

Evaluation of the floral rewards of
Aloe greatheadii var davyana
(Asphodelaceae), the most important indigenous
South African bee plant

by

Hannelie Human

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Philosophiæ Doctor (Entomology)

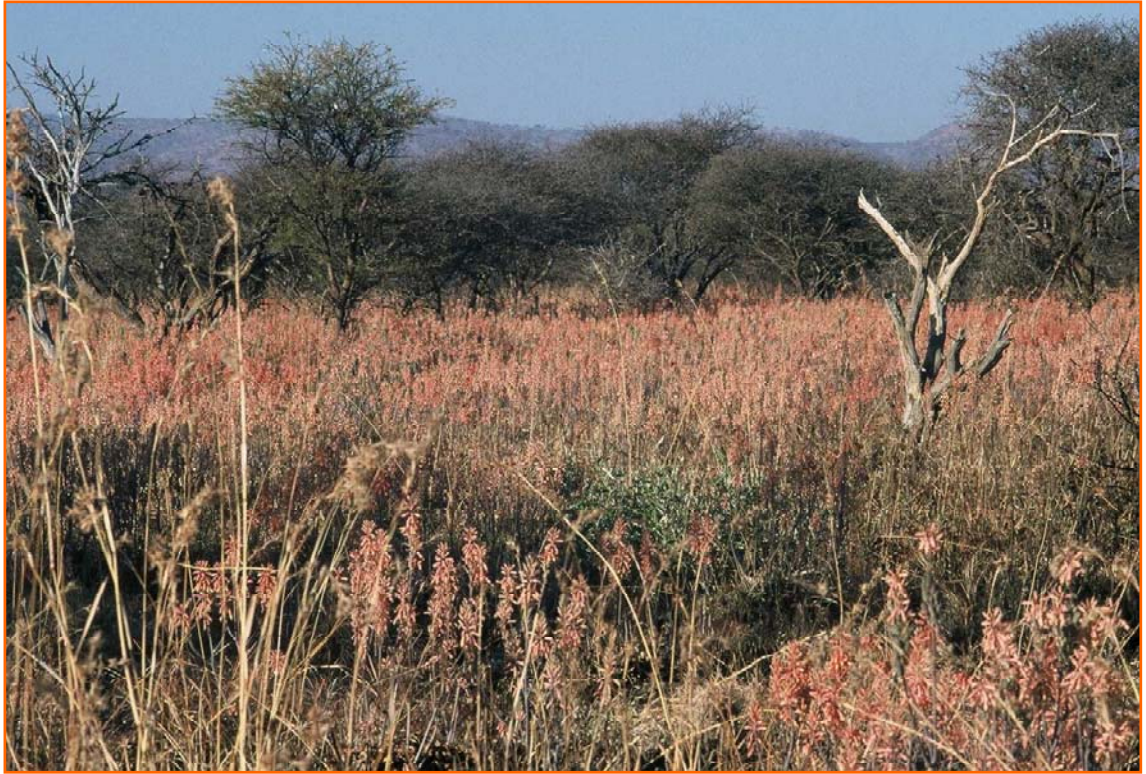
In the Faculty of Natural and Agricultural Science

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For my husband and children



"The larger the island of knowledge, the longer the shoreline of wonder" (Smith, 1996:4)

Smith, H. (1996) Foreword. In: Johnston, W. (Ed) *The Cloud of Unknowing and the Book of Privy Counselling*. Image Books, New York.

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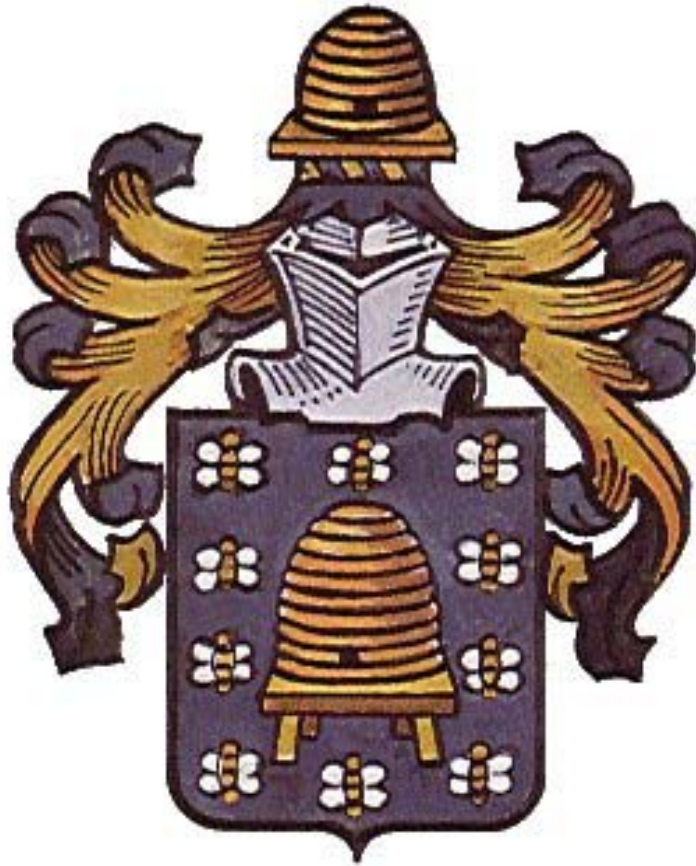
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Bezuidenhout coat of arms
(My maiden name was Bezuidenhout)

**Evaluation of the floral rewards of *Aloe greatheadii* var *davyana*
(Asphodelaceae), the most important indigenous South African bee plant**

Student: Hannelie Human
Supervisor: Prof. Sue W. Nicolson
Department: Zoology and Entomology, University of Pretoria
Degree: Philosophiae Doctor

Abstract

The most important indigenous bee plant in South Africa is the winter flowering *Aloe greatheadii* var *davyana*, with a widespread distribution across the summer rainfall region. Beekeepers commonly move their hives to the "aloe fields" during winter, using the strong pollen and nectar flow for colony growth, queen rearing and honey production. In spite of its importance for the bee industry, no complete pollen analysis is available and, except for the popular bee literature, little is known about nectar production or pollinators. The aim of the study was therefore to evaluate the floral rewards of this aloe and to investigate the importance of these resources for honeybees.

We analysed fresh, bee-collected and stored aloe pollen for its nutritional content (not previously done for any plant species). Addition of nectar and glandular secretions leads to an increase in water and carbohydrate content and a decrease in protein and lipid content. All the essential amino acids, except tryptophan, met or exceeded the minimum levels for honeybee development. In worker bees in queenright colonies, ovarian development is greater on aloe than on sunflower pollen, which may be explained by the exceptionally high protein content and high extraction efficiency during digestion.

In assessing the nectar resource, we investigated the nectary structure and nectar presentation of two species belonging to different sections of the genus *Aloe*, *A. castanea* and *A. greatheadii* var *davyana*, but anatomical differences were not related to the nectar production. We looked at variation in nectar volume and concentration of *A. greatheadii* var *davyana* on various levels, from within the flowers to across the summer rainfall area. Nectar was continuously available and, although dilute (mean

concentration 18.6%), the nectar of *A. greatheadii* var *davyana* is more concentrated than that of other *Aloe* species, making it an ideal source of energy and water for honeybees. Utilisation of dilute nectar by bees requires elimination of much excess water. We sampled crop contents of nectar foragers to determine if changes in nectar concentration occurred after collection and before unloading in the hive. Contrary to the common assumption that nectar is either unchanged or slightly diluted during transport, we observed a dramatic increase in concentration and a decrease in volume between the flowers and the hive. Bees may be foraging primarily to get enough water for their physiological needs. Using miniaturised data loggers, we showed that bees are able to adjust nest humidity within sub-optimal limits, in addition to efficient regulation of hive temperature. Humidity levels are influenced by trade-offs with regulation of temperature and respiratory gas exchanges.

Although the dilute nectar and pinkish red tubular flowers are characteristic of bird-pollination, exclusion experiments showed that bees are the primary pollinators of *A. greatheadii* var *davyana*. This contrasts with other *Aloe* species which are pollinated by sunbirds and other passerine birds, but highlights the two-way interaction between the bees and the aloes.

Table of contents	Page
Acknowledgements	3
Abstract	4
Table of contents	8
General Introduction	10 - 19
References	20 - 23
Chapter 1: Nutritional content of fresh, bee-collected and stored pollen of <i>Aloe greatheadii</i> var <i>davyana</i> (Asphodelaceae).	24 - 38
References	39 - 42
Chapter 2: Influence of <i>Aloe greatheadii</i> var <i>davyana</i> (Asphodelaceae) pollen quality on ovarian development in honeybees, <i>Apis mellifera scutellata</i>	43 - 55
References.....	56 - 58
Chapter 3: Nectary structure and nectar presentation in <i>Aloe castanea</i> and <i>A. greatheadii</i> var <i>davyana</i> (Asphodelaceae)	59 - 74
References	75 - 76
Chapter 4: Dilute nectar of <i>Aloe greatheadii</i> var <i>davyana</i> as a resource for honeybees, <i>Apis mellifera scutellata</i> , during dry South African winters.....	77 - 95
References	96 - 98
Chapter 5: Do honeybees, <i>Apis mellifera scutellata</i> , eliminate excess water from the dilute nectar of <i>Aloe greatheadii</i> var <i>davyana</i> before returning to the hive?.....	99 - 112
References	113 - 116

Chapter 6: Do honeybees, <i>Apis mellifera scutellata</i> , regulate humidity in their nest?	117 - 125
References.....	126 – 127
Chapter 7: Appearances can be deceiving: insect-pollinated <i>Aloe greatheadii</i> var <i>davyana</i> has an ornithophilous pollination syndrome	128 - 143
References	144 - 145
General conclusion	146 - 151
References	152 - 154
Appendix: Cytological features of <i>Aloe greatheadii</i> var <i>davyana</i> pollen: a comparison between fresh, bee-collected and stored pollen	155 - 161
References	162 - 163

GENERAL INTRODUCTION

Honeybee flora of South Africa: exotic and indigenous plants

Honeybees visit about 1000 plant species in South Africa for pollen and/or nectar. Only half of these plant species are indigenous (Illgner, 2002). Many South African beekeepers migrate with their hives over distances of hundreds of kilometres to certain crops as they flower, and use minor nectar sources to bridge periods between major nectar flows (Johannsmeier, 2001). For example, in the northern summer rainfall area, the cycle begins in spring with certain *Eucalyptus* species, followed by *Faurea saligna* (boekenhout) and *Fagopyrum esculentum* (buckwheat) in December. In January honeybees are used to pollinate *Phaseolus coccineus* (kidney beans) and *Helianthus annuus* (sunflowers) and in autumn *Cosmos bipinnatus* (cosmos) is available. *Eucalyptus grandis* flowers from April onwards while July and August are the important *Aloe* months (Keats, 1980).

The annual honey crop in South Africa is estimated at 3 500 tons, of which 1 800 tons is derived from *Eucalyptus*, 900 tons from crop plants and the remainder from weeds, indigenous and other plants (Johannsmeier, 2001). Of the exotic bee plants, the single most important nectar source for bees is *Eucalyptus* and beekeeping is considered impossible without it. There are 34 *Eucalyptus* species listed as honey plants in South Africa. These trees are highly attractive nectar and pollen producers, grow under a variety of conditions and have a widespread distribution with a year-round flowering period (Johannsmeier, 2001; Illgner, 2002). In South Africa a timber industry, based on fast growing trees such as pines and eucalypts, was established at the end of the nineteenth century, and by the early twentieth century the majority (80%) of trees grown were *Eucalyptus grandis*. These plantations provided additional and more reliable sources of nectar than the indigenous flora. However, since 1975 *Eucalyptus* nectar flows have declined to about one third of the previous average. Various factors may contribute to this deterioration; *Drosophila flavohirta* larvae utilising the nectar in *Eucalyptus* flowers (Herrmann, 1983; Nicolson, 1994) and the leaf sucking bugs *Thaumastocoris australicus* that cause defoliation, dieback of branches and even death of *Eucalyptus* trees (Jacobs & Naser, 2005). *Thaumastocoris australicus* may even

affect the flowering and nectar production of infested *Eucalyptus* trees (D. Jacobs, pers. comm.). Another explanation may be the genetic make up of the trees currently planted in plantations; *Eucalyptus* with fewer flowers are preferred, thus less nectar is available to honeybees (A. Schehle, pers. comm.). Government regulations (the Working for Water Programme) provide for the removal of certain *Eucalyptus* species in water catchment areas and along watercourses, and this presents another threat to beekeepers (Johannsmeier, 2001).

Of the indigenous plants, the major producers of honey are firstly the aloes, of which *A. greatheadii* var *davyana* is the most important species, followed by boekenhout (*Faurea saligna*), wilde peer (*Dombeya rotundifolia*), karee (*Rhus lancea*), wilde sering (*Burkea africana*), wit-olyf (*Cordia caffra*), as well as several *Acacia* and *Protea* species (Beyleveld, 1969; Fletcher & Johannsmeier, 1978; Schonfeld, 1983; Johannsmeier, 2001). An extensive list of bee plants in South Africa, including their distribution as well as flowering phenology, has since been compiled by Johannsmeier (2001) and Illgner (2002).

The genus *Aloe*

The genus *Aloe*, family Asphodelaceae, occurs across a wide range of habitats, from dry forests to scrublands in Africa, Madagascar, Arabia, the Canary and the Comoro islands. South Africa has the highest diversity of *Aloe* species with more than 100 species (Van Wyk & Smith, 1996; Glen & Hardy, 2000; Smith et al., 2000). These succulent plants grow well in warm climates and can tolerate drought. Few species, however, can withstand frost. The huge variation in size, leaf width, leaf markings, etc., has led to the division of the genus into 26 sections (Reynolds, 1969; Van Wyk & Smith, 1996; Glen & Hardy, 2000). The section Pictae (spotted aloes) is the largest, consisting of 32 species. These aloes are stemless or short-stemmed, have inflorescences that are branched and re-branched and their flowers, with conspicuous basal swellings, often have pale longitudinal stripes. Species are difficult to distinguish from each other e.g. the summer flowering *Aloe zebrina* and winter flowering *A. greatheadii* var *davyana* (Schönland) Glen & D.S. Hardy (Van Wyk & Smith, 1996; Glen & Hardy, 2000).

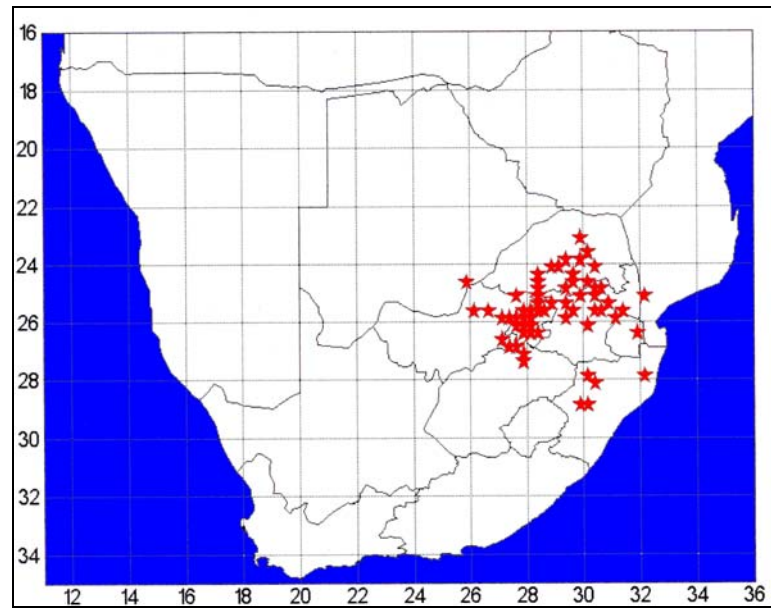
Aloe greatheadii* var *davyana* and honeybees, *Apis mellifera* *scutellata

The winter flowering *A. greatheadii* var *davyana* (Fig. 1) has a widespread distribution across the summer rainfall area (Fig. 2) and is very common in the Bushveld and on the Witwatersrand (Glen & Hardy, 2000; Van Wyk & Smith, 1996; Smith & Crouch, 2001). The plants grow well in rocky terrain and on grassy plains and occurring most densely in overgrazed areas (Clark, 1992). Plants are robust and grow singly or in groups of up to fifteen. They flower prolifically in mid-winter, from June to August, with flower colour ranging from pale pink to bright red. Flower abundance may vary throughout the flowering period and from year to year. Pronounced daily temperature changes characterise the winter flowering period with warm days and cold, sometimes frosty, nights.



Figure 1. *Aloe greatheadii* var *davyana*

A



B



Figure 2. (A) Distribution map of *Aloe greatheadii* var. *davyana* (redrawn from Glen & Hardy, 2000) and (B) map of South Africa (redrawn from www.safarinow.com) indicating study sites used in this study: 1. Rooideplaat Nature Reserve, 2. Rust de Winter, 3. Marble Hall and 4. Zeerust.

Aloe greatheadii var *davyana* is a major indigenous beeplant and it is widely known that beekeepers move their hives to the aloe fields north of Pretoria in winter (see Fig.3) to make use of the strong nectar and pollen flow from *A. greatheadii* var *davyana* (Fletcher & Johannsmeier, 1978). The first report on the utilisation of this aloe was that of Mr Krohn from Rustenburg in 1934 (Williams, 2002). However, it was only in 1950 that Mr E.A Schnetler realised the commercial value of *A. greatheadii* var *davyana*. While transporting bees along the Warmbaths (now Bela Bela) road to a new site, his truck broke down and the bees had to be unloaded. When it was time to reload the hives he realised that the bees were collecting pollen and nectar from *A. greatheadii* var *davyana* growing in the vicinity (Short, 1962; Keats, 1980).



Figure 3. Beehives among *A. greatheadii* var *davyana* in the Rust de Winter area in 2005.

Apparently honeybees become particularly aggressive and unmanageable during this nectar flow (Fletcher & Johannsmeier, 1978). Doull (1976) believed that this behaviour might be caused by certain properties of the pollen; he considered *A. greatheadii* var *davyana* to be a good source of pollen but a poor source of nectar. He observed few bees collecting nectar during the day, in spite of nectar being readily available, and suggested that the aggressive behaviour of honeybees is the result of the amount of food available and natural behavioural patterns of bees. On the other hand, Johannsmeier (2001) considers the nectar to be of medium to good quality. The best nectar and pollen is apparently produced from approximately July 17 to August 25 each year, and

thereafter the quality deteriorates; after this period beekeepers move their hives to the next available food source e.g. *Eucalyptus* or *Citrus* (J. Williams, pers. comm.).

Currently the entire beekeeping industry, as well as the agricultural industry in South Africa that depends on pollination services of *Apis mellifera scutellata* honeybees, is at threat by *A. mellifera capensis* bees. Prior to the translocation of *A. mellifera capensis* in 1991 into the interior of South Africa, *A. mellifera capensis* (Cape honeybees) and *A. mellifera scutellata* (African honeybees) remained separate races of honeybees. *Apis mellifera capensis* were only found along the Cape coast, their distribution roughly corresponding to that of the fynbos vegetation, but they are now distributed throughout the country. These two races of bees are incompatible since *A. mellifera scutellata* queens are unable to control and prevent *A. mellifera capensis* workers from reproducing. *Apis mellifera capensis* workers rapidly become laying workers and as soon as they start laying eggs, the African bees start looking after these bees as though they were queens, neglecting their own queen, which eventually dies. The new “queens” are not able to manipulate the colony with pheromones, thus causing the colony to dwindle and eventually die. The rich nectar and pollen flow from *A. greatheadii* var *davyana* seems to promote the *A. mellifera capensis* take-over. When *A. mellifera scutellata* beehives are moved to the aloes in winter, *A. mellifera capensis* are able to spread between these hives and apiaries (Allsopp, 1998; Kryger et al., 2000). Pollen of the aloes also activates the ovaries of *A. mellifera capensis* workers even in the presence of the queen (Kryger, et al., 2000).

In an attempt to eliminate and control the problem, the government divided the country into two sectors and prohibited movement of the two races of bees between these sectors (Johannsmeier, 2001). All *A. mellifera capensis* colonies and infested colonies north of the dividing line were legally required to be destroyed. Thousands of colonies have been lost due to this infestation, and many commercial beekeepers have been forced out of business. Beekeepers that lost their stock were financially supported in order to re-establish *A. mellifera scutellata* beekeeping but attempts to manage this problem have been unsuccessful and research will have to provide a permanent solution (Johannsmeier, 1997, 2001; Allsopp, 1998). Unfortunately, some wild populations of *A. mellifera scutellata* have also become infested with *A. mellifera capensis* (Johannsmeier, 1997).

Is there anything special about *Aloe greatheadii* var *davyana* pollen and nectar?

Beekeepers use the strong nectar and pollen flow of *A. greatheadii* var *davyana* not only for honey production but also to build up colonies, rear queens and increase colony numbers by division (Jackson, 1979; Williams, 2002). Pollen quality and quantity have a direct effect on the productivity of a bee colony. Proteins and amino acids are important for the growth and development of bees and insufficient quantities of protein (< 20% dry mass) may affect reproduction, brood rearing and longevity of honeybees and subsequently honey production (Kleinschmidt & Kondos, 1978; Moritz & Crailsheim, 1987). Pollen lipids are a source of energy and are important for the synthesis of reserve fat and glycogen as well as for the production of royal jelly (Singh et al., 1999; Loidl & Crailsheim, 2001; Manning, 2001).

Aloe greatheadii var *davyana* aloes are a major pollen source, and since the anthers are exerted beyond the floral tubes the orange pollen is readily available to honeybees. Very little is known about the pollen except that it has a high protein content (33.8% on a dry mass basis, based on a single measurement; Johannsmeier, 2001), and aloes in general are starchless according to Franchi et al. (1996). Beekeepers describe *A. greatheadii* var *davyana* pollen as “dry” with a sweet taste to it and the nectar as “reasonably dense and sweet” (Doull, 1976; Schönfeld, 1983; Williams, 2002).

It is possible that substances such as protein in the pollen may have a direct effect on the ovarian development of workers honeybees (Kryger, P., Wossler, T.C., Crewe, R.M., Moritz, F.A. & Johannsmeier, M.F., unpublished data) who are able to start reproducing in the absence of a queen (Velthuis, 1970). The relationship between dietary protein and ovarian development in adult workers has been well documented. Protein-rich diets promote ovarian development and oogenesis is restricted by a lack of protein (Hoover et al., 2006). Ovarian development is not only influenced by the quality of the diets but may also be affected by the time of year (Hoover et al., 2006). In the summer rainfall areas, cold winter nights (often below freezing) and low ambient temperatures early in the mornings result in overcrowding of the brood nest as well as delayed foraging; thereby aggravating the swarming tendency (a period with little or no brood) on the aloes (Steinhobel, 1976) thus allowing workers to use food reserves for development of their ovaries instead of taking care of the brood.

The other floral reward is nectar. Nectar concentrations can vary between 7% and 70% and great variation exists not only between species but also within species (Nicolson, 1998; Nepi et al., 2001). The mean nectar concentration tend to be less in flowers from the tropics and higher in hot and dry climates (Willmer & Stone, 2004). Composition and production rates of nectar vary with time of day, flower age, nutritional status of the plant and even location of the flower on the plant, and are also influenced by environmental parameters such as temperature and relative humidity (Corbet et al., 1979; Nicolson, 1994; Vesprini et al., 1999; Nepi et al., 2001).

Nectar of bird-pollinated flowers is usually relatively dilute. Birds are closely associated with aloes (Oatley & Skead, 1972) such as *A. ferox*, which has nectar with low concentrations (12.5%) and large volumes per flower (180 µl) (Hoffman, 1988). *Aloe* species produce very dilute (10-15%), hexose-dominant nectars (Van Wyk et al., 1993; Nicolson, 2002). The average nectar concentrations of bee-pollinated flowers tend to exceed 35% (Nicolson, 1998) and although honeybees collect nectar with a wide range of sugar concentrations (from 12-65% w/w, or 0.5 to 2.5M) they prefer nectar with 30-50% sugar content (Southwick & Pimentel, 1981). However, when honeybees need to cool the hive (through evaporation) they will collect nectar with lower concentrations or collect water. Honeybees are known to collect more dilute nectars during the dearth period in Israel (Eisikowitch & Masad, 1982). Since *A. greatheadii* var *davyana* flowers during the dry winter months the dilute aloe nectar can thus also serve as a source of moisture (Van Wyk et al., 1993; Tribe & Johannsmeier, 1996). This nectar contributes substantially to the honey crop (Williams, 2002); therefore large quantities of the dilute nectar must enter the hive and consequently may affect humidity regulation in the hive. Transforming nectar into ripe honey requires elimination of excess water. Utilisation of the dilute nectar of *A. greatheadii* var *davyana* (with a water content of 77%; Human and Nicolson, unpublished data) will thus require significant evaporation. Bees could begin this process en route to the hive, prior to the unloading of their crop contents. However, since the study by Park (1932), it has generally been assumed that changes in nectar concentration only occur in the hive during storage and the honey ripening process.

Pollination ecology

Animal pollinated flowers possess certain floral features such as colour, structure and scent that are believed to reflect the preferences of their pollinators. These associations were described by Faegri and Van der Pijl (1979) and became known as “pollination syndromes”. They included nectar volume in their descriptions while Baker and Baker (1983) extended the whole concept to include nectar composition. Pollination syndromes have been helpful in understanding plant-pollinator interactions. Recognition of certain floral features and reward types can help to predict pollinators but has to be applied critically otherwise it can be misleading. Pollinators are not restricted to visiting only one kind of flower; birds may sometimes visit flowers that are not typical bird flowers, while bees and butterflies may visit ornithophilous flowers (Robertson et al., 2005). As a result, flowers may be visited by a wide variety of pollinator types but may actually only be effectively pollinated by some visitors (Robertson et al., 2005). Most aloes appear to be typical bird pollinated plants, with red-orange tubular flowers with dilute nectar, but they may also be pollinated by bees and other insects (Stokes & Yeaton, 1995).

Few studies are available on pollination ecology of aloes (Holland, 1978) and little is known about nectar secretion rates, time of anthesis or visitors to most *Aloe* species. Although bees are noted visitors to aloes their contribution to pollination of *Aloes* has seldom been investigated as aloes were classically considered to be bird pollinated. Studies by Stokes and Yeaton (1995) and Ratsirarson (1995) showed that birds and not insects, were the primary pollinators of *A. candelabrum* and *A. divaricata* respectively while Tribe and Johansmeier (1996) consider sunbirds and honeybees as the major pollinators of three species of tree aloes, *A. dichotoma*, *A. pillansii* and *A. ramosissima*. The red tubular flowers of *A. ferox* indicate sunbird pollination but a study by Hoffman (1988) showed that both birds and bees may be pollinators of this aloe. The pinkish-red colour, tubular structure and lack of scent of *A. greatheadii* var *davyana* flowers, as well as the copious and relatively dilute nectar, fits the bird pollination syndrome (Faegri & Van der Pijl, 1979). However, since honeybees are known to utilise *A. greatheadii* var *davyana* pollen and nectar extensively they may also contribute to the pollination of this aloe.

Thesis organisation

The aim of this study was to evaluate the floral rewards of *Aloe greatheadii* var *davyana* (Asphodelaceae). In spite of the importance of *A. greatheadii* var *davyana* for the beekeeping industry, no complete analysis of its pollen is available and except for the popular bee literature little is known about its nectar concentration and production. It is also unknown whether honeybees, *Apis mellifera scutellata*, are the primary pollinators of *A. greatheadii* var *davyana*.

Each chapter is presented as a research article and consequently there is some overlap of information and references. I did not write the thesis in the first person but used the term "we" throughout the thesis due to the fact that some of the chapters are already published and that I were unable to do all the bee fieldwork on my own.

Chapter one (published as Human & Nicolson, 2006, *Phytochemistry*) deals with the nutritional value of fresh, bee-collected and stored pollen of *A. greatheadii* var *davyana*, examining whether there is anything special about the pollen that causes the rapid build-up of colonies when feeding on this aloe. Chapter two investigates the influence of pollen of *A. greatheadii* var *davyana* and sunflower, *H. annuus*, on ovarian development in normal queenright colonies in the field and compares the extraction efficiency of the two types of pollen. Chapter three (published as Nepi et al., 2006, *Plant Systematics and Evolution*) compares the nectary structure and nectar presentation in *A. castanea* and *A. greatheadii* var *davyana*. Chapter four deals with nectar production patterns of *A. greatheadii* var *davyana* and the dilute nectar as a resource for honeybees, *A. mellifera scutellata*, during dry winters. Chapter five investigates whether honeybees, *A. mellifera scutellata*, eliminate excess water from the dilute nectar of *A. greatheadii* var *davyana* before returning to the hive. Chapter six (published as Human et al., 2006, *Naturwissenschaften*) tries to answer the question whether honeybees, *A. mellifera scutellata*, regulate humidity in their nest and whether the dilute *A. greatheadii* var *davyana* nectar affects it. Chapter seven investigates the importance of birds and bees as pollinators of *A. greatheadii* var *davyana*, which exhibits an ornithophilous pollination syndrome. The general conclusion synthesises the main finding of the above mentioned chapters and provides ideas for future research.

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CHAPTER 1

Nutritional content of fresh, bee-collected and stored pollen of *Aloe greatheadii* var *davyana* (Asphodelaceae)

H. Human and S. W. Nicolson

Department of Zoology and Entomology, University of Pretoria, Pretoria 0002, South Africa



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Abstract

Aloe greatheadii var *davyana* is the most important indigenous South African bee plant. Fresh, bee-collected and stored pollen of this aloe was collected and analysed for its nutritional content, including amino acid and fatty acid composition. Highly significant differences were found between the three types of pollen. Collection and storage by the bees resulted in increased water (13 to 21% wet wt) and carbohydrate content (35 to 61% dry wt), with a resultant decrease in crude protein (51 to 28% dry wt) and lipid content (10 to 8% dry wt). Essential amino acids were present in equal or higher amounts than the known required minimum levels for honeybee development, with the exception of tryptophan. Fatty acids comprised a higher proportion of total lipid in fresh pollen than in bee-collected and stored pollen. This study is the first to compare the changes that occur in pollen of a single plant species after collection by honeybees.

Introduction

In South Africa, the winter flowering *Aloe greatheadii* var *davyana* (Schönland) Glen & D.S. Hardy is the most important indigenous plant utilised by migratory beekeepers (Johannsmeier, 2001). This species belongs to the largest section of the succulent genus *Aloe*, the Pictae or spotted aloes (Glen & Hardy, 2000). It has a widespread distribution across the northern summer rainfall area of South Africa, being very common in the bushveld and on the Witwatersrand (Short, 1962; Smith & Crouch, 2001). Migratory beekeepers commonly move their bees (*Apis mellifera scutellata*) to the aloe fields north of Pretoria in winter, when no other food source is available, in order to make use of the strong pollen and nectar flow of *A. greatheadii* var *davyana* (Fletcher & Johansmeier, 1978). Apart from honey production, beekeepers use the pollen flow to build up colonies, rear queens and increase colony numbers. Honeybees become particularly aggressive and unmanageable during this period (Doull, 1976; Fletcher & Johansmeier, 1978). This behaviour might be caused by some properties of the pollen, or it may be natural behaviour patterns of bees in a situation of abundant food and a large amount of sealed brood (Doull, 1976).

The quantity and quality of pollen collected by honeybees affects reproduction, brood rearing and longevity, thus ultimately the productivity of the colony (Kleinschmidt & Kondos, 1978). Apart from small quantities in nectar (Baker & Baker, 1983), honeybees obtain all the proteins, lipids, minerals and vitamins they need for brood rearing, adult growth and development from pollen (Day et al., 1990; Loidl & Crailsheim, 2001). The proportions of these nutrients can vary widely among pollens of different plant species (Todd & Bretherick, 1942; Stanley & Linskens, 1974; Roulston & Cane, 2000), but few complete analyses are available for the chemical composition of pollens.

Pollen analyses are generally carried out on bee-collected pollens because of the ease of collection (Stanley & Linskens, 1974). Bees do not consume fresh pollen. During collection and storage the pollen composition is changed through the addition of mainly nectar, but also glandular secretions (Winston, 1987; Roulston, 2005) and together with a specific bacterial flora associated with stored pollen, this increases the digestibility and nutritive value of pollen for honeybees (Herbert & Shimanuki, 1978). Addition of nectar to fresh pollen will affect all values obtained by chemical analysis, as illustrated

by the data of Louw and Nicolson (1983), who compared the chemical composition of *Virgilia divaricata* (Fabaceae) pollen with the pollen paste used by the carpenter bee, *Xylocopa capitata*, to provision larval cells.

Even though *A. greatheadii* var *davyana* is regarded as a very important bee plant, no analysis of its pollen is available, except for a single protein measurement (33.8% dry mass) (Johannsmeier, 2001). The only other nutritional information available for *Aloe* pollen is that *A. ferox* pollen has a crude protein content of 47% (Roulston et al., 2000) and that *Aloe* pollen in general, is starchless (Franchi et al., 1996). Pollen analysis is used here to determine the nutritional value of *A. greatheadii* var *davyana* pollen for honeybees, and to investigate whether there is anything special about the pollen that causes the rapid buildup of colonies. We have compared pollen from the flower, the bee corbiculae and the hive with each other and with *Eucalyptus* pollen. *Eucalyptus* species in South Africa provide more than 50% of the country's annual honey crop (Johannsmeier, 2001) and the abundant pollen and nectar also leads to strong build up of honeybee colonies (Fletcher & Johannsmeier, 1978).

Methods

Study site and plant species

Six beehives were moved in June 2005 to Roodeplaat Nature Reserve, 25km NE of Pretoria. Fresh pollen was collected from about 30000 *A. greatheadii* var *davyana* flowers by gently brushing the anthers with a small paintbrush. Bee-collected pollen was obtained directly from bees returning to the hives through a bottom fitting pollen trap. Stored pollen was removed from 10-15 adjacent cells in the brood frames of each of the six hives. These three types of pollen were subjected to scanning electron microscopy, and samples were frozen at -20°C for chemical analyses.

Scanning electron microscopy

Pollen was mounted on SEM stubs and sputter coated with gold, using a Polaron E5200 sputter coater (Watford, UK). Specimens were examined and pictures obtained with a JEOL 840 Scanning Electron Microscope (Tokyo, Japan) at the Laboratory for Microscopy and Microanalysis at the University of Pretoria.

Water content

Samples of the three pollen types (0.3 g of fresh pollen, 1 g of bee-collected and stored pollen) were dried to constant weight at 65°C to obtain water content as a percentage of fresh weight (AOAC, 2000).

Protein analysis

Crude protein content was determined, in duplicate, according to the Dumas method (AOAC, 2000). Total nitrogen content was determined using an elemental analyser (model FP-428; Leco instruments, Mississauga, Canada), calibrated against known standards. Pollen samples (0.2 g) were weighed into a combustion boat, and combusted at 950°C. To determine total crude protein, nitrogen values were multiplied by a conversion factor of 6.25 (Roulston et al., 2000).

Amino acid analysis

Pollen samples (10-20 mg) were analysed in duplicate for free amino acids as well as tryptophan in the Department of Biochemistry at the University of Pretoria. The samples were analysed by the Pico. Tag Column® method (3.9 mm x 15 cm) using a Waters HPLC amino acid analyser (Waters, Millipore Corp., Milford, MA). Samples were hydrolysed with 6N HCl, and then derivatised with phenylisothiocyanate (PITC) to produce Phenylthiocarbamyl (PTC) amino acids. These amino acids were analysed by reverse phase HPLC. Buffers used were 0.14M sodium acetate trihydrate and water-acetonitrile (60:40). Absorbance was detected at 254 nm using a UV spectrophotometer. The column operated at 46°C with a flow rate of 1ml/min (Bidlemeier et al., 1984).

Lipid content

Pollen grains were ground with a mortar and pestle to release all internal pollen lipids. Rupturing of the pollen grains was verified microscopically. Total lipid content was obtained by chloroform-methanol extraction, in duplicate, of the dried pollen using the method described by Folch et al. (1957), and the lipid fraction was estimated from the difference in weight.

Fatty acids

Standard procedures were used for methylation of lipids, using 0.7 g pollen per analysis, prior to determination of fatty acid composition (Genet et al., 2004). Fatty acids were

identified using a Varian (Varian Ass Inc 1985, USA) 3300 FID chromatograph, with WCOT fused silica capillary columns, CPSIL 88 (100 m, 0.25 mm). Column temperature was 140-240°C while the injector port and FID were maintained at 250°C. Helium was used as the carrier gas at an airflow rate of 50 ml/min. The fatty acids in samples were identified through a comparison with the relative retention times of fatty acid methyl ester peaks in standards obtained from Sigma (Taufkirchen, Germany).

Ash

Samples of the three pollen types (0.2 g each) were weighed into porcelain crucibles and placed in a temperature-controlled furnace that was preheated to 600°C for 2 h. Crucibles were transferred to a desiccator, cooled and weighed immediately thereafter (AOAC, 2000).

Statistical analysis

Data for nutritional content of fresh, bee-collected and stored *A. greatheadii* var *davyana* pollen (water content, crude protein, lipid, ash and carbohydrates) did not meet the assumptions for parametric statistics; variances were not homogeneous and data did not conform to a normal distribution. Statistical comparisons were therefore made using Kruskal-Wallis ANOVA by ranks and the Mann-Whitney U test (Zar, 1984). Analyses were performed with the program Statistica 6.0 (1984-2004). We compared the proportions of essential and non-essential amino acids and the proportions of saturated and non-saturated fatty acids of the different pollen types, based on the absolute amounts in each pollen type, using the Fischer-Exact test. Values are given throughout as means \pm SD.

Results

Fresh pollen grains vary in size from 44-50 μ m, are bilaterally symmetrical and have an elliptical shape with a deep furrow (Fig. 1A). The surface is perforated-reticulate and exine ornamentations are less visible on the tips of pollen grains where pollenkitt is more abundant (Fig. 1A). The low relative humidity during the flowering season contributes to the dehydrated status of fresh pollen grains. Bee-collected pollen grains are hydrated and swollen compared to fresh pollen. An increase in volume, especially in

the furrow area, is evident in both bee-collected (Fig. 1B) and stored pollen of *A. greatheadii* var *davyana* (Fig. 1C).

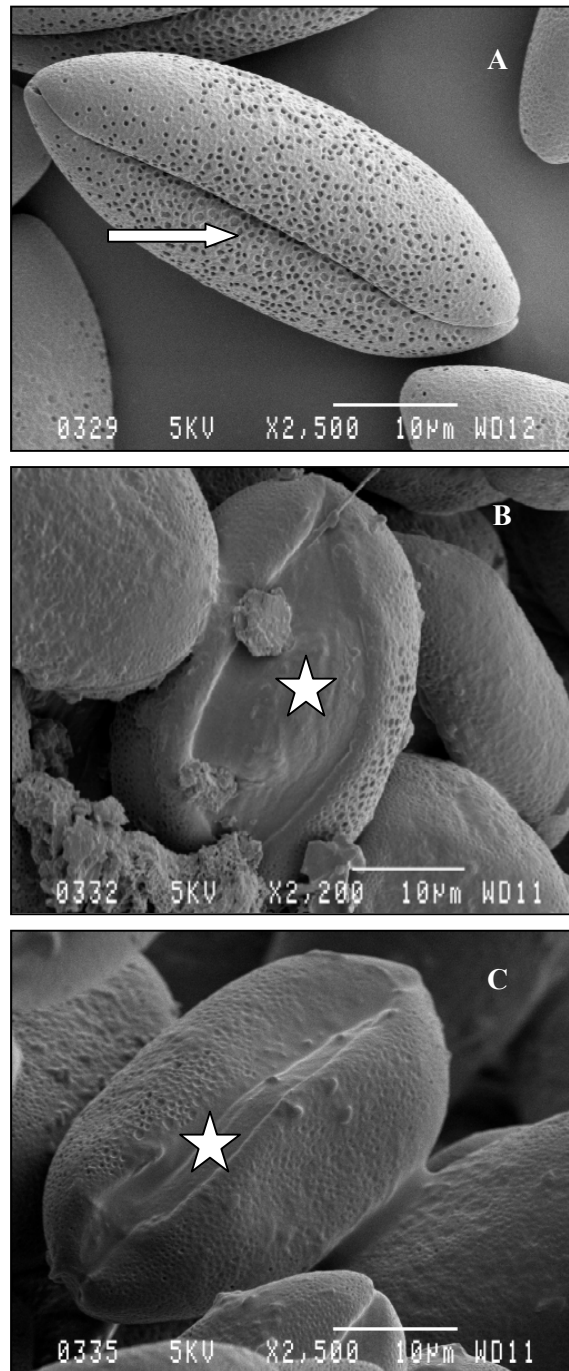


Figure 1. Scanning electron microscopy pictures of (A) fresh, (B) bee-collected and (C) stored *A. greatheadii* var *davyana* pollen. Arrows indicate deep furrow in fresh pollen. Bee-collected and stored pollen is swollen, especially in the furrow area (*) where the intine is exposed.

A summary of the nutritional content of the three types of *A. greatheadii* var *davyana* pollen is presented in Table 1. There are highly significant differences in chemical composition between fresh, bee-collected and stored *A. greatheadii* var *davyana* pollen: for water content ($H_{2,36} = 25.297$, $P < 0.001$), protein ($H_{2,36} = 31.207$, $P < 0.0001$), lipid ($H_{2,36} = 31.195$, $P < 0.001$) ash ($H_{2,36} = 23.457$, $P < 0.001$) and carbohydrate ($H_{2,36} = 24.532$, $P < 0.001$). The water content in fresh pollen is significantly lower than that of bee-collected and stored pollen (for both $z = -4.157$, $P < 0.001$). Similarly bee-collected pollen has significantly lower water content than stored pollen ($z = -2.078$, $P = 0.038$).

Table 1. Comparison of the nutritional content of fresh, bee-collected and stored pollen of *A. greatheadii* var *davyana*. Values are means \pm SD of 6 samples. Carbohydrate content was obtained by difference.

	Fresh pollen	Bee collected pollen	Stored pollen
	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$
Water content (% wet mass)	13.1 ± 1.4	18.8 ± 3.3	21.0 ± 2.4
Crude protein (% dry mass)	50.8 ± 2.7	31.4 ± 1.0	28.1 ± 1.6
Lipids (% dry mass)	10.0 ± 1.4	5.5 ± 1.0	7.6 ± 0.2
Ash (% dry mass)	4.5 ± 0.4	3.6 ± 0.2	3.6 ± 0.2
Carbohydrate (% dry mass)	34.7 ± 3.1	59.5 ± 1.3	60.7 ± 1.5

Crude protein content decreases significantly from 51% dry mass in fresh *A. greatheadii* var *davyana* pollen to 31% in bee-collected and 28% in stored pollen ($z = 4.157$, $P < 0.001$, for all paired comparisons). At least 18 amino acids are present in *A. greatheadii* var *davyana* pollen, including the 10 essential amino acids for honeybees (Table 2). The proportions of essential and non-essential amino acids did not differ significantly between fresh, bee-collected and stored pollen ($P = 1.0$).

Table 2. Amino acids in *A. greatheadii* var *davyana* pollen. Quantities are given as g/100g protein and compared with the minimal levels of essential amino acids necessary for honeybees and with royal jelly (De Groot, 1953).

<i>Aloe greatheadii</i> var <i>davyana</i> pollen					
	Fresh	Bee collected	Bee stored	Min levels required*	Royal jelly*
Essential amino acids					
Arginine	5.09	5.13	5.29	3.0	5.10
Histidine	1.89	2.12	2.25	1.50	2.20
Isoleucine	4.08	4.05	4.35	4.00	5.30
Leucine	6.96	7.04	7.48	4.50	7.70
Lysine	6.06	6.35	6.50	3.00	6.70
Methionine	1.53	2.17	2.25	1.50	1.90
Phenylalanine	4.15	4.13	4.44	1.50	4.10
Threonine	4.39	4.71	5.03	3.00	4.00
Tryptophan	0.14	0.16	0.16	1.00	1.30
Valine	4.82	4.89	5.16	4.00	6.70
Non-essential amino acids					
Alanine	5.02	6.01	5.85		
Aspartic acid	8.58	9.05	9.90		
Glutamic acid	9.75	9.37	10.78		
Glycine	3.97	4.26	4.51		
Hydroxyproline	0.92	0.90	0.92		
Proline	6.21	6.90	7.32		
Serine	5.25	5.40	5.78		
Tyrosine	2.75	2.75	2.81		

(*taken from De Groot, 1953)

Table 2 compares the quantities of essential and non-essential amino acids in the three pollen samples with those in royal jelly and the minimum requirements of honeybees (De Groot, 1953). Essential amino acids in *A. greatheadii* var *davyana* pollen are present in equal or higher amounts than the minimum requirements, with the exception of tryptophan. The levels of essential amino acids in all three types of *A. greatheadii* var *davyana* pollen are similar to those in royal jelly.

Lipid content is significantly higher ($z = 4.157$, $P < 0.001$) in fresh pollen than in bee-collected and stored pollen. At the same time bee-collected pollen contains significantly less lipid than stored pollen ($z = -4.157$, $P < 0.001$). The lipid fraction of *A. greatheadii* var *davyana* pollen includes 18 fatty acids and a number of minor unidentified peaks (Table 3). The four dominant fatty acids present in fresh pollen are palmitic acid, stearic acid (C-18), oleic acid and gadoleic acid (C-20:1). These four fatty acids compose 76% (fresh), 72% (bee-collected) and 65% (stored) of the total lipid content found in *A. greatheadii* var *davyana* pollen. The percentage of stearic acid decreases in bee-collected and stored pollen while the percentage of gadoleic acid increases, especially in bee-collected pollen. However, the proportions of saturated, monounsaturated and polyunsaturated fatty acids do not differ significantly between fresh, bee-collected and stored pollen of *A. greatheadii* var *davyana* ($P = 0.2$).

Carbohydrate is significantly lower in fresh than in stored pollen ($z = -4.157$, $P < 0.0001$). There are however no significant differences in carbohydrate content between bee-collected and stored pollen ($z = -1.617$, $P = 0.106$).

In both bee-collected and stored pollen ash content was significantly lower than in fresh pollen ($z = 4.157$, $P < 0.0001$) but did not differ significantly between bee-collected and stored pollen ($z = 0.346$, $P = 0.729$).

Table 3. Fatty acid composition of total lipid fractions extracted from *A. greatheadii* var *davyana* pollen. Data for individual fatty acids are given both as mg/g dry mass of pollen and as a percentage of the total fatty acids. A missing value indicates that the fatty acid was not detected.

Lo Long Chain Fatty Acids		Fresh pollen		Bee collected pollen		Bee stored pollen	
		mg/g	%FA	mg/g	%FA	mg/g	%FA
Myristic	C14:0	2.64	4.37	0.27	0.86	1.47	2.54
Palmitic	C16:0	14.37	23.76	4.39	14.03	12.85	22.19
Stearic	C18:0	10.27	16.99	1.52	4.85	3.51	6.07
Arachidic	C20:0	1.16	1.93	0.3	0.97	0.75	1.30
Behenic	C22:0	0.19	0.32	0.51	1.64	1.34	2.31
Lignoceric	C24:0			0.21	0.68	1.47	2.53
Total saturated		28.63	47.37	7.20	23.03	21.39	36.93
Palmitoleic	C16:1	1.25	2.06				
Oleic	C18:1 n-9	14.17	23.44	3.66	11.71	7.21	12.45
Gadoleic	C20:1	7.00	11.58	12.99	41.53	13.99	24.16
Total monounsaturated		22.42	37.08	16.65	53.24	21.20	36.61
Ricinoleic	C18:2 n-6			0.86	2.75	3.34	5.76
Linoleic	C18:2	1.26	2.08	2.55	8.15	2.16	3.73
Alphalinolenic	C18:3 n-3			0.05	0.17	0.12	0.21
Gamalinolenic	C18:3 n-6	2.18	3.61	2.38	7.61	3.91	6.74
Eicosadienoic	C20:2	0.54	0.90	0.12	0.40	0.50	0.87
Homo-g-linolenic	C20:3 n-6			0.02	0.06	0.04	0.07
Timnodonic	C20:5 n-3	1.31	2.16	0.13	0.42	0.62	1.06
Brassic	C22:2			0.16	0.52	0.20	0.34
DHA	C22:6 n-3	2.31	3.83				
Total polyunsaturated		7.60	12.58	6.27	20.08	10.89	18.78
Unidentified peak a		1.79	2.97	0.11	0.34	0.50	0.86
Unidentified peak b				0.57	1.82	0.46	0.79
Unidentified peak c				0.14	0.44	1.95	3.37
Unidentified peak d				0.16	0.51	0.74	1.27
Unidentified peak e				0.18	0.56	0.22	0.37
Unidentified peak f						0.59	1.01
Total unidentified peaks		1.79	2.97	1.05	3.68	4.46	7.67
Total fatty acids		60.44	100%	31.28	100%	57.94	100%

Discussion

The morphology of *A. greatheadii* var *davyana* pollen is similar to that described for other *Aloe* species (Steyn et al., 1998). Pollen shape changes during development, dispersal and arrival on the stigma due to loss and then uptake of water (Nepi et al., 2001). The increase in moisture content reflects the addition of nectar and glandular secretions. During rehydration, the intine and protoplasm absorb water and increase in volume while the exine stretches (Pacini, 1986), thereby enhancing the availability of nutrients for digestion, because exposure of the intine (Fig. 1B) presents a region for enzymatic penetration and subsequent processing of the pollen grain contents (Human & Nicolson, 2003, Nepi et al., 2005). Thus pollen handling by bees probably prepares the grains for efficient digestion.

The protein content of pollen is considered a direct and reliable measure of its nutritional value (Pernal & Currie, 2000; Cook et al., 2003). Roulston et al. (2000) compiled a database of the crude protein concentrations in hand-collected pollen of 377 plant species, either through their own analyses or from the literature. Contrary to expectation, pollen of animal-pollinated plants was not richer in protein than that of wind-pollinated plants. In spite of the importance of pollen protein to bees, honeybees collect pollens with protein contents ranging widely from 12 to 61% across all plant taxa (Roulston et al., 2000). The crude protein content of fresh pollen lies at the high end of the range of values in the literature and is comparable with that of buzz-pollinated plants (Roulston et al., 2000). The protein content in bee-collected *A. greatheadii* var *davyana* pollen is higher than that of most bee-collected pollens (20-33%) of *Eucalyptus* species (Kleinschmidt & Kondos, 1978; Rayner & Langridge, 1985; Somerville, 2001). Even though the summer flowering aloe *A. zebrina*, belonging to the same *Aloe* Section, is not considered an important bee plant, its fresh pollen also has a very high protein content (54.9% dry mass) (Human & Nicolson, unpublished data).

Amino acid composition, however, may define the nutritional value of pollen more accurately than protein content, since the nutritional value is reduced when inadequate amounts of the essential amino acids (De Groot, 1953; Cook et al., 2003) are present. Generally pollen contains all the essential amino acids but the amounts may vary

between plant species (Roulston et al., 2000). The most frequently lacking amino acid in many *Eucalyptus* species is isoleucine, while others have borderline concentrations (Somerville, 2001). Some *Eucalyptus* species are also reported to be limiting in tryptophan (Bell et al., 1983; Rayner & Langridge, 1985). The predominant amino acids in pollen of 62 species, including 20 *Eucalyptus* species reported by Somerville (2001) are glutamic acid, aspartic acid and proline, all non-essential amino acids.

Pollen lipids consist of internal cytoplasmic lipids and external lipids of the pollenkit, but lipid content reported in the literature is mostly that of lipids derived from the pollenkit of pollen and may comprise only a small fraction of total lipids (Roulston & Cane, 2000; Manning, 2001). Evans et al. (1987) demonstrated dramatic increases in lipid content after mechanically fracturing pollen grains of *Brassica napus*. Although we used ground pollen for lipid extractions, the pollen of *A. greatheadii* var *davyana* does not have a very high lipid content compared to the range of 0.8% to 31.7% reported in the literature (Farag et al., 1978; Evans et al., 1987; Roulston & Cane, 2000). The latter study recorded lipid content higher than 5% for at least 60% of the plant species. The lipid content of *A. greatheadii* var *davyana* pollen was much higher than average values reported for *Eucalyptus* species (< 2%, Somerville 2001; < 1.42%, Manning & Harvey 2002). For honeybees the lipids, including fatty acids and sterols, are important sources of energy, are used for the synthesis of reserve fat and glycogen, and contribute to the production of royal jelly (Singh et al., 1999; Loidl & Crailsheim, 2001; Manning, 2001; Manning & Harvey, 2002). According to a study by Singh et al. (1999) bees preferred pollens with the highest amount of lipids.

In addition to variation in lipid content, pollen also varies in the relative proportions of fatty acids and in their diversity (Manning, 2001; Markowicz Bastos et al., 2004). Fatty acids are important in the reproduction, development, and nutrition of honeybees (Manning, 2001; Farag et al., 1978). Certain fatty acids, such as linoleic, linolenic, myristic and lauric acids, have bactericidal and antifungal properties that are important for colony hygiene (Manning, 2001; Manning & Harvey, 2002). From the literature it seems that, in general, the dominant fatty acids present in pollens are palmitic (C-16), oleic (C-18:1), linoleic (C-18:2) and linolenic (C-18:3) acids (Manning, 2001). It is known that once pollen is stored, its fatty acid composition changes (Van der Vorst, 1982). The concentrations of individual fatty acids in *A. greatheadii* var *davyana* pollen

are close to values reported for pollens from 46 plant species by Manning (2001), the major difference being the high gadoleic acid content. Gadoleic acid is not listed as being present in other pollens.

Carbohydrate content varies widely in pollen: Todd and Bretherick (1942) recorded values from 1-37% of total dry mass in hand-collected pollen, and from 21–48% in bee-collected pollen. Particularly because of the added nectar, carbohydrate constitutes a large fraction of the nutritional content of *A. greatheadii* var *davyana* pollen. Not all carbohydrates are nutritionally useful, e.g. pectin is an important structural component of the cell wall and essential in plant growth and development but has no known nutritional value for bees (Aouali et al., 2001; De Halac et al., 2003). Pectin content in *A. greatheadii* var *davyana* is 7.1% in fresh pollen, and 8.5% and 8.3% in bee-collected and stored pollen respectively (Human & Nicolson, unpublished data). This increase in pectin content can be due to a response of pollen to hydration: water gain by pollen may initiate the mechanism of pollen germination, where the synthesis of pectins is required for the new wall construction (Shivanna, 2003).

According to Roulston and Buchman (2000), starch content in pollen ranges from 0-22%. Most pollen contain less than 5% starch: sunflower pollen, for example, has a starch content of 0.4% while *Aloe ferox* pollen is starchless (Roulston & Buchmann, 2000). Similarly, no starch is present in *A. greatheadii* var *davyana* pollen (Human & Nicolson, unpublished data). According to Todd and Bretherick (1942) and Herbert and Shimanuki (1978), ash content of pollen ranges between 0.9 and 6.4%. This is contrary to the study of Todd and Bretherick (1942), who determined no differences between the ash content of fresh and bee-collected pollen.

In general, studies on the nutritional content of pollen have focussed only on single aspects, with some exceptions (Roulston & Cane, 2000; Roulston et al., 2000; Manning, 2001; Somerville, 2001). Most of the analyses used bee-collected pollen. To our knowledge, this study is the first to compare the nutritional content of fresh, bee-collected and stored pollen in a single plant species and thus highlight the changes that occur in pollen after collection. The overall nutritional content of *A. greatheadii* var *davyana* pollen appears to be much better than that of *Eucalyptus* species. However,

except for the very high protein content and high concentration of gadoleic acid, there is no specific nutritional aspect of the pollen of *A. greatheadii* var *davyana* that can explain the aggression observed in bees on the aloe fields. The extremely high protein level and overall excellent nutritional content of *A. greatheadii* var *davyana* pollen, together with the movement of apiaries in winter to aloe fields north of Pretoria, contributes to the productivity of the migratory beekeeping industry. Further work needs to examine pollen digestion efficiency and the effect of this high protein content on ovarian and hypopharyngeal gland development.

Acknowledgements

We are grateful to Roodeplaat Nature Reserve for permission to move bees onto the Reserve, to Prof Robin Crewe for his bees and to Dr Per Kryger and Dr Vincent Dietemann for their assistance in working with the bees. Arnold and Danelle Human helped to collect the fresh pollen. The South African National Research Foundation, the Italian Ministry of Foreign Affairs (General Direction for Cultural Promotion and Cooperation) in the framework of the Italy/South Africa Science and Technology Agreement (2001-2004) and the University of Pretoria are thanked for funding this project. We thank Massimo Nepi, Department of Environmental Sciences, University of Siena, Italy, for guidance, the Laboratory for Microscopy and Microanalysis and UP Nutrilab, (Department of Animal and Wildlife Sciences), both at the University of Pretoria.

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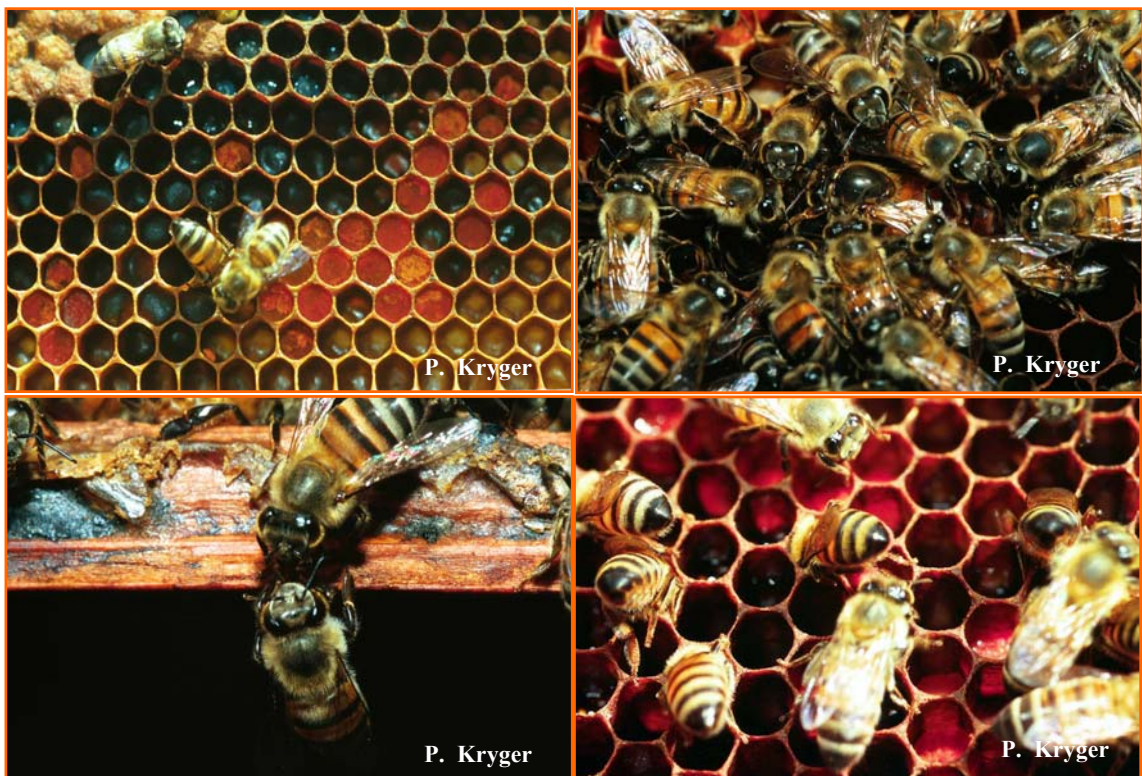
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CHAPTER 2

Influence of *A. greatheadii* var *davyana* pollen quality on ovarian development in honeybees (*Apis mellifera scutellata*)

H. Human, S.W. Nicolson and V. Dietemann

Department of Zoology and Entomology, University of Pretoria, Pretoria 0002, South Africa.



Abstract

In honeybee colonies there normally is a queen that lays eggs and workers that supply food and maintain the nest. However, worker bees are able to reproduce in the absence of the queen. A variety of factors, including temperature, food, pheromones and social interactions, have an influence on ovarian development. Protein-rich diets are known to promote ovarian and egg development and, since the main source of protein for honeybees is pollen, the quality and digestibility of the pollen may also have an influence. We have determined the effect of two types of pollen, sunflower and aloe, on ovarian development in queenright colonies in the field and in laboratory induced queenless groups. Extraction efficiency was determined for both pollen types.

Under queenright conditions worker bees exhibited higher ovarian development when feeding on aloe pollen than on sunflower pollen. However, in queenless groups, worker bees sustained on sunflower pollen had significantly more developed ovaries compared to bees fed with aloe pollen. In addition higher mortality was observed for bees fed aloe pollen. We observed higher extraction efficiency for aloe (80%) compared to sunflower (69%) pollen in the midgut of honeybees.

The higher ovarian development in workers of queenright colonies feeding on *A. greatheadii* var *davyana* may be attributed to the overall excellent nutritional content of this pollen and the high protein level (32% dry mass) in bee-collected pollen compared to the 15% in sunflower pollen. The higher extraction efficiency can be attributed to the structure and size of pollen grains, *A. greatheadii* var *davyana* pollen is bigger and smoother and better from a volume: surface point of view to digest compared to the smaller and more ornamented sunflower pollen. We explain the unexpected effect of aloe pollen on honeybee physiology in the queenless groups with the potential detrimental effects of protein in high concentrations.

Introduction

The division of reproductive labour is one of the major characteristics of social Hymenoptera. The queen lays eggs and workers supply food and maintain the nest. In worker bees, the reserves carried forward from larval nutrition that could have been used for ovarian development are instead used for brood care and foraging (Hunt & Nalepa, 1994). However, worker honeybees are able to reproduce in the absence of a queen (Velthuis, 1970): they possess ovaries and although they cannot mate they are able to lay unfertilised eggs which will develop into males with the exception of *Apis mellifera capensis* bees that are able to produce diploid female offspring (Neumann & Moritz, 2002).

Worker ovarian development in *A. mellifera* is influenced, indirectly or directly, by a variety of factors, including temperature, food, brood and queen pheromones, as well as aggression and trophallactic interactions with other workers (Hoover et al., 2006). In terms of nutrition, protein is essential for the normal growth and development of bees (Moritz & Crailsheim, 1987; Schmidt et al., 1995). It is well known that a protein-rich diet promotes ovarian development (Hoover et al., 2006; Lin & Winston, 1998) and contributes to egg development (Wheeler, 1996; Pernal & Currie, 2000).

Pollen is the main dietary source of protein for honeybees (Grogan & Hunt, 1979; Pernal & Currie, 2000). However, protein concentrations in pollen vary widely among different plant species, ranging between 2.5 and 60% dry mass (Todd & Bretherick, 1942; Stanley & Linskens, 1974; Roulston & Cane, 2000). *Aloe* pollen has the highest protein content recorded for South African pollens; the crude protein content in fresh *A. greatheadii* var *davyana* pollen amounts to 51% dry mass although it decreases to 31% in bee-collected and 28% in stored pollen (Chapter 1). The summer flowering aloe, *A. zebrina*, not considered to be an important bee plant, also has a very high protein content (54.9% dry mass) in its fresh pollen (Human & Nicolson, unpublished data). Bee-collected pollen of *A. greatheadii* var *davyana* has a higher protein content than that of pollens of most *Eucalyptus* species (20-33%) (Kleinschmidt & Kondos, 1978; Rayner & Langridge, 1985; Somerville, 2001).

In order to reach the nutrient rich cytoplasm, pollen feeders need to overcome the digestion obstacle presented by the walls of pollen grains (Klungness & Peng, 1984). There are six basic methods that can be used by insects and other animals to digest pollen: mechanical damage, piercing and sucking, external digestion, enzyme, osmotic shock and pseudogermination (Human & Nicolson, 2003). Pollen is not only digested in different ways but also to different extents (Crailsheim et al. 1992; Roulston & Cane, 2000). Numerous studies have investigated various aspects of pollen digestion in both honeybee larvae and adult workers (Mortiz & Crailsheim, 1987; Schmidt & Buchman, 1985; Crailsheim et al., 1992; Schmidt et al., 1995; Dobson & Peng, 1997). By adding nectar upon collection, bees start to "pre-digest" *A. greatheadii* var *davyana* pollen grains making it easier for individuals to digest the grain contents (Chapter 1).

Ovarian development may be influenced not only by the quality and digestibility of pollen but also by its seasonal availability (Hoover et al., 2006). In South Africa one would expect higher ovarian development in native *A. mellifera scutellata* bees in summer than in winter, when few floral resources are available. South African migratory beekeepers move their hives in midsummer to sunflower fields, *Helianthus annuus*, for pollination of the crops and simultaneously make use of the nectar and pollen flow. During winter the beekeepers move their hives to the "aloe fields" north of Pretoria. *Aloe greatheadii* var *davyana* has a widespread distribution across the northern summer rainfall areas (Glen & Hardy, 2000; Van Wyk & Smith, 1996) and flowers when little else is available. The abundant pollen and nectar of this aloe is used by beekeepers to build up colonies, rear queens and increase colony numbers (Williams, 2002).

The aim of this study was to determine: firstly, the effect of sunflower and aloe pollen on ovarian development in queenright colonies; secondly, the effect of these two pollen types on ovarian development in laboratory induced queenless workers, and lastly, the extraction efficiency of pollen digestion for both pollen types.

Methods

Study site and plant species

In February 2004, six queenright honeybee hives (*A. mellifera scutellata*) were maintained on sunflower (*H. annuus*) fields in the Bronkhorstspuit district (28° 39'E, 25° 54'S) in Gauteng Province. Thereafter the hives were moved to Roodeplaat Nature Reserve (size 795 ha; 28° 39'E, 25° 66'S) for the duration of the winter (June and July) where they were able to make use of the strong pollen and nectar flow of *A. greatheadii* var *davyana*.

During both the sunflower and aloe flowering periods in 2004, 70 bees were collected from frames of each of 6 queenright hives to determine ovarian development. In addition 50 bees were collected from each of three hives during both the sunflower and aloe flows to determine pollen extraction efficiency. Bees were stored at -20°C until they were dissected.

Ovarian development in queenright colonies

Dissections (n = 70 per colony) were performed in a small Petri dish with a layer of black wax under a binocular microscope with 30x magnification. The head and thorax of each bee was removed and the abdomen placed in a drop of water for dissection. The abdomen was opened with tweezers and the sternites pulled backwards to reveal the ovaries. Ovarian development was categorised according to Hess (1942). We classified ovarian development into five stages with stage 1 being undeveloped, and stage 5 being workers with fully developed ovaries with eggs (see Fig. 1). Stages 1 and 2 were combined as "undeveloped ovaries" and stages 3 to 5 as "developed ovaries" for data analysis (Mohammed et al., 1998).

Ovarian development in the laboratory (queenless groups)

Frames with capped worker brood were removed from 12 *A. mellifera scutellata* hives and placed in an incubator with a constant temperature of 34°C and relative humidity of 55%. After one day of incubation, newly emerged workers (0-24 h old) were obtained. One hundred and twenty bees of the same colony were placed together into a hoarding cage (11 x 8.5 x 7 cm) without a queen for the duration of the experiment. In order to test the effect of pollen (aloe versus sunflower) on ovarian development of queenless

workers, we prepared 12 cages. Six cages were fed bee candy containing *A. greatheadii* var *davyana* pollen and 6 cages received bee candy containing *H. annuus* pollen. Each hoarding cage was supplied with a vial of sugar water (sucrose; concentration = 1g / ml) and bee candy (honey and pollen in a 1:1 ratio, bound with icing sugar; method of Mohammadi et al., 1998). A piece of comb was attached to the upper part of each cage. The exposure of bees to daylight has been found to prevent ovarian development in laboratory studies (Velthuis, 1970); therefore, all cages were kept in darkness.

Every second day, the cages were checked for dead bees, which were removed and counted, and sugar water and bee candy were renewed if necessary. After 14 days, all surviving bees were killed by freezing at -20°C and stored at this temperature until dissection. Ovarian development was determined for 20 bees from each cage, as mentioned above.

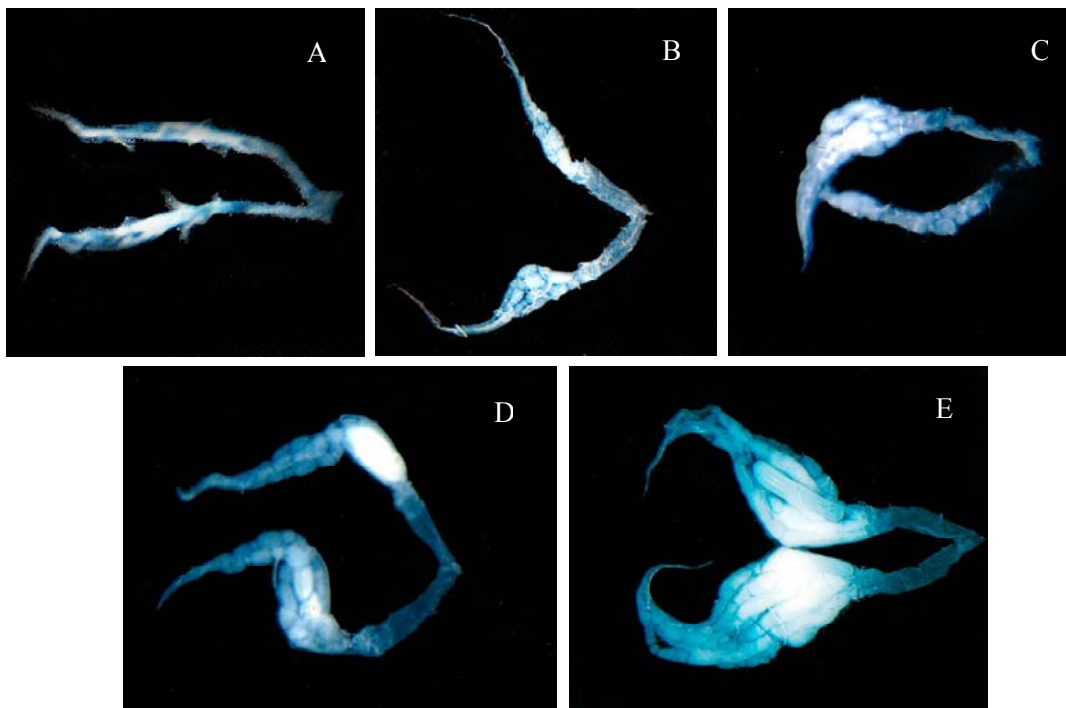


Figure 1. Ovarian development in worker bees categorised according to Hess (1942). (A) stage 1, (B) stage 2, (C) stage 3, (D) stage 4 and (E) stage 5. (Photos by V. Dietemann)

Pollen extraction efficiency

Pollen digestion by honeybees takes place in the midgut (Moritz & Crailsheim, 1987) and empty pollen grains accumulate in the rectum. Fifty worker bees were obtained from 3 colonies each during the sunflower and aloe flow. The midgut and hindgut were

dissected from each of these bees and the gut contents were released by rupturing the gut walls. The gut contents were then transferred to a microscope slide and stained with a drop of cotton lactophenol blue (this stains the cytoplasm blue, but leaves the cell walls unstained) and the samples sealed with a cover slip. The slides (one slide per bee) were examined under a light microscope and 100 grains were evaluated as full, half-full and empty. A full grain was defined as one that contained more than half of its cytoplasm and that was similar in shape and contents to the reference pollen. A half-full grain contained less than half of its cytoplasm and empty grains had no cytoplasm (Human & Nicolson, 2003).

An important factor to consider in calculations of extraction efficiency is that all fresh pollen samples contain some grains that are either partially or completely devoid of their contents. Fresh pollen was hand collected from 10 randomly selected sunflower and aloe plants (one flower per plant) to be used as reference pollen for comparison with pollen in the gut of honeybees. Fresh pollen was transferred onto a microscope slide, stained and evaluated as for gut contents.

The following formula was used to calculate extraction efficiency (Human & Nicolson, 2003) for sunflower and aloe pollen in the midgut and hindgut of each bee.

$$\text{Extraction efficiency} = \frac{\text{No. empty grains in gut} - \text{No. empty grains in fresh pollen}}{\text{No. full grains in fresh pollen}} \times 100$$

Statistical analysis

The frequency of individual workers with developed ovaries that fed on different pollens in queenright colonies in the field as well as in queenless groups in the laboratory, and the mortality rate in the laboratory, were assessed with the Fischer-Exact test. The data for pollen extraction efficiency met assumptions for parametric statistics. Student's t-tests were thus used to compare the extraction efficiency of aloe and sunflower pollen in the midgut and hindgut of bees.

Statistical analysis was performed with Statistica 6.0 (1984-2004). The level of statistical significance for all analyses was set at $P < 0.05$. Values are given throughout as means \pm SD.

Results

Ovarian development in queenright colonies

Under queenright conditions no bees showed fully developed ovaries (stage 5), only stage 3 and 4 development was observed. However, *Aloe* pollen had a significant effect ($P = 0.008$) on ovarian development, in that worker bees on the *Aloe* flow exhibited a higher percentage of stage 3 and 4 ovarian development as opposed to bees feeding on sunflower pollen (Fig. 2). Bees on the sunflower flow had a higher percentage of workers with undeveloped ovaries than bees feeding on aloe pollen (Fig. 2).

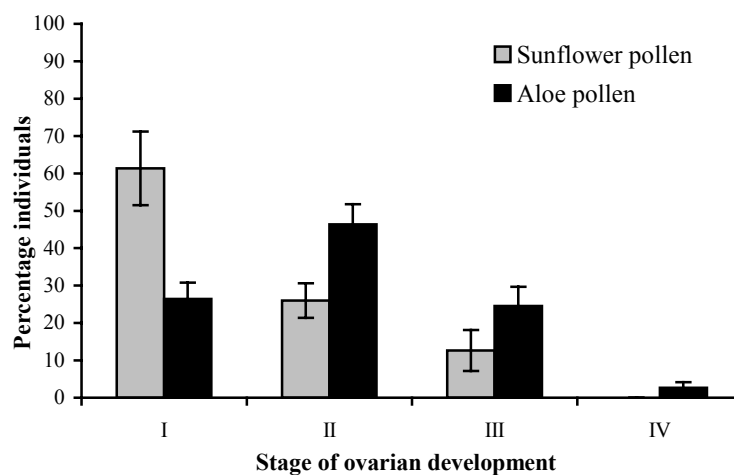


Figure 2. Worker ovarian development of bees from queenright colonies feeding on sunflower (*H. annuus*) or aloe (*A. greatheadii* var *davyana*) pollen. Data are given as means \pm SD, $n = 70$.

Ovarian development in the laboratory (queenless groups)

Worker bees in the cages supplied with sunflower pollen had significantly more developed ovaries than bees fed with aloe pollen ($P < 0.05$) (Fig. 3). This was in contrast to worker bees under queenright conditions. In addition, bees sustained on aloe pollen exhibited a significantly higher mortality ($34.1 \pm 17.3\%$) than those fed on sunflower pollen ($13.5 \pm 8.9\%$) ($P < 0.001$).

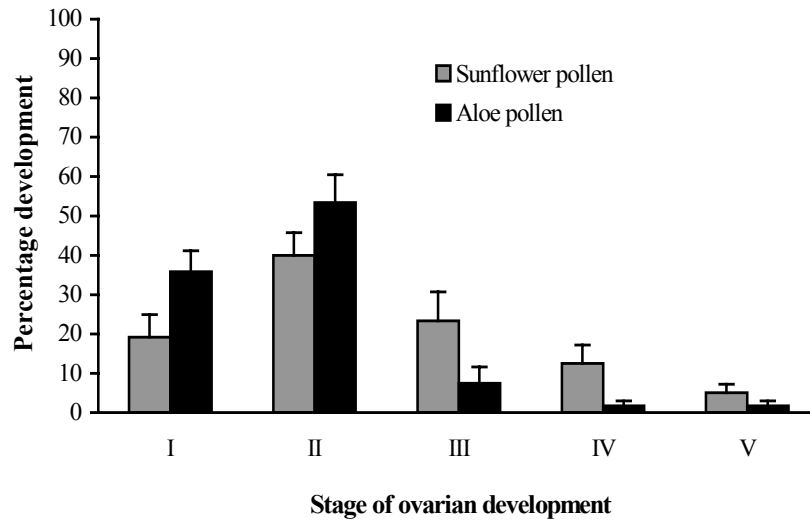


Figure 3. Comparison of ovarian development of individual queenless worker bees fed sunflower (*H. annuus*) and aloe (*A. greatheadii* var *davyana*) pollen in the laboratory. Data are given as means \pm SD, n = 20.

Pollen digestion

The pollen grains of *A. greatheadii* var *davyana* pollen are large (48 μ m in length) with a deep furrow (Fig. 4A). Sunflower pollen is round and smaller (29 μ m in diameter) than aloe pollen, with an ornamented exine (Fig. 4B).

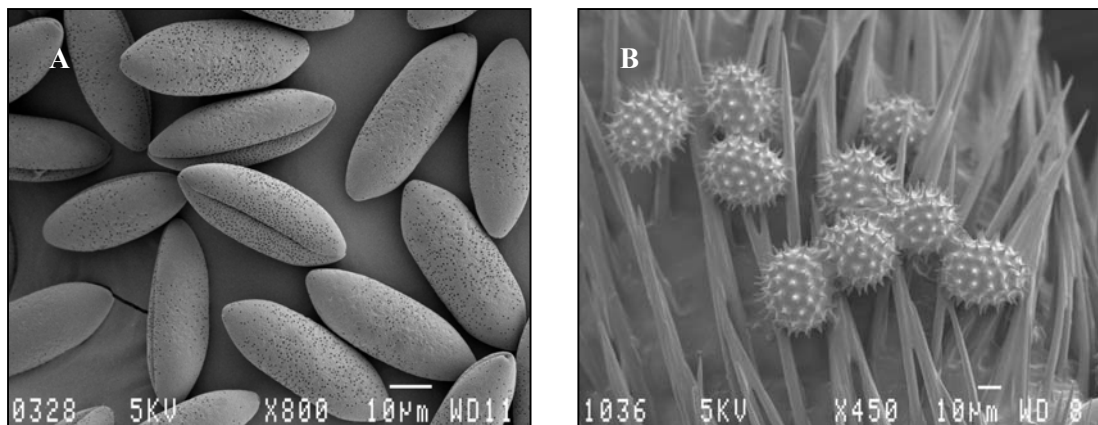


Figure 4. Scanning electron microscopy pictures of (A) aloe (*A. greatheadii* var *davyana*) pollen and (B) sunflower (*H. annuus*) pollen, attached to hairs on *A. mellifera* *scutellata* legs. Note different scales.

Aloe and sunflower pollen that morphologically resembled the control pollens was found in the midgut of the honeybees, confirming that these plants were the main source

of pollen during the experiment. A significantly higher percentage ($t = 7.826$, $df = 4$, $P < 0.01$) of aloe pollen grains were already empty in the midgut as compared to sunflower pollen (Fig. 5A). A few aloe and sunflower pollen grains were half-full in both the mid- and hindgut (Fig. 5A, B). The percentage of empty pollen grains increased in the rectum and was significantly higher for aloe than for sunflower pollen ($t = 14.872$, $df = 4$, $P < 0.001$) (Fig. 5B). Fresh hand-collected pollen from the two plants was used as a reference for pollen in the mid- and hindgut of bees. The percentage of empty pollen grains in fresh aloe pollen ($4.6 \pm 1.2\%$) was significantly lower ($t = -4.99$, $df = 18$, $P < 0.001$) than that in sunflower pollen ($6.7 \pm 2.5\%$).

Extraction efficiency of pollen of the two plant species was found to be significantly different in both the midgut ($t = 9.889$, $df = 4$, $P < 0.001$) and hindgut ($t = 16.179$, $df = 4$, $P < 0.001$). The contents of $80.2 \pm 10.2\%$ aloe pollen grains were already extracted in the midgut of honeybees compared to $69.2 \pm 4.9\%$ for sunflowers. The percentage of empty pollen grains of both plant species increased slightly in the hindgut.

Discussion

In a queenright colony there is a single, fertile queen and thousands of sterile workers that perform normal nest duties (Winston, 1987). Ovarian development of these workers is inhibited by pheromones produced either by the queen or the brood (Jay, 1972; Plettner et al., 1993; Mohammedi et al., 1998; Hoover et al., 2006). Queen pheromones do not completely inhibit ovarian development in workers. Normally a small number of egg-laying workers are present in a queenright colony producing 0.1% of the brood (Visscher, 1969) and there are also a large proportion of workers with developed ovaries that do not reproduce (Kropáčová & Haslbachová, 1969). Jay (1972) reported ovarian development, in spite of the presence of a queen, in a considerable number of workers when all brood was removed from the colony. In the absence of a queen, *A. mellifera* workers will initially try to rear an emergency queen; if this is unsuccessful, more workers will develop their ovaries and start to lay eggs and produce queenlike pheromones thereby becoming false queens. Workers of African races will start laying eggs much sooner after queen loss than bees of temperate regions, the extreme example being *A. mellifera capensis* bees that show ovarian development 3-6 days after queen loss (Plettner et al., 1993).

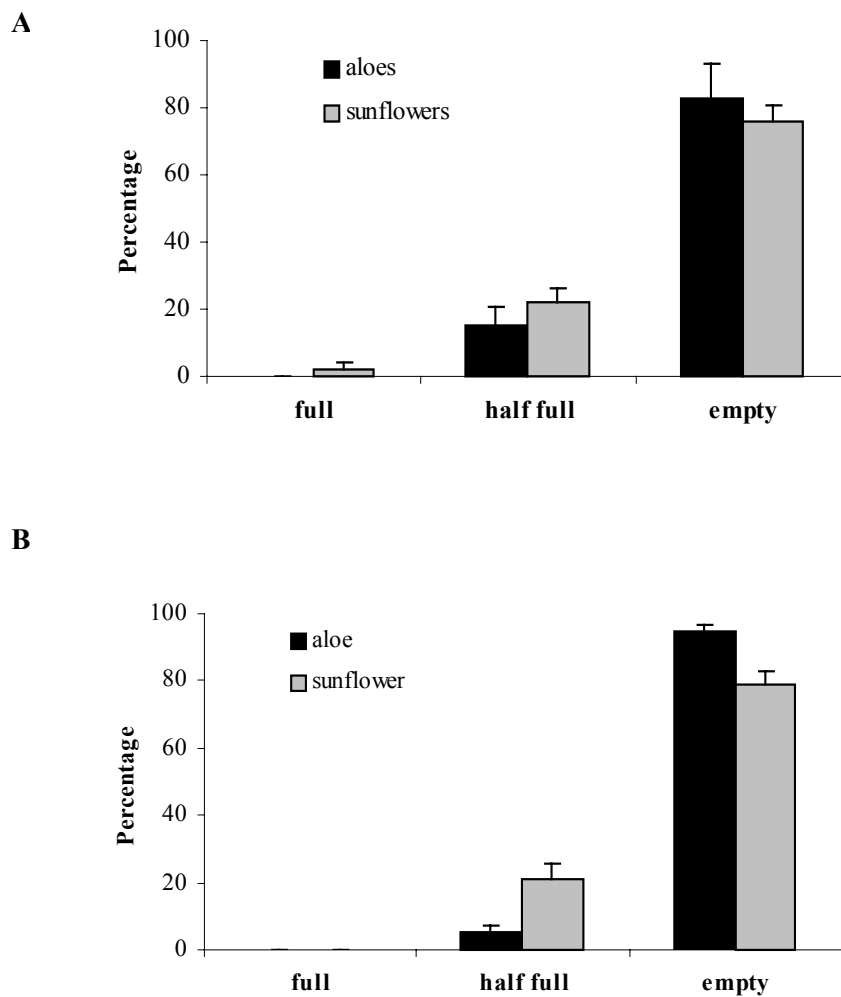


Figure 5. The percentage of empty, half full and full aloe (*A. greatheadii* var *davyana*) and sunflower (*H. annuus*) pollen grains in (A) the midgut and (B) the hindgut of honeybees (Data are given as means \pm SD, n = 50).

In this study we observed the highest ovarian development in worker bees of queenright *A. mellifera scutellata* colonies when these are feeding on *A. greatheadii* var *davyana* pollen. We observed less development in bees feeding on sunflower pollen. An explanation for the higher ovarian development related to *A. greatheadii* var *davyana* pollen may be found in the quality difference between the two pollen sources; *A. greatheadii* var *davyana* pollen has a much higher protein level (31% dry mass) in bee-collected pollen (Chapter 1) as opposed to sunflower pollen which is considered to be nutritionally poor, having a protein content of 15% (Schmidt et al., 1995). In spite of

this, honeybees readily utilise sunflower pollen and may also collect and consume other pollen species available at the same time. It has been shown that bees utilising only sunflower pollen may become stressed and have a shorter lifespan (Schmidt et al., 1995). Lin and Winston (1998) found ovarian development in worker bees feeding on mixed pollen and royal jelly diets, containing 22% and 13% protein respectively. The royal jelly may have a higher nutritive value, in spite of the lower protein content compared to pollen, due to the presence of adequate levels of amino acids. Adequate levels of amino acids in pollen grains are more important than the protein content (De Groot, 1953). The essential amino acids in *A. greatheadii* var *davyana* pollen are present in equal or higher amounts than those in royal jelly (Chapter 1).

There are three mechanisms by which honeybees can obtain the proteins necessary to sustain ovarian development. The first mechanism, although it is considered less important than adult nutrition, is by carrying larval reserves forward to later developmental stages (Hoover et al., 2006). The other mechanisms are based on adult nutrition, and involve feeding directly on pollen (solitary pathway) or producing queenlike pheromones in order to receive food (royal jelly) through trophallaxis (social pathway) (Hoover et al., 2006; Schäfer et al., 2006). After ingestion pollen is transported from the crop through the proventriculus to the midgut and then the hindgut (Crailsheim et al., 1992). According to Moritz and Crailsheim (1987), protein is digested mainly in the midgut of bees. It has been suggested by Kroon et al. (1974) that the change from high osmotic concentration in the crop of honeybees to lower osmotic concentration in the midgut may cause pollen grains to rupture, thereby initiating pollen digestion in the gut. However, although a high percentage of pollen grains of both species was digested in the midgut of bees in this study, the exines of pollen grains remained intact. Pollen was therefore probably digested enzymatically and not through osmotic shock (see Human & Nicolson, 2003). Values for extraction efficiency in the midgut and the observed increase in the hindgut in this study are in agreement with values given for adult honeybees by Peng et al. (1985) and Crailsheim (1992, 1993).

The high extraction efficiency for *A. greatheadii* var *davyana* pollen compared to that of *H. annuus* can be attributed to the structure and size of pollen grains. Aloe pollen is much better to digest from the point of view of volume: surface ratio. Moreover it is bigger, smooth and lacks a pollenkitt, compared to the more ornamented sunflower

pollen that is covered with a prominent pollenkitt layer thereby. Bees have to first digest the pollenkitt before they can digest the cytoplasm of the sunflower pollen. The high extraction efficiency for aloe pollen implies that bees are able to utilise a high percentage of the protein in the pollen.

The reduced ovarian development and high mortality observed in queenless workers that fed on *A. greatheadii* var *davyana* pollen, compared to those feeding on sunflower pollen, are not easily explained. Possibly they can be attributed to the fact that protein levels can either have a positive or detrimental effect on honeybees. Standifer et al. (1960) found that high protein levels increased hypopharyngeal gland development while lower levels prolonged lifespan. Herbert et al. (1977) observed higher mortality in caged workers fed large amounts of protein (50%) compared to those fed smaller amounts (5 and 10%). Their high protein diets were formulated with Wheast® that contains 57% protein and is produced by fermentation of cottage cheese whey by yeast. Bees fed 100% royal jelly died within 3 days (Lin & Winston, 1998). Preliminary results of a replicate study in which caged workers were fed water instead of sugar water resulted in increased lifespan for workers that fed on *A. greatheadii* var *davyana* pollen as well as increased ovarian development after 16 days (B. Langer, pers. comm.). According to D. Raubenheimer (pers. comm.) the observed mortality may be the result of excess protein in relation to other nutrients.

This study raises an interesting question about the effect of high dietary proteins in laboratory experiments. The exceptionally high nutritional value of *A. greatheadii* var *davyana* pollen and the consequent effect on ovarian development may facilitate research on other aspects of ovarian development, such as the correlation with mandibular gland pheromone production.

Acknowledgements

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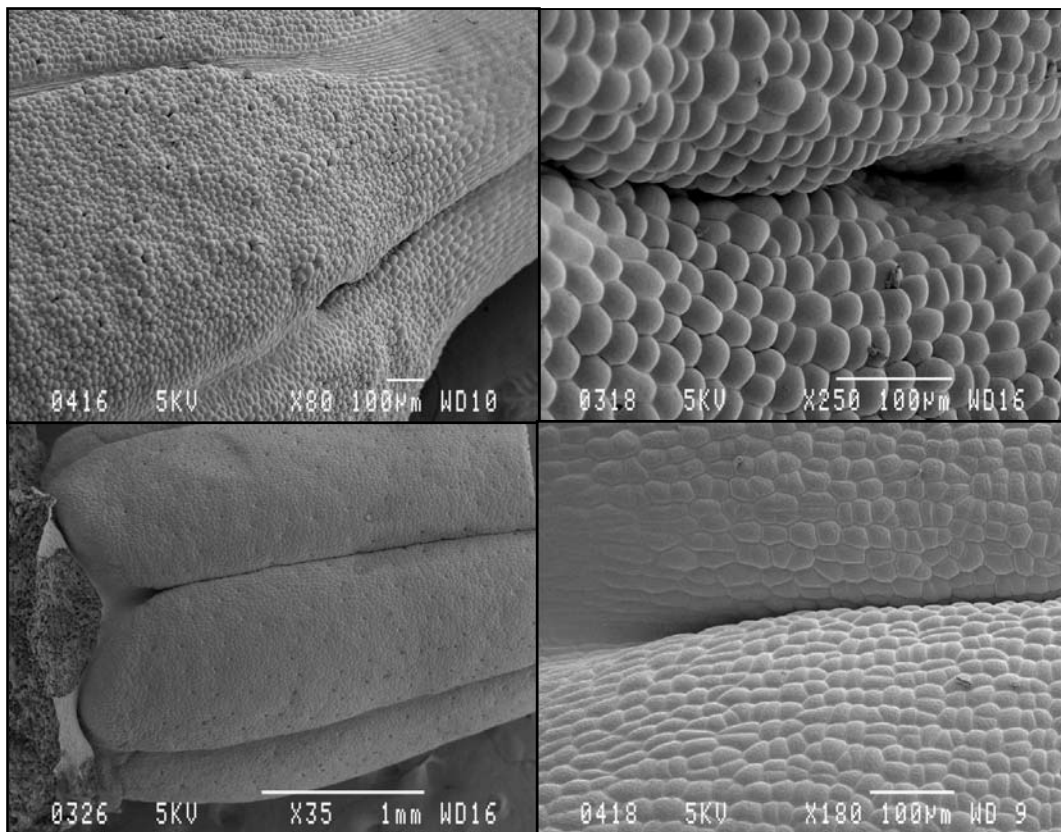
CHAPTER 3

Nectary structure and nectar presentation in *Aloe castanea* and *A. greatheadii* var *davyana* (Asphodelaceae)

M. Nepi¹, H. Human², S.W. Nicolson², L. Cresti¹, E. Pacini¹

¹Department of Environmental Sciences “G. Sarfatti”, University of Siena, Via Mattioli 4, 53100 Siena, Italy

²Department of Zoology and Entomology, University of Pretoria, Pretoria 0002, South Africa



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Abstract

This paper deals with the nectary structure and nectar presentation of two species belonging to different sections of the genus *Aloe*: *A. castanea* (Anguialoe) and *A. greatheadii* var *davyana* (Pictae). The development of the nectary was studied by means of bright field and fluorescence light microscopy and scanning electron microscopy (SEM) in three flower stages (young, intermediate, old). Both species have septal nectaries. In *A. castanea*, a subsidiary tissue, not present in *A. greatheadii* var *davyana*, was found beneath the nectary epithelium. This tissue accumulated starch that was hydrolyzed during secretion. Starch was slightly accumulated around the nectary in *A. greatheadii* var *davyana*. The distribution of chlorophyll in the ovary was also different in the two species. These anatomical differences are not, however, correlated with greater nectar production in *A. castanea*. In this species, the nectary seems to degenerate after secretion, while in *A. greatheadii* var *davyana* no sign of degeneration was observed. Differences in nectar presentation among the two species may account for different pollinators visiting their flowers.

Introduction

Fahn (1979) presented a topographical classification of floral nectaries, indicating nine different types. Among them, the “ovarial nectary” type includes nectaries that are placed in the septal region between adjacent carpels, the so-called septal nectaries or gynopleural nectaries as they have been more recently defined by Smets and Cresens (1988). Gynopleural nectaries are restricted to monocotyledons, where they represent the most common type of floral nectary (Smets et al., 2000). The gynopleural nectary, being a cavity inside the ovary, is not directly exposed to nectar-feeding animals and the site of nectar emission is often different from the site of nectar production (Smets et al., 2000). For this reason we can apply the terminology ‘secondary nectar presentation’ according to Pacini et al. (2003). Flower morphology and the site of nectar presentation, combined with nectar quantity and composition, are the main factors determining potential pollinators among nectar-feeding animals (Fægri & Van der Pijl, 1979; Baker & Baker, 1983; Proctor et al., 1996). An appropriate positioning of the nectar inside the flower ensures the efficiency of pollination: while exploiting the nectar, the visitor should inevitably contact the reproductive organs.

In this paper we describe the structure of the gynopleural nectaries of *Aloe castanea* (Schönland) and *A. greatheadii* var *davyana* (Schönland) Glen & D.S. Hardy. The genus *Aloe*, family Asphodelaceae, consists of about 350 species occurring across a wide range of habitats in Africa, Madagascar and nearby Comoro Islands, the Middle East, and the Canary Islands. The huge variation in size, length and width of leaves, leaf markings, raceme length and even flower size has led to the division of the genus into 26 sections (Reynolds, 1950; Holland, 1978; Van Wyk & Smith, 1996; Glen & Hardy, 2000). *Aloe greatheadii* var *davyana* belongs to the largest section, Pictae or spotted aloes, and exists either as solitary plants or in large colonies. This aloe produces pink tubular flowers during winter (from June to August) and has a widespread distribution range across the summer rainfall areas of South Africa. The plants grow well on rocky terrain and on grassy plains and are most dense in overgrazed areas (Van Wyk & Smith, 1996; Glen & Hardy, 2000). *Aloe greatheadii* var *davyana* is an extremely important bee plant in South Africa, and beekeepers are known to move their beehives to the aloe fields in winter to make use of the strong pollen and nectar flow (Fletcher & Johannsmeier, 1978).

Aloe castanea belongs to the section *Anguialoe*. This multistemmed aloe, 2-4 m tall with branched stems, has long curved inflorescences with subsessile orange-brown flowers and abundant nectar. It flowers from July to August and occurs in hot, dry thorny woodland in Mpumalanga and Limpopo Provinces of South Africa and in Swaziland (Van Wyk & Smith, 1996; Glen & Hardy, 2000). Aloes are very important nectar producers in dry habitats, but very little is known about their nectary structure and the manner of nectar presentation to pollinators. Due to the differences in flower morphology and potential pollinators of *A. castanea* and *A. greatheadii* var *davyana*, we would expect different mechanisms of nectar transport and presentation

Methods

Plant material

Ovaries of *A. castanea* (Schönland) were collected from plants growing in the Pretoria Botanic Garden (Fig. 1) and those of *A. greatheadii* var *davyana* (Schönland) Glen & D.S. Hardy from plants in Roodeplaat Nature Reserve, Gauteng (28° 39'E, 25° 66'S) (Fig. 5). Voucher specimen collection was therefore not necessary for *A. castanea* and *A. greatheadii* var *davyana* was identified in natural habitats by taxonomic experts. Three different flower stages were examined for each species: young flowers with the corolla starting to open but not all anthers dehiscent; intermediate flowers with all the anthers dehiscent and the corolla completely open; old flowers in which the corolla had started to wilt. Nectar production rate varies with age in a similar manner in the two species: increasing from young to intermediate stages and decreasing in old flowers (Nicolson & Nepi, 2005).

Light microscopy and histochemistry

Ovaries were dissected from flowers under a stereo microscope and fixed in 5 % glutaraldehyde in phosphate buffer (pH 6.9), dehydrated in an ethanol series and embedded in Technovit 7100 (Heraeus Kulzer GmbH). A complete series of semi-thin sections (3-5 µm) was obtained with an LKB 8800 microtome. Sections from corresponding parts of the ovary were stained for histochemistry with the following:

- Toluidine Blue O as general staining (O'Brien & McCully, 1981);
- PAS (periodic acid/Schiff's reaction) for total insoluble polysaccharides (O'Brien & McCully, 1981);

- c) Alcian Blue for pectins (O'Brien & McCully, 1981);
- d) IKI (iodine-potassium iodide or Lugol) for starch (Johansen, 1940);
- e) Auramine O for cuticle (Heslop-Harrison, 1977);
- f) Aniline Blue for callose (Johansen, 1940).

In addition, thin hand-cut sections (20-50 μm) of young ovaries were mounted in distilled water on slides and examined on a Zeiss Axiovert 200 inverted microscope (Carl Zeiss, Göttingen, Germany) at 10x magnification for autofluorescence of chloroplasts.

Scanning electron microscopy

Cross and longitudinal sections were made of the ovaries from the three different flower stages of *A. castanea* and *A. greatheadii* var *davyana*. In order to be able to distinguish between top and bottom, we left the upper part of each ovary with 1 mm of style still attached. The material was fixed in 2.5% glutaraldehyde and a sodium phosphate buffer (pH 7.4) for 1 h. Material was rinsed for three times for 10 min each in a sodium phosphate buffer prior to post-fixation with 1% aqueous osmium oxalate for 1 h. Thereafter the material was rinsed with distilled water for twice for 10 min each, and dehydrated in an increasing ethanol series (30, 50, 70, 90 and 100%) for 10 min each. The 100% ethanol was repeated three times before critical point drying in a Polaron critical point drier using carbon dioxide. Material was mounted on SEM stubs, sputter coated with gold and viewed with a JEOL 840 SEM (Tokyo, Japan) at the Laboratory for Microscopy and Microanalysis at the University of Pretoria.

Results

Flower morphology and nectar presentation

Flowers of *A. castanea* form dense inflorescences (Fig. 2). Each flower has an orange-brown, cylindric-campanulate perianth widely opened at the top and generally oriented upward or horizontally. There are six flattened filaments bearing anthers. All the filaments (and the anthers) are exerted from the perianth, the inner ones being longer than the outer ones. The superior ovary is orange (Fig. 3) and bears a long style ending with a tiny stigma positioned at the level of the anthers with longer filaments. Nectar accumulates at the top of the ovary (Fig. 3) filling the space between the filaments. Nectar is pale when just secreted but soon becomes dark reddish-brown (Figs 3, 4). The

pink-orange flowers of *A. greatheadii* var *davyana* form less dense inflorescences (Fig. 6). The orientation of the flowers changes according to their development: from upward as buds to downward just before anthesis, and up again after the end of anthesis and during fruit development (Fig. 6). The perianth is narrower than in *A. castanea* and forms a tube, from which the anthers of the longer filaments are exerted first (Fig. 7). The perianth enlarges at the base of the ovary to form a small bulb (Fig. 7). The disposition of gynoecium and androecium is very similar to *A. castanea*. The superior ovary is green (Fig. 8) and has a long style ending with a tiny stigma, slightly curved. Nectar accumulates around the inferior third of the ovary (Fig. 8), filling the bulb formed by the perianth enlargement. When secretion is particularly abundant, nectar flows along the filaments and the style and may appear at the mouth of the perianth tube where it becomes accessible for bees.

Nectary structure and development

The general anatomy of the nectary is similar in both species. The gynopleural nectaries consist of three clefts located in the septal region between adjacent carpels (Fig. 9). The cavities are lined by secretory epithelium characterised by small cells with dense cytoplasm and large nuclei (Fig. 10). A very thin and irregular cuticle is present on the surface of the epithelium (Fig. 11). Beneath the epithelium in *A. castanea* there is a subsidiary tissue composed of vacuolated cells which are smaller than cells in the other parts of the ovary parenchyma (Fig. 10). This tissue is not clearly evident in *A. greatheadii* var *davyana* and the nectar cavity is extremely reduced (Fig. 12). In this species, the outer tangential walls and the distal part of the radial walls of the epithelium cells appear thicker (Fig. 12) and are intensely stained by PAS (Fig. 13a) and Alcian Blue (Fig. 13b) and have a somewhat “corroded” appearance. In the old flower, the nectar cavity of *A. greatheadii* var *davyana* has almost completely disappeared and the walls described above are thicker and more intensely stained by PAS (Fig. 14) and Alcian Blue (Fig. 15a). In the same walls there is an irregular deposition of callose (Fig. 15b). The modifications of the walls are less evident in *A. castanea*, where there is a reduced thickening in young (Figs 16a, b) and old (Fig. 17) flowers and there is no deposition of callose in the old stage. The epithelial and subsidiary cells of *A. castanea* undergo cytological modification during development, being more vacuolated and with an irregular nuclear shape in the old flower stage (Fig. 18). Some cells in the subsidiary tissue seem to degenerate (Fig. 18). These modifications are not evident in *A.*

greatheadii var *davyana*, where epithelial cells maintain their initial shape even in the old flower stage (Fig. 14).

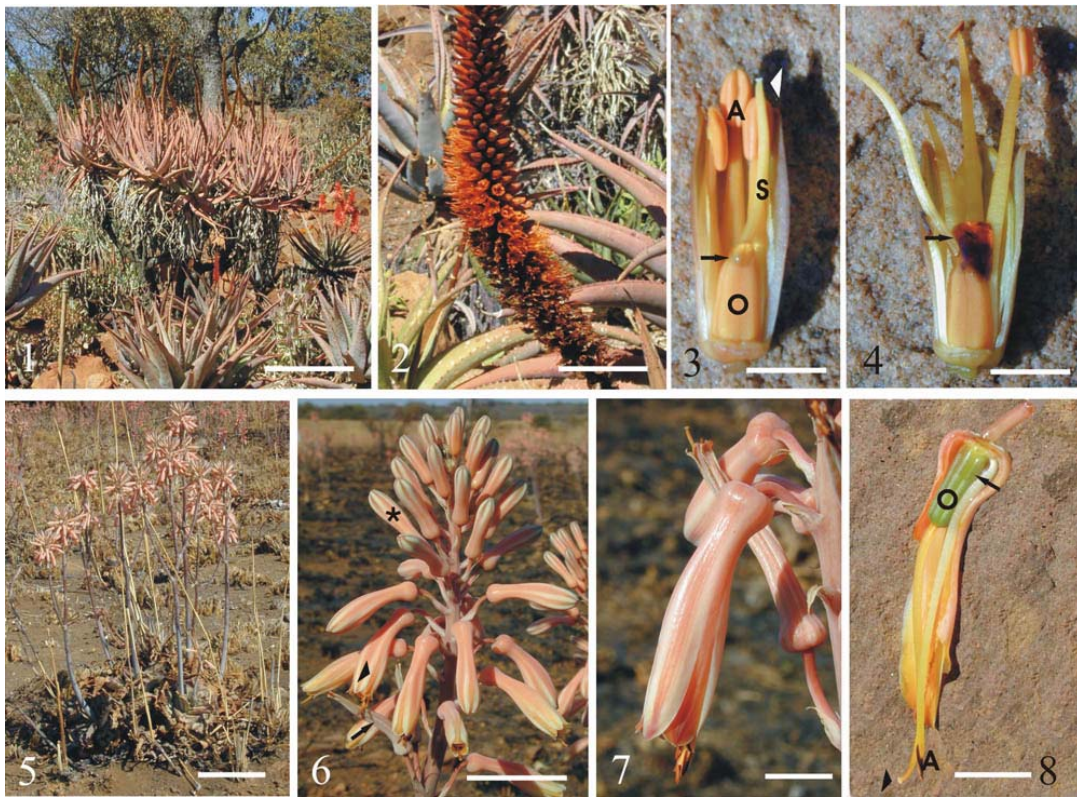


Plate 1

Plate 1. (Fig. 1) A large multi-stemmed plant of *Aloe castanea* in the Pretoria Botanic Garden. Bar = 1 m. **(Fig. 2)** The long curved inflorescence of *Aloe castanea* with densely-packed orange-brown flowers that bend sideways and upwards. Bar = 10 cm **(Fig. 3)** A flower of *Aloe castanea* in the young stage with the corolla and filaments partially removed. The superior ovary (O) is orange and has a long style (S) ending with a tiny stigma (arrowhead). Freshly secreted pale nectar is present at the top of the ovary (arrow). A = anthers. Bar = 0.5 cm. **(Fig. 4)** A flower of *Aloe castanea* in the middle stage with the corolla and filaments partially removed. As secretion proceeds, nectar (arrow) accumulates in the space between the filaments and becomes dark red-brown. Bar = 0.5 cm. **(Fig. 5)** Plants of *Aloe greatheadii* var *davyana* in Roodeplaat Nature Reserve, Gauteng. Bar = 15 cm. **(Fig. 6)** An inflorescence of *Aloe greatheadii* var *davyana*. The pink-orange flowers change in orientation during development. They are in an upward position before anthesis (asterisk), they bend downward just before anthesis (arrowhead) and upward again after the end of anthesis and during fruit development (arrow). Bar = 3 cm. **(Fig. 7)** Close-up of a flower of *A. greatheadii* var *davyana*. At anthesis the longer anthers are exerted from the perianth tube. The perianth is enlarged at its base. Bar = 1.5 cm. **(Fig. 8)** A flower of *Aloe greatheadii* var *davyana* in the middle stage with the corolla and filaments partially removed. The superior ovary is green (O). The long style ends with a tiny slightly curved stigma (arrowhead). A = anthers. Nectar accumulates around the base of the ovary (arrow). Bar = 1.5 cm.

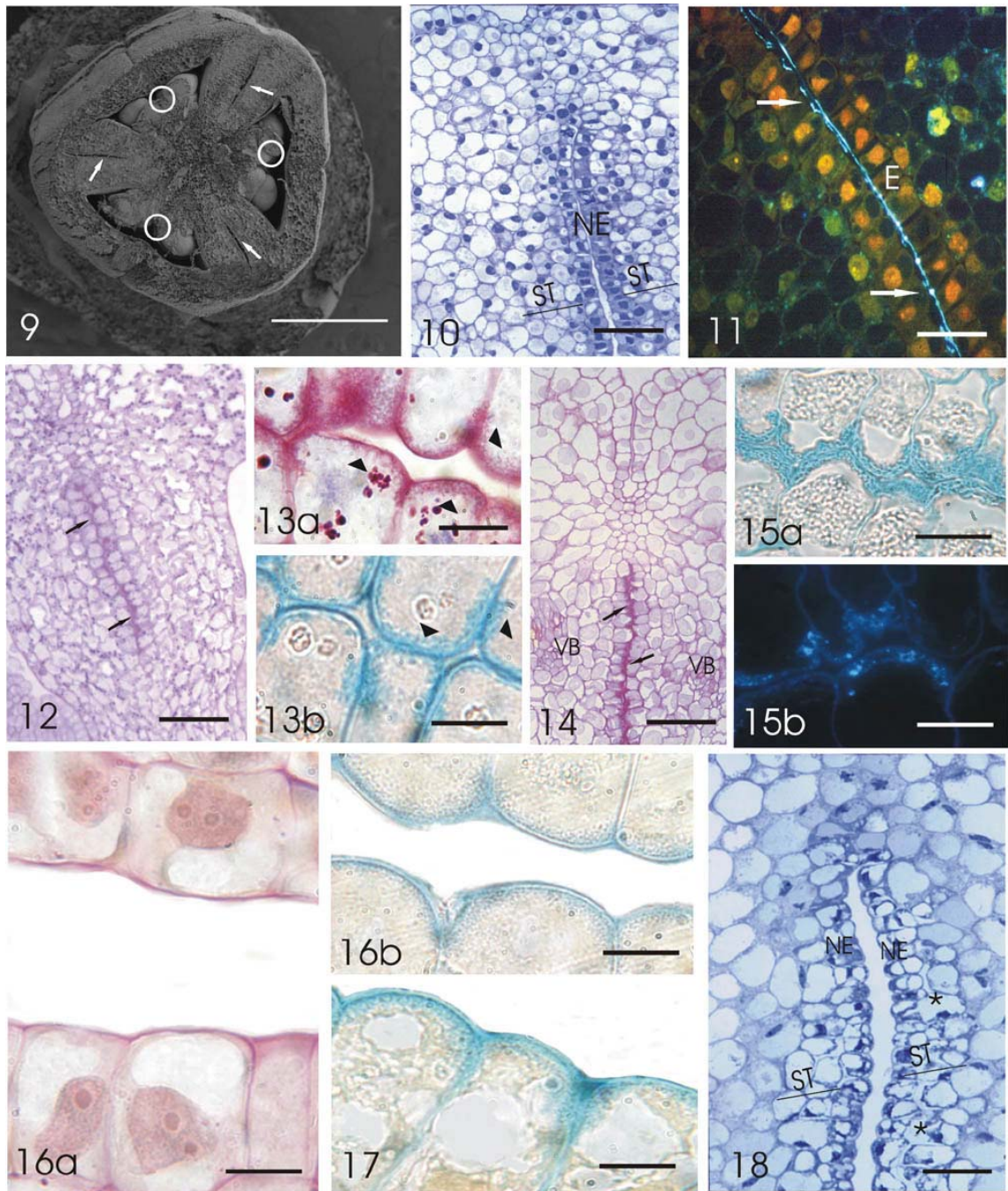


Plate 2

Plate 2. (Fig. 9) Scanning electron micrograph of a cross section of the ovary of *A. greatheadii* var *davyana* showing the localization of the three gynopleural nectaries (arrows) alternating with the ovary locules. O = ovules. Bar = 1 mm. **(Fig. 10)** Cross section of the ovary of an *A. castanea* flower in the young stage, stained with Toluidine Blue O. The nectary cavity is lined by an epithelium (NE) made of small cells with dense cytoplasm and relatively large nuclei. A subsidiary tissue (ST) is present around the nectary. Bar = 200 µm. **(Fig. 11)** Cross section of the ovary of an *A. castanea* flower in the young stage, stained with Auramine O. A very thin and irregular cuticle (arrows) is present on the surface of the epithelium. Bar = 100 µm. **(Fig. 12)** Cross section of the ovary of an *A. greatheadii* var *davyana* flower in the young stage, stained with PAS. The nectary cavity is reduced compared to that of *A. castanea* and the subsidiary tissue is not evident. The thick tangential outer walls (arrows) of the epithelial cells are intensely stained by PAS. Bar = 200 µm. **(Fig. 13)** Nectary epithelium cells in the young flower of *A. greatheadii* var *davyana* stained with PAS (**a**) and with Alcian Blue (**b**). The outer tangential walls and the distal part of the radial walls (arrow heads) are thicker than the other walls and have a somewhat corroded appearance. Bar = 30 µm. **(Fig. 14)** Nectary of *A. greatheadii* var *davyana* in the old flower stage stained with PAS. The nectar cavity is occluded and the outer tangential walls of the epithelium cells (arrows) are more densely stained by PAS than in the young stage. VB = vascular bundle. Bar = 200 µm. **(Fig. 15)** Nectary epithelium cells in the old flower of *A. greatheadii* var *davyana* stained with Alcian Blue (**a**) and with Aniline Blue (**b**). The outer tangential walls and the distal part of the radial walls of the epithelium cells appear thicker and more intensely stained by Alcian Blue than in the young stage (Fig 13b). The Aniline Blue reveals an irregular deposition of callose. Bar = 30 µm. **(Fig. 16)** Nectary epithelium cells in the young flower of *A. castanea*, stained with PAS (**a**) and Alcian Blue (**b**). The outer tangential walls and the distal part of the radial walls are less thick and less stained compared to the same stage in *A. greatheadii* var *davyana*. Bar = 15 µm. **(Fig. 17)** Nectary epithelium cells in the old flower of *A. castanea* stained with Alcian Blue. The outer tangential walls and the distal part of the radial walls are less thick and less stained compared to the same stage in *A. greatheadii* var *davyana*. Bar = 15 µm. **(Fig. 18)** Nectary of *A. castanea* in the old flower stained with Toluidine Blue O. The epithelium cells (NE) and the cells of the subsidiary tissue (ST) are more vacuolated than in the young stage. Some cells in the subsidiary tissue seem to degenerate (asterisk). Bar = 100 µm.

Starch is present in both species in the young stages, but with different localisation. In *A. castanea*, starch is present throughout the ovary but at higher concentration in the subsidiary tissue around the nectary (Fig. 19a). In *A. greatheadii* var *davyana*, starch is present mainly in the cortical part of the ovary and in very low quantity around the nectary (Fig. 20a). In both cases starch is completely hydrolysed in the old flower stage (Figs 19b and 20b).

Chlorophyll is present in the ovary of both species, although that of *A. castanea* appears deep orange. In this species chlorophyll has a homogeneous distribution throughout the ovary, being present also in the subsidiary tissue around the nectary (Fig. 21). In *A. greatheadii* var *davyana*, chlorophyll is present in the ovary wall while it is almost absent in the tissue around the nectary (Fig. 22). Vascular bundles containing phloem and xylem were observed around the nectary (see Fig. 14).

Nectar outlet

In *A. castanea*, each nectary cavity has a nectar outlet located just at the base of the style (Fig. 23). It is derived from the merging of an invagination of the cutinised epidermal surface, in continuity with the carpellary suture (Fig. 23), with the apical part of the nectary (Figs 24 and 25). Small cells are present in the vicinity of the merging point (Fig. 24).

In *A. greatheadii* var *davyana*, the carpellary suture is wide at the base of the ovary but it becomes deeper and narrower towards the top of the ovary (Fig. 26 and Figs 27a, b). At two-thirds from the top of the ovary, the invagination of the epidermal surface has tightly connivent margins, except in the inner part where a tubular structure is formed (Fig. 27c). The tubular structure becomes deeper towards the top of the ovary, where it merges with the apical part of the nectary (Fig. 27d). Small cells are present in the inner part of the tubular structure (Fig. 28). Although the tubular structure is in continuity with the outside, this communication is prevented by the presence of the cuticle that occludes the narrow space between the connivent margins of the epidermis (Fig. 29).

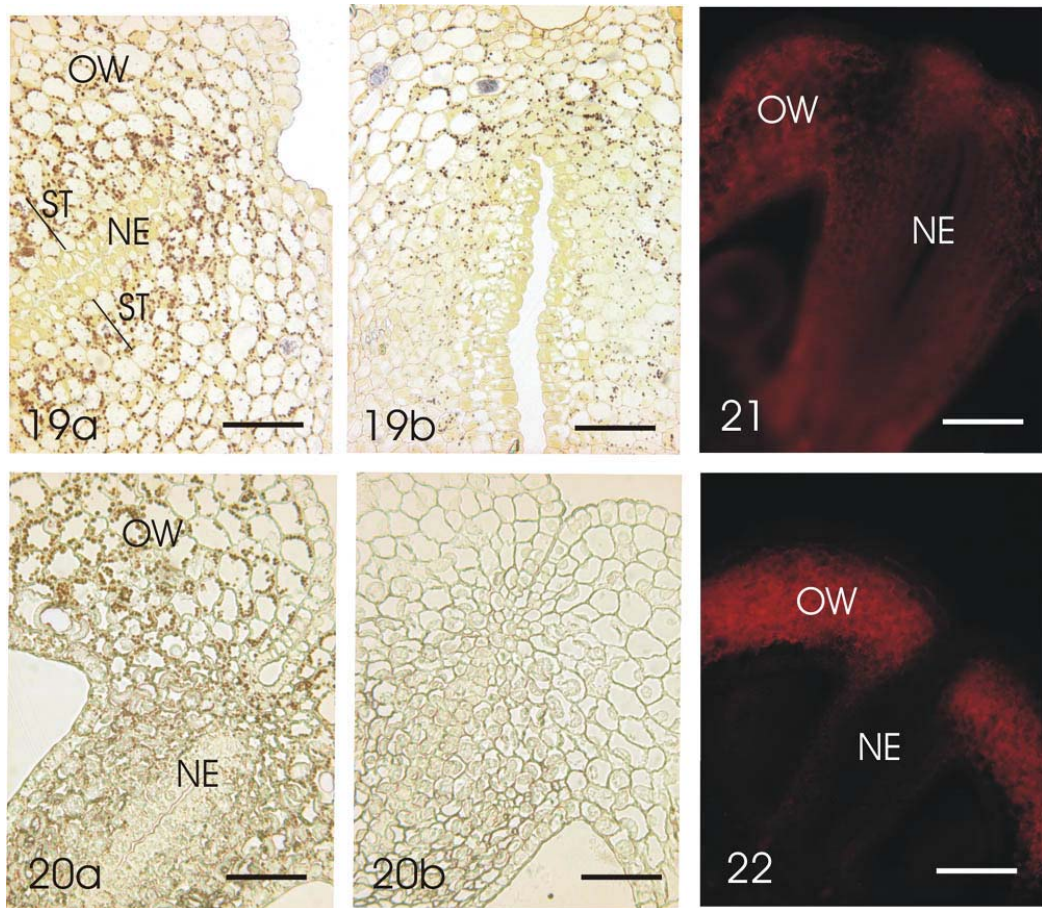


Plate 3

Plate 3. (Fig. 19) Nectary of *A. castanea* in the young (a) and old (b) flower stained with IKI. In the young flower starch is present in higher concentration in the subsidiary tissue (ST) around the nectary epithelium (NE). Starch is almost completely hydrolysed in the old flower. OW = ovary wall. Bar = 200 μ m. **(Fig. 20)** Nectary of *Aloe greatheadii* var *davyana* in the young (a) and old (b) flower stained with IKI. In the young flower starch is present in higher concentration in the ovary wall (OW). Starch is completely hydrolysed in the old flower. NE = nectary epithelium. Bar = 200 μ m. **(Fig. 21)** Chlorophyll autofluorescence in the ovary of *A. castanea*. Chlorophyll has a homogeneous distribution being present also in the subsidiary tissue around the nectary. NE = nectary epithelium; OW = ovary wall. Bar = 300 μ m. **(Fig. 22)** Chlorophyll autofluorescence in the ovary of *Aloe greatheadii* var *davyana*. Chlorophyll is present exclusively in the ovary wall (OW). NE = nectary epithelium. Bar = 300 μ m.

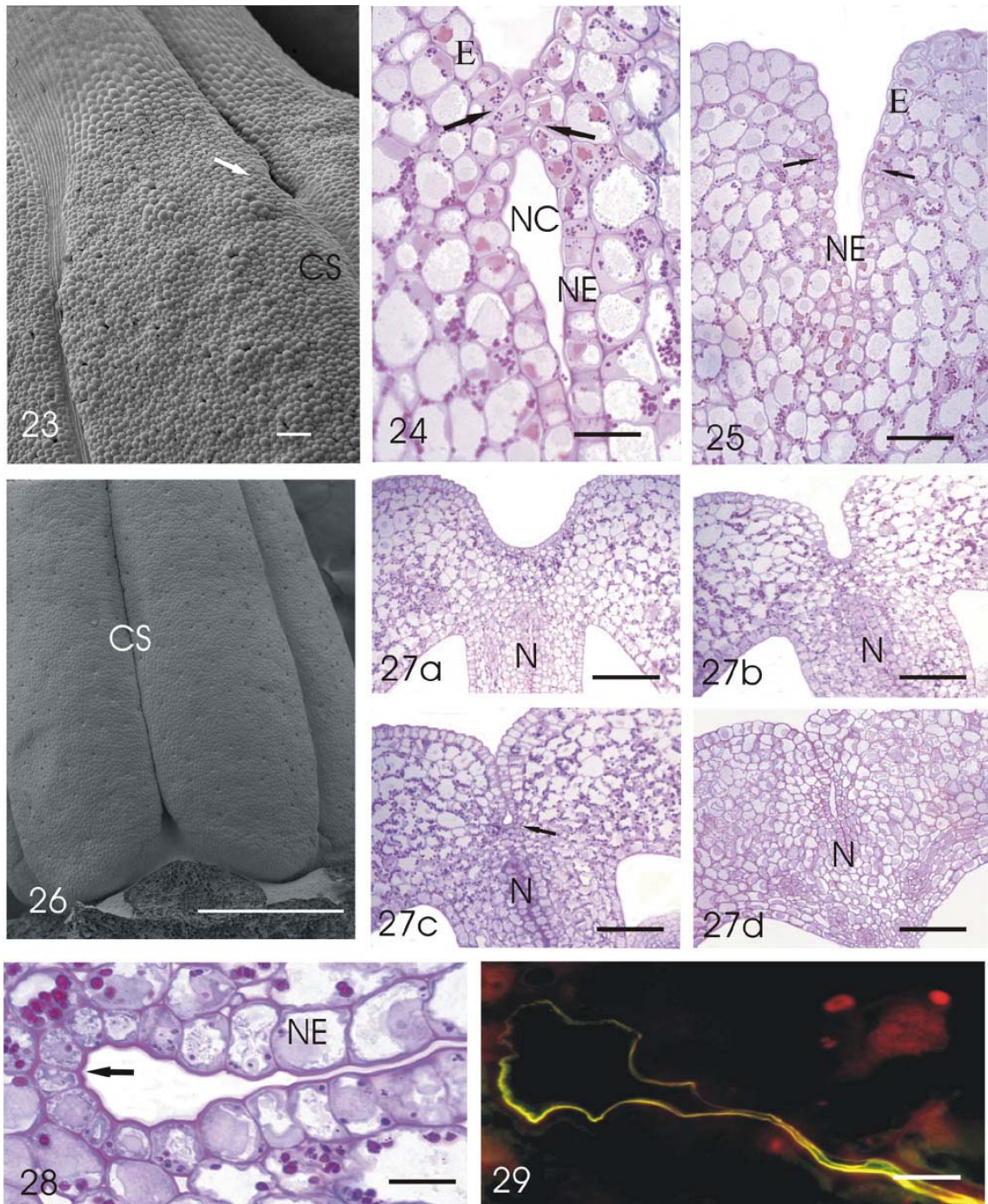


Plate 4

Plate 4. (Fig. 23) Scanning electron micrograph of the ovary of *A. castanea*. The nectary outlet (arrow) is located at the base of the style. CS = carpellary suture. Bar = 100 μ m **(Fig. 24)** Cross section of the apical part of the ovary of *A. castanea* stained with PAS and Coomassie Blue. A few small cells (arrows) are present between the nectary cavity and the epidermal invagination. E = epidermis; NE = nectary epithelium; NC = nectary cavity. Bar = 100 μ m. **(Fig. 25)** Cross section of the nectary outlet at the base of the style in *A. castanea*. Small cells (arrows) are present between the nectary epithelium (NE) and the epidermis (E). Bar = 200 μ m. **(Fig. 26)** Scanning electron micrograph of the base of the ovary in *A. greatheadii* var *davyana*. The carpellary suture (CS) is wide at the base of the ovary and becomes narrower towards the top of the ovary. Bar = 1 mm. **(Fig. 27) a-d.** Sequential cross sections of the ovary of *A. greatheadii* var *davyana* stained with PAS and Coomassie Blue. **a.** The wide carpellary suture at the base of the ovary becomes narrower and deeper towards the top of the ovary. **b.** At 1/3 of the distance from the top of the ovary, a deep epidermal invagination is present. **c.** This invagination became deeper and with connivent margins at two thirds from the top, forming a tube-like structure (arrow). **d.** This structure merged with the nectary cavity at the top of the ovary. N=nectary. Bar = 400 μ m. **(Fig. 28)** Cross section of the deep epidermal invagination in the ovary of *A. greatheadii* var *davyana* stained with PAS and Coomassie Blue. The invagination ends with a tube-like structure with small cells (arrow) in the inner part. Bar = 50 μ m. **(Fig. 29)** Cross section of the deep epidermal invagination in the ovary of *A. greatheadii* var *davyana* ending with a tube-like structure stained with PAS and Auramine O. The cuticle occludes the narrow space between the connivent margins of the epidermis. Bar = 50 μ m.

Discussion

Nectar anatomy and development

According to Smets et al. (2000), there are two main nectary types in monocotyledons: septal (i.e. persistent) and perigonal (i.e. caducous). In both *Aloe* species typical septal nectaries were found. The morphological characters of these nectaries correspond to the “liliad type” described by Schmid (1985) as “non-labyrinthine distinct septal nectaries” and are considered by this author to be a primitive character in the phylogeny of monocotyledons.

Development of septal nectaries follows two patterns that differ mainly in the fate of the nectary after the secreting phase. A breakdown of the nectary epithelium after secretion was demonstrated in *Musa paradisiaca* female flowers (Fahn & Kotler, 1972); while the transformation of the nectary tissue into parenchyma, by means of elongation of epithelium cells and occlusion of the nectary cavity, has been reported in *Aloe*, *Gasteria* and *Tillandsia* (Schnepf & Pross, 1976; Cecchi Fiordi & Palandri, 1982). Schnepf and Pross (1976) also demonstrated differentiation of transfer cells in the epithelium of the septal nectaries in some *Aloe* species. A short time before anthesis they form an elaborate system of wall protuberances along their outer walls. In the developing fruit they redifferentiate, lose the wall protuberances, increase in size, and become parenchymatous cells. The redifferentiation of transfer cells was accompanied by the transformation of amyloplasts into chloroplasts. The differentiation of transfer cells in septal nectaries is supposed to be an anatomical mechanism to increase nectar output (Schmid, 1985). According to our observations, the differentiation of epithelial cells into transfer cells most probably occurred in both *Aloe* species, but the transformation of the nectary tissue into parenchyma can be hypothesised only for *A. greatheadii* var *davyana*. The differentiation of thickened outer walls in the epithelium cells was already evident in the young stage in *A. greatheadii* var *davyana* where they have a somewhat “corroded” appearance (see figs 13a, b), as reported by Saunders (1890) for *Kniphofia*, an aspect that can be related to the differentiation of transfer cells (Schmid, 1985). In *A. greatheadii* var *davyana*, the elongation of epithelial cells is not evident, and moreover the nectar cavity is completely occluded in the old stage. The deposition of callose in the thickened outer walls signalled the end of secretion activity, as reported also by Schnepf and Pross (1976). In *A. castanea*, the vacuolation and elongation of

epithelial cells is evident in old flowers but the nectar cavity is still present; in addition, the thickening of the epithelial cells' outer walls is reduced in comparison to *A. greatheadii* var *davyana* and there is no deposition of callose in those walls.

The localisation of chlorophyll and starch storage sites overlap in both species. In *A. greatheadii* var *davyana* both chlorophyll and starch are concentrated in the ovarian wall. In *A. castanea* chlorophyll is also present around the nectary, where an increased starch accumulation was observed. These differences between *A. greatheadii* var *davyana* and *A. castanea* are related to the different extent of the subsidiary glandular tissue underlying the epithelium cells. This tissue is more developed in *A. castanea*, and evidently photosynthesising and able to store starch. Different extents of the subsidiary tissue were also observed in different species of *Tillandsia* (Cecchi Fiordi & Palandri, 1982) and were related to differences in nectar production. In both *Aloe* species almost all the starch was hydrolysed in the old flower stage, suggesting a correlation between nectar production and starch hydrolysis, as observed for other species secreting copious quantities of nectar (Nepi et al., 1996; Durkee et al., 1981; Pacini et al., 2003). The greater quantity of starch around the nectary does not, however, result in greater sugar production in *A. castanea* (mean volume per flower 44.6 μ l, concentration 16.0%) (Nicolson & Nepi, 2005) compared to *A. greatheadii* var *davyana* (mean volume per flower 30.7 μ l, concentration 23.5%) (Chapter 4).

Nectar presentation and pollinators

Just as pollen has primary and secondary presentation (Faegri & van der Pijl, 1979), the same was proposed for nectar by Pacini et al. (2003). The presentation is primary when the site of nectar production and the site of nectar emission are the same - the more common situation. When these sites are different the term secondary presentation is used. In this case nectar flows from the nectary and collects in another part of the flower. As in all plants having septal nectaries, *Aloe* species have secondary nectar presentation (Dauman, 1970; Smets et al., 2000). Nonetheless, nectar presentation is different in the two species we studied. *A. castanea* has primary nectar outlets located at the base of the style and nectar accumulates at the top of the ovary, sometimes filling the corolla tube. The system of secondary nectar presentation is more complicated in the case of *A. greatheadii* var *davyana*, where secondary drainage through a capillary duct is present and nectar is accumulated at the base of the ovary in a bulb formed by an

enlargement of the corolla. In *A. greatheadii* var *davyana* a primary nectar outlet is present at the top of the ovary, from where the nectar is transported by means of capillarity through the tubular structure formed by deep invagination of the epidermis. When this deep invagination enlarges, about at one third of the ovary length from its base, the nectar may flow into the bulb through a secondary outlet. These kinds of nectar ducts were reviewed by Vogel (1998) and were described in plants with septal nectaries or in plants where nectar accumulates in spurs or other narrow tubular containers. The ducts that we found in *A. greatheadii* var *davyana* are very similar, from a morphological point of view, to those described in *Milla biflora* (Alliaceae) (Vogel, 1998) although they are longer in the latter species.

Among aloes, the bulb at the base of the corolla is a common feature in the section *Pictae* (Glen & Hardy, 2000), and species belonging to this section probably have the same nectar presentation as described for *A. greatheadii* var *davyana*.

Because nectar composition is remarkably constant in species of *Aloe* (sucrose is almost absent and there are almost equal amounts of glucose and fructose; van Wyk et al., 1993), the flower morphology and secondary presentation of nectar, which affect nectar availability, may be important to potential animal visitors. According to our observations, honeybees collect only pollen from *A. castanea*, ignoring the very dilute nectar, but collect both pollen and nectar from *A. greatheadii* var *davyana*. Bees are probably effective pollinators in both cases. Bird visitors to *A. castanea* include sunbirds and larger, less specialised passerines while only sunbirds have been observed probing the tubular flowers of *A. greatheadii* var *davyana*.

Acknowledgements

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CHAPTER 4

Dilute nectar of *Aloe greatheadii* var *davyana* as a resource for honeybees, *Apis mellifera scutellata*, during dry South African winters

H. Human and S.W. Nicolson

Department of Zoology and Entomology, University of Pretoria, Pretoria 0002, South Africa.



V. Dietemann

Abstract

The winter flowering *Aloe greatheadii* var *davyana* is a major indigenous bee plant; with a widespread distribution across the northern summer rainfall areas of South Africa. Its highly nutritious pollen is utilised by migratory beekeepers for colony buildup and the strong nectar flow for honey production. We looked at variation in nectar (volume and concentration) on various levels in an assessment of this nectar resource. There were no significant differences in nectar volume and concentration between the bulb and the floral tube, only between flower stages. Nectar was continuously available, with both volume and concentration remaining relatively constant throughout the day. The average volumes and concentrations of nectar in screened flowers (30.7 μ l, 23.5% w/w) were significantly higher than those in unscreened flowers (14.7 μ l, 18.6%). Nectar volume was observed to be lowest and nectar concentration highest late in the flowering season. The volumes and concentrations of nectar measured across the distribution range of *A. greatheadii* var *davyana* were significantly lower at Marble Hall in the east compared to Roodeplaat (middle of the range) and Zeerust in the west. *Aloe greatheadii* var *davyana* nectar, although dilute from a bee perspective, is more concentrated than that of other *Aloe* species, and is an ideal source of energy and water for honeybees.

Introduction

An important winter pollen and nectar source for South African beekeepers in the northern summer rainfall regions of the country is the indigenous *Aloe greatheadii* var *davyana*. Beekeepers move their hives over hundreds of kilometers to the “aloe fields” north of Pretoria where the highly nutritious pollen of this aloe (Chapter 1) promotes colony growth and the copious nectar contributes substantially to the honey crop (Williams, 2002). *Aloe greatheadii* var *davyana* flowers prolifically in mid-winter (end June - mid August), when few other nectar sources are available. These aloes occupy rocky areas in grassland and thrive in disturbed areas (Glen & Hardy, 2000; Van Wyk & Smith, 1996). They grow equally well in full sun and in shade beneath trees.

Aloe species are known for their copious dilute nectar; an example is the nectar of *A. ferox* with an average volume and concentration of 180 µl and 12.5% respectively (Hoffman, 1988). Hoffman (1988) found that nectar and pollen were the chief floral rewards for birds and honeybees respectively. Tubular flowers with low nectar concentrations are associated with bird pollination, while bee-pollinated flowers have higher nectar concentrations (>35%, Pyke & Waser, 1981). However, honeybees are known to collect nectar with concentrations ranging from 15 to 65% (Visscher & Seeley, 1982), and when they need to cool the hive by evaporation they will collect water or dilute nectar (Eisikowitch & Masad, 1982).

Nectar concentrations vary widely (7-70%), not only between but also within species (Nicolson, 1998). For example, the low end of the range is *Eucalyptus incrassata* (Myrtaceae) with extremely dilute nectar (7%) and at the high end is *Carum carvi* (Apiaceae) producing nectar with an average concentration of 66.5% (Bond & Brown, 1979; Langenberger & Davis, 2002). Variation within a species is illustrated by the range of 2 to 62% observed in *Echium plantagineum* (Boraginaceae) and 4 to 72% in *Clintonia borealis* (Liliaceae) (Corbet & Delfosse 1984; Plowright, 1981). The volume and concentration of nectar are influenced by factors such as nectary activity, flower age, temperature and relative humidity, water availability and animal visitors.

Nicolson and Nepi (2005) investigated nectar production during the dry winter in open, campanulate flowers of *Aloe castanea*. The nectar of these flowers is more exposed than

that of aloes with tubular flowers. The authors observed variation in nectar concentrations of 8 to 10% between individual plants of *A. castanea*. We hypothesised that the tubular structure of *A. greatheadii* var *davyana* flowers would prevent evaporation of nectar. We looked at variation in nectar volume and concentration on various levels, from within individual flowers to across the summer rainfall area of South Africa, in order to assess the nectar resource being used by beekeepers. We compared nectar (volume and concentration) in the bulb and the tube, between different flower stages, in screened and unscreened flowers, and in flowers in the sun and shade. We sampled nectar over the flowering season as well as across the distribution range of *A. greatheadii* var *davyana*.

Methods

Nectar production in *A. greatheadii* var *davyana* was studied at Roodeplaat Nature Reserve (795 ha) (28° 39'E, 25° 66'S), at Rust de Winter (28° 23'E, 25° 12'S), Zeerust (26° 02'E, 25° 36'S) and Marble Hall (29° 17'E, 24° 59'S) during the winter months (June and July) of 2003-2005 (see Fig. 2B, Introduction). All areas have dense populations of *A. greatheadii* var *davyana*, especially Rust de Winter.

Nectar was collected in disposable haematocrit tubes (length 75 mm/75 µl). Volumes of nectar were determined from column length in haematocrit tubes and the concentrations measured as % w/w sucrose equivalents with a pocket refractometer (0-50%, Bellingham & Stanley Ltd, Tunbridge Wells, UK). Temperature and relative humidity were measured at flower height with either a hand-held thermohygrometer (Model TES 1365, TES Electrical Corp., Taiwan) or HOBO dataloggers (Onset Computer Corporation, Pocasset, MA, USA). The operating ranges of these loggers are -20°C to 70°C and 25 to 90 % for temperature and RH respectively.

Flower development and effect of flower age on nectar production

Twelve flower buds that were about to open (three each on four different plants) were tagged and flower development followed. During observations the duration of events such as filament and style elongation, anther dehiscence and the presence of nectar as well as floral characteristics were recorded. Observations were made every 30 min from 09.00 until 16.00 h on the first day, and again at 09.00 h on subsequent days.

The effect of flower age on nectar production (volume and concentration) was then determined by marking just opening flowers on five plants prior to nectar collection. Racemes with marked flowers were covered with gauze (2 mm mesh size) to exclude pollinators. This allowed for nectar sampling from five flowers of each stage (Fig. 1) from each of the five plants between 10.00 and 12.00 h on the following day.

All remaining measurements of nectar volume and concentration were made on stage 3 flowers (Fig. 1D) which showed the highest nectar production; see Results. Each flower was sampled only once.

Nectar in the bulb and floral tube

A characteristic feature of the family Pictae (spotted aloes), to which *A. greatheadii* var *davyana* belongs, is the distinct basal swelling (bulb) at the bottom of the tubular flowers (see Fig. 1). Five flowers from ten plants each were measured for bulb depth and length and total length of flower. Thereafter four flowers (stage 3) were picked from five plants each on three consecutive sampling days (11, 12 and 13 August 2004) at Roodeplaat Nature Reserve and at Rust de Winter, and the volume and concentration were measured separately for nectar in the bulb and in the floral tube. Two of these sampling days were at Roodeplaat Nature Reserve; one was a warm day and the other a cool, cloudy day. At Rust de Winter the weather was similar to the warm day at Roodeplaat. All other measurements were made on bulb and floral tube combined.

Screened and unscreened flowers

Nectar present in unscreened flowers (standing crop) represents the nectar encountered by floral visitors. Twenty plants were randomly marked and the inflorescences from 10 plants were covered in gauze (2 mm mesh size) while the remainder were left open. Nectar volumes and concentrations were measured hourly from 08.00 to 16.00 h in three flowers (stage 3) from each plant; gauze covers were replaced after each collection. Two HOBO dataloggers on one plant were used to measure temperature and humidity for the duration of the experiment: one was attached to an open raceme and the other to a raceme covered with gauze.

Plants in the sun and shade

Differences between nectar volume and concentration in plants growing in the sun and in the shade were also recorded. The volume and concentration of nectar from three unscreened flowers on six plants in the sun and six plants in the shade were recorded hourly from 08.00 until 17.00 h. Ambient temperature and humidity were also recorded hourly in dappled shade.

Nectar production during a flowering season

To determine whether nectar production varied during the flowering season, full day measurements of nectar volume and concentration were made at Roodeplaat Nature Reserve on 5 July, 26 July and 15 August 2003. On each date we sampled three unscreened flowers on each of 10 plants from 08.00 until 17.00 h.

Nectar production across the distribution range

In order to evaluate nectar production across the distribution range of *A. greatheadii* var *davyana*, full day nectar measurements were made (hourly from 08.00 until 17.00 h) of nectar volume and concentration early in the flowering season (on 5, 10 and 13 July respectively) at Zeerust, Roodeplaat and Marble Hall. Zeerust is at the western end of the distribution range of *A. greatheadii* var *davyana* and Marble Hall at the eastern end, with Roodeplaat being more central (see map on page 4 of Introduction). All flowers were sampled once.

Statistical analysis

The effects of flower age on nectar volume and concentration were compared within and between plants by multivariate ANOVA. The data met the assumptions for parametric statistics after the nectar volumes were \log_{10} transformed. Post hoc comparisons of nectar production between different flower stages were performed by Tukey tests (Zar, 1984).

Data for nectar measured in the bulb and floral tube met the assumptions for parametric statistics, therefore paired Student's t-tests were used to compare variation in nectar volume and concentration.

All other nectar data did not meet the assumptions for normality; variances were not homogeneous and data did not conform to a normal distribution. The effect of treatment (screened and unscreened flowers, plants in the sun or shade) and time on nectar volume and concentration as well as variation in nectar volume and concentration through the flowering season and across the distribution range were therefore assessed with Kruskal-Wallis ANOVA. Mann-Whitney U-tests were used for comparisons of the mean volumes and concentrations throughout the day.

Analyses were performed with the program Statistica 6.0 (1984-2004). The level of statistical significance for all analyses was $P = 0.05$. Values are given throughout as means \pm SD.

Results

Effect of flower age on nectar production

Flower stages for *A. greatheadii* var *davyana* are illustrated in Figure 1. Flowers opened throughout the day and had an average lifespan of four days. Nectar was collected from flowers of different stages between 10.00 and 12.00 h on a warm day and volumes are combined values for nectar available in the bulb and floral tube. The temperature increased from 19.4°C to 21.7°C and RH decreased from 41 to 29% during this time. Nectar was already present in stage 1 flowers (just opening), and remained present until the flowers wilted. The mean nectar volume increased to a maximum of 33.5 μ l in stage 3 flowers, and then decreased in wilted flowers (Fig. 2). The average concentration of nectar varied much less with flower age, with a maximum of 21.4% in stage 3. There were no significant differences in nectar volume and concentration between ($F = 1.767$, $df = 8$, $P = 0.08$) or within plants ($F = 0.934$, $df = 10$, $P = 0.503$), only between flower stages ($F = 41.943$, $df = 6$, $P < 0.001$). The results of Tukey tests for comparisons between stages are indicated in Figure 2. Nectar volume varied significantly between all flower stages while nectar concentration remained relatively stable across flower stages, but was significantly lower in stages 1 and 4 than stages 2 and 3 (Fig. 2).

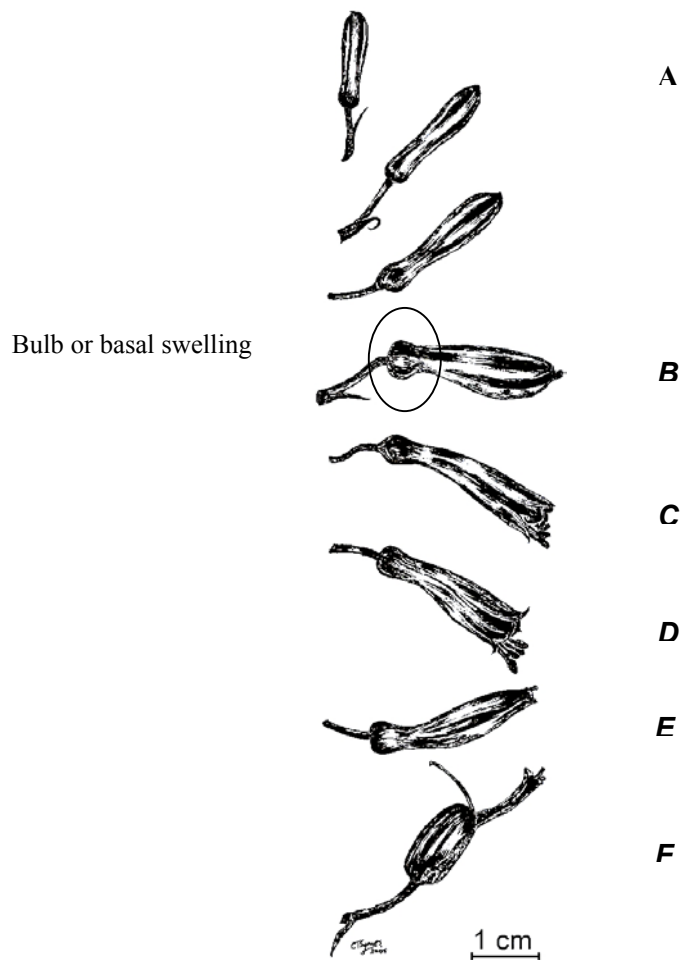


Figure 1. Flower development in *Aloe greatheadii* var *davyana*. Orientation of flowers changed from 0° to 120° then back to 80° with age. **(A)** Closed flower buds, with an upward orientation. **(B)** Flowers in which the corolla was just opening (stage 1), note the horizontal position. **(C)** Open flowers, 2-5 h, with the three long anthers exserted (stage 2), **(D)** The floral tube reached its maximum width, oriented downwards, with all six anthers exserted after 24 h (stage 3), **(E)** At 72 h the floral tube started to wilt while the style remained turgid (stage 4); flower in an upward position. Flowers were completely wilted after 96 h. **(F)** Fruit appeared approximately 3 weeks later.

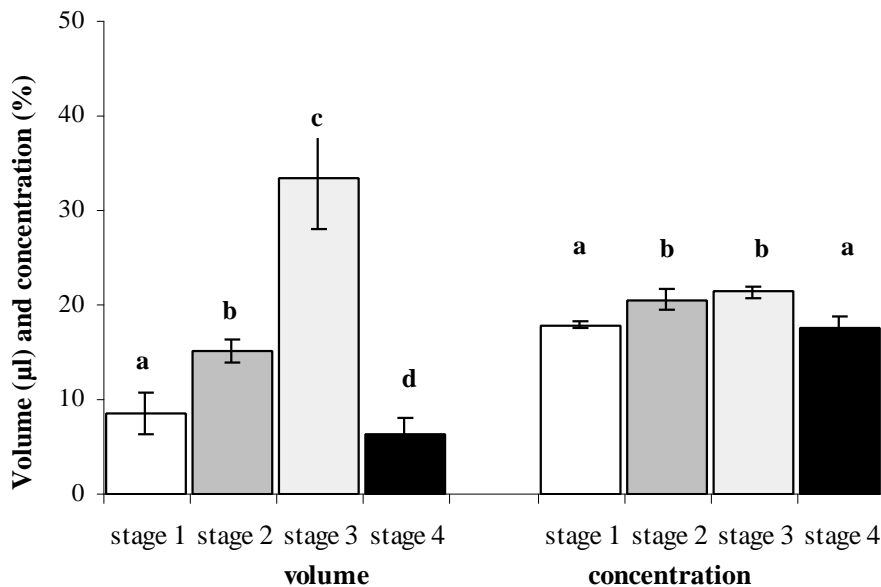
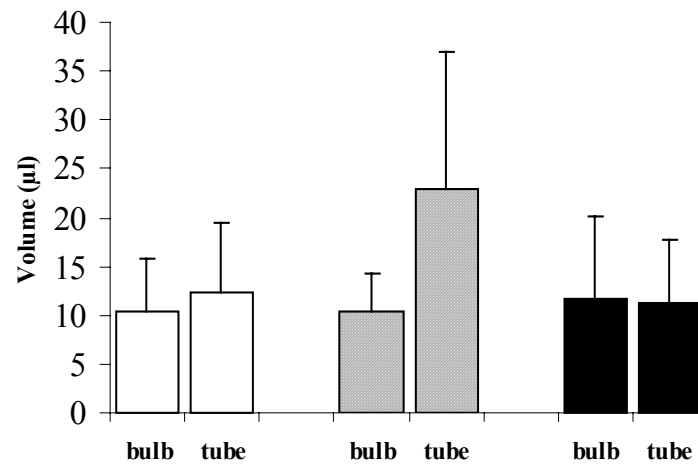


Figure 2. Average volume (combined values for nectar in the bulb and floral tube) and concentration of nectar produced in flowers of different flower stages (means \pm SD, $n = 5$ per flower stage). All plants were screened (bees denied access). No letters in common denote significant differences at $P \leq 0.05$.

Differences between the bulb and floral tube

The average length of flowers (bulb and floral tube) of *A. greatheadii* var *davyana* is 28.1 ± 4.7 mm with the average bulb width and length of the flowers being 5.2 ± 0.7 and 6.8 ± 0.5 mm, respectively. The differences between nectar volumes in the bulb and tube were not significant (Fig. 3, Table 1), with the exception of the higher volume of nectar in flowers measured on the cool day. Although nectar concentration in the floral tube (19-22%) was higher than that in the bulb (18-21%) at both sites, the differences were not significant (Table 1). Temperature and humidity measured at Roodeplaat Nature Reserve on the warm day was 22°C and 17%, and on the cool day 16°C and 39%. At Rust de Winter the temperature was 25°C and RH was 15%.

A



B

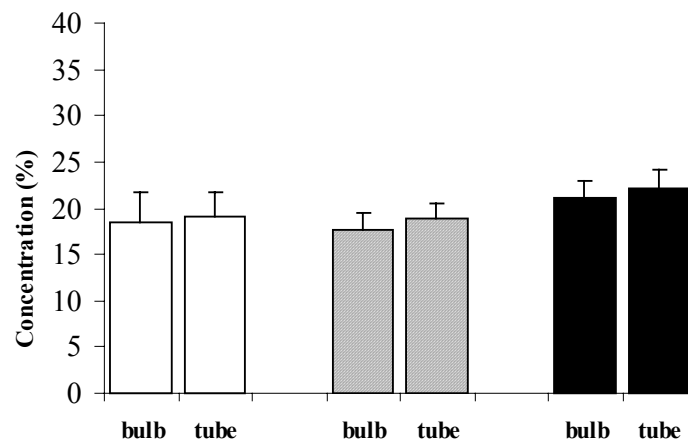


Figure 3. Average (A) volume and (B) concentration of nectar available in floral tube and bulb of *Aloe greatheadii* var *dayyana* flowers measured at Roodeplaat Nature Reserve on a warm day (empty bars) and a cool, cloudy day (hatched bars), and at Rust de Winter (solid bars) (means \pm SD, n = 20).

Table 1: Results of Student's t-tests comparing nectar volume and concentration measured in the bulb and floral tube of *A. greatheadii* var *davyana* flowers. Significance is shown by italics.

		t	df	P
Roodeplaat Nature Reserve (warm day)	Volume	1.02	38	0.32
	Concentration	0.70	38	0.50
Roodeplaat Nature Reserve (cool day)	<i>Volume</i>	<i>3.90</i>	<i>38</i>	<i>0.001</i>
	Concentration	1.91	38	0.07
Rust de Winter	Volume	-0.12	38	0.90
	Concentration	1.92	38	0.06

Screened and unscreened flowers

Treatment had a significant effect on both volume ($H_{1,480} = 266.361$, $P < 0.001$) and concentration of nectar ($H_{1,480} = 172.059$, $P < 0.001$). The average volumes and concentrations of nectar available throughout the day in screened flowers ($30.7 \pm 9.2 \mu\text{l}$, $23.5 \pm 4.4\%$) were significantly higher (for volume $U = 4024.0$, $P < 0.001$, and for concentration $U = 8906.5$, $P < 0.001$) than those in unscreened flowers ($14.7 \pm 7.1 \mu\text{l}$, $18.6 \pm 2.7\%$) (Fig. 4). The nectar volume available in screened flowers was slightly higher early in the morning and around noon while the volume in unscreened flowers showed a peak at 09.00 h. However no significant differences were observed (after Bonferroni adjustments) for hourly comparisons of nectar volumes or concentration throughout the day within screened and unscreened flowers.

These data were collected on a windless day. Temperature measured at the screened inflorescence throughout the day was not significantly higher ($\pm 4.5^\circ\text{C}$) than that measured at the unscreened inflorescence ($U = 21.0$, $P = 0.248$) (Fig. 4C). Relative humidity was not significantly lower in the screened inflorescence ($U = 15.0$, $P = 0.074$), but this seemingly constant RH in the screened raceme for most of the day is possibly due to the limited operating range of the HOBO dataloggers.

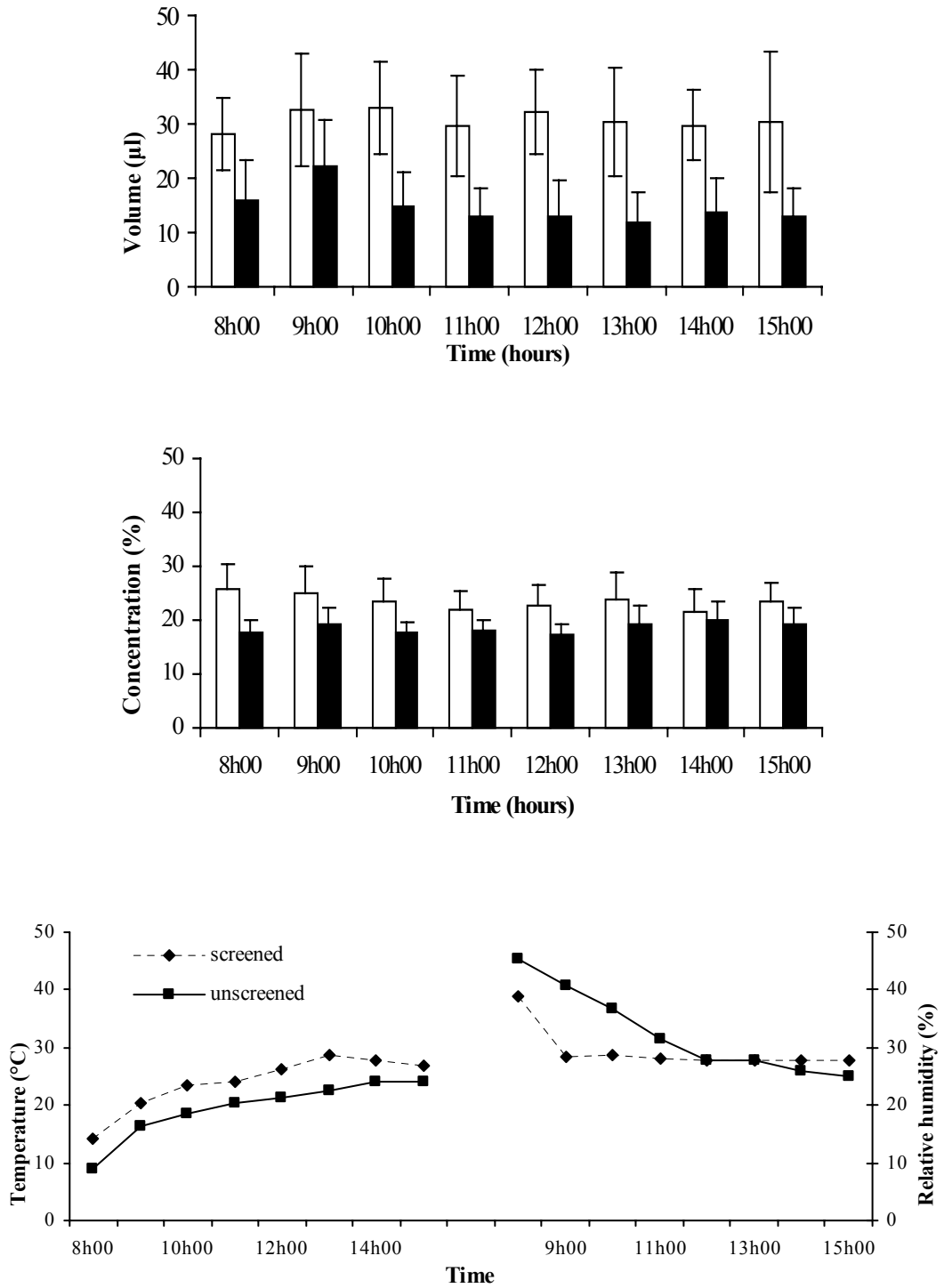


Figure 4. Nectar volume (A) and concentration (B) and temperature and humidity (C) measured in screened (blank bars) and unscreened flowers (solid bars) over a full day (means \pm SD, n=30).

Plants in the sun and shade

The average hourly volume and concentration of nectar secreted in the sun ($18.1 \pm 6.7 \mu\text{l}$, $19.4 \pm 3.2\%$) was significantly higher (for volume $H_{1,360} = 20.335$, $P < 0.001$; for concentration $H_{1,360} = 10.965$, $P < 0.001$) than that secreted in the shade ($14.6 \pm 6.3 \mu\text{l}$, $17.8 \pm 2.6\%$). Mann-Whitney U-tests showed that volumes of nectar for plants in the sun were higher in mid morning (11.00 h) and late afternoon (15.00 – 16.00 hrs) than for plants in the shade (volume at 11.00 h $U = 91.50$, $P = 0.026$; at 15.00 h $U = 82.50$, $P = 0.011$; at 16.00 h $U = 40.50$, $P < 0.0001$). Nectar concentration in plants in the sun was significantly higher between 08.00 and 12.00 h as compared to that in plants in the shade (concentration at 08.00 h $U = 94.0$, $P = 0.031$; at 10.00 h $U = 77.0$, $P = 0.007$; at 11.00 h $U = 91.50$, $P = 0.0257$). The temperature ranged between 11 and 25°C and RH decreased from 24 to 7% during the day.

Nectar production through the flowering season

Daily temperatures were very similar for the three sampling days at Roodeplaat Nature Reserve spaced throughout the flowering season. The mean minimum daily temperature was $9.8 \pm 0.8^{\circ}\text{C}$ and the maximum was $23.3 \pm 1.5^{\circ}\text{C}$. RH decreased through each day, with the average maximum value being 32.1% and the minimum value 5.3%.

The mean volume of nectar produced throughout the day was $14 \pm 2.6 \mu\text{l}$ early in the season, $17 \pm 1.9 \mu\text{l}$ in the middle and $12 \pm 1.8 \mu\text{l}$ late in the season. The concentration of nectar increased from $17 \pm 0.7\%$ to $21 \pm 1.7\%$ over the flowering season. These values were significantly different; for volume ($H_{2,900} = 68.956$, $P < 0.001$) and concentration ($H_{2,900} = 175.665$, $P < 0.001$).

The volume of nectar produced in the middle of the season was significantly higher than that produced early ($U = 35589.5$, $P < 0.001$) and late in the season (volume $U = 27153.5$, $P < 0.001$), while the volume of nectar early in the season was significantly higher than that produced late in the season ($U = 37061.5$, $P < 0.001$). The average concentration of nectar produced late in the season was significantly higher than the concentration of nectar produced early ($U = 16997.5$, $P < 0.001$) and in the middle of the season ($U = 28336.0$, $P < 0.001$). Nectar concentration early in the season was significantly lower than that of nectar in the middle of the season ($U = 34146.5$, $P < 0.001$).

Nectar production across the distribution range

There were significant differences in volume ($H_{2, 270} = 265.572$, $P < 0.001$) and concentration ($H_{2, 720} = 55.090$, $P < 0.001$) of nectar across the distribution range of *A. greatheadii* var *davyana* (see map page 4 of Introduction), from Zeerust in the west to Marble Hall in the east (Fig. 5A, B). Different populations may contribute to these differences. The volume and concentration of nectar were not significantly different at Zeerust and Roodeplaat (for volume $U = 26617.0$, $P = 0.151$; for concentration $U = 55582.0$, $P = 0.934$). However, the volume of nectar produced at Marble Hall was significantly lower than at Roodeplaat Nature Reserve ($U = 6542.5$, $P < 0.001$) and Zeerust ($U = 8396.5$, $P < 0.001$) (Fig. 5A). The average concentration of nectar available at Marble Hall was significantly lower than that of nectar at Roodeplaat Nature Reserve ($U = 12131.0$, $P < 0.001$) and Zeerust ($U = 11458.5$, $P < 0.001$).

Average daily temperatures were $15.9 \pm 3.8^{\circ}\text{C}$ at Zeerust, $18.0 \pm 4.4^{\circ}\text{C}$ at Roodeplaat Nature Reserve and $25.7 \pm 3.8^{\circ}\text{C}$ at Marble Hall. The range of relative humidity during the day was similar at Zeerust and Marble Hall (29.5 - 15.4% and 25.6 - 18.2% respectively) but lower at Roodeplaat Nature Reserve (23.7 - 6.7%).

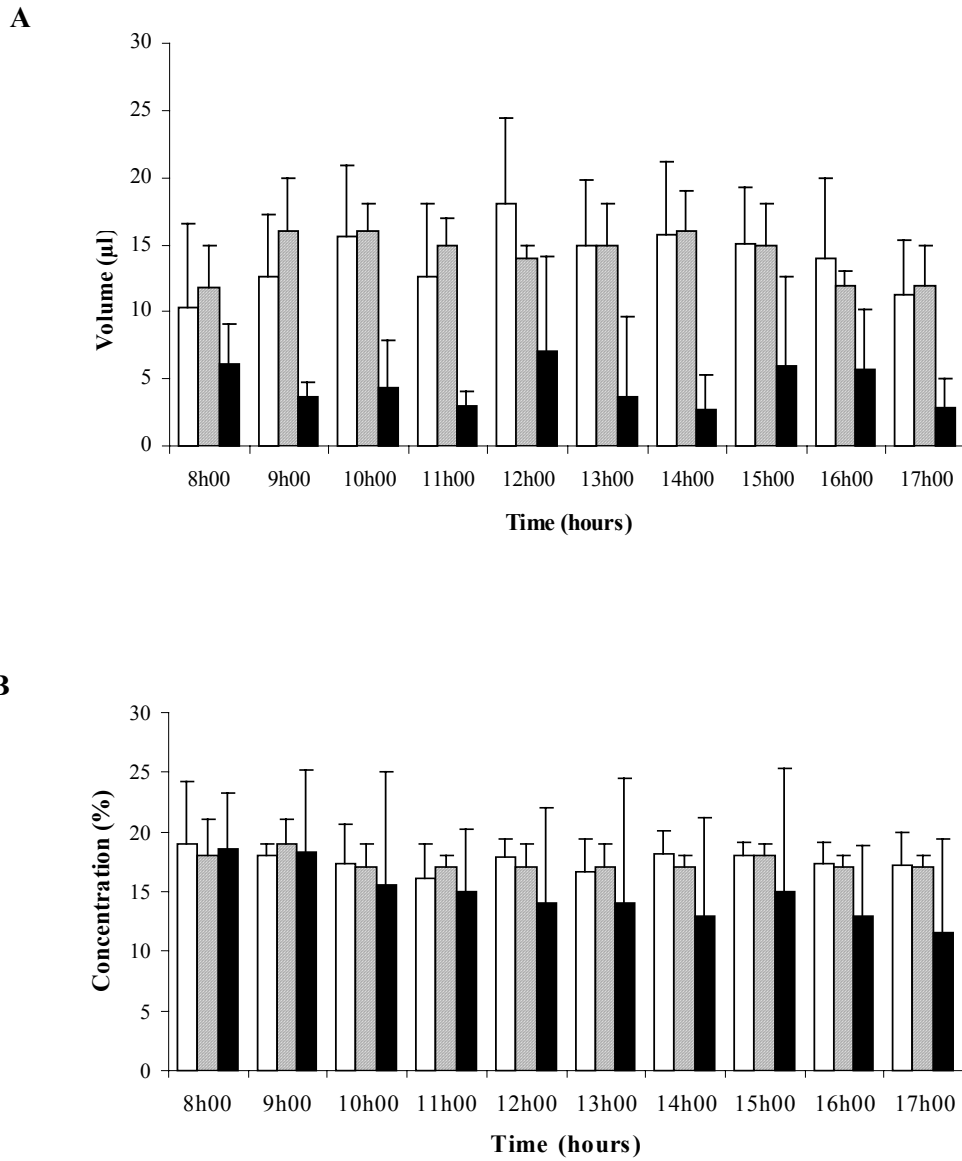


Figure 5. Nectar volume (**A**) and concentration (**B**) of *Aloe greatheadii* var *davyana* available throughout the day at Zeerust (blank bars), Roodeplaat Nature Reserve (hatched bars) and Marble Hall (solid bars). Nectar was sampled early in the flowering season (means \pm SD, n=30).

Discussion

The dilute nectar of *A. greatheadii* var *davyana* is available to foragers throughout the day in spite of extremely low ambient humidities during the flowering season. The low humidity is likely to increase evaporation from the nectar, but tubular flowers modify the humidity gradient and slow the exchange of water between the air and nectar (Plowright, 1987). The tubular flowers of *A. greatheadii* var *davyana* may explain the more constant concentration of its nectar compared to the greater variation seen in the more open flowers of *A. castanea* and the shorter tubular flowers of *A. ferox*, even though both the latter aloes have more dilute nectar (Hoffman, 1988; Nicolson and Nepi 2005). It is known that the shape of flowers helps to determine their nectar concentration. This is clearly illustrated by comparing nectar concentrations measured on a summer day: from an initial concentration of about 20%, in the tubular flowers of *Echium vulgare* concentration remain below 50% while it reaches 60% in the cup-shaped flowers of *Crataegus* (Corbet et al., 1979), while in the open umbelliferous flowers of *Heracleum* nectar evaporates freely, and even becomes crystalline (Willmer, 1983). The internal microclimate of more humid air in tubular flowers helps to slow the rate of equilibration of nectar with ambient conditions. Floral features that contribute to delayed evaporation from nectar include elongated corollas, hairs within and constrictions of the corolla beyond the nectary (Corbet et al., 1979; Nicolson, 2002). Therefore, although slow, some evaporation may occur from nectar in the tube of *A. greatheadii* var *davyana* flowers, explaining the slight difference in nectar concentrations between the bulb and floral tube.

Flower stages observed in *A. greatheadii* var *davyana* are similar to those in other *Aloe* species, e.g. *A. castanea* and *A. ferox* (Hoffman, 1988; Nicolson and Nepi 2005) and nectar volume also varied significantly between the different stages, reaching a peak in stage 3 flowers with a substantial decline in stage 4 flowers. Contrary to observations for *A. castanea* and *A. ferox*, the nectar concentration of *A. greatheadii* var *davyana* flowers remained more constant with flower age and declined only slightly in stage 4 flowers. Bernadello et al. (1994) and Torres and Galetto (1998) interpreted the decline in nectar volume, but not concentration, with age in flowers of *Combretum fruticosum* (Combretaceae) and *Mandevillea pentlandiana* (Apocynaceae) as an indication of reabsorption. Nectar of *A. greatheadii* var *davyana* flowers remains in contact with the

nectary, therefore the lower volume and concentration in stage 4 flowers may be suggestive of reabsorption.

Table 2. Volume and concentration of nectar (standing crop) measured in flowers of four *Aloe* species of the section *Pictae* (n = 10). Data presented as means \pm SD. (Human & Nicolson, unpublished data)

<i>Aloe</i> species	Volume	Concentration
Winter flowering		
<i>A. branddraaiensis</i>	8.0 \pm 4.1	17.2 \pm 1.3
<i>A. grandidentata</i>	22.7 \pm 21.1	12.5 \pm 1.7
<i>A. maculata</i>	21.6 \pm 8.5	14.6 \pm 1.0
Summer flowering		
<i>A. zebrina</i>	33.2 \pm 17.1	22.4 \pm 1.4

The nectar concentration of *A. greatheadii* var *davyana* corresponds to nectars taken by birds (Nicolson & Fleming, 2003), but is unusually high compared to most *Aloe* species. Nectar concentration of the summer flowering *A. zebrina* is also relatively concentrated. It was thought that the high concentration observed in these two species might have a phylogenetic basis rather than being an adaptation for pollinator type, but other spotted aloes have lower concentrations (Table 2). Other aloes with tubular flowers produce nectar with much higher volumes, e.g. *A. ferox* and *A. marlothii*, 180 μ l and 250 μ l respectively, and lower concentrations (12.5% and 12.1% respectively) (Hoffman, 1988; C.T. Symes, unpub data). These species have much larger flowers and nectaries than that of *A. greatheadii* var *davyana*, which explains the higher volumes of nectar (Opler, 1983). Flowers of *A. castanea*, on the other hand, are smaller and more open (campanulate) and the increased exposure might be expected to lead to more evaporation and higher nectar concentrations. However, this species has very dilute nectar of below 10% throughout the day (Nicolson & Nepi, 2005). Aloe flowers are frequented by sunbirds and larger passerine birds (Oatley & Skead, 1972) as well as by bees. The most dilute *Aloe* nectars appear to be associated with pollination by generalised passerines rather than by sunbirds (S.D Johnson & S.W. Nicolson, in prep).

It is foraging by bees that lead to substantially lower volumes of nectar in unscreened flowers than in screened flowers. Although the standing crop volume is low (15 μ l), a large proportion (10-12 μ l) of this nectar is inaccessible to bees and remains in the bulb of the flowers. The observed differences in volume and concentration of nectar between screened and unscreened flowers were similar to those observed by Corbet and Willmer (1981) and Wyatt et al. (1992). Even though insects may affect the volume of nectar, it is unlikely that they will have a direct effect on the concentration of the remaining nectar. The increased concentration of nectar in screened flowers may be the result of increased ambient temperature and decreased relative humidity in bags (Dafni, 1992), especially during the windless conditions of our study.

Variability in nectar rewards is also an effect of ambient conditions such as sun and shade. Higher ambient temperature may explain the significantly higher nectar volume of plants in the sun, and although the concentration was slightly higher in the sun the difference was not significant. In Israel Goldstein et al. (1987) observed higher volumes of nectar for *A. arborescens* in the shade compared to plants in the sun, but concentrations remained the same. They found that sunbirds preferred to feed on flowers in the sun in spite of the smaller volume of nectar and attributed this preference to energy saving. Nicolson and Nepi (2005) observed marked differences in flower development in *A. castanea* on the sunny and shady side of racemes, with higher volumes and lower concentrations in nectar of flowers in the shady side.

Seasonal patterns of nectar production have seldom been investigated. Pleasants (1983) observed a seasonal decline in nectar volume of *Ipomopsis aggregata* (Polemoniaceae), but no change in concentration. He attributed the decline in nectar volume to increased energy demands on plants as a result of seed development. McDade (2004) hypothesised that nectar production would be higher early in the season in order to entrain hummingbirds, while later in the season plants only need to produce enough nectar to keep them returning. According to local beekeepers, *A. greatheadii* var *davyana* nectar is more abundant at the end of the flowering season (A Schehle, pers. comm.); however, we measured the lowest volumes and highest concentration late in the season. The northern provinces of South Africa are summer rainfall regions with dry winters, therefore as winter progresses water stress increases and this may contribute to the increase in concentration. According to Carroll et al. (2001) and Wyatt et al. (1992),

drought indirectly influences floral rewards and thus pollinator visitation; plants experiencing water stress may produce less nectar. Leiss and Klinkhamer (2005) demonstrated a decrease in nectar production with low water availability with a resultant decrease in pollination. However, leaf succulence enables *A. greatheadii* var *davyana* to provide abundant nectar during winter when alternative sources are scarce thus making it an ideal resource for beekeepers.

Acknowledgements

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CHAPTER 5

Do honeybees, *Apis mellifera scutellata*, eliminate excess water from the dilute nectar of *Aloe greatheadii* var *davyana* before returning to the hive?

H. Human and S.W. Nicolson

Department of Zoology and Entomology, University of Pretoria, Pretoria 0002, South Africa.



Abstract

Aloe greatheadii var *davyana* flowers during the dry winter months across the northern summer rainfall areas of South Africa. Nectar is continuously available throughout the day, at an average concentration of about 20% w/w. The crop contents of nectar foragers were sampled at two sites in Gauteng Province to determine whether changes in nectar concentration occurred after collection and before unloading in the hive. Possibly because this nectar is so dilute for honeybees, relatively small volumes, $10.8 \pm 8.8 \mu\text{l}$ at Roodeplaat Nature Reserve and $15.8 \pm 6.6 \mu\text{l}$ at Rust de Winter, were transported back to the hive. We observed a significant increase in nectar concentration, accompanied by a decrease in nectar volume, between the flowers and the hive. At Roodeplaat Nature Reserve the nectar concentration increased from 21.3% in the flowers to 32.0% in the crops of honeybees captured at the flowers and at Rust de Winter from 21.8 to 35.6%. We observed a further increase in bees captured at the hive entrance. This dramatic increase in concentration of the crop contents between the flowers and the hive is unexpected in view of the common assumption that nectar is either unchanged or slightly diluted during transport.

Introduction

Aloe greatheadii var *davyana* is an extremely important indigenous plant for South African beekeepers, with a widespread distribution across the northern summer rainfall regions (Van Wyk & Smith, 1996; Glen & Hardy, 2000; Williams, 2002). This aloe flowers in mid-winter, from June to August, at a time when little else is flowering. It is common for beekeepers to move their hives to the “aloe fields” north of Pretoria during winter. The strong pollen and nectar flow is used for honey production, to rear queens, and to build up colonies and increase colony numbers by division (Jackson, 1979; Williams, 2002). The pollen of *A. greatheadii* var *davyana* is an excellent food source, having a very high protein content (Chapter 1).

The nectar of *A. greatheadii* var *davyana* is available to floral visitors throughout the day, with an average standing crop of $14.7 \pm 7.1 \mu\text{l}$ (volume) and $18.6 \pm 2.7\%$ (concentration) (Chapter 4). Although dilute for honeybees, this nectar is more concentrated than that of other *Aloe* species. In general, *Aloe* species produce copious quantities of very dilute (10-15%), hexose-dominant nectars (Van Wyk et al., 1993; Nicolson, 2002). For example, *A. ferox* produces large volumes (180 μl per flower) with a low concentration (12.5%) (Hoffman, 1988), while the concentration of *A. castanea* nectar remains below 10% throughout the day (Nicolson & Nepi, 2005).

Dilute nectar is associated with bird pollinators (Nicolson, 2002) while honeybees generally prefer nectar with 30-50% sugar (Southwick & Pimentel, 1981). However, honeybees have been shown to collect nectar over a much wider range of concentration (e.g. 15-65% w/w, Visscher & Seeley, 1982). Dilute nectars do not necessarily deter bees: when they need to cool the hive by evaporation they will collect nectar with lower concentrations (Eisikowitch & Masad, 1982; Ohguchi & Aoki, 1983). Lindauer (1955) observed honeybees collecting both water and nectar on a daily basis from spring until autumn. The water need of a colony is affected by the quantity of nectar; during a good nectar flow less water is needed (Lindauer, 1955). During winter, when the temperature is lower and the air is drier, the water needs of honeybee colonies may increase (Johansson & Johansson, 1978). In addition to providing energy, the nectar of *A. greatheadii* var *davyana* may serve as a source of moisture to honeybees during the dry winter months.

Utilising dilute nectar as a food source poses a problem to bees, by increasing the amount of excess water that needs to be eliminated during the ripening of honey. Extensive evaporation will be necessary with utilization of the dilute nectar of *A. greatheadii* var *davyana*. This process could begin before unloading of the crop contents in the hive. However, Park (1932) observed no increase in sugar concentration in the honeybee crop between the nectar source and the hive entrance when bees were collecting nectars of about 30%. Since then it has been generally accepted that the concentration of nectar in the forager's crop is an accurate indication of the nectar concentration of the flowers it has been visiting, and this has in fact been used as a method of sampling nectar (see Roubik & Buchmann, 1984; Roubik et al., 1995). In addition Oertel et al. (1951) observed a dilution of crop contents after the consumption of experimental syrups by honeybees and attributed it to the addition of glandular secretions and rapid inversion of sucrose.

Park (1932) trained field bees to feed at a feeder and starved the bees for at least 1 h before conducting his experiments. Oertel et al. (1951) placed bees in cages and starved them for 1.5 h before feeding them experimental syrups of varying concentrations. These periods of time were sufficient to ensure that the bees' crops were empty. Our study was conducted in natural field sites with dense stands of *A. greatheadii* var *davyana*, and bees were allowed to forage normally on the dilute hexose-rich (only 2% sucrose) nectar (Van Wyk et al., 1993). Since *A. greatheadii* var *davyana* flowers when nothing else is flowering, there could only be one source of the nectar brought back to the hive. Honeybees were captured on flowers and at the hive entrance to investigate whether nectar concentration in the crop changed between foraging at flowers and arrival at the hive.

Methods

Prior to the onset of flowering, six honeybee (*Apis mellifera scutellata* L) hives were moved to Roodeplaat Nature Reserve (795 ha) (28° 39'E, 25° 66'S) in Gauteng Province as part of a broader study of the interactions between the bees and the aloes. Observations on bee foraging were conducted at Roodeplaat Nature Reserve and also at Rust de Winter (28° 23'E, 25° 12'S). There were approximately 300 beehives at Rust de

Winter. Both areas have dense populations of *A. greatheadii* var *davyana*, especially Rust de Winter, and the flower patches and hives were less than 300 m apart.

We captured 30 bees leaving hives and another 30 bees returning to hives at each site, and an additional 30 swarming bees at Rust de Winter. We placed the bees on ice and weighed them in the laboratory with a Sartorius micro scale (Gottingen, Germany) to determine the mass of the nectar loads.

The crop contents of nectar foragers were sampled between 09.00 and 13.00 h on two consecutive days (11 - 12 July 2004). We sampled residual nectar from flowers that were visited and the crop contents of three categories of bees: foragers captured at the flowers, foragers arriving at the hive entrance, and swarming bees. On each day:

1. We captured 50 honeybees at flowers after they had collected nectar for >20 s (determined to be the average honeybee visit duration, pers. obs.), expressed and measured their crop contents and simultaneously removed the flowers that had been visited to measure the volume and concentration of residual nectar.
2. We blocked the hive entrances between 10.00 and 11.00 h and captured 50 returning foraging bees in Ziploc plastic bags. The bags were placed on ice to facilitate handling of the bees.
3. At Rust de Winter we also managed to capture and express the crop contents of 50 swarming bees.

Volumes of residual nectar were determined from column length in disposable haematocrit tubes (length 75 mm/75 μ l) and the concentrations measured as % w/w sucrose equivalents with a pocket refractometer (0-50%, Bellingham & Stanley Ltd, Tunbridge Wells, UK). Crop contents of all bees were extracted within 10 min of capture. Bees were induced to regurgitate by pressing the thorax dorsoventrally (Roubik & Buchmann, 1984) and the crop contents were then collected from the mouthparts in haematocrit tubes. The volume and concentration of the crop contents were measured as for nectar. Because the crop contents of swarming bees were extremely viscous, only concentration could be measured, using a high range pocket refractometer (40-85%, Bellingham & Stanley Ltd, Tunbridge Wells, UK).

Statistical analysis

Data for bee weights and for bee crop contents (volume and concentration) did not meet the assumptions for parametric statistics; variances were not homogeneous and data did not conform to a normal distribution. Due to significant differences between the two sites we were unable to pool the data and each site was analysed separately. Variation in volume and concentration of the crop contents of honeybees captured at the flowers and at the hive entrance, and nectar remaining in the flowers, were therefore assessed with Kruskal-Wallis ANOVA (Zar, 1984) and the level of significance was $P < 0.05$. Mann-Whitney U-tests were used for paired comparisons, including crop contents of swarming bees captured at Rust de Winter. Bonferroni corrections were applied for all paired combinations.

Analyses were performed with the program Statistica 6.0 (1984-2004). Values are given throughout as means \pm SD.

Results

As a result of low ambient temperatures during the winter flowering season, bee foraging only started at 09.00 h in the mornings and stopped after 16.00 h, amounting to a working period of only seven hours. Flowers opened throughout the day, so both pollen and nectar were continuously available (Chapter 4). Honeybees appeared to collect only nectar or pollen, not both. Bees obtained nectar by partially or completely entering the tubular flowers.

The average mass of returning foragers at Roodeplaat Nature Reserve was 73.6 ± 4.6 mg and that of foragers leaving the hive was 61.7 ± 2.8 mg, resulting in a mean weight of 11.6 ± 5.0 mg for nectar loads which may include small volumes at departure. At Rust de Winter the average weight of returning foragers was 77.7 ± 8.2 mg, and that of foragers leaving the hive was 62.9 ± 2.9 mg, resulting in a mean weight of 13.9 ± 7.9 mg for nectar loads. The average nectar loads include any initial honey consumed prior to leaving the hive. Swarming bees weighed on average 100.6 ± 10.6 mg resulting in a load mass of 37.7 ± 9.2 mg and a calculated volume of $27.9 \mu\text{l}$ for nectar loads (based on the density of the 71% sugar solution in their crops; Fig. 1B). There was a significant

difference between the body masses of foragers captured at Roodeplaat Nature Reserve and Rust de Winter ($U = 325.0$, $P > 0.05$).

Table 1. Results of paired comparisons of means for residual nectar and crop contents of bees (Mann-Whitney U-test). Adjusted P values after Bonferroni corrections are $P < 0.025$ for Roodeplaat and $P < 0.017$ for Rust de Winter. Significance is shown by italics.

Roodeplaat		Crop contents of bees at flowers	Crop contents of returning foragers
Residual nectar	Volume	<i>$U = 550.00$, $P < 0.0001$</i>	<i>$U = 505.50$, $P < 0.0001$</i>
	Concentration	<i>$U = 6.00$, $P < 0.0001$</i>	<i>$U = 5.00$, $P < 0.0001$</i>
Crops contents of bees at flowers	Volume		$U = 980.50$, $P > 0.05$
	Concentration		<i>$U = 401.50$, $P < 0.0001$</i>
Rust de Winter			
Residual nectar	Volume	$U = 1014.50$, $P = 0.105$	<i>$U = 651.00$, $P < 0.0001$</i>
	Concentration	<i>$U = 5.00$, $P < 0.0001$</i>	<i>$U = 0.00$, $P < 0.0001$</i>
Crops contents of bees at flowers	Volume		<i>$U = 600.00$, $P < 0.0001$</i>
	Concentration		<i>$U = 581.50$, $P < 0.0001$</i>
Swarming bees	Concentration	<i>$U = 0.00$, $P < 0.0001$ for all comparisons</i>	

We observed significant differences between the volume and concentration of residual nectar in the flowers and the nectar in the crops of bees captured at the flowers and returning to the hive at both Roodeplaat Nature Reserve (for volume $H_{2, 150} = 35.08$, $P < 0.001$; concentration $H_{2, 150} = 113.83$, $P < 0.001$) and Rust de Winter, including crop contents of swarming bees (volume $H_{2, 150} = 23.31$, $P < 0.001$; concentration $H_{3, 200} = 173.19$, $P < 0.001$). At Roodeplaat Nature Reserve the volume of residual nectar in individual flowers was significantly higher than the volume in the crops of bees captured at the flowers and in crops of foragers returning to the hive (Table 1, Fig. 1A). The crop volumes of bees captured at the flowers were not significantly higher than

those of foragers returning to the hive. There was a significant increase in concentration from the residual nectar to the crop contents of bees captured at the flowers, and a further increase in the crop of bees captured at the hive entrance (Table 1). The crop contents of returning foragers had a significantly higher concentration than those of bees captured at the flowers (Table 1).

The same pattern was observed at Rust de Winter (Fig. 1B). There was no significant difference between the volume of residual nectar in flowers and the crop volume of bees captured at the flowers, but the volume of residual nectar was significantly higher than the volume in the crops of returning foragers. The crop volumes of bees captured at the flowers were significantly higher than those of returning foragers (Table 1). The differences in concentration between the flowers and the crops of bees at the flowers, returning foragers and swarming bees remained highly significant after Bonferroni corrections. The concentration of residual nectar in the flowers was significantly lower than that in crop contents of bees captured at the flowers and of foragers returning to the hive (Fig. 1B). Nectar concentration in crops of returning foragers was significantly higher than that of bees captured at the flowers and the nectar concentration of swarming bees was significantly higher than all other measurements (Fig. 1B, Table 1).

Data at the two localities were collected on two consecutive days with similar weather. The temperature measured at Roodeplaat Nature Reserve increased from 22.2 to 28°C and RH decreased from 17.4 to 10.4%, and at Rust de Winter temperature increased from 18 to 25.4°C and RH from 27.6 to 15.1%.

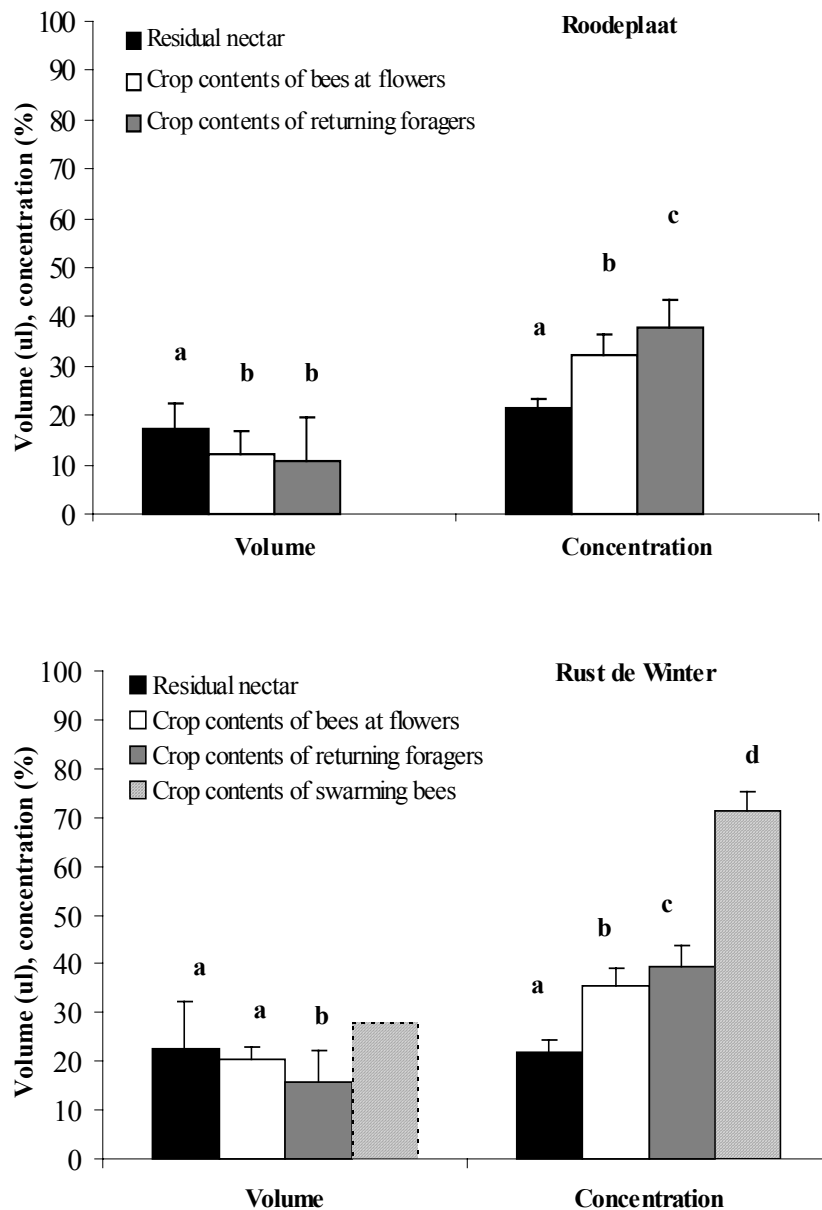


Figure 1. Nectar volume and concentration in residual nectar after honeybee visits and in crops of bees captured at the flowers and returning to the hive at (A) Roodeplaat and (B) Rust de Winter (means \pm SD, $n = 50$). Crop contents were collected and measured from bees captured at the flowers and at the hive entrance. Measurements at Rust de Winter included crop concentrations of swarming bees: the volume of crop contents for swarming bees was calculated (see text). No letters in common denote significant differences at $P < 0.025$ for Roodeplaat and $P < 0.017$ for Rust de Winter after Bonferroni corrections.

Discussion

Aloe greatheadii var *davyana* flowers when very few nectar and pollen sources are available. The flowers open throughout the day, ensuring constant availability of pollen to floral visitors. There is also constant availability of copious amounts of dilute nectar (Chapter 4). In spite of the nectar being more dilute than other bee-preferred nectars (30-50%, Southwick and Pimentel, 1981), this nectar flow contributes substantially to the honey crop (Williams, 2002) and serves as a source of water. According to Johansson and Johansson (1978), water availability may mean the difference between weak and strong colonies and access to water may result in increased brood rearing as observed in spring. The dilute nectar of *A. greatheadii* var *davyana* appears to meet the energy and water requirements of the bees during a dry period and may thus contribute to increased brood rearing.

Studies on crop loads carried by bees report both mass and volume. *Apis mellifera* *scutellata* bees in this study collected the most dilute nectar and carried the smallest loads compared to other reports (Table 2). According to Brosch and Schneider (1985), bees can store about 60 μ l in the crop with an unladen body mass of only 60 to 80 mg. The crop volumes measured in this study for bees at flowers (12 and 20 μ l) and those returning to the hive (11 and 16 μ l) are similar to the mean 13.6 μ l reported by Huang and Seeley (2003) for foragers when nectar was less abundant, but are much lower than those reported by Roubik & Buchmann (1984) for bees feeding on 45% sucrose solutions (59 μ l).

Foraging honeybees are able to regulate crop filling and it is known that the nectar load increases with a higher nectar flow rate (Núñez, 1970; Huang & Seeley, 2003) and with higher sugar concentrations (Roubik & Buchman, 1984). Seeley (1986) reported much larger crop loads (58 μ l) for bees returning from a feeder supplying a 55% sucrose solution than for bees returning from flowers (2 μ l). The observed partial crop loads in our study, in spite of readily available food sources and short flying distances to the hive, support the findings of Schmid-Hempel et al. (1985) who predicted partial crop loads for foragers flying short distances, thereby maximising their energetic efficiency rather than nectar delivery rate.

Table 2. Mass of bees and crop contents: departing foragers, crop contents after feeding on different diets and swarming bees.

Subspecies and locality	Diet	Body mass of honeybees (mg)	Mass of crop contents (mg)	Reference
<i>A. mellifera ligustica</i> Arizona	Feeder, 40%	73	23-29 max 70	Feuerbacher et al. (2003)
<i>A. mellifera ligustica</i> Japan	Natural sources Unknown	81	24 spring, summer 12 autumn	Fukuda et al (1969)
<i>A. mellifera scutellata</i> South Africa	<i>A. greatheadii</i> var <i>davyana</i> , 21%	62	12 - 14	This study
<i>A. mellifera scutellata</i> South Africa	<i>Eucalyptus</i> wide range	-	6 - 13	Hepburn & Magnuson (1988)
Africanised Various	Unknown	93	25 - 40	Winston (1987)
<i>A. mellifera</i> <i>lingustica</i> x <i>carnica</i> USA?	Natural sources 41%	-	40 - 80	Southwick and Pimentel (1981)
Swarming <i>A. mellifera</i> <i>scutellata</i> , South Africa	<i>A. greatheadii</i> var <i>davyana</i>	-	39	This study
Swarming Africanised Various	Unknown	-	30	Winston (1987)

An alternative reason for partial crop loads is a motivational one, where partial loads would instead serve to benefit the hive through increased exchange of information within the hive (recruitment of nestmates). The metabolic hypothesis as proposed by Schmid-Hempel et al. (1985) lost support when Moffat (2000) found that metabolic rates of bees were unrelated to the size of crop loads but were linearly related to the reward rate and might be controlled by a motivational drive. In contrast, Wolf et al. (1989) and Feuerbacher et al. (2003) reported increased metabolic rates for honeybees with an increased nectar load. Territorial male bees avoid carrying large crop loads, as illustrated by the small loads carried by male carpenter bees (20 μ l) compared to females (69 μ l) (Louw & Nicolson, 1983) and male *Anthophora plumipes* that only carry enough nectar (1-5 μ l) to meet their short term requirements, while females commonly carry 30 μ l nectar for their own needs and that of their offspring (Willmer & Stone, 2004).

The increase in concentration of the crop contents that we observed is already apparent in honeybees collected at the flowers. This increase in nectar concentration is contrary to the findings of Park (1932), who found that nectar is not concentrated in the crop of bees returning to the hive, but only in the hive itself during the storage and honey ripening process. It is known that departing foragers do not leave the hive with completely empty crops (Park, 1932; Lindauer, 1955; Winston, 1987) and the amounts in crops of departing bees may vary from bee to bee and with time (Park, 1932). However, both Park (1932) and Oertel et al. (1951) starved their bees for at least an hour before conducting their experiments. This meant that bees had no nectar or honey in their crops prior to feeding on the sugar solutions offered. According to Oertel et al. (1951), a decrease in concentration in the crop is to be expected since bees add dilute glandular secretions, including the enzyme invertase, to the crop contents, resulting in both dilution and hydrolysis of sucrose into glucose and fructose. However, *A. greatheadii* var *davyana* produces 98% hexose nectar (Van Wyk et al., 1993), and no hydrolysis is necessary.

Our findings regarding changes in crop concentration are in agreement with those of Willmer (1986, 1988), who also investigated changes in nectar concentration after collection. Willmer (1986) investigated mason bees, *Chalicodoma sicula*, collecting dilute *Lotus creticus* nectar (daily range 22-40%) growing on sand dunes in Israel. The bees rapidly increased their crop contents to about 58% after collection of nectar. Since a simultaneous dilution of the haemolymph was measured, water must have been transported from the gut into the haemolymph. Willmer (1988) also studied two species of carpenter bees, *Xylocopa sulcatipes* and *X. pubescens*, collecting nectar from *Calotropis procera* in southern Israel. Crop contents of the smaller *X. sulcatipes* bees (57%) were much more concentrated than nectar in the flowers or the crop contents of *X. pubescens* (46%). In *X. sulcatipes* bees, water moved from nectar in the gut into the haemolymph during flight, thus is lowering their haemolymph concentrations. The only other study that has examined changes in nectar concentrations in bee crops is that of Biesmeijer et al. (1999), who compared the crop contents of two *Melipona* species collecting 50% sucrose from an artificial feeder in Costa Rica. He observed that sugar concentration of the load increased by only 0.2% between the feeder and the hive.

The crop or honey stomach of bees is an expandable compartment that stores honey as well as nectar and water collected by foragers. Its primary function is to retain nectar or water and in addition it is the site where invertase is added to nectar to hydrolyse sucrose (Lindauer, 1955; Louw & Nicolson, 1983). However, foragers need enough energy to sustain flight. It is energetically advantageous for them to use fuels stored in the crop rather than reserves stored in fat body or muscle. It is assumed that bees can only use nectar stored in the crop when it passes through to the midgut, since the crop is impermeable (its cuticular lining prevents absorption of either sugar or water molecules; Lindauer, 1955). Crop emptying in bees is controlled through the osmolality of the food and haemolymph and adjusted to energy demands (Roces & Blatt, 1999). Foragers are able to adjust the rate at which sugar leaves the crop according to their metabolic rates (Blatt & Roces, 2002) and it is known that the metabolic rates in turn depend on the reward rate at the food source (Balderrama et al., 1992).

Excess water can be withdrawn from nectar either internally via the midgut or externally through evaporation from the mouthparts. "Tongue-lashing" is a process where nectar is regurgitated onto the tongue and evaporated, and is used by honeybees to achieve evaporative cooling of the body, in particular the head (Heinrich, 1980), a process effectively used by *A. mellifera caucasica* bees flying in the Sonoran desert (Cooper et al., 1985) or in *Xylocopa* bees to concentrate nectar before storage (Corbet & Willmer, 1980). The excretion of copious urine, whether in flight or when alighting on a flower, is conspicuous in carpenter bees, *Xylocopa* species (Willmer, 1988; Nicolson, 1990) and bumble bees, *Bombus lucorum* (Bertsch, 1984) and, according to Park (1932), it is well known that honeybees also excrete a colourless liquid, believed to be water, when transporting dilute nectar. Johansson and Johansson (1978) reported that water-collecting bees regurgitate only 70% of the water collected, while the remaining 30% is ingested and removed through excretion. The removal of water will explain the increase in concentration and the decrease in volume of the crop contents. The small volumes transported back to the hive in our study will aid the concentrating process since removal of water will have more of an effect on small volumes. However, since the crop is impermeable it is difficult to explain the removal of water from the crop contents without accompanying sugar.

Honeybees foraging on the dilute nectar of *A. greatheadii* var *davyana* are flying in very dry air, therefore evaporative losses during flight may be considerable. The bees may thus forage partly to get enough water for their physiological needs. The low concentration of *A. greatheadii* var *davyana* nectar is not a problem for water balance at the colonial level because evaporation of the dilute nectar is aided by low ambient humidities prevailing during the flowering season.

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CHAPTER 6

Do honeybees, *Apis mellifera scutellata*, regulate humidity in their nest?

H. Human, S.W. Nicolson and V. Dietemann

Department of Zoology and Entomology, University of Pretoria, Pretoria 0002, South Africa.



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Abstract

Honeybees are highly efficient at regulating the biophysical parameters of their hive according to colony needs. Thermoregulation has been the most extensively studied aspect of nest homeostasis. In contrast, little is known about how humidity is regulated in beehives, if at all. Although high humidity is necessary for brood development, regulation of this parameter by honeybee workers has not yet been demonstrated. In the past, humidity was measured too crudely for a regulation mechanism to be identified. We reassess this issue, using miniaturised data loggers that allow humidity measurements in natural situations and at several places in the nest. We present evidence that workers influence humidity in the hive. However, there are constraints on potential regulation mechanisms because humidity optima may vary in different locations of the nest. Humidity could also depend on variable external factors such as water availability, which further impairs the regulation. Moreover, there are trade-offs with the regulation of temperature and respiratory gas exchanges that can disrupt the establishment of optimal humidity levels. As a result, we argue that workers can only adjust humidity within sub-optimal limits.

Introduction

Honeybee colonies show efficient regulation of the biophysical parameters of their hive. Constant temperature is crucial for the normal growth and development of the immature stages (Himmer, 1927; Degrandi-Hoffman et al., 1993). Colony thermoregulation is well studied in honeybees and hive temperatures are adjusted through various mechanisms. During winter, honeybees form clusters to conserve heat generated by the shivering of their flight muscles (e.g. Stabentheiner et al., 2003). During summer, when the nest temperature exceeds the optimum range, workers collect water and spread droplets on the comb; fanning causes their evaporation and results in active cooling (Lindauer, 1955). This water is collected either by water foragers or incidentally through foraging for nectar (Lindauer, 1955; Kühnholz & Seeley, 1997).

In spite of the supposedly important role of humidity in brood development (Park, 1949; Lindauer, 1955), little is known of how this parameter is regulated by honeybees, if at all (Ribbands, 1953; Büdel, 1960; Simpson, 1961; Johansson & Johansson, 1979; Willmer, 1986). Earlier measurements of humidity were made in hives emptied of half the frames and occupants, or in an extra compartment placed on top of the hive, in order to accommodate large monitoring devices such as hygrothermographs (e.g. Oertel, 1949). Usually the measurements were of relative humidity, which is dependant on temperature (as the saturation vapour density of water in air increases with air temperature) and this led to the conclusion that humidity in beehives simply follows variations in temperature and that bees do not actively regulate it (Lindauer, 1955; Simpson, 1961). We have investigated whether honeybees regulate humidity in their hives using miniaturised technology that made it possible to measure this parameter in a biologically relevant manner.

Methods

We measured temperature, absolute humidity (AH) and relative humidity (RH) inside three *Apis mellifera scutellata* colonies containing approximately 20,000 bees each reared in Langstroth hives with one shallow super. AH was measured in order to exclude the effect of temperature and assess the water vapour density in the hive atmosphere. The apiary was located in the Roodeplaat Nature Reserve, Gauteng

Province (28° 39'E, 25° 66'S), in the summer rainfall area of South Africa. Monitoring occurred in the dry winter month of July 2005, during peak nectar flow of *Aloe greatheadii* var *davyana*. These conditions are ideal for our study as the dry atmosphere creates a stress to which colonies have to react, but the presence of abundant forage ensures that the colonies are healthy and can adjust to this natural stress. The hives were within one kilometre of a dam, providing them with a source of water.

Miniature HOBO H8 data loggers (61 x 48 x 20 mm, Onset Computer Corporation, Pocasset, MA, USA) were used for continuous recording of temperature, AH and RH (at 2-min intervals for four consecutive days). The operating ranges of the loggers for RH, AH and temperature are 25 to 90%, 0.3-157.4 g/m³ and -20 to 70°C respectively. Their accuracy is $\pm 5\%$, ± 0.8 g/m³ and $\pm 0.7^\circ\text{C}$. The data loggers were wrapped in metal gauze to prevent the bees covering the probes with propolis. The loggers were placed in the nectar stores and in the middle of the central brood comb of each hive (a piece of comb of the logger's size was cut out for this purpose). Although the loggers recorded the parameters as soon as they were embedded, we considered the data only after the brood temperature returned to 34.5°C, which suggested that the bees resumed normal activity. An empty hive without bees, brood or nectar comb served as a control for the effect of the hive itself on the parameters measured. After four days, the data loggers were removed and the data analysed. Cosinor analyses (Nelson et al., 1979) were performed to compare variations in AH and RH between colonies and between brood and nectar stores of each colony. For this, 15 consecutive 2-min interval measurements were averaged to obtain a point every half hour ($n = 192$) over the two days monitored. Bonferroni correction was applied when the parameters measured were compared for paired combinations of the three colonies. The level of significance adopted was 0.01.

Results

Control temperature varied from 3.7 to 30.7°C over the measurement period and was close to ambient conditions. Temperature in the nectar stores was higher and fluctuated to a lesser degree (14.6 to 38.1°C). Temperature in the brood area remained constant around 35°C (Fig. 1a). AH was low in the control hive and higher than ambient AH. In

the nectar stores, it was higher on average and fluctuated widely. In the brood, AH was again higher, and still fluctuated, but within a narrower range (Fig. 1b). RH in the nectar stores and brood area was higher than the control in two of the three colonies (Fig. 1c). Colony 3 had lower RH than the other colonies. In contrast to AH, RH was similar in the nectar stores and the brood area in two of the three hives. Colony 2 had a higher RH in the nectar stores (Fig. 1b and c). The inter-colonial variation in AH and RH patterns observed could not be explained on the basis of colony size. Cosinor analyses revealed significant differences in AH or RH between brood area and nectar stores of each colony ($df = 3$, $n = 378$, $F > 19.6$, $P < 0.001$ in all cases). There were also significant differences in AH between the brood areas of different colonies as well as between their nectar stores ($df = 3$, $n = 378$, $F > 17.8$, $P < 0.01$ after Bonferroni correction in all cases). The same was true for RH ($df = 3$, $n = 378$, $F > 23.3$, $P < 0.01$ after Bonferroni correction in all cases).

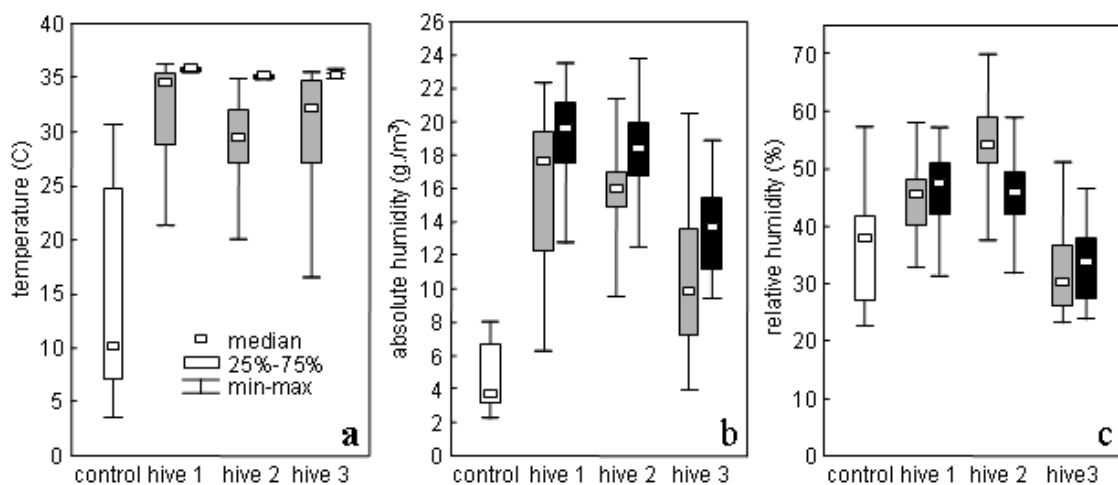


Figure 1. Summary statistics for microclimatic parameters in three colonies measured over two consecutive days with similar weather. Data shown are (a) temperature, (b) absolute humidity and (c) relative humidity in the nectar stores (grey bars) and in the brood area (black bars). Parameters measured in an empty hive are shown as a control (white bar).

Control temperature and AH followed the same daily pattern, rising after sunrise to plateau during the day and decreasing progressively in the late afternoon until sunrise the next day (Fig. 2a and b). The same patterns were evident in the nectar stores, but

with peak values being maintained for longer. In the brood, the trend for AH was opposite: AH increased in the late afternoon to drop the next morning (Fig. 2b). After a morning peak corresponding to dew formation, control RH decreased during the day due to the increase in temperature, then increased during the evening and night as temperature dropped (Fig. 2c). The pattern of in-hive variations in RH was similar to that of AH, but the difference between brood area and nectar stores RH was of lower amplitude (Fig. 2c). RH rose during the day in the nectar stores while it decreased in the brood area. At night, the trend was opposite (Fig. 2c).

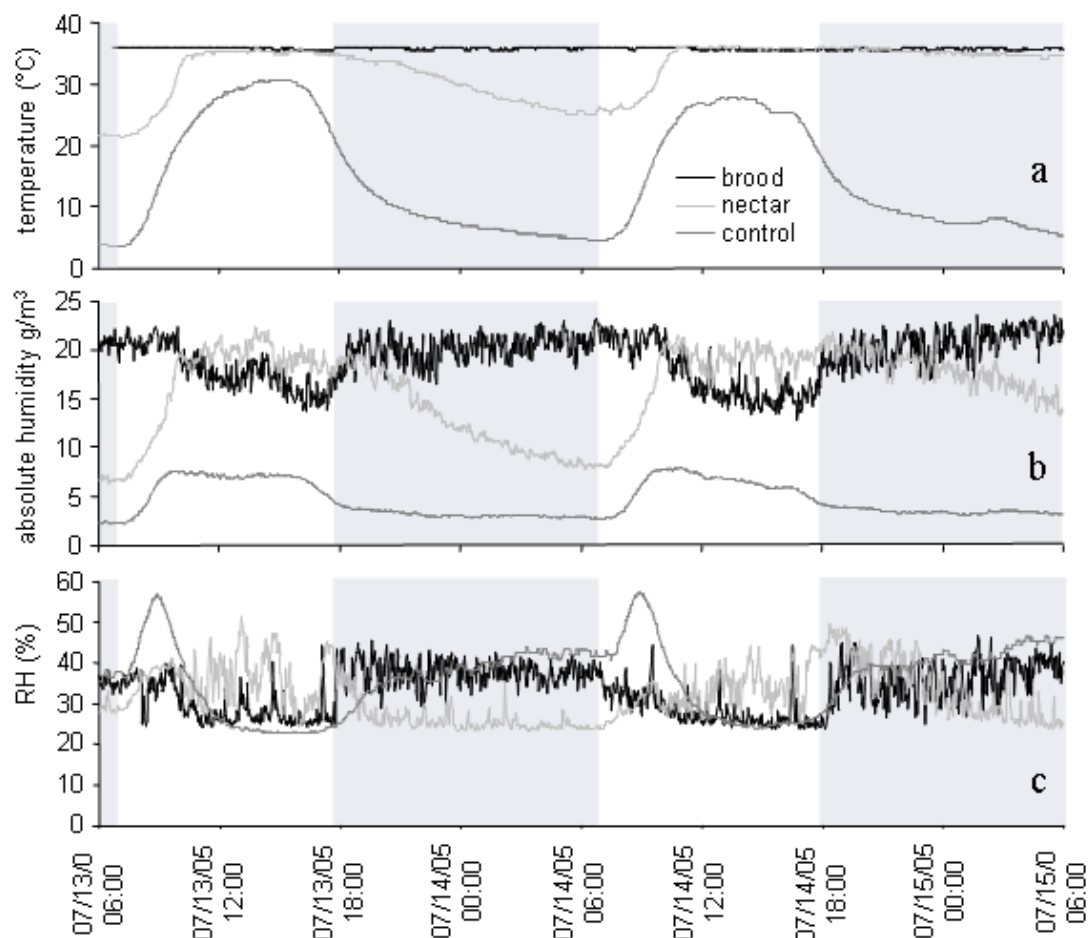


Figure 2. Variation in microclimatic parameters in a single colony over two consecutive days (only data for two days are presented for clarity). Data shown are (a) temperature, (b) absolute humidity and (c) relative humidity in the nectar stores and in brood area. Results obtained were similar for all three hives. Control parameters measured in an empty hive are also presented. Shaded areas represent night time.

Discussion

Large day-night fluctuations in temperature are characteristic of winters in Gauteng Province, South Africa. Minimum temperature was 3.5°C and maximum temperature was 31.3°C. Regardless of this high variation, *A. m. scutellata* bees were able to regulate brood temperatures with precision, confirming many previous studies (see literature in Moritz & Southwick, 1992; Heinrich, 1993).

Drought is another feature of the winters in this region. However, hive AH was always higher than control AH, indicating that it is not solely dependent on ambient humidity and that the humidity retention capacity of the hive does not explain the values measured. Although we found wide intercolonial variations, AH was always higher in the brood area where there is little nectar available as a source of water and a tendency for evaporation due to the high temperature maintained, but where there is also a high humidity requirement for optimal brood development (Doull, 1976). This suggests that humidity in this area is maintained at a high level by the workers. In contrast, AH in the nectar stores was lower, despite the high quantity of water evaporated during the honey ripening process (*Aloe greatheadii* var *davyana* nectar has a water content of 77%; (Chapter 4). Decreasing humidity in these stores would allow the evaporation of nectar in honey and prevent microbial growth. The different AH measured in these areas and the lower amplitude variations of brood AH suggest that humidity is regulated, although not precisely. RH was more similar between the two areas monitored than AH. This is due to differences in temperature combined with the differences in AH.

The daily fluctuations of humidity in the brood area and nectar stores could be due to the honey ripening process. The fanning necessary to evacuate surplus water vapour generated by nectar concentration could decrease humidity level in the brood, given that these two areas share the same atmosphere, but not the same potential water vapour sources (nectar or transpiration). Active concentration of nectar by tongue lashing (Lindauer, 1955) occurs just after unloading (Ribbands, 1953) and stops together with foraging at dusk. At this time brood humidity could be restored to optimal levels. At night, the difference in humidity between these areas could be exacerbated by transpiration from a higher number of workers aggregated on the brood combs than on the nectar combs and by their insulating effect.

Figure 1 shows that all colonies regulated their brood temperature with similar efficiency. In contrast, there is no detectable optimum for AH. Temperature in beehives can be adjusted with precision because of the insulating effect of the hive, honey stores (Lindauer, 1955) and the bees' bodies (Starks & Gilley, 1999). Furthermore, heat is produced by the bees themselves (Heinrich, 1993) and transmitted to the brood by direct contact (Bujok et al., 2002). As a consequence, bees do not rely on an external heat source or on air movement to transmit heat. In addition, optimal temperatures are the same for all hive regions: high temperature favours optimal brood development and honey ripening. In contrast, humidity modification necessitates water or nectar collection outside the hive and their evaporation, each step adding variability in the regulation mechanism. Limitations to humidity adjustment may also occur when no water is available (during droughts or at night) or when no water foragers are available (Wohlgemuth, 1957). Furthermore, humidity optima differ in the brood area and nectar stores (see above). The difficulty of regulating humidity independently in each area might result in sub-optimal humidity levels. Humidity can also depend on trade-offs with other biophysical parameters such as temperature or respiratory gases (e.g. Seeley, 1974; Korb & Linsenmair, 1998; Kleineidam & Roces, 2000; Wohlgemuth, 1957). For example, stale air has to be flushed out to allow clean air to enter the hive. Air at the optimal humidity will thus be expelled and replaced with air at ambient humidity. Humidity should thus be re-adjusted after each 'breathing' event (Southwick & Moritz, 1987). This could explain the ragged aspect of the nectar store and brood humidity curves in comparison to the control measurement (Fig. 2b and c).

Several facts have nurtured doubts about whether honeybees do regulate humidity in their hives or not (Ribbands, 1953; Büdel, 1960; Simpson, 1961; Johansson & Johansson, 1979; Willmer, 1986). Monitoring devices used in the past were too large to differentiate between areas with different humidities. Furthermore, humidity may be only partially regulated due to the constraints and trade-offs mentioned above, and the absence of clear optimal humidity values could have hindered the recognition of a regulation mechanism. According to our hypothesis of humidity regulation in a hive, the optimal RH level is close to 40% (high plateau of brood RH in Fig. 2c).

Humidity levels measured in this study corresponded with those measured by others (Büdel, 1960; Wohlgemuth, 1957), but RH was below the optimum levels for brood development ($> 90\%$) identified by Doull (1976). Although microclimate in the cells is influenced by hive atmosphere, the humidity at the bottom of the cells, where brood develops, may be higher than our measured values. High moisture could be generated by the jelly (which has a high water content) in which larvae float and by water deposited in cells by workers and maintained through the insulation provided by dense worker cover (Doull, 1976). Humidity in the brood area should then just be high enough to prevent desiccation of the cell atmosphere between the frequent visits of nurse bees (approx. every 9 min, calculated from Lindauer, 1953). We are currently investigating whether humidity is passively or actively regulated. Passive regulation could be based on transpiration of the hive's inhabitants and on the capacity effect of nectar (acting as a sink or source of water). Active regulation could be achieved by water collection and evaporation. Regulation of humidity would represent a sociophysiological mechanism that further contributes to the complex nest homeostasis of honeybees.

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CHAPTER 7

Appearances can be deceiving: insect-pollinated *Aloe greatheadii* var *davyana* has an ornithophilous pollination syndrome

H. Human, S.W. Nicolson and C.T. Symes

²Department of Zoology and Entomology, University of Pretoria, Pretoria 0002, South Africa.



Abstract

Aloe greatheadii var *davyana* (Asphodelaceae) is a winter flowering aloe with a widespread distribution in South Africa. It exhibits a characteristic bird pollination syndrome (conspicuous pinkish-red flowers, tubular corolla, copious, dilute and unscented nectar) and although visited by numerous occasional avian nectarivores, is an important plant in the bee industry. It is used by apiarists to build up honeybee colonies in winter and for honey production. Exclusion experiments were conducted to test pollinator efficiency during two flowering seasons. In both years fruit set indicated that honeybees were the most important pollinators. At least eleven species of birds; from the families Coliidae, Malaconotidae, Muscicapidae, Nectarinidae, Pycnonotidae, Ploceidae and Sturniidae were recorded visiting inflorescences, where they probed for nectar and ate flowers, but contributed very little to pollination success. Placement of honeybee hives at the flowering site in the second season significantly improved pollination success. Pollination was negligible when all pollinators were excluded, indicating no spontaneous pollination in *A. greatheadii* var *davyana*. Despite displaying the characteristics of a bird pollination syndrome, nectar characteristics measured in this study (volume $19.6 \pm 6.9 \mu\text{l}$ and concentration $21.1 \pm 2.8\%$) are identified as the single characteristic responsible for successfully attracting bees *en masse* as successful pollinators.

Introduction

South Africa has the highest diversity of *Aloe* species with more than 100 species occupying a variety of habitats (Van Wyk & Smith, 1996). These succulent plants grow well in warm climates and flower mostly during the winter months. The genus *Aloe* L. consists of 26 Sections, of which the section Pictae, or spotted aloes (characterised by their spotted leaves), is the largest. *Aloe greatheadii* var *davyana* belongs to this section and may exist as either solitary individuals or in medium-sized groups (up to 15 individuals). *Aloe greatheadii* var *davyana* has a widespread distribution in the northern summer rainfall areas of South Africa (Glen & Hardy, 2000; Van Wyk & Smith, 1996).

The pinkish-red colour, tubular structure and lack of scent of *A. greatheadii* var *davyana* flowers, as well as the large amount of relatively dilute nectar (mean volume $14.7 \pm 7.1 \mu\text{l}$ and concentration $18.6 \pm 2.7\%$, Chapter 4) typifies a bird pollination syndrome (Faegri & Van der Pijl, 1979). In South Africa at least 73 species of birds have been recorded visiting 14 species of aloes, as well as eight other flowering plants and trees, for nectar (Oatley & Skead, 1972). Sunbirds are closely associated with aloes, but bees and other insects also visit aloe flowers. From the few studies conducted it appears that sunbirds are important pollinators: in species such as *A. marlothii*, *A. ferox*, *A. mayottensis* and *A. divaricata* many opportunistic passerine birds have been recorded visiting flowers for nectar (Hoffman, 1988; Ratsirarson, 1995; Skead, 1967; Johannsmeier, 1976; Stokes & Yeaton, 1995; Pailler et al., 2002; Johnson et al., 2006; C.T Symes, pers. obs.). Birds visited *A. ferox*, although the most frequent floral visitors to flowers were honeybees (Hofman, 1988). Even though the red tubular flowers are suggestive of sunbird pollination honeybees were suggested to be important pollinators (Hoffman, 1988). According to Johnson et al. (2006) honeybees frequently visit *A. vryheidensis* but collect only pollen and do not attempt to collect nectar, due to its bitter taste. Sunbirds also find the nectar unpalatable, so that pollination of *A. vryheidensis* is carried out by short-billed birds.

Aloe greatheadii var *davyana* is commonly utilised by South African migratory beekeepers during winter months when few other nectar sources are available (Williams, 2002). The nutritious pollen (Chapter 1) and strong nectar flow (Chapter 4) are used to build up colonies, rear queens and obtain a substantial honey crop (Williams,

2002). Since *A. greatheadii* var *davyana* fits a typical bird pollination syndrome (see syndrome characters; Thomson et al., 2000), and is visited by honeybees and a host of bird species, the following questions are asked; 1) Is either of these pollinator guilds more effective than the other and if so what characteristics/ features determine any possible biased pollination success? 2) If honeybees are important pollinators is pollination success affected by changes in bee density?

Methods

Study site and plant species

Nectar secretion and pollination of *A. greatheadii* var *davyana* plants was studied during the peak flowering period at the Roodeplaat Nature Reserve (size 795 ha; 28° 39'E, 25° 66'S) in Gauteng Province, South Africa during the winter months (June and July) of 2003-2004. In the second year, six honeybee hives (*Apis mellifera scutellata*) were moved to the reserve prior to the onset of flowering. The hives were placed \pm 2 m apart within 500 m of the study site with the exclusion cages (shown later).

Population density

Population density of *A. greatheadii* var *davyana* was determined in 2004 for Roodeplaat Nature Reserve by counting the number of plants occurring in 15 plots (each 5 x 5 m) over a distance of 7 km, the plots being 500 m apart from each other. Forty five plants were randomly picked and the number of inflorescences and racemes counted as well as the number of flowers on each raceme, to estimate the number of flowers available for bee foraging in the Reserve.

Nectar production

Four flower stages can be recognised in *A. greatheadii* var *davyana* flowers. Flowers in which the corolla is just opening represent stage 1 and open flowers with the long stamens exerted stage 2. Stage 3 flowers can be recognised when the floral tube reaches its maximum width and all 6 anthers are exerted, and stage 4 flowers when the floral tube starts to wilt but the style remains turgid and exerted (Chapter 4, see Fig. 2). Nectar volume increases with flower age and reaches a peak in stage 3 flowers, thereafter it decreases dramatically. Nectar concentration follows the same pattern but less dramatic differences are observed (Chapter 4). Therefore all nectar measurements in this study

were made on unscreened, stage 3 flowers. Nectar production in *A. greatheadii* var *davyana* was investigated over a period of one week. Ten plants were randomly chosen and nectar was collected daily between 10.00 and 12.00 h from all stage 3 flowers, in haematocrit tubes (length 75 mm/75 µl). Volumes of nectar were determined from column length and the concentrations measured as % w/w sucrose equivalents with a hand-held pocket refractometer (Bellingham & Stanley Ltd, 0-50%, Tunbridge Wells, UK). Temperature and relative humidity were measured at flower height with a portable thermohygrometer (Model TES 1365, TES Electrical Corp., Taiwan).

Bird observations

In 2006, during peak flowering, the study site was visited to assess the activity of avian visitors to flowering *A. greatheadii* var *davyana*. Mist nets (12 m) were used to catch birds in the vicinity of flowering aloes to determine pollen loads on the face of potential flower visitors. For all specimens captured a swab of the crown and throat was obtained using sticky tape and placed on a slide mount for further inspection in the laboratory.

Exclusion experiments

In order to determine which pollinators were the most effective, twenty plants, at least 10 m apart, were arbitrarily chosen prior to bud opening for exclusion experiments. The experiment consisted of three treatments per plant: exclusion of birds, exclusion of birds and bees, and a control. A cage made of steel wire, with sufficient space around the florets (at least 5 cm) to prevent sunbird bills from reaching flowers, was placed over one raceme and attached to a steel rod with cable ties (Fig. 1). These cages excluded birds but allowed bees to enter and visit flowers (H. Human, pers. obs). Gauze bags (2 mm mesh size) were placed over a separate raceme to exclude all pollinators. The control raceme was marked and allowed access to all visitors. Racemes at different positions on the inflorescence were selected for each treatment in order to avoid allocating the terminal raceme with the most flower buds to a certain treatment. Racemes were caged prior to any flower opening. The length of each raceme was measured and the total number of flowers counted prior to caging. After fruit maturation all fruits were collected for each treatment and counted. Five fruits from each treatment were opened, and the number of seeds that developed in each fruit determined. All damaged and aborted flowers were collected and recorded. The experiment was repeated the following season after six beehives were placed at the reserve. These

exclusion experiment methods were similar to those described by Stokes and Yeaton (1995) and Ratsirarson (1995).



Figure 1. Growth form of a typical *Aloe greatheadii* var *davyana* plant showing exclusion treatments; (A) mesh bags to exclude all pollinators (i.e. birds and bees), (B) wire cages to exclude birds and, (C) control left open to all pollinators. (Height of cage support rod = 1, 20 m).

Statistical analysis

Data did not meet the assumptions for parametric statistics; variances were not homogeneous and data did not conform to a normal distribution. Student's t-tests were used for comparisons of flower length, bulb width and flower opening as well as

between flower length and bird bill lengths: this data met the assumptions for parametric tests. Differences in fruit set and seed set between treatments, sites and season were assessed with Kruskal-Wallis ANOVA and the Mann-Whitney U-test was used for all comparisons (Zar, 1984). Bonferroni corrections were applied for multiple paired combinations of the same data and the level of significance adopted for these comparisons was 0.0125, for all other analysis the level of significance was 0.05. Values are given throughout as mean \pm SD. Analyses were performed using Statistica 6.0 (1984-2004).

Results

Plant species

The colour of the tubular flowers varied from pale pink to bright red. Flowers have a conspicuous basal swelling, as do most of the aloes belonging to the section *Pictae* (Fig. 2 A) (Van Wyk & Smith, 1996). Flowers opened throughout the day and had an average lifespan of 4.0 ± 6.8 days. Fruits developed approximately 3 weeks later (Fig. 2A, B).

Population density

Aloe greatheadii var *davyana* grows equally well in the sun and under trees (Fig. 2C). Its density was estimated to be 104 ± 96 plants/100m² (n = 15). Plants had an average of 4 ± 2.1 racemes on each of 2 ± 0.5 inflorescences. Each raceme had 34.0 ± 16.9 flowers with approximately 340 flowers on each plant (n = 45).

Nectar production

The average length of the tubular flowers was 27.3 ± 1.7 mm, fitting the bill of one sunbird species but not other short-billed passerine bird species (see Table 1 and Fig. 3a). The average bulb width was 5.2 ± 0.7 mm and flower opening width 6.6 ± 0.8 mm, allowing honeybees to easily crawl into flowers, wedging themselves between the flower petals (Fig. 3B). The average daily volume of nectar (standing crop) was 19.6 ± 6.9 μ l per flower (total n = 365) and the average concentration of nectar was $21.1 \pm 2.8\%$. As a result of low ambient winter temperatures, bee foraging only started at approximately 09.00 h in the mornings and stopped after approximately 16.00 h. The average minimum daily temperature (between 10.00 and 12.00 h) measured was $10.6 \pm$

0.8°C and the maximum was $24.6 \pm 1.5^\circ\text{C}$. Relative humidity decreased during the day; the maximum RH measured was 32.1% and the minimum was 8.7%.



Figure 2. (A) Flower development of *Aloe greatheadii* var *davyana*, stages 1-4, (B) cluster of plants, and, (C) stand of *Aloe greatheadii* var *davyana* at Roodeplaat Nature Reserve, in peak flowering during July (period of exclusion experiments).

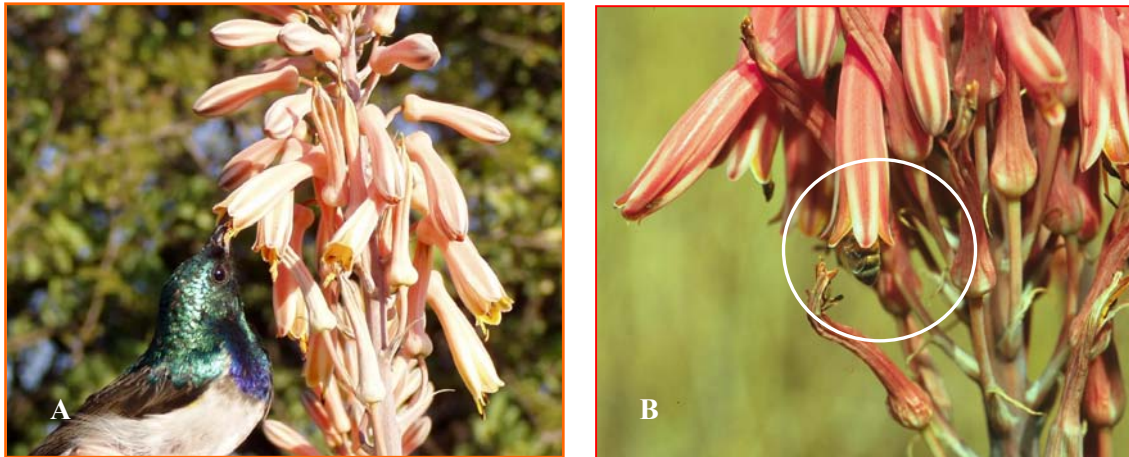


Figure 3. (A) Male sunbird *Cinnyrus talatala* collecting nectar from a flower of *A. greatheadii* var *davyana* and, (B) Honeybee *A. mellifera scutellata* nectar-forager crawling into the floral tube.

Avian visitors to Aloe greatheadii var davyana

We have no quantitative data for flower visitation by the birds and bees that visited *A. greatheadii* var *davyana* but did observe passerine birds being earlier visitors in the day. Bird species recorded at the study site, and visiting *A. greatheadii* var *davyana*, are summarised in Table 1. Bird species that probed aloe flowers for nectar did so by perching on the aloe pedicel and probing upwards into stage three flowers. A number of stage three flowers would be probed before the feeding bird moved on. Vigilance was maintained with flocks of birds (mixed species) concentrating feeding activity in a patch. Feeding evidence observed on birds was noted by the presence of pollen predominantly on the throat. Some pollen was also occasionally noted on the head. Evidence of flowers removed from inflorescences was observed although no flower feeding was recorded during observations.

Exclusion experiments

Fruit set

Flowering of the dense population of *A. greatheadii* var *davyana* was immense during both seasons. Honeybees were observed to move freely in and out of the exclusion cages. Minimal other insects were observed visiting the flowers. Results of exclusion experiments indicate that both birds and bees contribute to fruit set (Fig. 4).

Table 1. Bird species at Roodeplaat Nature Reserve recorded feeding on *Aloe greatheadii* var *davyana* during July 2006, Feeding guilds of respective species indicated as, ins = insectivore; frug = frugivore; nect = nectarivore; foli = folivore. Species previously recorded feeding on *Aloe* sp. indicated according to, 1 = Oatley (1964); 2 = Oatley & Skead (1972), * = recorded feeding on *A. greatheadii*. Bill length given as mean \pm SD (n) (Symes unpubl. data). Bill length compared with average *A. greatheadii* var *davyana* flower length, 27.3 ± 1.7 mm. Significance is shown by italics. (t = test independent samples).

Species	Guild	Observed	Pollen swab	Joe	Bill length	Comparison with aloe (t-test independent samples)
COLIIDAE						
Speckled Mousebird <i>Colius striatus</i>	frug > foli > nect	yes	-		12.3 ± 0.6 (9)	$T = 28.3, df = 57, P < 0.0001$
MALACONOTIDAE						
Crimson-breasted Shrike <i>Laniarius atrococcineus</i>	frug > ins	no	no		20.7 (1)	
PYCNONOTIDAE						
Dark-capped Bulbul <i>Pycnonotus tricolour</i>	frug > ins	yes	-	, 2 *	16.7 ± 1.4 (35)	$T = 33.7, df = 83, P < 0.0001$
CISTICOLIDAE						
Neddicky <i>Cisticola fulvicapilla</i>	ins	suspect	-	, 2 *	10.2 ± 0.6 (13)	$T = 38.3, df = 61, P < 0.0001$
Black-chested Prinia <i>Prinia flavicans</i>	ins	yes	-	, 2 *	10.1 ± 0.7 (24)	$T = 50.9, df = 72, P < 0.0001$
MUSCICAPIDAE						
White-browed Scrub-Robin <i>Cercotrichas leucophrys</i>	ins > frug	suspect	no	, 2	13.7 (1)	
STURNIDAE						
Cape Glossy Starling <i>Lamprotornis nitens</i>	frug = ins		-	, 2 *	18.1 (1)	
NECTARINIIDAE						
Amethyst Sunbird <i>Chalcomitra amethystine</i>	nect > ins	yes	-		27.6 ± 1.3 (12)	$T = 0.9, df = 60, P = 0.336$
White-bellied Sunbird <i>Cinnyris talatala</i>	nect > ins	yes	yes		20.9 ± 1.1 (20)	$T = 17.7, df = 68, P < 0.0001$
PLOCEIDAE						
Cape Weaver <i>Ploceus capensis</i>	ins > frug > nect	yes	yes	, 2	21.4 ± 1.3 (20)	$T = 16.2, df = 68, P < 0.0001$
Southern Masked-Weaver <i>Ploceus velatus</i>	ins > frug > nect	yes	yes	, 2 *	16.2 ± 1.2 (126)	$T = 52.7, df = 174, P < 0.0001$

However, bees played a significantly greater role in flower pollination. Racemes open to all pollinators produced the highest fruit set while racemes where birds were excluded produced slightly less. Exclusion of all pollinators resulted in little to almost no fruit set. Fruit set was significantly different between the three treatments, in 2003 ($H_{2,60} = 38.440$, $P < 0.001$) and 2004 ($H_{2,60} = 40.909$, $P < 0.001$). A comparison between the three treatments in both 2003 and 2004 showed significant differences between the treatment with no pollinator and the one open to all (2003, $U = 3.50$, $P < 0.001$ and 2004, $U = 0.00$, $P < 0.001$) and the no pollinator treatment and bee treatment (birds were excluded (2003, $U = 8.50$, $P < 0.001$ and 2004, $U = 0.00$, $P < 0.001$). There was no significant difference between the open to all treatment and the treatment where birds were excluded (2003, $U = 139.00$, $P = 0.1$ and 2004, $U = 194.50$, $P = 0.9$). When the treatments of the two seasons were compared no significant difference was observed in fruit set in the open to all treatment ($U = 156.00$, $P = 0.234$) but significant differences were observed in treatments that excluded all pollinators ($U = 74.00$, $P < 0.001$) and birds ($U = 99.00$, $P = 0.006$).

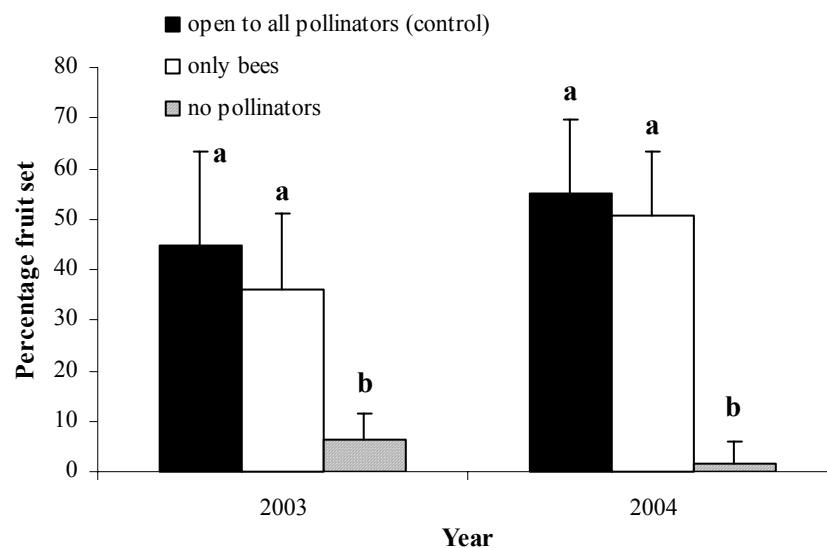


Figure 4. The average percentage fruit set (\pm SD) per raceme ($n = 20$) for *A. greatheadii* var *davyana* from pollinator exclusion experiments for two seasons (2003 and 2004). Six beehives were introduced to Roodeplaat Nature Reserve in 2004.

Seed set

Seed set was significantly different between the treatments in both years, for 2003 ($H_{2,60} = 15.941$, $P = 0.0003$) and for 2004 ($H_{2,60} = 42.484$, $P < 0.001$). In 2003 seed set was significantly different between the control treatment (open to all) and the treatment that allowed only bees ($U = 100.50$, $P = 0.007$) but there was no significant difference between the two treatments in 2004 ($U = 131.00$, $P = 0.06$). Seed set in treatments that excluded all pollinators was significantly lower than that of the control treatments (2003, $U = 67.00$, $P < 0.001$; 2004, $U = 0.00$, $P < 0.001$) and that of treatments that allowed only bees (2003, $U = 127.00$, $P < 0.05$; 2004, $U = 0.00$, $P < 0.001$). When seed set was compared between the two years significant differences were observed ($H_{2,120} = 55.616$, $P < 0.001$). Seed set was not significantly different between the control treatments (open to all) ($U = 156.00$, $P = 0.234$) but was significantly different for treatments that allowed only bees ($U = 99.00$, $P < 0.05$) and the treatments that excluded all pollinators ($U = 74.00$, $P < 0.001$).

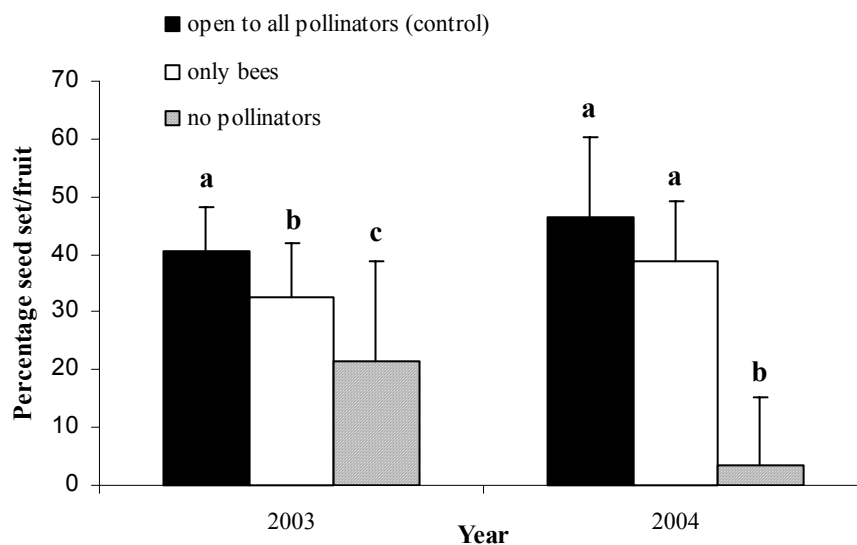


Figure 5. The average seed set (\pm SD) per fruit ($n = 5$) for *A. greatheadii* var *davyana* from pollinator exclusions for two seasons (2003 and 2004). Six beehives were introduced to Roodeplaat Nature Reserve in 2004.

Discussion

Aloe greatheadii var *davyana* flowers conform well to the requirements characteristic of a bird pollination syndrome (Table 2). However the availability of relative constant nectar (volume and concentration) throughout the day may serve to attract pollinators from divergent pollinator groups. Nectar of *A. greatheadii* var *davyana* was found to be continuously available with a relatively constant volume and concentration, available to visitors throughout the day (Chapter 4). In this study the average volume and concentration of nectar produced in unscreened flowers was $19.6 \pm 6.9 \mu\text{l}$ and $21.1 \pm 2.8\%$, a typical bird nectar (Nicolson, 2002). Plants may offer different rewards to pollinators; in *A. ferox* the chief reward for honeybees may be pollen while nectar may be the main reward for birds (Hoffmann, 1988). In *A. greatheadii* var *davyana* birds collect nectar while bees collect both pollen and nectar. This nectar flow contributes to a major part of the beekeepers' honey crop.

Just as birds sometimes visit flowers that are not typical bird flowers, other pollinators such as bees may visit ornithophilous flowers (Robertson et al., 2005). In spite of visitation by a wide variety of pollinator types only some of these visitors may actually be effective pollinators of the plant (Robertson et al., 2005). Aloes appear to be typical bird pollinated plants but bees and other insects may also pollinate them (Stokes & Yeaton, 1995). In this study, despite the typical bird pollination characteristics of *A. greatheadii* var *davyana* flowers, both birds and bees were responsible for its pollination; with the contribution of birds being significantly less than expected. The high percentage of fruit set in bird-excluded inflorescences suggests that bees are the primary pollinators of *A. greatheadii* var *davyana*, because only bees had access to flowers and the greater fruit set in the second season can be attributed to the increased density of bees at the study site, through the introduction of honeybee hives. However, these results do not show that birds are ineffective pollinators. This is in agreement with the findings of Tribe and Johannsmeier (1996) that considered both sunbirds and honeybees as the major pollinators of three species of tree aloes, *A. dichotoma*, *A. pillansii* and *A. ramosissima*. However, although stingless bees, *Trigona* species, were utilising pollen of *A. divaricata* they did not contribute to the pollination of this aloe while sunbirds, *Nectarinia souimanga* were considered to be the primary pollinators (Ratsirarson, 1995). Contrary to the results of this study, pollination of *A. vryheidensis*

is achieved by "occasional nectarivores" that use only a small quantity of the bitter tasting nectar in their daily diets while the contribution of bees to the pollination of this aloe is attributed to their numbers rather than their effectiveness (Johnson et al. in press). The primary pollinators of *A. candelabrum* (Stokes & Yeaton, 1995) and *A. divaricata*, an indigenous aloe from Madagascar (Ratsirarson, 1995), are birds.

Table 2. Comparison of bird pollination syndrome characters (Thomson et al., 2000) and flowers of *Aloe greatheadii* var *davyana*.

Bird pollination character	<i>Aloe greatheadii</i> var <i>davyana</i>
Red/orange coloured flowers	Flowers salmon pink to red
Absence of scent	No scent
Long floral tube	Flower length = 27.3 ± 1.7 mm; flower opening width = 6.6 ± 0.8 mm.
Exserted anthers and stigma	Anthers exserted before stigma, maximum nectar standing crop at male phase (stage 3)
Less pronounced landing platform	No landing platform
Inclined flower	Inclined at 120° at maximum nectar production (stage 3 flowers)
High volume of nectar	High volume- average 33.3 ± 9.8 μ l (low compared to <i>A. ferox</i> and <i>A. marlothii</i>)
Low concentration of nectar	Average $21.4 \pm 1.3\%$ (high compared to <i>A. ferox</i> and <i>A. marlothii</i>)

Aloe ferox and *A. maculata* are examples of aloes with flowers suggestive of a bird pollination syndrome that are pollinated by both birds and bees (Hoffmann, 1988; S.D. Johnson, pers. comm.). The percentage fruit set for the different treatments in *A. greatheadii* var *davyana* was much higher (45-55% for all pollinators) than observations on other species (Hoffman, 1988; Ratsirarson, 1995; Stokes & Yeaton, 1995). Pailler et al. (2002) reported only 30% for *A. mayottensis*, which has only one bird pollinator. There may be various explanations for the low effect of birds on fruit set in *A. greatheadii* var *davyana*. The low effect can be attributed to the fact that the more slender inflorescence stalks of *A. greatheadii* var *davyana* plants may not be as suitable for larger perching birds (e.g. > 35g bulbuls) as those of, for example, *A. marlothii*. The

nectar may furthermore be less accessible to larger passerine birds due to the downward orientation of flowers. But the rigid stems allow a place to perch to access these downward facing flowers – even more suggestive of ornithophily. Flower length of *A. greatheadii* var *davyana* allows sunbirds to get pollen on their throats when probing for nectar, thereby making them legitimate nectar feeders (Maclean, 1990). Subsequently only sunbirds (legitimate nectarivores) are able to contribute to pollination while other birds may remove and eat whole flowers (robbers) in order to get to the nectar (Oatley & Skead, 1972). This was noted during feeding observations, with bulbuls and mousebirds often seen removing whole flowers during visits to *A. greatheadii* var *davyana* inflorescences (Human & Symes, pers. obs).

Caging in this experiment did not negatively affect bee behaviour and subsequent fruit set, as was suggested by Celebrezze and Paton (2004). The differences in behaviour of pollinators and timing of their visits may have contributed to differences in their effectiveness. Foraging bees only started foraging at 09.00 h and stopped at 16.00 h compared to birds that were early morning visitors when bees were inactive. Birds may also have been more active on cooler days when bees were less active. Taking into account the number of foraging bees (20 000+), the amount of flowers they were visiting and the time spend on foraging compared to the number of birds may also have resulted in reduced effectiveness of subsequent visits by birds. Variation in the effectiveness of pollinators may be the result of the inability of the pollinator to pick up or deposit pollen onto the stigmas of a plant and may be influenced by flower shape and size as well as the fit of the visitor (Robertson et al., 2005).

Although nectar volume and concentration, in general, may be broadly associated with certain classes of visitors, Johnson et al. (in press) showed that secondary compounds in nectar, resulting in a dark colour and bitter taste of nectar, may serve to filter visitors from flowers. A few studies have tested whether ornithophilous pollination syndromes correctly predict the floral visitors and primary pollinators of plant species, other than *Aloes*. Varying results were obtained. Hingston and McQuillan (2000) concluded that pollination syndromes are mostly unreliable predictors of Tasmanian floral visitors. On the other hand, as predicted by its ornithophilous characteristics, *Protea roupelliae* Meisn is pollinated exclusively by birds (Hargreaves et al., 2004). Contrary to the study by Hargreaves et al. (2004) and its ornithophilous syndrome the major pollinators of

A. greatheadii var *davyana* flowers are honeybees. This is in agreement with Robertson et al. (2005) who observed both bees and birds to be the pollinators of two New Zealand mistletoes, *Peraxilla colensoi* and *P. tetrapetala*, which displayed a classical ornithophilous syndrome.

In this study the assigned floral syndrome could have led to inaccurate predictions about the plant's primary pollinator if it was not critically evaluated and investigated. The primary pollinator predicted by the syndrome only played a minor role. This confirms Hargreaves et al.'s (2004) conclusion that pollination syndromes are valuable for the development of testable hypothesis about pollination systems but should not be accepted as evidence on pollinators of a plant. Appearances can thus be deceiving.

Acknowledgments

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GENERAL CONCLUSION

Throughout the preceding chapters we have attempted to evaluate the floral resources of *Aloe greatheadii* var *davyana* utilised so keenly by honeybees and beekeepers. We have analysed the pollen for its nutritional value and investigated the effect of its exceptionally high protein content on the ovarian development of honeybees. In addition we have determined extraction efficiency during digestion of the pollen by honeybees. In order to assess the nectar resource available to honeybees, we have examined the nectary structure of *A. greatheadii* var *davyana* as well as the variation in nectar volume and concentration on various levels, from within individual flowers to across the northern summer rainfall region of South Africa. The ability of honeybees to deal with the excess water derived from utilising such dilute nectar was investigated, together with the effect of the dilute nectar on regulation of nest humidity. The floral characteristics of *A. greatheadii* var *davyana* are suggestive of bird pollination but little was previously known about its pollination. We determined the primary pollinators of this aloe through exclusion experiments. Here we summarise the key findings of each chapter and bring together the bees and the aloes, highlighting areas for future research.

Pollen of *Aloe greatheadii* var *davyana*: a rich floral reward

This study is the first, to our knowledge, to compare the changes that occur in pollen of a single species after its collection by honeybees. We have shown that the composition of pollen is indeed altered through the addition of nectar and glandular secretions by honeybees (Winston, 1987; Roulston, 2005). This is reflected in the increase in water and carbohydrate content of the pollen and the resultant decrease in its protein and lipid content. For meaningful comparisons of the nutritional value of pollens, standardised methods are needed. Although it is time consuming to collect fresh pollen compared with the ease of obtaining bee-collected pollen, it will give a better reflection of the true nutritional composition of pollen. The protein content of fresh *A. greatheadii* var *davyana* pollen (51% dry mass) is, with the exception of *A. zebrina*, the highest recorded yet for South African pollens. The comparison between the amino acid composition of pollen and royal jelly (De Groot, 1953) demonstrates the overall excellent nutritional content of this pollen.

Apis mellifera scutellata bees have a very low threshold response to intruders and become particularly aggressive and defensive during the aloe flow. In addition the number of bees available for colony defence greatly increases as a result of the availability of abundant pollen and nectar (Johannsmeier, 2001). Doull (1979a) suggested that the aggressive behaviour of bees may be due to chemical properties of the pollen of *A. greatheadii* var *davyana*. However, apart from the high protein content and high concentration of gadoleic acid, there is no obvious chemical explanation for the observed aggression in bees. The excellent nutritional quality of the pollen may contribute to increased egg production by the queen, resulting in a large amount of sealed brood present in the colonies. Winter weather delays foraging and results in short working days for forager bees (foraging only starts around 09.00 h in the morning), and hence overcrowding of the hive. Therefore the observed aggression may be the result of natural behaviour patterns in a situation of abundant food and several thousands of brood, all worth defending.

It is well known that protein is essential for the normal growth and development of bees (Moritz & Crailsheim, 1987) and the effect of dietary protein on ovarian development is well documented (Hoover et al., 2006; Lin & Winston, 1998; Pernal & Currie, 2000). Pollen is the main dietary source of protein for honeybees (Grogan & Hunt, 1979; Pernal & Currie, 2000), and protein concentrations vary widely among pollens of different plant species. The extent of pollen digestion also varies between plant species (Roulston & Cane, 2000) and is influenced by the type of consumer and the method of digestion. Extraction efficiency reflects the efficiency with which consumers extract pollen contents, but the proportion of empty pollen grains in fresh pollen is not always quantified in these calculations even though it can be substantial; according to Law (1992) up to 18% of fresh *Banksia* pollen grains are empty. Therefore in calculations of extraction efficiency one needs to adjust for the proportion of initially empty grains. Pollen digestion is furthermore influenced by pollen wall thickness and grain size (Roulston, 2005). Pollen consumers may be able to extract enough nutrients from grains even if they don't completely empty them. Less effort is needed to digest thin-walled pollen compared to ornamented pollen grains, and this is illustrated by the work of Suarez-Cervera et al. (1994) and Human and Nicolson (2003) as well as the present study.

The greater ovarian development in queenright worker bees sustained on aloe than on sunflower pollen may be attributed to the higher extraction efficiency (80%) during digestion and the exceptionally high protein content of bee-collected *A. greatheadii* var *davyana* pollen (31%). The contradictory results of workers with more developed ovaries in induced queenless groups sustained on sunflower pollen compared to aloe pollen, and the higher mortality for bees fed aloe pollen, may be explained by the detrimental effects of proteins available in high concentrations (Herbert et al., 1977; D. Raubenheimer, pers. comm.). This leads to an interesting question about the effect of high protein diets in laboratory experiments. Worker bees sustained on diets known to promote ovarian development will facilitate investigations on the correlation between ovarian development and production of mandibular gland pheromones.

Nectar of *Aloe greatheadii* var *davyana*: a water source in dry winter atmospheres

In assessing the nectar reward we also looked at the nectary structure and nectar presentation of an aloe species with open flowers belonging to a different section of the genus *Aloe*. However, anatomical differences between the nectaries of *A. castanea* and *A. greatheadii* var *davyana* were not correlated with the greater nectar secretion in *A. castanea* (Nicolson & Nepi, 2005). Variation in nectar (volume and concentration) of *A. greatheadii* var *davyana* was investigated on various levels, from within flowers to across its distribution range. The distinct basal swelling (bulb) at the bottom of the tubular flowers of *A. greatheadii* var *davyana* is a characteristic feature of the section Pictae (spotted aloes) (Glen & Hardy, 2000). The observed gradient in nectar concentration between the bulb and the tube can be explained by slight evaporation that may occur in the floral tube. The microclimate in the tubular flowers delays evaporation (Plowright, 1987), ensuring a constant concentration of the nectar available to foragers throughout the day, in spite of extremely low ambient humidities during the flowering period. The nectar, which from a bee perspective may be dilute (Southwick & Pimentel, 1981), is more concentrated than nectar measured in other *Aloe* species. Although the standing crop volume of nectar (15 µl) is lower than the mean residual nectar measured at two study sites after bee visitation (17 and 23 µl respectively), a large proportion of this nectar (10-12 µl) is inaccessible to honeybees and remains in the bulb of the flowers. The observed gradient between floral bulb and tube nectar adds a new level of variation in nectar studies.

Aloe greatheadii var *davyana* flowers in dry winter months when little else is flowering and when the water needs of honeybee colonies may increase (Johansson & Johansson, 1978). Typical winters in the summer rainfall area are characterised by pronounced diurnal temperature changes and very low relative humidity. Leaf succulence enables *A. greatheadii* var *davyana* to provide abundant dilute nectar that thus rewards honeybees with both energy and water, compensating for the low relative humidity and the evaporative costs associated with flying in dry air.

Utilisation of dilute nectar will require elimination of a greater amount of excess water by honeybees and it is possible that this process can begin in the field prior to unloading in the hive. Studies by Park (1932) and Oertel et al. (1951) contributed to the common assumption that the concentration of nectar is either slightly diluted or unchanged en route to the hive and only concentrated after unloading in the hive. Contrary to this assumption, we observed dramatic increases in nectar concentration between the flowers and the crops of bees captured at the flowers, with a further increase in returning bees captured at the hive entrance. Obviously, using the concentration of nectar in the forager's crop as an accurate indication of the nectar concentration of the flowers it has been visiting and therefore as a method of sampling nectar (see Roubik & Buchmann, 1984; Roubik et al., 1995) needs careful reconsideration.

The dilute nectar of *A. greatheadii* var *davyana* is not a problem for water balance at a colonial level, since low ambient humidities during the flowering season facilitate evaporation. Honeybees are highly efficient at regulation of hive temperature but little is known about the regulation of humidity in spite of its supposedly important role in brood development (Lindauer, 1955; Büdel, 1960; Doull, 1976b). Earlier measurements of humidity were made in hives with large monitoring devices such as hygrothermographs (e.g. Oertel, 1949) and led to the conclusion that humidity in beehives simply follows variations in temperature without active regulation (Lindauer, 1955; Simpson, 1961). Our use of miniaturised data loggers to measure humidity in different parts of the nest showed that bees are also able to adjust nest humidity within sub-optimal limits. The daily fluctuations in humidity that we observed could be the result of the honey ripening process (and timing thereof) and may also be influenced by the number of workers inside the hive at a given time. The regulation of humidity is influenced by trade-offs with regulation of temperature and respiratory gas exchanges

(e.g. Seeley, 1974; Korb & Linsenmair, 1998; Kleineidam & Roces, 2000). Further investigation into the regulation of humidity (actively or passively) is underway.

Pollination of *Aloe greatheadii* var *davyana* does not fit the pollination syndrome

Most of the characteristic floral features associated with bird pollination (Thomson et al., 2000) are exhibited by *A. greatheadii* var *davyana* flowers, such as conspicuous pinkish-red flowers, long tubular corollas, copious, dilute and unscented nectar. Even though these aloes are visited by at least 11 species of birds, exclusion experiments showed that honeybees are the primary pollinators. This is in contrast with other *Aloe* species that are pollinated by sunbirds and other passerine birds (Chapter 7) but similar to *A. ferox* which is, in spite of the expected sunbird pollination, pollinated by both birds and bees (Hoffman, 1988). Although it is common for bird-pollinated flowers to have flowers with a downward floral orientation (Aizen, 2003), the low effect of birds on fruit set in this study can be attributed to the combination of a downward orientation of the flowers on inflorescence stalks that may not be suitable for larger perching birds such as bulbuls thereby making the nectar less accessible to these birds and the eating of flowers by birds (pers. obs.). The slender inflorescence stalks of *A. greatheadii* var *davyana* are however, not a problem for sunbirds therefore they may be contributing to the pollination of this aloe. One should be aware that appearances could be deceiving. The usefulness of pollination syndromes in predicting pollinators has been questioned in recent literature (see Hargreaves et al., 2004). A critical evaluation and investigation of assigned floral syndromes is therefore needed in pollination studies.

This broad study of the interactions between honeybees and *A. greatheadii* var *davyana* serves to highlight the beneficial two-way interaction and simultaneously the paradox of *A. greatheadii* var *davyana* and bees. Honeybees are entirely dependent on the flowers of *A. greatheadii* var *davyana* in winter when little else is available. These aloes offer copious amounts of pollen that provides all the nutritional requirements of larval and adult honeybees and results in extensive brood production. Beekeepers depend on the aloe flowering season to increase their colony numbers by division. *Aloe greatheadii* var *davyana* flowers in an arid setting, but offers dilute nectar that serves as both water and energy rewards. In spite of the apparent ornithophilous syndrome and the fact that a large proportion of the nectar remains inaccessible to bees, hidden in the bulb, these

aloes ensure a constant availability of nectar at a concentration ideally suited to the needs of honeybees and that enables beekeepers to harvest a substantial honey crop. In return for the floral rewards they utilise, bees are the primary pollinators of the aloe.

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APPENDIX

Cytological features of the fresh, bee-collected and stored pollen of *Aloe greatheadii* var *davyana*

H. Human¹, M. Nepi² and S.W. Nicolson¹

¹Department of Zoology and Entomology, University of Pretoria, Pretoria 0002, South Africa.

²Department of Environmental Sciences “G. Sarfatti”, University of Siena, Via Mattioli 4, 53100 Siena,
Italy

Introduction

The pollen of *Aloe greatheadii* var *davyana* has excellent nutritional value and satisfies all the requirements of the developing honeybee brood (Chapter 1). Nutrients in the pollen cytoplasm are protected by the pollen grain wall which has an outer layer known as the exine, composed of, among other substances, sporopollenin (Heslop-Harrison, 1971; Stanley & Linskens, 1974). The exine is frequently perforated by pores leading to the inner layer or intine. These pores play an important role during dehydration, dispersal and rehydration of pollen grains and determine the amount of water loss or uptake during these processes (Roulston & Cane, 2000). The intine consists of cellulose, pectins, proteins and hemicellulose (Roulston & Cane, 2000), while the exine is an indigestible and chemically resistant bio-polymer (Nepi & Franchi, 2000).

Pollen grains can be digested either by the destruction of the outer wall or through the pores. Roulston and Cane (2000) reviewed the mechanisms used by pollen feeders to reach the substances contained in the pollen cytoplasm. Honeybees are different from other insects because they pre-digest pollen, which is a process of pollen manipulation that begins during foraging. Honeybees add nectar and glandular secretions to pollen for external transport as well as for preparation of larval food or “bee bread”, thereby altering the composition and nutritional value of the pollen (Chapter 1; Herbert & Shimanuki, 1978; Roulston, 2005). Manipulation of the pollen not only makes it digestion easier but also leads to increased digestion efficiency of *A. greatheadii* var

davyana pollen by adult worker bees (Chapter 2). Few studies have focussed on the efficiency of pollen digestion in adult bees but have been studied mainly in larvae (see Human & Nicolson, 2003).

The determination of the chemical composition of pollen grain cytoplasm (Chapter 1) requires biochemical methods while histochemistry and cytochemistry provide detailed information about the localisation of these substances. This information is difficult to obtain and sometimes one can only determine the presence or absence of certain substances with different stains. Therefore the two methods combined may be useful in understanding both the cytochemical and structural modifications that occur in pollen grains.

Methods

Fresh, bee-collected and stored *A. greatheadii* var *davyana* pollen was collected using the same methods as described in Chapter 1. Pollen samples were fixed in 2% glutaraldehyde in phosphate buffer at pH 7.2 and dehydrated in an ethanol series with increasing concentrations and embedded in LR white (London Resin Co. Ltd). Semi-thin sections (1-2 μ m) were obtained with an LKB 8800 microtome, mounted on slides and stained with the following stains: Toluidine blue (TBO) as a general stain (O'Brien & McCully, 1981); Auramine O for cuticle (Heslop-Harrison, 1977); Calcofluor for cellulose in intine (O'Brien & McCully, 1981); PAS (periodic acid Schiff reaction) for insoluble polysaccharides such as starch (O'Brien & McCully, 1981) and Alcian blue 8GX for pectins (Jensen, 1962). These sections were examined on a Zeiss Axiophot 200 inverted microscope (Carl Zeiss, Götingen, Germany) at different magnifications in the Department of Environmental Sciences, University of Siena, Italy.

Results

Mature pollen contains, among other substances, carbohydrate and lipid reserves. All insoluble polysaccharides can be detected by PAS (Franchi et al., 1996). The intine and cytoplasm of fresh, bee-collected and stored *A. greatheadii* var *davyana* pollen are visible with PAS staining (Fig. 1) but no starch was observed. Pseudo-germination was observed in a few stored pollen grains (not shown).

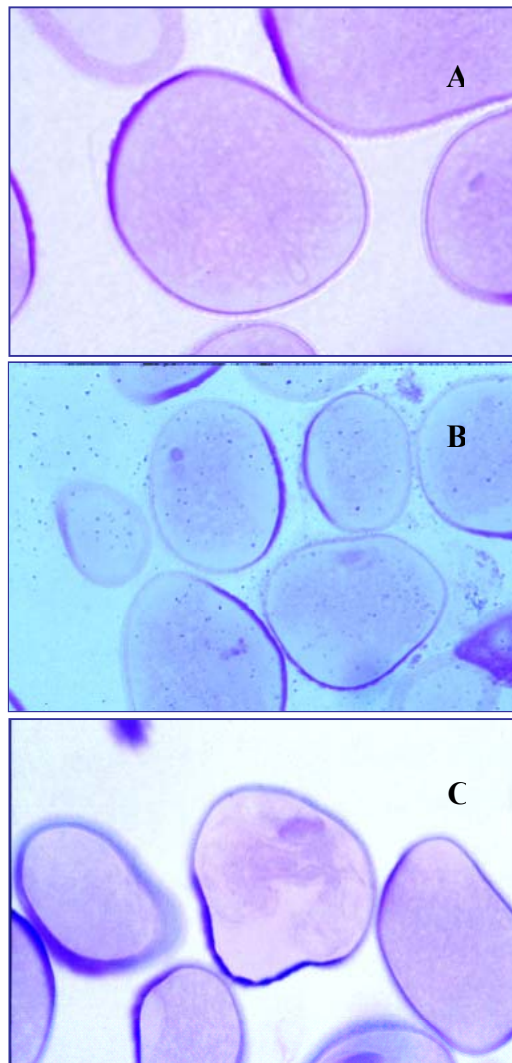


Figure 1. Indicates the intine and cytoplasm with PAS staining in (A) fresh, (B) bee-collected and (C) stored *A. greatheadii* var *davyana* pollen.

The exine contains sporopollenin that is fluorescent; therefore with autofluorescence microscopy one can distinguish between two non-continuous exine layers (Fig. 2A) without the use of any stains. The Auramine O stain intensifies the differences between parts of the exine such as the columellar structures in the outer exine layer (Fig. 2B, C).

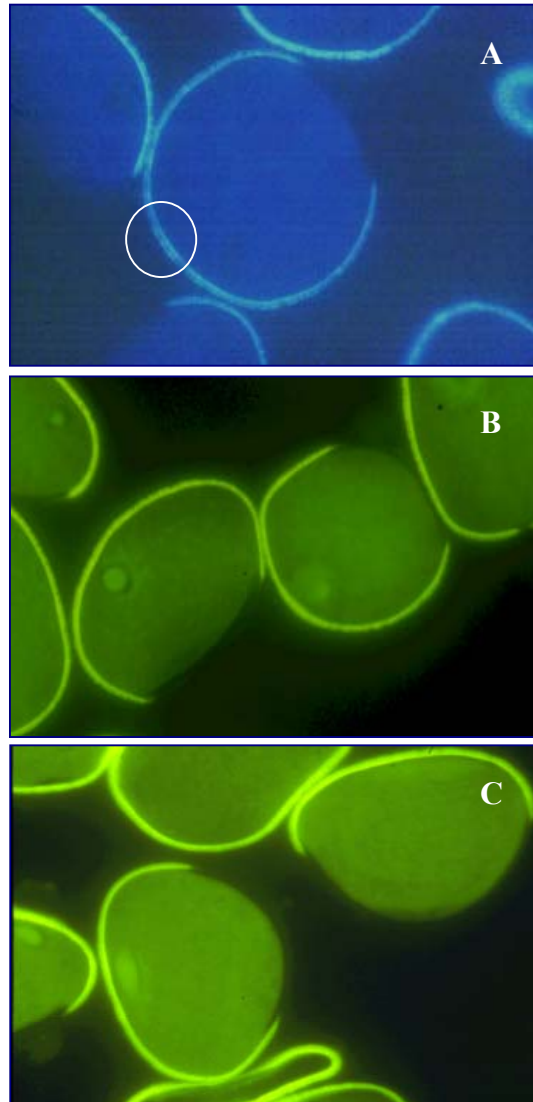


Figure 2. (A) With auto-fluorescence microscopy two non-continuous layers of the exine and a pore is visible in fresh *A. greatheadii* var *davyana* pollen. (B) Differences between parts of the exine are intensified with Auramine O in bee-collected pollen. (C) Stored pollen stained with Auramine O clearly showing the swollen intine.

The intine consists of cellulose and pectins; in this case a very thin layer of cellulose is observed in the inner part of the intine after staining the pollen grains with calcofluor (Fig. 3). Cellulosic intine appears with a more irregular profile in pollen grains stored in the hive compared to pollen from flower and bee corbiculae. Alcian blue stain showed the localisation of pectin in the outer intine layer. The surface of the outer intine layer became more irregular in pollen grains stored in the hive compared to grains in bee collected pollen (Fig. 4).

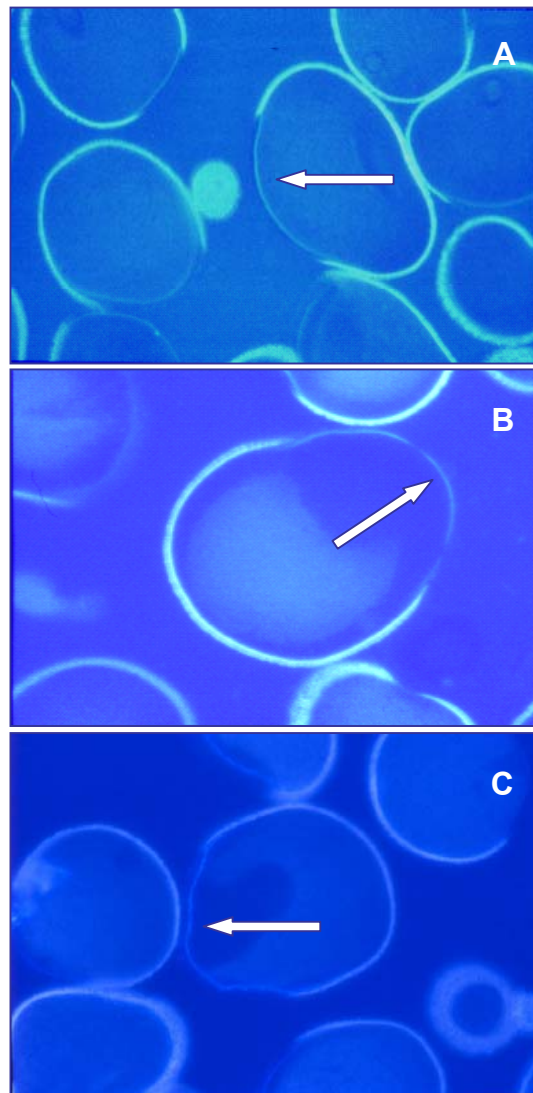


Figure 3. The obvious thin layer of cellulose in the intine of (A) fresh, (B) bee-collected and (C) stored *Aloe greatheadii* var *davyana* pollen.

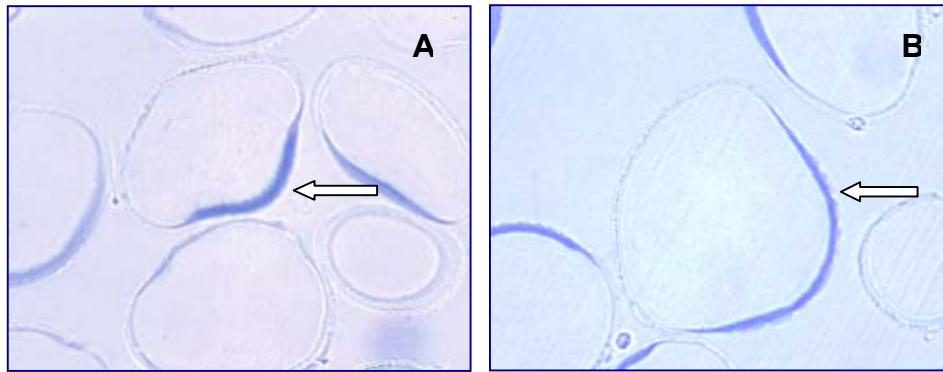


Figure 4. Alcian blue stain showed the localisation of pectin in the intine layer of **(A)** bee-collected and **(B)** stored *A. greatheadii* var *davyana* pollen

Discussion

Pollen shape changes during development, dispersal and arrival on the stigma due to loss and uptake of water in equilibrium with the surrounding environment. Mature pollen becomes dehydrated just before or during anther opening, thus increasing pollen fitness enabling it to withstand changes in environmental conditions. Ambient relative humidity and the number of pores may also have an effect on overall pollen volume. Upon landing on a compatible stigma, pollen rehydrates and germinates. This causes mechanical stress that must be sustained by the pollen walls, plasma membrane and protoplast (Nepi et al., 2001).

Aloe greatheadii var *davyana* flowers in winter, when relative humidity is low, which contributes to the dehydrated status of the fresh pollen grains. In this dehydrated state, the pollen wall is folded in the aperture regions and the indigestible exine is the only pollen wall component exposed to the external environment. Upon collection the nectar and glandular secretions added by honeybees supply moisture for pollen rehydration. During rehydration the intine absorbs water and increases in volume and surface area, especially in the furrow area, while the exine stretches. This demonstrates how the elasticity of the walls plays a role in the changes of volume and shape (Pacini, 1986). The pectin that is located mainly in the intine of *A. greatheadii* var *davyana* pollen may add to the hydration effects due to its hygroscopic properties (Aouli et al., 2001; De Halac et al., 2003).

The study by Suarez-Cervera et al. (1994) reported no ultrastructural changes in the pollen grain walls of stored pollen compared to fresh pollen. Added to this Klunness and Peng (1984 a, b) did not observe morphological changes in the pollen walls and protoplasm of stored pollen grains. Changes only occurred in the honeybee gut during digestion. In *A. greatheadii* var *davyana*, the only modification that occurred in the structure of the pollen wall, between fresh and stored pollen, appeared to be a slight change in the appearance of pectin and cellulose in the exposed intine. The suggestion that grains become compressed in the rectum as a result of the removal of certain structural components from the pollen wall through digestion (Klungness & Peng, 1984 a, b) may explain the occurrence of *A. greatheadii* var *davyana* pollen grains in the gut of honeybees (Chapter 2). The thin, exposed intine may contribute to the high digestion efficiency observed in honeybees for *A. greatheadii* var *davyana* pollen (Chapter 2).

The physiological state of *A. greatheadii* var pollen grains are deeply changed through hydration in that the intine is exposed, presenting a region for enzyme penetration during the digestive process. Thus pollen handling by honeybees probably “prepares” the pollen grains for efficient digestion.

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