Italian Journal of Animal Science

ISSN: 1828-051X (Online) Journal homepage: www.tandfonline.com/journals/tjas20

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To cite this article: Pier Paolo Danieli, F. Manganello, I. Ghaffar, F. Lazzari, F. Petrocchi Jasinski, V. Malagnini, N. Cherrered & A. Mohammedi (2025) Physiological responses of young Italian worker bees (*Apis mellifera ligustica* Spin.) induced by different pollen diets, Italian Journal of Animal Science, 24:1, 1781-1799, DOI: 10.1080/1828051X.2025.2540589

To link to this article: https://doi.org/10.1080/1828051X.2025.2540589

ITALIAN JOURNAL OF ANIMAL SCIENCE
2025, VOL. 24, NO. 1, 1781–1799
https://doi.org/10.1080/1828051X.2025.2540589

RESEARCH ARTICLE

OPEN ACCESS

Physiological responses of young Italian worker bees (*Apis mellifera ligustica* Spin.) induced by different pollen diets

Pier Paolo Danieli[a], F. Manganello[a], I. Ghaffar[b], F. Lazzari[a], F. Petrocchi Jasinski[a], V. Malagnini[c], N. Cherrered[d] and A. Mohammedi[b]

[a]Dipartimento di Scienze Agrarie e Forestali (DAFNE), Università degli Studi della Tuscia, Viterbo, Italy; [b]Laboratory of Valorization and Conservation of Biological Resources, Faculty of Sciences, University M'hamed Bougara of Boumerdes, Boumerdes, Algeria; [c]World Biodiversity Association, Verona, Italy; [d]Laboratory of Cellular and Molecular Biology, University of Sciences and Technology Houari Boumediene (USTHB), Algiers, Algeria

ABSTRACT

Adequate pollen nutrition plays a crucial role in honey bee development and resilience to stressors. This study investigated the physiological responses of newly emerged Italian honey bee workers (*Apis mellifera ligustica* Spin.) to pollen diets collected at different times throughout the beekeeping season in a suburban area of Central Italy. The objective was to evaluate how variation in pollen composition and diversity influences key physiological traits and potential resistance to *Vairimorpha* spp. infection of workers. Corbicular pollen loads were collected every three weeks from March to August, and characterised through palynological, proximate, and mineral analyses. In-cage feeding trials were conducted over two weeks, evaluating daily intake, body composition (crude protein and lipid content), hypopharyngeal gland development, ovary maturation, and *Vairimorpha apis* and *V. ceranae* spore loads using RT-PCR and sequencing. Results showed that pollen collected during the central part of the season (April–June) had higher botanical diversity and nutrient content, particularly in proteins and key minerals (Mg, P, Cu, and Zn). Honey bees fed with these pollens exhibited greater development of hypopharyngeal glands, higher protein and lipid body reserves, and reduced relative *Vairimorpha* spp. spore loads compared to controls. Notably, pollen diets enhanced physiological development even when infection was present, suggesting a buffering effect of high-quality nutrition. These findings confirm the importance of seasonal pollen quality and diversity in sustaining bee health. Future studies should focus on experimental infections and compare mono- versus multifloral diets to better define nutritional strategies for beekeeping under changing environmental conditions.

HIGHLIGHTS

- Seasonal pollen diversity influences honey bees (*Apis mellifera ligustica* Spin.) physiology and *Vairimorpha* spp. prevalence.
- Mid-season (April–June) pollens improve protein and lipid reserves and enhance hypopharyngeal gland development in young worker bees.
- High-quality multifloral pollen diets mitigate nutritional stress and support bee resilience under suboptimal conditions.

ARTICLE HISTORY
Received 16 May 2025
Revised 27 June 2025
Accepted 23 July 2025

KEYWORDS
Honey bee; pollen; artificial nutrition; protein diet; *Vairimorpha* spp.

Introduction

Adequate nutrition in western honey bee (*Apis mellifera* L.) (HB) is crucial for colonies growth and development (Brodschneider and Crailsheim 2010). Differently from other farmed species, where specific nutritional needs are well known and appropriate diets can be formulated, supporting the HB nutrition is challenging because every colony has specific needs depending on the landscape nutritional opportunities, development stage and its own health condition (Tsuruda et al. 2021). In addition, considering the complex organisation of HB colonies, the nutrition can be

CONTACT Pier Paolo Danieli danieli@unitus.it

Supplemental data for this article can be accessed online at https://doi.org/10.1080/1828051X.2025.2540589.

considered at three levels: colony nutrition, adult nutrition and larval nutrition (Brodschneider and Crailsheim 2010). In addition, foraging activities, and consequently the colony nutrition, is highly dependent on environmental constraints and on the floral composition of the landscape (Donkersley et al. 2014). While the recently main focus of HB research programs is often on the internal factors of the hive like disease profiles, queen fecundity, and varroa mite (*Varroa destructor*) control, overall little effort is made to understand and track the external factors of the hive (DeStefano 2015). For example, just few evidences are present in literature on the nutritive value of the pollen and nectar collected by the worker bees according to environmental drivers such as season, location, and climate changes (Avni et al. 2009; 2014).

It is well known that colony losses, that are occurring worldwide (Requier et al. 2018; Evans and Chen 2021), are the result of the impact of multiple stressors (Neumann and Carreck 2010; Steinhauer et al. 2018). However, it has been proposed that the combination of nutritional stress, infections by pathogens and pesticide exposure are the most important driving forces (Naug 2009; Dainat et al. 2012a; Smith et al. 2013; Tosi et al. 2018).

As far as the relationships between HB nutrition and their health status, it is well known how the consumption of pollen and its quality affect lifespan (Di Pasquale et al. 2013), immuno-competence (Alaux et al. 2010), behaviour of the division of labour in the colony (Ament et al. 2010), and resistance to pathogen infection (Alaux et al. 2011; Porrini et al. 2011). Among the pathogens that threaten HB health, *Varroa destructor*, RNA viruses (Dainat et al. 2012b), and the microsporidia *Vairimorpha apis* (Zander), formally *Nosema apis* according to Tokarev et al. (2020) and *Vairimorpha ceranae* (Fries) (Fries et al. 1996), formally *Nosema ceranae* according to Tokarev et al. (2020), have the most significant impact on colony losses (Higes et al. 2009; 2013). Huang (2012) has shown that spring pollen supplement can work as an insurance, when weather is poor, and can reduce the negative effects of *Vairimorpha spp.* infection. However, surprisingly, an *in vitro* study found that pollen-fed bees had higher levels of *Vairimorpha spp.* infection but also a longer lifespan than bees fed syrup alone (Jack et al. 2016). These conflicting results highlight the complex relationship between HBs and their parasites/pathogens in the context of other environmental factors (i.e. food availability). The impact of pollen intake on bee health is a complex issue that requires considering not only the quantity of pollen consumed, but also the quality and diversity of the pollen diet (Omar et al. 2017; Radev 2022).

Concerning the nutritional value of mono- or multifloral pollen just a few research has been carried out in the last years. As a matter of fact, previous researches on pollen quality, bee health and foraging behaviour relied only on measuring the protein content (Human et al. 2007; Kitaoka and Nieh 2009; Leonhardt and Blüthgen 2012) but, in some cases, inconsistent insights have been gained. Some studies (Schmidt and Hayden 1984; Schmidt et al. 1995; Alaux et al. 2010; Di Pasquale et al. 2013) have shown that polyfloral pollen is healthier for honey bees than pollens from single plant species. Mixed pollen administered to caged HBs made the bees' live longer than those on a single species of pollen (Huang 2012) suggesting that multi-source pollen has higher value than monofloral pollen to maintain HBs in good health status.

The diversity of pollen in the HB diet could not be directly associated with improved nursing physiology (Di Pasquale et al. 2013).

According to the increase of the immunocompetence in HBs fed with multifloral pollen, it was also demonstrated that *V. ceranae* parasitised workers fed with multifloral pollen, lived longer than bees fed with *Cistus* sp. monofloral pollen (Di Pasquale et al. 2013).

Overall, in the literature concerning the HB nutrition topic, poor attention was paid on the HB genetics, even though the subspecific gene assortment can play a role on the response of worker bees to their diet. For example, the Saharan bee (*A. m. sahariensis*) (Baldensperger 1932) and the Tellian bee (*A. m. intermissa*) (Buttel-Reepen 1906) use pollen differently. In fact, under laboratory conditions, Khedidji et al. (2022) showed that when they receive a protein diet, the Saharan bees promote the development of their hypopharyngeal glands, while the Tellian bees use the protein source for optimal development of their ovaries. It seems that from an evolutionary point of view, the use of pollen by a subspecies is oriented towards a very specific physiological function to improve its fitness.

The present work aimed to investigate how newly emerged Italian honey bee workers (*Apis mellifera ligustica* Spin.) respond to different pollen diets collected across the active beekeeping season in a suburban area, by assessing the impact of seasonal variation in pollen composition and diversity on key physiological parameters in order to evaluate the nutritional quality and functional role of pollen in honey bee health and development.

Material and methods

Pollen harvesting

Curbicolar pollen loads (CPLs) were collected from 10 Italian-Dadant bee hives, equipped with pollen traps (Anel, Athens, Greece) and located in the PLANT-B apiary at the Experimental-Educational Farm 'Nello Lupori' (Tuscia University, Viterbo, Italy) (42.4271454°E; 12,0796127°N; 302 m a.s.l.). From March to August 2021, every three weeks, the pollen traps were activated for 24 h and trapped CPLs from all the hives were collected and then pooled according to the harvesting month. Before pooling, a representative portion of CPL samples were retained for palynological analysis. In the following, the CPL monthly pools were subsequently used as pollen diets and labelled according to the progressive harvesting month numbers (PD03–PD08).

Diets formulation

For the following feeding trials with caged bee, two controls over the six PDs (Table 1) were used. The first control diet (SS50) was a sucrose/ASTM type I water solution (50:50 w/w), and the other one (HF) was formulated according to Haydak (1970). To ensure the same moisture of the PDs and HF control, they were levelled to the one with the lowest dry matter content (CPL pool of March, 610 g/kg) by adding suitable volumes of ASTM type I water (Table 1).

Palynological analysis, proximate composition, and mineral profiling of solid diets

Chemicals and reagents

All the reagents used were of technical grade or higher. Distilled water was used if not otherwise specified. For the mineral profiling of solid diets, all the reagents were of analytical grade and ASTM Type-I water was always used. Nitric acid (67%–69%) (VWR International, Radnor, USA) was used both for cleaning procedures and serial dilutions. All glassware and plastics were cleaned by overnight soaking in 10% HNO_3 before a final rinse with ASTM Type-I water. The stock solutions (1000 mg/L in 2% HNO_3) for calibration were purchased from Carlo Erba Reagents (Milan, Italy) (Na, Ca, K, Fe, and Zn) and from VWR International (Radnor, USA) (P, Cu, Mg, and Mn).

Palynological analysis

The CPLs collected per beehive and sampling date were grouped by colour obtaining a total of 228 representative samples. A CPL per representative sample was suspended in 1 mL of distilled water using an advanced vortex mixer ZX3 (VELP Scientifica, Usmate Velate, Italy), and 20 µL of suspension were dried at room temperature on a microscopic slide. A drop of glycerine jelly was placed on top of the dried pollen grains and then a glass slide was placed atop. Pollen grains were then observed under a B500PPH optical microscope (OPTIKA S.r.l., Ponteranica, Italy) at 400–1000× magnification. For each representative CPL, about 500 pollen grains were counted according to the "transect" method (Tamic et al. 2011). Pollen grains were identified at the lowest possible taxonomic level according to the available literature (Ricciardelli d'Albore 1998; Bucher 2004; El-Labban 2020) and the PalDat (2000) palynological databases. The identified pollens were classified following the

Table 1. Formulation of the experimental diets used in feeding trials with caged young worker bees.

	Ingredients (g/kg)					
Diet	Defatted soybean meal[a]	Sucrose	Brewer's yeast (wet)[b]	Whey powder[c]	CPL[d]	ASTM type I water
SS50	–	500.0	–	–	–	500.0
HF	272.7	272.7	272.7	90.9	–	90.9
PD03	–	–	–	–	1000.0	–
PD04	–	–	–	–	954.7	45.3
PD05	–	–	–	–	762.6	237.4
PD06	–	–	–	–	783.4	216.6
PD07	–	–	–	–	774.3	225.7
PD08	–	–	–	–	782.1	217.9

[a] Purchased from Bongiovanni Farine (Mondovì, Italy), fat = 10.0 g/kg, protein = 520.0 g/kg, carbohydrates = 300.0 g/kg, data declared by the producer.
[b] Purchased from Lesaffre Italia S.p.a. (Trecasali, Italy).
[c] Purchased from Fast Ingredients (Nizza Monferrato, Italy), protein = 800 g/kg, data declared by the producer.
[d] PD03 = CPLs harvested on 26/03/2021, PD04 = CPLs harvested on 30/04/2021, PD05 = CPLs harvested on 21/05/2021; PD06 = CPLs harvested on 11/06/2021, PD07 = CPLs harvested on 02/07/2021, PD08 = CPLs harvested on 23/07/2021 and 12/08/2021, and pooled.

"pollen types" nomenclature proposed by Persano Oddo and Ricciardelli d'Albore (1989).

Proximate composition of solid diets

Representative aliquots of solid diets (Table 1) were dried in an oven at 65 °C to constant weight, grinded through a ZM 200 Ultra Centrifugal Mill (Retsch, Haan, Germany) to pass a 0.5 mm screen and then stored in sealed polyethylene bottles until analysis. The dry matter content (**DM**, g/kg) was determined by gravimetry according to the AOAC method n. 934.01 (AOAC 2000). The gravimetric determination of the ash content (**ASH**, g/kg DM) was obtained after sample incineration by a muffle furnace (AOAC method n. 942.05) (AOAC 2000). Crude protein (**CP**, g/kg DM) and ethereal extract (**EE**, g/kg DM) contents were measured according to the AOAC methods n. 978.04 and 920.39 (AOAC 2000). In addition, as a measure of potentially undigestible carbohydrates in solid diets, the neutral detergent fibre corrected for the residual ash content ($\mathbf{aNDF_{om}}$, g/kg DM) (Van Soest et al. 1991) was determined by boiling 0.5 g of sample for 1 h in 100 mL of neutral detergent solution (Carlo Erba Reagents, Milan, Italy) with 0.05 mL of heat-stable α-amylase (Astori Tecnica s.r.l., Poncarale, Italy) and 0.5 g of sodium sulphite. Then, the non-fibrous carbohydrates content (NFC, g/kg DM), was calculated as follows (Bernabucci et al. 2009):

$$NFC = 1000 - ASH - CP - EE - NDF \tag{1}$$

Solid diet samples mineralisation

Aliquots of 0.7 g of solid diet samples were digested with 6 mL concentrated HNO_3 and 1 mL water using a Multiwave GO Plus microwave oven (Anton Paar Italia S.r.l., Italy). A Reagent Laboratory Blank (RLB) composed of HNO_3 2% was included in each mineralisation cycle (one out of eight positions per run). The samples' mineralisation program consisted of a 20 min temperature ramp followed by 10 min at 100 °C; a 10 min ramp followed by 5 min at 180 °C, and finally a 5 min ramp down to room temperature. After each mineralisation run, a standard cleaning procedure for the digestion Teflon vessels was performed by filling them with 4 mL of HNO_3 and 1 mL of water and by running a 10 min ramp up to and internal temperature of 180 °C, that was then held for 5 min. Once the cleaning procedure was completed, the vessels were emptied and then left to dry in a stove at 65 °C for two hours. Before mineralisation, all the samples and the reagents were handled with non-metallic materials carefully washed with 10% HNO_3.

Mineral profiling by atomic absorption spectrophotometry (AAS)

The Na, K, Ca, Mg, Mn, and Zn contents in solid diets were determined by flame atomic absorption spectroscopy (F-AAS) using a Shimadzu AA 7000 spectrophotometer equipped with a flame atomisation unit and an ASC-7000 autosampler (Shimadzu Corporation, Kyoto, Japan). Acetylene 6.0 grade (AirLiquide Italia SpA, Milan, Italy) was used as combustible gas and compressed air produced by an oil-less compressor as comburent. The elements Fe, Cu, Mn and P were analysed by graphite furnace atomic absorption spectroscopy (GF-AAS) using a Shimadzu GFA 7000 A (Shimadzu Corporation, Kyoto, Japan) with an ASC-7000 autosampler (Shimadzu Corporation, Kyoto, Japan). For the graphite furnace measurements, argon 6.0 grade (AirLiquide Italia SpA, Milan, Italy) and pyrolytic coated graphite tubes with platform (Shimadzu Corporation, Kyoto, Japan) were used. Multi-element (for Cd, Cr, Cu, Fe, K, Mg, Na, and Pb) or single element (for Zn and P) hollow cathode lamps (Hamamatsu Photonics K.K., Shizuoka, Japan), were used as radiation sources. A deuterium lamp (Shimadzu Corporation, Kyoto, Japan) was used for background correction for both the atomisation systems. For the AAS calibration, working standard solutions were daily prepared by diluting the standard stocks to 10 mg/L, then further diluted in HNO_3 (67%) to obtain the from three to five calibrators depending on the element. For F-AAS analysis, the calibration ranges varied between 0.1–0.5 mg Mg/L and 1.25–5.0 mg Ca/L, with a minimum linear fitting (R^2) of 0.994 (Ca). For the elements analysed by GF-AAS, the calibration ranges varied between 0.5–4.0 μg P/L and 1.25–10.00 μg Fe/L, with the lowest linear fitting (R^2) recorded for iron of 0.997. Each analytical data was the average of three consecutive AAS runs performed on the same sample, calibrator or RLB.

Due to the lack of a suitable pollen Standard Reference Material (SRM) allowing to assess the whole-method's accuracy for the investigated elements, non-fat milk powder (SRM 1549) from the National Institute of Standards & Technology (Department of Commerce, USA) was used following the same mineralisation/analysis procedure adopted for the solid diets' samples. From two replicates, the average recoveries were calculated (Table S1). The contents of the investigated elements were then corrected by the respective average recoveries.

Feeding trials with young workers

Queen caging and emerging workers' management

To obtain the young workers, a healthy colony housed in a B-BOX hive (Beiing Srl, Faenza, Italy) and maintained in an all-year round facility (Taber and Owens 1970) at the University of Tuscia under controlled environmental conditions (25.1 ± 3.2 °C) was used. To obtain a cohort of 0–24 h old workers, the laying queen, provided by a *A. m. ligustica* Spin. queen bee breeder (CREA 2023), was caged forcing her to lay on an comb inlaid in a plastic queen-excluder cage (Erboristeria Apistica, Sondrio, Italy). The brood development was monitored daily by noting the number of eggs, larvae, and capped cells. As the whole experimentation was carried out in duplicate, the first queen caging took place on 05/02/2022, and the second one took place on 10/03/2022. In case of outside adverse weather conditions during queen caging, the colony was fed at 100 mL sucrose solution (50:50) per day. About four days before the foreseen mass emergence of worker bees, the comb was moved in a net cage maintained into a laboratory incubator (Carbolite Gero Ltd, Hope, UK) at 33.6 ± 1.2 °C and 71.6 ± 3.4% RH. Emerging bees were provided with SS50 and tap water *ad libitum*.

Feeding trials set-up

Groups of 30 emerging workers were collected through a battery-powered vacuum mini-cleaner and then placed inside self-built 3D printed cages (6 cm × 4 cm × 7 cm; inner volume 168 cm^3) at a density of 17.8 bees/100 cm^3 (Bosua et al. 2018; Manganello et al. 2023). To limit any possible undesired effect of the plastic material on the bees, only polyethylene terephthalate (PET) and polylactate (PLA) filaments (Prusa Reseach a.s., Partyzánská, Czech Republic) were used in the 3D printing process. A total of twenty-four cages (three cages per diet/control) equipped with a removable bottom tray for monitoring the varroa mite fall and the collection of any debris (e.g. feces) were used. Each cage had two holes per side, suitably sized to fit 2 mL centrifuge tubes which contained the diet to be tested; the tubes were pierced (4.5 mm) on the bottom to allow for the bees to enter freely. The top of the cage was designed to allow fitting 1 mL graduated syringe barrels containing water or SS50. In the back side of the cage, a removable panel was fitted with a piece (4 cm × 5 cm) of embossed wax foundation to allow for quantifying the wax produced by the bees.

Each trial lasted two weeks along with dead bees were counted and removed daily. The daily intake of water (**WI**; µL/bee/day) and SS50 (**SSI**; µL/bee/day) were also recorded. The unconsumed solid diet was replaced every two days, dried at 65 °C for 24 h, and the average daily DM intake (**DMI**, mg DM/bee/day) was calculated by difference from the dry weight of diet administered, divided the average number of alive bees. At the end of each trial the bees still alive were narcotised by cooling and were stored in freezing conditions for further measurements. Also, at the end of the trial the wax removable panels were taken and weighed, and the average amount of wax produced daily (**DWP**, mg/cage/day) was obtained by subtracting the initial weight of each wax panel and dividing the increment by the number of trial days.

Worker bees' body proximate composition

Ethereal extract content

Eleven HBs per cage, thirty-three per treatment, were randomly selected from the end-trial alive ones. Each HB was dissected with a surgical steel scalpel respectively in head (**H**), torax (**T**) and abdomen (**A**); after that, the respective body part groups were dried in an oven at 65 °C to constant weight to determine the dry DM by gravimetry (AOAC method n. 934.01) (AOAC 2000). Subsequently, dry material was moved to 2 mL centrifuge tubes (Eppendorf, Enfield, USA) and crushed (3 min at 2500 oscillation per min) by using a Mini-BeadBeater 24 homogeniser (BioSpec Products, Inc., Bartlesville, USA) with one 4 mm diameter magnetic beads (Biosigma, Cona, Italy). Each pulverised sample, representative of a body part, was quantitative moved and weighted (*W1*), by an analytical balance OHAUS Explorer® Pro (Ohaus GmbH, Switzerland), inside a lipid-free filter bag (Astori Tecnica s.r.l., Poncarale, Italy), previously weighed (*W2*) with the same balance, and then sealed. The samples were then submitted to with a Soxhlet extraction according to AOAC method n. 920.39 (AOAC 2000). The EE content, separately of HBs' heads (**EE_H**), thoraxes (**EE_T**) and abdomens (**EE_A**) (g/kg DM), was calculated as follows:

$$EE = \frac{W1 + W2 - W3}{W1} \times 1000 \tag{2}$$

where *W3* was the residual sample dried weight (105 °C, 30 min) after the extraction. The method accuracy was estimated by a recovery exercise. Due to the lack of a suitable Standard Reference Material, whole milk powder (SRM 8435) from the National Institute of Standards & Technology (Department of Commerce, USA) was used. From three daily replicates, the average recovery was calculated to be from 110% to 112%. The data obtained in different working days were corrected for the respective average recoveries. The total body EE content (**EE_{TB}**; g/kg DM) was estimated as weighted mean based on the single body part weight.

Crude protein content

Due to limited amount of sample material, the CP content of HB body parts was assessed through the Kjeldahl method (AOAC method n. 978.04) (AOAC 2000) after the ethereal extraction. Sulphuric acid (0.1 N) was used to titre the total nitrogen of the samples by using a Flash automatised titrator (Steroglass, Perugia, Italy) to end-point pH 4.2. To consider the nitrogen content of the filter bags used, three empty filter bags for each mineralisation cycle, were analysed daily and the mean value subtracted from the titrant volume used to reach the endpoint; CP content of head (**CP$_H$**), thorax (**CP$_T$**) and abdomen (**CP$_A$**) samples (g/kg DM) was estimated as follows:

$$CP = \frac{(V_s - V_0) \times 0.0014}{W1} \times 6.25 \times 1000 \qquad (3)$$

where V_s was the volume in mL of H_2SO_4 added to reach the endpoint for a sample, V_0 was the mean H_2SO_4 titration volume needed for empty bags, $W1$ was the sample dry weight and 6.25 is a conventional factor applied to the net total nitrogen (Danieli et al. 2023). As in the case of the EE analysis, whole milk powder (SRM 8435) from the National Institute of Standards & Technology (Department of Commerce, USA) was used to assess the method accuracy. From three daily replicates, the average recovery was calculated to be from 97% to 117%; the data obtained were corrected according to the respective average recoveries. The total body CP content (**CP$_{TB}$**; g/kg DM) was estimated as weighted average based on the CP content measured in each body part.

Vairimorpha spp. spore load

At the end of each feeding trial, two survived HBs per cage were randomly selected and managed as a single sample. The DNA extraction was performed with the commercial kit QIAmp® DNA Mini (QIAGEN, Velno, Netherlands) according to Babin et al. (2022). Aliquots of 1 µL of the DNA extract were analysed using a Quant Gene 9600 RT-PCR (Bioer Thecnology, Hangzhou, China) with WizPure qPCR Master (SYBR) (Wizbiosolutions Inc, Seongnam, South Corea) and customs primers described by Chen et al. (2009) with the following amplification cycle: 5 min at 95 °C, followed by 45 cycles of 15 s at 95 °C, 30 s at 56 °C. As confirmation, six DNA samples from as many bee pairs, in double (two reference samples and four samples of bees which tested positive by RT-PCR) were subjected to the SANGER amplicon sequencing, performed by BioFab Research s.r.l. (Rome, Italy).

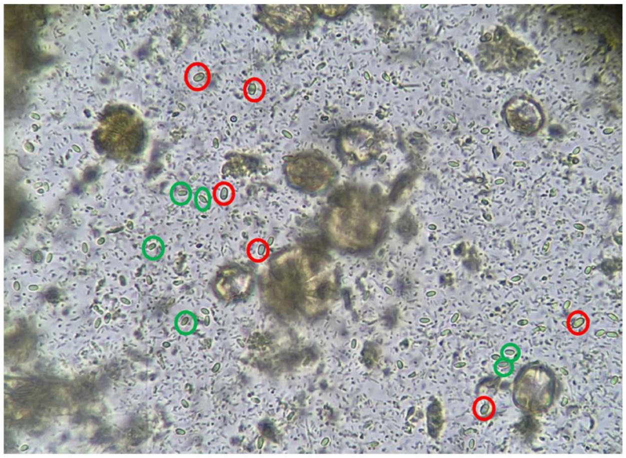

Figure 1. Reference sample with *V. ceranae* and *V. apis* spores respectively highlighted in green and red (optical magnification 400×).

To perform an absolute quantification of the bees' spore load, calibrations with single positive (*V. ceranae*) and double positive (*V. ceranae* and *V. apis*) reference samples, gently provided by Centro de Investigación Apícola y Agroambiental (CIAPA-IRIAF, Marchamalo, Spain), were performed. At first, the spore contents of the reference samples were quantified by using a Burker chamber (Marienfeld GmbH & Co., Lauda-Königshofen, Germany) under a Primo Star optical microscope (Carl Zeiss, Jena, Germany) at 400× magnification. According to the Texas Apiary Inspection Servicen.d. (accessed 14/10/2024), Fries et al. (1996, 2013) and Oliver (2007), the quota of *V. apis* spores in the double positive reference sample (Figure 1) was microscopically assessed on three technical replicates by the same operator as 34.9 ± 8.5%. After counting, the reference samples were then serially diluted from 1:1 to 1:256 in PCR grade water (spore densities ranging from 1.12×10^5/mL to 3.96×10^2/mL and from 3.56×10^4/mL to 1.38×10^2/mL, for *V. ceranae* and *V. apis* respectively) and then the total DNA was extracted. By using diluted reference samples as calibrators, three daily RT-PCR calibration runs were performed with the same experimental set-up. Dilutions were considered positive after checking the melting curve (Chen et al. 2009) and the LOD was established for $C_t \geq 42.59$ (dilution 1:8 for *V. apis*). Finally, the spore number detected was referred to a single bee. For *V. ceranae* (Equation (4)) and *V. apis* (Equation (5)) two logarithmic calibrations were obtained with R^2 of 0.98 and 0.96, respectively:

$$C_{tc} = -4.18 \ln(SN) + 74.08 \qquad (4)$$

$$C_{ta} = -3.12 \ln(SN) + 63.14 \qquad (5)$$

where C_{tc} referred to *V. ceranae*, C_{ta} referred to *V. apis*, and SN was the spore number obtained by microscopic count combined with the dilution factors adopted.

The limit of quantification (LOQ), which is the lowest DNA concentration detected in $\geq$ 95% of replicates, was assessed on 9 replicates of the lowest concentration of the calibrators, evenly distributed on three independent RT-PCR runs. The LOQ was valid if a specific amplification signal was detected (*i.e.* >LOD) in at least 8 replicates (Babin et al. 2022). For *V. ceranae* a LOQ of C_t=40.86 (sample dilution of 1:8), and for *V. apis* a LOQ of C_t=42.59 (sample dilution of 1:8) were estimated (Supplementary Table 2). For *V. apis*, the method LOD and LOQ coincided (C_t=42.59) according to Armbruster and Pry (2008). As with the reference samples, the DNA from the unknown ones was extracted and amplified by RT-PCR as above mentioned and the spore load (**SL**; n/bee) for *V. ceranae* (**SL$_c$**) and *V. apis* (**SL$_a$**) were extrapolated from Equations (4) and (5), respectively. Relative spore load per bee (**RSL,** %) was calculated considering the initial spore load found in a pool of two emerging workers per trial as follow:

$$RLS_c = \left(\frac{SL_c - ISL_c}{ISL_c}\right) \times 100 \tag{6}$$

$$RLS_a = \left(\frac{SL_a - ISL_a}{ISL_a}\right) \times 100 \tag{7}$$

$$RLS_{ca} = \left(\frac{SL_{ca} - ISL_{ca}}{ISL_{ca}}\right) \times 100 \tag{8}$$

where ISL_c, ISL_a and ISL_{ca}, were the initial spore loads for *V. ceranae* and *V. apis*, separately or joined, respectively. Prevalence of *V. ceranae* (***P$_c$***), *V. apis* (***P$_a$***) and co-infection cases (***P$_{ca}$***) was expressed in percentual term of positive bees' samples per diet.

Worker bees ovary and hypopharyngeal gland development

Eight frozen HBs per cage were shipped and analysed by the VALCORE Laboratory at the UMBB University (Boumerdes, Algeria). Each worker was dissected, and the weights of heads, thorax and abdomen were recorded using an analytical balance OHAUS Explorer® Pro (Ohaus GmbH, Nänikon, Switzerland).

The ovary development was studied under a binocular microscope using the classification of developmental stages. Five stages were differentiated: the two first stages (sizes 1 and 2) corresponded to undeveloped ovaries and the last three (sizes 3–5) to developed ovaries according to Velthuis (1970) and Khedidji et al. (2022) (Table S2).

The development of the hypopharyngeal glands (HPG) was assessed by calculating their weight. For this, HPG were removed with forceps through an incision in the front part of the head and put on a microscope slide in a droplet of ASTM Type-I water. The HPG were removed with a fine clip and dried for 4 h at 110 °C (Fluri et al. 1982). The dry weight of the individual HPG was determined by subtracting the weight of glass blade to the weight of dried HPG fixed on it.

Data processing and statistical analysis

Shannon (H') and Shannon Evenness (E) indexes were calculated to appraise the floral biodiversity of the pollen diets. The molar Ca/P and Cu/Fe ratios were calculated in order to consider possible interactions among these elements (Fadlalla 2022). For the daily recorded variables, such as **SSI**, **WI**, **DMI**, individual intake of CP (**CPI**; mg CP/bee/day), EE (**EEI**; mg EE/bee/day), and NFC (**NFCI**; mg NFC/bee/day), but also the HBs mortality rate (**MR**; %) calculated as the percentage of dead bees in fourteen days on the initial number per cage, and the varroa mite fall (**VMF**; n/day/cage) a repeated measures ANOVA was performed including diet (**D**; SS50, HF, PF03-PD08), time (**T**; 1–14 days), and diet × time interaction as fixed factor. The Tukey's test was used for *post-hoc* pairwise comparisons. When needed, linear contrasts were also performed. Significance was always declared at $p \leq 0.05$.

At the end of each feeding trial, the average daily gain (**ADG**; mg/day/bee) was calculated by the difference between the average weight of the alive bees at 14 days and the average weight of the bees at the beginning of the trial, divided by the trial days.

For the **ADG**, the average daily wax produced (**DWP**; mg/day), the HB body part and total body DM (g/kg), CP and EE contents (see Equations (2) and (3)), **HPGw** and head weight (**Hw**) (mg DM/cage) and the **HPGw/Hw** ratio, a General Linear Model was performed using **D** as fixed factor. To test any possible effect of daily recorded variables, on the end-trial variables, average SSI, WI, DMI, CPI, EEI and NFCI were entered as covariates in the model. The Tukey's test was used for pairwise comparisons, and significance was always declared at $p \leq 0.05$.

As the ovary development (**OD**, five stages), and the **RSL** for *V ceranae*, *V. apis,* and *V. ceranae* + *V. apis* were not normally distributed, the effect of the diet was tested by the non-paramentric Kruskal-Wallis test followed by the *post-hoc* Dunn's test for pairwise comparisons; significance was always declared at $p \leq 0.05$. The binomial output of ***P$_c$***, ***P$_a$*** and ***P$_{ca}$*** were used to

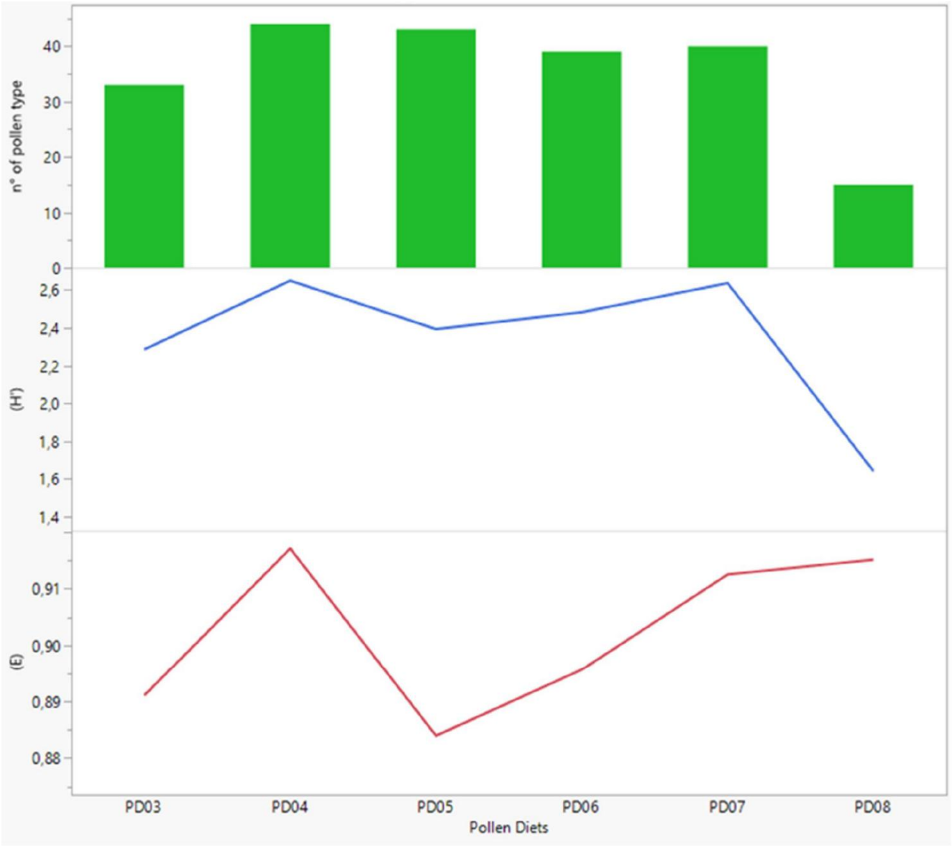

Figure 2. Biodiversity of pollen diets assessed by the number of different pollen types found (top), and by the Shannon (middle), and the evenness (bottom) indexes.

create a contingency table and tested with non-parametric independent test for qualitative categorical variables (Chi-square and Wilks' G^2). Pearson's moment correlation analysis was performed for all the physiological variables. In order to ensure that the parameters which values were percentages could (*i.e.* **MR**, **RLS_c**, **RLS_a**, **RLS_{ca}**) have a normal distribution for the statistical analysis, the arcsine transformation described by Zar (2014) was applied. All the analysis were performed with STATISTICA 10 software (Statosoft inc., Tulsa, USA).

Results

Palynological analysis of pollen diets

Pollen biodiversity resulted quite stable in pooled CPLs from April to July (i.e. PD04-PD07) (Figure 2), with the H' ranging from 2.39 to 2.65. The lowest CPL botanical diversity was observed in August (PD08; $H' = 1.64$), with only 6 pollen types observed. The Evenness peaked in April CPLs (PD04; $E = 0.92$), then decreased in May (PD05; $E = 0.88$), before rising again at the end of the season with August CPLs matching the value of April.

Table 2. Proximate composition (g/kg DM), macro (Ca, K, Mg, P; g/kg DM) and micro-mineral (Na, Zn, Mn, Fe, Cu, Cd, Cr, Pb; mg/kg DM) profiles of solid diets.

	HF	PD03	PD04	PD05	PD06	PD07	PD08
CP	367.4	249.4	247.4	209.1	279.5	255.0	196.1
EE	18.5	60.8	62.4	47.6	63.9	30.5	77.6
$aNDF_{om}$	78.1	57.6	44.7	34.4	114.3	70.5	33.1
NFC	502.1	601.4	615.1	679.9	519.8	620.4	669.4
ASH	33.9	30.7	30.4	29.0	22.4	23.6	23.8
Ca	1.2	1.4	1.3	1.3	1.3	1.4	1.3
K	22.3	10.6	11.8	11.3	13.2	10.3	10.7
Mg	1.1	1.0	1.1	1.2	1.2	1.1	0.8
P*	1.4	1.7	1.8	1.9	2.0	2.0	1.1
Na	229.1	183.0	135.1	61.1	52.1	97.5	44.0
Zn	29.9	61.2	48.3	51.3	55.3	63.2	25.1
Mn	4.1	10.5	9.5	9.7	12.4	14.9	5.1
Fe	3.3	5.2	4.2	3.5	4.8	4.6	3.9
Cu	3.6	5.2	5.5	5.1	5.5	5.4	3.4
Ca/P*	0.7	0.6	0.6	0.5	0.5	0.5	0.9
Cu/Fe*	1.0	0.9	1.2	1.3	1.0	1.0	0.8
Pb	0.001	0.062	0.051	0.053	0.034	0.039	0.045
Cr	0.0068	0.0134	0.0075	0.0046	0.0049	0.0068	0.0068
Cd	0.0079	0.0123	0.0093	0.0081	0.0107	0.0128	0.0129

Abbreviations: CP: Crude Protein; EE: Ethereal Extract; aNDFom: Neutral Detergent Fibre corrected for the ash content; NFC: Non-fibre carbohydrates; * molar ratios.

Proximate composition and mineral profile of pollen diets

Among the PDs, the highest CP level was found in PD06 and the lowest in PD08 (Table 2). However, the

HF control had about 53% more CP than the average value of PDs (239.4 g/kg DM). Among the pollen diets, the highest EE content was found in PD04, which was around 10% higher than PD03 and more than twice the diet with the lowest EE content (PD07); however, the HF had an EE content even lower, about 40% less than PD07. The PD06 had the highest aNDFom content, followed by HF, but the lowest value was found in the August PD. The NFC peaked in PD05, while PD07 and PD08 reached respectively 95% and 90% of this maximum; still the HF diet showed, in absolute terms, the lowest NFC content. The ASH content was about 30 g/kg DM in PD03 to PD05, while the HF diet was about 11% higher, on average, and fell to 22–24 g/kg DM in PDs including CPLs harvested later in the season (i.e. PD06-PD08).

The mineral profile differed across the diets. In particular, the PD04 had the highest Na content and the PD08 had the lowest. The Ca, K, Mg and P contents tended to be steady in all the pollen diets with the exception of a 5% and 41% lower level respectively for K and P in PD08 compared to the average value of the other PDs (11.3 ± 3.9 and 1.9 ± 0.1 g/kg DM, for K and P respectively); conversely, the K content in HF was about double than the average value of PDs (11.3 ± 1.0 g/kg DM). Except for HF, which had the highest content, sodium was from 2 to 3 times higher in PD03 and PD04 than in other PDs (63.7 ± 20.4 mg/kg DM). Zinc, manganese and copper concentrations were noticeably low in HF diet and in PD08. With minor exception (i.e. PD05), the iron content of PDs was higher in comparison to the HF control diet.

Worker bees' intakes

Both the factors diet and time affected the SSI, WI, DMI, CPI, EEI, and NFCI, but their interaction was always significant as well (Table 3). As for the SSI, although the overall effect was significant, no pairwise comparisons turned out to be significant. Higher values were observed during the first seven trial days for SS50, HF compared to other PDs (Figure S1A), even though the contrast analysis, extended to the whole experiment (Table S4), did not support a differential intake of SS between control and PD fed bees.

The WI resulted comparable across all groups except for PD07 (Table 3), but the contrast between controls (0.62 ± 0.01 μL/day) and PDs (0.36 ± 0.05 μL/day) was highly significant ($p < 0.001$) (Table S4), due to an overall higher water intake in controls compared to PDs during the first three trial days (Figure S1B).

Concerning the DMI, consistent increases were observed across all PD groups, with pairwise comparisons showing significant differences ($p < 0.01$) compared to the HF-fed groups (Table 3). Additionally, significant contrasts ($p < 0.001$) were found when comparing the DMI (2.71 ± 0.17 mg/day) of PD fed bees to those of the HF counterparts (Table S4). The CPI reflected the same pattern of DMI for the PD groups (Table 3), as well as for significant contrast analysis ($p < 0.001$) between HF and PD fed bees (0.64 ± 0.05 mg/day) (Table S4).

The EE intake resulted higher ($p < 0.01$) in PD fed bees compared to the HF fed ones, with the highest values recorded for PD03, PD06 and PD08, while PD07 showed an intermediate value (Table 3). Contrast analysis of PDs against HF, PD03, and PD07 highlighted a significant lower ($p < 0.001$) trend throughout the entire trials of these diets compared to the other PD groups (Table S4).

The NFC intake was comparable for the first three pollen diets, while higher values were recorded respectively for PD07 and PD06 ($p < 0.01$); even in this case the bees fed on HF showed the lowest intake compared to the PD fed bees ($p < 0.01$) (Table 3). Contrast analysis highlighted that HF and PD08 diets were consistently lower ($p < 0.001$) compared to the other PD groups (Table S4) throughout all trials.

Table 3. Effect of the diet on physiological variables measured on young worker bees.

	Diet (D)									*p*-value		
Variable	SS50	HF	PD03	PD04	PD05	PD06	PD07	PD08	SEM	D	T	D × T
SSI	21.88	22.43	19.89	21.37	22.43	21.1	19.36	19.10	1.28	<0.001	<0.001	<0.001
WI	0.62^{a}	0.62^{a}	0.56^{ab}	0.34^{ab}	0.45^{ab}	0.40^{ab}	0.17^{b}	0.26^{ab}	0.09	0.003	<0.001	<0.001
DMI	nd	0.41^{B}	1.97^{A}	2.93^{A}	3.27^{A}	2.99^{A}	2.48^{A}	2.60^{A}	0.35	<0.001	<0.001	<0.001
CPI	nd	0.15^{B}	0.49^{A}	0.72^{A}	0.68^{A}	0.83^{A}	0.63^{A}	0.51^{A}	0.09	<0.001	<0.001	<0.001
EEI	nd	0.01^{C}	0.12^{AB}	0.18^{A}	0.15^{AB}	0.19^{A}	0.07^{B}	0.20^{A}	0.02	<0.001	<0.001	<0.001
NFCI	nd	0.03^{D}	0.11^{C}	0.13^{C}	0.11^{C}	0.34^{A}	0.17^{B}	0.09^{C}	0.02	<0.001	<0.001	<0.001
VMF	0.54	0.07	0.19	0.31	0.17	0.23	0.00	0.24	0.13	0.193	<0.001	0.297
MR	9.5^{a}	2.0^{b}	6.3^{a}	3.2^{a}	4.4^{a}	5.4^{a}	8.6^{a}	2.4^{ab}	1.5	0.007	<0.001	0.220

Abbreviations: SSI: daily individual intake of SS50 (μL/day); WI: daily individual intake of water (μL/day); DMI: daily individual intake of DM of solid diet (mg/day); CPI: daily individual CP intake (mg/day); EEI: daily individual EE intake (mg/day); NFCI: daily individual NFC intake (mg/day); VMF: Varroa Mite Fall (n); MR: Mortality Rate (%); SEM: Standard Error of the Mean (pooled); T: Time; $^{A,B}p \leq 0.01$; $^{a,b}p \leq 0.05$.

Table 4. Effect of diet on physiological traits calculated at the end of the trial.

	SS50	HF	PD03	PD04	PD5	PD06	PD07	PD08	SEM	*p*-value
ADG	0.21^{b}	0.13^{b}	1.18^{a}	1.36^{a}	1.5^{a}	1.28^{a}	1.42^{a}	1.11^{a}	0.25	<0.001
DWP	2.46	1.46	3.63	1.00	2.29	4.24	3.18	2.67	0.74	0.066
Hw	10.1^{A}	9.4^{B}	10.5^{AB}	10.9^{A}	10.9^{A}	10.9^{A}	11.1^{A}	10.1^{AB}	0.3	0.002
HPGw	0.20	0.20	0.21	0.22	0.23	0.19	0.22	0.20	0.02	0.654
OD	1.02^{b}	1.04^{b}	1.13^{ab}	1.04^{b}	1.38^{a}	1.38^{a}	1.13^{ab}	1.21^{a}	0.10	0.014
HPGw/Hw	0.02	0.02	0.02	0.02	0.02	0.01	0.02	0.02	0.01	0.697

Abbreviations: ADG: Average Daily Gain (mg AI); DWP: Daily Wax Production (mg DM); Hw: Head weight (mg DM); HPGw: Hypopharyngeal glands weight (mg DM); OD: Ovary development (stage n.); SEM: Standard Error of the Mean (pooled); $^{A,B}p \leq 0.01$; $^{a,b}p \leq 0.05$.

Table 5. Effect of diet on HBs' body composition.

	SS50	HF	PD03	PD04	PD05	PD06	PD07	PD08	SEM	*p*-value
DM_H	308.6^{B}	302.4^{B}	328.3^{AB}	336.3^{AB}	342.8^{AB}	330.7^{AB}	355.1^{A}	319.8^{AB}	7.8	<0.001
DM_T	303.9	298.2	331.0	298.4	339.9	339.8	341.8	290.4	18.4	0.081
DM_A	161.7^{B}	140.2^{B}	227.0^{AB}	238.4^{A}	243.3^{A}	216.8^{AB}	267.1^{A}	207.9^{AB}	14.9	<0.001
CP_H	543.9^{b}	567.1^{b}	640.9^{a}	661.2^{a}	647.8^{a}	629.1^{a}	603.5^{a}	626.1^{a}	22.7	0.007
CP_T	629.8^{b}	657.6^{b}	715.1^{a}	764.1^{a}	778.1^{a}	715.02^{a}	707.9^{a}	716.1^{a}	28.7	0.018
CP_A	327.8^{B}	383.3^{AB}	409.3^{AB}	421.2^{AB}	464.1^{A}	460.0^{A}	385.3^{AB}	410.1^{AB}	23.3	0.007
CP_{TB}	218.3^{B}	241.9^{B}	332.3^{A}	352.5^{A}	384.8^{A}	357.8^{A}	337.2^{A}	323.3^{A}	15.9	<0.001
EE_H	122.9	124.9	123.7	113.5	119.6	97.2	104.7	114.6	14.2	0.902
EE_T	56.4	50.9	49.0	47.6	36.2	37.3	34.1	34.0	5.4	0.034
EE_A	132.4^{C}	155.9^{C}	232.4^{AB}	245.1^{A}	200.6^{B}	194.8^{BC}	123.5^{C}	258.8^{A}	9.2	<0.001
EE_{TB}	62.2^{C}	71.4^{C}	128.8^{A}	140.5^{A}	117.0^{A}	105.9^{B}	77.2^{C}	133.8^{A}	5.3	<0.001

Abbreviations: DM_H, DM_T, DM_A: Dry Matter content of the Head, Thorax and Abdomen respectively (g/kg); CP_H, CP_T, CP_A, CP_{TB}: Crude Protein content of the Head, Thorax, Abdomen and Total Body (g/kg DM); EE_H, EE_T, EE_A, EE_{TB}: Ethereal Extract content of the Head, Thorax, Abdomen and Total Body (g/kg DM).

As far as the mortality rate, it was affected by the diet and time, but the D × T interaction was not significant (Table 3). Notably, the MR in PD fed bees was always higher than in HF groups ($p < 0.05$), except for the PD08 one.

Worker bees' development

The diet played a role on the ADG, Hw and OD measured at the end of the trials, but not on the other physiological variables (Table 4). None of tested covariables (average SSI, WI, DMI, CPI, EEI and NFCI) had significant effect. As far as the ADG, the PDs fed bees exhibited a higher ($p < 0.05$) weight gains compared to the HF and SS50 counterparts (Table 4). Head weight showed a significant variation among diets with the bees fed on PD04 to PD07, as well as on SS50, that exhibited higher values compared to the HF controls ($p < 0.01$). Concerning the bees' ovaries, the SS50, HF and PD04 fed bees showed lower development ($p < 0.05$) than the PD06-08 fed counterparts (Table 4).

Worker bees' body composition analysis

The effect of the diet on the DM, CP and EE contents of the different HBs' body parts resulted always significant except for the DM_T and the EE_H (Table 5).

As far as the DM content in heads, only the PD07 fed bees showed a higher value ($p < 0.01$) compared to the controls, but as far as the abdomen DM content, it was also higher in PD04 and PD05 than in SS50 and HF fed bees. The contrast of HF versus PDs, was not significant for DM_H (Table S9).

The CP_H and CP_T contents in all PDs fed bees resulted higher compared to controls ($p < 0.05$), but as far as the CP_A content it was not the case for PD05 and PD06 (Table 5). However, the total body CP content of all the PD fed bees (348.2 ± 20.2) was higher ($p < 0.01$) than SS50 and HF groups (230.1 ± 11.8).

Concerning the abdomen, the bees fed on PD04 and PD08 showed the highest EE content (Table 5), followed by the PD05 bees ($p < 0.01$) and then by the PD07 fed bees that not differed from the controls ($p < 0.01$). Except for PD07 fed bees, the total body EE of all PD groups was higher in comparison to the controls ($p < 0.01$).

Conversely, the EE_T was higher in controls than in PD fed bees, with the lowest value recorded for PD08. Pairwise comparisons and contrast analysis did not result statistically significant (Table 5).

V. apis and V. ceranae infection prevalence and relative spore load

The 100% of newly emerged bees at the beginning of the trials were positive for *V. ceranae,* and 50% of them were also positive for *V. apis*. At the end of the trials, nine HB pools out of forty (22.5%) resulted co-infected, 20 (50.0%) were positive for *V. ceranae* only, and 11 resulted positives for *V. apis* (27.5%) only. For

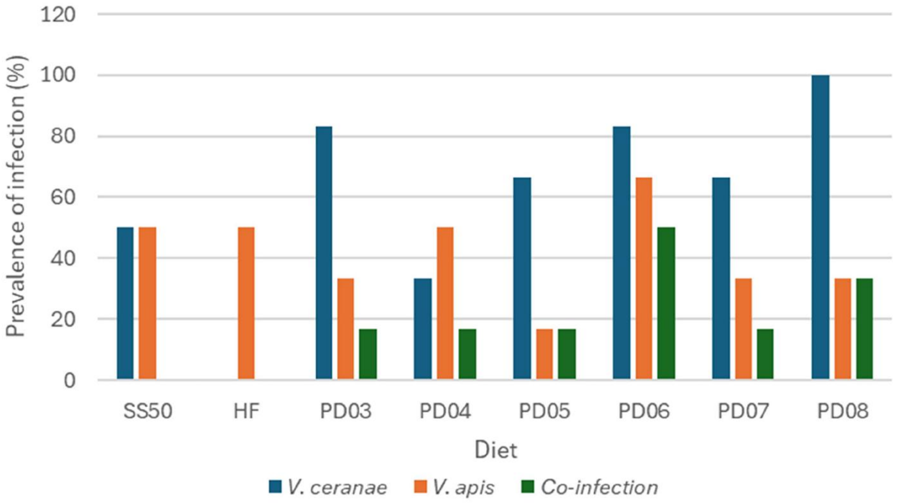

Figure 3. Prevalence of infection of *V. ceranae*, *V. apis* and co-infection.

Table 6. Independence tests between prevalence and treatments.

	Observed	Critical	DF	Alpha	*p*-value
Chi-square	23.741	36.415	24	0.05	0.476
Wilks' G^2	28.705	36.415	24	0.05	0.231

V. ceranae (Figure 3), the prevalence ranged from 0% in bees fed on HF up to 100% in bees fed on PD08, while *V. apis* prevalence ranged from 17% in PD05 up to 67% in PD06 groups (Figure 3). The frequency of double positive cases was overall low except for PD06 (50% of the total) and PD08 (Figure 3) and it was nil in the controls (SS50 and HF). However, the independence tests between infection state and diets did not result statistically significant (Table 6).

With the exception of coinfection (RLS_{ca}), the RSL for the cases of *V. ceranae* or *V. apis* infection were negative. However, the effect of the diet was significant just for RSL_c ($p < 0.05$) with total remission of spore load for HF-fed bees ($p > 0.01$), though not significantly different from diets PD03-PD05 and the SS50 control (Table 7).

Discussion

Adequate nutrition is essential for honey bee colony growth and stress resistance (Brodschneider and Crailsheim 2010). Though the honey bee nutrition strongly depends on seasonal and floral diversity, yet researches often overlook these environmental factors (Donkersley et al. 2014; DeStefano 2015; DeGrandi-Hoffman et al. 2018; Tsuruda et al. 2021). Multifloral pollen diets are reported to enhance longevity, immunity, and pathogen resistance in honey bees (Schmidt et al. 1995; Alaux et al. 2010; Di Pasquale et al. 2013), even though pollen diversity, among other factors, is strongly affected by seasonality (Al-Kahtani et al. 2020; 2021; Malagnini et al. 2022).

In this study, pollen biodiversity of CPLs was high in the first part of the production season (April-June) but markedly decreased in August (Figure 2). Accordingly, both H′ and E followed similar patterns during the season, except for August CPLs, due to the low number of pollen types (six) that resulted almost evenly represented (Figure 2). These results are roughly in line with Dimou and Thrasyvoulou (2007) who collected the highest number of different pollens in Greece between April and June (though almost double than in this study) and with Malagnini et al. (2022), that in Northern Italy observed higher pollen biodiversity of CPLs in March–May, though with 50% lower value of H′ compared to our findings. It is interesting to notice that three pollen types in this study (*i.e. Rubus*, *Quercus*, and *Echium* types) were always well represented in CPLs harvested from April to June, collectively representing 22% of the total pollen types of that period. Specifically, *Rubus* pollens accounted for 10.2%, *Echium* for 6.3%, and *Quercus* for 5.5%. Though the specific nutritional value of these pollen types are not fully documented in literature, their consistent availability could represent a reliable foraging base for the honey bee colonies in the region, as highlighted in a previous study (Donkersley et al. 2014).

Previous researches on pollen gathered by the honey bees, reported protein contents ranging from 20.0 to 600.0 g/kg, and fat contents between 10.0 and 200.0 g/kg (Roulston et al. 2000; Roulston and Cane 2000; Ruedenauer et al. 2019; Vaudo et al. 2020). Though the CP content of PDs, the average level was

Table 7. Effect of diets on *Vairimorpha* spp. relative spore loads (%).

Variable	SS50	HF	PD03	PD04	PD05	PD06	PD07	PD08	SEM	*p*-value
RSL$_c$	-50.09^{AB}	-100.00^{B}	-37.75^{AB}	-79.99^{AB}	-61.97^{AB}	-21.41^{A}	-24.46^{A}	-19.11^{A}	18.56	0.032
RLS$_a$	−39.54	−48.15	−62.91	−38.75	−89.91	−41.47	−36.47	−49.61	28.01	0.891
RLS$_{ca}$	0.00	0.00	10.60	11.82	11.53	37.82	18.91	31.81	13.40	0.421

Abbreviations: RSL$_c$: *V. ceranae* relative spore load; RLS$_a$: *V. apis* relative spore load; RLS$_{ca}$: relative spore load in co-infection cases; SEM: Standard Error of the Mean (pooled).

assessed as 239.4 g/kg DM, aligned with the mentioned data. Moreover, CP content (Table 2) exhibited a positive but not significant association with pollen biodiversity (H′) ($r = 0.70$, $p = 0.116$, data not shown).

The EE and ash contents of PDs, were consistent with literature findings (Roulston et al. 2000; Roulston and Cane 2000; Campos et al. 2008; Thakur and Nanda 2020; Vaudo et al. 2020). In particular, all PDs, but also the HF control, for both the constituents (Table 2) were close to the lower limit of the reported range, none of them showed any noteworthy correlations with other in trial tested variables and H′ (data not shown).

To date, no specific data are available for the NFC content in CPLs as assessed in this study. However, Herbert and Shimanuki (1978) found starch and pectin contents in corbicular pollen of 18.0 g/kg DM and 16.0 g/kg DM respectively. In another study (Bayram et al. 2023), total carbohydrate in Italian CPL samples ranged from 256.6 g/kg DM to 613.0 g/kg DM, whereas in Croatian samples it ranged from 433.9 to 479.0 g/kg DM (Prdun et al. 2021). In our study, the total carbohydrate content, calculated as the sum of NDF and NFC, ranged from 634.1 to 714.3 g/kg DM. These values are higher and not consistent with the aforementioned study on total carbohydrates and data on starch and pectin. Although significant variability in carbohydrate composition can be expected in pollen from different floral origins (Bonvehí and Jordà 1997; Bertoncelj et al. 2018) but methodological differences across studies may lead to apparent discrepancies.

The ash content of CPLs, according to Campos et al. (2008) and Thakur and Nanda (2020), can be variable from 20.0 to 60.0 g/kg. Our data, ranging from 22.4 to 30.7 g/kg DM for PDs, reflected the literature values and showed a decreasing trend as the season progressed, while HF control diet resulted higher compared to PDs (Table 2).

Concerning the macro element profile, the data on CPL of this study confirms literature data as the average values of 12.9 g/kg DM for K, 1.7 g/kg DM for P while, for microelements, Cu (4.81 mg/kg DM) falls within the reported ranges (K: 2.2–38.0 g/kg DM; P: 1.4–80.0 g/kg DM; Cu: 3.0–42.0 mg/kg DM)(Somerville and Nicol 2002; Valverde et al. 2023). Conversely, the average Na content in PDs (114.6 mg/kg DM, Table 2) was approximately twice the maximum value reported by Somerville and Nicol (2002). In contrast, the average Fe content in solid diets (4.2 mg/kg DM) was about 70% lower than the minimum detected by Somerville and Nicol (2002) (range: 14.0–520.0 mg/kg DM), and approximately 92% lower than the values reported by Valverde et al. (2023) (range: 58–134 mg/kg DM).

Differently from the main compositional traits, where only for CP, a slight association was found with H′ that was not statistically supported, moderate-to-strong positive associations of the variable content of P ($r = 0.93$, $p = 0.007$, data not shown), Mg ($r = 0.84$, $p = 0.036$), Cu ($r = 0.96$, $p = 0.002$), Mn ($r = 0.82$, $p = 0.047$), and Zn ($r = 0.81$, $p = 0.048$) in CPLs were found with the Shannon index. It is known that phosphorus and magnesium play an essential roles in regulating haemolymph osmotic pressure and maintaining inter- and intracellular fluid balance in honey bees (Valverde et al. 2023). On the other side, elements such as copper, manganese, and zinc have been recognised as crucial for bees' growth, development, and reproductive functions (Valverde et al. 2023). Based on the reported physiological effects of such macro and micro minerals on honey bees, it can be speculated that the positive association found in this study could, though partially, explain the widely recognised correlation in the literature (Schmidt et al. 1995; Alaux et al. 2010; Di Pasquale et al. 2013) between pollen diversity and the health of honey bee colonies.

Furthermore, the chemical composition analysis of the diets showed that the highest protein content was observed in the June diets (PD06), while the lowest was in August (PD08). PD08 was also poor in the same mineral elements correlated with H′ (Mg, P, ZN, Mn and Cu) just like HF diets; in addition, strong positive correlations between diets component and micro- and macroelements (Table S7) were registered for all of them (ranging from $r = 0.375$, $p < 0.05$ for NFC with Na to $r = 0.965$, $p < 0.001$ for NFC with Ca) with the exception of K and Na with EE. This results agree with previous studies emphasising the importance of pollen quality in honey bee health (Di Pasquale et al. 2013).

The role of macro- and micronutrients was also evident in the positive associations between protein and lipid contents in HB total bodies and the phosphorus and magnesium concentrations in pollen diets. In particular, CP presented strong correlation with Mg ($r = 0.608$, $p < 0.001$) and P ($r = 0.697$, $p < 0.001$) while lipid content showed positive, but less stronger compared to CP ones, correlations for magnesium and phosphorus (respectively $r = 0.396$, $p < 0.001$ for Mg and $r = 0.419$, $p < 0.001$ for P) (data not shown). As in honey bees magnesium is involved in muscle contraction and relaxation and participates in ATP metabolism (De Sousa et al. 2022) as well as for P that may function as energy sources for ATP synthesis (Hsu et al. 2007), our data confirm the importance of nutrient balance for bee health, as previously suggested by Brodschneider and Crailsheim (2010).

Finally, since in the past the protein content of pollen has been considered a direct and reliable measure of pollen quality (Pernal and Currie 2000), and it has been observed that the overall nutritional composition of pollen plays a fundamental role in determining its quality (Thakur and Nanda 2020; Oroian et al. 2022), it can be said the protein content, together with the aforementioned mineral elements and high pollen biodiversity, can be considered an indicator of higher-quality pollen diets.

Pollen fed groups showed higher intake of DM, and therefore of CP, EE and NFC, than the HF controls (Table 3). The low DMI of HF controls could be due to a poor palatability caused by the matrix structure or the absence of nectar in the formulation. Pollen is typically preferred by honey bees over pollen replacements (Ghramh and Khan 2023; Kim et al. 2024), not because it is richer in nutrients but rather because it contains specific carbohydrates, lipids, and amino acids (Bilisik et al. 2008) in suitable compositions that make it highly appealing to the honey bees (Huang 2012). In addition, foraging workers normally add regurgitated nectar or honey when packing pollen into their corbicula with further positive effects on its palatability (Boch 1982; Lan et al. 2021).

Diet is a crucial factor in honey bee development, directly influencing body composition, physiological functions, and overall health (Di Pasquale et al. 2013; Arien et al. 2020). Total body composition data for PD fed bees of this study are aligned with previous findings by Kunert and Crailsheim (1988) and Ghosh et al. (2016), who reported values ranging from 250 to 780 g/kg DM for CP, and from 69.0 to 160.0 g/kg DM for the EE content. Notably, not only the CP-free (SS50) but also the HF fed bees showed lower total body CP contents than the PD counterparts (Table 5): it is well known that during the first two weeks after emergence, worker bees gain body mass and proteins primarily due to their pollen diet (Haydak 1970; Di Pasquale et al. 2013). Although HF was higher in protein than PDs, the lower intake (Table 3) was most probably the main limitation of the CP accumulation in bees (Ghramh and Khan 2023; Kim et al. 2024).

The low lipid content of HF, combined with the reduced DMI (Table 3), resulted in a very low end-of-trial lipid accumulation in bees similarly to SS50-fed bees (Table 5). On the contrary, the higher EEI in PD-fed bees (Table 3) was reflected in a higher total body fat content, though some differences among the PD groups were detected ($r = 0.584$, $p < 0.001$, data not shown).

The evidence for CP and EE accumulation in the body of young workers paralleled the average daily gain of PDs-fed bees which were higher than SS50 and HF controls, especially for diets during the central months of the season (PD04-PD07; Table 4), reflecting ($r = 0.576$, $p < 0.0001$ for CP and $r = 0.317$, $p < 0.028$ for EE; data not shown) exactly the trend of DM intake (Table 3).

As far as the allocation of main nutrients (CP, EE) in the bees' body, CP in bees' heads and thoraxes was twice or more than in the abdomen (Zheng et al. 2014), that in turn was the main site of EE accumulation followed by the bees' head (Brejcha et al. 2023), though with no difference among the experimental groups (Table 5).

Despite a similar dry matter intake across all pollen diets (Table 3), the lower lipid content of PD07 (Table 2) resulted in a lower EEI compared to the other PD groups (Table 3), leading to a reduced lipid deposition in the bee's abdomen and thereby in whole body (Table 5). In fact a strong positive correlation has been observed between EE content in diets and EEI ($r = 0.794$, $p < 0.001$; data not shown); it is well known that the fat body develops more extensively in bees fed pollen diets (Alaux et al. 2010).

These findings highlight how diet composition influences the body composition of developing bees.

Proteins play a crucial role in the development of HPGs (DeGrandi-Hoffman et al. 2010; Sun et al. 2021; Khedidji et al. 2024), and it is known that honey bees with limited pollen intake or those fed protein-deficient pollen exhibit less developed HPGs (Crailsheim and Stolberg 1989; Khedidji et al. 2022). Our data suggest that multifloral diets demonstrated the ability to promote greater development of hypopharyngeal glands in PD fed bee compared to HF control (Table 4), as indicated by increased head weight (Hrassnigg and Crailsheim 1998; Rahman et al. 2014; Rinkevich et al. 2016).

In pollen fed bees, Alaux et al. (2010), observed an increase in glucose oxidase (GOX) activity, which may help explain the positive outcomes noted in hypopharyngeal gland (HPG) development, in diets PD06 and PD07, as well as for a slight increase in OD although they are always at the inactive stage (Table 4). GOX is produced within the hypopharyngeal glands (Ohashi et al. 1999) and facilitates the transformation of b-D-glucose into gluconic acid and hydrogen peroxide, thus playing an important role in bees immunity. These findings support the hypothesis that a multifloral diet can enhance immune functions, in agreement with Alaux et al. (2010).

Vairimorpha spp. infection and pollen nutrition are widely discussed topic (Martín-Hernández et al. 2011; Porrini et al. 2011; Jack et al. 2016). Since the microsporidia have a limited ability to produce ATP and the absence of most primary metabolite genes increase the metabolic cost for the host (Williams 2009; Azzouz-Olden et al. 2018), infected bees may compensate by feeding more, as energetic stress is shown to rise with higher spore doses (Martín-Hernández et al. 2011).

Even if no clinical symptoms of *Vairimorpha* spp. infection were observed, spore loads of 561 ± 741 and 2,850 ± 2,784 were detected for *V. apis* and *V. ceranae*, respectively, with maximum values reaching 2,455 and 10,950 spores per bee (data not shown). SL_c were consistent with data reported by Bourgeois et al. (2010) while SL_a resulted always higher (*V. apis* = 303 ± 38; *V. ceranae* = 12,768 ± 8,106), these spore loads could be sufficient to cause infection in honey bees (Malone and Stefanovic 1999).

The statistical analysis failed to reveal a significant relationship between diet type and *Vairimorpha* spp. prevalence (Table 6). This result may reflect the complexity of interactions between nutrition and infection by the microsporidians, as previously noted by Jack et al. (2016), who found higher infection levels in pollen-fed bees (prevalence range 23%–35%) compared to 50% of prevalence of *V. ceranae* and 27.5% of *V. apis* in our trials, but also greater longevity compared to those fed sugar syrup. The lack of relationship between diet type and *Vairimorpha* spp. prevalence (Table 6) underscores that while pollen may not directly prevent infection, it can enhance bees' physiological responses by supporting their metabolic and immune functions. Interestingly, a reduction in RSL_a and RSLc was observed across several diets, including not only pollen-based diets but also the sugar and artificial diet controls (Table 7). While this suggests that the reduction in spore load is not exclusive to pollen intake, certain pollen diets, specifically PD03, PD04, and PD05, achieved RSLc reduction values comparable to the HF group, which reached a total reduction of spore load between the beginning and the end of the trial (Table 7). This outcome suggests that some diets may indirectly still support spore reduction similarly to control diets. The reduction in sporal load observed in pollen diets could have contributed to promoting a high survival rate at the end of the trials, which was 94.77 ± 6.38% (data not shown), as reported in previous studies by Di Pasquale et al. (2013) and Khedidji et al. (2022). Nevertheless, in this study the mortality rate of the PD groups (5.23 ± 2.60%), with the exception of the PD08 one, was higher compared to the HF controls (Table 3). Mortality highlighted just in pollen diets may be explained by the evidences that curbicolar pollen could serve as vector of contaminants like pesticides (de Oliveira et al. 2016; Ruiz-Toledo et al. 2018), organic pollutants (Roszko et al. 2016) and heavy metals (Morgano et al. 2010). The higher dry matter intake from PD compared to HF (Table 3), along with the corresponding accumulation of CP in the total body (Table 5), could result in an increased intake of these toxic contaminants by bees. Although, for example, Pb content in pollen diets was higher than in HF control (Table 2), the differences observed in mortality rates (Table 3) cannot be directly attributed to variations in toxic elements concentrations among diets (Pb: $r = -0.101$, $p = 0.406$, Cr: $r = -0.185$, $p = 0.598$; Cd: $r = -0.215$, $p = 0.229$; data not shown).

In addition, a negative correlation between RLS_c and EE_{TB} was observed ($r = 0.328$, $p = 0.023$; data not shown), supporting the findings of Gilbert et al. (2024), who reported that infection of *V.ceranae* reduced the fat body lipid reserve in *Apis mellifera*.

Interestingly, and in contrast to recent reports indicating the declining prevalence, or even absence, of *V. apis* in Italy (Bordin et al. 2022; Pandiscia et al. 2024), this study reports the co-presence of *V.ceranae* and *V.apis* in *A. m. ligustica*. The methodological refinements applied in this study allow us to assert its persistence in tested honey bee colonies. Specifically, the use of species-specific primers selected from Chen et al. (2009), amplification of positive samples, Sanger sequencing of the resulting amplicons, and alignment with the NCBI database provide robust evidence supporting this finding, warranting further investigation on a wider scale.

Conclusions

In summary, the data suggest that pollen diets with diverse and high-quality composition can support honey bee physiology under nutritional and pathological stress (*e.g.* harsh summer temperatures, drought conditions, lower forage availability), as indicated by increased head weight (used as a proxy for HPG development) and by a general reduction in spore load of *Vairimorpha spp.*, especially *V. ceranae*, also observed in some pollen diets, although similar reductions were recorded in non-pollen diets as well.

Furthermore, curbicolar pollen collected during the central part (April-June) of the beekeeping season, with high quality composition and botanical biodiversity, seems to be more effective to induce positive physiological responses in young Italian honey bees. This kind of pollen supplementation in different times of the beekeeping season (*e.g.* late summer, pre-wintering) could support the nutritional requirements of colonies helping beekeepers counteract seasonal constraint in colonies' management.

However, the complexity of interactions between nutritional intervention and pathological stressors requires further studies, as for example challenging test with instrumental infection of *Vairimorpha* spp. in honey bees supplemented with mono- and multifloral pollen diets to fully understand these relationships and optimise colony management strategies, particularly in increasingly challenging environmental contexts.

Acknowledgements

Financial support has been provided by:

- PRIMA (grant number 1812/PLANT-B), a program supported by the European Union;
- PON—Ricerca e Innovazione 2014–2020 (DM1016/2021) for Manganello F. PhD fellowship.

Vairimorpha spp. positive sample were gently furnished by Centro de Investigación Apícola y Agroambiental (CIAPA-IRIAF, Marchamalo, Spain).

Disclosure statement

No potential conflict of interest was reported by the authors.

Data availability statement

The data presented in this study are available on request from the corresponding author.

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