1	Title Seasonality of salt foraging in honey bees (Apis mellifera)
2	Running Header Salt foraging in honey bees
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24 Abstract

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

While there are many studies regarding macronutrient requirements of
honey bees, few investigate micronutrient needs. From 2013-2015, we conducted
preference assays in both summer and fall.

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

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

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

44

45 Key-words minerals, nutrition, nutritional ecology, optimal foraging, seasonality

46

### 47 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, Simpson et al. 2009). Reaching nutrient- and intake-targets is not typically as simple as balancing the intake and output of energy—most organisms require a balance of complex macro (carbohydrates, proteins, lipids) and micro (vitamins, minerals) nutrients (Simpson and Raubenheimer 2011). Reaching this balance is made challenging by the fact that nutrient composition varies between food sources.

55 Thus, foraging organisms face a difficult balancing act which may be made 56 more difficult by both abiotic (e.g. temperature, precipitation) and biotic (e.g. floral 57 distribution and abundance, predators) factors (Raubenheimer, Simpson et al. 2009). 58 Social organisms, particularly eusocial insects, face yet another layer of complexity: 59 they have to obtain the right nutritional requirements for both themselves and their 60 nestmates, which often require different ratios of different nutrients depending on 61 caste and age (Lihoreau, Buhl et al. 2015). Although micronutrients are vital for all 62 physiological processes, nutrition is often studied with a focus on macronutrients 63 (Cohen 2004, Rupp 2015). To fully understand an organism's nutritional ecology, 64 however, it is important to investigate how organisms balance the intake of both 65 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

71 nutrition that can be manipulated and observed. For example, honey bee nutritional 72 requirements shift with age—dependent larvae require large amounts of protein while 73 the oldest adult workers feed mainly on carbohydrates (Haydak 1970, Paoli, Donley et 74 al. 2014). Importantly, studying honey bees allows for the investigation of fitness 75 effects at both the individual- and colony-level (Lihoreau, Buhl et al. 2015).

76 While we know that diet diversity is important to honey bee health (Alaux, 77 Ducloz et al. 2010), we do not know which nutrients in this diverse diet are important. 78 Bees raised on a polyfloral diet exhibit stronger baseline immunocompetence than 79 bees raised on a monofloral diet, even when the monofloral diet has a higher overall 80 protein content (%) than the polyfloral diet (Alaux, Ducloz et al. 2010). Thus, there is 81 more to developing a strong immune system than getting the right amount of protein. 82 While much is known about the macronutrients honey bees need to maintain a healthy 83 colony, very little is known about the micronutrients that are needed (Haydak 1970, 84 Cohen 2004, Huang 2010).

85 Honey bees tend to prefer compound-rich (hereafter called "dirty") water 86 sources over clean water sources (Butler 1940). Despite countless observations, there 87 is only one scientific field study (Butler 1940) regarding this specific behavior. Butler 88 (1940) concluded that honey bees prefer dirty water based on strong odor cues. While 89 this provides a proximate mechanism by which honey bees may find dirty water, it 90 does not provide an evolutionary explanation for this behavior. Similar to geophagy, 91 the intentional consumption of earth (Young, Sherman et al. 2011), dirty-water-92 seeking behavior likely has an adaptive purpose.

We hypothesize that honey bees have an optimal diet that includes nutritional
resources from both floral and water sources. Since the honey bee's main floral diet

95 only contains trace amounts of micronutrients (Somerville 2005, Brodschneider and 96 Crailsheim 2010), and sodium-specific foraging is a well-known behavior in social 97 insects (Botch and Judd 2011, Pizarro, McCreery et al. 2012) and across the animal 98 kingdom (Denton 1982, Young, Sherman et al. 2011, Starks and Slabach 2012), we 99 postulate that to obtain a well-rounded diet, honey bees selectively forage in soil and 100 water for minerals that their main floral diet may lack. As the honey bee colony is a 101 dynamic environment and honey bees live in temperate regions, our hypothesis leads 102 us to three main predictions.

103 Firstly, if honey bees have an optimal diet that is satisfied by both floral and 104 water sources, we predict that honey bees will show mineral preferences when 105 foraging for water. Minerals are essential for all physiological functions (e.g. muscle 106 movement and immunity) (Cohen 2004). Universally, sodium is a key player in 107 osmoregulation. While soil is known to contain significant amounts of sodium, the 108 above ground parts of land plants-the main food source for honey bees-rarely 109 contain a great amount of this important mineral (Oates 1978, Cohen 2004). In 110 insects, when coupled with potassium, sodium regulates cellular and body fluid pH. 111 We predict that honey bees will prefer both sodium and potassium in comparison to 112 deionized 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 and Xu 2015). In excessive amounts, however, calcium

119 can cause paralysis in honey bees (Somerville 2005). We predict that honey bees will 120 prefer magnesium but not calcium. Lastly, as phosphate is essential to the process of 121 bioenergentic activity and nitrogen is vital for cellular communication and waste 122 removal (among other things), we predict that honey bees will prefer both mineral 123 solutions relative to deionized water.

124 Secondly, if dirty water sources are coupled with floral resources to reach an 125 optimal diet, we predict that the strength of mineral preferences when foraging for 126 water will differ with the distribution and abundance of floral resources (i.e. the 127 seasons). We expect mineral preferences when foraging for water to complement 128 which minerals are available in floral resources (mainly pollen). Major minerals found 129 in bee-collected pollen are potassium, calcium, and magnesium; levels of each 130 mineral vary within and between summer and fall (Herbert and Miller-Ihli 1987, 131 O'Connor, Bonoan et al. unpublished data).

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 and Shimanukia 1978).

Although honey bee mineral (NaCl, MgCl<sub>2</sub>, KCl, Na<sub>2</sub>HPO<sub>4</sub>) preferences have recently been tested in the lab (Lau and 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 suchnutrient preferences may affect fitness.

144

#### 145 Materials and methods

146 This study was performed on the Tufts University Medford/Somerville 147 campus (equipped with eight 2-frame observation hives) during fall 2013, 2014, 2015 148 (Sep-Oct), and summer 2014, 2015 (Jul-Aug). Given general insect micronutrient 149 requirements, we tested preferences for six specific mineral solutions: sodium (NaCl), 150 calcium (CaCl<sub>2</sub>), potassium (KCl), magnesium (MgCl<sub>2</sub>), phosphorus (KH<sub>2</sub>PO<sub>4</sub>), and 151 nitrogen (NH<sub>4</sub>Cl) (Cohen 2004). Minerals were also chosen based on what honey bees 152 are likely to find in soil or dirty water where they often forage (O'Connor, Bonoan et 153 al. *unpublished data*).

154

### 155 Mineral preferences

156 Once the bees were trained (von Frisch 1967) to forage in an open, grassy 157 location (about 50 - 90 m from the observation hives), we conducted preference 158 assays two to five times a week (weather-permitting). We set up preference assays on 159 a 1.82 m long plastic table which was divided into two 4 x 4 (72 cm x 72 cm) grids 160 (Fig. 1). In addition to the six mineral solutions, a sucrose solution (10% during fall 161 assays, 20% during summer assays) served as the positive control and deionized water 162 served as the negative control (Pizarro, McCreery et al. 2012). Based on honey bee 163 supplemental feeding guidelines (Somerville 2005), all mineral solutions were a 1% 164 concentration. In the lab, honey bees respond to NaCl levels as low as 50 µM (de 165 Brito Sanchez 2011) and preferentially respond to 1.5% NaCl, 1.5% MgCl<sub>2</sub>, 0 – 1.5%

KCl, and 0.4 - 0.75% NaH<sub>2</sub>PO<sub>4</sub> (Lau and 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 at 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 each trial.

172 At the beginning of each trial, 50 ml falcon tubes were filled with 25 ml of the 173 appropriate solution and randomly allocated (using a random list generator, 174 www.random.org) to a numbered square on each grid (Fig. 1, inset). Once the tasting 175 table was set up, bees were allowed to forage for 5 - 7 hours (depending on weather). 176 At the end of each trial, we measured the amount (ml) of each solution remaining in 177 both the control and experimental grids. The change in volume of the control side 178 subtracted from that of the experimental side yielded the total volume consumed by 179 the foraging bees. In total, we conducted 33 preference assays in summer and 18 180 preference assays in fall. For each year (2013, 2014, 2015), new bees were installed 181 (thus, we have two true replicates for summer and three true replicates for fall) and 182 the eight observation hives, and thus eight colonies, were trained to forage at the 183 tasting table.

184

# 185 Colony fitness

To assess what preferences mean for colony fitness, we gave bees a colonyspecific, light-weight, colored powder mark during 2014 preference assays (Fig. 2) (Hagler, Mueller et al. 2011, Bonoan and Starks 2016). We constructed marking apparatuses from mesh cloth, filled with non-toxic acrylic colored powders (ECO

190 Pigments, Day-Glo Color Corporation). Using Velcro (Scotch Extreme Fasteners), we 191 affixed the marking apparatuses to each hive entrance such that foragers were dusted 192 with visible colored markings upon exiting (Bonoan and Starks 2016). As each hive-193 and thus colony—had a unique color, we could approximate the number of visits each 194 colony made to each solution during preference assays (Fig. 2). The approximate 195 number of visits indicates the intensity of colony-specific preference for each mineral 196 solution. We counted the number of bees from each colony at each solution every 15 197 minutes throughout the duration of the trial. Unmarked bees were classified as feral 198 and not included in analysis.

199 To see if colony-specific preferences correlated with internal colony 200 dynamics, we measured two colony fitness parameters three times a week beginning 201 in Jul 2014 and ending in Oct 2014. First, population estimates were recorded 202 according to Sammataro & Avitabile (2011): a standard deep frame entirely covered 203 by one layer of bees is roughly 2000 adult individuals; estimates were taken in 204 increments of 250 bees. Second, the total area of capped brood (i.e. the amount of 205 oldest honey bee brood) was calculated by first measuring the area of the smallest 206 rectangle to encompass the brood patch. As honey bee brood patches tend to be oval-207 shaped, we then subtracted the area of empty corners from the area of the original 208 rectangle. This was then multiplied by the quality of the queen's laying pattern which 209 was ranked on a scale of one to five depending on the spottiness of brood comb 210 (1=100-80% empty cells; 2=80-60% empty cells; 3=60-40% empty cells; 4=40-20% 211 empty cells; 5=20-0% empty cells). A similar scale was implemented by Vaudo et al. 212 (2011) as a proxy for honey bee colony health. Together, these calculations gave us 213 the total capped brood area of each colony.

214

### 215 Statistical analysis

216 For preference assays, data for all three years (2013, 2014, 2015) were pooled 217 by season as there was no significant effect of year on the amount of each solution 218 consumed. Prior to analysis, we removed data for sucrose, our positive control. To 219 determine whether or not there was an overall effect of season (summer versus fall) or 220 mineral solution, we ran a two-way ANOVA on natural log-transformed data. 221 Following this analysis, we ran two one-way ANOVAs (one for each season) with 222 contrasts. This allowed for the comparison of each mineral solution to our negative 223 control, deionized water. Again, data were natural log-transformed prior to analysis in 224 order to meet test assumptions.

225 To analyze the 2014 data for colony fitness, we combined the counts of visits 226 each colony made to mineral solutions (CaCl<sub>2</sub>, KCl, KH<sub>2</sub>PO<sub>4</sub>, NaCl, NH<sub>4</sub>Cl, MgCl<sub>2</sub>) 227 for each week. To standardize for colony size (colonies ranged from 0 - 7,000228 individuals throughout data collection), we calculated the approximate proportion of 229 the colony that made visits to mineral solutions each week by dividing the number of 230 visits to mineral solutions by the average colony population for that same week. These 231 calculations yield a colony-size adjusted measure of the intensity of preference for 232 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 nine days for an egg to develop into a 5<sup>th</sup> instar larvae and get capped over) and a three week offset for the adult population (it takes about twenty-one days for an egg to fully develop into an adult worker bee) (Winston 1987). Since colony fitness parameters are expected to naturally decrease inthe fall, we only used summer data for this analysis.

239 For both brood area and adult population, we ran a Poisson regression against 240 a null hypothesis of no effect of minerals (i.e. a line with a slope of 0). We did not 241 have enough measurements per colony to add colony as a random effect. Instead, we 242 standardized visits by calculating the proportion of each colony visiting minerals 243 rather than combined number of visits (see above). For brood area, we examined 244 whether or not the proportion of workers visiting minerals at week t affected the 245 brood area at week t + 1. Similarly, for adult population, we examined whether or not 246 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 247 248 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).

251

### 252 **Results**

253 Mineral preferences

When all three field years (2013, 2014, 2015) are analyzed 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 phosphorous in the fall compared to the summer (Fig. 4a).

When controlling for deionized water, an effect of season on mineral 260 261 preferences emerges (Fig. 3b). No matter the season, bees drank significantly more 262 sodium than deionized water (fall:  $t_{245} = 3.996$ , P < 0.001, summer:  $t_{455} = 4.008$ , P < 0.001,  $t_{45} = 0.008$ , P < 0.001,  $t_{45} = 0.008$ , P < 0.001,  $t_{45} = 0.008$ ,  $t_{45}$ 263 0.001). In the fall, bees drank significantly more potassium than deionized water ( $t_{245}$ 264 = 2.254, P = 0.025) and drank about the same of the two solutions in the summer (Fig. 265 3b). Though the effect was not significant, the bees did drink more calcium and 266 magnesium than deionized water in the fall and less than deionized water in the 267 summer (Fig. 3b). During the summer, bees drank significantly less nitrogen ( $t_{455} = -$ 2.251, P = 0.025) and phosphorous ( $t_{455} = -2.064$ , P = 0.040) than deionized water 268 269 (Fig. 3b). This trend was not observed in the fall.

270

### 271 Colony fitness

There was a significant effect of the approximate proportion of the colony visiting mineral solutions on capped brood area one week out ( $X^{2}_{1} = 13.2, P < 0.001$ ) and adult population three weeks out ( $X^{2}_{1} = 202.7, P < 0.001$ ). Although the effect is significant, the pseudo-R<sup>2</sup> 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).

278

### 279 Discussion

Honey bees showed mineral preferences and, in comparison to deionized 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 fall. 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 fall.

The seasonality in preferences was made apparent when the mean volume consumed was analyzed relative to deionized water. Relative to deionized water, bees drank significantly more potassium and tended to drink more calcium and magnesium in the fall (Fig. 3b). In the summer, however, there was no significant preference for potassium and bees tended to avoid calcium and magnesium.

292 This switch in preferences is particularly interesting since potassium, calcium, 293 and magnesium are three of the most prominent minerals found in pollen (Herbert and 294 Miller-Ihli 1987). Moreover, the levels of these minerals in pollen vary with season; 295 in the summer, there are high levels of these minerals in pollen while in the fall, there 296 are low levels of these minerals in pollen (Herbert and Miller-Ihli 1987). Taken with 297 our data, this suggests that honey bees are foraging for minerals in water based on 298 what their floral diet is lacking. The bees' preference for sodium no matter the season 299 also supports our hypothesis—the above ground parts of plants rarely contain much 300 sodium (Oates 1978, Cohen 2004) and herbivores are often sodium-limited (Denton 301 1982). Preliminary analysis of pollen our bees collected throughout the 2015 season 302 (Jul – Oct) shows that out of potassium, calcium, magnesium and sodium, sodium is 303 found at the lowest levels (0 - 4 ppm) (O'Connor, Bonoan et al. *unpublished data*).

It was unexpected that bees would avoid nitrogen and phosphorous. Since 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 and Jamieson 1948). Additionally, if honey bees are searching in soil

for nitrogen sources, nitrogen exists in various forms in the soil. It is possible that our specific nitrogen compound is not the nitrogen compound honey bees prefer. This is also a possible explanation for the unexpected avoidance of phosphorous.

311 Regarding minerals and colony fitness, it is hard to determine anything 312 conclusive with the current data. While stronger colonies do tend to visit more 313 minerals than weaker colonies (Supporting information), it is hard to say which came 314 first. Do strong colonies visit minerals more often because they are strong? Or, are 315 they strong because they visit minerals more often? Unfortunately, our regression 316 with the offset does not help answer the question. Although our data show a 317 significant effect of minerals on both colony fitness parameters, the model does not 318 reveal either a positive or a negative effect. This may be because we do not have 319 enough data or we should collect data from larger, more natural-sized hives.

Overall, our data suggest that honey bees forage at 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 lab 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

331 spring, floral resources are abundant, and thus honey bees do not need to seek out332 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 as well as larger Langstroth hives.

346 More generally, collecting data on mineral preferences in honey bees can 347 bolster our understanding of pollinator health and nutritional ecology. While there is 348 much known about insect nutrition and foraging in general, micronutrient (both 349 vitamin and mineral) requirements remain poorly understood (Cohen 2004), even 350 with the recent decline in pollinator populations. Micronutrients are a physiologically 351 important part of any organism's complex diet (Simpson and Raubenheimer 2011, 352 Simpson and Raubenheimer 2012, Rupp 2015), and should be studied for a more 353 complete understanding of the complex balancing act organisms face when foraging.

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448	<b>Fig. 1.</b> Tasting table with foraging honey bees on the experimental side of the table.
449	The mesh covering on the control side of the table allowed us to exclude bees and
450	account for volume change due to evaporation during the trial. Volume change in the
451	experimental side minus volume change on the control side gave us the amount honey
452	bees actually consumed. Inset: Tasting table grid that was used to randomly allocate
453	solutions for each trial.

454

455 Fig. 2. Yellow and pink powder-marked honey bees visiting a solution at the tasting
456 table. Counts of each color bee at each solution were made every 15 minutes.

457

458 Fig. 3. (a) Mean volume change of each mineral consumed (ml) by all eight hives 459 separated by season. Error bars are  $\pm 1$  standard error and show the variation in 460 volume consumed for each trial within each season. There was no significant effect of 461 season on the volume consumed however, there was an effect of mineral solution. (b) 462 Volume change for each mineral solution relative to volume change of deionized 463 water (negative control, x = 0.0), calculated for each field season. Anything above the baseline was preferred compared to deionized water, anything below the baseline was 464 465 avoided compared to deionized water. Bees significantly preferred sodium no matter 466 the season. In the fall, bees significantly preferred potassium. In the summer, bees 467 significantly avoided nitrogen and phosphorus. Error bars are  $\pm 1$  standard error and 468 show the variation in volume consumed for each trial within each season. (\*P < 0.05, 469 \*\*\**P* < 0.001)

470

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

477

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