

SHORT COMMUNICATION

Ratios rather than concentrations of nutritionally important elements may shape honey bee preferences for ‘dirty water’

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Abstract. 1. Honey bees require minerals for a complete diet. However, minerals from flowers can be inadequate in concentration and composition. Therefore, honey bees may drink ‘dirty water’ from natural sources such as puddles. Some research has attempted to simulate this through honey bee bioassays, but to date, these have tested minerals individually, not as mixtures as would occur in nature. Here, for the first time, we use honey bees in bioassays in which a range of mineral mixtures are presented together in choice experiments.

2. Six minerals (NaCl, KCl, CaCl₂, MgCl₂, NH₄Cl, and KH₂PO₄) were used in mixtures to simulate different mineral stoichiometries, which may occur in ‘dirty water’, such as puddles, from which honey bees often drink. Based on the honey bee mineral tolerance ranges from the literature, these mixtures were offered in aqueous solutions at low, medium, high, and mixed molar concentrations. Deionised water and sucrose were neutral and positive controls, respectively. Petri dishes were set up in containers in a laboratory. Twenty worker honey bees (*Apis mellifera* L.) were placed into each container and observed for drinking behaviour for 1 h.

3. Honey bees preferred the mixed molar treatment comprising a high Na:K ratio, a medium molarity of NaCl and a low molarity of the other minerals. This novel finding suggests that mixed mineral ‘dirty water’ should be investigated on a larger scale with multiple hives in the field and highlights the importance of stoichiometrically balanced honey bee diets.

Key words. ‘Dirty water’, elements, foraging, honey bees, minerals, stoichiometry.

Introduction

The honey bee, *Apis mellifera* L., is a crucially important pollinator and is currently facing multiple stressors making management more difficult such as additional pests and diseases, and reduced floral diversity (Brosi *et al.*, 2017; Requier *et al.*, 2017). Understanding the factors involved in maintaining or improving honey bee fitness remains crucial, such as their diets. Nutrient-rich ‘dirty water’ sources are a key aspect of honey bee diets to supplement the poor stoichiometric balance of elements

that their pollen diets sometimes lack (Bonoan *et al.*, 2017; Filipiak *et al.*, 2017).

Ecological stoichiometry considers building up and maintaining a body to involve a myriad of biochemical reactions, which are subject to limitation unless they are chemically balanced (Sterner & Elser, 2002). In an unbalanced state, a scarcity of specific atoms in reactants limits the reaction, which in living organisms leads to an inability to synthesise vital molecules even if food is unrestricted and unlimited. Therefore, unbalanced ratios of certain elements in food can limit honey bee growth and development due to the stoichiometric mismatch between nutritional demand and supply (Filipiak & Filipiak, 2020; Filipiak *et al.*, 2021). In particular, the scarcity of elements, such as Na, in honey bee diets can be important

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negative factors for honey bee health and fitness (Filipiak *et al.*, 2017).

Although honey bee mineral preferences have recently been tested in the field and laboratory as individual solutions (Dorian & Bonoan, 2016; Lau & Nieh, 2016; Bonoan *et al.*, 2017), no research has investigated honey bee preferences for combinations of these minerals at varying molar ratios. Therefore, the ecological processes investigated here were the extent to which honey bee workers can respond to small differences of mineral composition in their diet. We hypothesised that honey bee workers will exhibit a preference for a specific ratio of nutritional elements in a solution.

Materials and methods

Six minerals (NaCl, KCl, CaCl₂, MgCl₂, NH₄Cl, and KH₂PO₄) were used to compose four solutions each having various molar ratios of *Na:K* as well as different concentrations of Na, K, Ca, Mg, N, and P, based on work by Dorian and Bonoan (2016), Lau and Nieh (2016) and Bonoan *et al.* (2017). These solutions were low, medium, high, and mixed molar concentrations of the minerals, the latter of which contained a mix of medium molarity of NaCl and low molarity of the other five minerals. These were derived from Filipiak *et al.* (2017) as being important in limitation of honey bee health and fitness. For example, these elements play important roles in honey bee larval development (Herbert Jr. & Shimanuki, 1978), haemolymph, and antioxidant status (Zhang & Xu, 2015). Molar concentrations were used here in place of percentage concentrations because the latter are biased by elements having different molecular weights. Deionised water and 20% sucrose solution were neutral and positive controls, respectively. Details are provided in (Table 1).

Four millilitres of each treatment were poured into individual 60 mm/5 ml Petri dishes with a 60 mm diameter cotton pad placed on top of the solution, so that honey bees could land. These Petri dishes were then randomly placed (using a random number generator, www.random.org) 10 cm apart in a transparent W30 × D10 × H50 cm plastic container, with a 0.5 mm mesh-covered lid, within a W60 × D60 × H60 cm transparent cage (BugDorm 2120F; <https://shop.bugdorm.com/bugdorm-2120f-insect-rearing-tent-p-4.html>). This unit constituted the first replicate. A second replicate of the experiment was set up in a cage, next to the first, so that two independent replicates of the experiment were run simultaneously.

Different groups, each with 40 honey bee workers, were randomly collected from a hive on Ellesmere Junction Road, Lincoln, New Zealand (coordinates: 43°64'21.2"S, 172°46'78.6"E) every morning and afternoon across the 12 days that the experiment was run. The experiment had 12 morning and 12 afternoon sets of two replicates over January and February 2020. The 40 honey bees collected for the morning experiment were retained while the afternoon group was collected, so there was no mixing of the bees over 1 day. As honey bee hives contain tens of thousands of worker bees, the likelihood of the same worker individuals being used between days is extremely low. Prior to each experiment, honey bees were placed into a 4 °C refrigerator for a 15-min cooling period, then 20 bees were selected at

random as a group and placed into each mesh-covered container using forceps. Each group of 20 bees in a cage comprised one replicate, and the two replicates run simultaneously were combined prior to data analysis (as a simpler alternative to running a hierarchical statistical analysis with replicates nested within days, which would also have resulted in summation of the data from the two replicates run simultaneously). It would have been unrealistic to utilise more than 20 bees in the size of the BugDorm used here as intra-hive worker competition is unlikely in the field. A 45-min acclimatisation period was allowed, with the aid of a fan heat source 3 m away from the experiment to aid the honey bees to resume active foraging.

Following this period, honey bees in the two replicates were simultaneously continuously observed for 1 h. A 'drinking event' was characterised as honey bee proboscis extension onto the cotton pad and at least 3 s of abdominal pumping/contractions that characterised fluid intake (Lau & Nieh, 2016). To ensure that replicates were independent within and between experiments, sterilised equipment, fresh solutions, and new honey bee groups were used for each.

For each of the morning and afternoon data sets, counts were summed over the two replicates on each day (so counts were total visitations per hour per 40 bees), as mentioned earlier. For a 'whole-day' data set, counts were summed over the four replicates per day. The summations were carried out so that the statistical analysis was based purely upon variations between mornings (or afternoons, or days) rather being a hierarchical analysis with two error terms, one being variations between cages within mornings and the second being residual variations between mornings (or afternoons, or days). Each of these three variables was statistically analysed with terms 'treatment' and 'day' (a 'blocking' factor) using a generalised linear model (GLM) with a Poisson error term, a logarithm 'link function' and with the 'dispersion parameter' estimated rather than fixed, using GenStat Release 20.1. For *post hoc* pairwise comparisons of all pairs of the means, each GLM gave a 'prediction' of the means and estimated a least significant difference (LSD) at $P < 0.05$ for comparing each of the $15 = {}^6C_2 = \frac{6!}{(2! \times (6-2)!)}$ pairs of means (6C_2 is defined to be the number of ways you can choose 2 means from 6 means). These LSDs were relatively small for comparing the two smallest means, relatively large for comparing the two largest means, and intermediate in size for comparing a small and a large mean (see Table 2). In the case of the 'whole-day' data set, means and LSDs were divided by two so the units were the same as for the first two variables ('morning' and 'afternoon').

Results and discussion

Our emphasis here has been on mixed mineral solutions, unlike other work which has only used individual minerals. Overall, honey bees significantly preferred the mixed molar treatment that had both a high *Na:K* ratio and a high concentration of NaCl (Table 1) compared to the low, medium, and high molar treatments and deionised water (approximate $F_{5,55} = 52.64$, $P < 0.001$) (Table 2). Additionally, the honey bees preferred the mixed molar treatment with a high *Na:K*

Table 1. Low, medium, high, and mixed molar concentrations of six minerals used in this work were NaCl, KCl, CaCl₂, MgCl₂, NH₄Cl, and KH₂PO₄.

Mineral solution (treatment)	Na:K	Na:K:Ca:Mg:N:P	Na	K	Ca	Mg	N	P
Low molar conc.	8.3:1	12.5:1.5:2.5:2.5:1	0.25	0.03	0.05	0.05	0.05	0.02
Medium molar conc.	7.1:1	12.5:1.75:3.75:3.75:2.5:1	0.5	0.07	0.15	0.15	0.1	0.04
High molar conc.	7.7:1	14.3:1.9:4.3:4.3:2.9:1	1	0.13	0.3	0.3	0.2	0.07
Mixed molar conc.	16.7:1	25:1.5:2.5:2.5:2.5:1	0.5	0.03	0.05	0.05	0.05	0.02

Two further treatments were 20% sucrose solution and deionised water as positive and neutral controls, respectively. Na:K and Na:K:Ca:Mg:N:P are molar ratios of indicated elements. Na, K, Ca, Mg, N, and P are concentrations of indicated elements given as amount of moles per 1 litre of a solution.

Table 2. Mean number of honey bee drinking observations per hour (per 40 bees) for morning, afternoon, and 'whole-day' data sets.

Mineral solution (treatment)	Mean number of honey bee drinking observations per hour (/40 bees)		
	Morning	Afternoon	'Whole day'
1. Mixed molar conc.	5.75 a	7.25 a	6.50 a
2. Sucrose	4.67 a	4.00 b	4.33 b
3. Deionised water	1.50 b	1.17 c	1.33 c
4. Low molar conc.	0.75 bc	1.17 c	0.96 cd
5. Medium molar conc.	0.42 c	0.67 cd	0.54 d
6. High molar conc.	0.75 bc	0.33 d	0.54 d
LSD (5%) (trt 5 vs. 6)	0.59	0.55	0.44
LSD (5%) (trt 1 vs. 2)	1.76	1.84	1.40

Lettering was assigned on the basis of the 15 pairwise LSDs output by the GLM procedure. As examples, two LSDs are given: the smallest LSD is for comparing the two smallest means (treatments 5 and 6) and the largest LSD is for comparing the two largest means (treatments 1 and 2). Two treatments differ significantly ($P < 0.05$) if they have no letters in common.

LSD, least significant difference; GLM, generalised linear model.

ratio compared to sucrose solution; however, this preference was not observed in the morning (Table 2). Therefore, we hypothesise that honey bees first (in the morning) supplement their energy as sucrose presents a carbohydrate 'reward', and when this need is completed (in the afternoon) they forage for nutrients. This is because sucrose and nectar do not contain sufficient concentrations of limiting minerals to support honey bee diets alone.

Bees exhibited a preference for the mixed molar treatment, and this was the only treatment having high a Na:K ratio (Table 1). The medium molar treatment had a similar Na concentration as the mixed treatment but had a much lower Na:K ratio (Table 1). The high molar treatment had a higher Na concentration but a much lower Na:K ratio than the mixed treatment (Table 1). To draw ecologically and evolutionary relevant conclusions, we interpret observed preferences for a high Na:K ratio within the context of bee fitness. The Na:K ratio in herbivores' food influences survival and fitness and shapes the functioning of food webs because of unique features of both nutrients: (i) the fundamental differences in Na and K concentrations between animal and plant tissues (much lower Na concentration and much higher K concentration in case of plants) (Kaspari, 2020), and (ii) exceptional importance of Na for animals such as honey bees (Filipiak *et al.*, 2017). For example, acute bee paralysis may be caused by too low a Na:K ratio in honey

bee food (Horn, 1985). The importance of Na and K for honey bees extends far beyond the well-known mechanism of homeostasis regulation (Kaspari, 2020). Na regulates sensing and assimilation of other vital nutrients, that is, N and P from food, and Na–P cotransporters directly influence bee physiology (Werner & Kinne, 2001; Bergwitz & Jüppner, 2011). K levels regulate responses to extreme cold and heat (Dow, 2017). Theoretical modelling and feeding experiments utilising two bee species have shown that concentrations of both Na and K in bee food and therefore Na:K ratio strongly influence bee mortality, development, and ultimately fitness (Filipiak *et al.*, 2017; Filipiak & Filipiak, 2020; Filipiak *et al.*, 2021).

Our findings support previous research that has determined that honey bees display a preference for Na in 'dirty water' (Bonoan *et al.*, 2017), such as 0.29% NaCl over distilled water (Butler, 1940), and the highest proboscis extension reflex (PER) to 1.5% NaCl solutions (Lau & Nieh, 2016). However, the current work has found that the Na:K ratio of 'dirty water' mineral solutions is what drives honey bee preference rather than Na concentration, and therefore should be considered in future research. Na also occurs frequently at low concentrations in pollens (Bonoan *et al.*, 2018); however, this alone does not meet honey bee requirements for a stoichiometrically balanced diet (Filipiak *et al.*, 2017).

In the current work, unlike Na, honey bees showed limited preference for low ratios and concentrations of K, Ca, Mg, N, and P in 'dirty water' (Table 2). This supports previous research where honey bees have shown aversions to K concentrations above 1.5% (Butler, 1940; Waller *et al.*, 1972; Lau & Nieh, 2016). Similarly, Bonoan *et al.* (2017) found that although Ca consumption rates differed across seasons, honey bees drank less Ca than they did Na and avoided Ca during summer. In the case of Mg, honey bees can show a preference for low (Butler, 1940) and medium concentrations (Lau & Nieh, 2016). This variation may be dependent on the ratio of Mg rather than its concentration as well as the time of year (Bonoan *et al.*, 2018). The low honey bee preference of P in the current work is consistent with the findings of Butler (1940), who reported very few honey bee visitations at 1.42% Na₂HPO₄, and Lau and Nieh (2016), which observed that honey bee PER responses significantly declined above 0.75% of this compound. Bonoan *et al.* (2017) similarly observed decreased preference for P and N at 1% concentration. The above findings are likely because honey bees already receive near-adequate amounts of K, Ca, Mg, N, and P through floral resources depending on time of year and location (Auclair & Jamieson, 1948; Bonoan *et al.*, 2017; Filipiak *et al.*, 2017).

Honey bees' sense of time (*Zeitgedächtnis*) (Moore *et al.*, 1989) and foraging activity are highly adaptive and vary considerably through their foraging periods (Yang *et al.*, 2008). However, the current work was conducted in a laboratory with artificial light, although natural light shone through the windows, so the former condition may have over-ridden the processes mentioned by Moore *et al.*, (1989) and Yang *et al.*, (2008) (see Table 2). In addition to possible diurnal impacts on honey bees' foraging behaviour, it is likely that there will be seasonal differences in honey bee nutritional needs (Bonoan *et al.*, 2017). However, investigating this was beyond the scope of this work, which was an initial test of honey bee preferences of mixed mineral 'dirty water'.

In summary, our hypothesis was that honey bees show a preference for a particular mix of stoichiometric mineral solution. Although the honey bees used all came from one hive, these typically contain tens of thousands of worker bees, so the likelihood of the same bees being used across all replicates is very low. While generalised findings cannot be made due to the use of honey bees from one hive, this work suggests the approach of using mixed molar 'dirty water' to supplement honey bee diets should be further investigated on a larger scale. It would be beneficial to repeat the design *in situ* near multiple beehives in the field to further determine any such honey bee-preferences as well as seasonal differences. Such research on stoichiometric mineral solutions may contribute to improved diets of honey bees if adopted by the apicultural industry.

To date, studies have focused on concentrations of minerals in food and solutions. We have shown that element ratios may be a more important factor than mineral concentrations, shaping honey bee foraging preferences.

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Author Contributions

Prof Steve Wratten and Dr Morgan Shields conceived this research idea. Sarah Cairns and Dr Morgan Shields designed the work, and Dr Michał Filipiak calculated the stoichiometric mineral ratios in each solution. Sarah Cairns collected the data.

Sarah Cairns wrote the manuscript with the help of Prof Steve Wratten, Dr Morgan Shields, Dr Michał Filipiak, and Emiliano Veronesi. David Saville conducted the data analyses and Prof Steve Wratten obtained the research funding.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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