

Conversion of High and Low Pollen Protein Diets Into Protein in Worker Honey Bees (Hymenoptera: Apidae)

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ABSTRACT Adequate protein levels are necessary to maintain strong honey bee [*Apis mellifera* (L.)] colonies. The aim of this study was to quantify how pollens with different crude protein contents influence protein stores within individual honey bees. Caged bees were fed one of three diets, consisting of high-protein-content pollen, low-protein-content pollen, or protein-free diet as control; measurements were made based on protein content in hemolymph and fat body, fat body weight, and body weight. Vitellogenin in hemolymph was also measured. Bees fed with high crude protein diet had significantly higher levels of protein in hemolymph and fat bodies. Caged bees did not increase pollen consumption to compensate for the lower protein in the diet, and ingesting ≈ 4 mg of protein per bee could achieve levels of $20 \mu\text{g}/\mu\text{l}$ protein in hemolymph. Worker bees fed with low crude protein diet took more time in reaching similar protein content of the bees that were fed with high crude protein diet. The data showed that fat bodies and body weight were not efficient methods of measuring the protein status of bees. The determination of total protein or vitellogenin concentration in the hemolymph from 13-d-old bees and protein concentration of fat bodies from 9-d-old bees could be good indicators of nutritional status of honey bees.

KEY WORDS honey bees, pollen diets, protein, nutritional status, fat body

Pollen is the primary source of protein, lipids, vitamins, and minerals for bee nutrition. In cold temperate climates, honey bee (*Apis mellifera* L.) colonies must survive the winter and produce brood in the spring for the replacement of winter bees. To survive, the colony should be composed of long-living “winter bees” that emerge in the autumn (Matilla and Otis 2006). In a colony preparing for winter, workers eat pollen over a long period (Kleinschmidt 1983, Kunert and Crailsheim 1988), and the proteins play a major role in determining the length of life of honey bees (Amdam and Omholt 2002). Workers bees with low nutritional reserves or limited protein intake have a shorter life span as adults. Protein deficiency also affects the ability of honey bees to resist diseases (Matilla and Otis 2006), and a link between protein nutrition and immunity has been reported (Alaux et al. 2011); consequently, nutrition is suspected to be an important factor involved in Colony Collapse Disorder (Cox Foster et al. 2007).

The protein status in colonies and in body stores of individual workers is critical to ensure survival in win-

ter (Matilla and Otis 2006). The weight of workers, the quantity of their body reserves, the protein content in fat bodies and hemolymph, and the weight of hypopharyngeal glands have been considered as indicators of nutritional status (Fluri et al. 1982, Pernal and Currie 2000, Amdam and Omholt 2002).

After emergence, winter bees should store large amounts of proteins that have been synthesized in the fat body. The fat body consists mainly of thin layers of cells spread against the body wall of the abdomen, where the cells are organized in thin lobes (Snodgrass 1956). There is a constant exchange between the fat body and the hemolymph with alternate phases of release and absorption of proteins (Cruz-Landim 1983). Vitellogenin (a very high-density lipoprotein, VHDL) is the protein predominant in the hemolymph of insects (Fluri et al. 1977, 1982; Palli and Locke 1988; Amdam et al. 2004a), and a high correlation between total protein content in the hemolymph and vitellogenin has been reported (Amdam et al. 2005). A notable feature of long-lived worker bees is that they have high VHDL concentration (Fluri et al. 1977, 1982; Amdam et al. 2004a,b).

Fat body size increases in autumn bees and the storage proteins are very important for the survival of bees in cold temperate climates. Pollen feeding leads to fat body development (Maurizio 1954), and it influences the physiology of newly emerged bees.

Bitondi and Simoes (1996) investigated how the quantity of pollen ingested by honey bee workers in

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small groups in laboratory influences the level of protein in their hemolymph. Earlier studies on caged bees generally used a single pollen source or artificial diets and determined protein titers in hemolymph (Cremónez et al. 1998, Cappelari et al. 2009, De Jong et al. 2009). However, none of these studies has considered this phenomenon in bees emerged in autumn consuming pollen diets with different protein content. Protein concentrations of pollen collected by bees vary widely among plant species, ranging between 2.5 and 60% of dry mass (Stanley and Linskens 1974, Roulston and Cane 2000). To determine the value for honey bees, it is necessary to measure the effects of different pollen or protein diets on physiological parameters (Brodschneider and Crailsheim 2010). In addition, there are few studies with empirical data that relate fat body development and level of protein in the hemolymph with the quality in terms of crude protein of the pollen that the bees consume.

In this work, we measured the postfeeding changes in the levels of protein in the hemolymph and fat bodies, in honey bees fed with pollen of high and low content of crude protein. Vitellogenin in hemolymph was also measured, assuming that it is the principal protein (Amdam et al. 2004a). We also measured the weight of fat bodies and of adult bees to know which of all the parameters could be a proper indicator of colony nutrition status.

Material and Methods

The study was conducted at the University National of Centre of Buenos Aires Province in Tandil, Province of Buenos Aires, Argentina. All bees used were from European honey bee (*A. mellifera*) colonies headed by commercially produced queens. We used bees that were reared in autumn just before brood rearing ceased; these workers are presumptive winter bees (Matilla et al. 2001).

Pollen Collection. High-protein-content pollen (HCP) and low-protein-content pollen (LCP) were collected with pollen traps from honey bee colonies foraging on natural grassland before the start of the study and kept frozen for 3 mo. Previous studies examining the degradation of stored pollen over time indicated that the nutritional value did not decline in the first year of storage (De Groot 1953, Pernal and Currie 2000).

Estimates of CP concentration in pollen were derived from estimates of nitrogen concentration, measured by the Kjeldahl method, multiplied by 6.25 (Kirk 1980). The CP level was used because it is a good parameter for measuring pollen quality and is correlated with the physiological conditions of worker bees (Standifer et al. 1960, McCaughey et al. 1980, Pernal and Currie 2000). The CP concentration was 29% in HCP and 10% in LCP.

In HCP, pollen of *Echium plantaginum* was dominant. An adequate balance of amino acids for bee requirements has been recorded for *E. plantaginum* pollen (Somerville 2001, Somerville and Nicol 2006). LCP had a blend of pollen, and no type of pollen was

dominant. The LCP blend contained *Trifolium repens*, *Trifolium pratense*, *Vicia faba*, *Heliantus annuus*, *Eryngium* sp., *Baccharis* sp., *Pasiflora* sp., *Boraginaceae* spp., and *Umbelliferae* spp. Deficiency of amino acids has not been reported for *T. repens*, *T. pratense*, *H. annuus*, and *V. faba* in the literature (Somerville and Nicol 2006).

Bioassay Cages. One frame with capped worker brood was removed from each of eight colonies in autumn, and placed in an incubator at 32–34°C and 60% relative humidity (RH). Workers emerging were mixed, and equal proportions were placed in wooden cages (8 by 25 by 46 cm) with metal screened sides. Eight cages, each with 90 g of bees, were maintained in the incubator for 30 d at 30°C and 60–65% RH and fed ad libitum with water and 50% sucrose syrup solution. Three cages per each protein diet were used. Pollen was ground with a mortar to obtain a fine powder and small drops of water were added to obtain an adhesive consistency and to avoid spreading. Approximately 30 g of each pollen diet were placed in the cages, in a shallow plastic container, and the diets were replaced every 1 or 2 d.

The diets tested were as follows: 1) HCP + syrup solution, 2) LCP + syrup solution, and 3) a protein-free diet as control (50% syrup solution in water).

The quantity of diet consumed by caged bees was determined by the difference between the quantity of diet supplied and the amount left by the bees. The diet was given periodically at 1- or 2-d intervals, and at these times, the dead bees were removed and counted. The dead bees and the total number of bees in each cage were considered in the estimation of consumption. The results were expressed in mg of pollen per bee per day. Estimates of the protein consumed by bees were calculated multiplying the consumption values by the appropriate CP content.

The weight of 10 bees per cage was measured when bees were 2, 6, 9, 13, 16, 21, and 25 d old.

Hemolymph Extraction. Two pools of hemolymph from five newly emerged bees each (0–12 h old, called 0 d old) and from each cage were collected from beneath the third tergite before confinement to the incubator (Cremónez et al. 1998). After that the hemolymph of 2-, 6-, 9-, 13-, 16-, 21-, and 25-d-old workers was collected using a Drummond microcapillary previously washed with 0.1% phenylthiourea solution. Two pools of hemolymph from five bees each were collected for each age and cage and stored at –20°C for later determination of total proteins. Vitellogenin levels were estimated from hemolymph pools.

Fat Body Extraction. Fat bodies from workers 6, 9, 16, 21, 25, and 30 d old were extracted under a dissecting stereomicroscope (Leica S6D). Fat bodies from 0- and 2-d-old bees were not developed and could not be extracted. In total, five bees for each age and cage were dissected according to the method of Paes de Oliveira and Cruz-Landim (2004). Each fat body pool was weighed and stored at –20°C.

Total Protein Determination. Levels of protein in hemolymph and fat bodies were determined spectrophotometrically at 595 nm by the Bradford method

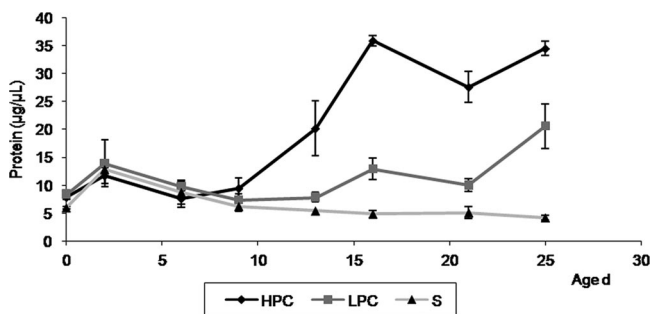


Fig. 1. Mean \pm SD protein titer ($\mu\text{g}/\mu\text{L}$) in hemolymph measured in *A. mellifera* workers from 0 to 25 d old, fed with a diet of high protein content (HCP: 29% crude protein), low protein (LCP: 10% crude protein), and control (C: 50% sucrose syrup solution in water). Each mean was estimated from protein measurements of two pools of five bees each from each of three repetitions in protein diets. Bees from 13 to 25 d old fed with HCP had significantly higher titers of protein than LCP and controls ($P < 0.001$). Protein levels in bees fed with LCP were significantly higher than the control only at 25 d old ($P < 0.001$).

(1976), using bovine serum albumin for the standard curve determination. The protein reagent consisted of 0.01% Coomassie Brilliant Blue G-250, 4.7% (wt:vol) ethanol, and 8.5% (wt:vol) phosphoric acid dissolved in water. One milliliter of protein reagent was used with 3 μL of hemolymph (diluted 1/10).

For levels of protein in fat bodies, the tissue was homogenized in 15 μL of buffer (Tris-HCl 20 mM, pH 7.5; ClNa 150 mM; phenylthiourea 1%) with protease inhibitor (Sigma-Aldrich). One milligram of tissue was mixed with 15 μL of buffer and centrifuged at 3°C, for 5 min at 10,000 rpm. Aliquots of the supernatant extracts were used for total protein quantification by the Bradford (1976) method.

Vitellogenin Quantification. Electrophoresis separation of the proteins in hemolymph was performed in 7% of polyacrylamide gels run under denaturing conditions by Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

After electrophoresis, the gels were stained with Coomassie Brilliant Blue G-250. Molecular weight markers (Fermentas, Rockford, IL) were used to determine the relative molecular mass of protein in the hemolymph. A primary antibody antivitellogenin obtained from rabbits diluted 1:2500 in phosphate-buffered saline was used for Western blot, to ensure that positive marks in the 180 kDa band corresponded to vitellogenin. The reaction was revealed with 3,3'-diaminobenzidine. Quantification of bands was made with TotalLab Quant software. The quantification was made in workers from 6 to 25 d old.

Statistical Analysis. An analysis of variance (ANOVA) with mixed modeling using repeated measures (SAS Institute 2010) was used to compare treatments. The protein concentration, weight of fat bodies, and bees was analyzed using a model with age of bees and treatments as factors. The interaction of time \times treatment was also tested; when the interaction was significant, differences of least squares means were estimated. Owing to the nested structure of the data, cage was used as a random effect, allowing for correlation between observations from the same cage.

Consumption of pollen between treatments was compared using *t*-tests.

Results

Protein in Hemolymph and Fat Bodies. A significant interaction between treatments and age was detected for the titer of protein in hemolymph ($F = 4.95$; $df = 14, 35$; $P < 0.001$). When effects between treatments for each day were analyzed separately, differences in titers of protein between treatments were not significant ($P > 0.05$) during the first 9 d (Fig. 1). Older bees (13–25 d old) fed with HCP had higher levels of protein in hemolymph than LCP and the control bees ($P < 0.001$). Mean protein levels in bees fed LCP were significantly higher than the control ($t = 7.41$; $df = 35$; $P < 0.001$) only at 25 d old (Fig. 1).

No significant interaction was detected between treatments and bee age when the protein titer in fat bodies was analyzed ($F = 1.01$; $df = 10, 24$; $P = 0.463$). The treatment effect was significant ($F = 16.22$; $df = 2, 5$; $P = 0.006$). When pairwise comparisons were applied, all treatments were significantly different ($P < 0.05$). The diet with HCP showed the highest levels of protein in fat bodies compared with LCP and control treatments (Fig. 2). The effect of age of honey bees was significant ($F = 3.28$; $df = 5, 24$, $P = 0.021$) owing to increase of the protein concentration in fat bodies from 9 d old to 30 d old for all diets.

Considering all ages together, the average levels of vitellogenin were $32.3 \pm 3.99\%$ in HCP, $17 \pm 1.23\%$ in LCP, and $16.9 \pm 0.52\%$ in the control (Fig. 3a). The vitellogenin band was found in all gels (Fig. 3b) and confirmed by Western blot (Fig. 3c). Another band at 70 kDa was found in all gels from the 2- to 25-d-old bees (Fig. 3b).

Weight of Fat Body and Bee Body. No significant interaction was detected between treatment and bee age when the weight of fat bodies was analyzed ($F = 1.59$; $df = 13, 32$; $P = 0.139$). For all treatments, the weight of fat bodies was similar ($F = 4.61$; $df = 2, 5$; $P = 0.073$, 0.44 ± 0.02 mg). The effect of age of bees (all

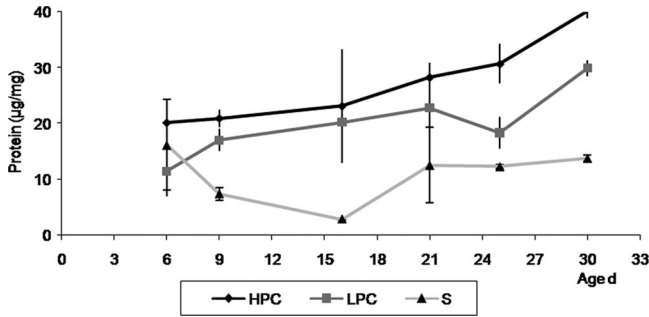


Fig. 2. Mean \pm SD protein titer ($\mu\text{g}/\text{mg}$) in fat bodies measured in *A. mellifera* workers from 6 to 30 d old, fed with diet with high protein content (HCP: 29% crude protein), low protein (LCP: 10% crude protein), and control (C: 50% sucrose syrup solution in water). Each mean was estimated from protein measurements of five bees from each of three repetitions in protein diets. Protein concentrations were greater in bees fed with HCP compared with LPC and control ($P = 0.04$ and $P = 0.002$, respectively). The titers of protein increased significantly from 9- to 30-d-old bees for all diets ($P = 0.002$).

treatments together) was significant ($F = 2.75$; $df = 7, 32$; $P = 0.023$). When the weight was analyzed for each age, a significant increase was detected between 6-d-old bees (0.27 ± 0.02 mg) to 9-d-old bees (0.38 ± 0.04 mg; $t = -2.59$; $df = 32$; $P = 0.014$) and the differences remained up to 30 d old ($P < 0.05$; 0.44 ± 0.05 mg).

The body weight of bees was significantly different between treatments ($F = 11.67$; $df = 2, 5$; $P = 0.013$). The weight of bees fed the HCP diet (107.8 ± 2.75 mg) was not different from bees fed the LCP diet (99.4 ± 2.28 ; $P >$

0.05). Both pollen diets produced heavier bees compared with the control (88.7 ± 3.42 ; $P < 0.05$).

Diet Consumption. No significant differences in total consumption of pollen between diets were detected ($t = 2.08$; $df = 20$; $P = 0.967$). The highest consumption for both protein diets was observed from the sixth to ninth day (14.33 mg per bee for LCP and 12.65 mg per bee for HCP diet); these values represented a 45 and 40%, respectively, of the total consumed pollen throughout the whole test. At 22 d old,

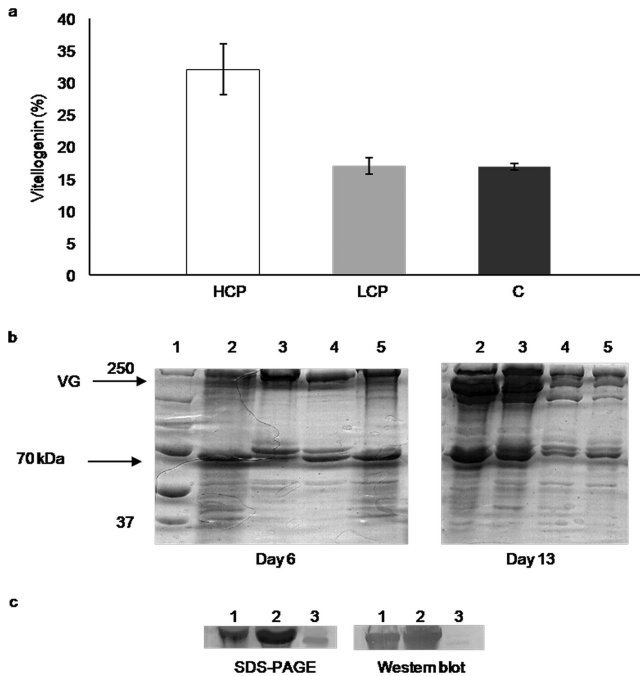


Fig. 3. (a) Mean vitellogenin titers (expressed as percentage of protein total) in the hemolymph of bees fed with high (HCP), low (LCP) protein content diet, and sucrose syrup as control. The mean value was obtained from two pools of five bees each, on worker bees of 6, 9, 13, 16, 21, and 25 d old. (b) SDS-PAGE from 6- and 13-d-old workers. Lane 1: molecular weight markers (weight is indicated on the left in kDa); Lanes 2–3: proteins from hemolymph samples of treatment HCP, lanes 4–5: proteins from hemolymph samples of treatment LCP. VG: vitellogenin. (c) SDS-PAGE and Western blot of hemolymph from workers. Lane 1: VG from hemolymph samples of treatment LCP. Lane 2: VG from hemolymph samples of treatment HCP. Lane 3: molecular weight markers (180 kDa band).

the pollen consumption was negligible. Total consumption of protein in 21 d was 3.2 mg per bee in LCP and 9.5 mg per bee in the HCP treatment. In this HCP treatment, 3.6 mg of protein per bee was consumed in the first 9 d.

Discussion

This is one of only few studies that consider the relation between CP ingested and build up levels of hemolymph proteins. The data show that the determination of total protein or vitellogenin concentration in the hemolymph from 13-d-old bees and the protein concentration in fat bodies from 9-d-old bees could be good indicators of nutritional status of bees. Conversely, fat bodies and body weight are not efficient methods of measuring the protein status of bees fed with HCP and LCP.

Our results show that bees that are not rearing brood can achieve levels of $\approx 20 \mu\text{g}/\mu\text{l}$ protein in hemolymph when they are ingesting ≈ 4 mg of protein. The vitellogenin represented a large fraction of the total protein in the hemolymph, and the highest titers were observed in bees fed HCP diet. We observed a dense band of 70 KDa corresponding to hexamerin (HEX 70 a) in the hemolymph of 6- to 25-d-old bees fed with all diets (Fig. 3b). Although we did not quantify it, in HCP diet the bands were very dense, suggesting that the accumulation of hexamerins of 70 KDa were associated with the diet. This is in agreement with Martins et al. (2008), who reported different transcription levels of HEX 70 associated with the diet in young workers. Although the function of this protein (HEX 70 a) is not known in the hemolymph of adult bees, it is possible that it represents an additional source of amino acids. Our data of protein and vitellogenin concentrations are also consistent with results from other experiments (e.g., Fluri et al. 1977, 1982; Bitondi and Simoes 1996; Cremónez et al. 1998; Amdam et al. 2005), although none of these other studies specifically compared diets that differed in crude protein content.

It is known that consumption of good quality pollen leads to greater fat body development (Maurizio 1954). However, in this study, feeding with an HCP diet did not cause a significant increase of fat body weight compared with the bees fed with the other diets. In fact, differential development was not observed when the extraction of fat bodies was performed. It is possible that the trophocytes increase in size, but not in number (Paes de Oliveira and Cruz Landim 2003) and thus limit weight increase. Our data show that the development of fat bodies in 30-d-old bees that are not feeding larvae (caged bees in this study) is apparently more associated with bee age than with diet. In fact, the weight of fat bodies in the control was similar to protein diets, but only a mean of $5.36 \mu\text{g}$ of protein in whole fat body was registered, indicating that they could store other substances. According to Paes de Oliveira and Cruz-Landim (2003), the fat body also stores carbohydrates and lipids. It is clear

that bees given a nonprotein diet have a physiological mechanism that allows build up fat bodies.

Notably, in both protein diets, the consumption was reduced at the same time and total pollen consumption per bee was similar in both diets; this suggests that caged bees do not increase pollen consumption to compensate for the lower protein in the diet. This is in agreement with Pernal and Currie (2000) and Alaux et al. (2011) who have worked with caged bees. Conversely, Kleinschmidt and Kondos (1978) reported an increase in pollen consumption when the level of pollen protein in colonies decreased by 10%. In fact, in colonies with brood, there may be a stimulus to increase the consumption to satisfy protein requirements when the protein level of pollen is low. However, in the absence of brood, as in our trial, bees have different physiological conditions and do not compensate for a protein deficit by increasing consumption.

The bees in our study might resemble winter bees in colonies, when little or no open brood is present. To determine the nutritional status in late autumn colonies, we should sample the bees 13 d after emergence of the last cycle of brood; at this time, there is a greater chance of finding workers that are not nursing and not foraging. This information would be an important management tool because knowledge of nutritional condition in the autumn is important to maintain vigorous colonies for overwintering.

Trophallactic contacts in the conditions of this study might resemble the interactions that occur in one frame cover of bees in a colony at the end of the autumn owing to the quantity of bees that we used in each cage. Even so, our study needs to be repeated in colonies to determine how the interactions at colony level can affect the workers' metabolism.

There are no prior published data that describe changes in proteins levels in the fat body and hemolymph after consumption of low and high levels of protein. Our study shows that bees fed with HCP increased protein titers in the hemolymph and fat bodies by a factor of 1.67 over protein titers in bees fed with LCP. Additional research is needed to establish the relation between role of vitellogenin and hexamerin as reserve proteins in broodless bees, especially in normal colonies entering winter.

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