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Crushing corn pollen grains increased diet digestibility and hemolymph protein content while decreasing honey bee consumption

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Abstract – A honey bee colony's ability to grow and develop is dependent on adequate nutrition. Bees collect pollen from flowers as a source of protein, fat, vitamins, and minerals. The crude protein content of corn pollen is considered low, around 15%; however, bees frequently visit the male flowers of the tassels for pollen. In this study, we aimed for the first time to improve the nutritious value of corn pollen by mechanically crushing its external pollen wall. We then compared the effect of feeding crushed vs. non-crushed corn pollen grains on honey bee diet consumption, digestibility, hemolymph protein content, hypopharyngeal gland (HPG) size, and thorax weight under laboratory conditions. We found that crushing corn pollen grains increased diet digestibility and hemolymph protein content while decreasing honey bee pollen consumption (–39.88%). Crushing pollen however had no effect on HPG size or thorax weight. These findings may be beneficial to beekeepers in areas where corn monoculture is prevalent. The effect of crushed corn pollen on larval development and growth, as well as colony development and vitality, should be investigated in future studies.

Honey bees / Monodiet digestibility / Hemolymph protein concentration / Hypopharyngeal gland / Nutrition / Corn/zea pollen

1. INTRODUCTION

The western honey bee (*Apis mellifera* L.) provides highly valued pollination services for a wide range of agricultural crops. They account for 80% of all insect pollinators (Klein et al. 2007; Khalifa et al. 2021a). However, in recent years, pollinator decline has been reported worldwide (Potts et al. 2010; Goulson et al.

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2015; Al Naggar et al. 2018). Colony losses are likely the result of the effect of multiple stressors (e.g., pesticides, pathogens, parasites, and poor nutrition) (Steinhauer et al. 2018; El-Seedi et al. 2022). Therefore, it has been proposed that the combination of nutritional stress, infections by pathogens, and pesticide exposure are among the most important driving forces (Naug 2009; Dainat et al. 2012; Tosi et al. 2017; Al Naggar and Baer 2019; Al Naggar and Paxton 2021; Alburaki et al. 2022; Filipiak et al. 2022).

Adequate nutrition is a honey bee colony's basis for growth and development (Brodschneider and Crailsheim 2010). Because landscapes are increasingly characterized by agriculturally intensive monocultures, and because honey bee pollination services frequently occur within a human-defined ecosystem, bees' nutritional needs may not be adequately met (Naug 2009). In this context, "food resource deficiency" refers to both a shortage of food and the lower nutritional quality of food sources that are available in inhabited habitats. Therefore, there are inconsistencies between what bees need and what their environment can give, which contributes to the decline of bee populations (Vaudo et al. 2015; Hemberger et al. 2021; Wilson et al. 2021; Filipiak et al. 2022). As a result, the question of whether and how bees should be provided with supplemental food when nutritional deficits occur arises.

Pollen is the main natural source of protein for adult bees after emergence (do Carmo Zerbo et al. 2001; Brodschneider and Crailsheim 2010). Pollen protein content and quality are also factors that influence honey bee colony longevity and performance (Dietz 1975; Omar et al. 2017). Pollen grains are collected by honey bees from a wide variety of plant species (Schmidt, 1984; Khalifa et al. 2021b), and the nutritional composition of pollen varies greatly depending on the plant species (Stanley and Linskens 1974; Roulston and Cane 2000; Omar et al. 2017). The protein content of bee pollen is the most important characteristic and is primarily used to categorize bee pollen quality as excellent (> 25%), average (20–25%), and poor (20%) (Somerville 2001). Protein

concentrations vary greatly between plant species, ranging from 2.5 to 61%, with an average of 25–45% (Roulston and Cane 2000; Weiner et al. 2010; Bryś et al. 2021). Cactaceae and Onagraceae plants have the lowest protein content (15–25%), whereas Melastomataceae, Cochlospermataceae, and Solanaceae have the highest protein content (over 51%) (Roulston and Cane 2002). According to Nicolson and Human (2013), bees kept near crop monocultures such as sunflowers, which have poor pollen quality, may have poor development. Pollen also contains some lipids, sterols, vitamins, minerals, and carbohydrates. Wind-pollinated plants, such as corn (Hofmann et al. 2014), which covers thousands of acres in different parts of the world International Grains Council (international organization) (2013), produce a lot of pollen, but it has a low nutrient protein content of about 15%, which is why it is considered poor pollen. As a result, the question of whether and how corn pollen's nutritional value could be increased arose.

Indeed, the pollen's nutritional value is primarily determined by the efficiency with which bees digest pollen. Pollen digestibility is thought to be affected by two factors: the thickness of the pollen wall, which is made of intine or exine, and the number of germination pores (Roulston and Cane 2000). The intine is mostly made up of pectin and cellulose. The exine layer is composed of sporopollenin and is coated by a pollenkitt, which is composed of proteins, sugars, and lipids (T'ai and Cane 2000). Bees can not digest pollen grains wall due to their chemical nature (Heslop-Harrison and Heslop-Harrison 1991), but they can digest and extract protein nutrients through the germination pore (Peng et al. 1985).

Because animals that feed on pollen vary in size, behavior, and physiology, there is a wide range of pollen digestion mechanisms: (1) mechanically crack open the pollen wall, (2) pierce the pollen wall with sharp mouthparts, (3) dissolve the pollen wall with enzymes, (4) induce germination or pseudo-germination, (5) osmotic shock bursts the pollen wall, and (6)

digestive enzymes penetrate the pollen wall (Roulston and Cane 2000). The first three of these methods are uncommon, and the last three are difficult to identify. Pollen digestion can be difficult due to the multiple cell wall layers that cover the nutritional cytoplasm. Kroon et al. (1974) hypothesized that differences in osmotic pressure between the bee crop and the midgut could cause pollen grains to break, exposing the nutrient cytoplasm. Other studies, however, rejected this hypothesis, finding a high number of undamaged pollen grains in the midgut (Klungness and Peng 1984a; Peng et al. 1985). Furthermore, Moritz and Crailsheim (1987) and Dobson (1988) reported that the external structure of the pollen grain wall, particularly exine, was found to be indigestible. As a result, the percentage of empty pollen grains in bee feces was used to assess digestion performance (Roulston and Cane 2000). Furthermore, Crailsheim et al. (1992) discovered a difference in the percentage of empty grains between two different types of pollen (*Castanea sativa* MILL. and *Trifolium repens* L.).

In this study, we attempted for the first time to improve the nutritious value of corn pollen, by crushing its external pollen wall mechanically. We then tested the effect of feeding bees with crushed vs. non-crushed corn pollen grains on bee diet consumption, digestibility, hemolymph protein content, hypopharyngeal gland (HPG) size, and thorax weight under laboratory conditions. We hypothesized that shattering corn pollen's exterior wall would modulate diet consumption and digestibility as well as honey bee worker physiology.

2. MATERIALS AND METHODS

The laboratory experiments were conducted in the Economic Entomology research laboratory of the Department of Plant Protection, Assiut University, in Assiut, Egypt, during the active season of honey bee colonies (May till July 2021).

2.1. Pollen loads collection

Pollen traps were used to collect pollen loads collected by bees from the entrance of hives every day during the corn flowering season. Pollen loads were separated based on color, then dried in an oven under 35 °C until weight consistency was achieved to remove all water from the pollen grains, which aids in preservation, and stored at −20 °C until use.

2.2. Preparation of crushed pollen

To rupture the pollen cell layers, the frozen pollen load was crushed with a ceramic mortar and pestle, then sieved and crushed several times until a fine powder was formed. Then, using a scanning electric microscope (JSM—5400) at the Electron Microscope Unit, Assiut University, Egypt, we scanned the surface of these fine and crushed pollen grains to ensure that the grinding was sufficient to disrupt their cell wall (Figure 1).

2.3. Honey bees

Three colonies of *A. mellifera carnica* hybrid maintained in the research apiary of the Plant Protection Department at the Faculty of Agriculture, Assiut University, Egypt, was used in the experiments from May to July 2021. Colonies had no visible honey bee diseases and had adequate bee bread, honey stores, and a large number of young honey bee workers. All colonies were treated with formic acid to control *Varroa* mites and *Varroa* levels have been checked before the experiment.

To obtain newly emerged honey bee workers (0–24 h old), three sealed brood combs ready to emerge from the three honey bee colonies were transferred and placed in an incubator overnight at 34.5 °C and 65% RH (Williams et al. 2013). The following day, we collected newly emerged bees from all brood combs, combined them, and then randomly assigned them to different treatments. All the cages used

Corn pollen grains

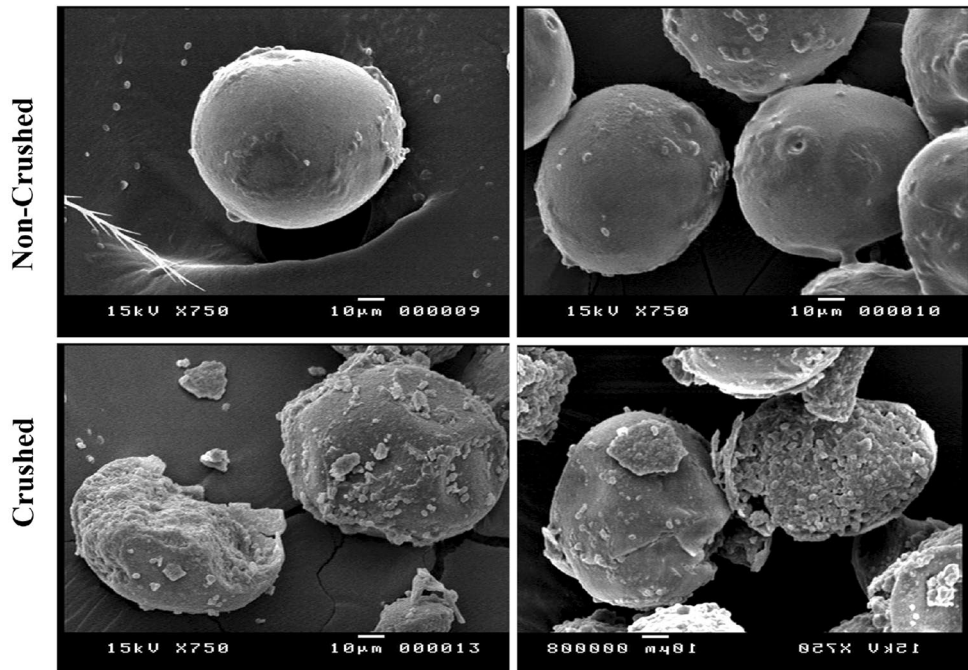


Figure 1. Scanning electron microscope photographs showing crushed and non-crushed corn pollen grains.

in this study were made from pellucid plastic cups (Evans et al. 2009). For ventilation, the spouts of the plastic cups were covered with an iron net. Cups were provided with wax comb to simulate the natural colony conditions for honey bees (Williams et al. 2013). Two holes were drilled in the cup: one in the bottom for introducing sugar solutions with a 5 ml syringe and one on the side for presenting protein diets with a half of a 2 ml Eppendorf type shaped like a dish (Figure 2A).

2.4. Experimental design

We carried out two separate experiments in similar manner to determine whether crushed pollen could modulate diet consumption and digestibility as well as honey bee worker physiology (Figure 2B).

2.4.1. Diet consumption and digestibility

This experiment consisted of two treatment groups: non-crushed and crushed corn pollen each with three cages containing 30 newly emerged bees. Bees were fed pollen diets (1 g per cage), either whole or crushed, and sugar syrup for 10 days, after which the pollen dish were removed, and the bees in all treatments were fed only sugar syrup ad libitum for another 4 days. Diet consumption was measured and renewed daily for 10 days. Dead bees were removed from the cages and considered to not have consumed pollen that day.

To assess diet digestibility, a 12-day-old worker was used because nurse bee consumes and digests more protein diets than older bees and to ensure that they had completed all the digestion and the rectum would not receive any more (Crailsheim et al. 1992; Omar et al. 2017). To determine digestibility, protein concentrations

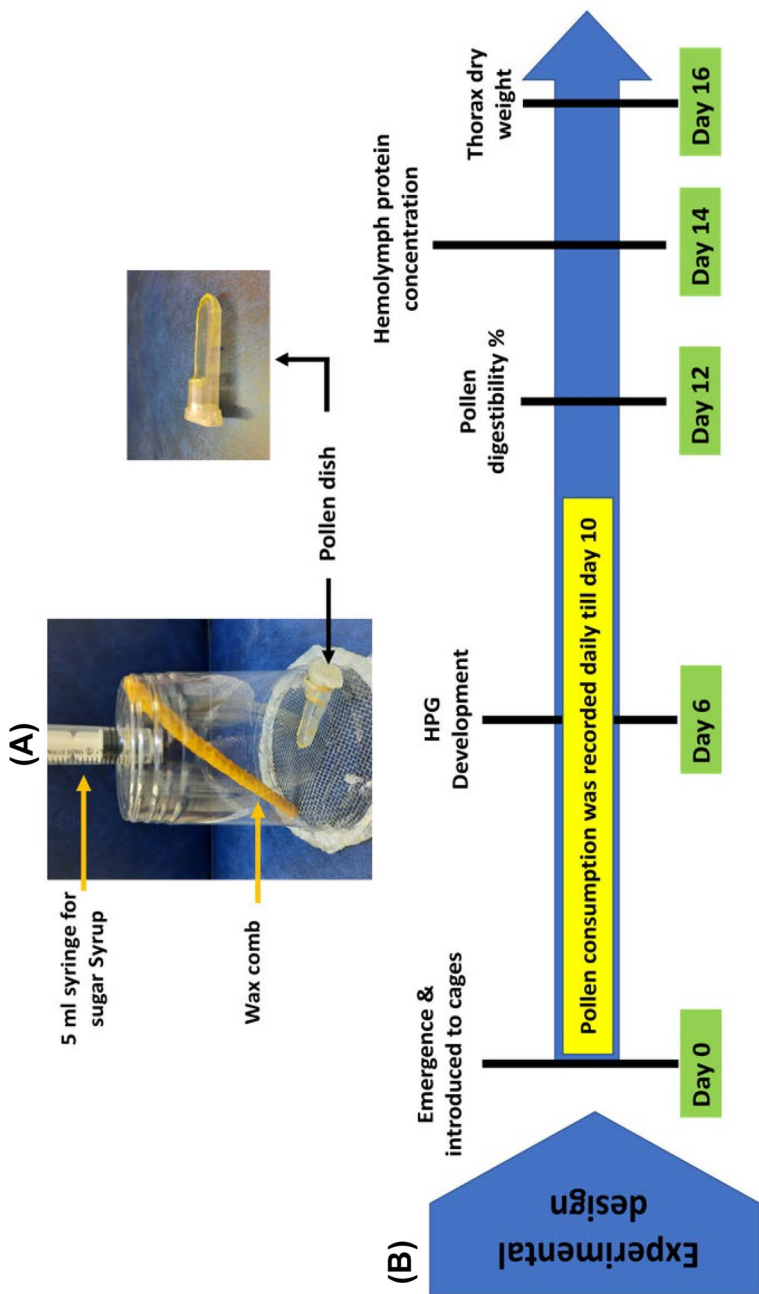


Figure 2. **A** The cages used in the experiments and **B** the experimental design, which show the duration of pollen feeding and sampling time of bees for HPG measurement, pollen digestibility, hemolymph protein concentration, and thorax dry weight.

in the hindgut contents of a 12-day-old worker were examined and compared to concentrations in maize pollen (DeGrandi-Hoffman et al. 2016). Five bees were randomly selected and dissected from each cage, then the hindgut of each bee was extracted and weighted, and a 1- μ l of the hindgut contents was mixed with 99 μ l of PBS and 1% EDTA-free HPIC. The solutions were then mixed, and 100 μ l of this combined solution was added to 900 μ l of PBS with 1% EDTA-free HPIC, which was then stored at 80 °C until total soluble protein was measured using a spectrophotometer (752 UV/Vis Spectrophotometer, Jing hua Instruments, Inc., Shanghai, China) at 595 nm (Bradford 1976; Cremonz et al. 1998). Protein concentrations in corn pollen were also measured using three samples chosen randomly from each diet. Using 1 mg of the diet added to 999 μ l of PBS with 1% EDTA-free HPIC and analyzed as described above. The approximate digestibility was calculated according to the following equation (Eq. 1) (Slansky and Scriber 1985; DeGrandi-Hoffman et al. 2016).

$$\text{Approximate digestibility} = \frac{\text{Protein conc. in diet} - \text{Protein conc. in hind gut}}{\text{Protein conc. in diet}} \times 100 \quad (1)$$

Then, we quantified the total protein content in the hemolymph of 14 days old bees. Bees were chosen randomly from each cage and hemolymph was collected using the method described by Borsuk et al. (2017). Briefly, the bee antenna was separated, then careful pressure was applied to the bee's abdomen. This caused a drop of hemolymph to appear at the base of the removed antenna, which was collected with a pipette. Hemolymph from each cage ($n \sim 20$ bees) was pooled and samples were stored at -80 °C. Total protein content was measured using 1 μ l of hemolymph sample that was mixed with 99 μ l of PBS and 1% EDTA-free HPIC and then analyzed as described above.

2.4.2. HPG development and thorax weight

We measured HPG acini volume and thorax weight to see if crushed pollen had any effect

on honey bee worker physiology. To do that, the same treatments as described above were tested, each with three cages, and each cage contained 30 newly emerged worker bees. Bees were fed pollen diets, either whole or crushed, and sugar syrup for 10 days, after which the pollen dish were removed, and the bees in all treatments were fed only sugar syrup ad libitum for another 6 days. Seven bees were chosen at random from each cage at the age of 6 days to measure the HPG acini volume using a 100-fold magnification ocular micrometer (SP1 10/0.25) microscope Reichard Diavar, as reported in Omar et al. (2017). We used 6-day-old bees because the maximum HPG development has been reported to be between 5 and 9 days (Moritz and Crailsheim 1987; Altaye et al. 2010; Omar et al. 2017).

At the end of the experiment, sub-samples of surviving bees ($n = 10$ bees per cage) were sampled at the age of 16 days to determine the fresh and dry weight of the thorax. Each bee sample was dissected into three sections:

head, thorax, and abdomen. Each thorax was weighted with wings and legs to determine fresh weight before being placed in a 55 °C oven for 5 days and reweighted to determine the dry weight to investigate the potential effect of diet on thorax muscles development (Brodschneider and Crailsheim 2010; Hendriksma et al. 2019).

2.5. Statistical analysis

The data were analyzed using GraphPad Prism 8.00 for Windows (www.graphpad.com). A Student *t*-test was used to compare the effects of crushed vs. non-crushed corn pollen grains on diet consumption, diet digestibility, hemolymph protein content, HPG acini volume, and thorax dry weight. A significance level of 0.05 was used to define a test's significance.

3. RESULTS

3.1. Diets consumption, digestibility, and hemolymph protein content

Diet consumption in bees fed crushed corn pollen grains was significantly lower (-39.88%) than in bees fed non-crushed corn pollen grains (Student t -test; $df = 4$, $P = 0.003$) (Figure 3a). There was a

significant increase in the percentage of diet digestibility in bees fed crushed corn pollen grains compared to bees fed non-crushed corn pollen grains (Student t -test; $df = 4$, $P = 0.02$) (Figure 3b). As a result of the increase in diet digestibility, total hemolymph protein content increased significantly in bees fed crushed corn pollen grains versus bees fed non-crushed pollen grains (Student t -test; $df = 4$, $P = 0.04$) (Figure 3c).

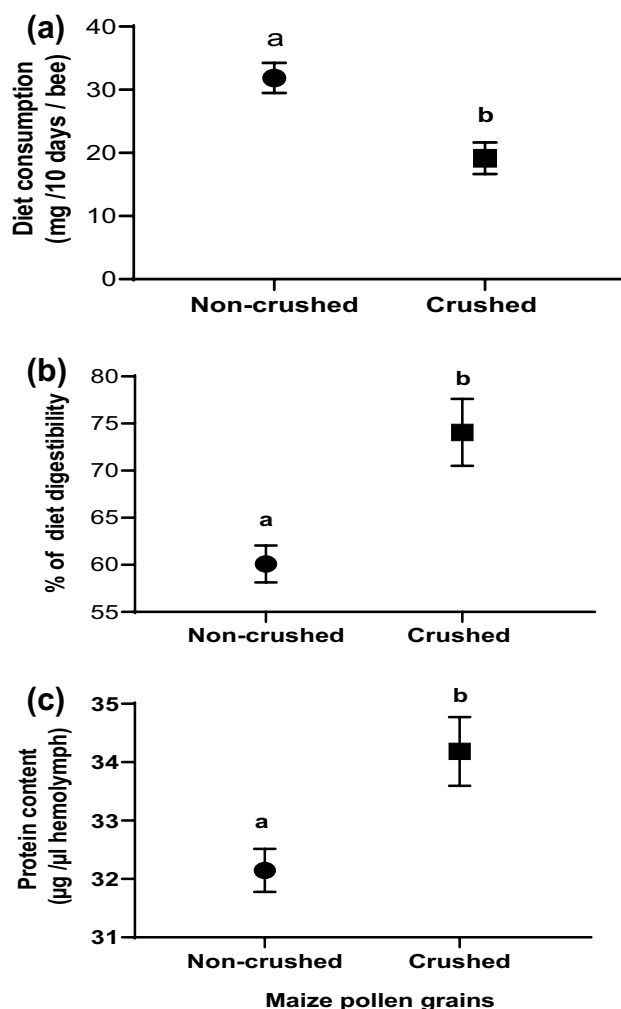


Figure 3. Showing means \pm standard error mean (SEM) of **a** diet consumption, **b** % of diet digestibility ($n=3$ pooled samples of 5 bees from each cage), and **c** hemolymph protein content ($n=3$ pooled samples of ~ 20 bees from each cage) in bees fed crushed vs. non-crushed corn pollen grains for 10 days. Different lowercase letters denote a significant difference between treatments (Student t -test, $P < 0.05$).

3.2. HPG development and thorax dry weight

The HPG acini volume was measured at age of 6 days old, and there was no significant difference between bees fed crushed vs. non-crushed corn pollen grains (Student *t*-test; $df = 38$, $P = 0.42$) (Figure 4a). We also quantified the dry weight of the thorax as an indicator of flight muscle development at the end of the experiment when bees are 16 days old and found no

significant difference between bees fed crushed vs. non-crushed corn pollen grains (Student *t*-test; $df = 38$, $P = 0.42$) (Figure 4b)

4. DISCUSSION

Nutrition is the term used to describe the process of obtaining nutrients from the outside in order to meet the energy requirements for maintaining vitality. Just like every living species,

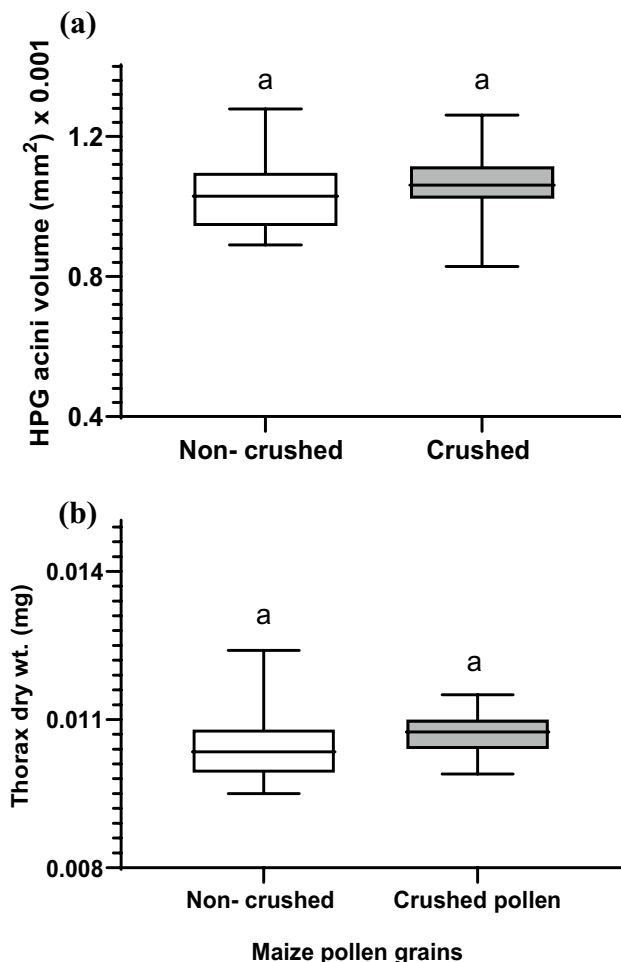


Figure 4. The effect of feeding bees crushed corn pollen grains vs. non-crushed grains for 10 days on **a** HPG acini volume of 6-day-old bees and **b** dry weight of thorax region of 16-day-old bees. Symbols on the box plot represent maximum and minimum values ($n=21$ bees) (whiskers: \top \perp), mean values (-), the top and bottom lines of the middle box represent the 25% and 75% quartiles. There was no significant difference between treatments (Student *t*-test, $P > 0.05$).

honey bees need nutrients to survive. Pollen is considered the primary source of protein for honey bees, supporting growth and development (Brodschneider and Crailsheim 2010). A worker bee in a colony needs 145 mg of pollen on average throughout its lifetime to complete its life cycle (Haydak 1970). In the current study, we crushed the external pollen wall of corn pollen, which is a poor protein source for bees as an attempt to increase its nutritional value. We found for the first time that crushing corn pollen grains increased diet digestibility and hemolymph protein content while decreasing honey bee consumption.

Animals that feed on pollen must overcome an indigestible or even chemically resistant pollen grain wall in order to reach the nutritious cytoplasm (Stanley and Linskens 1974). Pollen contains a variety of cell wall constituents such as cellulose, pectins, and sporollenin matrix, which may affect digestibility (Crailsheim et al. 1992; Roulston and Cane 2000). The current findings showed that total crushed corn pollen intake by bees during the nursing period was reduced by -39.89% when compared to bees fed normal corn pollen grains, while there was a significant increase in digestibility (23.24%) of crushed corn pollen versus non-crushed pollen. The destruction of the outer wall or diffusion through pores is required for the digestion of pollen grains. This could explain the increase in digestibility of crushed pollen grains observed in the current study. The digestibility of normal pollen grains ranges was reported to be from 30 to 70% (Klungness and Peng 1984b). This variation in digestibility of pollen grains depends on the degree of disruption of the intine and the exposure of pollen protoplasm to digestive enzymes (Klungness and Peng 1984a; Peng et al. 1985).

It is worth noting that the mechanism and effectiveness of pollen grain digestion differ between animals that feed on pollen and according to pollen species. Corn pollen, for example, breaks early in the midgut of maize beetles, unlike sunflower pollen, which has intact exines. It does, however, remain intact in honey bees' midguts (Human and Nicolson 2003), indicating

that osmotic shock is not as important to honey bees as previously assumed (Kroon et al. 1974) and pointing to another mechanism of digestion, such as enzymatic action. As a result, grinding corn pollen grains, which increased digestibility relative to non-grinded pollen in the current study, could be a new mechanical method to promote bee feeding and the nutritional value of low protein pollen sources. More research is required to explore the impact of crushed corn pollen on larval development and growth, even though larvae are fed a lot of jelly that has already been digested by nurse bees and digest pollen differently than adult bees because they do not have a crop (Roulston and Cane 2000).

The protein content of hemolymph is usually used to assess the efficacy of protein diets and pollen quality (Cremonz et al. 1998; Kunc et al. 2019; Smart et al. 2019; Noordyke and Ellis 2021). High protein concentrations increase bee lifespan, immunity, and resistance to pathogens such as *Nosema* spp. (Strachecka et al. 2015; Schulz et al. 2019; Skowronek et al. 2021). In the current study, bees fed crushed corn pollen grains had significantly higher total hemolymph protein content than bees fed non-crushed pollen grains. What is remarkable is that bees fed crushed corn pollen grains had higher protein content while consuming less than those fed non-crushed pollen grains. This could be attributed to the increased digestibility of ground pollen in the diet, as more amino acids are absorbed as a result of increased protein digestion (Wang et al. 2014). Because, according to the geometric framework (GF) for nutrition, animals require multiple nutrients at the same time and must achieve the optimal proportion of nutrients in their diet, they are forced to consume a variety of foods and/or make trade-offs by consuming too much or too little of certain nutrients in the foods they can eat (Raubenheimer and Simpson 1997).

On the other hand, when bees were fed a crushed pollen diet, the development of the HPG and thorax weight increased, but the difference was insignificant when compared to bees fed non-crushed pollen. Pollen consumption has been shown to be positively related to HPG

development (Hrassnigg and Crailsheim 1998; Corby-Harris et al. 2016) Furthermore, the type of pollen grains and its nutritional value influence HPG development and activity (Omar et al., 2017), hence further research into the effect of mechanical crushing on different types of pollen grain on HPG development and activity is required.

5. CONCLUSIONS

We revealed for the first time that crushing corn pollen grains, which is a poor protein source for bees, enhanced diet digestibility, and hemolymph protein content while reducing honey bee consumption. These findings could be useful for beekeepers in areas where corn monoculture predominates. Future studies should investigate the effect of crushed corn pollen on larval development and growth, as well as colony development and vitality.

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AUTHOR CONTRIBUTION

EMO: conceptualization, methodology, writing—original draft preparation. AO: pollen preparation and SEM photography. HD and HE: editing and review. YA: data analysis, visualization, writing—reviewing and editing.

DATA AVAILABILITY

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

CODE AVAILABILITY

Not applicable.

DECLARATIONS

Ethics approval No approval of the Research Ethics Committee was required to achieve the goals of this study, as the experimental work involved an unregulated invertebrate species (*Apis mellifera*).

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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