Macronutrient Regulation by the Desert Leafcutter Ant *Acromyrmex versicolor*

by

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ABSTRACT

Understanding how and why animals choose what to eat is one of the fundamental goals of nutritional and behavioral biology. We can scale this question to animals that live in social groups, including eusocial insects. One of the factors that plays an important role in foraging decisions is the prevalence of specific nutrients and their relative balance. This dissertation explores the role of relative nutrient content in the food selection decisions of a species that is eusocial and also agricultural, the desert leafcutter ant Acromyrmex versicolor.

I used a dietary choice assay, varying the relative amount of protein and carbohydrates in the available diets, to demonstrate that A. versicolor colonies regulate relative collection of protein and carbohydrates. I tracked the foraging behavior of individual workers to show that foragers vary in their relative collection of experimental diets and in their foraging frequency, but that there is no relationship between these key factors of foraging behavior. The high proportion of carbohydrates preferred by lab colonies suggests that they forage to nutritionally support the fungus rather than brood and workers. To test this, I manipulated the relative amounts of 1) fungus, and 2) brood (larvae) and assessed foraging response. Changing the amount of brood had no effect on foraging. Although decreasing the size of fungus gardens did not change relative P:C collection, it produced significant increases in caloric intake, supporting the assertion that the fungus is the main driver of colony nutrient regulation.

I also analyzed the nutritional content of naturally harvested forage material collected from field colonies, and measured recruitment to experimental diets with varying relative macronutrient content. Field results confirmed a strong colony

preference for high carbohydrate diets. They also indicated that this species may, at times, be limited in its ability to collect sufficiently high levels of carbohydrates to meet optimal intake. This dissertation provides important insights about fundamental aspects of leafcutter ant biology and extends our understanding of the role of relative nutrient content in foraging decisions to systems that span multiple trophic levels.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

THE DESERT LEAFCUTTER ANT ACROMYRMEX VERSICOLOR REGULATES RELATIVE COLLECTION OF PROTEIN AND CARBOHYDRATES

Introduction

Animals actively regulate their relative collection of specific nutrients, and this ability to meet relative nutritional needs has profound effects on fitness. Regulation of relative collection of the macronutrients, protein, carbohydrates, and lipids, has been especially well-evidenced for a diversity of species (Simpson and Raubenheimer, 2012). This regulation of relative macronutrient collection also scales to social groups, including highly eusocial species (nutrient regulation by ants reviewed in Csata & Dussutour, 2019; Dussutour & Simpson, 2009; Helm et al., 2017; Hendriksma et al., 2019). The goal of this study is to determine if leafcutter ants, a social species which practices agriculture, also regulate their relative collection of nutrients in the same manner as non-agricultural ant species using the desert leafcutter ant *Acromyrmex versicolor* as our study system.

Our understanding of the important role which specific nutrients and their relative balance play in foraging decisions has been facilitated by nutritionally discrete models. One of these models is the geometric framework, an approach to understanding nutritional decisions that focuses on relative consumption of nutrients (S. J. Simpson & Raubenheimer, 1995). A key finding of studies utilizing the geometric framework is that many animals defend an "intake target," an optimal relative consumption of the nutrients being studied to maximize some aspect of the animal's fitness, usually lifespan or reproductive output (for an overview see Simpson and Raubenheimer, 2012). This framework has been applied successfully to a diverse number of animal species including

locusts (e.g. Simpson and Raubenheimer, 2001), caterpillars (e.g. Lee et al., 2002), weevils (Wright et al., 2003), fruit flies (e.g. Kwang et al., 2008), spiders (e.g. Jensen et al., 2011), slugs (Jensen et al., 2013), fish (e.g. Ruohonen et al., 2007), mice (Sørensen et al., 2008), domestic dogs (Hewson-Hughes et al., 2013), domestic cats (Hewson-Hughes et al., 2013), wild boar (Senior et al., 2016), moose (Felton et al., 2016), baboons (Johnson et al., 2013), *Mycocepherus* fungus gardening ants (Shik et al., 2016), and leafcutter ants (Crumière et al., 2021, 2022; Shik et al., 2020). It has produced a deeper understanding of the specific relative nutritional needs of many different species, as well as the role which discrete nutrients play in foraging decisions.

Solitarily living animals make food consumption decisions based on their own current nutritional needs. Animals living in social groups that practice food sharing, however, should make foraging decisions that integrate the nutritional needs of group members across life stages, with their distinct and diverse nutritional needs (Lihoreau et al., 2014, 2015; Simpson et al., 2010).

Ants and honey bees forage cooperatively, meaning a subset of the adult workers collect the food that is eaten by the entire adult population as well as the brood (Hölldobler & Willson, 1990). This necessitates distribution of the collected food and that foragers gather information about the nutritional needs of the other members of the colony (Cassill & Tschinkel, 1999; Schmickl & Crailsheim, 2004; Seeley, 1989). Brood and adult ants have distinct nutritional needs, with the brood requiring primarily protein while the adults need mostly carbohydrates (Hölldobler & Willson, 1990; Markin, 1970; Sorensen & Vinson, 1981). Colonies of *Rhytidoponera* sp. (green-headed ants) regulate macronutrient consumption consistently, and the proportion of brood to adult ants drives

relative collection of protein (i.e. with an increased proportion of brood the colony collects a greater relative amount of protein) (Dussutour & Simpson, 2009).

Ant colonies have been established as a model system for studying communal regulation of nutrient collection (reviewed in Csata & Dussutour, 2019), and leafcutter ants offer valuable insights into social regulation of nutrient balance across trophic levels (Crumière et al., 2020, 2021, 2022; Shik et al., 2018). Leafcutter ants live in an obligate mutualism with a fungal symbiont. The ants provide food, primarily in the form of freshly cut leaf fragments, to the fungus, which in turn is consumed by the ants (Bass & Cherrett, 1995; Hölldobler & Wilson, 2010; Quinlan & Cherrett, 1979). This represents a rare scenario in which an animal is deciding which food items to collect not for their own consumption or for sharing with the other members of their social group, but rather to provide to an entirely different life form: their fungal symbiont.

This study system used for this project was the desert leafcutter ant *Acromyrmex versicolor*. This species is found in the Southwestern United States and Northern Mexico, with most colonies living in the Sonoran Desert (Gamboa, 1975).

Relative nutrient content of food items plays an important role in the foraging decisions of fungus gardening ants. Lower attines, ants that farm fungus but in a non-obligate symbiosis, regulate their relative nutrient collection to maximize their fungus gardens' production of edible growth. Field colonies collect food items to provide a protein to carbohydrate ratio that a laboratory experiment indicated supports maximal somatic growth, but suppresses energy invested into non-edible gametic production (Shik et al., 2016).

The relative balance of nutrients also has profound effects on the fitness of leafcutter colonies. Colonies of *A. versicolor* restricted to a diet rich in carbohydrates exhibit indicators of positive fitness effects, such as increased fungal area and increased foraging behavior, while colonies restricted to a diet with an increased relative protein content display indicators of negative fitness effects, including decreased fungal area, decreased foraging behavior, and in some cases colony mortality (Clark 2011). Fungus gardens collected from *Atta colombica* and grown in vitro on gels with varying protein to carbohydrate ratios grew best on nutrient substrates that have a high relative carbohydrate content, and are more sensitive to protein content than carbohydrate content (Crumière et al., 2021; Shik et al., 2020). While this does not indicate whether the ants actively regulate relative collection of nutrients, it does suggest they will respond to relative nutritional content of food items given the profound impacts of protein to carbohydrate ratios on colony health and survival.

To assess the role of relative nutrient content in the foraging decisions of *A. versicolor* we utilized a choice assay. Laboratory colonies were provided with pairs of experimental diets that varied in their relative protein to carbohydrate ratios, and their relative collection of the available diets was tracked across five diet pairings to determine if the ants modulated their collection of the diets to yield the same relative intake of these macronutrients.

Methods

Experimental colonies

The laboratory colonies used in the study were founded with newly mated *A. versicolor* queens collected from the Catalina Foothills in Southern Arizona and were approximately two years old at the start of experiments. Each colony was housed in a plastic nest box containing three 3.5 cm by 8.5 cm petri dishes. Each petri dish was lined with dental plaster that was watered biweekly to provide the humidity necessary for fungal growth. The nest boxes were connected to another plastic container that served as a foraging arena. Five colonies of approximately equal size, between 275 and 400 workers, were used for this experiment. Worker numbers were estimated using the methods described in (Clark & Fewell, 2014).

Experimental diets and protocol

We made our experimental diets for this study by mixing polenta, a coarsely ground cornmeal, with either xylose, a plant-based carbohydrate, or amisoy, a plant-derived protein (based on Clark, 2011). We generated diets with five distinct protein to carbohydrate ratios. These diets were presented to laboratory colonies in the following pairings: 1P:2C (indicating 1 gram of protein for every 2 grams of carbohydrates) with 1P:10C, 1P:4C with 1P:9C, 1P:4C with 1P:10C, 1P:6C with 1P:9C, and 1P:6C with 1P:10C. Each diet was uniquely colored with food dye for easy identification.

To feed colonies, we placed two small trays containing a pre-weighed amount of each diet into the foraging arena. The trays were placed near each other and an equal distance from the tube connected to the nest box, to ensure that foragers had equal access to both diets. If necessary, the diets were replenished by adding a pre-weighed amount of

diet to the same tray during the experimental period to ensure *ab libitum* access to both diets. After 72 hours, we removed the trays from the foraging arena, weighed them, and calculated the amount of each diet that had been collected by the ants. We converted the weights of each diet that had been collected into actual nutrient mass based on the composition of the diets. We then provided fresh trays of the two diets in swapped locations to counteract potential directional foraging biases (Hunt et al., 2014; Vallortigara & Rogers, 2005)

The five colonies were broken into two blocks, one consisting of three colonies and the other composed of the remaining two colonies. The order of the diet pairings was randomly assigned, and each of the blocks received the pairings in a different order. Diet pairings were presented to the colonies for 21 days, which resulted in 7 measurements per colony for each diet pairing.

While colonies were undergoing the experimental protocol, the ants formed mixed piles of the experimental diets inside the nest box. At the end of the three week experimental period for each diet pairing, we collected these piles from the nest box and divided them on the basis of color. We weighed the amount of each diet that had been placed in these piles, and calculated the weight of nutrients contained in the piles. This information was used to calculate the amounts of protein and carbohydrates provided to the fungus gardens during each diet pairing by subtracting the amount of nutrients stored in the piles from the total mass of nutrients collected.

Statistical Analyses

Statistical analyses were conducted using R (R Core Team, 2022). We divided the amount of protein collected by the combined mass of protein and carbohydrates collected to calculate the proportion of protein collected, and used this as the response variable for subsequent analyses. This response variable is not normally distributed (Shapiro-Wilks p-value < 0.05), and is bounded between 0 and 1, so we ran a generalized linear mixed model (GLMM) with a beta distribution using the lme4 package (Bates et al., 2015). Diet pairing, date, amount of food cached, and block were treated as fixed effects, while colony ID was a random effect. We ran models with all possible interaction combinations between variables (as well as models with and without the random effect) and found that the model without any interaction terms and no random effect had the lowest AIC and BIC values. In this model, only the effect of the diet pairing had a significant effect (Type II Wald $\chi 2$ test: $\chi 2$ = 12.655, df = 4, p-value < 0.05 for diet pairing, p-value for all other effects > 0.05). We therefore performed a nonparametric Kruskal-Wallis test between diet pairing and the proportion of protein collected.

We also performed post-hoc one-sample Wilcoxon tests to determine whether colonies were foraging randomly within each diet pair. The estimated median of the test was calculated separately for each diet pair, as the null expectation that the ants were choosing food randomly would have different results for each pair. For instance, in the diet pairing 1P:4C and 1P:9C, we would expect the output protein level of random choice to be the average of the two rails: (1+1)/2:(4+9)/2 = 1P:6.5C. However, for the diet pair 1P:6C and 1P:10C, the average would be 1P:8C. We adjusted p-values with the Holm-Bonferroni method to control for multiple comparisons.

To evaluate the effect of the same factors on the proportion of protein provided to the fungus, we followed the same statistical protocol, except we included a term for the amount of cached nutrients. The amount of cached protein and carbohydrates are highly collinear (VIF > 10), so we combined these two terms to create a total amount of cached nutrients rather than including the terms separately. The response variable is again not normal (Shapiro-Wilks p-value < 0.05), and we tested GLMMs with all possible interaction structures between predictor variables. We also performed a Kruskal-Wallis test and post-hoc one-sample Wilcoxon tests.

Finally, we tested whether the colonies preferentially collected the more carbohydrate rich diet in each pairing using the above protocol. We performed a Kruskal-Wallis test between diet pairing and the proportion of the more carbohydrate rich diet collected as well as a post-hoc one-sample Wilcoxon tests to see if colonies were choosing the more carbohydrate rich diet more or less than 50% of the time. We again adjusted p-values with the Holm-Bonferroni method to control for multiple comparisons.

Results

Diet pair had a significant effect on the proportion of protein collected (Kruskal-Wallis test: $\chi 2 = 24.915$, df = 4, p-value < 0.001). For 3 of the 5 diet pairs, the median proportion of protein collected by the ants were not significantly different from that of random choice (One-sample Wilcoxon rank sum test: Holm-Bonferroni-corrected p-values for 1P:2C and 1P:10C, 1P:4C and 1P:10C, and 1P:6C and 1P:10C > 0.05, p-values for 1P:4C and 1P:10C, 1P:6C and 1P:9C < 0.05) (Figure 1.1).

The most parsimonious GLMM that described the proportion of protein provisioned to the fungus never included the total amount of cached nutrients. Diet pair also had a significant effect on the proportion of protein provisioned to the fungus when we performed a Kruskal-Wallis test ($\chi 2$ = 24.915, df = 4, p-value < 0.001). The proportion of protein provided to the fungus was not significantly different than random for any of the five diet pairings. (one-sample Wilcoxon rank sum test: Holm-Bonferroni-corrected p-values for all diet pairs > 0.05) (Figure 1.2). However, since we only collected these mixed piles once at the end of each diet pairing, insignificance may be due to small sample sizes (n = 5 for each diet pairing). The proportion of protein in the mixed piles was not significantly different from the proportion of protein collected from the foraging arena (Pearson's cor = 0.75, df = 23, p-value < 0.001).

Diet pair had a significant effect on the relative collection of the more carbohydrate rich diet (Kruskal-Wallis test: $\chi 2$ = 14.065, df = 4, p-value < 0.01). The proportion of the more carbohydrate rich diet was significantly different from random in 2 of the 5 diet pairs (On-sample Wilcoxon rank sum test: Holm-Bonferroni-corrected p-values for 1P:2C and 1P:10C, 1P:6C and 1P:9C < 0.05, p-values for 1P:4C and 1P:10C, 1P:4C and 1P:9C, 1P:6C and 1P:10C > 0.05). Of note, this included the diet pairing in which the nutrient content of the two diets was most disparate, and which included the most proteinaceous diet (1P:2C and 1P:10C).

With the exception of the 1P:6C and 1P:9C diet pairing, protein collection varied less than carbohydrate collection (Figure 1.4). The mean relative collections of macronutrients are also loosely clustered together, with overlap in average relative collection of protein and carbohydrates.

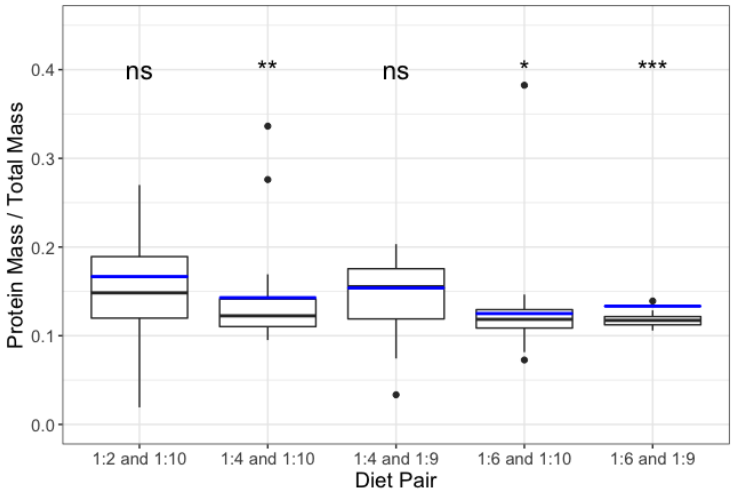

Figure 1.1. Proportion of protein collected by *A. versicolor* across diet pairings. Each blue point line is the proportion of protein that would be collected if the ants were choosing food items randomly. Stars above each boxplot represent significance levels from a one sample Wilcoxon test comparing the experimental results of a diet pairing to the random proportion (ns = not significant, *p<0.05, **p<0.01, ***p<0.001).

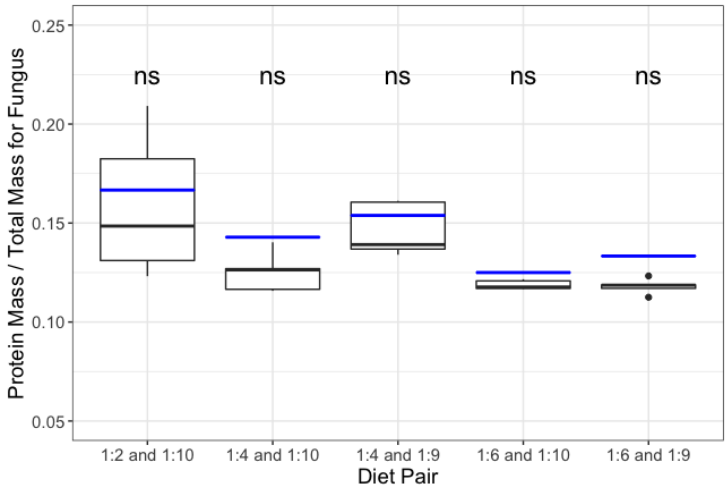

Figure 1.2. The proportion of protein provisioned to the fungus garden for each diet pairing. Each blue point line is the proportion of protein collected if the ants were

choosing food items randomly. Stars above each boxplot represent significance levels from a one sample Wilcoxon test comparing the experimental results of a diet pairing to the random proportion (ns = not significant, *p<0.05, **p<0.01, ***p<0.001). Insignificance may be due to low sample size (n = 5 for all diet pairings)

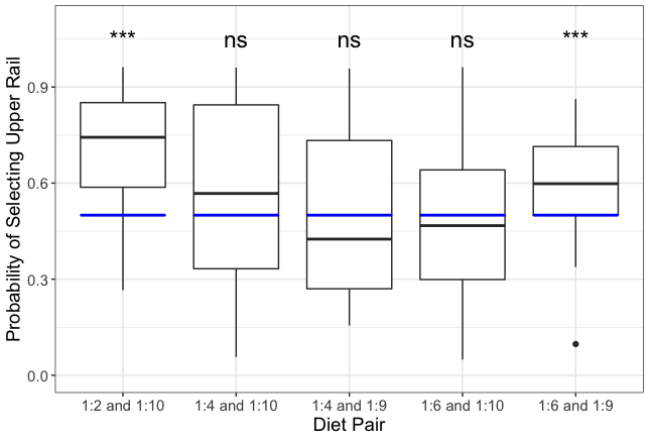

Figure 1.3. Proportion of higher carbohydrate diet collected across diet pairings. Each blue point line shows the probability of choosing each diet randomly. Stars above each boxplot represent significance levels from a one sample Wilcoxon test comparing the experimental results of a diet pairing to random (ns = not significant, *p<0.05, **p<0.01, ***p<0.001).

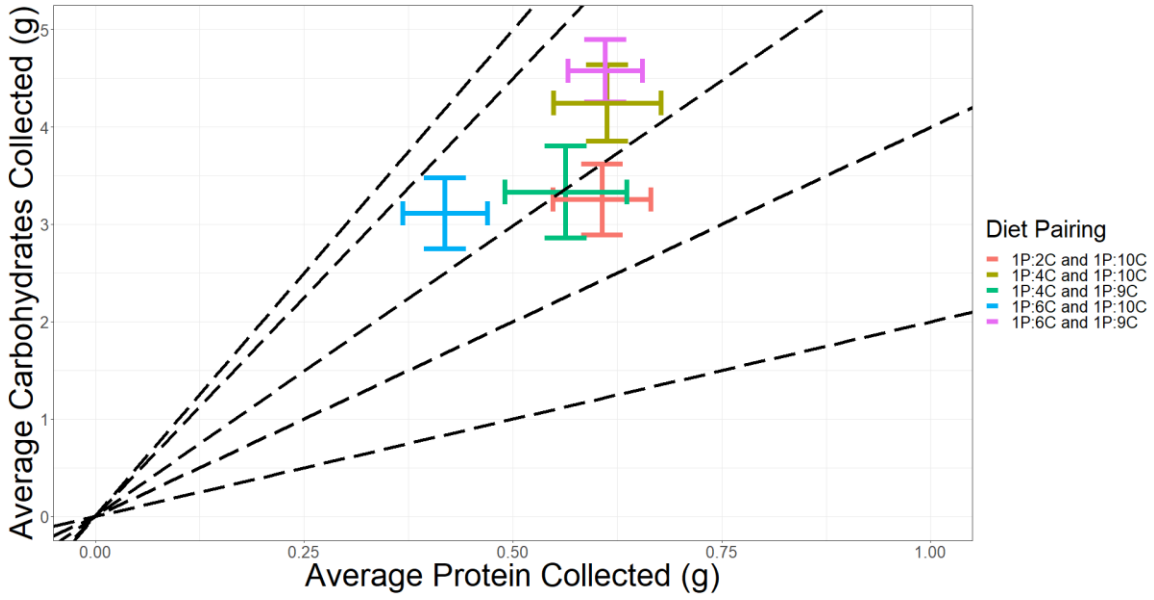

Figure 1.4. Average protein and carbohydrate collection by *A. versicolor* across diet pairings. The dashed lines represent the nutritional content of the experimental diets, and the colored crossbars represent the mean and standard error for the average collection by experimental colonies for each of the 3 day foraging bouts.

Discussion

A. versicolor colonies modulated their foraging behavior in response to the relative nutritional content of the available diets. For two of the diet pairings, most notably the 1P:2C and 1P:10C pairing, in which the most proteinaceous diet was paired with the most carbohydrate-rich diet, the amount of each diet that was collected was significantly different (Figure 1.3). With the exception of the 1P:6C and 1P:9C diet pairing, protein collection varied less than carbohydrate collection, and the average amount of protein collected was lower for the 1P:6C and 1P:9C diet pairing than the others (Figure 1.4).

Combined with other recent evidence from field studies of food items being collected by leafcutter colonies and the growth of leafcutter fungus in vitro on nutrient plates, these results suggest that relative nutrient content of food items plays an important role in the foraging decisions of leafcutter ants (Crumière et al., 2021, 2022), and that the fungus of leafcutter colonies is more sensitive to the protein content of its food than the carbohydrate content (Crumière et al., 2021; Shik et al., 2016).

Protein and carbohydrates were not placed into the mixed piles in the nest box in different relative amounts than they were collected from the foraging arena. Additionally, there was no difference between relative nutrient content of collected food items and relative provisioning to the fungus gardens (Figure 1.2). Field colonies make piles of naturally harvested forage material around the entrance of their colonies, and regularly move food items from these piles into the colony (personal observation). That there is no difference in the relative nutrient content of the piles formed by colonies within the nest box and the relative collection of nutrients from the foraging arena suggests that these piles may not play an active role in nutrient regulation by colonies, but instead serve some other function. However, whether this laboratory finding accurately reflects the role of food piles in the field requires further study.

Colonies did not collect a significantly different amount of the available diets for three of the diet pairings (Figure 1.3). The relative nutrient content of these diets was not especially dissimilar, and this result suggests an experimental design consideration for future choice experiments: the relative nutrient content of diets used in choice assays should be as disparate as possible to allow for more accurate assessment of active nutrient regulation. The original design for this study included providing colonies with diets that

contained protein in higher relative amounts, up to 3P:1C. However, pilot studies revealed that at a relative protein content greater than 1P:2C colonies collected diets in very small amounts that were difficult to accurately measure, or avoided collecting them entirely when other more carbohydrate rich options were available. Therefore, they were not included in this study. The rejection of diets with a greater than 1P:2C relative protein content when more carbohydrate rich options are available is not surprising given that the fungus gardens of fungus farming ants are not tolerant of high levels of protein, and grow optimally on diets high in carbohydrates (Crumière et al., 2021, 2022; Shik et al., 2016).

Nutritionally discrete models have deepened and expanded our understanding of how animals make foraging decisions. The relative nutrient content of food items plays a fundamental role in how many species of solitarily foraging animals decide what to eat (Simpson & Raubenheimer, 2012). This work has been extended to social systems, including highly social species like ants (Csata & Dussutour, 2019). The nutritional content of the substrate on which the fungus garden of a leafcutter ant colony is grown has profound effects on fitness (Clark, 2011, Crumière et al., 2021, 2022; Shik et al., 2016), and the results of this study show that when given food selection choices, relative nutrient content plays an important role in foraging decisions even in a context spanning trophic levels.

CHAPTER 2

NUTRIENT COLLECTION BY THE DESERT LEAFCUTTER ANT ACROMYRMEX VERSICOLOR IS DRIVEN BY FUNGUS GARDEN SIZE

Introduction

An animal's nutrient needs are shaped by a complex and interacting set of factors, including developmental trajectory, age, recent physical activity, and a plethora of other characteristics which can dramatically impact which foods will enhance an organism's evolutionary fitness. The nutrient needs of a social group of animals have an extra layer complexity, as the different members of the group may have diverse nutrient needs based on individual variation in life history traits and physiological status (Lihoreau et al., 2014, 2015; Simpson et al., 2010).

Agricultural species, like humans and leafcutter ants, collect food items to supply the crops they tend, rather than for direct consumption. In these systems, foraging decisions involve another layer of complexity if maximizing nutrient balance for the species being farmed involves different criteria than foraging directly for the individuals within the social group itself. Non-human agricultural species, such as leafcutting ants, provide a unique opportunity to explore how nutrient balance is prioritized in a social and agricultural context (Shik et al., 2016; Shik et al., 2014). They also allow us to tease apart the behavioral and communication strategies underlying these decisions(Crumière et al., 2020, 2021). In this study, I explore the question of how the desert leafcutter ant *Acromyrmex versicolor* makes differential foraging decisions around changes in colony

composition, including the fungus it tends, to better understand the role of nutrient balance in both social and agricultural systems.

Nutritionally discrete models consider the role of distinct nutrients, as opposed to a measure that combines multiple nutrients into a single currency such as calories, in foraging decisions and corresponding impacts on fitness. (Simpson & Raubenheimer, 2012; Sterner & Elser, 2003). They have become valuable tools which have deepened our understanding of nutritional interactions on the individual, group, and ecological levels. Implementing a nutritionally discrete model in thus study allows us to obtain a more nuanced understanding of how colony composition drives nutrient collection of leafcutter ant colonies by analyzing how both relative and absolute collection of discrete nutrients change following changes in colony demographics.

The geometric framework is a nutritionally discrete model that focuses on relative consumption of multiple nutrients, and has been particularly useful in studies focused on the macronutrients protein, carbohydrates, and lipids (Simpson & Raubenheimer, 1995). The application of this framework to a wide array of animal species has furthered our understanding of the relative nutritional needs of these organisms, as well as the underlying criteria that drive the foraging decisions of these species (Simpson & Raubenheimer, 2012). One of the key findings of this approach is that many animals regulate relative collection of nutrients to specific target ratios, and the ability to successfully reach these targets has profound impacts on fitness (Dussutour & Simpson, 2012; Kwang et al., 2008; Simpson & Raubenheimer, 2012).

The nutritional requirements of an ant colony are driven by the relative presence of different life history stages within the colony. Adult ants and immature brood have

distinct nutritional needs, with adults primarily requiring carbohydrates as a fuel source, while eggs and larvae need higher concentrations of protein to grow their developing bodies (Hölldobler & Willson, 1990; Markin, 1970; Sorensen & Vinson, 1981). This is also true for other social insects, including honey bees, in which brood primarily consume protein-rich pollen while adult workers consume carbohydrate-rich nectar (Al-Tikrity et al., 2015; reviewed in Brodschneider & Crailsheim, 2010; Eckert et al., 1994; Pankiw et al., 1998). There is also a direct relationship between the amount of pollen stored in a honeybee colony and brood production, and colonies appear to actively regulate pollen stores around a homeostatic setpoint, responding to reductions in pollen storage by increasing pollen foraging effort (Fewell & Winston, 1992).

When the composition of an ant colony is experimentally manipulated, there are corresponding shifts in relative nutrient collection. When brood are removed from a colony, fewer nutrients overall are collected, and relatively less protein is collected (Dussutour & Simpson, 2009). The presence of brood also drives pollen collection in honeybees, with an increase in the number of larvae resulting in an increase in pollen foraging (Al-Tikrity et al., 2015; Barker, 2015; Free, 1967; Jaycox, 1970; Todd & Reed, 1970).

In addition to brood and adult workers, leafcutter ant colonies have a third major colony component: they live in an obligate mutualism with a fungus. The ants farm this fungal symbiont by providing it with food, primarily in the form of freshly cut leaf fragments, and the fungus in turn produces nutritionally-rich hyphal swellings which are the primary food source of the ants (Bass & Cherrett, 1995; Hölldobler & Wilson, 2010; Quinlan & Cherrett, 1979). While most animals collect food to consume themselves, or

for the other members of their group to ingest in the case of social organisms, leafcutter ants are instead collecting food to provide to an entirely different lifeform. This raises the question of whether leafcutter ants alter collection of nutrients with changes in colony demography in the same manner as non-farming ants, or if there are unique drivers of nutrient collection as a result of fungus farming in this agricultural social system

There are some indications that fungus gardens play a key role in determining relative nutrient needs of leafcutter ant colonies. Lower attines, ants that farm fungus but in a non-obligate symbiosis, regulate their relative nutrient collection to provide a protein to carbohydrate ratio which supports maximal somatic growth of their fungal symbiont, but suppresses energy invested into non-edible gametic production (Shik et al., 2016). Restricting *A. versicolor* colonies to an array of diets with distinct protein to carbohydrate ratios revealed that consuming diets high in protein decreased fungus mass and in some cases colony mortality (Clark, 2011). The fungal symbiont of *Atta colombica* also displays differing growth rates based on the relative nutritional content of the substrate on which it is grown (Crumière et al., 2021).

Despite these insights, the role of the relative abundance of the components of a leafcutter colony in shaping colony nutritional needs and collection is not yet understood, and this study represents the first time the experimental approach of manipulating colony demography and measuring resulting changes in nutrient collection has been reported.

In this study we measured collection of diets with different relative nutrient compositions before and after levels of brood and fungus garden were manipulated in lab-reared colonies of *Acromyrmex versicolor*. We focused on the relative collection of protein and carbohydrates, as the effect of manipulating colony composition on these

macronutrients has already been studied in other social insect systems. Our study shows that colony composition does not play the same role in shaping nutrient collection by *A. versicolor* colonies that it does in non-agricultural ant speices, and highlights the central role that the fungus garden plays in driving the foraging behavior of leafcutting ants.

Methods

General approach

To assess how colony demography shapes nutrient collection by *A. versicolor,* we measured collection of two experimental diets that contained protein and carbohydrates in different relative amounts before and after experimentally manipulating the amount of brood and fungus in laboratory colonies.

Experimental colonies, diets, and foraging protocol

The laboratory colonies used in the study were founded with newly mated *A. versicolor* queens collected from the Catalina Foothills in Southern Arizona, and were approximately two years old at the start of the experiments. Each colony was housed in a plastic nest box containing three 3.5 cm by 8.5 cm petri dishes lined with dental plaster. These were watered biweekly to provide the humidity required for fungal growth. Each nest box was connected by a tube to another plastic box that served as a foraging arena.

To create experimental diets with distinct protein to carbohydrate ratios for this study we blended ground cornmeal with either xylose, a plant-based carbohydrate, or

amisoy, a plant-derived protein (based on Clark 2011). The two diets contained either a 1:9 ratio of protein to carbohydrates (1P:9C) or a 1:4 ratio (1P:4C). The two diets were uniquely colored for easy visual identification. To feed colonies, we placed two small trays containing a pre-weighed amount of each diet into the foraging arena. The trays were placed near each other and an equal distance from the tube connected to the nest box, to ensure that foragers had equal access to both diets. After 24 hours the trays were removed from the arena and weighed to determine how much of each diet had been collected by the ants. We then provided fresh trays of the two diets in swapped locations to counteract potential directional foraging biases (Hunt et al., 2014; Vallortigara & Rogers, 2005). This protocol was used for seven days prior to manipulating either brood number or fungus volume. The same foraging protocol was then used to monitor foraging behavior after each manipulation.

Colony demography measurements

To measure fungus garden size and mass, we took an overhead photograph of each nest box containing the three fungus chambers using a camera placed at a set height of 20 inches above the box. The fungus surface area on the lid of the chamber was manually traced using Fiji (Schindelin et al., 2012). We used the surface area of the fungus garden to calculate fungus mass using the equation $mass_{fungus} = 0.0997 \times area_{fungus}$, which was highly correlated to actual fungus mass in colonies that were measured using these estimates and then sacrificed to compare real values with the estimates ($r^2 = 0.9765$) (Clark & Fewell, 2014).

We estimated worker number by visually scanning the nest box and foraging arena and counting all visible ants with the assistance of a hand counter. These measurements were performed twice per colony, and the average worker number calculated. Many of the workers in a leafcutter colony are not visible, because they are visually obscured by the solid mass of the fungus gardens. Count numbers thus need to be converted to actual worker numbers and the corresponding worker mass using the equations $number_{workers} = 1.483 \times estimate_{workers}$ and $mass_{workers} = 0.00375 \times estimate_{workers}$, which were highly correlated with actual worker number and actual worker mass ($r^2 = 0.983$ and 0.9728, respectively) in colonies that were measured using these estimates and then sacrificed to compare real values with the estimates (Clark & Fewell, 2014).

To estimate brood number, we carefully opened each of the fungus chambers and counted all visible larvae and pupae using a hand counter. Fungus chambers were opened in a large plastic container that was lined with pre-moistened plaster to provide humidity and thus prevent desiccation of the brood or fungus. Egg number was not counted and included in brood number counts, because eggs are small and hard to reliably count. As conversion equations for brood number and mass were not generated in (Clark & Fewell, 2014), brood number could not be converted to absolute brood number or brood mass. However, as the same methodology was used to count brood number before and after the experimental manipulations, the resulting counts should reflect relative changes in brood number.

Experiment 1: Brood addition and reduction protocol

To assess the role of brood in driving the nutrient collection of leafcutter colonies, we measured collection of the experimental diets before and after we increased or decreased the level of brood in lab-reared colonies of *A. versicolor*. Six colonies of relatively equal size were used for this experiment, and they were randomly assigned into three pairs. We performed worker and brood counts on Days 1, 8, and 15 of the experiment. Brood levels were manipulated on Day 8 after brood counts were made.

To manipulate brood levels, we removed a number of larvae equal to 50% of the total brood counted (larvae + pupae) from one colony in a pair and placed them on the floor of one of the fungus chambers of the other colony (see Table 2.1 for numbers of larvae moved). In each case, the brood were moved from the colony with higher brood counts. Workers were observed picking the brood off the nest chamber floor and moving them into the fungus garden within half an hour of the brood addition.

Experiment 2: Fungus garden reduction protocol

To assess the role of the fungus gardens in driving the nutrient collection of leafcutter colonies, we measured collection of nutrients before and after we experimentally decreased the size of the fungus garden of lab-reared colonies of *A. versicolor*. Leafcutter colonies will only grow one strain of fungus in a colony at a time, so fungus addition manipulations could not be performed (Mueller et al., 2010). Four colonies of approximately equal size were used for the fungus reduction experiment. We performed

worker and brood counts on Days 1, 8, and 15 of the experiment. Photographs of the fungus chambers were taken on the same days. We took two photographs of the fungus gardens on Day 8, one before the fungus reduction and one after.

After the pre-treatment photograph of the fungus gardens had been taken on Day 8, one of the three fungus chambers was removed from the nest box of each colony and opened in a large plaster-lined container. The plaster had been pre-moistened to provide humidity to prevent desiccation of the fungus and brood while they were being separated. We relocated all of the eggs, larvae, and pupae from the fungus chamber that was having its fungus garden removed into the fungus chambers that remained intact in the colony. We placed an empty plaster-lined petri dish into the nest box to replace the fungus-containing chamber that had been removed.

Fungus surface area measurements from the photographs taken on Day 1 and Day 8 before fungus was removed were pooled as the "before" manipulation and the surface area measurements from photographs taken on Day 8 after fungus reduction and Day 15 were pooled as the "after" manipulation.

Table 2.1. Number of larvae moved in brood manipulation experiment. The number of larvae moved is equal to 50% of the total number of brood observed in the colony with a great brood number on the day that the brood were relocated.

Colony from which larvae were removed	Colony to which larvae were added	Number of larvae moved
NS173	NS171	35
NS1716	NS1740	98
NS1718	NS1714	64

Results

Brood Addition and Reduction

We used linear mixed effects models with colony as a random effect to test how colony demography changed as a result of our manipulations in both the brood manipulation and fungus reduction experiments. In the brood manipulation experiment, AIC and BIC values were lower for a model including an interaction effect of treatment and sequence than a model without an interaction effect. The interaction effect of treatment by sequence had a significant effect on total brood number ($p > 0.05$ for treatment and sequence, $p < 0.005$ for the interaction effect), indicating that the experimental manipulation successfully changed brood amounts (Figure 2.1). For worker number, again AIC and BIC values were lower for a model including an interaction effect than a model without an interaction effect. The linear model revealed that worker number did

not change as a result of brood addition or reduction ($p > 0.05$ for treatment, sequence, and interaction) (Figure 2.2).

We found no effect of manipulating brood amounts on nutrient collection. Neither brood addition nor reduction altered relative collection of nutrients by the colony (Two-way ANOVA, $P < 0.05$) (Figure 2.3). Altering brood number also had no effect on the total mass of macronutrients collected (Two-way ANOVA, $P > 0.05$) (Figure 2.4); colonies did not significantly increase or decrease foraging levels in response to changes in brood level.

Fungus Reduction

Our manipulation of the fungus garden produced a mean 24.3% decrease in total fungal mass (paired t-test, $P < 0.005$) (Figure 2.5). A linear mixed effects model was used to confirm that treatment, sequence, and their interaction did not have a significant effect on brood number ($p > 0.05$ for all three factors) (Figure 2.6) or worker number (Figure 2.7), indicating that our fungus manipulation did not alter the prevalence of either ant component of the colony.

As was observed in the brood manipulation experiments, reducing fungus mass did not affect relative macronutrient content by the colony (paired t-test, $p > 0.05$) (Figure 2.8). However, reducing fungal mass resulted in a 56% increase on average in the total mass of macronutrients collected (paired t-test, $p < 0.05$) (Figure 2.9).

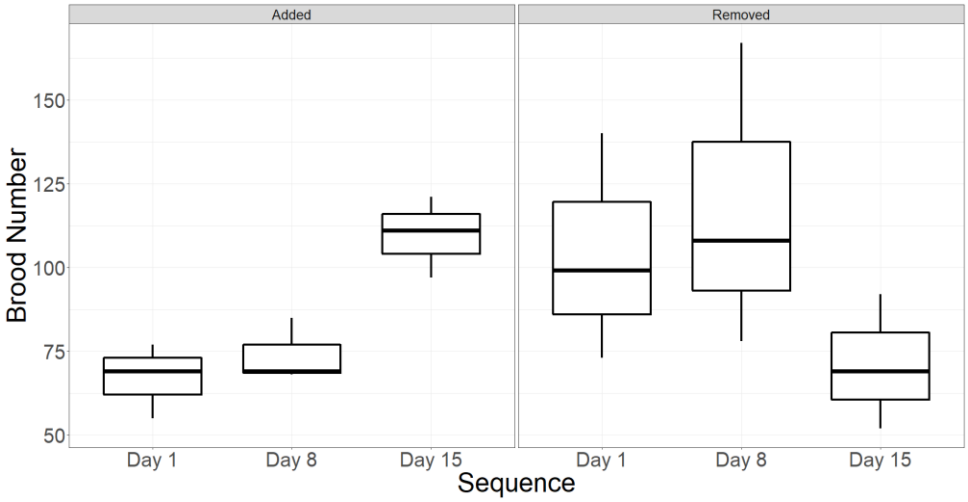

Figure 2.1. Brood number across brood addition and reduction experiment. A linear mixed effects model using colony as a random effect was used to confirm that the interaction effect of treatment by sequence had a significant effect on total brood number ($p < 0.005$). This indicates our manipulations significantly altered brood number.

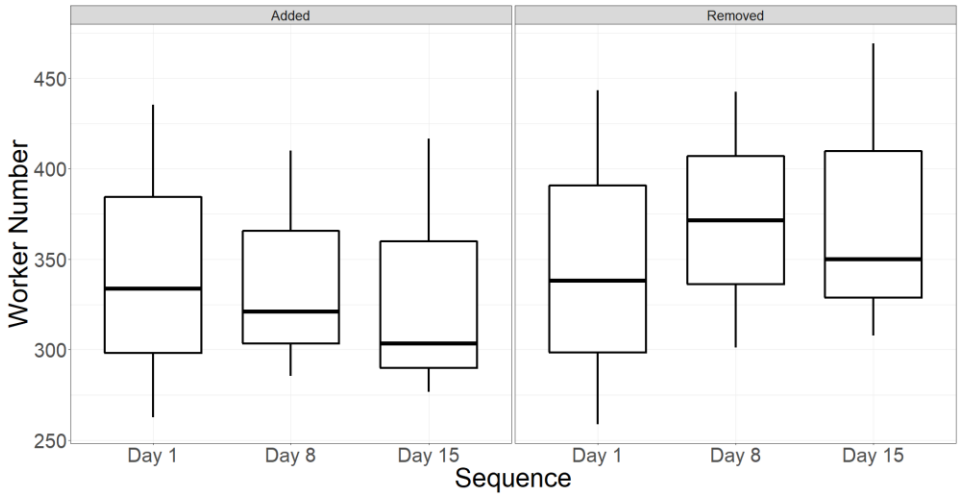

Figure 2.2. Worker Number throughout the sequence of the brood addition and reduction experiment. A mixed effects linear model indicated there was no effect of sequence, treatment, or the interaction of treatment and sequence on worker number ($p > 0.05$ in all cases). This indicates that neither treatment altered colony worker number.

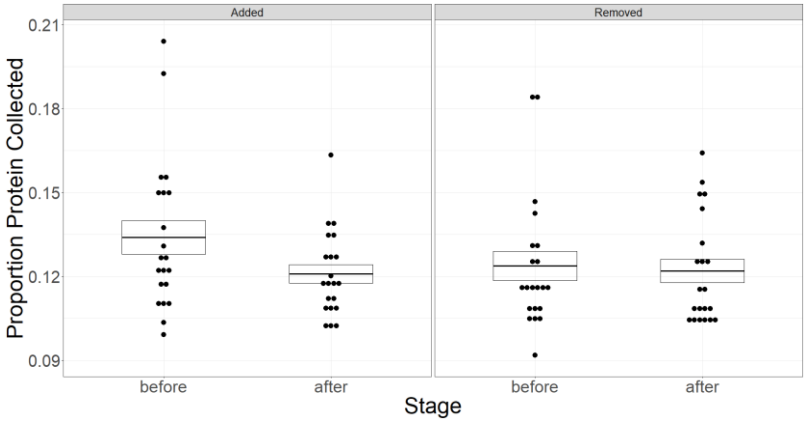

Figure 2.3. Proportion of protein collected before and after brood manipulation. Each dot represents the proportion of protein collected for one of the 24 hour foraging bouts, and the bar and box represent mean and standard error, respectively. The relative amounts of protein and carbohydrates (visualized as proportion of protein) collected by *A. versicolor* colonies did not change as a result of either brood addition or reduction (Two-way ANOVA, for treatment, stage, and stage by treatment interaction $p > 0.05$ in all cases).

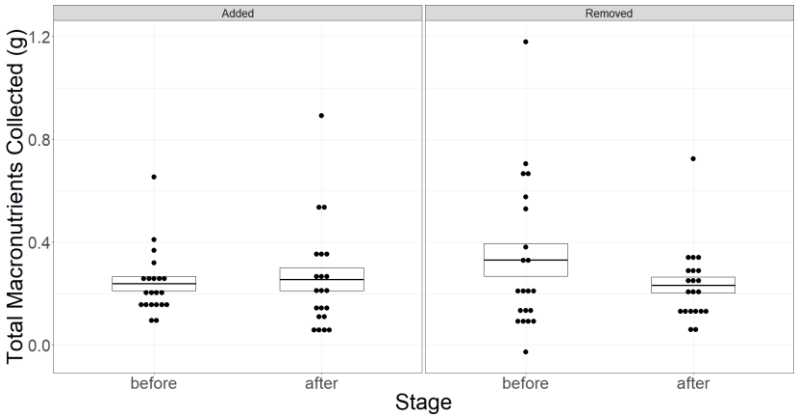

Figure 2.4. Mass of macronutrients collected before and after brood addition and reduction. Each dot represents the mass of macronutrients collected during one of the 24 hour foraging bouts, and the bar and box represent mean and standard error, respectively. The mass of macronutrients collected by *A. versicolor* colonies did not change as a result of either brood manipulation (Two-way ANOVA, for treatment, stage, and stage by treatment interaction $p > 0.05$ in all cases).

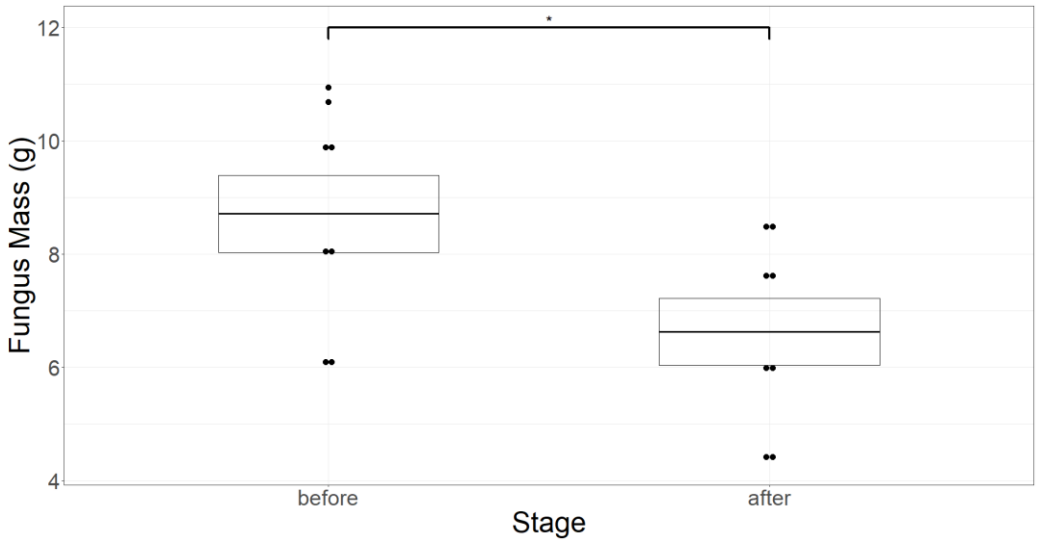

Figure 2.5. Fungus mass before and after fungus reduction. Removal of part of the fungus garden significantly decreased fungus mass (paired t-test, $p < .005$) by an average of 24.3%.

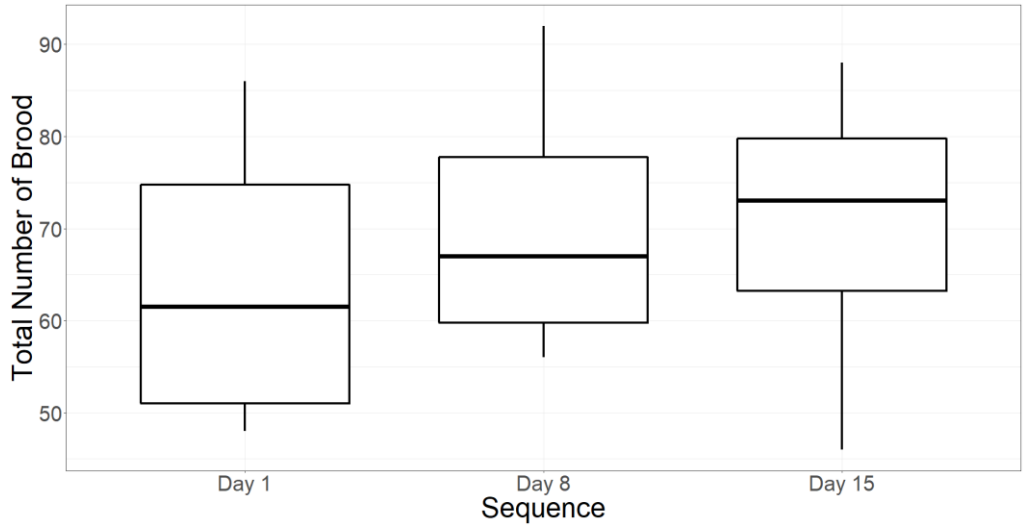

Figure 2.6. Brood number throughout the fungus reduction experiment. A linear mixed effects model indicated there was no effect of sequence on brood number ($p < 0.05$).

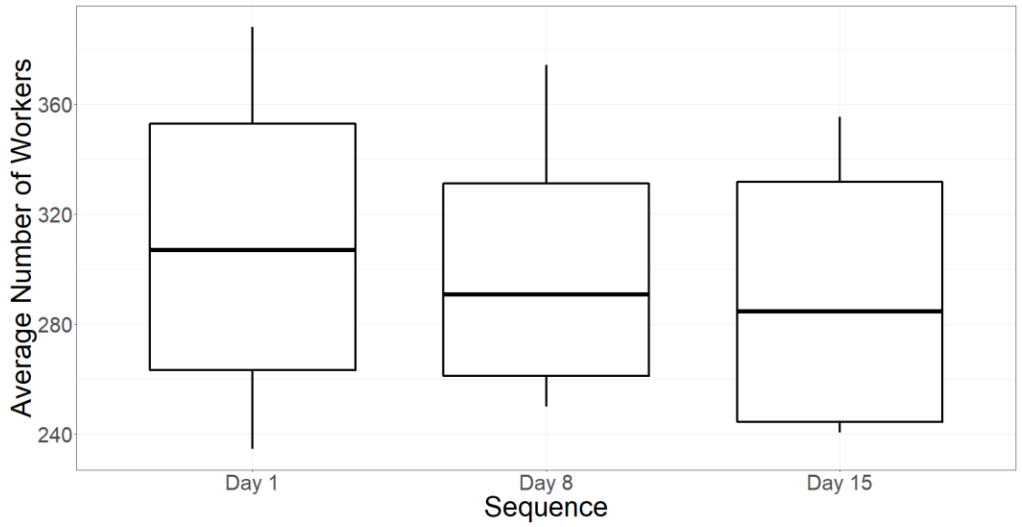

Figure 2.7. Worker number through the sequence of the fungus reduction experiment. A linear mixed effects model indicated there was no effect of sequence on brood number ($p < 0.05$).

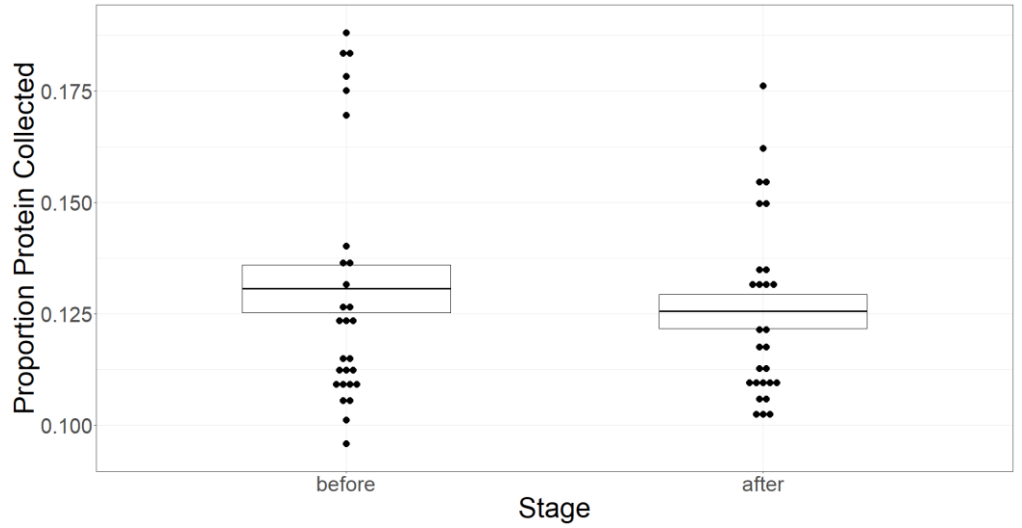

Figure 2.8. Proportion of protein collected before and after fungus reduction. Each dot represents the proportion of protein collected for one of the 24 hour foraging bouts, and the bar and box represent mean and standard error, respectively. There was no significant change in the relative collection of macronutrients (visualized as proportion of protein) collected after the mass of fungus in colonies was reduced (Paired t- test, $p > 0.05$).

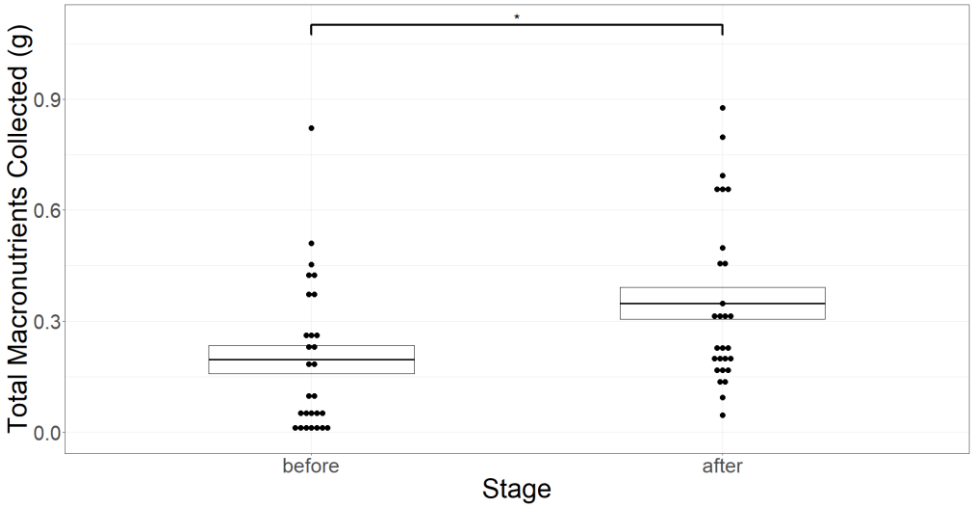

Figure 2.9. Mass of macronutrients collected before and after fungus reduction. Each dot represents the mass of macronutrients collected during one of the 24 hour foraging bouts, and the bar and box represent mean and standard error, respectively. Removal of roughly 24.3% of total fungus volume resulted in a 56% increase on average in overall collection of macronutrients (Paired t-test, $p = 0.03$).

Discussion

Altering the amount of brood in *A. versicolor* colonies did not affect collection of nutrients but decreasing the size of fungus gardens did have a significant effect on foraging behavior (Figures 2.3, 2.4, and 2.9). This result suggests that brood do not play the same central role in driving colony foraging behavior of *A. versicolor* that has been observed in non-agricultural ants. Altering levels of brood in colonies of non-farming ants results in changes of both overall collection of food and relative collection of nutrients (Dussutour & Simpson, 2009). In this study, decreasing fungus garden mass increased overall collection of nutrients, and therefore overall calorie collection, but

protein and carbohydrates were collected in the same relative amounts (Figures 2.8 and 2.9). This suggests that the fungus gardens of leafcutter ants do not replace the role of brood in non-agricultural ant species in shaping colony foraging behavior, but instead that colony composition plays a fundamentally different role in driving nutrient collection in this agricultural system. Altering the amount of fungus relative to the number of ants and brood in the colony did not alter relative collection of nutrients, which suggests that highly carbohydrate biased relative collection of protein and carbohydrates observed in this species (Smith et al in prep) is not a combination of ant and fungus needs, but rather reflects the nutritional needs of the fungus alone.

Decreasing the size of the fungus gardens resulted in an increase in the mass of nutrients collected by foragers (Figure 2.9). This suggests that the ants do not simply modulate the amount of nutrients being collected based on the current mass of the fungus gardens, as in that case the amount of nutrients collected should decrease to match the decreased size of the fungus gardens. Instead, the ants increase overall collection of food, likely to provide the excess calories needed to grow the fungus garden back to its pre-manipulation mass. The growth rates of the fungus garden and the ant population of an *A. versicolor* colony are closely tied, and maintaining a sufficiently large fungus garden to support the ant population has a dramatic effect on the survival of a nascent colony (Clark & Fewell, 2014). However, the underlying dynamics of how relative fungus mass, brood number, and adult worker numbers are monitored and maintained are not currently understood. The results of this study provide evidence that the size of the fungus garden relative to the ant population continues to be an important factor in more mature

leafcutter colonies, as preserving fungus garden size is actively maintained following disturbance.

This study was performed across a relatively short time span to allow for a more detailed understanding of how colonies immediately respond to changes in colony composition. It would be informative to monitor foraging behavior and colony demography over longer periods of time following colony demography manipulations, to determine if colony composition returns to approximately the original state, how long it takes to do so, and how foraging behavior changes over time until relative colony composition and/or the colony's rate of growth return to pre-manipulation values. This would yield a more detailed understanding of how actively the relative composition of the colony is maintained, and how long it takes for this composition to be reestablished following disturbance.

Leafcutter colonies increased their collection of food items following the reduction of their stored food source, the fungus (Figure 2.9). This result is analogous to honeybee colonies increasing foraging effort to return pollen storage to pre-manipulation levels following experimental reduction of stored pollen (Fewell & Winston, 1992). This suggests that leafcutter colonies may actively maintain a homeostatic fungus garden mass based on current colony size, much like honeybees do with stored pollen. However, the food store of a leafcutter colony, the fungus, has a mixed nutritional composition, unlike the single nutrient food stores of pollen (protein) and honey (carbohydrates) found in a honeybee colony. The results of this study indicate that one of the benefits of fungus farming may be that the fungus represents a method of storing food that does not require shifts in relative collection of nutrients following disturbance events. The ants collect

nutrients in the same relative amounts, but increase or decrease overall food collection based on fungus garden mass.

This study focused on the distinct roles of the components of a leafcutter ant colony in driving colony-level protein and carbohydrate collection. We focused on these nutrients as their relationship with colony composition has been studied in other social insect species, allowing for direct comparisons with our results (Al-Tikrity et al., 2015; Cassill & Tschinkel, 1999; Dussutour & Simpson, 2009; Fewell & Winston, 1992). However, it would be interesting to see whether other nutrients follow different patterns of collection following colony composition manipulations. For example, when *A. versicolor* colonies are restricted to diets with the same protein to carbohydrate ratio but varying levels of phosphorous, there is a resulting change in brood number but no effect on the fungus garden (Clark, 2011). Therefore, it would be interesting to study whether manipulating brood levels results in corresponding changes in phosphorous collection.

The results of this study suggest that the relative size of fungus gardens in leafcutter ant colonies drives foraging behavior, and that altering the amount of fungus garden in a colony results in a change of absolute but not relative collection of nutrients. This finding poses interesting new questions about the evolution of nutrient regulation in leafcutter ants and provides new insights about the potential benefits of fungus farming.

CHAPTER 3

THE DESERT LEAFCUTTER ANT ACROMYRMEX VERSICOLOR PREFERS FOODS THAT ARE RICHER IN CARBOHYDRATES THAN ITS NATURAL DIET

Introduction

Many animal species actively regulate their relative collection of specific nutrients, and their ability to meet their relative nutritional needs has significant effects on their evolutionary fitness (Simpson & Raubenheimer, 2012). Most of the studies that have identified the important role that relative nutrient content of food items plays in foraging decisions have been performed in a laboratory setting because of the many advantages of performing this type of experiment in a highly controlled environment. However, laboratory studies may not always be perfect reflections of nutrient collection decisions in the field, where a much wider array of factors including predation, limitations in food options, and seasonal variation in available food items may impact foraging decisions (Cook & Behmer, 2010; Raubenheimer et al., 2018). As a result, pairing laboratory studies with experiments performed in the field provides a deeper and more contextualized understanding of the role which specific nutrients and their relative prevalence in food items plays in the foraging decisions of diverse animal species.

Studies of nutrient regulation performed in the field have revealed that animals may be constrained in their ability to meet their relative nutritional needs because of limitations in the availability of specific nutrients in the local environment. The nitrogen content of plants, which exists primarily in the form of protein, has been connected to

preferential consumption by herbivores and their success for decades (Mattson, 1980). The generally accepted paradigm has been that herbivores are constrained by access to protein, and that increased nitrogen/protein content of plants will result in increased abundance and performance of herbivores (White, 1993).

However, more recent work has challenged this paradigm. Herbivores and omnivores more carefully regulate their consumption of protein than carbohydrates (S. J. Simpson & Raubenheimer, 2005), and they will overconsume carbohydrates in diets with low protein content, but avoid overconsumption of protein (Simpson & Raubenheimer, 2005; Simpson et al., 2003; Simpson et al., 2004). The herbivorous Australian plague locust *Chortoicetes terminifera* can experience inhibited development when feeding on a host plant with an excess ratio of protein to carbohydrates (Clissold et al., 2006), and the Asian locust *Oedaleus asiaticus* preferentially consumes diets with low protein content (Cease et al., 2012). Overconsumption of protein has also been linked with decreased lifespan in solitary living herbivores (Clissold et al., 2006) in the individuals that comprise social groups which are herbivorous (Pirk et al., 2010), and on whole social groups of omnivores (Dussutour & Simpson, 2012). Taken together, these results suggest that while some herbivores and omnivores may be protein limited, others may struggle to consume enough carbohydrates to fulfill their relative nutritional needs, or are calorie limited as they decrease overall food consumption to avoid overconsuming protein.

The goal of this study is to determine if a social species that practices agriculture faces nutrient or calorie limitations in a natural field setting, using the desert leafutter ant *Acromyrmex versicolor* as a study system. Leafcutter ants live in an obligate mutualism with the fungus *Leucoagaricus gongylophorous*. The ants provide the fungus with food,

primarily in the form of leaf fragments and other vegetation, to their fungal symbiont, which the ants in turn consume (Bass & Cherrett, 1995; Hölldobler & Wilson, 2010; Quinlan & Cherrett, 1979). Thus, this species is making food selection decisions about similar food types as an herbivore, but to supply to their farmed crop rather than for their own direct consumption.

There are several reasons to suspect that natural colonies of leafcutter ants may face either carbohydrate or calorie limitations. The fungus of leafcutter ants grows best on substrates that are relatively rich in carbohydrates and growth is suppressed on diets containing over 30% protein (Crumière et al., 2021), and colonies of *A. versicolor* restricted to a diet with a moderate protein content (1P:3C, indicating 1 gram of protein for every 3 grams of carbohydrates) experience reduced fungus growth and in some cases colony mortality (Clark 2011). A choice assay performed in a laboratory setting indicated that *A. versicolor* colonies regulate their relative collection of experimental diets to yield a highly carbohydrate skewed intake of macronutrients (Smith et al in prep). Given that *A. versicolor* prefers a diet that is relatively high in carbohydrates, and the dramatic negative effects this species experiences when forced to overconsume protein, studying whether this species experiences nutrient limitations in the field could provide powerful novel insights about the natural history of *A. versicolor*.

Two experiments were performed to achieve the goal of this study. First, naturally occurring food items that had been harvested by *A. versicolor* foragers was gathered from field colonies, and their relative nutritional content measured. Field colonies were then given access to a pair of experimental diets that varied in their relative protein and carbohydrate content, one with a relatively high carbohydrate content (1P:10C) and one

with a relatively low carbohydrate content (1P:2C), and the number of workers foraging from each diet was measured. This study was performed every other month over the course of a year, to allow for an assessment of whether nutrient limitations and/or recruitment to experimental diets varied seasonally.

Methods

Collection of naturally-harvested forage material

This experiment was performed using three field colonies located in Tonto National Forest near the Phon D Sutton recreation area, which is approximately half an hour outside of the Phoenix metropolitan area. One of the colonies was first located in 2014, so it was at least 4 years old at the time this data was collected, but the relative age of the other colonies was unknown. All three colonies exhibited signs of being relatively mature, with hundreds to thousands of foragers being observed outside of the colony during peak foraging times and alates (reproductive individuals) being observed near the mouths of the colonies in the spring preceding the annual mating flights.

Naturally harvested food material was collected every other month. The time of day at which *A. versicolor* workers perform activities outside of the nest, including foraging, varies throughout the year with changes in temperature (Gamboa, 1976). Each month that forage material was going to be collected, I travelled to the field colonies and assessed relative foraging activity at different times to determine when peak foraging was taking place. *A. versicolor* workers carry food externally in their mandibles, and they

forage both on trunk trails and individually (Gamboa, 1975). To collect forage material, I lay on a mat on the ground perpendicular to the main foraging trail to avoid disturbing the primary foraging activity of the colony, and using tweezers removed the forage material from the mandibles of foragers as they approached the colony entrance. Leafcutter workers have strong mandibles which are sufficiently powerful to hold their body weight, and in some cases workers did not release the forage material and were lifted into the air by hanging onto the food item. If this occurred, a twig was used to apply light pressure to the top of the ants' head, and then the food item was pulled from their mandibles. This procedure never resulted in any indications of injury, and ants resumed normal behavior quickly. This protocol was followed for 90 minutes during the previously assessed time of peak foraging activity for each field colony. The protocol was performed on all three field colonies within the same week, with the forage material of each colony being collected on a different day.

Recruitment to experimental diets

I measured the number of foragers on experimental diets using the same three field colonies from which naturally harvested food was collected. I performed this protocol after the natural forage protocol had been performed on all three colonies during the same months.

I made experimental diets for use in this study by supplementing polenta, a coarsely ground cornmeal, with either amisoy, a plant-derived protein, or xylose, a common plant sugar (based on Clark, 2011). The two diets used in this experiment

contained protein and carbohydrates in the relative ratios of 1P:2C (1 gram of protein for every 2 grams of carbohydrates) and 1P:10C. The diets were uniquely colored for easy identification.

I placed two weigh boats 12 inches to each side of the main foraging trail approximately 5 feet from the colony entrance. These trays were filled with a pre-weighed 10 gram amount of the two experimental diets. After 30 minutes, a length of time previously determined as adequate to allow colonies to find and potentially recruit to the trays of experimental diets, the number of ants on each weigh boat was recorded using a hand counter. I counted the number of workers on each tray a second time 15 minutes later, and then repeated this protocol within 72 hours with the side of the main foraging trail on which the trays were placed relative to the colony entrance switched to account for potential directional foraging biases (Hunt et al., 2014; Vallortigara & Rogers, 2005).

Statistical analyses were performed using R (R Core Team, 2022). A repeated measures two-way ANOVA was used to test whether there was an effect of diet, month, and the interaction of these two factors on the number of foragers observed on the experimental diets. Because analysis indicated that both factors and their interaction had a significant effect on forager number, pairwise t-tests with Bonferroni correction were then used to determine during which months collection of the two diets was significantly different. A one-way ANOVA was used to test the effect of month at each level of diet, followed by pairwise t-tests with Bonferroni correction to determine during which months the number of foragers on each diet was significantly different from forager number observed on the same diet during other months.

Results

Month, diet, and their interaction all had a significant effect on the number of foragers observed on the experimental diets (repeated measures two-way ANOVA, df = 5, 1, 5, respectively, $p < 0.05$ for all three factors). The number of foragers counted on the 1P:10C diet was greater than the number of workers observed on the 1P:2C during all six months in which this protocol was applied (pairwise t-test with Bonferroni correction, df = 5, $p < 0.05$ for all comparisons) (Figure 3.1).

There were four comparisons in which the number of foragers observed on the 1P:10C diet was significantly different across months, and the number of workers counted on the 1P:2C diet was significantly different in two comparisons across months (Table 3.1). However, there were no clear seasonal patterns in these differences, with the possible exception that the number of foragers observed on both diets was significantly higher in June than during the following observation period in August.

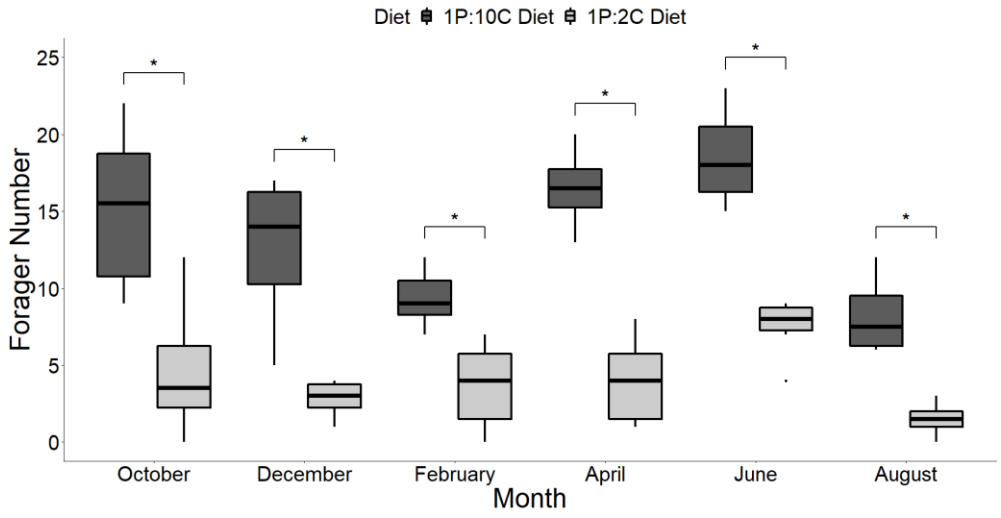

Figure 3.1. Forager number observed on experimental diets from field colonies of *A. versicolor*. A two-way repeated measures ANOVA indicated there was a significant effect of month, diet, and their interaction on forager number (df = 5, 1, 5, respectively, $p < 0.05$ for all three factors). Pairwise t-tests with Bonferroni corrections revealed that forager number was greater on the 1P:10C diet than the 1P:2C diet every month that data was collected (df = 5, $p < 0.05$ for all comparisons). Additional pairwise t-tests with Bonferri correction indicated that there was variation in forager number on each diet separately across months (e.g. collection of 1P:10C was significantly different between June and August), but there was no strong seasonal pattern in these results (Table 3.1).

Table 3.1. Significant differences in forager number between months. P-values were calculated using pairwise t-tests with Bonferroni corrections.

Diet	Month 1	Month 2	Df	Adjusted p -value
1P:10C	April	August	5	0.029
1P:10C	February	April	5	0.048
1P:10C	June	August	5	0.021
1P:10C	February	June	5	<0.005
1P:2C	June	August	5	0.017
1P:2C	December	June	5	0.040

Discussion

The number of foragers observed on the tray containing 1P:10C diet was higher than the number of foragers on the 1P:2C diet across all six months (Figure 3.1). This indicates that colonies have a preference for the more carbohydrate-rich diet, and that this preference is consistent across seasons. The natural forage material collected by leafcutter ants contains protein and carbohydrates in much less carbohydrate biased ratios than those which promote maximal growth of their fungus gardens (Crumière et al., 2021; Shik et al., 2018). Combined with the result of this study that ants prefer a highly carbohydrate biased diet, this strongly suggests that the growth of *A. versicolor* colonies may be constrained by access to carbohydrates. This finding contributes to the growing number of examples in which animals that primarily forage for plant material do not display evidence of protein limitation (e.g. Cease et al., 2012).

This study did not produce robust evidence of strong seasonal patterns of nutrient limitation. There were four comparisons across months in which the number of workers

observed on the 1P:10C diet were significantly different, and two comparisons across months in which the number of workers on the 1P:2C diet were significantly different (Table 3.1). However there was no clear seasonal pattern in these differences, with the possible exception that the number of workers observed on both the 1P:2C and 1P:10C diets in June was significantly higher than the following observation in August. Given that the Sonoran desert typically experiences a drought beginning in April and peaking in June before ending in July with the onset of the summer monsoons (Zolotokrylin et al., 2016), this may indicate that there are limitations in available natural food options during this drought period. A future application of this protocol on a more frequent basis from the period of May through August would allow for an assessment of whether such seasonal patterns of nutrient limitations exist.

CHAPTER 4

INTERINDIVIDUAL VARIATION IN FORAGING BEHAVIOR BY THE DESERT LEAFCUTTER ANT ACROMYRMEX VERSICOLOR

Introduction

There are many factors that can impact what an animal will decide to eat. The prevalence of specific nutrients, as well as their relative balance within food items, plays an important role in the food selection decisions of many species (Simpson & Raubenheimer, 2012). This result scales to social groups, where a group will modulate its relative collection of available food items based on their nutritional content to yield a consistent relative intake of specific nutrients (Dussutour & Simpson, 2009). Agricultural species that live in social groups also regulate their relative collection of nutrients (Smith et al in prep). However, the mechanisms by which individuals foraging as part of a social group coordinate food selection decisions to allow the group as a whole to consistently collect nutrients in the same relative amounts are not yet understood. The goal of this study is to assess how individual patterns of foraging behavior by *Acromyrmex versicolor* workers are coordinated to allow the colony to consistently collect nutrients in the same relative amounts. To achieve this goal, we uniquely marked the majority of foragers in laboratory colonies of *A. versicolor* and tracked individual foraging behavior when colonies were provided with pairings of experimental diets that varied in their relative nutrient content.

There are several aspects of living in a social group which add extra complexity to food selection decisions. Many animals that live in social groups share food items with other members of their group, which means that the animals collecting the food items need to gather information about the nutritional needs of other group members and incorporate them into their foraging decisions (Lihoreau et al., 2014, 2015; Simpson et al., 2010). Additionally, the members of a social group may have distinct nutritional needs based on differences in life history traits (Simpson & Raubenheimer, 2012). This means that the subset of individuals collecting food items for the social group need to make foraging decisions that will meet the unique and distinct nutritional needs of all group members.

Eusocial insects practice food sharing and forage cooperatively, meaning a subset of individuals perform foraging tasks for the entire group (Cassill & Tschinkel, 1999; Hölldobler & Willson, 1990; Seeley, 1989). The brood and adult ants also have different nutrient needs, with the brood requiring primarily protein while the adults need mostly carbohydrates (Hölldobler & Willson, 1990; Markin, 1970; Sorensen & Vinson, 1981). Despite the extra complexity this adds to the foraging decisions of ants, ant colonies still actively and accurately regulate their relative collection of macronutrients (Bazazi et al., 2016; Cook & Behmer, 2010; Dussutour & Simpson, 2008; Dussutour & Simpson, 2009) and their ability to do so can have profound effects on colony-level fitness (Dussutour & Simpson, 2012; Clark 2011).

The ability of animals that live in social groups to accurately regulate nutrient collection requires them to make foraging choices at both the individual and collective level. Historically researchers have tended to assume that individuals within a social

group all followed the same behavioral rules (e.g. Bonabeau et al., 1997), which reduces the need for coordination at the group level. However, work in recent years has indicated that individuals within a group may vary in their propensity to perform certain behaviors, and that even slight variation in individual behavioral phenotypes can play a core role in group-level decision making and behavior (Beshers & Fewell, 2003; Dussutour et al., 2005; Modlmeier et al., 2013; Pruitt & Pinter-Wollman, 2015)

The foraging decisions of leafcutter ants have a unique layer of complexity: foragers are not collecting food items to consume themselves or share with group members, but instead are gathering food to provision to their farmed fungus. Leafcutter ants live in an obligate mutualism with a fungal symbiont. The ants provide the fungus with food, primarily in the form of freshly-cut leaf fragments, and the fungus in turn produces nutritionally-dense hyphal swellings which are the primary food source of the ants (Bass & Cherrett, 1995; Hölldobler & Wilson, 2010; Quinlan & Cherrett, 1979). Relative nutrient collection has fitness effects on leafcutter ant colonies (Clark 2011), and fungus-farming ants actively regulate their nutrient collection (Shik, Santos, et al., 2014) The focal species of this study, the desert leafcutter ant *Acromyrmex versicolor*, also regulates nutrient collection at the colony-level (Smith et al in prep), but the mechanism by which colonies behaviorally regulate nutrient collection is not yet understood.

Although nutrient regulation by leafcutter ants is beginning to be understood on the colony level, the processes and criteria underlying individual foraging decisions, and how individual foraging decisions are coordinated at the colony level, remains poorly understood. There is some evidence that variation in foraging choices among workers of the same colony may play a role in colony-level nutrient collection of leafcutter ants.

Individual foragers from the same colony of *Acromyrmex octospinosus* consistently avoid collecting leaves containing defense compounds that harm their fungal symbiont, but vary in their collection of palatable leaves (Therrien, 1988). This suggests that separate decision-making processes may shape leaf selection: the presence or absence of plant secondary defense chemicals determines whether a leaf is collected, and how much of the leaf is harvested is based on other criteria, potentially nutritional content. That workers varied in their collection of palatable leaves suggests that individuals within a leafcutter colony do not follow the same decision-making rules when foraging, and that variation among foragers in food selection based on relative nutrient content may contribute to colony-level nutrient regulation.

The goal of this study was to analyze how patterns of individual foraging behavior change when the relative nutrient content of available food sources was shifted, and how this might allow for consistent relative nutrient collection at the group level. We observed how foraging frequency, the relative collection of available food sources, and interactions of these two factors changed as the nutrient content of the available food sources changed.

Methods

General methods

To study individual patterns of foraging behavior by *A. versicolor* workers, we uniquely marked the majority of foragers in three laboratory colonies and tracked their foraging

behavior as they were presented with three pairings of experimental diets which contained different relative amounts of protein and carbohydrates.

Colonies

Three colonies of *A. versicolor* were used in this study. These colonies were started with queens collected in the field during the summer mating flights and reared in the lab. The colonies were founded during the summer of 2016 and allowed to grow under stable laboratory conditions for 13 months before the start of this experiment. The colonies had populations of an estimated 288 individuals, 294 individuals, and 243 individuals at the beginning of the experiment, with worker estimates being made following the protocol outlined in (Clark & Fewell, 2014) (Table 4.1). Each colony was housed in a nest box that was connected to a separate foraging arena by a single centrally located tube. The nest box contained 3 petri dishes lined with dental plaster that was moistened with deionized water every 3 days to provide the humidity required by the fungus gardens. Colonies were kept in a room that was maintained at 30° C throughout the course of the experiment.

Paint Marking

We collected workers for paint marking from the foraging arena of each colony. Individuals actively carrying or manipulating food items were preferentially collected for marking. If no ants were actively foraging, we collected workers from the foraging arena

at random. We marked at least 63% of all workers in the experimental colonies (Table 24.1), which likely represents a larger proportion of the forager population as only a subset of workers are foragers and ants were only selected for marking from the foraging arena, avoiding the marking of workers that primarily perform behaviors within the nest box. Workers were secured to a piece of foam with two sewing pins that were placed parallel to the surface of the foam and which held their legs and antennae firmly in place. We used an oil paint pen to place a colored dot on the head, abdomen, and thorax of each ant. The color combination placed on each ant was a unique sequence, to allow for identification of individual workers. Forager mortality was higher than anticipated, so additional workers were marked at the halfway point of the experiment (Table 4.2)

Experimental Diets and Foraging Protocol

The diets used for this experiment were modified from Clark (2011). They consisted of coarsely ground cornmeal, and their relative macronutrient content was modified by adding either xylose, a common plant sugar, or amisoy, a plant-based protein source. Experimental diets were presented to colonies in three pairings: 1P:6C (indicating 1 gram of protein for every 6 grams of carbohydrates) and 1P:9C, 1P:4C and 1P:9C, and 1P:6C and 1P:10C (Table 4.2). Each diet was uniquely colored with food coloring to allow for identification during video analysis.

We placed a pre-weighed amounts of each diet in the foraging arena on pre-weighed weigh boats an equal distance from the tube leading to the nestbox. Diets were presented to colonies in the foraging arena for 1 hour and 15 minutes, and then removed.

The colony was then starved for 48 hours before the protocol was repeated. Each diet pairing was presented to each colony a total of four times (Table 4.2).

Video Recording and Analysis

A video camera was placed above the foraging arena at the location of the tube leading to the nest box. Recording was not started until 15 minutes after the experimental diets were placed into the foraging arena, to provide time for the colony to discover the experimental diets and to allow for the colony to recover from the potential disturbance caused by opening the foraging arena. Foraging activity was then recorded for one hour.

Video analysis was performed using Solomon Coder (Peter, 2019). Every time a marked forager carried a piece of experimental diet into the tube leading to the nest box, we recorded the time, sequence of paint marks on the ant, and which diet the ant was carrying (identified by color).

Table 4.1. Estimated colony sizes and number of marked foragers at the beginning and end of the individual variation in foraging behavior experiment

Colony	Beginning Colony Size	Number of Marked Foragers at Beginning of Study	Final Colony Size	Total Number of Foragers Marked by End of Study
NS165	288	184	232	243
AV165	294	220	280	259
NS163	243	171	209	229

Table 4.2. Timeline of diet pairings presented to colonies in individual variation experiment

Date	Diet Pairing
9/12/2017 and 9/15/2017	1P:6C and 1P:9C
9/18/2017 and 9/21/2017	1P:4C and 1P:9C
9/24/2017 and 9/27/2017	1P:6C and 1P:10C
9/30/2017 and 10/3/2017	1P:6C and 1P:9C
10/6/2017 and 10/9/2017	1P:4C and 1P:9C
10/12/2017 and 10/15/2017	1P:6C and 1P:10C

Results

Individuals varied in their foraging frequency, with most marked individuals making less than 5 foraging trips for each diet pairing, but with some individuals foraging up to over 100 times across the four foraging bouts for a single diet pairing. This result was consistent across all three diet pairings (Figure 4.1). Individuals also varied in their relative collection of the available diets (Figure 4.2).

There was no relationship between foraging frequency and the relative collection of the available diets (linear regression, $p > 0.5$, adjusted $R^2 < 0.1$ for all three diet pairings) (Figure 4.3). Because the diets were provided to the colonies in the same order (Table 2.2), we could not rule out a potential effect of previous experience on subsequent food selection decisions. As a result of worker mortality and because many uniquely marked workers only foraged in a limited number of recorded foraging bouts, we did not have a sufficiently large sample size to analyze whether unique individuals were consistent in their foraging frequency or the relative rate at which they collected the available diets.

This study was designed to study foraging behavior at the individual level rather than at the colony level, and as a result did not produce data which allow for robust statistical analysis of colony level responses to changes in the available diets. However, several interesting patterns of colony level foraging behavior were observed. First, when the relative nutritional content of the available diets was closer to one another, the number of foraging trips increased, with the greatest number of foraging trips being observed for the closest diet pairing (1P:6C and 1P:9C) for all three colonies (Figure 4.4). Additionally, the number of uniquely marked individuals observed foraging tended to increase as the relative nutritional content of the diets was closer together, and again the closest diet pairing (1P:6C and 1P:9C) elicited foraging from the largest number of uniquely marked foragers (Figure 4.5). An exact binomial test was performed comparing the relative collection of the available diets by each uniquely marked forager to collection by all individuals, which revealed that the number of individual foragers collecting the available diets in a relative proportion that was significantly different from the colony also tended to increase when the nutritional content of the available diets was closer (Figure 4.6). However, there was no clear trend in the proportion of individuals collecting diets at a rate significantly different than the colony as a whole (Figure 4.7).

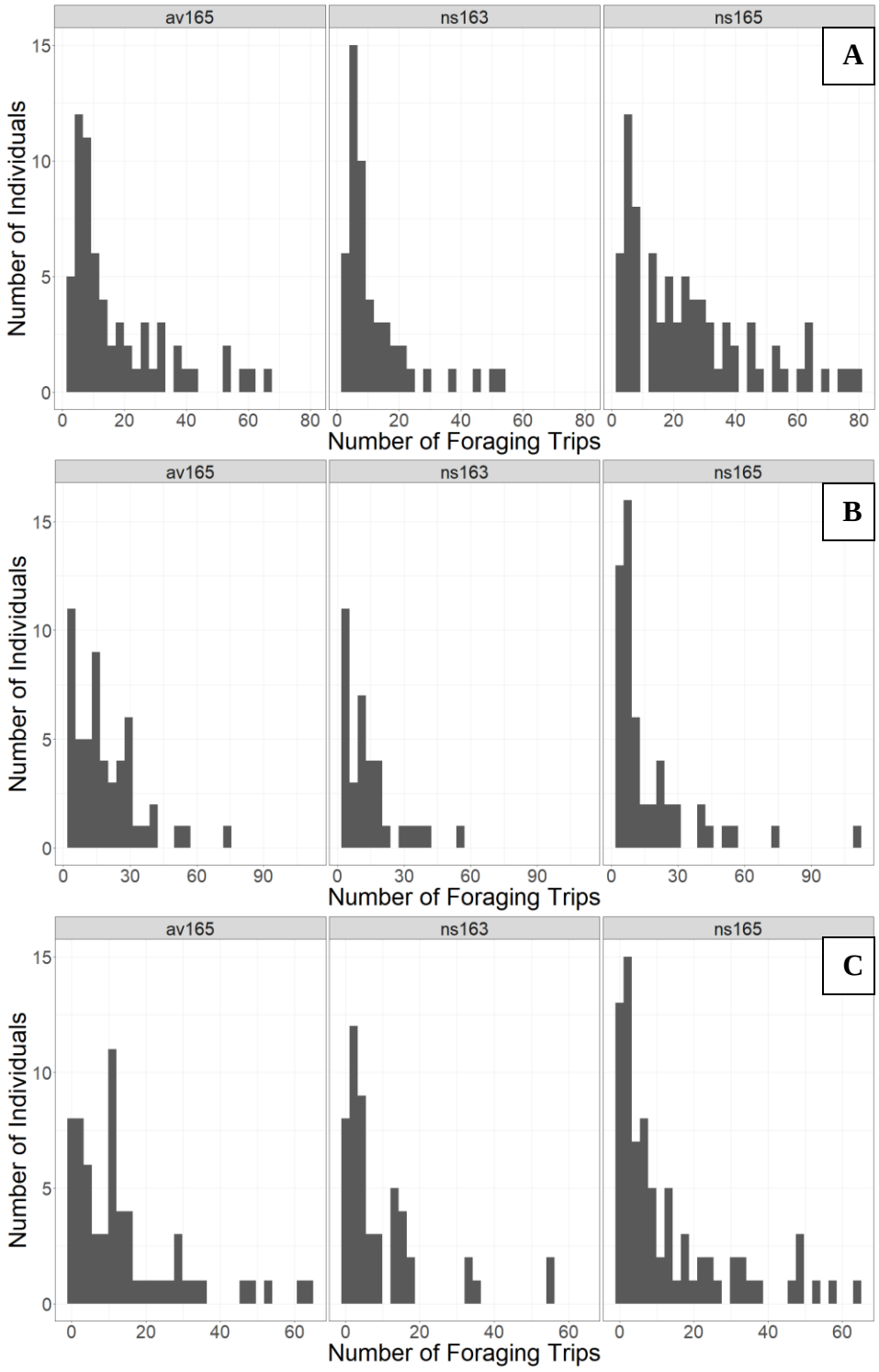

Figure 4.1. Variation in foraging frequency across the three experimental diet pairings. (A) 1P:6C and 1P:9C diet pairing, (B) 1P:4C and 1P:9C diet pairing, (C) 1P:6C and 1P:10C

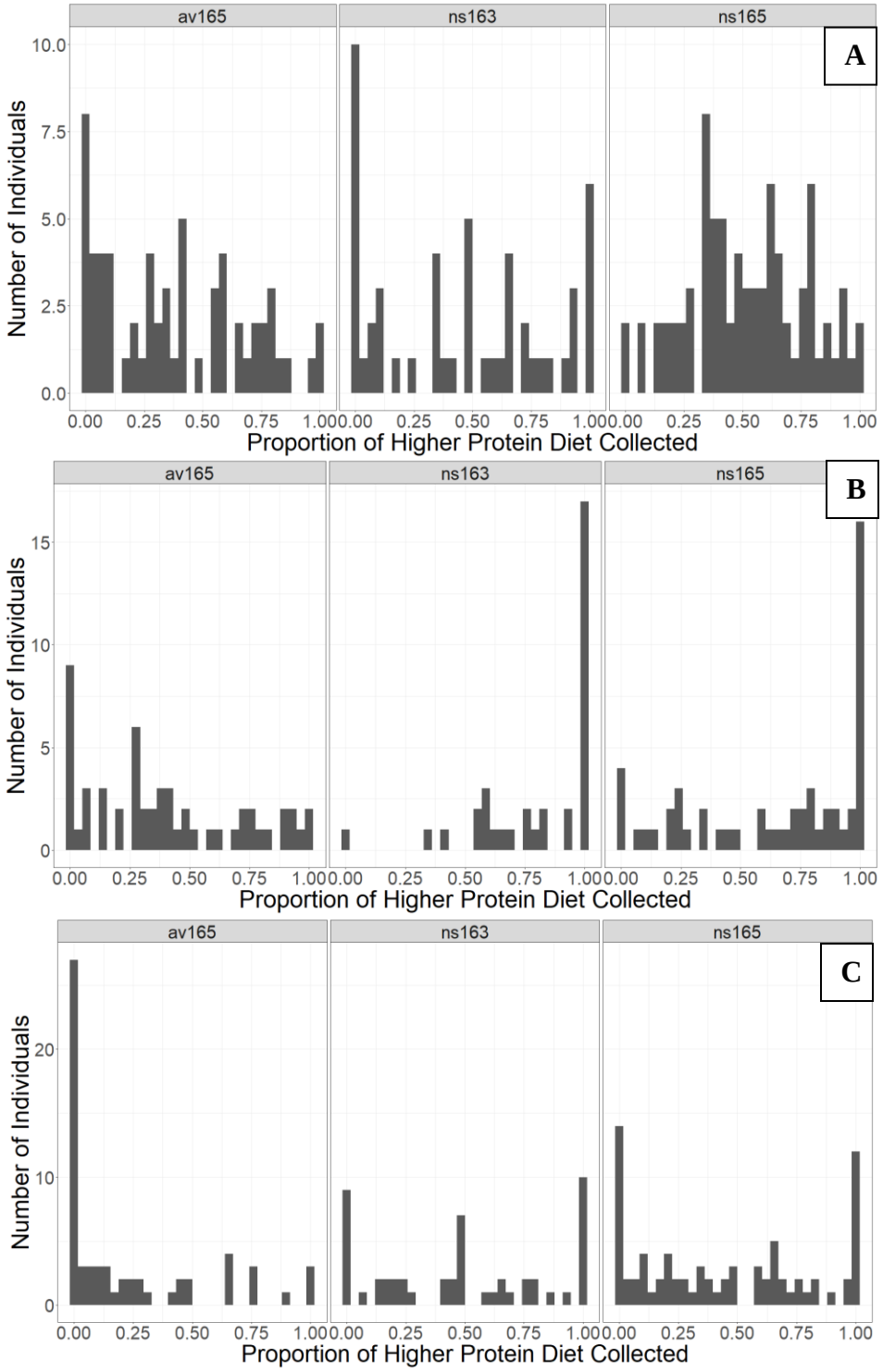

Figure 4.2. Relative collection of the available diets across the three experimental diet pairings. (A) 1P:6C and 1P:9C diet pairing, (B) 1P:4C and 1P:9C diet pairing, (C) 1P:6C and 1P:10C

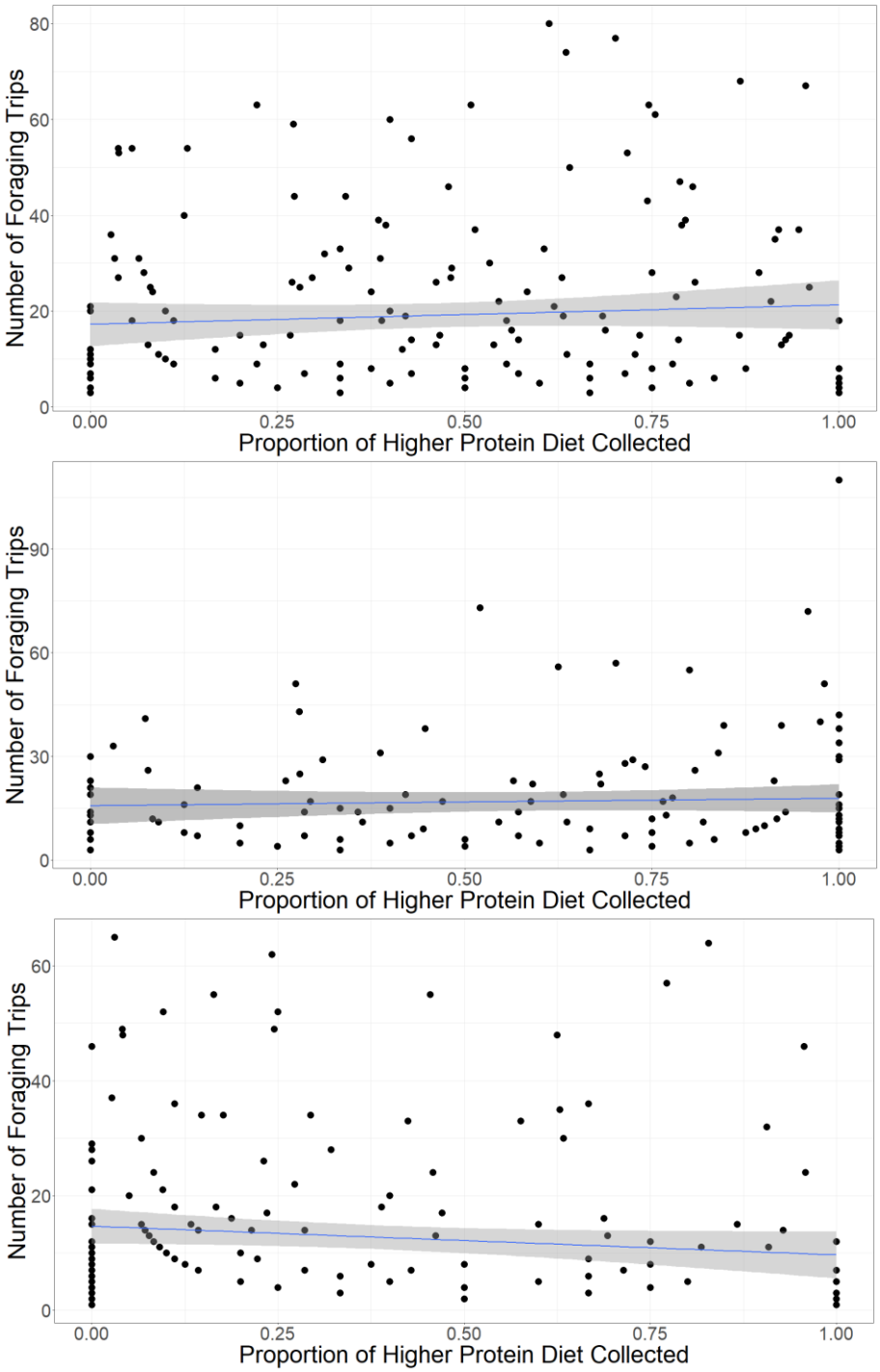

Figure 4.3. There is no correlation between foraging frequency and relative collection of experimental diets for the any of the three diet pairings (linear regression, $p > 0.05$, adjusted $R^2 < 0.1$ in all cases) (A) 1P:6C and 1P:9C diet pairing, (B) 1P:4C and 1P:9C diet pairing, (C) 1P:6C and 1P:10C

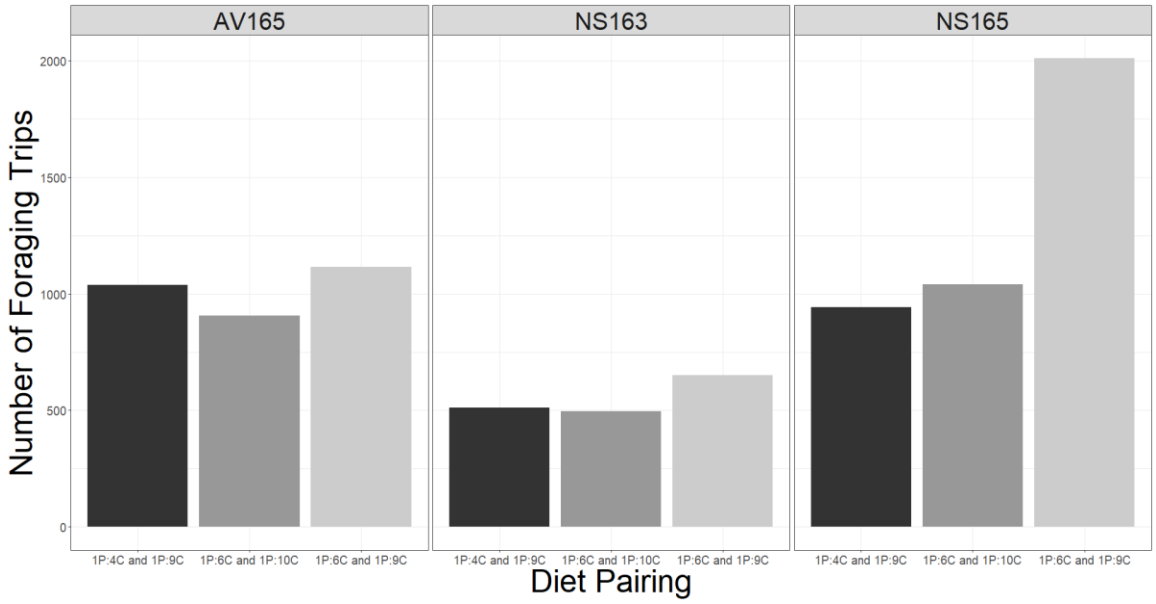

Figure 4.4. Total number of foraging trips taking by each *A. versicolor* colony during each diet pairing

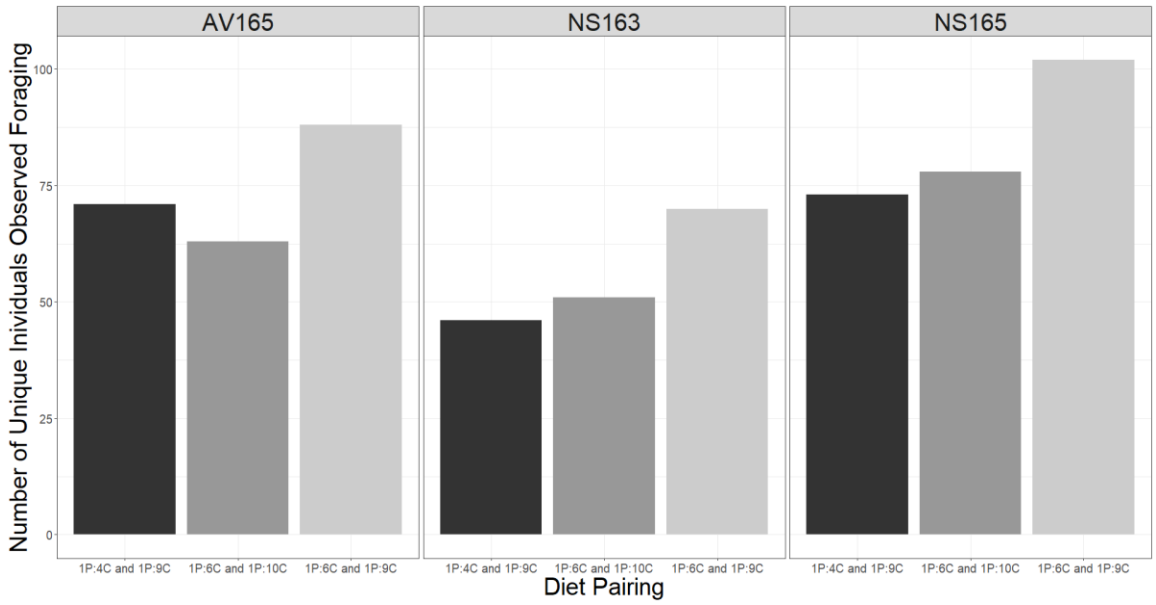

Figure 4.5. Number of unique individuals observed foraging in each *A. versicolor* colony during each diet pairing

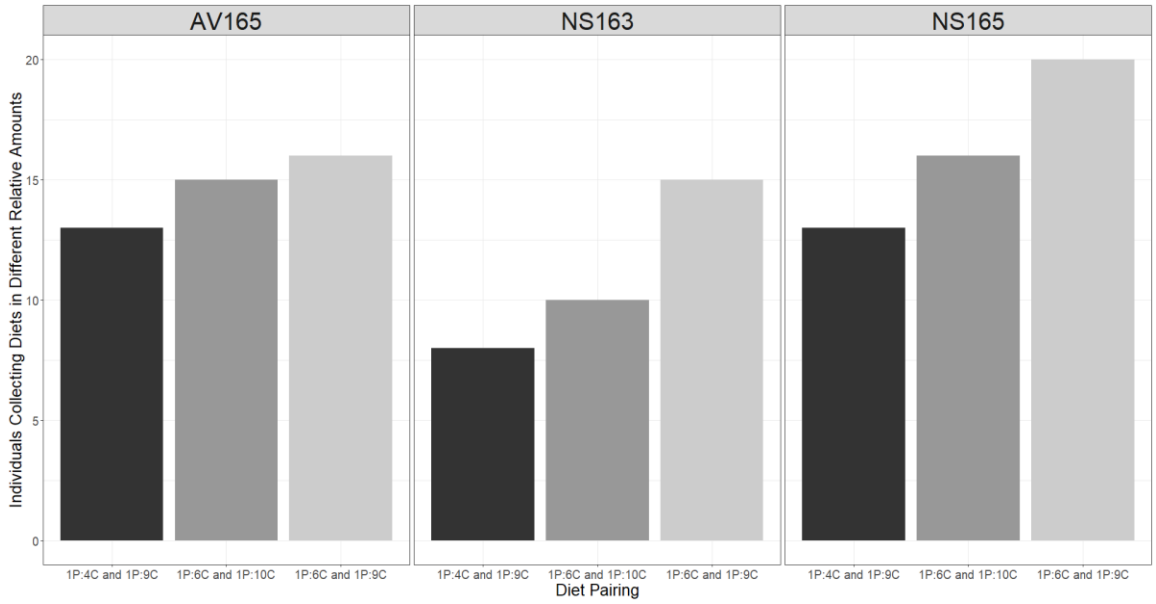

Figure 4.6. Number of individuals collecting diets in a relative amount that was significantly different from the group as a whole (Exact binomial test, $p < 0.05$ for individuals counted as collecting diets at a significantly different rate).

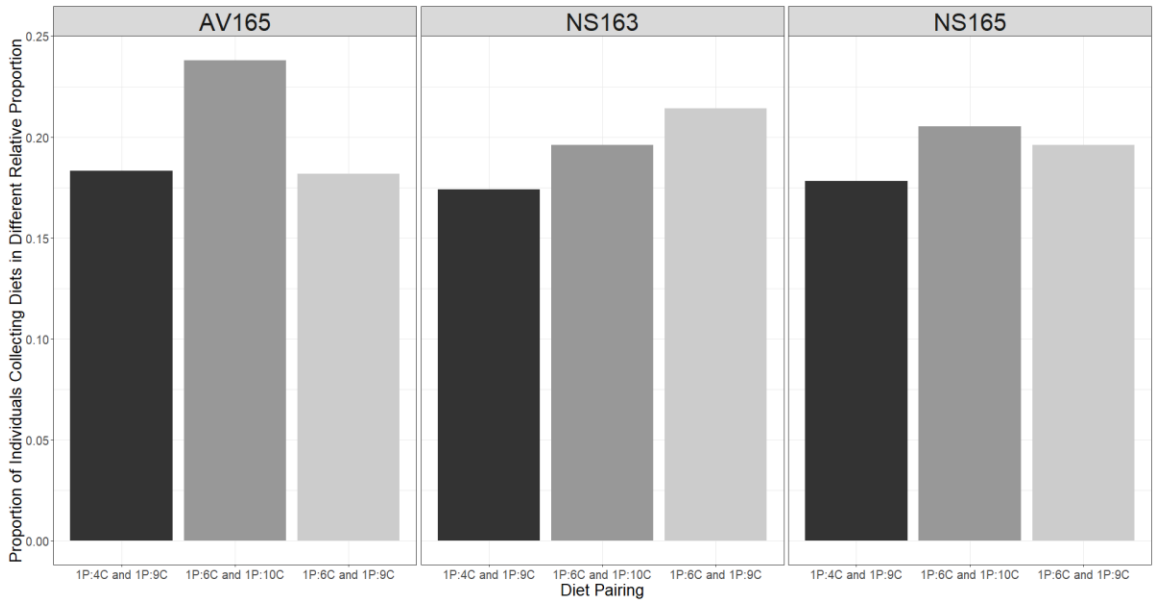

Figure 4.7. Proportion of individuals collecting diets in a relative amount that was significantly different than the group as a whole (Exact binomial test, $p < 0.05$ for individuals counted as collecting diets at a significantly different rate).

Discussion

Individuals varied in their foraging frequency (Figure 4.1) and in their relative collection of the available diets (Figure 4.2) Similar patterns of variation in foraging behavior were observed across all three diet pairings, which suggests that variation in these key characteristics of foraging may play an important role in the colony-level foraging strategy of *A. versicolor*.

Many animal make speed-accuracy trade-offs when foraging (Chittka et al., 2003, 2009; Latty & Beekman, 2011). If *A. versicolor* foragers were making similar trade-offs, we might expect a relationship between foraging frequency and relative collection of available diets, as assessing the available food choices and blending collection of the available diets to meet the colony's optimal needs might take additional time. However, no such relationship existed (Figure 4.3), suggesting that *A. versicolor* workers may not need to choose between foraging accurately or foraging quickly.

Although the trends observed at the colony level could not be statistically verified, they do provide interesting insight that warrants further study (Figures 4.4, 4.5, 4.6, and 4.7). Perhaps colony level changes in the number of individuals foraging and the overall number of foraging trips made by foragers interacts with two types of observed individual variation to allow colonies to flexibly respond to changing nutritional landscapes.

The results of this study led to a collaboration which resulted in a mathematical model based on having separate response thresholds for foraging and for whether an individual will collect a given diet based on relative nutrient content which produces

theoretical results that match the experimental data of this study (Lynch et al in prep, Smith et al in prep). The results of this model provide an additional possible mechanism by which the foraging behavior observed in this study would allow colonies to consistently collect protein and carbohydrates in the consistent relative amounts across different diet pairings.

This study was designed to provide detailed information about individual changes in foraging behavior in response to changes in the nutritional content of available food items. It would also be interesting to use RFID tags, which have been used to successfully track foraging behavior by ants, to study patterns of food collection from single diet pairings over long periods of time (Burchill et al in prep).

The results of this study provide evidence that the nutrient regulation strategy of *A. versicolor* colonies involves foragers that vary in their propensity to collect any given food item in multiple key ways. This result raises a new set of interesting questions revolving around how this variation is coordinated and organized at the colony level.

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69