

Omega-3 deficiency impairs honey bee learning

Yael Arien^a, Arnon Dag^b, Shlomi Zarchin^a, Tania Masci^a, and Sharoni Shafir^{a,1}

^aB. Triwaks Bee Research Center, Department of Entomology, Robert H. Smith Faculty of Agriculture, Food & Environment, The Hebrew University of Jerusalem, Rehovot 76100, Israel; and ^bGilat Research Center, Institute of Plant Sciences, Agricultural Research Organization, Mobile Post Negev 85280, Israel

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Deficiency in essential omega-3 polyunsaturated fatty acids (PUFAs), particularly the long-chain form of docosahexaenoic acid (DHA), has been linked to health problems in mammals, including many mental disorders and reduced cognitive performance. Insects have very low long-chain PUFA concentrations, and the effect of omega-3 deficiency on cognition in insects has not been studied. We show a low omega-6:3 ratio of pollen collected by honey bee colonies in heterogeneous landscapes and in many hand-collected pollens that we analyzed. We identified *Eucalyptus* as an important bee-forage plant particularly poor in omega-3 and high in the omega-6:3 ratio. We tested the effect of dietary omega-3 deficiency on olfactory and tactile associative learning of the economically highly valued honey bee. Bees fed either of two omega-3-poor diets, or *Eucalyptus* pollen, showed greatly reduced learning abilities in conditioned proboscis-extension assays compared with those fed omega-3-rich diets, or omega-3-rich pollen mixture. The effect on performance was not due to reduced sucrose sensitivity. Omega-3 deficiency also led to smaller hypopharyngeal glands. Bee brains contained high omega-3 concentrations, which were only slightly affected by diet, suggesting additional peripheral effects on learning. The shift from a low to high omega-6:3 ratio in the Western human diet is deemed a primary cause of many diseases and reduced mental health. A similar shift seems to be occurring in bee forage, possibly an important factor in colony declines. Our study shows the detrimental effect on cognitive performance of omega-3 deficiency in a nonmammal.

fatty acids | alpha-linolenic acid | *Apis mellifera* | associative conditioning | proboscis extension response

Omega-3 and omega-6 fatty acids are two families of polyunsaturated fatty acids (PUFAs). Fatty acids (FAs) are important in structuring membrane lipids, and, because these PUFAs cannot be synthesized by higher animals, they must be acquired in the diet (1). Alpha-linolenic acid (ALA) (C18:3n-3) and linoleic acid (LA) (C18:2n-6) are the major omega-3 and omega-6 FAs, respectively. ALA is found in seeds, oils, and pollen. Some fish and other sea life also contain longer chain omega-3 FAs, eicosapentaenoic acid (EPA) (C20:5n-3) and docosahexaenoic acid (DHA) (C22:6n-3). Long-chain omega-3 PUFAs are major constituents of mammalian brain, and deficiency in these PUFAs, coupled with a high omega-6:3 ratio, is associated with many diseases and neurological disorders (2, 3). Because long-chain PUFAs occur in very low concentrations in insects (4), and *Drosophila* have been found to lack the necessary enzymes to synthesize them (5), insects have not been considered good models for studying the effect of omega-3 deficiency on cognitive performance. Nevertheless, a few studies have addressed this issue in insects, mainly in *Drosophila*, concluding that, although human and fly brain differ in long-chain FAs, lipids and lipid signaling are to a large extent conserved and important for the neuronal health of *Drosophila* (6).

Bees provide crucial pollination services that support our food security, enrich our diet's nutritional value, and are highly valued economically (7). These services are threatened worldwide by declining populations of pollinators, including the all-important honey bee. Malnutrition is emerging as one of the leading suspected culprits for declining bee populations, and for the plight of the honey bee in particular (7–10). Bees require nectar, their

main carbohydrate source, and pollen, which provides proteins, lipids, vitamins, and minerals (11). Malnutrition may be due to low pollen quantity, quality, or diversity, a condition that is aggravated in agricultural monocultures (12–14), and in greenhouses (15). Malnourished bees have smaller hypopharyngeal glands (HPGs) (a source of queen and worker jelly) (9, 16), are more susceptible to deformed wing virus (16), are less tolerant to parasitism by *Nosema ceranae* (9), are more vulnerable to pesticides (17), have a compromised immune system (18), and have a shorter lifespan (19). Whereas diet quality is affected by amino acid content and composition, proteins alone cannot explain some of the effects of diet on bee health and colony functioning and deficits in additional nutritional factors: specifically, lipids are suspected (9). ALA and LA are generally considered essential fatty acids (eFAs) for most insects (20, 21), including bees (22).

Pollens of different plant species vary greatly in lipid concentration and in the composition of FAs, including ALA and LA (23). In a diverse habitat, colonies tend to collect pollen from a variety of sources (24). But in disturbed habitats and extensive agricultural monocultures, the breadth of the diet is reduced (25), and bees may suffer from a deficiency of eFAs. Proper functioning of a honey bee colony relies on adequate production of young bees and on integration of many behaviors requiring sophisticated cognitive abilities.

In the present study, we tested the effect of omega-3 dietary deficiency on the development of honey bee HPG and on the performance of bees in olfactory and tactile learning. Colonies were fed one of four artificial diets, two rich in omega-3 and two poor in omega-3. We found that omega-3-poor diets mainly reduced omega-3 levels in the body, and only slightly in the brain, and reduced HPG size. Omega-3 dietary deficiency greatly reduced performance in both olfactory and tactile associative learning assays. Our results show the influence of dietary omega-3 on cognitive performance in a model insect. Furthermore, we show a low omega-6:3 ratio of many wild flowers and of pollen collected by

Significance

Omega-3 is an essential polyunsaturated fatty acid (PUFA) that most animals need to acquire in their diet. Mammalian brains are rich in docosahexaenoic acid (DHA), a long-chain form of omega-3, whose deficiency, coupled with a high omega-6:3 ratio, leads to numerous cognitive disorders and mental diseases. Insects have only trace amounts of long-chain PUFAs, and the effect of omega-3 deficiency on cognition has not been studied. We found that omega-3 deficiency greatly impaired honey bee learning, extending the finding of the importance of omega-3 to a nonmammal, despite lack of DHA in its brain. Furthermore, our analyses of pollens suggest that many managed colonies are experiencing a shift in available forage toward a higher omega-6:3 ratio, which may be leading to colony declines.

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¹To whom correspondence should be addressed. Email: sharoni.shafir@mail.huji.ac.il.

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honey bee colonies, but a higher omega-6:3 ratio of some increasingly dominant cultivated plants, specifically almond and *Eucalyptus*. In a Petri dish experiment, olfactory associative learning of bees fed 1 wk on *Eucalyptus* pollen was greatly reduced. The reduction in omega-3 in modern human diet is deemed the most important global factor responsible for increased incidence of disease (2, 26). Likewise, our findings suggest that omega-3 deficits in bee nutrition, due to the limited diversity of pollen availability in transformed landscapes, may play a major role in decreased bee health and colony collapse disorder (CCD).

Materials and Methods

More detailed methods are provided in [SI Materials and Methods](#).

Bees and General Procedure—Experiments 1 and 2. Honey bee colonies were placed separately in netted enclosures. The hives were placed inside the enclosures for a week without additional food to finish their pollen reserves. Then, each hive foraged from a dish in the enclosure that contained 30 g of a specific diet every day, and leftover food from the day before was removed and weighed. Each hive had inside the enclosure ad libitum access to 50% sucrose solution and fresh water.

Diets. We used four powder diets based on toasted soy flour, which is rich in proteins and poor in omega-3 fatty acids (Tables S1 and S2). The omega-3-poor diets included corn or sesame oil, which are poor in omega-3. One of the omega-3-rich diets included an oil mixture, with also sage and flax oils, which are rich in omega-3. Another omega-3-rich diet included a pollen mixture instead of vegetable oils. All diets included some pollen as phagostimulant.

Experiment 1. We used 19 hives, five replicates for treatments corn, sesame, and pollen, and four replicates for the oil mixture treatment.

Olfactory Proboscis Extension Response Conditioning. Associative learning experiments were performed according to our standard proboscis extension response (PER) protocols (27–29). Thirty-five bees were taken from every hive each day of the experiment, 6 wk after the beginning of diet feeding. Bees were strapped into sectioned hollow plastic straws. After 45 min, bees were fed 1 μ L of a 50% wt/wt sucrose solution. After one more hour, we tested the appetitive motivation of the bees. We touched the antenna of each bee with a cotton swab soaked in a 51.3% (wt/wt) (1.5 M) sucrose solution (but without feeding the bees). Bees that did not respond with proboscis extension were removed from the experiment; 79% of bees were motivated, regardless of treatment ($\chi^2_{3,889} = 3.9$, $P = 0.27$). The experiment continued with 20 motivated bees, 5 from each treatment.

Bees were moved one at a time into position in front of an odor source. The odor was delivered for 4 s over the bee's antennae, followed by a reward for 3 s together with another 3 s of odor. Each bee was exposed to two odors in 12 conditioning trials, six to each odor, with an intertrial interval of 8 min. One odor was associated with a positive reward (odor A), and the other odor with a negative reward (odor B), in a pseudorandom sequence ABBABAABBA. The two odors were benzyl acetate and geranyl acetate, and their role as odor A or B was balanced across subjects. In trial A, bees received 0.4 μ L of a 51.3% sucrose solution as a positive reward (US+), administered by a Gilmont micro-syringe. In trial B, the negative reward consisted of touching the antennae with a cotton swab dipped in a 2-M NaCl solution.

Memory of conditioned stimuli (CS) was tested after 24 h in two trials. Each bee was presented on the first and second trials, respectively, with the positively or negatively associated CS. A correct response was defined as extending the proboscis in the first trial and not extending it in the second trial. After the second trial, the bee was fed sucrose solution as a test of appetitive motivation, and only bees that responded with proboscis extension were included in the statistical analysis.

Experiment 2. After the large effects we observed in experiment 1, we conducted a second experiment in which we controlled additional factors. In experiment 2, we tested marked bees of known age, we assessed the sucrose sensitivity of subjects before conditioning, and we tested both olfactory and tactile learning. We tested a total of eight colonies, two replicates per diet treatment.

Marked Bees. In experiment 1, we collected unmarked bees from the brood area, presumably nurse bees. To control for a potential effect of diet on the age of nurses, in experiment 2, we placed combs with sealed brood in an incubator and marked emerging bees up to 1 d old. We took combs from

outdoor colonies so that the bees developed in well-nourished colonies. We could thus assess the effect of diet in experiment 2 specifically on adult bees. We added marked bees to each of the experimental hives and collected them from middle combs after 4 wk for the olfactory learning test, and after two more weeks for the tactile learning test; no marked bees were seen foraging.

Sucrose Sensitivity in Learning Performance. When sucrose is the reward, learning performance is correlated with the sucrose sensitivity of subjects (30). To control for a possible effect of diet on sucrose sensitivity, which would only indirectly affect learning, in experiment 2, we first assessed the sucrose responsiveness of every subject. In place of the motivation test with 51.3% sucrose solution, we tested the response to being touched in the antennae by an ascending concentration of sucrose solution: 0%, 0.1%, 0.3%, 1%, 3%, 10%, and 30%. Each bee received a sucrose response score between 0 and 7, representing the total number of trials in which the bee extended the proboscis to the sucrose stimuli. Modifying the technique of Scheiner et al. (30), based on this score, we determined the sucrose concentration of the positive reward in the subsequent olfactory and tactile PER assays (Table S3).

Olfactory PER Conditioning. The olfactory PER conditioning test in experiment 2 was the same as that in experiment 1, except that we tested bees of known age (4 wk old) and bees were rewarded with sucrose solution concentrations according to their sucrose responsiveness.

Tactile PER Conditioning. The tactile PER conditioning test was similar to the olfactory PER conditioning one and was conducted 2 wk after the olfactory test. We first determined the sucrose responsiveness of 6-wk-old marked bees, from which the sucrose concentration of the reward for each subject during subsequent tactile PER conditioning was determined. In this test, bees were exposed to a positively reinforced tactile stimulus for six trials; there was no CS associated with a negative reinforcement. The CS was touching the bee's antennae with a 4 \times 4-mm-square piece of black sandpaper connected to the tip of an iron rod. We touched both antennae of each bee for 5 s, followed with a reward of 0.4 μ L of the appropriate sucrose solution. We noted whether the bee extended its proboscis during the 5-s presentation of the CS.

Fatty Acid Analysis: Body, Brains, and Pollen. All bees were frozen at -20 $^{\circ}$ C immediately after the learning experiment ended. Pollen was collected fresh from flowers and similarly frozen. Before analysis, samples were freeze-dried in a lyophilizer and then transmethylated in methanol with 1% HCl and with C17 as internal standard. Fatty acid methyl esters (FAMES) were extracted in organic phase with hexane and injected into an FID Agilent 7890A GC. Each bee body was analyzed individually, including the brain for the first experiment, and excluding the brain for the second experiment. Bee brains were dissected and pooled; each sample included 20 brains in the first experiment and 12 brains in the second.

Hypopharyngeal Gland Measurements. We measured HPGs only in experiment 1; we analyzed two bees per colony. HPGs were removed from each bee and gently placed on a microscope slide in a drop of distilled water. We measured the perimeter and the length of their minor axis length, which we refer to as diameter, of five neighboring acini per bee.

Experiment 3. After the effects we observed with artificial diets, we tested the effect on olfactory PER conditioning of feeding on *Eucalyptus* pollens. Due to the limited availability of pollen, we developed a Petri-dish protocol. Five 1-d-old bees were placed inside 9-cm-diameter Petri dishes with filter paper on the bottom to absorb excrements. Each dish contained three 1-mL Eppendorf tubes that supplied ad libitum diet, honey, or water, respectively. Dishes were kept in an incubator (34 $^{\circ}$ C, 50–60% humidity) for 1 wk, and then bees were tested in olfactory PER conditioning as in experiment 1 (but without the 24-h memory test). To test the validity of this protocol, we first ran an experiment in which we compared the four diets tested in experiments 1 and 2 (Table S4). We then ran an experiment in which diets consisted of powdered bee-collected pollen pellets of *Eucalyptus ficifolia* or *Eucalyptus camaldulensis*, which are poor in omega-3, and mixed pollen pellets, rich in omega-3 (Table S5).

Statistical Analyses. The effect of diet on FAs in bee brains and bodies was tested by ANOVA on arcsin square root-transformed proportions, to meet ANOVA requirements. The effect of diet on HPG size was tested by ANOVA on the mean size of five acini per bee. The effect of diet on learning performance was tested by ANOVA on Δ PE index (29), consisting of the difference between the sum of responses during the last three trials to the CS+ and to the CS-, when learning curves reach asymptotic values. ANOVA also compared responses during

the last three US+ trials. Differences in correct responses during the memory phase were analyzed by χ^2 (likelihood ratio) test. Distribution of sucrose sensitivity scores did not meet requirements of parametric tests and were therefore tested by the Kruskal–Wallis test. All statistics were done using JMP v. 10 (SAS Institute).

Results

Diet Collection and Effect on Hypopharyngeal Glands. There was no difference between diets in the amount collected by foragers ($F_{3,23} = 0.95$, $P = 0.43$) (Fig. S1), with colonies collecting a mean 6.2 g of diet per day. We measured HPG size mainly as an independent physiological measure of an effect of our diets. Dietary treatment affected gland diameter (Fig. 1A) ($F_{3,34} = 4.58$, $P = 0.009$) and perimeter ($F_{3,34} = 5.65$, $P = 0.003$). In particular, glands of bees fed an omega-3-rich diet were larger (diameter, $F_{1,34} = 11.1$, $P = 0.0021$; perimeter, $F_{1,34} = 15.5$, $P = 0.0004$) than those fed an omega-3-poor diet. None of our treatments supported much development of sealed brood, possibly due to high levels of soy flour in all diets, or other factors related to the conditions in the enclosures. Nevertheless, because reduced HPGs affect the ability of nurse bees to raise brood, with detrimental effects for colony development, our results suggest that omega-3 deficiency would hinder colony development.

Total Fatty Acids in Brain and Body. Total fatty acid (TFA) percent of sample dry weight was greater in bee brain than body in experiment 2 (Fig. 1B) ($F_{1,102} = 124.5$, $P < 0.0001$); in experiment 1, we lost some brain fat tissue during lyophilization and thus could not calculate this measure. However, honey bee brains were less rich in FAs compared with mammalian brains, with FAs constituting about 50% of dry matter (31, 32). TFA percent was not affected by diet treatment in either body (experiment 1, $F_{3,36} = 2.46$, $P = 0.078$; experiment 2, $F_{3,92} = 0.44$, $P = 0.72$) or brain (experiment 1, $F_{3,15} = 0.13$, $P = 0.94$; experiment 2, $F_{3,4} = 0.21$, $P = 0.88$). The main FAs in bees were the saturated palmitic acid (PA) (16:0) and stearic acid (SA) (18:0), the monounsaturated oleic acid (OA) (18:1n-9), and the two essential PUFAs, LA and ALA (Table S6). Concentrations of these FAs, as well as of many lesser ones, differed between brain and body tissue (Table S6). These main FAs in bee bodies constituted 89.2% of TFA, averaged over all treatments, and were similar to those reported by Haddad et al. (33), averaged over three older age-classes of worker bees, for bee thorax (94.0%) and abdomen (90.8%), which constitute most of the weight of the bee. In bee head, Haddad et al. (33) found that gamma-linolenic acid (GLA) (18:3n-6) constituted 12.8% of TFA. Interestingly, *Drosophila* lack GLA in their heads (6, 34). We found only 0.24% GLA in bee brains, suggesting that most GLA in bee heads is in nonbrain tissue. GLA was more rare in the thorax (0.17%) and abdomen (1.5%) in that study (33), and we did not detect it in bee bodies.

We found low concentrations of eicosatrienoic acid (ETra) (C20:3n-3 cis) in bee brains and bodies (Table S6). The consistency of this finding between the many samples and the tendency for greater concentrations in brains than in bodies increase our confidence in the presence of this omega-3 FA in bees. This finding also supports the hypothesis that C20 polyunsaturated FAs are present in most insects, but in very low concentrations, sometimes just around detection level (4, 35).

Brain and Body Essential Fatty Acid Composition. The percentages of omega-3 and omega-6 of total FAs in bee bodies and brains were similar in the two experiments (Fig. 1C–F and Table S6). Dietary treatment affected percent omega-3 in bee bodies (Fig. 1C) (experiment 1, $F_{3,36} = 4.58$, $P = 0.0081$; experiment 2, $F_{3,92} = 88.8$, $P < 0.0001$). In particular, omega-3 levels were greater in bodies of bees fed an omega-3-rich diet (oil mixture or pollen) relative to those fed an omega-3-poor diet (corn or sesame) (experiment 1, $F_{1,38} = 12.3$, $P = 0.0012$; experiment 2, $F_{1,94} = 110.9$, $P < 0.0001$). Omega-3 levels in bee brains (~30%) were much greater than in bee bodies (~4–9%), and stable; ANOVA showed no difference between dietary treatments (Fig. 1D) (experiment 1, $F_{3,15} = 0.50$, $P = 0.69$; experiment 2, $F_{3,4} = 1.36$,

$P = 0.37$). However, in both experiments, omega-3 levels in brains were slightly greater in both omega-3-rich diets than in the omega-3-poor diets. Permutation analysis showed that the chance of such a pattern occurring by chance is 0.028. This finding suggests that the honey bee brain is a primary sink for omega-3, maintaining a high proportion of this eFA, which may be only slightly affected by diet.

Dietary treatment also affected percent omega-6 in bee bodies, but to a lesser degree than omega-3 (Fig. 1E). There was no significant difference between the four treatments in experiment 1 ($F_{3,36} = 2.59$, $P = 0.068$), but omega-6 levels were lower in bodies of bees fed an omega-3-rich diet relative to those fed an omega-3-poor diet ($F_{1,38} = 7.83$, $P = 0.008$). In experiment 2, there was a significant treatment effect ($F_{3,92} = 3.95$, $P = 0.011$), specifically with corn having higher levels than sesame and oil mixture. There was no consistent effect between the omega-3-rich and omega-3-poor diets ($F_{1,94} = 0.0914$, $P = 0.76$). Opposite to the pattern of omega-3, omega-6 levels in bee brains (~5%) were lower than in bee bodies (~17–27%) and did not differ between treatments (Fig. 1F) (experiment 1, $F_{3,15} = 1.15$, $P = 0.36$; experiment 2, $F_{3,4} = 0.62$, $P = 0.64$).

Olfactory PER Conditioning. In experiment 1, bees quickly learned to respond to the CS+ and not to the CS– (Fig. 2A). Learning performance differed between the diet treatments ($F_{3,392} = 91.0$, $P < 0.0001$), with bees fed the omega-3-poor diets responding less to the CS+ relative to those fed the omega-3-rich diets ($F_{1,394} = 282$, $P < 0.0001$). Bees from all diet treatments responded to the US+ in almost all trials, yet performance differed statistically between treatments (Fig. 2B) ($F_{3,392} = 4.69$, $P = 0.003$), with lesser response for bees fed the omega-3-poor diets relative to those fed the omega-3-rich diets ($F_{1,394} = 13.6$, $P = 0.0003$). The following day, treatments differed in how well they discriminated between the CS+ and the CS– (Fig. 2C) ($\chi^2_{3,312} = 76.6$, $P < 0.0001$), with bees from the omega-3-rich treatments scoring higher than those from the omega-3-poor treatments ($\chi^2_{1,312} = 76.4$, $P < 0.0001$).

To control for a possible effect of diet on sucrose sensitivity, in experiment 2, we assessed the sucrose responsiveness of subjects

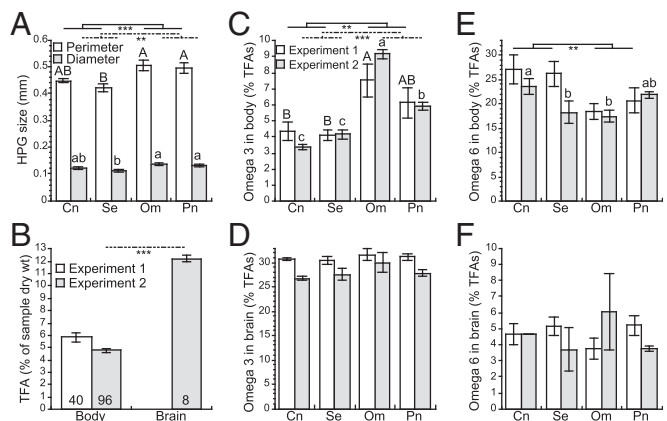


Fig. 1. (A) Hypopharyngeal gland perimeter and diameter (short axis) of bees fed four treatment diets. Horizontal lines compare between diets rich (Om, oil mixture; Pn, pollen) or poor (Cn, corn; Se, sesame) in omega-3. Sample sizes were 10 (8 for Om diet) bees per treatment. (B) Total fatty acid (TFA) percent of sample dry weight of bee body and brain. Numbers at bottom of bars are sample size; for brains, each sample is a pool of 12 bee brains. Data not available for TFA% of brains in experiment 1. (C and E) Percent essential fatty acids of total fatty acids (TFAs) in bee bodies and (D and F) brains. Sample sizes were 10 or 24 bees per treatment for body analyses, and five (four for Om diet) samples of 20 bees each or two samples of 12 bees each for brain analyses, in experiments 1 and 2, respectively. Different letters represent statistically significant differences between treatments (Tukey's test, $P < 0.05$). ** $P < 0.01$, *** $P < 0.001$. Error bars represent SE.

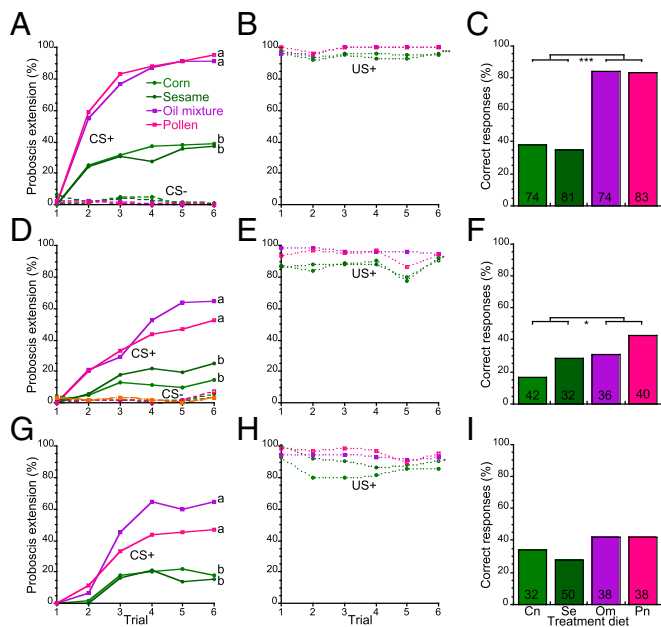


Fig. 2. Performance in olfactory (A–C, experiment 1; D–F, experiment 2) and tactile (G–I) PER conditioning of bees fed four treatment diets. The first figure in each row shows learning curves to a positively rewarded conditioned stimulus (CS+; full lines). Different letters represent statistically significant differences between treatments (Tukey's test, $P < 0.05$). The olfactory assay included a negatively rewarded conditioned stimulus (CS–; dashed lines). Bees quickly learned to respond to the CS+ and not to the CS–. In all three experiments, bees fed omega-3-rich diets (oil mixture and pollen) learned better than those fed omega-3-poor diets (corn and sesame). The second figure in each row shows the response to the sucrose reward (US+; dotted lines). Response percentages were high in all treatments but were statistically significantly lower in the omega-3-poor treatments. The third figure in each row shows performance in a memory test 24 h after conditioning; numbers in bars are sample sizes. * $P < 0.05$, *** $P < 0.001$.

before PER conditioning. Treatments did not differ in sucrose sensitivity (Fig. S2A) ($\chi^2 = 1.57$, $df = 3$, $P = 0.67$), nor did the omega-3-rich treatments differ from the omega-3-poor ones ($\chi^2 = 0.87$, $df = 1$, $P = 0.35$). As in experiment 1, performance differed between the diet treatments (Fig. 2D) ($F_{3,221} = 17.9$, $P < 0.0001$), with lesser performance for bees fed the omega-3-poor diets relative to those fed the omega-3-rich diets ($F_{1,223} = 49.9$, $P < 0.0001$). In experiment 2, subjects were of known age, 4 wk old, thus showing that these differences cannot be attributed to differences in the age of the tested bees. In experiment 1, all subjects were rewarded with a 50% sucrose solution whereas, in experiment 2, subjects were rewarded with sucrose solutions of between 0.3% and 30%, according to their sucrose sensitivity. Learning asymptotes were correspondingly lower, as they are related to the level of reward (27–29, 36). Response to US+ was similar in the four treatments (Fig. 2E) ($F_{3,221} = 2.19$, $P = 0.090$), yet bees fed the omega-3-poor diets responded less relative to those fed the omega-3-rich diets ($F_{1,223} = 6.30$, $P = 0.013$). The following day, the four treatments discriminated similarly between the CS+ and the CS– (Fig. 2F) ($\chi^2_{3,150} = 6.80$, $P = 0.079$), but bees from the omega-3-rich treatments scored higher than those from the omega-3-poor treatments ($\chi^2_{1,150} = 4.23$, $P = 0.040$).

In experiment 3, bees fed on the soy-based diets for a week in Petri dishes, and learning performance was affected as in experiments 1 and 2 (Fig. 3A) ($F_{3,115} = 9.7$, $P < 0.0001$). Bees fed on omega-3-poor *Eucalyptus* pollens had poor learning performance compared with those feeding on omega-3-rich pollen (Fig. 3B) ($F_{2,194} = 10.9$, $P < 0.0001$). Response to the US+ did not differ between treatments (soy-based diets, $F_{3,115} = 1.65$, $P = 0.18$; pollens, $F_{2,194} = 1.15$, $P = 0.32$).

Tactile PER Conditioning. To control for the possibility that diet may have affected olfactory sensitivity, rather than learning ability, we conducted a tactile PER conditioning test (30). We again assessed sucrose sensitivity before conditioning and found no effect of treatment on sucrose sensitivity (Fig. S2B) ($\chi^2_3 = 1.51$, $P = 0.68$), nor did the omega-3-rich treatments differ from the omega-3-poor ones ($\chi^2_1 = 1.14$, $P = 0.29$).

Tactile PER conditioning results were similar to those of olfactory PER conditioning. Performance differed between the diet treatments (Fig. 2G) ($F_{3,233} = 16.4$, $P < 0.0001$), with lesser performance for bees fed the omega-3-poor diets relative to those fed the omega-3-rich diets ($F_{1,235} = 42.8$, $P < 0.0001$). Response to US+ did not differ between the four treatments (Fig. 2H) ($F_{3,233} = 1.74$, $P = 0.16$), but bees fed the omega-3-poor diets responded less relative to those fed the omega-3-rich diets ($F_{1,235} = 4.42$, $P = 0.037$). The following day, the four treatments did not differ in their response to the CS+ (Fig. 2I) ($\chi^2_{3,158} = 2.68$, $P = 0.44$), nor did the omega-3-rich treatments differ from the omega-3-poor treatments ($\chi^2_{1,158} = 2.31$, $P = 0.13$).

FA Composition of Pollen. Pollens varied in composition of eFAs (Table 1 and Table S7). We found the highest omega-6:3 ratio in hand-collected *Eucalyptus* pollen, similar to that in bee-collected *Eucalyptus* pollen (Table S5). FA composition is generally similar between bee- and hand-collected pollen (37, 38). The *Eucalyptus* values are in the range of our corn and sesame treatments. Pollen of Rosaceae fruit trees was also relatively high in omega-6:3 ratio (Table 1 and Table S7).

Discussion

FA Composition of Bee Brain and Body Differ. We found a striking difference between brain and body eFA composition, with high ALA levels in the brain, possibly slightly dependent on diet. Whereas, in *Drosophila* heads, Yoshioka et al. (34) did not find ALA, Stark et al. (39) found up to about 15% ALA, attributing the difference between the two studies to differences in diet. In our study, LA levels in the body depended on diet and ranged between about 17% and 27%, in agreement with the general pattern in insects of changing FA composition in response to changing levels of dietary PUFAs (40). LA levels were around only 5% in the brain, regardless of diet. The greater variation around mean values of brain LA levels compared with ALA levels suggests that brain LA levels may be somewhat less tightly regulated. Overall, eFA composition in the brain is conserved relative to the body across taxa and diets (31, 41). In rats, even extreme omega-3 dietary deficiency results in small (~5–20%) changes in brain PUFA composition, in specific brain regions (42).

Bees, like *Drosophila*, may lack the delta-6/delta-5 desaturases of the common pathway of elongation of omega-3 FAs, widely spread throughout eukaryotes (5). However, we found low concentrations of ETrA, which is part of the alternative delta-8 pathway, found in some algae and protists, requiring a specific

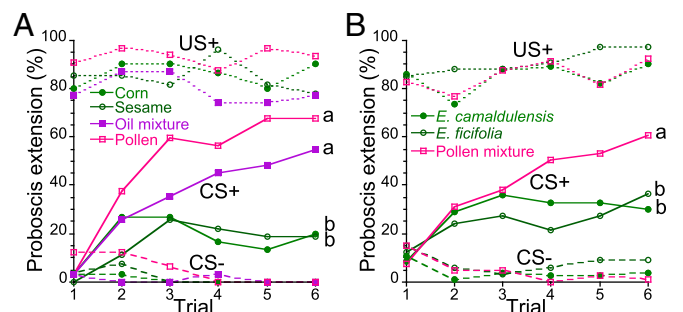


Fig. 3. Performance in olfactory PER conditioning of bees in experiment 3 fed four treatment diets as in experiments 1 and 2 (A) or pollen pellets of *Eucalyptus* or of a bee-collected mixture (B). Lines coded as in Fig. 2.

Table 1. Hand-collected pollens listed in order of increasing omega-6:3 ratio

Genus	Omega-6:3
<i>Echinops</i>	0.09
<i>Lupinus</i>	0.12
<i>Raphanus</i>	0.14
<i>Oxalis</i>	0.19 ± 0.05
<i>Carthamus</i>	0.20
<i>Sinapis</i>	0.22
<i>Solanum</i>	0.28
<i>Passiflora</i>	0.29
<i>Lilium</i>	0.35
<i>Gossypium</i>	0.37
<i>Hylocereus</i>	0.39 ± 0.12
<i>Zea</i>	0.39
<i>Helianthus</i>	0.47 ± 0.10
<i>Capsicum</i>	0.64 ± 0.05
<i>Allium</i>	0.82 ± 0.23
<i>Anethum</i>	1.24
<i>Leucophyllum</i>	1.52
<i>Foeniculum</i>	1.65
Rosaceae spp	1.74 ± 0.06
<i>Pistacia</i>	2.06
<i>Phoenix</i>	3.92
<i>Eucalyptus</i>	5.34

Where there were several independent samples, we provide means ± SE. The Rosaceae include seven species; species names and FA composition are shown in Table S7.

delta-9 elongase (43). Possibly, ETrA is produced, not by the bees themselves, but through their symbionts (44–46).

Associative Learning Affected by Omega-3 Deficiency. The artificial diets of experiments 1 and 2 allowed us to specifically manipulate FA composition by using different oils in the sesame, corn, and oil mixture treatments, while keeping all other ingredients the same. Experiment 3 extended our study to pollen that is especially poor in omega-3, but we cannot rule out additional effects of other nutritional differences, such as low isoleucine in *Eucalyptus* (11). We did not find an effect of diet on sucrose sensitivity. Bees from the omega-3-poor treatments in experiments 1 and 2 (but not in experiment 3) showed small reduction in response to the US during PER conditioning, which cannot explain the large reduction in learning that we found. In the PER assay, large differences in reward values between the US are necessary for achieving such large differences in performance (27–29, 36). Mice pups fed an omega-3-deficient diet, and whose mothers were also fed the same diet, had reduced sensitivity to sucrose (47). Such an effect is hypothesized to be due to reduced central dopaminergic function, leading to anhedonia, rather than reduced sensory perception (47, 48). Omega-3 deficiency did not affect synapse density in rat frontal cortex but greatly reduced dopaminergic vesicle compartments (49). Dietary omega-3 supplementation restored dopaminergic activity, in compromised mice that suffered from reduced activity (50), and increased dopamine and serotonin levels in rat brains (51). In insects, octopamine is the main chemically related biogenic amine that mediates the reward pathway of appetitive associative conditioning (52), specifically by activity of the ventral unpaired median (VUMmx1) neuron (53). It remains to be tested whether this pathway is compromised by omega-3 deficiency in bees and whether other forms of learning, other than appetitive conditioning, are similarly affected.

Interestingly, as in mammals (31, 42), despite the clear effect of dietary omega-3 deficiency on learning, diet affected body eFA composition more than that of the brain. The emerging field of honey bee nutrigenomics is finding important peripheral (in the abdomen, not the brain) pathways that are influenced by pollen diet, such as the insulin/TOR (target of rapamycin) pathway and

the insulin receptor substrate gene, which is associated with foraging behavior (54, 55). The nutrigenomics of dietary eFAs effects on learning remain to be revealed.

Reduced performance in an olfactory appetitive conditioning assay could be attributed to reduced sensitivity of the antennae, which are the main chemosensory organ (56). However, we found similar reduction in performance in a tactile appetitive conditioning assay (30). Thus, omega-3 deficiency could not have specifically damaged chemosensory receptors. The tactile assay also involved stimulating the antennae, which include mechanoreceptors on the flagellum and base of the antennae (56). General damage to antennal sensitivity can be precluded, however, because the main gustatory receptors are also found on the antennae (57). Thus, it seems that omega-3 deficiency mostly affects higher level processing rather than sensory sensitivity.

Performance during memory retrieval of the conditioned olfactory stimuli after 24 h largely resembled performance during conditioning. In tactile conditioning, performance of the control groups tended to decline. We conclude that omega-3 deficiency hinders acquisition, but we do not find an effect on early long-term memory retention (58).

Is the Omega-6:3 Ratio Landscape Changing? Humans have evolved on a diet consisting of an omega-6:3 ratio of about 1:1. The shift in the modern Western diet to a ratio of greater than 15:1 is deemed the most important global factor responsible for increased incidence of disease (2, 26, 59). In heterogeneous habitat, the omega-6:3 ratio in mixtures of pollen collected by honey bee colonies has been reported to be usually less than one, with a mean of 0.32 for 27 samples from three sites, one each in Poland, South Korea, and China (60), a mean of 0.76 for 16 samples from three sites in Romania (61), 0.77 in a sample from Florida (62), a mean of 0.87 for 54 samples from four sites in Israel (22), and 0.08 and 0.41 in the European pollen batches we used (Table S4). Pollens of most plants visited by bees have low omega-6:3 ratios (23) (Table 1). However, areas of diverse natural habitat have generally declined due to urbanization and expansion of agricultural landscapes, and these areas have become less heterogeneous (12–14). In modern beekeeping, colonies are often transported to vast agricultural monocultures for pollination or to exploit honey flows. For example, colonies are transported for honey production to forage on *Eucalyptus*, which is an attractive nectar source (63). Cultivated *Eucalyptus* forests have surpassed 20 million hectares worldwide (64), and it is a dominant pollen source for bees, also where it is an exotic plant (65). However, its pollen is extremely rich in LA and poor in ALA, with an omega-6:3 ratio ranging between 5 and 26 (66) (Table 1 and Table S5).

The Rosaceae include important monoculture crops (e.g., almond, apple, pear), and they are relatively high in the omega-6:3 ratio (23) (Table 1 and Table S7). In the United States, 60% of all honey bee colonies are placed in California almond orchards during bloom (67, 68). Early flowering of almonds and the sparsity of alternative pollen make it virtually the sole pollen source for the bee colonies, during the main period of colony growth. The omega-6:3 ratio of the Rosaceae is nevertheless similar to that of our oil mixture diet, which supported high learning performance. The consequences of a severalfold increase in the omega-6:3 ratio in bee landscapes deserve further attention.

Nutritional deficiencies probably contribute to the problems associated with less heterogeneous habitats, which can be mitigated by mixed-crops agriculture. As we gain knowledge of essential nutrients, such as eFAs and amino acids (22, 69), we could optimally design nutritionally balanced agricultural landscapes. Of the many stressors contributing to bee colony failure, Perry et al. (70) recently showed that supplying adequate feeding can best prevent vulnerable colonies from collapse.

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Supporting Information

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SI Materials and Methods

Bees and General Procedure—Experiments 1 and 2. Nucleus honey bee colonies containing four to five frames of bees, including two frames with brood, were placed separately in netted enclosures (2.5 × 4 × 2 m) according to a randomized block design. The hives were placed inside the enclosures for a week without additional food in order to finish their pollen reserves. Then each hive foraged from a dish in the enclosure that contained 30 g of a specific diet every day, and leftover food from the day before was removed and weighed. Each hive had inside the enclosure ad libitum access to 50% sucrose solution and fresh water. All hives were inside the enclosures for 7 wk in experiment 1, and for 9 wk in experiment 2.

Diets. We used four powder diets based on toasted soy flour, which is rich in proteins and poor in omega-3 fatty acids (Tables S1 and S2). The omega-3-poor diets included corn or sesame oil, which are poor in omega-3. One of the omega-3-rich diets included an oil mixture, with also sage and flax oils, which are rich in omega-3. Another omega-3-rich diet included a pollen mixture instead of vegetable oils. All diets included some pollen as phagostimulant. The diets were kept at −20° C and aliquotted daily to feed the bees.

Experiment 1. We used 19 hives, five replicates for treatments corn, sesame, and pollen, and four replicates for the oil mixture treatment.

Olfactory Proboscis Extension Response Conditioning. Associative learning experiments were performed according to our standard proboscis extension response (PER) protocols (27–29). Thirty-five bees were taken from every hive each day of the experiment, 6 wk after the beginning of diet feeding. Bees were collected from the brood cell areas in the middle combs and thus were likely to be nurse bees. Bees were placed in a freezer for about 5–6 min until motionless and then strapped into a sectioned hollow plastic straw by a 3-mm-wide strip of duct tape that wrapped around the straw and the (dorsal) thorax of the bee. After 45 min, bees were fed 1 μL of a 50% wt/wt sucrose solution. After one more hour, we tested the appetitive motivation of the bees. We touched the antenna of each bee with a cotton swab soaked in 51.3% wt/wt (1.5 M) sucrose solution (but without feeding the bees). Bees that did not respond with proboscis extension were removed from the experiment; 79% of bees were motivated, regardless of treatment ($\chi^2_{3,889} = 3.9$, $P = 0.27$). The experiment continued with 20 motivated bees, 5 from each treatment.

Bees were lined up on a ruler, at 4-cm intervals, with a partition between each subject and its neighbors. One bee at a time was moved into position in front of an odor source. The odor was delivered for 4 s over the bee's antennae, followed by a reward for 3 s together with another 3 s of odor. Each bee was exposed to two odors in 12 conditioning trials, 6 to each odor, with an intertrial interval of 8 min. One odor was associated with a positive reward (odor A), and the other odor with a negative reward (odor B), in a pseudorandom sequence ABBABAABABBA. The two odors were benzyl acetate and geranyl acetate, and their role as odor A or B was balanced across subjects. In trial A, bees received 0.4 μL of a 51.3% sucrose solution as a positive reward (US+), administered by a Gilmont microsyringe. In trial B, the negative reward consisted of touching the antennae with a cotton swab dipped in a 2-M NaCl solution. The bees were not fed the salt solution, which may cause malaise.

Memory of conditioned stimuli (CS) was tested after 24 h in two trials. Each bee was presented on the first and second trials, respectively, with the positively or negatively associated CS. A correct response was defined as extending the proboscis in the first trial and not extending it in the second trial. After the second trial, the bee was fed sucrose solution as a test of appetitive motivation, and only bees that responded with proboscis extension were included in the statistical analysis.

Experiment 2. After the large effects we observed in experiment 1, we conducted a second experiment in which we controlled additional factors. In experiment 2, we tested marked bees of known age, we assessed the sucrose sensitivity of subjects prior to conditioning, and we tested both olfactory and tactile learning. We tested a total of eight colonies, two replicates per diet treatment.

Marked Bees. In experiment 1, we collected unmarked bees from the brood area, presumably nurse bees. To control for a potential effect of diet on the age of nurses, in experiment 2, we placed combs with sealed brood in an incubator and marked emerging bees up to 1 d old. We took combs from outdoor colonies so that the bees developed in well-nourished colonies. We could thus assess the effect of diet in experiment 2 specifically on adult bees. We added 150 of these marked bees to each of the experimental hives. We collected marked bees from middle combs after 4 wk for the olfactory learning test, and after two more weeks for the tactile learning test; no marked bees were seen foraging.

Sucrose Sensitivity in Learning Performance. When sucrose is the reward, learning performance is correlated with the sucrose sensitivity of subjects (30). To control for a possible effect of diet on sucrose sensitivity, which would only indirectly affect learning, in experiment 2, we first assessed the sucrose responsiveness of every subject. In place of the motivation test with 51.3% sucrose solution, we tested the response to being touched in the antennae by an ascending concentration of sucrose solution: 0%, 0.1%, 0.3%, 1%, 3%, 10%, and 30% at interstimulus intervals of 4 min. Each bee received a sucrose response score between 0 and 7, representing the total number of trials in which the bee extended the proboscis to the sucrose stimuli. Modifying the technique of Scheiner et al. (30), based on this score, we determined the sucrose concentration of the positive reward in the subsequent olfactory and tactile PER assays (Table S4). Bees that did not respond at all were removed from the experiments.

Olfactory PER Conditioning. The olfactory PER conditioning test in experiment 2 was the same as that in experiment 1, except that we tested bees of known age (4 wk old) and bees were rewarded with sucrose solution concentrations according to their sucrose responsiveness.

Tactile PER Conditioning. The tactile PER conditioning test was similar to the olfactory PER conditioning one and was conducted 2 wk after the olfactory test. We first determined the sucrose responsiveness of 6-wk-old marked bees, from which the sucrose concentration of the reward for each subject during subsequent tactile PER conditioning was determined. In this test, bees were exposed to a positively reinforced tactile stimulus for six trials; there was no CS associated with a negative reinforcement. The CS was touching the bee's antennae with a 4 × 4-mm-square piece of black sandpaper connected to the tip of an iron rod. We touched both antennae of each bee for 5 s, followed with a reward of 0.4 μL

of the appropriate sucrose solution. We noted whether the bee extended its proboscis during the 5-s presentation of the CS.

Fatty Acid Analysis: Body, Brains, and Pollen. All bees were frozen at -20°C immediately after the learning experiment ended. Pollen was collected fresh from flowers and similarly frozen. Before analysis, samples were freeze-dried in a lyophilizer and then transmethylated in 2 mL of methanol with 1% HCl at 50°C for 4 h with 50 μg of C17 as internal standard. Fatty acid methyl esters (FAMES) were extracted in organic phase with 2 mL (1 mL for brain tissue) of hexane, and 1 μL was injected into an FID Agilent 7890A GC equipped with a DB-23 column (60 m, 0.25 mm) with 0.25-mm-thick film (J&W Scientific). The following temperature program was used: 150°C for 1 min, increasing by 5°C per min until 230°C , and 5 min at that temperature. He was used as carrier gas. Due to loss of some brain tissue in experiment 1 during lyophilization, samples were evaporated before injection to increase detection resolution. Each bee body was analyzed individually, including the brain for the first experiment, and excluding the brain for the second experiment. Bee brains were dissected and pooled; each sample included 20 brains in the first experiment and 12 brains in the second.

Hypopharyngeal Gland Measurements. We measured HPGs only in experiment 1; we analyzed two bees per colony. HPGs were removed from each bee and gently placed on a microscope slide in a drop of distilled water. The glands were not covered by a cover glass in order to maintain their approximately oval shape. We measured the perimeter and the length of their minor axis length, which we refer to as diameter, of five neighboring acini per bee with the Optika Vision Pro program, calibrated by an ocular micrometer slide (OB-M 1/100; Olympus).

Experiment 3. After the effects we observed with artificial diets, we tested the effect on olfactory PER conditioning of feeding on *Eucalyptus* pollens. Due to the limited availability of pollen, we developed a Petri-dish protocol. Five 1-d-old bees were placed inside 9-cm-diameter Petri dishes with filter paper on the bottom to absorb excrements. Each dish contained three 1-ml Eppendorf tubes that supplied ad libitum diet, honey, or water, respectively. Dishes were kept in an incubator (34°C , 50–60% humidity) for 1 wk, and then bees were tested in olfactory PER conditioning as in experiment 1 (but without the 24-h memory test). To test the validity of this protocol, we first ran an experiment in which we compared the four diets tested in experiments 1 and 2 (Table S5). We then ran an experiment in which diets consisted of powdered bee-collected pollen pellets of *Eucalyptus ficifolia* or *Eucalyptus camaldulensis*, which are poor in omega-3, and mixed pollen pellets, rich in omega-3 (Table S6).

Statistical Analyses. The effect of diet on FAs in bee brains and bodies was tested by ANOVA on arcsin square root-transformed proportions, to meet ANOVA requirements. The effect of diet on HPG size was tested by ANOVA on the mean size of five acini per bee. The effect of diet on learning performance was tested by ANOVA on the ΔPE index (29), consisting of the difference between the sum of responses during the last three trials to the CS+ and to the CS-, when learning curves reach asymptotic values. ANOVA also compared responses during the last three US+ trials. Differences in correct responses during the memory phase were analyzed by χ^2 (likelihood ratio) test. Distribution of sucrose sensitivity scores did not meet requirements of parametric tests and were therefore tested by the Kruskal–Wallis test. All statistics were done using JMP version 10 (SAS Institute).

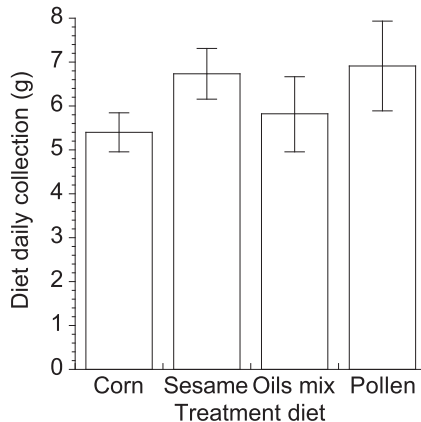


Fig. S1. Mean (\pm SE) weight of treatment diet collected per colony per day. Sample sizes were seven (six for oil mixture diet) colonies per treatment, in the two experiments combined.

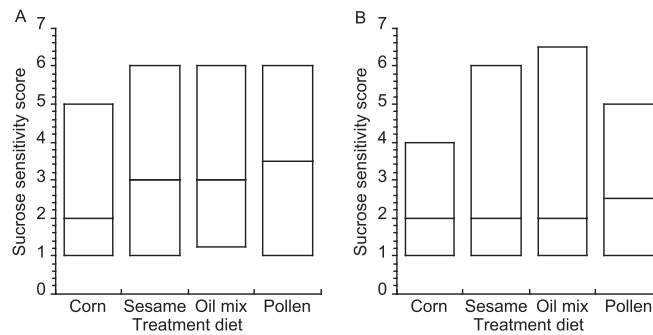


Fig. S2. Sucrose sensitivity scores of bees in experiment 2 fed four different diets before olfactory (A) or tactile (B) PER conditioning. Sucrose sensitivity did not differ significantly between treatments in either of the two groups ($\chi^2_3 = 1.57$, $P = 0.67$, and $\chi^2_3 = 1.51$, $P = 0.68$, respectively). There were no significant differences between diets rich (oil mixture and pollen) or poor (corn and sesame) in omega-3 ($\chi^2_1 = 0.87$, $P = 0.35$, and $\chi^2_1 = 1.14$, $P = 0.29$, respectively). Because sensitivity was tested before PER conditioning, the data could be combined into a single model; still, no significant differences were found between the omega-3-rich and the omega-3-poor diets ($\chi^2_1 = 2.08$, $P = 0.15$, $n = 470$). Box plots represent medians and interquartiles. $n = 51$ – 66 bees per diet in each group.

Table S1. The composition of the four soy-based diets showing percent of soy flour, pollen pellets mixture, and vegetable oil in the diet

Treatment diet/ingredient (%)	Corn	Sesame	Oil mixture	Pollen
Soy flour	75	75	75	50
Pollen pellet mixture	8	8	8	50
Corn oil	17	0	4.25	0
Sesame oil	0	17	4.25	0
Sage oil	0	0	4.25	0
Flax oil	0	0	4.25	0

Table S2. Fatty acid composition (% of TFA) of the four soy-based diets used in experiments 1 and 2, their omega-6:3 ratio, and %TFA of dry weight

Treatment diet/FA	Corn	Sesame	Oil mixture	Pollen
C16:0	11.40	10.02	9.65	6.73
C18:0	2.83	5.19	3.76	1.48
C22:0	0.26	0.32	0.25	0.21
C16:1	0.85	0.98	0.93	0.51
C18:1	25.26	32.82	23.08	8.73
C20:1n-9 c	0.20	0.19	0.17	0.09
C18:2n-6 c (LA)	53.85	46.08	41.82	21.41
C18:3n-3 c (ALA)	4.56	3.63	19.47	59.41
Total*	99.21	99.23	99.13	98.57
Omega-6:3	11.81	12.69	2.15	0.36
TFA, % of dry weight	3.71	9.25	5.28	4.87
Crude protein content, % [†]	22.80	19.60	26.52	20.54

Also included is crude protein content as percent of sample as is.

*FAs below 0.15% were considered below detection level.

[†]Crude protein content was analyzed using the Kjeldahl method, using a nitrogen-to-crude protein factor of 6.25, which is typically used for food protein.

Table S3. Sucrose solution concentrations used as the positive reward in the learning tests of experiment 2, depending on the sucrose sensitivity scores of each bee, and the distribution of bees in each category in the olfactory PER and the tactile PER tests, showing number of bees, and the percent in parentheses, in each score category

Sucrose sensitivity score	1	2	3	4	5	6	7
Sucrose concentration, %	30	30	30	15	10	1	0.3
Olfactory PER, no. (%)	67 (29)	41 (18)	23 (10)	17 (7)	19 (8)	22 (10)	42 (18)
Tactile PER, no. (%)	77 (32)	53 (22)	34 (14)	11 (5)	11 (5)	13 (5)	40 (17)

Table S4. Fatty acid composition (% of TFA) of the four soy-based diets used in experiment 3, their omega-6:3 ratio, and %TFA of dry weight

Treatment diet/FA	Corn	Sesame	Oil mixture	Pollen
C16:0	12.02	9.85	10.52	11.87
C18:0	1.99	5.35	3.42	3.40
C22:0	0.18	0.21	0.21	0.29
C18:1 trans-11	0.65	0.86	0.77	0.81
C18:1	26.40	35.40	22.53	20.24
C20:1n-9 c	0.25	0.22	0.21	0.25
C18:2n-6 c (LA)	55.88	41.64	40.15	39.43
C18:3n-3 c (ALA)	1.76	5.48	21.32	22.71
Total*	99.13	99.01	99.13	99
Omega-6:3 [†]	31.80	7.60	1.88	1.73
TFA, % of dry weight	12.35	17.32	9.37	9.86
Crude protein content, % [‡]	35.25	34.86	34.78	30.21

Also included is crude protein content as percent of sample as is.

*FAs below 0.15% were considered below detection level.

[†]The pollen diet omega-6:3 ratio was greater than in experiments 1 and 2 due to the lower ratio of the pollen mixture batch used in those experiments (0.08) relative to that used in experiment 3 (0.41). The batches were bee-collected pollens from Slovakia (batch 1) and Romania and Spain (batch 2), used by bumble bee-rearing companies.

[‡]Crude protein content was analyzed using the Kjeldahl method, using a nitrogen-to-crude protein factor of 6.25, which is typically used for food protein. Crude protein content in experiment 3 was greater than in experiments 1 and 2, largely due to the batch of soy flour used in experiment 3 being richer in protein (47%) than that used in experiments 1 and 2 (37%).

Table S5. Fatty acid composition (% of TFA) of the three pollen diets of experiment 3, and their omega-6:3 ratio

Treatment diet/FA	<i>E. ficifolia</i>	<i>E. camaldulensis</i>	Pollen mixture
C14:0	0.78	0.33	—
C16:0	19.21	21.29	9.79
C18:0	1.91	3.30	2.62
C20:0	1.57	2.63	0.30
C22:0	1.00	1.72	0.21
C24:0	0.51	0.74	—
C16:1	—	0.17	—
C18:1	6.89	4.48	5.78
C20:1n-9 c	—	—	0.19
C20:2	0.32	0.78	—
C20:3	0.18	0.40	—
C18:2n-6 c (LA)	60.76	52.42	23.28
C18:3n-3 c (ALA)	6.36	10.95	56.80
Total*	99.35	99.07	98.75
Omega-6:3	9.55	4.79	0.41
Crude protein content, % [†]	NA	20.75	14.26

*FAs below 0.15% were considered below detection level and are marked by an em dash (—).

[†]Crude protein content was analyzed using the Kjeldahl method, using a nitrogen-to-crude protein factor of 5.6, suitable for bee-collected pollen pellets (71). We did not have sufficient *E. ficifolia* pollen pellets for crude protein analysis (NA, not available), but values are similar in various *Eucalyptus* pollens, including *E. camaldulensis* (71), and similar to our value for *E. camaldulensis*.

