Seasonality of salt foraging in honey bees (*Apis mellifera*)

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Abstract. 1. Honey bees (*Apis mellifera*) prefer foraging at compound-rich, 'dirty', water sources over clean water sources. As a honey bee's main floral diet only contains trace amounts of micronutrients – likely not enough to sustain an entire colony – it was hypothesised that honey bees forage in dirty water for physiologically essential minerals that their floral diet, and thus the colony, may lack.

2. While there are many studies regarding macronutrient requirements of honey bees, few investigate micronutrient needs. For this study, from 2013 to 2015, a series of preference assays were conducted in both summer and autumn.

3. During all field seasons, honey bees exhibited a strong preference for sodium in comparison to deionised water. There was, however, a notable switch in preferences for other minerals between seasons.

4. Calcium, magnesium, and potassium – three minerals most commonly found in pollen – were preferred in autumn when pollen was scarce, but were avoided in summer when pollen was abundant. Thus, as floral resources change in distribution and abundance, honey bees similarly change their water-foraging preferences.

5. Our data suggest that, although they are generalists with relatively few gustatory receptor genes, honey bee foragers are fine-tuned to search for micronutrients. This ability likely helps the foragers in their search for a balanced diet for the colony as a whole.

Key words. Minerals, nutrition, nutritional ecology, optimal foraging, seasonality.

Introduction

Nutrition shapes all living organisms, and yet there are few studies that take into account the complexity of nutrition at the ecological level (Raubenheimer *et al.*, 2009). Reaching nutrient and intake targets is not typically as simple as balancing the

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intake and output of energy – most organisms require a balance of complex macronutrients (carbohydrates, proteins, lipids) and micronutrients (vitamins, minerals) (Simpson & Raubenheimer, 2011). Reaching this balance is made challenging by the fact that nutrient composition varies between food sources.

Thus, foraging organisms face a difficult balancing act which may be made more difficult by both abiotic (e.g. temperature, precipitation) and biotic (e.g. floral distribution and abundance, predators) factors (Raubenheimer *et al.*, 2009). Social organisms, particularly eusocial insects, face yet another layer of complexity: they have to obtain the right nutritional requirements for both themselves and their nestmates, which often require different ratios of different nutrients depending on caste and age (Lihoreau *et al.*, 2015). Although micronutrients are vital for all physiological processes, nutrition is often studied with a focus on macronutrients (Cohen, 2004; Rupp, 2015). To fully understand an organism's nutritional ecology, however, it is important to investigate how organisms balance the intake of both macro- and micronutrients in the field.

Honey bees (*Apis mellifera*) are an ideal study system for this area of research; research on honey bee foraging dates back to at least Charles Darwin (Darwin, 1872), and research on honey bee nutrition dates back to at least the 1930s (Haydak, 1934). Thus, there is a strong baseline understanding of honey bee nutrition and foraging. In addition, there is a suite of ecological factors that affect both honey bee foraging and nutrition that can be manipulated and observed. For example, honey bee nutritional requirements shift with age – dependent larvae require large amounts of protein while the oldest adult workers feed mainly on carbohydrates (Haydak, 1970; Paoli *et al.*, 2014). Importantly, studying honey bees allows for the investigation of fitness effects at both the individual and colony level (Lihoreau *et al.*, 2015).

While we know that diet diversity is important to honey bee health (Alaux *et al.*, 2010), we do not know which nutrients in this diverse diet are important. Bees raised on a polyfloral diet exhibit stronger baseline immunocompetence than bees raised on a monofloral diet, even when the monofloral diet has a higher overall protein content (%) than the polyfloral diet (Alaux *et al.*, 2010). Thus, there is more to developing a strong immune system than getting the right amount of protein. While much is known about the macronutrients honey bees need to maintain a healthy colony, very little is known about the micronutrients that are needed (Haydak, 1970; Cohen, 2004; Huang, 2010).

Honey bees tend to prefer compound-rich (hereafter called 'dirty') water sources over clean water sources (Butler, 1940). Despite countless observations, there is only one scientific field study (Butler, 1940) regarding this specific behaviour. Butler (1940) concluded that honey bees prefer dirty water based on strong odour cues. While this provides a proximate mechanism by which honey bees may find dirty water, it does not provide an evolutionary explanation for this behaviour. Similar to geophagy, the intentional consumption of earth (Young *et al.*, 2011), dirty water-seeking behaviour likely has an adaptive purpose.

We hypothesise that honey bees have an optimal diet that includes nutritional resources from both floral and water sources. As the honey bee's main floral diet only contains trace amounts of micronutrients (Somerville, 2005; Brodschneider & Crailsheim, 2010), and sodium-specific foraging is a well-known behaviour in social insects (Botch & Judd, 2011; Pizarro *et al.*, 2012) and across the animal kingdom (Denton, 1982; Young *et al.*, 2011; Starks & Slabach, 2012), we postulate that to obtain a well-rounded diet, honey bees selectively forage in soil and water for minerals that their main floral diet may lack. As the honey bee colony is a dynamic environment and honey bees live in temperate regions, our hypothesis leads us to three main predictions.

First, if honey bees have an optimal diet that is satisfied by both floral and water sources, we predict that they will show mineral preferences when foraging for water. Minerals are essential for all physiological functions (e.g. muscle movement and immunity) (Cohen, 2004). Universally, sodium is a key player in osmoregulation. While soil is known to contain significant amounts of sodium, the above-ground parts of land plants – the main food source for honey bees – rarely contain a great amount of this important mineral (Oates, 1978; Cohen, 2004). In insects, when coupled with potassium, sodium regulates cellular and body fluid pH. We predict that honey bees will prefer both sodium and potassium in comparison to deionised water.

Other minerals we chose for this study were two co-factors (magnesium and calcium) and two minerals that are important to all life forms (phosphate and nitrogen) (Cohen, 2004). Aside from its role as a co-factor, calcium is also important in the regulation of muscle movement, and plays an important structural role in invertebrates. Specifically in honey bees, calcium aids in pupation and total antioxidant capacity (Zhang & Xu, 2015). In excessive amounts, however, calcium can cause paralysis in honey bees (Somerville, 2005). We predict that honey bees will prefer magnesium but not calcium. Lastly, as phosphate is essential to the process of bioenergentic activity and nitrogen is vital for cellular communication and waste removal (among other things), we predict that honey bees will prefer both mineral solutions relative to deionised water.

Secondly, if dirty water sources are coupled with floral resources to reach an optimal diet, we predict that the strength of mineral preferences when foraging for water will differ with the distribution and abundance of floral resources (i.e. the seasons). We expect mineral preferences when foraging for water to complement which minerals are available in floral resources (mainly pollen). The major minerals found in bee-collected pollen are potassium, calcium and magnesium; the levels of each mineral vary within and between summer and autumn (Herbert & Miller-Ihli, 1987, L. D. O'Connor *et al.*, unpublished).

Lastly, if there is an optimal honey bee diet, we predict that deviations from the presumably well-rounded diet will adversely affect colony fitness. While the specific mineral requirements of honey bees are not known, honey bees likely need minerals to successfully rear brood. Bees fed a semi-synthetic diet with pollen ash (which contained potassium, sodium, and calcium) reared more brood than bees fed a completely synthetic diet of amino acids and vitamins (Herbert & Shimanukia, 1978).

Although honey bee mineral (NaCl, MgCl$_2$, KCl, Na$_2$HPO$_4$) preferences have recently been tested in the laboratory (Lau & Nieh, 2016), our study is the first to investigate the mineral specificity of honey bee foragers in the field. It is also the first to examine mineral preferences over ecological time (i.e. seasons) and how such nutrient preferences may affect fitness.

Materials and methods

This study was performed on the Tufts University Medford/Somerville campus (equipped with eight two-frame observation hives) during autumn 2013, 2014 and 2015 (September–October), and summer 2014 and 2015 (July–August). Given general insect micronutrient requirements, we tested preferences for six specific mineral solutions: sodium (NaCl), calcium (CaCl$_2$), potassium (KCl), magnesium (MgCl$_2$), phosphorus (KH$_2$PO$_4$) and nitrogen (NH$_4$Cl) (Cohen, 2004). Minerals were also chosen based on what honey bees are likely to find in soil or dirty water where they often forage (L. D. O'Connor *et al.*, unpublished).
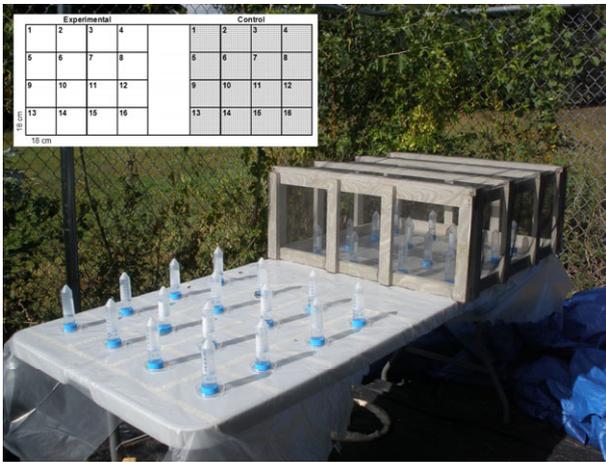
Fig. 1. Tasting table with foraging honey bees on the experimental side of the table. The mesh covering on the control side of the table allowed us to exclude bees and account for volume change due to evaporation during the trial. The difference in volume change between the experimental side and the control side gave us the amount that honey bees actually consumed. Inset: Tasting table grid that was used to randomly allocate solutions for each trial. [Colour figure can be viewed at wileyonlinelibrary.com].

Fig. 2. Yellow and pink powder-marked honey bees visiting a solution at the tasting table. Counts of each colour bee for each solution were made every 15 min. [Colour figure can be viewed at wileyonlinelibrary.com].

Mineral preferences

Once the bees were trained (von Frisch, 1967) to forage in an open, grassy location (about 50–90 m from the observation hives), we conducted preference assays two to five times a week (weather permitting). We set up preference assays on a 1.82-m-long plastic table which was divided into two 4×4 (72 cm $\times$ 72 cm) grids (Fig. 1). In addition to the six mineral solutions, a sucrose solution (10% during autumn assays, 20% during summer assays) served as the positive control, and deionised water served as the negative control (Pizarro *et al.*, 2012). Based on honey bee supplemental feeding guidelines (Somerville, 2005), all mineral solutions were a 1% concentration. In the laboratory, honey bees respond to NaCl levels as low as 50 μM (de Brito Sanchez, 2011) and preferentially respond to 1.5% NaCl, 1.5% $MgCl_2$, 0–1.5% KCl and 0.4–0.75% NaH_2PO_4 (Lau & Nieh, 2016); thus, it is likely that honey bees were able to taste our salt solutions. There were two tubes of each solution on each grid; bees were allowed to forage on one grid (the experimental grid) while the other grid was covered with mesh to exclude bees (the control grid) and account for volume change due to evaporation (Fig. 1). Control and experimental grids were alternated for each trial.

At the beginning of each trial, 50 ml falcon tubes were filled with 25 ml of the appropriate solution and randomly allocated (using a random list generator, www.random.org) to a numbered square on each grid (Fig. 1, inset). Once the tasting table was set up, bees were allowed to forage for 5–7 h (depending on weather). At the end of each trial, we measured the amount (ml) of each solution remaining in both the control and experimental grids. The difference in volume between the control side and the experimental side yielded the total volume consumed by the foraging bees. In total, we conducted 33 preference assays in summer and 18 preference assays in autumn. For each year (2013, 2014, 2015), new bees were installed (thus, we have two true replicates for summer and three true replicates for autumn) and the eight observation hives, and thus eight colonies, were trained to forage at the tasting table.

Colony fitness

To assess what preferences mean for colony fitness, we gave bees a colony-specific, lightweight, coloured powder mark during 2014 preference assays (Fig. 2) (Hagler *et al.*, 2011; Bonoan & Starks, 2016). We constructed marking apparatuses from mesh cloth, filled with non-toxic acrylic coloured powders (ECO Pigments, Day-Glo Color Corporation). Using Velcro (Scotch Extreme Fasteners), we affixed the marking apparatuses to each hive entrance such that foragers were dusted with visible coloured markings upon exiting (Bonoan & Starks, 2016). As each hive – and thus colony – had a unique colour, we could approximate the number of visits each colony made to each solution during preference assays (Fig. 2). The approximate number of visits indicates the intensity of colony-specific preference for each mineral solution. We counted the number of bees from each colony at each solution every 15 min throughout the duration of the trial. Unmarked bees were classified as feral and not included in analysis.

To see if colony-specific preferences correlated with internal colony dynamics, we measured two colony fitness parameters three times a week, beginning in July 2014 and ending in October 2014. First, population estimates were recorded according to Sammataro and Avitabile (2011): a standard deep frame entirely covered by one layer of bees is roughly 2000 adult individuals; estimates were taken in increments of 250 bees. Second, the total area of capped brood (i.e. the amount of oldest honey bee brood) was calculated by first measuring the area of the smallest rectangle to encompass the brood patch. As honey bee brood patches tend to be oval-shaped, we then subtracted the area of empty

corners from the area of the original rectangle. This was then multiplied by the quality of the queen's laying pattern which was ranked on a scale of one to five depending on the spottiness of brood comb (1, 100% to 80% empty cells; 2, 80% to 60% empty cells; 3, 60% to 40% empty cells; 4, 40% to 20% empty cells; 5, 20% to 0% empty cells). A similar scale was implemented by Vaudo *et al.* (2011) as a proxy for honey bee colony health. Together, these calculations gave us the total capped brood area of each colony.

Statistical analysis

For preference assays, data for all 3 years (2013, 2014, 2015) were pooled by season as there was no significant effect of year on the amount of each solution consumed. Prior to analysis, we removed data for sucrose, our positive control. To determine whether or not there was an overall effect of season (summer versus autumn) or mineral solution, we ran a two-way ANOVA on natural log-transformed data. Following this analysis, we ran two one-way ANOVAs (one for each season) with contrasts. This allowed us to compare each mineral solution with our negative control, deionised water. Again, data were natural log-transformed prior to analysis in order to meet test assumptions.

To analyse the 2014 data for colony fitness, we combined the counts of visits made by each colony to mineral solutions ($CaCl_2$, KCl, KH_2PO_4, $NaCl$, NH_4Cl, $MgCl_2$) for each week. To standardise for colony size (colonies ranged from 0 to 7000 individuals throughout data collection), we calculated the approximate proportion of the colony that made visits to mineral solutions each week by dividing the number of visits to mineral solutions by the average colony population for that same week. These calculations yielded a colony-size adjusted measure of the intensity of preference for each mineral source.

Based on what we know about the honey bee life cycle, we then used a 1-week offset for capped brood area (it takes about 9 days for an egg to develop into a fifth-instar larva and get capped over) and a 3-week offset for the adult population (it takes about 21 days for an egg to fully develop into an adult worker bee) (Winston, 1987). Because colony fitness parameters are expected to naturally decrease in the autumn, we only used summer data for this analysis.

For both brood area and adult population, we ran a Poisson regression against a null hypothesis of no effect of minerals (i.e. a line with a slope of 0). We did not have enough measurements per colony to add colony as a random effect. Instead, we standardised visits by calculating the proportion of each colony visiting minerals rather than the combined number of visits (see earlier). For brood area, we examined whether or not the proportion of workers visiting minerals at week t affected the brood area at week $t+1$. Similarly, for the adult population, we examined whether or not the proportion at week t affected the brood area at week $t+3$. For both models, pseudo-R^2 was calculated by dividing the residual deviance by the null deviance, and subtracting that value from 1.

All analyses were run in R version 3.2.4 (2016-03-10) using the mosaic package (R Development Core Team, 2008).

Results

Mineral preferences

When all three field years (2013, 2014, 2015) are analysed together, there is a significant effect of mineral solution on volume collected ($F_{6,700} = 11.803$, $P < 0.001$); however, there is no significant effect of season on volume collected ($F_{1,700} = 0.002$, $P = 0.967$) (Fig. 3a). The bees drank about the same amount of potassium, calcium, magnesium and nitrogen no matter the season. The bees drank less water, sodium and slightly less phosphorus in the autumn compared with the summer (Fig. 3a).

When controlling for deionised water, an effect of season on mineral preferences emerges (Fig. 3b). No matter the season, bees drank significantly more sodium than deionised water (autumn, $t_{245} = 3.996$, $P < 0.001$, summer, $t_{455} = 4.008$, $P < 0.001$). In the autumn, bees drank significantly more potassium than deionised water ($t_{245} = 2.254$, $P = 0.025$) and in the summer they drank similar amounts of the two solutions (Fig. 3b). Although the effect was not significant, the bees did drink more calcium and magnesium than deionised water in the autumn and less than deionised water in the summer (Fig. 3b). During the summer, bees drank significantly less nitrogen ($t_{455} = -2.251$, $P = 0.025$) and phosphorus ($t_{455} = -2.064$, $P = 0.040$) than deionised water (Fig. 3b). This trend was not observed in the autumn.

Colony fitness

There was a significant effect of the approximate proportion of the colony visiting mineral solutions on capped brood area 1 week out ($X_1^2 = 13.2$, $P < 0.001$) and adult population 3 weeks out ($X_1^2 = 202.7$, $P < 0.001$). Although the effect is significant, the pseudo-R^2 values (0.0016 for brood area, 0.0072 for adult population) suggest that our current data do not explain the model much better than a straight line (Fig. 4).

Discussion

Honey bees showed mineral preferences and, in comparison to deionised water, those preferences varied with season. This supports our hypothesis that honey bees forage in dirty water for minerals that their floral diet may lack. Although the mean volume consumed did not vary with season (Fig. 3a), there are more bees in the colony in the summer than there are in the autumn. Thus, on a per-bee basis, there is a difference in the amount of minerals a colony forages for. In the summer, bees receive less 'water-derived' minerals on a per-bee basis than in the autumn.

The seasonality in preferences was made apparent when the mean volume consumed was analysed relative to deionised water. Relative to deionised water, bees drank significantly more potassium and tended to drink more calcium and magnesium
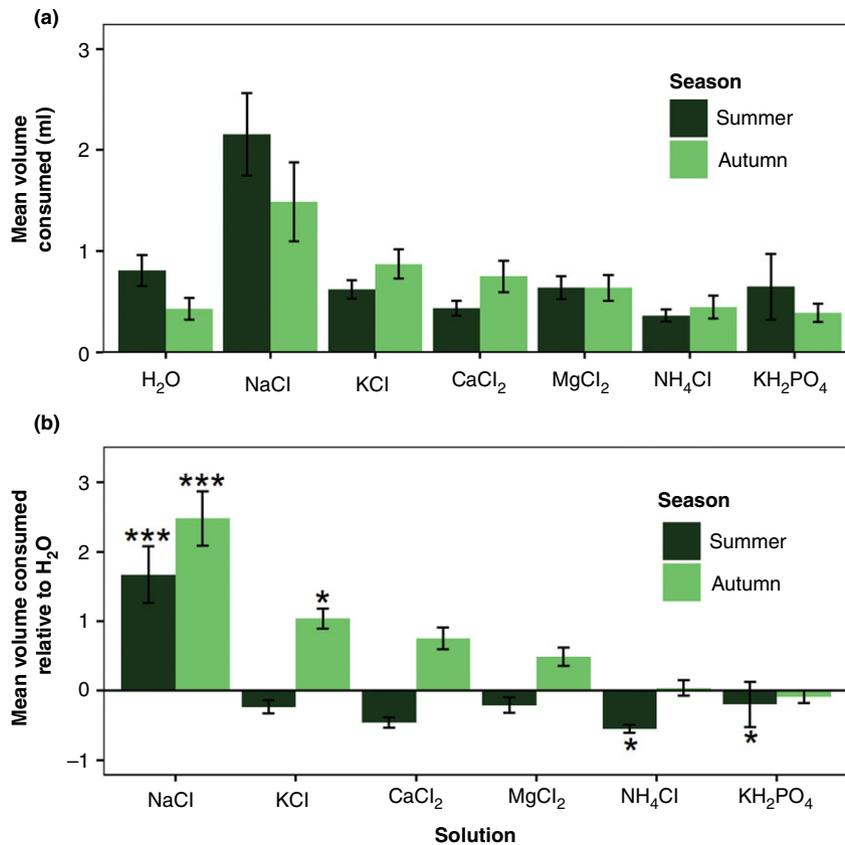
Fig. 3. (a) Mean volume change of each mineral consumed (ml) by all eight hives separated by season. Error bars are ±1 SE and show the variation in volume consumed for each trial within each season. There was no significant effect of season on the volume consumed; however, there was an effect of mineral solution. (b) Volume change for each mineral solution relative to the volume change of deionised water (negative control, $x = 0.0$), calculated for each field season. Anything above the baseline was preferred compared with deionised water, and anything below the baseline was avoided compared with deionised water. Bees significantly preferred sodium no matter the season. In the autumn, bees significantly preferred potassium. In the summer, bees significantly avoided nitrogen and phosphorus. Error bars are ±1 SE and show the variation in volume consumed for each trial within each season. (*$P < 0.05$, ***$P < 0.001$). [Colour figure can be viewed at wileyonlinelibrary.com].

in the autumn (Fig. 3b). In the summer, however, there was no significant preference for potassium and bees tended to avoid calcium and magnesium.

This switch in preferences is particularly interesting as potassium, calcium and magnesium are three of the most prominent minerals found in pollen (Herbert & Miller-Ihli, 1987). Moreover, the levels of these minerals in pollen vary with season; in summer, the levels are high, whereas in autumn they are low (Herbert & Miller-Ihli, 1987). Taken together with our data, this suggests that honey bees are foraging for minerals in water based on what is lacking in their floral diet. The bees' preference for sodium no matter the season also supports our hypothesis – the above-ground parts of plants rarely contain much sodium (Oates, 1978; Cohen, 2004) and herbivores are often sodium-limited (Denton, 1982). Preliminary analysis of pollen collected by our bees throughout the 2015 season (July–October) showed that, of potassium, calcium, magnesium and sodium, sodium is at the lowest levels (0–4 ppm) (L. D. O'Connor *et al.*, unpublished).

It was unexpected that bees would avoid nitrogen and phosphorus. As bees significantly avoided nitrogen in the summer,

it is possible that they receive adequate amounts of nitrogen from the abundant pollen sources; pollen is high in amino acid content (Auclair & Jamieson, 1948). Additionally, there are various forms of nitrogen in soil – where bees might be seeking a nitrogen source – and it is possible that our specific nitrogen compound is not the one that honey bees prefer. This might also explain the unexpected avoidance of phosphorus.

Regarding minerals and colony fitness, it is hard to determine anything conclusive with the current data. While stronger colonies do tend to visit more minerals than weaker colonies (Figure S1), it is hard to say which came first. Do strong colonies visit minerals more often because they are strong? Or, are they strong because they visit minerals more often? Unfortunately, our regression with the offset does not help to answer the question. Although our data show a significant effect of minerals on both colony fitness parameters, the model does not reveal either a positive or a negative effect. This may be because we do not have enough data or we should collect data from larger, more natural-sized hives.
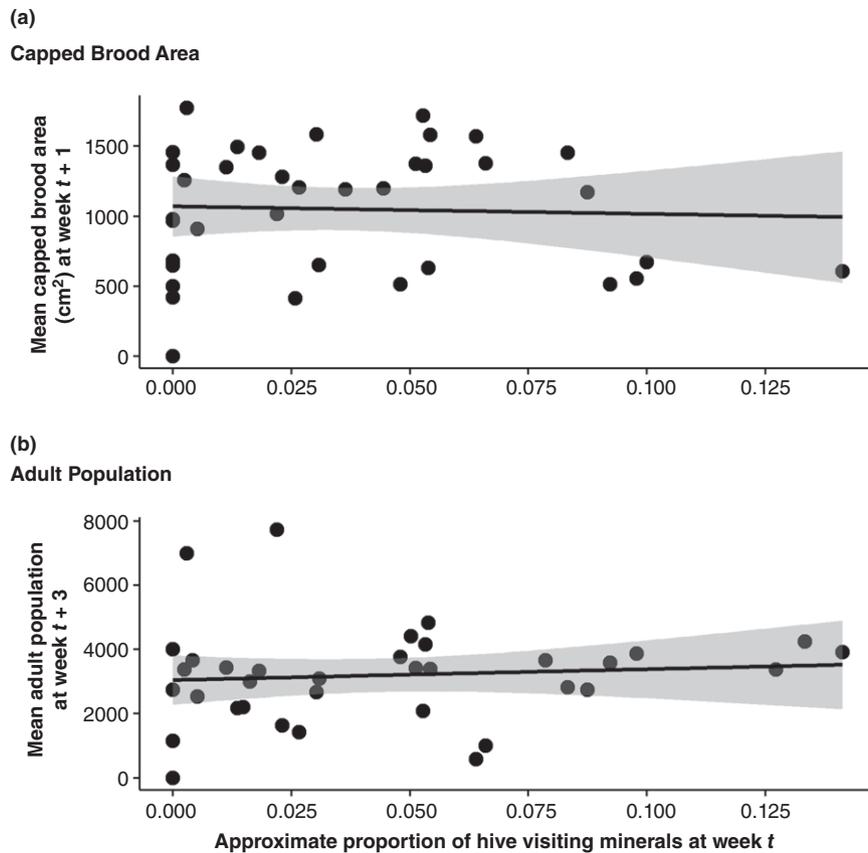
(a)

Capped Brood Area

(b)

Adult Population

Fig. 4. (a) Approximate mean proportion of hives visiting minerals in week t and the mean capped brood area of hives in week $t + 1$. (b) Approximate mean proportion of hives visiting minerals in week t and the mean adult population of hives in week $t + 3$. Offsets were determined based on the honey bee life cycle (Winston, 1987). Although both analyses gave significant results, the near-zero pseudo-R^2 values indicate that the model explains our data only slightly better than a straight line.

Overall, our data suggest that honey bees forage in dirty water sources for minerals that may be lacking in their floral diet. If such minerals are lacking in their floral diet, they are likely lacking in the colony as a whole. This is currently speculation, however, as our colony fitness data were inconclusive. The effects of minerals on colony fitness could be further tested either in the field with larger hives or in the laboratory with caged hives.

This is the second study to examine honey bee mineral preferences when foraging for water and the first study to examine such preferences on a seasonal basis. Butler (1940) did not find that honey bees exhibited mineral preferences when foraging for water; Butler's (1940) study was done in the spring. The fact that Butler (1940) did not find mineral preferences is in line with our hypothesis and our data; in spring, floral resources are abundant, and thus honey bees do not need to seek out minerals in water sources.

Our study has implications in applied and basic science. On the applied side, understanding the seasonality of honey bee mineral requirements can lead to the development of season-specific diet supplements, and better overall nutrition throughout the year for both managed honey bee hives (via diet supplements) and wild pollinator populations (via the planting of diverse flora).

Regarding honey bees specifically, our data show that despite having relatively few taste genes (de Brito Sanchez, 2011),

honey bees can discriminate water sources based on nutritional content. This ability likely helps foragers in their effort to obtain a balanced diet for both themselves and the colony.

Being a superorganism, honey bees provide the opportunity to investigate how mineral preferences may correlate with fitness. Although our data suggest that we need to collect more data regarding colony fitness, such parameters are easily measured in both observation hives and larger Langstroth hives.

More generally, collecting data on mineral preferences in honey bees can bolster our understanding of pollinator health and nutritional ecology. While much is known about insect nutrition and foraging in general, micronutrient (both vitamin and mineral) requirements remain poorly understood (Cohen, 2004), even with the recent decline in pollinator populations. Micronutrients are a physiologically important part of any organism's complex diet (Simpson & Raubenheimer, 2011, 2012; Rupp, 2015) and should be studied for a more complete understanding of the complex balancing act faced by organisms when foraging.

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Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/een.12375

Figure S1. Mean percentage change per week relative to baseline colony fitness parameters of a representative healthy (a) and unhealthy (b) hive. Anything above '0' represents a percentage increase compared with baseline and anything below '0' represents a percentage decrease compared with baseline. Approximate mean percentage of the same healthy (c) and unhealthy (d) hives that visited each mineral solution each week. For all graphs, week 0 is the beginning of the summer (7 July 2014) and week 10 is the end of the summer (19 September 2014).

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