$). Even at times when a large proportion of flowers had pollinaria removed, there were relatively few insertions (e.g. 10 February 2008; 18 December 2008), indicating high pollen losses occur during some flowering periods. PTE followed a similar trend to pollen deposition and was significantly correlated with the percentage of pollinated flowers (Spearman's rank = 0.91, $p < 0.05$, $n = 12$; Fig. 3). High pollen transfer efficiency values depended entirely on the frequency of depositions. For instance in a sample of flowers sampled at 18 May 2008 the percentage of removals were not significantly greater than at 28 February 2009 (proportions based t-test: $t_{(190)} = 0.67$; $p = 0.50$). However due to the significantly greater percentage of flowers with pollinaria deposited (proportions based t-test; $t_{(190)} = 4.55$, $p < 0.0001$) at 18 May 2008, the PTE at this date was much higher (62.1%) whereas the PTE on 28 February 2009 was zero.

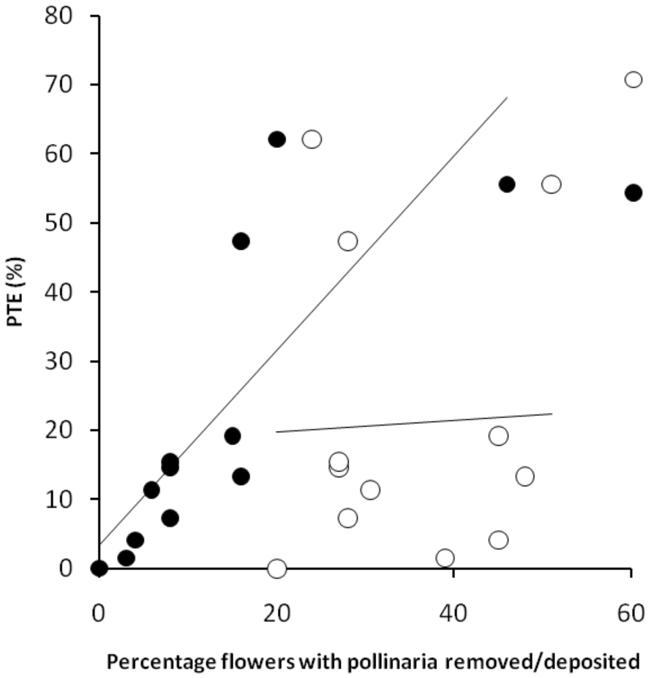


Figure 3: Relationship between pollen transfer efficiency and the percentage of flowers with pollinaria removed and deposited.

Morphological evidence for the presence of two different flower types in C. obtusifolium

In the samples of flowers collected to examine the occurrence of two flower morphs, measurements of individual characters showed that most were approximately normally distributed and correlated. However anther wing length and gynostegium height were distinctly bimodal frequency distributions, confirming my observations and those of Brown (1908). The bimodal distribution of anther wing length showed that very few flowers had anther wing lengths between 0.8 - 0.9mm (Figure 6); I therefore used the middle of this interval (0.85mm) as the boundary between LW and SW flowers. Flowers with anther wings equal to or below 0.85 were considered to have short anther wings (SW flowers = male flowers) and flowers with anther wings above this value were considered to have long anther wings (LW flowers = hermaphrodite flowers).

Principal component analysis (PCA) based on all 10 characteristics indicated that flowers with short and long anther wings formed two morphologically distinct groupings. There was some overlap with the 95% confidence in the PCA scores based on the entire data set of 10 flower characters (Fig. 4). Principal component 1 and 2 had the highest eigenvalues and combined explained nearly 69% of the variation in the data (Table 2, Fig. 4). I inspected the contribution of individual variables to the respective principal components by inspecting a biplot of the eigenvectors of the first two principle components and also a matrix of correlation values between the principal components and the individual variables (see Quinn and Keough, 2002). The size of the eigenvector and correlation values indicate the contribution of individual variables to the principle component. High values mean that the variable has a large contribution and vice versa (Quinn and Keough, 2002). This analysis indicated that the four gynostegium characters (anther wing length, gynostegium width, gynostegium height, alar fissure width) and corona lobe width contributed the most to PC1. This was further confirmed by the high correlation values of these individual variables with PC1. In addition petal length was also highly correlated with PC1, suggesting that flowers with long anther wings have longer petals.

Based on the above results showing the importance of the gynostegium characters for defining flowers with short and long anther wings, a second PCA was done using only gynostegium characters (i.e. by excluding measurements of petals, corona lobes and pollinaria) but using the same data set. This analysis showed a more distinct separation of long and short-wing flower phenotypes. The first principal component explained 83.31% of the data. In this analysis, flowers with short and long anther wings had nearly no overlap between in the principal component scores and 95% confidence intervals (Fig. 5). These results suggest that the gynostegia of flowers with long and short anther wings were more divergent. Again, this result was further supported by high correlation values between these characters and the first principal component.

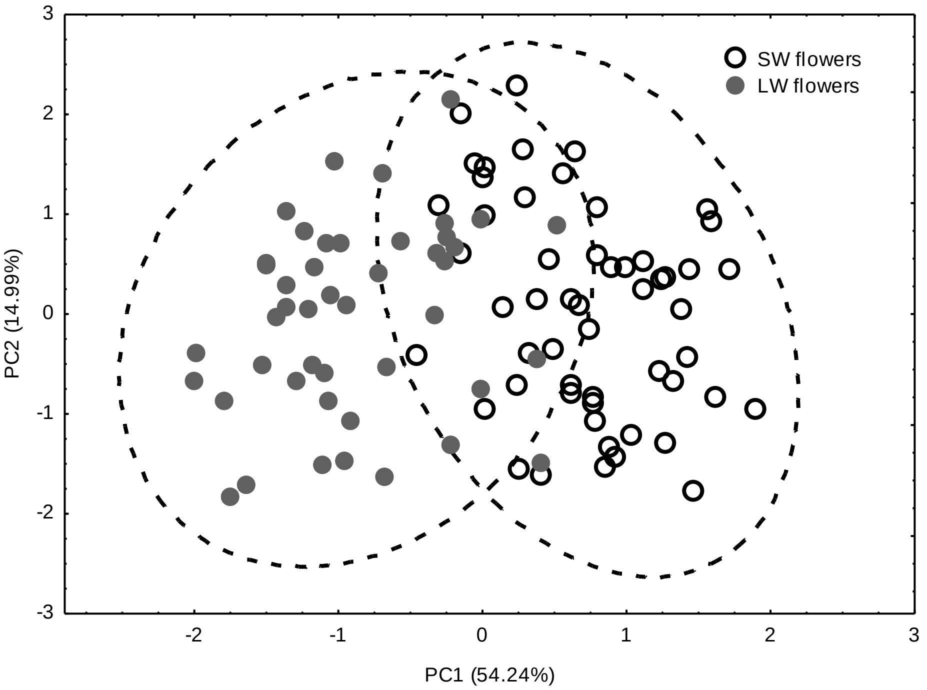


Figure 4: Biplot of first two principal components based on all measured flower characters showing separation PC scores between flowers with long anther wings (LW flowers) and flowers with short anther wings (SW) flowers into two morphological groupings (n = 100 flowers of 10 plants; dashed lines represent 95% confidence ellipses).

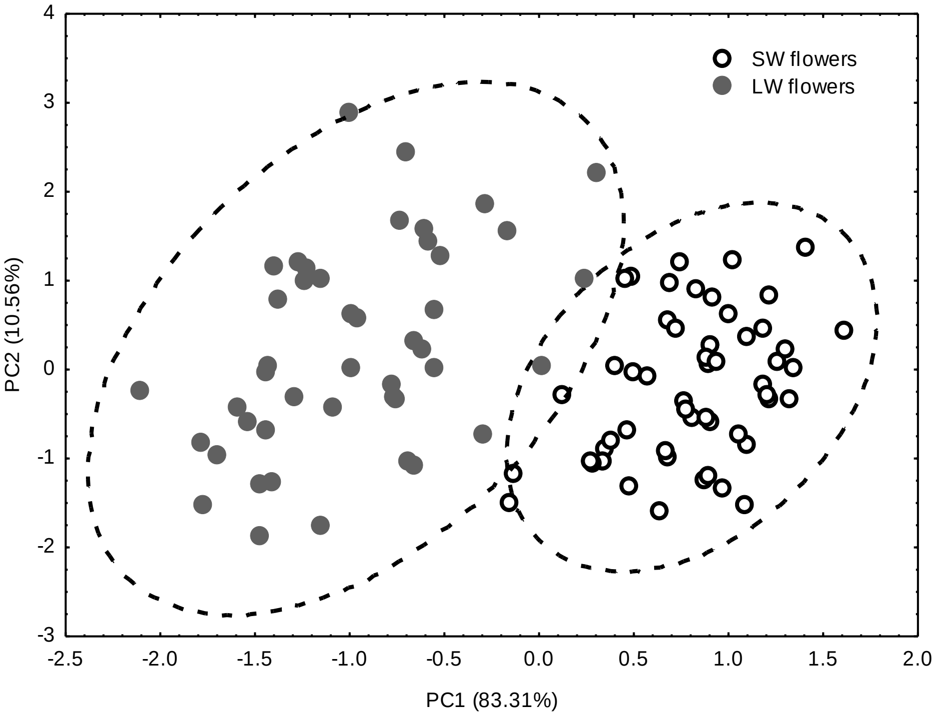
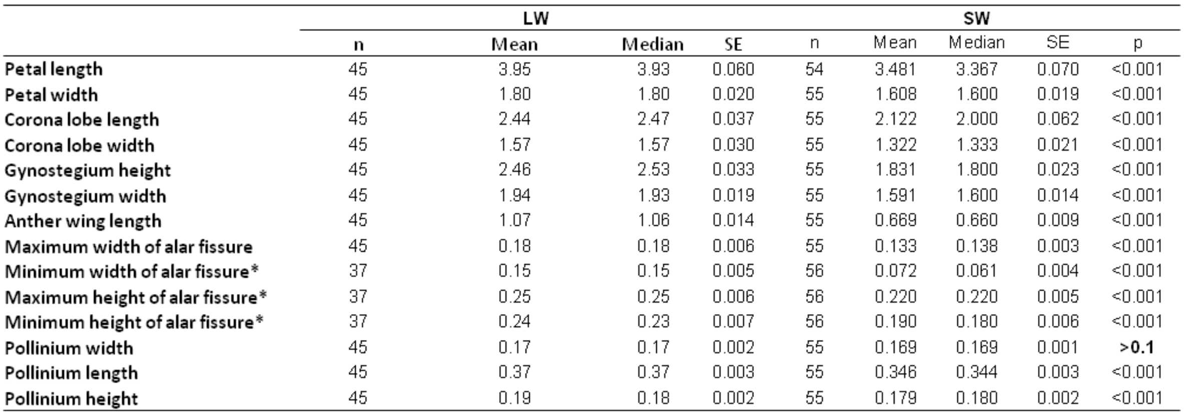


Figure 5: PCA biplot of first two principal components using four gynostegium characters (n=99 flowers of 10 plants; dashed lines represent 95% confidence ellipses).

The results of the PCA therefore confirmed my hypothesis that *C. obtusifolium* produces two distinct flower morphs that can be separated according to the length of the anther wings.

When flower morphs were grouped as flowers with long (LW) or short anther wings (SW) all but one character (pollinium width) were significantly larger in LW flowers than SW flowers (Table 3).

Table 3: Summary of differences between LW and SW flowers in 14 flower characters measured on flowers of *C. obtusifolium*. P-values indicate significant differences using Komolgorov-Smirnov two sample tests.



	LW				SW				
	n	Mean	Median	SE	n	Mean	Median	SE	p
Petal length	45	3.95	3.93	0.060	54	3.481	3.367	0.070	<0.001
Petal width	45	1.80	1.80	0.020	55	1.608	1.600	0.019	<0.001
Corona lobe length	45	2.44	2.47	0.037	55	2.122	2.000	0.062	<0.001
Corona lobe width	45	1.57	1.57	0.030	55	1.322	1.333	0.021	<0.001
Gynostegium height	45	2.46	2.53	0.033	55	1.831	1.800	0.023	<0.001
Gynostegium width	45	1.94	1.93	0.019	55	1.591	1.600	0.014	<0.001
Anther wing length	45	1.07	1.06	0.014	55	0.669	0.660	0.009	<0.001
Maximum width of alar fissure	45	0.18	0.18	0.006	55	0.133	0.138	0.003	<0.001
Minimum width of alar fissure*	37	0.15	0.15	0.005	56	0.072	0.061	0.004	<0.001
Maximum height of alar fissure*	37	0.25	0.25	0.006	56	0.220	0.220	0.005	<0.001
Minimum height of alar fissure*	37	0.24	0.23	0.007	56	0.190	0.180	0.006	<0.001
Pollinium width	45	0.17	0.17	0.002	55	0.169	0.169	0.001	**>0.1**
Pollinium length	45	0.37	0.37	0.003	55	0.346	0.344	0.003	<0.001
Pollinium height	45	0.19	0.18	0.002	55	0.179	0.180	0.002	<0.001

* Additional character measurements taken from a sample of flowers picked to estimate PTE on 28 February 2009. These characters were not included in the PCA.

Measurements made from flower samples collected from Grahamstown and Port Alfred indicated that the polymorphism is also present in individuals in these two populations and that the proportion of either flower morph may significantly exceed that of the other at different times. The frequency distributions of anther wing measurements for all three areas showed that there were few or no flowers with intermediate anther wing lengths (between 08 - 0.9mm; Fig. 6). In a sample (n = 57 flowers, 19 plants) of flowers collected in Grahamstown on 22 April 2008 to estimate PTE, 68.4% (n = 39) were SW flowers and 31.6% (n = 18) LW flowers. These percentages were significantly different (proportions based t-test, t $_{(55)}$ = 2.61; p = 0.0091). Similarly in a sample (n = 91 flowers, 31 plants) of flowers collected to estimate PTE on 6 April 2008 in Port Alfred, 70.5% (n = 63) of flowers were SW and 29.5% (n = 28) were LW. Again the proportions were significantly different (proportions based t-test, t $_{(89)}$ = 3.66; p < 0.001).

In contrast, the anther wing lengths of *Cynanchum ellipticum* were normally distributed and showed no evidence of bimodality as was seen in *C. obtusifolium*.

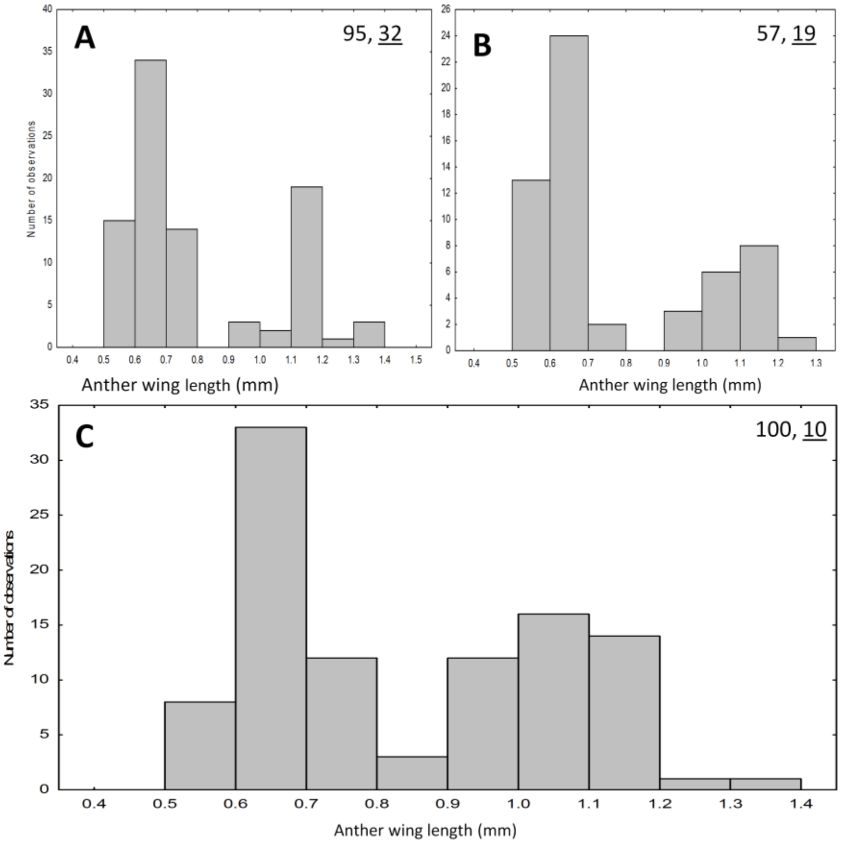


Figure 6: Frequency histograms showing the bimodal distribution of anther wing lengths in A) Port Alfred B) Grahamstown and C) Kenton-on-Sea (Number in parenthesis indicates the number of flowers and number of plants (underlined).)

Individual plants bear both types of flowers. From the ten plants that were sampled to collect flowers for morphological measurements, eight bore both long and short winged flowers. In a total of 100 flowers, 44 (44%) were long-winged flowers and the remaining fraction were short winged flowers. The percentage of flowers per individual that were long-winged ranged from 0 to 100% with average percentage of 44% of flowers per individual being long-winged types.

Difference in pollen export and receipt between LW and SW flowers

A significantly greater percentage of SW flowers had pollinaria removed than the percentage of LW flowers with pollinaria removed ($t_2 = 3.37$; $p = 0.04$; Fig. 7). Flowers with long anther wings generally had a higher proportion of pollinaria deposited than short wing flowers that received only one pollinium. The difference was however not statistically significant ($t_2 = 2.52$; $p = 0.09$; Fig. 7), owing to the low levels of pollinarium depositions in LW flowers during these sampling dates.

Figure 7: Differences in the proportion of flowers with pollinaria removed and the proportion of pollinated flowers in SW and LW flowers (bars = mean ±1SD).

Deposition in SW flowers is limited by the morphology of the anther wings which causes the alar fissure to be too narrow for pollinarium deposition in SW flowers. Flowers with long anther wings had significantly larger maximum and minimum alar fissure widths and higher maximum and minimum alar fissure heights (Table 3; previous section). Figure 8 plots the number of pollinaria that were deposited per flower against the maximum width of the alar fissure and shows that no pollinaria are deposited where maximum alar fissure widths are below 0.15 which corresponds closely to the lowest alar fissure width of LW flowers (See Figure 9A). However, the range of the maximum alar fissure width of SW flower types suggests that SW flowers have alar fissure widths wide enough to receive pollinaria and the lack of pollinium deposition in these flowers is likely due to the influence of additional morphological differences (see later).

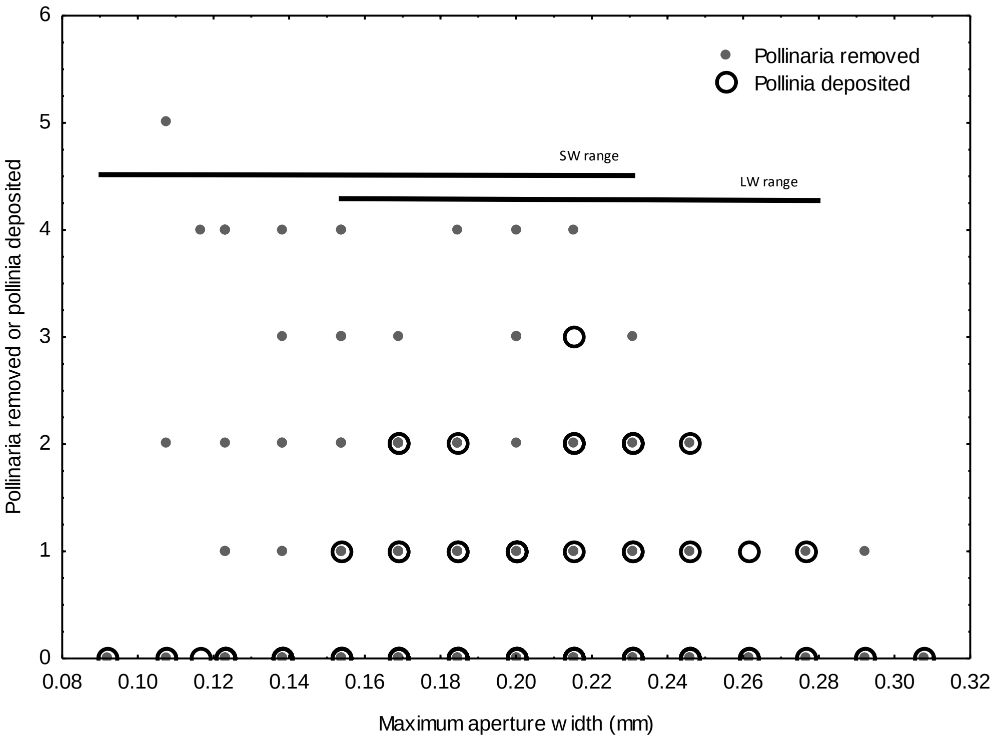


Figure 8: Comparison of pollinarium removal and deposition and the maximum width of the alar fissure in flowers of *C. obtusifolium* (n = 277 flowers). Lines indicate the range of the maximum width of the alar fissure in both SW and LW morphs.

Figure 9A indicates that all of the pollinia measured in this sample where wider than the average maximum alar fissure width of SW flowers (Fig. 9A). Most pollinia were narrower than the mean maximum aperture width of LW flowers. Pollinia widths were wider than the minimum alar fissure widths of both types of flowers (Fig. 9B), however the margin by which the pollinium width exceeded the minimum alar fissure width of SW flowers was much greater than for LW flowers. The height of pollinia was lower than the average maximum alar fissure heights of both LW and SW flowers (Fig. 9C) while the height of pollinia was lower than the minimum height of LW flowers. The height of pollinia was centred around the average minimum alar fissure height of SW flowers. These data present evidence that the lower levels of pollinarium deposition in SW flowers is the result of the smaller dimensions of the alar fissure that largely restricts pollinium deposition to LW flowers. The data however suggests that pollinia need not be smaller than the alar fissure to be inserted. For instance the minimum alar fissure width of LW flowers was lower than pollinium widths but in this species a large part of the alar fissure is composed of soft, pliable stigmatic tissue that makes up the bottom section of the alar fissure and can stretch outwards and envelope pollinia. Presumably this is easier in LW flowers as the maximum alar fissure exceeds the pollinium width allowing the pollinium to wedge into the front of the alar fissure and be inserted with force from the insect. Therefore a combination of a higher and broader alar fissure that may also stretch and envelope pollinia is responsible for facilitating pollinium insertion in LW flowers. Ideally the dimensions of freshly deposited pollinaria should be measured and related to the dimensions of the alar fissure, but rapid pollen tube growth alters the dimensions of such pollinia.

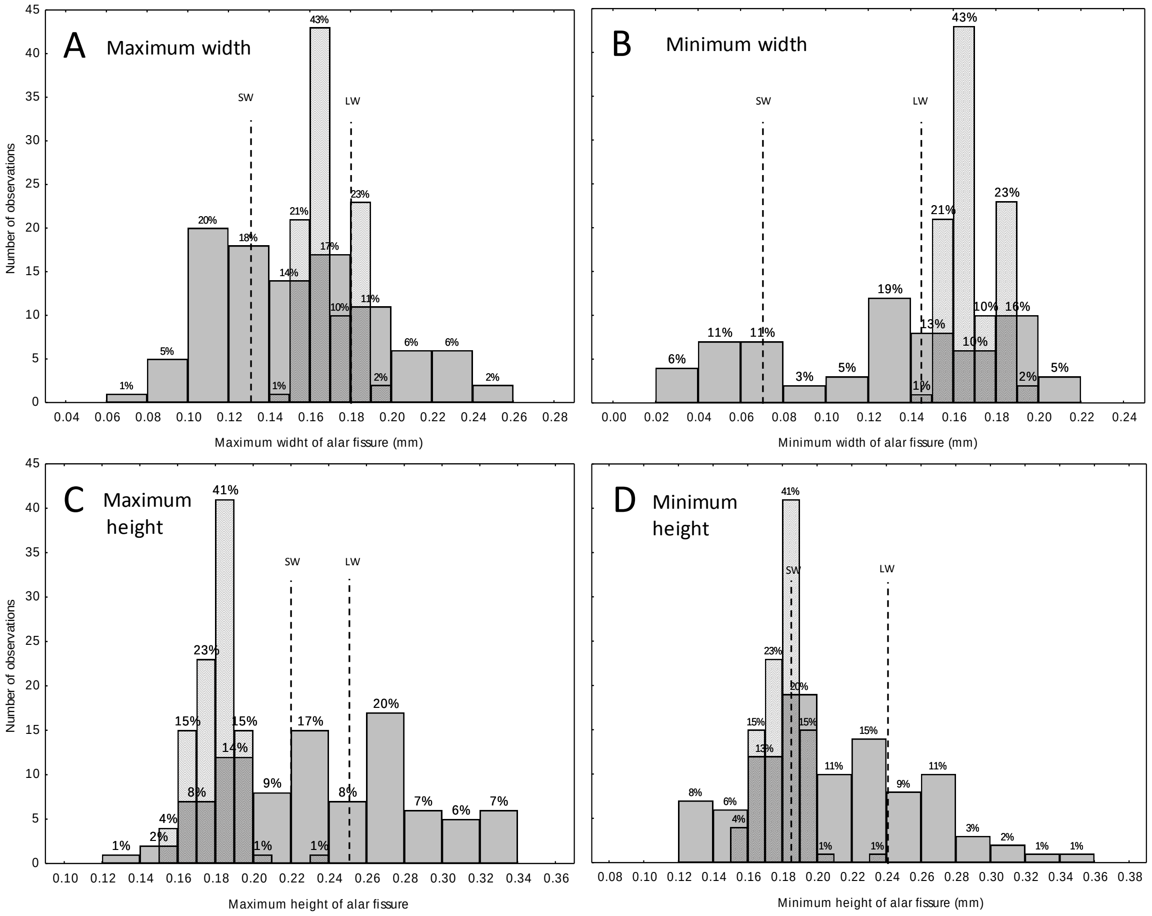


Figure 9: Frequency histograms of the maximum (grey bars; A) and minimum width of the alar fissure (grey bars; B) and the maximum and minimum height of the alar fissure (grey bars; C&D). In each case the frequency histogram is overlain with the histrogram of the width of pollinaria (hashed bars; A&B) and the height of pollinaria (hashed bars; C&D). Dashed lines in each figure refer to the average values for SW flowers (left) and average values for LW flowers (right).

Flowering phenology of andromonoecy in C. obtusifolium

Five of the 32 inflorescences produced both flower types and three bore both flower types at the same time. Thus inflorescences mostly only display one flower type at a time. There was also evidence that different flower morphs were produced at different positions along the inflorescence, as older inflorescences with evidence of several flowering episodes (by having multiple flower scars) typically produced only SW flowers (Coombs unpublished data).

Temporal variation in the ratio of LW and SW flowers and the relationship between pollen transfer efficiency and the proportion of LW flowers

Figure 10 shows that the ratio of LW and SW flowers was not constant throughout the year and varied at different sampling intervals. There was a significant negative relationship between the number of SW flowers and the sampling date (F (1, 2) = 24. 1; r^2 = 0.88, n = 4, p = 0.04), while during these four dates there was a significant increase in the number of LW flowers (F (1, 2) = 24. 1; r^2 = 0.88, n = 4, p = 0.05; Fig. 10). This trend should however be interpreted with caution as the sample size is very low and data is lacking for two sampling dates (November and December 2008) within this series of sampling dates. Figures 10 and Figure 11 indicates that PTE is unlikely to depend solely on the percentage of LW flowers as it is expected that increases in PTE would co-occur with increases in the number of pollen receiving flowers available. However the percentage of LW flowers steadily increased over the four sampling dates from October 2008 to March 2009 while during this time pollinarium removal, deposition and PTE varied unpredictably. I inspected the correlation between PTE and the percentage of LW flowers using data from six dates for which this data was available (Fig. 12). This relationship was weak and non-significant (Spearman's correlation co-efficient = 0.03; p > 0.05, n = 6) suggesting that the production of LW flowers is unlikely to be increased at times of high pollination success, however more data needs to be collected to test whether such a relationship exists.

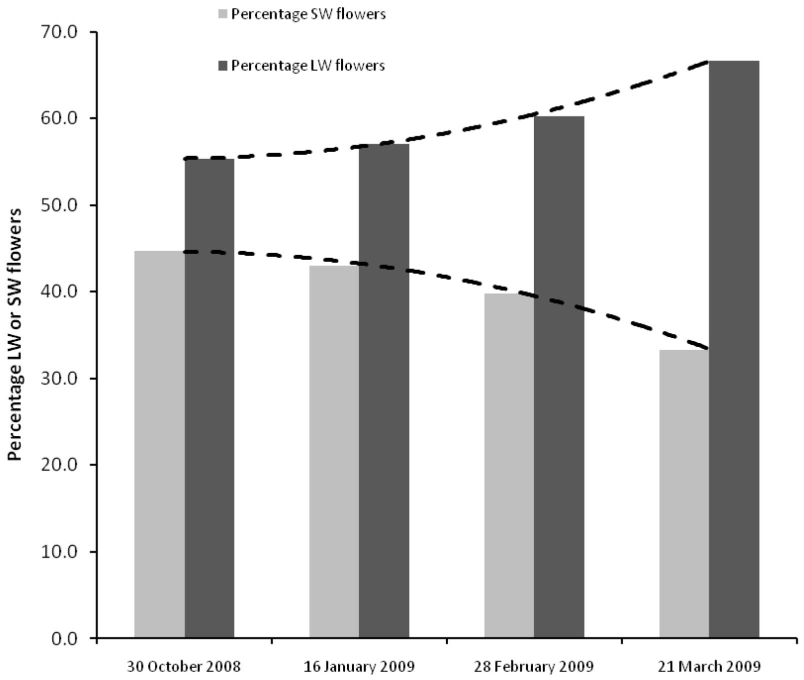


Figure 10: Percentage of LW and SW flowers recorded on four different dates in samples collected from the Kenton-on-Sea (Dashed lines above bars indicate polynomial function fitted to data points, see methods section).

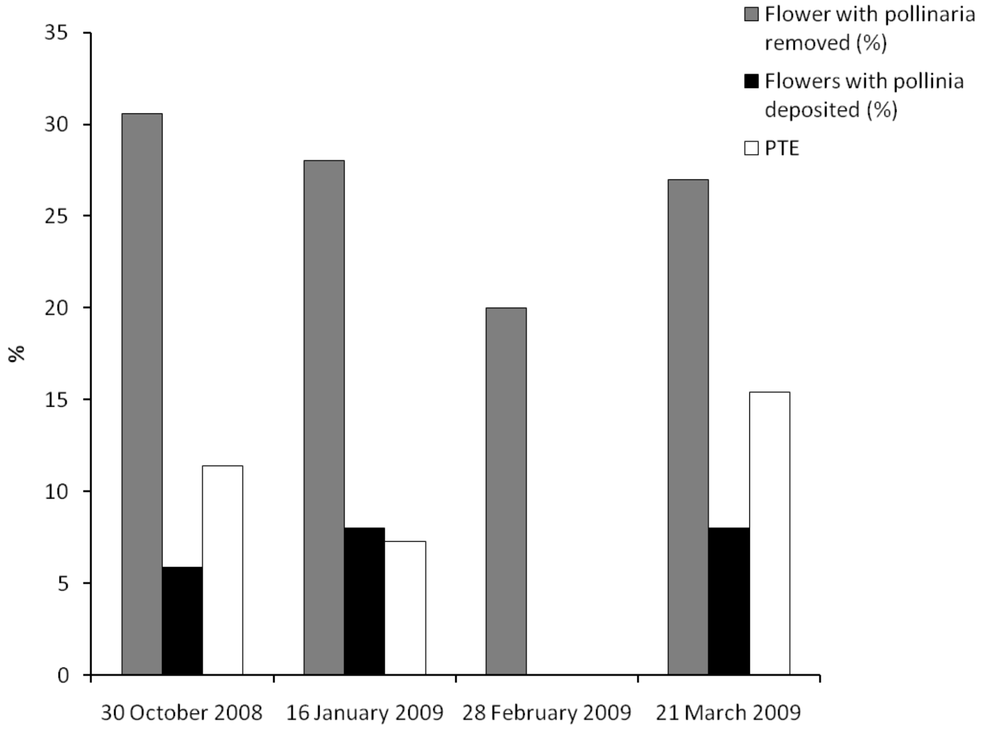


Figure 11: Percentage of flowers with pollinaria removed, pollinia deposited and PTE on four different dates at Kenton-on-Sea.

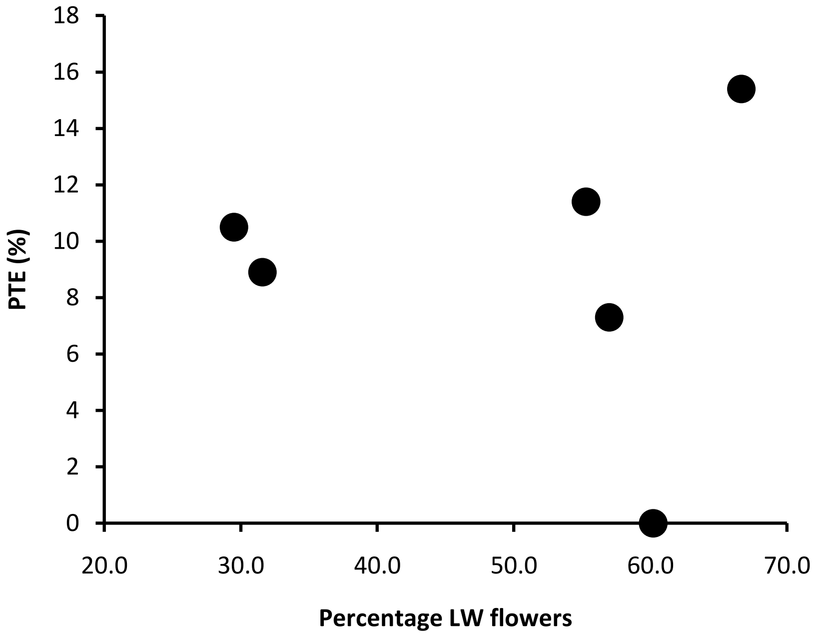


Figure 12: The correlation between PTE and the percentage of LW flowers was weak and non-significant.

Discussion

Cynanchum obtusifolium was mainly pollinated by honeybees at these three study sites in South Africa. During peak flowering periods of *C. obtusifolium* honeybees were common and reliable visitors, while other flower visitors were more patchy and unpredictable in their abundance. The wide variety of other insects that visit this species and bear pollinaria does however suggest that the system may be more generalized at certain times of the year when honeybees may prefer foraging on other flowers. None of the other hymenopteran visitors however carried pollinaria on the tarsi indicating that the morphology of honeybees may interact with flowers in a way that is more efficient at removing and depositing pollinia. Further studies would need to be carried out to compare the differences in pollinator effectiveness between the different insect visitors to *C. ellipticum* (*cf.* Fishbein and Venable, 1996) to establish the role of other insect taxa in pollinating this species at different times of the year. Some of the taxa collected, such as the different families of flies, are well known flower visitors and pollinators (Larson *et al.,* 2001). The flower visiting habits of South African Argidae is not well documented (Gess pers. comm.), but most of the hymenopteran taxa other than honeybees that were collected visiting *C. obtusifolium* are a group of generalized flowers visitors (Gess and Gess, 2003), these are likely to be opportunistic visitors that capitalize on the large nectar resource that this species provides during flowering periods. Adults of all these hymenopteran families such as Scoliidae (Coombs *et al.,* 2009), Halictidae (*e.g.* Momose *et al.,* 1998) and Vespidae (Forster, 1994; Coombs *et al.,* 2009) are well known nectar foragers and pollinators. The extended flowering period of *C. obtusifolium* undoubtedly contributes to the abundance of pollinators that visit this species by overlapping with the activity periods a wide range of pollinators (*cf.* Herrera, 1988).

There is not enough data available on the various pollinators of *Cynanchum* to generalize about pollinator interactions within the genus. Studies on the pollination of other *Cynanchum* species have revealed pollination by various wasp families (Vespidae; Scoliidae; Sphecidae) and bees (Halictidae) in this genus (Ollerton and Liede, 2003; Ollerton *et al.,* 2010). Some *Cynanchum* species (e.g. *Cynanchum harlingii*, Wolff *et al.,* 2008; *Cynanchum caudatum*, Yamashiro *et al.,* 2008) are involved in more generalized pollinator relationships.

The nectar concentration of *Cynanchum* has rarely been measured, however nectar concentrations of *C. obtusifolium* are within a similar range to that seen in other species of *Cynanchum* (*C. caudatum* = 22-24%; *C. willfordii* = 25%; *C. boudieri* = 20-22% sucrose;

Yamashiro *et al.,* 2008). It is difficult to infer that these nectar values are typical of bee-pollinated species as honeybee colonies typically consume a large range of nectar concentrations under natural conditions (Seeley, 1986). However, the *Cynanchum* species studied by Yamashiro *et al.* (2008), were also visited and pollinated by various bees (Anthophoridae and Apidae) and wasps (Vespidae and Scolidae) of similar families that were found visiting *C. obtusifolium*, suggesting that these these nectar concentration values may be typical of species pollinated by these Hymenoptera. The range in median nectar concentration values found in the wasp-pollinated *G. physocarpus* (16.2% - 38.4%) also suggests that wasps visiting this species consume nectar of similar concentration to that found in *C. obtusifolium* (Chapter 2).

The pollination success of *C. obtusifolium* varied between different sites and within sites over the course of the flowering season. Although such intra-seasonal variation in pollination success has been found in other species with long term flowering periods, these studies have found that pollination and fruiting success may either fluctuate unpredictably throughout the flowering season and may vary by several orders of magnitude at different times (Bullock *et al.,* 1983; Peter and Johnson, 2008). Such variation in pollination success of these species could be driven by several processes. For instance, variation in pollination success at different sites could be caused by variation in the abundance of different pollinators (*cf.* Herrera, 1998) and differences in pollinator visitation rates at different sites (Lundemo and Totland, 2007). Increases in pollination success may also coincide with peak periods of flowering intensity (Peter and Johnson, 2008). I suspected that increases in pollination success could co-occur with increased numbers of hermaphrodite flowers produced at certain periods in the flowering season, however my data showed no relationship between PTE and the percentage of hermaphrodite flowers in the population. Diggle (1993) found that the proportion of male flowers produced per plant increased in plants where flowers had been artificially pollinated, however larger sample sizes are needed to confirm whether *C. obtusifolium* alters the proportion of different flower morphs produced by plants at different times of the flowering season and whether this is related to levels of pollination and fruiting success within the population (e.g. Diggle, 1993). Data presented here indicated that pollen deposition increased directionally and peaked during September 2008 indicating that pollinator visits do not vary unpredictably throughout the season but may increase with flowering intensity at different flowering times. It is common for species that flower episodically to show large variation in the intensity of different flowering periods (Bawa, 1983). Further studies should investigate the role of environmental variables, flowering intensity and pollination success in *C. obtusifolium* as has been done in dioecious species (see later).

Andromonoecy in *Cynanchum obtusifolium*

The results of this study clearly showed that there is a flower polymorphism in *C. obtusifolium* with flowers with short anther wings being functionally male flowers that very seldomly receive pollinia, whereas flowers with long anther wings are functionally hermaphroditic as they both export and receive pollinaria. The single case where I found a pollinium deposited in a flower classified as a SW morph, indicates that this classification is artificial and that flowers with anther wing lengths close to the boundary may receive pollinaria even when classified as functionally male flowers using this method. Although I carried out breeding system analysis on this species using pollen from both LW and SW flowers (*e.g.* Solomon, 1985; Kouonon *et al.,* 2009), these failed due to very dry conditions at the end of 2009. Thus it has only been determined that *C. obtusifolium* is functionally andromonoecious and it is not known if flowers with short anther wings are capable of self-pollination. However autogermination of pollinia was very seldomly observed in either flower type and probably only occurs when pollinia are unseated without being removed.

Andromonoecy has been found in at least one other species of Asclepiadoideae. Tanaka *et al.* (2006) documented andromonoecy to be present in *Metaplexis japonica*. In both *C. obtusifolium* and *M. japonica* hermaphrodite flowers are larger and have a greater number of ovules. The male flowers of both *C. obtusifolium* and *M. japonica* have highly reduced stigmatic chambers (see Tanaka *et al.,* 2006). Although andromonoecy has been reported for nearly 4000 species within approximately 33 plant families (Miller and Diggle, 2003), to my knowledge this study and that of Tanaka *et al.* (2006) are the only reports of this breeding system in the Asclepiadoideae.

There was evidence to suggest that both flowers types are not equal in their ability to export pollen. Short winged flowers had significantly higher overall pollinarium removal than flowers with long-anther wings. Other than the larger size of LW flower, there are no obvious differences in external appearance between LW and SW flowers suggesting that honeybees are unlikely to discriminate between the two different flower types as they have been found to do in gynodioecious species (Ashman, 2000), making it unlikely that this is due to honeybees preferring either flower type. Rather I suspect the increased pollen export of SW flowers could be due to SW flowers having smaller flowers with nectar that is more easily accessible to a wider scope of insects including non-pollinating nectar thieves and occasional pollinators. The higher pollinarium removal in SW flowers does however agree with the hypothesis that male flowers are more effective at exporting pollen in andromonoecious species (“increased pollen donation hypotheses”; Vallejo-Marin and Rausher, 2006).

The relationship between pollination success and the proportion of flowers with pollinia deposited suggests that increases in PTE in this species is due to more efficient deposition at some periods during the flowering season. I found no relationship between the proportion of LW flowers present and PTE suggesting that increases in PTE are unlikely to be associated purely with increases in the numbers of hermaphroditic flowers. This is in contrast to findings by Diggle (2003) where artificial pollen supplementation was associated with increased production of hermaphrodite flowers. Changes in the levels of pollination success could likely be influenced by the presence of other flowering species resulting in competition for pollinators (e.g. Motten, 1986; Rathcke, 1988; Chittka and Shurkens, 2001). Such changes could also conceivably result from nectar robbers and less effective pollinators mostly removing pollen during some flowering periods while legitimate pollinators such as honeybees remove and deposit pollinia and hence cause increases in PTE when these are the main flower visitors.

Similar to findings by Miller and Diggle (2003), I found that the percentage of SW and LW flowers was variable and changed at different times throughout flowering period. Such phenotypic variability is common in andromonoecious species and may be caused by changing environmental conditions such as water availability, nutrients and shade (Solomon, 1985; Miller and Diggle, 2003). Fruit development has also been found to suppress hermaphrodite flower production and increase production of male flowers in

Solanum candidum and *S. ferox* (Miller and Diggle, 2003), suggesting that *C. obtusifolium* could produce a greater proportion of SW flowers at times following high pollination success. *Cynanchum obtusifolium* displays individual flowering characteristics of andromonoecy also found in genera like *Solanum* where much of the details of flowering phenology have been documented (Miller and Diggle, 2003). Findings by this study combined with those of others, suggest a common theme in andromonoecy is that male and hermaphrodite flowers are not produced randomly along an inflorescence but these flowers are typically produced at the base of the inflorescence whereas male flowers are produced more often in more distal sections of the inflorescence (Diggle, 1997; Solomon, 1985; Miller and Diggle, 2003). Results from a small pilot study combined with my observations suggest that the flowering patterns of *C. obtusifolium* is similar to that found in these studies whereby those inflorescences that produce LW flowers typically produce these flowers at the base of the inflorescence and SW flowers are produced at more distal portions of the inflorescence. Not all inflorescences produce both types of flowers and a fraction produced only SW flowers while others switched from initially producing LW flowers at the base of the inflorescence to producing more SW flowers in successive flowering events. I also observed several fruit carried at the base of the inflorescence, further confirming that LW flowers are generally produced in this region.

Conclusion

Results from this study indicated that *C. obtusifolium* is mainly pollinated by honeybees that maintain high levels of pollination success in this species. Levels of pollination success do however vary throughout the year, which is possibly related to the abundance of honeybees. The function of andromonoecy remains unclear in this species and its discovery within this genus opens up further avenues of research to answer other important questions relating to the phenology and evolution of this trait in *C. obtusifolium*. Particularly interesting would be to determine the role of nutrients and water as well as the influence of pollination on the ratio of SW and LW flowers produced by plants (e.g. Solomon, 1985, Diggle, 1993). The presence of andromonoecy in *C. obtusifolium* and its absence in *C. ellipticum* also raises a host of questions relating to differential resource allocation to reproduction between these two species. Andromonoecy is thought to have evolved in in species where resources limit fruit set and plants produce relatively large fruit (Bertin, 1982; Miller and Diggle, 2007 and references therein). The fruit of *C. obtusifolium* are much larger than that of *C. ellipticum* which could suggest that there exist fundamental differences in terms of resource allocation to reproduction in these two species. Andromonoecy may also function to increase the flower display size and subsequent pollinator visitation (Podolsky, 1992) through using male flowers that are relatively less costly to produce (Solomon, 1986; Vallejo-Marin and Rausher, 2007). Therefore, future studies should investigate changes in the production of male and hermaphrodite flowers in *C. obtusifolium* to determine whether this is related to resource availability or pollination success (*c.f.* Solomon, 1985).

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Part B: Degree of specialization in fly-pollinated Asclepiads

Chapter 6

Generalized fly pollination in *Ceropegia ampliata* (Apocynaceae-Asclepiadoideae), and the role of trapping hairs in pollen export and receipt

Abstract

Flowers of the genus *Ceropegia* display elaborate adaptations to trap pollinating flies. Flies are trapped within a bulbous base of the flower after moving through an elongated corolla tube lined with stiff hairs. When these hairs wilt after several days, insects held in the bulbous chamber at the base of the corolla tube are released. Despite such complex adaptations to trapping pollinators, key aspects of the pollination ecology including the identity of pollinators, presence or absence of nectar rewards, duration of pollinator trapping and pollination success remain undescribed for the majority of *Ceropegia* species. Importantly no studies have empirically tested the role that trapping hairs may have on pollen export and receipt. I documented the pollination biology of *Ceropegia ampliata* in two natural populations and found that *C. ampliata* can be considered a generalist being pollinated by flies from at least four families (Tachinidae, Sarcophagidae, Muscidae and Lauxaniidae). The duration of trapping phase lasted two to five days and flowers produce small quantities of nectar. Pollination success was highly variable but generally low with occasional peaks suggesting that flies likely visit this species sporadically. Flowers that had already proceeded beyond the trapping phase generally had a significantly greater number of pollinaria removed than flowers that were still in the trapping phase probably reflecting the longer exposure to pollinators. In contrast I found no differences in pollinarium removal

between flowers with trapping hairs present and flowers with hairs experimentally disabled. The role of trapping hairs in the pollination success of *C. ampliata* therefore remains uncertain although I propose that on the basis of this experiment trapping may be an adaptation to enhance female success through pollen deposition rather than pollen export. Given the low rates of natural pollen deposition, an experiment with a large number of replicates is required to test this hypothesis in *Ceropegia*.

Introduction

Flies are important and omnipresent insect pollinators (Larson *et al.* 2001). Much of our understanding of the interaction of flies with different flowers originates from examples of co-evolution between long-proboscid flies such as members of the Bombyliidae, Nemestrinidae and Tabanidae and the long-tubed flowers that these insects pollinate (Goldblatt and Manning, 2000; Anderson and Johnson, 2009; Pauw et al. 2009). Short tongued flies have been known to be important pollinators since their interactions with flowers were first described by Knuth (1905). Flies such as the Syrphidae (Pansarin, 2008; Jauker and Wolters, 2008; Kearns, 1992), Tachinidae (Lehnebag and Robertson, 2004; Griffin *et al.* 2009; Kearns, 1992), Anthomyiidae (Elberling and Olesen, 1999; Pellmyr, 1989; Kearns, 1992), Bibionidae (Johnson and Steiner 1994) and Muscidae (Kearns, 1992; Elberling and Olesen, 1999) have all been shown to act as effective pollinators. Other lesser known fly pollinating families include mosquitoes (Culicidae; Thien and Utech, 1970) and fungus gnats (Sciaridae and Mycetophilidae; Mesler *et al.* 1980; Okuyama *et al*. 2004; Goldblatt *et al.* 2004; Blanco and Barboza, 2005).

Flowers pollinated by short-tongued flies are highly diverse in shape, colour, size, scent and presence of reward (Larson *et al.* 2001). A common theme in some fly pollinated flowers is that pollinators are trapped inside the flowers through a combination of slippery surfaces, erect hairs and convoluted corolla tubes (Vogel, 1961; Faegri and van der Pijl, 1979; Proctor *et al.* 1996; Oelschlagel *et al.* 2009; Poppinga *et al*. 2010), although these trapping mechanisms are not restricted to sapromyophilous species. These carrion fly-pollinated flowers are typically leathery or hairy, have strong, rancid organic scents and may either be deceptive (e.g. dead horse arum; Stensmyr *et al.* 2002) or may reward pollinators with nectar (e.g. *Stapelia*; Bruyns, 2005; Herrera and Nassar, 2009).

The morphology of trapping flowers has been well described; in particular, several studies have given detailed morphological descriptions of the structures involved in trapping (Vogel, 1961; Oelschlagel *et al*. 2009). An important question that remains however is to determine the influence that these structures have on pollination success in these species. I am not aware of any studies that have manipulated traits associated with trap functioning to test the fitness consequences of trapping in terms of pollen export and receipt.

The degree of specialisation in fly trapping species varies. Several species are pollinated by a group of flies sharing similar morphology and behaviour (i.e. functional specialization *sensu* Fenster *et al.* 2004). These include midge trapping in *Aristolochia* spp. (Sakai, 2002) and *Arisaema* spp. (Vogel and Martens, 2000). Other species are pollinated by a taxonomically narrower range of pollinators (i.e. ecologically specialized, *sensu* Fenster et al. 2004) and include species like *Aristolochia pallida* (Rulik *et al.* 2008) and *Arum italicum* (Albre *et al.* 2003) that are pollinated by three morphospecies (*Magaselia* spp.) and two species

(*Phoridae*) respectively. Some species may also have truly generalized pollination systems and are visited and pollinated by numerous fly species that vary widely in morphology (e.g. *Aristolochia grandiflora*; Burgess *et al.* 2004). Little is known about the degree of pollinator specificity in *Ceropegia*. Ollerton *et al*. (2009) concluded that similar to other fly trapping flowers, the degree of specialization in *Ceropegia* varies, with some species having highly generalized pollination systems (e.g. *C. aristolochiodes ssp. deflersiana*) and other are more specialized (e.g. *Ceropegia linearis*). Few species of *Ceropegia* are pollinated en masse by small swarming dipterans (e.g. midges) and sampling large numbers of pollinators in the flowers of this genus is difficult, thus degree of specialization in these species remains uncertain (e.g. Heiduk *et al.* 2010; see review by Ollerton *et al*. 2009). Studies that collect large samples of pollinators over several seasons are therefore required to understand the pollinator specificity in *Ceropegia*.

The genus *Ceropegia* (Asclepiadoideae), a highly speciose asclepiad genus with approximately 180 currently recognized species (Ollerton *et al*. 2009), is well known for the morphology of flowers thought to function in trapping potential pollinators (Vogel, 1961, Proctor *et al*. 1996). Flowers are adorned with attractive structures that may contribute to the trapping mechanism of the flowers (Vogel, 1961). These accessory structures include moveable hairs (*Ceropegia ampliata*), vibratile corolla lobes (*Ceropegia bowkerri*) or a hood that covers the entrance, with the only access being provided through small lateral entrance apertures (e.g. *Ceropegia sandersoni*). Flower colours are diverse but white, green and dark purple predominate with various patterns such as spotted petals or a vein-like marking on the corolla (Faegri and van der Pijl, 1979). While variable, flowers of the genus *Ceropegia* have a common basic morphology (“Bauplan”) consists of a long tubular corolla with a bulbous base distally which is thought to serve as an imprisoning chamber for visiting flies (Vogel, 1961). Pollinating flies are kept within this chamber due to the stiff, erect hairs that line the entrance tube and which are presumably difficult for small insects such as these flies to part (Vogel, 1961). Passing through the barrier of hairs when entering the flower is facilitated as in many species the hairs angled slightly downwards. The presence of these hairs is temporary and after being erect for several days, they wilt releasing the flies held in the corolla bulb (Vogel, 1961).

Despite the fascinating pollination biology of many fly pollinated Asclepiads, even basic aspects of the pollination ecology such as the different types of pollinators, pollination success and even the presence or absence of nectar rewards remains largely unknown. The available data describes primarily flower visitors and pollinators and is restricted to specimens cultivated in greenhouses (e.g. Vogel, 1961) or studies of insects found within the flowers of herbarium specimens (Meve and Liede, 1994; Ollerton *et al.* 2009 and references therein; Masinde, 2004, Karuppusamy and Pullaiah, 2009; Heiduk *et al.* 2010). Few studies have collected pollinators in wild growing populations (e.g. Ollerton *et al*. 2009 and Masinde 2004) which is a consequence of the difficulty of doing natural history studies on wild plants due to their cryptic nature and low or patchy abundance and irregular flowering. To my knowledge no studies have manipulated floral traits involved involved in trapping pollinators to determine the influence of these structures on pollen removal and receipt. Asclepiads are ideal taxa for answering ecological questions, as pollen is presented as pollinia that allows the average levels of pollen removal and deposition to be relatively easily determined (Wyatt and Broyles, 1994). In this study I investigate the pollination biology of *Ceropegia ampliata*, a species that occurs in sufficient abundance in its habitat to offer the opportunity to describe various aspects of the pollination system. Specifically, I ask the following questions: 1) Which flies pollinate *C. ampliata* and how specialized is the pollination system; 2) are the flowers rewarding; 3) what are the natural levels of pollen removal and deposition; and 4) does imprisonment influence reproductive success?

Material and Methods

Study species and study site.

Ceropegia ampliata E. Mey is a scrambling member of the Asclepiadoideae (Apocynaceae). The flowers are light green and white and have a conspicuous cage-like structure at the opening of the corolla tube (Dyer, 1983). Flowers of this species produce a distinctly pungent spermous scent. The distribution of this species is continuous from Oudtshoorn in the Western Cape north-eastwards through Kwazulu-Natal including Zululand, where it is commonly found growing in dry scrub and thornveld to Mpumalanga (Dyer, 1983; Retief and Herman, 1997; Pooley, 1998).

This study was undertaken at two study sites in the vicinity of Grahamstown, Eastern Cape Province, South Africa. The first was a large population of *C. ampliata* growing in a small municipal reserve, the Ecca Pass Wildflower Reserve, 15 km North of Grahamstown. The second study site was a population growing along the edge of the abandoned “Old Queen’s road” approximately 13 km from Grahamstown on the Fort Beaufort road. These two study sites are separated by a distance of 4.2 km.

The vegetation type at both reserves is classified as Great Fish river Noorsveld thicket (Hoare *et al.* 2006). A recognizable characteristic of this vegetation type is the formation of bushclumps composed of both woody and succulent trees and shrubs. The areas between these bushclumps are dominated by low growing shrubs (Hoare *et al*. 2006) on which *C. ampliata* is most commonly found.

Pollinators and pollinarium loads

Pollinators were collected from both study sites by inspecting all open flowers for the presence of flies. Flowers containing flies were collected and the flies removed. Flowers collected for other purposes (see below) were also inspected for the presence of flies. Any flies that were found within the flowers were killed, pinned, identified to family level and the pollinarium load counted. Pollinia typically detach from the clip when deposited and therefore the entire pollen load is made up of whole pollinaria (no pollinia removed), ½ pollinaria (pollinaria with only one pollinium) and corpusculi (both pollinia deposited or groomed off). The total pollinarium load refers to the sum of all whole pollinaria, ½ pollinaria and corpusculae. The total time spent searching for pollinators, included planned visits to inspect flowers for pollinators as well as the time spent monitoring flower phenology, amounted to approximately 25 hrs.

Pollen removal, deposition and pollen transfer efficiency

To estimate pollination success, flowers were picked on 4 different dates during the 2007 and 2008 flowering seasons at the Ecca Pass site. These were 2 February 2007 (n = 31), 10 February 2008 (n = 8) and 20 February 2008 (n = 20). On each sampling date I picked one flower per plant. If more than one flower was present, other flowers were inspected for pollinators. For all flowers I recorded the number of pollinaria removed, the number of pollinia deposited and used this to calculate the pollen transfer efficiency (PTE), which is a population level estimate of the fraction of removed pollinaria that are deposited on stigmas (Johnson et al. 2005). In milkweeds this is calculated by dividing the average number of deposited pollinia by twice the average number of pollinaria removed per sample (there are two pollinia per pollinarium; also see Coombs et al. 2009).

Sampling was conducted 2-5 February 2010 at Ecca Pass, as well as the Old Queen's road population on 4 February 2010. Because of the small number of individuals in the Old Queen's road population, I sampled all open flower on a plant (range: 1 - 12, median = 3). In total we collected 47 flowers from 12 plants at this study site.

Do trapping hairs promote pollen export and receipt?

To examine the role of trapping pollinators in terms of pollen export and receipt, I compared pollen removal and deposition in flowers that had flaccid hairs and hence had already had the opportunity to trap and release pollinators to those that were still in the trapping phase with turgid hairs. This data was collected from the same sample of flowers collected for PTE from Ecca pass on 2 February 2007 and the sample of flowers collected on 4 February 2010 from the Old Queen's road population. The caveat of this approach is that flowers with flaccid hairs are older and may have had a more time to be visited by pollinators.

To test for the influence of trapping hairs on pollinarium removal and deposition while controlling for flowers age, I compared pollinarium removal and deposition in flowers with trapping hairs experimentally disabled to control flowers with trapping hairs present. This was done by bagging large buds that were close to opening on plants growing at Old Queen's road population. Once flowers had opened we removed the potential influence of trapping hairs by gently pressing a dissecting needle along the inside of the corolla tube which ruptures the hairs causing these to become flaccid. Control flowers consisted of marking between one to three open flowers per plant and harvesting these on the same date as flowers that were experimentally manipulated. Both control and experimental flowers were harvested once these had wilted and were near senescence. Owing to herbivory it was not always possible to get both treatment and control flowers on all plants. Plants were visited every two to four days and freshly wilted flowers were harvested to count the number of removed and deposited pollinaria. Using *t* statistics based on proportions, I then tested if the proportion of flowers that had pollinaria removed or deposited were significantly different between plants that had hairs experimentally removed versus control flowers.

Flowering trapping times, longevity and morphometrics

During 2007, I marked 41 flowering plants with numbered aluminium metal tags. On these plants we marked a total of 45 open flowers that were inspected daily to quantify natural levels of fruit set and herbivory. Using the same plants I tagged 1 to 4 buds with coloured tags. Starting at 4 February 2007, we inspected buds daily until 13 Feb 2007 and recorded the number of days spent in trapping and non-trapping phases.

To compare the shape and size of the flower to the morphometrics of its pollinators (e.g. Rulik *et al.* 2008), I measured the width and length of the flies and compared this to aspects of the floral morphology that might serve to restrict access by pollinators. The width of the fly was measured as the widest part across the thorax. For each flower I measured the width of the gap between one pair of the apical corona lobes, the minimum and maximum width of the trapping “bulb” as well as the length of the corolla tube, measured from the distal end where the corolla tube becomes dissected into the cage like fingers to the top of the trapping bulb at the proximal end. The height of the bulb was also measured, as well as the width of the corolla tube at the top and bottom. Data for flower measurements was collected from a sample of 23 flowers sampled from 19 plants.

Nectar, colour and scent producing area

To detect the areas where nectar and scent may be produced, I immersed three freshly harvested flowers in a solution of 1% Neutral red which stains physiologically active tissue such as nectaries and osmophores (Vogel 1990; Nepi 2007). Flowers were left in this solution for five minutes, removed and cut longitudinally through the corolla tube and "trapping" bulb to determine which regions had taken up the stain. Staining was performed on three flowers picked from two plants.

To investigate the presence of what was suspected to be minute amounts of nectar I used a micropipette puller (Narishige, Model PB 7, Tokyo) to make miniature needles small enough to be inserted into the putative nectaries at the base of the alar fissure. These needles were made from standard 5µl glass microcapillary tubes and had internal diameter of approximately 0.1 mm and external diameter of about 0.3mm at the needle tip.

The needle was attached to silicon tubing at the rear and the fluid was gently sucked from the putative nectaries of between – two and six flowers per plant. Once a large enough volume was obtained (~ 0.10µl), the fluid was expressed on the prism of an Otago 0-50% sucrose refractometer with a 1µl plate and the concentration of sugars determined.

The nectar volume was determined by weighing the volume of nectar contained in the micropipette on a five place electronic balance (Ohaus Discovery, model DV215CD, Ohaus Corporation, USA) and then dividing this value by the number of flowers from which the nectar was collected to calculate the weight of nectar per flower. This weight was then converted to volume by dividing the average weight of nectar by the appropriate density of a sucrose solution at the specific concentration (% w/v) as measured with the refractometer. In total, 19 flowers from 6 plants growing at the Old Queen's road population were used for nectar analysis.

Colour measurements were made on five flowers each sampled from a different individual plant. For each flower I measured the reflection spectra of the inside and outside of the corolla tube, inside and outside of the trapping bulb, inside of the green corolla lobes, as well the colour of the darker purple lining occurring on the inside of the corolla tube at the base of the long neck. Finally, I measured the reflection spectra of the small black triangular marking that occurs on the inside of the flower at the base of the green cage tips. All colour measurements were made with an USB 2000 photospectrometer. (Ocean Optics, Dunedin Florida, see Peter and Johnson 2008 for details).

Flower parasitism

While doing field work on *C. ampliata* I observed evidence of insect parasitism of the flower buds which was characterised by the corolla tube of parasitized flower buds becoming translucent, followed by the flower bud wilting shortly thereafter. To identify the insect parasitising flowers buds, we picked nine parasitised buds from three plants and placed these in small 250 ml plastic jars. The opening was then covered with fine nylon gauze and inspected daily. All flies that emerged were killed by freezing, mounted and identified.

Results

Pollinators and Pollinarium loads

In total 37 flies belonging to five different families were collected from the flowers of *C. ampliata* (Table 1). Flies with the largest pollinaria loads were Sarcophagidae and Tachinidae. Other flies such as Lauxaniidae and Muscidae also bore pollinaria but had smaller pollinarium loads than the Sarcophagidae and Tachinidae (Table 1; Fig. 1). The most numerous flies belonged to the family Sciaridae, of which 19 were collected at the Old Queen's road population. Despite their abundance, only two of a total of twenty of these flies collected at the two sites carried pollinaria (Table 1). None of the Sciaridae carried half pollinaria or corpusculae, which were regularly encountered on Tachinidae, Sarcophagidae, Muscidae and more rarely on Lauxaniidae. Pollinating insects typically carry ½ pollinaria and corpusculae, indicating that these insects may deposit pollinia. Two additional flies collected at Ecca pass also bore pollinaria, but could not be identified. Other occasional visitors that were also collected included, one unidentified moth, ants (Formicidae) and small beetles (Phalacridae).

Table 1: Pollinarium loads of different insect families visiting *C. ampliata* flowers.

Study population	Order	Family	Length (median)	Width (median)	No. of Individuals collected in flowers (no. carrying pollinaria)	Full pollinaria (mean ± SD)	1/2 pollinaria (mean ± SD)	Corpusculi (mean ± SD)	Total (mean ± SD)
Ecca pass	Diptera	Lauxaniidae	3	1	1 (1)	0	0	1	1
		Muscidae	4.19	1.52	2 (1)	0.50 ± 0.71	0	0	0.50 ± 0.71
		Sarcophagidae	5.53	1.8	2 (2)	4.0 ± 0	0.50 ± 0.71	0	4.5 ± 0.71
		Sciaridae	3.4	0.8	1 (1)	1	0	0	1
		Tachinidae	5.5	2.37	9 (7)	1.78 ± 1.56	0.67 ± 0.87	0	2.44 ± 1.51
		Unknown sp 1.	4.9	1.6	1 (1)	3	1	0	4
		Unknown sp 2.	3.3	1.1	1(1)	1	0	0	1
Old Queen’s road									
	Diptera	Lauxaniidae	2.98	1.32	2(1)	1.50 ± 2.12	0	0	1.50 ± 2.12
		Phalacridae	*ca.* 1-2mm	ca. 1mm	2 (0)	0	0	0	0
		Sarcophagidae	7.4	2.7	1 (1)	3	2	1	6
		Sciaridae	2.3	0.48	19 (1)	0.05 ± 0.23	0	0	0.05 ± 0.23
	Hymenoptera	Formicidae	*ca.* 2-4mm	< 1mm	3 (0)	0	0	0	0
	Lepidoptera	-	-	-	1 (0)	0	0	0	0
	Coleoptera	Phalacridae	-	-	2 (0)	0	0	0	0

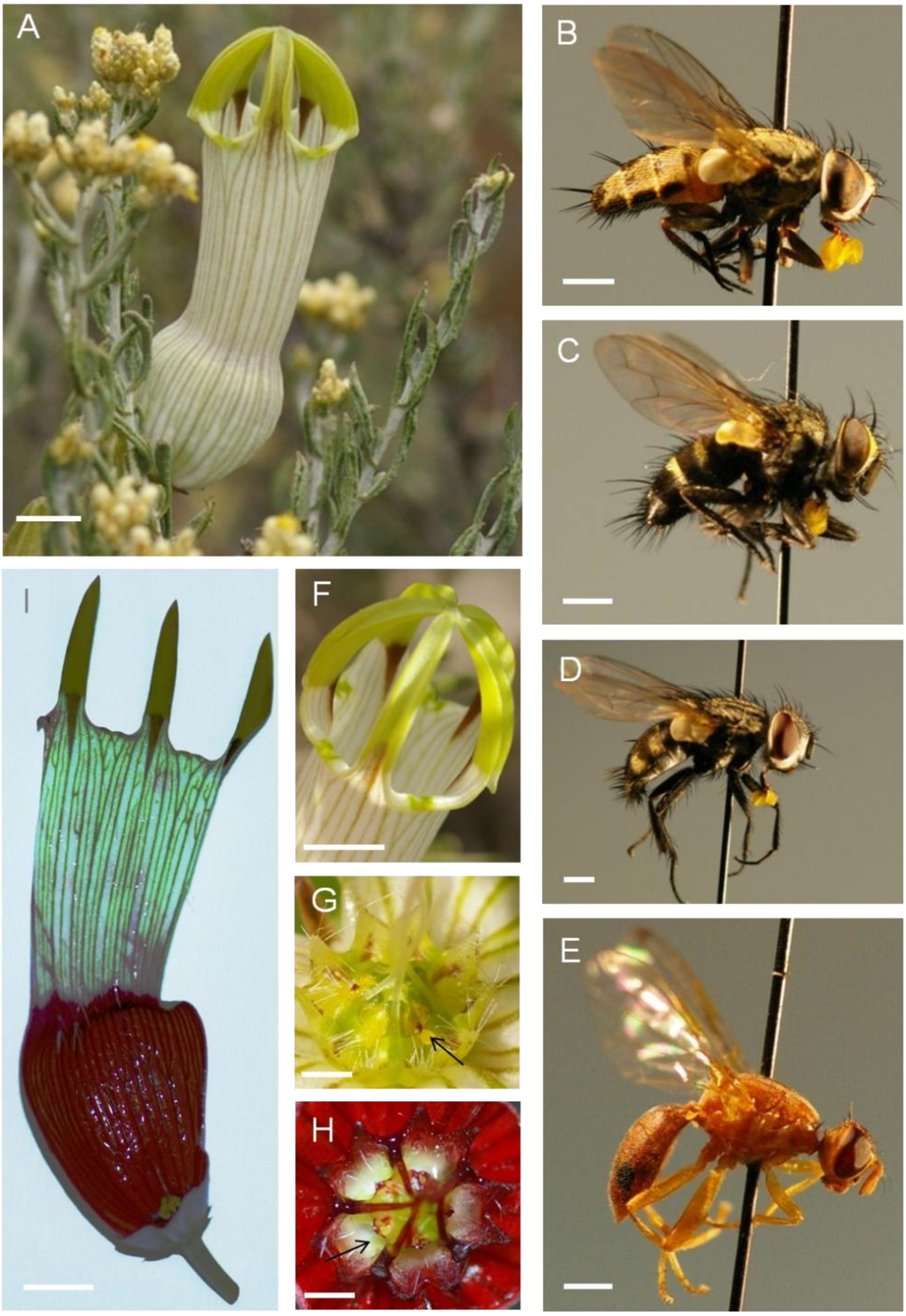

Figure 2: *Ceropegia ampliata* flowering at Ecca Pass reserve (**A**). The main pollinators of *C. ampliata* were Sarcophagidae (**B**) and Tachinidae (**C, D**). Flower buds are heavily parasitized by a small species of fruit fly, *Dacus apoxanthus* (Bezzi, Tephritidae, **E**). Pollinating flies are trapped inside the flower by initially entering the cage-like structure at the top of the flower and crawling down the corolla tube (**F**). Flies often deposit pollinia (arrow) while trapped inside the flower (**G**). In *C. ampliata* nectar is accumulated in the corona nectar cups at the base of the alar fissure (arrow; **H**). The region of scent production is located within the trapping chamber, staining red when exposed to neutral red (**I**). (Scale bars: **A, I & F** =5mm; All others = 1mm).

Pollen removal, deposition and pollen transfer efficiency

Estimates of pollination success indicated that reproductive success was highly variable both within the same season and between different seasons. The percentage of flowers with pollinaria removed was higher than the percentage of flowers with pollinia deposited. Pollinarium deposition was typically low and was frequently zero. The percentage of pollinated flowers was zero for the first two sampling dates and, like pollinarium removal, increased to 20% on 20 February 2008 (Fig. 2). These patterns of pollinarium removal and pollinia deposition resulted in PTE being zero at the first two sampling dates but 10% at the last sampling date (Fig. 2).

During 2010 the proportion of flowers with one pollinarium removed at the Ecca pass population was again low (7.7%, n = 13) and no flowers received pollinaria. Pollinarium removal rates at the Old Queen's road population were however high, with 31.9% of flowers having pollinaria removed and 12.8% having pollinia deposited. PTE of 13% was the highest recorded during the study period.

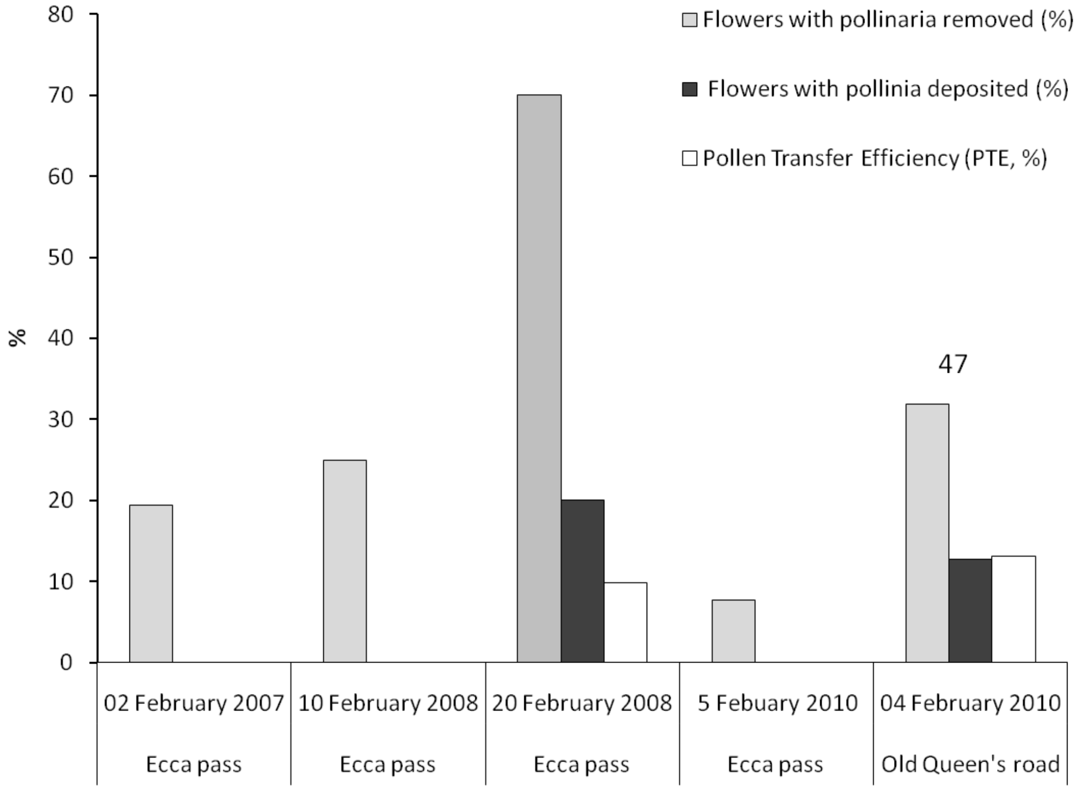

Figure 2: The percentage of flowers with pollinia removed, percentage of pollinated flowers and pollen transfer efficiency (percentage of removed pollinia that are deposited on conspecific stigmas) at different sampling dates for flowers of *Ceropegia ampliata.*

Do trapping hairs promote pollen export and receipt?

Flowers with flaccid hairs generally had a higher proportion of flowers with pollinaria removed and pollinia deposited. During 2007, the proportion of flowers with at least one pollinarium removed was 7.7% (n = 13) for flowers in the trapping phase and 27.8% (n = 18) for flowers in the non-trapping phase (Table 2). This difference was not statistically significant (t-test based on proportions, t_{29} = -0.14, p = 0.16). No pollinaria where deposited in either trapping or non-trapping flowers.

In the sample of flowers from the Old Queen's road from 2010, similar numbers were in the trapping (n = 22) and non-trapping (n = 25) phases. The percentage of flowers that had at least one pollinarium removed was significantly higher (proportions t-test, t_{45} = -3.66, p = 0.0002) in post-trapping phase flowers (59.1%) versus those with erect hairs (8%). Similarly, the percentage of flowers with pollinia deposited was higher in flowers that were in the non-trapping phase (22.7%) than in flowers in the trapping phase (4%), although this was marginally non-significant (proportions t-test, t_{45} = 1.85, p = 0.06). These results are consistent with the trend seen at Ecca Pass in 2007.

Flowers that had hairs experimentally removed had a higher percentage of removed pollinia (41.7%, 5 flowers of 12; 6 plants) versus control flowers (27.8 %, 5 flowers of 18; 8 plants) although this difference was not significant (proportions t-test, t_{28} = 0.79, p = 0.46). Depositions were low in both experimental (one pollinium, 8.3%) and control (two pollinia, 11.1%) flowers.

Flowering trapping times, longevity and morphometrics

The percentage of flowers in the trapping and non-trapping phase was 41.9 and 58.1 respectively (Table 2) and were not significantly different (proportions based t-test, t_{29} = 0.89, p = 0.38). This suggests that during the flowering period similar proportions of flowers are releasing or trapping pollinators. I also observed that flowers on the same plant have both trapping and non-trapping phases present simultaneously.

Due to extensive flower herbivory, data for the duration of trapping and non trapping phases was only obtained from 18 flowers of off 14 plants. Most flowers remained in the trapping phase for a longer period (median = 3.5, IQR = 2 - 5) than the relatively short period after which hairs become flaccid (median = 1, IQR = 1). Flowers with flaccid hairs and buds are not scented to the human nose.

Table 2: Summary of the percentage of flowers in the trapping and non-trapping phase from a sample of 31 flowers picked from *C. ampliata* and the corresponding differences in pollen removal and deposition of flowers in the trapping and non - trapping phases (Data collected during 2007).

	Phase	
	"Trapping"(%,n)	"Non-trapping"(%,n)
Flowers	41.94 (13)	58.06 (18)
Flowers with pollinaria removed	7.7 (13)	28 (18)
Percentage of pollinated flowers	0 (13)	0 (18)

Table 3: Summary of the fate of buds and flowers tagged on different plants during 2007.

	Flowers (%, n=45, flowers)	Buds (%, n=47 buds)
Parasatism	0.00	40.4
Herbivory	4.4	21.3
No damage	95.6	38.3
Fruit set	0	0

The flower bulb is approximately round in cross section with an average diameter of 13.20 mm (SD = 1.2) and slightly ovoid with an average height of 15.80 mm (SD = 1.9). The corolla tube has a slight reverse taper with the widest region at the opening of the trapping bulb (mean = 10, SD = 1.6) and the narrowest region at the constriction near the base (mean =

7.90, SD = 1). The width of the openings between the corolla lobes was 4.4 mm (SD = 0.9) and was wider than the median thorax width of Sarcophagids and Tachinids suggesting that that larger flies easily enter the flower through the corolla lobes. The average length of the corolla tube exceeded the length of the flies several fold and therefore flies have to crawl some distance before entering the basal bulb. The corolla tube is lined with soft-white hairs that are held erect by an inflated base filled with fluid that rapidly leaks out when the hair is squashed. Although the average length of the trapping hairs from different regions of the corolla tube was 2.8 mm (SD = 0.4, n = 23), I subsequently noticed that the length of the hairs lining the corolla tube were on average slightly shorter (average = 2.9 mm, SD = 0.6, n = 8 hairs from 2 flowers) than those at the constricted basal region (average = 4.4 mm, SD = 0.6, n = 8 hairs from 2 flowers). It is noteworthy that despite the longer hair that this species has at the base of the corolla tube, these hairs do not form a distinctly dense ring of long hairs that line the bulb constriction as is the case in some other *Ceropegia* species such as *C. macmasteri* (Dold 2006) and *Aristolochia* spp. (Proctor et al. 1996). The hairs lining the inside of the bulb were much finer presumably as these do not need contribute to imprisoning flies.

Nectar, colour and scent producing area

All flowers showed the same pattern of staining**.** The entire adaxial region of the bulbous base stained red as well as small amounts of staining between the cage-like tips of the corolla. The outside region of the flower remained unstained. Therefore the primary region of scent production appears to be inside the trapping bulb. There is still no certainty as to where the nectar is secreted in *Ceropegia* species however there is evidence suggesting that the region of nectar production occurs in the tissue behind the guiderails (Lock, Endress and Ollerton, unpublished data). I found no staining in this area, which could be due to either the viscosity of the dye or the presence of viscous nectar preventing dye from coming into contact with nectaries (Fig. 1H, I).

Using microcapillary tubes we detected minute quantities of nectar in the flowers of *C. ampliata*. Nectar concentration ranged between 14.5% and 21% sucrose equivalents with an average of 17.7% (SE = 0.9, n = 6). Nectar volumes were an average of 0.20 µl (SD = 0.087, n = 6) per flower (0.04 µl per nectary).

Both inside and outside the trapping bulb and corolla tube reflect UV. Other parts of the flower are UV absorbing.

(Fig. 3).

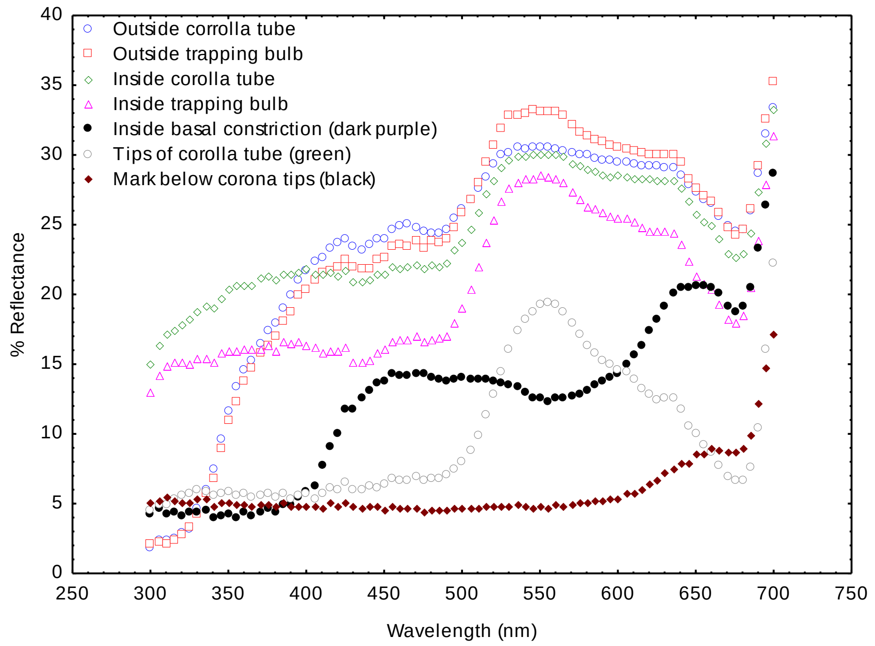

Figure 3: Average spectral reflection functions of different parts of the flower of *C. ampliata*.

Flower parasitism

The majority (61.7%, n = 29) of buds were either parasitised (40.4%, n = 19) by a small Terphritid fly (*Dacus apoxanthus* – Bezzi) or eaten by herbivores (21.3%, n = 10). Only 38.3% (n = 18) of buds opened as flowers. Relatively few of the open flowers marked for fruit set experienced herbivory (4.4%) and the vast majority (95.6%) remained open and undamaged until flower senescence (Table 3). None of the marked flowers went on to set fruit.

Six flies were hatched from the parasitized buds and all were identified as *Dacus apoxanthus* (Tephritidae, Fig. 1E). On one occasion we observed the egg laying behaviour of this species. The fly landed on the bud and tapped the posterior region of its abdomen on the tip of the bud. It then walked along the stem of the plant possibly searching for other buds.

Discussion

I demonstrate that *C. ampliata* has a generalized pollination system with the main pollinators consisting of a group of flies of the families Tachinidae, Sarcophagidae, Muscidae and Lauxaniidae that are the main pollinators of *Ceropegia ampliata* in its natural habitat. The large numbers of Sciarids found inside the flowers of *C. ampliata* are most likely just nectar thieves. Sciarids are common flower visitors to other fly pollinated species including trap flowers (e.g. Mesler *et al.* 1980; Rulik *et al.* 2008; Heiduk *et al.* 2010), and several South African species of *Ceropegia* (see supplementary material in Ollerton *et al.* 2009). Sciarids may also be involved in some highly specialized pollination interactions (e.g. *Lepanthes* spp., Orchidaceae; Blanco and Barboza 2005), but their abundance in this case is likely due to the attraction of these flies to the scent of rotting and decaying plant materials where females oviposit (Picker *et al.* 2002). Other flower visitors observed (i.e. leaf beetles (Phalacridae), ants, small unidentified wasp and spiders) were not considered suitable pollinators.

Similar to findings in other species of *Ceropegia* (see Ollerton *et al.* 2009), I found that the majority of pollinarium bearing flies were relatively large female flies that bore pollinaria exclusively on the proboscis. Female flies of all of the major pollinating fly families of *C. ampliata* are known to visit flowers, either to deposit eggs or to obtain nectar resources required for egg production, sexual maturation or physiological maintenance (Larson *et al.* 2001). Individual female flies are most likely lured to visit *C. ampliata* through the pungent scent that this species produces and feed on nectar contained in small nectar cups at the base of the gynostegium while imprisoned. No eggs were found deposited in the flowers as is often the case in brood site mimics (Meve and Liede, 1994), however due to the small size of these eggs it is possible that these were overlooked. None of the fly families reported here have been previously documented as common pollinators of other *Ceropegia* species. For instances in an analysis of 59 of the approximately 180 species of *Ceropegia*, Ollerton *et al.* (2009) found only one species to be pollinated by Tachinids and none were pollinated by Lauxaniids, Muscids, or Sarcophagids. Similarly Masinde (2004) did not find any of these families as pollinators of several Eastern African species of *Ceropegia*. Heiduk *et al.* (2010) reported Sciaridae as visitors to the flowers of *Ceropegia dolichophylla* in China. The studies by Ollerton *et al.* (2009) and Masinde (2004) found similar fly families as pollinators (Ceratopogonidae, Milichiidae and Chloropidae) none of which were found to visit *C. ampliata* in the current study, although the families Ceratopogonidae, Milichiidae and Chloropidae naturally occur in southern Africa (Scholtz and Holm, 1985).

The flowers of *C. ampliata* are in the trapping phase for between two and five days, a relatively long period when compared to some other species of trapping flowers. For instance some *Aristolochia* species only trap pollinators for one day after which they are released (Burgess *et al.* 2004). Trapping times of other *Ceropegia* species have been poorly documented, owing to the lack of natural history studies, but Muller (1926; cited in Proctor *et al.* 1996) reports that available evidence suggests that trapping times are variable and depend on the species. Species with relatively short trapping phase include *Ceropegia woodii* where flowers remain in the trapping phase for between one to two days (Muller 1926; cited in Proctor *et al.* 1996) and *Ceropegia dolichophylla* where flowers remain trapping for one day (Heiduk *et al.* 2010). Proctor *et al*. (1996) report that in a small subset of *Ceropegia* that were studied, the trapping phase lasts between one and four days.

I suspect that in part the longer trapping periods in some species may be a result of flowers waiting for longer periods of time as the arrival of pollinators is likely to be unpredictable in the arid environments of the study sites.

The permanency of entrapment in *Ceropegia* probably depends on what morphological features prevent flies from escaping. *Ceropegia* species in general prevent pollinators from escaping using a combination of the convoluted corolla tube and trapping hairs (Vogel, 1961). In *C. ampliata*, the corolla tube is slightly curved, and much wider than the width of most pollinating flies, and is therefore is an unlikely barrier to escape. The erect hairs lining the corolla tubes are however rigid and relatively dense and oriented slightly downwards, thereby providing resistance to escaping flies. Added to this the hairs may also optically disguise the exit hole as although these are relatively evenly distributed throughout the tube, they appear dense when viewed from below when the bulb is excised. The finer hairs lining the inside of the bulb do not function in trapping.

My data showed that flies may be trapped repeatedly by several different flowers which agrees with observations on other trapping flowers (Dafni, 1984). This was shown by pollinarium bearing flies frequently bearing more pollinaria on the proboscis than had been removed from the flower from which they were collected. Although we only recorded this data for a small sample (n = 9) of the flowers that contained pollinarium bearing flies, four of these flies bore more pollinaria than was removed from the flower. Flowers on the same plant are not produced synchronously thus both trapping and non-trapping phases are present simultaneously, increasing the possibility for self-pollination. Although pollinarium bending is thought to prevent self-pollination (Peter and Johnson, 2006) and does occur in the pollinaria of *C. ampliata*, it would be interesting to see whether this trend also holds in *C. ampliata* as the residency time of flies within a flower is likely to be relatively long.

Trapping pollinators increases the time that pollinators spend inside flower which increases the likelihood of removing and depositing pollen (Dafni, 1984). In *Ceropegia* this may increase pollen export and receipt - flies that are restrained have more time to remove and deposit pollinaria. My data gives some support for this hypothesis and indicated that trapping may promote pollen export as the flowers that were older and already in the non-trapping phase had more pollinaria removed than flowers that were still in the trapping phase. Judging from the results of these experimental manipulations, trapping hairs do not function to increase pollen export as similar percentages of flowers with hairs present and absent had pollinaria removed. However, the age of flowers confounded these interpretations and to confirm this larger sample sizes are needed and this experiment would have to be repeated for multiple seasons to quantify any long term pollination benefits that trapping may confer. The absence of any effect of trapping hairs on pollen removal also suggests that trapping hairs may function to promote female success as pollen depositions were generally lower than removals in all estimates of seasonal pollination success.

This study documents pollination success for the first time in the genus *Ceropegia*. My data indicated that pollen removal, deposition and pollen transfer efficiency is generally very low and may frequently be zero, but may increase suddenly under conditions favourable to pollinators. Fruit were also rarely found on plants in the field, further suggesting that fruiting success is generally low. Fruit set in *Ceropegia* provides a reliable estimate of seasonal reproductive success as *Ceropegia* differs from *Stapelia* in that this genus does not have delayed fruit maturation (Bruyns, 2005). Fruit set has also been found to be generally low and seasonally variable in other trap flower systems (e.g. *Aristolochia paucinervis*; Berjano *et al.* 2006; *Arum maculata*, Ollerton and Diaz, 1999). Estimates of pollination success in other fly-pollinated milkweeds include that by Herrera and Nassar (2009) where the authors found high rates of pollinarium removal and insertion in invading populations of the *Stapelia gigantea*, a sapromyophilous stapeliad in Venezuela. In this species the percentage of flowers with pollinaria removed was 60% and the percentage of flowers with pollinia deposited 35% which is comparable to the pollination success of *C. ampliata* at peak periods. Pollinarium removal, pollinia deposition and PTE in Hymenopteran-pollinated systems is higher and more consistent to that seen in *C. ampliata*. For instance the highest PTE that was recorded for *C. ampliata* was 13.1% whereas PTE for the honeybee pollinated *Cynanchum ellipticum* (Apocynaceae – Asclepiadoideae) regularly exceeded 40% (Chapter 4). PTE for the exotic honeybee pollinated *Aruajia sericifera* was also generally higher and the lowest PTE recorded for *A. sericifera* (12.8%) was close to the maximum of *C. ampliata* (Coombs and Peter, 2010). PTE for both these bee pollinated species was never zero. Estimates of pollination success in other non-sapromyophilous species pollinated by short-tongued Diptera include *Disa obtusa* (Orchidaceae) where 94% of flowers had pollinaria removed and 84% of flowers were pollinated (Johnson and Steiner, 1994). Van der Niet et *al.* (2010) also report high removal values in the orchid *Schizochilus angustifolius* where the percentage of flowers with removals approached 90% (89.7%) in at least one study population. The PTE for *S. angustifolius* was however quite low (1.4 – 3.7%). My data suggests that the pollination success of trapping flowers such as *C. ampliata* may be quite high but is seasonally unpredictable which owing to the patchy abundance of pollinators.

Ollerton and Diaz (1999) indicated that pollination success (measured as fruit set) in some populations of *Arum maculata* varied predictably throughout the season, peaking during peak flowering. Interestingly variation in pollination was not related to changes in fly abundance in this species (Ollerton and Diaz, 1999). This suggests that one reason why *C. ampliata* traps pollinators and why the trapping phase is relatively long is to make use of a variable and unpredictable pollinator fauna.

In conclusion, in this study I show that *C. ampliata* has generalized pollination system, being pollinated by flies from several different families which are rewarded with minute amounts of nectar. Pollination success in *C. ampliata* is generally low but may be high during certain times of the flowering season. I have not conclusively demonstrated the role that trapping hairs have on reproductive success of this species, although the trapping hairs had little influence on pollen export. Future studies should aim to carry out these experimental manipulations on larger samples of flowers over several seasons to test the hypothesis that trapping pollinators increases female reproductive success in this genus.

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Chapter 7

Specialized pollination biology, long term pollination success and population structure of a rare carrion fly-pollinated *Stapelia* (Apocynaceae - Asclepiadoideae)

Abstract

Flowers of the genus *Stapelia* (and related genera including *Orbea*, *Huernea* and *Caralluma*) typically attract carrion fly pollinators by producing foul, rancid odours, and being intricately coloured and textured to mimic the smell, colour and texture of decaying plant or animal material or animal excrement. Despite the presence of traits indicative of carrion fly pollination in *Stapelia*, little research has been conducted on the identity of pollinators and degree of specialization of different species and our knowledge of their pollination is largely constructed from observational notes from earlier workers scattered throughout the literature. Here I present the first multi-year study that documents the pollination biology of *Stapelia hirsuta* var. *baylissi* (Apocynaceae – Asclepiadoideae) a rare variety of *Stapelia hirsuta*. I describe aspects of the pollination syndrome such as what flies pollinate this species, flower colours and possible rewards and the degree of specialization of the pollination system. I also documented the pollination success in *Stapelia* by recording the average rate of pollen removal, pollen receipt and the pollen transfer efficiency of this species over a three year period. Due to its rarity, I documented the size class distribution of the study population as well as other indicators of reproductive success such as the levels of fruit set and fruit herbivory of *S. hirsuta* var. *baylissi*. The results indicated that *S. hirsuta* *var. baylissi* is specialized for pollination by only a few species of small anthomyiidae as pollinators. Pollinarium removal was higher than pollen deposition at all sampling dates. Pollen transfer efficiency was generally low and highly variable - an apparent feature of some fly-pollinated flowers. The demographic profile of *S. hirsuta* var. *baylissi* indicated that most individuals were single or multi-stemmed juveniles, with fewer adults and seedlings being present. The findings of this study suggest that despite low levels of pollination success and a specialized pollination system, this relatively isolated population of *S. hirsuta* var. *baylissi* is maintaining sufficient levels of pollination success and recruitment to maintain a viable population.

Introduction

Flies are common flower visitors and important pollinators (Larson *et al.,* 2001; Ssymank *et al.,* 2008). Despite their importance as pollinators, the extent to which flies specialize towards certain flowers is poorly known. It is assumed that most flies are generalist flower visitors (Faegri and van der Pijl, 1979; Ssymank *et al.,* 2008), however several examples exist of functional specialization (pollination by a group of insects with similar morphology and behaviour; *sensu* Fenster *et al.,* 2004) involving pollination by long proboscid flies belonging to the families Nemestrinidae (Johnson, 2006; Potgieter *et al.,* 2009; Goldblatt and Manning, 2000; Johnson and Steiner, 1997), Bombyllidae (Johnson and Dafni, 1998; Johnson and Midgley, 1997; Larson *et al.,* 2001) and Tabanidae (Johnson and Morita, 2006; Johnson and Steiner, 1997). Despite these well-known examples, there exists a wide diversity of short-tongued flies that are typically generalist pollinators of a wide range of flowers from several different plant families (Larson *et al.,* 2001; Proctor *et al.,* 1996).

Fly-pollinated flowers vary greatly both in the manner with which they attract flies and the actual mechanism of pollination (Larson *et al.,* 2001). Arguably the most conspicuous and well known of all fly-pollinated flowers are those pollinated by carrion flies which share floral traits such as rancid odours and other morphological features that serve to mimic putrefying plant or animal material or animal excreta (Faegri and van der Pijl, 1979; Proctor *et al.,* 1996; Meve and Liede, 1994). Sapromyiophily is particularly well represented within the Asclepiadoideae (Apocynaceae) where entire genera appear to use such deception (most notably *Ceropegia, Stapelia*, *Huernia* and *Caralluma*) by having flowers that display the visual, olfactory and tactile clues that resemble the food or brood sites of pollinating carrion flies (Meve and Liede, 1994). The petals of these flowers are often hairy and heavily ridged, mimicking the appearance of decaying plants or animals. Flower colours are typically dark hues of red, brown or purple or may consist of a lighter backround (*e.g.* yellow, white) dappled with dark spots or lines (e.g. *Stapelia verrucosa*; Meve and Liede, 1994; Meve *et al.,* 2004). The flowers of stapeliads emit rancid scents that mimic various dipteran breeding or feeding substrates (*e.g.* herbivore dung, urine, rotting carcasses; Jurgens *et al.,* 2006), although some do produce more sweet smelling scents and may be more broadly myiophilous (Bruyns, 2005).

Despite the diverse flower morphologies found in stapeliads (see Bruyns, 2005), few pollinators have been identified and most observations have relied on notes and observations gleaned from the literature (Meve and Liede, 1994; Ollerton and Liede, 1997).

There are few examples of specialized pollination relationships within Stapeliads however data by Jonkers (2010) suggest specialized pollination of *Desmidorchis impostor* by a single species of Carnidae (Diptera). Raspi *et al.,* (2009) also report specialized pollination of *Caralluma eoropaea* by a single species of *Milichiella lacteipennis*. Other aspects of the pollination biology of stapeliads remain almost entirely undocumented. It is surprising that we lack a basic understanding of the natural levels of pollination success (i.e. pollinarium removal, —deposition and pollen transfer efficiency) and fruit set in natural populations of *Stapelia*. Data on pollination success is fortunately easy to estimate in this genus as the pollen is presented as pollinia (aggregated, waxy pollen masses that attach to pollinator through a small mechanical clip known as a caudicle; Wyatt and Broyles, 1994).

Documenting the average levels of pollination success in *S. hirsuta* var. *baylissi* (L.C.Leach) Bruyns also provides information on pollinator interactions and pollination success in rare species. This species classifies as a 'classic rarity', which is characterised by it having a small geographic range and narrow habitat specificity but individuals occur in relatively dense populations (Rabinowitz, 1981). Given that plant reproductive success frequently depends on the size (Ward and Johnson, 2005; Brys *et al.,* 2004; Brys *et al.,* 2008, but see Chapter 2) or density of the population (Kunin, 1997), rare plant species are often expected to suffer from low levels of pollination success owing to their small population sizes (Spira, 2001). This suggests that similarly low levels of pollination may occur in *S. hirsuta* var. *baylissi*. Despite their low abundance, naturally rare species may compensate for low pollination services by setting fruit through automatic self-pollination (Kephart *et al.,* 1998; Dieringer, 1999; Neel, 2001) or clonal growth.

In addition to low pollination success, other ecological interactions such as herbivory may also reduce reproductive output in rare species (Brigham, 2003). Although there are few studies on fruit herbivory in rare plants, several studies have reported high rates of pre-dispersal seed predation in rare plants (Menges *et al.,* 1986; Hegazy and Eesa, 1999; Timmerman - Eskine and Boyd, 1999; Kery *et al.,* 2001; Robson, 2010), which is likely to have similar effects on recruitment as fruit herbivory. For instance, Hegazy and Eesa (1991) report that seed predation by beetles reduced seed set in the rare *Ebenus armitagei* (Leguminoseae) by 94.4%. I am not aware of any studies that have documented natural levels of fruit herbivory in different species of *Stapelia*. Based on field observations that suggested that a large proportion of the fruit of *S. hirsuta* var. *baylissi* were eaten by herbivores, I quantified the levels of fruit herbivory in this species to determine the likelihood that this could limit recruitment in this species.

This work was motivated by the near absence of comprehensive studies on the pollination biology of charismatic fly-pollinated genus *Stapelia.* I examine in detail the pollination biology and demography of a single *Stapelia* species, *Stapelia hirsuta* var. *baylissi* and ask the following questions: 1) what flies pollinate *S. hirsuta* var. *baylissi* and is the species a generalist or specialist; 2) what is the long term natural pollination success in terms of pollinarium removal, deposition, pollen transfer efficiency and fruit set of this species; 3) what is the demographic structure of the population of this rare variety and 4) what proportion of fruit are eaten by herbivores ?

Methods

Study species and distribution

Stapelia hirsuta L. is a widespread species with five recognized varieties (*S. hirsuta* L. var. *gariepensis* (Pillans) Bruyns, - var*. tsomoensis* (N.E.Br.) Bruyns, var. *hirsuta*, var. *vetula* (Masson) Bruyns and var. *baylissii* (L.C.Leach) Bruyns). Collectively the distribution of S. *hirsuta* extends from the south-western corner of Namibia and bordering parts of South Africa (var. *gariepensis*) to the former Transkei (var*. tsomoensis*; Bruyns, 2005). One of these varieties was originally named *Stapelia praetermissa* by Leach (1984). Bruyns' (2005) revision reclassified the species as a variety of *Stapelia hirsuta* and named it *Stapelia hirsuta* var. *baylissi*. To date this variety has only been described from four localities (Bruyns, 2005). The geographical distribution of *S. hirsuta* var*. baylissi* is confined to the Kariega River and its tributaries (Leach, 1984). Within this region the habitat consists of dry rocky outcrops located above small cliffs overlooking a river (Bruyns, 2005).

Stapelia hirsuta var. *baylissi* is a short stem succulent, branching at ground level. Flowering starts in February and ends in late April. This variety is rare and was placed by Victor and Dold (2003) in the category of vulnerable (VU D2) on the IUCN red listed plant species. The study population occurred on a farm south of Grahamstown (33^{o} 18' 20"S, 26^{o} 31' 28" E) and was relatively dense with approximately 100 flowering individuals occurring in a total area of approximately 50 X 50m. The upper boundary of the population started from where the first individuals were found at a distance of approximately 40m from the edge of the cliff. From this point plants were common right up to the cliff edge. Several individuals were also found growing on the cliff face. Due to the rarity of this variety the exact location of this study population will be withheld.

Pollinator behaviour and pollinarium loads

Observations for pollinators were carried out on 20 days over a period of two years (2007 = 14 days, 2008 = 6 days). Observation periods were not fixed and varied from 20 minutes to 4 ½ hours. Observation periods occurred throughout the day, starting as early as 9:00 in morning sessions and ending as late as 18:00 for afternoon periods. In addition to flies seen visiting the flowers of *Stapelia hirsuta* var. *baylissi,* I also caught flies that were commonly seen perching on the rocks around plants. Observations were also made of the behaviour of flies when visiting flowers. In addition to catching pollinators with insect nets, I caught flies using baited fly traps (Red Top™) that were suspended from metal holding rods, which were stationed at different positions within the population. I set two traps in 2007 and three in 2008 and baited these with cow pats.

Flower colours and reward

I measured the colour spectra of five flowers each from a different plant. For each flower I removed one petal and measured the colour spectra at three places: the tip, middle and at the base near the gynostegium. This was done using an Ocean Optics USB 2000 photospectrometer (Ocean Optics, Dunedin Florida, see Peter and Johnson 2008 for details).

I inspected several flowers for the presence of nectar droplets in the nectar cavity which is below the anther wings.

Pollinaria removal, deposition and pollen transfer efficiency

Flowers were collected on two dates during 2007, four dates during 2008 and three dates during 2009. Sampling dates were spaced approximately two weeks apart. On every sampling date, I selected between 15 and 26 plants and picked one flower randomly per plant. For each flower the number of pollinaria that was removed and deposited was counted and used to calculate the pollen transfer efficiency (PTE), the fraction of removed pollinaria that reach conspecific stigmas (Johnson *et al.,* 2005). Pollen transfer efficiency (PTE) is calculated by dividing the average number of deposited pollinia by twice the number of removed pollinaria as there are two pollinia per pollinarium.

Flower production and fruit set

Flower production and fruit set was measured from a sample of between 39 - 45 flowering plants marked during 2007 and 2008. Plants were marked with numbered aluminium tags and during 2007, I counted the number of stems, and the number of flowers and buds per plant on all inflorescences per plant. The number of fruit that each plant produced was counted for two years (2007, 2008) in July following the completion of flowering, giving sufficient time for the fruit to mature. The loss of some tags (via burial or becoming detached from plants) meant that I had to mark additional plants during 2008. The majority (29) of individuals were used in both years. I investigated the relationship between plant size and reproductive output (number of flowers and buds and number of fruit) using simple nonparametric univariate correlation analysis. This was done by first doing a correlation analysis with stem number as a predictor of the number of flowers and buds (“flowers + buds”) and then using flowers and buds as a predictor of the number of fruit per plant.

Demography

The demographic characteristics of the population were measured in 2007 and 2009. In both years I established three parallel line-transects, starting from near the top edge of the population, extending downhill through the population to the edge of the cliff, that delimited the end of the transect. Transects were each one meter wide with adjacent transects spaced two metres apart. Individual transects ranged from 8.5 to 20m. During 2007, I recorded the following parameters for up to five randomly chosen stems per individual encountered along the transect: height (measured as the vertical height from the base of the stem), width (narrowest width between two parallel sides of the stems that are roughly square in cross section), number of stems per individual, flowers present or absent, and for single stemmed individuals the number of leaf rudiments (*sensu* Bruyns, 2005) on the stem. These data was used to define individuals into 4 stage classes similar to what is required to construct transition matrices (see Caswell, 2001):

1) Seedlings (S) - Following Bruyns (1995; 2005), the seedlings of *Stapelia species* are characterised by a single small stem (hypocotyl) and several relatively minute primary and secondary leaves that are bunched together and emerge from the growing tip. A single pair of cotyledons are positioned on either side of this growing tip and become reduced leaf

rudiments as the seedling grows. I therefore classified seedlings as plants with only one pair of leaf rudiments and a tip of primary and secondary leaves.

2) Single stemmed juveniles (SSJ) - stems are thicker and bear more leaf rudiments than the previous class, non-flowering.

3) Multi-stemmed juveniles (MSJ) - multi-stemmed, no evidence of current or previous flowering.

4) Flowering adults (FA) - represented by individuals that were either flowering or showing signs of previous flowering.

I repeated the demography during 2009 and overlapped transects as closely as possible to those used during 2007. I then counted the number of individuals in each stage class.

Fruit herbivory

While quantifying fruit set I noticed that a number of fruit had apparently been consumed by small mammals. The number of fruit per plant was scored on three dates in 2007. These were 25 June, 27 July 2007, and finally on the 10 October 2007. Fruit herbivory was again scored on two dates during 2008 with fruit set initially counted on the 29 June 2008 and recounted on 16 August 2008. Similar to the fruit of other stapeliads (Bruyns, 2005) the fruit of *S. hirsuta* var. *baylissi* take a long time to mature therefore I allowed at least 3-4 weeks before scoring fruit set. To identify the herbivore responsible for eating the fruit I set hair traps (*e.g.* Castro-Arellano *et al.,* 2008) following the end of flowering in 2008. These consisted of placing 2-3 wood sticks (*ca.* 30mm wide and 600mm high) near 5 fruit bearing plants in such a position so as an animal needs to brush against these sticks in order to access fruit. Sticks were covered with a thin coating of Plantex^{Tm} (tacky substance used to capture insects) in order to collect hair.

Results

Pollinator behaviour and pollinarium loads

I spent a total of 47 hours observing and catching pollinators of *Stapelia hirsuta* var*. baylissi*. During this time seven individuals of an *Anthomyia* species (5 netted, 2 trapped) were caught. All of these flies were apparently the same species (Fig 1, C&F). Six of these bore pollinaria. In addition I observed, but failed to catch a further 4 pollinarium bearing individuals of what is assumed to be the same species of *Anthomyia*. This was in sharp contrast to a total of 55 other flies belonging to 6 families of which only four Sarcophagids bore pollinaria (Table 1). One sarcophagid was seen (but not caught) removing a pollinarium (Table 1). The proportion of sarcophagids that bore pollinaria was significantly less than the proportion of Anthomyiidae bearing pollinaria (0.11 vs. 0.86, proportions based t-test, t_{41}= -4.30; $p < 0.0001$). Anthomyiidae were also the only flies that bore ½ pollinaria and corpusculae, indicating that these flies are likely to deposit more pollinia than sarcophagids.

Sarcophagids were very common in the area and are likely secondarily important as pollinators. Observations of 13 visits from large sarcophagids indicated that these flies would initially land on a rock or vegetation near the plant before finding flowers through short flights. Sarcophagids would often sit for several minutes around the flower before visiting a flower. These visits were not timed, but they were typically short duration visits, being less than about 10 seconds. Only on one occasion was a sarcophagid observed removing a pollinarium. All four of the pollinarium bearing sarcophagids carried a single full pollinarium.

Table 1: Summary of the different fly families that visited or were caught nearby *S. hirsuta* var. *baylissi*.

Family	Number of individuals caught.	No of individuals bearing pollinaria.	Average no. whole pollinaria (±1 SE).	Average no. 1/2 pollinaria (±1 SE).	Average no. corpusculae (±1 SE).
Anthomyiidae	7	6	1.14 ± 0.26	0.14 ± 0.14	0.14 ± 0.14
Sarcophagidae	36	4	0.11 ± 0.053	0	0
Muscidae	11	0	0	0	0
Calliphoridae	1	0	0	0	0
Lauxanidae	1	0	0	0	0
Rhinophoridae	3	0	0	0	0
Tachinidae	3	0	0	0	0

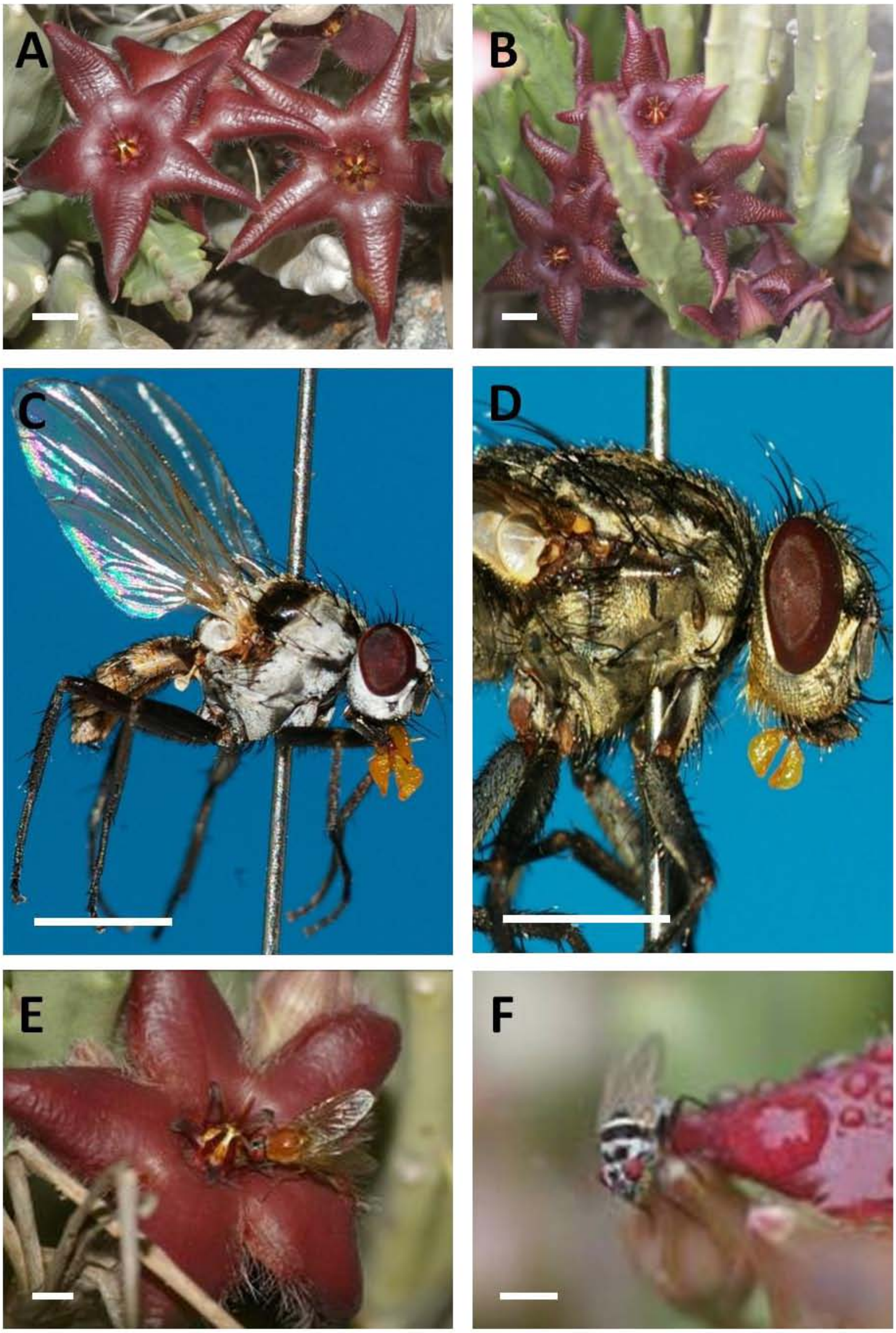

Figure 1: Flowering individuals of *Stapelia hirsuta* var. *baylissi* at a population south of Grahamstown (A&B). Flowers are coloured deep red and are produced on small pedicels (See Bruyns, 2005) that grow directly from the stem (A&B). Flies from several different families were seen visiting the flowers of *Stapelia hirsuta* var. *baylissi* (C-F). These include Anthomyiidae (C&F), Sarcophagidae (D) and Muscidae (E). Only anthomyiids (C) and sarcophagids (D) bore pollinaria. Scale bars: A & B =10mm, C, D & F = 2mm, F = 5mm.

On two separate occasions I observed *Anthomyia* species either removing or depositing pollinaria. On 14 March 2007 a fly was observed removing a pollinarium when it was suddenly trapped when the proboscis became wedged within the corpusculum as the fly probed at the gynostegium. The fly then vigorously pulled at the pollinarium until the pollinarium detached. After flying off and landing on one of the stems of the plant, the fly repeatedly rubbed its forelegs over the pollinarium apparently trying to remove the pollinarium. On 22 April 2007 I also observed an anthomyiid deposit a pollinarium. The fly was initially perched on a grass blade near the plant, after which it visited the flower. The fly moved in short-jerky motions on the petals as it approached the gynostegium. While probing at the base of the gynostegium the fly became trapped in a similar fashion to that described above. However in this case I timed the duration of the visit, which exceeded 10 minutes, after which I tried to capture the fly but it escaped. Inspection of the flower revealed that a whole pollinarium had become detached from the fly's proboscis, but only one pollinium was deposited in the stigmatic groove.

Flower colours and reward

All three parts of the flower showed a constant low reflectance of less than 2.5% between 300-600nm followed by a small but sharp increase between 600 - 700nm up to *ca.* 20% reflectance. At least one individual in the population had yellow/white coloured ridges on the petals, but this appears to be an uncommon floral phenotype in this species. There was no UV reflectance and the human interpretation of the colour is accurate.

Despite regularly inspecting the flowers during pollinator observation sessions I never saw nectar droplets accumulate near the base of the gynostegium, suggesting that *S. hirsuta var. baylissi* either produces no nectar or produces quantities so minute as to be unnoticeable during inspections. In other species of *Stapelia*, nectar has been reported to be hidden in a “nectar cavity”, located at the base of the guide rails (Bruyns, 2005). While trying to determine the breeding system of this species a small amount of fluid was seen at the base of the anther wings, however the volume of this fluid was too small to analyze.

Pollinaria removal, deposition and pollen transfer efficiency

When data from all sampling dates were grouped together, the average PTE was 3% for the population over a period of three years. The percentage of flowers with at least one pollinarium removed ranged from 12 to 61% on different sampling dates. The percentage of pollinated flowers (flowers with at least one pollinium deposited) was substantially lower and ranged from 0 - 17%. Pollinarium deposition was zero for six out of nine of the sampling dates. Pollen transfer efficiency tracked pollen deposition and was similarly low and very variable. Pollen transfer efficiency was zero for six of the nine sampling dates but increased to 7.1% and 8% on 8 April 2007 and 31 March 2008 respectively (Fig. 2). Flies almost always break the caudicle of the pollinarium and all depositions consisted of only a single pollinium wedged in the alar fissure, except for the instance described above where a fly was observed depositing a whole pollinarium (see Pollinator behaviour and pollinarium loads above).

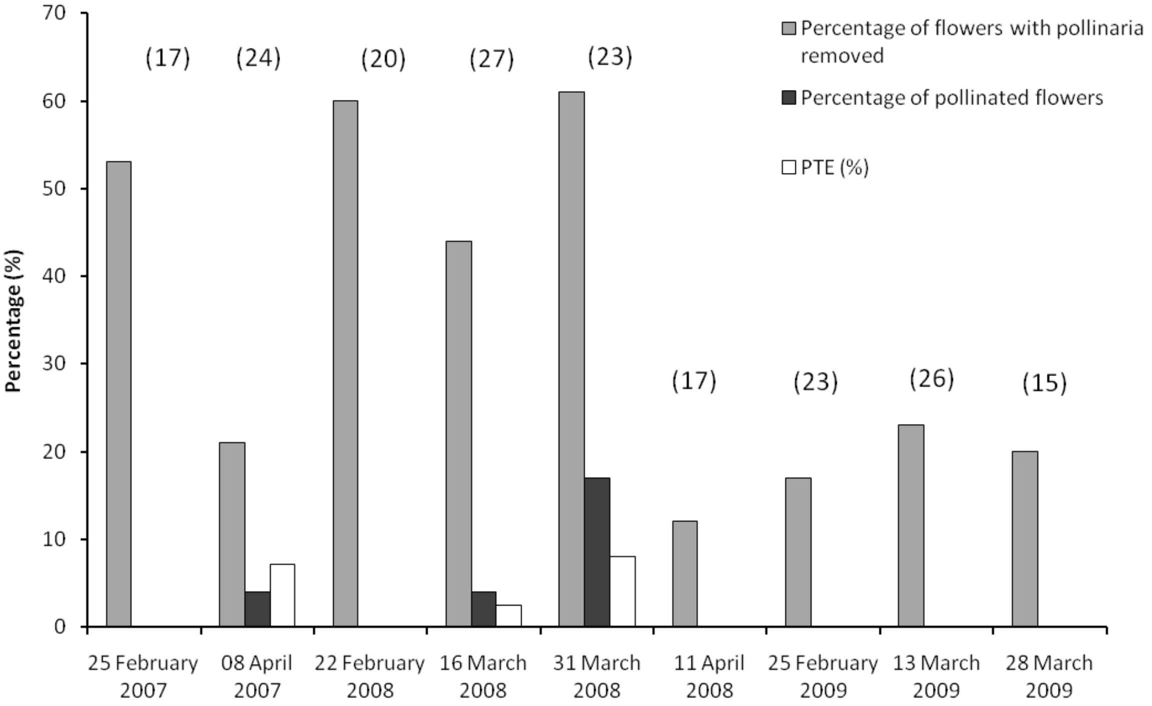

Figure 2: Pollination success measured in a population of *Stapelia hirsuta* var. *baylissi* measured over a three year period. The proportion of flowers with pollinaria removed always exceeded that of flowers with pollinia deposited. Pollen transfer efficiency was generally low and was zero for most of the dates (numbers in parenthesis indicate sample size).

Flowering phenology and fruit set

Fruit set per plant ranged from zero to five during 2007 and 2008. During both years, about half of the flowering individuals produced no fruit (49% (n = 25) in 2007 and 51% (n = 24) in 2008; Fig. 3). A slightly smaller percentage of individuals produced one fruit (42% in 2007, n = 18 and 44% (n = 19) in 2008). A few relatively large plants (n = 4, 2007; n = 2, 2008) produced more than one fruit with a maximum of five fruit being borne by the same large individual during both years. Of the 29 marked plants that could be found in both years 7 individuals (24%) set fruit in both years. During 2007 fruit set was significantly correlated to the number of flowers and buds produced by plants in that year (Fig. 4; Spearman's rank correlation coefficient, $r_s = 0.61$, $p < 0.05$, $n = 39$). The number of flowers and buds produced was also significantly correlated to the number of stems (Fig. Spearman's rank correlation coefficient, $r_s = 0.61$, $p < 0.05$, $n = 39$).

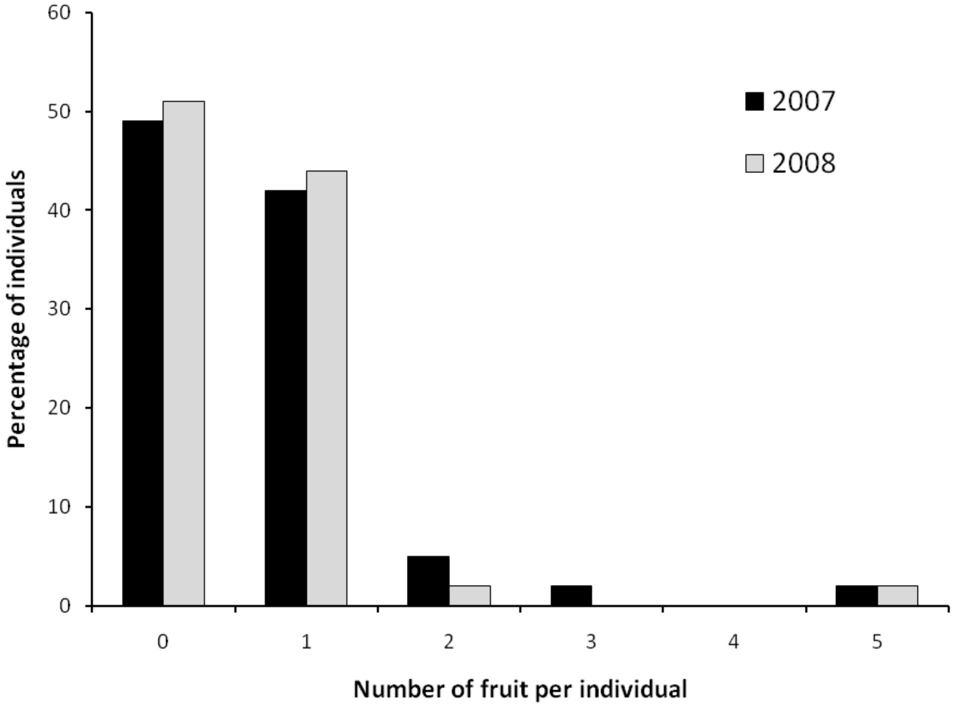

Figure 3: Frequency histogram of the number of individuals that bore one or more fruit measured over two flowering seasons. Most plant bore no fruit but slightly less than half of the individuals produced at least one fruit.

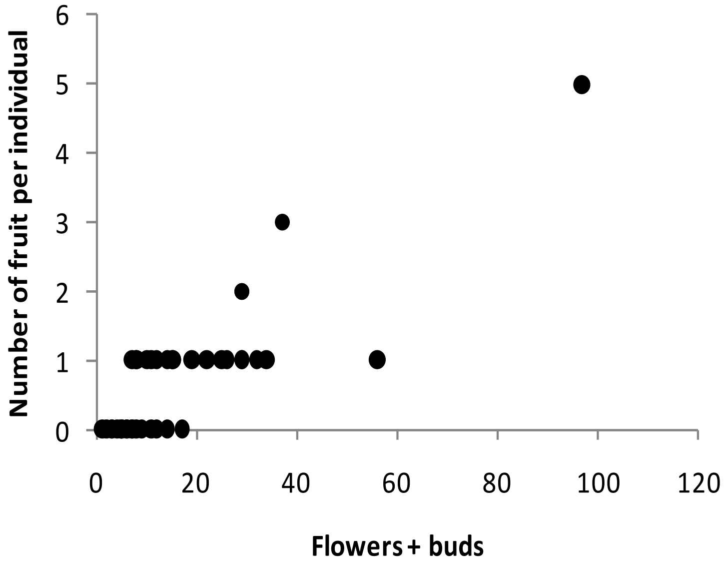

Figure 4: The number of fruit produced per individual was positively correlated with the number of flowers and buds produced per plant.

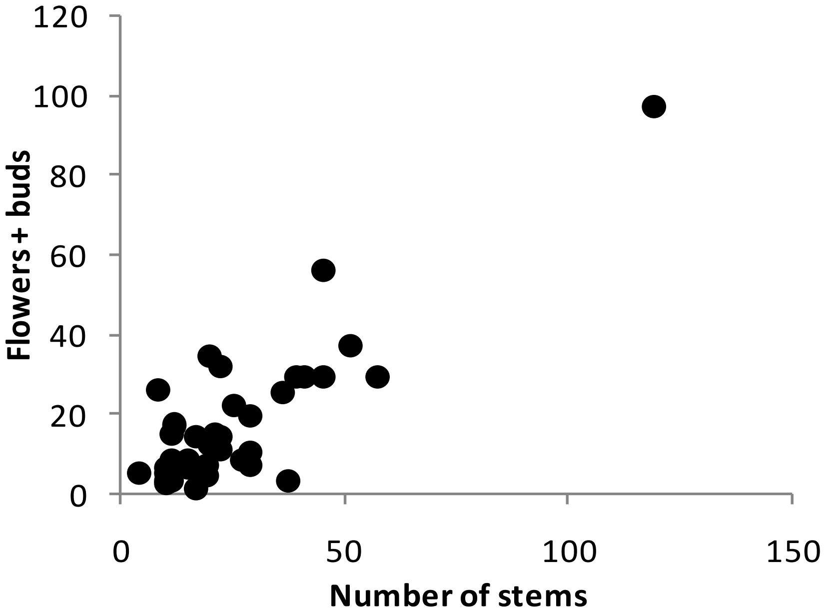

Figure 5: The number of flowers and buds per plant varied positively with plant size.

Demography

In total I counted and measured 171 individuals during 2007 and 127 individuals during 2009. In both years the majority of individuals were either single - or multi-stemmed juveniles. In 2007, 40% (n = 68) were single stemmed juveniles and 39% (n = 66) were multi-stemmed juveniles (39%, n = 66) (Fig. 6). In 2007 reproductive adults made up 17% (n = 29) of the individuals measured while only 5% (n = 8) were seedlings. During 2009, the demographic profile of the population was similar to that seen in 2007 with the exception that no seedlings were found during this year. Single-stemmed juveniles comprised 45% (n = 57) and multi-stemmed juveniles were 35% (n = 44) of the population. No seedlings were found during 2009 but the percentage and number of reproductive adults (20%, n = 26) was very similar to that found in 2007.

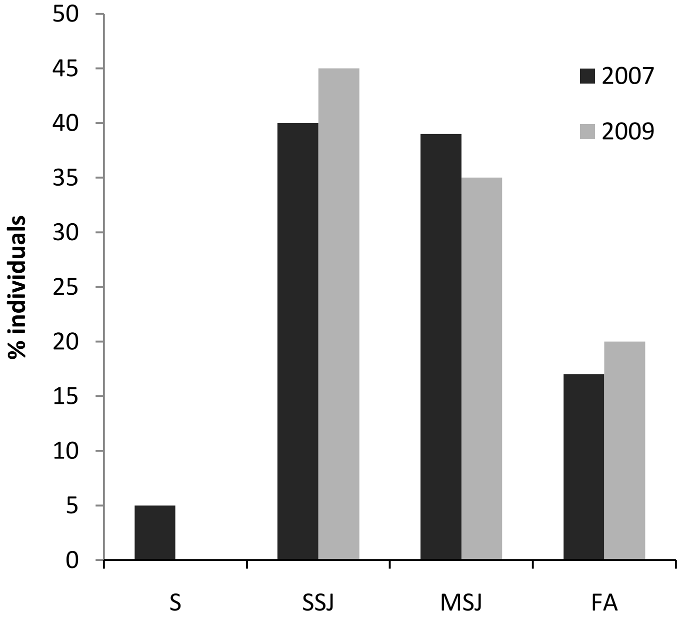

Figure 6: Frequency histogram of the stage-class distribution of *Stapelia hirsuta* var. *baylissi* measured for two years at a population near Grahamstown. Most individuals were either single-stemmed juveniles or multi-stemmed juveniles, with relatively fewer flowering adults.

Fruit herbivory

Fruit herbivory was high in both years. A total of 30 fruits were present on marked plants in 2007 and 17 were either completely or partially eaten. Similarly during 2008, 17 of the 25 fruit that were present at the first counting showed signs of herbivory. Thus 55% of fruit were eaten during 2007 and 66 % in 2008. This figure is likely to be an underestimate of fruit herbivory due to more fruit potentially being consumed subsequent to sampling dates. I only managed to collect a small sample (< 10 strands) of hair from one of the hair traps where the fruit had obviously been eaten. Conclusive identification could not be made using hair prints. The hair was however visually very similar to hair collected from rock hyrax (*Procavia capensis*) from a small colony near Rhodes University. Given the habit of *S. hirsuta* var. *baylissi* of growing on cliffs and the appearance of tooth scars on the fruit and stems it is most likely rock hyrax that consume the fruit and gnaw at the stems. Rock hyraxes were also frequently seen on the cliffs in the immediate vicinity of the population.

Discussion

Degree of pollinator specialization in Stapelia hirsuta var. baylissi

My results indicate that *Stapelia hirsuta* var. *baylissi* is specialised for pollination by a small anthomyiid flies (probably a single species) with a species of Sarcophagidae being secondarily important. Other flies that visited the flowers of this species included Muscidae, Calliphoridae, Luaxaniidae and Rhinophoridae and Tachinidae. The data therefore suggests that *S. hirsuta* var. *baylissi* is specialised to pollination by anthomyiids as the presence of ½ pollinaria and corpusculae further indicates that these flies are also likely to deposit pollinia.

None of the sarcophagids that bore pollinaria bore any ½ pollinaria or corpusculae suggesting that sarcophagids are frequent visitors but relatively ineffectual pollinators. Other varietes of *Stapelia hirsuta* are reportedly visited by sarcophagids and calliphorids (Meve and Liede, 1994). Sarcophagidae, Muscidae and Calliphoridae are also common flower visitors of other species of *Stapelia* (*Stapelia pillansii*, *S. flavirostris*, *S. gigantea*, *S. grandiflora* and *S. gariepensis*; Meve and Liede, 1994; Ollerton *et al.* 2010) and other sapromyphilous asclepiad genera such as *Orbea*, *Pachycymbium* and *Piaranthus* (Meve and Liede, 1994). Although these large sarcophagids were regularly seen visiting the flowers of *S. hirsuta* var. *baylissi* the proportion of individuals carrying pollinaria was significantly less than the proportion of pollinarium bearing anthomyiids.

The degree of specialization in sapromyiophilous systems is variable. Some brood site mimics may be specialized to one or a few species of pollinator (e.g. *Aristolochia*, Rulik *et al.,* 2008, Sakai, 2002), while others may have more generalized pollination systems (e.g *Ceropegia ampliata*, Chapter 6; Ollerton *et al.,* 2009). Due to a lack of thorough natural history studies, the degree of specialization in different species of *Stapelia* is uncertain and most of our knowledge of the diversity of pollinators in *Stapelia* is constructed entirely from incidental observations and field notes (Meve and Liede, 1994). The available data, poor as it may be, suggests that most species for which pollinator information is available, have relatively generalized pollination systems (Meve and Liede, 1994; Bruyns, 2000), at least at the level of species (*cf*. Johnson and Steiner, 2000). There are however some examples of stapeliads that appear to have specialized pollinators. For instance Jonkers (2010) found that *Desmidorchis impostor* was mainly visited (and presumably pollinated) by a single species of Carnidae, *Meoneura nitidiuscula*. There is some evidence to suggest that there may be a degree of functional specialization in stapeliads (Jurgens *et al.,* 2006), as flowers may be grouped according to differences in scent chemistry (Jurgens *et al.,* 2006) and morphologies (Bruyns, 2000) which could correspond to pollination by certain groups of flies that share similar brood sites (*e.g.* fungus gnats, Bruyns, 2000). Several authors have made observations of Calliphoridae, Sarcophagidae and Muscidae as visitors to the flowers of different *Stapelia* species (Meve and Liede, 1994; Herrera and Nassar, 2009), however specific information on pollinarium loads and the relative efficacy of different flies as pollinators remains unknown. This study is therefore the first to report such specialized fly pollination in a stapeliad, however understanding the basis of pollinator specifity of *S. hirsuta var. baylissi* requires investigating other features such as scent chemistry and combining these observations with those made in other populations.

To my knowledge, highly specialized pollination by anthomyiids has only been reported in *Trollius europaeas* (Ranunculaceae) where the flowers are exclusively pollinated by four species of *Chiastocheta* that pollinate the flowers while mating and laying eggs (Pellmyr, 1989). Specialization to anthomyiids has not been reported for any other South African plants, although anthomyiids are well known flowers visitors to generalised fly-pollinated flowers (*e.g.* Elberling and Olesen, 1999; Scobie and Wilcock, 2009; Strakosh and Ferguson, 2005). Little is known about the degree of specialization in the Anthomyiidae, although most are assumed to be generalist pollinators, similar to other short-tongued flies (Larson *et al.,* 2001; Proctor *et al.,* 1996, but see Elberling and Oleson, 1999). All the studies that have documented pollination by Anthomyiidae suggest that they are mostly attracted to myiophilic flowers with sweet scents (Proctor *et al.,* 1996). I am not aware of other reports of anthomyiids pollinating carrion fly-pollinated plants including asclepiads and none are mentioned in the reviews by Meve and Liede (1994) and Ollerton and Liede (1997) or have been catalogued in the ASCLEPOL database by Ollerton (2010).

It is suspected that the pollination of *S. hirsuta* var. *baylissi* may also be linked to the brood site of its pollinators. Anthomyiids appear to be highly attracted to the scent of fresh herbivore dung and on several occasions while re-baiting traps these anthomyiids would rapidly appear and visit the fly traps. Many anthomyiids with similar coloration (i.e. grey backround with black bands across thorax) were collected from rock hyrax (*Procavia capensis*) middens at a small colony near Rhodes University. There were also very few sarcophagids present at these middens. Many rock hyrax were present on the cliffs near this population and it is possible that these flies use these middens as breeding grounds. I plan to test the hypothesis that *S. hirsuta* var. *baylissi* mimics the brood site of it pollinators by examining the scent compounds emitted by the flowers and comparing these to the odours emitted by rock hyrax middens.

Pollination success, fruit set, and fruit herbivory

Pollinarium removal was generally higher than pollinarium deposition at all sampling dates, and varied stochastically along with pollen transfer efficiency. These measures of reproductive success showed no definite pattern of increasing or decreasing throughout the season. The high ratio of pollinarium removal versus deposition at all sampling dates confirms the general trend of marginal returns of exported pollinia in asclepiads (Ollerton *et al.,* 2003). However pollination success in *S. hirsuta* var. *baylissi* is generally lower than has been found in other pollinarium bearing species pollinated by small flies. For instance, the pollination efficiency in other fly-pollinated species such as *Disa obtusa* by *Bibio turnerri* was higher and Johnson and Steiner (1994) report a pollination success of 84% of flowers and nearly all (94%) had one or more pollinaria removed. The percentage of flowers with pollinaria removed in *Habenaria obtusata* was also higher and varied between 31.4% and 44.8% (Thien and Utech, 1970). The lack of long term studies on the pollination success of other sapromyiophilous taxa prevents a comparison between these findings and others, however higher rates of pollinarium removal and deposition were measured in invasive populations of *Stapelia gigantea* in Venezuela, where 60% of plants had pollinaria removed while 35% of flowers were pollinated (Herrera and Nassar, 2009). The low pollen transfer efficiency of *S. hirsuta* var. *baylissi* is probably not a consequence of its rarity and population size as the many flowering individuals in the study population make up a sizeable display albeit relatively cryptic to human observers. It is surprising that the pollination efficiency of sapromyiophilous stapeliads have received so little attention particularly, considering how well known the group is in the pollination literature and the relative ease with which pollination success may be quantified in the asclepiads (Ollerton and Liede, 1997; Wyatt and Broyles, 1994).

The highly variable pollen removal and deposition in *S. hirsuta* var. *baylissi* translated into low fruit set per individual. Most individuals produced either no fruit or only one fruit. As a result, it seems likely that most plants are pollen limited, but given that the life history of this species coupled with the dry environments (i.e. dry, sandy soils) in which stapeliads are usually found (Bruyns, 2005), resources such as water could limit seed set as has been found in other plants (Lee and Bazzaz, 1982). The extent to which this species is pollen limited is not known, however carrying out pollen limitation studies (e.g. Ashmann *et al.*, 2004) on plants in wild populations would be practically difficult due to the small pollinaria that usually require pollinia to be inserted into the stigmatic cleft while viewing flowers under a stereo microscope (Chapter 2). In addition to the above limits to fruit production, over 60% of the fruit produced were eaten by herbivores, which could limit seedling recruitment. However the demographic profile of this species shows large numbers of juveniles present, suggesting that the ultimate impact of fruit herbivory is likely to be marginal. Alternatively, seedling establishment is relatively unpredictable and dependent on years when good rainfall and low levels of herbivory coincide.

Levels of fruit and seed predation in rare species vary, with some species having high rates of pre-dispersal seed predation that could negatively influence recruitment (e.g. Hegazy and Eesa, 1999; Menges *et al.,* 1986; Kery *et al.,* 2001; Timmerman - Eskine and Boyd, 1999; Robson, 2010) however to determine whether rates of fruit predation influence the population viability of *S. hirsuta* var. *baylissi*, the influence of herbivory on actual seedling establishment and subsequent population growth would need to be determined (e.g. Louda, 1982).

Rock hyraxes are common herbivores in the study area and their foraging areas are nucleated around cliffs (Fourie, 1983). The diet of these small herbivores varies seasonally and the predation of the fruits of *S. hirsuta* var. *baylissi* occurs from July to August- a time when the quality of preferred food sources (e.g. grasses, small shrubs and trees) is reduced (Fourie, 1983). Rock hyrax most likely locate the fruit of this species by scent as many of the fruit that were bagged with white nylon bags were eaten after the bag was purposefully torn off by the animals. Deep tooth scars were often seen on the stems of adult plants suggesting that rock hyraxes also feed on other parts of the plants. Interestingly the majority of flowers remained completely untouched despite their relatively large size and fleshy tissue. In light of the hypothesis that the scent of the flowers mimics the scent of rock hyrax middens, it will be interesting to determine if these animals find the flowers of *S. hirsuta* var. *baylissi* unappealing based on their scent (e.g. Lev - Yadun *et al.,* 2009).

Pollination success and rarity

Despite this species being a very narrow endemic, levels of pollination success were similar to that documented in other sapromyiophilous species with a wider distribution (e.g. *Ceropegia ampliata*, Chapter 6). Ideally, a phylogenetically controlled comparison should be done to compare the pollination success of *S. hirsuta* var. *baylissi* to other more common and widespread *Stapelia* species (e.g. Rymer *et al.,* 2004). Although rare species are considered likely to suffer from a lack of pollinator visits, there are several examples where rare species showed no signs of pollen limitation (Tepedino *et al.,* 1999; Hill *et al.,* 2008; Petanidou *et al.,* 1995). In the case of *S. hirsuta* var. *baylissi*, multiple observations of pollinators suggest that pollinators are visiting the population but visitation is likely to be sporadic. The relatively low pollination success is likely to reflect the patchy abundance of these pollinators and does not suggest a case of a chronic lack of pollinators resulting in pollination failure as has been found in some rare species (Steiner, 1993)

Demography and conservation

The demography of this species suggests that recruitment is taking place, but annual seedling establishment may be low (5% of individuals were seedlings) or is highly variable and dependent on suitable conditions for seedling establishment. The size-class profile and positive correlation between plant size, flowers and buds and the reproductive output of this species is in agreement with expectations of species that take a long period to reach sexual maturity, have low adult mortality and maintain relatively low annual reproduction (Franco and Silvertown, 1997). Given that all of the reproductive adults that were marked during the 2007 flowering season were still flowering during 2009, adults are likely to be long lived and annual adult mortality is probably low. This suggests that that the turnover of mature individuals is low and population growth in *Stapelia hirsuta* var. *baylissi* is likely to be slow. Unfortunately very little is known about the demography and population characteristics of other *Stapelia* species (but see Bruyns, 2005). It is therefore not possible to compare my data with any other species in this genus (including more common species). Although I did not quantify the exact number, a few reproductively mature individuals did not flower every year; these could easily be identified by the presence of flowering scars on the parent plant. A possible explanation for this could be that producing fruit in these species is physiologically depleting causing a reduction in fecundity in following flowering seasons (*e.g.* Crone *et al.,* 2009; Zimmerman and Aide, 1989). However, nearly a quarter of adult plants bore fruit on both years suggesting that such depletion is not severe.

Stapelia hirsuta var. *baylissi* is classified as vulnerable (VU D2) by Victor and Dold (2003). The study population is well conserved as it is located on private farmland. Observations on plants throughout the three field seasons indicate that plants frequently die if stepped on or knocked over (great care was taken to avoid this!). This species will therefore certainly suffer greatly under frequent trampling (e.g. Maschinski *et al.,* 1997) resulting from the activity of grazing or browsing live stock. White and Sloane (1933) made similar observations and cautioned that stapeliads in general are vulnerable to herbivory from live stock such as ostriches, sheep, cattle and goats. The rocky terrain habitat and the tendency of plants to grow on the edge of steep river valleys may serve to protect the species from herbivory by some livestock such as sheep and cattle, but not goats.

The succulence of this species indicates that it is unlikely to be fire adapted and land managers should not burn areas where populations occur. Fire will also remove the thick grass cover, that is important for the establishment of juveniles. Bruyns (2005) has made similar observations on the necessity of ‘nurse plants’ for seedling establishment for other stapeliads.

None of the abovementioned threats are of concern at the study location and the current land management strategy is conserving the species adequately. The demography of the species indicated that in both years the vast majority of individuals were immature, which suggest that despite the low pollination success and pervasive fruit pilfering, the species is recruiting successfully and is likely to persist unaided into the future.

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Chapter 8

Generalized fly-pollination and extensive inflorescence herbivory in *Chlorocyathus lobulata,* a rare endemic Periplocoid milkweed (Periplocoideae-Apocynaceae)

Abstract

Studies on the pollination biology of rare plants have shown that some rare species suffer from chronic pollen limitation, owing to a lack of pollinator visits. Thus natural history studies documenting the pollination biology of rare species can contribute to understanding whether reproduction in these species is low due to the break-down of a specialized pollinator mutualism or a result of some other aspect of their biology. There are relatively few studies documenting aspects of the pollination biology of periplocoids (Apocynaceae – Periplocoideae) to determine the degree of specialization in different species, and levels of pollination success. I studied the pollination biology and rates of flower herbivory in *Chlorocyathus lobulata*, a rare endemic milkweed that is restricted to a single site along the Kap Rver, Eastern Cape, South Africa. *Chlorocyathus lobulata* is fly-pollinated, with flies from the families Tachinidae, Diopsidae and Tephritidae bearing translators. Average levels of pollination success were relatively high with the percentage of flowers with translators removed being 54.35% and 44.83% in 2007 and 2008 respectively. The percentage of flowers with tetrads (pollen) deposited was 54.35% during 2007 and 51.72% during 2008. Pollen transfer efficiency was 8% in 2007 and 9% in 2008. The demographic profile of *C. lobulata* indicated that most individuals (87 of 111) were non-flowering juveniles, suggesting

that recruitment is taking place. Results from this study indicate that despite the rarity of *C. lobulata*, the species is reproducing successfully and its reproductive biology, at least over the two years of this study, does not explain the extreme rarity of this species.

Introduction

Natural history studies that document the ecological interactions of rare species are needed to identify which factors could potentially limit reproduction in these taxa (Schemske and Horvitz, 1994). Studies on the pollination biology of rare plants have revealed that some species suffer from chronic pollination failure (*e.g.* Steiner, 1993). Such pollination failure is considered more likely in plants that have highly specialized pollination mutualisms (Bond, 1994; Johnson and Steiner, 2000) as annual fluctuations in the abundance of certain pollinator species may be large and hence cause pollination success to fluctuate widely between seasons (Herrera, 1988). Plant species with more generalized pollination systems are expected to suffer less from seasonal fluctuations in specific species of pollinators (Waser *et al.,* 1996). One example of a species that suffers from chronic pollen limitation owing to a lack of pollinators include the work of Steiner (1993) and Steiner and Whitehead (1996) where the authors reported pollination failure in some populations of *Ixianthes retzioides* (Scrophulariaceae), caused by the absence of the specialist pollinator (oil collecting bees, *Rediviva gigas*) within these populations.

Studies documenting ecological interactions of rare plants have also revealed that herbivory may contribute to their rarity (see review by Brigham, 2003). For instance, extensive fruit and flower herbivory has been reported in rare plant species (Kery *et al.,* 2001; Johnson *et* *al.,* 2004a; Münzbergová, 2005). In some species, flower and seed herbivory have been shown to have greater impacts on endemic species relative to their more widespread congeners (Münzbergová, 2005; Lavergne *et al.,* 2005), suggesting that herbivory may restrict the distribution of these endemics through its influence on the number of seeds produced and subsequently the dispersal and recruitment abilities of these plants (Lavergne *et al.,* 2005). Natural history studies documenting these interactions are however relatively scarce (Brigham, 2003), but needed to determine the influence that such herbivores may have on the reproduction in these plants.

Few studies have documented the pollination biology of members of the Periplocoideae (Apocynaceae). Ollerton and Liede (1997) found that pollinators have only been recorded for eight of the approximately 180 species within the subfamily (Ollerton and Liede, 1997; Ollerton *et al.,* 2010). In this study, I documented the pollination biology and demography of *Chlorocyathus lobulata*, a rare endemic member of the Periplocoideae with an extremely localised distribution, being restricted to a single population along the Kap River in South Africa (Venter *et al.,* 2006). Specifically, I document aspects of the pollination biology of *C. lobulata* to determine whether the highly localized distribution of this species is caused by the collapse of a highly specialized pollination mutualism resulting in the absence of recruitment. To establish whether this species is recruiting successfully, I also document other population characteristics such as the size class distribution of this species and the extensive flower herbivory that was observed during this study and could limit recruitment in this species.

Methods

Study species

Chlorocyathus lobulata (Venter & R.L.Verh.) Venter (Apocynaceae - Periplocoideae) is a rare endemic with a highly restricted distribution (see Appendix 1B for description). The species is a perennial climber and has only been found growing within the forest on the eastern bank of the Kap River reserve, Eastern Cape, South Africa (33° 29' 00"S, 27° 04' 48"E; Venter *et al.,* 2006) despite extensive searchers by AP Dold in other similar sites.

Chlorocyathus lobulata is one of only two members of the genus *Chlorocyathus* (Venter, 2008). It was originally described as *Kappia lobulata* to acknowledge its restricted distribution. The flowers are produced in determinate umbels, each divided into one to six clusters bearing between one and five buds. Typically only one flower is open at a time (Venter *et al.,* 2006). Flowering starts during January and ends in late April. The flowers are coloured bright green and emit a pleasant fruity scent similar to watermelon.

Pollinators

Pollinators were caught using an insect net and with the use of fly traps. Two trapping methods were used to collect flies. The first trapping method consisted of suspending baited fly traps near flowering individuals of *C. lobulata*. Traps were baited with water melon and winter melon. These baits were used as they smell similar to that of the flowers of *C. lobulata* to humans. The second trapping method consisted of placing small green coloured sticky traps near the flowers of *C. lobulata*. These were made of (50mm X 50mm) rectangular green cardboard cards and were covered with a thin layer of Plantex^{Tm} (a highly tacky substance used to exclude ants from citrus trees). When catching pollinators with a hand net, I monitored several inflorescences on the same plant for the duration of the observation period. Any flies seen visiting the flowers as well as flies sitting in close proximity to flowers were caught and inspected for the presence of translators on their proboscides. Observation periods were typically three to four hours long and started from between 8:00 and 9:00 am. Pollinator observations were made for a total of approximately 23 hrs of observation time and were carried out over the three year study period (2007, 2008 and 2009).

Pollen removal, deposition and pollen transfer efficiency

The subfamily Periplocoideae is distinguished from other subfamilies within the family Apocynaceae (Asclepiadoideae and Secamonoideae) by not having pollinia (Verhoeven and Venter, 2001). Pollen of most periplocoids is presented as large pollen grains (tetrads) in a cup-like structure (translator) which is attached to the pollinators with a sticky pad (viscidium), similar to that of orchids (Johnson and Edwards, 2000). To quantify pollination success, I picked between one and five flowers per plant in 2007 (n = 14 plants) and 2008 (n = 13 plants) and counted the number of translators that were removed and the number of pollen tetrads that were deposited per flower in each sample. In order to quantify the amount of pollen tetrads that were removed, I calculated the average number of tetrads per translator from a subsample of 36 flowers in the samples of flowers collected for PTE during 2007. A single translator was removed from each flower and pressed flat on a microscope slide to force the pollen tetrads to spread out from the pollen translator. The average number of tetrads that were removed was determined by counting the number of translators removed per flower in each sample and multiplying this value by the average number of tetrads per translator (see above). Tetrad deposition was counted as all pollen tetrads present on the stigmatic surface after a pollinarium had been removed. In cases where the translator was still present, I only counted the number of pollen tetrads deposited if the pollinarium had been obviously disturbed and become unseated from its natural position in the gynostegium. Pollen transfer efficiency (i.e. the number of removed pollen grains deposited on stigmas, *sensu* Johnson *et al.,* 2005) was calculated by dividing the average number of tetrads deposited per flower by the average number of tetrads removed.

Flower colours and rewards

I measured the reflection spectra from 12 flowers picked from 10 plants. For each flower a single measurement was made in the centre of the petal. All measurements were done with an ocean Optics USB 2000 spectrophotometer (following Peter and Johnson, 2008). I plotted the spectral reflection function of flowers alongside the reflectance function measured from five leaves to determine the contrast between flowers and the typical background to which these are displayed.

Flower herbivory

The flowers of *C. lobulata* were heavily parasitized by caterpillars. In order to identify this moth, I collected several inflorescences showing signs of caterpillar infestation during 2007, and incubated these in small 250ml plastic jars sealed with gauze. In order to confirm whether predation in 2008 was by the same species, I set up a sampling protocol that consisted of picking between one and five infested umbels per plant from 13 plants in total and again incubated these in gauze-covered glass jars. Field observations were also made on the appearance of the caterpillars and what parts of the plants were mostly attacked.

During 2009, I conducted a more detailed investigation which consisted of marking between 10-20 inflorescences on six plants and counting the number of buds, parasitized buds, open flowers and closed flowers per inflorescence at five dates spaced between 7-10 days apart. Furthermore, I limited data analysis to those inflorescences where these were present for at least the first three sampling dates. Due to buds rapidly abscising once the flower bud has been parasitized, I counted the number of parasitized buds as those buds that had signs of herbivory and were still attached to the inflorescence. The total number of buds present on the inflorescence was counted as the number of infested and uninfested buds. This technique provides a more conservative estimate of the number of buds that are parasitized.

To estimate the percentage of inflorescences within the population that were infested with the larvae of *B. onychinalis*, I selected 12 plants that were accessible and on each plant selected up to five inflorescences per plant and counted the total number of inflorescences that were either infested or not.

Demography

The demographic profile of this species was constructed by establishing six transects (3 m x 30 m) that started from approximately three metres from the edge of the river and extended perpendicularly to the river into the forest. Along these transects, I counted the number of non-flowering juveniles and adults. In addition to this I walked a single, continuous 140m transect along a footpath running along the side of the river and identified all adults growing within 10m of the footpath. This data was combined with that from the previous transects to determine whether *C. lobulata* is restricted to growing in certain tree species. I also noted the tree species that these were growing in, and visually estimated the height of all trees.

Results

Pollinators

The low number of flies seen visiting this species suggests that fly visitation is sporadic and unpredictable. During the entire observation period, I only observed three visits by flies and two visits by butterflies to the flowers of *C. lobulata*. One fly visited the flower the flowers of *C. lobulata* by initially landing on the leaves of the plant and approaching the flowers with short search flights, before alighting on the petals and probing at the fleshy corolla lobes (Fig. 1A). Three flies were caught in sticky traps (2 Tachinidae and 1 Platypezidae), none of which bore translators. Flies caught in baited fly traps were 6 Muscidae, 11 Cecidomyiidae and 8 Drosophilidae. All other flies were caught by hand net. Other small unidentified insects that were caught included one ant, a small beetle and an unidentified moth. None of these bore translators and were not considered potential pollinators. Two of the flies that were collected bearing the pollinaria of *C. lobulata* were caught with insect nets while visiting the flowers of *C. lobulata*, while the other two flies were caught sitting on leaves near the flowers of *C. lobulata*. Pollinarium bearing flies belonged to the families Tachinidae (Fig. 1B & D; Table 1), Diopsidae (Fig. 1C) and Tephritidae. Other smaller species such as the

Cecidomyiidae and Drosophilidae are considered too small to remove the translators of *C. lobulata* and are likely attracted to scent of the baits traps as brood sites.

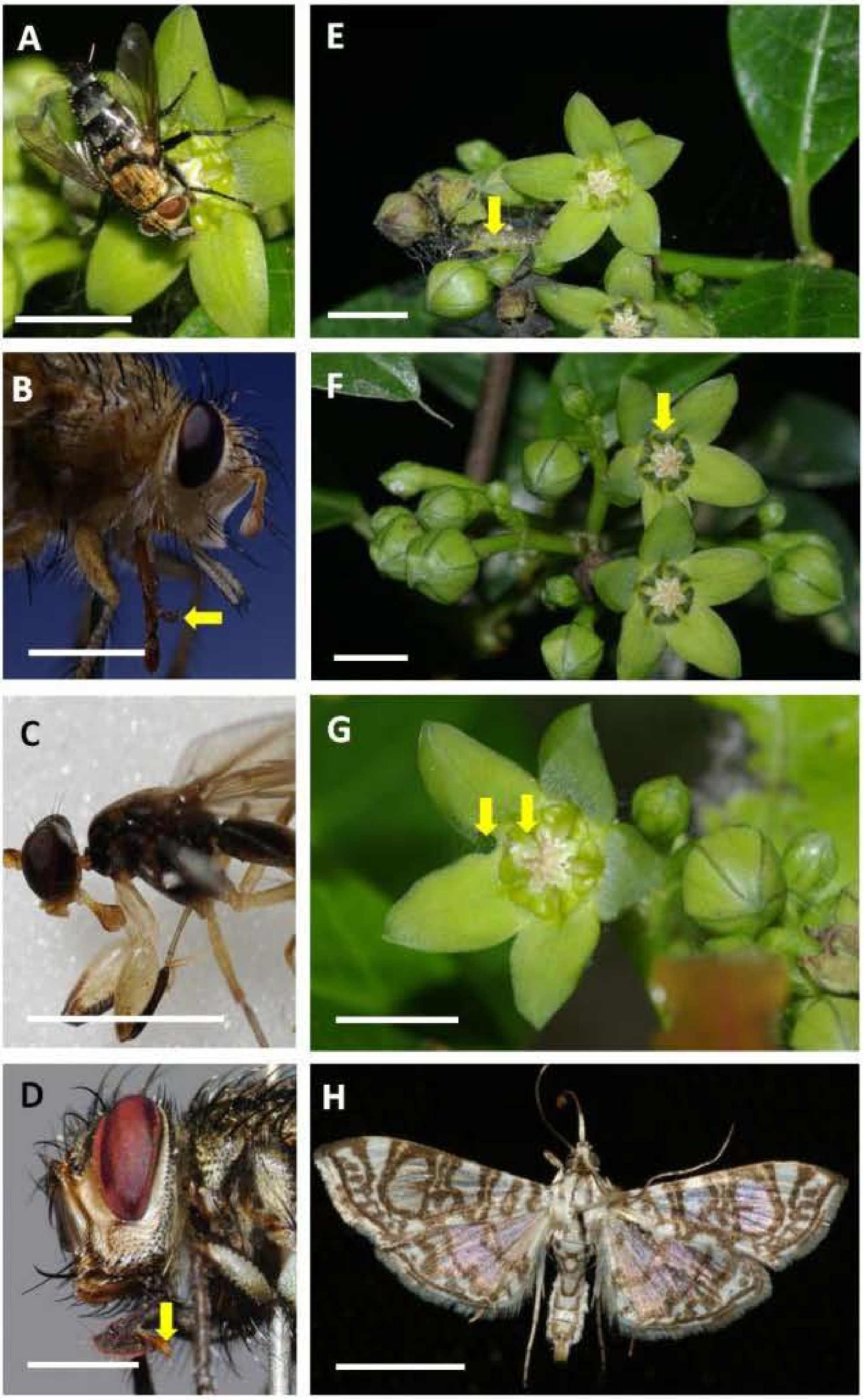

Figure 1: *Chlorocyathus lobulata* is pollinated by short-tongued flies (A). Translators of *C. lobulata* are attached to the pollinator when a fly probes into the cavities between the corolla lobes. Flies that bore translators include Tachinidae (B, D) and Diopsidae (C). The larvae of *Bocchoris onychynalis* feed on the flowers of *C. lobulata* (arrows; E, F, G). Signs of infestation are a black colour present on the corolla lobes (arrow, F). Adult of *B. onychynalis* (H). Arrows in images B and D point to translators. Scalebars: A, E - H = 5mm; B - D = 2mm.

Table 1: Flies collected either visiting or in the near vicinity of the flowers of the study species in Kap River forest using either fly traps or insect nets.

Family	Number of individuals	No of individuals bearing pollinaria.	Average number of translators (±1 SE).
Cecidomyiidae	11	0	0
Diopsidae	1	1	1
Drosophilidae	8	0	0
Lauxanidae	1	0	0
Muscidae	10	0	0
Phoridae	1	0	0
Platypezidae	2	0	0
Stratiomyidae	2	0	0
Syrphidae	1	0	0
Tachinidae	8	2	0.63 ± 1.41
Tephritidae	2	1	0.5 ± 0.71

Pollen removal, deposition and pollen transfer efficiency

The percentage of flowers with translators removed and tetrads deposited was relatively high in both years. In both years nearly half of all flowers had translators removed and slightly more than half of all flowers had tetrads deposited. PTE was similar for both years and was 8% in 2007 to 9% during 2008 (Table 2). It is not known what percentage of these pollen tetrads is self-pollen, but it is likely to be relatively large as tetrads contained in the translator may be pressed down onto the stigmatic surface when a flower visitor probes the top of the translator.

Table 2: Translators removed, deposited and pollen transfer efficiency for *C. lobulata* measured over two flowering seasons.

Year	Number of plants	Number of flowers	% flowers with translators removed	% flowers with tetrads deposited	Average no. tetrads removed	Average no. of tetrads deposited	PTE (%)
2007	14	46	54.35	54.35	287.42 ±52.85	26.0 ±6.0	9
2008	13	29	44.83	51.72	264.43 ±67.74	20.27 ±5.27	8

Flower colours and rewards

There was no obvious indication of the presence of flower rewards although the cavities between the corolla lobes have a glossy sheen which could indicate that a small amount of reward is secreted, however this amount would be very small. Flowers were generally brighter than the background leaves and are likely to contrast against the backround vegetation (Fig. 2). One important constituent of the scent of this species is cucumber aldehyde (Coombs, Peter and Johnson, unpublished data), which smells similar to watermelon and may be an important scent compound in attracting flies.

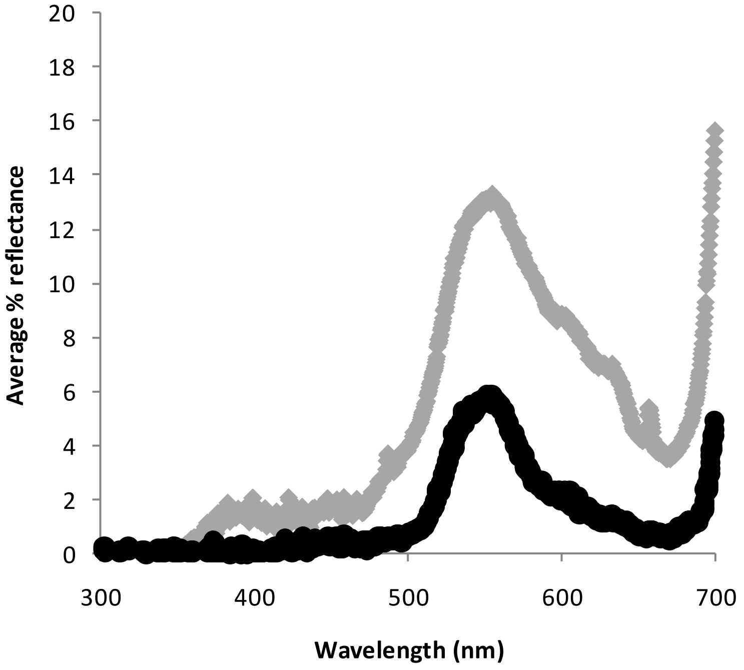

Figure 2: Average spectral reflection traces for the leaves (black) and flowers (grey) of *C. lobulata* (see text for details).

Demography

In total, I measured 111 individuals of which 87 were non-flowering juveniles. Most juveniles (62%) were less than 10cm in height. The majority of non-flowering juveniles occurred near flowering adults with 85% being within a distance five metres from the nearest flowering adult. *C. lobulata* was not restricted to climbing on certain tree species. The most common trees in which this species was found were *Harpephyllum caffrum* (17%), *Trichocladus ellipticum* (13%) and *Euphorbia triangularis* (11%; Fig. 3).

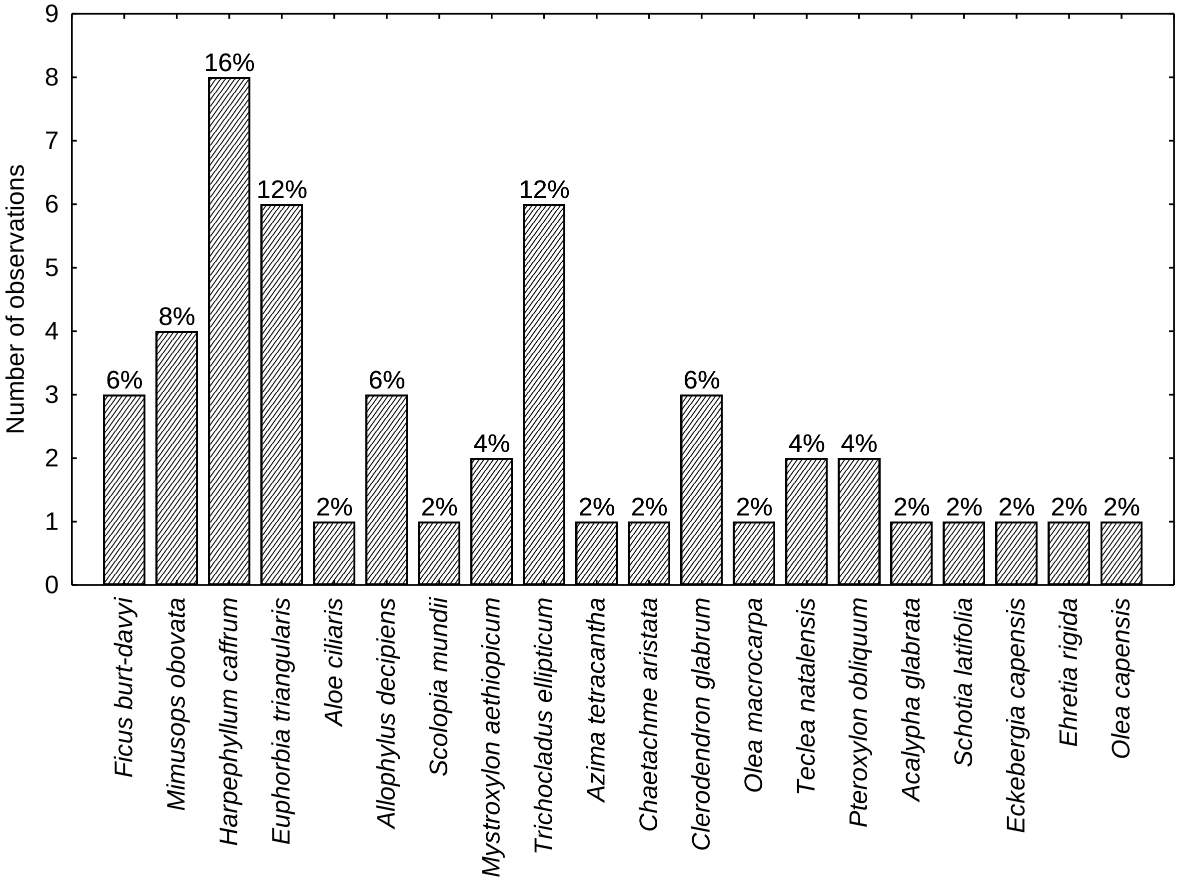

Figure 3: Percentage of individuals of *C. lobulata* growing in different tree species throughout the Kap River forest.

Flower herbivory

The lepidopteran larvae that consume the flowers and buds of *C. lobulata* were all identified as *Bocchoris onychynalis* (Heterocera – Pyralidae - Pyrustinae, Guenee, 1854) by W. Mey (Fig. 1H). Cocoons of *Bocchoris onychynalis* consisted of a leaf folded over and joined by silk strands. Caterpillars are small (< 20 mm), bright green on the ventral side and brown on the dorsal side (Fig. 1E). On the host plant, infection started when there was a noticeable blackening of the corolla lobes (Fig. 1F). Thereafter buds and flowers were either consumed (Fig. 1F, G) or many buds aborted. The blackened appearance is a result of the dried, damaged tissue (Fig. 1F). All parts of the inflorescences are eaten including the pedicle but caterpillars do appear to prefer flowers and buds. In all cases the inflorescences are completely destroyed once a caterpillar is present.

The number of buds per inflorescence rapidly reduced across all five dates and went from an average of 12.2 (SE = 0.7) on the first sampling date to 1.2 (SE = 0.3) buds on the final sampling date (Fig. 4). The number of buds that showed signs of herbivory increased from the first date and peaked at a maximum on the 31 March 2009. During this time the average number of open and closed flowers per inflorescence remained constantly low indicating that the reduction in buds was not due to buds maturing into flowers (Fig. 5).

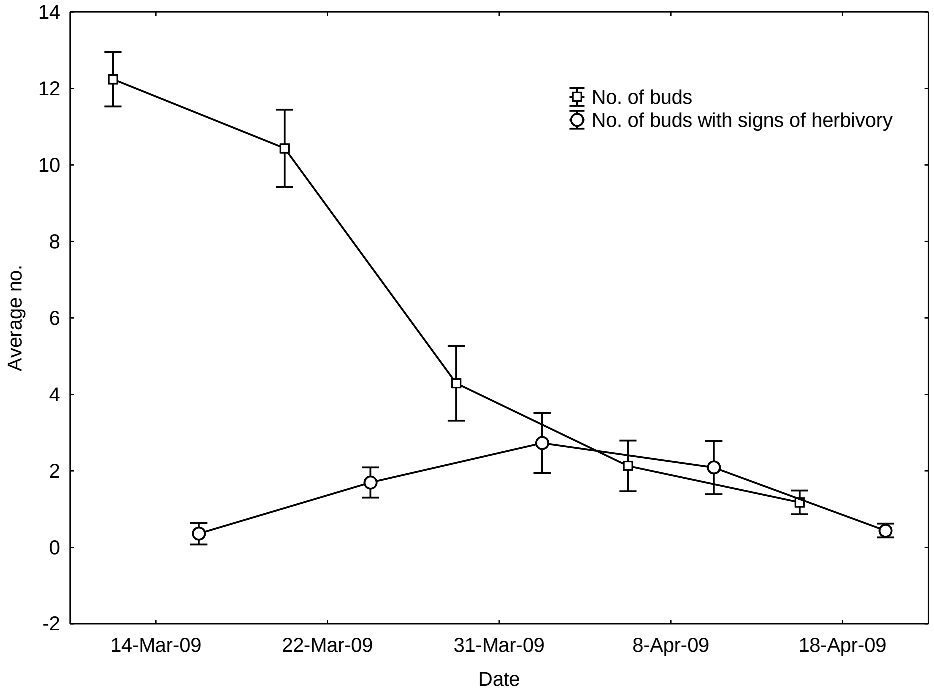

Figure 4: Average number of buds showing signs of herbivory measured at five different dates in inflorescences infested by *Bocchoris onychynalis* (Error bars = ± 1 SE).

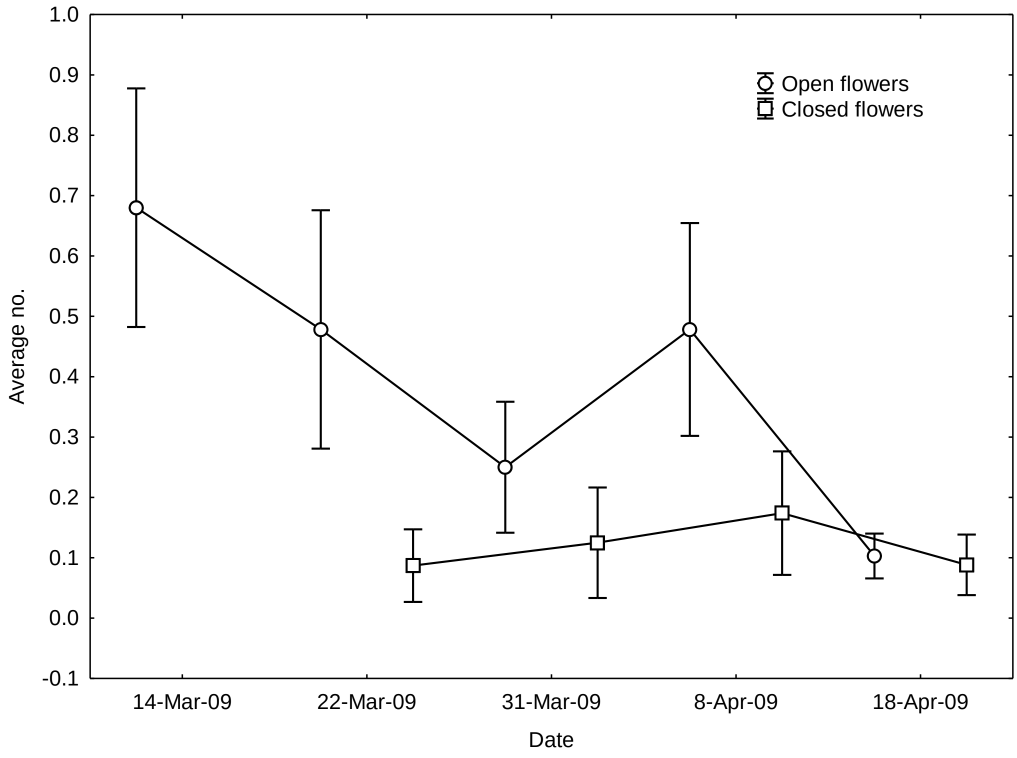

Figure 5: Average number of open flowers and closed flowers measured at five different dates in inflorescences infested by *Bocchoris onychynalis* (closed flowers were not scored on the first date, due to the difficulty of distinguishing closed flowers from buds near anthesis, Error bars = ± 1 SE).

In total, I marked 161 umbels on 12 plants of which 130 (81%) were infested with larvae of *B. onychinalis*. Most individuals of *C. lobulata* (98 %) were flowering in trees higher than 2 metres.

Discussion

These data suggest that *C. lobulata* is pollinated by several different species of short-tongued flies. Families of flies found visiting this species are well known generalist flower visitors (Larson *et al.,* 2001) but have thus far not been reported as pollinators of periplocoids. The limited data that is available on the pollination of periplocoids also records hymenopteran and lepidopteran pollination in the subfamily (Ollerton and Liede, 1997 and references therein; Ollerton *et al.,* 2010). In *C. lobulata* effective removal of translators requires that the proboscis of the visiting insect needs to be sufficiently long to be extended into the cavities between the corolla lobes and reach the viscidium which is located near the bottom this cavity. Therefore the flowers of *C. lobulata* show some characteristics that may limit effective pollination to a smaller subset of fly pollinators, and although flowers of the Periplocoideae are considered to be open access flowers (Ollerton and Liede, 1997), these features combined with the relative diversity of flies that bore pollinaria suggests that *C. lobulata* may be functionally specialized (*sensu* Fenster *et al.,* 2004). Judging from field observations and pollinator records, short-tongued flies with slightly longer proboscid lengths (*e.g. Degenea*) are morphologically well suited to efficiently removing pollinaria from the flowers of *C. lobulata*. Pollination by long-tongued flies (Bombyliidae) had been reported in *Rhaphionacme hirsuta* (Venter unpublished data: Ollerton *et al.,* 2010).

More extreme examples of morphological adaptation has been commonly documented in flowers pollinated by long-tongued flies (Goldblatt and Manning, 2000; Johnson and Steiner, 1997; Anderson and Johnson, 2009), but with the exception of fly trapping pollination systems (e.g. *Ceropegia* species; Vogel, 1961; Ollerton *et al.,* 2009; Masinde, 2004; Chapter 6) and sapromyiophilous species (e.g. *Stapelia, Huernea, Duvalia,* Meve and Liede, 1994; Meve *et al.,* 2004; Chapter 7), much less is known about the role of certain flower structures in flowers pollinated by short-tongued flies. Fly pollination is probably widespread in the subfamily Periplocoideae, and has been reported in other genera such as *Periploca* (Endress, 1994). Schick (1982; cited in Ollerton and Liede, 1997) reports fly pollination by Sarcophagidae and Calliphoridae in *P. sepium* and *P. graeca*. There are however very few records available on the pollination of members of this subfamily (Ollerton and Liede, 1997; but see Faria Vieira *et al.,* 1999).

Flower characteristics associated with pollination by short-tongued flies are extremely variable (Larson *et al.,* 2001). The bright green flower colours of *C. lobulata* contrasts against the darker leaves and may serve to advertise flowers to flies. The minute hairs present on the petals have also been reported in other fly-pollinated milkweeds (*e.g. Schyphostelma* species; Wolff *et al.,* 2008), but is also present species of *Raphionacme*, a closely related genus (Venter, 2009), raising the possibility that this trait may be ancestral in the subfamily. The role of the darker green corolla lobes is not known but these may be involved in scent production. The flower morphology of some members (e.g. *Raphionacme zeyheri*) of the closely related genus *Raphionacme* displays similar flower coloration and morphology (Venter, 2009), suggesting that these species may also be pollinated by short-tongued flies.

Given the somewhat cryptic colouration of the flowers, the scent of *C. lobulata* likely to be important. Myiophilous flowers are often sweetly scented (Proctor *et al.,* 1996). To the human nose, the flowers of *C. lobulata* smell similar to the scent of watermelon and analysis of the flower scent of *C. lobulata* identified one of the main volatiles as *cucumber aldehyde* (Coombs, Peter and Johnson, unpublished data). Similar reports on fruit flies being attracted to such sweet smelling flowers have been reported by Keng-Hong and Nishida (2005) who showed that the flowers of *Bulbophyllum apertum* produce sweet smelling odours such as raspberry ketone, that attracts fruit flies of the genus *Bactrocera* which pollinate this orchid.

The average levels of pollination success in *C. lobulata* suggests that levels of translator removal are high but as a consequence of the low number of tetrads that are deposited per stigma, the PTE is quite low. Approximately ten percent of removed tetrads are deposited on stigmas. This is comparable to the pollination success measured in other fly-pollinated asclepiads such as *Ceropegia* and *Stapelia* (Chapter 6 & Chapter 7) respectively. Unfortunately, owing to the practical difficulty of obtaining flowers and the high incidence of flower herbivory, PTE could not be traced at different intervals throughout the flowering season. I know of no other estimates of pollination success in the Periplocoideae and none are mentioned in the review by Harder and Johnson (2008).

No evidence was found in this study to support the hypothesis that the restricted distribution of *C. lobulata* may be the result of the break-down of a specialized pollination mutualism. Average levels of translator removal were relatively high and frequently approached 50% suggesting that pollinators regularly visit this species, although observations on pollinator activity indicated that pollinator visitation is likely to be patchy and unpredictable. This type of pollinator activity is typical of fly-pollinated species (Faegri and van der Pijl, 1979; Chapter 6 & 7). In species where pollination failure has been reported (e.g. Steiner, 1993; Johnson *et al.,* 2004b), visitation rates where exceedingly low and fruit set increased several fold with artificial pollinations (Steiner, 1993; Johnson *et al.,* 2004b). In species with highly specialized pollination mutualisms, the negative impact on reproduction caused by the absence of pollinators may be estimated by comparing the pollination success of populations where the pollinator is present to those where the pollinator is absent. For instance Steiner and Whitehead (1996) reported that fruit set in a population of *I. retzioides* where pollinators regularly visited plants was between five to eight fold higher than fruit set in populations where the pollinator was absent (Steiner, 1993). Although I did not carry out experiments to determine the extent of pollen limitation in *C. lobulata*, the high percentage of flowers with translators removed and pollen tetrads deposited combined with the large number of juveniles plants leads to the conclusion that this species receives sufficient pollinator services to maintain adequate levels of recruitment. The generalized pollination system of *C. lobulata* may thus further contribute to preventing pollination failure as has been suggested by other studies (Martin Rodriguez and Fenster, 2010). Future studies should document the breeding system of *C. lobulata* to determine whether this species may be buffered from pollination failure through setting fruit by means of autonomous self-pollination (e.g. Eckert and Schaefer, 1998; Kephart *et al.,* 1998; Dieringer, 1999; Neel, 2001). Carrying out breeding systems and quantifying levels of pollen limitation in this species would however be challenging given the difficulty of accessing flowers in the canopy. It is also worth noting that rare species are not necessarily predisposed to extreme pollen limitation (Tepedino *et al.,* 1999; Hill *et al.,* 2008; Petanidou *et al.,* 1995).

Flower herbivory in *C. lobulata* was extensive and occurred consistently in all three years that I studied this population. Flower parasitism by lepidopteran larvae is common in milkweeds where several lepidopteran species use milkweeds as host-plants for their larvae (See Dickinson and Kroon, 1987). The association between *C. lobulata* and the larvae of *B. onychinalis* is not a highly specialized mutualism however as *B. onychinalis* has been recorded on other milkweed species (*e.g. Gomphocarpus fruticosa*; H.K. Munro, 1925). I have also found high rates of bud herbivory and parasitism occurring in *Ceropegia ampliata* (Chapter 6), although in this species it results from a dipteran parasite.

The rate of inflorescence herbivory was relatively rapid causing most of the flowers and buds on infested inflorescences to fall off within five weeks of becoming infested by the larvae of *B. onychinalis*. It is uncertain whether flower and bud herbivory could limit seed set to the point where the distribution of this species is limited by such herbivory, however this seems unlikely given the high numbers of juveniles present. Other studies have however found that inflorescence herbivory may significantly reduce seed production (Galen, 1990; Louda and Potvin, 1995), and could effectively limit range expansion in some species (e.g. *Polemonium viscosum*; Galen, 1990). Future studies could determine the cost in terms of flower production through excluding moth caterpillars with the use of insecticides (*e.g*. Louda and Potvin, 1995), however care should be taken the application of insecticides does not affect pollinators. Despite the obvious cost of reducing the number of buds and flowers, flower herbivory may also indirectly influence pollination success in this species through reducing the number of pollinator visits (McCall, 2008; Suarez *et al.,* 2009; Krupnick *et al.,* 1999).

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Chapter 9

Conclusion

Pollination success

Despite evidence suggesting that plant reproductive success varies positively with population size in some plant species (e.g. Groom, 1998; Cappucino, 2004; Chapter 1), I found that in *G. physocarpus* there was no such relationship, which was surprising given that this species was found to be self-incompatible. The high pollination success in this species is therefore likely the consequence of the relatively generalized (but functionally specialized *sensu* Fenster *et al.,* 2004) pollination system.

The successful establishment of *Araujia sericifera* in South Africa has been facilitated by the ability of this species to attract native honeybees as pollinators (Chapter 2). The example of *A. sericifera* invading South Africa and *G. physocarpus* being an invasive species in Australia (Forster, 1994), shows that while milkweed flowers are relatively complex in terms of morphology, this does not prevent novel pollinators from pollinating the flowers of these species. There are an increasing number of studies that have found that plants with relatively specialized pollination systems are capable of invading new ranges, provided that similar functional groups of pollinators are present within the invaded range (Rodger *et al.,* 2010; Liu and Pemberton, 2010).

Both *A. sericifera* and *G. physocarpus* are invasive milkweeds in different parts of the world, which opens up the possibility to investigate whether these species have shifted their breeding systems in the invasive ranges. While *G. physocarpus* is self-incompatible in South Africa, there have been suggestions that this species is self-compatible in Australia (M. Ward, University of Queensland, pers. comm. 2006), where it is an invasive species (Forster, 1994). *Araujia sericifera* is capable of pollinator facilitated self-pollination in South Africa and it would be interesting to determine whether this species is self-incompatible in its native range. Rambuda and Johnson (2004) reported a presumed shift in the breeding system of *Lilium formosanum* that is capable of automatic self-pollination in South Africa but is apparently an obligate out-crossing species in its native range of Taiwan.

The success with which *A. sericifera* has co-opted native honeybees as its pollinators in South Africa was demonstrated by the relatively high level of pollination success which was comparable to the native honeybee pollinated *C. ellipticum*. Average levels of pollinarium removal, deposition and PTE was consistently high in *C. ellipticum* but varied throughout the season and frequently approached the maximum attainable value (50%), indicating that pollinarium loading in *C. ellipticum* may function to increase pollen transfer efficiency. Future research should aim to document the level of self-pollination in this system. Pollination success in the andromonoecious *C. obtusifolium* indicated that pollination success is highest during peak flowering periods which is more likely to be due to peaks in pollinator activity rather than increased percentages of hermaphrodite flowers.

Data on the pollination success of sapromyiophilous species showed that pollination success in these species is patchy and unpredictable and undoubtedly also linked to the abundance of pollinators in relatively arid areas. In both *C. ampliata* and *S. hirsuta var. baylissi*, pollinarium removal could reach relatively high levels (60 - 70%) which is comparable to pollinium removal rates in other pollinarium bearing species that are pollinated by short-tongued flies (e.g. *Schizochilus* species, Van der Niet *et al.,* 2010; *Disa obtusa*; Johnson and Steiner, 1994). Pollinarium deposition and pollen transfer efficiency was however much lower, suggesting that a large proportion of removed pollinaria are not deposited and are likely to be lost as a result of being groomed off or pollinators rapidly leaving the population and not visiting flowers within that population again.

The low pollination success in sapromyiophilous species suggests that trapping may function to increase the chances of successfull pollination through increasing the residency time of the pollinator within the flower (Dafni, 1984; Chapter 6). In contrast to this hypothesis, my results on the influence of trapping hairs on pollen export and receipt showed no significant effects of trapping hairs on pollination success. I did however find that older flowers had significantly higher levels of pollinarium removal which could support the idea that trapping at least increases pollen export. It is worth noting however that my sample sizes were small and owing to the unpredictable levels of pollination success in *C. ampliata*, more data are required, preferably over several seasons, to determine the influence of trapping hairs on pollination success. Unfortunately these types of studies are difficult to carry out on wild populations of *Ceropegia* unless a large and relatively dense population can be found. Therefore experiments using potted plants grown from cuttings might be a more practical for such experiments. The success of these types of experiments also relies on sufficient number of pollinators being present, which is not always the case.

Several aspects of the pollination ecology of *C. ampliata* (and other *Ceropegia* species) remain unknown and could be the focus of future research. The large variation in the degree of pubescence and the distribution of hair in the corolla, could suggest that hairs function to trap pollinators in some species while in other species they may merely function to assist with deception. In *C. ampliata* hairs are not as dense and localized as is the case in other species of *Ceropegia* (e.g. *Ceropegia macmasteri*, Dold, 2006). Manipulating the presence of these structures could reveal the functional basis of this variation, but would be difficult due to the small population sizes and scarce pollinators of different *Ceropegia* species. An alternative method that could be used is to construct artificial flowers to investigate the role of different flower morphologies in terms of pollinator trapping.

Given the low levels of pollination success in *C. ampliata*, it would be interesting to determine the breeding system of this species to determine whether it relies on automatic self-pollination as a means of reproductive assurance during times of low pollinator abundance (*e.g.* Eckert and Schaefer, 1998), however data on fruit set suggests that this is unlikely in wild populations.

While there are several studies that have documented the trapping times of different species of *Ceropegia* (see Chapter 6), little information is available on the actual residency times of pollinators within the flowers. In some species of *Aristolochia* (*Aristolochia grandiflora*), the release of pollinators is closely tied with the simultaneous wilting of trapping hairs and perianth tube through which flies must move in order to enter and exit the flowers (Burgess *et al.,* 2004). My data indicated that pollinators may escape the flowers of *C. ampliata* even while the flowers are within the trapping phase. There is no information on the microclimate within these flowers which may be important to ensure the survival of pollinators that are held within flowers for several days (Dafni, 1984). Experiments comparing temperature on the inside and outside of flowers could indicate whether temperatures inside the flowers are generally lower, which could be achieved by the light coloured reflective colours of the corolla tube. Temperatures at Ecca-pass are frequently extreme and could conceivably be fatal to flies that are trapped inside flowers for several days (see Dafni, 1984). Other ecological studies on *C. ampliata* could determine the distances that pollinators move between flowers, which could be done by dusting the inside of flowers with fluorescent dye powders (Dafni, 2008 pers. comm.).

The pollination success in *C. lobulata* and *Stapelia hirsuta* var. *baylissi* also provided an opportunity to investigate whether these two rare species maintain sufficient levels of pollination success to maintain viable populations. Rare plants frequently occur in small, isolated populations or sparse populations within a relatively narrow distribution range (Rabinowitz, 1981). Both types of distribution may generate what is known as the Allee effect (Allee, 1931) where small or sparse populations have reduced reproductive success as a result of a break down in cooperative interactions such as pollinator services (Courchamp *et al.,* 1999; Stephens *et al.,* 1999; Chapter 1). In both instances these plant populations are thought to provide insufficient rewards to pollinators, causing reduced rates of pollinator visitation which in turn results in reduced reproductive success (Oostermeijer, 2003). However, as shown by both studies on rare species in this study, there was no indication that these two rare species were suffering from a collapse of pollinator services. The likely explanation for this is that the interaction between pollinator and flowers in small populations is more complex and some species may have small populations but compensate for this by producing large floral displays (Moran and Hopper, 1987; cited in Brigham, 2003) while other species such as *Chlorocyathus* that do not occur in such dense may be regularly visited due to the presence of other flowering species (Laverty, 1992; Ghazoul, 2006)

An interesting direction of research may be to establish whether Allee effects are equally likely for species with different pollination syndromes, as these pollinators are likely to differ in their response to the spatial organization of plants. For instance short-tongued flies do not have the same energetic demands as social Hymenoptera (Faegri and van der Pijl, 1979) and observations on fly-pollinated milkweeds in this study indicated that visitation patterns in fly-pollinated species differs to that seen in hymenopteran pollinated milkweed (see below). Evidence for reduced reproduction in small or sparse populations has however been found in species pollinated by several different taxa. Allee effects have been demonstrated in species pollinated by small mammals and birds (e.g. *Banksia goodii*, Lamont *et al.,* 1993) and long proboscid flies (e.g. *Brunsvigia radulosa*; Ward and Johnson, 2005) as well as in more generalist pollinated species such as *Panax quinquefolius*, which is pollinated mainly by syrphid flies and halictid bees (Hackney and McGraw, 2001; Schlessman, 1985). Davis *et al.* (2004) have also reported Allee effects in wind pollinated species. Although there is still limited data, these studies suggest that the pollination syndrome of a species may not influence whether it suffers reduced reproductive success when occurring in small populations.

Pollination systems and specialization

In the species studied in this thesis that were pollinated by Hymenoptera, there was evidence that flowers were pollinated by a smaller subset of the total complement of visitors suggesting some degree of specialization towards specific pollinators. Similarly, *G. physocarpus* was functionally specialized (*sensu* Fenster *et al.,* 2004) towards pollination by just two genera of Vespidae (Chapter 2). For instance both species of *Cynanchum* were visited by a wide diversity of pollinators from several different orders but were primarily pollinated by honeybees (Chapters 4 & 5). Hymenopteran pollinated milkweeds vary from highly generalized species of *Asclepias* (*e.g. Asclepias verticillata*; Ollerton and Liede, 1997) to more specialized wasp-pollinated systems such as that of *Pachycarpus asperifolius, Pachycarpus appendiculatus, Pachycarpus grandiflorus,* (Shuttleworth and Johnson, 2006; Shuttleworth and Johnson, 2009a; Shuttleworth and Johnson, 2009b; see also Shuttleworth and Johnson, 2009c). The diversity of different species visiting flowers of both species of *Cynanchum* is likely due to the relatively open flowers of these species where nectar is relatively exposed. In *C. ellipticum* there may be some filtering of pollinators due to the cup-like corona forming a short (*ca.* 2 mm) coronal tube (Chapter 4).

The function of different corona morphologies in *Cynanchum* is not known for most species, although the progression from open to closed coronas is thought to be associated with increasing specialization towards a narrower set of pollinators with longer tongues and to exclude nectar thieves (Ollerton and Liede, 2003; Yamashiro *et al.,* 2008). The cup-like corona structure of *Cynanchum ellipticum* could function in a similar way and may explain the abundance of lepidopteran visitors to this species.

In *C. obtusifolium* the corona lobes of hermaphrodite flowers were relatively longer than those of smaller male flowers, and could represent partial specialization where both pollinating and non-pollinating insects may remove pollinaria from male flowers while only those insects with sufficiently long proboscides and are strong enough to part corona lobes (*e.g*. honeybees) can effectively deposit pollen in hermaphrodite flowers.

In both species of *Cynanchum* there appears to be an interaction between the gynostegium and corona which results in divergent pollination mechanisms in species that use the same pollinator species, *Apis mellifera*. In one species, *C. ellipticum*, the tubular corona combined with pollinaria that form chains, results in the accumulation of large pollinarium balls on these pollinators (see later). In *C. obtusifolium*, the pollinaria do not form such long chains, however the differences in gynostegium morphology generates a functional flower polymorphism.

Although both species of *Cynanchum* co-occur and flowering bouts frequently overlap, my observations indicate that honeybees rarely shift between these two species. Kephart and Theiss (2003) found similar specialization by certain pollinators visiting mixed patches of *Asclepias incarnata* and *A. verticillata* (Kephart and Theiss, 2003). The floral constancy of honeybees visiting both species of *Cynanchum* certainly suggests that *C. obtusifolium* and *C. ellipticum* differ in terms of the quality of their rewards and bees can distinguish between the two species as discussed below. I never found pollinaria of one species being deposited in the alar fissure of the other species.

Honeybees could potentially distinguish between these species through differences in the scent between both species which is distinguishable to humans. The role of scent in attracting pollinators has been well established in bee-pollinated species (Dotterl and Vereecken, 2010) and may be a cue by which honeybees distinguish between these two species. Differences in flower colours may also be important by providing a cue for discrimination between these two species. The colours of the corona of *C. ellipticum* and *C. obtusifolium* are similar, although the petals of *C. obtusifolium* appear to play a greater role in display as these are mostly coloured green with a dark purple area near the base of the petal. In contrast, the petals of *C. ellipticum* are similar in colour are quite narrow and their role in display is likely to be overshadowed by the relatively larger tubular white corona.

I noticed that the visitation pattern of honeybees visiting *Cynanchum ellipticum* was patchy, whereas visits by honeybees to *C. obtusifolium* appeared more reliable. I suspect that such variation could be caused by the presence of co-flowering species as plants frequently compete for pollination services (Levin and Anderson, 1970; Zimmerman, 1980; Motten, 1986; Rathcke, 1988; Chittka and Schurkens, 2001). There may also be differences in the nectar chemistry between *C. ellipticum* and *C. obtusifolium*, which may cause honeybees to selectively forage on *C. obtusifolium*. Such differences are unlikely to be caused by differences in the nectar concentration alone between these two species, as both species had similar nectar concentration values (*C. ellipticum* = 31.16 % sucrose equivalents; C. obtusifolium = 22. 49 and 30. 43% sucrose equivalents).

In the three fly-pollinated systems I found evidence for both specialized and highly generalized pollinator relationships. In *Stapelia hirsuta* var. *baylissi* my data suggests specialization towards pollination by Anthomyiidae. The degree of specialization in different species of *Stapelia* is uncertain and appears to vary between different species, although some specialized relationships have been reported (Jonkers, 2010, Raspi *et al.,* 2010). Therefore the relationship between *S. hirsuta* var. *baylissi* and anthomyiid flies requires further investigation which would require analysing the scent of this species and comparing this to the scent of rock hyrax (*Procavia capensis*) middens where it occurs (Chapter 7). Studies analysing the flower odours in asclepiads have indicated that certain flower odours can be separated according to the substrate that the species is mimicking (Jurgens *et al.,* 2006). The degree of specialization in sapromyiophilous systems is likely to be influenced by the specific substrate that the flowers are mimicking. For instance substrates such as rotting meat, may attract a diversity of different fly families, but further specialization may be achieved through differences in the flower morphology (see Meve *et al.,* 2004 for description of isolating mechanisms in stapeliads).

In contrast to the specialized relationship seen between anthomyiids and *Stapelia hirsuta* var. *baylissi*, the pollinator records of *Ceropegia ampliata* and *Chlorocyathus lobulata* suggested highly generalized relationships in these taxa which agree with most data on fly pollination relationships in milkweeds (Ollerton and Liede, 1997; Ollerton *et al.,* 2009; Wolff *et al.,* 2008; Yamashiro *et al.,* 2008).

Although the flower morphology of sapromyiophilous milkweeds mimics the brood or feeding substrate of flies, these flowers are generally thought to be rewarding although the evidence is limited (Meve and Liede, 1994; Lock, Endress and Ollerton, unpublished data; Bruyns, 2000). Due to the small volumes of nectar that is produced and the difficulty extracting this nectar from the flower, the concentration of the sugars in these nectars have rarely been measured. However the technique used in chapter seven provides a new method which is very effective at extracting minute volumes of nectar without damaging the flower tissue which can alter the concentration of small nectar volumes through introducing cell contents into the nectar (see Morrant *et al.,* 2009). I am only aware of one other study that quantified the sucrose concentration contained in the nectar of sapromyiophilous milkweed. Herrera and Nassar (2008) found that the nectar concentration of *Stapelia gigantea* was low at 13.81% sucrose equivalents however this species produces remarkably large nectar volumes of 25.41 µl per flower. *Ceropegia ampliata* produces minute volumes of nectar (0.20 µl per flower) of a relatively low concentration (17.7%). These data suggest that fly-pollination requires relatively dilute nectar solutions as reward however there is insufficient information to generalize about the sucrose content of rewards in different sapromyiophilous milkweeds.

Pollinator abundance in different systems

The behaviour of pollinators indicated that in bee- and wasp-pollinated species, visits were relatively common (provided that the weather conditions were suitable). In *G. physocarpus*, *A. sericifera*, and both species of *Cynanchum*, large samples of pollinating insects could be collected with few hours of sampling effort. The importance and efficacy of honeybees as pollinators is well known and wasps have also been reported to be highly effective pollinators of other asclepiads (Shuttleworth and Johnson, 2006; Vieira and Shepherd, 1999; Foster, 1994). This high pollinator abundance also translated in to relatively high and consistent pollination success (see later).

Although Hymenoptera are the most common pollinators of asclepiads, fly-pollination is common in the family (Ollerton and Liede, 1997). In species that were fly-pollinated, visitation rate was generally low and highly unpredictable, although there are some fly-pollinated species within the subfamily Secamonoideae (Apocynaceae) with higher visitation rates (e.g. *Secamone filiformis,* G. Coombs and C.I. Peter unpublished data). When bees and wasps visited the flowers of the study species these insects visited several flowers on the plant in quick succession, while in fly-pollinated species visit periods by the main pollinators were relatively long and unpredictable.

Gynostegium structure and pollinarium loading

Variations in the patterns of pollen loading between *C. obtusifolium* and *C. ellipticum* is likely to be achieved through differences in the flower morphology, particularly the structure of the gynostegium and pollinaria. *C. ellipticum* employs a pollination mechanism where the morphology of pollinaria is adapted to highly efficient chaining, whereas in *C. obtusifolium* pollinaria never forms such chains. Different milkweed species place pollinaria on different parts of the insect body. For instance the pollinaria of *Pachycarpus asperifolius* attach to the labial palps of large pompilid wasps (*Hemipepsis* species; Shuttleworth and Johnson, 2009a) wheras some beetle pollinated milkweeds attach pollinaria haphazardly to hairs and spines on various part of the body (termed "messy pollination" by Ollerton *et al.,* 2003). While it has been found that the pollinaria of *C. ellipticum* and *C. obtusifolium* attach selectively to the mouthparts of honeybees, the functional role of such large pollinarium accumulation on one part of the pollinators body remains unclear, but I hypothesize that this may be an adaptation to increase pollination success (Chapter 4). Ollerton *et al.* (2003) found no apparent advantage in terms of pollination success in species that load pollinaria on various parts of the body, however the high pollination success of *C. ellipticum* suggests that large pollinarium loads loaded onto one part of the body may function to increase pollination success.

The morphology of the corona and gynostegium determines the interactions of different parts of the pollinator with the gynostegium. For example, Yamashiro *et al.* (2008) suggested that the morphology of the corona in *Cynanchum* and *Vincetoxicum* acts to allow nectar to accumulate below the guide rails causing pollinaria to be placed on the mouthparts. In *Ceropegia* nectar accumulates in corona cups below the alar fissure (Chapter seven), an arrangement that causes pollinaria to attach to the proboscis of flies (Lock, Endress and Ollerton, unpublished data). In species such as *Gomphocarpus physocarpus* (Chapter 2); wasps consume nectar from the corona hoods, while pollinaria are loaded mainly on the tarsi that pass through the alar fissure situated between the corona hoods (see also Yamashiro *et al.,* 2008). In other wasp-pollinated species (e.g. *Oxypetalum*, Vieira and Shepherd, 1999) pollinaria are loaded on the mouthparts. The example of the two species of *Cynanchum* suggest that with the positioning of nectar being placed at the base of the gynostegium coupled with apparently minor structural variations in the morphology of the gynostegium and pollinaria, dramatic variation in patterns of pollinarium loading and pollen receipt in flowers can be generated.

The gynostegium structure of milkweeds not only influences the pattern of pollinarium loading but the structure of the gynostegium chamber of *C. ellipticum* suggests that some species may limit the amount of pollen that can potentially be received and in so doing limit the amount of self-pollination that may otherwise occur. This may be particularly adaptive in species where pollinaria do not re-configure once these are removed from the flower, as pollinarium reconfiguration is thought to promote outcrossing (Peter and Johnson, 2006).

While pollinarium reconfiguration occurs in most milkweed species that have been studied to date (*e.g. Asclepias*, Wyatt and Broyles, 1994; *Pachycarpus*, Shuttleworth and Johnson, 2006; *Gomphocarpus*, Coombs *et al.,* 2009), including sapromyiophilous taxa such as *Stapelia* and *Ceropegia* (Chapters 5 & 6), it is curiously absent in both study species of *Cynanchum*. Given that pollinaria become easier to insert once they have undergone re-configuration (Wyatt, 1976; Coombs *et al.,* 2009), it would be interesting to determine how the gynostegium structure of *C. ellipticum* and *C. obtusifolium* differ in order to facilitate insertion of pollinaria that have not undergone re-configuration. Non-reconfiguring pollinaria may also attach slightly differently to pollinators and clip onto the appendage of pollinators in such a way that these are already positioned to be inserted.

I did not inspect whether the translators of *Chlorocyathus lobulata* (Chapter 8) undergo reconfiguration in the same way as do orchid pollinaria (Johnson and Edwards, 2000), and further studies need to be carried out to see whether pollinium bending occurs in the Periplocoideae. I am not aware of any studies that have determined whether pollinarium re-configuration occurs in the Periplocoideae.

Variation in the structure of the gynostegium may alter the pattern of pollinium receipt shown by differences in the dimensions of the anther wings and alar fissure generating a functional flower dimorphism in *C. obtusifolium* (Chapter 5). Not only does this species limit pollen receipt in male flowers but the dimensions of the male flowers suggest that smaller flowers are more efficient at exporting pollen as well. However, as highlighted in Chapter five this finding may be confounded with relatively shallow male flowers allowing for a greater diversity of insects to access the nectar and in so doing remove pollinaria.

There is no evidence that the pollinarium loads of *C. ellipticum* have any effect on the foraging speeds of honeybees. This was in contrast to the findings by Morse (1981) that showed that the presence of *Asclepias* pollinaria on the proboscides and tarsi of pollinating bumblebees significantly slowed the foraging speed of these insects. There was however some evidence that honeybees do eventually become frustrated when the pollinarium load becomes too large and some bees were observed attempting to groom pollinaria off.

There exist several examples of mutualsims where some cost is incurred to one of the mutualists (Bronstein, 2001). Most examples involving pollination mutualisms include those where the pollinators disadvantage the plant (*i.e*. pollinating seed parasites; Pellmyr, 1997). Examples of the cost to pollinators are relatively more scarce, however some orchids attach pollinaria to the eyes of pollinating hawkmoths (Johnson and Liltved, 1997), that undoubtedly influences the visual abilities of these insects (Johnson pers. comm). Morse (1981) presented a similar argument when describing the pollinator interactions between bumblebees and *Asclepias syriaca* as a “high-cost” system as in order to obtain a reward, bumblebees are physically injured or their foraging rates were decreased as a result of being attached to their mouthparts and tarsi. Thus, for pollinators, foraging on certain types of milkweeds – particularly species with large rigid anther wings, the energetic reward gained in terms of nectar consumption comes at the cost or risking physical injury or death. A similar situation was reported by Shuttleworth and Johnson (2009a) where in the highly specialized pollination mutualism between *Hemipepsis* wasps and the flowers of *Pachycarpus appendiculatus* the labial palps (important sensory organs) are frequently cut off by the rigid anther wings of this species.

The pollinarium loading in *C. ellipticum* and *A. sericifera* provided evidence for both negligible effects of milkweed pollinaria on pollinator foraging as well as profound negative effects. My results indicated that while honeybees that feed on *C. ellipticum* accumulated large numbers of pollinaria on the proboscides, this apparently had little effect on their foraging behaviour. In contrast, the larger flowers and rigid anther wings found in *A. sericifera* frequently restrain and kill moths and occasionally honey bees that visited and pollinated this species (Chapter three).

The pattern of pollinarium loading on the proboscides of honeybees opens up new avenues for research on the pollination system in *C. ellipticum*. Particularly interesting would be to quantify what levels of self-pollination occur in this species, however the difficulty of labelling milkweed pollinia remains a significant obstacle to tracking pollen fates of milkweeds in a similar way as has been done on orchids (Kropf and Renner, 2008). The pollinia of milkweeds are covered by a thick waxy layer (Wyatt and Broyles, 1994) which makes it difficult to stain the pollinium using histochemical stains. Furthermore, the position of pollinia behind the anther wing makes it difficult to place dye directly on pollinia. I attempted a new technique for pollinium staining in milkweeds that involved piercing pollinia *in situ* with a micropipette with the tip drawn into a needle with a micropipette puller (see Chapter 6). It was found that pollinia that were removed from the flower and pierced with a needle would readily take up histochemical stains such as Sudan IV. This technique did however prove to be unsuccessful practically as pollinia that were pierced while inside the flower were relatively more difficult to extract.

To conclude, this thesis has investigated a diverse range of topics in the pollination biology of milkweeds and grouped these under two relatively broad themes; the first deals with ecological interactions in species pollinated by Hymenoptera while the second examines specialization in fly-pollinated species. In each of these studies I have measured the degree of pollinator specialization, average levels of pollination success, nectar rewards and in some species I measured other population characteristics such as the demography. I have found different patterns of pollinarium loading between congeneric species and also describe how an invasive species co-opts native honeybees as its pollinator. I have shown that the degree of specialization in different species was variable with both hymenopteran- and dipteran-pollinated species having species that were either generalist pollinated (e.g. Chapters 2 & 6) and species that were relatively more specialized (e.g. Chapters 4 & 7). I show that pollination success in hymenopteran-pollinated species is generally higher and more consistent than the pollination success in dipteran-pollinated species which was generally low and patchy. Given that pollination success is so low in fly-pollinated species, I also determined that the trapping hairs in the flowers of *Ceropegia ampliata* may increase pollen removal. Several of these studies were also more broadly applicable to plant pollination ecology, for example, I find that in both rare (Chapter 7 & 9) and common species (Chapter 2) small populations did not suffer from pollination failure. These studies demonstrate the potential diversity of pollination systems that exist in milkweed pollination systems and the value of using milkweeds as model species to investigate various aspects of plant reproductive ecology (c.f. Wyatt and Broyles, 1994).

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Appendix 1: Related papers

Appendix 1A: The study was done during 2006 and determines the role of nectar guards in preventing nectar theft by sunbirds visiting the flowers of *Strelitzia reginae*. Similar to the approach taken in Chapter 6 I removed these structures in some flowers and compared nectar consumption by sunbirds visiting flowers where nectar barriers were experimentally removed to flowers where these were still present. Both studies emphasize the role of manipulating the presence of morphological features in flowers in order to examine the function of these features in terms of their interaction with flower visitors or their influence on pollination success (Chapter 6).

Appendix 1B: This paper was accepted in Flowering Plant of Africa and provides a brief description of the taxonomy and ecology of *Chlorocyathus lobulata*.

Appendix 1A: Coombs and Peter (2009) – *Importance of nectar barriers in limiting nectar theft by sunbirds visiting flowers of* Strelitizia reginae

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Short communication

Do floral traits of *Strelitzia reginae* limit nectar theft by sunbirds?

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Abstract

The shape of flowers frequently corresponds to the morphology of pollinators but some floral traits may also function to prevent non-pollinating flower visitors from stealing flower rewards. Despite the presence of such structures few studies have demonstrated their efficacy in limiting the nectar intake by nectar thieves. The flowers of *Strelitzia reginae* are regularly visited by sunbirds that do not effect pollination and act solely as nectar thieves. In this species, the nectary is covered by the convoluted bases of the petals ("nectar barriers"). In this study we investigate how non-pollinating sunbirds interact with these nectar barriers and whether nectar barriers play a role in limiting the amount of nectar sunbirds can steal. We quantified the volume of nectar that sunbirds consume while visiting flowers where nectar barriers were present and in flowers where these were experimentally removed. We found that sunbirds consume a median of 106.8 µl of nectar when visiting flowers with nectar barriers present and consumed a significantly greater volume of nectar (median = 158.03 µl) in flowers without nectar barriers. These results suggest that the convoluted petals that cover the nectary of *S. reginae* may function to reduce nectar theft but are likely to be more effective against insect nectar thieves. This is one of the first studies to quantitatively demonstrate the role of flower features that may function to limit nectar theft.

Keywords: Nectar theft; Robbery; Sunbirds; *Strelitzia reginae*

1. Introduction

Nectar is a readily available source of energy (Heinrich, 1981) and consequently many flower visiting animals steal nectar without "payment" in the form of pollination (Inouye, 1983; Maloof and Inouye, 2000). Most groups of flower visiting animals have members that steal nectar (Inouye, 1983; Proctor et al., 1996). Inouye (1983) defines nectar robbery as the consumption of nectar from flowers without contacting the sexual parts of the flower by physically damaging the flower in a way that is not done by legitimate pollinators. The best known nectar robbery is undertaken by *Xylocopa* and *Bombus* bees that pierce the tissue of the base of the corolla tube to access the nectar (Inouye, 1983). In contrast, nectar theft is when nectar is freely accessible but without the pollinators coming into contact with the anthers and stigma as a result of a morphological mismatch between the pollinator and flower (Inouye, 1980). While this terminology clearly separates the two forms of nectar pilfering, both terms are commonly used interchangeably in the literature (Inouye, 1980) as well as in every-day language. In this paper we will primarily use the term nectar "theft" as this is what occurs in our study system.

Due to the negative impacts of nectar theft on plant fitness (Wyatt, 1980; Traveset et al., 1998; Irwin and Brody, 1998; Maloof and Inouye, 2000; Irwin et al., 2001), plants have evolved physical and chemical mechanisms that prevent nectar theft (Rhoades and Bergdahl, 1981; Inouye, 1983; Johnson et al., 2006). Such features include longer corolla tubes (Lara and Ornelas, 2001), or thicker corolla tissue (*Thunbergia grandiflora,* Acanthaceae; Inouye, 1983), while in some species, the nectar may be distasteful to potential nectar thieves (Johnson et al., 2006).

Few studies have quantified the volumes of nectar removed by nectar stealing visitors to test whether these mechanisms are indeed effective at limiting the volume of the nectar removed (but see Irwin et al., 2004). One example is that of

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Appendix 1A: Coombs and Peter (2009) – *Importance of nectar barriers in limiting nectar theft by sunbirds visiting flowers of* Strelitizia reginae

Johnson et al. (2006) who found that the high concentrations of phenolic compounds in the nectar of *Aloe vryheidensis* was distasteful to sunbirds that avoided consuming the nectar of this species.

Sunbirds are the dominant group of obligate nectar feeding birds in South Africa with 20 species present in the region (Skead, 1967; Oatley and Skead, 1972). Although sunbirds pollinate numerous African plants (Johnson, 1995; Pauw, 1998; Anderson et al., 2005; Johnson and Nicolson, 2008) they may also be nectar thieves of some species where the flowers are not morphologically adapted for sunbird pollination (e.g. Johnson et al., 2006). *Strelitzia reginae* is one such example and despite sunbirds being regular visitors to this species both in wild and cultivated plants, studies by Coombs et al. (2007) found that the vast majority of these visits entail the birds perching on the spathe of the flower and hence not touching the anthers or stigma, functioning solely as nectar thieves. This is in contrast to the legitimate weaver bird pollinators that visit the flowers infrequently and alight on the fused blue petal, making contact with the anthers and stigma (Rowan, 1974; Skead, 1975; Coombs et al., 2007). In light of these findings showing that the nectar thieving sunbirds more frequently visit the flowers of *S. reginae* than the legitimate weaver pollinators, our attention was drawn to the possibility that the relatively tough convoluted petals that cover the nectary ("nectar barriers") may function to prevent such nectar theft.

We therefore set out to quantify nectar theft by sunbirds on *S. reginae* and ask: 1) do visits by sunbirds significantly reduce the volume of nectar present in flowers and 2) does the presence of the convoluted petal bases serve as barriers to sunbird access to the nectary, reducing the volume of nectar consumed by these birds?

2. Methods

2.1. Study species and study site

The iconic *S. reginae* is a short, stemless evergreen perennial that is found along the coast of the Eastern Cape province of South Africa between Port Elizabeth and Port St. Johns and approximately 60 km inland. The inflorescence consists of a peduncle arising from the axils of the leaves and produces a coriaceous bract, the spathe, from which individual flowers arise sequentially. The flowers of this species are bisexual and consist of 3 orange sepals and 3 blue petals. The lower two blue petals are fused forming a triangular sheath that enclose the five anthers (Archer, 2000). The nectar is situated in a short corolla tube at the base of the three blue petals. The two fused petals form a convoluted covering at the base of the petals enclosing the nectary. For purposes of this study we call these "nectar barriers" (Fig. 1A). The third petal is smaller and concave, forming a hood over the entrance to the nectary.

All experiments were conducted in 3 large naturalized stands of *S. reginae* growing in the Grahamstown Botanical Gardens, Eastern Cape, 13 km from the nearest wild population. Visits were observed from hides approximately 2 m from the experimental patches.

2.2. Is nectar removed by other means?

The experimental manipulations described below require the introduction of known volumes of nectar prior to the experiment. These experiments assume that nectar volume and concentration do not change due to other factors such as reabsorption (e.g. Burquez and Corbet, 1991), evaporation or leaking from flowers. In all cases nectar volume was determined using 50 µl micropipettes, while nectar concentration was measured with an Atago refractometer.

To test these assumptions, we tested for changes in the volume and concentration of nectar, 3½ h after a known amount of nectar was introduced into bagged flowers. The initial nectar volume and concentration of all open flowers (n=9) on 5 plants were measured between 10:00 and 12:30. The average volume of nectar was then replaced by injecting nectar from a stock solution of nectar collected the previous afternoon from approximately 40 individuals of *S. reginae* growing within the gardens of Rhodes University, a few hundred meters from our study plants. Nectar was removed or injected either through a small hole pierced in the sidewall of the corolla tube or by parting the nectar barriers and inserting the micropipette directly into the nectar. The nectar volume and concentration were again measured from 16:00 in the afternoon to determine if any changes in nectar volume and concentration had occurred.

2.3. Determining nectar theft

Flowers were observed on 7 days during May and July 2006. On each observation day, we selected at least 5 individuals within line of sight from the hide and used all newly opened flowers on these plants for the experiment. This resulted in between 7 and 11 flowers being used per observation session. To prevent sunbirds from visiting flowers before experiments, inflorescences were bagged in the late afternoon of the previous day. Using the method described above, the initial standing volume and concentration of nectar per flower were determined and the nectar replaced with a similar volume of nectar from the nectar stock collected from other plants rounded to the nearest 5 µl. In later experiments we standardized the injected nectar volume to 160 µl per flower (rounded average of mean morning nectar volume= 156.7 µl, SD=77.4, n=27 flowers).

Flowers were immediately harvested following a sunbird visit and the volume and concentration were measured. Observations made on the first day only tested whether sunbirds reduce the volume of nectar within unmodified flowers. On all other days we compared nectar consumption by sunbirds visiting both unmodified flowers and flowers where nectar barriers were removed. The absence of nectar barriers was simulated by removing a small square section of the barriers (Fig. 1B). This procedure does not alter the morphology of the flower in any way other than allowing for more direct access to the nectar. During observation periods we attempted to assign equal numbers of flowers to either treatment (i.e. nectar barrier present or absent) and on 4 of the observation dates also bagged 2 controls of each treatment (total of 8 per treatment) to quantify whether nectar barriers serve to

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Fig. 1. The nectary of *S. reginae* is covered by the convoluted bases of the two fused petals forming a barrier to the opening of the corolla tube (A). To simulate the absence of this structure we removed a small square section of the protective petals (B). Sunbirds visit the flowers of *S. reginae* and access the nectar without contacting the anthers or stigma (C). Scale bars: A & B = 5 mm, C = 100 mm.

prevent evaporative loss of nectar. To increase the sample size of controls, the same protocol as before was used to bag an additional 4 controls on 2 days after observations on nectar thievery were completed. This culminated in a total of 12 controls for each treatment. The nectar volume and concentration of controls were measured at the end of each observation period. Nectar concentration was measured to assess if part of the nectar loss could be the result of evaporation. All observation periods occurred between 7:30 and 14:30 pm, and typically lasted between 1 and 3½ h.

2.4. Statistical analysis

In order to express the volume of nectar consumed by sunbirds as positive values we calculated the difference between the final and initial volume of nectar during the experimental period by subtracting the final volume from the initial volume, therefore negative values indicate an increase in final volume. The change in nectar concentration was calculated by subtracting the initial concentration from the final concentration, therefore negative values indicate lower final concentrations.

Because variances were heterogeneous we used non-parametric ANOVA (PERMANOVA, see Anderson, 2001) to analyse for the effect of sunbirds and nectar barriers on the volume and concentration of nectar consumed. This statistical method does not require normality of response variables and is robust to mild departures from variance homogeneity (Anderson, 2001), as was the case in our data. We carried out the all data analysis on Box–Cox transformed data which further improved homogeneity. We performed two-way fixed effects ANOVA with bird present or absent and nectar barrier present or absent as the two main effects. A Type III sum of squares model was used (Anderson, 2005a). Data analysis was done using the program DISTLM (v.5) and distances between data values were calculated as Euclidean distances. We obtained both *p*-values and *t*-values (see later) using 999 permutations to achieve adequate precision of the *p*-value at the 5% level of significance (see Anderson, 2001). A second *p*-value was derived using the Monte–Carlo sampling option provided by the program. The Monte–Carlo method provides a more precise *p*-value when sample sizes are small (Anderson, 2005b). In order to specifically address question 2 (stated above), we performed two pre-planned post-hoc tests (Sokal and Rohlf, 1995). These were to test if sunbirds consumed significantly more nectar when visiting flowers with nectar barriers absent (upper-tailed *t*-test) and to test for any significantly different nectar volumes in control flowers with or without nectar barriers present (two-tailed *t*-test). Methods for obtaining *t*-values are discussed in Anderson (2001). For easier interpretation, we plotted box-plots with untransformed data in order to show the spread of the data.

3. Results

3.1. Is nectar removed by other means?

The volume of nectar within flowers did not differ significantly between the initial (10:00–12:30) and final (16:00–18:00) sampling periods ($t_{16}=-0.040$, $p=0.41$) suggesting that nectar is not re-absorbed and does not undergo significant evaporative losses using this method. The average injected volume of nectar was 205.2 (SD=61.7) and the final volume was 206.5 (SD=83.5). The average concentration also showed no significant change ($t_{16}=1.10$, $p=0.30$).

3.2. Nectar thieving

The only sunbirds that visited *S. reginae* during our observation periods were either Greater Double-collared sunbirds (*Cinnyris afer*) or Southern [Lesser] Double-collared sunbirds (*Cinnyris chalybeus*, Fig. 1C). Amethyst [Black] sunbirds (*Chalcomitra amethystina*) and Grey sunbirds (*Cyanomitra veroxii*) were less common in the vicinity and rarely visited *S. reginae* and did not visit any of the flowers used for the nectar thieving experiments. Malachite sunbirds (*Nectarinia famosa*) were never observed visiting *S. reginae* despite frequently visiting *Aloe ferox* in the immediate vicinity. In total 33 visits were seen of which only 3 were by females. We did not distinguish between Southern and Greater Double-collared sunbirds as this can be difficult when birds are viewed from a distance or not trapped to confirm identity. The change in the nectar volume that was consumed was significantly greater in flowers that had been visited by sunbirds than control flowers ($p=0.001$, Fig. 2A; Table 1). Sunbirds significantly reduced the volume of nectar both in flowers that had nectar barriers present and in the treatments where these were removed ($p=0.001$, Table 1). The sunbirds consumed a median of 106.8 µl (UQ=154.22, LQ=40.90, IQR=113.32) from flowers with nectar barriers present and a median of 158.03 µl (UQ=188.60, LQ = 127.82, IQR=60.80) where nectar barriers were absent. In both control treatments with no sunbirds the median nectar values where negative indicating that nectar was produced. The interaction effect between the two main factors was marginally non-significant ($p=0.062$, Table 1), but was significant when using Monte–Carlo *p*-values ($p=0.046$). However, post-hoc tests indicated that sunbirds consumed significantly more nectar from flowers without nectar barriers ($t_{30}=2.21$, $p<0.05$). Sunbirds consumed on average 93.7% of the nectar in flowers where nectar barriers were removed and 63.4% where nectar barriers were present. There was no difference in the amount of nectar produced by control flowers with or without nectar barriers ($t_{22}=0.40$, $p>0.5$, Fig. 2A). The final sample size for nectar concentration was lower than that for nectar volumes as we could not measure the nectar concentration

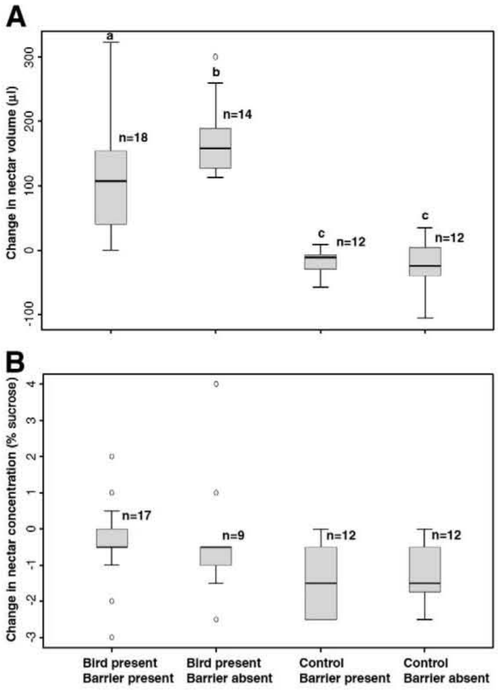

Fig. 2. Change in (A) nectar volume and (B) nectar concentration of experimental and control flowers (open circles indicate outliers). The negative values in control flowers indicate that these typically contained a greater volume or more dilute nectar in the final measurements (Letters indicate significant differences using post-hoc *t*-tests.

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Table 1
Summary of non-parametric ANOVA (PERMANOVA) testing for the effect that nectar barriers have on limiting the intake of nectar by sunbirds.

Volume	SS	df	MS	*F*	*p*
Bird present/control	52898.44	1	52898.44	103.40	0.001 (0.001)
Barrier present/absent	1753.20	1	1753.20	3.43	0.077 (0.056)
Bird*barrier pres./abs.	1921.35	1	1921.35	3.76	0.062 (0.046)
Residual	25611.62	52	492.53		
Total	82184.61	55			
Concentration					
Bird present/control	6.81	1	6.81	6.56	0.014 (0.007)
Barrier present/absent	0.33	1	0.33	0.32	0.57 (0.57)
Bird*barrier pres./abs.	0.0028	1	0.0028	0.0027	0.96 (0.97)
Residual	47.42	46	1.03		
Total	54.56	49			

p-values in brackets are Monte–Carlo *p*-values.

of 6 flowers ($n=1$ for nectar barriers present; $n=5$ for nectar barriers absent) where all the nectar was consumed by visiting sunbirds. The only significant difference in nectar concentration was between control and experimental flowers ($p=0.014$) as the control flowers had significantly more dilute nectar (median change$_{\text{bird present}}=-0.50$ (UQ=0.0, LQ=−1.0, IQR=1.0), median change$_{\text{control}}=-1.50$ (UQ=−0.5, LQ=−2.0, IQR=1.5). We did not perform any post-hoc tests on nectar concentration differences as none of the changes in concentration were considered extreme enough to suggest that significant evaporation had taken place.

4. Discussion

Our results indicate that sunbirds significantly reduce the standing crop of nectar following visits to the flowers of *S. reginae* while not contributing to the pollination of this species. The behaviour of sunbirds indicates that they are nectar thieves and can manipulate the nectar barrier with their beaks to gain access to the nectar without causing obvious damage to the flowers of *S. reginae* (pers. obs.). This differs from nectar robbing birds such as the flower pierces (genus *Diglossa*) that access nectar by piercing through the sidewall of flowers (Arizmendi et al., 1996; Traveset et al., 1998). Sunbirds consumed significantly greater volumes of nectar when visiting flowers without nectar barriers. Our field observations suggest however that sunbirds have learnt to carefully insert the beak under the convoluted nectar barriers and still drink a substantial fraction of the nectar even when nectar barriers are present. This suggests that even if sunbirds consume significantly less nectar with barriers present, the effect may be small. If nectar barriers specifically functioned to prevent nectar theft by sunbirds, we expect that the differences in volumes consumed between flowers either possessing or lacking nectar barriers would have been greater. The removal of nectar barriers did however appear to reduce the variability of nectar that was being consumed. Such a large range of volumes consumed by sunbirds visiting flowers with nectar barriers also suggests that the effect of nectar barriers *per se* at restricting the volume of stolen nectar is likely to be overshadowed by the high variation in nectar consumed by individual sunbirds (see Kohler et al., 2006). Nectar barriers may therefore be more effective against insect nectar thieves such as honeybees (*Apis mellifera*) and ants that were also observed attempting to rob nectar on warm mornings. These insects are obviously weaker than sunbirds and cannot easily access the nectar by piercing or biting through the protective petals as larger bees such as Carpenter bees (*Xylocopa* spp.) and Bumblebees (*Bombus* spp.) are capable of doing (Inouye, 1983).

Ideally we would like to have been able to demonstrate experimentally that the nectar barriers do not limit nectar foraging by the legitimate weaver bird pollinators of *S. reginae* (Skead, 1975; Coombs et al., 2007) that have short, stout bills that we believe can easily separate the nectar barriers to reach the nectary. This approach would however also have its limitations, as weavers may be relatively clumsy nectar consumers due the bill being unspecialized for nectar feeding (Oatley and Skead, 1972). Weaver visits are also sporadic and fleeting making their arrival difficult to predict. Typically a flock of weavers descend on the population and visit every flower within 5 or 10 min. This may only happen once in a week, or less frequently (Rowan, 1974; Skead, 1975; pers. obs.) so the chances of legitimate visits to prepared flowers with known nectar volumes are low. Our observations of weaver visits to the flowers of *S. reginae* indicate that weavers do not drink nectar from every flower that they visit, and nectar may be secondarily important to the main reward of pollen (Coombs et al., 2007). Therefore the absolute amount of nectar that is present is perhaps not as important as the presence of at least some nectar to prevent weavers from leaving the patch of plants in search of water after visiting several flowers. Data to support this is limited but sunbirds consumed all the nectar from 5 (36%) of the flowers without nectar barriers but only completely emptied one (6%) of the flowers with barriers present.

The morphological features of the flowers of *S. reginae* that may serve to reduce nectar thieving may also function to limit the evaporation of nectar during warm periods as has been shown by others (Petanidou et al., 2000). Natural populations of *S. reginae* occur frequently in arid succulent thicket and we therefore cannot rule out that these structures also function to limit evaporative loss of nectar. However the lack of any large positive changes in nectar concentration in the control treatment where nectar barriers were removed suggests that this is unlikely. We did however limit our observations to shorter

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periods in the morning and an experiment testing for the role of nectar barriers in preventing evaporation would have to trace flowers with both treatments (i.e. nectar barriers present and absent) throughout the day. *S. reginae* also flowers in winter when temperatures are more moderate and given that environmental temperatures may influence the volume and concentration of nectar (Nicolson and Nepi, 2005), evaporation may play little role during this time. Plants may also compensate for evaporative losses of nectar by having a constant rate of secretion (Nicolson and Nepi, 2005), and may increase secretion when nectar is removed (Navarro, 1999). Judging from the above evidence, nectar barriers may function to limit nectar thievery in *S. reginae*, but are less effective at limiting nectar theft by the primary nectar thieves — sunbirds. Comparative studies are however lacking and it would be interesting to see other studies addressing the same question on other flowers that are subject to frequent nectar pilfering. Our study serves to demonstrate the importance of carrying out manipulative experiments to show that structures commonly invoked to prevent nectar theft really do limit the volume of nectar that thieves consume.

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Appendix 1B:

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PLATE …….

CHLOROCYATHUS LOBULATA

Eastern Cape, South Africa

APOCYNACEAE

Chlorocyathus lobulata (Venter and R.L.Verh.) Venter, comb. nov. in South African Journal of Botany 74: 288 (2008); *Raphionacme lobulata* (Venter and R.L Verh.): 603 (1988); *Kappia lobulata* (Venter *et al.*): 529 (2006).

Chlorocyathus lobulata was first discovered by R. A. Dyer in 1936 and was found growing approximately 5 km from the Fish River mouth (Figure 1), 'near the confluence of the Kap and Fish rivers' (Venter *et al*. 2006; Venter and Verhoeven, 1988). The loose description of its original locality was one of the reasons why it took nearly 70 years for the species to be rediscovered. The type specimen, Dyer 3381, cites the locality as "4 miles from Fish River mouth, near Kap River in low forest". The plant was found growing in Euphorbiaceous thicket (Venter and Verhoeven, 1988), now called Kowie Thicket (Hoare *et al*. 2006), and was reportedly very rare as Dyer stated that "It was not seen more than the once and was rather inconspicuous, so it would need luck to rediscover it" (Dyer 1987 quoted in Venter & Verhoeven, 1988: 603). Venter revisited the type locality but was unable to find the plant and postulated that the cryptic nature of this species, coupled with large scale agricultural transformation of the habitat, had probably resulted in its disappearance (Venter & Verhoeven 1988). The latter part of Dyer's statement later turned out to be true. It was only by chance that Tony Dold, who had spent many weeks searching the Kap River Reserve unsuccessfully, found an individual in the crown of a fallen tree. In this way *C. lobulata* was rediscovered in 2003, nearly 70 years after it was originally collected.

Rediscovering *C. lobulata* was a challenge and naming it turned out to be equally difficult given its unusual characters. Professor Johan Venter immediately recognized Dyer's type specimen lodged in PRE as a new taxon within the Periplocoideae (Apocynaceae) but features such as its forest habitat, climbing habit, leaf morphology, crown-like interpetiolar stipules and the presence of hair on the inner surface of the petal excluded it from most genera (Venter *et al.* 2006). It was however, sufficiently similar to *Raphionacme* to warrant its initial inclusion in this genus and was named *Raphionacme lobulata* (Venter & Verhoeven 1988). The original classification was based on the incomplete type specimen and its re-discovery in 2003 enabled flowers and fruit to be collected for the first time. It was now apparent that *C. lobulata* was in fact distinct from *Raphionacme* and related genera such as *Batesanthus*, *Baseonema*, *Mondia* and *Stomatostemma* (Venter *et al*. 2006). Based on this new information it was described as the monotypic genus *Kappia*, named after the type locality, the Kap River reserve (Venter *et al.* 2006). The species epithet refers to the distinctive hemispherical coronal lobes that distinguish this species from *Raphionacme* (Venter & Verhoeven, 1988). Venter (2008) finally assigned the species to the genus *Chlorocyathus* that he describes as having similar fleshy root tubers and "*Raphionacme*-like" flowers (Venter 2008). The genus *Chlorocyathus* was originally described by Oliver in 1887, but was later considered by N. E. Brown (1907) to be synonymous with *Raphionacme*. The genus was resurrected by Venter (2008) to accommodate *C. lobulata* and a newly described species, *C. monteiroae*.

Habitat, distribution and ecology — *Chlorocyathus lobulata* is a rare endemic liana that has thus far only been found within the riparian forest occurring only on the eastern bank of the Kap River near its confluence with the Fish River (Venter *et al*. 2006). This is the only known population of this highly localized Albany Centre endemic (Van Wyk & Smith 2001) that occurs in an area of less than 5km^2 and has thus been assessed as Vulnerable (Victor & Dold 2002). The species does not occur throughout the forest and is restricted to mature riparian forest and is uncommon at the forest edge. It is however not confined to the immediate vicinity of the river and is frequently found growing on the steep inclines of the river valley. Within the forest *C. lobulata* grows in common forest trees such as *Trichocladus ellipticus, Euphorbia triangularis, Harpephyllum caffrum*, *Celtis africana* and *Mimusops obovata*. The maximum height of mature individuals varies between 12-15 m but this depends on the size of supporting tree (Coombs pers. obs. 2009). The thickened stems of large individuals are sometimes seen hanging from trees and climbing for several metres into the forest canopy where plants typically flower. Individuals growing on smaller trees may also flower at lower levels under the forest canopy (Coombs pers. obs. 2009). Stems may be identified by the characteristic interpetiolar stipules that form a crown of tooth-like projections surrounding the stem (Venter *et al*. 2006). Interpetiolar stipules are more distinct in younger parts of the stem. The fleshy tubers (Plate 1) are frequently hidden below ground, but may be seen when they are exposed above ground in areas with a steeper slope where the underlying substrate is rockier.

Pollination biology and flower herbivory —Flowering starts in January and continues until late April. Pollen is contained in 5 cup-like translators that are arranged radially within the flower and surround the gynostegium (Venter *et al*. 2006). Translators overlay the stigmatic surface and contain approximately 300 pollen tetrads per translator (Coombs *et al*.

unpublished data.). Observations of pollinators indicate that this species is pollinated by flies that visit the small, bright green flowers (Plate 1: 5). Flowers attract flies by emitting a pleasant fruity scent similar to that of watermelon. Flies visiting the flowers probe into the cavities separating the fleshy coronal lobes (Plate 1: 5), and in so doing remove the entire pollinarium when the translator attaches to the proboscis of the pollinating insects by means of a white, sticky pad similar to the viscidium found in orchid pollinaria (Coombs *et al*. in prep.; Endress, 2001; Johnson & Edwards, 2000). Pollinarium removal also exposes the stigmatic surface onto which pollen is deposited when a fly carrying pollinaria probes the flower. Flies that have been found carrying pollinaria include one individual of a species *Degenea* (Tachinidae), one *Ceratitis* species (Tephritidae) and an unidentified species of Tachinidae.

Most inflorescences are infested by larvae of the moth *Bocchoris onychinalis* (Pyralidae-Pyraustinae). Initial signs of infestation are a blackening of the corona lobes after which the entire flower is either consumed or becomes a dark brown-black colour (Coombs *et al*. in prep.). Small, light-green larvae are sometimes seen on infested flowers and buds, spinning loosely woven silk strands between the flowers and buds. Data by Coombs *et al*. (in prep.) collected during 2009 indicate that approximately 80% of inflorescences are infested with larvae and due to these larvae consuming flowers and a large proportion of buds.

Description (partly after Venter *et al.* 2006).—Perennial liana up to 15 m long. *Roots* slender, with tubers strung along the roots (Plate 1: 1). *Stems* up to 15 mm diameter. *Leaves* simple, opposite, glabrous, petiolate; petiole glossy, reddish-brown, 8–12 mm long; interpetiolar stipules fleshy, sub-spherical, dentate; blade ovate to elliptic, coriaceous, 60–70×20–35mm, adaxial surface glossy, dark green, abaxial surface pale green, margin entire

(Plate 1: 2). *Inflorescence* cymose, monochasial branches up to 10-flowered (Plate 1: 2). *Flowers* actinomorphic, bisexual, pentamerous (Plate 1: 5). *Sepals* free, broadly triangular, 2–3×2 mm, margins membranous to ciliate, apex acute (Plate 1: 2, 5). *Corolla* funnel-shaped (Plate 1: 3), 6–10 mm long, semi-succulent; tube 2–3 mm long, glabrous; lobes ovate, 4–7×3–5 mm, apex acute, outside glabrous, apple green flushed pale reddish-brown towards the base, hirsute on inside with white hairs, apple green, hirsute (Plate 1: 4). *Corona:* columns: 5, yellow, fleshy; lobes from apices of coronal columns, broadly obcordate, 0.5–1.0×2 mm, yellow-green, tinged pale maroon (Plate 1: 5). *Stamens* directly below corona lobes; filaments fused to inner base of coronal columns, linear, ±0.5 mm long; anthers fused to stylehead, angular-ovate, ±1 mm long. *Fruit* paired, 45° divergent follicles, or single and erect as illustrated here (Plate 1: 4); follicles ellipsoid with rounded apices, 65–80×23–25 mm, smooth, glossy green becoming longitudinally wrinkled and pale straw coloured when ripe. *Seeds* oblong-obovate, flattened, concavo-convex, 10–13×3–5 mm, yellow-brown becoming dark brown, rugulose with dark central ridge along the inner surface. Plate 0000.

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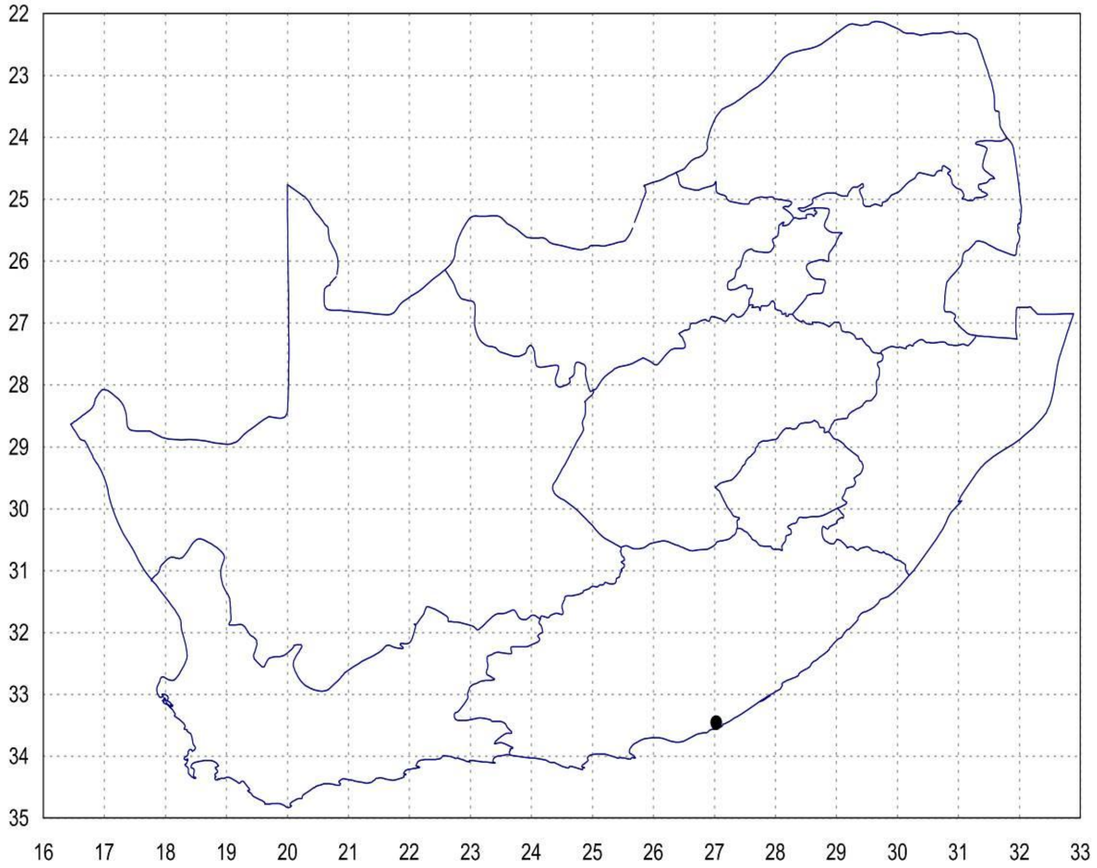

FIGURE 1.—Known geographical distribution of *Chlorocyathus lobulata.*

Plate0000.—1, Fleshy root tuber , x 0.5; 2, young stem showing interpetiolar stipules and inflorescence, x 1; 3, Longitudinal section through flower showing funnel shaped corolla; 4, fruit with paired follicles, x 2; 5, Flower showing distinctive coronal lobes, x 6;. Voucher Specimen: Dold 4461 in Selmar Schonland Herbarium (GRA), Grahamstown. Artist: Susan Abraham.