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### ► To cite this version:

Cédric Alaux, François Ducloz, Didier Crauser, Yves Le Conte. Diet effects on honeybee immunocompetence. *Biology Letters*, 2010, 6 (4), pp.562-565. 10.1098/rsbl.2009.0986 . hal-02668801

**HAL Id: hal-02668801**

**<https://hal.inrae.fr/hal-02668801>**

Submitted on 31 May 2020

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## DIET EFFECTS ON HONEYBEE IMMUNOCOMPETENCE

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Running title: Nutrition and immunity in honeybees

## Abstract

The maintenance of the immune system can be costly, and a lack of dietary protein can increase the susceptibility of organisms to disease. However, few studies have investigated the relationship between protein nutrition and immunity in insects. Here, we tested in honeybees (*Apis mellifera*) whether dietary protein-quantity (monofloral pollen) and diet diversity (polyfloral pollen) can shape baseline immunocompetence by measuring parameters of individual immunity (haemocyte concentration, fat body content and phenoloxidase activity), and glucose oxidase activity, which enables bees to sterilize colony and brood food, as a parameter of social immunity. Protein feeding modified both individual and social immunocompetence but increases in dietary protein-quantity did not enhance immunocompetence. However, diet diversity increased immunocompetence levels. In particular, polyfloral diets induced higher glucose oxidase activity compared to monofloral diets, including protein-rich diets. These results suggest a link between protein nutrition and immunity in honeybees, and underscore the critical role of resource availability on pollinator health.

**Keywords:** Immunocompetence, diet, pollen, honeybee, social immunity

## Introduction

The maintenance of the immune system is one of the most costly physiological systems in animals (Lochmiller & Deerenberg 2000; Schmid-Hempel 2005) and deficient nutrition can impair immune function and increase the susceptibility of individuals to disease. In humans, dietary protein deficiency reduces the concentrations of most amino acids in plasma and compromises the immune system (Li *et al.* 2007). Therefore, an adequate provision of proteins is required to sustain normal immunocompetence (IC), defined as the capacity of an organism to mount an immune response (Wilson-Rich *et al.* 2008). However, beside a few studies showing that the dietary protein-quantity and quality can enhance immune

functions (Lee *et al.* 2006; Lee *et al.* 2008) this relationship has been poorly investigated in insects. Also, to our knowledge, it is unknown whether diet diversity also affects IC in insects.

To fill this gap, we tested the effect of both dietary protein-quantity and diet diversity on honeybee (*Apis mellifera*) IC by feeding bees with mono- and polyfloral pollen diets. The honeybee is a valuable model for such studies, because they usually pollinate a variety of plants but sometimes are forced to feed on single crops (monocultures). In addition, pollen, which is the main source of dietary protein and contains essential amino acids for their development (de Groot 1953), can influence longevity, the development of hypopharyngeal glands (HPG) and ovaries (Pernal & Currie, 2000), and the susceptibility to pathogens (Rinderer & Elliott 1977; Rinderer *et al.* 1974); but to what extent pollen can affect IC is not known. Finally, honeybee populations have been declining over the last years and a current idea suggests that honeybee colonies may suffer from a compromised immune system (van Engelsdorp *et al.* 2008), which could be related to poor nutrition commonly associated with colony losses (van Engelsdorp *et al.* 2008). Such a study will provide a framework to better understand how the abundance and diversity of environmental resources can shape an organism's immunity.

The effects of pollen diet on the capacity for disease resistance were empirically tested by measuring haemocyte concentration and fat body content as indirect assessments of cellular and humoral IC, respectively, and phenoloxidase (PO) activity, which is involved in both. Haemocytes are involved in the phagocytosis and encapsulation of parasites, the latter process requiring PO activity, and fat body is the main site of antimicrobial peptide synthesis. As social organisms, honeybees depend not only on individual immunity, but also on the overall functioning of the hive. So, we also analyzed glucose oxidase (GOX) activity as a parameter of social immunity. Mainly expressed in the hypopharyngeal glands (HPGs) (Ohashi *et al.* 1999), GOX catalyses the oxidation of  $\beta$ -D-glucose to gluconic acid and hydrogen peroxide, the latter having antiseptic properties. The antiseptic products are secreted into larval food and

honey, which contributes to colony-food sterilization (White *et al.* 1963), and hence, to the prevention of disease contamination at the group level.

## Material and methods

Experiments were performed in Avignon (France) with local hybrid colonies (*A. m. ligustica*/*A. m. mellifera*). For controlling the pollen intake, one-day old bees were reared in cages in the dark at 32°C and 70% RH. They were obtained from honeycombs containing late-stage pupae removed from source colonies. Bees were fed *ad libitum* with candy (30% honey from the source colonies, 70% powdered sugar), water and one of the pollen diets prepared with 1/10 water. Pollen diets were replaced every day for 10 days and bees were collected at day 5 and 10 for IC sampling. To simulate as much as possible the colony rearing conditions, cages were exposed to a Beeboost® (Pherotech), releasing one queen-equivalent of queen mandibular pheromone per day.

### Pollen diets

Seven blends of fresh pollen commonly found in France and with a respective predominance of *Acer*, *Castanea*, *Cistus*, *Erica*, *Quercus*, *Salix* and *Taraxacum* pollen were obtained from Pollenergie (France) and frozen at -20°C. To produce monofloral pollen diets, we sorted, by colour, pellets of the predominant pollen from each of the commercial blend. Nitrogen and then protein contents of the different pollens were determined by microkjeldahl analysis using a Vapodest 45 (Gerhardt) and according to the procedures described in ISO 5983 (1997). In order to test the effect of protein quantity and pollen diversity, groups of 80 bees were respectively fed with monofloral diets that differed in the quantity of protein or polyfloral diets that had the same amount of protein as monofloral diets (Table 1). Control groups had no pollen. The experiment was repeated on 5 different colonies.

### Immunocompetence

Immunocompetence was assessed indirectly in the absence of an actual pathogen challenge. To determine the haemocyte concentration, haemolymph was extracted with micro capillaries (10  $\mu$ l) from the second abdominal tergite and diluted 1:5 in ice cold PBS (ph 7.4). The number of haemocytes/ $\mu$ l of haemolymph was counted using a phase contrast microscope (x200) with haemocytometer. Fat body mass was estimated using an ether extraction method according to Wilson-Rich *et al.* (2008), then the relative mass of fat body was given as the percent change in abdominal weight after the ether wash. There are no data yet showing how the fat body size correlates with immune response, but this tissue is the main site of immunoproteins synthesis, energy and protein storage used for brood food, and vitellogenin synthesis involved in longevity (Amdam & Omholt 2002). PO and GOX activity were respectively measured on whole abdomen and head (see supplementary information). Diet and age effects on IC were determined using two-way ANOVA followed by Newman-Keuls post-hoc tests.

## Results

The amount of pollen consumed per bee and per day did not differ between the diets (one-way ANOVA:  $F_{5,204}=0.863$ ,  $p=0.51$ ,  $5.6\pm 2.1$  mg/bee/day) but we found a significant effect of pollen diet on the different immune parameters. Control bees had a higher haemocyte concentration compared to bees fed with pollen ( $F_{6,489}=5.27$ ,  $p<0.001$ ; figure 1a). Regarding PO activity, a slight pollen-diet effect was observed ( $F_{6,428}=2.54$ ,  $p=0.02$ ), which was due to a higher PO activity in bees fed with the protein-richest polyfloral blend compared to control bees (figure 1b). The fat body contents increased with pollen diets ( $F_{6,489}=6.74$ ,  $p<0.001$ ). In addition, the first polyfloral blend induced a higher fat body content compared to its monofloral counterpart (same protein content) and the protein-lowest monofloral diets (figure 1c). Pollen consumption also greatly increased GOX activity, and furthermore, the type of diet had a pronounced effect on its activity ( $F_{6,446}=19.9$ ,  $p<0.001$ ). Both, polyfloral diets induced a higher GOX activity compared to the same protein-level monofloral diets and the protein-richest diet (figure 1d). Finally, for each immune parameter, no difference was observed between the different monofloral diets (figure 1a-d).

A significant age effect was also observed on the different immune parameters. Haemocyte concentration and fat body contents decreased with age ( $F_{1,489}=19.1$ ,  $p<0.001$  and  $F_{1,489}=25.2$ ,  $p<0.001$ , respectively) contrary to PO and GOX activity, which increased with age ( $F_{1,428}=24.9$ ,  $p<0.001$  and  $F_{1,446}=123.6$ ,  $p<0.001$ , respectively). In addition, the effect of diet on IC was consistent between 5 and 10 day-old bees, as no significant interaction between the diet and age factors was found ( $p>0.05$  for each immune parameter).

## Discussion

In this study, we provided experimental evidences for a link between pollen nutrition and baseline IC in honeybees. These results suggest that the abundance and diversity of environmental resources can have a direct impact on pollinator's health.

Haemocyte concentration was augmented in bees fed a diet with no protein. Higher haemocyte concentrations are expected to be associated with protein supply and higher resistance to disease. However, bees fed with protein might invest more resources in certain types of haemocytes (e.g. granulocyte or plasmatocyte) at the expense of others types. An investment in type of haemocytes, that are more costly to produce, would ultimately lead to an overall decrease of haemocyte numbers. Or, since the metabolic activity of haemocytes increases with a pollen diet (Szymaś & Jędruszek 2003), haemocyte concentration might increase in bees fed without protein to compensate for the lower metabolic activity. This age decrease in concentration was also found by Schmid *et al.* (2008). However, Wilson-Rich *et al.* (2008) observed a lower haemocyte concentration in one-day-old bees compared to foragers, perhaps because collected bees were very young and in the process of reaching the maximum haemocyte concentration. Pollen feeding did not clearly affect PO activity. Similar results were found in bumblebees, where limited nutrition did not affect the encapsulation response mediated by PO (Schmid-Hempel &

Schmid-Hempel 1998). Perhaps, the costs induced by pathogens would reveal a critical role of protein diet on PO activity. On the contrary, GOX activity, which does not provide an immune protection to the bee itself but allows prevention of infectious disease in the colony, was greatly affected by the protein diets, suggesting that bees would invest more resources in social rather than individual immunity. Indeed, honeybees possess only one-third the numbers of immune response genes known for solitary insects (Evans et al. 2006), which indicates that others types of defense against pathogens might be important (e.g. GOX).

Regarding the diet quality, the different monofloral diets did not induce changes in the IC levels. This could be explained by a nutritive compensation of bees fed with protein-poor pollen; however, the amount of pollen consumed per bee did not differ between the diets. Interestingly, a comparable range of protein content induces differences in ovary and HPG development (Pernal & Currie 2000), suggesting that IC in bees is not sensitive to the amount of protein. However, we cannot exclude the possibility that other pollen with poorer or richer-protein content or challenges with pathogens would induce differences in IC.

Interestingly, polyfloral diets enhanced some immune functions compared to monofloral diets, in particular GOX activity, meaning that the diversity in floral resources confers bees with better in-hive antiseptic protection. This demonstrates that diet diversity is important, and that a minimal nutrient diversity may not meet all honeybee needs. Because nitrogen content was equal between the mono- and polyfloral diets, additional properties might be present in the pollen mix. For example, essential amino-acids from protein digested are required in specific proportions to complete their normal growth and development (de Groot 1953). Low pollen diversity might represent a major limiting factor for honeybee's development, but a polyfloral diet might increase the diversity and/or the proportion of specific proteins and amino acids required for tissue development (fat body and HPGs). This assumption



is supported by the study of Tasei & Aupinel (2008) showing that bumble bee larvae fed with a polyfloral blend were heavier than larvae fed with monofloral diets of higher protein content.

If nutrition is a critical factor in immune response, then malnutrition is likely one of causes of immunodeficiency in honeybee colonies. This work also emphasizes the importance of diet diversity and underscores the need for further studies to test different blends of proteins and identify protein “cocktails” essential for developing normal immune function.

## Acknowledgements

We thank Pollenergie for pollen supply, J.L. Brunet, D. Feuillet, F. Mondet, M.R. Schmid and B. Vaissière for helps and advices and A. Brockmann, C.M. McDonnell and S.F. Pernal for comments that improved the manuscript. Funding was provided by HFSP (RGP0042/2007). C. Alaux was supported by an INRA young researcher position (INRA SPE department).

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**Table 1:** Mono- and polyfloral diets of pollen. The percentage of the different pollens used for each polyfloral blend is given. The protein content of *Acer*, *Erica* and *Salix* pollen was respectively 25.9, 17.1 and 25.8%.

Diet	Pollen type	% protein	Blend composition
Monofloral	<i>Cistus</i>	15.5	/
	<i>Taraxacum</i>	19.8	/
	<i>Castanea</i>	23.6	/
	<i>Quercus</i>	29.6	/
Polyfloral	1 <sup>st</sup> pollen blend	19.8	32% <i>Erica</i> , 28% <i>Cistus</i> , 16% <i>Castanea</i> , 12% <i>Salix</i> , 12% <i>Acer</i>
	2 <sup>nd</sup> pollen blend	23.6	24% <i>Quercus</i> , 20% <i>Salix</i> , 20% <i>Taraxacum</i> , 18% <i>Acer</i> , 18% <i>Cistus</i>

## Figure legend

**Figure 1:** Effect of pollen diet on IC in 5 (open bars) and 10 days-old bees (filled bars). (a) Haemocyte concentration, (b) Phenoloxidase activity, (c) Fat body mass and (d) Glucose oxidase activity. 8 bees per cage for each experimental group were analyzed for each immune parameter. The protein percentage of each pollen diet is indicated on the x-axis. Each letter indicates significant differences between diets ( $p < 0.05$ , Newman-Keuls post-hoc tests). No significant interaction between the diet and age factors was found ( $p > 0.05$  for each immune parameter). Data show mean  $\pm$  se.

