

RESEARCH ARTICLE

The determination of nest depth in founding queens of leaf-cutting ants (*Atta vollenweideri*): idiothetic and temporal control

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SUMMARY

Leaf-cutting ant queens excavate a founding nest consisting of a vertical tunnel and a final horizontal chamber. Nest foundation is very time consuming, and colony success depends on the excavated depth. Although shallow nests may be energetically cheaper to dig, queens may be more exposed to the changing environment. Deeper chambers, in contrast, may be climatically more stable, but are more expensive to dig. We investigated the mechanisms underlying the control of nest depth in queens of the leaf-cutting ant *Atta vollenweideri*. We focused on the use of internal information for the control of nest depth, and therefore maintained the soil and environmental conditions invariant during the different laboratory experiments. We compared the tunnel lengths excavated by queens that were able to complete their nests earlier, faster or slower than under standard conditions. An earlier and faster nest completion was obtained by offering queens either pre-excavated tunnels of different lengths, soils at different temperatures, or soft sandy soils. A slower nest excavation was induced by offering queens harder dry soils, and by delaying the start of digging several days after the nuptial flight. Results indicate that the determination of nest depth was a regulated process involving the use of internal references: queens excavated their tunnels either until a particular depth was reached or for some predetermined length of time. Queens appear to monitor their movements while walking up and down the tunnel, and to compare this sensory information with a motor command that represents a preset tunnel length to be excavated before switching to chamber digging. In addition to this form of idiothetic control, results indicate that the elapsed digging time also feeds back onto the control system. It is argued that the determination of nest depth, i.e. the transition from tunnel to chamber digging, is initiated either after a preset tunnel length is reached, or as soon as a maximal time interval has elapsed, irrespective of the excavated tunnel length. A control system using both idiothetic and temporal information, as demonstrated in the present study, allows queens to flexibly react to different soil conditions, and therefore avoid excessive time and energy investments. Possible mechanisms underlying the control of chamber size are also discussed.

Key words: nest building, behavior, mechanism, time control, proprioception, tunnel, depth, chamber size, digging.

INTRODUCTION

As in most ant species, sexuals of *Atta* leaf-cutting ants fly away from the mother nest in large numbers for the so-called 'nuptial flights' (Ihering, 1898) and, after mating, single queens land on the ground, break off their wings and start to dig a founding nest. This consists of a vertical tunnel and a more or less horizontal chamber at its end (Eidmann, 1935). Nest excavation requires two consecutive digging patterns: at the very beginning, the queen excavates with the mandibles a vertical tunnel slightly wider than her body diameter. She transports the excavated pellets backwards while extending the tunnel, and deposits them around the tunnel entrance. When a given depth is reached, the queen starts to enlarge the end of the tunnel to one side and initiates the excavation of the founding chamber. As the enlargement of the nest chamber proceeds, the queen is able to turn around and moves forward when carrying the single loads. At this stage the queen starts to close the tunnel opening from the inside, using the pellets excavated from the chamber, hence initiating a claustral phase. Claustral ant queens rely exclusively on their body reserves to support themselves and to raise the fungus and the brood until the first eclosed workers initiate foraging (Autuori, 1941; Ihering, 1898; Janet, 1907).

Digging of a founding nest by *Atta* queens requires several hours and therefore demands a significant energy investment. For ants in general, little is known about the mechanisms underlying the determination of the tunnel length, and therefore the final nest depth. Intuitively, the excavation of a shallow chamber is expected to require less time and energy, yet the queen would be in the long term more exposed to changing environmental variables like temperature or soil moisture (Alvalá et al., 2002; Bollazzi et al., 2008; Lapointe et al., 1998). Deeper chambers may counteract these effects and offer a more stable microclimate, but they require more time and energy for their construction, with the concomitant extended exposure of the queens to predators while digging (Autuori, 1950). In addition, although it does not compromise the rate of offspring production, a larger digging effort by queens significantly decreases the probability of their survival after nest founding (Camargo et al., 2011).

As indicated above, nest founding by queens requires two digging patterns: the excavation of a vertical tunnel, and the excavation of a final lateral chamber. It is unknown how this behavioral transition is controlled, i.e. how queens determine the depth of the excavated tunnel so as to initiate the enlargement of

the lateral chamber. Four different, not necessarily mutually exclusive control mechanisms for the determination of the tunnel depth are conceivable *a priori*, which may rely on external or internal feedback loops. First, queens may extend the tunnel being excavated until certain soil conditions (e.g. temperature, moisture or a particular composition) are found. Under this scenario, the feedback loop involved in the control of nest depth would be completely dependent on external variables. Second, queens may monitor their movements while walking up and down the tunnel, and compare this sensory information with a motor command that represents a preset tunnel length to be excavated before switching to chamber digging. This mode of idiothetic control, as known in the context of insect navigation (Seyfarth et al., 1982), is based on spatial information derived by reference to previous movements, and is therefore independent of external cues. Third, queens may possess a mechanism that measures the time elapsed since the start of digging, and triggers the switch from tunnel to chamber digging as soon as a given time interval has elapsed, irrespective of the tunnel depth reached. And fourth, queens may assess their energy expenditure while digging, and so stop the tunnel enlargement once a given amount of energy has been spent.

Previous investigations on *Atta* queens suggest that the control system regulating nest depth may involve idiothetic cues. The first experimental studies were performed in the leaf-cutting ant *Atta sexdens*, by presenting single queens a tube filled with soil in the laboratory (Ribeiro, 1972; Ribeiro, 1995). By experimentally manipulating the tunnel length while the queens were excavating, individuals were observed to dig more after an experimental shortening, and less after an extension of the tunnel, before switching to chamber digging. Even though the precise underlying mechanisms remained elusive, these observations were consistent with an idiothetic control system similar to that involved in the control of nest depth in digger wasps (Brockmann, 1980): ant queens appear to dig until they reach a given tunnel length, although some flexibility exists (Ribeiro, 1995). For instance, *A. sexdens* queens dig much shorter tunnels in denser soils (Stein and Xavier, 1984), as also known for *Acromyrmex* leaf-cutting ant queens (Montenegro, 1973) and wasps (Evans and West Eberhard, 1970), suggesting that time and/or energy costs may also feed back into the system controlling nest depth. Interestingly, comparisons of previous reports show that under natural field conditions, *A. sexdens* queens excavate shorter tunnels (Autuori, 1941) than those dug in the laboratory (Ribeiro, 1972; Ribeiro, 1995), yet the queens in both groups spent roughly similar times for digging. Although these results point at the involvement of some temporal control, a compelling hypothesis on the mechanisms underlying the control of nest depth in *Atta* leaf-cutting ant queens that accounts for the results described so far is lacking.

The aim of the present study was to investigate the mechanisms underlying the control of nest depth in queens of the leaf-cutting ant *Atta vollenweideri* (Forel 1893). We focused on the use of internal information for the control of nest depth, and therefore maintained the soil and environmental conditions invariant during the distinct experimental series. To identify the involved variables, laboratory experiments were designed in which single founding queens were able to excavate their complete nests earlier, faster or slower than under standard conditions, and both the excavated tunnel lengths and the time invested until the switch from tunnel to chamber digging were recorded. An earlier and faster nest completion was obtained by offering queens, in independent assays, either pre-excavated tunnels of different lengths, soils at higher temperatures (in which they are expected to excavate more rapidly but to spend

more energy per unit time) or soft sandy soils (which can be excavated at higher speeds). A slower nest excavation was attained by offering queens dry soils, which are harder to excavate, and by delaying the start of digging several days after the nuptial flight. Records of the depths excavated by queens in natural soils in the field allowed comparisons with the laboratory investigations. Additionally, because the complete tunnel length is closed from the inside with soil pellets arising from the chamber being excavated, a relationship between the tunnel length and the final chamber size may intuitively be expected. This relationship was evaluated across the experiments, and also using a literature survey, to shed light on the mechanisms involved in the determination of chamber size.

MATERIALS AND METHODS

Experiments were conducted at the biological field station of the Reserva Ecológica El Bagual in Formosa province, Chaco region of northern Argentina, in 2005, 2006 and 2008. The nuptial flights of the leaf-cutting ant *A. vollenweideri* occur on sunny days after heavy rainfalls shortly before dawn, from the end of October to the beginning of November.

Nest founding in the field

Eighteen and 24 field nests were marked with numbered sticks 1 day after the nuptial flights in 2005 and 2006, respectively. The identification of the recently founded nests could be easily accomplished on the following morning owing to the unique size of the soil pellets and their circular distribution around the closed nest entrance. These nests were opened after 2 to 4 weeks to measure the tunnel lengths (cm) from the soil surface to the end of the tunnel, without including the chamber height.

Laboratory experiments

Experiments were performed in the laboratory of the field station under standardized conditions in 2005, 2006 and 2008. For the experiments, mated queens were collected shortly after they landed on the ground and detached their wings, before they started to excavate their nests. This occurred in darkness, around 20:00–21:00h local time. The number of available queens varied in the different years because of difficulties in collection. Queens were maintained individually in plastic cups together with a piece of wet cotton until the beginning of the experiments, as described below.

Experimental setup and general procedure

The experimental setup consisted of a plastic tube filled with soil (11 cm in diameter; 80 or 120 cm in height depending on the experimental series), with a plastic cup (9 cm in diameter; 250 ml) fitted to the soil surface. A single queen was placed into the cup and could directly contact the soil of the tube only through a central opening (2 cm in diameter) at the cup bottom, thus preventing the queens from digging along the tube's edges. The time elapsed since the beginning of digging until the entrance of the excavated tunnel was closed with a first layer of pellets was recorded as digging time. At this time, the tunnel construction was considered as finished because queens could turn around in the chamber. Three days thereafter, the plastic tubes were opened and the excavated tunnel length was measured, from the soil surface to the end of the tunnel, without including the chamber height. The so-called 'closed tunnel length' was also measured, where the closed tunnel corresponds to the tunnel segment completely filled with tightly packed soil pellets. Finally, height, length and width of the excavated chamber were measured, and chamber volume obtained using the simple method of water displacement after a plaster cast of the chamber was made.

Digging under standard conditions

Because of potential differences in the performance of the queens collected in the different years, each experimental series was run with a simultaneous series under standard conditions. Queens dug in tubes filled with soil from an abandoned *Atta* nest, which has a large proportion of clay (Cosarinsky and Roces, 2007). The collected dry clayish soil (hereafter 'clay') was added with water until the mixture reached a standardized soil moisture of $17.47 \pm 1.61\%$ ($N=66$). Water content of the soil mixture was measured for each experimental series. The digging tubes were placed in a temperature-controlled room, and the temperature of the soil inside, measured with Tinytag data loggers (Gemini Dataloggers, Sussex, UK), was maintained at 20.5 ± 0.6 and $20.6 \pm 1.2^\circ\text{C}$ in the years 2005 and 2008, respectively, but raised to $24.0 \pm 0.6^\circ\text{C}$ due to an electrical power outage in 2006, both for the experimental series and the corresponding series under standard conditions.

Experimental series

To evaluate whether queens would stop the excavation of the nest earlier, as soon as a preset tunnel length was reached, they were offered pre-excavated tunnels of different lengths, either 15 cm ($N=6$) or 80 cm ($N=7$), and their digging responses quantified. The offered tunnels were pre-excavated with a drill [14 mm in diameter, in the range of the natural tunnel diameters (Ribeiro 1972)] in the middle of the filled tube. The experiments and the corresponding series under standard conditions, i.e. without a pre-excavated tunnel ($N=9$), were performed 5 days after the second nuptial flight in 2006.

Faster nest excavation was achieved by presenting queens, in independent series, either with soils at a higher temperature, or with digging tubes filled with sand. To achieve higher soil temperatures, tubes filled with soil were placed in a thermostatic chamber (ca. 1 m^3 in size) maintaining a mean (\pm s.d.) temperature of $27.2 \pm 0.5^\circ\text{C}$ ($N=10$). Experiments under standard conditions ($N=10$) were run in the laboratory room, at a controlled $20.5 \pm 0.6^\circ\text{C}$. Both series were performed 1 day after the nuptial flight in 2005.

Experiments with plastic tubes filled with sand ($N=9$), and the corresponding series under standard conditions ($N=9$) were conducted 5 days after the second nuptial flight in 2006. Before filling the tubes, the sand was moistened to a water content of $7.94 \pm 2.60\%$ to avoid a later collapse of the excavated structure during data collection.

Slower nest excavation was obtained by offering queens dry soils for excavation. Dry soil was prepared by using the same clayish soil as in the standard experiments, but after a drying period in the sun of 4–5 days, until a water content of $7.18 \pm 2.52\%$ was reached. Five experiments with dry soil and seven under standard conditions were performed 5 days after the first nuptial flight in 2006.

Because *Atta* queens are known to excavate shorter tunnels at a slower pace the longer they are prevented from digging (Ribeiro, 1972; Ribeiro, 1995), we compared the digging behavior of queens that were maintained in a plastic cup and only allowed to excavate their nests 1 ($N=14$), 5 ($N=8$) or 16 days ($N=14$) after their nuptial flight in 2008.

Statistical analysis

Differences between two groups were compared using a Mann–Whitney *U*-test ($\alpha=0.05$). Comparisons involving more than two groups were made with Kruskal–Wallis *H*-tests and a Bonferroni-corrected *post hoc* Mann–Whitney *U*-test (two tests per data set, $\alpha=0.025$).

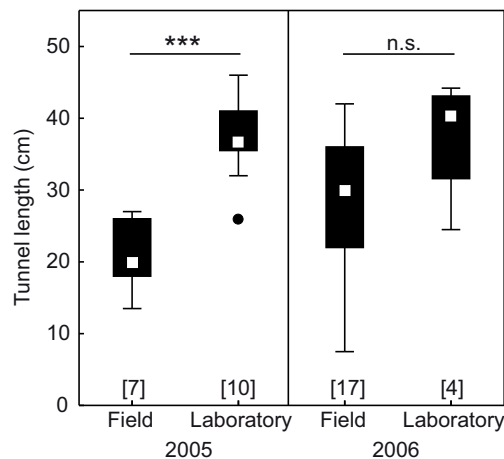


Fig. 1. Tunnel lengths from nests dug by *Atta vollenweideri* queens in the field and the laboratory under standard conditions (2005 and 2006). Numbers of nests measured are given in brackets. Data shown are medians (squares) and upper and lower quartiles (box); error bars indicate the range without outliers (***) $P < 0.001$, n.s., not significant). Outliers (black circle) correspond to observations outside the interquartile range.

RESULTS

Digging behavior of queens

Queens removed small soil particles with their mandibles through repeated bites. The particles were then pressed together to form a large pellet that the queen picked up and transported to the outside. To give an example of the average queen's excavation rate and the time investment in the laboratory tubes, pellets collected from one queen 16 and 20 h after the start of the experiment had a mean (\pm s.d.) mass of $203.75 \pm 51.65\text{ mg}$ ($N=26$). To dig a tunnel with an average length of 32.7 cm, which corresponds to an excavated soil mass of 89.33 g, the queen removed 438 pellets over a total digging time of 18 h 3 min, thus reaching an average digging speed of 1.81 cm h^{-1} .

Nest founding in the field

Out of the 18 founding nests marked in 2005, three collapsed because of flooding and in eight of them the tunnels were closed, but no distinguishable chamber was found after their excavation. Seven nests were completely constructed, with a vertical tunnel and a horizontal chamber on its end. The single queens were found in the chamber, and they had already regurgitated the fungus carried in the buccal pocket and had laid eggs. The excavated tunnels attained a median length of 20.0 cm, ranging from 13.5 to 27 cm (Fig. 1, left). Nests dug by queens under controlled laboratory conditions, in contrast, had significantly longer tunnels, with median lengths of 36.8 cm, and a minimal and maximal length of 26 and 46 cm, respectively (*U*-test, $N=17$, $U=1.5$, $P < 0.001$).

The queen mortality rate was also high for the field nests marked in 2006. From the 24 nests initially marked, three could not be located anymore, and four of them had no chamber. The remaining 17 nests were completely constructed, but in seven of them the queen was found dead. The tunnels in these nests attained a median length of 30 cm, with a minimal and maximal value of 7.5 and 42 cm, respectively (Fig. 1, right). Nests dug by queens under controlled laboratory conditions did not differ in tunnel length, with a median length of 40.4 cm (range=24.5–44.2 cm; *U*-test, $N=21$, $U=14.5$, $P > 0.05$). Comparing both years, although the depths of the field nests showed large variation, there were no statistical differences between them (*U*-test, $N=24$, $U=29.5$, $P=0.06$). Similarly, nests

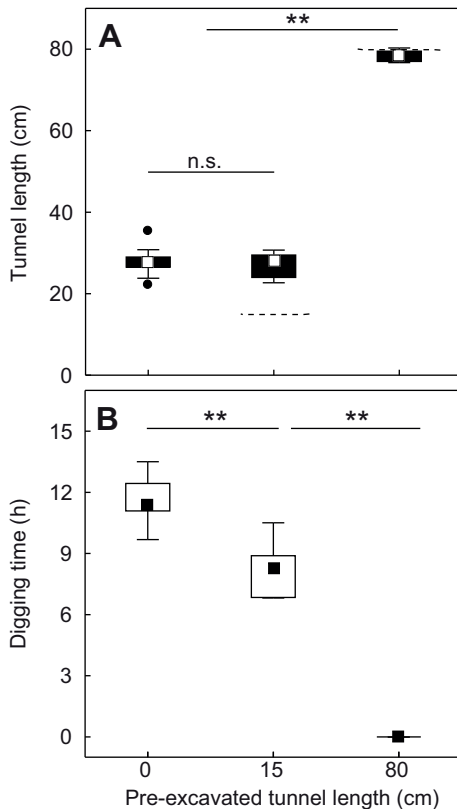


Fig. 2. Reaching the nest depth in advance. Tunnel lengths (A) and digging times (B) of *A. vollenweideri* queens when pre-excavated tunnels of different lengths were offered. The pre-excavated tunnel lengths are indicated by the dashed lines in the upper graph. Experiments were performed 5 days after the second nuptial flight in 2006 (0 cm, i.e. standard conditions: $N=9$; 15 cm: $N=5$; 80 cm: $N=6$). Data shown are medians (squares) and upper and lower quartiles (boxes); error bars indicate the range without outliers (** $P<0.01$, n.s., not significant). Outliers (black circles) correspond to observations outside the interquartile range.

excavated in the laboratory did not differ between years (U -test, $N=14$, $U=15$, $P>0.05$).

Reaching the nest depth in advance

The results of the experiments offering pre-excavated tunnels of different lengths are presented in Fig. 2. Queens offered no pre-excavated tunnel (standard conditions) dug tunnels with a median length of 27.8 cm (range=22.5–35.6 cm), and spent a median time of 11 h 25 min (range=9 h 41 min to 13 h 30 min; Fig. 2). When a pre-excavated tunnel of 15 cm was offered, which corresponds to nearly one-half of the tunnel length excavated under standard conditions, queens extended the offered tunnel to a total median length of 28 cm, not significantly different from the previous one (H -test, $H_{2,20}=12.00$; *post hoc* U -test after Bonferroni correction, 15 vs 0 cm, $N=14$, $U=22$, $P>0.025$; Fig. 2A). Only one queen in this series was observed to immediately start to close the tunnel, so that its chamber was built at a depth of 15 cm. This value was not considered for statistical analysis, as there was no tunnel enlargement. As the queens in this series dug only approximately one-half of the tunnel by themselves, the measured digging time was significantly shorter than that under standard conditions (H -test, $H_{2,20}=16.54$; *post hoc* U -test after Bonferroni correction, 15 vs 0 cm, $N=14$, $U=1$, $P<0.01$; Fig. 2B).

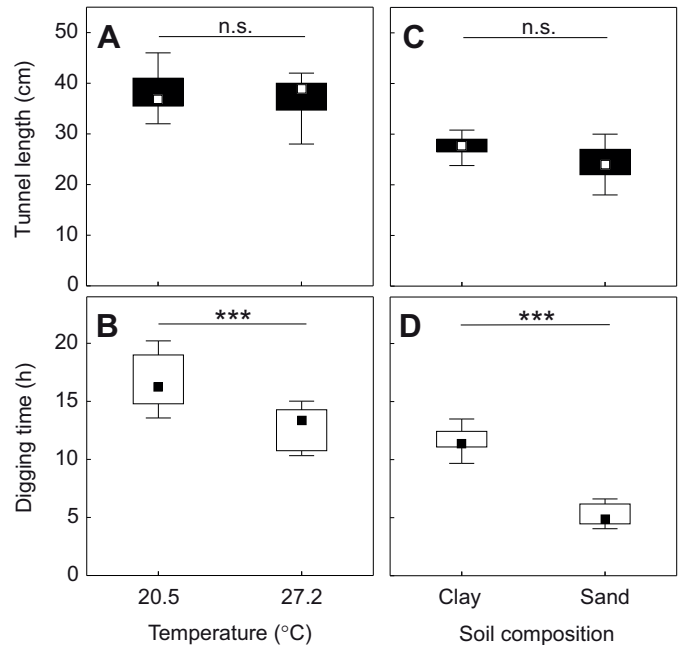


Fig. 3. Digging at a faster pace. Tunnel lengths and digging times of *A. vollenweideri* queens measured at soil temperatures of 20.5°C (standard conditions) and 27.2°C (A,B), as well as in clay (standard conditions) and sand (C,D). Temperature experiments were performed 1 day after the nuptial flight in 2005 ($N=10$ each), and soil-condition experiments 5 days after the second nuptial flight in 2006 ($N=9$ each). Data shown are medians (squares) and upper and lower quartiles (boxes); error bars indicate the range without outliers (** $P<0.001$, n.s., not significant).

Finally, when an 80 cm tunnel length was offered, which corresponded to nearly 2.8 times the tunnel length excavated under standard conditions, no single queen enlarged the existing tunnel. Instead, queens immediately began the excavation of the lateral chamber at the final 2–3 cm of the pre-excavated tunnel. The tunnels were significantly longer than those excavated in the other series (U -test after Bonferroni correction, 80 vs 0 cm, $N=15$, $U=0$, $P<0.001$; 80 vs 15 cm, $N=11$, $U=0$, $P<0.01$). Digging time could not be measured because the offered tunnel was not enlarged, and was therefore assigned a value of zero (U -test after Bonferroni correction, 80 vs 0 cm, $N=15$, $U=0$, $P<0.001$; 80 vs 15 cm, $N=11$, $U=0$, $P<0.01$). Interestingly, one queen started the excavation of the chamber at a tunnel depth of 48 cm. Because observations inside the tunnel were not possible, it is unknown whether this queen reached the bottom and then climbed up the tunnel to initiate the chamber, or never reached the bottom of the tunnel. For this reason, data from this queen were not considered for statistical analysis.

Nest digging at a faster pace

Fig. 3 summarizes the results of the two different experimental series in which queens excavated either in soil at higher temperatures or in sand. Queens dug similar tunnel lengths at the two experimental temperatures, 20.5 and 27.2°C (U -test, $N=20$, $U=46.5$, $P>0.05$; Fig. 3A), but spent shorter times digging at the higher temperature (U -test, $N=20$, $U=7$, $P<0.001$; Fig. 3B).

When excavating in a soft soil composed of pure sand, queens dug tunnel lengths that were similar to those excavated in clay (standard conditions, U -test, $N=18$, $U=23$, $P>0.05$; Fig. 3C), but spent shorter times digging (U -test, $N=18$, $U=0$, $P<0.001$; Fig. 3D).

Note that a temperature of 20.5°C and the use of clay, as presented in Fig. 3A,B, represent the standard conditions. Nonetheless, the tunnel lengths differed, with shorter tunnels excavated in 2006 (U -test, $N=19$, $U=10$, $P<0.025$). Considering that no effect of the year on the excavated length was observed (Fig. 1), it is likely that the shorter tunnels are the result of the time elapsed between the nuptial flight and the start of the experiments (5th vs 1st day; see also experiments on delayed digging, below).

Nest digging at a slower pace

Queens excavating their founding nests in dry clay dug shorter tunnels than queens digging in clay under standard conditions (U -test, $N=12$, $U=0$, $P<0.01$; Fig. 4A), but spent the same total time digging (U -test, $N=12$, $U=9$, $P>0.05$; Fig. 4B).

A similar pattern was observed in queens that were allowed to start their nest excavation after long time delays (Fig. 5). Queens digging on days 1, 5 and 16 after the nuptial flight dug shorter tunnels the longer their waiting time (H -test, $H_{2,36}=27.64$; *post hoc* U -test after Bonferroni correction, 1st vs 5th day, $N=22$, $U=6$, $P<0.001$; 1st vs 16th day, $N=28$, $U=0$, $P<0.001$; 5th vs 16th day, $N=22$, $U=9$, $P<0.001$; Fig. 5A). Their time investment for digging, however, was similar for the three different delays, with the exception of the comparison between days 1 and 5 (H -test, $H_{2,36}=8.43$; *post hoc* U -test after Bonferroni correction, 1st vs 5th day, $N=22$, $U=17$, $P<0.01$; 1st vs 16th day, $N=28$, $U=54$, $P>0.05$; 5th vs 16th day, $N=22$, $U=39$, $P>0.05$; Fig. 5B).

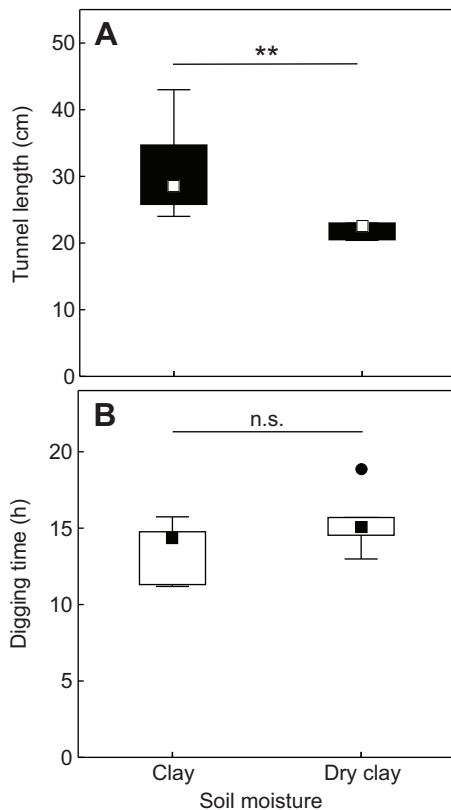


Fig. 4. Digging at a slower pace. Tunnel lengths (A) and digging times (B) of nests excavated by *A. vollenweideri* queens in clay (standard conditions, $N=7$) and dry clay ($N=5$). The experiments were performed 5 days after the first nuptial flight in 2006. Data shown are medians (squares) and upper and lower quartiles (boxes); error bars indicate the range without outliers (** $P<0.01$, n.s., not significant). Outliers (black circle) correspond to observations outside the interquartile range.

The control of chamber size

As mentioned above, the final nest chamber is initiated by the enlargement of the bottom of the tunnel towards one side. The available space thus becomes larger, so that the queen can turn around and so initiate the closing of the tunnel with the excavated soil pellets, starting at the nest entrance and tightly packing them over the complete tunnel extension. As a consequence, there should be a relationship between the excavated tunnel length and the final chamber size. This potential relationship was evaluated using data from the previous experiments in which variability in the excavated lengths was observed, i.e. excavation in dry clay, with an 80 cm pre-excavated tunnel, and after different delays.

In all but one experiment, the tunnels were closed within the first 5 cm from the soil surface until the last 5–10 cm from the bottom. There was a positive relationship between the closed tunnel length, i.e. the extent of the tunnel completely packed with soil pellets, and the excavated tunnel length ($y=-6.4555+0.9106x$, $R^2=0.69$, $N=29$, $P<0.001$; Fig. 6A), which means that the larger the chamber excavated, the larger the closed tunnel length ($y=7.513+0.8133x$, $R^2=0.78$, $N=33$, $P<0.001$; Fig. 6B). An interesting exception to this relationship is represented by the series in which a pre-excavated 80 cm tunnel was offered. Queens were observed to close the tunnels not over their entire length, but in two to three sections (Fig. 6A, right). By adding these sections, the total length of the closed tunnel was comparable to that of the 1st day (U -test, $N=10$, $U=6$, $P>0.05$; Fig. 6A). Thus, the resulting chamber size did not differ from that

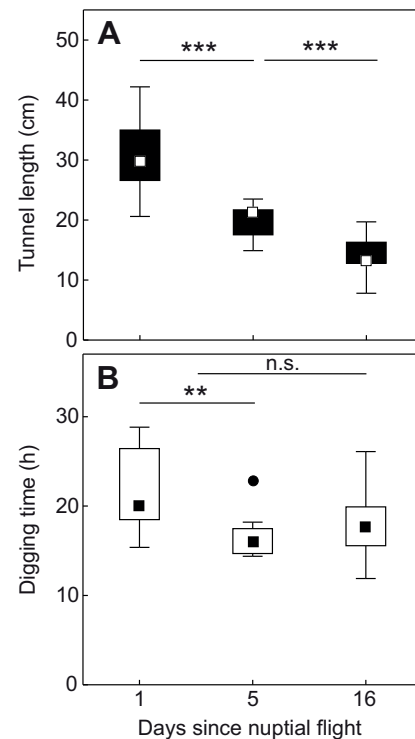


Fig. 5. Delayed digging. Tunnel lengths (A) and digging times (B) in *A. vollenweideri* nests built under standard conditions on the 1st ($N=14$), 5th ($N=8$) or 16th day ($N=14$) after the nuptial flight in 2008. Data shown are medians (squares) and upper and lower quartiles (boxes); error bars indicate the range without outliers (** $P<0.01$, *** $P<0.001$, n.s., not significant). Outliers (black circle) correspond to observations outside the interquartile range.

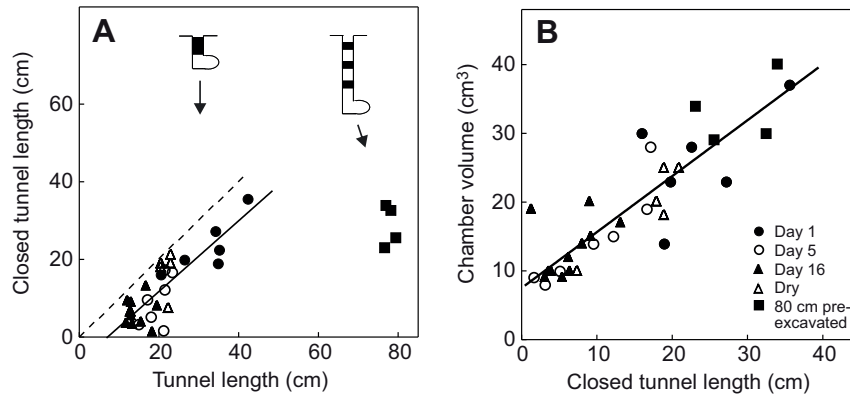


Fig. 6. Control of chamber size in nests dug by *A. vollenweideri* queens. (A) Extent of tunnel closing as a function of the excavated tunnel length. The regression line ($y = -6.4555 + 0.9106x$, $R^2 = 0.69$, $N = 29$, $P < 0.001$) takes into account data from four experimental series (see key on the right): under standard conditions, performed on days 1, 5 and 16 after the nuptial flight in 2008 ($n = 6, 7$ and 11 , respectively) and with dry clay ($N = 5$). The series with an 80 cm pre-excavated tunnel ($N = 4$) was not considered for the regression analysis because of the experimentally added tunnel length. The sample sizes were smaller compared with those used for the analysis of tunnel lengths, because of the exclusion of experiments in which the chambers reached the tube wall or were not completely plastered out. The dashed line describes the 1:1 relationship between the two variables. The drawings on the top depict the founding nests and the different ways of tunnel closure as observed: either in one segment, or in two to three sections (series with an 80 cm pre-excavated tunnel). (B) Relationship between the length of the closed tunnel and the final chamber volume, obtained using the method of water displacement, for the five experimental series as indicated in the legend: $y = 7.513 + 0.8133x$ ($R^2 = 0.78$, $N = 33$, $P < 0.001$).

of the experiments performed 1 day after the nuptial flight (U -test, $N = 10$, $U = 4.5$, $P > 0.05$; Fig. 6B).

Fig. 7 summarizes the relationship between average tunnel length and average chamber volume for field and laboratory nests of *A. vollenweideri*, as well as for three independent data sets taken from two published studies on the related species *Atta capiguara* (Mariconi, 1974; Pereira da Silva et al., 1982). Chamber volume was observed to positively depend on the length of the excavated tunnel, i.e. the longer the excavated tunnel, the larger the chambers dug.

DISCUSSION

The control of nest depth: underlying mechanisms

In this study we examined the mechanisms involved in the determination of nest depth by leaf-cutting ant queens. We advanced four different hypotheses for the possible control systems, one of them involving the use of external information, and the other three requiring internal information about the queen's own movements, the time elapsed since the beginning of digging or the total energy spent while digging. Our results provide clear evidence that the excavation of the founding nest by *A. vollenweideri* queens is a regulated process involving the use of internal information. Under specific experimental conditions, queens were observed to excavate the tunnel either until a preset depth was reached or for some predetermined length of time, indicating that both idiothetic information about the queen's movements as well as an estimate of the elapsed time are involved. The hypothesis that queens estimate their energy expenditure while digging and stop the tunnel enlargement once a given amount of energy was spent can be ruled out, as will be described below. Because the soil and environmental conditions were maintained invariant during the experiments, we cannot completely exclude the involvement of external variables during the control of nest depth. Physical features of the soil (i.e. moisture, composition or sediment boundaries) are known to influence the digging behavior of ant workers, and thus the shape of the excavated structures (Espinoza and Santamarina, 2010; Minter et al., 2011; Toffin et al., 2010). However, it appears unlikely that queens may respond to environmental soil conditions while

digging across the soil profile, and switch to chamber digging once a physical discontinuity, or a specific temperature, moisture or soil composition, is encountered. Similarly, digger wasps are known to enlarge their tunnels until a preset tunnel length, and not until some specific value of soil temperature, moisture or light intensity inside the burrow is reached (Brockmann, 1976; Brockmann, 1980).

Evidence that queens are enlarging the tunnel until a preset depth is reached arose from the experiments in which tunnels of different pre-excavated lengths were offered. Queens enlarged shorter tunnels, or started immediately with the excavation of the chamber when the presented tunnels were longer than those excavated under standard conditions. Queens were therefore able to distinguish between the different offered lengths, and so to obtain an estimate of the walked distance. Desert ants (genus *Cataglyphis*) are known to travel long distances during foraging, and proprioceptive information obtained while walking appears to be stored by a 'stride integrator', thus providing the ants with a distance estimate (Wittlinger et al., 2006; Wittlinger et al., 2007). Ants, like most insects, have mechanoreceptive hair plates (Markl, 1963; Mittelstaedt, 1962; Pringle, 1938) located at different joints, which provide information about the position of the body parts relative to each other. It is suggested that leaf-cutting ant queens are also able to gain proprioceptive information through walking the tunnel up and down, and so to obtain an estimate of the walked distance that may be compared with a motor command. It is important to note that a queen performs more than 400 walks up and down the tunnel before starting the excavation of the lateral chamber. It is unknown whether the obtained proprioceptive information while walking is monitored every time the queen is walking the tunnel, or whether there are temporal windows along the digging process within which information processing takes place.

Further evidence on the existence of idiothetic control was provided by the experiments in which queens were able to excavate their complete nests faster than under standard conditions. Queens digging in warmer or less dense (sandy) soils switched to chamber digging after having reached a tunnel length similar to that of queens

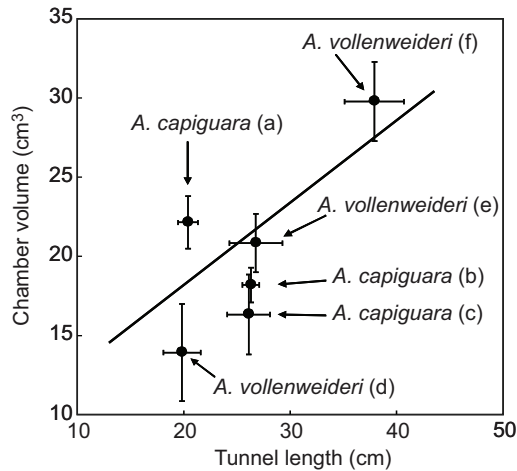


Fig. 7. Chamber volume as a function of tunnel length for founding nests of *Atta capiguara* (data from the literature) and *Atta vollenweideri* (present study). All chamber volumes were calculated with the formula of an ellipsoid ($V=\pi/6lhw$), because literature data were only available as chamber length, width and height. Data are means \pm s.e.m. The regression line is based on all single data points ($N=92$): $y=7.8428+0.5185x$ ($R^2=0.21$, $P<0.001$). Numbers of nests for each species are as follows: (a) *A. capiguara*, $N=37$ (Pereira da Silva et al., 1982); (b,c) *A. capiguara*, $N=16$ and 12, respectively (Mariconi, 1974); (d) *A. vollenweideri*, $N=6$ field nests in 2005; (e) *A. vollenweideri*, $N=15$ field nests in 2006; (f) *A. vollenweideri*, $N=6$ laboratory nests dug by queens 1 day after the nuptial flight in 2005.

digging under standard conditions, although their time investment was significantly shorter. Besides providing evidence for the existence of idiothetic control, comparisons of the digging behavior at the different temperatures assayed (20.5 and 27.2°C) allowed us to reject the hypothesis that queens would stop enlarging the tunnel after the consumption of a certain amount of energy. Based on the quantitative relationship between metabolic costs of cutting and temperature for leaf-cutting ants (Krumme, 1997), or locomotory activity and temperature (Bollazzi and Roces, 2011), it can be calculated that the expected fractional increase in activity for a temperature increase of 10°C, i.e. the Q_{10} value, should average 1.9 for the temperature range between 20.5 and 27.2°C, as in our experiments. As a consequence, queens excavating at 27.2°C are expected to spend more energy per unit time, on average by a factor of 1.9, than queens excavating at 20.5°C. Based on the hypothesis that queens in both groups should stop the tunnel enlargement once a given amount of energy was spent, it follows that queens excavating at 27.2°C should excavate for a time interval that corresponds to that invested by queens at 20.5°C divided by the same factor (i.e. 1.9). Based on the median times spent at 20.5°C (16 h 14 min; Fig. 3B), queens at 27.2°C should have spent 8 h 33 min digging. The median digging time of queens at 27.2°C was 13 h 20 min (Fig. 3D), i.e. ca. 56% longer than expected under the hypothesis that queens stop the enlargement of the tunnel after the consumption of a given amount of energy.

In addition to the use of idiothetic control, unequivocal evidence for the involvement of an additional temporal control was provided by the experiments in which queens excavated their complete nests slower, or were only allowed to initiate digging after long time delays of several days. When digging in dry soils, queens dug shorter tunnels than under the standard conditions, yet invested a similar total time for digging. That means that as soon as a maximal prescribed length of time is spent, queens would stop the

enlargement of the tunnel and switch to chamber digging, even though the preset tunnel length was not reached. The hypothesis about the involvement of a temporal control is in addition supported by the results obtained in the experimental series with delayed digging. Queens precluded for several days to initiate digging showed a slower digging rate, probably as a result of partial exhaustion or desiccation, and excavated tunnels of reduced length, yet they invested a similar time, roughly 20 h, until switching to chamber digging. Analogous results were recently obtained in *A. sexdens* in another context: queens that successively excavated two or three founding nests dug significantly shorter tunnels than those of their first nest, yet they spent a similar time digging (Camargo et al., 2011).

Whatever the precise mechanisms involved in the temporal control, the ability to estimate a time interval is needed. For the possibility of time perception in ants, it is important to distinguish between two different phenomena that have been shown to underlie behavioral timing in animals: a phase sense, which refers to the ability of animals to anticipate events that recur at a fixed time of the day (at a fixed phase of the day–night cycle), and an interval sense, which refers to the ability to respond to something that repeatedly comes a fixed amount of time after an event that occurs at varying points in the day–night cycle (Gallistel, 1990). The studies on temporal learning so far reported for honeybees (Wahl, 1932) and nectar-feeding ants (Harrison and Breed, 1987; Schatz et al., 1994), which focused on the finding of food at a certain time of the day, dealt with the phase sense. The ability to assess time intervals, i.e. the interval sense, has already been implicitly acknowledged in early studies on honeybee foraging (Núñez, 1966), and proposed for honeybees in the context of food unloading (Seeley, 1989; Wainseboim et al., 2003). *Camponotus rufipes* foraging ants are able to estimate time intervals in the range of minutes: workers accustomed to collect nectar at slow rates waited at the feeder longer than did ants collecting nectar at higher rates, and their waiting times in tests in which an exhausted feeder was offered were similar to those observed during training (Schilman and Roces, 2003).

The conclusion that a feedback loop providing temporal information is involved in the control of nest depth in *Atta* queens, however, does not necessarily mean that an interval sense is involved, as nest digging is not a recurrent event in the life of a queen – it takes place only once. A more likely explanation is that the queen's digging behavior is controlled by a motor program that is not simply run until completion, but uses both temporal and idiothetic information as feedback. In this line of argument, all results obtained so far can be accounted for by postulating the following algorithm underlying the control of nest depth in leaf-cutting ant queens (Fig. 8). It is argued that idiothetic information gained while walking the excavated tunnel, as well as the elapsed time, feeds back onto the control system as follows: a queen starting to dig a founding nest has to perform a series of repeated behavioral sequences (soil removal, pellet carriage) until one of the criteria triggering the change from tunnel digging to chamber digging is met, i.e. either a preset tunnel length is reached or a prescribed time interval is finished. As long as neither of these criteria is met, the queen is expected to continue enlarging the tunnel. The variability in the tunnel lengths measured for several *Atta* species in the field [7.5–12 cm in *A. colombica* (Weber, 1972); 6.5–13 cm in *A. cephalotes* (Weber, 1937); 15–25 cm in *A. texana* (Moser, 1967); 20–40 cm in *A. sexdens* (Ihering, 1898); 8–9 cm in *A. bisphaerica* (Araújo et al., 2003); 11–34 cm in *A. capiguara* (Mariconi, 1974; Pereira da Silva et al., 1982); and 9–15 cm in *A. insularis* (Bruner and Valdés Barry, 1949)] may be the result of variable soil

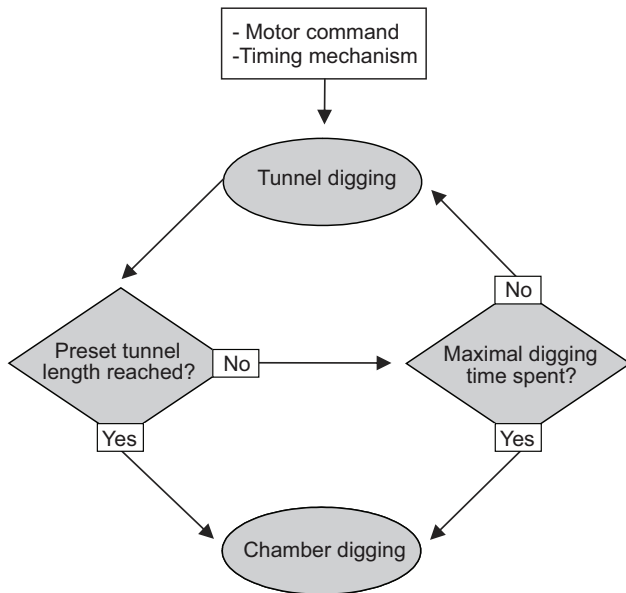


Fig. 8. Hypothetical algorithm underlying the digging behavior of *A. vollenweideri* queens. Digging behavior is the output of a motor command that is initiated simultaneously with a timing mechanism. The proposed control system involves two regulatory feedbacks: one monitoring the queen's movements during the excavation and conveying proprioceptive information, and the other requiring an estimate of the elapsed time. The transition from tunnel to chamber digging would be initiated as soon as one of the criteria is fulfilled, i.e. either a preset tunnel length is reached or maximal digging time is spent.

conditions and/or the prevalence of one of the two different feedback loops proposed in the present study.

Digging of a founding nest by ant queens appears to follow a fixed sequence and, like other behavioral sequences that occur once in an animal's life span (Hansell, 1984), this sequence is expected to be rather inflexible and substantially controlled by internal cues. Rigid pre-programmed behaviors, once initiated, are unfolded until their conclusion without regard to changes in the environment. In contrast, animals performing a building sequence repeatedly in their life often show behavioral flexibility (Koenig, 1951), being able to re-build their nests back into the original condition after damage, repeatedly build the same nest structure, or complete half-finished nests because they respond to stimuli offered by the construction itself as the building process proceeds; this form of stimulus-response mechanism was originally called 'stigmergy' (Grassé, 1959). Based on the present results, it is argued that nest founding by leaf-cutting ant queens follows a rigid sequence of tunnel digging followed by the excavation of a chamber, but that the behavioral transition between these two digging patterns is controlled *via* two feedback loops involving temporal and proprioceptive information. A hypothetical control system that would trigger the excavation of a chamber only after a preset tunnel length is reached, i.e. lacking temporal control, would have the disadvantage that in harder soils, longer digging times and higher digging efforts need to be spent: queens would be exposed to predators for longer, and the increasing digging efforts may compromise their survival (Camargo et al., 2011). A control system using both idiothetic and temporal information, as demonstrated in the present study, allows a flexible reaction to different soil conditions so as to preclude excessive time and energy investments.

To what extent similar control systems underlie the digging behavior of ant workers, thus providing them with spatial and temporal references while excavating the soil, remains completely unknown.

The control of chamber size

Considering that during the excavation of the chamber the queen completely plugs the tunnel from the inside with the removed soil pellets, a relationship between tunnel length and final chamber size should be observed. Our results and data from the literature on a different *Atta* species are consistent with this hypothesis. Queens are therefore expected to stop chamber enlargement as soon as the nest tunnel is completely closed. Any further enlargement of the chamber would have the consequence that the resulting pellets must be deposited within the chamber being enlarged, thus reducing its volume and turning the queen's behavior into a Sisyphus work. An exception for the relationship between tunnel length and chamber size is represented by the experiments in which pre-excavated tunnels of 80 cm length were offered, i.e. 2.8 times longer than the tunnels excavated under standard conditions. If the tunnels were completely closed during the excavation of the chamber, the final chamber would have been 2.8 times larger in size than those excavated under standard conditions. However, queens did not close the complete tunnel length, but only two or three separate sections. By adding their lengths, a total closed tunnel length similar to that observed under standard conditions was obtained. This result indicates that an additional yet unknown feedback mechanism is involved in the control of chamber size. Queens may also use their bodies as a template, as described for nest building in weaver birds (Collias and Collias, 1962), and so set an upper limit to the chamber size to be excavated. In addition, a temporal control, as we described for the control of tunnel length, may underlie the regulation of chamber size.

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