Manuscript on interspecific variation in nutritional requirements across bees

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Denis Michez[1], Alexandre Barraud[1], Lena Barascou[2], Victor Lefebvre[1], Deborah Sene[2], Yves Le Conte[2], Cedric Alaux[2], Francesca-Vittoria Grillenzoni[3], Francesca Corvucci[3], Giorgia Serra [3] & Cecilia Costa[3]

[1]*University of Mons, Research Institute for Biosciences, Laboratory of Zoology, Place du Parc 20, 7000 Mons, Belgium*

[2] *INRAE, Abeilles et Environnement, Avignon, France*

[3] *CREA Research Centre for Agriculture and Environment, Via Di Saliceto 80, 40128, Bologna, Italy*

PoshBee

Pan-european assessment, monitoring, and mitigation of stressors on the health of bees

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Summary

With approximately 2,000 species currently recorded in Europe, bees are a highly diversified and efficient group of pollinating insects. They obtain their nutrients from the nectar and pollen of flowers. However, the chemical composition of these resources, especially of pollen (e.g. protein, lipid, amino acids, fatty acids or sterol content), is highly variable among plant species. While it is well known that bees show interspecific variation in their floral choices, there is a lack of information on the nutritional requirements of different bee species. We therefore developed original experiments in laboratory conditions to evaluate interspecific variation in bee nutritional requirements. We analysed the chemical content of eight pollen blends, which differed in terms of protein, lipid, amino acid and sterol total concentration and profiles. Each pollen blend was provided to four different model bee species: honey bees (*Apis mellifera*), bumble bees (*Bombus terrestris*), and mason bees (*Osmia bicornis* and *Osmia cornuta*). For each species, specific protocols were used to monitor their development (e.g. weight, timing, survival) and resource collection. Overall, we found that the nutritional requirements across these species are different, and that a low-quality diet for one species is not necessarily low-quality for another. While honey bees are negatively impacted by diets with a high protein content (~40%), bumble bees and mason bees develop normally on these diets but struggle on diets with a low total amino acid and sterol content, specifically with low concentrations of 24-methylenecholesterol and β-sitosterol. Overall, our study supports the need for conserving and/or introducing plant diversity into managed ecosystems to meet the natural nutritional preferences of bees at species and community levels.

1. Introduction

With more than 2,000 species recorded in Europe (Rasmont et al., 2017), bees represent a highly diverse group of pollinators (Michener, 2007; Danforth et al., 2013). These species show wide variability in various traits such as body size (i.e. from 0.3mm to 4.5cm in Europe), social behaviour (e.g. cleptoparasitic, solitary, eusocial), nesting behaviour (e.g. cavity- or soil-nesting), foraging strategies (e.g. pollen generalist or specialist) and phenology (e.g. uni- or bivoltine) (Michener, 2007; Michez et al., 2019). This diversity is crucial for the successful sexual reproduction of wild and domesticated plants, but it is also critical to understand this variability to implement efficient conservation programs (Nieto et al., 2014). Indeed, bees are the dominant pollinators of crops and wild plants in most ecosystems, visiting more than 90% of crop varieties (Potts et al., 2016). Some generalist bee species have been domesticated and are now used for crop pollination, such as the western honey bee (*Apis mellifera*), the buff-tailed bumble bee (*Bombus terrestris*) (Banda and Paxton, 1991; Velthuis and Doorn, 2006), and a few solitary species (Gruber et al., 2011; Pitts-Singer and Cane, 2011). However, unmanaged species are still key pollinators as there are many genus-specific plant-pollinator interactions, linking wild plant diversity to wild bee diversity (Ollerton, 2017). Moreover, wild bees have been shown to increase crop production by up to twice as much as honey bees, underlining the importance of wild bees even in agro-ecosystems (Garibaldi et al., 2013; Weekers et al., 2022).

Losses and declines in managed and wild bee populations have been reported worldwide (Cameron et al., 2011; Goulson et al., 2015; Duchenne et al., 2020). Habitat loss and agricultural intensification, resulting in landscape simplification, have been identified as important drivers of pollinator decline (Winfree, 2010; Persson et al., 2015; Vray et al., 2019). These factors can directly or indirectly affect the quality, quantity and diversity of floral resources and thus the food sources of bees (e.g. Roger et al., 2017). This may make the abundance, distribution/availability, quality and diversity of these resources a main proximal pressure in explaining bee population trends (Roulston and Goodell, 2011; Vaudo et al., 2015).

Bees obtain their carbohydrate nutrient intake mainly from nectar, and their protein and lipid from pollen (Roulston and Cane, 2000; Nicolson, 2011). The chemical composition of pollen is highly variable across floral species, between 2-60% and 1-20% for protein and lipid contents, respectively (Roulston

and Cane, 2000; Vaudo et al., 2020). Field and semi-field studies showed that this chemical composition can be related to bee health (e.g., honey bee *A. mellifera*: Alaux et al., 2010; Brodschneider and Crailsheim, 2010; Di Pasquale et al., 2013; mason bee *Osmia bicornis*: Bukovinszky et al., 2017). Generalist bees seem able to assess pollen chemical quality and balance multiple macronutrient resources when making foraging decisions (Vaudo et al., 2016, 2018; Kraus et al., 2019; Ruedenauer et al., 2020). Based on a large quantity and diversity of samples, Vaudo et al. (2020) showed that honey bees collected pollens between 1:1 and 2:1 protein to lipid (P:L) ratio. This species appears to occupy a different nutritional space compared to *Bombus impatiens* and *Osmia cornifrons*, which collect at P:L ratios of 4:1 and 2:9, respectively. Furthermore, to satisfy the food intake of colonies with numerous individuals, honey bees must collect large amounts of pollen. Therefore, honey bees collect pollen from generalist, open floral morphologies such as mass blooming trees (e.g. *Quercus* sp., *Salix* sp., *Prunus* sp.) and wild herbs with high production of pollen (e.g. Asteraceae), which may have a nutritional make up that falls in the lower P:L values (i.e. 1-3:1 P:L) (Vaudo et al., 2020). Bumble bees appear to be much more picky in their choices, since many species mainly forage on Fabaceae pollen showing a high P:L ratio value (3.8 ± 0.5) (Leonhardt and Blüthgen, 2012; Wood et al., 2021). In contrast to honey bees and bumble bees, *Osmia cornifrons*, a solitary foraging bee with a short flight period, has mixed preferences for Rosaceae and Fabaceae pollen (Haider et al., 2014; Nagamitsu et al., 2018), with average P:L ratios of 1.6 ± 0.3 and 3.8 ± 0.5, respectively.

Regarding chemical profiles, particular lipids and proteins also seem to be important in bee nutritional requirement. For example, sterols (e.g. β-sitosterol) are essential to synthesise ecdysteroid, which is involved in the moulting of the larvae and the maturation of the ovaries of female imago. In the case of sterol deficiency, a delay in moulting can be observed (Regali, 1996). Additionally, a good amino acid balance is crucial for bee development (Moerman et al., 2016). Amino acids are involved in growth, survival, flight ability or in immunity (Regali, 1996; Carter et al., 2006; Moerman et al., 2016). Some amino-acids (methionine, lysine, threonine, histidine, leucine, isoleucine, valine, phenylalanine, tryptophan) and sterols (24-methylenecholestrol and β-sitosterol) cannot be synthesised by the bee and are therefore considered as essential, meaning that it is necessary to obtain them through pollen consumption (De Groot, 1953; Behmer and Nes, 2003; Svoboda et al., 1978).

Experimental studies in controlled conditions have confirmed that the nutritional quality of pollen (e.g. the concentration of protein, and lipids, sterols and amino acids) can have an impact on the development and mortality of bumble bees (e.g Taseï and Aupinel, 2008; Vanderplanck et al., 2014; Moerman et al., 2016, 2017; Barraud et al., 2020; Carnell, Hulse and Hughes, 2020) and mason bees (Sedivy et al., 2011; Eckhardt et al., 2014). The floral diversity of pollen diet does not seem to be the major factor of quality, as bumble bees develop better on high-quality monofloral diets compared to low-quality polyfloral diets (Moerman et al., 2017; Carnell et al., 2020). The pattern for honey bees appears to be similar at an individual level, with pollen quality (reflected by protein content) having an impact on the physiology and survival of adult honey bees (Brodschneider and Crailsheim, 2010; Di Pasquale et al., 2013; Frias et al., 2016; Omar et al., 2017; Li et al., 2019).

Overall, these results suggest that a loss of a part of the plant community, especially the families covering a specific physiological requirement (e.g., Fabaceae), is more likely to affect bumble bees and solitary bees than honey bees (Leonhardt and Blüthgen, 2012). The more generalised the foraging behaviour of a particular bee species, the more likely it is to be able to switch to alternative host plants and persist in an area, even if those host plants are of a lower nutritional quality (Roger et al., 2017b). However, there are very few studies evaluating and comparing the development of various generalist bee species in controlled conditions on the same pollen diets (Moerman et al., 2016), and no study considering a broad diversity of bee clades (e.g., different bee tribes or bee families).

To address these knowledge gaps, we evaluated the effect of 8 pollen mixes of different qualities on key life-history traits regulated by pollen consumption in four European bee species (2 Apidae species: *Apis mellifera* (Apini) and *Bombus terrestris* (Bombini); 2 Megachilidae species: *Osmia bicornis* and *O. cornuta*). We first conducted palynological and chemical analysis (total protein, total lipid, amino acid

and sterol content) on these pollen blends to characterise their quality composition. We then developed experiments in controlled conditions and monitored the key life-history traits in bees fed with these pollen diets (e.g., survival for honey bees, brood production for bumble bees and larva development for mason bees). We finally investigated which nutritional factors better explain bee health and development across the four species. Our hypothesis was that bee nutritional requirements are different across species.

2. Methodology

2.1 Overview of the study

According to the grant agreement, we were planning to develop protocols for three model bee species (*A. mellifera*, *B. terrestris* and *O. bicornis*) to assess the effect of nutrition on development and health. We additionally considered *O. cornuta*. We also planned to focus on pollen as the diet, since its chemical composition is much more variable than that of nectar and it is known to affect bee health (e.g. *A. mellifera*, Alaux et al. 2010; *B. terrestris*, Roger et al. 2017; *O. cornuta*, Eckhardt et al. 2014). We originally considered 3 different pollen sources from crops regularly visited by generalist bees: *Phacelia tanacetifolia*, oilseed rape (OSR) and sunflower, which show a gradient in protein concentration, high in *Phacelia*, intermediate in OSR and low in sunflower, but these pollens were not available pesticide free in sufficient quantities. We therefore worked on 8 other pesticide free diets. We organised the characterisation of the chemical profile of the 8 pollen diets (total protein, amino acid profile, total lipids, sterols; e.g. Roger et al. 2017). The presence of agrochemical residues in pollen was determined by CREA. We considered the following parameters for *Apis* and *Bombus* species as end point parameters: (i) number, mass and mortality of offspring (larvae), (ii) mortality and fat body of workers, (iii) total syrup and pollen consumed, (iv) sperm quality of males. We did not evaluate fat body because preliminary results showed that this trait did not vary much. As time was really limited due to COVID pandemia we decided to not evaluate this parameter. Regarding sperm quality, it was not possible to study it on honey bees as the selected protocol considered females only. We did analyse the impact of pesticide on sperm quality on bumble bees but we had to do it in a specific experience (longer to have male emergence from the microcolonies). We did not include the study of sperm quality in the first batch of experiments because it was too timing consuming due to access restrictions associated with COVID pandemia. The results on sperm quality will be analysed and published after the deadline of this deliverable. For *Osmia*, we wanted to study for each developing bee: (i) the amount and rate of pollen consumption, (ii) speed of larval development, (iii) larval mortality, (iv) pupal mortality, (v) rate of depletion of the fat body, (vi) time until emergence from the cocoon, (vii) weight at emergence, and (viii) sperm quality of males. For the same reasons as for the previous species, we did not consider sperm quality (i.e., experience only with females). Preliminary data from other experiments with *O. bicornis* however show that fat body depletion can be robustly approximated by measuring cocoon weight. Instead of measuring fat body, genotype expression analysis in bees exposed to different nutrition and specific pesticides at different doses was performed (included in the deliverable 5.2). This greatly contributed the mechanistic understanding of impacts detected in other PoshBee *Osmia* experiments, and thus their impact and relevance. The protocols and analyses are described below. This study was published in 2022 under the following reference:

Barraud A., Barascou L., Lefebvre V., Sene D., Le Conte Y., Alaux C., Grillenzoni F.-V., Corvucci F., Serra G., Costa C., Vanderplanck M., Michez D. 2022. Variation in nutritional requirements across bee species. Frontiers in Sustainable Food Systems, 6: 824750. https://doi.org/10.3389/fsufs.2022.824750

2.2 Bee model species

This study was conducted on four common pollen generalist bee species recorded in Europe which are foraging in the same habitat for part of the year (Michez et al., 2019). We selected the Western honey bee *Apis mellifera* (Hymenoptera, Apidae, Apini), a domesticated eusocial species; the buff tailed bumble bee *Bombus terrestris* (Hymenoptera, Apidae, Bombini), a wild social species (Rasmont et al., 2008); and two mason bees (*Osmia bicornis* and *O. cornuta*; Hymenoptera, Megachilidae, Osmiini), wild solitary species. They are commonly used as model species because of their easy management in laboratory conditions.

2.3 Characterisation of pollen diet

Eight organic blends of honey bee-collected pollen were purchased from the company "*Abeille heureuse*" (France). Each pollen blend was gamma irradiated to avoid parasite infection, homogenized to reduce the risk of variation in palynological composition in each pollen treatment, and stored at -80°C before the experiment. In addition, a fraction of each pollen diet was lyophilized and stored at -20°C for palynological and chemical analyses (see below). Each pollen mix was named based on their palynological analysis, using the first letter of the dominant pollen species (see Table 1).

2.3.1 Pesticide analyses

For each pollen diet, the presence of pesticide residues was determined by liquid chromatography–tandem mass spectrometry (LC-MS/MS) with a limit of quantification of 0.01 mg/kg and a limit of detection of 0.005 mg/kg following the EN 15662:2018 procedure. Residues of 2,4 dimethylformamidine (DMF, degradation products of amitraz) and tau-fluvalinate were detected in all pollen blends but were below the limit of quantification. These compounds, used as chemical treatments against the honey bee parasite *Varroa destructor*, are consistently found in honey bee collected pollens (47.4% and 88.3% of trapped pollens for amitraz and tau-fluvalinate, respectively; Mullin et al., 2010; Calatayud-Vernich et al., 2019) and are considered as relatively safe for honey bees with an oral LD50 of 75 µg/bee for amitraz (contact exposure) and 45 µg/bee for tau-fluvalinate (oral exposure) (US EPA, 2021).

2.3.2 Palynological analyses

One gram of pollen sample was inserted and centrifuged in a 50 ml centrifuge tube and then dissolved in 20 ml of distilled water. Using a Pasteur pipette, a drop of sediment was placed on a microscope slide and spread out over an area of about 18 x 18 mm. After drying, the sediment was included in one drop of glycerine jelly and covered with the cover slip. Examination under the microscope was performed with 400X magnification. After a first general check to identify all the pollen types in the slide, a second read of the slide was carried out until 500 pollen grains were counted. Abortive, irregular or broken pollen grains were still counted if they could be identified.

Recognition of pollen type was based on comparison between the observed pollen forms and those present in the CREA-AA collection of reference slides (built from anthers of identified plants). For each pollen type, the percentage of each species with respect to the total number of counted pollen grains was calculated.

2.3.3 Protein analyses

Pollen protein concentration was measured using the Bradford assay according to Vaudo et al. (2020). We added 1.5mL of 0.1M NaOH to ~1mg of pollen sample (dry weight), and conducted the Bradford assay with the Bio-Rad Protein Assay Kit microassay 300 µL microplate protocol using bovine γ-globulin as the protein standard (Bio-Rad Laboratories, Inc., Hercules, CA). We used three technical replications for each biological replication and measured absorbance at 595nm using a SpectraMax 190 spectrophotometer (Molecular Devices, LLC, Sunnyvale, CA). Protein concentrations were calculated using polynomial 2nd analysis from the protein standards.

2.3.4 Lipid analyses

Pollen lipid concentrations were determined using a modified protocol from Van Handel and Day, 1988. In 2.0mL microcentrifuge tubes, we added 200µL 2% sodium sulfate and 1.6mL chloroform/methanol to ~1mg of each pollen sample (dry weight) before a 5 min centrifugation. Supernatant was transferred to a clean glass tube with 600µL deionised water, and centrifuged for 5 min. We separated the top carbohydrate/water/methanol fraction and the remaining chloroform fraction was used for lipid analysis. The lipid/chloroform fraction was left overnight in a fume hood to completely evaporate the solvent. We added 200µL sulfuric acid to the sample and heated at 100°C for 10min. Then, 5mL vanillin/phosphoric acid reagent was added. We used three 300µL technical replications for each biological replication and measured absorbance at 525nm. Lipid concentrations were calculated using polynomial 2nd analysis from vegetable oil standards. Pollen concentrations of protein and lipids are reported as µg nutrient/mg pollen, and subsequent P:L ratios were determined for each diet.

2.3.5 Amino acid analyses

For the analysis of total amino acids, 1mL of hydrolysis solution (6N HCl, 0.1% phenol and 500 µM norleucine) was added to 3–5 mg (dry weight) of pollen (Vanderplanck et al., 2014) and then incubated for 24 hours at 110°C. The hydrolysate was evaporated until dryness under vacuum in a boiling bath at 100°C. Afterwards, 1 mL of the sodium citrate buffer pH 2.2 was added into the tube. The sample solution was poured into an HPLC vial after filtration (0.2 µm filter), and each amino acid was measured separately with an ion-exchange chromatograph. A post-column ninhydrin reaction produced coloured derivatives, which was monitored via a UV detector. For amino acid quantification, norleucine was used as the internal standard. This analysis includes essential amino acids that bees cannot synthesize, as well as the non-essential ones. The essential amino acids were established by De Groot (1953) for honey bees; namely arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine.

2.3.6 Sterol analyses

Before each analysis, pollen samples were divided into a minimum of three samples (i.e., 20 mg (dry weight) per analytical replicate). Sterols were quantified by GC-FID after extraction and purification according to the method described by Vanderplanck et al. (2011). The multi-step procedure can be summarized as follows: (i) saponification with 2M methanolic potassium hydroxide, (ii) extraction of the unsaponifiable portion with diethylether and several water washings, (iii) solvent evaporation, (iv) fractionation of the unsaponifiable portion by TLC, (v) trimethylsilylation of the sterols (scraped from the silicagel) and (vi) separation by GC. The total sterol content was determined considering all peaks above the limit of quantification ((LOQ); LOQ = 9.6 ng/1.2 µl injected) whose retention time were between cholesterol and betulin (internal standard). Individual sterols were quantified on the basis of peak areas from analyses. Under the present analytical conditions applied, campesterol and 24-methylenecholesterol co-eluted. Therefore, the results are pooled for these two compounds. Compounds were identified according to their retention times by comparison with those of sunflower oil as reference. The identifications were corroborated by GC-FID (Vanderplanck et al., 2011).

2.4 Experimental protocols

As the three genera (i.e. *Apis*, *Bombus* and *Osmia*) show very different life cycles and behaviour, they could not be tested following the same protocol in laboratory conditions. Thus, we developed a different experimental setup for each of the three bee genera (Figure 1).

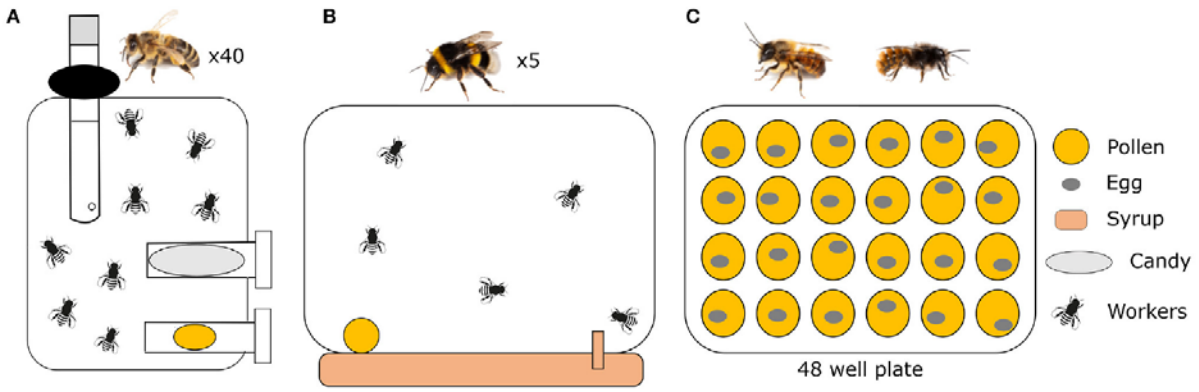

Figure 1: Experimental set-ups for (A) honey bees, (B) bumble bees and (C) mason bees. Caged honey bees were provided with water, candy and one of the pollen diets. Five bumble bees (workers) were placed in plastic boxes provided with pollen and syrup. Eggs of mason bees developed in cell culture plates of 48 wells filled with 400mg of pollen.

2.4.1 Honey bee (*Apis mellifera*)

In adult honey bees, pollen consumption is largely limited to young worker bees and queens. Its consumption in workers enables the development of mandibular glands (Camilli et al., 2020) and hypopharyngeal glands, where jelly is produced to feed larvae, the queen and drones (Crailsheim, 1992; Crailsheim et al., 1992). We therefore tested the influence of pollen quality at the individual level on the fresh weight of individual heads, which is highly correlated to the volume of acini from the hypopharyngeal glands (Hrassnigg and Crailsheim, 1998). We also measured the survival rate of bees. To obtain one-day-old bees, brood frames of eight colonies (*Apis mellifera ligustica* x *Apis mellifera mellifera*) containing late-stage pupae were placed overnight into an incubator under controlled conditions (34°C, 50-70% of relative humidity (RH)). The next day, newly-emerged bees (less than 1 day old) were collected, mixed and groups of 40 bees were placed in cages (10.5 cm x 7.5 cm x 11.5 cm) (Pain, 1966). Caged bees, kept in an incubator (30°C and 50-70% RH), were provided *ad libitum* with water, candy (Apifonda® + powdered sugar) and one of the pollen diets (n = 10 cages per experimental group) (Figure 1A). Pollen diets were replaced every day for 10 days. To simulate as much as possible colony rearing conditions, caged bees were provided with a Beeboost® (Ickowicz, France), releasing one queen-equivalent of queen mandibular pheromone per day. Each day, pollen diets were weighed to determine the amount of pollen consumed per day and per bee. Pollen collection was corrected for evaporation, which was estimated by placing two samples of each pollen mixture in the same incubator for 24 hours. Bee mortality was recorded every day for 44 days by counting and removing dead bees from cages. On day 7, 9 bees were sampled from each cage and stored at -80°C. The fresh weight of heads was then measured for individual bees (n = 9 bees per cage giving a total of 90 bees per experimental group).

2.4.2 Bumble bee *(Bombus terrestris*)

We tested the impact of pollen on bumble bees at the micro-colony level. Such a method to test the nutritive value of pollen diets has been shown to be a good estimate of queenright colony development at least under laboratory conditions with food *ad libitum* (Taseï and Aupinel, 2008b). A total of five queen-right colonies of 100 *Bombus terrestris* workers each were used to build up 80 queen-less micro-colonies of five workers, placed in plastic boxes (8 x 16 x 16cm) (Figure 1B). This number of individuals per micro-colony has been repeatedly used and is assumed to be the most reliable for assessing diet effects (Gradish et al., 2013; Moerman et al., 2016; Roger et al., 2017a; Vanderplanck et al., 2018; Klinger et al., 2019). Micro-colonies were then distributed in the different conditions (n = 10 micro-colonies for each experimental treatment). All micro-colonies were maintained in the same room in constant darkness at 26 ± 2°C with a relative humidity of 60-65%. They were manipulated under red light to minimize disturbance (Sadd, 2011) for a period of 28 days. Pollen diets were provided *ad libitum* to the

micro-colonies as candies (mixed pollen with sugar syrup). New pollen candies were provided every two days, while the previous ones were weighed to assess pollen collection. Pollen collection was corrected for evaporation by monitoring the weight of two samples of each diet placed in the rearing room for 48h. To estimate the performance and development of bumble bee micro-colonies, we measured: (i) the total pollen and syrup collections, which can impact brood production and development (e.g. Plowright et al., 2008; Sutcliffe and Plowright, 2008); and (ii) colony growth after 28 days of development [i.e. mass of individuals from all brood stages (eggs, larvae, pupae, non-emerged and emerged males)] (Vanderplanck et al., 2014, 2018). For each micro-colony, all the measured parameters were divided by the total mass of the five workers to standardize the results and avoid potential effects of worker activities related to their size (i.e., consumption and brood care) (Cnaani and Hefetz, 1994). Additionally, we calculated the pollen efficacy as the mass of total offspring divided by the total pollen collection to estimate the colony performance.

2.4.3 Mason bee *(O. cornuta and O. bicornis*)

We tested the impact of the pollen diet on the two species of mason bees at the larval stage. Standard mason bee nesting plates were installed close to the laboratory on the campus of the University of Mons (Belgium). A total of 1,000 individuals were released next to the nests. At regular time intervals, nests were opened and investigated for brood cell production. After three weeks, offspring were collected at the egg stage to avoid the consumption of the original pollen supply by the freshly emerged larvae. In the laboratory, cell culture plates of 48 wells were filled with 400 mg of prepared pollen (mixed pollen with sugar syrup) (Figure 1C). A fine brush was used to pick the egg from its original brood cell, and a single egg was carefully placed onto each pollen provision (n = 35-40 eggs per treatment group). Plates were then placed into an incubator under controlled conditions (23°C, 60 % RH). Developmental stage of larvae was assessed every day for one month and categorised as egg, larvae, feeding larvae, feeding and defecating larvae, spinning larvae, light cocoon and cocoon. The time required to reach cocoon stage was used for the analyses. On average 90 days after cocoon development, each individual was taken out of the brood cells and weighed. Plates were then kept at 12°C for 4 days and at 4°C for ~120 days to mimic hibernation. After 140 days, all cocoons were again kept at 12°C for 4 days before moving them into an incubator (25°C, 60 % RH) to elicit emergence. After emergence, adults were weighed (fresh weight) and determined as male or female.

2.5 Statistical analyses

Statistical analyses were run using statistical software R version 3.3.3 (R Core Team, 2017). To analyse the influence of pollen diets on honey bee survival, the number of dead bees per day and cage throughout the experiments were transformed into a survival table. A Cox proportional-hazards regression model was then used to compare the different diets, with R functions (coxph) and the package survival (Cox, 1970), considering the censored data of the bees that were alive at the end of the study. Pollen consumption and fresh weight of heads were analysed using Kruskal–Wallis followed by Dunn's multiple comparison tests since data were not normally distributed.

For statistical analyses on bumble bee and *Osmia* data, two-way crossed analyses of variance (Two-Way crossed ANOVA) were conducted to evaluate the effect of diet. Since it is a parametric test, homoscedasticity (Bartlett test) and normality of the residuals (Shapiro test) were checked prior to the analyses. When violation occurred, data were log- or z-transformed to normality of residuals ("ztransform"function, R-package "GenABEL", Lenth, 2009) prior to the test. Multiple pairwise comparisons were conducted using Tukey HSD post-hoc tests when ANOVA analyses detected a significant difference between pollen diets (p-value < 0.05).

Differences in nutritional content were assessed using either Kruskal–Wallis followed by Dunn's multiple comparison tests for proteins, lipids and P:L ratios, or perMANOVA for sterols and amino acids (Euclidean distance, 999 permutations, "adonis" command) after testing for multivariate homogeneity ("betadisper" command) (R-package vegan, Oksanen et al., 2020). Given the number of replicates, it was not possible to run multiple pairwise comparisons on the amino acid and sterol data. Differences were

visually assessed on UPGMA clusters using Euclidean distance and multiscale bootstrap resampling to calculate p-values for uncertainty in hierarchical cluster (R- package pvclust, Suzuki, Terada and Shimodaira, 2019). Indicator compound analyses were performed to identify nutrients that were indicative of the groups defined based on the hierarchical cluster ("indval" command) (R-package labdsv, Roberts, 2019). All these analyses were conducted using data expressed as mg/g.

We analysed the influence of each macro-nutrient in diets (i.e. proteins and lipids) on species performance using Response Surface Models (RSM). As it is standard for geometric analyses of nutrition, the models included the linear and quadratic components for protein and lipid intake as well as the interaction term between proteins and lipids as explanatory variables. Regarding the response variable, we used the proportion of individuals that survived for each diet treatment for *Apis mellifera*, the pollen efficacy for *Bombus terrestris* and the adult mass for *Osmia bicornis* and *O. cornuta*. As our response variables were measured in different units, we standardized each response variable to a mean of zero and a standard deviation of one using a Z transformation prior to analysis (« ztransform » function from the GenABEL R-package ; Ronnegard et al., 2016). We performed these analyses using the « rsm » function from the rsm R-package (Lenth, 2009), first considering lipid and protein content (RSM 1), then sterol and amino acid content (RSM 2).

3. Results

3.1 Palynological analyses of pollen blend

The pollen mixtures were analysed to verify the dominant pollen (Table 1). Analyses confirmed the palynological origin indicated by the seller for only two out of eight samples (samples C, S). Only four out of eight samples showed the presence of a dominant pollen (samples C, MS, BQ, SP); the other samples were found to be more or less heterogeneous mixtures of three or more pollens.

3.2 Chemical analyses of pollen blend

Pollen mixes had different chemical composition (Table 2, perMANOVA, $p < 0.001$). Protein content varied from 209.61 to 397.66 mg/g of pollen ($p < 0.001$). C and S pollen blends had the significantly lowest protein content compared to other mixes (209.61 ± 9.13 and 214.86 ± 10.5 mg/g, respectively, all $p < 0.03$), whereas TSo pollen mix had the highest protein content (397.66 ± 11.5 mg/g, all $p < 0.001$,). SP pollen mix had the lowest lipid content (45.72 ± 2.42 mg/g, all $p < 0.001$), while ST and QS pollen mixes had the significantly highest lipid content (89.93 and 82.87 mg/g, respectively, all $p < 0.03$). P:L ratio ranged from 2.93-3.44 for ST, C and S pollen mixes, to 6.01-6.19 for SP and TSo diets (all $p < 0.001$).

Table 1. Frequency (%) of most represented pollen species in the 8 pollen blends

Pollen mixes	Dominant pollen species	Frequency (%)
C	Cistaceae : *Cistus ladanifer*	95
MS	Rosaceae : *Malus/Pyrus f.*	40
	Salicaceae : *Salix*	27
TSo	Asteraceae : *Taraxacum*	21
	Fabaceae : *Sophora*	17
QS	Fagaceae : *Quercus robur gr.*	51
	Salicaceae : *Salix*	29
BQ	*Brassicaceae*	36
	Fagaceae : *Quercus robur gr.*	35
SP	Salicaceae : *Salix*	43
	Rosaceae : *Prunus f*	34
S	Salicaceae : *Salix*	89
ST	Salicaceae : *Salix*	64
	Asteraceae : *Taraxacum*	21

Total and essential amino acid content ranged from 109.73 mg/g (SP diet) and 233.28 mg/g (SP diet) to 52.57 mg/g (C diet) and 125.04 mg/g (C diet), respectively. UPGMA analyses identified two clusters: one composed of the C diet only (Cluster A, Figure S1A) with a higher proline concentration ($p = 0.018$), and a second cluster composed of all other diets (Cluster B, Figure S1A) with a higher content of every amino acid except proline (all $p < 0.017$). The different diets displayed concentrations of total sterol from 4.54 mg/g (C diet) to 14.38 mg/g (ST diet). UPGMA analyses identified two clusters: one composed of the ST and SP diets (Cluster B, Figure S1B) with a higher 24-Methylenecholesterol concentration compared to the second cluster composed of all other diets (Cluster A, Figure S1B, $p = 0.021$). Other significant differences between these clusters were identified: higher concentrations of Cholesterol and δ7-Stigmasterol in the TSo diet ($p = 0.024$ and 0.01, respectively), a higher concentration of Stigmasterol in the BQ diet ($p = 0.007$) and higher concentration of δ7-Avenasterol in the ST diet ($p = 0.007$). Despite the low concentrations of 24-Methylenecholesterol and δ5-Avenasterol observed in the C diet compared to other diets (Table 2), they were not defined as significant by our analytical model, probably due to the lack of replicates. Those differences were therefore considered as tendencies.

3.3 Diet effect on bees

Honey bees (*Apis mellifera*)

Significant differences in bee survival between pollen diets were observed with the following order from the least to the most beneficial pollen: TSo < MS = BQ = C < SP < ST = QS = S (Figure 2A). Pollen diets were not consumed equally ($p < 0.01$, Figure 2B). Bees consumed significantly more of the QS and BQ than the TSo and S pollen mixes. The head weight was also affected by the type of pollen ($p < 0.001$, Figure 2C). Bees fed with the TSo pollen diet had a lighter head than bees fed with any of the S, QS, BQ or ST pollen mixes. After normalization to the amount of consumed pollen, head weights were the lowest for QS and BQ pollens and the highest for TSo and S pollen mixes ($p < 0.001$; fig. S2).

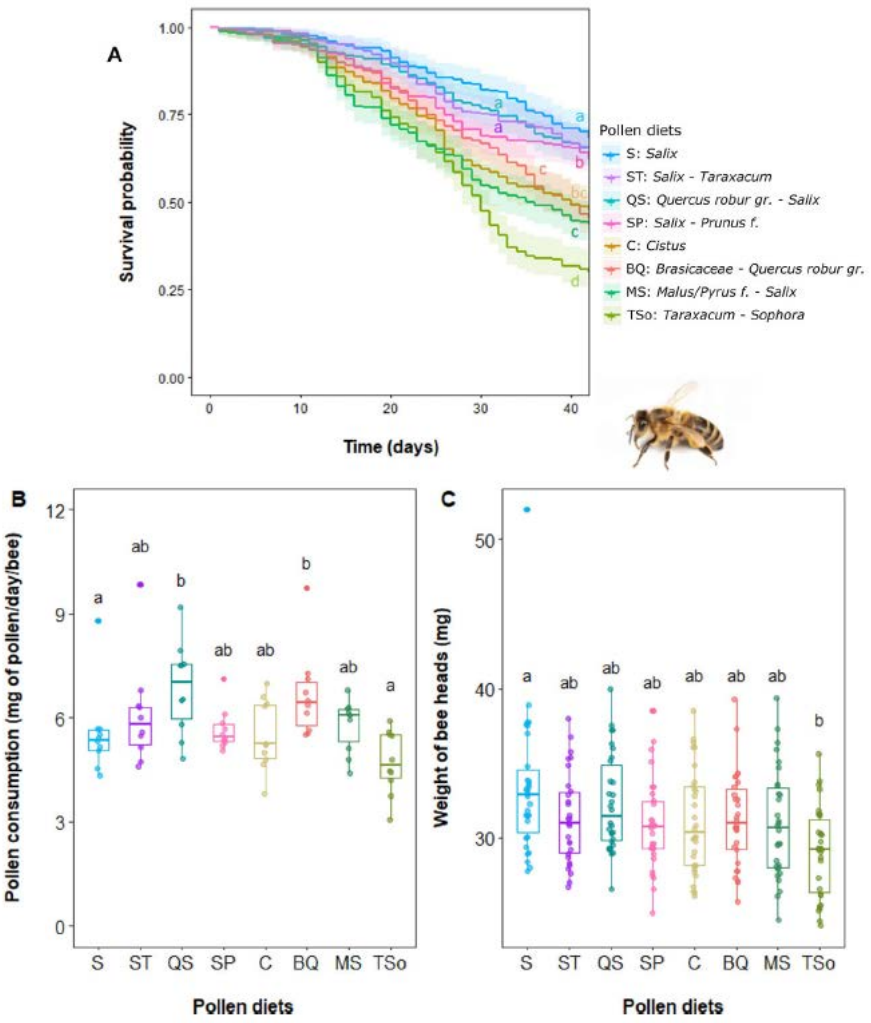

Figure 2: Influence of pollen diets on *Apis mellifera*. (A) Survival probability (n = 30 bees per cage and 10 cages per pollen regime), (B) pollen collection and (C) head weight. Boxes indicate the 1st and 3rd interquartile range with line denoting median (n = 30 pools of 3 bees per pollen). Whiskers include 90% of the individuals, beyond which each outlier are represented by circles. Different letters indicate significant differences between pollen diets (Kruskal-Wallis tests followed by Dunn's multiple comparison tests: $p < 0.01$).

Table 2. Chemical composition and P:L ratios of the 8 pollen blends. Mean values are presented with their standard deviation. Maximum and minimum values for each chemical are in bold as indicative. Different letters indicate significant differences (Kruskal-Wallis tests followed by Dunn's multiple comparison tests, $p < 0.05$).

Chemicals (mg/g)	ST	C	S	QS	BQ	MS	SP	TSo
Protein content	263.73 ± 26.3 b	**209.61 ± 9.13 a**	**214.86 ± 10.5 a**	285.10 ± 12.4 b	283.89 ± 7.23 b	287.49 ± 9.00 b	274.94 ± 18.1 b	**397.66 ± 11.5 c**
Lipid content	**89.93 ± 3.20 d**	64.33 ± 1.15 b	62.63 ± 4.3 b	82.87 ± 2.75 d	74.98 ± 2.53 c	68.55 ± 2.74 bc	**45.72 ± 2.42 a**	64.24 ± 2.4 b
Protein:Lipid ratio	2.96 a	3.26 ab	3.44 ac	3.44 ac	3.79 bc	4.19 c	6.01 d	**6.19 d**
Total amino acids	184.05 ± 7.69	**125.04 ± 13.39**	181.35 ± 25.04	194.18 ± 5.39	174.54 ± 24.48	218.37 ± 17.07	**233.28 ± 16.01**	198.57 ± 25.42
Essential amino acids	88.66 ± 1.89	**52.57 ± 5.03**	86.46 ± 10.45	90.45 ± 3.28	85.63 ± 8.05	103.54 ± 5.54	**109.73 ± 7.48**	91.54 ± 12.3
Alanine	11.14 ± 0.21	**7.87 ± 0.97**	10.21 ± 1.28	10.87 ± 0.31	10.73 ± 1.27	12.79 ± 0.62	**13.1 ± 0.87**	11.28 ± 1.46
Arginine	10.53 ± 0.18	**5.94 ± 0.27**	12.43 ± 1.28	12.39 ± 0.09	11.48 ± 0.75	12.33 ± 0.73	**14.88 ± 0.76**	10.4 ± 1.18
Asparagine	18.79 ± 1.53	**10.4 ± 2.01**	20.05 ± 2.96	20.62 ± 0.46	17.46 ± 3.51	22.52 ± 2.4	**25.51 ± 1.58**	19.68 ± 2.41
Glutamate	19.97 ± 3.76	**11.3 ± 3.87**	22.88 ± 4.55	25.32 ± 1.64	18.3 ± 5.51	24.65 ± 5.3	**30.56 ± 3.8**	24.06 ± 4.45
Glycine	8.7 ± 0.33	**4.01 ± 1.51**	8.17 ± 1.03	8.43 ± 0.26	7.39 ± 1.43	9.14 ± 0.79	**10.31 ± 0.86**	8.73 ± 1.02
Histidine	**9.83 ± 1.34**	**5.11 ± 0.79**	6.22 ± 1.15	7.17 ± 0.15	7.22 ± 0.58	6.1 ± 2.09	7.54 ± 0.68	7.95 ± 1.11
Isoleucine	7.57 ± 0.09	**4.81 ± 0.3**	7.76 ± 0.96	8.16 ± 0.36	7.7 ± 0.86	10.16 ± 1.14	**10.36 ± 0.82**	8.6 ± 1.3
Leucine	13.69 ± 0.34	**9.34 ± 0.42**	13.99 ± 1.79	14.82 ± 0.9	13.61 ± 1.48	**18.4 ± 2.46**	18.33 ± 1.84	15.6 ± 2.8
Lysine	16.68 ± 0.39	**8.25 ± 1.71**	14.65 ± 1.25	14.71 ± 0.24	14.58 ± 1.59	16.29 ± 1.41	**17.77 ± 1.06**	14.55 ± 1.13
Methionine	4.19 ± 0.15	**2.84 ± 0.25**	4.53 ± 0.37	4.25 ± 0.22	4.23 ± 0.47	5.38 ± 0.16	**5.42 ± 0.35**	4.57 ± 0.42
Phenylalanine	8.48 ± 0.39	**5.35 ± 0.62**	8.61 ± 1.14	9.7 ± 0.73	8.72 ± 1.1	**12.42 ± 2.4**	11.83 ± 1.55	10.37 ± 2.08
Proline	14.2 ± 0.59	**28.41 ± 1.42**	11.52 ± 0.63	14.55 ± 0.15	14.8 ± 1.34	**20.59 ± 2.25**	17.19 ± 0.96	18.87 ± 1.56
Serine	9.31 ± 1.52	**5.09 ± 1.54**	9.4 ± 1.76	10.14 ± 0.4	8.52 ± 2.24	11.17 ± 1.69	**11.79 ± 1.14**	10.74 ± 1.32
Threonine	7.65 ± 0.3	**4.72 ± 0.67**	8.03 ± 1.07	8.38 ± 0.34	7.93 ± 1.18	9.39 ± 0.72	**10.13 ± 0.84**	8.48 ± 0.99
Tyrosine	6.69 ± 0.26	**3.95 ± 0.39**	6.9 ± 1.23	7.59 ± 0.62	6.57 ± 1.18	9.08 ± 1.51	**9.46 ± 0.96**	8.05 ±1.55
Valine	10.04 ± 0.19	**6.2 ± 0.17**	10.23 ± 1.49	10.87 ± 0.45	10.17 ± 0.98	13.07 ± 1.12	**13.47 ± 1.02**	11 ± 1.76
Total sterols	14.38 ± 1.42	**4.54 ± 2.06**	8.22 ± 2.09	6.31 ± 1.6	6.86 ± 2.06	6.51 ± 0.16	12.68 ± 4.59	7.45 ± 0.44
Cholesterol	0.14 ± 0.02	0.08 ± 0.02	0.1 ± 0.02	0.05 ± 0.03	0.06 ± 0.08	**0.04 ± 0**	0.06 ± 0.02	**0.52 ± 0.14**
24-Methylenechol/camp	**8.37 ± 1.21**	**1.43 ± 0.56**	2.48 ± 1.53	2.5 ± 0.76	3.21 ± 3.47	4.85 ± 0.15	10.26 ± 3.64	2.61 ± 1.71
Stigmasterol	0.11 ± 0.02	0.1 ± 0.08	0.1 ± 0.02	0.08 ± 0	**0.79 ± 1.44**	0.08 ± 0.01	**0.02 ± 0.02**	0.23 ± 0.1
β-Sitosterol	1.44 ± 0.1	**0.73 ± 0.5**	2.42 ± 0.48	**2.57 ± 0.66**	1.5 ± 1.3	**0.73 ± 0.04**	1.2 ± 0.46	2.28 ± 1.16
*δ*5-Avenasterol	0.96 ± 0.07	**2.15 ± 0.87**	1.41 ± 0.36	0.96 ± 0.17	1.27 ± 2.81	**0.75 ± 0.04**	1.05 ± 0.39	0.97 ± 0.5
*δ*7-Stigmasterol	0.24 ± 0.04	**0 ± 0**	0.02 ± 0.04	0.07 ± 0.05	**0 ± 0**	0.02 ± 0.01	0.03 ± 0.05	**0.61 ± 0.15**
*δ*7-Avenasterol	**3.12 ± 0.34**	**0.04 ± 0.04**	1.69 ± 0.25	0.08 ± 0.02	**0.04 ± 0.02**	0.05 ± 0	0.06 ± 0.02	0.22 ± 0.03

Bumble bees (*Bombus terrestris*)

Brood production was impacted by the diet ($p < 0.01$). Microcolonies fed with MS and S diets were more developed, in term of total brood mass after 28 days, compared to those fed with the C diet ($p = 0.026$ and $p = 0.022$, respectively, Figure 3A). Pollen efficacy was also influenced by the different pollen diets. Microcolonies with bumble bees fed with MS, TSo, SP, S and ST pollen diets produced more brood per gram of pollen consumed in 28 days compared to those fed with the C pollen diet (all $p < 0.04$, Figure 3B). The different pollen mixes had a limited impact on resource collection. Bumble bees consumed more of the TSo diet than the SP diet ($p = 0.024$, Figure 3C). No significant differences were observed between other diets nor regarding syrup collection ($p > 0.05$, Figures 3C-D).

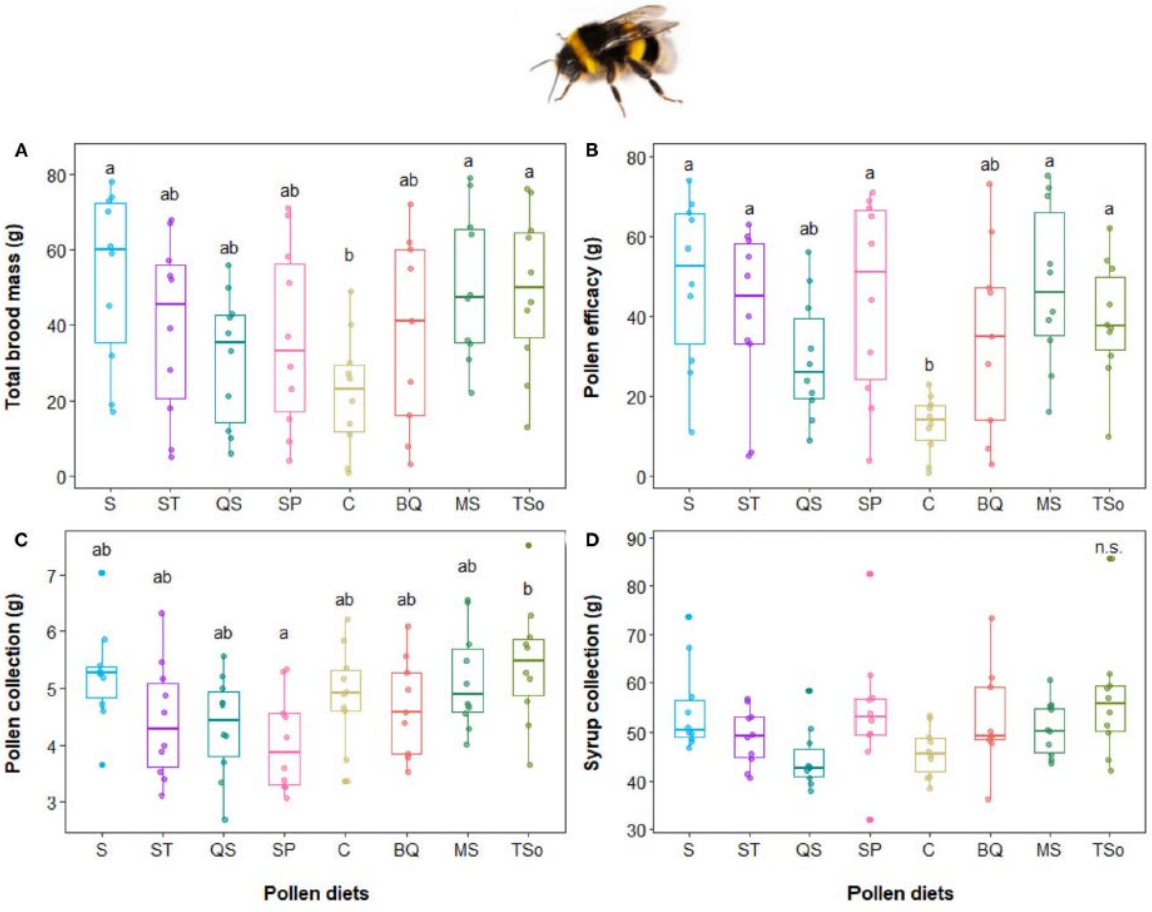

Figure 3: Influence of pollen diets on *Bombus terrestris*. (A) Brood mass production (g), (B) pollen efficacy (ratio between brood mass and pollen collection), (C) pollen collection (g) and (D) syrup collection. Boxes indicate the 1st and 3rd interquartile range with line denoting median (n = 10 micro-colonies with 5 workers per pollen diet). Different letters indicate significant differences between pollen diets (ANOVA tests followed by Tukey's multiple comparison tests: $p < 0.05$).

Mason bees (*Osmia bicornis* and *O. cornuta*)

Osmia bicornis developed differently depending on their diet ($p < 0.001$). Bees fed with the C diet had a significantly higher development time compared to any other diets (all $p < 0.027$), with approximately 25 and 31 days required to reach cocoon stage for females and males, respectively (Figure 4A). In contrast, bees fed with the BQ diet had the lowest development time (all $p < 0.004$), with 20 and 23 days required to reach cocoon stage for females and males, respectively (Figure 4A). Development time on

other diets range from 22 to 27 days. Cocoons of larvae fed with the C diet were significantly lighter compared to any other diets (all $p < 0.002$), with a mean weight of 0.118 ± 0.011 and 0.095 ± 0.012g for females and males, respectively. Cocoons of larvae fed with the MS diet were approximately 29%, 12% and 11% heavier compared to those fed with C, TSo or ST diets (all $p < 0.02$), respectively (Figure 4C). Adult weight showed similar differences (Figure 4E). The time required to reach cocoon stage for *Osmia cornuta* was significantly lower when fed with the BQ diet compared to any other diets (all $p < 0.02$, Figure 4B). No significant differences were observed among the other diets (all $p > 0.05$). Cocoons of larvae fed with the C diet were significantly lighter compared to any other diets (all $p < 0.021$), with a mean weight of 0.102 ± 0.005 and 0.094 ± 0.011g for females and males, respectively. Cocoons of larvae fed with the SP diet were approximately 27% heavier compared to those fed with the C diet, with a mean weight of 0.135 ± 0.012 and 0.115 ± 0.021g for females and males, respectively. They were also significantly heavier than cocoons of larvae fed with QS, BQ, ST or TSo diets (all $p < 0.003$, Figure 4D). Adult weight showed similar differences, but with no significant differences between the C, TSo and ST diets (all $p > 0.07$, Figure 4F).

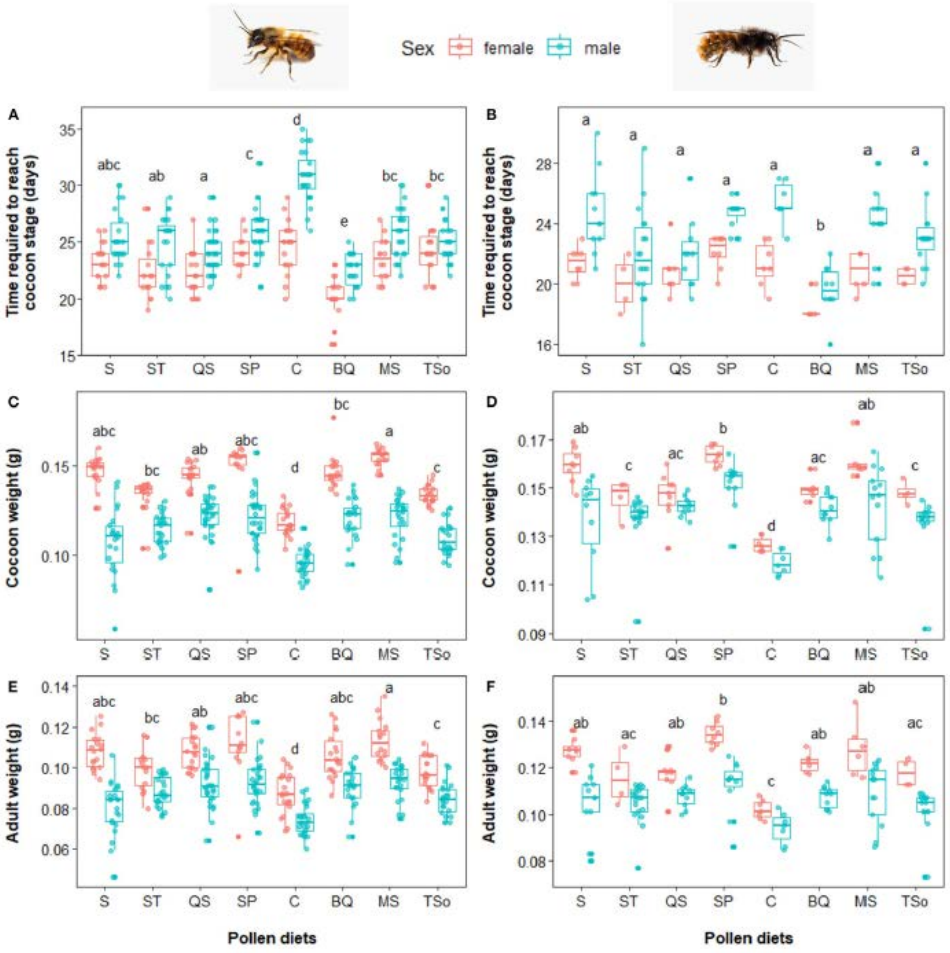

Figure 4: Influence of pollen diets on (left) *Osmia bicornis* and (right) *Osmia cornuta*. (A, B) Time required for larvae to reach the cocoon stage (days), (C, D) cocoon weight (g) and (E, F) adult weight (g). Boxes indicate the 1st and 3rd interquartile range with line denoting median (n = 40 per pollen diet). Different letters indicate significant differences between pollen diets (ANOVA tests followed by Tukey's multiple comparison tests: $p < 0.05$).

3.4 Geometric analyses

RSM analyses showed that variation in *A. mellifera* survival across diets was not explained by total amino acids (AAT) or sterols (RSM 2, Table S1), but rather by protein and lipid content (RSM 1, Table S1). On the other hand, observed differences between diet-dependent pollen efficacies in *B. terrestris* were not explained by protein or lipid content (RSM 1, Table S1), but rather by AAT and sterols (RSM 2, Table S1). *O. bicornis* female mass variation was explained by the protein content only, while male mass was explained by proteins, AAT and sterols (RSM 1, Table S1). Finally, *O. cornuta* adult mass variations between diets were explained in the same way as for *O. bicornis*: proteins for females (RSM 1, Table S1); proteins, AAT and sterols for males (RSM 2, Table S1).

4 Discussion

Variation in chemical composition of pollen diets

Chemical composition of the tested diets was different, despite some observed similarities. First, none of the pollen diets had a protein content lower than 21%, which is already an average amount: approximately 25 plant genera contain less than 15% of protein in pollen (Roulston and Cane, 2002). On the other hand, the protein-richest diet of our experiments had nearly 40% protein content (TSo diet). With more than 60 plant genera containing more than 40% of protein in pollen (Roulston and Cane, 2002), our diets are in the range of what can be found in nature, but no pollen with a particularly low or high protein content was tested here. Since we used pollen blends and not monofloral diets, it was expected that no extreme values would be observed (Roger et al. 2017). Lipid analyses also showed differences between the diets. Previous analyses showed that total lipid content in pollen can range from approximately 1% to 20% (Roulston and Cane, 2000; Roulston et al., 2000). With a range of 4.6% to 9% of total lipid content, our diets are again in range with no extreme values. Note that the lipid analysis method may not completely lyse the pollen grains. There may therefore be a slight difference between the results of the analysis and what can be assimilated by the bee.

Amino acid analyses showed that amino acids profiles were similar between pollen blends. These results are in line with previous studies, suggesting that amino acid profiles are conserved among plants (Roulston and Cane, 2002; Weiner et al., 2010; Vanderplanck et al., 2014). However, differences between our diets were observed in terms of total and essential amino acids, and the amount of specific amino acids. In this regard, we have observed that the C diet contains 54-72% less amino acids and 48-61% less total and essential amino acids, compared to other diets.

Finally, among our different diets, total sterol concentration and sterolic profiles were different, with a lower total sterol content in the C diet. These results corroborate with previous studies that showed a high variability in sterolic compounds concentrations across plant species (Vanderplanck et al., 2014, 2018).

Relationship between bee performance traits and pollen chemical composition

B. terrestris, *O. bicornis* and *O. cornuta* worst performances were observed when fed with the C diet, whereas a different result was recorded for *A. mellifera*, for which worst performance was observed when fed with the TSo diet. *A. mellifera* performance fed with C diet was average, while the TSo diet was good and average for *B. terrestris* and *Osmia* species, respectively. Diet effects were similar between *O. bicornis* and *O. cornuta*. As both species are included in the same genus, it can be expected that nutritional preferences are conserved.

Several studies pointed out protein and/or protein:lipid ratio as an indicator for pollen quality regarding developmental performances (Roulston and Cane, 2002; Smeets and Duchateau, 2003; Alaux et al., 2010; Nicolson, 2011). However, observed results and RSM analyses on *Bombus terrestris* showed that performance cannot be explained by protein, lipid or P:L ratio, while adult female mass of *Osmia* species is only slightly affected by protein content. Some good diets (see the S pollen mix) and bad diets (see

the C pollen mix) share the same protein, lipid, content and P:L ratio. These results indicate a difference in nutritional abilities compared to *A. mellifera*, for which RSM analyses highlighted an importance of protein and lipids for their survivability. Despite the common assumption that proteins are positively related to performance (Regali and Rasmont, 1995; Génissel et al., 2002; Roulston and Cane, 2002; Smeets and Duchateau, 2003; Alaux et al., 2010; Nicolson, 2011; Stabler et al., 2015), in our case, the only observed effects that can be related to protein content were a negative one on *Apis mellifera* when fed with a protein-rich diet (39.8%, TSo pollen mix) and a slight one on *Osmia* species that is difficult to identify as positive or negative, as both pollen with the highest (TSo) and lowest (C) protein content resulted in lighter adults compared to other diets. Interestingly, Roulston and Cane (2002) observed a higher mortality rate when *Lasioglossum zephyrum* individuals were fed with a diet containing more than 39% of total protein content. Moreover, it has already been shown that in laboratory conditions, mortality and ovary development in *A. mellifera* workers were negatively impacted when fed with pollen containing 32% protein compared to bees fed with 15% protein (Human et al., 2007). Other authors observed similar mortality results with protein-rich diets (Standifer, 1967; Herbert et al., 1977), suggesting that high levels of proteins can lead to deleterious effects.

Differences in amino acid content between the different diets did not impact *A. mellifera*, but seem to explain results observed with *B. terrestris* and *Osmia* species, with more than 3 times less pollen efficacy for *B. terrestris*, and up to -22% and -26% adult weight for *O. bicornis* and *O. cornuta*, respectively, when fed with the C diet, which had the lowest amount of total and essential amino acids. These results are similar to those of Archer et al. (2021), who concluded that amino acid intake was positively correlated with bumble bee body mass, and underlined that the effects of total amino acid intake may depend on the blend of individual amino acids. Interestingly, bumble bees can perceive some amino acids via chemotactile antennal stimulation and distinguish different concentrations without being able to differentiate each amino acid (Ruedenauer et al., 2019). This way, bumble bees could therefore assess the overall quantity of some amino acids and adapt their foraging decisions. However, sucha strategy could also lead to wrong decisions, i.e. a rich pollen with non-favourable amino acids proportions.

Finally, sterol analyses explained observed results regarding *B. terrestris* and *O. cornuta* performance traits. Some sterolic compounds have already been reported to be positively correlated with larval growth (Vanderplanck et al., 2014). 24-methylenecholesterol is known to influence moulting and ovary development (Svoboda et al., 1978, 1983; Human et al., 2007), while β-sitosterol and δ5-avenasterol are supposedly involved in metabolic pathways of *B. terrestris* or have a phagostimulant effect (Regali, 1996). Sterols in the C diet did not affect *A. mellifera* performances. However, these results could be explained by the performance traits tested in our experiment, which did not take into account reproductive performance. In this regard, it could be interesting to test the effect of diet on larval development and not only on adults, to better evaluate the effect of sterols, which are known to have a positive effect on the larval development on other bee species. Unfortunately, we did not have the expertise to run this kind of experiment in laboratory conditions. Nevertheless, the TSo diet, which caused the lowest survival rate in *A. mellifera*, contained relatively high concentrations of cholesterol and δ7-stigmasterol. While no negative effect has previously been reported for cholesterol in the literature, it is possible that this compound was in excess for *A. mellifera*. On the other hand, δ7-stigmasterol havsalready been reported as being not beneficial for bumble bees (Vanderplanck et al., 2018). *A. mellifera* could therefore be more sensitive to the presence of this compound compared to other species. Finally, *O. bicornis* reached the cocoon stage quicker when fed with the BQ pollen mix. This diet has a relatively high concentration of stigmasterol, suggesting a potential role in the larval development of this species. It has recently been reported by Ruedenauer et al. (2021) that bees cannot taste sterols, limiting the ability to forage on plants with beneficial sterol composition or to avoid detrimental ones. Moreover, this work and previous studies showed that pollen consumption is not adjusted to compensate low nutritional quality of a diet (see Vanderplanck et al. (2014) for *B. terrestris* and Corby-Harris et al. (2018) for *A. mellifera*), suggesting that a diet with a detrimental sterol composition could be regularly consumed by bees without them perceiving it and being able to adapt their consumption.

Good diets for everyone

Our results show that, while the 8 diet effects are different among species, similarities can be noted. Because the best performances of each species were shared across different diets, we could not define only one specific favourable diet. However, a global profile could be defined by comparing the nutritional factors of these diets. Based on this and previous studies, a generally good diet would require a high concentration of amino acids, and of 24-methylenecholesterol, β-sitosterol and δ5-avenasterol (Vanderplanck et al., 2014). P:L ratio did not impact measured parameters in this study. While Vaudo et al. (2016) have shown an impact of the P:L ratio on foraging behaviour, the effects of this ratio on colony development remain relatively unclear. More studies should be done to see if the foraging strategy in favour of the P:L ratio results in effects on colony development, by artificially modifying the protein and lipid content of the same pollen without affecting the other compounds. Low protein content did not affect bees in this study. However, as all diets contained more than 20% protein, we cannot conclude that proteins are not playing a role in diet quality. Regarding the literature, we hypothesise that protein content is important up to a certain threshold that can be close to 20% depending on the species (Regali and Rasmont, 1995; Génissel et al., 2002; Taseï and Aupinel, 2008b; Alaux et al., 2010), and can become detrimental if in excess (Standifer, 1967; Herbert et al., 1977; Roulston and Cane, 2002; Human et al., 2007). Once the minimum protein requirements are met, this work suggest that amino acid and sterol compositions are playing a key role in reproductive performance.

This study does not consider every element that can alter the quality of a pollen, such as pollenkit digestibility or secondary metabolites (alkaloids, lactones, diterpenes or cyanogenic glycosides) (Peng et al., 1985; Detzel and Wink, 1993; London-Shafir et al., 2003; Williams, 2003; Gunduz et al., 2008; Kempf et al., 2010; Sedivy et al., 2012; Gosselin et al., 2013), and did not evaluate directly the sterol effect on *A. mellifera* due to the parameters we measured (only adults were evaluated). Further research is therefore needed to fully understand the importance of each pollen-quality driver and interspecific differences. However, our study overall supports the need for conserving/introducing plant diversity into agro-ecosystems to meet the nutritional preferences of different bee species.

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6 Supplementary Material

Table S1. RSM analyses, showing how the relative dietary content of protein, lipids, and their interaction (RSM1) or total amino acids (AAT), total sterols and their interaction (RSM2) affect response variables (survival for *A. mellifera*, pollen efficacy for *B. terrestris* and adult mass for *O. bicornis* and *O. cornuta,*). Asterisks indicate statistically significant differences (*p < 0.05; **p < 0.01 and_***p < 0.001). Non-significant overall models (p > 0.05) were not interpreted.

Species	Response variable	Explanatory variable	Estimate	Std. Err.	t	*p*	R2	Lack of fit (*p*)
A. mellifera	Survival	***RSM 1***				< 0.001	0.1982	0.08078
		Proteins	1.73682	0.85650	2.028	0.04618*		
		Lipids	8.72062	3.86690	2.255	0.02708*		
		Proteins²	-0.00525	0.00374	-1.401	0.16539		
		Lipids²	-0.23017	0.08121	-2.834	0.00592**		
		Proteins:Lipids	-0.21138	0.11119	-1.901	0.06118		
		RSM 2				0.2383	0.02403	< 0.001
B. terrestris	Pollen efficacy	***RSM 1***				0.3076	0.01402	< 0.001
		RSM 2				< 0.001	0.238	0.81716
		AAT	-2.18255	1.23905	-1.761	0.0823		
		Sterols	24.56773	10.36600	2.370	0.0204*		
		AAT²	0.07074	0.03789	1.867	0.0659		
		Sterols²	-5.85449	2.52980	-2.314	0.0235*		
		AAT:Sterols	-0.64601	0.32321	-1.999	0.0494*		
O. bicornis	Female mass	***RSM 1***				< 0.001	0.2254	< 0.001
		Proteins	-0.09003	0.58418	-0.154	0.878		
		Lipids	-3.50154	2.64091	-1.326	0.187		
		Proteins²	-0.01098	0.00255	-4.306	< 0.001***		
		Lipids²	0.00621	0.05706	0.109	0.913		
		Proteins:Lipids	0.11931	0.07575	1.575	0.118		
		RSM 2				< 0.001	0.275	< 0.001
		AAT	-0.51061	0.82594	-0.618	0.538		
		Sterols	8.66404	6.93936	1.249	0.214		
		AAT2	0.02056	0.02536	0.811	0.419		
		Sterols²	-2.34178	1.67529	-1.398	0.165		
		AAT:Sterols	-0.21728	0.22256	-0.976	0.331		
	Male mass	***RSM 1***				< 0.001	0.2788	0.34067
		Proteins	0.50724	0.39692	1.278	0.203		
		Lipids	-0.83979	1.77209	-0.474	0.639		
		Proteins²	-0.01040	0.00183	-5.693	< 0.001 ***		
		Lipids²	0.01028	0.03797	0.271	0.787		
		Proteins:Lipids	0.02404	0.05093	0.472	0.637		

		RSM 2				< 0.001	0.2243	< 0.001
		AAT	1.62015	0.60480	2.679	0.00803**		
		Sterols	-11.74348	5.10167	-2.302	0.02241*		
		AAT²	-0.04234	0.01854	-2.284	0.02347*		
		Sterols²	3.65963	1.26602	2.891	0.00429**		
		AAT:Sterols	0.21793	0.15956	1.366	0.17359		
O. cornuta	Female mass	***RSM 1***				0.001135	0.1041	0.17674
		Proteins	0.07095	0.66160	0.107	0.9148		
		Lipids	-2.28756	3.02614	-0.755	0.4522		
		Proteins²	-0.00710	0.00286	-2.483	0.0149*		
		Lipids²	0.04486	0.06290	0.713	0.4776		
		Proteins:Lipids	0.05782	0.08639	0.669	0.5041		
		RSM 2				0.02429	0.08512	0.07179
		AAT	0.50706	1.02562	0.494	0.622		
		Sterols	-2.50243	8.56049	-0.292	0.771		
		AAT²	-0.01293	0.03113	-0.415	0.679		
		Sterols²	0.22061	2.09538	0.105	0.916		
		AAT:Sterols	0.10975	0.25891	0.424	0.673		
	Male mass	***RSM 1***				< 0.001	0.3332	< 0.001
		Proteins	0.31470	0.84398	0.373	0.71095		
		Lipids	-2.02060	3.74212	-0.540	0.59183		
		Proteins²	-0.01097	0.00397	-2.765	0.00816**		
		Lipids²	0.00690	0.08052	0.086	0.93210		
		Proteins:Lipids	0.05723	0.10953	0.522	0.60385		
		RSM 2				< 0.001	0.6109	0.13866
		AAT	-1.85262	0.89624	-2.067	0.04438*		
		Sterols	19.69040	7.45737	2.640	0.01127*		
		AAT²	0.05616	0.02772	2.026	0.04857*		
		Sterols²	-6.98024	1.82124	-3.833	< 0.001***		
		AAT:Sterols	-0.28526	0.24593	-1.160	0.25206		

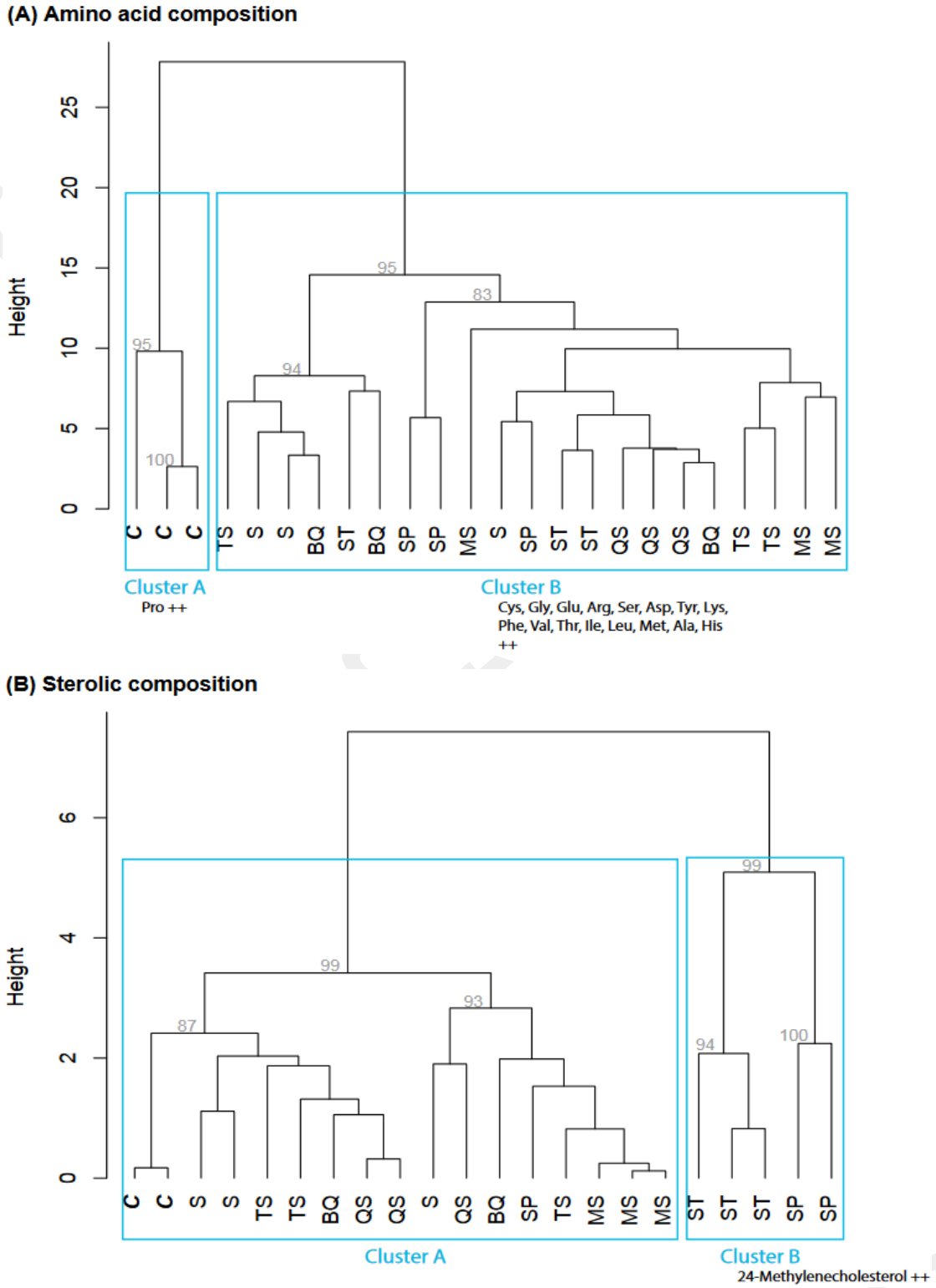

Figure S1. UPGMA models with identified clusters and their indicative nutrients in relation to (A) amino acid composition or (B) sterolic composition.

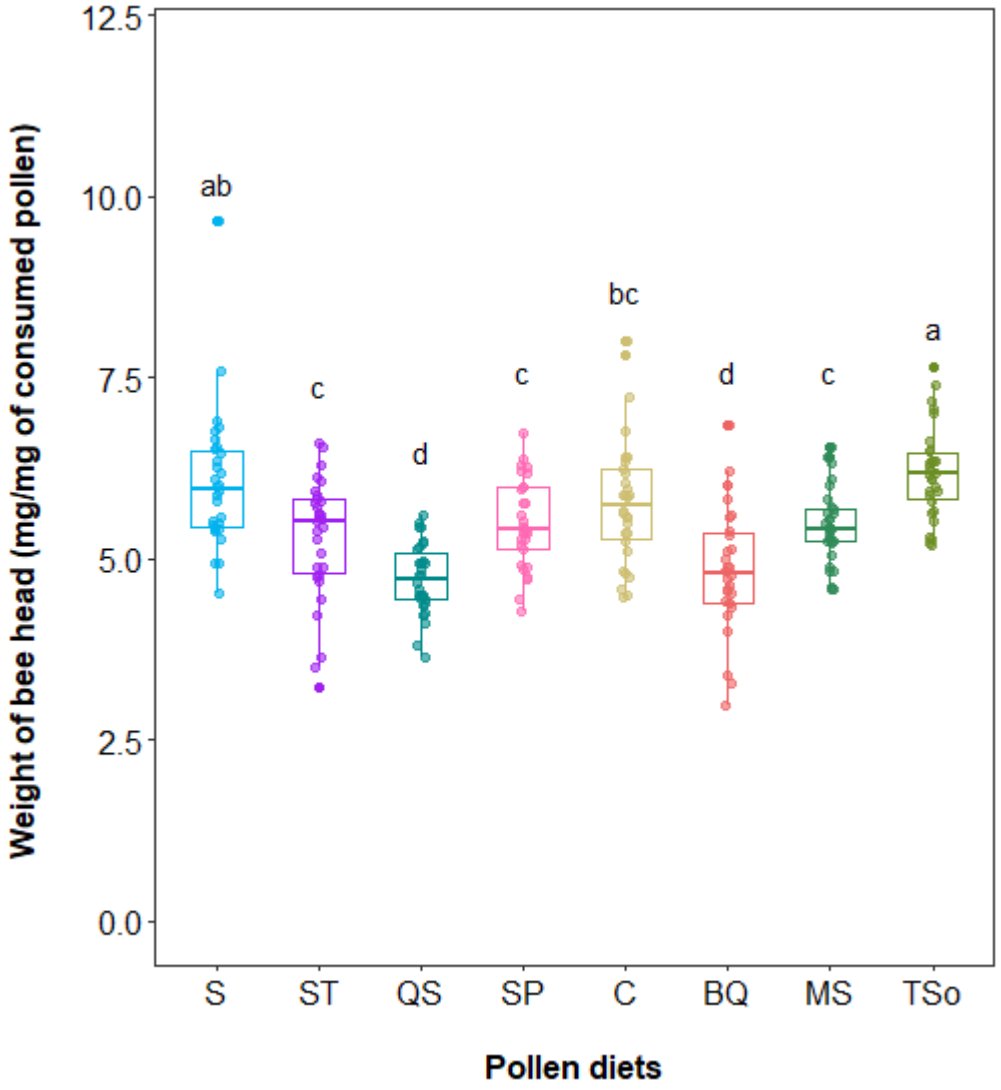

Figure S2. Weight of bee heads normalized to the amount of pollen consumed. Boxes indicate the 1st and 3rd interquartile range with line denoting median (n = 30 pools of 3 bees per pollen). Whiskers include 90% of the individuals, beyond which each outlier are represented by circles. Different letters indicate significant differences between pollen diets (Kruskal- Wallis tests followed by Dunn's multiple comparison tests: $p < 0.001$).