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LETTERS

Best be(e) on low fat: linking nutrient perception, regulation and fitness

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Abstract

Preventing malnutrition through consuming nutritionally appropriate resources represents a challenge for foraging animals. This is due to often high variation in the nutritional quality of available resources. Foragers consequently need to evaluate different food sources. However, even the same food source can provide a plethora of nutritional and non-nutritional cues, which could serve for quality assessment. We show that bumblebees, *Bombus terrestris,* overcome this challenge by relying on lipids as nutritional cue when selecting pollen. The bees 'prioritised' lipid perception in learning experiments and avoided lipid consumption in feeding experiments, which supported survival and reproduction. In contrast, survival and reproduction were severely reduced by increased lipid contents. Our study highlights the importance of fat regulation for pollen foraging bumblebees. It also reveals that nutrient perception, nutrient regulation and reproductive fitness can be linked, which represents an effective strategy enabling quick foraging decisions that prevent malnutrition and maximise fitness.

Keywords

bee decline, foraging, nutrition, plant–insect interactions, pollen quality, PER, resource use.

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INTRODUCTION

Malnutrition resulting from the consumption of inadequate or nutritionally inappropriate food resources has severe health, performance and fitness consequences for most organisms (Su & Gao 2007; Lee *et al*. 2008; Simpson & Raubenheimer 2012; Arganda *et al*. 2017; Vaudo *et al*. 2018). In fact, malnutrition may contribute to or enhance widespread population declines, such as currently observed in insects in general and bees in particular (Naug 2009; Potts *et al*. 2010; Goulson *et al*. 2015; Hallmann *et al*. 2017; Seibold *et al*. 2019). Whether or not a resource is appropriate for an organism largely depends on species-specific requirements and thus on the nutritional quality of food, i.e. the composition and quantity of nutrients. For example, high intake of fat and deviations from ideal fatty acid (FA) ratios (Simopoulos 2002) can impair learning (Arien *et al*. 2015) and shorten lifespans in honeybees (Haddad *et al*. 2007; Manning *et al*. 2007). Selecting and consuming appropriate food resources through behavioural and physiological adaptations (e.g. differentiation by taste, memorisation of valuable resource patches, nutrient-selective foraging) can strongly increase individual health and reproductive fitness.

The intake of ideal nutrient ratios is however challenged by the high degree of variation in the amounts and ratios of different micro- and macronutrients in different food resources (Simpson & Raubenheimer 2009; Simpson & Raubenheimer 2012; Biesalski 2017). Avoiding malnutrition and obtaining nutritionally appropriate diets consequently requires nutrient-sensitive foraging and/or consumption (Raubenheimer & Simpson 1993, 1999; Mayntz *et al*. 2005; Jensen *et al*. 2011). In fact, many animals, e.g. bumblebees (*Bombus terrestris*) (Ruedenauer *et al*. 2016) or trap-jaw ants (*Odontomachus hastatus*) (Bazazi *et al*. 2016), rapidly adapt their foraging behaviour and resource intake to changes in the nutritional quality of food. These prompt behavioural responses indicate that these species are capable of sensing nutritional differences between different food sources likely by means of specific taste receptors (Abisgold & Simpson 1988; Simpson *et al*. 1991). However, different food sources vary in the composition of a plethora of nutritional cues and signals, not all of which are meaningful in each context. This may explain why many animal species regulate the intake of one specific macronutrient, but not the intake of others (Simpson & Raubenheimer 2012). As a consequence, animals readily consume too low or excessive amounts of other nutrients in order to reach the intake target of the regulated nutrient (Simpson & Raubenheimer 2012). For example, some omnivores (e.g. humans) and herbivores (e.g. herbivorous primates and insects (e.g. locusts, caterpillars)) regulate protein intake, while many predators (e.g. minks, carabid beetles) regulate carbohydrate and/or fat intake (Gosby *et al*. 2011; Bray *et al*. 2012; Simpson & Raubenheimer 2012). Nutritional quality is thus largely defined by the content of the regulated nutrient. Interestingly, we hardly know the perceptional mechanisms underlying nutritional quality assessment (Abisgold & Simpson 1988;

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Simpson *et al*. 1991; Simpson & Raubenheimer 2012). For example, it is widely unknown which nutritional cues are perceived or whether they are linked to regulated nutrients.

In this study, we elucidate nutritional quality assessment and perception and determine whether it can be linked to nutritional target regulation in the bumblebee *Bombus terrestris*. We studied bees because they are important pollinators, obtain most nutrients from nectar and pollen as main food sources and appear to regulate protein intake (like other herbivores) (Pirk *et al*. 2010; Vaudo *et al*. 2016b). While nectar is mainly a source for carbohydrates, pollen contains most other required nutrients (protein, fat, minerals and vitamins (Roulston & Cane 2000)). Besides nutrients, pollen additionally contains several other compounds such as secondary metabolites (Palmer-Young *et al*. 2019a; Palmer-Young *et al*. 2019b) and scent compounds (Dobson *et al*. 1996; Dobson *et al*. 1999). Pollen thus represents a complex chemical mixture with many different volatile (e.g. terpenoids and benzenoids (Dobson *et al*. 1999)) and non-volatile (e.g. nutrients and plant secondary metabolites) cues. Among these cues, bumblebees appear to use non-volatile nutritional cues for nutritional quality assessment, as they can differentiate between two pollen types differing in nutritional quality only when they can taste pollen (Ruedenauer *et al*. 2015). Besides their own individual perception, bumblebees, like other social insects, could additionally rely on behavioural or chemical feedback from relatives, which process (e.g. nurse bees) or consume (e.g. offspring) allocated food (Dussutour & Simpson 2008; Grüter *et al*. 2013; Ruedenauer *et al*. 2016).

Moreover, the nutritional quality of pollen and in particular its protein content seems to largely determine bumblebee colony development (Kämper *et al*. 2016; Moerman *et al*. 2017; Roger *et al*. 2017) as well as bumblebee immune defence (Di Pasquale *et al*. 2013; Brunner *et al*. 2014; Roger *et al*. 2017). Pollen protein content and amino acid profiles furthermore correlate with foraging preferences (Kitaoka & Nieh 2009; Leonhardt & Blüthgen 2012; Kriesell *et al*. 2017). However, more recent studies suggest that, in addition to protein, fat also plays an important role in nutrient regulation for bumblebees (Vaudo *et al*. 2016a; Vaudo *et al*. 2016b).

We performed a range of behavioural experiments with *B. terrestris* to determine (1) the nutritional cues perceived and thus potentially used for nutritional quality assessment, (2) whether these cues were linked to nutrient regulation and (3) whether these cues affected *B. terrestris* health and reproductive fitness. We focused on protein and fat, the two main pollen macronutrients apparently regulated by bumblebees (Pirk *et al*. 2010; Vaudo *et al*. 2016b). However, most protein and fat molecules are likely too large for taste receptors (Solms 1969), rendering smaller molecules, e.g. amino acids (AAs) and fatty acids (FAs), more likely candidates for reception and thus perception. In fact, the content of free AAs positively correlates with the total protein content of pollen ($r = 0.40$, $P < 0.001$; data obtained from Weiner *et al*. 2010) and negatively with its fat content (for bee-collected pollen, Ruedenauer *et al*. 2019). We consequently predicted that bees would use both AAs and FAs in pollen as nutritional cues to obtain information on the content of their regulated macronutrient protein. Because bees were suggested to avoid excessive protein/AA intake (Helm *et al*. 2017), we further expected bumblebees to reduce collection of pollen enriched with AAs. As animals mostly regulate only one nutrient group and because bees were suggested to be particularly sensitive to pollen protein content, we predicted that they should show no differences in collection between pure pollen and pollen enriched with FAs. We finally hypothesised that colonies fed pollen enriched with excessive amounts of AAs or FAs would show reduced survival and reproduction, as overconsumption of nutrients is generally toxic (Simpson & Raubenheimer 2009; Pirk *et al*. 2010).

MATERIALS AND METHODS

Bee colonies

We purchased 24 *Bombus terrestris* colonies from a commercial supplier (Behr, Kampen, Germany) between February 2017 and April 2018. Six of these colonies were transferred into two-chambered wooden boxes (240 × 210 × 110 mm per chamber, where one chamber served as brood chamber and the other chamber as foraging chamber). These colonies were used for learning experiments. The other 18 colonies, kept in the original boxes (270 × 240 × 200 mm) provided by the supplier, were used as source colonies for the feeding experiments. All animals were kept in a climate chamber (25°C, 50-% humidity, 12/12 h light/dark-cycle) and fed *ad libitum* Apiinvert (Südzucker AG, Mannheim, Germany; a mixture of sucrose, fructose, and glucose, delivered with the colonies) and honeybee-collected pollen (Naturwaren Niederrhein GmBH, Goch-Asperden, Germany).

Preparation of pollen diets

For all experiments, pollen was prepared in large quantities by mixing and grinding the same bee-collected pollen (as fed to the source colonies) with a coffee mill (CM 800, Graef, Arnsberg, Germany). The bee-collected pollen was relatively diverse in colours (personal observation) and can comprise pollen from up to 15 different genera (Ruedenauer *et al*. 2016). For each diet, we mixed 48 g of the resulting powder with 11 ml (for pure pollen) or 13 ml (for pollen subsequently 'enriched' with AAs and FAs) of deionised water to create a paste that sticks to the copper plates used in the learning experiments (Fig. S1). We subsequently added powdery AAs or FAs to 'nutritionally enrich' the latter pollen–water mixture. The slightly different amounts of water were used to achieve a similar pollen paste texture across diets. Prepared pollen diets were kept frozen until usage. Consequently, all diets of one experimental series were produced from the same batch of pollen and differed only in the amount and type of admixture added. Note that bee-collected pollen is mixed with regurgitated nectar by honeybees, which does not, however, elicit a spontaneous PER in unconditioned bumblebees.

We tested pure bee-collected pollen and bee-collected pollen enriched with 0.5, 5 or 10x the natural mean concentrations of (1) eleven AAs (0.5x/5x/10x AA) (Table S1, means were calculated from the dataset of Weiner *et al*. (2010)) or (2) seven FAs (0.5x/5x/10x FA) (Table S2, means calculated

from the dataset of Manning (2006)) in learning and feeding experiments. The AA and FA concentrations tested occur in natural pollen, except for the 10x concentration, which exceeds the upper limits of known concentrations (Roulston & Cane 2000; Manning 2006; Weiner *et al*. 2010). AA and FA contents of the bee-collected pollen used in our experiments were analysed as described in Methods S1. Contents were within the natural range as calculated for hand-collected pollen (Table S1 and S2).

Note that we did not include oleic acid and linoleic acid in the FA mixture, because these FA modify the pollen paste texture in a way that prevents testing. However, as oleic and linoleic acid are both beneficial to bees and were found to support cognitive performance in bees (Arien *et al*. 2015; Arien *et al*. 2018; Muth *et al*. 2018), we tested them separately in the learning experiments (see below).

Learning experiments

We used a recently established technique, chemotactile differential conditioning of the proboscis extension response (PER) (Ruedenauer *et al*. 2015), to test whether *Bombus terrestris* workers can differentiate pollen differing in its AA and FA content. The PER learning assay is based on classical conditioning (Pavlov 1927) and allows testing whether or not bees can learn to differentiate between two sensory cues (e.g. food with two different concentrations of the same nutrient). While other methods, such as electroantennographic measurements or single-sensillum recordings investigate cue processing at the receptor level, PER conditioning ultimately tests perception and thus the effect of specific cues on actual behavioural responses. In contrast, signals measured at the receptor level can be modified or even nullified on their way from receptors to processing centres in the brain (Eltz & Lunau 2005) and thus have no or little impact on an animal's behaviour. PER conditioning thus enabled us to test the bees' ability for preingestive perception by means of the antennae and consequently the bees' assessment of pollen nutritional quality-based on specific nutrients.

The experimental setup of the PER experiments was based on Sommerlandt *et al*. (2014) and Ruedenauer *et al*. (2015). Bumblebees covering the full size spectrum from small to large workers were caught from the foraging chamber and chilled on ice for 15 min. Thereafter, they were placed inside tubes made of plastic pipette tips (Hartenstein, Würzburg, Germany) and fixed with 'yokes' made of paper clips (Sommerlandt *et al*. 2014; Ruedenauer *et al*. 2015). Head and forelegs were unfixed to allow movement and a proper PER. The bees were then placed in a rack equipped with damp cloth and fed with a 0.5 M sucrose solution. The rack was placed in a climate chamber (see above) for 25 h. On the next day, each individual was tested for a proper PER by holding a toothpick soaked with 0.5 M sucrose solution to its antennae. Only bumblebees showing a proper PER were used for the experiments. We used a standardised PER protocol (e.g. Bitterman *et al*. 1983; Laloi *et al*. 1999) with differential chemotactile conditioning (Ruedenauer *et al*. 2015). This method uses two different conditioned stimuli (CS), one rewarded (CS+) with sucrose solution (unconditioned stimulus, US) and one unrewarded (CS−). Bees were placed in a test rack and allowed to rest for 15 s, before a CS was presented to the antenna for 6 s using a copper stick mounted on a micromanipulator (Ruedenauer *et al*. 2015). As soon as the bee touched the CS, the 6 s time interval was started to test for a PER. In case of the CS+, a tooth pick soaked with sugar water (US) was held to the antenna for the last 3 s of the CS presentation, and the bee was allowed to lick. When a CS− was presented a blank tooth pick was held near the antenna to account for a possible bias due to the tooth pick movement and thus prevent bees from using visual cues for learning. After stimulus presentation, the bee was allowed to rest for another 15 s and the next bee was tested. The intertrial interval (ITI), the time between trials of the same individual, was 8 min (Bitterman *et al*. 1983). One experimental series consisted of 20 trials presenting CS + and CS− in the following order: CS+, CS−, CS+, CS+, CS−, CS+, CS−, CS−, CS+, CS−, CS+, CS−, CS+, CS+, CS−, CS−, CS+, CS−, CS−, CS+. This order does not allow for any inferences on the next stimulus and remains stable across trials. Each individual was only tested in one experimental series. Each stimulus was tested as both CS+ and CS− in two different experimental series (referred to as "reversed meaning").

For presentation of the CS, a wet filter paper was placed on the copper sticks and about 50 mg of pollen paste was applied to this paper. The pollen and filter paper were renewed after every stimulation (each bee was stimulated with fresh pollen and filter paper). The plates were cleaned in 99% ethanol (Hartenstein, Würzburg, Germany) after each stimulation.

At first, we tested pure bee-collected pollen (PP) (henceforth referred to as pollen) against the same pollen enriched with (a) 10x AA or (b) 10x FA. Note that, despite adjusting water amounts, pollen paste texture was practically identical for pollen enriched with different amounts of FAs and only slightly different for pure pollen. To nevertheless ensure that the bees actually learned differences in nutrient concentrations and not in texture, we tested pollen enriched with 0.5x the natural mean concentrations of the seven FA (0.5x FA) against the 10x FA. We additionally tested pollen enriched with different concentrations of only linoleic and only oleic acid separately (0.5x against 10x), because these two FAs are abundant in pollen and beneficial for bees (Manning 2006), but could not be included in the mixture (see above). To finally determine whether differentiation was based on all or only specific FAs out of the nine FAs tested in total, we additionally tested each of the nine FAs against pure pollen.

Despite the use of concentrations naturally found in pollen, the addition of AAs could have exceeded the natural detection/perception range of bumblebees. To account for this possibility, we repeated the AA experiment using pollen which was first diluted with cellulose (pollen:cellulose 1:10) before adding AAs (0.5x and 10x AA) and testing each concentration against diluted pollen. The pollen types used thus contained overall lower AA concentrations which were still within the natural concentration range of pollen and provided similar relative differences as tested above. Cellulose can neither be neither smelled nor tasted by bumblebees and should therefore not affect perception (Mapalad *et al*. 2008; Ruedenauer *et al*. 2015).

Feeding experiments

We additionally tested for the effect of pollen enriched with AAs or FAs on consumption and thus changes in bee foraging behaviour post-ingestion using feeding experiments with microcolonies (Ruedenauer *et al.* 2016; Vaudo *et al.* 2016b). These queenless colonies, which consist exclusively of workers, have been proven to be fully comparable to queenright colonies for measuring nutritional requirements and fitness effects (Génissel *et al.* 2002; Tasei & Aupinel 2008).

The experimental approach followed Ruedenauer *et al.* (2016). Based on 18 mother colonies, we prepared overall 336 queenless microcolonies, each consisting of 20 randomly selected *Bombus terrestris* workers all obtained from the same mother colony. This resulted in ~ 19 microcolonies per mother colony (Table S3). Microcolonies were kept in small two-chambered wooden boxes (14.5 × 13 × 10 cm per chamber) covered with clear acrylic glass. Food was provided in one chamber (foraging chamber) and the second chamber was used for nesting (nest chamber). The boxes were kept in the laboratory in a 12/12 h light/dark cycle at 25°C. Bees had *ad libitum* access to a 2 M sucrose solution.

We performed two types of feeding experiments: choice and no choice diet experiments (Table S3). In no choice diet experiments, the bees were provided with one pollen type/one diet only, which allowed us to compare the effect of each diet on the worker bees' survival and reproduction used as measure for fitness. In choice diet experiments, bees were offered two different diets (pure pollen and enriched pollen) simultaneously to measure differences in consumption and thus foraging choices between the two diets. The choice diet experiment consequently provided information on whether or not bees discriminated between pure and enriched pollen when they could probe and ingest the pollen. In contrast, the PER learning experiments only provided information on pre-ingestive discrimination. To finally determine whether the workers relied on larval feedback for their foraging decisions, we performed each choice diet experiment with half of the microcolonies being allowed to rear brood (brood treatment) and brood removed from the other half (no brood treatment). Larval feedback could, in theory, interfere with individual worker assessment as requirements can differ between larvae and adults, resulting in different resource intake between colonies with and without brood. As microcolonies were queenless, they only produced male offspring.

Fresh pollen was provided daily in small petri dishes placed in the centre of the foraging chamber containing either one diet only (no choice diet experiments) or two different diets (choice diet experiments). For the choice diet experiment, the position of the two petri dishes was randomised across days. Each day, the dishes were weighed to quantify the food uptake from each diet by each microcolony. Evaporation was taken into account by calculating the weight loss of dishes (containing the same diets) placed outside the colonies and correcting weights of experimental dishes accordingly. As bees died over the course of the experiments and the number of individuals varied between colonies, we always divided overall food collection by the number of individuals present in each microcolony per day. We determined the effect of pollen enriched in AA or FA content on the longevity and reproductive success of bumblebees in microcolonies in the no choice diet experiments. For the survival analysis, dead individuals were recorded each day. To analyse differences in reproductive success, we recorded the number of egg clumps, larval cells, pupae and hatched drones per day.

A complete overview of the feeding experiments, treatments (no choice or choice diet, pollen type(s) offered and brood or non-brood) and numbers of microcolonies tested in each setup is shown in Table S3. In order to produce robust results, we repeated each feeding experiment at least twice between February 2017 and April 2018.

Data analysis

To analyse differences in learning performance (PER experiments), we used the number of positive responses (i.e. proboscis extension reactions) to each conditioned stimulus. First, we checked whether response numbers depended on whether a substance was used as CS+ or CS− (reversed meanings) using a Mann-Whitney U test (because data were not normally distributed). As we found no differences in any of the experiments (Table S4), data were combined following standard PER conditioning procedures (Laloi *et al.* 1999; Sommerlandt *et al.* 2014). For the comparison of responses towards the CS+ and CS− we used paired U tests to account for the fact that CS+ and CS− values were obtained from the same individual.

For each feeding experiment, we always tested first for significant effects of the "experimental period" and the "mother colony" by including these factors as random effects in a generalised linear mixed effect model (GLMM) and comparing the GLMM to a generalised linear models (GLM) without random effects (both with Gaussian distribution), following Zuur *et al.* (2009). When both or one of the random factors explained a significant proportion of the observed variance, as assessed through a likelihood ratio test for model comparison (Table S9), we performed a GLMM (*lme4* package (Bates *et al.* 2015)) with the respective random factor(s). When there was no significant effect of either one of the random factors, we performed a GLM (*lme4* package). We always tested for the effect of diet (fixed effect) on the mean pollen consumed per individual and microcolony over the whole experiment, either within one treatment (choice diet) or between treatments (no choice diet).

Differences in reproductive success between microcolonies offered different pollen diets in the no choice diet experiment were analysed either with a GLMM or a GLM (see above) analysing differences in the number of egg clumps, larval and pupal cells (newly produced or removed in relation to the previous day) between diets (fixed effect).

Differences in the survival rate of bees in the no choice diet experiment were analysed with Kaplan-Meyer survival statistics by comparing median survival times between each diet pair using log-rank tests (*survival* (Therneau & Grambsch 2013) & *KMsurv* package (Klein & Moeschberger 2006)). As this involved multiple testing, we adjusted the α-level using Bonferroni.

All statistical analyses were performed using the program R v3.5.0 (R Core Team 2018).

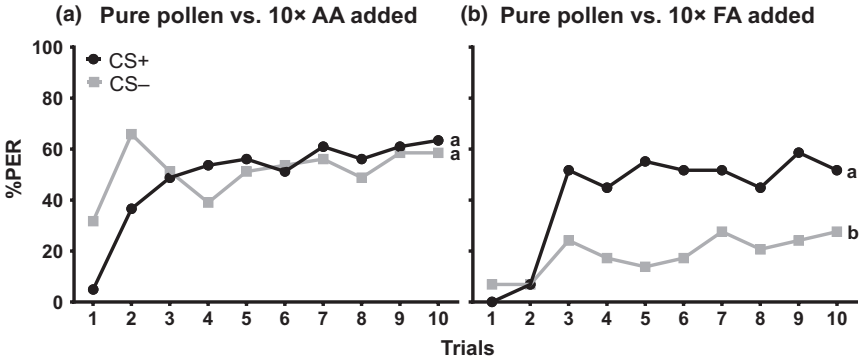

Figure 1 Percentage of proboscis extension responses (%PER) shown by *Bombus terrestris* individuals ($N = 100$) in differential chemotactile conditioning to bee-collected pollen enriched with (a) 10x the natural concentration of amino acids (AA, $N = 41$) and (b) 10x the natural concentration of fatty acids (FA, $N = 58$). CS+ (black) represents the rewarded conditioned stimulus, CS− (grey) the unrewarded conditioned stimulus. Both stimuli were used as CS+ and CS−. As there was no significant difference in learning performance between these reversed meanings (Table S4), both groups were combined. Different letters next to each line indicate a significant difference between stimuli ($P < 0.05$)

RESULTS

Learning experiments

Bumblebees differentiated between pure pollen and pollen enriched with FAs already after the first three CS+ and three CS− trials (10x FA, Fig. 1b, Table S4), while they did not learn to differentiate between pure pollen and pollen enriched with AAs even after 20 trials (Fig. 1a, Table S4). Bumblebees were also not able to differentiate between diluted pollen and diluted pollen enriched with AAs (Fig. S2). However, pollen enriched with the 0.5x FA and 10x FA mixtures as well as with linoleic and oleic acid (0.5x and 10x) were again clearly differentiated by the bumblebees after six CS+ and six CS− trials (Fig. S3, Table S4). Bumblebees could also discriminate between pure pollen and pollen enriched with each one out of the nine FAs at similar levels as found for pollen enriched with all FAs (Fig. S4, Table S4).

Feeding experiments

Adding different concentrations of AAs did not affect overall pollen consumption of microcolonies, neither in the choice diet (Fig. S5, Table S5) nor in the no choice diet experiment (Fig. 2a, Table S5). Bumblebees in all treatments consumed on average between 11 mg (± 3 mg) and 17 mg (± 3 mg) per individual and day. Consequently, bumblebees consumed more AAs in the high AA no choice diet experiments (Fig. 2c, Table S6). However, this did not affect their reproduction (Table S7) or survival (Fig. 2e, Table S8).

In contrast, bumblebees showed clear preferences for pure pollen over pollen enriched with FAs at ecologically relevant (low) concentrations in the choice diet experiment, independently of the presence of brood (Fig. 3, Table S5). While they consumed on average 18 mg (± 5 mg) of pure pollen, they only consumed on average 7 mg (± 3 mg) per individual and day of pollen enriched with FAs. Consequently, consumption of FAs decreased (to less than 2 mg (± 0.5 mg) per individual and day) with increasing pollen FA content (Fig. 2d, Table S6). Pollen enriched with FAs also significantly reduced reproduction (Table S7) and survival (Fig. 2f, Table S8). Survival decreased by 80% in the high FA treatment compared to pure pollen. Colonies in the middle and high FA treatments were only able to produce four egg clumps in total, none of which developed into larvae.

DISCUSSION

Our results demonstrate that *Bombus terrestris* workers focus perception on and thus learn one particular nutrient group, fatty acids (FAs), while ignoring others, e.g. amino acids (AAs), when assessing pollen nutritional quality. Moreover, while FAs are essential for bees (Arien *et al*. 2015; Annoscia *et al*. 2017), increased FA concentrations in pollen had a more detrimental effect on survival and reproductive fitness than AAs. Our results consequently suggest that, when assessing pollen nutritional quality, *B. terrestris*, and potentially also other bees, 'prioritise' perception of one particular nutritional cue, which also appears to be the nutrient with the strongest fitness consequences.

Links between nutrition and fitness, here defined as individual and microcolony survival and reproduction, have been repeatedly demonstrated in several insects, including bees (i.e. Keller *et al*. 2005; Alaux *et al*. 2010; Brodschneider & Crailsheim 2010; Archer *et al*. 2014; Roger *et al*. 2017). However, none of these studies investigated the role of nutrient perception or how perception may be linked to nutrient regulation and individual/colony fitness.

Queenright colonies and queenless microcolonies (as used in our feeding experiments) are comparable in terms of nutritional intake and reproductive behaviour (Génissel *et al*. 2002; Tasei & Aupinel 2008). Moreover, unlike different castes in honeybees, bumblebee workers, queens and drones receive food of equal nutritional composition (Pereboom 2000), which suggests that they have the same (or at least similar) nutritional requirements and are therefore similarly affected by food of inappropriate quality (e.g. of high fat content).

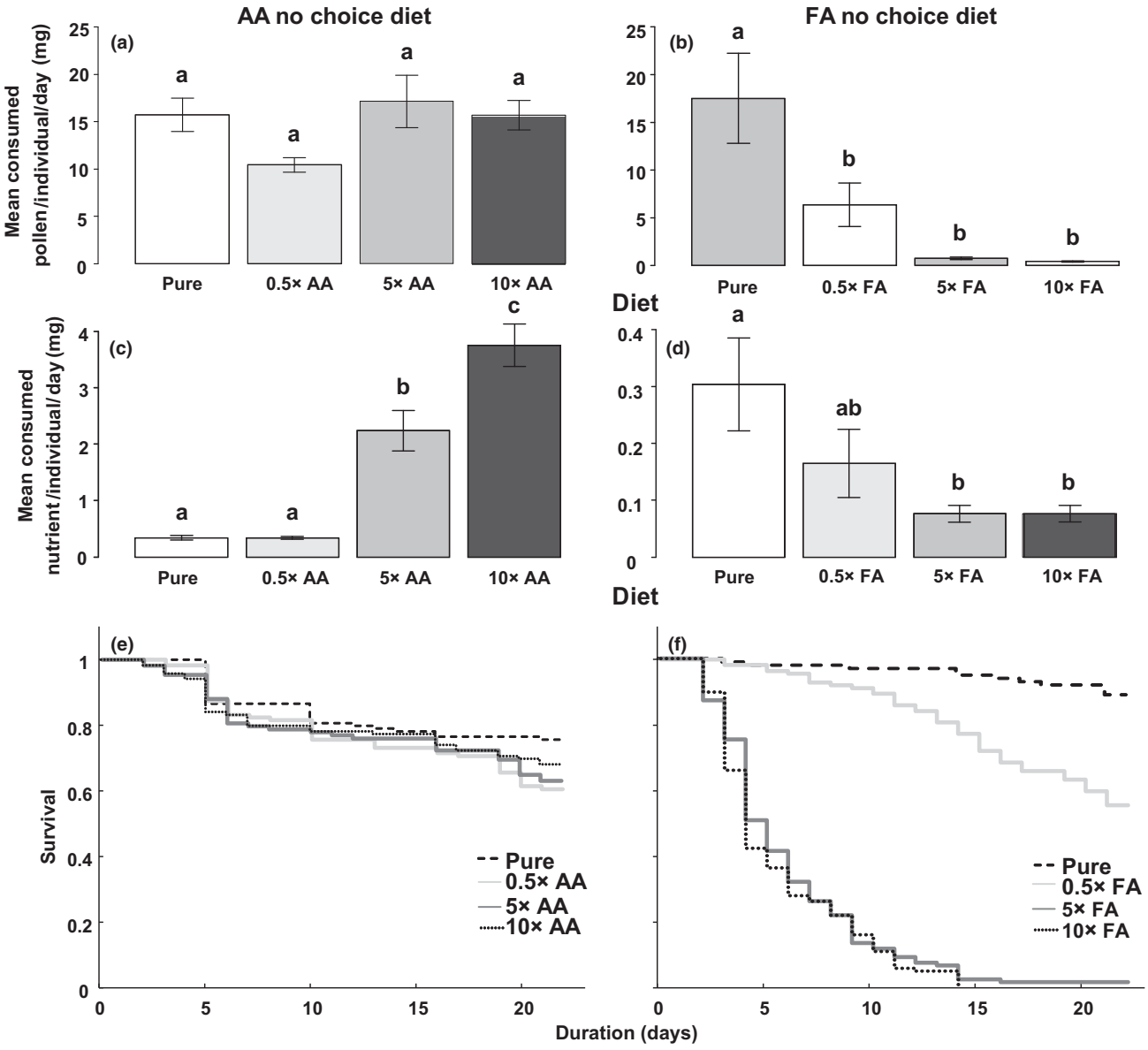

Figure 2 Consumption of pollen and nutrients and survival of *Bombus terrestris* in six microcolonies in no choice diet experiments ($N = 48$). Average daily (a and b) food and (c and d) nutrient collection [μg/individual ± SD] of different pollen diets. Bees were offered pollen enriched with (a, c, e) different concentrations of amino acids (AAs) or (b, d, f) different concentrations of fatty acids (FAs). Different letters above the bars indicate significant differences ($P < 0.05$) between pollen diet/nutrient consumption according to Tukey post hoc pairwise comparisons (see Tables S5 & S6). (e and f) Average survival probability of *Bombus terrestris* individuals. There was no difference in the survival of (e) individuals fed with different AA diets (Table S9, S10), but (f) individuals fed with 0.5x, 5x and 10x FA diets died faster compared to individuals fed pure pollen, and individuals fed with 5x and 10x FA diets died faster compared to individuals fed 0.5x FA diets (Table S8)

Fitness effects (i.e. effects on worker reproduction and survival) observed in microcolonies do consequently most likely apply to queenright colonies and to the reproduction of virgin queens and drones (Génissel *et al.* 2002; Tasei & Aupinel 2008). Moreover, workers are largely responsible for provisioning the colony. If they avoid specific pollen sources in the field or die faster as a consequence of inappropriate quality (e.g. high fat content), the colony as a whole will be affected and potentially starve. We are therefore confident that the negative fitness consequences found in our experiments with microcolonies fed pollen enriched with FAs can be directly related to queenright colonies.

Similar negative effects of FAs on survival were also shown for honeybees (*Apis mellifera*) by Manning *et al.* (2007). In fact, high FA concentrations in food can limit the uptake rate of FAs by midgut cells (as reviewed by Canavoso *et al.* 2001), which can subsequently damage cell membranes (Haddad *et al.* 2007) and may explain why *B. terrestris* workers strongly avoided consuming pollen enriched with FAs (Fig. 2b and d). Consequently, the observed negative survival and fitness effect of FAs in pollen were most likely due to a combination of both intoxication with excessive FA amounts (Canavoso *et al.* 2001; Haddad *et al.* 2007; Manning *et al.* 2007) and a lack of other essential nutrients as a consequence

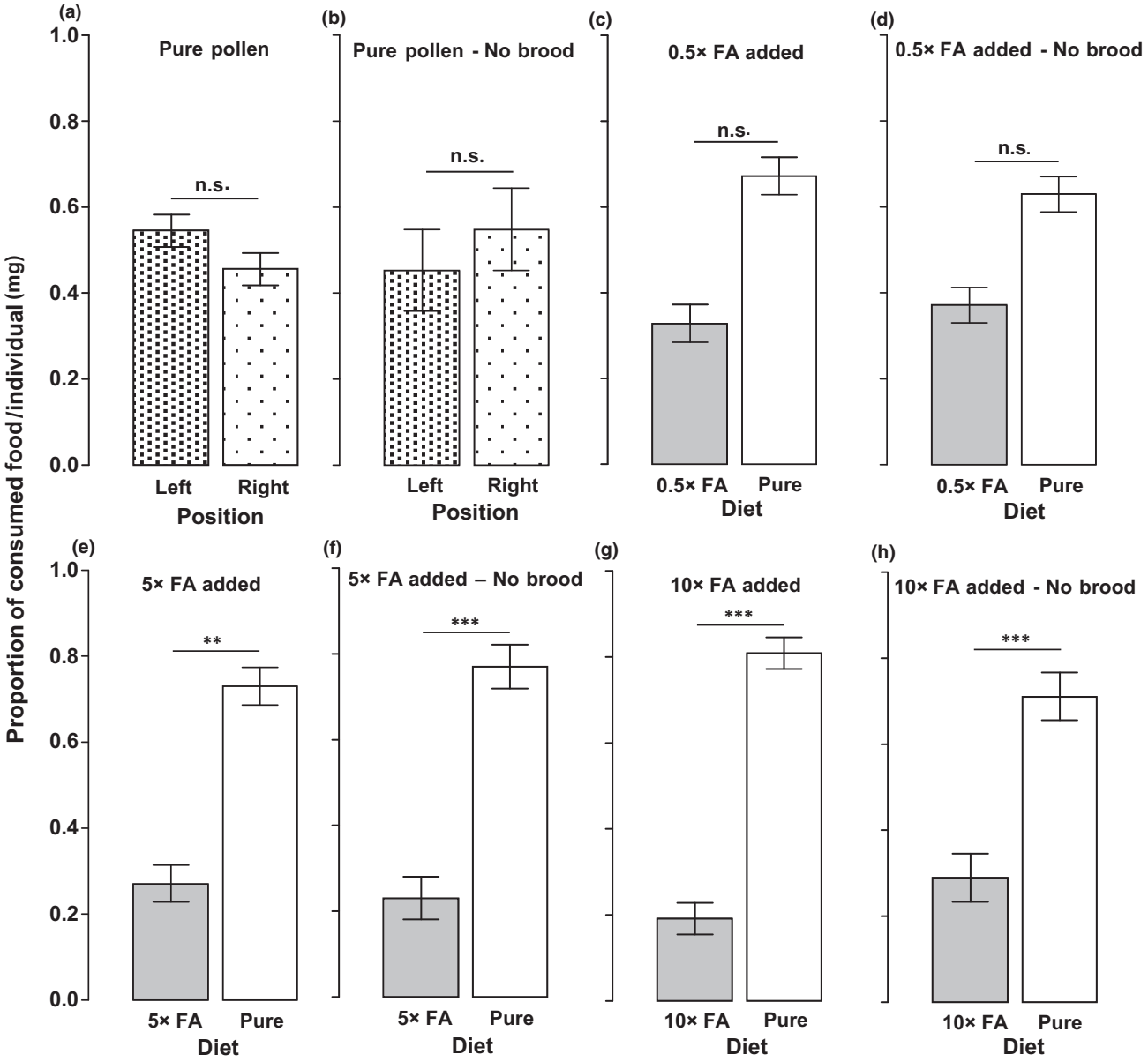

Figure 3 Proportion of daily food collected [mean proportion food collected/individual ± standard deviation (SD)] from each of two different pollen diets offered to individuals of 12 *Bombus terrestris* microcolonies in choice diet feeding experiments ($N = 96$). Bees were offered a choice between (a and b) two pure pollen diets as control, (c and d) pure pollen and pollen enriched with 0.5x the natural concentration of fatty acids (FAs), (e and f) pure pollen and pollen enriched with 5x the natural FA concentrations and (g and h) pure pollen and pollen enriched with 10x the natural FA concentration. In half of the treatments, colonies were allowed to raise brood, while the in the other half of the treatments, egg clumps were removed daily (No brood). Significance levels: n.s. = not significant, $^{**}P < 0.01$, $^{***}P < 0.001$

of reduced overall pollen consumption (Rodriguez *et al.* 1993; Simpson & Raubenheimer 2012) due to fat avoidance. Both intoxication and a lack of nutrients will ultimately impact on reproduction (Human *et al.* 2007; Pirk *et al.* 2010) and reduce survival.

Our findings seem to contradict the frequently discussed importance of pollen protein and AAs for bumblebee foraging, and suggest an at least equally important role of fat/FAs. However, most previous studies only considered protein/AA content but rarely fat/FA content. It is possible that contents of both macronutrients are naturally correlated in pollen, e.g. due to linked biosynthesis pathways. For example, a negative correlation between protein and fat, as found by Ruedenauer *et al.* (2019) for pollen collected by bees, might enable bees to select a high P:L ratio (Vaudo *et al.* 2016b) through focusing on a reduced fat/FA intake.

Notably, bumblebees are capable of receiving and perceiving specific AAs and of learning differences in AA concentrations, at least when AAs are dissolved in water and not in pollen (Ruedenauer *et al.* 2019). Likewise, honeybees appear to use AAs to select nectar rich in (essential) AAs, which they prefer over nectar poor in (essential) AAs (Alm *et al.* 1990;

Hendriksma *et al*. 2014). Such context- or food resource-dependent cue perception suggests that bees are not only sensitive to the nutritional quality of collected food, but also adjust their sensory perception to the nutritional profile and dietary role of specific food resources. In fact, different types of food (e.g. pollen vs. nectar) appear to be subject to different nutritional quality measures (e.g. pollen quality may be mostly assessed by its fat, nectar quality by its sugar and AA content), likely because they have different dietary roles (e.g. pollen provides protein, fat and micronutrients, while nectar is the main sugar and thus energy source). When regulated nutrients occur in combination with other nutrients, as is e.g. the case for fat(ty acids) in pollen, perception of cues directly related to regulated nutrients and reproductive fitness seems to be 'prioritised'. Reception of other nutrients, e.g. AAs, at the receptor level may however still take place. A simple mechanism explaining this 'perceptional prioritisation' would be that the FA input, as soon as present, is overlaying the AA input, either through receptive reinforcement (Abisgold & Simpson 1988; Simpson *et al*. 1991) or through adaptations of the received information at the brain level (Eltz & Lunau 2005), which may lead to a modification or even full extinction of "non-relevant" (nutritional) cues. Such processing would enable a specific and context-dependent nutritional quality assessment, as observed in our study.

Our results are thus in contrast with our expectations and with assumptions of previous studies suggesting that bees, like many other herbivores, regulate protein intake when collecting pollen. We suggest that, instead, *Bombus terrestris* workers, and potentially also other bees, focus on fat regulation when collecting pollen and use FAs as major nutritional cue for nutritional quality assessment. Moreover, we show, for the first time in insects, that perception, nutrient regulation and fitness can be linked for a specific resource (Fig. S6). 'Prioritised perception' of nutritional cues/nutrients, which are most closely linked to fitness, may represent a most valuable, highly efficient and evolutionary beneficial strategy for foraging animals.

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CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHORSHIP

SDL, JS, FAR and DR conceived the experimental concepts. FAR, DKB, NGM and LN performed the experiments. FAR analysed the data. FAR, SDL and JS drafted the manuscript. All authors discussed the results, commented on the draft and agreed to the final version.

DATA AVAILABILITY STATEMENT

All relevant data available on: https://osf.io/ry2wp/?view_only = d255d00d3bb74ca5a34f85fcfa4b11b3

REFERENCES

Abisgold, J.D. & Simpson, S.J. (1988). The effect of dietary protein levels and haemolymph composition on the sensitivity of the maxillary palp chemoreceptors of locusts. *J. Exp. Biol*., 135, 215–229.

Alaux, C., Ducloz, F., Crauser, D. & Le Conte, Y. (2010). Diet effects on honeybee immunocompetence. *Biol. Let*., 6, 562–565.

Alm, J., Ohnmeiss, T.E., Lanza, J. & Vriesenga, L. (1990). Preference of cabbage white butterflies and honey bees for nectar that contains amino acids. *Oecologia*, 84, 53–57.

Annoscia, D., Zanni, V., Galbraith, D., Quirici, A., Grozinger, C., Bortolomeazzi, R. *et al*. (2017). Elucidating the mechanisms underlying the beneficial health effects of dietary pollen on honey bees (*Apis mellifera*) infested by *Varroa* mite ectoparasites. *Sci. Rep*., 7, 6258.

Archer, C.R., Pirk, C.W.W., Wright, G.A. & Nicolson, S.W. (2014). Nutrition affects survival in African honeybees exposed to interacting stressors. *Funct. Ecol*., 28, 913–923.

Arganda, S., Bouchebti, S., Bazazi, S., Le Hesran, S., Puga, C., Latil, G. *et al*. (2017). Parsing the life-shortening effects of dietary protein: effects of individual amino acids. *Proceedings of the Royal Society B: Biological Sciences*, 284.

Arien, Y., Dag, A., Zarchin, S., Masci, T. & Shafir, S. (2015). Omega-3 deficiency impairs honey bee learning. *Proc. Natl. Acad. Sci*., 112, 15761–15766.

Arien, Y., Dag, A. & Shafir, S. (2018). Omega- 6: 3 ratio more than absolute lipid level in diet affects associative learning in honey bees. *Front. Psychol*., 9, https://doi.org/10.3389/fpsyg.2018.01001

Bates, D., Mächler, M., Bolker, B. & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *J. Stat. Softw*., 67, 1–48.

Bazazi, S., Arganda, S., Moreau, M., Jeanson, R. & Dussutour, A. (2016). Responses to nutritional challenges in ant colonies. *Anim. Behav*., 111, 235–249.

Biesalski, H.K. (2017). *Unsere Ernährungsbiografie: Wer sie kennt, lebt gesünder*. Albrecht Knaus Verlag, München.

Bitterman, M.E., Menzel, R., Fietz, A. & Schäfer, S. (1983). Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). *J. Comp. Psychol*., 97, 107–119.

Bray, G.A., Smith, S.R., de Jonge, L., Xie, H., Rood, J., Martin, C.K. *et al*. (2012). Effect of dietary protein content on weight gain, energy expenditure, and body composition during overeating: a randomized controlled trial. *JAMA*, 307, 47–55.

Brodschneider, R. & Crailsheim, K. (2010). Nutrition and health in honey bees. *Apidologie*, 41, 278–294.

Brunner, F.S., Schmid-Hempel, P. & Barribeau, S.M. (2014). Protein-poor diet reduces host-specific immune gene expression in *Bombus terrestris*. *P. Roy. Soc. B: Biol. Sci*., 281.

Canavoso, L.E., Jouni, Z.E., Karnas, K.J., Pennington, J.E. & Wells, M.A. (2001). Fat metabolism in insects. *Annu. Rev. Nutr*., 21, 23–46.

Di Pasquale, G., Salignon, M., Le Conte, Y., Belzunces, L.P., Decourtye, A., Kretzschmar, A. *et al*. (2013). Influence of pollen nutrition on honey bee health: do pollen quality and diversity matter? *PLoS ONE*, 8, e72016.

Dobson, H.E.M., Groth, I. & Bergstrom, G. (1996). Pollen advertisement: chemical contrasts between whole-flower and pollen odors. *Am. J. Bot*., 83, 877–885.

Dobson, H.E.M., Danielson, E.M. & Wesep, I.D.V. (1999). Pollen odor chemicals as modulators of bumble bee foraging on *Rosa rugosa* Thunb. (Rosaceae). *Plant. Spec. Biol*., 14, 153–166.

Dussutour, A. & Simpson, S.J. (2008). Carbohydrate regulation in relation to colony growth in ants. *J. Exp. Biol*., 211, 2224–2232.

Eltz, T. & Lunau, K. (2005). Antennal response to fragrance compounds in male orchid bees. *Chemoecology*, 15, 135–138.
Génissel, A., Aupinel, P., Bressac, C., Tasei, J.N. & Chevrier, C. (2002). Influence of pollen origin on performance of *Bombus terrestris* micro-colonies. *Entomol. Exp. Appl.*, 104, 329–336.
Gosby, A.K., Conigrave, A.D., Lau, N.S., Iglesias, M.A., Hall, R.M., Jebb, S.A. *et al.* (2011). Testing protein leverage in lean humans: a randomised controlled experimental study. *PLoS ONE*, 6, e25929.
Goulson, D., Nicholls, E., Botías, C. & Rotheray, E.L. (2015). Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science*, 347.
Grüter, C., Segers, F.H.I.D. & Ratnieks, F.L.W. (2013). Social learning strategies in honeybee foragers: do the costs of using private information affect the use of social information? *Anim. Behav.*, 85, 1443–1449.
Haddad, L.S., Kelbert, L. & Hulbert, A.J. (2007). Extended longevity of queen honey bees compared to workers is associated with peroxidation-resistant membranes. *Exp. Gerontol.*, 42, 601–609.
Hallmann, C.A., Sorg, M., Jongejans, E., Siepel, H., Hofland, N., Schwan, H. *et al.* (2017). More than 75 percent decline over 27 years in total flying insect biomass in protected areas. *PLoS ONE*, 12, e0185809.
Helm, B.R., Slater, G., Rajamohan, A., Yocum, G.D., Greenlee, K.J. & Bowsher, J.H. (2017). The geometric framework for nutrition reveals interactions between protein and carbohydrate during larval growth in honey bees. *Biol. Open.*, 6, 872–880.
Hendriksma, H.P., Oxman, K.L. & Shafir, S. (2014). Amino acid and carbohydrate tradeoffs by honey bee nectar foragers and their implications for plant–pollinator interactions. *J. Insect Physiol.*, 69, 56–64.
Human, H., Nicolson, S.W., Strauss, K., Pirk, C.W.W. & Dietemann, V. (2007). Influence of pollen quality on ovarian development in honeybee workers (*Apis mellifera scutellata*). *J. Insect Physiol.*, 53, 649–655.
Jensen, K., Mayntz, D., Toft, S., Raubenheimer, D. & Simpson, S.J. (2011). Nutrient regulation in a predator, the wolf spider *Pardosa prativaga*. *Anim. Behav.*, 81, 993–999.
Kämper, W., Werner, P.K., Hilpert, A., Westphal, C., Blüthgen, N., Eltz, T. *et al.* (2016). How landscape, pollen intake and pollen quality affect colony growth in *Bombus terrestris*. *Landscape Ecol.*, 1–14.
Keller, I., Fluri, P. & Imdorf, A. (2005). Pollen nutrition and colony development in honey bees: part 1. *Bee World*, 86, 3–10.
Kitaoka, T.K. & Nieh, J.C. (2009). Bumble bee pollen foraging regulation: role of pollen quality, storage levels, and odor. *Behav. Ecol. Sociobiol.*, 63, 501–510.
Klein, J.P. & Moeschberger, M.L. (2006). *Survival analysis: techniques for censored and truncated data*. Springer, New York, New York.
Kriesell, L., Hilpert, A. & Leonhardt, S.D. (2017). Different but the same: bumblebee species collect pollen of different plant sources but similar amino acid profiles. *Apidologie*, 48, 102–116.
Laloi, D., Sandoz, J.C., Picard-Nizou, A.L. & Pham-Delegue, M.H. (1999). Olfactory conditioning of the proboscis extension reflex in the bumble bee *Bombus terrestris*. *Ann. Soc. Entomol. Fr.*, 35, 154–158.
Lee, K.P., Simpson, S.J., Clissold, F.J., Brooks, R., Ballard, J.W.O., Taylor, P.W. *et al.* (2008). Lifespan and reproduction in Drosophila: New insights from nutritional geometry. *Proc. Natl. Acad. Sci. U.S.A.*, 105(7), 2498–2503
Leonhardt, S.D. & Blüthgen, N. (2012). The same, but different: pollen foraging in honeybee and bumblebee colonies. *Apidologie*, 43, 449–464.
Manning, R. (2006). *Fatty acid composition of pollen and the effect of two dominant fatty acids (linoleic and oleic) in pollen and flour diets on longevity and nutritional composition of honey bees (Apis mellifera)*. PhD thesis, Murdoch University, Perth.
Manning, R., Rutkay, A., Eaton, L. & Dell, B. (2007). Lipid-enhanced pollen and lipid-reduced flour diets and their effect on the longevity of honey bees (*Apis mellifera* L.). *Australian Journal of Entomology*, 46, 251–257.
Mapalad, K.S., Leu, D. & Nieh, J.C. (2008). Bumble bees heat up for high quality pollen. *J. Exp. Biol.*, 211, 2239–2242.
Mayntz, D., Raubenheimer, D., Salomon, M., Toft, S. & Simpson, S.J. (2005). Nutrient-specific foraging in invertebrate predators. *Science*, 307, 111–113.
Moerman, R., Vanderplanck, M., Fournier, D., Jacquemart, A.-L. & Michez, D. (2017). Pollen nutrients better explain bumblebee colony development than pollen diversity. *Insect Conserv. Divers.*, 10(2), 171–179.
Muth, F., Breslow, P.R., Masek, P. & Leonard, A.S. (2018). A pollen fatty acid enhances learning and survival in bumblebees. *Behav. Ecol.*, 29, 1371–1379.
Naug, D. (2009). Nutritional stress due to habitat loss may explain recent honeybee colony collapses. *Biol. Conserv.*, 142, 2369–2372.
Palmer-Young, E.C., Farrell, I.W., Adler, L.S., Milano, N.J., Egan, P.A., Irwin, R.E. *et al.* (2019a). Secondary metabolites from nectar and pollen: a resource for ecological and evolutionary studies. *Ecology*, 100, e02621.
Palmer-Young, E.C., Farrell, I.W., Adler, L.S., Milano, N.J., Egan, P.A., Junker, R.R. *et al.* (2019b). Chemistry of floral rewards: intra- and interspecific variability of nectar and pollen secondary metabolites across taxa. *Ecol. Monogr.*, 89, e01335.
Pavlov, I.P. (1927). *Conditioned reflexes*. Oxford University Press, Oxford.
Pereboom, J.J.M. (2000). The composition of larval food and the significance of exocrine secretions in the bumblebee Bombus terrestris. *Ins. Soc.*, 47, 11–20.
Pirk, C.W.W., Boodhoo, C., Human, H. & Nicolson, S. (2010). The importance of protein type and protein to carbohydrate ratio for survival and ovarian activation of caged honeybees (*Apis mellifera scutellata*). *Apidologie*, 41, 62–72.
Potts, S.G., Biesmeijer, J.C., Kremen, C., Neumann, P., Schweiger, O. & Kunin, W.E. (2010). Global pollinator declines: trends, impacts and drivers. *Trends Ecol. Evol.*, 25, 345–353.
R Core Team. (2018). R: A language and environment for statistical computing. In: R Foundation for Statistical Computing. R Foundation for Statistical Computing Vienna, Austria.
Raubenheimer, D. & Simpson, S.J. (1993). The geometry of compensatory feeding in the locust. *Anim. Behav.*, 45, 953–964.
Raubenheimer, D. & Simpson, S.J. (1999). Integrating nutrition: a geometrical approach. *Entomol. Exp. Appl.*, 91, 67–82.
Rodriguez, A.E., Tingey, W.M. & Mutschler, M.A. (1993). Acylsugars of *Lycopersicon pennellii* deter settling and feeding of the green peach aphid (Homoptera: Aphididae). *J. Econ. Entomol.*, 86, 34–39.
Roger, N., Michez, D., Wattiez, R., Sheridan, C. & Vanderplanck, M. (2017). Diet effects on bumblebee health. *J. Insect Physiol.*, 96, 128–133
Roulston, T.H. & Cane, J.H. (2000). Pollen nutritional content and digestibility for animals. *Plant Syst. Evol.*, 222, 187–209.
Ruedenauer, F.A., Spaethe, J. & Leonhardt, S.D. (2015). How to know which food is good for you: bumblebees use taste to discriminate between different concentrations of food differing in nutrient content. *J. Exp. Biol.*, 218, 2233–2240.
Ruedenauer, F.A., Spaethe, J. & Leonhardt, S.D. (2016). Hungry for quality—individual bumblebees forage flexibly to collect high-quality pollen. *Behav. Ecol. Sociobiol.*, 70, 1209–1217.
Ruedenauer, F.A., Spaethe, J., van der Kooi, C.J. & Leonhardt, S.D. (2019). Pollinator or pedigree: which factors determine the evolution of pollen nutrients? *Oecologia*, 191, 349–358.
Seibold, S., Gossner, M.M., Simons, N.K., Blüthgen, N., Müller, J., Ambarlı, D. *et al.* (2019). Arthropod decline in grasslands and forests is associated with landscape-level drivers. *Nature*, 574, 671–674.
Simopoulos, A.P. (2002). The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed. Pharmacother.*, 56, 365–379.
Simpson, S.J. & Raubenheimer, D. (2009). Macronutrient balance and lifespan. *Aging-US*, 1, 875–880.
Simpson, S.J. & Raubenheimer, D. (2012). *The nature of nutrition: a unifying framework from animal adaptation to human obesity*. Princeton University Press, Princeton.
Simpson, S.J., James, S., Simmonds, M.S.J. & Blaney, W.M. (1991). Variation in chemosensitivity and the control of dietary selection behaviour in the locust. *Appetite*, 17, 141–154.

Solms, J. (1969). Taste of amino acids, peptides, and proteins. *J. Agr. Food. Chem.*, 17, 686–688.

Sommerlandt, F.M.J., Rossler, W. & Spaethe, J. (2014). Elemental and non-elemental olfactory learning using PER conditioning in the bumblebee, *Bombus terrestris*. *Apidologie*, 45, 106–115.

Su, J.W. & Gao, F. (2007). Growth rate and anticipated longevity of *Chrysopa phyllochroma* Wesmael (Neuroptera : Chrysopidae) larvae fed on *Aphis gossypii* Glover (Homoptera : Aphididae). *Appl. Entomol. Zoolog.*, 42, 41–47.

Tasei, J.-N. & Aupinel, P. (2008). Validation of a method using queenless *Bombus terrestris* micro-colonies for testing the nutritive value of commercial pollen mixes by comparison with queenright colonies. *J. Econ. Entomol.*, 101, 1737–1742.

Therneau, T.M. & Grambsch, P.M. (2013). *Modeling Survival Data: Extending the Cox Model*. Springer, New York, New York.

Vaudo, A.D., Patch, H.M., Mortensen, D.A., Tooker, J.F. & Grozinger, C.M. (2016a). Macronutrient ratios in pollen shape bumble bee (*Bombus impatiens*) foraging strategies and floral preferences. *Proc. Natl. Acad. Sci.*, 113(28), E4035–E4042

Vaudo, A.D., Stabler, D., Patch, H.M., Tooker, J.F., Grozinger, C.M. & Wright, G.A. (2016b). Bumble bees regulate their intake of the essential protein and lipid pollen macronutrients. *J. Exp. Biol.*, 219(24), 3962–3970.

Vaudo, A.D., Farrell, L.M., Patch, H.M., Grozinger, C.M. & Tooker, J.F. (2018). Consistent pollen nutritional intake drives bumble bee (*Bombus impatiens*) colony growth and reproduction across different habitats. *Ecol. Evol.*, 8, 5765–5776.

Weiner, C.N., Hilpert, A., Werner, M., Linsenmair, K.E. & Blüthgen, N. (2010). Pollen amino acids and flower specialisation in solitary bees. *Apidologie*, 41, 476–487.

Zuur, A.F., Ieno, E.N., Walker, N., Saveliev, A.A. & Smith, G.M. (2009). *Mixed Effects Models and Extensions in Ecology with R*. Springer Science & Business Media, Berlin.

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