CHAPTER 2

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

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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 Bronkhorstspruit district (28 39'E, 25 54'S) in Gauteng Province. Thereafter the hives were moved to Roodeplaat Nature Reserve (size 795 ha; 28º 39'E, 25º 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 (Mohammedi 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 34ºC 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 Mohammedi 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.

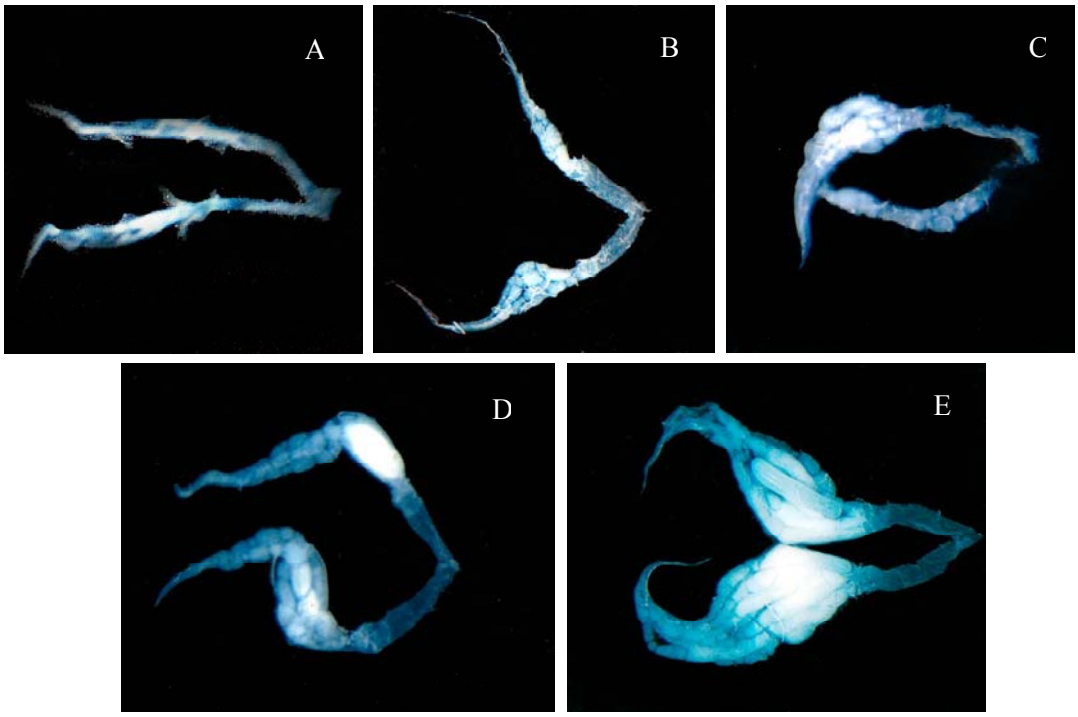

Figure 1. Ovarian development in worker bees categorised according to Hess (1942). **(A)** stage 1, **(B)** stage 2, **(C)** stage 3, **(D)** stage 4 and **(E)** stage 5. (Photos by V. Dietemann)

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.

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

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 ± 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).

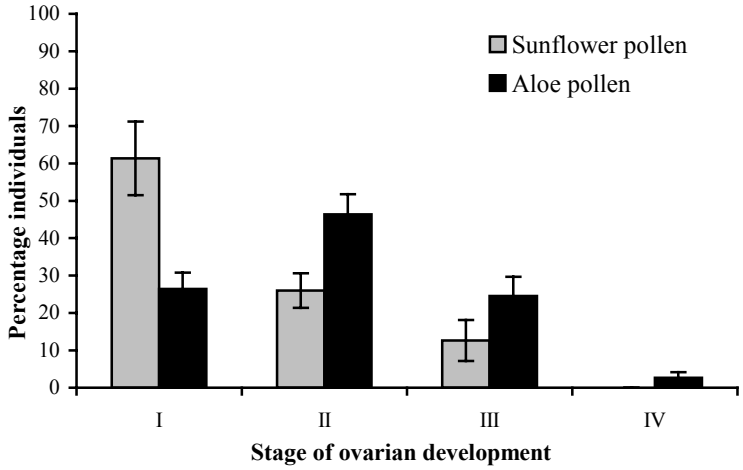

Figure 2. Worker ovarian development of bees from queenright colonies feeding on sunflower (*H. annuus*) or aloe (*A. greatheadii* var *davyana*) pollen. Data are given as means ± SD, n = 70.

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 ± 17.3%) than those fed on sunflower pollen (13.5 ± 8.9 %) (P < 0.001).

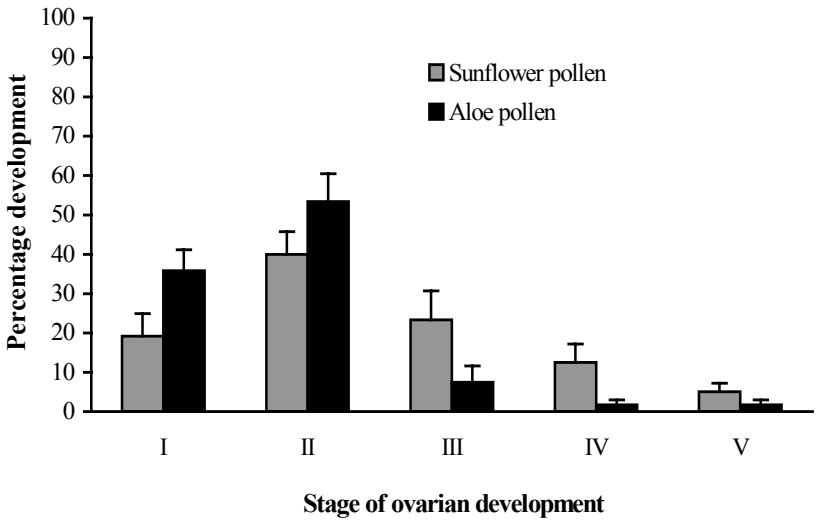

Figure 3. Comparison of ovarian development of individual queenless worker bees fed sunflower (*H. annuus*) and aloe (*A. greatheadii* var *davyana*) pollen in the laboratory. Data are given as means ± SD, n = 20.

Pollen digestion

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

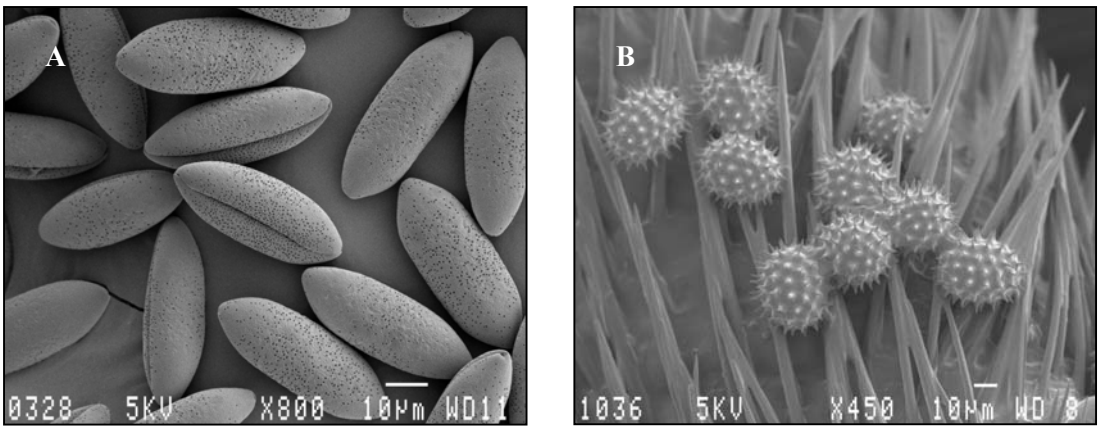

Figure 4. Scanning electron microscopy pictures of **(A)** aloe (*A. greatheadii* var *davyana*) pollen and **(B)** sunflower (*H. annuus*) pollen, attached to hairs on *A. mellifera scutellata* legs. Note different scales.

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 ± 1.2%) was significantly lower ($t = -4.99$, $df = 18$, $P < 0.001$) than that in sunflower pollen (6.7 ± 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 ± 10.2% aloe pollen grains were already extracted in the midgut of honeybees compared to 69.2 ± 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 (Kropácová & 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).

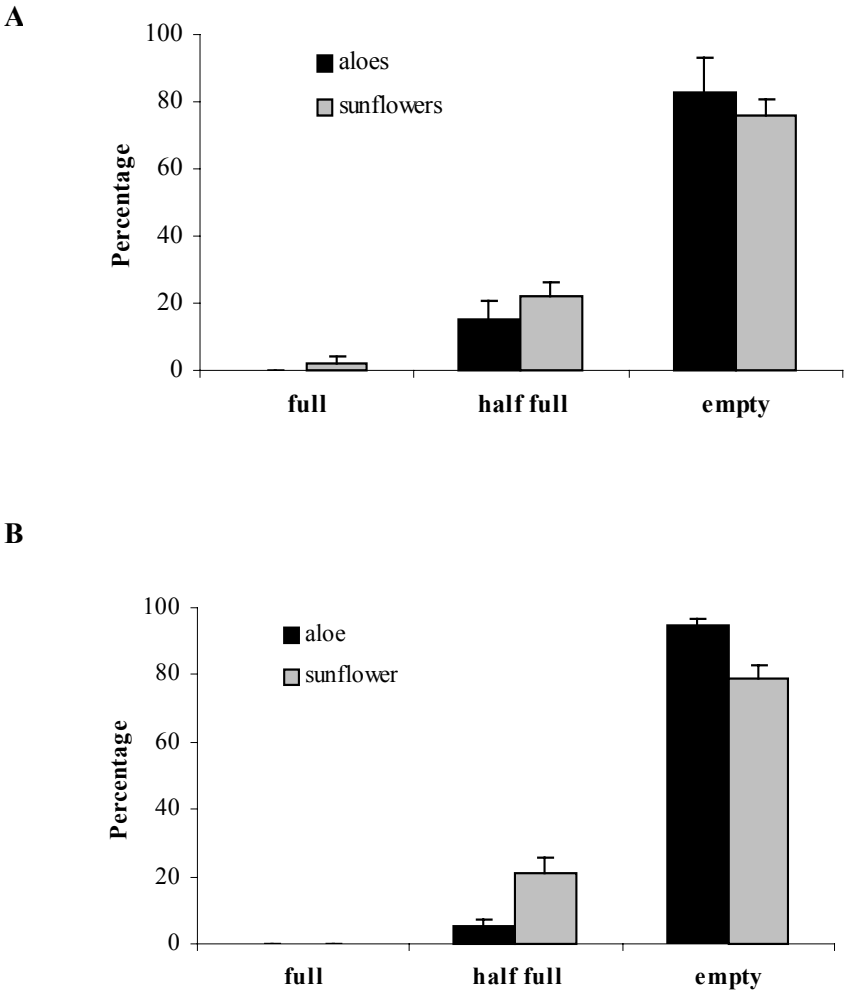

Figure 5. The percentage of empty, half full and full aloe (*A. greatheadii* var *davyana*) and sunflower (*H. annuus*) pollen grains in **(A)** the midgut and **(B)** the hindgut of honeybees (Data are given as means ± SD, n = 50).

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.

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