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Diet effects on bumblebee health

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ABSTRACT

Among physiological processes, the maintenance of immunity is one of the most energetically costly in invertebrates. Disease resistance can be quantified by measuring immunocompetence, which is defined as the ability of an organism to mount an immune response, either in cellular, humoral or behavioural forms. In insects, immune capacity can be affected by a variety of factors including pesticides, genetic diversity or diet. Here we focus on an important species of domesticated pollinator, *Bombus terrestris*, and the potential impact of a poor pollen diet (low nutritional content and toxic) on its health. We investigate three responses at both colony and individual levels: behavioural, humoral and cellular. Our results show that poor pollen diets decrease larval and pupal masses and increase larval ejection as well as adult constitutive immunity (i.e., prophenoloxidase assays). The susceptibility of bumblebees to disease and infection might therefore be greater after a nutritive stress. These findings raise the importance of available plant hosts, especially floral plant species providing pollen with suitable nutritive quality (i.e., nutrient pollen content) for bumblebees.

1. Introduction

The maintenance of immunity is one of the most energetically costly physiological processes in invertebrates (Schmid-Hempel, 2005), and investment in this life history strategy is directly related to disease resistance (Minchella, 1985; Godfray and Hassell, 1987). Disease resistance can be quantified by measuring immunocompetence (IC), which is defined as the ability of an organism to mount an immune response, either in cellular, humoral or behavioural form (Wilson-Rich et al., 2008). In invertebrates, immune responses may be activated through a variety of pathways (Imd-Immunodeficiency, Toll and Jak-STAT; Kingsolver et al., 2013) upon detection of pathogen- or damage-associated molecular patterns (PAMPs, DAMPs), and result in antimicrobial peptide production, phagocytosis and/or melanisation (Sheridan et al., 2014). Melanisation is a key component of the innate immune repertoire in invertebrates (Cerenius and Söderhäll, 2004), and is initiated by the activation of phenoloxidases (active PO, i.e., active form of phenoloxidase) through proteolytic cleavage of prophenoloxidases (proPO, i.e., constitutive form of phenoloxidase) found in zymogen form in insect haemolymph. This eventually results in the deposition of a melanin coat and encapsulation of non-self features (e.g., parasites, pathogens, foreign objects).

In insects, immune capacity can be affected by a variety of factors including pesticides (Di Prisco et al., 2013; Brandt et al., 2016), genetic diversity (Whitehorn et al., 2011) or diet (Alaux et al., 2010). Among these threats, decrease of diet quality has been of major concern for the conservation of many groups of animals, especially pollinators such as bees (Goulson et al., 2015). In fact, some studies have already shown that pollen quality and diversity significantly impact honeybee immunocompetence (Alaux et al., 2010; Di Pasquale et al., 2013), and that a protein-poor diet significantly reduces host-specific immune gene expression during immunological challenge in bumblebees (Brunner et al., 2014) but does not impact the level of encapsulation (i.e., immune defense; Schmid-Hempel and Schmid-Hempel, 1998). In addition, immune responses represent an energetic cost for which insects must compensate, notably by increasing food consumption. For example, Tyler et al. (2006) revealed that *Bombus terrestris* increased its pollen consumption during immunological challenge, underlying the importance of food availability for habitat quality (Schmid-Hempel and Schmid-Hempel, 1998).

All these studies clearly indicate that diet quality and quantity interface with immunocompetence and pollinator health. Clear assessment of the effects of food quality in the absence of immune challenge (i.e., constitutive immunity) is still lacking, however,

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despite its prime importance in the face of ongoing global environmental changes leading to disturbed areas with simplified ecosystems (i.e., poorly diversified) (Goulson et al., 2015). In order to fill this gap, we aim to determine how pollen quality impacts the health of *B. terrestris*. Our study is based on monitored rearing of micro-colonies of *B. terrestris* fed on pollen diets possessing different nutritive content, taking into account changes in behavioural, humoral and cellular response as well as brood health. Our hypothesis is that a high nutritive diet could enhance bumblebee immunity at both individual and colony level.

2. Material and methods

2.1. Bumblebee model

The bumblebee, *Bombus terrestris* L. (Hymenoptera, Apidae), was selected as our species model because it is a domesticated and social species that is easy to rear. This species is one of the most generalist species (i.e., wide diet breadth) in the genus *Bombus* (e.g. Kleijn and Raemakers, 2008; Somme et al., 2015) and it is potentially impacted by environmental changes leading to diet shift (Roger et al., 2016). As bumblebees do not modify the nutritive composition of their pollen diet (Pereboom, 2000), pollen diet directly impacts bumblebee health, including brood and imago. Colonies of *B. terrestris* were provided by the company Biobest *bvba* (Westerlo, Belgium).

2.2. Pollen diets

We selected three pollen diets displaying different chemical compositions, as well as different efficacy (i.e., mass of total offspring divided by mass of pollen collection) for *B. terrestris* colony development, namely, *Salix*, *Cirsium* and *Cistus* diets (Table S1). *Salix* pollen is a major spring resource for newly emerged queens (Aupinel et al., 2001; Moquet et al., 2015) and is known to positively impact bumblebee colony development (Vanderplanck et al., 2014; Moerman et al., 2015). By contrast, *Cirsium* pollen impedes colony development and leads to high pollen intake for low brood mass (Vanderplanck et al., 2016). *In natura*, this plant species is mainly exploited for nectar by bumblebees (Goulson et al., 2005), but *Cirsium* pollen can be included in low proportion (i.e. 5–10%) in the bumblebee pollen diet and particularly by *B. terrestris* (Crowther et al., 2014; Somme et al., 2015). We made the hypothesis that this proportion can increase in a poorly diversified ecosystem with dominance of *Cirsium*. Finally, *Cistus* pollen was considered for its intermediate quality (Vanderplanck et al., 2014; Moerman et al., 2015). These three diets are expected to have different effects on bumblebee immunocompetence.

Cistus and *Salix* pollens were purchased from the companies "Pollenergie France" and from "Ruchers de Lorraine", respectively. Pollen loads were sorted according to their colour after microscopic confirmation. *Cirsium* pollen was collected using honeybee hives equipped with pollen traps in areas where *Cirsium* was dominant. Pollen loads of *Cirsium* were then identified under a light microscope and isolated from non-*Cirsium* pollens based on their purple colour. As genera within the Asteraceae family are quite hard to differentiate based on pollen shape, identification verification was performed through molecular sequencing and DNA barcoding. Chemical composition of these pollen blends was analysed (Table S1).

2.3. Experimental setup

Experiments were performed at the University of Mons (Mons, Belgium) in a dark room at 26–28 °C and 65% relative humidity to simulate natural colony-rearing conditions. Bumblebees were fed *ad libitum* with inverted sugar syrup (BIOGLUC®, Biobest) and pollen candies were provided every two days to avoid degradation of pollen nutritive content. All manipulations (e.g., pollen supplies) were performed under red light to avoid disturbing colonies, as bees do not detect this range of the light spectrum. Two different protocols were used because the various monitored parameters required different experiment durations and different ways in which to sacrifice workers.

Experiment 1: Evaluation of behavioural and humoral responses – Two-day-old *B. terrestris* workers were divided into 30 micro-colonies (ten micro-colonies for each diet) of five workers and placed in different plastic boxes (10 × 16 × 16 cm). These micro-colonies were fed for 3 weeks with specific pollen candies. In each micro-colony, one of the workers became dominant laying drone eggs (i.e. haploid egg), the overall group functioning as an early stage bumble bee colony (Genissel et al., 2002). The survival of workers, as well as total syrup and pollen intakes, were controlled throughout the experiment. After three weeks, six different parameters were evaluated to test for a potentialdiet impact on colony health. These parameters were: (i) mean fresh mass of one isolated larva (i.e., total mass of isolated larvae divided by number of isolated larvae), (ii) mean fresh mass of one pupa (i.e., total mass of pupae divided by number of pupae) and (iii) larval ejection percentage (i.e., total ejected larvae divided by total number of offspring) and (iv) abdominal fat body content (i.e. fat body mass divided by the total abdomen mass) of the 23-day-old workers. Larval body size (i.e., body mass) has been proven to influence immunocompetence in *Eupoecilia ambiguella*, with smaller larvae being more vulnerable to infection than larger ones, and is obviously a factor that should be taken into account for insect immune system measurement in general (Vogelweith et al., 2013). Larval ejection is a social and immunological behaviour that ensure ejection of infected and dead larvae (Spivak and Reuter, 2001). Abdominal fat body content of workers was measured according to Ellers (1996) to test for a potential diet impact on imago. Isolated abdomens of three workers per micro-colony (n = 30 per pollen diet) were weighed after drying at 70 °C for 3 days. Dried abdomens were then placed into 2 ml of diethyl ether for 24 h to extract fat, rinsed twice and weighed again after drying at 70 °C for 7 days. Thoracic fat dry mass was determined as the difference between initial and final abdominal dry mass, standardised by abdomen mass before extraction to avoid biases linked to worker size. Finally, we calculated (v) the weighted pollen intake and (vi) the weighted syrup intake as the total pollen and syrup collection respectively divided by the mass of living individuals in each micro-colony (i.e. mass of workers, larvae and pupae). These two parameters were estimated to detect potential nutritional stress which could act as a trigger of an immune stress (Brunner et al., 2014).

Experiment 2: Evaluation of cellular response – Three queenright colonies of 60 two-day-old *B. terrestris* workers were fed a *Salix* diet for 5 days. 15 workers from each colony were then collected and snap frozen in liquid nitrogen to stop all enzymatic reactions, and then stored at −80 °C until IC evaluation analyses. The 45 remaining workers from each colony were split into 3 new queenless micro-colonies of 15 workers and placed in different plastic boxes (10 × 16 × 16 cm). Each of the 3 newly split colonies were fed with *Salix*, *Cistus* or *Cirsium* candies for 5 more days to have 3 test-cases: *Salix-Salix* (no diet change, as control), *Salix-Cistus* and *Salix-Cirsium*. All workers were then collected, fixed and stored as above for future IC evaluation experiments.

PO assays were performed following methods adapted from Whitehorn et al. (2011). Thoraces were isolated from snap frozen workers and individually homogenised in 700 µl phosphate-buffered saline (PBS; 8.74 g NaCl, 1.78 g $Na_2HPO_4{\cdot}H_2O$, 1000 ml

distilled water, pH = 6.5) with an adapted pestle in a cold room before being vortexed and centrifuged at 4000*g* (4 °C for 10 min) to recover the supernatant (i.e., diluted haemolymph). For the active PO measurements (n = 3 per worker), reaction mixtures were prepared in a 96-well plate on ice by mixing 140 µl of MQ water, 20 µl of PBS, 20 µl of supernatant and 20 µl of L-DOPA solution (4 mg ml^{-1} in MQ water). Reaction mixtures for the total PO (proPO plus the active PO) (n = 3 per worker) contained 120 µl of fresh distilled water, 20 µl of fresh PBS, 20 µl of supernatant and 20 µl of bovine α-chymotrypsin solution (2 mg ml^{-1} MQ water), and were incubated for 5 min at room temperature. 20 µl of L-DOPA solution were then added to the reaction mixture. For both measurements (active and total PO), blank and control mixes were prepared without L-DOPA and without supernatant, respectively, by adjusting the total volume with fresh distilled water. Plates were placed in a preheated (30 °C) plate reader (FLUOstar Optima, BMG Labtech) and absorbance was measured every 20 s for 50 min at 480 nm (OPTIMA control v 2.20). Enzyme activity was measured as the slope (V_{max} value) of the reaction curve during the linear phase of the reaction. All PO measures were normalised to the protein concentration of haemolymph per sample (determined by Bradford assay with a bovine serum albumin standard).

The variation of active and total PO over the last 5 days was characterised for each of the diets as $\frac{PO10d}{\overline{PO5d}}$ where *PO*10*d* is the PO measure of each bumblebee at 10 days and $\overline{PO5d}$ is the average of PO measures of the 15 initially sacrificed workers after 5 days.

2.4. Statistical analyses

Mass of larvae and pupae (i.e., used for the mean mass parameters) were standardised to the total mass of workers in the micro-colonies to avoid potential bias resulting from worker activities (e.g., differences in brood care). The different parameters were compared between diets through an one-way analysis of variance (ANOVA) followed by Tukey post hoc tests using arcsine transformation on data in percentage form (i.e., larval ejection) and rank transformation on non-parametric data (i.e., violation of normality of residuals and/or homoscedasticity). Active and total PO measurements were compared separately between diets using one-way ANOVA followed by Tukey post hoc tests on rank-transformed data. For each diet, the variation of active and total PO were compared to each other using t-tests on rank-transformed data to assess the variation of prophenoloxidase pools after diet change. Fat body content was compared between diets using one-way ANOVA followed by Tukey post hoc tests on rank-transformed data.

3. Results

3.1. Diet effects on physiological and behavioural response

A diet impact was observed on the mean mass of one isolated larva ($F_{2,27} = 15.21$, $p < 0.001$), which was significantly lower for broods fed with the *Cirsium* diet than those fed with *Salix* or *Cistus* diets (Fig. 1A, Table 1). No difference was found for the mean mass of one pupa ($F_{2,27} = 2.36$, $p = 0.114$), however, although broods fed on *Cirsium* never displayed pupae (Fig. 1A, Table 1). A significant diet effect was also detected for the larval ejection percentage ($F_{2,27} = 11.57$, $p < 0.001$), with micro-colonies fed with *Cirsium* pollen displaying a significantly higher rate of larval ejection than those fed with *Salix* and *Cistus* diets (Fig. 1B, Table 1). All the ejected larvae were found dead during the dissection of the micro-colonies (i.e., after 3 weeks of experiments). Weighted pollen intake was significantly different among diets ($F_{2,27} = 34.89$, $p < 0.001$) with *Cirsium* diet showing a higher weighted pollen intake than the two other diets ($p < 0.001$ for *Salix* and *Cistus* diets). Same trend was observed for weighted syrup intake ($F_{2,27} = 34.89$, $p < 0.001$) with higher weighted syrup intake for *Cirsium* than for the two other diets ($p < 0.001$).

3.2. Diet effects on humoral response

Despite a trend of lower fat body mass for *Cirsium*-fed *B. terrestris*, no significant impact of diet was found on the fat body content of workers ($F_{2,87} = 2.35$, $p = 0.101$) (Fig. 1C, Table 1).

3.3. Diet effects on cellular response

No significant difference in the variation of the levels of both active PO ($F_{2,123} = 0.53$, $p = 0.587$) and total PO ($F_{2,122} = 0.96$, $p = 0.377$) was observed among diets (Fig. 1D, Table 1). Furthermore, for bumblebees fed on the *Salix-Salix* diet (i.e. no diet change), the total PO variation was significantly higher than the active PO variation ($p = 0.023$). Active PO did not vary (i.e., ratio almost equal to one) although the total PO tripled indeed. A similar tendency was found for the *Salix-Cistus* diet, but it was not significant ($p = 0.134$). Active and total PO variations were nearly equal for the *Salix-Cirsium* diet ($p = 0.346$) (Fig. 1D, Table 1).

4. Discussion

4.1. Diet effects on physiological and behavioural response

Our results show that poor quality diet, such as one consisting of *Cirsium* pollen, might weaken the health state of the colony at two levels. First, in regard to diet impact on brood health, *Cirsium* pollen significantly increased larval ejection (i.e., mortality) in comparison to the other diets. Similar negative effects of Asteraceae pollen on larval development have been highlighted for unspecialised bee species like *Chelostoma* and *Hoplitis* genera (Megachilidae) (Praz et al., 2008; Sedivy et al., 2011). In bumblebees, Genissel et al. (2002) observed 100% larval ejection in micro-colonies fed with *Taraxacum* pollen. These results could be explained in two different ways: (i) larval mortality is natural and workers clean up the colony by ejecting dead larvae or (ii) workers, due to some Asteraceae pollen content, eject living larvae to compensate for the nutrient deficit (i.e. a reduced number of larvae needs less nutrient at colony level) (Plowright and Plowright, 1999). If the first hypothesis is true, mortality could be linked to a potential presence of toxic compounds in the Asteraceae pollen or to a nutrient deficit due to a lesser pollen grain digestibility by larvae or to a lesser larval feeding efficacy by workers (Praz et al., 2008). In contrast, the second explanation is in accordance with a study by Ruedenauer et al. (2015) where it was proven that bumblebees are able to discriminate foods of different concentrations using chemosensory perception (i.e., taste). However, as we did not evaluate the ejection of larvae on daily basis, we cannot confirm any of the two hypotheses.

Second, in addition to the observed increase in larval ejection, micro-colonies fed with the lowest quality pollen (i.e., *Cirsium*) tended to produce smaller larvae. Similar results were found in colonies fed with pollen blends of *Helianthus* (Tasei and Aupinel, 2008). This impact on larval size directly influences the size of newly emerged adults, which is related to offspring production, brood care, foraging efficiency and immune ability (Cnaani and Hefetz, 1994; Vogelweith et al., 2013). The lack of pupal development in *Cirsium*-fed *B. terrestris* is in accordance with previous studies on honeybees where no brood was produced when hives were fed with *Taraxacum* pollen (Loper and Berdel, 1980) and adult longevity was reduced when hives were fed with *Helianthus* pollen (Schmidt et al., 1995). The difference of *Cirsium* diet in chemical composition could partly explain this negative effect. Even if the

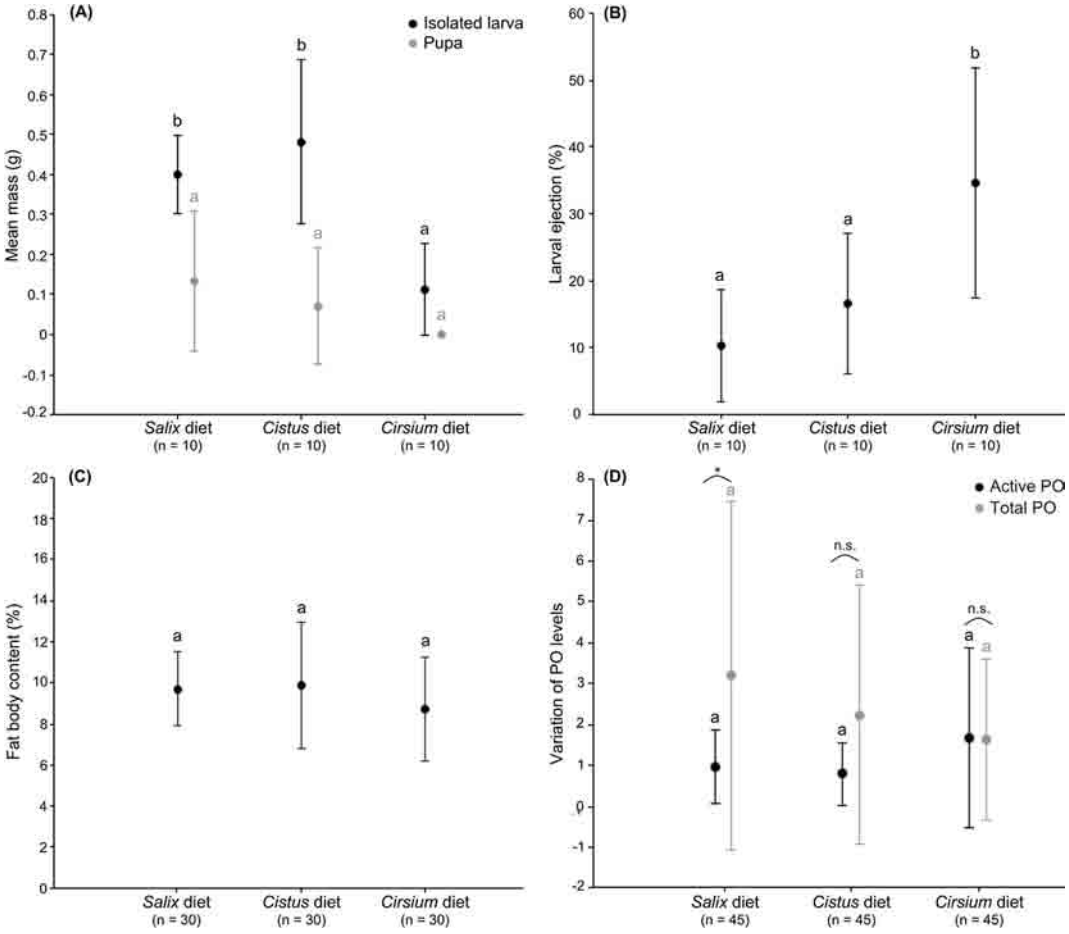

Fig. 1. The impact of different pollen diets (*Salix*, *Cistus* and *Cirsium*) on (A) mean fresh mass of isolated larvae or pupae after 3 weeks of rearing, (B) larval ejection after 3 weeks of rearing, (C) fat body content of workers after 3 weeks of rearing and (D) variations in active and total phenoloxidase (PO) activities of workers after diet change (*Salix*-*Salix*, *Salix*-*Cistus*, *Salix*-*Cirsium*). Groups differing significantly from each other in post hoc tests are marked with different letters, with shared letters indicating a non-significant difference. Within one diet case, groups differing significantly from each other in *t*-tests are marked with the following code: n.s. = non-significant difference; $* = p < 0.05$.

Table 1
Monitored parameters from the bioassays of the three pollen diets investigated in this study, expressed as mean (SD) (PO = phenoloxidase). Groups differing significantly from each other in post hoc tests are marked with different letters, with shared letters indicating a non-significant difference.

Monitored parameters	*Salix* diet		*Cistus* diet		*Cirsium* diet	
Weighted pollen intake (g/g) (n = 10)	1.24 (0.18)	a	1.22 (0.20)	a	2.39 (0.61)	b
Weighted syrup intake (g/g) (n = 10)	5.61 (2.42)	a	5.35 (1.78)	a	21.62 (6.68)	b
Workers fat body (%) (n = 30)	9.71 (1.80)	a	9.88 (3.09)	a	8.71 (2.51)	a
Larval ejection (%) (n = 10)	10.23 (8.38)	a	16.53 (10.46)	a	34.60 (17.19)	b
Mean mass of isolated larvae (g) (n = 127, 78, 27)	0.40 (0.10)	b	0.48 (0.21)	b	0.11 (0.11)	a
Mean mass of pupae (g) (n = 16, 3, 0)	0.13 (0.18)	a	0.07 (0.15)	a	0 (0)	a
Variation of active PO (n = 45)	0.97 (0.90)	a	0.79 (0.79)	a	1.67 (2.20)	a
Variation of total PO (n = 45)	3.19 (4.27)	a	3.23 (3.16)	a	1.63 (1.97)	a

total amino acid concentration is similar to which of *Salix* diet, essential amino acid concentration is lower in the *Cirsium* diet (Table S1) which can explain the negative effect on larval development (Moerman et al., 2015). To determine a potential negative effect of high δ7-sterols on bumblebee health, monitored bioassays could be performed on micro-colonies fed with a *Salix* diet supplemented with different concentrations of these phytosterols.

4.2. Diet effects on humoral response

No diet effect was detected on fat body content of workers. Fat body may potentially be maintained during stress or adaptation periods (such as adapting to a new diet) through increased foraging activity and nutritional intake, even if the new diet is not suitable for the nutritive requirements of bumblebees. It is the case in this study as the weighted pollen and syrup intakes were higher for *Cirsium* pollen despite a lower mean mass of isolated larvae. This hypothesis is in line with previous work which showed that bumblebees increased their food consumption when immunologically challenged (Tyler et al., 2006). The hypothesis for the maintenance of immune system by food compensation is also supported by the failure of gene upregulation (i.e., immune response) found in infected bumblebees because of pollen deprivation (Brunner et al., 2014).

4.3. Diet effects on cellular response

The variation in PO activity (i.e., total or active) resulting from diet change did not differ according to the pollen provided. This is in line with the findings that proteic nutritive stress did not affect the encapsulation response of *B. terrestris* (Schmid-Hempel and Schmid-Hempel, 1998). Similarly, Alaux et al. (2010) showed that pollen diet did not impact honeybee basal immunocompetence, regardless of the protein concentration. PO activity seems to be linked to the carbohydrate intake (Lee et al., 2008a; Cotter et al., 2011) which is in accord with our results since workers had access to syrup *ad libitum*.

Our bioassays provide the first evidence that active and total PO variations may differ from each other following a nutrition shift to poor diet. While the *Salix-Salix* diet group (i.e., no diet shift) had significant variation of total PO than active PO, this was not the case for the two other groups (i.e., *Salix-Cistus* and *Salix-Cirsium*), for which total PO increased proportionally with active PO. One explanation could be that no proPO is produced when workers are fed on poor pollen because all energy is used to maintain other physiological processes. Such trade-off between immunity and other physiological traits (e.g., detoxification, lifespan and reproduction) is in line with previous studies on insects (Lee et al., 2008b; Cotter et al., 2011; Ponton et al., 2013).

Overall, these findings suggest that the constitutive immunity of *B. terrestris* increases when individuals have unrestricted access to a high quality diet, which may prevent risks of infection by pathogens such as *Crithidia bombi* (Brown et al., 2003). Conversely, a diet shift to unsuitable pollen content leads to a decrease in proPO stock as it is directly used as active PO, which indicates that an unsuitable diet is a trigger for an immune response in bumblebees.

5. Conclusion

Our bioassays, coupled with estimates of immunological parameters, clearly demonstrate the energetically costly nature of the maintenance of satisfactory health state to ensure colony development in bumblebees, as previously observed for other invertebrates. The susceptibility of bumblebees to disease and infection might then be greater after the nutritive stress of shifting to a poor diet. Collectively, these findings confirm the importance of pollen nutritive content in allowing bumblebees to produce strong immune responses to parasites and diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jinsphys.2016.11.002.

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Supplementary information

Table S1. Chemical composition (i.e. total amino acid, essential amino acid and sterol content), efficacy (i.e. pollen collection mass/brood mass) and digestibility (i.e. percentage of open pollen grain in worker' faeces) of the three pollen diets investigated in this study, expressed as mean (SD) (according to Vanderplanck et al. in press). Groups differing significantly from each other in post-hoc tests are marked with different letters, with shared letters indicating a non-significant difference.

Diet quality parameters	*Salix* diet		*Cistus* diet		*Cirsium* diet	
Total amino acid content (%) (n=5)	14.42 (0.61)	a	13.61 (1.02)	a	14.55 (0.58)	a
Essential amino acid content (%) (n=5)	5.01 (0.34)	a	4.47 (0.29)	ab	4.23 (0.51)	b
Sterol content (%) (n=5)	0.44 (0.05)	a	0.26 (0.02)	b	0.14 (0.01)	c
Pollen efficacy (g/g) (n=10)	0.72 (0.13)	a	0.60 (0.09)	a	0.19 (0.16)	b
Pollen digestibility (%) (n=30)	95.17 (1.41)	a	60.48 (5.75)	b	18.78 (2.81)	c