



Variations in Nutritional Requirements Across Bee Species

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With 2,000 species currently recorded in Europe, bees are a highly diversified and efficient group of pollinating insects. They obtain their nutrients from 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 the interspecific variations in bee nutritional requirements. We analyzed the chemical content of eight pollen blends, different in terms of protein, lipid, amino acids, and sterols total concentration and profiles. Each pollen blend was provided to four different bee model species: honey bees (*Apis mellifera*), bumblebees (*Bombus terrestris*), 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 those species are different, and that a low-quality diet for one species is not necessarily low-quality for another one. While honey bees are negatively impacted by diets with a high protein content (~40%), bumblebees 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 of conserving and/or introducing plant diversity into managed ecosystems to meet the natural nutritional preferences of bees at species and community level.

Keywords: bees, pollen, nutrition, bumble bee, honey bee, *Osmia*, nutrients

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 a wide variability in various traits such as body size (i.e., from 0.3 mm to 4.5 cm in Europe), social behavior (e.g., cleptoparasitic, solitary, eusocial), nesting behavior (e.g., cavity- or soil-nesting), foraging strategies (e.g., pollen generalist or specialist), or 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 crop 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 like the western honey bee (*Apis mellifera*), the buff-tailed bumblebee (*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, the quantity and the diversity of floral resources and thus the food sources of bees (e.g., Roger et al., 2017b). This makes the abundance, distribution/availability, quality and diversity of these resources potentially a main proximal pressure 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). Bumblebees look 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 bumblebees, *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 seem more also important in bee nutritional requirement. For example, sterols (e.g., β -sitosterol) are essential to synthesize ecdysteroid, involved in the molting of the larvae and the maturation of the ovaries of female imago. In case of sterol deficiency, a delay in molting can be observed (Regali, 1996). Additionally, a good amino acid balance is also crucial for the bee development (Moerman et al., 2016). They 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-methylenecholesterol and β -sitosterol) cannot be synthesized by the bee and are therefore considered as essential, meaning that it is necessary to obtain them through pollen consumption (De Groot, 1953; Svoboda et al., 1978; Behmer and Nes, 2003).

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 bumblebees (e.g., Tasei and Aupinel, 2008a; Vanderplanck et al., 2014; Moerman et al., 2016, 2017; Barraud et al., 2020; Carnell et al., 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 bumblebees 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 specific physiological requirement (e.g., Fabaceae), is more likely to affect bumblebees and solitary bees than honey bees (Leonhardt and Blüthgen, 2012). The more generalized the foraging behavior 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 multiple 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 characterize 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 bumblebees, 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.

MATERIALS AND METHODS

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 bumblebee *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. Bumblebee colonies were provided by Biobest NV (Westerlo, Belgium); honey bees were obtained from local apiaries at the “Institut National de la Recherche pour l’Agriculture, l’Alimentation et l’Environnement” (INRAE) in Avignon (France) and the mason bees were provided by Wildbiene + Partner (Switzerland).

Characterization of Pollen Diets

Eight organic blends of honeybee-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 dominant pollen species (see Table 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 honeybee parasite *Varroa destructor* are consistently found in 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 honeybees with an oral LD50 of 75 $\mu\text{g}/\text{bee}$ for amitraz (contact exposure)

TABLE 1 | Frequency (%) of most represented pollen species in the 8 pollen blend.

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

and 45 $\mu\text{g}/\text{bee}$ for tau-fluvalinate (oral exposure) (US EPA, 2021).

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 18×18 mm. After drying, the sediment was included in one drop of glycerine jelly and covered with the cover slip. Examination under the microscope were 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.

Protein Analyses

Pollen protein concentration was measured using the Bradford assay according to Vaudo et al. (2020). We added 1.5 mL of 0.1 M NaOH to ~ 1 mg 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 595 nm using a SpectraMax 190 spectrophotometer (Molecular Devices, LLC, Sunnyvale, CA). Protein concentrations were calculated using polynomial 2nd analysis from the protein standards.

Lipid Analyses

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

Amino Acids Analyses

For the analysis of total amino acids, 1 mL of hydrolysis solution (6N HCl, 0.1% phenol and 500 μ M norleucine) was added to 3–5 mg (dry weight) of pollen (Vanderplanck et al., 2014) and then incubated for 24 h 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 in an HPLC vial after filtration (0.2 μ m filter), and each amino acid was measured separately with an ion-exchange chromatograph. A post-column ninhydrin reaction produced colored derivatives, which was monitored via a UV detector. For amino acid quantification, norleucine was used as internal standard. This analysis includes essential amino acids that bee 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.

Sterols 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 μ l 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).

Bee Nutrition Studies

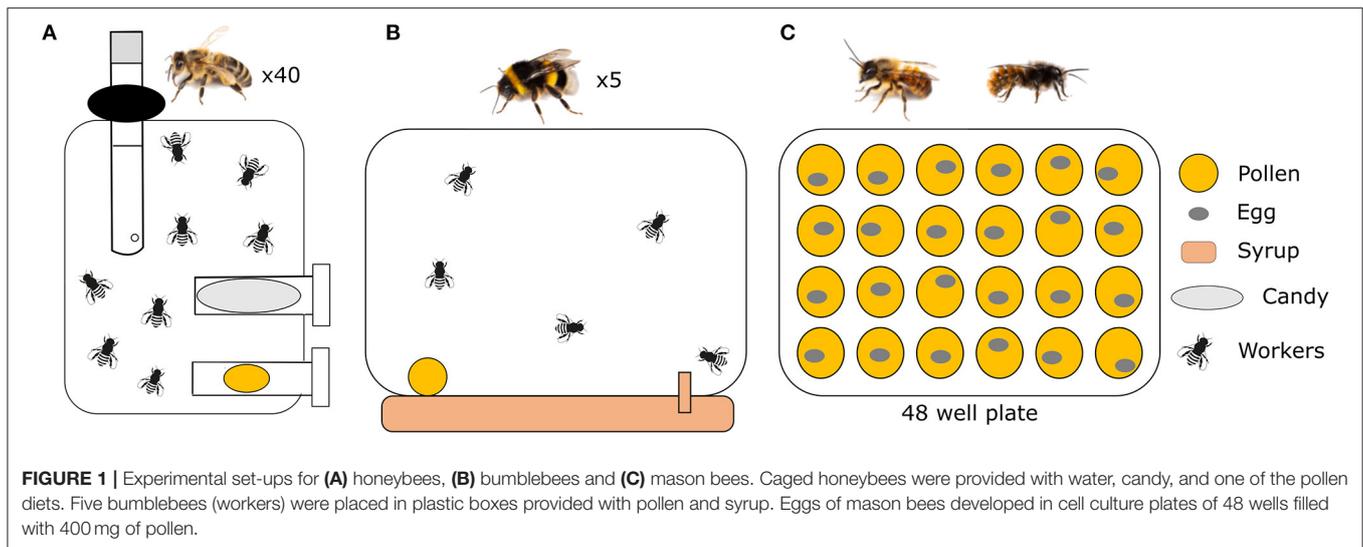
As the three genera (i.e., *Apis*, *Bombus*, and *Osmia*) show very different life cycles and behavior, they could not be tested following the same protocol in laboratory conditions. Thus, we developed different experimental setup for each of the three bee genera.

Honeybees (*Apis mellifera*)

In honeybees, pollen is mostly consumed by young adult bees. Its consumption enables the development of mandibular glands (Camilli et al., 2020) and especially hypopharyngeal glands (Crailsheim et al., 1992), where jelly is produced to feed larvae, the queen and drones (Crailsheim, 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. Experiments were performed in the spring. To obtain 1-day-old bees, brood frames containing late-stage pupae were taken from eight healthy colonies normally treated against the parasitic mite *Varroa destructor* (*Apis mellifera ligustica* \times *Apis mellifera mellifera*), and were placed overnight into an incubator under controlled conditions [34°C, 50–70% of relative humidity (RH)]. The next day, newly-emerged bees (<1 day old) were collected, mixed and groups of 40 bees were placed in cages (10.5 \times 7.5 \times 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 h. 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 on individual bees ($n = 9$ bees per cage giving a total of 90 bees per experimental group).

Bumblebees (*Bombus terrestris*)

We tested the impact of pollen on bumblebees at 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* (Tasei and Aupinel, 2008b). A total of five queen-right colonies of 100 *Bombus terrestris* workers were used to build up 80 queen-less micro-colonies of five workers, placed in plastic



boxes ($8 \times 16 \times 16$ cm) (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 $26 \pm 2^\circ\text{C}$ 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 2 days, while the previous ones were weighed to assess the pollen collection. Pollen collection was corrected for evaporation by monitoring the weight of two samples of each diet placed in the rearing room for 48 h. To estimate the performance and development of bumblebee 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); (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 effect 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.

Mason Bees (*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 3 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 placed cautiously onto each pollen provision ($n = 35\text{--}40$ 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 1 month and categorized into 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 of them 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.

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 analyzed using Kruskal–Wallis followed by Dunn’s multiple comparison tests since data were not normally distributed.

For statistical analyses on bumblebees 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 detected significant difference between pollen diets ($p < 0.05$).

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 a multiple pairwise comparisons on amino acids and sterol data. Differences were then 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 et al., 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 analyzed 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 ($\ll ztransform \gg$ function from the GenABEL R-package; Ronnegard et al., 2016). We performed these analyses using the $\ll rsm \gg$ function from the *rsm* R-package (Lenth, 2009), first considering lipid and protein content (RSM 1), then sterol and amino acid content (RSM 2).

RESULTS

Palynological Analyses of Pollen Blends

The pollen mixtures were analyzed to verify the indication of the dominant pollen (Table 1). Analyses confirmed the palynological origin indicated by the seller only for 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 pollen (see Supplementary Figure S1).

Chemical Analyses of Pollen Blends

Pollen mixes had different chemical composition (Table 2, perMANOVA, $p < 0.001$). Protein content varies 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 to 3.44 for ST, C and S pollen mixes, to 6.01–6.19 for SP and TSo diets (all $p < 0.001$).

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 C diet only (Cluster A, Supplementary Figure S1A) with a higher proline concentration ($p = 0.018$), and a second cluster composed of all other diets (Cluster B, Supplementary 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 ST and SP diets (Cluster B, Supplementary Figure S1B) with a higher 24-Methylenecholesterol concentration compared to the second cluster composed of all other diets (Cluster A, Supplementary Figure S1B, $p = 0.021$). Other significant differences out of these clusters have been identified: higher concentrations of Cholesterol and $\delta 7$ -Stigmasterol in TSo diet ($p = 0.024$ and 0.01 , respectively), a higher concentration of Stigmasterol in BQ diet ($p = 0.007$) and higher concentration of $\delta 7$ -Avenasterol in ST diet ($p = 0.007$). Despite the low concentrations of 24-Methylenecholesterol and $\delta 5$ -Avenasterol observed in 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.

Diet Effects on Bees

Honeybees (*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 TSo and S pollen mixes. The head weight was also affected by the type of pollen ($p < 0.001$, Figure 2C). Bees fed with TSo pollen diet had a lighter head than bees fed with 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 ($p < 0.001$; Supplementary Figure S2).

Bumblebees (*Bombus terrestris*)

The 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 C diet ($p = 0.026$ and $p = 0.022$, respectively, Figure 3A). Pollen efficacy was also influenced by the different pollen diets. Microcolonies with bumblebees 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 C pollen diet (all $p < 0.04$,

TABLE 2 | Chemical composition and P:L ratios of the 8 pollen blends.

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
δ5-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
δ7-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
δ7-Avenasterol	3.12 ± 0.34	0.04 ± 0.04	1.69 ± 0.25	0.08 ± 0.02	0.04 ± 0.02	0.05 ± 0	± 0.02	0.22 ± 0.03

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, $p < 0.05$).

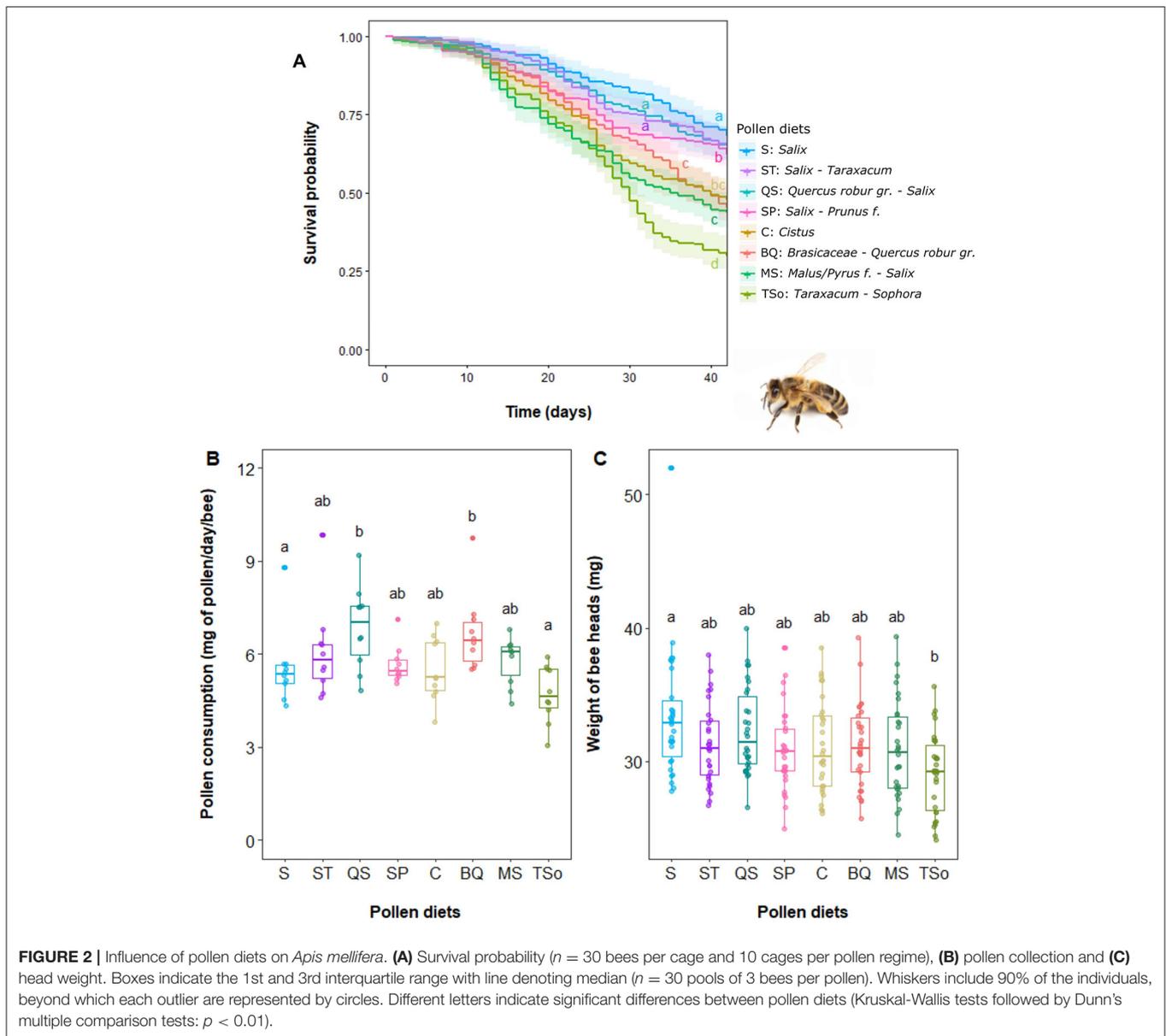
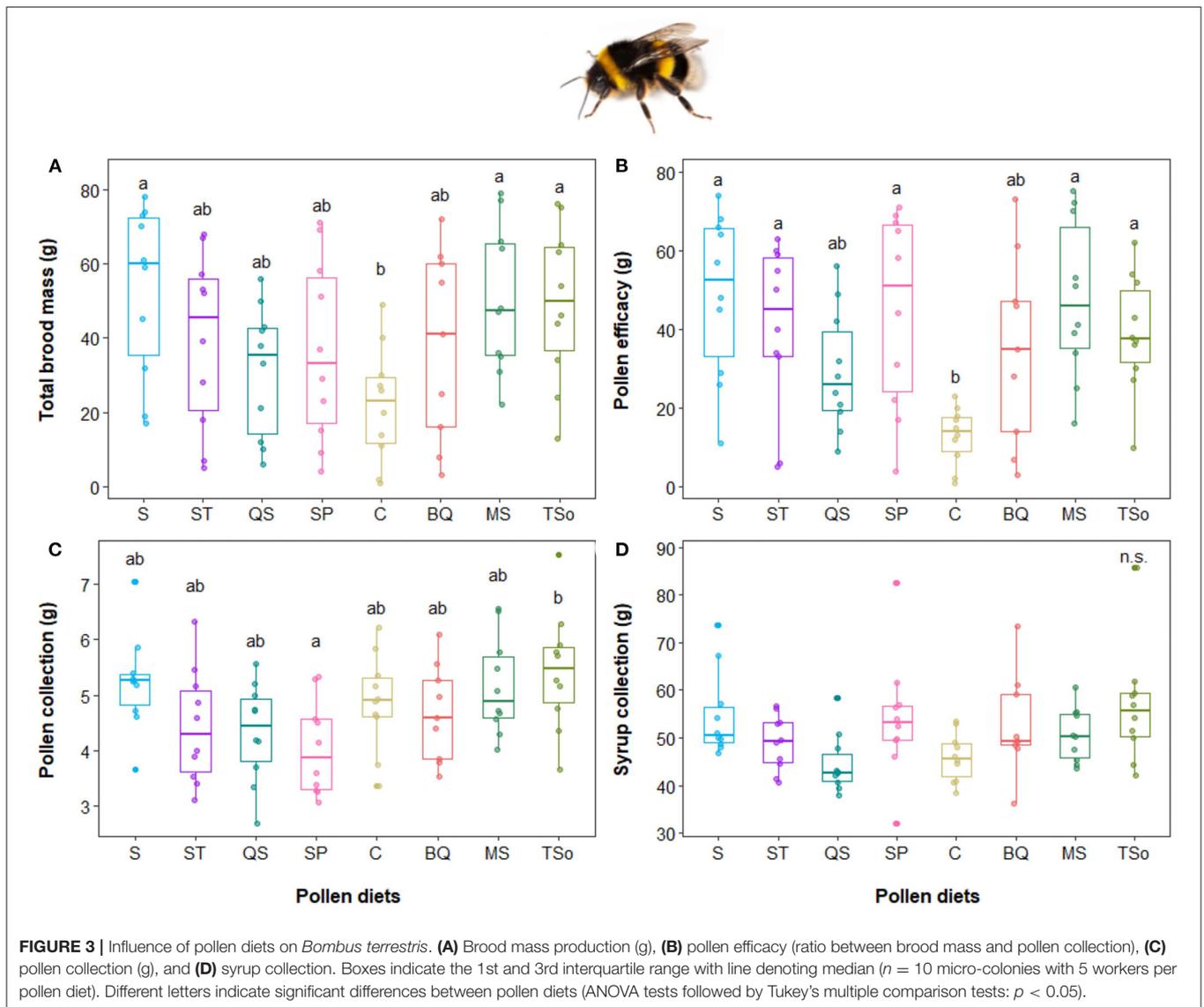


Figure 3B). The different pollen mixes had a limited impact on resources collection. Bumblebees consume more of the TSo diet than the SP diet ($p = 0.024$, **Figure 3C**). No significant differences were observed between other diets nor regarding syrup collection ($p > 0.05$, **Figures 3C,D**).

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

Osmia bicornis developed differently depending of their diet ($p < 0.001$). Bees fed with C diet had a significantly higher development time compared to any other diets (all $p < 0.027$), with ~ 25 and 31 days required to reach cocoon stage for females and males, respectively (**Figure 4A**). In contrast, bees fed with 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 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 ± 0.012 g for females and males, respectively. Cocoons of larvae fed with MS diet were ~ 29 , 12, and 11% heavier compared to those fed with C, TSo or ST diet (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 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 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 ± 0.011 g for females and males, respectively. Cocoons of larvae fed with SP diet were $\sim 27\%$ heavier compared to those fed with C diet, with



a mean weight of 0.135 ± 0.012 and 0.115 ± 0.021 g for females and males, respectively. They were also significantly heavier than cocoons of larvae fed with QS, BQ, ST, and TSo diet (all $p < 0.003$, **Figure 4D**). Adult weight showed similar differences, but with no significant differences between C, Tso, and ST diets (all $p > 0.07$, **Figure 4F**).

Geometric Analyses

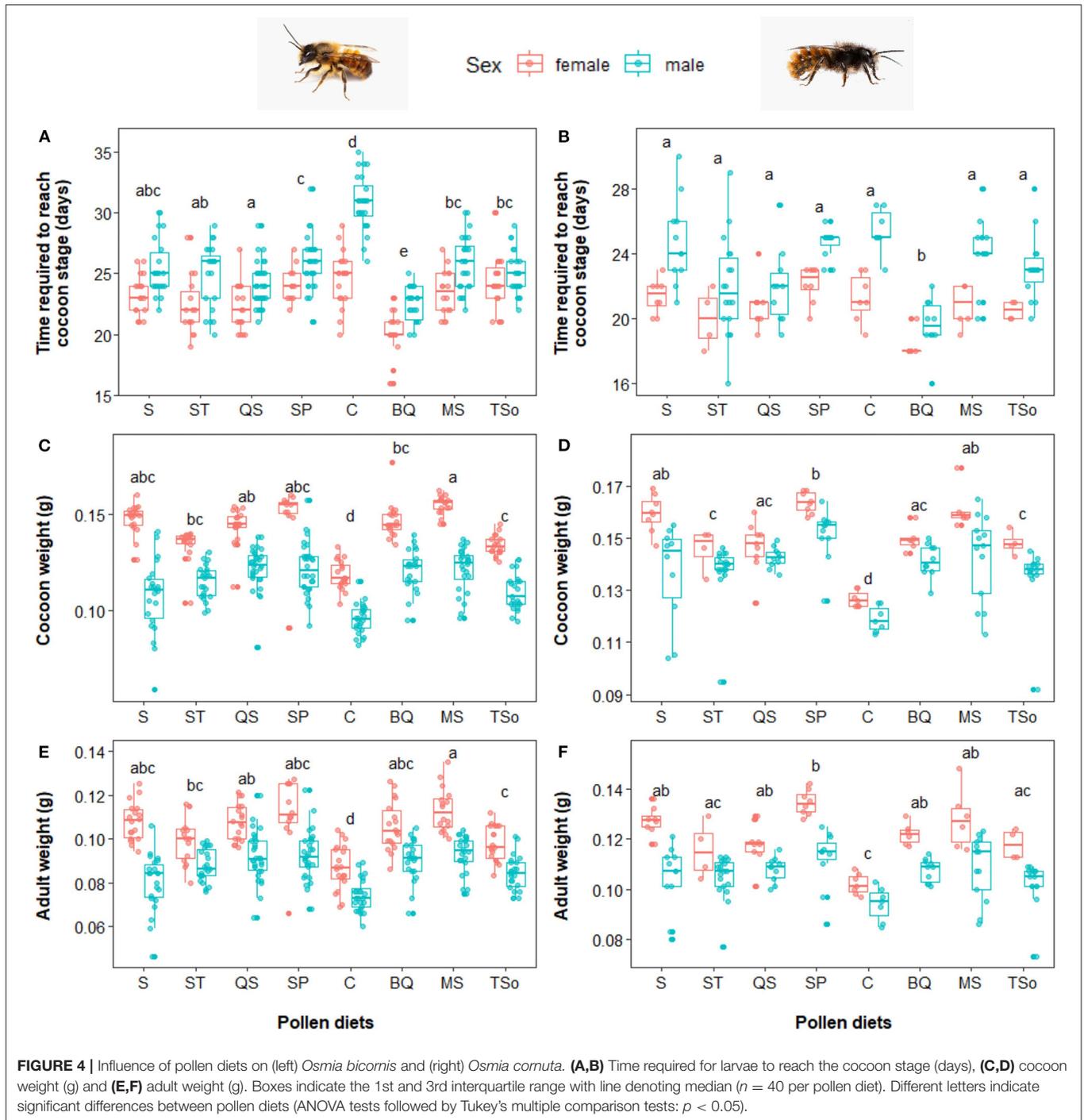
RSM analyses showed that *A. mellifera* survival variations between diets were not explained by total amino acids (AAT) or sterols (RSM 2, **Supplementary Table S2**), but rather by protein and lipid content (RSM 1, **Supplementary Table S2**). On the other hand, observed differences between diet-dependant pollen efficacies in *B. terrestris* were not explained by protein or lipid content (RSM 1, **Supplementary Table S2**), but rather by AAT and sterols (RSM 2, **Supplementary Table S2**). *O. bicornis* female mass variations were explained by the protein content

only, while male mass was explained by proteins, AAT and sterols (RSM 1, **Supplementary Table S2**). Finally, *O. cornuta* adult mass variations between diets were explained in the same way as for *O. bicornis*: proteins for females (RSM 1, **Supplementary Table S2**); proteins, AAT and sterols for males (RSM 2, **Supplementary Table S2**).

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: ~ 25 plant genera contain $< 15\%$ of protein in pollen (Roulston and Cane, 2002). On the other hand, the protein-rich diet of our experiments contained nearly 40% of proteins



(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 natura*, but no pollen with a particularly low or high protein content was tested here. As we used pollen blends and not monofloral diets, it was expected that no extreme values would be observed (Roger et al., 2017b). Lipids analyses also showed differences between the diets. Previous analyses showed that lipids can range from ~ 1 to 20%

in total content (Roulston and Cane, 2000; Roulston et al., 2000). With a variation from 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 acids analyses showed similar 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 have been observed in term of total and essential amino acids, and the amount of specific amino acids. In this regard, we have observed that 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. Those 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 C diet, whereas a different result was recorded for *A. mellifera*, for which worst performance was observed when fed with TSo diet. *A. mellifera* performances fed with C diet were average, while 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 slightly affected by protein content. Some good diets (see S pollen mix) and bad diets (see C pollen mix) share the same protein, lipid, content and P:L ratio. Those 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 performances (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% of proteins compared to bees fed with 15% of proteins (Human et al., 2007). Other authors observed similar mortality results with protein-rich diet

(Standifer, 1967; Herbert et al., 1977), suggesting that high levels of proteins can lead to deleterious effect.

Differences in amino acids content between the different diets did not impact *A. mellifera*, but seems 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 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 bumblebee body mass, and underlined that the effects of total amino acids intake may depend on the blend of individual amino acids. Interestingly, bumblebees 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, bumblebees could therefore assess the overall quantity of some amino acids and adapt their foraging decisions. However, such strategy could also lead wrong decisions, i.e., a rich pollen in non-favorable amino acids proportions.

Finally, sterols analyses could explain observed results regarding *B. terrestris* and *O. cornuta* measured 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 molting and ovary development (Svoboda et al., 1978, 1983; Human et al., 2007), while β -sitosterol and δ 5-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 performances. 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 sterols effect, known to have a positive effect on the larval development on other bee species. Nevertheless, TSo diet, which caused the lowest survival rate in *A. mellifera*, contained relatively high concentrations of cholesterol and δ 7-stigmasterol. If no negative effect has been reported for cholesterol in the literature, maybe this compound was in excess for *A. mellifera*. On the other hand, δ 7-stigmasterol have already been reported as not beneficial for bumblebees (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 cocoon stage quicker when fed with 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 can't taste sterols, limiting the bee 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 the bees without them perceiving it and being able to adapt their consumption.

Good Diets for Everyone

Our results show that, if 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 favorable 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 δ 5-avenasterol (Vanderplanck et al., 2014). P:L ratio did not impact measured parameters of this study. While Vaudo et al. (2016) have shown an impact of the P:L ratio on foraging behavior, the effects of this ratio on colony development remain relatively unclear. More studies should be done to see if the foraging strategy in favor 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% of protein, we cannot conclude that proteins are not playing a role in diet quality. Regarding the literature, we hypothesize 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; Tasei 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 performances.

This study does did 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 sterols effect on *A. mellifera* due to measured parameters (only adults evaluated). Further research is therefore needed to fully comprehend the importance of each pollen-quality driver and interspecific differences. However, our

study overall supports the need of conserving/introducing plant diversity into agro-ecosystems to meet the nutritional preferences of different bee species.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

AB and LB wrote the manuscript and conducted experiments on the bees. VL did all nutrient analyses. DS was involved in *Apis* experiments. YL and CA supervised *Apis* experiments and redaction. F-VG, FC, GS, and CC performed the palynological/pesticide residues analyses and wrote results about it. MV and DM supervised the whole work. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fsufs.2022.824750/full#supplementary-material>

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