ON THE ROLE OF LOCAL INFORMATION IN THE SPATIAL ORGANISATION OF COLLECTIVE NEST DIGGING IN THE LEAF-CUTTING ANT *ATTA VOLLENWEIDERI* (FOREL, 1893)

A Thesis submitted in partial fulfillment of the requirements for the degree of
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For my family

Aut inveniam viam aut faciam.

— *attributed to Hannibal*

SUMMARY

Many ant species excavate underground nests. One of the most impressive examples is the Chaco leaf-cutting ant *Atta vollenweideri* from the Gran Chaco region in South America. The nests excavated by the workers of that species are among the largest insect-built structures on the planet. They are ecavated over years possibly involving millions of working individuals. However, the mechanisms underlying the organisation of collective nest digging in ants remain largely unknown. Considering the sheer dimensions of the nest in comparison to the size and presumably limited perceptual and cognitive abilities of the single worker, the assumption can be made that organising mechanisms are mostly based on responses of individuals to local stimuli within their perceptual range. Among these local stimuli that guide nest digging we can expect environmental variables, stimuli that relate to the requirements of the colony, and stimuli related to the spatial coordination of collective effort. The present thesis investigates the role of local stimuli from these three categories in the organisation of collective digging behaviour in the Chaco leaf-cutting ant. It describes experiments on (1) how workers respond in the context of digging to differences in soil moisture, which comprises an important environmental variable; (2) how available nest space influences nest enlargement; (3) and how the spatial coordination of excavating workers is implemented by responding to stimuli arising from nest mates while engaged in digging behaviour.

The experiments on soil water content show that workers prefer to dig in moist materials that allow for fast excavation and transport rates. Accordingly, an unequal distribution of water in the soil around a nest can influence how the nest shape develops. On the other hand, results also indicate that workers strongly avoid excavating in extremely moist materials. Regarding the abundant occurrence of flooding events in the Gran Chaco region, the latter can be interpreted as an adaptation to avoid water inflow into the nest.

In the experiments on the effect of nest space, the ants excavated less when presented with larger nests. When a large amount of space was suddenly added to the nest during the digging process, excavation rates decreased according to the new volume. These observations confirm the hypothesis that digging activity is regulated according to space requirements, possibly because crowding conditions inside the nest influence excavation behaviour. However, observations also indicate an intrinsic decrease of digging motivation with time. Moreover, excavation rates correlate with nest size only when comparing nests of similar shape. Distributing a similar nest volume to three

smaller chambers, instead of one, resulted in drastically decreased digging rates. A possible explanation for that observation lies in the distribution of workers inside the nest that may vary according to nest geometry: a different distribution of individuals can lead to in different local crowding conditions in similar nest volumes.

Furthermore, two different stimuli are described that are used in the spatial coordination of collective digging effort. First, fresh soil pellets deposited close to the digging site on their way from the surface increase the probability that arriving workers join excavation efforts at the same site. The deposition of pellets on the way is a consequence of sequential task partitioning during soil transport. The pellets are carried in transport chains that closely resemble the modalities of leaf transport observed at the surface. Second, workers stridulate while digging. The short-ranged vibrational signals produced thereby also attract nest mates to excavate at the same location. Accordingly, two mutually complementing mechanisms are described that allow to concentrate excavators at one location. In both cases, a local stimulus that is generated by current close-by excavation activity increases the probability of the stimulus receiver to dig close to other excavators. In an environment otherwise poor in digging stimuli, these mechanisms can be especially important to give collective digging efforts a common direction. As a consequence it can be argued that the spatial organisation of collective digging is based on choice copying. Individuals copy nest mate decisions on where to excavate by responding to local stimuli provided by nest mate digging activity.

Taken together, responses to local stimuli can determine the direction of nest growth, aid in preventing the inflow of surface water into the nest, guide the adjustment of nest size to colony requirements and spatially coordinate collective digging efforts. Even though it cannot be ruled out that digging responses based e.g. on spatial memory or long-term experience exist, the results presented here clearly demonstrate that responses to local information account for many important aspects of nest development.

ZUSAMMENFASSUNG

Viele Ameisenarten graben unterirdische Nester. Eines der wohl eindrucksvollsten Beispiele ist die Chaco-Blattschneiderameise *Atta vollenweideri* aus der Gran Chaco Region in Südamerika. Die Nester dieser Art gehören zu den größten von Insekten gebauten Strukturen auf unserem Planeten, und an ihrer Konstruktion sind vermutlich Millionen von Individuen über mehrere Jahre beteiligt. Die Mechanismen, die der Organisation des kollektiven Nestgrabeverhaltens zu Grunde liegen, sind weitgehend unbekannt. Berücksichtigt man allerdings allein die Dimensionen des gebauten Nestes im Vergleich zur Größe, und den vermutlich begrenzten Sinnes- und Kognitionsleistungen der einzelnen Arbeiterin, so liegt die Vermutung nahe, daß den Organisationsmechanismen überwiegend Reaktionen auf lokale Reize innerhalb der Wahrnehmungsreichweite zu Grunde liegen. Zu diesen lokalen Reizen gehören vermutlich Umweltvariablen, Reize, die mit den Bedürfnissen der Kolonie zusammenhängen, und Reize, die der räumlichen Koordination gemeinsamer Bemühungen dienen. Die vorliegende Dissertation untersucht die Rolle lokaler Reize in der Organisation des kollektiven Grabeverhaltens bei *Atta vollenweideri* auf diesen drei Ebenen. Sie beschreibt Experimente (1) zur Reaktion grabender Arbeiterinnen auf Unterschiede in der Bodenfeuchte, die in diesem Zusammenhang eine wichtige Umweltvariable darstellt, (2) zum Einfluß der verfügbaren Nestgröße auf die Nesterweiterung und (3) zur räumlichen Koordination grabender Arbeiterinnen durch Reize, die von anderen grabenden Arbeiterinnen ausgehen.

Die Versuche zur Bodenfeuchte zeigen eine Präferenz für feuchte Materialen, die hohe Grabe- und Transportraten ermöglichen. Demzufolge kann die Verteilung des Wassers im Boden um ein Nest erheblichen Einfluß auf die Entwicklung der Nestform haben. Andererseits zeigen die Ergebnisse aber auch, daß Arbeiterinnen vermeiden, in extrem nassen Materialen zu graben. In Anbetracht der regelmäßigen Überflutungen in der Gran Chaco Region lässt sich diese Ablehnung als eine Adaptation interpretieren, die hilft, Wassereinbrüche ins Nest zu vermeiden.

In den Experimenten zum Einfluß der Nestgröße gruben die Ameisen weniger, wenn ihnen ein größeres Nest zur Verfügung stand. Wenn das Nest im Laufe des Grabeprozesses künstlich schlagartig vergrößert wurde, passte sich die Graberate dem neuen Volumen an. Diese Beobachtungen bestätigen die Hypothese, daß die Grabeaktivität abhängig vom Platzbedarf reguliert wird, vermutlich, weil die Individuendichte im Nest das Grabeverhalten beeinflusst. Andererseits zeigen die Beobachtungen aber auch eine zeitabhängige, intrinsische Abnahme der Motivation zu graben. Zudem korreliert die Graberate

nur dann mit der Nestgröße, wenn Nester von vergleichbarer Form in Betracht gezogen werden. Die Verteilung eines ähnlichen Nestvolumens auf drei kleinere Kammern statt einer größeren führte zu deutlich niedrigeren Graberaten. Eine mögliche Erklärung für diese Beobachtung liegt in der Verteilung der Arbeiterinnen im Nestinnern, die möglicherweise mit der Nestgeometrie variiert. Unterschiede in der Verteilung der Individuen können bei gleichem Nestvolumen unterschiedliche lokale Individuendichten zur Folge haben.

Des weiteren werden zwei verschiedene Stimuli beschrieben, die zur räumlichen Koordination des kollektiven Grabeverhaltens genutzt werden. Zum einen erhöht sich die Wahrscheinlichkeit, daß eine ankommende Arbeiterin sich den Grabearbeiten an einer bestimmten Stelle anschließt, wenn auf dem Weg dorthin, nahe der betreffenden Stelle, frisch ausgegrabene Bodenpartikel, sogenannte *pellets*, abgelegt wurden. Das passiert in Folge der sequentiellen Arbeitsteilung beim Transportieren der Erde, denn der Transport der *pellets* erfolgt in Arbeitsketten, ähnlich wie der Transport von Blattfragmenten an der Oberfläche. Zum anderen stridulieren die Arbeiterinnen beim Graben und erzeugen dadurch Vibrationssignale von kurzer Reichweite, die ebenfalls Nestgenossinnen zum Graben an derselben Stelle motivieren. Es werden also zwei sich gegenseitig ergänzende Mechanismen beschrieben, die es erlauben, grabende Arbeiterinnen an einer bestimmten Stelle zu konzentrieren. In beiden Fällen erhöht ein Reiz, der durch aktuelle Grabeaktivität in unmittelbarer Nähe generiert wird, die Wahrscheinlichkeit, daß der Reizempfänger in der Nähe anderer grabender Arbeiterinnen zu Graben beginnt. Besonders in einem ansonsten, in Bezug auf das Grabeverhalten reizarmen Umfeld können derartige Mechanismen wichtig sein, um den kollektiven Grabebemühungen eine gemeinsame Richtung zu geben. Dementsprechend lässt sich argumentieren, daß die räumliche Organisation des Grabeverhaltens auf der Nachahmung von Entscheidungen basiert. Individuen kopieren die Entscheidungen ihrer Nestgenossinnen, wo gegraben wird, indem sie auf Reize reagieren, die von der Grabeaktivität anderer Arbeiterinnen ausgehen.

Lokale Reize können also die Richtung des Nestwachstums bestimmen, zur Vermeidung von Wassereinbrüchen ins Nest beitragen, die Anpassung der Nestgröße an die Koloniegröße steuern und das kollektive Grabeverhalten der Kolonie räumlich koordinieren. Wenngleich auch ein Einfluß von räumlichem Gedächtnis oder längerfristiger Erfahrung nicht ausgeschlossen werden kann, zeigen die hier präsentierten Ergebnisse eindeutig, daß Antworten auf lokale Information für viele wichtige Aspekte der Nestentwicklung verantwortlich sind.

RESUMEN

Muchas especies de hormigas excavan nidos subterráneos. Uno de los ejemplos más impresionantes es el de la hormiga cortadora de hoja del Chaco, *Atta vollenweideri*, que habita en la región del Gran Chaco en Sudamérica. Los hormigueros excavados por las obreras de esta especie son unas de las estructuras construidas por insectos mas grandes del mundo. Se cree que su construcción requiere del trabajo de millones de individuos. Sin mebargo los mecanismos que subyacen la organización de la excavación de estos nidos siguen siendo en gran parte desconocidos. En vista de las dimensiones del nido en comparación con el tamaño y presumiblemente limitadas capacidades perceptivas y cognitivas de las obreras individuales, hace suponer que los mecanismos de dicha organización se basan principalmente en respuestas ante estímulos locales que se encuentran dentro del rango de percepción de los individuos. Entre los estímulos locales que guía la excavación del hormiguero podemos esperar encontrar variables ambientales, estímulos relacionados con los requisitos de la colonia y otros relacionados con la coordinación espacial del esfuerzo colectivo. Esta tesis investiga el rol que juegan los estímulos locales de estas tres categorías en la organización de la excavación colectiva en *Atta vollenweideri*. La tesis describe experimentos en los que se investiga (1) cómo las obreras responden a diferencias en la humedad del suelo, que representa una variable ambiental importante; (2) cómo el espacio disponible en el nido influye su ampliación; (3) y cómo la coordinación espacial de las obreras surge como respuesta a estímulos relacionados con la actividad de excavación de otras compañeras de nido.

Los experimentos sobre el contenido de agua del suelo muestran que las obreras prefieren excavar en materiales húmedos que permiten tasas de excavación y transporte altas. En consecuencia, los patrones de distribución de agua en el suelo, alrededor del hormiguero, pueden influir el desarrollo de la forma del nido. Por otro lado, los resultados indican que las obreras evitan excavar en materiales extremadamente húmedos. Dados los frecuentes eventos de inundación en el Gran Chaco, este comportamiento se puede interpretar como una adaptación para evitar la entrada de agua al nido.

En los experimentos sobre el efecto de la disponibilidad de espacio, las hormigas excavaron menos al presentarse en nidos más grandes. Cuando una gran cantidad de espacio se añadió repentinamente al nido durante el proceso de excavación, la tasa de excavación disminuyó de acuerdo con el nuevo volumen ofrecido. Estas observaciones confirman la hipótesis de que la actividad excavadora se regula de acuerdo con los requisitos espaciales, posiblemente debido a que

las condiciones de hacinamiento generadas en el interior del nido influencian el comportamiento de excavación. Sin embargo, las observaciones también indican una disminución intrínseca de la motivación por excavar con el tiempo. Por otra parte, la tasa de excavación se correlaciona con el tamaño del nido sólo al comparar nidos de formas similares. La distribución del volumen de un nido de una unica cámara en uno de tres cámaras más pequeñas, se tradujo en una disminución drástica de la tasa de excavación. Una posible explicación para esta observación es la distribución diferencial de las obreras en el interior del nido de acuerdo a su geometría: una distribución diferente de los individuos puede conducir a condiciones de hacinamiento locales distintas en volúmenes similares.

Además, otros dos estímulos se describen que son utilizados en la coordinación espacial del esfuerzo de excavación colectiva. En primer lugar, las particulas de suelo frescas, excavadas y depositadas cerca del sitio de excavación en su camino desde la superficie, aumentan la probabilidad de que una obrera comience a excavar en el mismo sitio. La deposición de particulas en el camino es una consecuencia de la partición tarea secuencial durante el transporte de suelo. En este sentido, el transporte del suelo se asemeja estrechamente a las modalidades de transporte de la hoja que se observan en la superficie. En segundo lugar, las hormigas obreras estridulan durante la excavación. Estas señales vibratorias de corto alcance atraen a otras obreras para excavar en el mismo lugar. Mutuamente complementarios, estos dos mecanismos le permiten a las excavadoras concentrarse en un lugar determinado. En ambos casos, un estímulo local que se genera por la misma actividad de excavación, aumenta la probabilidad de que el receptor del estímulo excave cerca de otras excavadoras. En una situación carente de otros estímulos que induzacan la excavación, estos mecanismos pueden ser especialmente importante para guiar los esfuerzos de excavación colectivos una dirección común. Como consecuencia se puede afirmar que la organización espacial de la excavación colectiva se basa en la coordinación por medio copia de decisiones. Individuos que copian las decisiones de otros respecto a donde excavar, responden a estímulos locales proporcionados por la actividad de excavación misma.

En conjunto, las respuestas a los estímulos locales pueden determinar la dirección en la que el hormiguero se amplia, contribuir a evitar la entrada de agua superficial, guiar el ajuste del tamaño del nido según los requisitos de espacio de la colonia, y espacialmente coordinar los esfuerzos colectivos de excavación. Apesar de que no se puede descartar a existencia de respuestas de excavación basadas por ejemplo, en memorias espaciales o en experiencias a largo plazo, los resultados presentados aquí demuestran claramente que las respuestas a información local son relevantes en muchos aspectos importantes del desarrollo del hormiguero.

PUBLICATIONS

This thesis is based on the following manuscripts:

- S. Pielström and F. Roces. Vibrational communication in the spatial organization of collective digging in the leaf-cutting ant *Atta vollenweideri*. *Animal Behaviour* 84:743–752, 2012.
- S. Pielström and F. Roces. Sequential soil transport and its influence on the spatial organisation of collective digging in leaf-cutting ants. *PLoS ONE* 8: e57040, 2013.
- S. Pielström and F. Roces. Soil moisture and excavation behaviour in the Chaco leaf-cutting ant (*Atta vollenweideri*): digging performance and prevention of water inflow into the nest. (Submitted)
- S. Pielström and F. Roces. Investigations on the regulation of nest enlargement in the leaf-cutting ant *Atta vollenweideri*. (In preparation)

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Part I

REVIEW

1

UNFORESIGHTFUL ARCHITECTS

Considering the size of their builders, underground ant nests can reach remarkable dimensions. In the earliest comprehensive work written on ant behaviour, the *Recherches sur les Moeurs des Fourmis Indigènes*, the Swiss entomologist Pierre Huber stated in the very first sentence of his first chapter:

> *"Le premier objet qui frappe nos sens en commençant à étudier les moeurs des fourmis, c'est l'art avec lequel elles construisent leur habitation, dont la grandeur paroît souvent contraster avec leur petitesse ;"*
>
> "The first thing that astonishes our senses, when beginning to study the habits of ants, is the skill with which they build their dwellings, the magnitude of which appears often to contrast with their puniness;"
>
> — *Huber (1810, p. 17)*

Yet, in all his astonishment about that contrast, Huber did not even know of the largest ant colonies and their constructions. The nests of the *Atta* leaf-cutting ants are, along with those of some mound-building termites, the largest constructions built by insects. In a colony of the Chaco leaf-cutting ant, *Atta vollenweideri* (Forel, 1893), an estimated 9.5 metric tons of material are excavated in the underground to create chambers and tunnels, and heaped up at the surface to constitute a nest mound (Jonkman, 1980; Jonkman, 1980). In terms of the mass of relocated building material, that nest is 3 billion times larger than a single one of the organisms building and inhabiting it. Thus, if we imagined a hypothetical horror film featuring mutated leaf-cutting ants as large as a human being, and if we simply ignored all effects of scaling and allometry, the nests of these film ants would have to be 30 times larger than Khufu's Great Pyramid at Gizeh[1]. And this achievement is not the exceptional deed of some ancient god king and his subjects, to be remembered for millennia, it is, rather, just the ordinary life style of these ants, emerging wherever a colony of this species appears.

However, it is not only the extreme dimensions of the construction

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[1] A single worker weighs about 3 mg on average, as opposed to 9.5 metric tons of building material, giving a ratio of 1:3 billion. In comparison, when king Khufu had his people build the Great Pyramid, about 6 million metric tons of material were moved (Romer, 2007, p. 157). Estimating the body mass of the average Egyptian peasant in the early bronze age to be about 60 kg, we can calculate a building-to-organism mass ratio of 1:100 million.

that makes this feat remarkable, but also the limitations of the individual builders' abilities that calls for totally different way of coordinating collective effort. In human construction projects coordination of individual workers is based on the foresight of planning individuals. They define in advance what the construction is supposed to look like and then precisely communicate their plan to other workers who themselves can foresee how their actions will contribute to the envisaged outcome. In social insects however, we have to assume that things work completely different. It seems very unlikely that individuals are able to foresee the future state of their construction, or even to oversee its current state. Considering the gigantic dimensions of the nest relative to an individual, *Atta vollenweideri* is probably a good example for Hansell's notion that

> "[...] for many builders, the completed structure extends well beyond the organisms sensory range."
>
> — *Hansell (2005, p. 98)*

But how is it possible then for millions of individuals to collectively create a giant, coherent structure with functionally differentiated substructures with no plan, no foresight, and not even much knowledge about current outline of the nest? The key to understanding the nature of ant nests is, in my opinion, to analyse the requirements that drive building responses, and to identify the behavioural mechanisms that satisfy those particular requirements. In doing this, one especially has to regard one specific aspect of building behaviour that applies to social insects. Many animal constructions, like a bird nest or spider web, are first built, and then they serve their function. Neither are of much use until completed and there is little development of size and shape after their builders have started to use them for their main purpose (Hansell, 2005). That is not true for an ant nest. Once the founding chamber is completed, there is no stage in further nest development that can be considered "complete" or "incomplete" from the inhabitants' point of view. When we look at an ant nest during a particular stage of the colony life cycle, we do not see a "complete construction", but the current results of the adjustments made so far in response to changing conditions. These adjustments probably take place as long as the nest exists. Thus, rather than being a comprehensive construction, the ant nest can be seen as the accumulated remnants of behavioural responses to requirements that changed over time.

A rough outline of these requirements and the nature of the responses can be made if considering that the number of requirements, as one would expect, grows with increasing social complexity during evolution (Tab. 1). Being considered by some authors as the current climax of social evolution in Hymenoptera (Hölldobler and Wilson, 2009, 407), the *Atta* leaf-cutting ants can be expected to have a great

Table 1: Rough systematisation of hypothetical steps in the evolution of collective nest building and related aspects of building behaviour.

EVOLUTIONARY STEP	BEHAVIOURAL ASPECT
Short-term nests	Responses to environmental templates
Long-lasting nests	Regulatory responses to the environment
Evolution of sociality	Regulatory responses to social variables
	Coordination of individual responses

range of behavioural adaptations related to building behaviour.

Even in a solitary species that only uses a simple nest temporarily to rear a single generation of brood, we can expect that builders have to respond in some way to environmental templates, e.g. by choosing a particular type of soil for excavation[2]. There are various examples on how solitary wasps choose a location to build an underground nest according to environmental factors (Evans, 1966). Ants, even though they use their nests collectively and for extended periods, reportedly still rely on environmental templates. Known examples include the commonly observed excavation of tunnels along roots or chambers below flat stones, thereby increasing the stability of the structures. In some ant species, workers reportedly dig along the planes in between soil layers (Minter et al., 2012); in others, they prefer to excavate in soils with a particularly high moisture content (Thomé, 1972). Regarding the environment during nest building can serve multiple functions: it can be advantageous for the building process itself, e.g. by selecting a material that is particularly easy to manipulate; the builder can choose a location particularly well suited to the protection of the later inhabitants against predators or parasites; and it can choose a location that promises to provide a particularly suitable micro environment, i.e. one that reduces the variation of environmental conditions in a way that is beneficial for the inhabitant. The latter is possibly one of the most important evolutionary benefits of building behaviour (Hansell, 2005).

Using the same nest for extended periods of time would be expected to come with more variation in environmental conditions around and inside the construction. For example, the full annual temperature variation is far larger than that of a day or a month. On the other hand, if the builder stays in that nest, it can make subsequent adjustments of nest architecture to new environmental conditions, e.g. by excavating deeper when the temperature close to the surface becomes too hot or too cold. Such behaviour can be described as a regulatory response: it is triggered by an unfavourable change of the

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[2] Even if the builder works without reference to environmental templates most of the time, it must at least rely on them when initially choosing a location to start the work.

environment inside the nest, but as soon as that change has been compensated for, it would not be adaptive to continue the response behaviour any more. The manipulation of the environment through a building response gradually eliminates the stimulus that initially triggered it. An example for such a feedback mechanism can be seen in the regulation of air humidity inside the nests of grass-cutting ants (Bollazzi and Roces, 2007, 2010), but similar mechanisms can be imagined to exist as well in solitary nest builders, as long as they use their constructions for extended periods.

A social lifestyle creates in itself additional requirements on building behaviour. Division of labour creates a situation where it is no longer sufficient for builders to respond only to their own needs. The activity of the builders has to account for the needs of the other individuals too. Thus, it becomes necessary for nest building to also be performed as a response to social variables, i.e. to stimuli that relate to the needs of nest mates[3]. For example, if the offspring is reared in brood cells, then non-reproductive builders of these cells are required to build them at a rate corresponding to the current fecundity of the reproductive individuals. Laboratory experiments suggest that ants adjust their nest to colony population (Rassé and Deneubourg, 2001) and, in leaf-cutting ants, to the space requirements of the symbiotic fungus (Fröhle, 2009; Fröhle and Roces, 2009). Both are examples for regulative responses to social variables.

Additionally, social conditions facilitate the development of coordination among the individuals. Besides meeting the needs of their nest mates, workers are now able to respond to the building behaviour of nest mates, which allows for the temporal and/or spatial coordination of individual efforts. Coordinated behaviour, such as digging at the same location to create a new chamber instead of several individuals each excavating a chamber on their own, can be adaptive, as it allows the creation of a functional result more rapidly. Additionally, interactions among building individuals can hypothetically result in emergent properties of the structure, i.e. in properties that cannot be created by single individuals, no matter how long they work. Little evidence exists on coordinating mechanisms in excavation behaviour in ants. Some observations suggest that workers respond to pheromones when deciding where to excavate (Wilson, 1958; Blum and Warter, 1966), and theoretical considerations indicate that at least some kind of recruitment mechanism may be used in the spatial organisation of collective digging (Deneubourg and Franks, 1995). However, empirical knowledge about the organising mechanisms remains sparse.

In many other examples for nest building in social insects the coor-

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[3] I will use the term "environmental variable", if it is potentially important for both social and solitary builders, "social variable", if it is exclusively important for social species.

dination of collective building can be understood in terms of self-organisation (Camazine et al., 2001; Hansell, 2005). Self-organisation in social insects is commonly understood as

> "[...] a set of dynamical mechanisms whereby structures appear at the global level of a system from the interactions among its lower-level components. The rules specifying the interactions among the system's constituent units are executed on the basis of purely local information, without reference to the global pattern, which is an emergent property of the system, rather than a property imposed upon the system by an external ordering influence."
>
> — *Bonabeau et al. (1997)*

In underground ant nests, self-organisation has been suggested as one of the main mechanisms accounting for the emergence of observable patterns (Deneubourg and Franks, 1995; Toffin et al., 2009). Self-organised processes feature multistability, i.e. they have multiple, equally probable outcomes (Bonabeau et al., 1997; Nicolis and Prigogine, 1977). Accordingly, if a structure is built in a self-organised process, the precise result is by definition unforeseeable. Thus, foresight is no prerequisite for self-organised building. Another aspect of the definition that makes the concept particularly interesting for explaining the emergence of a giant underground nest from the actions of millions of tiny ants, is the assumption that the acting units act only on the basis of local information. It has been shown that even solitary species often rely on local stimuli when building structures, thereby circumventing the need to assess the current state of the construction in order to know what to do next (Hansell, 2005, p. 110).
Accordingly, we can expect that workers of the Chaco leaf-cutting ant perform excavation behaviour in response to local stimuli in three different ways: (1) As a response to environmental templates, (2) as a regulatory response to environmental and social variables that change during nest development, and (3) in order to coordinate their behaviour with the activity of other digging workers. With the present work, I intend to contribute to the understanding of nest architecture and ontogeny in *Atta vollenweideri* by investigating the role of local stimuli in worker excavation behaviour considering all three points. As an introduction to the research field, I will give a short outline of the research history and current knowledge on underground nest building in ants and on the biology of the Chaco leaf-cutting ant in Chapters 2 and 3 respectively. The empirical part of the work will begin in Chapter 4 with a descriptive outline of preliminary observations that are the basis for the experimental designs described later. Chapter 5 is aimed at investigating the use of soil moisture as an environmental template and examines possible adaptive benefits. In

Chapter 6 I will move on to social variables, and analyse the role of available nest space on digging rates. The least well known aspect of collective nest digging, the spatial and temporal coordination of individuals, will be the topic of Chapters 7 and 8. Here, I will describe how digging decisions are influenced by stimuli that indicate nest mate digging activity. Finally, in Chapter 9, the reported results will be discussed in a larger context in order to examine how they can help our understanding the of nest ontogeny as the cumulative result of local responses to different local stimuli, and how a large and complex nest can be built in this way without any foresight on behalf of the builders.

2

ANT ARCHITECTURE AND BUILDING BEHAVIOUR: AN OVERVIEW WITH A FOCUS ON LEAF-CUTTING ANTS

The vast majority of ant species construct nests that are at least partially subterranean (Sudd, 1969; Hölldobler and Wilson, 1990). In contrast to the commonness of underground nesting in ants, comparatively few studies provide accurate descriptions of underground architecture and excavation behaviour in ants. As I am writing this text, there are 12,762 scientifically described ant species according to the taxonomic database of the Ohio State University[1]. On the other hand, reliable reports on nest architecture that are based on the excavation of entire nests and go beyond qualitative and often idealised descriptions are available for only about 30 of these species in the scientific literature[2] (Tab. 2). This is possibly due to the fact that both the structures, and the behaviours generating them, exist underground and hidden from the observers' eye. The study of field nest structure through excavation is a destructive and remarkably laborious task, and the observation of excavation behaviour is, with some exceptions, almost impossible in the field. In this chapter I will describe the current knowledge about the internal structure of subterranean ant nests and the excavation behaviour of ant workers.

2.1 THE INTERNAL STRUCTURE OF UNDERGROUND ANT NESTS

Research history

In the early literature the architecture of subterranean ant nests was described in terms of general impressions and idealised patterns (Huber, 1810; Wheeler, 1910). The knowledge was based on more or less accidental observations, rather than on planned excavations of nests with the explicit intention to examine their internal structure (Tschinkel, 2003). If architectural features were studied intentionally, nest were only partially opened in order to examine particular questions outside the context of nest building behaviour. One early examples of a more detailed report of observations made during the intentional excavation of ant nests is the work of Moggridge (1873, p. 21), who first described the granaries in the nests of the seed-harvesting ant

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[1] For current numbers, see `http://osuc.biosci.ohio-state.edu/hymenoptera/tsa.sppcount?the_taxon=Formicidae`

[2] Admittedly, there are probably more studies that I was unable find. The given number is based on what is available to me, and roughly represents the scope of studies that is regarded in modern literature.

Table 2: Thorough descriptions of underground ant architecture in the scientific literature.

SPECIES	COUNTRY*	REFERENCE
Acromyrmex subterraneus	Brazil (11)	Santos Lopes et al. (2011)
Acromyrmex rugosus	Brazil (12)	Verza et al. (2007)
Aphaenogaster ashmeadi	USA (12)	Tschinkel (2011)
Aphaenogaster floridana	USA (25)	Tschinkel (2011)
Aphaenogaster treatae	USA (8)	Tschinkel (2011)
Atta bisphaerica	Brazil (6)	Moreira et al. (2004b)
Atta cephalotes	Suriname (7)	Stahel and Geijskes (1939, 1941)
	Peru (3)	Weyrauch (1942)
Atta laevigata	Brazil (3)	Moreira et al. (2004a)
Atta sexdens	Suriname (3)	Stahel and Geijskes (1939, 1941)
	Peru (3)	Weyrauch (1942)
	Brazil (1)	Autuori (1942)
Atta texana	USA (1)	Moser (2006)
Atta vollenweideri	Paraguay (6)	Jonkman (1980)
Camponotus socius	USA (24)	Tschinkel (2005)
Camponotus xerxes	Kasakhstan (1)	Dlussky (1974)
Cataglyphis flavigastra	Kasakhstan (2)	Dlussky (1974)
Cataglyphis foreli	Kasakhstan (1)	Dlussky (1974)
Cataglyphis pallida	Kasakhstan (4)	Dlussky (1974)
Cataglyphis setipes	Kasakhstan (2)	Dlussky (1974)
Formica pallidefulva	USA (25)	Mikheyev and Tschinkel (2004)
Lasius neoniger	USA (6)	Wang et al. (1995)
Messor aralocaspius	Kasakhstan (2)	Dlussky (1974)
Messor arenarius	Algeria (1)	Délye (1971)
Messor ebenius	Lebanon (23)	Thomé (1972)
Messor variabilis	Kasakhstan (1)	Dlussky (1974)
Monomorium barbatulum	Kasakhstan (1)	Dlussky (1974)
Monomorium gracillimum	Kasakhstan (1)	Dlussky (1974)
Odontomachus brunneus	USA (17)	Cerquera and Tschinkel (2010)
Pheidole morrissi	USA (2)	Tschinkel (2003)
Pheidole oxyops	Brazil (7)	Forti et al. (2007)
Pogonomyrmex badius	USA (57)	Tschinkel (1999, 2004)
Prenolepis imparis	USA (24)	Tschinkel (1987)
Solenopsis invicta	USA (11)	Cassill et al. (2002)
Tetramorium inerme	Kasakhstan (1)	Dlussky (1974)
Tetramorium schneideri	Kasakhstan (1)	Dlussky (1974)

*Number of excavated nests indicated in parentheses.

Messor barbarus[3]. He thereby finally confirmed the long discussed hypothesis that these ants feed on seeds and store supplies of them over prolonged periods. It was about the same time that Belt (1874, p. 64) gave the first account of fungus culturing in chambers of the nests of *Atta*[4] leaf-cutting ants. His description of the nest however, was again a very idealised one.

It was also the genus *Atta* that was the subject of the first real systematic studies on the internal nest structure itself. In this genus, though being notorious for building extremely large nests that are particularly laborious to excavate, studies on nest architecture were for the first time conducted as meticulously as an archaeological excavation. The main reason may be found in the role these ants have as an agricultural pest in many parts of Latin America. Accordingly, there has always been a strong interest in finding new methods to destroy their nests. Albeit that the appropriateness of destroying *Atta* nests for pest control can be questioned from the conservational point of view, after all *Atta* ants are considered as keystone species in many ecosystems (Fowler et al., 1989), the desire to destroy them resulted in extensive studies on *Atta* nests and produced the first comprehensive qualitative and quantitative descriptions of underground architecture in ants.

The first scientists that tried to produce a thorough description of *Atta* architecture were Eidmann (1932, 1935) and Jacoby (1935, 1936, 1937, 1953, 1955, 1960). Relying on the method of casting *Atta* nests with concrete before excavation to preserve the internal structure during the procedure, these authors concentrated on qualitative descriptions of the structures and their interconnections. This cement-casting method has also been employed by other authors (Moreira et al., 2004a,b; Moser, 2006). In other studies researchers preferred to excavate the nest without casting it in cement, in order examine the content of the particular cavities. They rather relied on creating several consecutive cross sections of the nest beginning from a trench beside it, and documenting the internal features by drawing them on a scaled grid (Stahel and Geijskes, 1939, 1941; Autuori, 1942; Weyrauch, 1942; Jonkman, 1980).

During the 1970's, publications appeared on the internal nest structure in desert-dwelling ant species (Délye, 1971; Thomé, 1972; Dlussky, 1974). Here, the description of both methodology and observations were generally less sophisticated than in the formerly mentioned studies on *Atta*, indicating that the authors who wrote in French and

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[3] Throughout his text, Moggridge refers to *Messor barbarus* as *Atta barbara*, a classification that is now outdated.

[4] Belt calls the genus *Oecodoma* which is a junior synonym of *Atta* (Roger 1863) that is no longer in use. He refers to no particular species, and in his days the *Acromyrmex* species were included in the genus *Atta* until finally raised to genus by Emery in 1913. Nevertheless, Belt describes leaf-cutting ants with large nest mounts, which must be interpreted as a description of a true *Atta* species.

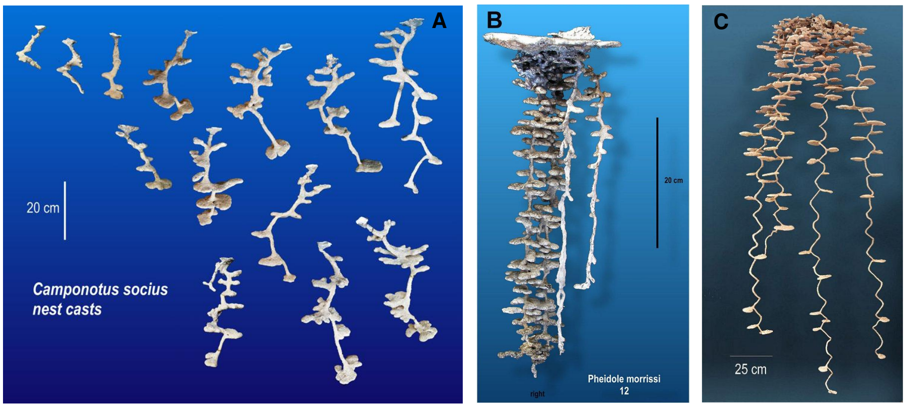

Figure 1: Nest casts made by Walter Tschinkel. (A) *Camponotus socius*, originally published by Tschinkel (2005). (B) *Pheidole morrissi*, originally published by Tschinkel (2010). (C) *Pogonomyrmex badius*, originally published by Tschinkel (2004). The orginal figures have been published *open access* under *Creative Commons Attribution* 3.0 licence.

Russian may have been unaware at that time about the published work on *Atta* nests, which was written mostly in German, Spanish or Portuguese. They mostly relied on scaled drawings of entirely excavated nests that are presumably idealised by projecting their three-dimensional structure onto a single two-dimensional plane. The described nests include species from the genera *Messor*, *Camponotus*, *Cataglyphis*, *Monomorium* and *Tetramorium*.

Underground ant architecture as a research field was revived by Tschinkel (1987), who was the first to systematically study the nests of an ant species from North America, and authored or co-authored several publications on the nests of ant species from Florida thereafter (Tschinkel, 1993, 2004, 2005, 2011; Cassill et al., 2002; Mikheyev and Tschinkel, 2004; Cerquera and Tschinkel, 2010). In contrast to former studies, he examined not only the internal structure in different stages of nest development, but also the sizes of the single cavities, the distribution of nest space along the depth gradient and the distribution of workers and brood inside the nest. A year later, Williams and Lofgren (1988) described a method of casting ant nests with dental plaster. In the years since then, the nest architectures of several ant species have been studied at great detail, and a range of alternative nest casting methods has been developed and described by Tschinkel (2010). Field studies during the past two decades concentrated on species from the Sao Paulo state, Brazil, and from Florida, USA[5].

General patterns

According to Wheeler (1910, p. 199), the subterranean structures excavated by ants (for examples, see Fig. 1A-C) during nest building can be generally divided into two categories: chambers, and galleries or tunnels; the former being more spacious and flat on the floor, whereas the latter are more or less tubular. However, distinction according to these idealised morphological features is not always that easy. Especially when built horizontally, tunnels can be relatively flat and broad rather than tubular (for examples see photographs in Moreira et al., 2004a,b). Chambers on the other hand can themselves vary considerably in terms of size and shape. One other possibility for discrimination between the two types of structures is the way they are used. Chambers are generally used to store brood, and, in some special cases, fungus gardens or seed supplies. Queens usually spend most of their time in a chamber, so we can say that chambers are the locations in the nest were we find most of the temporarily stationary assets of the colony. Tunnels apparently serve as connections between chambers, and to the surface. Their main purpose is to allow free movement, placing stationary assets in a tunnel would obstruct movements and therefore be not adaptive, especially in large colonies with high traffic rates. So one can possibly make a functional distinction simply by saying that chambers are the locations where things stay, while tunnels are the parts of the nest where things move[6].

A very common pattern found in many ant species is a nest composed of a single, more or less vertical tunnel connecting the nest to the surface through a single nest opening, with several chambers built alongside it (e.g. Tschinkel, 1987, 2004, 2011; Mikheyev and Tschinkel, 2004; Forti et al., 2007). Wheeler stated that:

> "In several species, the chambers have the appearance of being strung along a single vertical gallery like beads on a thread."
>
> — *Wheeler (1910, p. 199)*

Tschinkel (2003) introduced the term *shish-kebob* unit to describe such a structure, and stated in compliance with Dlussky (1974) that most subterranean ant nest can be described as being a single, or an accumulation of several such units. For example, in the Florida harvester ant *Pogonomyrmex badius*, young nests feature a single vertical unit, while a maximum of four units was reported the largest nests (Tschinkel, 2004). The vertical distribution of nest volume is top

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[5] The only exemption is the study by Wang et al. (1995) on *Lasius neoniger* from Wisconsin, USA.

[6] From that perspective, one could hypothesise that the nests of larger colonies with higher traffic rates show a clearer morphological differentiation between tunnels and chambers.

heavy in many ant species, i.e. they have more chambers, or larger chamber close to the surface (e.g. Wang et al., 1995; Tschinkel, 1999; Mikheyev and Tschinkel, 2004). In *Pogonomyrmex badius*, the vertical shafts are typically helical and chambers project laterally from one point at the outside of the tunnel (Fig. 1c). In other species, tunnels can be more irregularly inclined, as for example in *Camponotus socius* (Fig. 1a Tschinkel, 2005), or more or less straight, as for example in *Pheidole oxyops* (Forti et al., 2007), *Pheidole morrissi* (Tschinkel, 2010), or *Aphenogaster floridana* (Tschinkel, 2011). In *Aphaenogaster floridana*, the internal structure suggests that the nests are built by adding storeys over time. The straight, vertical tunnels are not always oriented along a single line. They usually end in the centre of a disc-like chamber, and the next tunnel segment leading further down often starts at a different position at the floor of that chamber. The situation is different in *Pheidole oxyops*, a species remarkable for its habit to construct nest openings that serve as pitfall traps. Below that trap, the nest is composed of a single, relatively straight vertical tunnel. The chambers are again shaped like flat, round discs, with the tunnel projecting through their centres.

Pheidole morrissi (Fig. 1b), on the other hand, inhabits nest composed of multiple *shish-kebob* units with more irregular chambers, that often merge with the chambers from neighbouring units. In some cases, authors have described underground ant nests as “diffuse networks of galleries” (e.g. Wang et al., 1995). One example for an architecture that features particularly intricate interconnections is the nest of the imported red fire ant *Solenopsis invicta* Buren (Cassill et al., 2002). However, Tschinkel (2003) pointed out that when it comes to the subterranean part of the nests, even the complex structures found in fire ants can be described as multiple, closely packed vertical units with interconnections arising from chambers merging into each other.

The significance of the vertical tunnel as the principal unit of nest excavation remains speculative, but it is conceivable that exploring the variation of conditions along the soil profile can be advantageous in allowing the workers to find a location for chamber building that provides suitable conditions. In some desert ants, authors have assumed that the vertical shafts even reach down to the groundwater table (e.g. Thomé, 1972; Dlussky, 1974).

According to Dlussky (1974), another architectural feature found in several species of desert-dwelling ants is a system of horizontal tunnels, mostly located close to the surface, but sometimes also in deeper soil layers. Such tunnel systems often connect different vertical units of the nest, in some cases as distant from each other as to appear to be separate nests. Additionally, horizontal galleries close to the surface might serve as passageways that allow foragers to access more distant areas while minimising the risks that come with moving outside the nest.

The nest of the Atta leaf-cutting ants

Various horizontal tunnels are found in the nests of the *Atta* leaf-cutting ants that, to the present day, seem to defy any attempts of being understood in terms general patterns. Even though they closely resemble the characteristic *shish-kebob* unit *sensu* Tschinkel (2003) in their young stages (Jacoby, 1936, 1937; Jonkman, 1980), mature nests have an astonishingly complicated three-dimensional structure. Reports from different regions and from different authors are often contradictory, even when describing a nest of the same species, indicating a strong variability in nest architecture, and possibly in the authors' interpretations of observed structures.

The *Atta* leaf-cutting ants live in large colonies that can have several million individuals in their mature stage. Their underground nests are, in terms of the amount of excavated soil, by far the largest subterranean ant nests (Bollazzi et al., 2012). The soil excavated underground is deposited around several nest openings, in many species constituting a single nest mound of considerable dimensions. Moreira et al. (2004a) reported one particular *Atta laevigata* (Smith) nest where the soil removed from the underground covered an area of 67.2 m^2 at the surface. The most apparent feature that distinguishes the underground architecture of all Attini[7] species from that of other ants is the voluminous fungus chambers with their highly domed ceilings, where workers cultivate symbiotic fungus on collected plant material (Belt, 1874, p. 64). A single *Atta* nest can have up to 8,000 fungus chambers (Moreira et al., 2004a) that have remarkably smooth outer walls and are relatively uniform in size and shape (e.g. Jacoby, 1953, 1955; Jonkman, 1980).

The authors of older publications (e.g. Eidmann, 1935; Weyrauch, 1942; Jonkman, 1980) often reflected the view that there is an "active zone" and a "less active zone" in mature *Atta* nests. Not all fungus chambers in a mature nests are used simultaneously, and they hypothesise that the colony constantly excavates new chambers at the periphery of the nest and uses them for fungus culturing, while older chambers are abandoned over time. Moser (2006) refers to completely empty chambers in an *Atta texana* nest as "dormancy cavities", and assumes that they "serve as storage areas for excess workers". Eidmann (1932) reports the unused chambers and tunnels in less active zone occasionally being filled with soil pellets, indicating that the workers sometimes opportunistically dispose of excavated material in unused underground cavities. I made a similar observation in a mature nest of *Atta vollenweideri*, however, only some of several unused chambers and tunnels were filled with soil pellets in that case.

Empty fungus chambers, or, more precisely, cavities that resemble fungus chambers in size and shape but do not contain fungus gar-

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[7] The *Attini* tribe includes several other genera of fungus-growing ants related to *Atta*.

den, are often used for waste disposal. With the exemption of *Atta colombica*, all *Atta* species maintain cavities underground to dispose of dead individuals and depleted material from the fungus gardens. According to Moser (2006), old fungus chambers, unused tunnels and “irregular cavities“ are utilised for refuse disposal in *Atta texana*.

Other publications describe specific refuse chambers; more or less bell- or bottle-shaped, much larger than the fungus chambers, and located in a deeper soil layer; according to the authors close to the ground water table (Stahel and Geijskes, 1939, 1941; Amante, 1964; Jonkman, 1980). Stahel and Geijskes, investigating the subterranean architecture of *Atta sexdens* and *Atta cephalotes* in Suriname, hypothesised that such cavities are used by the colony to gain access to water, before they are filled with refuse later on. Accordingly, they named these structures *Zisternen* (cisterns) and distinguished them from the refuse chambers. This topic will be discussed in Chapter 5.

Recent field studies from southern Brazil revealed another type refuse chamber to be found in *Atta laevigata*: utricular, vertical chambers that reach several meters underground are located in what appears to be a small filial nest containing no fungus gardens, and being located some metres from the main nest [8].

Besides fungus and refuse chambers, there are some types of cavities appearing in particular reports that are much more difficult to interpret. Most prominent among these, Eidmann (1932, 1935) and Jacoby (1955) both describe a single, large, vertical structure with an irregular surface, located centrally in between the fungus chambers, that they call the *Zentralraum* (central cavity). This central cavity contained neither fungus, nor brood, nor refuse, but due to the fact that they found similar structures in several *Atta sexdens* nests, Eidmann and Jacoby assume it to be important for the colony. Moser (2006) also found a central cavity in *Atta texana*, here it was located relatively deeply, and hypothesised that the ants only use that cavity during the winter to accumulate all the fungus material of the colony in one place in order to concentrate all the metabolic heat of the colony. This explanation seems rather unlikely for *Atta sexdens* in southern Brazil, especially considering the fact that the described irregularity of the central cavity is in strong contrast to the reported regularity of the fungus chambers. Possibly, many unusual shapes that appear in *Atta* nests originate just from former digging activity of other animals, or from the degradation of dead tree roots that remained in the soil.

There is even more ambiguity in literature concerning the tunnels in mature Atta nests. Eidmann (1932) went so far as to distinguish four principal types of tunnels in *Atta* nests: foraging tunnels, round tunnels, oval tunnels and ventilation shafts. The longest one are the foraging tunnels (*Schleppkanäle*) that project up to 200 m from the nest. Foraging tunnels that are used by workers to carry leaf material from

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[8] Flavio Roces, pers. com.

distant patches to the nest have been reported for *Atta laevigata*, *Atta capiguara*, *Atta bisphaerica*, *Atta sexdens*, *Atta saltensis* and *Atta opaciceps* (e.g. see Moreira et al., 2004a,b) Secondly, at the nest itself, Eidmann discriminates between round, and oval/flat tunnels according to their cross section that, as he states mirrors the shape of the nest opening leading into the tunnel. Round tunnels are more or less vertical, while horizontal tunnels are usually of the oval type; and Eidmann even admits that round tunnels, once they take turn into horizontal direction, become oval tunnels, and vice versa. This may indicate that both types serve the same function, and that under different conditions, i.e. vertical or horizontal position, different shapes serve that function. Eidmann thinks in that direction when hypothesising that workers move faster when walking on the ground, than when hanging on the ceiling, and that the flat shape might therefore allow more workers to move fast. Regarding the observation that both tunnel types transform into each other, one could argue that discrimination between them may be obsolete.

The round and oval tunnels in the upper part of the nest follow no obvious pattern. In the publications of Eidmann and Jacoby, this part of the nest is called the *Kanalgewirr* (tangle of channels). According to them, the fungus chambers are located below that section. Descriptions vary on how the fungus chambers are connected to the tunnels. Authorities seemingly agree that smaller side[9] tunnels lead from the main tunnels[10] to the chambers. One side tunnel can sometimes lead to multiple fungus chambers, and sometimes fungus chambers are connected to each other through additional small tunnels. The side tunnels are usually curved upwards in *Atta sexdens* according to Eidmann and Jacoby, and merge into the fungus chambers at their bottom side. The main tunnels in chamber zone are mostly vertical, but inclined towards a point below the nest centre. In the photographs of *Atta laevigata* and *Atta bisphaerica* nest casts provided by Moreira et al. (2004a,b), chambers are often located laterally to the main tunnels, and not above it. Some of the main tunnels seem to run horizontally straight through the chamber zone. Below the chamber zone, Stahel and Geijskes (1939, 1941) found large horizontal tunnels, while Eidmann and Jacoby in their publications described the main tunnels running down from the chamber zone to describe a curve below the chambers just to run upwards at the other side. In an idealised sketch, Jacoby (1955) even assumed that the main tunnels are principally U-shaped, and possibly meet at the deepest point of the nest. During nest growth, the author hypothesises, workers add a new system of U-tunnels at the outer perimeter of the nest, that envelopes the old nest. Eidmann furthermore speculates about the existence of a ring

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[9] It should be mentioned here that these side tunnels are not included into Eidmann's four different tunnel types.

[10] Either round or oval.

tunnel that surrounds the entire chamber zone and connects it to the foraging tunnels. It must be noted however that it requires a certain amount of goodwill to derive these interpretations from sketches of the actual field observations provided by the authors. Accordingly, both assumptions must be treated as pure speculation.

Besides foraging tunnels, round tunnels and oval tunnels, Eidmann (1932) reports the discovery of long, straight, vertical tunnels that were too thin to be filled with cement, and rarely used by the ants. He hypothesised these tunnels to serve as ventilation shafts that lead directly upwards from the fungus chambers. These shafts seem to be unknown to the authors working in other areas, and it remains an open question even if they originate from ant excavations, or other causes.

This example and others mentioned above highlight the intricacy of the internal structure of *Atta nests*. They are can hardly be described in terms of interconnected vertical units, the inter and even the intra specific variability seems to be very high, and perhaps it is often difficult in a nest cast to differentiate between structures excavated by the ants, structures that have been in the soil already before the foundation of the colony, and older structures encountered by the ants modified by them afterwards. Much more fieldwork will be necessary to produce a thorough understanding of the *Atta* nest and its development.

2.2 EXCAVATION BEHAVIOUR

The architectural features of underground ant nests described above are a visible result of the workers' excavation behaviour. In the earlier literature descriptions of excavation behaviour in ants concentrated on delineating details of soil manipulation on the individual level (see e.g. in Huber, 1810; Wheeler, 1910; Sudd, 1969). Excavation was described as a process that involves three consecutive steps: (1) *grabbing*, i.e. loosening the soil with the mandibles, (2) *raking*, i.e. aggregating the loosened soil beneath the mesosoma by means of mandible and leg movements, to create a soil pellet that can be picked up and held with the mandibles, and (3) *transport*, i.e. carrying the soil pellet away and depositing it outside the nest. This repetitive sequence of behavioural steps on the individual level is the very basis of underground nest building in ants. However, its knowledge hardly allows to understand the development of nest shapes on colony level.

Leaders, followers and social enhancement

Scientists started early on hypothesising that explaining nest shape and development is possible only on the basis of understanding the interactions of the individual workers. Well into the 20th century, there was some ambiguity among writers about the question if hierarchical structures underlie the organisation of collective work in ant colonies. A quote on ants in the Bible indicates that the absence of hierarchical organisation in ant societies was advocated by some even back in ancient times:

> "Go to the ant, o sluggard; consider her ways, and be wise. Without having any chief, officer, or ruler, she prepares her bread in summer and gathers her food in harvest."
>
> — *Proverbs VI.6-8*

On the other hand, Belt for example hypothesises as late as 1874 that some aspects of the seemingly reasonable behaviour he observed in leaf-cutting ants can be explained due to the leadership of the major workers:

> "The largest class of what are called workers are, I believe, the directors and protectors of the others. They are never seen out of the nest, excepting on particular occasions [...]. The stately observant way in which they stalk about, and their great size, compared with the others, always impressed me with the idea that in their bulky heads lay the brains that directed the community in its various duties. Many of their actions [...] can scarcely be blind instinct. [...] I can imagine a young ant getting a severe earwigging from one of the *major-domos* for its stupidity."
>
> — *Belt (1874, p. 66)*

The view that instinctive stimulus-response patterns rather than reasoning and hierarchy underlie the organisation of collective behaviour in insect societies became more accepted at the beginning of the 20th century (Wheeler, 1910, pp. 518-530). Nonetheless, the interpretation of ant behaviour in terms of hierarchical structures is still reflected in Chen's laboratory study on digging activity in isolated workers and small groups of *Camponotus japonicus* Mayr, 1866 (Chen, 1937a,b). He found a strong variability in the digging motivation of isolated workers. Moreover, individual workers excavated significantly more when kept in small groups, and that effect was particularly strong for the individuals that had displayed very low excavation rates under isolated conditions. Chen interpreted that effect of social enhancement as a result of hierarchical structures in the ant society, denoting the more active individuals *leaders*, the less active ones *followers*.

Two consecutive studies on social enhancement in excavator groups reported a contrary effect in different *Formica* and *Polyergus* species. Here, workers excavated less, when kept in groups, than under isolated conditions (Sakagami and Hayashida, 1962; Sudd, 1971, 1972a).

However, another study by Imamura (1982) on *Formica yessensis* Forel supported Chen's social enhancement hypothesis. Here, the effect was even present when only two workers excavated close to each other separated by a fine mesh. Imamura speculated that the excavators produce recruitment pheromones, that can pass though the mesh and stimulate other excavators.

Spatial patterns

Beside these studies that concentrated on the quantitative effects of social interaction, Sudd described, beside other contributions to the topic (Sudd, 1968, 1969, 1971, 1972a, 1975), experiments on the directionality of digging behaviour. He reported that isolated workers excavate tunnels with roughly species-specific patterns. In *Lasius niger* tunnels are spreading and convoluted and often branch out under the surface, while tunnels excavated by *Formica* workers tend to be vertical, straight and unbranched (Sudd, 1970b). In another study, tunnel direction in *Formica* is directly influenced by gravity: when rotating an experimental setup that contains tunnelling ants around a horizontal axis, tunnel sections excavated afterwards are in alignment with changes in the direction of gravity (Sudd, 1972b). Sudd also reported a set of experiments on the response of workers to already existing tunnels (Sudd, 1970a). Workers presented with existing tunnels mostly started excavating at the tunnel end. A smaller proportion of the ants was observed to start excavating randomly along the tunnel at some distance to the end. This proportion was higher in *Lasius niger* than in *Formica*, possibly explaining why the tunnel systems excavated by the former are more branched and irregular.

This concludes the overview on the research published up to the end of the 20th century on ant nest excavation. Some further contributions that were published in the past 13 years will be introduced where appropriate in the following chapters. In the next Chapter I will introduce my study species, the Chaco leaf-cutting ant.

3

ON THE NATURAL HISTORY OF THE CHACO LEAF-CUTTING ANT

The study species of the present thesis, *Atta vollenweideri* (Forel, 1893), is one of the most intensively studied ant species from South America. It has been subject to several field studies[1], laboratory experiments on foraging, learning and nest building behaviour (Röschard and Roces, 2002; Kleineidam et al., 2007; Fröhle and Roces, 2012; Cosarinsky and Roces, 2012), observations on the biomechanics of leaf transport (Moll et al., 2010, 2012, 2013), and even neurophysiological (Ruchty et al., 2009) and neuroanatomical studies Kelber et al. (2010); Kuebler et al. (2010).

3.1 TAXONOMY, SYSTEMATICS AND BIOGEOGRAPHY

In the Guaraní language that is native to Paraguay and northern Argentina, the Chaco leaf-cutting ant is called the *ysaú vidrio*, in contrast to other *Atta*[2] species that people native to the same areas simply refer to as *ysaú* (Schade, 1973). Accordingly, it can be claimed that the Chaco leaf-cutting ant has been recognised as a distinct taxonomic entity by the people inhabiting its distribution area long before its first scientific description. Nonetheless, the *ysaú vidrio* has been first described scientifically by Forel (1893) as *Atta sexdens* r. *vollenweideri*, a subspecies of *Atta sexdens*, and finally raised to species by Borgmeier (1950). The molecular phylogeny of the genus *Atta* places *Atta vollenweideri* into the *Epiatta* subgenus, together with *Atta capiguara*, *Atta bisphaerica*, *Atta laevigata*, *Atta opaciceps*, and *Atta saltensis*, the latter being the species most closely related to *Atta vollenweideri* (Bacci et al., 2009).

The geographical distribution of *Atta vollenweideri* is mainly restricted to the Gran Chaco region in South America. This savannah type habitat covers the majority of western Paraguay and parts of south-eastern Bolivia, south-western Brasil and northern Argentina. Populations of *Atta vollenweideri* can be found along Río Paraná and lower Río Uruguay as far south as Santa Fe (Vittar and del C. Cuezzo,

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[1] By Bucher and Zuccardi (1967), Jonkman (1976, 1978, 1979a,b); Jonkman (1980); Jonkman (1980), Kleineidam and Roces (2000), Kleineidam et al. (2001), Röschard and Roces (2002, 2003a,b, 2011), Cosarinsky and Roces (2007), Sosa and Brazeiro (2010) and Fröhle and Roces (2012)

[2] According to Wild (2007), the Guaraní speaking people of rural Paraguay also distinguish, as does modern taxonomy, in general between the genera *Atta* (*ysaú*) and *Acromyrmex* (*akeké*).

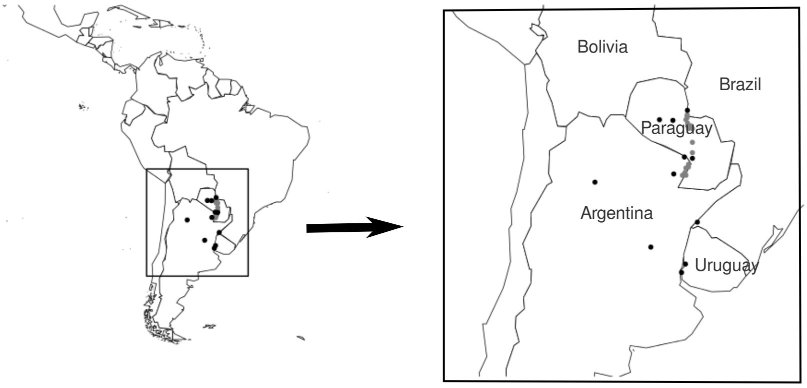

Figure 2: Biogeography of *Atta vollenweideri*: known populations based on the references listed in Tab.5. Black dots indicate the locations of populations specifically mentioned in the literature. Grey dots indicate coordinates estimated from Fig. 5 in Jonkman (1976) which is based on the results of an aerial photoraphic survey.

2008) and western Uruguay[3], as far as Porto Murtinho, Brazil to the north (Carvalho and Tarragó, 1982). The species is restricted to flat areas with clayish alluvial soils, as can be found close to large rivers. In hilly territory, for example in eastern Paraguay, it *Atta vollenweideri* does not occur. Here, *Atta sexdens*, *Atta capiguara*, and *Atta laevigata* are abundant (Fowler, 1983, 1985; Jonkman, 1976; Wild, 2007). In sandier soils within the Gran Chaco, like for example the region of the Parque Nacional Copo (Santiago del Estero, Argentina), *Atta saltensis* occurs regularly while *Atta vollenweideri* does not[4]. The Pantanal in southern Brazil, another flat lowland region neighbouring the Gran Chaco to the north, is also not included in the geographical distribution of *Atta vollenweideri*. Here, *Atta sexdens* is the most common *Atta* species (Corrêa et al., 2006). To the west, the distribution reaches over the entire lowland of central South America. *Atta vollenweideri* is still abundant in the province of Tucumán (Bucher and Zuccardi, 1967; Bucher, 1974), and in west Formosa, at the limit with Salta[5]. Accordingly, the reported distribution ranges at from 22°S to 33°S on the north-south axis, and from 57°W to 65°W on the east-west axis (Fig 2; Tab. 5).

Atta vollenweideri is the only *Atta* species in its habitat. However, in our study area at the *Reserva El Bagual*, close to San Francisco de Laishi, Formosa, Argentina (26°17′08″S; 58°49′43″W), it is sympatric with at least four *Acromyrmex* species: *Acromyrmex lundi*, *Acromyrmex*

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[3] Martin Bollazzi, pers. com.

[4] Flavio Roces, pers. com.

[5] Flavio Roces, pers. com.

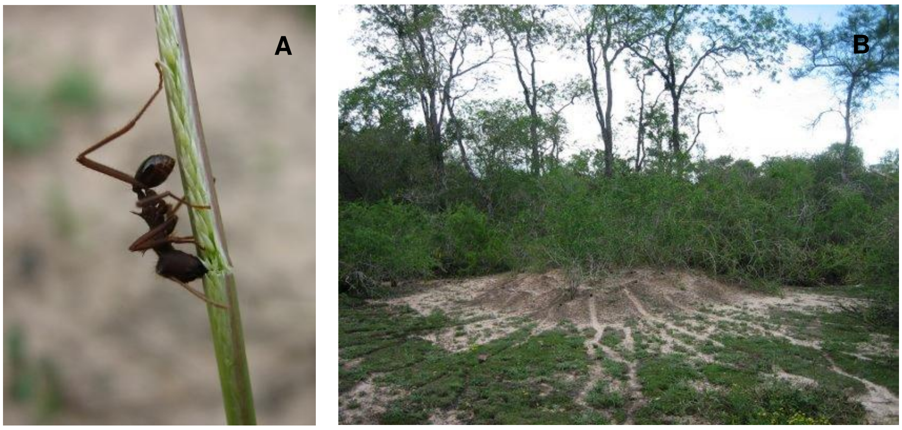

Figure 3: Foragers and foraging trails. (A) A forager cutting grass. (B) Foraging trail around a nest. All pitcures were taken at the *Resereva El Bagual*, Formosa, Argentina.

fracticornis, *Acromyrmex heyeri*, and *Acromyrmex striatus*, the former two being common in the area, while I only found a single nest of each of the two latter species. The density of *Atta vollenweideri* colonies can be surprisingly high at suitable patches in the landscape. Analysing aerial photographs from Paraguay, Jonkman (1979a) identified 45 living and 131 dead nests within a study area of 150 ha. On aerial photographs of the same study area that were 29 years older, he identified only 12 living and 9 dead nests, indicating an abrupt population increase that Jonkman ascribed to the increased land use. Nests rarely occur on areas covered with undisturbed high grass, whereas grazing and regular burning of the pastures may provide the ants with suitable nest sites and more tender young leaf material.

3.2 FEEDING, FORAGING AND ORIENTATION

Like other *Epiatta* species, *Atta vollenweideri* collects predominantly grass fragments (Fig. 3A), but also leaves, or even fruits occasionally. Unlike many other *Atta* species, *Atta vollenweideri* does not build underground foraging tunnels radiating from the nest (see Chapter 2). The workers move on numerous foraging trails that are cleared from vegetation and radiate from the nest, and can reach several hundred metres long (Fig. 3B).

The trails are marked with pheromones. Neurobiological studies on *Atta vollenweideri* indicate that some workers possess particular neuroanatomical adaptations to respond to trail pheromones that are not present in others. Among the workers, two different phenotypes have been identified concerning the number of olfactory glomeruli in the antennal lobes. The two antennal lobes present the first olfactory neuropils in the insect brain, i.e. the first locations where olfactory information from the antennae is processed. They contain several olfactory glomeruli, and each odour presented to the antennae produces a characteristic activation pattern in the these glomeruli. Among *Atta vollenweideri* workers, there are HN (high number) phenotype, that has 440 glomeruli, and LN (low number) phenotyope with only about 390 glomeruli. Additionally, some workers have a single enlarged glumerulus, a so-called macroglomerulus in a region that responds to the trail pheromone (MG phenotype), while in other workers all glomeruli are equally large (RG phenotype). The majority of small workers belongs to the LN-RG, most large workers to the HN-MG phenotype, indicating that larger workers possess special neuronal adaptations related to the use of trail pheromones. The difference between the phenotypes manifests during pupal development. Accordingly, task specialisation in *Atta vollenweideri* workers it at least partially promoted by early neuronal developments (Kelber et al., 2010; Kuebler et al., 2010).

Even though trail pheromones seemingly play an important role in the foraging behaviour of *Atta vollenweideri*, workers also display the remarkable learning ability with possible implications for individual orientation. Laboratory studies demonstrated that workers are not only able to precisely detect the direction of thermal radiation with their antennae (Ruchty et al., 2009), they can also be trained to use thermal radiation as an orientation cue to find a food reward (Kleineidam et al., 2007).

The blades of grass are cut by the workers into transportable units, and if cut close to the nest, usually carried home by the cutter itself. At longer distances, the ants rely on transport chains: the cutters remain at the patch while 2-5 carriers, that are smaller at average than the cutters, carry the materials to the nest. All carriers but the last one after some distance simply drop their load on the trail, where they remain without any obvious pattern or accumulation, to be picked up by another individual. The first carrier in the transport chain usually covers only a short distance and returns to the patch immediately, the last carrier covers the largest proportion of the carrying distance (Röschard and Roces, 2003a). On the way to the nest, the grass fragments are often cut into even smaller pieces with the effect that, with increasing proximity to the nest, the load size matches the size of its carrier increasingly well, thereby allowing for enhanced transport rates (Moll et al., 2012; Röschard and Roces, 2002, 2003b).

Grass fragments are generally held by carriers at one end rather than in the centre. Longer fragments are held more vertically than shorter fragments. The angle of the load is adjusted by means of head movements to maintain stability even when walking on a slope (Moll et al., 2010, 2013). When increasing only load mass, walking speed of the carriers remains constant, but metabolic rates increase.

Figure 4: Nuptial flights. (A) View of the nest surface before the nuptial flight (Photo: Jenja Kronenbitter). (B) Young queens waiting in the nest opening. (C) A young queen. All pitcures were taken at the *Resereva El Bagual*, Formosa, Argentina. All depicted persons gave consent to the use of the photographies.

The energy costs for carrying the load are similar to those for carrying a unit of body mass. When increasing load length, on the contrary, the metabolic rate remains equal, but carriers move at a significantly slower pace because they have to maintain load stability (Moll et al., 2012). Accordingly, cutting the fragments shorter on their way and matching them with the size of the carriers can contribute significantly to maximising transport rates. On the other hand, due to the fact that loads stay on the ground unmoved in between carrying events, the sequential mode of transport is still slower than a single worker covering the entire distance. Remarkably, more fragments are passed from one worker to another, i.e. the sequentiality of the task is more pronounced, for loads of higher quality. This suggests that involving many workers in a task that could be done by one individual is, in this case, not aimed at maximising transport rates, but at distributing information about the collected material and thereby maximising information transfer among foragers (Röschard and Roces, 2011).

3.3 COLONY FOUNDING, NEST DEVOLPMENT AND ARCHITECTURE

The foundation of new *Atta vollenweideri* colonies takes place after the nuptial flights during the early wet season of the year, in October and November. According to my own observations in 2005, 2008, and 2009, there is usually more than one nuptial flight in each year. While the first is largest in terms of the number flying individuals, some males and females tend to remain in the nests and join later, often much smaller nuptial flights. They begin soon before dusk, seemingly on hot days with high air humidity, and usually about one day after a rainfall. Such an event is rather conspicuous, because birds aggregate around the nests, and small mammals, such as foxes and armadillos, can sometimes be seen to approach the nest sites, presumably to feed on the large males and queens. The nest mound, which is usually relatively free of workers aside from the openings and foraging trails, is covered with ants that will instantly attack any kind of intruder. Hundreds of males and young queens wait in the central nest openings, where they can be seen during warm nights up to several days before the nuptial flight. The males are the first to leave the openings, assemble on the nest mound, and start to fly away in a nearly vertical direction (Fig. 4A-C). It takes only minutes until the majority of males have started. It is only when almost all the males have left that the young queens begin to follow. This view is accompanied by a variety of birds that fly through the ascending swarm to prey on the ants. Many young queens without gasters can be found on the ground in this stage of the nuptial flight, indicating that some of the birds eat only the highly nutritious gasters and simply drop the rest of their prey in order to catch another one.

After sunset, the young queens can be found in open areas, where they land and immediately break off their wings by means of their posterior legs. The wings of an *Atta vollenweideri* queen are relatively firm and elastic when force is applied to their flat side, but they are stiff and break easily if only little force is applied in an edge-to-edge direction. To break them off a queen will hold its wing close to the body, the long axis parallel to the main body axis. They orientate the flat of the wing sideways, rather than upwards, and then employ their posterior legs to hook the upper edge of the wing and pull it downwards, with the result that the wing breaks.

After it breaks off its wings, the young queens spend only a few minutes finding a place to excavate a founding nest. The behaviour of the queen during the construction of the founding nest has been investigated at great detail by Fröhle and Roces (2012). The queens excavate a vertical tunnel that is on average 20 cm deep and so narrow that the queen has to walk backwards when carrying out excavated material. Once the tunnel is finished, the queen builds a small chamber at its end. The length of the initial tunnel is a compromise between the benefits that come with a deeper chamber, such as reduced temperature variation, and the risk posed by predators that exists as long as the queen has to move outside regularly. Laboratory experiments have demonstrated that the ants try to reach a particular depth, but switch to chamber building even at a shorter tunnel length if it takes too long to go deeper. When excavating the chamber, the queen can be observed to leave the tunnel head first when disposing of soil pellets, as the chamber provides sufficient space for the queen to turn around. The soil pellets are deposited in a circle around the nest opening. After some time, the queen stops carrying out soil pellets and starts using them to seal the tunnel from the inside. On the morning after a nuptial flight the holes dug by the queens and the pellet circles surrounding them are easily recognisable. A few days later the rains have usually erased all traces of them.

Atta vollenweideri queens perform claustral nest founding, meaning once the nest is sealed they never leave it again and rely entirely on their body's energy resources until the first generation of workers emerges from the nest and starts foraging. Mortality during this early stage of the colony seems to be particularly high. In 2008, I marked 43 founding nests the day after a major nuptial flight in an area of about 1 ha. That area featured 3 mature nests, an indicator that soil properties and environment were not unsuitable for nest sites. However, none of these founding colonies survived to the next year.

If the colony survives till the hatching of a first worker generation, these workers then start to enlarge the nest. During this early stage, it has only a single opening in the centre of a small crater that is about 10 cm high. Workers forage individually. In both aspects the young *Atta vollenweideri* colonies strongly resemble the sympatric colonies of *Arcomyrmex fracticornis*. I excavated a nest in that stage of development in 2008. Underground, it was composed of a single, relatively straight vertical tunnel that reached 2 m deep. The nest had 11 chambers branching off from the vertical shaft. Some of these chambers were connected via smaller tunnels, while others originated from the main shaft directly. Seven of the chambers were filled with fungus; two smaller chambers, both located further away from the main shaft, were filled with refuse; the two remaining chambers were of the same size as the fungus chambers, but empty.

Later in the nest development, a second opening appears at the edge of the crater and workers begin to deposit excavated soil around it (Fig. 5A-B). As nest development continues, additional openings are excavated at the edges of the existing nest mound. Nest growth occurs predominantly during the spring and summer season (Jonkman, 1980; Jonkman, 1980). In the winter many nest openings are temporarily closed again. Once a colony has reached 3 years of age, its remaining life expectancy is, on average, another 7 years (Jonkman, 1979a).

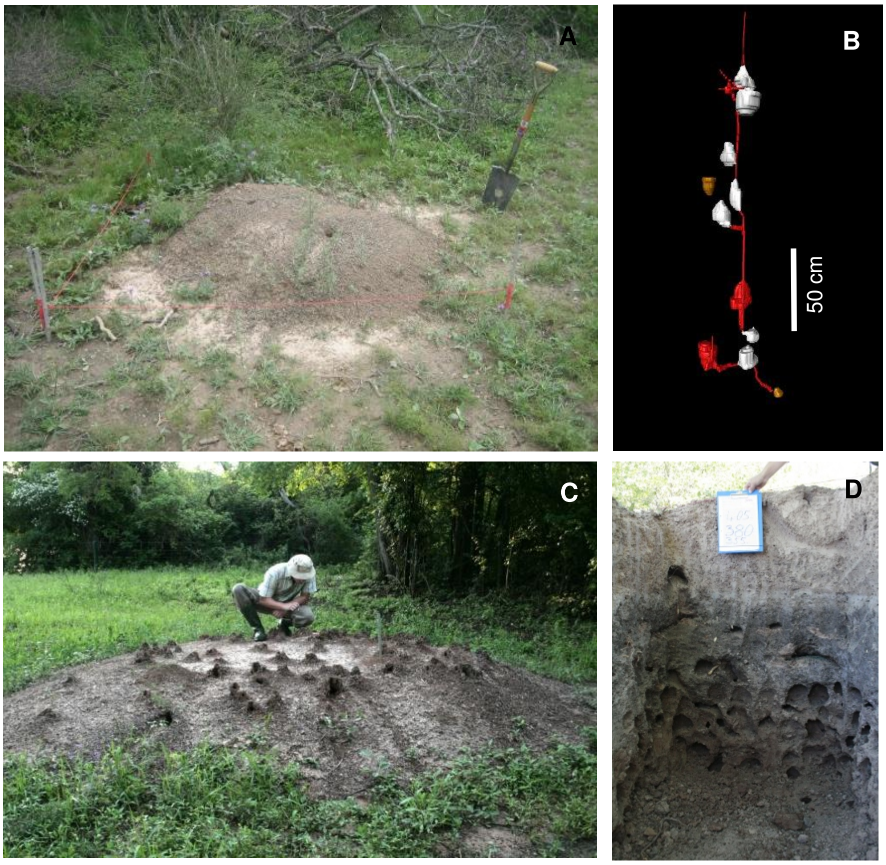

Figure 5: *Atta vollenweideri* nests. (A) Young nest with only two openings. (B) *3D*-Reconstruction of a young nest with only one opening that was excavated in 2008. Red areas were empty, white areas contained fungus garden, yellow areas colony refuse. (C) Mature nest (Photo: Jenja Kronenbitter). (D) Cut into the garden zone of a mature nest. All pitcures were taken at the *Resereva El Bagual*, Formosa, Argentina. All depicted persons gave consent to the use of the photographies.

The mature nest of the Chaco leaf-cutting ant (Fig. 5c-d) is different from other *Atta* species especially in two architectural features (Schade, 1973; Jonkman, 1980). The first is the complete absence of underground foraging tunnels, as mentioned above. But even more prominently, many larger nests have chimneys of clay erected by the workers above the central nest openings on top of the mound. These *ventilation turrets* enhance the gas exchange between the environment and the underground nest by taking advantage of the Bernoulli effect (Kleineidam and Roces, 2000; Kleineidam et al., 2001). They are constructed from materials selectively imported from the immediate vicinity of the nest opening, including, most prominently, soil pellets from underground formerly deposited there and also plant material (Cosarinsky and Roces, 2007, 2012).

Like in other *Atta* species, nests can grow impressively large. The soil excavated from underground is heaped in a mound that can be 1 m high and 8 m wide in a mature nest (Jonkman, 1980; Jonkman, 1980). The sheer amount of soil colonies of this species move from deeper layers to the surface makes them a major factor shaping the composition of the vegetation in the Gran Chaco region (Bucher and Zuccardi, 1967; Jonkman, 1976, 1978; Sosa and Brazeiro, 2010).

In contrast to that supposedly important impact the excavation behaviour of *Atta vollenweideri* has on colony life, and even on the development of the entire landscape in the Gran Chaco, the mechanisms guiding that behaviour remain largely unknown. In the following chapters, I will describe experimental approaches aimed at investigating how local stimuli influence digging decisions in workers of the Chaco leaf-cutting ant.

Part II

RESEARCH

4

SOME OBSERVATIONS ON STRUCTURAL CUES AND GRAVITAXIS: IMPLICATIONS FOR EXPERIMENT DESIGN

ABTRACT When designing experiments on a certain aspect of excavation behaviour, factors other than those investigated need to be considered. In this chapter, I report preliminary observations on factors that influence excavation behaviour. These observations, even though mostly anecdotal and not subject to thorough investigation, provide the basic knowledge that influenced the design of many experiments described in the following chapters. *Atta vollenweideri* workers respond to transitions from softer to harder materials, to surface irregularities, pre-formed structures and to gravity, when deciding where to excavate. Possible functions of the described responses under natural conditions are discussed.

Introduction

Designing a manipulative experiment for behavioural investigations is often a matter of trial and error. Several designs that appear promising to investigate a particular hypothesis prove useless when put to the test, not because they yield unexpected results, but because they yield no result at all. Often they simply turn out unsuitable to observe and quantify the behaviour originally intended to study. In other cases, the experiments reveal conceptual weaknesses later on, or they are simply abandoned in favour of simpler and/or more elegant ideas. These designs, in publications referred to as *preliminary experiment*, are usually, apart from vague remarks, not documented further. However, the experiences gained in such preliminary experiments are often critical for later designs. The aim of this chapter is to describe some of these preliminary observations that are important for understanding the design of several experiments described in the later chapters.

Transitions from softer to harder material

One feature regularly observed in laboratory experiments is that ants, if kept in a soil-filled container, strongly prefer to excavate along the confinements of the container, e.g. along the outer wall of a plastic box. This preference often results in an extended network of tunnels running along the outer walls of the box. In nature, a similar behaviour can be observed: tunnels are often excavated along roots, and many species excavate chambers under flat stones. Most likely, that preference to dig at the border to harder materials increases the stability of the structures, and is therefore favoured by natural selection. Another explanation for the effect in the laboratory can be the attempt to maintain a certain direction during tunnel excavation. Encountering a hard obstacle, the workers hypothetically try to dig around that object, a strategy that works with stone in the underground, but possibly leads to erratic tunnelling along the walls in a plastic box.

For experimental design, the preference to dig along harder material can create problems. Laboratory designs are required to confine the ants to a limited space. Having them excavate in plastic boxes, buckets or thin soil layers between glass plates, often results in constructions that have little resemblance to field nest architecture. Additionally, in choice experiments, the necessary confinements constitute competing stimuli. Especially when testing vibrational stimuli, it is desirable to have the ants excavate away from confinements, because harder materials, like plastic, behave very differently than soil in terms of wave propagation and resonance. Possible ways of dealing with that issue will be described later, in Chapter 8.

Surface irregularities

One possible way of motivating workers to start digging away from the confinements is hiding the material transition as well as possible, and offering another stimulus at the location intended for excavation. Herein, one can rely on another regularly observed preference: the preference to dig at irregularities in the soil surface. A small scratch in smooth clay surface can be a strong stimulus to attract excavators. In *Atta vollenweideri* for example, we observed that irregularities along the wall of a tunnel provoke excavation behaviour. The observation was made in a setup originally intended as a choice experiment to test the effect of stridulation signals on the decision for where to start excavating.

A laboratory colony of *Atta vollenweideri* was connected to a feeding box through a clay tunnel. The workers foraged for leaf material through a tunnel leading through a massive body of clay. In the centre of that tunnel, two spots located at the opposing lateral walls, were scratched with a nail. The transition from plastic tube to clay tunnel was hidden as well as possible by smearing these places from the inside with wet clay (Fig. 6). In an advanced version of the setup, the scratches were marking two opposing spots (circular shape, 25 mm diameter) of coloured clay inside the tunnel. The spots marked locations where the tunnel walls were thinned out, and vibrational stimuli was applied to the outside for the ants inside the tunnel to respond to. The aim of the experiment was to apply a stimulus to

Figure 6: Digging at surface irregularities. This is an early version of the tunnel experiment without coloured clay spots. Rather than digging randomly along the smooth wall tunnel, the workers excavated at the two scratches in the centre.

one of the two alternative locations, both scratched, but marked with different colours, and then to measure the response by counting the number of coloured soil pellets carried out of the tunnel.

Unfortunately, the setup failed to serve its original purpose, for excavation activity was very high at both sides. Possibly, the stimulus provided by the scratch was too strong, and the number of workers near by too large, to observe differences in the attractiveness of alternative locations relying on that method. Nonetheless, the experiment demonstrated that workers start to excavate more likely at a scratch, than at other locations along the tunnel. This is even more evident in the experiments with the coloured spots. Here, the coloured pellets carried out by the workers originate from excavation activity at the scratches, while undyed pellets originate from digging at other locations. The proportion of coloured soil pellets was 54.1±15.6% (mean±standard deviation, $n = 9$) of all pellets carried out; more than half of the pellets originated from two circular spots of 25 mm diameter in a tunnel that was 10 cm long and, itself 25 mm in diameter. Together, the coloured spots covered about 10 cm^2 of the internal tunnel surface of 78.5 cm^2. Accordingly, 54% of the pellets were excavated at the 13% of the available surface where irregularities had been encountered.

Existing structures and gravity

Another aspect of nest digging behaviour that can be regularly observed and utilised for experiment design, is the tendency to enlarge existing structures. If ants find a spherical cavity underground, workers allocate a large proportion of their excavation effort towards enlarging that chamber, if encountering a tunnel that ends blindly, the ants will, in most cases, further elongate that tunnel (see e.g. Sudd, 1970a). Existing chambers and tunnel ends are, in most cases, preferred over alternative locations when choosing a place to excavate, as long as there are no material transitions or surface irregularities that attract attention. Accordingly, it is possible to create a situation in which workers exclusively dig at the end of a tunnel, as long as the rest of the tunnel has regular, smooth walls, and as long as there is a smooth transition in between materials, for example if the ants enter the experiment via a plastic tube. Examples of such a spatially selective nest enlargement, and for experiments taking advantage ot that effect will be reported in Chapters 6 and 8.

The direction of tunnel excavation, if elongating a pre-given structure, follows gravitactic patterns in a three-dimensional environment (see Sudd, 1972a). This was shown for *Atta vollenweideri* in the following experiment: a plastic box was filled with clay (20% water content), and a tunnel 2 cm long and 1 cm wide was drilled into the soil either from below, from above, or from the side. A laboratory colony of *Atta vollenweideri* was connected to that tunnel with tubing of 1 cm inner diameter. Entering the soil from above, workers exclusively dug to extend the tunnel further downwards ($n = 3$). If the ants, on the contrary came from below, they extended the tunnel upwards ($n = 3$). If entering from the side into a horizontal tunnel, workers extended the tunnel curving upwards until reaching an angle between 70° and 85° ($n = 3$). Only if the tunnel reaches the top wall of the box will workers start to dig tunnels along the plastic. Therefore, it can be assumed that in the laboratory workers of *Atta vollenweideri* display negative gravitaxis if extending a horizontal tunnel or a vertical tunnel at the upper end, but they show positive gravitaxis if extending a vertical tunnel at the bottom end.

Discussion

Both observations, the tendency to dig even at small irregularities encountered at relatively homogeneous surfaces, and the tendency to further enlarge an existing tunnel, demonstrate the eagerness of *Atta vollenweideri* workers to continue the building work begun by other individuals, and to follow the spatial decisions of nest mates as to where to build a structure.

In nature, irregularities at the internal surface of a nest can be expected to exist where other workers have already started to excavate. By responding to scratches in the tunnel wall, workers are possibly copying spatial decisions of their nest mates. A consensus among many workers about where to excavate can enable a colony to allocate the efforts of many individuals to one location, resulting in the cooperative excavation of large structures. Comparable observations have been reported for termites, where workers similarly respond to surface irregularity in tunnel walls (Lee et al., 2008a), and eagerly enlarge pre-formed tunnels (Lee et al., 2008b).

Searching for a potential mechanism that allows coordinated collective nest building in social insects, Grassé (1959) introduced the concept of *stigmergy*. According to his concept, individuals do not coordinate their building behaviour with that of its nest mates on behalf of information directly communicated by other individuals, but by responding the existing structures. Accordingly, the building activity of one worker creates the cue used by other workers to decide what to do next. Each individual modulates its building behaviour according to the structures it currently encounters. The cues guiding building decisions are the structures already present. These cues are constantly altered by the activity of nestmates and the deciding individual itself. For example, when constructing the wall of a termite nest workers can cooperate without communicating. One individual places the first soil pellet, the next worker entering the site responds to the already placed pellet by placing its own pellet right beside the first one, or on top of it. If all workers follow that rule, a wall will result from the combined efforts of the individuals.

The observed digging responses to surface irregularities and existing structures can be interpreted in the same way. According to the presented observations of *Atta vollenweideri*, an unfinished tunnel leading nowhere provides by its presence alone a strong stimulus motivating workers to extend that tunnel. In nature, a smaller irregularity may be a hint for workers that excavation activity has already been initiated at a certain locality, thus motivating an individual to “join” in the process. It can be concluded that stigmergy, in this case the use of already built structures as a cue guiding digging activity, does contribute to the spatial organisation of collective nest excavation in *Atta vollenweideri*.

Accordingly, two types of structural cues can be distinguished in nest digging behaviour: structural cues provided by the environment, such as transitions from soft to hard material, allow ants to seize opportunities to build more stable structures; and structural cues provided by previous worker activity, such as unfinished tunnels or surface irregularities, that can be interpreted as stigmergic cues that are used to spatially coordinate collective nest digging.

Gravitactic tunneling behaviour has been reported by Sudd (1972a) for *Formica lemani* Bondroit and *Myrmica longinodis* Nylander. Wang et al. (1995) reported a similar phenomenon in *Lasius neoniger*: in vertical, two-dimensional laboratory setup workers coming from above first completed a single vertical tunnel, leading all way down to the bottom, until starting to excavate at other locations. In most ant species, subterranean nests are constructed around vertical tunnels that connect the chambers to the surface (Tschinkel, 2003). Possibly the first instinct of an ant worker, before starting to excavate, is to search for a vertical tunnel leading to the surface, where excavated material can be deposited. *Atta vollenweideri* colonies queens found new nests *claustrally*, i.e. they hide in underground founding chambers with no connection to the outside until the first workers hatch (Fröhle and Roces, 2012). It can be expected that the first workers have to dig upwards and create a nest opening. The internal structure of the young nests (Jonkman, 1980) suggests that the next step is to extend the nest downwards. Having a nest that reaches through several layers of soil may be advantageous for the colony because it allows the workers to choose the locations for chamber building from a gradient of environmental conditions. For example, soil temperature conditions change with varying depth, something that reportedly influences digging behaviour in *Acromyrmex* leaf-cutting ants (Bollazzi et al., 2008). It may be that all ant workers, even in older colonies, follow the same pattern when confronted with the task of nest excavation: they search for an exit to the surface, that has to be located upwards under natural conditions. If there is none, they excavate one. If there is, they dig downwards, to find the most suitable soil layer for chamber building.

Whatever the potential functions in nature, responses to gravity and to structural cues, seem to have a strong influence on the behaviour of ant workers in artificial, experimental environments. Even though this has not been investigated in deliberate experiments with a proper sample size, the phenomena described here were regarded in the design of several experiments described in the following chapters. The consideration of both structural cues and gravitaxis allowed the prediction of the response of the ants to particular experimental designs, and the appropriate manipulation of local stimuli at the location of expected excavation activity.

5

SOIL MOISTURE AND NEST EXCAVATION

This chapter is based on the manuscript: S. Pielström and F. Roces. Soil moisture and excavation behaviour in the Chaco leaf-cutting ant (Atta vollenweideri): digging performance and prevention of water inflow into the nest. (Submitted)

ABSTRACT The Chaco leaf-cutting ant *Atta vollenweideri* is native to the clay-heavy soils of the Gran Chaco region in South America. This habitat is characterised by strong annual variations in precipitation and moisture conditions. The aim of this study was to characterise nest digging behaviour in *Atta vollenweideri* in soils of varying moisture. This was done in a series of laboratory experiments using standardised, plastic clay-water mixtures with gravimetric water contents ranging from 14%, where the material was relatively brittle, to 26%, where the clay was close to the liquid limit. In a choice experiment, soils with higher water contents were chosen more often for digging workers than dryer soil. Nonetheless, the ants mostly avoided to excavate in the highest available moisture. Maximal group-level excavation rates occurred at a lower moisture, around 20%, close to the point in the moisture scale where the clay swells and becomes increasingly sticky. At a water content of 18% and more, pellets collected from carriers afar from the digging site were larger than those freshly excavated. Both digging rate and pellet transport rate were observed to be higher at higher moistures. Digging rate reached a plateau at 22% water content and remained at that level with increasing moisture. Transport rates began to increase at 18%, had a maximum at 20%, and then decreased again, but stayed above the level observed at dry soils of 14% and 16% water content. When simulating a situation in which workers excavated an upward tunnel below accumulated surface water, ants stopped digging about 12 mm below the interface soil/water, a behaviour representing a possible adaptation to the threat of water inflow field colonies are exposed to while digging under seasonally flooded soils. Possible roles of soil water in the temporal and spatial pattern of nest growth are discussed.

5.1 INTRODUCTION

In a recent publication, van Gils et al. (2010) proposed the adaptation to specific soil properties as a major explaining factor for the distribution pattern of leaf-cutting ant nests, an idea termed the *suitable soils* hypothesis by the authors. From that perspective and with regard to the current knowledge on the geographic distribution of *Atta vollenweideri*, alluvial alfisols presumably are the exclusive habitat of the Chaco leaf-cutting ant.

Besides the chemical composition, moisture can be an important factor making a soil suitable or unsuitable for particular the occurrence of particular ant species (Johnson, 1998; Tschinkel et al., 2012). In *Atta vollenweideri*, the range of distribution is mostly subtropical in areas characterised by high annual variability in moisture conditions. While the winters are mostly dry in the wet Chaco of the Formosa province in Argentina - precipitation reaches an annual minimum with an average of 40 mm in July - heavy rainfalls occur periodically in the spring and summer seasons. From October to April, precipitation is at least three to five times higher than the annual minimum (Gorleri, 2005).

Long-term observations revealed that young nests of *Atta vollenweideri* grow primarily during that wet spring and summer season (Jonkman, 1980). This can be attributed to a higher availability of suitable plant material for fungus culturing, but possibly also to differences in soil moisture conditions that affect building behaviour. Even in older, reproductively active nests, wet soil pellets deposited around the nest openings can be observed especially in the nights following a rain (Fig. 7A). Clay tends to be harder when it contains less water, which results in higher time and/or energy costs when ants excavate in dryer soils (Schelter, 2009). It has been demonstrated for several ant species that excavation rates increase with increasing soil water content in laboratory experiments (Sudd, 1969; Mikheyev and Tschinkel, 2004; Espinoza and Santamarina, 2010). Moreover, workers of the desert-dwelling harvesting ant *Messor ebenius* Forel were reported to even excavate their nest according to water gradients: they remove wet sand when water has percolated into the nest, but stop as soon as only dry soil is left around them. Accordingly, nest shape in this species is mostly determined by the infiltration behaviour of the water (Thomé, 1972).

In many soil types, moisture content has a strong influence on the physical properties, especially on the plasticity, of the material (Marshall et al., 1996), which are expected to strongly influence ant digging behaviour.

As explained earlier, excavation behaviour in ants can be described as a repetitive sequence of grabbing, raking and transport. Grabbing, i.e. loosening the soil at the digging face is, in the particular case of *Atta vollenweideri* workers manipulating clay, achieved by virtually biting and cutting into the material. The soil is raked into pellets by means of the mandibles and front legs. When *Atta vollenweideri* workers rake moist clay, they rely mostly on pulling the material together with closed mandibles in the manner of an ice-cream scoop. As soils tend to be softer at higher water content, both grabbing and raking can be expected to be easier at higher moisture. The resulting soil pellets are then transported to the surface. In some ant species that inhabit sandy desert soils, workers can rely on specialised structures (e.g. psammophores) to keep their loads of sand grains together. Nonetheless, it is only in moist sand that particles can be aggregated for transport, probably resulting in much higher transport rates (Délye, 1957, 1971). In *Atta vollenweideri*, loads are transported sequentially, i.e. the excavator disposes of the carried soil pellet close to the digging site, where other individual picks a single pellet up for further transport (see Chapter 7). Workers do not have psammophores or comparable structures to carry soil pellets. They simply hold their loads in between their mandibles. Accordingly, the ants can only carry more than single crumbs of soil if they are moist enough to stick together.

During grabbing and raking the soil, and even slightly before, *Atta vollenweideri* workers stridulate to attract nest mates to excavate at the same location (see Chapter 8). The range of a vibrational communication signal is another factor potentially influenced by the physical properties of the substrate as conductor material. As moister soils tend to be softer, it can be expected that they have higher attenuation rates for substrate vibrations, resulting in a decreased range of signal transmission.

In addition to the potential influences on the excavation process, soil moisture remains an important environmental factor once the construction is completed. Thereby, it can hypothetically also serve as a cue that allows workers to enlarge the nest at suitable locations, and avoid digging in unsuitable soils. Leaf-cutting ants prefer high air humidities for fungus culturing (Roces and Kleineidam, 2000). Establishing fungus chambers in a soil with an extremely low water potential may therefore be disadvantageous. On the other hand, excess water can also be disadvantageous for nest building, if, as a result, water penetrates and accumulates inside nest cavities. Some ant species are known to avoid locations with high soil moisture for nest building (Brian et al., 1966; Elahi, 2004). Workers of the red fire ant *Solenopsis invicta* Buren were even demonstrated to have an increased mortality at higher soil water content (Xu et al., 2009).

In the Gran Chaco region, the clayish soil does not allow large quantities of rainwater to rapidly percolate, regularly resulting in temporal flooding of wide areas. Flooded pastures and even deeper temporal ponds regularly appear in the immediate vicinity of *Atta vollenweideri* nest mounts (Fig. 7B-D). While the clayish soil structure may prevent water percolation into the underground nest cavities, it can be expected that architectural and behavioural adaptations are necessary to prevent water inflow via the nest openings. In mature colonies of *Atta vollenweideri*, most of the nest openings are usually located above the local high water mark, particularly because they occur on top of the nest mound, or at least on the slopes (Jonkman, 1980). Small colonies close all their nest openings during rainfalls, with the side effect that carbon dioxide concentration inside the nest increases significantly (Kleineidam and Roces, 2000).

It is unknown when and how new tunnels and nest openings are

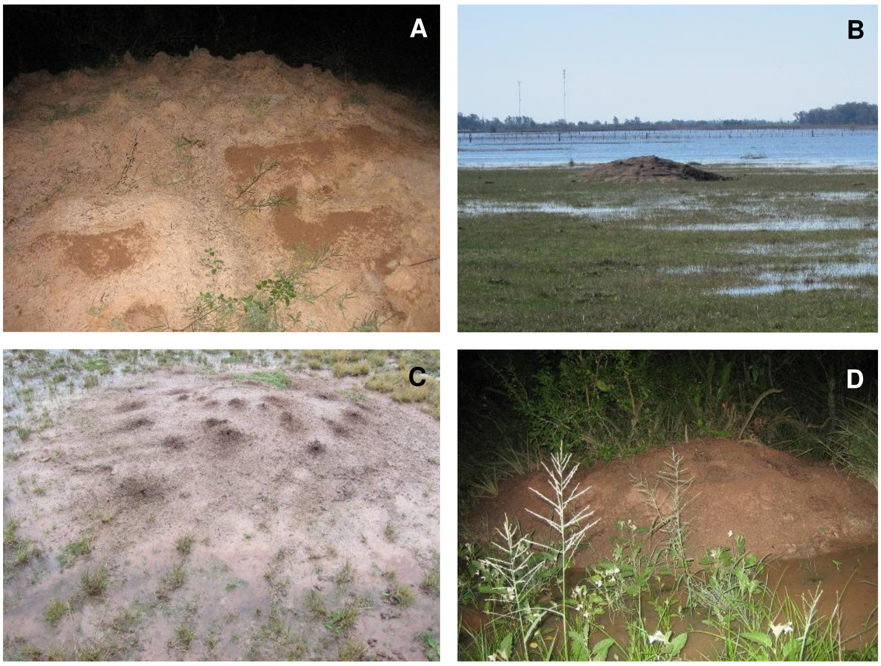

Figure 7: Water around and inside *Atta vollenweideri* nests. (A) A nest mound at the Reserva El Bagual, Formosa, Argentina. Visible are the accumulations of freshly deposited, moist soil pellets carried up from the underground by workers during the night. The moisture of the freshly deposited pellets is often higher than that of the surrounding soil at the nest surface, giving them a darker colour. (B) *Atta vollenweideri* nest in a flooded meadow near Villa Soriano, Uruguay (Photo: Martin Bollazzi). (C) Close up of another nest mound in the same region. The nest openings remain above the water level in the flooded meadow (Photo: Marin Bollazzi). (D) Another nest from Formosa, located beside a seasonal pond that is filled with water after spring rain.

constructed during nest growth. However, if nests mostly grow in the seasons associated with temporary accumulations of surface water, there might be an imminent risk that new nest tunnels excavated from below hit on ponded water, with a concomitant water inflow into deeper nest chambers. Workers excavating new tunnels could hypothetically avoid the excavation underneath ponded water if their spatial memory and orientation abilities allow them to know the locations of water ponds at the surface when digging in the underground. That seems unlikely, given the size and geometrical complexity of a mature *Atta vollenweideri* nest. Alternatively, the ants could use the steep increase in soil moisture close to surface water as a cue to decide where to stop excavating. This leads to a situation in which a vertical moisture gradient would provide information influencing the workers' decisions as to where to excavate.

The present work is aimed at investigating the influence of soil moisture on the excavation and soil transport behaviour of *Atta vollenweideri* workers. This was done in a series of laboratory experiments using a standardised set of seven clay mixtures with increasing water content, representing a gradient ranging from relatively hard to almost fluid material, but generally between the plastic limit and the liquid limit. To evaluate the variation of physical properties within the used moisture range, we compared the mixtures in terms of bulk density, toughness, and tensile strength, all variables that potentially influence the ability of the workers to loosen the material and form pellets. The ability of these pellets to stick together for transport was quantified, as well as the attenuation rate of vibrational signals in the different mixtures.

To investigate how soil moisture influences digging decisions, the response of ants to the different materials was investigated in terms of individual digging preferences, group-level excavation rates, and propagation range of vibrational signals produced while digging. The latter was considered as a measure of recruitment intensity. To investigate whether the observed digging responses can be exclusively interpreted as a preference for materials easy to manipulate, we evaluated the influence of soil moisture on excavation performance considering both digging and soil transport. Finally, we investigated whether workers stop excavating an upwards tunnel when approaching accumulated surface water, to prevent water inflow into the nest. Thus, groups of workers were allowed to extend a tunnel upwards in a clay tube with accumulated water on top of the clay.

5.2 METHODS

Animals and Materials

Animals for the behavioural experiments were obtained from two different laboratory colonies of *Atta vollenweideri*, excavated at an age of approximately 8 months at a privately owned site, the *Reserva El Bagual*, San Francisco de Laishi, Formosa, Argentina (26°17′08″ S; 58°49′43″ W), with the permission of Pablo Götz (owner) and Alejandro Di Giacomo (supervisor of the *Reserva*). *Atta vollenweideri* is widespread in the area and not protected under the Convention on International Trade in Endangered Species (CITES). After excavation, colonies were transferred to the laboratory and kept under controlled conditions at 25°C and a 12:12h LD cycle.

Experiments were conducted using 7 different standardised clay-water mixtures, made from industrial clay with a maximum particle size of 0.5 mm (CLAYTEC *Lehm gemahlen 10.001*) and varying only in water content. The gravimetric moisture contents (u) in each of the mixtures were 14%, 16%, 18%, 20%, 22%, 24% and 26% of the overall mass. Within this moisture spectrum, the physical properties of the material change considerably (Fig. 8A-D). However, it represents the maximum range of water contents that allows for the construction of the experimental setups described below. At 14% soil water content, the material used in this study was dry and tough, but it could still be formed and pressed into vessels without breaking. With less water, the material becomes very brittle and impossible to mould homogeneously into the experimental setups. At the maximum soil water content of 26%, the soil was sticky with a clearly developed water film at the surface, but still consistent enough to stay in place in an experimental setup. With higher water content, the soil resembled a thick fluid, making it impossible to create firm cavities. Accordingly, the moistures contents used in the experiment likely represent most of the range from the plastic limit to the liquid limit of the material. The described properties and moistures are not directly comparable to field conditions (Cosarinsky and Roces, 2007). Within the distribution range of *Atta vollenweideri* some variation in the properties of the soils can be expected, they are probably harder or softer at the same water content according particle size distributions at a specific location. Therefore, results obtained from the experiments described here do not allow for a quantitative extrapolation to field conditions. Depending on the location, soils of a specific moisture content can be expected to have other physical properties. The range of clay mixtures used in this study was intended to represent a moisture spectrum in which the most important transitions in physical properties take place. In other soils, these changes likely happen at lower or higher soil water contents. Some specific physical properties were determined in a series of experiments described below.

Statistical analysis was done in R. 2.14.1 (`http://www.R-project.org`). For the sake of clarity, specific methodological details of the different experiments will be presented together with their results.

Material properties

First, we quantified bulk density, toughness, ultimate tensile strength, pellet stickiness, and the attenuation rate of vibrational signals for each of the used clay mixtures.

Bulk density was determined by filling a 200 ml vessel with the material and by measuring the mass at nearest 0.1 mg. Density was calculated dividing the measured mass by 200 ml. Measurements were repeated five times for each of the seven mixtures.

To acquire an estimate of toughness, a plastic box of 9 x 9 x 6 cm was filled with the material to be measured. Then, an aluminium cuboid with a footprint of 1 x 1 cm was pushed into the soil from above with a constant force of 10 N. The penetration depth of the cuboid was measured at nearest 1 mm and used as a measure for soil toughness. Again, each measurement was repeated five times for each of the seven mixtures.

To measure ultimate tensile strength, two plastic tubes with an inner diameter of 22.1 mm were taped together and filled with the material. Then, the tubes were held vertically and the upper one was fixed on a retort stand. The tape was removed so that the lower of the two tubes was connected to the other one and held above the ground exclusively by the clay. A plastic box was attached to the bottom tube and slowly filled with sand. When the clay connection between the two tubes broke, the entire mass of the lower part of the construction, i.e. of the clay-filled tube plus the attached box and the amount of sand inside it, was measured at nearest 0.1 mg. This mass was considered the limit of the tensile force that can be held by the material. To calculate strength in N/cm^2, this mass was multiplied by a gravitational force of $9.806\ m/s^2$ and then divided by the cross section of $3.85\ cm^2$.

The stickiness of the clay mixtures was assessed by determining if pellets of a particular moisture content adhered to the surface of the same material when held upside-down. For this experiment, a standard plastic Petri dish was filled with clay and the surface was smoothed. In separate box, ants were allowed to excavate the same material. Fresh pellets were taken and dropped onto the surface in the Petri dish from a height of about 1 cm. One hundred pellets were placed in that manner in one Petri dish. The Petri dish was then turned upside-down and held with the pellet-covered soil surface facing downwards. The proportion of pellets sticking to the soil surface without falling down was counted. This measurement was repeated 10 times for each of the seven moistures.

The attenuation rate of stridulatory signals, which are involved in digging behaviour, was determined for the different materials by measuring the remaining amplitude of an artificial wave of known original amplitude at different distances from the source. Attenuation in dB was then calculated at each distance, so as to determine a distance-dependent damping rate by regression analysis. The procedure will be described in detail in Chapter 8.

Moisture preference

To investigate the preferred clay moisture to initiate digging in *Atta vollenweideri* workers, their responses when presented with different soil moisture contents were quantified in two ways. In a choice experiment, all seven mixtures were presented simultaneously to determine the ants' preference to initiate digging. In a separate no-choice experiment, workers were presented with a single clay mixture and their stridulation rate while excavating was examined as a measure of recruiting intensity.

To investigate moisture preference, seven sample holders were prepared by cutting 1 cm long pieces from the top ends of photometric plastic vials. Each sample holder was filled completely with one of the clay mixtures. The clay faces were smoothed and the sample holders circularly arranged in a closed 5.5 cm diameter Petri dish (a picture of the setting is shown in Fig. 9A). The dish had a hole in the centre of the bottom side that allowed the connection of flexible tubing with an inner diameter of 1 cm. The sample holders were arranged in a gapless circle with their clay faces oriented towards the centre hole and held in place with small pieces of play dough. Within that arrangement, the clay samples were sorted according to soil water content, the moisture increasing in clock-wise direction. The ants entered the experiment through a tubing that connected an entire laboratory colony to the hole in the bottom of the Petri dish. Each replicate of the experiment was started by connecting the colony to the setup. The first occurrence of excavation behaviour at one of the samples was considered as a decision for this particular mixture.

This method, considering only the first response of the first worker within a group, allowed the observation of individual decisions without separating individuals from the colony and removing them from their social context. After a decision was observed, all parts of the setup were cleaned, the sample holders were refilled and the procedure repeated. Workers that had been within the setup during one replicate of the experiment were kept separately from the colony for the duration of the entire series. After each replicate, the position of the clay mixtures relative to the laboratory colony was rotated counter clockwise by 1/7 of the full circle, to rule out possible effects of the workers' walking direction when coming towards the set up on their preferred digging reaction. The whole procedure was repeated for $n = 56$ times.

Stridulation rates of excavating workers were measured for each moisture by carefully separating excavators in plastic vials, recording stridulation by means of an attached *Bruel & Kjær* Type 4333 accelerometer, and counting the stridulation chirps produced per second while digging. The employed method will be described more detailed in Chapter 8, that specifically deals with stridulation behaviour. For each mixture, $n = 10$ workers were observed.

Differences in signal propagation that occur with varying moisture complicate measurement of stridulation rate. In materials with higher attenuation rates, stridulation signals are more likely to remain below the noise level and thereby be hidden from the observer. To compensate for the potential effects of different propagation properties, a reference measurement was conducted that replaced the ants in the experiment by a standardised playback signal. This artificial signal was applied to the clay surface in the experimental setup by means of an aluminium rod (55 mm long, 5 mm in diameter) connected to a *Bruel & Kjær* Type 4810 mini shaker. The volume of the reference track was scaled so that the strongest signal in the track had a peak-to-peak amplitude of 8 cm/s^2. The playback sample was 10 s long and contained 138 stridulation chirps. With each of the clay mixtures, the sample track was recorded again, and stored in a wave format audio file. The sound files were blinded for analysis, i.e. the files were randomly renamed by one person, while another person counted the signals. While no signal loss occurred at 14%, 16% and 18%, the number of counted signals decreased from the original 138 to 126 at 20%, 116 at 22%, 107 at 24% and 88 at 26% soil moisture content. The numbers were divided by the maximum of 138. This proportion was provided the mixture-specific correction factor for signal counts.

Group-level performance

To measure group-level excavation rate as a function of soil water content, subcolonies, i.e. groups of workers with a fungus garden, were detached from the main colony and allowed to excavate in one of the mixtures, without the possibility to choose alternative moistures.

In addition to the preference of workers that were able to choose, as described above, we investigated how group-level excavation rate is influenced by soil moisture if only a single mixture was available to the ants. Comparable experiments in several ant species, usually distinguishing only 2-3 levels of water content, reported faster excavation rates at higher moistures (Sudd, 1969; Mikheyev and Tschinkel, 2004; Espinoza and Santamarina, 2010). We temporarily separated subcolonies with a box of fungus garden from the large laboratory colonies, and connected single subcolonies to a box entirely filled with a given clay mixture. The amount of soil removed from that clay box within 48 h was measured.

Subcolonies were obtained by detaching small plastic boxes of 9 x 9 x 6 cm that were completely filled with fungus garden from a large colony. Accordingly, both the number of workers and the amount of fungus in the subcolonies was only roughly standardised. We preferred that method over precisely determining the size of the subcolonies, because opening the fungus chambers to obtain material and separating ants from fungus for counting is so disturbing that the fungus may have reduced growth or even die if would have been done too often. To have a rough estimate of the number of workers in such a box, one was opened, the fungus taken apart and the number and size distribution of the workers inside investigated. As an example, it contained 912 workers, with a size distribution strongly biased towards small individuals. Even though this subcolony housed 397 workers larger than 0.9 mg, with 0.9 mg being the smallest worker size observed digging in later experiments, this number was considered as a sufficient work force for single digging experiments. The boxes were connected to the main colony for at least three days in advance before separation, then connected to the experimental clay box (18 x 9 x 6 cm) and an empty box for soil deposition (9 x 9 x 6 cm). The clay box was weighed before the experiment and 48 h after the subcolony had been connected. The experiment was repeated 51 times.

During the 48 h experiments, the clay in the boxes desiccated considerably. Because of variability among the replicates, even though the clay was initially prepared with defined moisture, it was impossible to assign each result to one of the initial well-defined mixtures, as for the other experiments. Therefore, moisture inside the boxes was measured after the experiments employing the "wet and dry" method, i.e. taking a sample of about 400 g and determining its wet mass, its dry mass and comparing the two.

Individual work performance

The effects of varying moisture and the associated changes in physical properties on individual work performance were investigated by comparing pellet size and working speed in both soil excavators and soil carriers. Excavators usually carried the pellets for few centimetres only. Other workers picked them up later on and covered the remaining distance to the nest opening. Accordingly, when looking at individuals, it is useful to distinguish soil excavation and soil transport as separate tasks[1]. We quantified digging and transport rates, tasks that require measuring the speed at which each item is processed (i.e. excavated or carried), and the mass of the item, using three different experimental setups.

In the first experiment, both pellet mass and pellet digging rate were quantified for excavating individuals. Seven plastic boxes of 9 x 9 x 6 cm were used, each one filled with one of the clay mixtures. The clay in the boxes was 2 cm high only, leaving sufficient space for ant workers to move in the box on the clay surface where they still could be gently removed with tweezers for further measurements. A laboratory colony was connected to the box, whereupon workers entered it and started excavating. This construction allowed for the observation of individual excavators. The time they needed to excavate a soil pellet was measured, the animals and the produced soil pellets were collected and weighed. The number of observed workers at each moisture was $n = 20$, with the exception of 20%, where $n = 40$ workers were collected. Additional pellets were collected directly from their excavators for a more accurate measure of average pellet mass in between behavioural observations. As a result, about 70 pellets from each clay mixture were available for weighing.

The observation of soil transport speed was done in a different setup that allowed workers to excavate at the end of a clay tunnel and then

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[1] See Chapter 7

walk through that tunnel carrying the excavated pellet. This tunnel was prepared in a clay layer between two horizontal glass plates of 20 x 10 cm. The tunnel started at the centre of one of the short sides of the construction, leading inwards parallel to the long sides for 15 cm. The tunnel had a quadratic cross section of 1 x 1 cm, and was located at the upper glass plate. The entire clay layer was 1.5 cm deep, thereby leaving 5 mm of soil between the ground of the tunnel and the bottom glass plate for the ants to walk on. Through the top glass plate ant workers were observed carrying soil pellets along the tunnel. This allowed the distance and duration that pellets were carried to be measured and the walking speed of the carriers to be calculated. The experiment allowed a large number of transport events to be observed ($n = 83$ at 14% and 18%, $n = 84$ at 24% and $n = 100$ at 16%, 20%, 22% and 26%), but not allowed the pellets to be collected afterwards for weighing.

Carried pellets were sampled, in yet another setup, by filling entire plastic boxes with clay. These were connected to a second, open plastic box via 50 cm of tubing. The ants had to pass this second box on their way back from the clay box to the colony. Here, pellets were collected when carried out of the tubing and weighed. The rates at which pellets were carried out of the tubes differed considerably according to soil water content, resulting in different sample sizes between 11 and 24 for each clay mixture.

The results of these measurements showed no linear or exponential dependence on moisture. The water content was therefore treated as a factorial variable during the statistical analysis of these experiments, and the seven moisture grades from 14% to 26% as levels of that factor. As a result, the influence of moisture on the measured variables was tested with ANOVA, and differences in between the particular levels with Bonferroni-corrected pair-wise t-tests. Comparison of the masses of excavated and carried pellets was done by directly comparing the data sets level-wise with Welch two-sample t-tests. All data sets were ln-transformed in advance to compensate for light skewness.

Prevention of water inflow

To investigate whether ant workers would continue to dig upwards irrespective of increasing soil moisture until meeting accumulated water or would stop before doing so, the situation of tunnelling upwards towards a seasonal accumulation of surface water was simulated in a laboratory experiment. Plastic tubes of 10 cm height and 4.5 cm inner diameter were filled 6 cm high with clay of 20% soil water content. A 1 cm clay layer with 26% moisture was added on top, and water was then carefully poured 2 cm high onto the soil. In a control series, no water was added on top of the soil. A drawing of the setting is shown in Fig. 5c. Before the experiment started, the filled tubes were stored for 24 h to establish a moisture gradient. The tubes were covered with plastic wrap for that time to minimise evaporation losses. For each replicate, 100 ant workers were placed in a plastic box of 9 x 9 x 6 cm. The box was connected to a T-shaped tube, one end pushed into the clay from below, allowing the ants to dig into the clay, the other end pointing downward. The bottom opening of the tube was closed with a fine metal wire netting, so that potentially inflowing water would not drown the ants. After 24 h, the ants and the water layer were removed from the setup. Beginning at the top, the soil was removed centimetre by centimetre. From each centimetre of clay, a sample was taken to determine soil water content, and it was noted how far the workers had excavated upwards. In addition, the dry mass of the excavated soil pellets deposited in the plastic box was determined. Sample size was $n = 17$ for the experiments with water, $n = 16$ for the control without water.

5.3 RESULTS

Material properties

Bulk density decreased from 2.2±0.1 g/ml (median±interquartile range, $n = 5$) at 14% to 1.9±0.1 g/ml ($n = 5$) at 26% water content. This relationship was not linear. While decreasing constantly at a low rate from 14% to 18% and from 20% to 26%, bulk density was reduced considerably between 18% and 20% water content, indicating that the clay swells within that moisture range (Fig. 8E).

When measuring toughness, there was no measurable penetration of the material at moistures below 20%. At 20%, the aluminium penetrated the clay 1±0 mm (median±IQR, $n = 5$) deep. Penetration depth increased to 5±1 mm ($n = 5$) at 22% and finally to 23±3 mm ($n = 5$) at 24%. The 26% mixture was so soft that the aluminium, as well as the weight providing the force, sunk into the material completely until reaching the bottom of the clay box. The 26% clay must therefore be considered outside the scale of measurements.

Tensile strength decreased linearly[2] from 0.59±0.04 N/cm^2 ($n = 5$) at 14% to 0.32±0.03 N/cm^2 ($n = 5$) at 26% water content. Regarding pellet stickiness, 8.5±0.5% (median±IQR, $n = 10$) of the pellets adhered to the surface even at lowest moisture of 14%. The proportion increased to reach 100% at 22% soil water content and remained at 100% for higher moistures (Fig. 8E).

The method for measuring the attenuation rates of substrate vibration was successful at moistures ranging from 14% to 20%. Within that range, the attenuation rate increased from 0.5 dB/cm at 14% soil water content, over 1.1 dB/cm at 16% and 18%, to 2.6 dB/cm at 20%.

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[2] Linear regression: $TS = 0.97 - 0.02 * u$, $n = 35$, $R^2 = 0.40$, $p < 0.001$

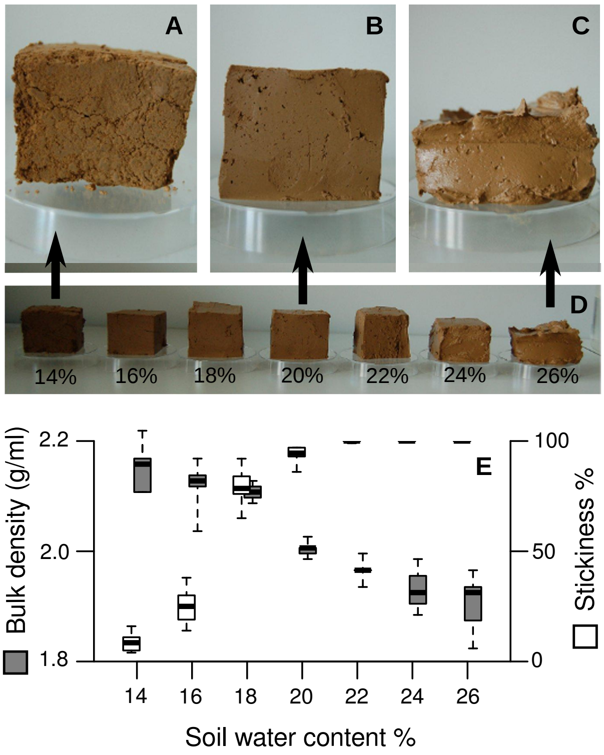

Figure 8: Clay mixtures used in the laboratory experiments. The soil water content in the seven mixtures ranged from 14% to 26% of the overall mass. (A-D) Samples of clay with 14% (A), 20% (B) and 26% (C) water content in close up, and the entire spectrum of mixtures used in this study (D), ranging from relatively hard and brittle to very soft and sticky material. The samples depicted here varied in mass. (E) Change of physical properties as a function of water content within the investigated range. Grey boxes represent the material bulk density of the material, scaled at the left Y-axis, white boxes represent the stickiness, i.e. the percentage of pellets from that mixture that stick to a surface of the same material. Stickiness values are scaled at the right Y-axis. Bars within the boxes indicate median values. The box shows the range from 25% to 75% quartile. Whiskers indicate minimum and maximum values without outliers. Sample size was $n = 10$ for each measurement.

The average signal peak-to-peak amplitude of a stridulation from a *Atta vollenweideri* digging worker was 9.6 cm/s^2, measured at a distance of 1 cm from the source. The reported maximum amplitude averages 21.7 cm/s^2 (see Chapter 8), with a minimum physiological detection threshold for surface vibrations of about 4 cm/s^2 in *Atta cephalotes* (L.) workers (Markl, 1970). Accordingly, the range of an average signal decreased with increasing moisture from 8.6 cm at 14% soil water content to 2.5 cm at 20%. The potential range of the strongest signal decreased from 15.7 cm to 3.8 cm within the same moisture range.

At higher moistures, amplitudes remained below the noise level beyond a distance of one centimetre, so that the measurements were insufficient to calculate attenuation rates from regression analysis.

Moisture preference

When presented with all seven mixtures simultaneously (Fig. 9A), ants mostly chose 24% clay moisture. This occurred 21 times, equalling 37.5% of the 56 experiments. 22% soil water content was chosen second most often, 17 times altogether (i.e. in 30% of the experiments). In general, preference, i.e. the number of decisions for a particular mixture, increased with increasing moisture (Fig. 9B), with the remarkable exception of 26% clay, that was chosen only 3 times (5% of the replicates). The observed distribution of choices deviated significantly from an even distribution (χ^2-test for goodness of fit: $\chi^2 = 46.2$, $df = 6$, $p < 0.001$). In summary, preference depended on soil water content and increased with increasing moisture, but was very low at the highest soil water content of 26%.

Stridulation rates decreased with increasing moisture (linear regression, signal counts were *ln*-transformed to obtain normal distribution in the residuals: $F_{1,68} = 10.5$, $p = 0.002$). Average stridulation rate decreased from 9.7±3.1 signals/s (median±IQR, $n = 10$) at 14% moisture to 6.4±2.0 signals/s ($n = 10$) at 26%.

Group-level performance

Concerning the group-level excavation rate, results showed an increase of the cumulative excavated mass towards the middle of the tested moisture range, with a maximum of 136 g at 20.3% soil water content, and a minimum of only 4 g at the highest moisture of 26.0%. In four cases, fungus had been relocated into the clay box resulting in small cavities emerging around the fungus garden in the soil. As the direct presence of fungus can be expected to influence excavation rates (Fröhle, 2009; Fröhle and Roces, 2009), these replicates were not further considered.

Identification of the moisture leading to the highest average group-

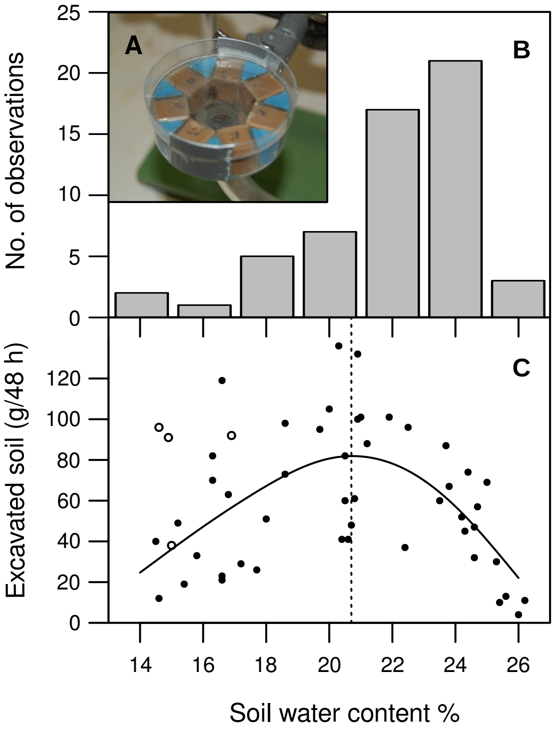

Figure 9: Individual preference and group-level performance. (A) Experimental setup for choice experiments on moisture preference. Seven soil samples of different water content were presented simultaneously to the colony. The first digging reaction on one of the freshly prepared samples was counted as a decision for that moisture. N = 56 decisions were observed. (B) Number of decisions for one of the presented soil samples as a function of moisture. (C) Group-level excavation effort as a function of soil moisture. White circles represent replicates in which workers relocated part of the colony fungus into the excavation chamber. These were not considered for statistical analysis. The black line shows the prediction of the generalized additive model fitted to the data (Deviance explained = 41.1%, GVC score = 772.13, Scale estimate = 711.7, N = 47). The dotted line depicts the maximum predicted by the model.

level excavation rates was achieved by fitting a generalized additive model (GAM) and computing the maximum of the predicted values and the corresponding moisture. Moisture was treated as a linear explanatory variable for the amount of excavated soil. According to the model, the excavated amount of soil was significantly influenced by soil water content ($n = 47$, $F_{2.7,3.3} = 8.0$, $p < 0.001$). The fitted curve (the model explains 41.4% of the deviance) reached a maximum at 20.7% water content with a predicted value of 81.9 g (Fig. 9c).

Individual work performance

Excavated and carried pellets had a similar mass of 2.2±2.1 mg at 14% and 16% soil water content (median±IQR, $n = 174$). At moistures larger than 16%, carried pellets were significantly heavier when transported than when freshly excavated (Fig. 10a; for statistical details see Appendix A, Tab. 6), with a mass of 4.9±3.2 mg ($n = 94$) for carried, and 2.7±2.3 mg ($n = 353$) for excavated pellets. In both excavated and carried pellets, mass was significantly influenced by moisture (one-way ANOVA for freshly excavated pellets: $F_{6,486} = 8.7$, $p < 0.001$; carried pellets: $F_{6,121} = 5.3$, $p < 0.001$). The heaviest pellets were excavated and carried in mixtures with 22% moisture, with the excavated pellets averaging 3.7±2.7 mg ($n = 74$), the carried ones 5.2±3.1 mg ($n = 20$).

The time spent to excavate a pellet was also influenced by moisture (one-way ANOVA: $F_{6,137} = 23.9$, $p < 0.001$). It took longest at 18% soil water content, in average 60.3±41.6 s (median±IQR, $n = 20$). In dryer soils, the time invested to form a pellet was only 40.7±24.4 s ($n = 40$), while at higher moistures, it decreased with increasing water content, to 9.3±4.1 s ($n = 20$) at 26% (Fig. 10b; see Appendix A, Tab. 7 for detailed statistics).

The individual excavation rate for each pellet, calculated by dividing pellet mass by the time spent to excavate it, also depended on moisture (one-way ANOVA: $F_{6,137} = 23.9$, $p < 0.001$). The rate decreased from a median of 0.07±0.13 mg/s (median±IQR, $n = 20$) at 14% moisture to 0.03±0.03 mg/s ($n = 20$) at 18%. At higher moistures, the rate increased again, remaining relatively constant at 0.20±0.16 mg/s ($n = 64$) at a moisture equal or higher than 22% (Fig. 10c; see Appendix A, Tab. 8 for detailed statistics).

Median worker mass was 3.0±1.8 mg (median±IQR, $n = 160$). After *ln* transformation of body mass, a negative correlation of worker mass and moisture could be demonstrated by linear regression analysis ($F_{1,158} = 85.3$, $p = 0.022$), yet, though significant, the effect was weak[3]. Median body mass decreased from 3.1±1.1 mg ($n = 20$) at 14%, to 2.3±0.9 mg ($n = 20$) at 26% soil water content.

Walking speed of pellet carriers was also influenced by moisture (one-

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[3] $m = 4.39 * e - 0.02 * u$, $R^2 = 0.03$

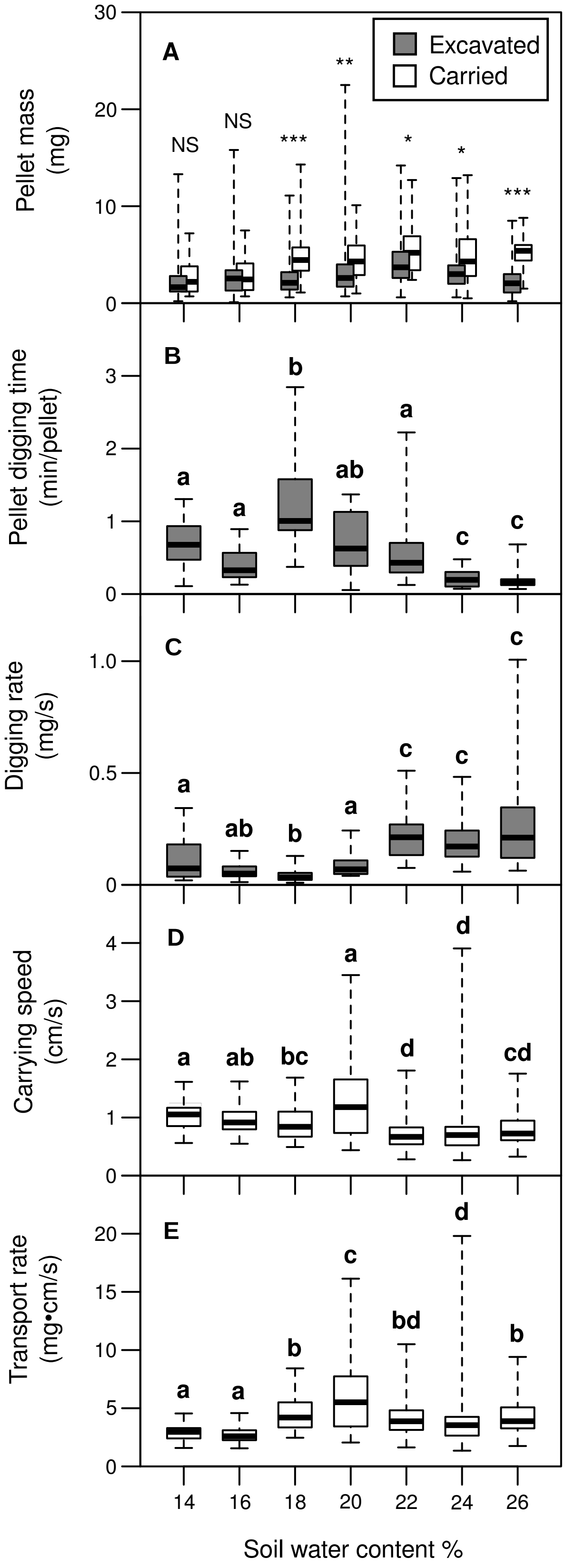

Figure 10: Excavation and transport performance as a function of soil water content. See Appendix A, Tab. 6-10 for detailed statistics. composed the underground the workers had to walk on. (E) Transport rate calculated as the product of carrying speed and average pellet mass. Boxes labelled with the same letter are not significantly different. Details on the *post-hoc* comparisons are provided in the Appendix A, Tab. 7 – 10.

way ANOVA: $F_{6,643} = 33.5, p < 0.001$), the maximum being 1.2±0.9 cm/s at 20% (median±IQR, $n = 100$). On average, carriers moved at a slightly faster pace (0.9±0.4 cm/s, $n = 266$) at moistures below 20% than they did at moistures above 20% (0.7±0.3 cm/s, $n = 284$; Fig. 10D; see Appendix A, Tab. 9 for detailed statistics).

As it was not possible to weigh the pellets actually carried during the speed measurements, transport rates had to be calculated by multiplying the measured walking speeds with the moisture-dependent median pellets masses for carried pellets described above. The transport rates depended on soil water content too (one-way ANOVA: $F_{6,643} = 49.1, p < 0.001$). As for the carrying speeds, the maximum was observed at 20%, in this case 6.0±3.0 mg*cm/s (median±IQR, $n = 100$). However, transport rates were generally lower in dry soil of 16% water content and less, averaging 2.7±0.9 mg*cm/s ($n = 183$), as opposed to 4.0±2.2 mg*cm/s ($n = 467$) at moistures of 18% or higher (Fig. 10E; see Appendix A, Tab. 10 for detailed statistics).

Prevention of water inflow

Simulating a situation with temporal accumulation of surface water above an underground nest structure in the laboratory (Fig. 11A-C), the observed excavated structures were exclusively vertical tunnels leading upwards through the clay. This tunnel reached the surface in all but two replicates (14 of 16) of the control series, while excavation was always stopped below the surface when water was present, at an average depth of 1.2±0.5 cm (median±IQR, $n = 17$; Fig. 11D-E), with the resulting tunnels being significantly shorter than the control ones (Wilcoxon rank sum test: $W_{17,16} = 1127, p < 0.001$). However, the amount of soil excavated in the control series was not significantly higher (a mean of 15.1±11.7 g with water, 13.8±8.4 g without; Welch two-sample t-test: $t_{29.017} = 0.4, p = 0.727$), indicating that tunnels were not shorter just because the workers excavated at a lower rate under the conditions encountered when water was present at the surface. Soil water content was 26.5±1.0% (median±IQR, $n = 17$) in the uppermost soil layer when covered with water. One centimetre below, moisture strongly decreased to 20.5±0.3% ($n = 17$), evincing a very steep moisture gradient. In the controls, the moisture gradient started with 22.7±1.0% ($n = 16$) at the surface and decreased to 19.8±1.1% ($n = 16$) at 1 cm depth. To calculate the moisture value at which the ants stopped tunnel excavation in the experimental series, a four-parameter logistic model was fitted with the measured soil water contents (u in % of the wet mass) as the dependent variable, and average depth (d in cm below the surface) as linear predictor. The upper asymptote was assumed to equal a water content of 100% above the soil layer. As soil water content was measured as average values for a layer of 1 cm, the mean between the upper and the lower limit of the measured layer was used as a value for average depth, e.g. 0.5 cm for the upper layer from 0 – 1 cm, 1.5 cm for the next layer between 1 and 2 cm, and so on. According to the model, soil water content at 1.2 cm depth, where the tunnels ended in average, had an expected value of 21.1%[4].

5.4 DISCUSSION

Transport rate and colony-level performance

Our results showed that, within the offered range of clay moistures, group-level excavation rate reaches a maximum at 20% soil water content. However, in the choice experiment, workers clearly preferred 22% and 24% moisture over 20%, indicating that the maximum group-level excavation rate cannot simply be explained by individual preferences. Likewise, digging rate at the individual level was still comparably low at 20%, not reaching its plateau until a moisture of 22%. While neither preference, nor digging performance can account for the highest group-level excavations rates that occur at 20%, transport rate, intriguingly, had its maximum at the very same moisture. It is therefore argued that the rate at which ant groups remove soil from a box, a measure regularly used in laboratory experiments to asses ant excavation rates (Mikheyev and Tschinkel, 2004; Rassé and Deneubourg, 2001; Bollazzi et al., 2008; Chen, 2009), is limited mainly by the rate of soil transport rather than by the rate of loosening it from the surrounding walls. This view is supported by the observation that soil pellets accumulate inside nests when workers excavate (Fröhle, 2009; Fröhle and Roces, 2009), indicating that soil pellets are usually produced at a rate higher than their rate of removal from the digging site.

Transport rates are probably higher at higher soil water contents due to soil pellets sticking together, thereby allowing the formation of larger loads. Material stickiness strongly increases between 16% and 18% water content in the laboratory clay mixture. Comparing the masses of freshly excavated pellets and carried loads revealed the latter to be significantly larger, but only for moistures of 18% or larger, indicating that soil particles are carried in larger loads if they are sticky enough to be aggregated. In our study, that increase of load size had, for six of seven tested mixtures at least, a stronger effect on transport rates than the observed differences in carrier walking speed. The only exception was the clay with 20% water content, where large loads additionally combined with exceptionally fast carrying, resulted in the highest average transport rates observed during the experiments. It is possible that carriers moved at the fastest pace

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[4] Four-parameter logistic model: $u = A + ((B - A)/(1 + e^{(-(D-d)/C))})$; with $A = 100$, $B = 20.2$, $C = -0.38$, $D = 0.35$; $F_{2,118} = 274.4$, $p < 0.001$

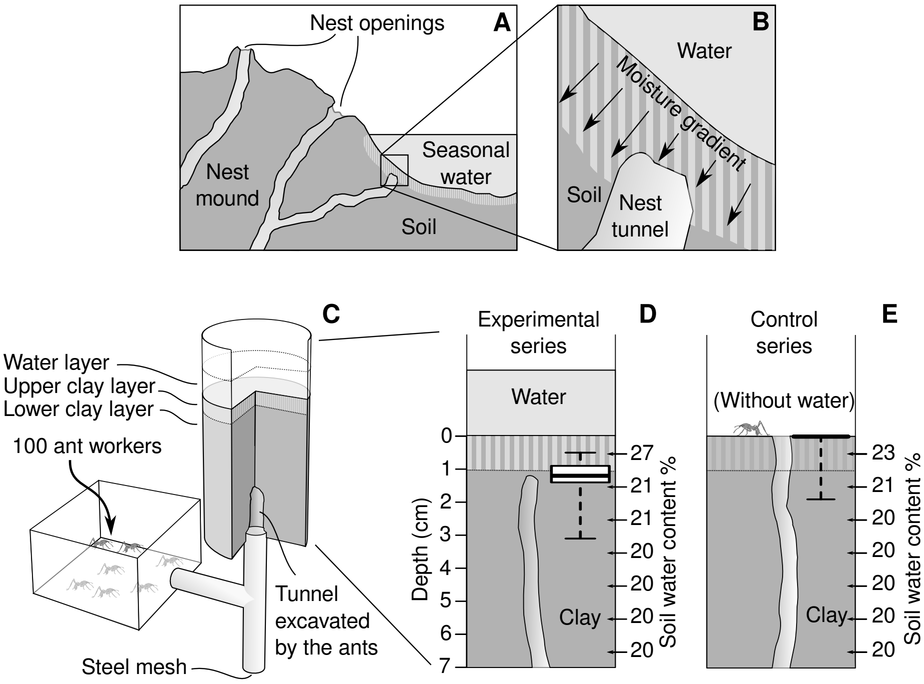

Figure 11: Prevention of the inflow of surface water. (A and B) Hypothetical situation in a field nest when water remains at the surface after a rain: a tunnel being excavated upwards to the surface reaches a layer of soil infiltrated by surface water. Further extension of the tunnel would result in water inflow into the deeper parts of the nest. (C) Experimental setup to simulate that situation in the laboratory. (D and E) Result of the experimental series and the control series without water: position of the tunnel end after 24 h. The left Y-axis represents the depth below the soil surface, the right Y-axis the median soil water content. Bars within the boxes indicate median values. The box shows the range from 25% to 75% quartile. Whiskers indicate minimum and maximum values without outliers. Sample size was $n = 17$ for the experiment and $n = 16$ for the control. Ant drawings were made by Karin Moll.

on the 20% soil due to improved adhesion/detachment while walking, which would also be influenced by soil water content. However, it remains an open question.

Recruitment by means of stridulation

Atta vollenweideri workers stridulate while excavating and thereby attract nestmates to dig at the same location (see Chapter 8). Our experiments have shown a negative influence of soil moisture on signal transmission. Maximum range decreased from about 15.7 cm to 3.8 cm with increasing moisture contents from 14% to 20%. In moister soils, attenuation was even too strong to be measured with the employed method. A positive influence of enhanced transmission of recruitment signals on group-level excavation rate was not observed. Considering the high group-level excavation rates at moistures of 20% and higher, it is concluded that hampered recruitment due to high attenuation rates at high moistures does not have a strong effect on overall group performance.

Moreover, the measured stridulation rates decreased slightly with increasing moisture, even after correction for signal loss due to moisture-dependent attenuation rates. Stridulation intensity thereby did not mirror the preferences of the workers. The weak decrease can possibly be explained by considering a poorer body-to-ground conduction on softer material: workers would apply more force when manipulating harder material, perhaps resulting in a better transmission of vibrations from the body to the material, while more signals are lost when the soil is softer. Such an effect cannot be completely compensated with the adjustments applied to the measured stridulations.

On the other hand, there are possible interpretations of increased stridulation rates in dry soils. Lower stridulation rates in moist soils can be interpreted as an adaptive response to poor transmission properties, if assuming that workers vary their stridulatory behaviour based on the actual soil properties, i.e. they stridulate less when the environment is less suitable for vibrational recruitment. Alternatively, high stridulation rates in dry soils can be seen as a reaction to materials that are harder to excavate, so that workers may increase their recruitment efforts to attract workers. Finally, the influence of soil moisture on digging stridulations can also be interpreted without considering any communicational aspect of stridulation behaviour. Spangler (1973) hypothesized that stridulation in digging Hymenoptera was a mechanism that mechanically facilitated the loosening of the soil to be excavated. Following that hypothesis, the excavation of dryer, harder soils possibly requires more stridulatory vibrations. Digging stridulations have been demonstrated to serve a communicational function in *Atta vollenweideri* [25], nonetheless, an additional mechanical function cannot be ruled out. However, regard-less of all the efforts to compensate for moisture-dependent attenuation effect in the present study, the observed influence of moisture on stridulation rates can still be a direct result of different propagation properties.

Inflow prevention

Workers excavating an upward tunnel below accumulated surface water were observed to stop digging before reaching the interface soil/water. Why do workers stop digging before reaching the accumulated water? The results of the previous series indicated that both excavation and transport rate are equally high at moistures higher than 20% soil water content. The choice of the individuals, if presented with different moistures, seemed to be dominated by the preference for materials that can be excavated at higher rates. In the choice experiments, most workers selected clay of 22% and 24% water. Both values fall into the moisture range that allows the highest excavation rate, indicating that preferring such a soil over a dryer alternative for digging can save time and, due to the decreased toughness and tensile strength, possibly even reduce energy investments. But, although soils of 26% were excavated and transported at the same rate, workers rarely preferred that mixture. Additionally, the lowest group- level excavation rates were observed close to 26% soil water content, most probably as a result of avoidance by the workers, according to the above considerations.

Avoidance of the highest moisture presented cannot be simply explained by physical properties and their influence on work performance. It can be, nevertheless, possibly explained by considering high moistures as an indicator of proximity to water accumulations such as seasonal ponds, so that further excavation could in some cases provoke water inflow into the nest. Our experiment on the prevention of water inflow, which confronted workers with a very steep moisture gradient, demonstrates the ability of *Atta vollenweideri* workers to stop tunnelling upwards before reaching ponded water at the surface. Interestingly, although the 26% clay mixture was not completely rejected during the choice experiments, workers stopped digging at much lower moistures in all of the gradient experiments, around 21.1% based on our calculations, so that water inflow was successfully prevented. The average water content in the clay mixture decreased from 27% in the first centimetre to 21% in the second. It is therefore suggested that workers responded to the moisture gradient rather than to a specific threshold moisture to stop digging, and that they were able to distinguish moisture changes at a relatively small spatial scale. The comparison of the observed value, i.e. 21.1%, with the results of the choice experiment, where clays of 22% and 24% were highly preferred, suggests that workers indeed respond to the gradient rather than to a particular threshold value when deciding to stop excavation.

Admittedly, our measurements of the moisture gradient, yielding centimetre-wise averages, were of limited precision, and gave a poor representation of the moisture changes over distance. Nevertheless, the measurements show that the standing water does not percolate very deep into the clay, reinforcing the idea that ants need to respond to small-scale moisture gradients.

The situation discussed here, a tunnel excavated upwards in the direction of standing water that seasonally accumulates at the surface, is certainly a hypothetical one. Nonetheless it is plausible considering field observations, and the results of the gradient experiment clearly demonstrate the ants' ability to avoid inflow of surface water when digging towards accumulated water. It remains an open question how new nest openings develop during colony growth, but the experiment demonstrates that tunnels can be built from the underground to the surface even in the rainy season without the risk of excavating upwards underneath water that may inflow into the nest. It is important to emphasize that clayish soils, on the one side, may prevent surface water to enter the nest, but on the other side preclude a rapid water percolation in the case of partial nest flooding, rendering the flooded areas of the nest useless for prolonged periods. Because of the observation of water-free nest cavities immediately adjacent to ponded water in field *Atta vollenweideri* nests, Jonkman (1980) supposed that the inner walls of nest chambers were waterproof, coated with organic matter as reported for termites. However, micromorphological analyses showed no lining of the chamber walls at all (Cosarinsky and Roces, 2007). The capillary forces in the clay-heavy soil of the Gran Chaco are probably sufficient to prevent extensive water movement through soil layers. Other phenomena have been reported that can be interpreted as mechanisms to prevent water inflow in *Atta vollenweideri* nests. Young colonies with shallow nest mounds were observed to close their nest openings completely during rainfalls (Kleineidam and Roces, 2000). Older nests have higher nest mounds and the openings are usually located above the ground level. Within the nest mounds, blindly ending tunnels indicate former nest openings that have been closed completely and never used again during the ontogeny of the nest (Jonkman, 1980; Jonkman, 1980).

Water and nest ontogeny

Our observation that *Atta vollenweideri* workers prefer to excavate in moist soils that allow for high digging and transport rates, but avoid excavation beyond a particular point along an increasing moisture gradient, provides a framework for the understanding of some morphological features previously reported for *Atta* nests. Two old publications documenting systematic excavations of *Atta* nests described vertical tunnels leading into soil layers below the fungus chambers (Weyrauch, 1942; Moser, 2006). Other studies even described large cavities in a comparable position, roughly bell-shaped (Stahel and Geijskes, 1939, 1941; Jonkman, 1980), generally located at a soil layer deeper than the fungus chambers, and often morphologically different from the latter. Such vertical structures below the fungus chambers, with or without cavities, were not found in a number of other studies on the same and on other *Atta* species (Autuori, 1942; Jacoby, 1953, 1955; Moreira et al., 2004b,a; Andrade et al., 2005), and it remains an open question whether they occur only depending on particular local soil conditions. Remarkably, all these studies were conducted in southern Brazil, in the states of São Paulo and Rio de Janeiro. The authors of the studies that described such deeper structures, with or without cavities, excavated their nests in other regions (e.g. Suriname and Peru), and hypothesised that they reach down to the groundwater table, or at least to the depth the groundwater level had at the time of their construction. Stahel and Geijskes (1939, 1941) reported that deeper nest cavities in *Atta sexdens* and *Atta cephalotes* nests were often filled with water or at least reached into layers of moist soil. Some of them were reported to be filled with the colony's refuse, and accordingly referred to as refuse chambers, as Jonkman (1980) did with that type of cavity in *Atta vollenweideri*. Stahel and Geijskes (1939, 1941) distinguished the empty cavities from the refuse chambers, calling them *Zisternen* (cisterns), and argued that they represented water reservoirs for colony needs. They suggested that by having access to an underground water source, the colony would be able to regulate air humidity in the fungus gardens by carrying either water or wet soil up into the fungus chambers.

In the light of these descriptions, it can be assumed that under some circumstances, the extension of vertical tunnels in both directions, upwards and downwards, until reaching either the surface or a soil layer with drastically increasing water content, is a general pattern in the construction of *Atta* nests. A moisture gradient in the soil can be expected to occur close to the groundwater table as well. While stopping an upwards excavation after contacting very moist soil can prevent water inflow into the nest, as demonstrated in our experiment, a similar response during a downward excavation can account for the supposed pattern of refuse chambers or cisterns reaching down to the groundwater level. The described bell-shape of the cavities may be a result of the ants excavating downwards in the moisture gradient. As in our experiments, they are likely to excavate more at higher moistures but to avoid the layers surpassing a particular threshold, thus excavating further centrifugally from the end of the tunnel, following their moisture preferences. This mechanism provides, at least in some cases, a hypothetical explanation for the occurrence of giant chambers in *Atta* nests, which may remain empty or be used for the deposition of refuse. In that case, one environmental variable influencing the shape of the *Atta* nests would be the distribution of water in the soil, as Thomé (1972) suggested for the nests of *Messor ebenius*. Nevertheless, even though such responses to groundwater provide a persuasive interpretation of some aspects of *Atta* nest architecture reported in literature, the authors of the excavations poorly described how they actually measured the depth of the groundwater table, or whether they simply assumed its existence from their observations of the nest architecture. Thus, such interpretations remain purely hypothetical, and further investigations on the structure of field nests and soil features are required to fully understand the effect, if any, soil moisture has on the morphology of the deeper parts of the nest.

Atta vollenweideri nests were reported to grow mostly during the rainy season (Jonkman, 1980). Beside seasonal variation in the growth rate of the colony because of increased harvesting, our results suggest that increased growth may also result from workers digging faster at higher soil water content. Additionally, our results demonstrate that a moisture gradient provides a cue that prompts workers not to excavate further when tunnelling towards surface water. Accordingly, it can be argued that soil moisture influences nest growth in *Atta vollenweideri* in two ways: its annual variation is probably an important factor for nest growth in general by influencing excavation rates, and strong increases in moisture provide a local cue for excavating workers so as to stop tunnelling and prevent accidentally flooding the nest, the latter aspect likely being an important adaptation to the specific habitat of the Chaco leaf-cutting ant.

6

REGULATION OF NEST ENLARGEMENT

ABSTRACT According to field excavations, there is a correlation of nest size and colony size in subterranean ant nests. Laboratory studies have reported that excavation rates are regulated according to space demands that are in leaf-cutting ants determined by both worker population and the amount of symbiotic fungus maintained in the nest. Based on experiments with varying numbers of workers excavating nests, these studies assume that the increase of nest space due to digging activity acts as a negative feedback on the excavating individuals. Here, we present a laboratory study on the regulation of nest enlargement in the Chaco leaf-cutting ant that (1) presents a constant number of workers with prepared nests of different initial dimensions. (2) Tests for the existence of a negative feedback imparted by increasing space by artificially increasing nest volume during the digging process. (3) Investigates the effect on nest enlargement of the distributing a similar amount of space over three chambers instead of one. This was done in three-dimensional artificial nests that resemble young field nests, composed of a vertical tunnel and a spherical chamber. The response in terms of chamber and tunnel enlargement was observed for ten days with different initial conditions. Offering four different initial nest volumes, we observed both chamber and tunnel enlargement to depend on initial nest size, as long as there was a fungus garden in the nest. Artificially increasing nest space resulted in a reduction of digging rates, indicating that the increase of space during nest digging constitutes a negative feedback mechanism that regulates nest enlargement. However, the effect of present space on digging rates diminished over time, suggesting the involvement of other unknown feedback mechanisms. Finally, distributing space over three chambers led to drastically decreased excavation rates. This result is discussed regarding both possible effects of nest geometry on local conditions and the possibility that spatial memory influences digging decisions.

6.1 INTRODUCTION

Many ant species excavate nests in soil, which are usually organized in chambers big enough to hold food, brood items and the workers that tend them, and tunnels that connect chambers to the surface and to each other (Sudd, 1967, 1969). Considering the amount of soil ant colonies relocate during nest excavation, an important proportion of the colony's time and energy investments are expected to be devoted to digging behaviour. Since the space requirements change with the growth of the colony, i.e. with the increase in both worker and brood numbers, nest size should be adjusted over time to accommodate the increased population. Field excavations of ant nests revealed a strong correlation between population size and nest size in different species[1]. A similar positive relationship between worker numbers and nest size was also reported in laboratory studies on different ant species (Rassé and Deneubourg, 2001; Buhl et al., 2004; Halley et al., 2005; Fröhle, 2009; Fröhle and Roces, 2009), indicating that ants, at the level of the colony, are able to regulate the size of the nest in response to changes in the number of colony members. Possible regulatory mechanisms that may underlie the adjustment of nest size to population size, as suggested by Deneubourg and Franks (1995) on theoretical grounds, involve positive feedback at the beginning, and negative feedback in the later stages of the digging process. In leaf-cutting ants, nest-space requirements differ from those of other ant species because of the ants' agricultural habits (Belt, 1874; Weber, 1972): workers need to provide the fungus not only with the appropriate plant substrate, but also with sufficient space to allow its growth. As a consequence, colony growth does not only result from an increase in the number of ants, but also from a nearly continuous growth of the underground fungus gardens as workers forage for plant material. The housing of a growing structure (the fungus garden) in leaf-cutting ant nests adds a variable, beyond the population size, that is expected to markedly influence the control of the final nest size. Recent studies have clearly demonstrated a strong quantitative influence of fungus volume on nest size in the leaf-cutting ant *Acromyrmex lundi* (Guérin-Méneville), suggesting that the fungus garden acts as a physical template for the determination of chamber size (Fröhle, 2009; Fröhle and Roces, 2009).

All laboratory studies reported so far in the literature used a similar basic procedure for the study of nest-size regulation: groups of ants varying in size were allowed to excavate in an arena of limited space, and both digging rates and the generated space were quantified. Because of the inevitably space constraints imposed by the digging arenas, it remains an open question whether the digging dynamics and/or the resulting structures are representative of the ant behaviour observed under natural conditions. More important, the structure of a natural nest both grows and differentiates over time as colony develops, so that they do not normally have the need to excavate a complete new nest at once as in the laboratory experiments, with the exception of *Pogonomyrmex badius* colonies that are known to abandon their old nest and excavate a complete new nest similar to

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[1] *Prenolepis imparis* (Say), Tschinkel (1987); *Solenopsis invicta* Buren, Tschinkel (1993); *Pogonomyrmex badius* (Latreille), Tschinkel (1999, 2004); *Formica pallidefulva* Latreille, ?; *Camponotus socius* Roger, Tschinkel (2005); *Odontomachus brunneus* (Patton), Cerquera and Tschinkel (2010).

the old one in a couple of days (Tschinkel, 2004, 2013).

In the present study, we investigated to what extent the available nest space influences excavation rates in the Chaco leaf-cutting ant, *Atta vollenweideri* (Forel, 1893), and whether the presence of a fungus garden stimulates digging. Unlike the majority of the formerly published experiments (Rassé and Deneubourg, 2001; Buhl et al., 2004; Halley et al., 2005) we focused on varying the available space rather than the number of workers. Taking into account the complex architecture and the highly three-dimensional chamber geometry typical for our study species, we aimed at offering varying nest space in a setup resembling a young natural nest as closely as possible under standardised laboratory conditions.

Field nests develop from a small founding chamber dug by the queen itself at the bottom end of a vertical tunnel (Fröhle and Roces, 2012). Here, the queen remains without access to the surface until the first workers start to enlarge the nest. One year old nests have only one nest opening. They consist of a vertical tunnel about 2 m deep, and several fungus and refuse chambers along this main tunnel (Jonkman, 1980). Our aim was to mimic the conditions of such a young nest by presenting ants with a vertical tunnel that connects to an empty spherical chamber, and then leads further down to end blindly. Workers may enlarge the existing chamber, extend the tunnel further downwards, or even excavate additional tunnels. Preliminary observations had revealed that *Atta vollenweideri* workers prefer to elongate existing tunnels and enlarge existing chambers over excavating at alternative locations, if there are no other major irregularities in the internal surface. In our experiments, workers with a fungus garden were given access to such artificial nests, and the amount of material they excavated was monitored for ten days. Presenting the ants with coloured clay at the tunnel end allowed us to distinguish clay pellets excavated during chamber enlargement from those originating from tunnel digging. Four different initial chamber sizes were offered in this way to groups of 50 workers and 1g of fungus, which workers relocated into the available nest. Control series with workers without fungus were performed for three of the four different nest volumes.

In order to investigate whether an increase of nest space feeds back on excavation rate independently from the workers prior digging efforts, we conducted a series of experiments in which digging workers were confronted with a sudden increase in chamber volume not caused by their own work. In a setup with an artificial nest similar to those offered in the former series, we prepared a second large chamber, not connected to the initial nest, but close to the small initial chamber, so that digging workers enlarging the initial chamber were expected to break through and therefore be exposed to the additional space.

Finally, the effect of distributing the same available space in a different nest shape was investigated by observing nest enlargement in artificial nests with three small chambers that together had the same volume as one of the single chambers offered in the former experiments. The digging rates observed in the spatially more distributed nest were compared to those in the more concentrated nest.

6.2 METHODS

Construction of artificial nests

The construction of artificial nests was performed as follows: a vertical plastic tube, 25 cm high and 10 cm wide, comprised of two half pipes taped together, were filled with industrial clay (CLAYTEC *Lehm gemahlen 10.001*, maximum particle size: 0.5 mm) containing 19% water (Fig. 12A). On top of the cylindrical body, a straight marking line was cut into the clay surface connecting the two points at which the plastic half pipes met. On this line, about 2 cm from the edge, a steal rod with a diameter of 1 cm was pushed through the clay parallel to the central axis of the cylinder, and afterwards pulled out with a constant drilling movement, thus creating a straight, 1 cm broad tunnel from the top to the bottom side of the cylinder (Fig. 12B). After removing the tape from the plastic tube, the whole construction was cut in half along the marking line on the top side and the along the tunnel by means of a piece of wire (Fig. 12 C), thereby creating two half cylinders of clay with half the tunnel in each of the cutting surfaces (Fig. 12D).

To create a chamber, two corresponding half spheres were cut into the cutting surface, with the sphere's centre 12.5 cm below the surface, and the outer perimeter reaching into the tunnel thereby merging with the outer tunnel wall (Fig. 12E). The major part of the chamber volume was roughly cut into the clay with a spoon. A wooden ball of the desired volume was used as a template for chamber form and size. It was first wetted and then and then put into the rough half chamber, there it was turned hereby smoothing the inner surface of the chamber. Afterwards, irregularities were removed with a spoon of filled up with clay before inserting the ball again. The whole procedure was repeated until a smooth, half-spherical structure that could contain exactly half of the template ball was formed.

Finally, the steel rod initially used to drill the tunnel was wetted laid in one of the half tunnels. Along the sides of the rod, a small ridge of very moist clay (about 24% water content) was pressed against the steel, with the intent to seal the tunnel after re-assembling the half cylinders. The same type of ridge about 5 mm high was created at the edges of the half chamber. The surfaces that had to stick together when reassembled were wetted, and the two half cylinders were rejoined and taped together. Then, the steel rod was carefully pulled

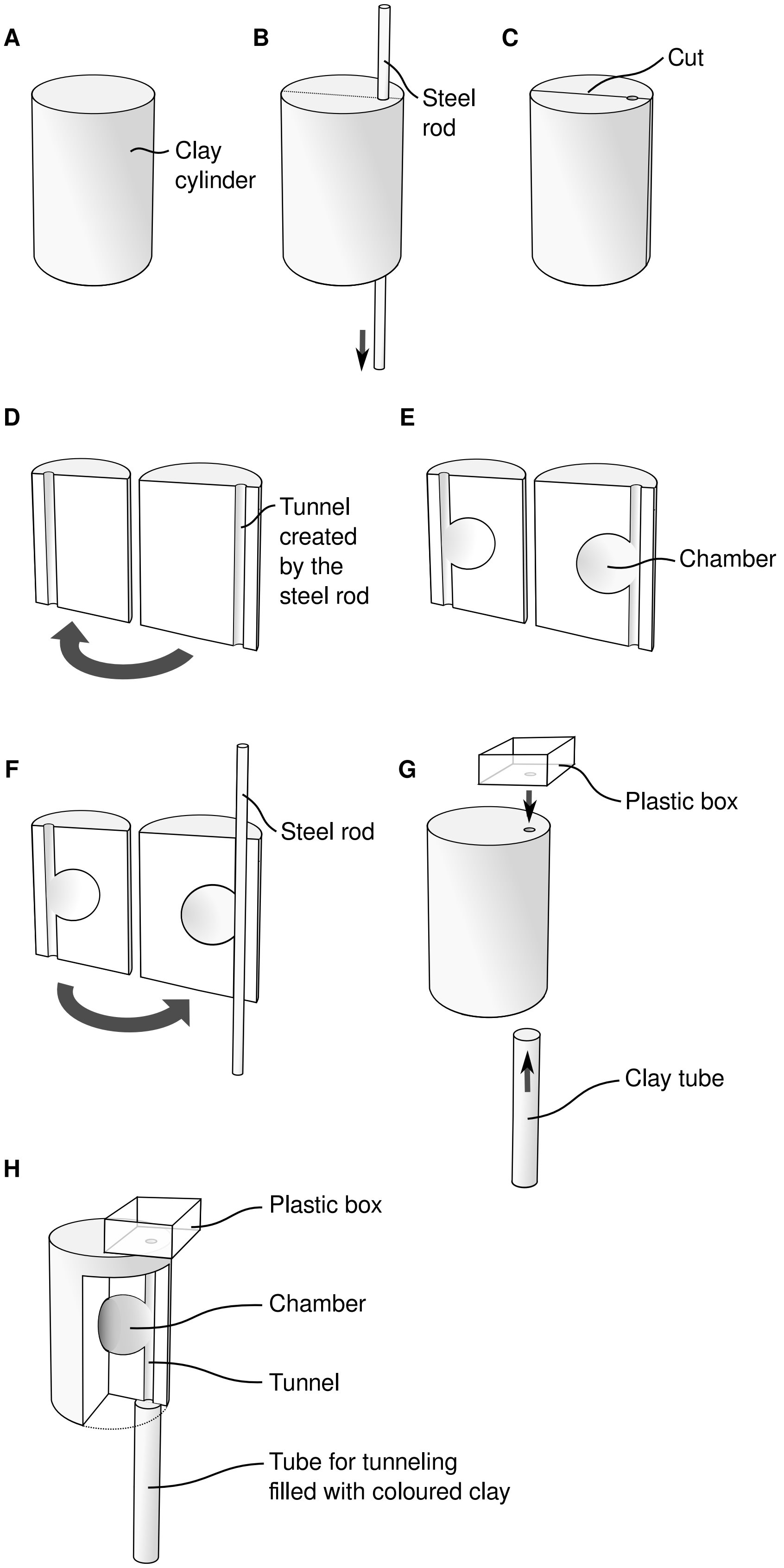

Figure 12: Construction of three-dimensional artificial nests.

out under constant drilling movements (Fig. 12F). The result was a three-dimensional artificial nest with smooth inner surfaces, comprised of a vertical tunnel that laterally hits a spherical chamber on half its way down.

For the largest nest size tested in this study, the plastic cylinders were replaced by a plastic bucket equally high but 26 cm in diameter. For no wooden balls were available as a template in that size, were used a round plastic disc with 15 cm diameter that was held vertically in the half chamber and repeatedly turned around its vertical axis to create a half-spherical cavity.

On top of the cylinder we placed a plastic box of 9 x 9 x 6 cm with a round hole in the centre, where a tube of 2 cm length a 1 cm inner diameter gave access to the entrance clay tunnel below. We will refer to it as the *depositing box* throughout the text. The setup was placed on a table with a round hole, 3 cm wide, the bottom end of the tunnel placed above that hole, for 24 h. During that time, the water film on the internal surfaces, a result of wetting the surfaces for smoothing, evaporated. Then, to provide the ants with the possibility to further extend the tunnel downwards, a thin plastic tube filled with clay was inserted into the hole in the table so that the clay surface on its top sealed the end of the vertical tunnel (Fig. 12G-H). This tube was created by removing the bottoms from 50 ml *Falcon* tubes, 3 cm in diameter, and plunging them together, so that it could be extended during the experiment as needed. This tube was filled with coloured clay. As the ants were expected to further extend the tunnel downwards at this location and thereby to excavate in the coloured clay, this measure allowed the separate assessment of chamber and tunnel enlargement by quantifying the proportion of coloured clay pellets carried out by the ants each day. The three upper tunnel segments were filled with clay containing 19% water and about 2% *Syria Green* powder pigment (*Malzeit Künstlerpigmente, Creativ Discount*, Art. no. 586680). The fourth, last segment was filled with clay dyed red (*Vermeil Red* Art. no. 586641). The observation of red pellets carried out by the ants was an indication that workers already reached the last tunnel segment, and addition of further segments for digging became necessary. Preliminary experiments indicated no noticeable effect of the dye on ant's excavation behaviour.

Experimental procedure

For each experiment, 50 workers were taken from one of two *Atta vollenweideri* colonies. For those experiments conducted with a fungus garden given to the workers, 1 g of a recently-established fungus garden was taken from the main colony and all workers but the smallest fungus gardeners were removed. The smallest workers in *Atta vollenweideri*, the fungus gardeners, do not excavate, but their absence would have negatively affected the survival of the fungus garden. For that reason, additional 20 gardeners were added to the experimental subcolony. According to Fröhle (2009), the fungus garden in *Acromyrmex lundi* has a relative density of about 0.14 g/ml, indicating that 1 g of fungus, if completely incorporated into the offered nest chamber, could occupy about 7 ml of nest space.

Single experiments were started by transferring the subcolony, i.e. workers and fungus, into the depositing box. Here the ants were daily provided with water and 1:1 honey-water solution. For the first 24 h, the lid of the box remained closed to avoid strong desiccation of the fungus garden. The lid was removed from the depositing box after 24 h, and in the case of an incomplete incorporation of the fungus into the nest, the remaining fungus was removed after 48 h. Each day, the excavated material was collected from the depositing box, dried and weighed. To estimate how much of the excavated clay originated from chamber enlargement or tunnel extension, respectively, a sample of 0.5 ml (about 40 -120 pellets) was taken from the excavated material. The number of dyed and undyed pellets in the sample was counted, dyed pellets originating from tunnel digging and undyed pellets from chamber enlargement. Together with the overall dry mass of clay excavated at each day, this proportion allowed to calculate how much material had been excavated as a result of chamber enlargement and tunnel enlargement respectively for each day. After the daily collection of the clay pellets, the subcolony was fed with 10 round blackberry (*Rubus fruticosus*) leaf discs, each 6 mm in diameter. Dead individuals were counted and replaced.

On the tenth day after starting, the experiment was finished and the setup opened. Fungus was removed from the nest and weighted. If the structures excavated by the ants encountered the outer wall of the setup in some location, or inaccuracies during the construction of the setup had left slits or discontinuities in the nest, or most of the workers were dead, the experiment was cancelled, i.e. it was not considered at all. The calculation of chamber and tunnel enlargement was reliable only if tunnel building took place exclusively at the bottom end of the prepared tunnel. Tunnel building in any other location would take place in uncoloured material, thereby making it impossible to discriminate between clay pellets originating from chamber enlargement and tunnel enlargement in the plastic box. Therefore, an experiment was not considered for the calculation of chamber and tunnel enlargement if side tunnels were projecting from the chamber (Fig. 13A-B). Any structure projecting from the original chamber or tunnel at a place other than the lower end of the original was considered to be a side tunnel. Finally, tunnel length was measured as the distance the ants had excavated further down from the initial tunnel end. All experiments were done at 25°C under an 12:12h light:dark cycle. Six to 7 experiments were run at the same time. In each of them,

Table 3: Number of replicates for the different initial nest size conditions tested.

Condition	*Fungus*	*Started*	*cancelled*	*n*	*Side tunnels*	*no ST*
8 ml	yes	7	0	7	3	4
8 ml	no	6	0	6	3	3
22 ml	yes	10	3	7	2	5
22 ml	no	6	0	6	2	4
113 ml	yes	10	2	8	2	6
113 ml	no	8	1	7	3	4
1740 ml	yes	9	2	7	2	5
8+113 ml	yes	7	1	6	3	3
3x8 ml	yes	9	0	9	2	7
Sum		72	9	63	22	41

Given are for each experimental condition the number of replicates originally started (*start*), the number of replicates *cancelled* as a consequence of escaped workers, the remaining sample size (*n*) and the number of replicates with and without side tunnels projecting from the chamber.

different experimental conditions were tested in parallel to rule out seasonal effects.

Experimental series

To investigate the influence of available space on excavation rate, four different initial chamber volumes were offered. Different chamber sizes were generated by using different templates when creating the chambers. The smallest chamber had a volume of 8 ml, i.e. the template was a ball of 2.5 cm diameter. The other sizes were 22 ml (3.5 cm in diameter), 113 ml (6 cm) and 1740 ml (15 cm), with the largest offered chamber being roughly similar to the size of a fungus chamber in a mature field nest. All chamber sizes, in independent assays, were offered to subcolonies composed of workers and a fungus garden. Control series with nests offered to workers without a fungus were performed for three of the four chamber sizes, 8, 22 and 113 ml.

In order to investigate the role of available nest space in influencing excavation rates once the process of nest enlargement was started, we performed a series of experiments in which workers actually engaged in digging were confronted with a sudden increase in chamber volume. To achieve this, a second large chamber not connected to the initial nest was prepared close to the small initial chamber, so that digging workers enlarging the initial chamber were expected to break through and therefore be exposed to the additional space. A condition was offered in which ants were initially offered a chamber of only 8 ml, but nest size suddenly increases some time after excavation started. To achieve this, a second chamber of 113 ml was prepared in the setup, with no connection to the nest at the beginning, but with only 5 mm of clay between the small chamber initially belonging to the nest and the large one. When the workers started to enlarge the small chamber, they soon broke though into the larger one, thus merging both chambers. This sudden increase in nest space was expected to happen within the first or second days of the experiment. Therefore, excavation rates in this condition were compared to both the standard 8 ml and 113 ml condition on the first, second and third day.

In a final experimental series, the effect of distributing the same available space in a different nest shape was investigated by observing nest enlargement in artificial nests with three small chambers that together had the same volume as one of the single chambers offered in the former experiments. The digging rates observed in the spatially more distributed nest were compared to those in the more concentrated nest. A nest with three small chambers of 8 ml each was offered instead of a single chamber of similar volume. The overall chamber volume of the distributed nest was 24 ml, very close to the chamber volume of 22 ml condition. The preparation procedure was similar to the 8 ml condition, but with two additional chambers of the same size. They were centered 5 cm above and 5 cm below the central one, and their outer sides oriented to the opposite direction, to prevent the growing chambers from merging into each other.

The subcolonies' responses to the different experimental conditions were compared in terms of the cumulative mass excavated within ten days, and in terms of the maximum excavated rates observed within that period. These measures served as dependant variables, the initially presented condition as the predicting variable. When comparing the different initial chamber sizes, this was done in a linear regression analysis using *log*-transformed overall nest volume as a linear predictor. When there was no linear relationship between the variables, or treating the different conditions as a linear rather than categorical variable made no sense, groups were compared in an ANOVA with *post-hoc* pairwise two-sample Welch *t*-tests and Bonferroni correction. Sample sizes were small for conditions without a fungus garden, and the 1740 ml chambers were not tested without a fungus garden at all. Accordingly, regressions were done for conditions with and conditions without fungus separately, as analysing everything together in single ANCOVAS would have led to unbalanced designs. To compare result from experiments with fungus and those from experiments without, measurements at each initial nest size were directly compared, again using two-sample Welch t-tests. Further statistical procedures are described in the respective parts of the results section. Sample sizes, the numbers of replicates not considered for the calculation of chamber and tunnel enlargement, and the number of replicates not considered at all, are summarised in Table 3. All statistical calculations were done in R v. 2.14.1 (`http://www.r-project.org`).

6.3 RESULTS

Nest sizes and excavation rates

During the experiments, fresh clay pellets were found in the plastic box on almost every day. Only few pellets remained inside the nest at the end of the experiments, mostly single items sticking to the wall of the tunnel. In all the offered nest conditions, subcolonies enlarged the chambers, predominantly at their sides and bottom. Upward enlargement was observed only in the two smaller of the initial chamber sizes, the 8 ml and the 22 ml conditions. The chambers extended mainly laterally, i.e. from the tunnel away. Examples of chamber enlargement are shown in Fig. 13C-F. Side tunnels projecting from the chamber to the outer confinements of the setup were constructed in 37.5% of the experiments (for more details, see Tab. 3). The occurrence of side tunnels was independent of initial chamber size, presence or absence of a fungus garden (two-way GLM for quasibinomial data, with fungus as factorial, initial chamber size as a linear predictor: *F textsubscript3,6 = 2.4, p = 0.242*).
At average, 5.4±5.7 dead workers (mean±standard deviation, $n = 480$) had to be replaced each day. Cumulative mass of material excavated within 10 days was slightly, but significantly influenced by the number of workers that had died within that time, each dead individual reducing the excavated mass by 0.4 g at average (linear regression: $F_{1,46} = 8.6, p = 0.005$). The number of dead ants was not influenced by the presented chamber volume (one-way ANOVA: $F_{3,44} = 0.9, p = 0.435$), but significantly higher in experiments without a fungus garden (two-way ANOVA with fungus and presented volume as predicting factors: $F_{6,41} = 13.9, p < 0.001$). Subcolonies provided with a fungus garden relocated it into the nest chamber, in most cases only partially (Fig. 13G-H). The amount of fungus material left outside was no linear function of initial nest volume (linear regression: $F_{1,25} = 0.1, p = 0.746$), but there was a significant difference among conditions (one-way ANOVA: $F_{3,23} = 4.8, p = 0.009$). The smallest amount of fungus, an average of 22±47 mg (mean±standard deviation, $n = 8$) remained outside in the 113 ml experiments. The amount of fungus found inside the chambers after the experiments (Fig. 14A) differed, like the amounts of fungus left outside, depending on initial nest volume (ANOVA: $F_{3,25} = 6.0, p = 0.003$). The largest fungus gardens were maintained in

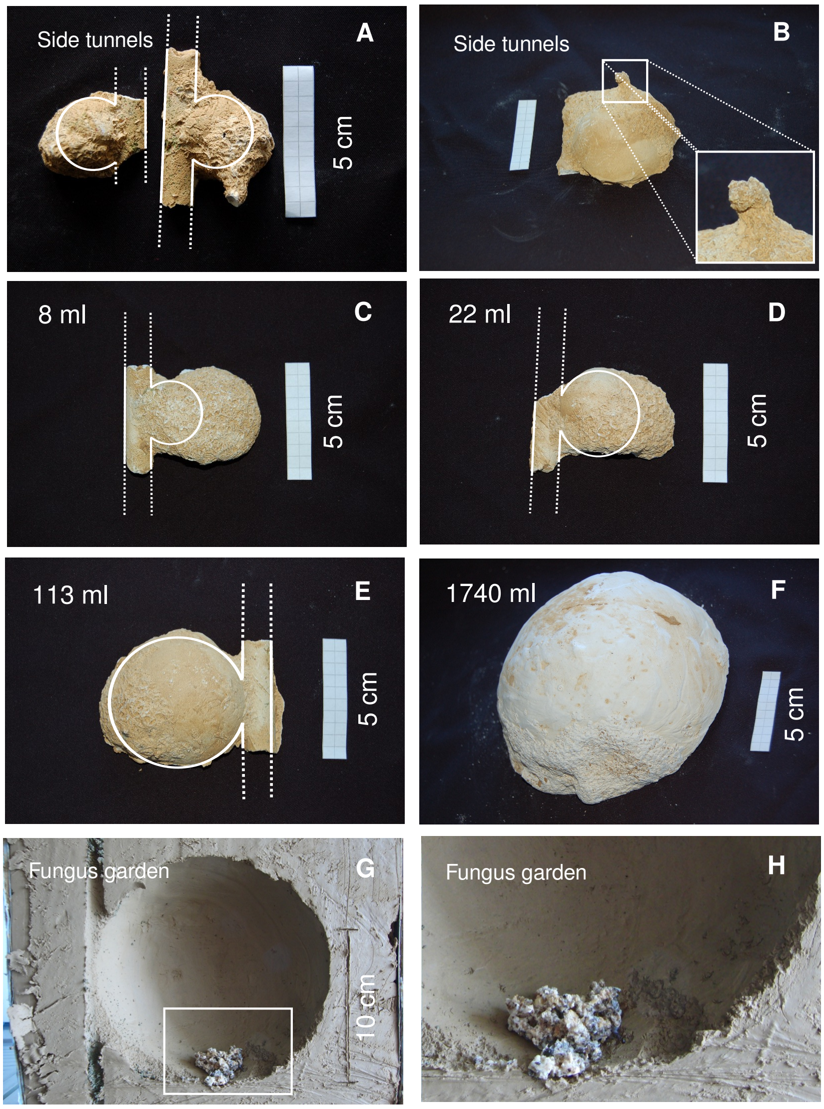

Figure 13: Examples of excavated structures. (A) Example for a side tunnel projecting from an enlarged 8 ml chamber. White lines indicate the initial structure. The chamber has been cut in two halves until a cast has been made from plaster. (B) Example for a side tunnel projecting from an enlarged 113 ml chamber. The initial structure is not indicated, because the cast has not been photographed from top view. (C-E) Examples for chambers with initial volumes of 8, 22 and 113 ml at the end of the experiment. Shown are chambers enlarged by subcolonies with a fungus garden. White lines indicate the nest shape as it was initially presented. (F) Example half of a 1740 ml chamber at the end of the experiment cast in plaster. (G-H) Overview and close up of a fungus garden in a 1740 ml chamber at the end of the experiment.

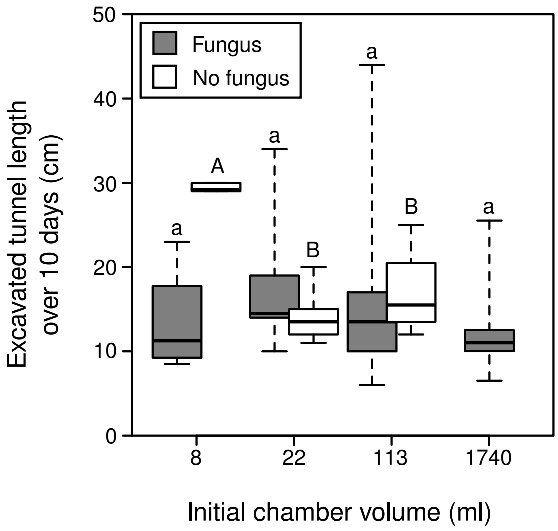

Figure 14: Final tunnel elongation after ten days as a function of initial chamber volume. Lengths were measured from the bottom end of the pre-given tunnel, after ten days. Only values for experiments without side tunnels are indicated. For sample sizes, see Tab. 3. The box shows the range from 25% to 75% quartile. Bars within the boxes indicate median values. Whiskers indicate minimum and maximum values without outliers. Boxes marked with the same letter are not statistically different from each other. Grey boxes represent measurements from subcolonies with, white boxes experiments without a fungus garden. Boxes marked with the same letter are not statistically different from each other.

chambers with an initial volume of 113 ml, here fungus gardens had a mass of 1.36±0.30 g (median±IQR, $n = 8$). Often, especially in the 1740 ml chambers, the fungus garden occupied only a small proportion of the overall chamber volume. Here, the fungus garden had a mass of 0.18±0.10 g (mean±standard deviation, $n = 7$) at average, i.e. it occupied approximately 1.3 ml of a 1740 ml chamber. Even in that condition, signs of excavation activity were visible on the chamber floors (Fig. 13G-H).

All of the pre-given tunnels were elongated downwards during the experiments (Fig. 14). Final tunnel elongation, measured from the lowest point of the pre-given tunnel, was 14.5±10.5 cm (median±IQR, $n = 31$). Linear regressions indicated no linear relationship between tunnel depth an *log*-transformed initial nest volume (with fungus: $F_{1,18} = 0.3, p = 0.604$; without fungus: $F_{1,9} = 2.2, p = 0.174$). Nevertheless, treating the initial volumes as independent groups in a one-way ANOVA revealed initial nest size to have a significant influence on tunnel depth when no fungus is present (with fungus: $F_{3,16} = 0.3, p = 0.798$; without fungus: $F_{2,8} = 11.6, p = 0.004$). The experiments with

8 ml chambers and no fungus featured the deepest tunnels, with a median length of 29.0±0.5 cm ($n = 3$). Only here, in the 8 ml condition, tunnel elongation differed significantly between replicates with a fungus garden, and those without ($t_{3.1} = -4.8, P = 0.016$; see Tab. 11 for more details).

For subcolonies provided with a fungus garden, the cumulative mass of material excavated within ten days (Fig. 15B) was negatively influenced by initial nest volume (Linear regression with *log*-transformed initial nest volumes: $F_{1,27} = 31.3, P < 0.001$). In the absence of symbiotic fungus, the cumulative mass excavated was independent of the offered nest size ($F_{1,17} = 0.7, p = 0.421$), and significantly smaller than that of subcolonies with a fungus garden, for the offered chamber sizes 8 ml and 113 ml (Tab. 11).

Chamber enlargement, i.e. the cumulative mass excavated to enlarge the chamber within ten days, showed a comparable pattern (Fig. 15C). Again, regression analysis revealed a significant negative influence of *log*-transformed initial nest volume when a fungus garden was present ($F_{1,18} = 17.6, p < 0.001$), but not in the absence of the latter ($F_{1,9} = 0.1, p = 0.730$). A significant difference between experiments with, and those without fungus was observed only for 8 ml initial chamber size ($t_{3.6} = 6.0, p = 0.005$; see Tab. 11 for more details). Tunnel enlargement (Fig. 15D) was also negatively influencedby nest size, but again only in the presence of a fungus garden (with fungus: $F_{1,18} = 18.6, p < 0.001$; without fungus: $F_{1,9} = 2.7, p = 0.134$). However, concerning tunnel enlargement in terms of cumulative mass of clay excavated during tunnel building there was no significant difference between replicates with and replicates without fungus, if comparing them for each volume separately. This was true even for the 8 ml experiment, were subcolonies without a fungus gardens excavated tunnels that were significantly longer, but doing so they did not remove significantly more material than subcolonies with a fungus garden, indicating that their tunnels were also narrower in general (Tab. 11).

Excavation rates were in general higher within the first days of the experiment, but reached their maxima at different days (Fig. 15E). Maximum medians were obtained at the first day in 8 ml experiments with fungus, 1740 ml, 22 ml without fungus and 113 ml without fungus. The 22 ml and 113 ml experiments with fungus both had their maximum at the second day; the 8 ml experiments without fungus reached their maximum excavation rate not until the fourth day. Maximum rates were also negatively influenced by initial nest size when the subcolonies had a fungus garden ($F_{1,27} = 18.0, p < 0.001$), but not in the absence of the latter ($F_{1,17} = 0.7, p = 0.404$). Direct comparisons between conditions of equal initial nest sizes with and without fungus revealed no significant differences (Tab. 2). Excavation rates decreased towards the end of the experiment, reached an overall median of 2.2±3.1 ($n = 41$) at day 10. Here, initial nest volume had no

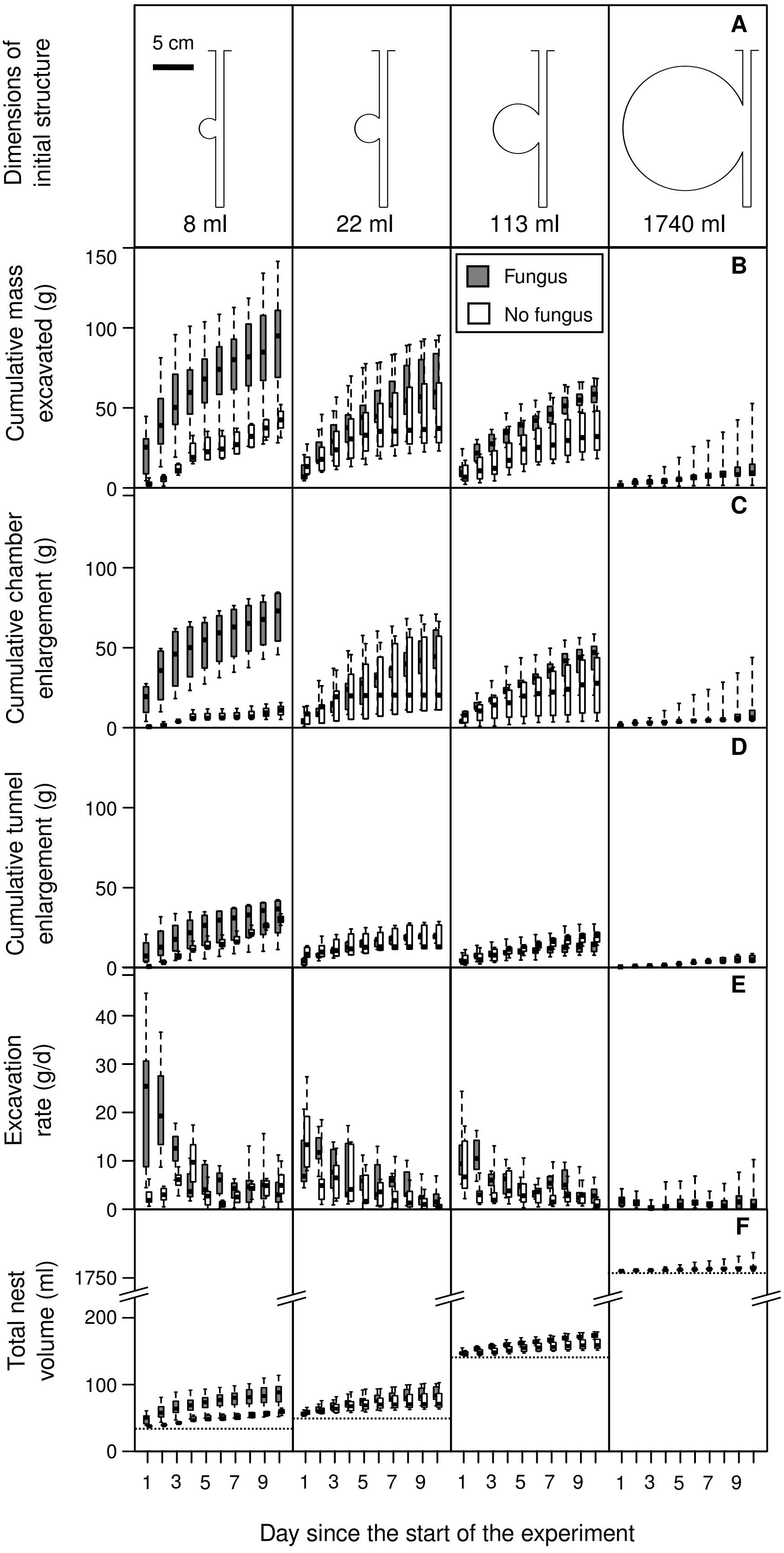

Figure 15: Quantitative effects of initial chamber volume with and without fungus.

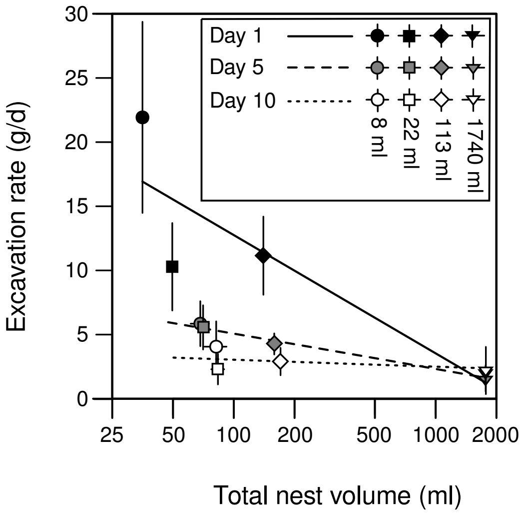

Figure 16: Excavation rate (*r*) as a function of nest volume (*v*). Volume was *log*-transformed for regression analysis. Given are values from experiments with 8 ml (circles), 22 ml (squares), 113 ml (rhomboids) and 1740 ml (triangles) initial chamber size with a fungus garden after one day (black symbols, solid regression line; $r=31.2-9.2*log(v)$, $R^2 = 0.32$, $n = 29$, $p < 0.001$), five days (grey symbols, dashed regression line; $r=10.5-2.7*log(v)$, $R^2 = 0.26$, $n = 29$, $p = 0.004$) and ten days (white symbols, dotted regression line; $r=4.1-0.6*log(v)$, $R^2 = 0.01$, $n = 29$, $p = 0.604$). The data the regressions are based on are not completely represented in this graph. Each point shows the mean value for one day and initial chamber size, vertical and horizontal lines projecting from the data points show standard errors. Details on the regressions for all days are given in Tab. 12

effect any more in the presence of fungus ($F_{1,27} = 0.6$, $p = 0.443$), but a positive effect in its absence ($F_{1,17} = 5.6$, $p = 0.030$). Nonetheless, no significant differences were observed direct comparisons between groups with and without fungus (Tab. 11).

Final nest volumes after 10 days were different (one-way ANOVA: $F_{3,25} = 2.8*10^4$, $p < 0.001$), ranging from 87.2±22.9 ml ($n = 7$) in the 8 ml, to 1772.3±3.8 g ($n = 7$) in the 1740 ml condition (Fig. 15F). To further analyse the effect of current nest size on nest enlargement over time, a series of day-wise regressions on the data obtained from experiments with a fungus garden were performed. Digging rate was taken as the dependant variable; the actual nest volume at the beginning of the day was used as a linear predictor. Analysing the data that way revealed a negative correlation between digging rate and available nest volume that diminished over time (Fig. 16, Tab. 12): while there was a clear negative influence of nest volume at day 1 ($F_{1,27} = 12.6$, $p < 0.001$), the effect was not significant any more at day 10 ($F_{1,27} = 0.3$, $p < 0.604$).

Sudden increase of chamber volume

In all experiments offering the 8+113 ml condition, the initially available 8 ml chamber was merged with the 113 ml chamber after 10 days (Fig. 17A-B). Excavation took place at all sides of the smaller 8 ml chamber. At the larger 113 ml chamber the top wall remained untouched by excavation activity in all experiments. Nest growth was comparable to the 22 ml and 113 ml experiments (Fig. 12C). The cumulative mass excavated within 10 days was 49.9±16.9 g (median±IQR, $n = 6$). When comparing to the four different standard volumes, 8 ml, 22 ml, 113 ml and 1740 ml, the condition had a significant influence on the excavated mass (ANOVA: $F_{4,30} = 9.4, p < 0.001$). However, *post-hoc* comparisons of the 8+113 ml conditions with each of the standard volumes revealed a significant difference only to the 1740 ml chambers (Tab. 13).
At the beginning of the experiment, the nest in the 8+113 ml condition was identical to an 8 ml nest, after the chambers merged; nest size is much more comparable to that of a 113 ml nest. Thus, we directly compared the excavation rates of these three conditions, 8 ml, 113 ml and 8+113 ml, at the first three days of the experiment (Fig. 17D), assuming that the chambers must have had merged until the third day. At the first day, excavation rate in the 8+113 ml condition was 16.7±8.9 g/d (median±IQR, $n = 6$). It was higher than in the 113 ml conditions, but did not differ significantly from it or from the 8 ml condition (one-way ANOVA: $F_{2,18} = 2.1, p = 0.154$). At the second day, excavation rate in the 8+113 ml condition decreased to 8.4±3.0 g/d ($n = 6$). At this day, excavation rate was significantly influenced by the experimental condition (one-way ANOVA: $F_{2,18} = 6.6, p = 0.007$), with the rates in the 8+133 ml experiments being lower than in the 8 ml, but not different from the 113 ml condition (see Tab. 14 for *post-hoc* comparisons). By day 3, excavation rate had decreased significantly as compared to the first day, to 5.8±1.8 g/d ($n = 6$; two-sample Welch t-test: $t_{5.3} = 2.9, p = 0.030$). In the same time, the excavation rate in the 8 ml condition did not change significantly (two-sample Welch t-test: $t_{6.9} = 1.6, p = 0.146$). At the third day, excavation rates for both 8+113 ml and 113 ml were significantly lower than in the 8 ml condition (one-way ANOVA: $F_{2,18} = 13.8, p < 0.001$; see Tab. 14 for *post-hoc* comparisons).

Distributed nest space: the three-chamber experiment

With three chambers of 8 ml each, only the bottommost of them was used to store fungus and was visibly enlarged by the ants, while the other two chambers occasionally showed scattered signs of digging behaviour on their inner walls, but no visible increase of size (Fig. 18B). The workers excavated 22.6±16.4 g (median±IQR, $n = 9$) within ten days (Fig. 18C). Compared to the values of the standard

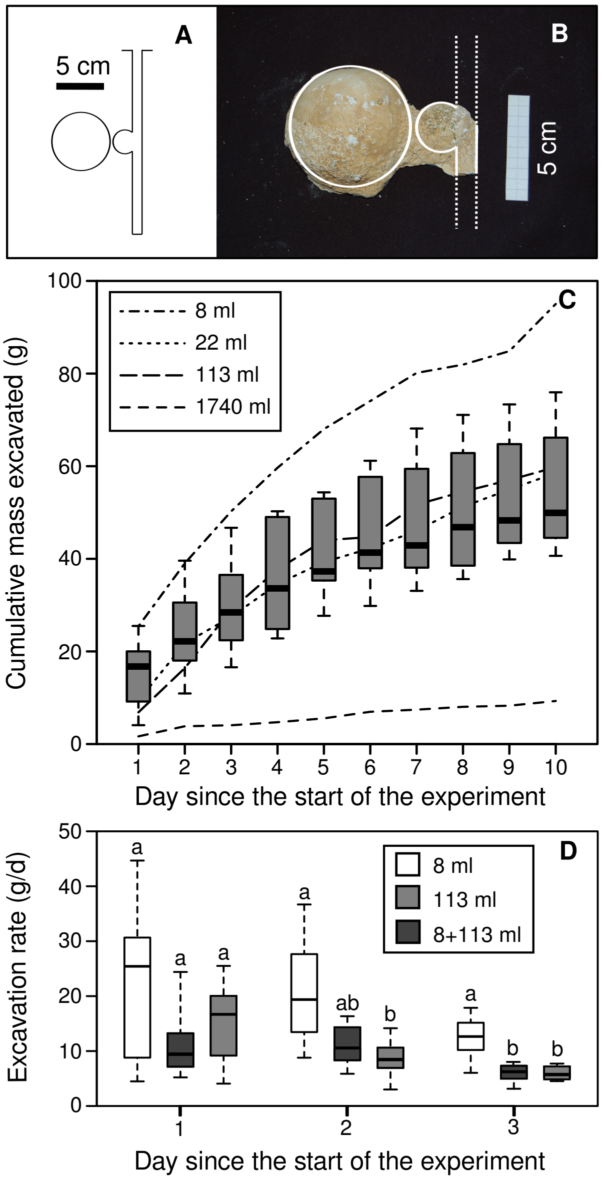

Figure 17: Nest enlargement with a sudden increase of nest volume. (A) Schematic depiction of the initial structure in the 8+113 ml condition. (B) Example plaster cast of chamber in the 8+113 ml condition after ten days. White lines indicate the nest shape as it was initially presented. (C) Cumulative excavated mass in the 8+113 ml condition as a function of time. To compare with results from the other experiments, lines in the background represent the median cumulative excavated masses of the experiments with fungus, as already indicated with their dispersion in Figure 15B. (D) Direct comparison of excavation rates for 8 ml (white boxes), 113 ml (dark grey) and 8+113 ml (light grey) for the first three days. Within each day, groups sharing the same letter are not significantly different from each other (Tab. 14). Values for the 8 ml and 113 ml treatment are identical to those in Figure 15E. The box shows the range from 25% to 75% quartile. Bars within the boxes indicate median values. Whiskers indicate minimum and maximum values without outliers. Sample size was $n = 6$ for the 8+113 ml treatment.

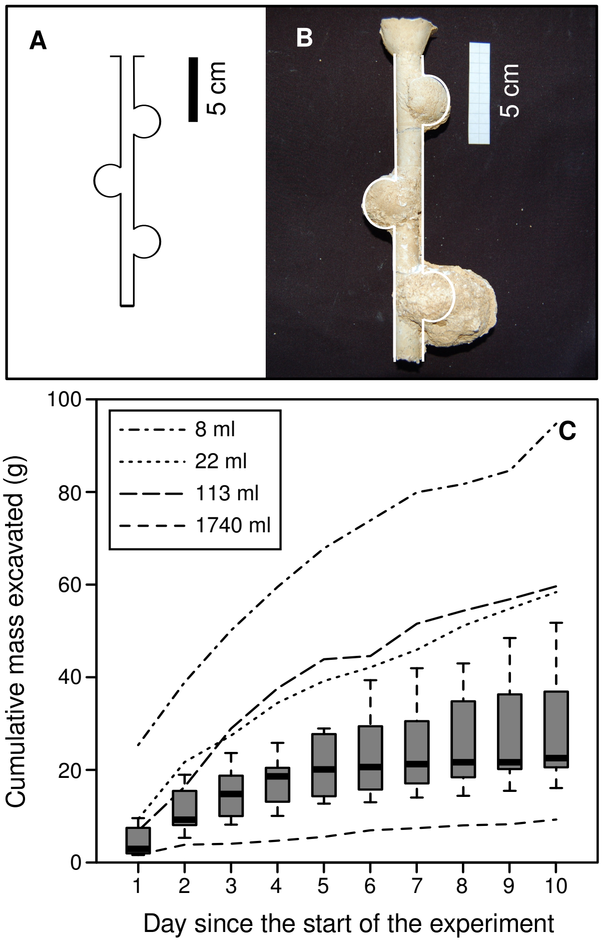

Figure 18: Nest enlargement when space is distributed to three chambers. (A) Schematic depiction of the initial structure in the three chambers condition. (B) Example plaster cast after ten days. White lines indicate the nest shape as it was initially presented. Note that the funnel-shaped structure at the upper end of the tunnel is an artefact of casting the tunnel in plaster and does not represent a structure of the nest itself. (C) Cumulative excavated mass in the three chambers condition as a function of time. To compare with results from the other experiments, lines in the background represent the median cumulative excavated masses of the experiments with fungus, as already indicated with their dispersion in Figure 15B. The box shows the range from 25% to 75% quartile. Bars within the boxes indicate median values. Whiskers indicate minimum and maximum values without outliers. Sample size was $n = 9$.

volumes, this cumulative mass was significantly smaller than all other but the 1740 ml condition ($F_{4,33} = 13.2, p < 0.001$; see Tab. 15 groupwise comparisons).

6.4 DISCUSSION

The influence of available space on digging

Regarding the inhibitory effect of space on excavation behaviour, our results not only confirmed the negative correlation between digging activity and *per capita* nest space reported in former laboratory studies (Rassé and Deneubourg, 2001; Buhl et al., 2004; Halley et al., 2005; Fröhle, 2009; Fröhle and Roces, 2009). We also demonstrated that artificially increasing nest volume during the digging process results in an adjustment of excavation rate to the new nest volume. We thereby demonstrate the existence of a negative feedback mechanism that regulates digging behaviour according to space requirements.

The negative feedbacks acting as the nest grows may also be to worker density: although a high worker density initially stimulates digging activity of individuals, density decreases over time as excavation proceeds because of the generated space, thus reducing the stimulus intensity that elicits digging (Deneubourg and Franks, 1995). The density-dependent stimulus situation may be related to the number of interactions a worker experiences at the digging[2], to the occurrence of nestmate odours or respiratory CO_2 (Hangartner, 1969), to stigmergic responses, in which the soil pellets deposited by the excavators at the digging site spatially directs digging responses (see Chapter 7), or to the presence of pheromones or other recruitment signals. It has been argued on theoretical grounds that digging workers may deposit pheromones that initially stimulate digging as a positive feedback, and that excavation may later decrease or even stop as nest space is generated because of the propagation properties of the supposed chemical recruitment involved (Deneubourg and Franks, 1995; Buhl et al., 2005). Although empirical evidence for the involvement of digging pheromones is scarce[3], we recently showed that vibrational signals produced by workers while digging indeed attract nestmates to the digging size (see Chapter 8), so that the involvement of communication signals in the feedback loop regulating digging activity remains to be determined.

In our control series without a fungus garden, digging activity was generally lower, mostly due to reduced chamber excavation. In leafcutting ant nests, space demands of the colony are determined by

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[2] For the role of worker interaction in task decisions, see e.g. Gordon et al. (1993); Gordon and Mehdiabadi (1999); Greene and Gordon (2007); Gordon (2010).

[3] Only two published studies touch that topic, see (Wilson, 1958; Blum and Warter, 1966). Both report digging reactions to alarm pheromones from the mandibular gland, but do not futher investigate their role in actual nest excavation.

both the number of workers and the volume of fungus garden (Fröhle, 2009; Fröhle and Roces, 2009). Thus digging rates in our experiments with fungus can be interpreted as the result of a combined effect of both workers and fungus and their space demands. This combined effect was possibly strongest in the 8 ml condition, where chamber volume was relatively close to the volume of the fungus garden, whereas other conditions clearly offered sufficient space to accommodate the fungus garden from the beginning of the experiment. The same condition without a fungus garden yielded another remarkable result: here, the vertical tunnels were excavated deeper within 10 days than in any other experimental condition with or without fungus. However, even though the tunnels were deeper, the workers did no remove more soil during tunnel building, indicating that the tunnels also must have been narrower than in other conditions.

Our results indicate that the influence of space on digging behaviour diminished with ongoing time during the experiment: in all tested conditions, digging rates decreased to a common base-line level with no reference to the achieved nest volume. Likewise, Rassé and Deneubourg (2001) observed that workers excavate smaller nests when tested in the same experiment a second time, also indicating that prior exposure to digging material or to the activity itself has a negative effect on excavation behaviour. As a consequence we can assume that during the down regulation of digging activity space-imparted negative feedback is complemented by an intrinsic feedback due to prior experience.

Distributing nest space to three small chambers instead of one large cavity resulted in a significant reduction of digging activity, even if compared to to single-chamber condition much larger regarding both volume and internal surface area. We can only speculate about explanations for this surprising result. It is conceivable that the distribution of nest space has an effect on the distribution of individuals inside. If the individual's motivation to dig is influenced by the presence of other workers at the site, a different distribution of individuals inside the nest may result in different excavation rates even at similar overall nest sizes. Our results indicate that the presence of a fungus garden leads to increased chamber digging. In the three chamber experiments, fungus culturing took place exclusively in the bottom chamber, and that chamber was the only one enlarged by the workers. This is not surprising, because it is likely that workers were distributed over the three different chambers, resulting a local relaxation of worker density in the fungus chamber. With the one exception mentioned above, tunnel excavation was relatively similar throughout the tested conditions. Similarly, tunnel excavation was with the one exception mentioned above, relatively constant throughout the tested conditions. As only a relatively small number of workers can be present and excavate at the bottom end of the tunnel the same time, the locally perceived crowding situation/space demand in the tunnel that stimulates digging may be similar throughout the conditions. Accordingly, our observations in the three chamber experiment can be explained as a result of the workers responding only to strictly local information.

Another possible explanation for the low digging activity in the three-chamber experiment would assume quite the opposite: if workers remember the occurrence of chambers/structures in the close vicinity of an actual digging site, this could possibly negatively influence their digging motivation. Evidently, the founding queens of *Atta vollenweideri* are able to estimate the length of their excavated tunnels when deciding where to build their founding chambers (Fröhle and Roces, 2012). Therefore, it is not impossible that even workers of that species rely also on spatial memory, rather than exclusively on local information, when making digging decisions.

Differentiation and growth in natural nests

In some of our experiments workers instead of simply enlarging the chambers excavated new tunnels projecting from the chamber walls. Toffin et al. (2009) observed a transition from chamber to tunnel building, when *Lasius niger* workers enlarged an artificial chamber. While initially only enlarging an existing cavity into a round chamber, the workers automatically switched to tunnel building when a particular chamber size was excavated. The threshold for this self-organised transition was identified as a specific proportion of worker number to chamber size. As a consequence it could be expected for our experiments that new tunnels are built more often in experiments with larger chamber volumes. We observed side tunnels emerging from chamber walls in 35% of all experiments, but, in contrary to prediction, their occurrence was not influenced by nest size. Admittedly, our experiments were not aimed at investigating that aspect, and the overall number of replicates was inadequate to allow for a reliable analysis of a binary response variable (i.e. "side tunnels" vs. "no side tunnels").

Concerning the differentiation and growth of underground nests another important aspect should be considered. In the laboratory, we at once present a given number of workers with a given set of conditions, usually implying a complete lack of space, and observe their response. With the exception of *Pogonomyrmex badius*, a species from Florida that regularly constructs completely new underground nests (Tschinkel, 2004, 2013), field nests in most ant species grow gradually. The fact that both colony and nest growth occur gradually poses an important question concerning the regulation of nest space according to colony size. Our results, as well as those reported by Rassé and Deneubourg (2001), indicate a negative effect of prior experience on nest digging. In our experiments, excavation rates dropped to a baseline level independent of available nest space within only 10 days. With the mostly gradual nest growth in the field, workers are exposed to soil, and possibly to excavation activity, for their entire life. Therefore, even though we expect that the feedback mechanisms observed in the laboratory come at least to some effect in nature too, it remains an open question how that permanent exposure influences the workers' digging response to the colony's space demands in their natural environment.

The other important aspect is the variability in several environmental and social factors an ant colony supposedly faces during its life cycle. Several publications describe factors other than nest size and worker population, which influence the rate of excavation in isolated worker groups under laboratory conditions. Reported factors include soil particle size (Espinoza and Santamarina, 2010), soil temperature (Mikheyev and Tschinkel, 2004; Bollazzi et al., 2008), soil water content (Mikheyev and Tschinkel, 2004; Espinoza and Santamarina, 2010), season and prior experience (Rassé and Deneubourg, 2001). Now, with our results, we can add to that list encountered nest shape and the resulting spatial distribution of space. Additionally, our results indicate that even though excavation rates are regulated at some degree according space availability, this regulating mechanism does not lead to the abandonment of digging behaviour once a particular nest size is achieved that matches colony size. The magnitude of the remaining space-independant base line activity is perhaps strongly influenced by these other factors. Moreover, some of them vary considerably during colony development, with the expected result that nests occasionally grow independent of space requirements.

Thus, the match between nest size and colony size that can be observed in the field irrespective of the several other factors that influence nest growth, cannot in all cases be explained by a systematic adjustment alone. On the other hand, nest size, shape, depth, the number of chambers and tunnels, environmental characteristics like temperature and moisture and intrinsic condition of the workers expectedly are roughly comparable in similar stages of nest ontogeny, and that is possibly the reason why nest volume and colony size often match so strictly in nature.

We argue that in many stages of colony development the presence of environmental stimuli that positively influence digging activity is probably an important driving force of nest growth. Besides, a current demand for space may in some stages of colony development may also stimulate digging activity, for instance when colonies experience periods of population growth, or when available nest space suddenly decreases due to the collapse of a cavity. In such cases, we expect that the generated nest space acts as a negative feedback on the digging behaviour, so that excavation activity decreases again afterwards. In many other situations an increase in digging activity might be stimulated by environmental variables. After a rainfall, for instance, enhanced soil moisture that allows for higher excavation rates and soil transport rates can lead to higher excavation rates at reduced energy costs. Digging activity stimulated by environmental factors can be expected to provide sufficient, or even surplus space in many stages of nest growth. It remains an open question whether this surplus space is generated as tunnels, or whether cavities are excavated in advance to provide space for future fungus chambers. In this line of arguments, nest size in nature is a function of colony size, not only due to systematic nest-size regulation by means of space-imparted negative feedback on digging behaviour. That relationship is caused by an interplay of intrinsic factors, nest shape, and space requirements that occur during nest development. Nest growth should therefore be seen as a complex, prolonged interplay with mutual interrelations between environmental templates, space requirements, intrinsic motivation and current nest geometry that relates to functional differentiation. Within that process, digging responses to local environmental conditions may often be much more influential on nest growth than overall nest size and space requirements.

7

SEQUENTIAL SOIL TRANSPORT

This chapter has been originally published in: S. Pielström and F. Roces. Sequential soil transport and its influence on the spatial organisation of collective digging in leaf-cutting ants. PLoS ONE 8: e57040, 2013.

ABSTRACT The Chaco leaf-cutting ant *Atta vollenweideri* (Forel) inhabits large and deep subterranean nests composed of a large number of fungus and refuse chambers. The ants dispose of the excavated soil by forming small pellets that are carried to the surface. For ants in general, the organisation of underground soil transport during nest building remains completely unknown. In the laboratory, we investigated how soil pellets are formed and transported, and whether their occurrence influences the spatial organisation of collective digging. Similar to leaf transport, we discovered size matching between soil pellet mass and carrier mass. Workers observed while digging excavated pellets at a rate of 26 per hour. Each excavator deposited its pellets in an individual cluster, independently of the preferred deposition sites of other excavators. Soil pellets were transported sequentially over 2 m, and the transport involved up to 12 workers belonging to three functionally distinct groups: excavators, several short- distance carriers that dropped the collected pellets after a few centimetres, and long-distance, last carriers that reached the final deposition site. When initiating a new excavation, the proportion of long-distance carriers increased from 18% to 45% within the first five hours, and remained unchanged over more than 20 hours. Accumulated, freshly-excavated pellets significantly influenced the workers' decision where to start digging in a choice experiment. Thus, pellets temporarily accumulated as a result of their sequential transport provide cues that spatially organise collective nest excavation.

7.1 INTRODUCTION

When excavating subterranean nests, animals have to dispose of the removed soil by transporting it from the underground to the surface (Hansell, 2005). With increasing nest size and depth, builders invest a large proportion of their time and energy budget for soil transport, especially when underground nests are not built by solitary individuals, but constructed collectively by animal societies. In termites and ants for instance, the constructions can reach gigantic dimensions as compared to the body size of the builders, and the amounts of soil to be relocated as well as the transport distances are substantial (Emerson, 1938; Weber, 1972). However, the organisation of underground soil transport in animal societies remains largely unexplored, with the exception of a detailed study on soil transport in the naked mole rat *Heterocephalus glaber* Rüppell, in which workers were observed to utilize sequential task partitioning to move soil to the surface (Jarvis and Sale, 1971). The term task partitioning denotes a process in which a single task, e.g. the transport of one particular food item or soil particle, is split up between different worker groups (Jeanne, 1986a,b; Anderson and Ratnieks, 2000; Anderson et al., 2002). Despite the fact that ant colonies move considerable amounts of soil while excavating underground nests, published works on ants refer only to the organisation of soil disposal and the emergence of mounds around the nest entrances (see references in Cosarinsky and Roces, 2012), with some early observations on task partitioning during the deposition of soil at the surface in *Cataglyphis longipedem* (Eichwald)[1] and *Messor barbarus* (L.) (Hingston, 1929, p. 296)[2]. One ant species that has been studied intensely in terms of construction and functionality of external nest architecture is the leaf-cutting ant *Atta vollenweideri* (Forel) from the Gran Chaco region in South America. Workers of this species usually build turrets on top of the nest openings in the centre of their nest mound. These turrets, which enhance wind-induced nest ventilation (Kleineidam & Roces, 2000; Kleineidam et al. 2001), are mainly built with materials collected from the soil deposits around the nest openings, i.e. materials previously transported to the surface from the underground or collected from adjacent areas (Cosarinsky and Roces, 2007, 2012). In colonies of this species, the effort made to relocate soil is expected to be particularly high. The nests of *Atta vollenweideri* are among the largest structures built by social insects. At a depth of around 1-2 m, workers excavate thousands of closely-packed fungus chambers that contain the symbiotic fungus as well as the queen and brood. At deeper soil layers, larger chambers are excavated and used as deposits for the colony refuse. An intricate arrangement of tunnels connects the underground chambers to the surface, where the soil excavated from underground is heaped in a mound that can be 1 m high and 8 m wide in a mature nest (Jonkman, 1980; Jonkman, 1980). The sheer amount of soil colonies of this species move from deeper layers to the surface makes them a major factor shaping the composition of the vegetation in the Gran Chaco region (Bucher and Zuccardi, 1967; Jonkman, 1976, 1978; Sosa and Brazeiro, 2010). In a nest of these dimensions, with transport ways over large distances and against the gravitational force, workers are expected to incur large time and energy costs in carrying soil. Similar to desert ants that are known to lift and carry sand with the aid of their psammophores or even maxillary palps (Délye, 1957, 1971; Spangler and Rettenmeyer, 1966), *Atta vollenweideri* workers actively form aggregations of fine material (Cosarinsky and Roces, 2007), so called *pellets* (Sleeman and Brewer, 1972), but carry them in between the mandibles without the aid of any specialised structures. The transport rates of soil pellets, which

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[1] Formerly known as *Myrmecocystus viaticus setipes* Forel.

[2] Reviewed in Anderson and Ratnieks (2000).

can vary in size considerably, may be higher if size matching occurs between worker size and load size, as known for foraging ants and even *Atta vollenweideri* workers excavating soil to built ventilation turrets (Lighton et al., 1987; Röschard and Roces, 2002; Cosarinsky and Roces, 2012). In addition, it is uncertain that an ant excavating soil will travel across the nest to deposit the excavated load outside, and then find again the excavation site in the complex nest structure. The distances between an excavation site and the closest nest opening can be expected to regularly exceed 1-2 m, and the way may involve several tunnel forks.

In the context of long-distance leaf carriage, *Atta vollenweideri* and other *Atta* species have been reported to rely on sequential transport. Leaf fragments are often not carried into the nest by the cutting individual, but dropped on the foraging trail where they are picked up by other workers (Fowler and Robinson, 1979; Hubbell et al., 1980; Röschard and Roces, 2003b, 2011). Sequential transport of soil loads, as known in the context of leaf carriage, may provide advantages regarding the spatial orientation of the involved workers, as compared to an individual mode of transport. Interestingly, accumulation of soil pellets inside leaf-cutting ant nests has regularly been observed (Jonkman, 1980; Jonkman, 1980; Autuori, 1942; Fröhle, 2009; Fröhle and Roces, 2009), suggesting that excavators do not necessarily carry the excavated soil to the outside, and that sequential soil transport may occur under natural conditions. If temporary deposits of freshly excavated soil occur, accumulated soil pellets may provide local information to other workers about current digging activity in the close vicinity, thus acting as stigmergic cues that may lead arriving workers to search for the digging site. Stigmergy is a regulatory process aimed at explaining how collective building behaviour can be organised without central control (Grassé, 1959)[3]: by changing the environment through building behaviour, the structure created by one individual passively provides a cue that influences the behaviour of other individuals, i.e. coordination is achieved through the structure workers are actually building. When deciding where to excavate, workers of *Atta vollenweideri* might use pellets deposited by other excavators as a cue. Thereby, sequential soil transport may be beneficial not only for the orientation of the carriers, but also for the spatial organisation of further excavation activity.

The aim of this study was to characterise the transport of soil pellets in the leaf-cutting ant *Atta vollenweideri*, and to investigate whether soil pellets are temporarily deposited inside the nest and influence the excavation behaviour of nearby workers. In a first experiment, pellet mass was compared to carrier body mass to investigate a possible size matching. Then, the occurrence of sequential transport was quantified under controlled laboratory conditions by observing soil

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[3] Reviewed in Theraulaz and Bonabeau (1999).

pellets from the excavation site until they were deposited outside the nest. The behaviour of individual excavators was observed in a separate experiment, measuring how fast they produced pellets and observing where they deposited them. The response of other excavators to deposited pellets was evaluated in three different choice experiments, aimed at investigating whether single, fresh and old soil pellets influence the spatial decision of workers as where to initiate digging.

7.2 METHODS

Size matching during soil transport

To characterise soil transport and size-matching in *Atta vollenweideri*, workers carrying soil pellets were collected in the field and in the laboratory, and both body mass and load mass were measured at the nearest 0.1 mg. In the field, samples were collected at nest openings, where the carriers were expected to have already covered a transport distance of up to several meters. In the laboratory, two different samples of loaded workers were taken. In the first sample, loaded workers were collected after having walked through a 2 m long tube. Since workers were not individually marked, it was unknown whether the observed carriers excavated their pellets, or collected pellets previously excavated by other workers. In the second sample, pellets and the corresponding excavators were collected directly at the excavation site. Thus, the different samples allowed size-matching between loads and body masses to be comparatively analysed for both excavators and carriers.

Size-matching in the field was investigated during a field trip on November 17th, 2008. At the *Reserva El Bagual*, San Francisco de Laishi, Formosa, Argentina, 7 mature field nests were selected from three different areas at a maximum distance of 1.5 km from each other. At each nest, a random sample of soil carriers was collected by selecting one opening, closely observing it and picking each individual coming out with a soil pellet by means of a forceps. Only 10 workers were picked from each nest to be able to move to the next nest in time, and thus to sample all the nests the same day. Workers still holding their pellets were immediately transferred into *Eppendorf* vials. After collection, carrier body mass, wet pellet mass and dry pellet mass were weighed, and soil water contents were calculated. Eleven samples were damaged in the transport bag and not considered for further analysis. For the collection of loaded workers in the laboratory under conditions comparable to those in the field, two laboratory colonies were connected to open plastic boxes (9 x 9 x 6 cm), called *collecting boxes*, that allowed the collection of workers with forceps. From such a plastic box the ants were able to enter a 2 m long flexible transparent tubing with an inner diameter of 10 mm. At the distal end, the tube was connected to a second box of the same dimensions entirely filled with a soil mixture composed of 60% clay, 20% sand and 20% water. The colonies were connected at the evening and the next day, 40 carriers with their loads were collected from each colony after having walked over 2 m, transferred into *Eppendorf* vials, and weighed as described.

A comparable procedure was used to collect pellets and their excavators. The clay was directly placed in the collecting box to a depth of about 1 cm, and no tube and second box were necessary. Workers were selected while digging at the surface and caught with forceps after having finished a pellet. Again, 40 workers with their corresponding pellets were collected from each colony and transferred into Eppendorf vials for weighing. To evaluate the relationship between pellet mass and carrier body mass, both measures were used as variables in a regression analysis.

Transport chains – 'Pelletograms'

The second experiment was intended to characterise the entire transport process of a single pellet from excavation to deposition outside the nest. In analogy to an ethogram, which characterises all behaviours and movements observed in an animal within an observation period, we created so-called *pelletograms* by observing single soil pellets and recording their transports, the number of carriers involved in the entire transport process, their waiting times, overall transport speed, as well as walking velocities and transport distances of the participating carriers. To observe the entire transport of excavated pellets from excavation to their deposition outside the nest, a laboratory colony of *Atta vollenweideri* was attached via a T-shaped connector to a box for pellet deposition at one side, and a 200 cm long transparent tube with an inner diameter of 10 mm on the other. At the distal end, the tube was connected to a second tube completely filled with moist clay (20% water content), so that the ants, when reaching the end of the tube, were able to excavate in clay. Along the way from the clay to the depositing box, a centimetre scale was drawn on the outside of the tube to allow for fast assessment of the position of dropped pellets.

Time counting was started with the beginning of excavation activity. Subcolonies were allowed to continuously excavate over two days, to evaluate whether the measured parameters change over time when the excavation site became known to the ants. For each observation, a digging worker was selected and the fate of its excavated pellet was followed. Every movement of that pellet was noted in terms of pick-up time and as well as dropping time and position, until it finally reached the depositing box. That way, we monitored for each pellet, how much time it had spent to cover a distance of 200 cm, how many workers had been involved in the transport process, how long it had lain on the way in between two transport events, and what distance had been covered at what speed in each transport event.

After starting the experiment, single pellets were observed over a period of 6 to 7 h. The next day, new clay was pushed into the tube from the distal end before continuing the observations, to keep the distance from the digging face to the depositing box constant, i.e. to compensate the tunnelling progress the ants had made over night. The whole procedure of offering an excavation site previously unknown to the ants, i.e. of connecting a fresh tube leading to a new excavation site to the colony, and observing pellets during their transport process for two days, was repeated six times, each experiment yielding observational data for about 20 pellets.

To test for temporal changes in the observed parameters, time since the experiment had been started was taken as the predicting variable in regression analysis. Transport duration, number of carriers involved and waiting time were each *ln*-transformed to achieve normal distribution of residuals, and tested for each day separately.

Three different functional worker groups were distinguished: excavators, defined as the workers carrying their own excavated pellet, short-distance carriers, defined as the workers that pick up a pellet and carry it for a distance, but not out into the depositing box, and long-distance carriers that cover the last part of the distance and deposit the pellets outside. These functional groups were analogous to those described during sequential transport of leaves in foraging ants: cutters, which cut the leaves and often carry the fragments only for short distances, short-distance carriers that drop their fragments on the way, and long-distance carriers that cover the remaining distance to the nest (Röschard and Roces, 2003a).

The three groups were, again day wise, compared in terms of the distance they covered and their walking speed. While the latter comparison was done using an ANCOVA, in the former case no transformation generated normally distributed residuals due to the strong bifurcate skewness of the data. Therefore, we utilized a resampling technique to compare the groups (Freedman, 1981). Confidence intervals for the means and regression coefficients were bootstrapped by recalculating both values for 104 random subsamples of $n = 20$ each. From those distributions, quantiles were calculated to determine confidence intervals. The effect of time was considered significant when the estimated 95% confidence intervals of the regression coefficients did not include 0. To compare the means of all groups with each other, three intergroup comparisons were necessary. Therefore, the significance level α was corrected from 1/20 to 1/60. The 98.3% confidence intervals were estimated for all means. Mean values with overlapping 98.3% confidence intervals were considered not significantly different from each other.

Finally, the percentage of long-distance carriers within all carriers, i.e. long-distance plus short-distance carriers, was calculated for each hour. A generalized linear model (GLM) with quasibinomial error distribution was used to analyse changes of this percentage as a function of time.

Quantifying pellet excavation – Ethograms

The observation of individual workers while excavating soil pellets was conducted with subcolonies, i.e. groups of workers without a queen, separated from the rest of the colony, but maintaining their own fungus garden. The subcolonies were allowed to excavate in a thin layer of soil between two horizontal glass plates. The setup made it possible to directly observe individually marked workers over an extended period of time, with the aim of assessing how long a single worker is engaged in digging behaviour, how many soil pellets it can form in that time, and where it deposits the pellets after producing them.

The subcolonies were assembled in small transparent plastic boxes (9 x 9 x 6 cm) connected to the laboratory colony during the weeks before the experiments. In this time, workers filled the boxes with fungus garden. These boxes, henceforth referred to as *fungus gardens*, were separated from the main colonies and opened to remove all workers but the fungus gardeners. Afterwards, each fungus garden was connected with a T-shaped tube (10 mm inner diameter) to two additional boxes of the same size, one daily replenished with fresh leaves, water and 1:1 honey water solution, from now referred to as the *feeding box*, the other one empty. The empty box was the *depositing box*, intended as location for the ants to deposit soil pellets. The depositing box was later connected to the *excavation arena*, a 4 mm layer of soil composed of 60% clay, 20% sand and 20% water, placed between two horizontal glass plates of 400 x 200 x 3 mm each. The connecting tube from the depositing box directly lead into a preformed tunnel 1 cm wide and 4 cm long, leading from the centre of one short side of the excavation arena directly to the centre of the rectangle in an angle of 90°. This was the place where workers from the subcolony started excavating (Fig. 19A). Until the experiment started, the connection between depositing box and excavation arena was kept closed.

For each subcolony, 200 intermediate size workers were chosen from the main colony and added to the separated fungus garden. 75 of these workers were individually marked using a colour code based on three dorsal marking spots on the ant's body: one on the posterior part of the head, the second on the two anterior segments of the mesosoma, the third on the two anterior gaster tergites. The colours

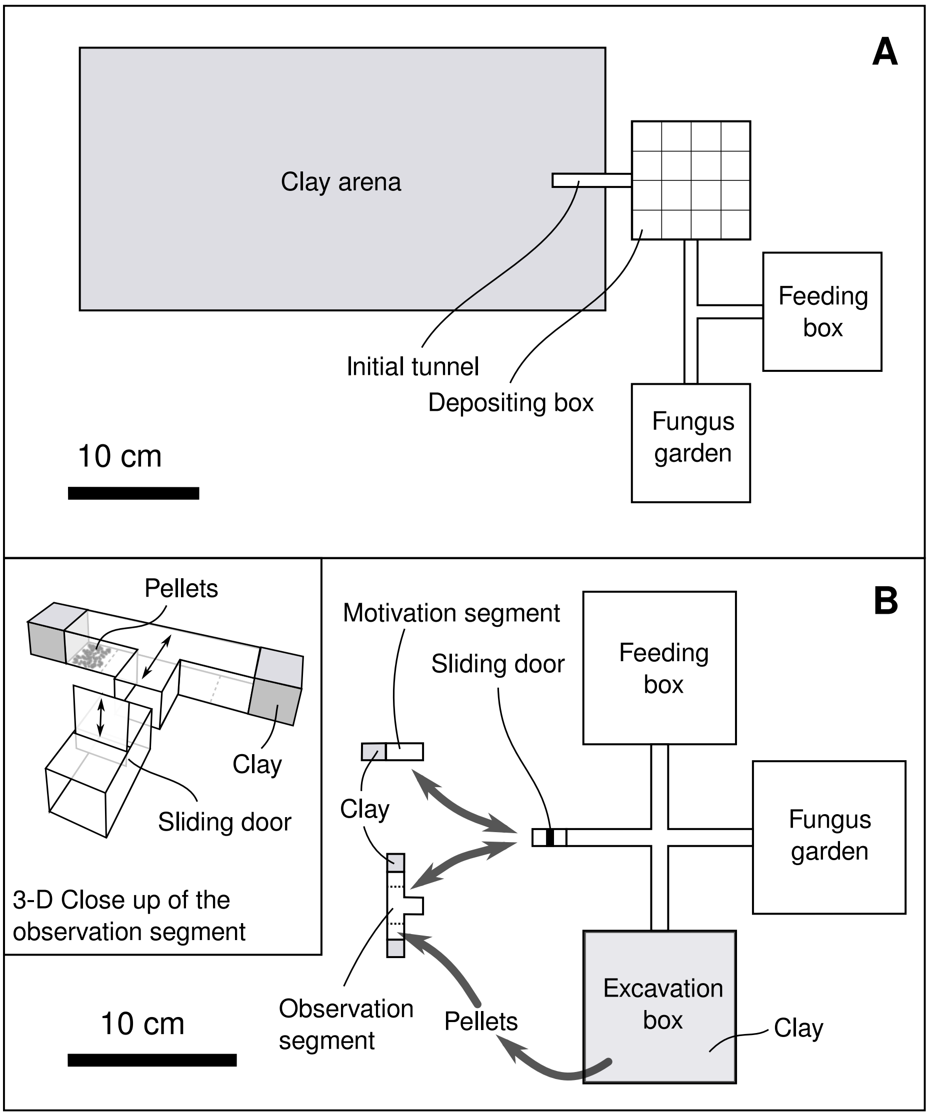

Figure 19: Experimental setups for ethograms and choice experiments. (A) Setup for observations on individual workers while excavating in soil and depositing pellets. Observations of individually marked workers were done both inside the clay arena, where soil pellets were excavated, and in the depositing box, where the most pellets were deposited. (B) Setup for choice experiments to test the influence of deposited pellets on the workers' decisions where to excavate. The motivation segment was connected to the subcolony to stimulate ants to excavate at that location, while soil pellets from the excavation box were placed at one side in the observation segment for the subsequent choice situation. After replacing the motivation segment with the observation segment (inset), it was observed at which side the ants started to dig first.

used were from *Edding-750* markers, applied to the marking spots by means of the tip of a thin iron wire. After assembling, the subcolonies were given time to calm and familiarize with the new environment for two days. On the third day, experiments were started.

Each experimental day started with the preparation of a new excavation arena and with connecting it to the subcolony. Dead individuals were replaced to keep group size and proportion of marked individuals constant. When the ants had started to dig in the excavation arena, one marked individual entering the arena and joining the excavation was chosen for close observation. Behaviours were recorded as a real-time ethogram in the software *BioLogic* (v. 1.0.0). Four different types of events were defined in the program: *start digging* to denote that the observed individual had started to excavate, *stop digging* for the moment the ant ceased excavation, and *pick* and *drop* to record pellet transportation activity the same way. This way it was possible to record when and how long an individual was engaged in either pellet digging or pellet carrying. Additionally, the location where pellets were dropped was recorded distinguishing *arena* for the excavation arena, *tube* for the connecting tube and *box* for the depositing box. The floor area of the depositing box itself was divided into 16 equal squares. If a pellet was deposited there, it was also noted in which of the squares the pellet had been dropped. This allowed for the calculation of the Index of Dispersion for each individual, to evaluate whether a worker drops its excavated pellets in an equal-, random- or aggregated distribution.

On each day, observations were conducted for up to 7 h, and each worker was observed as long as possible. Only when leaving the arena and depositing box for fungus garden or feeding box and staying absent for more than 10 min, observations for this worker were finished, and another one was chosen. The observation of an individual was finished without considering it for analysis if the worker did not reach the termination criterion of 10 min absence until the end of the day. However, this was never the case. Twelve individuals from two different subcolonies were observed. *Indices of Dispersion* were calculated for 8 workers, because workers were included in the analysis only if the exact locations of all their dropping events could be recorded.

Dropped pellets as cues for digging decisions – Choice experiments

To investigate whether deposited pellets act as cues that attract workers and spatially guide their decision where to dig, two alternative locations to excavate were offered simultaneously in a choice experiment, one with pellets deposited in front of the soil face, the other without. To avoid disturbance or overcrowding due to the separation from the colony, subcolonies were allowed to excavate at a particular spot that was later replaced by a T-shaped choice setup offering two alternative locations to excavate (Fig. 19B). In this setup, small groups of 5-6 animals were separated from the tube leading to the subcolony by using a sliding door, so as to avoid overcrowding. Only the first reaction of one of the 5-6 ants, i.e. the first occurrence of digging behaviour at one of the two alternative sites, was counted as a response. The measured responses can therefore be considered as individual choices rather than group responses, even though the choosing individual was not completely isolated from other workers. The subcolonies used for the experiments were created by separating a small fungus box including workers from the main colony and connecting it to a cross-shaped tube. Through that tube, the ants could enter a feeding box turning to one side at the junction, or, turning to the opposing direction, a similar sized box filled about 1 cm high with clay (18% water content) hereafter referred to as the *excavation box*. Workers excavating in that box provided the pellets used as a stimulus in the choice experiment. Pellets were collected from the excavation box using forceps, that were cleaned with 100% ethanol after each use, and stored into the *evaporation tube*, another tube of 10 mm inner diameter that was closed at one end using a plug with a small hole (about 3 mm diameter) in the centre. The other end was connected to a pump pushing air through the evaporation tube. In between the pump and the evaporation tube, a water bubbler was connected so as to saturate the circulating air. Hereby it was possible to allow chemicals on the pellets to evaporate and to prevent desiccation. Pellets for the *one hour* condition were kept here for one hour, those used in the other conditions put into the evaporation tube as well to rule out any effects that may come due to the treatment itself, but were removed after only one minute. Moving straight ahead from the fungus garden, the tube was connected through a small sliding door to a tunnel section with a square-shaped cross section that had been created by removing the bottom from 4 x 1 x 1 cm photometric plastic vial. The end of this tunnel was plugged with another piece of clay. We will call this part of the setup the *motivation segment* throughout the text. Here, as well as inside the excavation box, ant workers soon began to dig.

When digging activity was observed inside the motivation segment, the motivation segment was replaced with the *observation segment*, a T-shaped tunnel segment built from the same material, but branching to end in two equal clay walls located in opposite directions at a distance of 4 cm from each other. At one side, the square centimetre on the bottom immediately in front of the clay was covered with pellets from the excavation box. The clay surface was on both sides marked with an X-shaped scratch. Assuming that surface irregularities can motivate ants to start digging at them, as reported for termites (Lee et al., 2008a), scratching the surfaces was aimed at shortening the response time of the workers to gain more replicates within the same time. A sliding door prevented additional workers from entering the segment. The fact that the observation segment replaced another segment in which workers had dug already ensured a number of workers with a high probability of resuming excavation behaviour to return to that location.

After connecting the observation segment to the setup, the sliding door was closed as soon as 5-6 workers had entered. Hereby, on the one hand, inconclusive observations caused by ants digging simultaneously at both alternative sides under crowed conditions were avoided; on the other hand the workers were not torn out of their social environment. Preliminary observations had shown us that individual workers completely isolated from nestmates wait much longer until they start excavating, if they excavate at all. The isolated worker groups in our experiment were observed for 10 minutes. As soon as the first of the observed workers started to excavate at one side, this was counted as a decision for that side. Workers were then removed from the observation segment and kept separately until the experiment was finished. The observation segment was again replaced by the motivation segment for a period of at least 15 minutes. The observation segment was cleaned with 100% ethanol and the side with the pellets switched after each observation.

The percentage of choices for the side with the pellets was tested for deviation from a theoretical 1:1 distribution with a binomial test.

7.3 RESULTS

Size matching during soil transport

Pellets collected in the field (Fig. 20B) ranged from 2.7 mg to 75.8 mg with an average mass of 15.8±12.1 mg, containing 14.0±5.0% water (median±IQR range, $n = 67$). Pellets were predominantly carried by workers of intermediate size, the smallest weighing 1.5 mg, the largest 15.9 mg, the median being 5.2±4.0 mg ($n = 67$). Pellet size significantly depended on the size of the worker carrying it ($F_{1,57} = 16.21$, $p < 0.001$) with the carriers, especially the smaller ones, usually carrying more than their own body mass: the median loading ratio, i.e. the proportion of load mass to carrier mass was 2.9±2.1. Carried pellets (Fig. 20C) collected in the laboratory at 2 m distance to the digging site had an average mass of 4.0±3.7 mg (median±interquartile range, $n = 80$), containing 18.8±0.1% ($n = 80$) water. The body mass of carrying workers was 3.9±2.2 mg ($n = 80$). Again, pellet size significantly depended on carrier size ($F_{1,78} = 24.09$, $p < 0.001$). Here, loading ratio was lower than in the field, with median of 1.0±0.7 ($n = 80$), indicating that workers carried loads roughly equivalent to their own body mass. Freshly excavated pellets (Fig. 20D) weighed

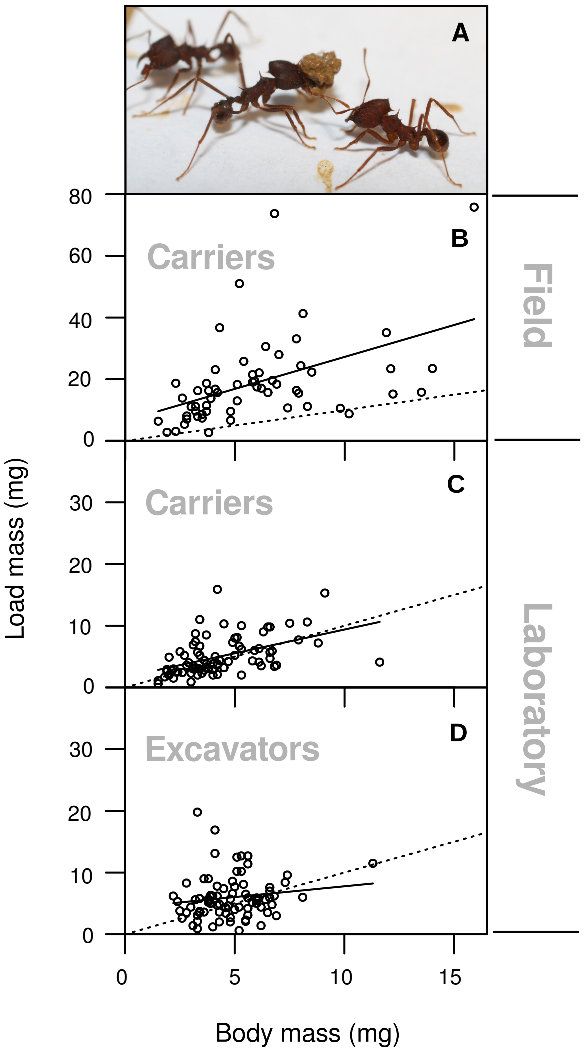

Figure 20: Relationship between pellet mass (m_P) and worker body mass (m_W). In each graph, the solid line represents the regression line and the dotted line a hypothetical 1:1 ratio of load mass to carrier mass. Data points above that line correspond to carriers less heavy than their loads. (A) *Atta vollenweideri* worker carrying a soil pellet in the laboratory (Photo: Andrew I. Bruce). (B) Pellets and carriers collected at the openings of field nests ($m_P = 6.5+2.1\ m_W$, $R^2 = 0.22$, $n = 60$, $p < 0.001$). (C) Pellets and carriers collected from laboratory colonies at 2 m distance from the excavation site ($m_P = 1.7+0.8m_W$, $R^2 = 0.24$, $n = 80$, $p < 0.001$). (D) Pellets and excavators collected directly at the excavation site ($m_P = 4.3+0.3m_W$, $R^2 = 0.02$, $n = 80$, $p = 0.186$).

5.5±3.8 mg (median±interquartile range, $n = 80$) with 20.9±0.1% ($n = 80$) water content. The body mass of the excavators was 3.8±1.8 mg ($n = 80$). Regression analysis showed no significant dependence of pellet size on excavator size ($F_{1,78} = 1.78, p = 0.186$).

Transport chains – 'Pelletograms'

The time it took for one pellet to be transported over the whole distance of 200 cm changed during the observation period. While it took 35.2±12.0 min (median±interquartile range, $n = 10$) for pellets produced during the first hour after starting the experiment, median transport duration was 7.3±4.1 min ($n = 73$) only on the second day (Fig. 21A). The transport duration decreased as a function of time during the first day ($F_{1,58} = 48.2, p < 0.001$) and, at a smaller rate, also at the second day ($F_{1,71} = 4.1, p = 0.047$).
All pellets were transported by more than one individual. Those produced during the first hour were carried by 7.0±2.5 workers (median±interquartile range, $n = 10$), while only 3.0±1.0 workers ($n = 73$) contributed to the transport of each pellet at the second day (Fig. 21B). Again, this value decreased at a higher rate during the first day ($F_{1,58} = 19.4, p < 0.001$) than during the second ($F_{1,71} = 4.4, p = 0.039$). In between two transport events, the items were simply deposited on the ground of the tube. There was no particular location for load dropping; a homogeneous layer of pellets covered the ground of the tube, this layer beginning some centimetres from the excavation site and continuing for about 30-40 cm. The waiting times of the pellets, i.e. the time a pellet remained at one location in between two transport events, decreased on the first ($F_{1,57} = 6.6, p = 0.013$) and on the second day ($F_{1,71} = 9.3, P = 0.003$), the median being 182±263 s ($n = 41$) during the first hour, and only 64±89 s ($n = 185$) at the second day (Fig. 21C).
Three different types of workers contributed to the transport of pellets. The excavators themselves, 133 were observed during the experiment, carried their pellets for a distance of 9.5±5.5 cm (median±interquartile range, $n = 133$) until dropping them. The intermediate short-distance carriers, 281 of which were observed, transported the particles for another 9.0±14.0 cm ($n = 281$) each, until they were picked up by long-distance carriers, bringing them out into the deposition box with a significantly longer (see Tab. 16 in Appendix A for detailed statistics) median transport distance of 168.0±34.6 cm ($n = 133$) (Fig. 22A). Additionally, the three different worker types, excavators, short-distance carriers and long-distance carriers, moved at a significantly different walking speed (influence of 'type' on walking speed on the first day: $F_{2,286} = 119.3, p < 0.001$; second day: $F\ textsubscript2,254 = 92.9, p < 0.001$), with the short-distance carriers moving slower than the other groups.

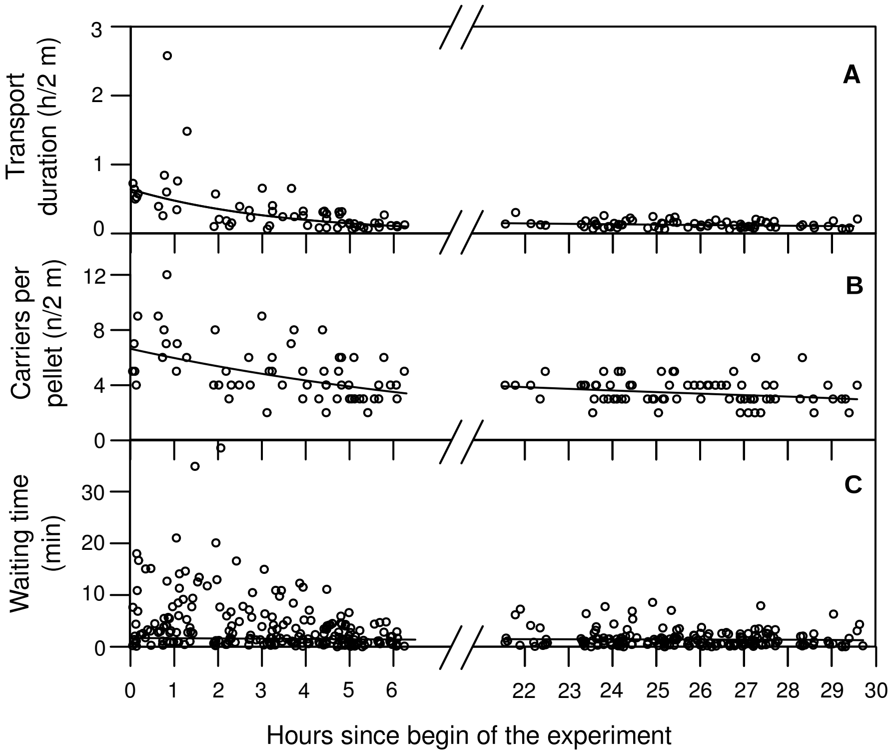

Figure 21: Characteristics of pellet transport as a function of time. Transport duration (*d*), number of carriers involved (*N*) and the waiting times (*w*) between transport events are plotted as a function of time (*t*). (A) Time needed for a pellet to be sequentially carried over a distance of 2 m from the excavation site. Solid lines show the results of regression with *ln*-transformed *d*-values on both days (first day: $d = 0.6*e^{-0.3*t}$, $R^2 = 0.45$, $n = 60$, $p < 0.001$; second day: $d = 0.4*e^{-0.04*t}$, $R^2 = 0.05$, $n = 73$, $p = 0.047$). (B) The number of workers involved in the transport of single pellets. Solid lines show the results of regression with *ln*-transformed Y-values on both days (first day: $N = 6.6*e^{-0.1*t}$, $R^2 = 0.25$, $n = 60$, $p < 0.001$; second day: $N = 7.9*e^{-0.03*t}$, $R^2 = 0.06$, $n = 73$, $p = 0.039$). (C) Time a pellet remained on the floor between two transport events. Solid lines show the results of regression with *ln*-transformed Y-values on both days (first day: $w = 3.8*e^{-0.2*t}$, $R^2 = 0.10$, $n = 225$, $p < 0.001$; second day: $w = 5.5*e^{-0.1*t}$, $R^2 = 0.02$, $n = 177$, NS).

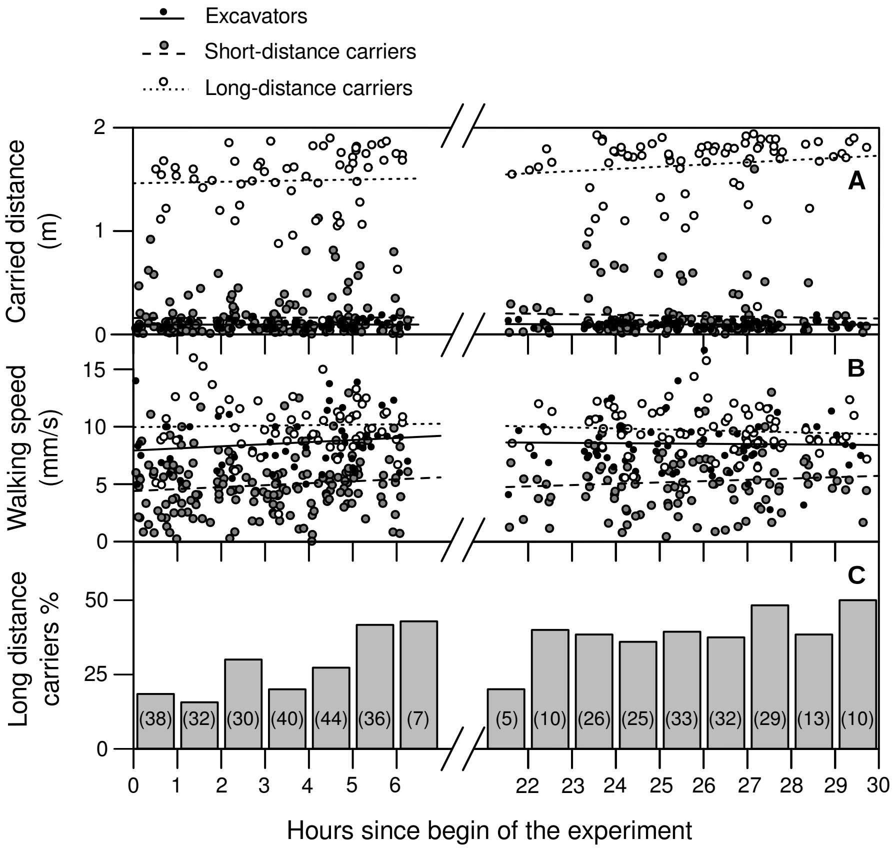

Figure 22: Distances walked (*l*) and speeds (*v*) measured in the different carriers involved in pellet transport as a function of time (*t*). Black dots and solid regression lines represent excavators, grey dots and dashed regression lines short-distance carriers, and white dots and dotted regression lines long-distance carriers. (A) Distances covered (in cm) during one transport event by excavators (first day: $l = 7.7 + 0.53*t$, *NS*; second day: $l = 11.4 - 0.07*t$, *NS*), short-distance carriers (first day: $l = 14.0 + 0.71*t$, *NS*; second day: $l = 32.8 - 0.58*t$, *NS*) and long-distance carriers (first day: $l = 142.0 + 1.98*t$, *NS*; second day: $l = 108.8 + 2.13*t$, *NS*; for 1.6% confidence intervals see Tab. 16). (B) Walking speed in each transport event for excavators (first day: $v = 7.9 + 0.18*t$, $R^2 = 0.02$, $n = 60$, *NS*; second day: $v = 9.2 - 0.003*t$, $R^2 = 0.001$, $n = 73$, *NS*), short-distance carriers (first day: $v = 4.4 + 0.17*t$, $R^2 = 0.01$, $n = 168$, *NS*; second day: $v = 2.3 + 0.11*t$, $R^2 = 0.008$, $n = 113$, *NS*) and long-distance carriers (first day: $v = 9.9 + 0.04*t$, $R^2 = 0.001$, $n = 60$, *NS*; second day: $v = 11.9 - 0.008*t$, $R^2 = 0.007$, $n = 73$, *NS*). (C) Proportion of long-distance carriers among individuals that picked up pellets. The number of observed pick-up events is shown in parentheses for each time interval.

Their average walking speed was 5.0±2.4 mm/s ($n = 281$), as compared to 8.5±2.2 mm/s ($n = 133$) for excavators, and 10.0±2.2 mm/s ($n = 133$) for long distance carriers. Walking speed did not change significantly during the observation period (influence of time on walking speed on the first day: *0.02 (mm/s)/h*, $F_{1,284} = 3.4$, $p = 0.067$; second day: *-0.003 (mm/s)/h*, $F_{1,253} = 0.06$, $p < 0.812$; Fig. 22B).
The percentage of long-distance carriers among individuals picking up pellets increased significantly during the first day ($F_6 = 10.2$, $P = 0.024$), from 18% ($n = 38$) in the first hour to a constant proportion of 40% ($n = 188$) during the second day (Fig. 22C).

Quantifying pellet excavation – Ethograms

The 12 individuals observed in the laboratory excavated soil pellets in relatively uniform behavioural sequences, the most marked interindividual difference being the overall period of time workers spent performing the task, ranging from 14 to 269 min with an average of 121±79 min (mean±standard deviation, $n = 12$). Within this period, the observed individuals excavated pellets at an average rate of 25.7±10.4 pellets/h ($n = 12$). During repeated sequences, the animals approached the soil and started manipulating it by what Sudd (1969) has described as 'grabbing': the mandibles were inserted into the clay, a behaviour that, in the observed case of excavating in wet clay, much resembles cutting behaviour in other materials. The workers virtually bite into the tough material. After that, the soil was 'raked' together by using both forelegs and the closed mandibles that were used much like an ice-cream scoop to aggregate soil beneath the body in an anterioposterior pulling movement. After one or more sequences of grabbing and raking, the accumulated body of soil was picked up with the mandibles, carried away and dropped at another location. The average time spent to form one pellet was 41.0±30.0 s ($n = 536$). After dropping the pellet the worker had produced itself, it occasionally picked up one or two other pellets and relocated them before starting to excavate the next pellet. Thus, between two consecutive excavation sequences workers relocated between one and three pellets. Therefore, besides producing the pellets, excavators also relocated them at a mean rate of 25.8±6.2 pellets/h (Tab. 4). The majority of pellets were relocated directly into the depositing box. Within the depositing box, the pellets appeared to be evenly distributed on the ground without apparent aggregations. However, at the individual level workers preferred to drop all their pellets within one particular area. As the high Indices of Dispersion, averaging 7.3±1.1 ($n = 8$), indicate, all the pellets carried by a single individual were not dropped in the box randomly, but placed in an aggregation (Fig. 23), each worker aggregating its own loads at a different place.

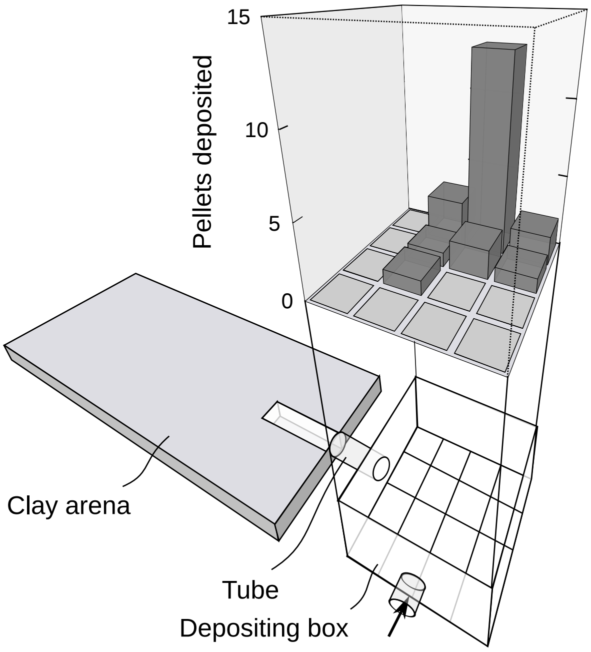

Figure 23: Example of the distribution of pellets deposited by a single worker in the depositing box. Shown are the clay arena, where individually marked workers excavated soil pellets, and the depositing box, where most pellets were dropped by the observed individuals. The thick arrow indicates the location where the animals entered the setup. To reconstruct the individual pattern of pellet deposition for each observed worker, the ground of the depositing box was divided into 16 equal squares. The number of pellets dropped in each square by one individual was counted. Each bar in the graph represents one of the possible 16 areas distinguished inside the box, with the height of the bar indicating the number of pellets dropped inside this field by the observed individual. The worker observed for this example (worker nr. 7) placed 23 pellets in the box within a period of 44 min. The resulting index of dispersion was 7.2. The three-dimensional barplot is based on a script by Michal J. Figurski (`http://gallery.r-enthusiasts.com/graph/Presentation_style_3D_barplot_161`).

ANT	TIME (MIN)	DUG	CARRIED	SPEED (S/PELLET)	DI
1	14	5	4	28.0±20.4	-
2	180	63	60	61.0±40.1	-
3	269	71	88	35.7±23.0	-
4	78	29	32	51.8±40.0	6.0
5	180	63	57	37.4±32.0	9.0
6	189	85	98	41.7±32.4	6.2
7	44	21	26	29.4±15.0	7.2
8	64	29	27	46.0±30.1	6.6
9	156	65	67	40.6±23.2	8.3
10	150	41	72	33.1±18.3	8.3
11	18	17	11	33.2±25.4	-
12	108	47	47	32.5±17.4	6.7

Table 4: Long-term observations of individual excavators. Shown are, for each of the 12 observed workers, the duration of its continuous excavation activity, the number of pellets excavated and the number of pellets carried within that time, the resulting digging and transport rates, the average time spent for excavating a single pellet (mean±SD), the locations where the pellets were deposited and, for those pellets deposited in the box, the Index of Dispersion (DI) for their distribution on the 16 square areas. A DI close to 0 is an indicator of a regular distribution. Values around 0.5 characterise random distributions, while values close to 1 and higher indicate an aggregated distribution. For four individuals (1, 2, 3 and 11) not every pellet dropping could be observed, and therefore no dispersion indices were assigned to them.

Dropped pellets as cues for digging decisions – Choice experiments

Using a layer of fresh pellets as a stimulus in the choice situation, excavation was first initiated at the side where the pellets were presented in 70% (56 of 80) of the experiments. The ants significantly ($n = 80$, $p < 0.001$) preferred to dig at that side. If pellets were offered as stimulus after a waiting period of one hour, there was no significant effect: the side with the pellet layer was chosen in 59% (47 of 80) of the trials ($n = 80$, $p = 0.146$). With only one fresh pellet as a stimulus, the corresponding side was chosen in 55% (44 of 80) of the trials and again, the effect was not significant ($n = 80$, $p = 0.434$, Fig. 24).

7.4 DISCUSSION

Our results indicate that underground soil transport strongly resembles the long-distance sequential transport of leaf fragments by

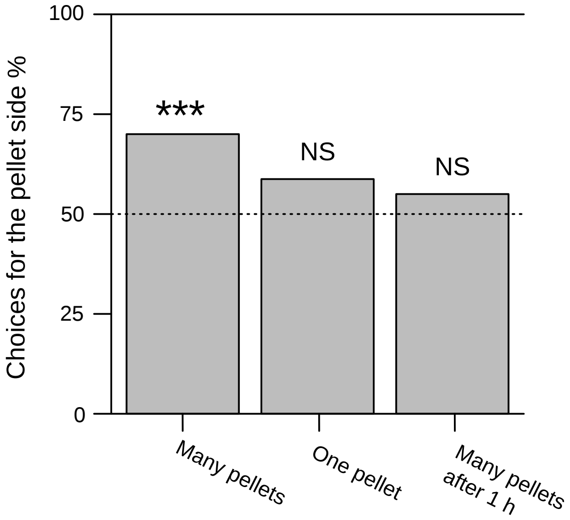

Figure 24: Choices between two alternative locations to excavate. To different locations were offered for excavation. At one side, pellets were deposited on the ground (see Fig. 19). The sample size was 80 for all conditions. Significance levels for binomial tests are indicated above the bars ('NS' not significant, '*' $p < 0.05$, '**' $p < 0.01$, '***' $p < 0.001$).

foraging leaf-cutting ants, and that task partitioning during transport results in accumulations of fresh pellets that provide a cue influencing other workers in their decision where to excavate. Single pellets were not carried to the surface by the excavator directly, but transported by several individuals and deposited on the way in between transport events. Excavators themselves carried their pellets for about 9.5 cm only and they were selective in choosing a location where to drop them, usually aggregating their pellets individually at one spot. Several short-distance carriers picked the items up and carried them at low speeds over short ranges before dropping them on the ground. Finally, the long-distance carriers picked up the loads again, and covered the rest of the way until the final deposition outside the nest. The number of short-distance carriers contributing to the transport of each pellet was higher during the establishment of excavation activity at a new location, and dropped over time, indicating that more workers carried the pellets directly to the outside as the digging process progressed.

During soil transport, load size was shown to depend on carrier size as also reported for leaf transport (Röschard and Roces, 2003b). This effect was only observed in carriers, but not in excavators, which walk only very short distances before returning to the excavation site. Size matching in carriers suggests that they are either highly selective when picking up pellets for transport, or actively manipulate the sticky material to create a load roughly adjusted to their body size. This may suggest that task partitioning among carriers facilitates size-matching, and is therefore aimed at maximizing material transport rates, as discussed for leaf foraging (Lighton et al., 1987; Röschard and Roces, 2002; Roces and Bollazzi, 2009), even though we did not find direct support for this hypothesis because excavators and long distance carriers walked at the same speed. It is important to mention that the loading ratios observed in the field were larger than those from the laboratory. While workers in the laboratory carried loads roughly equivalent to their body mass, field workers carried loads that were in average three times heavier than their bodies, a phenomenon that may be related to the physical properties and/or humidity of the heterogeneous natural soils that influence the aggregation of the material to be carried as a pellet. Sequential task partitioning in foraging *Atta vollenweideri* workers follows a similar pattern (Röschard and Roces, 2003a). As described for the excavators, cutters contribute little to the transport of the leaf fragment they harvested. Short-distance carriers cover small portions of the overall distance when the leaf fragment is close to its origin, until a long-distance carrier picks the item up to carry it for the rest of the way to the nest. Again, there is no particular location on the way for dropping. Leaf fragments are transported significantly slower in transport chains than when carried directly by one worker only (Röschard and Roces, 2003a, 2011).

Two hypotheses have been suggested to account for the advantages of sequential transport in foraging leaf-cutting ants (Anderson et al., 2002; Röschard and Roces, 2003a, 2011; Roces and Bollazzi, 2009). First, sequential load transport may have been favoured during evolution because of a faster load delivery rate. These arguments are the core of the so-called *economic-transport hypothesis*, with *economic* in this context referring to the maximization of the transportation speed of a leaf fragment, which at the colony level may result in an increased overall rate of resource delivery (Röschard and Roces, 2003a). A faster material transport rate may be achieved via an improved size-matching, as discussed above, or because each contributing worker restricts its task to a certain part of the way, which may improve its orientation and indirectly its walking speed. It remains an open question whether a worker harvesting a leaf fragment may succeed in carrying it back to the nest for about 100 m, and then in finding its way back to the cutting site. If not, sequential transport would be absolutely necessary to exploit a distant leaf patch as fast as possible. Second, leaf fragments may inform other foragers either about type and quality of a newly exploited food patch, or simply about the fact that such a food patch can be found in that direction. In this case, the behavioural response of transferring fragments would have been selected for because of its positive effect on the information flow. This *information-transfer hypothesis* states that workers may trade off material transport rate for enhanced information transfer during social foraging, and field experiments on foraging *Atta vollenweideri* leaf-cutting ants provided support for these arguments (Röschard and Roces, 2011).

With regard to the described similarities between leaf transport and soil transport, both hypotheses might be valid for soil transport as well. We observed task partitioning between short-distance and long-distance carriers. Both groups differed not only in the distances they covered, but also in their carrying speed, which was significantly slower in short-distance carriers. We can only speculate about the function of short-distance carriers during soil transport. Possibly, the parts of the tunnel where pellets are deposited constitute a challenge to the ants' orientation abilities. The constant loading and unloading activity may result in a permanent change of chemical and physical cues, requiring specialized individuals, each familiar with a short section of the changing local environment, to carry the loads. Slow walking speeds of short-distance carriers may result from slow orientation movements and/or walking difficulties on a pellet-covered surface.

Another possibility to account for the observed slow walking speed is that short-distance carriers move at a slower pace to contact workers and so to enhance the information flow about the current digging activity. Encounter rates with workers carrying a pellet might inform nest mates about current digging activity, in addition to the effect of deposited pellets on workers' digging choices. Interaction patterns are known to influence task allocation in insect societies, by informing individuals about the activities of nest mates (Gordon, 1996). Hypothetically, encounter rates with soil carriers can inform workers about both the fact that nest mates are excavating and the direction leading to the place where nest enlargement is happening. The fact that the proportion of short-distance carriers contributing to the transport of each pellet decreases within the first few hours, as shown in our experiments, appears to support the hypothesis that they may contribute to the recruitment of nestmates to the excavation site, for recruitment would be more important when excavation activity begins at a new location than later on. Finally, our observations indicate that in between two excavation events, some excavators occasionally pick up other pellets and relocate them. We need therefore to assume that a significant proportion of the short-distance carriers observed in our pelletograms were in fact excavators temporarily working as carriers. Since excavators are known to actively recruit nest mates to the digging site by producing stridulation signals while forming their pellets (see Chapter 8), their behaviour as short-distance carriers, and the spatial arrangement of the carried pellets, could also be considered as part of the recruitment system.

In our experiments, excavators formed pellets at a relatively constant rate for an average period of 2 h. They deposited their pellets close to the digging site being highly selective about the location where to drop them. With several excavators working in parallel, individual deposition patterns resulted in a homogeneous layer of pellets covering the ground nearby the excavation site. The observations that individuals aggregate their pellets, but no obvious aggregation results at the group level, may appear contradictory if we expect pellets to act as stigmergic cues. But they can be interpreted in the terms of maximizing individual working rates: the individual selectivity of dropping points by the excavators may improve their individual performance by allowing them to quickly return and to find their excavation site.

We demonstrated in our choice experiments that a number of fresh pellets deposited close to a potential digging site increases the probability of other workers to prefer it over an alternative site when deciding where to dig. It is therefore likely that primary underground deposits of soil pellets act as stigmergic cues. It has been recently demonstrated in the same species that stridulation signals produced by digging individuals attract nestmates to excavate at the same location (see Chapter 8). The advantage of one individual preferring to excavate close to other individuals is the possibility of a spatial organisation and coordination of collective digging activity: many individuals choose the same location to excavate, which results in a faster emergence of a larger structure. Pellets deposited close to excavation sites, which were shown in the present study to be attractive to other workers and to act as stigmergic cues, may have a comparable effect. Being spread out along the tunnel for up to 40 cm, they may even complement stridulation signals, which are effective only at distances shorter than 6 cm (see Chapter 8).

Our results indicate that the effect of deposited pellets as a cue depends on two variables: quantity and time since its excavation. A single pellet in the tunnel had no measurable effect on the worker's decision where to excavate. If we hypothesize that sequential transport of soil pellets has been favoured during evolution to provide cues aimed at aggregating excavators at one spot, a large number of pellets may more likely originate from a large number of excavators, and should therefore be more attractive as a stigmergic cue. The second variable important for the effectiveness of deposited pellets as a cue was the time since its excavation. After one hour, the pellets, even in large numbers, had lost their effect in the choice experiment. It remains an open question if this was due to chemicals on the pellet surface that evaporated in this period. Only two cases have been reported in which pheromones induced excavation behaviour in ants (Wilson, 1958; Blum and Warter, 1966). In both cases, the substances were described as alarm pheromones and their use during regular nest excavation was not further investigated. However, both substances were extracted from the mandibular gland: hypothetically, they could be applied onto a pellet while digging without any marking behaviour obvious to the observer. Another possibility would be that saliva or traces of cuticular hydrocarbons from the excavator's mandibles remain on the pellet only for some time after excavation. Whatever the mechanisms leading to the lack of attractivity over time, responding only to fresh pellets as cues allows workers to join the current excavation activity. It is due to task partitioning in the process of soil transport that excavated pellets are left on the way to the excavation site, thereby providing cues influencing the excavation behaviour of nearby workers. As a consequence, one important advantage of sequential transport, besides its potential benefits for the orientation in the complex environment of a giant underground nest, can be seen in providing these cues. Hereby, task partitioning during soil transport likely contributes to the spatial organisation of collective excavation behaviour that leads to the complex architecture of leaf-cutting ant nests.

8

VIBRATIONAL COMMUNICATION

This chapter has been originally published in: S. Pielström and F. Roces. Vibrational communication in the spatial organization of collective digging in the leaf-cutting ant Atta vollenweideri. Animal Behaviour 84:743–752, 2012.

ABSTRACT Subterranean ant nests are structures that emerge from collective building. The mechanisms used by individual ant workers to coordinate their building behaviour are largely unknown. We discovered that isolated workers of the Chaco leaf-cutting ant *Atta vollenweideri* (Forel) stridulate while excavating in soil, and investigated the possibility that workers communicate *via* vibrational signals in the context of collective nest excavation. Workers were observed to stridulate at similar rates, not only while manipulating soil with their mandibles, but also several seconds before grabbing the material. The measured attenuation rate suggests a detectable signal range of less than 6 cm. When presented with stridulation signals in an experimental arena offering distinct locations to excavate, workers were more likely to dig close to the source than at an alternative site. The probability for a worker to dig at a location increased with the intensity of the vibrational signals produced at this location. Group-level stridulatory activity was monitored in the context of nest enlargement, in which ants excavating a tunnel were allowed to suddenly break into an existing chamber. Recordings before and after having gained access to the chamber showed that workers stridulated while excavating, but gradually discontinued signal production when nest space was available. Our results indicate that beyond the previously-described use of vibrational signals in the contexts of food recruitment and alarm communication, workers stridulate while engaged in nest digging and attract nestmates to join excavation activity at the same location, thus contributing to the spatial organization of collective nest building.

8.1 INTRODUCTION

building activity of workers.

It is conceivable that workers within an ant group may initiate, modulate or cease building activity by responding to cues arising from the abiotic environment, from the social environment, for instance the encounters with colony members, or by responding to communication signals produced by nestmates. It has been demonstrated that environmental variables such as air flow and soil temperature provide cues used by leaf-cutting ant workers to make decisions about nest building (Bollazzi and Roces, 2007; Bollazzi et al., 2008; Bollazzi and Roces, 2010). A correlation between colony size and nest volume observed in several ant species also suggests worker-per-volume ratio as an important variable modulating excavation behaviour (Rassé and Deneubourg, 2001; Buhl et al., 2004; Mikheyev and Tschinkel, 2004). Concerning communication in the context of nest building, much less evidence is available. Based on behavioural observations of reactions of the Florida harvester ant *Pogonomyrmex badius* (Latreille) to secretions of its mandibular gland, citetWilson1958 suggested the existence of a pheromone inducing excavation behaviour. Blum and Warter (1966) reported workers of *Dorymyrmex pyramicus* (Roger) to respond with digging to high concentrations of their alarm pheromone. Even though chemical communication is a common and well studied phenomenon in ants (Hölldobler and Wilson, 1990), no further studies have been published to demonstrate a clear causal relationship between pheromones and nest excavation in ants.

Besides chemical communication, vibrational communication by means of stridulation is common in many ant taxa (Markl, 1973). In leaf-cutting ants, the stridulation organ is located at the joint between gaster and postpetiolus, and vibrations are produced by slightly moving the gaster up and down (Markl, 1968). Vibrations are transmitted both through the air and through the substrate, but they are only sensed as substrate-borne signals, since ants are unable to perceive airborne sound (Roces and Tautz, 2001). Stridulation during excavation behaviour has been described for workers of the western harvester ant *Pogonomyrmex occidentalis* (Cresson) (Spangler, 1967). In addition to this, colonies of the red fire ant *Solenopsis invicta* Buren show an increased colony-level stridulation rate when excavating in soil (Rauth and Vinson, 2006). However, since no effects of the vibrational signals on the excavation behaviour of nestmates were observed (Spangler, 1967), Spangler (1973) advanced the hypothesis that stridulation during excavation provides mechanical support by loosening the soil, and it has therefore no communicative function. In the leaf-cutting ant *Atta cephalotes* (L.), stridulation was described in a different context. Markl (1965, 1967) observed workers to stridulate whenever prevented from moving freely. If trapped under a layer of soil, workers stridulate to attract nestmates, which respond by excavating the trapped individuals. In this case, stridulation is clearly used for communication, with workers responding to the signals with excavation behaviour. In accordance with its production in aggressive encounters, stridulation was interpreted primarily as an alarm signal (Markl, 1965, 1967).

Later studies revealed additional communicational functions of stridulation in ants. Foragers of the European seed harvesting ant *Messor capitatus* (Latreille) stridulate while at food sources and inside the nest to recruit nestmates (Baroni-Urbani et al., 1988). Leaf-cutting ant foragers (*Atta*) stridulate while cutting leaves, thus producing a short-range recruitment signal that travels along the branches of the plant and attracts other workers to cut material at the same area of

the plant (Roces et al., 1993; Roces and Hölldobler, 1996). Additionally, stridulation is used as a means of communication between leaf carriers and hitchhikers (Roces and Hölldobler, 1995).
Hence, in leaf-cutting ants, stridulatory vibrations are known to attract nestmates in the contexts of alarm, in which they elicit excavation behaviour, and food recruitment. In two other ant taxa, stridulation behaviour has been observed in the context of nest excavation, but its function remains elusive. This raises the question as to whether leaf-cutting ants may also use stridulatory vibrations as communication signals during nest excavation. The aim of this work was to explore the use of vibrational communication in the context of collective nest excavation in the leaf-cutting ant *Atta vollenweideri*. We first performed a detailed observation of excavation behaviour of individuals and explored the production of vibrational signals as correlated to their actual digging movements. We then evaluated the attenuation of vibrational signals on the soil surface to quantify the potential range of signal detection by nearby workers. In order to investigate whether stridulatory vibrations guide workers when deciding where to initiate excavations, we additionally performed choice experiments: one series using alarm stridulations, and the other series using digging stridulations as stimuli. Finally, to rule out the possibility that individual workers stridulated because of having been isolated during the measurements, group-level stridulatory activity was monitored in subcolonies in the context of excavation to relocate the fungus garden into a new chamber.

8.2 METHODS

All experiments and measurements were conducted on vibration absorbing surfaces. To prepare such a surface, an iron board of 110 x 50 cm and approximately 80 kg was mounted on four fully inflated wheelbarrow rubber tires. To measure vibrations, a *Bruel & Kjær* Type 4333 accelerometer was connected to a *Bruel & Kjær* Type 2635 charge amplifier. The sensor output was recorded as a sound file with a computer sound card (*Creative SoundBlaster X-Fi* or *Creative SoundBlaster Audigy 2 ZS*) at 96 kHz sample rate, and stored in 16-bit pcm uncompressed *Wave Audio File* format. The recorded signal amplitude, scaled in dB, was converted in acceleration units as follows: a 1 kHz vibration was produced by connecting a *Bruel & Kjær* Type 4810 mini shaker to a sine wave generator. An accelerometer was mounted on top of the mini shaker with a screw, and was connected to an oscilloscope, where the output amplitude was calibrated to 1, 5, 10, 50, 100 and 500 cm/s^2. Then, vibrations of these amplitudes were recorded as sound files and used for calibrations. Records were processed and analysed in *Audacity* 1.2.6. (`http://audacity.sourceforge.net`). *LabView* 8.2. was used to obtain the Fast Fourier Transformation. Calculations, determination of sound sample amplitudes and statistical analysis were made in R 2.11.1. (`http://www.R-project.org`).

Observation of digging individuals

To measure the vibrations produced by individual ants while excavating in soil, single workers had to be isolated from the colony while excavating. To achieve this, a laboratory colony of *Atta vollenweideri* was connected to a horizontal tunnel made out of plastic vials of 10 x 10 x 45 mm in size. The last tunnel section, henceforth called 'observation vial', was a vial filled with clay at the posterior end (Fig. 25A). Thus, the tunnel ended blindly in a clay wall at which ants could excavate. The tunnel segment before the observation vial was equipped with a small sliding door, thus allowing us to control the access of workers into the observation vial. To avoid noise originating from the colony, the observation vial was slightly detached from the preceding tunnel segment, and mounted on a separate retort stand. A clamp holding the observation vial pressed the recording accelerometer against the top side of the vial, thus allowing measurement of vibrations produced inside.

Workers coming from the colony were initially allowed to excavate in the observation vial for two minutes. Then the sliding door was closed and the observation vial was moved away from the tunnel. This eliminated any mechanical contact between the observation vial and the rest of the colony, and stridulations originating from the colony could not disturb the measurement. Additionally, moving the observation vial allowed us to access its opening and so we could carefully remove all ants but the one currently engaged in digging. If the remaining worker continued excavating after the removal of all nestmates, its behaviour was videotaped and its stridulations produced were simultaneously recorded in the audio track. The worker was observed until it ceased excavation, and then removed from the experiment.

In each video sample of an individual digging ant, the excavation of the last soil pellet was selected for close observation. According to Sudd (1969), ant excavation behaviour can be described as a repetitive behavioural sequence comprised of 'grabbing' soil with the mandibles, and then 'raking' it with mandibles and legs to accumulate material for 'transport'. In addition to grabbing, raking and transport, the approaching phase before the mandibles contacted the soil was classified as an additional phase of the excavation behaviour, giving a total of four phases: approaching, grabbing, raking and transport. For each of the observed workers, the repetition rate of stridulations was measured in the four phases. To allow for swift identification and separation of stridulation signals, the tracks were opened in spectral view, single signals were counted and then divided by the

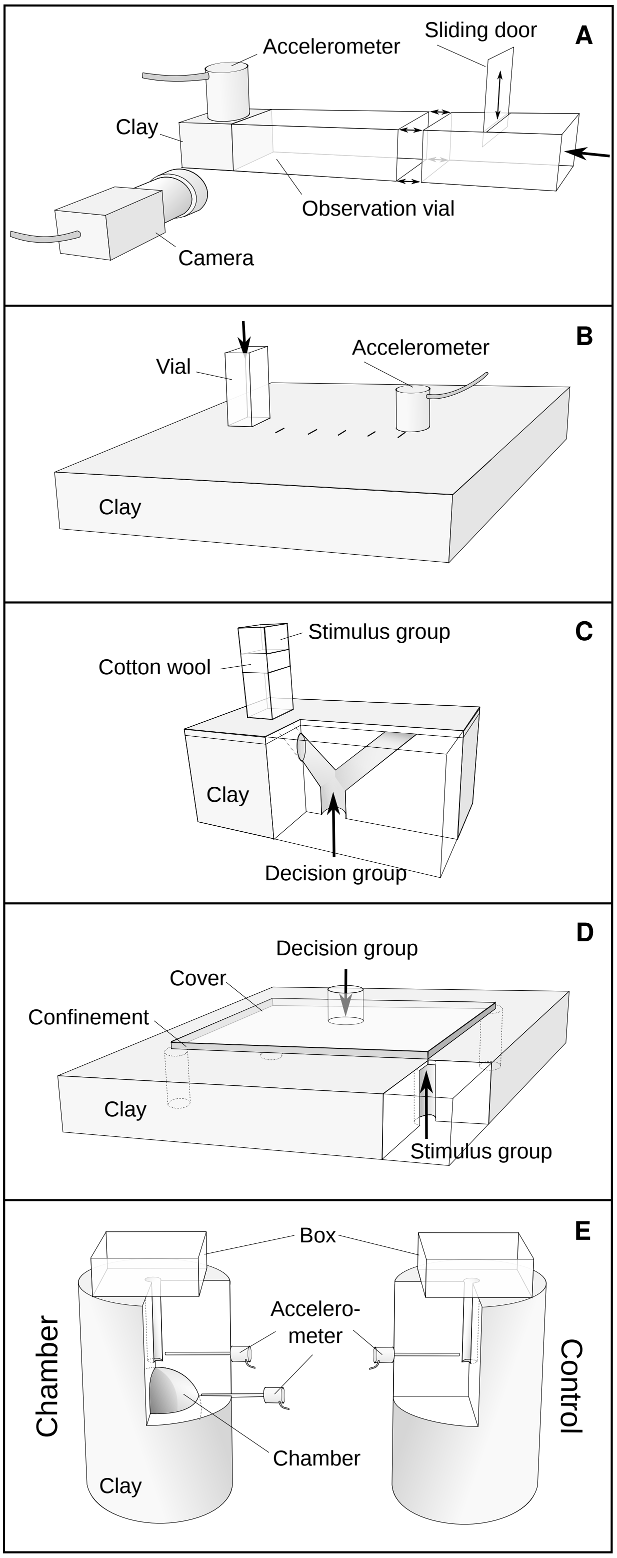

Figure 25: Experimental setups used in this study. Thin arrows indicate the moving directions of movable parts, thick arrows indicate where the animals entered the experiment.

length of the section. The sound files were blinded for signal counting, i.e. a person unfamiliar with the experiment randomly renamed the data files, while a second person quantified the signals. This procedure allowed us to compare stridulation rates in the four different phases. Twenty one individuals with a mean body mass of 5.1±2.1 mg were videotaped during excavation, and their behaviour analysed as described above.

To test for statistical differences in the rate of signal production, a mixed-effects ANOVA model was fitted onto the data set, applying the four phases of behaviour as a fixed factor and the number of the observed worker as a random factor to heed the repeated-measures design of the experiment. An α correction ($\alpha = 0.008$) was used for *post-hoc* paired t-test comparisons.

Signal attenuation over distance

Two different approaches were used to evaluate how the amplitude of the stridulatory signals on the soil surface decreases as a function of distance. First, signals directly measured while ants were excavating at the surface were evaluated, to quantify the distance-dependent decrease of mean signal amplitude. Second, the attenuation of artificially-generated 1 kHz sine waves was measured. This approach allows for a much higher and well defined initial amplitude, since signals produced by ants varied in amplitude, and thus for a more precise calculation of the attenuation properties of the material.

For the first approach, a laboratory colony of *Atta vollenweideri* was connected to a plastic vial, which had had its bottom removed, *via* a pipe. The vial was held vertically about 0.5 mm above the surface of a clay block of 20 x 20 x 10 cm, avoiding any physical contact (Fig. 25B). Thus, ants coming from the colony were given access to a square of 1 x 1 cm on the clay surface. According to preliminary observations, 4-5 workers excavate simultaneously at a surface of that size. The vial was positioned without any physical contact between the clay and the colony. This avoided potential interference from stridulation signals produced in the nest. At a distance of 1, 2, 3, 4 and 5 cm from the excavating ants, 10 sound samples of 5 s each were recorded and stored on the computer. The whole procedure was repeated on 5 different clay blocks. In each sample file, stridulation signals were subsequently identified in the spectral view, and their amplitude was measured. The samples were blinded before analysis.

To quantify the noise level, 10 control sound samples were recorded on each of the 5 clay blocks with no ants on their surface. The maximum amplitude was measured in every control sample. The 95% quantile of the control amplitudes was taken as the minimum amplitude considered significantly larger than the background noise, and this value was referred to as the *noise level*.

For the second approach, sine waves were produced by a wave generator connected to a *Bruel & Kjær* Type 4810 mini shaker. Vibrations were transmitted to the surface of a 20 x 20 x 10 cm clay block via a 55 mm aluminium rod of 5 mm in diameter. A 1 kHz vibration with initial peak-to-peak amplitude of 20 cm/s^2 was applied to the surface, calibrated by attaching the accelerometer directly to the mini shaker with a screw. The remaining amplitudes were measured at a distance of 1, 2, 3, 4 and 5 cm from the source, and the output was visualized with an oscilloscope. Attenuation in dB was calculated for each distance. The whole procedure was repeated four times, each time on a new clay block.

Behavioural response to stridulatory signals

Similar to Spangler (Spangler, 1967), we investigated if the perception of stridulation signals spatially guides the excavation behaviour of nestmates. For that, workers were presented with stridulatory signals in two different choice experiments, and their preferred location to start digging was evaluated.

The first experiment offered a binary choice of locations to initiate excavation. Preliminary observations revealed a clear preference of ants to excavate upwards if introduced from the side or from below into a container filled with soil. Therefore, a vertical, Y-shaped tunnel of approximately 1 cm diameter was pricked out in a clay cuboid of 8 x 4 x 2 cm. Workers were able to enter the tunnel through an opening at the bottom end. About 1 cm above this entrance, the vertically-oriented tunnel forked into two tunnels leading further upwards at an angle of 45 (Fig. 25c). The top ends of both tunnels were closed with a 4 mm layer of clay. On top of one of the tunnel ends, a plastic vial was applied vertically to the surface, with the open end standing on the 4 mm clay layer. In this vial, a number of ant workers, henceforth called the *stimulus group*, was trapped by slightly compressing them at the end of the vial behind a piece of cotton wool until they were unable to move freely. In this situation, the workers stridulated strongly. Stridulations were transferred from the tube onto the clay and into the tunnel, while any diffusion of pheromones was prevented by the wet clay (Currie, 1984). Experiments were performed with vials containing either 20 workers ($n = 105$) or 40 workers ($n = 37$) as stimulus groups, and no workers as a control ($n = 82$). Measurements of the stimulus group to quantify the stridulation intensity inside the tunnels showed clear differences between the two tunnel ends concerning amplitude and signal repetition rate, for both the 20 and the 40 workers stimulus group (see Appendix A: Tab. 17).

The experiment was started by connecting a tube containing 20 ant workers, hereafter called the *decision group*, to the tunnel, and applying the vial containing the stimulus group to the surface above one of the tunnel branches. It is important to mention that due to space constraints, no more than 4-5 workers from the decision group were expected to be able to excavate simultaneously. The side at which the stimulus group was applied changed in every experiment. As no direct observation of the decision group inside the tunnel was possible (Fig. 25C), the surface of the 4 mm covering layer was observed and the breaking through of the first ant on one of the sides was scored as decision. If the decision group broke through at both sides or dug at locations other than the two tunnel ends, the experiment was not considered. If no breakthrough occurred, the experiment was finished after 4 h. Results were tested against a random distribution of choices (1:1) with a binomial test.

If given access to a square-shaped confined soil surface, ant workers have a strong preference to excavate at the corners. The second experiment offered a confined clay area with four corners to the decision group. The stimulus group was applied to one of the four corners from below (Fig. 25D). The vibrational signals used as stimulus were stridulations produced by nestmates engaged in digging during the essays. Additionally, the setup allowed for direct observation of the decision group, so that the start of digging by the first ant in the group could be used as an indicative response. The four-corner-choice experiment was performed with a colony of a different species, *Atta cephalotes* (collected in Gamboa, Panama, in 2003), because colonies of *Atta vollenweideri* were not available at that time.

A rectangle of 15 x 15 cm was confined with walls of polyvinyl chloride (PVC) 15 mm high and 2 mm thick on the surface of a cuboid body of clay 28 x 28 x 8 cm in size. On top of this confinement, a cover of acrylic glass allowed observations. Below each corner of the rectangle, a tube with an inner diameter of 2 cm was inserted into the clay block, to create a vertical tunnel ending 5 mm below the surface (Fig. 25D).

Each experiment ($n = 32$) was started by connecting the *Atta cephalotes* colony to the setup with two tubes. The first tube was connected to one of the tunnels entering the clay block from below. There, the workers started digging upwards and stridulating while doing so, thus providing the stimulus group for the experiment. According to preliminary observations, about 4-5 workers were able to excavate simultaneously at an area of 1 cm^2. Therefore, the number of ants from the stimulus group digging at the same time was expected to vary between 25-30 individuals.

The other independent connection allowed workers, the decision group, to access the clay rectangle through a 1 cm opening in the acrylic glass cover. When approximately 80 workers entered the area, the connection to the colony was cut to prevent overcrowding. Counts of the decision group after each experiment showed an average group size of 66±17 ants. The location where the first worker from the decision group started digging was noted as a decision. If no excavation behaviour was observed, the experiment ended after 20 min. In no case did the stimulus group break through the surface within this time. For each new experiment, the position of the stimulus group was changed to the next corner clockwise, and the upper millimetre of the clay surface was renewed.

For analysis, four equal quadrants were defined on the clay surface, and the number of runs in which the first excavation event took place in that quadrant was counted. The number of decisions for the quadrant with the stimulus group was compared to the number of decisions for the other quadrants, by testing against a random distribution (1:3) using a binomial test.

Group-level stridulation in the context of nest digging

To rule out the possibility that individual workers stridulated in the previous experiments due to isolation during the measurements, group-level stridulatory activity was monitored in subcolonies (groups of workers separated from the main colony but maintaining their own fungus garden) in the context of nest excavation, in which workers excavated to relocate the fungus garden into a new chamber. The experimental setup allowed ants excavating a tunnel to suddenly break into an existing chamber, offering space to relocate the fungus. Stridulatory activity was recorded before and after having gained access to the chamber. Control series offered no chamber, so that ants continued to excavate a tunnel, and no fungus relocation took place.

To achieve this, a vertical tube of 25 cm height and 10 cm diameter was filled with clay. In the centre of the tube a 1 cm-wide tunnel ran downwards 5 cm deep into the soil (Fig. 25E). In the experimental series, a spherical chamber (6 cm in diameter) was offered 6 mm below the end of the tunnel. In the control series, no chamber was offered. According to preliminary observations, workers dig further downward if given access to a vertical tunnel from above. Therefore, ants in the experimental series were expected to excavate downwards until reaching the chamber, resulting in a sudden increase of available space.

Vibrations were measured at two different points in the chamber experiments, one close to the end of the tunnel presented to the ants, the other close to the wall of the chamber (Fig. 25E). To reach these points, two 55 mm aluminium rods of 5 mm diameter were attached to accelerometers and pushed into the clay through holes at the side of the tubes until their distant ends were approximately 4 mm from the tunnel or chamber wall. By comparing the amplitudes of corresponding stridulation signals at the two measuring points, it was possible to determine the moment when the excavating ants reached the chamber, for, in that moment, the signal amplitude measured at the lower sensor suddenly increased in comparison to that at the upper sensor. In the control series, only the upper measuring point was used.

For each run of the experiment, we assembled subcolonies of 200 workers and 1 g of fungus in a plastic box placed on top of the clay tube (Fig. 25E). After an establishment phase of 24 h, the lid of the box was removed to threaten the fungus garden with desiccation, and a hole in the bottom of the box was opened to give the ants access to the clay tunnel. After opening the tunnel, observations were made every 20 minutes for 6 hours. Each observation involved measuring transport rates of excavated clay pellets to the outside, and of incorporated fungus pieces into the excavation. Further, 10 s sound samples were recorded at the two different measuring points simultaneously. After the end of each chamber experiment, the subcolony was left in the setup for 18 h. After that period of time, the setup was opened to check if a fungus garden had been established inside the offered cavity. Stridulation rate was counted for each of the samples, which were blinded before computer analysis. The sample size was 20 for the chamber experiment and 14 for the control. Stridulation rates were normalized by square-root transformation and tested for differences statistically in a mixed-effects ANCOVA. The number of the experiment was used as a random factor with regard to the repeated-measures design. Time was treated as a linear predictor and treatment, i.e. chamber or control, as a fixed factor. To test for differences between chamber and control series at each observation point, *post-hoc* t-tests were applied to the transformed data sets. Taking into consideration the number of group comparisons, the significance level was corrected to $p = 0.003$ (Fig. 25E).

8.3 RESULTS

Observation of digging individuals

All 21 workers observed during the experiments stridulated while excavating. They produced signals with a dominant frequency around 1 kHz and a repetition rate of about 4 signals per second (Fig. 26). Similar to the description Sudd (1969) gave for other ant species, the excavation behaviour observed in *Atta vollenweideri* workers can be described as a repetitive behavioural sequence. The workers approached the soil surface with open mandibles and touched it with their antennae (Fig. 27A). They used their mandibles to cut into the surface, similar to the behaviour described as grabbing (Sudd, 1969), and then began raking by pulling the mandibles backwards and aggregating material with their mandibles and legs beneath the mesosoma. After that, the ants slightly turned laterally and again touched

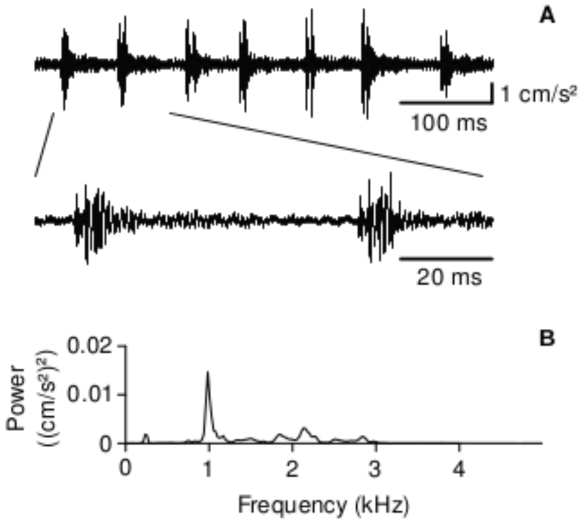

Figure 26: Production of stridulation by an isolated *Atta vollenweideri* worker excavating in soil, recorded on the outer side of the observation vial. (A) Measured acceleration values over time. The calibration bar (acceleration in cm/s^2) is valid for both plots. Recordings were filtered below 500 Hz. (B) Power spectrum of the signal track illustrated above. Filter type: Hanning. FFT size: 4096. Resolution: 23.44 Hz.

the soil with opened mandibles until initiating the next cut. After one or more cuts, an aggregation of soil could be seen beneath the workers' mesosoma. This aggregation, which may be further compacted before loading, was called a *pellet*. After the last cut and pull sequence, the pellet was lifted up with the mandibles for transport (Fig. 27A).

Figures 27B and 27C show an oscillogram and spectrogram of signals produced during the excavation sequence. The repetition rate in the different phases of excavation behaviour was equal in three of the four phases (Fig. 27D). The repetition rate was similar during the approach before making contact with the soil, the cut and the pull phase of excavation behaviour, averaging 5.7±4.3 signals per second. It was only when leaving the digging site that most ants ceased stridulating (influence of the phase on stridulation rate: $F_{3,20} = 21.2$, $p < 0.001$, see Tab. 18 in Appendix A material for details of *post-hoc* comparisons).

Signal attenuation over distance

The measured acceleration amplitude of stridulation signals produced by excavating ants decreased asymptotically with distance towards the noise level of 5.4 cm/s^2 measured from peak to peak. At a

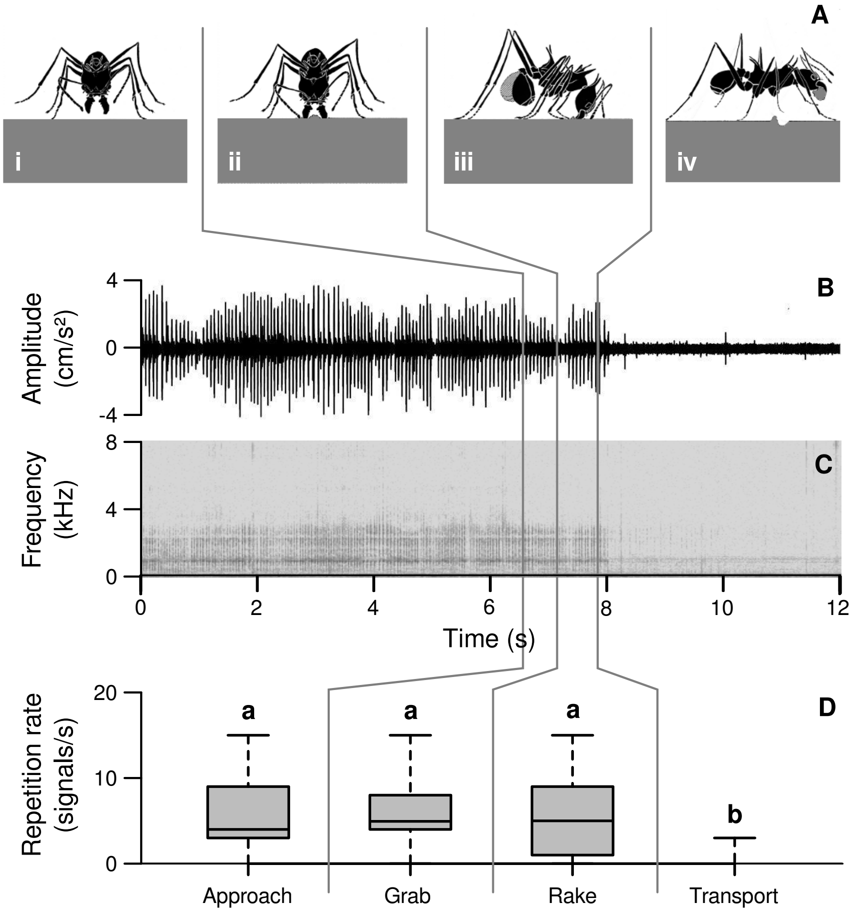

Figure 27: Stridulation rates of workers in the different phases of digging behaviour. (A) The four different phases of the behavioural sequence: the *approach* phase (i), *grabbing* (ii), *raking* (iii) and *transport* (iv) of a soil pellet. (B) and (C) Waveform and spectral view of an example sound record. (D) Signal repetition rate in the different phases of excavation behaviour. Boxes sharing the same letter are not statistically different from each other. Bars within the boxes indicate median values. The box shows the range from 25% to 75% quartile. Whiskers indicate minimum and maximum values. Drawings made by Malu Obermayer.

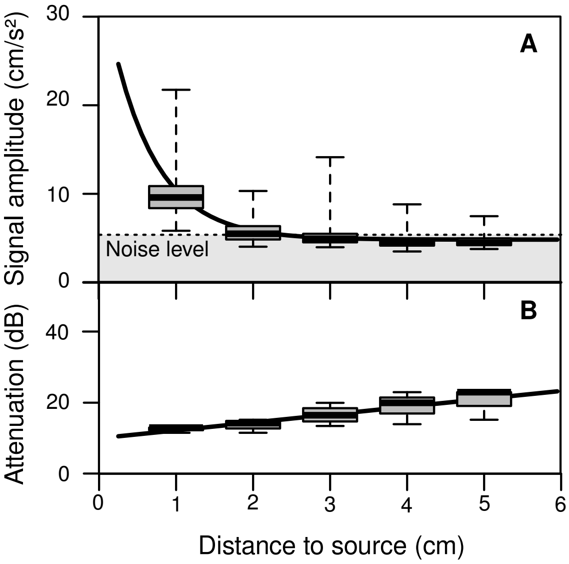

Figure 28: Attenuation of stridulation signals over distance to the source. (A) Peak-to-peak acceleration amplitude of detected signals produced by digging *Atta vollenweideri* workers. (B) Attenuation of an artificial 1 kHz wave of 20 cm/s². Bars within the boxes indicate median values. The box shows the range from 25% to 75% quartile. Whiskers indicate minimum and maximum values without outliers.

distance of 1 cm to the signal source, the median acceleration amplitude was 9.6 cm/s². At 2 cm distance, median acceleration amplitude decreased to 5.5 cm/s² to reach the 95% noise level at 3 cm distance with an acceleration amplitude of 4.9 cm/s² (Fig. 28A).

The attenuation of 1 kHz sinus waves over the surface increased linearly from an average of 12.6±0.7 dB at 1 cm distance to 21.1±3.9 dB at 5 cm. Thus, the signals were attenuated stronger between 0 and 1 cm, where the mean attenuation rate was 12.6±0.7 dB/cm, than between 1 and 5 cm, where a linear regression gave an average damping rate of only 2.2 dB/cm (Fig. 28B).

The much higher attenuation rate between 0 and 1 cm can be explained by energy losses during the transmission of the vibration from the shaker onto the surface. The damping rate of 2.2 dB/cm calculated by regression can therefore be taken as an average attenuation rate, for it does not take into account the theoretical attenuation value of 0 dB at 0 cm.

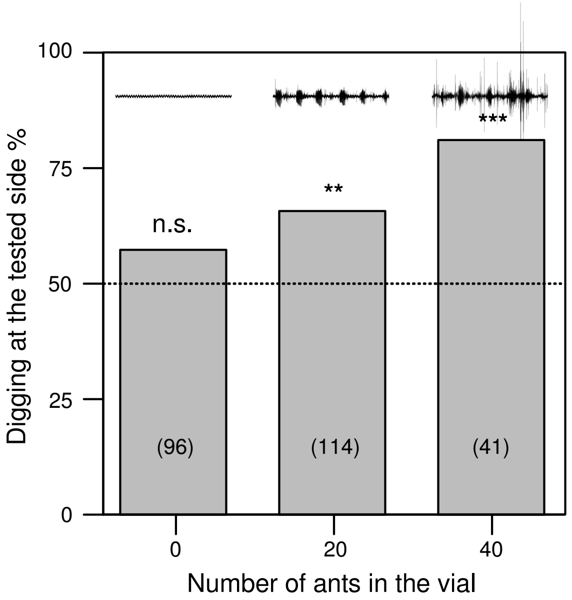

Figure 29: Response to stridulation singals, measured as the proportion of ant groups (*decision groups*) digging towards the vial in a Y-shaped binary choice experiment, for stimulus groups of 0, 20 and 40 workers. Sample sizes are indicated inside the bars. Significance levels by symbols above the bars (n.s.: not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$). At the top end of each bar, an example of the vibrational signals produced by the stimulus group is indicated. Tracks were filtered below 500 Hz.

Behavioural response to stridulatory signals

In the binary choice experiment with *Atta vollenweideri*, while empty vials had no significant effect on the choice of direction ($n = 82$, $p = 0.22$), vials containing 20 stridulating workers had a significant influence ($n = 105$, $p = 0.002$). The side with the vial was chosen in 66% of the experiments. With 40 stridulating workers, the preference for the vial side increased to 81% $n = 37$, $p < 0.001$; Fig. 29).

When there were no ants in the stimulus group, workers carried out the first pellet after a mean of 29±24 min ($n = 82$). It took 27±21 min ($n = 105$) with a stimulus group of 20 and 48±41 min with a stimulus group of 40 workers ($n = 37$). Thus, the latter displayed a significantly longer delay until starting to excavate (ANOVA after *log*-transformation: $F_{2,246} = 8.5$, $p < 0.001$). The average duration of excavation activity before breaking through the surface was for 98±51 min for the empty vial experiments, 96±48 min for the 20 and 88±42 min for the 40 worker stimulus group (ANOVA: $F_{2,246} = 0.3$, $p = 0.75$), thus indicating that the digging speed of workers was not influenced by the intensity of stridulation (for details see Tab. 19 in the Appendix A).

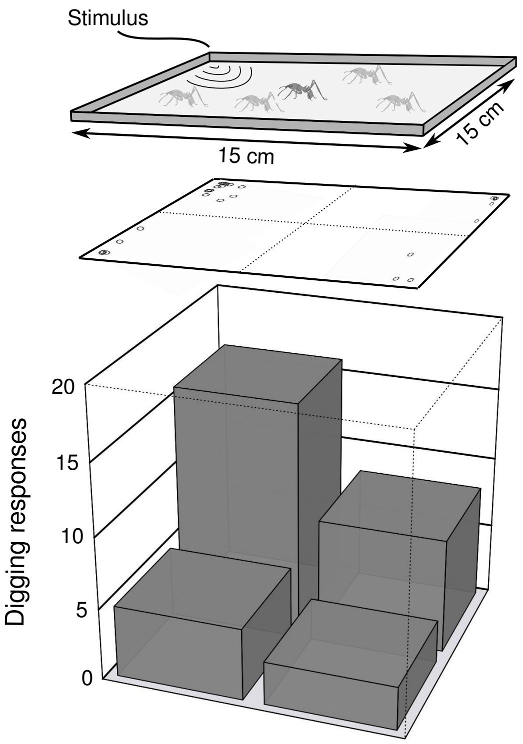

Figure 30: Decisions recorded for each of the four quadrants in the four-corner choice experiment. The drawing on top illustrates the setup with workers of *Atta cephalotes* moving freely on a clay surface, and deciding where to initiate digging. The corner where the stimulus group is applied is indicated by a label. Below this, the actual locations of the recorded first digging reactions in each single run are shown. The three-dimensional bar plot shows the distribution of the observed responses. Ant drawings made by Karin Moll. The three-dimensional barplot is based on a script by Michal J. Figurski (`http://gallery.r-enthusiasts.com/graph/Presentation_style_3D_barplot_161`).

In the four-corner experiment with *Atta cephalotes*, the first digging activity was observed 16 times in the quadrant with the stimulus group (Fig. 30), this corner being chosen significantly more often than the others ($n = 32$, $p = 0.003$).

Group-level stridulation in the context of nest digging

In the chamber experiments, it took the subcolonies 138±44 min ($n = 20$) to reach the chamber. The rate of soil removal increased within 30 min after starting the experiment to an average of 3.5±3.3 pellets/min. In the control experiments it took approximately 130 min to reach a comparable rate of soil removal. From there on it remained constant during the experiments (Fig. 31A). In the chamber

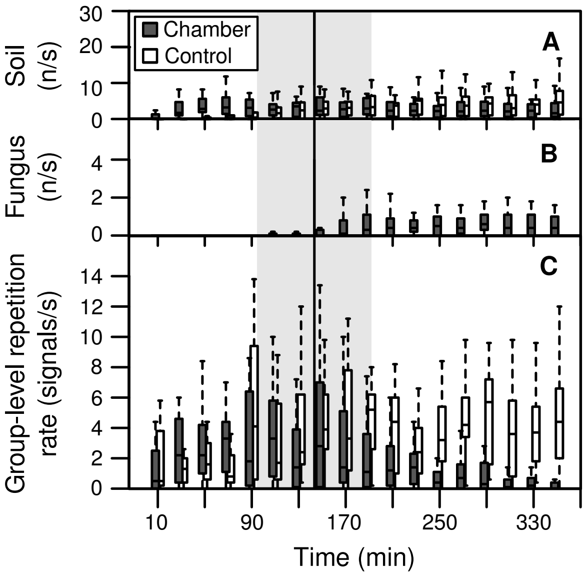

Figure 31: Production of stridulatory signals during collective digging. (A) Rate of soil removal over time, as number of pellets carried per minute. (B) Rate of fungus carriage inside the nest over time, as number of fungus pieces carried per minute. (C) Group-level stridulation rate. Grey boxes correspond to the chamber experiments, where a chamber was available after digging 6 mm deep. White boxes correspond to the control experiments without a chamber. Bars within the boxes indicate median values. The box shows the range from 25% to 75% quartile. Whiskers indicate minimum and maximum values without outliers. The mean time needed by workers until reaching the chamber is marked by the vertical line in the background. The light grey field shows its standard deviation.

experiment, soon after reaching the chamber the ants started relocating fungus into the entrance at an average rate of 0.7±1.1 pieces/min. In the control experiment, no fungus was relocated (Fig. 31B). The group-level repetition rate of stridulation signals was not constant during the experiments [1]. Within 30 min after starting the experiment, stridulation activity raised to an average of 2.7±3.0 signals/s. When the chamber was reached, stridulation activity decreased afterwards, while it remained constant in the control experiments [2] (Fig. 31C). According to *post-hoc* comparisons, at the 250 minute mark and later, the median repetition rates of chamber and control group differed significantly (Appendix A: Tab. 20). After 24 h, the subcolonies had established fungus gardens inside the offered cavities in all chamber conditions.

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[1] Influence of time as a predicting variable: $F_{1,33} = 7.5$, $p = 0.006$.

[2] Influence of treatment as a predicting variable: $F_{1,33} = 8.9$, $p = 0.006$.

8.4 DISCUSSION

Stridulation has been described in leaf-cutting ants as an alarm signal (Markl, 1965, 1967), and as a short-range recruitment signal in the context of leaf harvesting (Roces et al., 1993; Roces and Hölldobler, 1995, 1996). Our results demonstrate the use of stridulation behaviour in a novel situation: the context of nest digging. Our observations clearly demonstrate that leaf-cutting ant workers stridulate during excavation. The signals did not differ from those reported during leaf-cutting or in alarm situations in frequency or amplitude (Markl, 1968; Roces et al., 1993). Stridulation intensity in terms of signal repetition rate was high during the manipulation of the soil and even before contact with the material. On the clay used for our experiments, the acceleration amplitude of measured stridulation signals decreased to the noise level within a few centimetres, with an average attenuation rate 2.2 dB/cm.

Two different choice experiments demonstrated the influence of stridulation on the decision of other workers as to where to start excavating, i.e. stridulation signals attracted other workers and thereby directed their digging efforts. This was demonstrated in two different experiments using alarm stridulations as well as vibratory signals produced by digging nestmates, and also for two different species: *Atta vollenweideri* and *Atta cephalotes*. The probability for a worker to dig at a location increased with the intensity of the vibrational signals produced at this location, which correlated with the number of stridulating ants. While these results clearly indicate that substrate-borne stridulatory vibrations trigger digging activity at the spot, it is an open question whether the vibrations also act as modulatory signals, for instance by lowering the threshold to respond to other stimuli, or by increasing the intensity or duration of the digging response. In the foraging context, stridulatory signals alone can elicit a recruitment response in the recipient ants (Roces et al., 1993), but it is known that chemical recruitment communication can also be enhanced by substrate-borne stridulations, which are part of a multimodal recruitment system that lowers the response threshold of the receiver for the releasing component of the chemical signal (Hahn and Maschwitz, 1985; Baroni-Urbani et al., 1988). In the honeybee *Apis mellifera* L., for instance, vibrations act as non-specific, modulatory communication signals that cause an increase in activity or alter the responsiveness to different stimuli (reviewed by Schneider and Lewis, 2004).

Stridulation behaviour was observed to occur during regular nest excavation in the context of fungus relocation. Therefore, it appears very unlikely that workers isolated during our experiments stridulated as an alarm reaction because of their confinement, or that the choice experiments merely reflect the decision of the ants to free buried nestmates. The group-level stridulation rate strongly decreased when workers gained access to an existing chamber and began to transport the fungus into it. Interestingly, the rate at which soil pellets were carried out remained constant for at least three hours after the decrease in stridulation rate. This may indicate that either excavation continued while the ants decreased their stridulatory activity, or that excavation activity decreased but transport of clay pellets continued because of their accumulation inside the chamber. Concerning this question, no final conclusion can be drawn from our results.

Spangler (1973) proposed stridulation as a mechanism to produce vibrations in order to mechanically facilitate soil manipulation with no communicative function. In an experimental setup comparable to our four-corner choice experiment, he investigated whether workers of *Pogonomyrmex occidentalis* prefer to dig close to the source of a stridulation signal, and observed no effect (Spangler, 1967). While species-specific differences as compared to leaf-cutting ants may exist, it seems that the lack of preferences resulted from stridulation signals having been applied at the centre of a sand box, while ants usually prefer to excavate at the edges. Our choice experiments clearly demonstrated that *Atta* workers prefer to start digging close to the source of stridulation signals, thus indicating that stridulations produced by digging workers guide the decisions of nestmates where to start digging. We cannot rule out the hypothesis that stridulations may in addition provide a mechanical aid during soil removal. However, stridulations are not only produced during the actual soil removal, but also during the approach of the workers to the digging site, which would not be relevant as a mechanical aid. Investigations on their use as a mechanical aid may require comparative measurements of digging effort in stridulating and non-stridulating ants, or observations of stridulatory activity in contexts where no communication is needed. An analogous approach to evaluate the function of stridulations in foraging leaf-cutting ants was employed by Roces and Hölldobler (1996), but as far as no comparable information in the context of digging is obtained, support for the hypothesis that stridulatory vibrations mechanically facilitate digging remains elusive.

Our results clearly demonstrate that vibrational communication underlies the spatial organization of collective digging in ants. Workers preferred to dig close to other digging workers, thus leading to a spatial concentration of the excavation efforts, much like foragers of *Lasius niger* (L.) concentrate on one pheromone trail by following the strongest olfactory signal (Beckers et al., 1990). A comparable mechanism has been described in the drywood termite *Cryptotermes secundus* (Hill). In this species, feeding and nest enlargement are equivalent, since workers feed on wood while extending their nest. Workers stridulate while feeding, and other workers are attracted by the vibrational signals (Evans et al., 2007), so that stridulation can be interpreted as a communication mechanism that helps to concentrate nest enlarging activities at one place.

An alternative communication mechanism for the spatial organization of excavating ants may be the use of pheromones to communicate the location of current digging activity to nestmates. Wilson (1958) described workers of *Pogonomyrmex badius* reacting to extracts from the mandibular gland with excavation behaviour, and therefore proposed the existence of a digging pheromone. Workers of *Doronomyrmex pyramicus* respond with digging to high concentrations of 2-Heptanone, their own alarm pheromone (Blum and Warter, 1966). No other evidence on pheromones emitted during excavation or on pheromones inducing excavation behaviour is known for ants. However, the use of trail pheromones, or other chemical marking, to recruit workers to a digging place cannot be ruled out.

The use of vibrational communication has a clear advantage over pheromones as workers are able to easily determine the direction of a signal source, even in a very confined space. Unlike a chemical signal, a stridulation signal has a direction of propagation apparent from the decrease in its amplitude over distance. The stronger this decrease, the shorter the minimum distance between two measuring points at which a difference can be perceived, so as to determine the direction of the signal source. Therefore, the origin of a signal with a relatively short range can be easily located, an advantage especially inside a nest where a large number of nearby workers is expected to move around.

To estimate the effective range of a vibrational signal, the ability of workers to perceive it must be taken into account. Markl (1970) reported a minimal sensitivity threshold, measured electrophysiologically in the leg nerves of *Atta cephalotes* workers, averaging a peak-to-peak acceleration amplitude of 4 cm/s^2, a value only slightly below the noise level measured in our study. We calculated an average attenuation rate 2.2 dB/cm. With these two values, we can take the acceleration amplitudes of the ant stridulations we measured at different distances, and calculate the distance at which signal amplitude decreases below the physiological detection threshold. According to our observations, stridulation signals should be detectable within a mean range of 3.7 ± 1.1 cm; the strongest signal we measured would theoretically be perceived up to 6.2 cm. However, these are estimates based on an absolute minimum threshold obtained from electrophysiological measurements, rather than behavioural observations, so that the real effective range of the signal remains unknown. We suggest an estimated maximum range of about 6 cm, with the actual values strongly dependent on the soil properties.

Two other reports are known from literature concerning stridulation signal attenuation and effective range in ants. Markl (1968) measured an attenuation rate of 6 dB/cm for the alarm stridulations of *Atta cephalotes* workers buried under loose sand or earth. This attenuation value is much higher than the one we calculated from our measurements, but it was measured under different conditions with the aim to assess how good alarm stridulations of a buried worker completely covered with soil are transmitted as longitudinal waves through the soil types typical for the habitat of *Atta cephalotes*. Our measurements, on the contrary, evaluated the damping rate of transversal waves on the surface of clay. In our experiments, body-to-ground contact was restricted to the legs and mandibles, the natural situation of a digging worker producing stridulatory signals. In *Pogonomyrmex occidentalis*, maximum detectable ranges of alarm stridulations were reported to vary between 12 cm and 30 cm depending on moisture and compression of the soil (Spangler, 1974). The detectable range was the maximum distance at which stridulation could be heard when connecting the sensor to a loudspeaker. All stridulation signals had amplitudes above the noise level at up to 5 cm from the signal source. However, different substrates, methods of signals recording, and of applying the ants to the surface were used, so that comparisons are complicated, e.g. workers were either pushed into the soil by means of forceps, or held in a slit in a plastic strip (Spangler, 1974). Both methods give a body-to-ground conduction very different to that in our experiments, which may result in much higher initial signal amplitudes.

Markl (1973) hypothesized that stridulatory communication in ants evolved as an alarm mechanism for trapped individuals calling for help after cavity collapses, and discussed the occurrence of the stridulatory organ in the Formicidae. He argued that its existence could be understood as a widespread adaptation predominantly present in species building subterranean nests. Our results, in accordance with former observations in other ant species (Spangler, 1967; Rauth and Vinson, 2006), indicate that stridulation is indeed a common behaviour in underground dwelling Myrmicines, and suggest that its origin and adaptive value may as well be as a communicative function to spatially coordinate collective nest building.

Part III

CONCLUSION

9

THE SCOPE OF LOCAL STIMULUS-RESPONSE PATTERNS

The experiments described in the last four chapters demonstrated in various examples how excavation behaviour in *Atta vollenweideri* is influenced by local stimuli. As supposed in the first chapter of the thesis, the nature of these local stimuli ranges from environmental variables that are expectedly important also for a solitary nest builder to communication signals that allow for the coordination of collective behaviour, and possible denote a later step in the evolution of cooperative mechanisms.

Soil water content, an environmental variable possibly significant for all underground nest builders both social and solitary, is a local cue that can potentially determine the direction of nest growth in many situations. Additionally, it can aid to prevent the inflow of surface water into the nest. Crowding conditions inside the nest possibly provide cues that allow excavators to adjust nest size to colony requirements. This regulatory response to the condition of the entire colony can be seen as an important adaptation of nest building behaviour to the requirements of a social life style.

Choice copying, social enhancement and response thresholds

Furthermore, even some form of coordination among several digging individuals can be achieved in *Atta vollenweideri* colonies with the participants acting solely on behalf of local information: both fresh soil pellets deposited on the way to a digging site, and short-range vibratory signals emitted by digging workers increase the probability that other individuals will excavate at the same location. This enables groups of excavators to concentrate at one spot even in the complete absence of other guiding cues. This also implicates that the coordination of individuals in the group does not require communicating complex information; it is rather a matter of some individuals taking a decision randomly, or based, for example, on environmental cues, and other individuals copying the decision of the majority. Wheeler noted on communication in ants that

> "[...] much of the interchange of impressions in an ant colony is to secure cooperation through imitation."
>
> — *Wheeler (1910, p. 536)*

In other words: in many cases ants exchange information to inform nest mates about their own decisions in order to allow others copying their decisions. Following that thought it comes to no surprise that the cues and signals involved in the coordination of collective digging are relatively unspecific and allow for little coding. Their role is simply to indicate current activities and their location.

This is particularly interesting in connection with the observations of Chen (1937a,b). He described the occurrence of more active *leaders* and less active *followers*, and the social enhancement of follower digging activity in groups of *Camponotus japonicus* workers excavating in the laboratory (see Chapter 2). From the current point of view, assuming a hierarchical organisation of workers during the performance of a task, as Chen supposedly did with his terminology, appears rather questionable. Nonetheless, the idea of social enhancement does not; it can be explained, without assuming any hierarchy, on the basis of response thresholds models. The idea behind that concept is that task allocation within an insect colony is based on the variability of individual response thresholds to the different stimuli that trigger task performance (Bonabeau, 1998).

When Chen reported that some workers excavate a lot while other excavate little or nothing under isolated conditions, this reflects the differences in the workers' response thresholds to the digging stimuli present in the experiment. The results presented in Chapters 7 and 8 indicate that digging behaviour itself generates new digging stimuli. Accordingly, the leader *sensu* Chen (1937a,b) can be interpreted as an individual with low response threshold for example to environmental digging stimuli. The follower, on the other hand, is possibly an individual with a high response threshold to the environmental stimulus the leader responded to, but a low threshold for the cues and recruitment signals produced by the excavating leader. This view is further supported by Chen's notion that groups composed entirely of individuals that had been identified as leaders in former experiments excavated in a more scattered pattern, while follower-only groups tended to stay close together and dig at the same location.

In the light of response threshold models, Chen's findings hint towards the existence of at least some degree of specialisation among excavating workers. Hypothetically, some individuals respond to digging stimuli that relate to environmental variables or colony requirements, other workers respond more strongly to nest mate digging behaviour, often not assessing the need for further nest excavation by themselves, but relying on nest-mate decisions. It remains an open question if that specialisation is related to genetics, age, former experience or other factors. Concerning the evolution of collective digging, it is apparent that the leader-type behaviour must have existed first. There would be no nest at all without individuals that take the initiative and start excavating. The follower-type behaviour represents a later development in the evolution of nest building that allows for cooperative task performance by means of nest mates copying leader decisions.

The self-organisation paradigm – how helpful is it to understand underground nest building in ants?

As a consequence of workers copying each other's decisions on a local level, many digging patterns observed on group level expectedly emerge from the interactions of the individuals. When patterns in a multi-component system, such as an insect colony, arise from multiple, rather simple local interactions of the components, this is often interpreted as an instance of self-organisation (Bonabeau et al., 1997). An *Atta vollenweideri* colony can be seen an enormously large multi-component system, and the nest as a pattern generated by the actions and interactions of numerous components. The observations reported in the previous chapters indicate that many of these actions and interactions on the component level are performed purely on behalf of local information without any reference to the global pattern. Accordingly, from this abstract point of view, the process of collective nest excavation bears several signatures of self-organisation.

Other aspects of social insect behaviour have been described and successfully analysed in the light of that concept. In collective foraging, for instance, when ant colonies have multiple potential ways leading to a food source, the dynamics of individuals trail-laying and trail-following behaviour result the concentration of foragers on a single trails due to the amplification of local fluctuations of trail-pheromone concentration (Beckers et al., 1990). The reactions of *Atta vollenweideri* workers to deposited soil pellets and stridulation signals, as described in Chapters 7 and 8, urge comparison to these amplification mechanisms known from foraging trails.

Former studies have surmised or demonstrated instances self-organisation in ant digging behaviour. Regarding the adjustment of nest size to colony size, Deneubourg and Franks (1995) suggested a mechanism based on positive feedback due to recruitment activity of excavating individuals: if nest space is small in comparison to colony size, distances between individuals are rather short, resulting in high recruitment success and self-amplification of digging activity. If nest space is large, the communication distances are too, which diminishes recruitment success. Experimental evidence has been interpreted in favour of thit hypothesis (Rassé and Deneubourg, 2001; Buhl et al., 2005), that is particularly interesting from the perspective of self-organisation, because the positive feedback involved in the mechanism is one of its basic ingredients (Bonabeau et al., 1997). It allows for the amplification of local fluctuations in digging activity which, in a homogeneous environment, results in self-organised emergence of nest shapes.

The principal possibility of pattern emergence from collective digging activity under certain conditions in the laboratory has been elegantly demonstrated by Toffin et al. (2009). In their experiment, workers accessed a thin layer of soil through an opening in the centre. At the beginning, the workers had little space, and therefore excavators were closely packed along the entire outer wall of the cavity. At a nest volume specific for the number of workers tested, the number of excavators was not sufficient any more to dig simultaneously along the entire digging frontier. Probably due to the positive feedback described above, excavators concentrated at particular locations and, as a consequence of that concentration, excavated tunnels rather than further enlarging the chamber. This shape transition from a round cavity to *fingered* structure can definitely be interpreted as an instance of self-organisation.

However, the more important question, in my opinion, is to what extend the variability of nest shapes observed the field can be explained by that self-organisation. The original purpose of the concept was to explain processes with multi-stable outcomes in an homogeneous environment. While many processes in nature have a strictly predictable outcome, for instance a chemical reaction that starts with an amount of educts and ends with a particular amount of products, others are, like the crystallisation of a snow flake, highly unpredictable, even though the responsible processes on the molecular level are simple and well understood. The self-organisation paradigm elegantly solves that problem, it explains the emergence of unpredictable patterns as multistable outcomes of multiple interactions in a homogeneous environment (Haken, 1977; Nicolis and Prigogine, 1977; Nicolis and Nicolis, 2007).

Applying that concept to nest digging does, though legitimate, entail an important issue: soil is not homogeneous environment. On the contrary, soils provide a broad spectrum of environmental templates that influence digging behaviour, and these templates possibly strongly limit the multistability of the digging process and make the outcome relatively predictable. This does not affect the assumed existence of feedback mechanisms in collective nest digging. They are evident and certainly necessary for the adjustment of nest architecture to the requirements of the colony, but a decentralised multi-agent regulation mechanism is not in itself a self-organised process. It just provides many prerequisites for self-organisation, but as long as the only result of process is the predictable adjustment of one variable to another, the global process is no more complex than a simple chemical reaction.

The merits of the self-organisation concept in understanding complex processes are evident (Camazine et al., 2001). Nevertheless, in the case of collective underground nest building it is likely that much of the observed variability is determined by other factors. The presence of feedback mechanisms in collective excavation behaviour necessarily results in self-organised pattern generation where other determining factors are absent, but the margins of the self-organisation effect are possibly small in many situations. As homogeneous conditions are more like encountered on a smaller scale, it can be argued that self-organisation accounts primarily for small-scale variability in underground architecture. For instance, the depth, at which workers excavate a chamber along a vertical tunnel might be determined by the environment when a particular depth offers a suitable environment. Meanwhile, the orientation of the chamber, i.e. the side of the tunnel at which it is constructed will be determined by self-organisation, as long as the soil is homogeneous in all directions.

The question is, in my opinion, if the rather complex concept of self-organisation is necessary and useful particularly for the understanding of underground ant nests development. In nature, it possibly accounts only for small scale variability of the overall system. On the contrary, explaining nest digging in ants as a combined result of environmental templates, decentralised regulatory responses, and coordination of individuals by choice copying possibly provides an easier and more understandable concept.

Outlook

According to results presented in this thesis, the spatial organisation of collective nest digging, and consequently the direction of nest growth results from local responses local stimuli in multiple cases. Some of the investigated factors, such as soil moisture and nest space, represent opportunities or necessities that change with time, but provoke immediate digging responses. Accordingly, the internal structure of the nest at a given point of time can be seen as the cumulative result of past adjustments to the conditions. The experiments reported here will hopefully provide a contribution to the understanding of these adjustment processes. However, to completely understand the processes that determine nest development in ants, further research will be necessary.

Most importantly, to understand the development of the internal structure of ant nests, it will be inevitable to gain more knowledge about the internal structure of ant nests. The presently available data on ant architecture are, though growing rapidly, barely adequate at the moment for an estimation of interspecific and intraspecic variability in nest architecture. After all, at the moment we hardly know to what extend interspecific variability observed in the field is caused by genotypical differences, or simply by habitat choice and phenotypical adjustments to different environments.

As pointed out above, environmental templates expectedly have a strong influence on how nests grow. Identifying all these templates,

investigating their distribution in the field, and testing their effect on digging behaviour will be one necessary step towards a thorough understanding of nest architecture. Another is possibly to understand how different factors interact, especially when they have conflicting effects on the excavators, and how threshold distributions and specialisation among individuals become involved in the process.
Furthermore, digging decisions and organising mechanisms that go beyond local responses cannot be ruled out. *Atta vollenweideri* queens, when excavating their founding nest, reportedly regard the length of their tunnel in when deciding where and when to contruct the founding chamber (Fröhle and Roces, 2012). As these tunnels supposedly extend beyond the queens perceptual range, it can be argued that spatial memory rather than local information influences a digging decision in that case. Even though the currently known aspects of excavation behaviour in workers generally allow for an interpretation as local responses, there is no reason to rule out in general the use, for instance of spatial memory, by workers for digging decisions.

Whatever the further mechanisms involved, the results in the present thesis demonstrate that digging responses to local information can be adaptive in many ways. That rather simple type of mechanisms can aid in the prevention of water inflow during flooding events, regulate nest size according to colony requirements and even coordinate multiple individuals in a cooperative action. Even if there are other mechanisms not based on the use of local information, responses to local stimuli probably account for an important proportion of the variability observed in the underground nest architecture of the Chaco leaf-cutting ant.

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Part IV

APPENDIX

A

TABLES AND STATISTICS

Table 5: Reported populations of *Atta vollenweideri* with references.

LOCATION	COORDINATES	REFERENCES
ARGENTINA		
Reserva *El Bagual*, Formosa	26°17′S, 58°50′W	Kleineidam and Roces (2000) Kleineidam et al. (2001) Röschard and Roces (2002) Röschard and Roces (2003b) Cosarinsky and Roces (2007) Fröhle and Roces (2012)
Rio Pilcomayo, Formosa	25°04′S, 58°07′W	Kleineidam et al. (2001) Röschard and Roces (2003a)
Cruz Alta, Tucumán	26°54′S, 65°00′W	Bucher and Zuccardi (1967) Bucher (1974)
Santa Fé	31°23′S, 60°25′W	Flavio Roces, pers. com.
BRAZIL		
Porto Murtinho	29°45′S, 57°05′W	Carvalho and Tarragó (1982)
PARAGUAY		
Limpio	25°10′S, 57°30′W	Jonkman (1980); Jonkman (1980)
Filadelfia	22°21′S, 60°02′W	Jonkman (1980); Jonkman (1980)
Puerto Casado	22°17′S, 57°56′W	Jonkman (1976)
Central Paraguay	22°24′S, 59°00′W	`http://www.antweb.org` CASENT 173819 CASENT 173820
Central Paraquay	24°45′S, 57°30′W *	Jonkman (1976)
Central Paraguay	24°00′S, 57°30′W *	Jonkman (1976)
Central Paraguay	25°36′S, 57°48′W *	Jonkman (1976)
Central Paraguay	23°00′S, 57°30′W *	Jonkman (1976)
Central Paraguay	23°00′S, 57°48′W *	Jonkman (1976)
Central Paraguay	22°48′S, 57°36′W *	Jonkman (1976)
Central Paraguay	22°28′S, 57°52′W *	Jonkman (1976)
Central Paraguay	22°36′S, 57°52′W *	Jonkman (1976)
Central Paraguay	22°12′S, 57°54′W *	Jonkman (1976)
Central Paraguay	22°04′S, 57°54′W *	Jonkman (1976)
Central Paraguay	22°00′S. 57°54′W *	Jonkman (1976)
Southern Paraguay	26°24′S, 58°00′W *	Jonkman (1976)
Southern Paraguay	26°24′S, 58°16′W *	Jonkman (1976)
Southern Paraguay	26°12′S, 58°04′W *	Jonkman (1976)
Southern Paraguay	25°48′S, 57°48′W *	Jonkman (1976)
Southern Paraguay	25°52′S, 58°00′W *	Jonkman (1976)
URUGUAY		
Rio Negro	32°52′S, 58°02′W	Sosa and Brazeiro (2010)
Villa Soriano	33°29′S, 58°20′W	Martin Bollazzi, pers. com.

* Coordinates estimated from Fig. 5 in Jonkman (1976) which is based on the results of an aerial photographic survey.

Table 6: Mass of excavated and carried pellets at different moistures.

Soil water content	*Excavated*[1]	*Carried*[1]	*t*	*df*	*p*
14%	2.8±2.8 (70)	2.8±2.0 (14)	0.6	20.8	0.536
16%	3.0±2.8 (70)	2.8±1.8 (20)	0.6	41.5	0.572
18%	2.8±2.4 (69)	5.0±3.0 (24)	4.6	46.3	<0.001
20%	3.3±3.2 (70)	4.7±2.5 (19)	2.9	33.0	0.006
22%	4.6±3.0 (74)	5.8±2.9 (20)	2.3	36.0	0.027
24%	3.5±2.5 (70)	5.1±3.0 (20)	2.2	28.7	0.036
26%	2.4±1.7 (70)	5.4±2.0 (11)	5.9	21.0	<0.001

Excavated and carried pellets were compared at each moisture by means of Welch two sample *t*-tests, data sets were *ln*-transformed to approximate normal distribution.

[1] Mean pellet mass in mg ±standard deviation, sample sizes are indicated in parentheses behind the values.

Table 7: Digging duration as a function of moisture: *post-hoc* comparisons.

Soil water content	14%	16%	18%	20%	22%	24%
14%						
16%	0.145					
18%	0.036	<0.001				
20%	1.000	0.347	0.153			
22%	1.000	1.000	<0.001	1.000		
24%	<0.001	0.025	<0.001	<0.001	<0.001	
26%	<0.001	0.005	<0.001	<0.001	<0.001	1.000

Measurements were grouped according to soil water content and *ln*-transformed. Given are the adjusted *p* values of *post-hoc* pair-wise Welch two-sample tests.

Table 8: Excavation rate as a function of moisture: *post-hoc* comparisons.

Soil water content	14%	16%	18%	20%	22%	24%
14%						
16%	1.000					
18%	0.028	0.633				
20%	1.000	1.000	0.002			
22%	0.003	<0.001	<0.001	<0.001		
24%	0.022	<0.001	<0.001	<0.001	1.000	
26%	0.005	<0.001	<0.001	<0.001	1.000	1.000

Measurements were grouped according to soil water content and *ln*-transformed. Given are the adjusted *p* values of *post-hoc* pair-wise Welch two-sample tests.

Table 9: Carrying speed as a function of moisture: *post-hoc* comparisons.

Soil water content	14%	16%	18%	20%	22%	24%
14%						
16%	0.124					
18%	0.008	1.000				
20%	1.000	0.008	<0.001			
22%	<0.001	<0.001	<0.001	<0.001		
24%	<0.001	<0.001	<0.001	<0.001	1.000	
26%	<0.001	<0.001	0.081	<0.001	0.124	0.736

Measurements were grouped according to soil water content and *ln*-transformed. Given are the adjusted *p* values of *post-hoc* pair-wise Welch two-sample tests.

Table 10: Transport rate as a function of moisture: *post-hoc* comparisons.

Soil water content	14%	16%	18%	20%	22%	24%
14%						
16%	0.161					
18%	<0.001	<0.001				
20%	<0.001	<0.001	0.031			
22%	<0.001	<0.001	0.370	<0.001		
24%	0.005	<0.001	<0.001	<0.001	0.386	
26%	<0.001	<0.001	1.000	<0.001	1.000	0.033

Measurements were grouped according to soil water content and *ln*-transformed. Given are the adjusted p values of *post-hoc* pair-wise Welch two-sample tests.

Table 11: The effect of the presence of fungus garden on different parameters of nest growth.

Variable	*Condition*	*Fungus*[1]	*No fungus*[1]	t	*df*	p
Tunnel elon-	8 ml	13.5±6.5(4)	29.3±20.6(3)	-4.8	3.1	0.016*
gation (cm)	22 ml	18.3±9.3(5)	14.5±4.0(4)	0.8	5.7	0.446
after 10 days	113 ml	17.3±13.7(6)	17.0±5.6(4)	0.1	7.1	0.958
Cumulative	8 ml	89.2±39.4(7)	42.3.5±7.7(6)	3.1	6.5	0.019*
mass exc. (g)	22 ml	64.4±22.9(7)	54.0±29.0(6)	0.7	9.5	0.495
after 10 days	113 ml	58.4±6.9(8)	37.4±20.1(7)	2.6	7.2	0.033*
Chamber enlar-	8 ml	69.1±18.6(4)	10.6±5.3(3)	6.0	3.6	0.005**
gement (g)	22 ml	45.0±23.1(5)	38.8±27.1(4)	0.4	6.0	0.728
after 10 days	113 ml	43.4±13.5(6)	26.6±20.4(4)	1.4	4.8	0.210
Tunnel enlar-	8 ml	31.8±14.4(4)	30.2±3.6(3)	0.2	3.5	0.840
gement (g)	22 ml	19.1±2.4(5)	20.1±8.9(4)	-0.2	3.4	0.840
after 10 days	113 ml	15.0±6.8(6)	19.1±3.8(4)	-1.2	7.9	0.258
Maximum dig-	8 ml	21.9±14.9(7)	10.3±4.9(6)	2.0	7.5	0.089
ging rate (g/d)	22 ml	12.3±3.6(7)	15.2±8.0(6)	-0.8	6.7	0.437
in 10 days	113 ml	11.0±3.8(8)	9.0±6.0(7)	0.8	10.0	0.464
Final digging	8 ml	4.1±3.9(7)	5.3±3.0(6)	-0.6	10.9	0.532
rate (g/d)	22 ml	2.3±2.4(7)	0.9±0.8(6)	1.5	7.5	0.182
at day 10	113 ml	2.9±2.1(8)	1.2±0.9(7)	2.0	9.8	0.071

[1] Mean±standard deviation, sample sizes are indicated in parentheses.
*$p < 0.05$; ** $p < 0.01$

Table 12: Day wise regression analysis for excavation rate as a function of nest volume.

Day	*Regression equation*	r^2	$F_{1,27}$	*p*
1	r=31.19-9.22v	0.32	12.6	<0.001***
2	r=32.56-9.59v	0.48	25.0	<0.001***
3	r=20.77-6.29v	0.52	29.0	<0.001***
4	r=13.23-3.65v	0.22	7.5	0.011*
5	r=10.54-2.73v	0.26	9.6	0.004**
6	r=9.07-2.21v	0.15	4.7	0.038*
7	r=9.16-2.30v	0.26	9.7	0.004**
8	r=9.69-2.35v	0.14	4.4	0.044*
9	r=5.63-1.04v	0.03	0.8	0.377
10	r=4.14-0.55v	0.01	0.3	0.604

For each day, the excavation rate, the relationship between excavation rate (r, in g/d) and *log*-transformed nest volume at the beginning of the day (v, in ml) was analysed separately. Sample size is $n = 29$ for all regressions.

*$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Table 13: Day-wise *post-hoc* comparison of the 8+113 ml condition with the 8 ml and 113 ml on the first three days.

Day	*Comparison*	*t*	*df*	*p*
1	8+113 ml vs 8 ml	-1.0	9.3	1.000
	8+113 ml vs 113 ml	1.1	9.3	0.889
	8 ml vs 113 ml	1.8	7.7	0.338
2	8+113 ml vs 8 ml	-3.0	7.8	0.050*
	8+113 ml vs 113 ml	-1.2	11.1	0.750
	8 ml vs 113 ml	2.4	7.5	0.130
3	8+113 ml vs 8 ml	-3.9	7.5	0.015*
	8+113 ml vs 113 ml	-0.04	11.9	1.000
	8 ml vs 113 ml	-3.9	7.7	0.014*

Groups were compared with two-sample Welch t-tests. p-values are adjusted with the Bonferroni correction method.

*$p < 0.05$

Table 14: *Post-hoc* comparison of the 8+113 ml condition with the standard volumes.

Condition	*t*	*df*	*p*
8 ml	-2.2	7.6	0.249
22 ml	-1.0	10.0	1.000
113 ml	-0.6	6.9	1.000
1740 ml	4.6	11.0	0.003**

Groups were compared with two-sample Welch *t*-tests. *p*-values are adjusted with the Bonferroni correction method.

** $p < 0.01$

Table 15: *Post-hoc* comparison of the three-chamber condition with the standard volumes.

Condition	*t*	*df*	*p*
8 ml	-3.9	7.0	0.023*
22 ml	-3.7	8.8	0.019*
113 ml	-6.1	12.6	<0.001***
1740 ml	1.7	10.8	0.125

Groups were compared with two-sample Welch *t*-tests. *p*-values are adjusted with the Bonferroni correction method.

* $p < 0.05$

*** $p < 0.001$

Table 16: Statistics for carrying distance and its temporal dynamics in the three types of workers: excavators, short-distance carriers and long-distance carriers.

Worker type	*n*	*Mean distance (cm)*	*Regression estimate (cm/h)*
FIRST DAYS			
Excavators	60	9.6^{a} (7.6 to 11.6)	0.53^{NS} (-0.33 to 1.32)
Short-distance carriers	168	16.2^{a} (7.9 to 28.1)	0.71^{NS} (-4.80 to 6.26)
Long-distance carriers	60	149.3^{b} (128.9 to 165.3)	1.98^{NS} (-7.11 to 9.47)
SECOND DAYS			
Excavators	73	9.7^{a} (8.1 to 11.4)	-0.07^{NS} (-0.82 to 0.75)
Short-distance carriers	113	18.0^{a} (8.4 to 33.0)	-0.58^{NS} (-5.42 to 5.12)
Long-distance carriers	73	164.1^{b} (146.2 to 177.6)	2.13^{NS} (-4.83 to 8.13)

Shown are the number of observations, mean distance, and slope of the relationship between carrying distance and time, for the first and the second day separately. The 95% confidence interval for the means and the 98.3% confidence interval for the slopes, both determined by bootstrapping, are indicated in parentheses. Mean values with non-overlapping confidence intervals are considered significantly different. Values that are not significantly different are marked with the same superscript letter. Slopes are considered significant, if their confidence intervals do not include zero. Significance levels are indicated in superscripts (NS not significant; * $p < 0.017$).

Table 17: Mean repetition rate and amplitude of the stridulations used as stimulus in the binary choice experiment, for stimulus groups of 20 and 40 workers.

Stimulus	*Time*	*Repetition rate*	*(singals/s)*	*Amplitude*	*(cm/s^2)*
		Vial	No vial	Vial	No vial
20 ants	Start	17.3±3.8	6.9±3.7	37.5±5.4	7.0±0.4
	End	8.0±4.9	2.4±2.3	5.0±0.0	5.0±0.0
40 ants	Start	19.5±0.1	8.2±2.7	75.0±14.1	12.5±3.5
	End	14.1±3.4	4.5±4.2	32.5±10.6	5.0±0.0

Values are given for the first (start) and the last (end) measurement during the duration of 4 h for both sides: *vial* with the stimulus group, and *no vial*. The noise level averaged 5 cm/s^2, and it was therefore the minimum measurable amplitude. Sample size was 10 for all measurements

Table 18: Pair wise comparisons of repetition rates of stridulatory vibrations (in signals per second) during the four sequences of excavation behaviour: approaching, grabbing, raking and transport.

Comparison	$\Delta_{mean \pm SD}$	t_{20}	*p*
approach vs grab	-1.0±2.4	-0.2	0.859
approach vs rake	-0.05±3.7	-0.1	0.953
approach vs transport	5.4±4.5	5.5	<0.001
grab vs rake	0.05±2.9	0.1	0.942
grab vs transport	5.5±4.2	6.0	<0.001
rake vs transport	5.5±4.8	5.2	<0.001

Shown are the differences in the repetition rates, and the output of *post-hoc* paired *t*-tests between the groups. Twenty ants were observed ($n = 20$). Six group-wise comparisons were made, so that the commonly used significance level of 0.05 was divided by 6 and thereby adjusted to $\alpha = 0.008$.

Table 19: Timing of excavation behaviour of the decision group in the binary choice experiment, for the stimulus groups of 0 , 20 and 40 ants.

Stimulus	*Delay (min)*		*Duration (min)*	
	Vibrating	*Non Vibrating*	*Vibrating*	*Non Vibrating*
0 ants	28.2±23.2(47)	30.3±24.5(35)	92.7±50.8(47)	104.2±51.4(35)
20 ants	28.6±22.4(69)	23.8±18.1(36)	91.6±49.1(69)	103.9±47.4(36)
40 ants	49.2±43.8(30)	42.3±28.4(7)	87.4±44.7(30)	87.7±29.3(7)

Given are the mean delay from gaining access to the setup to carrying out the first pellet, and the mean duration of excavation activity from carrying out the first pellet to breaking through the surface. Values for experiments where the decision group dug at the side at which the stimulus group was applied (“vibrating”) are given separated from those experiments where the decision group decided for the other side (“non vibrating”). Sample sizes are indicated in parenthesis.

Table 20: Comparisons between group-level stridulation rates (in signals per second) in the context of nest digging.

Time (min)	*Chamber*	*Control*	*t*	*df*	*p*
10	0.83±0.87	1.01±0.88	-0.6	28.0	0.5775
30	1.35±0.87	1.09±0.82	0.9	29.1	0.3763
50	1.50±0.80	1.36±0.78	0.5	28.6	0.6157
70	1.57±0.84	1.11±0.95	1.4	25.7	0.6492
90	1.43±1.10	1.96±1.14	-1.4	27.5	0.1878
110	1.63±0.99	1.53±1.16	0.3	25.2	0.7947
130	1.22±1.04	1.77±0.80	-1.7	31.7	0.0963
150	1.50±1.23	1.89±0.82	-1.1	31.9	0.2758
170	1.37±0.97	1.92±1.00	-1.6	27.5	0.1182
190	1.23±1.07	2.01±0.77	-2.5	32.0	0.0188
210	1.09±0.82	1.71±1.03	-1.9	23.9	0.0709
230	1.14±0.83	1.52±0.94	-1.2	26.0	0.2384
250	0.75±0.76	1.74±0.84	-3.5	26.1	0.0017
270	0.79±0.78	1.99±0.68	-4.7	30.3	<0.001
290	0.69±0.67	2.15±0.84	-5.4	23.9	<0.001
310	0.48±0.65	1.73±0.90	-4.4	22.2	<0.001
330	0.59±0.76	1.80±0.69	-4.9	29.7	<0.001
350	0.39±0.74	1.95±1.01	-5.0	22.6	<0.001

The measurements were normalized by square-root transformation and then compared in multiple t-tests. The table indicates the transformed stridulation rates (mean±SD) for both chamber and control conditions, as well as the statistics for each point of time. With 18 comparisons, the significance level was adjusted to $\alpha = 0.003$.

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