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Original article

Pollen quality of fresh and 1-year-old single pollen diets for worker honey bees (*Apis mellifera* L.)

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Abstract – Newly-emerged honey bees were placed in cages and fed sucrose syrup and one of the following single-pollen diets: *Malus domestica* Borkh., *Brassica campestris* L., *Phacelia tanacetifolia* L., *Melilotus officinalis* (L.) Pall., *Helianthus annuus* L., *Pinus banksiana* (Lamb.), artificial supplement (Bee-Pro®) or nothing. Hypopharyngeal gland protein was determined at intervals of 0, 3, 8 and 14 days and ovary development was visually scored on day 14. The development of hypopharyngeal glands and ovaries varied with diet and, collectively, proved to be sensitive measures of protein utilization and pollen quality. For workers fed 1-year-old *Phacelia* pollen, protein was utilized in a differential fashion, promoting the development of ovaries over that of hypopharyngeal glands. Development of glands and ovaries was strongly correlated with the amount of protein workers consumed from pollen diets, and to a lesser extent, the crude protein content of diets. Storing pollen for 1 year by freezing did not affect gland or ovary development.

***Apis mellifera* / hypopharyngeal gland / ovary / nutrition / protein / pollen**

1. INTRODUCTION

Pollen is the only source of protein in the diet of the honey bee, *Apis mellifera* L., and it also contains all of the lipids, vitamins and minerals necessary for normal growth and development of the colony. Numerous studies have evaluated the quality of bee-collected pollen by direct measurement of nutritional factors [1, 14, 21, 24, 30, 32, 43, 50, 53, 54, 56, 59, 60, 65, 66, 69, 76, 78, 82, 85, 86, 90, 93, 94, 97, 101, 103], or by such parameters as: brood production; the growth, longevity, survival or protein

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content of workers; or the development of hypopharyngeal glands and ovaries [6, 15, 22, 23, 25, 28, 29, 31, 42, 45–47, 49, 52, 57, 59, 62, 77–79, 81, 87–89, 95, 99]. The direct measurement of nutritional factors, alone, may be misleading, as the importance of the non-protein components of pollen is not well understood [58, 91, 97].

Evaluating pollen quality by measuring colony growth and development would provide the most pertinent information about its potential impact on honey bee fitness. However, the collection of large quantities of pollen for use in studies on full-size colonies is not feasible at present. Measuring factors that are related to the workers' ability to utilize pollen, such as hypopharyngeal gland and ovary development, could address any inherent differences in the efficiency of pollen digestion and its relative assimilation into tissues of young workers or brood. Because young worker bees are responsible for feeding all castes and age cohorts within a colony [7–9, 11], measurements of hypopharyngeal gland development could also provide information about the amount of protein potentially disseminated to the rest of the colony, relative to a particular pollen source. Furthermore, by feeding similar groups of caged workers single pollen diets, relative consumption can be measured and the palatability of pollen diets may be evaluated.

Hypopharyngeal gland development is influenced by the quantity and quality of protein ingested by workers [25, 28, 35, 47, 57, 59, 87–89]. The size of the hypopharyngeal glands, as measured by acinal diameter, is related to their total protein content [5]. In nurse bees, the protein content of the hypopharyngeal glands can also be used as an indication of gland activity [37, 68]. Examination of the hypopharyngeal glands from caged newly-emerged workers is a reliable measure of dietary protein assimilation, because these glands will develop in the absence of brood and their total protein content is unaffected by brood quantity [10, 37]. Therefore, the protein content of the hypopharyngeal glands is an effective physiological parameter for evaluating the quality of pollen consumed by newly-emerged workers.

A second physiological parameter used to evaluate the quality of a pollen diet is the extent of ovary development in newly-emerged workers. Pollen that is protein-rich usually promotes ovary and egg development in workers that are caged without queens [42, 51, 57, 62, 63], and a lack of pollen protein can retard or prevent ovary development [26]. Pollen protein promotes growth of the fat body [57], and haemolymph vitellogenin titre has been linked to the level of pollen in the diet of workers [3, 12], thereby establishing the importance of pollen protein for egg development. Hence, ovarian development is another direct measure of pollen protein utilization by workers, and could indicate the potential for development of ovaries and production of eggs by queens.

In honey bee workers, the question of whether different physiological systems have different nutritional requirements has not been adequately studied. Maurizio [57] showed similar trends in the hypopharyngeal gland and ovary development for bees fed different pollen diets. Haydak [28], using similarly designed tests, showed that the development of hypopharyngeal glands was more sensitive to reductions in pollen quality than that of mandibular glands. If unequal partitioning of protein between developing hypopharyngeal glands and ovaries exists, it would affect our judgement of how these indicators should be used in making assessments of pollen quality.

Ambiguity also remains over the value of stored pollen as a protein source for honey bees. Most studies examining the nutritional value of pollen after periods of storage are confounded by the use of diets with mixed, and often unidentified, pollen species. In addition, poor descriptions of the techniques used to store pollen, and incomplete

information about the conditions maintained during storage are common. Thus, it is difficult to assess the effects of specific storage parameters on pollen quality, and whether such effects are consistent among species.

The age of pollen used to feed honey bees can influence worker growth and development, or the production of brood by a colony. Workers fed dried pollen that is 1-year-old or older have smaller hypopharyngeal glands and lower rates of weight gain than workers fed fresh pollen [28, 57]. Colonies fed diets composed of 2-year-old dried pollen rear less brood than colonies fed freshly-dried pollen [29]. The amount and age of pollen in formulated diets also can affect its utilization by workers. Hagedorn and Moeller [25] determined that newly-emerged workers, fed pollen supplement containing small quantities of dried or frozen 1-year-old pollen, have similar hypopharyngeal gland development to those fed supplement mixed with fresh pollen. When fed supplements containing pollen stored for more than 1 year, however, workers have smaller glands. The rate of thoracic weight gain in workers fed supplement containing dried pollen also decreases when the pollen component has been stored for extended periods.

In contrast, other studies show that the age of pollen fed to bees does not affect at least some of the physiological measures indicative of pollen quality. Ovarian development of queenless workers fed dried 1-year-old pollen [62], and the lifespan of caged bees fed dried 2-year-old pollen [23], is similar to that found in bees fed fresh pollen. Dietz and Stevenson [15] showed that drying and freezing pollen can extend its nutritional value for bees to some extent. Brood production in colonies fed freshly-collected dried pollen does not differ from that in colonies fed dried frozen pollen. Dried frozen pollen can support limited brood rearing even after 11 years; without freezing, similarly-aged dried pollen is completely ineffective. The combination of drying and freezing is important, as pollen stored by freezing is less adequate for long-term brood production than pollen that is first dried and then frozen [15]. However, drying pollen may not adequately prevent the degradation of lipids that are nutritionally important for honey bees, or prevent changes in those lipids influencing the palatability of pollen [73]. Freezing pollen, in combination with storage in an oxygen-reduced environment, might prevent degradation of pollen protein while simultaneously preventing the oxidation of other non-protein constituents.

A nutritional comparison between fresh and 1-year-old stored pollen, using several identified pollen species would provide a precise and meaningful comparison of the quality of pollen following storage and its utilization by different physiological systems. This would provide useful information for beekeepers, who typically use the previous year's pollen to feed colonies, as well as for researchers interested in aspects of pollen nutrition and consumption.

In this study, we evaluated the quality of several single pollen diets and one pollen substitute for honey bees. The parameters we chose to measure were physiological indices of pollen utilization in newly-emerged workers: i.e., the development of the hypopharyngeal glands and ovaries. We also determined whether the pattern of hypopharyngeal gland development differed from that of the ovaries, to ascertain whether one parameter was a more sensitive indicator of pollen protein utilization, or whether the hypopharyngeal glands and ovaries utilized protein in a differential fashion. In addition, we examined the relationship between the quality of a pollen as a food source for bees, and its protein content. Finally, we evaluated whether freezing pollen and storing it for 1 year in an oxygen-reduced environment would cause a reduction in its quality.

2. MATERIALS AND METHODS

2.1. Pollen collection

During the summers of 1993 and 1994, pollen was collected from blooming trees and field crops using honey bee (*Apis mellifera*) colonies fitted with OAC pollen traps [83]. Four colonies, housed in standard Langstroth hives, were placed in isolated plots containing the following species: *Malus domestica* Borkh. (mixed var.) (apple), *Brassica campestris* L. var. 'Goldrush' (oilseed rape), *Phacelia tanacetifolia* L. var. 'Angelia' (phacelia), *Melilotus officinalis* (L.) Pall. var. 'Norgold' (yellow sweetclover), and *Helianthus annuus* L. var. 'Sigco 954' (sunflower). Pine pollen was obtained by collecting male cones from pine trees, *Pinus banksiana* (Lamb.) (jack pine), drying them at 35 °C for 1 d, and then shaking the dried cones. *Malus* pollen was collected from the orchards of the Agriculture and Agri-Food Canada Research Centre in Morden, Manitoba. Pollen from field crops was collected at the University of Manitoba Glenlea Research Station, or on co-operating producers' farms in southern Manitoba. *Pinus* pollen was collected from the Sandilands Provincial Forest in eastern Manitoba.

The following protocol was observed for the collection, handling and storage of all pollen samples to preserve the integrity of external pollen lipids, and to minimize oxidation and desiccation of pollen. Surfaces of OAC pollen traps were rinsed with *n*-pentane, and the pollen collection trays of the traps were lined with tinfoil, to ensure that pollen only came into contact with lipid-free surfaces. All laboratory tools and surfaces used for the handling and sorting of pollen were also carefully cleaned so that they were lipid-free, and care was taken to avoid contacting pollen by hand. Pollen was separated from non-pollen debris manually, and corbicular loads were sorted on the basis of pollen load colour [33, 44]. Pollen from each colour cohort was mounted in glycerine jelly [19] and examined under a compound microscope at 400 × to confirm the identity of the pollen species [13]. Fresh, undried pollen that was not immediately used for the preparation of pollen diets was readied for storage by placing it in glass vials (26 by 60 mm, 23 mL). After vials were filled with pollen, any air remaining was displaced with nitrogen. Vials were immediately sealed with teflon-lined screw-caps and kept frozen at –30 °C.

2.2. Experimental design

The experimental design consisted of two factors that were tested: the age of pollen (or pollen substitute) fed to bees, and its species. The age of pollen was defined as being 1-year-old or freshly collected. One-year-old pollen (or pollen substitute) was collected during the previous summer and stored frozen, using the previously-described protocol. Freshly-collected pollen was taken and used immediately, or was temporarily frozen until needed. This short period of freezing was necessary to preserve early-season species (*Malus, Pinus*) under optimal conditions until late-season species (*Helianthus*) could be collected, at which time all diets were evaluated simultaneously. Eight different pollen diets were tested, each formulated from a single pollen species or pollen substitute. Individual trials consisted of the 8 pollen diets of the same pollen age fed to bees for a duration of 14 days. Trials were performed concurrently by staggering the starting dates at 2-day intervals. Six individual trials were performed, enabling all treatment combinations to be replicated three times.

2.3. Bioassay cages

Worker bees were confined in bioassay cages and provided a single diet. Cages (12.7 by 17.5 by 6.4 cm) were constructed of 1.3-cm thick spruce plywood, and were covered on one face with fibreglass screening (1.1 mm openings) (Fig. 1). Each cage contained a piece of plastic comb (10.1 by

14.9 cm) (Perma Comb Systems®, Woodland Hills, CA, USA), which formed the rear interior wall. Cages also contained a small (1.6 by 1.6 by 8.9 cm) plexiglass diet tray for feeding bees, and a 125-mL feeder bottle which provided a 2-M sucrose solution. Both the pollen diets and sucrose solution were fed ad libitum.

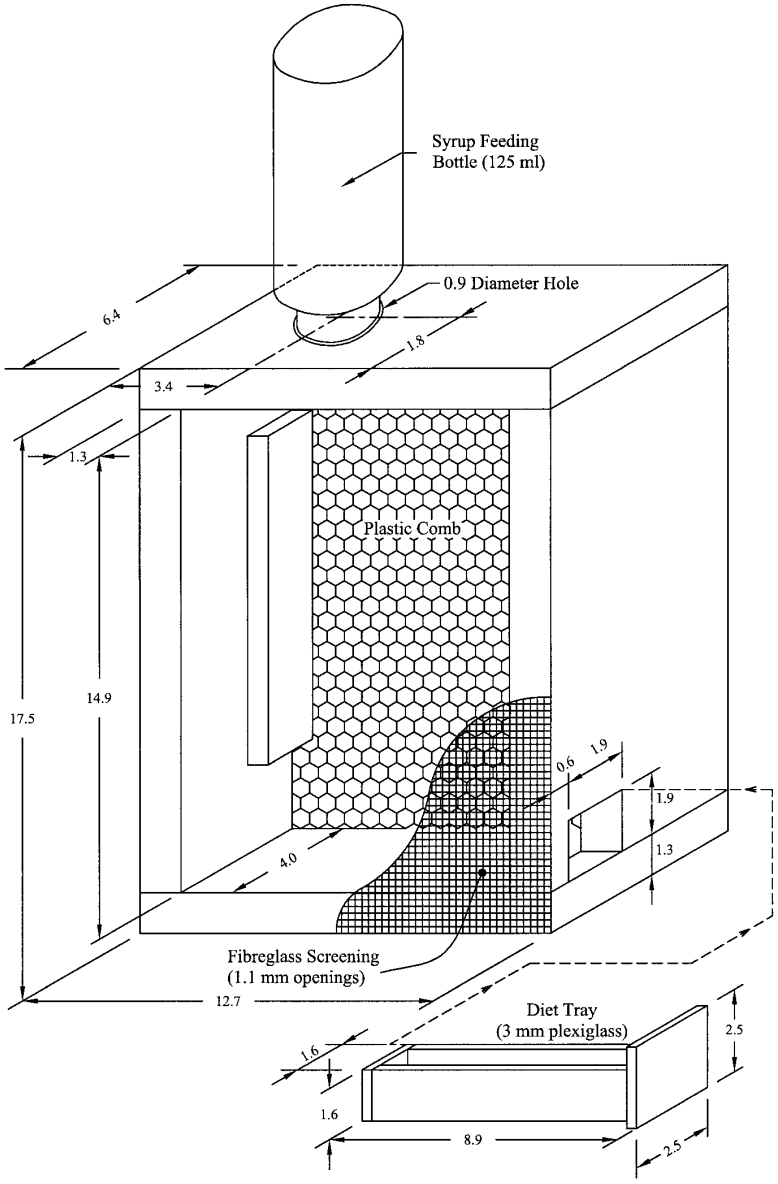

Figure 1. Bioassay cage used for experimental treatments, constructed from 1.3-cm thick spruce plywood and fibreglass screening. Not shown is Tergal® drapery lining material (200-µm openings) stretched across neck of sucrose feeder bottle. Plexiglass diet tray was inserted into opening at bottom side wall of cage. Unit of measurement for all dimensions is centimeters.

2.4. Diet preparation

Six of the 8 diets were prepared from the previously described pollen species; one was prepared from a commercial honey bee pollen substitute (Bee-Pro®, Mann Lake Supply, Hackensack, MN, USA), and the final diet contained nothing. To simplify descriptions, the pollen substitute Bee-Pro® will, hereafter, be referred to as a pollen diet. Honey bee-collected pollen was formulated into diets by mixing pollen with water to obtain a paste-like consistency. Each diet was prepared from a pooled sample containing equal amounts of pollen from each collection date. For Bee-Pro® and *Pinus* pollen, sucrose (30% w/w dry mass) was added before mixing with water. This amount is equivalent to the total weight of sugar normally found in bee-collected pollen, as determined over a wide range of plant species [60, 74, 85, 94].

2.5. Bioassay protocol

Frames of *A. mellifera* capped brood were collected and incubated overnight at 30 °C to obtain newly-emerged adult workers. Cohorts of 150 newly-emerged adult workers, collected within 12 h of emergence, were then placed in bioassay cages along with weighed portions of prepared diet mixtures and were incubated at 30 ± 1 °C and 70% RH. To estimate the percentage of water lost by diets to evaporation, equal portions of the same diet mixtures used in bioassay cages were placed in an additional set of diet trays. These trays were placed in the same incubator as the respective bioassay cages. All diet trays were reweighed, cleaned, and subsequently replenished with a known mass of freshly prepared diet mixture on days 3 and 8. On day 14, the conclusion of the experimental trials, diets were not replenished. The mortality of honey bees within treatments was evaluated by removing and counting dead bees from cages on days 3, 8 and 14.

Hypopharyngeal glands were removed from randomly selected bees on days 0, 3, 8 and 14. These intervals were chosen to establish gland size. Hypopharyngeal glands become large and biosynthetically active by the time workers are 3 days old [5, 27, 36], but decrease in size and activity with age [10, 38, 48]. On day 0, 80 bees were selected from the pool of newly-emerged workers to establish the extent of their gland development. On days 3, 8 and 14 of each trial, 10 bees were removed from each bioassay cage, killed by crushing the thorax, and then decapitated. Decapitated heads were immediately bisected with a razor blade (from the ocelli to the mandibles) and both hypopharyngeal glands were removed by dissection in phosphate-buffered saline (PBS), pH 7.3 [18]. Glands were then placed in microcentrifuge tubes (1.5 mL) containing 0.1 mL of PBS, and frozen until subsequent protein analysis.

On day 14 of the trials, ovaries were dissected from 25 bees, randomly selected from each bioassay cage. These bees were used to establish a mean value for ovary development per treatment. Bees were killed by crushing the thorax, pinned in a dish containing *Apis* saline [5], and dissected. Ovaries were examined using a binocular dissecting microscope, and their stage of development was visually scored using a 5-point scale, modified from Velthuis [96]. A single, whole number score was assigned per ovary, after determining the stage of development to which the majority of its ovarioles belonged. Ovaries were classified as: 0: undeveloped (completely resting ovary, small ovarioles close to each other); 1: oogenesis starting (cells swelling the top of the ovariole and descending); 2: slight development (eggs distinguished from trophocytes with the nutritive follicle volume not exceeding that of the egg); 3: moderate development (egg volume exceeding that of the nutritive follicle); 4: highly developed (eggs fully elongated –'sausage-shaped' in appearance, with only a remnant of the trophocytes behind the eggs).

2.6. Hypopharyngeal gland protein

Hypopharyngeal gland development was assessed using a modified Bradford dye-binding assay for total protein [4]. Reagents for the Bradford dye-binding assay were prepared using Coomassie brilliant blue G-250 dye (Pharmacia LKB Inc., Uppsala, Sweden) according to the protocol outlined in Peterson [64]. A bovine serum albumin (BSA) protein standard solution (0.56 mg/mL) was prepared from lyophilized crystals (Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) and double-distilled water. This solution was prepared with 1 mg/mL of sodium azide (Mallinckrodt Specialty Chemicals Canada, Inc., Mississauga, ON, Canada) and was kept frozen in 15-mL aliquots until used.

For protein determination, the frozen hypopharyngeal glands were thawed and mechanically ground in their microcentrifuge tubes using a pestle. Twenty-five μL of 50% (w/v) aqueous *n*-octyl-β-D-glucanopyranoside (OG) (ICN Biomedicals, Inc., Costa Mesa, CA, USA) was added to each sample to solubilize membrane-bound proteins [20]. Individual samples were then mixed by vortexing, and were centrifuged for 10 min at 8 850 × *g*. A 10-μL aliquot of the supernatant was then diluted 10-fold in PBS (pH 7.3), or by an appropriate value so that the concentration of the each sample fell within the range of the standard curve. An appropriate amount of OG was added in diluted samples to reach a final concentration of 0.2% (w/v) in the dye reagent [20].

Two 10-μL aliquots of each diluted sample were pipetted into separate wells of a microassay plate. Standards, ranking in concentration from 0.05 to 0.5 μg/μL of BSA, were prepared and added to the microassay plates, along with reagent blanks. Dilute Coomassie dye reagent (200 μL) was then added to all wells containing samples and standards. The microassay plate was then agitated on an orbital plate shaker, and incubated at 20 °C for 5 min. Optical densities of samples were read immediately in a microplate reader (Model 450, Bio-Rad Laboratories, Hercules, CA, USA) set to 595 nm. Absorbances were plotted against the linear portion of the standard curve for calculation of protein concentrations in the unknowns.

2.7. Protein determination of pollen diets

Duplicate samples of fresh and 1-year-old pollen, as well as Bee-Pro®, were analyzed to determine total crude protein. Samples were weighed before and after drying in an oven at 70 °C for 24 h, to ascertain dry weight and water content. Dried samples were ground with a mortar and pestle to achieve a powder-like consistency, and then stored in a desiccator until analyzed. Total nitrogen content of the samples was determined using an elemental analyzer (Model FP-428, Leco Instruments Ltd., Mississauga, ON, Canada), calibrated against known nitrogen standards. To determine total crude protein, nitrogen values were multiplied by a conversion factor of 5.6 [65].

2.8. Calculation of diet consumption

The net weight of the pollen diet consumed within treatments was calculated for each treatment and time interval by weighing diet trays before and after bees consumed the diet, and then correcting for the amount of water lost between time intervals. Water loss between time intervals was estimated using a duplicate set of diets placed inside incubators, which were not consumed by bees. Sugar content of bee-collected pollen diets (sugar is added to pollen during its collection by bees), was assumed to be equal [74].

2.9. Statistical analysis

Analyses were conducted on mean values of hypopharyngeal gland protein, ovary

score and diet consumption for each bioassay cage rather than for each individual bee, to provide comparisons based on the correct experimental unit. Differences in hypopharyngeal gland protein and diet consumption among treatments were examined using a split-plot design ANOVA with pollen age, replicate and diet as main factors, and time as a repeated measure [70]. Honey bee mortality was analyzed using the same design, but counts of dead bees per time interval were converted to cumulative proportions of the initial population. Proportions were then transformed using an angular transformation [84] prior to analysis. Ovary development was analyzed as a factorial ANOVA, with pollen age, replicate and diet as factors. For all analyses, comparisons between means were made either with single degree of freedom contrasts or least-significant difference tests (LSD) [70].

3. RESULTS

Consumption of experimental diets was independent of any interaction between diet type and age ($F = 1.74$; df = 6, 12; P = NS) (Fig. 2). For all diets, consumption differed with the age of pollen used ($F = 9.20$; df = 1, 12; $P < 0.05$), with workers consuming slightly more fresh than 1-year-old pollen. This preference continued to remain evident after Bee-Pro® (a pollen substitute) was excluded from the previous analysis ($F = 5.41$; df = 1, 10; $P < 0.05$). The quantity of diet consumed also varied with the type of pollen fed ($F = 62.44$; df = 6, 12; $P < 0.0001$), as workers ate substantially more *Malus*, *Melilotus*, *Phacelia*, *Brassica* and *Helianthus* pollen than that of Bee-Pro® or *Pinus*.

Temporal patterns of pollen consumption were influenced by the type of pollen

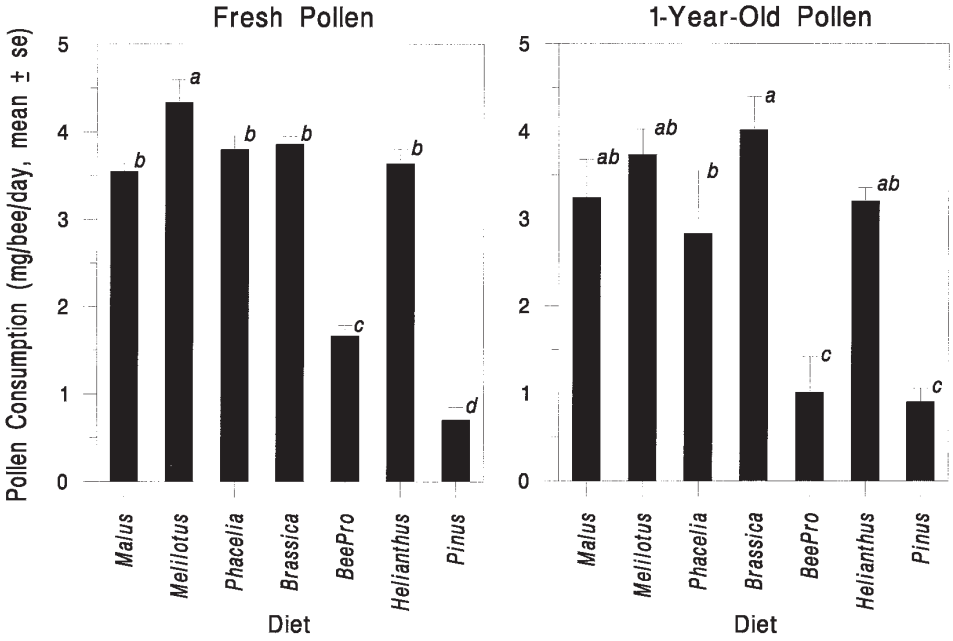

Figure 2. Pollen consumption for worker bees fed freshly collected and 1-year-old pollen. Values represent the mean amount of pollen consumed within treatments per worker bee per day. Mean numbers of bees in treatments were calculated from the populations in cages at the midpoint of each experimental time interval, corrected for sampling loss and mortality, and weighted by the duration of the interval. Significant differences between diets are indicated by different letters (LSD, $\alpha = 0.05$).

diet fed to workers ($F = 26.51$; df = 12, 36; $P < 0.0001$), but not its age ($F = 2.58$; df = 2, 36; P = NS) (Fig. 3). Over both ages of pollen, the consumption of *Malus*, *Melilotus*, *Phacelia*, *Brassica*, *Helianthus* and Bee-Pro® differed among time intervals ($F = 679.61$; df = 2, 30; $P < 0.0001$). For these diets, maximum consumption occurred during days 0–3, exceeding values for the remaining time intervals ($F = 1237.99$; df = 1, 30; $P < 0.0001$). Consumption during the 3–8-day interval substantially declined relative to the 0–3-day interval ($F = 623.30$; df = 1, 30; $P < 0.0001$), but was greater than that for the 8–14-day interval ($F = 121.23$; df = 1, 30; $P < 0.0001$). The consumption of *Pinus* pollen did not vary with time ($F = 1.81$; df = 2, 36; P = NS). Within each time interval, Bee-Pro® and *Pinus* were consumed less than other pollen diets, over both ages of pollen.

Table I shows the total crude protein content of each pollen diet and its accompanying water content. There was no linear relationship between the crude protein content of a pollen diet and its consumption by worker bees ($r^2 = 0.054$; P = NS). Estimates of the actual amount of protein consumed by bees in treatments were also calculated by converting consumption values to dry mass quantities and multiplying by the appropriate crude protein conversion. The consumption of dietary protein was influenced by the interaction between diet type and age ($F = 4.96$; df = 6, 12; $P < 0.01$) (Fig. 4). Workers consumed greater quantities of protein from fresh *Phacelia* ($F = 29.79$; df = 1, 12; $P < 0.0001$) or Bee-Pro® ($F = 7.14$; df = 1, 12; $P < 0.05$) diets than those that were 1 year old. However, protein consumption from *Malus*, *Melilotus*, *Brassica*, *Helianthus* and *Pinus* diets did not differ between ages of pollen ($F = 3.08$; df = 1, 10; P = NS).

Temporal patterns of protein consumption varied with the type of pollen diet fed to workers (F =23.51; df = 12, 24; $P < 0.0001$), but not its age ($F = 2.68$; df = 2, 24; P = NS)

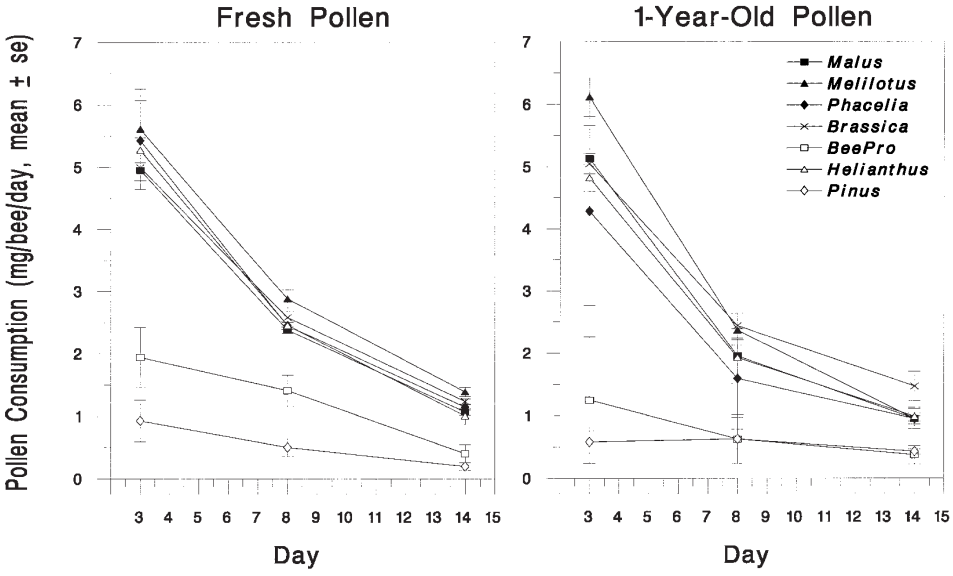

Figure 3. Pollen consumption during each experimental time interval for worker bees fed freshly collected and 1-year-old pollen. Values represent mean consumption per bee per day, based on populations in cages at the midpoint of the time interval, corrected for sampling loss and mortality.

Table I. Protein and water content of pollen diets.

Pollen diet		Age of pollen	% Protein[a], µg/100 µg dry mass	% Water, µg/100 µg wet mass
Species	Common name			
Malus domestica	Apple	Fresh	25.12	10.71
Melilotus officinalis	Yellow sweetclover	Fresh	24.15	23.48
Phacelia tanacetifolia	Phacelia	Fresh	30.10	14.56
Brassica campestris	Oilseed rape	Fresh	25.72	29.53
Bee-Pro®	Bee-Pro®	Fresh	29.92	5.89
Helianthus annuus	Sunflower	Fresh	14.86	17.66
Pinus banksiana	jack pine	Fresh	14.03	7.50
Malus domestica	Apple	1-year-old	24.29	11.50
Melilotus officinalis	Yellow sweetclover	1-year-old	23.92	13.88
Phacelia tanacetifolia	Phacelia	1-year-old	26.02	18.43
Brassica campestris	Oilseed rape	1-year-old	24.67	19.99
Bee-Pro®	Bee-Pro®	1-year-old	29.89	5.80
Helianthus annuus	Sunflower	1-year-old	15.00	23.94
Phacelia banksiana	jack pine	1-year-old	14.00	7.31

[a] Protein determination for Bee-Pro® and jack pine was performed after the addition of 30% (w/w dry mass) sucrose.

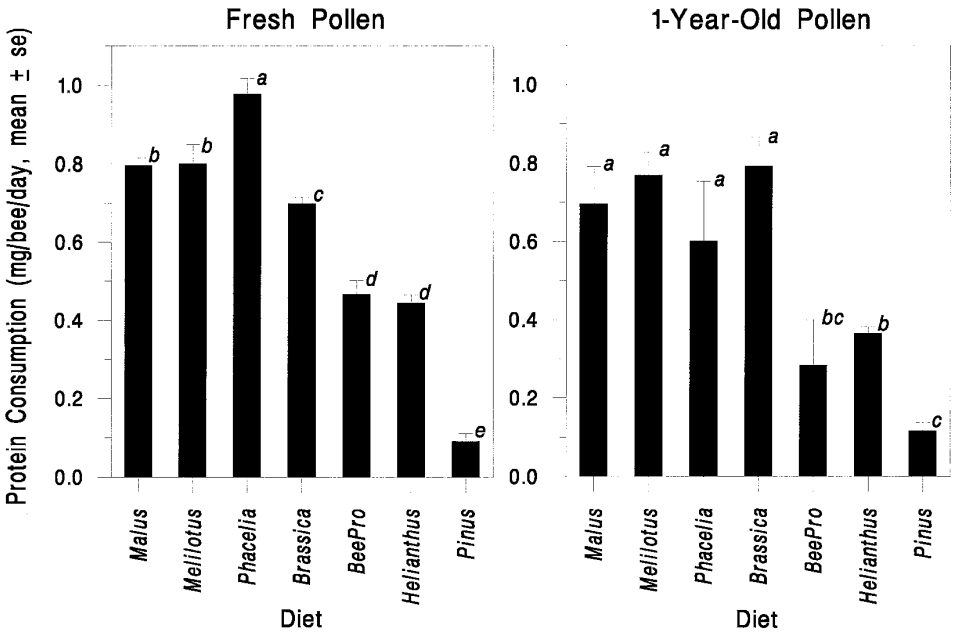

Figure 4. Protein consumption for worker bees fed freshly collected and 1-year-old pollen. Values for consumption were calculated using the protein content of each diet. Mean numbers of bees in treatments were calculated from populations in cages at the midpoint of each experimental time interval, corrected for sampling loss and mortality, and weighted by the duration of the interval. Significant differences between diets are indicated by different letters (LSD, $\alpha = 0.05$).

(Fig. 5). Over both ages of pollen, the consumption of *Malus*, *Melilotus*, *Phacelia*, *Brassica*, *Helianthus* and Bee-Pro® differed among time intervals (F = 531.10; df = 2, 20; $P < 0.0001$). For these diets, maximum consumption occurred during days 0–3, significantly exceeding values for the remaining time intervals (F = 964.46; df = 1, 20; $P < 0.0001$). Consumption during the 3–8-day interval declined relative to the 0–3-day interval (F = 481.88; df = 1, 20; $P < 0.0001$), but was greater than that for the 8–14-day interval (F = 97.74 ; df = 1, 20; $P < 0.0001$). Protein consumption from *Pinus* pollen diets did not vary with time (F = 0.64; df = 2, 24; P = NS). Within each time interval, less protein was consumed from *Helianthus*, Bee-Pro® or *Pinus* pollen than from all other diets, over both ages of pollen.

Overall worker bee mortality, analyzed as the cumulative proportion of bees dying per cage, was similar in bees fed different ages of pollen ($F < 0.01$; df = 1, 14; P = NS), different pollen diets (F = 2.27; df = 7, 14; P = NS) and between different time periods (F = 2.38; df = 2, 94; P = NS). The mean cumulative proportion of dead bees at the end of the experiment was 3.6 ± 0.5%, with values ranging from 0–16%.

The overall analysis of samples from 1 600 newly-emerged workers clearly shows that the type of pollen consumed by bees had a pronounced effect on their hypopharyngeal gland development (F = 23.22; df = 7, 14; $P < 0.0001$) (Fig. 6). Furthermore, these effects were consistent between freshly-collected and 1-year-old pollen diets (F = 0.54; df = 7, 14; P = NS). The age of pollen used to feed bees did not affect the degree of hypopharyngeal gland development (F = 0.28; df = 1, 14; P = NS). For both ages of pollen, diets composed of *Malus*, *Melilotus*, *Phacelia* and *Brassica* pollen promoted greater hypopharyngeal gland development than diets prepared from *Pinus* or no pollen, with Bee-Pro® and *Helianthus* diets being intermediate.

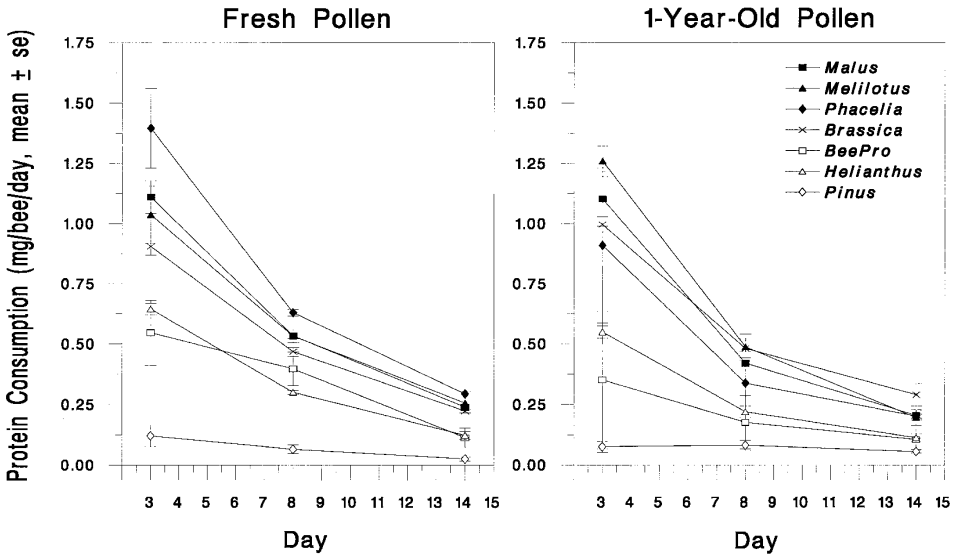

Figure 5. Protein consumption during each experimental time interval, for worker bees fed freshly collected and 1-year-old pollen. Values for consumption were calculated using the protein content of each diet. Mean bee numbers per cage are based on populations at the midpoint of the time interval, corrected for sampling loss and mortality.

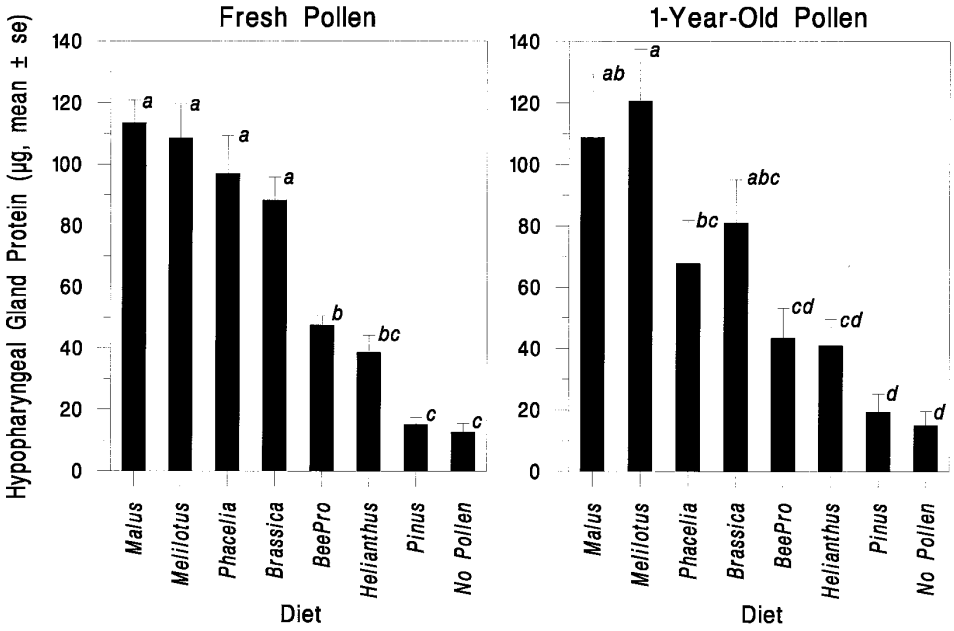

Figure 6. Hypopharyngeal gland development for worker bees fed freshly collected and 1-year-old pollen. Values for hypopharyngeal gland protein represent mean development, averaged over the time intervals ending on days 3, 8 and 14. Significant differences between diets are indicated by different letters (LSD, $\alpha = 0.05$).

Temporal patterns of hypopharyngeal gland protein content changed with the age of pollen fed to workers ($F = 18.27$; df = 2, 74; $P < 0.0001$) and were also dependent on the type of diet consumed ($F = 2.64$; df = 14, 74; $P < 0.01$) (Fig. 7). For workers fed freshly-collected pollen, the hypopharyngeal glands contained an average of 44.5 ± 3.2 µ protein on day 0, with values ranging from 14.2–80.5 µ. Workers fed fresh *Malus*, *Melilotus*, *Phacelia*, *Brassica* and *Helianthus* pollen developed glands that varied in protein content among time intervals ($F = 35.86$; df = 2, 16; $P < 0.0001$). Workers consuming *Malus* pollen diets developed glands that were largest during the 0–3-day interval ($F = 8.94$; df = 1, 28; $P < 0.001$). However, those fed *Melilotus*, *Phacelia*, *Brassica* and *Helianthus* pollen developed glands that were smallest during days 0–3 ($F = 59.37$; df = 1, 12; $P < 0.0001$), peaking in size during the 3–8-day interval ($F = 68.64$; df = 1, 12; $P < 0.0001$). For workers fed Bee-Pro®, *Pinus* or no pollen, gland protein did not vary significantly over time ($F = 3.71$; df = 2, 12; P = NS). Within individual time intervals, workers fed *Malus*, *Melilotus*, *Phacelia* and *Brassica* pollen consistently developed larger glands than bees fed other diets; workers fed *Pinus* or no pollen showed the least development.

For workers fed 1-year-old pollen, hypopharyngeal gland development showed slightly different temporal patterns (Fig. 7). The hypopharyngeal glands from these bees contained an average of 38.9 ± 2.9 µ protein on day 0, with values ranging from 14.1–67.3 µ. For all diets, the mean values for gland protein were greatest during the 0–3-day interval; however, only those for *Malus*, *Melilotus*, Bee-Pro® and *Helianthus* showed significant changes over time

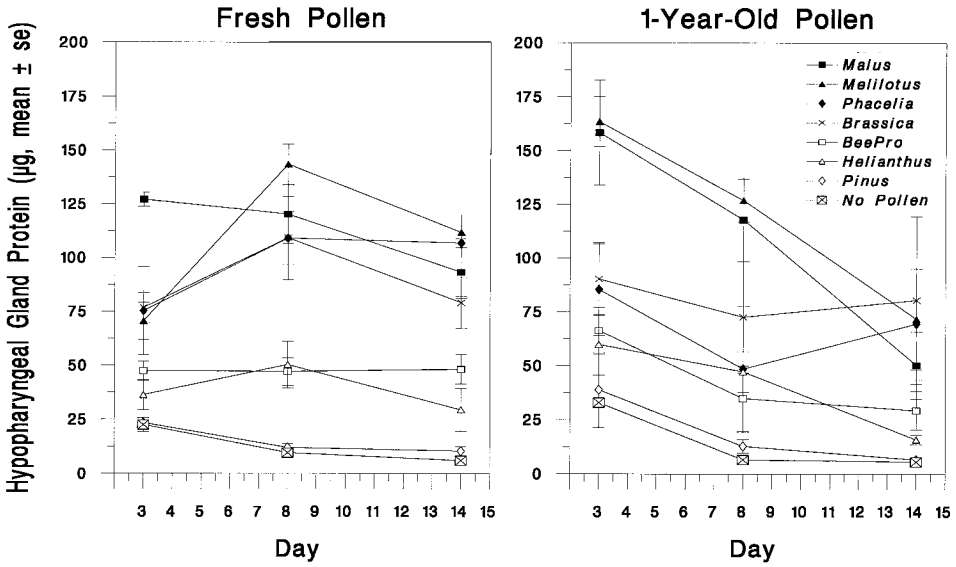

Figure 7. Hypopharyngeal gland development during each experimental time interval, for worker bees fed freshly collected and 1-year-old pollen.

(F = 20.28; df = 2, 12; P < 0.0001). For these diets, hypopharyngeal gland development was greatest during the 0–3-day interval (F = 27.54; df = 2, 12; P < 0.001), and declined as the experiment progressed. Protein levels from bees fed the remaining pollen diets showed little variation over time, or possessed greater variability per time interval. The relative ranking of 1-year-old diets within time intervals showed some similarity to fresh pollen diets, but fewer consistent patterns among groups of diets were seen across time periods. Workers fed *Malus* and *Melilotus* diets during the 0–3-day interval developed larger hypopharyngeal glands than bees fed all other diets, and continued to develop larger glands than with most remaining diets during the 3–8-day interval. Workers fed *Pinus* or no pollen had low hypopharyngeal gland protein during the 0–3-day interval, and during the 3–8-day and 8–14-day time intervals, produced the lowest hypopharyngeal protein of all diets. All other 1-year-old diets were similar for hypopharyngeal gland development, within all time periods.

The analyses of pooled ovary scores from 1 200 dissections showed similar patterns to those indicated by hypopharyngeal gland development. The overall analysis showed that ovary development was greatly affected by the type of pollen fed to workers (F = 10.39; df = 7, 47; P < 0.0001) (Fig. 8). The age of pollen used to feed newly-emerged workers had no effect on the extent of their ovarian development (F = 0.72; df=1, 47; P =NS), and no interaction between pollen age and diet type existed (F = 0.34; df = 7, 30; P = NS). For both ages of pollen, diets composed of *Malus*, *Melilotus*, *Phacelia* and *Brassica* pollen promoted greater ovary development than diets prepared from *Pinus* or no pollen. Bee-Pro® and *Helianthus* diets produced intermediate ovary development.

The linear relationship between ovary score and hypopharyngeal gland protein is shown in Figure 9. These two measures of protein utilization are well correlated (r^2 = 0.904; P < 0.0001). The crude protein content of diets was also found to be

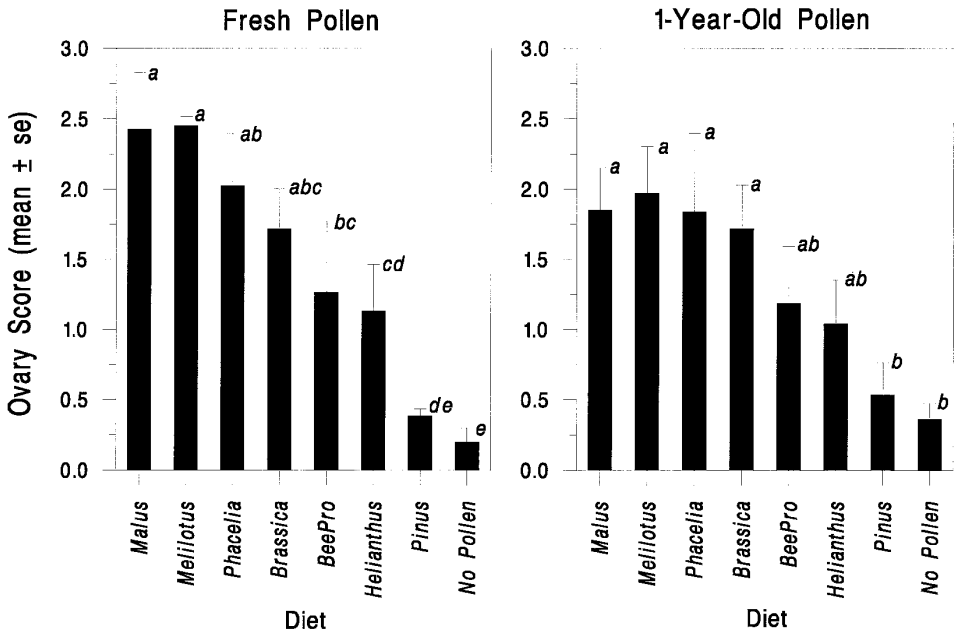

Figure 8. Ovary development for worker bees fed freshly collected and 1-year-old pollen. Values for ovary score represent the mean extent of ovary development for bees on day 14 of the experimental period. Significant differences between diets are indicated by different letters (LSD, $\alpha = 0.05$).

correlated with hypopharyngeal gland protein ($r^2 = 0.471$; $P < 0.01$) and ovary score ($r^2 = 0.601$; $P < 0.001$). However, the strongest linear relationships were found between protein consumption and hypopharyngeal gland development ($r^2 = 0.869$; $P < 0.0001$) (Fig. 10a), and between protein consumption and ovary score ($r^2 = 0.905$; $P < 0.0001$.) (Fig. 10b).

4. DISCUSSION

The development of the hypopharyngeal glands and ovaries in newly-emerged worker bees both appear to be reliable and sensitive measures of protein utilization, and when used together provide a good indication of the quality of the pollen that is being consumed. This conclusion is supported by a strong correlation between the amount of protein consumed from pollen diets and the extent of hypopharyngeal gland or ovary development.

Hypopharyngeal gland and ovary development were chosen for analysis in this study because they were considered the best indicators of how pollen diets affect colony-level fitness. Hypopharyngeal gland development in nurse bees is positively correlated with pollen consumption [35], and protein synthesis from these glands utilizes protein derived from pollen [8]. In a colony, nurse bees actively consume and digest the largest quantities of stored pollen [11] and secrete it as brood food from their hypopharyngeal and mandibular glands [80]. The quantity and quality of brood food produced by nurse bees have important ramifications for the fitness of the colony as a whole. Most brood food is fed to the developing larvae within the colony; however, a significant proportion is also fed to the adult members of each caste [9]. The quality of food

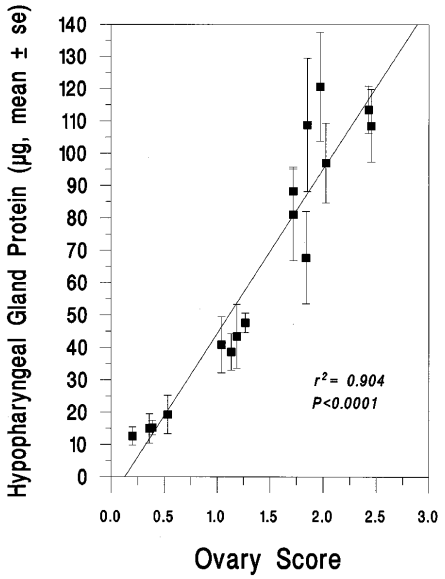

Figure 9. Relationship between mean ovary score and hypopharyngeal gland protein for worker bees fed freshly collected and 1-year-old pollen. Values for hypopharyngeal gland protein represent mean development, averaged over the intervals ending on days 3, 8 and 14. Values for ovary score represent the mean extent of ovary development for bees on day 14 of the experimental period.

received by the brood and the queen, especially, has the potential to influence the overall rate of colony growth. In addition, the survival of workers is directly affected by the total amount of pollen protein consumed [45, 46, 77]. Therefore, diets that enhance hypopharyngeal gland development in nurses potentially influence colony population size, a variable that is related to such colony-level fitness components as increased colony survival and reproductive performance [71]. In contrast, worker ovarian development provides a direct measure of the ability of bees to convert pollen proteins into vitellogenin [3, 12], a lipoprotein which is required for egg-laying. Greater or more efficient production of vitellogenin in queens could increase fecundity, colony population size, and therefore colony-level fitness.

Although the utilization of pollen protein by the hypopharyngeal glands and ovaries closely paralleled each other, our results showed that there are important differences in the way that the ovaries and hypopharyngeal glands assimilate protein from some pollen sources. Workers fed

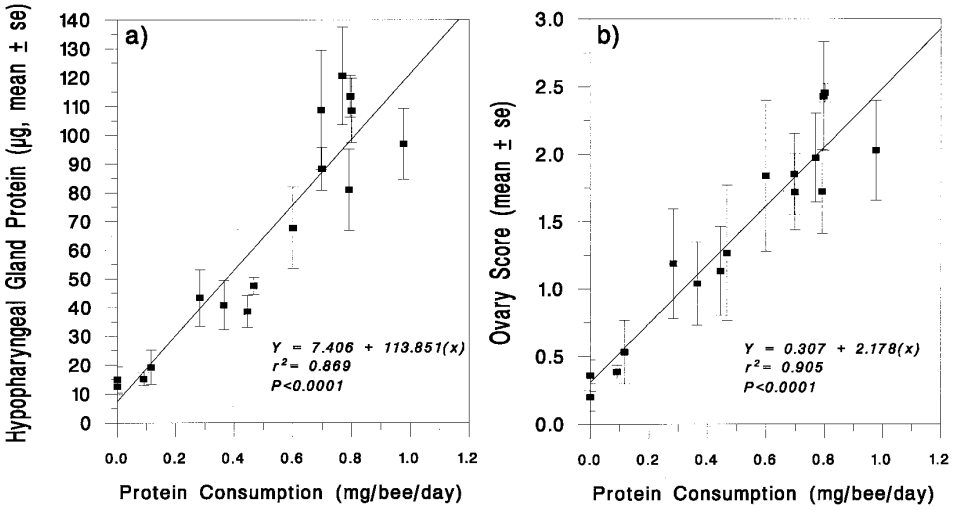

Figure 10. Correlations between protein consumption per worker (from Fig. 4) and indices of worker development, for freshly collected and 1-year-old pollen diets. **a**) Hypopharyngeal gland protein. Values represent mean development, averaged over the intervals ending on days 3, 8 and 14. **b**) Ovary development. Values are the mean extent of development on day 14 of the experimental period.

1-year-old *Phacelia* pollen developed smaller hypopharyngeal glands than workers fed fresh *Phacelia* pollen, while bees fed other diets had no change in gland size between both ages of pollen. In contrast, ovary development for workers fed fresh or 1-year-old *Phacelia* pollen was similar, even though bees consumed less 1-year-old pollen. It is likely that decreased consumption of 1-year-old pollen limited the protein available for metabolic processes in these workers. As a result, protein may have been utilized according to a physiological priority, or sink, which favoured the development of the ovaries over that of the hypopharyngeal glands. If this is true, such differential utilization of protein would not occur under queenright conditions, where ovary development is naturally suppressed, but hypopharyngeal gland development is not [40, 41]. Our results suggest, that under queenless conditions, more than one physiological index of protein utilization should be used to evaluate honey bee nutrition. Such measures should include ovarian development.

The significant positive correlation between the crude protein content of pollen diets and hypopharyngeal gland or ovary development in workers indicates that, in the absence of conducting a nutritional bioassay, crude protein content could be used as a general guideline for evaluating pollen quality. Although most species of pollen that have been quantitatively analyzed exhibit similar amino-acid profiles and contain the minimum levels of essential amino acids [23] necessary for normal honey bee growth and development [14, 21, 23, 59, 66, 85, 102], protein content is important. The developmental rate of the hypopharyngeal glands in workers is not related to the essential amino-acid composition of the pollen consumed [59], but is correlated with the level of protein in the diet [59, 88] and the amount of protein that is ingested [59, 89]. Furthermore, the addition of essential amino acids has often proven to be unsuccessful at improving the nutritional status of specific pollen diets [2, 23, 52]. Even for a species such as dandelion (*Taraxacum officinale* Weber ex Wigg.), which does not support brood rearing because of amino acid deficiencies [31, 52], crude protein content is characteristically low (9.9%) [53]. Therefore, our results support the use of crude protein content as a parameter for evaluating the quality of a pollen diet.

Freezing pollen and storing it in an oxygen-reduced environment for up to 1 year did not degrade its nitrogen content, or change its nutritional value for honey bee workers. Although workers consumed slightly more fresh than 1-year-old frozen pollen, overall hypopharyngeal gland and ovary development did not differ between fresh or stored pollen diets. Although the crude protein content among species of pollen used in this study differed by up to 16%, protein levels for fresh and 1-year-old conspecific pollen differed by less than 1% (except for *Phacelia* at 4.1%). These results are in agreement with studies that have reported little change in the content of proteins, minerals, carbohydrates, and lipids in pollen after storage by freezing [14, 103]. However, this is in contrast with reports of reductions in digestible proteins [93], deterioration or lowered availability of proteins [29], decreased vitamin content [24], or increased mortality and loss of brood-rearing capacity [15] of stored pollen. Our 1-year-old pollen samples may have benefitted from a lower storage temperature (–30 °C) than that used in other studies, and also the presence of an oxygen-reduced atmosphere. The pollen species chosen for this study may also have inherently influenced the nature of our results. Lack of proper taxonomic identification of pollen species confounds the interpretation of results from many other studies, as mixed or unidentified pollen sources have often been utilized. Our findings with pure pollen samples of known origin indicate that the use of 1-year-old frozen pollen for use as feeding supplements in colonies could be nutritionally equivalent

to that of fresh pollen if improved storage protocols such as those used in this study were followed.

Reduced palatability of stored pollen for worker bees may be responsible for the slight reduction in consumption of 1-year-old pollen relative to freshly collected pollen observed in this study. The attractiveness and palatability of pollen is affected by its lipid composition [16, 17, 34, 39, 55, 73, 78, 92]. Although the lipid composition changes when pollen is stored in the hive [54, 98], it has not been demonstrated whether such changes occur under other storage conditions. Changes in pollen lipids could explain the slight reductions in pollen consumption in studies such as ours, where protein content has been shown to remain stable in storage. Reduced palatability of stored pollen occurs when pollen has been dried and stored continuously at room temperature [15, 95].

The species of pollen we examined in this study, with the exception of *Pinus*, are usually collected in large quantities by honey bees. The relative differences in protein content among these species show that foraging honey bees collect pollen that varies greatly in quality. Our mean crude protein values for *Malus domestica* (24.7%), *Melilotus officinalis* (24.0%), *Helianthus annuus* (14.9%), *Brassica campestris* (25.2%) and *Pinus banksiana* (14.0%) are comparable to other studies that have analyzed bee-collected pollen, after standardizing their data for the protein conversion factor of 5.6. From these studies, it is apparent that protein may vary within a particular species, or a closely related group of species. Reported protein values for apple (*Malus pumila* Mill.) range between 22.7–24.6%, while those for *Trifolium* species vary between 17.6– 18.4% [59, 87]. Oilseed rape pollen is reported to contain between 19.4–22.7% protein for *Brassica campestris* [87, 94], 24.3% for *Brassica napus* L. [66], and 21.6% for an unidentified mixture of both species [86]. In addition, *Helianthus annuus* pollen has been described to contain 15.8% protein [66]. Low protein values among various species of *Pinus* (1.9–12.1%) have also been reported for bee-collected and hand-collected samples [14, 23, 45, 66, 85, 93, 94]. Published values for the crude protein content of *Phacelia tanacetifolia* pollen were not found; in our study it contained the highest level of crude protein (28.1%) of all natural pollen diets. Although variation in protein content occurs between geographic locations and with different collection techniques [53], our reported values compare favourably with other studies. The polylectic nature of honey bee foraging may be an adaptation to prevent colonies from becoming dependent on a small number of pollen sources lacking in protein, and lessen the impact of vitamin, mineral or lipid deficiencies, or toxin overloads [72], associated with individual species.

An interesting finding in this study concerns the lack of relationship between the crude protein content of the diets tested and their relative consumption by newly-emerged workers. This lack of relationship suggests that young worker bees, particularly nurses, may have no inherent mechanism through which they can discriminate the protein content of the diet that they are consuming. If workers could discriminate this aspect of pollen quality, it would be expected that larger quantities of some pollen species, such as *Helianthus* and *Pinus*, would have been consumed to compensate for their low protein content [100]. In colonies, large increases in pollen consumption occur when the level of pollen protein decreases by 10%, in an apparent attempt to meet protein requirements [46]. In our study, which used caged queenless workers, consumption did not increase with a decrease in pollen protein. *Helianthus* pollen, which is low in protein, was consumed in the same proportions as higher-protein pollens, and *Pinus* pollen was consumed in small quantities. In contrast with our findings, Schmidt and Johnson [75] showed a weak positive correlation between worker feeding preference and the protein

content of pollen diets. Their results are, however, consistent with our findings in showing that workers do not increase pollen consumption to compensate for reductions in dietary protein. Schmidt and Johnson's work also suggests that consumption may be influenced by physical or chemical cues that are unrelated to pollen quality.

Factors other than protein may be important in determining the amount of pollen consumed by individual workers. The amount of pollen consumed is, in part, also a measure of how attractive and palatable a diet is. Bee-Pro®, a pollen substitute containing no natural pollen, is as high in protein content as *Malus*, *Melilotus*, *Brassica* or *Phacelia* pollen. However, Bee-Pro® consumption was low relative to natural pollen. This may have occurred because Bee-Pro® lacks phagostimulants normally associated with the lipid components of pollen [67, 73]. The lack of phagostimulants or the presence of repellents in *Pinus* pollen, an anemophilous species, may also explain its low level of consumption. Many anemophilous species of pollen are readily collected by bees [61], but others remain unpreferred or contain toxic compounds [77]. Our study demonstrates that *Pinus* pollen is not readily consumed by workers, and confirms the poor value of *Pinus* pollen for honey bee development and longevity [23, 93]. Its lack of nutritional value does not appear to be the result of deficient amino-acid composition as the supplementation of amino acids that are absent, or present in low concentrations in *Pinus* pollen, do not improve its quality [23]. Therefore, the existence of compounds that reduce the palatability of *Pinus* pollen likely influence its utilization by honey bees.

Modern agricultural systems entail the use of large monocultures, severely limiting floral diversity for bees. Our findings suggest that the colony performance of honey bees is not likely to be adversely affected if bees are limited to foraging on monocultures of *Malus domestica*, *Melilotus officinalis*, *Brassica campestris* or *Phacelia tanacetifolia*. However, honey bee colonies restricted to foraging on *Helianthus annuus* during its bloom period may suffer a slight loss of fitness. Pollen supplements made from Bee-Pro® might benefit from the addition of pollen, or phagostimulants from pollen, that bees normally prefer to consume. *Pinus banksiana* pollen is nutritionally deficient, and is not palatable to bees. Although sometimes marketed as a pollen substitute, it should not be used as a feed supplement for honey bees.

In conclusion, the development of hypopharyngeal glands and ovaries in newly-emerged honey bee workers is strongly correlated with the amount of pollen protein consumed from diets, and to a lesser extent, with the crude protein content of the diets themselves. For certain pollen diets, workers physiologically allocate protein in a differential fashion, promoting greater development of the ovaries over that of the hypopharyngeal glands. Over the species we have tested, the consumption of pollen by workers appears to be unrelated to its nutritional content, and may be more influenced by the presence or absence of phagostimulants or repellants. We also ascertained that pollen quality remains unaffected after storage for 1 year, following our protocol of freezing in an oxygen-reduced environment. Although pollen quality is best evaluated in single-pollen nutritional bioassays, the crude protein content of pollen can be used as a general guideline for evaluating the quality of pollen collected by foragers.

Résumé – Qualité de différents pollens monofloraux, frais et d'un an, comme nourriture pour les ouvrières d'abeilles domestiques (*Apis mellifera* L.). Des groupes de 150 abeilles naissantes ont été placées dans des cages en bois, nourries avec une solution de saccharose à 2 M et avec l'un des six pollens suivants : *Malus domestica* Borkh., *Brassica campestris* L., *Phacelia tanacetifolia* L., *Melilotus officinalis* (L.) Pall., *Helianthus annuus* L. et

Pinus banksiana (Lamb.). Un succédané commercial de pollen, Bee-Pro®, et un régime sans pollen ont également été testés. Le pollen fourni aux abeilles encagées était soit du pollen fraîchement récolté, soit du pollen congelé à –30 °C pendant un an et conservé dans des récipients en verre sous atmosphère réduite en oxygène. Au début de l'expérience, puis aux jours J3, J8 et J14, dix abeilles ont été prélevées dans chaque cage et leurs glandes hypopharyngiennes extraites. La teneur totale en protéines des glandes a été déterminée par le test de Bradford et utilisée comme mesure du développement et de la taille des glandes. A la fin de l'expérience (J14), 25 abeilles de chaque cage ont été disséquées et leur développement ovarien classé visuellement de 0 à 4. Le développement des glandes hypopharyngiennes et des ovaires a varié en fonction du régime et s'est comporté, dans l'ensemble, comme une mesure fiable et sensible de l'utilisation des protéines et de la qualité du pollen. Pour les ouvrières nourries avec du pollen de phacélie d'un an, les protéines ont été utilisées différemment : elles ont plus favorisé le développement des ovaires que celui des glandes hypopharyngiennes. Le développement des ovaires et des glandes était fortement corrélé avec la quantité de protéines consommées issues du pollen et, à un moindre degré, avec la teneur brute en protéines des pollens. La conservation du pollen par congélation durant un an n'a eu aucun effet sur le développement des glandes ni des ovaires. Le pollen de tournesol et le succédané Bee-Pro® devraient être complétés par d'autres espèces de pollen lorsqu'ils sont donnés en nourrissement ; le pollen de pin devrait être évité.

***Apis mellifera* / glande hypopharyngienne / ovaire / nourrissement / protéine / pollen**

Zusammenfassung – Qualität von frischem und ein Jahr altem unifloralen Pollen als Nahrung von Honigbienenarbeiterinnen (*Apis mellifera* L.). Gruppen von jeweils 150 frisch geschlüpften Honigbienen (*Apis mellifera* L.) wurden in hölzernen Testkäfigen mit 2 M Zuckerlösung versorgt und mit jeweils einer der folgenden Pollennahrungen gefüttert: *Malus domestica* Borkh., *Brassica campestris* L., *Phacelia tanacetifolia* L., *Meliotus officinalis* (L.) Pall., *Helianthus annuus* L. und *Pinus banksiana* (Lamb.). Weiterhin wurden zusätzlich die kommerzielle Pollenersatznahrung Bee-Pro® und eine pollenfreie Ernährung untersucht. Den gekäfigten Bienen wurde entweder frisch gesammelter Pollen verfüttert oder Pollen, der ein Jahr lang bei –30 °C in Glasgefäßen in einer sauerstoffreduzierten Atmosphäre gelagert worden war. Zu Beginn des Experiments, nach 3, 8 und 14 Tagen (dem Versuchsende) wurden jeweils 10 Arbeiterinnen aus jedem Käfig entnommen und die Hypopharynxdrüsen herauspräpariert. Zu jedem dieser Zeitpunkte wurde die Nahrungsaufnahme gemessen, der Roheiweißgehalt jeder Nahrung wurde mit einer Elementaranalyse bestimmt. Anhand des Untersuchungsverfahrens von Bradford wurde der Gesamtproteingehalt der Drüsen bestimmt und als Maß für den Entwicklungsgrad und die Größe der Drüsen benutzt. Weiterhin wurden am 14. Tag des Experiments 25 Bienen präpariert und die Entwicklung der Ovarien visuell in 5 Stufen eingeteilt. Der Entwicklungszustand der Hypopharynxdrüsen und der Ovarien war bei den verschiedenen Ernährungen unterschiedlich und erwies sich als zuverlässige und empfindliche Messgröße für die Eiweißnutzung und die Pollenqualität. Bei der Verfütterung von ein Jahr lang gelagertem *Phacelia* Pollen wurde das Eiweiß allerdings abweichend genutzt und förderte die Entwicklung der Ovarien stärker als die der Hypopharynxdrüsen. Die Entwicklung der Drüsen und der Ovarien war stark mit der Menge von Eiweiß korreliert, die die Arbeiterinnen mit der Pollennahrung aufnahmen, zu einem geringeren Grad hing sie mit dem Roheiweißgehalt der Nahrung zusammen. Die tiefgekühlte Lagerung des Pollens über ein Jahr hatte auf die

Entwicklung der Ovarien und Drüsen keinen Einfluss. *Helianthus* Pollen und die Bee-Pro Pollenersatznahrung sollten zur Fütterung von Bienenvölkern mit anderen Pollenarten angereichert werden; der Pollen von *Pinus* sollte vermieden werden.

***Apis mellifera* / Hypopharynxdrüse / Ovarien / Ernährung / Protein / Pollen**

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