1 Inter-individual variation in nutrient balancing in the honeybee (*Apis mellifera*)

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15 **Abstract**

16 The Geometric Framework approach in nutritional ecology postulates that animals
17 attempt to balance the consumption of different nutrients rather than simply maximizing
18 energetic gains. The intake target with respect to each nutrient maximizes fitness in a specific
19 dimension and any difference between individuals in intake targets therefore represents
20 alternative behavioral and fitness maximization strategies. Nutritional interactions are a central
21 component of all social groups and any inter-individual variation in intake targets should
22 therefore have a significant influence on social dynamics. Using the honeybee colony as an
23 experimental model, we quantified differences in the carbohydrate intake targets of individual
24 foragers using a capillary feeder (CAFE) assay. Our results show that the bees did not simply
25 maximize their net energetic gain, but combined sugar and water in their diet in a way that

brought them to an intake target equivalent to a 33% sucrose solution. Although the mean intake target with respect to the nutrients sucrose and water was the same under different food choice regimens, there was significant inter-individual variation in intake target and the manner in which individuals reached this target, a variation which suggests different levels of tolerance to nutrient imbalance. We discuss our results in the context of how colony performance may be influenced by the different nutrient balancing strategies of individual members and how such nutritional constraints could have contributed to the evolution of sociality.

Key Words

Nutritional Geometry; Feeding Behavior; Social Behavior; Inter-individual Variation; Eusociality; Honeybee

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Introduction

All animals must obtain a specific combination of different nutrients to optimize different life history traits. For instance, an animal that is maximizing growth or reproduction may require a larger proportion of protein in its diet, while an animal that is more concerned about survival is likely to maximize the intake of carbohydrates as a quickly available fuel source. This is in contrast to what is predicted by optimal foraging theory (Charnov, 1976), which has traditionally considered energetic gain as the primary currency driving the foraging decisions of animals. Nutritional geometry, a bottom-up, state-space modeling approach specifically developed to address this issue, explains foraging behavior in terms of satisfying a ratio among different nutrients that maximizes fitness (Simpson and Raubenheimer, 1993, 2012). The level of a nutrient that provides the maximum contribution to a given life history trait is defined as the intake target for that nutrient, thus requiring an animal to satisfy a multidimensional intake target. Faced with different food items that vary in their nutritional compositions, an animal is therefore confronted with the complex problem of how to reach or approach this multidimensional target in a way that achieves a nutritional balance for maximum fitness.

Nutritional geometry has been shown to be a robust model for explaining how animals regulate their foraging to balance the intake of different nutrients. While the Geometric Framework has been used to explain nutrient balancing with respect to different fitness parameters in a variety of species (Simpson and Raubenheimer, 2012), whether such nutritional regulation plays any role in social dynamics is only beginning to be considered (Behmer, 2009; Cook et al., 2010; Lihoreau et al., 2014). Social insect colonies of honeybees and ants have been shown to behave in a manner consistent with the Geometric Framework of nutrient balancing, regulating their nutrient intake at a collective level (Dussutour and Simpson, 2008, 2009; Hendriksma and Shafir, 2016). In an interesting contrast to what might be expected from optimal foraging theory, ant colonies were found to switch from consuming a concentrated sugar solution to a more dilute solution with time, which suggests that they were balancing their diet with respect to the nutrients sugar and water, rather than simply maximizing their energetic intake (Dussutour and Simpson, 2008).

77 Within any group such as a social insect colony, one can expect a substantial amount of
78 inter-individual variability in intake targets not only between members of different behavioral
79 groups (Paoli et al., 2014), but also within a behavioral group with each individual possessing
80 different physiological dispositions. For example, it has been shown that pollen foragers have
81 higher metabolic rates than non-pollen foragers (Feuerbacher et al., 2003) and these foragers
82 with higher metabolic demands could exhibit a higher carbohydrate intake target than other
83 foragers. Individuals varying in their overall foraging efforts might also be driven in part by how
84 closely they monitor their own intake target, which has been referred to as an individual's
85 'nutritional latitude' (Senior et al., 2015). It is therefore important to understand the nature of
86 such variation within a colony and how it might impact the nutritional intake at the colony level,
87 which in turn might have played a role in the evolution of social behavior itself.

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89 The capillary feeder (CAFE) assay, originally developed to examine the prandiology of
90 the fruit fly, *Drosophila melanogaster* (Ja et al., 2007), is a technique that allows precise
91 measurement of liquid food consumption by individual animals and can be applied to both short-
92 and long-term feeding experiments (Deshpande et al., 2014). Because the diet of adult workers in
93 social insect colonies consists primarily of carbohydrates (Altaye et al., 2010; Ihle et al., 2014;
94 Paoli et al., 2014), we used a modified CAFE assay to investigate the variation in carbohydrate
95 | intake target and nutrient balancing strategy with respect to sucrose and water among individual
96 honeybee foragers. In the absence of any substantial fat reserves, these foragers critically rely on
97 their nectar based carbohydrate diet, consisting mainly of water and sucrose, to meet their large
98 energetic requirements for flight and foraging performance (Sacktor, 1970; Candy et al., 1997),
99 subjecting them to strong selection for managing their carbohydrate budgets. By removing a
100 forager from the colony and allowing her to choose between two different concentrations of
101 sucrose solutions, we were able to examine the variation in how an individual bee regulates her
102 nutritional requirements, independent of the nutritional state of the colony.

2.1

Methods

2.1.1

Gustatory Responsiveness Assay

We collected returning honeybee (*Apis mellifera*) foragers from five different colonies, noting whether or not they were carrying pollen, and chilled them on ice just enough to allow them to be harnessed into plastic straws. The gustatory sucrose sensitivity of each bee was assessed by stimulating its antennae first with water and then with an ascending series of sucrose concentrations up to 60% (0, 0.1, 0.3, 1, 3, 10, 30, 45 and 60%) and testing for the extension of its proboscis, the Proboscis Extension Response (PER). All bees were stimulated with water between the presentations of two successive sucrose concentrations in order to reduce the effects of any potential sensitization to sucrose. The concentrations of all sucrose solutions in this study were prepared and reported as w/w sucrose solution: weight (g) sucrose / (weight (g) sucrose + (g) water). A Gustatory Responsiveness Score (GRS) was calculated for each bee as the sum of the PERs elicited to the initial presentation of water and the eight sucrose concentrations (Scheiner et al., 2001). The GRS scores in this experiment therefore has a range of 0-9, a score of 0 indicating that the bee did not respond to any of the stimuli, including the first presentation of water, while a score of 9 indicates that the bee responded to the initial water presentation and all the sucrose concentrations.

2.1.2

CAFE Assay

Immediately following the GRS assay, each bee was fed until satiation with a 30% sucrose solution (to equalize their energetic states) and subjected to a 16-hour CAFE assay to determine its individual intake target with respect to sucrose and water. Each bee was placed in a clear acrylic chamber (3 cm ID and 3 cm tall) with ventilation holes and two glass capillary feeding tubes (152 mm long, 1.12 mm ID; World Precision Instruments, item number: TW150-6), each filled with 110 μ l of sucrose solution of a different concentration, representing two

alternative food choices. The two solutions were enhanced with either blue or yellow food coloring to enable their discrimination during analysis and the two colors were alternated between the two concentrations and the two sides of the chamber in different replicates to correct for any potential color or side bias. The chambers were placed in an incubator set at 25° C and 60% Relative Humidity (RH) and a camera with an automatic timer was used to record the level of the solution in each capillary at hourly intervals to measure the consumption of each solution. We conducted two series of CAFE assays, one in which the two sucrose solutions provided were 45% and 5%, and another in which the two solutions were 45% and 1%. Each replicate of the assay also included a control chamber identical to the others, but without a bee in it, to account for any evaporative loss of the solutions.

2.1.3

Statistical Analysis

The hourly consumption of each solution by each bee was calculated after subtracting the average hourly rate of evaporation from the control chambers, and from this the total amounts of sucrose and water consumed were used to calculate the hourly intake and the final intake target for each bee, expressed as sucrose concentrations. A one-sample t-test was used to compare the average final intake target across all bees to an intake target equivalent to the mean concentration of the two solutions. A two-sample t-test was used to compare the intake targets in the two treatments. An F-test of variance was used to compare the variation in the amount of water consumed to the amount variation in sucrose consumed. The nutritional latitude of a bee was calculated as the mean absolute difference between its final intake target and its intake target at each hour, given by $(\sum |IT_{final} - IT_{hour}|) / n$, where n is the number of hourly observations for the bee. Kolmogorov-Smirnov goodness of fit tests were used to compare the distributions of individual intake targets and nutritional latitudes with expected normal distributions. Pearson's correlations were used to investigate the relationships between gustatory responsiveness, forager type, and the final intake target of each bee. All statistical analyses were conducted using R (version 3.1.1).

3.1

Results

3.1.1

Intake target

A total of 200 bees, which completed the CAFE assay without exhausting either of the sucrose solutions, were used in the analysis to ensure that all of them had a choice between the two foods during the entire assay. At the end of the 16-hour assay, the bees in the two CAFE assays, consisting of different pairs of sucrose concentrations, converged on the same, statistically indistinguishable intake target (Welch Two-sample t-test: $t_{188} = 0.15$, $p = 0.88$, Fig. 1A). The intake target for the experiment in which the bees had a choice between 1% and 45% solutions was 0.33 ± 0.009 and the intake target observed in the experiment with 5% and 45% solutions was 0.33 ± 0.01 , both equivalent to a 33% sucrose solution. The two intake targets were significantly different from the mean of the two concentrations in both treatments (1% vs. 45%: $t_{109} = 2485.51$, $p < 0.0001$; 5% vs. 45%: $t_{89} = 2351.48$, $p < 0.0001$), demonstrating that the bees were not simply feeding randomly. In both treatments, the pattern of hourly intake indicates an initial bias followed by a decline in the consumption of the high concentration solution, such that by the end of the assay the two solutions were being consumed in a specific ratio (Fig. 1B). This pattern of consumption suggests that the bees were actively regulating their intake target.

Figure 1

Figure 1: Intake target of bees (mean $\pm$ SE) with respect to sucrose and water determined by a 16-hour CAFE assay, with data represented as (A) final amount of sucrose and water consumed, the three different lines representing the concentrations of the different sucrose solutions in the two experiments, and (B) hourly intake target of bees, calculated as consumption within each hourly interval, in the two treatments.

Although the intake targets realized by the two groups of bees in the two treatments were the same, there was considerable inter-individual variation with a significantly higher variation in the amount of water consumed than in the amount of sucrose consumed by the bees (1% vs. 45%: $F_{109, 109} = 1.95$, $p < 0.001$; 5% vs. 45%: $F_{89, 89} = 2.5$, $p < 0.001$, Fig. 2A). This resulted in individual intake targets showing a significant departure from a normal distribution (Kolmogorov-Smirnov Test: $D = 0.16$, $n = 200$, $p < 0.001$, Fig. 2B), with a larger number of bees demonstrating high intake targets.

Figure 2

Figure 2: Inter-individual variation in the final intake target among bees in the two treatments showing (A) a higher variation on the water than on the sugar axis, and (B) a significant departure from an expected normal distribution.

3.1.2

Nutritional Latitude

Individuals can reach similar intake targets but remain true to the target or stray away from it by different extents throughout the assay, the magnitude of which is defined as one's nutritional latitude. A low nutritional latitude is seen as a relatively straight trajectory to the intake target while a high latitude is seen as a more meandering trajectory to the target, punctuated by large changes in the slope of the line (Fig. 3A). Nutritional latitudes were normally distributed (Kolmogorov-Smirnov test, $D = 0.07$, $n = 200$, $p = 0.28$, Fig. 3B), and the

208 nutritional latitude of a bee did not show any correlation with her intake target (Pearson's
209 correlation: $t_{198} = 0.03$, $p = 0.97$, Fig. 3C).

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Figure 3

Figure 3: Nutritional latitudes of bees illustrated by (A) cumulative consumption of four representative individuals with high or low intake targets and high or low latitudes. Each point represents the cumulative intake at a given time point; circles represent a bee with high latitude and triangles represent a bee with low latitude, filled shapes indicate a bee with high intake target and open shapes represent a bee with low intake target, the points overlap during hours that a bee did not consume either of the two solutions, (B) their normal distribution among individuals, and (C) their non-significant correlation with intake target.

3.1.3

Gustatory Responsiveness

Pollen foragers had higher gustatory responsiveness scores (GRS) than non-pollen foragers ($t_{158} = 3.07$, $p < 0.01$) and the GRS of an individual bee was positively correlated with its intake target (Pearson's correlation: $t_{192} = 2.86$, $p < 0.01$, Fig. 4).

Figure 4

Figure 4: Intake target (mean $\pm$ SE) and Gustatory Responsiveness Score ($n = 194$) of individual bees are significantly correlated. The number of bees with a given GRS is indicated above each data point.

4.1

Discussion

In this study we used the principles of nutritional geometry to examine the nutritional intake and nutrient balancing strategies of individual honeybee foragers with respect to sucrose and water. This is the first time, to the best of our knowledge, that a CAFE assay has been used to investigate the variation in the fine-scale feeding behavior among individual honeybees. Our results show that individual bees balance their nutritional intake independent of the colony context and that there is a substantial amount of inter-individual variation in both intake targets and how they reach these targets.

Honeybee foragers vary in terms of their gustatory responsiveness to sucrose (Page et al., 1998; Scheiner et al., 2001) and individual bees have been shown to use gustatory information to discriminate between different diets (Hendriksma and Shafir, 2016). Gustatory information is known to play a key role in nutrient balancing, helping an animal to assess the suitability of a food resource with respect to its intake target and thereby inform its foraging decisions (Simpson and Raubenheimer, 1993, 1996). The positive correlation between GRS and intake target observed in this study could indicate that a higher sensitivity to sucrose may be key to obtaining a higher intake target with respect to the carbohydrate requirements of individual honeybees. Since individuals with higher GRS are known to be pollen foragers (Pankiw and Page, 2000), who also have higher metabolic rates (Feuerbacher et al., 2003), it seems likely that the higher intake targets observed in these bees could be associated with their task-related metabolic needs.

In both the treatments in our study, the bees were required to choose between two alternative food resources, and remarkably the bees consistently combined the available foods in a way that allowed them to achieve an intake target equivalent to a 33% sucrose solution. That the bees were not feeding randomly is demonstrated both by a final intake target greater than the average of the two solutions offered and by the change in relative consumption of the two solutions over time. The fact that there was greater variation in the amount of water consumed in comparison to that of sucrose, suggests that the bees were prioritizing the sucrose component of

the available food resources. However, they were also not merely trying to maximize their net energetic gain, as would have been indicated had they consumed all of the high concentration sucrose solution before consuming any of the low concentration solution, in fact not a single bee exhibited this behavior. Rather, the bees combined the two different foods in a way which indicates that a 33% sucrose solution represents an ideal homeostatic target (Simpson and Raubenheimer, 1993; Köhler et al., 2012) for honeybee foragers. This is in contrast to what might be predicted by energy-maximizing optimal foraging models and shows that honeybees also actively regulate their water intake and even when considering a simple carbohydrate and water diet, nutritional geometry provides a more comprehensive description of the feeding behavior of animals. Few studies on Geometric Framework have considered water as a nutrient, but those which do, find that water has direct effects on fitness traits (Raubenheimer and Gäde, 1994; Fanson et al., 2012; Köhler et al., 2012).

The variation in nutrient balancing observed among individual bees is of particular interest because behavioral variation is considered to be adaptive in most biological systems, providing social groups with the flexibility to respond to environmental challenges (Mattila and Seeley, 2007; Pruitt and Riechert, 2011). The foraging behavior of each member in a social insect colony is limited by her own energetic and nutritional demands (Wolf et al., 1989; Feuerbacher *et al.*, 2003), which suggests that the difference among individual foragers in terms of their contribution to the colony (Pankiw and Page, 2000) could be a reflection of differences in their intake targets. As the subjects in our CAFE assays were kept at a constant and ideal temperature and allowed little movement, our results represent a close approximation of their carbohydrate requirement for maintaining a basal metabolic rate and the observed variation in intake targets probably reflects the intrinsic difference among individuals with respect to it. The variation in intake target can be expected to be substantially higher within the context of natural colonies due to difference in activity levels and task dependent metabolic demands among individuals and our future work aims to address such differences. Individuals with higher intake targets with respect to sucrose could also have higher survival as indicated by bees surviving longer on diets with a higher carbohydrate bias (Altaye et al., 2010; Paoli et al., 2014).

We defined nutritional latitude as the mean deviation of an individual bee from its own intake target, which translates to the degree to which that individual can tolerate a nutrient excess or deficit associated with ingesting a nutritionally imbalanced food (Senior et al., 2015). This latitude may arise from the action of several independent mechanisms such as the physiological cost of sustenance on an imbalanced diet, the travel cost between different food resources, or the level of competition for each of these resources. Each of these factors differs in terms of how it is expected to act on behavior, for example a high physiological cost of imbalance should induce an individual to switch more frequently between resources, leading to a lower latitude, while a high travel cost between resources should discourage such a switch, leading to a higher latitude. The nutritional latitude of an individual is therefore an outcome of the relative magnitudes of these different forces. Since in our experiment there is no competition for food and the magnitude of travel cost can be considered negligible, the observed nutritional latitude of an individual is a likely outcome of its physiological tolerance to unbalanced food. Bees with a higher latitude are therefore likely to be individuals with a higher tolerance for physiological imbalance than bees with a lower nutritional latitude. It would be interesting to ask if bees with different levels of physiological tolerance to unbalanced diets have different levels of resilience during times of resource scarcity or have different amounts of flexibility in terms of taking advantage of a wider variety of food resources in a natural ecological setting.

An unbalanced diet is costly to animals as any excess nutrient must be stored or excreted, both of which might require energetic expenditure. In insects, an excess amount of sugars can be toxic, especially as the insect ages (Garrido et al., 2015). This may be a relevant factor in the transition of a honeybee into the role of forager, which is accompanied by a shrinkage of the fat bodies (Toth et al. 2005) and a possible decrease in the ability to store excess sugars. However, an ability to unload any such excess sugars into a communal food cache for group level benefits may have contributed to the evolution of cooperative living in the honeybee. It has been shown that the nutritional needs of adult bees shift towards a carbohydrate biased diet when they make the transition from within-hive duties to foraging (Paoli et al., 2014). However, it would be interesting to determine if there is also a decrease in nutritional latitude with age that would suggest a decrease in physiological tolerance to high concentrations of sugar and if this is related

to the propensity of an individual to share food with its nestmates.

Nutritional interactions are a central component of all social groups. A social insect colony, generally comprised of a single reproductive queen and thousands of her offspring who forego their own reproduction in order to work for the benefit of the group, is often referred to as a superorganism (Wilson, 1971; Moritz and Southwick, 1992). The traditional viewpoint regarding cooperative foraging and food sharing in social insects is centered on the idea that the behavior of individual foragers is primarily regulated by the nutritional state of the colony (Seeley, 1995). However, recent research show that foraging decisions are also regulated at the level of the nutritional state of the individual (Toth et al., 2005; Mayack and Naug, 2013), leading to the idea that altruistic foraging in eusocial insects is driven by regulatory mechanisms that have been evolutionarily co-opted from solitary insects (Toth and Robinson, 2007). The current study supports these previous findings by demonstrating that individual honeybees are sensitive to their own nutritional requirements independent of the colony, especially in terms of carbohydrate which serves as a primary component of the adult honeybee diet and directly affects their performance and survival. We therefore propose that any inter-individual variation in the intake target among foragers should reflect in their performance and contribution toward the intake target of the colony and that understanding how a collection of individuals with different intake targets might drive social dynamics can contribute to our understanding regarding the evolution of social behavior.

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