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Flight and digging effort in leaf-cutting ant males and gynes

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Abstract

The nuptial flight and nest digging are high intensity activities which consume body reserves. The flight and digging effort was quantified by measuring the carbohydrate and total lipids content in males and females before and after the nuptial flight, and the queen's digging effort during the foundation. The digging effort was quantified by experimentally stimulating the queens to dig a nest – one, two or three consecutive times – compared to the queens that did not dig. The colorimetric method was used to determine the soluble carbohydrates and extraction method of immersion was used to determine the total lipids. The results showed significant loss of carbohydrates and total lipids in males and females after the flight. On average the males contained 0.027 mg of soluble carbohydrates before the nuptial flight, and 0.005 mg after the nuptial flight, and the females contained 0.129 mg of soluble carbohydrates before the nuptial flight, and 0.079 mg after the nuptial flight. For the males the percentage of lipids decreased from $5.27 \pm 1.07\%$ to $2.60 \pm 0.63\%$ and for females from $36.46 \pm 4.86\%$ to 32.62% after the nuptial flight. The digging effort of the queen caused a slight reduction in total carbohydrates, it was without digging 0.054 mg, normal digging 0.055 mg, double digging 0.045 mg (decrease of 20,22 %), and triple digging 0.044 mg (decrease of 20 %) per queen. Based on our results we conclude that the carbohydrate content is the main energetic resource used for the nuptial flight and nest digging, for males and gynes of leaf-cutting ants.

Introduction

Annually, mature *Atta* colonies produce male and female winged ants that leave the colony to form new colonies and thereby, continue and perpetuate the species (Hölldobler & Wilson, 1990). The nuptial flight of leaf-cutting ants may occur in the morning or afternoon according to species, controlled by the first rains starting the rainy season (Staab & Kleineidam, 2014 for *Atta vollenweideri*), with the males forming “swarms” of 200 m in diameter and heights over 150 m above ground level for *Atta capiguara* (Amante, 1972). The flying distance varies according to species and the air flow speed. It is known that females of *Atta texana* can fly at a speed of 5.33 ms^{-1} , indicating a distance of 10.4 km (Moser, 1967); *Atta sexdens* at a speed of 1.57 ms^{-1} , with a possible distance of 11.1 km (Jutsum & Quinlan, 1978), and field observations have shown a dispersion of queens of 9.6 km for this species (Cherrett, 1968). After the nuptial flight and copulation, a queen of *Atta sexdens* digs a vertical tunnel of about

15 cm and a chamber within which she is enclosed, caring for fungus culture and brood (Autuori, 1942).

The nest digging takes on average about 6 to 10 hours (Autuori, 1942), with about 300 trips to stack the excavated soil by the queen, each trip lasting from 30 seconds to 30 minutes (Ribeiro, 1972). Because this activity is very intense, it is assumed that the energetic cost of the excavation is high. There is a few data available in the literature regarding the amount of energy required by the queen for digging the nests (Camargo & Forti, 2013 A). The energy required is removed from carbohydrate reserves, because the lipid content is spared when queen dug the nest (Camargo & Forti, 2013 B).

The digging effort significantly affects the survival of queens of *Atta sexdens rubropilosa*. At the first week after an extra digging effort, (successively digging of two or three nests) there is a higher mortality in queens. It can probably be stated that the mortality of the queens was caused by the depletion of body reserves (Camargo et al., 2011). Besides, in the claustral





Fig 1. Behavioral sequence of nest digging by the queen, illustrating the “turning point”, in other words, queen digs with head down, and when it can turn around into the chamber, it carries the soil pellet with head up until enclosure.

phase, queens lose about 40% of their body weight after 60 days (Della Lucia et al., 1995), reaching minimum body mass before the first workers begin to forage. When foraging starts, the queen recovers her body mass, due to nourishment in the colony (Della Lucia et al., 1990, Camargo et al., 2013).

The goal of the present study was to measure the soluble carbohydrate and lipid contents of gynes and males consumed before and after the nuptial flight, and soluble carbohydrate consumed before and after the nest excavation by the queen.

Material and Methods

*Collecting *Atta sexdens rubropilosa* females and males before and after the nuptial flight.*

We collected females and males (pre-nuptial flight) from three nests of *A. sexdens rubropilosa* over a mound of loose-soil before the nuptial flight. These insects (N = 40) were taken to the laboratory, immediately killed by freezing and analyzed for lipid content.

The mated females (post-nuptial flight) were collected after shedding their wings and beginning to dig start their new colonies (N=20), and males after their death (N=20). After the collection, the queens were immediately stored in plastic containers (11 cm in diameter, 8 cm high), containing 1 cm of plaster at the bottom to keep the air moist. The queens and males were transported to the Laboratório de Insetos Sociais-Praga - FCA/UNESP - Botucatu, SP, Brazil, this travel took 30 minutes.

Digging Effort

This experimental set up was previously performed according to Camargo et al. (2011) and Camargo and Forti (2013 B). One hour after the nuptial flight, the queens were placed in tubes filled with soil (25 cm high and 10 cm in diameter), removed at 60 cm in depth, at a density of 1.6 g/cm³. The queens did not start laying eggs before the experiments. Four experimental series were performed, as follow:

1) Without digging: Queens did not dig at all, and were directly placed singly in a plastic container;

2) Single digging: Queens were allowed to dig in the tube filled with soil, until they sealed the excavated tunnel;

3) Double digging: Queens were allowed to dig twice in the tube filled with soil, by being immediately removed from the dug chamber and again met in a new digging tube, in which they started to excavate a second nest;

4) Triple digging: Queens were allowed to dig thrice in the tube filled with soil, by being met immediately in two new digging tubes after the first excavation.

For all four groups, the queens were removed from the tubes and frozen in order to determine the soluble carbohydrates.

In addition to the soluble carbohydrate content, the length of the excavated tunnel was determined, as well as the excavated chamber dimensions (length, width and height). The volume of excavated soil was also measured by direct weighing in a semi-analytical balance. The queens' digging time was measured to assess their performance and effort. The queens' digging time was observed in 3 ways: First – time spent in tunnel excavation, Second – time spent in chamber excavation, and Third – time spent in tunnel and chamber excavation, when the observer lost queen's turning point (Fig 1). This behavioral sequence was studied by Fröhle and Roces (2012).

Determination of carbohydrates

The colorimetric determination of the soluble carbohydrates was achieved by the method of Dubois et al. (1956), based on the reaction of carbohydrates, sulfuric acid and phenol. The reaction generates a yellow-orange color which is sensitive and stable. The absorbance was determined on a Beckman DU640 spectrophotometer. The blank received water in place of the extracts. Glucose was used as standard ($C=90.531Abs + 3.7125$, $R^2=0.992$). The values were expressed as mg of total sugars.

Determining total lipid content

Males and females before and after the nuptial flight were killed by freezing and then subjected to the experimental procedure used by Seal (2009). The procedure was as follow: 1) Fresh mass of males and females was individually determined; 2) Queens were dried for 24 hours at 70°C and their dry mass was determined; and 3) The lipid content was extracted with petroleum ether (bp 40-60°C) for 24 hours and the queens weighed again. This procedure was repeated for 72 hours of extraction. The percentage of total fat content was calculated

using the formula: $100x (DM - FFDM)/DM$, where DM is the dry mass and FFDM is dry and fat free mass. The energy content of the ants was obtained by multiplying their lean mass by 18.87 J mg⁻¹ and their fat mass by 39.33 J mg⁻¹ (Peakin, 1972).

Statistical analysis

The total lipid and soluble carbohydrate content of males and queens before and after the nuptial flight and nest digging effort were compared using a paired t-test ($\alpha=0.05$). The data of the excavation effort were subjected to the ANOVA of repeated measures ($\alpha=0.05$), comparing the variable total digging time, overall tunnel length and excavated soil of each experimental series. The analysis was performed using the SigmaPlot 11.0 program.

Results

Nuptial flight effort

The average fresh masses of male and females of *A. sexdens rubropilosa* before nuptial flight were 103.6±18.6 mg and 656.7±75.1 mg, whereas the masses of male and females after nuptial flight which had mated and flew were 154.3±7.9 mg and 631.2±38.8 mg, respectively (Table 1).

For males and females we verified a significant loss of soluble carbohydrate after the flight. On average the males contained 0.027 mg of soluble carbohydrates before the nuptial flight, and 0.005 mg after the nuptial flight (reduction of 81.48%) (t test=7.661, d.f.=18, P< 0.0001). On average the females contained 0.129 mg of soluble carbohydrates before the nuptial flight, and 0.079 mg after the nuptial flight (reduction of 38.76 %), differing significantly between them (t test, t=4.718, d.f.=18, P< 0.0001).

Lipids represented 36.46±4.86 % and 5.27±1.07% of the body mass of females and males before nuptial flight, respectively. This percentage decreased to 35.00±2.28% in females and 2.60±0.63% in males who mated and flew (Table 1). As expected, significant differences were detected in the percentage of lipids of males before and after the nuptial flight (ANOVA, $F_{1,19}=89.051$, P<0.001). But, the percentage of lipids of females who had and had not flown were not significantly different (ANOVA, $F_{1,19}=1.400$, P=0.242). The energy content was 8563.24±1668.76 J for females before nuptial flight, 8413.37±802.56 J for females who had flown (Table 1).

Table 1. Mean and standard deviation (m±sd) of masses (mg), percentage of fat and energy content (J) in the bodies of males and female of *Atta sexdens rubropilosa* before and after nuptial flight.

	Male before NF (n=20)	Male after NF (n=20)	Female before NF (n=20)	Female after NF (n=20)
Fresh Mass (mg)	103.68±18.61	154.29±7.96	656.72±75.05	631.18±38.81
Dry mass (mg)	35.17±5.60	53.02±2.74	323.69±53.64	322.85±25.20
Lean mass (mg)	33.31±5.32	51.63±2.54	203.68±25.75	209.39±11.63
Lipid content (mg)	1.86±0.51	1.39±0.41	120.01±31.39	113.46±15.77
Lipid percentage (%)	5.27±1.07	2.60±0.63	36.46±4.86	35.00±2.28
Energy content (J)	-	-	8563.24±1668.76	8413.37±802.56

Digging effort

In the single digging, the average time spent for the queens was of 383.21 ± 59.01 (N=14) minutes to dig the tunnel, and 42.21 ± 40.46 (N=14) minutes for the initial chamber. It was not possible to observe the turning point for some queens, they took an average of 523.75 ± 164.79 (N=14) minutes to build the entire nest. In the double digging, the average spent time for the queens was of 310.35 ± 137.94 (N=20) minutes to dig the tunnel, and 77.35 ± 95.19 (N=20) minutes for the initial chamber. Some went into claustral confinement without the observation of the queen's turning point, thus the total digging time was of 305.0 ± 184.59 (N=5) minutes to build the entire nest. In this experimental series, five queens did not do any digging. In the triple digging, the average spent time for the queens was of 331.21 ± 169.90 (N=10) minutes to dig the tunnel, and 79.30 ± 89.87 (N=10) minutes for the initial chamber. Some of them enclosure without the observation of the queen's turning point, thus the total digging time was of 347.67 ± 73.21 (N=10) minutes to build the entire nest. In this experimental series, eight queens did no digging.

The statistical analysis showed that there were differences at double digging (ANOVA, $F_{1,52}=12.47$, $P<0.001$), where the queen's total digging time was higher at first than in the second digging time. Moreover, there was difference in the triple digging (ANOVA, $F_{1,77}=5.85$, $P<0.05$), where the first founding nest in the triple digging experiment takes significantly longer than two following ones (Tukey post test, first *versus* second, $P<0.05$; first *versus* third, $P<0.01$, second *versus* third, non significant).

The overall of average removed soil dry mass was 35.66 ± 6.86 g for the single, 59.21 ± 15.38 g for double and 87.90 ± 16.98 for triple digging. There were significant differences among dry mass of soil excavated by the queen, being this amount higher for the triple digging, followed by double digging in the excavated soil (ANOVA, $F_{2,87}=70.95$, $P<0.0001$, Tukey post test: first *versus* second, $P<0.01$; first *versus* third, $P<0.01$; second *versus* third, $P<0.01$).

The overall tunnel length dug by the queens was 12.00 ± 2.02 cm for the single, 19.32 ± 4.58 cm for double and 26.62 ± 6.12 cm for triple digging. There were differences in the excavated tunnel length (ANOVA: $F_{2,87}=51.84$, $P<0.0001$, Tukey post test: first *versus* second, $P<0.01$; first *versus* third, $P<0.01$; second *versus* third, $P<0.01$).

Queen's digging effort caused a slight reduction in total carbohydrates when they excavated two and three nests. Queens that did not dig presented 0.054 mg of total carbohydrates, and after a single digging, 0.055 mg. On the other hand, after double and triple digging they showed 0.045 mg and 0.044 mg, respectively, which represents a decrease of about 20,22 % of total carbohydrates (ANOVA, $F_{3,36}=3.1096$, $P=0.0376$).

Discussion

As expected, total soluble carbohydrate and lipid contents reduced in males and total soluble carbohydrate reduced in gynes of *Atta sexdens rubropilosa* after the nuptial flight. This result is similar that found by Jutsum and Quinlan (1978) in *Atta sexdens*. The authors found that 21% of the dry mass of the winged is carbohydrate, which is completely consumed after the nuptial flight. Similarly, in a study of *Formica lugubris*, it was demonstrated that carbohydrate (stored as glycogen) is the main energy source for the nuptial flight (Passera et al., 1989). Furthermore, both studies state that lipids are not used as energy for the females' flight, as observed in our study (Table 1) and in Camargo & Forti (2013 B). In *Cataglyphis cursor* and *Iridomyrmex humilis*, the males have higher carbohydrate content when compared with females (Passera & Keller, 1990). From these studies, it can be assumed that the first energy source to be depleted in the nuptial flight is the carbohydrate reserves. Lipids and proteins degrade at slower rates when compared to carbohydrates, preserving these reserves for the activities after nuptial flight.

It is known that the symbiotic fungus is a rich source of carbohydrates, especially glucose. According to Silva et al. (2003), the glucose concentration in the fungus garden was of 27.7 mg.g^{-1} , about two and a half times higher than that found in plant leaves (10.8 mg.g^{-1} , for *Eucalyptus alba*) and about six and a half times higher than that found in symbiotic fungi mycelia grown in culture medium (4.3 mg.g^{-1}). Thus, the nourishment from the fungus garden allows the sexual forms to acquire sufficient reserves for the nuptial flight (Fujihara et al., 2012), and the subsequent excavation of the nest by the founding queen, as showed in our study when queens dug two and three time.

With regards to the digging effort, under the experimental conditions, leaf-cutting ant queens excavated the nest (Table 2), as previously described in the literature (Eidmann, 1935; Cunha, 1968; Camargo et al., 2011; Fujihara et al., 2012), first a tunnel, and then a chamber (Fig 1). The depth of the initial chamber, ranging from 8.5 to 15 cm (Autuori, 1942; Ribeiro, 1972).

Table 2. Mean and standard deviation (cm) of tunnel length and dimensions of the initial chamber (cm) of initial nests of *Atta sexdens rubropilosa*.

Experimental series	Tunnel	Chamber		
		Length	Height	Width
Single digging	12.0±2.02	3.93±0.71	2.11±0.38	2.78±0.38
Double digging I	10.82±2.48	3.35±0.70	2.09±0.40	2.78±0.73
Double digging II	8.50±2.35	3.73±0.98	1.98±0.26	2.83±0.68
Triple digging I	10.47±2.52	3.22±0.82	1.95±0.32	2.66±0.56
Triple digging II	8.43±1.99	2.95±0.54	1.83±0.34	2.23±0.44
Triple digging III	7.72±2.27	3.87±0.57	1.99±0.30	2.64±0.51

Our results show that carbohydrate content of the queens of *Atta sexdens rubropilosa* were affected by the increased digging effort, in other words, by the successive excavations experimentally induced. Additionally, Camargo et al. (2013) found that a single worker (body weight 9.65 ± 1.50 mg) dug on average 0.85 ± 0.27 g of soil in 24 hours and consumed approximately 0.58 ± 0.23 J, a significant energy cost by the workers. Thus, there is a digging cost directly detected by the consumption of total carbohydrates. The queens dug 35.66 g in the single digging, and when subjected to triple excavation, dug about 87.90 g.

Furthermore, it is known that the oviposition rate is not affected by the digging effort (Camargo et al., 2011), but it does accentuate mortality of these queens. The authors state that there is no clear justification for the queen's mortality based on the depletion of body reserves. There may be two consequences of the successive effort. First, excessive cuticle abrasion due to the increasing digging, with associated water losses as described for harvesting ants (Johnson, 2000; Johnson & Gibbs, 2004) and second, the accumulation of oxidative damage associated with the intense initial digging activity, as known for flying insects (Magwere et al, 2006; Sohal & Buchan, 1981).

Based on our results it can be concluded that the amount of carbohydrates is the main energetic resource used for the nuptial flight for males and gynes, and digging nest for queens of leaf-cutting ants, when they dug two or three times.

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