Building behavior and nest climate control in leaf-cutting ants: How environmental cues affect the building responses of workers of *Atta vollenweideri*

Bauverhalten und Kontrolle des Nestklimas bei Blattschneiderameisen: Wie Umweltreize die Bauaktivität von Arbeiterinnen der Art *Atta vollenweideri* beeinflussen

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Summary

The present work investigates the influence of environmental stimuli on the building behavior of workers of the leaf-cutting ant *Atta vollenweideri*. It focuses on cues related to the airflow-driven ventilation of their giant underground nests, i.e., air movements and their direction, carbon dioxide concentrations and humidity levels of the nest air. First, it is shown that workers are able to use airflow and its direction as learned orientation cue by performing learning experiments with individual foragers using a classical conditioning paradigm. This ability is expected to allow workers to also navigate inside the nest tunnels using the prevailing airflow directions for orientation, for example during tasks related to nest construction and climate control.

Furthermore, the influence of carbon dioxide on the digging behavior of workers is investigated. While elevated CO_2 levels hardly affect the digging rate of the ants, workers prefer to excavate at locations with lower concentrations and avoid higher CO_2 levels when given a choice. Under natural conditions, shifting their digging activity to soil layers containing lower carbon dioxide levels might help colonies to excavate new or to broaden existing nest openings, if the CO_2 concentration in the underground rises.

It is also shown that workers preferably transport excavated soil along tunnels containing high CO_2 concentrations, when carbon dioxide levels in the underground are elevated as well. In addition, workers prefer to carry soil pellets along outflow tunnels instead of inflow tunnels, at least for high humidity levels of the air. The material transported along tunnels providing outflow of CO_2-rich air might be used by workers for the construction of ventilation turrets on top of the nest mound, which is expected to promote the wind-induced ventilation and the removal of carbon dioxide from the underground.

The climatic conditions inside the nest tunnels also influence the structural features of the turrets constructed by workers on top the nest. While airflow and humidity have no effect on turret structure, outflow of CO_2-rich air from the nest causes workers to construct turrets with additional openings and increased aperture, potentially enhancing the airflow-driven gas exchanges within the nest.

Finally, the effect of airflow and ventilation turrets on the gas exchanges in *Atta vollenweideri* nests is tested experimentally on a physical model of a small nest consisting of a single chamber and two nest tunnels. The carbon dioxide clearance rate from the underground was measured depending on both the presence of airflow in the nest and the structural features of the built turrets. Carbon dioxide is removed faster from the physical nest model when air

moves through the nest, confirming the contribution of wind-induced flow inside the nest tunnels to the ventilation of *Atta vollenweideri* nests. In addition, turrets placed on top of one of the tunnel openings of the nest further enhance the CO_2 clearance rate and the effect is positively correlated with turret aperture.

Taken together, climatic variables like airflow, carbon dioxide and humidity levels strongly affect the building responses of *Atta vollenweideri* leaf-cutting ants. Workers use these environmental stimuli as orientation cue in the nest during tasks related to excavation, soil transport and turret construction. Although the effects of these building responses on the microclimatic conditions inside the nest remain elusive so far, the described behaviors are expected to allow ant colonies to restore and maintain a proper nest climate in the underground.

Zusammenfassung

Die vorliegende Arbeit untersucht den Einfluss von Umweltreizen auf das Bauverhalten von Blattschneiderameisen der Art *Atta vollenweideri*. Dabei wird der Fokus auf Luftströmungen und deren Richtung, sowie CO_2-Konzentration und Feuchtigkeitsgehalt der Luft gelegt, welche alle im Zusammenhang mit dem wind-induzierten Ventilationssystem der riesigen, unterirdischen Nester stehen.

Zunächst wird experimentell mit Hilfe von klassischer Konditionierung gezeigt, dass Arbeiterinnen während des Furagierens lernen können, Luftströmungen sowie deren Richtung zur Orientierung zu nutzen. Diese Fähigkeit sollte Arbeiterinnen auch die Navigation im Nest anhand der auftretenden Strömungsrichtung der Luft, zum Beispiel während Tätigkeiten im Kontext des Nestbaus und der Klimakontrolle, ermöglichen.

Weiterhin wird der Einfluss von Kohlenstoffdioxid auf das Grabeverhalten von Arbeiterinnen untersucht. Obwohl CO_2 kaum die Grabe-Rate der Ameisen beeinflusst, graben Arbeiterinnen bevorzugt an Orten mit niedrigerer Konzentration und vermeiden höhere Konzentrationen, wenn möglich. Unter natürlichen Bedingungen könnte das Verlagern der Grabeaktivität in Bodenschichten mit niedrigerer CO_2-Konzentration Kolonien dabei helfen, neue Nestöffnungen zu graben oder bestehende zu erweitern, wenn die CO_2-Konzentration unter der Erde zunimmt.

Zusätzlich wird gezeigt, dass Arbeiterinnen ausgegrabene Erde vornehmlich entlang Tunnel transportieren, die eine hohe CO_2-Konzentration aufweisen, wenn die CO_2-Konzentration im Untergrund ebenfalls erhöht ist. Zudem bevorzugen Arbeiterinnen den Transport von Erdmaterial entlang Ausstrom- anstatt Einstrom-Tunnel, zumindest für hohe Luftfeuchtigkeiten. Material, welches entlang Nesttunnel transportiert wird, aus denen CO_2-haltige Luft ausströmt, könnte Arbeiterinnen zum Bau der Ventilationstürme an der Nestoberfläche dienen, was die wind-induzierte Belüftung der Nester verstärken und die Abfuhr von CO_2 aus dem Nest fördern sollte.

Die klimatischen Bedingungen in den Nesttunneln beeinflussen auch die strukturellen Eigenschaften der Ventilationstürme, die von Arbeiterinnen oberhalb des Nests errichtet werden. Während Luftströmungen und Luftfeuchtigkeit keinen Einfluss auf die Struktur der Türme haben, veranlasst das Ausströmen von CO_2-haltiger Luft aus dem Nest Arbeiterinnen dazu, Türme zu bauen, die mehrere Öffnungen und eine vergrößerte Öffnungsfläche besitzen, was den strömungsinduzierten Gasaustausch im Nest begünstigen könnte.

Abschließend werden die Auswirkungen von Luftströmungen und Ventilationstürmen auf den Gasaustausch in den Nestern der Blattschneiderameise *Atta vollenweideri* mit Hilfe eines physikalischen Modells eines kleinen Nests, bestehend aus einer einzelnen Nestkammer und zwei Nesttunneln, untersucht. Die Abfuhr-Rate von CO_2 aus dem Untergrund wurde abhängig vom Vorhandensein von Luftströmungen und den strukturellen Eigenschaften der errichteten Ventilationstürme gemessen. CO_2 wird schneller aus dem physikalischen Modell entfernt, wenn Luft durch das Nest strömt, was den Beitrag von Luftbewegungen in den Tunneln zur Ventilation der Nester von *Atta vollenweideri* bestätigt. Ventilationstürme an einer der Nestöffnungen platziert, verstärken zusätzlich die Abfuhr-Rate von CO_2 aus dem Nest und dieser Effekt nimmt mit zunehmender Öffnungsfläche der Türme zu.

Zusammengefasst beeinflussen Klimavariablen wie Luftströmungen, Kohlenstoffdioxid und Luftfeuchtigkeit stark das Bauverhalten von Blattschneiderameisen der Art *Atta vollenweideri*. Arbeiterinnen nutzen diese Umweltreize zur Orientierung im Nest während Tätigkeiten, die im Zusammenhang mit Grabeverhalten, dem Transport von Erdmaterial und dem Bau von Ventilationstürmen stehen. Obwohl die Auswirkungen dieser Bauantworten auf die mikroklimatischen Bedingungen im Nest zunächst noch unklar sind, wird angenommen, dass die beschriebenen Verhaltensweisen es Kolonien erlauben, ein geeignetes Nestklima wiederherzustellen und aufrechtzuerhalten.

I. General introduction

1. The construction of nests in social insects

Nest construction is widely spread in the animal kingdom and can be found in a variety of different taxa including mammals, birds, reptiles and fish (Hansell, 2005). However, the most elaborate nests of all animals are found within the social insects, a group that mainly comprises species belonging to the orders Hymenoptera (ants, bees and wasps) and Isoptera (termites). Colonies of social insects may contain several million individuals that collectively shape the architecture of their nest and create structures that far exceed the size of their builders (Hölldobler and Wilson, 2009). The function of their nest is generally the same as in all animals, since it offers protection to the members of the colony against both predators and unfavorable environmental conditions (Hansell, 2005). Structurally, however, nests of social insects may vary tremendously, ranging from nest cavities created in soil or between rocks, for example in many ant species (Hölldobler and Wilson, 1990), to the construction of hanging mud or paper nests like in wasps (Theraulaz and Bonabeau, 1995; Hozumi and Inagaki, 2010). Among the largest and most complex nests within the social insects are certainly the giant underground nests of leaf-cutting ants in the Neotropics and the huge cathedral mounds of African and Australian termites. Despite their structural complexity, the mechanisms underlying the construction of such elaborate nests result from rather simple interactions of individuals with each other and with the environment (Hölldobler and Wilson, 2009). As individual workers generally lack a global representation of the final structure, their building responses depend only on local information (Theraulaz and Bonabeau, 1995). Complex structures emerge via self-organized processes based on stigmergy and the use of environmental templates (Theraulaz *et al.*, 1998). Such mechanisms have originally been described especially for termites (Grassé, 1959), which have been extensively studied due to their impressive nest architecture (Emerson, 1938; Harris, 1956) and their ability to regulate the climatic conditions inside their nest mounds (Lüscher, 1956; Korb and Linsenmair, 2000). However, self-organized processes have been identified to underlie the construction and modification of nest structures for example in ants as well (Buhl *et al.*, 2005; Khuong *et al.*, 2016). Simple feedback mechanisms involving interactions with nest mates or with environmental stimuli allow insect colonies for example to regulate the size of their nests, as shown for different ant species (Deneubourg and Franks, 1995; Rasse and Deneubourg, 2001; Halley *et al.*, 2005; Fröhle and Roces, 2009; Römer and Roces, 2014). While the use of environmental cues to regulate the nest climate depending on the current needs of the colony has been demonstrated in bumble bees (Weidenmüller *et al.*, 2002; Weidenmüller, 2004), it is still largely unknown how environmental stimuli influence the behavioral responses in other social insects, for example in ants. Therefore, the aim of the present work is to investigate the behavioral responses of ants to environmental stimuli using a species of leaf-cutting ants, *Atta vollenweideri*, as a model organism.

2. The biology of leaf-cutting ants

The term leaf-cutting ants refers primarily to ants of two genera, namely *Atta* and *Acromyrmex*, which cut fragments out of live plant material that serve as a substrate for the cultivation of a symbiotic fungus in their nest (Weber, 1972b). Leaf-cutting ants belong, together with all other known fungus-growing ants, to the Neotropical tribe of the Attini. Their distribution covers almost the entire South American continent, with the exception of Chile, and ranges across Central America and even parts of North America, including Mexico and the southernmost regions within the US (Weber, 1972b; Cherrett and Peregrine, 1976; Fowler, 1983; Mayhé-Nunes and Jaffé, 1998). The ecological success of the leaf-cutting ants as one of the dominant herbivores in the Neotropics can be attributed mainly to the symbiotic relationship with their fungus, enabling colonies to indirectly exploit a variety of different plant species as food sources, including crop plants (Weber, 1972b; Wirth *et al.*, 2003). Thus, leaf-cutting ants are often considered pests in large parts of their geographical range as they are responsible for huge crop damages estimated at a total economic loss of more than one billion US dollars annually (Cramer, 1967; Hölldobler and Wilson, 1990). Depending on the species, mature colonies may harvest between one kilogram and several hundred kilograms of plant material per year (Jonkman, 1980a; Robinson and Fowler, 1982; Wirth *et al.*, 2003), and largely affect plant community composition of their surrounding environment (Jonkman, 1978; Moutinho *et al.*, 2003). Due to the enormous amount of plant biomass that is collected and transported into their underground nest, leaf-cutting ants also strongly contribute to the flow of elements and nutrients in the soil and serve as important drivers for bioturbation (Haines, 1978; Mead *et al.*, 2008). During their lifetime, a single colony may move up to 40 tons of soil, corresponding to a nest volume of more than 20 m^3 (Weber, 1966). Depending on the species, mature nests of *Atta* may contain several million individuals and between 1000 and 8000 chambers, most of them in a depth between 1-3 meters (Jonkman, 1980b; c, Moreira *et al.*, 2004a; b). In *Acromyrmex,* colonies are generally smaller with only several thousands of workers, and hundreds or even fewer nest chambers, excavated in shallower soil layers (Hölldobler and Wilson, 1990). In the majority of these chambers leaf-cutting ant workers cultivate their symbiotic fungus using the harvested plant material as substrate, while the remaining chambers are either empty or may contain the colony waste.

Leaf-cutting ant workers express a remarkable size-polymorphism with larger, media-sized workers engaging mainly in tasks related to nest construction or foraging, while smaller, minima workers take care of the brood and the fungus garden (Weber, 1972b). The plant material collected by foragers is further cut in the nest and incorporated into the fungus garden by the smaller ants. Fecal droplets of workers serve as manure and are added to the plant fragments before pressing them into the fungal structure (Weber, 1966). For the proper growth of the fungus in the underground, suitable microclimatic conditions inside the nest are crucial. The leaf-cutting ant fungus, *Attamyces bromatificus*, has strict demands of high humidity and temperatures between 20 and 25°C (Quinlan and Cherrett, 1978; Powell and Stradling, 1986) and workers avoid unfavorable conditions for the cultivation of their fungus (Roces and Kleineidam, 2000; Bollazzi and Roces, 2002). Besides temperature and humidity, other abiotic factors, like carbon dioxide levels are expected to influence fungal growth as well. Due to the respiration of both the fungus and the ants, huge amounts of CO_2 are produced in the nest. In addition, the soil acts as natural source for carbon dioxide (Schwartz and Bazzaz, 1973; Reardon *et al.*, 1979; Davidson and Trumbore, 1995), generally resulting in hypoxic (low O_2) and hypercapnic (high CO_2) conditions in the underground. While low concentrations of carbon dioxide might stimulate fungal growth (Bécard and Piché, 1989) and leaf-cutting ant workers prefer slightly elevated CO_2 levels for the cultivation of their fungus (Römer *et al.*, 2017), excessive amounts of carbon dioxide accompanied by a reduced availability of oxygen are expected to be detrimental for both the fungus (Kleineidam and Roces, 2000) and the ants. In order to maintain the balance between a suitable nest temperature, high humidity levels and low carbon dioxide concentrations in the nest, leaf-cutting ant colonies show specific building responses that help regulating the microclimatic conditions inside the nest. In *Acromyrmex ambiguus*, for example, workers plug their nest tunnels with leaf fragments when dry air enters the nest in order to avoid desiccation (Bollazzi and Roces, 2007). In *Acromyrmex heyeri*, outflow of humid air from the nest triggers the deposition of building material in order to seal leaks in the nest thatch (Bollazzi and Roces, 2010a). In the same species, increased temperatures inside the nest cause workers to create additional nest openings that are again closed in favor of humidity control (Bollazzi and Roces, 2010b). Similar mechanisms are expected to occur in the larger colonies of *Atta* as well, however, only few studies have dealt with the building responses and especially the influence of climatic cues on building responses in their giant underground nests so far.

3. The nests of *Atta vollenweideri*

Named after the Gran Chaco region in central South America, *Atta vollenweideri* (Forel, 1893) is one of the most studied species among the Attini. It is the southernmost species of *Atta* inhabiting mainly the lowland savannas across Paraguay, Brazil, Uruguay and Argentina (Weber, 1966; Jonkman, 1979; Farji Brener and Ruggiero, 1994). As their habitat is dominated by pastures and shrublands, colonies of *Atta vollenweideri* forage mostly on grasses, but occasionally harvest leaves as well (Jonkman, 1976). Their foraging trails, often clearly visible, may extend up to 100 m from the nest and are regularly cleared of vegetation by workers (Jonkman, 1976).

Adult nests of *Atta vollenweideri* can occur in relative high densities of 1-3 colonies per hectare and are easily identifiable due to their enormous, dome-shaped mounds, consisting of soil excavated in the underground (Jonkman, 1979). The nest mounds can reach a height of 1 m and cover an area of more than 40 m² (Jonkman, 1980b). Several nest openings lead as tunnels from the surface to the underground part of the nest, where up to 2000 chambers are located in a depth of 2-3 m (Jonkman, 1980c). Most of the nest chambers are a spherical-shaped, about 20 cm high and contain the fungus garden. Below the fungus chambers, workers excavate larger, cone-shaped refuse chambers up to 2 m high, in which the colony waste is deposited (Jonkman, 1980c).

Atta vollenweideri is limited to clay-heavy soils and is replaced by other *Atta* species in soils with higher sand content (Jonkman, 1976). Due to the high clay content of the soil, diffusive gas exchanges between the nest and the environment are limited and colonies are often faced with reduced O_2 levels and high CO_2 concentrations of up to 6% in the underground (Kleineidam and Roces, 2000). In order to ensure the supply of oxygen and the removal of carbon dioxide from the underground, nests of *Atta vollenweideri* rely on a wind-induced ventilation mechanism that facilitates the gas exchanges with the environment (Kleineidam *et al.*, 2001). Surface wind passes over the nest mound, creating a pressure difference between the air inside and outside the nest caused by the Bernoulli principle (Vogel and Bretz, 1972). As a result, used, CO_2-rich air is dragged out of nest openings on the center of the mound, followed by an inflow of fresh air through openings on the periphery of the nest mound (Kleineidam *et al.*, 2001). In addition, workers construct small turrets on top of central outflow openings using soil excavated in the underground or plant material collected from the immediate vicinity as building material (Cosarinsky and Roces, 2007; 2012). The construction of turrets is assumed to further enhance the ventilation effect of the nest by elevating the tunnel opening and exposing it to increased surface wind speeds. Besides long-term modifications of nest structures, colonies of *Atta vollenweideri* also elicit short-term building responses like the closing of nest openings during the winter months (the dry season) or heavy rains (Jonkman, 1980b; c). It is expected that such building responses depend on a colony's current demand for nest ventilation and therefore are influenced by the climatic conditions inside the nest, however so far information about the environmental stimuli that act as cues for workers and their influence on the regulatory building responses in the nests of *Atta vollenweideri* is lacking.

4. Thesis aims and experimental approach

This work comprises seven chapters that investigate the use of airflow, carbon dioxide and air humidity levels inside the nest of *Atta vollenweideri* as potential cues for workers during orientation as well as during tasks related to nest digging, soil transport and turret construction. Chapter II investigates the ability of workers to use air movements and their direction as learned orientation cues, as they are expected to provide reliable navigational information in the nest tunnels. For that, single foragers were trained to a food source using airflow and its direction as conditioned stimulus in a classical conditioning paradigm.

Chapter III is aimed at quantifying the effect of carbon dioxide on the digging behavior of workers. High CO_2 levels in the underground should indicate poor nest ventilation and workers might counteract hypercapnic conditions in their nest by excavating new or broadening existing nest tunnels. For that, the digging rates of worker groups exposed to different CO_2 levels were measured, and the preferences of workers for specific carbon dioxide concentrations during excavation were evaluated using binary choice tests.

Chapter IV investigates whether workers use carbon dioxide and airflow directions inside the nest tunnels as orientation cue during soil transport. The transport of soil along tunnels containing outflow of CO_2-rich air and its deposition around nest openings on the center of the mound is expected to facilitate the wind-induced ventilation of the nest and might promote the construction of turrets on the mound. For that, single workers engaged in soil transport were tested for their preference for both carbon dioxide as well as airflow directions using binary choice tests.

In Chapter V, the influence of the climatic conditions in the nest tunnels on the construction of ventilation turrets was investigated, since workers are expected modify turret structure as a response to unfavorable climatic conditions in the nest. Simulating a natural nest arrangement, building material for the construction of turrets was offered to a subcolony exposed to different airflow conditions, humidity levels and carbon dioxide concentrations, and the structural features of the resulting turrets were measured.

Chapter VI was aimed at estimating the effect of surface wind as well as turret structure on the nest ventilation in *Atta vollenweideri*. Using a physical model of a small nest consisting of two tunnels and a single nest chamber, removal rates of carbon dioxide from the nest were measured under the presence of airflow in the nest and turrets placed at one of the tunnel openings.

Chapter VII finally integrates the results of the previous chapters and discusses the influence of environmental variables on the building responses shown by workers of *Atta vollenweideri* as compared to previous findings in leaf-cutting ants and social insects in general.

II. Anemotactic orientation: Workers use the presence of airflow and its direction as learned orientation cue

Abstract

The large underground nests of *Atta vollenweideri* leaf-cutting ants are ventilated via a passive, wind-induced mechanism, with air moving through nest tunnels to ensure gas exchanges with the external environment. Surface wind drags air out of central nest openings, which causes air to enter through openings at the periphery, resulting in a clear separation between inflow and outflow tunnels in the nest. Workers are able to detect the airflow inside the nest and are expected to use their directional information for orientation in the underground. In this chapter, we investigated the ants' ability to use airflow and its direction as learned orientation cue in two separate experiments based on a classical conditioning paradigm. In both experiments, single ant workers were branched off the main foraging trail and trained to a food source offering oat flakes as reward. While approaching the reward, ants were exposed to airflow with a velocity of 10 cm/s as conditioned stimulus (CS). After nine training trials, workers were branched off to a T-maze and tested for airflow preference in a binary choice situation. In the first experiment, we tested whether workers were able to learn the location of the food source using airflow as orientation cue. When approaching the food source during training, one group of workers was exposed to frontal airflow, the second group to airflow coming from behind the ant. A third group of foragers experienced frontal airflow filtered through activated charcoal to exclude the possibility of workers using an odor cue instead of the airflow itself as conditioned stimulus. During the test, single workers were given the choice between the same stimulus they experienced during training (CS) on one side and no airflow stimulus on the other side of the T-maze. In all groups, workers significantly preferred the side where the CS was presented during the test, indicating that they associated the airflow stimulus, not an odor cue, with the food reward during training, and that learning occurred for both airflow directions equally. In the second experiment, we tested whether workers simply used the presence of airflow as orientation cue or if they were able to use specifically the direction of the airflow as a stimulus. Again, one group was trained using frontal airflow and a second group using airflow from behind as CS. During the test, workers were given the choice between walking towards the side with the CS or towards the other side of the T-maze having the same airflow yet with opposing direction. In a third group, workers were not exposed to airflow during training, but to both airflow directions during the test in

order to control for eventual innate preferences for airflow directions. Workers showed no innate preference for airflow direction, but significantly preferred the CS to the alternate airflow stimulus when trained using frontal airflow. However, when exposed to airflow from behind during training, workers were unable to differentiate between airflow directions in the test. We propose that workers of *Atta vollenweideri* might use air movements and their direction for orientation to locate inflow or outflow tunnels inside their nest.

1. Introduction

The giant subterranean nests of the leaf-cutting ant *Atta vollenweideri* are among the largest structures found in the animal kingdom. In order to maintain a suitable microclimate in the underground, nests rely on a passive, wind-induced ventilation mechanism that facilitates the removal of carbon dioxide from the underground and supplies the colony with fresh air (Kleineidam *et al.*, 2001). The aboveground nest mound of this species is covered with up to 200 nest openings, which are not all used as entrances or exits for the ants (Jonkman, 1980b), but rather serve the purpose of nest ventilation. Air moves through the nest tunnels and the direction of the airflow depends on the position of the tunnel openings on the nest mound, with central openings serving as outflow and peripheral ones as inflow conduits. The mechanism underlying the wind-induced ventilation system relies on the Bernoulli principle (Vogel and Bretz, 1972) and is similar to the one found in burrows of prairie-dogs (Vogel *et al.*, 1973) and other ground dwelling animals (Brickner-Braun *et al.*, 2014). As surface wind is passing over the nest mound, an air pressure difference is created on top of the mound, dragging air out of central nest openings. The resulting decrease in air pressure inside the nest is then compensated by an inflow of fresh air through nest openings at the periphery of the mound. On top of central outflow openings ants construct small turrets from soil excavated in the underground (Jonkman, 1980b; c) and imported building materials (Cosarinsky and Roces, 2007; 2012), which are expected to further enhance nest ventilation by elevating the nest opening and exposing it to higher wind speeds (Kleineidam and Roces, 2000; Kleineidam *et al.*, 2001). Depending on surface wind, airflow velocity inside the nest may vary, with average values between 1-5 cm/s (Kleineidam *et al.*, 2001), thus workers are expected to frequently encounter air movements during a variety of tasks inside the nest. Recently, it was shown that leaf-cutting ants are able to perceive airflow (Bollazzi and Roces, 2007; Halboth and Roces, 2017), an ability widely spread across different arthropod taxa, including mosquitos (Bässler, 1958), scorpions (Linsenmair, 1968), beetles (Linsenmair, 1969), cockroaches (Bell and Kramer, 1979) and other ants (Wehner and Duelli, 1971; Van Vorhis Key and Baker, 1982; Wolf and Wehner, 2000). In *Atta vollenweideri*, single workers showed behavioral responses to airflow stimuli with a velocity between 2 cm/s and 20 cm/s (Halboth and Roces, 2017), similar to the speed of the air currents they should encounter across the nest tunnels under natural conditions. In the same study, workers previously exposed to an airflow stimulus of a given direction during digging showed later on a strong preference for the airflow direction experienced before. Concerning the mechanism underlying this experience-dependent choice for airflow direction, the formation of short-term memory in workers using airflow as learned orientation cue was suggested, however, alternative explanations could not be excluded.

Therefore, the aim of this chapter was to investigate whether workers are indeed able to use the direction of an airflow stimulus as a learned orientation cue, by performing learning experiments with foragers of *Atta vollenweideri* in a classical conditioning paradigm. Classical conditioning, as described by Pavlov (1927), is a widely used method to test stimulus sensitivity and learning abilities of animals, and the paradigm has been used in many studies throughout the literature. It involves the pairing of two stimuli: an unconditioned stimulus (US), capable of eliciting an unconditioned response without prior training, and a conditioned stimulus (CS) not eliciting a response. By temporarily pairing the two stimuli during the conditioning procedure, an association is created between them, resulting in the elicitation of a conditioned response, resembling the unconditioned response originally elicited (Alloway, 1972). Thus, classical conditioning is a convenient method to test the ability of an animal to perceive certain stimuli. In insects, classical conditioning is commonly applied using the proboscis extension response, for example in blowflies (Frings, 1941) or honey bees (Frings, 1944), where harnessed individuals are trained to associate a certain stimulus with a food reward. Although similar methods have been developed for ants (Guerrieri and d'Ettorre, 2010), conditioning experiments are more often performed with freely moving workers in a foraging context. In previous studies, memory formation in ants has been shown mostly for olfactory or visual stimuli (Roces, 1994; Cammaerts, 2004; Josens *et al.*, 2009), but also thermal radiation (Kleineidam *et al.*, 2007). In this chapter, we used a classical conditioning paradigm to test whether workers of *Atta vollenweideri* were able to use the presence of air movements and, more specifically, the direction of the airflow they are exposed to as learned cue for anemotactic orientation.

2. Methods

2.1. General setup

Prior to the experiments, small queenless subcolonies of *Atta vollenweideri* were established by removing a fungus chamber, containing fungus garden, workers and brood, from the mother colony and connecting it to a waste chamber (20 x 10 x 10 cm) and an open box (20 x 20 x 10 cm) via transparent PVC tubes (Ø3 cm). The subcolony was connected via a wooden bridge (100 x 4 cm) to a foraging arena (20 x 20 x 10 cm) where the ants could forage freely on fresh leaves of blackberry (Fig. 1). A small platform (4 x 5 cm) was attached to the main trail from which single foragers walking towards the previously-established food source in the foraging arena were gently rerouted either towards a training site (wooden bridge: 6 x 1 cm) or a test site (T-maze: 4 x 1 cm, side arms: 4 x 1 cm) via a wooden side trail (20 x 1 cm). At the training site, foragers encountered a piece of oat flakes as a reward (US) while being exposed to airflow as conditioned stimulus (CS). At the test site, no food item was placed, and workers could choose between two sides of the T-maze, containing the same stimulus they experienced during training (CS) on one side and either no airflow stimulus or a stimulus with opposing direction on the other side, depending on the experiment. The airflow was generated by two connected miniature vane pumps (Schwarzer Precision 135 FZ, power supply: Base-Tech BT-305) and transferred to the training and test sites via rubber tubes (Ø3 mm). Airflow speed was measured using an anemometer (AVT Model 8475) and set to 10 cm/s, close to the values measured in the tunnels of field nests of *Atta vollenweideri* (Kleineidam *et al.*, 2001).

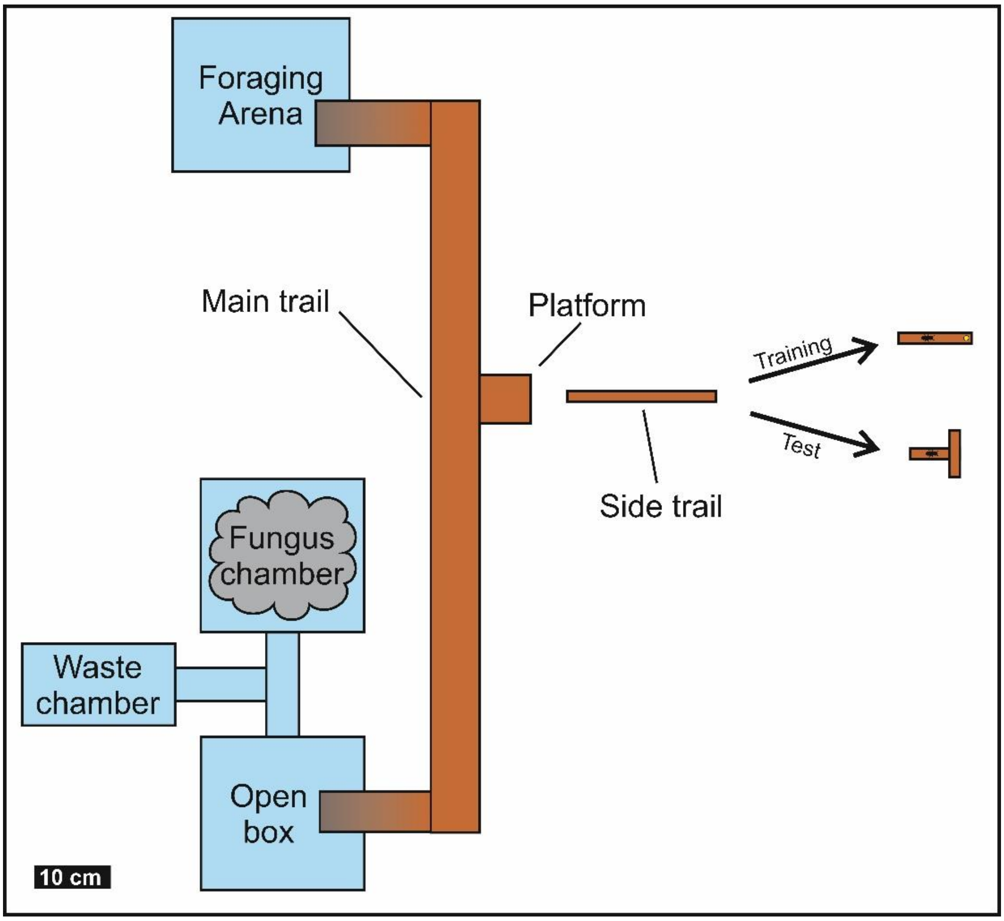


Figure 1: General setup used during both experiments (top view). The subcolony, consisting of a fungus chamber, a waste chamber and an open box, was connected to a foraging arena via a 1 m long wooden bridge serving as main foraging trail for ants. Single workers were branched off a platform attached to the main bridge onto a side trail and subsequently rerouted first to a training site offering airflow as conditioned stimulus (CS) and a small oat flake as a reward (US). After nine consecutive training trials, foragers were rerouted to a T-maze and tested for airflow preferences, in order to see whether they associated the CS with the US.

2.2. Experimental procedure

The general training and test procedures were practically identical in both experiments to be described below. While the majority of workers kept foraging on the main trail, a single scout ant occasionally left the main trail and entered the wooden platform, from which she was gently rerouted via the side trail to the training platform. Here, the ant encountered a small oat flake as reward (US), while being exposed to an airflow stimulus (CS), depending on the experiment. After picking up the reward, the ant turned around and walked back again onto the side trail, where she was cautiously marked on either one leg, the back of the head or the thorax using a toothpick dipped in permanent marker ink of different colors (edding®). Marking an individual forager had no visible effect on the behavior of nest mates or on the marked ant itself, and preliminary tests revealed that marked ants released back into the colony appear to normally participate in colony tasks and can be found even several weeks after marking (personal observation).

After marking, the ant was gently rerouted back to the platform attached to the main foraging trail. On the main trail, the scout usually dropped its load after a few centimeters and returned to the side trail to continue foraging at the newly discovered food source. This behavior is commonly observed in leaf-cutting ants, when new food sources apart from the main foraging trail are exploited (Fowler and Robinson, 1979; Hubbell *et al.*, 1980). A similar technique has already been used for the classical conditioning of ants to thermal stimuli in an earlier study on this species (Kleineidam *et al.*, 2007) and it allowed us to get the same ant to forage at the training site for multiple subsequent trials. Occasionally, foragers did not pick up the food item at the training site, but returned to the main trail unloaded. However, after brief interactions with other workers on the main trail, the marked scout again searched for the side trail and was rerouted back to the training site to continue foraging. Therefore, also foraging trips of unloaded workers were counted as successful training trials, as workers nevertheless recruited nest mates on the main foraging trail.

After nine consecutive training trials, the marked ant was rerouted to a T-maze for the test. Here, foragers could choose between the two sides of the T-maze, one providing a stimulus situation similar to the one presented during training (CS), and the other providing a different stimulus situation, depending on the series. On each side arm of the T-maze a line was previously drawn 1 cm away from the center and a worker’s choice for one side was only counted when the ant completely crossed the line on either side. Afterwards, tested foragers were removed from the colony to avoid pseudo-replications. For each worker we recorded the choice during the test, the duration to complete the entire training and test procedure, and the inter-trial interval, i.e., the time elapsed between a loaded worker entered the main trail and returned to the training site for the next trial. Since our technique allowed workers to forage freely, the inter-trial interval varied between workers, however individuals that did not return to the training site within 15 min were excluded from the data.

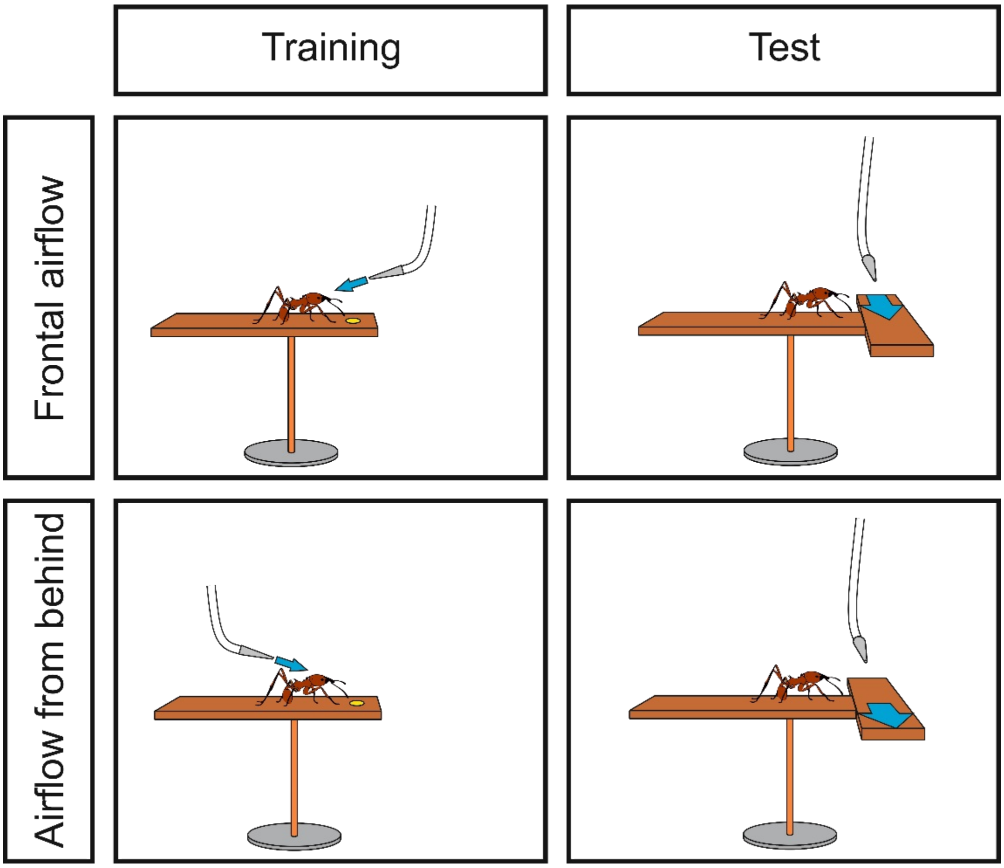


Figure 2: Airflow directions (blue arrow) in the first experiment during training and test using airflow with a velocity of 10 cm/s as conditioned stimulus. Single foragers were branched off the main trail and transferred to a training site. Here, they encountered a small oat flake as reward (yellow spot) while being exposed either to frontal airflow, i.e., walking upwind (top left) or to airflow from behind, i.e., walking downwind (bottom left). After nine consecutive training trials, workers were rerouted to a T-Maze and tested for airflow preference. During the test, airflow was present only on one side of the T-maze and directed either towards (top right) or away from the center (bottom right) according to the series. An additional series using again frontal airflow was performed with charcoal-filtered air to exclude potential olfactory cues from the airflow that may influence workers' choices.

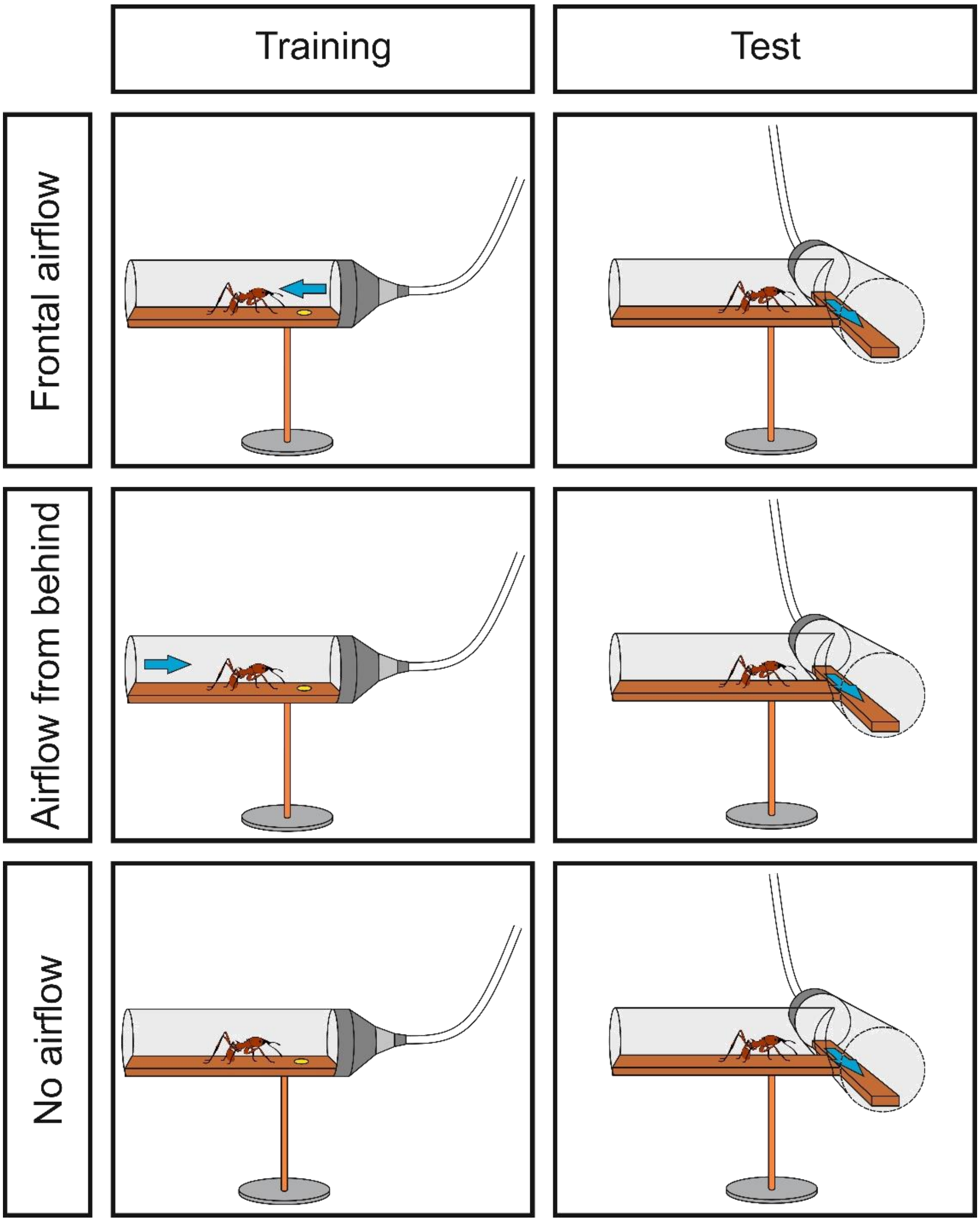


Figure 3: Airflow directions (blue arrow) in the second experiment during training and test using airflow with a velocity of 10 cm/s as conditioned stimulus. Single foragers were branched off the main trail and transferred to a training site. Here, they encountered a small oat flake as reward (yellow spot) while being exposed either to frontal airflow (top left), airflow from behind (center left) or no airflow (bottom left). After nine consecutive training trials, workers were rerouted to a T-Maze and tested for airflow preference, where they were given the choice between two airflow stimuli of opposing direction, i.e., workers could walk either upwind or downwind (right column). Note: For illustrative purposes, the rubber tubes transferring airflow to and out of the T-Maze are only depicted for the left side arm.

2.3. Experimental series

In the first experiment, we tested the workers' ability to associate the reward (US) with an airflow stimulus (CS) at the training site. The airflow was emitted from the rubber tubes ending in pipette tips (Ø1 mm) that were placed above the training site (wooden bridge: 6 x 1 cm) in a way that foragers approaching the food source were exposed to a continuous airflow with a velocity of 10 cm/s. We performed a total of three series. In the first series, workers encountered frontal airflow as a training stimulus, i.e., they walked upwind towards the food source (Fig. 2, top left), while in the second series the airflow stimulus was applied from behind the ant, i.e., workers walked downwind towards the reward (Fig. 2, bottom left). At the test site, airflow was applied only to one side of the T-maze (wooden bridge: 4 x 1 cm, side arms: 4 x 1 cm), while no airflow was detectable on the opposing side. The direction of the airflow matched the one during the training, i.e., workers would either walk upwind or towards no wind on the T-maze (Fig. 2, top right), or downwind or towards no wind (Fig. 2, bottom right), depending on the series. This was achieved by placing the pipette tips above the T-maze in a way that airflow was either directed from the end of one side arm towards the center in the first, or from the center towards the end in the second series. A third, control series was performed in order to exclude the possibility of foragers using olfactory cues within the airflow as conditioned stimulus during training or test. Again, frontal airflow with a velocity of 10 cm/s was used as training stimulus, yet the pumped air was previously filtered using activated charcoal.

Since in the first experiment foragers might learn the location of the food reward based only on the presence of airflow and not necessarily on its directional information, we performed a second experiment to test whether workers were able to specifically use the direction of the airflow as orientation cue. Workers were again exposed to airflow of 10 cm/s during training, but in contrast to the first experiment, they could choose between two airflow stimuli with opposing direction at the T-maze. This was achieved by creating a continuous airflow from one side of the T-maze to the other, resulting in the possibility for workers to walk either upwind or downwind during the test (Fig. 3, right column). We performed again a total of three series. In the first series, workers were exposed to frontal airflow, i.e., they walked upwind while approaching the reward (Fig. 3, top left). In the second series, workers were exposed to airflow coming from behind, i.e., they walked downwind towards the food source (Fig. 3, center left). A third series was performed, where workers were trained without experiencing airflow at the food source (Fig. 3, bottom left), but were given the choice between both airflow directions at the T-maze in order to control for a potential innate preference for airflow direction. Both the training platform as well as the T-maze were placed inside plastic tubes (Ø2.1 cm), ensuring shielding of the training and test platform against air turbulences of the room air and allowing for a more steady flow of air, especially from one side to the other side of the T-maze. In order to minimize the effect of chemical contaminants inside the plastic tubes, the air was again filtered using activated charcoal during both training and test.

In all experiments, the side providing the conditioned stimulus was switched randomly for each individual to rule out a possible side bias of the ants. In order to minimize effects of perturbations in the room air, training and tests were always performed under a shielded wooden box (85 x 70 x 50 cm) with only one open side to allow access to the experimental setup.

2.4. Statistical analysis

For all experiments, we compared in each series the distribution of workers’ choices during the test to a 1:1 random distribution using G-tests of goodness of fit (McDonald 2014). Inter-trial intervals were compared between the beginning (first to second trial) and the end of the training (last trial to test) via paired t-test. Additionally, in the first experiment, the distribution of workers’ choices in the series using frontal airflow either with filtered or unfiltered air were compared to each other using a G-Test of independence (McDonald, 2014). The significance level in all experiments was $\alpha = 0.05$.

3. Results

Although not all scouts that were rerouted to the training site picked up the reward or started recruiting at the main trail, the majority of ants (93%, n = 353) that returned to the training site after the first trial completed the training and test procedure. On average, foragers completed the entire training and test procedure after 36 ± 13 min (mean ± SD, n = 330) with an average inter-trial interval of 4 ± 2 min (mean ± SD, n = 330). Workers spent 5 ± 3 min (mean ± SD, n = 330) between the first and second training trial and only 3 ± 2 min (mean ± SD, n = 330) between the last training trial and the test trial. Therefore, the overall time between individual trials significantly decreased during training as workers returned to the side trail faster between the last training trial and the test than between the first and the second training trial (Paired t-test: t = 8,51, df = 329, $p < 0.001$). During the test, workers often quickly approached the choice point of the T-maze but then stopped and hesitated before moving on. In most cases, ants could be observed antennating and occasionally changing the direction they were facing in the center of the maze, before choosing to walk towards the end of one of the two side arms. In both experiments, the side of the T-maze containing the conditioned stimulus was switched randomly after each test and overall workers did not show a side preference for the left side (52%) or the right side (48%) of the T-maze (G-Test: G = 0.33, p = 0.56, n = 330).

3.1. Airflow as learned orientation cue

Ants exposed to airflow either frontal or from behind during training were able to associate the stimulus with the location of a food reward. Workers significantly preferred the side providing the airflow stimulus during the test for all three series (Fig. 4). In the series using frontal airflow (unfiltered air), i.e., workers walked upwind towards the food source during training, the majority of workers (70%) chose the side containing the airflow stimulus and walked upwind during the test as well (G = 4.94, p = 0.026). Accordingly, the remaining 30% chose the opposing side of the T-maze where no airflow stimulus was presented. Similarly, when exposed to airflow from behind, i.e., workers walked downwind during training, more foragers (73%) chose the airflow side during the test as well and preferred to walk downwind instead of choosing the side without an airflow stimulus (G = 6.79, p = 0.009). When using frontal airflow with filtered air, again 73% of the ants preferred the side containing airflow over the side where no airflow was present (G = 6.79, p = 0.009).

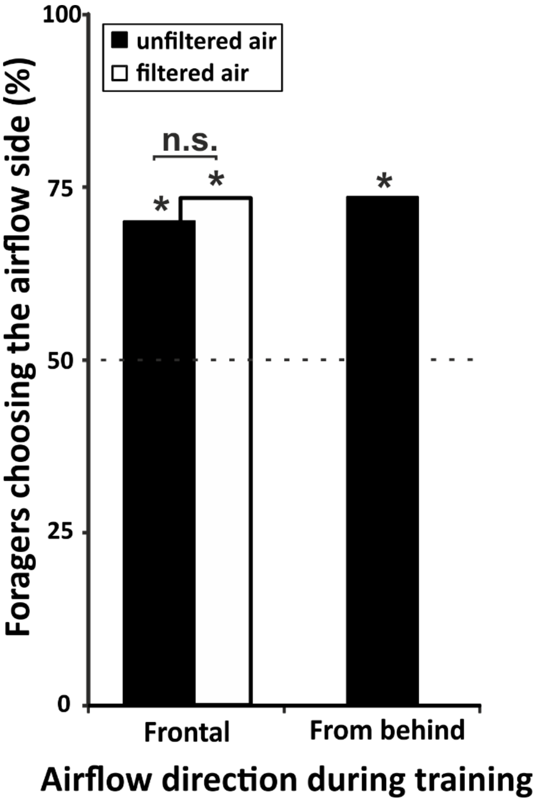


Figure 4: Airflow preferences of foragers during the test situation in the first experiment. Workers were trained using either frontal airflow or airflow from behind as conditioned stimulus and given the choice between the CS and no airflow during the test on a T-maze (black bars). An additional series was performed using frontal airflow with filtered air (white bar) to rule out the influence of olfactory cues on workers' choices. In each series n = 30 individual ants were tested. Asterisks indicate significant differences compared to a 1:1 random distribution depicted as dashed line.

3.2. Airflow direction as learned orientation cue

Workers' preferences for airflow direction during the test differed between the series, depending on the airflow conditions workers experienced during training (Fig. 5). When using frontal airflow as training stimulus, i.e., workers walked upwind towards the food source, 68% of the ants chose the upwind side as well during the test, while only 32% of the ants chose the downwind side later on (G = 5.01, p = 0.025). However, when exposed to airflow coming from behind, i.e., workers walked downwind towards the food source during training, 40% of the tested individuals chose the upwind side and 60% chose the downwind side of the T-maze during the test, which was not statistically different from a random distribution (G = 1.61, p = 0.204). When no airflow stimulus was present during training, workers showed no innate preferences for airflow direction, as 52% of the ants walked upwind and 48% walked downwind during the test (G = 0.10, p = 0.752).

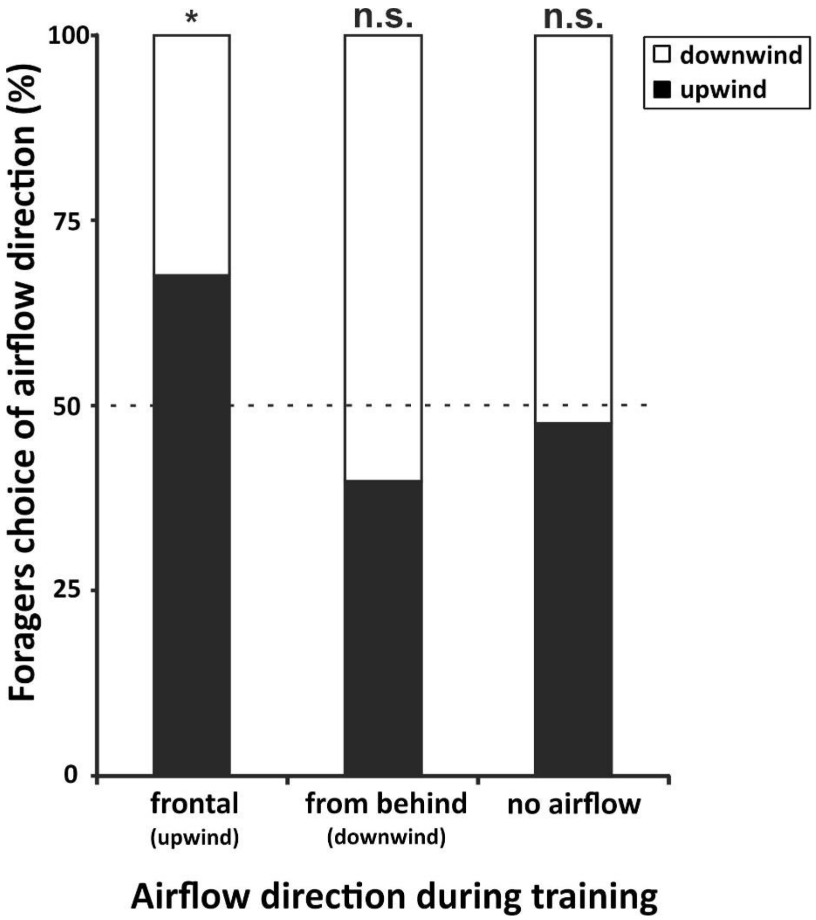


Figure 5: Airflow preferences of foragers during the test situation in the second experiment depending on the airflow conditions presented during training. Workers were exposed to frontal airflow, airflow from behind or no airflow during training and could either walk upwind (black bars) or downwind (white bars) during the test. In each series n = 40 individual ants were tested. Asterisks indicate significant differences compared to a 1:1 random distribution depicted as dashed line.

4. Discussion

In our experiments, we trained single foragers to a food source while exposing them to airflow as conditioned stimulus. Afterwards, we tested the workers for airflow preferences in a two-choice situation, in order to investigate whether they associated the food reward with the airflow experienced during training and used it as orientation cue during the test. We performed two different experiments in which we tested the ants' ability to use the presence of airflow as orientation cue and their ability to use specifically the direction of the airflow stimulus for orientation.

In the first experiment, workers of *Atta vollenweideri* learned the location of the food source based on the airflow stimulus presented at the training site and subsequently used this information for anemotactic orientation during the test. The ability to use airflow for orientation has been demonstrated for other ants as well, for example the argentine ant *Linepithema humile* (Van Vorhis Key and Baker, 1982) and the desert ant *Cataglyphis fortis* (Wehner and Duelli, 1971; Wolf and Wehner, 2000). However, in both species, workers used anemotaxis in combination with chemotaxis to locate a food source, thus also relying on olfactory cues within the airflow. In our experiments, ants memorized the stimulus based on the air movement itself and did not rely on chemotaxis to locate the food source, as the use of activated charcoal precluded olfactory cues from the air. In addition, a potential influence of olfactory cues emitted from the food reward itself can also be excluded, since workers also learned the location of the food source while walking downwind and in addition, no reward was present during the test on the T-maze.

In our first experiment, ants were successfully trained using both frontal airflow as well as airflow from behind as CS, indicating that the ants' ability to detect the airflow stimulus was independent of its direction. However, since workers were always given the choice between the CS and no airflow in the T-maze, it is possible that ants only associated the movement of air, but not the direction of the airflow with the location of the food source. Therefore, ants were given the choice between two airflow stimuli with opposing direction in the second experiment, i.e., workers could either walk upwind or downwind during the test. Workers preferred frontal airflow during the test when previously exposed to frontal airflow during the training as well, indicating that they associated not only the presence of airflow, but also its direction at the training site with the reward. However, workers failed to use the direction of the airflow for orientation when the airflow was coming from behind during training. Since the ants showed no preference for either direction when airflow was absent during training, this result was not simply based on an innate preference of leaf-cutting ants for frontal airflow masking the successful learning performances of workers in the second series. Instead, it is more likely that a frontal airflow stimulus is easier to detect for workers compared to an airflow stimulus coming from behind. Like in many other arthropods, the detection of air movements in ants most likely occurs via their antennae, more precisely the Johnston's organ located in the second antennal segment (Masson and Gabouriaut, 1973; Keil, 1997; Yack, 2004). Since the antennae of an ant are usually facing forward in the direction of movement, the ant's own body might act as a shield and block or deflect airflow coming from behind while walking towards the food source. Thus, the velocity or directionality of the airflow stimulus coming from behind might be perceived differently by a worker as compared to a frontal stimulus. Judging from the fact that workers were able to use airflow coming from behind for orientation in the first experiment, it is safe to assume that the stimulus was still detectable for the ants, but possibly provided only equivocal directional information.

Nevertheless, our results demonstrate that *Atta vollenweideri* leaf-cutting ants are sensitive to air movements and are able to use the presence of airflow and its direction as learned orientation cue, as proposed in previous experiments (Halboth and Roces, 2017). In the field, workers are expected to frequently encounter air movements within the underground as their nests are ventilated by a wind-induced mechanism (Kleineidam *et al.*, 2001). Since the direction of the airflow inside a given tunnel should be largely reliable, depending on the location of the tunnel opening on the surface of the nest mound, workers might use this information as learned orientation cue during several tasks inside the nest. Not all tunnel openings are used as entrances or exits by the ants as foragers usually leave the nest via peripheral, inflow tunnels (Jonkman, 1980b), while central, outflow tunnels rather serve the purpose of nest ventilation. Therefore, workers leaving the nest might use the direction of the airflow for example to locate peripheral nest openings for their foraging trips. Although speculative, foraging leaf-cutting ants might even rely on the directional airflow information provided by the surface wind outside the nest, for example to locate food sources or return to the nest, similar to the use of visual, olfactory or even magnetic cues (Vilela *et al.*, 1987; Jaffé *et al.*, 1990; Riveros and Srygley, 2008). It should be noted, though, that the wind speed on the surface can reach values of several m/s (Geiger, 1956) and therefore far exceeds the average velocities of ca. 1-5 cm/s measured inside the nest (Kleineidam *et al.*, 2001). Thus, the stronger air movements on the surface might rather prevent leaf-cutting ants from foraging, since especially loaded workers carrying leaf-fragments are negatively affected by wind (Anderson and Jadin, 2001; Alma *et al.*, 2016a; b) and risk being blown away (Weber, 1972a). However, in the latter case, workers might even use the directional information of the wind to compensate for the passive displacement by a wind gust, as shown for other ant species (Wystrach and Schwarz, 2013).

Within their nest, workers of Atta *vollenweideri* might also use the direction of the airflow as orientation cue to locate the central nest openings for example during tasks related to nest digging, soil transport or turret construction. On their nest mound, the ants construct ventilation turrets only on top of outflow tunnels (Jonkman, 1980b; Kleineidam *et al.*, 2001) using soil that is excavated in the underground and carried to the surface via transport chains (Pielström and Roces, 2013). Workers engaged in soil transport might memorize the prevailing airflow conditions at the digging site and in the nest tunnels and use this information not only to locate central outflow openings, but also to quickly return to their initial position inside the nest.

All in all, it is still an open question in what context and to which extent leaf-cutting ants rely on airflow-related cues in the field, but the present results nevertheless demonstrate their ability to use anemotactic orientation, implying the biological relevance of air movements for *Atta vollenweideri*.

III. Nest excavation: Influence of carbon dioxide levels on digging performance and digging choice

Abstract

The giant nests of *Atta vollenweideri* leaf-cutting ants are among the largest found in the animal kingdom and may reach a depth of several meters. During colony growth, ants excavate new tunnels and chambers for the rearing of fungus and brood. In the underground, the ants are confronted with elevated carbon dioxide concentrations due to nest depth and colony respiration that are expected to be harmful for brood and fungus. However, mature nests of *Atta vollenweideri* are usually well-ventilated via a wind-induced mechanism and contain multiple nest tunnels that connect the underground part to the surface, thus enabling the movement of air inside the nest and facilitating the removal of carbon dioxide from the underground. We hypothesized that the excavation of ventilation tunnels is triggered by increasing carbon dioxide concentrations in the nest, and that workers prefer to excavate at locations where CO_2 levels are elevated to facilitate gas exchanges.

In this chapter we first tested whether increased carbon dioxide concentrations affect digging performance in *Atta vollenweideri* leaf-cutting ants. Groups of workers contained in an arena partially filled with a clay/sand mixture were exposed to CO_2 levels of 0.04%, 2%, 4%, 6% and 10%, and the amount of building material excavated during a period of 5 hours was measured. Additionally, we recorded the number of ants engaged in excavation, soil transport or other activities, and quantified the amount of building material deposited inside and outside of the arena, as more workers might transport excavated material away from the digging sites under elevated CO_2 conditions.

The digging performance of ants was not affected by elevated carbon dioxide levels up to 6%, but significantly declined at 10% CO_2. Thus, increasing carbon dioxide did not enhance the digging response of workers, but rather resulted in reduced digging performances at the highest concentration. Most of the excavated material was deposited inside the digging arena and only small amounts were transported to the outside. The number of workers engaged in excavation was highest at the beginning of the experiment and declined after ca. 3 hours, while the number of workers engaged in soil transport was low at the beginning, but increased over time.

In a second experiment, we tested whether workers show a preference for certain carbon dioxide concentrations while digging in the nest, by offering a group of ants two digging

arenas containing different amounts of carbon dioxide, namely 0.04%, 1% or 5%, in a two-choice experiment. After 4 hours, we measured the amount of building material excavated in each arena. In addition, we counted the number of ants present in each arena every hour during the experiment.

Workers mostly showed a significant digging preference for a lower CO_2 concentration when presented with the choice between 0.04% and 5% CO_2, and tended to prefer to excavate at 1% over 5% CO_2. Workers, however, usually were equally distributed between the two digging arenas, i.e., there were no innate preferences for high or low CO_2 concentrations.

We propose that elevated CO_2 levels in the nest do not attract workers of *Atta vollenweideri* to excavate, but the presence of a strong CO_2 gradient leads to ants shifting their digging activity to sites with lower CO_2 levels, a mechanism possibly related to the excavation of new nest tunnels.

1. Introduction

The construction of stationary nests is a common feature in ants and in most species, colonies excavate their nests in the soil (Sudd, 1969). Size and structural features of such nests may vary tremendously among ant species and while some ants construct semi-superficial nest mounds (McCook, 1877; Farji-Brener, 2000) or dwell in shallow soil layers (Wang *et al.*, 1995; Mikheyev and Tschinkel, 2004), others excavate larger nests deep down in the ground that might even reach ground water levels (Dlusskii, 1981). Among the largest nests among all ant species are the giant underground nests of *Atta* leaf-cutting ants native to the Neotropics. In some species, these nests can reach a depth of up to 7 m (Moreira *et al.*, 2004a) and may contain several million individuals (Weber, 1972a). During colony growth the ants move about 10 tons of soil (Jonkman, 1980c) to excavate a complex network of tunnels and chambers in the underground. Most of these chambers are used to cultivate a symbiotic fungus on collected plant material, while others serve as refuse chambers, in which the colony disposes their waste. Depending on the species, the number of fungus chambers that can be found in a single nest may vary substantially, and mature nests may possess as much as 8000 of them (Moreira *et al.*, 2004a).

For the proper growth of the fungus, rather stable climatic conditions inside the nest are required, since the fungus has strict demands for high humidity levels and temperatures between 25°C and 30°C (Quinlan and Cherrett, 1978), and deviations might have detrimental effects on the symbiotic fungus (Powell and Stradling, 1986). In addition, the carbon dioxide levels in the underground are elevated due to soil depth (Reardon *et al.*, 1979) or colony respiration (Kleineidam and Roces, 2000) and the resulting hypercapnic (high CO_2) and hypoxic (low O_2) conditions are also expected to negatively influence fungal growth. Measurements in larger leaf-cutting ant nests revealed that CO_2 levels may reach values between 1.5% and 4.5% for *Atta capiguara* or *Atta laevigata* (Bollazzi *et al.*, 2012) and up to 5.7% in *Atta vollenweideri* (Kleineidam and Roces, 2000). Especially in the latter species, the removal of carbon dioxide from the underground and the supply of the nest with oxygen pose a major problem for colonies, since *Atta vollenweideri* inhabits the Gran Chaco region in South America, where the soils are particular clay-heavy. Clay-heavy soils only show low porosity and air permeability (Currie, 1984) and thereby hinder a diffusion-based gas exchange between the nest and the surrounding soil. As a result, the nests of *Atta vollenweideri* are ventilated by a wind-induced mechanism that facilitates the gas exchange between the underground nest and the environment (Kleineidam *et al.*, 2001). The aboveground nest mound is covered with up to 200 nest openings connecting the underground nest to the surface, that are not all used as entrances or exits (Jonkman, 1980b; c), but rather serve ventilation purposes. Air leaves the nest through openings located on the center of the mound, while fresh air enters through openings at the periphery, leading to a separation of inflow and outflow channels (Kleineidam *et al.*, 2001). Most nest openings are about 3-4 cm in diameter (Jonkman, 1980b) while others might become particularly large with up to 10 cm (Cosarinsky and Roces, 2007). Not all openings remain visible throughout the whole year, as most of them are closed during the winter months (Jonkman, 1980b; c), probably to avoid humidity loss from the nest. However, closure of nest openings prevents the wind-induced ventilation mechanism and leads to an accumulation of carbon dioxide in the underground (Kleineidam and Roces, 2000), and colonies are often faced with a trade-off between humidity control and gas exchange.

Additionally, as nests continue to grow and new fungus chambers are excavated in the underground, the overall nest structure changes and certain parts of the nest might not be as efficiently ventilated as others. The increasing number of ants and the size of fungus gardens also contributes to the rise of CO_2 in the underground and workers are expected to perceive and respond to these changes in the nest.

The ability to detect CO_2 is widely spread among insects (Nicolas and Sillans, 1989; Stange, 1996; Stange and Stowe, 1999) and there is evidence also in multiple ant species, for example the fire ant *Solenopsis saevissima* (Wilson, 1962) or the desert ant *Cataglyphis fortis* (Buehlmann *et al.*, 2012). Electrophysiological studies on leaf-cutting ants revealed that workers of *Atta cephalotes* and *Atta sexdens* possess specialized receptor cells in their antennae that allow them to detect both absolute CO_2 levels and gradual changes in the carbon dioxide concentration (Kleineidam and Tautz, 1996; Kleineidam *et al.*, 2000). Sensitivity to CO_2 also has been shown for *Acromyrmex lundii*, where workers prefer slightly elevated, but avoid high concentrations of carbon dioxide for the rearing of their fungus (Römer *et al.*, 2017). In the fire ant *Solenopsis geminata*, carbon dioxide released from trapped workers attracts nest mates and acts as a releaser for digging behavior in workers (Hangartner, 1969). However, elevated CO_2 levels did not affect nest excavation in the wood ant *Formica pallidefulva* (Mikheyev and Tschinkel, 2004) or the harvester ant *Pogonomyrmex badius* (Tschinkel, 2013).

For the leaf-cutting ant *Atta vollenweideri*, it is expected that carbon dioxide serves as an indicator for poor nest ventilation and that workers counteract the increase of CO_2 in the underground by adjusting the nest’s openings state according to their current ventilation needs. Workers might re-open or widen existing nest openings or excavate new ventilation tunnels in order to maintain a proper gas exchange between the nest and the environment. In this chapter, we therefore investigated whether carbon dioxide influences the digging behavior of *Atta vollenweideri* leaf-cutting ants and whether workers prefer to excavate at locations where the carbon dioxide concentrations are elevated. The results are discussed in the context of nest ventilation and collective climate control in this species.

2. Methods

All experiments were carried out at the University of Würzburg, Germany, on a queenright laboratory colony of *Atta vollenweideri* founded 2004 in Formosa, Argentina. The ants were maintained under controlled conditions at ~25°C, ~50% air humidity a 12h:12h LD cycle and fed daily with fresh leaves of blackberry (*Rubus fruticosus*) and dog rose (*Rosa canina*). At the time of the experiments the colony contained ca. 56 l fungus garden distributed to 14 plastic boxes (20 x 20 x 10 cm) interconnected with PVC tubes (Ø3 cm). Additionally, a plastic box (20 x 20 x 10 cm) partially filled with a mixture of clay and sand was connected to the colony. The mixture always consisted of two parts clay powder (CLAYTEC "Lehm gemahlen 10.001", particle size: ≤ 0.5 mm) and one part sand (DORSILIT® 9 "Kristall II", particle size: 0.6 – 1.2 mm) with a water content of 19% of the total mass. All ants from the colony gained access to the box for at least one hour prior to the experiments where they could freely dig in order to establish a digging context in the colony. For data collection only workers engaged in either digging or transporting soil were collected from the box over a period of ca. 20-30 min, and immediately transferred to the experimental setup in order to perform the experiments with groups of workers that are more likely motivated to excavate.

2.1. Influence of CO_2 on the digging performance of worker groups

In order to establish a digging arena for the experiments (Fig. 6) a Plexiglas container (10 x 10 x 2 cm) was partially filled with a clay/sand mixture of the same composition as mentioned before. We weighed the arena containing the clay/sand mixture to the nearest 0.01g before the experiment. Afterwards, the digging arena was covered with a Plexiglas plate (10 x 10 x 0.3 cm) and sealed with duct tape to prevent the desiccation of the clay/sand mixture. It was then connected to an "ant release box" (10 x 10 x 6 cm) using a transparent PVC tube (Ø 1.5 cm). Prior to the experiments, a stable carbon dioxide concentration of the air inside the digging arena was established by injecting a constant flow of air (75 ml/min) containing varying amounts of CO_2, depending on the series. The flow of air and carbon dioxide was adjusted using a mass flow meter (Sable systems flow bar 8) and then transferred to the arena through two transparent PVC tubes (Ø 3 mm), resulting in an inflow of air with approximately 37.5 ml/min and 4 cm/s per tube.

In total, we conducted five different experimental series using carbon dioxide concentrations of 0.04%, 2%, 4%, 6% and 10% CO_2. The series using 0.04% CO_2 acted as control series using atmospheric levels, while 2% CO_2 represent values measured in well-ventilated field nests of *Atta vollenweideri* in 2m depth (Kleineidam and Roces, 2000). Carbon dioxide levels of 4% and 6% have been be found in nests that lack sufficient ventilation, for example due to the closure of nest openings (Kleineidam and Roces, 2000), while levels of 10% represent extreme values that have never been measured in the field. The concentrations in the digging arena were measured before the start of each trial using a CO_2 analyzer (Gasmitter®, Sensor Devices).

Each trial started after we transferred 50 workers previously engaged in digging or soil transport to the ant release box, from which they could freely move into the digging arena. After half an hour, we counted every 30 min the number of ants currently present in the digging arena and assigned them to one of three categories according to their current task, i.e., excavating at the digging site, transporting excavated pellets or other behavioral activities. After the last counting (after 5 hours), the digging arenas were opened and all ants were removed. Using scraper and brush we removed all of the excavated material and loose pellets from the digging arena and weighed the remaining clay/sand inside the arena to the nearest 0.01 g. In order to calculate the amount of material the ants excavated during the course of 5 hours, we subtracted the weight of the arena at the end of the trial from the weight of the arena at the beginning of the trial. We also separately noted the weight of the removed pellets deposited either inside the arena or outside the arena in the ant release box. We used four separate replicates of the arena shown in Fig. 6, which allowed us to perform four simultaneous trials each day.

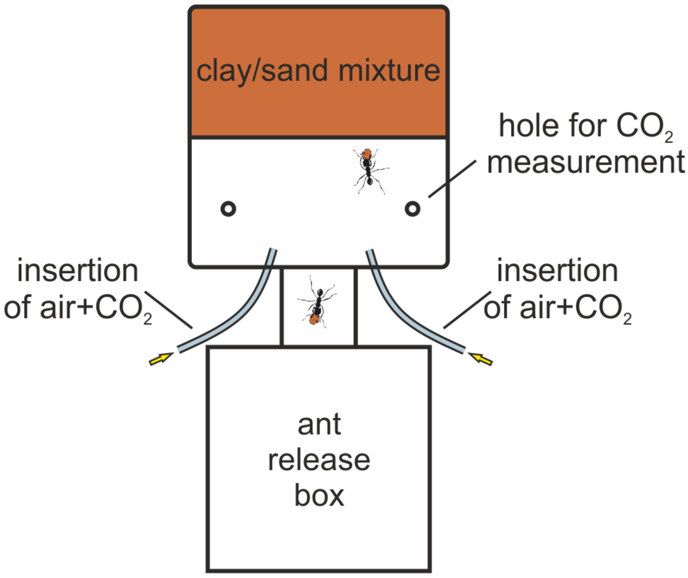


Figure 6: Experimental setup used in the first experiment for measuring the influence of carbon dioxide on digging performance. A group of 50 workers was released to the setup and ants could freely excavate for 5 hours while being exposed to different carbon dioxide concentrations in the digging arena.

2.2. Workers' preference for carbon dioxide during digging

Two digging arenas (9 x 9 x 3 cm) were partially filled with a clay/sand mixture, sealed and weighed analog to the first experiment, and connected to the ant release box via a Y-shaped PVC tube (Ø 2 cm) to resemble a two-choice experiment (Fig. 7). In contrast to the digging arenas from the first experiment, a gas permeable separating wall consisting of a perforated Plexiglas disc was inserted vertically into the digging arenas through a slit in the arena roof. This allowed air to move freely in the digging arena but prevented ants from physical contact with the clay mixture as long as the separating wall was lowered. Similar to the first experiment the carbon dioxide concentrations in both arenas were manipulated by injecting a mixture of air and CO_2 into the digging arenas through PVC tubes, generating an inflow of air with approximately 50 ml/min and 3 cm/s per arena. However, this time the CO_2 concentration of both arenas was adjusted separately, by using two valves (Sierra: 0-100 ml for CO_2, 0-1000 ml for air) connected to a first mass flow controller (Sable Systems 2 Channel MFC v1.0) for one arena, and two valves (Sierra: 0-100 ml for CO_2, 0-1000 ml for air) connected to a second mass flow controller (Sable Systems MFC-4) for the other arena.

In total, we conducted 5 experimental series using concentrations of 0.04% (atmospheric level), 1% (low CO_2) and 5% CO_2 (high CO_2). In a control series we offered 0.04% CO_2 in both arenas to rule out that workers show a preference for one of the two digging arenas independent of the prevailing carbon dioxide concentrations. In three series we tested different amounts of CO_2 in both arenas, namely 0.04% versus 1% CO_2, 0.04% versus 5% CO_2 and 1% versus 5% CO_2 in order to test whether workers show a preference for a certain carbon dioxide concentration during excavation. A second control series was conducted, where both arenas contained CO_2 levels of 5% to check whether a potential preference for CO_2 in the other series simply resulted from physiological constraints and reduced digging activity of the ants due to elevated carbon dioxide levels. The carbon dioxide concentration in each arena was again measured prior to each trial similar to the first experiment.

At the beginning of the trial 100 workers previously engaged in digging or soil transport were collected from the colony and transferred to the ant release box. The ants then could freely move inside the setup, but were unable to access the clay mixture as the separating walls were lowered for the first 60 min of each trial. This allowed ants to first distribute themselves among the two arenas according to their own carbon dioxide preference before digging, instead of randomly choosing one arena and immediately starting to excavate. After 1 hour, the number of ants present in each arena was counted and the separating wall was lifted, giving the workers access to the clay mixture for the remaining 3 hours of the trial. Every 60 min the number of ants in both arenas was counted again. After each trial, all ants and any loose material were removed before weighing the arena a second time in order to calculate the amount excavated per arena, analog to the first experiment. Again, four separate replicates of the setup were used parallel in order to perform four trials simultaneously.

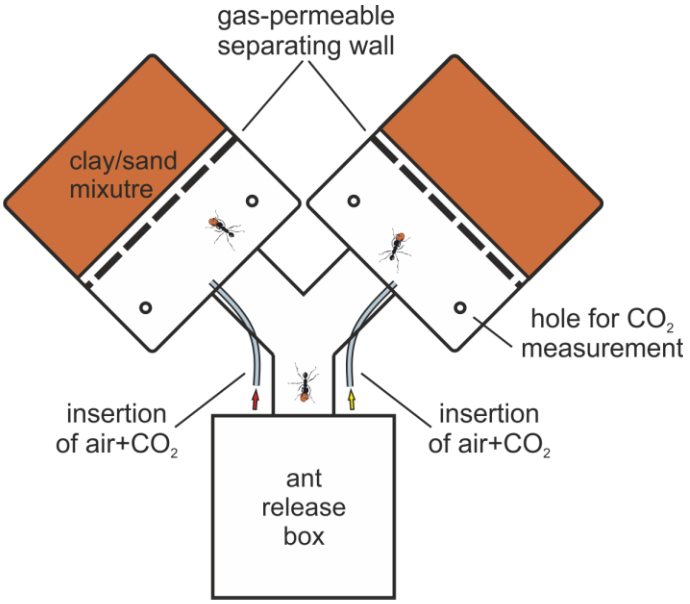


Figure 7: Experimental setup used in the second experiment for measuring the preference of workers for carbon dioxide during digging. A group of 100 workers was released to the setup where they could distribute themselves between two digging arenas while being exposed to different CO_2 concentrations. After one hour, workers gained access to the clay-mixture filled section of the digging arenas where they could freely excavate for another 3 hours.

2.3. Statistical analysis

All data were first tested for normality using the Shapiro-Wilk normality test. Accordingly, the number of ants engaged in digging or soil transport were compared between the beginning and the end of the trials, using Mann-Whitney-U tests, as well as between all experimental series at three different time points using Kruskal-Wallis test followed by Dunn's multiple comparison test. The data for the amount of excavated material and the deposition of pellets in the first experiment were analyzed either with ANOVA followed by Tukey's multiple comparison test (post-hoc) or Kruskal-Wallis test followed by Dunn's multiple comparison test. In the second experiment, the distribution of ants among the digging arenas over the course of the trials was compared using Wilcoxon-signed-rank tests, while the amount of excavated material was compared between both arenas in all series with Mann-Whitney-U tests. Detailed statistics are presented in the Appendix. The significance level was set to $\alpha = 0.05$ for all tests.

3. Results

3.1. Influence of CO_2 on the digging performance of worker groups

After being released into the setup, workers began to spread out and distribute themselves more or less equally between the ant release box and the digging arena. Inside the digging arena, some ants quickly discovered the digging surface and started excavating. During excavation workers first grabbed material out of the digging surface using their mandibles and then aggregated it into pellets using their front legs. Pellets were then transported away from the digging site either by the excavating ant itself or by a different worker. Over the course of each trial, the number of ants engaged in excavation and soil transport was similar among the series, but largely varied with increasing duration of the trial (Fig. 8). For the first 2.5 hours, the number of ants digging in the arena remained rather constant, with approximately 10-15 ants excavating. Afterwards, the number of ants declined and was significantly lower at the end of the trial (5 hour mark) compared to the beginning (0.5 hour mark), for all series (Fig. 8a). In contrast, the number of ants engaged in soil transport was low after 0.5 hours, with ca. 0-1 workers, but significantly increased in all series until the end of the trial to approximately 2-4 ants (Fig. 8b). The number of ants performing other tasks besides excavation and soil transport also increased during the course of the trial in all series except the 0.04% CO_2 series (Fig. 8c). In order to test the effect of the CO_2 concentration on the number of workers performing tasks in the arena, we compared the number of ants engaged in excavation, soil transport and other tasks for all series at the beginning (0.5 h), the middle (2.5 h) and the end (5 h) of the experiment (Fig. 9). The carbon dioxide concentration affected the number of ants excavating after 0.5 hours (Kruskal-Wallis test: $H_{4,60} = 11.73$, $p = 0.019$), after 2.5 h (Kruskal-Wallis test: $H_{4,60} = 16.44$, $p = 0.003$) and after 5 hours (Kruskal-Wallis test: $H_{4,60} = 18.25$, $p = 0.001$). More ants excavated at 0.5 hours in the 4% CO_2 series compared to the 0.04% CO_2 series, while at 2.5 hours the number of excavators at 4% CO_2 was higher compared to all the other series. After 5 hours, the number of ants excavating was only lower for 10% CO_2 compared to 0.04% and 6% CO_2 (Fig. 9a). The number of ants engaged in soil transport was influenced by the CO_2 levels after 0.5 hours (Kruskal-Wallis test: $H_{4,60} = 15.13$, $p = 0.004$), after 2.5 hours (Kruskal-Wallis test: $H_{4,60} = 11.02$, $p = 0.026$) and after 5 hours (Kruskal-Wallis test: $H_{4,60} = 9.98$, $p = 0.041$), but was only lower at 0.5 hours for 10% CO_2 compared to 4% and 6% CO_2. In all other cases, the number of ants engaged in carrying soil pellets was the same for all series at all time points (Fig. 9b). For ants participating in other tasks, there was an effect of the carbon dioxide levels for 0.5 hours (Kruskal-Wallis test: $H_{4,60} = 21.80$, p

< 0.001), for 2.5 hours (Kruskal-Wallis test: $H_{4,60} = 20.03$, $p < 0.001$) and 5 hours (Kruskal-Wallis test: $H_{4,60} = 17.19$, $p = 0.002$). At 0.5 hours, more ants were engaged in other tasks at 0.04% compared to 4%, 6% and 10% CO_2, while at 2.5 hours the number was only higher for 0.04% compared to 6% CO_2. In contrast, the number of ants was lower in the 0.04% CO_2 series compared to 4% and 10% CO_2 after 5 hours (Fig. 9c).

The amount of material excavated by the ants during each trial was affected by the prevailing carbon dioxide concentration (Fig. 10) and differed among the series overall (ANOVA: $F_{4,55} = 6.37$, $p < 0.001$). Less material was excavated in the 10% series, but there was no statistical difference between the series 0.04%, 2%, 4% and 6% CO_2. In the 0.04% CO_2 series, i.e., under atmospheric levels, workers excavated 51.48 ± 6.47 g (mean ± SD) over the course of the trial. When CO_2 levels where elevated, the amount of material excavated by the ants was 51.94 ± 4.76 g (mean ± SD) for the 2% series, 49.38 ± 5.06 g (mean ± SD) for the 4% series and 51.23 ± 4.33 g (mean ± SD) for the series containing 6% CO_2. At 10% CO_2 workers only excavated 43.32 ± 3.40 g (mean ± SD).

Most of the pellets excavated by the ants were deposited inside the digging arena, and much less material was transported outside and dropped in the ant release box (Fig. 11). The amount of pellets inside the arena depended on the CO_2 concentration and differed among the series (ANOVA: $F_{4,55} = 3.73$, $p = 0.009$). In the 0.04% series, ants deposited 37.73 ± 4.69 g (mean ± SD) of pellets inside the arena. When carbon dioxide levels were elevated, the amount of pellets inside the digging arena was 38.78 ± 3.44 g (mean ± SD) for 2% CO_2, 36.68 ± 2.21 g (mean ± SD) for 4% CO_2, 34.55 ± 5.94 g (mean ± SD) for 6% CO_2 and 33.32 ± 2.76 g (mean ± SD) for 10% CO_2.

However, the only statistical significant difference was found between the 2% and 10% CO_2 series. The amount of pellets carried out of the digging arena was always lower, but also depended on the series (Kruskal-Wallis test: $H_{4,60} = 11.00$, $p = 0.027$). Workers transported 2.30 ± 7.35 g (median ± IQR) in the 0.04% series, 2.10 ± 2.90 g (median ± IQR) in the 2% series and 3.95 ± 3.40 g (median ± IQR) in the 4% series. In the series using 6% CO_2 the amount of clay deposited outside the digging arena was highest with 7.80 ± 6.08 g (median ± IQR), which was significantly higher than in the series using 10% CO_2 with 1.75 ± 0.93 g (median ± IQR) pellets.

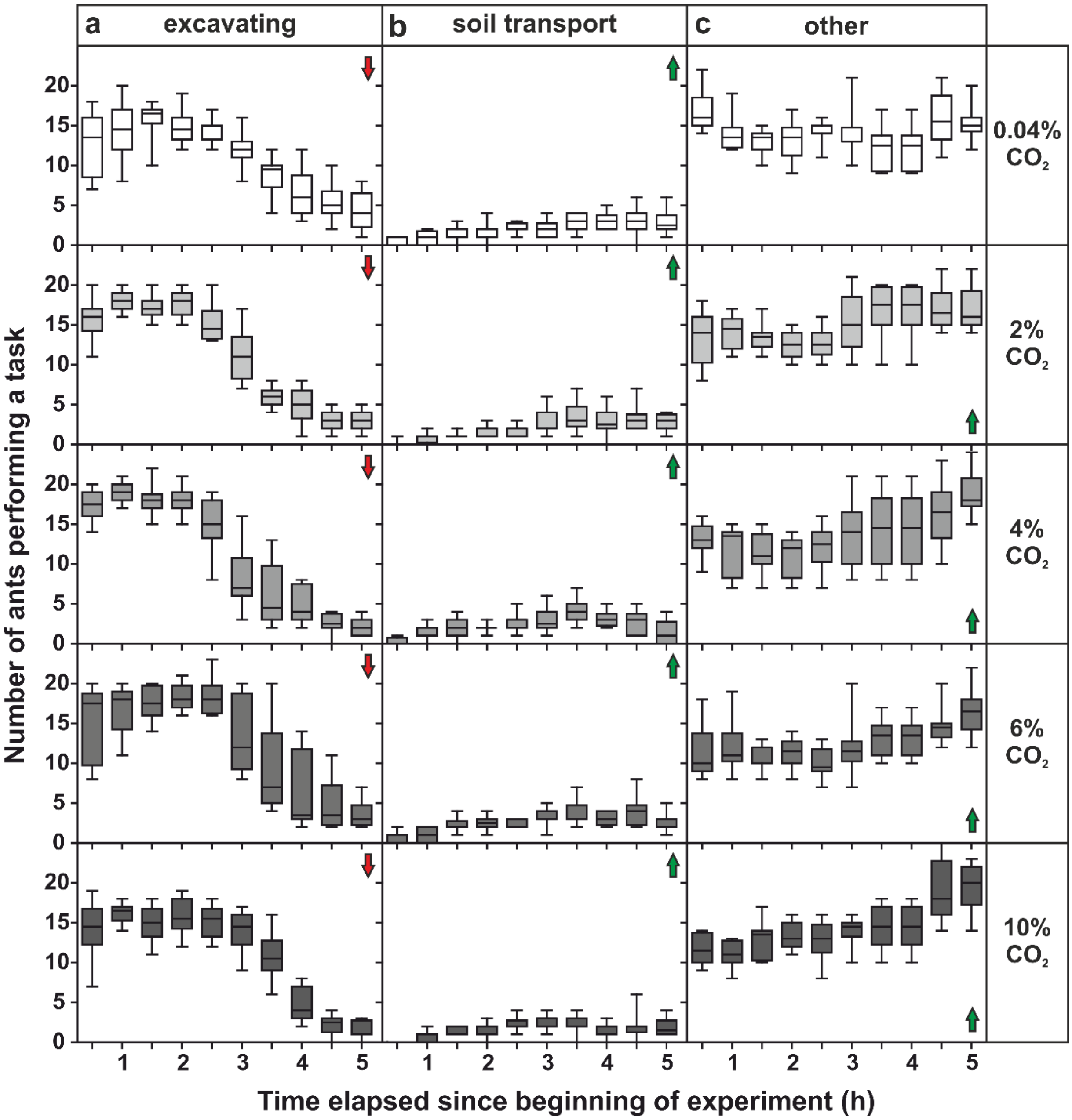


Figure 8: Number of ants engaged in a) excavating, b) soil transport and c) other activities over the course of the experiment, for the different carbon dioxide concentrations. Each series contains n = 12 replicates. Shown are medians (horizontal bars), 25-75% percentiles (boxes) and min-max values (whiskers). Arrows indicate either a statistically significant increase (green, upward) or a decrease (red, downward) in the number of ants performing a task after 5 hours, as compared to 0.5 hours. Detailed statistics can be found in A1.

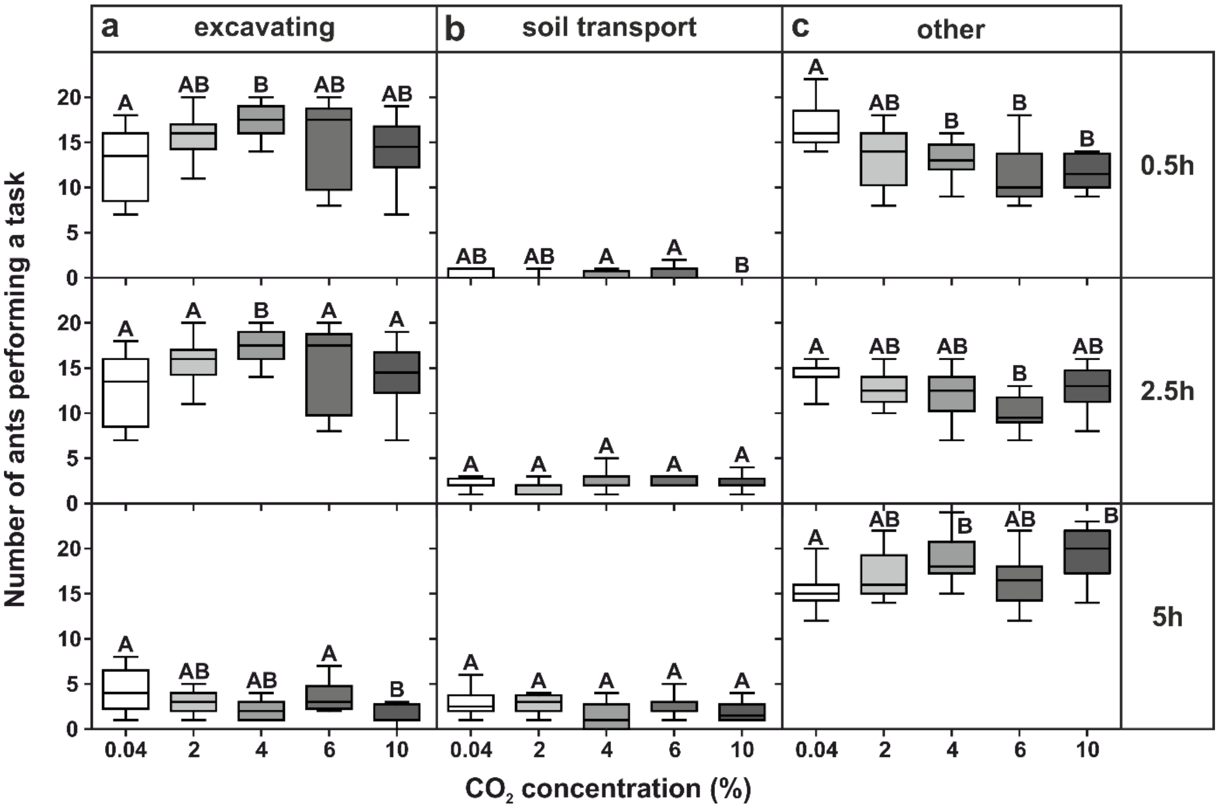


Figure 9: Number of ants engaged in a) excavating, b) soil transport and c) other activities depending on CO2 concentration at 0.5 hours, 2.5 hours and 5 hours after the beginning of the experiment. Each series contains n = 12 replicates. Shown are medians (horizontal bars), 25-75% percentiles (boxes) and min-max values (whiskers). Groups sharing the same letter are not statistically different. Detailed statistics can be found in A2, A3 and A4.

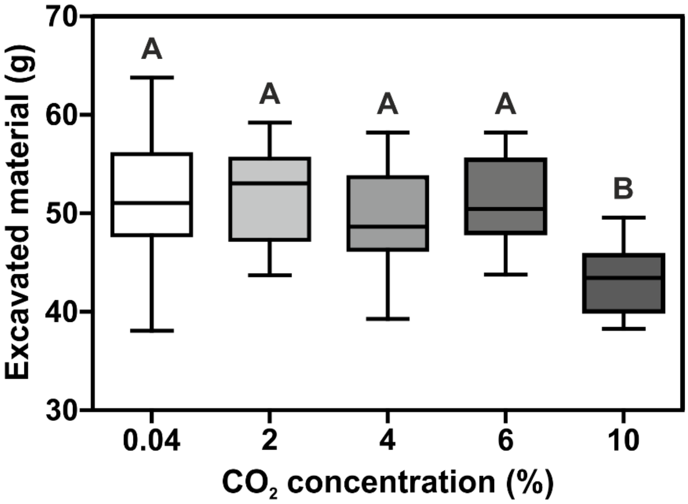


Figure 10: Influence of carbon dioxide concentration on the amount of material excavated by a group of 50 workers over the course of 5 hours. Each series contains n = 12 replicates. Shown are medians (horizontal bars), 25-75% percentiles (boxes) and min-max values (whiskers). Groups sharing the same letter are not statistically different. Detailed statistics can be found in A5.

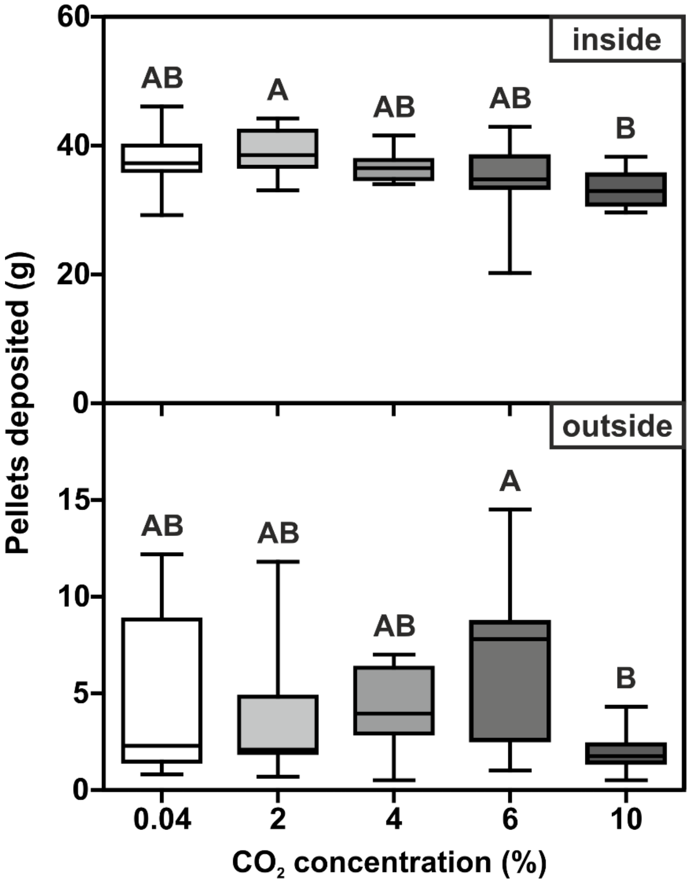


Figure 11: Total mass of all pellets deposited inside the digging arena (top) and outside of the digging arena (bottom) over the course of 5 hours. Each series contains n = 12 replicates. Shown are medians (horizontal bars), 25-75% percentiles (boxes) and min-max values (whiskers). Groups sharing the same letter are not statistically different. Detailed statistics can be found in A6, A7.

3.2. Workers' preferences for carbon dioxide during digging

After being transferred to the release box, workers began spreading out across the setup and distributed in both digging arenas. Overall, the number of workers present in the digging arena was mostly the same for both sides in each series, independent of the time elapsed since the beginning of the trial (Fig. 12). Approximately 25-30 workers were usually present in each of the digging arena at all times. Only on two occasions more ants were present in the arena with the lower carbon dioxide concentration, i.e., in the 0.04% vs. 5% series after 2 hours, and the 1% vs. 5% series after 4 hours. Apart from that there were no statistically significant differences among the groups, indicating that workers did not prefer or avoid one particular carbon dioxide concentration, but rather randomly distributed among both arenas in all series.

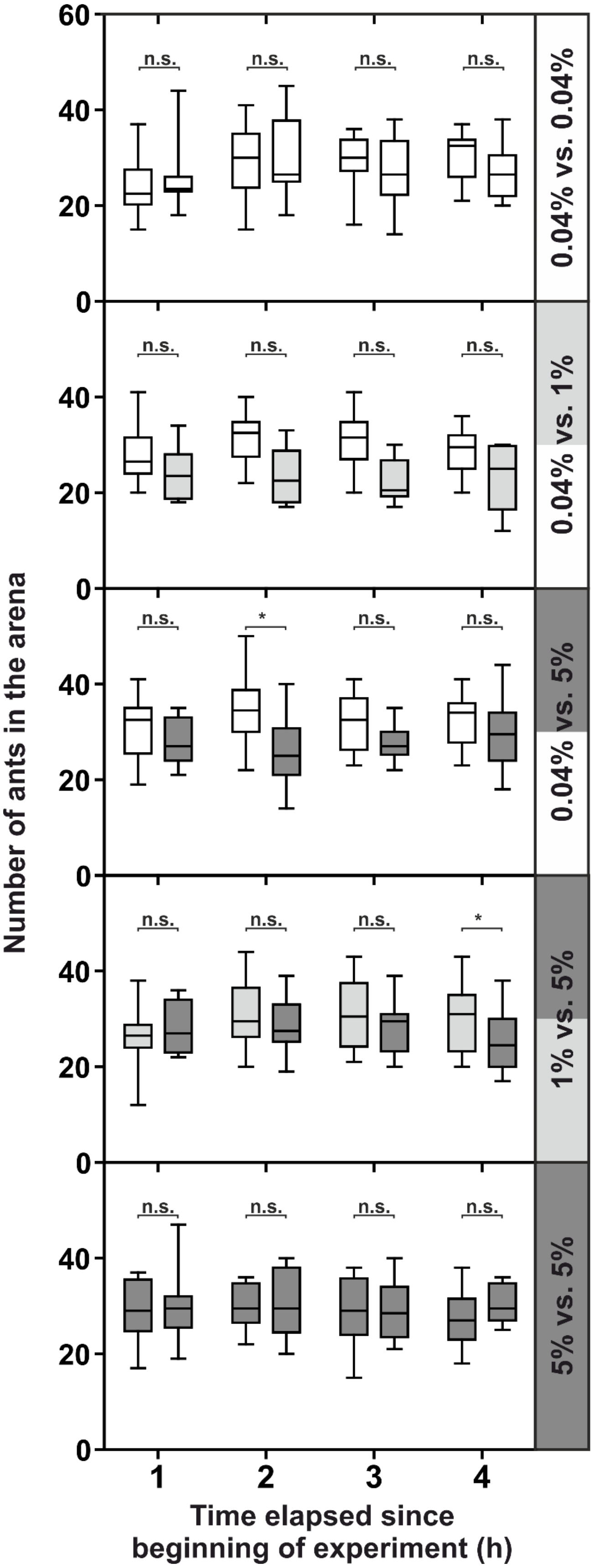


Figure 12: Number of ants present in each digging arena over the course of the trial. Each series contains n = 16 replicates. Shown are medians (horizontal bars), 25-75% percentiles (boxes) and min-max values (whiskers). Groups sharing the same letter are not statistically different. Detailed statistics can be found in A8.

There were, however, differences between both tested arenas concerning the amount of excavated material throughout the 3 hour period in which workers had access to the clay mixture (Fig. 13). In the control series (0.04% vs. 0.04% CO_2), workers excavated the same amount in both arenas with 11.1 ± 7.2 g (median ± IQR) on the left and 9.7 ± 3.9 g (median ± IQR) on the right side. In the series 0.04% vs. 1% CO_2, workers tended to excavate more in the arena with atmospheric levels, with 11.5 ± 5.4 g (median ± IQR), than in the arena with 1% CO_2 levels, with 6.6 ± 8.1 g (median ± IQR). However, a statistical significant difference was only found between 0.04% vs 5% CO_2, when workers excavated with 12.6 ± 5.7 g (median ± IQR) more of the clay mixture at atmospheric levels than at 5% CO_2 levels, with 5.4 ± 9.0 g (median ± IQR). In the series 1% vs 5% CO_2 the amount of material excavated in both arenas, i.e., 9.5 ± 12.4 g (median ± IQR) at 1% CO_2 and 7.9 ± 5.5 g (median ± IQR) at 5% CO_2, were the same for both carbon dioxide concentrations. In the second control, in which the carbon dioxide concentration was elevated to 5% on both sides, workers excavated 11.7 ± 4.8 g (median ± IQR) on the left and 9.2 ± 5.4 g (median ± IQR) on the right side, thus showing a statistical preference for the left digging arena.

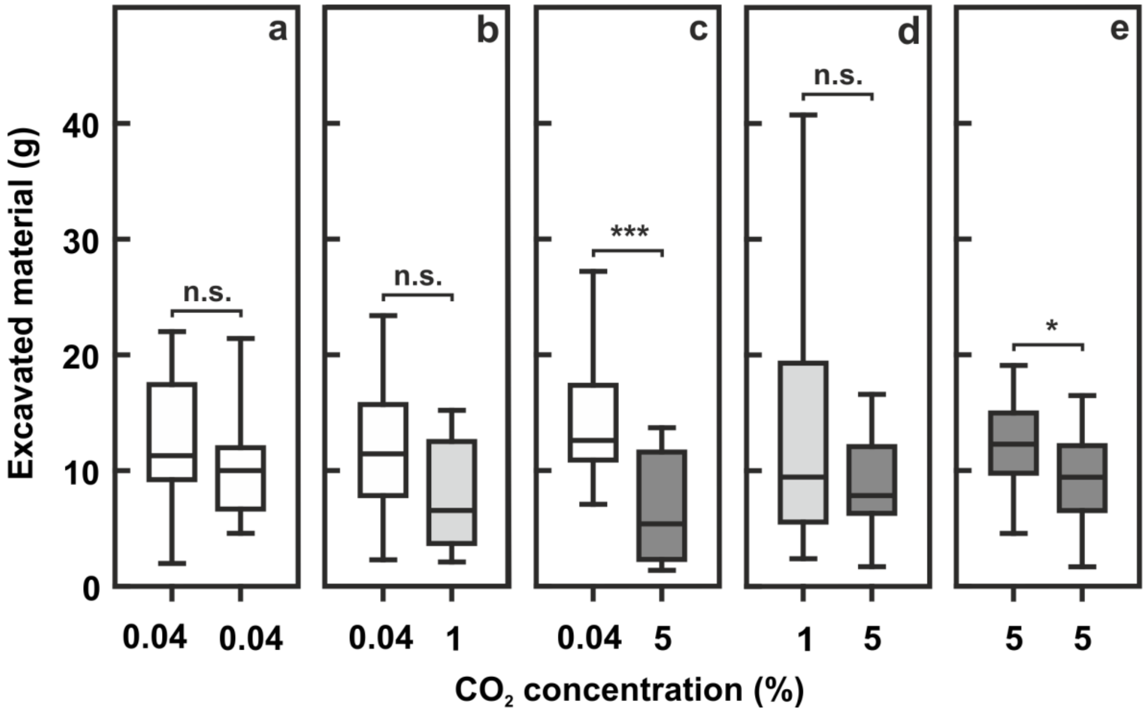


Figure 13: Amount of material excavated in a two-choice experiment by a group of 50 workers over the course of 3 hours. Each series contains n = 16 replicates. Shown are medians (horizontal bars), 25-75% percentiles (boxes) and min-max values (whiskers). Asterisks indicate significant differences between groups. Detailed statistics can be found in A9.

4. Discussion

4.1. Influence of CO_2 on the digging performance of worker groups

Independent of the carbon dioxide concentration, the number of workers engaged in digging was high for the first 2.5 hours, but decreased for the remaining duration of the experimental trial. In contrast, the number of workers carrying excavated soil pellets increased over time. Obviously, as time progressed and workers kept excavating at the digging surface, more and more pellets accumulated inside the arena. While the majority of pellets was deposited inside the digging arena, some workers carried pellets out of the arena into the open box. During nest excavation ant workers engage in sequential soil transport (Pielström and Roces, 2013; Tschinkel *et al.*, 2015), similar to the aboveground transport chains observed during foraging in leaf-cutting ants (Hubbell *et al.*, 1980; Röschard and Roces, 2003). Soil pellets are usually not carried away from the digging site by the excavator itself, but rather transported by other individuals (Pielström and Roces, 2013). The distribution of work among several workers, or task partitioning, is a common feature in ants and has been extensively discussed throughout the literature (Hölldobler and Wilson, 1990; Beshers and Fewell, 2001; Gordon, 2015). Some members in a group of ants usually are more inclined to perform tasks like excavation, soil transport or foraging and tend to keep performing a particular task over several hours, or sometimes days or weeks (personal observation). In our experiment, however, we only counted the number of ants performing a task and did not mark individual workers or follow them over a longer period of time. Thus it remains unclear whether most of the digging was done only by a small number of ants or whether workers switched between tasks. Similarly, it was impossible for us to distinguish between a decline of digging ants and an increase of soil carrying ants after 2.5 h due to ants switching between tasks or due to excavators simply stopping, while ants previously engaged in other activities began the transport of pellets.

Concerning the influence of carbon dioxide on digging performance, differences were only observed at 10% CO_2, the highest concentration tested. While the number of ants engaged in digging varied between all series, there were no consistent differences between the groups throughout the experiment. Fewer ants were observed excavating at 10% CO_2 only and the end of the trial, but not during other time points, yet workers excavated less material in the 10% CO_2 series compared to all other series. This indicates that while the high carbon dioxide concentration did not deter workers from entering the digging arena or starting to excavate, it nevertheless reduced their overall digging performance, i.e., the amount of excavated material by the worker group over the course of 5 hours.

The excavation of soil in ants can be described as a series of three repetitive steps, (1) grabbing, (2) raking and (3) transporting of material (Sudd, 1969). First, workers thrust their opened mandibles into the substrate, close them to grab a cluster of soil particles and remove it from the digging surface. When enough material is loosened, ants make raking movements using their mandibles and forelegs to collect the material and form it into a soil pellet. After that the workers turn away from the digging surface and either drop the pellet in their close vicinity or carry the pellet over a short distance before its release.

Since we did not investigate the behavior of individual workers, it is unclear whether the differences in digging performance result from the fact that an ant grabbed less material during a single event or whether it simply took longer for an ant to complete one cycle of the excavation process. Nevertheless, it can be assumed that the high carbon dioxide concentration in the arena in the 10% CO_2 series negatively affected the digging ability of the ants, as elevated carbon dioxide concentrations have been shown to affect many aspects of insect physiology and can lead to reduced muscle activity, changes in respiratory rates and water loss (Nicolas and Sillans, 1989). Workers use their mandibles during excavation in a similar manner to the cutting of leaves, which is one of the most energy-consuming tasks for ants (Roces and Lighten, 1995) and excessive digging effort has been shown to increase the mortality rate at least in queen leaf-cutting ants (Camargo *et al.*, 2011). Despite the fact that leaf-cutting ants are constantly confronted with elevated CO_2 concentrations in the nest, and both ants and fungus are expected to have adapted to this, it is likely that extremely high levels of carbon dioxide make soil excavation even more difficult for ants, thus resulting in an overall lower digging performance.

Besides the reduced digging rate at 10%, carbon dioxide had no influence on the amount of material excavated in each series, indicating that elevated CO_2 levels alone did not enhance the digging performance of workers.

Previous studies have shown that the excavation rate of workers is affected by the worker density and the amount of available space inside the nest, as crowding and an aggregation of ants result in an increased digging activity (Buhl *et al.*, 2005; Römer and Roces, 2015). By creating additional space, however, the worker density is again reduced, which is known to inhibit further nest excavation (Pielström, 2013). Since in our experiments the overall number of workers present in the digging arena was roughly the same during the entire duration of the trial, worker density could only have been reduced by the creation of additional space inside the digging arena. Over time, workers removed some clay/sand mixture from the digging arena and transported the pellets into the open box, thus increasing the amount of available space inside the arena.

The amount of soil pellets deposited outside of the digging arena was mostly unaffected by the carbon dioxide concentration. While it was lower in the 10% CO_2 series compared to the 6% CO_2 series, this effect can most likely be attributed to the reduced digging activity of the ants at 10% CO_2. Although not statistically significant, the median amount of pellets deposited outside the digging arena appeared to be higher at 4% and 6% CO_2 compared to 0.04% and 2% CO_2. In field nests of *Atta vollenweideri*, elevated carbon dioxide levels of 4-6% indicate poor nest ventilation (Kleineidam and Roces, 2000) and colonies are expected to take countermeasures by promoting the wind-induced ventilation of the nest, thus facilitating the removal of carbon dioxide from the underground. Hypothetically, by increasing the amount of soil transported to the surface workers might contribute to this effect by (1) removing material from tunnel walls, effectively increasing tunnel diameter, or (2) increasing the amount of building material available for the construction of ventilation turrets on top of outflow openings. In both cases, the movement of air through the nest would likely be enhanced, leading to a reduction of carbon dioxide in the underground nest. However, the results obtained from our experiment do not suggest that this is the case.

4.2. Workers’ preferences for carbon dioxide during digging

In contrast to the first experiment, workers were given a choice between two digging arenas containing different carbon dioxide concentrations. For the first hour of each trial, workers had no access to the digging sites, but were allowed to freely spread among the arenas. Independent of the prevailing carbon dioxide concentrations, workers always distributed evenly between both digging arenas. Even after getting access to the clay mixture, the number of ants present in one digging arena usually matched the number of ants in the other arena, indicating that there was no innate preference or avoidance of the ants for one particular CO_2 concentration. Contrary to the findings of studies on other arthropods, workers of *Atta vollenweideri* were not attracted by elevated carbon dioxide levels. However, the context in which CO_2 is relevant for leaf-cutting ants might be completely different compared to other invertebrates. Elevated carbon dioxide levels often indicate the presence of food sources for various solitary arthropods (Nicolas and Sillans, 1989; Stange, 1996) and are important for example during plant selection in moths (Thom *et al.*, 2004) or host-selection in ticks (Holscher *et al.*, 1980) and mosquitos (Gillies, 1980). Even in ants, the effect of carbon dioxide on worker behavior strongly depends on the context. In fire ants (*Solenopsis*), CO_2

released by trapped workers attracts nest mates and triggers digging behavior in order to free them (Wilson, 1962; Hangartner, 1969), while desert ants (*Cataglyphis*) use the carbon dioxide emitting from their nest as orientation cue during homing (Buehlmann *et al.*, 2012). Leaf-cutting ants possess the rare ability to respond to both the absolute CO_2 levels as well as changes in the carbon dioxide concentration (Kleineidam and Tautz, 1996; Kleineidam *et al.*, 2000), indicating the importance of CO_2 perception for the ants. However, for leaf-cutting ants CO_2 might not necessarily serve as a cue that attracts workers, but instead act as an indicator for poor nest ventilation. In addition, workers might possess no carbon dioxide preference for themselves, but rather prefer slightly elevated CO_2 levels for the rearing of their symbiotic fungus, as recently shown in *Acromyrmex lundii* (Römer *et al.*, 2017).

Concerning the digging behavior, workers did not excavate more material in locations where carbon dioxide levels were elevated, but rather significantly preferred digging under lower concentrations for the series 0.04% vs. 5% CO_2 and marginally preferred 0.04% over 1% CO_2. In the series where the CO_2 levels were 1% in one and 5% in the other arena, workers again excavated the same amount on both sides, showing no carbon dioxide preference during digging. It seems more likely that workers preferred excavation at lower carbon dioxide levels instead of avoiding digging under 5% $CO_{2.}$ In our first experiment, ants were able to maintain the same digging performance for all carbon dioxide concentrations between 0.04% and 6% $CO_{2,}$ proving that in this range carbon dioxide does not inhibit the capability of workers to excavate. In addition, workers only showed a preference for lower CO_2 levels when the contrast between both arenas was the highest (0.04% vs. 5%), but overall excavated a similar amount, i.e., roughly 21 g combined, in the second control (5% vs. 5%) compared to in the first control series (0.04% vs. 0.04%). This indicates that instead of simply avoiding to dig at high carbon dioxide concentrations, workers might have preferred to excavate at the location with the lower CO_2 concentration. This view is further supported by fact that while the amount of material excavated differed between arenas, the number of workers present throughout the entire trial was mostly the same for both sides. Instead of orienting towards the source of CO_2, like fire ants (Wilson, 1962; Hangartner, 1969), workers of *Atta vollenweideri* might prefer digging in a direction away from the higher carbon dioxide concentration. Digging along a decreasing carbon dioxide gradient in the underground would cause workers to excavate upwards under natural conditions, as the CO_2 levels in shallow soil layers are lower than those in deeper layers. This mechanism could lead to the excavation of new nest tunnels as a result of poor nest ventilation, which in turn might facilitate the removal of carbon dioxide from the underground.

It seems unlikely that the preference to dig at atmospheric CO_2 levels is related to the excavation of new nest chambers, since leaf-cutting ants, at least in the species *Acromyrmex lundii*, actually avoid atmospheric, but prefer slightly elevated carbon dioxide levels of 1-3% for the cultivation of their symbiotic fungus (Römer *et al.*, 2017).

It is important to note that while there were also differences in the amount of material excavated between both arenas in the second control series (5% vs. 5% CO_2), no such difference was found for the first control series (0.04% vs. 0.04% CO_2). It is therefore unlikely that the existence of a side bias is responsible for the observed digging preferences in the series using different carbon dioxide levels in both arenas. However, in order to verify that workers specifically prefer digging along a carbon dioxide gradient towards the lower concentrations, further experiments should be performed in which workers are for example confronted with varying CO_2 levels along a continuous digging surface, simulating the carbon dioxide gradient that can be found across different soil layers in nature.

IV. Underground soil transport: Influence of CO_2, airflow direction and humidity on workers' preference for pellet deposition

Abstract

Gas exchanges in the giant underground nests of the leaf-cutting ant *Atta vollenweideri* are facilitated by a wind-induced ventilation mechanism, with fresh air entering and CO_2-rich air leaving the nest through several openings on the nest mound. On top of outflow openings, workers construct small ventilation turrets using soil excavated in the underground and transported to the surface. We hypothesized that climatic cues inside the nest tunnels are used by workers for orientation in order to locate outflow openings during soil transport. In the laboratory, we tested the preference of workers engaged in pellet transport for climatic variables in two binary choice experiments. In the first experiment, a group of ants was exposed during transport of soil pellets along a tunnel to either 0.04%, 1% or 5% CO_2, simulating atmospheric levels, good or poor nest ventilation in the underground. Single ants carrying pellets then were rerouted and confronted with the choice between two tunnels, each providing different CO_2 levels. When carbon dioxide in the underground was elevated, workers significantly preferred to transport pellets along tunnels containing higher levels of CO_2, as well. When exposed to atmospheric or low CO_2 levels, workers showed no preference during the test. In the second experiment, single workers carrying pellets were rerouted and given the choice between two tunnels, one providing inflow, and the other providing outflow of air, with air velocities of either 0, 5 or 10 cm /s, and at either 30% or 70% relative humidity. Workers preferred the pellet transport along tunnels providing outflow of humid air for 5 and 10 cm/s, as well as outflow of dry air for 5 cm/s. When dry air at 10 cm/s was used, workers however preferred the transport of pellets along the inflow tunnel. In summary, workers used the climatic conditions inside the tunnels for orientation and preferred the transport of excavated pellets along tunnels containing high CO_2 levels and outflowing air, corresponding to the prevailing conditions at central nest openings on field nests. We argue that the deposition of soil pellets around central outflow openings promotes the wind-induced nest ventilation and might be related to the construction of ventilation turrets on top of the nest. For inflowing dry air at 10 cm/s, however, pellet deposition may be a workers' response aimed at closing the inflow openings to prevent nest desiccation.

1. Introduction

The giant underground nests of *Atta vollenweideri*, a leaf-cutting ant species native to the Gran Chaco region in South America, are among the largest structures created by social insects. Their nests consist of a complex network of tunnels and chambers that may cover an area of 40 m² and reach a depth of about 2 m (Jonkman, 1979; 1980c). A mature colony may contain several million workers (Weber, 1972a) and during the excavation of their nest ants may move up to 10 tons of soil (Jonkman, 1980c). Workers remove the excavated soil from the underground by transporting it to the surface, thereby facilitating soil turnover rates and reshaping vegetation structure and composition of the surrounding area (Jonkman, 1978). Above the ground, the soil is piled up around the nest openings to form a conical-shaped mound up to 1 m high and 8 m wide that is permeated with up to 200 openings leading to the underground chambers (Jonkman, 1980b; c).

However, nest openings are not all used as entrances or exits by foraging workers, but some rather serve ventilation purposes. For soil-dwelling animals, proper ventilation of the subterranean nest is often a challenging task, as the exchange of respiratory gases are limited in the underground (Hansell, 2005). Due to the respiration of the colony members and - in case of the leaf-cutting ants - the symbiotic fungus, large amounts of oxygen are consumed and carbon dioxide is produced in the underground. In addition, the soil acts as a natural source for CO_2 and its levels rise with increasing depth (Schwartz and Bazzaz, 1973; Reardon *et al.*, 1979; Davidson and Trumbore, 1995). As a result, the CO_2 levels inside the nests of *Atta vollenweideri* usually vary between 1-2% compared to the atmospheric levels of currently 0.04%, but may reach values of up to 5.7% when nest openings are closed (Kleineidam and Roces, 2000), for example during the winter months (Jonkman, 1980b; c).

Depending on nest size and soil properties, a diffusive air exchange may not be sufficient for the supply of fresh air and the removal of CO_2 from the underground (Withers, 1978). Therefore, nests of the leaf-cutting ant *Atta vollenweideri* are ventilated via a wind-induced mechanism with air entering and leaving the nest through openings on the nest mound (Kleineidam *et al.*, 2001). The underlying mechanism relies on the Bernoulli principle and viscous entrainment (Vogel and Bretz, 1972), similar to the ventilation system found in burrows of prairie-dogs (Vogel *et al.*, 1973). In Atta *vollenweideri*, surface wind is accelerated while passing over the nest mound, resulting in pressure differences between the elevated center and the periphery of the mound (Kleineidam *et al.*, 2001). As a consequence, air is sucked out of central nest openings, creating an outflow of used, CO_2-rich air from the underground. With a short delay that depends on wind speed, inflow of fresh, O_2-rich air through openings on the mound periphery follows, resulting in a largely predictable separation of inflow and outflow tunnels in the nest, independent of surface wind direction.

On top of central nest openings, workers additionally construct conspicuous turrets that are expected to promote the ventilation of the nest by further elevating the nest opening and by exposing it to higher surface wind velocities. For their construction, workers mainly use excavated soil from the underground, but reinforce the structures by incorporating twigs or plant fragments collected from the surrounding area (Cosarinsky and Roces, 2007; 2012). During the transport of material from the underground, workers engage in sequential soil transport (Pielström and Roces, 2013), similar to the aboveground transport chains observed in foraging leaf-cutting ants (Hubbell *et al.*, 1980; Röschard and Roces, 2011). While some ants excavate soil from the underground digging sites, other workers pick up the pellets and carry them towards the nest exits over more or less shorter distances, before they are dropped again (Pielström and Roces, 2013; Tschinkel *et al.*, 2015). Lastly, long-distance carriers transport the pellets all the way to the surface and deposit them outside the nest.

The ventilation turrets are constructed using mostly excavated material from the underground and they can only be found in the center of the nest mound. Therefore, it is tempting to assume that workers carrying soil pellets to the surface prefer the transport of material along tunnels leading to central nest openings in order to promote the construction of ventilation turrets there. We hypothesized that during soil transport, workers use the climatic variables inside the nest tunnels, i.e., airflow direction and carbon dioxide levels, as orientation cues in order to locate central nest openings. Workers might prefer the transport of pellets along tunnels providing outflow instead of inflow, and having high instead of low CO_2 levels. In addition, workers' choices for high or low carbon dioxide during soil transport might depend on the current need for ventilation in the nest, i.e., their preferences might be stronger, when the CO_2 levels inside the nest are higher, as increased turret building activity could counteract poor nest ventilation.

We tested preferences for environmental cues in leaf-cutting ants engaged in soil transport in the laboratory, by confronting single workers carrying excavated pellets with a choice between two opposing tunnel segments that provided either high vs. low carbon dioxide levels (first experiment), or inflow vs. outflow of air (second experiment). Additionally, in the first experiment workers were exposed to different CO_2 levels before the choice test, to simulate the effect of poor nest ventilation.

2. Methods

Experiments were carried out at the University of Würzburg, Germany, on a queenright laboratory colony of *Atta vollenweideri* founded 2004 in Formosa, Argentina. The colony was maintained under controlled conditions at ~25°C, ~50% air humidity, a 12h:12h LD cycle and fed daily with fresh leaves of blackberry (*Rubus fruticosus*). At the time of the experiments, the colony contained ca. 70 l fungus garden distributed to 19 plastic boxes (20 x 20 x 10 cm) interconnected with PVC tubes (Ø3 cm). Additionally, a plastic box (20 x 20 x 10 cm) partially filled with a mixture of clay and sand was connected to the colony. The mixture always consisted of two parts clay powder (CLAYTEC “Lehm gemahlen 10.001”, particle size: ≤ 0.5 mm) and one part sand (DORSILIT® 9 “Kristall II”, particle size: 0.6 – 1.2 mm) with a water content of 19% of the total mass. All ants from the colony gained access to the box for at least one hour prior to the experiments where they could freely excavate in order to establish a digging context in the colony. All loose material excavated by workers during this time was collected and used in the subsequent experiments, henceforth referred to as pellets. For the experiments, only workers engaged in either digging or transporting pellets were collected from the box within 15-20 min and immediately transferred to the setup as a group of workers that are more likely motivated to continue their excavation activity.

2.1. General setup

The rationale of the experiment was to investigate the transport of excavated soil pellets in *Atta vollenweideri* workers along a nest tunnel and to test whether climate-related parameters in the nest tunnel, namely carbon dioxide levels, airflow and humidity, affect the choice of workers for the pellet deposition. In our experimental setup we simulated an inclined nest tunnel connecting the underground part of the nest to the surface, by linking together multiple Plexiglas tube segments (Ø 3 cm) to form a ca. 40 cm long tunnel leading to an open plastic box (10 x 10 x 6 cm) for pellet deposition (Fig. 14a). In order to prevent ants from escaping the setup, the walls of the open box were coated in Fluon, while the bottom end of the nest tunnel was sealed using rubber stoppers. The bottom section of the nest tunnel was filled with ca. 30g of moistened pellets, previously excavated by ants of the same colony as mentioned before, in order to simulate a digging site in the underground.

At the beginning of each trial, a group of 50 workers previously engaged in digging or soil transport, was collected from the colony and transferred into the experimental setup. For 4-5 hours workers were allowed to freely move inside the setup and they could pick up and transport the offered pellets along the tunnel. During this time, some ants picked up a pellet from the pile and carried it along the tunnel towards the surface, i.e., into the open box for pellet deposition, where it was dropped.

A sliding segment in the center of the tunnel allowed the displacement of single ants that entered the tunnel segment carrying a soil pellet to a second Plexiglas tunnel located above the first one (Fig. 14b). During this transition, the first tunnel segment ([1]) was replaced by an equivalent tunnel segment ([2]), closing the gap and allowing the remaining workers in the setup to continue moving along the initial tunnel leading to the box for pellet deposition. At the end of the displaced tunnel either a Y-maze (experiment 1; Fig. 14c), or a T-maze (experiment 2; Fig. 14d) was placed, offering a two-choice situation for the transferred ant to continue the transport and deposit the pellet. Both mazes consisted of ca. 4.5 cm long side arms of the same material as used for the setup, arranged in either a 120° angle (Y-maze) or a 180° angle (T-maze). In the Y-maze both side arms ended in two open boxes for pellet deposition (10 x 10 x 6 cm), while in the T-maze they ended blindly. The two side arms offered different climatic conditions as a choice, depending on the experimental series. As soon as the transferred worker dropped the pellet in one of the two side arms or in the open boxes at their end, it was counted as decision and both ant and pellet were removed from the setup. If an ant dropped the pellet before or at the decision point, both were excluded from the data collection. Afterwards, another worker carrying a soil pellet along the nest tunnel was rerouted with the sliding tunnel segment as described, and tested in the same way. In order to prevent subsequent workers from following a potential pheromone trail laid by the previous ant along one of the side arms, the inner walls of the mazes were covered with a plastic foil, which was replaced after every individual.

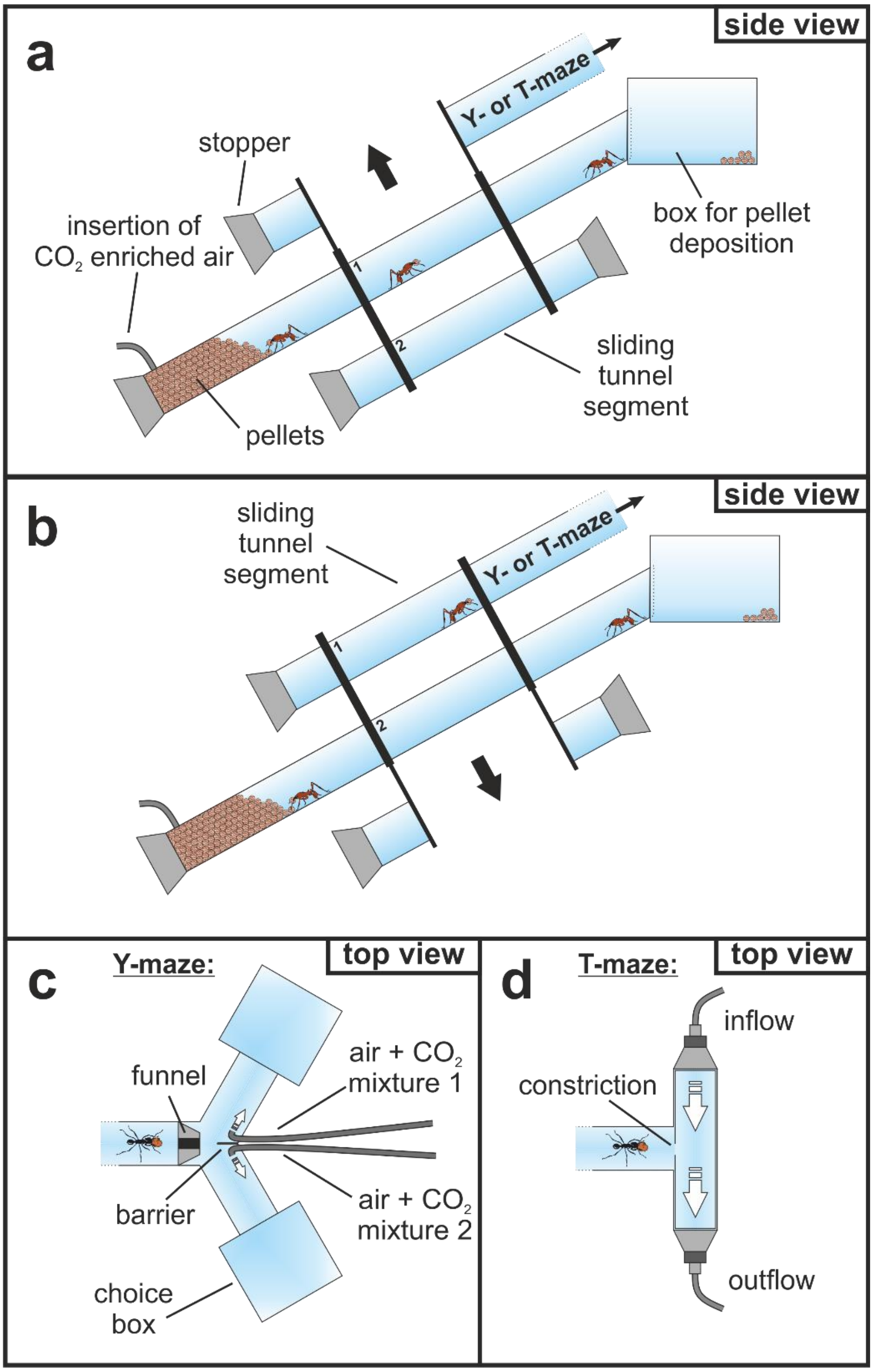


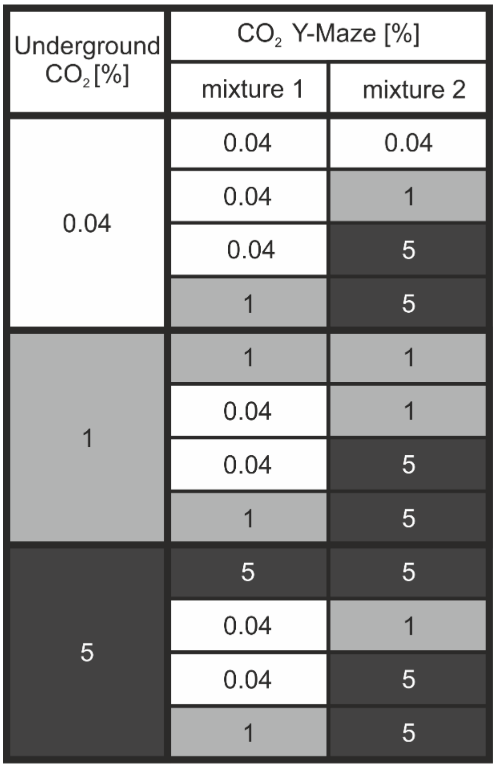
Figure 14: Experimental setup for the observation of soil transport in ant workers. a) An inclined nest tunnel was simulated in which a group of 50 workers was offered pellets in the underground. b) Single workers carrying pellets to the surface were rerouted to a second tunnel via a sliding segment and tested in a two-choice situation either for carbon dioxide preference in a Y-Maze (c) or for preference for airflow direction in a T-Maze (d).

2.2. Influence of CO_2 on pellet transport

In the first experiment, we investigated whether the carbon dioxide concentration in the underground affected the soil transport in leaf-cutting ants, i.e., whether workers prefer the transport of pellets along tunnels with certain CO_2 levels. Therefore we manipulated the carbon dioxide levels inside the experimental setup by inserting CO_2 enriched air via a small opening into the bottom part of the tunnel, where the pellets were offered, henceforth referred to as “underground” (Fig. 14a, b). For the workers’ choice, two different mixtures of air and carbon dioxide were inserted in each side arm of the Y-maze (Fig. 14c). In this way, we tested whether the carbon dioxide concentration in the underground affected the choice of individual workers later on during the test.

The gas mixtures were generated using compressed air and CO_2 from the laboratory lines regulated separately for each side with a multi-channel mass flow meter (Flow Bar 8 - Sable Systems) before being transferred to the setup via rubber tubes (Ø 3 mm) inserted in the center of the Y-maze. As a consequence, both gas mixtures were injected as close to the decision point as possible, one to the left and one to the right side of a separating barrier that was placed in the center of the Y-maze in order to prevent both gas mixtures from interfering with each other. Additionally, the openings of the tubes inside the Y-maze were aligned in a way that ensured the direction of the airflow from the insertion point towards the outer end of the tunnel, on both sides. The gas mixtures were injected at a rate of approximately 150 ml/min at the Y-maze, resulting in an airflow velocity of ca. 9 cm/s directly at the insertion point (Ø 3 mm). However, since the cross-section of the adjacent tunnel was much larger (Ø 20 mm), the airflow velocity on each side was lower than 1 cm/s and therefore unlikely to negatively affect the workers. In order to increase the probability of workers to perceive both gas concentrations in the Y-maze prior to their decision and prevent ants from choosing one side simply by walking along the tunnel wall, workers had to pass through a narrow funnel (Ø 0.5 cm) before entering the bifurcation.

We performed 12 experimental series using different combinations of carbon dioxide concentrations (Fig. 15), to simulate atmospheric levels of 0.04%, low CO_2 levels of 1% as encountered in well-ventilated ant nests, and high CO_2 levels of 5% as measured in poorly-ventilated nests (Kleineidam and Roces, 2000). For each CO_2 concentration, a control series was performed where workers were given the choice between the same levels on both sides of the Y-maze, i.e., 0.04% vs. 0.04% CO_2 when previously exposed to 0.04% in the underground, 1% vs. 1% CO_2 when previously exposed to 1% in the underground and 5% vs 5% CO_2 when previously exposed to 5% in the underground. In the remaining series workers were given the choice between two different concentrations, a lower and a higher one, at the Y-maze.



Underground CO_2 [%]	CO_2 Y-Maze [%]	
	mixture 1	mixture 2
0.04	0.04	0.04
	0.04	1
	0.04	5
	1	5
1	1	1
	0.04	1
	0.04	5
	1	5
5	5	5
	0.04	1
	0.04	5
	1	5

Figure 15: Overview of the 12 tested combinations of carbon dioxide in the first experiment. CO_2 levels were manipulated in the underground, where the pellets were offered (left column) and independently in both side arms of the Y-maze (middle, right column). Colors represent atmospheric values (0.04%, white), low (1%, grey) or high (5%, dark grey) carbon dioxide levels.

2.3. Influence of airflow properties on pellet transport

In the second experiment, we investigated whether the airflow properties in the tunnel, i.e., the direction and the humidity of the airflow, influenced the transport of soil pellets in *Atta vollenweideri*. In contrast to the first experiment, the CO_2 levels in the underground, where the pellets were offered, were invariant and maintained at atmospheric levels. Single ants carrying a pellet along the tunnel were again rerouted to the second tunnel and confronted with the choice between two opposing sides of a T-Maze, one providing inflow of air, the other one providing outflow of air (Fig. 14d). The airflow was generated using miniature vane pumps (Schwarzer Precision 135 FZ, power supply: Base-Tech BT-305) and transferred to a T-maze via rubber tubes (Ø 3 mm). In the series using high air humidity the airflow was humidified to approximately 70% RH by leading it first through a glass wash bottle and then to the T-maze. In the series using dry air, the humidity of the airflow corresponded with the humidity of the room air (ca. 30% RH). Similar to the first experiment, workers had to enter the side arms of the T-maze through a narrow constriction (Ø 0.5 mm) to prevent workers from choosing a side by simply walking along the tunnel wall.

Overall, we performed 6 series, using humid or dry air with a speed of 0, 5 or 10 cm/s to simulate no airflow, low or high airflow velocities, respectively. The series using 0 cm/s acted as a control group to test whether workers show a side bias when choosing one side of the T-maze. However, for better understanding, both sides are still referred to as “inflow” or “outflow” tunnel, although no air movements were present.

2.4. Statistical analysis

In both experiments, the distribution of workers’ choices in the Y- or the T-maze was compared for each series to a 1:1 random distribution using the G-Test of Goodness of fit (McDonald, 2014). In addition, we compared the series using airflow velocities of 10 cm/s for dry and humid air using the G-Test of independence (McDonald, 2014). The level of significance in all tests was $\alpha = 0.05$.

3. Results

Shortly after being transferred to the experimental setup, the ants discovered the offered pellets in the bottom segment of the tunnel and started to excavate and move material along the tunnel. Some ants grabbed a pellet, turned around and dropped it again a few centimeters away from the pick-up location, while others carried the pellet along the entire tunnel and deposited it in the open, “outside” box. Afterwards, those workers often returned to the pile of pellets in the “underground” in order to pick up another pellet and repeat the process, a common behavior of leaf-cutting ants engaged in tunnel excavation (personal observation).

3.1. Influence of CO_2 on pellet transport

Workers were first exposed to either 0.04%, 1% or 5% carbon dioxide in a tunnel partially filled with pellets and afterwards tested for CO_2 preference during soil transport to the surface. Workers’ choices for pellet deposition strongly differed between the series depending on the carbon dioxide levels in the underground as well as in the Y-maze (Fig. 16).

When exposed to atmospheric CO_2 levels in the underground, workers showed no preference during the test. Their choices for the left and the right side of Y-maze were not statistically different from a 1:1 distribution in all series (Fig. 16a). In the control series (0.04% vs. 0.04% CO_2), 55% of workers chose the left side and 45% chose the right side of the Y-maze. In the series 0.04% vs. 1% CO_2, 45% of the ants deposited their pellets at 0.04% and 55% of the ants chose the 1% side, while in the series 0.04% vs. 5% CO_2 exactly 50% of the workers dropped their pellet on either side. When offered the choice between 1% and 5% CO_2, 40% of the ants transported their load along the tunnel containing the lower concentration and 60% chose the higher concentration.

When workers were exposed to a low CO_2 concentration of 1% in the underground, they again showed no preference for of the two sides of the Y-maze for the control series (1% vs. 1% CO_2), as 45% chose the left and 55% chose the right side for pellet deposition (Fig. 16b). Similarly, ants showed no preference for certain carbon dioxide levels both in the series using 0.04% vs. 1% CO_2 (50% vs. 50% of workers) and 0.04% vs. 5% CO_2 (45% vs. 50% of workers). However, when offered the choice between 1% and 5% CO_2 significantly more ants (75%) transported their pellet along the tunnel containing 5% CO_2 compared to 1% CO_2 (25% of workers).

When the group of ants was exposed to high carbon dioxide levels of 5% in the underground, there again was no difference in the distribution of workers’ choices for the control series (5% vs. 5% CO_2), as 55% chose the left and 45% chose the right side of the Y-maze (Fig. 16c).

However, in all other series, significantly more workers transported their load along the tunnel containing the higher CO_2 concentration. In the series 0.04% vs. 1%, 25% of the ants chose the lower concentration and 75% the higher concentration. For the series 0.04% vs. 5% CO_2, only 10% of the ants chose atmospheric levels and 90% of the ants carried the pellet along the tunnel containing 5% CO_2. When offered the choice between 1% and 5% CO_2, the majority of workers (80%) deposited their pellet on the side with 5% CO_2 and only 20% chose the side containing 1% CO_2. Overall, whenever workers showed a preference, they always preferred the transport of pellets along tunnels containing the highest carbon dioxide levels offered as choice, and this only happened if the CO_2 levels they experienced in the underground were higher than the atmospheric ones.

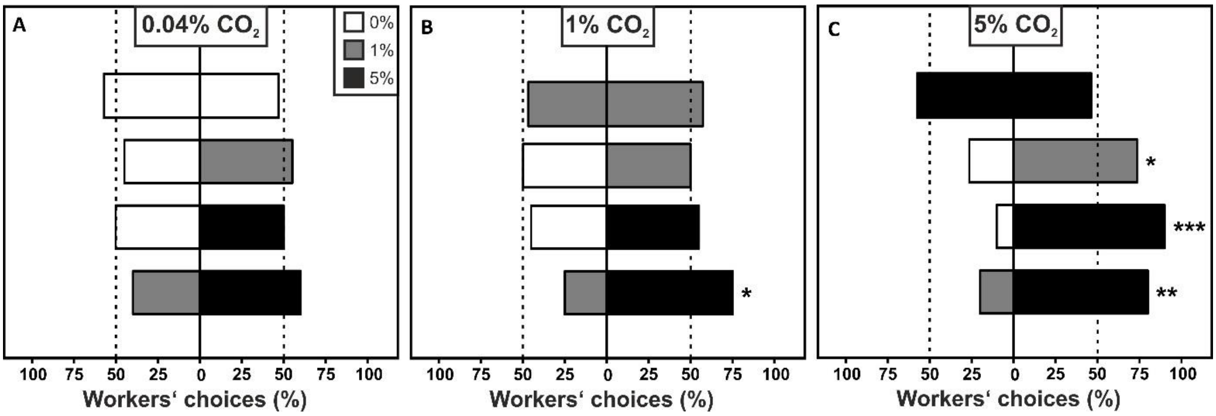


Figure 16: Distribution of workers' choices for carbon dioxide levels during pellet deposition. Workers were exposed to a) 0.04%, b) 1% or c) 5% CO_2 in the underground. Colors indicate the different CO_2 levels tested in the Y-maze, with the lower concentration of the pair depicted on the left, and the higher concentration on the right side of the x-axis. The legend is valid for all three graphs. Each series comprised n = 20 workers. Dashed lines indicate 50% values for no preference. Asterisks indicate statistically significant differences ($p < 0.05$) compared to a 1:1 random distribution. Detailed statistics can be found in A10.

3.2. Influence of airflow properties on pellet transport

We confronted workers carrying soil pellets with the choice between two tunnels, providing either inflow or outflow of either dry (30% RH) or humid air (70% RH) with a velocity of 0, 5 or 10 cm/s.

Workers' choices for pellet deposition were dependent on both airflow velocity and air humidity and differed between the series (Fig. 17).

When humidified air was used, workers showed no preference for airflow direction in the control series (0 cm/s), as 57% of workers chose the inflow side and 43% chose the outflow side, but significantly preferred outflowing air rather than inflowing air for 5 cm/s (27% vs. 73%, respectively) during soil transport (Fig. 17a). At the highest airflow velocity tested, ants showed a marginally significant tendency to deposit their pellet on the outflow side (67%) instead of the inflow side (33%).

When dry air was used, workers again showed no preference for airflow direction in the series using 0 cm/s (control), as 50% of the ants chose the inflow side and 50% chose the outflow side of the T-maze (Fig. 17b). At low airflow velocities of 5 cm/s, workers significantly preferred the outflow side (70%) over the inflow side (30%). However, when airflow velocity was increased to 10 cm/s, workers instead showed a marginally significant tendency to deposit their pellet in the inflow tunnel (67%) instead of the outflow tunnel (33%). This result was in contrast to the series using humid air at 10 cm/s, in which workers tended to prefer the outflow side. Comparing the choices of workers at 10 cm/s for dry air vs. humid air revealed a significant difference between the airflow preferences (G = 6.80, p = 0.009).

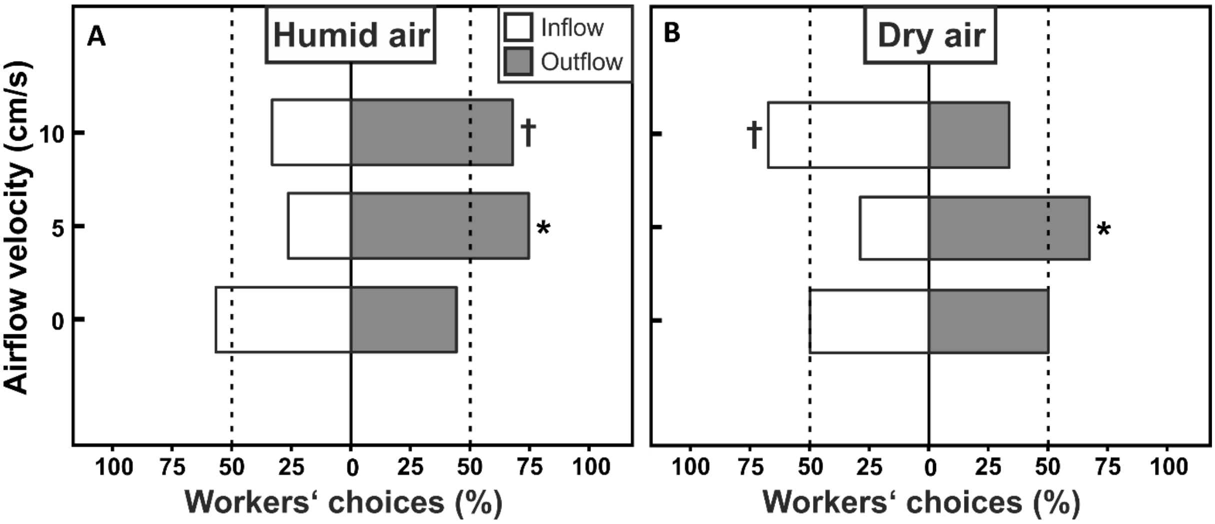


Figure 17: Distribution of workers’ choices for inflow and outflow during pellet deposition depending on airflow velocity, using a) humid air of approximately 70% RH or b) dry air of approximately 30% RH. The legend is valid for both graphs. Each series comprised n = 30 workers. Dashed lines indicate 50% values for no preference. Asterisks indicate statistically significant differences ($p < 0.05$), daggers indicate marginally significant tendencies ($p < 0.1$) compared to a 1:1 random distribution. Detailed statistics can be found in A11.

4. Discussion

4.1. Influence of CO_2 on pellet transport

Our results provide evidence that workers of *Atta vollenweideri* use the climatic conditions inside the nest tunnels for orientation during the transport of excavated soil from the underground to the surface. Workers preferred the transport of pellets along tunnels containing higher instead of lower CO_2 levels, when the concentration of carbon dioxide at the underground pile of pellets was elevated as well. However, when exposed to atmospheric CO_2 levels, workers did not prefer any particular concentration later on during the test, but rather randomly deposited their pellet on either one of the sides of the Y-maze. This indicates that the preference of workers for tunnels containing high carbon dioxide levels depends on the current concentration of carbon dioxide in the underground, which may be an indicator of the demands for ventilation in the nest. In well ventilated *Atta vollenweideri* nests, the CO_2 levels in the underground usually range between 1-2%, (Kleineidam and Roces, 2000), and ants are expected to have adapted to slightly elevated CO_2 levels during evolution. Atmospheric (0.04%) or slightly elevated carbon dioxide levels (1%) in the underground therefore should imply sufficient gas exchange with the environment and a functioning nest ventilation system. However, exposure of workers to higher CO_2 levels (5%) in the underground should indicate poor nest ventilation, as carbon dioxide levels in field nests may reach values up to 5.7%, when nest openings are closed (Kleineidam and Roces, 2000). An overall increase in CO_2 due to poor nest ventilation should elevate the CO_2 levels primarily in tunnels where air leaves the nest, but to a lesser extent in tunnels where fresh air enters the nest (Bollazzi *et al.*, 2012). Thus, a preference for the transport of soil pellets along tunnels containing high carbon dioxide concentration might ensure that more excavated material is deposited around the central outflow openings, potentially facilitating the wind-induced ventilation of the nest. By heaping up soil around a tunnel at the surface, the nest opening is further elevated relative to the ground and thereby exposed to higher surface wind speeds. This mechanism, based on the Bernoulli principle and viscous entrainment (Vogel and Bretz, 1972; Kleineidam *et al.*, 2001), is the driving force behind the ventilation of the nests of many burrowing animals, including moles (Olszewski and Skoczen, 1965), prairie-dogs (Vogel *et al.*, 1973) or termites (Weir, 1973). For the leaf-cutting ant *Atta capiguara* it has been shown that nest openings elevated by heaps of soil serve as outflow openings while openings on the ground level serve as inflow openings or contain no airflow (Bollazzi *et al.*, 2012). Removing the heaps of soil around nest openings also influences the airflow conditions inside the nest tunnels and placing the soil heap around inflow openings reverses the airflow direction accordingly.

It remains an open question whether the deposition of pellets around outflow openings also facilitates the construction or modification of ventilation turrets that can be found on top of *Atta vollenweideri* nests, since their construction results not only from a passive deposition of soil, but rather from a particular arrangement of soil pellets. In the field, workers are known to actively import building material from the surrounding area to support their construction (Cosarinsky and Roces, 2007; 2012). Thus, the soil pellets used for the construction of ventilation turrets might not only be the result of pellet transport along outflow tunnels from the underground, but also from the deposition of imported material predominantly around outflow openings. In fact, workers importing soil pellets prefer to deposit them around openings where air leaves the nest, not where air enters the nest (Oppermann, 2011). Apart from that, the factors that trigger the construction of turrets in leaf-cutting ants are still largely unknown. Reports indicate that colonies of *Atta vollenweideri* show an increased turret building activity predominantly after heavy precipitation (Jonkman, 1980b). In addition, colonies usually close the majority of their nest openings during rain (Jonkman, 1980b), possibly to avoid water inflow into the nest, a common risk for nests of this species (Pielström and Roces, 2014). The closure of all nest openings as observed in small field nests, however, results in an increase of the underground CO_2 concentration (Kleineidam and Roces, 2000), and workers are expected to open them soon afterwards to leave for foraging and to allow air exchanges with the environment. Whether the increased turret building activity after heavy rains is linked to the high demand for nest ventilation after nest closure or simply results from the improved workability of wet soil remains elusive, so far.

It is important to note that the preference of workers for high carbon dioxide levels during the soil transport is not necessarily related to the total amount of material carried to the surface. In fact, as shown in Chapter III, exposure of workers to CO_2 concentrations up to 6% during excavation caused no increase in the amount of excavated material transported out of the digging arena. In addition, workers showed no preference for higher carbon dioxide levels, but instead preferred lower concentrations during the excavation in the nest. This indicates that CO_2 might only modify the ants' preferences without acting as an attractant for workers of *Atta vollenweideri*, in contrast to the findings on, for example, fire ants (Wilson, 1962; Hangartner, 1969). However, since we tested the same number of workers for all series and only recorded their carbon dioxide preferences, the results obtained in the first experiment do not allow us to draw any further conclusions concerning the total number of workers engaged in soil transport or the amount of soil pellets transported to the surface.

4.2. Influence of airflow properties on pellet transport

Apart from the carbon dioxide concentration, workers also were able to use the direction of the airflow in the tunnels as orientation cue during the transport of pellets from the underground to the surface. Ants carrying pellets preferred the transport along tunnels providing outflow of air, instead of inflow, for both dry and humid air at a velocity of 5 cm/s. Due to the clear separation of inflow and outflow channels in *Atta vollenweideri* nests (Kleineidam *et al.*, 2001), the air currents in the nest should provide a largely reliable orientation cue for the ants in the underground. A recent study has shown that workers are not only highly sensitive to air movements down to 2-5 cm/s, but also use their direction for anemotactic orientation during excavation in the nest (Halboth and Roces, 2017). Consequently, it is most likely that soil carrying workers are able to differentiate between tunnels leading to central or peripheral openings on the nest mound based on the directional information provided by the air movements inside the tunnel. After the deposition of their load, leaf-cutting ants often return to the digging site in order to pick up the next pellet and repeat the process (personal observation). Therefore, airflow directions in the tunnel might also help unloaded workers orient when returning to the digging site in the underground. As shown in Chapter II, foraging leaf-cutting ants can learn the location of a food source using the prevailing airflow conditions for orientation, at least for airflow velocities of 10 cm/s. This was true for ants walking both upwind as well as downwind. Consequently, foragers should be able use the direction of the airflow in peripheral nest tunnels for orientation when exiting or returning to the nest. In a similar manner, workers carrying soil pellets should encounter outflow of air when leaving the nest through central tunnels or experience reversed airflow directions when returning to the digging sites.

It is important to note that while for humid air (70% RH) workers tended to prefer the transport along outflow tunnels, their preference shifted to a tendency towards inflow tunnels when dry air (30% RH) with a velocity of 10 cm/s was used. Since the symbiotic fungus in the nest is prone to desiccation and workers show strong preferences for the rearing of their fungus at humidity levels close to saturation (Roces and Kleineidam, 2000), leaf-cutting ants are often faced with a trade-off between nest ventilation and humidity control. Colonies of *Acromyrmex ambiguus* build their nests in superficial soil layers and use plant fragments to plug their nest tunnels only when dry air enters the nest, not humid air (Bollazzi and Roces, 2007). In the thatch-building ant *Acromyrmex heyeri*, outflow of humid air triggers building responses, as workers try to seal the leak (Bollazzi and Roces, 2010a). For *Atta vollenweideri*, it is known that colonies often close their nest entrances during the winter months, i.e., the dry season (Jonkman, 1980b; c). Therefore, inflow of dry air into the nest might result in workers transporting excavated soil preferably along tunnels leading to openings located at the periphery of the nest mound in order to close the opening and prevent humidity loss in the nest. Interestingly, inflow of dry air at 5 cm/s did not elicit the same response in workers, instead they preferred the transport of soil along the outflow tunnels. This can be explained by the reduced risk of humidity loss from the nest at lower velocities, since the evaporation rate of water is positively correlated with airflow velocity (Pauken, 1999).

In general, the response of workers was less univocal for airflow velocities of 10 cm/s compared to 5 cm/s, i.e., ants only showed marginally significant tendencies for certain airflow directions. Measurements in well-ventilated nests of *Atta vollenweideri* have shown average airflow velocities of 1.25 to 4.62 cm/s with maximum values of ca. 8 cm/s, depending on surface wind speed (Kleineidam *et al.*, 2001). While both airflow velocities in our experiment (5 and 10 cm/s) should indicate a functioning ventilation system, values of 10 cm/s might already be beyond the response threshold of workers for building responses, thus preventing workers from depositing further material around the outflow openings, which otherwise may result in even higher airflow velocities. In this line of argument, it would be interesting to explore whether workers of *Atta vollenweideri* also show specific building responses such as the closure of nest openings, when the airflow in the nest is too strong, thereby actively obstructing the wind-induced ventilation in the nest.

V. Construction of ventilation turrets: Carbon dioxide levels in the nest tunnels, but not airflow or air humidity, influence turret structure

Abstract

Nest ventilation in the leaf-cutting ant *Atta vollenweideri* is driven via a wind-induced mechanism. On their nests, workers construct small turrets that are expected to facilitate nest ventilation. We hypothesized that the construction and structural features of the turrets would depend on the colony's current demands for ventilation and thus might be influenced by the prevailing environmental conditions inside the nest. Therefore, we tested whether climate-related parameters, namely airflow, air humidity and CO_2 levels in the outflowing nest air influenced turret construction in *Atta vollenweideri*. In the laboratory, we simulated a semi-natural nest arrangement with fungus chambers, a central ventilation tunnel providing outflow of air and an aboveground building arena for turret construction. In independent series, different climatic conditions inside the ventilation tunnel were experimentally generated, and after 24 hours, several features of the built turret were quantified, i.e., mass, height, number and surface area (aperture) of turret openings. Turret mass and height were similar in all experiments even when no airflow was provided in the ventilation tunnel. However, elevated CO_2 levels led to the construction of a turret with several minor openings and a larger total aperture. This effect was statistically significant at higher CO_2 levels of 5% and 10% but not at 1% CO_2. The construction of a turret with several minor openings did not depend on the strong differences in CO_2 levels between the outflowing and the outside air, since workers also built permeated turrets even when the CO_2 levels inside and outside were both similarly high. We propose that the construction of turrets with several openings and larger opening surface area might facilitate the removal of CO_2 from the underground nest structure and could therefore be involved in the control of nest climate in leaf-cutting ants.

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1. Introduction

The construction of underground nests provide animals with protection against predators and unfavorable climatic conditions, yet compromise the air exchanges between the nest environment and the atmosphere. To cope with such reduced air exchanges, two kinds of adaptive responses of the inhabitants are conceivable: first, a physiological adaptation, such as an increased tolerance to hypercapnic and hypoxic conditions, i.e., to high CO_2 and to low O_2 levels, respectively. And second, a behavioral adaptation, such as responses that help ventilating the nest via active or passive mechanisms, like fanning in bumblebees (Weidenmüller *et al.*, 2002) or the construction of specific ventilatory nest structures (Hansell, 2005).

Leaf-cutting ants of the genus *Atta* offer a particularly interesting model system for the study of such adaptations, since their nests are among the largest structures found in the animal kingdom. Depending on the species, leaf-cutting ant nests consist of a vast number of underground tunnels and chambers (Moreira *et al.*, 2004a; b), in which the ants rear a symbiotic fungus as main food source for their brood. The fungus has strict demands of high humidity close to saturation and temperatures between 25°C and 30°C (Quinlan and Cherrett, 1978), and deviations from those ranges might be detrimental for fungal growth (Powell and Stradling, 1986). Colonies are able to maintain suitable microclimatic conditions inside the nest in two different ways, i.e., short-term behavioral responses and long-term modifications of the nest architecture.

Short-term behavioral reactions to unfavorable microclimatic conditions in leaf-cutting ants may include the collection of water in order to increase nest humidity (Ribeiro and Navas, 2008) or the relocation of brood or fungus along temperature gradients in the nest (Bollazzi and Roces, 2002), a behavior also found in other ant species (Roces and Núñez, 1989). In the long-term, workers might modify existing underground structures, i.e., enlarge tunnels or chambers, or excavate new ones when the current climatic conditions inside the nest are suboptimal. In *Acromyrmex lundii*, workers stop digging when soil temperature is lower than 20°C or rises above 30°C, and shift their digging activity to locations with more suitable temperatures (Bollazzi *et al.*, 2008). This mechanism is likely to be involved in the determination of nest depth as leaf-cutting ants tend to avoid superficial soil layers and prefer to excavate their nests in deeper and cooler layers.

However, nesting in deeper soil layers might also entail disadvantages, as the carbon dioxide concentrations in the underground drastically increase with increasing depth (Reardon *et al.*, 1979). Additionally, large amounts of carbon dioxide are produced underground due to the respiration of the colony and the decomposition of organic matter in the nest, and recent studies characterized the contributions of ant communities to the CO_2 efflux from subterranean nests to the atmosphere, not only for leaf-cutting ants (Sousa-Souto *et al.*, 2012), but for other ant species as well (Hasin *et al.*, 2014). Measurements in the field showed that, depending on soil properties like moisture and porosity, carbon dioxide concentrations in ant nests can reach values of 0.2% in *Pogonomyrmex badius* (Tschinkel, 2004), about 1.5 to 4.5% in *Atta capiguara* or *Atta laevigata* (Bollazzi *et al.*, 2012) and up to 5.7% in *Atta vollenweideri* (Kleineidam and Roces, 2000), and far exceed the atmospheric levels of currently 0.04% CO_2. Especially for *Atta vollenweideri*, a species native to the clay-heavy soils of the Gran Chaco region in South America, the elevated carbon dioxide levels in the nest pose a major problem. Clay-heavy soils show low porosity and low air permeability (Currie, 1984), which hinders the removal of CO_2 from underground chambers and the supply of the nest with oxygen via diffusion with the surrounding soil. To facilitate gas exchanges, nests of *Atta vollenweideri* rely on a wind-induced ventilation mechanism taking advantage of the Bernoulli principle (Kleineidam *et al.*, 2001), similar to that involved in the ventilation of prairie dogs' burrows (Vogel *et al.*, 1973). The nests possess an aboveground nest mound permeated with up to 200 nest openings that are not all used as exits or entrances by the ants (Jonkman, 1980b; c). Inflow and outflow of air through the nest openings depend on their location on the nest mound. Surface wind is dragging air out of central tunnels, followed by an inflow of air at the periphery (Kleineidam *et al.*, 2001). On top of central nest openings, the ants construct conspicuous turrets that are expected to enhance nest ventilation by elevating the tunnel opening and exposing them to greater wind velocities (Fig. 18a, b).

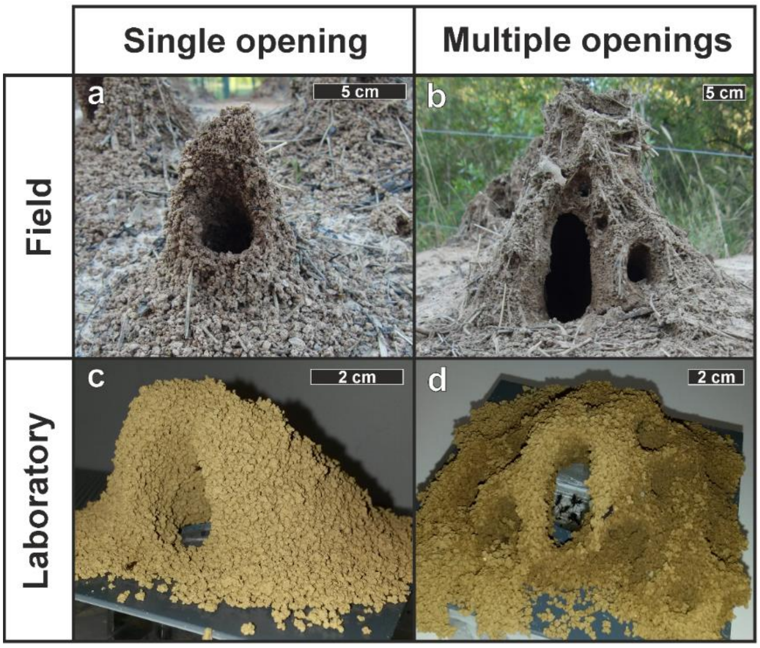

Figure 18: Ventilation turrets of *Atta vollenweideri*. Top row: Ventilation turrets of field nests (Formosa, Argentina) with a) one single opening and b) multiple openings. Bottom row: turrets constructed in a laboratory colony with c) one single opening and d) multiple openings. Note the different scale bars.

Most of the building material used for the construction of turrets originates from the excavation of nest structures in the underground. Workers carry soil pellets from underground digging sites to the surface and deposit them around the nest openings, resulting in the formation of crater-like soil heaps, a common feature of ant nests in warmer climates (Sudd, 1982). Additionally, workers rearrange the soil pellets and import building material like twigs and grasses from the immediate vicinity to form such particular structures (Cosarinsky and Roces, 2007; 2012). The variables that lead to turret construction and influence the turret shape, however, are still largely unknown. Jonkman observed an increase in turret height on *Atta vollenweideri* nests after heavy precipitation, and also the closure of some turret openings during rain or during the winter months (Jonkman, 1980b), indicating that turret construction is influenced by environmental conditions.

We hypothesized that the ants' building behavior and the resulting structure of the turrets would depend on the colony's current ventilation demands and thus might be influenced by the prevailing climatic conditions inside the nest. In the present study, we investigated turret-building behavior in the leaf-cutting ant *Atta vollenweideri* and the influence of climate-related parameters, i.e., airflow, air humidity and carbon dioxide levels in the outflowing nest air on the construction and structural features of the ventilation turrets.

2. Methods

Experiments were carried out at the University of Würzburg, Germany from January to April 2015 and January to March 2016 using a laboratory colony of *Atta vollenweideri* founded in the year 2004 in Formosa, Argentina. The colony was kept under controlled conditions at 24.7 ± 0.5°C, 40 ± 5% air humidity and a 12h:12h LD cycle, and fed daily with fresh leaves of blackberry (*Rubus fruticosus*) and dog rose (*Rosa canina*). At the time of the experiments, the colony contained ca. 60-70 l fungus garden, distributed in 16 (at the end 19) fungus chambers made of plastic boxes. For data collection, individual fungus chambers were detached from the colony and connected to the experimental setup. Experiments were performed simultaneously on two setups containing four fungus chambers each. Since we used only one colony, both setups were located on opposite sides and connected to the same foraging arena. However, the two setups were treated as independent replicates, since we never observed the exchange of building material between them. In between experimental trials, the position of two of the four fungus chambers used in each setup was exchanged and in addition, one of the chambers was replaced by another fungus chamber from the main colony on a weekly basis to randomize sampling during data collection. Overall, we used 18 fungus chambers arranged in 16 different configurations. The two setups were connected with the main colony via PVC tubes during weekends, in order to allow the exchange of workers within the colony.

2.1. General setup

The rationale of this study was to investigate turret construction and the influence of different environmental conditions inside the nest tunnels on the colony's building response. We performed different experimental series focusing on some of the most important environmental variables prevailing in *Atta vollenweideri* leaf-cutting ant nests, i.e., the presence or absence of airflow in the nest tunnels, and both the relative humidity and the carbon dioxide concentration of the air leaving the nest.

By performing experiments in the laboratory, we were able to manipulate one parameter at a time without changing the others. We used an experimental setup (Fig. 19) that simulated the spatial arrangement of compartments observed in field nests, i.e., an underground area with fungus and waste chambers connected to the surface by tunnels that are either used by foragers as exits/entrances or serve ventilation purposes (Jonkman, 1980b). A similar setup has already proven suitable for the observation of turret construction in the studied species (Cosarinsky and Roces, 2012).

The experimental nest consisted of four fungus chambers (20 x 20 x 10 cm) and a waste chamber (20 x 10 x 10 cm) interconnected by transparent PVC tubes (Ø 3 cm) as depicted in Figure 19. A foraging arena was available at a distance of 120 cm, where the colony was provided daily with water and fresh leaves. Approximately 30 cm away from the nest a vertical plastic tube (Ø 3.3 cm, h = 15 cm) led to an open building arena (65 x 45 x 12 cm), simulating a vertical nest tunnel leading to the surface. The walls of the building arena were coated with Fluon® to prevent the ants from escaping. Inside the arena 300 g of building material was offered to the ants, consisting of a 2:1 mixture of clay powder (CLAYTEC "Lehm gemahlen 10.001", particle size: ≤ 0.5 mm) and sand (DORSILIT® 9 "Kristall II", particle size: 0.6 – 1.2 mm) with a water content of 19% of the total mass. In order to prevent desiccation, the material was kept in a closed plastic box (10 x 10 x 9 cm) accessible to the ants only through a small opening in the front (Ø 2 cm). A building platform (12 x 12 x 0.1 cm) was placed on top of the nest opening to allow the removal of the constructed turret at the end of the trial without damaging its structure.

At the beginning of each trial, access to the building arena was given to the colony and all ants could freely move across the setup for the entire duration of the trial. After a short time, workers started to excavate small particles from the offered clay mixture and deposited the pellets around the tunnel opening, ultimately leading to the construction of a dome-shaped turret on the building platform. After 24 hours, the turret was removed and its height and the number of openings quantified. Opening aperture was estimated by measuring its diameter horizontally (d_h) and vertically (d_v), and by calculating opening surface using the formula for the area of an ellipse: $A = \pi \times \frac{dh}{2} \times \frac{dv}{2}$

Total turret aperture was calculated as the sum of the aperture of all turret openings. Additionally, turret mass was measured to the nearest 0.1 g using precision scales (Mettler PM3000) after overnight drying in an oven.

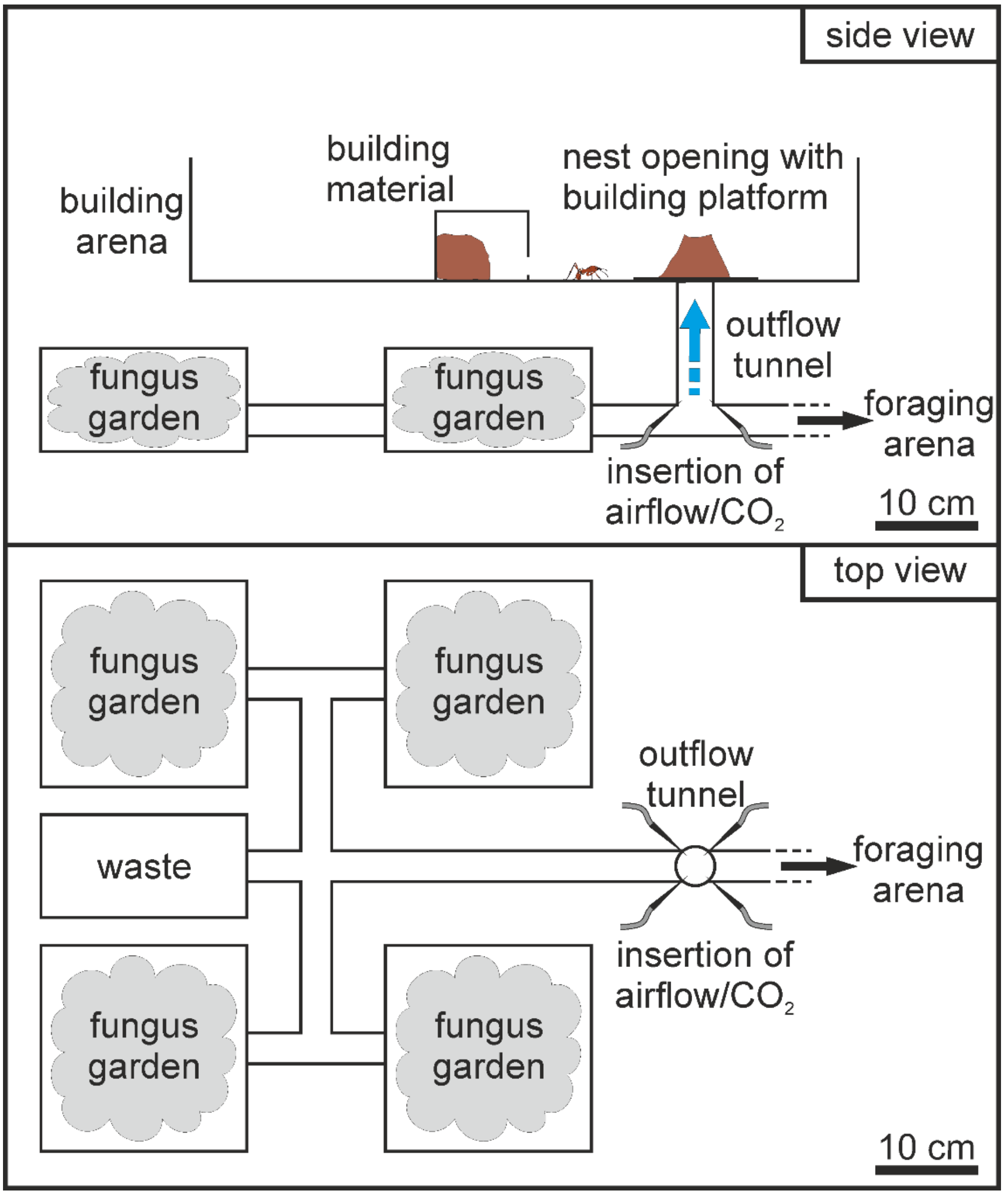

Figure 19: Experimental setup for the investigation of turret construction at an opening located above the nest. Side view: The nest was connected to a vertical tube leading to a building arena where a mixture of clay and sand was offered as building material. Inside the tube, an airflow with high or low humidity, and with varying levels of carbon dioxide depending on the experiments, was generated to investigate the effect of different environmental conditions in the outflowing nest air on the structure of the turret to be constructed on the building platform. Top view: Waste and fungus chambers simulating the natural arrangement of different nest compartments connected to an outflow tunnel, as observed in field nests. Note: The building arena is not depicted for the sake of clarity.

2.2. Turrets constructed on nest openings containing no airflow, humid or dry air

In the first experiment, we tested whether the presence or absence of outflowing air in a nest tunnel, and its humidity, influence the construction and structure of ventilation turrets. In order to simulate an outflow tunnel, airflow inside the vertical tube was generated using compressed air from the laboratory line and adjusted to 2500 ml/min (0.15 m^3/h) using a mass flow meter (Flow Bar 8 - Sable Systems). The airflow was directed to the experimental setup via rubber tubes (Ø 3 mm) ending in pipette tips that were inserted into the bottom part of the vertical tunnel (Fig. 19), thus creating an outflow of air from the nest with a velocity of ca. 5 cm/s, close to the values measured in outflow tunnels of field nests (Kleineidam *et al.*, 2001). Depending on the series the airflow was humidified by leading it first into a glass wash bottle and then into the vertical tunnel. Air humidity inside the vertical tunnel was measured prior to the trials with a thermo-hygrometer (range: 10-99%), and the adjusted airflow velocity was checked with an anemometer (Testo 405-v1, range 0-5 m/s, resolution: 0.01 m/s). Three series were conducted that differed in the velocity and humidity of the airflow present in the vertical outflow tunnel. In the first series, no airflow was added to the nest tunnel, and air humidity in the nest was kept high (0 cm/s, 80% RH). In the second series, an outflow of humid air (5 cm/s, 80% RH) was generated inside the nest tunnel, in order to test the effect of the airflow itself as compared to the previous series. In the third series, we tested the effect of reduced air humidity by generating an outflow of dry air (5 cm/s, 40% RH) in the tunnel.

2.3. Turrets constructed on nest openings with outflowing air containing different carbon dioxide levels

In the second experiment, we tested whether different carbon dioxide concentrations inside the nest tunnel, as may occur because of varying degrees of nest ventilation, affect the workers building behavior and the resulting turret structure. While lower carbon dioxide levels of 1-2% are common in shallow soil layers and in well-ventilated underground nests, higher levels around 6% usually indicate poor nest ventilation (Kleineidam and Roces, 2000). In order to simulate variable demands for nest ventilation, carbon dioxide was added to the outflowing air in different concentrations, depending on the experimental series, via the laboratory gas line. The concentration was regulated with a mass flow meter as in the first experiment. The mixture of air and CO_2 was humidified with the help of glass wash bottles to reach approximately 80% relative humidity. Prior to the experiments, the concentration inside the outflow tunnel was measured using a hand-held carbon dioxide meter (Vaisala

CARBOCAP® GM70). Even though the airflow containing carbon dioxide was inserted in the nest at the bottom part of the vertical tube (Fig. 19), no increase of carbon dioxide could be detected in adjacent tunnels or other compartments of the nest, i.e., CO_2-enriched air could only be perceived by workers either inside the vertical tube or close to the tube opening. We conducted three different series, all providing outflow of humid air (5 cm/s, 80% RH) yet with different levels of carbon dioxide, i.e., 1% CO_2, 5% CO_2 and 10% CO_2. One of the previous series from the first experiment was taken into account for comparisons, since it investigated the effect of atmospheric levels of carbon dioxide (ca. 0.04%) at the same conditions (airflow of 5 cm/s, 80% RH).

2.4. **Turrets constructed on nest openings with outflowing air containing similar carbon dioxide levels to those of the outside air**

In the previous experiment, ants constructed turrets with multiple openings when the outflowing air contained high CO_2 levels, as it will be presented in the Results. Therefore we investigated whether the construction of turrets with several minor openings depended on the strong differences in CO_2 levels between the inside and the outside of the nest tunnel experienced by workers. In order to investigate whether a CO_2 gradient is necessary for the construction of turrets with several openings, we performed four different series using the same setup as previously described. In the first two series, we provided outflowing air in the vertical nest tunnel with either atmospheric or 5% CO_2-levels using an open building arena (Fig. 20, left column), similar to the conditions of the previous experiment. In the third and fourth series (Fig. 20, right column), we removed the CO_2 gradient between the inside and the outside of the tunnel by covering the building arena with a Plexiglas plate (50 x 70 cm). This method allowed for a homogenous distribution of carbon dioxide in the whole arena. The third series provided outflow of atmospheric air in the closed arena (Fig. 20, top right) and served therefore as a control for a potential effect of the cover on building responses. The fourth series provided outflow of 5% CO_2 (Fig. 20, bottom right) and no gradient between the inside and outside of the tunnel because of the cover. The carbon dioxide concentrations inside the building arena were measured for all four series prior to the experiments at different measuring points in a distance of ca. 3 cm around the tunnel opening (Fig. 20). For the series using closed arenas, small holes, drilled in the cover of the building arena and closed during the actual trials, allowed the insertion of the hand-held carbon dioxide meter.

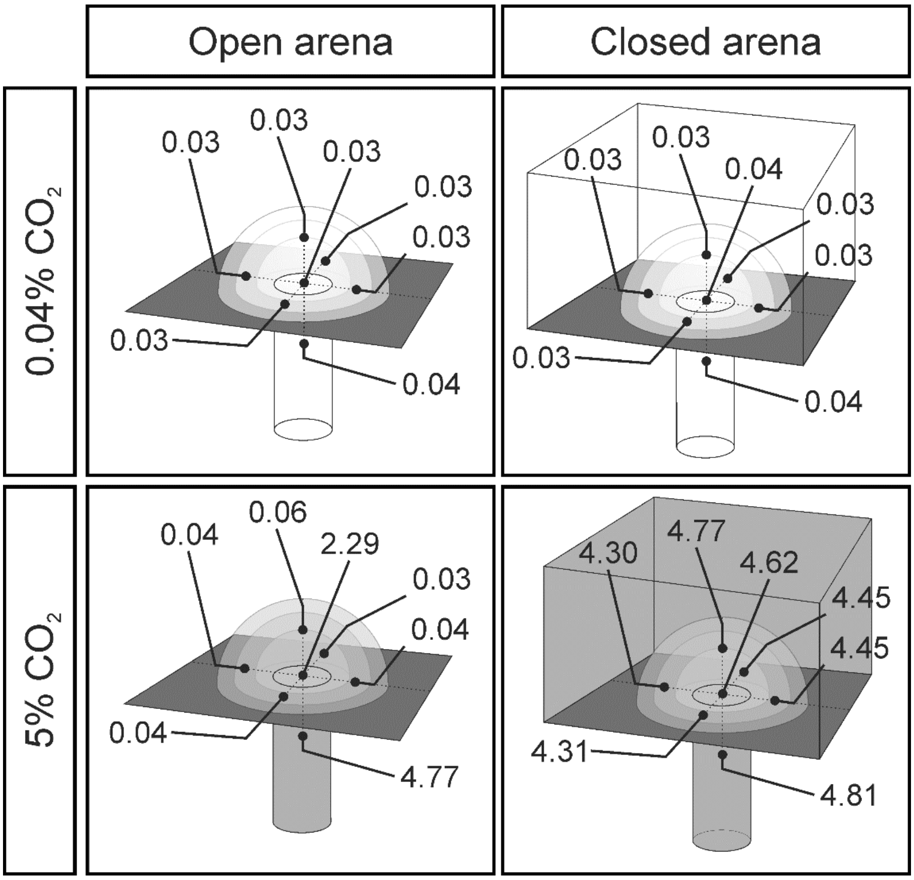

Figure 20: Schematic drawing of the experimental series in the third experiment. Indicated is the carbon dioxide gradient at the building platform (rectangle) on top of the outflow nest tunnel (vertical tube). Black dots represent measuring points and the recorded mean CO_2 levels (%), when gradients where either present (open arena, left column) or absent (closed arena, right column), both for the series using atmospheric (0.04%) or elevated CO_2 levels (5%) in the outflowing air (top and bottom rows, respectively).

2.5. Statistical analysis

All data was tested for normality using the Shapiro-Wilk normality test and consequently analyzed using either ANOVA followed by Tukey’s multiple comparison test (post-hoc) or Kruskal-Wallis test followed by Dunn’s multiple comparison test (post-hoc). Accordingly, either mean ± SD or median ± IQR values are presented for normally distributed or non-normally distributed data, respectively. However, for uniformity purposes, most results are graphically shown as boxplots representing median (horizontal bars), 25-75% percentiles (boxes) and min-max values (whiskers). The significance level was set to $\alpha = 0.05$ for all tests.

3. Results

Soon after allowing ants to enter the building arena they discovered the offered building material and initiated turret construction, i.e., workers started excavating small pellets from the clay/sand mixture that were subsequently transported, by themselves or other workers, to the building platform and placed around the nest opening. Initially, a ring of pellets was formed around the nest opening, which was later elevated to a conically-shaped structure containing a central opening, usually 2-3 cm in diameter. As the structure grew, however, the opening sometimes shifted laterally or several smaller openings emerged at the turret walls, depending on the series (Fig. 18c, d). Experiments were finished after 24 hours, since the ants' building activity gradually decreased and less workers were observed incorporating pellets into the turret. Preliminary observations of turret construction over 48 or 72 hours showed only slight increases in turret size as compared to that reached after 24 hours, and sporadic deposition of dead fungus and unsuitable plant material on the building arena.

3.1. Turrets constructed on nest openings containing no airflow, humid or dry air

The overall building activity of the ants was roughly the same for all series performed, resulting in turrets of similar size and shape. Neither airflow, nor humidty affected the turret mass (Fig. 21a). Turret dry mass was 151.2 ± 78.15 g (median ± IQR) when no airflow was generated inside the vertical tunnel, 175.3 ± 64 g (median ± IQR) when the air was humidified and 171.6 ± 46.5 g (median ± IQR) when dry air was presented in the outflow tunnel (for statistics see Figure caption).

The height of the constructed turrets, however, showed significant differences among the series (Fig. 21b). Without airflow the average turret height was 3.50 ± 0.76 cm (mean ± SD), but significnatly increased to 4.60 ± 1.18 cm (mean ± SD) when outflow of humid air was present at the nest opening. Outflow of dry air at the nest opening resulted in the construction of intermediate sized turrets averaging 4.42 ± 0.41 cm (mean ± SD), a value not statistically different from those of the two other series.

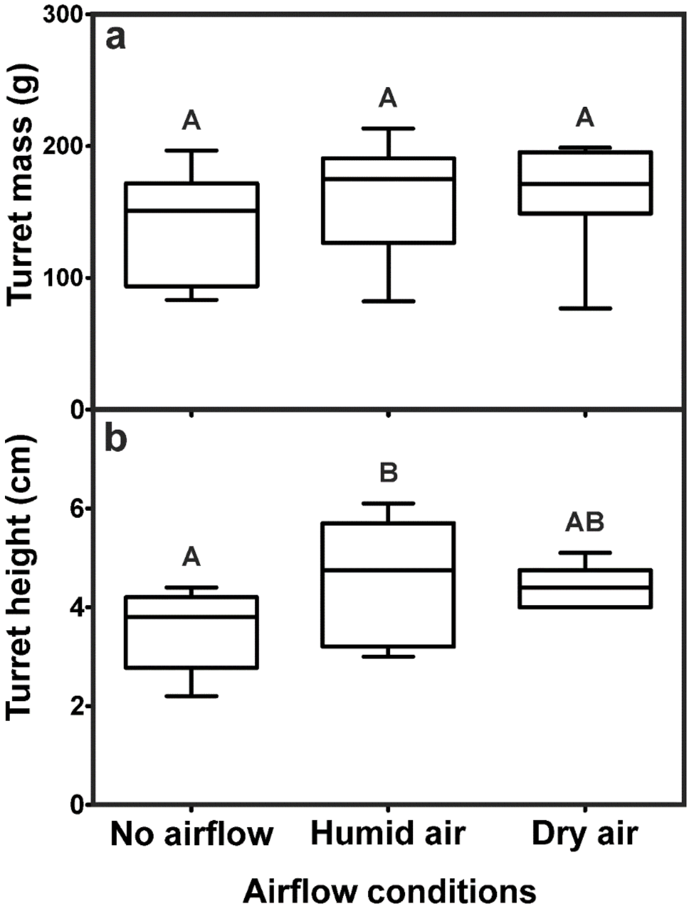

Figure 21: Influence of airflow and humidity on the construction of ventilation turrets. a) Turret mass (Kruskal-Wallis test: $H_{2,30} = 1.99$, $p = 0.37$). b) Turret height (ANOVA: $F_{2,27} = 4.90$, $p = 0.015$; Tukey's Multiple Comparison test: No airflow vs. Humid air: $p = 0.019$, No airflow vs. Dry air: $p = 0.055$, Humid air vs. Dry air: $p = 0.883$). Each series comprises n = 10 replicates. Shown are medians (horizontal bars), 25-75% percentiles (boxes) and min-max values (whiskers). Groups sharing the same letters are not statistically different.

Concerning the structural features of the turrets, no statistical differences were found among the series, neither for the number of turret openings (Fig. 22a), nor the total turret aperture (Fig. 22b). Turrets had 1.0 ± 0.25 (median ± IQR) openings in the no airflow series and 1.0 ± 0.25 (median ± IQR), and 1.5 ± 1 (median ± IQR) openings in the humid air and dry air series, respectively.

Total turret aperture, i.e., the sum of the aperture of all openings, was also similar in all series with 5.37 ± 1.26 cm^2 (mean ± SD) in the series without airflow, 6.23 ± 1.41 cm^2 (mean ± SD) in the series with humid air, and 5.23 ± 2.69 cm^2 (mean ± SD) in the series with dry air.

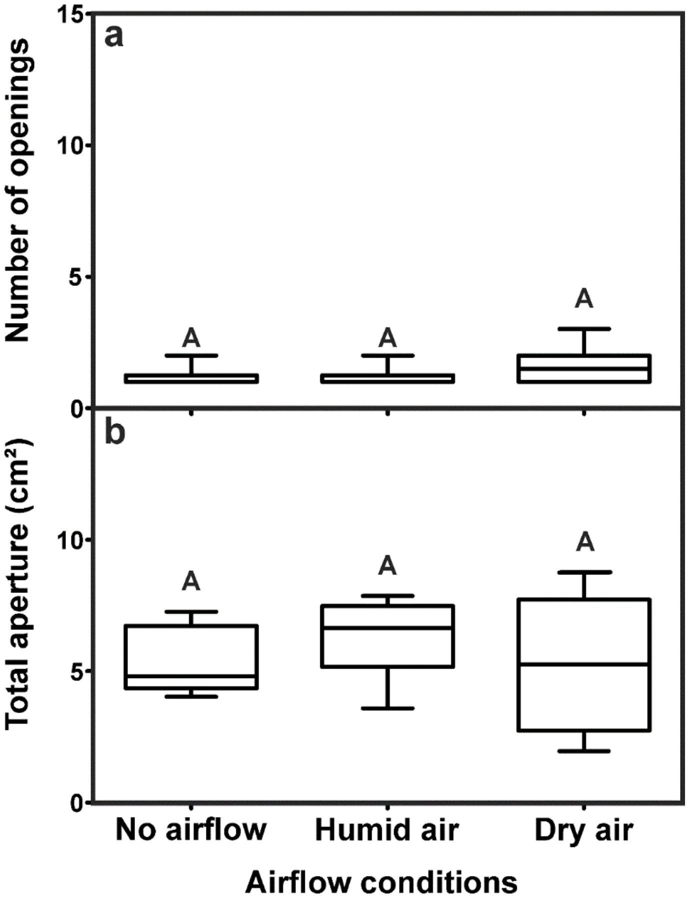

Figure 22: Influence of airflow and humidity on the structure of the ventilation turret. a) Number of turret openings (Kruskal-Wallis test: $H_{2,30}$ = 3.10, p = 0.21). b) Total turret aperture (ANOVA: $F_{2,27}$ = 0.81, p = 0.46). Each series comprises n = 10 replicates. Shown are medians (horizontal bars), 25-75% percentiles (boxes) and min-max values (whiskers). Groups sharing the same letters are not statistically different.

3.2. <u>Turrets constructed on nest openings with outflowing air containing different carbon dioxide levels</u>

In the second experiment we simulated conditions of well-ventilated nests by adding 1% carbon dioxide, or of poorly-ventilated nests by adding either 5% or 10% carbon dioxide to the outflow in the vertical tunnel. The overall building activity of the ants was similar for all series, resulting in turrets of similar size and shape. As in the previous experiment, there were no differences in the amount of building material used for turret construction among the series (Fig. 23a). Mean turret mass was 162.34 ± 41.22 g (mean ± SD) for the series using humid air without adding carbon dioxide (atmospheric CO_2), 172.19 ± 18.94 g (mean ± SD) for 1% CO_2, 158.33 ± 18.39 g (mean ± SD) for 5% CO_2 and 163.28 ± 23.55 g (mean ± SD) for 10% CO_2.

Turret height, however, was affected by the carbon dioxide concentration in the outflow tunnel (Fig. 23b). Turrets of the series with atmospheric levels were the tallest, and turret height decreased with increasing carbon dioxide concentrations at the nest opening, resulting in the smallest turrets in the 10% CO_2 series. The values averaged 4.60 ± 1.18 cm (mean ± SD) for atmospheric levels, 4.29 ± 0.47 cm (mean ± SD) for 1% CO_2, 3.68 ± 0.56 cm (mean ± SD) for 5% CO_2 and 3.38 ± 0.22 cm (mean ± SD) for the 10% CO_2 series.

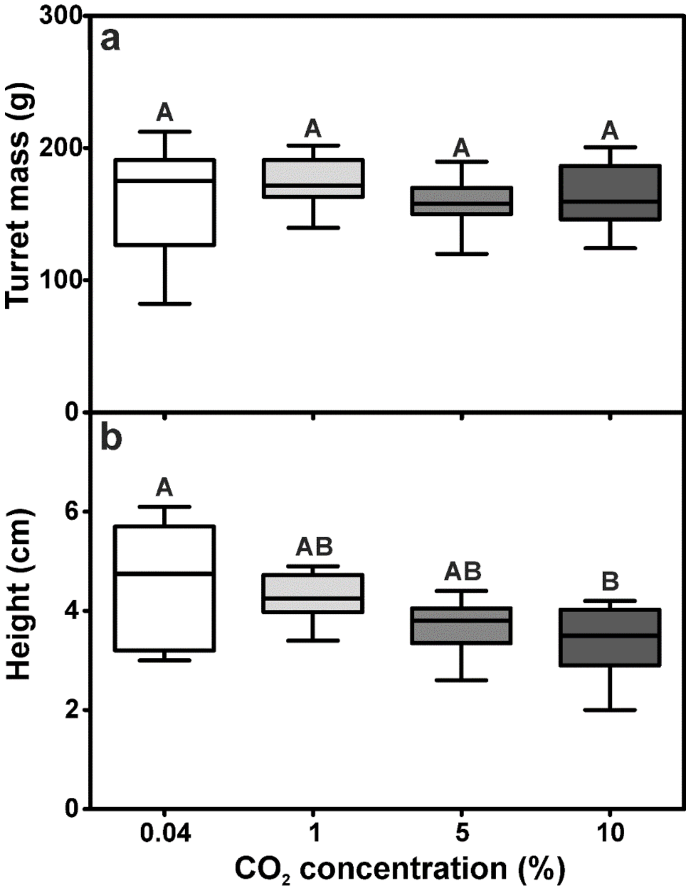

Figure 23: Influence of carbon dioxide levels on the construction of ventilation turrets. a) Turret mass (ANOVA: $F_{3,36} = 0.46$, $p = 0.71$). b) Turret height (ANOVA: $F_{3,36} = 5.19$, $p = 0.004$). Each series comprises n = 10 replicates. Shown are medians (horizontal bars), 25-75% percentiles (boxes) and min-max values (whiskers). Groups sharing the same letters are not statistically different. Detailed statistics can be found in A12.

The structural features of the ventilation turrets were strongly influenced by the carbon dioxide concentration at the nest opening, as the number of turret openings differed among the series (Fig. 24a). While most turrets constructed under atmospheric CO_2 levels had just one opening, turrets constructed under elevated carbon dioxide levels usually possessed one larger and several smaller openings. Turrets had 1.00 ± 0.25 (median ± IQR) openings for the series using atmospheric levels and 3.00 ± 1.50 (median ± IQR) openings for 1% CO_2, but the number significantly increased to 5.00 ± 3.25 and 6.00 ± 7.00 (median ± IQR) openings for 5% CO_2 and 10% CO_2, respectively.

Alongside with the number of turret openings, the total aperture of the ventilation turrets was also affected by the different levels of carbon dioxide in the outflowing air (Fig. 24b). Turret aperture was similar for low carbon dioxide concentrations and was 6.23 ± 1.41 cm^2 (mean ± SD) in the series with atmospheric levels and 6.06 ± 1.58 cm^2 (mean ± SD) for 1% CO_2. However, it was significantly larger in the 5% CO_2 series with 10.61 ± 1.20 cm^2 (mean ± SD), but not in the 10% CO_2 series with 7.76 ± 2.61 cm^2 (mean ± SD).

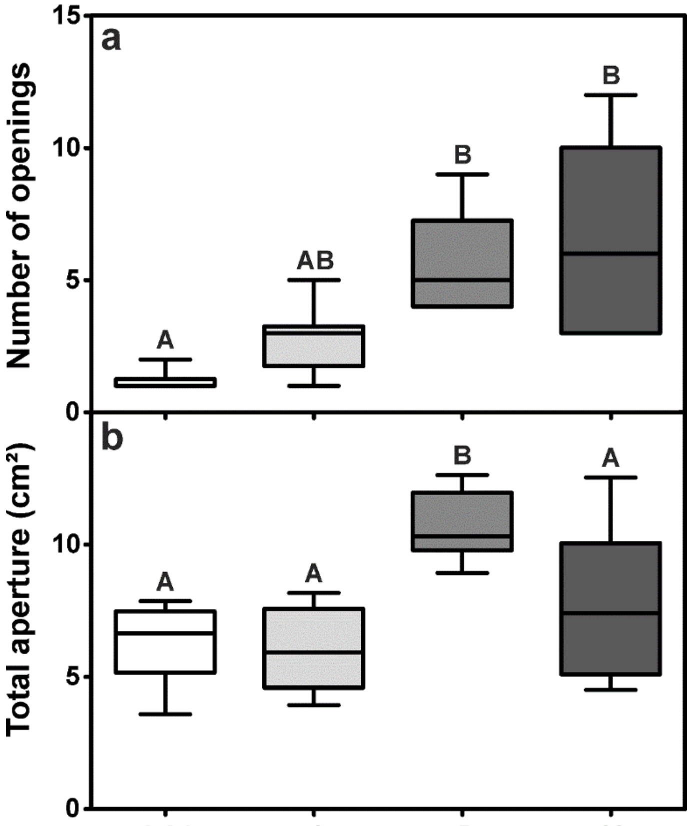

Figure 24: Influence of carbon dioxide levels on the structure of the ventilation turret. a) Number of turret openings (Kruskal-Wallis test: $H_{3,40} = 27.81$, $p < 0.001$). b) Total turret aperture (ANOVA: $F_{3,36} = 13.95$, $p < 0.001$). Each series comprises n = 10 replicates. Shown are medians (horizontal bars), 25-75% percentiles (boxes) and min-max values (whiskers). Groups sharing the same letters are not statsitically different. Detailed statistics can be found in A13, A14.

Total turret aperture comprised the aperture of the turrets' largest opening and the aperture of the several smaller openings. Increasing carbon dioxide levels significantly influenced the aperture of the largest opening (Fig. 25a). Comparable to the total turret aperture, the largest opening size was observed in the group containing 5% CO_2 (7.54 ± 1.38 cm²; median ± IQR), while the main opening was smaller for the series with 1% and 10%, with 4.90 ± 2.57 cm² (median ± IQR) and 4.71 ± 1.96 cm² for 10% CO_2, respectively, and intermediate for the series with atmospheric levels, with 6.54 ± 2.01 cm² (median ± IQR).

Besides the largest opening, most turrets possessed several smaller openings that largely contributed to the total aperture of the ventilation turrets (Fig. 25b). When the outflowing air contained atmospheric CO_2 levels, turrets hardly had more than one large opening, resulting in the lowest average total aperture. However, the construction of several minor openings led to an increase of total turret aperture in the other series. The increase was especially prominent in the 5% CO_2 and 10% CO_2 series, where the carbon dioxide levels were elevated beyond the levels occuring in well-ventilated field nests. By the addition of several minor openings, turret aperture increased by 0.00 ± 0.12 cm² (median ± IQR) for atmospheric levels, 0.70 ± 1.42 cm² (median ± IQR) in the 1% CO_2 series, 2.99 ± 1.99 cm² (median ± IQR) in the 5% CO_2 series and 1.47 ± 3.22 cm² (median ± IQR) in the 10% CO_2 series. The addition of multiple smaller openings therefore accounted for an increase in total turret aperture of up to ~30% as compared to the conditions when CO_2 levels in the outflowing air were atmospheric (Fig. 25b).

The size of the single largest turret opening strongly varied among trials in each series, yet it was markedly larger than the size of minor openings (Fig. 26, top and bottom row, respectively). The median size of minor openings was 0.71 cm² for atmospheric levels, 0.44 cm² for 1% CO_2, 0.38 cm² for 5% CO_2 and 0.38 cm² for 10% CO_2.

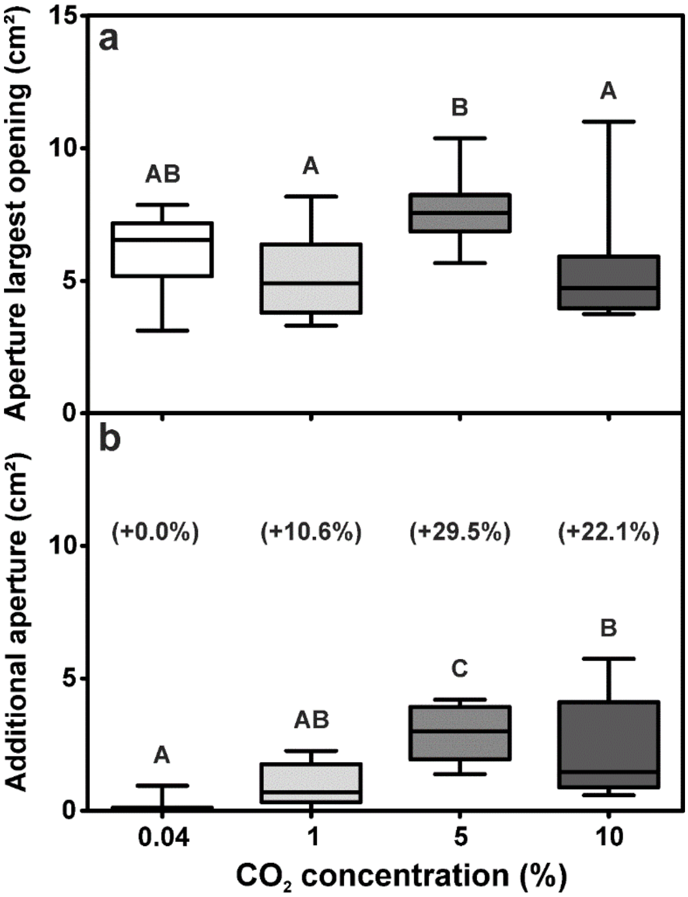

Figure 25: Influence of carbon dioxide levels on total turret aperture. a) Aperture of the single main opening (Kruskal-Wallis test: $H_{3,40}$ = 13.43, p = 0.004). b) Sum of the apertures of all minor openings (Kruskal-Wallis test: $H_{3,40}$ = 24.60, $p < 0.001$). Values in parentheses indicate the median increase in total turret aperture achieved by the addition of smaller openings. Each series comprises n = 10 replicates. Shown are medians (horizontal bars), 25-75% percentiles (boxes) and min-max values (whiskers). Groups sharing the same letters are not statistically different. Detailed statistics can be found in A15, A16.

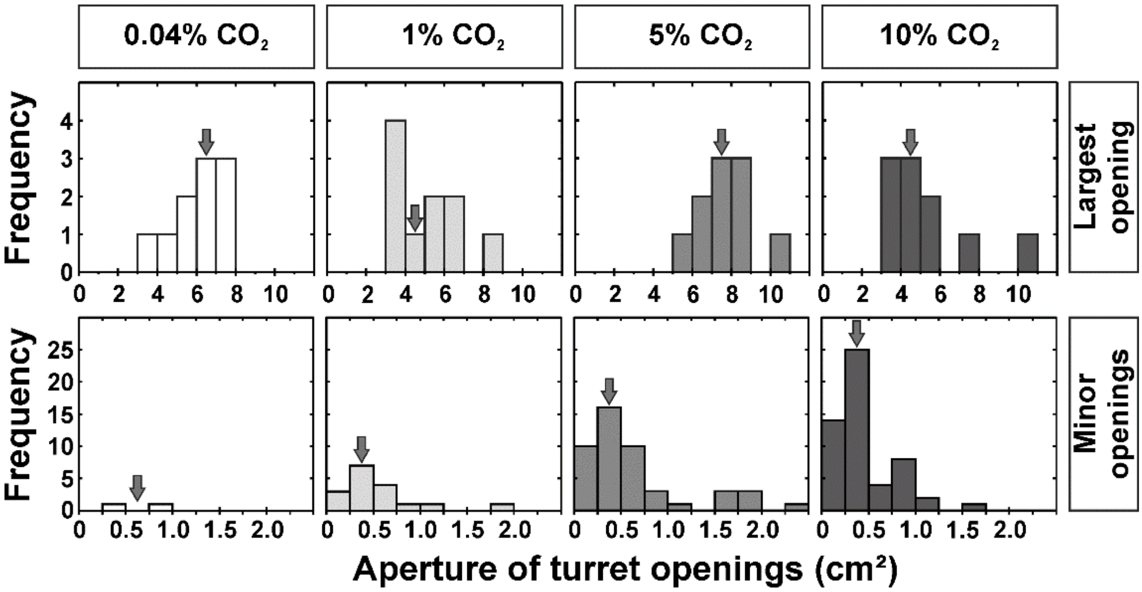

Figure 26: Size distribution of the largest opening (first row) and minor openings (second row) measured on turrets constructed on openings with outflowing air of different carbon dioxide levels. Arrows indicate median turret aperture, calculated for the largest openings (n = 10 each) and minor openings of the series using atmospheric CO_2 levels (n = 2), 1% CO_2 (n = 17), 5% CO_2 (n = 47) and 10% CO_2 (n = 54). Due to the differences in sample sizes, no statistical analysis was performed. Note the different scale on both x- and y-axis between the largest and the minor openings.

3.3. Turrets constructed on nest openings with outflowing air containing similar carbon dioxide levels to those of the outside air

In the third experiment we investigated whether the construction of a turret with several minor openings depended on the strong differences in CO_2 levels between the outflowing and the outside air. Covering the building arena to remove the CO_2 gradient did not affect the overall building activity of the colony, as similar-sized dome-shaped turrets were constructed in all series. The mass of the turrets was similar in all four series (Fig. 27a). When the building arena was open, turret mass averaged 157.30 ± 80.20 g (median ± IQR) for atmospheric CO_2 levels and 134.80 ± 46.30 g (median ± IQR) for 5% CO_2. Turret mass in closed arenas averaged 130.30 ± 99.07 g (median ± IQR) and 103.70 ± 73.70 g (median ± IQR) for atmospheric levels and 5% CO_2, respectively.

Turret height was also independent of the presence of a CO_2 gradient under most conditions (Fig. 27b). There was only a significant difference in height between turrets constructed under atmospheric CO_2 levels with a CO_2 gradient (open arena: 4.46 ± 0.69 cm; mean ± SD) and without a gradient (closed arena: 3.50 ± 0.71 cm; mean ± SD). When carbon dioxide levels were elevated to 5%, turret height was 3.92 ± 0.31 cm (mean ± SD) for open and 3.69 ± 0.41 cm for closed arenas.

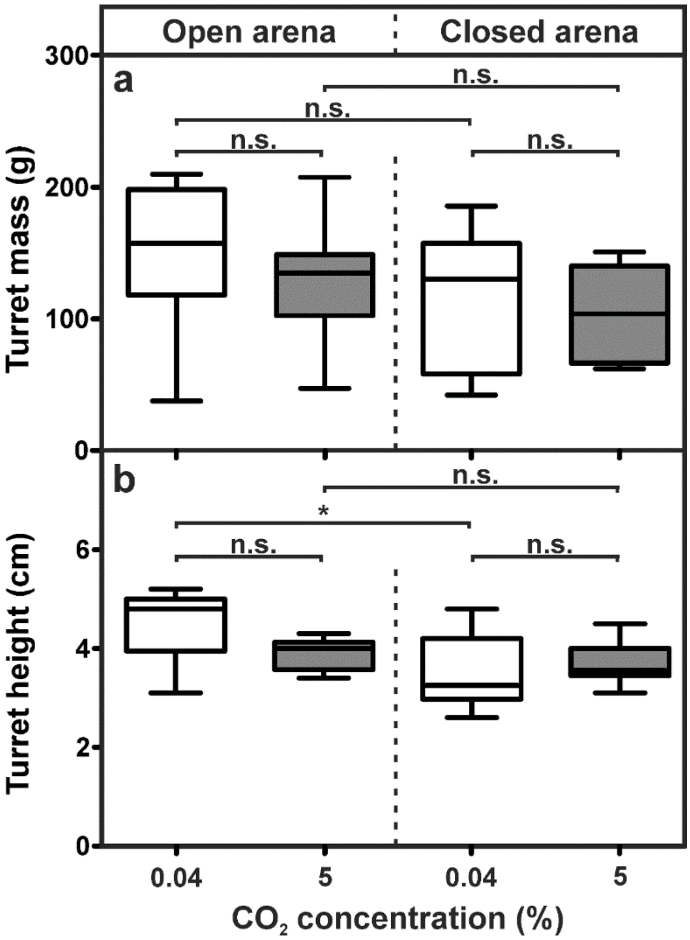

Figure 27: Influence of a carbon dioxide gradient on turret construction. a) Turret mass (Kruskal-Wallis test: $H_{3,40} = 5.04$, $p = 0.169$). b) Turret height (ANOVA: $F_{3,36} = 5.55$, $p < 0.003$). Each series comprises n = 10 replicates. Shown are medians (horizontal bars), 25-75% percentiles (boxes) and min-max values (whiskers). Asterisks indicate statistically significant differences between groups. Detailed statistics can be found in A17.

Similar to the second experiment, the structural features of the turrets significantly varied among the series. The absence of a gradient, however, did not affect the number of turret openings, as there were no differences between open and closed arenas (Fig. 28a). Differences were only observed between series with atmospheric levels and the 5% CO_2 series, as expected: in open arenas with a CO_2 gradient, the number of turret openings was 1.00 ± 1.00 (median ± IQR) for atmospheric levels and 3.50 ± 1.50 (median ± IQR) for 5% CO_2. When the gradient was removed, turrets had 2.00 ± 1.25 (median ± IQR) openings in the series using atmospheric levels and 5.00 ± 1.50 (median ± IQR) openings in the 5% CO_2 series.

The increase in the number of turret openings was also accompanied by an increase in turret aperture (Fig. 28b). Again, turret aperture was only larger when carbon dioxide concentrations were elevated. Covering the building arena, i.e., removing the gradient at the tunnel opening, did not affect turret structure and no difference was observed between series with open and covered arenas when CO_2 levels were equal. Total turret aperture in open arenas was 4.28 ± 1.38 cm² (mean ± SD) for atmospheric CO_2 and 6.91 ± 2.50 cm² (mean ± SD) for 5% CO_2. Turrets constructed in covered arenas had a total aperture of 3.06 ± 1.43 cm² (mean ± SD) in the series with atmospheric values and 5.50 ± 1.78 cm² (mean ± SD) in the 5% CO_2 series.

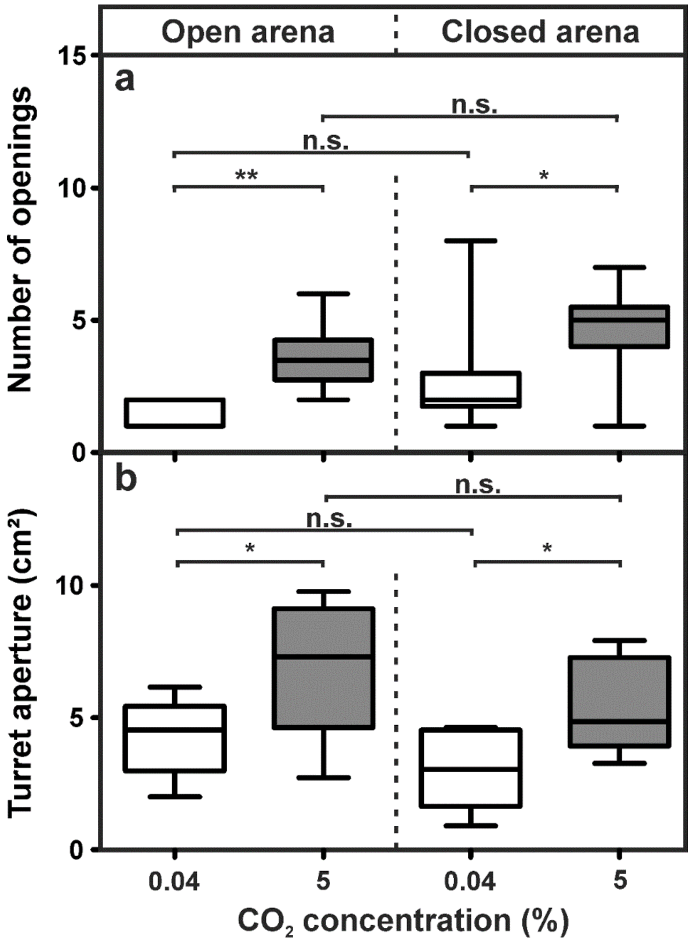

Figure 28: Influence of a carbon dioxide gradient on turret structure. a) Number of turret openings (Kruskal-Wallis test: $H_{3,40}$ = 21.13, p < 0.001). b) Total turret aperture (ANOVA: $F_{3,36}$ = 8.14, p < 0.001). Each series comprises n = 10 replicates. Shown are medians (horizontal bars), 25-75% percentiles (boxes) and min-max values (whiskers). Asterisks indicate statistical significant differences between groups. Detailed statistics can be found in A18, A19.

4. Discussion

4.1. Building behavior and turret construction

In all experiments, workers excavated and formed pellets from the offered building material and transported them to the building platform, where they constructed a dome-shaped turret around the nest opening. Although single pellets were occasionally dropped by workers in other places, construction behavior was never observed at locations in the building arena other than around the nest opening. Usually, a worker did not just drop the transported pellet at the nest opening, but rather searched for a suitable spot at the construction site and incorporated its pellet into the existing structure by pressing it onto the turret wall using their mandibles and front legs. A similar behavior is shown by leaf-cutting ant workers when soil pellets are deposited at the walls of underground tunnels or chambers during nest excavation (Römer and Roces, 2015) or during the incorporation of plant material into the fungus garden (Garrett *et al.*, 2016). After the deposition of the pellet, workers often returned to the digging site in order to excavate or pick up another pellet and repeat the process. Although turret construction was quite slow at the beginning, more and more ants participated in excavation and transport and after a couple of hours, the turret grew in both height and diameter. Turret height and turret mass after 24 hours were considered as a reliable indicator for the colony's overall building activity, as turrets usually had a similar size and shape across the experiments. However, the structural features of the turrets were much more variable among the series, depending on the prevailing climatic conditions at the nest opening. The carbon dioxide concentration and not the presence of airflow or the air humidity strongly affected the construction of the turrets and led to differences in both the number of turret openings and the total turret aperture among the series.

Noteworthy, workers always constructed turret-shaped structures on top of the nest opening in our experiments, independent of the climatic conditions inside the nest tunnel. This indicates that workers do not require outflow of air, a humidity gradient or carbon dioxide gradient at the nest opening in order to initiate turret construction. Although speculative, other factors might be used as cues for the initiation of turret construction by workers, as well. For example, volatiles, like colony odors or pheromones might leave through nest openings even without outflowing air and could be used by workers on the mound as orientation cue for the deposition of building material imported from the surrounding area.

By offering the clay mixture only in the building arena outside of the nest, we were able to investigate turret construction independently of nest excavation, as workers had to actively import building material from the outside instead of just piling up the pellets excavated underground around the nest opening, a common feature of soil-nesting ants (McCook, 1877; Jonkman, 1980b; Hölldobler and Wilson, 1990; Franks *et al.*, 2004). Under natural conditions, however, turrets are mainly constructed using soil pellets from underground excavation sites that are carried to the surface. It is unknown whether workers that carry soil pellets over long distances to reach the nest surface (Pielström and Roces, 2013) may select tunnels based on their airflow direction, humidity or carbon dioxide levels. However, we recently demonstrated that leaf-cutting ants engaged in nest digging use airflow directions for orientation in the underground (Halboth and Roces, 2017). In such a case, the putative underground selection of environmental parameters by pellet carriers might explain why nest openings with inflowing air or no airflow hardly possess turrets in the field. Hypothetically, lack of a possibility to select tunnels with particular environmental conditions from the underground might be the reason why workers importing building material constructed a similarly-sized turret even under conditions of no airflow in our experiments.

It is important to note that we manipulated the environmental conditions only inside a single ventilation tunnel, not in the adjacent nest compartments. Consequently, changes in the building behavior of workers can only be attributed to their response to local conditions at the nest opening or inside the final tunnel section. It is an open question whether workers also react to unsuitable environmental conditions that occur inside the fungus chambers and engage in turret-building behavior. On the one side, this may appear unlikely because of the larger distances between the fungus chambers and the nest surface. On the other side, the environmental conditions inside the fungus chambers are by far steadier than those in the nest tunnels and so more reliable as cues for a long-term building response such as the construction of a turret, since airflow through the tunnels is unable to directly reach the fungus chambers (Bollazzi *et al.*, 2012).

4.2. Turrets constructed on nest openings containing no airflow, humid or dry air

The presence of airflow at the nest opening hardly affected the construction of turrets, i.e., the amount of building material used by the workers was the same with or without outflowing air from the nest tunnel. In addition, the structural features of the turrets were similar irrespective of the presence of airflow, humid or dry air as turrets from all series usually only had a single large opening and a similar total turret aperture. Under natural conditions, the ants construct turrets mainly on top of nest openings located centrally on the mound. Due to the wind-induced ventilation of the nests, central openings serve as outflow tunnels, while peripheral ones serve as inflow tunnels (Kleineidam *et al.*, 2001). Since turret construction usually occurs on top of openings where air leaves the nest, it is tempting to assume that workers use the airflow as a cue during the construction of ventilation turrets and deposit more pellet material around outflow channels rather than around openings where air enters the nest or no airflow is present at all. In our experiments, the amount of building material deposited by workers around the nest opening was the same for all three series and was not influenced by the presence or absence of airflow. However, turrets were higher when humid or dry air left the nest compared to those of the series without air movements, indicating that the pellets were arranged in a slightly different way around the outflow tunnel. Even though the overall building activity of the colony and the amount of material import were unaffected, workers might have preferred to drop their load in close vicinity to the perceived outflow of nest air, resulting in a deposition of pellets closer to the actual opening and thereby an increase in turret height and a decrease in turret diameter. A similar behavior was reported during nest construction in termites of the genus *Zootermopsis*, in which workers use air movements as a cue for the deposition of building material (Howse, 1966), and during climate control in leaf-cutting ants of the genus *Acromyrmex* (Bollazzi and Roces, 2007). In the latter, only inflow of dry air into the nest evoked the deposition of material as a building response, while inflow of humid air was not sufficient to trigger material deposition. However, it is worth noting that in both cases workers deposit material in order to seal leaks in the nest structure or close nest openings to avoid desiccation, while in *Atta vollenweideri* workers might deposit material around nest openings in order to facilitate the outflow of nest air. It is possible though that such a building response depends on other factors as well, since workers might also respond to air humidity (high vs. low), airflow direction (inflow vs. outflow) or different according to their current location (inside vs. outside the nest).

In our experiments, however, the relative humidity of the air leaving the nest tunnel had no apparent influence on turret construction, although it is known to affect the workers' building behavior in several leaf-cutting ant species. For example, workers of *Acromyrmex heyeri* close some of the openings on their nest mound when the nest interior desiccates, i.e., when the relative air humidity drops from 98% to 50%, similar to the humidity values of 80% (humid air) and 40% (dry air) used in our experiments (Bollazzi and Roces, 2010c). In *Atta vollenweideri*, humidity as well as heat loss from the nest are expected to be the reason why colonies close the majority of the nest openings during the winter months. Additionally, higher levels of air and soil moisture due to precipitation might be responsible for an increased building activity of workers on the nest surface that results in higher turrets after heavy rains (Jonkman, 1980b). In fact, the moisture of the soil has been shown to influence the excavation behavior of *Atta vollenweideri*, as workers prefer digging in moist clay with 20-22% water content, but avoid excavation in soils of lower or higher moistures (Pielström and Roces, 2014). Although the moisture of the clay mixture offered was constant (19% water content) in our experiments, higher air humidity might have facilitated turret construction as pellets should desiccate at a slower rate and therefore be more likely to stick to each other when workers press them into the existing turret structure. In fact, albeit not statistically significant, turret height was slightly higher when the outflowing air in the nest tunnel was humid. Partly for this reason, we used humidified air at 80% RH during the subsequent experiments.

4.3. Turrets constructed on nest openings with outflowing air containing different carbon dioxide levels

Size and shape, i.e., mass and height of the constructed turrets were mainly unaffected by carbon dioxide and were roughly the same for all series using carbon dioxide levels between 0.04% and 10%. Although elevated CO_2 levels might attract ants (Wilson, 1962) and are known to evoke digging behavior in *Solenopsis* fire ants (Hangartner, 1969), our results indicate that the release of carbon dioxide rich air from the central nest openings does not promote the construction of turrets per se. However, ants built turrets that usually contained a larger number of openings and thus a larger total aperture at higher CO_2 levels in the outflowing air than atmospheric ones.

Leaf-cutting ants, like other underground nesting animals, are continuously exposed to elevated carbon dioxide levels of the soil air, especially in the giant underground nests of species belonging to the genus *Atta* (Kleineidam and Roces, 2000; Bollazzi *et al.*, 2012). An increase in carbon dioxide levels is usually accompanied by a decrease in oxygen levels. High levels of CO_2 as those sometimes measured in field nests are known to negatively influence fungus respiration (Kleineidam and Roces, 2000); workers' respiration is known to be affected at very low O_2 levels (Hebling *et al.*, 1992). Leaf-cutting ants are therefore expected to cope with such unfavorable climatic conditions in the nest by facilitating nest ventilation and thus the gas exchange with the environment.

The construction of turrets with several minor openings might be one way to improve the removal of carbon dioxide from the underground nest by increasing the turret's open surface area. A larger opening's total surface area should facilitate both diffusive and induced airflows and therefore increase the volume of air that can be dragged out of the nest by surface wind, thus allowing a better gas exchange with the environment as observed in ant nests with entrances of different size (Hasin *et al.*, 2014) and in the respiratory organs of animals (Forster, 1957). The increase of turret aperture due to the excavation of several smaller openings was especially prominent in the series using 5% CO_2 and to a lesser extent in the series with 1% and 10% CO_2. A concentration of 1-2% is commonly found in well-ventilated nests of leaf-cutting ants and is therefore unlikely to be detrimental for both fungus and workers' or brood respiration, as they are expected to have adapted to slightly increased CO_2 levels. This could explain why the construction of several openings was not as prominent as compared to the other series containing high levels of carbon dioxide. In fact, it has been shown that workers of *Acromyrmex lundii* actually prefer slightly elevated carbon dioxide concentrations of 1-3% when relocating fungus (Römer *et al.*, 2017).

On the other hand, a carbon dioxide concentration of 5.7% is the highest concentration that has been reported for nests of *Atta vollenweideri* (Kleineidam and Roces, 2000) and it strongly indicates a poor nest ventilation, for example due to the closure of most nest openings. Although a concentration of 10% CO_2 would indicate an even worse nest ventilation, the response of the ants was not stronger than in the 5% series. While the constructed turrets did also possess several minor openings, their total surface area was lower than in the 5% CO_2 series. Several explanations might account for this effect. First, 10% CO_2 exceeds by far the concentrations measured in *Atta vollenweideri* nests in the field (Kleineidam and Roces, 2000) and may represent a very artificial situation. Second, while electrophysiological studies on the response characteristics of CO_2 receptors in leaf-cutting ants revealed a working range of 0-10% CO_2, sensory neurons showed a stimulus overload and saturation for concentrations of 7% and higher (Kleineidam, 1999). Therefore, any further increase of the carbon dioxide levels does not necessarily elicit a stronger neuronal response and might consequently cause similar behavioral reactions of workers to different stimulus intensities. Third, elevated carbon dioxide levels around 10% are known to have detrimental effects on different aspects of insect physiology (Nicolas and Sillans, 1989), possibly influencing the workers' building behavior in our experiments as well. This is further suggested by the fact that the height of the ventilation turrets tended to decrease with increasing carbon dioxide concentration. Alternatively, adding multiple openings to the ventilation turrets might simply decrease turret stability and thereby limit the potential height of the structure, leading to the construction of smaller turrets. Our results do not allow distinguishing among these possibilities and all of them might have contributed to the observed effect as well.

It is interesting to note that in our experiments, the increase in total aperture for outflowing air with 5% CO_2 was achieved by the addition of several minor openings instead of by the enlargement of the single, initial turret opening. For similar total turret aperture, it is unlikely that a single large opening may facilitate nest ventilation stronger than several minor openings of the same total area might do, since both the overall airflow velocity and the air volume passing through the turret should be the same. However, several smaller openings might provide advantages as compared to a single, larger opening of the same surface area. The smaller the opening, i.e., the smaller the gallery across the turret wall, the easier it should be for workers to close it using soil pellets or leaf material as compared to a single large turret opening. This might allow colonies to rapidly close or re-open nest openings depending on the current ventilation needs or the temperature or humidity conditions inside the nest.

Alternatively, the addition of several small turret openings might just be a side effect of the workers' digging behavior. When the carbon dioxide concentration inside the nest tunnel increases, workers might engage in digging behavior at the turret wall and remove previously deposited soil pellets. Depending on the diameter of the nest tunnel and the interior of the turret, one could imagine individual workers initiating excavation at separate spots, resulting in the appearance of multiple holes at different locations on the turret's surface as opposed to the formation of a single larger opening. However, the pattern of emergence of the tunnel opening remains elusive, since we neither focused on the behavior of individual workers during turret construction, nor documented the time of the emergence of individual turret openings.

Regarding the aperture of the minor turret openings, it appeared that the size of single turret openings slightly decreased with increasing carbon dioxide concentration. It is unclear whether this effect is linked to turret stability as stated above, since increasing the carbon dioxide concentration led to an increase in the total number of openings. The unequal sample sizes between the series precluded any statistical analysis, so that the question remains unanswered so far.

In addition to the expected increase in the size of the turret opening under natural conditions, workers from field colonies might also broaden the outflow tunnels and therefore increase the volume of the nest air that can be exchanged with the environment over a given time period. Since outflow channels are not used as entrances/exits for foragers, the size of the tunnel openings should be related to climate control rather than to ant traffic. The average size of the nest openings on mounds of *Atta vollenweideri* has been reported to vary between 3 and 5 cm (Jonkman, 1980b; c), yet single openings can also be quite large, with diameters up to 10 cm (Cosarinsky and Roces, 2007). While in our experiments we chose the diameter of the opening and ventilation tunnel accordingly, the ants could not increase its diameter and were therefore limited to changing the opening size of the turret.

4.4. **Turrets constructed on nest openings with outflowing air containing similar carbon dioxide levels to those of the outside air**

Covering the building arena, thereby removing the CO_2 gradient at the building platform in the series using 5% CO_2 in the outflowing air, did not affect the construction of turrets, i.e., turret height and turret mass were the same in open and closed arenas. This indicates that workers do not rely on the difference in carbon dioxide concentrations between the inside and the outside of the nest tunnel when turrets containing multiple openings are constructed.

However, the number of openings and the total aperture were different for series containing atmospheric levels compared to series containing 5% CO_2, independent of the presence of a gradient. This confirms the results of our second experiment, where increased carbon dioxide levels caused an increase in number of turret openings as well as turret aperture.

It is known that leaf-cutting ants possess the rare ability to respond to both the absolute CO_2 levels as well as to the changes of the carbon dioxide concentration (Kleineidam and Tautz, 1996) using specialized receptor cells on the tip of the antennae (Kleineidam *et al.*, 2000). This feature would allow them to monitor the absolute carbon dioxide concentration in the nest continuously, indicating its biological relevance for leaf-cutting ants. In most insects, carbon dioxide serves as an orientation cue and is used for example by moths to find plants (Stange, 1992) or by ticks to find hosts (Holscher *et al.*, 1980). It might be sufficient for an animal to find a certain location by following increasing CO_2 levels compared to the background air. Although leaf-cutting ants could also use carbon dioxide to orientate in the underground, it might even be more important to assess the absolute carbon dioxide concentration instead of relying on a strong CO_2 gradient between the inside and the outside of the nest tunnel to trigger the construction of a turret as a long-term response.

Overall, it remains unclear whether the building responses that workers showed in our experiments as a reaction to increased carbon dioxide concentrations also occur in field colonies. While turrets constructed under natural conditions may also contain several minor openings similar to the turrets built in the laboratory (Fig. 18b, d), it is unknown whether their structural features are also related to the carbon dioxide concentration in the outflowing nest air. In addition, it needs to be investigated whether and to which extent the addition of several minor openings at high CO_2 levels actually improves the gas exchange with the environment and enhances nest ventilation in *Atta vollenweideri* field nests.

VI. Wind-induced nest ventilation: Effect of airflow and turret structure on the gas exchanges in a physical nest model

Abstract

Nest ventilation in the leaf-cutting ant *Atta vollenweideri* is passively driven by a wind-induced mechanism that facilitates the supply of fresh air and the removal of carbon dioxide from the underground. On the surface, ants pile up excavated soil to form a conically-shaped nest mound that is permeated with several openings through which air moves. CO_2-rich air leaves the nest through openings on the center of the mound, while fresh air enters through peripheral openings. In addition, workers construct small turrets on top of central outflow openings that are expected to further improve the ventilation of the nest. In previous experiments, it was shown that the structural features of these turrets are highly variable and depend on the local climatic conditions inside the tunnels leading to the nest surface, as turrets constructed under elevated CO_2 levels possessed more openings and an increased total aperture. In this chapter we tested whether the presence and structure of turrets affect nest ventilation in *Atta vollenweideri* under the presence or absence of airflows. For that, we established a physical model of a small nest, consisting of a single nest chamber and two tunnels, with one providing inflow, the other one providing outflow, depending on the series. We increased the carbon dioxide levels inside the chamber to 5% and measured its removal from the nest over two hours for opened/closed tunnel openings, with/without airflow and with/without a turret placed on the outflow opening. The clearance rate of CO_2 was low under windless conditions for both opened or closed nest openings, but significantly increased when airflow was present inside the nest tunnels. The presence of both airflow and a turret resulted in the highest CO_2 clearance rate, as almost all carbon dioxide was removed from the nest chamber within two hours. Turret structure partially affected the ventilation of the nest, as the CO_2 clearance rate increased with increasing turret aperture, but was independent of turret height or number of turret openings. We argue that the construction of turrets with increased aperture might be an effective mechanism to promote wind-induced ventilation and so to counteract the elevated underground carbon dioxide levels in the nests of *Atta vollenweideri*.

1. Introduction

The majority of ants construct their nests in soil (Hölldobler and Wilson, 1990), as it offers both protection against predators and unfavorable environmental conditions (Hansell, 2005). However, the subterranean lifestyle comes at the expense of nest ventilation, as the gas exchanges with the environment, i.e., the supply of oxygen and the removal of carbon dioxide, are often limited in the underground. For leaf-cutting ants, this poses a major problem, as their nests are among the largest structures found in the animal kingdom. Depending on the species, nests may contain several thousand underground chambers and reach a depth of several meters (Jonkman, 1980c; Moreira *et al.*, 2004a; b). In soil, carbon dioxide levels rise with increasing depth (Davidson and Trumbore, 1995) and due to ant and fungal respiration, colonies usually are exposed to hypoxic (low O_2) and hypercapnic (high CO_2) conditions inside their nests (Kleineidam and Roces, 2000; Bollazzi *et al.*, 2012). In order to cope with these conditions, inhabitants of subterranean nests often show either physiological adaptations, such as increased tolerance to carbon dioxide (Nicolas and Sillans, 1989) or behavioral adaptations that facilitate the ventilation of the nest (Hansell, 2005). Since ants lack the ability to actively ventilate their nests like other social insects, for example via fanning in bees (Seeley, 1974; Weidenmüller *et al.*, 2002), they often rely on passive mechanisms that promote the gas exchanges with the environment. In the underground, diffusion is an important driver of nest ventilation, at least in terrestrial substrates (Withers, 1978), and it has been identified as the underlying mechanism of gas exchanges for example in the egg clutches of mound-building birds, sea turtles and crocodiles (Ackerman, 1977; Seymour and Ackerman, 1980). However, the rate of diffusion strongly depends on soil characteristics (Withers, 1978; Currie, 1984) and nest type (Wilson and Kilgore, 1978), and it is often too low to solely account for the exchange of respiratory gases, as in the case of the leaf-cutting ant *Atta vollenweideri*. In this species, colonies predominantly nest in clay-heavy soils with low porosity and thus low permeability to air, hindering diffusive flows in the underground.

Besides diffusion, other mechanisms may facilitate the removal of CO_2 and the supply with fresh air in animal nests. Temperature gradients within a nest, resulting either from solar radiation or the production of metabolic heat, might also contribute to the exchange of respiratory gases, an effect that has been investigated extensively in termites (Lüscher, 1956; Korb, 2003; King *et al.*, 2015; Ocko *et al.*, 2017). In their nests, warmer air, enriched with CO_2, rises and leaves through the top, while cooler, fresh air enters at the bottom of their mound, resulting in the renewal of the nest air via conductive heat flow. Although initially also proposed for the giant nests of leaf-cutting ants (Hölldobler and Wilson, 1990), evidence supporting a thermal-induced nest ventilation in *Atta* is lacking. Instead, their nests have been shown to rely on a wind-induced ventilation mechanism that promotes the gas exchanges between the nest and the environment (Kleineidam *et al.*, 2001; Bollazzi *et al.*, 2012). Airflow-driven ventilation has been demonstrated mainly for multi-entranced borrows of mammals, including moles (Olszewski and Skoczen, 1965), kangaroo rats (Birukow, 1958), prairie-dogs (Vogel *et al.*, 1973) and jirds (Brickner-Braun *et al.*, 2014), but also in the single-opening nests of skinks (Turner and Pinshow, 2015) and bee-eaters (White *et al.*, 1978; Ar and Piontkewitz, 1992). In social insects, wind-induced gas exchanges have been described for termites of the genus *Macrotermes* (Weir, 1973; Darlington *et al.*, 1997) and are expected to also occur in the mud nests of the wasp *Polybia spinifex* (Hozumi and Inagaki, 2010) and in the cliff dwelling ant *Malagidris sofina* (Helms *et al.*, 2014). The physical mechanism underlying a wind-induced ventilation depends on the size and structure of a burrow. While nests with a single opening rely on turbulent air gusts, multi-entranced burrows are mainly ventilated by unidirectional or oscillating airflow (Brickner-Braun *et al.*, 2014; Turner and Pinshow, 2015). In burrows that are ventilated by unidirectional airflow, wind is not forced directly into the nest. Instead, air exchanges result from pressure differences between lower and elevated nest entrances by taking advantage of the Bernoulli principle and viscous dragging (Vogel and Bretz, 1972).

In the leaf-cutting ant *Atta vollenweideri*, excavated soil from the underground is heaped up to form a conically-shaped nest mound that is covered with up to 200 nest openings (Jonkman, 1980b; c). Surface wind is passing over the mound, dragging air out of openings on the center followed by an inflow of fresh air through openings on the periphery of the mound (Kleineidam *et al.*, 2001). Consequently, even large nests are generally well-ventilated with average carbon dioxide levels of 1-2% in the underground, due to the unhindered movement of air inside the nest tunnels (Kleineidam and Roces, 2000). However, when colonies close the majority of their nest openings, for example during rain or during the winter months (Jonkman, 1980b; c), CO_2 concentrations in the underground may quickly rise up to 5%, and drop again when the nest tunnels are re-opened (Kleineidam and Roces, 2000). Workers of *Atta vollenweideri* have been observed to additionally modify the structure of their mound by constructing small turrets on top of central nest openings, especially after rains (Jonkman, 1980b). These turrets are expected to enhance nest ventilation as their presence further elevates the outflow openings relative to the ground and thus exposes them to higher wind velocities. Turrets are mainly built using excavated soil from the underground, but workers might also collect leaves and twigs from the immediate vicinity that are incorporated to reinforce the structure (Cosarinsky and Roces, 2007; 2012).

The structural features of the turrets are highly variable and, as shown in Chapter V, strongly depend on the prevailing climatic conditions inside the tunnels leading to the nest surface. When carbon dioxide levels in the nest tunnels were elevated, the constructed turrets possessed more openings and an increased turret aperture compared to turrets constructed under atmospheric levels. We hypothesized that this modification of turret structure further improves the air exchanges between the nest and the environment by facilitating the removal of carbon dioxide from the underground. In this chapter, we investigated the nest ventilation in the leaf-cutting ant *Atta vollenweideri*, using a physical model of a small nest consisting of two nest tunnels and a single chamber. We measured the removal rate of carbon dioxide from the nest chamber depending on both the presence of a turret on one of the tunnel openings and the occurrence of airflow. In addition, we tested whether structural features of the turret affected the CO_2 clearance from the nest.

2. Methods

2.1. Experimental setup

The aim of this study was to quantify the effect of airflow in the nest tunnels as well as the presence and structure of ventilation turrets on the removal of carbon dioxide from the underground part of leaf-cutting ant nests. Therefore, we performed experiments in the laboratory at the University of Würzburg on a simplified, artificial leaf-cutting ant nest that mimicked the arrangement of a natural field nest of *Atta vollenweideri*, using its internal structure as a template (Fig. 29). The nest model consisted of a single nest chamber (20 x 20 x 10 cm) that was connected to a U-shaped tunnel system ending in two separate nest openings at the surface. The vertical tunnels consisted of Plexiglas tubes (40 cm, Ø 3.3 cm) that were connected to a horizontal plastic tube (Ø 3 cm) at a distance of 30 cm apart from each other. The nest surface consisted of 2 Plexiglas plates (50 x 70 cm) stabilized horizontally using retort stands and clamps. We simulated surface wind by creating a continuous airflow at a velocity of ca. 40 cm/s above the nest opening by setting up a PC-case fan (WALLAIR Model 40966, 12V, 3000 rpm) at a distance of 10 cm from one of the nest openings. Air was not forced into the opening but instead dragged out of the tunnel, based on the Bernoulli principle and viscous entrainment (Vogel and Bretz, 1972), resulting in an inflow of air at the second opening. Airflow velocity inside the tunnels was measured prior to the experiments using a hand-held anemometer (Testo 405-v1, range 0-5 m/s, resolution: 0.01 m/s). It varied between 2-10 cm/s, close to the range of airflow velocities measured in field nests of *Atta vollenweideri* (Kleineidam *et al.*, 2001).

In order to simulate increased carbon dioxide levels in the underground, we inserted CO_2 from the laboratory gas line into the nest chamber via a PVC tube (Ø 1 cm). The carbon dioxide concentration inside the chamber was continuously measured throughout the experiment using a hand-held carbon dioxide meter (Vaisala CARBOCAP® GM70). Prior to the start of each trial, the CO_2 levels inside the nest chamber were elevated to 5%, close to the values measured in poorly ventilated field nests (Kleineidam and Roces, 2000). In order to quantify the rate of CO_2 clearance, i.e., the amount of carbon dioxide removed from the artificial nest chamber, we recorded the carbon dioxide concentration inside the nest chamber every 15 min, for a period of 120 min.

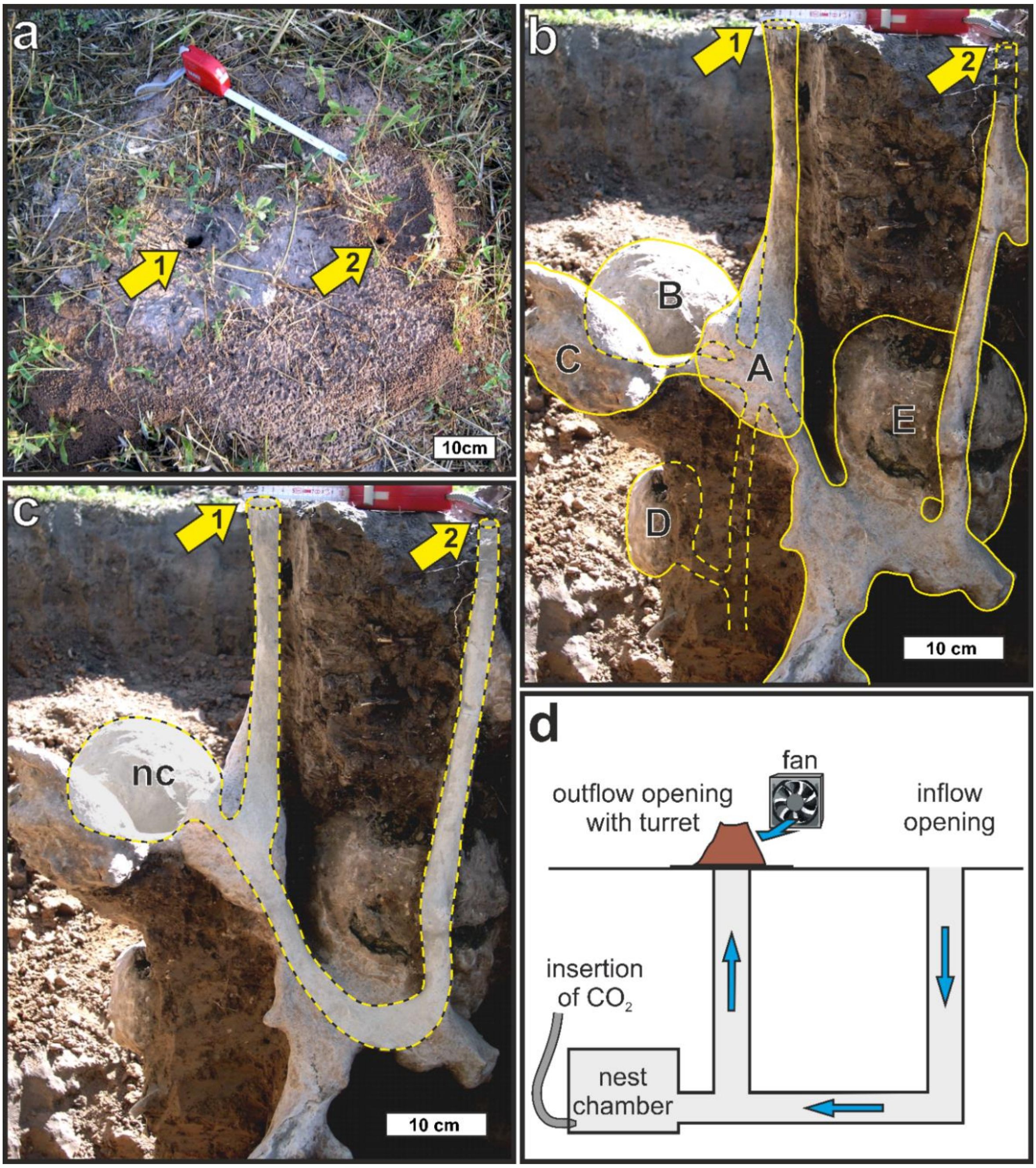

Figure 29: Nest structure of a small field nest of *Atta vollenweideri.* a) External view of a small nest with two nest openings, indicated by arrows. Note the heaps of excavated soil around the openings. b) Cement cast of the same nest, showing the internal structure of the nest, outlined in yellow. Dotted lines indicate structural features not visible in the picture. The two openings, marked by arrows, lead as separate tunnels to the underground nest, consisting of the further-enlarged initial queen chamber (A), as well as several fungus chambers (B, C, D, E) excavated during colony growth. c) The outlined part of the same nest, i.e., the connection of the tunnels to a single nest chamber (nc), served as template for the physical model of the nest. All photos were taken by Flavio Roces in Formosa, Argentina. d) Simplified nest model consisting of a single chamber and two vertical tunnels, one providing inflow and the other one outflow at the nest opening. CO_2 was inserted into the nest chamber to generate increased underground carbon dioxide levels, and the CO_2 clearance rate was measured depending on the presence of airflow and a ventilation turret at the outflow opening.

We performed four experimental series, differing from each other concerning opening state of the nest, the presence of airflow as well as the presence of a turret at the outflow opening. In the first series, both tunnel openings were closed using rubber stoppers to quantify the loss of carbon dioxide from the nest chamber over the 120 min period in a closed model system. In a second series, both nest openings were opened, but no airflow was generated at the nest surface. In the third series, we tested whether the presence of airflow in the nest influenced the rate of carbon dioxide removal from the chamber by generating surface airflow with a velocity of approximately 40 cm/s on top of one of the openings. The surface airflow resulted in outflow of air at the selected opening, and inflow of air at the other tunnel opening with a velocity of ca. 2-10 cm/s. In the fourth series, we tested whether the presence of both, airflow and a ventilation turret at the outflow opening further improved the removal of carbon dioxide from the nest model.

Turrets used in the fourth series were naturally-constructed by ants beforehand in the laboratory, by offering a mixture of clay and sand on a building platform in an open arena to a laboratory colony of *Atta vollenweideri*, a method that has been established in previous experiments (see Chapter V for a detailed description). After 24 hours, both the turret and the supporting building platform were removed from the arena, cleared of all remaining ants, and placed on top of the outflow opening of the nest model as shown in Figure 29d. As a result of the natural building process by the ants, the constructed turrets were not uniform, but varied greatly concerning their structural features. This variability, however, allowed us to additionally quantify the influence of structural features, i.e., turret height, number of turret openings and total turret aperture on the removal of carbon dioxide from the artificial nest.

2.2. Statistical analysis

For all series, the carbon dioxide levels in the nest chamber were log-transformed and analyzed using ANCOVA with time as covariate. Post-hoc between-group comparisons were conducted using Bonferroni tests. The influence of turret structure on the CO_2 removal from the nest chamber was analyzed using linear regressions. The significance level in all tests was $\alpha = 0.05$.

3. Results

In all four series the carbon dixode levels measured inside the nest chamber decreased over time (Fig. 30). At the beginning of the experiment, the CO_2 levels inside the nest chamber averaged 5.03 ± 0.02% (mean ± SD) in the series with closed tunnel openings, 5.03 ± 0.07% (mean ± SD) in the series with opened tunnels, 5.01 ± 0.03% (mean ± SD) in the series with opened tunnels and airflow, and 5.02 ± 0.06% (mean ± SD) when tunnels where opened and both airflow and a turret were present (Fig. 30a). After 120 min, at the end of the experiment, the carbon dioxide concentration had decreased to 3.47 ± 0.17% (mean ± SD) in the closed series and to 2.74 ± 0.24% (mean ± SD) in the opened series without airflow. When airflow was generated at the nest opening, the carbon dioxide concentration dropped to 1.22 ± 0.27% (mean ± SD) without a turret and to 0.56 ± 0.36% (mean ± SD) with a turret at the outflow opening. Since the decrease of CO_2 followed a non-linear decay, the measured carbon dioxide concentrations were log-transformed to allow for a linear regression analysis (Fig. 30b). The CO_2 levels inside the nest chamber significantly decreased over time (ANCOVA: $F_{1,373} = 504.71$, $p < 0.001$) and the clearance rate significantly differed among the series (ANCOVA: $F_{3,373} = 166.88$, $p < 0.001$). The lowest CO_2 clearance rates, i.e., the highest CO_2 levels at the end of the experiment, were recorded without the presence of airflow, when the nest openings were either closed or opened, with no statistically significant difference between the series. In contrast, when airflow was generated in the tunnels and at the nest opening, the carbon dioxide clearance rate was significantly higher, i.e., less CO_2 remained in the nest chamber at the end of the experiment. The highest clearance rate was recorded for the series where both airflow as well as a turret were present at the nest opening. Here, almost all carbon dioxide that was initially injected into the chamber was removed within the duration of the experiment.

In contrast to the first three series, the carbon dioxide levels in the nest chamber in the fourth series using turrets displayed a high level of variance (see Fig. 30, black circles), as the CO_2 clearance rates within the series were different depending on the structural features of the turrets (Fig. 31).

The height of the naturally-built turrets varied between 3.5 and 4.8 cm (Fig. 31a), yet no statistically significant relationship between turret height and CO_2 clearance rates was observed (Linear regression: $F_{1,10} = 1.46$, $p = 0.255$). Constructed turrets had between 1 and 4 openings (Fig. 31b), but the number of openings had no influence on the CO_2 clearance rates (Linear regression: $F_{1,10} = 1.06$, $p = 0.328$). However, the carbon dioxide levels in the nest chamber were strongly affected by the total aperture of the turret, which ranged from 1.8 to

6.7 cm² (Fig. 31c), as the CO$_2$ clearance rates were higher, the larger the total aperture of the turret (Linear regression: $F_{1,10} = 10.85$, $p < 0.01$). The calculated log transformed CO$_2$ clearance rates for the smallest (1.8 cm²) and the largest turret aperture (6.7 cm²) correspond to values of 1.97 and 2.48, respectively, meaning that by increasing the turret aperture by a factor of 3.7, the removal of carbon dioxide from the nest was increased by approximately 25%.

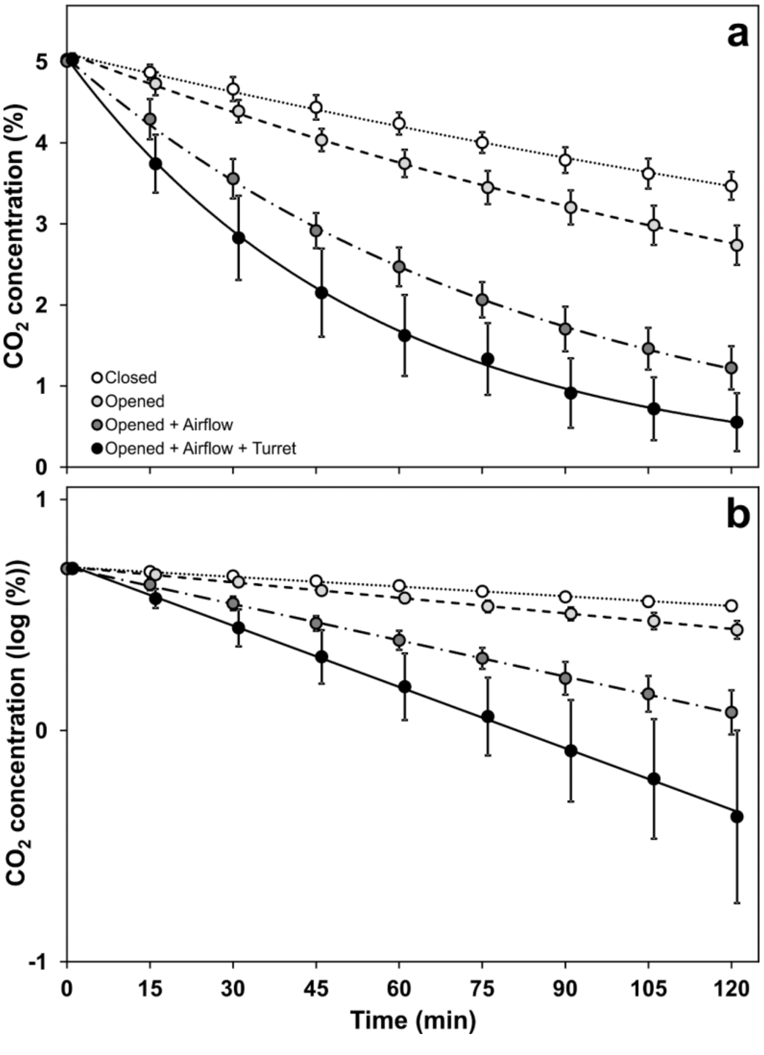

Figure 30: CO$_2$ levels inside the nest chamber as a function of time for all four experimental series. Nest openings were either closed (white circles, n = 10), opened (light grey circles, n = 10), opened containing airflow (dark grey circles, n = 10), or opened containing both airflow and a turret at the outflow opening (black circles, n = 12). a) Measured CO$_2$ concentrations in percent (mean ± SD) decreased exponentially over time for all series: closed ($y = 5.098{*}e^{-0.003x}$), opened ($y = 5.107{*}e^{-0.005x}$), opened + airflow ($y = 5.043{*}e^{-0.012x}$) and opened + airflow + turret ($y = 5.045{*}e^{-0.018x}$). b) Log-transformed CO$_2$ levels (mean ± SD) decreasing linearly over time for the series closed ($y = -0.001x + 0.707$, $R^2 = 1.00$), opened ($y = -0.002x + 0.706$, $R^2 = 1.00$), opened + airflow ($y = -0.005x + 0.704$, $R^2 = 1.00$) and opened + airflow + turret ($y = -0.009x + 0.710$, $R^2 = 1.00$). For clarity, data points are slightly offset horizontally. Detailed statistics can be found in A20.

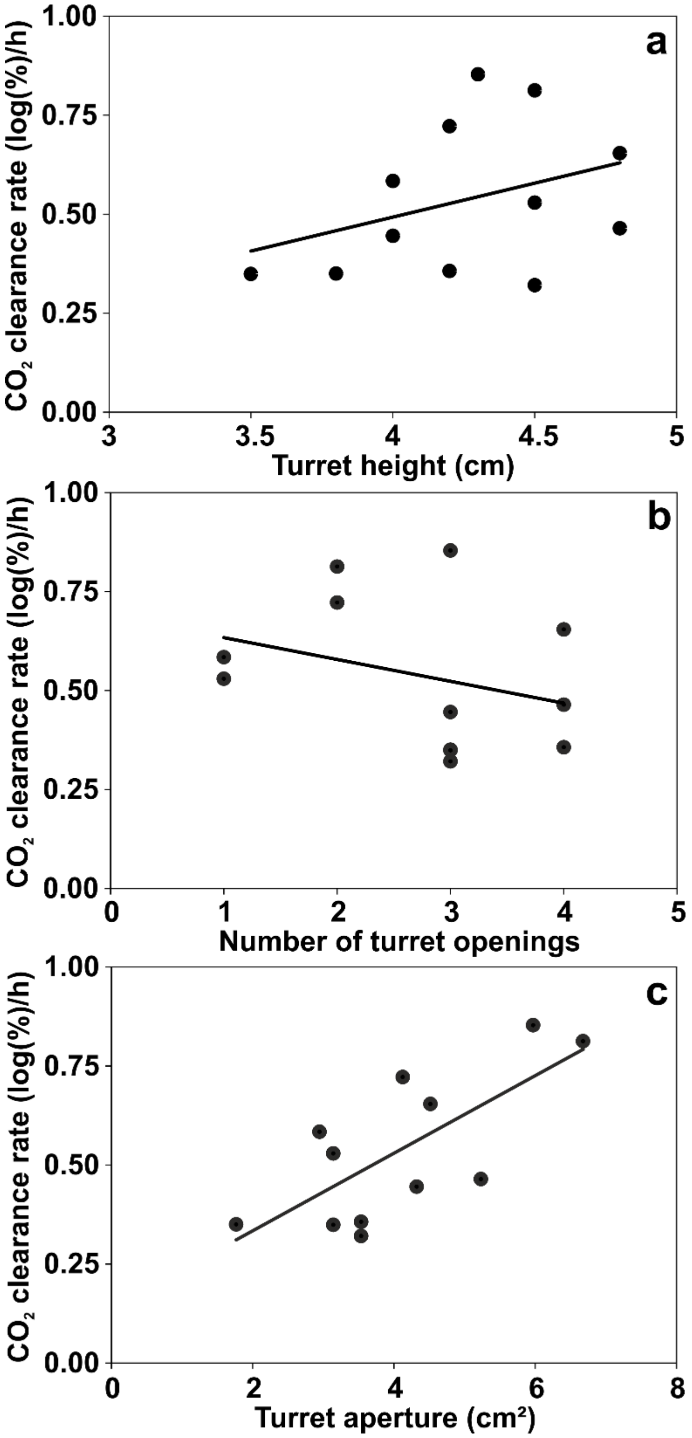

Figure 31: Influence of turret structure on the rate of CO_2 clearance inside the nest chamber. Log-transformed CO_2 clearance rates per hour were depicted as a function of a) Turret height ($y = 0.171x - 0.193$, $R^2 = 0.13$, $n = 12$), b) Number of turret openings ($y = 0.06x + 0.689$, $R^2 = 0.10$, $n = 12$) and c) Total turret aperture ($y = 0.098x + 0.138$, $R^2 = 0.51$, $n = 12$).

4. Discussion

In our physical model of a small *Atta vollenweideri* nest, the carbon dioxide levels inside the nest chamber continuously decreased over time and the rate of CO_2 removal from the chamber was strongly affected by both the presence of airflow and a ventilation turret at the nest opening. Without air moving through the tunnels, the carbon dioxide concentration in the nest decreased only slowly and most of the CO_2 remained inside the nest chamber at the end of the experiment. Opening or closing both nest tunnels had no strong effect on the carbon dioxide clearance rate, indicating that only a small amount of the gas left the nest model via the tunnel openings under windless conditions. Instead, the CO_2 that escaped the chamber was possibly absorbed by the material of the artificial nest or leaked out through other parts of the physical model. The low efflux of CO_2 via the tunnel openings might result from the higher density of carbon dioxide compared to air, causing it to accumulate at the bottom of the nest model. In an underground nest undisturbed by air movements, this can result in the emergence of a density gradient between shallow and deeper soil layers (Turner and Pinshow, 2015). However, completely windless conditions are rather exceptional in nature and usually there is a considerable amount of carbon dioxide that is emitted from subterranean ant nests via the nest openings (Sousa-Souto *et al.*, 2012; Hasin *et al.*, 2014).

In our experiments, the amount of carbon dioxide that was removed from the nest model increased when a continuous surface wind was generated above one of the nest openings. Due to differences in air pressure between the two openings, air was dragged out of the first tunnel followed by an inflow of air at the second tunnel. This mechanism, based on the Bernoulli principle and viscous entrainment (Vogel and Bretz, 1972), has been shown to facilitate the ventilation in underground nests of leaf-cutting ants (Kleineidam and Roces, 2000; Kleineidam *et al.*, 2001). It is important to note that air moving through the tunnels does not enter the fungus chambers directly, but instead contributes to the ventilatory effect by acting as an O_2 source and CO_2 sink (Bollazzi *et al.*, 2012). This is further supported by the fact that the carbon dioxide levels in the nest chamber decreased exponentially instead of linearly over time. Since the CO_2 gradient between the tunnel air and the chamber air is steeper at the beginning of the experiment, diffusion rates should be higher at the beginning as well, but decrease towards the end of the experiment as more CO_2 is removed from the nest chamber.

The efflux of carbon dioxide from the nest model was further increased when a turret was placed above the outflow tunnel. Turrets were previously constructed from a mixture of clay and sand by workers of a laboratory colony of *Atta vollenweideri*. The presence of both airflow and a turret at the tunnel opening resulted in the highest clearance rate, as almost all the CO_2 was removed within two hours, i.e., carbon dioxide levels inside the chamber were well below 1% at the end of the experiment. Under natural conditions, the construction of a turret on a nest tunnel is expected to promote the wind-induced ventilation by elevating the nest opening relative to the ground and exposing it to increased surface wind speeds (Kleineidam *et al.*, 2001). Although the speed of the airflow generated by the fan was the same with or without the presence of a turret, the air passing over the tunnel opening is forced to flow around the turret, potentially increasing its velocity. As a result, the pressure gradient between the air above the turret and inside the tunnel might have been increased, further enhancing the outflow of CO_2-rich air from the nest model.

The CO_2 clearance rate from the chamber was affected by one of the structural features of the turret placed on the tunnel opening, namely the total turret aperture. Interestingly, turret height had no significant effect on the removal of carbon dioxide, despite being an important factor contributing to the ventilation of a nest under natural conditions. As mentioned before, the construction of a turret on top of the outflow tunnel elevates the nest opening and exposes it to higher wind speeds. In our experiments, however, the velocity of the airflow that was locally generated at the nest surface was set to approximately 40 cm/s and did not increase with increasing height. Although the air passing over the nest opening was forced to flow around the turret, thus increasing its velocity, the influence of the turret's height on this mechanism might have been negligible in our model system. While there was some variation concerning the height of the constructed turrets, they never exceeded 5 cm and the difference between the shortest and the tallest turret only was 1.3 cm. In contrast, turrets constructed on field nests of *Atta vollenweideri* may reach a height of 20-30 cm (Jonkman, 1980b). In combination with the height of the nest mound itself, turret openings can be elevated up to 1 m above the ground level and thus should be exposed to even higher surface wind speeds of several m/s (Geiger, 1956).

While the number of turret openings per se had no effect on the CO_2 clearance rate, a larger total aperture of the turret significantly enhanced the removal of carbon dioxide from the nest chamber. By increasing the aperture of the turrets, workers effectively increase the surface area of the nest entrance, which has been shown to positively influence the CO_2 efflux from ant nests (Hasin *et al.*, 2014). A larger surface area of the nest opening might facilitate the exchange of gases with the environment either by increasing the amount of air that can be dragged out of the nest tunnel, or by increasing diffusion between the nest air and the environment, similar to the mechanism in the respiratory system of animals (Forster, 1957). As previously shown, turrets with an increased total aperture are predominantly constructed when the carbon dioxide levels in the nest are elevated up to 5%, indicating poor ventilation of the nest (see Chapter V). Together with the present findings, these results support the hypothesis that workers of *Atta vollenweideri* specifically modify the structure of their turrets to counter increasing CO_2 levels in the nest air and improve the wind-induced ventilation. In the field, colonies of *Atta vollenweideri* are known to close the openings of their turrets for example during rain (Jonkman, 1980b), resulting in a rapid increase of carbon dioxide in the nest (Kleineidam and Roces, 2000). After rains, workers re-open their nest entrances and show an increased turret building activity (Jonkman, 1980b), allowing for a renewal of the nest air. While it is unlikely that workers again reduce the aperture of the turret openings as soon as the CO_2 concentration has reached a tolerable level, the trade-off between gas exchanges and humidity or temperature loss from the nest might cause workers to continuously regulate the opening state of their nest depending on the prevailing climatic conditions. Although purely speculative, being able to frequently adjust the aperture of their turret openings might be the reason why workers construct turrets with multiple smaller openings instead of a single large opening (see Chapter V). While hypothetically having the same effect on ventilation, smaller openings should be easier to close or re-open than larger ones.

In our experiments, we simulated the ventilation in small nests of *Atta vollenweideri* consisting of a single nest chamber and two connected nest tunnels. While unidirectional airflow through the nest requires only two nest openings, both the conically-shaped nest mound and the conspicuous turrets that facilitate the wind-induced ventilation are predominantly found in larger, mature colonies. Therefore, it might be more difficult for smaller colonies to prevent the accumulation of carbon dioxide in the underground and maintain a proper nest climate, especially until enough soil is excavated in the underground to form a nest mound on the surface, which results in elevation differences between their nest openings. During the first year, nests of *Atta vollenweideri* only consist of a few chambers and a single nest opening (Jonkman, 1980c), rendering an effective ventilation via unidirectional airflow highly unlikely. However, as their few chambers are likely located in more superficial soil layers compared to those of adult nests, they might not be exposed to very high carbon dioxide levels in the underground. Thus it remains elusive whether small, single-opening nests require such an elaborate ventilation mechanism in the first place.

Other fungus-growing ants inhabiting single-entranced nests are known to erect walls around their nest opening to prevent flooding (Navarro and Jaffé, 1985; LeBrun *et al.*, 2011) or build funnel-like structures of unknown purpose (Mueller and Wcislo, 1998), and small chimneys on top of nest entrances have also been reported for young *Atta* nests (Weber, 1972b). Thus, the construction of a turret might be advantageous for small colonies of *Atta vollenweideri* as well, especially since their nests are also prone to inundation without the presence of an elevated nest mound (Pielström and Roces, 2014). Whether the gas exchanges with the environment in a nest with a single tunnel opening would benefit from the presence of a turret, however, remains to be investigated.

VII. General discussion

1. Recapitulation of main findings

Before discussing the results in the next paragraphs, a brief summary of the main findings of the thesis is given. The present work was aimed at exploring the environmental stimuli that act as cues for workers of the leaf-cutting ant *Atta vollenweideri* and influence their building responses in the nest. It focused on climatic variables related to the airflow-driven gas exchanges in the underground, i.e., air movements and their direction, as well as carbon dioxide and humidity levels in the nest air.

It was shown that workers were able to use air movements and their direction as learned orientation cue during foraging, which should also allow them to navigate in the underground during tasks related to climate control as well to use the airflow directions they experience inside the nest tunnels as cues (Chapter II).

Elevated carbon dioxide levels did not affect the digging rate of worker groups over a biologically relevant range, but ants mostly preferred to excavate at lower CO_2 concentrations when given a choice, a behavior potentially involved in the formation of new or broadening of existing nest openings (Chapter III).

During soil transport, workers preferably carried their pellets along tunnels containing elevated carbon dioxide levels when previously exposed to increased CO_2 levels. Additionally, more pellets were transported along tunnels providing outflow instead of inflow of humid air, a mechanism that might promote the construction of ventilation turrets on the nest. However, workers preferred the transport of soil along inflow tunnels when facing inflow of dry air with a high velocity (Chapter IV).

The construction of turrets was mainly influenced by carbon dioxide, not by airflow or humidity, as turrets constructed under elevated CO_2 concentrations had more openings and a larger total aperture compared to turrets constructed under atmospheric levels (Chapter V).

This modification of turret structure most likely facilitates air movements inside the nest and results in an increased rate of carbon dioxide removal from the underground, as demonstrated using a physical model of a small nest (Chapter VI).

Taken together, the results indicate that workers of the leaf-cutting ant *Atta vollenweideri* react to the environmental conditions inside their nest with specific building responses that might help restoring and maintaining a suitable nest climate in the underground.

2. Climatic variables and regulatory responses in social insects

Behavioral responses to unfavorable climatic conditions aimed at maintaining a suitable nest climate are common among social insects and have been reported for bees, wasps, ants and termites (Jones and Oldroyd, 2006). In bees, workers respond to low nest temperatures by aggregating and increasing metabolic heat production (Kronenberg and Heller, 1982; Stabentheiner, 2005). Increased temperatures or elevated carbon dioxide levels in the nest, on the other hand, cause bees to respond with wing fanning behavior in order to improve nest ventilation (Seeley, 1974; Weidenmüller *et al.*, 2002). In addition, fanning prevents overheating and desiccation of the brood via the evaporation of previously collected water (Lindauer, 1955), a behavior also observed in wasps (Kasuya, 1982). While the production of heat via metabolic activity or heat transfer inside the nest have also been described for army ants (Franks, 1989) or wood ants, respectively (Horstmann and Schmid, 1986; Rosengren *et al.*, 1987; Frouz, 2000; Kadochová *et al.*, 2017), ants as well as termites lack the ability to ventilate their nest for example via fanning. Instead their colonies mostly rely on the architecture of the nest in order promote the gas exchanges with the environment or maintain suitable climatic conditions in the nest. Thus, colonies show specific building responses and modify structural features of their nests depending on environmental cues encountered by workers. In termites of the subfamily Macrotermitinae, ambient temperature patterns influence the structure of their giant mounds and thus whether they are predominantly ventilated via temperature or airflow-driven mechanisms (Korb and Linsenmair, 2000). For some species of *Acromyrmex* leaf-cutting ants, soil temperature can determine whether colonies construct superficial or subterranean nests (Bollazzi *et al.*, 2008) and the humidity levels in the nest air determine whether nest entrances are opened or closed (Bollazzi and Roces, 2007, 2010a). The results obtained in this work indicate that the environmental variables encountered by workers of *Atta vollenweideri* elicit similar building responses for the purpose of restoring and maintaining suitable climatic conditions inside their nest.

3. The regulation of nest climate in *Atta vollenweideri*

For leaf-cutting ants, proper growth of the symbiotic fungus in the nest is crucial for colony survival. Colonies are expected to maintain a suitable microclimate in the nest and avoid conditions that might be detrimental for fungal growth in the underground. Besides temperature limitations, the risk of fungus desiccation due to low humidity levels in the nest air, as well as the inhibition of its respiration due to hypoxic and hypercapnic conditions, pose some of the main problems for leaf-cutting ant colonies. Usually, both factors cannot be regulated independently, as the manipulation of one factor influences the other, often creating a trade-off between humidity control and gas exchanges inside the nest of leaf-cutting ants (Bollazzi and Roces, 2007). The present work identified some of the building responses of workers that may aid the maintenance of a suitable nest climate, i.e., facilitate the ventilation or prevent the desiccation of the nest. Integrating these results together with information available from the literature, a flow diagram is proposed aimed at describing the potential mechanisms underlying the regulation of the nest climate in *Atta vollenweideri* (Fig. 32).

Due to the respiration of the fungus as well as soil depth, oxygen levels are decreased while carbon dioxide levels are increased in the underground (Kleineidam and Roces, 2000; Bollazzi *et al.*, 2012). An increase in carbon dioxide concentrations in the nest causes workers to shift their digging activity to areas in the nest with lower CO_2 levels (Chapter III) possibly resulting in an increased excavation in more shallow soil layers or even the creation of new nest openings. In addition, the soil excavated in the underground is preferably transported along tunnels providing outflow of air and high carbon dioxide levels (Chapter IV), where it might be used to elevate nest openings and promote the construction of ventilation turrets on the center of the mound. The ability of workers to differentiate between inflow and outflow channels and to learn locations based on the prevailing airflow directions (Chapter II) most likely helps workers during this task. Outflow of CO_2-rich air from the nest also causes workers to construct turrets with multiple openings and an increased aperture (Chapter V). Resulting from the workers' building responses, the wind-induced ventilation and thus airflow within the nest should be promoted, removing CO_2 from the underground (Chapter VI) and ensuring the supply of the nest with fresh air.

However, increasing the airflow in the tunnels might come at the expense of high humidity levels inside the nest. Especially during the winter months (dry season), inflow of dry air and outflow of humid air from the nest can cause desiccation of the symbiotic fungus. Inflow of dry air at high velocities thus causes workers to transport soil not along outflow, but inflow tunnels instead (Chapter IV). Workers might use the building material here to reduce the inflow of dry air by closing the nest openings, a behavior already observed in field colonies of *Atta vollenweideri* (Jonkman, 1980b) and laboratory colonies of *Acromyrmex ambiguus* (Bollazzi and Roces, 2007). While the closing of nest entrances and exits prevents the loss of humidity from the nest air, gas exchanges between the nest and the environment are also hindered, resulting again in the rapid accumulation of carbon dioxide in the underground (Kleineidam and Roces, 2000), especially in the clay-heavy, low air-permeable soils inhabited by *Atta vollenweideri* (Jonkman, 1976). As a consequence colonies might be constantly faced with the trade-off between gas exchange and humidity control in their nest and are required to balance airflow, carbon dioxide and humidity levels in the underground.

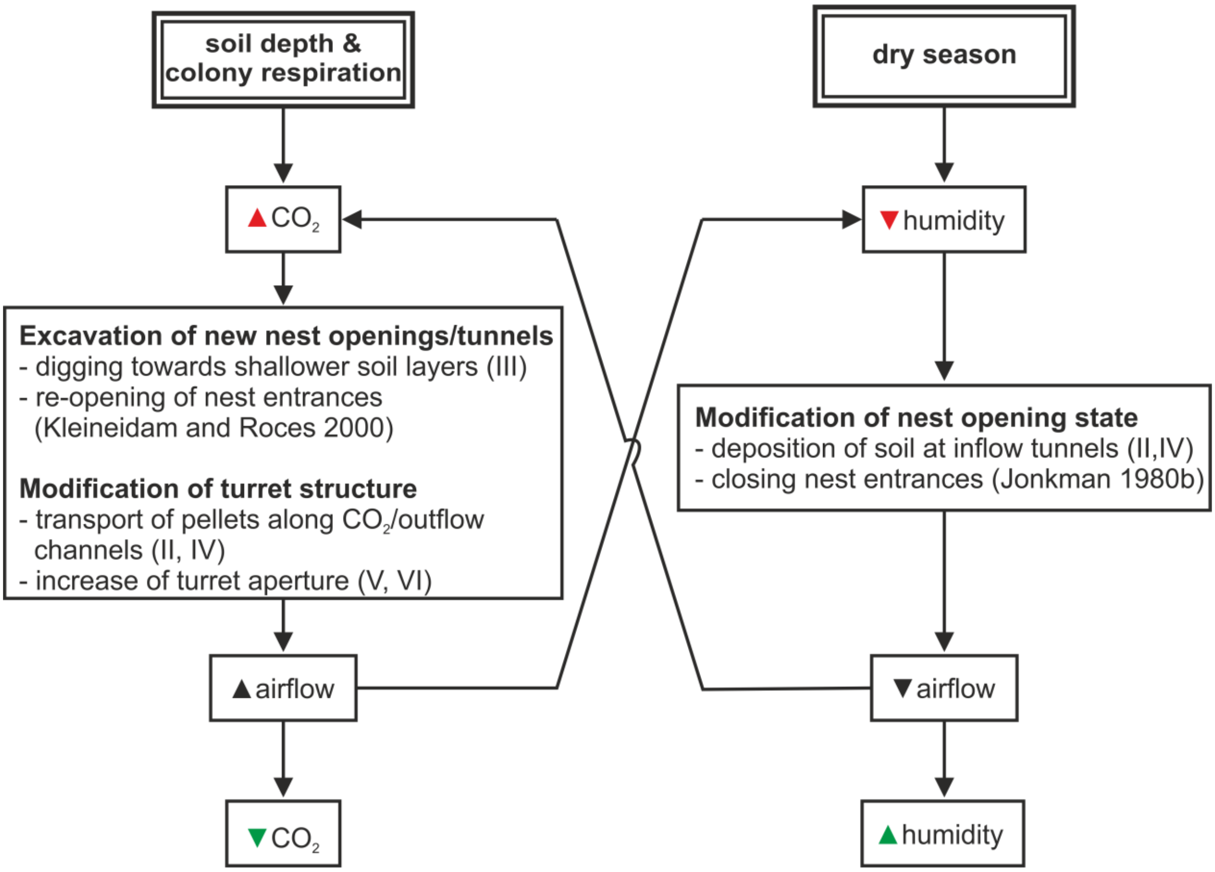

Figure 32: Flow diagram of the regulation of nest climate in the leaf-cutting ant *Atta vollenweideri* based on information from the literature and the results obtained in this work. Left side: Regulation of carbon dioxide levels. Right side: Regulation of humidity levels. Roman numerals indicate the chapters describing the respective building responses in detail. Triangles indicate an increase (▲) or decrease (▼) in airflow, carbon dioxide or humidity levels inside the nest, with positive (green) or negative (red) effects on the nest climate.

4. Outlook

The present work investigated some of the environmental stimuli that act as cues and affect the building responses of workers of the leaf-cutting ant *Atta vollenweideri*. Since their nests rely heavily on a wind-induced ventilation mechanism, this work focused on cues related to airflow, carbon dioxide levels and air humidity inside the nest. However, under natural conditions, other cues like temperature or moisture gradients in the underground are important for fungal growth as well and might also act as environmental cues for workers influencing their building responses in the nest. Thus, the proposed mechanisms for the regulation of the nest climate in the leaf-cutting ant *Atta vollenweideri* need to be extended accordingly. Furthermore, while specific building responses depending on the environmental cues inside the nest of leaf-cutting ants have been identified in this work, their presumed effect on the climatic conditions in nests of *Atta vollenweideri* should be experimentally verified, especially in field colonies. Finally, while there is some understanding of the regulation of the environmental conditions in mature colonies, almost no information is available concerning the climate regulation in smaller nests of *Atta vollenweideri* leaf-cutting ants or if it is necessary at all, since they usually lack the structural features required for an elaborate wind-induced nest ventilation, as found in adult colonies.

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Appendix

A1: Comparison of the number of ants performing a task after 0.5h vs. after 5h (Mann-Whitney-U test):

	Excavating		**Soil transport**		**Other**	
Series	**U**	**p**	**U**	**p**	**U**	**p**
0.04%	4.50	< 0.001	7.00	< 0.001	48.00	0.167
2%	0.00	< 0.001	1.00	< 0.001	33.00	0.024
4%	0.00	< 0.001	34.50	0.019	2.50	< 0.001
6%	0.00	< 0.001	14.00	< 0.001	17.50	0.002
10%	0.00	< 0.001	0.00	< 0.001	1.50	< 0.001

A2: Group-wise comparison of the number of ants performing a task at 0.5 hours depending on carbon dioxide levels (Dunn's Multiple Comparison test, post-hoc).

Series	**Excavating**	**Soil transport**	**Other**
0.04% vs 2%	p = 1.000	p = 0.901	p = 0.155
0.04% vs 4%	p = 0.030	p = 1.000	p = 0.048
0.04% vs 6%	p = 0.397	p = 1.000	p < 0.001
0.04% vs 10%	p = 1.000	p = 0.177	p = 0.001
2% vs 4%	p = 1.000	p = 1.000	p = 1.000
2% vs 6%	p = 1.000	p = 0.553	p = 0.901
2% vs 10%	p = 1.000	p = 1.000	p = 1.000
4% vs 6%	p = 1.000	p = 1.000	p = 1.000
4% vs 10%	p = 0.118	p = 1.000	p = 1.000
6% vs 10%	p = 1.000	p = 0.0947	p = 1.000

A3: Group-wise comparison of the number of ants performing a task at 2.5 hours depending on carbon dioxide levels (Dunn's Multiple Comparison test, post-hoc).

Series	Excavating	Soil transport	Other
0.04% vs 2%	$p = 1.000$	$p = 1.000$	$p = 0.737$
0.04% vs 4%	$p = 1.000$	$p = 0.970$	$p = 0.245$
0.04% vs 6%	$p = 0.003$	$p = 0.728$	$p < 0.001$
0.04% vs 10%	$p = 1.000$	$p = 1.000$	$p = 0.901$
2% vs 4%	$p = 1.000$	$p = 0.185$	$p = 1.000$
2% vs 6%	$p = 0.022$	$p = 0.128$	$p = 0.096$
2% vs 10%	$p = 1.000$	$p = 1.000$	$p = 1.000$
4% vs 6%	$p = 0.050$	$p = 1.000$	$p = 0.334$
4% vs 10%	$p = 1.000$	$p = 1.000$	$p = 1.000$
6% vs 10%	$p = 0.028$	$p = 0.970$	$p = 0.073$

A4: Group-wise comparison of the number of ants performing a task at 5 hours depending on carbon dioxide levels (Dunn's Multiple Comparison test, post-hoc).

Series	Excavating	Soil transport	Other
0.04% vs 2%	$p = 1.000$	$p = 1.000$	$p = 1.000$
0.04% vs 4%	$p = 0.116$	$p = 0.369$	$p = 0.032$
0.04% vs 6%	$p = 1.000$	$p = 1.000$	$p = 1.000$
0.04% vs 10%	$p = 0.005$	$p = 1.000$	$p = 0.006$
2% vs 4%	$p = 1.000$	$p = 0.100$	$p = 0.648$
2% vs 6%	$p = 1.000$	$p = 1.000$	$p = 1.000$
2% vs 10%	$p = 0.128$	$p = 0.349$	$p = 0.200$
4% vs 6%	$p = 0.339$	$p = 1.000$	$p = 0.375$
4% vs 10%	$p = 1.000$	$p = 1.000$	$p = 1.000$
6% vs 10%	$p = 0.019$	$p = 1.000$	$p = 0.105$

A5: Group-wise comparison of the amount of material excavated among all series (Tukey's Multiple Comparison test, post-hoc):

Series	**0.04%**	**2%**	**4%**	**6%**
2%	p = 0.999			
4%	p = 0.830	p = 0.704		
6%	p = 1.000	p = 1.000	p = 0.885	
10%	p = 0.001	p < 0.001	p = 0.030	p = 0.002

A6: Group-wise comparisons of the total mass of pellets deposited inside the digging arena among all series (Tukey's Multiple Comparison test, post-hoc):

Series	**0.04%**	**2%**	**4%**	**6%**
2%	p = 0.968			
4%	p = 0.967	p = 0.705		
6%	p = 0.314	p= 0.091		
10%	p = 0.070	p = 0.013	p = 0.263	p = 0.944

A7: Group-wise comparisons of the total mass of pellets deposited outside the digging arena among all series (Dunn's Multiple Comparison test, post-hoc):

Series	**0.04%**	**2%**	**4%**	**6%**
2%	p = 0.939			
4%	p = 0.996	p = 0.994		
6%	p = 0.417	p = 0.103	p = 0.232	
10%	p = 0.261	p = 0.708	p = 0.457	p = 0.004

A8: Group-wise comparison of the number of ants present per arena over the course of the trial (Wilcoxon-signed-rank test):

	1h		**2h**		**3h**		**4h**	
Series	**Z**	**p**	**Z**	**p**	**Z**	**p**	**Z**	**p**
0.04% vs 0.04%	0.057	0.955	0.052	0.959	0.879	0.379	1.836	0.066
0.04% vs 1%	0.052	0.959	1.060	0.289	1.601	0.109	0.682	0.496
0.04% vs 5%	1.306	0.191	2.044	0.041	1.939	0.052	0.982	0.326
1% vs 5%	0.682	0.496	0.724	0.469	1.193	0.233	2.101	0.036
5% vs 5%	0.126	0.900	0.052	0.959	0.170	0.865	1.422	0.155

A9: Group-wise comparison of the amount of material excavated per arena (Mann-Whitney-U Test):

Series	U	p
0.04% vs. 0.04%	101.5	0.327
0.04% vs. 1%	78.00	0.062
0.04% vs. 5%	38.50	< 0.001
1% vs. 5%	100.50	0.309
5% vs. 5%	73.00	0.040

A10: Comparison of workers' choices for carbon dioxide during pellet transport (G-Test Goodness of fit):

	Underground CO_2					
	0.04%		**1%**		**5%**	
Series	**G**	**p**	**G**	**p**	**G**	**p**
Control	0.20	0.654	0.20	0.654	0.20	0.654
0.04% vs. 1%	0.20	0.654	0.00	1.000	5.23	0.022
0.04% vs. 5%	0.00	1.000	0.20	0.654	14.72	< 0.001
1% vs. 5%	0.81	0.369	5.23	0.022	7.71	0.005

A11: Comparison of workers' choices for airflow direction depending on airflow velocity and air humidity (G-Test Goodness of fit):

	Humid air		**Dry air**	
Series	**G**	**p**	**G**	**p**
0 cm/s	0.20	0.654	0.00	1.000
5 cm/s	6.79	0.005	4.94	0.026
10 cm/s	3.40	0.065	3.40	0.065

A12: Group-wise comparison of turret height depending on CO_2 concentration (Tukey's Multiple Comparison test, post-hoc).

Series	0.04%	1%	5%
0.04%			
1%	p = 0.807		
5%	p = 0.054	p = 0.307	
10%	p = 0.006	p = 0.057	p = 0.821

A13: Group-wise comparison of number of turret openings depending on CO_2 concentration (Dunn's Multiple Comparison test, post-hoc).

Series	**0.04%**	**1%**	**5%**
0.04%			
1%	$p = 0.501$		
5%	$p < 0.001$	$p = 0.057$	
10%	$p < 0.001$	$p = 0.053$	$p = 1.000$

A14: Group-wise comparison of total turret aperture depending on CO_2 concentration (Tukey's Multiple Comparison test, post-hoc).

Series	**0.04%**	**1%**	**5%**
0.04%			
1%	$p = 0.996$		
5%	$p < 0.001$	$p < 0.001$	
10%	$p = 0.239$	$p = 0.161$	$p = 0.006$

A15: Group-wise comparison of largest opening aperture depending on CO_2 concentration (Dunn's Multiple Comparison test, post-hoc).

Series	**0.04%**	**1%**	**5%**
0.04%			
1%	$p = 1.000$		
5%	$p = 0.250$	$p = 0.008$	
10%	$p = 1.000$	$p = 1.000$	$p = 0.010$

A16: Group-wise comparison of additional opening aperture depending on CO_2 concentration (Dunn's Multiple Comparison test, post-hoc).

Series	**0.04%**	**1%**	**5%**
0.04%			
1%	$p = 0.471$		
5%	$p < 0.001$	$p = 0.032$	
10%	$p = 0.002$	$p = 0.424$	$p = 1.000$

A17: Group-wise comparison of turret height depending on the presence of CO_2 gradients (Tukey's Multiple Comparison test, post-hoc).

Series	**0.04% open**	**5% open**	**0.04% closed**
0.04% open			
5% open	p = 0.148		
0.04% closed	p = 0.002		
5% closed		p = 1.000	p = 1.000

A18: Group-wise comparison of number of turret openings depending on the presence of CO_2 gradients (Dunn's Multiple Comparison test, post-hoc).

Series	**0.04% open**	**5% open**	**0.04% closed**
0.04% open			
5% open	p = 0.003		
0.04% closed	p = 0.324		
5% closed		p = 1.000	p = 0.046

A19: Group-wise comparison of total turret aperture depending on the presence of CO_2 gradients (Tukey's Multiple Comparison test, post-hoc).

Series	**0.04% open**	**5% open**	**0.04% closed**
0.04% open			
5% open	p = 0.011		
0.04% closed	p = 0.583		
5% closed		p = 0.369	p = 0.021

A20: Group-wise comparison of CO_2 clearance rates between all experimental series (Bonferroni Test, post-hoc).

Series	**Closed**	**Opened**	**Opened + Airflow**
Opened	p = 0.165		
Opened + Airflow	p < 0.001	p < 0.001	
Opened + Airflow + Turret	p < 0.001	p < 0.001	p < 0.001

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Publications

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Aug. 2016	**Poster presentation at the European IUSSI congress 2016** in Helsinki, Finland
Mar. 2015	**Poster presentation at the IV. Central European Meeting of the IUSSI 2015** in Lichtenfels, Germany
Dec. 2015	**Workshop “Frontiers in insect behavior II”** in Tempe, Arizona, United States
Sept. 2014	**Poster presentation at the 107th Annual Meeting of the German Zoological Society** in Göttingen, Germany
May 2014	**Workshop “Frontiers in insect behavior I”** in Würzburg, Germany
Sept. 2013	**Poster presentation at the 5th Central European Workshop of Myrmecology** in Innsbruck, Austria

Würzburg, January 2018 Florian Halboth

Affidavit/Eidesstattliche Erklärung

I hereby confirm that my thesis entitled “*Building behavior and nest climate control in leaf-cutting ants: How environmental cues affect the building responses of workers of Atta vollenweideri*” is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Würzburg, January 2018

Place, Date Florian Halboth

Hiermit erkläre ich an Eides statt, die Dissertation „*Bauverhalten und Kontrolle des Nestklimas bei Blattschneiderameisen: Wie Umweltreize die Bauaktivität von Arbeiterinnen der Art Atta vollenweideri beeinflussen*“ eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Würzburg, Januar 2018

Ort, Datum Florian Halboth