Digestion of maize and sunflower pollen by the spotted maize beetle *Astylus atromaculatus* (Melyridae)

by

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Declaration

I declare that the thesis, which I hereby submit for the degree Master of Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at another university.

Hannelie Human

Date .....

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Abstract

Digestion of maize and sunflower pollen by the spotted maize beetle *Astylus atromaculatus* (Melyridae).

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The relationship between beetles and flowers is often mutually beneficial. Flowers provide not only edible rewards and favourable micro-environments, but may also be sites for mating and egg-laying activities. Even though beetles consume various parts of flowers, including pollen, and can sometimes cause considerable damage, they are in fact important pollinators of many flowers. Pollen was once considered indigestible but is actually a highly nutritious food source for many animals (including insects, birds and mammals) which use a variety of methods to digest it. Six basic methods are discussed in detail: mechanical damage, piercing and sucking, external digestion, enzymatic action, osmotic shock and pseudogermination.

In this study I investigated the mechanism and efficiency of pollen digestion of two different kinds of pollen, that of maize *Zea mays* and sunflower *Helianthus annuus*, by the spotted maize beetle *Astylus atromaculatus* (Melyridae) an economically important pest in South Africa. Histological observations were made of the gut contents and

faeces of spotted maize beetles that fed on maize and sunflower. A high percentage of maize pollen grains (88%) was found to be empty and ruptured in the anterior midgut of these beetles, while sunflower pollen, although the contents were removed from most of the grains (84%), remained intact. Osmotic shock was apparently involved in digestion of maize pollen while another method, such as enzymatic action, may be used for the digestion of sunflower pollen by this beetle. Digestion efficiency of pollen, which corrects for the number of initially empty grains, was determined for the spotted maize beetle (67% for sorghum, 72% for sunflower and 74% for maize) and was found to be high in comparison to values for various mammals, birds and insects consuming pollen of other plant species.

Kroon et al. (1974) proposed osmotic shock as a prerequisite for pollen digestion in honeybees and this hypothesis was accepted into the literature without question. I investigated the effect of osmotic shock on maize pollen by looking at behaviour of pollen grains under varying osmotic concentrations. Given that rainwater can sometimes cause irreversible damage to pollen grains, distilled water was used to simulate rain and sucrose solutions the stigmatic exudates of flowers. A small number of studies have focused on differences between cultivars, therefore *in vitro* studies were carried out subjecting maize pollen of different cultivars to different sucrose and glucose:fructose concentrations, using distilled water as a control. Results of this study indicated that maize pollen might burst in distilled water and sugar solutions of various concentrations did not decrease the amount of rupturing compared to that in water.

Few studies have looked at pollen of a single plant species being digested by different animals. I compared the efficiency and mechanism of maize pollen digestion by

honeybees (*Apis mellifera*) and spotted maize beetles. Digestive efficiency was high (80%) in beetles compared to that of bees (21%). Maize pollen bursts early in the midgut of maize beetles but remained intact in honeybees: this suggests that osmotic shock is not as important for bees as previously suggested.

Contents

Chapter 1

Pollen nutritional content and digestion by animals: a literature review.

"The use of the attire of flowers to be not only ornament and distinction to us but also food for a vast number of little animals who have their peculiar provisions stores up in these attires of flowers: each becoming their lodging and their dining-room, both in one" Nehemia Grew 1671 (Yorks 1980)

1.1 Beetles as pollinators and pollen feeders

Successful pollination of plants depends on the attractiveness of flowers to their pollinators and the ability of the pollinators to deposit enough pollen on the stigmas (Frankel and Galun 1977). There is a tendency for certain animal groups, morphologically and ethological similar, to be strongly associated with specific floral classes. Consistent patterns were observed and defined as pollination syndromes by the German pollination biologist Stefan Vogel in 1954 (Faegri and Van der Pijl 1979).

The result is that flowers with a sweet smell became known as "bee-flowers" and red and orange coloured flowers with a mild odour as "butterfly flowers". "Beetle flowers" may either be large and bowl or urn-like in shape, or may consist of small flowers, often unisexual, condensed on a branch or in inflorescences (Faegri and Van der Pijl 1979). Both these flower types can accommodate several beetles at the same time (Bernhardt 2000). Beetles are attracted not only to dull (cream to greenish) but also to blue-violet and red-orange flowers (Dafni et al. 1990, Picker and Midgley 1996). Floral odours said to attract beetles may vary from faecal to musky to honey-like; however in a large proportion of beetle-pollinated plants floral scents are undetected or faint (Bernhardt 2000). Pollination syndromes have also been described for flower-visiting vertebrates including birds, bats and rodents. These syndromes were turned into stereotypes by biologists underestimating the complexity and variability of relationships existing in nature: although the concept of pollination syndromes may be inaccurate, it still is a useful tool for biologists (Buchmann and Nabhan 1996).

Even though the relationship between beetles and flowers is one of the earliest examples of floral specialization, Faegri and Van der Pijl (1979) considered pollination by beetles as largely accidental and beetles as sometimes able to cause severe damage to flowers. Many beetles, due to their short mouthparts, prefer flowers with exposed pollen where they will crawl around in the flowers consuming pollen and nectar, or chew on other parts of the flower. In flowers with less accessible food, bigger beetles deal with the problem by chewing their way through and sometimes destroying floral organs, in order to reach the hidden pollen or nectar (Faegri and van der Pijl 1979, Barth 1985). In their search for food and mates they casually transfer pollen of their host plants. This activity has been called "mess and soil pollination" (Faegri and Van der Pijl 1979). In fact beetles are important pollinators and are estimated to pollinate a remarkable 88.3% of all flowering plants (Buchman and Nabhan 1996).

Beetles visit flowers for three reasons that are difficult to separate. Examples illustrate that they are interconnected:

1. *Edible rewards*. Beetles eat almost all parts of plants, and many are known to eat pollen and flower petals and even to suck nectar. Monkey beetles (Scarabaeidae: Melolonthinae: Hopliini), of which 62% are endemic to South Africa, are known to feed on pollen directly from the anthers and may possibly be feeding on nectar as well (Johnson and Nicolson 2001). Adult Mexican corn rootworms (Chrysomelidae) feed on leaves, silks, pollen and immature seeds of corn (Jones and Coppedge 2000). The blister beetle, *Nemognatha* (Meloidae) feed entirely on nectar with mouthparts resembling those of butterflies and bees, their suctorial tongues, however, incapable of rolling up (Percival 1965, Gottsberger 1990).

2. *Site for mating and egg-laying activities.* Curculionid beetles of the genus *Elleschodes* pollinate the flowers of *Eupomatia* (Magnoliidae) and use these flowers as a brood place to lay their eggs. The larvae develop in the flowers and use them as food (Endress 1994). Hundreds of small curculionids, *Phyllotrox* sp., are attracted by the odour produced by the flowers of the Panama hat palm, *Carludovica palmata* (Cyclanthaceae). Upon arrival the beetles feed on the staminodes, copulate and crawl deeper into the inflorescence to where the female flowers are hidden. Most of the beetles remain here during the day and become loaded with pollen grains. When they move on to the next attracting inflorescence they cause pollination (Gottsberger 1990).

3. *Favourable micro-environment.* The interior of some flowers may offer an environment preferable to that outside (Bernhardt, 2000). In the tropics scarab beetles (Dynastinae) are attracted to the floral chambers of *Annonaceae* (Magnoliidae). These floral chambers provide a safe environment and fulfill the basic requirements of the beetles: food, shelter against predators and daylight, and mating opportunities, and in return the beetles render their services as pollinators (Gottsberger 1989, 1990, Endress 1994). A South African example is that of adult pollen beetles, *Pria* sp. (Nitidulidae), which arrive with the onset of flowering on male flowers of *Leucadendron xanthoconus* (Proteaceae) to feed and mate. Apparently they prefer male plants but also visit female flowers for heat and protection and perhaps mating sites. The visits to female flowers doubled in colder weather, i.e. overcast and rainy days (A. Hemborg pers. comm.).

The consumption of pollen may be an ancestral trait in the evolution of beetles: examination of fossils and gut impressions shows that beetles already ate pollen millions of years ago (Faegri and Van der Pijl 1979, Gottsberger 1990, Bernhardt 2000). Their chewing mouthparts make it difficult to distinguish between their motives for visiting the flowers. Because of their mouthpart structure, it is generally accepted that beetles will be able to mechanically crack open pollen grains, but the method of digestion used by beetles feeding on pollen is not clear. Evidence for beetles cracking the pollen wall with their mandibles to enable them to get to the contents of the pollen grain is lacking.

Pollen feeding studies in Coleoptera have been carried out on the Mexican corn rootworm (Chrysomelidae) and the boll weevil (Curculionidae). Unfortunately only the quantities and types of pollen ingested were investigated, not the method of digestion (Jones et al. 1993, Jones and Coppedge 2000). Johnson and Nicolson (2001) studied pollen digestion by protea beetles (Cetoniinae) and monkey beetles (Hopliinae). They examined different gut regions histochemically to determine the appearance of pollen grains and removal of nutrients, and suggested that digestive enzymes might be the most likely method used by these beetles to obtain the nutritious contents of pollen grains.

1.2 Pollen as a floral reward

Whether wind dispersed or ingested and excreted by insects, pollen often remains recognizable for millions of years, sometimes even identifiable to source plant taxa (Labandeira 1997). Pollen is produced to ensure the reproduction of plants and

therefore it needs to be resistant to desiccation and consumption by other organisms. To achieve this, the nutrient-rich cytoplasm of pollen grains is surrounded by a protective outer layer, the sporoderm or pollen wall. This consists of two layers: the exterior exine and the interior intine. The exine of the pollen grain is composed mostly of sporopollenin, a highly resistant polymer of carotenoids that does not react with most chemicals. The exine, either smooth or sometimes sculptured in distinctive ways, presents an obstacle to pollen feeders and is referred to as being "indigestible" or even "chemically resistant" by some authors (Stanley and Linskens, 1974). The intine is composed of protein, including hydrolytic enzymes, and cellulose and pectin (Stanley and Linskens 1974, Heslop-Harrison 1979a, Nepi and Franchi 2000). The pollen surface of entomophilous plant species may be covered with an oily substance, the pollenkitt, making the pollen sticky and thus suitable for adhering to the body of the pollinator (Frankel and Galun 1977, Rebelo 1987, Faegri and Van der Pijl 1979, Nepi and Franchi, 2000). Apart from adding additional nutritional value to the pollen grain, other functions of the pollenkitt are to impart colour or odour to the pollen (Roulston and Cane 2000, Pacini 2000).

Specialized regions known as apertures (pores) occur in the pollen wall, the intine being thicker and the exine thinner in the vicinity of an aperture. These apertures differ in form, structure and number within different plant species. Pollen tube emission, and the exchange of water and other substances, occurs at these sites (Heslop-Harrison 1979a, 1979b, Nepi and Franchi 2000). Pollen grains are known to dehydrate after anthesis and before dispersal. During dispersal they are in equilibrium with ambient conditions and they rehydrate after coming in contact with the stigma of the compatible plant. The pollen walls, plasma membrane and protoplast are able to sustain the

mechanical stress due to changes in volume as a result of the loss or uptake of water (Bassani et al. 1994, Nepi, et al. 2001).

Chemical analyses have revealed the highly nutritious contents of pollen grains. The cytoplasm contains, apart from micronutrients, the following macronutrients: protein (up to 61% of total dry weight), carbohydrates (up to 56%), which may or may not include starch (0-23%), and lipids (up to 19%). The proportions of these nutrients can vary widely among different plant species (Todd and Bretherick 1942, Stanley and Linskens 1974, Roulston and Cane 2000).

The protein in pollen is intended for the growth of the pollen tube and is therefore very appropriate for the developmental stages of insect consumers. Most pollen contains all the common amino acids necessary for ovarian development, although the specific amino acid, tyrosine, which is needed for moulting may sometimes be lacking. The nitrogen in pollen protein is an essential part of the dietary requirements of some social insect species where parental care occurs and pollen forms the principal food source of the young (Gilbert 1972, Haslett 1983, Roulston and Cane 2000). However, dandelion pollen is considered valuable brood-rearing pollen but has a low protein content (11%), while blue gum pollen on the other hand is not so highly valued and has above the average protein content (26%) (Todd and Bretherick 1942). Variation in protein content will have an effect on the amount of pollen needed (Roulston and Cane 2000). Some authors feel that the nutritive value of pollen is not related to protein content, for example Roulston and Cane (2000) stated that no other chemical constituent of pollen grains has been shown to influence as many aspects of a pollen consumer's performance as well as protein.

Pollen from entomophilous flowers (pollen that is actively gathered/ transferred by insects) is richer in nutrients and has a higher caloric content than anemophilous pollen (pollen that is transported by the wind) (Petanidou and Vokou 1990). This supports Baker and Baker (1979) who found that anemophilous species, in general, produce pollen grains rich in carbohydrates while the pollen of entomophilous species on the other hand is lipid-rich (the latter would have a higher energy content since lipids contain more energy per gram than starches). But all researchers do not share this conclusion, since some of the most lipid-rich pollens are from anemophilous species, e.g. *Juglans nigra* (18%), *Alnus incana* (13%) and *Fagus sylvatica* (12%) (Roulston and Cane 2000). More anemophilous species than entomophilous species produce starchy pollen, e.g *Zea mays* (17%) vs. *Helianthus annuus* (0.3%) making the anemophilous pollen an inferior caloric reward. This has led to the assumption that insects will avoid such pollens and show a preference for starchless pollen, but Roulston and Buchmann (2000) found insufficient evidence for this theory. Other studies also indicate that honeybees as well as other bee species can digest starch and that many do collect starchy pollen (Simpson and Neff 1983, Klungness and Peng 1984).

Many researchers have analyzed pollen for specific components and therefore pollen analyses are not always complete for a species. Analysis is frequently done on bee-collected pollen since it is easy to collect. Todd and Bretherick (1942) were careful to distinguish between bee-collected and hand-collected pollen in presenting data from their analyses. Bee-collected pollen is said to have a higher nutritive value than hand-collected pollen (Stanley and Linskens 1974). Both bumble bees and honeybees add nectar sugar to their pollen load for external transport, thereby increasing the soluble

carbohydrate content (Roulston 2003). Apart from increasing pollen weight, all calculations in chemical analysis will be affected. This is illustrated by the data of Louw and Nicolson (1983), who compared the chemical composition of *Virgilia divaricata* pollen with the pollen paste used by the carpenter bee, *Xylocopa capitata,* to provision larval cells (Table 1). The female carpenter bee regurgitates nectar to moisten pollen grains and shape the food mass.

TABLE 1 Chemical composition of *Virgilia divaricata* (Fabaceae) pollen and the pollen-nectar paste used by *Xylocopa capitata* to provision larval cells. Data reproduced from Louw and Nicolson (1983).

Pollen	*Virgilia*	Pollen paste
Water content	33.5 ± 1.9	20.9 ± 0.8
Protein (%)	25.0 ± 2.0	15.9 ± 0.9
Lipid (%)	6.9 ± 0.7	4.6 ± 0.3
Crude fibre (%)	7.1 ± 1.1	5.4 ± 0.4
Ash (%)	5.4 ± 0.7	4.6 ± 0.7
Carbohydrate (%, by difference)	55.6	69.5

All values are expressed as mean ± S.E. and as % dry mass apart from water content (% wet mass).

1.3 Pollen digestion

Pollen was formerly assumed to be indigestible but can actually be digested by a surprising variety of animals. Sometimes ingestion of pollen grains can be incidental to other feeding, with pollen being an admixture of the food, for example pollen taken up with nectar or when anthers are eaten (Stanley and Linskens 1974, Faegri and Van der Pijl 1979, D'Arcy 1996). In order to reach the nutritious cytoplasm pollen feeders need

to overcome the obstacle presented by the pollen grain wall. The utilization of pollen presumes either digestion through existing pores or the destruction of the outer pollen grain wall.

Differences in pollen wall structure and composition, the size of pollen grains, as well as gut passage rates of pollen feeders, will result in differences in the digestibility of pollen types (Law 1992, Crailsheim et al. 1993). A considerable variation in the structure of pollen walls of different plant species was demonstrated. The cellulose content of pollen of 28 plant species was reported to vary from 1 to 15% dry weight, and their sporopollenin content from 2 to 23% (Klungness and Peng 1984). This variation may cause differences in osmotic lysis and permeability. The type and size of pollen grains ingested play an important role in determining gut passage time. For example, the passage of smaller pollen grains was slowed down by large pollen grains, therefore larger pollen grains will have a longer gut passage time although digestion efficiency will not necessarily increase (Law 1992). Variation in extraction of pollen grain contents from different plant species as well as variation in gut passage rates has been demonstrated for different animals (Turner 1984, Richardson et al. 1986, Herrera and Martinez del Rio 1998).

The variation in size, behaviour and physiology among pollen consumers leads to an interesting variety of methods used to digest pollen. Some of these methods are very rare and apply to few of the animals digesting pollen, and other methods are difficult to distinguish. Six basic methods used by insects and other animals to extract pollen contents are described in the literature:

1.3.1 Mechanical damage

Insect mandibles may be used to crack the pollen wall (Samuelson 1994). Grinding mandibles are found in some pollen feeding beetles and micropterygid moths (Crowson 1981, Barth 1985, Thien et al. 1985). Roulston and Cane (2000) failed, however, to find any study demonstrating whole pollen grains entering the mandibles and crushed pollen leaving them. Claims in the literature seem to be derived from morphological analysis correlating mandible structure with a pollen diet (Roulston and Cane 2000).

Instead of using their mouthparts to crack pollen grains, some pollen-feeding beetles crack the grains in their digestive tracts. One species of scarab beetle, *Cyclocephala amazona*, consumes the trichomes of its food host plant, the peach palm *Bactris gasipaes*, before ingesting the pollen from the same plant. The trichomes have highly lignified cell walls but no nutritive value, and are apparently used as gastroliths to grind the pollen grains in the digestive tract (Rickson et al. 1990).

1.3.2 Piercing and sucking

Pollen feeding is reported to be widespread among flower thrips (Thysanoptera). Due to their small size these species are incapable of ingesting whole pollen grains (Kirk 1984). They use their forelegs to hold a grain and then pierce the grain with their mandibles and suck out the fluid contents. Empty pollen grains are discarded. Depending on the size of the grain, the temperature and the thrips species, this process takes 2-20 seconds per grain with daily feeding rates ranging from 29 to 843 grains per thrips per day (Kirk 1984, 1987).

The females of some species of ceratopogonid flies usually feed on nectar but need the nourishment of pollen grains, instead of the typical blood or hemolymph meals used by other biting flies, for their oocyte development. It was shown that the females of *Atrichophogon pollinovorus* press the tip of their rostrum against the pollen grain and then suck out the contents, the whole process taking 6 seconds (Downes 1955).

1.3.3 External digestion

A form of external digestion is found in *Heliconius* butterflies (Nymphalidae). Collected pollen adheres to the ventral side of the proboscis, near the head. The pollen is then wetted by a clear liquid exuded from the proboscis tip and kneaded for hours through the coiling and uncoiling of the proboscis. The pollen grains swell and the contents start to leak out through the pores in the pollen wall, allowing the butterflies to imbibe the nutrients without swallowing the pollen grains (Gilbert 1972). Pollen processing behaviour similar to the above has also been found in papilionid butterflies *(Parides* and *Battus*) from the Costa Rican rainforests (De Vries 1979) as well as the eucalypt nectar fly, *Drosophila flavohirta* (Nicolson 1994). The gathering of pollen by *D. flavohirta* results in a pollen mass on the anterior surface of the extended proboscis. The period of pollen gathering is then followed by a period of pollen processing when the fly, otherwise motionless, moves its proboscis up and down. During this process the pollen grains become swollen with subsequent release of the contents (Nicolson 1994).

1.3.4 Enzymes

Enzymes can be used to dissolve the pollen wall or to penetrate it. The enzyme exinase is found only in collembolans, which are capable of breaking down the exine and fully digesting the pollen grain. When *Juniperus* pollen was fed to the collembolan *Ontchiurus pseudofimetarius,* the pollen wall ruptured and then completely disappeared during its passage through the gut (Scott and Stojanovich 1963). However this digestive ability is not possessed by all collembolans (Roulston and Cane 2000).

Recent work on pollen digestion by scarab beetles (Johnson and Nicolson 2001) shows that these beetles use their maxillae as pollen brooms to sweep the pollen into their mouths. Grains are ingested intact and nutrients such as lipids and proteins are efficiently removed from the pollen grains during passage through the gut. Digestive enzymes may penetrate the wall through pores to process the contents inside the grain: digested material would then exude out through the pores to be absorbed (Simpson and Neff 1983, Roulston 2003). Pollen grains were modified by digestive enzymes in passing through the gut of *Osmia cornuta* (Hymenoptera: Megachilidae) larvae (Nepi et al. 1997). Pollen enzymes already present in the cell wall may also have an influence on the digestion process (Grogan and Hunt 1979, Roulston and Cane 2000).

Various researchers have looked at pollen digestion by vertebrates. Van Tets (1997) demonstrated that small mammals feeding on the pollen of *Protea* species in the Cape fynbos are capable of extracting the contents of ingested pollen grains. In Australia similar observations have been made for *Synconycteris australis* and *Rattus fuscipes*, both feeding on *Banksia* pollen (Law 1992, Van Tets and Whelan 1997). The animals

were observed feeding on pollen and the number of empty grains in their faeces determined. Methods of digestion were not specifically investigated, but the presence of partially digested pollen grains, together with intact exines and the absence of germinating or burst grains, suggests enzymatic digestion of the protoplast through the pollen wall (Van Tets, 1997).

1.3.5 Osmotic shock

Pollen grains may burst when transferred from a medium of high osmotic concentration to one of low osmotic concentration, as was demonstrated *in vitro* by Kroon et al. (1974). These authors proposed that this might happen in the gut of pollen feeders such as honeybees. Adult bees have a crop, divided by the proventriculus from the midgut, and there may be substantial differences in osmotic concentration between these gut regions, so that the pollen is subjected to a large and sudden change in osmotic concentration. Kroon et al. (1974) suggested that this osmotic differential was enough to burst pollen grains, making the contents accessible to digestion. Nicolson (1990) demonstrated that osmotic concentrations could vary dramatically in the alimentary canal of the carpenter bee, *Xylocopa capitata:* the osmolarity of the crop contents (5381 ± 240 mOsm/l) was 5.6 times higher than that of the midgut contents (956 ± 68 mOsm/l).

1.3.6 Pseudogermination

Germination of pollen grains can be induced *in vitro* by warm sugar solutions, into which proteins and free amino acids are released. Optimum conditions for germination

of pollen grains vary for different plant species. Temperatures ranging between 20 °C and 30 °C, sucrose solutions varying from 0.75% to 40% and a weakly acid environment (pH 5.5 - 6.5) have been suggested as optimum conditions for many species (Stanley and Linskens 1974, Turner 1984). The instant forming of pollen tubes, similar to those of germinating pollen, but more suddenly, is called pseudogermination (Stanley and Linskens 1974). Nectar is sometimes stored in the crop of insects and may stimulate the germination or pseudogermination of pollen grains in the crop. Larvae of megachilid bees *Osmia cornuta* are fed on pollen–nectar provisions. The nectar may provide conditions for pseudogermination: protrusions of cytoplasm were observed in some stored pollen types and digestion of these was more complete than in pollen that did not show these protrusions. These protrusions might be a preparation for the digestion of pollen in the gut of these larvae (Suarez-Cervera et al. 1994). The digestive enzymes in the gut can then act on the pollen tube without needing to penetrate the exine.

Hoverflies (Diptera: Syrphidae) require both nectar and pollen in their diets. Pollen remains intact until it reaches the midgut. Here most of the pollen grains extrude their contents through a germination pore, in a manner reminiscent to pseudogermination. When these grains reach the hindgut they are empty (Holloway 1976, Gilbert 1981, Haslett 1983). A large sac-like diverticulum is found in false blister beetles, Oedomeridae, where nectar is stored, and this may induce germination (Arnett 1968, Samuelson 1994). Contents of pollen grains were shown to gradually extrude in the midgut of *Stenostoma coerulea*, being mainly empty in the hindgut (Crowson 1981). Only initial stages of germination of pollen grains were observed in adult honeybees *Apis mellifera* after the partial modification of the pollen grains by digestive enzymes

(Klungness and Peng 1984, Peng et al. 1985, 1986). Dobson and Peng (1997) observed the gradual extrusion of pollen protoplasm through the germination pores in the gut of *Chelostoma florisomne* larvae.

According to Turner (1984) nectar may also be present in the alimentary canals of some flower-feeding marsupial species. She reported that ingested *Banksia* pollen was 95-100% digested by the honey possum *Tarsipes rostratus*, and found cytoplasmic protrusions similar to pollen tubes in the lower stomachs, although Richardson et al. (1986) in their studies of *T. rostratus* did not observe protrusions in the gut pollen.

Various species of birds have been found to consume pollen while imbibing nectar. The simultaneous intake of nectar may result in pollen germination and thus digestion. Some Darwin's finches digest approximately 90% of pollen grains in this way (Grant 1996). Lesser double-collared sunbirds, *Nectarinia chalybea,* digest only 19% of ingested pollen grains, but this is enough for pollen to be a useful nitrogen source for these birds since they have exceptionally low nitrogen requirements, 6.8 mg N day^{-1}. Only 4% germinating pollen grains were observed, not enough to have an effect on digestion (Van Tets and Nicolson 2000). On the other hand, no germination of *Banksia* pollen grains occurred upon consumption with a sugar solution by the New Holland honeyeater, *Phylidonyris novaehollandiae* (Paton 1981). Nectar found in the stomachs of some nectarivorous bats provides conditions for pseudogermination (Howell 1974). The pteropid bat, *Syconicteris australis* was found to digest about 50% of ingested *Banksia* and *Callistemon* pollen grains, and when supplied with pollen as the sole source of protein this bat was able to maintain a positive nitrogen balance. However the

exact mechanisms used by bats to digest pollen have not yet been established (Martinez del Rio 1994).

1.4 Description of plants

Pollen dispersal can be either abiotic, usually by wind, or biotic, involving animal pollinators. In anemophilous plants flowers are usually situated high on the plant and produce large quantities of small, light and dry pollen with a smooth surface (Ackerman 2000). The pollen is exposed to the air upon anther opening and carried away by the wind. Unlike entomophilous plants these plants do not have to wait for the arrival of insect vectors in order to achieve pollination, even though the large quantities of pollen may attract insects in search of food (Faegri and Van der Pijl 1979): for example *Astylus atromaculatus* on maize. This type of pollination is considered random, imprecise and wasteful, neither as efficient nor as effective as animal pollination (Rebelo 1987).

An example of an anemophilous species is maize, *Zea mays* (Graminaceae), which originated in the Americas. The main stalk terminates in a male inflorescence that is made up of many small flowers, the spikelets, each bearing anthers. The female inflorescence is carried separately on the stalk enclosed in modified leaves. Maize plants produce large amounts of pollen (Nepi et al. 2001). Pollen shed in the morning contains more water than pollen shed at noon (Barnabas and Rajki 1981). The composition of maize pollen has been given as 20% protein, 37% carbohydrates, 7% lipids, 6% water, 3% ash, but this leaves a high percentage, 31%, of undetermined

material (Todd and Bretherick 1942). Another anemophilous species is grain sorghum, *Sorghum vulgare* (Graminaceae), native to Asia and Africa, and a grass similar to maize in vegetative appearance. Sorghum flowers are borne on a head or panicle, and the flowers that begin to open are pollinated soon after the panicle has completely emerged. Pollen shedding begins at the top of the panicle and progresses downward. The sorghum pollen grains are similar in appearance to those of maize except that the grains are smaller.

Flowers of entomophilous plants generally are large and colourful, with pollen being well exposed and readily available to insect vectors (Frankel and Galun 1977). A good example is the sunflower *Helianthus annuus,* an annual crop plant native to southwestern America, which belongs to the largest plant family, the Asteraceae (in which beetle pollination is common). This plant usually flowers in midsummer and pollinators, of which the honeybee *Apis mellifera* is regarded as the most important, are needed to achieve sufficient seed set (Du Toit and Holm 1992). The sunflower is composed of 1000 to 4000 individual florets surrounded by showy yellow sterile flowers, the outer or ray florets. These ray florets serve as a visual attraction, while the less conspicuous florets provide pollen and nectar, the energy reward. Bees collect the pollen freely along with nectar at the flower base. Sunflowers produce large amounts of pollen and nectar (Minckley et al. 1994, Kamler 1997).

1.5 The spotted maize beetle, *Astylus atromaculatus*

The family Melyridae, containing many brightly coloured beetles, is divided into four subfamilies, of which the Dasytinae, Melyrinae and Malachiinae are most important in

southern Africa (Scholtz and Holm 1985). The spotted maize beetle *Astylus atromaculatus* is the most commonly encountered member of the Dasytinae and attracts attention because of its mass aggregations on various plants. These beetles are native to South America and were introduced to South Africa by accident (Annecke and Moran 1982). The main distribution of *A. atromaculatus* in South Africa seems to be in Mpumalanga, North West Province, Free State and Gauteng (Pick 1996).

Adult beetles are 12-15 mm long and slender, with black markings on yellow elytra which cover the entire abdomen. They show a seasonal occurrence: adults appear in mid December from pupal cells underneath the ground and numbers increase rapidly, with the most severe infestation in January and February. Thereafter numbers decrease in April, with almost no beetles present in May. At first they can be found on the leaves of young maize and sorghum plants and on certain grasses and weeds. As soon as maize pollen becomes available the beetles move over to the maize fields where they cause damage to the maize plumes, cobs and kernels. They also damage sorghum by feeding on the anthers, flowers and developing grains (Drinkwater et al. 1997). *Astylus atromaculatus* utilizes at least 24 plant species including agricultural plants, trees, weeds, flowers and grasses (Esterhuizen 1997).

The larvae can cause considerable damage to seeds, thereby reducing germination with a reduced plant stand as a result. The damage to maize, groundnuts, sorghum and sunflower seeds developed into a serious problem and during the 1970s effective control measures became necessary (Pick 1996). Currently the spotted maize beetle is regarded as a "minor and sporadic pest of maize and several other crops" (Annecke and Moran 1982). However these beetles are also beneficial: they feed on the spores of rust

and the fungi causing leaf blight on sunflowers (Drinkwater 1997) and also act as significant pollinators on sunflowers (Du Toit 1990).

Apart from the damage mentioned above, the beetles are poisonous. The toxic substance is unidentified but appears not to be cantharidin (Kellerman et al. 1972). Livestock deaths were reported from the Marico district (Northwest Province) in 1970 after cattle consumed large numbers of spotted maize beetles (Kellerman et al. 1972), and again in 1992 on the Springbok Flats (Drinkwater 1997). Guinea fowl may be the only possible natural enemies of these beetles since the poisonous substance does not affect them (Esterhuizen 1997).

1.6 Aim of this study

Even though beetles pollinate a remarkable 88.3% of all flowering plants (Buchman and Nabhan 1996), little is known about pollen feeding in beetles. Comparative work on pollen digestion is rare and studies have focused on digestion of different pollens by different animals and, in insects, even different life stages. Therefore one can only agree with Roulston and Cane (2000) that "...literature on pollen digestion contains more variables (species examined and pollen characteristics) than equations (experimental observations)", with the result that studies on pollen digestion remain inconclusive.

To help move the study of pollen digestion from observation to hypothesis testing, the aim of this study was to look at one insect species, the spotted maize beetle *Astylus atromaculatus*, utilizing two very different kinds of pollen: that of maize, *Zea mays* and

sunflower, *Helianthus annuus*. Few studies (see Holloway 1976) have examined the digestion of anemophilous pollen by insects. I further compared the digestion of one pollen type (maize) by two different insect species, the spotted maize beetle and the honeybee, *Apis mellifera*.

Questions addressed by this study:

1. Which pollen is digested more efficiently: that of sunflowers, *Helianthus annuus* (an entomophilous species), or that of maize, *Zea mays* (an anemophilous species)? Both these plants are economically important crops utilized by the spotted maize beetle.

2. Does the spotted maize beetle, *Astylus atromaculatus*, use osmotic shock as the method of pollen digestion?

3. Since Kroon et al. (1974) proposed osmotic shock as the mechanism of pollen digestion used by honeybees, *Apis mellifera,* I compared the method and efficiency of digestion of maize pollen by both honeybees and the spotted maize beetle.

Chapter 2

The role of osmotic shock in digestion of maize and sunflower pollen in the spotted maize beetle, *Astylus atromaculatus* (Melyridae)

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2.1 Introduction

The "mess-and-soil" pollination by beetles has been considered largely accidental (Faegri and Van der Pijl 1979) but they are nevertheless responsible for the pollination of a remarkable 88.3% of all flowering plants (Buchmann and Nabhan 1996). Beetles may consume nearly all floral parts, including pollen and nectar (Gottsberger 1990), and are attracted to flowers for food, safe environments for mating and egg laying and other rewards that include oils and perfumes (Kevan and Baker 1983, Rebelo 1987, D'Arcy 1996, Buchmann and Nabhan 1997, Bernhardt 2000).

The pollen wall presents an "indigestible" or even "chemically resistant"(Stanley and Linskens, 1974) obstacle that pollen feeders need to overcome in order to reach the nutritious cytoplasm. Utilization of pollen grains presumes either digestion by diffusion through pores or the destruction of the outer grain wall. The variation in size, behavior and physiology among pollen feeders leads to an interesting variety of methods used to digest pollen. Some of the methods used are rare and others are difficult to distinguish.

1) *Mechanical damage:* It is suggested that beetles use their chewing mouthparts to mechanically damage pollen grains. Grinding mandibles are found in some pollen feeding beetles (Crowson 1981, Barth 1985, Thien et al. 1985, Samuelson 1994, Bernhardt 1996), but Roulston and Cane (2000) failed to find any study demonstrating that whole pollen grains entered the mandibles and crushed pollen left them. Mechanical damage to pollen grains can occur in the gut as a result of trichomes ingested together with grains (Rickson et al. 1990).

2) *Piercing and sucking*: Flower thrips (Thysanoptera) are known to pierce and suck out the contents from pollen grains (Kirk 1984, 1987).

3) *External digestion:* Some nymphalid and papilionid butterflies from the Costa Rican rainforests gather pollen on the proboscis, which is then wetted with a clear liquid exuding from the proboscis tip and kneaded through the coiling and uncoiling of the proboscis. The pollen grains swell and contents start to leak out through the pores, enabling the butterflies to imbibe the nutrients without swallowing the grains (Gilbert 1972, De Vries 1979). Similar pollen processing behavior was found in the eucalypt nectar fly, *Drosophila flavohirta* (Nicolson 1994).

4) *Digestive enzymes:* Enzymes can be used either to dissolve the pollen wall or to penetrate the pollen wall through pores and process the contents inside the grain. Digested material then exudes out through the pores to be absorbed in the gut (Simpson and Neff 1983). The enzyme exinase is found only in some collembolans, which are capable of breaking down the exine and fully digesting the pollen grains (Scott and Stojanovich 1963).

5) *Pseudogermination:* The instant forming of pollen tubes, similar to those of germinating pollen, is called pseudogermination (Stanley and Linskens 1974). Nectar is sometimes stored in the crop of insects and may stimulate the germination or pseudo-germination of pollen grains in the crop of, for example, hoverflies (Gilbert 1981, Haslett 1983).

6) *Osmotic shock*: This may be an interesting mechanism of pollen digestion. When pollen grains are transferred from high to low osmotic concentration they may open at the pores or burst, allowing the protoplasm to subsequently stream out. Kroon et

al. (1974) proposed that this might occur in the gut of pollen feeders such as honeybees.

Little comparative work has been carried out on pollen digestion: studies have focused on digestion of different pollens by different animals and, in insects, even different life stages. The aim of this study was to look at one insect species, the spotted maize beetle, *Astylus atromaculatus*, utilizing different kinds of pollen: maize, *Zea mays* (an anemophilous species) and sunflower, *Helianthus annuus* (an entomophilous species). The spotted maize beetle *Astylus atromaculatus* (Melyridae, subfamily Dasytinae) is native to South America and was introduced to South Africa by accident. It is regarded as a minor and sporadic pest of maize and several other crops including sunflower (Annecke and Moran 1982). Du Toit (1990) demonstrated that these beetles are also beneficial as pollinators of sunflowers. Adult beetles are known to be primarily pollen feeders and seasonally attract attention with their mass aggregations.

Questions addressed by this study were, firstly, to determine which pollen is digested more efficiently: that of sunflowers, *Helianthus annuus* or that of maize, *Zea mays*. Secondly, which method of pollen digestion does the spotted maize beetle use? Mechanical and chemical mechanisms were considered, with particular emphasis on the role of osmotic shock in digestion of maize pollen, by both maize beetle beetles and honeybees.

2.2 Materials and Methods

2.2.1 Morphological examinations: pollen and beetles

Most insect pollinators are typically covered with a dense coat of hairs that have a large number of teeth and hooks to which pollen can adhere. Furthermore they have external adaptations to carry pollen such as setae, combs, baskets etc. It was decided to investigate the presence of such structures and the location of pollen grains on the body of *Astylus atromaculatus*. Thirty spotted maize beetles were randomly collected from both maize and sunflower plants in the Bronkhorstspruit district (28°39'E, 25°54'S) in Gauteng Province and immediately killed with ethyl acetate. Handling was limited to minimize the accidental removal of pollen grains from their bodies. The beetles were left to dry for 4 days at room temperature, then mounted with the aid of conductive carbon cement (CCC) on SEM stubs and sputter coated with gold using a Polaron E5200 sputter coater (Watford, England). Specimens were examined with a JEOL 840 Scanning Electron Microscope (Tokyo, Japan) at the Laboratory for Microscopy and Microanalysis at the University of Pretoria.

Mouthparts are of particular interest in the consumption and digestion of pollen, and the proventriculus has been shown to be involved in pollen filtration in some insects. The mouthparts and proventriculus of ten spotted maize beetles were dissected in a fixative consisting of 2.5% glutaraldehyde and a sodium phosphate buffer (pH 7.4). The proventriculus was cut open. Fixation was carried out for 1 h. Material was rinsed in the same buffer as used in the fixative for 3 x 10 min. Post fixation was achieved with 1% aqueous osmium oxalate for 1 h and then rinsed in three changes of distilled water

for 10 min each. Material was dehydrated in an ethanol series consisting of 30, 50, 70 and 90% solutions of ethanol in distilled water for 10 min in each. The 100% ethanol was repeated 3x before critical point drying using carbon dioxide in a Polaron critical point drier. Material was mounted on SEM stubs using CCC and sputter coated with gold and viewed with the JEOL 840 SEM.

Additional dissections were done in order to examine the gross morphology of the alimentary canal.

2.2.2 Extraction Efficiency

Fresh pollen was hand-collected from 10 randomly selected maize and sunflower plants to be used as reference pollen for comparison with pollen in the gut and faeces of the beetle. Fresh pollen was stained with a drop of cotton lactophenol blue and counterstained with basic fuchsin. Cotton lactophenol blue stained the cytoplasm dark blue but the cell walls were left unstained. Basic fuschin stained the cell wall pink. On a microscope slide a cube of gelatin jelly (2 x 2 x 2 mm) (Wooller et al. 1983) was melted and a drop of pollen mixture put into the gelatin,which was then sealed with paraffin wax and a coverslip to ensure a more permanent slide. This was repeated with pollen from each of the plants. Using a light microscope at 20x and 40x magnification, 100 pollen grains from each microscope slide were counted and evaluated as either full, half-full or empty. A full grain was defined as one that contained 51-100% of its contents and was similar in shape and contents to the reference pollen. In a half full grain 50% and less of the contents were present and in an empty grain all of the

contents were removed. The number of empty grains is important in calculations of extraction efficiency.

In order to determine extraction efficiency of pollen, 150 spotted maize beetles were collected from maize plants in the Bronkhorstspruit district (28°39'E, 25°54'S) and from sunflowers in the Delmas district (28°39'E, 26°08'S) during March and April 2001. Ninety of these beetles were killed immediately and frozen, while the rest were kept alive.

Thirty beetles each were allowed to feed on fresh maize tassels and sunflowers. After feeding for three hours the beetles were placed individually in vials and fresh faecal pellets were collected.

This procedure was repeated with spotted maize beetles collected from sorghum, *Sorghum vulgare* (Graminaceae), another anemophilous species, in the Bronkhorstspruit district during February 2002. Faecal pellets were placed in a drop of distilled water on a microscope slide and gently mashed to disintegrate the pellets. The sample was stained with cotton lactophenol blue, counterstained with basic fuchsin and sealed with gelatin jelly and a cover slip. Once again 100 grains were scored on each slide with respect to their condition (30 slides per plant species).

Dissections were performed under a dissection microscope in a small Petri dish with a layer of black wax on the bottom. Beetles were pinned to the wax, ventral side down, and both the elytra and membraneous wings removed. The midgut and hindgut were dissected out of 30 beetles each that fed on maize, sunflowers and later on sorghum.

The contents of the midgut were removed by rupturing the walls of the midgut, while contents of the hindgut were gently extruded. These gut contents were transferred to microscope slides with a micropipette, stained with a drop of cotton lactophenol blue and counter stained with a drop of basic fuchsin. Basic fuchsin stains the exine of pollen grains pink and is useful for counting thin walled empty pollen grains such as maize and sorghum, which are otherwise difficult to observe. These samples were sealed with gelatin jelly and a cover slip and examined under a light microscope. One hundred pollen grains were scored in relation to their condition (full, half-full or empty as described above for fresh pollen) on each slide. Ninety slides (the two gut regions and faeces) were evaluated for beetles feeding on each plant species. Representative digital imaging photographs of pollen grains in various regions were taken with a Nikon DXM 1200 digital camera (Nikon Corp, Tokyo, Japan).

All fresh pollen samples contain grains that are either completely or partially devoid of their contents. This is a factor that needs to be taken into consideration in extraction efficiency calculations. The following formula was used for extraction efficiency (% digested) (Brice et al. 1989, Herrera and Martinez del Rio 1998).

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

Statistical comparisons were made using a non-parametric Kruskal-Wallis one-way Analysis of Variance (ANOVA) of ranks cited as ($H_{(df,n)}$) and the Mann-Whitney U test

cited as (Z , Zar 1984). For all statistical analyses the level of significance was $P \leq 0.05$.

2.2.3 Osmotic shock: *in vitro* experiments using maize pollen

Apart from considering mechanical mechanisms as a possible method of digestion, chemical options were also investigated. Kroon et al. (1974) proposed osmotic shock as a possible mechanism of pollen digestion in honeybees, after subjecting pollen first to honey (simulating high osmotic pressure in the crop) and then to insect Ringer (low osmotic pressure in the midgut). *In vitro* experiments were carried out by subjecting fresh maize pollen to changes in osmotic concentration. After extensive pilot studies, sucrose (20%, 40% and 60% w/w) and a 50:50 glucose and fructose solution (20%, 40% and 60% w/w) were the solutions selected. It is assumed that sucrose is hydrolyzed to equal amounts of glucose and fructose. Fresh maize pollen was hand-collected from 10 randomly selected plants from each of two different cultivars, PAN 6256 and PAN 6568, on the farm Puntlyf in the Bronkhorstspruit district (28°39'E, 25°54'S). These are standard agricultural cultivars planted in Gauteng Province. Fresh pollen was used immediately.

Pollen (about 0.05 g) was placed in 0.5 ml of the various sucrose and hexose solutions. A relatively high concentration of pollen was used to minimize settling of pollen grains. After 15 min in the sugar solution 1.5 ml distilled water was added to dilute the sugar solution. After another 10 min a sample of this pollen mixture was transferred with a micropipettte to a microscope slide and 100 grains were immediately scored as full, half-full or empty as previously described. Control pollen was treated with distilled

water only. Once again 100 grains were evaluated using a light microscope at 20x and 40x magnification. No staining methods were used, in order to minimize variables that may influence the reaction of pollen grains.

The effects of different concentrations and sugar types on pollen of the two maize cultivars were statistically compared using the non-parametric Kruskal-Wallis one-way Analysis of Variance (ANOVA) of ranks and the Mann-Whitney U test (Zar 1984).

Eisikowitch and Woodell (1975) demonstrated the bursting of *Armeria maritima* pollen grains in distilled water unless preexposed to high relative humidity, while Knox (1993) demonstrated that grass pollens rupture by osmotic shock in rainwater. Therefore to further investigate osmotic shock, *in vitro* experiments were carried out on maize pollen in distilled water. Pollen was randomly collected from 10 different maize plants each from four standard cultivars PAN 6256, PAN 6332, PAN 6568 and 4549 BTG. Pollen (about 0.05 g) was placed in 2 ml of distilled water and after 10 min a sample of this mixture was placed on a microscope slide and 100 grains were immediately scored as being full, half full or empty as for extraction efficiency.

Statistical comparisons were made between the percentage of empty or ruptured grains for the different cultivars using the non-parametric Kruskal-Wallis one-way Analysis of Variance (ANOVA) of ranks and the Mann-Whitney U test (Zar 1984).

A small amount of maize pollen was placed in approximately 0.05 ml water on a microscope slide with a coverslip and sealed with colourless nail varnish. Slides were viewed on a Zeiss Axiovert 200 inverted microscope (Carl Zeiss, Göttingen, Germany)

using 10x and 20x objectives. Micrographs demonstrating reactions of pollen grains were immediately obtained employing a Nikon DXM 1200 Digital camera (Nikon Corp, Tokyo, Japan).

2.2.4 Feeding experiments comparing pollen digestion in beetles and bees

Observations by Klungness and Peng (1984) suggested that initial pollen digestion might take place in the anterior midgut of honeybees and that osmotic shock may be one of the mechanisms involved. It therefore seemed relevant to compare the digestion of maize pollen by honeybees and the spotted maize beetle.

Newly emerged worker bees were caught and confined in bioassay cages with two glass sides (12.5 x 15 x 8 cm). Cages with 40 bees each were placed in constant darkness in an incubator at 30 ± 1°C and the bees starved overnight to facilitate clearance of their guts. According to Corbet and Willmer (1980) bees show a preference for nectars with 50-60% sucrose and seldom collect more concentrated nectars. Therefore fresh maize pollen was suspended in a 50% sucrose solution (0.25 g pollen per 10 ml solution) and supplied *ad libitum* inside the cages for periods of 1, 2 and 3 h (n = 40 bees for each). After each time period 20 bees were collected and killed and the gut dissected out as for beetles (section 2.2.2). The 60 remaining bees were dissected only for crop contents.

Beetles were randomly collected from maize plants on the farm Witfontein in the Bronkhorstspruit area (28°39'E, 25°54'S) and placed in a glass container with a lid (22 x 16 x 15 cm) and starved overnight. Beetles were then supplied with fresh maize

pollen suspended in a 50% sucrose solution (0.25 g pollen per 10 ml solution) and allowed to feed for 1, 2 and 3 h (n = 20 beetles for each time period). Thereafter beetles were captured, killed and the gut dissected out.

Pollen grains in midgut contents of bees and beetles were transferred with a micropipette to microscope slides, stained with cotton lactophenol blue, counterstained with basic fuchsin and the sample was sealed with gelatin jelly and a cover slip. For the two insect species 100 grains from each of the 120 slides were evaluated.

A non-parametric Kruskal-Wallis one-way Analysis of Variance (ANOVA) of ranks and the Mann-Whitney U test (Zar 1984) were used to make statistical comparisons between extraction efficiency for different time intervals between and within insect species.

2.3 Results

2.3.1 Morphological examinations: pollen and beetles

Sunflower pollen is round, small in size (29 µm in diameter) and has an ornamented exine. The pollen grains are sticky due to the pollenkitt surrounding them and have a tendency to form clumps (Fig. 1a, b). By contrast maize pollen (97 µm in diameter) is larger, with a smooth surface and no pollenkitt (Fig. 1c, d). Only one aperture is evident in maize pollen while none are visible in sunflower pollen (Fig. 1c).

Pollen of both plant species was transported on the body surfaces of the beetles. In beetles captured on maize flowers pollen was found only in the dorsal regions, at the junction between thorax and abdomen and on the anterior parts of the elytra (Fig. 2a). Sunflower pollen was much more abundant on beetles than maize pollen. Numerous pollen grains were attached to the hairs on beetles captured on sunflowers and grains were observed around the eyes, on the legs and in the grooves on the elytra of the beetles (Fig. 2 b, c) (see also Fig.9).

Spotted maize beetles have short, prognathous mouthparts enabling them to probe into flowers for pollen (Fig. 3a). The mouthparts, shown in ventral view in Fig. 3b, consist of a labrum (dorsal), two pairs of jaws - the maxillae and the mandibles (latero-ventral), a labium (ventral) and a median tonguelike hypopharynx. The labrum (upper lip) is a hairy flap that closes the pre-oral cavity in front with its inner surface forming the roof of the oral cavity. On this inner surface a hairy groove is visible (Fig. 4a). The mandibles are sclerotized and apically pointed. Their molar surface has parallel, fine-toothed ridges with valleys between (Fig. 4b) and bears on the outside long setae, resembling a brush-like structure (Fig. 4c). Ventral to the main cutting edge, a hairy mandibular lobe is present (Fig. 4d). The maxillae are situated between the mandibles and labium and consist of a hairy, brushlike inner lacinia and an outer galea (maxillary brushes). Lateral to the galea a maxillary palp is present (Fig. 4e). The labium (lower lip) closes the pre-oral cavity to the rear and consists of hairy lobes: the inner glossae and outer paraglossae with labial palpi that are attached laterally to the prementum (Fig. 4f).

Dissections of the alimentary canal (Fig. 5a) showed that the mouth cavity opens into the oesophagus with a proventriculus, but no crop, between the oesophagus and midgut. The proventriculus is a pear shaped bulb, with a hairy internal armature, consisting of long filiform hairs in rows (Fig. 5b, c). In honeybees the proventriculus serves as a filter for nectar (Peng and Marston 1986). The midgut is a straight undifferentiated transparent tube with the outer surface set with regenerative nidi. Inside the midgut a peritrophic membrane encloses the solid food material. Posteriorly the midgut becomes narrower and is followed by the hindgut, forming a loop, and anus.

2.3.2 Extraction efficiency

Fresh hand-collected pollen from the food plants was used as a control for comparison with pollen in the mid- and hindgut as well as faeces of the beetles and was treated the same way as ingested pollen. The cytoplasm stained blue with cotton lactophenol blue (e.g. Fig. 6a) while the exine stained pink with basic fuschin (e.g. Fig. 6c). Most of the maize pollen grains were already ruptured and empty (Fig. 6a) in the anterior midgut of spotted maize beetles. The line of rupture bore no relation to the position of the single pore. Pollen grains became more empty and compressed in the hindgut and the exines of some empty maize pollen grains remained intact (Fig. 6c). A few pollen grains remained full. In faecal samples pollen grains were empty (Fig. 6e) with few grains remaining either full or half full.

After the observations of ruptured maize pollen grains, the extraction efficiency of pollen from sorghum (*Sorghum vulgare*), also an anemophilous plant, was also measured. Sorghum is a grass similar to maize in vegetative appearance with pollen

similar to that of maize except that it is smaller (31 μm in diameter). Sorghum pollen grains were also observed to be ruptured and empty in the anterior midgut and extraction of the contents continued with passage of pollen through the gut (not shown).

Sunflower pollen that morphologically resembled the control pollen was found in the anterior midgut of spotted maize beetles (Fig. 6b). A high percentage of these grains were already empty with all the exines intact, and no protrusions were observed. Once again more pollen grains were empty in the hindgut and faecal samples with a few grains remaining half full or full (Fig 6d, f).

The relatively low percentage of empty pollen grains present in fresh pollen of maize, sunflower and sorghum (Fig. 7) was taken into consideration in the determination of extraction efficiency.

Extraction efficiency was compared for the midgut, hindgut and faeces (Fig. 7). The contents of a very high percentage of grains were already extracted in the anterior midgut: 74% for maize, 72% for sunflower and 67% for sorghum. This percentage increased slightly in the hindgut, but decreased in faeces of beetles feeding on sunflower and sorghum. Extraction efficiency for pollen of the three plant species was significantly different in the midgut ($H_{(2, 90)} = 17.31$, $p < 0.001$), but not in the hindgut ($H_{(2, 90)} = 2.27$, $p = 0.322$) and was significantly different in the faeces ($H_{(2, 90)} = 57.42$, $p < 0.001$).

The same extraction efficiency data was compared with respect to species (Fig. 8). Extraction efficiency of pollen in the midgut, hindgut and faeces was then compared for

each plant species. Significant differences were found for all three plant species except for maize pollen where there was no significant difference between the hindgut and faeces ($z = 1.54$, $p = 0.123$). SEM examinations of sunflower pollen on beetles offered a possible explanation for the decreased extraction efficiency in faecal samples: fresh pollen sticking to the hairs around the anus, so that faeces would become 'contaminated' with fresh pollen (Fig. 9a, b). This would explain the decrease in percentage extraction efficiency in the faecal samples from beetles feeding on sunflower and sorghum pollen.

2.3.3 Osmotic shock: *in vitro* experiments using maize pollen

Maize pollen grains from two different cultivars, PAN 6568 and PAN 6256, were immersed in different concentrations of sucrose and glucose:fructose for ten minutes before distilled water was added to the mixture. Grains became shrunken, and as soon as water was added they immediately rehydrated. The reaction of pollen grains to this treatment was immediate: some grains remained intact and full (Fig. 10a), the contents of some grains immediately started to stream out through pores (Fig. 10b, c) and others burst (Fig. 11a - c). These reactions occurred within in seconds. Protrusions resembling pseudogermination were not observed in either the sucrose or glucose:fructose solutions.

The two cultivars, PAN 6568 and PAN6256 discussed in more detail: The percentage of empty or ruptured pollen grains of cultivar PAN 6568 (i.e. those that either ruptured or lost their contents through the pores) decreased from 77% to 62% as sucrose concentration increased from 20 - 60% w/w. Almost exactly the same decrease, from

78% to 62%, was observed with increased glucose:fructose (20 - 60% w/w) concentrations (Fig. 12). In the control solution (distilled water) 80% of grains were empty due to the outflow of contents. The percentages of empty pollen grains were mostly significantly different ($H_{(6, 70)} = 42.23$, $p < 0.001$) between the different sucrose and glucose: fructose concentrations, except that the percentages in 20% sucrose and 20% glucose:fructose were not significantly different to the control treatment (Table 1). For cultivar PAN 6256 the same tendency of a decrease of empty or ruptured grains with an increase in sugar concentration was observed: for sucrose it decreased from 82% to 57% and for the glucose:fructose the percentages decreased from 86% to 61% (Fig. 12). These differences were also significant ($H_{(6, 70)} = 60.30$, $p < 0.001$). In the control solution (distilled water) 90% of grains were empty due to the outflow of contents. The percentages of empty grains between the different sugar concentrations were all significantly different to one another (Table 2) with only that of 20% sucrose not different from 40% glucose: fructose and 60% sucrose not different from 60% glucose: fructose. Only the 20% glucose: fructose did not have a significant percentage of empty of ruptured grains compared to the control treatment. The number of empty or ruptured grains differed significantly between the two cultivars in distilled water ($H_{(1, 20)} = 13.62$, $p < 0.001$), 60% sucrose ($H_{(1, 20)} = 5.70$, $p = 0.017$), 20% glucose:fructose ($H_{(1,20)} = 5.01$, $p = 0.025$), and 40% glucose:fructose ($H_{(1, 20)} = 12.15$, $p = 0.001$).

The effect of distilled water on pollen from four different maize cultivars, PAN 6256, PAN 6332, PAN 6568 and 4549 BTG were looked at in more detail. The reaction of pollen grains to water was immediate: grains either burst, remained full, or the contents started to flow out through the pores. Pollen grain bursting was completed in a matter

of seconds but the outflow of contents continued over time. In grains that burst the line of rupture was not in relation to the position of the pore.

The average number of grains that remained full after 10 minutes in all four cultivars tested was 24%, while the contents of 56% grains start to flow out through pores and about 21% burst (Fig. 13). The percentage of burst grains of Cultivar PAN 6568 was significantly less than in the other three cultivars ($H_{(3,\ 40)} = 11.96$, $p = 0.008$).

2.3.4 Feeding experiments comparing pollen digestion in beetles and bees

Results of maize pollen feeding experiments differed dramatically between bees and beetles. Very few maize pollen grains (range 1 – 4 grains) were found in the crops of 60 bees dissected for crop contents. The crops were full, but only the 50% sucrose solution was present. Pollen grains found in the midgut of honeybees after different time periods were counted as full, half full or empty (Fig. 14). The exines of empty grains were still intact: no ruptured grains were observed, in contrast to the beetles, nor were there any signs of protrusions from the pollen grains.

Even though spotted maize beetles lack a crop, the majority of maize pollen grains found in the midgut of these beetles were empty and ruptured. Once again the line of rupture bore no relation to the position of the single pore.

Most digestion in beetles (71%) occurred in the first hour after feeding. Within three hours of feeding, the proportion of digested pollen grains increased to 80%. These

values can be compared with 15% in bees after the first hour and 21% after three hours (Fig. 14). Extraction efficiency for the three time intervals was subsequently determined for bees and beetles (to compensate for the number of empty pollen grains in fresh pollen). Extraction efficiency was significantly higher ($H_{(5, 90)} = 76.54$, $p < 0.001$) in beetles. Extraction efficiency was also significantly different between time intervals within each group: for bees ($H_{(2, 45)} = 13.14$, $p < 0.001$) and for beetles ($H_{(2, 45)} = 25.43$, $p < 0.0001$).

2.4 Discussion

In spite of being considered as an economically important pest species the spotted maize beetle *Astylus atromaculatus* is a beneficial pollinator and also an opportunistic pollen feeder that utilizes many available food sources. I shall consider, in turn, which of the various possible mechanisms of pollen digestion are used by the spotted maize beetle digesting pollen of two plant species: maize and sunflower. Secondly, I shall focus on the role of osmotic shock in pollen digestion. Both rainwater and distilled water are known to cause the bursting of pollen grains from certain plant species, therefore the effects of distilled water and different sugar solutions on maize pollen will be assessed. Thirdly, I shall investigate the importance of osmotic shock for pollen digestion by honeybees while by comparing the digestion of pollen of one plant species, maize, by two insect species, maize beetles and honeybees, *Apis mellifera*.

What are the mechanisms of pollen digestion in maize beetles?

Mouthparts of insects vary in structure and function according to diet (Daly et al. 1981) and in beetles, in particular, mandible structure is closely related to feeding habits (Nel and Scholtz 1990). Mann and Crowson (1981) considered the mandibular cracking of pollen grains by certain beetles to be common, but Roulston and Cane (2000) were unable to find a study that demonstrated whole pollen grains entering and crushed pollen leaving the mandibles of beetles. Beetles that use trichomes to crack pollen grains in their gut rather than with their mouthparts have been found in a single instance (Rickson et al. 1990).

Based on their maxillary and mandibular structure, the mouthparts of spotted maize beetles seem to be adapted for pollen feeding but not for pollen chewing. Numerous setae are present on all mouthparts although they are not as dense as in protea beetles that are more specialized pollen feeders (Johnson and Nicolson 2001). The maxillae lack cutting edges and the lobes are dense brushes. The mandibles appear unable to provide sufficient mechanical force to crush and grind pollen grains and have brush-like setae on the outside, together with a very hairy mandibular lobe lying ventral to the cutting edge. This lobe probably serves to pick up pollen grains and might aid in pollen eating itself (Crowson 1981, Nel and Scholtz 1990). Barth (1985) described beetles bearing dense brushes as pollen-sweeping machines with pollen brooms. It is therefore likely that pollen grains are not chewed by spotted maize beetles but rather ingested intact, and no trichomes are ingested with pollen grains that can cause mechanical damage to the grains in the gut. The fact that pollen is ingested intact rules out not only mechanical damage, but also piercing and sucking as well as external digestion as

possible mechanisms of pollen digestion. The only explanation for the presence of ruptured maize pollen grains in the anterior midgut therefore seems to be osmotic shock, while intact sunflower pollen grains in the same part of the gut suggest that another mechanism of digestion may also be used. Undigested exines of both maize and sunflower pollen are present in the faeces, therefore these beetles do not secrete the enzyme exinase, used by collembolans to break down the pollen wall (Scott and Stojanovich 1963).

Reduction or absence of the crop, common in beetle larvae, is generally associated with more or less continuous feeding and rapid food passage. Without a crop there is no place where nectar and pollen can be stored simultaneously long enough for germination to be induced in *A. atromaculatus*. Moreover no pollen tubes were observed in the midgut, so pseudo-germination is ruled out as a possible mechanism of digestion. Many Coleoptera have a well-developed proventriculus between the crop and midgut but this has not been reported in pollen-feeding beetles (Crowson 1981). The proventriculus is a sclerotised and muscular structure, with a sphincter-like valve at the posterior end that in bees serves as a filter extracting pollen from nectar. Peng et al. (1985, 1986) described this process in honeybees: pollen grains are caught in the hairs and lips of the proventriculus and passed into the midgut, while solutions such as nectar are retained in the crop. The flow of food to the midgut is regulated and at the same time backflow of food from the midgut is prevented. The proventriculus seems to be well developed in spotted maize beetles but the exact role it plays in this pollen feeding beetle has not been established yet.

Recent work on pollen digestion by scarab beetles (Johnson and Nicolson 2001) shows that pollen grains are ingested intact and the lipid and protein content is efficiently removed, probably by enzymatic action, during passage through the gut. Enzymes acting on the exines of pollen grains will weaken the exine, resulting in rupturing of grains further along the gut. The contents of maize and sorghum pollen grains consumed by *A. atromaculatus* were already very effectively removed (maize 74% and sorghum 67%) in the anterior midgut. The consistent presence of empty, ruptured maize as well as sorghum pollen grains, in the anterior midgut, indicates that the most likely method of digestion used by spotted maize beetles seems to be osmotic shock and not enzymatic digestion. Even though the contents of sunflower pollen are already very efficiently removed in the anterior midgut (72%), the intact exines of the grains indicate that sunflower pollen is not digested through osmotic shock and that enzymes may play a role.

The role of osmotic shock and pollen hydration in pollen digestion

Kroon et al. (1974) demonstrated *in vitro* that pollen grains may readily burst under certain osmotic conditions. Pollen that passes from the crop to the midgut of honeybees is subjected to a sudden drop in osmotic concentration and the entry of water by osmosis might cause grains to burst. Osmotic concentrations can vary dramatically in the alimentary canal of the carpenter bee *Xylocopa capitata,* with a 5.6 times difference between crop and midgut contents: the osmolality of the crop contents and midgut being 5381 ± 240 (± SE, n = 6) mmol kg^{-1} and 956 ± 68 mmol kg^{-1} respectively (Nicolson 1990). Kroon et al. (1974) hypothesized that osmotic shock was a prerequisite for pollen digestion and their opinion has been accepted in the literature

without confirmation by subsequent studies, in particular without confirmation in vivo (Peng et al. 1986). The pollen used for the *in vitro* studies of Kroon et al. (1974) was a mixed collection of unnamed bee-collected pollen, some stored in a freezer for more than one year. Methods were not explained clearly. Replication of these experiments was difficult since there was no standardization of concentrations or amounts used, neither for pollen nor for the solutions in which it was immersed. Although Kroon et al. (1974) did not describe a control treatment in their experiments, using distilled water as a control treatment seemed appropriate and served as an indication of the response of pollen grains to water.

Pollen grains rehydrate upon contact with water, and pollen of some species, when exposed to water or rain, may burst or lose its contents and become irreversibly damaged (Eisikowitch and Woodell 1975, Corbet and Plumridge 1985, Knox 1993, Dafni 1996, Cruden 2000, Pacini, 2000, Nepi et al. 2001). According to Heslop-Harrison (1979b) the inflow of water into a grain upon rehydration may be rapid, although there may not be a continuous expansion of the grain. The movement of water into the pollen grain will be met with some resistance from the exine and apertural pathways at first, but the apertural resistance will fall as greater areas of intine are exposed. All of these changes cause mechanical stress that has to be sustained by the pollen wall, membranes and protoplast (Bassani et al. 1994, Nepi et al. 2001) and the eventual fate of the pollen grain - swelling or rupturing - depends on this. The water content of pollen grains at the time of dispersal varies among species (Heslop-Harrison 1979b). Thin-walled pollen such as that of *Zea mays* loses water just before and/or just after anther opening and as a result the pollen shape changes. Other pollen with a thicker exine, e.g. that of *Cucurbita pepo*, decreases in volume with water loss but does

not change shape (Nepi et al. 2001). Mature maize pollen has a relatively high water content (45 - 60% wet weight) with a high capacity to absorb water so that it can germinate very quickly (Heslop-Harrison 1979b, Barnabas and Rajlki 1981). Sunflower pollen has a low water content (< 30%), enabling the pollen to adapt to environmental conditions of low humidity and survive longer (Nepi et al. 2001).

Other factors that have an effect on the size, shape and behaviour of pollen grains are temperature and relative humidity (Bassani et al. 1994). Fluctuating ambient temperatures and a decrease in relative humidity will increase osmotic concentration within pollen grains and thus limit water loss, while an increase in relative humidity will result in a decrease in osmotic concentration in the grains (Pacini 2000). Thunderstorms, for example, are associated with heavy rainfall and a decrease in temperature and will therefore result in decreased osmotic concentration in pollen grains. Knox (1993) described a series of events that coincided in Melbourne, Australia, when epidemics of asthma were associated with thunderstorms. All of the patients suffering from these asthma attacks suffered from hay fever due to grass pollens. Allergen-containing particles exist in ragweed and grass pollen, but experiments showed that whole pollen grains could not induce asthma. Knox (1993) demonstrated that osmotic shock caused the rupturing of rye grass pollen washed out of the air by rainwater. This rupturing ensured the release of allergen-containing starch granules into the atmosphere and subsequently induced asthma.

The variation that exists between pollens of different species may also exist between pollens of different cultivars. The study by Peng et al. (1987) on pollen variation in alfafa (*Medicago sativa*) is one of few studies that have focused on pollen variation

within cultivars. Their studies of honeybee pollination of alfafa cultivars were complicated due to the difficulty of identifying the cultivar of the pollen concerned, and that led to an investigation of the possibility of distinguishing among cultivars. In the present study pollen from different maize cultivars did react differently to *in vitro* tests even though only one cultivar was significantly different from the others. All maize cultivars used were hybrid cultivars. PAN 6256, PAN 6568 and PAN 6332 are yellow cultivars while 4559 BTG is a genetically engineered white maize cultivar, containing the *Bacillus thuringiensis* gene. The main differences between these cultivars are the growth period and amount of pollen production. PAN 6256 produces the least pollen; PAN 6568 more pollen; PAN 6332, an older generation cultivar, produces the most pollen; and 4559 BTG also has abundant pollen that is available over a longer period than in other cultivars (A. Pretorius, pers. comm.).

Reactions of maize pollen in distilled water and various sugar solutions are interesting because of the probability of rain during the flowering season in South Africa. Results of this study indicate that maize pollen may rupture in distilled water due to osmotic shock. The osmolality of glucose:fructose mixtures is much higher than that of sucrose solutions with equivalent sugar on a percentage w/w basis (Nicolson 1998, 2002) but neither the sucrose nor the hexose solutions decreased the amount of rupturing compared to that observed in water. This concurs with findings by Dafni (1996) who exposed pollen grains of various plant species to sucrose concentrations (0 – 50%). Pollen of typical summer flowering species burst in both distilled water and low sucrose concentrations, with no bursting in concentrations of 20% and higher. No bursting was observed for species that flower at the end of summer or in the rainy season. Eisikowitch and Woodell (1975) looked at osmotic concentrations to which

pollen grains of a widely distributed coastal plant, *Armeria maritima,* were exposed. These plants grow on salt marshes, maritime rocks and cliffs as well as inland habitats. These plants are exposed to different ecological conditions: flowers of the plants growing on salt marshes are sometimes covered with sea-water, tidal zone flowers are exposed to sea water of varying concentrations while inland flowers are sometimes exposed to rain. They tested the effect of various concentrations of sea water, a series of sucrose concentrations, and distilled water on *A. maritima* pollen. The pollen of these flowers is very tolerant to varying concentrations of sea water but bursts in sucrose solutions up to 50% as well as in distilled water.

Since some pollen grains have the ability to withstand high osmotic concentrations but are sensitive to low ones, it is likely that the osmotic concentration in the midgut of spotted maize beetles may be low enough to induce an osmotic shock to thin-walled pollen grains such as those of maize and sorghum. Even though pollen is not, in the absence of a crop, suddenly transferred from a high to a low osmotic concentration, as described by Kroon et al. (1974). It is important to realize that osmotic shock may not only occur when pollen is transferred from a high to a low osmotic concentration, but also when pollen is transferred from a low to a high osmotic concentration. The bursting of grains may not be the only consequence of osmotic shock: the opening of pollen grains at their apertures and retraction of the cytoplasm from the pollen wall may be others (Cresti et al. 2001).

Digestive efficiency of spotted maize beetles: comparison with honeybees and other animals

Honeybees primarily utilize pollen from entomphilous species, but are also known to utilize pollen from anemophilous species, including many grasses (Poaceae) and anemophilous types of the composites (Asteraceae) (J.O. Schmidt, pers. comm.). It was therefore relevant to compare the digestion of maize pollen by beetles and honeybees. This study illustrated that the mechanism used by bees for digestion of maize pollen is not only different to that of *A. atromaculatus* but also much less efficient. Dramatic differences in digestion were already observed between bees and beetles after the first hour of feeding. Only 15% of maize pollen grains were empty in bees and none were ruptured, even though the pollen grains had been subjected to a change in osmotic concentration (food = pollen suspended in 50% sucrose) during passage from the crop to the midgut. In contrast, the majority of maize pollen grains (71%) were already empty and ruptured in the anterior midgut of spotted maize beetles. Digestion of maize pollen progressed with time but even after three hours neither ruptured grains nor any protrusions were observed in the midgut of bees and the percentage of empty grains (21%) remained low.

Bees do not use osmotic shock or pseudogermination as mechanisms of digestion for maize pollen. Observations by Peng et al. (1985, 1986) on digestion of thin-walled alfafa pollen by honeybees indicated that this pollen did not respond to changes in osmotic concentrations between the crop and the midgut, which also ruled out osmotic shock as a mechanism. They did not find any burst pollen grains in the anterior midgut of the honeybee and reported that dandelion (*Taraxacum officinale)* and alfafa

(*Medicago sativa)* pollen slowly degraded and lost its contents during passage through the midgut, with the pollen wall breaking in the posterior midgut (Peng et al. 1985, 1986). Despite these observations Peng et al. (1986) do not exclude the possibility that some thin-walled pollen may burst to release cytoplasm. If bees do not use osmotic shock to digest maize pollen then they probably do not use it at all. It is more likely that pollen grain contents gradually exuded from the grains through the pores, due to the action of digestive enzymes.

Pollen is known to contain proteolytic enzymes and according to Grogan and Hunt (1979) these enzymes may be active in digestion of pollen protein based on quantities of these enzymes found in both pollen and in bee guts. Crailsheim (1993) illustrated that abdominal temperatures of insects may also affect pollen digestion. Utilization of *Castanea* and *Trifolium* pollen by honeybees remained the same in the midgut but decreased from 44% to 20% and 35% to 16% respectively in the hindgut with an increase in age of honeybees from 1 to 23 days old. Young bees stay near the brood, the warmest place in the hive, while foragers stay on the edges or even outside during the night, thus having lower abdominal temperatures that could reduce the effect of midgut enzymes (Crailsheim 1992). Maize beetles are abundant during the warm summer months; they are basking in the sun whether sitting on maize plumes or on sunflowers. Therefore their abdominal temperatures may be quite high, with increased effectiveness of midgut enzymes that may play a role in the digestion of sunflower pollen.

Table 3 illustrates the problems discussed by Roulston and Cane (2000). Different digestibilities of pollen among plant species as well as different digestive abilities of pollen consumers may be the cause of the considerable variation in digestive efficiency.

The digestive efficiency measured in the anterior midgut of maize beetles (74-80% for maize, 72% for sunflower and 67% for sorghum) is high compared to that seen in most mammals, birds and other insects (Table 3). The digestive efficiency of pollen consumers has been estimated in many studies through collection of faeces and the scoring of pollen grains as full or empty. Digestive efficiency ranges widely from 5 to 100%. However, some of the percentages may be to high because most of these studies have not corrected for the number of empty grains found in fresh pollen, a number that can be substantial. Digestive efficiency may also be underestimated if only completely empty pollen grains are used for the calculation, because animals may still extract nutrients from pollen grains even when they do not completely empty these grains.

Spotted maize beetles are much more efficient at digesting pollen than most birds investigated. In comparison to mammals and other insects, these beetles have the same or even higher digestive efficiency (Table 3). The digestive efficiency of spotted maize beetles is slightly higher for maize pollen, equal for sunflower and less for sorghum pollen than the digestive efficiency found in winter honeybees on sweet chestnut pollen (Crailsheim 1992) and solitary bee larvae on pear pollen (Cresti 2001). Honeybees in the present study had a much lower digestive efficiency than both summer and winter bees investigated by Crailsheim (1993), but this author made no correction for the number of initially empty grains.

Digestive efficiency of pollen in birds is very low compared to that of mammals. A limited amount of literature, however, is available on pollen digestive efficiency in birds. An exception to the low values is the high percentage found for Darwin's finches (>90%; Grant 1996), although this study did not correct for the number of initially

empty pollen grains it still is high. Numerous observations have been made on pollen feeding by birds (Brice et al. 1989), but unfortunately there is only three studies on pollen digestion in birds of which two were concerned with *Eucalyptus* pollen. Although lorikeets are well known as pollen feeders, they have a digestive efficiency of only 5-7% when feeding on *Eucalyptus calophylla* pollen (Brice et al. 1989), compared to the 19% found in nectarivorous sunbirds consuming the same pollen (Van Tets and Nicolson 2000). On the other hand adult, nonnectarivorous cockatiels, also reported to be pollen feeders in the wild, have a digestive efficiency of 18% and their nestlings 38%, for *E. calophylla* pollen (Brice et al 1989). Brice et al. (1989) found digestive efficiencies of lorikeet and cockatiel nestlings to be at least double the values for their parents. This is a contradiction since a more complete digestion of nutrients is normally seen in older birds, in which passage time slows down with age and further development of the gut (Brice et al 1989).

Digestive efficiencies for bats range between 31 and 90%, for African rodents between 58 and 83% and for Australian marsupials between 37 and 100% (Table 3). Except for the study by Turner (1984), studies on Australian marsupials have not included corrections for the number of initially empty grains found, therefore these percentages should be lower. Law (1992) and Herrera and Martinez del Rio (1998) made corrections for the number of initially empty grains in their bat studies. Most of the studies on pollen digestion by mammals have involved Australian mammals consuming *Banksia* pollen, and wide variation in digestive efficiencies has been found. Nectarivorous bats fed a diet containing a single pollen species, *Banksia integrifolia*, were more efficient at pollen digestion than those fed with a diet containing mixed pollen (Law 1992). Herrera and Martinez del Rio (1998) used two species each of

nectarivorous and frugivorous bats in their study. They found a higher digestive efficiency for bats that regularly include pollen in their diets than for bats that only consume pollen seasonally. Values were also higher than for the bats feeding on *Banksia* pollen (53-55%; Law 1992). The digestive efficiency of *Leptonycteris caurasoae* (90%; Herrera and Martinez del Rio 1998) is close to that of the marsupial *Tarsipes rostratus* (95-100%; Richardson et al. 1986) and this level of pollen extraction was achieved with a shorter gut passage time than that of *T. rostratus* (< 2h and 6h respectively). High digestive efficiencies also occur in nectarivorous Darwin's finches (>90%; Grant 1996) but further comparisons are limited since Grant (1996) did not provide any gut transit times. Digestive efficiencies were similar for *Cercartetus nanus* and *Petaurus breviceps* consuming *Banksia* pollen (Turner 1984, Goldingay 1987, Van Tets and Whelan 1997).

African rodents seem to be fairly efficient in their digestion of *Protea* pollen (Van Tets 1997), with values similar to that of some Australian mammals consuming *Banksia* pollen. Both *Banksia* and *Protea* belong to the Proteaceae, a large family common in the southern hemisphere. A high propoertion of the pollen digestion studies presented in Table 3, concern Proteaceae. Among the Proteaceae there are some species that are in flower at various times of the year e.g. *Banksia,* while others e.g. *Protea* generally flower between autumn and early summer. There are a variety of animals, including many species of insects, birds and small mammals that visit the generalized and accessible flowers of the Proteaceae. Knowledge regarding the animal visitors, pollination and pollen consumption for species of the Proteaceae is still incomplete (Collins and Rebelo 1987).

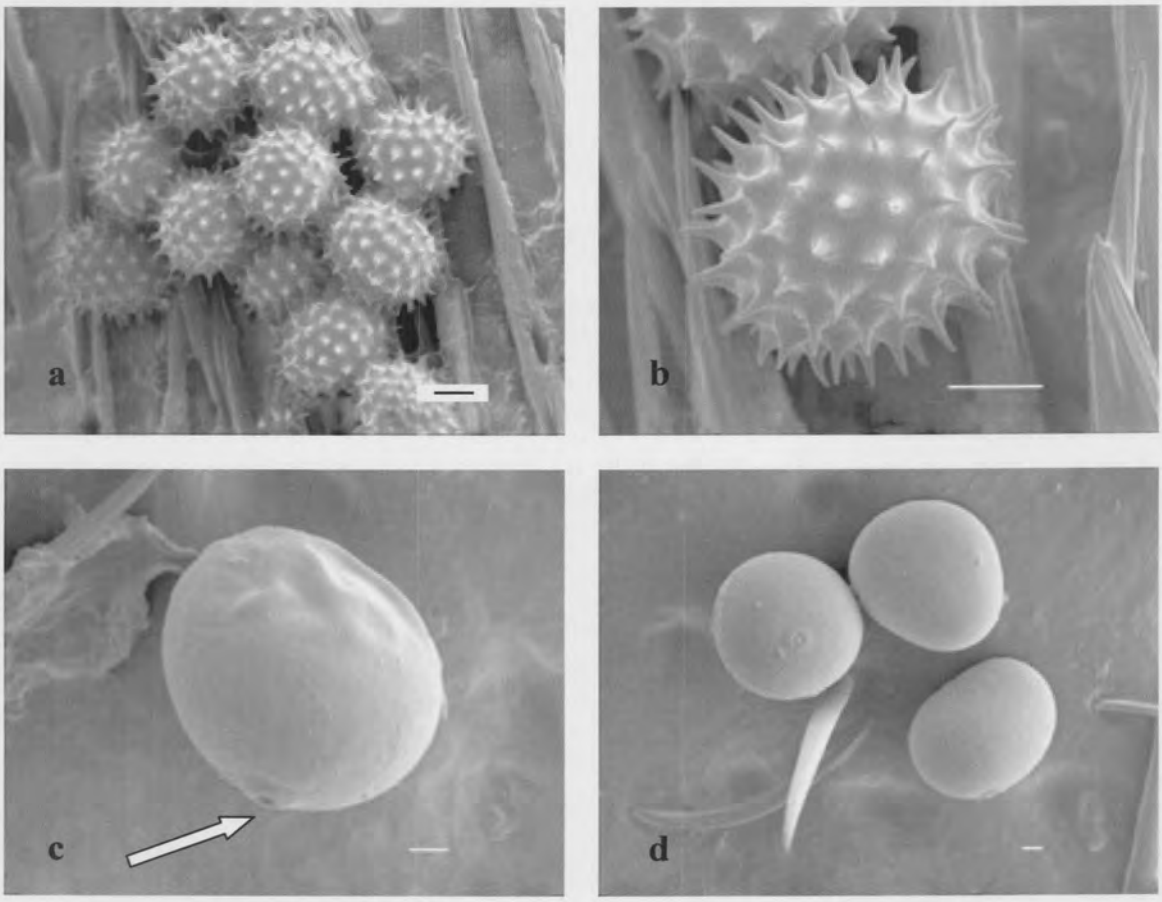


Fig. 1 a - d: Scanning electron micrographs (SEM) comparing the structure and size of fresh sunflower pollen, *Helianthus annuus* (a,b) and maize pollen, *Zea mays*, (c, d) on the spotted maize beetle, *Astylus atromaculatus*. a: Clumps of sunflower pollen glued together by the sticky pollenkitt. b: Enlargement showing the sculptured exine of sunflower pollen. c: Maize pollen grains have a smooth surface. The single aperture (pore) indicated by the arrow. d: Maize pollen grains on the elytra. Scale bars = 10 μm.

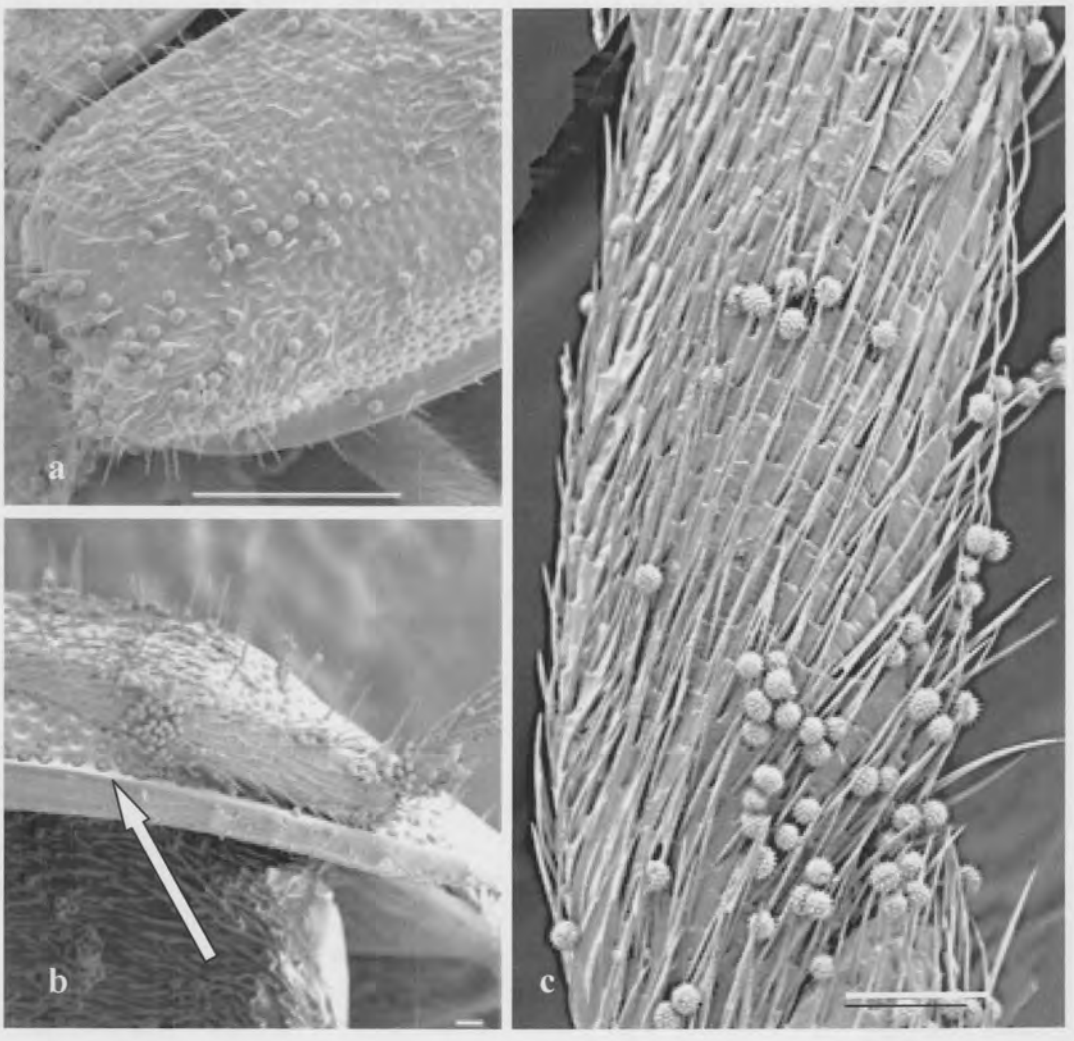
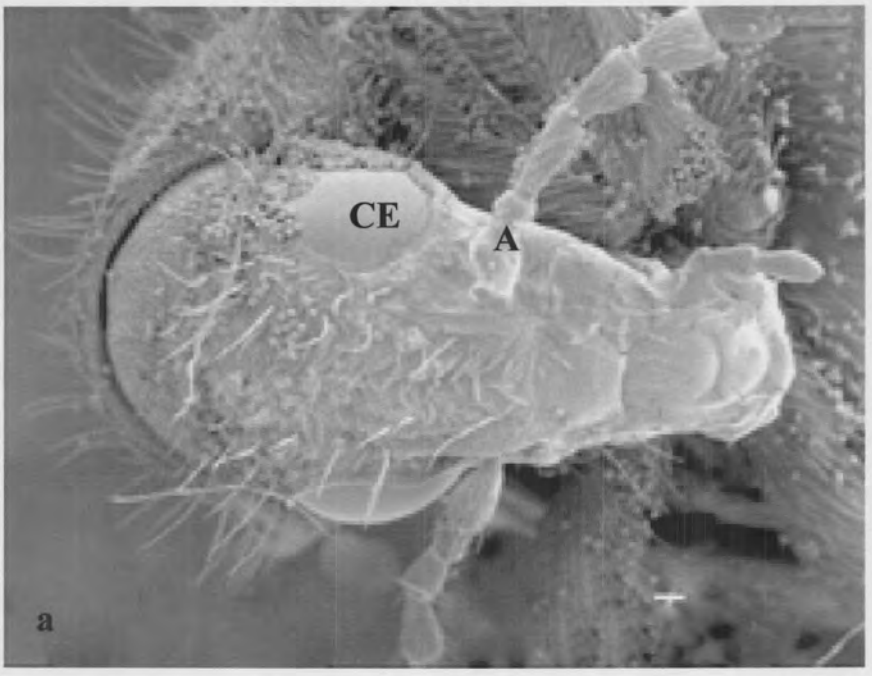


Fig. 2 a - c: SEM micrographs illustrating pollen grain presence on various regions of the body of spotted maize beetles. a: Dorsal view of the anterior elytra and thorax with maize pollen captured between the hairs. b: Sunflower pollen in grooves on the posterior elytra (see arrow). c: Sunflower pollen sticking to hairs on the hindleg.

Scale bars a = 1 μm; b, c: = 100 μm.



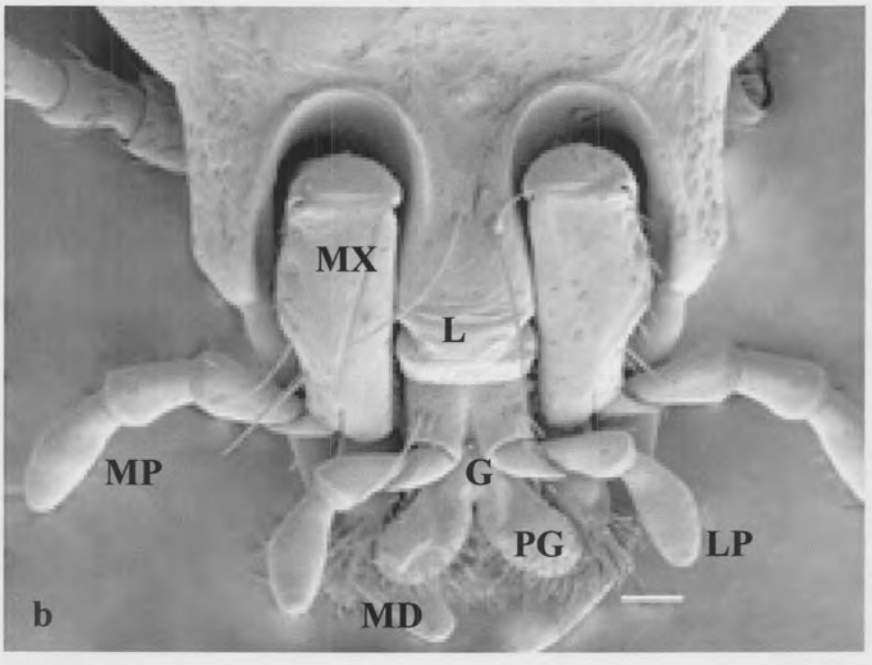


Fig. 3 a - b: SEM micrographs of the head and mouthparts of the spotted maize beetle, *Astylus atromaculatus*. a. Anterior view of head with prognatous mouthparts: CE compound eye; A antenna. b: Ventral view of intact mouthparts: G glossa; L labium; LP labial palp; MD mandible; MP maxillary palp; MX maxilla; PG paraglossa. Scale bars = 100 µm.

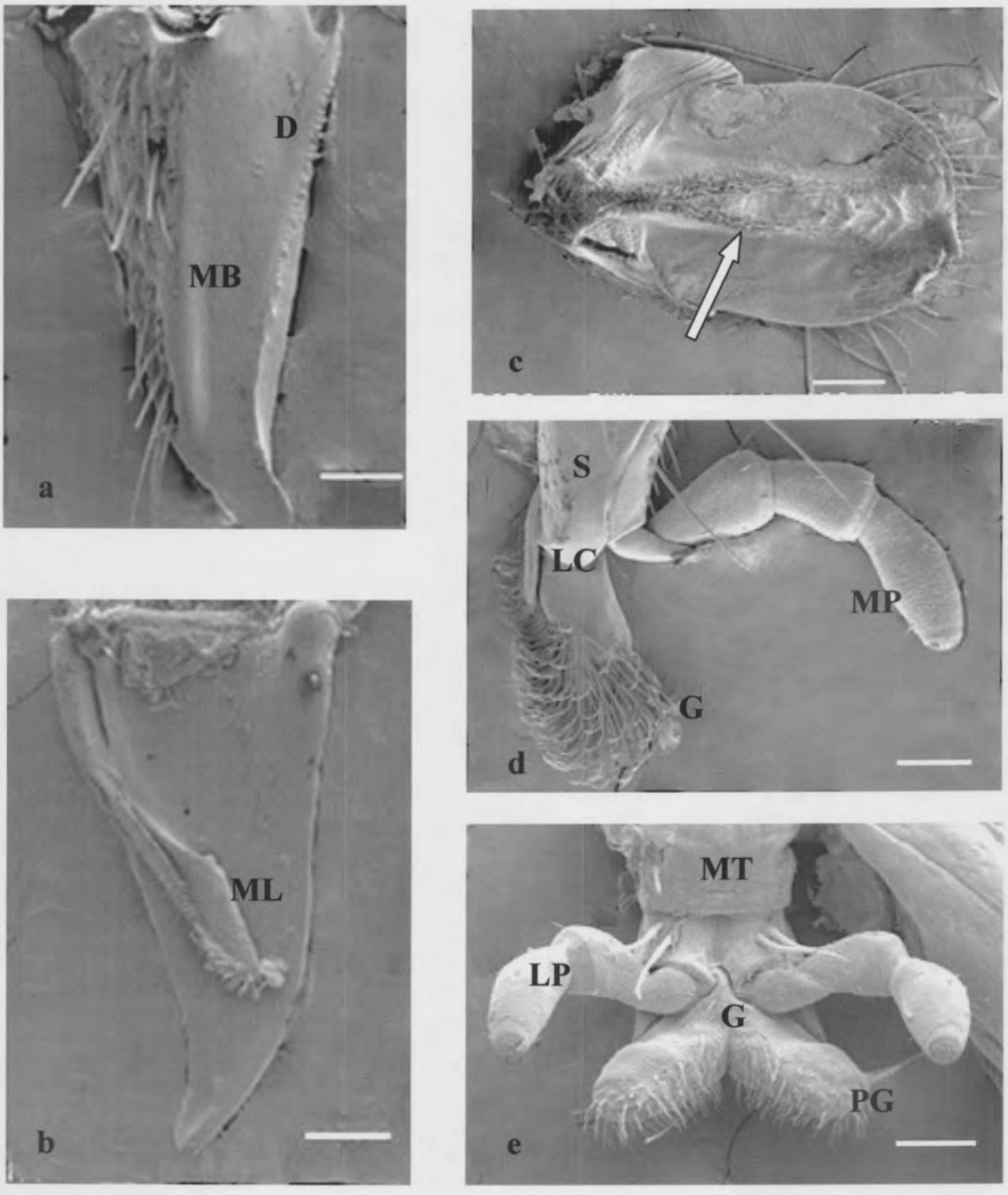


Fig. 4 a – e: SEM micrographs of the mouthparts of the spotted maize beetle.

a: Mandible: D dentes; MB mandibular brush. b: Mandible: ML hairy molar lobe.

c: Labrum with a hairy groove (see arrow) and hairs around the anterior end.

d: Maxilla: G galea with brush; LC lacinia (maxillary brush); MP maxillary palp.

S stipes. e: Ventral view of labium: PG paraglossa and G glossa; LP labial palp;

MT mentum. Scale bars a - e = 100 μm.

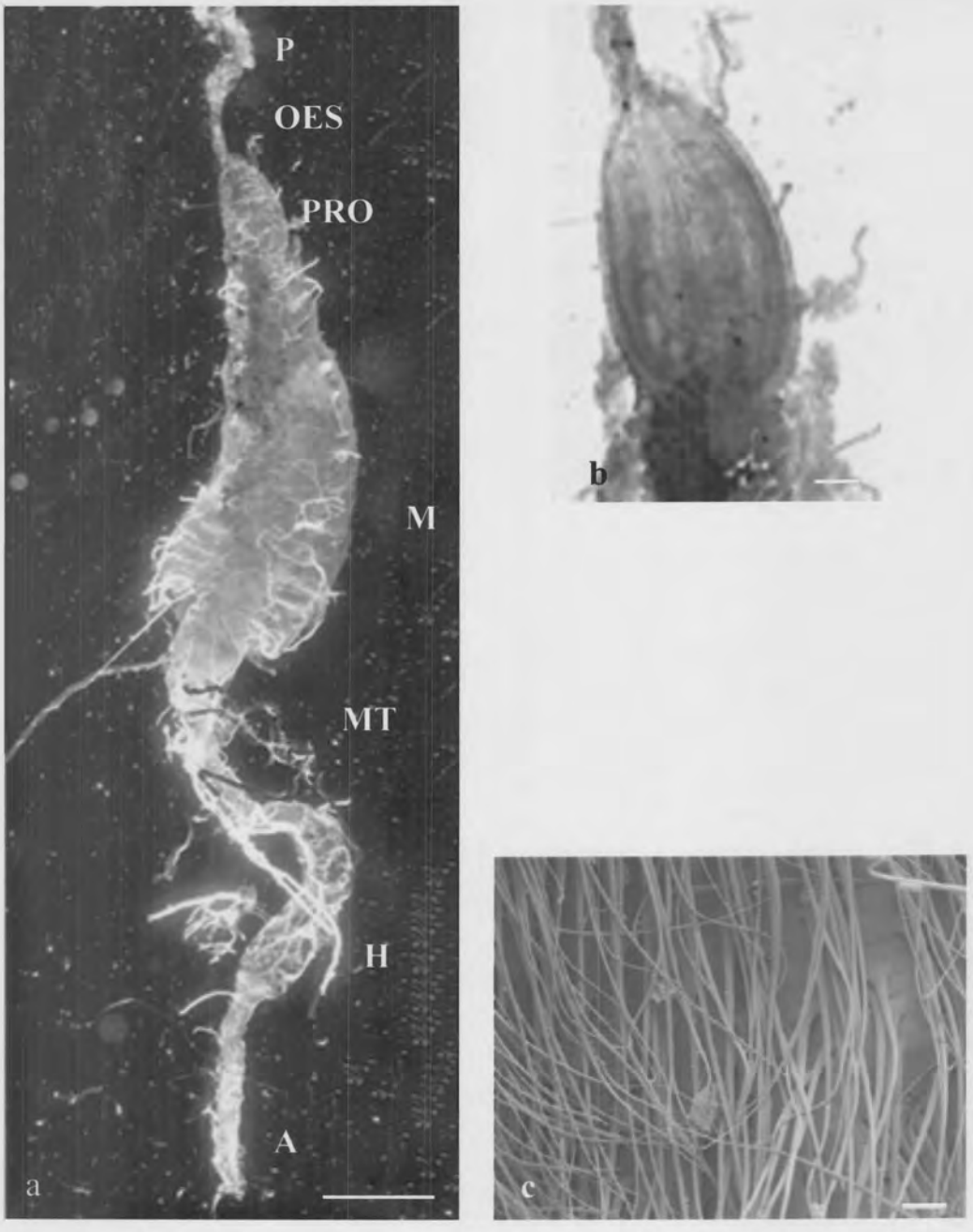


Fig 5 a – c: Light microscope (LM) and SEM micrographs of the alimentary canal of a spotted maize beetle. a: LM micrograph of an intact alimentary canal: A anus; H hindgut; M midgut; MT malphigian tubules; OES oesophagus; P pharynx; PRO proventriculus. b: Close up view of the proventriculus. c: SEM micrograph showing the hairy interior of the proventriculus.

Scale bars a, b = 1mm, c = 10 μm.

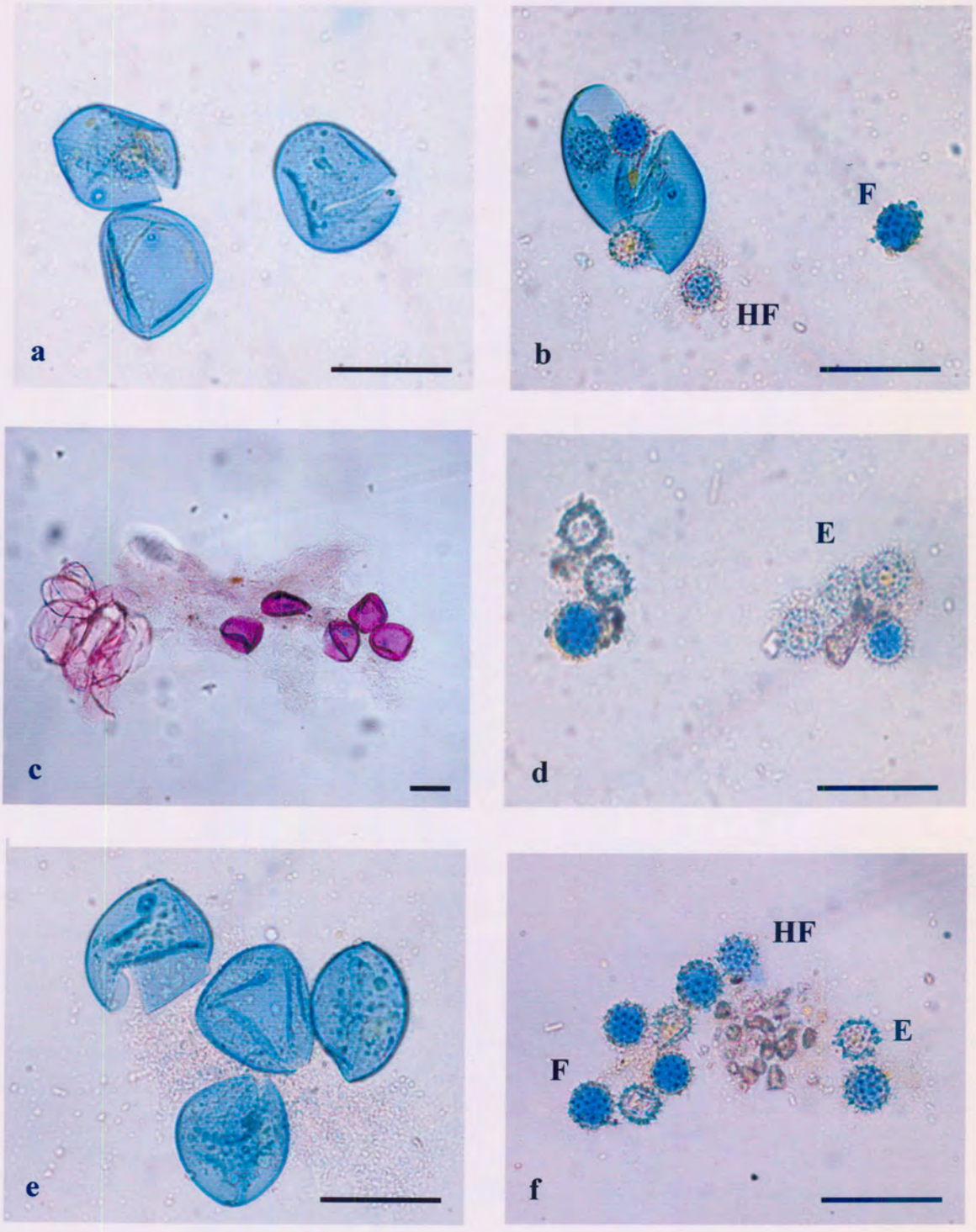


Fig 6. a - f: LM micrographs of samples from the midgut (a, b), hindgut (c, d) and faeces (e, f) containing maize and sunflower pollen; grains stained blue with cotton lactophenol blue and pink with basic fuchsin. a: Empty maize pollen grains, two ruptured, in the midgut, with evident pores. b: Sunflower pollen from the midgut (and single ruptured maize pollen grain). c: Compressed exines of maize pollen from the hindgut and empty maize pollen grains to the right. d: Sunflower pollen from the hindgut, two empty and a full grain to the left, a half full and empty grains to the right. e: Maize pollen from faecal samples. f: Sunflower pollen from the faeces. F = full, HF = half full, E = empty. Scale bars a – f = 100 μm.

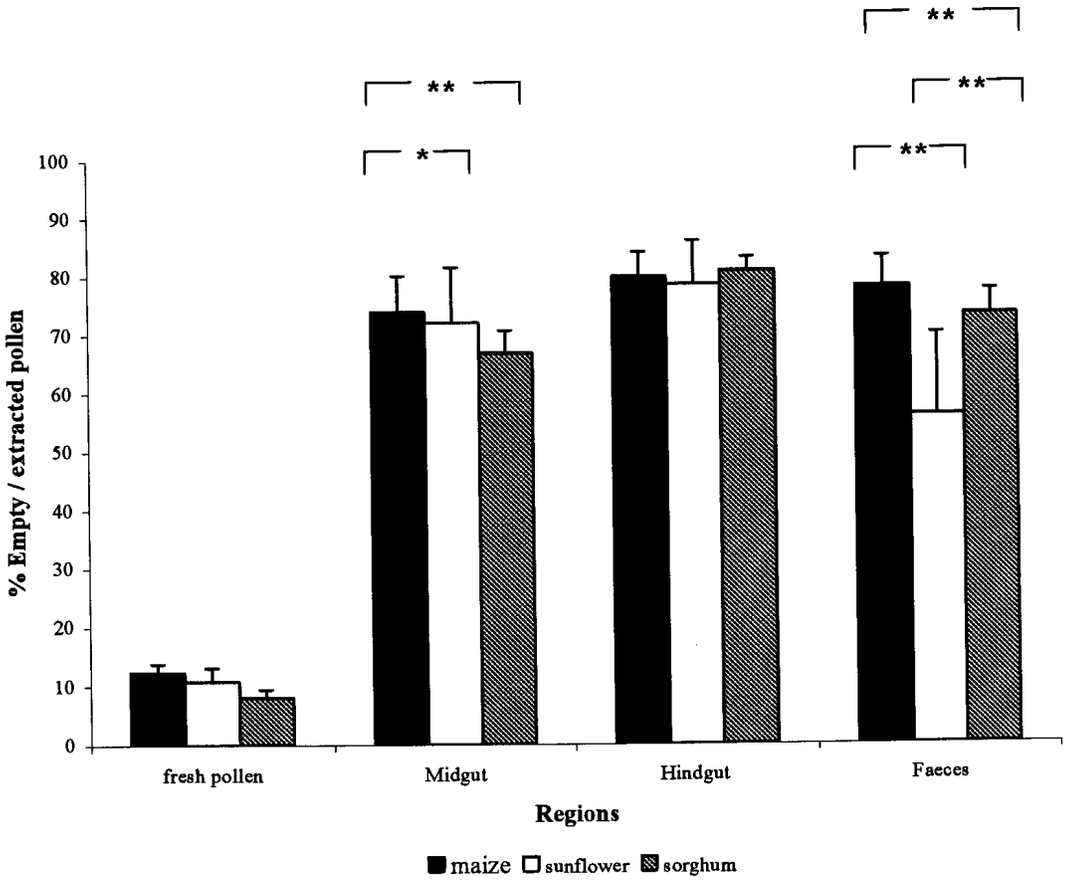


Fig. 7. Extraction efficiency for pollen of maize, sunflower and sorghum in the midgut, hindgut and faeces of spotted maize beetles. Percentage of empty pollen grains is also shown. Data points are means ± SD. A Kruskal-Wallis ANOVA was conducted between different plant species for the midgut, hindgut and faeces. Significant differences are indicated between extraction efficiency for sorghum and maize as well as sorghum and sunflower in the midgut, and between all three pollen types in the feacal samples (Mann-Whitney U test). * $P < 0.005$, ** $P < 0.0005$.

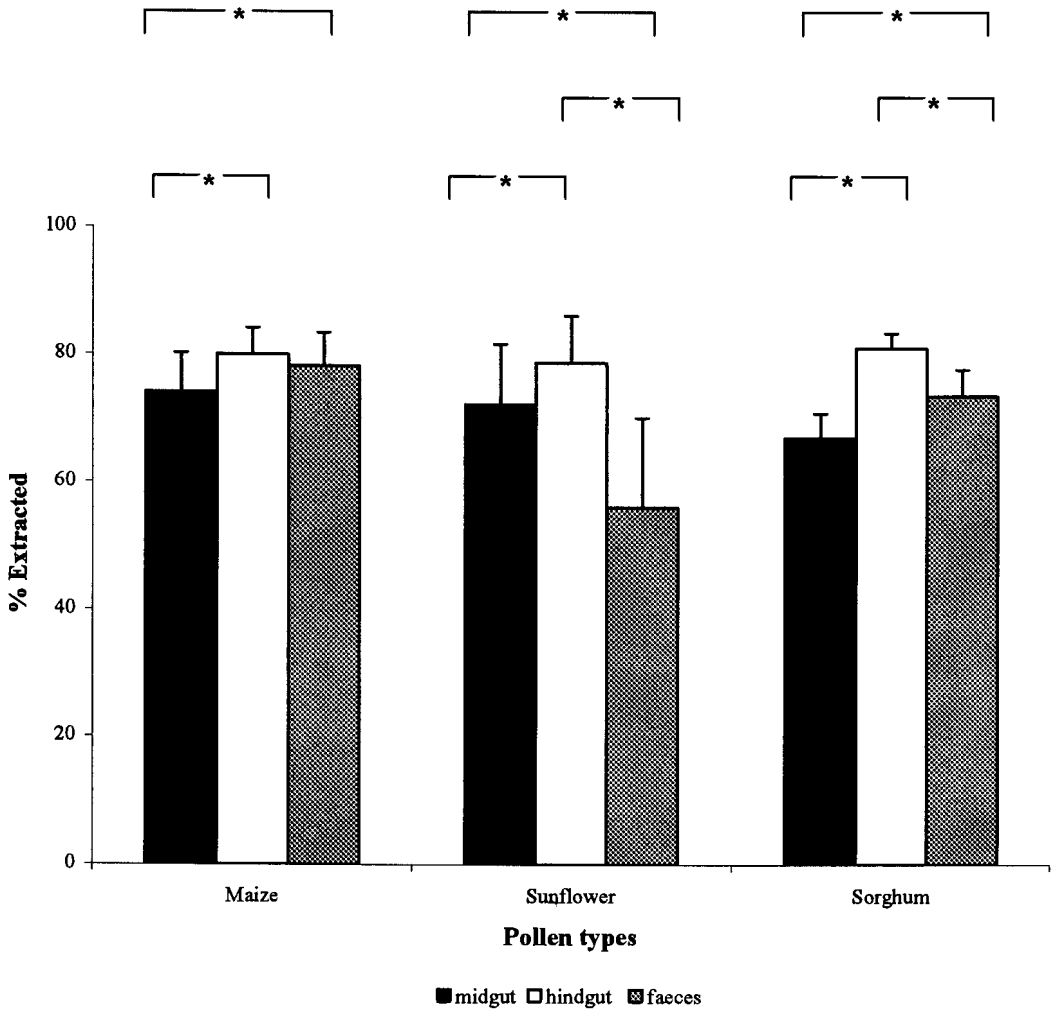


Fig. 8. Extraction efficiency of pollen in midgut and hindgut and faeces of beetles feeding on three plant species. Data points are means ± SD. A Kruskal-Wallis ANOVA was conducted between midgut, hindgut and faeces for each plant species. Highly significant differences are indicated between extraction efficiencies for midgut and hindgut and midgut and faeces for maize pollen, and significant differences between all three regions for both sunflower and sorghum pollen (Mann-Whitney U test). * $P < 0.0005$.

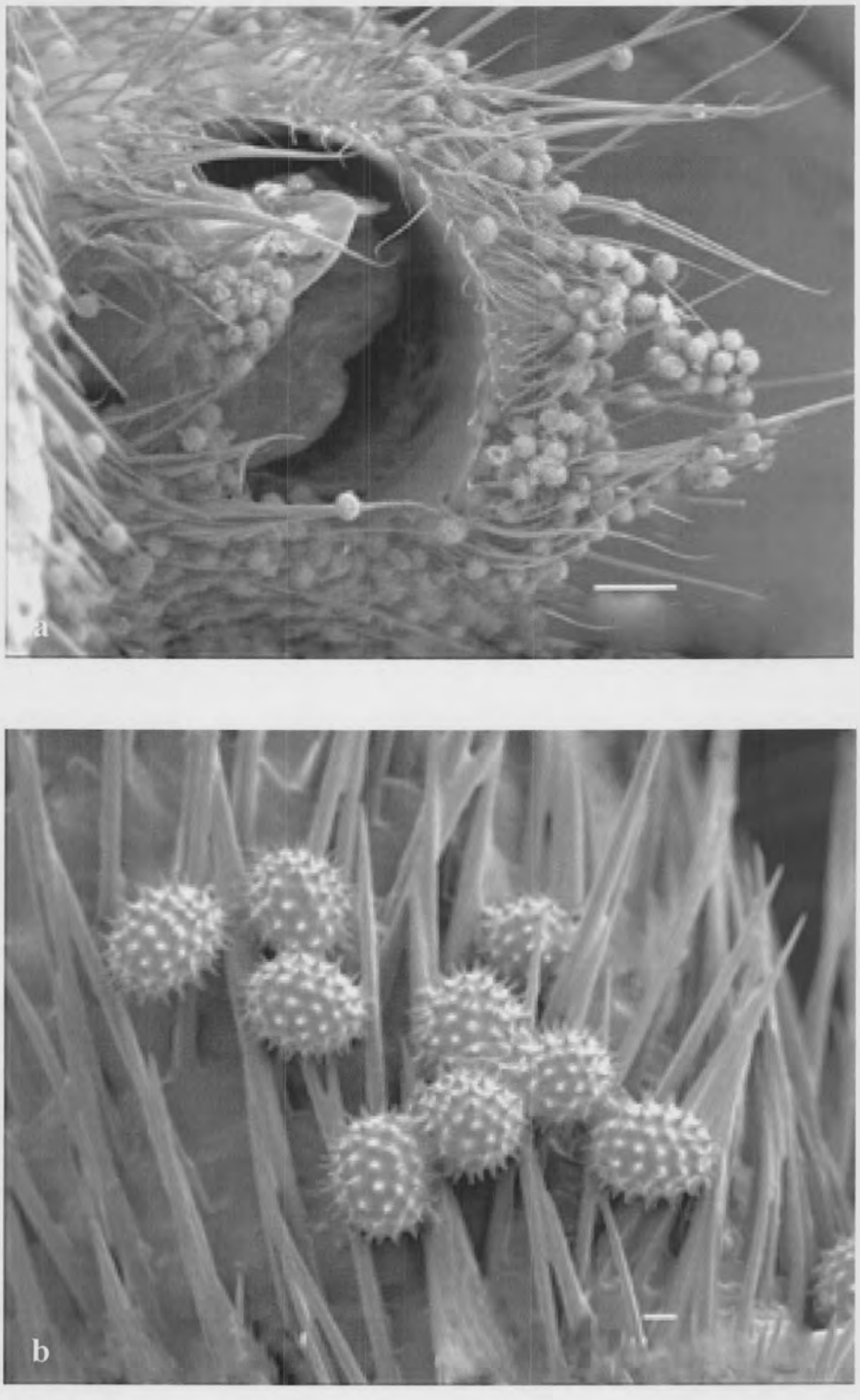


Fig. 9. a - b: SEM micrographs of sunflower pollen grains adhering to hairs on the body of the spotted maize beetle. a: Pollen grains adhering to hairs around the anus, a likely cause of 'contamination' of faecal samples with fresh pollen. b: Pollenkitt enabling pollen grains to adhere to hairs on the insects' body.

Scale bars a = 100 µm, b = 10 µm.

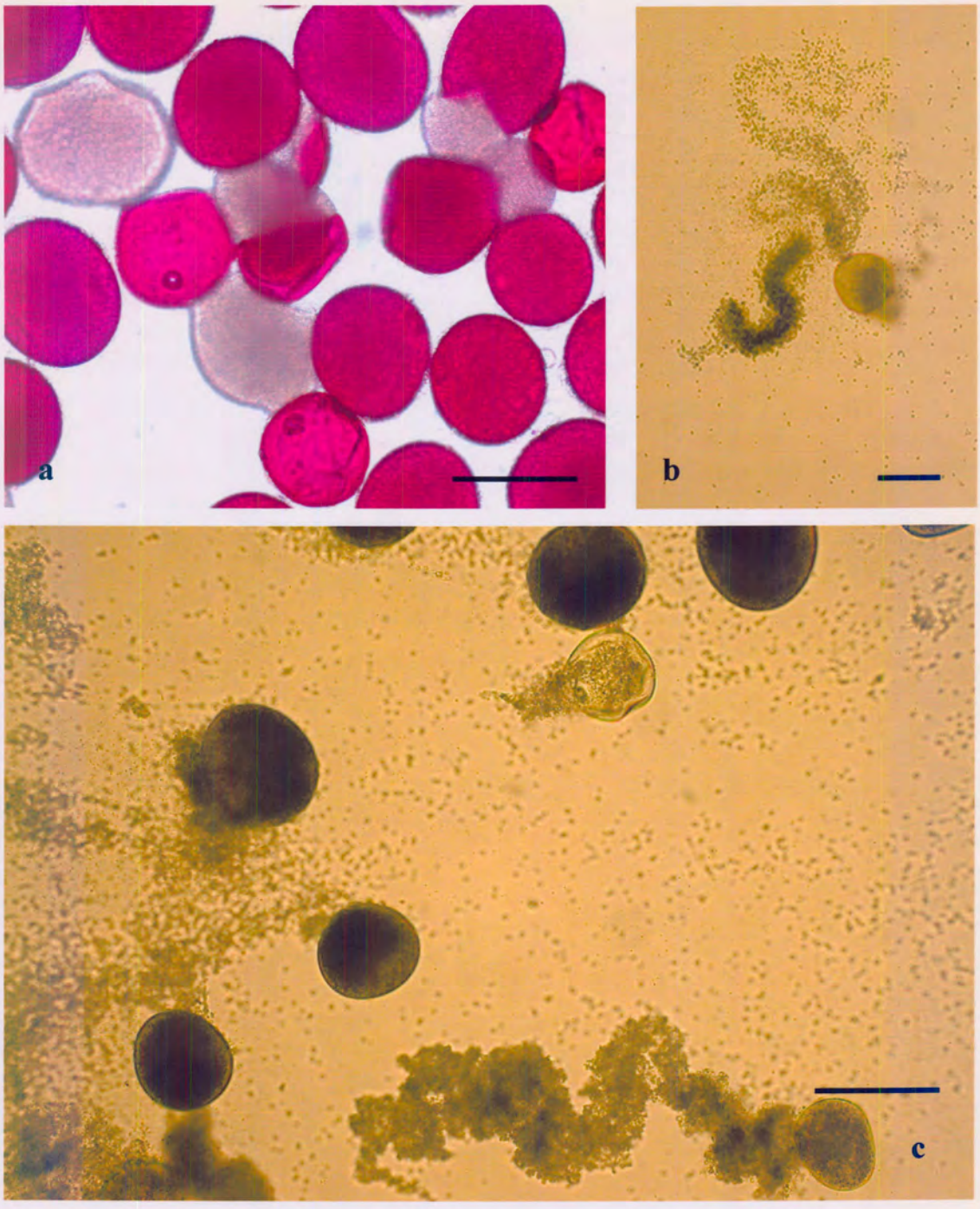


Fig. 10 a - c: LM micrographs demonstrating the fast outflow of grain contents through pores of maize pollen. a: Full pollen grains. b: Pollen grain contents streaming out through pore. c: Left, contents are just beginning to flow out from grains: top right, grain is almost empty: and bottom right, grain is half empty. Scale bars a – c = 100 μm.

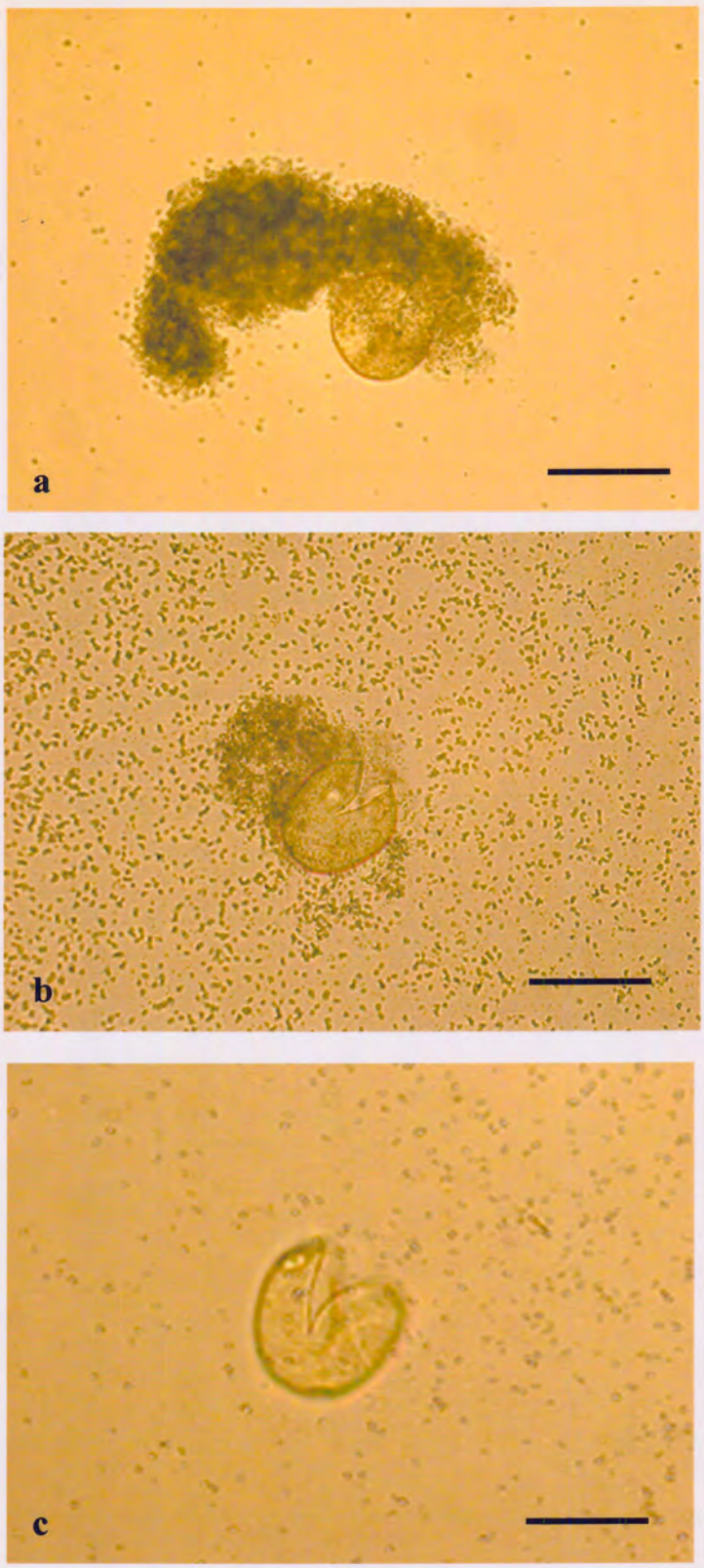


Fig 11 a- c: LM micrographs illustrating the bursting of a maize pollen grain in distilled water. a: Grain bursting. b: Contents 50% removed, contents have a granularly consistency/ starch granules from contents surrounding pollen grain. c: Pollen grain is 100% empty. Scale bars a – c = 100 μm.

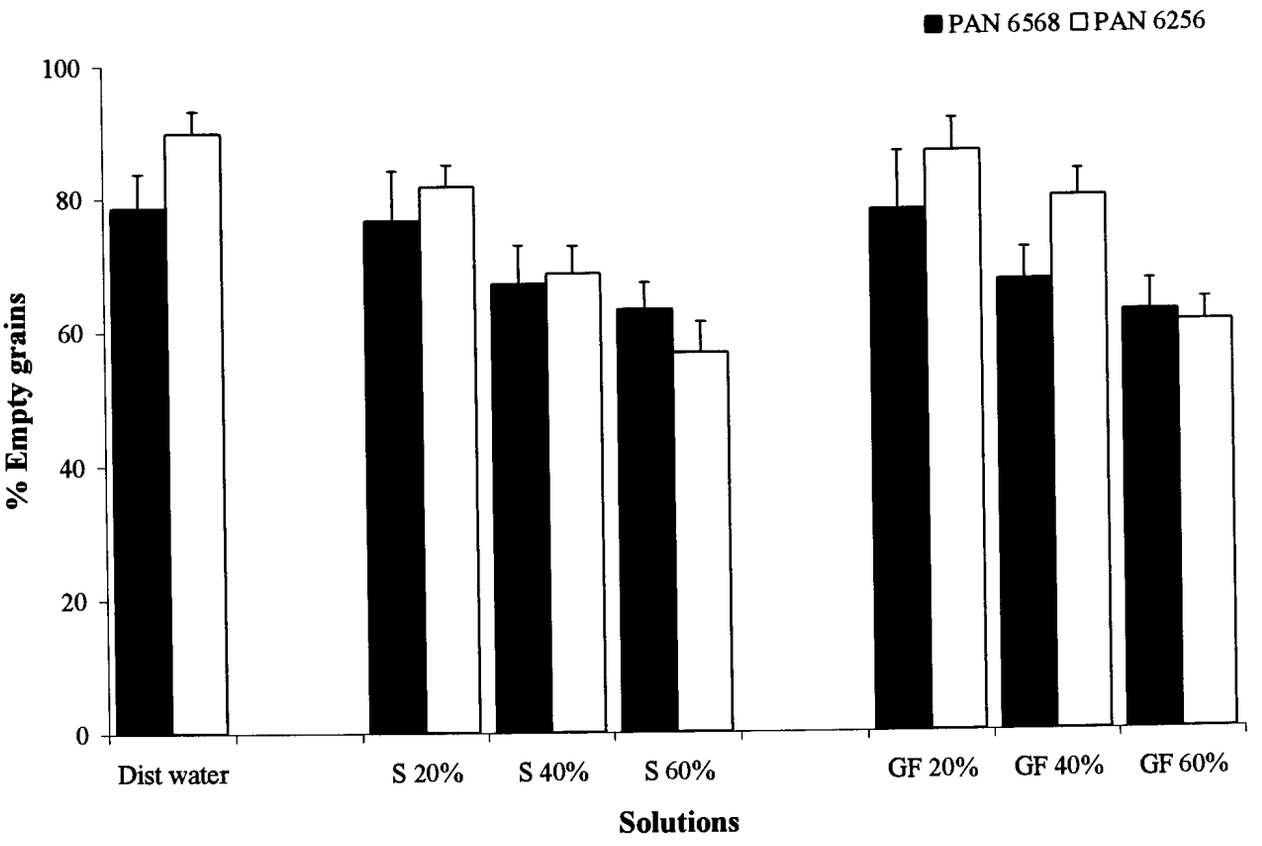


Fig 12. Percentage of empty grains of two maize cultivars immersed in distilled water (control) and different concentrations of sucrose (S) and glucose:fructose (GF) solutions for 10 minutes before distilled water was added to mixtures. Data points are means ± SD.

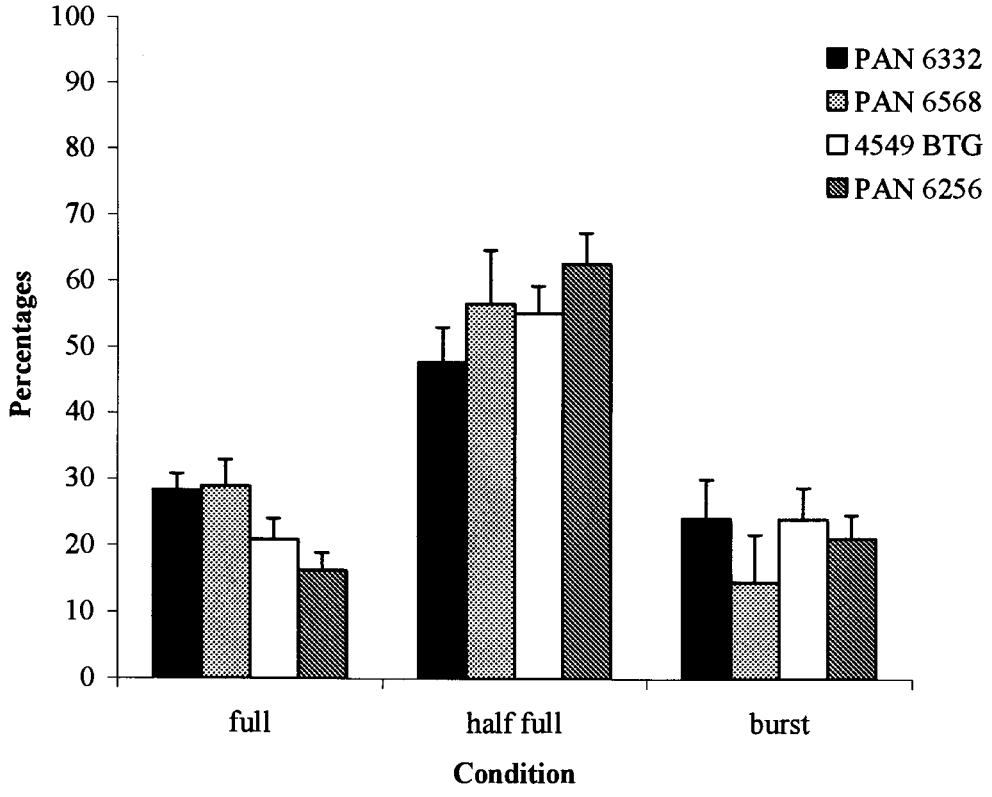


Fig 13. Condition of maize pollen grains from four different cultivars subjected to distilled water for 10 minutes. Data points are means ± SD.

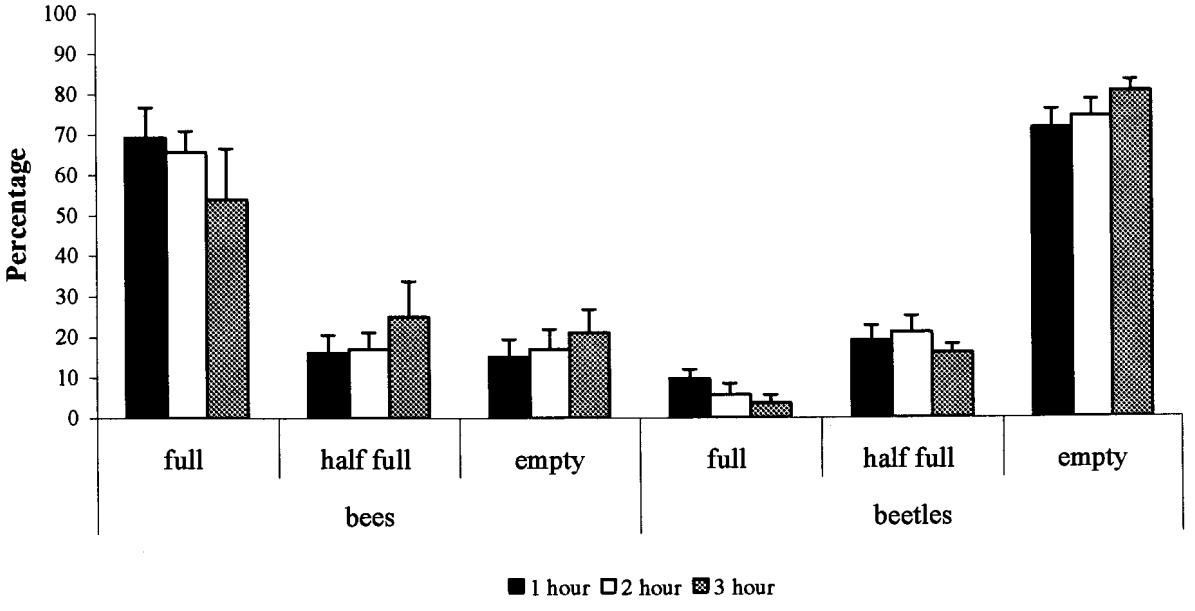


Fig. 14. Condition of maize pollen grains in the midgut of honeybees, *Apis mellifera* and spotted maize beetles, *Astylus atromaculatus*, at different times after feeding. Data points are means ± SD.

Table 1. The effect of different concentrations of sucrose (S) and glucose:fructose (GF) solutions on rupturing of maize pollen (cultivar PAN 6568). Significant P-values are printed in bold (Mann Whitney U-test).

	20% S	40% S	60%S	20% GF	40% GF	60% GF
20% S						
40% S	**0.0015**					
60% S	**0.0002**	0.0944				
20% GF	0.5687	**0.0115**	**0.0005**			
40% GF	**0.0007**	0.790	0.0679	**0.0125**		
60% GF	**0.0004**	0.0521	0.5430	**0.0007**	**0.0185**	
0% (control)	0.1830	**0.0009**	**0.0002**	1.0000	**0.0009**	**0.0002**

Table 2. The effect of different concentrations of sucrose (S) and glucose:fructose (GF) solutions on rupturing of maize pollen (cultivar PAN 6568). Significant P-values are printed in bold (Mann Whitney U-test).

	20% S	40% S	60%S	20% GF	40% GF	60% GF
20% S						
40% S	**0.0003**					
60% S	**0.0002**	**0.0001**				
20% GF	**0.0524**	**0.0002**	**0.0002**			
40% GF	0.3056	**0.0001**	**0.0002**	**0.0089**		
60% GF	**0.0002**	**0.0005**	0.0581	**0.0002**	**0.0002**	
0%	**0.0002**	**0.0001**	**0.0001**	0.0798	**0.0004**	**0.0000**

Table 3 Digestive efficiency of different pollen types by various animals.

Animal species	Plant species	Digestive efficiency	References	Remarks
Insects				
Honeybees (*Apis mellifera*)	Sweet chestnut (*Castanea sativa*)	Summer 40 Winter 73	Crailsheim 1992	Natural diet, from hives Evaluation: gut contents
Honeybees (*A. mellifera*) Spotted maize beetles (*Astylus atromaculatus*)	Maize (*Zea mays*)	21 80	This study*	Laboratory-fed with hand-collected pollen Evaluation: gut contents
Solitary bee larvae (*Osmia cornuta*)	Pear (*Pyrus communis*)	73	Cresti et al. 2001	Adults induced to feed on pollen before egg deposition Evaluation: gut contents
Spotted maize beetles (*Astylus atromaculatus*)	Sorghum (*Sorghum vulgare*) Sunflower (*Helianthus annuus*) Maize (*Zea mays*)	67 72 74	This study*	Beetles captured on food plants Evaluation: faeces & gut contents
Birds				
Darwin's finches (*Geospiza scandens, G. fortis*)	Prickly pear (*Opuntia echios*)	> 90	Grant 1996	Natural diet in the wild Evaluation: faeces
Rainbow lorikeets (*Trichoglossus haematodus* Moluccan lorikeets (*T. h. moluccanus*)	*Eucalyptus calophylla* *E. calophylla* *Prunus spp* .	5 adults 7 nestlings 26 adults 13 nestlings 12	Brice et al 1989*	Laboratory-fed with hand- and bee-collected pollen Evaluation: faeces
Anna's hummingbirds (*Calypte anna*) Cockaticls (*Nymphicus hollandicus*)	*E.calophylla* *Callistemon citrinus* *E. calophylla*	7 5 adults 18 nestlings 38		
Lesser double collared sunbirds (*Nectarinia chalybea*)	*E. calophylla*	19	Van Tets & Nicolson 2000*	Laboratory-fed with bee-collected pollen Evaluation: faeces

Mammals				
Bats (*Syconycteris australis*)	*Banksia integrifolia*, bottlebrush (*Callistemon viminalis*) mixed pollen: *Banksia* spp., *Grevillea* spp. Myrtaceae	53 55 37 32 31	Law 1992*	Laboratory-fed with hand-collected pollen & mixed pollen (commercially available) Evaluation: faeces
Bats (*Leptonycteris caurasoae* *Anoura geoffroyi* *Artibeus jamaicensis* *Sturnira lilium*)	Unidentified spp of columnar cactus	90 46 68 32	Herrera & Martinez Del Rio 1998*	Laboratory-fed with hand-collected pollen Evaluation: faeces
African rodents: (*Rhabdomys pumilio* *Aethomys namaquensis* *Mus minutoides*) Elephant-shrew: (*Elephantulus edwardii*)	*Protea humiflora*, *P.subulifolia* *P. laurifolia*,	58 60 83 49	Van Tets 1997	Natural diet in the wild Evaluation: faeces
Australian Marsupials: (*Cercartetus nanus* *Petaurus breviceps* *Antechinus stuartii*) Australian rodent: (*Rattus fuscipes*)	*Banksia* spp.	65 66 37 55	Van Tets & Whelan 1997	Natural diet in the wild Evaluation: faeces
Australian Marsupials: (*Cercartetus nanus*)	*Banksia integrifolia*, *B. serrata* *B. spinulosa*	79 65 64	Turner 84*	Natural diet in the wild as well as laboratory-fed with *Banksia* inflorescences Evaluation: faeces
Australian Marsupials: (*Tarsipes rostratus*)	*Banksia spp*	95-100	Richardson et al. 1986	Laboratory-fed Evaluation: faeces
Australian Marsupials: (*Petaurus breviceps*)	*Banksia spinulosa*	53	Goldingay et al. 1987	Natural diet in the wild Evaluation: faeces

* these studies corrected for the number of initially empty pollen grains in fresh pollen

Synthesis

Synthesis

Pollen is a nutritious food and the major floral attractant for many beetles. Although it has been considered indigestible, many animals have found mechanisms to overcome the obstacle presented by the pollen wall. Factors that may influence the digestibility of pollen as well as the efficiency of digestion are the plant species, thickness of the pollen wall and the presence and number of germination pores, as well as the digestive ability of pollen consumers. This study has clearly demonstrated that an insect species digests pollen of different plant species differently and that the same pollen can be digested through different mechanisms with varying efficiency in different insects. Studies on pollen digestion are not straightforward: some of the important considerations that researchers need to keep in mind will be discussed in more detail.

Digestion efficiency is the percentage of empty grains in the gut contents or faeces and should be adjusted to correct for the number of empty pollen grains found in fresh pollen (Roulston and Cane 2000). The number of empty pollen grains in fresh pollen can be substantial (up to 18% for *Banksia* pollen; Law 1992). Some researchers (e.g. Brice et al. 1989, Turner 1984, Law 1992, Herrera and Martinez del Rio 1998, Van Tets and Nicolson 2000 and the present study) account for this factor while others do not. Empty pollen grains are not always quantified in calculations of digestion efficiency: only completely empty grains may be used or grains partially devoid their contents may also be considered as empty and included in calculations. No formal definitions have been given in the literature for either digestive ability or the digestibility of pollen. Both these

terms are rather vague. Digestive ability can be seen as the competence of the consumer to extract the contents of pollen grains while digestibility of pollen is the degree to which pollen can or can not be digested. Pollen consumers may extract enough nutrients from pollen grains even when they do not completely empty the grains. However, digestion efficiency remains the preferred term.

Pollen wall thickness and pollen grain size may play an important role in digestion (Roulston 2003). Suarez-Cervera et al. (1994) illustrated that the degree of digestion in larvae of *Osmia* bees may be determined by the thickness of the intine of pollen grains consumed by these larvae. The exine of the pollen wall remained unbroken after digestion in all the pollen types examined in this study. The thin intine of *Sonchus oleraceus* and *Cistus* spp. pollen disappeared after digestion in the faeces while the thick intine of *Quercus ilex* and *Prunus dulcis* pollen appeared unaltered. Thin walled maize pollen (Nepi et al. 2001) is considered large (97 µm in diameter) compared to sunflower pollen that is medium in size (29 µm). Sorghum, on the other hand, is another grass and its pollen looks like maize pollen: it is also thin walled but is smaller than maize (31 µm). Less "effort" is required in the digestion of maize and sorghum pollen since osmotic shock is a quick and easy way to release 100% of the pollen grain contents. Another mechanism might be needed to overcome the barrier presented by the thicker wall of sunflower pollen in order to reach the contents. Law (1992) looked at digestion efficiency and followed the passage of different sized pollen grains through the gut of bats. He used bee-collected pollen consisting of a mixture of three main pollen types: large *Grevillea* pollen (80 µm), intermediate *Banksia* pollen (50 µm) and small pollen of Myrtaceae (25

µm). Digestion efficiency was 38% for *Banksia* pollen and 32% for both the other pollen types, and gut passage times were similar for all three pollen types, an indication that there was no selection for size in this case.

Regarding the nutritional value of pollen, chemical analyses of pollen need to be standardized. Hand collection of enough fresh pollen needed for analyses is difficult, therefore many researchers use pollen pellets taken from bees or combs. These pollen pellets can easily be collected in large amounts but unfortunately are not pure pollen. Bees add, among other substances, regurgitated nectar or honey to pollen for transport on their legs. Enzymes such as invertase may even be added to pollen. Invertase is known to hydrolyse sucrose and depolymerizes fructans: the result is a loss of sucrose and fructans and an increase in the osmotic pressure of pollen (Pacini 2000). It was believed that bees add a fatty acid, 10-hydroxy-2-decenoic-acid, with antibiotic properties (occurring in their mandibular glands) to pollen either during pollen collection or during storage but Verhoef et al. (1986) disputed this. All of these substances can affect not only the mass of the pollen but also all calculations of pollen composition; for example sugars present in bee-collected pollen may account for up to 40% of the dry mass of pollen pellets (Roulston and Cane 2000). This is clearly illustrated in Table 1 (Page 9 of this thesis): *Virgilia* pollen has a carbohydrate content of 56% compared to 70% in the pollen-nectar paste (Louw and Nicolson 1983). Therefore it is better to collect pollen directly from the anthers for chemical analysis.

Pollen variation among cultivars is another important factor that needs to be taken into consideration. The *in vitro* experiments described here indicated that pollen from different cultivars of the same species may react differently to germination conditions and to conditions in the gut lumen of the insect. Relative humidity (RH) and temperature are known to have an influence on reactions of pollen grains (Eisikowitch and Woodell 1975, Bassani et al. 1994), an aspect that also needs to be taken into consideration in storage conditions of pollen before usage in experiments.

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