



Underground nest building: the effect of CO₂ on digging rates, soil transport and choice of a digging site in leaf-cutting ants

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Received: 6 February 2018 / Revised: 12 March 2018 / Accepted: 14 March 2018 / Published online: 22 March 2018
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Abstract

By reacting to local environmental stimuli, ant workers excavate a nest that offers suitable climatic conditions. Workers may face increasing CO₂ levels while digging across the soil profile, and it is an open question whether these levels are used as cues during nest excavation. Here, we explored the influence of different underground CO₂ concentrations on digging behavior in the leaf-cutting ant *Acromyrmex lundii*. We first quantified digging rates and transport of excavated material as a function of CO₂ levels, ranging from atmospheric values to 11% CO₂. The mass of both the excavated soil and the pellets transported out of the digging chamber were quantified. CO₂ preferences for excavation were investigated in a second experiment, in which workers were presented with a choice of two digging sites that differed in their CO₂ levels, ranging from atmospheric to 4% CO₂, and the mass of excavated material was quantified. Digging rates were similar for all tested CO₂ levels up to 7%, and only significantly lower for 11%. The transport of excavated soil increased with increasing CO₂ levels up to 7%, and then decreased at 11%. Workers preferred digging at 1% CO₂ and avoided levels of 4%. We suggest that the observed CO₂ preferences are likely driven by the requirements of their symbiotic fungus, and could be one of the reasons why colonies of *A. lundii* excavate superficial nest chambers.

Keywords Carbon dioxide · Nest excavation · Cue · Self-organization · Nest architecture

Introduction

Social insects build the most complex nests in the animal kingdom. While building is a costly process, it is advantageous for the inhabiting colony. Erecting a barrier between the colony members and the physical environment provides a better defense against predators, helps to organize workers' tasks and dampens climatic fluctuations to some degree (Hansell 1984). Excavating underground nests, as colonies of many ant species do, further aids climatic control. Climatic conditions underground, while still influenced by precipitation and solar radiation, fluctuate less and are

not characterized by extreme high and low temperature or humidity conditions as aboveground.

Despite the complex nature of social insect nests, they result from simple responses of building workers to local biotic or abiotic stimuli (Deneubourg and Franks 1995; Theraulaz et al. 1998; Bonabeau 1998), without workers having an overview of the complete process or a central control. For example, the presence of other nestmates as biotic cues triggers nest digging behavior in ants, and decreasing local worker density leads to cessation of excavation (Deneubourg and Franks 1995; Rasse and Deneubourg 2001). Other biotic stimuli help to intensify the excavation process. In the leaf-cutting ant *Acromyrmex lundii*, locally-deposited brood and symbiotic fungus increase worker density at these sites as they are attractive to workers (Römer and Roces 2014). Vibrational signals produced by digging workers attract more workers to the site, leading to stronger aggregation (Pielström and Roces 2012). Building can also be organized by stigmergic responses to the abiotic environment without a direct interaction among workers (Grassé 1959; Theraulaz and Bonabeau 1999). For example, the deposition of freshly excavated soil pellets can influence ant workers' decision

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where to engage in excavation and it, therefore, spatially organizes building (Pielström and Roces 2013).

Microclimatic factors are important abiotic cues for nest building as nests should provide adequate temperature or humidity ranges for their inhabitants as well as for the performance of colony tasks, especially the raising of brood (Roces and Núñez 1989; Bollazzi and Roces 2002; Falibene et al. 2016), as it ensures colony growth. Excavating or enlarging a nest where proper conditions are encountered should provide the colony with an appropriate climate. Indeed, leaf-cutting ants show coinciding preferences for nest excavation (Bollazzi et al. 2008; Pielström and Roces 2014) and for brood and fungus rearing (Roces and Kleineidam 2000; Bollazzi and Roces 2002), i.e., between 20 and 30 °C and high soil moistures, well-suited for the development of the symbiotic fungus these ants grow (Quinlan and Cherrett 1978; Powell and Stradling 1986). As a general rule, climatic conditions underground fluctuate more strongly close to the soil surface, and temperature decreases and moisture increases with depth (Hillel 1998). To some degree, the microclimate of underground nests can also be adjusted by building responses, i.e., the opening and closing of nest openings to hinder airflows and to counteract humidity losses of the nest air (Bollazzi and Roces 2007).

Building an underground nest or controlling ventilatory airflows through building responses in an existing nest presents the colony with a problem. Depending on soil characteristics, the gas exchange may be hindered, leading to underground hypercapnic (high carbon dioxide) and hypoxic (low oxygen) conditions. Decaying organic material in the soil as well as the respiration of the inhabiting colony itself further increase the CO₂ levels. Generally, the underground CO₂ levels close to the soil surface are much higher than the atmospheric levels, currently around 0.04% CO₂. Close to the surface of sandy soils, for instance, CO₂ levels range from 0.2 to 0.4% CO₂ (Tschinkel 2013), while levels of 1–2.7% CO₂ can be detected in more organic soils (unpublished data). At deeper soil layers, levels of 5–7% can be reached (Kleineidam and Roces 2000; Bollazzi et al. 2012), as the underground CO₂ concentration increases with depth (Schwartz and Bazzaz 1973; Hamada and Tanaka 2001).

Leaf-cutting ants are an ideal organism to study the potential influence of underground CO₂ concentrations on nest excavation. They build the most complex nests among ants, with nests of some species having thousands of chambers and encompassing millions of individuals (Moreira et al. 2004a, b). In addition, they are the only insects known equipped with antennal sensilla that respond to changes and to absolute CO₂ levels (Kleineidam and Tautz 1996; Kleineidam et al. 2000). High CO₂ concentrations inside the nest may influence colony growth by negatively influencing the respiration rate of the fungal symbiont (Kleineidam and Roces 2000), the sole food source of the brood. In our recent

study, we showed that workers of *A. lundii* avoided high CO₂ concentrations to maintain their fungus, and rather preferred intermediate CO₂ values as found at levels close to the soil surface (Römer et al. 2017).

As of yet, it is unclear whether long-term exposure to the CO₂ levels encountered in ant nests might affect growth or development of their brood, as observed in some Lepidoptera (Coelho Junior and Parra 2013; Coelho Junior et al. 2017). Much higher CO₂ values, as used for instance for pest control or anesthesia, can negatively affect not only brood development, but also some aspects of the adult physiology (Beckmann 1974; Nicolas and Sillans 1989; Guerenstein and Hildebrand 2008; Klaiber et al. 2013). Leaf-cutting ants show distinct CO₂ preferences for the placement of their brood (unpublished data) and also show specific, regulatory building responses when constructing nest turrets in response to high CO₂ levels inside nest tunnels (Halboth and Roces 2017).

Considering both the negative effect of CO₂ on fungus growth and the observed CO₂ preferences for the placement of fungus and brood inside the nest, leaf-cutting ants are expected to use CO₂ as a cue when they excavate their nests. We, therefore, asked whether CO₂ influences the digging behavior of workers of the leaf-cutting ant *A. lundii*, and whether workers select a digging site based on the prevailing CO₂ levels. To answer the first question, we offered a group of workers single digging chambers with different CO₂ concentrations, ranging from atmospheric values to 7% CO₂. As leaf-cutting ants are tolerant to very low O₂ concentrations (Hebling et al. 1992), we also asked whether they may still excavate at the extreme levels of 11% CO₂, although such high levels are unlikely to occur in their nests. We quantified both the total amount of soil excavated at different CO₂ levels and the removal of excavated soil pellets after 5 h. The selection of a digging site depending on the CO₂ levels was explored by presenting workers with a binary choice setup offering two digging chambers with different CO₂ concentrations. Here, atmospheric, 1% CO₂ (as occurring at superficial soil layers) and 4% CO₂ (as found at deeper soil layers) were offered, and the total amount of excavated soil was quantified.

Materials and methods

Study animals

All experiments were performed with worker groups of the leaf-cutting ant *A. lundii*. Founding colonies were collected near Montevideo, Uruguay, and raised in the Department of Behavioral Physiology and Sociobiology at the University of Würzburg, Germany. Colonies were kept under constant temperature (25 °C) and relative humidity (50%), under a

12:12 h LD cycle. At the time of the experiments, all colonies were mature and at least 4 years old. They consisted of eight nest boxes (19×19×9 cm) filled with symbiotic fungus, a feeding box and a box for waste disposal. Food (*Rubus fruticosus* leaves, diluted honey) and water were provided ad libitum.

Experimental setups and procedure

Effect of CO₂ on digging rates

A digging chamber (9.5×9.5×3 cm) was connected to a second, open box (Fig. 1a; ant release box, 19×19×9 cm) by a piece of plastic tubing (diameter 1.5 cm). The back half of the digging chamber was filled with a clay–sand mixture (2:1, Claytec Baulehm gemahlen 0–0.5 mm, Viersen,

Germany and Dorsilit Kristall-Quarzsand 0.1–0.5 mm, Hirschau, Germany; 16% water content) for excavation. To generate a constant CO₂ concentration inside the digging box, a small rubber hose (diameter 0.3 cm) was inserted through the back-wall of the chamber and buried up to half-way into the clay. Extending from the rubber hose, a small tunnel was drilled into the clay through which the airstream was pumped (flow rate 50 ml min⁻¹) into the empty part of the digging chamber. To prevent desiccation of the clay mixture by the inflowing air, the airstream was directed through a wash bottle filled with water before the hose was inserted into the arena. To maintain a stable CO₂ concentration, the chamber air was pumped out (miniature vane pump, 135 FZ, Schwarzer Precision, Germany) at an equal flow rate of 50 ml min⁻¹ through two small rubber hoses (diameter 0.3 cm) inserted in the entrance wall of the chamber. To quantify digging rates at atmospheric values (ca. 0.04% CO₂), the standard laboratory compressed air was used as source. Elevated CO₂ concentrations were generated by mixing compressed air with pure carbon dioxide using gas-mixing devices (Mass Flow Controller MFC-4 and Mass Flow Controller v1.0, Sable Systems International, USA).

On the day of the experiment, 100 media-sized workers (mean body mass: 5.3 ± 1.2 mg SD, *n* = 80) were collected from 1 of 5 mother colonies. We started the assay only after the establishment of the chosen CO₂ level inside the chamber (CO₂ sensor: Gasmittler, Sensor Devices, Germany; range 0–10%, resolution: 0.01%), since a local CO₂ stimulus could lead to ant aggregation at the spot and to subsequent digging activity because of the increased ant density, and not as a result of the offered CO₂ concentration. Then, a group of 100 workers was placed into the ant release box from where ants could freely explore the whole setup and start to dig. After 5 h, all ants were removed and the total amount of excavated material was quantified by weighing the digging chamber, cleared of all excavated soil pellets, to the nearest 0.1 g (1 g of the clay mixture corresponds to an excavated volume of 0.41 cm³), and by subtracting the weight from the full arena weighed at the beginning of the assay. As leaf-cutting ants may not remove all excavated material from the digging site, but use it to subsequently reduce the created nest space (Römer and Roces 2015), we also separately quantified to the nearest 0.1 g the mass of excavated clay pellets carried out of the digging chamber.

Six series were performed. First, we quantified digging rates at atmospheric CO₂ levels as a control baseline. Further, we assayed concentrations of 1 and 2%, to investigate the effects of levels measured close to the soil surface (unpublished data), where nest chambers of *A. lundii* are usually found (Zolessi and González 1978). Additionally, levels that occur at deeper soil layers, 4 and 7%, were tested. Finally, we assayed an extreme CO₂ value of 11%, over-reaching the highest levels measured around leaf-cutting ant

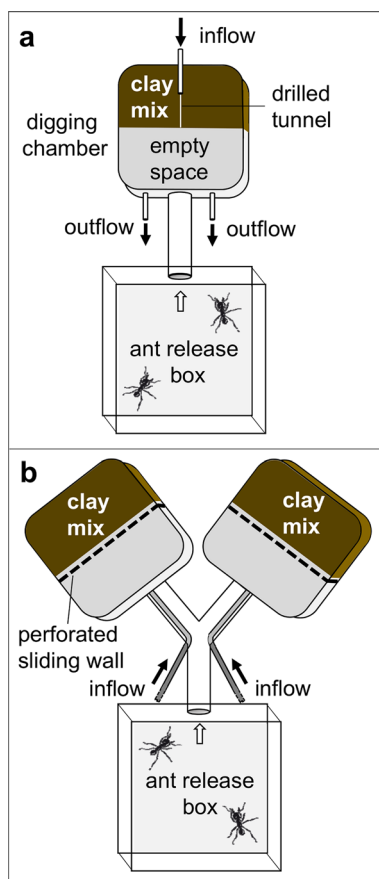


Fig. 1 Experimental setups **a** to quantify digging rates: digging chamber connected to an ant release box. Inflow of air with different CO₂ concentrations through rubber hose inserted into the clay mixture through the back chamber wall. Outflow of air through hoses located in the front wall if the chamber, **b** choice of a digging site: two digging chambers connected by a Y-shaped tube to an ant release box. Inflow of air with different CO₂ concentrations into the chambers through rubber hoses inserted at the bifurcation point of the Y-shaped tunnel leading to the nest chambers. The injected air left passively through the upper openings of the sliding wall

nesses, to test workers' tolerance levels. We chose this level because workers of the mangrove-dwelling ant *Camponotus anderseni* switch to anaerobic respiration during nest flooding at this concentration (Nielsen et al. 2006). We had also used this concentration in previous unpublished studies on leaf-cutting ants and detected no signs of lethargy by workers during current tasks or deaths. It is important to indicate that the O₂ concentrations of the CO₂-enriched gas mixtures used in the present series only decreased by 3% over the assayed CO₂ range, from 20.95% O₂ at atmospheric CO₂ levels, to 18.1% O₂ at 11% CO₂. Since leaf-cutting ant workers show respiratory regulation and are not affected by O₂ levels down to 10% (Hebling et al. 1992), we are confident that any effect of the used gas mixtures should be attributed to the experimentally increased CO₂ levels.

Choice of a digging site based on CO₂ levels

Two digging chambers (9.5 × 9.5 × 3 cm) were connected to a third, open box (ant release box, 9.5 × 9.5 × 5.5 cm, Fig. 1b) by a Y-shaped plastic tube (diameter 1.7 cm). The back half of each chamber was filled with a clay–sand mixture (2:1) with a water content of 19%. A perforated, sliding wall was inserted in each lid of the digging chambers, effectively preventing initial digging but allowing the diffusion of volatiles from the clay mixture. For the establishment of the different CO₂ concentrations in the digging chambers, specific CO₂ mixtures were pumped into each of the two digging chambers through two small rubber hoses (diameter 0.3 cm; flow rate 50 ml min⁻¹), inserted into the Y-shaped tube at the bifurcation point and terminating in each chamber. Measurements of air humidity inside the digging chambers without ants revealed values between 70 and 90%, which prevented desiccation of the clay mixture.

Analogous to the previous experiment, 100 media-sized workers were collected from one of three mother colonies. When the chosen CO₂ concentrations were established in both digging chambers, workers were placed into the ant release box. From there they had free access to both digging chambers, but were initially prevented from initiating excavation at once by the sliding walls in front of the clay mixture. After a familiarization period of 1 h, the sliding walls were lifted and ants could excavate for 3 h. Afterwards, the ants were removed and the total amount of excavated material was quantified to the nearest 0.1 g (1 g of the clay mixture corresponds to an excavated volume of 0.28 cm³). As the aim of this experiment was to determine worker preferences for the task of nest digging, pellet transport out of the digging arenas was not recorded.

We tested different combinations of three CO₂ concentrations, namely atmospheric levels, 1% and 4%, as they occur at superficial and deep soil layers, respectively. Four different series were performed offering the following choices:

atmospheric vs. atmospheric levels (control for side biases), atmospheric vs. 1% CO₂, atmospheric vs. 4% CO₂, and 1% CO₂ vs. 4% CO₂.

Data analysis

Normal distribution of data was tested using the Shapiro–Wilk test. Digging rates and pellet transport were compared using either ANOVA and Scheffé post hoc tests or the Kruskal–Wallis test and multiple Dunn comparisons with a Bonferroni–Holm correction as post hoc tests. Datasets of digging choices were compared using paired *t* tests. For the sake of homogeneity, data on digging rates and pellet transport are presented as boxplots in Fig. 2, although data were analyzed based on the outcome of the test of normal distribution. All graphs were created with SigmaPlot 10 (Systat Software, Inc., San Jose, California, USA) and later

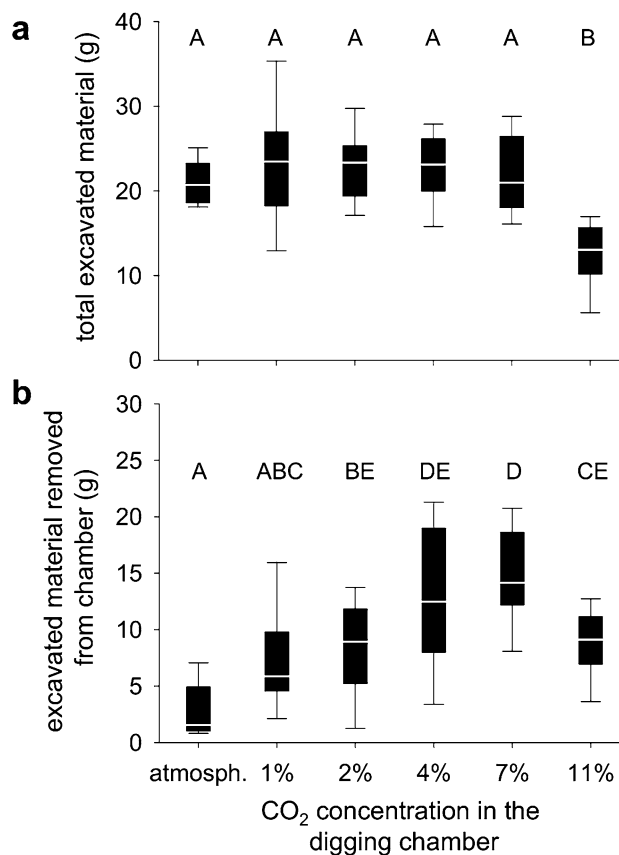


Fig. 2 Digging behavior **a** mass of excavated material (in g) over 5 h, depending on the prevailing CO₂ concentration: ANOVA and Scheffé post hoc tests, *n* = 20 per series, **b** excavated material removed from the chamber, depending on the CO₂ concentration: Kruskal–Wallis test and multiple Dunn comparisons with Bonferroni–Holm correction as post hoc test, *n* = 20 per series, line: median, box: 25–75% percentiles, whiskers: min–max values, presentation without outliers, data groups sharing the same letters do not differ statistically, $\alpha \leq 0.05$

edited using Microsoft PowerPoint (Microsoft Corporation, Redmond, Washington, USA).

Results

Effect of CO₂ on digging rates

Not all released ants were present in the digging arena during the experiment (usually between 25 and 40 workers), and not all ants present in the arena engaged in digging. Rather, workers moved between the release box and digging arena, so that a large proportion of ants was also present either in

the connecting tube or in the release box at any given time. The total mass of material excavated over 5 h was similar in the range from atmospheric values to 7% CO₂ (Fig. 2a; atmospheric: 21.17 ± 0.72 g mean ± SE, 1%: 23.6 ± 1.59 g, 2%: 23.03 ± 1.0 g, 4%: 23.09 ± 0.93 g, 7%: 21.76 ± 1.04 g; sample size of each group = 20). Only digging activity at the extremely high value of 11% CO₂ (12.36 ± 0.84 g) was significantly lower (ANOVA: $F = 16.19$, $p < 0.0001$, Table 1).

The mass of excavated pellets removed from the digging site increased with increasing CO₂ concentration. Only at 11%, at which a low digging rate was displayed as indicated above, did the amount of removed pellets significantly decrease (Fig. 2b; atmospheric: 1.6 g median, 1%: 5.95 g, 2%: 9 g, 4%: 12.45 g, 7%: 14.2 g, 11%: 9.15 g; sample size = 20 in each group). The statistical comparisons among levels indicated that pellet transport was comparable between atmospheric values and 1%, but increased significantly at 2% CO₂. There were no differences between 1 and 2%, but pellet transport differed between 1 and 4% as well as 7%. Transport at 2% was significantly lower than at 7%, but comparable to 4%. In summary, most material was removed from the chambers at 4 and 7%, followed by 2 and 11% CO₂ (Kruskal–Wallis test: $H = 54.86$, $p < 0.00001$; Table 1).

In relation to the total amount of material excavated (Fig. 2a), only 7% of the pellets were removed outside of the digging chamber at atmospheric values, while nearly 70% of them were removed at the highest levels of 7% CO₂ and 11% CO₂. Exemplary photos of digging chambers showing areas of excavation and pellets still left there at the end of the assay are presented in Fig. 3a–c.

Choice of a digging site based on CO₂ levels

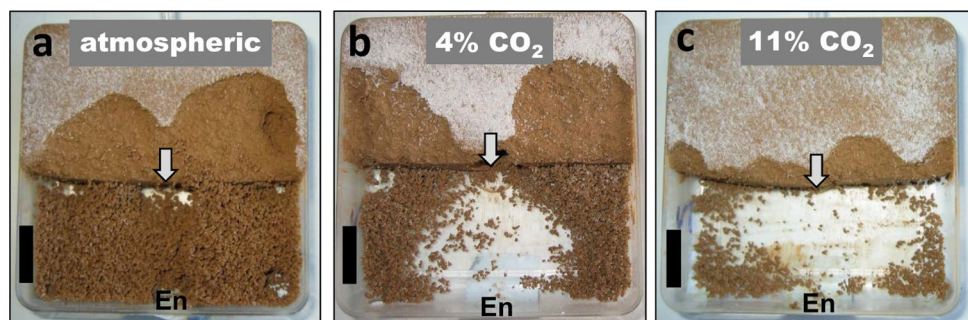
During this experiment, most workers were inside the digging chambers and only a small proportion was seen, at any given time, in the ant release box. Worker numbers in the chambers also fluctuated throughout the time of the assay. Similar to the first experiment, not all workers at the digging sites actually engaged in excavation. Workers showed no side preference when presented with the control choice situation offering atmospheric CO₂ values at

Table 1 Statistical details of experiment 1

Groups	Digging rates		External pellet transport	
	Post hoc test: Scheffé		Post hoc test: Multiple Dunn comparisons, Bonferroni–Holm corrected	
	<i>F</i>	<i>p</i>	<i>Z</i>	<i>p</i>
Atmospheric vs. 1%	0.53	0.76	2.66	0.054
Atmospheric vs. 2%	0.31	0.91	3.26	0.011
Atmospheric vs. 4%	0.33	0.89	5.43	<0.0001
Atmospheric vs. 7%	0.03	1	6.78	<0.0001
Atmospheric vs. 11%	6.96	<0.0001	3.6	0.004
1 vs. 2%	0.03	1	0.6	1
1 vs. 4%	0.02	1	2.76	0.046
1 vs. 7%	0.31	0.91	4.12	<0.001
1 vs. 11%	11.32	<0.0001	0.94	1
2 vs. 4%	0.0003	1	2.17	0.18
2 vs. 7%	0.15	0.98	3.53	0.005
2 vs. 11%	10.21	<0.0001	0.34	1
4 vs. 7%	0.16	0.98	1.36	0.7
4 vs. 11%	10.32	<0.0001	1.83	0.34
7 vs. 11%	7.89	<0.0001	3.18	0.013

Left digging rates, right external pellet transport, sample size $n = 20$ for each group

Fig. 3 Exemplary photos of digging chambers showing the excavated areas (upper chamber half) and the pellets remaining in the chamber (lower chamber half), **a** digging at atmospheric values, **b** digging at 4% CO₂ concentration, **c** digging at 11% CO₂ concentration, En = Entrance to chamber, gray arrow = direction of air inflow, black bar = 2 cm



both digging chambers, i.e., digging activity was equally distributed (Fig. 4a; paired t test: $t = -2.04$, $n = 12$, $p = 0.07$). More material was excavated under atmospheric conditions than in the chamber with elevated CO₂ levels, whether these were 1 or 4%, yet the differences were not statistically significant (Fig. 4b, c; paired t test: atmosph. vs 1%. $t = 1.32$, $n = 12$, $p = 0.21$; atmosph. vs. 4%. $t = 1.82$, $n = 12$, $p = 0.1$). There was a significant preference when two different CO₂ levels that occur underground were presented as choice, i.e., 1 vs. 4%. Workers excavated more in the chamber with 1% CO₂, a level found at shallow soil strata, and avoided 4% CO₂, a value expected to occur at deep soil layers (Fig. 4d; paired t test: $t = 3.8$, $n = 12$, $p = 0.003$).

While no comparisons between the two experiments were planned because of their different aims, note that the measured digging rates at a given CO₂ level largely differed between the experiments. For instance, an average of 5 g/h at atmospheric levels in Exp. 1—digging rates—(Fig. 2, left), and of 14 g/h (or a mean of 7 g/h in each of the two chambers at atmospheric levels, Fig. 4a) in Exp. 2—digging choices—were excavated. The higher digging rates in the choice experiment likely resulted from the higher water content of the offered clay mixture (19%, compared to 16% in the first experiment), which significantly increases both excavation speed and mass of the excavated pellets (Pielström and Roces 2014).

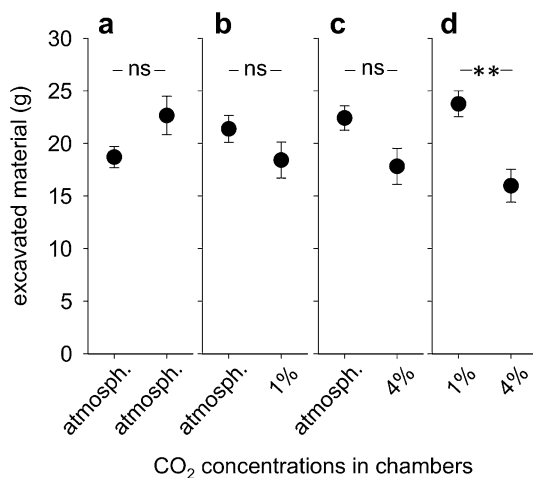


Fig. 4 Choice of a digging site based on CO₂ levels, **a** atmospheric vs. atmospheric (control), **b** atmospheric vs. 1% CO₂, **c** atmospheric vs. 4% CO₂, **d** 1 vs. 4% CO₂, expressed as excavated material per chamber after 3 h, $n = 12$ in each group, round symbols—means, whiskers—standard error, ns not significant, *** $p < 0.01$

Discussion

Underground CO₂ levels and digging rates

Digging rates of *A. lundii* were only negatively influenced by the very high CO₂ concentration of 11%. Such high CO₂ levels have never been measured in or around leaf-cutting ant nests, not even for deep nests of the genus *Atta*, which can reach 5–7 m (Moreira et al. 2004a) and levels of 6–7% CO₂ (Kleineidam and Roces 2000; Bollazzi et al. 2012). It is unknown under which conditions leaf-cutting ants would face such high levels of 11%. It is also unclear whether such high levels lead to a reduction in individual digging performance, or in the number of ants that engage in digging because of individual differences in response thresholds. However, this shows that leaf-cutting ants are not only tolerant to decreasing levels of O₂ down to 10% (Hebling et al. 1992), but also to high levels of CO₂, and that they are still able to excavate to some degree and engage in transport under such high hypercapnic conditions.

Increased CO₂ levels are known to act as a releaser of digging behavior in the ant *Solenopsis geminata* (Hangartner 1969). Our digging experiments did not reveal this effect, as digging rates at levels between 1 and 7% were similar to atmospheric levels. However, *S. geminata* workers were attracted to a local CO₂ source and aggregated closely to such a source. The local increase of CO₂ at the site could have led to a self-organized digging response triggered by the increased ant aggregation at the site, rather than a direct effect of CO₂ on excavation activity. This is the reason why we started the assays only when the CO₂ level had been established inside the digging chamber and, therefore, prevented workers to initially respond to the CO₂ provided at the local outflowing site on the digging face.

Underground CO₂ levels and the transport of excavated material

While there was no detectable effect of CO₂ on digging rates at CO₂ levels surrounding natural leaf-cutting ant nests, the same cannot be said about the effect on transport of the excavated soil pellets. Workers carried more of their excavated material outside of the digging chamber, the higher the CO₂ concentration. Usually, not all excavated soil pellets are transported outside of the nest. A recent study found that when digging over 2 days, up to 30% of soil pellets is deposited inside the excavated nest space and reworked (Römer and Roces 2015), either to create narrow tunnels or reduce cavity space. Probably due to

the short experimental time of 5 h, ants did not appear to use the material to build or form certain structures in the present experiments, and simply placed the excavated pellets on the ground a few centimeters behind the active digging zone.

As we did not observe individual workers, it is unclear whether all excavated pellets were deposited inside the digging chamber by the initial excavators and then picked-up and carried away by different ants, or if some, if not all excavators carried their pellets further away themselves. Pellet transport in leaf-cutting ants seems to be organized analogous to leaf-fragment transport into transport chains (Röschard and Roces 2003) where the original excavators only carry their loads a very short distance before dropping it, and short-distance and long-distance carriers pick the pellets up and transport them further away from the digging site (Pielström and Roces 2013).

Two scenarios may account for the increased removal of excavated material from the chamber with increasing CO₂ concentration. CO₂ could have, as mentioned above, a negative effect on digging activity, even at concentrations of 1–7%, and increasing numbers of ants could have stopped excavating, with increasing CO₂ concentration. However, as workers were not individually observed, we cannot rule out that alternative workers gaining access to the digging face kept overall digging rates at high levels. Digging rates would only be noticeable reduced when not enough ants could replace the ones that gave up digging. This might have been the case at the CO₂ level of 11%. Ants no longer engaging in excavation could then have switched to soil transport, so that an increasing number of workers transported soil pellets out of the digging chamber, with higher CO₂ concentration. This would be an analogous behavioral switch known from foraging leaf-cutting ants, which change from leaf cutting to leaf transport when their mandibles are worn down and they are no longer physically able to cut leaves (Schofield et al. 2011). On the other hand, removing the pellets from the digging site could be a regulatory response at unsuitable, high CO₂ concentrations aimed at generating more free space to increase local airflows and so to facilitate nest ventilation. As O₂ values in our experiments only slightly decreased at high CO₂ levels, as indicated above, the observed pellet transport is unlikely to be a response to changes in O₂ values.

CO₂ preferences for the selection of a digging site

Acromyrme lundii displayed CO₂ preferences for the selection of a digging site, yet only when the offered concentrations in both chambers corresponded to values that occur underground. When one chamber offered atmospheric values as occurring aboveground, digging activity was equal in both chambers. This is not due to the inability of the ants to perceive the difference between the two CO₂ concentrations,

since leaf-cutting ants are able to detect very low absolute CO₂ values (Kleineidam and Tautz 1996; Kleineidam et al. 2000). Also, a recent study showed that *A. lundii* does display CO₂ preferences in experiments with atmospheric levels offered as an alternative to elevated values, and avoids atmospheric values to maintain the fungus (Römer et al. 2017). It remains unknown why ants showed no preferences for excavation between chambers with atmospheric and underground CO₂ values in the present study. Interestingly, the displayed preference for digging at 1% is similar to the CO₂ preference shown for the maintenance of the fungus (Römer et al. 2017), i.e., such digging preference may lead to the excavation of chambers at suitable levels for this crucial nest task.

The effects of carbon dioxide on nest excavation and determination of nest depth in leaf-cutting ants

Ants should excavate their nests at soil strata that offer suitable environmental conditions to successfully perform their inside-nest tasks. Indeed, leaf-cutting ants do also display preferences for other microclimatic variables when they excavate (Bollazzi et al. 2008; Pielström and Roces 2014). As climatic variables change with soil depth, ants could use gradients in the soil as cues to initiate nest excavation. Many ant nests have a very distinct stratification of their underground chambers, for example the Florida harvester ant *Pogonomyrmex badius*, with a distinct top-heavy chamber stratification (Tschinkel 2004). As CO₂ increases with depth, ants could use this gradient as a template for nest excavation. However, experimentally reversing the CO₂ gradient did not lead to the excavation of upside-down nests by *P. badius* (Tschinkel 2013). Lack of use of the soil CO₂ gradient may be due to the better aerated soil type in which *P. badius* excavates their nests. This sandy soil does not have a steep CO₂ gradient with depth, whereas in the more organic and clayish soil many leaf-cutting ant species dig their nests in, CO₂ levels increase very steeply with depth (Bollazzi et al. 2012).

Our study revealed that leaf-cutting ants do use the carbon dioxide levels in the soil as a cue for self-organized nest excavation. Based on previous findings for temperature and moisture levels, the range of the reported microclimatic preferences for digging is broader (Bollazzi et al. 2008; Pielström and Roces 2014) than the range for the placement and maintenance of brood and fungus (Roces and Kleineidam 2000; Bollazzi and Roces 2002). The same seems to be the case for leaf-cutting ants' CO₂ preferences. Here, they not only show narrow, but also distinct preferred CO₂ ranges for fungus and brood tending (between 1 and 3% for fungus: Römer et al. 2017; 3% for brood, unpublished results). Avoiding too high CO₂ values, as they occur at deep soil layers, but accepting elevated ones as found closer to the soil surface (our present results),

would provide workers with broader microclimatic ranges that enable short-term responses to changing environmental conditions, i.e., brood or fungus relocation across different nest depths to follow their preferred values.

Even though preferences for specific environmental variables may concentrate digging activity at certain sites across the soil profile, they may not be the direct cause for the emergence of a nest chamber, since brood or fungus need to be first placed at a selected site, as demonstrated in a recent study (Römer and Roces 2014). Environmental variables determine where brood and fungus are placed (Roces and Kleineidam 2000; Bollazzi and Roces 2002; Römer et al. 2017). The relocated items, which are attractive to workers, lead to their aggregation at the site, which in turn triggers digging behavior (Rasse and Deneubourg 2001). Other factors, such as vibrational communication signals produced by workers engaged in digging (Pielström and Roces 2012), stigmergic responses to excavated soil pellets (Pielström and Roces 2013), and microclimatic preferences for digging could further increase digging activity at the selected site.

The observed choices for a CO₂ concentration found closely to the soil surface also help to understand *A. lundii*'s subterranean nesting biology, with nest chambers excavated in the uppermost soil layers. Since the respiration of the fungus is compromised at high CO₂ levels as occurring deep underground (Kleineidam and Roces 2000), avoidance of 4% CO₂ and preference for 1% during excavation could, therefore, be a behavioral adaptation of *A. lundii* to provide a long-term, suitable environment for fungus farming in nest chambers dug at superficial soil levels. Colonies of other *Acromyrmex* species and of most species of the genus *Atta* excavate much deeper nests with chambers found at 4 m (Verza et al. 2007) or even at 7 m (Moser 1963; Moreira et al. 2004a) below the surface. Deeper underground, some environmental variables such as humidity or temperature might be stable and more critical for brood and fungus survival than others. Depending on location and soil characteristics, ants are, therefore, expected to trade-off their competing microclimatic preferences when excavating new chambers at the best suitable sites. This aspect of self-organized nesting behavior remains to be explored in the future.

Acknowledgements We would like to thank David Urbaniec, Lisa Weidner and Baris Düdükü for their help during the experiments, and Griselda Roces for the ant drawing. We are grateful to two anonymous reviewers, whose comments greatly improved the manuscript. Daniela Römer was supported by a grant from the Postdoc Plus funding program of the Graduate School of Life Sciences (GSLs) of the University of Würzburg, Germany, and a Grant of the Agencia Nacional de Investigación e Innovación (ANII, Grant Number PD_NAC_2015_1_108641), Uruguay. Florian Halboth was supported by a Grant from the German Excellence Initiative to the Graduate School of Life Sciences, University of Würzburg.

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