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# Colony-level macronutrient regulation in ants: mechanisms, hoarding and associated costs

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Social and nonsocial organisms both require a suite of nutrients in correct amounts and ratios to promote growth and fitness, but the nutrient profiles of available foods are rarely optimal. Nutrient acquisition in insect societies is more complex compared to that of nonsocial organisms however, because foraging is restricted to only a proportion of the colony, and these members must satisfy their own nutritional requirements and those of other members having distinct nutritional needs. In this study we used laboratory colonies of the fire ant *Solenopsis invicta* to quantify how ants regulate their protein–carbohydrate intake when restricted to diets with different fixed protein–carbohydrate (p:c) ratios, and to quantify, at both the individual and colony level, behaviours and costs associated with nutrient regulation when feeding on these foods. We found that ants were most attracted to foods with equal or moderately protein-biased p:c ratios. However, colonies on these two treatments created large hoards, and the p:c ratios of these foods differed from that of collected food. In general, carbohydrates were extracted and protein was retained. As a result, carbohydrate intake on all diets except the extremely protein-biased diet was similar. However, carbohydrate regulation on diets with equal and moderately protein-biased p:c ratios may be costly through elevated worker activity and mortality, and through reduction of worker lipid reserves. For colonies feeding on heavily protein-biased food, energy production may have been achieved via gluconeogenesis. We discuss our results in relation to how dietary p:c imbalances in naturally encountered foods may be driving ant foraging behaviour in the field.


Insect societies, especially ant colonies, have been described as superorganisms sharing many functional parallels with individual organisms (Wheeler 1911; Hölldobler & Wilson 2009). Among these is the fundamental need to acquire a suite of nutrients in the correct amounts and ratios promoting growth and fitness. However, there are a number of factors that make nutrient acquisition at the colony level more challenging compared to that for a solitary individual. First, division of labour in ant societies results in foraging responsibilities, and thus nutrient retrieval, being assigned to only a subset of adult colony members. Second, most ant colonies contain overlapping generations composed of mature and immature individuals having different nutrient requirements (Hölldobler & Wilson 1990; Cassill & Tschinkel 1999a). Therefore the challenge for foraging workers is that they must harvest food such that it satisfies their own nutritional requirements while also addressing the nutritional needs of the other members of the colony, including the queen, the larvae, and the other workers. An additional consideration is foraging costs, including energetic expenditure and potential mortality of workers when foraging (Wolf & Schmid-Hempel 1990; Beauchamp 1992).

The decision of a forager to retrieve an encountered food item is shaped by both internal and external factors, occurring at both the individual and colony level. At the individual level, a worker's current physiological condition (e.g. endogenous stores of lipids; Blanchard et al. 2000) may prompt foraging behaviour, and attractiveness of an encountered food item may be affected by the perceived relative availability and/or abundance of food items (Stein et al. 1990; Hahn & Wheeler 2002; Kay 2004), and their novelty (Howard et al. 1996). At the colony level, feedback related to larval nutritional demands (especially for protein), transmitted through a 'chain-of-demand' between brood, nurse workers and foragers, and created through colony member food sharing is likely to be important (Cassill et al. 1998; Cassill & Tschinkel 1999b; Behmer 2009). However, the strength of both internal and external cues directing worker foraging decisions can vary temporally, compounding the task of nutrient retrieval especially in the face of potential resource shortfalls (e.g. seasonal variation in resource availability). Extensive research of ant nutritional biology has helped elucidate potential determinants guiding collection of

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resources by workers, including, but not limited to, resource preference of different colony members (Cassill & Tschinkel 1999b; Portha et al. 2004), temporal shifts in resource availability and worker preference (Cannon & Fell 2002), and food distribution among colony members (Vinson 1968).

Two critical nutrients that influence ant foraging behaviour and performance are protein and carbohydrates (Cassill et al. 1998; Cassill & Tschinkel 1999b). However, researchers often investigate the effects of these two nutrients in isolation from one another, even though all naturally available food items encountered by ants (including honeydew) contain a mixture of macro- and micronutrients. The key is that various foods contain different absolute amounts and ratios of nutrients, at both a spatial and temporal scale. Additionally, few studies of ant nutritional biology have linked changes in macronutrient availability to alterations of behaviour and physiological condition of individuals (but see Kay et al. 2006), and even fewer have focused on colony-level behaviours of ants associated with nutrient regulation in a framework of imbalanced nutrient availability, and the costs of these behaviour (but see Dussutour & Simpson 2008a, 2009).

How animals simultaneously regulate the intake of multiple nutrients, and how the nutritional content of food affects animal behaviour and physiology can readily be explored in an integrated fashion using the experimental approach of the 'geometric framework' (reviewed by Behmer 2009). A key strength of this approach is that it allows visualization of how an organism prioritizes the intake of particular nutrients when it is restricted to a food with a defined, and fixed, nutrient profile. In particular, this approach reveals the extent to which an organism will overeat nutrients present in excess of requirements, in order to increase their intake of nutrients that are in short supply. And when these compromises are measured across a range of foods with different nutritional profiles, insights about global rules governing nutrient regulation can be elucidated (Raubenheimer & Simpson 1999; Simpson et al. 2004). The geometric framework was originally designed for use with insect herbivores (reviewed by Behmer 2009), but it has also been used to study nutrient regulation in a broad range of organisms, including chickens (Raubenheimer & Simpson 1997), rats (Simpson & Raubenheimer 1997), mice (Sørensen et al. 2008), fish (Ruohonen et al. 2007), and even humans (Simpson et al. 2003). Quite recently it has also been used to study nutritional regulation in ants (Dussutour & Simpson 2008a, 2009).

In the current study we used the experimental approach of the geometric framework to explore nutrient regulation at the colony level in the red imported fire ant, *Solenopsis invicta*. We did this by giving experimental ant colonies ad libitum access to foods with different protein–carbohydrate ratios, then we quantified the amounts of food collected over a 7-week period and expressed these data in terms of protein–carbohydrate intake. We also investigated whether the amounts of protein and carbohydrate collected were equal to the amounts of these macronutrients consumed by the colony (see Dussutour & Simpson 2009). Furthermore, we quantified potential costs associated with procurement of food, including worker foraging activity, worker mortality and alterations to worker physiological condition (measured in terms of amounts of endogenous lipid stores). We discuss how results from our study may elucidate important mechanistic linkages associated with nutrient availability under more natural conditions.

## METHODS

### *Experimental Colonies*

We collected polygynous colonies of *Solenopsis invicta* from 10 source colonies at the Riverside campus of Texas A&M University, U.S.A., between 19 April and 5 May 2008, and maintained them in their original nest soil in buckets for 2 days; during this time we provided colonies with fresh-frozen crickets, 20% (v/v) honey solution, and water. We then used a drip-floatation method (Banks et al. 1981) to remove workers, brood and queens from the soil, and then left these colonies separately in large tubs overnight before forming monogynous experimental colonies. At this time we also haphazardly collected small groups of workers from each source colony and placed them into vials containing silica gel desiccant in preparation for lipid extraction (see below).

Each monogynous experimental colony consisted of a single wingless queen, 1750 mg workers (haphazardly chosen), 250 mg larvae and 125 mg pupae. This generated a worker-to-total brood ratio of ~5:1, which mimicked natural *S. invicta* colonies during mid-spring (Cassill 2002). Larvae and pupae included in experimental colonies ranged in size and developmental stage, but we purposely avoided selecting larvae and pupae of reproductives. The 10 sources colonies produced different total numbers of experimental colonies; often source colonies did not provide adequate amounts of workers and/or larvae (and in some instances numbers of queens) to generate five experimental colonies (one for each treatment). Nevertheless, each experimental treatment had experimental colonies from at least four source colonies.

Each experimental colony was housed in a 24.6 × 19.2 × 9.5 cm plastic box, provided with a 15 cm diameter lidded and covered petri dish for use as an artificial nest chamber, filled approximately half-full with hardened Castone® dental stone (Castone Corp., Opelika, AL, U.S.A.), and an ad libitum water source. Colonies were housed in an insectary at 26 °C, kept at ambient humidity of 45–60%, and maintained under a 12:12 h light:dark cycle (using fluorescent lighting). We kept the nest chamber humidity levels high by regularly moistening the Castone® substrate (Cassill & Tschinkel 2000).

### *Experimental Diets*

Experimental diets consisted of five dry, granular synthetic foods modified from both Straka & Feldhaar (2007) and Dussutour & Simpson (2008b), and their total combined protein (p) and digestible carbohydrate (c) content ranged from 80 to 83%. The five diets, expressed as the percentage of diet total dry mass, were: (1) p14:c69, (2) p21:c62, (3) p41:c41, (4) p60:c20 and (5) p67:c13. The dietary protein component was an approximate 1:1 mixture of whey protein concentrate and calcium caseinate, with a small but constant amount of protein provided by whole egg powder (which was also a source of essential lipids, including sterols). The sole source of digestible carbohydrate (henceforth only carbohydrate) in our experimental diets was sucrose. The amount of each dietary ingredient used in making each experimental diet is shown in Table 1. According to product nutritional information, each component in Table 1 contained impurities that reduced the amounts of whey protein by 15%, calcium caseinate by 8%, and sucrose by 7%. We also added a small amount of methyl 4-hydroxybenzoate to each diet (0.5 mg) to retard microbial growth (Dussutour & Simpson 2008b).

We combined all the dry dietary components and homogenized them using an electric mixer. We dissolved water-soluble vitamins in water, and we mixed insoluble beta-carotene by shaking it vigorously for 30 s before adding it to dry components, and then added it to the original dry ingredients. After complete mixing, diets were spread evenly on a large plastic weighing dish and placed in an oven set at 35 °C until dry. We formed 1 mm diameter granules by grinding dried food through a no. 18 U.S.A. Standard Sieve. Workers of all body sizes readily collected this granule size in preliminary trials (personal observation).

**Table 1**
Components of experimental diets*

| Component | Diet | | | | |
|---|---|---|---|---|---|
| | p14:c69 | p21:c62 | p41:c41 | p60:c20 | p67:c13 |
| Whey protein | 1.3 | 4.3 | 13.4 | 22.4 | 25.3 |
| Calcium caseinate | 1.2 | 3.9 | 12.1 | 20.3 | 23.0 |
| Whole egg powder | 15.6 | 15.6 | 15.6 | 15.6 | 15.6 |
| Sucrose | 54.5 | 49.1 | 32.7 | 14.9 | 10.9 |
| Wesson salts | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 |
| Vitamin mix | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 |
| Water (ml) | 10 | 10 | 15 | 20 | 20 |

Amounts are based on 60 g macronutrient total weight (minus product impurities, see text). The whole egg powder contributed a constant amount of 6.8 g of lipids (fats and sterols) to each diet. See Straka & Feldhaar (2007) for vitamin mix composition.

* Straka & Feldhaar 2007; Dussutour & Simpson 2008a, b.

*Food Collection*

We presented experimental diets to colonies in 3.5 cm diameter lidded petri dishes with three small holes, which provided access to foraging ants. We allocated an approximately equal amount of diet to each food dish, allowed these foods to dry for 24 h in a 35 °C drying oven, then weighed them using a microbalance (to the nearest 0.01 mg). We replaced food dishes weekly, except in cases where ants removed most of the food before the end of the week. When this occurred, we added new food dishes (prepared as described above), making sure that colonies were never without access to food. At the end of each week, we retrieved food dishes with remaining diet, dried the contents in situ, and reweighed food dishes to obtain the amount of each diet collected. In some colonies we noticed piles of 'hoarded' food inside the nests. These piles were clearly different from waste dumps that contained dead ants and other material (e.g. cotton) located outside the nests. We collected this hoarded food at the end of 7-week experimental period (so as not to disturb the colonies during the experiment), dried and then weighed it. From the above data, we calculated, for each colony, both the total amount of food collected and the amount hoarded.

*Protein and Carbohydrate Analysis of Hoarded Foods*

After hoarded food had dried, we ground it into a fine powder using a small amount of liquid nitrogen in a mortar and pestel. We then redried the hoarded food in preparation for total protein and carbohydrate analysis using spectrophotometric techniques (see below for details). We conducted these analyses to measure the extent to which ants manipulate the protein and carbohydrate content of the hoarded food (Dussutour & Simpson 2009). From each of the hoarded food samples, we weighed two 100 mg aliquots (one for protein analysis, the other for carbohydrate analysis), and placed each aliquot separately into 15 ml plastic centrifuge tubes (VWR International, West Chester, PA, U.S.A.). For the protein analysis we added 10 ml of tris-buffered saline (TBS) (20 mM Tris, 500 mM NaCl; pH = 7.5), and for the carbohydrate analysis we added 10 ml of nano-pure de-ionized water. Samples were then thoroughly mixed to dissolve miscible components. Next we pipetted two 1 mm aliquots from each sample and placed each of these 1 mm aliquots into separate 1.5 ml microcentrifuge tubes. These subsamples were then centrifuged at 10 000 revolutions/min for 8 min. We used two samples of the supernatant from each tube as replicates in spectrophotometric assays to measure protein and carbohydrate content using a coomassie dye protocol (CB-Protein assay; G-Biosciences, Maryland Heights, MO, U.S.A.) and a modified phenol-sulphuric acid protocol (Taylor 1995), respectively. We estimated the amount of protein and carbohydrate in each hoarded food pile using a linear regression equation that had been constructed using the experimental diets, which were treated in the same manner as the hoarded food samples. (The $r^2$ values for protein and carbohydrate regressions were both in excess of 0.97.)

*Worker Behaviour and Performance*

We also recorded worker behaviour and performance each week during the 7-week experimental period. To test for effects of dietary treatment on weekly worker foraging intensity, we recorded, using spot checks, the number of workers actively foraging inside experimental food dishes. Spot checks were completed every other day, and approximately every 20 min during 2 h in the morning (~0900–1100 hours), and 2 h in the afternoon (~1400–1600 hours). We define 'actively foraging' as being in direct contact with the test food. Data were compiled and expressed as the number of workers per observation. To test for effects of dietary treatment on weekly worker mortality, at the end of each week, we aspirated dead bodies from containers and measured mortality by counting worker heads under a stereoscope.

*Lipid Extraction from Workers*

Lipids were extracted from workers of source colonies and experimental colonies, the latter at the end of the 7-week experiment. We used a chloroform extraction protocol modified from Loveridge (1973) to extract whole-body lipids. One hundred individuals (dried to a constant mass) were placed in pre-weighed glass test tubes and weighed to the nearest 0.01 mg. Next, we added 2 mm of chloroform to each sample, then we covered test tubes with a small glass marble to minimize chloroform evaporation. After 24 h we aspirated the chloroform from each sample with a glass pipette, and added fresh chloroform to the tubes. A total of three chloroform soaks were completed. After these extractions, we allowed the worker remains to dry completely, then we reweighed the tubes containing worker remains. The difference in weight of worker bodies before and after lipid extraction gave us the weight of lipids in the samples.

*Statistical Analyses*

We used parametric statistics to conduct all analyses. Prior to each analysis, data were checked for normality and equal variances. If these criteria were not met, data were transformed (typically by log transformation), and subsequently analysed using ANOVA, repeated measure ANOVA or standard least-squares regression analyses. For repeated measure ANOVA, each of the 7 weeks of the experimental period represented different, but correlated conditions (in terms of the nutritional status of the colony and colony demographics), and thus, repeated measurements of weekly quantified data were not independent. Analyses using transformed data are signified in the text. All analyses were conducted using the software package JMP 7.02 (SAS Institute, Inc., Cary, NC, U.S.A.).

## RESULTS

*Food Collection and Protein–Carbohydrate Intake*

The total amount of food collected by foragers of *S. invicta* colonies was significantly affected by dietary p:c ratio (Table 2). Collected amounts were highest on the p41:c41 and p60:c20 diets, and equally low on the remaining diets (Table 3). Colonies collected significantly more food during the first weeks of the experiment relative to the last few weeks (Table 2, Fig. 1), but there was no significant diet-by-week interaction (Table 2).

**Table 2**
Results from repeated measure ANOVA investigating between-subject (diet) effects, within-subject (week) effects, and between-subjects by within-subjects interaction effects

| Variable | Source | *F* | *df* | *P* |
|---|---|---|---|---|
| Total food collected | Diet | 4.13 | 4 | 0.010 |
| | Week | 6.20 | 6 | <0.001 |
| | Diet*week | 1.03 | 24 | 0.401 |
| Protein collected | Diet | 20.55 | 4 | <0.001 |
| | Week | 6.13 | 6 | <0.001 |
| | Diet*week | 1.07 | 24 | 0.401 |
| Carbohydrate collected | Diet | 11.36 | 4 | <0.001 |
| | Week | 6.13 | 6 | <0.001 |
| | Diet*week | 1.07 | 24 | 0.401 |
| Foraging intensity | Diet | 7.92 | 4 | <0.001 |
| | Week | 2.63 | 6 | 0.050 |
| | Diet*week | 1.32 | 24 | 0.185 |
| Worker mortality | Diet | 6.20 | 4 | 0.001 |
| | Week | 10.13 | 6 | <0.001 |
| | Diet*week | 3.45 | 24 | <0.001 |

Food collection, expressed as amounts of total protein and carbohydrate collected over 7 weeks, is shown for each diet in Fig. 2. When analysed using a repeated measure ANOVA, protein collection was significantly affected by dietary p:c ratio, and by week, but there was no diet-by-week interaction (Table 2). Tukey post hoc comparisons showed that protein collection was greatest on the p60:c20 diet, but did not differ statistically compared to diets p41:c41 and p67:c13 (Fig. 2). Protein collection was equally low on the p14:c69 and p21:c62 diets, and amounts collected were statistically different compared to the equal-ratio and protein-biased diets. Carbohydrate collection was also significantly affected by the p:c ratio of the diet, and by week, but again there was no diet-by-week interaction (Table 2). Carbohydrate collection was greatest on p41:c41 diet, but did not differ statistically compared to p14:c69, p21:c62, and p60:c20 diets (Fig. 2). Carbohydrate collection on the p67:c13 diet was significantly reduced compared to the other four diets.

For some colonies, however, some of the collected food was hoarded inside the ants' artificial nests. This hoarding behaviour was observed on all treatments, and although colonies feeding on p41:c41 or p60:c20 diets were observed hoarding most often, the frequency of hoarding did not differ significantly between diets (Fisher's exact test: likelihood ratio = 6.65, $P = 0.156$; Table 3). We did, however, observe significant differences between diets in the amounts of hoarded food (ANOVA of log-transformed data: $F_{4,12} = 3.55$, $P = 0.039$). Tukey post hoc analysis revealed that colonies on the p41:c41 and p60:c20 diets had the largest hoards; hoard size was intermediate on diets p14:c69 and p67:c13, and lowest on diet p21:c62 (Table 3).

**Table 3**
Total amounts of experimental diets collected, the percentage of colonies demonstrating hoarding behaviour and the total amounts of experimental diets hoarded by these colonies for the 7-week experimental period

| Diet | Total amount collected* (mg) | % Colonies that hoarded | Total amount hoarded (mg) |
|---|---|---|---|
| p14:c69 | 2880 (±371)$^{c}$ | 50$^{a}$ | 2431 (±594)$^{ab}$ |
| p21:c62 | 4004 (±659)$^{bc}$ | 33$^{a}$ | 1393 (±1066)$^{b}$ |
| p41:c41 | 8320 (±1334)$^{a}$ | 83$^{a}$ | 5313 (±903)$^{a}$ |
| p60:c20 | 7979 (±1453)$^{ab}$ | 83$^{a}$ | 3093 (±617)$^{ab}$ |
| p67:c13 | 4339 (±1433)$^{bc}$ | 33$^{a}$ | 1871 (±484)$^{b}$ |

* Superscript letters refer to significant differences in Tukey post hoc tests of log-transformed data.

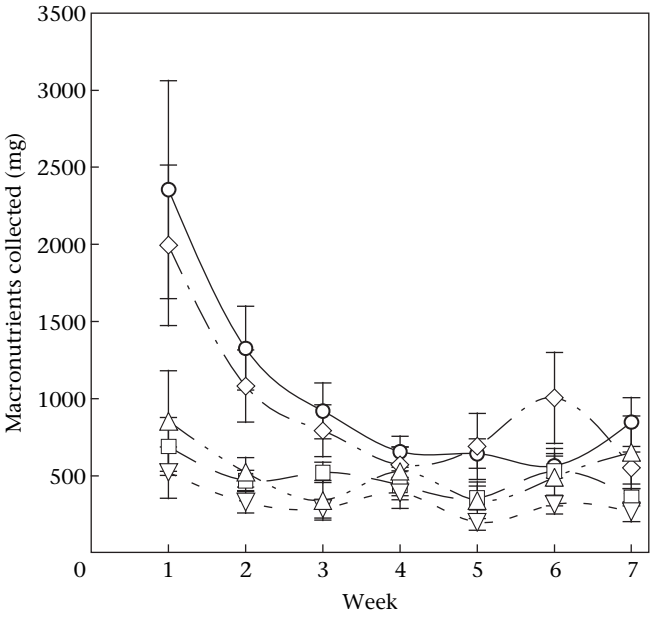


**Figure 1.** Mean ± SE weekly amounts of total food collected for each of 7 weeks. Symbols and lines represent experimental diets: - - ▽ - -: p14:c69; — □ —: p21:c62; —○—: p41:c41; — · ◇ —: p60:c20; — · · △ —: p67:c13.

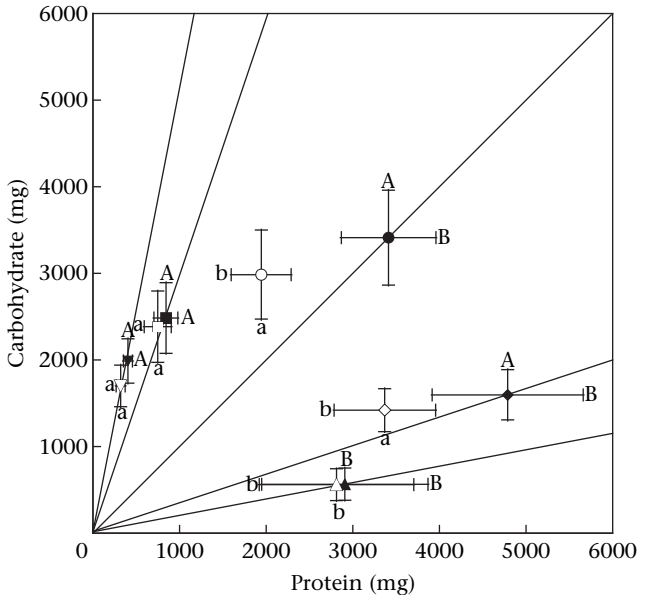


**Figure 2.** Mean ± SE amounts of protein and carbohydrate collected from food dishes (closed symbols) and consumed (open symbols) after accounting for dietary manipulation by experimental colonies over a 7-week period. Symbols represent experimental diets: ▽,▼: p14:c69; □,■: p21:c62; ○,●: p41:c41; ◇,◆: p60:c20; △,▲: p67:c13. Each food is also depicted as a 'rail' running outward from the origin. Because ants were confined to a single food having a fixed protein an carbohydrate ratio, data points representing the amounts of these macronutrients collected necessarily lie on top of each food rail. Deviations of open symbols from these rails represent the estimated amounts of protein and carbohydrate remaining in hoarded foods. Different uppercase letters to the right and above error bars of closed symbols indicate significant differences between treatments in the amounts of protein and carbohydrate collected, respectively. Different lowercase letters to the left and below error bars of open symbols indicate significant differences between treatments in the amount of protein and carbohydrate collected, respectively, after taking into account dietary manipulation of collected foods.

Previous work had demonstrated that ants manipulate the nutritional content of collected food (Dussutour & Simpson 2009). Analyses of protein and carbohydrate content of hoarded foods indicate that *S. invicta* also manipulates the nutritional content of collected food. Median values of protein, carbohydrate and non-macronutrient content of the hoarded food piles (expressed as a percentage of the dry mass) are shown in Fig. 3, and from these data, three main trends emerged. First, the protein content of hoarded foods was similar to that of the respective experimental diet, expect for diet p67:c13; here the protein content of the hoarded food was about 20%. Second, the carbohydrate content of hoarded foods was always reduced compared to the carbohydrate content of the respective experimental diet. Finally, the non-macronutrient content of hoarded foods was always higher than that of the respective experimental diet (which was relatively constant across all diets at ~16–20%).

Estimates of protein and carbohydrate content of hoarded food, together with nutritive content of collected food allowed us to estimate actual protein and carbohydrate consumption (since it would be incorrect to estimate consumption simply from amounts collected) (shown as open symbols in Fig. 2). Across diets, both the amount of protein and carbohydrate consumed differed significantly (ANOVA of log-transformed data: $F_{4,25} = 17.39$, $P < 0.001$; $F_{4,25} = 9.96$, $P < 0.001$, respectively). Our results demonstrate that ant colonies on the p14:c69, p21:c62 and p67:c13 diets ingested most of the protein and carbohydrates that they collected. Ant colonies on the p41:c41 and p60:c20 diets also consumed most of the carbohydrates they collected, but in contrast to the other three treatments (one heavily protein-biased, the other two carbohydrate-biased), a large amount of the protein they collected was not consumed. Selective extraction of the carbohydrates from collected foods in colonies on diets p41:c41 and p60:c20 significantly reduced the p:c ratio of consumed foods relative to that of collected foods (diet p41:c41: p:c ratio of collected and consumed food = 1.00 and 0.69, respectively; *t* test: $t_5 = -2.64$, $P = 0.023$: diet p60:c20: p:c ratio of collected and consumed food = 3.00 and 2.46, respectively; *t* test: $t_5 = -3.95$, $P = 0.005$). The p:c ratio of consumed foods did not differ significantly from that of collected foods for the remaining diets.

### *Worker Behaviour and Performance*

Worker foraging intensity, measured as the number of ants observed feeding on experimental foods for each observation, was similar between morning (mean ± SE = 5.05 ± 0.54) and afternoon (4.56 ± 0.47) observational periods (ANOVA of log-transformed data: $F_{1,39} = 1.79$, $P = 0.196$), and no significant interaction between observational period and food p:c ratio was found from a repeated measure ANOVA of log-transformed data (observation period-by-diet interaction: $F_{4,35} = 0.47$, $P = 0.754$). Given the lack of difference between morning and afternoon observational periods, data were pooled for a subsequent repeated measure ANOVA. This analysis revealed that worker foraging intensity was significantly affected by dietary p:c ratio (Table 2). Total worker foraging intensity was highest for colonies feeding on p60:c20 diets, intermediate on p41:c41 and p21:c62 diets, and lowest on the two remaining extremely unbalanced diets (Fig. 4). Foraging intensity also differed significantly over the 7-week experimental period, and weekly patterns were highly variable (data not shown). However, there was no significant diet-by-week interaction (Table 2).

### *Worker Mortality*

Total worker mortality, analysed using a repeated measure ANOVA of log-transformed data, showed a significant diet-by-week interaction (Table 2). Colonies feeding on carbohydrate-biased diets (i.e. p14:c69 and p21c:62) experienced the lowest levels of worker mortality, and their mortality rates were consistent on a weekly basis (Fig. 5). In contrast, colonies feeding on the p41:c41 and protein-biased diets (i.e. p60:c20 and p67:c13) showed greater, and increasing worker mortality over the same period (Fig. 5). For nearly all diets (except p21:c62) worker mortality decreased, in

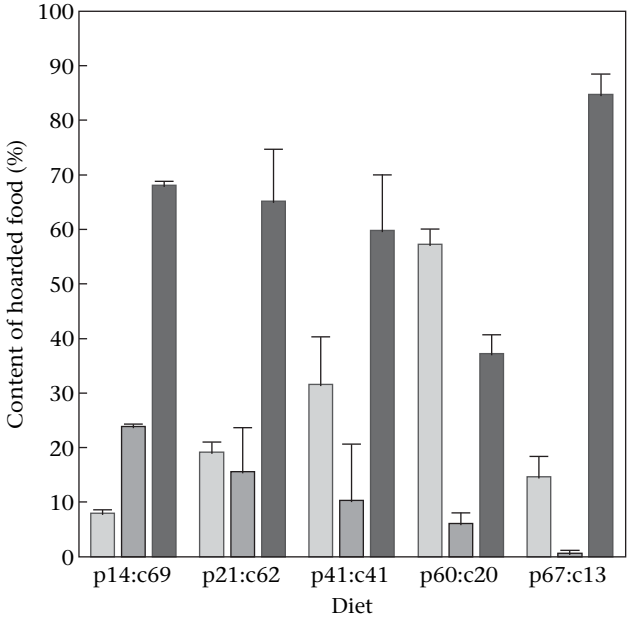


**Figure 3.** Median (±median absolute deviations) amounts of protein (▒), carbohydrate (▒), and 'nonmacronutrient' (■) expressed as a percentage of the dry weight of hoarded foods. The degree of dietary manipulation of hoarded foods by colonies can be determined from the hoarded food protein-to-carbohydrate ratios (p:c), which can be estimated from percentage values of protein and carbohydrate.

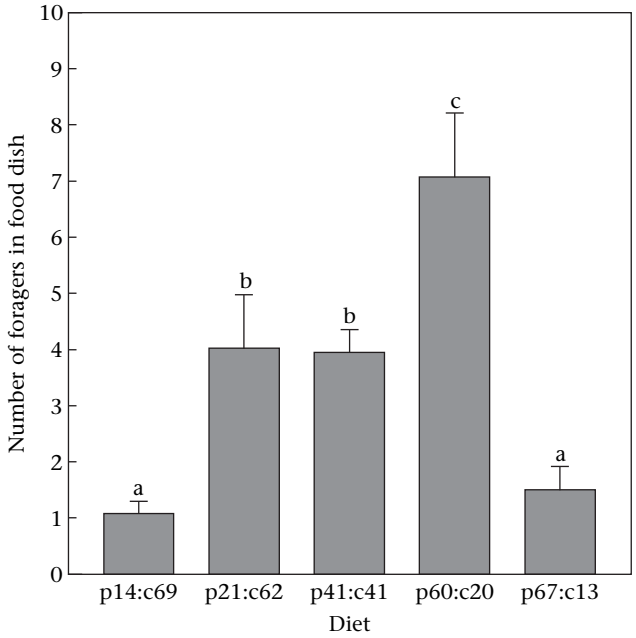


**Figure 4.** Mean + SE number of workers observed on experimental foods (from all spot checks combined). Different letters above columns indicate statistically significant differences between treatments from Tukey post hoc tests with alpha at 0.05.

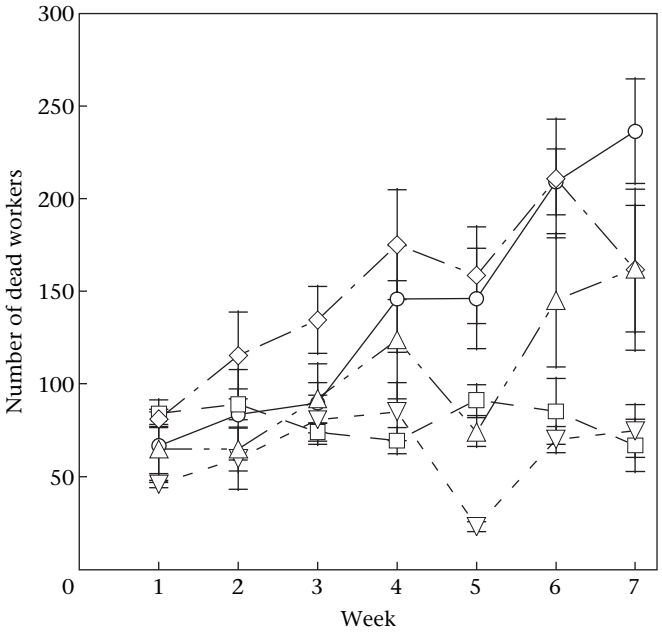


**Figure 5.** Mean ± SE number of dead workers from colonies on each treatment for each week over the duration of the experiment. Symbols and lines represent experimental diets: - - ▽ - -: p14:c69; — □ —: p21:c62; —○—: p41:c41; — · ◇ —: p60:c20; — · · △ —: p67:c13.

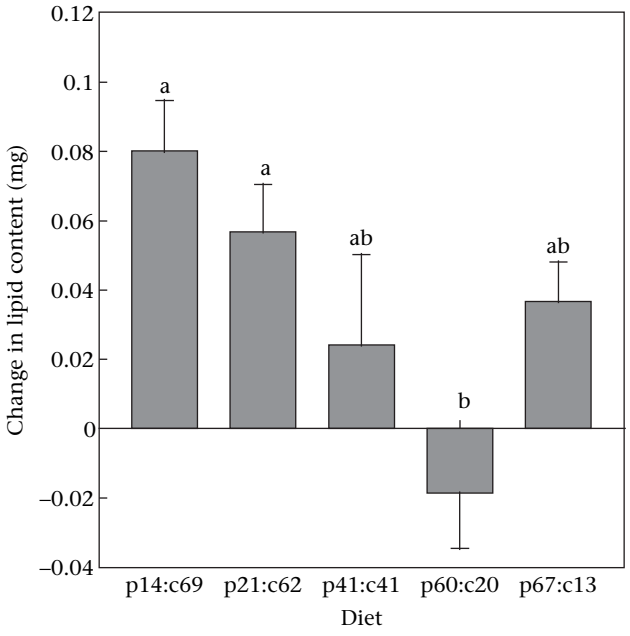


**Figure 6.** Mean + SE change in lipid content of workers at the end of the experiment. This variable was calculated as the average lipid mass of 100 workers from each experimental colony at the end of the experiment minus the average lipid mass of 100 workers from the source colony at the start of the experiment. Different letters above columns indicate statistically significant differences between treatments from Tukey post hoc tests with alpha at 0.05.

some cases sharply, from week 4 to week 5, but then increased again during weeks 6 and 7. A standard least-squared regression analysis of log-transformed worker foraging intensity and mortality data revealed that colonies with the most active workforce also had the highest levels of worker mortality ($r^2 = 0.38$, $F_{1,28} = 17.13$, $P < 0.001$).

### *Lipid Extraction from Workers*

Postexperimental whole-body worker lipid content, relative to whole-body lipid content of workers from source colonies (prior to the start of the experiments), was significantly affected by dietary p:c ratio (ANOVA: $F_{4,25} = 4.72$, $P = 0.006$; Fig. 6). Tukey post hoc tests revealed that lipid content of workers was statistically similar on the p14:c69, p21:c62, p41:c41 and p67:c13 diets. Worker lipid content was statistically similar on the latter two diets and the p60:c20 diet, but lipid content of workers on this last diet differed significantly from that of workers on the two carbohydrate-biased diets (Fig. 6). Relative worker lipid content significantly increased on the p14:c69 ($t$ test: $t_5 = 4.18$, $P = 0.009$), p21:c62 ($t_5 = 5.48$, $P = 0.003$) and p67:c13 ($t_5 = 3.21$, $P = 0.024$) diets. There was no significant change in relative worker lipid content on the p41:c41 ($t_5 = 0.92$, $P = 0.398$) or the p60:c20 ($t_5 = -1.19$, $P = 0.291$) diet.

Both log-transformed worker foraging intensity and log-transformed worker mortality were negatively correlated with relative worker whole-body lipid content (worker foraging intensity: $r^2 = 0.17$, $F_{1,28} = 5.61$, $P = 0.025$; worker mortality: $r^2 = 0.18$, $F_{1,28} = 6.34$, $P < 0.001$).

## DISCUSSION

Foraging ants need to attend to both their own nutritional needs (typically carbohydrates for energy) and those of their nestmates (carbohydrates for additional workers, but protein for queens and growing larvae). In this study we investigated, at the colony level, how ants regulate their protein–carbohydrate intake when they are constrained to feeding on solid, singly available diets having fixed p:c ratios. We also quantified, at both the individual and colony levels, behaviours and costs associated with feeding on these same diets. Our results revealed a number of significant findings. First, foods with balanced to moderately protein-biased p:c ratios were most attractive (measured in terms of amounts collected), while those with low p:c ratios were least attractive. However, across all dietary treatments, and consistently on the balanced and moderately protein-biased diets, colonies did not consume all the collected food, but instead hoarded the excess inside their nests. Interestingly, we found that ants extracted carbohydrates from the food they hoarded, but they only extracted protein from the high protein-biased diet (p67:c13). These data, when combined with the collection data, suggest that ants in our study prioritized carbohydrate intake over protein intake. Second, we found that the p:c ratio of the foods affected the number of foragers that collected food. Food collection was highest on the moderately protein-biased food, but equally low on the foods with extreme p:c ratios (p14:69 and p67:c13). Finally, we found that worker mortality was highest on diets with equal or high p:c ratios, and that both foraging and mortality rates were inversely correlated with worker lipid reserves. This finding suggests that a worker's physiological condition might be tightly linked to its behaviour and performance.

When foods contain key nutrients in low concentrations, animals can respond by increasing the amount of food they consume (e.g. Lavoie & Oberhauser 2004; Simpson et al. 2004; Berner et al. 2005). In the current study we interpret the collection of large amounts of equal-ratio (p41:c41) and protein-biased foods (p60:c20 and p67:c13) as an attempt to meet the colony-level carbohydrate requirements when restricted to feeding on carbohydrate-poor foods. Previous studies have shown that carbohydrate-deprived ants will ingest more carbohydrates when they encounter them (Sorensen et al. 1985; Josens & Roces 2000), and will also recruit more heavily to carbohydrate sources (Mailleux et al. 2006; also see below). Ants are also known to regulate

carbohydrate intake through increasing consumption of diluted carbohydrate sources (Dussutour & Simpson 2008a). In contrast to the aqueous carbohydrates in previously described studies, the carbohydrates in our study occurred in solid form, and were mixed with proteins at various concentrations. Despite the more complex nature of our experimental foods, workers of *S. invicta* were recruited to, and collected and hoarded, large amounts of our relatively carbohydrate-poor foods.

Prioritizing carbohydrates over protein by colonies in this study is further supported by the extent to which the colonies manipulated their hoarded food. Ants are regarded as eminent hoarders, collecting and caching excess resources, including liquid foods, seeds, leaves and arthropod prey (Hölldobler & Wilson 1990; Hart & Ratnieks 2000; Reyes-Lopez & Fernandez-Haeger 2002; Gayahan & Tschinkel 2008). A nutritional component often accompanies hypotheses of why and under what circumstances ants hoard resources, implying the need of colonies to provision resources in light of changing environmental conditions affecting resource availability (e.g. Judd 2006). Cues regulating hoarding behaviour should thus be distinct from those dictating immediate food choice (Vander Wall 1990). In the present study, and probably in most instances where hoarding has been observed, the hoarded food was not simply excess collected food retained for periods when a particular resource present in the food was required by the colony. Rather, workers chemically manipulated hoarded food through selective extraction of carbohydrates (see below), which was probably used to satisfy immediate physiological needs. Thus, although this cached, manipulated food may not reflect true hoarding (sensu stricto Vander Wall 1990), it nevertheless appeared to retain apparent nutritional value. We observed consistent hoarding by colonies on the p41:c41 and p60:c20 diets, and chemical analysis of the hoarded foods from these treatments showed that they both retained a significant amount of protein. A cache of protein-rich food would be of great value for developing larvae and reproductively active queens. Observations of postcollection manipulation of diets and caching of remains suggest that, given a nutritionally complex food item, decisions directing hoarding may be in response to both satisfying immediate needs as well as possible long-term requirements of the colony.

Extraction of significant amounts of carbohydrates from solid foods demonstrates a novel physiological mechanism that ants can use to meet their energy requirements. To extract carbohydrates, workers altered the consistency of the food from solid to a semi-liquid state (personal observations) probably through regurgitation of crop contents, and mixed with enzymes (e.g. invertase) secreted from glands (Ayre 1967; Ricks & Vinson 1972). The chemical analysis of the hoarded p67:c13 diet also suggests that ants, when restricted to foods with low carbohydrate content, may combine efficient extraction of carbohydrates with differential utilization of ingested proteins, via gluconeogenesis, to meet their energy needs. That gluconeogenesis might be occurring is also supported by the finding that relatively inactive colonies feeding on the p67:c13 diet, in contrast to those on the p41:c41 and p60:c20 diets, gained lipid content over the course of the experiment. Generating energy via gluconeogenesis has been observed in a number of insect herbivores (e.g. Raubenheimer & Simpson 2003; Thompson & Borchardt 2003), but to our knowledge this is the first study providing evidence that gluconeogenesis might also occur in ants, and is likely to be facilitated by the larvae (Hölldobler & Wilson 1990). The high protein content of the hoarded food piles on the p41:c41 and p60:c20 treatments, and the absence of lipid accumulation in workers from these two treatments suggests, however, that gluconeogenesis may only be triggered by diets that have extremely high p:c ratios.

Such mechanisms of nutrient regulation in ants can be costly, particularly when colonies are restricted to feeding on nutritionally imbalanced diets. These may be measured in relation to the energetic costs associated with food collection (Beauchamp 1992; Fernández et al. 2002), and also the physiological costs of processing excess dietary components (Raubenheimer et al. 2005; Boersma & Elser 2006; Dussutour & Simpson 2009). Additionally, energetic costs of extracting carbohydrates in solid form are likely to be elevated relative to those incurred when feeding on aqueous carbohydrates (see DiMeglio & Mattes 2000). For social insects, costs associated with nutrient regulation are felt colony-wide. However, acting as both the collective mouth, and to some degree the gut (Dussutour & Simpson 2009), cumulative costs of nutrient regulation appear to be incurred to a large extent by the worker caste, and expressed at a colony level through elevated worker mortality (Dussutour & Simpson 2009). High worker mortality is probably a side effect of carbohydrate limitation (Kay et al. 2006), and perhaps also from ingestion of excess soluble proteins (Lee et al. 2008; Dussutour & Simpson 2009). In this study, high worker mortality was probably partly due to a combination of these factors. However, costs associated with dietary manipulation (e.g. heightened foraging activity and possible negative effects of water loss) probably compounded factors negatively impacting worker longevity.

The continued decline in potential workforce recruits and the persistent collection and hoarding of often relatively large amounts of the p41:c41 and p60:c20 food suggest that continued collection of large amounts of food was performed by a single, highly motivated workforce, rather than a populous workforce. Motivation of a social insect colony may be measured in terms of recruitment of additional workers to a resource, and prompted by perceived richness of resource (Cassill 2003), the value of which varies with colony starvation (Mailleux et al. 2006; Rodrigues 2006). Motivation to forage at the colony level is a sum of individual worker motivation to leave the nest. Factors determining the membership of a social insect foraging workforce are thought to follow a colony-level phenological sequence guiding individual worker occupation (reviewed in: Traniello 1989: social insect foraging; Beshers & Fewell 2001: division of labour), and including worker age (reviewed in Robson & Beshers 1997), perceived life expectancy (Moron et al. 2008), social interactions (Robinson et al. 2009) and individual physiological condition, most notably body lipid reserves (O'Donnell & Jeanne 1995; Blanchard et al. 2000; Robinson et al. 2009). Furthermore, colony size is associated with the distribution of task assignment among individuals in insect societies (Jeanson et al. 2007; Dornhaus et al. 2008); a factor that is likely to be correlated with some aforementioned points.

In this study, increased individual motivation to forage was probably affected by a combination of the above factors. However, it particularly appeared to be driven by both increased value perceived by carbohydrate-starved workers of equal-ratio and moderately protein-biased diets, and for the latter treatment, relatively poor individual physiological condition of workers (i.e. decline in relative worker lipid content). The physiological condition of workers feeding on the moderately protein-biased diet (p60:c20) may have triggered foraging behaviour of a greater proportion of available workers from these colonies. Conversely, elevated lipid content of workers from colonies feeding on the other diets may have arrested the foraging behaviour of a greater number of available workers, as exemplified from physiological condition and foraging behaviour of colonies feeding on foods with the extreme p:c ratios. Focusing on the first hypothesis, research has shown that foragers of ant colonies tend to be leaner individuals (Blanchard et al. 2000; Robinson et al. 2009), and perhaps leanness in ants is regarded as an indication of impending death, thus triggering the more risky behaviour of foraging outside the nest (see Moron et al. 2008). Such physiological determination of behaviour deserves more study, including how the seasonal

changes to the physiological condition of social insects affects timing of foraging behaviour of individuals.

Macronutrient regulation probably occurs regularly in social insects under field conditions (e.g. Cook & Behmer, in press), and observations made in the present study show that *S. invicta* colonies can redress a diminishing carbohydrate supply through increased collection of carbohydrate-poor foods, and extraction of carbohydrates from these foods, attests to their capability. Additionally, nutrient availability and concomitant responses in foraging behaviour and physiological condition of individuals hint to these as other possible determinants of natural seasonal changes in nutrient preference and foraging intensity of natural populations of ants. We are currently addressing this latter issue by investigating seasonal shifts in nutrient regulation and associated behaviour and performance of colonies with disparate a priori resource backgrounds.

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