Original Article

COMPARISON OF DIFFERENT POLLEN SUBSTITUTES FOR THE FEEDING OF LABORATORY REARED BUMBLE BEE (*BOMBUS TERRESTRIS*) COLONIES

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Abstract

In bumble bee colonies, pollen is the only protein source for larval feeding and its shortage causes a distress in larval development. Adult bumble bees need pollen for the development of glands and the reproductive system. In bumble bee rearing, honey bee collected pollen is used as the main protein source, either as fresh-frozen or dried pellets, and pollen provisioning is the most problematic and expensive aspect of mass rearing. In honey bee breeding, pollen substitutes are used during the period of food shortage or to stimulate colony strength. We tested different protein diets (five commercial pollen substitutes and two natural protein sources) for the maintenance of bumble bee colonies in captivity. We further mixed Feedbee®, one of the substitutes that gave the best results, with different amounts of pollen to evaluate the optimal amount needed for the whole colony development. Although none of the pure protein diets alone were adequate, diets with a 1 to 1 and 1 to 3 ratio of Feedbee to pollen were both suitable for colony development and queen production. The colony consumed between 2 and 4 g per day of the Feedbee mixed diets, corresponding to a protein consumption of 0.75-0.85 g day^{-1}. Nevertheless, the consumption rate of the pure pollen showed that a mean amount of protein between 0.4 and 0.5 g day^{-1} was enough to allow colony development indicating the suitability of Feedbee mixed diets.

Keywords: artificial diet, *Bombus terrestris*, bumble bee, pollen substitute, protein source

INTRODUCTION

In bumble bee colonies, pollen is the only protein source for larval feeding. Bumble bee workers feed their larvae a mixture of pollen and nectar for the duration of their development and produce no other protein secretions to give them, as with honey bees (Pereboom, 2000). There is a strong correlation between the weight of bumble bee larvae and the amount of pollen they ingest (Ribeiro, Duchateau, & Velthuis, 1996). Pollen shortage in bumble bee colonies causes a delay in larval development (Sutcliffe & Plowright, 1990), the production of fewer and/or smaller workers (Sutcliffe & Plowright, 1988; Schmid-Hempel & Schmid-Hempel, 1998) and sexuals. Under extreme conditions of food shortage, larvae are ejected from the nest. Pollen is important also for adult bumble bees. Although their diet mainly consists of nectar, they consume also pollen throughout the adult life for the development of glands and reproductive system (Duchateau & Velthuis, 1989; Pereboom, 2000; Stabler et al., 2015). Pollen deprivation leads to a reduction in adult longevity and reproduction (Smeets & Duchateau, 2003).

The buff-tailed bumble bee *Bombus terrestris*

is a generalist pollinator that lives in colonies of a few hundred individuals. It is a "pollen storer" species and in nature the workers deposit pollen in pollen storage pots, from which they either consume it for themselves or use it to feed their larvae (Smeets & Duchateau, 2003). *B. terrestris* is an important pollinator of some greenhouse crops, especially tomatoes and is extensively used in commercial pollination systems. Since the 1980s its colonies have been mass reared and sold for pollination purposes in all the countries where it is native (Velthuis & Van Doorn, 2006).

In bumble bee laboratory rearing, honey bee collected pollen is used as the main protein source, either as fresh-frozen or dried pellets (Ptacek, 2000). The two kinds of pollen give similar results in terms of larval and adult development, but colonies fed on fresh-frozen pollen produce gynes with greater size and longevity, which result in larger-size colonies, than those fed on dried pollen (Ribeiro, Duchateau, & Velthuis, 1996). Other studies have demonstrated that different kinds of commercially available pollen perform better in different phases of the colony cycle (Yoon et al., 2005). The characteristics of pollen varies widely according to the plant of origin, and the pollen nutritive value, estimated by the crude protein (CP) concentration, ranges between 25 mg g^{-1} and 610 mg g^{-1} dry mass (Roulston, Cane, & Buchmann, 2000). Bumble bees are able to discriminate between high- and low-quality pollen and to regulate their foraging activity according to this factor (Kitaoka & Nieh, 2009).

When comparing the efficacy of pollens from different botanical origin on laboratory reared bumble bee colonies, those containing higher protein percentage give the best rearing success (Aupinel et al., 2000; Genissel et al., 2002; Tasei & Aupinel, 2008a). Protein concentration is not the only relevant feature for pollen nutritive value, but also the kind of amino acids and such chemicals as sterolic compounds contained in the pollen play an important role in bumble bee larval development (Vanderplank et al., 2014; Moerman et al., 2015, 2017).

If pollen nutritive value is one of the key factors in bumble bee mass rearing, pollen provisioning is the most problematic aspect of this commercial activity. Pollen collected by honey bees is very expensive, its production can be very variable in terms of quantity and quality, and it can contain pollutants, such as insecticides and herbicides, (Chauzat et al., 2006; Bernal et al., 2010; Kasiotis et al., 2014; Tosi et al., 2018) and pathogens from honey bees (Singh et al., 2010; Graystock et al., 2013). For all these reasons, the development of an adequate pollen substitutes for bumble bee mass rearing would be of great economic relevance.

In honey bee breeding, pollen substitutes are used during the period of food shortage or to stimulate colony strength. A number of investigations have been performed on the effects of different protein sources, and other additives on bee productivity have been tested (reviewed in: Herbert, 1997; Black, 2006). In addition, beekeeping practices often include the use of different natural protein sources to be administered to bees, according to local or individual customs. However, despite many decades of beekeeping research and practice, pollen substitutes remain less effective than most sources of fresh pollen, particularly for brood rearing. Several commercial products are sold as pollen substitutes for honey bee colonies, but most still contain variable percentages of pollen, added to alternative protein sources. Contrarily, no researches are known on the development of an alternative protein diet for bumble bees and no commercial products for bumble bee nutrition are currently on the market (Graystock et al., 2016).

The aim of the study is to test different protein diets for the maintenance of bumble bee colonies in captivity. While all previous studies on the nutritive value of protein source for bumble bee colony development have been performed in micro-colonies (Genissel et al., 2002; Tasei & Aupinel, 2008a, 2008b), we decided to assess the development of whole queenright colonies in order to evaluate the effect of different diets on different timing and features of colony development.

MATERIAL AND METHODS

This study was carried out over two consecutive assays on a total of 153 second-generation laboratory colonies of *Bombus terrestris* (125 colonies in the first assay, twenty-eight in the second one).

Colony rearing and management

Colonies were reared from commercial colonies (Bioplanet S.c.a., Cesena, Italy) following the procedure described by Bogo et al. (2017). Obtained colonies were reared in a climate room (28 ± 1 °C and 60 ± 10% RH, continuous darkness) and fed on fresh frozen pollen and sucrose solution (1:1 w/w). When the first brood emerged, the colonies were moved to bigger plastic boxes (25 × 15 × 14 cm) and the protein source was shifted to the test diets. Before diet shifting, we estimated the colony size by counting the number of egg cells, larvae, pupae and adults. We assigned the colonies to the different test diet groups making sure that all the groups were composed by colonies similar in size. Three times a week the colony boxes were cleaned, and food was refreshed (both protein source and sucrose solution).

Registered parameters

Three times a week we recorded test diet consumption (in weight), number of dead adults, number of dead larvae, date of colony ending (i.e. no workers left and/or complete absence of brood), date of gyne and male emergence and number of emerged gynes. After colony ending, we calculated the number of emerged workers in the period of food substitution as the number of alive adults at the end of the trial plus the number of dead adults during the trial, minus the number of pupae and adults at the beginning of food substitution. Finally, we calculated the colony lifespan, from the beginning of the test to colony ending.

In order to compare colony output in all the groups, we considered the above parameters only during the first twenty-four days of the assay trial and during the first twenty-five days of the second assay (see below), since these were the lifespans reached by the shortest-lasting colonies, respectively in the two assays.

Test diets

We performed the first assay with eight commercial pollen substitutes sold for honey bees colonies during periods of pollen shortages (Karya Ari Keki®, Calcalar Ltd, Muğla, Turkey; Bee Food®, Melissokomiki Athinon S.A., Schimatari, Greece; Candipolline®, Enolapi Srl, Verona, Italy; Feedbee® (two types: powder and paste) and Nutri-Bombus® (three types: A, B and C; Nutrifeed Canada Inc., Ajax, Canada) and two natural protein sources (brewer's yeast and chestnut flour) (Tab. 1). The powdery-formulated diets were mixed with sugar syrup, obtained by dissolving the sugar in water in a proportion of 1: 2 (1 kg sugar in 2 litres water). Fresh frozen pollen was used as a positive control, administered in either honey bee pollen pellets alone or mixed with sugar syrup (pollen paste).

In the second assay, we tested again the pollen substitute which had given in the first assay the best result and took into consideration also costs and market availability, namely Feedbee powder, mixed with 50% and 75% of pollen paste. The characteristics of the different diets are described in Tab. 1.

Analyses of test diets for crude protein concentration and sugars

In order to determine the nitrogen concentration (N), the Kjeldahl method (Bradstreet, 1954) was applied, following Conti et al. (2016). A minimum quantity of 300 mg per sample (one for each diet) was used. Crude protein (CP) was estimated by multiplying N concentration by 6.25 (Jones, 1931). For the determination of sugar (summation of sucrose, glucose and fructose) concentrations, one sample (15 g) for each diet was processed according to Conti et al. (2016).

Statistical analysis

Statistical analyses were performed using multivariate analysis of variance (MANOVA), one-way analysis of variance (ANOVA) and discriminant analysis (DA) (Podani, 2000;

Table 1.
Characteristics of the diets used for the two assays and the corresponding number of treated colonies

Diet	Raw material consistency	Final consistency	Sugar syrup (%)	N° of colonies
First assay				
Pollen paste	Pellets	Paste	23	16
Pollen pellets	Pellets	Pellets	0	8
Feedbee® powder	Powder	Paste	33	17
Feedbee® paste	Paste	Paste	0	10
Candipolline®	Paste	Paste	0	9
Bee Food®	Paste	Paste	0	9
Karya Ari Keki®	Paste	Paste	0	9
Chestnut flour	Powder	Paste	45	7
Brewer's yeast	Powder	Paste	43	8
Nutri-Bombus® A	Paste	Paste	0	11
Nutri-Bombus® B	Paste	Paste	0	11
Nutri-Bombus® C	Paste	Paste	0	10
Total				125
Second assay				
Pollen paste	Pellets	Paste	18.5	10
25% Feedbee® + 75% Pollen paste	Paste	Paste	13.9	9
50% Feedbee® + 50% Pollen paste	Paste	Paste	9.3	9
Total				28

Sokal & Rohlf, 2010). In order to control type I error for the subsequent separate univariate ANOVAs following MANOVA, we corrected the experiment-wise error rate by the sequential Dunn-Šidak method (Sokal & Rohlf, 2010). The DA was used to investigate the nature of multivariate effects of different diets and how the dependent variables together discriminate the treatments (Podani, 2000). The DA was carried out using Canoco for Windows 4.5 (Lepš & Šmilauer, 2003; Ter Braak & Šmilauer, 2002) and a manual forward selection of variables, corrected by the sequential Dunn-Šidak method, was used to choose the best explanatory ones after 9999 permutations under a reduced model (Anderson & Legendre, 1999). Furthermore, the selected variables were evaluated for the performance in the correct classification of different bumble bees into their respective diets by estimating the probabilities of misclassification with cross validation.

Effect sizes within MANOVA, ANOVAs and DA were estimated, respectively, with adjusted ξ^2, Cohen's ω^2 and R^2. Given an evidently different number of replicates per treatment, a posteriori comparison of individual means was based on the minimum significant difference (MSD) method obtained from the Hochberg's GT2 statistic (Sokal & Rohlf, 2010).

Data were subjected to a logarithmic transformation (Sokal & Rohlf, 2010) before analysis, which effectively homogenized variances (Levene's test) and produced normal distributions (Shapiro-Wilk test) (Sokal & Rohlf, 2010). Consequently, we chose to present the median values instead of the arithmetic means.

Covariance was homogenous as well (Box's M test) (Podani, 2000). In order to compare the different diets for their similarity in sugar and CP concentration, a principal coordinate analysis (PCoA) was performed on a matrix obtained by Goodall probabilistic similarity indices (Goodall, 1966; Podani, 2000).

RESULTS

First assay: effects of test diets on colony development

Only diets consisting of pollen (either pellets alone or pellets with syrup) allowed the normal development of bumble bee colonies and their subsequent production of gynes and males. The numerical parameters for these diets were not included in the analysis because they would have been out of scale compared to those of the other diets. With all the other test diets, the colonies ceased their growth at an early stage of development and did not produce males or queens.

Using Pillai's trace, food type significantly affected the colony duration, number of emerged workers and number of dead larvae and adults after twenty-four days ($V = 1.49$, $F_{36, 364} = 5.99$, $p \leq 0.001$). The dependent variables had a large effect size being the adjusted $\xi^2 = 0.31$. The separate univariate ANOVAs on the outcome variables revealed that food type significantly affected the colony duration ($F_{9, 91} = 5.59$, $p \leq 0.001$), dead adults ($F_{9, 91} = 16.0$, $p \leq 0.001$), dead larvae ($F_{9, 91} = 4.88$, $p \leq 0.001$) and emerged workers ($F_{9, 91} = 2.18$, $p \leq 0.05$). Colonies fed with Feedbee had a significantly longer duration than those fed with Bee Food, Karya Ari Keki, Feedbee paste and chestnut flour but not from the others. Colonies fed with chestnut flour had a significantly shorter duration than all the others except for those fed with Bee Food, Karya Ari Keki, Feedbee paste and Nutri-Bombus A. The effect size was moderately large ($\omega^2 = 0.29$) as colonies fed with Feedbee whose longest duration lasted 1.5 times longer than those fedwith y chestnut flour which lasted the least (Tab. 2).

Given high variation in the number of dead adults, the effect size was remarkably high for this variable ($\omega^2 = 0.57$). The number of dead adults within twenty-four days under Karya Ari Keki, Bee Food and Candipolline feeding was the highest and significantly different from all other treatments (Tab. 2). Candipolline treatment was, however, not significantly different from Fedbee powder, Chestnut flour and Feedbee paste. All three Nutri-Bombus treatments had the least negative effects on adults survival and were significantly different from all the other treatments except for Feedbee powder, Brewers' yeast and Chestnut flour.

The number of dead larvae within twenty-four days in colonies fed by Feedbee paste was the highest and significantly different from all the treatments except from Nutri-Bombus A and C. All the other treatments were not significantly different between them. Nevertheless, the effect size was moderately large ($\omega^2 = 0.26$) mainly because the number of dead larvae in colonies fed by Feedbee paste was from 1.5 to 3 folds higher than in other colonies. Due to considerably high variation in number of emerged workers within each treatment, only Candipolline and Nutri-Bombus B treatments were significantly different (Tab. 2). Consequently, also the effect size was much smaller ($\omega^2 = 0.10$).

Although MANOVA was highly significant and with large effect size, the single ANOVAs, separately for each variable, were generally unable to discriminate well among the different pollen substitutes. Therefore, as a follow up MANOVA we applied discriminant analysis (DA), a multivariate constrained ordination technique, that takes account of the correlation between the dependent variables. Discriminant analysis revealed four discriminant functions of which the first two explained 84% of the variance (Fig. 1A). The canonical R^2 of the first and second discriminant functions were, respectively, 0.68 and 0.39 in accordance with the high value of the adjusted ξ^2 from MANOVA. The correlations between outcomes and the discriminant functions revealed that the number of dead adults loaded significantly ($p \leq 0.001$) highly in the first function ($R = 0.84$) (Fig. 1A). The number of dead larvae and colony duration loaded sig-

Table 2.
Effects of different pollen substitutes on number of dead larvae within 24 days, number of dead adults within 24 days, colony duration, and number of emerged workers of artificially reared bumble bees

Pollen substitute	Colony duration (days)	Dead adults (n)	Dead larvae (n)	Emerged workers (n)
Candipolline®	80 ± 3 ab	12 ± 2 ab	18 ± 3 b	2 ± 1 b
Bee Food®	70 ± 1 bc	23 ± 4 a	32 ± 5 b	13 ± 4 ab
Feedbee® powder	89 ± 3 a	8 ± 2 bcd	30 ± 4 b	11 ± 3 ab
Karya Ari Keky®	70 ± 1 bc	25 ± 4 a	25 ± 4 b	8 ± 3 ab
Brewers' yeast	81 ± 6 ab	4 ± 1 cd	23 ± 4 b	7 ± 2 ab
Chestnut flour	59 ± 2 c	8 ± 2 bcd	19 ± 4 b	4 ± 1 ab
Feedbee® paste	70 ± 2 bc	10 ± 2 bc	60 ± 4 a	8 ± 3 ab
Nutri-Bombus® A	75 ± 5 abc	3 ± 1 d	35 ± 7 ab	12 ± 4 ab
Nutri-Bombus® B	86 ± 4 ab	3 ± 1 d	34 ± 5 b	17 ± 6 a
Nutri-Bombus® C	79 ± 6 ab	3 ± 1 d	40 ± 8 ab	7 ± 3 ab

Different letters indicate significant differences among treatments ($p \leq 0.05$, GT2-method). Values are median ± SE.

nificantly ($p \leq 0.001$) and moderately evenly in the the second function with opposite effect, with canonical variate correlation coefficients of -0.61 and 0.69, respectively (Fig. 1A). On the contrary, the number of emerged workers loaded negligibly but significantly ($p \leq 0.05$) on both functions, with canonical variate correlation coefficients of -0.12 and -0.06, respectively (Fig. 1A).

Relatively to the centroids of the different

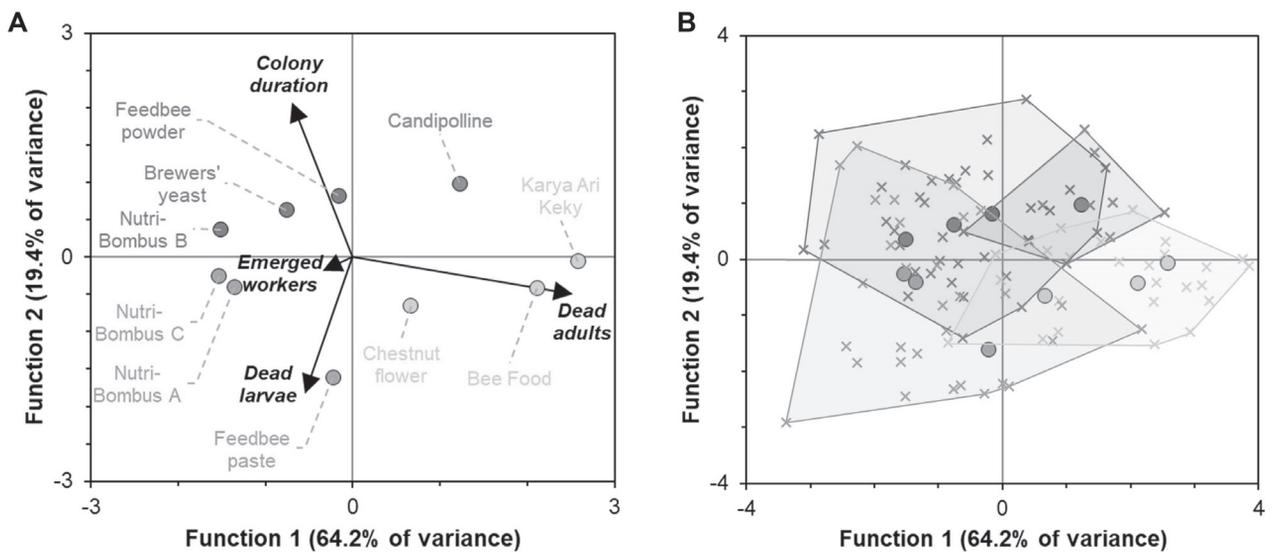

Fig. 1. Projection on the first two discriminant functions of different pollen substitutes for the feeding of artificially reared bumble bees. Circles represent treatment centroids of each pollen substitute. (A) Correlation biplot based on discriminant analysis (DA). Differently discriminated centroids of pollen substitutes are in different DA quadrants. Explanatory variables are shown as arrows magnified three times for graphical purposes. (B) Canonical scores of individual colonies (crosses) on first two discriminant functions. Different groups are shown with different grayscale crosses and enclosed by an envelope.

diets, the discriminant combined-treatment plot showed that the first function discriminated Feedbee powder, Feedbee paste, brewer's yeast, and all three Nutri-Bombus substitutes from all the other treatments (Fig. 1B). The second function differentiated Feedbee powder, brewer's yeast and Nutri-Bombus B from Nutri-Bombus A and C, and Feedbee paste. Finally, the second function differentiated also Candipolline from Karya Ari Keky, Bee Food and Chestnut flour (Fig. 1B). The discriminant function based on significant variables on the four group of diets, defined as above, displayed overall 67% classification success after cross-validation. More precisely, 84%, 67%, 61% and 44% classification success were displayed, respectively, for single groups comprising (i) Karya Ari Keky, Bee Food and Chestnut flour, (ii) Feedbee powder, brewer's yeast and Nutri-Bombus B, (iii) Nutri-Bombus A and C, and Feedbee paste, and (iv) Candipolline alone (Fig. 1B). Thus a partial overlap of some diets was present (Fig. 1).

Second assay: effects of test diets on colony development and male and gyne production

From the results of the first assay, we selected Feedbee as the pollen substitute which gave the best results, taking into account all the observed parameters. The only other substitutes which gave a similar effect were brewers' yeast and Nutri-Bombus B, but the former is more expensive while the latter at the moment was not yet available on the market. Therefore, in the second assay we mixed Feedbee with different amounts of pollen paste (50 and 75%), to evaluate the optimal amount needed to achieve whole colony development, in comparison to pollen alone.

The difference in larvae mortality was not significant among treatments ($F_{2, 25}$ = 0.08) (Tab. 3). On the contrary, adult mortality was significantly affected by different supplements ($F_{2, 25}$ = 4.28, $p \leq 0.05$) and the effect size was moderately large (ω^2 = 0.19). Indeed, colonies fed by pollen paste had significantly ($p \leq 0.05$) fewer dead adults (on average three folds) than those fed by Feedbee mixed with only 50% pollen paste (Tab. 3). However, there was no significant difference in dead adults between colonies fed by pollen paste and Feedbee mixed with 75% pollen paste, and between the two Feedbee treatments.

All three supplements allowed the whole development of bumble bee colonies and their subsequent production of sexuals. We then further compared the number of produced gynes, the pre-switch point period and the pre-gyne point period (i.e. the days elapsed between the deposition of the first egg cell and the switch point and gyne point respectively; Bogo et al., 2018) among these three test diets (Tab. 3) and found no significant differences after ANOVA tests ($F_{2, 25}$ = 1.30, 1.99 and 0.01 for, respectively, produced gynes, the pre-switch point period and the pre-gyne point period).

Table 3.

Number of dead larvae, dead adults and produced gynes, and pre-switch and pre-gyne point period in colonies fed with pure pollen paste and pollen paste mixed with 25% and 50% Feedbee®

Diet	Dead larvae	Dead adults	Number of produced gynes	Pre-gyne point period	Pre-switch point period
Pollen paste	4 ± 1	1 ± 0.2 b	134 ± 23	51 ± 3	56 ± 3
25% Feedbee® + 75% Pollen paste	4 ± 2	2 ± 0.4 ab	157 ± 17	52 ± 3	47 ± 4
50% Feedbee® + 50% Pollen paste	5 ± 2	3 ± 1 a	109 ± 18	51 ± 1	56 ± 3

Different letters indicate significant differences among treatments ($p \leq 0.05$, GT2-method). Values are median ± SE.

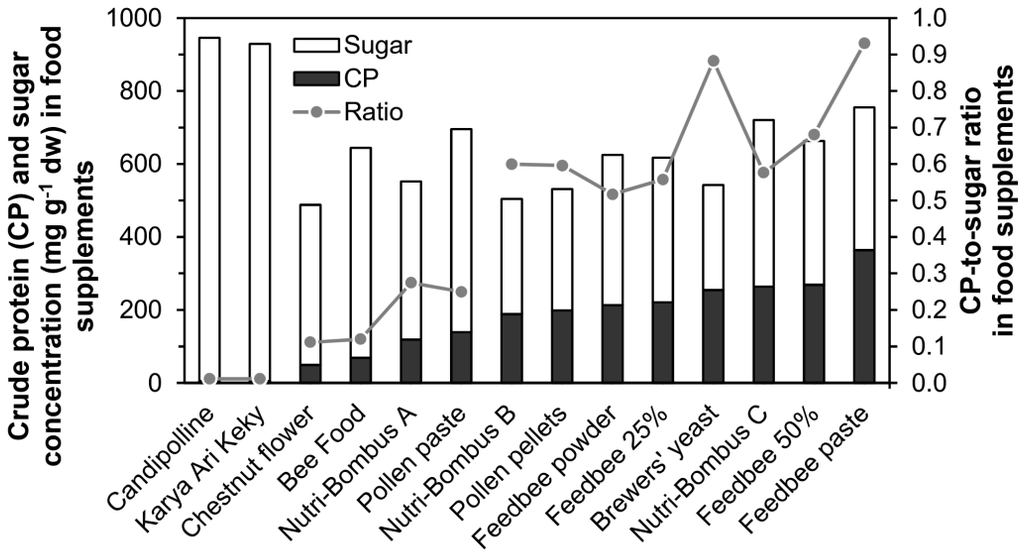

Fig. 2. Crude protein (CP) and sugar (summation of sucrose, glucose and fructose) concentration, and CP-to-sugar ratio of the tested diets. Diets were shown in ascending order of CP concentration. CP-to-sugar ratios connected by a line identified diets belonging to the same cluster after principal coordinates analysis (PCoA) on Goodall probabilistic similarity index.

Crude protein and sugar concentrations of test diets

The fourteen different diets were clearly distinguished into three clusters by their mean CP-to-sugar ratios (Fig. 2). Candipolline and Karya Ari Keky belonged to the cluster with the smallest mean CP-to-sugar ratio (0.005) while Brewer's yeast, Feedbee 25% and 50%, Feedbee paste and powder, Nutri-Bombus B and C, and Pollen pellets to the cluster with the highest mean CP-to-sugar ratio (0.7). Finally, Bee Food, Chestnut flour, Nutri-Bombus A and Pollen paste belonged to the cluster with an intermediate mean CP-to-sugar ratio (0.2) (Fig. 2).

Diet consumption

The mean total daily consumption significantly ($p \leq 0.001$) differed between the test diets (Fig. 3) and with a very large effect size ($\omega^2 = 0.71$). The most consumed diet was Feedbee 25% although not significantly more than Feedbee 50%, the two diets containing only pollen and Candipolline. The other test diets were significantly lower from Feedbee 25% and Pollen

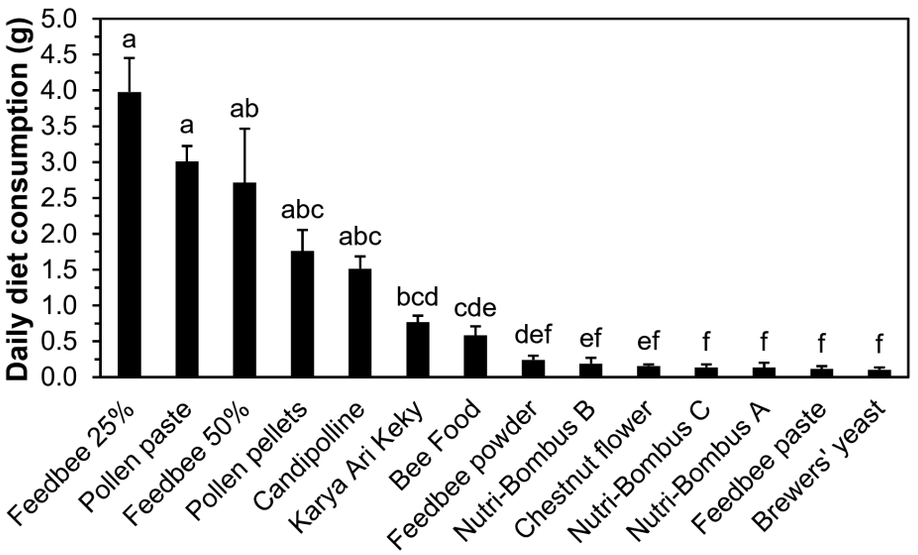

Fig. 3. Daily diet consumption of different test diets in descending order of consumption. Different letters indicate significant differences in concentration among test diets ($p \leq 0.05$, GT2-method). Values are median + SE, n comprised between 7 and 26.

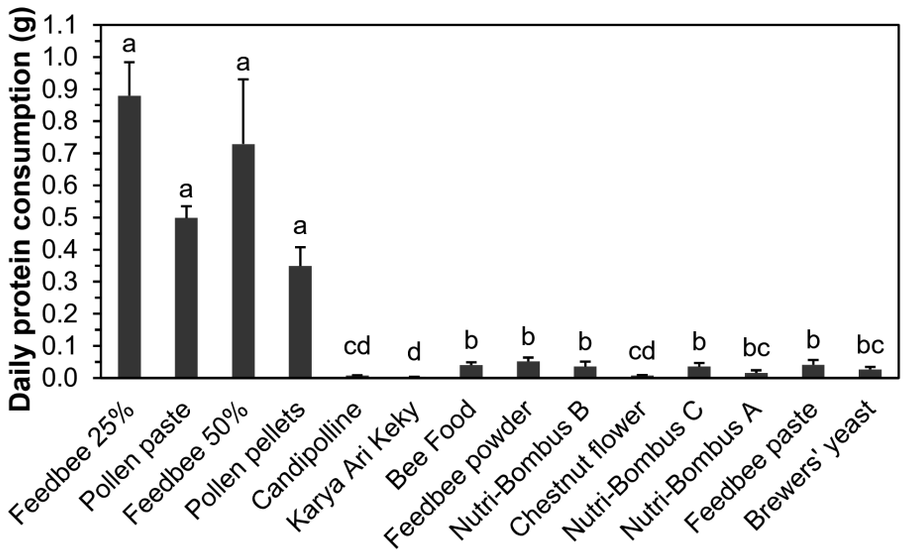

Fig. 4. Daily protein consumption of the different test diets. Different letters indicate significant differences in concentration among test diets ($p \leq 0.05$, GT2-method). Values are median + SE, n comprised between 7 and 26. For comparisons purposes, diets were ordered according to descending order of daily total consumption as in Fig. 3.

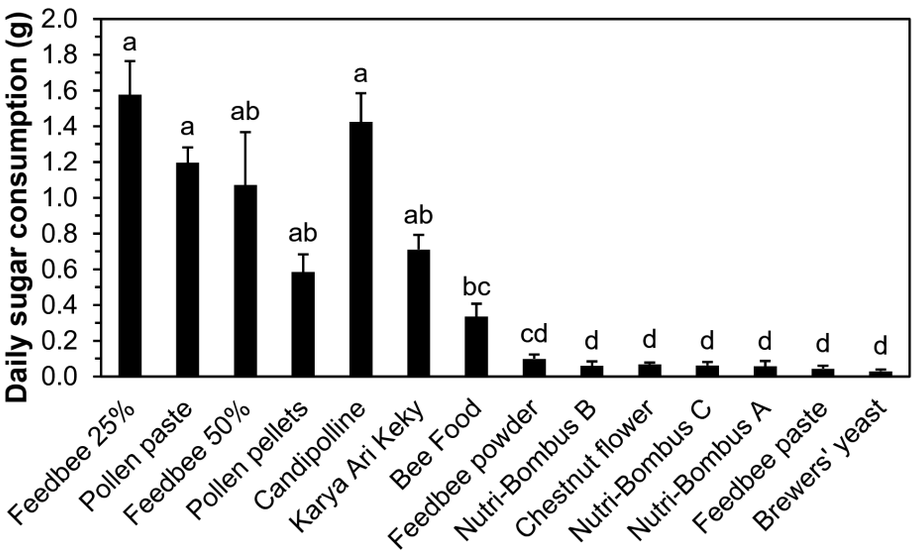

Fig. 5. Daily sugar consumption of the different test diets. Different letters indicate significant differences in concentration among test diets ($p \leq 0.05$, GT2-method). Values are median + SE, n comprised between 7 and 26. For comparisons purposes, diets were ordered according to descending order of daily total consumption as in Fig. 3.

paste and had a daily consumption lower than one gram and from 2 to 38 folds lower than the Feedbee 25 and 50%, Pollen and Candipolline diets (Fig. 3). However, Karya Ari Keky was not significantly different from Feedbee 50%, Pollen Pellets and Candipolline, and Bee Food was not significantly different from Pollen Pellets and Candipolline (Fig. 3).

When we combined total mean daily consumption with the percent of protein and sugar in each diet, calculating the amount of CP and sugar respectively achieved with each diet, we also obtained significant ($p \leq 0.001$) differences between test diets and with a very large effect size ($\omega^2 = 0.80$ and 0.74 for CP and sugar, respectively) (Figs 4 and 5). The significantly ($p \leq 0.05$) highest amount of protein was achieved by diets containing Feedbee 25% and 50% and the two diets containing only pollen which had from 7 to 229 folds higher amount of protein than other diets (Fig. 4). Among the remaining diets, Candipolline, Karya Ari Keky and Chestnut flour

had the lowest amount of protein, although not always to a significant extent (Fig. 4).
Sugars obtained by Feedbee 25%, Pollen paste and Candipolline were the highest and significantly ($p \leq 0.05$) different from all the others diets except for Feedbee 50%, Pollen pellets and Karya Ari Keky (Fig. 5). Feedbee 50%, Pollen paste and Karya Ari Keky were also not significantly different from Bee Food, which was significantly ($p \leq 0.05$) different from all the remaining diets except Feedbee powder (Fig. 5). Feedbee 25% and 50%, the diets containing only pollen, Candipolline and Karya Ari Keky had 3 to 53 folds differences from other significantly different diets (Fig. 5).

DISCUSSION

The main result of our study is that pollen is absoultely necessary for the development of brood in the bumble bee colony. None of the tested diets was able to replace pollen in leading brood development and many of them were even unsuitable to maintain adult survival. This is not surprising since most of them (except for Nutri-bombus) were designed to sustain honey bee colony in a period of pollen shortage and not to feed bumble bee colonies during the whole development.
According to the number of dead larvae and adults, colony duration and emerged workers, we selected Feedbee in powder formulation and Nutri-Bombus B as the most promising among the commercial pollen substitutes, and brewers' yeast among the natural protein sources. However, brewer's yeast is quite expensive (approximately 6 € per kg against 0.5 € per kg of Feedbee) and once mixed with the syrup, the formulation dries up very quickly, while Nutri-Bombus B is not yet available on the market. Therefore, we selected Feedbee powder to test the minimum needed pollen amount to be added to the diet in order to sustain colony development. The results showed that both diets with 50% or 25% of Feedbee were suitable for bumble bee rearing, with no significant difference in colony parameters between these two diets and with the "whole pollen" diet, except for a slightly but significantly higher mortality of adults with 50% Feedbee. Nevertheless, an overall view of the measured parameters and CP and sugar daily consumption, a diet with 50% pollen mixed with Feedbee would be enough to sustain the complete colony development and queen production. We did not test 75% Feedbee diet because the Feedbee-to-pollen ratio it is too close to Feedbee alone to expect substantial differences. Although in the first assay Feedbee resulted the best diet, it was still not able to sustain colony development. From the analysis of sugars and protein of the different diets and the total and relative consumption, the diets that allow colony development and queen production (Feedbee 25% and 50%, pollen pellets and pollen paste) had a protein concentration between 200 and 300 mg g^{-1}. They were also the most consumed ones, confirming for bumble bees the high palatability of Feedbee reported also in honeybees (Saffari et al., 2010), and those who provided the highest amount of proteins. Brewer's yeast and Nutri-Bombus C, that had also a protein percentage included in this range, and Feedbee paste, where the protein concentration was even greater, were however little consumed and the total amount of protein they provided was very low.
On the contrary, the commercial pollen substitutes with a very low CP concentration and CP-to-sugar ratio (Candipolline, Karya Ari Keky) had relatively high consumption rates. However, the amount of protein they could provide was too low to allow brood development. Bee Food and chestnut flour also had a low CP concentration and CP-to-sugar ratio. Bee Food had an intermediate consumption rate , but the amount of provided protein was not high enough. Chestnut flour was little consumed and provided one of the lowest protein amounts among all diets, together with Candipolline and Karya Ari Keky. These four latter diets had the worst performances according to the discriminant analysis.
The Feedbee mixed diets used in the second assays were those with the highest protein consumption (slightly lower than 1 g per day), but the consumption rate of the two "whole pollen"

diets showed that a mean amount of protein between 0.4 and 0.5 g per day was enough to allow colony development. All the other diets that provided an amount of protein far below 0.1 g per day are, thus, to be considered inadequate. The lack of clear correlation between the amount of protein assumed with the diet and the colony performance demonstrates that the amount of protein alone is not a sufficient parameter to explain the success of a diet formulation. Other such factors as the kind of amino acids, and other such nutrients as lipid, vitamins and macro and micro nutrients are probably important to sustain larval growth. These parameters have not been considered in our work and would deserve a more thorough investigation.

Besides brood development, the diet in bumble bee colonies is important for other factors, such as health and immunity. Poor pollen diets reduce adult constitutive immunity, augmenting the susceptibility to disease and infections (Roger et al., 2017), and a lack of protein in the diet significantly reduces host-specific immune gene expression (Brunner, Schmid-Hempel, & Barribeau, 2014). Accordingly, pollen consumption in bumblebee is augmented during immunological challenge (Tyler, Adams, & Mallon, 2006). On the other hand, pollen pellets collected by honeybees, used for feeding bumble bee colonies in mass rearing facilities, are often contaminated by pathogens (Singh et al., 2010; Graystock et al., 2013). Those parasites, both from honey bees and bumble bees, can easily spread through the pollen from wild bees to artificially reared colonies of bumble bees. The use of a hygienic artificial pollen substitute represents a solution against the spreading of parasites. In a recent study, the use of Nutri-Bombus as hygienic food was compared to untreated, irradiated or ozone-treated fresh pollen, for the capability of transmitting the main bumble bee parasites and virus to a group of adult workers (Graystock et al., 2016). The results showed no infections of any parasites in workers fed with Nutri-Bombus, while in workers fed with both treated or untreated pollen a variable prevalence infection of the main pathogens was recorded. Therefore, the current irradiation and sterilisation method of pollen proved to be ineffective in preventing parasite and pathogens transmission, with the additional risk to reduce the nutritional value of pollen.

Another problem of honey bee pollen pellets is represented by pesticide residues, as a three year survey of Italian honey bee collected pollen revealed that 62% of samples contained at least one pesticide and 38% of samples had multiresidual contaminations (Tosi et al., 2018). Comparable results were obtained from similar investigations in France (Chauzat et al., 2006) and Greece (Kasiotis et al., 2014).

For all these reasons, the development of a protein replacement for bumble bees would represent a good solution. In our study the tested pollen substitutes alone were insufficient to sustain colony development, while at least 50% pollen was necessary to allow a complete rearing cycle. This could represent a valid compromise combining the advantages of the pollen component necessary for larval growth and of the hygienic value and lack of pesticide contamination of the protein substitute.

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