

## RESEARCH ARTICLE

# Unbalanced fatty acid diets impair discrimination ability of honey bee workers to damaged and healthy brood odors

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## ABSTRACT

Nutrition supports social insect colonies by regulating both individual performance and colony growth. In honey bee colonies, task-related behaviors such as nursing and foraging are partially mediated by nutrition. Young workers (nurses) consume almost all of the pollen in the hive, while foragers consume mostly nectar. Pollen provides vital proteins and lipids, consumed by nurse bees for approximately 1 week post-eclosion. The role that lipids play in the physiology and behavior of adult bees is gaining significant attention. Recent research suggests that diets with balanced ratios of fatty acids increase olfactory learning in honey bees. Olfaction is crucial for young worker bees to perform brood care and cell cleaning behaviors, which is important for hive health and disease control. Thus, we targeted the early adult, pollen-feeding stage to examine how fatty acids affect cognition to hive-relevant odors. We fed young workers (days 0–9) diets balanced or unbalanced in their ratio of essential fatty acids ( $\omega$ -6:3) sourced from pollen or cooking oils. We then measured their ability to learn healthy and damaged brood odors, as well as their ability to discriminate between the two. Workers fed balanced diets could learn and discriminate between brood odors better than workers fed unbalanced diets. Consumption of both diet types decreased with age, but their cognitive effects remained. These results suggest that diet affects young worker cognitive development, which may affect task-related behaviors and colony hygiene.

**KEY WORDS:** Pollen, Cognition, PER, Nutrition, Olfaction

## INTRODUCTION

Honey bees consume pollen and nectar from a variety of plants to meet the dynamic nutritional needs of the colony. Nectar provides carbohydrates, while pollen supplies protein, lipids and many micronutrients. Adult bees consume pollen during the first 2 weeks of their life before transitioning to mostly nectar as they take on foraging roles later in life. Pollen contains essential nutrients missing from nectar that are necessary for the development and health of adult workers. The protein and lipid percentages in plant pollen vary between species, but usually range between 1% and 10% total lipid and 10% and 40% protein (Feás et al., 2012; Nogueira et al., 2012; Vaudo et al., 2020). The dietary pollen consumed by

young workers activates the growth of brood food-producing glands (hypopharyngeal and mandibular) and the fat body, an organ with roles in both physiology and nutrient storage. Large glands and fat bodies enable young workers to provision the brood and queen with gland secretions, an activity called ‘nursing’. Young workers deprived of pollen have smaller glands and fat bodies (Corby-Harris et al., 2014, 2016, 2019). They are also less resistant to stressors such as infection and pesticides (Alaux et al., 2010, 2011; Di Pasquale et al., 2013; Wahl and Ulm, 1983; DeGrandi-Hoffman et al., 2010; Crone and Christina, 2021; Annoscia et al., 2017). While protein and amino acids have been discussed in the literature for decades, the importance of lipids is only recently gaining attention.

Beyond the individual effects of pollen limitation, nutrition is also essential for hive function. Brood rearing diminishes when pollen is limited (Haydak, 1970; DeGrandi-Hoffman et al., 2008; Keller et al., 2005). Pollen scarcity results in young nurses begging more frequently for food from others, making fewer visits to developing brood, and even cannibalizing eggs and larvae (Haydak, 1970; Schmickl and Crailsheim, 2001). Nutritional stress also accelerates behavioral development of workers from in-hive to out-of-hive duties (Corona et al., 2019 preprint; Schulz et al., 1998). Lipid stores are more closely related to behavioral task than to age, and precocious foragers have low lipid levels despite their young age (Toth and Robinson, 2005). Habitat fragmentation, climate change and monoculture landscapes are all common scenarios that can cause poor nutrition or lack of pollen diversity. These scenarios are significant problems for honey bees because pollen availability and diversity are linked to colony survival and disease resistance (Huang, 2012; Di Pasquale et al., 2013, 2016; Requier et al., 2017; Annoscia et al., 2017).

It is clear pollen is important for behavior and colony health, although the nuances of lipid nutrition are not as straightforward as lipid quantity alone. A recent analysis of plant protein to lipid ratios (P:L) and pollinator interactions shows honey bees tend to forage for pollen with lower P:L ratios, 1:1 to 2:1 (Vaudo et al., 2020). Young worker bees consume most of the pollen to develop hypopharyngeal glands and produce brood food, playing a significant role in regulating the nutritional titers of the hive. One study showed young workers actively regulate their intake of lipids relative to amounts of protein, consuming diets to achieve P:L ratios of ~1.25:1 (Stabler et al., 2021). Whether foragers are responding to colony deficiencies by preferentially foraging on pollens with certain P:L ratios is debated. If the colony is deficient in fatty acids, one study showed workers did not necessarily preferentially forage for pollen higher in fatty acids, but did prefer pollen with higher P:L ratios (Corby-Harris et al., 2021). Ratios of fatty acids such as alpha-linolenic acid ( $\omega$ -3) and linoleic acid ( $\omega$ -6) are a class of lipids that are important for bee health and behavior. Surveys of stored pollen (bee bread) have found that  $\omega$ -3 is the most abundant fatty acid (Kaplan et al.,

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2016; Čeksterytė and Jansen et al., 2012). Broadly speaking, fatty acids are a source of energy (Metzler, 2003) and are components of cell membranes. While many fatty acids are synthesized *de novo*, others must be consumed via pollen and are known as essential fatty acids. The fatty acid composition in pollen varies with the species of plant (Roulston and Cane, 2000; Manning, 2001). For example, pollens from the genus *Eucalyptus* or *Phoenix* have high  $\omega$ -6:3 ratios, and *Echinops* and *Lupinus* have low  $\omega$ -6:3 ratios (Arien et al., 2015).

Young worker behavior is less well understood than that of foragers but is equally important, especially in terms of brood care and disease control. Young bees rely on olfaction to detect social pheromones and diseased brood within the hive (Masterman et al., 2001; Gramacho and Spivak, 2003; McAfee et al., 2018; Wagoner et al., 2019). These olfactory cues can stimulate behaviors such as caring for brood and cleaning out infected cells. These olfactory guided behaviors could be subject to change based on nutritional effects. Cognitively,  $\omega$ -3 is important for learning odors in honey bees (Arien et al., 2015), although the ratio of  $\omega$ -6:3 seems to be as important, if not more so, to learning and discrimination of floral odors (Arien et al., 2018). Workers first locate diseased brood, then uncap the cell so the brood can be removed by other workers (hygienic behaviors). Bees that engage in uncapping have higher olfactory sensitivity than those removing the brood from the cell (Gramacho and Spivak, 2003).

Here, we asked whether fatty acid nutrition influences the ability of young worker bees to detect in-hive odors. Because the first 6 days of development is primarily the pollen-feeding stage, we focused on the cognitive capacities of younger workers. We tested two hypotheses: that the balance of omega essential fatty acids in the diet influences the ability of young bees (1) to learn damaged brood odors and (2) to discriminate between healthy and damaged brood odors. Bees are known to uncap brood damaged by wax moth larvae, but only during a narrow window of pupal development (white–purple eye stage) (reviewed in Spivak and Danka, 2021). Thus, we chose healthy and mechanically damaged pupae occupying this pupal stage to test discriminatory abilities of young worker bees. The diet was manipulated by adding cooking oils with different essential fatty acid ratios to a base diet, or by providing bees with corbicular pollens with different essential fatty acid ratios. Newly emerged bees were fed these diets for 9 days and their ability to learn and discriminate damaged brood odors was assessed at 3, 6 and 9 days.

## MATERIALS AND METHODS

### Animal care and cage maintenance

Honey bee, *Apis mellifera* Linnaeus 1758, colonies were maintained at the Carl Hayden Bee Research Center (CHBRC) in Tucson, AZ, USA, and all experiments were performed during the spring season (March–June 2021). Natural spring pollens include *Fraxinus* sp. (ash; 45%), *Prosopis* sp. (mesquite; 19%), *Pistacia* sp. (Chinese pistache; 11%), Brassicaceae (10%) and *Eucalyptus/Melaleuca/Eugenia* sp. (8%) (Corby-Harris et al., 2018). Two to three sealed

brood frames from five colonies were placed in a hive body in a temperature-controlled incubator (33±1°C) each week for 12 weeks. All adults that emerged over an 18 h period were placed into vented plastic cages (11.5 cm×7.5 cm×16.5 cm). The cages were kept in an incubator at 34°C with a pan of water at the bottom to maintain humidity at approximately 65–70%. Bees were fed *ad libitum* in cages equipped with two diet plugs containing diet patty, a water bottle and 30% sucrose solution. Each cage contained 200 bees and diets were replaced with fresh diet every 3 days. Diets were weighed before and after they were placed inside the cages. Consumption was analyzed per bee across days, with dead bees as censored values.

### Diet composition

Caged bees were fed balanced or unbalanced fatty acid diets at ~4% of their total diet. Balanced diets had a ~3:5 ratio of  $\omega$ -6:3, and unbalanced diets had a >5:1 ratio of  $\omega$ -6:3 (Table 1,  $n=10$ –11 cages per treatment). Previous studies showed that these diets affect olfactory learning in honey bees (Arien et al., 2018). The diet ingredients included defatted soy flour (43%), granulated sugar (25%), drier sugar (25%), water, and cooking oils or pollen (4%). Cooking oils allowed us to achieve specific  $\omega$ -6:3 fatty acid ratios as flaxseed oil (~73% polyunsaturated fatty acids) contains a more ‘balanced’ ratio of  $\omega$ -6:3, while corn oil (~60% polyunsaturated fatty acids) contains a more ‘unbalanced’ ratio of  $\omega$ -6:3 (Table 1). We replicated these diets using corbicular pollen containing similar omega fatty acid ratios: pollen in the spring of 2018 in Arizona at the CHBRC for the balanced diet (Corby-Harris et al., 2018) and a sample of commercial pollen for the unbalanced diet (Durham’s Bee Farm Inc., Walls, MS, USA). Dry mass was recorded for pollen samples and they were broken up using a mechanical bead beater (BioSpec Products, Bartlesville, OK, USA). Total lipid content of corbicular pollen was measured using a sulfo-phospho-vanillin assay (Van Handel, 1985; Corby-Harris et al., 2019). We surveyed multiple bags of pollen at the CHBRC and discovered a bag of commercially available pollen (Durham’s Bee Farm Inc.) that contained a comparable, unbalanced ratio of  $\omega$ -6:3 to that of corn oil. Using total lipid values of pollen obtained using the sulfo-phospho-vanillin assay, we calculated how many grams of pollen to add to a patty to achieve 4% total lipids of the diet. Control diets had no added oils and only a small amount of *Brassica* pollen (2%) to encourage feeding behaviors.

Three samples taken from diet patties containing pollen or cooking oil were analyzed to assess their fatty acid levels via fatty acid methyl ester (FAME) analysis using GC-MS. Samples were homogenized in an Eppendorf tube containing zirconium beads with a Folch reagent (2:1 v:v chloroform:methanol) using a mechanical bead beater (BioSpec Products, Bartlesville, OK, USA). Each sample had 45  $\mu$ l of internal standard, cis-10-heptadecenoic acid (Sigma-Aldrich, St Louis, MO, USA; MFCD00133175), to account for extraction efficiency and amount of lipid injected into the GC-MS. The samples were then partitioned with 210  $\mu$ l of 0.25% KCl, followed by removal of the Folch layer. The remaining

**Table 1. Omega fatty acid ratios in balanced and unbalanced diet patties**

Diet	Source	$\omega$ -3 $\mu$ g g <sup>-1</sup>	$\omega$ -6 $\mu$ g g <sup>-1</sup>	$\omega$ -6:3 ratio
Balanced	Flaxseed oil	112,313±20,042	73,952±10,058	0.65
	Pollen (spring Arizona)	143,616±7517	70,404±10,730	0.49
Unbalanced	Corn oil	19,869±3594	248,843±47,321	12.52
	Pollen (commercial)	59,871±1647	299,855±1045	5.00

Data are means±s.e.m.

sample was dried in a vacuum centrifuge (Savant SPD2010 SpeedVac) and reconstituted in 100  $\mu$ l toluene. Samples were then esterified with 1000  $\mu$ l of 8% methanolic HCl at 45°C for 16 h. The FAMES were mixed with hexane and underwent three washes with distilled water to partition out the acid residues. We sampled the hexane layer and analyzed FAME compounds using EI GC-MS on an HP 7890A gas chromatograph and HP 5975D mass spectrometer detector (Agilent, Inc., Santa Clara, CA, USA). Samples were injected into helium gas at 1.2 ml min<sup>-1</sup> inside a HP-5MS column (30 m×0.25 mm×0.25  $\mu$ m film; Agilent, Inc.). Temperatures ramped at 10°C min<sup>-1</sup> from 35°C with an initial 1 min hold to 230°C, holding for 8 min, followed by a ramp to 320°C, finishing with a 0.5 min cool down. The chromatograms were analyzed using Mass Hunter software (Agilent, Inc.). FAME compounds were identified by comparing the retention times of the mass spectra with esterified standards (Sigma-Aldrich) and compounds were quantified by comparing sample mass fragments (*m/z*) with known amounts of standards.

### Learning and discrimination assays

Learning trials and discrimination assays were conducted using a proboscis extension response (PER) associative conditioning assay, which is commonly used to assess honey bee cognition (Smith and Burden, 2014). Bees of known ages (3, 6, 9 days) were taken from the cages and secured in custom-made plastic harnesses. Sample sizes were comparable between balanced (oil *N*=79; pollen *N*=76) and unbalanced diets (oil *N*=79; pollen *N*=80), sampling equally across ages (average of 25 bees per age 3, 6, 9 days). Bees were fed 5  $\mu$ l of 1 mol l<sup>-1</sup> sucrose solution and left for 1 h in the dark to acclimate to the harnesses. Bees that did not exhibit PER were removed from the experiment. Learning assays took place in a clear acrylic case with a custom-built air delivery system. The air flow was controlled using an Arduino mega (Sparkfun Electronics, Boulder, CO, USA) with a motor shield to run a 3-way solenoid (12 V). Odors tested were from three pre-melanized healthy pupae or from three damaged pupae (decapitation and damage to thorax), at the pink–purple eye stage. This stage was selected because bees will uncap pupa in this developmental stage if mechanically damaged by wax moth larvae (reviewed in Spivak and Danka, 2021). The brood was carefully taken straight from the frames each morning of an experiment day. Pupae were kept in glass Pasteur pipettes until the start of the experiment approximately 1 h later. This was so that if any of the healthy pupae showed melanization or were leaking hemolymph from accidental injury, they could be discarded and replaced with new healthy pupae. A large plastic pipette tip was fastened on the end of the glass Pasteur pipette to direct the airflow at the bee. A push button controlled 4 s of air stream blowing over the pupae. Airflow was controlled by a Whisper 10 gallon air pump (Tetra U.S., Blacksburg, VA, USA) and delivered through ¼ inch tubing. Air was evacuated to remove odors using 3 inch diameter dryer tubing connected to a metal funnel secured to the back of a fan (Honeywell HT-900 TurboForce Air Circulator Fan).

Bees were placed in the learning arena individually for learning trials using established protocols (Bitterman et al., 1983; Takeda, 1961; Smith and Burden, 2014). Individual trials lasted 60 s for a single bee and the inter-trial interval was 10–12 min. A differential conditioning procedure was used in which bees learn two conditioned stimuli (CS), one of which was rewarded with 2 mol l<sup>-1</sup> sucrose (+) while the other was punished with a salt solution (-). After odor exposure, salt was touched to the tongue briefly for the punished odor while 2–3  $\mu$ l sucrose was fed for the

rewarded odor. In the current study, we used healthy-brood odors as the punished stimulus (CS-), and injured-brood odors as the rewarded stimulus (CS+). Odors were presented in a pseudo-randomized order for 5 trials each and the order of presentation was kept constant throughout the experiment (Smith and Burden, 2014).

### Statistical analyses

The data from the diet consumption and behavioral assays were analyzed using R (<http://www.R-project.org/>) and RStudio (version 1.4.1106). We used a two-way ANOVA to analyze how diet consumption was impacted by age (1–4, 5–9 days), fatty acid ratio (balanced versus unbalanced) and essential fatty acid source (pollen, oil). We performed pairwise comparisons using a Tukey's HSD *post hoc* test on the Type II ANOVA table by running the CRAN package *emmeans* (estimated marginal means; <https://CRAN.R-project.org/package=emmeans>) function. A Shapiro–Wilk test for normality indicated the data were normally distributed (*W*=0.9855, *P*=0.39).

We used a generalized linear model (GLM) with a binomial error distribution and a logit link to compare how diet affects learning acquisition to damaged and healthy brood odors. We analyzed discrimination ability by calculating the discrimination index (DI) (Masterman et al., 2000). DI was calculated for each individual trial by subtracting the sum of responses to the CS- from the sum of responses to the CS+. For each trial, a higher DI means more bees discriminated between CS+ and CS-, and thus display better discrimination ability (a DI >1 indicates more bees correctly identified the CS+, while a DI <1 indicates more bees incorrectly responded to the CS-). We compared discrimination ability between the two odors across trials (1–5). The response variable was whether the bee exhibited PER, recorded as a 1 or a 0. In the discrimination assay, the predictor variables were diet (balanced or unbalanced), age (day 3, 6, 9), odor (healthy or damaged), trial (1–5), and fatty acid source (oil or pollen). Cage and hive were random effects.

## RESULTS

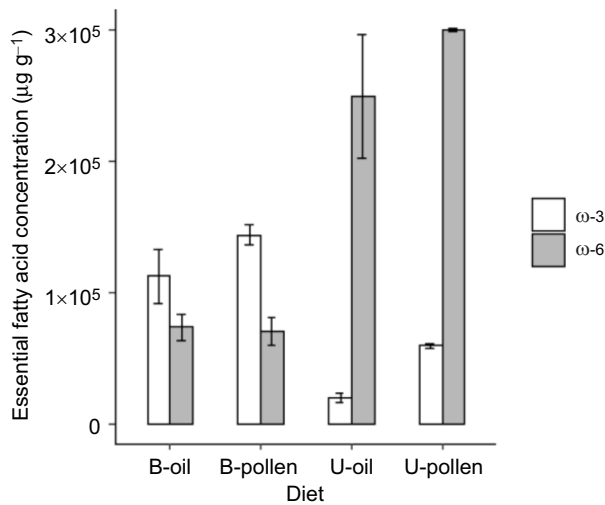
### Diet composition and consumption

To achieve balanced and unbalanced diets, we first analyzed the three core samples from patties enriched with  $\omega$ -3 and  $\omega$ -6 levels from cooking oils and corbicular pollen. We then mixed the pollen or oils into the diet patties to achieve ~4% total lipids in the patty. The amount and ratio of  $\omega$ -3 and  $\omega$ -6 in the patties was measured using GC-MS (Table 1). Flaxseed oil and spring Arizona pollen had low  $\omega$ -6:3 ratios, while corn oil and commercial pollen had high  $\omega$ -6:3 ratios (Fig. 1).

Across age groups, bees consumed the unbalanced and balanced diets equally (Fig. 2; ANOVA;  $F_{3,86}=1.156$ , *P*=0.331). Bees ate less of the diets as they aged beyond 4 days (Fig. 2;  $F_{1,86}=71.835$ , *P*<0.001), but consumption was not affected by essential fatty acid source ( $F_{3,86}=0.102$ , *P*=0.959).

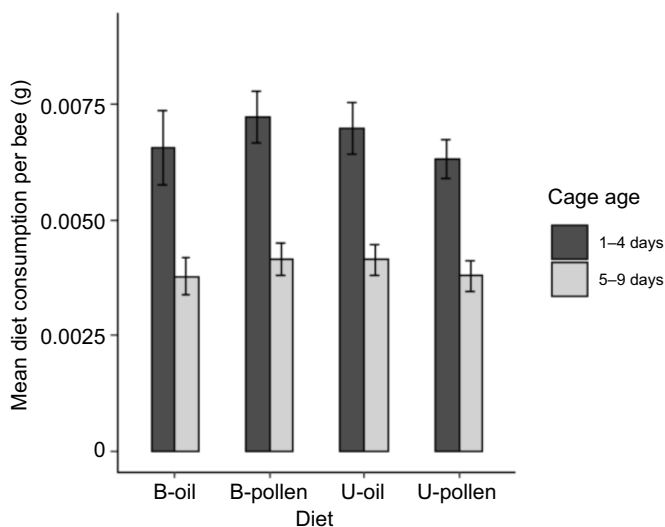
### Learning acquisition and discrimination ability

Our results supported the hypothesis that diet (balanced or unbalanced) affects the response to brood odors (damaged or healthy brood). In the following results, a high proportion of responses to a conditioned stimulus (CS- or CS+) indicates learning and a significant difference between the proportion of responses to CS- and CS+ suggests that bees learned the two odors differently. We found a significant interaction between essential fatty acid ratio and odor on learning acquisition (Fig. 3;  $\chi^2=75.69$ ,



**Fig. 1. Mean levels of essential fatty acids found in patties supplemented with oils or pollen.** Diets with balanced  $\omega$ -6:3 essential fatty acid ratios are indicated with a 'B' (B-oil, flaxseed oil; B-pollen, spring Arizona pollen), and diets with unbalanced ratios are indicated with a 'U' (U-oil, corn oil; U-pollen, commercial pollen). Bars represent means ( $\pm$ s.e.m.).

d.f.=2,  $P<0.001$ ). Bees fed either of the balanced diets responded to CS+ significantly more than to CS− as early as trial 2 (oil:  $Z=4.729$ ,  $P<0.001$ ; pollen:  $Z=3.555$ ,  $P<0.001$ ), and while responses to CS+ increased to over 80%, responses to CS− leveled off at ~20% (Fig. 3A). In contrast, bees fed the unbalanced diets learned the two odors equally ( $Z=-2.836$ ,  $P=0.551$ ), showing no significant difference in learning acquisition between CS− and CS+ for the first two trials. (Fig. 3B). By trial three, bees fed balanced diets could discriminate between odors (oil:  $Z=5.732$ ,  $P<0.001$ ; pollen:  $Z=1.357$ ;  $P<0.001$ ), and bees fed the unbalanced diets (oil:  $Z=0.709$ ,  $P=0.478$ ; pollen:  $Z=1.357$ ,  $P=0.174$ ) or control diet ( $Z=-1.620$ ,  $P=0.1052$ ) could not discriminate between odors. This suggests that both odor type and diet affect learning acquisition. Therefore, bees fed balanced diets respond to damaged brood odors more quickly than bees fed unbalanced diets.



**Fig. 2. Average consumption of pollen patty diets by caged bees in incubators.** Mean ( $\pm$ s.e.m., calculated across multiple cages) consumption of diets by honey bees in days 1–4 days and 5–9.

**Table 2. Discrimination index analysis of deviance table (Type II tests)**

Factor	LR $\chi^2$	d.f.	Pr(> $\chi^2$ )
Age	28.30	2	$P<0.0001^*$
Odor	268.87	1	$P<0.0001^*$
Trial	661.01	4	$P<0.0001^*$
Diet type	9.87	1	$P<0.0001^*$
Ratio	7.43	2	$P<0.024^*$
Odor×trial	81.51	4	$P=0.001^*$
Age×diet type	5.76	2	$P=0.056$
Age×ratio	18.34	4	$P=0.001^*$
Odor×ratio	75.69	2	$P<0.001^*$
Trial×ratio	14.48	8	$P=0.070$
Odor×diet type×ratio	10.95	1	$P<0.001^*$
Age×diet type×ratio	8.76	2	$P=0.012^*$

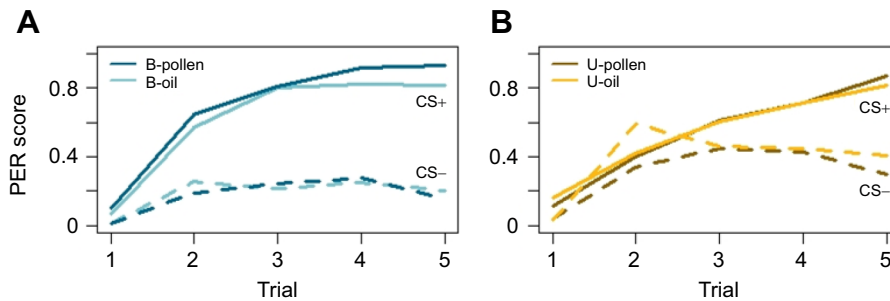
Generalized linear model results showing an effect of diet and trial, but no two-way interactions between diet and age. Significance based on 95% confidence interval, significant  $P$ -values are indicated with an asterisk.

While a differential response to CS− and CS+ would suggest discrimination ability, a more direct test was explored using the DI. Our results also supported the hypothesis that discrimination ability was affected by diet ratio. A GLM revealed that DI was significantly affected by trial (Table 2;  $\chi^2=661.0$ , d.f.=4,  $P<0.001$ ) and by diet ratio (balanced or unbalanced) ( $\chi^2=7.43$ , d.f.=2,  $P<0.001$ ). We found a two-way interaction between diet ratio and age on the DI ( $\chi^2=18.34$ , d.f.=4,  $P<0.001$ ). When ages were pooled, balanced diets had significantly higher DI than unbalanced diets as early as trial 2 (Fig. 4;  $Z=7.010$ ,  $P<0.001$ ). This trend continued across the remaining trials: trial 3 ( $Z=6.903$ ,  $P<0.001$ ), trial 4 ( $Z=4.394$ ,  $P<0.001$ ) and trial 5 ( $Z=3.229$ ,  $P=0.0012$ ) (Fig. 4). Thus, bees on balanced diets could discriminate odors earlier and overall better than bees fed unbalanced diets. Overall, we found a strong effect of balanced versus unbalanced diets that was relatively consistent across ages.

To assess how age affected learning and discrimination of brood odors with respect to diet, we compared the PER of bees at 3, 6 and 9 days (Figs S1, S2, S3). We found a significant two-way interaction between age and odor ( $\chi^2=6.041$ , d.f.=2,  $P=0.048$ ), and significant interactions with age and trial ( $\chi^2=13.461$ , d.f.=2,  $P=0.001$ ). Interestingly, the PER was affected by a three-way interaction between age, essential fatty acid source (oil or pollen) and diet (balanced or unbalanced) ( $\chi^2=8.984$ , d.f.=2,  $P=0.011$ ). These interactions could be explained when breaking down the intra-trial learning differences between the diets and essential fatty acid source. By trial 2, 6 day old bees fed the balanced-oil (B-oil) diet had a higher PER to odors than did 3 day old bees fed unbalanced pollen (U-pollen) diets ( $Z=3.493$ ,  $P=0.024$ ). However, as the age effect was marginally significant, along with inconsistent results across diets, we think the age effects in this study were minimal. Overall, we found a strong effect of balanced versus unbalanced diets that was relatively consistent across ages.

## DISCUSSION

We found that unbalanced diets impair young worker bees' ability to discriminate between damaged and healthy brood odors. Our study is the first to test the effects of balanced and unbalanced fatty acid ratios on olfaction and learning using hive odors. Bees fed the balanced diets could discriminate between brood odors in earlier trials than those fed the unbalanced diets, results consistent with other studies that discovered balanced and unbalanced diets affect discrimination ability to floral odors (Arien et al., 2015, 2018). We found day 9 bees had similar cognitive abilities to day 3 and 6 bees,



**Fig. 3. Learning acquisition curves of bees on balanced and unbalanced diets.** Data are the proportion of proboscis extension responses (PERs) to odors over five trials for bees on the balanced diet (A; B-pollen and B-oil) and unbalanced diet (B: U-pollen and U-oil), with ages pooled. Solid lines represent data for damaged-brood odors rewarded with sucrose (CS+), and dashed lines indicate data for healthy-brood odors punished with salt (CS-).

even though they ate less of the diet between 6 and 9 days. Whether fatty acids eaten by young bees and stored in tissues are later utilized in performing behaviors as bees age is an emerging hypothesis from this study.

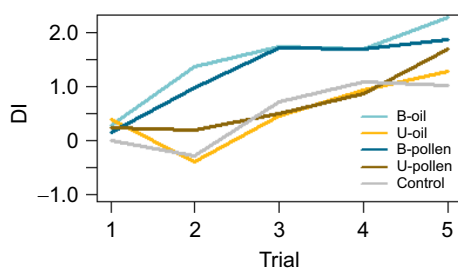
A diverse nutritional landscape can offer honey bees a variety of pollen types. Arien et al. (2015) collected pollen from 28 species across time and space of plant species and found ratios of  $\omega$ -6:3 ranging between 0.09 and 5.34. Even though fatty acids vary considerably across species, honey bee colonies can find themselves in areas lacking nutritional diversity, such as in monocultures or holding yards (temporary areas for colonies while in transport). Some trees from the genus *Rosaceae*, such as almond and apples, carry pollen with high  $\omega$ -6:3 ratios (Arien et al., 2015). We found a mixture of corbicula pollen collected in the Arizona spring contained low  $\omega$ -6:3, and workers consumed similar amounts compared with bees fed diets made with commercial pollen high in  $\omega$ -6:3. Although we used cages in our study, some evidence suggests nurse bees do not select pollen based on nutritional value (Corby-Harris et al., 2018), and prefer fresh over stored pollen (Carroll et al., 2021). Nutritional landscapes are in continual flux, especially with climate change, and the repercussions for honey bee colony survival and individual development is an important area of study. Our results support the general hypothesis that fatty acid ratios influence the cognitive abilities of young worker bees, and this can be seen as early as 3 days old. In sum, we now know that fatty acid diets affect discriminatory ability across ages to both floral and brood odors, suggesting these diets affect cognition more generally. If olfactory cognition is compromised, there could be numerous downstream effects on individual behavior and colony health.

We showed that fatty acid diets can impact the ability of young bees (3–9 days old) to discriminate between injured- and healthy-brood odors. What task-related repercussions could a fatty acid imbalance have for a honey bee colony? Olfaction likely plays a

major role in hygienic behavior, as genetic lines of hygienic bees are highly sensitive to diseased brood odors (chalkbrood infected pupae) compared with unselected or wild-type bees (Masterman et al., 2001; Gramacho and Spivak, 2003; McAfee et al., 2018). Electroantennogram recordings show bees displaying hygienic behavior have higher olfactory sensitivity to brood odors (Masterman et al., 2000). A recent study suggests hygienic bees may also detect *Varroa*-parasitized brood via changes in cuticular hydrocarbon profiles (Wagoner et al., 2019). If indeed fatty acids affect olfactory learning, then diet may have downstream effects on hygienic task-related stimuli and the ability of hives to prevent disease from spreading through the colony. Not all nurse bees engage in hygienic behaviors and there is natural variation in the number of individuals performing these behaviors (Arathi et al., 2000, 2001; Panasiuk et al., 2010). As with many behaviors, hygienic behavior has a genetic component (Spivak and Gilliam, 1998) but a relatively low heritability (0.23–0.37; Facchini et al., 2019), suggesting that the environment, and possibly nutrition, influences the trait.

Honey bees engage in hygienic behaviors at a range of ages. Arathi et al. (2000) and Arathi and Spivak (2001) found that bees start performing hygienic behaviors at approximately 15–17 days old, but with considerable variation around this average ( $15.4 \pm 7.4$  days). Panasiuk et al. (2010) found that these behaviors can be observed as early as 6 days old. In our study, we observed that bees as young as 3 days old fed balanced diets had the ability to discriminate between injured- and healthy-brood odors. This trend continued to 9 days, even though they were eating less of the diet after 4 days. Discriminating between healthy and injured brood odors is likely an important part of performing hygienic behaviors, but it is unclear whether these diets also affect uncapping or removal behaviors in the hive, an area that we are currently investigating.

Our behavioral assays suggest that bees fed balanced diets learn and discriminate between healthy and damaged brood odors better than bees fed unbalanced diets, but the mechanism behind this difference is unclear. Fatty acids may affect cognition, olfaction, or both. The rapid and sustained cognitive responses to fatty acid intake that we observed may involve neural or metabolic pathways. We know that sucrose perception is not significantly impacted by unbalanced diets (Arien et al., 2015), although it is possible that taste and olfactory sensitivity could still be differentially affected. In the brain, olfactory stimuli are sent directly to the antennal lobes and then transmitted to the mushroom bodies and lateral horn, so higher order structures, such as the mushroom bodies, may be affected by  $\omega$ -3 ratios. The importance of lipids for social insect brain function and cognition is an expanding area of research. More data are needed to determine how lipids regulate individual and group behaviors within a colony.



**Fig. 4. Discrimination index (DI) of bees fed balanced or unbalanced diets.** The DI was calculated for each of the five trials by subtracting the sum of responses to the CS- from the sum of responses to the CS+. Data are for bees on the balanced, unbalanced and control diets, with ages pooled.

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### Competing interests

S.S. assigned his rights to Yissum research development company of the Hebrew University of Jerusalem Ltd, which, together with University of Newcastle Upon Tyne and University of Pretoria, submitted a patent application on 16 November 2015 entitled 'Bee nutrition'. The remaining authors declare no competing or financial interests.

### Author contributions

Conceptualization: M.B., S.S., B.H.S., V.C.-H.; Methodology: M.B., M.C., V.C.-H.; Validation: M.B.; Formal analysis: M.B., A.C.W., V.C.-H.; Investigation: M.B., V.C.-H.; Resources: V.C.-H.; Data curation: M.B., A.C.W., V.C.-H.; Writing - original draft: M.B., V.C.-H.; Writing - review & editing: M.B., A.C.W., M.C., S.S., B.H.S., V.C.-H.; Visualization: M.B., A.C.W., V.C.-H.; Supervision: V.C.-H.; Project administration: M.B., V.C.-H.; Funding acquisition: M.B., V.C.-H.

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