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Seasonality of honey bee (*Apis mellifera*) micronutrient supplementation and environmental limitation

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ABSTRACT

Honey bees (*Apis mellifera*) obtain micronutrients from floral resources and "dirty", or turbid, water. Past research suggests that honey bees drink dirty water to supplement the micronutrients in their floral diet, however, there is no research that directly investigates how floral micronutrient content varies with water preferences, or how micronutrients in honey bees themselves vary seasonally. In this study, we used chemical analyses (ICP-OES) to investigate seasonal variation of micronutrients in honey bee workers and floral resources in the field. We found that honey bees likely use mineralized water to supplement their floral diet and may be limited by availability of calcium and potassium. Our results also suggest that honey bees may seasonally seek specific micronutrients, perhaps in preparation for overwintering.

1. Introduction

All organisms require nutrients to grow, survive, and reproduce. Whether through active foraging or passive absorption, an organism must interact with its environment in order to obtain its required nutrients. Due to both biotic and abiotic factors, nutrients in the environment vary within and between food items, as well as spatially and temporally. Determining how organisms meet specific nutrient goals in an ever-changing environment is a central goal of nutritional ecology (Raubenheimer et al., 2009).

To achieve this research goal, we must explore dietary needs of animals interacting with their environment over time, in as natural a setting as possible. Such studies require close observation and analysis of an animal's foraging behavior. Comprehensive dietary monitoring is often time consuming: observations for a single study can last several years (e.g. Slabach et al., 2015). To go beyond behavioral studies and to better understand how an animal's nutrient requirements may or may not be met in the environment, we must sample organisms as well as their food items. Behaviors such as inconspicuous foraging, migration (Barrett et al., 2007), or overwinter dormancy, can make sampling for an extended period of time difficult.

These problems can be overcome by using honey bees (*Apis mellifera*) as a model organism. Honey bees have a relatively short generation time and are active year-round (Winston, 1987). Furthermore, honey bees reside in temperate regions where their floral sources vary in distribution and abundance over the course of a year (Lindter, 2014), and changes in bee-collected floral sources can be identified to the genus and/or species level (e.g., Richardson et al., 2015). Lastly, honey bee foragers bring nutritional resources (both floral and non-floral) back to the hive, which provides a central location for observation of behavior, and sampling of organisms and food items. These characteristics have helped scientists gather a strong baseline understanding of honey bee nutrition and foraging since the 1930s (Haydak, 1937, 1970; deGroot, 1953; Somerville, 2005; Brodschneider and Crailsheim, 2010; Huang, 2010).

Floral sources are the honey bee's source of macronutrients: pollen provides the primary source of lipids and proteins, while nectar provides carbohydrates (Haydak, 1970). Nutritional values of both are variable across plant species (Somerville, 2005) and time of year (Standifer et al., 1978). Foragers selectively seek macronutrients the colony may lack, likely in an attempt to reach an optimal macronutrient target (Hagler, 1990; Hendriksma and Shafir, 2016).

Pollen, nectar, and water contain trace amounts of micronutrients that are as variable, if not more variable, than macronutrients (Herbert and Miller-Ihli, 1987; Somerville, 2005). Micronutrients are as important as macronutrients (Rupp, 2015), and yet they are understudied when it comes to nutrition. Honey bees likely get micronutrients from two main sources: pollen (Herbert and Miller-Ihli, 1987; Filipiak et al., 2017) and "dirty", or turbid, water (Bonoan et al., 2016). While nectar does contain trace amounts of micronutrients, levels are higher in pollen and likely co-vary with micronutrient levels in nectar (Herbert and Shimanuki, 1978). Also, corbicular, or bee-collected, pollen contains nectar-based regurgitation (Thorp, 2000) and thus, micronutrient content of corbicular pollen can serve as a proxy for the micronutrient

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Table 1
Predicted and observed micronutrient content in corbicular pollen compared between summer and fall. If honey bees are able to obtain an optimal micronutrient diet, micronutrient content of corbicular pollen should complement water preferences as determined by Bonoan et al. (2016). Arrows and colors represent the direction of preference and/or mineral content, double arrows and darker colors represent a strong directionality. Asterisks indicate which relationships were not complementary.

		Water Preference	*Predicted Pollen Content*	*Observed Pollen Content*
Calcium	Summer	↓	↑	↑
	Fall*	↑	↓	↑
Magnesium	Summer	↓	↑	↑
	Fall	↑	↓	↓
Potassium	Summer*	↓	↑	↓
	Fall*	↑	↓	↑↑
Sodium	Summer	↑↑	↓↓	↓↓
	Fall	↑↑	↓↓	↓↓

content in bee-collected floral resources.

Although there have been various observations of honey bees drinking from dirty water sources when clean sources are available (Butler, 1940; Abou-Shaara, 2012), little research has been done regarding water as a nutrient source. Our research shows that honey bee water preferences are a likely mechanism for micronutrient supplementation (Bonoan et al., 2016), similar to geophagia in other animals (Starks and Slabach, 2012). We showed that honey bee foragers prefer water containing micronutrients over deionized water, and that such preferences vary with season. Interestingly, the seasonality in mineral preferences when foraging for water coincided with micronutrients lacking in corbicular pollen as determined in the mid-Atlantic region (Herbert and Miller-Ihli, 1987). Here, we 1) further explore mineralized water as a diet supplement by analyzing the micronutrient content of corbicular pollen of *our* bees *during* our water preference assays, and 2) examine how adult bee micronutrient requirements and/or availability may vary throughout the year.

First, if honey bees are able to obtain an optimal micronutrient diet utilizing both floral and non-floral resources, we predict that water preferences and micronutrient content in pollen will be complementary (Table 1). For example, when a micronutrient preference is strong in water, we would expect that micronutrient content to be relatively low in pollen (Table 1).

Alternatively, it may be that some micronutrients are limiting in nature, making it challenging for the animal to obtain the required amount. In this case, we would expect to see complementarity in one direction, i.e. a preference will always be detected (Table 1). The reverse is also true: if a chemical is detrimental, we would expect it always to be avoided. Such compounds, however, would not fall under a typical optimal diet framework.

Second, if honey bees are able to obtain an optimal micronutrient diet utilizing both floral and non-floral sources, we predict that adult bees will exhibit relatively stable levels of micronutrients throughout the year. Variation in adult bee micronutrient content throughout the year may point to a micronutrient that is unavailable in the environment or a micronutrient that is necessary for a specific physiological function.

This study is among the first to investigate micronutrient foraging in bees and is the first to investigate variation of insect micronutrient content over time, in the field. Current studies investigate how micronutrient content varies between social insect castes (e.g. Judd and Fasnacht, 2007), how micronutrient content differs throughout the insect digestive tract (e.g. Stewart et al., 2011), and the difference in micronutrient content of insects used to feed primates in captivity (e.g. Rothman et al., 2014). In this study, we sample corbicular pollen and adult bees from the field for chemical analyses (ICP-OES) in the lab. Our study's field setting allows us to detect possible mismatches between honey bee nutritional requirements and environmental availability, and how such mismatches may shift with the seasonal distribution and abundance of food items.

2. Materials and methods

2.1. Subjects

For pollen collection and analysis, honey bee colonies were kept in 8 two-frame observation hives (53 cm × 48 cm × 5 cm) inside a temperature controlled facility with a single access tube to the outside (see Bonoan and Starks (2016) for a diagram of the hives). Hives were located on the Tufts University Medford/Somerville campus in Massachusetts. All 8 hives were queenright, and each frame contained mixed brood and food stores. These were the same colonies used for preference assays in Bonoan et al. (2016).

For collection of adult honey bees, 3 queenright honey bee colonies were kept in standard field hives (Langstroth hives), each with two 10-frame deep boxes (50.5 cm × 40.6 cm × 24.4 cm) full of mixed brood and food stores, and one 10-frame medium box (50.5 cm × 41.3 cm × 16.8 cm) where the bees drew comb, and stored nectar and honey. Hives were kept under trees at the northern edge of a clearing in Grafton, Massachusetts at the Cummings School of Veterinary Medicine at Tufts University.

Fig. 1. Pollen trap on observation hive entrance. The 5-mesh hardware cloth pollen trap knocks corbicular pollen (yellow pellets on the pictured bee's back legs) off returning bees. Corbicular pollen was caught in a petri dish below the trap. Photo: Rachael E. Bonoan. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.2. Sample collection and storage

Corbicular pollen was collected from observation hives over a 10-week period (July 12–September 24) in 2015. Water preference assays (see Bonoan et al. (2016) for details) were conducted 2–3 times/week on these same colonies during this same period. To collect pollen, we secured a pollen trap (5-mesh hardware cloth, Scotch Extreme Fastener 1″ × 10′) at each hive entrance for a maximum of one hour each week during peak pollen foraging (8:30–10:30 AM) (Abou-Shaara, 2014) (Fig. 1). Due to variation in hive activity, we did not always collect pollen from all 8 hives but we did always collect pollen from at least 4 hives. After collection, we stored the pollen in 2.0 mL microcentrifuge tubes at −20 °C until digestion (see "Sample preparation") and analysis.

Adult honey bees were collected from all 3 Langstroth hives over a 17-week period (July 12–November 19) in 2016. Each week, we collected worker bees by gently scooping individuals off the brood comb and into a 50 mL falcon tube. Collected bees were chilled, submerged in 95% ethanol, and transported to the lab in a cooler (Human et al., 2013). In the lab, the bees were separated and individually stored in 1.5 mL centrifuge tubes with 95% ethanol at −20 °C until digestion and analysis (Judd and Fasnacht, 2007). Since all adults were collected from the center of the brood comb, all bees were estimated to be 3–12 day old nurse bees (Winston, 1987).

Although corbicular pollen and adult honey bees were collected in different years, from different locations, we feel confident in using our 2016 adult bees as a proxy for the seasonality of micronutrient levels in honey bees in the Northeastern U.S. in general: our 3 Langstroth hives were kept on an unmanaged wildflower field near forested habitat, and were only disturbed once a week for sampling.

2.3. Sample preparation

Corbicular pollen was lyophilized and grouped into 2-week periods by hive. Samples were crushed and homogenized with a mortar and pestle, and 0.07 g of the sample was added to a 7 mL polytetrafluroacetate (PFA) vial (Savillex, PFA vial) with 1 mL concentrated nitric acid (Maher et al., 2001). Four PFA vials were placed into a 120 mL PFA closed digestion vessel (Savillex, PFA digestion vessel, plain buttress threaded closure): 3 PFA vials containing samples were placed on top of 1 empty PFA vial, which sat in 10 mL deionized water. The digestion vessel was microwaved following Maher et al (2001). The digested sample was diluted to 10 mL with deionized water and stored at 4 °C until analysis.

Honey bees were oven dried at 35 °C until they reached a constant mass (Judd and Fasnacht, 2007). Dried bees were placed in individual glass vials containing 0.3 mL concentrated nitric acid. The vials were incubated at 37 °C in a shaking water bath until the samples were dissolved. The digested sample was diluted with deionized water to a final volume of 10 mL. The diluted liquid was centrifuged and the supernatant was run through a microfilter (Naglene, 0.45 µm SFCA membrane). Samples were stored at 4 °C until analysis.

2.4. Sample analysis

Ion Coupled Plasma Optical Emission Spectrum (ICP-OES; Prodigy ICP, Teledyne Leeman Labs, Hudson, NH) was used to quantify the presence and total concentration of sodium, calcium, potassium, and magnesium ions (Judd and Fasnacht, 2007). Calibration curves for the elements of interest were created using standards (Inorganic Ventures) ranging from 0.1 µg/mg to 30 µg/mg. These concentrations were chosen based on preliminary analyses of samples. Emission for each ion was detected at the following wavelengths: Na, 589.592 nm; Ca, 317.933 nm; K, 766.491 nm; and Mg, 279.533 nm.

2.5. Statistical analysis

To analyze the effect of time on micronutrient concentration in corbicular pollen, we ran a linear mixed model that tested for independent and interaction effects of date and element on total micronutrient concentration (concentration = date × element), with hive added as a random effect. To fit assumptions, the model was a Gaussian (normal) family with a log link. To determine significance, we used marginal hypothesis tests, implemented with the Anova() function.

Similarly, we ran a Gaussian family, log link, linear mixed model that tested for independent and interaction effects of date and element on total micronutrient concentration in bees (concentration = date × element), with hive added as a random effect. Again, to determine significance, we used marginal hypothesis tests, implemented with the Anova() function.

All statistical analyses were run in R version 3.2.2 using MASS, car, and lme4 (R Development Core Team, 2008).

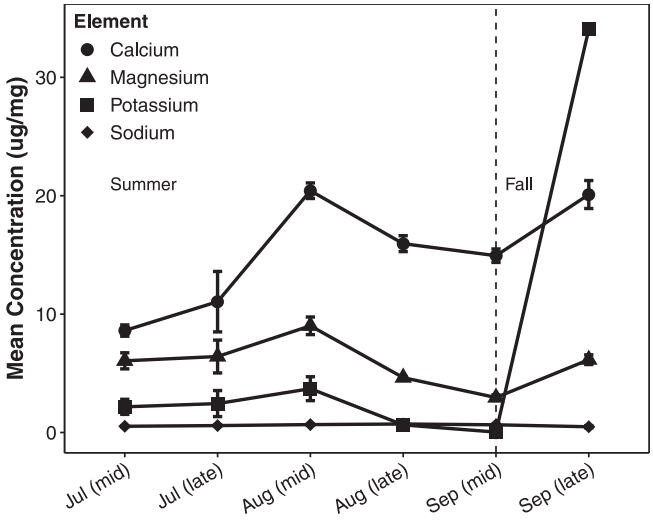

Fig. 2. Mean concentration of micronutrients in corbicular pollen over time (two-week increments). Each point represents pollen samples from 4 to 8 hives as not all hives brought pollen back during the sampling period. Error bars are ± 1 SE and show the variation between hives.

3. Results

3.1. Corbicular pollen

Micronutrient concentrations in corbicular pollen varied significantly over time (ANOVA on log-linked LMM, $X^2 = 311.25$, $df = 5$, $p < 0.0001$) and there was a significant interaction of element and time (ANOVA on log-linked LMM, $X^2 = 415.73$, $df = 15$, $p < 0.0001$) (Fig. 2). Sodium, which was preferred by water foragers in both summer and fall (Bonoan et al., 2016), was consistently the least abundant element in corbicular pollen, ranging between 0 and 1 μg/mg (Fig. 2). In both summer and fall, sodium water preferences complement sodium content in corbicular pollen (Table 1).

In the summer, calcium and magnesium levels in corbicular pollen were relatively high and complemented the avoidance of both minerals in our water preference assays (Bonoan et al., 2016). Calcium, which was the most avoided micronutrient during summer water foraging, was the most abundant in corbicular pollen collected in the summer, ranging from 10 to 20 μg/mg (Fig. 2). Despite summertime avoidance of potassium during our preference assays, potassium was relatively low in corbicular pollen in the summer (Fig. 2). Thus, during the summer, potassium content in corbicular pollen did not complement water preferences (Table 1).

In the fall, magnesium levels in corbicular pollen were relatively low and complemented the preference shown for water with magnesium. Both calcium and potassium were preferred during our fall preference assays (Bonoan et al., 2016) however, there was no complementation: levels of both increased in corbicular pollen (Fig. 2). Fall potassium levels in corbicular pollen increased beyond the upper limit of our pre-determined standards (30 μg/mg).

3.2. Adult bees

Micronutrient concentrations significantly varied with time (ANOVA on log-linked LMM, $X^2 = 408.89$, $df = 16$, $p < 0.0001$) and again, there was a significant interaction of element and time (ANOVA on log-linked LMM, $X^2 = 1366.33$, $df = 48$, $p < 0.0001$) (Fig. 3). In the summer, sodium, calcium, and magnesium were found in similar concentrations, between 3 and 7 μg/mg, and varied similarly (Fig. 3). During late fall, however, calcium became more concentrated and more variable, increasing from 3.24 ± 0.18 μg/mg to 20.90 ± 1.01 μg/mg; most of this variation was between hives. Potassium was often five times more concentrated than other elements and exhibited the greatest variation between hives (Fig. 3).

4. Discussion

Micronutrient content in both corbicular pollen and adult bees varied over time. Below, we discuss 1) micronutrient content of corbicular pollen in the context of water foraging preferences and 2) micronutrient content of young adult bees in the context of nutrient availability and physiology. Using both data sets, we speculate which micronutrients may be limiting in the environment.

4.1. Corbicular pollen

Micronutrient content in corbicular pollen varied over time and complemented five of the eight preferences found for micronutrients in water (Bonoan et al., 2016) (Table 1). These data support our prediction that honey bees likely use micronutrients in water to supplement certain micronutrients lacking in corbicular pollen, and thus floral resources (Herbert and Shimanuki, 1978; Thorp, 2000). As such, our data suggest that honey bees regulate micronutrient intake by foraging at multiple, diverse resources.

Based on past research, this conclusion makes sense: honey bees forage for specific macronutrients in floral sources (Hendriksma and Shafir, 2016), and have been found to alter nectar foraging based on potassium content (Hagler, 1990). Alternatively, micronutrient content

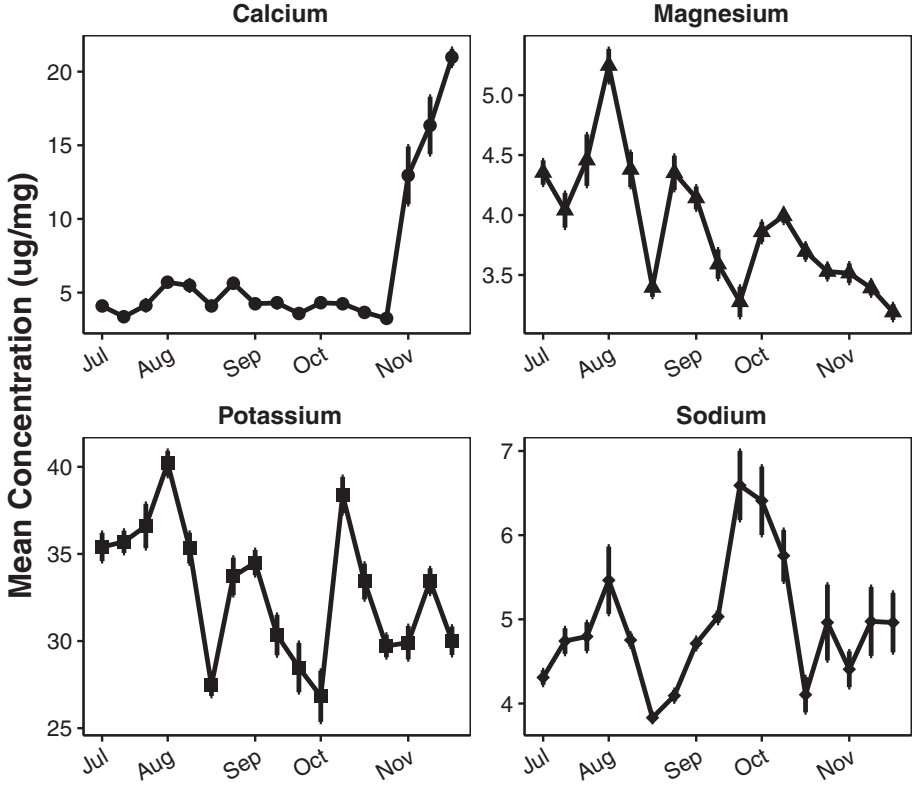

Fig. 3. Mean concentration of each micronutrient in adult honey bees over time (one-week intervals). Each point represents data from 15 bees sampled from 3 hives. Error bars are ± 1 SE and represent variation between individual bees. Note the y-axis scales are not uniform.

of corbicular pollen may simply reflect what is available in the environment. We have collected pollen from floral sources within a 6 km radius of our observation hives to test this alternative hypothesis. This study is ongoing.

As expected based on honey bee water preferences and plant biology in general, sodium content was low in corbicular pollen in both summer and fall (Fig. 2). Water preferences for magnesium also complemented magnesium levels in corbicular pollen in both summer and fall (Table 1).

Calcium and potassium concentrations of corbicular pollen in fall, however, did not seem to complement water preferences as we expected (Table 1). For both micronutrients, potassium in particular, concentrations in corbicular pollen *and* preferences for water increased into the fall (Fig. 2, Table 1). This suggests that honey bees may require high levels of calcium and potassium and may have to take advantage of various nutritional resources to obtain such levels.

4.2. Adult bees

Contrary to our prediction, micronutrient content in adult bees varied over time. This suggests that honey bee micronutrient requirements shift with season, or that certain micronutrients are limiting in the environment.

Sodium is an important osmo- and pH regulator in insects and magnesium is an important co-factor for various physiological processes (Cohen, 2004); concentrations in bees exhibited peaks and troughs at similar points in time, particularly in late summer, suggesting the two co-vary in the environment. Although the concentration range (3.0–7.5 μg/mg) is small, relative variation in the two micronutrients is drastic: magnesium content exhibits drops more than 50% between summer and fall, and sodium exhibits multiple drops of more than 50% (Fig. 3). Thus, as the fall approaches, honey bees may continue to balance their micronutrient intake (as shown in the corbicular pollen data) but these micronutrients may not bioaccumulate in the same way throughout the year. While our current data do not account for excretion of micronutrients, future studies could build upon our data and collect bee excrement throughout the year to calculate actual bioaccumulation.

Both calcium and potassium were found in large amounts in adult honey bees and over a much greater range (25–40 μg/mg) than sodium and magnesium (Fig. 3). In fall, calcium increased in all three areas: honey bee water preference, corbicular pollen concentration, and adult bee concentration (Table 1, Fig. 3). Each of our hives experienced a spike in calcium concentration at different time points in the fall; this may indicate a behavioral shift away from brood rearing toward overwintering (Mattila et al., 2001). Honey bees survive winter by forming a cluster around the queen and actively generating heat via muscle contractions (Omholt, 1987; Stabentheiner, 2003). The increase in stored calcium could be due to its use in muscle movement.

Additionally, the calcium concentration increase in our hives corresponds with the last brood reared before winter. Winter workers are physiologically different than summer workers and survive longer (6–8 months instead of 1 month) (Fluri et al., 1982). Thus, these individuals may have different nutrient requirements. Kunert and Crailsheim (2015) found that successful winter workers had higher macronutrient stores than summer workers. Our results suggest to survive through the winter, workers may need certain micronutrient stores as well. The increase in calcium content of adult bees could also be due to changing floral resources in the environment, it is possible that the only floral source available at this time of year is high in calcium.

An alternative hypothesis, which we do not favor, is that the practice of “sweetening” lawns using calcium carbonate during the fall led to an increase in calcium in both corbicular pollen and bees via a form of contamination. We do not favor this hypothesis because our bees were located adjacent to large unmanaged wildflower fields, which were surrounded by woods and were great distances from managed lawns. Additionally, if calcium were contaminating the corbicular pollen at our observation hives, we would expect a decrease in preferences for calcium in foraged water, and instead we saw an increase.

Similar to calcium, honey bee potassium concentrations were much higher than expected based on corbicular pollen concentrations and water preferences. Although we cannot explain the source of this “extra” potassium with our current data, one possible explanation is nectar. High concentrations of potassium can occur in nectar (Nicolson, 2011; Afik et al., 2014). Both royal and worker jelly also have high concentrations of potassium (Wang et al., 2015) and thus, this micronutrient may enter individuals via feeding during the larval stage. Potassium can act as a phagostimulant, which is beneficial to developing larvae (Cohen, 2004). Thus, the high potassium content we identified may be an artifact of our sampling procedure: we sampled young, and likely recently emerged, workers. These young workers are tasked with feeding the developing larvae and could have had recently secreted royal and/or worker jelly from their hypopharyngeal glands (Winston, 1987). Our processing and analysis protocol did not allow us to determine if nutrients were simply present in/on the bee at the time of sampling or if the nutrients were assimilated. It would be interesting to investigate how honey bee potassium content might vary with age or with digestive organ. It may be that younger adults have more stored potassium than older adults or that the hypopharyngeal glands, the organ used to produce royal and worker jelly, has a higher potassium content than the digestive tract, where nutrient absorption occurs (House, 1974).

4.3. Conclusions

Our data suggest that honeybees may actively regulate micronutrient intake with respect to some micronutrients, while others may be limiting in the environment. This conclusion is consistent with research done by Filipiak et al. (2017): possible environmental limitations to honey bee growth and development resulted mainly from the scarcity of micronutrients, such as sodium and potassium, in corbicular pollen. As such, the diet of honeybees appears to be more dynamic than previously considered. Current supplemental diets available to beekeepers (e.g. Vitamin B Healthy, Honey Bee Healthy) do not account for this dynamic nature, and thus may not be sufficient for year-round use.

This study also highlights the importance of understanding nutrient requirements in a natural environment, especially in temperate regions where nutritional resources, as well as nutrient requirements, likely shift throughout the year. To our knowledge, this is first study to examine variation of micronutrient content in insects and their food over time, in the field. Using honey bees as a model system, we provide a framework for filling a gap in nutritional ecology where currently, micronutrient requirements are understudied relative to macronutrient requirements.

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Author contributions

REB and LDO contributed equally to this work. PTS aided in conceptualization, experimental design, and data interpretation. All authors wrote, read, and approved the final manuscript.

Conflict of interest

The authors declare that they have no potential conflict of interest in relation to the study in this paper.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jinsphys.2018.02.002.

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