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ORIGINAL ARTICLE

Is non-host pollen suitable for generalist bumblebees?

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Abstract Current evidence suggests that pollen is both chemically and structurally protected. Despite increasing interest in studying bee–flower networks, the constraints for bee development related to pollen nutritional content, toxicity and digestibility as well as their role in the shaping of bee–flower interactions have been poorly studied. In this study we combined bioassays of the generalist bee *Bombus terrestris* on pollen of *Cirsium*, *Trifolium*, *Salix*, and *Cistus* genera with an assessment of nutritional content, toxicity, and digestibility of pollen. Microcolonies showed significant differences in their development, non-host pollen of *Cirsium* being the most unfavorable. This pollen was characterized by the presence of quite rare δ7-sterols and a low digestibility. *Cirsium* consumption seemed increase syrup collection, which is probably related to a detoxification mixing behavior. These results strongly suggest that pollen traits may act as drivers of plant selection by bees and partly explain why Asteraceae pollen is rare in bee generalist diet.

Key words bee–flower interactions; generalist bees; pollen defences

Introduction

Bees rely exclusively on plant resources for their reproduction and store quantities of pollen and nectar in their nests to feed their larvae. Like other herbivore guilds, bees forage on plethora of highly diverse host plants with different host breadth, from strict specialization (i.e., oligolecty) to broad generalization (i.e., polylecty) (Robertson, 1925; Cane & Sipes, 2006; Dötterl & Vereecken, 2010). Among worldwide free-living bee species (i.e., excluding cuckoo bees), the half part is polylectic (e.g., *Colletes nigricans* visiting the flowers of up to 15 different plant families) (Westrich, 1989; Minckley & Roulston, 2006; Müller & Kuhlmann, 2008). Polylecty is considered advantageous in reducing dependence upon a limited number of pollen sources. However, generalist bees have to face variation in floral morphologies and floral resources (i.e., quantity and quality) (Wcislo & Cane, 1996).

Pollen varies in its primary and secondary metabolites as well as in its wall resistance. All these pollen traits seem to be related to taxonomy, plant life history traits (e.g., anemophilous versus zoophilous pollens), genetics and environment (Petanidou & Vokou, 1990; Karise *et al.*, 2006). These variations of pollen properties may act as so many defence mechanisms to prevent excessive pollen harvesting by generalist bees. Indeed generalist bees do not forage randomly on all available plants but they are limited to a range of suitable host plants that cope with their abilities either neurological,

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physiologial or both (Sedivy *et al.*, 2011; Haider *et al.*, 2013; Vanderplanck *et al.*, 2014a). For example, Asteraceae are ubiquitous in most temperate habitats (Funk *et al.*, 2005) where they host a large number of specialized bee species but are only occasionnaly exploited by polylectic species such as in *Bombus* (Goulson *et al.*, 2005; Kleijn & Raemakers, 2008) and *Colletes* (Müller & Kuhlmann, 2008). This Asteraceae avoidance by polyleges cannot be explained by neural limitation since compound inflorescences ensure an easy access to both pollen and nectar over an extended time period without the need for special handling techniques nor specialized morphological structures (Müller & Kuhlmann, 2008). In contrast, the failure of several unspecialized bee species to develop on Asteraceae pollen clearly indicates that utilization of Asteraceae pollen requires special physiological adaptations, possibly leading to a narrowing of the spectrum of pollen-feeding visitors (Levin & Haydak, 1957; Guirguis & Brindley, 1974; Génissel *et al.*, 2002; Williams, 2003; Praz *et al.*, 2008; Sedivy *et al.*, 2011).

Despite increasing interest in studying bee–flower networks, the role of pollen properties in shaping interactions has been poorly investigated. Moreover, experimental bioassays combined with chemical analyses remain sparse, limiting a full understanding of pollen constraints for bee development. Here we assess at once the constraints related to pollen nutritional content, toxicity and digestibility, which are generally studied in isolation, to provide a complete picture of pollen defences. Two main questions are addressed: (i) Do plant species avoided by generalist bees produce unsuitable pollen for their requirements? As host foraging is probably driven by resource quality and then bee requirements (Leonhardt & Blüthgen, 2012), we expect that microcolonies of the buff-tailed bumblebee (*Bombus terrestris*) forced to feed on naturally unforaged pollen will display a decreased fitness. (ii) Which pollen trait might be unfavorable to generalist bees? As bumblebees seem to select their host plants based on their pollen quality (e.g., Hanley *et al.*, 2008), we hypothesize that plant species avoided by *B. terrestris* will have different physical or chemical properties to those foraged by this bumblebee species.

Materials and methods

Study design

Bee model We selected *Bombus terrestris* L. (Hymenoptera: Apidae) as a model generalist bee species to test our hypotheses. This social species forages on hundreds of different plant species and numerous plant families (Kleijn & Raemakers, 2008; Rasmont *et al.*, 2008; Leonhardt & Blüthgen, 2012). Nutritive values of different pollen are reported to impact directly on colony development since individual workers do not change the diet composition they supply to the brood, unlike honeybees (Pereboom, 2000).

Pollen diets According to Goulson *et al.* (2005), Fabaceae appear to be the major pollen source for most bumblebee species whereas Asteraceae are only visited for nectar. These 2 plant families are therefore herein selected to assess unfavorable pollen properties with *Trifolium* genus as Fabaceae model and *Cirsium* genus as Asteraceae model. In addition, 2 diet controls, namely *Salix* and *Cistus* pollen, were used during the bioassays in the same way as described in previous studies (Vanderplanck *et al.*, 2014a; Moerman *et al.*, 2015).

To collect enough pollen (i.e., around 250 g) for the bioassays, bumblebee rearing experiments are performed using honeybee pollen loads following previous studies in nutritive ecology (e.g., Génissel *et al.*, 2002; Tasei & Aupinel, 2008a; Baloglu & Gurel, 2015; Ruedenauer *et al.*, 2015; Ruedenauer *et al.*, 2016). Although honeybee pollen loads and floral pollen may differ in their chemical compositions for a same plant species, our experimental design remains consistent as all analyses were conducted on pollen candies to avoid misinterpretation of bioassays. The pollen diets were provided as a candy, a mix of honeybee pollen loads and commercial inverted sugar syrup, containing methyl and propyl hydroxybenzoate at 0.6 % (BIOGLUC®, Biobest Westerlo, Belgium). Such addition of BIOGLUC syrup allows preventing from potential effects of microorganisms on diet quality. *Salix* and *Cistus* pollen loads were provided by Ruchers de Lorraine (*Salix* sp.) and Pollenergie France (*Cistus* sp.). *Trifolium* and *Cirsium* pollen loads were collected using 2 honeybee hives with pollen traps placed during 3 weeks in areas where the 2 target plants were dominant. Pollen loads were sorted according to their color after microscopical confirmation. As Asteraceae genera are quite hard to differentiate based on pollen shape, identification was performed through molecular sequencing and DNA barcoding. DNA from 2 pollen pellets was extracted using Food DNA extraction Kit (Macherey Nagel Hoerdt, France) according to the manufacturer's protocol. The nrITS (ITS1), *trn*L (UAA) intron and *rbc*L regions were amplified with universal primers (Taberlet *et al.*, 1991; Chase *et al.*, 1993; Chen *et al.*, 2010) using standard polymerase chain reaction (PCR) protocol. All amplification reactions were performed in a volume of 25 μL. The PCR mix contains 0.2 μmol/L of each primers (Sigma-Aldrich Saint-Quentin Fallavier, France), 200 μmol/L dNTP (Promega Charbonnières-les-Bains,

France), 0.025 U/μL Go Taq (Promega Charbonnières-les-Bains, France), 1× PCR buffer, and 10–50 ng of template DNA. The PCR conditions included an initial denaturation at 95 °C for 5 min, followed by 34 cycles of 30 sec at 94 °C for template denaturation, 40 sec at 54 °C (ITS), 55 °C (*rbc*L) and 60 °C (*trn*L) for primer annealing, 1 min at 72 °C for extension, and finished with an extension step of 10 min at 72 °C. Amplified products were sequenced on an ABI3730XL automated sequencer (Genoscreen, Lille, France). Sequences obtained were aligned using the Geneious Alignment (Geneious v. 9.0.5) and compared with GenBank BLASTn.

Rearing experiment Two-day-old workers of *Bombus terrestris* were provided by Biobest *bvba* (Westerlo, Belgium). They were divided into 40 microcolonies (10 microcolonies for each diet) of 5 workers and placed in different plastic boxes (10 cm × 16 cm × 16 cm). These microcolonies were reared in a dark room at 26–28 °C and 65% relative humidity. They were fed *ad libitum* with inverted sugar syrup (BIOGLUC®, Biobest Westerlo, Belgium) and pollen candies during a 12-d period following the first episode of egg-laying of a worker. New pollen candies were provided every 2 d (0.5 g, 1.0 g or 1.5 g depending on the age of the microcolony) to avoid nutrient alteration. Syrup and pollen supplies as well as microcolonies monitoring were done in the darkroom under red light in order to avoid disturbing colonies, as bees do not detect this range of the light spectrum.

Such a method using queenless *Bombus terrestris* microcolonies for testing the nutritive value of pollen diets has already been validated and is accepted as good estimate of queenright colony development (Tasei & Aupinel, 2008a).

Bumblebee performance

Microcolony development Diet performance and bumblebee feeding response have been evaluated based on: (i) composition and fresh weight of brood (i.e., eggs, non-isolated larvae, isolated larvae, and pupae), (ii) percentage of ejected larvae, (iii) pollen collection (i.e., amount of pollen consumed and stored) (fresh matter), (iv) pollen efficacy (i.e., ratio between the weight of brood and the pollen collection), and (v) syrup collection (i.e., amount of syrup consumed and stored) (parameters adapted from Tasei & Aupinel, 2008b). Pollen and syrup collections were measured by weighing pollen candies and syrup container before their introduction into the microcolony and after their removing (i.e., every 2 d). All weight parameters (i.e., brood weight, pollen collection, and syrup collection) were standardized by the total weight of workers in the microcolonies to avoid potential bias from worker activities (i.e., consumption and brood care).

Worker fat body and digestibility Abdominal fat body content of workers was measured according to Ellers (1996). Isolated abdomens of 3 workers per microcolony ($n = 30$ per pollen diet) were weighed after drying at 70 °C for 3 d. Dried abdomens were then put into 2 mL of diethyl ether for 24 h to extract fat, rinsed twice and weighed again after drying at 70 °C for 7 d. The fat mass was defined as the abdominal weight loss during this process, standardized by abdomen weight before extraction to avoid biases linked to worker size.

Digestibility analysis was adapted from the study of Fernandes-Da-Silva and Serrão (2000). For each microcolony, worker faeces were collected and homogenized. A fraction was diluted with a methylene blue solution and embedded in gelatin for microscope slide mounting ($n = 3$ per microcolony, i.e., $n = 30$ per diet). Digested (i.e., light-colored or uncolored with only contour line visible) and undigested (i.e., dark-colored and fully filled) pollen grains were counted at random under a microscope in different slide fields until a total of 200 grains had been counted (DIFF 15, Compact multifunction cell counter). Digestibility was defined as percentage of empty pollen grains (Fernandes-Da-Silva & Serrão, 2000).

Diet characterization

Nutritional content *Amino acids*: Amino acid content was assessed based on 5 mg of pollen candy (fresh mass, $n = 5$) according to Vanderplanck *et al.* (2014b). Total amino acid extracts were analyzed using an ion exchange chromatograph and norleucine as internal standard for further amino acid quantification. Essential amino acids for the honeybee were determined by De Groot (1953); namely arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. Only tryptophan was omitted in the present analyses because its isolation requires a separate alkaline hydrolysis and it is hardly ever a limiting essential amino acid (Standifer *et al.*, 1980).

Nitrogen: Nitrogen content of pollen candy was determined according to the Kjeldahl method (Kirk, 1950). In a 350 mL flask, 7.2 mL of concentrated H_2SO_4 (10N) and a catalyst tab (Kjetltabs; 1.5 g K2SO4, 0.045 g CuSO4.5H2O, 0.045 g TiO2) were added to 100 mg of pollen candy (fresh mass, $n = 5$). Digestion was performed in a Tecator Block Digester at 360 °C for 2 h, which leads to ammonium sulphate production. Both distillation and titration steps were conducted using a

Foss 2300 Kjeltec analyzer distiller unit. Through alkalinization with 10N NaOH, the ammonia was displaced from the ammonium sulphate and overdistilled (i.e., steam distillation) into a boric acid receiver with bromocresol green/methyl red mixed indicator. This solution was then titrated with 0.02N H_2SO_4 to determine the nitrogen content in the sample.

Sterols: Sterols were extracted and purified from 40 mg samples of pollen candy (fresh mass, $n = 5$) according to Vanderplanck *et al.* (2011). Sterolic extracts were analyzed by gas chromatography coupled to a flame ionization detector (GC-FID) using betulin as internal standard for sterol quantification (see Appendix S1 for analytical details).

Toxicity *Alkaloids*: Alkaloid extraction was performed using 1.5 mg samples of pollen candy weighed into a 1.5-mL tube (fresh mass, $n = 5$). We added 100 μL of extraction solution (methanol: milliQ water: formic acid, 70:30:0.5) and 4 glass beads (2 mm diameter). The tube was then shaken at 30 Hz for 4 min and centrifuged at 18 407 *g* for 4 min. To improve the recovery of supernatant without pollen grains, the whole sample was transferred to a microtube (i.e., 200 μL) and centrifuged again at 10 625 *g* for 5 min. 80 μL of supernatant were finally transferred in a vial and analyzed by ultra-high liquid chromatography coupled with quadrupole time of flight mass spectrometer (UHPLC-(ESI)-Q-ToF/MS) according to the method described in Gosselin *et al.* (2013) (see Appendix S2 for analytical details).

Saponins: Saponin content of pollen candy was determined using the method adapted from van Dyck *et al.* (2011). A total of 200 mg of pollen candy (fresh mass, $n = 5$) was shaked at 25 Hz for 5 min with 1 mL methanol (0.05 mg/mL ginsenoside as internal standard) and zirconium beads (0.5 mm diameter, 400 mg) in a 2 mL tube to ensure saponin extraction. The whole sample was transferred in a 10 mL falcon and the 2 mL tube was rinsed with 5 mL methanol to improve sample recovery. The centrifuged extract (1000 r/m for 5 min, 4.2 mL) was adjusted at 70% by addition of 1.8 mL H_2O. A first purification was performed by liquid–liquid partitioning, successively against n-hexane (v/v), dichloromethane (v/v), and chloroform (v/v). The final extract was evaporated under reduced pressure using a rotary evaporator (Heidolph Rotary Evaporator, Laborota 4001) and dissolved in water in order to undergo a last partitioning against isobutanol (v/v). The isobutanolic fraction, containing the purified saponins, was evaporated to dryness dissolved with 400 μL of water (HPLC grade). Purified extracts were tested for their hemolytic activities following the method described in Demeyer *et al.* (2014). Idenfication and quantification of saponins were performed using respectively MALDI–MS (matrix-assisted laser desorption/ionization–mass spectrometry) and LC–MS (liquid chromatography–mass spectrometry) methodologies (see Appendix S3 for analytical details).

Data analysis

Food collections (i.e., pollen and syrup) (g), brood masses (g), ejected larvae (%), worker fat body contents (%), diet digestibilities (%), and nutrient contents (mg/g) were compared using one-way analyses of variance (one-way ANOVA). Prior to these analyses, percentage data were arcsine-transformed to achieve variance stabilization. Since one-way ANOVA is a parametric test based on a *F*-distribution, the following assumptions were checked: (i) independent observations, (ii) normality of the residuals (Shapiro test), and (iii) homoscedasticity (Bartlett test). When assumption violation occurred even after possible transformations, permutational ANOVA (i.e., data set with deviation from normality only) or Kruskal–Wallis tests (i.e., data set with deviation from homoscedasticity regardless of normality) were used instead of ANOVA. If a significant effect of the diet was detected, multiple pairwise comparisons (*post hoc* test) were conducted with *P*-value adjustments to avoid increases in type error I due to multiple testing. All analyses were performed in R version 3.0.2 (R Core Team, 2013).

In order to detect differences between diet compositions (i.e., sterol and amino acid profiles) and brood compositions, perMANOVAs were performed using Bray–Curtis distances and 999 permutations ("adonis" command, R-package vegan, Oksanen *et al.*, 2013). Prior to this permutational analysis of variance, the multivariate homogeneity of within-group covariance matrices was verified using the "betadisper" function implementing Marti Anderson's testing method. When the returned *P* value was significant, multiple pairwise comparisons were conducted on the data with Bonferroni's adjustment to the *P* values. Differences in diet composition were visually assessed on a non-metric multidimensional scaling (nMDS) ordination using a Bray–Curtis similarity matrix, 2 dimensions and 50 runs. Statistics were conducted in R using functions from ecodist (Goslee & Urban, 2007). Indicator compound analyses were also performed in R using the "indval" function from the labdsv package (Roberts, 2013) to identify the compounds that were indicative of 1 diet. All multivariate analyses were conducted in R version 3.0.2 (R Core Team, 2013) using data expressed as percentage of total content for each sterolic compound or amino acid and as percentage of total brood mass for each developmental stage (relative abundances).

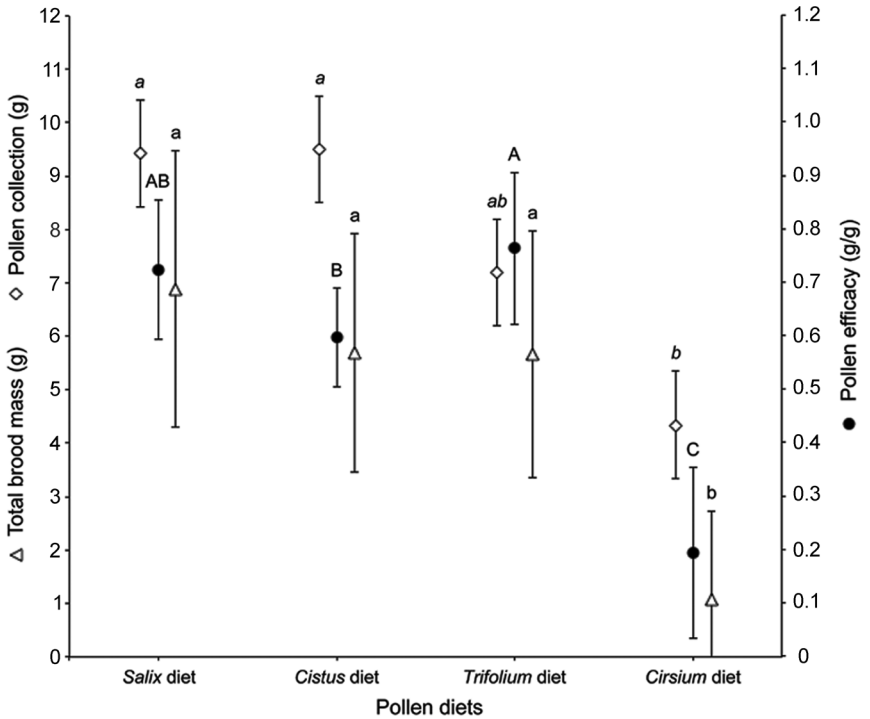

Fig. 1 Efficacy of the different pollen diets with regards to pollen collection and brood mass at the end of bioassays ($n = 10$ per diet). Different letters indicate significant difference between diets (*post hoc* tests, $P < 0.05$).

Results

Bumblebee performance

Brood masses (i.e., eggs, larvae and pupae cumulated) were significantly different between the microcolonies (ANOVA, $F_{3,36} = 13.33$, $P < 0.001$) with *Cirsium* pollen supporting lower offspring production compared to the other diets (Fig. 1, Table S1). A detailed study of the number of specimens as well as of the global mass for each developmental stage revealed that broods developed on *Cirsium* pollen displayed smaller non-isolated larvae as well as less isolated larvae compared to the broods fed on the other pollen diets. Actually microcolonies fed on Asteraceae diet showed significantly slower dynamics (perMANOVA, $F_{3,36} = 4.97$, $P < 0.002$) as their broods never displayed pupae and were characterized by large proportion of eggs and non-isolated larvae by contrast to the other pollen diets (i.e., presence of pupae and isolated larvae as dominant proportion of brood) (Table 1). This slower development went along with a significantly higher percentage of larval ejection (ANOVA, $F_{3,36} = 7.88$, $P < 0.001$) (Table S1).

Unlike broods, workers were not impacted by the diet as none died during bioassays and all of them displayed a fat body content surrounding 9%–10% regardless to the diet (Kruskal–Wallis, $H = 7.49$, df $= 3$, $P = 0.0578$) (Table S1). However, microscopical study of bumblebee faeces revealed an incomplete digestion of *Cirsium* pollen grains. Actually the digestibility differed significantly between all the diets (ANOVA, $F_{3,36} = 802.33$, $P < 0.001$), from the most digestible *Salix* diet (95.18% ± 1.41%) to the least digestible *Cirsium* diet (18.78% ± 2.81%). Both *Cistus* (60.48% ± 5.75%) and *Trifolium* (88% ± 3.02%) diets displayed intermediate digestibility (Table S1).

Pollen collection seemed also affected by the diet (ANOVA, $F_{3,36} = 8.38$, $P < 0.001$), with a lower collection for *Cirsium* (4.34 ± 1.83 g) and *Trifolium* (7.19 ± 2.17 g) diets compared to *Salix* (9.42 ± 3.09 g) and *Cistus* diets (9.49 ± 3.25 g) (Fig. 1, Table S1). However, microcolonies fed on *Cirsium* diet displayed a larger pollen collection per gram of offspring, significantly different from *Salix* and *Trifolium* diets (Kruskal–Wallis, $H = 25.36$, df $= 3$, $P < 0.001$). Both brood mass and pollen collection were capsulated in the efficacy parameter which was significantly lower for the Asteraceae diet compared to the other diets (perANOVA, $F_{3,36} = 37.77$, $P < 0.001$) (Table 1).

Monitoring of total syrup collection revealed that the energy intake was not impacted by the diet (ANOVA,

Table 1 Brood composition with the different stages expressed as percentage of total brood mass (i.e., dynamics of microcolony development) ($n = 10$ per diet).

Parameters	*Salix* diet		*Cistus* diet		*Trifolium* diet		*Cirsium* diet	
Egg ($n = 10$)	0.52 ± 0.34 (0 – 0.96)	ab	0.20 ± 0.24 (0 – 0.70)	b	0.42 ± 0.45 (0 – 1.17)	ab	7.09 ± 10.95 (0 – 35.77)	a
Non-isolated larvae ($n = 10$)	22.94 ± 15.78 (0 – 45.24)	b	31.59 ± 26.54 (10.78 – 100)	ab	22.07 ± 29.01 (2.88 ± 96.68)	b	61.91 ± 32.66 (0 – 100)	a
Isolated larvae ($n = 10$)	70.20 ± 11.88 (54.36 – 83.78)	b	67.23 ± 25.92 (0 – 89.22)	a	63.29 ± 25.93 (3.32 – 92.61)	ab	31 ± 35.15 (0 – 99.44)	b
Pupae ($n = 10$)	6.33 ± 9.46 (0 – 21.30)	b	0.99 ± 1.99 (0 – 5.68)	a	14.22 ± 18.33 (0 – 45.44)	a	0 ± 0 (0 – 0)	a

Note: Mean ± SD (min–max). Different letters indicate significant difference between diets (*post hoc* tests, $P < 0.05$).

$F_{3,36} = 1.30$, $P = 0.290$). However, when weighted by brood masses, significant differences were detected as microcolonies fed on *Cirsium* diet displayed the largest syrup collection per gram of offspring (Kruskal–Wallis, $H = 19.77$, df = 3, $P < 0.001$) (Table 1). In the same way, syrup collection weighted by pollen collection highlighted that bumblebees fed on *Cirsium* collected a significantly higher amount of syrup per gram of pollen compared to the other pollen diets (ANOVA, $F_{3,36} = 13.17$, $P < 0.001$).

Diet nutrients

Nitrogen content of diet differed significantly ($F_{3,16} = 103$, $P < 0.001$) with a decreasing trend from *Trifolium* diet, *Salix* diet, *Cirsium* diet, and *Cistus* diet, respectively (Table 2).

Total amino acid concentration was significantly different for *Trifolium* diet only, which displayed the highest concentration (ANOVA, $F_{3,16} = 27.99$, $P < 0.001$) (Table 2). The amount of essential amino acids also differed between the diets (ANOVA, $F_{3,16} = 25.41$, $P < 0.001$), with *Trifolium* diet displaying the highest concentration and *Cirsium* diet the lowest. No differences were detected between *Salix* and *Cistus* diets ($P = 0.18$), which had intermediate concentrations, and between *Cistus* and *Cirsium* diets ($P = 0.80$) (Table 2). Although amino acid compositions were significantly different among the 4 diets (perMANOVA, $F_{3,16} = 14.40$, $P < 0.001$; all pairwise comparisons $P < 0.05$), no amino acid deficiency has been highlighted (i.e., any diet missed any essential amino acids).

Sterol content of diets differed significantly (ANOVA, $F_{3,16} = 65.95$, $P < 0.001$) with a decreasing trend among *Salix* diet, *Trifolium* diet, *Cistus* diet, and *Cirsium* diet, respectively (Table 2). All diets differed in their sterolic compositions (perMANOVA, $F_{3,16} = 1140.8$, $P < 0.001$; all pairwise comparisons $P < 0.05$). Indicator compound analysis revealed that *Cirsium* diet was significantly associated with high abundance of quite rare phytosterols, namely δ7-stigmasterol (IndVal = 0.98, $P = 0.009$) and δ7-avenasterol (IndVal = 0.58, $P = 0.009$), as well as with abundance of cholestenone (IndVal = 0.76, $P = 0.009$) (Table 2, Fig. 2).

Diet toxicity

Despite the high sensitivity and the low detection limit of the analytical method (0.2 ng EA/mL), no trace of alkaloid was detected in any pollen diet.

With regards to saponins, no hemolytic activity was detected in the different pollen extracts, even in

Table 2 Chemical composition of the pollen diets.

Chemicals	*Salix* diet	*Cistus* diet	*Trifolium* diet	*Cirsium* diet
Nitrogen content (mg/g)	25.52 ± 1.38 b	19.60 ± 0.69 d	28.82 ± 0.67 a	22.10 ± 0.66 c
Total amino acids (mg/g)	144.23 ± 6.11 b	136.06 ± 10.15 b	185.04 ± 13.05 a	145.48 ± 5.82 b
Essential amino acids (mg/g)	50.14 ± 3.40 b	44.66 ± 2.92 bc	62.75 ± 4.38 a	42.34 ± 5.13 c
Alanine (%)	5.44 ± 0.02	4.99 ± 0.10	5.18 ± 0.02	5.45 ± 0.13
Arginine (%)	7.35 ± 0.11	6.12 ± 0.26	5.66 ± 0.14	4.33 ± 0.13
Asparagine (%)	10.85 ± 0.24	10.85 ± 0.75	9.51 ± 0.21	9.27 ± 0.35
Glutamate (%)	12.55 ± 0.11	13.02 ± 2.58	11.15 ± 0.16	10.60 ± 0.26
Glycine (%)	4.40 ± 0.02	3.84 ± 0.05	3.89 ± 0.01	3.24 ± 1.52
Histidine (%)	3.39 ± 0.03	3.68 ± 0.08	3.20 ± 0.03	4.61 ± 0.10
Isoleucine (%)	4.47 ± 0.07	4.03 ± 0.16	4.49 ± 0.06	3.73 ± 0.11
Leucine (%)	7.57 ± 0.07	7.18 ± 0.13	7.33 ± 0.06	6.18 ± 0.10
Lysine (%)	8.48 ± 0.17	7.94 v 0.16	7.72 ± 0.13	8.41 ± 0.09
Méthionine (%)	0.57 ± 0.30	0.20 ± 0.21	0.29 ± 0.32	0.24 ± 0.22
Phenylalanine (%)	4.81 ± 0.07	4.16 ± 0.54	4.89 ± 0.09	4.15 ± 0.14
Proline (%)	10.32 ± 0.62	14.63 ± 6.04	17.90 ± 0.54	22.50 ± 1.28
Serine (%)	5.74 ± 0.07	6.41 ± 2.68	5.27 ± 0.06	4.93 ± 0.15
Threonine (%)	4.92 ± 0.03	4.42 ± 0.19	4.74 ± 0.08	4.29 ± 0.12
Tyrosine (%)	3.68 ± 0.08	3.58 ± 0.87	3.65 ± 0.09	3.25 ± 0.14
Valine (%)	5.45 ± 0.09	4.94 ± 0.19	5.13 ± 0.03	4.82 ± 0.08
Total sterols (mg/g)	4.35 ± 0.51 a	2.58 ± 0.16 c	3.32 ± 0.65 b	1.35 ± 0.14 d
Cholesterol (%)	0.34 ± 0.05	0.50 ± 0.05	0.52 ± 0.10	1.38 ± 0.39
Desmosterol (%)	0.38 ± 0.03	0.46 ± 0.07	0.78 ± 0.05	1.39 ± 0.10
24-Methylenechol./ camp. (%)	58.41 ± 1.91	22.33 ± 2.26	46.12 ± 2.14	6.94 ± 1.88
Stigmasterol (%)	1.54 ± 0.83	0.72 ± 1.04	0.74 ± 1.00	0.89 ± 0.06
β-Sitosterol (%)	21.84 ± 0.67	45.86 ± 1.77	12.08 ± 1.03	6.11 ± 1.10
δ5-Avenasterol (%)	15.04 ± 0.56	27.64 ± 0.88	37.87 ± 1.81	4.88 ± 1.18
Cholestenone (%)	0.53 ± 0.08	0.87 ± 0.08	0.36 ± 0.18	5.43 ± 0.40
δ7-Stigmasterol (%)	0.58 ± 0.08	0.60 ± 0.06	0.20 ± 0.05	67.80 ± 2.35
δ7-Avenasterol (%)	1.34 ± 0.08	1.03 ± 0.10	1.31 ± 0.12	5.18 ± 0.22
Saponins (mg/g)	ND	ND	ND	ND
Alkaloids (mg/g)	ND	ND	ND	ND

Note: Mean ± SD. Different letters indicate significant difference between diets (*post hoc* tests, $P < 0.05$). ND, not detected.

3 times more concentrated samples. Moreover, there was no visible saponin signal on the MALDI-(+)-MS spectra suggesting that the different diets are saponin free (Table 2). Nonetheless major peaks were detected at *m/z* 757 in *Salix* extract and at *m/z* 787 in *Cirsium* extract. They were tentatively identified as flavonoid glycoside ions mainly based on their mass fragmentation behaviors. As observed in the MS/MS spectrum (Fig. 3), mass selected parent ions at *m/z* 757 generate upon collisional activation fragment ions at *m/z* 611, 449, and 287, that could arise from the formal consecutive losses of rhamnose, glucose and glucose residutes, sequentially affording $[M+H\text{-}Rham]^+$, $[M+H\text{-}Rham\text{-}Glc]^+$, and $[M+H\text{-}Rham\text{-}Glc\text{-}Glc]^+$. This sequence of decomposition, together with the data from the accurate mass measurement (measured *m/z* 757.2258/theoretical *m/z* 757.2191) allows the tentative assignment of the *m/z* 757 ions to a rhamnosyl dihexosyl luteolin/kaempferol derivative ($C_{33}H_{40}O_{20}$) (Fig. 3) (Hong *et al.*, 2006). Similar MS-based experiments were also conducted on the *m/z* 787 ions, but the obtained data did not afford a clearcut evidence on the nature of the corresponding molecule (measured *m/z* 787.3719/theoretical *m/z* 787.3694). We hypothetically identified the molecule as a quercetin derivative ($C_{45}H_{55}O_{12}$) with a fragmentation pattern produced by successive losses of 2 monosaccharide residues, that is, losses of 146 units and an unidentified 178 unit loss. Such presence of flavanoids in pollen has been reported previously and could play a role in plant reproduction and fertility (Falcone Ferreyra *et al.*, 2012 and references therein).

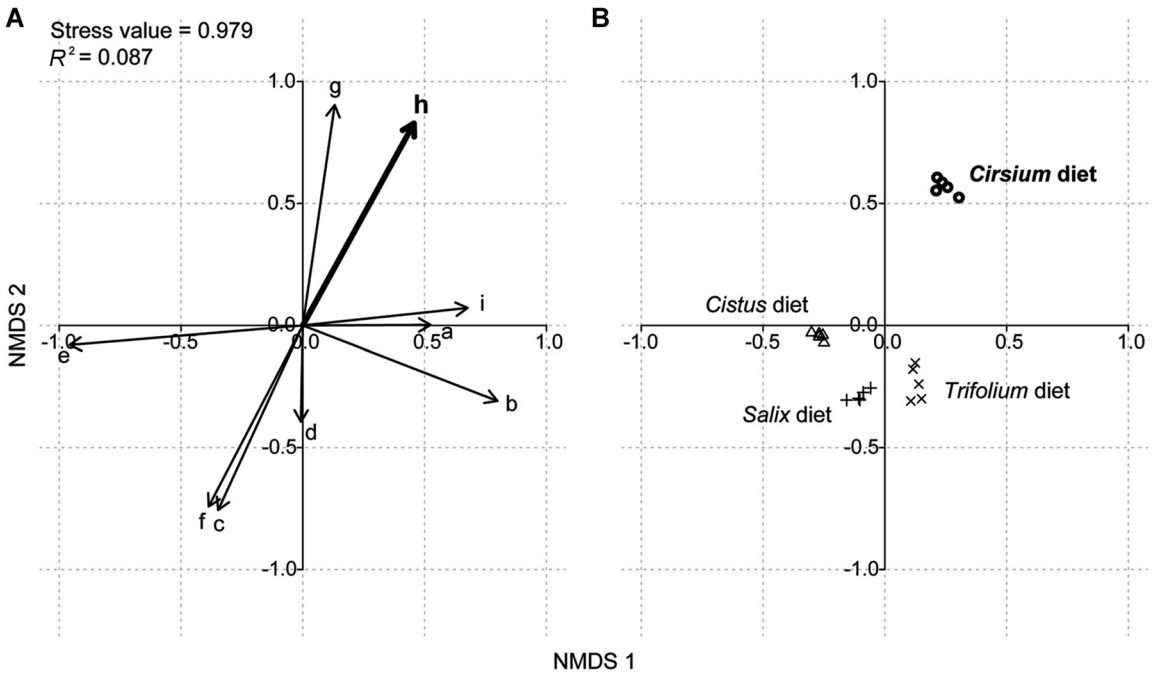

Fig. 2 nMDS ordination plot based on Bray–Curtis distances calculated on relative abundances of sterols in pollen diets showing (A) sterolic vectors with a, cholesterol; b, desmosterol; c, 24-methylenecholesterol/campesterol; d, stigmasterol; e, β-sitosterol; f, δ5-avenasterol; g, cholestenone; h, δ7-stigmasterol, and i, δ7-avenasterol and (B) replicates within pollen diet ($n = 5$).

Discussion

Impeded development of bumblebees on non-host pollen

The results of this study demonstrate that *Cirsium* pollen differs from the pollen of the other plants in being less suitable for the microcolony development of *B. terrestris*. Microcolonies of *B. terrestris* that fed a *Cirsium* pollen diet showed a significantly slower development, a higher larval mortality (i.e., larval ejection) and a considerably lower larval production compared with microcolonies fed with the other pollen diets.

Such low suitability of pollen for *B. terrestris* has been already highlighted for *Calluna vulgaris*, *Cistus* sp., and *Taraxacum* sp. (Génissel *et al.*, 2002; Tasei & Aupinel, 2008b; Vanderplanck *et al.*, 2014a). The present results may account for why Asteraceae pollen is rarely a component of diet bumblebees including the most polylectic species (e.g., *Bombus terrestris*) (Kleijn & Raemakers, 2008) despite the diversity and the abundance of this plant family (Funk *et al.*, 2005).

Our results are consistent with other studies suggesting that Asteraceae pollen is unfavorable for *Bombus terrestris* (Génissel *et al.*, 2002) and for non-specialist solitary bee species like Megachilidae (Herbert *et al.*, 1970; Müller & Kuhlmann, 2008; Praz *et al.*, 2008; Sedivy *et al.*, 2011). However, the negative impact of Asteraceae seems lower in bumblebees than in previously tested solitary bees as no strong larval mortality was observed unlike in *Chelostoma* and *Hoplitis* genera (Praz *et al.*, 2008). Such mitigation may be due to specific bee behaviors comprising a "social detoxification system" through pollen mixing (i.e., toxin dilution) and colony food processing (i.e., microbial fermentation) that reduces the number or quantity of ingested chemicals requiring detoxification by larvae (Berembaum & Johnson, 2015). This hypothesis of social detoxification strategy is supported by our finding that bumblebees fed on *Cirsium* pollen added a higher quantity of nectar (i.e., syrup) to the pollen, resulting in a nectar-mixing behavior assumed to dilute the toxins.

Unfavorable pollen properties

Characterization of the different diets revealed that *Cistus* pollen and *Cirsium* pollen did not significantly differ in their total and essential amino acid concentrations. *Cirsium* pollen even showed higher concentration in nitrogen than *Cistus* pollen. These results are in contradiction with the common claim that development

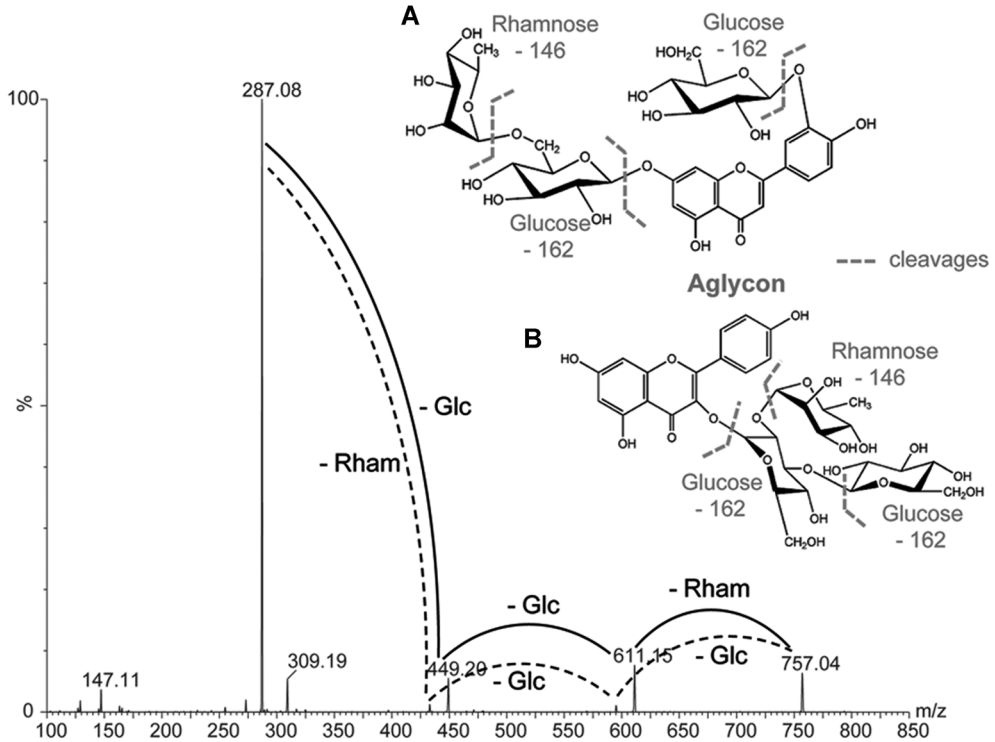

Fig. 3 Positive ion MALDI-MS/MS spectrum obtained from the peak at *m/z* 757 $[M+H]^+$ (*Salix* extract). Fragmentation pathways are labeled as -Glc (loss of a glucose residue), -Rham (loss of a rhamnose residue). Two isomers were highlighted from the mass fragmentation pattern, corresponding to 2 flavonoid glycosides namely (A) luteolin 7-rutinoside-3′-glucoside and (B) kaempferol 3-(3G-glucosylneohesperidoside).

performance of bees on pollen diet is positively associated to high amino acid concentration and/or protein content (e.g., Buchmann, 1986; Day *et al.*, 1990; Regali & Rasmont, 1995; Génissel *et al.*, 2002; Roulston & Cane, 2002; Smeets & Duchateau, 2003; Alaux *et al.*, 2010; Nicolson, 2011; Stabler *et al.*, 2015). While the development failure of honey bees fed exclusively on *Taraxacum* pollen (Herbert *et al.*, 1970) has been attributed to its lack in tryptophane and phenylalanine (Auclair & Jamieson, 1948) and its deficience in arginine (Herbert, 1992) (i.e., essential amino acids), the composition and concentration of essential amino acids in the pollen of *Cirsium* appears to be sufficient for a successful development. Recent studies have already showed that proteins and amino acids were not the only nutrients impacting pollen suitability but presence of other components may provide additional properties (Vanderplanck *et al.*, 2014a,b; Baloglu & Gurel, 2015). Actually, *Cirsium* pollen displays abundance of quite rare phytosterols (i.e., δ7-sterols). Moreover, microscopical analyses of feces suggest that workers encountered higher difficulties in extruding protoplasm when fed on *Cirsium* diet. Such traits might account for the lower suitability of *Cirsium* pollen (i.e., by limiting the development of *Bombus terrestris* microcolonies) highlighted in our bioassays.

The fact that δ7-sterols occur in *Cirsium* pollen in seemingly higher proportion than more common and usuable sterols (i.e., 24-methylenecholesterol, β-sitosterol and δ5-avenasterol) may account as a defence mechanism against excessive pollen harvesting. Although further studies are needed to clarify to what extent this pecular sterolic composition impedes microcolony development (i.e., slowing down of microcolony development and increase of larval mortality), recent research has already hypothized that such profil is not beneficial for bumblebees (Vanderplanck *et al.*, 2014a). Likewise, δ7-sterols are assumed to function as a unique defence against herbivory in other insect groups. Behmer and Elias (1999a) showed that such sterols negatively impacted grasshoppers at the cellular and/or hormonal levels. This is strengthened by the finding that grasshoppers have very sensitive behavioral responses (including learning) to food sterol profiles, which lead to aversions to plants with unsuitable δ7-compounds like chenopods (Chenopodiaceae) and

cucurbs (Cucurbitaceae) (Champagne & Bernays, 1991; Behmer & Elias, 1999b; Behmer *et al.*, 1999). Performance on diets containing phytosterols with δ7-configurations has been also examined in 2 lepideptoran species, the generalist *Heliothis zea* (Ritter & Nes, 1981) and the Brassicaceae specialist *Plutella xylostella* (Behmer & Grebenok, 1998). Neither grew particularly well, which was quite unexpected for the generalist feeding on a wide range of plants. Evidence is that the preferred host ranges of generalists tend to primarily contain suitable sterols, which support growth and development (Nes *et al.*, 1997).

The last physiological contsraint relates to secondary pollen compounds, which may be either directly toxic or interfere with nutrient assimilation (Arnold *et al.*, 2014; Irwin *et al.*, 2014). Although Praz *et al.* (2008) hypothesized that pigments (e.g., flavonoids) occurring in the Asteraceae pollen (either in the pollenkit or in the pollen wall) might contribute to defence against pollen feeders, there was no evidence for such pigment accumulation in the body of larvae raised on *Cirsium* pollen (no hue). Moreover, previous study has already shown that flavonoids display no or very little toxic properties for honeybee even though some deterring effects could be observed (e.g., catechin) (Detzel & Wink, 1993). This is due to a specific subfamily of cytochrome P450 monooxygenases (i.e., CYP6AS) implicated in efficient flavonoid detoxification processes in bees (Mao *et al.*, 2009; Feyereisen, 2012; Johnson, 2015). Additionally, bees may benefit from the presence of quercetin and other flavonoids in food because of their antioxidant or antimicrobial activity (Treutter, 2005). Our results demonstrated that we can also exclude pyrrolizidine alkaloids and saponins as possible candidates for *Cirsium* pollen toxicity, albeit high alkaloid concentrations in the pollen of other Asteraceae species such as *Senecio jacobaea* (Budde *et al.*, 2004).

Besides these chemical traits, an important proportion of the pollen grains of *Cirsium* were found to be still intact in the feces of adult bumblebee workers, leading to the conclusion that *Cirsium* grains could not be completely emptied by the workers. Similar difficulties in extracting nutrients have been reported for honeybee fed *Taraxacum* (Peng *et al.*, 1985) and could be behind the failure of *Chelostoma rapunculi* (oligolectic on *Campanula* genus), *C. florisomne* (oligolectic on *Ranunculus* genus), and *Hoplitis adunca* (oligolectic on *Echium* genus) to develop on Asteraceae pollen (Praz *et al.*, 2008). This incomplete digestion might result from structural properties of pollen that need specific enzymes for digestion of the intine and then might inhibit the extraction of nutrients (Roulston & Cane, 2000; Praz *et al.*, 2008). This hypothesis of digestibility constraint is supported by transmission electron microscopy since it has been revealed that Asteraceae species possess a thick multilayer pollen wall which retains sporopollenin and ensure not only mechanical but also biochemical protection of the pollen content (Meier-Melikyan *et al.*, 2003). Moreover, Williams (2003) suggested that the high amounts of pollenkit typical of Asteraceae could interfere with the nutrient assimilation process, rendering such pollen digestion difficult.

The observed patterns clearly point to the importance of chemical and mechanical defenses in shaping the relationships between bees and flowers, as highlighted in traditional plant-herbivore interactions (Ehrlich & Raven, 1964).

Pollen mixing as generalist response

Assuming that perfect chemical balance (i.e., abundance of limiting nutrients and rarity of toxins) is never met in pollen of a single plant species and that generalists can hardly fit tightly with the nutritional and allelochemical content of a several host plants, polylectic bees have to face a trade-off between toxicity and dietary deficiencies (Janz & Nylin, 2008; Singer, 2008). Recent research provided evidence that pollen mixing in the generalist solitary bee *Osmia cornuta* might be a strategy to complement nutrient deficiencies and/or mitigate against the harmful effects of secondary metabolites (Eckhardt *et al.*, 2014). Likewise, bumblebees provide their progeny with a mixture of pollen from different plant taxa (Brian, 1951; Free, 1970; Goulson, 2003; Leonhardt & Blütghen, 2012). This pollen mixing behavior might be a possible strategy to optimize larval food quality in bumblebees and is supported by findings that *Bombus* workers are able to assess pollen quality during foraging (Leonhardt & Blütghen, 2012). Pollen mixing might also reduce the bees'dependency upon hosts with favorable pollen, which would be advantageous under food shortage or phenological mismatching between the flowering period of preferred hosts and the flight period of generalist bees.

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Disclosure

The authors have no conflict of interest to declare.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Details on the gas chromatographic procedure used to determine sterolic composition of pollen.

Appendix S2. Details on the UHPLC-QTOFMS procedure used to determine alkaloid composition of pollen.

Appendix S3. Details on analytical procedures used to determine saponins composition of pollen, namely matrix-assisted laser desorption/ionization–mass spectrometry (MALDI-MS) and liquid chromatography–mass spectrometry (LC–MS) methods.

Table S1. Bumblebee performance on the 4 diets. Mean ± SD (min–max). Different letters indicate significant difference between diets (post hoc tests, $P < 0.05$); NA (i.e., non applicable) means no significant difference has been detected by the main statistical test (ANOVA or Kruskal–Wallis).