Responses to nutritional challenges in ant colonies

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Abstract

In social insects, food collection for the entire colony relies on a minority of its workers. How can the colony performance in the choice of resources, the allocation of workers, and the flexibility of food storage strategies emerge from the foraging decisions taken only by a minority? We addressed this question by posing nutritional challenges in the trap-jaw ants *Odontomachus hastatus* and explored their response in term of survival, foraging behaviour and energy storage. In the first challenge, ants alternated between long periods of confinement to a high protein diet to short periods of confinement to a high carbohydrate diet. In the second challenge, ants alternated between long periods of confinement to a high carbohydrate diet to short periods of confinement to a high protein diet. In the third challenge, ants were given simultaneously the high protein diet and the high carbohydrate diet. First, we showed that (i) mortality increased with protein consumption (ii) short access to a high carbohydrate diet lessened the negative consequence of high protein consumption (iii) ants given a choice of complementary diets regulated intake to minimize mortality. Second, we demonstrated that ants are using an energy saving strategy to overcome challenging nutritional environments. Third, we demonstrated that ants have an extraordinary capacity to regulate the amounts of food entering the nest: (i) at the collective level by allocating more workers to foraging on a high protein diet (ii) at the individual level by collecting more food on a high carbohydrate diet. Our study provides new insights into the strategies used by ants facing nutritional challenges and deepens our understanding of the nutritional ecology of ants, thereby, their vast ecological success.

Introduction

Animals live in a heterogeneous environment and their nutritional needs are likely to change over time, given the different demands for growth and reproduction. Thus maintaining an appropriate balance of nutrients is a major challenge for all animals. Individuals alone regulate their nutritional intake by selectively choosing the quality and quantity of food that meet their needs (Behmer 2009, Simpson and Raubenheimer 2012). The complication for animals living in groups, such as social insects, is that a minority of workers collects the food for the colony, and these individuals have very different nutritional requirements to other members of their colony (Brian and Abbott, 1977; Cassill and Tschinkel, 1999; Holldobler and Wilson, 1990; Sorensen et al., 1985, reviewed in Feldhaar 2014). For example, the egg-laying queen and the larvae have a much higher need for protein than do workers. Consequently, social insects exhibit a striking division of labour where nutritional feedbacks emanating from brood and workers exist and must be integrated by foragers during food harvesting (Dussutour and Simpson, 2008a; Dussutour and Simpson, 2009). Therefore, it is essential to understand how individuals modulate their behaviour in order to obtain the nutrients necessary for the survival, development and reproduction of not only themselves but all members of the group.

Important questions about animal nutrition can only be answered using an integrative approach, which takes into account several attributes of the environment (for example protein and carbohydrate content of the food) and of the animal (physiology, behaviour, etc.), and enables to study the interactions among these components. The Geometric Framework (GF), a state-space modelling approach, has been specifically designed to address such questions (Simpson and Raubenheimer 2012). This framework enables to accurately decipher the distinct and interactive roles of protein (P) and carbohydrates (C) on animal physiology and behaviour. Its application to the study of nutrition in a wide range of animals, from slime moulds to humans (Simpson & Raubenheimer 2012), has helped to answer important questions in nutritional ecology and biology, from the distribution of animal communities to the vulnerability of humans to obesity (Simpson & Raubenheimer 2012).

Using the GF, recent studies have shown that, as with other non-social organisms, ant colonies regulate separately their intake of both protein and carbohydrate when nutritionally imbalanced but complementary diets are available (Cook and Behmer, 2010; Cook et al., 2009; Dussutour and Simpson, 2008a; Dussutour and Simpson, 2009). When restricted to a single nutritionally imbalanced diet, food intake is driven essentially by carbohydrate, increasing as the percentage of carbohydrate in the diet decreases (Dussutour and Simpson 2009). Therefore, when ants are confined to a high protein–low carbohydrate diet, they compensate for the

shortage of carbohydrate by increasing drastically their food intake to meet their requirements in carbohydrate (Dussutour and Simpson, 2009; Dussutour and Simpson, 2012). However, this compensation comes at a cost; when ants consume a higher proportion of protein than required, mortality is higher, as observed in other insects (Hamilton et al., 1990; Lee et al., 2008; Maklakov et al., 2008; Pirk et al., 2010).

It remains unclear how being confined to a high-protein diet increases mortality rates. Firstly, such a decrease in longevity could result from a deficit in energy reserves. In insects, lipid storage reserves tend to decrease as they feed on a high protein diet while they tend to increases as they feed on a high carbohydrate diet (Behmer et al 2002). In most insects, carbohydrates are converted to lipid by lipogenesis and stored in the fat body (Canavoso et al 2001). Cook et al. (2010) suggested that generating energy store from protein *via* gluconeogenesis is possible in ants but might incur a cost at the physiological level and ultimately affect colony survival. Secondly, an increase in effort spent in foraging activities on a high protein diet may weaken ants and explain the decrease in survival probability (Houston and McNamara, 2014; Straka et al., 2014). It has been shown in previous studies that ants fed a high protein diet significantly increase their foraging activity to collect more food in order to compensate for the lack of carbohydrate (Dussutour and Simpson, 2012, Cook et al 2010). Therefore, a key step in building links between high protein diet and lifespan is then to clarify how nutritional challenges such as carbohydrate scarcity or/and protein excess affect physiological (energy store) and behavioural (foraging activity) traits and relate to survival. However, contrary to survival, until now, foraging behaviour (Dussutour and Simpson, 2012, Cook et al 2010) and energy storage (Cook et al. 2010) have only been measured at the colony level. However, because insect colonies can be composed of individuals radically different in form and function (Oster and Wilson 1978, Jeanson and Weidenmüller 2014), it is essential to determine what happens at the individual level to better understand the strategy observed at the colony level.

In this paper, we set about confining ant colonies to nutritional challenges such as carbohydrate scarcity and/or protein excess and explored their response at both the individual and collective level in term of survival, foraging behaviour and energy storage. The experiments were conducted with colonies of *Odontomachus hastatus*, a predatory ant species originally from French Guyana. In this species, workers are relatively large, which allows us to equip ants with passive microtransponders and automatically record their foraging activity (Jeanson, 2012; Moreau et al., 2010). In addition, their large sizes make them ideal to measure fat storage at an individual level.

Materials and Methods

(a) Study species and rearing conditions

In this study we use the arboreal trap-jaw ant *O. hastatus* (Hymenoptera: Formicidae). These ants measure approximately 13 mm long and are monomorphic. They live in the tropical forests of Central and South America and nest in the roots of epiphytic plants or in leaf litter accumulated at the base of palm trees. In this species, colonies' size varies between 20 to 500 individuals (Jeanson personal observation). Their diet is composed mainly of arboreal arthropods (Camargo and Oliveira, 2012). We collected 12 colonies in French Guiana (GPS: 4°05′N, 52°41′W) in January 2012. In French Guiana colonies of *O. hastatus* have one single reproducing queen (monogynous) (Jeanson 2012). We housed each colony in a plastic box inside which we placed several test tubes acting as nests. The tubes contained a reservoir of water held with a cotton plug and were surrounded by black paper, thereby recreating the dark, humid conditions found in nature. Before starting the experiments, we supplied colonies *ad libitum* with water and a mixed diet of vitamin-enriched food containing a 1:2 ratio of total proteins to digestible carbohydrates. (Dussutour and Simpson, 2008b), replenished every two days.

Before starting the experiments, we subdivided the 12 colonies in experimental queenless sub-colonies. We placed the ants in an experimental nest that was connected to a foraging arena with plastic tubes. The nest consisted of a square petri dish (10 cm x 10 cm x 1 cm) with a 5 mm layer of moist cotton, which we remoistened every two days to keep the nest chamber humidity levels high. We covered the nest compartment with a cardboard cover. The foraging arena consisted of a similar petri dish divided into two compartments (10 cm x 5 cm each) using wire mesh (10 cm x 1 cm) preventing displacements between compartments. A plastic tube (7 mm diameter) connected each foraging compartment to the nest (see Fig. S1). We placed the ants in experimental nests 3 days before the start of experiments to allow them time to habituate to the set-up. We carried out the experiments under red light with the nest cardboard cover removed, as foraging activity in this species is concentrated at night (Camargo and Oliveira, 2012). We maintained all the colonies under a 12:12 light/dark photoperiod at ambient humidity and temperature.

(b) Synthetic Foods

We used two artificial diets varying in the ratio of protein and carbohydrate with a fixed total macronutrient of 200 gL^{-1}: a high protein diet, P, with a protein to carbohydrate ratio of

5:1, and a high carbohydrate diet, C, with a protein to carbohydrate ratio of 1:5 (Dussutour and Simpson 2012). We used casein (Nutrimuscle), whey protein (Nutrimuscle) and whole egg as source of protein and glucose as a source of carbohydrate. The quantity of whole egg was identical in each diet to keep the same concentration of fat and minerals. The diets contained 0.5% vitamins (Vanderzant vitamin mixture for insects; Sigma). We were offered the nutrients in a 1 per cent agar gel. Each diet had total P + C of 200 $g.L^{-1}$ (for further preparation details see Dussutour and Simpson 2008, 2012).

(c) Experiment 1

In the first experiment, we investigated the effect of various nutritional challenges on the longevity, lipid stores and foraging activity of a carnivorous ant.

We confined 24 experimental colonies consisting of 30 ants and 8 larvae originating from 8 colonies to one of three diet treatments (8 experimental colonies per treatment). In the first one (treatment P), we explored the responses of colonies when switched from long periods of confinement to a high protein diet (day1 to day 16, day 18 to day 33) to short periods (day 17 and day 34) of confinement to a high carbohydrate diet. In contrast, in the second treatment (treatment C), we explored the responses of colonies when switched from long periods of confinement to a high carbohydrate diet (day1 to day 16, day 18 to day 33) to short periods of confinement to a high protein diet (day 17 and day 34). In both treatments, we confined colonies to an imbalanced food; hence, ants could either meet their requirements for protein or meet their requirements for carbohydrates. The switches gave us the opportunity to assess the ants' ability to compensate and recover for the imposed imbalances. In the third treatment (treatment P&C), we offered colonies a choice between a high protein diet (P) and a high carbohydrate diet (C) for 34 days, to establish whether they regulate their intake of protein and carbohydrate. In this treatment, the switch consisted in swapping the diets position on day 17 and 34, allowing us to distinguish between spatial and nutritional specialization (Fig S1). After 34 days, we sacrificed 12 colonies (four per treatment) and we continued the experiment for the remaining colonies until day 100 but without further switches i.e colonies were confined only to diet P (P treatment), only to diet C (C treatment) or simultaneously to both P and C diets (P&C treatment). The experiment was carried out in two consecutive series, with 12 colonies in each series. The food was provided in the foraging arena in both compartments for only three hours a day every day.

The effect of nutritional challenges on ant longevity

To assess mortality, we counted the number of dead ants within each experimental colony every day for 34 days. We then removed the corpses from the colony and stored them at -20°C. After day 34, we sacrificed the ants in 12 experimental colonies (four per treatment) and stored them at -20°C. We continued to feed the remaining twelve colonies the same diet until day 100. After day 100, we sacrificed all surviving ants and stored them at -20°C.

The effect of nutritional challenges on ant lipid stores

We determined the quantity of lipid content in all individual ants used in the experiment, using a lipid extraction protocol modified after (Cook et al 2010). We dried all ants in an oven overnight at 70°C, weighed to the nearest 0.001 mg and placed in an eppendorf tube. Chloroform (2 ml) was added to each tube. After 24 hr. we removed the chloroform and we added 2 ml of fresh chloroform. We completed a total of three chloroform soaks. We then dried the ants again at 70°C and reweighed. The difference in the weight of the ant before and after lipid extraction gave us the weight of the lipids in the samples. For each ant sample we then calculated the proportion lipid weight as a function of original dry weight.

Ants that died naturally might have remained up to 24 hours inside the nest after death before being frozen, as they were collected only once a day. Conversely, ants that survived were sacrificed and frozen immediately. Thus, as a control, using ants picked up randomly in 2 colonies, we examined whether there was a difference between ants that were sacrificed and left 24 hours in the nest (N=60), and ants that were sacrificed and immediately frozen (N=60). We found no difference in the proportion of lipid content (LME, $F=1.68$, $P=0.418$, N=120 ants).

The effect of nutritional challenges on ant behaviour

We equipped each ant in the experiment with a RFID (radio-frequency identification) tag, glued to their thoraxes using superglue (Jeanson 2012). Using two transponder readers placed next to the connecting tubes (Fig. S1), we could automatically identify the ants when they left the nest to forage or when they returned to the nest. We filmed each experimental set-up from above using a video camera (Sony HD-CX700V). Tag detection and filming was carried out every two days. We analysed the directional movements of all ants moving between the nest and foraging arena using our RFID tag detection data. We found there to be a 5% error in these RFID data due to lack of tag detection if an ant passed one of the two readers too quickly. We corrected this error using the video footage of ant movements and we used only these corrected data for our analyses. Using these RFID data we were able to measure the number of visits between the nest and the foraging arena and the time spent in the nest and the

foraging arena between visits.

Using the video footage we monitored ant behaviour in the foraging arena. For all experimental colonies we examined the behaviour of 5 ants (or as many that were present up to 5 ants) for each compartment and each observation day. We recorded, if an ant fed or not during a visit, the duration of the ants' first meal (uninterrupted feeding event) which reflects taste responses to the food (Simpson and Raubenheimer, 2000), and the total time spent feeding (total time spent feeding during a visit to the foraging arena) giving us an idea of the quantity of food ingested (Dussutour and Simpson, 2012). During the course of the experiment, ants were removing food from the food source and storing it as pellets in the nest. We recorded whether or not an ant was holding a pellet for every ant returning to the nest for the 3-hours trials, for each day and each colony.

(d) Supplementary Experiments

Experiment 2: The effect of external temperature on ant longevity

Due to the limited number of experimental set-ups available, we carried out Experiment 1 in two consecutive series. The mean ambient temperatures for the two series were significantly different from each other (two-sample T-test, T=-3.3, P=0.001, N=68 days, mean maximum temperature ± SD = 23.7±4.34 and 28.01±3.30 for series one and two, respectively). As presented in the result section below, mortality was significantly different between the two series but only for treatment P. In order to examine more precisely if temperature was the factor affecting ant mortality on treatment P we performed a second experiment to examine the effect of temperature on the mortality of ants fed on a high protein diet. Due to a limited number of ants, we chose to conduct this experiment at an individual level. We placed 60 individual ants from 10 colonies into separate circular petri dishes (1.5 cm x 8.5 cm diameter) and placed them in a controlled temperature environment of either 24°C (N=30) or 28°C (N=30). Each petri dish contained a water supply (through a cotton plugged test-tube with a water reservoir), a food supply of high protein food and some tissue paper for shelter. Food was provided *ad libitum* and replaced every two days. We recorded ant mortality every day for 34 days.

Experiment 3: The effect of food restriction on ant longevity and lipid content

Our lipid analyses results from Experiment 1 revealed that surviving ants on treatment P were fatter than ants on the C and treatment P&C suggesting an energy saving food storage strategy in response to carbohydrate shortage. In order to test the hypothesis that carbohydrate shortage was responsible for the increase in lipid reserves observed on treatment P, we

conducted a third experiment. We first confined 12 experimental colonies each consisting of 20 to 30 ants, to a rearing diet for 30 days (pre-treatment). During this 30-day period we assessed mortality every day and stored all the dead ants to assess their lipid content. After 30 days, we randomly sacrificed a total of 30 ants from all the colonies and performed lipid extraction. We then assigned the colonies to one of two treatments. The first group of 6 experimental colonies received the P diet with a feeding period of three hours for 34 days except on days 17 and 34 when they received the diet C (treatment P), similarly to experiment 1. In an attempt to restrict carbohydrate access, the second group of 6 experimental colonies received the C diet but this time with a feeding period of only one hour for 34 days, except on days 17 and 34 when they received the P diet for an hour also (treatment C-restricted). We carried out the experiments at 24°C and we assessed mortality every day for 34 days. We extracted lipid content for all ants that died during the experiment and for all ants that survived after 34 days.

Experiment 4: Correlation between foraging activity and lipid stores

We equipped 4 experimental colonies of 30 ants with RFID tag and fed them the P diet for three hours a day for 34 days, except on day 17 and 34 when we fed them the C diet for three hours, but this time we recorded their foraging activity for 24 hours a day instead of 3 hours. We measured ant lipid content when ants died or at the end of the experiment for those that survived in an attempt to relate foraging activity to lipid stores.

(e) Statistics

For all the experiments, we compared longevity data across treatments using a Cox regression analysis, with treatment, series (when needed) and colony as categorical variables and relative activity (when needed) as continuous variables. Treatment, relative activity and series were included in the analysis as factors. We included colony as a clustered term *i.e.* a group of sampling units because ants from the same experimental colony are unlikely to be independent. In the first experiment, to investigate the switch effect we compared the probability of dying before and after the switch using a mixed linear model. We included treatment, switch (before and after) and series in the analysis as fixed factors, whereas we included colony as a random factor.

For the first, the third and the fourth experiment, lipid data across treatments were compared using a linear mixed effect model (LME) or generalized linear mixed effect model (GLMM), with treatment, death (survived or died), series (when needed), ant weight, time

(when needed) and colony as variables. Series, treatment, time, death and weight were included in the analysis as fixed factors, whereas colony was included as a random factor.

All variables related to foraging behaviour and feeding behaviour were compared using generalized linear mixed effect model (GLMM) with day (when required), series, treatment, ants, (when required) and colony as variables. Series, treatment, diet and day were included in the analysis as fixed factors, whereas colony and ants ID were included as a random factor. To control for visit distribution between the two compartments of the foraging arena we used a one-sample t test considering only the ants that made more than 5 visits to the foraging arena throughout the whole experiment. For all experiments, normality was assessed using a Kolmogorov and Smirnov test and data were transformed to meet the assumption of normality when needed. For each dependent variable, we obtained a minimal model by a stepwise backward elimination procedure, i.e., by successively removing from the model the non-significant terms. All statistical tests were conducted with SPSS (v. 20, SPSS Inc. Chicago, U.S.A).

Results

Experiment 1:

Long exposure to a high protein diet shorten lifespan

Workers mortality was significantly affected by nutritional challenges. Until day 34, ants lived longer on both treatment C and P&C than on treatment P (Fig. 1A; Cox regression, Wald=35.88, P<0.001, N=720 ants) and the difference in ant mortality between both treatments C and P&C was not significant (P=0.384). When observed until day 100, ants lived longer on treatment P&C than on treatment C (Fig. 1B; Cox regression, Wald=12.19, P<0.001, N=240 ants). The difference between treatments C and P&C arose after about 45 days. We found that ants lived longer during the first series of experiments than during the second series of experiments but only for treatment P (Cox regression, Wald=32.99, P<0.001, N=720 ants). Lastly, treatment did not have any significant effect on larvae mortality (Cox regression, Wald=4.91, P=0.054, N=192 larvae).

Short exposure to the C diet lessen the dire effects of the P diet

To investigate the effect of the food switch on ant mortality, we compared the survival probabilities 4 days before the switch to 4 days after the switch. This period of measurement was chosen to correspond with the time required to respond again to nutrient scarcity (Mailleux et al 2006). The probability of dying on treatment P decreased after the switch, while it remained

unaffected by the switch for both treatments C and P&C (Fig. 1C; GLMM, treatment: F=15.65, P<0.001; switch: F=5.45, P=0.021; treatment*switch F=4.81, P=0.009; N=192).

Long exposure to a high protein diet lead to high lipid stores

We distinguished the ants that died naturally during the experiment from those that survived and that were sacrificed after 34 days. Ants that died naturally during the experiment were leaner on treatment P than those on the C and treatment P&C. Conversely, ants that survived the experiment were fatter on treatment P than on treatments C and P&C (Fig. 1D; GLMM, treatment: F=0.48, P=0.618; treatment*death F=70.09, P<0.001; N=343 ants). For all treatments, ants that died naturally during the experiment were leaner than the ants that survived (death: F=110.61, P<0.001). The weight of the ant after lipid extraction did not have a significant effect on lipid reserves (weight: F=0.29, P=0.586).

No spatial or nutritional specialization, ants switch between diets

Ants visited both compartments of the foraging arena and did not express any spatial preference at the individual level for all treatments (Fig S2A, one-sample t test, t=-1.45 P=0.152, N=89; t=-1.6, P=0.112, N=100; t=0.321, P=0.749, N=105; for P, C and P&C respectively). On treatment P&C, ants visited the compartment containing the C diet as many times as the compartment containing the P diet suggesting that ants did not specialized on a particular nutrient (Fig S2B, one-sample t test, t=0.19, P=0.848, N=105).

Weak task specialisation, many ants contribute to foraging

Over the course of the experiment, almost all ants were observed making at least one visit to the foraging arena (GLMM, treatment: F=2.53, P=0.107, N=24; mean proportion of ants observed foraging at least once: mean±CI95 0.87±0.06, 0.72±0.14 and 0.82±0.10 respectively for P, C and P&C treatment). However, on treatment P, the same ant was more likely to be observed on multiple days visiting the foraging arena (GLMM, treatment: F=10.88, P<0.001, N=586; mean proportion of days a same ant was observed foraging over the course of the experiment: mean±CI95: 0.45±0.05, 0.33±0.08 and 0.36±0.06, for P, C and P&C treatments, respectively).

Long exposure to the P diet lead to high foraging activity at the collective level while long exposure to the C diet lead to high foraging activity at the individual level

At the collective level, the daily colony foraging activity (defined as the number of daily visits to the foraging arena at the colony level divided by colony size) was higher on treatment P than on treatment C (Fig. 2A; GLMM, treatment: F=11.76, P=0.001, N=253) even when we switched diets (Fig. 2A; switch: F=0.07, P=0.787). This was due to a higher proportion of different ants engaged in foraging behaviour on treatment P in comparison to treatment C (Fig. 2B; GLMM, treatment: F=21.18, P<0.001; switch: F=0.07, P=0.796; N=253)

At the individual level, ants did fewer visits per day on treatment P than on treatment C (Fig. 2C; GLMM, treatment: F=53.03, P<0.001; switch: F=4.48, P=0.034; N=1654). The lower number of visits on treatment P was not attributable to longer visits to the foraging arena (Fig. 3A; GLMM, treatment: F=0.62, P=0.429; switch: F=13.49, P<0.001; N=1394) but to longer time spent in the nest after a visit to the foraging arena (Fig. 3B; GLMM, treatment: F=4.23, P=0.040; switch: F=0.73, P=0.392; treatment*switch F=10.75, P<0.001; N=1041). The number of visits decreased on treatment C when we switched the diet (ants were offered the P diet instead of the C diet) while it remained low when we switched the diet on treatment P (ants were offered the C diet instead of the P diet).

When offered simultaneously P and C diets (P&C treatment), foraging activity, proportion of ants engaged in foraging, number of daily visits per ant and visit duration did not differ significantly between the compartment offering the P diet and the compartment offering the C diet (Fig. S3A-C, Fig. 3C). Interestingly, as seen for treatment P, ants spent more time in the nest after a visit to the compartment offering the P diet than a visit to the compartment offering the C diet (Fig. 3D; GLMM, diet: F=10.88, P<0.001, switch: F=0.55, P=0.458; N=550).

The P diet is not attractive

The proportion of ants feeding when encountering the food during a visit to the foraging arena was lower on treatment P than on treatment C (Fig. 4A; GLMM, treatment: F=1.10, P=0.295; switch: F=2.09 P=0.149; N=553). This pattern was reversed on switch days when the ants on treatment P were offered the C diet instead of the P diet and the ants on treatment C were offered the P diet instead of the C diet (treatment*switch: F=28.72, P<0.001). Likewise, on treatment P&C the proportion of ants feeding was higher in the compartment offering the C diet than in the compartment offering the P diet (Fig. 5A; GLMM, diet: F=12.79, P<0.001, switch: F=1.32, P=0.251; N=337).

The P diet is not appetizing

First meal duration which often indicates food palatability, was shorter on treatment P than on treatment C and this pattern was again reversed on switch days when the diet were switched (Fig. 4B; GLMM, treatment: F=42.30, P<0.001; switch: F=0.53, P=0.469; treatment*switch F=182.82, P<0.001; N=420). On treatment P&C ants had longer first meals on the C diet than on the P diet (Fig. 5B; GLMM, diet: F=39.08 P<0.001; switch: F=2.01, P=0.157; N=265).

Short exposure to C diet lead to an increase in food consumption

The time spent feeding during a visit, which indicates food consumption at the individual level, did not differ significantly between P and C treatments, except the days we switched the diets. On treatment P, when ants fed the P diet were switched to the C diet, they doubled the time spent feeding (Fig. 4C; treatment: F=33.29, P<0.001; switch: F=3.88 P=0.050; treatment*switch F=17.14, P<0.001; N=420). On treatment P&C, ants fed for longer on the C diet than on the P diet (Fig. 5C; GLMM, diet: F=5.95, P=0.016; switch: F=0.78, P=0.783; N=232).

Ants bring less food to the nest when encountering the P diet

The proportion of ants returning a pellet of food was lower on treatment P than on treatment C. The proportion of ants returning a pellet increased when ants fed the P diet were switched to the C diet; this pattern was reversed for the ants fed the C diet and switched to the P diet (Fig. 4D; GLMM, treatment: F=22.93, P<0.001; switch: F=0.14, P=0.708; treatment*switch F=77.78, P<0.001; N=1183). Similarly, on treatment P&C the proportion of ants returning a pellet was higher on the C diet than on the P diet (Fig. 5D; GLMM, diet: F= 46.78, P<0.001; switch: F=1.77, P=0.183; N=578).

Ants collect the same quantity of carbohydrates no matter the nutritional challenges

To sum up the previous results, we computed an index to estimate the quantities of both protein (i_P) and carbohydrate (i_C) collected daily at the individual level for each treatment and plotted them in a nutritional landscape (Fig. 6). The indexes i_P and i_C were computed as follow:

$$i_P = ((p_d * n_d) / N_d) * T_d * P$$

$$i_C = ((p_d * n_d) / N_d) * T_d * C$$

Where p_d is the proportion of ants feeding during a visit to the foraging arena on day d, n_d is the number of visits to the foraging arena on day d, N_d is the colony size on day d (to adjust for ant mortality), T_d is the time spend feeding while visiting the foraging arena, P the concentration

405 in protein in the diet and *C* the concentration in carbohydrate in the diet. Estimated food
406 collection indexes i_P and i_C over 34 days are shown for each treatment in Fig. 6. The computed
407 indexes indicated that all colonies managed to collect almost the same quantity of carbohydrates
408 at the end of the experiment regardless of the diet that they were fed. On treatment P, collection
409 of food increased on switch days to provide limiting carbohydrate (indicated by the pronounced
410 kink upward in the consumption trajectory).
411
412 *High foraging activity leads to high mortality, except when ants feed on the P diet*
413 In order to explain the variability in lifespan in relation to foraging activity, we
414 examined the relative individual activity, which can be regarded as the 'density' of visits to the
415 foraging arena within the ant's entire period of observation. Thus, for each individual we
416 computed the relative activity as the number of its visits to the foraging arena within the entire
417 period of observation divided by lifespan. When considering all treatments we found that
418 relative activity was an important predictor of survival, namely ants that were more active died
419 earlier than less active ants (Cox Regression, relative activity: Wald=26.05 P=0.001; relative
420 activity*treatment: Wald=8.38 P=0.018; N=720). However within a treatment, this was true
421 only for treatments C and P&C (Cox Regression, Wald=12.21, P=0.010, N=240 and
422 Wald=20.41, P=0.003, N=240, respectively), but not for treatment P (Cox Regression,
423 Wald=1.6 P=0.246, N=240). To simplify, we illustrated this result in Fig. 7 by distinguishing
424 again ants that died naturally during the experiment from ants that survived.
425
426 **Experiment 2**
427 *High temperature shorten lifespan*
428 As seen above, mortality rate was significantly different between both series of
429 experiment but only for treatment P (mean half life 28.25 and 13.5 for series 1 and 2
430 respectively) while it had no effect on lipid content and behaviour. Thus we performed a second
431 experiment at an individual level to examine more precisely if temperature was the factor
432 affecting ant mortality on treatment P. We confirmed that high temperature shorten lifespan of
433 ants fed the P diet (Fig. 8; Cox regression, Wald=8.10, P<0.001, N=60; mean half life 30 and
434 17 days for series 24° and 28° respectively).
435
436 **Experiment 3**
437 *Carbohydrate restriction increases lipid stores*

Ants lived longer on treatment C-restricted than on treatment P (Fig. 9A; Cox regression, Wald=9.3, P=0.002, N=319). On both treatments the ants that died naturally during the experiment were again leaner than the ants that survived (Fig. 9B; GLMM, death: F=35.19, P<0.001; death*treatment F=4.1, P=0.044; N=278). Ants that survived on treatment P were as fat as the ants that survived on the C-restricted treatment (treatment: F=1.06, P=0.303). The ants that survived on treatment P and treatment C-restricted, respectively, were fatter after the treatment than before the treatment (time: F=55.70, P<0.001, Fig. S4). Again the lipid content did not depend on ant weight after removal of fat store (weight: F=0.18, P=0.674).

Experiment 4

No Correlation between foraging activity and lipid stores

Similarly to the results of Experiment 1, ants that died naturally during the experiment were leaner than the ants that survived (LME, death: F=83.21, P<0.001, N=107) and individual relative activity (number of visits/lifespan) did not affect lifespan (Cox Regression, Wald=6.07, P=0.091, N=107). The level of lipid stores at the end of the experiment did not depend significantly on relative activity (LME, activity: F=0.04 P=0.850, N=36 ants), meaning that ants that visited the foraging arena often did not get leaner or fatter.

Discussion

In this investigation we carried out a series of experiments on colonies of *O. hastatus* ants to determine how nutritional challenges interacts with lifespan, energy storage and behaviour.

Our longevity study revealed that long exposure to a high protein diet causes the highest mortality in ants, consistent with previous studies (Cook and Behmer, 2010; Dussutour and Simpson, 2009, 2012). While a high carbohydrate diet proved better than the high protein diet in terms of longevity, ultimately throughout 100 days, ants lived longest when presented with both types of diets. When offered two complementary diet P and C, ants select a diet composition P:C of 1:2. This protein-to-carbohydrate ratio did not change over time (Fig. 6), indicating that ants regulate both protein and carbohydrate on a relatively short-term basis. The balance achieved in *O. hastatus* is more protein biased than in the black garden ants *Lasius niger* (Dussutour and Simpson 2012). This is expected knowing that *L. niger* feed mainly on honeydew (Fiedler et al. 2007), a carbohydrate rich resource while *O. hastatus* feed mainly on arthropods (Camargo and Oliveira, 2012), In addition, we have found that switching ant colonies to a high carbohydrate diet alleviates the negative effects of a high protein diet. Such

beneficial effects are found after only one day of exposure to the high carbohydrate diet. Conversely, when ants were previously fed high carbohydrate food and switched for a day to a high protein diet, we did not observe the negative effects of the high protein diet later on. This result differs from that obtained by Dussutour and Simpson (2012) on *L. niger*. They showed that only one day of exposure to a high-protein diet had dire consequences for the ants, reducing colony size by more than 20 per cent. The difference in diet habits between these two species, might again explain the difference in high protein diet susceptibility. *O. hastatus*, as other predators (Wilder 2011), might be better adapted to protein-rich diets and might over-consume protein for short periods with lesser consequences their omnivorous counterpart.

Furthermore, environmental temperature and diet have an interactive effect on ant longevity. Here we show that mortality increases with increasing temperature only when ants were confined to high protein foods. This result is unlikely to be due to an increase in activity as a result of higher temperatures (Schmid-Hempel et al., 1985), as we did not observe any differences in foraging behaviour between our two series. Moreover, we have shown that a higher relative activity at the individual level is associated to a shorter lifespan only within treatments C and P&C. Ants that died or survived on treatment P did not display any difference in individual relative foraging activity. Therefore, temperature might have affected longevity through alterations of metabolic rate, increase of water loss and decreased tolerance to toxins and diseases (Calabi and Porter, 1989; Fan and Wernegreen, 2013; Simpson and Raubenheimer, 2012; Bouchbeti et al 2015).

In this study we also investigated how nutritional challenges affects energy storage in ants, which are likely to affect mortality. We observed that all ants that died naturally during the experiment were leaner than the ants that survived, and ants that died on treatment P were the leanest. This indicates that ants' death is correlated with lipid store depletion and second that lipid mobilization is higher on treatment P. Interestingly, ants that survived on treatment P were the fattest. These results may suggest that ants that had greater lipid reserves at the start of the experiment survived better on the high protein diet using an energy saving strategy. Alternatively it is possible that ants accumulate lipids throughout the experiment in response to carbohydrate shortage using an energy storage strategy. We were able to distinguish between these two strategies in our third experiment, which examined the effect of carbohydrate scarcity, either due to a low concentration of carbohydrate in the food (P treatment) or to a temporal restriction in the exposure to carbohydrate rich food (C restricted diet). In this experiment, lipid stores were measured before and after the treatment, and we were able to show that ants accumulated lipids throughout the experiment (Fig. S5). Limitations in the abundance of

carbohydrate may result in ants employing physiological mechanisms to increase their lipid reserves. Lipids can be synthesised from carbohydrates, as well as proteins (Arrese and Soulages, 2010, Cook et al 2010, Thompson 1998, Thompson and Redak 2000). Insects adapted to energy-poor diets may often store rather than metabolize energy (Warbrick-Smith et al. 2006). Storage response as an adaptation to episodic food availability is often related with an opportunistic predator strategy (Jensen et al. 2010, Wilder 2011, Jensen et al. 2012). Although *O. hastatus* is essentially carnivorous, it also feeds on sugary fluids gathered from fruit secretions (Camargo and Oliveira 2012). However, *O. hastatus* colonies inhabit tropical rainforest understory habitat where carbohydrate rich food such as fruits may be spread out in time and space, leading to unpredicted carbohydrate availability (Camargo and Oliveira 2012). Such a strategy ultimately results in better survival. Interestingly, only a few ants were able to increase their lipid stores and survived treatment P. This difference could be due to ant age and/or foraging activity. However, as opposed to a previous studies in social insects (Blanchard et al., 2000, Toth and Robinson, 2005), we did not see any correlation between lipid stores and foraging activity in ants confined to a P treatment, hence, only the age factor remains to be tested.

At the behavioural level, ant colonies fed on a high protein diet had a higher daily foraging activity than colonies fed on a high carbohydrate diet, presumably in an effort to maintain a constant carbohydrate intake. Such compensatory responses to nutrient composition have been demonstrated across a variety of insect groups including ants (review in: Dussutour and Simpson, 2008a; Dussutour and Simpson, 2009; Dussutour and Simpson, 2012; Simpson and Raubenheimer, 2012). In most ants, few members of the colony venture outside to collect food for the entire colony. Hence, foragers must adapt food collection strategy to meet every member nutritional requirements within the nest. Ants can do this at the collective level by modulating the number foragers or, at an individual level, by modulating the quantity of food harvested (Dussutour and Simpson, 2008a).

In this study, we confirmed that ants modify, at the collective and individual level, their feeding behaviour according to carbohydrate and protein concentration (Dussutour and Simpson 2008, Arganda et al., 2014). At the collective level, on the high protein diet, twice as many more ants were engaged in foraging activity than on the high carbohydrate diet indicating some sort of motivation to forage at the level of the colony. At the individual level, when encountering a high protein diet foragers had a low probability of: (i) feeding, (ii) bringing food back to the nest and (iii) leaving the nest for another foraging visit. These behaviours strongly suggest a lack of motivation at the individual level in response to an unappetizing food source.

In ponerinae ants, food cannot be exchanged via trophallaxis, therefore, in our experiment the only way to obtain food for the workers staying in the nest was via the food pellets brought back to the nest by their foraging congeners. However, as just mentioned, when encountering an unappetizing food foragers brought few pellets and did very few visits to the nest. These individual behaviours, by preventing an easy access to food for the ants staying in the nest, might have been behind the mobilization of a greater proportion of available workers at the collective level. Indeed, due to limited access to food, the ants that stayed in the nest might have been forced to take their turn in foraging to satisfy their own nutritional needs, thus increasing the foraging activity at the collective level. In this sense, collective foraging behaviour was affected by individual needs, which regulate, via negative or positive feedback, food-source exploitation. Hence, when colonies were confined to a high protein diet, food collection relied on numerous hungry ants rather than a few dedicated foragers while we observed the opposite when colonies were confined to a high carbohydrate diet.

Previous studies have shown that when offering carbohydrate to carbohydrate-deprived ants, they will recruit more heavily (Dussutour and Simpson 2008) and will ingest more carbohydrates (Sorensen et al. 1985; Josens & Roces 2000). On the switch day when the food was changed from a high protein diet to a high carbohydrate diet, the bigger proportion of ants foraging remained unchanged on treatment P. In contrast, at the individual level, we observed a more spontaneous and flexible behaviour in response to the diet composition: ants that switched from a high protein diet to a more palatable high carbohydrate diet had a higher probability of feeding and brought more food back to the nest. Therefore, combining a robust high foraging activity at the collective level when fed a high protein diet and an increase in feeding activity at an individual level when switched to a high carbohydrate diet, colonies managed to collect the same quantity of carbohydrates regardless of the treatment.

In conclusion, first, we have shown that excess dietary protein resulted in poor survival at the colony level. Second we have shown that the ants that survived the high protein diet treatment stored large amount of lipids, showing that the amount of body fat that an ant stores is a critical parameter for its survival. Third, we demonstrate that in ants, the compensation for limiting carbohydrate occurs at both a collective and an individual level depending on the protein and carbohydrate balance. Overall, this study provides new insights into the strategies used by ants facing nutritional challenges. Further experiments should identify the physiological processes underlying the ability of species feeding mostly on protein-rich food sources to compensate nutrient imbalance through a sporadic access to carbohydrate resources. Deciphering such regulatory mechanisms is expected to significantly deepen our understanding

of the nutritional ecology of predatory species and, thereby, to illuminate their vast ecological success.

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Figure captions

Fig. 1: The effect of the dietary protein-to-carbohydrate ratio on lifespan and fat storage. **A** Ant lifespan for different diets. The response to the ratio of protein (P) to carbohydrate (C) in the diet and to the choice of protein and carbohydrate diet (P&C) for 34 days. Experimental colonies of 30 individuals per treatment (n=8 colonies for each treatment). Mortality dynamics were consistent between colonies. **B** The response of exposure to treatment C in comparison to a choice of protein and carbohydrate diets (treatment P&C) for 100 days (n=4 colonies for each treatment). **C** Probability of surviving from one day to another according to treatment. The diets were switched for a day on day 17 and day 34 (indicated by the dotted line). Ants on treatment C received the P diet while ants on treatment P received the C diet. Ants on the P&C diet received the same diets, only their positions in the foraging arena were switched. The dynamics were consistent between series and colonies. **D** Proportion of lipids according to the treatment. Here, we distinguished two groups of ants, the ones that died naturally during the observation period of 34 days and the ones that survived and were sacrificed at the end of the observation period. Both series and colony have no effect on the proportion of lipids ($28<N<179$ for each data point. $P=0.584$ and $P=0.105$ for series and colony, respectively).

Fig. 2: The effect of the dietary protein-to-carbohydrate ratio on foraging behaviour. (**A**) Foraging activity defined as the number of daily visits to the foraging arena at the collective level (total number of visits per day) divided by colony size for all treatments. (**B**) Proportion of different ants visiting the foraging arena per day defined as the number of different individuals observed visiting the foraging arena divided by colony size for all treatments. As we know the ID of each ant entering the foraging arena we can identify which ant is engaged in foraging behaviour and which ant is not. Here we do not look at the number of visits but just at who is in charge of foraging on a particular day (**C**) Number of daily visits to the foraging arena per ant defined as the number of times the same ant was observed visiting the foraging arena per day for all treatments. N=8 colonies for each treatment. Switch indicates the days (17 and 34) when the diet was exchanged. The ants fed the P diet received the C diet while the ants fed the C diet received the P diet. The ants fed the P&C diet received the same diets, only their positions in the foraging arena were switched.

Fig. 3: The effect of the dietary protein-to-carbohydrate ratio on activity. Time spent in the foraging arena during a visit (in seconds) for P and C treatments (**A**) and P&C treatment (**C**). Time spent inside the nest between two visits to the foraging arena (in seconds) for P and C treatments (**B**) and P&C treatment (**D**). N=8 colonies for each treatment. Switch indicates the days (17 and 34) when the diet was exchanged. The ants fed the P diet received the C diet while the ants fed the C diet received the P diet. The ants fed the P&C diet received the same diets, only their positions in the foraging arena were switched.

Fig. 4: The effect of the dietary protein-to-carbohydrate ratio on feeding behaviour for treatment P and C treatment. A Proportion of ants feeding during a visit to the foraging arena **B** Duration of the first meal in seconds. Ants can have multiple meals during a single visit to the foraging arena **C** Total time spent feeding (in seconds) during a visit to the foraging arena defined as the sum of time spent in all meals taken during a visit to the foraging arena. **D** Proportion of ants returning food back to the nest as pellets. N=8 colonies for each treatment. Switch indicates the days (17 and 34) when the diet was exchanged. The ants fed the P diet received the C diet whereas the ants fed the C diet received the P diet.

Fig. 5: The effect of the dietary protein-to-carbohydrate ratio on feeding behaviour for treatment P&C. A Proportion of ants feeding during a visit to the foraging arena **B** Duration of the first meal in seconds. **C** Total time spent feeding (in seconds) during a visit to the foraging arena. **D** Proportion of ants returning food back to the nest as pellets. N=8 colonies for each treatment. Switch indicates the days (17 and 34) when the diet was exchanged. The ants fed the P&C diet received the same diets, only their positions in the foraging arena were switched. (see legend Fig 4 for details)

Fig. 6: Estimated Cumulative Protein and Carbohydrates Collected by Ants. Colonies were provided with one of three treatments at 2-day intervals over 34 days. Each treatment is represented as a line in the protein/carbohydrates plane. Within each time interval, the estimated nutrient collection points are connected with dotted black lines to form collection arrays, which demonstrate the nutrient balancing strategy. The indexes were computed every day for each colony as the probability to have a meal on day *d* during a visit, multiply the total number of visit during a day *d*, multiply the concentration in nutrient in g.L-1 (P or C), multiply the duration of a meal on day *d* in seconds, divided by the colony size on day *d* to adjust for

mortality. Error bars represent the standard error of the mean. Switches are indicated by the change in marker line colour.

Fig. 7: Relative activity in relation to survival time. Relative activity is defined as the number of visits to the foraging arena throughout the all observation period divided by the survival time. Here, we distinguished two groups of ants, the ones that died naturally during the observation period of 34 days and the ones that survived and were sacrificed at the end of the observation period.

Fig. 8: The effect of the temperature on lifespan. Effect of temperature on survival for single ants fed the P diet (n=30 for each temperature).

Fig. 9: The effect of food restriction on lifespan and fat storage. A Response to carbohydrate shortage using either the P diet or the C restricted diet for 34 days. Mortality dynamics were consistent between colonies. **B** Proportion of lipids before (pre-treatment) and after the experiment (colony effect $p=0.261$) ($30<N<85$ for each data point).

Supplementary figure captions

Fig. S1: Experiment 1 set-up. The diagram shows the nest, containing 30 *Odontomachus hastatus* workers marked with RFID tags and 8 larvae, connected to the foraging area by two tubes emanating from each side. Each tube is lined by two readers to detect ants that travel between the nest and food. Ants were fed with one of three artificial dietary treatments for 3 hours a day: a high protein diet (food containing a protein to carbohydrate ratio of 5:1 was placed on both compartments of the foraging area); a high carbohydrate diet (food containing a protein to carbohydrate ratio of 1:5 was placed on both compartments of the foraging area), and a balanced diet with high protein and high carbohydrate foods (5:1 food placed on one compartment and 1:5 food placed on the other compartment of the foraging area). The

experimental set-up was also filmed using a digital video camera. Tag detection and filming was carried out every two days.

Fig. S2: Memory for a compartment and specialisation for a diet. **A**. Distribution of ant visits between the right and the left compartments of the foraging arena. (N=89, N=100 and N=105 for P, C and P&C treatment respectively). Only ants that visited the foraging arena at least 5 times were considered. **B**. Distribution of ant visits between the P and C diet for treatment P&C. The P diet and the C diet were in the same compartment for 34 days except for switch days when their positions were switched (N=105). Only ants that visited the foraging arena at least 5 times were considered.

Fig. S3: The effect of the dietary protein-to-carbohydrate ratio on foraging behaviour for treatment P&C. (**A**) Foraging activity defined as the number of daily visits to each diet at the collective level divided by colony size for all treatments. (**B**) Proportion of different ants visiting each diet per day defined as the number of ants observed visiting a diet divided by colony size for all treatments. As we know the ID of each ant entering each compartment we can establish which ant is engaged in foraging behaviour on a particular diet and which ant is not (**C**) Number of daily visits to the foraging arena per ant defined as the number of times the same ant was observed visiting the same diet. N=8 colonies. Switch indicates the days (17 and 34) when the diets were switched. (statistics: GLMM, diet : F=0.16, P=0.686, N=272; F=0.25, P=0.616, N=272; F=2.83 P=0.092, N=1099; for foraging activity, proportion of ants engaged in foraging, and number of daily visits per ant respectively).

Fig. S4: Fat storage distribution for Experiment 3. 12 colonies were first confined to a standard laboratory rearing diet for a month (control pre-treatment). Then, they were separated in two groups of six colonies. One group was confined to a P diet for 3 hours per day (P treatment). Another group was confined to the C diet but with reduced food access of only 1 hour per day for 34 days (C-restricted treatment). We measured the proportion of lipid content in ants before and after the treatments. N=278 in total.

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