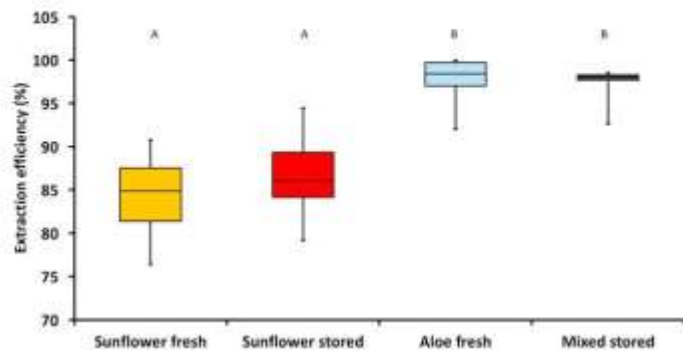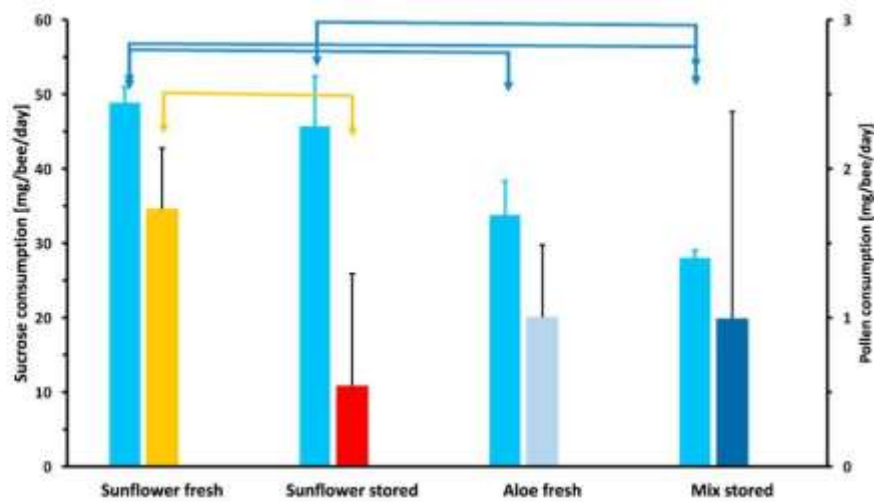# Digestibility and nutritional value of fresh and stored pollen for honey bees (*Apis mellifera scutellata*)

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Graphical abstract

Highlights

• Storage of pollen by honey bees may improve its digestibility and nutritional value.

• We measured consumption of fresh and stored pollen together with sucrose solution.

• Bees did not compensate for lower pollen protein or for sugar added during storage.

• Pollen extraction efficiency was high but did not increase as a result of storage.

ABSTRACT

Pollen, the main protein source for honey bees, is mixed with regurgitated nectar or honey during collection and then stored as 'bee bread' before its consumption, mainly by young nurse workers. It has been suggested that storage of pollen improves its nutritional value and digestibility, but there is little evidence for such changes. We fed two fresh pollen types of different protein content (aloe and sunflower), and two stored pollen types (sunflower and a mixed pollen), to young caged worker bees. We measured daily consumption of pollen and sucrose solution, and survival after 14 days. At day 14 we recorded ovarian activation and extraction efficiency, by counting empty pollen grains in the rectal contents. Extraction efficiency is a measure of pollen digestibility. Contrary to our predictions, bees did not consume more fresh sunflower pollen than fresh aloe pollen to compensate for the lower protein content of sunflower pollen. In addition, they did not consume less sucrose solution when fed stored pollen diets that are already enriched in sugar. Consumption of stored sunflower pollen resulted in a low protein to carbohydrate (P:C) intake. Survival and ovarian activation were higher on diets giving higher P:C intakes. Extraction efficiency was high (up to 99%) for all pollen diets, and comparison of fresh and stored sunflower pollen showed that storage did not make it easier to digest. Changes to pollen during storage do not confer obvious benefits to honey bees.

Keywords: fresh pollen, stored pollen, consumption, survival, ovarian activation, pollen extraction efficiency

1. Introduction

Pollen is essential for the development of honey bees (*Apis mellifera* L.), providing nutrients such as proteins, lipids, minerals and vitamins (Brodschneider and Crailsheim, 2010; Wright et al., 2018). In particular, high pollen consumption by adult worker bees in the first few days after emergence (Crailsheim et al., 1992) enables the development of their mandibular and hypopharyngeal glands which produce jelly for feeding brood and other colony members (Crailsheim, 1992; Haydak, 1970; Hrassnigg and Crailsheim, 1998; Lass and Crailsheim, 1996; Winston, 1987). It is well established that the quality of pollen diets, which is frequently equated to their protein content, and the quantity of pollen ingested affects the performance of honey bees, both in cage experiments and under field conditions. In these studies diets are evaluated by measuring fitness parameters such as lifespan, hypopharyngeal gland and ovarian activation, haemolymph protein content, or colony growth and susceptibility to disease (DeGrandi-Hoffman et al., 2016; Di Pasquale et al., 2016; Frias et al., 2016; Hoover et al., 2006; Human et al., 2007; Pernal and Currie, 2000). Most studies of this nature have used diets based on bee-collected pollen pellets. Stored pollen packed into the comb, known as bee bread, has seldom been tested for its effects on performance of honey bees. DeGrandi-Hoffman et al. (2013) compared haemolymph protein concentrations in bees fed bee bread from colonies of Africanized and European honey bees. Carroll et al. (2017) showed that bees prefer to consume freshly-stored pollen, but found no differences in body mass or hypopharyngeal gland protein levels when bees were fed fresh or aged diets. However, bees fed aged stored pollen show deleterious changes in the gut microbiome (Maes et al., 2016). It is not clear whether storage of pollen leads to any improvement in its nutritional value or digestibility.

Analyses of pollen chemistry have demonstrated great variation between plant species in nutritional content, such as protein varying between 2.5% and 61% dry mass (Roulston and Cane, 2000; Roulston et al., 2000). This variation was shown to be related to plant phylogeny in the hand-collected pollens analysed by Roulston et al. (2000). However, analyses of pollen composition are usually done on bee-collected pollen rather than fresh pollen (Serra Bonvehí and Escolà Jordà, 1997; Somerville and Nicol, 2006; Vanderplanck et al., 2014). Foragers moisten pollen grains with regurgitated nectar or honey for transport back to the hive (Harano et al., 2013; Thorp, 1979). The amount of sugar added during collection can be substantial - up to 50% dry mass, but usually unknown - and this alters the macronutrient content of the pollen (Nicolson, 2011; Roulston et al., 2000). This addition of sugar to pollen also occurs in stingless bees (Leonhardt et al., 2007). In two

plant species we have compared nutrients in fresh pollen, honey bee-collected pollen and stored pollen removed from the comb: *Aloe greatheadii* var *davyana* (aloe) and *Helianthus annuus* (sunflower) (Human and Nicolson, 2006; Nicolson and Human, 2013). Fresh aloe pollen has a much higher percentage of crude protein than fresh sunflower pollen (51% vs 26% dry mass), but for both species this percentage is greatly reduced in stored pollen (28% and 13% respectively). Most of the decrease in protein and increase in carbohydrate occurs during collection, with little difference between bee-collected and stored pollen (Human and Nicolson, 2006; Nicolson and Human, 2013). Protein to carbohydrate (P:C) ratios are important in bee diets, and previous application of the geometric framework approach to macronutrient regulation of caged worker bees has shown that intake targets are strongly biased towards low P:C ratios (Altaye et al., 2010; Archer et al., 2014b; Paoli et al., 2014). This contrasts with the widespread assessment of pollens by beekeepers according to their protein content (e.g. Schmidt et al., 1987; Johannsmeier, 2001; Somerville and Nicol, 2006). The low protein content of sunflower pollen has labelled it as a poor resource for honeybees (Schmidt et al., 1987; Somerville and Nicol, 2006) while aloe pollen is favoured as a winter resource by South African beekeepers (Johannsmeier, 2001).

The nutrients in pollen are not easily obtained. The walls of pollen grains are made up of three main layers, the pollenkitt, exine and intine, with the exine being the main barrier to digestion for honey bees and other pollen feeding animals (Roulston and Cane, 2000; Stanley and Linskens, 1974). In honey bees, it has been suggested that osmotic shock may occur when pollen grains move from a high osmotic concentration in the crop to a low one in the midgut, so that the pores open and release the cytoplasm (Kroon et al., 1974). However, Human and Nicolson (2003) found that maize pollen grains remained intact in the honey bee midgut, demonstrating that osmotic shock is not involved in digestion of these thin-walled pollen grains. Penetration of digestive enzymes such as proteases through germination pores in the pollen grain wall is probably the most important mechanism for pollen digestion by honey bees (Simpson and Neff, 1983). These enzymes process the contents which then leak out through the pores, the walls remaining intact (Peng et al., 1986; Roulston and Cane, 2000). The durability of the pollen grain wall enables measurement of extraction efficiency by counting the numbers of full and empty pollen grains in the faeces of pollen consumers and comparing with fresh pollen (Brice et al., 1989). From data on pollen grain condition in Crailsheim et al. (1992), Roulston and Cane (2000) calculated extraction efficiencies of between 50% and 98% in honey bees, with nurse bees utilising pollen better than foragers. This corresponds with age-related variation in protease activity in the honey bee midgut (Moritz and Crailsheim, 1987).

The experiment of Crailsheim et al. (1992) was carried out with newly emerged workers that were marked and returned to the colony. They were thus consuming comb-stored pollen. It has been assumed that stored pollen undergoes fermentation and nutrient conversion by microbes (Gilliam, 1997), making it more nutritious and more easily digestible than the fresh pollen collected by bees. This has been called the 'beebread maturation hypothesis' (Carroll et al., 2017). However, Herbert and Shimanuki (1978) found only minor changes in nutrient composition, other than a breakdown of starch, and Anderson et al. (2014) demonstrated an absence of microbes in stored pollen. Our analyses of aloe and sunflower pollen (Human and Nicolson, 2006; Nicolson and Human, 2013) show little change in nutrients with storage; however, it is possible that the pollen becomes easier to digest (Kwong and Moran, 2016; Lee et al., 2015). The hydration state of pollen may also be important for digestion: when nectar is added during pollen collection, pollen grains rehydrate and swell, so that previously sunken pores become exposed (Human and Nicolson, 2006; Nepi et al., 2005).

The aim of this study was to compare consumption and digestion of fresh and stored pollen of aloe and sunflower by caged worker bees. For bees on the same diets we also measured consumption of sucrose solution, survival over 14 days and ovarian activation. We made the following predictions. Bees would consume more sunflower than aloe pollen to compensate for its lower protein content, and similarly would consume more stored than fresh sunflower pollen due to the dilution of protein with added sugars. Bees would consume more sucrose solution when fed fresh pollen diets because half of the stored pollen is already sugar. We predicted that survival and ovarian activation would reflect dietary protein content in opposite ways, with survival being reduced by higher dietary protein, and ovarian activation being enhanced. Finally, pollen extraction efficiency would be higher for stored pollen because of possible microbial action, but would differ between aloe and sunflower pollens because of differences in pollen morphology.

2. Materials and methods

2.1. Pollen collection and study sites

Helianthus annuus flowers were collected in March 2015 from a commercial sunflower farm in the Mookgophong area (24°40′S 29°0′E), formerly known as Naboomspruit, Limpopo. Fresh pollen was harvested daily by brushing flowers from multiple plants with a paint brush. Stored *H. annuus* pollen

was collected from hives used for sunflower pollination on the same farm. *Aloe greatheadii* var *davyana* pollen was collected from plants in Roodeplaat Nature Reserve, 25 km NE of Pretoria (25°66'S 28°39'E), Gauteng, during the months of July and August 2015. Permission was granted by the Department of Agriculture and Rural Development of Gauteng to set up several hives in Roodeplaat Nature Reserve during the *A. g.* var. *davyana* flowering season. Hives were placed in July and removed in early September. Stored pollen was collected from these hives before their removal. Fresh and stored pollen was kept frozen at -20°C before use in feeding experiments.

Upon examination of the gut contents of the honeybees fed 'stored aloe pollen', we observed that the pollen was not that of *A. g.* var *davyana* but instead a mixture of five pollen types, with a monocot pollen, probably belonging to one of the liliaceous families, occurring at high frequency (74.5% of 1000 grains examined). We refer to this below as mixed stored pollen. For comparison with our previous analyses of aloe and sunflower pollen, its protein content was obtained by determining total nitrogen using an elemental analyser at the Southern African Grain Laboratory in Pretoria. Nitrogen values were multiplied by a conversion factor of 6.25 (Roulston et al., 2000) to give crude protein as 20.3% dry mass.

2.2. Consumption, survival and ovarian activation

Capped worker brood was removed from five *A. mellifera scutellata* colonies and placed in an incubator at 35°C and ~50% RH in complete darkness. Within 24 h groups of 100 newly emerged workers from the same colony were collected and placed into standard hoarding cages (11 x 8.5 x 7 cm; Köhler et al., 2013) in a second incubator under the same conditions. Twenty cages were prepared, five for each pollen type, namely: sunflower stored, sunflower fresh, mixed stored and aloe fresh. Each cage was supplied with a piece of comb hanging from the top and three feeding tubes; these were 15 ml plastic tubes with screw on lids and feeding holes cut into the tubes (1 x 0.3cm). Each cage received a tube with one of the pollen types, a tube with 50% w/w sucrose solution and a tube of fresh water. The tubes containing the pollen and sucrose solution were weighed daily in order to measure consumption and the contents were replaced. Consumption of sucrose was obtained by halving the consumption of 50% sucrose solution. The water was replaced as needed. Dead bees were removed every day and mortality was recorded. Measures of consumption were adjusted for the number of surviving bees. After 14 days all surviving bees were frozen at -20°C for later dissection.

Five bees from each replicate were dissected, giving a total of 25 bees per treatment. Dissections were performed and the ovaries graded according to Hess (1942) and Schäfer et al. (2006). Ovarian development was categorised into three stages: inactive, intermediate, and activated with some mature oocytes. The contents of the rectum of each bee were removed for determination of pollen extraction efficiency.

2.3. Pollen extraction efficiency

The rectal contents of each honey bee were placed on a slide, mixed with a droplet of distilled water and stained with cotton lactophenol blue, which stains the cytoplasm blue, but not the cell walls (Human and Nicolson, 2003). The mixture was then transferred to a haemocytometer and sealed with a cover slip. Squares from the counting chamber were randomly chosen and 100 grains in each were examined under a light microscope and scored as full, half empty or empty. As defined by Human and Nicolson (2003), full grains contain more than half the cytoplasm and are similar in shape to the reference pollen (fresh pollen), half empty grains contain half or less than half of the cytoplasm and empty grains have no cytoplasm. Five counts of 100 grains each were done for every dissected bee (25 bees per treatment). The same counting procedure was carried out for the fresh aloe and sunflower pollen to determine the number of initially empty grains. For the mixed stored pollen, grains were counted and scored as full, half empty or empty regardless of species. In calculating extraction efficiency the half empty grains in diet and faecal samples were also taken into account:

$$\text{Extraction efficiency (\%)} = \frac{(empty\ faecal - empty\ diet) + 1/2(half\ empty\ faecal - half\ empty\ diet)*100}{(full\ diet + 1/2half\ full\ diet)}$$

2.4. Statistical analysis

Since the consumption data did not fulfil the assumptions for parametric statistics (Pirk et al., 2013), we compared the consumption of pollen and sugar and the extraction efficiency among the different treatments using Kruskal-Wallis ANOVA followed by multiple comparisons of mean ranks for all groups for pairwise comparison as a post hoc comparison. To assist in judging differences between sample medians the 95% confidence interval (CI) for the median was used (McGill et al. 1978). The effect of the different pollen types on survival was tested using Kaplan-Meier survival analysis with the Gehan-Wilcoxon test for pairwise comparison. All statistical analyses were conducted in STATISTICA 12® (Statsoft, 2000).

3. Results

3.1. Pollen and sucrose consumption

Pollen consumption was significantly different among the four pollen types ($H_{3,280}$ = 10.7, p = 0.014; Table 1), being higher in fresh sunflower pollen compared to stored sunflower pollen (Fig. 1, Table 1). Likewise sucrose consumption was significantly different among the four pollen types ($H_{3,280}$ = 35.9, p <0.001). Sucrose consumption was lower in mixed stored pollen compared to both sunflower fresh and stored pollens and lower in aloe fresh pollen than in sunflower fresh pollen (Fig 1, Table 1).
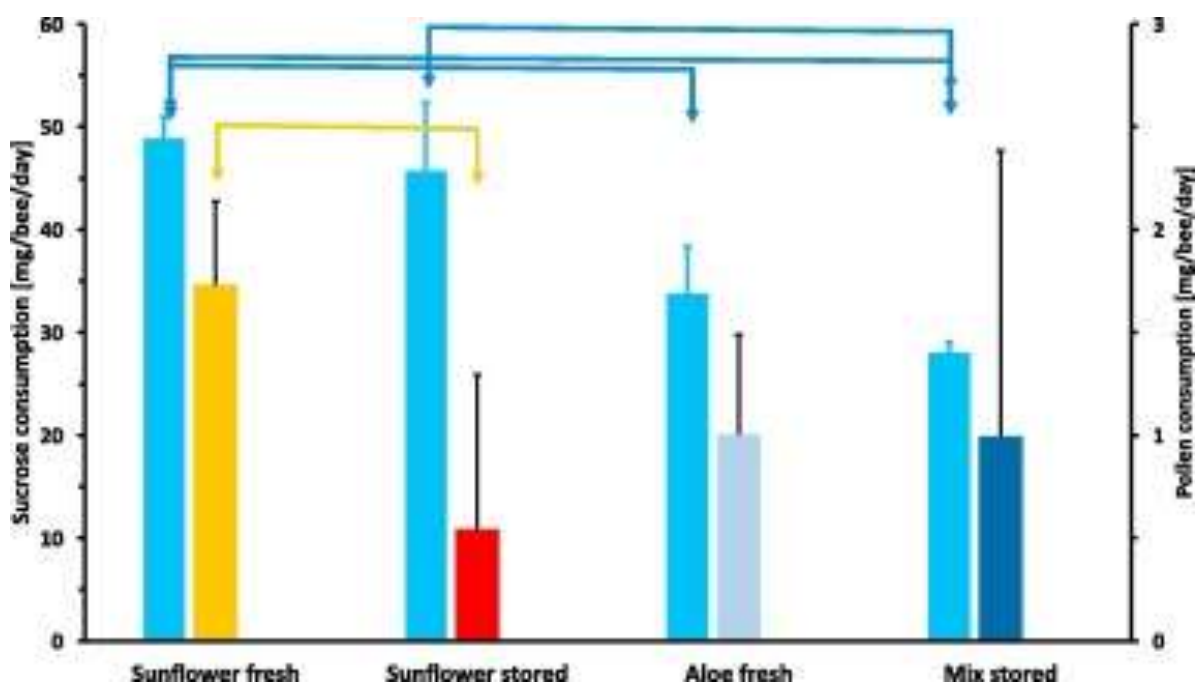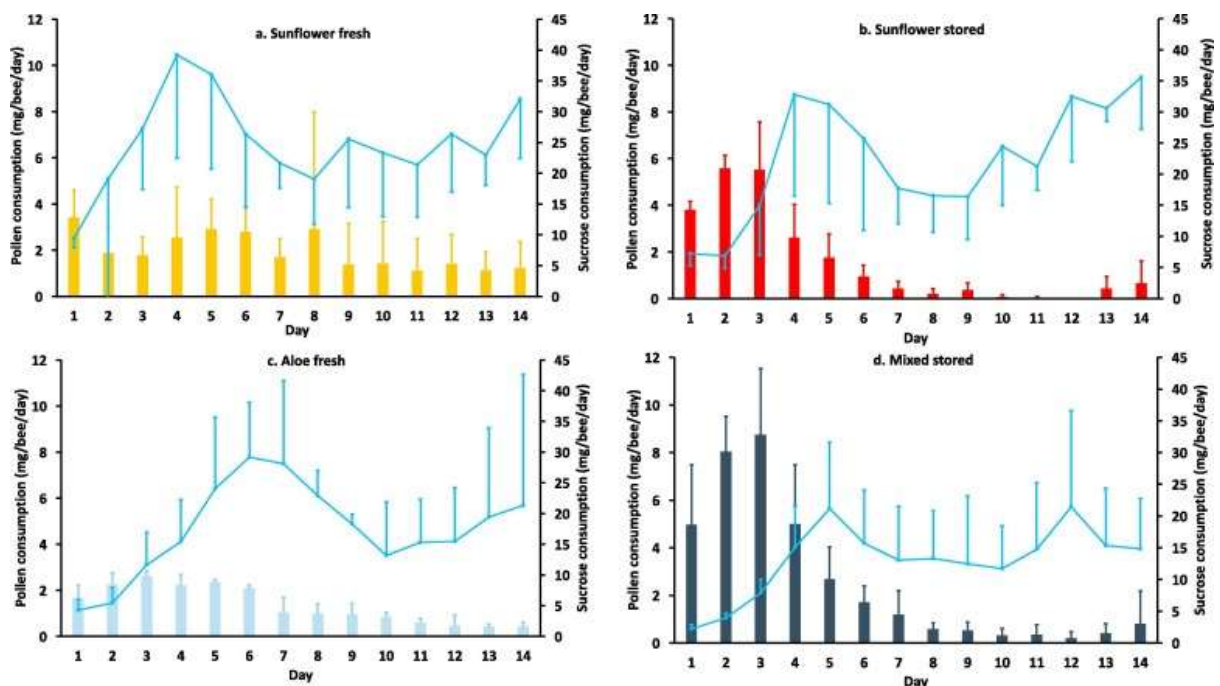

Figure 1. Consumption of pollen and sucrose on the four pollen treatments. Values are medians for consumption in mg/bee/day and whiskers represent the 95% confidence interval (CI); N = 5 cages from 5 colonies). Arrows show significant differences between pollen types in consumption of pollen and of sucrose (see Table 1). Groups of 100 bees were fed fresh and stored sunflower pollen, fresh aloe pollen and stored mixed pollen, together with 50% w/w sucrose solution. Consumption of sucrose was obtained by halving the consumption of 50% sucrose solution.

Table 1. Pollen and sucrose consumption (mg/bee/day) on four pollen treatments. Multiple comparisons for Kruskal-Wallis ANOVA, significant differences in bold.

Pollen	Sunflower fresh	Sunflower stored	Aloe fresh	Mixed stored
Sunflower fresh		**P < 0.01, Z = 3.22**	P = 1 , Z = 0.43	P = 1, Z = 1.04
Sunflower stored			P > 0.1, Z = 2.25	P > 0.6, Z = 1.64
Aloe fresh				P = 1, Z = 0.61
Mixed stored				

| Sucrose	Sunflower fresh	Sunflower stored	Aloe fresh	Mixed stored
Sunflower fresh		P = 1 , Z = 0.65	**P < 0.003, Z = 3.49**	**P < 0.001, Z= 5.48**
Sunflower stored			P > 0.5, Z=1.73	**P < 0.001, Z = 4.32**
Aloe fresh				P > 0.08 , Z = 2.43
Mixed stored				

Figure 2. Time course of pollen and sucrose consumption on the four pollen treatments. Bars represent pollen consumption and lines are sucrose consumption (both in mg/bee/day, mean ± SD, N = 5).

Pollen and sucrose consumption both varied significantly over the course of the experiment (pollen: $H_{13,280}$ = 196, p <0.0001; sucrose: $H_{13,280}$ = 76, p <0.0001). Pollen consumption was much higher in

the first week than in the second (Fig 2), and for the two stored pollens consumption in the second week was negligible (Fig. 2b, d). Sucrose consumption followed the same trends whether bees were fed fresh or stored pollen, with consumption increasing to a peak on days 4-6 then falling, to increase again in the second week (Fig. 2).

Cumulative intake of carbohydrate is plotted against that of protein for the four treatments in Fig. 3. We used data on the protein and carbohydrate content of fresh aloe pollen (Human and Nicolson, 2006) and fresh and stored sunflower pollen (Nicolson and Human, 2013), as well as the measured 20.3% protein for mixed stored pollen. Because this value is intermediate between the protein contents of stored aloe and sunflower pollens, we also assumed intermediate values for the carbohydrate content (70.4% dry mass) and water content (18.5% wet mass) of mixed stored pollen. Values for pollen protein and carbohydrate content were converted to a fresh mass basis, and carbohydrate intake in Fig. 3 is the sum of carbohydrate in the pollen consumed and half the mass of sucrose solution consumed. The steep rise in the second week reflects the predominance of sucrose consumption over pollen consumption, especially for the two stored pollens. P:C ratios at days 7 and 14, obtained from the same data, are presented in Table 2.
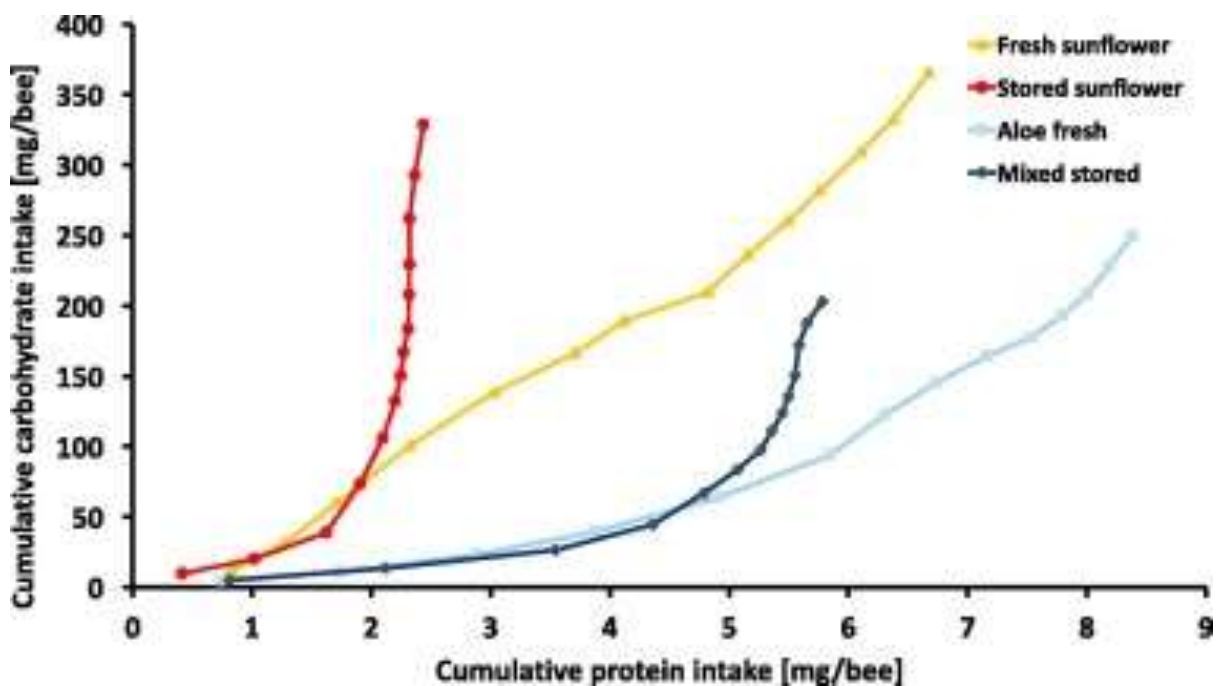

Figure 3. Cumulative intake of carbohydrate and protein for bees on the four pollen treatments. Protein is calculated from the protein content of each pollen type; carbohydrate is the sum of pollen carbohydrate and sucrose consumption.

Table 2. Protein to carbohydrate (P:C) ratios based on cumulative consumption on days 7 and 14 of the experiment (see Fig. 3).

Pollen	Day 7	Day 14
Sunflower fresh	1:46	1:55
Sunflower stored	1:60	1:135
Aloe fresh	1:19	1:30
Mixed stored	1:18	1:35

3.2. Survival

Survival of bees fed different pollen diets was significantly different (χ^2 = 70, df = 3, p <0.001) (Fig. 4). Survival of bees fed stored and fresh sunflower pollens was not significantly different (T = -1.1, p = 0.25). All other comparisons were significant, with the highest survival on fresh aloe pollen, followed by the two sunflower pollens, and the mixed stored pollen being the treatment with the least survival: sunflower fresh and aloe fresh (T = 5.9, p <0.001); sunflower fresh and mixed stored (T = 2.4, p <0.015); sunflower stored and mixed stored (T = 3.3, p <0.001); aloe fresh and sunflower stored (T = 6.1, p <0.001); and aloe fresh and mixed stored (T = 7.7, p <0.001).
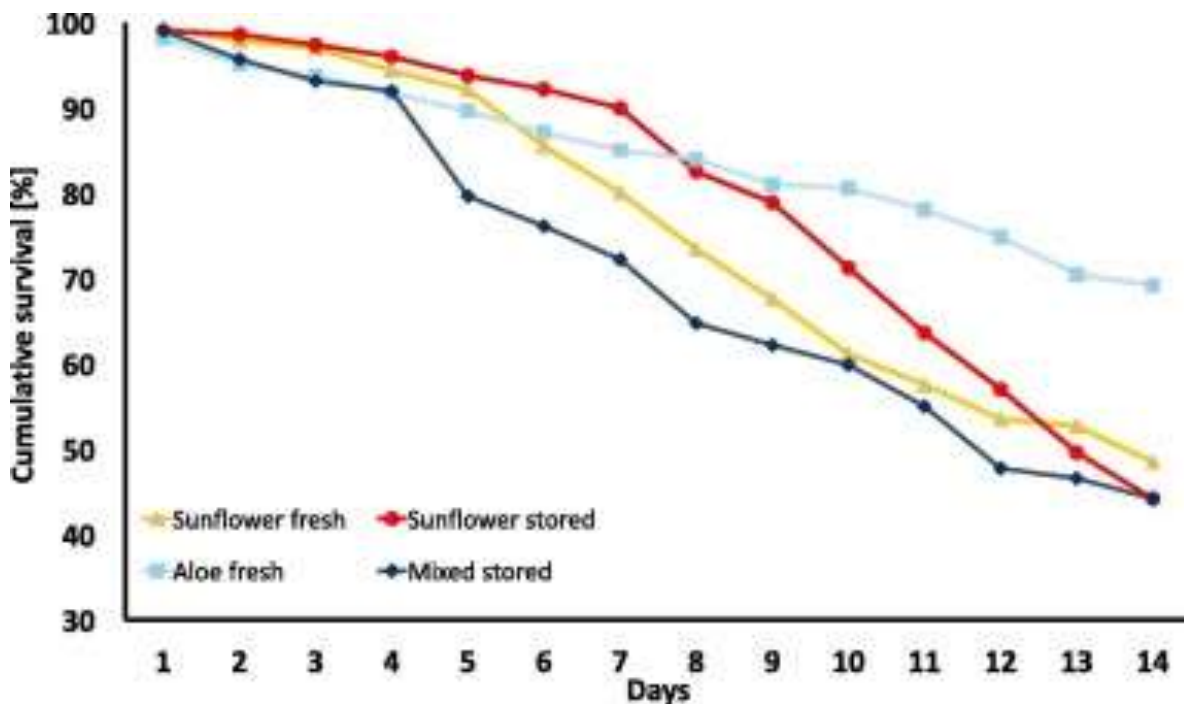
Figure 4. Cumulative survival of workers on the different pollen treatments.

3.3. Ovarian activation

Ovarian activation in bees fed different pollen diets was significantly different ($H_{3,645}$ = 89, p <0.001).
Bees fed mixed stored pollen had higher levels of ovarian activation than the other three groups (for
all three comparisons Z >3.859, p <0.001) (Fig. 5).
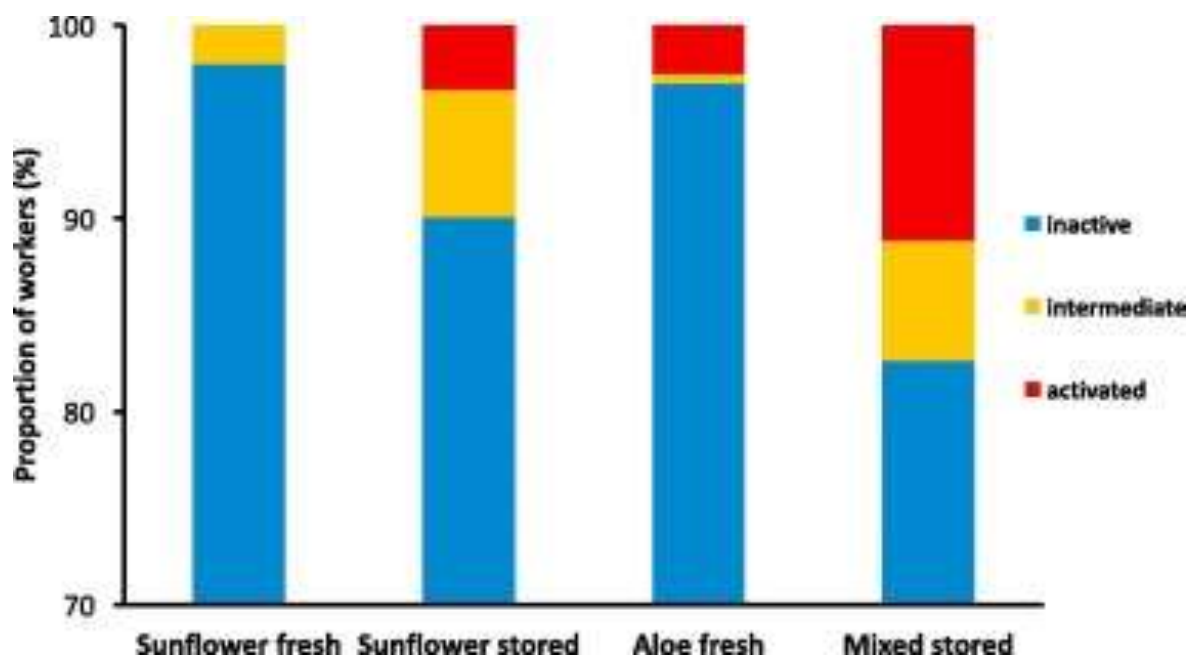
Figure 5. Ovarian activation of workers fed the different pollen types. Ovarian activation was assessed
according to Hess (1942) and Schäfer et al. (2006). The figure shows the proportion of workers showing
inactive, intermediate or activated ovaries.

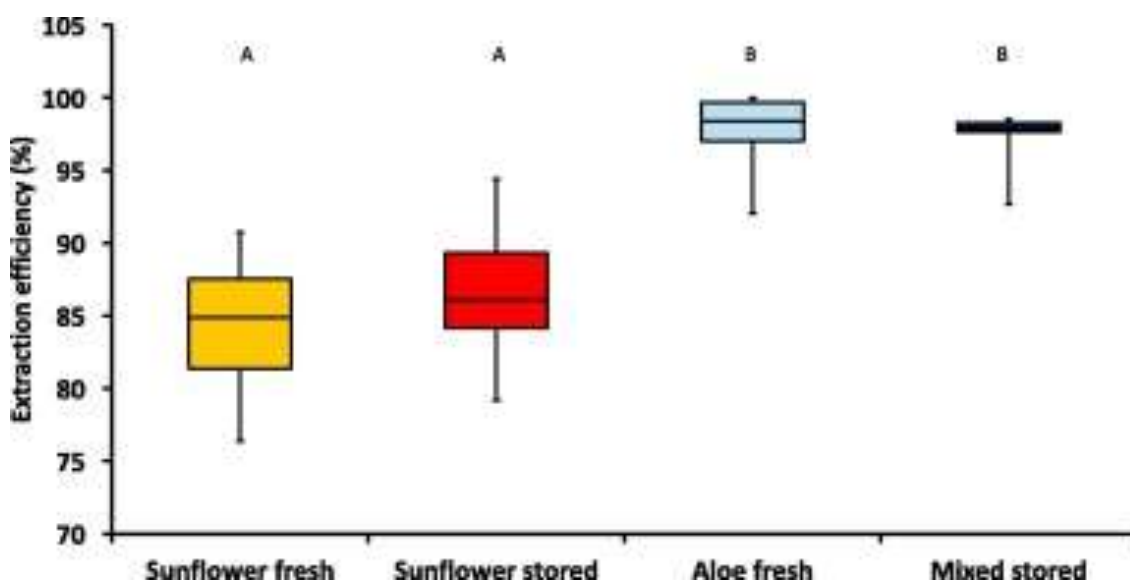### 3.4. Pollen extraction efficiency

Table 3 shows the percentages of full, half-full and empty pollen grains in the rectal contents of bees
fed the various pollen types. The percentage of half-full grains was much higher in the rectum of
bees fed sunflower pollen, both fresh and stored. There was a significant difference in extraction
efficiency between the different pollen types ($H_{3,200}$ = 147, p <0.001; Table 4). Fresh aloe pollen and
mixed stored pollen were digested with higher efficiency than sunflower pollen, but there was no
difference between fresh and stored sunflower pollens (Fig. 6).

Table 3. Percentages (± SD) of full, half-full and empty pollen grains in the reference pollen (before digestion) and the rectal contents of dissected honeybees (after digestion).

	Full	1/2 full	Empty
Sunflower fresh: reference	98.41 ± 1.65	0.25 ± 0.47	1.34 ± 1.61
Sunflower fresh: rectum	1.02 ± 1.72	28.96 ± 7.94	70.02 ± 8.02
Sunflower stored: rectum	1.47 ± 2.22	23.83 ± 12.20	74.70 ± 11.97
Aloe fresh: reference	94.77 ± 4.65	0.34 ± 0.72	4.89 ± 4.37
Aloe fresh: rectum	0.10 ± 0.44	1.34 ± 2.04	98.56 ± 2.09
Mixed stored: reference	80.30 ± 6.74	1.20 ± 1.66	18.50 ± 7.70
Mixed stored: rectum	0.02 ± 0.43	0.70 ± 2.35	99.28 ± 2.28

Table 4. Differences in extraction efficiency between the different pollen types. Multiple comparisons for Kruskal-Wallis ANOVA, significant differences in bold.

	Sunflower fresh	Sunflower stored	Aloe fresh	Mixed stored
Sunflower fresh		P = 1, Z = 1.006	**P < 0.0001, Z = 9.46**	**P < 0.0001, Z = 8.62**
Sunflower stored			**P < 0.0001, Z = 8.45**	**P < 0.0001, Z = 7.61**
Aloe fresh				P = 1, Z = 0.84

Figure 6. Extraction efficiency for the four pollen types in the rectum of honey bees. Data are given as median and IQR (N = 5).

4. Discussion

This study compared consumption and digestion of fresh and stored pollens differing in protein content. Because bees at the aloe field site collected other pollens, we are unable to compare fresh and stored aloe pollen. Comparing fresh and stored sunflower pollen shows that our predictions for consumption of pollen and sucrose were not met. Bees ate more of the fresh than stored sunflower pollen and consumption of sucrose solution did not differ for the two sunflower pollens. Survival was greatest on the fresh aloe pollen, in spite of its high protein content, and ovarian activation was highest on mixed stored pollen. Extraction efficiency was extremely high on fresh aloe and mixed stored diets, and lower on both sunflower diets; however, storage of sunflower pollen did not make it easier to digest.

Although stored sunflower pollen has half the protein content of fresh sunflower pollen (Nicolson and Human, 2013), bees did not increase overall consumption of the stored pollen to compensate for this. Instead, consumption of fresh sunflower pollen was higher. However, we observed that some of the fresh pollen provided to the bees was discarded inside the cages and could not be accurately weighed due to contamination with impurities such as wax. Consumption of fresh pollen could therefore have been overestimated. Feeding experiments are not usually carried out with fresh pollen because of its powdery consistency and the difficulty of collecting enough of it. *Pinus* pollen has been mixed with 30% sucrose solution for feeding to honey bees (Pernal and Currie, 2000), and used to study foraging of bumblebees at artificial flowers, where they collect less pollen when sugar rewards are absent or of lower concentration, probably because of the need for sugar in pollen packing in the corbiculae (Konzmann and Lunau, 2014). Similar to our findings, previous studies have also found that caged bees do not alter their consumption of bee-collected pollens according to protein content (Basualdo et al., 2013; Pernal and Currie, 2000). Overall consumption of sucrose solution did not differ between bees provided with the two sunflower pollens, even though added sugar is already present in the stored pollen.

Consumption patterns varied over the 14 days of the experiment. Pollen intake peaked early, then consumption of the two stored pollens was very low during the second week. A similar pattern has been found for consumption of bee-collected *Castanea sativa* pollen and other pollen diets (Omar et al., 2016). In all treatments, sucrose consumption peaked during the first week, dropped, then increased again during the second week. Caged worker bees provided with amino acids as protein source also consume more sucrose in the second week (Paoli et al., 2014). Pollen consumption is

reduced when honey bees transition from nurse bees to hive bees and then to foragers (from about 12 to 18 days after eclosion); more sugar than protein is necessary for wax secretion by older hive bees (Hepburn et al., 2014) and in preparation for foraging flights (Crailsheim et al., 1992; Winston, 1987). We have previously observed wax glands becoming active in caged bees (Altaye et al., 2010), and in a study of honey bee responses to nectar nicotine, where pollen was not provided, we found that higher sucrose uptake in the second week was due to storage of nectar in pieces of comb provided in the cages (Köhler et al., 2012). In the present study we did not examine the comb at the end of the experiment. Stored pollen was consumed in greater quantities than fresh pollen by very young bees; it may be that stored pollen is more palatable than fresh pollen because the added sugar imparts a sweeter taste (Konzmann and Lunau, 2014).

The cumulative intakes of carbohydrate and protein represented in Fig 3 confirm that consuming stored pollens does not lead to higher overall carbohydrate intake, and show that protein intake varies more than carbohydrate intake. There is also no convergence of P:C ratios on the different protein diets. Trajectories for the two sunflower pollens were similar in the first few days, after which bees virtually stopped eating the stored sunflower pollen and intake was mostly carbohydrate (shown by the near vertical line). The geometric framework modelling approach (Simpson and Raubenheimer, 2012) allows determination of intake targets in choice experiments with two or more imbalanced complementary diets. Previous studies with honey bees and bumble bees have shown intake targets, expressed as P:C ratios, biased towards carbohydrate, and that protein regulation is less strong than that of carbohydrate. These intake targets vary with the protein sources used, such as casein in agar-based diets or essential amino acids in liquid diets (Altaye et al., 2010; Archer et al., 2014a; Archer et al., 2014b; Stabler et al., 2015). Intake targets also vary with worker age, becoming more biased towards carbohydrate in the second week of adult life (Paoli et al., 2014). We also found reduced P:C ratios in the second week of adult life, due to the reduction in pollen consumption in all pollen treatments, especially stored sunflower pollen. Instead of complementary artificial diets, our experiment allowed bees to select a mix of pollen (their natural protein source) and sucrose solution. Importantly, the P:C ratios achieved on our pollen treatments are broadly comparable to those in other studies on both honey bees and bumble bees provided with casein-based diets. For honey bees, these include P:C ratios ranging from 1:6.5 (Archer et al., 2014b; Démares et al., 2016) to 1:150 (Paoli et al., 2014). Although the study of Vaudo et al. (2016) was focused on protein-to-lipid ratios, their data on consumption show P:C ratios of 1:69 and 1:85 in two bumblebee species.

Survival was similar across treatments for the first four days, when pollen consumption was high. The fresh aloe pollen with the highest protein content (and moderate consumption of sucrose solution) resulted in the highest survival at the end of the experiment. This is surprising because other studies have shown that artificial diets high in protein or essential amino acids shorten the lifespan of honey bees (Archer et al., 2014b; Paoli et al., 2014; Pirk et al., 2010). Although we previously obtained poor survival on no-choice diets containing pollen of *Aloe g.* var *davyana*, this may have been due to inadequate carbohydrate intake (Pirk et al., 2010). High protein pollens have been shown to lengthen the life of honeybees exposed to *Nosema*, but in the same study the quality and diversity of pollen diets did not affect survival of healthy bees (Di Pasquale et al., 2013). Using pollen mixes, Frias et al. (2016) found that survival increased with higher dietary protein but decreased in the case of mixes with a higher percentage of Asteraceae pollen, due to lower consumption of these mixes. Also using pollen mixes, Di Pasquale et al. (2016) found lowest survival when the pollen was collected during mass flowering of maize: this pollen is deficient in histidine (Höcherl et al., 2012).

Ovarian activation in worker honeybees is normally suppressed by the pheromones produced by the queen, brood and other workers (Pirk et al., 2011). However, with a high protein diet, especially under queenless conditions, workers can activate their ovaries (Hoover et al., 2006; Human et al., 2007; Lin and Winston, 1998; Pirk et al., 2011; Velthuis, 1970). We observed the highest ovarian activation in honeybee workers fed mixed stored pollen and the lowest in those fed fresh sunflower pollen. In a previous study we compared the ability of aloe and sunflower pollen to support ovarian activation of queenless workers (Human et al., 2007). Three colonies maintained in a sunflower field exhibited a significantly smaller number of workers with developed ovaries than when they were later moved to the aloes at Roodeplaat Nature Reserve. In the laboratory, however, the results differed: caged bees fed bee-collected sunflower pollen in bee candy showed higher ovarian development than those fed aloe pollen (Human et al., 2007). We suggested that in the field honeybees were supplementing their diet with nectar to achieve a balanced macronutrient ratio and this resulted in increased ovarian activity (Human et al., 2007). Mixed stored pollen, which gave the best ovarian activation, was also the diet that led to the lowest P:C intake ratio (Table 2). Increased dietary protein does not necessarily enhance ovarian activation (Frias et al., 2016), but this might be an artefact of the low protein content (8-18%) of the pollen used. We have previously shown that diets based on royal jelly result in higher ovarian activation than those based on other protein sources (Altaye et al., 2010; Pirk et al., 2010). It is possible that mixed stored pollen, which

supported the highest ovarian activation, may have contained more of some nutrients, similar to the ones found in royal jelly, required for ovarian activation.

The extraction efficiency of honeybees fed fresh aloe pollen was extremely high at 99%, and values for stored aloe pollen, if available, could not have exceeded this. Extraction efficiencies for stored and fresh sunflower pollen were 87% and 85% respectively, and there was no improvement with storage. In bees fed sunflower pollen (fresh and stored), the rectal contents had a high proportion of half-full grains (see Table 3) so for this plant species including half full grains in the formula is important. Without including half full grains, Human et al. (2007) reported a lower value of 70% for the rectal contents of honeybees sampled from hives on sunflower fields. The higher extraction efficiency for aloe pollen than for sunflower pollen may be due to pollen grain morphology. Aloe pollen grains are larger, so that more cytoplasm is extracted after enzymes have penetrated the pores; they are also smoother with much less pollenkitt and exine ornamentation than sunflower pollen and Asteraceae pollen in general (Human and Nicolson, 2003; Human et al., 2007). The dominant liliaceous pollen collected by colonies in Roodeplaat Nature Reserve had similar features to aloe pollen grains (smooth, no pollenkitt or ornamental exine) but was smaller. Fresh aloe and mixed stored pollens were digested with similar efficiency. The structure of the pollen grains may play a bigger role in digestibility than whether the pollen has been stored or not.

Extraction efficiencies provide a measure of pollen digestibility. Comparing the extraction efficiencies for fresh and stored sunflower pollen (the first time this has been done for the same species) shows that storage of sunflower pollen did not facilitate digestion. Storage also does not improve its nutritional value (Nicolson and Human, 2013). In stingless bees, which also store pollen in the colony, Fernandes da Silva and Serrão (2000) found no difference between bee-collected and stored pollen in terms of apparent digestibility, or hypopharyngeal gland development of the bees that consume it. Our findings are in agreement with those of Anderson et al. (2014), who found no difference in bacterial richness and diversity between newly collected and stored pollens and concluded that the stored pollen environment is not suitable for microbial growth, but is rather a preservative environment, like honey. These authors also showed that stored pollen maintained its structural integrity. Honey bees prefer to consume freshly stored pollen and do not obtain nutritional advantages when consuming older stored pollen (Carroll et al., 2017). In contrast, stingless bees prefer stored pollen to corbicular pollen (Vollet-Neto et al., 2017). Consumption of relatively fresh pollen by honey bees appears to be better for colony microbial health (Maes et al.,

2016). It is clear that we need to know much more about the changes to pollen during processing and storage, and whether these have nutritional and health benefits for bees.

Conflicts of interest: none

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