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The Impact of Pollen Diets and Pollen Substitutes on Some Physiological Traits of Honeybee Workers

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Abstract

Nutrition plays an important role in the health and development of honeybee castes. A protein-enriched diet improves bees' health and physiological condition. In this study, the effects of four different diets: (polyfloral pollen, monofloral pollen, soybean, and candy as a control) on the physiological characteristics of worker bees (hypopharyngeal glands, HPG), dry weight of thorax, longevity, and food consumption) were investigated. The data were recorded three times (4, 8, and 12 days) post-exposure to the tested diets. The results revealed that food consumption by worker bees was significantly higher in the pollen-tested diets (polyfloral and monofloral pollen) than in soybean and candy diets. Regarding longevity, there was no statistically significant difference among the four diets. There was a statistically significant difference in HPG and dry weight; the highest values were observed in pollen diets. These results suggest that diets containing protein and lipids support the health and development of honeybee workers.

Introduction

As the most economically significant pollinators, honeybees are essential to agricultural productivity and ecological stability worldwide (Garibaldi et al., 2011). However, the honeybee colony faces many challenges and threats that affect bee health. Pathogens, parasites, extensive pesticide usage, and climate change are contributing to honeybee losses (Larsen et al., 2019). Nutrition is a key factor regulating a range of phenomena in honeybee biology and development, including caste differentiation, growth, immunity, behavioral development, and lifespan (Mutti et al., 2011; Wang et al., 2013). Nectar or honey provides honeybees with carbohydrates, their energy source, while pollen provides the nutrients required for the development of their internal organs (Haydak, 1970). Pollen is the primary source of protein, lipids, vitamins, and minerals for bee nutrition. Low-protein nutrition decreases the lifespan of workers, honeybees' ability to resist disease (Matilla & Otis, 2006; Zahra & Talal, 2008), and immunity (Alaux et al., 2011; Larsen et al., 2019). Diets containing polyfloral pollen induced higher glucose oxidase activity (as a parameter of immunity) compared with monofloral pollen diets (Alaux et al., 2011). Bees fed with a high crude protein diet have significantly higher levels of total protein in hemolymph and fat bodies (Basualdo et al., 2013). During periods of pollen scarcity, protein-rich pollen substitutes, such as soybean and milk powder, may be used to feed the hives (Zahra & Talal, 2008; DeGrandi-Hoffman et al., 2010; Al-Ghamdi et al., 2011). The protein content of pollen varies between 2.5% and 61% (Roulston et al., 2000). The rearing of a larva requires 25 mg to 37.5 mg of protein. An adult bee consumes an average of 3.4–4.3 mg/day of pollen (Crailsheim et al., 1992). Feeding plays an important role in HPG development, and different diets affect gland development to varying degrees, depending on their nutritional values (Pinto et al., 2014). The activation

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of the HPG depends on the existence of a protein-based food. The availability of environmental nutrients is a key determinant of an organism's growth and survival (Li et al., 2007). Providing colonies with protein sources year-round, especially during pollen dearth periods, is critical. Recently, several studies have focused on formulating supplementary diets or substitutes to compensate for the lack of the natural protein source (Younis, 2019).

The current study evaluates the effects of pollen and pollen substitutes on body weight, longevity, food consumption, and the developmental stages of hypopharyngeal glands.

Materials and Methods

Honeybee race and rearing conditions

The experimental research was carried out under laboratory conditions at the Applied Entomology and Zoology Department, Faculty of Agriculture, Alexandria University, Egypt. The hybrid of the Italian (*Apis mellifera ligustica*) bee race was chosen to start the planned experiment. Frames of brood containing last-stage pupae were removed from a healthy hive and kept in an incubator under control conditions (30 ± 1 °C; 50-70% relative humidity) until the bees emerged.

Newly emerged workers were collected and held in groups of 40 individuals in perforated plastic cages (15 x 9 cm), with white muslin covering the tops of the cages (Renzi et al., 2016; Jones et al., 2018). The workers were randomly assigned to the different treatment groups. Four cages for each treatment were used. Each cage was equipped with a small vial as a feeder and a piece of wax to provide water and as a clustering plate for the bees. Bees were sampled at newly emerged (0 days), 4, 8, and 12 days old. Randomly, five bees from each cage were selected to estimate the following parameters: dry weight, longevity, food consumption, and HPG development.

Diet preparations

Four different diets were tested, as shown in Table 1. Candy was the first diet to contain only sugar and honey as a control. The second diet was a monofloral pollen diet containing 10% Egyptian clover pollen pellets. The third was polyfloral pollen, which contained 10% poly pollen pellets. The final one has 10% soybean. Each vial contained 15 g of diet to determine the amount of food consumed by bees. By weighing the leftover food and deducting it from the amount provided, the intake of the diet was calculated.

Table 1 - Types of the tested diets.

Diet	Sugar	Substance (10%)	Honey
Candy	75.0 gm	-----	25 gm
Monofloral	64.5 gm	10.5 gm (Egyptian clover)	25 gm
Polyfloral	64.5 gm	10.5 gm (polyfloral pollen)	25 gm
Soybean	64.5 gm	10.5 gm (soybean)	25 gm

Chemical analysis of Diets

Crude protein (%): The protein content was determined using the Kjeldahl method. Specifically, 0.20 g of ground diet samples were weighed and placed in digestion tubes. The samples were first digested with sulfuric acid in the presence of a catalyst. To accelerate the combustion reaction, 2 g of catalyst tablets were added, and the combustion was started. After combustion, the tubes were used in the Gerhardt apparatus, and the amounts of nitrogen and hydrochloric acid consumed were determined by titration. The nitrogen percentage obtained was multiplied by 5.6 to estimate the protein content.

Crude fat (%): The diet samples were dried in an oven at 50 ºC, and then, 2 g were placed on filter paper. The samples were then placed in the oil extraction apparatus for 6 hours, after which the oils were evaporated, and the remaining samples were weighed and calculated (Günesdogdu, 2024).

Measurements of the hypopharyngeal gland development

Bees at 4, 8, and 12-day-old were subjected to dissection for hypopharyngeal glands (n=5 bees per cage). Bees were frozen until dissection. HPG was removed from the head capsule and placed in a phosphate buffer solution. The HPGs were removed and separately spread on a glass slide for each bee. The external chitinous exoskeleton of the facial region of the head was removed between the compound eyes. The HPG was examined microscopically at X40 magnification.

An assessment of each gland was carried out to determine its developmental stage according to Maurizio (1954), as shown in (Fig. 1). For each individual bee, the developmental stage was recorded as the mean value of the two glandular lobes (left and right). The Gland Development Index (GDI) for each replicate was subsequently calculated using the weighted average formula:

$$GDI = \sum(i \times n_i)/N$$

where i is the developmental stage (1–4), n_i is the number of bees at that stage, and N is the total number of bees examined per replicate. Then, averages greater than 2.5 were considered well-developed HPGs. In contrast, averages below 2.5 indicated that HPGs were undeveloped. This formula converted qualitative development stages into quantitative (GDI) to facilitate objective statistical analysis between experimental groups.

Longevity

To measure longevity, the dead bee samples were collected and counted daily from each cage during all experiments to determine the number of days required for 50% of the population to die (El Barbary et al., 1980; Wang et al., 2014).

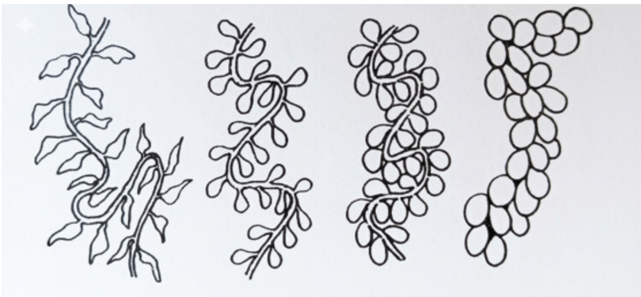

Fig.1. development stages of hypopharyngeal glands (HPGs). Stage 1. Main and side canals readily visible, gland lobes (acini) undeveloped, irregular, often forming nodes, transparent.
Stage 2. Main and side canals visible, gland lobes irregularly rounded, with clearly visible spaces between them, transparent.
Stage 3. Main canal visible, side canals mostly concealed by the tightly filled gland lobes. Gland lobes are transparent with small spaces between them.
Stage 4. Main and side canals are completely concealed, with no spaces visible between the gland lobes. Gland lobes developed to the maximum, milky -white or yellowish, and fully functional.

Dry weight

The samples were collected at specific times on the fourth, eighth, and twelfth days. Five bees were taken out of each cage; the thorax, including the legs and wings, was cut off with iris micro scissors. The thoraxes were then dried in an oven set to 102 ºC for 24 hours until they reached a consistent weight, after which they were weighed. (El Barbary., 1980).

Statistical Analyses

Statistical analysis was performed using GraphPad Prism software (version 5.01, GraphPad Software, Inc., 2007). Data are expressed as mean ± standard deviation. The Kolmogorov-Smirnov test was used to assess the normality of the parameters under study. The analysis of variance (ANOVA) was used, followed by the Bonferroni test, to compare mean values between and within treatment groups. Differences were considered statistically significant at p-value < 0.05.

Results

Nutritional analysis for crude protein and fat content

The nutritional evaluations of the studied diets are shown in Table 2. Polyfloral pollen had the lowest protein levels (20.19 %), and soybean had the highest protein levels (51.25 %). Regarding fat content, the highest values (5.93%) were found in polyfloral pollen, while the lowest value (2.50%) was found in soybean.

Table 2. Composition of the chemical analysis of diets.

Diet	Crude protein (%)	Crude fat (%)
Monofloral pollen	30.70	3.30
Polyfloral pollen	20.19	5.93
Soybean	51.25	2.50

Longevity of bees fed on different diets

The longevity of worker honeybees was longer when they were fed on polyfloral and monofloral pollen diets (23.75 days), followed by a decrease in longevity when fed on a soybean diet (23 days) (Figure 2). The shorter longevity was observed in bees that were fed only candy (22.25 days). No significant differences were recorded among all treatments. The result showed that the lifespan of bees fed on polyfloral, monofloral pollen, and soybean diets was longer than that of bees fed on candy by just one day, but without significant differences between them.

Food consumption

The results of dietary consumption are shown in Fig. 3. Total daily consumption and consumption over the entire study period differed significantly among the experimental groups ($p < 0.05$). The overall mean diet consumption was highest when feeding on polyfloral pollen (14.45 mg/bee/day) and monofloral pollen (14.47 mg/bee/day), while the

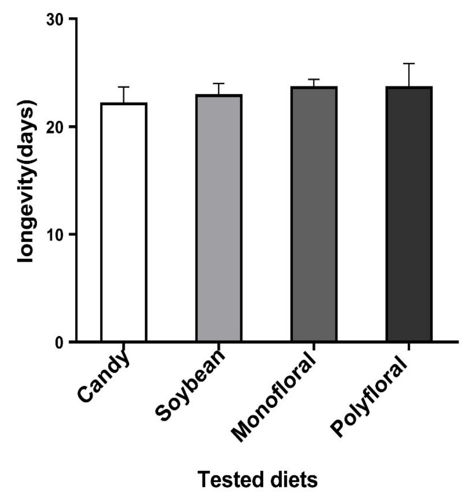

Figure 2. Effect of feeding different diets on the longevity of worker honeybees.

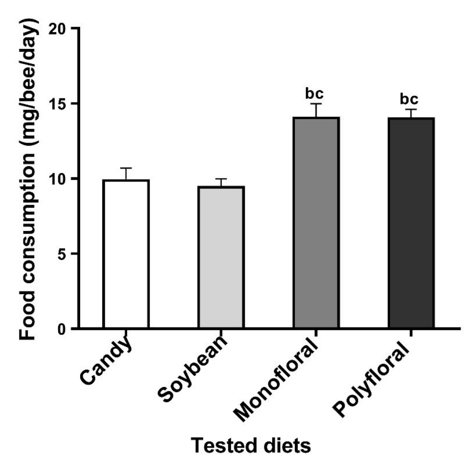

Figure 3. Mean consumption rate of tested diets by worker honey bees. Data presented as Mean±SD. n=4, b: significantly different from the candy-supplemented group, c: significantly different from the soybean-supplemented group; by ANOVA, followed by Bonferroni ($P < 0.05$).

lowest consumption was recorded at feeding on soybean (9.54 mg/bee/day) and candy (9.76 mg/bee/day). Also, the polyfloral and monofloral diets were more significant than the soybean and candy diets. Whereas there is no significant difference between the tested diets of polyfloral and monofloral pollen, as well as between soybean and candy.

Hypopharyngeal glands (HPGs) development

The influence of tested diets on the development of worker honey bees' HPG at 0-,4-,8-, and 12-day-old. The results clearly showed significant differences ($P < 0.05$) in the degree of HPG development among workers fed the tested diets as shown in Fig. 4. The highest mean development of HPG was found among worker honeybees fed on polyfloral pollen (3.1), followed by monofloral pollen (3) and soybean (2.7), and the lowest mean was recorded for the candy diet (2.2). The most significant and optimal HPG development occurred in 12-day-old worker honeybees, followed by that of 8- and 4-day-old worker honeybees. Statistical analysis revealed there were significant differences between pollen diets (polyfloral, monofloral), and candy across the three ages, as well as between pollen diets and soybean. Although there were no significant differences between polyfloral and monofloral pollen across the three ages. At 4 days old, no significant differences were observed among the four tested diets. Whereas at 8 and 12 days old, there were no significant differences between soybean and candy. The results indicate that the tested diets across the three ages showed greater statistical significance than the newly emerged samples (designated as time zero and untreated samples), with an average of 1.6, as shown in Fig. 5.

Dry weight

Worker bees fed on different diets showed significant variation in their thorax dry weight. Diets rich in protein and essential nutrients generally lead to heavier thoraxes compared to carbohydrate diets (candy) and newly emerged samples.

Honeybee diet and age significantly impact their dry weight. Young bees, especially those receiving sufficient protein from pollen, experience an increase in dry weight that later decreases with age and as they transition to foraging roles. Pollen is crucial for protein, lipid, and vitamin intake, which are essential for body mass development. In contrast, protein-deficient diets led to reduced dry weight and shorter lifespans.

The polyfloral and monofloral diets recorded the highest dry weight per bee thorax (10.1 mg), followed by the lowest dry weights, which were associated with the soybean diet (9.5 mg) and candy diet (9.2 mg), which was used as

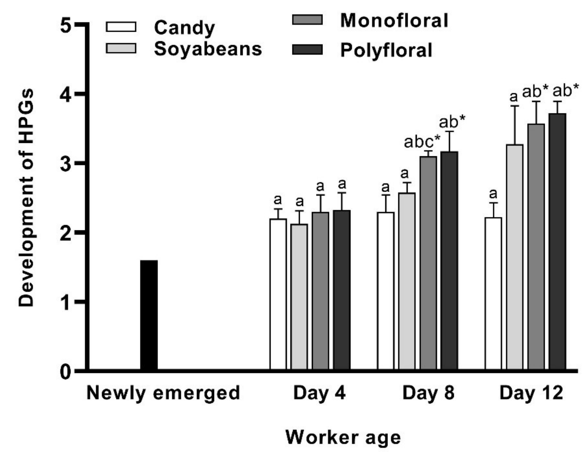

Figure 5. Development of hypopharyngeal glands in worker honeybees fed on different diets at different ages.
Data presented as Mean± SD. n=4, a: significantly different from the results of newly emerged, b: significantly different from the candy-supplemented group c: significantly different from the soybean-supplemented group, *: significantly different from the results of day 4; by ANOVA, followed by Bonferroni ($P < 0.05$).

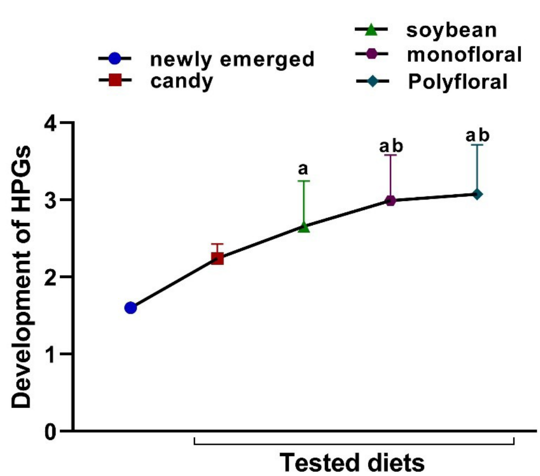

Figure 4. Effect of different dietary treatments on HPGs in honey bee workers
Data presented as Mean± SD. n=4, a: significantly different from the results of newly emerged, b: significantly different from the candy-supplemented group; by ANOVA, followed by Bonferroni ($P < 0.05$).

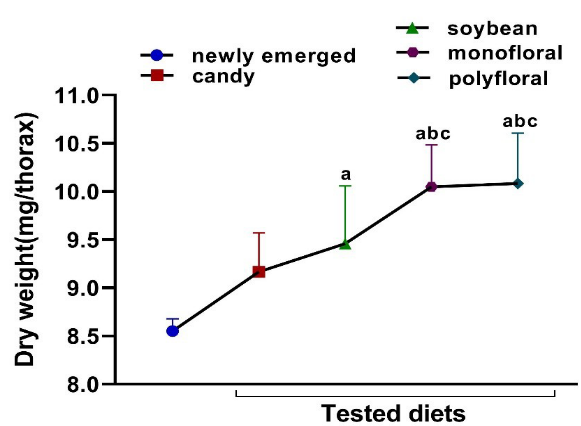

Figure 6. Effect of different dietary treatments on dry weight in honeybee workers
Data presented as Mean± SD. n=4, a: significantly different from the results of newly emerged, b: significantly different from the candy-supplemented group c: significantly different from the soybean-supplemented group; by ANOVA, followed by Bonferroni ($P < 0.05$).

a control. There were no significant differences between the two types of pollen diets. Following the same trend, there was no significant difference between soybean and candy. However, both soybean and candy diets resulted in significantly lower dry weights compared to the pollen diets, as shown in Fig. 6. The thorax dry weight of a newly emerged bee weighed 8.6 mg. Then it increased with advancing age.

Based on the honeybee-day-old, workers of 12-day-old had the highest weight, followed by those of 8-day-old and 4-day-old. Statistical analysis indicated significant differences between newly emerged and all tested diets across the three interval times, as shown in Fig. 7. There were no significant differences among the three interval times, whereas a significant difference was observed between 4-day-old and 12-day-old for the soybean diet. At 8-day-old, there were significant differences between the polyfloral pollen and candy diets.

Discussion

Nutritional analysis for crude protein- and fat- content

The nutritional analysis of the tested diets revealed a significant divergence in macronutrient composition, which directly impacts honeybee physiological development. In the present study, the crude protein content of polyfloral pollen was 20.19%, whereas the soybean substitute exhibited a substantially higher value of 51.25%. These findings are consistent with Roulston et al. (2000), who reported that crude protein levels in various pollens can range broadly from 2.5% to 61%. Notably, the protein level of the polyfloral pollen (20.19%) aligned with the 20% minimum threshold recommended for a functional diet (Li et al., 2012). This suggests that while protein concentrations varied by floral origin, the natural pollen used

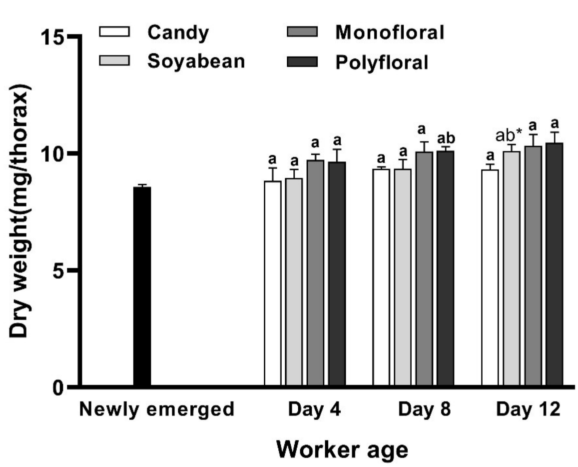

Figure 7. The thoracic dry weight of worker bees of varying ages and fed on various diets.
Data presented as Mean± SD. n=4, a: significantly different from the results of newly emerged, b: significantly different from the candy-supplemented group, *: significantly different from the results of day 4; by ANOVA, followed by Bonferroni ($P < 0.05$).

in this study provides the baseline nutritional requirements for colony growth and worker longevity.

Regarding lipid content, the polyfloral pollen yielded the highest value (5.93%), while the soybean diet contained the lowest (2.50%). These values fall within the documented range of 1% to 20% for pollen lipids, depending on the plant species (Roulston et al., 2000; Szczesna, 2006). The elevated lipid levels in the polyfloral diet are of functional importance, as dietary lipids play a vital role in honeybee health and immune capacity (Crone & Grozinger, 2021).

Furthermore, the higher lipid concentration may influence consumption patterns. Ghosh et al. (2020) and Stabler et al. (2021) indicated that bees tend to consume significantly more when diets are lipid-rich, exhibiting a clear preference for sources that balance high protein with high lipid content.

Ultimately, the availability of these macronutrients is a critical determinant of honeybee survival. Inadequate nutrition is known to reduce workers' physical capacity, thereby increasing the colony's susceptibility to a broad spectrum of pathogens (Li et al., 2007). The ability of bees to recognize and prioritize such nutrient-dense food sources as the polyfloral pollen analyzed here was likely mediated through an integrated system of sensory mechanisms and the perception of host-released odors (Borkakati et al., 2019).

Longevity of bees fed with different diets

The current findings indicated that, while polyfloral and monofloral pollen diets resulted in slightly longer longevity than soybean and candy diets, these differences were not statistically significant. These findings align with Ricigliano et al. (2022), who reported that high-quality pollen and formulated protein substitutes can yield comparable longevity in honeybee workers. Along the same lines, De Grandi-Hoffman et al. (2008) indicated that certain processed soy proteins reach consumption levels comparable to those of natural pollen when supplemented with specific lipids.

In contrast, Amro et al. (2019) reported that bees fed on soybean-based pollen substitutes recorded the lowest longevity among bee workers. In line with Manning et al. (2007), the low longevity of bees fed soybean diets suggests that the protein content of soybean meal is not effectively metabolized and appears to negatively impact the physiological status of the workers. Škerl & Gregorc (2014) reported that newly emerged bees fed a protein-enriched diet exhibited increased longevity compared to those maintained solely on sugar candy.

The effects of pollen and protein substitutes vary from one diet to another. So, Paray et al (2021) mention that protein is a limiting factor in diets. In addition, pollen or high-quality protein substitutes serve as the primary determinants of adult worker bee longevity.

Food consumption

Food consumption is a primary indicator of diet quality and is modulated by various biological factors (Kim et al., 2024). In this study, the significantly higher consumption of polyfloral (14.45 mg/bee/day) and monofloral pollen (14.47 mg/bee/day) compared to soybean (9.54 mg/bee/day) and candy (9.76 mg/bee/day) highlights the superior palatability and nutritional attractiveness of natural pollen. These agreed with (Chakrabarti et al., 2019) noted that honeybees possess a sensory bias toward natural pollen due to specific phytosterols and amino acid profiles that substitutes struggle to replicate. Furthermore, the lack of a significant difference between the two natural pollen types suggests that diet-pollen-based bees maintain high intake levels to meet physiological demands. On the other hand, the biogenic amines and phytosterols inherent in natural pollen that act as primary phagostimulants are typically absent or deficient in soybean-based substitutes. Accordingly, the correlation between high consumption and improved hypopharyngeal gland (HPG) development, as reported by Radev et al. (2014), demonstrated that the biological value of a diet was directly proportional to its palatability and subsequent physiological uptake. In contrast, De Grandi-Hoffman et al. (2008) found that certain highly processed soy proteins could reach consumption levels comparable to those of natural pollen when supplemented with specific lipids. This suggests that the low soybean consumption in our study may be specifically linked to the absence of certain attractants rather than to the protein source itself. These results support the hypothesis that carbohydrate-dominated diets fail to trigger feeding behavior as effectively as protein-rich pollen. The alignment of our data with Filipiak et al. (2020) and Ghosh et al. (2020) confirmed that natural pollen remains the gold standard for nutrition.

Hypopharyngeal gland development

A pollen diet is essential for nurse bees to develop hypopharyngeal glands (HPGs) and produce royal jelly to feed larvae, which is more important in the caste determination and development of the colony (Renzi, 2016). The maturation and development of HPG are considered important criteria for evaluating the suitability of natural pollen diets or protein supplements fed to young bees (Maurizio, 1954; Haydak, 1970). So, the four tested diets, particularly polyfloral pollen and monofloral pollen, had significantly greater effects on 8- and 12-day-old bees than on newly emerged and 4-day-old bees, which showed lower averages (Fig. 5). Our results were consistent with those of Amro et al. (2019). According to their findings, bees fed bee bread had the highest degree of HPG development (3.78), while bees fed a soybean meal-based diet had the lowest (2.14). Pinto et al. (2014) also reported that worker bees fed on pollen had larger acini area than workers fed on honey or soybean. Omar et al. (2016) evaluated different types of diets, monofloral pollen and polyfloral pollen, on the development of HPG and found that HPG was more developed in the two cases than when using sugar solution only. However, in our study, the total protein in monofloral (30.7%) was higher than in polyfloral (20.19%), and the HPG was more developed in polyfloral (3.1) than in monofloral (3). According to Schmidt (1982), the preference of honeybee workers for a type of pollen was not dependent on its protein content. Cook et al. (2003) added that amino acid composition determined the nutritional value of pollen rather than its total protein, and that any reduction in essential amino acids reduced the nutritional value of pollen. The HPG development of bees fed on the polyfloral blend was not different from that of bees provided with the monofloral diets (Di Pasquale et al., 2013).

Dry weight

The superiority of pollen-based diets is likely attributable to their complex profile of essential amino acids, lipids, and vitamins, which are indispensable for the structural development of body mass. While previous literature suggested that soybean flour could maximize thorax weight compared to simple sugar syrups (Ullah et al., 2021), our data indicate a clear physiological advantage for natural pollen over soybean substitutes. This aligns with the findings of Wang et al. (2014), who noted that while substitutes can sustain life, they often fail to match the biomass accumulation triggered by natural pollen. Newly emerged bees (8.6 mg) underwent a significant weight increase during the first 12 days of life. This maturation phase is critical, as higher thorax mass reflects enhanced flight muscle capacity, which is a fundamental determinant of colony fitness and foraging efficiency (Ricigliano et al., 2022). Our observation that 12-day-old workers attained peak weight supports the assertion by Hrassnigg & Crailsheim (2005) and Hendriksma et al. (2019) that young bees prioritize protein absorption for tissue growth before transitioning to foraging roles. These results reinforce the positive correlation between dry weight and lifespan (Eischen et al., 1982), suggesting that high-quality early-life nutrition, such as that provided by polyfloral diets, is a prerequisite for long-term worker survival (Di Pasquale et al., 2013; Manning et al., 2007).

Conclusion

Our results demonstrate the importance of assessing the effects of pollen and pollen-substitute diets on bee health. Bee pollen is considered superior in enhancing HPGs, dry weight, and food consumption. However, the effects of pollen and soybean diets were comparable with respect to bee longevity. In Egypt, more studies are needed to evaluate bee nutrition under both cage and field conditions.

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Authors' contributions

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M.A.S.: Conceptualization, methodology, writing – original draft, writing – review & editing
M.A.K.: Conceptualization, methodology, writing – original draft, writing – review & editing
M.E.D.: Conceptualization, methodology, writing – original draft, writing – review & editing
A.A.Z.: Conceptualization, methodology, writing – original draft, writing – review & editing
F.R.H. Investigation, formal analysis, writing – original draft, writing – review & editing

Conflicts of interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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