Where and how to build?

Influence of social and environmental cues on nest building behavior in leaf-cutting ants

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Photo title page: Cement cast of Atta laevigata nest in Botucatu, Brazil; source: W. Thaler

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Summary

This thesis explores the influence of social and environmental cues on the nest building behavior of leaf-cutting ants. Especially, the investigations are aimed at evaluating the mechanisms of nest building and how the nest environment can spatially guide building responses that lead to an adaptive nest architecture.

Many species of leaf-cutting ants build underground nests, where they rear a symbiotic fungus and the colony brood in spherical or dome-shaped nest chambers. These are connected to the soil surface by a system of tunnels. It has been hypothesized that workers excavate empty nest chambers in advance at underground sites where they encounter environmental conditions suitable for brood and fungus rearing. However, for such a cavity to emerge, a high local ant density at such places would be needed. Climatic conditions alone might not be sufficient to trigger ant aggregation at a particular site within the nest, as they are expected to prevail across a wider spatial range. In contrast to the hypothesis, it could be experimentally demonstrated in Chapter 2 of the present work that leaf-cutting ants (*Acromyrmex lundi*) do not excavate a chamber at sites where they encounter only suitable climatic conditions. Rather, when presented in the laboratory with a choice between two otherwise identical digging sites, offering suitable environmental conditions, but one containing brood, they displayed a higher excavation activity at the site where they encountered the putative content of a chamber. The shape of the excavated cavity was also more round and chamber-like. It is proposed that the self-organized digging activity was spatially guided by the increased ant aggregation around worker-attracting brood and fungus, and aided by the use of the symbiotic fungus as a template during chamber excavation. It is concluded that leaf-cutting ants respond to social cues during nest building.

Excavation is a costly process and colonies have to spend a part of their energy stores on nest building, so that regulatory responses for the control of nest excavation are expected to occur. It is known from the literature that the overall size of ant nests correlates with worker numbers, so that larger colonies inhabit larger nests. It has been proposed that when colonies grow, workers react to the increased space demands by enlarging the existing nest. The voluminous fungus of leaf-cutting ants as well as the large mass of colony brood should increase the colony's space demands even further. However, when the effect of in-nest stores was experimentally evaluated in Chapter 3, their presence did not influence the ants' digging intensity. Stored brood and fungus did however influence the architecture of the excavated nest, resulting in more efforts for chamber excavation, and less for tunnel excavation. The ants' initial density at the beginning of the building process also influenced the ants' motivation to excavate.

In addition, workers appeared to create nest space exceeding the colonies' needs. This was attributed to the self-organized nature of nest building, where positive and negative feedback mechanisms lead to the gradual initiation and cessation of digging. Workers secondarily regulated nest size by the opportunistic refilling of unused space with excavated soil pellets, which they otherwise would have transported outside of the nest. While self-organized mechanisms appear to be involved in the nest building process, the social cues of the ants' environment during building clearly influence the nest architecture and lead to an adjustment of the nest size to the current space needs of the colony.

Leaf-cutting ants prefer warm and humid conditions for fungus culture, its proper growth being the basis for the survival of the colony. The ants' behavioral responses to different temperature and humidity values are thought to lead to the excavation of nests in soil layers that offer proper conditions for brood and fungus rearing. As the respiratory gas CO_2 is known to hinder fungus' respiration and to show a clear gradient in the soil, it was evaluated in Chapter 4, whether workers of the leaf-cutting ant *A. lundi*, when choosing a place for fungus culture, also make a decision based on the CO_2 concentration of a nest site. Workers did indeed avoid high CO_2-levels for fungus rearing but actually preferred CO_2-values in the range encountered close to the soil surface, where this species excavates their nests. Building responses according to existing CO_2-levels could therefore lead to the excavation of fungus chambers in proper depth for fungus culture. A negative effect of increasing CO_2 concentrations on digging activity was hypothesized. However, as presented in Chapter 5, different CO_2-levels did not affect excavation. It is therefore proposed that it is the workers' CO_2-preference for fungus rearing that leads to chamber excavation at proper CO_2 concentrations. The deposition of fungus by workers at a given site should influence local ant aggregation around these worker-attracting items. This should in turn trigger building responses at this site.

While fungus chambers make up a big part of a leaf-cutting ant nest, most leaf-cutting ants of the genus *Atta* also spent part of the colony's energy on excavating large, voluminous chambers for waste disposal, rather than scattering the material aboveground. Analog to the guiding of fungus chamber excavation by environmental factors, it is expected that leaf-cutting ants also show environmental preferences for waste management. Waste deposition under different environmental conditions was evaluated in Chapter 6. *Atta laevigata* workers did indeed show climatic preferences for the environment in which they establish a dumpsite. They preferred deposition in a warm and dry environment and showed no preference for specific CO_2-levels. The disparity of environmental preferences for fungus rearing, for which they prefer warm, humid and CO_2-specific sites, should lead to a physical separation of nest

chambers for these tasks. Judging by the results of Chapter 7, where the use of olfactory cues during waste management was evaluated, the continued accumulation of waste particles in a waste chamber seems to be based on the use of volatiles. These originate from the waste itself, and seem to be used as an orientation cue by workers relocating the material. The ensuing large accumulation of waste at one site should result in the emergence of more voluminous chambers for waste disposal.

Taken together, the findings presented in this thesis show that the workers' task-dependent behavioral responses to their abiotic and social nest environment, mediated by processes of self-organization, can lead to the collective construction of a nest offering conditions suitable for the colony's needs.

Zusammenfassung

Diese Arbeit erforscht den Einfluss von sozialen Hinweisen und Hinweisen aus der Umwelt (*cues*) auf den Nestbau bei Blattschneiderameisen. Insbesondere sollen die Mechanismen des Nestbaus ergründet werden, und wie die Umwelt des Nestes den Vorgang des Nestbauens räumlich beeinflussen kann, so dass eine adaptierte Nestarchitektur entsteht.

Viele Arten von Blattschneiderameisen bauen unterirdische Nester, in welchen sie ihren symbiontischen Pilz und die Brut der Kolonie in kugel- oder kuppelförmigen Kammern aufziehen. Diese sind durch ein System von Tunneln mit der Erdoberfläche verbunden. Es wurde vermutet, dass die Arbeiterinnen leere Nestkammern im Voraus graben, an einer Stelle, an der sie Umweltbedingungen vorfinden, die geeignet sind, um ihren Pilz zu züchten und ihre Brut aufzuziehen. Damit ein Hohlraum gegraben wird, ist jedoch eine hohe lokale Ameisendichte nötig. Klimatische Bedingungen sind vermutlich nicht ausreichend, um eine hohe Ameisenaggregation an einer bestimmten Stelle im Nest hervorzurufen, da diese über einen weiten, räumlichen Bereich bestehen. Entgegengesetzt der Hypothese konnte in Kapitel 2 dieser Arbeit gezeigt werden, dass Blattschneiderameisen (*Acromyrmex lundi*) keine Kammern an Orten graben, an denen sie lediglich geeignete klimatische Bedingungen vorfinden. Statt dessen konnte experimentell gezeigt werden, dass sie eine höhere Grabeaktivität an einem Ort zeigen, an dem sie den voraussichtlichen Inhalt einer solchen Nestkammer, Brut und symbiontischen Pilz, vorfinden. Ansonsten waren die Umweltbedingungen an beiden, simultan angebotenen Grabeorten identisch und für Brut und Pilz geeignet. Auch war die Form des gegrabenen Raumes rund und ähnlicher einer Nestkammer. Vermutlich steigert die Ablage attraktiven Pilzes und Brut die Aggregation der Arbeiterinnen, wodurch deren selbstorganisierte Grabeaktivität räumlich beeinflusst wird. Zusätzlich wurde die Entstehung einer Kammer durch die Benutzung des Pilzes als ‚Vorlage' durch die Arbeiterinnen unterstützt. Es wird geschlussfolgert, dass Blattschneiderameisen während des Nestbaus auf soziale Hinweise in ihrer Umgebung reagieren.

Das Graben eines Nestes ist ein energetisch aufwendiger Prozess. Um ihn zu bewerkstelligen muss die Kolonie einen Teil ihrer Energiereserven einsetzen. Daher ist anzunehmen, dass das Nestgraben durch Kontrollmechanismen reguliert wird. Es war bereits bekannt, dass die Gesamtnestgröße mit der Anzahl an Arbeiterinnen einer Kolonie korreliert, so dass größere Kolonien auch größere Nester bewohnen. Daher wurde vermutet, dass bei Koloniewachstum die Arbeiterinnen auf den erhöhten Platzbedarf durch Erweiterung des Nestes reagieren. Der sehr voluminöse Pilz der Blattschneiderameisen sowie die große Masse an Brut sollten den Platzbedarf einer Kolonie zusätzlich erhöhen. Wie im Kapitel 3 dargestellt,

hatte das Vorhandensein dieses potentiellen Nestinhalts jedoch keinen Einfluss auf die Grabeintensität von *A. lundi*. Allerdings war ein Einfluss auf die Architektur des gegrabenen Nestes zu beobachten, der dazu führte, dass mehr Kammervolumen und weniger Tunnelvolumen gegraben wurde. Auch beeinflusste die Ameisendichte zu Beginn des Grabeprozesses die Grabemotivation der Ameisen. Auch gruben die Arbeiterinnen Nester, die den Platzbedarf der Kolonie vermutlich überstieg. Dies wurde auf die Selbstorganisation des Nestgrabens zurückgeführt, in dem positive und negative Rückkopplungsmechanismen dazu führen, dass es zu einer graduellen Auslösung sowie Einstellung des Grabens kommt. Die Arbeiterinnen regulierten die Nestgröße sekundär, indem sie opportunistisch den unbenutzten Platz mit ausgegrabenen Lehm-Pellets auffüllten, welche sie ansonsten aus dem Nest heraus transportiert hätten. Offensichtlich sind Mechanismen der Selbstorganisation am Prozess des Nestbaus beteiligt, allerdings beeinflussen soziale Hinweise aus der Umgebung die Architektur des gegrabenen Nestes und führen zu einer Anpassung der Nestgröße auf den aktuellen Platzbedarf der Kolonie.

Blattschneiderameisen bevorzugen warme und feuchte Bedingungen, um ihren symbiontischen Pilz zu züchten, dessen Wachstum die Lebensgrundlage der Kolonie darstellt. Die Verhaltensantworten der Ameisen zu verschiedenen Temperaturen oder Umgebungsfeuchtigkeiten scheinen zu einem Aushub des Nestes in geeigneten Erdschichten zu führen, die gute Wachstumsbedingungen für den Pilz und die Brut der Kolonie bieten. Da bekannt ist, dass das Gas Kohlendioxid (CO_2) die Atmung des symbiontischen Pilzes negativ beeinflussen kann, und einen deutlichen Gradienten im Untergrund aufweist, wurde in Kapitel 4 untersucht, ob Arbeiterinnen der Art *Acromyrmex lundi*, wenn sie einen geeigneten Ort für das Kultivieren ihres Pilzes suchen, eine Entscheidung anhand der vorherrschenden CO_2-Konzentration an diesem Ort treffen. Tatsächlich vermieden die Arbeiterinnen hohe CO_2-Konzentrationen zum Anbau des Pilzes, zogen aber CO_2-Werte vor, die nahe der Erdoberfläche vorherrschen, wo die Nester von *A. lundi* zu finden sind. Grabereaktionen entsprechend der vorherrschenden CO_2-Konzentrationen könnten also zum Graben eines Nestes in geeigneten Tiefen zur Züchtung des Pilzes führen. Daher wurde ein negativer Einfluss von CO_2 auf die Grabeaktivität vermutet. Allerdings, wie in Kapitel 5 zu sehen, haben unterschiedliche CO_2-Konzentrationen keine Einfluss auf die Grabeaktivität der Ameisen. Aufgrund dessen wird postuliert, dass es die CO_2-Präferenz der Arbeiterinnen beim Anbau des Pilzes ist, die zum Graben einer Kammer bei geeigneten CO_2-Werten führt. Die Ablage von Pilz durch die Arbeiterinnen gemäß ihrer CO_2-Präferenz für die Zucht des Pilzes sollte dazu führen, dass es an dieser Stelle im Nest zu einer Ansammlung von Arbeiterinnen kommt, da Pilz auf sie sehr attraktiv wirkt. Die hohe

Ameisendichte sollte wiederum zu einer höheren Grabeaktivität an dieser Stelle führen.

Pilzkammern machen einen großen Teil eines Blattschneider-Nestes aus. Allerdings beinhalten die meisten Nester der Blattschneiderameisen der Gattung *Atta* auch große, voluminöse Kammern zur Abfallentsorgung, für die sie ebenfalls einen Teil des Energiehaushaltes der Kolonie aufwenden, anstatt das Material auf der Erdoberfläche zu verstreuen. In Kapitel 6 wurde postuliert, dass analog zur Beeinflussung des Grabens einer Pilzkammer durch Umweltfaktoren, Blattschneiderameisen ebenfalls Umweltpräferenzen bei der Abfallentsorgung zeigen. Daher wurde die Entsorgung von Abfall unter verschiedenen klimatischen Bedingungen untersucht. Tatsächlich zeigten Arbeiterinnen der Art *Atta laevigata* bevorzugte klimatische Bedingungen, unter denen sie eine neue Abfall-Deponie etablierten. Hierfür präferierten sie eine warme und trockene Umgebung, zeigten jedoch keine Präferenzen für spezifische CO_2-Konzentrationen. Der Unterschied zu den präferierten Umwelt-bedingungen für die Haltung des Pilzes, für die sie warme, feuchte und CO_2-spezifische Orte bevorzugen, sollte zu einer räumlichen Separation von Nestkammern für diese unterschiedlichen Aufgaben führen. Basierend auf den Ergebnissen von Kapitel 7, in dem die Nutzung von olfaktorischen Hinweisen bei der Abfallentsorgung untersucht wurde, ist die kontinuierliche Anhäufung von Abfallpartikeln in der Abfallkammer auf die Wahrnehmung der Arbeiterinnen von Volatilen zurückzuführen. Diese gehen vom deponierten Abfall selbst aus und dienen den Arbeiterinnen zur Orientierung, wenn diese mit neuem Abfall beladen einen Ablageort suchen. Die daraus resultierende große Ansammlung von Abfall an einem Ort sollte zur Entstehung einer voluminösen Kammer zur Abfallentsorgung führen.

Zusammengefasst konnten die in dieser Arbeit präsentierten Ergebnisse zeigen, dass die abiotische und soziale Umwelt zu aufgabenspezifischen Verhaltensantworten bei den Arbeiterinnen führt. Diese führen, basierend auf selbstorganisierten Prozessen, zu kollektivem Nestbau. Das auf diese Weise entstehende Nest bietet der Kolonie geeignete Bedingungen für dessen Fortbestand.

Excavation of a cement-filled Atta laevigata nest in Botucatu, Brazil; Source: M. Bollazzi

Chapter 1

1. General Introduction

1.1 Natural history and nesting behavior of leaf-cutting ants

Leaf-cutting ants earned their name because they cut live plant material from trees, shrubs and grass to use as a substrate to rear a symbiotic fungus (Cherrett, 1989). The ability to farm one's own food source has only developed in two other systems on the planet, humans and termites. In termites, fungus rearing can be found in the family of Macrotermitine, while in ants the tribe Attine with its 230 species in 13 genera (Hölldobler and Wilson, 2011) has developed this trait ca. 50 million years ago in South America (Mueller et al., 2001). But only two genera, *Acromyrmex* and *Atta*, which have a well-developed worker polymorphism (Cherrett, 1989), cut live plant material.

The fungal cultivar co-evolved with the fungus-growing ants in a climate that was distinctly tropical; warm, humid and without seasonal changes (Ortiz-Jaureguizar and Cladera, 2006), and the symbiosis likely originated in the Amazon Basin (Kusnezov, 1963; Weber, 1972). However, over time the climate changed and leaf-cutting ants radiated and dispersed so that they now can be found from Louisiana and Texas in North America to the more arid and seasonal climate of southern South America. To achieve this, the ants need to build a nest that offers the fungus, which sustains the colony brood and in part the colony workers (Cherrett, 1989; Bass and Cherrett, 1995), suitable environmental conditions despite different local climate. This fungus grows optimally between temperatures of 25-30°C (Quinlan and Cherrett, 1978; Powell and Stradling, 1986) and high air humidity, as it is susceptible to desiccation (Roces and Kleineidam, 2000). Through the group effort of these social insects, they manage to build nests that have, at least to some degree, the ability to dampen environmental fluctuations and to gain some control over their local environment (Hansell, 2005).

The genus *Acromyrmex* with its colony sizes of a few tens to a few hundred thousand

individuals builds smaller, but more diverse nests (Bonetto, 1959; Verza et al., 2007). Sometimes the same species switches from building a superficial nest with a thatch to a subterranean nest, depending on the latitude where the species occurs (Bollazzi, 2008; Bollazzi et al., 2008). The genus *Atta* has large colony sizes with millions of individuals and builds huge underground nests reaching into deeper soil strata (Moser, 1963; Jonkman, 1980a; Moreira et al., 2004a; Moreira et al. 2004b), where the climatic fluctuations are dampened by the surrounding soil, but exchange of respiratory gases is likely to be hindered.

The nests of leaf-cutting ants are composed of two basic building units found in most ant nests in general, i.e., chambers and tunnels. Chambers are utilized to house colony workers, brood or food stores. Unlike the chambers in non-fungus growing ant species (Tschinkel, 2005; Cerquera and Tschinkel, 2010), Attine nest chambers are not flat but have a spherical shape to house the voluminous fungus (Jonkman, 1980a; Solomon et al., 2004; Bollazzi et al., 2012). The nest's tunnel system maintains the colony's traffic, including laden workers returning from foraging with leaf fragments, and connects the nest chambers to the soil surface. The internal nest architecture, i.e., the quantity and spatial arrangement of the nest chambers and tunnels can vary, so such so that species can be identified by this extended phenotype (Moser, 1963; Zolessi and Gonzalez, 1978; Jonkman, 1980a; Moreira, 2004a; Verza et al., 2007; Lopes et al., 2011). As complex as some nest types can be, they are thought to result from simple stimulus responses of workers triggered by local cues without a central control of overall nest building.

1.2 Nest building mechanisms

The local stimuli workers are expected to respond to can originate either from other nestmates or from the environment (Deneubourg and Franks, 1995; Bonabeau, 1998; Theraulaz et al., 1998), as the ants' sensory system allows them only the access to local information, without the ability to survey the complete structure. Yet complex, collectively built structures emerge, through which the survival of the colony as a whole is promoted.

Biological structures can be built using different work-organizing mechanisms. For nest building in social insects the main mechanisms are thought to be templates, stigmergy and self-organization. The use of a template does not necessitate any interactions between the colony's workers, whereas stigmergy and self-organization depend on either interactions between workers and their work-in-progress or worker-worker interactions.

Templates can exist as physical structures, pheromone concentrations or climatic gradients in the soil and may determine the final shape of a structure. For example, clusters of brood in the ant *Leptothorax tuberrointerruptus* are used as a template while the workers build a wall around it, encompassing their simple one-chambered nest (Franks and Deneubourg,

1997). In this situation, no interaction of nestmates would be needed to lead to the construction of the wall as each individual takes reference from the centrally deposited brood cluster. Leaf-cutting ants are also known to use such a physical template when they enlarge their fungus chambers. The size of the fungus itself determines chamber size and shape. When the fungus grows and the distance between fungus and the chamber wall diminishes, workers are stimulated to excavate around it, until they have created enough space to give workers unhindered access to the fungal mass to supply it with plant material (Fröhle, 2009; Fröhle and Roces, 2009).

The majority of the spatial organization of nest building seems to be self-organized by local interactions between the workers. Two kinds of interactions are differentiated, indirect interactions through the by-product of their work or direct interaction between workers. Interacting through modifications of the environment is known as 'stigmergy' (Grassé, 1959; Theraulaz and Bonabeau, 1999). The changing of the environment, as a result of a building response of a worker, triggers the building response of another, which may be a similar response or a different one leading to a next step in the construction process (Theraulaz et al., 1998). The term 'stigmergy' was first used to describe the emergence of pillars during nest construction in termites of the genus *Bellicositermes*, where deposited soil pellets trigger a response in other workers to also deposit pellets there, leading to pillar formation (Grassé, 1959). Stigmergic building responses can also be found in workers of the leaf-cutting ant *Atta vollenweideri*. During nest enlargement workers deposit excavated soil pellets near the excavation site, influencing the spatial organization of the building process and stimulating other workers to deposit pellets nearby (Pielström and Roces, 2013).

The direct interaction between workers, irrespective of interactions with the emerging structure, also lead to coordinated building responses. It is comprised of repeated stimulus-response steps of workers according to simple rules and also to workers' individual behavior depending on their genetic programs (Camazine et al., 2001). Individuals vary in their predisposition to perform certain tasks (Gordon, 1996), i.e., have different stimulus-response thresholds. Once a building response is triggered in one worker, the process is amplified by positive feedback (Theraulaz et al., 1998), when other nearby workers are also stimulated to engage in building. For example, during nest excavation, workers of *Atta vollenweideri* stridulate, which attracts other workers to the site (Pielström and Roces, 2012). The increasing local density in turn triggers building behavior of the recruited workers and leads to amplification of the digging activity. Positive feedback can therefore be seen as a recruitment process that impacts on the response of an individual to the stimulus (Deneubourg and Goss,

1989). For building behavior to be regulated, an inhibitor for building-responses is needed in the form of negative feedback (Rasse and Deneubourg, 2001; Buhl et al., 2005). The excavated space itself can act as such an inhibitor. For instance, a group of *Lasius niger* ants in the process of excavation, first create a large round cavity. However, by creating space the ants disperse and can no longer occupy all possible digging sites along the created cavity wall. Different sites begin competing with each other for workers (spatial competition), so that worker clusters form at some of the digging sites, leading to the ramification of the once round cavity, and as a last consequence, to the emergence of tunnels at these sites (Toffin et al., 2009), the other main architectural structure of nests. As a consequence, different nest structures can emerge through the different spatial distribution of workers, even though the individual digging behavior, i.e., cutting into the soil with the mandibles and forming the 'cut-out' material into pellets, is always the same.

The final nest structure may even become more complex by the interaction of the different mechanisms with one another. While the wall of a *L. tuberrointerruptus* nest is built at a certain distance from a brood cluster, i.e, via the use of a template, the deposited sand grains as building material of the wall itself are attractive to the ants and will trigger grain deposit nearby, i.e., a stigmergic, self-organized positive feedback is involved (Franks and Deneubourg, 1997). The cluster of brood itself also emerged through a self-organized process, when, after placement of pieces of brood at a suitable site, workers reacted to this stimulus by depositing more brood nearby, leading to the amplification of a heterogeneity in the environment.

So far, there are pieces of experimental evidence, and also theoretical accounts, which highlight the involvement of the described mechanisms in the emergence of complex nest structures in ants. However, the final structure, i.e., the nest, has to provide the inhabiting colony with certain functions such as protection and climate control. Therefore, the constraints of the environment to which the species is adapted should impact the process of nest building (Bonabeau, 1998), and whatever the mechanisms involved, they need to be investigated in the framework of adaptive nesting behavior. As the nests of leaf-cutting ants should provide the inhabiting colonies with better suited conditions to culture their fungus and rear their brood, it is therefore expected that the ants' behavioral responses to their environment help to spatially organize the nest building process, leading to a structure capable to support the colony's needs.

This present thesis is aimed at experimentally evaluating the mechanisms leaf-cutting ants use during the nest building process and what role social cues, i.e., brood and fungus, play during nest construction. In addition, the orientation responses to environmental cues during different in-nest tasks are evaluated, as they are expected to also impact on nest building and

help to spatially guide the emergence of nest structures at suitable conditions for the inhabiting colony. The studies were performed with two leaf-cutting ant species with disparate nesting habits, *Acromyrmex lundi* and *Atta laevigata*. The former species has a small colony size with only a few tens of thousands individuals and inhabits shallow subterranean nests with one to only a few fungus chambers, while the latter has a few million workers and excavates deep nests with thousands of fungus chambers.

1.3 Thesis aim and experimental approach

The present work is composed of eight chapters. Chapters 2 to 7 are organized in sections analog to scientific publications with an abstract, introduction, methods, results and a chapter-specific discussion. Chapter 8 intends to join the findings of the previous chapters and presents a general discussion about the relevance of the presented findings for the understanding of the nest architecture of leaf-cutting ants.

The first part of the thesis, Chapters 2 and 3, is aimed at exploring the mechanisms used by *Acromyrmex lundi* leaf-cutting ants during nest excavation. Specifically, the role of brood and fungus for the emergence of new nest chambers in leaf-cutting ants was evaluated in Chapter 2. It was unclear, whether new nest chambers are excavated in advance, at specific sites where ants encounter suitable microclimatic conditions for brood and fungus rearing, or whether chamber excavation is triggered by brood and fungus initially deposited at the potential excavation site. Before this could be experimentally evaluated, it first needed to be quantified, whether workers prioritize the relocation of brood over the relocation of fungus, and whether a brood cluster could act as a spatial orientation cue for a subsequent deposition of fungus. The final experiment was a binary digging experiment where the excavated volume and shape of emerging structures at two identical digging sites, one containing brood and the other empty, but both offering suitable microclimatic conditions, was evaluated. The experiment was then extended with another series that evaluated subsequent fungus relocation into the two sites.

Chapter 3 is aimed at evaluating the processes involved in the regulation of nest size, as the nest space is expected to be adjusted not only to the colony size, as known from the literature, but also to the current space needs of the colony, i.e., the rearing of the voluminous fungus and colony brood. To that aim, digging experiments were performed with worker groups of *Acromyrmex lundi* of similar size, and the influence of parameters other than worker numbers was investigated. For instance, the offered available space at the beginning of the digging process, which should impact ant density, was varied, in the presence or absence of brood or fungus. The resulting digging activity was quantified as well as the resulting nest sizes. Also evaluated were the sizes of the different architectural nest structures, chambers and tunnels, as

workers should adjust the sizes of these structures to the actual needs of the experimental colony. As leaf-cutting ants have also been shown to deposit part of the excavated soil inside the nest, successfully downsizing it, internal soil pellet deposition in absence or presence of fungus and brood was also quantified.

The second part of the thesis, Chapters 4 to 7, explores the role of environmental cues for two main tasks of a leaf-cutting ant colony, fungus culture and waste management as the ants' responses should affect nest building.

It was already known that the respiration of the symbiotic fungus is negatively influenced at high CO_2-levels existing at deeper soil strata. Chapter 4 therefore assesses leaf-cutting ants' preferences for the CO_2 concentrations of their nest environment when rearing the symbiotic fungus. The experiment was performed with *Acromyrmex lundi*, as the species excavates shallow subterranean nests. Workers in the process of relocating exposed fungus, prone to desiccation, from unsuitable environmental conditions, could choose between two nest chambers in a binary-choice setup. The CO_2-levels inside the chambers differed, while the temperature and humidity values offered identical and well suited conditions for fungus rearing. The CO_2-preferences for fungus rearing as well as for the workers themselves were evaluated.

In Chapter 5 it was evaluated whether the excavation of shallow nests in *Acromyrmex lundi* could be due to CO_2-dependent ants' digging responses, as this respiratory gas increases with depth and it was hypothesized that workers would excavate less, the higher the CO_2-levels. To this aim, a group of workers was exposed to different CO_2 concentrations while digging in a test chamber, levels ranging from atmospheric values to 11%. In addition, the deposition of excavated soil pellets in the chamber was evaluated, as the accumulated material inside the nest influences the free nest space and consequently the nest ventilation.

Chapter 6 is aimed at quantifying the microclimatic preferences for waste disposal in the ant *Atta laevigata*, which builds underground waste chambers. The evaluated preferences for a certain temperature, humidity or CO_2-range should influence the ants' decision where the waste chambers should be excavated in the soil. In addition, it might help explain why the waste chambers are separate from the fungus chambers. For that, a fungus garden with workers from a laboratory colony was connected to a binary-choice setup with two chambers, which either offered two different temperatures, humidities or CO_2-levels. Colony waste was added to the garden to trigger waste relocation towards the offered chambers, and the environmental preferences for waste management were assessed.

Ants have well-developed olfactory systems and odor perception plays a fundamental role in the contexts of foraging communication and plant selection. It seems therefore likely

that volatiles should also be used to organize work inside the nest. Chapter 7 investigated whether olfactory cues are used for orientation during waste management, as volatiles originating from fungus or waste would provide reliable information to decide where to accumulate this potentially pathogen-laden material. The volatiles were presented in an experimental arena to both sides of an artificially established waste heap. The relocation of the heap towards one of the sides emitting volatiles was quantified.

Chapter 8 integrates the findings of the present work, and discusses how the interaction between the investigated digging mechanisms and the stimuli arising from the abiotic and social environment could lead to the emergence of nest structures beneficial to colony survival.

Cement cast of fungus chambers, Atta laevigata nest; Source: W. Thaler

Chapter 2

2. Nest enlargement in leaf-cutting ants: relocated brood and fungus trigger the excavation of new chambers

Abstract

During colony growth, leaf-cutting ants enlarge their nests by excavating tunnels and chambers housing their fungus gardens and brood. Workers are expected to excavate new nest chambers at locations across the soil profile that offer suitable environmental conditions for brood and fungus rearing. It is an open question whether new chambers are excavated in advance, or will emerge around brood or fungus initially relocated to a suitable site in a previously-excavated tunnel. In the laboratory, we investigated the mechanisms underlying the excavation of new nest chambers in the leaf-cutting ant Acromyrmex lundi. Specifically, we asked whether workers relocate brood and fungus to suitable nest locations, and to what extent the relocated items trigger the excavation of a nest chamber and influence its shape. When brood and fungus were exposed to unfavorable environmental conditions, either low temperatures or low humidity, both were relocated, but ants clearly preferred to relocate the brood first. Workers relocated fungus to places containing brood, demonstrating that subsequent fungus relocation spatially follows the brood deposition. In addition, more ants aggregated at sites containing brood. When presented with a choice between two otherwise identical digging sites, but one containing brood, ants' excavation activity was higher at this site, and the shape of the excavated cavity was more rounded and chamber-like. The presence of fungus also led to the excavation of rounder shapes, with higher excavation activity at the site that also contained brood. We argue that during colony growth, workers preferentially relocate brood to suitable locations along a tunnel, and that relocated brood spatially guides fungus relocation and leads to increased digging activity around them. We suggest that nest chambers are not excavated in advance, but emerge through a self-organized process resulting from the aggregation of workers and their density-dependent digging behavior around the relocated brood and fungus.

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2.1 Introduction

Leaf-cutting ants build the most complex underground nests among ants. Their nests may consist of up to eight thousand underground chambers housing their symbiotic fungus, brood embedded within the fungus and in several species, also the colony's refuse (Stahel and Geijskes, 1939; Stahel and Geijskes, 1941; Jonkman, 1980b). Huge nests with millions of individuals and thousands of fungus chambers are generally excavated by colonies of the genus *Atta* (Moreira et al., 2004a; Moreira et al. 2004b), while colonies of the genus *Acromyrmex* excavate smaller nests composed of one or up to tens of chambers (Bonetto, 1959; Verza et al., 2007) with mature colony sizes between a few thousand (Clark and Fewell, 2014) and one to two hundred thousand individuals (Pereira-da-Silva et al., 1981; Fowler et al., 1986). All these nests are composed of two kinds of structures: oblong, narrow tunnels and spherical chambers with a flat bottom and a dome shaped ceiling, but each species has its own specific nest architecture by which it can be identified (Zolessi and Gonzalez, 1978; Jonkman, 1980a; Moreira et al., 2004a; Moreira et al., 2004b; Verza et al., 2007; Lopes et al., 2011; Bollazzi et al., 2012). *Atta* nests consist of a net of main tunnels leading downwards to deeper soil regions. Nest depths of 8 m have been reported for *Atta laevigata* (Moreira et al., 2004a; Bollazzi et al., 2012). These main tunnels connect to the nest chambers, which are oriented laterally to tunnels, mostly by one short and narrow branched off tunnel called peduncle, which end in the lower part of the chamber (Jacoby, 1953; Moreira et al., 2004a; Bollazzi et al., 2012). The main tunnels can have blind endings and a recent study using cement casts from *Atta laevigata* and *Atta capiguara* nests showed that these tunnels may have the beginnings of branched off peduncles, which end blind without excavated chambers (Bollazzi et al., 2012). There are also tunnels that lead farther downwards than the fungus chamber zone and are thought to reach the water table (Stahel and Geijskes, 1939; Jacoby, 1953; Moser, 1963), as well as horizontal foraging tunnels of considerable length (Moser, 1963; Moreira et al., 2004a; Bollazzi et al., 2012). *Acromyrmex* nests are generally shallower, with fungus chambers found close to the soil surface (5 – 50 cm) (Zolessi and Gonzalez, 1978; Wetterer et al., 1998; Camargo et al., 2004; Lopes et al., 2011), but nest depths also reaching 2-5 m have been reported for some species (Navarro and Jaffe, 1985; Lapointe et al., 1998; Verza et al., 2007). The nest tunnel system, while not as complex as that of *Atta* nests, also extends beyond the existing garden zone and some tunnels end blind.

Whether mature nests consist of thousands (*Atta*) or just a few (*Acromyrmex*) chambers, the founding nest is a single, downward leading tunnel of 10-30 cm in length connected to a small chamber, which is excavated by a new queen after her mating with several males

(Hölldobler and Wilson, 1990; Fröhle and Roces, 2012). Mating flights take place in spring during the hot months and after heavy rains (Jonkman, 1980a; Diehl-Fleig and Lucchese de Paula, 1992), when both the temperature and humidity of the soil are high and conditions are well suited to successfully rear fungus gardens and brood. Information on how nests are enlarged after this first step is scarce though more is known for *Atta* than for *Acromyrmex* species. The process of nest enlargement in ants is not centrally coordinated and appears to be self-organized with workers reacting to local stimuli without knowledge of the complete structure (Franks and Deneubourg, 1997; Rassé and Deneubourg, 2001; Buhl et al., 2005). When the first leaf-cutting ant workers appear 8-12 weeks after colony founding (Jacoby, 1937; Camargo et al., 2011), they are responsible for further nest enlargement, achieved by the excavation of tunnels, mostly leading downwards, and the excavation of new fungus chambers at deeper soil layers (Jacoby, 1936; Jacoby, 1937). *Acromyrmex* species are thought to enlarge their nests by building a few interconnected fungus chambers close to the surface. For example *A. lundi*, which has relatively simple mature nests with a large (diameter 50 cm) central chamber linked by tunnels to a few satellite chambers, had only created a small central chamber with tunnels originating from it, but no satellite chambers, within 1-2 years after colony foundation (Zolessi and Gonzalez, 1978).

Ants may increase the size of their nests in two ways, either enlarging existing chambers or excavating new ones. Mature fungus chambers in *Atta* species usually have a diameter of ~30 cm while chambers in more superficially nesting *Acromyrmex* species may reach a diameter of ~50 cm. The extent of chamber enlargement seems to have an upper limit. For example in a field nest of *Atta*, where chamber density was observed to be high, sometimes neighboring chambers were only separated by a very thin layer of soil (Jacoby, 1955; Bollazzi et al., 2012). Fusing the chambers together could have been achieved by the ants at a low energetic cost, yet this barrier was not breached. It remains to be discovered what the limiting factors are for enlarging an existing chamber. Cassill et al. (2002) for example proposed that smaller chamber sizes benefit colony communication in the fire ant, *Solenopsis invicta*. Large fungus chambers may have a reduced supply of fresh air, because of the diffusive movement of respiratory gases that need to reach the center of the fungus garden (Kleineidam and Roces, 2000; Bollazzi et al., 2012). As a result, at least at one time and likely at many intervals in the development of these colonies, their growth trajectory will exceed the space available within a single chamber and a new one must be constructed.

It is an open question whether new nest chambers are excavated in advance as colonies grow, or whether they emerge around an incipient cache of brood and/or fungus. In addition to

the stimulus resulting from insufficient space there are three other non-mutually exclusive scenarios for the relocation of brood and fungus from an existing chamber, and the potential excavation of a new chamber around them. First, pathogens may infect a fungus garden, and workers may remove and relocate healthy fungus pieces and brood. Second, the microclimatic conditions inside the fungus chamber may become unsuitable for brood and fungal development. Third, even when the conditions are not unsuitable, workers may find, or search for, more favorable conditions at a different location. All these four scenarios would potentially lead to brood and fungal deposition at a new site in the nest, i.e. in an existing tunnel, and to the subsequent excavation around them to create a chamber. However, empty chambers have been reported in field nests of a number of leaf-cutting ant species (Stahel and Geijskes, 1939; Jacoby, 1960; Jonkman, 1980b; Lapointe et al., 1998; Moreira et al., 2004a; Moreira et al., 2004b; Moser, 2006; Verza et al., 2007). One possibility is that such chambers were constructed around relocated items, and later emptied because of changing environmental conditions (Lapointe et al., 1998), presence of pathogens or fungus decay. Alternatively, ants might start the excavation of a chamber in advance upon finding a suitable place for their fungus and brood, as a direct reaction to local abiotic stimuli such as temperature or humidity. Ideal conditions for in vitro fungus rearing are temperatures between 20 and 30°C (Powell and Stradling, 1986; Quinlan and Cherrett, 1978) and in fact, leaf-cutting ants choose places with temperatures between 21 and 25°C when they relocate fungus and brood (Bollazzi and Roces, 2002). They also prefer relative humidities close to saturation for fungus rearing (Roces and Kleineidam, 2000). Given a choice between alternative sites, leaf-cutting ants prefer to dig at temperatures between 20-30°C, which may lead to a concentration of digging activity in soil layers of the preferred temperature range (Bollazzi et al., 2008). The nest enlargement in many *Atta* and some *Acromyrmex* species also takes place at deeper soil layers, which have a higher moisture content (Jacoby, 1936; Jacoby, 1937). More superficially nesting *Acromyrmex* species might conserve moisture in the soil surrounding their nests by accumulating leaf-litter on the nest surface, by plugging nest entrances, or by modifying the structure of the nest mound (Weber, 1966; Lopes et al., 2011; Bollazzi and Roces, 2007; Bollazzi and Roces, 2010c). To ensure proper conditions for brood and fungus rearing, and with it the survival of the colony, leaf-cutting ants even track their preferred temperature and humidity values across an existing nest and brood and fungus are relocated accordingly (Weber, 1957; Moser, 1963; Lapointe et al., 1998; Bollazzi and Roces, 2002).

The question arises whether abiotic environmental stimuli alone are sufficient to trigger digging of a new nest chamber in advance at a suitable location. Under controlled laboratory

conditions, workers of *Acromyrmex lundi* with neither brood nor fungus excavated only tunnels, but not chambers (Fröhle, 2009; Fröhle and Roces, 2009). Chambers were excavated as soon as the ants were allowed to relocate symbiotic fungus inside a digging arena, and digging activity concentrated around the deposited fungus. This suggests that beyond abiotic stimuli, contents to be stored are needed for the emergence of a nest chamber. We hypothesize that a suitable microclimate at a potential chamber location is not sufficient to trigger the excavation of a chamber, but that the contents to be stored, brood or fungus, are needed at this location to initiate chamber excavation. We propose that if chamber content is relocated to an already existing tunnel, excavation to generate further space should follow. To investigate this two-step process (relocation followed by excavation), we designed a series of experiments that first investigate the relocation of brood and fungus and then quantify the digging activity and chamber formation triggered by the relocated items.

The separate analysis of the relocation and excavation processes was necessary because it was unknown whether brood and fungus would be relocated simultaneously or sequentially, either of which might have distinct influences on subsequent digging behavior. Relocation comprises the removal of items at one place and their deposition at another. We first investigated the removal of items by exposing brood and fungus to unsuitable environmental conditions, using low temperature in a first experiment, and low air humidity in a second. We found that the ants exhibited a strong preference to remove the brood first. Because brood and fungus are maintained together in natural nests (as the young brood need to feed on the fungus), we expected that the subsequently removed fungus would be deposited near the relocated brood. We evaluated fungus deposition in binary-choice experiments offering two sites with suitable environmental conditions, only one containing brood. Two last experimental series were designed to evaluate whether chamber content triggers chamber excavation by quantifying the digging activity and shapes of excavated structures at two suitable sites offered in binary-choice experiments. One site contained brood, while the other did not, both in the presence or absence of fungus. Based on our findings we propose a density-dependent mechanism for the emergence of nest chambers through a self-organized process, with relocated brood and fungus acting as cues that elicit worker aggregation at their deposition sites, indirectly influencing the intensity of digging activity.

2.2 Materials and Methods

Experiments were performed in the laboratory between June 2010 and December 2011 with leaf-cutting ants of the species *Acromyrmex lundi*. This species is not protected under the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES).

Colonies were collected in Argentina in 2007 on privately owned land with the owner giving permission for their collection. They were reared at the Biocenter of the University of Würzburg, Germany in a walk-in environmental chamber at 25°C, 50% air humidity and a 12L:12D cycle. To control for possible effects of body size on behavioral performance, only medium sized workers (mean body mass calculated from a ubiquitous sample of medium sized workers taken from the colonies for weighing: 5.3 mg ± 1.2 mg SD, n=80) were used in the experiments described below. All experiments were performed with worker groups from large laboratory colonies. We realize that colonies of this species build relatively superficial nests with a few and sometimes just one nest chamber. However, we argue that colonies from this species, probably as well as from all leaf-cutting ant species, are confronted during their ontogeny with the need to enlarge their nests either by increasing the size of an existing chamber, or by excavating a new one, or both. Also, previous related studies from our lab were conducted on *A. lundi* (Fröhle, 2009; Fröhle and Roces, 2009), so that direct comparisons are possible.

After each assay the worker groups were not reintroduced into the colonies, so that each assay was considered independent from each other. To control for possible colony differences, 3 colonies were used and, if not otherwise stated, worker groups from all 3 colonies were used for each experiment. We also tested for possible colony effects, and the results of these tests are included in the appropriate figure captions. Since no colony effects were found, data from all colonies was pooled for statistical analysis.

(a) Determination of relocation preference for brood or fungus

As previously indicated, the sequence of brood and fungus relocation in natural nests is unknown. Since individual workers necessarily relocate single brood items and pieces of fungus separately, it is an open question whether workers prefer to relocate brood or fungus first when presented with a choice. The kind of items relocated first may distinctly influence the subsequent digging behavior at the deposition site. To evaluate the worker's preferences during relocation we first evaluated, in two independent experimental series, whether ants prioritize one item over the other during relocation to suitable nest conditions or if they were relocated simultaneously. If the former is the case, the preferentially removed item would be present at a new site first, i.e., within a nest tunnel, and might initially trigger the subsequent chamber excavation. In both series, removal was induced by exposing brood and fungus to unsuitable conditions, either low temperature or low air humidity, and their relocation quantified. Based on the outcome of these experiments we chose the item to be used as trigger for the excavation of a chamber in the digging experiments.

Temperature induced relocation experiment

In the first series, the dynamics of fungus and brood transport were quantified when both were simultaneously exposed to a low temperature to initiate relocation behavior (Experiment 1**, Fig. 2.1a** and **2.1b**). The experimental setup was as follows. To simulate a small nest, two round plastic arenas (diameter 15 cm, height 1 cm, henceforth called nest site 1 (S1) and nest site 2 (S2)) were filled with moist clay (Claytec Baulehm gemahlen 0–0.5 mm, Viersen, Germany, water content 18%, air humidity in nest site 99.9%) and connected to each other with a piece of tubing (length 10 cm). Two separate nest sites were necessary because site S1 was exposed to a lower temperature during the experiment, while site S2 was maintained at room temperature to offer a suitable microclimate. In nest site S1, we artificially constructed a main tunnel (7x1x1 cm), a short side tunnel (1x1x1 cm) and a chamber (diameter 5 cm) by cutting these spaces out of the clay (**Fig. 2.1b**). In nest site S2 only a tunnel (4x1x1 cm) was cut out. Prior to the experiment 0.5 g of fungus (without brood and gardening workers) and 20 pupae, both freshly removed from one of the large colonies, were placed inside the chamber in nest site S1. This site was then connected to a foraging area that consisted of two boxes (19x19x9 cm), linked by a wooden bridge. The first box contained an ample supply of water as well as honey water and will therefore be called 'foraging arena'. When restricted to only one foraging box, workers tend to spoil their food supply by mixing it with excavated clay pellets, which would negatively influence the workers' survival rate during the experiments. To prevent this situation a second box was added for soil deposition (**Fig. 2.1a**).

At the beginning of each assay 100 workers, collected from one of the three colonies, were released in the foraging arena. They could move freely across the wooden bridge into the soil deposition site and from there into and out of the nest. After two hours of familiarization time, the temperature in site S1, which had been placed on a cooling plate connected to a water bath, was lowered from room temperature (ca. 20°C) to 10°C. This temperature was chosen to induce relocation because previous work indicated that workers of the related species *Acromyrmex heyeri* avoid this temperature and relocate brood or fungus to warmer places (Bollazzi and Roces, 2002). Fifteen replicates were performed, using 5 worker groups from each colony. After 22 hours, the amount of relocated fungus into nest site S2 was weighed to the nearest 0.1 mg, and the number of relocated brood were counted. These were then converted to proportion (%) of the total content placed in nest site S1.

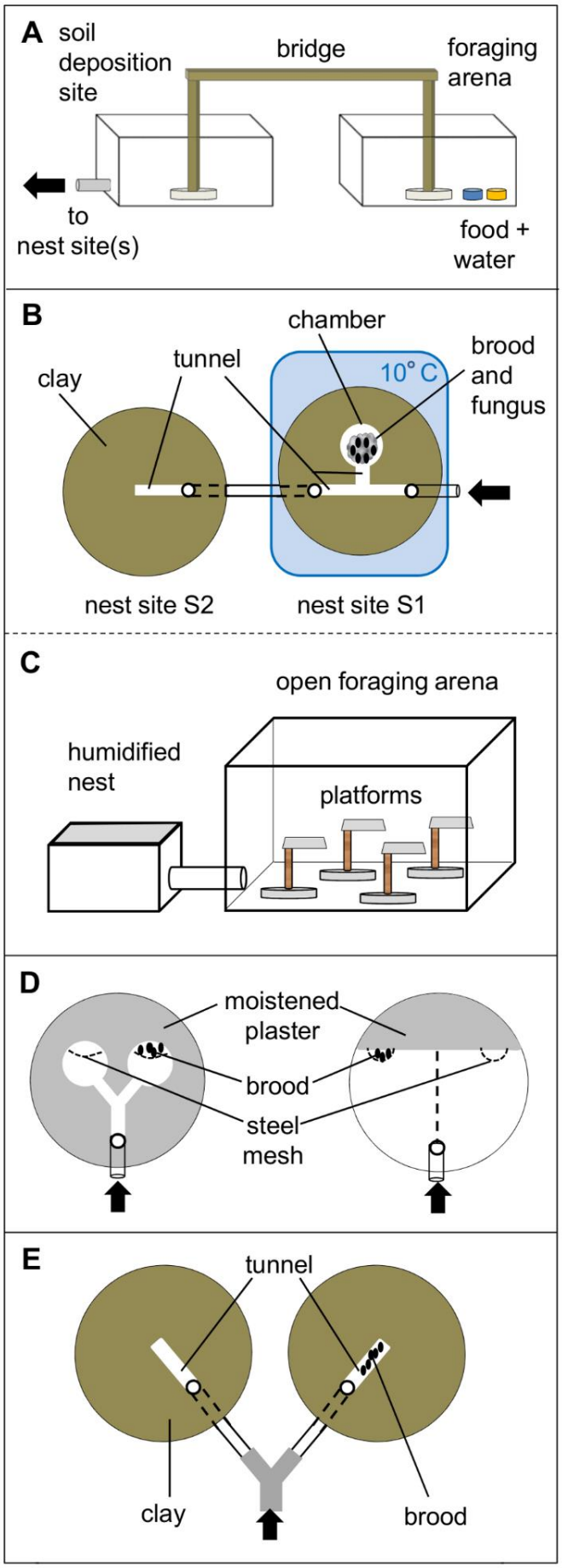

Figure 2.1: Experimental setups (arrows indicate the direction of entering ants). **(a)** Foraging area consisting of a soil deposition site (left) and a foraging arena (right). Here the arrow indicates the entry to experimental arena(s). **(b)** Nest sites for the temperature induced relocation experiment (Experiment 1): left – nest site with cut-out tunnel, at room temperature; right – nest site with cut out tunnels and chamber containing fungus and brood, placed on a cooling plate. **(c)** Setup for humidity induced relocation experiment (Experiment 2): left – humidified nest with moistened pebbles; right – open foraging arena with 4 experimental platforms. Ants were placed into the foraging arena at the beginning of the experiment. **(d)** Plaster nest sites for fungus relocation experiment: left – nest site with 2 small chambers, one containing brood (Experiment 3); right – nest site with 1 big chamber, one side containing brood (Experiment 4). **(e)** Clay nest sites for digging experiments, only one tunnel containing brood (Experiments 5 and 6).

Humidity induced relocation experiment: individual choices

It is important to note that the fungus in chambers of natural nests is a dense connected mass of hyphae, and that ants need to cut a transportable piece from the large mass for relocation. Brood might therefore be relocated first not necessarily because of a preference, but simply because they are just easier to pick-up and remove. To control for this effect, we performed the second experimental series using small, transportable pieces of fungus and observed the removal decision in real time. As low air humidity was observed to initiate a quick removal of items in preliminary experiments, it was used instead of low temperature as an unsuitable environmental factor to trigger removal. Because the experiment was easier to implement outside of the nest, one pupa and one piece of fungus were simultaneously exposed in a foraging arena (Experiment 2, **Fig. 2.1c**). Removal preferences of single workers were quantified in individual choice experiments. A plastic box with a lid (9x9x6 cm) acted as a nest site, with its bottom filled with moistened pebbles to offer humid conditions (air humidity levels in the nest site, close to saturation, 99.9%). It was connected to a foraging arena (an open box 19x19x9 cm) with humidity levels corresponding to room conditions (~50%), at which fungus and brood faced the threat of desiccation. Four platforms, each consisting of a plastic square (1.5x1.5 cm) glued on top of a 4.5 cm high wooden stick were placed in the foraging arena. In each assay 50 workers were released there and could explore it as well as the nest site for 1 h. Then, a piece of fungus and a brood item were placed on a randomly chosen platform. The mass of a brood item was 7.2 ± 0.18 mg (mean ± SE), and that of a fungus piece 13.9 ± 0.33 mg (mean ± SE). An ant, upon walking up the wooden stick to the platform, would encounter both items simultaneously. It was then noted which item was picked up first (and relocated to the nest site), and the time it took for the second item to be picked up by a different worker. Workers that relocated items were carefully removed with forceps after depositing their load in the nest. Tests were performed over 1h, with the platforms chosen at random each time. In total, 6 assays were performed using 3 different colonies (2 assays per colony) and a total of 101 pupa/piece of fungus pairs (pairs per colony: 41, 30, 30) were tested. Both experiments (1 and 2), although using different stimuli to trigger the ants' responses, were designed to evaluate the removal preference and not the final deposition of the items.

(b) Brood as a cue for fungus relocation

The deposition of items during a relocation process was evaluated in the next two experiments. Because workers showed a preference for brood relocation in the previous experiments, which could lead to the presence of brood at an alternative site first, only the influence of deposited brood on the subsequent fungus relocation was investigated. The brood in a fungus chamber is

usually embedded into the fungal mass (Weber, 1966; Lopes et al., 2005), with workers planting hyphae on the larval body (Armitage et al., 2012). As a consequence, we would expect that workers transport fungus pieces to a site where brood had been previously relocated to. Brood may therefore act as an orientation cue for workers relocating fungus. The subsequent accumulated fungal volume around the brood should then have an influence on the excavation of space at this site. Therefore, it was important to first demonstrate that relocation of fungus, as we expected, will follow relocation of brood. A set of two experimental series was performed without the involvement of digging activity. In the first experiment, ants were induced to relocate fungus from unsuitable conditions (low air humidity) and had the choice between two nest sites, one containing brood and the other without brood (Experiment 3, **Fig. 2.1d**, left), both offering suitable environmental conditions (temperature ~25°C, air humidity close to saturation). The rationale of offering two sites instead of one was to mimic more natural conditions, since natural nests may offer more than one site for a potential relocation.

The nest site consisted of a round plastic arena (diameter 15 cm, height 1 cm) filled with plaster (Sakret Bau- und Hobbygips, Berlin, Germany). We chose this material to prevent the ants from digging. A Y-shaped tunnel with a nest chamber (diameter 5 cm) at each end was cut out of the material, and pieces of steel mesh were fastened into the plaster to separate a part of each chamber (**Fig. 2.1d**, left). The plaster was remoistened with 10 ml of demineralized water (resulting air humidity levels 99.9%) and 20 pupae were placed behind the mesh in one of the nest chambers, so as to prevent their removal when workers entered the chamber during the assays. The nest site was then connected to a foraging arena (an open plastic box, 19x19x9 cm) containing an ample supply of water and honey water. At the beginning of each assay, a group of ants consisting of 50 medium and 10 minima workers (mean size 0.87 mg ± 0.29 mg SD; calculated from a ubiquitous sample of minima workers taken from the 3 colonies for weighing, n=60) was released in the foraging arena with free access to the nest. The mesh partition enabled medium workers to antennate the brood behind it, and minima workers to walk through and care for them. The side of the brood-containing nest chamber was alternated between assays. Familiarization time was 18 hours, after which the number of ants that aggregated in each chamber was counted, and 0.5 g of fungus, freshly collected from the same colony as the ants, was added in the foraging arena. The unfavorable low humidity (~50%) in the open box prompted ants to relocate the fungus inside the more humid nest. An assay was finished when workers relocated all fungus from the foraging arena into the nest. Afterwards, the fungus in each nest chamber was weighed to the nearest 0.1 mg. A total of 12 replicates were performed. Due to a limited number of available laboratory colonies, the experimental series as well as the

next series described below were performed with workers, brood and fungus from a single colony. It is therefore unclear if the outcome of this experiment can be considered as representative for the response of other colonies.

The second experimental series was aimed at evaluating whether the deposition of fungus at sites containing brood was actually a direct response to the brood presence. In the previous series fungus-carrying workers may have found the brood pile by following, for instance, pheromone markings left by ants as they aggregated in the brood containing chamber or by colony odors left on tunnel or chamber walls. By shortening the time span within which pheromones or colony odors could accumulate and offering brood at one spot in a relatively spacious chamber, fungus accumulation around this spot should be considered as a direct response to the brood presence. This may suggest that cues originating from the brood (i.e., pheromones, released CO_2) could also modulate the response threshold to engage, for instance, in digging, which might be relevant for chamber emergence and for the digging experiments described below.

The experimental set-up offered a nest site consisting of a single, spacious chamber (a line drawn on the chamber floor virtually divided the chamber in two halves), and ants were allowed to familiarize with it for a shorter period (Experiment 4, **Fig. 2.1d**, right). A similar round plastic arena (diameter 15 cm, height 1 cm) was used as a nest site, which was only partly filled with plaster forming a straight wall. The two mesh enclosures were fastened at opposite ends into the plaster wall. Entering workers could easily move across the single chamber and reach the enclosures. The plaster was remoistened with 5 ml of demineralized water and 20 freshly collected pupae were placed in one of the enclosures. The side of the brood-containing mesh was alternated between assays. Then a foraging arena (an open plastic box, 19x19x9 cm) was connected to the nest site. At the beginning of each assay 50 medium and 10 minima workers were released in the foraging arena. After a 2h familiarization period the number of workers present in each chamber 'half' was counted. Then 0.5 g fungus was placed into the foraging arena, but not in a single large piece as in the former series. It had been divided into 20 equally-sized, transportable pieces. The nest side to which the first 10 fungus pieces were relocated was noted and 13 replicates were performed.

(c) Chamber excavation as a response to the presence of brood and fungus

In order to evaluate whether the presence of brood or fungus at a site leads to the excavation of a chamber around them, workers' digging activity was quantified in a binary-choice experiment offering two suitable digging sites (temperature ~25°C, air humidity close to saturation). Two

different experimental series were performed (Experiments 5 and 6, **Fig. 2.1e**), presenting either brood or brood plus fungus as stimuli.

Brood stimulus

In the first series (Experiment 5), the brood was offered as a stimulus at one of the nest excavation sites, because of the observed preferences for brood relocation (further details in the Results). The setup for the first series was as follows. For each assay two round nest sites (diameter 15 cm, height 1 cm) were filled with moist clay (water content 18%) and a single tunnel (4x1x0.5 cm) was cut out of the material in each (**Fig. 2.1e**). Twenty pupae were placed in a preformed tunnel of one of the digging sites (alternated between assays). The digging sites were connected with each other and the two-box setup described in Experiment 1. The use of two separate nest sites connected via tubing, instead of a Y-shaped tunnel cut out in a single nest site allowed excavation only to occur at the two small tunnels, and therefore enabled a clear quantification of the emerging structures. At the beginning of each assay, a group of 100 workers was released in the foraging arena, and from there workers had access to the soil deposition site and both digging sites. After 24 hours, the amount of excavated clay in each nest site was quantified to the nearest 0.1 g and the excavated volume (cm^3) calculated (1 cm^3 = 1.8 g of clay). Fifteen replicates were performed, 5 replicates per colony.

Brood and fungus stimulus

In the second series (Experiment 6), we quantified the effect of a subsequent fungus deposition at the digging site on chamber emergence. The setup was identical to that used in the previous experimental series, with 20 pupae placed in one tunnel and a worker group of 100 ants released in the foraging arena. Then, 1 hour after workers started to dig and excavated clay pellets were deposited in the connecting tube, 0.5 g of freshly collected fungus was placed in the foraging arena. The low humidity there (~50%) caused the ants to relocate the fungus into the more humid nest sites (values close to saturation, 99.9%). After 24 hours, fungus and excavated material at each digging site were quantified to the nearest 0.1 mg and 0.1 g respectively, and the excavated volume was calculated. A total of 15 replicates were performed, 5 per colony.

(d) Shape of excavated structures

Even when comparable amounts of soil are excavated from digging sites, the shape of the resulting structure might vary from an intricate tunnel system to a more chamber-like, round cavity. To obtain a measure of the circularity of the excavated structures, i.e. of their cavity-like shape, their form factor was calculated. The form factor is the ratio of the area of an object

to the area of a circle with the same perimeter as the object (Ritter and Cooper, 2009), as follows:

$FF = (4\pi \text{ Area})/\text{Perimeter}^2$

The form factor varies from 0 to 1; the higher the value, the more circular the structure. To determine the area and the perimeter of the excavated structure, a plaster cast of the excavation was made of both digging sites at the end of each assay (for both experimental series), which were digitized with a scanner. Then, area and perimeter were measured using the software ImageJ (version 1.44p, National Institutes of Health, USA) and the form factor was calculated. Because of the offered preformed nest tunnel and entrance hole, the starting form factor of each excavated structure was not 0, but 0.28, the baseline from which the shape of the excavated structures could develop.

2.3 Results

(a) Determination of relocation preference for brood or fungus

No relocation of brood or fungus was observed before the cooling of the nest (Experiment 1). After 24 hours, the proportion of relocated brood was significantly higher than that of relocated fungus (**Fig. 2.2a**; Wilcoxon matched pair test; T=0.00; Z=3.41; $p<0.001$; n=15). In each of the 15 assays, ants relocated all live pupae (100%) into nest site 2, but only a median of 1.36% (25-75% percentiles = 0-2.78%) of the original 0.5 g fungus mass (0.0068 g; 25-75%= 0-0.0139 g). In 4 of the 15 assays no fungus at all was relocated. Single ants also showed a preference for brood relocation when a pupa and a piece of fungus were offered side by side (**Fig. 2.2b**, Experiment 2). In 92 (91.1%) of 101 observations, the first item picked up was brood (binomial test; $p<0.001$; **Fig. 2.2c**). The mass of a brood item (7.2 ±0.18 mg, mean ±SE), and that of a fungus piece (13.9 ±0.33 mg, mean ±SE) equals a burden (= (ant mass + load mass/ant mass); load size expressed in relative terms) of ~2-3.5. Leaf-cutting ants can carry a burden of up to 7.5 when they forage leaf-fragments (Rudolph and Loudon, 1986), indicating that both items were easily transportable in our experiment. Upon discovering both items on the platform, ants were observed to antennate them with slightly opened mandibles and protruded labium, and then to pick up one item and to carry it into the nest. The time lapse between the first and second item being picked up was measured in 60 of the performed 101 observations (fungus as the second item picked up: n=53; brood as the second item picked up: n=7). Fungus was picked up 47.7 s (± 29.3 SE) after the brood item, and brood was picked up 92.2 s (± 20.2 SE) after the fungus. Never was the second item not picked up by another worker, indicating that both brood and fungus pieces were healthy and undamaged.

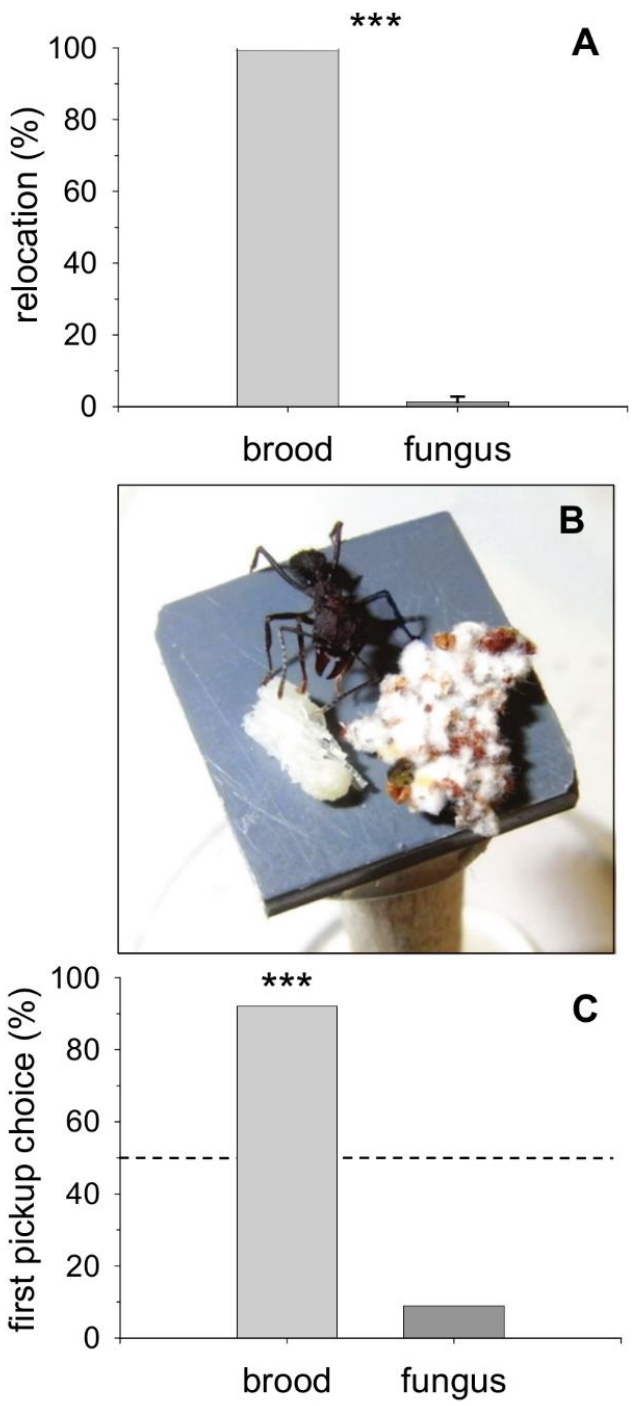

Figure 2.2: Brood and fungus relocation experiments. **(a)** Experiment 1: Relocation of items from cold stress (10°C) by a worker group after 24 h, presented as percentage of the total offered amount (20 pupae and 0.5 g fungus) (median ± 25-75% percentiles), ***p<0.001 (Analysis of colony effects: Kruskal-Wallis-Test; Brood relocation; H=0.0; p=1; n=5; Fungus relocation; H=0.66; p=0.72; n=5; n.s.; no colony effects found). **(b)** Experiment 2: Relocation of items from desiccation by single workers: an *A. lundi* worker encounters a pupa and a piece of fungus on an experimental platform (1.5x1.5 cm). **(c)** Score of first item relocated, expressed as percentage of total observations (n=101), ***p<0.001 (Analysis of colony effects: Fisher's Exact test for 3x2 contingency tables; p=0.24; no colony effects found).

(b) Brood as a cue for fungus relocation

When workers could choose to relocate fungus into an empty nest chamber or one with brood, the majority of the relocated fungus was deposited in the chamber containing the brood (Experiment 3; **Fig. 2.3a**; Wilcoxon matched pair test; T=3.0; Z=2.824; p<0.01; n=12). The median fungus deposit in the brood chamber was 0.345 g (25-75%= 0.276-0.428 g) and 0.038 g (25-75%= 0.017-0.129 g) in the empty chamber. Most of the pieces were placed side by side on the chamber floor, and rapidly filled the available space in the brood chamber. It is therefore likely that further fungus relocation was not possible because of lack of space, and it was shifted

to the alternative, brood-less chamber. Time differences of occurrence of the first fungus deposit in the chambers seem to support this view (in the brood chamber; median= 7.5 min; 25-75%= 5.5-11 min; in the empty chamber; median= 17 min; 25-75 %= 13-27.5 min; Wilcoxon matched pair test; T=5.000; Z=2.667; p<0.01; n=12). In addition, in 10 of the 12 assays the very first piece of fungus relocated inside was deposited in the brood chamber. A difference in the magnitude of worker aggregation in the two chambers was also observed. Significantly more ants were located in the brood chamber before the fungus was placed in the foraging arena and relocation took place (**Fig. 2.3b**; workers in brood chamber; median= 23; 25-75%= 16-24; workers in empty chamber; median= 3; 25-75%= 2-4; Wilcoxon matched pair test; T=0; Z=3.06; p<0.01; n=12).

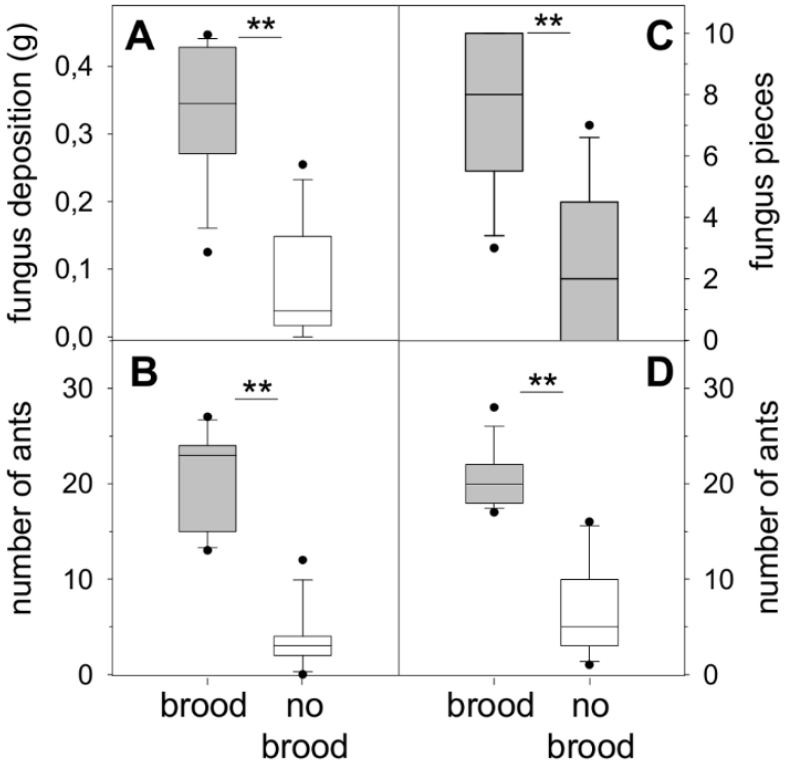

Figure 2.3: Fungus relocation experiment in non-digging setup. **(a)** Experiment 3: Fungus deposition in chambers, only one containing brood (n=12). **(b)** Number of *A. lundi* workers in chambers with and without brood (n=12). **(c)** Experiment 4: Fungus deposition at brood and empty side (n=13). **(d)** Number of workers at brood and empty side (n=13). Boxplots: median ± 25-75% percentiles, min max values and outliers, **p<0.01.

Even when a nest site with one big chamber instead of two small separate ones was offered (Experiment 4; **Fig. 2.3c**), and the familiarization period was shortened from 18 to 2h, significantly more fungus pieces were deposited on the brood side of the chamber (Wilcoxon matched pair test; T=5.5; Z=2.63; p<0.01; n=13). Of the 130 deposited pieces (first 10 of each assay, n=13), 97 were deposited on the brood, 33 on the empty side of the chamber. There also was a significant skew in ant aggregation in favor of the brood side. A median of 20 ants (25-75%= 18-21) were present on the brood side while a median of 5 ants (25-75%= 3-8) were present on the empty side (**Fig. 2.3d**; Wilcoxon matched pair test; T=0; Z=3.18; p<0.01; n=13).

(c) Chamber excavation as a response to the presence of brood and fungus

When given the possibility to either dig at a nest site with brood or at an empty one with no fungus present, ants excavated at both sites, but more material was removed from the nest site with brood (Experiment 5; **Fig. 2.4a**; Wilcoxon matched pair test; T=3.0; Z=3.24; p<0.01; n=15). The median excavated volume at the brood nest site was 21.5 cm³ (25-75%= 15.39-27.11 cm³; min-max= 10.44-37.28 cm³) and at the site without brood 9.72 cm³ (25-75%= 7.39-13.28 cm³; min-max= 3.67-15.89 cm³). Workers were observed to continue with excavation even when the experiment was stopped after 24 hours, although the space created around the brood looked more than sufficient to house both workers and brood. In fact, the brood pile took up very little space, and 10 piled pupae, as an example, occupy a mean area of 0.95 cm² (±0.25 SD) on the floor and a volume of less than 1 cm³.

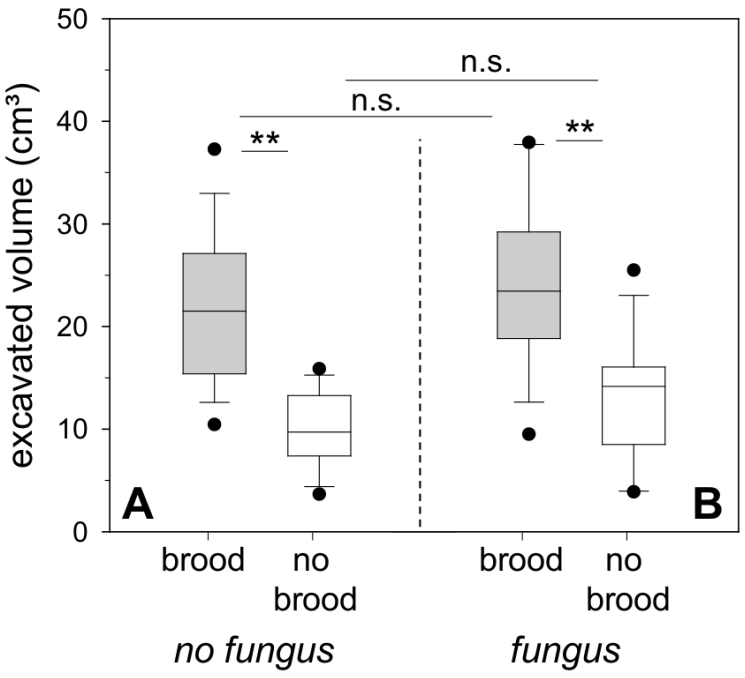

Figure 2.4: Digging activity (excavated volume) at nest sites with and without brood after 24 h (median ± 25-75% percentiles, min max values and outliers, n=15). **p<0.01; n.s.= not significant, p>0.05 **(a)** Experiment 5: Digging activity with brood only (Analysis of colony effects: ANOVA; brood; F=1.91; p=0.19; n=5; no brood; F=0.25; p=0.79; n=5; n.s.; no colony effects found) **(b)** Experiment 6: Digging activity with brood and fungus (Analysis of colony effects: ANOVA; brood and fungus; F=0.64; p=0.54; n=5; fungus; F=0.22; p=0.8; n=5; n.s.; no colony effects found).

Regarding the additional relocation of fungus inside excavated nest sites with and without brood, the amount of relocated fungus did not differ significantly between the sites (Experiment 6; **Fig. 2.5**; brood side: median= 0.248 g; 25-75%= 0.105-0.395 g; min-max= 0.060-0.489 g; non-brood side: median= 0.087 g; 25-75%= 0.001-0.244 g; min-max= 0-0.355 g; Wilcoxon matched pair test; T=29; Z=1.76; p>0.05; n=15), although in 3 of the 15 performed assays no fungus at all was deposited at the non-brood nest site. As in the previous series, digging activity

concentrated at the site containing brood and relocated fungus, and a significantly higher volume was excavated there (Experiment 6; **Fig. 2.4b**; brood side; median= 23.44 cm³; 25-75%= 18.83-29.22 cm³; min-max= 9.5-37.94 cm³; non-brood side; median= 14.17 cm³; 25-75%= 8.5-16.06 cm³; min-max= 3.89-25.5 cm³; Wilcoxon matched pair test; T=10.0; Z=2.84; p<0.01; n=15), despite the equal fungus deposit in both. Brood and fungus pieces were placed together; sometimes pupae were placed on top of the fungus.

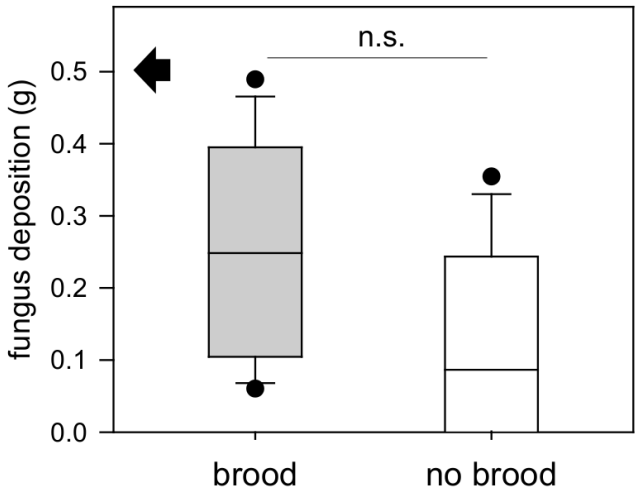

Figure 2.5: Experiment 6**:** Fungus deposition at nest sites (median ± 25-75% percentiles, min max values and outliers, n=15); black arrow indicates maximum possible fungus deposit; n.s.= not significant, p>0.05 (Analysis of colony effects: Kruskal-Wallis test; brood site; H=1.94; p=0.38; n=5; non-brood site; H=2.2; p=0.33; n=5; n.s.; no colony effects found).

Although the more voluminous fungus pieces are expected to take up more space than pupae, the volume excavated when fungus was present was not higher, but similar to that from the series with only brood present (**Fig. 2.4**; brood vs. brood and fungus: Mann-Whitney U Test; U=97.0; p>0.05; n=15; empty vs fungus: Mann-Whitney U Test; U=76.0; p>0.05; n=15). Examples of the excavated nest sites of all 4 different types (i.e., brood, empty, brood + fungus and fungus) are presented in **Fig. 2.6**.

(d) Shape of excavated structures

In the presence of brood, the shape of the excavated structure was more circular and therefore more chamber-like than without brood (Experiment 5; **Figs. 2.7a and 2.7c**; Form Factor (FF) brood site; median= 0.74; 25-75%= 0.56-0.84; FF empty side; median= 0.51; 25-75%= 0.45-0.54; Wilcoxon matched pair test; T=0.00; Z=3.41; p<0.001; n=15). The lower form factor for the non-brood site indicates a very high ratio of perimeter to area of the structure, i.e., a more tunnel-like shape.

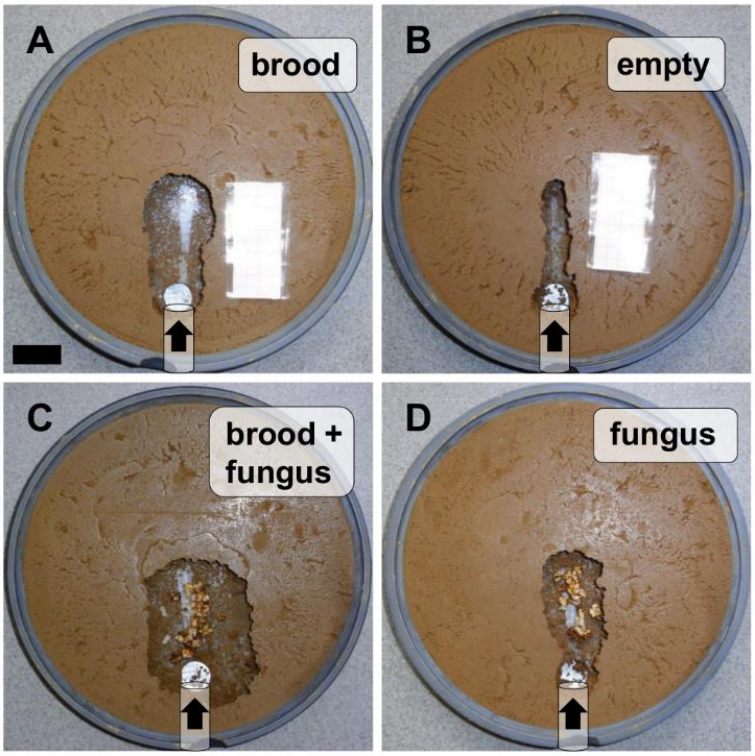

Figure 2.6: Pictures of digging sites at the end of the experiments. Experiment 5: **(a)** Brood. **(b)** Empty. Experiment 6: **(c)** Brood and fungus. **(d)** Fungus. Black bar = 2 cm; black arrows indicate the direction of entering ants.

In the series with additional fungus relocation, the excavated shapes at the nest sites containing brood were again significantly more circular and chamber-like than at the non-brood site (Experiment 6; **Figs. 2.7b and 2.7d**; FF brood site (fungus present); median= 0.79: 25-75%= 0.77-0.87; FF non brood site (fungus present); median= 0.60; 25-75%= 0.50-0.78; Wilcoxon matched pair test; T=9.0; Z=2.90; $p<0.01$; n=15), even though a similar amount of fungus, which is known to influence the shape of a chamber in accordance to its volume (Fröhle and Roces, 2009), was relocated to both sites. When comparing the shapes excavated at the brood and the non-brood site between the two different series, i.e., with or without additional fungus relocation, it was evident that the presence of fungus had a positive effect on the roundness of the excavated shape. The excavated structures at nest sites with both brood and fungus were rounder than at those with only brood (**Figs. 2.7c and 2.7d**; Man-Whitney U Test; U=62.0; $p<0.05$; n=15), and the excavated structures at sites with only fungus were rounder than at those with neither brood nor fungus (Man-Whitney U Test; U=61.50; $p<0.05$; n=15), with the least circular shapes being excavated at the latter, empty nest site.

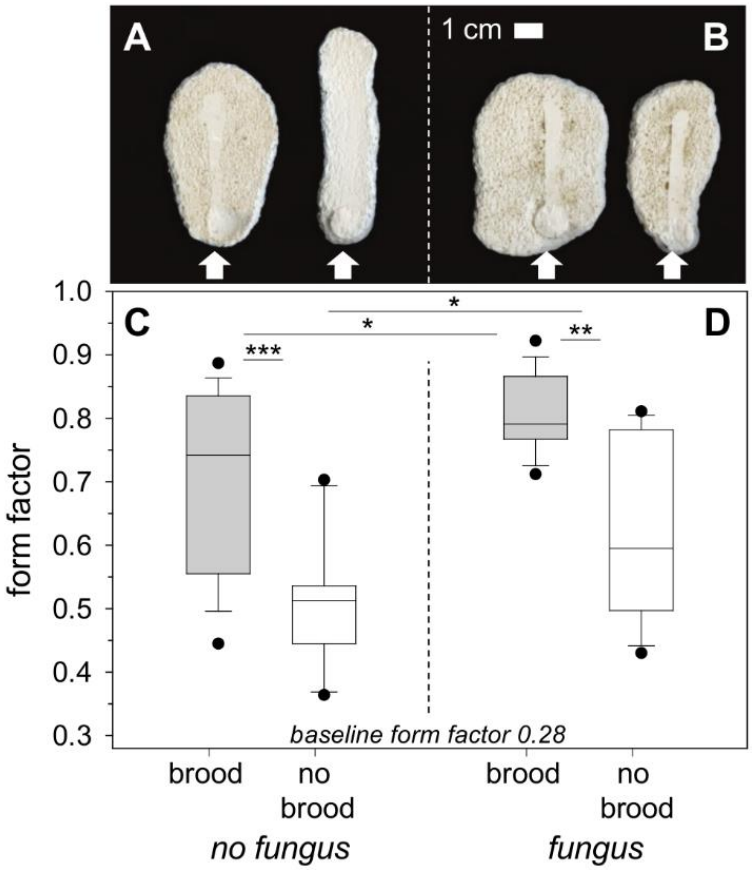

Figure 2.7: Evaluation of excavated shapes. **(a)** Plaster molds of excavation, brood only series (Experiment 5), Arrows indicate the direction of entering ants; view from below. **(b)** Plaster molds of excavation, brood and fungus series (Experiment 6). **(c)** Calculated form factor of brood only series (Analysis of colony effects: ANOVA; brood site; F=0.71; p=0.5; n=5; non-brood site; F=0.14; p=0.87; n=5; n.s.; no colony effects found). **(d)** Calculated form factor of brood and fungus series (Analysis of colony effects: ANOVA; brood and fungus site; F=1.24; p=0.32; n=5; fungus site; F=1.67; p=0.23; n=5; n.s.; no colony effects found). The y-axis starts from a baseline form factor of 0.28 (preformed structure: entrance hole and tunnel) (median ± 25-75% percentiles, min max values and outliers, n=15), *p≤0.05; **p<0.01; ***p<0.001.

A regression analysis was performed to evaluate whether the circularity of the excavated shapes depended on the excavated volume, i.e., the more material excavated, the rounder the resulting structures (**Figs. 2.8a and 2.8b**). Only at the nest sites with items (brood, fungus, or brood and fungus) was there a positive correlation between excavated volume and circularity (brood; r^2=0.56; p<0.01; fungus; r^2=0.42; p<0.01; brood and fungus; r^2=0.42; p<0.01).

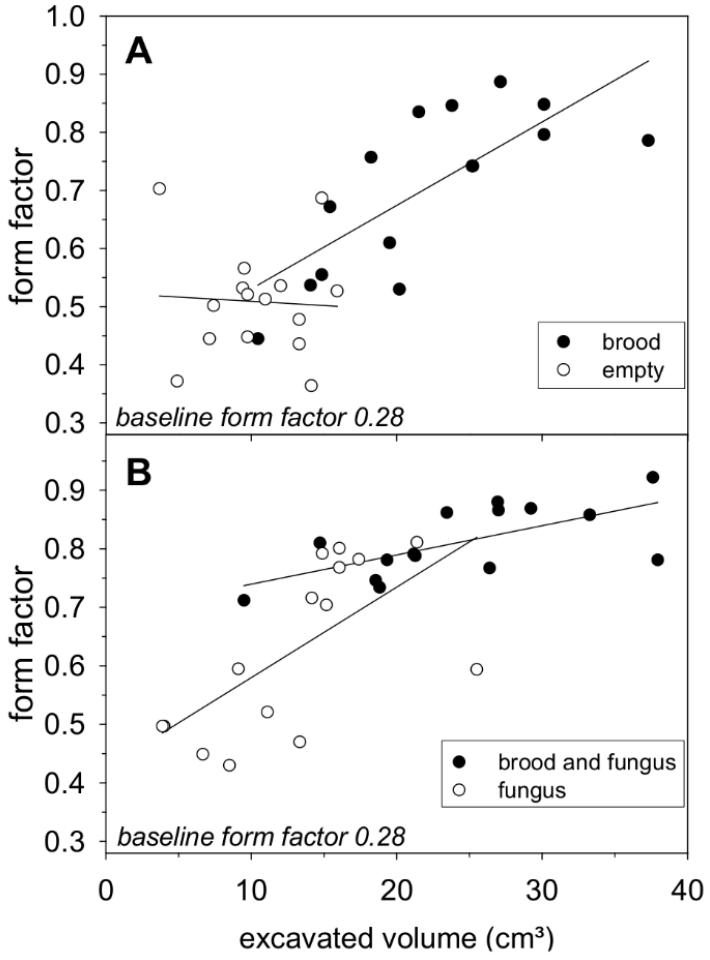

Figure 2.8: Relationship between the digging activity, measured as the excavated volume (x-axis), and the excavated shape, expressed as the form factor (y-axis). **(a)** Experiment 5: brood only series. Closed circles: brood; y=1E-6x+0.387; open circles: no brood (empty tunnel); y=1E-07x+0.523. **(b)** Experiment 6: brood and fungus series. Closed circles: brood and fungus; y=5E-07x+0.689; open circles: fungus; y=2E-06x+0.425; y-axis starts from a baseline form factor of 0.28.

When the nest site was empty, the excavated shapes did not increase in circularity (**Fig. 2.8a**; empty; r^2=0.04; p>0.05), although excavation ranged from 4-15 cm³. At the sites with items, however, an increase of 11 cm³ of excavated space led to a clear increase in circularity.

2.4 Discussion

Traditionally, abiotic factors such as humidity and temperature gradients have been described as the local stimuli workers use to choose a place for rearing their brood and fungus (Roces and Kleineidam, 2000; Bollazzi and Roces, 2002). The excavation of new fungus chambers at locations with suitable environmental conditions would therefore ensure proper development of the fungus and brood. However, the questions arises whether such suitable conditions suffice to trigger the excavation of a new chamber in advance, without the presence of fungus and brood at the spot. It is known that the symbiotic fungus, when relocated into a preformed, round chamber providing insufficient space, triggers the enlargement of the chamber in laboratory colonies of *Acromyrmex lundi* (Fröhle, 2009; Fröhle and Roces, 2009). Workers excavated

around the relocated fungus, thus extending the size of the initial chamber to accommodate all fungus. Without the presence of fungus, workers only excavated tunnels (Fröhle, 2009; Fröhle and Roces, 2009). While chambers can be enlarged when its content outgrows the offered space, probably by using the fungus as a template, there appears to be a maximal chamber size, so that at a given time new chambers need to be excavated. The abandoning of chambers because of unsuitable climatic conditions or the presence of contaminants should also go along with the excavation of new chambers.

Our results extend the knowledge about the emergence of nest structures by showing that the emergence of a new chamber can be triggered by the presence of brood at a site, with only tunnels being excavated at an alternative location without brood. These results are consistent with the hypothesis that nest chambers are not excavated in advance. Suitable microclimatic conditions alone do not appear to be sufficient to trigger chamber excavation. Based on the present results we propose a density-triggered mechanism of cavity excavation by which chambers emerge as functional structures when brood and fungus, i.e., the items that are expected to be stored in these cavities, are relocated and present at a given spot. Brood and fungus appear to serve as cues that attract workers and draw them away from other, environmentally suitable digging sites, thus leading to a high worker density around these items. Even though *A. lundi* inhabits nests with only a few chambers (Zolessi and Gonzalez, 1978), usually two to three, and sometimes only one, their shape is similar to that of other leaf-cutting ant species (Bonetto, 1959). We suggest that the proposed mechanism of chamber emergence and nest enlargement via relocated items is likely to be a common local mechanism that could also underlie the growth of the multi-chambered nests of *Atta* leaf-cutting ants, irrespective of their total number of chambers.

(a) Determination of relocation preference for brood or fungus

A. lundi workers showed a significant preference to relocate brood before relocating fungus, when exposed to a low temperature (Experiment 1) known to impair brood and fungal development (Powell and Stradling, 1986). This tendency to remove more brood than fungus was also observed in *Acromyrmex heyeri* workers (Bollazzi and Roces, 2002), which were in the process of carrying brood or fungus and exposed to a temperature of 10°C. As a result, they relocated more brood (40%) than fungus pieces (20%). In our experiments, it was surprising that so little fungus (in some assays none at all) was relocated to temperatures above 20°C. There was no apparent indication that during the experiment the fungus was damaged or died, because it would have been removed to the foraging area (foraging arena and soil deposit site), as observed in other experiments. While it could be argued that some of the fungus was infected

with pathogens and therefore was not relocated, the uniformly low fungus relocation would then imply that all the fungus collected from three different colonies and different gardens had been infected. It seems unlikely that fungus mortality or a pathogen infection was the reason for the reduced rate of fungus relocation in our assays. It is possible that the higher relocation rate of fungus in *A. heyeri* (Bollazzi and Roces, 2002) was due to workers already carrying the fungal pieces when exposed to the low temperatures, while in our experiments ants needed to cut free a piece of fungus first. The low temperature itself did not negatively influence the activity of the ectothermic ants, because the process of brood relocation was not affected and occurred completely.

There are other possible reasons why brood were relocated before the fungus. From an energetic perspective, brood might be more costly to produce than fungus. In the laboratory, we could observe that development from eggs to pupae took several weeks while the ants managed to create a new fungus garden (~1.3 l) in a week with ad libitum feeding. The second reason could be that there were microscopic traces of fungus left on the pupae we used in the experiments, so that fungus would be indirectly relocated with the brood. It is known that *Acromyrmex* pupae usually have a mycelial cover, not only from being embedded in the fungus garden, but also because workers actively plant these covers on the brood (Armitage et al., 2012). We removed any visible traces of fungus mycel from the pupae before the experiments, but there might have been microscopic traces left. It is unknown whether these traces would have been enough for the ants to start a new fungus garden. However, all fungus was still relocated into the nest site in Experiment 2, if only after the brood had been removed. If a mycelial cover influences the ants not to relocate fungus, they also should not have done so in Experiment 2. Third, brood might be easier to handle than fungus. Cutting out a piece of fungus takes time, which may lead ants to transport first the easily transportable items in a situation of rapidly changing environmental conditions. It has been reported that in a partly flooded field nest of *Atta sexdens*, part of the colony brood had been deposited in an upper, safe nest chamber, but none of the fungus gardens were relocated there (Stahel and Geijskes, 1941). That the time-consuming fungus removal was not the reason for the workers preference for brood relocation could be demonstrated when fungus was offered in small, easily transportable pieces (Experiment 2). Yet in over 90% of the pickup decisions, workers favored brood to be relocated first from desiccation. On the other hand this could indicate that brood is more prone to desiccation than fungus. However, the air humidity inside the clay nest sites in Experiment 1 was close to saturation, yet brood relocation was also preferred. Offered pupae and fungus pieces also differed in mass, a piece of fungus was twice as heavy as a pupa. Although the

masses are in the range of naturally foraged loads (Rudolph and Loudon, 1986) it could still be energetically more advantageous to carry a pupa. When ants picked up an item on top of the platform they were never observed to lift one item, put it down again and picking up the other item, as if the chosen item was too heavy to transport, a mechanism that is thought to lead to size-matching between carriers and their leaf fragments during foraging (Anderson and Jadin, 2001). Because ants always chose only one item and relocated it, we rule out differences in mass as a reason for the preferred brood relocation.

The motivation of ants to relocate exposed items from the foraging arena into the nest likely differs from that to relocate them within the nest. A stronger motivation to protect the items against exposition to the unsuitable outside environment may have been the reason for the complete relocation of fungus in the second series, yet brood were removed before the fungus. This seems to indicate that early brood removal in Experiment 1 was not just due to the physical restraints of the interconnected fungal mass on transportation. We therefore argue that *A. lundi* leaf-cutting ants seem to have a preference for brood relocation from sites having unsuitable conditions, and suggest that the observed pattern of relocation from the foraging arena (Experiment 2) reflects a transportation pattern that is also expected to occur within the nest. This was the reason why brood was tested as a possible trigger for chamber emergence in the digging experiments performed later.

(b) Brood as a cue for fungus relocation

Because of the association of brood and fungus in nest chambers (Lopes et al., 2005; Armitage et al., 2012), it was hypothesized that when brood is removed to other places in the nest, fungus pieces should be also relocated to these spots. The results of the fungus relocation experiments (Experiments 3 and 4) are in accordance with this hypothesis. Workers deposited more fungus at a location containing brood, irrespective whether the brood occurred in a separate chamber or at one side of a single chamber, suggesting that workers directly responded to the presence of brood. It remains an open question whether brood also directly influences the intensity of digging activity of those workers, which would later engage in the excavation of a chamber around it. Fungus would benefit from being relocated to a brood side because they have the same microclimatic demands on temperature and humidity. When workers of *A. heyeri* relocating brood and fungus could chose a deposition site along a temperature gradient, they selected temperatures from 21 to 25°C for both (Bollazzi and Roces, 2002), values that are known to ensure optimal fungal growth (Powell and Stradling, 1986). As a consequence, the presence of brood as a spatial cue for fungus deposit at such a site should also benefit fungal development.

Brood likewise might benefit from being surrounded by fungus. There could be several reasons: reduction of water loss through its not yet hardened cuticle, better insulation against temperature fluctuations, or reduction of risks of pathogen transmission by the barrier created by the fungus between brood and chamber floor. The experiments also highlighted how attractive brood was to workers, which will be of importance for our proposed mechanism of chamber excavation explained below. On average, 5-6 times more ants aggregated at the nest site that contained brood, so ant density clearly increased at this spot. Ant density likely plays an important role in nest excavation because high density is thought to stimulate workers to dig (Deneubourg and Franks, 1995, Rassé and Deneubourg, 2001). Such a density-triggered excavation behavior would result in the enlargement of a nest whenever the population increases, thus leading to a temporary increase in ant density, which would decline, once more space has been excavated. The fact that the size of many ant nests correlates with the number of ants inhabiting it, and larger colonies inhabit larger nests (Rassé and Deneubourg, 2001; Mikheyev and Tschinkel, 2004), support this hypothesis. Increased ant aggregation at brood deposition sites could result in a higher number of ants excavating at such a site, or an increased per capita excavation activity of workers, variables that were not quantified in our study.

(c) Chamber excavation as a response to the presence of brood and fungus

The results of the digging experiments indicate that brood presence leads to a spatial shift of digging activity towards a nest site containing brood, thus resulting in the excavation of rounder, more chamber-like shapes at this site (Experiment 5). It is likely that the presence of brood at the digging site caused a higher aggregation of workers at the site, as demonstrated in the fungus-relocation experiments mentioned in the previous section (Experiments 3 and 4), and that more workers engaged in digging there. Due to the opacity of the digging material, the number of ants engaged in digging could not be directly counted because parts of the excavation occurred under a layer of clay. Since significantly more ants aggregated at the brood site than at the site without brood in the fungus-relocation experiments (Experiments 3 and 4), it seems reasonable to infer that the effect of brood on worker aggregation should have been similar in the digging experiments. As a consequence, more workers present at a nest site with brood would lead to a higher excavated volume and a rounder cavity. In addition, brood could directly influence the intensity of digging in individual workers. While there are no comparative measurements of chamber shapes in field nests of leaf-cutting ants, nest tunnels are by definition long and narrow, meaning they have a horizontal cross-section with higher perimeter-to-area ratio than chambers, which are spherical and not lobed, in accordance with our results.

Regarding the hypothetical excavation of new nest chambers in advance, triggered

solely by a suitable microclimate, it is important to note that the environmental conditions offered at both nest excavation sites during our experiments were identical, and suitable for brood and fungus development. If environmental factors were the only cues ants use to decide where to initiate the excavation of a chamber, the shapes of the excavated structures should have been similar to one another. However, at the site with no brood or fungus present, ants excavated structures with shapes that resembled the preformed offered tunnel, and typically concentrated their digging at the tunnel tip. This was likely due to a disparity of worker number in favor of the site containing brood.

As previously indicated, it is known that the presence of fungus leads to chamber excavation (Fröhle, 2009; Fröhle and Roces, 2009). Workers excavated a cavity around the relocated fungus that was slightly bigger than the actual fungal structure and also shaped according to its proportions. When the fungus grew, so did the size of the chamber. Fungus in this regard was used as a dynamic template for the size and shape of the nest chamber. The use of brood as a template to shape the nest is known in the ant *Leptothorax tuberointerruptus* that inhabits very simple nest cavities. The amount of brood deposited in the middle of a worker cluster acts as a template for the erection of a surrounding wall that embodies all colony members (Franks and Deneubourg, 1997). In our experiments however, considering the large amount of space being excavated around brood, and the brood pile often being located not centrally in the excavated structure but to the side, it seems unlikely that the presence of brood alone is used as a template for chamber excavation.

Using fungus as a template is likely not the only variable involved in the determination of chamber size in leaf-cutting ant nests. The cavities excavated in our experiments with both brood and fungus (Experiment 6) were not of equal size, although an equal amount of fungus, i.e., a template of comparable size, had been deposited at both nest sites. Excavation at the brood (and fungus) site was higher than at the site with only fungus, probably because a higher number of workers were already present at the site with brood. Fungus relocation in this digging experiment did not follow the brood deposition, contrary to the expectations based on the relocation experiments with plaster nests (Experiments 3 and 4). This was probably due to the lack of space in the digging experiments, in which only small tunnels were offered. These results indicate that leaf-cutting ants, instead of letting the fungus die, relocate it to other suitable sites that they otherwise might not have chosen. The observed excavation of rounder shapes at the nest site containing only fungus emphasizes that fungus deposition triggers chamber emergence by influencing workers' digging activity in a way that rounder, more chamber like structures are excavated, even without brood.

The presence of brood and fungus concentrates excavating workers at the spot, leading to an evenly spread digging activity around it. This is highlighted by the existing correlation of excavated volume with the circularity of the excavated shapes only when at least either brood or fungus was present. A nest excavation site without relocated items might increase in excavated volume, but, likely because the ant workforce is not concentrated at a particular spot while digging, a less round and more tunnel-like structure is expected to emerge. Two mechanisms influencing ant aggregation, and therefore local ant density during nest digging, were recently described in leaf-cutting ants: a short-range vibrational signal, and even the presence of excavated soil pellets. Leaf-cutting ants stridulate while excavating, which attracts nearby workers to the digging site (Pielström and Roces, 2012). This effect could have led to an amplification of ant aggregation (and excavation) at the nest site where brood had been placed and workers had already started excavating and stridulating. The concentration of digging activity at a particular spot would be further guided by the presence of freshly-excavated pellets, deposited close to the excavation site, because they significantly influence the workers' decision where to start digging (Pielström and Roces, 2013). While each mechanism could work on its own to lead to ant aggregation influencing digging activity, they may also have had additive effects, as follows. Workers may have initially been attracted to one nest site because of brood, and started to dig because of the increased ant density there. The resulting presence of stridulating workers may have attracted more ants to the site, which led to further excavation there and the accumulation of soil pellets. This prompted even more workers that were present to engage in digging, leading to the significant difference in the volume and roundness of the excavated structures we observed.

(d) Mechanism of chamber emergence

We suggest the following mechanism underlying the emergence of a new chamber in a leaf-cutting ant nest. Because of space requirements or unsuitable conditions, brood and/or fungus are expected to be relocated from an existing nest chamber to a more suitable location in a nest tunnel. Due to the attractiveness of both brood and fungus, workers aggregate at the site, thus leading to a local increase of ant density. Workers would then excavate in a density-dependent manner until sufficient space is generated, thus leading to the emergence of a new nest chamber. Digging activity is thought to positively depend on ant density (Rasse and Deneubourg, 2001; Toffin et al., 2009; Toffin et al., 2010). Crowding may lower the behavioral threshold triggering digging, and lead to a higher number of digging workers at the site, with a larger space being excavated there. The results of the fungus relocation experiments (Experiments 3 and 4) suggest a direct response to the presence of brood, i.e., cues originating from the brood may influence

other behavioral responses. Whether brood also has, for example, a stimulating effect on the per-capita excavation rate of workers remains to be investigated.

This postulated mechanism for chamber emergence could be solely based on the effect of worker density on digging activity, or also on an additional direct stimulating effect of brood on digging responses. In both scenarios, brood and fungus appear to act as 'ant aggregators' that concentrate the workforce at a suitable place in the nest, leading to the excavation of chambers with circular shapes. Therefore, not the mere presence of brood but the resulting increase in ant density would be the determining factor that leads to the excavation of more chamber-like shapes.

When ant workers are spread out across a large nest area with a wide digging face, only scattered digging sites are occupied and less circular shapes emerge. This effect was observed in a digging experiment with workers of the ant *Lasius niger*. The ants had access to a digging arena through a hole in the arena lid, without preformed space inside (Toffin et al., 2009; Toffin et al., 2010). They first excavated in a centrifugal way, creating a circular cavity that later became ramified as tunnels started to develop from the cavity wall. These results seem to indicate that chambers can emerge without the presence of any chamber items, contrary to the findings of our study and the arguments advanced above. It is important to indicate that *L. niger* workers had access to only one possible digging site inside the arena, so that all workforce was initially concentrated there, with the ants likely aggregating first at the entrance hole. The increased ant density at this spot, even without the presence of brood, likely stimulated more ants to engage in digging (Deneubourg et al., 1995), so that a round structure was excavated. When the cavity grew, a decrease in ant density occurred, probably leading to a 'competition' of alternative, spatially-separated digging sites that attracted workers (Minter et al., 2012) and resulted in ramification of the excavated cavity and tunneling. As a consequence, it is likely that the initial cavity excavated by *L. niger* workers (Toffin et al., 2009; Toffin et al., 2010) is not a functional structure aimed at generating nest space to house workers or brood, but resulted from the initial crowding effects and further dynamics of digging.

The importance of worker aggregation and the concomitant increase in ant density for the excavation of nest chambers, irrespective of the presence of brood, needs to be evaluated in further studies using for example single digging arenas in which available space and worker numbers, with and without brood items, should be manipulated. In a natural nest, ants should spread out across their nest space, as long as they do not encounter any stimuli triggering aggregation. A lower ant density would induce fewer ants to start excavating, with no concentration of digging activity at a particular spot. Therefore, chamber-like cavities should

not be excavated there. We propose a distinction between calling an excavated space a chamber or a cavity, with the former term being used only when actual items usually housed in a chamber, i.e., brood, fungus or food, are present when the structure is excavated.

We suggest that the empty chambers that make up part of leaf-cutting ant nests (Stahel and Geijskes, 1939; Jacoby, 1960; Jonkman, 1980b; Lapointe et al., 1998; Moreira et al., 2004a; Moreira et al., 2004b; Moser, 2006; Verza et al., 2007) were not excavated in advance, but rather initially excavated around relocated items, brood and fungus. They were found empty likely because of fungus decay, pathogen threat, or relocation of their contents to more suitable nest locations. Likely, it is energetically disadvantageous to engage in costly digging (Sudd, 1969) in advance, before the actual need for chamber space arises, i.e., to excavate cavities that may not necessarily be used. Rather, we propose that chamber excavation is a self-organized process triggered by the aggregation of workers, i.e., by the increased ant density around relocated brood and fungus, which leads to a concentrated excavation at the deposition site and to the emergence of a chamber as a functional structure. Such a mechanism could hypothetically underlie the emergence of chambers in nests of other leaf-cutting ant species, and also in nests of non-fungus-growing ants that store brood or food, although the behavioral rules that lead to their species-specific architecture remain to be investigated.

Cement casts of fungus chambers and tunnels in a nest of Atta laevigata, source: W. Thaler

Chapter 3

3. Available space, symbiotic fungus and colony brood influence excavation and lead to the regulation of nest size in leaf-cutting ants

Abstract

The size of underground ant nests positively correlates with their worker number, suggesting that during colony growth, workers respond to increased space demands by enlarging the existing nest. In leaf-cutting ants, the presence of brood and a growing symbiotic fungus is expected to generate spatial demands workers should respond to. We investigated how these variables influence the regulation of nest size in the leaf-cutting ant Acromyrmex lundi. In the laboratory, worker groups were offered nest sites for excavation with either reduced or ample space, into which they could move and store brood and fungus. In the presence of brood, nests were more enlarged when less space was available. The relocation of fungus and brood into a chamber did not influence digging intensity when ample space was available, but larger chambers and fewer tunnels were excavated compared to the enlargement of empty cavities. During the process of nest enlargement, workers were observed to initially excavate space in excess, which was refilled with part of the removed soil pellets, thus leading to a reduction of the final nest size. Pellet deposition seemed to be opportunistic, with workers refilling unused space. Results indicate that the final nest size does not simply depend on the number of inhabiting workers. Rather, workers adjust the final nest size and excavate tunnels or chambers depending on the existing space and presence of stored items.

3.1 Introduction

Social insects build complex nests that protect their colonies and damp environmental fluctuations (Hansell, 1984). Unlike the majority of eusocial bee, wasp and termite species, which usually construct their nests out of wax, carton and other building materials above ground, most ant species excavate and inhabit subterranean nests (Hölldobler and Wilson, 1990). By removing soil, workers create the basic architectural structures of a nest, i.e., chambers and tunnels. Chambers are usually horizontally oriented, flat and have a round base. Here workers store the brood, food, and in some species, waste material (Jonkmann, 1980; Tschinkel, 2004; Mikheyev and Tschinkel, 2004). The chambers of fungus-growing ants are spherical and dome shaped (Antonialli and Giannotti, 2001; Bollazzi et al., 2012; Stahel and Geijskes, 1936; Tschinkel, 2003), because they are used to house a voluminous, sponge-like fungus that the ants farm (Weber, 1966). Nest chambers are connected to each other and to the soil surface through oblong and narrow tunnels. They accommodate the colonies traffic as well as contribute to nest ventilation (Bollazzi et al., 2012).

It is argued that the architecture of ant nests emerges through a self-organized process in which cooperating individuals only have access to local information (Deneubourg and Franks, 1995). While these arguments are well established in the literature, few studies have identified the specific environmental stimuli and decision-making processes involved in complex excavation behaviors. Ants may react to local stimuli originating from their environment, other nearby workers, and the by-products of the building process itself (Bollazzi et al., 2008; Rasse and Deneubourg, 2001; Pielström and Roces, 2013). The enlargement of a nest from its founding state, which in most ant species is comprised by a short, downward leading tunnel with a small chamber, appears to be a tightly regulated process. Nest size increases with time and is correlated with colony population (Tschinkel, 1987; Deneubourg and Franks, 1995; Tschinkel, 2004; Mikheyev and Tschinkel, 2004; Buhl et al., 2005), and these observations have led many to conclude that worker number is the primary factor responsible for nest size determination.

For example, in the ant *Lasius niger*, when ant groups of different sizes could excavate in an experimental arena, larger groups excavated larger nests (Rasse and Deneubourg, 2001). When more ants were added to simulate colony growth, ants responded with enhanced excavation, which led to the enlargement of the nest. A density dependent stimulation of digging activity is thought to be one of the possible underlying mechanisms of this response, where increased worker density at the beginning of nest enlargement initiates excavation. Through positive feedback, excavation activity could be maintained or increased at this site,

and later be counteracted via negative feedback, by which excavation would diminish or cease. Positive feedback may also occur through stigmergic responses to excavated soil pellets (Pielström and Roces, 2013) or through stridulating excavators (Pielström and Roces, 2012), which draws a workforce to a site where they excavate. When space has been created, the reduced worker density could act as a negative feedback, without requiring any explicit measure of nest and/or population size by workers. Nest excavation could therefore be self-regulated via a positive feedback, leading to nest enlargement, and the space generated causing inhibition of the process (Rasse and Deneubourg, 2001; Halley et al., 2005). A further hypothetical mechanism involved in the regulating of nest enlargement is workers obtaining spatial knowledge about the size of the excavated structure itself, by using idiothetic information, as founding queens of leaf-cutting ants do (Fröhle and Roces, 2012). Additionally, digging activity may act as a negative feedback, because workers may simply excavate less or stop digging as time progresses because of exhausted energy stores or changes in their response thresholds, as known for leaf-cutting ant queens (Camargo et al., 2011; Fröhle and Roces, 2012). This would lead to a final nest size without explicit information about the space already created or the presence of nestmates.

In addition to the self-organized behaviors documented during nest digging in ants, the use of templates to adjust the size of the nest chambers has also been reported. Leaf-cutting ants are known to shape and size their chambers according to the shape and volume of the symbiotic fungus housed in these chambers (Fröhle and Roces, 2009). In addition, it was demonstrated in *Acromyrmex lundi* that the other items stored in a leaf-cutting ant nest, including the colony's brood, could positively influence the size and shape of the nest structures excavated by workers (Römer and Roces, 2014). Brood presence led to the excavation of more round, chamber-like structures as those excavated at an alternative site without brood. Fungus presence had a similar effect, with the excavated chamber-like structures even larger if brood was present. A recent study on *Atta sexdens*, which excavates deeper nests in contrast to the more superficially nesting *Acromyrmex lundi*, also reported the same effect of brood and fungus presence on chamber excavation (Camargo and Forti, 2014). Since it triggers the excavation of nest chambers, the presence of brood and fungus should also influence the regulation of nest size.

In this work, we investigated how the available space and the presence of brood and fungus as stored items influence excavation and size regulation of leaf-cutting ant nests. If created space acts as a negative feedback, then a larger space should negatively influence excavation activity of the ants. The presence of brood and fungus, on the other hand, should lead to a larger increase of nest space than in the absence of these in-nest stores. In a first laboratory experiment,

we presented groups of *Acromymrex lundi* workers with a nest site either with reduced or ample space, into which they could move and store brood. In a second experiment, the influence of fungus was investigated when ample space was available. In both experiments, we quantified the magnitude of excavation as well as the size of the resulting nest and its different structures, i.e., chambers and tunnels. Since leaf-cutting ants were observed to deposit part of the removed soil inside the excavated structures (Pielström and Roces, 2013), we also evaluated the potential reduction of the final nest size through the deposition of excavated soil pellets.

3.2 Materials and Methods

All experiments were performed in the laboratory between June 2010 and June 2011 with 3 colonies of the leaf-cutting ant *Acromyrmex lundi*, founded in 2006. They were reared in a climate chamber at the University of Würzburg, Germany in the Department of Behavioural Physiology and Sociobiology, at 25°C, 50% humidity and a 12L:12D cycle on an ad-libitum diet of blackberry (*Rubus fruticosus*) leaves, honey water and water. Each colony was kept in a system of plastic boxes (19x19x9 cm) which served as artificial fungus chambers, a waste disposal box and a feeding arena, all interconnected by transparent plastic tubing.

Each assay was performed with a subcolony consisting of 150 medium sized workers (mean body mass calculated from a ubiquitous sample of 80 medium workers = 5.3 mg ± 1.2 mg SD), 75 of which were collected out of a randomly chosen fungus box and 75 out of the feeding arena. When brood or fungus was offered in a series, they were collected from the same fungus box as the workers. Workers, brood and fungus were not reintroduced into their colonies after the assays. All visible traces of fungus mycelium were removed from the brood and the fungus mass was cleaned of brood and workers.

(a) Regulation of nest size: effects of available space and brood presence

To evaluate whether the size of the available nest space influences excavation and leads to the regulation of nest enlargement, worker groups had the opportunity to enlarge an existing nest structure inside a digging arena, which provided either a reduced or ample space. The "reduced nest space" only consisted of a tunnel, while the "ample nest space" consisted of a tunnel and a cavity, whose size was chosen based on the space needs of the later performed experiment 2, where the cavity should be able to accommodate the volume of the offered symbiotic fungus (mean fungus volume: 20 cm^3). As the presence of stored items had been shown to influence a workers decision where to enlarge a nest structure (Römer and Roces, 2014), nests with reduced or ample space were combined with presence or absence of brood. Four different series were performed in experiment 1. The experimental setup was prepared as follows.

The two-dimensional nest site (30x30x1 cm), where ants could excavate, was made out of clear polycarbonate with an entrance hole (diameter 1 cm) in the arena bottom and a removable lid. The arena was filled with moist clay (Claytec Baulehm gemahlen 0–0.5 mm, Viersen, Germany) with a water content of 21%, to allow for easy excavation (Pielström and Roces, 2014). Some of the clay was then removed to create a free nest space in two different sizes. For the setup called 'reduced space', a 7 cm long tunnel (width 1 cm, height 1 cm, 7 cm^3) was cut out of the clay starting from the entrance hole (**Fig. 3.1a**).

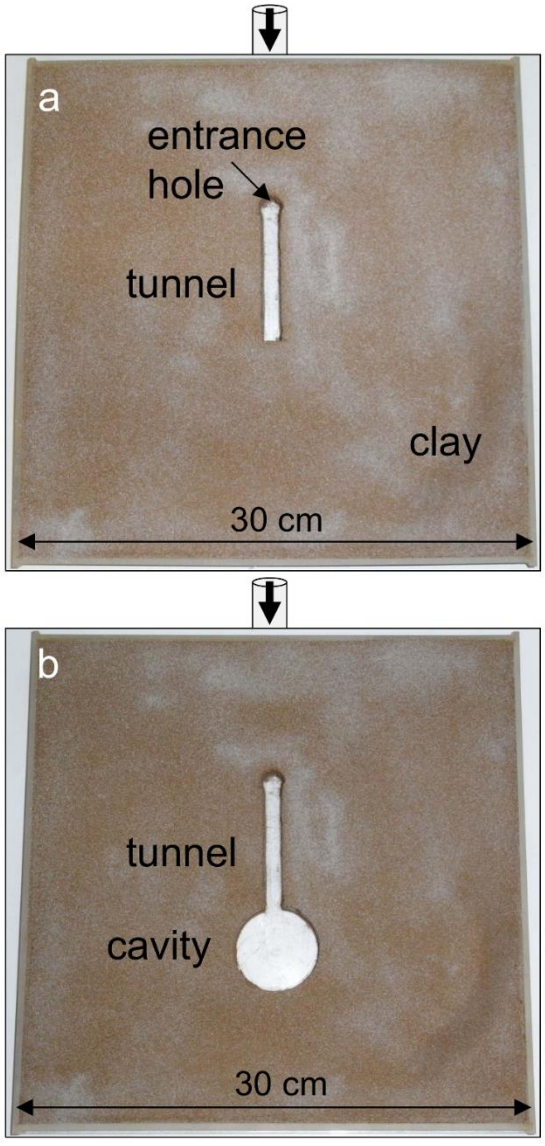

Figure 3.1 Experimental nest sites: **(a)** setup 'reduced space' with a tunnel (7x7x1 cm) as offered nest space; **(b)** setup 'ample space' with a tunnel and cavity (diameter 5 cm, height 1 cm) as offered nest space. The arrows indicate the direction of the ants coming from the feeding and soil-deposition box.

For the setup called 'ample space', a round cavity (diameter 5 cm, 21.6 cm^3) was removed in addition to the tunnel (**Fig. 3.1b**). The horizontally placed nest site was connected to a pellet deposit box (19x19x9 cm) and a feeding box (19x19x9 cm). By separating the soil deposit from the food supply, we ensured that the food source (honey water and water ad libitum) would

remain unspoiled by dropped-in soil pellets, which would negatively influence worker survival during the experiment. Both boxes were connected by a wooden bridge.

The four different series were as follows: (a) reduced space with brood (series 1a, n=15), (b) ample space with brood (series 1b, n=36), (c) reduced space without brood (series 1c, n=9) and (d) ample space without brood (series 1d, n=12). In both series with brood, the items were offered in increasing numbers to evaluate if brood quantity and not just its presence influences excavation and nest size regulation. In the series with reduced space, brood was offered in 5-brood increments, from 5 to 75 items (n=15). In the series with ample space, either 10, 30 or 75 pieces of brood were offered, and 12 assays for each brood amount were performed.

At the beginning of each assay, 150 workers (and brood) were placed in the feeding box and could explore the whole setup. Digging would usually begin within the hour. The running time of each assay was 48 hours, starting with the release of the ants. A few hours after workers had explored the setup and discovered the nest site, the brood would be transported into the more humid nest space.

After the end of the assay, the excavated nest was photographed with a piece of millimeter paper as size reference. Excavated soil pellets (the product of a grab-rake sequence during digging, Sudd, 1969), which had partly been deposited in the nest (usually adhered to the sides of chamber or tunnel walls), were carefully removed and weighed separately (chamber or tunnel) to the nearest 0.1 g. Chambers could easily be distinguished from tunnels because in the former, the soil across the arena height had been removed by the ants. Tunnels were shallower, usually excavated in the upper half of the clay layer. Then, a plaster cast was made of the emerged or enlarged chamber, and its volume measured by water displacement to the nearest 0.5 cm^3. In addition, both the final cavity area (i.e., the area remaining free after the deposition of soil pellets inside) and the initially excavated area (visualized after the removal of the deposited pellets) were measured (mm^2) using photographs and the software ImageJ (version 1.44p, National Institutes of Health, USA). The total digging activity, calculated as the excavated volume (cm^3), was quantified via the mass of the excavated clay.

(b) Regulation of nest size: influence of fungus and brood

To evaluate whether the presence of the symbiotic fungus, which is known to be used as a template to shape the fungus chamber, influences the regulation of nest size, digging experiments with fungus were performed. Only the setup ‘ample space’ with a tunnel and chamber was used for experiment 2 (see **Fig. 3.1**). In this experiment, two series were performed, one with fungus (series 2a, n=12) and one with fungus and additional brood (series 2b). The latter was done to evaluate if digging activity and nest size depend on the presence of

brood in the fungus garden. Brood was offered in two different quantities, either 30 (n=12) or 75 (n=16) items. The implementation of the assays was identical to the first series of experiments with 2.86 g freshly collected fungus (and brood) being placed in the feeding area at the beginning of the assay. This amount of fungus was chosen as it equals a mean fungus volume of 20 cm^3 (based on an evaluation of Fröhle, 2009), where 0.18 g of the symbiotic fungus of *A. lundi* equals 1.26 cm^3), so workers should be able to deposit it completely in the offered cavity (21.6 cm^3). At the end of the assay, the fungus relocated into the nest was collected and weighed to the nearest 0.1 mg, and the brood items counted. The results of both fungus series were then compared to series d of experiment 1 ('ample space without brood'), which served as a control.

(c) Measured variables

Four different nest parameters were compared to investigate the mechanisms of nest size regulation. (a) The excavated nest volume, defined as the space created through the digging activity of the workers. (b) The gross nest volume, defined as the excavated nest volume plus the initial space of the offered structures, either a tunnel or a tunnel and an empty cavity. (c) The space occupied by the internal deposit of soil pellets, as this reduced the usable nest space. (d) The final nest volume, calculated as the gross nest volume minus the space occupied by soil pellets, i.e., the final free nest space at the end of the experiment.

3.3 Results

After workers discovered the free space offered to them in the clay-filled arena, the first excavated pellets started to appear in the pellet deposit box within one hour. The offered items, brood and fungus, were readily transported inside and deposited either at the end of the offered tunnel (brood in the series with "reduced space"), where a chamber was excavated, or in the cavity initially offered (brood or fungus in the "ample space" series). Most of the digging activity took place straight ahead from the entrance hole, indicating that ants maintained their heading when coming from the outside area into the arena to excavate. Once the workers encountered the opposite arena wall, they excavated along it, forming u-shaped tunnels around the location of the offered structures. Part of the excavated soil pellets were not transported outside, but deposited inside the excavated nest and initially offered structures. **Figure 3.2** presents an example of the excavation observed in series 1a with reduced space and brood, in which the pellets deposited inside visibly narrow and smooth the wider and more irregular tunnels initially excavated.

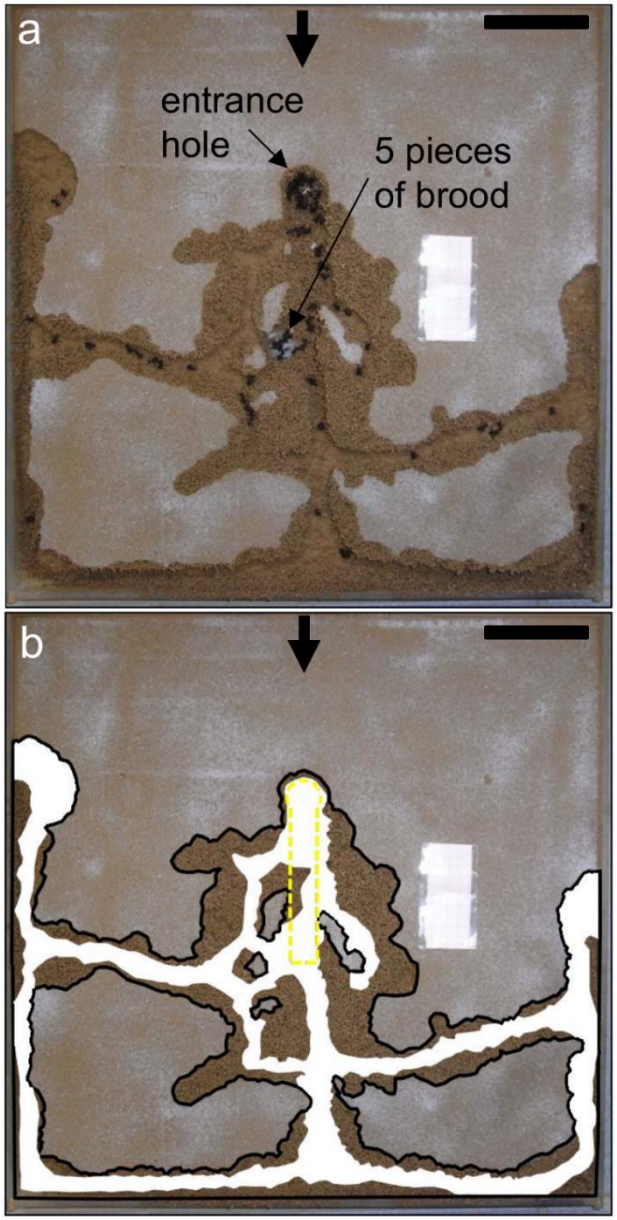

Figure 3.2: **(a)** Example of an excavated nest site with 5 pieces of brood after 48 h; **(b)** same picture; black line: boundary of the excavated nest; White area: final (free) nest space; yellow dashed line: outline of offered tunnel and entrance hole. The arrows indicate the direction of the ants coming from the feeding and soil-deposition box. Scale of black bar= 5 cm.

(a) Regulation of nest size: effects of available space and brood presence

In both series 1a and 1b, the excavated nest volume was independent of the number of brood items present (Series 1a: Regression analysis: r^2= 0.125, p>0.05, n=15; series 1b: Regression analysis: r^2=0.0005, p>0.05, n=36), so that the values were pooled within each series for further comparisons across series. The excavated nest sizes did differ among the four series (**Fig. 3.3a**, grey symbols, p<0.001, see figure captions for statistics). Ants in the setup 'reduced space with brood' (series 1a) excavated the largest volume of all four (mean 179.8 cm^3±28.8 SD). When the ants started with ample space and relocated brood, they excavated significantly less (mean 127.7 cm^3±25.9 SD). Starting with reduced space without brood did not lead to a statistically significant reduction in excavated volume (mean 150.4 cm^3±23.0 SD) compared to the equal space series with brood. However the excavated volume was also not significantly different to the ample space series without brood (mean 121.9 cm^3 ±16.9 SD).

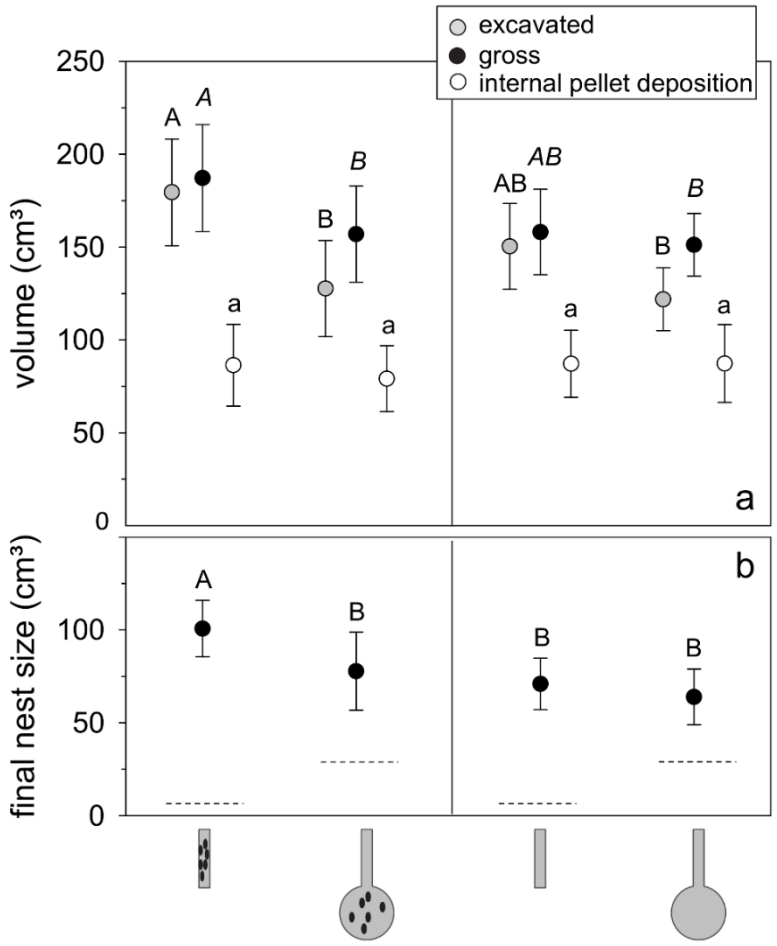

Figure 3.3: **(a)** Excavated volume (grey symbols), gross nest volume (black symbols), and internal pellet deposition (white symbols) of all 4 series of experiment 1: from left to right, reduced space with brood (series 1a, ample space with brood (series 1b), reduced space without brood (series 1c) and ample space without brood (series 1d). (Statistics: Excavated volume: one-way ANOVA, $F=18.03$, $p<0.001$, post-hoc test: Scheffé: reduced space with brood vs. without brood, $p=0.06$; $n_{1a}=15$, $n_{1c}=9$; ample space with brood vs. without brood, $p=0.92$, $n_{1b}=36$, $n_{1d}=12$; reduced space with brood vs. ample space with brood, $p<0.001$; reduced space with brood vs. ample space without brood, $p<0.001$; reduced space without brood vs. ample space with brood, $p=0.12$; reduced space without brood vs. ample space without brood, $p=0.09$; Gross nest volume: one way ANOVA, $F=6.38$, $p<0.001$, post hoc test: Scheffé: reduced space with brood vs. ample space with brood: $p<0.01$, reduced space with brood vs. ample space without brood: $p<0.01$; ample space brood vs. without brood: $p=0.92$; reduced space brood vs. without brood: $p=0.06$; without brood reduced space vs ample space: $p=0.94$; reduced space without brood vs ample space with brood: $p=0.99$; Internal pellet deposition: one-way ANOVA: $F=0.98$, $p=0.41$), **(b)** Final nest size after pellet deposition for the 4 series of experiment 1. The dashed line marks the volume of offered space at the beginning of the excavation. (Final nest volume: one way ANOVA: $F=10.5$, $p<0.001$; post hoc test Scheffé: reduced space with brood vs. without brood, $p<0.01$; ample space with brood vs. without brood, $p=0.17$; brood, reduced space vs. ample space, $p<0.01$; without brood, reduced space vs. ample space $p=0.86$; ample space with brood vs reduced space without brood; reduced space with brood vs. ample space without brood, $p<0.001$; line: median, box: 25-75% percentils, whiskers: min-max values). Groups with the same letter do not differ statistically; $p<0.05$.

The gross nest volumes were independent of brood quantity (Regression analysis: series 1a: r^2=0.125, p>0.05, n=15; series 1b: r^2=0.0005, p>0.05, n=36), and data from the assays with different brood numbers were therefore pooled. The gross nest volumes differed among the series (**Fig. 3.3a**, black symbols, p<0.001). The highest gross nest volume was exhibited when space was reduced and brood was present (Series 1a, mean 187.2 cm^3±28.8 SD) and there was a statistically significant reduction in volume when ample space was offered (Series 1b: mean 157 cm^3±25.9 SD). Analogous to the excavated volume, the gross nest volume in the reduced space series without brood (Series 1c: mean 158.1 cm^3±23.1 SD) did not differ statistically from the reduced space with brood series, but also not from the ample space without brood series (Series 1d: mean 151.2 cm^3±16.9 SD).

The space occupied by deposited soil pellets was the same, whether workers started out with less space or more space or whether ants stored brood in the nest site (**Fig. 3.3a**, white symbols; p=0.41).

The final nest sizes were also independent of the brood quantity (Regression analysis: series 1a: r^2=0.04, p=0.47; series 1b: r^2=0.0002, p>0.05, n=36), and data were therefore pooled. The final nest sizes were comparable to one another, except for the reduced space with brood series, which displayed a higher final nest size (**Fig. 3.3b**, Series 1a: mean= 100.8 cm^3±15.2 SD, series 1b: mean= 77.7 cm^3±21 SD, series 1c: mean= 70.9 cm^3±13.9 SD, series 1d: mean= 63.8 cm3±15 SD).

(b) The effect of brood on cavity area

Ants excavated a chamber in all 15 assays with reduced space and brood. When no brood was present, a cavity also emerged in 8 out of 9 performed assays. Chamber size was larger when brood was present (**Fig. 3.4**, black symbols; $mean_{series1a}$=28.1 cm^2±10.7 SD, $mean_{series1c=}$17.8 cm^2±3.6 SD), and independent of brood quantity (Regression analysis: r^2=0.134, p=0.18). In both series, the excavated area was significantly reduced by the deposition of soil pellets. The final area was larger when brood was stored in the chamber (**Fig. 3.4**, white symbols; $mean_{series1a}$=19.8 cm^2±10.4 SD, $mean_{series1c=}$7.9 cm^2±3.2 SD), with the pellets adhered to the chamber wall. Without brood the excavated cavity was almost completely filled with pellets so that only a tunnel structure remained (**Figs. 3.5a and 3.5b**: with brood; **Figs. 3.5c and 3.5d**: without brood). As recently proposed, only round structures excavated around stored items should be called ‘chambers’, while otherwise round, chamber-like structures, which sometimes

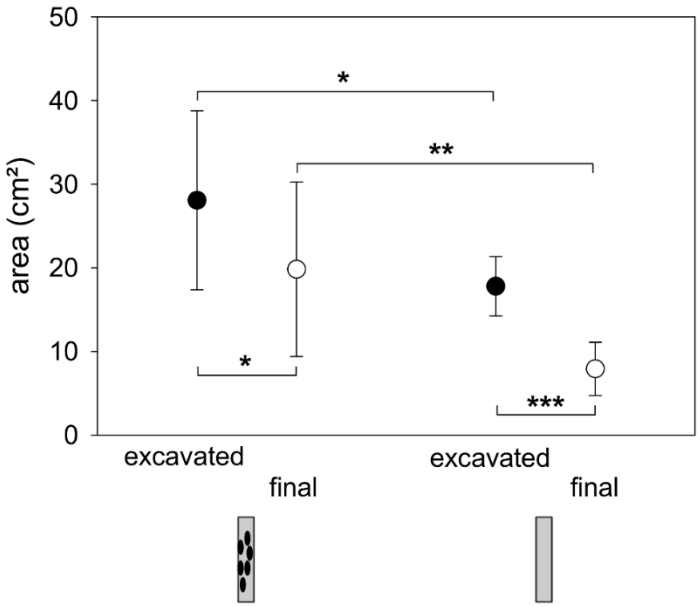

Figure 3.4: Excavated chamber or cavity area (cm²) depending on brood presence. Left: with brood (series 1a); right: without brood (series 1c). Excavated area (black symbols) and final area depending on pellet deposition (white symbols) are depicted for both series. (Unpaired t-test, comparison excavated area between series 1a and 1c: $t=2.62$, $df=21$, $p<0.05$, $n_{1a}=15$, $n_{1c}=8$; comparison final area between series 1a and 1c: $t=3.12$, $df=21$, $p<0.01$, $n_{1a}=15$, $n_{1c}=8$; reduction of area after pellet deposition in each series: paired t-test: series 1a: $t=2.75$, $df=14$, $p<0.05$, $n=15$; series 1c: $t=5.5$, $df=7$, $p<0.001$, $n=8$; circle: mean, whiskers: standard error); *=p<0.05; **=p<0.01; ***=p<0.001.

emerge in laboratory experiments without items being present, should be called ‚cavities' (Römer and Roces, 2014). In the present study, the terms are used accordingly and refer to the offered, round ample space as 'cavity'.

(c) Regulation of nest size: effects of fungus and brood

Irrespective of the presence of a voluminous fungus, the excavated volumes in the series offering ample space were statistically similar (**Fig. 3.6**, grey box plots; p=0.25; for detailed statistics see figure caption), as were the gross nest volumes (**Fig. 3.6**, black symbols; p=0.25). However, the final nest size differed among series (**Fig. 3.6**, white symbols; p<0.001). Without stored items, the final nest size was significantly reduced by a higher amount of deposited soil pellets inside these nest sites (series 1d vs. series 2a, p<0.001; series 1d vs series 2b, p<0.001). The presence of brood within the fungus did not influence the size of the final nest (series 2a vs. series 2b, p=0.7).

The separate analysis of the excavated structures, either chambers/cavities or tunnels, showed that the equal overall digging activity was differently allocated to chamber and tunnel excavation, depending on the presence of stored items. There was a marked difference in the enlargement of the offered cavity (21.6 cm³) depending on the presence of fungus (**Fig. 3.7**, grey box plots, white background, p<0.001). The offered cavity was significantly more enlarged when fungus was stored in it, and in the absence of fungus, the cavity was filled with excavated pellets, with only a free tunnel left as a passage connecting to the additional tunnels. **Fig. 3.8**

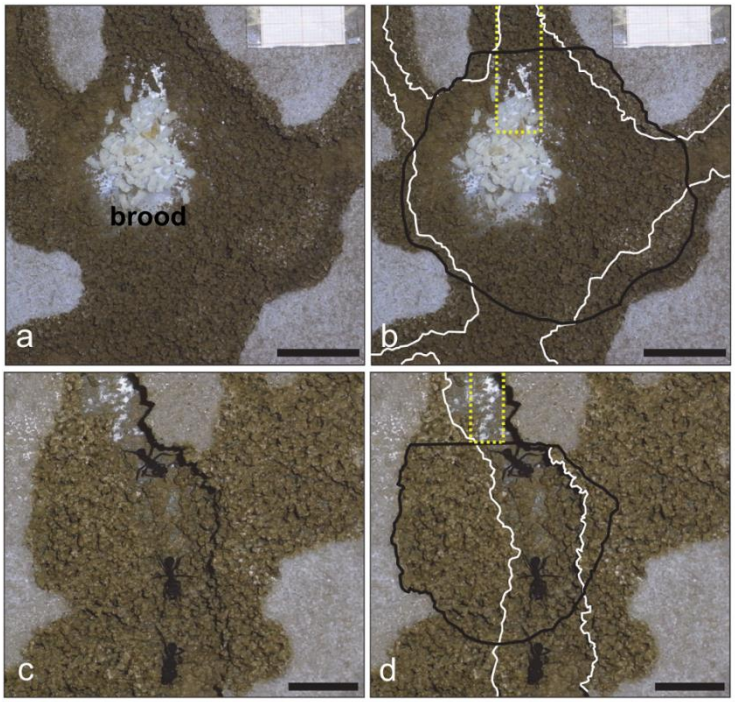

Figure 3.5: Examples of self-excavated chambers or cavities in both series with reduced space (Series 1a and 1c) after 48 h. **(a)** Brood present at nest site: A large chamber has been excavated and slightly reduced by pellet deposition. **(b)** Same image, with excavated chamber size (black line) and free final chamber size after pellet deposition outlined (white line). **(c)** No brood present at nest site: A smaller cavity was excavated and significantly reduced through pellet deposition, so that only a tunnel remained. **(d)** Same image, with cavity size (black line) and free final structure after pellet deposition outlined (white line). The yellow dashed line outlines part of the initially offered tunnel. Scale of black bar=2 cm.

and 3.9 present two examples of the excavated structures, without and with relocated fungus, respectively. The presence of additional brood did not lead to an increase in chamber enlargement ($p=0.31$). The presence of the symbiotic fungus also affected the magnitude of excavated tunnels (**Fig. 3.7**, grey symbols, green background, $p<0.001$). When no items were available to be stored in the nest site, a significantly larger tunnel volume was excavated (series 1d vs. series 2a, $p<0.05$; series 1d vs. series 2b, $p<0.001$). The presence of additional brood stored in the fungus did not lead to an increase of created tunnel volume (series 2a vs. series 2b, $p=0.09$).

The gross chamber volumes and the gross tunnel volumes (**Fig. 3.7**, black symbols) differed statistically, analogous to the excavated chamber and tunnel volumes (see figure caption for statistical details). Both structures were reduced by pellet deposit.

The final chamber volumes, which resulted from the deposition of pellets inside the enlarged and offered cavity space, differed statistically (**Fig. 3.7**, white symbols, white background, $p<0.001$), and were larger when fungus was stored in the nest site (series 1d vs. series 2a, $p<0.001$; series 1d vs. series 2b, $p<0.001$). The presence of additional brood did not result in a larger final chamber (series 2a vs. series 2b, $p=0.26$). The volume of the tunnels initially excavated was reduced via the deposition of pellets in different magnitudes, with the strongest reduction taking place when no stored items were present (**Fig 3.7**, white symbols, green back-

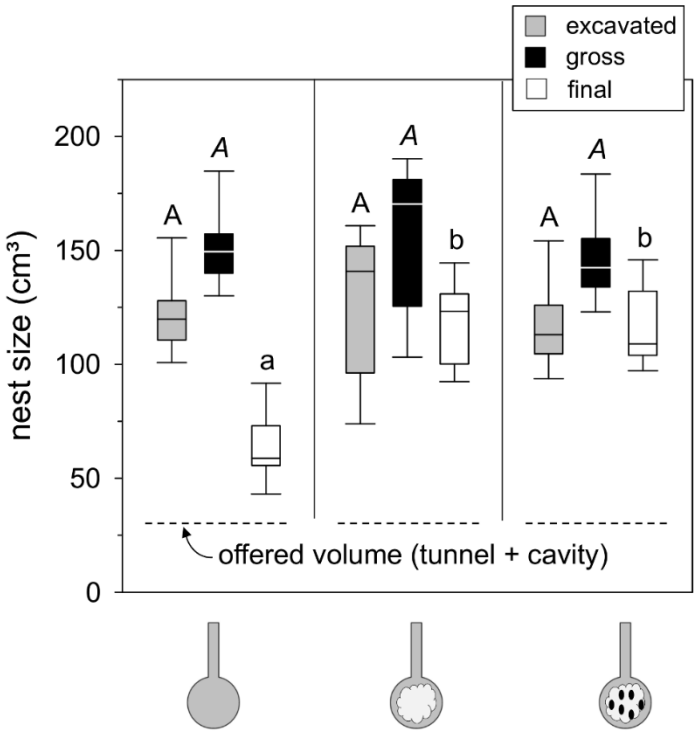

Figure 3.6 Nest sizes, depending on presence or absence of stored items: Excavated nest size (grey symbols), gross nest size (black symbols) and final, resized nest size (white symbols) in ample space setup. From left to right: without stored items (series 1d), with fungus (series 2a), with fungus and brood (series 2b). The dashed line marks the volume of offered space at the beginning of excavation (Excavated nest size: median$_{1d}$=119.8 cm³, 25-75% percentils=110.7-127.9, min-max= 97.1-163.8, n=12; median$_{2a}$=140.8 cm³, 25-75% percentils=96.2-151.8, min-max= 67.8-163.9, n=12; median$_{2b}$=113 cm³, 25-75% percentils=104.6-125.9, min-max= 78.1-156.8, n=28; Kruskal-Wallis test: H=2.78, p=0.25; Gross nest size: Kruskal-Wallis test: H=2.78, p=0.25; Final nest size: median$_{1d}$=58.8 cm³, 25-75% percentils=55.6-73.1, min-max= 39.6-95.9, n=12; median$_{2a}$=123.3 cm³, 25-75% percentils=100.1-130.9, min-max= 91-144.8, n=12; median$_{2b}$=108.9 cm³, 25-75% percentils=103.9-132.1, min-max= 83.4-173.4, n=28; Kruskal-Wallis test: H=26.6, p<0.001; post hoc test: Dunn, Bonferroni-Holm corrected: series 1d vs series 2a, p<0.001; series 1d vs series 2b, p<0.001; series 2a vs series 2b, p=0.7), line: median, box: 25-75% percentils, whiskers: min-max values). Groups with the same letter do not differ statistically; p<0.05.

ground). The resulting final tunnel volumes were comparable to one another, whether items were stored in the nest site or not (**Fig. 3.7**, white symbols, green background, p=0.07; for detailed statistics see figure caption).

3.4 Discussion

In our experiments, a group of workers, separated from their source colony, was able to excavate nest structures of varying size, consisting of tunnels and rounder, more chamber-like shapes, depending on variables other than worker number. The already existing nest space at the initiation of excavation as well as the presence of stored items, brood and fungus, affected

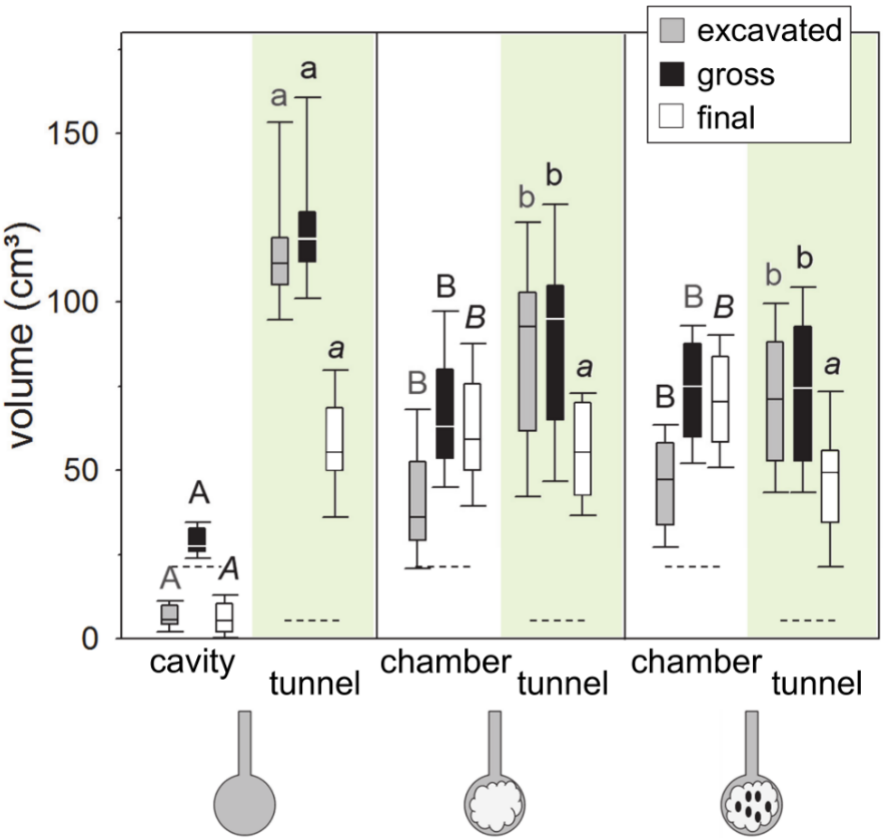

Figure 3.7: Excavated volume (grey symbols), gross nest volume (black symbols) and final nest size (white symbols) as in Fig. 6, but distinguishing between chamber/cavity (white background) or tunnel volume (green background). From left to right: without stored items (series 1d), with fungus (series 2a), with fungus and brood (series 2b). Dashed lines mark the size of offered cavity volume (21.6 cm³) and tunnel volume (7 cm³). (Enlarged chamber volume: median$_{1d}$= 5.8 cm³, 25-75% percentils=4.4-10, min-max= 2-11.2, n=12; median$_{2a}$= 36.2 cm³, 25-75% percentils=29.2-52.6, min-max= 18-70.7, n=12; median$_{2b}$= 47.3 cm³, 25-75% percentils=34-58.3, min-max= 16.2-73.7, n=28; Kruskal-Wallis test: H=27.7, p<0.001; post hoc test: Dunn, Bonferroni-Holm corrected: series 1d vs. series 2a, p<0.001; series 1d vs. series 2b, p<0.001; series 2a vs. series 2b, p=0.31; Excavated tunnel volume: median$_{1d}$=111.6 cm³, 25-75% percentils=105.1-119.1, min-max= 92.2-161.8, n=12; median$_{2a}$=92.9 cm³, 25-75% percentils=61.7-102.9, min-max= 38.6-129.2, n=12; median$_{2b}$=71.3 cm³, 25-75% percentils=52.9-88, min-max= 24.4-113.2, n=28; Kruskal-Wallis test: H=23.06, p<0.001; post hoc test: Dunn, Bonferroni-Holm corrected: series 1d vs. series 2a, p<0.05; series 1d vs. series 2b, p<0.001, series 2a vs. series 2b, p=0.09; Final chamber/cavity volume: median$_{1d}$=5.8 cm³, 25-75% percentils=4.4-10, min-max= 2-11.2, n=12; median$_{2a}$=36.2 cm³, 25-75% percentils=29.2-52.6, min-max= 18-70.7, n=12; median$_{2b}$=47.3 cm³, 25-75% percentils=34-58.3, min-max= 16.2-73.7, n=28; Kruskal-Wallis test: H=28.4, p<0.001; post hoc test: Dunn, Bonferroni-Holm corrected: series 1d vs. series 2a, p<0.001; series 1d vs. series 2b, p<0.001; series 2a vs. series 2b, p=0.26; Final tunnel volume: median$_{1d}$=55.4 cm³, 25-75% percentils=50-68.6, min-max= 32.8-82.3, n=12; median$_{2a}$=55.5 cm³, 25-75% percentils=42.7-70.1, min-max= 35.4-73.1, n=12; median$_{2b}$=49.2 cm³, 25-75% percentils=34.6-56, min-max= 18.3-84.3, n =28; Kruskal-Wallis test: H=5.36, p=0.07) (line: median, box: 25-75% percentils, whiskers: min-max values). Groups with the same letter do not differ statistically; p<0.05.

nest excavation and nest architecture. Not all of the excavated and initially offered space was used for storing items or facilitating the traffic flow. Rather, unused space, which may have arisen because of an initially crowding of workers that triggered high digging activity (Römer and Roces, 2014), was opportunistically refilled by the deposition of excavated soil pellets. The spatial demands of stored items, particularly the symbiotic fungus, led to a flexible adjustment of nest space, with the resulting final nest size not simply determined by the number of inhabiting workers, but adjusted to the current conditions in the nest.

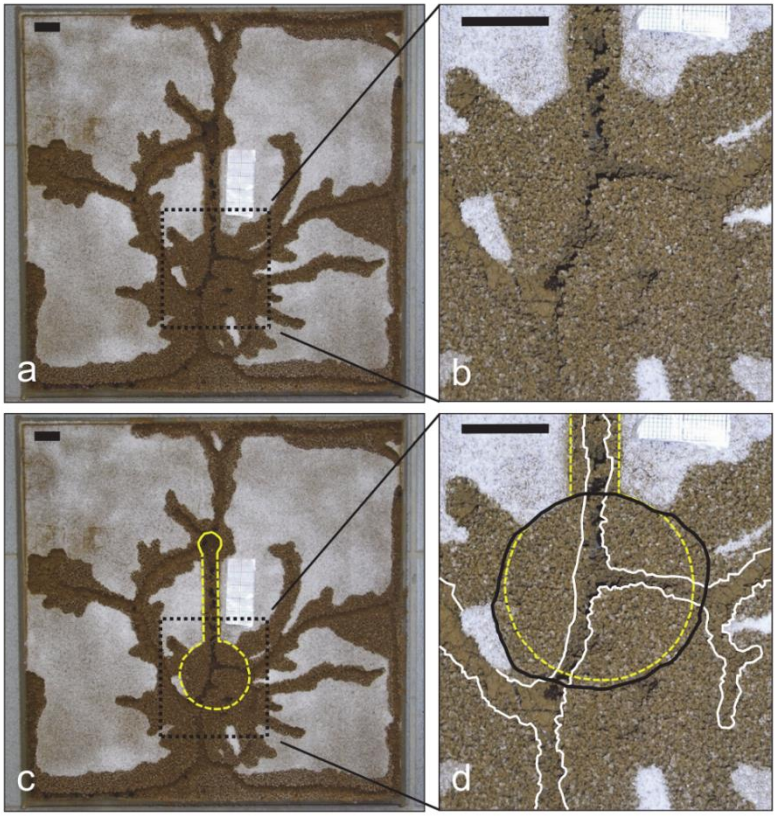

Figure 3.8: Example of enlargement of the offered cavity and tunnel excavation in the setup "ample space", without stored items (series 1d). The offered cavity and the excavated tunnels were secondarily reduced by the deposition of soil pellets. **(a)** Digging arena; **(b)** Close-up around the offered cavity, to better visualize the deposited pellets; **(c)** same pictures as *(a)*, with the offered structures marked with dashed, yellow line; **(d)** same picture as *(b)*, with enlargement of offered cavity marked with a black line, and the margin of deposited soil pellets marked with a white line. Scale of black bar=2 cm.

(a) Feedback mechanisms during nest excavation

Digging experiments in the laboratory have shown that the digging activity of ants (*Lasius niger*), once triggered, increases and then diminishes over time (Rasse and Deneubourg, 2001; Buhl et al., 2005). This implies the existence of feedback mechanisms that stimulate ants to dig, and also to cease digging after some time has passed. Because workers should create more space the longer they are excavating, nest space should act as an inhibitor (Rasse and Deneubourg, 2001; Buhl et al., 2005), leading to the cessation of nest enlargement. A similar inhibitory effect of nest space on excavation has been found in the leaf-cutting ant *Atta vollenweideri* (Pielström, 2013). While in our study both series that had been offered ample space displayed the lowest

digging activities, reduced space only led to a clearly increased digging activity when brood was present. Nest excavation does not seem to be regulated by one single feedback mechanism, i.e. available space, but other factors also influence digging behavior. The presence of stored items, in this case brood, could be one of these factors. This could be based on the attractiveness of brood to workers (Römer and Roces, 2014). More ants could have been present around the relocated brood in the small space of the offered tunnel compared to the series with an empty tunnel. Crowded conditions, i.e., high local ant density, is considered to increase the digging activity of ants (Rasse and Deneubourg, 2001; Toffin et al., 2009; Toffin et al., 2010). The offered ample space might have reduced this brood-induced crowding effect sufficiently to result in less excavation. The quantity of brood items present did not influence the excavation intensity; ants appear to only react to the presence of brood. We would like to point out that as a result of performing the experiments in the laboratory, the exhibited digging activity may be increased compared to their natural rates. In the experiments, a large number of workers was suddenly confronted with space of insufficient size, while colonies that naturally grow by incremental degrees may respond gradually by expanding the nest accordingly.

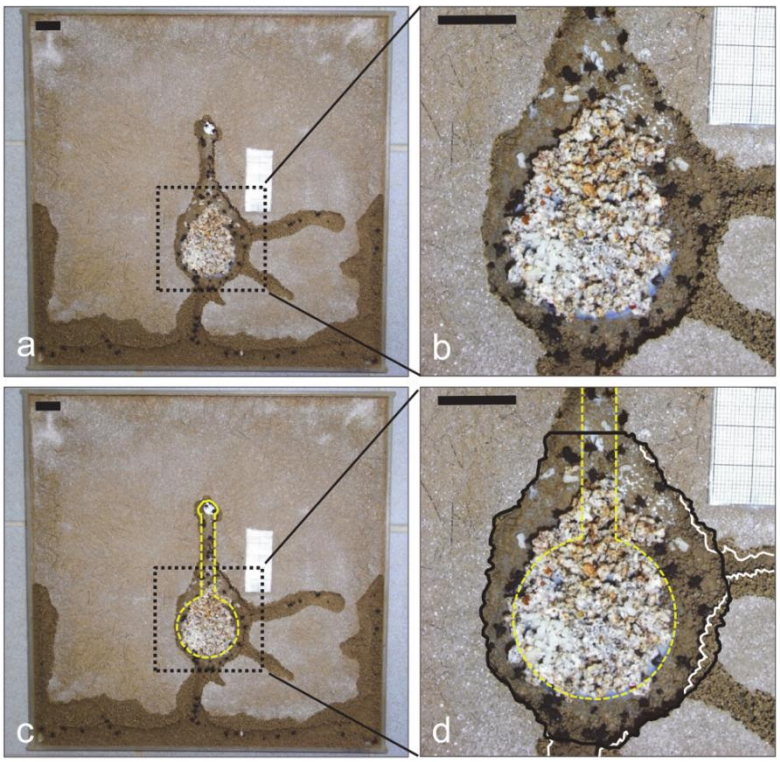

Figure 3.9: Example of enlargement of the offered cavity and tunnel excavation in setup 'ample space, with stored items' (fungus and brood, series 2b); the offered cavity was majorly enlarged and only a small tunnel volume was excavated. Tunnels were also reduced by the deposition of soil pellets. **(a)** Digging arena; **(b)** Close-up around the enlarged cavity; **(c)** same picture as *(a)*, with the offered structures marked with a dashed, yellow line; **(d)** same picture as *(b)*, with the cavity enlargement marked with a black line, and the margin of deposited soil pellets marked with a white line. Scale of black bar=2 cm.

Ants appear to be able to perceive space (Pratt and Pierce, 2001), but the means by which they judge the size of the available space remains unclear. Scouts of the rock-dwelling ant *Leptothorax albipennis* seem to assess the area size of a potential new nest by using the intersection frequency of their own, pheromone marked path (Mallon and Franks, 2000). Leaf-

cutting ant queens use idiothetic information to judge the length of the tunnel connecting the founding chamber with the surface (Fröhle and Roces, 2012). By walking around, or back and forth, workers could also hypothetically gain spatial knowledge about the existing structures. The number of ants present in the existing space, i.e., the ant density, may also be a cue that indirectly informs ants about space needs. An assessment of ant density is also hypothesized to operate in quorum sensing during collective-decision making in house hunting ants (Pratt, 2005), although its precise mechanisms remain to be elucidated. The larger the number of individuals in a colony, the higher the degree of crowding. Workers should start excavating as soon as an individual's behavioral threshold triggering digging is exceeded, based on ant density or encounter rates as stimuli. High ant density therefore should work as a positive feedback, and workers should keep excavating as long as density is high or the available nest space is low. During the excavation process, the enlarged nest space should lead to a higher dispersal of workers, i.e., a lower ant density, and less ants should start or continue excavating, as their thresholds are being undercut. The rate of encounters with nestmates may be one of the cues that inform workers about the local ant density (Gordon et al. 1993).

The mechanisms underlying nest enlargement do not seem to be solely based on the regulation of excavation. We could demonstrate that leaf-cutting ants, while excavating to create space, refill parts of the excavated nest with soil pellets, effectively reducing the final nest space. That excavation of nest space exceeds the current spatial needs of the colony could be due to the self-organizing nature of the digging process, where digging diminishes slowly and not abruptly. However, refilling seems to be opportunistic, rather than an active means to regulate or reduce the final nest space. In a study with *Atta vollenweideri* it was shown that the excavating ant transported the soil pellet a short distance, and usually deposited it within the excavated nest site (Pielström and Roces, 2013). These pellets would either be picked up by another worker and redeposited within the nest, or removed outside. Soil transport by workers is sequential (Pielström and Roces, 2013), and soil carriers appear to simply deposit their loads at places where the movements of nestmates are not obstructed or items are stored, i.e., at unused space. The increased internal pellet deposit in empty cavities when offering ample space, which led to a reduced final nest volume, supports this assertion. Regarding natural fungus-growing ant nests, there have also been reports about cavities refilled with soil (Autuori, 1942; Solomon et al., 2011; Moser, 1963; Moreira et al., 2004a; Moreira et al., 2004b). In addition, pellets are often deposited in formerly excavated tunnels (Sudd and Franks, 1987), which is in accordance with observations from our experiments. Sometimes short, rudimentary tunnels were completely refilled with pellets.

Chambers or cavities were resized to a different extent, depending on the presence of stored items. The size of the remaining structure was larger in brood presence. Ants possibly did not deposit soil pellets on brood items to prevent contact with harmful pathogens, which could be present in the surrounding soil. In the absence of stored items not only the size of the structure was majorly reduced, the initially round cavity also changed its shape to an oblong, narrow tunnel. This was caused by the deposition of the pellets alongside the walls of cavities or tunnels, where they were manipulated into position with the ants' mandibles or front legs. From an energetic perspective, refilling unused space with pellets may be the most economical way to dispose of the soil, without the need to carry the pellets outside of the nest.

Functionally, refilling might help to promote a more stable microclimate, better suited for brood or fungal development. Reducing chamber space and narrowing tunnels might lead to a reduction of airflow through the nest leading to loss of air humidity or more humid conditions close to brood and fungus, when moist pellets are placed nearby. The reduction of tunnel width could also lead to a smoother traffic flow. Pellet deposition in fungus-filled chambers was very low, probably because most of the space inside the chamber was either occupied by the fungus or used for worker traffic. This further indicates that workers only utilize unused space for pellet deposit. In addition to unused cavities, the excavated tunnel system was also greatly adjusted by pellet deposition. The final tunnel volumes were equal, despite workers excavating more tunnels when no items were stored and the initial offered cavity was only marginally enlarged. This seems to indicate that the total volume of the nest tunnel system was adjusted to the worker numbers.

Because of the possible increased excavation activity caused by the laboratory experiments, the amount of soil pellets and with it the internal pellet deposit might have been increased compared to natural conditions. However, in laboratory experiments with vertical arenas (Fröhle, 2009) or in 3-dimensional digging tubes (Pielström, 2012; personal observations), there were also pellet deposits in cavities or tunnels, although to a lesser extent.

(b) The formation of nest chambers and tunnels

Our study also gives further insight into the possible mechanisms of chamber and tunnel emergence. When ants stored brood items in the nest site, they excavated a larger, rounder structure than when no items were stored. However, a cavity was also excavated (when offered space was small, i.e. a tunnel) in the latter situation, which was later downsized to a narrow tunnel via the deposition of soil pellets. The emergence of a cavity without brood or fungus being present seems to contradict the hypothesis of chamber emergence proposed in a recent work with the same species (Römer and Roces, 2014). Here chambers only emerged around

deposited brood, while only tunnels were excavated at an alternative, empty site. This could be due to many ants aggregating at one site (around the worker-attracting brood), resulting in chamber-like shapes being excavated. In the present experiments, only single nest sites were offered for excavation, so that ants could only aggregate there and all digging would be concentrated at one site. The same cavity emerging effect was found in experiments with *Lasius niger*, where workers excavated at only one nest site without brood present (Toffin et al., 2009; Toffin et al., 2010). Workers first excavated in a centrifugal way, leading to the emergence of a round cavity, and later dug tunnels.

The excavation of a much larger chamber when brood items were stored is possibly due to a stimulating effect of the brood on an individuals' digging performance, perhaps through increased CO_2-levels emitted by the brood (Hangartner, 1969), or a higher number of workers aggregating around brood (Römer and Roces, 2014). Because part of the excavation took place under a layer of clay, ant aggregation could not be quantified. To further clarify this point, the ants present at a digging site should be counted in future experiments.

The presence of fungus also affected chamber size because ants used it as a template, thus leading to a comparable enlargement of the offered chamber, regardless of the additional presence of brood. The chambers were large enough for fungus storage and for the worker traffic around it.

Workers not only excavated a chamber or cavity, or enlarged the offered cavity, but they also excavated a tunnel system. Nest space was differently allocated into chamber and tunnel volumes, depending on the presence of stored items. The nests' tunnel system was larger, the less chamber volume workers excavated, because of the lack of stored items. This could be attributed to a change in ant density from high to low, and to an increase in available space, as observed in *Lasius* ants (Toffin et al., 2009; Toffin et al., 2010). In a larger space, *Lasius* workers could disperse more and could no longer occupy all possible digging sites. At the sites where excavation was still taking place, buds formed at the formerly smooth cavity walls. Tunnels emerged because digging concentrated at those buds that were further extended. Such a mechanism would explain the differences in excavated tunnel volumes depending on the presence of stored items or lack thereof in our study. When fungus, which markedly attracts workers (Römer and Roces, 2014), was relocated in the offered nest space, ants should have aggregated around the fungal mass, leading to an even expansion of space according to the spatial needs of the voluminous fungus. Without it, workers should have dispersed more, resulting in an only marginal enlargement of the cavity and an earlier initiation of tunnel excavation. Ants could then have excavated tunnels for a longer time period compared to

workers that had to excavate additional chamber space to house the fungus. Also, by placing the nest sites horizontally, ants could not use gravitation to orient themselves during excavation which could have led to a more dispersed tunneling pattern. Ants using gravity for orientation usually create a less branched tunnel system (Sudd, 1972).

Our study indicates that the regulation of nest size and internal architecture does not simply depend on the number of workers that inhabit a colony. Rather, nest size results from the nonlinear interactions between different dynamic stimuli. The mechanisms underlying the determination of nest size are flexible and seem to be affected by the available nest space and the presence of in-nest stores. They involve positive and negative feedback loops, such as crowding around stored items, and inhibition via the generated space, thus leading to a self-regulated onset and lessening of excavation. Another important mechanism by which ants adjust the size of their nests is the opportunistic deposition of excavated soil pellets at unused spaces, effectively downsizing their nest. While the deposition of pellets clearly indicate that workers recognize nest space that is not currently in use, its adaptive value remains elusive.

Fungus-filled nest chamber close to the soil surface in Atta bisphaerica; source: F. Roces

Chapter 4

4. Underground carbon dioxide levels: Preferences for fungus culturing by leaf-cutting ants

Abstract

Leaf-cutting ants rear a symbiotic fungus that shows reduced respiration rates at high CO_2-levels inside the nest (hypercapnia). When establishing a new underground garden, ants should therefore select soil depths with CO_2-levels suitable for fungus rearing. We investigated whether workers of Acromyrmex lundi discriminate between two potential nest sites for fungus rearing based solely on the actual CO_2-levels. In the laboratory, workers could relocate fungus, prone to desiccation, from an open box and select between two humid nest chambers with different CO_2 concentrations. When offered 4% CO_2 vs. atmospheric values, workers avoided rearing fungus at the highest levels, as expected. However, they preferred 1% CO_2 when the alternative site offered atmospheric values. If only high levels of 4% CO_2 were available, workers still relocated the exposed fungus into the humid chambers, showing that they accept unsuitable, otherwise avoided CO_2-levels to prevent fungus desiccation. Counts of workers in the chambers before and after fungus relocation suggest that the observed preferences during fungus relocation reflected the workers' preferences for CO_2-levels. It is argued that the observed CO_2-preferences should influence the ants' decisions where to excavate new fungus chambers across the soil profile and therefore impact nest architecture.

4.1 Introduction

Leaf-cutting ants live in a symbiosis with a fungus they rear on foraged plant material. It is the sole food source of the colony brood (Quinlan and Cherrett, 1979) and therefore crucial to colony survival. The underground nests leaf-cutting ants build help to dampen the fluctuations of the abiotic environment and therefore offer more stable conditions for fungus and brood development. Nests can be superficial with only a few chambers (Weber, 1966), or multi-chambered reaching deeper soil layers. The latter are mostly build by species of the genus *Atta*, whose colonies can comprise millions of individuals (Bonetto, 1959; Jonkman, 1980; Moreira et al., 2004a; Moreira et al., 2004b). The symbiotic fungus grows best at temperatures between 20°and 30°C (optimum 25°C; Powell and Stradling, 1986; Quinlan and Cherrett, 1978) and high air humidity, because it is very susceptible to desiccation (Roces and Kleineidam, 2000). The conditions of the soil surrounding the nest influence its climate, and workers will encounter environmental gradients across the soil profile, mainly of temperature, moisture and soil gases. Temperature and soil moisture are also known to vary diurnally and seasonally. Workers should therefore choose places to rear their fungus gardens that offer the best possible growth conditions for the fungus. When the nest is enlarged, either because of space demands or to search for better suited environmental conditions for their fungal cultivar, workers should orient their excavation based on environmental cues. Indeed, workers of the species *Acromyrmex lundi* use soil temperature as an orientation cue, preferring a temperature range between 20 and 30°C for digging (Bollazzi et al., 2008), which is well suited to fungal growth.

In addition to temperature and humidity, the carbon dioxide content of the nest air is expected to influence the growth of the fungus. High levels of carbon dioxide negatively influence the respiration rate of the leaf-cutting ants' fungus (Kleineidam and Roces, 2000). A similar negative effect of high CO_2 concentration on fungal growth has also been found in fungus-rearing termites of the genus *Macrotermes* (McComie and Dhanarajan, 1990). Underground-nesting ants produce large amounts of CO_2 while using up O_2 (Lighton, 1989; Jilková and Frouz, 2014). In leaf-cutting ants, the metabolism of the fungus likely generates large amounts of CO_2. In addition, the carbon dioxide concentrations in the soil air, even in superficial soil layers, are considerably increased when compared with the atmospheric CO_2-level of 0.03%. Decaying organic matter in the soil as well as microbial and root respiration are the main sources of CO_2 emission within the soil phase. The CO_2-levels can also vary locally, depending on changes in soil temperature (Hamada and Tanaka, 2001; Risk et al., 2002; Bekele et al., 2007), soil compaction (Currie, 1984) and moisture (Bekele et al., 2007; Currie, 1984), which can hinder gas exchange. Generally, underground CO_2 concentration increases with

depth (Schwartz and Bazzaz, 1973; Hamada and Tanaka, 2001; Bekele et al., 2007). Because the surrounding soil is such a massive source of CO_2, the levels inside underground nest chambers are in dynamic equilibrium with and closely correspond to the levels of the soil phase (Bollazzi et al., 2012). Increasing CO_2-levels with depth are associated with a corresponding decrease in O_2-levels (Bollazzi et al., 2012). In large and deep nests of *Atta* leaf-cutting ants, CO_2 concentrations of up to 6% have been reported, even at depths of 1.2 m (Kleineidam and Roces, 2000; Bollazzi et al., 2012). In the more superficial nests of *A. lundi*, measurements inside the main fungus chamber of four field nests fluctuated around 2% CO_2, varying between 1% and 2.7% (Martin Bollazzi, personal communication). Leaf-cutting ants are known to perceive not only the relative, but also the absolute CO_2 concentration with a special type of chemoreceptor, the sensilla ampullacea (Kleineidam and Tautz, 1996; Kleineidam et al., 2000), located on the last flagellar segment of the antennas.

To excavate a nest suitable to rear fungus and brood, leaf-cutting ant workers have to create nest space at soil layers providing adequate temperature, moisture and concentration of respiratory gases. Nest building behavior has been shown to be influenced by the temperature and the moisture of the soil, and conditions that would promote fungal growth are preferred (Bollazzi et al., 2008; Pielström and Roces, 2014). Proper parameters for fungal growth are rarely all at their optimum at one site inside the nest, and ants often need to trade off the control of one environmental variable for another. For example, *Acromyrmex ambiguus*, a sympatric species of *A. lundi*, builds superficial nests in sandy soils. Workers plug nest entrances with leaf fragments to prevent humidity losses, a behavior that would compromise the renewal of nest air and influence the levels of respiratory gases inside the nest (Bollazzi and Roces, 2007). Even though it is known that high CO_2-levels negatively influence the respiration of the leaf-cutting ant fungus (Kleineidam and Roces, 2000), it is unclear whether the soil CO_2 concentration is a relevant factor for the selection of suitable places for fungus rearing. Values that negatively influence the growth of the fungus should be avoided, if possible, analogue to avoiding unfavorable temperature or humidity values. This could either be achieved by relocating the fungus between existing chambers with different CO_2 concentrations, or by excavating new chambers during nest growth at soil depths with more suitable CO_2-levels.

In the present work it was investigated whether workers of the leaf-cutting ant *A. lundi*, which usually locates their fungus chambers at depths around 30-50 cm in heavy clayish soils, choose chambers for fungus rearing based solely on the CO_2 concentration of the nest site. In the laboratory, workers were induced to relocate fungus by exposing it to desiccation in an open box. Then, two similar humid nest chambers that differed in their CO_2-levels were offered in a

binary-choice setup, as places for fungus rearing. One chamber offered atmospheric values while the other offered 1% CO_2, a value measured in the superficial soil layers where their nests can be found. To determine whether workers avoid higher values, a CO_2-level of 4% was offered in a new series. This series was repeated with colonies of the sympatric species *A. ambiguus*, which excavates in sandy soils more superficial (Bollazzi et al., 2008) and likely better aerated nests than *A. lundi*, to test whether the expected avoidance of high CO_2-levels represents a generalized response in *Acromyrmex* leaf-cutting ants. In a final series, we investigated whether *A. lundi* would trade off high, unsuitable CO_2-levels for high humidity inside the chambers. Deposition of fungus in the chambers was quantified as well as worker aggregation during the assays, as it might influence the fungus relocation responses.

4.2 Materials and Methods

(a) Animals

All assays were performed with worker groups collected from laboratory colonies of the leaf-cutting ant *A. lundi*. The colonies were reared in a climatic chamber at 25°C, 50% air humidity and a 12L:12D cycle and fed ad libitum with blackberry leaves (*Rubus fruticosus*), water and honey water. The colonies were mature (being at least 4 years old at the time of the experiments) and were kept in a system of closed plastic boxes (19x19x9 cm) as artificial fungus chambers, a waste disposal box and a feeding arena, all connected by transparent plastic tubing. The worker groups were collected from the colonies on the day of the assay.

(b) Experimental Setup

In each assay, a group of ants was induced to relocate fungus by exposing it to desiccation and by offering two potential nest chambers that had equal temperature and humidity values, but differed in the CO_2 concentration.

The experimental setup was as follows: A square (9.5x9.5x5.5 cm), open plastic box, called the ant release box, was connected with a y-shaped tube (y-arm length 6 cm, y-stem length 7 cm, diameter 1.7 cm) to two nest chambers (**Fig. 4.1**). Each nest chamber consisted of a clear plastic ring (diameter 10 cm, height 3 cm), attached to a glass pane bottom (12x10 cm), and a lid made out of clear plastic. A moistened piece of round filter paper (diameter 10 cm) was placed on the bottom of each chamber to increase the air humidity to values well suited for fungus rearing (preferred humidity levels close to saturation; Roces and Kleineidam, 2000). The moistened paper increased the air humidity to 99.9% in the chambers (n=8). To establish two different CO_2 concentrations in the chambers, two small rubber tubes (diameter 0.3 cm) were inserted into the y-tube at the bifurcation point, running along an inner wall of the y-tube

and terminating in a chamber. Air with two different CO_2 concentrations was then continuously pumped into each chamber at a flow rate of 50 ml*min^{-1}. To create near atmospheric values (from now on referred to as 'atmospheric values' for simplification) a gas cylinder filled with synthetic air (79.5% N_2, 20.5% O_2, 0% CO_2) was connected to a tube leading to one of the chambers. The different CO_2 concentrations were generated by connecting a gas cylinder, filled with 100% CO_2, and another with synthetic air, to two valves, controlled by a gas mixer (Mass Flow Controller MFC-4, Sable Systems International, USA). The output of the gas mixer was

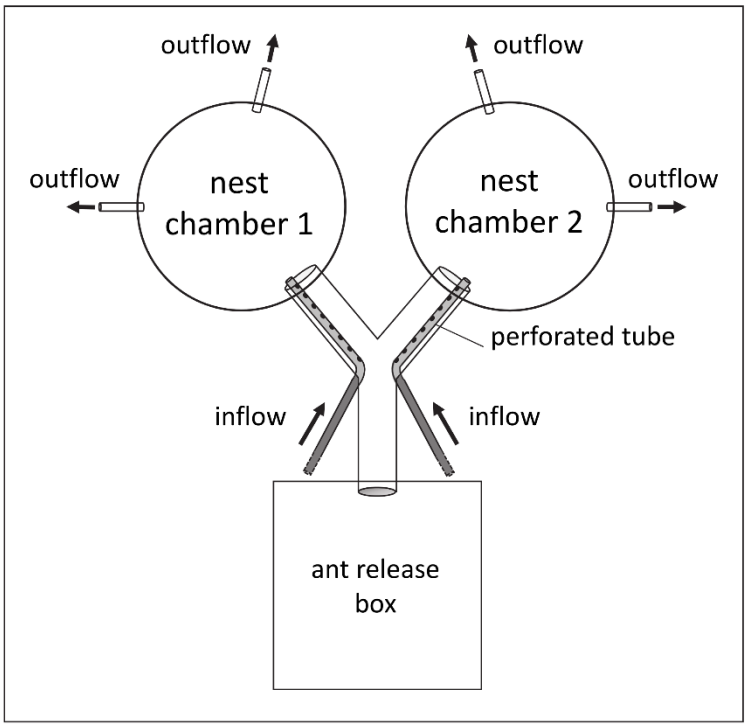

Figure 4.1: Experimental setup with ant release box and y-shaped tube leading to two nest chambers.

connected to a tube leading to the other chamber. To ensure that walking ants would encounter different CO_2-levels at the bifurcation point before entering one of the chambers, the entire length of rubber tube running inside the y-shaped tube was perforated (**Fig. 4.1**). To reach stable levels of CO_2, the atmosphere in the chambers was also continuously pumped out (miniature vane pump, 135 FZ, Schwarzer Precision, Germany), with an equal flow rate of 50 ml*min^{-1} by two rubber tubes (diameter 0.3 cm) inserted in the opposite chamber wall of the entrance hole.

(c) General Procedure

The experiments were performed as follows. One hundred media-sized workers were collected in equal numbers out of the feeding box and a randomly chosen fungus garden box of a colony. In addition, a piece of fungus was removed from the fungus garden and all ants and brood situated in it were carefully removed. A portion of 1 g cleaned fungus was separated from the

collected piece, and kept in a tightly closed petri dish on a piece of moistened filter paper, to prevent desiccation of the fungus. Before the workers were placed in the ant release box, the CO_2 concentrations in the two nest chambers were validated using a CO_2 sensor (range 0-10%, resolution: 0.02%; Gasmitter, Sensor Devices, Germany). The ants were only released when the levels had reached the appropriate values for the experiment. Otherwise, the CO_2 concentrations were adjusted and measured again. After the release of the ants, they could explore the whole setup for 1h and gain information about the different CO_2 concentrations in the two nest chambers. To evaluate whether the workers themselves (not in the context of relocating fungus) avoid high CO_2 concentrations or elevated values in general, the number of ants present in each chamber was counted after the familiarization period of 1h. Afterwards, the piece of cleaned fungus was placed into the open ant release box. Here it was exposed to normal room conditions with air humidity between 30-45% and atmospheric CO_2-levels, which should cause the fungal mass to desiccate and should lead to the relocation of pieces of fungus by the ants. In fact, pieces of fungus eventually not carried into the experimental nest appeared dry and brittle at the end of the assay. Workers had 3 hours to relocate the fungus inside one or both chambers. CO_2-levels in the chambers were measured again at the end of the assay. In addition, the number of workers present in each chamber was counted. The fungus transported into each chamber was collected and dried for 24h at 50°C and the weight of the dry fungus measured to the nearest 0.1 mg. Only assays in which the ants relocated 50% or more of the offered fungus mass into the nest chambers, weighed later as dry mass, were considered for further analysis.

(d) Experimental series

Series 1: It was investigated whether workers, when relocating their fungus, discriminate between atmospheric values and 1% CO_2, as this concentration is expected to occur in superficial soil layers. Because some mixing of the two airflows at the bifurcation point of the y-tube occurred, the CO_2-levels inside the chamber were not atmospheric (i.e., 0.03%), but in average 0.07% (mean ± 0.02 SD). The mean concentration in the other chamber was 1.01% (± 0.07 SD). The chambers assigned to each CO_2 concentration were alternated between assays. The series was performed with worker groups and fungus originating from 3 colonies of *A. lundi* (n=15, 5 assays per colony).

Series 2: To test whether ants avoid high CO_2 concentrations for fungus rearing as they would encounter at deep soil layers, workers were presented with a choice between 4% CO_2 (mean 4.25% ± 0.28 SD) in one chamber and atmospheric values (mean 0.13% ± 0.03 SD) in the other. The side of the high CO_2 concentration was alternated between assays. Assays were performed

with worker groups and fungus originating from 4 colonies of *A. lundi* (n_{all}=22, n_1=5, n_2=5, n_3=9, n_4=3). This series, 4% vs. atmospheric values, was repeated with 6 colonies of the sympatric species *A. ambiguus*, to investigate whether the observed CO_2-preference for fungus rearing is a generalized response within the *Acromyrmex* genus (n_{all}=17, n_1=4, n_2=2, n_3=4, n_4=3, n_5=2, n_6=2). The experimentally established levels were: 4% chamber: mean 3.92% ± 0.6 SD; atmospheric chamber: mean 0.24% ± 0.16 SD.

Series 3: As higher CO_2-levels are expected to be avoided, we investigated whether *A. lundi* workers accept unsuitable high CO_2-levels as a trade-off for the high humidity levels inside the nest chambers. For that, a concentration of 4% was offered in both chambers (left chamber: mean 4.23% ± 0.52 SD, right chamber: mean 4.24% ± 0.46 SD), so that workers were forced either to accept the high CO_2-levels, or to maintain the fungus under dry conditions in the open box. This series also served as a control for any side preference, since both nest chambers offered identical conditions. This series was performed with worker groups and fungus originating from 4 colonies (n_{all}=12, n_1=3, n_2=2, n_3=4, n_4=3).

4.3 Results

After their release in the open plastic box, workers immediately started exploring the y-shaped tubing and both nest chambers. The workers did not stay inside the more humid binary setup, but moved back and forth between it and the ant release box. At any given time, only a portion of the ants was present in both nest chambers while others were moving in the y-shaped tubing or were present in the box.

When the fungus was placed in the ant release box, many workers from the box and others coming from inside the chambers were observed to explore and aggregate near the fungus. The start of fungus removal into the chambers did not occur immediately, but could take up to 2 hours.

Workers of *A. lundi* deposited more fungus in the chamber with 1% CO_2 than at atmospheric values (Series 1, **Fig. 4.2a-c**; Wilcoxon matched pair test: Z=2.44, n=15, p=0.01). In 3 of 15 assays no fungus at all was placed at atmospheric levels. There was also a difference in the number of ants present in the nest chambers, depending on the CO_2 concentration. More ants aggregated in the chamber with 1% CO_2 1h after the assay began as well as at the end of the assay after 4h (**Fig. 4.2d**; after 1h: paired t-test, Bonferroni corrected at $p \leq 0.025$: t=2.73, df=14, n=15, p=0.02; after 4h: paired t-test: t=2.98, df=14, n=15, p=0.01). The number of ants in each chamber did not increase throughout the assay (**Fig. 4.2d**; paired t-test, Bonferroni

corrected at p≤0.025: chamber with 1% CO_2: t=0.2, n=15, p=0.84; chamber with atmospheric values: t=-0.76, n=15, p=0.46).

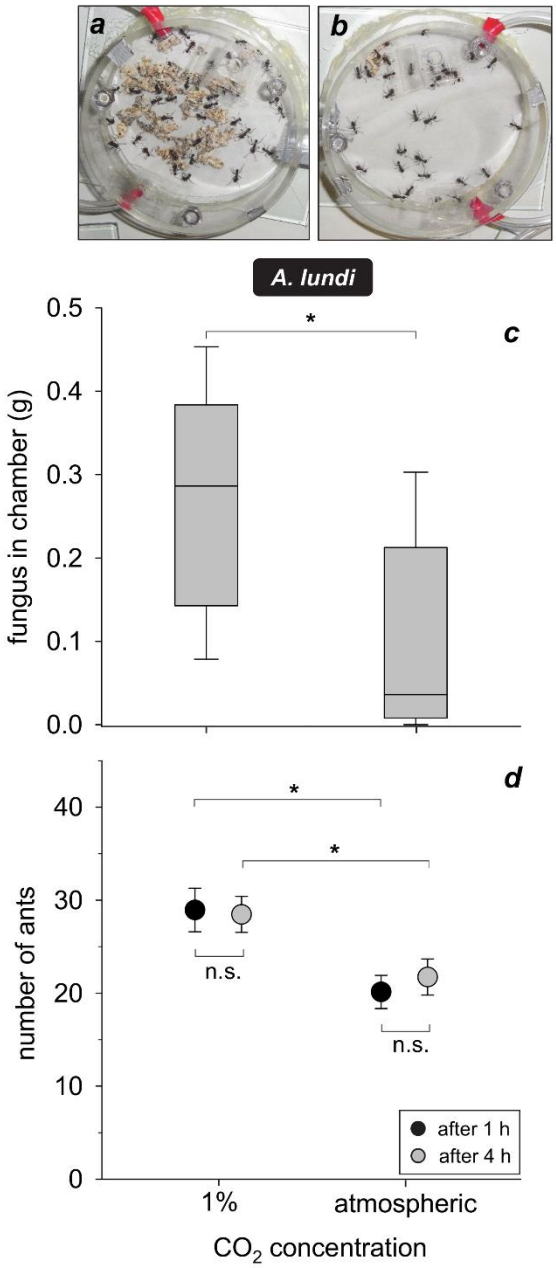

Figure 4.2: Series 1, *Acromyrmex lundi*, 1% CO_2 versus atmospheric values; **(a)** and **(b)** pictures of relocated fungus after 4h in chambers with either 1% CO_2 or atmospheric CO_2-values, **(c)** amount of relocated fungus in the chambers, depicted as medians (black line), 25-75% percentiles (box) and min-max values (whiskers), **(d)** number of ants aggregating in each chamber, 1h and 4h after the start of the assay. Depicted are means (round symbols) and SE (whiskers); n=15; *p ≤ 0.05; **p<0.01; n.s., not significant.

When workers of *A. lundi* had to choose between a nest chamber with 4% CO_2 and one with atmospheric values for fungus rearing, a significantly higher amount of fungus was deposited in the chamber with atmospheric values (Series 2, **Fig. 4.3a**; Wilcoxon matched pair test: Z=4.04, n=22, p<0.0001). In 13 of the 22 assays no fungus at all was deposited in the chamber with a high CO_2-level. After 4h, at the end of the assay, more ants aggregated in the chamber with near atmospheric values (**Fig. 4.3b**; paired t-test: t=-5.11, df=21, n=22; p<0.0001), together with the fungus. Unfortunately, the counts after 1h were missing.

A. ambiguus also avoided rearing fungus at high CO_2-levels and showed a preference for the nest chamber with atmospheric CO_2-values (Series 2; **Fig. 4.4a**; Wilcoxon matched pair

test: Z=2.11, n=17, p=0.035). In 15 of the 17 assays performed, all fungus was relocated into only one chamber. In 13 assays all fungus was deposited at atmospheric CO_2-values while in 2

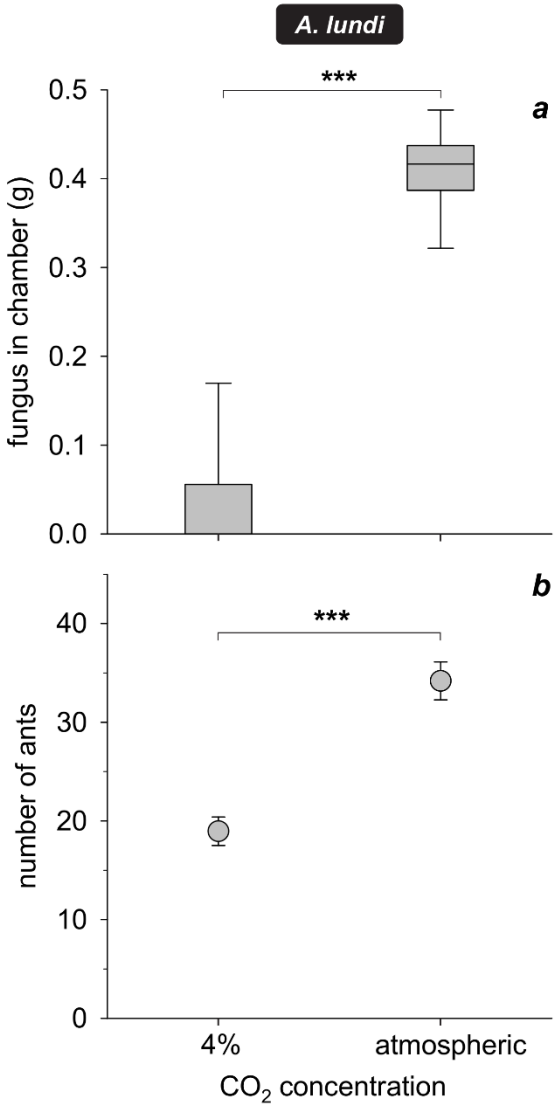

Figure 4.3: Series 2, *Acromyrmex lundi*, 4% CO_2 versus atmospheric values; **(a)** amount of relocated fungus, depicted as medians (black line), 25-75% percentiles (box) and min-max values (whiskers), **(b)** number of ants aggregating in each chamber, 1h and 4 h after the start of the assay, depicted are means (round symbols) and SE (whiskers). n=22; ***p < 0.001.

assays all fungus was relocated into the high CO_2 environment. The number of ants aggregating in the chambers changed throughout the assay. After 1h, ants were distributed evenly between both chambers (**Fig. 4.4b**; Wilcoxon matched pair test, Bonferroni corrected at p≤0.025: Z=2.01, n=17, p=0.04). At the end of the assay after 4h, when fungus had already been relocated into the chambers, more ants were present at atmospheric values, where most of the fungus had been deposited (Wilcoxon matched pair test, Bonferroni corrected at p≤0.025: Z=2.84, n=17, p=0.005). The number of ants in the chamber with 4% CO_2 did not change throughout the assay, while it did significantly increase in the atmospheric chamber (**Fig. 4.4b**; Wilcoxon matched pair test, Bonferroni corrected at p≤0.025: chamber with 4% CO_2: Z=1.27, n=17, p=0.21; chamber with atmospheric values: Z=3.41, n=17, p=0.0006).

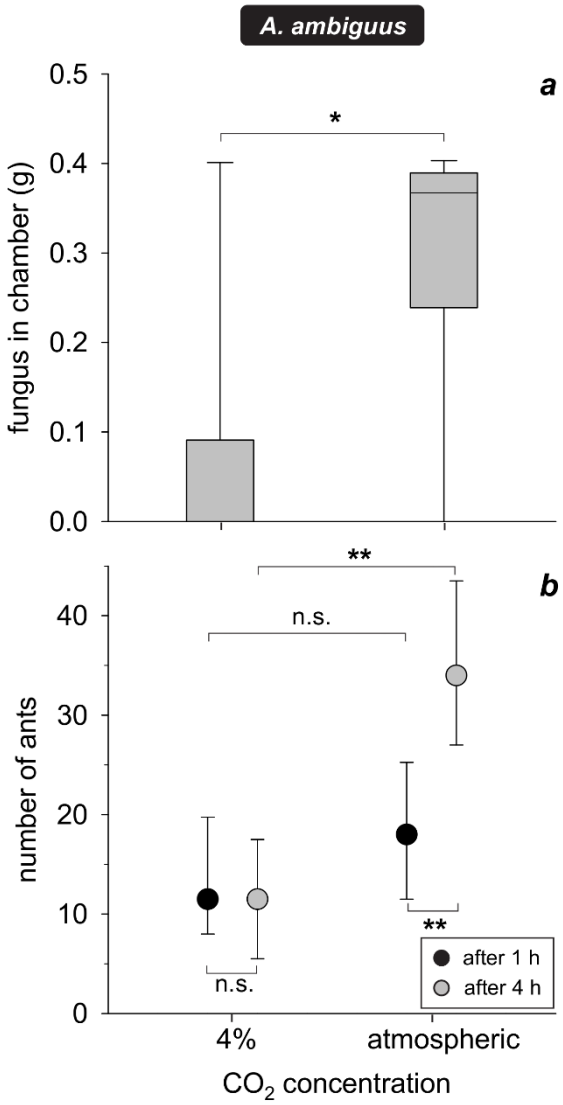

Figure 4.4: Series 2, *Acromyrmex ambiguus*, 4% CO_2 versus atmospheric values; **(a)** amount of relocated fungus in the chambers; n=17, **(b)** number of ants aggregated in a chamber, 1h and 4 h after the start of the assay; n=16. Depicted are medians (black line), 25-75% percentiles (box) and min-max values (whiskers); n=17; **p < 0.01; n.s., not significant.

Ants accepted the high concentration of 4% to relocate the fungus when the alternative was the exposition to unfavorable, dry conditions. Even though the median fungus mass was equally distributed into the two chambers (Series 3, **Fig. 4.5a**; Wilcoxon matched pair test: Z=1.18, n=12, p=0.24), there was a large variation per assay. In 8 of the 12 assays performed, the majority of the relocated fungus was placed in one nest chamber (left chamber, n=5; right chamber, n=3). An equal number of ants aggregated in the chambers, both 1h after release of workers as well as at the end of the assays after 4h (**Fig. 4.5b**; after 1h: paired t-test, Bonferroni corrected at p≤0.025: t=-1.07, df=11, n=12, p=0.31; after 4h: paired t-test: t=-1.43, df=11, n=12, p=0.18). The number of workers per chamber did not change, once fungus had been relocated into the chambers (**Fig. 4.5b**; paired t-test, Bonferroni corrected at p≤0.025: 4% CO_2 left chamber: t=-1.13, n=12, p=0.28; 4% CO_2 right chamber: t=-1.44, n=12, p=0.18).

4.4 Discussion

(a) Preferences for carbon dioxide levels for fungus rearing

This study demonstrates that *A. lundi* leaf-cutting ants, choosing a place for rearing of their fungus, make a decision based on the CO_2 concentration of the nest site. CO_2-values as high as 4% were avoided, but levels around 1% were actually preferred against atmospheric values.

A. lundi colonies inhabit shallow subterranean nests, 30-50 cm underground (Zolessi and Gonzalez, 1978). If elevated CO_2-levels are, in general, detrimental to fungal growth, ants

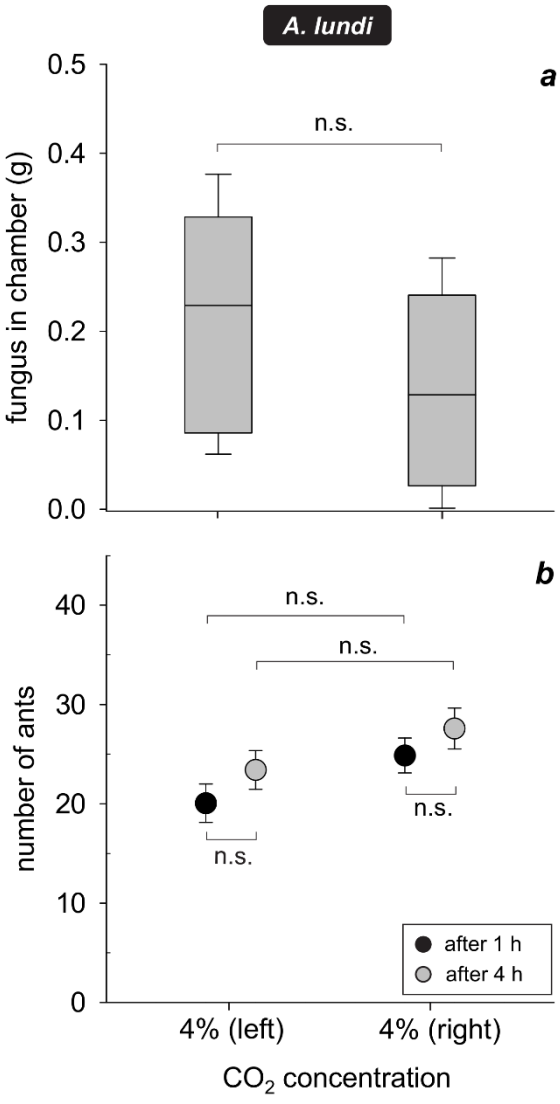

Figure 4.5: Series 3, *Acromyrmex lundi*, 4% CO_2 in both chambers **(a)** amount of relocated fungus in the chambers, depicted as medians (black line), 25-75% percentiles (box) and min-max values (whiskers); n=12, **(b)** number of ants aggregating in a chamber, 1h and 4h after the start of the assay, depicted are means (round symbols) and SE (whiskers). n=12; n.s., not significant.

should have also avoided 1%-levels for fungus rearing. It is unclear why workers in our experiment did relocate the fungus into the chambers offering 1% CO_2 and avoided atmospheric levels. On the one side, workers may avoid atmospheric levels because they may indicate exposition of the fungus to an open environment. On the other side, since the preferred 1%-

level corresponds with values measured in field nests (average 1-2%, Martin Bollazzi, personal communication), levels of this magnitude could have acted as a nest cue for the ants. In fact, *Atta* leaf-cutting ants and *Cataglyphis* desert ants use CO_2 as a cue that facilitates the orientation towards the nest entrance (Kleineidam, 1999; Buehlmann et al., 2012). The preference might also be due to an adaptation of *A. lundi* and its fungus to the CO_2-values they experience in the surrounding soil of their nest. Carbon dioxide levels higher than atmospheric values are also known to promote plant growth and CO_2 is known to be used as 'fertilizer' in industrial greenhouses to grow crops (Wittwer and Robb, 1964). While there are no *in vitro* measurements for the growth of leaf-cutting ant fungus under different CO_2 atmospheres, the growth of other fungi appears to be first stimulated with increasing CO_2 concentrations, and then hindered at even higher concentrations (Wells and Uota, 1969; de Reu et al., 1995). Whether the preference of leaf-cutting ants for 1% CO_2 is a response that has been selected during evolution to promote fungal growth, or as an orientation response to locate the nests, remains elusive.

Carbon dioxide levels as high as 4%, which can be encountered deeper underground, were avoided by *A. lundi* for fungus rearing. As workers of *A. ambiguus*, a sympatric species that builds more superficial, likely well aerated nests in sandy soils, also avoided 4% CO_2 for fungus culture, this behavior appears to be a general, robust response of *Acromyrmex* leaf-cutting ants to high CO_2-levels. It is noteworthy that workers, while avoiding 4% CO_2, needed in turn to accept atmospheric levels (Series 2), values that were otherwise avoided when offered together with 1% CO_2 (Series 1). There may be two reasons why *Acromyrmex* workers avoid high CO_2-levels. On the one hand, workers may not be adapted to such high levels that are never expected to occur in the soil layers where the nests are located. On the other hand, the reason for avoidance of high CO_2-levels could be a direct effect on the fungus, as high levels of carbon dioxide negatively influence fungal respiration and appear to hinder fungus growth (Kleineidam and Roces, 2000). Unlike winged social insects such as bees, which actively decrease the nest's CO_2-levels by fanning (Seeley, 1974), leaf-cutting ants can only passively influence the nest's CO_2 concentration to some extent. Even though the raised nest mound facilitates a wind-driven exchange of nest air (Kleineidam et al., 2001; Bollazzi et al., 2012), the actual CO_2 concentrations in the underground chambers are still strikingly high as compared to atmospheric values.

Carbon dioxide is known to be attractive to insects (Nicolas and Sillans, 1989) and can in addition lead to increased activity in ants (Burkhardt, 1991). As a consequence, the observed preference for fungus rearing may reflect the workers' own preferences for CO_2, and not necessarily a response to favor fungus growth under proper environmental conditions, as

indicated by our results for *A. lundi*. Solely *A. ambiguus* showed an equal worker distribution at the beginning when 4% was tested against atmospheric values, and workers aggregated more at the atmospheric levels later, together with the relocated fungus. Therefore, the workers' own CO_2-preferences cannot be ruled out as a factor strongly influencing the selection of sites for fungus culture. Once the fungus was present in a chamber, a large number of workers aggregated there as expected, since the fungus is known to strongly attract ants (Römer and Roces, 2014).

A suitable CO_2-atmosphere is not the only factor on which the proper fungal growth depends. Appropriate humidity and temperature levels are also required (Powell and Stradling, 1986; Quinlan and Cherrett, 1978; Roces and Kleineidam, 2000). Yet environmental conditions underground can vary depending on factors such as season, precipitation, soil type and depth. Presumably, not all environmental conditions that would promote fungal growth will be suitable at a given site. Rather, the ants will probably have to trade off their preference for one environmental factor for another, rearing their fungus under the best possible conditions, even if fungal growth is limited. This was demonstrated in the series with an equal CO_2 concentration of 4% in both chambers, where the ants did relocate all fungus inside, even though these high CO_2-levels were avoided when an alternative with lower values was available. This indicates that workers accept unsuitable, otherwise avoided CO_2-levels because they trade off the high CO_2 concentrations with high humidity of the surrounding air, so as to prevent fungus desiccation. Likely because fungus only develops properly under humidity values close to saturation (Powell and Stradling, 1986), nest humidity values should be kept high. For example, workers of the thatching leaf-cutting ant *Acromyrmex heyeri* regulate the temperature and humidity of their nest by opening and closing vents on the nest thatch (Bollazzi and Roces, 2010a). If the environmental air is dry, ants close openings to prevent air inflow and therefore avoid fungus desiccation, even if the resulting increased nest temperature is suboptimal for fungus rearing. It is therefore likely that leaf-cutting ants relocate and rear fungus at deep soil layers for a better control of humidity, even at the cost of tolerating high CO_2-levels.

(b) Implication of CO_2-preference for nest location and architecture

It is an open question whether leaf-cutting ants show a graded digging response to the varying CO_2-levels they would find across the soil, as they show to soil temperature and moisture (Bollazzi et al., 2008; Pielström and Roces, 2014). In the ant *Solenopsis geminata*, CO_2 is known to trigger digging behavior, which results in excavation towards trapped ants that release CO_2 (Hangartner, 1969). Using carbon dioxide as an orientation cue while digging and having preferences for particular levels could lead to a long-term response for the control of CO_2, i.e.,

the excavation of nest chambers at a depth appropriate for fungus rearing. However, laboratory experiments with different CO_2 concentrations did not show such a graded digging response to varying CO_2-levels in *A. lundi* (see Chapter 5). Florida harvester ants, *Pogonomyrmex badius*, the nests of which are vertical and have a very distinct top-heavy distribution of chambers, were thought to build their nests according to the occurring CO_2-gradient across the soil. When forced to excavate a new nest under an experimentally reversed soil CO_2-gradient, i.e., with levels decreasing with depth, workers still excavated a nest similar to the formerly inhabited one, and not one with inverted architecture (Tschinkel, 2013). This indicates that harvester ants do not respond in the long-term by using CO_2 as a cue for nest excavation.

Because of the lack of the long-term response of excavating nest chambers depending on the CO_2-levels, leaf-cutting ant workers can only respond to varying CO_2-levels in the short term when they search for new sites to relocate and rear their fungus, as demonstrated in the present study. However, the observed CO_2-preferences for fungus rearing could indirectly lead to a long-term digging response. A recent study indicated that the putative contents of a nest chamber, brood or fungus, have first to be present or relocated at a site to trigger the excavation of a chamber at this spot (Römer and Roces, 2014). If leaf-cutting ants deposit their relocated fungus in a tunnel where they encounter suitable CO_2 concentrations for fungal growth, as can be assumed from the experimental results, workers should aggregate around the worker-attracting fungus (Römer and Roces, 2014). As aggregation triggers digging responses, differences in underground CO_2-levels should have an impact on the excavation of new chambers and consequently on the final nest architecture. However, as mentioned above, the preferred lower values occur in upper soil layers, where other environmental factors are probably less suited for fungal growth. Different species of leaf-cutting ants seem to have adapted differently to cope with such environmental constraints. Many *Acromyrmex* species accumulate leaf-litter or plant material on top of their nests, which might help to prevent humidity loss and lessen environmental fluctuations through insulation (Weber, 1966; Bollazzi and Roces, 2007; Bollazzi and Roces, 2010b; Lopes et al., 2011). Also, their fungus might have evolved a higher humidity or temperature tolerance like the more cold-tolerant symbiont of *Atta texana* at the species' northern distribution frontier (Mueller et al., 2011). It is tempting to speculate that colonies of the genus *Atta* rear a fungus adapted to tolerate higher CO_2-levels, which enables workers to excavate chambers at deeper soil layers as compared to *Acromymrex*-species, likely searching for an environment with better-suited temperature and humidity levels.

It is argued that besides adaptive nest structures and behavioral responses for the control of nest climate (Bollazzi and Roces, 2007; Bollazzi and Roces, 2010a; Bollazzi and Roces,

2010b; Cosarinsky and Roces, 2012) and physiological adaptations of the fungal symbiont (Mueller et al., 2011), the prevailing differences in environmental conditions in the soil phase largely influence nest building behavior and climate control, as already shown for soil temperature and moisture (Bollazzi et al., 2008; Bollazzi et al., 2012; Pielström and Roces, 2014). The preference for lower CO_2-values for fungus rearing may draw chamber excavation upwards, closer to the soil surface, while moisture and temperature levels, although depending on latitude, may draw excavation behavior downwards. The spatial concentration of digging efforts, i.e., the resulting nest architecture, likely represents a compromise in the search for the most suitable environmental conditions for fungus rearing at the nesting site.

Fungus-filled nest chamber in a nest of Atta laevigata; source: W. Thaler

Chapter 5

5. Underground carbon dioxide levels: The effect on digging behavior of leaf-cutting ants

Abstract

The digging activity of leaf-cutting ants is influenced by the microclimatic conditions of their underground environment, mostly temperature and soil moisture. This is thought to lead to nest excavation at soil depths suitable to rear their symbiotic fungus and colony brood. It was investigated whether the level of CO_2 workers experience while digging, affects their excavation rates as CO_2 concentration is known to increase with soil depth. The superficial nesting habits of the leaf-cutting ant Acromyrmex lundi may be the result of workers avoiding excavation at high CO_2-levels or workers being metabolically constrained to excavate at these values. This was analyzed in independent assays using groups of medium-sized workers allowed to dig in a chamber with different CO_2 concentrations, ranging from atmospheric values to 11%. Both the total amount of excavated soil and the amount of soil pellets deposited inside the chamber were quantified after 5 hours. There was no difference in the excavation intensity observed at atmospheric or elevated CO_2 levels. Only the highest concentration of 11% negatively influenced digging activity. At atmospheric levels, workers deposited most of the excavated pellets inside the open chamber space. However, the pellet deposition steadily declined with increasing CO_2 concentration. This suggests that either workers removed more pellets at high CO_2-levels to promote a better nest ventilation, or workers, unable to excavate because of hypercapnia, switched from digging to pellet transport. As overall digging intensity remained level, it is suggested that other workers, not yet impaired by the raised CO_2-levels take over excavation. As there was no relationship between digging intensity and CO_2 concentration, it is concluded that the superficial nesting habits of A. lundi cannot be simply explained by an increased workers' digging activity at shallow depths characterized by suitable, low CO_2-levels.

5.1 Introduction

The nest excavation of ants is thought to be a self-organized process without a central control. Rather, each worker reacts to local stimuli, which can trigger and terminate digging activity. These stimuli can be of biotic or abiotic nature. Local aggregation of co-workers can stimulate ants to start digging while a decreasing local density may lead to cessation of excavation (Deneubourg and Franks, 1995; Rasse and Deneubourg, 2001). Density-modulated digging behavior also seems to be influenced by the location of brood and symbiotic fungus within the nest, which are attractive to workers (Römer and Roces, 2014). Vibrational signals of leaf-cutting ants, originating from workers engaged in digging, attract other ants to a site, which increases the probability of these ants to start excavating there (Pielström and Roces, 2012). The soil workers excavate and deposit in the form of pellets close to the active digging zone can also influence digging behavior of ants. In a study with the leaf-cutting ant *Atta vollenweideri* it was shown that the placement of freshly excavated pellets influences a workers decision where to start excavating (Pielström and Roces, 2013).

The environmental conditions encountered while digging in the soil are also very important for colony survival, as the symbiotic fungus leaf-cutting ants farm has specific microclimatic demands to grow properly. In in-vitro experiments, temperatures between 25-30°C incited optimal fungal growth (Quinlan and Cherrett, 1978; Powell and Stradling, 1986), a temperature range the ants do indeed prefer for culturing the fungus (Bollazzi and Roces, 2002). Workers also prefer an environment with near saturated humidity levels as the symbiotic fungus is also very susceptible to desiccation (Roces and Kleineidam, 2000). The humidity and temperature conditions in the soil are not stable, but change with soil depth, time of day, season or soil composition.

Carbon dioxide levels in the soil increase steeply with depth, compared to the levels of ~0.03% in the atmosphere. Even in superficial soil layers of 30-50 cm, the CO_2 concentration can reach levels between 1-2% (Martin Bollazzi, personal communication), depending on soil moisture and porosity. In the fungus garden zone of deeper-nesting *Atta* species (2-4 m), CO_2 concentrations of up to 6% have been reported (Kleineidam and Roces, 2000; Bollazzi et al., 2012). These highly elevated carbon dioxide levels seem to negatively influence fungal growth as a decrease in fungus respiration can be detected under these conditions (Kleineidam and Roces, 2000). Workers of *Acromyrmex lundi* indeed avoid such high concentrations for fungus culture (see Chapter 4). However, they prefer rearing the fungus at 1% CO_2, which is encountered in superficial soil layers, where their nests naturally occur (Zolessi and Gonzalez, 1978). It is therefore relevant where the ants excavate the nest, as such a structure can only

dampen environmental fluctuations to a certain degree, so that conditions in the surrounding soil will affect the growth of the colony's food source.

The climatic conditions at the excavation site also influence the digging activity of workers. *A. lundi* showed their highest digging activity at a temperature of 25°C (Bollazzi et al., 2008), optimal for fungal growth. A high water content of the soil also positively influenced the digging activity of *Atta vollenweideri* (Pielström and Roces, 2014), which should lead to increased humidity levels in the air of the excavated structure because of evaporation from the soil phase.

It is unclear to what extent CO_2 influences the digging behavior of leaf-cutting ants. In the ant *Solenopsis geminata*, CO_2 released by trapped workers triggered excavation behavior in nestmates and led to the rescue of these ants (Hangartner, 1969). It may be possible that the superficial nests of *A. lundi* emerged as a result of the ants graded digging response to different CO_2-levels in the soil. Moderate levels might stimulate the ants to dig, while higher levels may inhibit digging and are expected to be avoided. Therefore digging behavior of *A. lundi* workers was investigated under different CO_2 concentrations, ranging from atmospheric levels to 11% CO_2.

5.2 Materials and Methods

(a) Study animals

The experiment was performed between March and May 2013 with 5 laboratory colonies of the leaf-cutting ant *Acromyrmex lundi*. They were reared in a climatic chamber at 50% humidity, a temperature of 25°C and a 12L:12 D cycle. Each colony was housed in a system of 6-7 fungus filled plastic boxes (19x19x9 cm), a feeding arena and a box for waste disposal, all interconnected by transparent rubber tubing (diameter 2.5 cm). Water, honey water and blackberry leaves (*Prunus fruticosus*) were given daily.

(b) Experimental setup

To test the effect of CO_2 on digging behavior, leaf-cutting ant workers were exposed to different CO_2-levels while excavating. An open plastic box (19x19x9 cm), from now on called the ant release box, was connected with a 10 cm long piece of transparent rubber tube (diameter 1.0 cm) to the digging chamber (**Fig. 5.1**; 9x9 cm, height 2 cm). After the back half of the chamber was filled with a clay-sand mixture (2:1, Claytec Baulehm gemahlen 0–0.5 mm, Viersen, Germany and Dorsilit Kristall-Quarzsand 0.1-0.5 mm, Hirschau, Germany; 16% water content), the chamber lid was tightly sealed with tape. The CO_2 gas mixture was pumped into the digging chamber through a small rubber tube (diameter 0.3 cm) inserted into the posterior wall of the

plastic box and buried to a depth of 2 cm into the soil mixture. For the gas to reach the open space in the digging chamber, a small tunnel was drilled into the soil, originating from the gas tube. To generate the different CO_2 gas mixtures a gas cylinder containing synthetic air (79.5% N_2, 20.5% O_2, 0% CO_2) and a cylinder containing 100% CO_2 were connected to two valves controlled by a gas mixer (Mass Flow Controler MFC-4, Sable Systems International, USA).

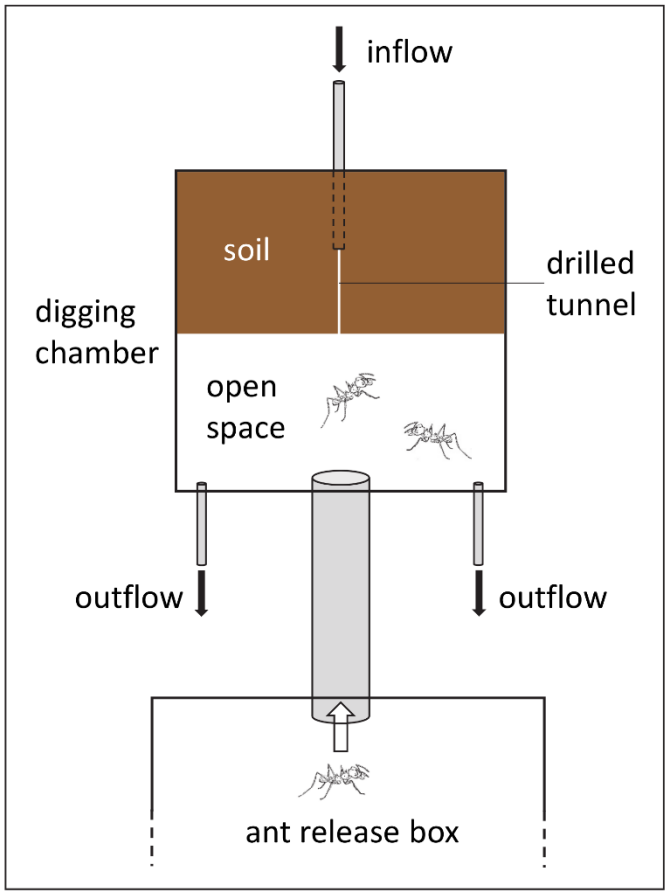

Figure 5.1: Experimental digging chamber with CO_2-inflow tube buried in the soil; black arrows mark the in- and outflow of CO_2-enriched air, the white arrow marks the entry direction of ants coming from the ant release box.

The inflow of the gas mixture was adjusted to 50 ml*min^{-1}, a flow rate causing no perturbation. To ensure a consistent CO_2 concentration throughout the assay, the air of the experimental chamber was pumped out continuously at a flow rate of 37.5 ml*min^{-1} (miniature vane pump, 135 FZ, Schwarzer Precision, Germany) through two small rubber tubes (diameter 0.3 cm) inserted in the anterior chamber wall at both sides of the entrance hole (distance to entrance: 4 cm). Choosing a smaller outflow than inflow rate should cause a slight backlog of the inflowing gas, and led to more stable CO_2 concentrations during the assays.

(c) Experimental procedure

For an assay, 100 media sized workers were collected from a laboratory colony. The CO_2 concentration inside the digging chamber was measured by sampling air through two small rubber tubes (diameter 0.3 cm) inserted into the arena lid, one for outflow, the other for inflow, and by passing it through a CO_2-analyzer (Gasmitter, resolution: 0.02%; Sensor Devices, Germany). When the appropriate CO_2-levels had been established, workers were released in the ant release box connected to the digging chamber. For 5 hours, workers could dig freely inside the chamber and were able to deposit the excavated soil pellets inside the free space of the digging chamber or 'outside' in the ant release box. Before the assay was finished, the CO_2 concentration inside the chamber was measured again. As leaf-cutting ants are known to adjust their internal nest space by depositing a prominent part of their excavated soil pellets inside the nest (see Chapter 3), the amount of soil pellets left in the digging chamber was collected and measured to the nearest 0.1 g. The total amount of excavated soil was determined to the nearest 0.1 g by weighing the digging arena before and after (without pellets) the assay.

Six series were performed, at 0.03% (atmospheric), 1%, 2%, 4%, 7% and 11% CO_2 concentration. **Table 5.1** presents the measured CO_2 values at the beginning and end of the series.

Table 5.1: CO_2 concentrations used in the different experimental series, with the values measured at the beginning and end of each assay.

	CO_2 concentration (%) Beginning of assay		**CO_2 concentration (%) End of assay**	
Series	median	25-75% percentiles	median	25-75% percentiles
atmospheric 0.03%	0.09	0.07-0.12	0.07	0.06-0.1
1%	1.04	0.98-1.07	1.01	0.99-1.04
2%	2.3	2.19-2.36	2.34	1.9-2.39
4%	4.11	3.95-4.31	4.21	3.87-4.69
7%	7.2	7.12-7.34	7.23	6.92-7.3
11%	11.35	10.0-12.2	11.47	10.67-11.82

Twenty assays were performed per series, i.e., 4 assays with each of the 5 colonies used. Workers that participated in a given experiment were not reintroduced into their colonies.

5.3 Results

The digging activity of the ants did not decrease with increasing CO_2 concentration, except for the highest tested concentration of 11% (**Fig. 5.2a**; one way ANOVA: F=16.19, n=20, p<0.001; post hoc test: Scheffé: p>0.05, except: 0.03% vs. 11%, 1% vs. 11%, 2% vs. 11%, 4% vs. 11%, 7% vs. 11%: p<0.001). Here, workers only excavated half as much as at the other concentrations. A negative effect of increasing CO_2 on the pellet deposition behavior of the workers could be observed (**Fig. 5.2b**; one-way ANOVA: F=31.5, n=20, p<0.001; for post-hoc test statistics see figure caption). Had they deposited 92.6% (median, 25-75% percentiles= 78.8-94.2%) of the excavated soil pellets within the digging chamber at near atmospheric values, they deposited less pellets inside, the higher the carbon dioxide levels (**Fig. 5.2c**). At 11%, only

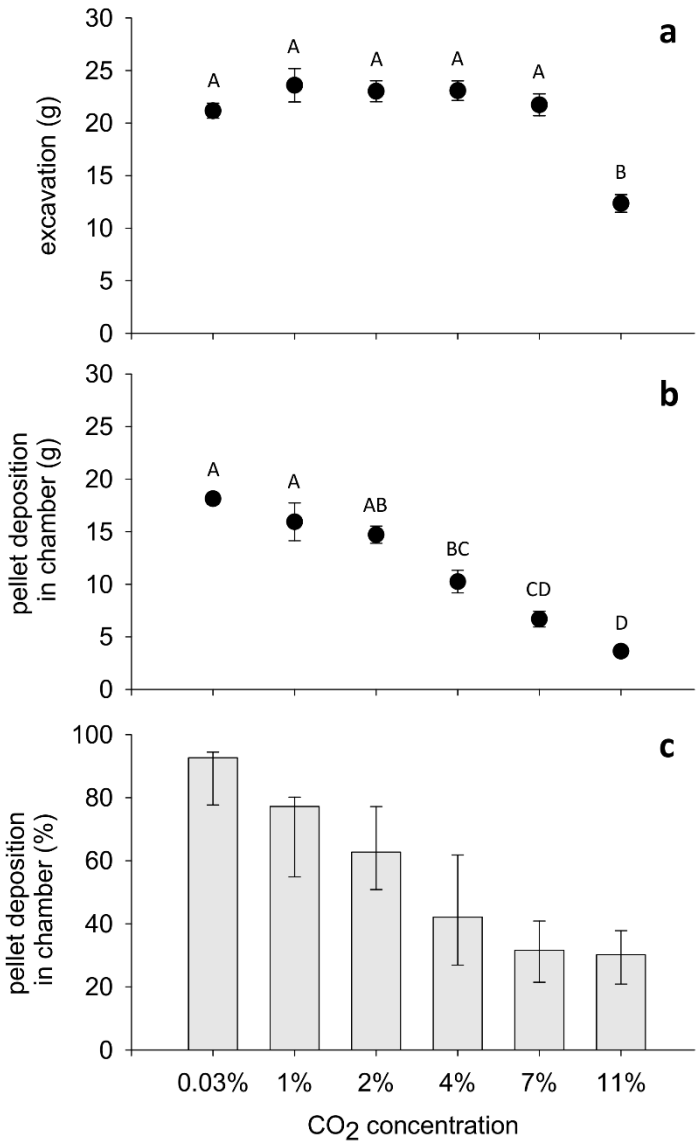

Figure 5.2: **(a)** Digging activity as a function of CO_2-levels within the chamber. Post-hoc test: Scheffé, series with the same letters do not differ statistically, p≤0.05, **(b)** amount of soil pellets deposited in the digging chamber as a function of CO_2-levels; post-hoc test: Scheffé, series with the same letters do not differ statistically, p≤0.05 (0.03% vs. 1% p=0.8, 0.03% vs. 2% p=0.34, 0.03% vs. 4% p=0.00, 0.03% vs. 7% p=0.00, 0.03% vs. 11% p=0.00; 1% vs. 2% p=0.98, 1% vs. 4% p=0.01, 1% vs. 7% p=0.00, 1% vs. 11% p=0.00; 2% vs. 4% p=0.09, 2% vs. 7% p=0.00, 2% vs. 11% p=0.00; 4% vs. 7% p=0.29, 4% vs. 11% p=0.001; 7% vs. 11% p=0.47), **(c)** percentage of soil pellets deposited in the digging chamber as a function of CO_2-levels. Depicted are means and SE in (a) and (b) and medians and 25-75% percentiles in (c).

30.2% (median, 25-75% percentiles= 21.0-37.0 %) of the pellets were not transported outside (**Fig. 5.3a-c**).

5.4 Discussion

Carbon dioxide does affect the digging activity of leaf-cutting ants, but only at extremely elevated levels. Such high levels of CO_2 have not been reported in chambers or in the soil around leaf-cutting ant nests, not even for the deep nests of the genus *Atta*. It is concluded that leaf-cutting ants, at least regarding their digging behavior, are not impaired by the CO_2-levels they encounter in their natural habitat.

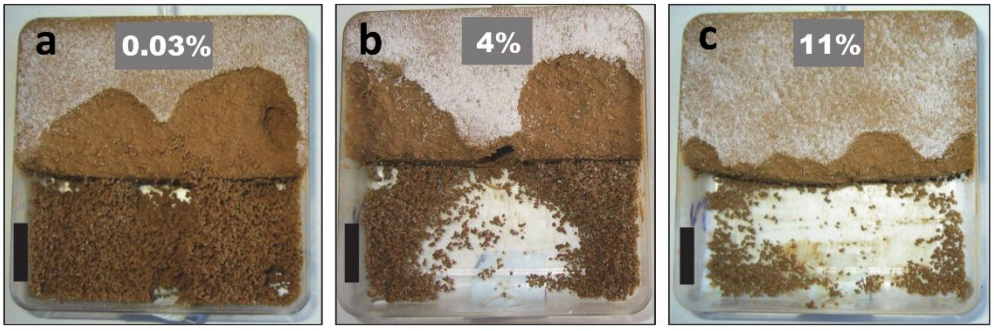

Figure 5.3: Examples of digging arenas after 5h **(a)** at atmospheric values; the open space of the chamber was used for pellet deposition **(b)** at 4%; the pellet deposition declined **(c)** at 11%; less digging activity and internal pellet deposition in the open space of the chamber was observed; black bar= 2 cm.

There also does not seem to be a CO_2 concentration that stimulates the ants digging responses to a greater degree. Would *Acromyrmex lundi* workers simply react to the CO_2-levels in the surrounding soil when they excavate their nest, they should excavate nest chambers in superficial soil layers, characterized by low CO_2-levels, as well as in deeper layers (2-4 m) where levels of 7% have been reported (Bollazzi et al., 2012). The superficial nesting habits of *A. lundi* can therefore not be explained by a stimulating or hindering effect of CO_2 on the digging activity of the workers.

The Florida harvester ant *Pogonomyrmex badius* builds characteristic, deep nests with the majority of chambers found in the upper soil regions. The distribution of the chambers seem to be correlated with the CO_2-gradient in the surrounding soil, which suggests the use of the gradient as a building template (Tschinkel, 2013). Yet, when the CO_2-gradient was artificially reversed and ants excavated a new nest, the nest's architecture was not reversed. The majority of chambers was not found in the lower part of the nest, but rather presented the species-specific

top-heavy chamber distribution. Carbon dioxide might still affect the nest architecture of leaf-cutting ants by its effect on the symbiotic fungus, as it negatively affects the respiration of the fungus at levels around 4-7% (Kleineidam and Roces, 2000), suggesting an encumbrance of fungal growth. Fungus chambers may arise by two different mechanisms. First, chambers could be excavated in advance as a digging response of workers to environmental conditions triggering digging (Bollazzi et al., 2008; Pielström and Roces, 2014). Second, chambers could also emerge around relocated brood and fungus, where these worker-attractive items increase local ant density at a site and lead to increased excavation (Römer and Roces, 2014). *A. lundi* seems to prefer levels of 1% CO_2 to rear their symbiotic fungus (see Chapter 4). This could lead to the fungus deposition at preferred CO_2-values and excavation of the fungus chambers in superficial soil layers, where these concentrations occur (Martin Bollazzi, personal communication).

It is unclear why overall digging activity declined so steeply at 11% while it remained at similar levels from atmospheric values to 7%. It is possible that not all workers of the introduced group took part in the excavation, possibly because space at the active digging zone was limited. Some of the excavating workers might have stopped, because they had a preference for specific concentrations of this respiratory gas while digging, and stopped, when they perceived others. Other ants, with different CO_2-preferences might have still be stimulated to excavate and could then have taken over the free digging sites becoming available. That way the overall digging activity would have been kept level until the majority of workers stopped excavating, because their CO_2-preferences were all exceeded at a concentration of 11%. This could be investigated in future experiments by counting the number of digging workers present per unit time and marking workers individually.

Another aspect of digging behavior, the sequential transport of soil pellets, was clearly negatively influenced by CO_2. At low values, almost all excavated soil pellets were deposited in the free part of the digging chamber. This deposition steadily decreased with increasing CO_2. Two possible scenarios seem plausible to explain this observation. Leaf-cutting ants are able to perceive the absolute carbon dioxide levels of their environment (Kleineidam and Tautz, 1996) and therefore should have been able to perceive the existing CO_2 concentrations. As a reaction to the increased levels of CO_2, which can have detrimental effects on the physiological condition of insects in general (Nicholas and Silans, 1989), ants could have removed pellets to increase the free space in the chamber to ensure better ventilation. Other building responses to increase nest ventilation are known for *Atta vollenweideri*, whose construction of turrets above nest openings can lead to a promoted gas exchange (Kleineidam et al., 2001). Alternatively, the

augmented pellet transport out of the digging chamber with increasing CO_2-levels could also be based on task-switching. For example, leaf-cutting ants with worn mandibles are physiologically (anatomically) impaired to cut leaves and a number of them switch to transporting leaf-fragments instead (Schofield et al., 2011). There is a mechanistic similarity between leaf-cutting and digging in the soil as the latter is also performed with the mandibles. The ant 'cuts' into the soil and then drags the loosened material below their thorax, where a succession of grab-rake sequences forms a soil pellet, a process also compared to ice cream scooping (Cassill et al., 2002). If the increased CO_2 concentration physiologically (metabolically) impairs the ants ability to dig, they might have analogously switched to transport. Usually soil pellets are transported sequentially, with the original excavator depositing the pellet only a short distance away from the active digging zone and a succession of short distance and long distance carriers managing the transport (Pielström and Roces, 2013). At the assays with atmospheric CO_2-levels, many excavators could be observed depositing their soil pellets on the floor of the chamber and then returning to their digging site to continue excavating. As a reaction to the increased CO_2-levels, more and more excavators, unable to continue digging because of hypercapnia, might have switched to transporting excavated pellets out of the digging arena.

Another effect of CO_2 on worker behavior could be observed. Ants excavated preferably at the small inflow tunnel drilled into the soil and towards the tube buried in the clay. This might have been a response to the mechanical irregularity in the soil surface created by the inflow tunnel, the workers response to the airflow or was due to the attractiveness of CO_2 to ants in general (Hangartner, 1969). Some ants even crawled into the inflow tube itself. This gas is not only emitted from the soil, originating there from decaying organic matter or root respiration, but is also produced as respiratory gas by the fungus and colony members. *A. lundi* also prefers to rear its symbiotic fungus in chambers with CO_2-levels higher than atmospheric values (see Chapter 4). An increased CO_2-level might be a nest cue to the ants that workers use when orienting towards the nest.

Worker of a laboratory colony of Atta laevigata carrying a piece of spent fungus into the waste chamber

Chapter 6

6. Waste management in leaf-cutting ants: The use of environmental cues in the selection of a dumpsite

Abstract

Leaf-cutting ants rear large amounts of a symbiotic fungus to nourish the colony's brood and sustain its workers. Once the fungus is exhausted, it is removed from the fungus chambers and, in most species of the genus Atta, accumulated in underground waste chambers. The ants could use environmental cues for orientation to decide where to deposit the colony waste. The material is also composed of unsuitable plant fragments, dead ants and weeded-out pathogenic fungi, and therefore poses a health risk to the colony. Accumulating the colony waste under climatic conditions that hinder the growth of alien fungi and bacteria could be a means to control pathogens. To determine if the ants use abiotic environmental cues during waste deposition, binary choice experiments were performed, offering two otherwise identical nest chambers with either different temperatures, relative humidities or CO_2 concentrations. Atta laevigata workers preferred storing the colony waste at warm temperatures of 25°C and at dry relative humidities of 33% or lower. The concentration of carbon dioxide was not used as an orientation cue, although workers preferred to aggregate at elevated CO_2-levels. The consequences of the discovered climatic preferences for leaf-cutting ants' nest architecture are discussed as well as possible strategies to deal with pathogen threats.

6.1 Introduction

Leaf-cutting ants are the dominant herbivores in their natural habitat because of their obligate symbiosis with a fungus (Basidiomycetes: *Leucoagaricus gongylophorus*; Möller, 1893), which enables them to use many different plant species to 'feed' their colony. The vegetal material is used to farm the symbiotic fungus, which in turn sustains the colony's brood and is part of a workers diet (Cherrett, 1989; Bass and Cherrett, 1995). Freshly cut plant material is continuously planted on top of the fungus garden while the exhausted fungus is removed from the bottom and transported out of the fungus chambers. Because of this symbiosis and their large colony sizes, the ants produce copious amounts of waste, consisting of exhausted fungus, non-digested leaves and the colonies dead. Leaf-cutting ants have developed two forms of disposing of their colony waste. The genus *Acromyrmex*, which in general has smaller colony sizes and builds nests with fewer fungus chambers, deposits their colony waste outside of the nest in above ground waste heaps (Navarro and Jaffe, 1985; Ballari et al., 2007). The other genus of leaf-cutting ants, *Atta*, whose colonies can have millions of individuals and underground nests with thousands of fungus chambers, generally deposits their colony's waste in large, underground waste chambers (Stahel and Geijskes, 1939; Stahel and Geijskes, 1941; Amante, 1964; Amante, 1667; Jonkman, 1980b; Bollazzi et al. 2012), with the exceptions being *Atta colombica* and *Atta mexicana*, which have external waste heaps (Rojas, 1989; Hart and Ratnieks, 2002; Herz et al., 2007). The waste chambers are usually different in shape to the spherical fungus chambers and are cone-, bottle- or irregularly-shaped.

The foraged leaf material for the symbiotic fungus can be infected with pathogenic fungi, for example the fungus garden pathogen *Escovopsis*, a genus of parasitic fungi (Currie et al., 1999; Reynolds and Currie, 2004; Rodrigues et al., 2008) that may rapidly overgrow whole fungus gardens, because the symbiont is a clonal monoculture especially susceptible to parasites (Mueller et al., 2010). This would weaken or even threaten the survival of the whole colony. To protect their colony from pathogen threat, leaf-cutting ants have developed various prophylactic or hygienic behaviors. Adaptations to prevent microbial contamination starts at the foraging stage where workers avoid harvesting leaf substrate with high amounts of endophytic fungi (Coblentz and Van Bael, 2013). While the leaf fragments are being transported back to the nest, small workers ride on the fragments, cleaning and licking the leaf surface thereby reducing the microbial load (Vieira-Neto et al., 2006; Griffiths and Hughes, 2010). Self- and allo-grooming of workers, especially foragers, is also thought to be a behavior aimed at controlling fungal pathogens (Richard and Errard, 2009; Reber et al., 2011). Leaf-cutting ants also use a chemical defense against pathogens, originating from the metapleural

gland, whose extracts they spread among their nestmates and also among the fungus gardens by grooming (Poulsen et al., 2002; Fernández-Marin et al., 2006). These measures might reduce pathogen infection originating from outside the colony, but are unlikely to be as effective as to eradicate all contaminants. In the fungus chambers, the hygienic behavior continues as workers groom the symbiont of pathogenic spores, or weed out infected fungus pieces (Currie and Stuart, 2001; Abramowski et al., 2011). The groomed spores as well as the infected fresh fungus pieces are also part of the colony waste. With removing spores and infected material to the waste dumpsite (external or internal), the threat is removed from the fungus chambers but accumulated at the waste site (Bot et al., 2001). The pathogen threat of the dumpsite is demonstrated by the higher mortality of workers in contact with waste (Bot et al., 2001; Hart et al., 2002; Brown et al., 2006; Lacerda et al., 2010).

Probably to reduce worker mortality and the risk of infecting the colony with pathogens, leaf-cutting ants have evolved a system of division of labor in waste management. There seems to be a division of labor between waste workers and foragers (Waddington and Hughes, 2010) as well as division of waste transport and work on the accumulated waste heap (Hart and Ratnieks, 2001; Hart and Ratnieks, 2002; Lacerda et al., 2006; Ballari et al., 2007). In addition, there is aggressive behavior directed towards waste workers trying to re-enter the colony (Hart and Ratnieks, 2001; Ballari et al., 2007).

Waste heaps are established outside of the nest on the soil surface near the nest entrance and underground waste chambers can be found below or to the side of fungus chambers (Hart and Ratnieks, 2002; Jonkman, 1980b). So far, it is unclear what cues ants use to decide where to establish a site for waste disposal. Underground, ants are exposed to gradients in the soil environment, which could be used as orientation cues should workers show specific environmental preferences for waste deposition, as already known for the selection of a site for fungus rearing (Roces and Kleineidam, 2000; Bollazzi and Roces, 2002).

Regarding the fungus, workers prefer to rear gardens at warm temperatures (25°C; Bollazzi and Roces, 2002) and a relative humidity close to saturation (98%; Roces and Kleineidam, 2000), climatic conditions which ensure proper fungal growth (Quinlan and Cherrett, 1978; Powell and Stradling, 1986). Carbon dioxide concentrations also could influence fungus farming, as high concentrations, encountered in deeper soil regions, negatively influence the respiration rate of the leaf-cutting ants' fungus (Kleineidam and Roces, 2000). When given the choice, workers of the leaf-cutting ant *Acromyrmex lundi* do indeed try to avoid high carbon dioxide concentrations (see Chapter 4) for fungus rearing. Fungal pathogens of the symbiotic fungus should be adapted to the conditions of their host to be able to parasitize it.

Accumulating waste material under environmental conditions not well suited for fungal growth could act as an additional hygienic behavior against pathogens to improve colony survival. Indeed, the leaf-cutting ant *Atta sexdens rubropilosa* does prefer chambers with low relative humidity for waste deposition when given the choice between 25% and 95% (Ribeiro and Navas, 2006).

It was investigated, whether workers of the leaf-cutting ant *Atta laevigata* use environmental cues when choosing a dumpsite. For that, three series of binary choice experiments were performed, offering workers in the process of relocating waste particles two potential sites for waste deposition with different (1) temperatures, (2) relative humidities and (3) different CO_2 concentrations. If ants choose abiotic environmental conditions that negatively influence the growth of pathogens and parasitic fungi in the waste, they should be expected to deposit the waste at low temperatures, low relative humidities and elevated CO_2-levels, conditions that are known to be unsuitable for the growth of the symbiotic fungus.

6.2 Materials and Methods

(a) Study animals

The experiments were performed between April 2012 and April 2014. Two laboratory colonies of the leaf-cutting ant *Atta laevigata* were used. This species builds the largest and deepest-reaching underground nests among leaf-cutting ants. Colonies can have millions of individuals, thousands of fungus chambers (Moreira et al., 2004a) and huge, cylindrical waste chambers (Roces and Forti, unpublished observations). The laboratory colonies were founded 2006 and 2007 and consisted of 15 fungus filled boxes (19x19x9 cm) each, a feeding arena and a large bucket for waste disposal, all interconnected by transparent, plastic tubing. The colonies were fed with fresh blackberry leaves (*Prunus fruticosus*), water and honey water and maintained under a 12L:12D cycle. For each assay a subcolony, consisting of a fungus filled box (= fungus chamber), was disconnected from the rest of the colony and connected to the specific binary test setup. After the assay, the fungus garden was re-connected to its mother colony.

(b) Temperature choice experiment: setup and procedure

A t-shaped plastic tube (diameter 2.5 cm) was connected to two experimental nest chambers (clear plastic, with lid, 9x9x6 cm). The chambers had a metal bottom for better thermal conduction. Each chamber was placed on a heating plate connected to a water bath (Jubalo Labortechnik GmbH, temperature adjustable to 0.1°C) as heat or cooling source (**Fig. 6.1a**).

Once the temperatures to be tested were established in the chambers, a subcolony, consisting of a fungus garden, including all workers within, was connected to the end of the t-

shaped tubing. No plant material, water or honey water was given to the ants during the assays. The ants present in the fungus garden could then explore the experimental setup freely. After 1h, 4 g of waste material taken from the mother colony, with all waste workers removed, was introduced into the fungus garden through a second entrance in the side wall of the box, to increase the amount of waste inside the fungus garden and so to trigger a removal response. For the next 23h, ants could relocate the waste material into the offered experimental nest chambers (total experimental time starting from connection of fungus garden, 24h) and choose between the two different temperatures. At the end of the assay, the fungus garden was disconnected and all ants carefully removed from the setup. The deposited waste material was collected separately for both chambers and weighed to the nearest 0.1 mg. As waste material has a high and variable water content, the waste particles, consisting mostly of pieces of fungus, were dried for 24h at 50°C and again weighed to the nearest 0.1 mg. All statistical analyses were performed using data of waste dry weight.

Four different temperature-choice series were performed: (1) 15°C vs. 25°C (n=20), as the first represents a cooler temperature the ants might encounter at deeper soil layers underground because waste chambers can be situated below the colonies fungus chambers, and the latter as the preferred temperature to rear symbiotic fungus. (2) 20°C vs. 25°C (n=12), as the first is the lower margin for proper symbiotic fungus growth, and to investigate whether workers are able to discriminate a 5°C-difference between the chambers when depositing waste. (3) 20°C vs. 30°C (n=20), as these temperatures represent the lower and upper limit of symbiotic fungus growth (Powell and Stradling, 1986). (4) 20°C vs. 20°C (n=12), as a control for any side preferences.

(c) Humidity choice experiment: setup und procedure

A t-shaped plastic tube (diameter 2.5 cm) was connected to two round experimental nest chambers (diameter 10.5 cm) covered with a glass plate (**Fig. 6.1b**). To create different relative humidities (RH) in each chamber, its bottom was made out of a fine plastic mesh. The chamber was then placed on top of another round dish of equal diameter (10.5cm), filled with different saturated saline solutions or silica gel (based on methods used by Roces and Kleineidam (2000) and Ribeiro and Navas (2006)), which led to the creation of different air RH above the dish. The following humidities were established in the chambers: 98% RH: $K_2Cr_2O_7$; 43% RH: K_2CO_3; 33%: $MgCl_2$. To create the RH of 10%, the dish was filled with silica gel. The humidity inside the chambers was measured with a hygrometer (Model H1, Testo AG) to the nearest 0.1%.

After the RH had settled in the chambers, the assay started by connecting a subcolony

to the setup. After a familiarization time of 1h, 4 g of additional waste was added to the fungus box analogous to the temperature experiment. The assay continued for 23h. The humidity inside the chamber was measured again to the nearest 0.1%. The ants were removed and the deposited waste material collected and processed as in the temperature experiment.

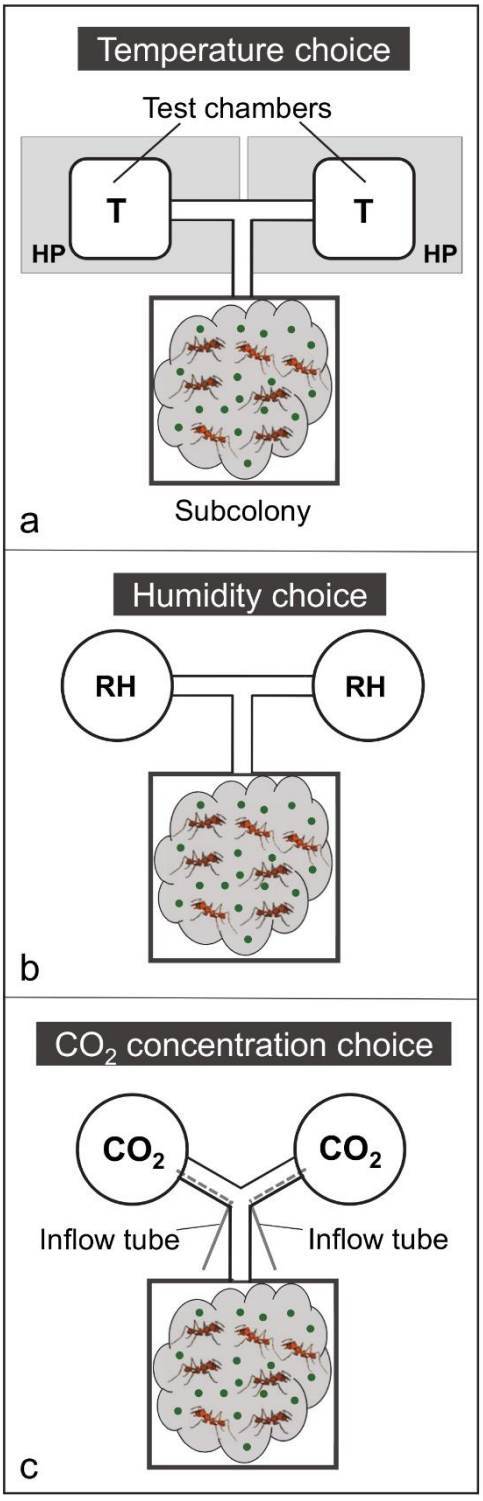

Figure 6.1: Experimental setups **(a)** Temperature choice experiment: Subcolony consisting of a fungus garden connected to two experimental nest chambers with different temperatures; T=Temperature, HP= heating plate, **(b)** Humidity choice experiment: Subcolony connected to experimental nest chambers with two different relative humidities (RH), **(c)** CO_2 concentration choice experiment: Subcolony connected to experimental nest chambers with different CO_2 concentrations (CO_2); inflow tube starting at the bifurcation point of the Y-tunnel.

Five different RH choice experiments were performed: (1) 33% vs. 98% (n=12), to investigate whether ants discriminate between clearly different dry and humid environments when removing waste, as they do for fungus rearing, for which they prefer a humidity near saturation (Roces and Kleineidam, 2000), (2) 33% vs. 43% (n=12), to evaluate whether workers are able to discriminate a 10%-difference between the chambers when depositing waste, (3) 10% vs. 33% (n=12), to evaluate whether workers chose the chamber with the lowest possible RH they encounter in their environment, (4) 10% vs. 43% (n=12), to increase the offered, higher RH value to moderate levels and (5) 33% vs. 33% (n=12), as a control for any side preferences.

(d) CO_2 concentration choice experiment: setup and procedure

A Y-shaped plastic tube (diameter 2.5 cm) was connected to two round experimental nest chambers (diameter 10 cm, clear plastic, with a lid) and a subcolony, recently removed from its mother colony. A closed gate between tunnel and garden entrance prevented ants from entering the setup before different CO_2 concentrations were established. To create two different CO_2 concentrations in the chambers, two small rubber tubes (diameter 0.3 cm) were inserted at the bifurcation point and running along an inner wall of the Y-tube, terminating in a chamber (**Fig. 6.1c**). To ensure that walking ants would encounter different CO_2-levels at the bifurcation point before entering one of the chambers, the entire length of small tube running inside the bigger Y-shaped tube was perforated. To create near atmospheric values (from now on referred to as 'atmospheric values' or 'atm' for simplification) a gas cylinder filled with synthetic air (79.5% N_2, 20.5% O_2, 0% CO_2) was connected to a tube leading to one of the chambers. The different CO_2 concentrations were generated by connecting a gas cylinder, filled with 100% CO_2, and another with synthetic air, to two valves, controlled by a gas mixer (Mixer: Sable Systems MFC-4 + Flow Adjust; valves: Sierra Mass Flow Controller, model No 840L-10-OV1-SV1-D-V4-S4 and model No 840L-2-OV1-SV1-D-V4-S4). The output of the gas mixer was connected to a tube leading to the other chamber. The inflow rate into the chambers was adjusted to 50 $ml*min^{-1}$ so as not to disturb the ants. To establish stable CO_2 concentrations throughout the experiment, the atmosphere in the chambers was continuously pumped out (miniature vane pump, Schwarzer Precision, Germany) with an equal outflow as inflow rate. CO_2 concentrations were measured to the nearest 0.1% with a CO_2-sensor (resolution: 0.02%; Gasmitter, Sensor Devices, Germany).

Once the atmosphere had settled in the chambers, the gate between fungus garden entrance and Y-tube was opened, allowing the workers to explore the setup. Because elevated carbon dioxide levels have been shown to be attractive to ants (*Solenopsis geminata*; Hangartner, 1964), the aggregation of workers in the nest chambers 1h after connection of the

fungus garden was quantified. Afterwards, 3 g of colony waste material was added to the fungus garden, and workers could relocate the waste for 4h. The running time of the assays was shortened compared to the other two series, because of the observation of very rapid responses. For that, less additional waste material was given in this experiment. At the end of the assay, the ants were removed and the deposited waste per chamber collected and processed as described for the previous series.

Five different series were performed: (1) atmospheric values (atm) vs. 1% CO_2 (n=20), as workers of the superficially dwelling leaf-cutting ant species *Acromyrmex lundi* preferred this elevated CO_2 value for the rearing of symbiotic fungus (see Chapter 4), (2) atm vs. 4% CO_2 (n=19), as this concentration is similar to the levels measured inside fungus chambers of deeper dwelling *Atta laevigata* and *Atta vollenweideri* nests (Kleineidam and Roces, 2000; Bollazzi et al., 2012), which negatively influences the respiration of the fungus (Kleineidam and Roces, 2000), (3) atm vs. 10% CO_2 (n=20), to present ants with highly hypercapnic conditions, (4) 4% vs. 10% CO_2 (n=20), to offer a choice between two different yet elevated CO_2-levels and (5) 4% vs. 4% CO_2 (n=20), as a control for any side preferences.

(e) Statistical analysis

Data was tested for normality using the Shapiro-Wilk test. Not all datasets of a series were normally distributed. For uniformity reasons, the Wilcoxon matched-pair test was used to check for statistical differences, even when the data was normally distributed. Accordingly, all data was presented as box-plots.

6.3 Results

Soon after connecting the fungus garden to the experimental setup, before additional waste was added to the garden, workers started to transport waste particles out of the garden to deposit it in one of the offered chambers. The waste was not simply dropped on the chamber floor, but accumulated by many ants, forming an organized heap. There they worked their single loads into place with the mandibles and front legs before returning to the fungus chamber. Other ants rearranged already deposited waste particles on the heap.

(a) Temperature-dependent waste deposition

When ants could choose between a chamber offering a cooler temperature of 15°C and a warmer one of 25°C, more waste was deposited at the warm temperature (**Fig. 6.2a**; Wilcoxon matched-pair test: T=46, Z=2.2, n=20, p=0.027). Once the temperature difference was reduced to 20°C in one chamber and 25°C in the other, ants still preferred deposition at the warmer temperature (**Fig. 6.2b**; Wilcoxon matched-pair test: T=15, Z=1.96, n=12, p=0.05). This changed

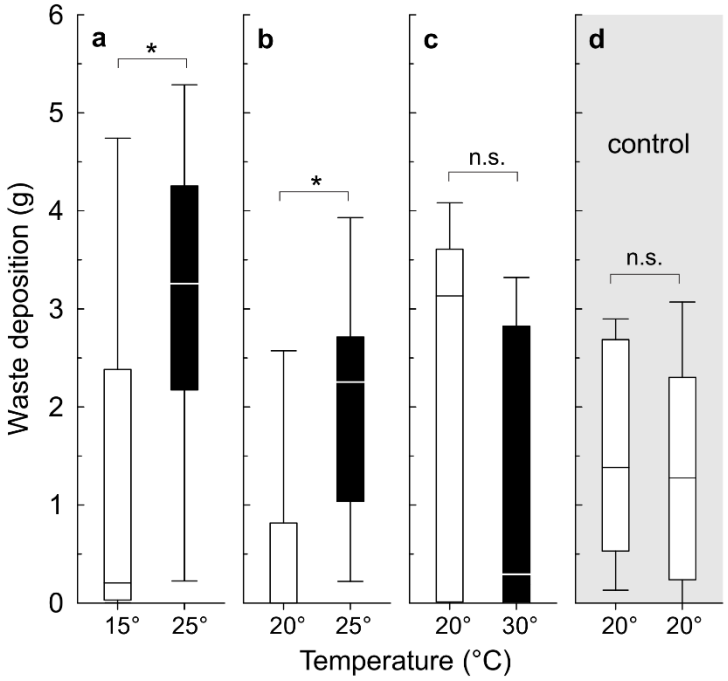

Figure 6.2: **(a)** – **(d)** Waste deposition in the binary-choice temperature experiment of the 4 series. Boxplots: median (line), 25-75% percentiles (box) and min max values (whiskers) without outliers; *p≤0.05, n.s.= not significant.

when workers no longer had the option to deposit waste at 25°C, but had to choose between 20°C and 30°C. Here, the deposited amount of waste was equally distributed between the two chambers (**Fig. 6.2c**; Wilcoxon matched-pair test: T=55, Z=1.87, n=20, p=0.062). The same equal waste distribution could be found in the control series with 20°C in both chambers (**Fig. 6.2d**; Wilcoxon matched-pair test: T=37, Z=0.16, n=12, p=0.875).

(b) Humidity-dependent waste deposition

When simultaneously offering a chamber with low relative humidity of 33% and one close to saturation at 98% RH, workers preferred to deposit the waste material in the dry chamber and avoided deposition at near saturated RH (**Fig. 6.3a**; Wilcoxon matched-pair test: T=0.0, Z=3.05, n=12, p=0.002). Even if there was only a 10% difference between lower humidity (33% RH) and higher humidity (43% RH) in the chambers, ants still deposited significantly more waste at the lower humidity value (**Fig. 6.3b**; Wilcoxon matched-pair test: T=9.0, Z=2.35, n=12, p=0.019). When both offered humidities were low (33% and 10%), a ubiquitous amount of waste was deposited in the chambers (**Fig. 6.3c**; Wilcoxon matched-pair test: T=28.0, Z=0.86, n=12, p=0.39). When instead of 33%, 43% was offered in one chamber and 10% in the other, workers again preferred deposition in the drier chamber (10%) than in the one with moderate humidity (**Fig. 6.3d**; Wilcoxon matched-pair test: T=2.0, Z=2.9, n=12, p=0.004). A control series with 33% RH in both chambers showed no difference in waste disposal (**Fig. 6.3e**; Wilcoxon matched-pair test: T=30.0, Z=0.71, n=12, p=0.48).

(c) Carbon dioxide dependent worker aggregation and waste deposition

In all 3 series offering atmospheric CO_2-levels against elevated levels (either 1%, 4% or 10%), the workers themselves preferred to aggregate in the latter, even at the extreme level of 10% (**Fig. 6.4a-c**; atm. vs. 1% CO_2: Wilcoxon matched-pair test: T=34.5, Z=2.22, n=20, p=0.03; atm. vs. 4% CO_2: Wilcoxon matched-pair test: T=18.0, Z=3.1, n=19, p=0.002; atm. vs. 10% CO_2: Wilcoxon matched-pair test: T=18.5, Z=3.07, n=20, p=0.002). When both chambers offered elevated CO_2-levels, workers were distributed evenly, whether the elevated levels were different (4% vs. 10%) or similar (control, 4% vs. 4%) (**Fig. 6.4d-e**; 4% CO_2 vs. 10% CO_2; Wilcoxon matched-pair test: T=88.5, Z=0.62, n=20, p=0.54; 4% vs 4%: Wilcoxon matched-pair test: T=70.0, Z=1.31, n=20, p=0.19).

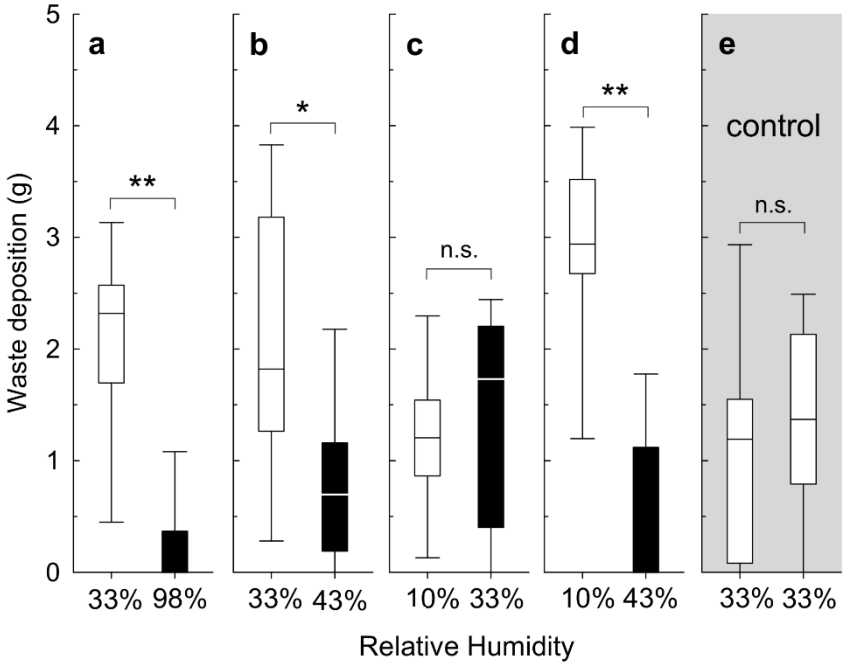

Figure 6.3: **(a) – (e)** Waste deposition in the binary-choice humidity experiment of the 5 series. Boxplots: median (line), 25-75% percentiles (box) and min max values (whiskers) without outliers; *p≤0.05, **p<0.01, n.s.= not significant.

Despite a skew of worker aggregation towards chambers with higher than atmospheric CO_2-levels, the deposited waste material was evenly distributed between atmospheric levels and elevated ones (**Fig. 6.4f-h**, atm. vs. 1% CO_2: Wilcoxon matched-pair test: T=57.0, Z=1.79, n=20, p=0.07; atm. vs. 4% CO_2: Wilcoxon matched-pair test: T=70.0, Z=1.01, n=19, p=0.31; atm. vs. 10% CO_2: Wilcoxon matched-pair test: T=56.0, Z=1.83, n=20, p=0.07). When the CO_2-levels in both chambers were elevated, either at different levels (4% vs. 10%) or similar ones (4% vs. 4%), the waste material was also evenly distributed between the chambers (**Fig. 6.4i-j**; 4% CO_2 vs. 10% CO_2; Wilcoxon matched-pair test: T=65.0, Z=1.49, n=20, p=0.14; 4% vs 4%: Wilcoxon matched-pair test: T=95.0, Z=0.37, n=20, p=0.71).

6.4 Discussion

Young colonies of *Atta laevigata*, before reaching the first year, dispose of their waste material by transporting it to the outside and by dropping the particles around the nest entrance (F. Roces, personal communication), as most mature *Acromyrmex* colonies, *Atta colombica* and *Atta mexicana* do (Navarro and Jaffe, 1985; Rojas, 1989; Hart and Ratnieks, 2002; Ballari et al., 2007). Only when colonies get older, workers switch to internal waste disposal in waste

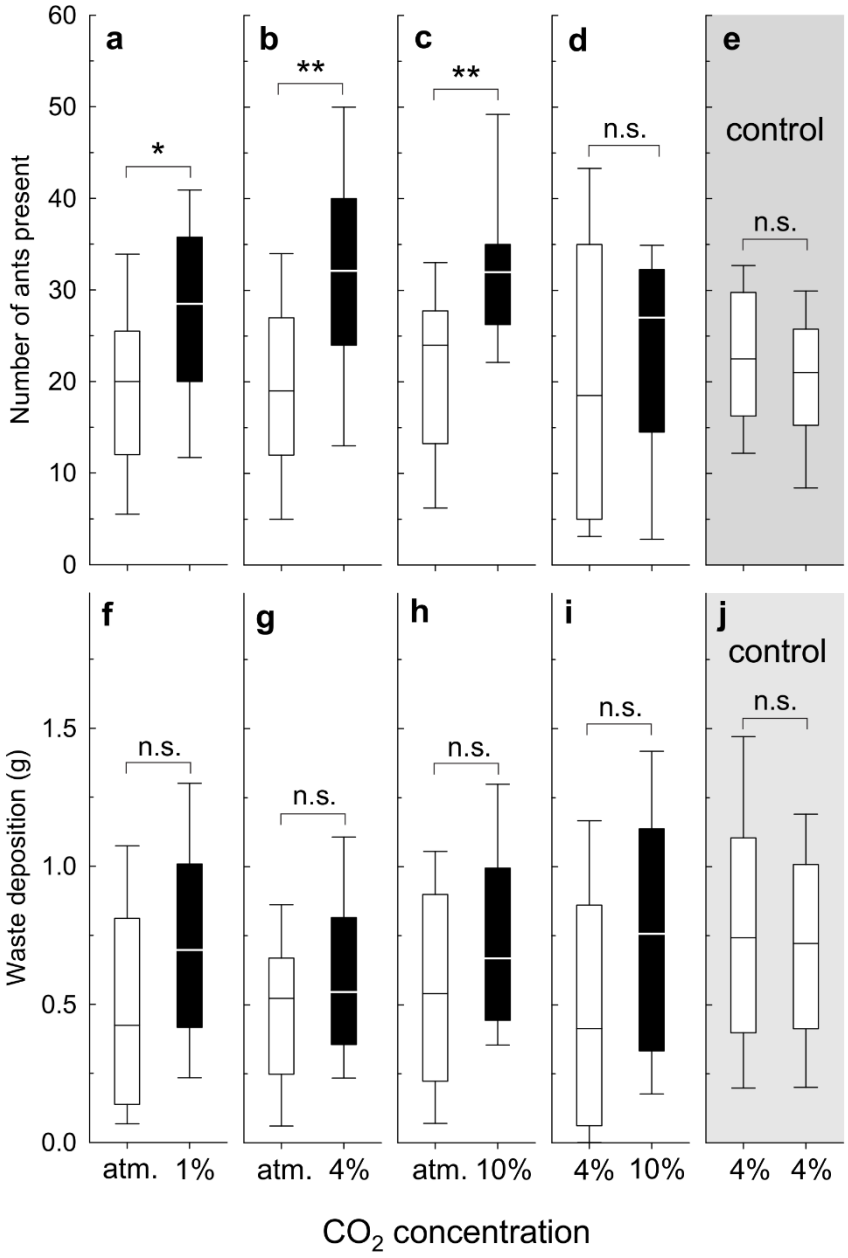

Figure 6.4: Results of the binary-choice CO_2 concentration experiment, 5 series were performed; **(a)** – **(e)**: Number of ants present in chamber; **(f)** – **(j)**: Waste deposition in chamber. Boxplots: median (line), 25-75% percentiles (box) and min max values (whiskers) without outliers; *p≤0.05, **p<0.01, n.s.= not significant.

chambers. This work could demonstrate that ants chose places to establish a dumpsite, based on abiotic cues of their environment. They preferred to deposit their waste under warm and dry conditions. *Atta sexdens rubropilosa* does display the same preference for a dry environment at

the dumpsite (Ribeiro and Navas, 2006). Supposedly, the climatic preferences displayed in this study are generally valid for leaf-cutting ants or at least species of the genus *Atta* that deposit their waste in underground chambers.

The preference of a humid environment for fungus chambers (Roces and Kleineidam, 2000) and a dry one for waste disposal should lead to the separation of fungus and waste chambers, as these environmental conditions are unlikely to occur at the same place in the nest. The waste chambers of *Atta* nests are usually situated to the side or below the fungus chambers (Stahel and Geijskes, 1939; Stahel and Geijskes, 1941; Amante, 1964; Amante, 1667; Jonkman, 1980b; Bollazzi et al. 2012). Carbon dioxide concentration would be a reliable depth cue to workers, as its levels increase with depth. However, the results of the CO_2 concentration experiment do not indicate the use of carbon dioxide levels for waste deposition.

A separation of the waste chambers from the fungus chambers could also emerge if workers used a simple cue not related to climatic conditions, distance, as observed in the ant *Myrmica rubra* (Diez et al., 2012). Leaf-cutting ant workers could establish waste chambers separated from the threatened fungus chambers, if waste-transporting workers would deposit it after they have walked a certain distance. However, a clear preference for a distance-dependent deposition of waste could not be detected in laboratory experiments (unpublished data). Based on the discovered preference for certain environmental factors it is therefore argued that the separation of waste and fungus chambers originates from the preferred climatic conditions at the dumpsite rather than being based on distance, although a distance-effect cannot be ruled out completely.

The separation should also hinder the spread of diseases and pathogenic fungi as it reduces the probability of contact between waste and workers involved in inside-nest tasks (Pie et al., 2003). The nest architecture of *Atta* leaf-cutting ants can therefore also be seen as part of a colony's prophylactic behavior aimed at reducing pathogen threat originating from a dumpsite.

The ants preferred warm temperatures of 25°C for waste deposition and not cooler temperatures as expected. Very high temperatures, caused by solar radiation, could also be detrimental to fungal growth and waste storage under these conditions could therefore be a well suited prophylactic behavior. It has been suggested that the high, fluctuating temperatures caused by sunlight help control the growth of microorganisms in the external waste heaps of *Acromyrmex lobicornis* (Ballari and Farji-Brener, 2006). *A. laevigata* seems to have a preference for 25°C, rather than depositing the waste at the warmest temperature they encounter. When the workers could deposit their waste material at 30°C, they distributed their

waste evenly between the chamber with the warm and the cooler temperature of 20°C. Perhaps accumulating waste at high temperatures underground is not feasible, because it is detrimental to the survival of ants that work on the waste heap. In addition, temperatures on the soil surface are expected to greatly exceed temperatures subsisting underground because of direct exposure to solar radiation. It can only be hypothesize, based on the preference for one of the offered temperatures, that depositing waste under these conditions could be advantageous to the colony. Perhaps it promotes a proper decomposition of the material, which cooler or hotter temperatures could hinder. It could also be the optimal temperature for the activity of the ants, so that the waste heap is better managed, leading to a higher decomposition and prevention of pathogenic fungal growth.

It is expected that the workers' preference for dry conditions at the site for waste disposal should be detrimental to alien fungus because of their susceptibility to desiccation. The preference does not seem to be specific to a certain humidity value, rather, once a threshold of low humidity has been reached, waste is preferably disposed of there. In the here performed series, workers were able to perceive humidity differences of 10%. The relative humidity value of 43% was avoided for the deposition of waste material. However, this moderate humidity value would not be suitable for fungus rearing either, as such RH would still result in its desiccation (personal observation). Perhaps the rejection of dumpsites with moderate and high humidity levels is based on the influence of the waste itself on the RH value of its environment. The waste of leaf-cutting ants consists for the most part of exhausted symbiotic fungus, which has a water content of 40-50% (personal observation). Water could evaporate at the deposition site and therefore increase the humidity content of the chamber air considerably. The measured humidity values inside the experimental nest chambers after waste deposition were always higher, compared to the beginning of the assay, especially in the chamber where most of the waste had been deposited. The same was noted in experiments by Bot et al. (2001). Perhaps the surrounding soil of natural waste chambers helps to absorb some of the moisture, keeping RH values at moderate levels. Accumulating waste at a site that already has moderate humidity content might lead to an unfavorably high humidity at the site.

Workers do not simply drop the waste particles at the dumpsite, but carefully manipulate them with their mandibles and front legs and, when a heap starts to form, build tunnels through the material (Cerqueira, 1983; Galeska Leite, 1990; Bot et al., 2001). These tunnels could help 'airing' out the waste, removing moisture from the waste when air flows through it. This could also help to reduce the temperature inside the waste mass, as within-waste decomposition processes should lead to an increase in temperature. Workers also continuously re-shift the

particles on the waste heap (Bot et al., 2001), which should also aid in decreasing the waste mass' humidity and temperature.

As high levels of CO_2 negatively influence the respiration rate of the leaf-cutting ants' fungus, these conditions would also influence the respiration of parasitic fungi. Contrary to the expectations, workers did not primarily deposit their waste material at high CO_2 concentrations in any of the performed series. Waste deposit was rather independent of the existing levels in the chambers. This was not due to the workers inability to detect the elevated levels. In fact, leaf-cutting ants cannot only detect the relative, but also the absolute carbon dioxide concentration of their environment, as they have a specialized chemosensillum on their antennae (Kleineidam and Tautz, 1996; Kleineidam et al., 2000). Workers preference to aggregate at the elevated CO_2-levels also demonstrates them perceiving the offered carbon dioxide levels and confirms the attractiveness of the gas to ants (Hangartner, 1969). However, waste distribution was not influenced by this attractiveness to workers. This experiment also indicates that leaf-cutting ants have a very high tolerance for elevated carbon dioxide levels as ants even preferred to aggregate at 10% CO_2 rather than in the chamber with atmospheric values. The decomposition processes inside the waste material could produce large amounts of CO_2, so that the levels will increase in a waste chamber, whether it was established at a site with low or high CO_2-levels. While *Atta* leaf-cutting ants have developed methods to passively ventilate their giant nests (Kleineidam and Roces, 2000; Kleineidam et al., 2001; Bollazzi et al., 2012), it is unclear whether the ventilation only aerates the fungus garden zone or also reaches the waste chambers.

Perhaps one of the most effective ways to counteract the pathogens in the waste is to facilitate the waste decomposition. Warm temperatures and moderately increased humidity levels (originating from the waste) could accelerate decomposition of the material and the pathogens existing within. Waste material has an increased share of anaerobic bacteria compared to the fungus garden (Scott et al., 2010). This could allow them to decompose the refuse under high CO_2-levels, likely associated to low O_2 concentrations, as well as under low CO_2-levels.

The switch from external waste disposal to internal dumpsite during the early ontogeny of an *A. laevigata* colony suggests that this type of waste management is advantageous to the colony. Environmental factors can probably be better controlled underground. This could aid the management of the huge amounts of produced colony waste, the consequence of the otherwise successful symbiotic lifestyle. So far, it can only be speculated that waste disposal under certain environmental conditions is a form of social immunity (Cremer et al., 2007) of

leaf-cutting ants, i.e., to spatially confine pathogen threat originating from the site of waste deposition. An evaluation of the growth of pathogenic fungi or the decomposition of leaf-cutting ant waste under different climatic conditions would contribute to a better understanding of the evolution of waste management strategies in these ants.

To be able to deposit their waste underground, leaf-cutting ants have to excavate space for waste disposal. Therefore, the evaluated preferred environmental conditions should also be of consequence to leaf-cutting ant nest architecture as behavioral responses to these conditions during waste chamber excavation can be expected.

Nest of Atta laevigata in Botucatu, Brazil; waste chamber zone on the left; nest mound and fungus garden zone on the right; source: L.C. Forti

Chapter 7

7. Waste management in leaf-cutting ants: The use of olfactory cues in the selection of a dumpsite

Abstract

Leaf-cutting ants have developed an elaborate waste management system, as their farmed symbiotic fungus is susceptible to pathogens and parasitic fungi. Exhausted or pathogenically infected fungus, dead nestmates and rejected plant material are continuously transported out of the nest's fungus chambers and disposed of, either in waste heaps on the soil surface or in large underground waste chambers. It is unclear which cues ants use to determine where to deposit this potentially dangerous material, but considering that ants possess very well developed olfactory systems, it is likely that olfactory cues may be involved. It was therefore investigated whether volatiles are used as cues during waste management. In binary choice experiments, workers of Atta laevigata were exposed to fungus and waste volatiles emanating from the sides of an artificially established waste heap. The relocation of the waste material towards the side of one of the volatile sources was recorded as indicative response of the use of olfactory cues. Unexpectedly, workers did not remove the waste heap from the side located above the fungus volatiles when only exposed to them. When workers were only exposed to waste volatiles, they relocated the waste towards the side emitting these volatiles. Exposure to both volatiles simultaneously also led to heap relocation towards waste volatiles and in addition to an increased removal of waste particles from above the fungus volatiles. Results indicate that leaf-cutting ants do use olfactory cues during waste disposal. The ants' preference to dispose of waste at a site where they perceive waste volatiles is expected to lead to a massive accumulation at one site within the nest.

7.1 Introduction

Hygienic behavior is important to individuals living in social groups as the close proximity of the group members abets disease and pathogen transmission. In addition, social groups produce large amounts of waste, an ideal breeding ground for diseases. South American leaf-cutting ants not only live in large colonies, they also rear a symbiotic fungus as a food source. As a consequence of fungus farming, copious amounts of exhausted mycelium are discarded as waste, as well as dead nestmates and unused plant fragments. The fungal symbiont is susceptible to pathogens, for example to the fungus garden parasite *Escovopsis* (Currie et al., 1999; Reynolds and Currie, 2004; Rodrigues, 2008). To defend against fungus garden parasites and other pathogens, which threaten the survival of the colony, the ants have developed various hygienic behavioral responses. Workers start removing possible pathogens on foraged plant material when it is being transported to the nest (Vieira-Neto et al., 2006; Griffiths and Hughes, 2010) and spread antimicrobial extracts, originating from their metapleural glands, across the fungus during grooming (Poulsen et al., 2002; Fernández-Marin et al., 2006). They also weed out infected mycelium from the underground fungus garden (Currie and Stuart, 2001; Abramowski et al., 2011).

This potentially hazardous material, as well as the exhausted fungus pieces and corpses, are removed to separate waste disposal sites. Waste is deposited either in waste heaps on the soil surface (Navarro and Jaffe, 1985; Rojas, 1989; Hart and Ratnieks, 2002; Herz et al., 2007; Ballari et al., 2007) or in large, underground waste chambers (Stahel and Geijskes, 1939; Stahel and Geijskes, 1941; Amante, 1964; Amante, 1667; Jonkman, 1980b; Bollazzi et al. 2012). The higher mortality of workers in prolonged contact with colony waste, i.e., ants transporting waste or ants working on the heap, demonstrates the pathogenicity of this material (Bot et al., 2001; Lacerda et al., 2010). Preventing workers with contact to waste from entering the colony is thought to be a possible mechanism to hinder pathogen transmission to the colony (Hart and Ratnieks, 2001). Finding a suitable site for waste disposal should therefore affect the state of a colony and its survival.

When searching for appropriate places to dispose of the colony waste, the ants could use cues of their abiotic environment. Indeed *Atta* leaf-cutting ants that excavate underground waste chambers prefer drier conditions for waste disposal as opposed to fungus rearing (Roces and Kleineidam, 2000; Ribeiro and Navas, 2006; also see Chapter 6). Preferring different environmental conditions for the tasks should result in the spatial separation of waste and fungus chambers.

Leaf-cutting ants have well-developed olfactory systems and show a high sensitivity to

odorants (Andryszak et al., 1990). Olfactory information is used in nestmate recognition (Hernández et al., 2002; Richard et al., 2007), marking the nest area (Hölldobler and Wilson, 1986) and orientation during foraging (Kleineidam et al., 2007). It has been suggested that especially in the nest, where no visual cues can be used, ants can still orient themselves along the tunnel system using differences in odor alone (Vilela et al., 1987).

Necrophoric behavior in ants, i.e., the removal of dead workers out of the nest and to the waste heap, also appears to be volatile triggered (Wilson et al., 1958; Howard and Tschinkel, 1976). Workers of the ant *Messor sanctus* relocated and rearranged corpse piles in reaction to a laminar air flow across these piles, suggesting that ants reacted to corpse volatiles within the airflow (Jost et al., 2007). Leaf-cutting ants also seem to detect waste material by chemical, probably volatile cues. Filter paper added with solvent extracts of spent leaf-cutting ant fungus elicits a similar relocation behavior as actual spent fungus pieces in *Atta cephalotes* and *Acromyrmex octospinosus* (Jaffé, 1982).

It was investigated, whether *Atta laevigata* leaf-cutting ants choose a place for waste disposal with the aid of olfactory cues. As disposing of potentially dangerous waste near the symbiotic fungus may threaten the fungus' health, it was evaluated, using choice experiments, whether workers remove waste particles away from a site emitting fungus volatiles and use waste volatiles as a cue where to deposit waste.

7.2 Materials and Methods

(a) Study Animals

The experiments were performed in October 2012 and January 2013 with two laboratory colonies of the leaf-cutting ant *Atta laevigata*. One colony was 7 years, the other 8 years old at the time of the experiments. The ants were reared in a nest consisting of 15 plastic boxes (19x19x9 cm) housing the colony fungus and brood, a bucket for waste disposal and a feeding box, all interconnected by rubber tubing (diameter 2.5 cm). They were provided daily with blackberry (*Prunus fruticosus*) leaves, honey water and water. For each assay, a subcolony, consisting of a fungus filled box, was disconnected from one colony and connected to the experimental setup. At the end of each assay, the box was reconnected to the main nest. Each fungus box was only used once per series.

(b) Experimental Setup

The experimental setup consisted of a triple-layered round experimental tower (diameter 7 cm, total height 7 cm) made out of three clear plastic rings (**Fig. 7.1a**). The top layer contained the experimental arena (height 3 cm), to which the fungus box was later connected, the second layer

two containers for fungus or waste material as sources of volatiles (height 2 cm) and the third, bottom layer two containers for water (height 2 cm). The ring of the experimental arena had an entrance hole of 2.1 cm diameter and a bottom made of a fine plastic mesh. The mesh size was

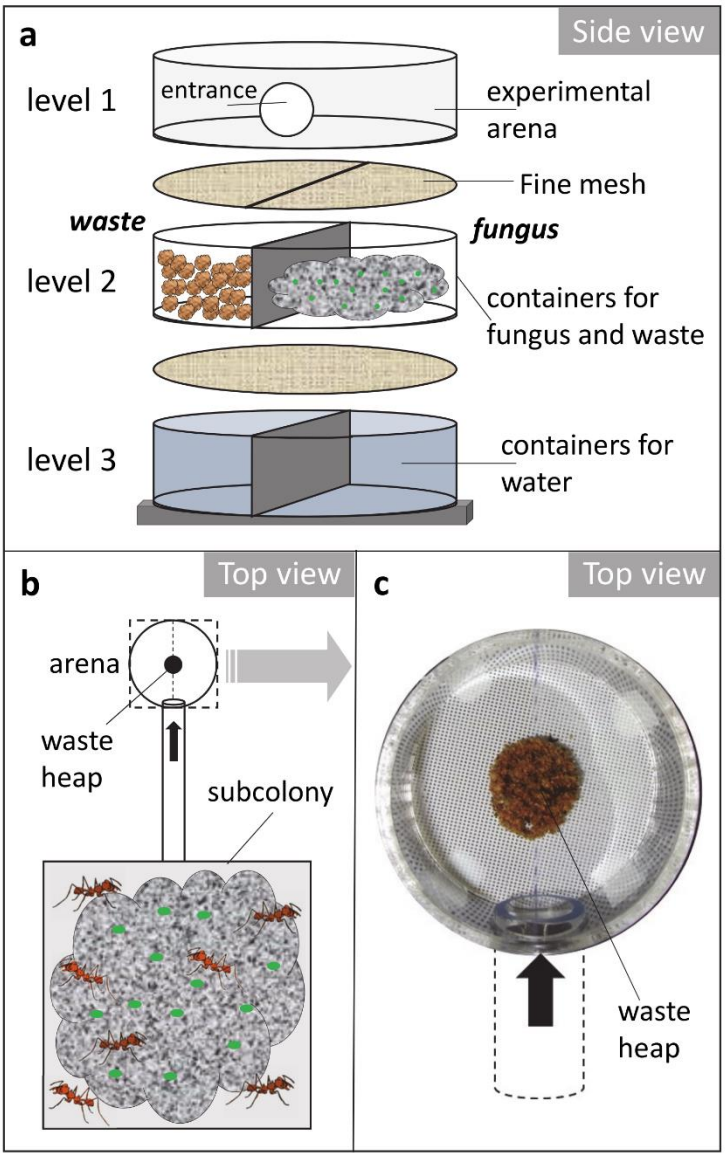

Figure 7.1: Experimental setup **(a)** Side view of experimental tower (diameter 7 cm, height 7 cm), **(b)** top view of subcolony connected to the experimental tower, **(c)** prepared experimental arena with waste heap; black arrows mark the direction of entering ants.

permeable to allow volatiles from this level to reach the experimental arena, yet small enough to prevent workers from passing through. During the assay the arena was covered with a glass plate to prevent ants from escaping. The second level was divided by a plastic wall (height 2 cm) into two equal halves, where fungus or waste material could be deposited as volatile sources. The bottom layer of the tower was filled with water, as symbiotic fungus is very susceptible to desiccation. A fine plastic mesh allowed humid air to reach the upper levels. The water container was also divided into two equal halves by a plastic wall, because it could not be ruled out that small fungus or waste particles would drop through the fine mesh into the

water source and spread across its surface where it could release volatiles. Although it has been demonstrated that leaf-cutting ants prefer a drier environment for waste deposition (Ribeiro and Navas, 2007; see also Chapter 6) the container under the non-fungus half was also filled with water to establish a similar level of air humidity throughout the experimental arena. The floor of the bottom layer was a 7x7 cm plastic square, fixed to the plastic ring.

(c) Experimental Procedure

On the day of the assay, a fungus garden box was disconnected from the laboratory colony and connected to the entrance hole in the experimental tower with a 30 cm long piece of rubber tubing (diameter 2.1 cm; **Fig. 7.1b**). Depending on the volatiles to be tested in the series, the containers in the second layer had been with filled with fungus or colony waste of the same colony (to 2/3 of the container height), or were empty. The fungus was collected from another fungus garden of the colony and had been cleaned of ant brood and garden workers. The waste material had been collected from the refuse bucket shortly before the assay. All live workers present in the waste were removed from the material. With the aid of a round metal ring (diameter 2 cm), a heap of 0.5 g of colony waste was placed in the center of the experimental arena, so that the waste amount was ubiquitously distributed between the arena halves (**Fig. 7.1c**).

Each assay began with the connection of the fungus-filled box to the experimental tower. All workers could explore the setup freely. Despite thousands of workers being presumably present in the fungus garden, based on a one-time count of all workers in a fungus garden box of *Atta colombica*, only a small number of ants, never more than 50, were present in the arena at any given time. The workers could relocate or reorganize the offered waste heap towards one of the sides providing the volatiles for 4 hours, during which some workers also transported waste material (mostly exhausted fungus pieces) out of the fungus garden and placed it on the heap. After 4 hours, the tower was disconnected and all ants were carefully removed from the arena, so that waste particles were not accidentally moved. Then, the waste present in each arena half was collected separately and dried for 24 h at 50°C, as colony refuse can have variable moisture content. The dried waste material was weighed to the nearest 0.1 mg.

Four different series were performed:

(1) Control: no volatiles, both containers empty
(2) Exposure to fungus volatiles: fungus volatiles vs. empty container
(3) Exposure to waste volatiles: waste volatiles vs. empty container
(4) Simultaneous exposure: waste volatiles vs. fungus volatiles

In each series, 24 assays were performed, 12 assays per colony. The sides where the different volatiles were offered were alternated between assays.

Normality of the data was analyzed using the Shapiro-Wilk test. A Wilcoxon matched pair test was used to evaluate differences in waste deposition between both arena halves. To evaluate differences in relocation behavior between the series, separately for either the waste side or the non-waste side, a Mann-Whitney U test was used.

7.3 Results

When no olfactory cues were presented, workers distributed the offered waste heap ubiquitously between both arena halves (**Fig. 7.2a**; Wilcoxon matched pair test: T=143.0, Z=0.2, n=24, p=0.84). However, when comparing the relocation per assay, the waste heap was majorly shifted towards one arena half in 19 of the performed 24 assays (n=8 towards right side, n=11 towards left side).

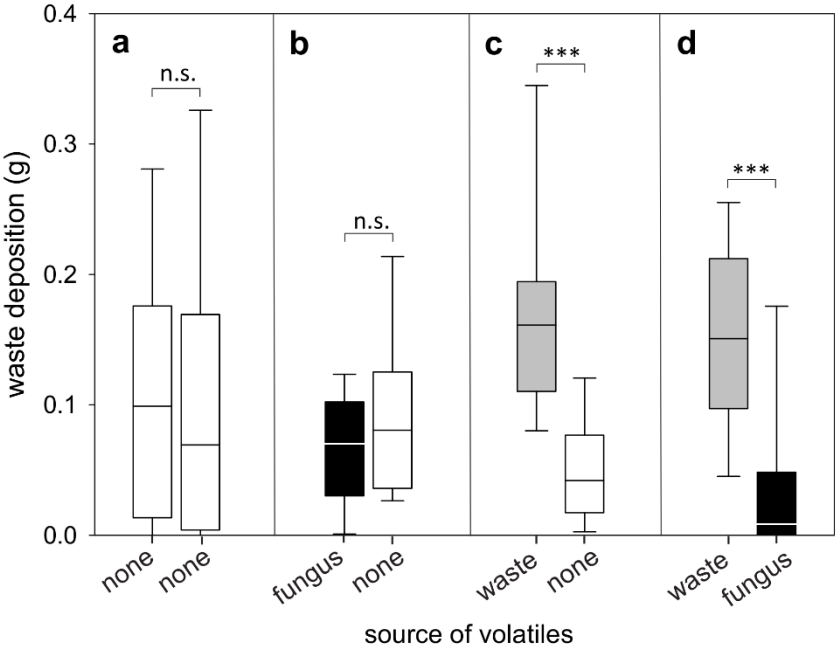

Figure 7.2: Waste deposition in arena half (box colors: white= no volatile source; black= fungus; grey= waste), **(a)** No odor cues. **(b)** Volatile: fungus. **(c)** Volatile: waste. **(d)** Volatiles: waste vs. fungus. ***p<0.001, n.s.= not significant.

When fungus volatiles were present as a possible orientation cue, workers did not remove the part of the waste heap sitting on top of the fungus volatiles and there was no significant difference in the distribution of waste material (**Fig. 7.2b**; Wilcoxon matched pair test: T=114.0, Z=1.03, n=24, p=0.3). Only in 13 of 24 assays was the majority of the waste heap shifted to one arena side (n=9 towards the empty side, n=4 towards the fungus volatile side).

In the presence of waste volatiles as a possible orientation cue, the majority of the waste was relocated towards the side emitting the volatile (**Fig. 7.2c**; Wilcoxon matched pair test: T=5.0, Z=4.14, n=24, p<0.0001). The same relocation of waste material towards waste volatiles

could be observed when both volatiles were offered simultaneously (**Fig. 7.2d**; Wilcoxon matched pair test: T=32.0, Z=3.37, n=24, p<0.001). In 8 of the 24 assays the waste was completely removed from the side releasing the fungus volatiles.

Workers relocating waste from the offered heap did not pick up material and deposited it further away. Rather, a waste particle was usually picked up at one side of the heap and deposited at the opposing edge of the heap, on the side where the preferred volatiles were provided. Sometimes the whole heap was 'moved' sideward across the arena floor until it accumulated against the arena wall.

Using data of the previous series, it was also evaluated whether the magnitude of waste relocation was different when both volatile sources were presented as compared with the presentation of only waste volatiles. Therefore, the amount of waste above the arena half emitting waste volatiles and the one not emitting the waste volatiles was compared separately. The amount of relocated waste at the site emitting waste volatiles did not differ between the two series (**Fig. 7.3a**; Mann-Whitney-U test: U=254, Z=0.70, n=24, p=0.48).

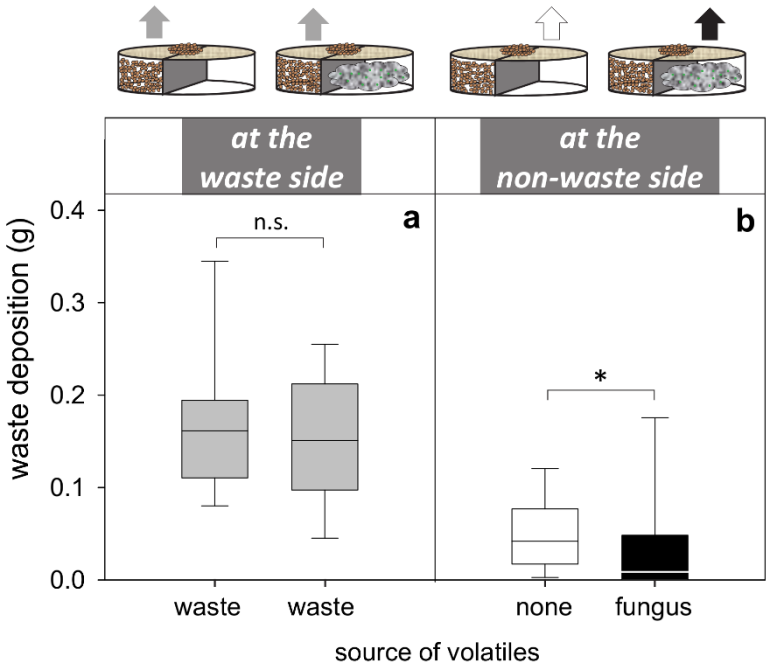

Figure 7.3: Comparisons of the amount of relocated waste in both series with waste volatiles (waste vs. empty and waste vs. fungus), independently for **(a)** the side emitting waste volatiles and **(b)** the side not emitting any waste volatiles (either no volatiles or fungus volatiles). *p≤0.05, n.s.= not significant.

However, less waste particles remained on the side not emitting the waste volatiles when fungus volatiles were presented (**Fig. 7.3b**; Mann-Whitney-U test: U=492, Z=192, n=24, p=0.048).

7.4 Discussion

Our results indicate that leaf-cutting ants use olfactory cues when they choose a site for waste disposal. Workers do not remove a waste heap in the presence of fungus volatiles, yet they

orient their waste deposition towards perceived waste volatiles, i.e., towards a site where waste has already been deposited.

The lack of a removal reaction from above fungus volatiles appears counterintuitive, as waste material could potentially transmit pathogens to the fungus garden (Currie et al., 1999a; Rodrigues, 2008). As a prophylactic behavior, potentially dangerous material should be moved away from sensitive items like the fungus as monoculture or the colony brood. For example, in experiments with the ant *Myrmica rubra*, workers removed ant corpses away from their brood and out of the nest (Diez et al., 2014). The removal behavior resulted in higher survival rates of colony workers and larvae. However, the direct physical contact between workers and larvae was not restricted, so that pathogens could have been transmitted from dead ants to the colony brood. Since the access to the symbiotic fungus was restricted in our experiments, there might have been no threat of pathogen transmission and therefore no waste removal.

While workers did not remove waste particles from above fungus volatiles, they clearly relocated the initial pre-given waste heap towards the site emitting waste volatiles. Waste relocation towards waste was also observed in the leaf-cutting ant *Atta cephalotes*. Workers transporting waste particles out of a laboratory nest rather deposited their loads on an artificially established waste heap directly next to the nest rather than walking a greater distance towards a naturally established heap at the edge of a laboratory table (Jaffé, 1982). Even unlikely, it is not clear whether workers in this study did not also react to visual cues provided by the waste pile. It should not have been the case here, as the main waste mass to emit the volatiles was situated below the fine mesh. Had the workers perceived the small, offered waste heap in the middle of the experimental arena as a visual cue, it should not have been removed from its place in the arena center in any of the experiments. It is therefore proposed that the observed waste relocation of the heap towards other colony waste is based on a stigmergic response mediated by waste volatiles. Stigmergy is known to play an important part in the self-organized nest construction of social insects, by which individuals interact with other colony members through the products of their work (Grassé, 1959; Theraulaz and Bonabeau, 1999). As a result, workers encountering such a stigmergic cue either continue with the same behavior as the previous ants, or perform a different behavior. Workers of *Atta vollenweideri*, for example, deposited excavated soil pellets during nest excavation near other, already deposited pellets within the nest (Pielström and Roces, 2013). The stigmergic response of *A. vollenweideri* could be based on a chemical or olfactory cue by saliva or gland secretions on the pellets. The results suggest the involvement of stigmergy during waste management in leaf-cutting ants.

For a stigmergic relocation of waste to occur, the cue, i.e., a place with accumulated

waste has to be already present. When leaf-cutting ants have to establish a new place for waste disposal, they choose a site based on abiotic stimuli, preferring dry and warm conditions (Ribeiro and Navas, 2006; see also Chapter 6) This should lead to the spatial separation of a waste disposal site and nest sites where fungus is reared, as very humid conditions are preferred for this task (Roces and Kleineidam, 2000). Olfactory cues might be used during the continuing waste disposal, after the sites have been established. The deposition of waste at an already established site, identifiable by the volatiles it emits, could lead to the accumulation of large quantities of waste material at one or only a number of sites within the nest structure. Indeed leaf-cutting ant nests can have thousands of fungus chambers (Moser, 1963; Moreira et al., 2004a, Moreira et al., 2004b), but only a few waste chambers, which are also larger than the fungus chambers, reaching heights of a few meters (Stahel and Geijskes, 1939; Stahel and Geijskes, 1941; Jonkman, 1980a). The more concentrated deposition of waste, either in a few chambers or on a large aboveground waste heap, might also be a prophylactic device against potential disease transmission through the waste.

Worker of an Acromyrmex lundi laboratory colony walking on a wooden bridge

Chapter 8

8. General Discussion

The present studies explored how social and environmental cues influence the workers behavioral responses and how this, aided by mechanisms of self-organization can lead to the building of a nest adapted to support a colony's needs.

The present thesis could experimentally demonstrate that in leaf-cutting ants (1) the placement of brood and fungus triggers the excavation of a new nest chamber, (2) the internal nest architecture is adjusted to the presence of putative chamber items, (3) the nest size is secondarily regulated after the initial excavation by opportunistic refilling of unused space, (4) workers prefer certain CO_2-levels inside the nest for fungus rearing, which is not reflected in workers CO_2-dependent digging behavior, (5) workers prefer environmental conditions for waste disposal different from those chosen for fungus rearing, and (6) workers use olfactory cues during waste management.

Taken together, the experiments demonstrate that while specific nest structures can emerge from self-organized local building responses alone, the final nest architecture aimed at providing suitable conditions for the colony needs only emerges as a consequence of adaptive workers' responses tuned to these local environmental stimuli.

8.1 How to build a nest – the mechanisms of collective nest building in ants

Models have described the process of nest building in social insects as a self-organized one, where workers with a rather simple behavioral repertoire react to local stimuli (Deneubourg and Franks, 1995; Franks and Deneubourg, 1997; Rasse and Deneubourg, 2001), sometimes also aided by templates in the environment or stigmergic cues (Franks and Deneubourg, 1997; Theraulaz and Bonabeau, 1999). Exploring the physical sequence of nest construction is important, as these are the basic mechanisms underlying communal nest building in social insects. However, nests are adaptive structures that provide their inhabiting colonies with proper

conditions for their in-nest tasks. To fully understand nest construction, the adapted behavioral responses to the workers' local environment need also to be quantified.

A group of ants, introduced into a homogenous medium, usually sand or clay, excavates cavities and tunnels, the basic building components of a nest (Buhl et al., 2004; Toffin et al., 2009; Toffin et al., 2010). This is thought to be based on a self-organized building process through density-dependent digging behavior and the existence of response thresholds in the workers (Rasse and Deneubourg, 2001). A worker engages in a certain task when its behavioral threshold for this task is reached (Bonabeau et al., 1998). Some ants will be stimulated to excavate at a site, which in turn stimulates other workers to do the same, amplifying the communal digging process by this positive feedback. A high local ant density leads to the excavation of cavities, as many ants excavate at every possible digging site along the wall of a structure, as demonstrated in Chapters 2 and 3. Nest tunnels also emerge through a self-organized process initiated because the generation of space during digging and the concomitant reduced worker density negatively feeds back on chamber digging. This leads to the lessening of excavation as only a low number of workers are stimulated to dig, so that digging activity is concentrated at discrete, spatially-separated digging sites that develop therefore as tunnels, as presented in Chapter 3.

This could lead to the conclusion that the nest architecture is merely a product of changing ant densities within the nest and their effects on digging behavior. One should take into account that without workers reacting to specific cues, ant aggregation at a certain place within the nest might not necessarily occur, as most ant nests consist of a large number of tunnels along which the colony workforce could disperse. Even environmental gradients in the soil phase, which ants could use as aggregation cues according to their preferences, are usually dispersed across a wider area. An external influence on the system is needed to lead to a proper spatial organization of building behavior, so that the emerging nest is adapted to the colony's needs. One of these external influences seems to be the use of brood as a template, as reported for *Leptothorax tuberointeruptus* ants (Franks and Deneubourg, 1997) or the use of symbiotic fungus in leaf-cutting ants (Fröhle and Roces, 2009). The findings of the present Thesis clearly indicate that the presence of colony brood and fungus cannot only act as a template in the nest environment, but also influences local ant density as brood and fungus strongly attract workers. As a consequence, the local worker aggregation leads to the excavation of chamber-like structures, as demonstrated in Chapter 2. Only in the presence of these social cues did workers not only excavate but also maintain these structures, as evinced in Chapter 3. While the presence of these items leads to a change in nest architecture adapted to the colony's needs, i.e., providing

suitable conditions to house brood and fungus, the mechanism by which this is accomplished is the same that leads to the emergence of non-functional, chamber-like cavities. Brood and fungus influence digging activity only in a way that the positive feedbacks, concentrating workforce at a spot, are stronger and persist over a longer time, indirectly leading to less tunnel excavation. While this underlying mechanism has only been explored in leaf-cutting ants, the same mechanism of density-influencing placement of colony brood could also lead to the excavation and emergence of nest chambers in other, non fungus-growing ants.

Ant nests are complex spatiotemporal structures, which have evolved to offer the colony proper developmental conditions for the colonies offspring and, in leaf-cutting ants, also for the symbiotic fungus. The workers' environmental preferences for the performance of in-nest tasks are also expected to spatially guide building responses, as they should be adapted to the colony's needs through evolutionary processes.

8.2 Where to engage in nest building?

The nest of an ant colony is part of the phenotype of the species. Aside from the morphology of an animal, its behavior as well as the modifications of the environment it causes through its behavior can be seen as an 'extended phenotype' (Dawkins, 1999; Turner, 2000; Minter et al., 2011). Analyses of the nest, its spatial distribution underground and evaluation of the nest climate can render inferences about the species that inhabits it. Vice versa, observation of the environmental preferences for in-nest tasks the colony workers perform should allow for predictions about the nest architecture of a species.

In the present work, the environmental preferences for two in-nest tasks, fungus rearing and waste management, were investigated to draw conclusions about the expected building of fungus and waste chambers in the nests of the species under scrutiny. It had been shown in previous work that the proper in-vitro temperature and humidity values (Quinlan and Cherret, 1978; Powell and Stradling, 1986) correspond very well with the behavioral preferences displayed by workers for fungus rearing (Bollazzi and Roces, 2002; Roces and Kleineidam, 2000), and also match the nest building responses to these stimuli (Bollazzi et al., 2008; Pielström and Roces, 2014). The carbon dioxide gradient in the soil could also be an important cue for building responses, as it negatively affects the respiratory rate of the fungus (Kleineidam and Roces, 2000). The leaf-cutting ant species *A. lundi*, which excavates shallow subterranean nests, does avoid culturing its fungus in a CO_2-environment prevalent at deeper soil layers, while actually preferring levels that coincide with the levels present in superficial soil layers, as demonstrated in Chapter 4. Nevertheless, the expected building responses did not match the observed CO_2-preferences for fungus rearing, as shown in Chapter 5. This might appear

contradictory to the hypothesized nest building responses based on environmental preferences for in-nest tasks. However, the mechanisms by which nest structures emerge have to be taken into account, as presented in Chapter 2. According to the discovered mechanisms for the excavation of a new nest chamber, workers should first deposit relocated fungus pieces according to their preferred CO_2 concentrations for fungus rearing. The resulting increased ant aggregation around these worker-attracting items would then trigger excavation around them. As chambers appear to emerge as a self-organized reaction to the placed items, a graded digging response to varying CO_2-levels might have not evolved. In fact, some of the results presented in Chapters 4 and 6 point to a high CO_2-tolerance of leaf-cutting ants. As observations in Florida harvester ants (*Pogonomyrmex badius,* Tschinkel, 2013) and mangrove dwelling ants (*Polyrhachis sokolova*, Nielsen et al., 2003; *Camponotus anderseni,* Nielsen et al., 2006) suggest, ants living in an environment with increased CO_2-levels appear to be very tolerant to high concentrations of this respiratory gas.

Not much attention has been paid in the literature to waste management as an important factor influencing nest architecture. This is surprising, as underground waste chambers are usually very much larger than the fungus chambers (Stahel and Geijskes, 1939; Amante, 1964; Jonkman, 1980b; Andrade et al., 2005; Bollazzi et al. 2012), and colonies would have to spend a lot of resources and energy to excavate such a structure. Workers prefer certain environmental conditions at the dumpsite, as demonstrated in Chapter 6. The preferred temperature of 25°C, most likely not suitable to hinder pathogen growth in the waste material, might benefit the emergence of these large structures as ants also display their highest digging rates at this temperature (Bollazzi et al., 2008). However, the preferred dry conditions for the dumpsite would probably require excavation in dry soil, which has been shown to be less attractive to workers and to negatively influence digging intensity (Pielström and Roces, 2014). Based on the observed mechanisms of cavity or chamber emergence, as presented in Chapters 2 and 3, it is not expected that workers excavate these large cavities in advance. Workers do not prefer to excavate in a dry environment, so ant aggregation in dry soil layers should be low and such a large cavity should not emerge. High aggregation of ants would be necessary to excavate such voluminous structures. Based on the observations that some nest tunnels were filled with waste in an *Atta texana* nest (Moser, 2006), and that the entrances to waste chambers are usually found on top of these chambers (Jonkman, 1980b), and not at the side of it, as is the case for fungus chambers (Bollazzi et al., 2012), it is likely that these cavities might emerge around waste material, deposited in tunnels leading downwards.

The ants' behavioral responses to volatiles released by the waste suggest that any

subsequent waste disposal would be oriented towards an established site, which should lead to the self-organized accumulation of waste material, as shown in Chapter 7. Waste chambers could therefore be enlarged in successive digging bouts, once the formerly excavated space is occupied by further waste accumulation. Colony waste is not simply dropped and abandoned in these waste chambers, instead a number of workers is present in these chambers and engage in reworking and shifting of the waste heap. When free space in the chamber decreases by fill-up with waste, the increasing density of the workers present on the waste could trigger more digging behavior, analog to the enlargement of a fungus chamber. Enlargement of the chamber might stop when waste deposition comes too close to the fungus garden zone, as this could increase the risk of infecting the colony with pathogens thriving in the waste.

8.3 Outlook

The studies summarized in the present Thesis demonstrated how environmental preferences, adapted through evolutionary processes, guide self-organized building behavior to ensure the emergence of nest structures suited to the species' needs. Leaf-cutting ants, although originating from a tropical climate, have radiated throughout evolutionary times across a wide range of climatic zones in South and North America. The different species-, and sometimes intra-species-, dependent nest architecture might allow for a certain control of the necessary nest climate to successfully rear fungus and brood. However, many species occur in the same habitat, yet display different nest architectures. Evolutionary processes might not only have affected their nests' architecture, but also led to adaptation of both workers as well as their clonal cultivars. This would have aided the dispersal of different species across different habitats as seen in the cold-tolerant fungus of *Atta texana* at the northern periphery of its distributional range (Mueller et al., 2011). The existence of additional adaptations to cope with changing environmental conditions should be investigated, as these would contribute to the understanding of the emergence of different nest types not only in ants, but also in social insects in general.

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Publications

Full Papers

Römer D and Roces F (2014) Nest enlargement in leaf-cutting ants: relocated brood and fungus trigger the excavation of new chambers. *PloS One* ***9****, e97872.*

Römer D and Roces F. Available space, symbiotic fungus and colony brood influence excavation and lead to the regulation of nest size in leaf-cutting ants. *Under review.*

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I confirm that I have obtained permission from both the publishers and the co-authors for legal second publication.

I also confirm my primary supervisor's acceptance.

Daniela Römer

Würzburg, December 16th 2014 Daniela Römer

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Erklärung

Gemäß § 4 Abs. 3 Ziff. 3, 5 und 8

der Promotionsordnung der Fakultät für Biologie der

Bayerischen Julius-Maximilians-Universität Würzburg

Hiermit erkläre ich ehrenwörtlich, die vorliegende Dissertation in allen Teilen selbstständig und nur mit den angegebenen Quellen und Hilfsmitteln angefertigt zu haben.

Die Dissertation wurde bisher weder vollständig noch teilweise einer anderen Hochschule mit dem Ziel einen akademischen Grad zu erwerben, vorgelegt.

Im November 1997 wurde mir von der Universität Würzburg der akademische Grad ‚Diplom-Biologin Univ. verliehen. Des Weiteren verlieh mir die Universität Jena im Juli 2002 den akademischen Grad ‚Master of Environmental Sciences' im Zusammenhang des postgradualen Studienganges
Ecotechnie'. Weitere akademische Grade habe ich weder erworben noch versucht zu erwerben.

Würzburg, den 16.12.2014 *Daniela Römer*