Behavioral and Nutritional Regulation of Colony Growth in the Desert

Leafcutter Ant *Acromyrmex versicolor*

by

Rebecca Clark

A Dissertation Presented in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

Approved June 2011 by the
Graduate Supervisory Committee:

Jennifer Fewell, Chair
Ulrich Mueller
Juergen Liebig
James Elser
Jon Harrison

ARIZONA STATE UNIVERSITY

August 2011

ABSTRACT

Like individual organisms, complex social groups are able to maintain

predictable trajectories of growth, from initial colony foundation to mature

reproductively capable units. They do so while simultaneously responding

flexibly to variation in nutrient availability and intake. Leafcutter ant colonies

function as tri-trophic systems, in which the ants harvest vegetation to grow a

fungus that, in turn, serves as food for the colony. Fungal growth rates and colony

worker production are interdependent, regulated by nutritional and behavioral

feedbacks. Fungal growth and quality are directly affected by worker foraging

decisions, while worker production is, in turn, dependent on the amount and

condition of the fungus. In this dissertation, I first characterized the growth

relationship between the workers and the fungus of the desert leafcutter ant

Acromyrmex versicolor during early stages of colony development, from colony

foundation by groups of queens through the beginnings of exponential growth. I

found that this relationship undergoes a period of slow growth and instability

when workers first emerge, and then becomes allometrically positive. I then

evaluated how mass and element ratios of resources collected by the ants are

translated into fungus and worker population growth, and refuse, finding that

colony digestive efficiency is comparable to digestive efficiencies of other

herbivorous insects and ruminants. To test how colonies behaviorally respond to

perturbations of the fungus garden, I quantified activity levels and task

performance of workers in colonies with either supplemented or diminished fungus gardens, and found that colonies adjusted activity and task allocation in response to the fungus garden size. Finally, to identify possible forms of nutrient limitation, I measured how colony performance was affected by changes in the relative amounts of carbohydrates, protein, and phosphorus available in the resources used to grow the fungus garden. From this experiment, I concluded that colony growth is primarily carbohydrate-limited.

DEDICATION

I wish to dedicate my dissertation to all of the friends and family members who supported and encouraged me as I worked on it, most especially my parents; my brother and sister; members of the Scrabble Society; members of the Extreme Picknicking Society; Scott Walters; Kathy Moum; and Emma Clark.

ACKNOWLEDGMENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1

TRANSITIONING FROM UNSTABLE TO STABLE GROWTH DYNAMICS

DURING EARLY COLONY ONTOGENY IN THE DESERT LEAFCUTTER

ANT *ACROMYRMEX VERSICOLOR*

Growth is a defining characteristic of all levels of biological organization,

from individual cells through populations, and selective pressures during growth

determine the range of possible phenotypes of organisms. As individual organisms

grow, they respond resiliently to environmental constraints while following

intrinsic growth plans. The interplay between environmental inputs such as

nutrient intake and this internal developmental program collectively determine the

organism's phenotype (Glazier, 2005; Doi et al, 2010). A similar interplay

between environmental influence and intrinsic rate of growth shapes the ontogeny

of insect societies, as they grow from one or a few individuals to a size of

thousands or millions (Oster and Wilson, 1978, Nonacs, 1991, Kay, 2004, Kay et

al, 2006, Dussutour and Simpson, 2009, Cook et al, 2010, Hou et al, 2010).

Despite recognition that development constrains social phenotype

(Fernández-Marín et al, 2004), surprisingly few studies have characterized how

complex social groups navigate developmental challenges as they move from

foundation through maturity. This characterization is critical for understanding

how colonies integrate regulatory mechanisms at different levels of developmental

organization (Yang, 2007). Leafcutter ant colonies provide a unique opportunity to track nutrient balancing with colony growth within an enclosed trophic system because the ants directly regulate the growth of the fungus, the colony's immediate food source. Here, we study the growth dynamics of newly initiated colonies of the desert leafcutter ant *Acromyrmex versicolor* to understand how the fungus garden / worker ant relationship unfolds as colonies develop over the first six months of life.

Models of colony growth are often based on ecological models of population growth that make the general assumption that all individuals within the growing population are equivalent (Oster and Wilson, 1978). While such models may effectively predict growth patterns for larger colony sizes, they are less useful for predicting events in small colonies, where internal organizational and demographic factors may play an important role in determining growth rates (Jeanne, 1999). In smaller colonies, individual worker actions assume greater importance with respect to the overall well-being of the colony (Jeanne and Nordheim, 1996, Jeanne, 1999, Jeanson et al, 2007). Ontogenetic constraints of younger colonies derive from three features: (1) a colony must perform multiple tasks with few workers, (2) the age structure of the colony is restricted to younger workers, and (3) worker size variation is limited, or worker morphologies differ categorically between small and mature colonies. All three of these constraints are present in extreme forms during early growth in leafcutter ant colonies: leafcutter

2

ant workers collectively perform as many as 30 different tasks (Wilson, 1980, Camargo et al, 2007); workers exhibit age-based polyethism, shifting from within-nest tasks to outside tasks across hundred-day lifespans (Camargo et al, 2007); and small leafcutter ant colonies possess restricted worker size distributions relative to larger colonies (Wilson, 1983). Collectively, this suggests that further work is needed to characterize growth dynamics in young leafcutter ant colonies.

Prior studies of fungus-growing ants (tribe Attini) have found that workers in small colonies perform the same task repertoire as large colonies (Wilson, 1983, Augustin and Santos, 2008) but it is not clear how task performance translates into division of labor or efficiency at the colony level, characteristics that can be difficult to measure meaningfully. In the case of leafcutter ant colonies, however, the fungus garden biomass can serve as a direct indicator of the colony's nutritional condition and growth efficiency because it serves as the ants' immediate food source and garden size is directly regulated by the worker population. If increases in division of labor and task subdivision occur as colonies grow, they are expected to lead to increases overall colony efficiency and robustness (Roschard and Roces, 2003), with consequences for the ability to regulate the growth of the fungus garden. Efficiency, for a growing colony, can be operationally measured as the ratio of the units of workers produced relative to the resources consumed by the colony. Robustness is associated with a colony's capability to homeostatically regulate its response to perturbations, and is related

to the degree of variability observed in the relationship between the two growing components of the colony, the fungus and the ants. Within the leafcutter ant system, any increase in efficiency or robustness could translate into a shift in the worker-fungus population relationship as colonies grow, such that larger colonies contain fewer, or less variable, units of fungus per unit of worker. This does assume that the standing fungal crop is proportional to the rate of fungus production and consumption by the ants.

As with many attine ants, queens of *A. versicolor* are semi-claustral (Fernández-Marín et al, 2004); after mating, digging a new nest, and expelling a fungus pellet as a starter culture for the new garden, queens leave the nest occasionally to forage for leaves as substrate for the fungus garden, which in turn provides food for the developing brood (Rissing et al, 1989). As workers emerge, queens gradually reduce their behavioral repertoire and remain inside the nest to lay eggs, while workers assume the tasks of collecting and preparing leaves for the fungus garden, raising brood, tending the fungus garden, and removing waste materials from the nest (Cahan and Julian, 1999). Colonies are relatively long-lived and may grow over the course of several years to a size of around 10,000 workers without reproduction, making it possible to study growth processes without the confounding effects of a colony's switch from growth to reproduction (Julian and Cahan, 1999). Previous work on the colony-founding period in both this species (Cahan and Julian, 1999) and in *Trachymyrmex septentrionalis* (Seal and

Tschinkel, 2007) suggested that the relationship between the worker population and the fungus garden biomass is unstable and/or negatively allometric immediately after the first cohort of workers emerges: colonies with larger initial worker populations have proportionally smaller fungus gardens. Unless fungus gardens in larger colonies can grow at a faster rate, or workers in larger colonies consume less fungus per capita, this negative relationship cannot be sustained over the long term. Therefore, work is needed to clarify if, and when, any kind of homeostatic balance between the ants and fungus is achieved. j

In this study, I used non-invasive methods to correlate the growth trajectories of the worker population and the fungus garden biomass in colonies from foundation through the first six months of development. I show that, in surviving colonies, the worker-fungus relationship undergoes a qualitative shift from negative, variable allometry (a hypometric relationship with less fungus per ant as the number of ants increases) to isometry, where the amount of fungus per ant is constant, regardless of colony size. I identify the critical worker population size threshold above which colonies presumably begin to effectively allocate efforts among tasks, leading to the formation of an isometric relationship between the worker population and fungus population.

Methods

To initiate colonies, I collected newly mated queens of the desert leafcutter ant, *Acromyrmex versicolor*, immediately after mating and just before they began to excavate new nests. Queens were collected on 8 August 2005 from underneath a mating flight along N Swan Road just north of N Plaza del Baron, in Tucson, AZ. This species normally forms pleometrotic foundress groups and mature colonies are thought to remain polygynous (Rissing et al, 1989, Rissing et al, 2000). Queens were housed in pleometrotic groups of four. Nests were comprised of two circular plastic dishes (internal dimensions: 8.4 cm diameter x 3.3 cm high) connected with vinyl tubing (~3 cm long x 1/4-inch inner diameter). The bottom of one chamber was lined with plaster of Paris and was used by colonies to house the fungus; the other chamber served as a foraging arena. Throughout the study, colonies were provided with an *ad libitum* mixture of palo brea (*Cercidium praecox*) leaves, polenta (coarsely ground cornmeal), and oatmeal, and humidity levels were maintained in the fungus chamber by watering the plaster with 2-4 mL of water once a week. The colonies were kept in a room maintained at 30-32°C from August through October, and at 25°C from November through March, with natural lighting from a bank of windows along one wall.

Colony worker population and fungus sizes were estimated once a week for 30 weeks using noninvasive methods, to minimize disturbance to the nests across repeated measurements. I visually estimated worker numbers by scanning

the nest and counting individuals with a hand counter. By week 30, surviving

colonies reached an average size of 182 workers, and population sizes appeared to

be consistently increasing from week to week (on average, 17 new workers were

added per week for the last four weeks).

Fungus area was measured from overhead photographs of the fungus

chamber taken from a set distance to standardize size. Fungus area was calculated

using the software program ImageJ (http://rsbweb.nih.gov/ij/). I validated both

worker and fungus estimation methods by repeating them on a separate set of 21

age-matched nests from which I subsequently separated out the fungus from all

workers, larvae and pupae. I then counted the total number of workers and

weighed the entire worker population (excluding brood) and fungus garden

(including brood). Fungus area was strongly and positively correlated with total

wet mass of the fungus garden (r^2=0.902, n=21 colonies, $mass_{fungus}$ =

$0.113area_{fungus}$-0.630, Figure 1A). Similarly, worker number estimates were

strongly positively correlated with actual worker numbers (r^2=0.897, n=21

colonies, Figure 1B) as well as total worker wet mass (r^2=0.831, n=21,

$mass_{workers}$=3.750$estimate_{workers}$+0.847).

I also assessed colony survival rates. The obligate mutualistic relationship

between the leafcutter ants and the fungus means that neither fungus nor ants can

survive and reproduce in the absence of the other. Therefore, death of a colony

was defined by the death of the fungus garden. Analyses of ant-fungus dynamics

included only colonies for which both the original fungus garden and the ant population survived the entire study period.

Data analysis. Data were analyzed using the program R, version 2.11.1 (R Development Core Team, 2010). Data for fungus area and worker population size were converted to wet mass units (grams) based on the correlations described above, then linearized and normalized by natural-log-transformation. To assess whether all colonies follow the same or different general growth pattern, I first constructed an overall simple linear model for each given size measure (fungus, ants) over time, using data for all colonies. The simple linear model was compared to a linear mixed-effects model, where colony identity was included as an additional random factor in the model. In the case of a significant difference between models, I selected the most parsimonious model with higher explanatory value, based on lower Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) indices (Sakamoto et al, 1986, Schwarz, 1978). In the event that the linear mixed-effects model was selected, post-hoc comparisons between colonies were conducted by constructing separate simple linear models for each individual colony.

Preliminary analyses of the overall mass relationship between the fungus and workers suggested that the relationship might not necessarily follow a simple linear trend, but might be exponential instead. To test for this possibility, I compared the AIC and BIC indices of a second-order, mixed-effects polynomial

8

regression against the linear mixed-effects model and selected the model with the lower AIC and BIC indices.

Results

Survival and population growth. A total of 174 colonies were initially established and monitored. Colonies experienced high mortality rates prior to first worker emergence, and then intermediate mortality rates thereafter. By week nine, towards the end of the period when workers began to emerge (see below), 132 of those colonies (75.9%) lost their fungus gardens and were therefore considered dead (Figure 2). A total of 32 colonies (18.4%) survived through week 9 but died before the end of the study at week 30, and thus only 12 colonies (6.9%) survived the entire time period. Mortality rates of over 90% are not unusual for field colonies (Cole, 2008) and it is interesting that even under conditions designed to be ideal (e.g. excess food) that mortality was so high. Data from 11 colonies that survived all the way through week 30 were used for further analysis. One excluded colony experienced rapid fungus garden losses during the final weeks of the study, indicative of imminent garden death.

In surviving colonies, workers emerged beginning during week 6 (the average day of first worker emergence was during week 7), and at least one worker had emerged in all colonies by week 10. Worker populations grew exponentially over time, with an overall growth exponent of 0.12 (t=8.53, p<0.0001, Figure 3a).

9

Colony identity was an important predictor for worker population growth rates (likelihood ratio = 280.7, p<0.0001), with individual colony growth exponents varying threefold, over a range from 0.07 to 0.21. In the mixed-effects model, this translated as a weighted effect of 0.05 on worker population growth rates.

Overall, the fungus gardens also grew exponentially over time, with an overall growth exponent of 0.098 (t=12.03, p<0.0001, Figure 3b). There were significant differences in garden growth between colonies (likelihood ratio = 241.5, p<0.0001). Fungus growth rate exponents also varied between colonies across a threefold range, from an exponent of 0.05 to 0.14. In the mixed-effects model, this represented a weighted effect of 0.03. Interestingly, shortly after workers emerged, many fungus gardens experienced a period with no growth, or a transient decline, before continuing to increase in size.

Worker-Fungus Population Dynamics. The relationship between the worker population size and fungus garden area did not remain consistent across the entire study period, but instead was best described by the polynomial mixed-effects model. This model accounted for variation between colonies and provided a significantly better fit than the corresponding linear mixed-effects model (likelihood ratio = 49.4, p<0.0001; Figures 3c and 4). The majority of colony second-order polynomial coefficients were significantly positive (seven out of 11 coefficients), ranging from 0.44 to 1.41. The coefficients for the remaining four colonies were not significantly different from zero.

Discussion

I found a transition in the worker-fungus biomass relationship that occurs during early colony development of the desert fungus-growing ant *Acromyrmex versicolor* (Figure 4): small colonies had little initial ant or fungus population increases, but once ant populations started to increase strongly, fungal mass also increased. This translated into an increase in the colony's worker production per unit fungus above a threshold colony size of about 50 workers. This type of transition is the inverse of general expectations for organismal growth: early stages of organismal development tend to occur rapidly, with little variation in developmental patterns, while later stages show higher degrees of phenotypic variability. While such developmental patterns may be representative for complex multicellular organisms, the present findings suggest that a different developmental possibility exists for biological entities at higher levels of organization.

The mathematical structure for scaling of the worker-fungus relationship in the present study differs from those reported by Seal and Tschinkel (2007a) for small and large colonies of *Trachymyrmex septentrionalis*. Seal and Tschinkel show an initial inverse relationship between worker number and fungus size (i.e. more workers with less fungus), but do not indicate how this translates into colony growth rate. In contrast, the data presented here indicate that this relationship is associated small initial worker and fungus growth rates. However,

11

both the present study and Seal and Tschinkel's analysis do indicate the presence of a similar qualitative transition between small and large colonies. Such a shift in a colony's worker production efficiency could be associated with ontogenetic changes in: (1) the colony's resource intake and processing, (2) fungus growth characteristics, or (3) efficiency in worker task performance.

Resource intake quantities and qualities can clearly affect fungus growth rates (Seal and Tschinkel, 2007b, Camargo et al, 2008) and colony mortality rates (Vieira-Neto and Vasconcelos, 2010), but that is unlikely to be the case in the present study, where an *ad-libitum* supply consisting of multiple fungus food types was available, and where all colonies had access to the same food sources. However, shifts in fungus food preparation methods, either through finer leaf degradation or more regular application of the fecal droplets used to aid leaf breakdown, could increase the leaf nutrients accessible to the growing fungus garden, and consequently increase the size of the growing ant population that could be supported per unit of fungus (Erthal et al, 2009). Evidence also suggests that pruning behavior by worker ants alters the growth of the fungus (Bass and Cherrett, 1996), leading it to produce larger amounts of staphylae, specialized structures that seem to be more nutritious than fungal hyphae (Bass and Cherrett, 1995). However, flexibility in fungus-tending behavior has not been observed directly in intact, growing colonies for any fungus-growing ant (Muscedere et al, 2010), making it difficult to assess the relative contributions of these behaviors to

changes in colony growth efficiency.

Ontogenetic changes in the worker size distribution and in task allocation could also produce shifts in overall colony growth efficiency. Wilson (1983) showed that young, small colonies of *Atta cephalotes* produced a nearly uniform size distribution that expanded as colonies increased in size and became right-skewed because colonies began to produce a small number of large majors in addition to maintaining the initial size distribution. Although comparable sociogenetic measurements have not been performed for other attine species and although size distribution patterns vary substantially between species (Wetterer, 1999), it is likely that size distributions shift in *Ac. versicolor* colonies as colonies grow, especially when comparing early stages of growth following the production of the first set of workers to later stages of growth. Shifts in the worker size distribution affect colony-level efficiency of task performance for tasks including leaf-processing (Burd and Howard, 2005), fungus care (Wilson, 1983, Augustin and Lopes, 2008), and brood care (Tschinkel and Porter, 1985), with consequences for overall colony performance and fungal production efficiency. For example, larger workers tend to specialize on, and are presumably more efficient at, leaf-cutting, whereas smaller workers specialize on fungus care, including weeding confined spaces in the fungus garden to remove parasites and pathogens that could affect the garden's food production (Bass and Cherrett, 1996, Oster and Wilson, 1978). Previous modeling work supports the idea that general

13

maintenance costs tied to colony growth should scale with negative allometry as well (Jeanson et al, 2007) and a mathematical model developed in parallel with the present study supports the idea the idea that division of labor affects the stability of the growth relationships between the workers and fungus (Kang et al, *submitted*).

In addition to changes in the overall size structure, as colonies begin to grow, there should be a shift in the colony's age distribution, with consequences for task distribution. Early in life, leafcutter ant workers specialize in tasks that are performed within the nest, such as brood care and fungus tending, whereas older workers perform tasks outside of the nest (Camargo et al, 2007). The earliest group of workers does not forage in *Ac. octospinosus* (Fernández-Marín et al, 2003) and in incipient colonies Fernández-Marín and colleagues observed a gradual transition from queen foraging, to queen and worker foraging, to worker foraging across the first three months of colony establishment, with a complete cessation of queen foraging at a colony size of approximately nine workers. Therefore, as a colony is established, the worker population may initially be biased towards the performance of tasks within the nest, without contributing to the growth of the fungus through foraging, causing a lag between the time when workers first begin to emerge and the time when they begin to contribute to the growth of the fungus garden via foraging.

Altogether, the observed survival and growth patterns suggest that leafcutter ant colonies experience a secondary population bottleneck that occurs during the transition from colony foundation to exponential growth. Further studies of colony organizational characteristics should clarify why this constraint exists and will help to explain how such large, complex societies have evolved.
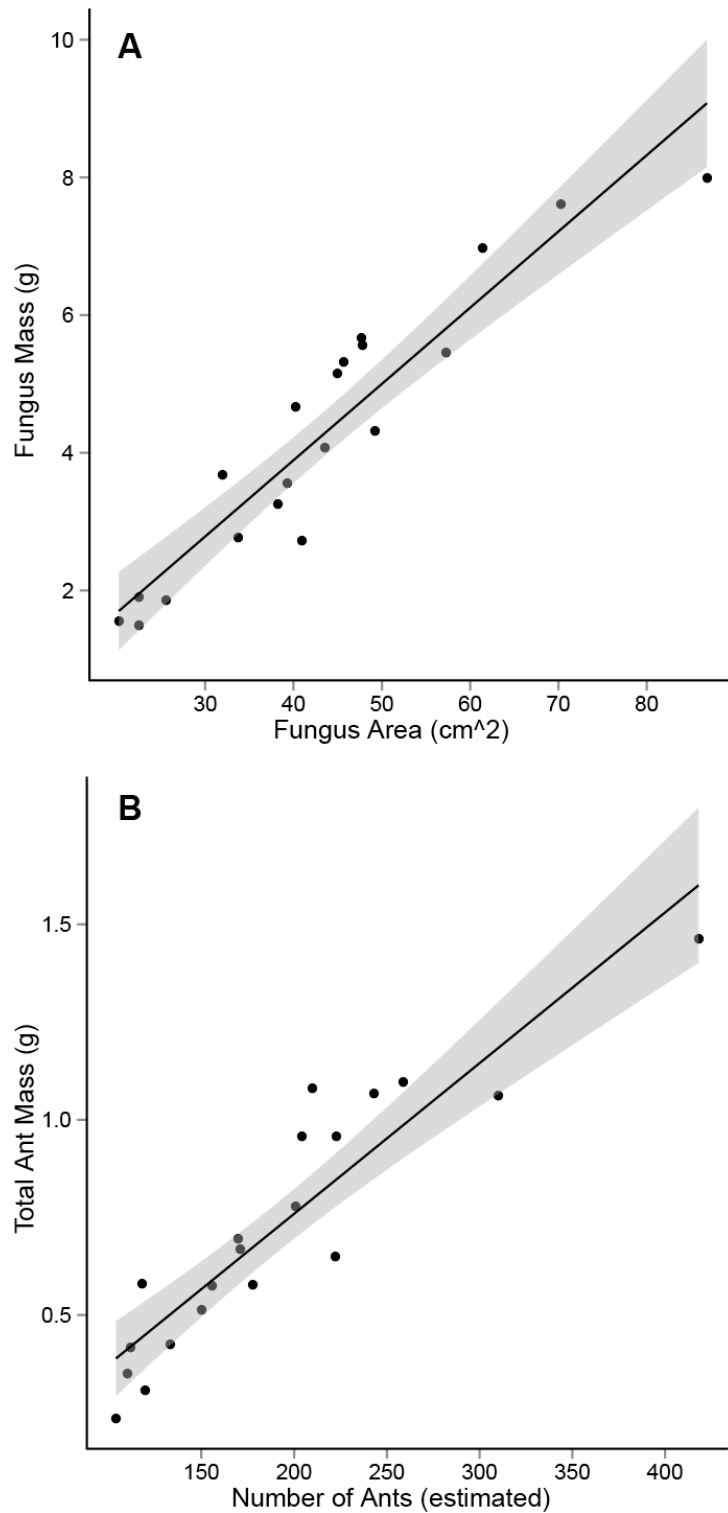
Figure 1. A) Correlation between fungus area and total fungus garden biomass (r^2=0.89). B) Correlation between the estimated number of workers and total worker wet biomass (r^2=0.88). Twenty-one age-matched colonies were used.

16
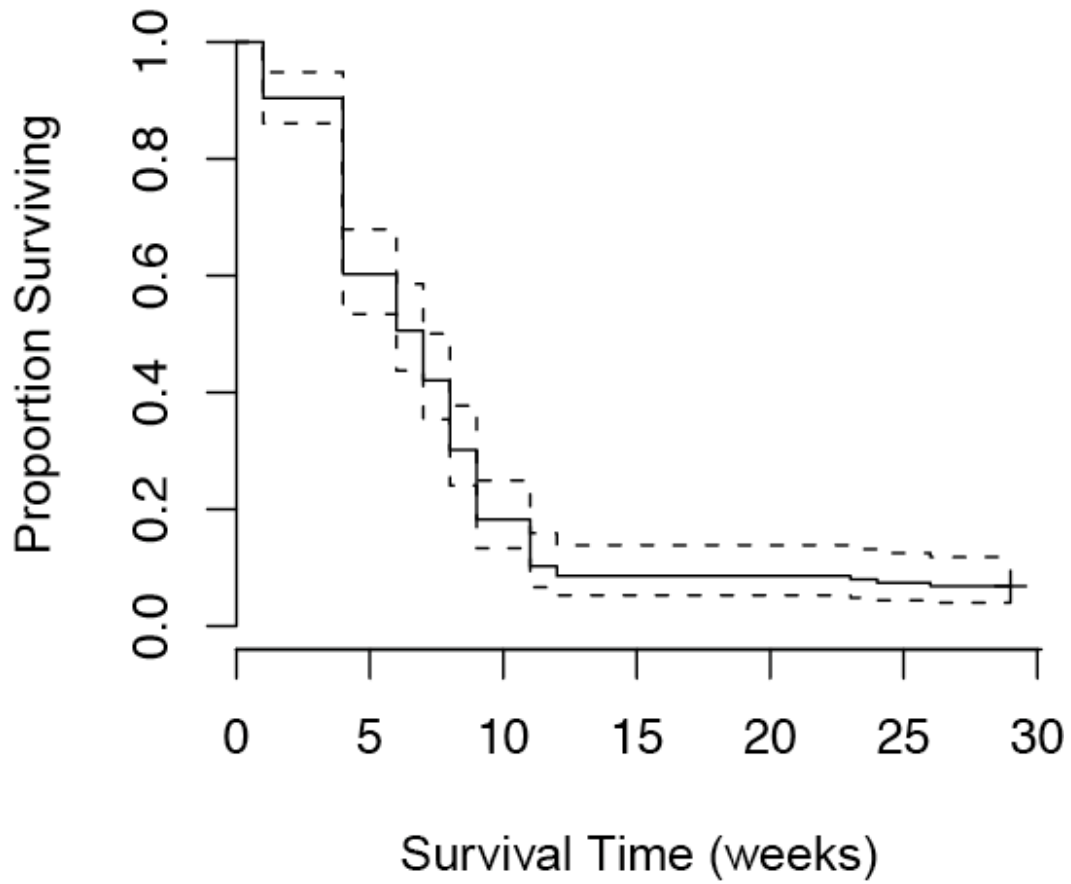
Figure 2. Survival curve for an initial population of 174 established nests.
Colony death date was defined by the death of the symbiotic fungus garden.
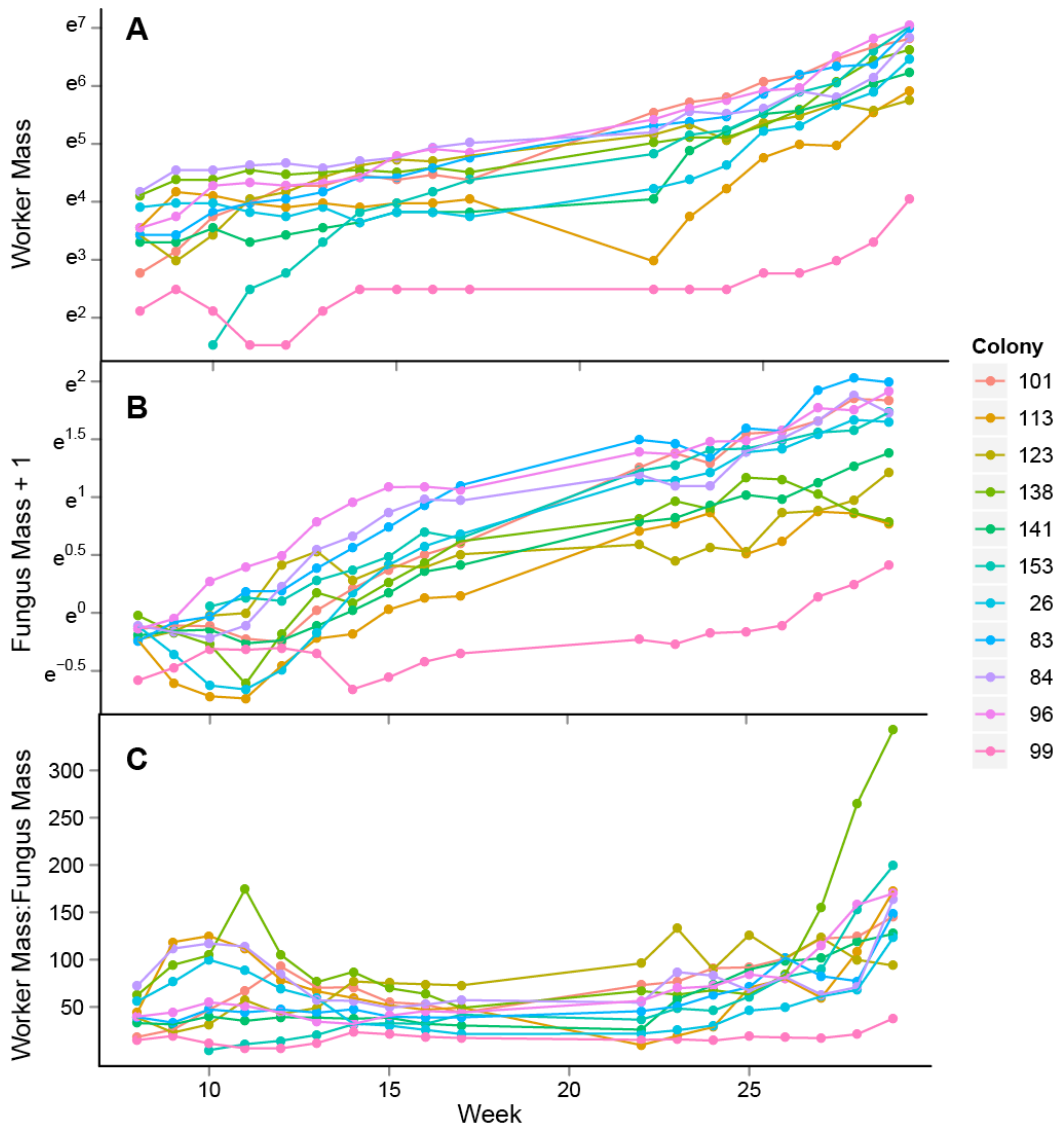
Figure 3. Growth relationships of workers and fungus during early colony development across a set of 11 colonies, from first worker emergence (day 55) through day 205. Each colony is identified by number and represented by a differently-colored line. A) Changes in the total worker population mass. B) Changes in the total fungus garden mass. C) The ratio of worker mass to fungus mass.

18
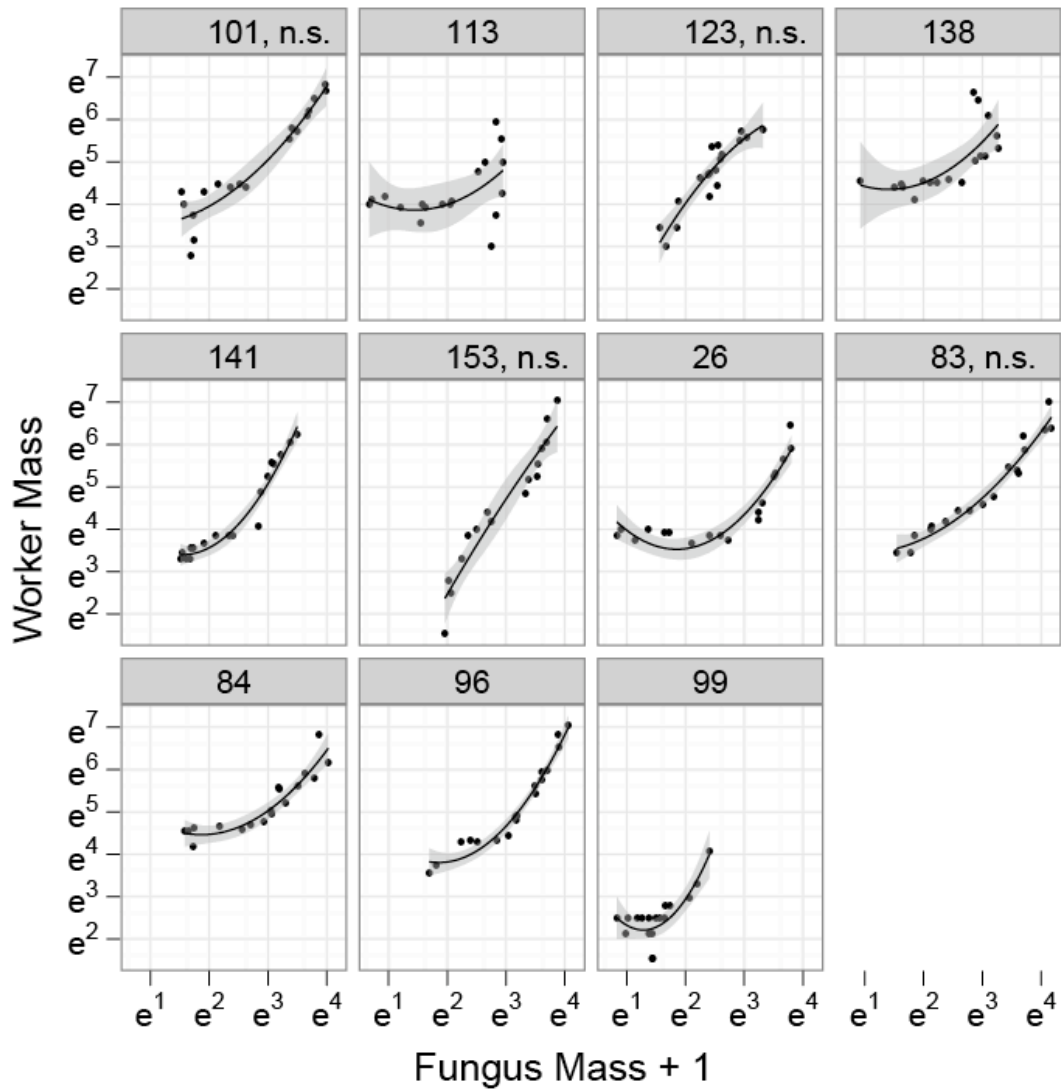
Figure 4. Log-log plots of the relationship between the fungus garden mass and total worker mass, fit to second-order polynomial models. The gray title boxes list colony identities, and colonies where polynomial fits were non-significant are labeled as n.s.. Shaded grey regions within the plots represent model 95% confidence intervals for polynomial models.

Chapter 2

ENERGY AND ELEMENT BUDGETS OF LEAFCUTTER ANT COLONIES

Ants are a large fraction of global animal biomass, and as such are thought to exert important influences on terrestrial ecosystem processes such as element and nutrient cycling (Davidson et al, 2003). To understand the ecological impact that ant colonies have, their foraging and feeding ecology need to be explicitly linked to colony growth. Leafcutter ants are an interesting candidate species for this because their evolved nutritional strategy causes them to function as herbivores: they harvest leaves to cultivate subterranean fungus gardens that feed the growing worker ant population (Wirth et al, 2003). This trophic structure makes leafcutter ant colony nutrition and growth an informative case that can be compared with studies of the foraging, feeding, and growth of individual herbivorous animals.

As with other herbivores, leafcutter ants are highly selective of their food sources, and base their choices on leaf nutritional content (Cherrett, 1972, Berish, 1986, Howard, 1987, Meyer et al, 2006, Mundim et al, 2009). Several central questions for leafcutter ant biology include whether, or which, forms of nutrient limitation occur, and how leaf inputs actually translate into growth. Two major hypotheses have been put forward for nutrient limitation, favoring either nitrogen (N, protein) or carbon (C, carbohydrate) limitation, depending on the evidence under consideration. Observational studies determined that colonies tend to select N-rich forage (Berish, 1986). On the other hand, colony refuse materials are also

20

high in N (Hudson et al, 2009), and foraging workers show stronger preferences for leaves with higher sugar content versus leaves with higher amino acid content (Meyer et al, 2006). There is also evidence that N-fixing bacteria found within the fungus garden produce N that is utilizable by the ants (Pinto-Tomas et al, 2009), which would reduce the need for external N inputs but could also increase demand for energy. A third, potentially overlooked, nutrient that could limit growth rates is phosphorus (P), which has been suggested to limit net primary productivity in some terrestrial ecosystems, and may thereby influence herbivore growth because of its role in growth-related biomolecules such as ribosomal RNA (Elser et al, 2000, 2007, Schade et al, 2003, Perkins et al, 2004, Hillebrand et al, 2009).

One useful method for assessing growth efficiency and determining how nutrients limit growth is to examine how biomass balances across all of the components of a system. Mass balancing involves estimating the intake, excretion and assimilation rates of bulk mass and of elements of interest such as C, N and P. Comparing the relative elemental composition of food against the composition and growth rate of the consumer should reveal which elements potentially constrain growth. Specifically, limiting resources are expected to be selectively absorbed, and minimally excreted in comparison to non-limiting resources. Mass-balance methods have long been applied to trace the fates of resources in individual organisms (e.g. Larsson and Tenow, 1979, Slansky and Fogal, 1985, Woods et al, 2002, Meehan and Lindroth, 2009) and ecosystems

(e.g. Nixon, 1995), and so should be useful in understanding the nutritional ecology across complex social systems as well.

In this study, I measured and compared mass balancing properties across entire colonies by monitoring resource intake for laboratory colonies of the desert leafcutter ant *Acromyrmex versicolor*. Colonies were provided with one of two single-food diets, palo brea leaves (*Cercidium praecox*) or polenta, both of which are relatively high-quality food for this species (Tibbets and Faeth, 1999, Weser, 2005). Resource intake measurements were coupled with measures of the biomass production of the fungus garden and ant population, and waste production (Figure 5). I also measured the C, N, and P contents of all colony components to ascertain how, and where, colonies utilize these potentially growth-limiting elements. I used these measures to assess major sinks for different resource types, and to provide an overall perspective for thinking about colony-level nutrition and growth in this tritrophic system.

Methods

Colony Growth and Mass Balancing. Data from colonies used for the present study came from two experiments originally designed to test for the effects of phosphorus limitation on colony growth, one involving a pure palo brea (*Cercidium praecox*) diet, and one involving a pure polenta diet. Both groups of colonies were established with *Acromyrmex versicolor* foundress queens collected from underneath mating swarms found at the intersection of Swan Rd. and E

22

Camino de la Brinca in Tucson, AZ (32°19'0" N, 110°53'33" W). Colonies fed on the palo brea leaf diet (n=20) were collected on 8 August 2005. Palo brea colonies had lost their original fungus strain over the course of colony development and were re-inoculated on 31 January 2007, with fungus that came from a single colony that had also been initiated in August 2005. Prior to the experiment, colonies were maintained on *ad libitum* diets consisting of polenta, oat flakes, and fresh palo brea leaves. These colonies were two years of age at the time of study, but were similar in size to six-month-old colonies due to prior fungus loss. Colonies fed the polenta diet (n=27) were established from queens collected on 1 August 2010. These colonies were five months old at the time of study.

Throughout both experiments, colonies were kept in a room maintained between 25-30°C with natural window lighting. Nests consisted of a series of round Petri dishes (Pioneer Plastics, 8.4 cm diameter x 3.3 cm high internal dimensions) that were interconnected with short segments of vinyl tubing (~2 cm long x ¼-inch inner diameter). Palo brea nests consisted of two adjacent dishes lined with ~1 cm of pottery plaster (fungus chambers) that were watered weekly with 1-3 mL distilled water to maintain high humidity and promote growth of the ants' fungus garden. One of the plaster-filled Petri dishes was also attached to a set of three circular foraging arenas, a central foraging arena and two side foraging

arenas (also 8.4 cm diameter x 3.3 cm high). Polenta nests consisted of a single

fungus chamber attached to a single foraging arena.

The palo brea colonies were given sets of pre-weighed palo brea leaflets

once a day in the two side foraging arenas. Leaflets were taken from branches that

had been freshly cut and soaked for 24 hours in either a monobasic potassium

phosphate solution (pH=6.0, 20 mmol/liter KH_2PO_4) or water adjusted to a pH of

6.0. The following day, all of the leaves remaining in the foraging arenas were

removed and re-weighed. Six sets of leaves (three sets soaked in potassium

phosphate and three sets soaked in water) were also placed in a container without

any ants present to permit an estimate of total leaf daily water loss, and were re-

weighed along with leaves left over from the colonies. Twenty-seven control leaf

samples were further dried for 3 days at 60°C, and then re-weighed to calculate

the mean proportion of leaf dry weight, which was 0.419±0.007. The proportion

of leaf dry weight did not differ between leaves soaked in water and leaves soaked

in the potassium phosphate solution, and colonies offered a choice between

control and KH_2PO_4-soaked leaves ended up collecting both leaf types equally.

Polenta colonies were fed once a week with pre-weighed amounts of

control or phosphorus-enriched polenta. At the end of each week, left-over

polenta was removed and reweighed and the difference in mass was calculated to

be the amount collected by the ants. Polenta diets were prepared by adding 100 g

of polenta to 400 mL of boiling water, either without any other additives (control,

n=14) or along with 1 g of potassium phosphate (P-enriched, n=13). Potassium phosphate addition raised the P content of polenta to match the P content of palo brea leaves (see Figure 8). Polenta and water mixtures were stirred at a moderate boil for four minutes to evenly mix ingredients, poured into a glass dish and dried at 70°C for two days, and then ground to mixtures of coarse particles with a corn mill.

Each week in both experiments, hand counters were used to noninvasively estimate the total number of adult workers in each nest and overhead digital photographs of the fungus chambers were taken at a standard distance to measure the area of the fungus garden. The area of the fungus was measured from photographs with the software program ImageJ and was used to calculate the total fungus garden mass. At the end of the palo brea experiment, to obtain relationships between estimation methods and actual wet mass units (Figure 6), I used probes and soft forceps to remove all of the workers, larvae, and pupae from the fungus gardens, and then separately weighed the fungus garden and the entire worker population. I also counted the exact number of workers, larvae, and pupae present. To convert from wet to dry mass units, I dried fungus gardens at 60°C for three days and reweighed them. With a separate set of workers, I measured individual ant wet masses, then froze the ants and dried them for three days, and reweighed them.

A. versicolor leafcutter ants create an external trash pile where they deposit multiple types of debris, including dead ants; old, dead fungal material; excavated plaster; and tiny fragments of processed leaves that have been rejected from the fungus garden. Entire trash piles were collected by aspiration and were sorted into separate piles: used fungus material plus rejected leaves; dead workers; dead larvae; and plaster pieces. Dead workers and larvae were counted, and used fungus material was weighed as a measure of the colony's total solid waste output.

Three of the colonies in the palo brea study showed mass losses over the course of the study, generally driven by reductions in fungus garden mass. These colonies were dropped from remaining analyses because we wished to focus on the mass-balance of growing colonies.

Mass Balance Calculations. Mass balancing calculations were performed for individual colonies. Calculations used to convert measurements into dry mass units are summarized in Table 1. Leaf intake, in dry mass units, was calculated as:

$$M_l \text{ (dry g day}^{-1}) = \Sigma \left([M_{l,i} - (M_{l,f}/ \text{FDWL})]*\text{DWR}) / t \right. \tag{1}$$

where $M_{l,i}$ and $M_{l,f}$ represent the initial and final leaf mass, respectively, in wet g; FDWL is the fractional change in leaf mass attributed to water loss over the day

(calculated as final/initial weight for control leaves); DWR is the average ratio of dry to wet mass for leaves (0.419 ± 0.007, n=21); and t is time, in days.

The growth of the fungus garden, in dry mass units, was calculated as:

$$\Delta M_f \text{ (dry g day}^{-1}) = [(A_{f,f} - A_{f,i})*0.0435 - 0.0459] / t \tag{2}$$

where $A_{f,f}$ and $A_{f,i}$ are the final and initial fungus garden areas (in cm^2), respectively, and t is time in days, as above. The conversion coefficient is based on the relationship between the fungus garden area and dry mass that was calculated at the end of the palo brea study from direct measurements, as described in the Methods section and shown in Figure 6.

The growth of the worker population, in dry mass units, was calculated by converting numeric worker estimates to wet mass estimates. The wet mass estimate was converted to a dry mass estimate, as follows:

$$\Delta M_w \text{ (dry g day}^{-1}) = [(N_{ew,f}-N_{ew,i})*0.00149+0.0190] / t \tag{3}$$

where $N_{ew,f}$ and $N_{ew,i}$ are the final and initial visually estimated worker population sizes, respectively. Conversion coefficients are based on relationships between estimated worker numbers and actual worker wet masses, and, individual worker wet versus dry mass (Figures 6C and 6D). This ignores workers found in the trash

pile, which were a small fraction of total colony size (< 3% of the worker population size). After sorting and removing dead workers, larvae, and plaster, trash production was measured directly in dry mass units and divided by the duration of the study to obtain the dry mass of trash produced per day.

Elemental Analysis. Carbon (C), nitrogen (N) and phosphorus (P) were measured in samples of palo brea leaves, polenta, larvae, pupae, and ants collected from separate colonies maintained in the lab under identical conditions to those colonies used for the mass balance studies, with the exception that these colonies were provided with a mixed diet of polenta and palo brea leaves. Dried fungus and trash samples from the palo brea experiment were used for measures of fungus and trash elemental composition. The fungus cannot be easily separated from the leaf material it is growing on (substrate), so fungus samples consisted of both fungal material and substrate.

All sample types (palo brea leaves, polenta, fungus, larvae, pupae, ants, and trash) were prepared for analysis by drying at 60°C for at least three days. Due to small individual masses (0.053-2.641 mg), larvae were grouped into small, medium, or large groups for analysis. Groups contained 22, 10, and 7 individuals, respectively. Pupae were similarly classified as small or large and put into groups containing 9 and 5 individuals, respectively. Leaves, polenta, fungus, grouped larvae, grouped pupae, and trash samples were homogenized to a fine powder

with a ball mill (Spex 8000D Dual Mixer/Ballmill, Metuchen, NJ). Adult workers were measured individually.

Total carbon and nitrogen contents of samples were measured with combustion analysis using a Perkin-Elmer CHN Analyzer (Waltham, MA). For P measurements, ants were weighed and ground into powder with a stirring rod directly within glass test tubes used for sample analysis. The remaining dried and homogenized sample types were weighed on an electronic balance (Mettler MT5, $\pm$ 0.1 µg) and placed into analysis tubes. Total P contents were measured with persulfate digestion and ascorbic acid colorimetry, as described by Clesceri et al (1998). Each assay also included duplicate P standards (0, 25, 50, 100, 200, 400, and 800 nmol KH_2PO_4 added as different volumes of a 500 µmol l^{-1} stock solution), with P concentrations selected to fall in a linear range of absorbance at 880 nm (absorbance < 0.6 units). Duplicate samples of finely ground apple leaves were included as internal standards (NIST standard no.1515, 0.154 %P by dry mass).

Results

Colony Size Properties. Differences in colony size could influence other colony characteristics, such as foraging rates, fungus growth, worker population growth rates, and refuse production. Therefore, I first tested, within diets, whether there were significant relationships between colony size and: (1) leaf

material or polenta harvested, (2) worker population growth, (3) fungus garden growth, and (4) the amount of refuse produced by the colony. Comparisons were made on estimates converted to dry masses (in g) per day. For both palo brea and polenta diets, colony size predicted the amount of leaf material collected by the colony, worker population growth, and refuse production, but did not predict changes in fungus garden size (Figure 7; statistics given in Table 2). However, residuals from the analysis of collected leaf mass, and residuals from the analysis of worker population growth, were positively associated with fungus growth rates (Table 3).

Because size was an important predictor of other colony characteristics, biomass comparisons between diets (below) were made using analyses of covariance (ANCOVAs) with colony size as the covariate. Colonies fed palo brea leaves collected significantly more leaf material per day than colonies given polenta (Figures 7A and B; $F_{1,44}=64.9$, $p<3.4\text{x}10^{-10}$). Worker populations and fungus gardens also grew more when fed palo brea leaves (Figures 7C through F; $F_{1,44}=120.2$, $p<3.6\text{x}10^{-14}$; $F_{1,44}=11.8$, $p=0.0013$). However, both treatments had equal rates of refuse production (Figures 7G and H; $F_{1,42}=0.001$, $p=0.99$).

To determine how the amount of leaves harvested by the workers translated into colony growth, I calculated assimilation rates of leaf material and polenta, which is equal to the proportion of resources that are consumed but not excreted and equivalent to approximate digestibility (Slansky and Scriber, 1985,

30

Schoonhoven et al, 1998). In this case, the refuse material was taken to represent

excreted material, as it contained egested leaves (small, rejected leaf fragments) and

used fungus substrate. Assimilation rates were not a function of colony size for

either diet ($F_{1,18}$=0.69, p=0.42 for palo brea; $F_{1,23}$=0.16, p=0.70 for polenta), and

were higher overall for the palo brea diet compared to the polenta diet ($F_{1,42}$=10.1,

p=0.0028; mean$_{palo\ brea}$= 94.7±4.1%, mean$_{polenta}$=86.0±2.3%). I calculated leaf

conversion efficiencies as:

$$CE = (\Delta M_{w,d} + \Delta M_f) / (M_l - M_r) \qquad\qquad (4)$$

using the mass variables as defined previously and in Table 2 (Slansky and Scriber,

1985, Schoonhoven et al, 1998). Conversion efficiencies were similar between

diets ($F_{1,42}$=0.35, p=0.56), and the overall mean conversion efficiency was

28.9±3.1% (SEM). Conversion efficiency did not vary with colony size (palo

brea: $F_{1,18}$=2.5, p=0.13; polenta: $F_{1,23}$=1.1, p=0.30). However, the amount of leaf

material that was assimilated (M_l-M_r) was correlated with growth rate (palo brea:

$F_{1,18}$=17.3, p=0.00059; polenta: $F_{1,23}$=22.4, p=9.1x10^{-05}).

Elemental Composition. Mismatch between the elemental composition

of resources and consumers can provide insights into potential forms of nutrient

limitation occurring in trophic systems. I compared the carbon (C), nitrogen (N),

and phosphorus (P) contents, along with C:N and C:P ratios, between colony

31

components, including foraged resources and refuse material, to understand the nature of elemental mismatch within the leafcutter ant system. Elemental composition varied significantly between colony components (Figure 8; for C: $F_{6,137}=169.2$, $p<2.2 \times 10^{-16}$; for N: $F_{6,137}=575.0$, $p<2.2 \times 10^{-16}$; for P: $F_{6,87}=121.3$, $p<2.2 \times 10^{-16}$). Adult ants had the highest C and N contents, which were 4% and 6% higher than the next-highest components, respectively. Palo brea leaves had higher C content than the fungus garden, which in turn had higher C content than the colony refuse pile (Figure 8A). Polenta, in contrast, had lower C content than the fungus, but higher C than colony refuse.

In contrast to what was observed for carbon contents, nitrogen contents were similar across the palo brea leaves, fungus garden, and colony refuse (Figure 8B). Developing larvae and pupae contained lower levels of N relative to adult workers, but were higher in N than the leaves and fungus garden. Polenta had low N compared to all other components. Finally, P contents were high across all ant life stages, relative to fungus, polenta, leaf, and refuse P. Interestingly, even after dead ants had been removed, colony refuse (rejected leaves + used fungus material) had higher P than the fungus, palo brea leaves, and polenta.

Elemental ratios could also provide insights into the nature of nutrient limitation, so, where possible, C:N and C:P elemental ratios were calculated. As could be anticipated, given the differences in elemental contents, C:N and C:P ratios also varied across colony components (Figure 9; for C:N, $F_{6,119}=105.1$,

32

$p<2.2x10^{-16}$; for C:P, $F_{4,23}=20.7$, $p=2.4x10^{-7}$). Fungal substrates and the fungus garden had the highest C:N and C:P ratios. Refuse material had a lower C:N than either the fungus or substrates, and ants had the lowest C:N ratios (Figure 9A). Larval, pupal, and refuse C:P were all similar to each other (Figure 9B).

Mass Balance of Carbon, Nitrogen, and Phosphorus. The mean foraging rates, growth rates, and refuse production rates measured in this study were used in conjunction with knowledge of elemental compositions to estimate how colonies utilized the carbon, nitrogen, and phosphorus they collected. Estimation efforts focused on colonies fed with palo brea leaves because most elemental measurements were made on colonies with palo brea-biased diets. Based on these measurements, colony growth and refuse production were calculated to account for 29% of ingested C for colonies fed palo brea leaves. Colonies feeding on palo brea leaves collected an average of 12.2 mmol of C per day, per g colony mass, of which 1.6 mmol were converted into fungus, and 0.66 mmol were converted into new workers. Meanwhile, 0.48 mmol day^{-1} g colony mass^{-1} of C were deposited as refuse material.

Overall foraging, assimilation, and refuse patterns were similar for nitrogen, with growth and refuse production accounting for 42% of the N obtained from palo brea leaves. Colonies collected an average of 0.91 mmol day^{-1} g colony mass^{-1} of nitrogen, while converting 0.015 mmol of N into new fungus material and

33

0.015 mmol into workers. An average of 0.05 mmol day^{-1} g colony mass^{-1} of N

was removed as refuse.

Phosphorus does not have a gaseous form, so it was possible to estimate P

consumption in two ways for each diet: (1) based on the difference between how

much mass was consumed and excreted plus the P content of consumed/excreted

materials, and (2) based on knowledge of fungus and worker growth rates and their

P compositions. P intake estimated to be 28% higher based on

consumption/excretion rates relative to estimates based on growth rates

($F_{1,89}$=35.4, p=5.2x10^{-8}; mean$_{c/e}$ = 0.128±0.019 mg/day; mean$_{growth}$ = 0.027±0.004

mg/day). P consumption rates were also estimated to be 26% higher for colonies

fed palo brea leaves in contrast to estimates based on the polenta diet ($F_{1,89}$=46.7,

p=9.9x10^{-10}; mean$_{palo\ brea}$=0.145±0.020 mg/day; mean$_{polenta}$=0.037±0.004 mg/day).

Overall, this suggests that P intake fell somewhere in the range between 0.027 and

0.145 mg/day.

Discussion

Overall, the mass and element balances presented here indicate that

leafcutter ant colony foraging and growth characteristics follow patterns that are

comparable to foraging and growth patterns observed for individual organisms,

with one major distinction: young, growing colonies incorporate and/or use most

of the leaf material they collect, leading to extremely high assimilation rates

relative to typical assimilation rates for other individual organisms. High

assimilation distinguishes colonies from individual organisms because most individual organisms are incapable of physically retaining as much of their food all at once. The assimilation measured here is also much higher than the 28.7% assimilation estimated by Wirth et al (2003) for large colonies of *Atta colombica* in Neotropical rainforests. The extreme difference between the present estimate and Wirth's estimate could be due to differences in the sizes and growth stages of the colonies studied. Larger colonies may reach an equilibrium point where leaf inputs begin to balance against refuse outputs. While assimilation rates were surprisingly high, colony conversion efficiencies were not nearly so extreme and instead fell within the same range as conversion efficiencies described for other polyphagous insects and of ruminants (Slansky and Scriber, 1985, Li and Hou, 2007). This could indicate that what appears to be a high overall rate of assimilation by the colony could also be influenced by the presence of other organisms within the fungus garden that do not necessarily directly contribute to the growth of the worker population (Suen et al, 2010).

Palo brea is higher in carbon, nitrogen, and phosphorus than polenta, and yet colonies collected more of it while maintaining the same growth rate and refuse production rate as the polenta-fed colonies. This could be due to differences in the forms in which nutrients occur in the two food sources. Palo brea trees, which are leguminous, are likely to contain more N-based plant defense compounds, including alkaloids (Cates and Rhoades, 1977), as compared to polenta. Palo brea's higher C content is also probably largely due to C present in hard-to-digest materials such as lignins and hemicellulose (Ramirez et al,

1990), and it is unclear whether these resources are utilizable by the fungus and ants (Abril and Bucher, 2002, Suen et al, 2010). Therefore, despite having higher total C and N, resources in palo brea leaves could be much more difficult for colonies to digest and use. If colonies cannot use all of the available C and N, collection of relatively more palo brea per given colony size could reflect a form of compensatory foraging to reach a target nutrient intake level, as has been observed in other herbivorous insects (Slansky and Wheeler, 1989, 1991).

The high nitrogen content in the refuse, as well as its lower C:N ratio compared to leaf, polenta, and fungus C:N, suggests that colonies selectively remove C relative to N, in support of the idea that colonies are C-limited overall. This is also supported by the fact that mass-balancing calculations accounted for more of the collected N and P than collected C. Carbon limitation is likely to correspond with carbohydrate limitation by the ants, an assertion supported by worker foraging preferences for carbohydrate-rich resources (Meyer et al, 2006). The patterns for phosphorus also support this conclusion; P content and C:P ratios followed similar patterns as nitrogen, including higher levels of P in refuse relative to the fungus and harvested materials.

Mass-balancing comparisons for N and P both accounted for roughly one-third of the N and P collected by colonies, meaning that the fate of the remaining two-thirds of the collected P still need to be accounted for. There are four potential explanations. First, the fact that P estimates based on intake/excretion rates are higher than estimates based on worker and fungus growth rates suggests that leaf and polenta intake rates could have been overestimated. Such errors are

36

thought to be relatively common in gravimetric studies of insect herbivore feeding

rates on leaves (Van Loon et al, 2005), but would not explain overestimates based

on the polenta diet. Secondly, the elemental composition of the fungus garden

could be labile. If this is the case, the N and P content of a fungus garden

provided with only polenta should be lower than the fungus garden N and P

content estimates reported here, and estimates should be higher for fungus

gardens provided with palo brea leaves. Similarly, the elemental composition of

refuse material may be dependent on the elemental composition of the leaves and

fungus, as appears to be the case for leafcutter ant colonies in pastures (Tadey and

Farji-Brener, 2007). Finally, N and P could be lost from the system components

measured here via excretion and leaching into the plaster lining the fungus

chamber. Future efforts will focus on identifying if these potential sinks can

account for the remaining N and P balance.

$$\text{Leaf intake} = \Delta \text{Fungus biomass} + \Delta \text{Ant biomass} + \text{Trash output}$$

+ Respiratory losses (as carbon dioxide)

+ Leaching and Excretion
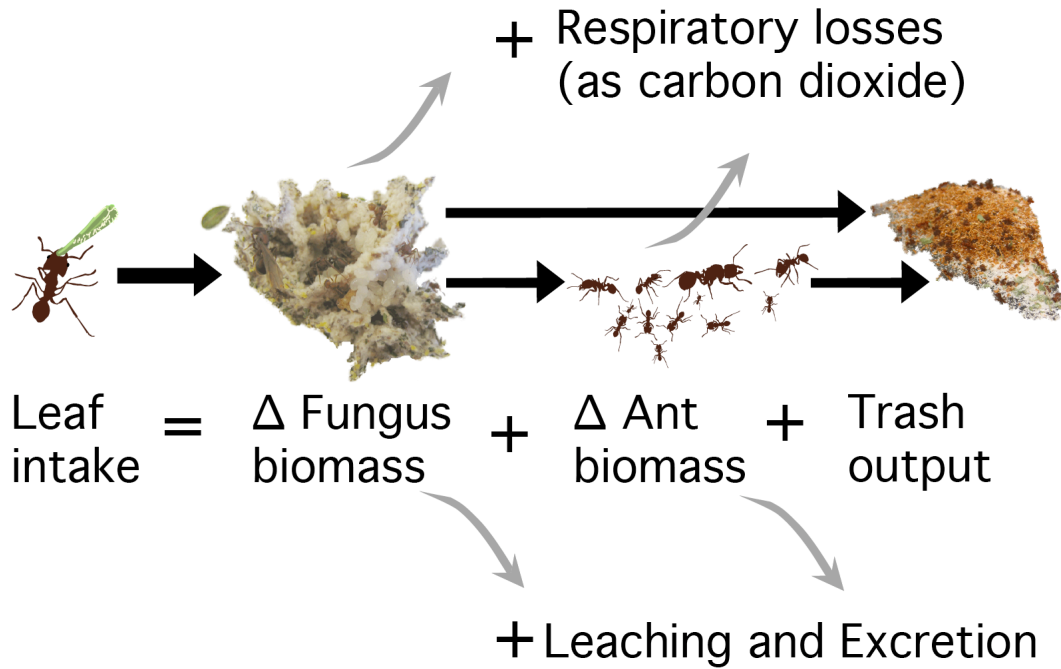
Figure 5. Schematic diagram of mass flows (dry mass) through leafcutter ant colonies. Worker forage for leaves, which they bring back to the nest, cut into small fragments, and apply to their fungus garden. As the fungus grows, it digests the leaf material and produces staphylae, specialized structures that the ants consume and feed to developing brood. Once leaf resources have been consumed, the exhausted leaf substrate and fungal remnants are pruned from the fungus garden and discarded in a trash pile, along with dead workers and rejected leaf material. Carbon is lost from the system as carbon dioxide via metabolic processes, and other minerals may be lost through leaching or excretion by the fungus, brood, and workers.
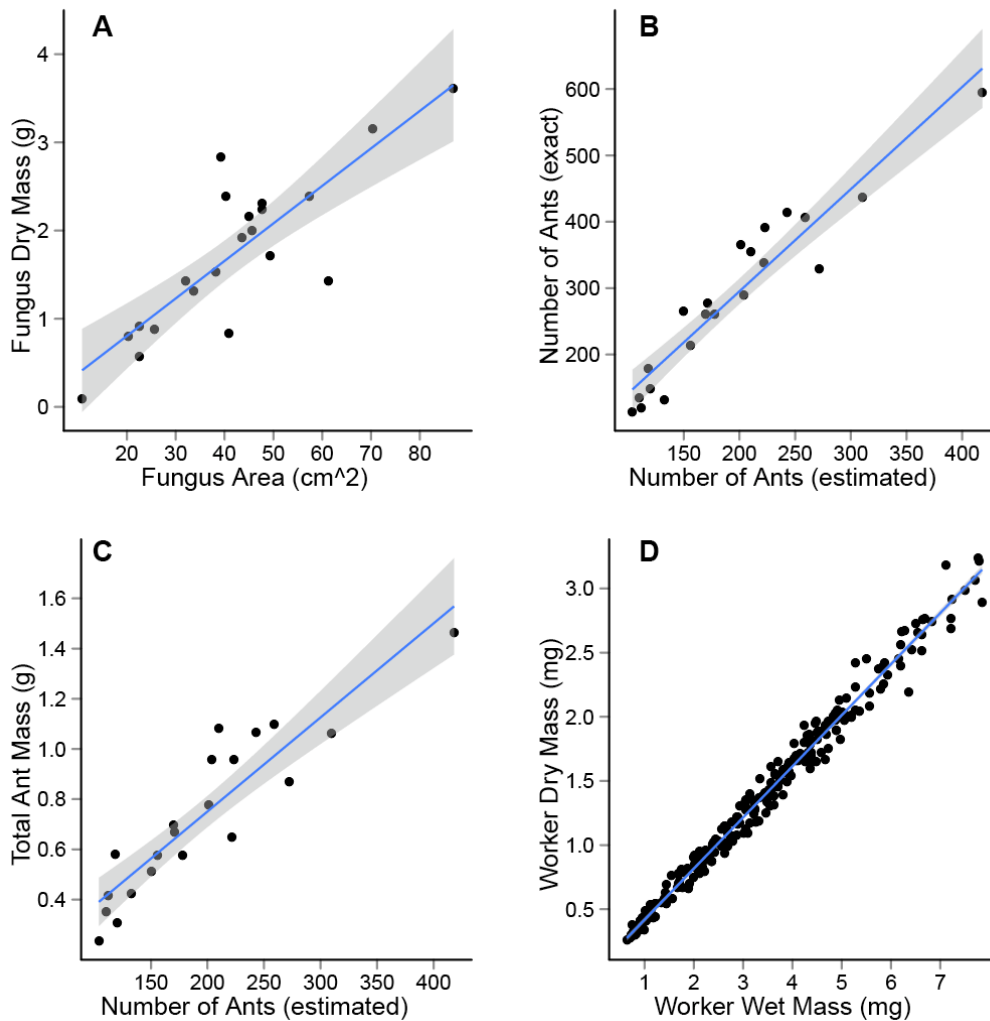
Figure 6. Correlations used to convert estimates to dry mass units. Summary statistics for correlations are listed in Table 1. A) The correlation between fungus area and the total fungus garden dry biomass. B) The correlation between worker population estimates and the actual number of workers present in each colony. C) The correlation between worker population estimates and the total worker population wet mass. D) The correlation between an individual worker's wet mass and her corresponding dry mass. The data presented are for workers sampled from four colonies that not part of the rest of this study.
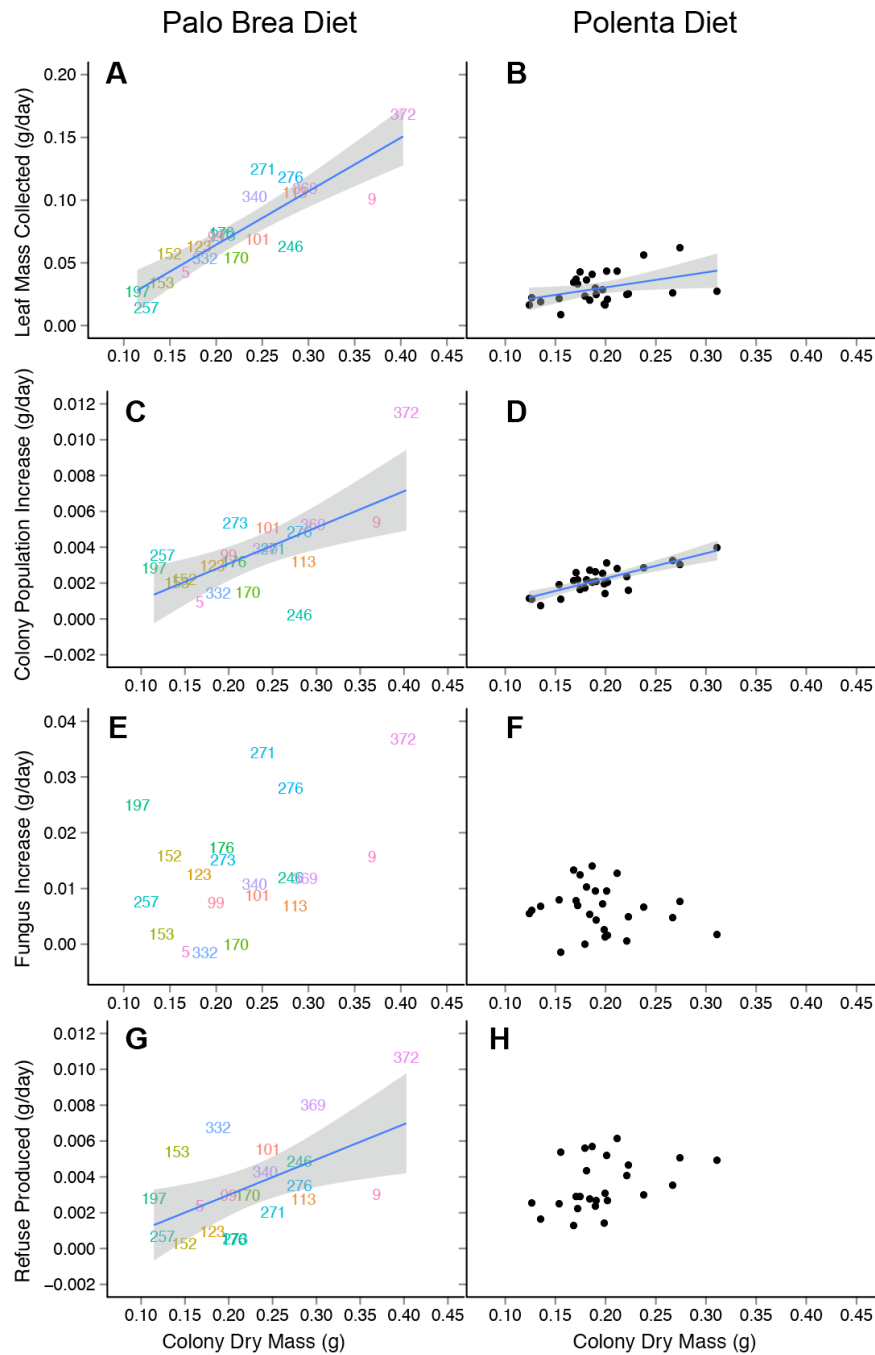
Figure 7. Relationships between colony size and leaf mass harvested (A, B), changes in the worker population size (C, D), changes in the fungus garden mass (E, F), and the amount of refuse produced (G, H), for colonies provided with diets consisting of either palo brea leaves (panels A, C, E, and G) or polenta (panels (B, D, F, and H). For colonies on the palo brea leaf diet, individual points are labeled with individual colony identity numbers.

40
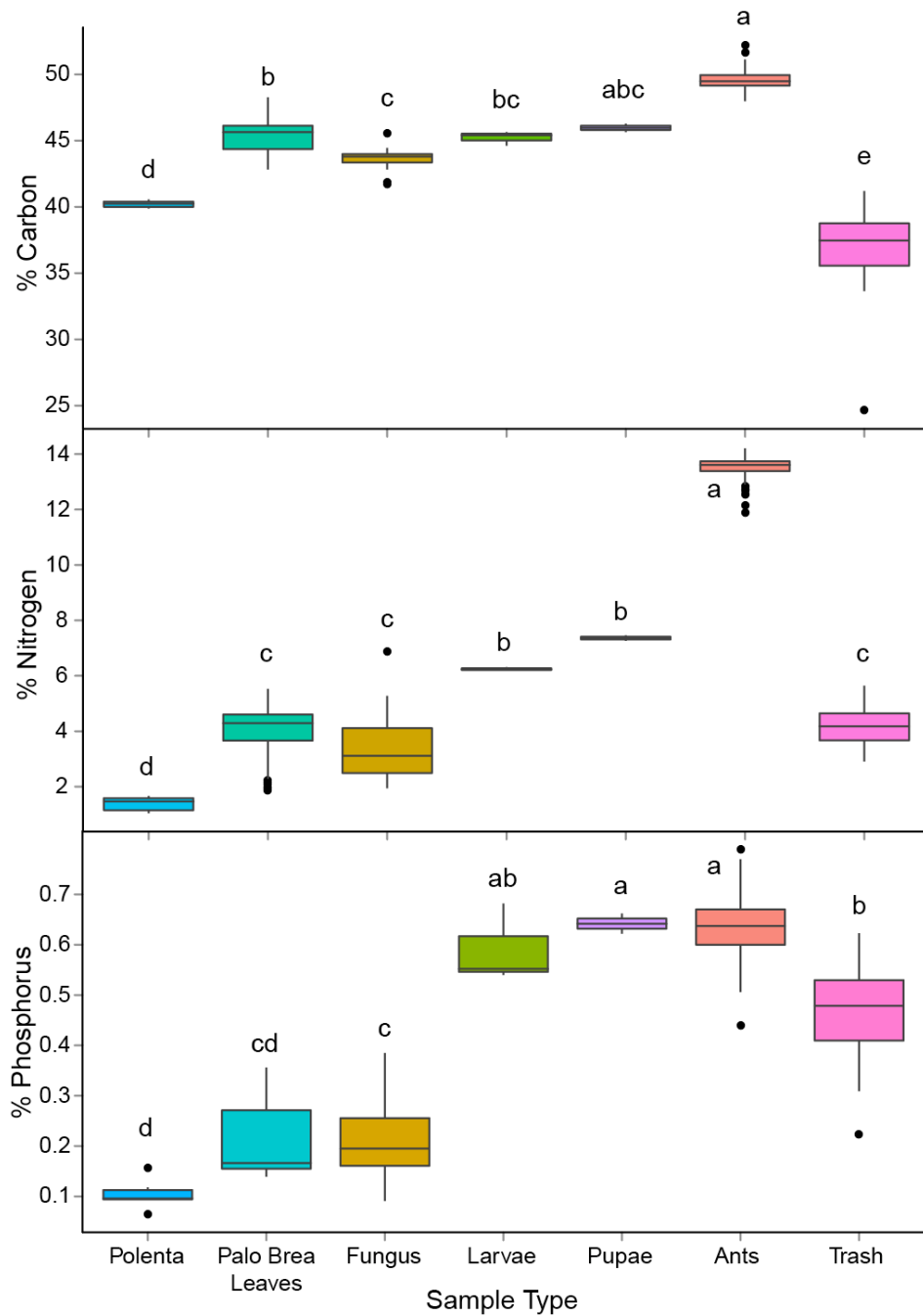
Figure 8. A) Carbon, B) Nitrogen, and C) Phosphorus composition of all leafcutter ant colony components, including material harvested to grow fungus (polenta and palo brea leaves); the fungus; ant larvae, pupae, and adult workers; and trash (used fungus substrate). Fungus measurements are of both the fungus itself and the material it is using to grow (fungus substrate). Element measures represent the percentage of dry mass composed of that element.
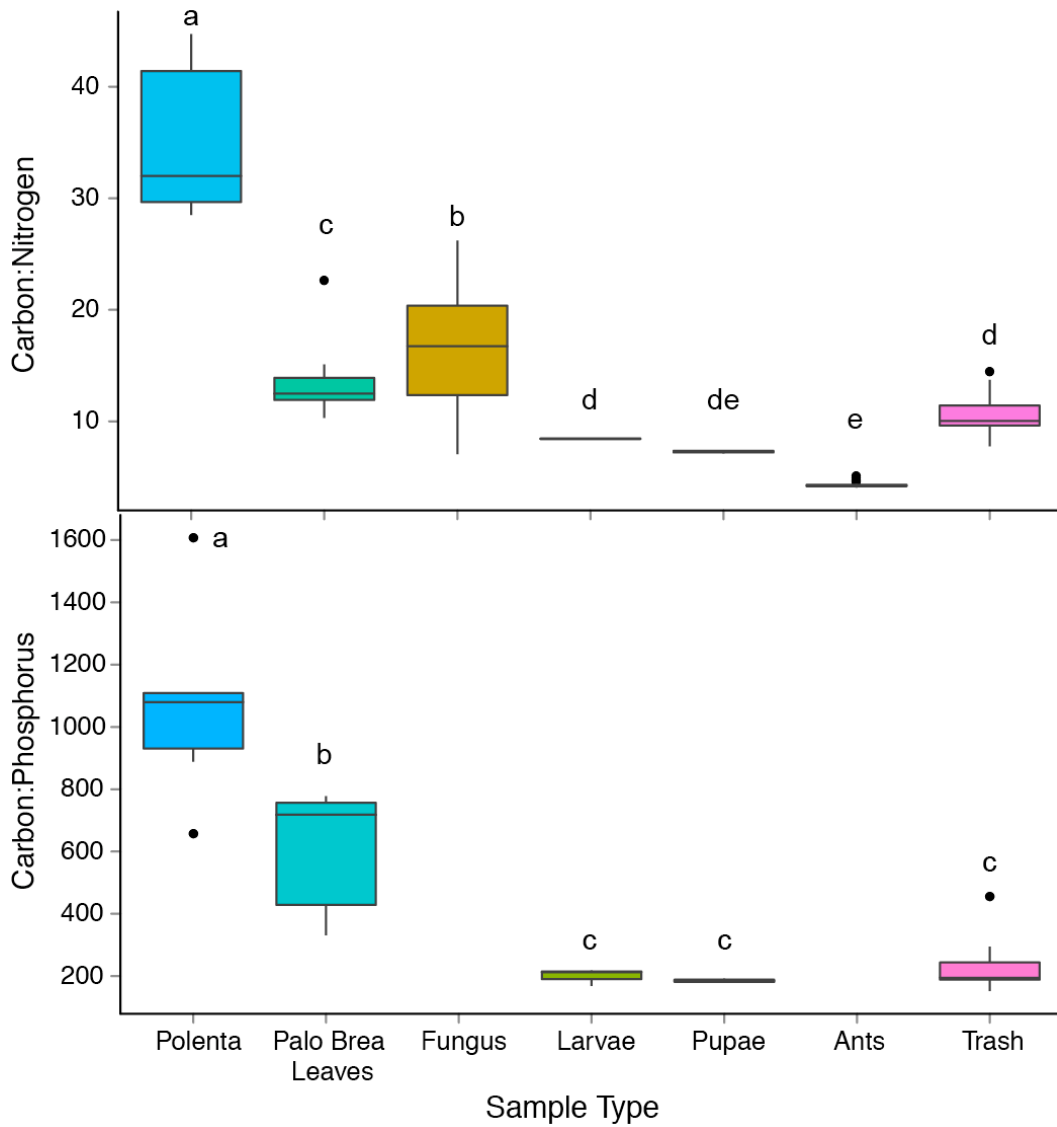
Figure 9. Ratios of A) Carbon:Nitrogen and B) Carbon:Phosphorus across leafcutter ant colony components. Carbon:Phosphorus ratios were only calculated for samples that were large enough to permit measurement of both carbon and phosphorus on the same sample, so measurements are not included for fungus or ants.

Table 1

Summary of equations used to convert fungus and worker estimates to dry mass units.

Variables	Relationship	r^2	F	df	p-value
Fungus:					
Wet Mass ($M_{f,w}$, g), Area (A_f, cm^2)	$M_{f,w} = 0.1125529(A_f) - 0.629872$	0.9017	174.3	1, 19	5.089×10^{-11}
Dry Mass ($M_{f,d}$, g), Area (A_f, cm^2)	$M_{f,d} = 0.042546 (A_f) - 0.045940$	0.7054	45.5	1, 19	1.913×10^{-6}
Workers:					
Estimated (N_{ew}), Actual Numbers (N_{aw})	$N_{aw} = 1.5396(N_{ew}) - 12.7037$	0.897	165.4	1, 19	7.95×10^{-11}
Wet Mass ($M_{w,w}$, g), Estimated Number (N_{ew})	$M_{w,w} = 0.0037496 (N_{ew}) + 0.0008464$	0.8311	93.48	1, 19	9.03×10^{-9}
Dry ($M_{w,d}$, g), Wet Mass ($M_{w,w}$, g)*	$M_{w,d} = 0.398667 (M_{w,w}) + 0.018683$	0.9846	15833	1, 247	$<2.2\times10^{-16}$

*Conversions were based on masses of individual workers instead of entire colonies.

43

Table 2

Summary of linear relationships between colony mass and other colony growth characteristics.

Characteristic	Slope (+ 95% Confidence Interval)	Intercept (+95% Confidence Interval)	r^2	F	df	p
Colonies fed Palo brea leaves						
Leaf Mass Collected (M_l, dry g day^{-1})	0.427 (0.306, 0.547)	-0.021 (-0.050, 0.008)	0.74	55.4	1, 18	6.8×10^{-7}
Population Growth ($\Delta M_{w,d}$, dry g day^{-1})	0.0202 (0.008, 0.032)	-0.0010 (-0.0038, 0.0019)	0.39	13.0	1, 18	0.0020
Fungus Growth (ΔM_f, dry g day^{-1})	0.0606 (-0.028, 0.043)	-0.0006 (-0.0159, 0.0147)	0.14	4.0	1, 18	0.062
Refuse Produced (M_r, dry g day^{-1})	0.0197 (-0.010, 0.021)	-0.0010 (-0.0045, 0.0025)	0.27	8.1	1, 18	0.011
Colonies fed Polenta						
Polenta Mass Collected (M_p, dry g day^{-1})	0.120 (0.011, 0.229)	0.006 (-0.015, 0.028)	0.14	5.1	1, 25	0.032
Population Growth ($\Delta M_{w,d}$, dry g day^{-1})	0.0140 (0.010, 0.018)	-0.0005 (-0.0014, 0.0003)	0.63	44.5	1, 25	5.5×10^{-7}
Fungus Growth (ΔM_f, dry g day^{-1})	-0.016 (-0.055, 0.024)	0.009 (0.001, 0.017)	0.01	0.66	1, 25	0.42
Refuse Produced (M_r, dry g day^{-1})	0.0132 (-0.0003, 0.0267)	0.0009 (-0.0018, 0.0037)	0.11	4.08	1, 23	0.056

44

Table 3

Summary of regression statistics between residuals from size regression analyses

Comparison	r^2	F	df	p
Colonies fed Palo brea leaves				
M_l versus ΔM_f	0.32	10.0	1, 18	0.005
$\Delta M_{w,d}$ versus ΔM_f	0.25	7.3	1, 18	0.015
M_l versus $\Delta M_{w,d}$	0.15	4.4	1, 18	0.050
Colonies fed Polenta				
M_p versus ΔM_f	0.56	34.0	1, 25	4.5×10^{-6}
$\Delta M_{w,d}$ versus ΔM_f	0.18	6.6	1, 25	0.016
M_l versus $\Delta M_{w,d}$	0.10	3.8	1, 25	0.062

Chapter 3

LEAFCUTTER ANTS ADJUST FORAGING BEHAVIOR AND ACTIVITY

LEVELS IN RESPONSE TO CHANGES IN FUNGUS GARDEN SIZE

Individual organisms respond to changes in energetic and nutritional

stores by altering multiple aspects of their biology, ranging from shifts in

digestion and metabolism to changes in foraging and feeding behavior. Similarly,

one would expect social groups such as social insect colonies to have multifaceted

regulatory responses to changes in levels of stored resources. To do so they must

reallocate the effort of individual workers across tasks, with potential trade-offs

between allocation to food collection with colony growth and maintenance.

Because they are distributed systems, this requires the coordination of multiple

individuals, each of whom possesses only local information about the colony's

state. Here, I focus on the question of how social insect colonies regulate work

allocation and effort around resource availability, by examining how the worker

populations within leafcutter ant colonies respond to changes in levels of their

primary food source, the colony's fungus.

Previous work in honey bees, bumble bees, and wasps supports the notion

that colonies adjust foraging effort in response to levels of stored resources

(Cartar, 1992, Fewell and Winston, 1992, Fewell and Bertram, 1999, O'Donnell,

1998). But performance of one task such as foraging cannot change

independently of allocation to other work requirements (Gordon, 1996); increases

in foraging effort are likely to have consequences for the performance of other tasks such as nest maintenance and brood production, as well as consequences for overall colony activity levels. Regulation of task allocation is hypothesized to be driven by a combination of intrinsic variation in individual task preference, and variation in stimulus levels for different tasks (Julian and Cahan, 1999, Julian and Fewell, 2004). Task stimuli may consist of indirect environmental cues, as in the case of stored resources, or they can be social interactions, such as recruitment signals that stimulate individuals to perform specific tasks. Collectively, these mechanisms can generate flexible division of labor at the level of the colony, allowing the colony to respond to changing conditions, such as food availability.

Leafcutter ants are a useful system for focusing on the interface between task allocation and resource availability because their fungus gardens function as a single stored, internal resource that is easily quantified. Fungus growth and worker behavior are regulated relative to each other; fungus growth patterns are dependent on the foraging and cultivation behavior of the ants (Chapter 1, Bass and Cherrett, 1996, Bass, 1997) while ant foraging decisions are driven by factors affecting the condition of the fungus garden (Ridley et al, 1996, Bass, 1997, North et al, 1999, Weser, 2005, Herz et al, 2008). This sophisticated feedback system between the ants and fungus is critical; if ant population growth outstrips fungal production, the colony will die.

Acromyrmex versicolor leafcutter colonies maintain stable fungus garden sizes relative to worker numbers present in the colony as they grow above 50

workers (Chapter 1). The stability of this relationship suggests that the presence of homeostatic feedback mechanisms between ant and fungus populations, likely driven by changes in the workers' behavior based on the colony size and fungal condition (Kang et al, *submitted*). In this study, I manipulate the levels of fungus available to colonies, to test the hypothesis that the workers are sensitive to and regulate the size of the fungus garden via changes in task allocation to maintain levels that permit colony maintenance and growth. If colonies regulate overall colony task allocation around fungal production, as expected by a trade-off model, then I would expect changes in fungus availability to cause corresponding shifts in task allocation. Particularly, I would expect that conditions of low fungus availability would require upregulation of behaviors directly promoting fungal growth, with potential downregulation of other activities. Because the fungus serves as the only food source in these colonies, the study also allows consideration of whether fungus availability energetically constrains work output, such that colonies with lower fungus levels show lower overall activity while increasing the amount of fungus (and thus energy availability) should allow increased activity.

Methods

   Colonies. Newly mated *Acromyrmex versicolor* queens were collected from underneath mating swarms on 19-20 July 2007 along E. Sunrise Dr. in northern Tucson, AZ. This species normally forms pleometrotic associations

(Rissing et al, 2000), so queens were housed in groups of three to establish laboratory colonies for study. The nest containers consisted of two circular plastic dishes (Pioneer Plastics; internal dimensions: 8.4 cm diameter x 3.3 cm height), connected with a short piece of vinyl tubing (1/4-inch inner diameter). The bottom of one container was lined with dental plaster (Darby Dental) to serve as a fungus-growing chamber, while the second chamber served as a foraging arena. As colonies grew, the available space for the fungus garden was expanded by attaching additional fungus chamber dishes to the nests with tubing. High humidity levels were maintained in fungus chambers by watering the plaster through a small hole in the lid (1.5 mm diameter) once or twice a week with two to four mL of deionized water. Colonies were given *ad libitum* polenta (coarsely ground cornmeal) and palo brea leaves (*Cercidium praecox*) in the foraging arena, and were allowed to grow until they reached sizes of at least 100 workers, with a mean worker population size of 781 workers by the start of the experiment and a size range of 118-2078 workers (Figure 10). A total of ten colonies were tested two at a time over the course of three months, so they were between the ages of nine months to one year old at the time of testing.

At the start of each round of the experiment, each colony's workers were counted and fungus gardens were weighed to assess baseline relationships between worker population size and fungus garden biomass. To minimize desiccation and exposure to foreign pathogens, workers were carefully removed from the fungus garden with flame-sterilized soft forceps and a probe, and were

individually counted and placed into a separate holding container. Work was conducted over a container lined with a large slab of moistened plaster. Fungus pieces were kept as intact as possible during this procedure and were placed into 50-mL tubes for weighing. Between uses, the container used to separate fungus and workers was sterilized with 95% ethanol and then dried to reduce potential microbial contamination.

To observe individual worker behavior, approximately 60 workers were haphazardly withdrawn from the holding container and individually paint-marked with unique four-color combinations of Pactra Racing Finish paint on the head, thorax, and abdomen. Once ants were marked and fungus gardens weighed, the entire colony was re-assembled in a fresh set of nest containers, consisting of two fungus chambers connected to a foraging arena. The new nest containers were large enough to include room for fungal growth in an empty fungus chamber and also permitted observations of ant activities within as much of the fungus garden as possible. The colony was then allowed to recover for 24 hours.

Fungus garden manipulations and behavioral observations. Colonies were paired by size for observations and fungus manipulations. This made it possible to transfer an appropriate amount of fungus from one colony into the other to manipulate garden size, while also ensuring that fungus gardens were in direct contact with ants over the experiment's duration. During manipulation, approximately 50% of one colony's fungus garden, as determined by its mass, was removed and transferred into the other colony using the same methods as

described for initial censuses, above, with the exception that only the transferred fungus material, not the entire fungus garden, was taken out of the colony. A fraction of leafcutter ant brood are distributed in the fungus garden in such a way that it is difficult to remove them without significant disruption of fungal material (i.e. damaging hyphae). Therefore, a small fraction of brood (< 20% of a colony's brood pile) were transferred with the fungus material.

I observed worker behavior in colonies over a period of eight days following initial painting and recovery, to determine how colonies responded to short-term changes in fungus garden availability. The first two days of observations were used to obtain baseline behavioral measurements (20 scans total), with colonies retaining extant worker-to-fungus relationships. At the end of the second day, the fungus garden of one of the two paired colonies was reduced by approximately half and transferred into the paired colony, increasing its fungus garden to roughly 150% of baseline mass (Figure 10B). Manipulated colonies were then observed for four days (40 scans total). Then, to measure carryover effects, fungus gardens were restored to their initial sizes by transferring back as much of the original garden as possible, and two more days of observations were completed (20 scans).

I performed ten scan sampling periods per day between the hours of 0800 and 1800 during which I recorded workers' behaviors and locations. An hour before observations began, colonies were given fresh palo brea leaves, to control for the effects of introducing fresh leaf material on foraging cycles (Muscedere et

al, 2011). Behaviors of marked workers were measured with a scan-sampling method, where efforts were made to find close to all of the marked workers during each scan period. Once workers were located and identified, their behavior was observed for several seconds and recorded. As many different types of behavior as possible were identified during scans; behaviors were then pooled into task groups or other activities as appropriate by their co-occurrence in sequence (for example moving items to trash pile and trash sorting) or by similar focus of effort (on fungus, brood, leaf material, or waste; Table 4; 37 distinct behaviors were seen). A total of 34,901 behaviors were observed over the course of the experiment, out of a total of 39,120 possible observations, translating into an average of 1163 observations per colony over eight days. Individual ants were observed 89.2% of possible times across scan samples.

Data analysis. Data analysis was conducted with the software program R, version 2.11.1 (R Development Core Team, 2010). Scan observations were first categorized into eight behavioral classes (Table 4): inactive, walking, foraging, fungus care, brood care, grooming/communicating, trash removal, and remaining miscellaneous behaviors that were not otherwise clearly classifiable. Individual worker observations were pooled within each colony for each behavioral class within each of the three time periods (Pre-manipulation, Manipulation, and Post-manipulation). To compare overall colony activity levels across different phases of the experiment, the four tasks of foraging, fungus care, brood care, and trash removal were combined into an overall measurement of task

52

performance. To measure changes in task allocation, the relative frequencies of each of these four tasks within the task performance category were calculated. No major changes in walking or rates of grooming or communicating were observed, so these behaviors were not included in subsequent analyses.

Individual colonies varied considerably in intrinsic inactivity and task performance levels. Therefore, to assess whether the experimental treatments affected behavior over the course of the experiment, linear mixed-effects models were constructed, using colony identity as a random factor (lme4 package, Bates and Maechler, 2010). Log-likelihood ratios of models with "experiment phase" as a fixed factor and colony identity as a random factor were then compared against models where the "experiment phase" term was dropped. Where significant differences were identified between models, the model with lower Akaike Information Criterion and Bayesian Information Criterion values was selected. Post-hoc differences between experimental phases were determined by computing univariate asymptotic p-values for each phase with the "cftest" function in the "multcomp" package (Hotharn et al, 2010). Lack of a significant difference between models was taken to indicate that the experimental treatment did not affect that particular behavior.

Results

Activity Budgets and Colony Activity Levels. Overall, observations of inactivity varied considerably between colonies and across observation periods,

ranging between 5%-40% of total observations. Task performance also varied

between 5- 40% of total observations per colony-treatment phase (mean task

performance=22.6%). Colonies significantly increased activity in response to

increased fungus gardens, and continued to show increased activity following the

return to baseline (Figure 11; log-likelihood χ^2=13.5, df=2, p=0.001). These

colonies also increased overall task performance rates (log-likelihood χ^2=12.4,

df=2, p=0.002). In contrast, there were no consistent patterns to variation in

inactivity or task performance in response to decreased fungus gardens (Figure

11; for inactivity, log-likelihood χ^2=5.3, df=2, p=0.072; for task performance,

χ^2=3.7, df=2, p=0.16).

The majority of task activities across all colonies were directed towards

fungus tending (mean fungus-tending frequency = 0.546±0.125 SEM), followed

by foraging (mean frequency = 0.261±0.107 SEM). Refuse removal and brood

care comprised the remaining 10.4% (±9.6%) and 9.0% (±5.8%) of tasks,

respectively. Colonies with increased fungus increased their rates of fungus care

and refuse removal but did not make consistent adjustments to foraging or brood

care (Figure 12; fungus care log-likelihood χ^2=8.2, df=2, p=0.017; refuse χ^2=14.5,

df=2, p=0.0007; foraging χ^2=4.5, df=2, p=0.103; brood care χ^2=2.3, df=2,

p=0.321). In contrast, colonies with decreased fungus significantly increased

foraging rates (χ^2=11.4, df=2, p=0.003, and decreased fungus and brood care

(χ^2=7.9, df=2, p=0.019; χ^2=6.8, df=2, p=0.033, respectively). Rates of refuse

removal in these colonies did not change in a consistent manner (χ^2=1.7, df=2, p=0.422).

Discussion

Although models of optimal foraging often consider behavioral decisions within the primary context of food intake, in reality organisms have to multitask; they must make decisions about allocation of effort to behaviors that enhance food intake or stores around the need to perform other tasks related to growth, reproduction and maintenance. Social insect colonies face the same allocation decisions, but can respond by distributing workers in different frequencies or rates across different tasks. Here I ask about the mechanisms by which leafcutter colonies distribute work in relation to food availability by examining shifts in total work output and task allocation as levels of their primary resource - their fungus garden - are changed. Colonies shifted both activity levels and task performance in response to manipulations of fungus garden availability, but responded in different ways depending on whether size of the garden was increased or decreased relative to baseline. Specific behavioral responses were especially apparent in colonies with decreased fungus gardens, which adjusted by upregulating foraging and leaf-processing rates, the two activities directly promoting growth of the fungus garden. There was not, however, a consistent downregulation of foraging in response to increased fungus availability.

55

Colonies integrated changes in task allocation with variation in overall activity levels. In particular, colonies offered high levels of fungus had higher overall task performance without necessarily adjusting allocation across tasks. These coupled results suggest food availability may impose some limitation on metabolic or energy use; when food availability falls, colonies reallocate across tasks to build food reservoirs without increasing total energy cost. When faced with higher food availability, colonies convert it to increasing overall work output. If this is the case, we would expect that the activity levels would be downregulated as the fungus garden is reduced back to baseline levels.

Although the observed behavioral patterns fit well with a model that integrates fungus availability with task performance and activity levels, it remains possible that added fungus is not considered the same as fungus already present in the nest. While effort was made to pair colonies with genetically similar fungus gardens for garden transfers, and to separate the introduced fungus from the colony's extant fungal crop, the introduced fungus could still contain chemicals or olfactory cues that lead colonies to reject foreign fungus material (Poulsen and Boomsma, 2005). While this could potentially explain the observed increases in refuse removal rates, it does not completely explain the behavioral changes in these colonies, given that one colony also went so far as to move the majority of its brood onto the introduced garden. Heightened activity levels for colonies with increased fungus could be driven by either increased effort by individual workers, or the recruitment of normally inactive workers.

Food availability and mechanisms of division of labor. Clearly, fungus garden size plays a critical role in shaping the division of labor in growing colonies. A similar connection between food intake and the division of labor has been observed in honey bee colonies, which homeostatically regulate pollen stores around a setpoint to ensure adequate supplies for developing brood (Fewell and Winston, 1992, Fewell and Bertram, 1999). Although the colony-level response patterns are qualitatively similar for bee pollen stores versus ant fungus levels, the mechanisms underlying the responses could differ considerably because resources are used differently by the two species. Specifically, honey bees can decouple the collection and storage of resources used for colony growth (pollen fed to brood) from resources used to meet energy needs (nectar stored as a buffer against seasonal variation; Fewell and Winston, 1995). Adult bees do not directly consume pollen, so they must rely more strongly on external cues to make foraging decisions, through direct assessments of pollen stores, brood hunger levels, and interactions with other bees.

In contrast, both nutrients for growth and energetic needs are acquired through the fungus garden for leafcutter ant colonies (Wetterer et al, 2001). Therefore, for colonies with less fungus, individual hunger levels could be an important stimulus for increased foraging efforts, in addition to influences by brood, social interactions, signals from the fungus, or combinations of factors. Colonies with low brood levels and/or without fungus appear to downregulate foraging efforts and task performance (personal observations), supporting the idea

that both factors contribute to foraging decisions. Additionally, the highly coordinated nature of the onset of foraging suggests that social interactions guide colony foraging (Bollazzi and Roces, 2011). The factors triggering the initiation of foraging bouts in leafcutter ant colonies are as of yet unknown, but information-gathering and communication are prioritized over leaf load-size during early foraging stages. Similarly, Schafer et al. (2006) and Greene and Gordon (2007) demonstrated that social interactions modulate foraging activity in *Pogonomyrmex barbatus* seed-harvester ants: both returning patrollers and successful returning foragers stimulate further foraging efforts by colonies. Together with the present results, these findings suggest that leafcutter ants use, at minimum, a combination of social interactions and information from the nest environment to promote foraging activity, with the intensity of foraging potentially driven by information about the size and condition of the fungus garden.

Given the increase in foraging rates, it would be useful to determine if resource selection is also modulated by the urgency of foraging needs, as is the case in honey bees (Fewell and Winston, 1992) and *Messor barbarus* ants (Reyes-Lopez and Fernandez-Haeger, 2002). If foraging demand does influence resource selection, it could partially explain some of the puzzling leaf selection patterns observed in tropical leafcutter ant species, where colonies often bypass highly preferred plants in pursuit of others that are only slightly preferred (Roces, 2002). The question still remains of how foraging rates increased, whether they

correspond to increases in individual foraging efforts, recruitment of additional

workers to foraging-related tasks, or both, as occurs in bumblebees (Cartar, 1992).
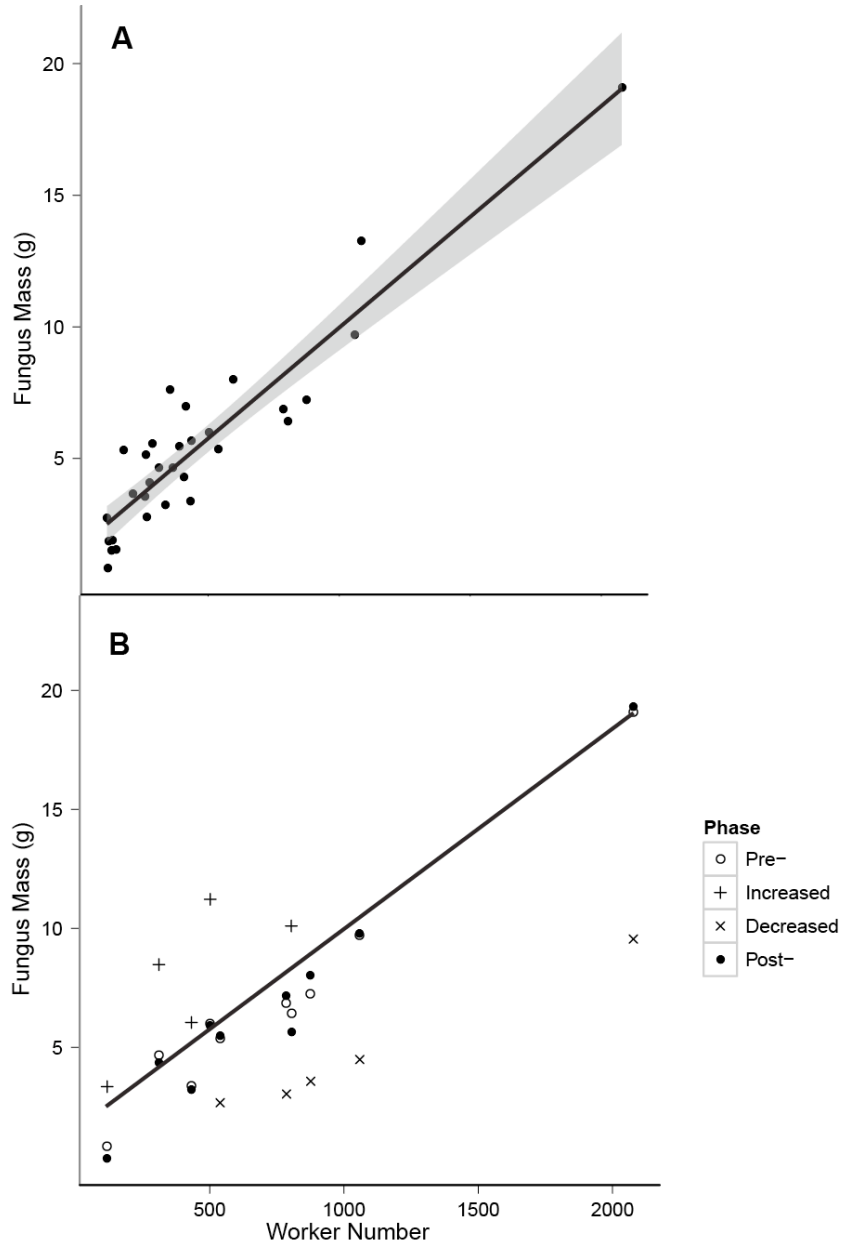
Figure 10. Relationship between the worker population size and the fungus garden mass. A) The natural relationship observed for laboratory colonies across the studied size range (Fungus Mass = 0.008 * Worker Number + 1.555, $F_{1,29}=181.4$, $p=5\times10^{-14}$, $r^2=0.86$). The shaded grey region represents 95% confidence intervals for the relationship B) Summary of the effects of the worker-fungus manipulation across the ten experimental colonies. The line represents the relationship depicted in part A.
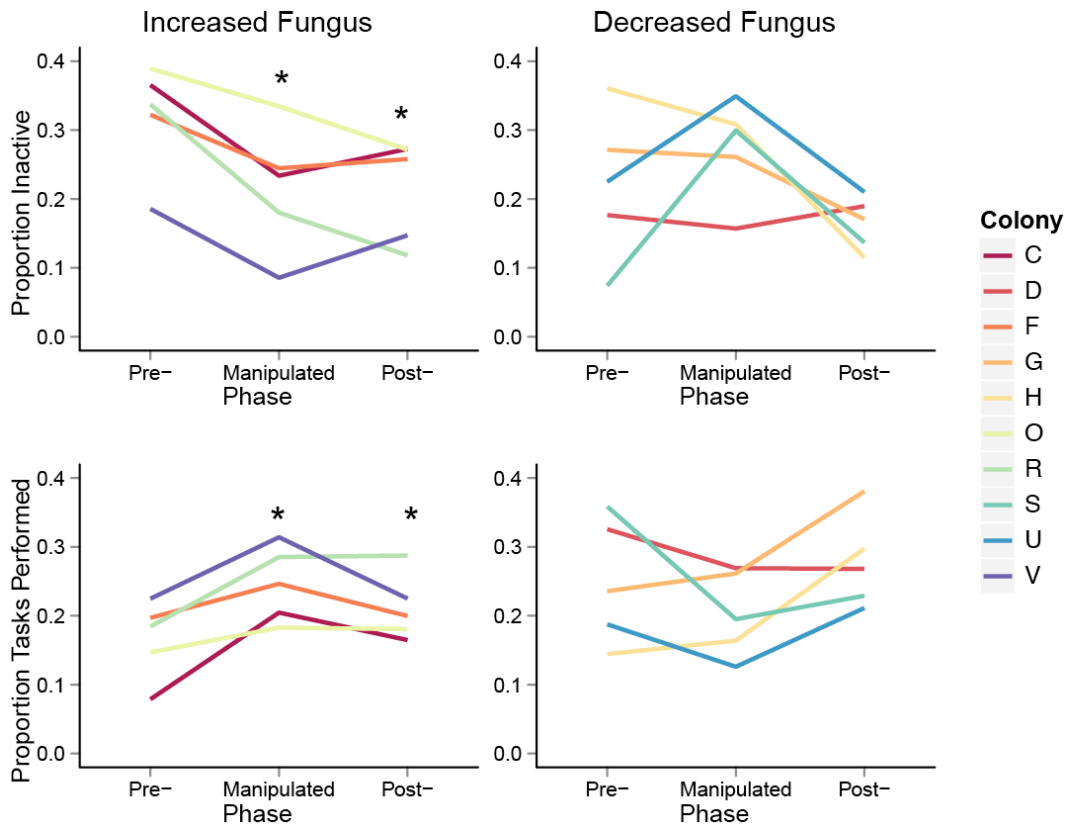
Figure 11. Frequencies of inactivity and task performance over the course of the experiment in colonies experiencing either decreases or increases in fungus garden areas during the "Manipulated" phase (n=5 colonies per treatment). Asterisks indicate time periods where there were significant shifts in frequencies, as indicated by significant differences in log-likelihood ratios between linear mixed-effects models that included and excluded a term for the experiment phase (univariate asymptotic $p<0.05$ for the coefficient estimate at that time period).
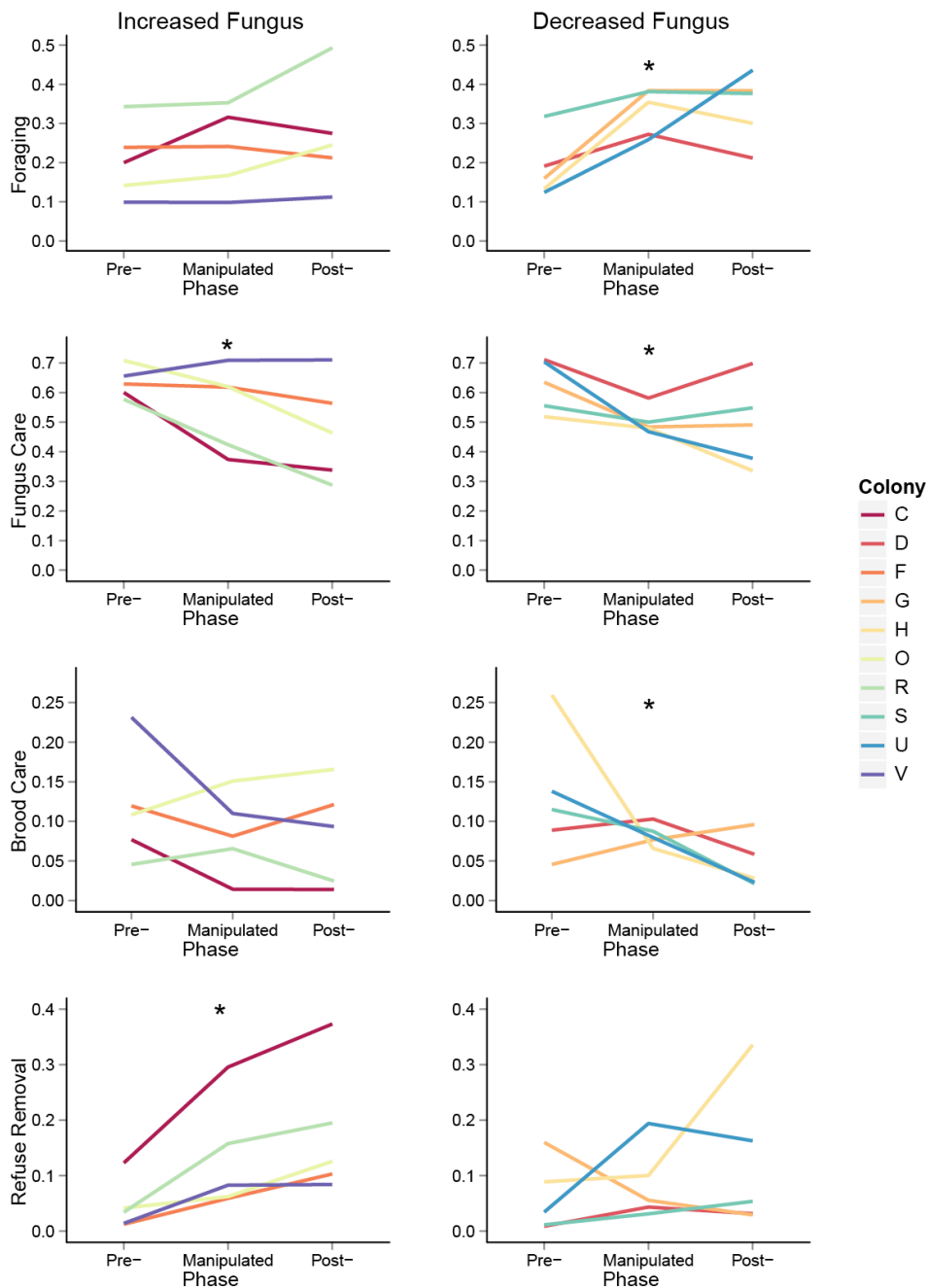
Figure 12. Relative frequencies with which four tasks were performed over the course of the experiment in colonies experiencing either decreases or increases in fungus garden areas during the "Manipulated" phase (n=5 colonies per treatment). Asterisks indicate times periods where there were significant (univariate, asymptotic p<0.05) shifts in frequencies, as indicated by significant differences in log-likelihood ratios between pairs of linear mixed-effects models that included and excluded a term for the experiment phase.

Table 4

Colony activity budgets over the course of the experiment.

Category	Behavior Description/List	Percentage of all observations
Inactive	Ant remained completely immobile	22.2%
Walking	Walking workers were not observed to be engaged in any specific tasks	25.6%
Foraging	Carrying leaf fragments; antennating leaves; licking leaves to prepare for addition to fungus garden; attaching leaves to fungus, or attaching fungus to freshly added leaf material	6.0%
Fungus-tending	Antennating fungus, licking fungus, or carrying fungus fragments	13.5%
Brood-care	Antennating, carrying, licking, or feeding brood	2.3%
Grooming / Communicating	Antennating, allogrooming, autogrooming, receiving grooming, or engaging in trophallaxis with other workers or the queen	21.1%
Trash removal	Carrying old fungus material, dead workers, plaster fragments, or other debris; licking or antennating trash	2.1%
Miscellaneous / Other	Antennating the plaster or container; licking or biting the plastic nest container or plaster; biting other ants (workers or brood); or carrying/being carried	7.3%

Chapter 4

EFFECTS OF CARBOHYDRATE, PROTEIN, AND PHOSPHORUS

SUPPLEMENTATION ON LEAFCUTTER ANT COLONY GROWTH

To successfully grow and reproduce, animals must obtain the appropriate

amounts and combinations of nutrients from the environment. Yet for most

organisms, the nutrients in available food do not match needs, requiring strategies

to deal with surfeits or deficits of key nutrients. As integrated units, growing

eusocial colonies must also effectively regulate growth around varying resources.

They do so in ways comparable to those used by individual organisms: selective

foraging, processing and allocating resources according to individual and colony

needs, and selectively absorbing or excreting different nutrients (Kay, 2004,

Dussutour and Simpson, 2008, Cook et al, 2010). These behavioral decisions and

physiological processes must be closely aligned with mechanisms of colony

growth, but the connection between resource intake and processing and growth

has not been well explored (Nonacs, 1991).

Here, I assess how colony performance is influenced by the relative

availability of potential limiting nutrients by providing a series of colonies with a

single food source but with varying nutrient ratios. When only one food is

available, an organism cannot balance food selections to achieve optimal intake of

multiple nutrients. Instead, if the available food does not contain an ideal nutrient

blend, the organism must compromise across potentially conflicting requirements,

overeating some nutrients while falling short for others (Raubenheimer and Simpson, 1993). Two ways in which organisms could compensate for nutrient limitation and achieve the same performance as on balanced foods include changes in feeding behavior and shifts in digestion (reviewed in Behmer, 2009). If the concentration of a key nutrient is diluted, organisms often respond by eating more food to reach the same total intake (Slansky and Wheeler, 1989, 1991). Some organisms also increase their digestive capacity, and/or retain food for longer to absorb more nutrients from it (Timmins et al, 1998, Raubenheimer and Bassil, 2007). In contrast, organisms cope with excess nutrients by eating less, absorbing less, and excreting more (Zanotto et al, 1993, Timmins et al, 1998).

Carbohydrates and proteins are often carefully regulated by insects because either can limit growth (Chapman, 1998). The influence of nutritional variation on colony growth has been directly assessed for carbohydrate and protein intake for two ant species, each of which employed specific coping methods to regulate growth in the face of imbalanced food availability (Dussutour and Simpson, 2008, Dussutour and Simpson, 2009, Cook et al, 2010). *Rhytidoponera metallica* workers mitigated variation in carbohydrate availability via compensatory foraging on an individual level; although more workers were recruited to solutions with higher sucrose concentrations, the amount of solution that each worker collected was inversely related to sugar concentration, indicating that colonies regulate sucrose intake around a specific nutritional target (Dussutour and Simpson, 2008). Interestingly, red imported fire ant workers were attracted

to, and collected more, foods with equal or moderately protein-biased ratios of protein to carbohydrates (Cook et al, 2010). In both species, however, food within the nest underwent further processing, such that colonies maintained constant carbohydrate and protein intake in the face of widely varying resources (Dussutour and Simpson, 2009, Cook et al, 2010).

In the above examples, colonies could directly assess and manipulate carbohydrate and protein intake levels. But in many systems this is not the case. Leafcutter ant colonies are of particular interest for expanding understanding of nutritional regulation and growth because of their distinct feeding ecology: colonies function as tri-trophic systems, with workers harvesting leaves and using them to grow a fungus garden, which serves as the colony's food source. The ants are choosy about the leaves they collect, tending to prefer young or wilted leaves that are less tough, higher in sugar and nitrogen, and lower in plant defense compounds, than surrounding vegetation (Cherrett, 1972, Hubbell et al, 1984, Berish, 1986, Mintzer, 1994, Vasconcelos and Cherrett, 1996, Howard, 1987, 1988, Wetterer et al, 2001, Meyer et al, 2006, Seal and Tschinkel, 2007). Leafcutter ant colonies are also highly polyphagous; laboratory colonies provided with single food sources often underperform colonies provided with multiple food types (Weser, 2005, Seal and Tschinkel, 2007, although see Tibbets and Faeth, 1999). This suggests that the ants normally use compensatory foraging methods to limit exposure to plant secondary compounds and to obtain a balance of nutrients.

While we know a fair amount about leafcutter ant foraging decisions and preferences, little is known about whether, or how leafcutter colony performance changes in response to variation in leaf nutritional quality. The few studies that have attempted to measure performance differences compared treatments where resources varied in quality along multiple dimensions simultaneously, including differences in nutrient content, moisture, and plant defense compounds (Tibbets and Faeth, 1999, Weser, 2005, Seal and Tschinkel, 2007). This makes it difficult to assess exactly how nutritional variation affects colony performance.

Nutritional effects on colony (worker) growth in leafcutter ants are directly mediated by nutrient effects on fungus garden growth. The leafcutter ant fungus is related to other fungal species that function as decomposers (Chapela et al, 1994), which adjust energy use around the relative availability of key nutrients, particularly nitrogen and phosphorus (Manzoni et al, 2010). Therefore, a full analysis of nutritional constraints on growth for this system should include changes in the relative availability of phosphorus, as well as protein (a nitrogen source) and carbohydrates (an energy source). If compensation in terms of energy use allows the composition of fungal tissues to be relatively constant, it could simply translate into differences in fungal growth rates under different forms of nutrient limitation, without affecting fungal quality. In contrast, if the leafcutter fungus selectively takes up and stores more abundant nutrients, differences in relative nutrient availability could be passed along to the ants, with the same kinds of consequences as outlined above.

The current experiments test for the effects of nutrient limitation in terms of the relative availability of carbohydrates, protein, and phosphorus, using a single food type (polenta) that can be directly supplemented with potentially limiting nutrients. Previous work indicated that polenta is lower in its relative amounts of carbon, nitrogen, and phosphorus as compared to palo brea leaves, another food source which is beneficial for colony performance (Chapter 2, Weser, 2005). If any one of these three dietary components limits colony performance, colonies are expected to respond to supplementation with altered foraging behavior, increased fungus growth rates, and increased population growth rates. Changes to foraging behavior will depend on the mechanisms colonies use to counteract nutrient limitation; if colonies engage in compensatory foraging, increasing a limiting nutrient would be expected to cause a decrease in the total amount of food collected. In contrast, a limiting nutrient could function as a phagostimulant, such that increased levels of that nutrient lead to an increase in foraging. Further, if colonies are able to selectively remove and excrete nutrients, limiting nutrients should appear in relatively lower amounts in the colony refuse material, while excess nutrients should occur at higher levels.

Methods

     Colonies. Newly mated *Acromyrmex versicolor* queens were collected as they were digging nests on 1 August 2010 in Tucson, AZ. *A. versicolor* queens usually establish colonies in groups (Rissing et al, 2000), so queens were initially

housed in groups of three to establish laboratory colonies. Nests were maintained

in a room with natural light levels from large windows. From November through

early March, room temperature was maintained at 25 degrees Celsius, to

approximate normal desert winter temperatures. Subsequently, the room

temperature was increased to 30° C to encourage growth for the final six weeks of

the experiment.

Lab nests were constructed from pairs of circular plastic dishes (Pioneer

Plastics; 8.4 cm diameter x 3.3 cm height, internal dimensions) joined with vinyl

tubing (1/4-inch internal diameter). One dish was lined with dental plaster (Darby

Dental) to serve as a foraging chamber, with the second chamber serving as a

foraging arena. Colonies were watered weekly with 1-2 mL of deionized water

during their establishment and throughout the experiment. Colonies were

provided with *ad libitum* polenta (coarsely ground cornmeal) and palo brea leaves

(*Cercidium praecox*) once a week, for the first three months of growth. After that

point, colonies were maintained on polenta only, as palo brea trees begin to lose

leaves at that time (November) and do not replace them until the following

February/March. When colonies reached an average size of 85 workers, colonies

with multiple surviving queens were subdivided into single-queen colonies, to

control for the effects of queen number on colony growth rates. Colonies were

then given an additional month to recover before dietary manipulations began.

Diets. Colonies were divided into four treatment groups, each of which

received a single diet over the course of the experiment: control, xylose, nitrogen,

69

and phosphorus (abbreviated as C, X, N, and P, respectively). Manipulations of carbohydrate, nitrogen, and phosphorus contents of the three experimental diets were based on the elemental composition of palo brea leaves, which possess higher levels of all three elements compared to polenta (Chapter 2), and are known to promote colony growth (Weser, 2005).

The leafcutter fungus primarily digests carbohydrates, including xylose, a predominant form of plant sugar found in leaves (Schiott et al, 2008, Moller et al, 2011). The ants themselves do not express enzymes for xylose breakdown (Gomes de Siquiera, 1998, Erthal et al, 2004), so xylose was used to alter the relative carbohydrate content of the polenta to match the percentage of potentially utilizable carbon of palo brea leaves. It was not possible to elevate net carbon content of polenta with a carbohydrate-based (non-sugar) carbon source, because pure carbohydrates contain a lower % carbon than polenta and palo brea. The xylose (X) diet was prepared by adding 13.5 g of xylose (Sigma-Aldrich) and 100 g of polenta to 400 mL of boiling deionized water. The nitrogen-supplemented (N) diet was created by adding 21.2 g Amisoy (soy protein acid hydrolysate, Sigma-Aldrich) per 78.8 g polenta to boiling water. For the phosphorus (P) diet, I added 1 g of potassium phosphate (dibasic, Sigma-Aldrich) to each 99 g of polenta. Each mixture was stirred at a moderate boil for four minutes to evenly mix the ingredients. The control (C) diet simply contained 100 g of polenta cooked in 400 mL of water without other additives. Each cooked diet was poured into a glass dish and placed in a drying oven at 70°C for two days to dry it

completely. Dried polenta was ground to a coarse mixture of heterogeneously-sized particles with a corn mill, weighed out, and provided to the colonies in a small weigh boat. At the end of each week, left-over polenta was removed and reweighed to measure how much had been collected by the ants, and was replaced with freshly prepared polenta.

Colony worker populations and fungus gardens were measured weekly for 14 weeks using noninvasive methods. Worker numbers were visually estimated by scanning the nest and counting individuals with a hand counter. Fungus garden areas were measured for weeks 1, 11, 12, 13 and 14. Fungus garden area, which correlates closely with fungus garden mass (Chapter 1), was measured from overhead photographs of the fungus chamber.

Small colonies produce refuse slowly, so refuse piles were collected during weeks 5, 10, and 14. Dead workers were hand-sorted from the fungus material and counted. The fungus refuse material was dried at 60°C for three days and then weighed. After total refuse dry mass was measured, refuse material was homogenized to a fine powder with a ball mill (Spex 8000D Dual Mixer/Ballmill, Metuchen, NJ). The total carbon and nitrogen contents of refuse samples were measured using the same methods as described in Chapter 2. Phosphorus contents of the refuse from a subset of the control and P-enriched colonies were measured for the second and third collection dates, again with the methods described previously (Chapter 2).

71

Statistical analyses. Many of the factors examined in this study are influenced by colony size, which ranged between 13-145 workers at the beginning of the experiment, and from 23 to 313 workers by the end. Ant-fungus dynamics are unstable in colonies with fewer than 50 workers (Chapter 1), so four colonies with fewer than 50 workers at the end of the experiment were excluded from analysis. Where appropriate, measures were corrected for differences in colony size by dividing the measure by the total number of initial adult workers in the colony. Developing brood make up a small fraction (< 3%) of a colony's biomass and are difficult to measure, so they were not included in colony size corrections. The number of workers is strongly correlated with the colony's total worker mass (Chapter 2).

In general, overall measurements made across the experiment were compared across treatment groups with analysis of variance, and post-hoc comparisons were made with Tukey's Honestly Significant Difference (HSD), using the software program R, version 2.11.1 (R Development Core Team, 2010). Measurements of colony dynamics throughout the course of the experiment represent within-subjects repeated measurements, so where relevant, linear mixed-effects (lme) models were constructed with "colony" as a random effect, and with "time" and "treatment" tested as fixed-effect factors. The appropriate model was selected by comparing model log-likelihood ratios between models with the factor "treatment" present and dropped, and choosing the more parsimonious of the two models as indicated by lower AIC and BIC indices

(package "lme4," Bates and Maechler, 2010). Where this method is used, it is described in the results as an "lme comparison", and the outcome of the log-likelihood ratio comparison is presented as a chi-square statistic and associated p-value. In cases where the "treatment" term was significant, post-hoc comparisons between treatments were assessed by examination of asymptotic p-values with the function "cftest" from the package "multcomp" (Hothorn et al, 2008).

Colonies in the amisoy (N) treatment initially contained slightly fewer workers than colonies in the other three treatment groups (Figure 13). This pattern remained consistent throughout the experiment, so, where appropriate, between-colony comparisons were made by dividing the relevant metric by the initial colony size (i.e. the initial number of workers).

Results

To characterize colony responses to the four dietary treatments, I compared colony survival, foraging rates, fungus growth, worker population growth rates and refuse production across treatments. If any of the three tested nutrients is limiting, supplementation with that nutrient should lead to shifts in foraging (either an increase or decrease), higher fungus growth rates, and/or faster worker population growth. Further, that nutrient is expected to show up in lesser or equal relative amounts in refuse materials.

Colony Survival, Foraging Rates and Fungus Growth. For leafcutter ants, the complete loss of the fungus garden leads to the imminent death of the

colony. In the final week of the experiment, three out of the 21 colonies from the amisoy (N) treatment experienced complete losses of their fungus gardens. The three colonies had worker population sizes of 86, 89, and 168 workers at the time of garden loss. These colonies were removed from subsequent analyses. In contrast, no colonies from the other treatment groups lost fungus gardens.

To measure overall foraging rates, we compared the amount of polenta collected each week for colonies in each of the four treatment groups. Colonies provided with the xylose (X) diet collected more polenta per worker over the course of the experiment, compared to the other three treatments (Figure 14; ANOVA for total amount collected, $F_{3,66}=27.4$, $p=1.2 \times 10^{-11}$). Similarly, to assess if the differences in foraging rates translated into changes in fungus growth dynamics, we compared changes in fungus garden sizes from the first week to the final week of the experiment. Dietary treatments also significantly affected fungus growth rates (Figure 15; lme model comparison, $\chi^2=12.8$, df=3, p=0.0052). When fungus growth rates were corrected for differences in colony size by dividing by the final number of workers, size changes were found to be lowest for colonies in the amisoy (N) treatment, intermediate for control and P-enriched colonies, and highest for xylose-enriched colonies (Figure 15b; ANOVA for overall size-corrected changes in fungus area with Tukey's HSD, $F_{3,71}=7.1$, p=0.00031).

Worker Population Growth and the Worker-to-Fungus Ratio. To determine if nutrient limitation of fungus garden growth affected worker

population growth, we compared worker population sizes between treatments. Overall, colony worker population growth rates were similar across all four treatments (Figure 13; lme model comparison χ^2=7.4, df=3, p=0.059). Worker population sizes stayed relatively constant across the first eight weeks of the experiment, and then, following the temperature increase, colonies in all four treatment groups grew larger over the last six weeks (lme model comparison, χ^2=1171.8, df=13, p<2.2x10^{-16}). When colony growth rates were corrected for worker population size, growth rates were best explained by a simplified model that included time and the colony identity but not treatment group as factors (Figure 13b; lme model comparison, χ^2=2.1, df=3, p=0.56).

Overall, there was a trend towards increasing worker-to-fungus ratios across the last four weeks of the experiment, relative to initial conditions, but ratios did not differ between treatments (Figure 16; lme comparison for treatment term, χ^2=3.6, df=3, p=0.31, lme comparison for time, χ^2=133, df=4, p<2.2x10^{-16}).

Refuse Production and Elemental Composition. First, to determine if a relationship existed between colony size and the amount of refuse produced, I performed linear regressions of colony size versus refuse production for each of the three periods where refuse was collected. Larger colonies produced proportionally more refuse across the first two time periods, although colony size only explained ~17% of the variation in refuse production (Figure 17; linear regressions; first five weeks: $F_{1,42}$=11.4, p=0.0016, r^2=0.19; next five weeks: $F_{1,44}$=8.4, p=0.0057, r^2=0.14). When I then tested to see whether a relationship

existed between the amount of refuse produced and experimental treatments, I found no difference in per-worker refuse production between treatments during these time periods (ANOVA for first five weeks: $F_{3,40}$=1.3, p=0.30, next five weeks: $F_{3,40}$=0.36, p=0.78). The relationship between colony size and refuse production was not significant for the final sampling period (Figure 17; $F_{1,44}$=0.44, p=0.51, r^2=0.01). Per-worker refuse production rates remained similar between treatments during this time period (Figure 17; $F_{3,42}$=2.2, p=0.10).

The elemental composition of the refuse material could serve as an indicator of limiting resources, as well as indicating the degree to which different nutrients have been used by the colony. Refuse carbon content increased across sampling periods, but was only marginally different between treatment groups (Figure 18a; lme for treatment, χ^2=7.1, df=3, p=0.070; lme for time, χ^2=12.7, df=2, p=0.0017). Over the course of the experiment, refuse produced by colonies in the amisoy treatment increased in N content, while N content remained constant for the other treatments (Figure 18b; lme for treatment, χ^2=93.3, df=3, p<2.2×10^{-16}; lme for time, χ^2=16.0, df=3, p=0.00034). This additionally led to a lower refuse C:N ratio for amisoy colonies, as compared to refuse from colonies in the other treatments, so that, by April, the C:N ratio of refuse in amisoy colonies was similar to the C:N ratio of the polenta provided to them (Figure 18c; lme comparison for treatment, χ^2=72.5, df=3, p<1.3×10^{-15}; lme comparison for time, χ^2=14.3, df=2, p=0.00077). At the same time, C:N in refuse increased for colonies in the xylose treatment.

Despite a threefold increase in polenta phosphorus content for colonies receiving the P-enriched diet, refuse P contents were the same for control and P-enriched colonies across the two months where P contents were measured (Figure 19a; for treatments: $F_{1,44}=0.0003$, p=0.99; for month: $F_{1,44}=2.7$, p=0.11). Interestingly, P contents of refuse samples were higher than P contents of the polenta provided for both control and P-enriched treatments, most likely due to selective removal of other nutrients from the polenta. The P contents of refuse samples measured here were similar to refuse P contents measured in a previous study as well (Figure 19a; Chapter 2). Refuse C:P ratios were also the same across control and P-enriched treatments (Figure 19b; for treatments: $F_{1,43}=0.060$, p=0.81, for month: $F_{1,43}=3.2$, p=0.080). Refuse N:P ratios were the same across treatments, but were collectively lower in the second measurement period, compared to the first (Figure 19c; for treatments: $F_{1,43}=0.11$, p=0.75; for month: $F_{1,43}=10.7$, p=0.0021).

Discussion

This experiment tested whether leafcutter colony growth and performance were influenced by nutrient limitation, by testing for the effects of carbohydrate, protein, and phosphorus supplementation on colony fungus gardens and worker populations. The observed patterns for foraging, fungus growth, and refuse composition empirically demonstrate that fungus growth is primarily carbohydrate-limited, and not limited by nitrogen or phosphorus availability. The

positive effects of carbohydrate supplementation and negative effects of protein supplementation on the fungus garden further indicate that colonies benefit overall from diets with higher carbohydrate:protein ratios. A similar form of carbohydrate limitation has been suggested for leafcutter ant colonies in the field, which produce refuse that is higher in nitrogen and phosphorus than surrounding leaf litter (Haines 1978, Hudson et al 2009), and whose workers are more responsive to a leaf's sugar content than to its amino acid content (Meyer et al 2006). As shown here, ants that collect leaves with higher carbohydrate content promote better growth and nutrient utilization by the fungus garden, leaving excess nitrogen and phosphorus behind in waste material.

Evidence against nitrogen limitation. Amisoy-supplementation led to decreased foraging rates, slowed fungal growth, and decreased colony survival, suggesting that either extra protein or the lower carbohydrate:protein ratio of the diet negatively impacted the fungus, which secondarily negatively affects the ants. The fact that these colonies had smaller fungus gardens coupled with larger amounts of refuse and higher refuse N and C levels suggests that the additional protein caused problems when it was overcollected. High refuse C could mean that colonies are prematurely discarding fungus garden pieces, before using all of the potentially utilizable resources. This situation is comparable to what happens to *Drosophila* feeding on high-protein, low-carbohydrate diets; flies consume both protein and carbohydrates to reach a target caloric intake, but on a high-protein diet overconsumption of protein leads to decreased longevity (Lee et al,

78

2008). The data also fit with the idea that colonies attempted, unsuccessfully, to compromise across conflicting carbohydrate and protein requirements by decreasing foraging and increasing nutrient processing rates (Raubenheimer and Simpson, 1993, Zanotto et al, 1993).

Nutritional effects on colony growth. Colony fungus growth rates are tied to worker growth rates (Chapter 1). Clear positive effects of xylose and negative effects of nitrogen on fungus growth therefore lead to an expectation of similar patterns for worker population growth rates. Yet worker population growth rates were the same between treatments over the course of the experiment. This results, at least in part, from a lag between nutritional effects on fungus growth and subsequent effects on the workers - an effect which could not be completely captured based on the duration of the experiment. The lag between fungal growth and effects on the worker population growth can be illustrated by the nitrogen-supplemented colonies that completely lost their fungus gardens; these colonies will eventually inevitably die, but it can take several weeks to months before the workers and queen are all dead.

The effects of xylose supplementation on ant population growth also depends on how xylose stimulates fungal growth. Added xylose could lead to an increase the fungus garden's metabolic output, and it could simply contribute to increased fungal energy stores. An increase in metabolic rate could lead to an increase in the amount of fungus, without changing its quality, whereas increased fungal energy stores could potentially be passed along to workers.

79

Consequences of nutrient limitation for element cycling. Feeding

decisions made by herbivores can have important influences on ecosystem

functioning, either by changing the abundances of different plant species, or by

altering nutrient cycling patterns (Schmitz, 2008, Zhang et al, 2011). Tropical

leafcutter ants have been estimated to harvest one-fifth to one-sixth of the total

plant biomass consumed by herbivores, and leave behind nutrient-rich refuse piles

(Wirth et al, 2003, Hudson et al, 2009), so leaf selection decisions and colony

performance have important implications for the impacts of leafcutter ants on

nutrient cycling in tropical forests. In a field survey of attine colonies located in

pastures experiencing different levels of grazing pressure, Tadey and Farji-Brener

(2007) found no difference in colony densities across fields, but did find that the

nitrogen and phosphorus content of colony refuse piles corresponded with pasture

nutrient availability. We found a similar pattern for the effects of N

supplementation, but found that P supplementation did not alter refuse P content,

which was consistently higher than input P levels. These findings argue against

selective absorption of P, but do not clarify why refuse P contents were equal

across treatments. It could be that P-supplemented polenta has not transitioned

completely through the system, or the supplemental P could be lost via leaching

into the plaster lining the fungus chamber.

Altogether, correspondence between the observational field data reported

from other studies and the experimental outcomes from the present study suggests

that leafcutter ant colonies serve as a good controlled system for studying the

effects of dietary balance on growth and performance across trophic levels. The

fact that colonies demonstrate integrated nutrient balancing across trophic levels

within the colony also supports the use of leafcutter ant colonies as a study system

for comparison with nutrient balancing in human agricultural systems.
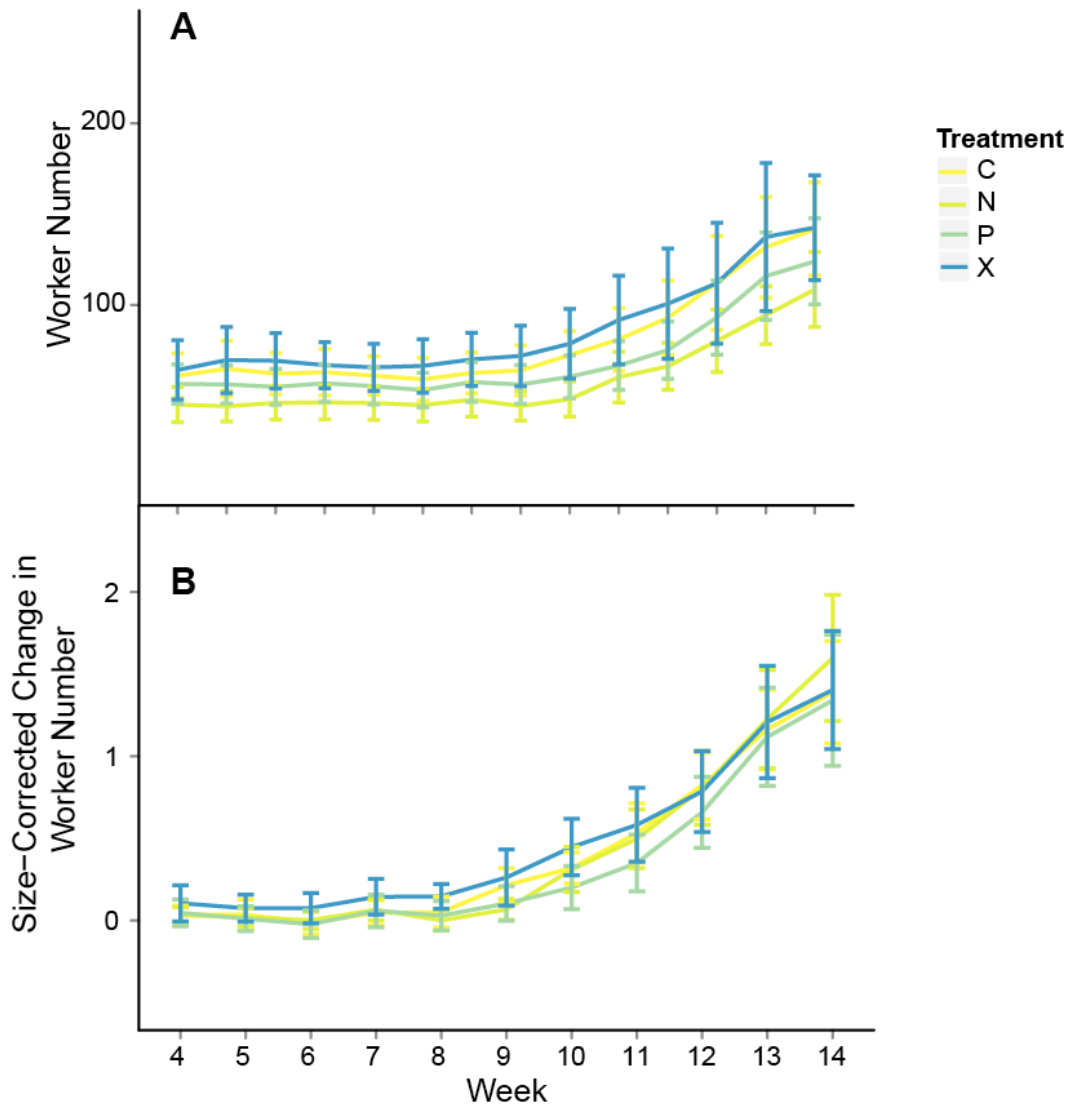
Figure 13. Summary of worker population growth over the course of the experiment in each of the four treatment groups (control - C, or supplemented with amisoy - N, potassium phosphate - P, or xylose - X). A) Mean worker number (±SEM, based on visual estimates) within colonies. B) Size-corrected changes in the worker population sizes, calculated by taking the difference in size at each week from the starting colony size, divided by the starting colony size. Amisoy-supplemented colonies were initially significantly smaller, but there were no differences between treatments in size-corrected changes in worker numbers.
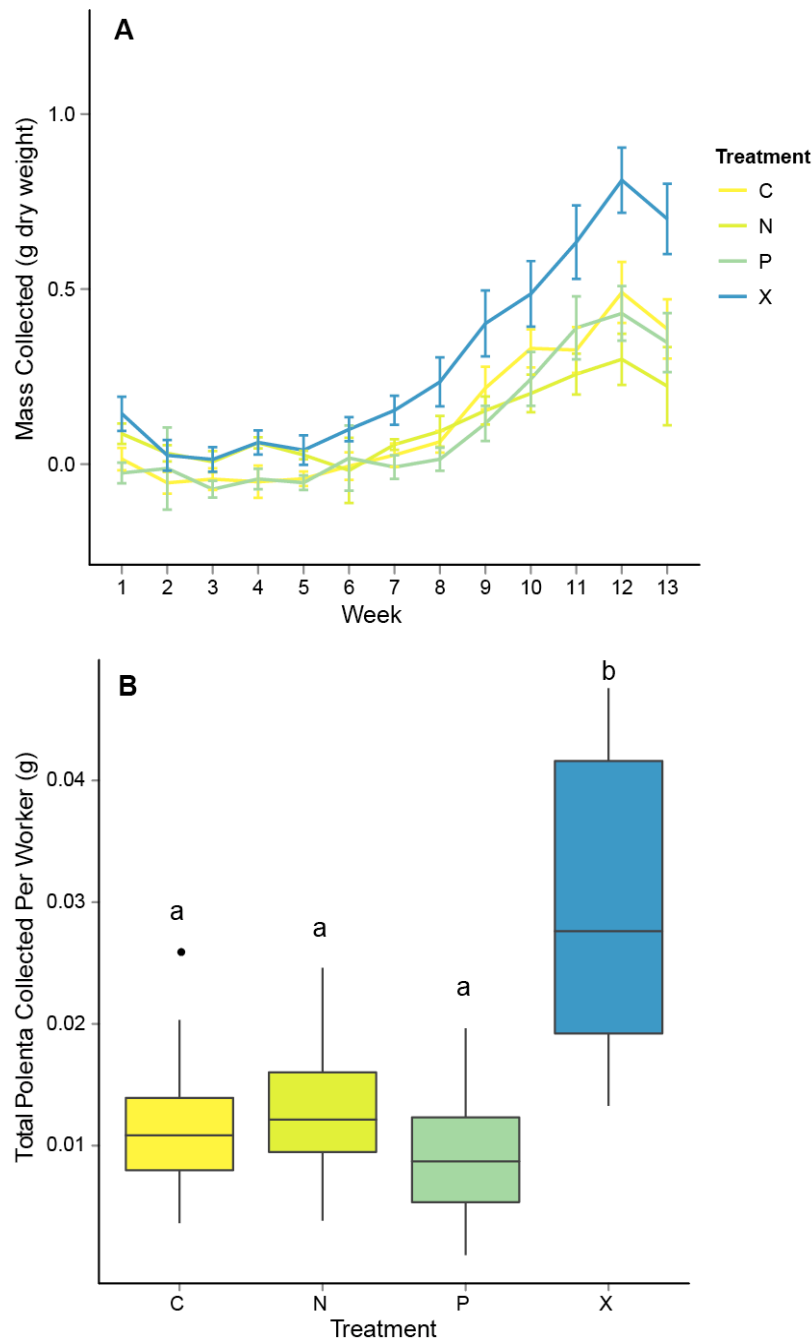
Figure 14. A) Mean amount of polenta (±SEM, in g of dry weight) collected each week, for colonies provided with polenta diets that were supplemented with xylose (X), amisoy (N), potassium phosphate (P), or control diets (C). The sample sizes for each treatment were 19, 17, 19, and 21 colonies, respectively, for X, N, P, and C. B) Boxplots of the total amount of polenta collected over the course of the experiment by colonies in each treatment group, corrected for total colony size. Treatments determined to be significantly different via post-hoc analysis (Tukey's HSD) are noted with different letters.
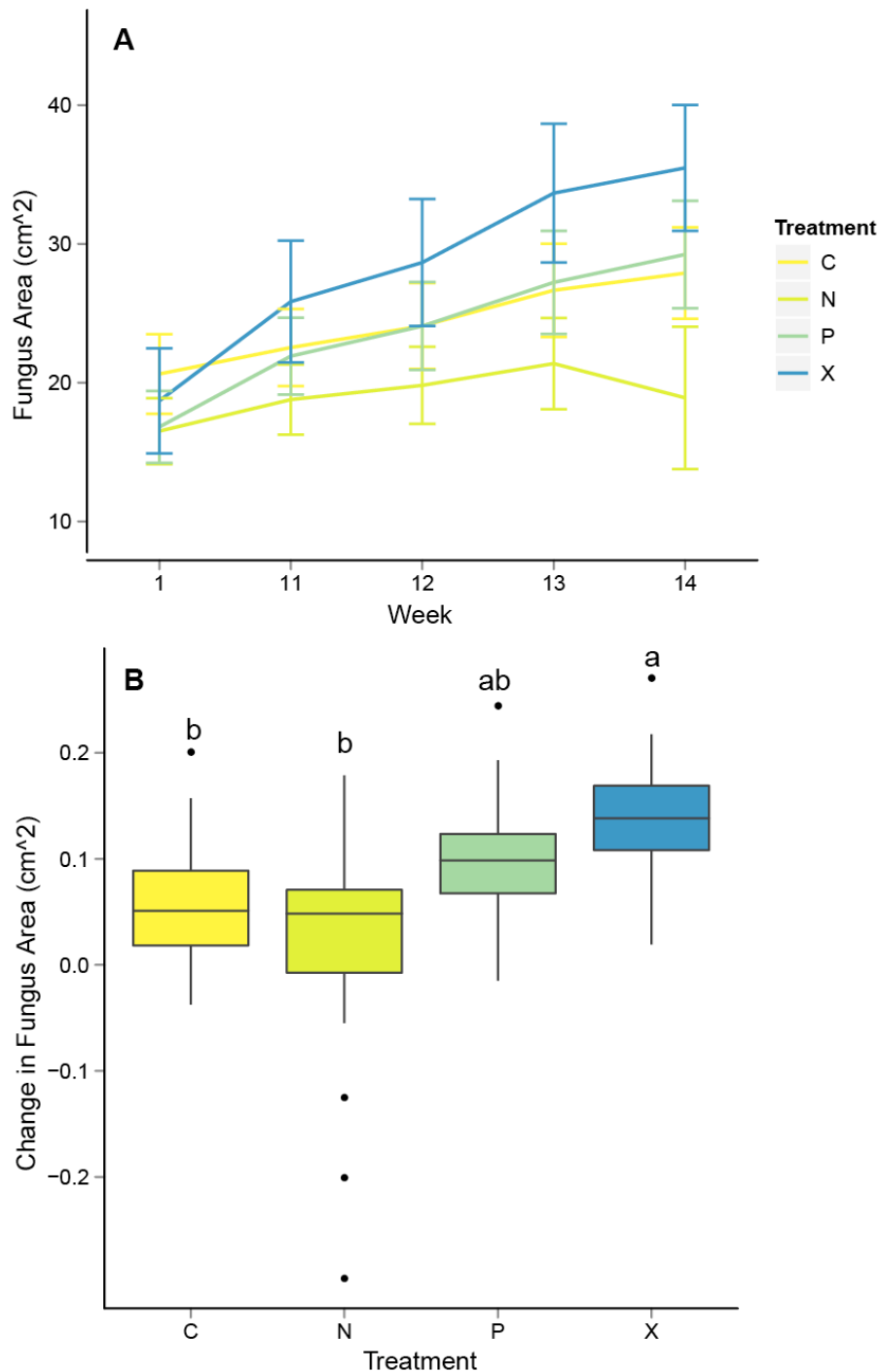
Figure 15. A) The mean area (±SEM) of fungus gardens during weeks 1 plus 11-14 for colonies provided with polenta diets supplemented with xylose (X), amisoy (N), potassium phosphate (P), or control diets (C). Sample sizes were 19, 17, 19, and 21, respectively. B) Boxplots of the total change in fungus garden areas from week 1 to week 14. Different letters indicate significant differences determined by ANOVA and post-hoc Tukey's HSD tests.
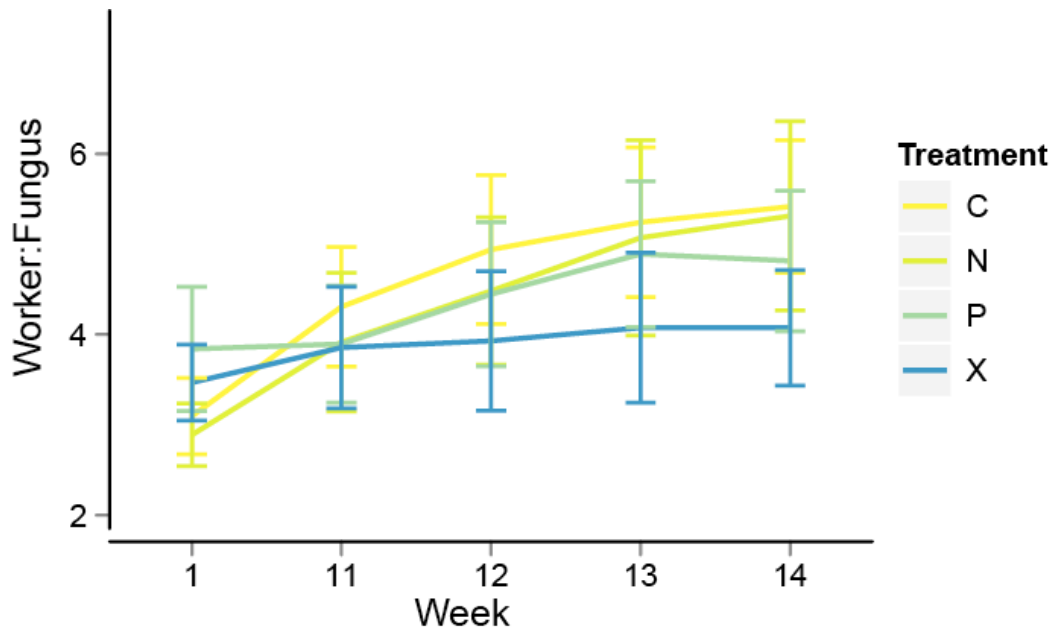
Figure 16. Mean ratios (±SEM) of the number of workers in each colony to the area of their fungus garden for weeks 1 and 11-14. Ratios were not significantly different between treatments.
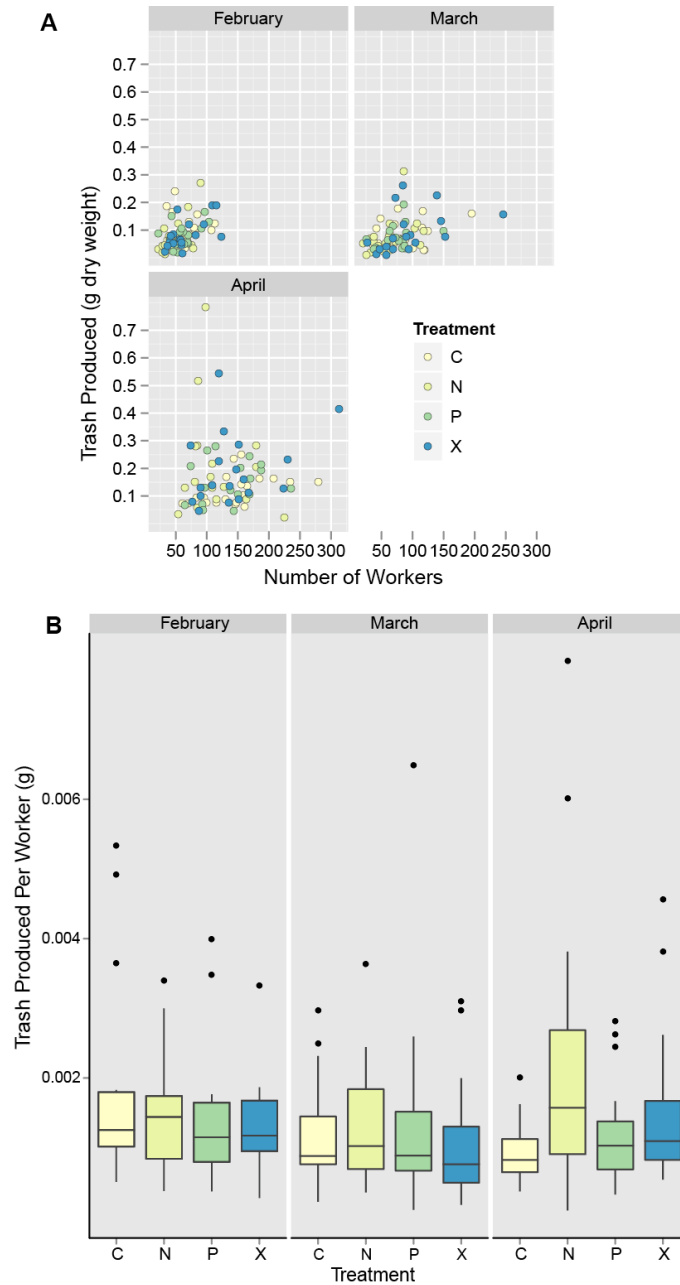
Figure 17. A) Relationship between colony size (estimated number of workers) and refuse production (g dry weight) for each of the three time periods when refuse was collected. Colony size predicted refuse production across the first two time periods, but not the third (statistics provided in text). B) Boxplots of the amount of refuse produced per worker across each of the four treatments for three time periods. There were no significant differences between treatments.
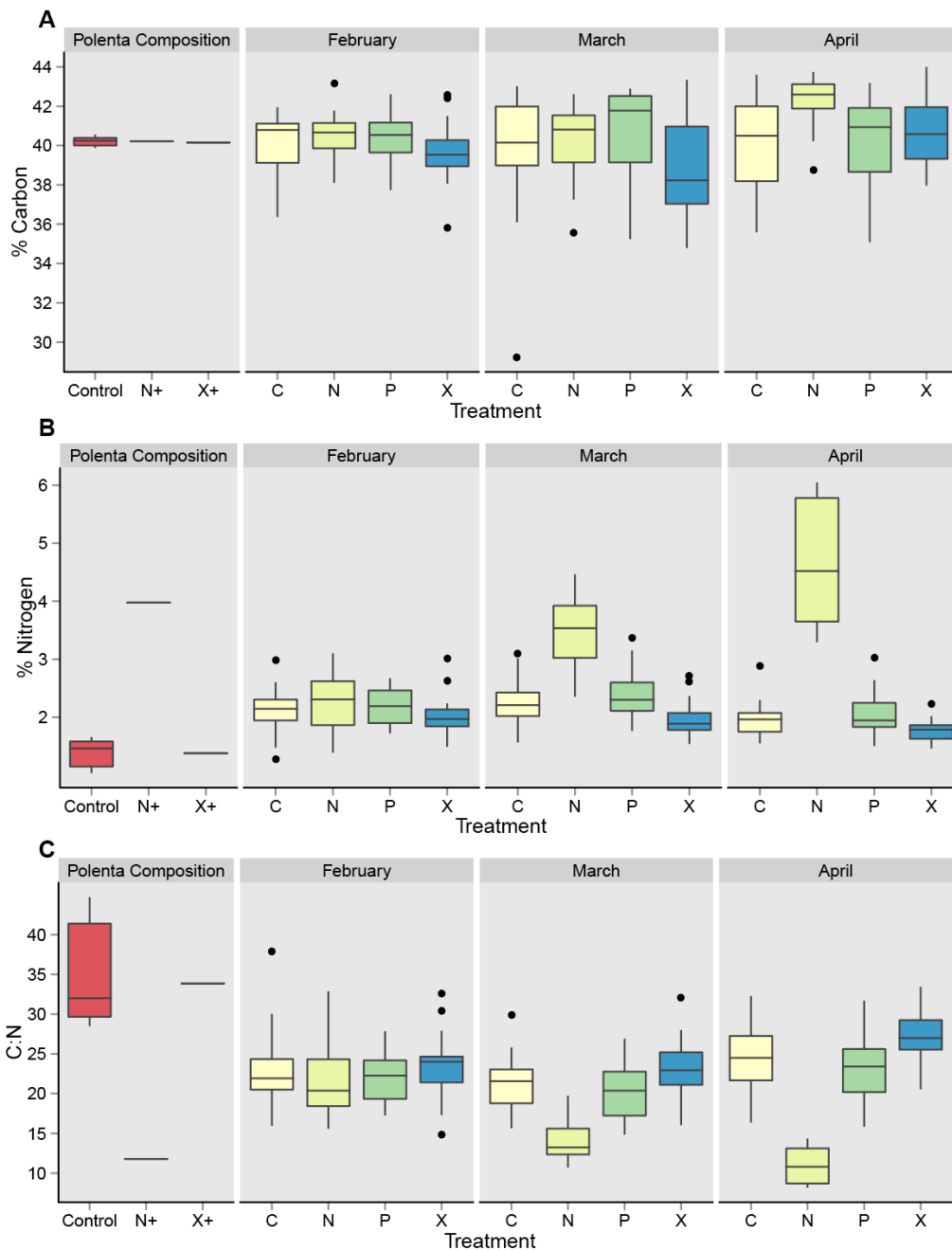
Figure 18. Carbon and nitrogen contents of the polenta provided to colonies receiving the control, xylose- or amisoy-supplemented diets, as well as the carbon and nitrogen contents of refuse samples collected from colonies at three timepoints over the course of the experiment. A) Carbon content, B) Nitrogen content, and C) Carbon-to-nitrogen ratios. Refuse carbon content increased over time, but did not differ between treatments. Nitrogen content increased for colonies in the amisoy treatment, also leading to significantly lower C:N ratios. Statistics are provided in the text.
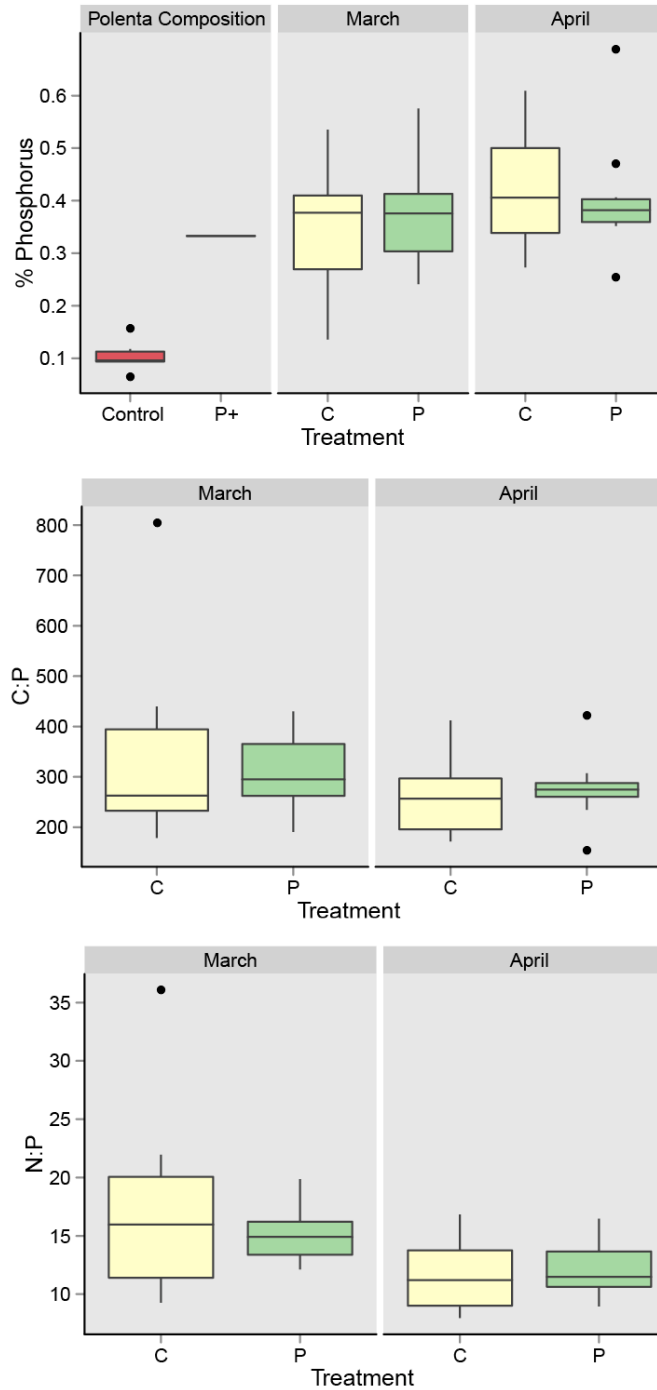
Figure 19. A) Phosphorus contents of polenta provided to colonies receiving control or phosphorus-supplemented diets, plus P contents of refuse collected at two timepoints over the course of the experiment B) C:P ratios of refuse. C) N:P ratios of refuse.

REFERENCES

Abril, A., & Bucher, E. (2002). Evidence that the fungus cultured by leaf-cutter ants does not metabolize cellulose. *Ecology Letters*, *5*, 325-328.

Augustin, J., & Santos, J. (2008). Behavior of early generations of *Atta sexdens* (Hymenoptera: Formicidae) workers during preparation of leaf substrate for symbiont fungus gardens. *Sociobiology*, *51*(1), 265-281.

Bass, M. (1997). The effects of leaf deprivation on leaf-cutting ants and their mutualistic fungus. *Ecological Entomology*, *22*(4), 384-389.

Bass, M., & Cherrett, J. M. (1995). Fungal hyphae as a source of nutrients for the leaf-cutting ant *Atta sexdens*. *Physiological Entomology*, *20*, 1-6.

Bass, M., & Cherrett, J. M. (1996a). Leaf-cutting ants (Formicidae, Attini) prune their fungus to increase and direct its productivity. *Functional Ecology*, *10*, 55-61.

Bass, M., & Cherrett, J. (1996b). Fungus garden structure in the leaf-cutting ant *Atta sexdens* (Formicidae, attini). *Symbiosis*, *21*(1), 9-24.

Bates, D., & Maechler, M. (2010). *lme4: Linear mixed-effects models using S4 classes*. Retrieved from http://CRAN.R-project.org/package=lme4

Behmer, S. (2009). Insect Herbivore Nutrient Regulation. *Annual Review of Entomology*, *54*, 165-187. doi:10.1146/annurev.ento.54.110807.090537

Berish, C. (1986). Leaf-cutting ants (*Atta cephalotes*) select nitrogen-rich forage. *American Midland Naturalist*, *115*(2), 268-276.

Bollazzi, M., & Roces, F. (2011). Information Needs at the Beginning of Foraging: Grass-Cutting Ants Trade Off Load Size for a Faster Return to the Nest. *PLOS ONE*, *6*(3). doi:10.1371/journal.pone.0017667

Burd, M., & Howard, J. (2005). Global optimization from suboptimal parts: foraging *sensu lato* by leaf-cutting ants. *Behavioral Ecology and Sociobiology*, *59*(2), 234-242. doi:10.1007/s00265-005-0029-4

Cahan, S., & Julian, G. (1999). Fitness consequences of cooperative colony founding in the desert leaf-cutter ant *Acromyrmex versicolor*. *Behavioral*

Ecology, 10(5), 585-591.

Camargo, R., Forti, L., Lopes, J., & de Matos, C. (2008). Growth of populations and the fungus garden of *Atta sexdens* rubropilosa (Hymenoptera, Formicidae) response to foraged substrates. *Sociobiology, 52*(3), 633-643.

Camargo, R., Forti, L., Lopes, J., Andrade, A., & Ottati, A. (2007). Age polyethism in the leaf-cutting ant *Acromyrmex subterraneus* brunneus Forel, 1911 (Hym., Formicidae). *Journal of Applied Entomology, 131*(2), 139-145. doi:10.1111/j.1439-0418.2006.01129.x

Cartar, R. (1992). Adjustment of foraging effort and task switching in energy-manipulated wild bumblebee colonies. *Animal Behaviour, 44*(1), 75-87.

Cates, R., & Rhoades, D. (1977). Patterns in the production of antiherbivore chemical defenses in plant communities. *Biochemical Systematics and Ecology, 6*, 185-193.

Chapela, I. H., Rehner, S. A., Schultz, T. R., & Mueller, U. G. (1994). Evolutionary History of the Symbiosis Between Fungus-Growing Ants and Their Fungi. *Science, 266(5191)*, 1691-1694.

Chapman, R. (1998). *The Insects: Structure and Function (4th ed.)*. New York: Cambridge University Press.

Cherrett, J. M. (1972). Some factors involved in the selection of vegetable substrate by *Atta cephalotes* (L.)(Hymenoptera: Formicidae) in tropical rain forest. *The Journal of Animal Ecology, 41*(3), 647-660.

Clesceri, L., Greenberg, A., & Eaton, A. (1998). *Standard Methods for the Examination of Water and Wastewater* (20th ed.). New York: American Public Health Association.

Cook, S., Eubanks, M., Gold, R., & Behmer, S. (2010). Colony-level macronutrient regulation in ants: mechanisms, hoarding and associated costs. *Animal Behaviour, 79*(2), 429-437. doi:10.1016/j.anbehav.2009.11.022

Davidson, D., Cook, S., Snelling, R., & Chua, T. (2003). Explaining the abundance of ants in lowland tropical rainforest canopies. *Science, 300*(5621), 969-972.

Doi, H., Cherif, M., Iwabuchi, T., Katano, I., Stegen, J., & Striebel, M. (2010). Integrating elements and energy through the metabolic dependencies of gross growth efficiency and the threshold elemental ratio. *OIKOS, 119*(5), 752-765. doi:10.1111/j.1600-0706.2009.18540.x

Dussutour, A., & Simpson, S. (2008a). Carbohydrate regulation in relation to colony growth in ants. *Journal of Experimental Biology, 211*(14), 2224-2232. doi:10.1242/jeb.017509

Dussutour, A., & Simpson, S. (2008b). Description of a simple synthetic diet for studying nutritional responses in ants. *Insectes Sociaux, 55*(3), 329-333. doi:10.1007/s00040-008-1008-3

Dussutour, A., & Simpson, S. (2009). Communal Nutrition in Ants. *Current Biology, 19*(9), 740-744. doi:10.1016/j.cub.2009.03.015

Elser, J. J., Bracken, M., Cleland, E., Gruner, D., Harpole, W., Hillebrand, H., Ngai, J., et al. (2007). Global analysis of nitrogen and phosphorus limitation of primary producers in freshwater, marine and terrestrial ecosystems. *Ecology Letters, 10*, 1-8.

Elser, J. J., Fagan, W., Denno, R., Dobberfuhl, D. R., Folarin, A., Huberty, A., Interlandi, S., et al. (2000). Nutritional constraints in terrestrial and freshwater food webs. *Nature, 408*, 578-580.

Erthal, J., Silva, C., & Samuels, R. (2004). Digestive enzymes of leaf-cutting ants, *Acromyrmex subterraneus* (Hymenoptera: Formicidae: Attini): Distribution in the gut of workers and partial characterization. *Journal of Insect Physiology, 50*, 881-891.

Erthal, M., Silva, C., Cooper, R., & Samuels, R. (2009). Hydrolytic enzymes of leaf-cutting ant fungi. *Comparative Biochemistry and Physiology B - Biochemistry and Molecular, 152*(1), 54-59. doi:10.1016/j.cbpb.2008.09.086

Fernandez-Marin, H., Zimmerman, J., & Wcislo, W. (2004). Ecological traits and evolutionary sequence of nest establishment in fungus-growing ants (Hymenoptera, Formicidae, Attini). *Biological Journal of the Linnean Society, 81*(1), 39-48.

Fernandez-Marin, H., Zimmermann, J., & Wcislo, W. (2003). Nest-founding in *Acromyrmex octospinosus* (Hymenoptera, Formicidae, Attini): demography and putative prophylactic behaviors. *Insectes Sociaux*, *50*(4), 304-308. doi:10.1007/s00040-003-0687-z

Fewell, J., & Bertram, S. (1999). Division of labor in a dynamic environment: Response by honeybees (*Apis mellifera*) to graded changes in colony pollen stores. *Behavioral Ecology and Sociobiology*, *46*, 171-179.

Fewell, J., & Winston, M. (1992). Colony state and regulation of pollen foraging in the honey bee, *Apis mellifera* L. *Behavioral Ecology and Sociobiology, 30*, 387-393.

Fewell, J., & Winston, M. (1995). Regulation of nectar collection in relation to honey storage levels by honey bees, *Apis mellifera*. *Behavioral Ecology*, *7*(3), 286-291.

Glazier, D. (2005). Beyond the "3/4-power law": variation in the intra- and interspecific scaling of metabolic rate in animals. *Biological Reviews*, *80*(4), 611-662. doi:10.1017/S1464793105006834

Gomes de Siqueira, C., Bacci, J., Pagnocca, F., Bueno, O., & Hebling, M. (1998). Metabolism of plant polysaccharides by *Leucoagaricus gongylophorus*, the symbiotic fungus of the leaf-cutting ant *Atta sexdens* L. *Applied and Environmental Microbiology*, *64*(12), 4820-4822.

Gordon, D. (1996). The organization of work in social insect colonies. *Nature*, *380*(6570), 121-124.

Greene, M., & Gordon, D. (2007). Interaction rate informs harvester ant task decisions. *Behavioral Ecology*, *18*(2), 451-455. doi:10.1093/beheco/arl105

Haines, B. (1978). Element and energy flows through colonies of the leaf-cutting ant, *Atta colombica*, in Panama. *Biotropica*, *10*(4), 270-277.

Herz, H, Holldobler, B, & Roces, F. (2008). Delayed rejection in a leaf-cutting ant after foraging on plants unsuitable for the symbiotic fungus. *Behavioral Ecology*, *19*(3), 575-582. doi:10.1093/beheco/arn016

Hillebrand, H., Borer, E., Bracken, M., Cardinale, B., Cebrian, J., Cleland, E., Elser, J., et al. (2009). Herbivore metabolism and stoichiometry each

constrain herbivory at different organizational scales across ecosystems. *Ecology Letters*, *12*(6), 516-527. doi:10.1111/j.1461-0248.2009.01304.x

Hothorn, T., Bretz, F., & Westfall, P. (2008). Simultaneous inference in general parametric models. *Biometrical Journal*, *50*(3), 346-363.

Hou, C., Kaspari, M., Vander Zanden, H. B., & Gillooly, J. F. (2010). Energetic basis of colonial living in social insects. *Proceedings of the National Academy of Sciences*, *107*(8), 3634-3638. doi:10.1073/pnas.0908071107

Howard, J. (1987). Leafcutting ant diet selection: The role of nutrients, water, and secondary chemistry. *Ecology*, *68*(3), 503-515.

Howard, J. (1988). Leafcutting and diet selection: Relative influence of leaf chemistry and physical features. *Ecology*, *69*(1), 250-260.

Hubbell, S., Howard, J., & Wiemer, D. (1984). Chemical leaf repellency to an Attine ant: Seasonal distribution among potential host plant species. *Ecology*, *65*(4), 1067-1076.

Hudson, T., Turner, B., Herz, H, & Robinson, J. (2009). Temporal patterns of nutrient availability around nests of leaf-cutting ants (*Atta colombica*) in secondary moist tropical forest. *Soil Biology and Biochemistry*, *41*(6), 1088-1093. doi:10.1016/j.soilbio.2009.02.014

Jeanne, R. L. (1999). Group size, productivity, and information flow in social wasps. *Information Processing in Social Insects* (pp. 3-30). Basel, Switzerland: Birkhauser Verlag.

Jeanne, R., & Nordheim, E. (1996). Productivity in a social wasp: Per capita output increases with swarm size. *Behavioral Ecology, 7(1)*, 43-48.

Jeanson, R., Fewell, J., Gorelick, R., & Bertram, S. (2007). Emergence of increased division of labor as a function of group size. *Behavioral Ecology and Sociobiology*, *62*(2), 289-298. doi:10.1007/s00265-007-0464-5

Julian, G., & Cahan, S. (1999). Undertaking specialization in the desert leaf-cutter ant *Acromyrmex versicolor*. *Animal Behaviour*, *58*, 437-442.

Julian, G., & Fewell, J. (2004). Genetic variation and task specialization in the desert leaf-cutter ant, *Acromyrmex versicolor*. *Animal Behaviour, 68*, 1-8.

Kay, A. (2004). The relative availabilities of complementary resources affect the feeding preferences of ant colonies. *Behavioral Ecology*, *15*(1), 63-70.

Kay, AD, Rostampour, S., & Sterner, R. (2006). Ant stoichiometry: elemental homeostasis in stage-structured colonies. *Functional Ecology*, *20*(6), 1037-1044. doi:10.1111/j.1365-2435.2006.01187.x

Larsson, S., & Tenow, O. (1979). Utilization of dry matter and bioelements in larvae of *Neodibrion sertifer* Geoffr. (Hym., Diprionidae) feeding on Scots Pine (Pinus sylvestris L.). *Oecologia*, *43*, 157-172.

Lee, K., Simpson, S., Clissold, F., Brooks, R., Ballard, J., Taylor, P., Soran, N., et al. (2008). Lifespan and reproduction in Drosophila: New insights from nutritional geometry. *Proceedings of the National Academy of Sciences, U.S.A., 105*, 2498-2503.

Li, D., & Hou, X. (2007). Effect of fungal elimination on bacteria and protozoa populations and degradation of straw dry matter in the rumen of sheep and goats. *Aisian-Australasian Journal of Animal Sciences*, *20(1)*, 70-74.

Manzoni, S., Trofymow, J., Jackson, R., & Porporato, A. (2010). Stoichiometric controls on carbon, nitrogen, and phosphorus dynamics in decomposing litter. *Ecological Monographs, 80(1)*, 89-106.

Mattson, W. (1980). Herbivory in relation to plant nitrogen content. *Annual Review of Ecology and Systematics*, *11*, 119-161.

Meehan, T., & Lindroth, R. (2009). Scaling of individual phosphorus flux by caterpillars of the whitemarked tussock moth, *Orygia leucostigma*. *Journal of Insect Science*, *9*.

Meyer, S., Roces, F., & Wirth, R. (2006). Selecting the drought stressed: effects of plant stress on intraspecific and within-plant herbivory patterns of the leaf-cutting ant *Atta colombica*. *Functional Ecology*, *20*(6), 973-981. doi:10.1111/j.1365-2435.2006.01178.x

Mintzer, A. (1994). Diet of the leafcutting ant, *Atta mexicana* (Hymenoptera: Formicidae), in a Sonoran desert habitat. *Journal of the Arizona-Nevada Academy of Science*, *28*, 1-8.

Moller, I. E., De Fine Licht, Henrik H., Harholt, J., Willats, W. G. T., & Boomsma, Jacobus J. (2011). The Dynamics of Plant Cell-Wall Polysaccharide Decomposition in Leaf-Cutting Ant Fungus Gardens. (J. Chave, Ed.) *PLoS ONE*, *6*(3), e17506. doi:10.1371/journal.pone.0017506

Mundim, F., Costa, A., & Vasconcelos, H. (2009). Leaf nutrient content and host plant selection by leaf-cutter ants, Atta laevigata, in a Neotropical savanna. *Entomologia Experimentalis et Applicata, 130(1)*, 47-54.

Muscedere, M. L., Berglund, J. L., & Traniello, J. F. A. (2010). Polymorphism and Division of Labor During Foraging Cycles in the Leaf-cutting Ant *Acromyrmex octospinosus* (Formicidae; Attini). *Journal of Insect Behavior*, *24*(2), 94-105. doi:10.1007/s10905-010-9239-3

Nixon, S., Granger, S., & Nowicki, B. (1995). An assessment of the annual mass balance of carbon, nitrogen, and phosphorus in Narragansett Bay. *Biogeochemistry*, *31*(1), 15-61.

Nonacs, P. (1991). Less growth with more food: How insect-prey availability changes colony demographics in the ant, *Camponotus floridanus*. *Journal of Insect Physiology*, *37*(12), 891-898.

North, R., Jackson, C., & Howse, P. (1999). Communication between the fungus garden and workers of the leaf-cutting ant, *Atta sexdens* rubropilosa, regarding choice of substrate for the fungus. *Physiological Entomology*, *24*(2), 127-133.

O'Donnell, S. (1998). Effects of experimental forager removals on division of labour in the primitively eusocial wasp *Polistes instabilis* (Hymenoptera: Vespidae). *Behaviour*, *135*, 173-193.

Oster, G., & Wilson, E. (1978). *Caste and Ecology in the Social Insects*. (R. May, Ed.) Monographs in Population Biology. Princeton, NJ: Princeton University Press.

Perkins, M., Woods, H., Harrison, J., & Elser, J. (2004). Dietary phosphorus affects the growth of larval *Manduca sexta*. *Archives of Insect Biochemistry and Physiology*, *55*, 153-168.

Pinto-Tomas, A., Anderson, M., Suen, G., Stevenson, D., Chu, F., Cleland, W., Weimer, P., et al. (2009). Symbiotic Nitrogen Fixation in the Fungus

Gardens of Leaf-Cutter Ants. *Science*, *326*(5956), 1120-1123. doi:10.1126/science.1173036

Porter, S., & Tschinkel, W. (1985). Fire ant polymorphism: The ergonomics of brood production. *Behavioral Ecology and Sociobiology, 16*, 323-336.

Poulsen, M., & Boomsma, J. J. (2005). Mutualistic fungi control crop diversity in fungus-growing ants. *Science, 307*, 741-744.

R Development Core Team. (2010). *R: A language and environment for statistical computing.* Vienna, Austria: R Foundation for Statistical Computing. Retrieved from http://www.R-project.org

Ramirez, R., Rodriguez, A., Tagle, L., DelValle, A., & Gonzalez, J. (1990). Nutrient content and intake of forage grazed by range goats in norhteastern Mexico. *Small Ruminant Research, 3(5)*, 435-448.

Raubenheimer, D., & Bassil, K. (2007). Separate effects of macronutrient concentration and balance on plastic gut responses in locusts. *Journal of Comparative Physiology B - Biochemical Systemic and Environmental Physiology, 177(8)*, 849-855.

Reyes-Lopez, J., & Fernandez-Haeger, J. (2002). Food storage in the nest and seed selectivity in the harvester ant *Messor barbarus* (Hymenoptera: Formicidae). *Sociobiology*, *39*(1), 123-128.

Ridley, P., Howse, P., & Jackson, C. (1996). Control of the behaviour of leaf-cutting ants by their "symbiotic" fungus. *Experientia*, *52*(6), 631-635.

Rissing, S., Johnson, R., & Martin, J. (2000). Colony founding behavior of some desert ants: Geographic variation in metrosis. *Psyche, 103*, 95-101.

Rissing, S., Pollock, G., Higgins, M., Hagen, R., & Smith, D. (1989). Foraging specialization without relatedness or dominance among co-founding ant queens. *Nature*, *338*(6214), 420-422.

Roces, F. (2002). Individual complexity and self-organization in foraging by leaf-cutting ants. *Biological Bulletin*, *202*(3), 306-313.

Roschard, J., & Roces, F. (2003). Cutters, carriers and transport chains: Distance-dependent foraging strategies in the grass-cutting ant *Atta vollenweideri*.

Insectes Sociaux, 50(3), 237-244. doi:10.1007/s00040-003-0663-7

Sakamoto, Y., Ishiguro, M., & Kitagawa, G. (1986). *Akaike Information Criterion Statistics*. Dordrecht, Holland: Reidel.

Schade, J., Kyle, M., Hobbie, S., Fagan, W., & Elser, J. (2003). Stoichiometric tracking of soil nutrients by a desert insect herbivore. *Ecology Letters*, *6*, 96-101.

Schafer, R., Holmes, S., & Gordon, D. (2006). Forager activation and food availability in harvester ants. *Animal Behaviour*, *71*, 815-822. doi:10.1016/j.anbehav.2005.05.024

Schiott, M., Licht, H., Lange, L., & Boomsma, J. (2008). Towards a molecular understanding of symbiont function: Identification of a fungal gene for the degradation of xylan in the fungus gardens of leaf-cutting ants. *BMC Microbiology, 8*. doi:10.1186/1471-2180-8-40

Schmitz, O. (2008). Herbivory from Individuals to Ecosystems. *Annual Review of Ecology Evolution and Systematics*, *39*, 133-152. doi:10.1146/annurev.ecolsys.39.110707.173418

Schoonhoven, L., Jermy, T., & van Loon, J. (1998). Plants as insect food: Not the ideal. *Insect-Plant Biology* (pp. 83-120). New York: Chapman & Hall.

Schoonhoven, L. (2005). *Insect-Plant Biology* (2nd ed.). New York, NY: Oxford University Press.

Seal, J. N., & Tschinkel, W. R. (2007a). Complexity in an obligate mutualism: Do fungus-gardening ants know what makes their garden grow? *Behavioral Ecology and Sociobiology*, *61*(8), 1151-1160.

Seal, J., & Tschinkel, W. (2007b). Energetics of newly-mated queens and colony founding in the fungus-gardening ants *Cyphomyrmex rimosus* and *Trachymyrmex septentrionalis* (Hymenoptera: Formicidae). *Physiological Entomology*, *32*(1), 8-15. doi:10.1111/j.1365-3032.2006.00534.x

Simpson, S., & Raubenheimer, D. (1993). A multi-level analysis of feeding behaviour: The geometry of nutritional decisions. *Philosophical Transactions: Biological Sciences, 342*, 381-402.

Slansky, F, & Fogal, W. (1985). Utilization of dry-matter and elements by larvae of the sawflies *Neodiprion sertifer* (Geoff) and *Diprion similis* (Hartig) feeding on Scots pine needles. *Canadian Journal of Zoology - Revue Canadienne de Zoologie, 63(3)*, 506-510.

Slansky, F, & Wheeler, G. (1989). Compensatory increases in food consumption and utilization efficiencies by velvetbean caterpillars mitigate impact of diluted diets on growth. *Entomologia Experimentalis et Applicata, 51(2)*, 175-187.

Slansky, F, & Wheeler, G. (1991). Food consumption and utilization responses to dietary dilution with cellulose and water by velvetbean caterpillars, *Anticarsia gemmatalis*. *Physiological Entomology, 16(1)*, 99-116.

Slansky, Frank, & Scriber, J. M. (1982). Selected Bibliography and Summary of Quantitative Food Utilization by Immature Insects. *Bulletin of the ESA, 28*, 43-56.

Suen, G., Scott, J., Aylward, F., Adams, S., Tringe, S., Pinto-Tomas, A., Foster, C., et al. (2010). An Insect Herbivore Microbiome with High Plant Biomass-Degrading Capacity. *PLoS Genetics*, *6*(9), e1001129. doi:10.1371/journal.pgen.1001129

Tadey, M., & Farji-Brener, A. (2007). Indirect effects of exotic grazers: livestock decreases the nutrient content of refuse dumps of leaf-cutting ants through vegetation impoverishment. *Journal of Applied Ecology*, *44*(6), 1209-1218. doi:10.1111/j.1365-2664.2007.01338.x

Tibbets, T., & Faeth, S. (1999). *Neotyphodium* endophytes in grasses: Deterrents or promoters of herbivory by leaf-cutting ants? *Oecologia*, *118*, 297-305.

Timmins, W., Bellward, K., Stamp, A., & Reynolds, S. (1988). Food-intake, conversion efficiency, and feeding-behavior of tobacco hornworm caterpillars given artificial diet of varying nutrient and water-content. *Physiological Entomology, 13(3)*, 303-314.

Van Loon, J.J.A., Casas, J., & Pincebourde, S. (2005). Nutritional ecology of insect-plant interactions: Persistent handicaps and the need for innovative approaches. *OIKOS 108(1)*, 194-201.

Vasconcelos, H. L., & Cherrett, J. M. (1996). The effect of wilting on the

selection of leaves by the leaf-cutting ant *Atta laevigata*. *Entomologia Experimentalis et Applicata*, *78*, 215-220.

Vieira-Neto, E., & Vasconcelos, H. (2010). Developmental changes in factors limiting colony survival and growth of the leaf-cutter ant *Atta laevigata*. *Ecography*, *33*(3), 538-544. doi:10.1111/j.1600-0587.2009.05875.x

Weser, J. T. (2005). *Foraging and information flow in two desert ant species*. Tempe, AZ: Arizona State University.

Wetterer, J. K., Himler, A. G., & Yospin, M. M. (2001). Foraging ecology of the desert leaf-cutting ant, *Acromyrmex versicolor*, in Arizona (Hymenoptera: Formicidae). *Sociobiology*, *37*(3B), 633-649.

Wetterer, J. (1999). The ecology and evolution of worker size-distribution in leaf-cutting ants (Hymenoptera: Formicidae). *Sociobiology*, *34*(1), 119-144.

Wilson, E. (1980). Caste and division of labor in leaf-cutter ants (Hymenoptera, Formicidae, Atta) .1. The overall pattern in *Atta sexdens*. *Behavioral Ecology and Sociobiology*, *7*(2), 143-156.

Wilson, E. (1983). Caste and division of labor in leaf-cutterants (Hymenoptera, Formicidae, Atta) .4. Colony ontogeny of *Atta cephalotes*. *Behavioral Ecology and Sociobiology*, *14*(1), 55-60.

Wirth, R., Herz, Hubert, Ryel, R. J., Beyschlag, W., & Holldobler, Bert. (2003). *Herbivory of Leaf-Cutting Ants: A Case Study on* Atta Colombica *in the Tropical Rainforest of Panama*. Ecological studies. Berlin: Springer.

Woods, H., Perkins, M., Elser, J., & Harrison, J. (2002). Absorption and storage of phosphorus by larval *Manduca sexta*. *Journal of Insect Physiology*, *48*, 555-564.

Yang, A. (2007). Thinking outside the embryo: The superorganism as a model for EvoDevo studies. *Biological Theory*, *2*(4), 398-408.

Zanotto, F., Simpson, S., & Raubenheimer, D. (1993). The regulation of growth by locusts through post-ingestive compensation for variation in the levels of dietary protein and carbohydrate. *Physiological Entomology, 18*, 425-434.

Zhang, G., Han, X., & Elser, J. (2011). Rapid top-down regulation of plant C:N:P stoichiometry by grasshoppers in an Inner Mongolia grassland ecosystem. *Oecologia, 166*(1), 253-264. doi:10.1007/s00442-011-1904-5

BIOGRAPHICAL SKETCH

Rebecca Clark was born in Seattle, WA in 1981, and grew up and attended high school at Holy Names Academy. She moved to Boston to attend Tufts University, where she earned a BA in Bio-Psychology while studying impulsivity and aggression in rats in 2003. She began her doctoral degree program studying the behavior, physiology, and ecology of ants shortly thereafter. In her spare time, she cooks, bakes, rows, reads, creates ceramics, and goes on extremely long bike rides.