Journal of Economic Entomology Advance Access published September 18, 2015



# Growth Rate of Bumblebee Larvae is Related to Pollen Amino Acids

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J. Econ. Entomol. 1–6 (2015); DOI: 10.1093/jee/tov279

**ABSTRACT** The use of *Bombus terrestris* L. commercial colonies for outdoor and greenhouse crop pollination is currently widespread. Colony breeding includes bumblebee feeding, mostly by using the honeybee pollen loads of diverse palynological composition. Because the chemical content of pollen is highly variable, the choice of commercial blend should not be random but has to be carefully selected to ensure the optimal development of workers and then pollination efficacy. In this work, we compared the impact of three common commercial blends on the development of bumblebee microcolonies, namely, *Actinidia deliciosa* L., *Cistus* sp., and *Salix* sp. We focus on amino acids (i.e., composition and amount), as they are currently used as an indicator of diet performance. Five parameters were used to determine microcolonies growth rate: 1) number of eggs, 2) number of alive larvae, 3) number of ejected larvae, 4) number of pupae, and 5) total number of offspring. Syrup collection was also monitored to estimate energetic requirement for colony growth. Results revealed that the three commercial blends chemically differed in their amino acid contents, with those displaying higher concentrations (i.e., *Salix* sp. and *A. deliciosa*) accelerating microcolony development along with an increase of syrup collection. The advantages of rearing bumblebee commercial colonies using a pollen diet with content are discussed.

**KEY WORDS** amino acid, bumblebee, growth, microcolony, pollen

Bumblebees are known to be very efficient pollinators of entomophilous crops (e.g., Zhang et al. 2015). *Bombus terrestris* L. is now supplied from large rearing facilities in Europe for pollination of a large variety of crops (Velthuis and van Doorn 2006). They are not only used in glasshouses but also outdoors; for example, ~15,000 nests per year are used in the United Kingdom for soft fruit pollination in open field or open-sided polytunnels (Goulson 2010).

Breeding of bumblebees is highly dependent for food resources on pollen loads collected from honeybee hives (Rasmont et al. 2005). From a quantitative point of view, it has been shown that a low food supply increases the duration of larval development (Sutcliffe and Plowright 1990), whereas starvation forces workers to partly eject larvae in order to ensure a sufficient nutrition for the remaining ones (Tasei and Aupinel 2008a). From a qualitative point of view, chemical composition has been shown to have major impacts on colony and worker sizes (Roulston et al. 2000, Roulston and Cane 2002, Tasei and Aupinel 2008a, Vanderplanck et al. 2014b). However, the impact of diet content on colony growth rate remains poorly understood, while this parameter is essential in breeding system.

Protein content is generally used to estimate pollen quality (Buchmann 1986), and it is highly variable among plant species, ranging from 2.5 to 61% of dry mass (Roulston et al. 2000, but see Vanderplanck et al. [2014a] for discrepancy in definitions). Pollen displaying high protein concentration (e.g., Salicaceae and Fabaceae) has been shown to positively impact bumblebee colony size. However, amino acid composition seems more accurate to determine the amount of pollen required by bees (Nicolson 2011). Moreover, it is nowadays accepted that this chemical feature is a good indicator for diet performance, as a lack of essential amino acids can stop bee growth and development (De Groot 1953, Nation 2002).

Our study aims to help the breeders to choose pollen, promoting faster development of colonies. In the current study, we considered three commercial blends of pollen with various chemical qualities to evaluate the relation between bumblebee colony growth rate and amino acids pollen content. We hypothesized that higher amino acid concentration accelerates larval development.

## Materials and Methods

**Experimental Design.** To evaluate the impact of amino acid content on *Bombus* colony growth rate, we

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reared *B. terrestris* queenless microcolonies from Biobest bvba (Westerlo, Belgium) on different diets, following the method developed by Regali and Rasmont (1995). Each microcolony was composed of five 1-d-old workers reared in plastic containers (8 by 16 by 16 $cm^3$). Such a method using queenless *B. terrestris* microcolonies for testing the nutritive value of pollen diets was shown to be a good estimate of queenright colony development at least under laboratory conditions (Tasei and Aupinel 2008a). All microcolonies were reared in a dark room at 28°C and 65% relative humidity (RH). They were fed ad libitum with sugar syrup (BIOGLUC, Biobest bvba, Westerlo, Belgium) and pollen candies during a 12-d period following the first episode of egg laying of a worker.

We fed 30 microcolonies with three commercial blends (10 each): *Actinidia* dominant blend (Actinidiaceae), *Cistus* dominant blend (Cistaceae), and *Salix* dominant blend (Salicaceae). These pollen diets are currently used to rear bumblebee and were selected because of their reported different amino acid content, respectively, 18.1, 14.4, and 20% (Génissel et al. 2002, Tasei and Aupinel 2008b). Commercial blends were mixed with sugar syrup (80% / 20% in weight) to form candies which were used to feed the microcolonies. New pollen candies were provided every 2 d to avoid the deterioration of nutrients.

Five parameters were used to estimate colony size: 1) number of eggs, 2) number of live larvae (nonisolated larvae were distinguished from isolated larvae), 3) number of ejected larvae, 4) number of pupae, and 5) total number of offspring (here considered like the total production of eggs, larvae, and pupae). As syrup collection is linked to colony energetic needs (Rehor et al. 2013), we used it as a proxy to estimate the colony growth. The growth rate of a colony was therefore evaluated by measuring the syrup collection every 3 d.

**Amino Acid Content.** Amino acid content was assessed based on 3–5 mg of candy (dry weight) following the method of Vanderplanck et al. (2014a). First, we added 1 ml of hydrolysis solution (6N HCl, 0.1% phenol, and 500 µM norleucine). The tube was put under nitrogen for 1 min to avoid methionine degradation and then incubated for 24 h at 110°C. The hydrolysate was vacuum dried in a boiling water bath at 100°C. Afterward, 1 ml of buffer pH 2.2 was added into the tube. The sample solution was mixed and poured in a high-performance liquid chromatography (HPLC) vial after filtration (0.2 µm). Amino acids were measured with an ion exchange chromatograph (Biochrom 20 plus amino acid analyzer). Norleucine was used as the internal standard allowing further amino acid quantification.

**Statistical Analyses.** We performed a one-way analysis of variance (one-way ANOVA) to test the null hypothesis of no difference in tested criteria and total amino acid content between the three diets. This test calculates an F statistic by taking the ratio of among-group sums of squares to within-group sums of squares. Because it is a parametric test based on an F distribution, the following assumptions were checked: 1) independent observations, 2) normality of the residuals (normal QQ plot and Shapiro test), and 3) homoscedasticity (Bartlett test). If normality or homoscedasticity was violated, we performed a nonparametric equivalent test. When these tests were significant, we performed multiple pairwise comparisons.

Both similarities and dissimilarities in essential amino acid compositions among the three diets were visually assessed using nonmetric multidimensional scaling ordinations (nMDS) based on Bray–Curtis dissimilarity matrix calculated on concentrations expressed in mg/g (absolute abundances). All nMDS plots were generated in R employing two dimensions (applying a conventional cutoff of <0.2 for the stress value) and 50 runs, using functions from Ecodist (Goslee and Urban 2007) and BiodiversityR (Kindt and Coe 2005). To test differences in these essential amino acid compositions, a perMANOVA was performed using the Bray–Curtis dissimilarity matrix and 999 permutations (Adonis command, R-package Vegan; Oksanen et al. 2010). Prior to this permutation analysis of variance, the multivariate homogeneity of within-group covariance matrices has been verified using the betadisper function implementing Marti Anderson's testing method. When perMANOVA returned a significant *P*-value ($P < 0.05$), multiple pairwise comparisons were conducted on the data to detect precisely the differences, and *P*-values were adjusted using Bonferroni's correction to avoid type I errors due to multiple testing.

To study the dynamics of syrup collection (i.e., growth rate of the colony), we performed linear regression on the total syrup collection over the time of each microcolony (i.e., 10 linear regressions per diet) as well as global linear regressions for each diet. For each linear model, normality of residuals (Shapiro test) as well as absence of autocorrelation (Durbin–Watson test) were tested. All analyses were performed in R version 2.2.1 with Sciviews R Console (version 0.9.2; R Development Core Team 2005).

## Results

**Microcolonies Development.** Microcolonies fed on *Salix* blend produced significantly more offspring compared with the other blends ($F = 5.8$; df = 2, 27; $P < 0.01$). This difference was mainly attributable to egg production, which was higher for the *Salix* blend ($F = 6.8$; df = 2, 27; $P = 0.004$), and to a lesser extent to pupal cell production, which was higher for the *Salix* blend but not significantly different from the *Actinidia deliciosa* blend according to post hoc test ($P = 0.21$; Supp Table 1 [online only] and Fig. 1).

Microcolonies reared on diets of *Salix* sp. and *A. deliciosa* collected a greater quantity of syrup (respectively, 36.1 ± 1.9 g and 35.1 ± 2.5 g) than microcolonies reared on *Cistus* sp. diet (31.2 ± 3.2 g; $F = 9.7$; df = 2, 27; $P < 0.01$; Supp Table 1 [online only] and Fig. 1).

Global linear regressions showed that dynamics of syrup collection is different according to the diet (Fig. 2). Regression slopes revealed that syrup collection increased significantly faster for microcolonies fed on *Actinidia* and *Salix* blends than on *Cistus* blend ($H = 10.3$, $P = 0.006$; Fig. 1).

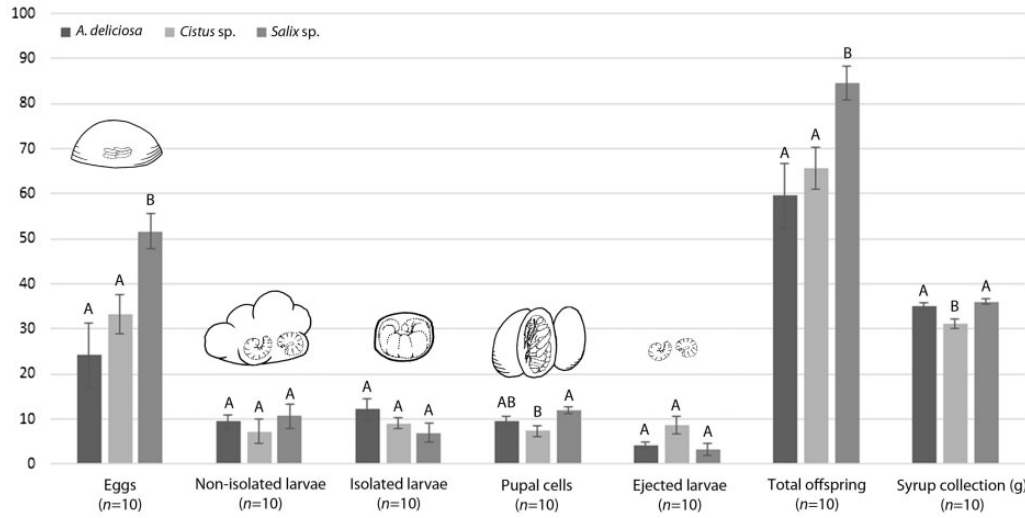


**Fig. 1.** Brood composition and syrup collection for microcolonies fed on the different diets. Groups differing significantly from each other in post hoc test (see results) are indicated with different letters, shared letters indicating nonsignificant differences.

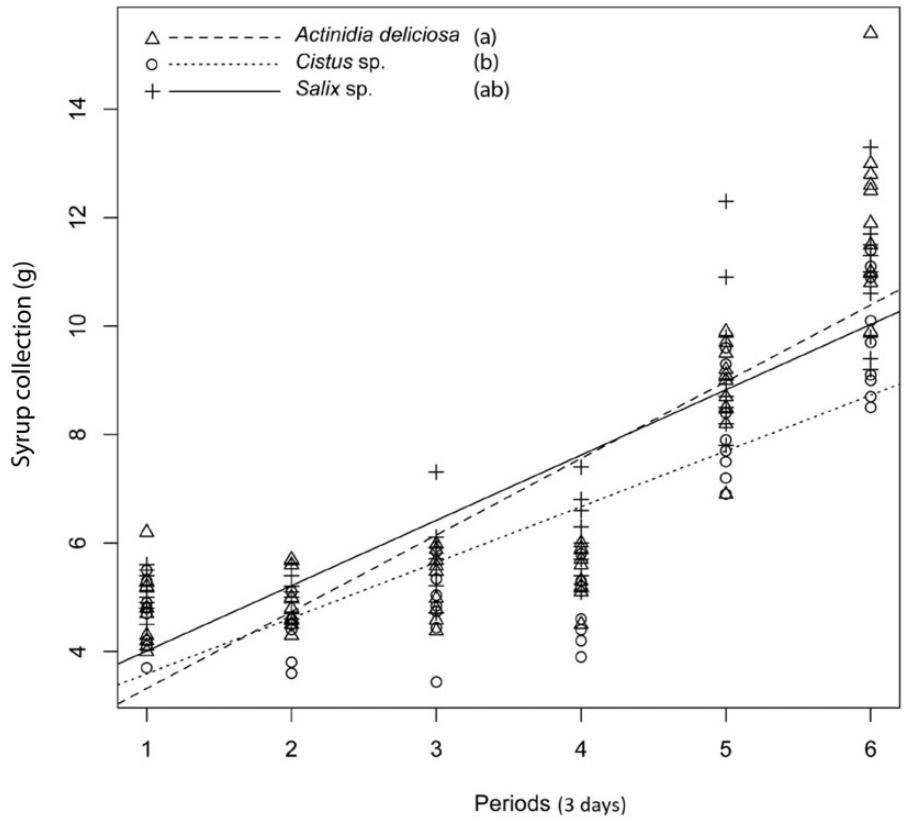


**Fig. 2.** Dynamics of syrup collection for microcolonies reared on pollen of *Salix* sp. ($n = 10$; solid line, $y = 2.3\ x - 2.6$, $R^2 = 0.98$), *A. deliciosa* ($n = 10$; dashed line, $y = 2.2\ x - 3.1$, $R^2 = 0.97$), and *Cistus* sp. ($n = 10$; dotted line, $y = 1.9\ x - 2.1$, $R^2 = 0.94$). Differences across diets were significant (K–W: $H = 10.34$, df = 2, $P = 0.006$). Groups differing significantly from each other in post hoc tests are indicated with different letters.

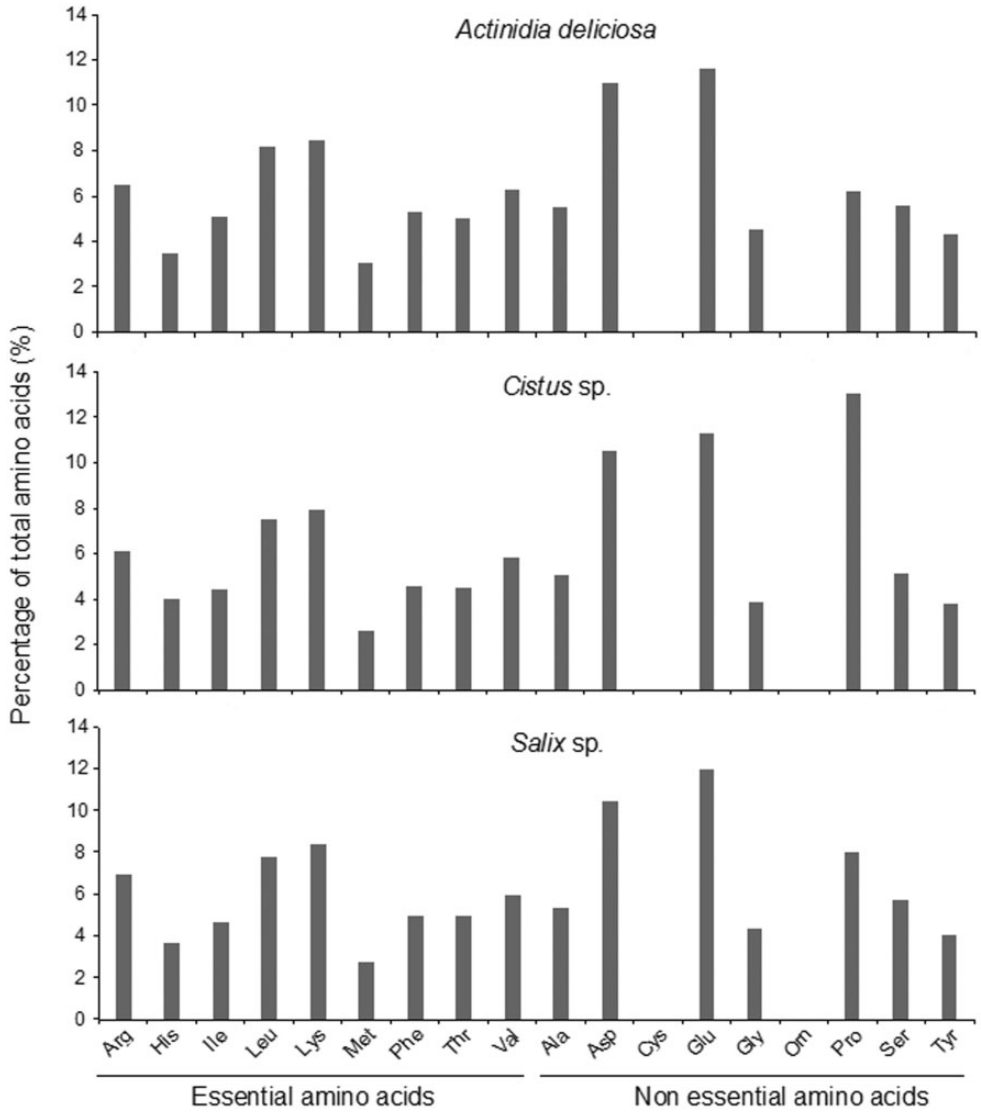


**Fig. 3.** Amino acid composition of the three diets (%) Arg, arginine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Thr, threonine; Val, valine; Ala, alanine; Asp, aspartic acid; Cys, cysteine; Glu, glutamic acid; Gly, glycine; Orn, ornithine; Pro, proline; Ser, serine; Tyr, Tyrosine.

**Amino Acid Content.** Total amino acid contents of *Salix* and *Actinidia* blends were similar (~200 mg/g), whereas it was significantly lower for *Cistus* blend (~138.3 mg/g; $F = 11.1$; df = 2, 6; $P = 0.01$; Supp Table 1 [online only]). Statistical analyses also detected a significant difference in essential amino acid content between diets ($F = 14.9$; df = 2, 6; $P < 0.01$), with greater concentrations in *Salix* and *Actinidia* blends (~100 mg/g) compared with *Cistus* blend (65 mg/g; Supp Table 1 [online only]). Despite this variability in total and essential amino acid contents, relative abundance remained quite similar between the three blends (Fig. 3).

PerMANOVA detected a significant difference in composition of essential amino acids between the pollen diets ($F = 18.68$; df = 2, 6; $P = 0.009$). Pairwise comparisons arranged the different diets into two groups: 1) one with *A. deliciosa* and *Salix* sp. ($P = 0.095$) and 2) one with *Cistus* sp. (*Cistus* sp.–*A. deliciosa*, $P = 0.048$; *Cistus* sp.–*Salix* sp., $P = 0.045$). The difference in concentrations of essential amino acids among the diets was well reflected by the gradient along nMDS 1 on nMDS ordination ($R^2 = 1$, stress value = 0.008; Fig. 4).

## Discussion

**Colony Size.** Three size parameters significantly differed among the diets: 1) the number of eggs, 2) the number of pupal cells, and 3) the number of offspring.

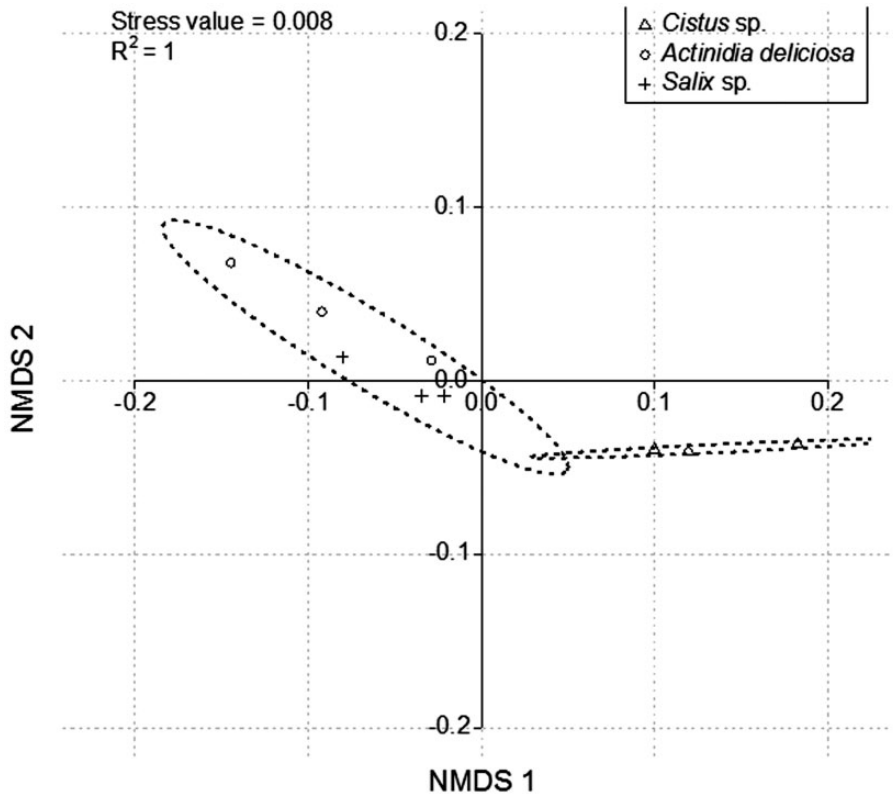


**Fig. 4.** Nonmetric multidimensional scaling (nMDS) plot based on Bray–Curtis distances calculated on absolute amounts (mg/g) of essential amino acids in diets ($R^2 = 1$, stress value = 0.008). *A. deliciosa* did not significantly differ from *Salix* sp. ($F = 1.79$, $P = 0.095$), whereas *Cistus* sp. displayed lower concentrations ($P < 0.05$).

Our results confirm that microcolonies fed on *Salix* sp. pollen (with higher concentration of amino acids) produced relatively more eggs (Génissel et al. 2002). However, some discordance arises between both studies, as these authors found that number of larvae depended on the diet, whereas this parameter remained constant for all the diets tested herein. This parameter is still up for discussion, as other studies suggest that pollen origin influences the weight of larvae rather than their number (Tasei and Aupinel 2008b, Vanderplanck et al. 2014b). With regards to larval ejection, Tasei and Aupinel (2008b) noticed that workers are stimulated to eject a proportion of larvae in order to feed the remaining individuals adequately. Given that bumblebees were supplied ad libitum with pollen candies and that the number of larvae was similar for all diets, the absence of significant difference for larval ejection we observed was as expected.

**Colony Growth Rate.** Based on our results, it seems that high pollen quality (i.e., high amino acid content) accelerates the production of eggs and the maturation of larvae. This higher production of eggs could be linked with a faster maturation of the ovarioles of the dominant worker promoted by protein-rich diet (Nation 2002). Such relation has already been demonstrated for the close related honey bee (Schäfer et al. 2006). Moreover, in the same way that low amounts of pollen imply a longer developmental time with only small bee production (Ribeiro 1994), low quality of pollen might negatively impact colony growth rate.

After 12 d of development, colonies fed on rich amino acid pollen showed a higher rate of syrup collection. This high collection level is associated with higher growth rate of the microcolonies. As microcolonies reared on *Salix* sp. and *A. deliciosa* have a faster development, workers required more energy for brood care, which is reflected by the increase of syrup collection. This result suggests that abundant source of syrup is complementary to beneficial pollen content as well as to poor pollen content (Vanderplanck et al. 2014b).

Herein, only pollen amino acids were investigated, but evidence is that other unevaluated parameters could impact on colony growth rate as toxic compounds or other limiting nutrients. In particular, it is well known that poorly digestible pollen (e.g., *Cistus* sp., R.M., unpublished data) might impact reproductive capacity of *Aphis mellifera* and so could reduce bumblebee colony growth too (Human et al. 2007).

**Breeding Efficiency.** Commercial pollen blends with higher concentration of amino acids seem to accelerate the colony growth. Through such nutritive advantage, colonies can produce an early emergence of workers which can forage faster in greenhouses or outdoors cultures.

Our results underline the importance of using commercial pollen with high amino acid contents along

with abundant syrup source to provide enough energy for optimal colony growth. Caution has nevertheless to be paid, as only one nutrient category was considered. Actually additional investigations are still needed to shed more light on the best commercial pollen blend for a better rearing efficiency of bumblebee colonies.

## Supplementary Data

Supplementary data are available at *Journal of Economic Entomology* online.

## Acknowledgments

We thank Isabelle Van de Vreken and Dimitri Evrard for technical support as well as David Notton (Natural History Museum, London) for reviewing the manuscript. This research was supported by grants from the "Fonds de la Recherche Fondamentale et Collective" (FRFC 2.4613.12, Web impact project; Belgium). Maryse Vanderplanck and Nathalie Roger received financial support from the "Fonds de la Recherche Scientifique" (FRS-FNRS) and from the "Fonds pour la Recherche dans l'Industrie et l'Agriculture" (FRIA).

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*Received 26 May 2015; accepted 29 August 2015.*