“Building behaviour and the control of nest climate in *Acromyrmex* leaf-cutting ants”

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Summary

The present work was aimed at experimentally studying whether climatic variables act as environmental cues for workers' building behaviour in leaf-cutting ants of the genus *Acromyrmex*, and to what extent building responses account for the maintenance of nest climate in a proper range for the inhabiting colony.

Specifically, this work presents independent analysis in different *Acromyrmex* species with disparate ecology and nesting habits, aimed at understanding to what extent: *i)* temperature and humidity act as cues for workers' building behaviour, *ii)* inter- and intraspecific differences in the nesting habits observed in South American *Acromyrmex* are based on distinct building behaviours and on the variation in regional climate across continent, *iii)* differences in nest architecture account for the maintenance of nest climate in a proper range for colony members and, *iv)* climatic variables trigger building responses aimed at controlling short-term changes in nest climate.

It is first experimentally shown that soil temperature acts as a cue for workers' digging behaviour. *Acromyrmex lundi* workers were observed to respond to both soil temperature as well as its changes, and to decide accordingly where to start or whether to stop digging. The soil temperature range preferred by workers to dig, between 20°C and maximally 30.6°C, matches the range at which colony growth is expected to be maximized. Temperature-sensitive digging might therefore lead to the establishment of the fungus chambers in soil layers with a proper range of temperatures for colony growth. Based on that, it was hypothesized that nest depth in *Acromyrmex* largely depends on the depth at which this temperature range is located across the soil profile, i.e., the higher the temperature in the superficial soil layers, the deeper the nest location, since soil temperature decreases with increasing depth. A bibliographic survey on nesting habits of 21 South American *Acromyrmex* species confirmed that the warmer the soil temperature at 50 cm depth throughout the South American continent, the higher the number of species presenting subterranean nests, compared with those inhabiting superficial nests. Temperature-sensitive digging in *Acromyrmex* would therefore explain the geographical distribution of nesting habits observed for this genus in the South American continent, i.e., subterranean in the northern tropical regions, and superficial in the southern temperate ones.

In addition, results showed that *Acromyrmex* colonies from temperate regions indeed achieve thermoregulatory benefits through the determination of nest depth based on thermoregulatory needs. In sympatrically-occurring colonies of the grass-cutting ant *A. heyeri,* temperature inside superficial thatched nests was higher, and more suitable for colony growth, than that inside subterranean nests. This temperature surplus was even higher in spring, at the time of production of sexual brood, than in winter or summer. It was demonstrated that such temperature surplus was brought about by the low thermal diffusivity of the nest thatch, which prevents diurnal nest overheating by the incoming solar radiation, and avoids losses of the accumulated daily heat into the cold air during night, thus leading to high average nest temperatures.

Although highly advantageous for colonies in terms of nest temperature, the determination of nest depth based on thermoregulatory needs may differentially affect nest ventilation and humidity depending on how nest exposition influences the exchange of nest air with the outside air. For instance, colonies with a superficial nesting habit might benefit from improved nest ventilation, but be at risk of desiccation due to their exposition and the consequent humidity losses into the dry outside air. Results demonstrated that in two *Acromyrmex* species, short-term regulatory building responses triggered and spatially organized by climatic variables occur, and may counteract undesired changes in internal nest humidity. Workers of the thatching grass-cutting ant *A. heyeri*, for instance, closed a number of nest-thatch openings as a response to desiccation of the outside air, even at a nest temperature that otherwise triggered the response of opening them so as to reduce nest temperature. In the leaf-cutting ant *A. ambiguus,* the direction of the airflow inside nest tunnels was shown to act as a cue for spatially guiding the building behaviour of plugging nest entrances. However, workers only responded if the humidity content of the circulating air was low, trading therefore nest ventilation for humidity maintenance.

Taken together, these results showed that climatic variables act as cues for workers' building behaviour in *Acromyrmex* colonies, principally by triggering and spatially organizing both digging and building responses. They may account for the maintenance of nest climate in a proper range for the inhabiting colony. In an attempt to integrate these findings into a scenario that considers building behaviour as a potentially force driving speciation, it was finally hypothesized that the use of climatic variables as cues for nest building can be considered as one additional factor that explains the high species richness of the genus *Acromyrmex* in the subtropical areas of the South American continent.

Zusammenfassung

Die vorliegende Arbeit untersucht, inwiefern das Bauverhalten von Blattschneiderameisen der Gattung *Acromyrmex* durch klimatische Variablen beeinflusst wird und dem Erhalt für die Ameisen geeigneter klimatischer Bedingungen dient.

Betrachtet werden verschiedene *Acromyrmex*-Arten, die sich in ihrer Ökologie und ihren Nistgewohnheiten unterscheiden. Ziel ist es zu verstehen, in wie fern: i) Temperatur und Feuchtigkeit als Reize das Bauverhalten der Arbeiterinnen beeinflussen, ii) Unterschiede im Bauverhalten und die regionale Variation des Klimas über den südamerikanischen Kontinent die beobachteten, inter- und intraspezifischen Unterschiede zwischen den Nesttypen südamerikanischer *Acromyrmex*-Arten erklären, iii) unterschiedliche Nestarchitekturen für die Aufrechterhaltung für die Ameisen geeigneter klimatischer Bedingungen im Nest sorgen, iv) klimatische Variablen Verhaltensweisen auslösen, die der Kontrolle kurzfristiger Änderungen des Nestklimas dienen.

Zunächst wird experimentell gezeigt, dass die Bodentemperatur ein Reiz ist, der das Bauverhalten von Ameisen beeinflusst. Es wurde beobachtet, dass *Acromyrmex lundi*-Arbeiterinnen sowohl auf Temperaturen als auch auf Temperaturänderungen reagieren, und, abhängig von diesen Variablen, über die Aufnahme oder den Abbruch des Grabeverhaltens entscheiden. Der Temperaturbereich im Boden, in dem die Arbeiterinnen zu Graben bevorzugen, also zwischen 20°C und maximal 30.6°C, entspricht dem Temperaturbereich, bei dem ein maximales Koloniewachstum erwartet werden sollte. Zudem legen die Ergebnisse nahe, dass die Orientierung des kollektiven Grabenverhaltens an der Bodentemperatur den Ameisen ermöglicht, Nestkammern in Bodenschichten zu etablieren die geeignete Temperaturbedingungen bieten.

Es wird angenommen, dass die Nesttiefe bei *Acromyrmex* stark davon abhängt, wie tief im Boden geeignete Temperaturbedingungen anzutreffen sind. Je höher die Temperatur in den obersten Bodenschichten, desto tiefer das Nest, denn die Bodentemperatur sinkt mit zunehmender Tiefe. Literaturdaten zu den Nistgewohnheiten von 21 südamerikanischen *Acromyrmex*-Arten wurden verglichen. Hierbei bestätigte sich, dass über den südamerikanischen Kontinent mit zunehmender, mittlerer Bodentemperatur in einer Tiefe von 50 cm auch der Anteil

der Arten zunimmt, die ausschließlich unterirdische Nester bauen im Verhältnis zu den Arten mit Oberflächennestern zunimmt. Temperaturabhängiges Graben würde die geographische Verteilung der Nistgewohnheiten von *Acromyrmex* in Südamerika erklären: Unterirdische Nester überwiegen in den nördlichen, tropischen Regionen und Oberflächennester in den gemäßigten Regionen im Süden.

Zudem konnte gezeigt werden, dass *Acromyrmex*-Kolonien der gemäßigten Regionen tatsächlich ihre Nesttemperatur durch Anpassung der Nesttiefe an klimatische Bedingungen regulieren. Bei der Grassschneiderameise *A. heyeri*, bei der Kolonien mit unterirdischen Nestern und solche mit oberflächlichen Hügelnestern sympatrisch vorkommen, war die Temperatur in den Oberflächennestern höher, und für das Koloniewachstum günstiger, als in unterirdischen Nestern. Dieser Temperaturvorteil war im Frühling, der Zeit, in der die Geschlechtstierbrut herangezogen wird, größer als in Winter oder Sommer. Es wurde gezeigt, dass dieser Vorteil durch die niedrige Wärmeleitfähigkeit der Nesthügels bedingt ist. Tagsüber verhindert der Nesthügel zunächst die Überhitzung durch Sonneneinstrahlung, und minimiert dann während der Nacht den Wärmeverlust an die kalte Umgebungsluft. Dies führt zu hohen Durchschnittstemperaturen innerhalb solcher Nester.

Neben dem Vorteil, den eine geringe Nesttiefe in diesem Fall für die Temperatur in der Nestkammer bietet, spielen auch weitere Aspekte eine Rolle. Kolonien mit oberflächlichen Nestern profitieren zwar von der vergleichsweise guten Nestventilation, setzen sich dabei aber einem Erhöhten Risiko aus, durch den Verlust von Feuchtigkeit an die Außenluft auszutrocknen. Bei zwei *Acromyrmex*-Arten zeigen die Ergebnisse das Auftreten regulatorischer Bauaktivität, die, ausgelöst und räumlich organisiert durch klimatische Variablen, einem unerwünschten Feuchtigkeitsverlust innerhalb des Nestes entgegenwirkt. Arbeiterinnen der hügelbauenden Grassschneiderameise *A. heyeri* verschlossen Öffnungen im Nesthügel als Antwort auf die Austrocknung der Aussenluft, und das selbst bei einer Nesttemperatur, auf die unter anderen Umständen mit der Öffnung derselben zur Reduzierung der Nesttemperatur reagiert worden wäre. Bei der Blattschneiderameise *A. ambiguus*, die unter bestimmten Bedingungen ihre Tunnel mit Pflanzenmaterial verschließt, wurde gezeigt, dass die Richtung der Luftbewegung in den Nestgängen das Verschließen der Eingänge räumlich beeinflusst. Dennoch reagierten Arbeiteinnen nur, wenn der Feuchtigkeitsgehalt der zirkulierenden Luft niedrig war, sie beschränkten somit die Nestventilation um die Feuchtigkeit aufrecht zu erhalten.

Zusammen genommen zeigten die Ergebnisse, dass klimatische Variablen Reize für das Bauverhalten der Arbeiterinnen in *Acromyrmex*-Kolonien darstellen. Grundsätzlich können sie Grabe- und Bauverhaltenweisen, die der Aufrechterhaltung eines geeigneten Nestklimas dienen, sowohl auslösen als auch räumlich organisieren. In einem Versuch, diese Erkenntnisse in ein Szenario zu integrieren, das das Bauverhalten als einen entscheidenden Faktor der Speziation in Betracht zieht, wird schließlich die Hypothese aufgestellt, dass die Nutzung klimatischer Reize beim Nestbau als zusätzlicher Faktor sein könnte, der den Artenreichtum der Gattung *Acromyrmex* in den subtropischen Regionen des südamerikanischen Kontinents zu erklärt.

1. Introduction and general aim

Ants of the neotropical myrmicine tribe Attini cultivate a fungus inside their nests in an obligate symbiotic relationship, which represents the sole food source for the colony brood (Bass and Cherrett 1995; Quinlan and Cherrett 1979; Weber 1972). The Attini genera *Atta and Acromyrmex* comprise the subgroup known as leaf-cutting ants, with polymorphic workers that are able to cut pieces of fresh leaves and use them as a substrate for the cultivation of the symbiotic fungus (Cherrett 1989). While *Atta* colonies may contain many millions of individuals (Fowler et al. 1986b) and inhabit giant nests up to 8 meters depth (Jonkman 1980; Moreira et al. 2004), *Acromyrmex* colonies have only several thousands of workers (Fowler et al. 1986b) and inhabit smaller and shallower nests (Gonçalves 1961). In South America, the leaf-cutting ant genus *Acromyrmex* have a wide distribution range, which extends between the 10°N up to the 44°S. *Acromyrmex* species occur therefore from the northern humid tropical forests and the dry tropical savannas, to the colder southern temperate Pampas and the dry sub Andean region, confronting thus a great variety of climates (Cherrett 1989; Weber 1972). Despite their ample distribution and the variety of climates they confront*, Acromyrmex* colonies are expected to maintain high humidity and temperatures around 25°C inside their nests, since these are the conditions that ensure the proper growth of the symbiotic fungus (Powell and Stradling 1986; Quinlan and Cherrett 1978). Given that behavioural responses are often the primary and sometimes the crucial mean by which insects copes with unsuitable climatic conditions (Bennet 1987; Chown and Nicolson 2004), *Acromyrmex* colonies are expected to have developed behavioural responses to maintain nest temperature and humidity in a proper range for the symbiotic fungus. It might be, at first, achieved by long-term responses related with building behaviour. One of the most common attributes of nests built by social insects is that they extend the control of the builder over some aspect of the environment, for instance, by protecting the nest inhabitants against extreme temperatures and the risk of water losses (Hansell 2005). For ants and termites, nest architecture has in fact been shown to influence nest ventilation, and to account for the maintenance of suitable nest temperatures and humidity (Frouz 2000; Kleineidam et al. 2001; Korb 2003; Rosengren et al. 1987; Turner 1994; Turner 2001). Therefore, *Acromyrmex* leaf-cutting ant colonies are expected to build nests that contribute to the maintenance of internal temperature and humidity between proper ranges for the symbiotic fungus and colony members. In addition, *Acromyrmex* workers may exhibit short-term responses to maintain fungus and brood under the proper range of temperature and humidity. For instance, by behavioural tracking the appropriate climatic conditions within the nest, similarly to other ant workers that carry the motionless brood to locations with proper temperature for growth (Anderson and Munger 2003; Cole 1994; Roces and Núñez 1989; Seeley and Heinrich 1981).

For nest construction, social insect workers do not use any reference of the final extent and arrangement of the nest they are building (Hansell 2005). The organization of workforces during nest building by social insects has been the focus of intensive research, mostly theoretical, over the last fifty years (see reviews in: Bonabeau et al. 1997; Camazine et al. 2001; Hansell 2005; Theraulaz et al. 1998). By considering stigmergic mechanisms (Theraulaz and Bonabeau 1999), self-organizing mechanisms (Theraulaz et al. 1998), and the use of templates (Bonabeau et al. 1998; Cox and Blanchard 2000), theoretical models have emphasized that the structure of a social insect nest results from a succession of stimulus-response steps involving the environment, the workers, and the by-product of their activities (Deneubourg and Franks 1995). Besides the structure being constructed, climatic variables, such as temperature and humidity, have also been considered as local cues that may trigger a building response. The final outcome of the collective building effort, as organized by climatic variables, is therefore assumed to be a nest with a stable and suitable climate for colony members (Turner 2000b). Up to date, however, it remained experimentally unexplored which climatic variables actually act as cues for collective building, and to what extent the observed building behaviour represent a regulatory response for the control of nest climate.

The present work is therefore aimed at experimentally studying whether climatic variables act as environmental cues for workers' building behaviour in leaf-cutting ants of the genus *Acromyrmex*, and to what extent the resulting building responses account for the maintenance of nest climate in a proper range for the inhabiting colony.

1.1. Specific aims and experimental approach

The present work is composed of 7 chapters. Chapters 2 to 6 present independent analysis in different *Acromyrmex* species with disparate ecology and nesting habits, aimed at understanding to what extent: *i)* temperature and humidity act as cues for workers' building behaviour, *ii)* inter- and intraspecific differences in the nesting habits observed in South American *Acromyrmex* are based on distinct building behaviours and on the variation in regional climate across the continent, *iii)* differences in nest architecture account for the maintenance of nest climate in a proper range for colony members and, *iv)* climatic variables trigger building responses aimed at controlling short-term changes in nest climate. While chapters 2 to 6 are organized in sections like scientific publications, with a summary, introduction, methods, results and a specific discussion, chapter 7 intends to integrate the reported findings into an scenario that considers nest building as an adaptation that may have influenced speciation within the genus *Acromyrmex*.

Specifically, chapter two is aimed at assessing the temperature range preferred by *Acromyrmex* workers to locate and tend the symbiotic fungus and the brood. This temperature range, together with the known humidity range preferred by leaf-cutting ant workers to cultivate the fungus, is assumed to correspond to the climatic conditions that *Acromyrmex* workers prefer inside their natural nests. For that, small groups of *A. heyeri* workers were confronted with a temperature gradient in the laboratory, and their thermopreference for the location of brood and fungus was assessed.

Chapter three is aimed at evaluating whether the use of soil temperature as a local cue during collective digging allows colonies to establish the fungus chambers in soil layers with proper temperatures for colony growth. For that, *Acromyrmex lundi* workers were confronted with soils at constant or changing temperatures, and the soil temperatures at which workers preferentially excavated, or decided to stop digging and search for alternative digging places, were evaluated. In addition, this chapter explores whether such temperature-sensitive digging may account for the interspecific differences observed in the depth of *Acromyrmex* nests throughout the South American continent, i.e., superficial nests in southern temperate areas, and subterranean ones in the northern tropical areas. For that, a bibliographic survey on 21 South American *Acromyrmex* species was carried out to establish the relationship between nest types and the South American soil temperature regimes at the geographical locations where they occur.

Chapter four is aimed at investigating whether *Acromyrmex* colonies achieve thermoregulatory benefits by showing plasticity in the nest type they construct as a function of the regional climatic conditions, either by building a thatched nest or digging a subterranean one. For that, long-term comparative measurements of the thermal relations between the fungus garden and the environment were performed in neighbour *A. heyeri* colonies inhabiting either superficial or subterranean nests.

Chapters five and six are aimed at assessing whether nest modifications are triggered and spatially organized by nest temperature, humidity and the direction of circulating air, and to what extent they counteract unsuitable short-term variations of nest climate. For that, *A. ambiguus* and *A. heyeri* colonies were confronted with controlled changes of the internal nest climate, and the resulting building responses quantified.

Chapter seven explores the adaptive value of the building behaviours described in the previous chapters for the different species considered. It specifically addresses the question whether distinct building behaviours might contribute to explain the latitudinal pattern in species richness shown by the genus *Acromyrmex* throughout the South American continent.

2. Thermal preference for fungus culturing and brood location by workers of the thatching grass-cutting ant *Acromyrmex heyeri*

Summary Thermal preferences for location of brood and fungus by workers of the thatching grass-cutting ant *Acromyrmex heyeri* were investigated in the laboratory, using a temperature gradient between 10ºC and 37ºC. Workers from a large laboratory colony were motivated to pick up exposed brood and fungus by removing a small part of the protective nest thatching. Single workers carrying pieces of fungus or brood items were then gently removed from the colony and placed at one end of the temperature gradient, either at 10 or 37°C. They were allowed to relocate the fungus or brood following their temperature preference. Both the probability of relocating an item and the temperatures selected were recorded after 45 minutes. The probability of relocation largely depended on the starting temperature, being higher for workers starting at 37°C than at 10°C, for both fungus and brood-carrying workers. For those relocated items, the selected temperatures averaged 25°C and 24°C for fungus and brood, respectively, when workers started from the hot end of the gradient. When workers started from the cold end of the gradient, the selected values were slightly lower, 22°C and 21°C for fungus and brood, respectively. These values match closely the temperature range in which maximal growth of the isolated attine ant fungus is achieved, between 20 and 25°C, as reported in the literature, indicating that the thermoregulatory behavioral responses of workers maximize fungal growth. Thermal preferences and the construction of thatch mounds are discussed as regulatory responses for the control of fungus growth in leaf-cutting ants inhabiting temperate regions.

2.1. Introduction

In ant colonies, the control of adequate microclimatic conditions for brood development is achieved though both the construction of a nest and the behavioral tracking of the appropriate conditions inside the nest by nurse workers that carry the motionless brood (Seeley and Heinrich, 1981; Roces and Núñez, 1995). Although the nest architecture can help to stabilize the climatic fluctuations inside the nest, brood transport by nurse workers ultimately represents a fine-tuning response to cope with excessive deviations from the adequate values.

The New World fungus-growing ants, tribe Attini, live in symbiosis with a fungus they cultivate, which provides the colony members with food. Although workers cover only a portion of their energetic demands by feeding on the fungus staphyllae, the fungus represents the unique food source for the developing larvae (Bass and Cherrett 1995; Quinlan and Cherrett 1979). As a consequence, workers are expected to maximize the growth of their symbiotic fungus not only by selecting suitable vegetable substrate, but also by an active choice of proper locations for culturing, according to the prevailing microclimatic conditions inside the nest. For instance, workers of a laboratory colony of *Atta sexdens rubropilosa* were observed to relocate the fungus garden along a humidity gradient, choosing the environments with the highest humidity (Roces and Kleineidam 2000). With regard to temperature, it is known that the isolated attine ant fungus has strict demands of temperature for proper growth *in vitro* (Powell and Stradling 1986; Quinlan and Cherrett 1978). But remarkably, there are no published studies dealing with thermal preferences of attine ant workers for fungus culturing, even though they are responsible for the relocation of the fungus gardens when temperature varies beyond suitable values.

It has been suggested that field colonies of the genus *Atta* move the fungus to different subterranean chambers according to their temperature (Eidmann 1935; Weber 1972). This appears to be a long-term response, since leaf-cutting ant species building large and complex subterranean nests are expected to achieve a more or less natural control of temperature in deep chambers buffered by surrounding soil (Kleineidam and Roces 2000). In contrast, leaf-cutting ant species constructing more simple nests closer to the surface, particularly those inhabiting the southernmost latitudes of the attine ant distribution range (e.g., *Acromyrmex lobicornis*, *A. ambiguus*, *A. lundi, A. heyeri*; (Farji-Brener and Ruggiero 1994)), may be more exposed to marked changes in environmental temperature.

Colonies of the thatching grass-cutting ant *Acromyrmex (Moellerius) heyeri* occur in open habitats of Uruguay, Brazil and the Pampean grasslands of Argentina (Bonetto 1959).They construct a thatch mound with dry grasses and soil that protects a central and single fungus garden usually located at the general soil level (Gonçalves 1961). Mound-building in *Acromyrmex* is unique among fungus-growing ants, and seems to represent an adaptation to low temperatures (Weber 1972). As a consequence, behavioral control of fungus temperature is expected to be particularly developed in mound-building *Acromyrmex* species inhabiting areas that undergo large temperature variations.

In the present study, temperature preferences by workers of the thatching grass-cutting ant *Acromyrmex heyeri* for the location of both brood and fungus were investigated in the laboratory along a temperature gradient. The removal of brood and fungus from either 10°C or 37°C, and the temperatures selected to relocate them, were evaluated under otherwise constant conditions. This schedule allowed us to investigate the response of workers to low and high temperature values that are experienced by colonies in the field.

2.2. Methods

Workers, brood and fungus used in the experiments were taken from a colony of *A. heyeri* collected in September 2000 in Joanico, Canelones, Uruguay (34º33´26´´S, 56º15´59´´W), and maintained in the laboratory at 25ºC under a LD cycle of 12:12h. This temperature was chosen based on reports of highest growth rates of isolated attine ant fungus (Powell and Stradling 1986).

Temperature preference of *A. heyeri* workers to locate brood and fungus were analyzed in a controlled temperature gradient. It consisted of an acrylic box (30 cm long, 10 cm wide and 2 cm in height) placed on an aluminium base and located in a room at 22ºC. The temperatures of the two ends of the box floor could be independently regulated through Peltier-elements attached to a self-constructed thermostatic circuit. One end of the box floor was maintained at 10°C (± 0.5°C), and the other end at 37°C (± 0.5°C). Using this procedure, a roughly linear gradient of about $1^{o}C*cm^{-1}$ was achieved. Temperatures were measured directly at the box floor using thermistor-thermometers (Beckmann+Egle, Germany, Model MD 3150), at a resolution of 0.01°C. Six thermometers were placed equidistantly along the box floor, thus allowing a long-term control of temperature. A relative humidity of 84% was maintained inside the box by placing a saturated solution of KCl (Solomon 1951) in a narrow plastic container all along the box. This humidity was chosen, and not the most preferred one of 98% (Roces and Kleineidam 2000), as it proved to be difficult to maintain a constant humidity at highest values because of the extreme temperature differences at the nest ends. Ants were prevented to contact the solution by covering the container with nylon gauze. Humidity inside the box was measured with a hygrometer at a resolution of 1% (measuring range at 25°C: between 2 and 98%).

Prior to each assay, thirty workers from the colony were placed inside the gradient-box and allowed to inspect the complete arrangement. This was essential to provide the box with colony odors, in order to avoid alarm behaviors by the workers during the assays. After at least three hours, workers were removed from the box and returned to the nest. Each assay was then initiated as follows. Workers from the large colony were motivated to pick up exposed brood and fungus by removing a small part of the nest thatching. As a reaction to this disturbance, single workers picked up either a fungus piece or a brood item, and searched for an appropriate place to relocate them. At this moment, five workers carrying fungus or a larvae, depending on the assays to be performed, were gently removed with forceps, put in a vial and placed together at one of the ends of the gradient-box, either 10ºC or 37ºC in independent assays. If workers showed signs of disturbance or alarm reactions, the complete group was removed and the procedure started again with the collection of five new ones. After 45 minutes, both the proportion of items moved from the end of the gradient-box and the temperatures selected to place the items were recorded. The items were usually piled and therefore occupied a small area of approximately 1 cm^2, so that the center of their distribution was used as the mean temperature chosen.

A total of 30 independent assays were performed with brood-carrying workers, each one using five new workers and items. Fifteen assays started with the workers being located at the cold end of the gradient, and other 15 were initiated from the hot end. Cold-end and hot-end assays were performed in random order. The same randomization schedule was used for the assays with fungus-carrying workers.

2.3. Results

Ants placed at the end of the temperature gradient with either fungus or brood quickly began to walk along the gradient searching for a suitable place to relocate the items. Usually after 15 minutes the items were either transported and placed at the selected temperature, or left at the starting point with no further removal. The observation time of 45 minutes assured that the behavioral responses completely stabilized, and that no transient responses were recorded.

The probability of relocating an item from the starting point largely depended on the starting temperature, being higher for workers moving from 37°C than from 10°C, for both fungus and brood-carrying workers (Fig. 2.1). Most of the fungus and the brood (81.3 and 97.4%, respectively) were relocated from 37°C (Fig. 2.1, black bars; for fungus: $\chi^2 = 66.13$, df = 1, $p < 0.001$; for brood: $\chi^2 = 94.74$, df = 1, $p < 0.001$). Worker responses from 10°C differed for fungus and brood. Less than 20% of the fungus was relocated, and as a consequence, the final distribution of the fungus pieces could not be statistically distinguished from the initial. In contrast, ca. 40% of the brood was relocated from the cold end, so that their final distribution was significantly different from the initial (Fig. 2.1, white bars; for fungus: $\chi^2 = 3.13$, df = 1, $p = 0.07$, NS; for brood; $\chi^2 = 17.08$, df = 1, $p < 0.001$).

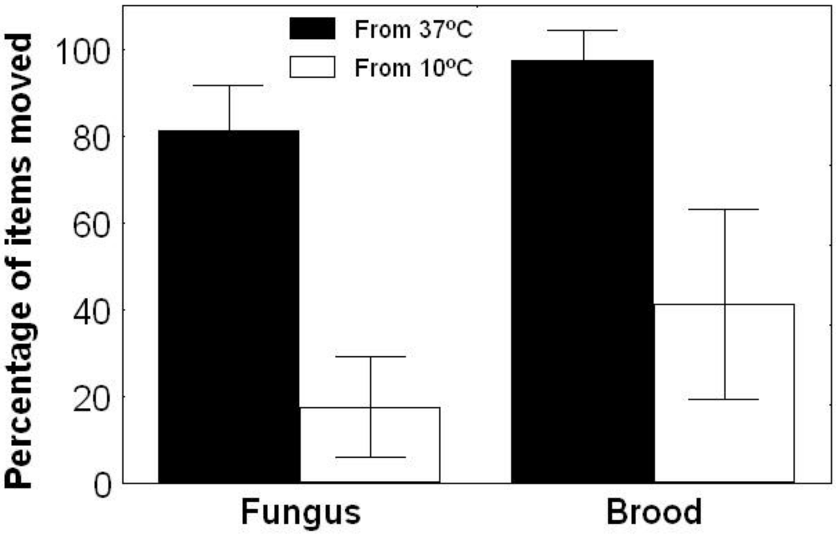

Figure 2.1: Proportion of items, fungus pieces or brood, transported along the thermal gradient by workers of A. heyeri (mean ± SD, n= 15). Workers started either from 37°C or from 10°C.

For the relocated items, the selected temperatures for both fungus and brood were similar when workers moved from 37°C, i.e., 24.96°C ± 1.80 for fungus and 24.35ºC ± 2.05 for brood (Fig. 2.2; mean ± SD; t-test for independent samples, t = 0.88, df = 28, p = 0.385, NS). The selected temperatures were also similar for both fungus and brood for workers starting at 10°C: 22.11ºC ± 3.70 for fungus and 20.82ºC ± 3.90 for brood (Fig. 2.2, t = 0.87, df = 25, p = 0.389, NS). However, the temperatures selected for the brood depended on the starting point, being higher when workers started at 37°C (Fig. 2.2, t = 3.07, df = 27, p = 0.005), whereas temperature selection for the fungus was slightly different, but statistically independent of the starting point (Fig. 2.2, t = 2.65, df = 26, p = 0.013).

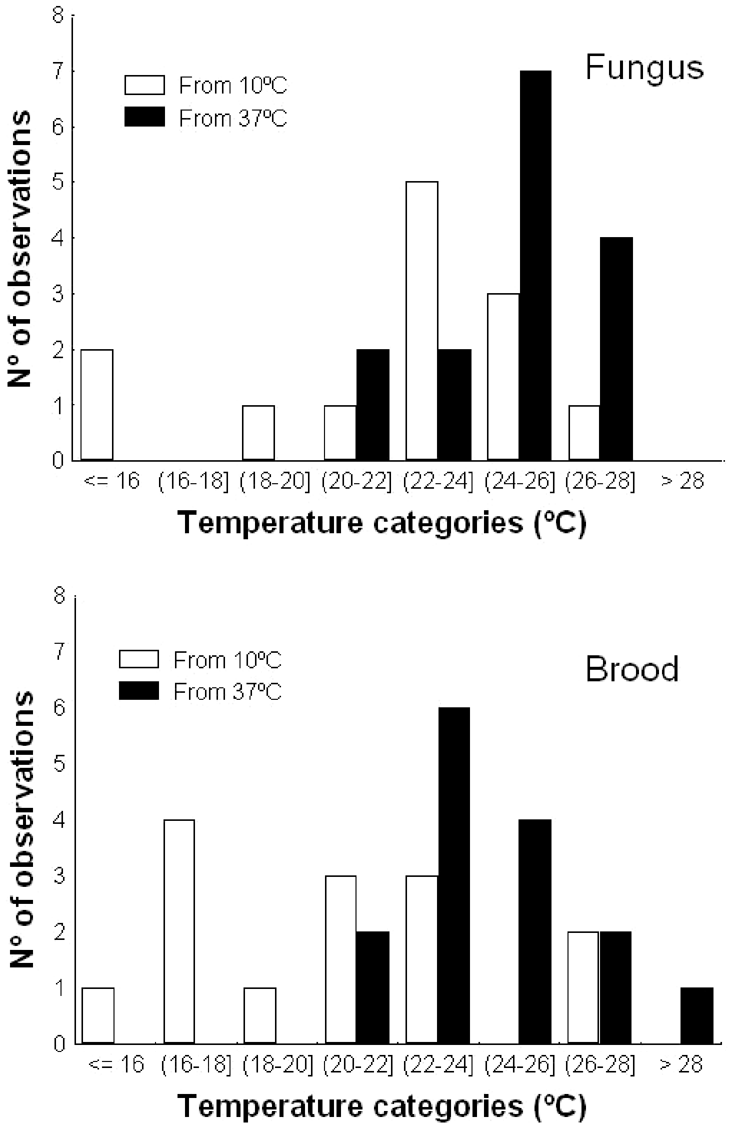

Figure 2.2: Temperature selected by *A. heyeri* workers to locate the fungus (a) or brood (b). Workers started either from 37°C or from 10°C, which represent the temperatures of the two ends of the gradient. In each assay, the relocated items were grouped by the workers at the selected temperature, so that each observation represents the response of five workers. For graphic representation of the frequency distribution, observations were grouped in intervals of 2°C.

2.4. Discussion

For both fungus- and brood-carrying workers, the probability to relocate items was strongly dependent of the temperature initially experienced by the workers. All items were quickly removed from 37°C, and the values selected to place them were less variable than those chosen when workers removed items from 10°C. These results clearly indicate the importance of avoiding high temperatures that are expected to be harmful for both fungus and brood. In fact, temperatures above 30°C are lethal for the isolated attine ant fungus (Powell and Stradling 1986), and it has been observed that a slight temperature increase causes workers of a laboratory colony of *Acromyrmex octospinosus* to move the fungus to a lower value (Quinlan and Cherrett 1978).

On the other hand, brief exposure to low temperatures seems not to compromise the growth and development of fungus. Powell and Stradling (1986) reported that the growth of the isolated fungus can be stopped at low temperatures, but it continues when the temperature is increased again. In addition, even small colonies of *Acromyrmex lundi* with brood kept at 5-8°C for 5 days recover and behave normally when temperature increases again (Weber 1972). As for the brood transport, *A. heyeri* workers responded to both starting temperatures, even though the probability of relocating brood was markedly lower when workers moved from 10°C. This is unexpected if is considered that in insects, the developmental rate of the brood positively depends on temperature (Gilbert and Raworth 1996), and workers are expected to try to maximize the growth rate of the brood. But on the other side, field colonies are sometimes exposed to even lower values for short periods (personal observations), so that immediate responses may not be as critical as it is the case for high temperatures. Chilling is unlikely to be responsible for the lower percentage of items moved observed in the experiments, as workers were not observed to be chilled or to move particularly slow at 10°C, and field observations indicate that they are active even at lower temperatures.

The selected temperatures averaged 25°C and 24°C for fungus and brood when workers started from the hot end of the gradient, and were slightly lower, 22°C and 21°C, when they started from the cold end. These values match very well the temperature range in which maximal growth of the isolated attine ant fungus is achieved, as reported in the literature. Powell and Stradling (1986), working with the symbiont fungus of three attine ant species, found highest growth rates between 20 and 25°C. The *A. octospinosus* isolated fungus grew best at 24.5ºC, and hardly at all at 10ºC and 37ºC (Quinlan and Cherrett 1978). Similar results were obtained by Cazin *et al.* (1989) working with *Atta cephalotes* isolated fungus, which grew better at 25ºC than 30ºC, and not at all at 37ºC. Therefore, these results show for the first time in leaf-cutting ants that the thermoregulatory behavioral responses of workers indeed maximize fungal growth. The behavioral preferences are also congruent with reports about fungus temperatures recorded in field colonies: 25-28°C for *Atta sexdens* (Eidmann 1935; Stahel and Geijskes 1940), 27.5°C for *Atta vollenweideri* (Kleineidam and Roces 2000), 25.6°C for *Acromyrmex coronatus* (Parra et al. 1974), and around 27°C for *A. heyeri* (Zolessi and Abenante 1998).

What is the ecological relevance of the thermoregulatory responses by workers of *A. heyeri*? This species has a distribution area that includes the temperate Neotropical zone (Fowler et al. 1986a), so that extreme air temperatures are usual and they are expected to largely influence nest temperature. For instance, mean temperatures of 10 and 28°C were measured in the fungus garden of the mound-building leaf-cutting ant species *Acromyrmex lobicornis* in winter and summer, with soil surface temperatures of 4 and 31ºC, respectively (Quiran and Pilati 1998). Results suggest that in winter, workers may first try to move the brood to more protected areas inside the nest, and then the fungus. Or they may even relocate the fungus on an hourly-basis following the daily changes of temperature, as suggested by Zolessi and Abenante (Zolessi and Abenante 1998), or seasonally, as reported for other fungus-growing ant species (Lapointe et al. 1998; Navarro and Jaffé 1985; Weber 1957). In this context, it is unknown whether worker thermal preferences vary following a daily pattern, as reported for the ant *Camponotus mus* (Roces 1995).

It is important to note that the particular architecture of the *A. heyeri* nest, with a singly fungus garden located at the ground level and covered with thatch, may on the one hand limit the possibility of fungus and brood relocation following temperature changes. But on the other hand, mound construction would be highly favorable on the long-term, by stabilizing nest temperature in a proper range for fungus and brood growth.

3. Soil temperature, digging behaviour, and the determination of nest depth in South American species of *Acromyrmex* leaf-cutting ants

Summary Since soil temperature is strongly influenced by soil depth, ants inhabiting underground nests are expected to excavate them at the soil depth that provides proper temperatures for the colony. This work was aimed *i)* at experimentally investigating whether *Acromyrmex* leaf-cutting ants use soil temperature as a cue for the determination of nest depth while digging, and *ii),* at elucidating the causes underlying the geographical distribution pattern of the different nest types of *Acromyrmex* in the South American continent: mostly subterranean nests in the northern tropical regions, and superficial, thatched mounds in the southern temperate regions. Results indicate that *A. lundi* workers use soil temperature as an orientation cue to decide where to start digging, and respond to rising and falling soil temperatures by moving to alternative digging places, or by stopping digging, respectively. The soil temperature range preferred by workers to dig, between 20°C and maximally 30.6°C, matches the range at which colony growth is expected to be maximized. A bibliographic survey on nesting habits of 21 South American *Acromyrmex* species showed that the warmer the soil temperature at 50 cm depth, the higher the number of species presenting subterranean nests, compared with superficial nests. It is suggested that nest depth in *Acromyrmex* largely depends on the depth at which the temperature range preferred by workers for digging is located in the soil profile, i.e., the higher the temperature in the superficial soil layers, the deeper the nest location. Temperature-sensitive digging in *Acromyrmex* would therefore help explaining the geographical distribution of nest types observed for this genus in the South American continent.

3.1. Introduction

For ants inhabiting nests excavated in soil, the microclimatic conditions faced by the colony largely depend on the soil depth at which the nests are located (Seeley and Heinrich 1981; Sudd 1982), since temperature, humidity, and air composition strongly vary with soil depth (Hillel 1998). Therefore, ants are expected to dig their nests at those soil layers providing proper conditions for colony development, as shown for instance in ants inhabiting deserts, which dig extremely deep nests in order to reach the water table (Dlussky 1974).

The selection of an adequate soil layer for nest location should be particularly relevant for leaf-cutting ants. Leaf-cutting ant workers of the genera *Atta* and *Acromyrmex* cut leaves as a substrate for a symbiotic fungus they cultivate inside the nest chambers, which represents the food source for the developing brood and to a lesser extent, for the adults (Bass and Cherrett 1995; Quinlan and Cherrett 1979; Weber 1972). Since this fungus has strict demands of high humidity and temperatures between 25 and 30°C for proper growth (Powell and Stradling 1986; Quinlan and Cherrett 1978), workers are expected to provide it with adequate nest microclimatic conditions. In the leaf-cutting ant genus *Acromyrmex,* nests are composed by one or multiple fungus chambers interconnected by tunnels, and show interspecific differences regarding their depth (Fowler and Claver 1991). Some species construct mound-shaped superficial nests, with the fungus garden located on the soil surface level and covered by a thatch mound composed of soil and plant fragments. On the contrary, other species inhabit nests with multiple chambers excavated up to a depth of three meters (Bonetto 1959; Fowler 1985; Gonçalves 1961; Lapointe et al. 1998). The differences in nest depth have commonly been seen as a long-term adaptation aimed at providing the symbiotic fungus garden and developing brood with suitable climatic conditions inside nests (Farji-Brener 2000; Lapointe et al. 1998; Navarro and Jaffé 1985). For instance, South American *Acromyrmex* species occurring in the southernmost distribution range often inhabit shallower nests, when compared to those species inhabiting northern areas of the continent (Farji-Brener 2000). It clearly suggests that the lower the average environmental temperature, because of the latitudinal increase, the higher the number of species that build superficial nests.

In ants, extreme temperatures negatively affect colony growth and survival (Porter 1988; Porter and Tschinkel 1993; Roces and Núñez 1989), and leaf-cutting ant colonies are therefore expected to avoid inhabiting those soil layers with extreme temperatures (Campbell 1977; Hillel 1998). Avoidance of unsuitable temperatures would not only be achieved by relocating the brood and fungus (chapter 2) (Bollazzi and Roces 2002) to deeper or superficial nest chambers as soon as such values are reached, but also by selecting soil layers with adequate temperatures to dig new chambers during colony growth. Given that in leaf-cutting ants proper growth of both fungus and brood strongly depends on temperature, and soil temperature largely depends on soil depth, it is proposed that *Acromyrmex* leaf-cutting ants sense and use soil temperature as an environmental cue to decide where to excavate their nests. As a consequence, nests should be excavated at those soil depths at which the proper range of temperatures for colony growth is found across the soil profile.

To test this hypothesis, the influence of soil temperature on workers' digging performance was investigated in the laboratory by confronting small groups of workers with soils at different temperatures. Second, it was investigated whether workers show thermopreferences and use soil temperature as a cue to decide where to start digging, when simultaneously confronted with two soils at different temperatures. Third, to assess whether digging workers sense and respond to continuous changes in soil temperature, workers were presented with either increasing or decreasing soil temperatures while digging, and their performance and thermal threshold to give up were determined.

Based on the hypothesis outlined above, it is further suggested that the two general types of nest morphology observed in the genus *Acromyrmex*, i.e., superficial and subterranean nests as indicated above, represent adaptations to the long-term temperature regimes experienced by populations over evolutionary time. Thus, at a geographic-scale level it may be expected that *Acromyrmex* colonies dig for instance deeper nests, the higher the soil temperatures at the superficial level, and viceversa. To evaluate this hypothesis, a bibliographic survey on 21 South American *Acromyrmex* species was carried out, and the relationship between nest types and soil temperature regimes at the geographical locations where they occur was established.

Thus, this work uses an empirical approach to evaluate the extent to which the use of soil temperature by digging workers accounts for the determination of nest depth in *Acromyrmex* leaf-cutting ants. Such an approach is aimed at elucidating the causes underlying the geographical distribution pattern of nest types, superficial and subterranean, observed for this genus in the South American continent.

3.2. Methods

The laboratory experiments were performed with one one-year old colony of the leaf-cutting ant *Acromyrmex lundi* collected in Sarandi del Yi, Uruguay (33°20'25''S, 55°37'53''W), and transported to the Department of Behavioural Physiology and Sociobiology at the University of Würzburg, Germany. *A. lundi* was chosen because colonies show plasticity in their nesting habits that seems to depend on the soil temperature experienced by the colonies. For instance, colonies dig subterranean nests (Fowler 1985) in the hot soils of Paraguay (Van Wambeke 1981), but may nest between aerial tree roots within a thatch mound of leaf fragments and debris (Bonetto 1959) in the milder soils of west Argentine (Van Wambeke 1981).

The soil used during the laboratory experiments was a mixture of sand and clay (2:1) with a mass water content of 12 % (range 10-15 %).

3.2.1. Effect of soil temperature on worker's digging performance

In order to assess how soil temperature affects workers digging performance, groups of 5 workers (mean body mass = 2.46 mg) were presented with soils at either 10, 15, 20, 25, 30, 35 or 40°C, and the total amount of soil excavated over four hours was recorded. A plastic tube of 10 cm length and 1 cm diameter filled with soil was used as a digging tube (Fig. 3.1a, D). It was fitted within an aluminium plate fixed to a thermostatic plate (Fig. 3.1a, Th and a), maintained at the desired temperature by means of a thermoregulated water bath (Fig. 3.1a, Tb). As soon as the soil reached the temperature to be tested, the five ants were gently introduced in a small box attached to the digging tube (Fig. 3.1a, A), and allowed to dig without disturbance. A total of 24 replicates were done for each soil temperature.

3.2.2. Soil temperature selected by workers to start digging

In order to know whether workers decide where to start digging depending on the sensed soil temperature, groups of 3 workers were simultaneously confronted with two soils at different temperatures, and their preference was recorded. For that, a simultaneous binary choice between a soil at 25°C and a soil at either 15, 20, 30 or 35°C was presented to the workers. The experimental setup consisted of three interconnected boxes (45 x 11 x 11 mm each) (Fig. 3.1b). The two lateral ones, called the digging boxes (Fig. 3,1b, D1 and D2), were filled with soil and placed on separated thermostatic plates independently thermoregulated by two thermal baths attached to them (Fig. 3.1b, Th and Tb). When the soil inside the digging boxes reached the two temperatures to be tested, three workers were gently introduced in the middle box (Fig. 3.1b, A). The selected temperature to start digging was defined as that of the digging box where workers started and continued digging over 30 minutes, since workers, once having selected a box, were not observed to change the side. In control assays both soils were maintained at 25°C, and side preferences were recorded in the same way. Forty replicates were carried out for each pair of tested temperatures.

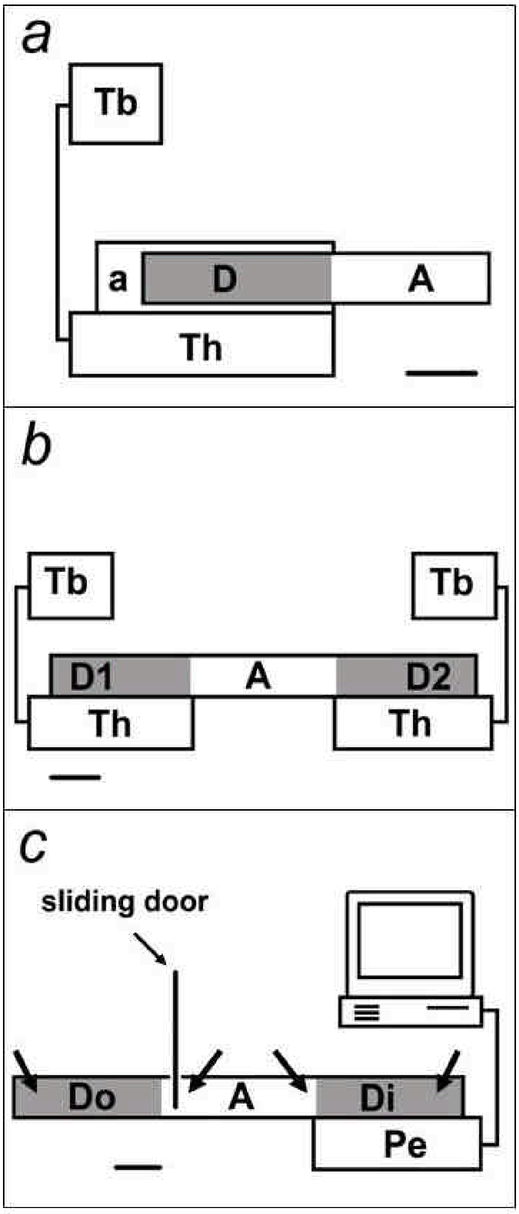

Figure 3.1: Setups used for the following experiments: **a)** Effect of soil temperature on workers' digging performance. **b)** Soil temperature selected by workers to start digging. **c)** Workers' response to increasing and decreasing soil temperatures. A = ant box; a = aluminium plate; D = digging tube; Th = thermostatic plate attached to a thermal bath (Tb) to regulate its temperature; D1 and D2 = digging boxes with different temperatures; Di and Do = initial digging box and optional digging box; Pe = computer-controlled Peltier element. The arrows in **c** show the locations where temperature was measured during the experiments with increasing and decreasing soil temperatures **(c)**. Grey areas represent the soil. In all figures the scale bar represents 2 cm (only the digging boxes and digging arenas are in scale).

3.2.3. Workers responses to increasing and decreasing soil temperatures while digging

Soil temperature at the superficial layers changes continuously over time, so that workers are expected to be exposed to changing temperatures while engaged in digging under natural conditions. In order to asses whether workers respond to soil temperature changes experienced during digging, they were confronted with either decreasing or increasing soil temperatures while digging, and their performance was recorded. Workers' performance was quantified as the rate of soil pellets removed from the soil with changing temperatures, compared to that from a soil with constant temperature. The experimental setup used to perform the experiments is presented in the figure 3.1c. A group of 5 workers was placed inside an ant box (Fig. 3.1c, A) located between two digging boxes (45 x 11 x 11 mm each) filled with soil initially maintained at room temperature, 24°C. Workers could initially start digging only inside one of them, the so-called initial digging box (Fig 3.1c, Di), because the access to the opposite one, called the optional digging box (Fig 3.1c, Do), was blocked by a sliding door (Fig. 3.1c, sliding door). Thirty minutes after workers started digging, this door was opened, so that workers could choose between either continuing digging inside the initial digging box or moving to the optional digging box. Digging activity was monitored over 100 min (see below). Through this procedure, it was possible to assess whether workers, once they started digging, show site fidelity to the initial digging place when soil temperature remained unchanged, even when they have the option to move to an alternative digging place. This series was considered the control one. In two other independent experimental series, 30 min after the assay's begin the soil temperature at the initial digging place was either increased or decreased at a rate of 0.1°C*min^{-1} for the following 100 min, and the sliding door was opened. Thus, the soil temperature changed either from 25 to 35°C, or from 25 to 15°C. Inside the optional digging box the soil was maintained at the same temperature workers experienced in the initial box at the beginning (ca. 24°C). By measuring workers' digging activity in the initial and optional digging boxes, the temperature at which workers stop digging at the initial box and eventually moved into the optional box was assessed. As a measure of digging activity, the number of soil pellets removed by workers from each digging box over time was recorded with four video cameras placed at each side of both digging boxes (not shown in Fig 3.1c). From the video recording, the number of pellets removed every 5 minutes throughout the final 100 minutes of the assay (with constant, increasing or decreasing temperature) was counted. A very accurate change of soil temperature inside the initial box was achieved using a computer-controlled Peltier element located inside an aluminium plate, above which the initial digging box was placed (Fig. 3.1c, Pe). Soil and air temperature were recorded inside the initial and optional digging boxes using a temperature data logger equipped with four wire sensors (Voltcraft K204). Twelve replicates of the series with increasing and decreasing temperatures, as well as the control ones, were performed.

Finally, in order to know whether the threshold temperature at which workers would stop digging may compromise worker survival, mortality rates of *A. lundi* workers as a function of temperature was evaluated in the laboratory. For that, independent groups of 50 workers (mean body mass = 2.43 mg) were placed in Petri dishes inside an incubator at 98% RH, and exposed to air temperatures ranging from 25 to 42°C, in 1°C-steps, i.e., a total of 18 groups were assayed. After 6 hours, the number of dead workers in each group was counted.

3.2.4. Soil temperature and observed nest depths in South American species of the genus *Acromyrmex*

In order to investigate whether soil temperature regimes have influenced, over evolutionary time, the determination of nest depths in *Acromyrmex* and so the geographical distribution of the two main nest types (subterranean and superficial), the relationship between nest location across the soil profile and soil temperature regimes was evaluated for 21 South American *Acromyrmex* species.

First, a bibliographic survey of publications on South American *Acromyrmex* species was carried out so as *i)* to determine the locations where species have usually been recorded, and *ii)* to classify the species as having one of two possible nest types, subterranean or superficial, as indicated above. Nests were assigned to one of these two categories depending at which depth the fungus chambers are located in the soil profile. In superficial nests, a single or multiple chambers are either located on the soil surface level or over it, i.e., an imaginary line representing the soil surface would cut through a fungus chamber at a given point. In such cases, the fungus gardens are usually covered by a thatch of plant fragments and soil, as in *A. heyeri* (Fig. 3.2). On the contrary, in subterranean nests one or multiple fungus chambers are entirely located under the soil surface level, as in *A. striatus* (Fig. 3.2).

Second, the locations where the surveyed *Acromyrmex* species have been recorded were assigned as having one of two possible “Soil Temperature Regimes”, i.e., the mean annual soil temperature at a depth of 50 cm. The data of Van Wambeke (1981) and the Global Soil Temperature Regimes map (USDA 2005) were used to assign locations as having one of these two categories of soil temperature regimes. To the first category, called thermic soils, belong both the thermic and isothermic soils of the classification system applied by the USDA (1975). In these soils, the mean annual soil temperature is 15°C or higher, but lower than 22°C. In thermic soils, the difference between mean summer and mean winter soil temperatures is more than 6°C, whereas in isothermic soil, it is less than 6°C. To the second category, called hyperthermic soils, belong both the hyperthermic and isohyperthermic soils of the classification system applied by the USDA (1975). In these soils the mean annual soil temperature is 22°C or higher. In hyperthermic soils, the difference between mean summer and mean winter soil temperatures is more than 6°C, whereas in isohyperthermic soils it is less than 6°C. Since vegetation cover alters the soil temperature regime, usually by diminishing soil temperature variation at the soil surface (Alvalá et al. 2002; Rosenberg et al. 1983; Weber 1959), locations were also classified depending on vegetation cover, either grasslands or woodlands. The bibliographic survey was limited to Argentina, Brazil, Colombia, Paraguay, Venezuela and Uruguay, since the most complete investigations regarding nesting habits of *Acromyrmex* species have only been made in these countries.

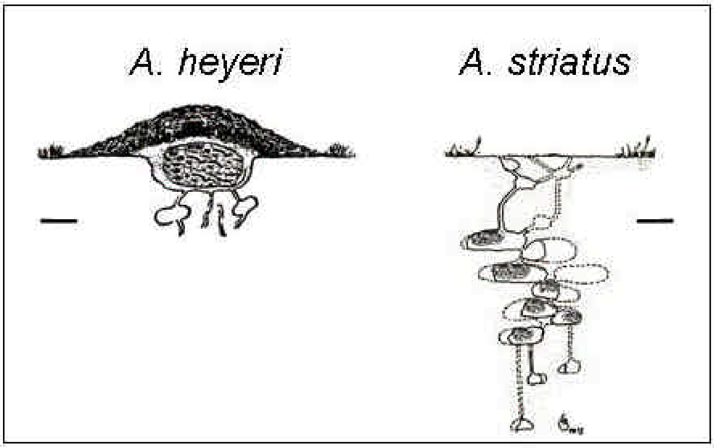

Figure 3.2: Examples of superficial and subterranean nest types in *Acromyrmex* leaf-cutting ants. Left side: the superficial nest built by *A. heyeri*, taken from Bonetto (1959). Right side: the subterranean nest excavated by *A. striatus*, taken from Carbonell (1943). The scale bar represents 10 cm.

3.3. Results

Soil temperature strongly influenced digging performance of *A. lundi* workers. Workers dug more in soils at 25°C than in soils at lower or higher temperatures (Fig. 3.3). The amount of soil excavated at both 20 and 30°C did not differ from the amount excavated at 25°C. Workers did not dig at all at 40°C, and at 10°C, the excavated amount was less than 20% of that excavated at 25°C (Fig 3.3, statistics at the figure caption).

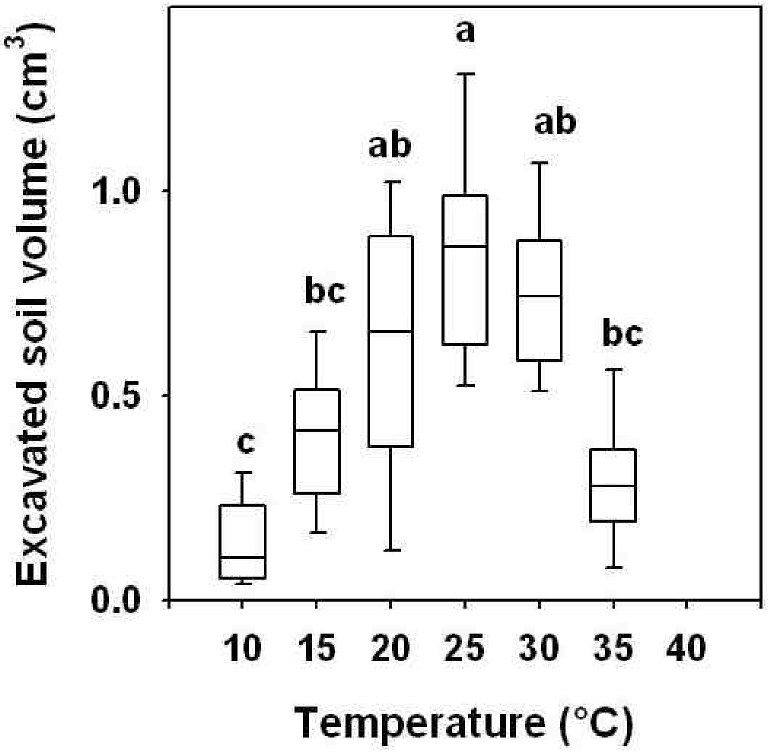

Figure 3.3: Digging performance measured as the volume excavated over 4 hours by five *A. lundi* workers in soils at different temperatures (Y-axis: median, box: 25%-75%, whisker 5%-95%) (Kruskal-Wallis-Test: H = 156.9, df = 6, p < 0.001, Post-hoc: Nemenyi-test, p<0.001). Values sharing the same letter are not statistically significant.

When presented with a choice between two constant temperatures, i.e., 25°C and one alternative, workers preferred to start digging in soils at 25°C over soils at 15 and 35°C, and there were no differences in preference in the range from 20 to 30°C (Fig. 3.4, statistics at the figure caption).

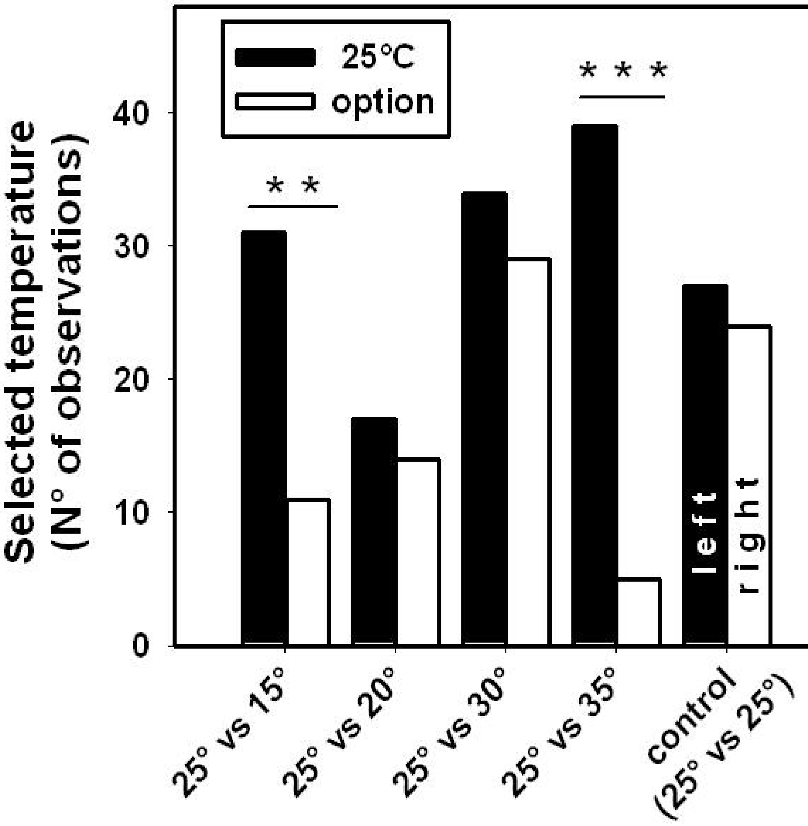

Figure 3.4: Soil temperature selected by groups of three *A. lundi* workers to start digging. Dual choice tests presenting both 25°C and an optional temperature (15, 20, 30 or 35°C) were performed, as indicated on the abscissa. The black bars show the preference for 25°C, and the white bars that for the optional temperature. The asterisks indicate the statistical significance at a p-level of 0.05 (*) or 0.001 (**), after a X^2-test. The control assays were aimed at controlling for side preferences (25°C vs. 25°C, “left” and “right” bars).

Workers clearly responded to changes in soil temperature experienced while digging, but qualitatively different to increasing and decreasing temperature. Figure 3.5 shows the number of soil pellets removed by workers every 5 minutes inside both the initial and the optional digging boxes, after the sliding door was opened.

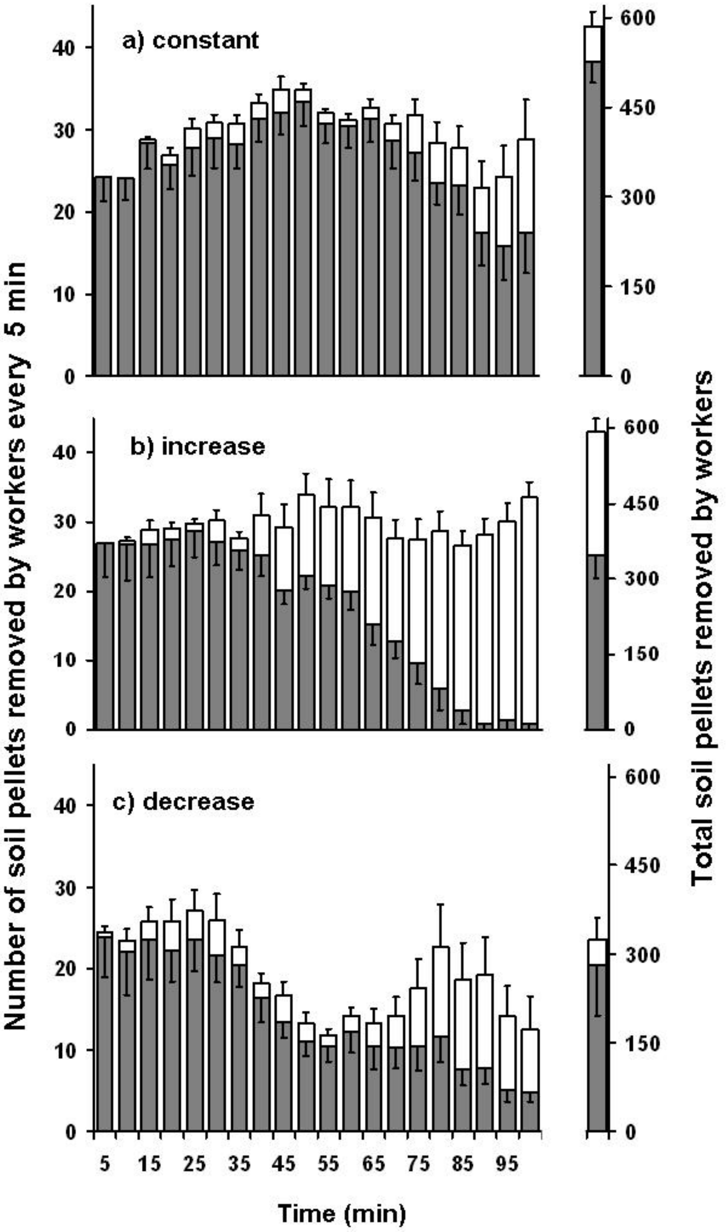

Figure 3.5: Workers responses to changes in soil temperature during digging. Number of soil pellets removed by workers either from the initial digging box (grey portion of the bars) or the optional digging box (white portion) every 5 minutes, over the 100 minutes of the experiments. **a)** Control experiments with constant temperature in both digging boxes, **b)** experiments with increasing soil temperature in the initial digging box, and constant in the optional one **c)** experiments with decreasing soil temperature in the initial digging box, and constant in the optional one. The bars at the right side represent the total digging activity in the initial and optional digging boxes. During the control series **(a)**, the soil and air temperature (mean ± SE, N=12) inside the initial box remained unchanged at 23.7 ± 0.04 and 24.0 ± 0.08°C. During the experiments with increasing soil temperature **(b)**, the starting soil and air temperature in the initial box were 24.3 ± 0.16 and 24.01 ± 0.17°C,while for the experiments with decreasing soil temperature **(c)**, they were 24.2 ± 0.16 and 24.03 ± 0.09°C, respectively. The measured rates of change in soil and air temperature inside the initial digging box during the experiment "**b**" were 0.10 ± 0.01 and 0.08 ± 0.007°C/min, while for the experiment "**c**" they were 0.10 ± 0.008 and 0.05 ± 0.006°C/min, respectively. During all series **(a,b,c)**, the soil and air temperature inside the optional box remained at 23.9 ± 0.03 and 24.3 ± 0.06°C.

During the control experiments with no temperature change, workers mostly continued digging at the initial digging place after the sliding door was opened (Fig. 3.5a). Only after 80 minutes workers dug at the optional digging place to some extent, yet digging activity was still much concentrated at the initial digging place. By the contrary, workers exposed to increasing temperature at the initial digging place moved to the optional digging place after a while (Fig. 3.5b), and their digging activity almost completely concentrated on the optional digging box at the end of the experiment. In the experiments with decreasing soil temperature (Fig. 3.5c), digging activity at the initial box decreased as temperature changed, but no concomitant increase of activity in the optional box was observed. As a consequence, the total digging activity was lower than that observed in the two previous experiments (Fig. 3.5, right ordinates, white plus grey portions of the bars) (One-way ANOVA, $F_{(34,2)}$ = 17.26, $p<0.001$, Post-hoc Scheffe Test at $p<0.001$). There was no difference in the total digging activity between the control series and the series with increasing temperatures (One-way ANOVA, $F_{(34,2)}$ = 17.26, $p<0.001$, Post-hoc Scheffe Test at $p<0.001$).

For the series with increasing temperatures, the average threshold temperature at which workers decided to give up and to change the digging place was evaluated by plotting digging activity in the initial digging box (as percentage of the total activity in the two boxes) as a function of the actual temperature (Fig. 3.6a). The sigmoidal fit (solid line) shows that the soil temperature at which digging activity decreased to 50% at the initial box was 30.3°C. Figure 3.6b presents the relationship between worker mortality and temperature established in the laboratory assays. The sigmoidal fit shows that the lethal air temperature (50% mortality) was 39.7°C.

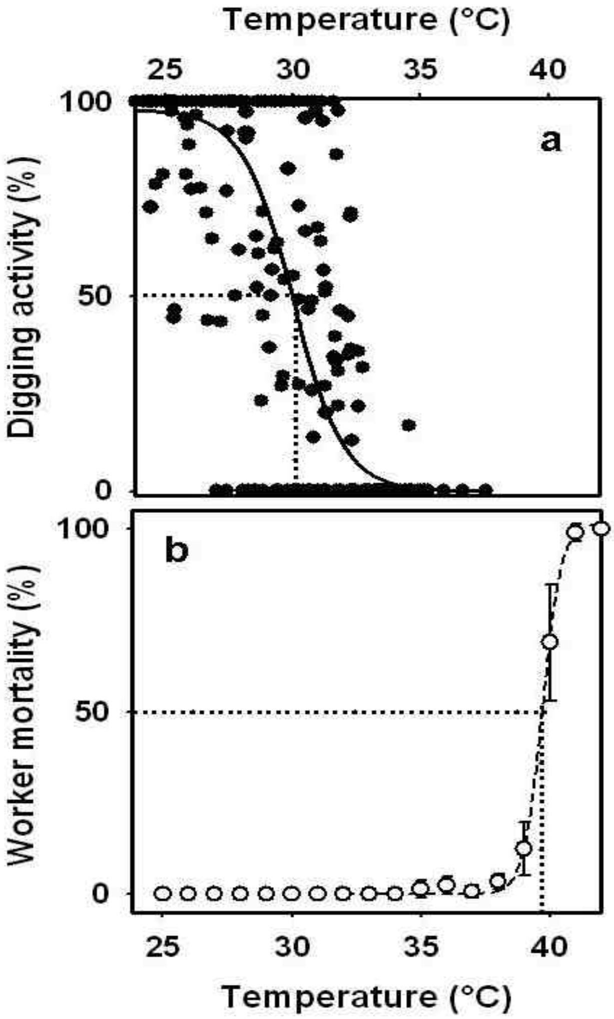

Figure 3.6: **a)** Temperature threshold for giving up digging activity at the initial digging box, as temperature increased. Each black point is the number of pellets removed from the initial box as a percentage of the total digging activity from both digging boxes, at the temperatures reached every 5 minutes (data from the grey/white bars, Fig. 3.5b). The continuous line represents the non-linear sigmoidal regression of digging activity on soil temperature (Digging activity (%) = 98.13 [1+exp(-(T(°C)-30.31)/-1.67)], R^2 = 0.59, p<0.001). The doted line shows the soil temperature at which digging activity fell to 50%. **b)** Worker mortality as a function of air temperature in 1°C-steps (N=7, ± SE). The dashed line represents the non-linear sigmoidal regression of worker mortality on air temperature (Mortality (%) = 101.63 [1+exp(-(T(°C)-39.72)/-0.36)], R^2 = 0.98, p<0.001). The doted line shows the air temperature at which worker mortality reached 50 %.

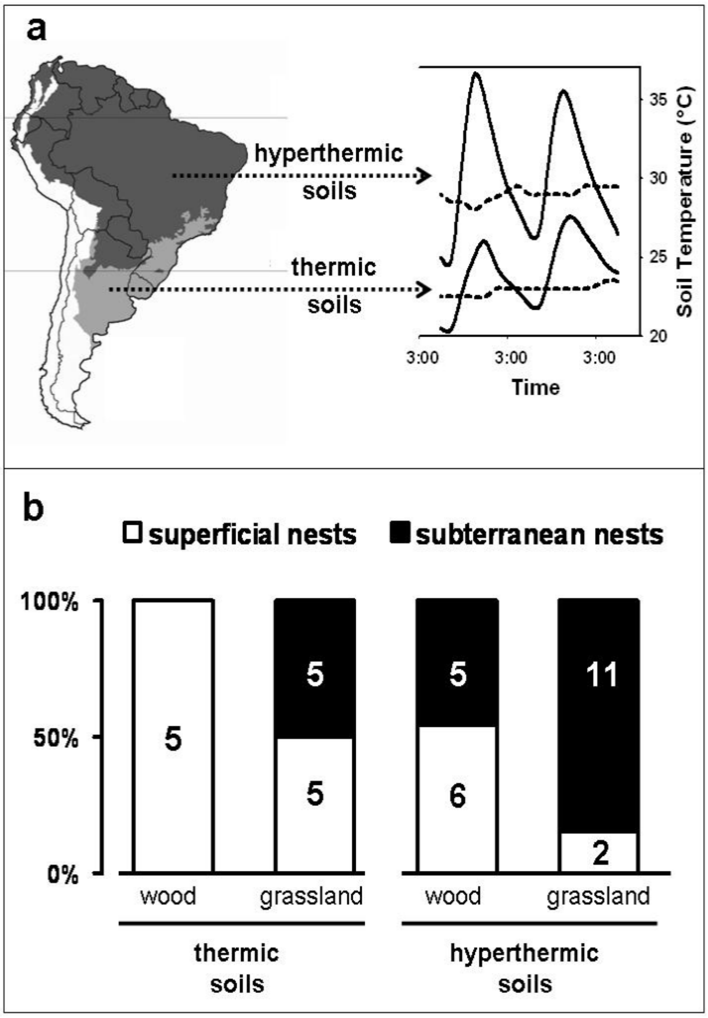

Figure 3.7: **a)** Left: map showing the distribution of thermic and hyperthermic soils in South America, partially based on the Global Soil Temperature Regimes map (USDA 2005). Right: soil temperature at 10 cm depth (continuous line) and 40 cm depth (dashed line) over 48 h of a hyperthermic and a thermic soil in spring 2005. Thermic soil: Julio Castilhos, RS, Brazil, 29°17’ S, 53°69’ W. Hyperthermic soil; Coaceral, BA, Brazil, 10°56’ S, 45°69’ W. Data obtained from the PCD Program (CPTEC 2006). **b)** Nest types recorded for 21 *Acromyrmex* species depending on soil temperature regime (thermic or hyperthermic soil) and vegetation cover (grass land or wood land) at the site of occurrence. Detailed information for each of the 21 species surveyed is given in the Table 1.

Figure 3.7a presents the distribution of thermic and hyperthermic soil temperature regimes in South America, and examples of the soil temperature variation over 48 hours at locations subjected to either a hyperthermic or a thermic regime. Figure 3.7b summarizes the nest types described for the South American *Acromyrmex* species, as well as the soil temperature regimes (thermic or hyperthermic soil) and vegetation cover (grassland or woodland) at the site of occurrence. Results show that the higher the average soil temperature, the higher the number of subterranean nests. In hyperthermic soils covered by grass lands, 11 species were recorded as having subterranean nests, and 2 with superficial nests, whereas in woodlands, 5 species presented subterranean and 6 superficial nests. In the case of thermic soils covered by grasslands, both nest types were equally frequent, while inside woodlands only superficial nests occurred (Names of the corresponding species and their associations with soils and vegetation covers are provided in Table 3.1).

	Hyperthermic Soils		**Thermic Soils**	
	open lands	**woodlands**	**open lands**	**woodlands**
A. ambiguus			subterranean	superficial
A. aspersus		subterranean	superficial	superficial
A. balzani	subterranean		subterranean	
A. coronatus		superficial		
A. crassispinus	subterranean	subterranean	superficial	superficial
A. diasi		superficial		
A. disciger		superficial		superficial
A. fracticornis	subterranean			
A. heyeri	superficial		superficial	
A. hispidus fallax	subterranean		subterranean	
A. hispidus			superficial	
A. histryx		superficial		
A. landolti	subterranean			
A. laticeps laticeps	subterranean			
A. lundi lundi	subterranean	superficial	subterranean	superficial
A. lobicornis	superficial		superficial	
A. niger	subterranean	subterranean		
A. octospinosus	subterranean	subterranean		
A. rugosus	subterranean	superficial		
A. striatus	subterranean		subterranean	
A. subterraneus		subterranean		

? superficial	**2 (15.4 %)**	**6 (54.5 %)**	**5 (50.0 %)**	**5 (100 %)**
? subterranean	**11 (84.6 %)**	**5 (45.5 %)**	**5 (50.0 %)**	**0 (0 %)**

Table 3.1: Nest types recorded for the 21 surveyed *Acromyrmex* species depending on soil temperature regime (thermic or hyperthermic soil) and vegetation cover (grass land or wood land) at the site of occurrence. The last two rows summarize the results, the number between parentheses indicate percentages. The publications considered for the different countries were: Argentina (Bonetto 1959; Farji-Brener 1994; Farji-Brener 2000; Kusnezov 1956; Kusnezov 1978), Brazil (De Andrade 1991; Fernandes Soares et al. 2006; Gonçalves 1961; Guerra de Gusmao 1998; Link et al. 2001a; Link et al. 2001b; Link et al. 2001c), Colombia (Lapointe et al. 1998), Paraguay (Fowler 1985; Fowler and Claver 1991), Venezuela (Espina and Timaure 1977; Navarro and Jaffé 1985), Uruguay (Carbonell 1943; Zolessi and Abenante 1973; Zolessi and González 1974; Zolessi and González 1978; Zolessi and Philippi 1998).

3.4. Discussion

3.4.1. Behavioural responses to soil temperature

The laboratory investigations clearly showed that *A. lundi* workers use soil temperature as an orientation cue to decide where to start digging, and respond to rising and falling soil temperatures by either moving to alternative digging places, or by stopping digging, respectively. This is the first experimental demonstration that temperature is used as a cue in the context of collective nest digging in ants. For leaf-cutting ants in particular, temperature has previously been shown to be used as a cue during brood and fungus relocation (chapter 2) (Bollazzi and Roces 2002) and even for food location (Kleineidam et al. 2007), indicating that temperature sensitivity in leaf-cutting ants is widespread and functional in different contexts.

What could be the effects of a temperature-sensitive digging activity on the location of underground ant nests? For ants in general, young colonies seem to enlarge their nests by preferentially adding chambers at the upper soil layers, thus resulting in a top-heavy distribution of nest volume regarding soil depth (Tschinkel 2003). This distribution pattern is known for several ant species, such as *Acromyrmex landolti*, *Lasius flavus*, *Pogonomyrmex badius, P. montanus, P. subnitidus, P. rugosus, Prenolepis imparis, Formica pallidefulva, F. exsectoides, F. yessensis, Solenopsis invicta, Pheidole morrissi*, *Odontomachus sp* and *Chonomyrma sp* (Bristow et al. 1992; Dlussky 1981; Ito 1973; Lapointe et al. 1998; Markin et al. 1973; Mikheyev and Tschinkel 2004; Tschinkel 1999; Tschinkel 2003; Tschinkel 2004). It has been suggested that nest’s volume distribution in underground nests might be associated to an environmental factor found in the soil (Mikheyev and Tschinkel 2004), because the main determinants of the soil environment such as temperature, moisture and air composition strongly vary with soil depth (Campbell 1977; Hillel 1998; Rosenberg et al. 1983). Based on the present results, it is suggested that the depth at which *Acromyrmex* nests are located largely depends on the occurrence across the soil profile of the temperature range preferred by workers for digging.

In *Acromyrmex*, nest excavation begins as a superficial founding chamber excavated by the mated queen at 5 to 20 cm depth, depending on the species considered (Bruch 1923; Camargo et al. 2004; Montenegro 1973). Whether workers excavate afterwards a subterranean or a superficial nest during colony ontogeny will depend on the soil temperature regimes they experience during colony growth. For instance, in open lands of tropical areas, where soil temperatures in the first centimeters can largely exceed 30°C during daily oscillations (Passerat de Silans et al. 2006) (Fig. 3.7), workers are expected to dig downwards to avoid these extreme temperatures. The results support this view, because workers avoided a soil at 35°C to start digging, yet started in a soil at 25°C. In addition, workers engaged in digging in a soil with increasing temperature stopped digging between 30 and 32°C, but continued digging at an alternative location at 25°C without diminishing their digging activity. Across the soil profile, it would have the consequence that workers' digging effort is directed downwards, because workers tend to leave the superficial soil layers with continuously changing and extreme temperature values.

But the avoidance of the superficial soil layers during nest enlargement, which might occur in colonies inhabiting hyperthermic soils covered by grasslands, is expected to be not too strong in hyperthermic soils from woodlands or in thermic soils. There, the location of the founding chamber in the upper soil layers already offers a suitable temperature range for colony survival, and temperatures are not expected to reach extremely high values (Fig. 3.7). Therefore, workers should be able to enlarge the nest without the need to go deeper into the soil profile, where soil temperature is probably lower than near the surface. This idea is also supported by the obtained results. Workers avoided to start digging in soils at 15°C, and when confronted with decreasing soil temperatures, diminished digging activity and did not show a tendency to change digging places. As a consequence, *Acromyrmex* superficial nests would be expected to be composed of one single large chamber because of the concentration of digging activity at a single place, and this is indeed the fact for 9 species with the only exception of *A. lobicornis,* which excavates multiple small chambers inside a solid mound constructed with soil and debris.

In the same line of arguments, growth of subterranean *Acromyrmex* nests should result from the addition of new discrete units, the fungus chambers, probably excavated around an incipient fungus garden initially placed in a tunnel (see discussion below). As a consequence, mature *Acromyrmex* subterranean nests are expected to be composed of more or less vertical tunnels with multiple fungus chambers attached to them. This general nest arrangement has been observed in all *Acromyrmex* subterranean nests (Bonetto 1959; Fowler 1985; Gonçalves 1961; Lapointe et al. 1998), and it is a common characteristic of ant subterranean nests (Tschinkel 2003). If colonies tend to avoid extreme soil temperature oscillations during nest growth, most nest chambers of those *Acromyrmex* species inhabiting hyperthermic soils should be located below 50 cm depth, since the daily temperature variation at this depth is strongly attenuated in most soil types (Campbell 1977). However, at which soil depth thermal variations are damped depends on soil physical properties such as thermal conductivity and specific heat, which largely vary with soil particle size and water content (Campbell 1977; Hillel 1998; Rosenberg et al. 1983). The occurrence of empty nest chambers above 50 cm depth (Guerra de Gusmao 1998; Lapointe et al. 1998; Weber 1972), suggests that they were excavated early during colony ontogeny and then abandoned as soon as deeper and so most suitable fungus chambers were excavated.

The determination of nest depth in mature colonies, besides its expected dependence on soil temperature as discussed above, might also be influenced by colony size. Since it has been demonstrated in ants that the excavated nest volume depends on colony size (Buhl et al. 2005; Deneubourg and Franks 1995; Halley et al. 2005; Rasse and Deneubourg 2001), it could be argued that colonies with a single nest chamber will simply go deeper as they grow because of the increase in chamber size. If so, inter- and intraspecific differences in nest depth could merely be regarded as a by-product of differences in colony sizes. Evidence for *Acromyrmex* leaf-cutting ants, however, does not support this view. For instance, maximal nest depth in colonies of *A. rugosus* inhabiting hyperthermic soils in north Brazil is not related to colony size (Fernandes Soares et al. 2006). In addition, while *A. rugosus* colonies excavate subterranean nests, *A. heyeri* colonies build superficial nests on the thermic soils of south Brazil, even though they have a worker population ten times higher than colonies of *A. rugosus* (Diehl-Fleig and Droste 1992; Guerra de Gusmao 1998). These observations also agree with results obtained by Mackay (1981) in three *Pogonomyrmex* species, and by Mikheyev and Tschinkel (2004) in *Formica pallidefulva*, in which colony size and nest depth were not correlated.

Finally, it has been shown that ant colonies belonging to those species inhabiting temperate areas are larger than those occurring in tropical areas (Kaspari and Vargo 1995). Assuming this trend for *Acromyrmex* species, and considering that nest depth would depend on colony size as indicated above, *Acromyrmex* nests should occur deeper in temperate areas than in tropical ones. However, the obtained results did not support this hypothesis, since the deeper nests were usually recorded in the tropical areas, where hyperthermic soils are predominant, and the shallower nests in the temperate areas, where thermic soils are predominant.

3.4.2. The determination of nest depth in *Acromyrmex*

The idea that nest-depth determination in the genus *Acromyrmex* depends on the soil depth at which the preferred temperatures for digging are found, supported by the present laboratory investigations in *A. lundi*, is further reinforced by the results obtained from the bibliographic survey on the nesting habits of South American *Acromyrmex* species. They suggest that soil temperature influences nest depth, since the warmer the soil temperature, either as a consequence of soil temperature regime or vegetation cover, the higher the number of surveyed species having subterranean nests compared with superficial ones. For instance, 85% of the *Acromyrmex* species inhabiting hyperthermic soils covered by grasslands, which are expected to have the hottest superficial soil layers, dig subterranean nests. By the contrary, *Acromyrmex* species only inhabit superficial nests in thermic soils covered by woodlands, which are expected to have colder deep soil layers. However, both nest types occur in hyperthermic soils covered by woodlands, or thermic soils covered by grasslands, both having mild superficial soil layers. Taken together, these results indicate that the deeper the temperature range preferred by workers to dig is located across the soil profile, the deeper the location of the nests in the different *Acromyrmex* species. Such a trend, however, does not preclude the existence of further mechanisms, yet unknown, influencing the determination of nest depth. It is also an open question why some *Acromyrmex* species build the same nest type irrespective of the soil temperature regime, as *A. balzani, A. fracticornis, A. heyeri, A. hispidus fallax, A. lobicornis,* and *A. striatus,* (Table 3.1).

Soil moisture may intuitively be expected to play a role in influencing nest depth, as indicated in the introduction for desert ants. For leaf-cutting ants, Lapointe and Serrano (1998) suggested that soil moisture has also an effect on the determination of nest depth in *Acromyrmex landolti*. They found that the depth of underground nests was negatively correlated with soil moisture content, yet colonies were never observed to build superficial nests. In *A. crassispinus*, on the other hand, intraspecific variation in nest types seems to strongly depend on soil temperature regimes and not on soil moisture content. Colonies build subterranean nests in the hot soils of Paraguay, but superficial nests in the colder thermic soils of central Argentina and south Brazil (Bonetto 1959; Fowler 1985; Gonçalves 1961; Guerra de Gusmao 1998; Link et al. 2001c), although both regions present the same soil moisture regime (Van Wambeke 1981), i.e., a measure of how many days soils remain dry throughout a year (USDA 1975). In soils, the extent of desiccation as a function of depth largely depends on soil temperature (Hillel 1998; USDA 1975), and furthermore, soil moisture content affects soil temperature (Hillel 1998; Rosenberg et al. 1983). Therefore, it would be in general difficult to dissect out the effects of moisture and temperature on nest depth in the field, since both variables are highly associated and also depend on other soil characteristics, such as soil particle size (Hillel 1998).

Reports on intraspecific variation in nest types from two other *Acromyrmex* species further support the idea that it strongly depends on the soil temperature regimes. *Acromyrmex rugosus,* a typical inhabitant of hyperthermic soils, has been recorded as having subterranean nests in open lands, but inhabiting shallower nests between the root system of trees in woodlands, where soil temperatures are expected to be milder (Fowler 1985). The same pattern has been observed for *A. lundi* and *A ambiguus* inhabiting either hyperthermic or thermic soils in Argentina, Brazil, Uruguay and Paraguay (Bonetto 1959; Fowler 1985; Gonçalves 1961; Zolessi and González 1978). Interestingly, intraspecific variation in nesting habits as a function of soil temperature is not exclusive of *Acromyrmex* leaf-cutting ants. The North American ant *Prenolepis imparis*, for instance, dig subterranean nests 3 times deeper in the hyperthermic soils of Florida (Tschinkel 1987) than in the thermic and mesic soils of Missouri and Ohio (Talbot 1943a; Talbot 1943b; USDA 2005). Thus, the fact that soil temperature also seems to influence nest depth in *Prenolepis imparis* (Tschinkel 1987) suggests that its use as a cue to determine nest depth would be widespread in ants.

3.4.3 Nest depth as an adaptation for the maintenance of proper nest temperatures

In ants, it has already been demonstrated that colonies are highly dependent on proper temperatures for their growth (Brian 1973; Elmes and Wardlaw 1983; Pontin 1960; Porter 1988; Southerland 1988), survival (Callcott et al. 2000; Korzukhin et al. 2001; Pontin 1963) and offspring production (Brian and Brian 1951; Markin et al. 1974). For instance, mean annual air temperature, which is highly related to soil temperature (Van Wambeke 1981), is the most important variable influencing occurrence and density of Paraguayan leaf-cutting ants (Fowler 1983a). Therefore, temperature can be regarded as an important selective force on the mechanisms leading to the determination of nest depth by workers in *Acromyrmex* species. The Attini ant-fungus mutualism originated in South America during the early Cenozoic, 45-65 million of years ago (Mueller et al. 2001), when South American climate was warm, wet and non-seasonal (Ortiz-Jaureguizar and Cladera 2006). While these conditions remained unchanged in northern South America, in regions southern than 15°S the climate became dryer and seasonal, and the biomes changed from tropical forests to steppes (Ortiz-Jaureguizar and Cladera 2006). Leaf cutting-ant colonies, which rely on fungus growing for survival, are therefore expected to have developed adaptations to maintain their symbiotic fungus under proper climatic conditions, for instance by using soil temperature for the determination of nest depth. The adaptive value of nest-depth determination is evidenced by the fact that those species with mound (superficial) nests have more southerly limits than those species inhabiting subterranean nests (Farji-Brener 2000). The threshold temperature to give up a digging place when temperature increased, i.e., 30°C as determined in this study, also emphasizes the adaptive value of using soil temperature for the determination of nest depth. This temperature was not lethal at all for workers, but it matched the temperature at which brood development in ants starts being negatively affected, as reported for two common South American ants from subtropical and temperate areas, *Camponotus mus* and *Solenopsis invicta* (Porter 1988; Porter and Tschinkel 1993; Roces and Núñez 1989). Temperatures above 30°C were in addition found to be lethal for *in vitro* cultivars of the symbiotic fungus *Attamyces bromatificus* isolated from colonies of *Acromyrmex octospinosus*, *Atta cephalotes* and *Trachymyrmex urichi* (Powell and Stradling 1986). Therefore, workers seem to avoid, when digging, the soil temperature range that is unsuitable for both fungus and brood development. It suggests that fungus and brood may play an important role in influencing the determination of nest depth. In *Acromyrmex heyeri*, workers confronted with a temperature gradient ranging from 10 to 37°C prefer a temperature of ca. 24°C to locate brood and fungus (chapter 2) (Bollazzi and Roces 2002). Considering this thermal preference, it is conceivable that during nest enlargement workers first relocate fungus and brood to nest tunnels that provide the range of preferred temperatures, and afterwards, brood and fungus might stimulate further digging at such location, leading to the formation of a fungus chamber.

Although nest depth has been seen as an adaptation aimed at achieving a suitable nest microclimate (Dlussky 1968; Hansell 2005; Seeley and Heinrich 1981; Sudd and Franks 1987), it was up to date unknown how ant colonies were able to adjust nest depth to local environmental conditions. Temperature-sensitive digging behaviour in *Acromyrmex* would therefore help colonies to achieve a proper nest climate for fungus and brood development, and so to maximize colony growth rates.

4. Adapting to temperate climate through building behaviour: comparative thermal biology in thatched and subterranean grass-cutting ant nests (*Acromyrmex heyeri*)

Summary In South American temperate areas, sympatrically-occurring colonies of the grass-cutting ant *Acromyrmex heyeri* inhabit one of two different nest types, either thatched or subterranean nests, offering the unique opportunity to comparatively investigate whether ant colonies adapt to temperate climate by building thatched nests. This work was aimed at assessing the benefits that *A. heyeri* colonies achieve through their building behavior in terms of thermoregulation by *i)* comparing thermal relations with the environment in neighbor colonies inhabiting both nest types, *ii)* studying how the thermal properties of the material used by workers to build the thatch influences such thermal relations. Results showed that temperature in subterranean nests did not differ from that of the environment, but it was significantly lower that that of thatched nests. The temperature surplus in thatched nests was higher in spring, at the time of production of sexual brood, than in winter or summer. Such surplus was brought about by the low thermal diffusivity of a nest thatch built with plant fragments. It prevents diurnal nest overheating by the incoming solar radiation, and avoids losses of the accumulated daily heat into the cold air during night, thus leading to high average nest temperatures. Throughout the year, the extent to which daily maxima of the thatched nests exceeded that of the soil continuously increased until maximal soil temperatures around 24°C, and then decreased, being lower for maximal soil temperatures beyond 31°C. This emphasizes the importance of workers' building behaviour for ants inhabiting temperate regions, since temperatures between 20 and 25°C maximizes the growth of the symbiotic fungus cultivated by leaf-cutting ants, and those higher than 30°C are lethal. Building a thatched nest, as opposed to excavate underground chambers, enables *A. heyeri* colonies to thermoregulate within the range of proper temperatures for colony development.

4.1. Introduction

In ants, the construction of thatched, mound-shaped nests has commonly been considered as a behavioural adaptation to low environmental temperatures, since inside-mound temperatures are more stable, and usually higher, than those of the environment (Frouz 1996; Frouz 2000; see reviews in: Heinrich 1993b; Seeley and Heinrich 1981). Although there exists extensive information regarding nest temperatures in thatching ants, all studies so far refer to species inhabiting the northern hemisphere, particularly wood ants of the genus *Formica*. However, thatching ants are also present in the southern hemisphere, and include for instance several species of the South American leaf-cutting ant genus *Acromyrmex* (Bonetto 1959; Fowler and Claver 1991; Gonçalves 1961; Weber 1972). In the thatched nests of *Acromyrmex,* the fungus gardens are located at the soil surface level and covered by a thatch made of plant fragments (Fig. 4.1). Thatched nests built by *Acromyrmex* have also been discussed as an adaptation to low environmental temperatures, since those species that build a thatch have more southerly distribution than those inhabiting subterranean nests (Farji-Brener 2000). Thus, *Acromyrmex* thatched nests in the southern hemisphere appear to have the same function as the *Formica* mound-shaped nests in the northern hemisphere (Seeley and Heinrich 1981), i.e., the thatch helps the colony to achieve higher and more stables temperatures than those of the environment. In *Formica*, such temperature surplus inside the nest is supposed to result from the interplay between an internal source of heat, which appears to be related to the presence of colony members, and the insulating properties of the thatch, while the contribution of solar radiation is unclear (Frouz 1996; Frouz 2000; see reviews in: Gösswald 1989; Heinrich 1993b; Seeley and Heinrich 1981).

Unambiguous evidence about the thermoregulatory benefits provided by the construction of a thatched nest, compared to living in an underground nest, could ideally be obtained by comparing colonies of the same species that facultatively inhabit the two different nest types under otherwise similar environmental conditions. Three different *Acromyrmex* leaf-cutting ant species, *A. ambiguus, A. heyeri,* and *A. crassispinus,* have commonly been reported as having either a thatched or a subterranean nest type (Diehl-Fleig and Droste 1992; Fowler 1985; Gonçalves 1961; Guerra de Gusmao 1998). They offer the unique opportunity to perform intraspecific comparisons of the colony's thermal biology in relation to the nest type inhabited, and to explore how a specific nest structure, the nest thatch, influences heat exchanges with the environment.

This study was therefore aimed at elucidating whether the construction of thatched nests in *Acromyrmex* leaf-cutting ants represents a thermoregulatory adaptation to the climatic conditions occurring in the South American temperate regions. For that, it was quantified the thermal relations between the environment and the fungus garden for both thatched and subterranean nests of the grass-cutting ant *Acromyrmex heyeri,* and evaluated the influence of the incoming solar radiation and the colony's inhabitants on nest temperature. For thatched nests, it was furthermore investigated how the thermal properties of the thatch material influenced the heat exchanges with the environment.

4.2. Methods

The present study was performed on colonies of the grass-cutting ant *Acromyrmex heyeri*, which occur in temperate grasslands of southern South America (Fowler 1988). *A. heyeri* colonies usually build a thatched, mound-shaped nest with grass fragments, but have also been reported as inhabiting subterranean nests consisting of a single fungus chamber excavated in the soil (Diehl-Fleig and Droste 1992). A schematic representation of both nest types is given in Fig. 4.1. A thatched nest is referred here as an above-ground structure constructed with collected plant material and soil particles on the nesting site, which is permeated by galleries and chambers and inhabited by the colony. It differs from uninhabited mounds in a number of ant species that result from the deposition of the excavated soil. In leaf-cutting ants, the thatch material is arranged by workers so as to form a chamber inside which the fungus garden is cultivated. Thatch and fungus garden are separated structures, i.e., the fungus it is not cultivated inside the thatch, yet the thatch covers the fungus garden like a roof.

All field measurements were carried out on neighbor subterranean and thatched nests of *A. heyeri* located in a farm in Joanico, south Uruguay (34° 33´ 26'' S; 56° 15' 59'' W), from June 2001 to February 2005. The colonies were located in cattle stockyards covered by short grasses and bushes. Only colonies that were not covered by bushes, i.e., fully exposed to the sun, were used during the study.

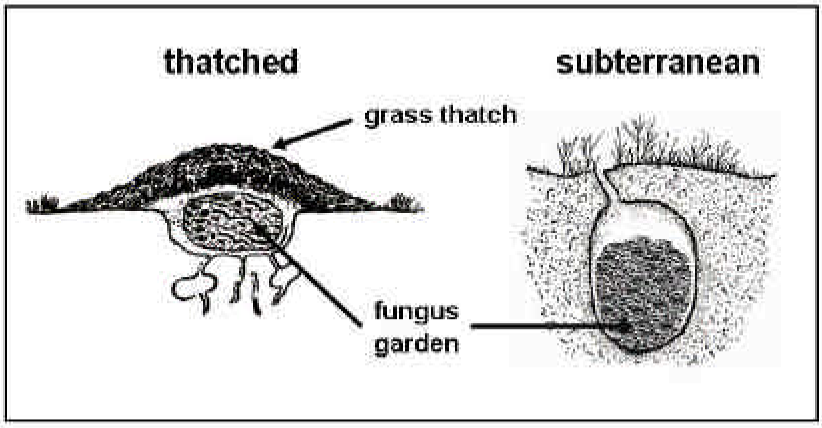

Figure 4.1: Schematic drawing of a thatched and a subterranean nest of *Acromyrmex heyeri* as investigated in the present study. The thatched nest is partially based on Bonetto (1959).

4.2.1. Fungus garden temperature in both thatched and subterranean nests

In order to quantify the thermal relations between the nest and the environment, temperature was simultaneously recorded in the fungus chamber of colonies inhabiting the two different nest types, thatched and subterranean, and correlated with parallel measurements of soil and air temperatures.

Temperature measurements were performed during the years 2001 and 2002. Each year, a different pair of one thatched and one subterranean nest was simultaneously monitored. Subterranean nests revealed to be scarce, so that only one mid-size subterranean nest could be found in the study area each year. Measurements were performed continuously over two periods: from June 27th 2001 until January 24th 2002, and from July 25th until December 23rd 2002. For both nest types, fungus garden temperature was measured in the central portion of the fungus garden. Air temperature was measured at 1 m height, and soil temperature at 2-3 cm depth. In all cases, temperatures were recorded every 30 minutes using Tinytag® data loggers. Data was downloaded to a notebook every week.

Mean soil temperatures at 50 cm depth, a maximal limit usually observed in subterranean *A. heyeri* nests (Bonetto 1959; Diehl-Fleig and Droste 1992; Gonçalves 1961), were calculated for the study area for winter, spring and summer. For that, the Newhall Simulation Model used by Van Wambeke (1981) to determine soil temperature regimes of South America was applied. Details about the calculations are given in the Appendix (4.A.1).

4.2.2. The effect of incoming solar radiation and colony presence on fungus temperature of thatched nests

In order to know to what extent the temperature inside thatched nests is related to the incoming solar radiation, the correlation between fungus garden temperature and the daily accumulated income of direct solar radiation was evaluated. For the two years of measurements, the daily accumulated income of direct solar radiation (kJ/day/cm^2) was estimated using the Angström equation modified by Page (Goswami et al. 2000). Details about the calculations are given in the Appendix (4.A.2).

The effect of incoming solar radiation on fungus garden temperature in thatched nests was also experimentally evaluated by comparing fungus chamber temperature in experimentally-shaded nests, with the temperatures measured before and after shading. Four thatched nests were covered by a wooden roof for 20 days, using an 80 x 80 cm wooden plate placed 15 cm above the mound top. A 30 cm-thick layer of dry plants was placed on the wooden plate to avoid its heating by sunshine. Fungus chamber temperature was measured one week before the roof was installed, during shading, and one week after removal of the roof.

The contribution of the colony (fungus garden and ants) to the observed nest temperatures was evaluated by measuring nest temperatures before and after the colony was poisoned. For that, four thatched nests were first covered by a roof for 20 days as described above, to control for the effects of solar radiation. Simultaneously with the placement of the roof, the colonies were poisoned using a commercial citrus-pulp-based bait containing 0.03% Sulfluramid®. This experiment was carried out as a part of the annual pest management performed by local farmers during the summer 2003. Two days after being poisoned, colonies stopped cutting grasses and died after one week, yet the thatch structure remained undamaged. Fungus chamber temperature was measured during one week before the roof was placed, i.e., on untreated colonies, over 20 days after the poisoning, in which nests were shaded, and during a final week after removal of the roof.

4.2.3. Thermal properties of thatch and soil

The thermal diffusivity of the nest thatch was measured to evaluate how the thatch influences the heat flows between the colony and the environment. The thermal diffusivity of a given material is the ratio between the heat it conduces, which depends on its thermal conductivity (k), and the amount of heat it stores per unit volume, which depends on its heat capacity (C). The larger the thermal diffusivity, the faster the propagation of heat into the medium (Cengel 2003). Therefore, if the thatch material facilitates heat exchange between the colony and the environment, a high thermal diffusivity of thatch material should be expected. By the contrary, a low thermal diffusivity should be expected if the thatch material limits heat exchanges. Moreover, thermal diffusivity of the thatch ultimately depends on the material that *A. heyeri* workers select to build the thatch, either soil or grass fragments. In order to explore how material selection by workers may influence thatch's thermal properties, the thermal diffusivity of the soil surrounding the nest was also determined, and compared to that of the thatch built by workers using grass fragments.

In order to measure the thermal diffusivity of the thatch, ca. 500 cm^3 of thatch material were collected every 2 days from the upper portion of different thatched nests during February 2005. It was done for two weeks, totalizing 8 thatch samples. Simultaneously, samples of the soil surrounding the nests were collected. Each of these soil samples consisted of a mixture of ca. 30 cm^3 taken at 2, 10, 20 and 40 cm depth. Immediately after collection, water mass content of all thatch and soil samples was measured using a gravimetric method (Smith and Mullins 2001). Since water content influences thermal diffusivity (Campbell 1977), the relationship between thermal diffusivity and water content of the thatch material and surrounding soil was obtained. Therefore, soil and thatch samples were milled and dried for 1 week at 50°C. Thermal diffusivity was measured in the dried samples, assigned as having 0% water mass content. Afterwards, water was added to the samples until they successively reached 10, 20 and 30% water mass content. Thermal diffusivity was successively measured in the probes with different water contents. A Dual-Probe Heat Capacity Sensor (DPHC) (Campbell et al. 1991) was constructed for the measurements. Details about the construction of the DPHC and methodology are given in the Appendix (4.A.3).

4.3. Results

4.3.1. Fungus garden temperature: seasonal comparisons between thatched and subterranean nests

During winter, daily mean fungus garden temperature in both nest types slightly differed from that of the soil (Fig. 4.2, upper portion) or of the air (Fig. 4.3, upper portion), with most measurements lying directly on the isotherms relating fungus vs. soil or air temperature. Nevertheless, fungus garden temperature in thatched nests was in average 2-3°C higher than in the subterranean nests (Fig. 4.2, upper portion).

In spring, fungus garden temperature inside the thatched nest was clearly higher than that of the soil and the air. By the contrary, fungus temperature inside the subterranean nest did not differ so markedly from that of the environment (soil or air, Figs. 4.2 and 4.3, middle portion). As a consequence, the temperature of the thatched fungus was in average 6°C higher than that of the subterranean fungus. It reached 26.8°C (N=90, SD=2.5) in spring 2001, and 25.1°C (N=21, SD=2.6) in spring 2002, exceeding the temperature recorded in the subterranean fungus, 20.9°C (N=90, SD=2.8) and 19.4°C (N=21, SD=2.6), respectively (Figure 4.2, middle portion). It is important to indicate that spring 2002 was considered to extend until October 12th, because the colony inhabiting the subterranean nest started to build a thatch. The following days were included in the summer period for analysis, so as to differentiate between the periods in which the colony inhabited a subterranean nest and a partially-constructed thatched nest. Detailed descriptions of nest morphology and the change of the subterranean nest into a thatched one are given in the Appendix (4.A.4).

In both years, alate sexuals were observed from October in the monitored thatched nests, but not in the subterranean nests. Three nuptial flights were recorded in year 2001 and two in 2002. All of them occurred during the first week of November in the first morning hours, agreeing with observations by Diehl-Fleig (1993).

In summer, the daily mean fungus garden temperature in the thatched nests was in average 2°C higher than that of the subterranean nests (Figure 4.2, lower portion). In year 2002 the colony inhabiting the subterranean nest started to build a thatch, and it was observed that its fungus garden temperature turned out to be higher than that of the environment. This is evident at the lower right portion of the figures 4.2 and 4.3, because the values corresponding to the partially-constructed thatched nest (black circles) are closer to those of the thatched nest (white circles), compared to the previous seasons. However, the temperatures recorded in the partially-constructed thatched nest were still lower than those of the thatched nest.

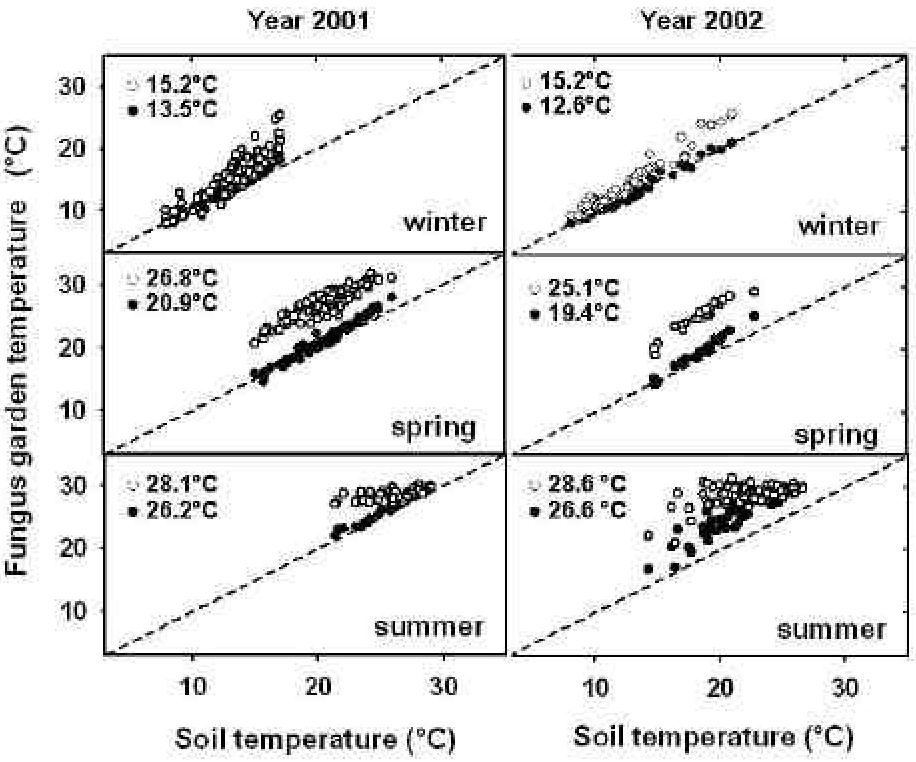

Figure 4.2: Fungus garden temperature as a function of soil temperature in thatched nests (white circles), and subterranean nests (black circles). Each point represents a daily mean. Left: year 2001. Right: year 2002. The diagonal dashed line represents the isotherm of fungus garden temperature versus daily mean soil temperature. The numerical values in each graph are the average daily mean temperature for the corresponding season.

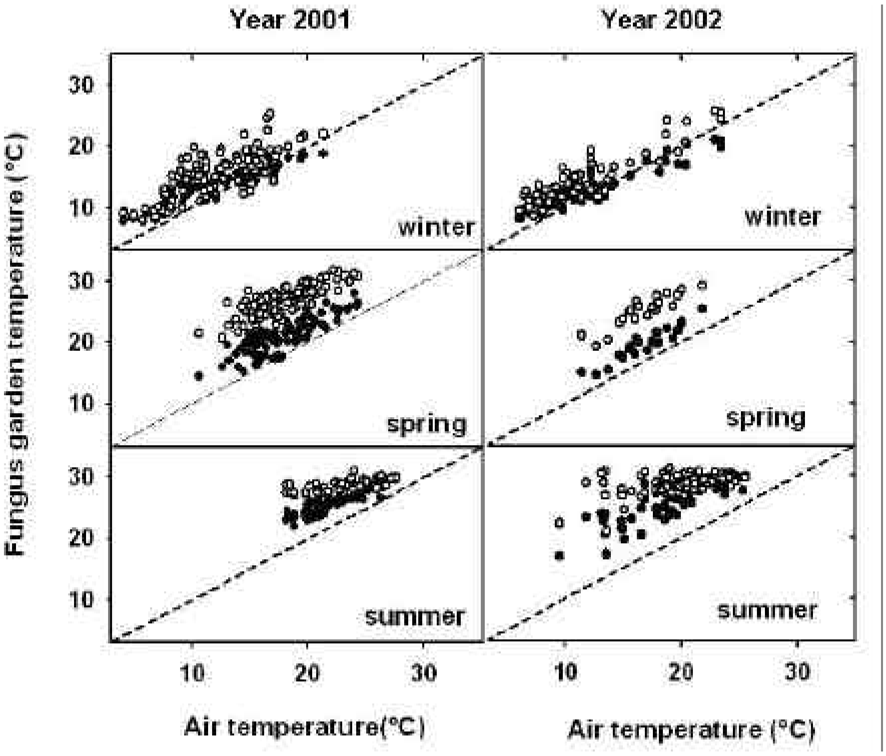

Figure 4.3: Fungus garden temperature as a function of air temperature in thatched nests (white circles), and subterranean nests (black circles). Left: year 2001. Right: year 2002. Each point represents a daily mean. The diagonal dashed line represents the isotherm of fungus garden temperature versus daily mean air temperature.

4.3.2. The effect of incoming solar radiation on daily changes of fungus garden temperature in both nest types

Daily mean temperature in both nest types, as shown in the figs. 4.2 and 4.3, is expected to depend on the daily maximum and minimum reached by the environment and nest exposition to these temperatures. Figure 4.4 presents examples of the daily variation of temperature over 48 h, as it was recorded for field nests in winter, spring and summer 2001. While the temperatures in the subterranean fungus roughly corresponded to that the soil, the thatched fungus showed a different pattern. The amplitude of the daily temperature changes was observed to decrease from spring to summer, with daily maximal temperatures below those of the soil, the air and the subterranean fungus.

The daily variation of temperature depicted in Fig. 4.4 for both nest types and the soil depends on the magnitude of heat gains and losses throughout the day. During the day, the incoming solar radiation drives the temperature of the soil and both nest types until its daily maximum. At night, the rate at which the gained heat is lost to the cooler environment (the air) determines the minimal temperature reached at the beginning of the following day (next-day minimum).

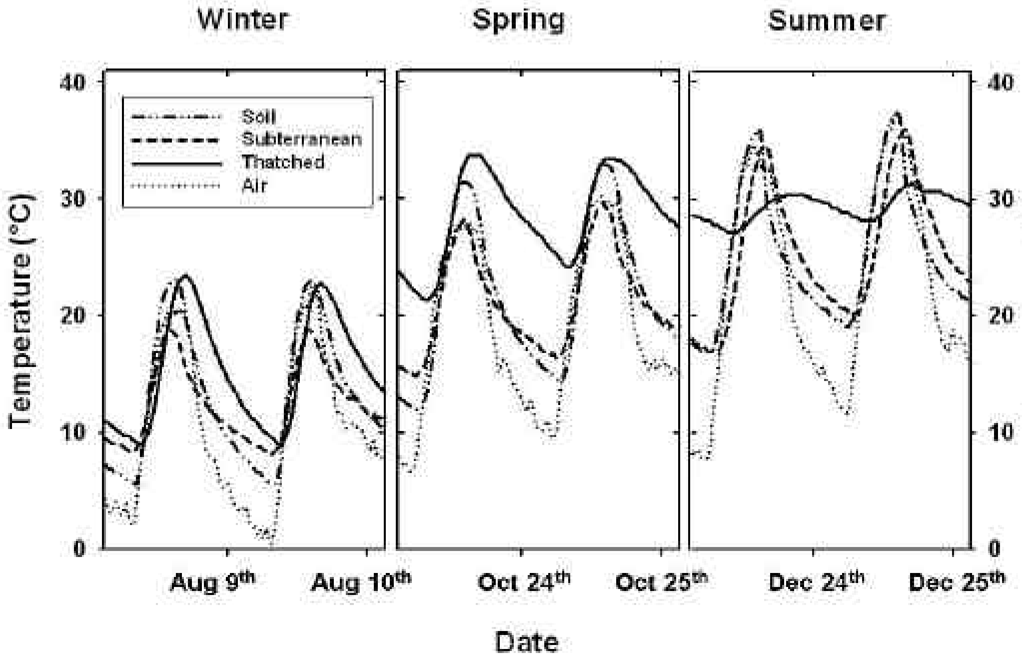

Figure 4.4: Examples of temperature variation in fungus gardens of a thatched and a subterranean nest, as well as in the soil, over 48 h in winter, spring and summer 2001.

Being more exposed, a thatched nest is a priori expected to experience steeper heat gains than the subterranean nest, as well as a faster loss of any gained heat at night. Figure 4.5 presents the measured rates of temperature change, both increasing and decreasing, as a function of the daily accumulated solar radiation, for the soil and the two nest types. Contrary to the assumption formulated above, the higher the incoming solar radiation, the lower the rate at which the thatched fungus gained heat during the day, compared to the subterranean fungus and the soil (Figure 4.5, upper portion). Furthermore, the thatched fungus lost its gained heat at night at a slower rate than the subterranean fungus and the soil (Figure 4.5, lower portion).

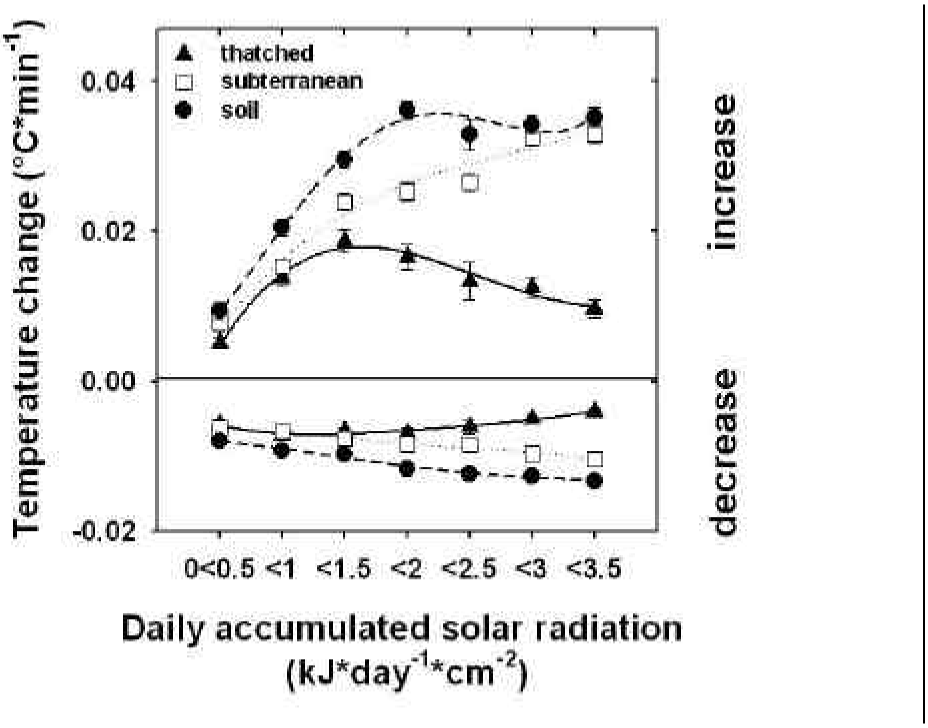

Figure 4.5: The rate at which temperature increased between daily minima and maxima at day (upper portion), and decreased between daily maxima and next-day minima at night (lower portion), in the thatched fungus (?), the subterranean fungus (?), and the soil (?), as a function of daily accumulated solar radiation (categories). Data from 2001 and 2002 were pooled, since Figs. 4.2 and 4.3 showed that the thermal relations between both nest types and the environment were similar in both years.

The effect of the lower rates of heat gain and loss, as exhibited by the thatched fungus regarding the subterranean fungus and soil, on daily nest temperatures is explored in detail in the Fig. 4.6. It depicts the daily mean, next-day minimum and maximum as a function of the daily accumulated solar radiation, for the two nest types and the soil. All these relationships were positive (Fig. 4.6, statistics at the figure caption). The slope of the daily mean as a function of solar radiation was slightly steeper for the thatched fungus than for the subterranean fungus and the soil (Fig. 4.6, upper portion, statistics at the figure caption). The slope of the next-day minima as a function of daily solar radiation was also significantly steeper for the thatched fungus than for the subterranean fungus and the soil. However, the slope of the daily maxima was significantly lower (Fig. 4.6, middle vs. lower portion, statistics at the figure caption). These results indicate that the surplus of temperature observed in the thatched fungus increases with increasing solar radiation (Fig. 4.6, upper portion) because of the low degree of heat losses. It follows that the extent to which next-day minimum of the thatched fungus surpasses that of the subterranean fungus and the soil directly depends on the daily incoming of solar radiation (Fig. 4.6 middle portion). Although the thatched fungus and soil maxima were positively related to solar radiation, the daily maxima of the thatched fungus exceeded that of the soil, and therefore that of the subterranean fungus as well, only for low accumulated solar radiation (< 1 kJ*day*cm^{-2}), being equal or lower at greater values (Fig. 4.6, lower portion).

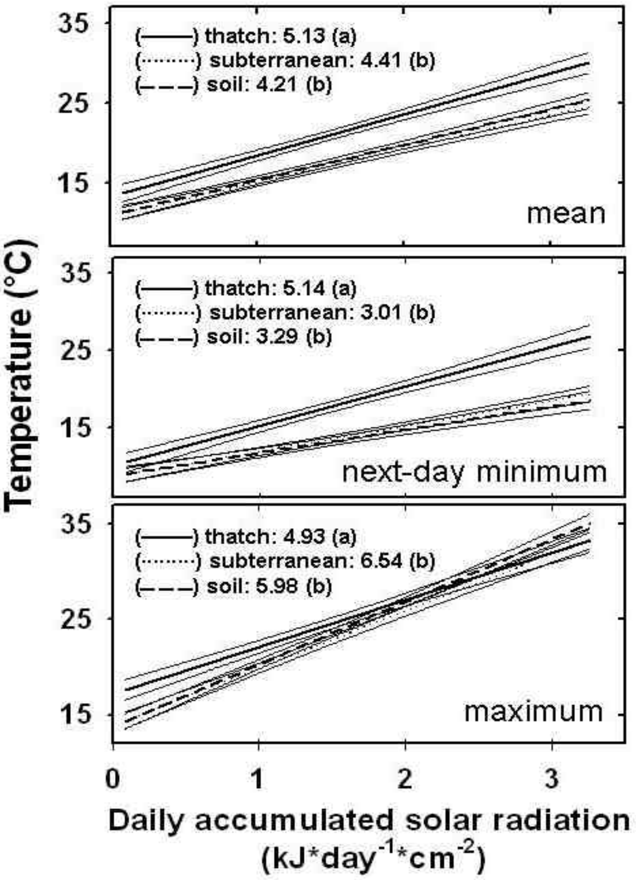

Figure 4.6: Regression lines (95% confidence limits) of the daily mean, the daily next-day minimum, and the daily maximum of the thatched fungus (continuous line), the subterranean fungus (dotted) and the soil (dashed) vs. daily accumulated solar radiation. Data from 2001 and 2002 were pooled, since Figs. 2 and 3 suggest that the thermal relations between both nest types and the environment were similar in both years. All regressions are significant at $p<0.01$ (T-test) (N=292 for each regression). Regression coefficients of the thatched, subterranean and soil for the daily means: 0.44, 0.50, 0.51; for the next-day minima: 0.38, 0.26, 0.37; for the daily maxima: 0.43, 0.70, 0.64. The slopes are given in the insets of each graph. Regression slopes followed by the same letter are not significantly different. t-Test, at $p<0.08$ for daily means, at $p<0.01$ for next-day minimum and maximum (Fowler et al. 1998).

The relationship between the maximum temperatures of thatched fungus and soil is explored in Fig. 4.7. The extent to which fungus daily maxima exceeded that of the soil continuously increased up to maximal soil temperature of 24°C. Beyond this value, such a difference decreased and turned out to be inverse for maximum soil temperatures higher than 30°C. The difference between fungus and soil maxima was highest at an average maximal soil temperature of 23.9°C (year 2001) and 24.1 (year 2002) (Fig. 4.7, statistics at the figure caption). Fungus and soil maxima were positively related only for values of soil maxima lower than 24°C, yet negatively correlated for higher values (Fig. 4.7, statistics at the figure caption). Furthermore, maximum fungus temperature became lower than that of the soil when maximal soil temperature surpassed a value of ca. 31.6°C and 30.5°C, for the years 2001 and 2002, respectively (Fig. 4.7, statistics at the figure caption). Moreover, maximum fungus temperature exceeded that of the soil only when maximum soil temperature was higher than 14.7°C and 15.1°C for years 2001 and 2002, respectively (Fig. 4.7, statistics at the figure caption).

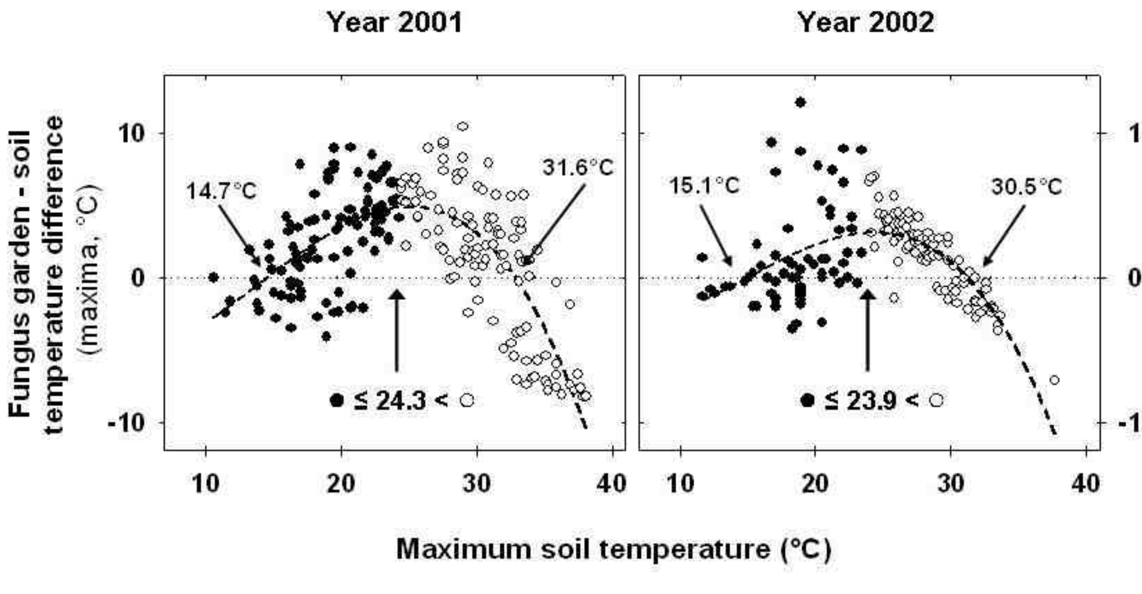

Figure 4.7: Temperature difference between the fungus garden and soil for the thatched nests (daily maxima), as a function of maximum soil temperature during the years 2001 and 2002 (left and right graph, respectively). The dashed curve line is the best-fit polynomial regression line; year 2001: $f(x) = -5.08-0.43x+0.08x^2-0.002x^3$; $R^2=0.54$, $p<0.001$; year 2002: $f(x) = -1.27-0.75x+0.08x^2-0.002x^3$; $R^2=0.37$, $p<0.001$. Temperature values marked by the arrows show the T (°C) at which $f(x)=0$, i.e., the soil temperatures at which there was no difference between them and that of the fungus. The highest temperature difference between soil and fungus was obtained by calculating the first derivative, $f'(x)=b+2cx+3dx^2$. The values at which $f'(x)=0$ were calculated for both years, being 24.3°C in 2001 and 23.9°C in 2002, and are

indicated in the corresponding graph. The black points (?) represent those values at which f'(x) < 0, the white points (?) represent those values at which f'(x) = 0. The regression coefficients and slopes for the data at which f'(x) = 0 are: year 2001, R^2 =0.36, b=0.52; year 2002, R^2 =0.15, b=0.43. The regression coefficients and slopes for the data at which f'(x) < 0 are: year 2001, R^2 =0.58, b= -1.04; year 2002, R^2 =0.76, b= -0.83.

4.3.3. The effect of colony presence on the temperature of thatched nests

Figure 4.8 shows the daily temperature difference between the fungus chamber and the soil over time, for both control and poisoned colonies, when the thatched nests were shaded.

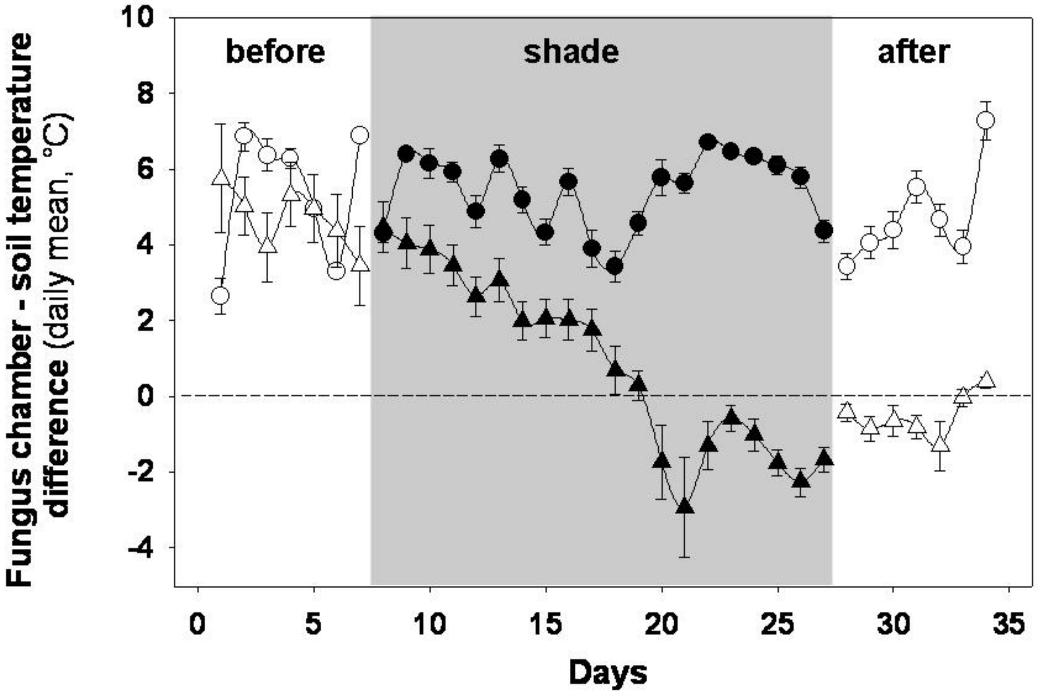

Figure 4.8: Temperature differences between fungus chamber and soil (daily mean ± SE) during the experiments in which the thatched nests were experimentally shaded, as well as for a control colony (circles) and a colony that died after poisoning (triangles). Since the fungus garden of the poisoned colony collapsed during the measuring period, the measured values are referred to as chamber (and not fungus) temperatures.

Before the experimental shading, the fungus chamber temperature (daily mean) exceeded that of the soil by either 5.3°C (SE=0.6, N=7) or 4.7°C (SE=0.2, N=7) (Fig. 4.8, before, circles vs. triangles), values that are not statistically different (T-test,

t_{12}=0.89, p=0.40). During the weeks of experimental shading, nests with living colonies still showed a fungus temperature higher than that of the soil, yet fungus chamber temperature in poisoned nests strongly decreased over time, and reached that of the soil after 14 days (Fig. 4.8, shaded, circles vs. triangles). After the wooden roofs were removed, the temperature of the nest with living colonies did not differ from that recorded before the experimental shading (Fig. 4.8, circles, after vs. before, T-test for matched pairs, t_6=1.1, p=0.31), yet it was clearly lower in the poisoned ones (Fig. 4.8, triangles, after vs. before, T-test for matched pairs, t_6=1.1, p<0.001). Consequently, the temperature difference between the fungus chamber and the soil strongly differed between both treatments after removal of the roofs (Fig. 4.8, after, circles vs. triangles, T-test, t_{12}=9.95, p<0.001). Taken together, these results indicate that the temperature surplus of a thatched fungus regarding the soil largely depended on the colony presence and not on the direct solar radiation that reached the thatch surface.

4.3.4. Thermal properties of the thatch material and soil

Thermal diffusivity of both the thatch material and the soil largely depended on their water mass content (Fig. 4.9). For extremely dry conditions, thermal diffusivities of thatch material and soil were roughly similar, yet at moderate and high water contents, the thermal diffusivity of the thatch material was clearly lower than that of the soil. Although thermal diffusivity of the thatch material increased at high water contents, probably due to the deposition of water in the air spaces that increases thermal conductivity, it was still lower than that of the surrounding soil. It occurred because at high water contents, heat capacity increased more than thermal conductivity and therefore thermal diffusivity of both, soil and thatch, decreased (Campbell 1977). Figure 4.9 also presents the estimated ranges of thermal diffusivity for the thatch material and surrounding soil from the field nests. It was calculated by substituting the water content of the soil and thatch samples collected in the field in the corresponding equations (Fig. 4.9, dotted and continuous lines).

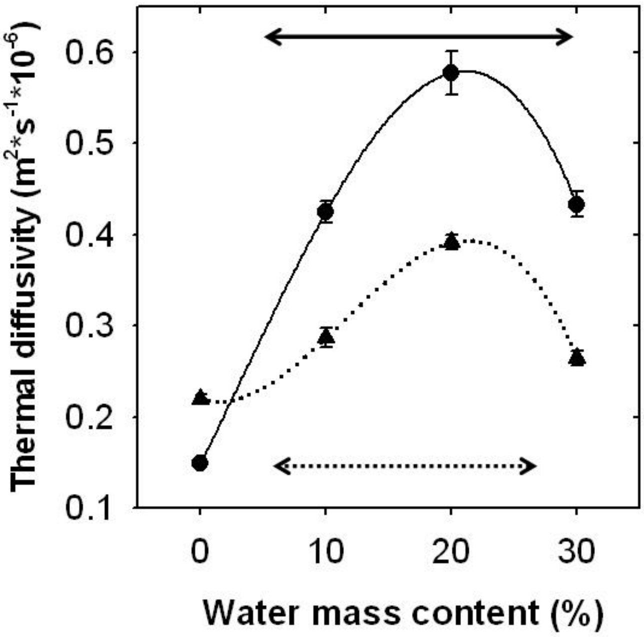

Figure 4.9: Thermal diffusivity of the thatch material and soil as a function of water mass content (%). The black circles are the measured thermal diffusivity for the soil (mean ± SE); the continuous line is the best-fit line regression, $D_{soil} = 0.15x10^{-6} + (0.28x10^{-7})(?) + (0.24x10^{-9})(?)^2 - (0.29x10^{-10})(?)^3$, $r^2 = 0.91$, $p<0.001$. The black triangles are the measured thermal diffusivity for the thatch material (mean ± SE); the dotted line is the best-fit line regression, $D_{thatch} = 0.22x10^{-6} - (0.40x10^{-8})(?) + (0.15x10^{-8})(?)^2 - (0.45x10^{-10})(?)^3$, $r^2 = 0.73$, $p<0.001$. The dotted and continuous straight lines show the range of soil moisture content found under field conditions for the thatch material and soil respectively.

4.4. Discussion

4.4.1. Temperature in thatched and subterranean nests: the thatch limits heat exchanges with the environment

Taken together, results indicate that the construction of a thatched nest, as compared to live in a subterranean nest, provides *A. heyeri* colonies with a thermoregulatory advantage. The average fungus garden temperature in thatched nests was higher than in subterranean nests, and also higher than the environment, mostly in spring (Figs. 4.2 and 4.3). Such thermal interactions of the thatched nest with the environment can be explained if it is assumed that the thatch reduces heat transfer between the environment and the inhabiting colony, i.e., the thatch material acts as an insulator and not as a heat transmitter. In fact, the measured thermal diffusivity of the *A. heyeri* thatch, which ranged from 0.22 x 10^{-6} to 0.28 x 10^{-6} (m^2/s), was similar to that of materials considered as insulators, such as some woods (Cengel 2003), and slightly lower than that of the mound material from *Formica polyctena* nests in Netherlands (Brandt 1980). Thus, these results in *A. heyeri* indicate that the grass fragments used by workers to build the nest thatch strongly limited the heat flow between the nest and the environment. These results agree with those obtained by Frouz (1996) with *Formica polyctena* in the Check Republic. Nest insulation would not be achieved if workers built the nest mounds using the readily available surrounding soil, since it has a greater thermal diffusivity than grasses (Fig. 4.9). Interestingly, field observations of *A. heyeri* nests indicate that fungus gardens are not directly placed on the soil in the single nest chamber, but on a 5-10 cm thick layer composed of exhausted grass fragments. This organic material is also expected to have lower thermal diffusivity than the surrounding soil (Cengel 2003), and should therefore contribute to limit the heat exchanges between the fungus garden and the underlying soil even more.

A hypothetical representation of the heat flows between the environment and the nest is presented in Fig. 4.10, comparatively for thatched and subterranean nests. During the day, the incoming solar radiation directly reaches the soil and the exposed thatched nest (Fig. 4.10, DAY, continuous line), but due to its low thermal diffusivity, the thatch material may limit the heat flow into the fungus garden (Fig. 4.10, DAY, dashed line). Consequently, the thatched fungus gains heat at a lower rate than the subterranean one and the environment (as shown in Fig. 4.5, upper portion), which precludes nest overheating by limiting the maximal daily temperatures that can be reached (as shown in Fig. 4.6, lower portion). During the night, the thatch's low thermal diffusivity prevents losses of the internally accumulated heat into the air (Fig. 4.10, NIGHT, thatched nest). Consequently, the thatched fungus losses heat at a lower rate than the subterranean one and the environment (Fig. 4.5, lower portion). It follows that the greater the amount of heat stored at the beginning of the cooling phase, the higher the next-day minimum (as shown in Fig. 4.6, middle portion). Besides, the mulch of exhausted fungus material on which the fungus garden is placed may also prevent heat losses into the soil and so contribute to insulate the fungus garden (Fig. 4.10, NIGHT). Since the subterranean fungus is not surrounded by insulating material, heat losses may occur throughout the cooling phase, as far as its temperature is higher than that of the surrounding soil and air (Fig. 4.10, NIGHT, subterranean nest).

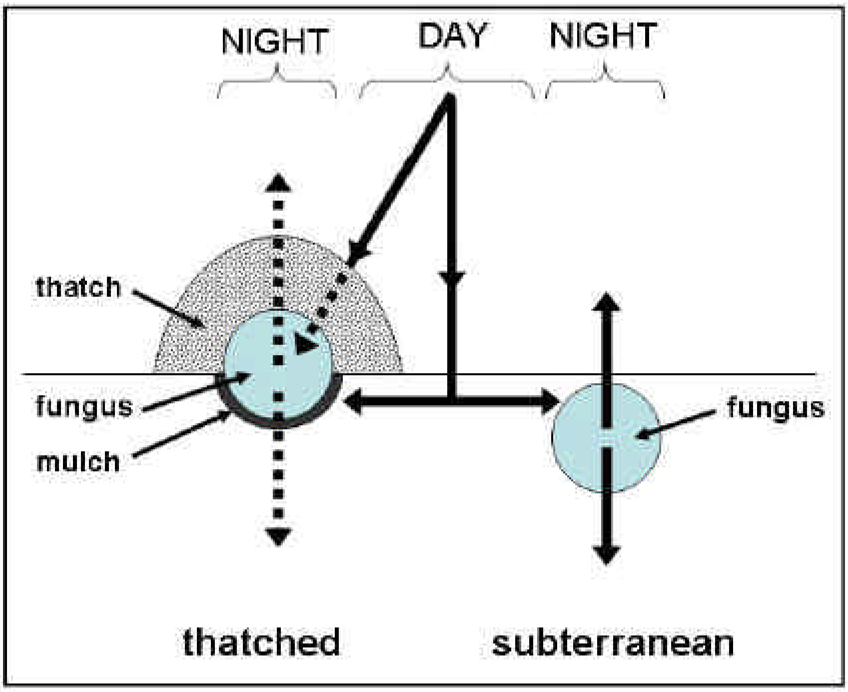

Figure 4.10: Thermal relations between the fungus garden and the environment in a thatched nest and a subterranean nest during day and night. The arrows show the directions of the heat flow. The dashed and continuous lines indicate limited and non-limited heat flows, respectively. Drawings are not made at scale.

Thus, it is suggested that the achievement of a temperature surplus above the environment in thatched nests of *A. heyeri* results from the prevention of heat losses through the thatch into the cool air, and not from the nest exposure to direct solar radiation. The measurements on shaded nests, showing that even complete shading did not influence such temperature surplus, clearly supported this view (Fig 4.8, circles). The maintenance of such high temperature not only depended on the properties of the nest thatch, but also on the presence of the colony, as the results with the poisoned colonies showed (Fig. 4.8, triangles). Raigner (1948) obtained similar results with *Formica polyctena* in France. There are at least two possible explanations for these results. First, a contribution of an internal source of heat. Metabolic heat production was already suggested for *Formica spp* in the northern hemisphere (Frouz 2000; Gösswald 1989; Horstmann 1990), although its origin, either the colony members or the decaying nest material, is still unclear (Frouz 2000;

Horstmann 1990). Second, the interplay between the insulating properties of the thatch material and the high heat capacity of the fungus. The fungus may store large heat amounts during the day and loose them slowly because of the insulating properties of the thatch, so being the cause for the observed high daily minima in thatched nests. The obtained results do not allow to elucidate which of these two mechanisms may account for the observed thermal dynamics, since colony poisoning eliminated the colony members and therefore also affected fungus maintenance and health.

4.4.2. The maintenance of proper temperature in thatched nests

As discussed above, it seems likely that thatched nests achieve higher daily mean temperatures, when compared to subterranean nests and the soil, because the thatch material with its low thermal diffusivity passively avoids losses of the heat stored during the previous day into the cold air at night (Fig. 4.10). However, the fact that fungus daily maxima became lower than the soil daily maximum from spring to summer (see the examples of the Fig. 4.4), suggests different effects of the thatch depending on season. In fact, whether the daily maxima of the thatched fungus exceeds that of the soil depended on the maximal values the soil reached (Fig. 4.7). The threshold value at which maximum fungus temperature became lower than that of the soil, ca. 30°C, matches the temperature beyond which ant brood development is negatively affected, as reported for two common South American ant species inhabiting subtropical and temperate areas, *Camponotus mus* and *Solenopsis invicta* (Porter 1988; Porter and Tschinkel 1993; Roces and Núñez 1989). Furthermore, temperatures above 30°C were found to be lethal for in vitro cultivars of the symbiotic fungus *Attamyces bromatificus* isolated from colonies of *Acromyrmex octospinosus, Atta cephalotes* and *Trachymyrmex urichi* (Powell and Stradling 1986). Moreover, the estimated maximum soil temperature at which the difference between fungus and soil daily maxima started decreasing, ca. 24°C (Fig. 4.7), matches the temperature preferred by workers for fungus and brood location, around 24.1°C (chapter 2) (Bollazzi and Roces 2002). The present results agree with those obtained by Rosengren et al. (1987) on *Formica aquilonia* and *F. grankullensis* in Finland, and by Frouz (2000) on *F. polyctena* in the Czech Republic. They found out that under certain conditions, mound and environmental temperatures were negatively correlated, so that colonies maintained some degree of thermal homeostasis. In *Formica* it is known that colonies might regulate inside-mound temperatures by reducing mound height, creating and enlarging nest openings, and reducing worker clustering (Horstmann and Schmid 1986).

In other *Acromyrmex* leaf-cutting ant species, similar regulatory responses were described. For instance, the presence of openings permeating the nest thatch has been described for *A. heyeri* and *A. lobicornis* (Bonetto 1959; Gonçalves 1961). Results obtained using laboratory colonies of *A. heyeri* showed that the higher the internal nest temperature, the greater the number of ventilation holes opened by workers on the mound (chapter 5). It suggests that the observed prevention of extreme daily temperature maxima could be the result of active changes in the thatch structure made by workers, so as to favor nest ventilation and hence heat exchanges with the outside. Besides making holes, avoidance of highest temperatures may also be achieved by increasing the thatch thickness, and hence, by reducing heat inflow from the thatch's surface into the fungus garden even more. Such response may however drastically reduce heat losses during night as well, thus leading to an increase in both the daily minima and mean. The facts that the daily rate of temperature increase, as well as the daily rate of temperature decrease, markedly diminished with increasing solar radiation over the year (Fig. 4.5), suggest that workers might counteract extremely high daily maxima by increasing thatch thickness.

4.4.3. Thermoregulatory benefits of thatched nests

Ant colonies are highly dependent of proper ranges of temperature for their growth (Brian 1973; Pontin 1960; Porter 1988; Southerland 1988), survival (Brian 1956; Callcott et al. 2000; Korzukhin et al. 2001; Pontin 1963) and rate of offspring production (Brian and Brian 1951; Markin et al. 1974). Therefore, they exhibit marked thermopreferences for brood location inside the nest. In *A. heyeri,* the preferred values to locate brood and fungus average 24.1°C (Chapter 2) (Bollazzi and Roces 2002), which closely matches the temperature of 25°C at which the growth rate of the symbiotic fungus of leaf-cutting ant is maximized (Powell and Stradling 1986). The results suggest that the thatch contributes to maintain fungus garden temperature in a proper range for colony growth, and that the subterranean nests fail to do so. For instance, fungus garden temperature in the thatched nests reached, and even exceeded, the preferred value of 24.1°C in spring 2001 and early spring 2002, while it was clearly lower in the subterranean nest in the same periods (Figs. 4.2 and 4.3). Given that subterranean fungus and soil temperature were almost similar, and soil temperatures at the study area averaged 17.9°C in spring (based on a 30-years record, see ESM for further details), fungus gardens from subterranean colonies are expected to have similar values and so to be unable to reach the suitable, preferred temperatures around 24.1°C. The benefit that *A. heyeri* colonies achieve by inhabiting a thatched nest is emphasized by the fact that only the colonies inhabiting thatched nests were observed to produce sexuals. It is in accordance with observations made by Bonetto (1959) in Argentina, where colonies of several *Acromyrmex* species inhabiting thatched-superficial nests were observed to produce sexuals before subterranean colonies. Moreover, sexuals were observed earlier in nests with a thatch mostly composed of grass fragments than in those with a high percent of soil in the thatch (Bonetto 1959). It emphasized the importance the selection of building material by workers, dry grass fragments instead of soil, has for the maintenance of proper temperatures inside thatched nests.

A nest temperature higher than the environmental one has often been recorded for the well known mound-building ant genus *Formica* in the northern hemisphere. For instance, in *Formica aquilonia* and *F. grankullensis* in Finland (Rosengren et al. 1987), *F. ulkei* in USA (Scherba 1962), and *F. polyctena* in Germany (Heimann 1963; Kneitz 1970). However, the present contribution is the first in addressing the question how ant colonies adapt to temperate climate through their building behaviour, and what are the mechanisms underlying such adaptation. By comparing simpatrically-occurring thatched and subterranean nests of the same species, this work further demonstrates that building of a thatched nest, as opposed to excavating underground chambers, enables *A. heyeri* colonies to achieve a proper temperature range for colony growth and reproduction. It further emphasizes the importance of workers' building behaviour for thermoregulatory needs in ants inhabiting temperate regions.

4.A. Appendix

4.A.1. Fungus garden temperature in both thatched and subterranean nests

The Newhall Simulation Model used by Van Wambeke (1981) to determine soil temperature regimes of South America was applied to calculate average soil temperature at 50 cm depth at the study site.

Daily mean air temperatures for the years 1972 to 2001, obtained from Las Brujas Meteorological Station, situated 15 kilometers west of the study area, were used for calculations (INIA-MGAP 2007). The mean summer temperature was calculated by averaging the three summer monthly air temperatures (December, January and February), and adding 2.5°C to this average. One sixth of the difference between the summer ad winter temperatures is then subtracted from this average to take into account the decrease in seasonal amplitude at 50 cm depth. The mean winter temperature was calculated by averaging the three winter monthly air temperatures (June, July and August) and adding 2.5°C to this average. One sixth of the difference between the summer and winter temperatures was then added to this average to take into account the decrease in seasonal amplitude at 50 cm depth.

Estimation of the temperatures at 50 cm soil depth gave average values of 15.0, 17.9 and 22.7°C for winter, spring and summer respectively

4.A.2. The effect of incoming solar radiation and colony presence on fungus temperature of thatched nests

The daily accumulated income of direct solar radiation ($kJ*day*cm^{-2}$) for the two years of measurements was estimated using the Angström equation, modified by Page (Goswami et al. 2000).:

$$\mathbf{R_s = R_o (a+b\ n/N)}$$

R_s and R_o are the horizontal terrestrial and extraterrestrial radiation levels averaged for a determined period of time, in this case 24 h.

n is the daily measured hours of sunshine,

N is the daily astronomically possible hours of sunshine.

a and b are constants experimentally obtained for a given area. Those adequate for the study area were obtained from Corsi (1980).

Daily values of n, N, and R_o for south Uruguay were obtained from the GRAS program of the Ministry of Agriculture of Uruguay (INIA-MGAP 2007).

4.A.3. Thermal properties of thatch and soil

A Dual-Probe Heat Capacity Sensor (DPHC, Fig. 4.A.1) (Campbell et al. 1991) was constructed for the measurements. Thermal diffusivity was calculated by dividing thermal conductivity by heat capacity. The DPHC consisted of a small plastic body with a heater and a thermocouple placed parallel to each other. The heather was a ceramic pt100, (Pt100 “Messwiderstand”, Electhortherm®) of 10 mm length, 2 mm width and 0.5 mm thickness; the sensor was a wire sensor (Typ K NiCrNi, B+B Thermo-Technik) connected to a data logger (Voltcraft® K204, Conrad Electronics). The heater and the sensor were inserted into the material to be tested tightly filling the space between them.

A brief current pulse (5s) was applied to the heater and the temperature of the thermocouple was monitored with the data logger every second. The response of the thermocouple to the heat pulse was used to simultaneously determine the heat capacity, *C*, and thermal conductivity, *k*, of the probe (Campbell et al. 1991; Ham 2001; Ham and Benson 2004).

The heat capacity ($kJ*kg^{-1}*°C^{-1}$) was calculated as follows:

$$\mathbf{C = q / (?er^2T_{max})} \quad \text{(eq. 1)}$$

where q is the applied power per length of heater probe (J/m),

r is the distance between the heater and the sensor (m),

and T_{max} is the temperature increase at the thermistor probe (°C).

The heat loss to the opposite side of the thermocouple due to the use of a plate-shaped heather instead of a cylindrical-shaped one, was corrected by dividing the numerator q by 2.

The thermal conductivity of the soil ($W*m^{-1}*°C^{-1}$) was calculated as follow:

$$\mathbf{k = r^2C / (4t_m)} \quad \text{(eq. 2),}$$

where C is the calculated heat capacity
and t_m (s) is the elapsed time required to reach T_{max}

The duration of the pulse (5s) was controlled by a computer-switched relay board placed between the current source and the heater (Relay board eight-fold serial, Conrad®). After a pulse, a new one was applied only after the sample temperature decreased again to room temperature, 23°C. Five pulses were applied to each of the 8 soil and thatch samples.

The DPHC calibration was performed using water-free glycerin at 23°C as a standard reference material. Thus, the obtained values of C and k for the water-free glycerin (C = 2.216 kJ*kg^{-1}*°C^{-1}, SE=0.054; k = 0.31 W*m^{-1}*°C^{-1}, SE=0.009) were similar to those obtained by Fontana (1999) using a DPHC (at 23°C, C = 2.215 kJ*kg^{-1}*°C^{-1}; k = 0.29 W*m^{-1}*°C^{-1}), and those given by Rahman (1995) (at 27°C, C = 2.42 kJ*kg^{-1}*°C^{-1}; k = 0.286 W*m^{-1}*°C^{-1}) and Cengel (2003) (at 20°C, C = 2.31 kJ*kg^{-1}*°C^{-1}).

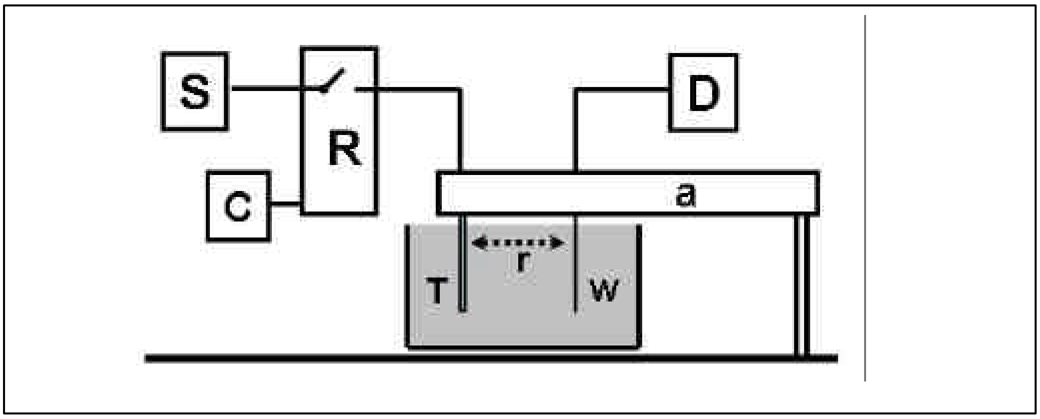

Figure 4.A.1: Dual-Probe Heat Capacity Sensor (DPHC) used to estimate thermal diffusivity of thatch material and soil. P=container for the sample of soil or thatch material; T=heather; W=wire sensor, D=data logger, R=computer-controlled relay-board; C=computer; S=power source, a=plastic body where the heather and the sensor were fixed, attached to a table, r=distance between the heater and the wire sensor. The DPHC scheme is not made at scale.

4.A.4. Nest morphology of thatched and subterranean nests

By carefully opening by hand a small window on the nest thatch, it was possible to repeatedly observe the fungus chamber and assess its size, without damaging the colony. After the manipulations, workers were observed to rapidly repair the nest thatch by relocating nest material. The thatched nests investigated during both years presented a single ball-shaped fungus chamber, ca. 42-45 cm in diameter, located on a soil depression at 20-25 cm depth. However, the fungus garden was not in contact with the soil, but placed on a 10 cm mulch layer composed of exhausted grass fragments. Thus, more than half of the fungus garden was positioned above the soil surface level, covered by a thatch. In summer, the mound height reached ca. 35 cm, with the upper portion of the thatch ca. 10-13 cm thick and composed of recently-cut grass fragments. In winter, the thatch was clearly thinner, around 5-7 cm, and composed of dry grass fragments. The lower portions of the thatch sides were made of a mixture of grass fragments and soil. Inside the thatch there were many tunnels, empty in spring and summer, but filled with small fungus gardens during winter, mostly at the northeast side. During winter, the fungus garden occupied only half of the available chamber volume, yet it occupied the whole chamber during spring and summer.

The investigated subterranean nest in the year 2001 had a single fungus chamber. It was tube-shaped, 30 cm high and 20 cm wide, and its base was located at 35 cm depth. During winter, less than a half of the chamber was occupied with a fungus garden located in its upper part, hanging from both the chamber top and the upper portion of the walls. During spring, the fungus garden occupied the whole chamber, while it decreased in volume again in summer, and was observed located at the bottom of the chamber. In year 2002, the subterranean nest consisted of a single chamber. It was ball-shaped with a diameter of 20 cm, and its base placed at 25 cm depth. In winter, the fungus garden occupied only one half of the chamber volume. Interestingly, in early spring the colony inhabiting the subterranean nest started building a mound-shaped, thatched nest. The nest morphology began to change around October 12th 2002. At first, it was observed that the nest entrance was completely covered by a mound made of soil, ca. 20 cm wide and 5 cm high. Some days later, workers placed dry and fresh grass fragments on that mound. Workers increased the size of the mound by adding grass fragments and, to a lesser extent, soil excavated from the nest interior. Simultaneously, the fungus grew upwards, beyond the soil surface, and was covered by a thatch constructed by workers mostly composed of grass fragments. Therefore, the fungus chamber reached a diameter of 35 cm in only four weeks, with its base placed at 25 cm depth on a mulch of exhausted grass fragments. It was covered by an 8-10 cm thick thatch of grass fragments, and the resulting mound nest reached a height of 18-20 cm.

5. Building behaviour for climate control in leaf-cutting ants: *Acromyrmex heyeri* workers trade off thermoregulation for humidity control

Summary In ants, building behaviour can increase colony's survival in a given habitat, if it improves colony's abilities for controlling the internal climatic conditions, providing that a building response for controlling a given parameter, such as temperature or humidity, do no interfere with the control of one another. In thatching species of the leaf cutting-ant genus *Acromyrmex*, the presence of openings permeating the nest thatch has been commonly reported. In this study, it is first addressed the question whether workers of the thatching ant *A. heyeri* modify the number of openings as a function of the internal nest temperature. And second, it is investigated to what extent such response to nest temperature interacts with the control of nest humidity, and whether workers trade off one of the regulatory responses for the other. Results show that when confronted to nest temperatures ranging from 20 to 30°C, *A. heyeri* workers permeated the thatch with more openings, the higher the internal nest temperature. This is thought to be aimed at restoring nest temperature to the preferred value of 25°C, by regulating heat exchanges between nest and the outside. In addition, responses to relative humidity interacted with those based on temperature. During an experimental desiccation phase, *A. heyeri* workers closed openings as a response to air desiccation, even at a nest temperature that otherwise triggered the opposite response. It demonstrates that *A. heyeri* workers trade off a response related to thermoregulation for the maintenance of internal nest humidity. This trade-off is discussed as an adaptation that allowed some thatching *Acromyrmex* species to expand and survive in temperate and semi-arid South American regions.

5.1. Introduction

In the leaf-cutting ants genus *Acromyrmex,* some species inhabiting South American temperate areas, such as *A. heyeri, A. hispidus, A lobicornis* and *A. crassispinus*, construct mound-shaped superficial nests, in which the mostly single fungus garden is located on the soil surface and covered by a thatch composed of plant fragments and, to a lesser extent, soil particles (Fig. 5.1; right, upper corner) (Bonetto 1959; Fowler 1985; Gonçalves 1961). In these nests, the single fungus garden rests directly on the soil surface, while its sides and upper part are covered by the thatch material that forms a chamber surrounding the fungus garden. For ants inhabiting temperate areas, thatched nests have commonly been considered as a long-term adaptation to low environmental temperatures, since the temperature inside mounds is usually higher, and more stable, than that of the environment (Frouz 1996; Frouz 2000; see reviews in: Heinrich 1993b; Seeley and Heinrich 1981) (see also chapter 4). Although the thatch itself may help stabilize the climatic conditions inside the nest, workers may also counteract short-term unfavourable climatic changes, for instance by modifying the tunnels that permeate the nest mound.

In *Formica polyctena*, workers were observed to modify the nest thatch as a response to an experimental increase in both external and internal nest temperature (Horstmann and Schmid 1986; Lange 1959). With increasing internal temperatures, workers reduced the mound height, enlarged the existing nest openings and made new ones, which resulted in the maintenance of nest temperature below 35°C because of the facilitated heat exchanges with the environment. The presence of openings permeating the nest thatch has also been reported for some mound-builder *Acromyrmex* leaf-cutting ants, such as *A. heyeri and A. lobicornis*, (Bonetto 1959; Gonçalves 1961; Zolessi and González 1974) (Fig. 5.1, left). These openings are distributed in the upper portion of the thatch and are not used as nest entrances, which are located at the basal portion of the thatched mound. Up to date, it is unknown whether these openings promote exchanges of gas, heat or humidity with the outside and whether workers respond to changes in the nest climate or the environment by altering the number of openings.

Figure 5.1: **Right, upper:** two filed nests of *A. heyeri* at the location where the experimental colony was collected (south Uruguay, 34° 33´ 26'' S; 56° 15' 59'' W). Scale bar: 20 cm. **Left:** three opened ventilation holes (shown by the arrows) on the thatch of a field *A. heyeri* nest. Scale bar: 1 cm. **Right, lower:** an open ventilation hole, as it was observed during the laboratory experiments. The arrow shows the white fungus garden inside the nest. Scale bar: 1 cm.

However, permeating the nest thatch with openings to promote air exchanges, and so to get rid of excessive heat, may compromise the maintenance of internal nest humidity. In large nests of *Atta* leaf-cutting ants, nest ventilation do not appear to compromise the humidity conditions inside the underground fungus chambers, since the relative humidity in the chambers averages saturation levels (Stahel & Geijkes 1940; Kleineidam & Roces 2000). The situation in the more exposed *Acromyrmex* thatched nests may be substantially different, because the inflowing air will directly circulate through the single fungus garden and therefore increase its risk of desiccation. In fact, it has recently been experimentally demonstrated that *Acromyrmex* leaf-cutting ants show regulatory responses to counteract the risk of colony desiccation (Bollazzi and Roces 2007) (see also chapter 6) . *Acromyrmex* *ambiguus* workers use leaf fragments to close only those nest tunnels through which dry air enters the nest, reducing nest ventilation and therefore the risk of fungus desiccation. They show no responses to the circulation of humid air under otherwise similar conditions.

It is conceivable that at least two competing regulatory responses may underlie the control of nest microclimate in colonies of *Acromyrmex* thatching ants: workers may be expected to construct openings as soon as the fungus temperature increases beyond a critical point, but at the same time to close them if the maintenance of internal nest humidity is compromised.

The present study investigates whether workers of the thatching grass-cutting ant *Acromyrmex heyeri* respond to the competing demands for temperature and humidity control by modifying the architecture of the thatch. For that, it is first experimentally addressed the question whether workers modify the number of openings permeating the thatch as a function of the internal nest temperature. Second, it is explored the extent to which the control of nest humidity interacts with the thermoregulatory responses, and whether workers trade off one of the regulatory responses for the other.

5.2. Methods

The laboratory experiments were performed with one one-year old colony of the thatching grass-cutting ant *Acromyrmex heyeri* collected in Joanico, Canelones, Uruguay (34° 33´ 26’’ S; 56° 15’ 59’’ W), and transported to the Department of Behavioural Physiology and Sociobiology at the University of Würzburg, Germany. Since colonies vary greatly in size and worker-size distribution, as well as harvesting motivation, a standardization of colony responses would be very difficult to achieve, and pooling data from different colonies may mask the effects of the variables studied. For these reasons, only one colony was used throughout the experimental series.

The rationale of the experimental design was the following: at the beginning of the experiment, the laboratory colony of *A. heyeri* was maintained over three days at constant nest temperature and high humidity of outside air, and the number of openings permeating the nest thatch was counted. Thereafter, the outside air was dried, while nest temperature was maintained as in the previous days, and the number of thatch openings was further recorded over three additional days. This procedure was repeated for three different temperatures controlled experimentally, in independent series. As a consequence, both the number of thatch openings as a function of nest temperature, and the influence of humidity on that response, were experimentally evaluated.

The experimental colony was placed inside a Perspex box (50 x 50 x 50 cm) with its floor made of glass and side walls coated with Fluon to prevent ant escape. A 3-cm-thick layer of plaster covered the box floor, except at the central portion, where a circular area (20 cm in diameter) remained free. The experimental colony, consisting of a single fungus garden averaging a volume of 2 l, was placed on this area in contact with the glass floor. Dry grasses and wet clay were offered to the colony as building material. After a week, the colony built a mound-shaped thatch on the fungus, 30 cm in diameter and 20 cm in height. Six holes on the Perspex box cover, fitted with a metal mesh, allowed air exchanges. During the experiments, the box containing the colony was placed on a thermostatic plate regulated by a thermal bath attached to it. This thermostatic plate allowed us to adjust the internal nest temperature without altering the outside (room) temperature, which was maintained at 25°C in the controlled room.

Three different series were performed, each lasting 6 days, with the nest temperature set at either 20, 25 or 30°C. During the first three days, called the “humid phase”, the relative humidity of the air inside the box was maintained above 98% by continuously adding water to the plaster floor and keeping the box closed. During the following three days, called the “desiccation phase”, the box cover was removed and the air inside the box turned out to be dry because of the inflow of the controlled outside air from the experimental room, with an average low humidity of 35%. The number of nest openings was counted on digital photos taken at the end of the humid phase, and every day during the desiccation phase.

Ten replicates were performed for each assayed temperature. The internal nest temperature, as well as the temperature and the RH of the air surrounding the nest, were recorded every 30 min using RH and temperature data loggers (Tinytag®). Measurements of internal humidity using a RH-sensor placed inside the fungus chamber were not possible because of its saturation, thus indicating that internal RH was indeed very high and likely approached saturation values, as reported for leaf-cutting ant field colonies (Kleineidam and Roces 2000; Stahel and Geijskes 1940)

To give an idea of the temperature and humidity conditions experienced by field colonies of *A. heyeri*, both fungus and outside temperature, as well as the relative humidity measured directly above the thatch surface, were recorded for a field nest located in Uruguay using RH and temperature data loggers. Similarly to the laboratory nest, measurements of RH inside this field nest using a RH-sensor placed inside the fungus chamber were not possible.

5.3. Results

After the three days of the humid phase, *A. heyeri* workers permeated the thatch with openings at all temperatures assayed, yet the number of openings was higher, the higher the internal nest temperature (Fig 5.2, day 3, humid: Friedman's ANOVA, $Chi^2_{(10,3)}$= 19.53, $p < 0.001$. 20 vs. 25°C: Wilcoxon-Test, Z=2.66, N=10, p=0.0076. 30 vs. 25°C: Wilcoxon-Test, Z=2.80, N=10, p=0.0050). The observed openings had ca. 1 cm in diameter. Sometimes, it was even possible to see the fungus garden through the thatch openings (Fig. 5.1; right, lower corner).

During the entire desiccation phase, the humidity of the outside air at the thatch surface averaged 50% (Fig. 5.2, top). At such low humidity, workers were observed to close a number of thatch openings. The number of openings at the end of the desiccation phase was significantly lower than at the beginning, for the three temperatures assayed, although the outside relative humidity did not change (Fig. 5.2, days 4 to 6; after Friedman's ANOVA; 20°C: $Chi^2_{(10,3)}$= 20.57, p= 0.001; 25°C: $Chi^2_{(10,3)}$= 28.60, $p < 0.001$; 30°C: $Chi^2_{(10,3)}$= 28.65, $p < 0.001$). At the end of the experiment (day 6), more ventilation holes remained open for a nest temperature of 30°C than for 20°C (Wilcoxon-Test, Z=2.49, N=10, p=0.012) or 25°C (Wilcoxon-Test, Z=2.42, N=10, p=0.015), yet no difference was observed between 20 and 25°C (Wilcoxon-Test, Z=0.76, N=10, p=0.45). Interestingly, openings appeared at the same locations in the different independent replicates, indicating that many of them were repeatedly closed and opened.

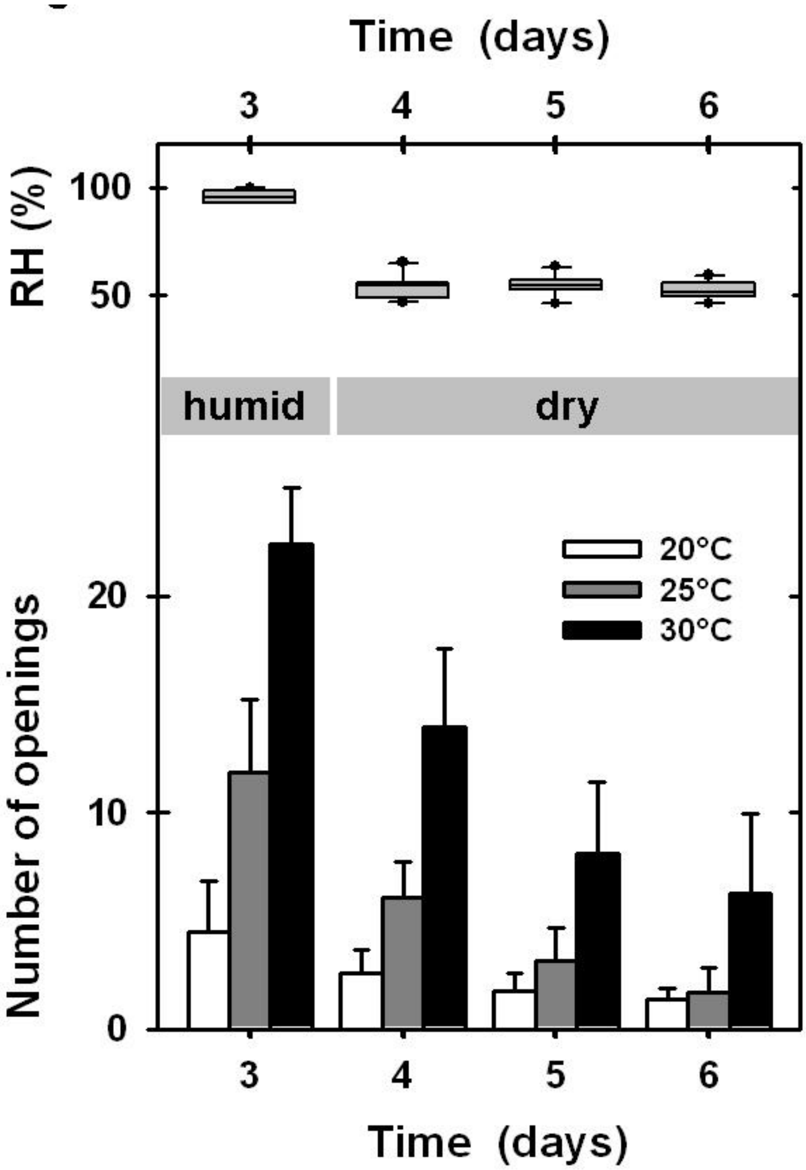

Figure 5.2: **Top**: Relative humidity (median, ± 25-75%) in the nest box during the two experimental phases, called "humid" and "dry". **Bottom:** number of openings (mean ± SD, N=10) recorded in the nest thatch over the days 3 to 6, depending on internal nest temperature. The day 3 (humid) is the final of a three-day-long humid phase, during which the outside air was maintained at ca. 98% RH. The day 4 to 6 (dry) correspond to the desiccation phase, with an outside air humidity maintained at ca. 50%.

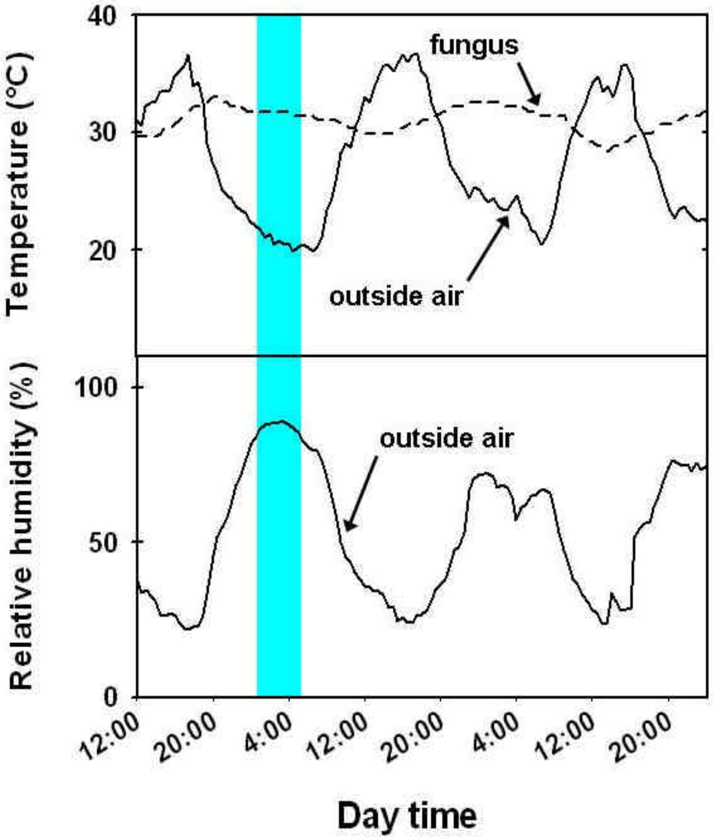

Figure 5.3: **Upper portion:** Temperature inside the fungus garden of a field *A. heyeri* nest and in the outside air at 1 m high, over three summer days in a grassland in Uruguay (5th to 8th March 2005) (33°20'25''S, 55°37'53''W). **Lower portion:** Relative humidity at 1 m above the nest thatch. The grey area shows the day time at which the relative humidity of outside air exceeded the minimum value of 84%, which has been shown to be preferred by leaf-cutting ant workers to tend brood and fungus (Roces and Kleineidam 2000).

Figure 5.3 (top) shows the daily temperature changes recorded in a field nest over three summer days, compared to the environmental values. While outside air temperature showed ample temperature variations, between 20 and 35°C, the temperature inside the nest showed a strongly damped oscillation around 30°C. The relative humidity of the outside air (Fig. 5.3, bottom) varied between 25 and 80%, being negatively related to the outside temperature as expected. The grey area indicates the day times at which humidity was higher than 84%, thus lying in the range preferred by leaf-cutting ant workers to tend brood and fungus (Roces and Kleineidam 2000). At those times, any air exchange with the outside through existing nest openings would not significantly affect water balance inside the nest.

5.4. Discussion

Results showed that *A. heyeri* workers always permeated the nest thatch with openings, but their number was higher, the higher the internal nest temperature. As soon as the relative humidity of the surrounding air decreased, workers were observed to close a number of openings. At the end of the desiccation phase, the number of existing openings was significantly lower than during the humid phase for all temperatures. It suggests that workers trade off a thermoregulatory response, i.e., the opening of holes on the thatch at high temperatures, for the maintenance of internal nest humidity.

The occurrence of more openings on the nest thatch at high temperatures opens the question whether workers' responses were also triggered by differences in the concentration of respiratory gases inside the nest, which are expected to occur because of the increased colony metabolism at higher temperatures. If so, a large number of nest openings at higher temperatures may be a response to, for instance, get rid of the accumulated CO_2 via diffusion and/or convective ventilation, and not necessarily a response to facilitate heat losses. This seems, however, unlikely. Even a complete nest dosing should not affect gas exchanges, because the composition and structure of the nest thatch resembles that of the organic mulch used in agriculture for preserving soil heat and moisture, which nevertheless allows freely air movements between the soil and the atmosphere (Hillel 1998; Oliveira et al. 2001; Tilander and Bonzi 1997). Based on these arguments, it is argued that the observed changes in the number of nest openings are a response aimed at influencing internal nest temperature and humidity.

In *A. heyeri,* it is known that workers locate brood and fungus at temperatures averaging 25°C when they have a choice between temperatures ranging from 10 to 37°C (chapter 2) (Bollazzi and Roces 2002). It might be therefore plausible to assume that any thermoregulatory response under field conditions should be aimed at reaching and maintaining a nest temperature around 25°C. For instance, workers would preclude air movements between the nest interior and the outside by closing thatch openings if in field conditions low environmental temperatures drives nest temperature bellow 25°C. Closing openings would avoid heat losses and so maintain internal nest temperature. This response corresponds to that observed in the experiments at 20°C, even though the outside air temperature was higher than the internal one. Such response seems to be exclusively based on the sensed internal nest temperature. Otherwise, workers should have opened more holes on the thatch so as to favor the inflow of warm outside air (25°C) into the nest.

But if in field conditions high environmental temperature drives nest temperature beyond 25°C, workers try to increase nest ventilation by making more thatch openings, as observed in the experiments at 30°C. As mentioned above for *Formica polyctena*, increasing the number of nest openings, or enlarging existing ones, prevented overheating by facilitating the inflow of colder outside air (Horstmann and Schmid 1986). The response to high nest temperature observed in the laboratory experiments would be the most conspicuous part of a more complex regulatory response showed by field colonies. In field nests, it was repeatedly observed that *A. heyeri* workers continuously picked-up, transported and rearranged the thatch's material at night, when nest temperature is typically higher than that of the outside air (Fig 5.3, upper portion). Thus, material turnover so as to loosen the thatch structure may increase nest ventilation, and facilitate heat losses to the cooler outside. Although temperature has previously been shown to be used by leaf-cutting ants as a cue during brood and fungus relocation (chapter 2) (Bollazzi and Roces 2002), and even for food location (Kleineidam et al. 2007), this study shows for the first time that temperature triggers building responses aimed at maintaining the internal nest climate in a proper range for colony.

In addition, these results show that responses to relative humidity interact with those based on temperature, so that *A. heyeri* workers trade off thermoregulation for humidity control. During the desiccation phase, workers closed a number of thatch openings even at a nest temperature that otherwise triggered the opposite response, for instance at 30°C. The fact that workers gradually closed additional openings over the 3 days of the desiccation phase, even though the humidity of the outside air remained unchanged, suggests that they did not responded directly to it. It suggests that they responded to the gradual decrease of inside-nest water content drive by the dry outside air, whatever the mechanism involved. The present results, and those obtained with *Acromyrmex ambiguus* (Bollazzi and Roces 2007) (see also chapter 6), indicate that the closing of nest openings by *Acromyrmex* workers is usually related to the desiccation of nest interior.

Based on the field measurements, it is suggested that field colonies may be often confronted with a trade off between temperature and humidity control. For instance, the nest reached temperatures around 30°C in a summer day (Fig. 3, upper portion), which are 5°C higher than the value preferred by *A. heyeri* workers to tend brood and fungus (chapter 2) (Bollazzi and Roces 2002). Values greater than 30°C are in addition lethal for the fungus (Powell and Stradling 1986). Therefore, workers are expected to permeate the thatch with openings so as to reduce nest temperature, since the outside air is colder than that of the nest (Fig. 5.3, upper portion). However, humidity of outside air rarely reached values higher than 84% RH, which preferred by leaf-cutting ant workers to tend brood and fungus (Roces and Kleineidam 2000). Consequently, workers are expected to trade off their responses related to thermoregulation, i.e., making more thatch openings, for the maintenance of internal nest humidity. Nevertheless, *A. heyeri* workers can also trade humidity control for thermoregulation when nest temperature reaches lethal values of fungus, as observed in the experiments. More thatch openings have been recorded at 30°C than at 20°C and 25°C after exposing the *A. heyeri* colony to dry outside air over 72 h (Fig. 5.2, dry, day 6).

Although in social insects the question of how climatic variables are used by colonies as cues during collective building has been the focus of intensive theoretical research (Bonabeau et al. 1997; Camazine et al. 2001; Theraulaz et al. 1998), the question of what climatic variables triggers and spatially guide local building behaviour remained experimentally unexplored. In ants, even though that digging behaviour, workers' activity and the dynamics of corpse aggregation are known to be influenced by climatic variables (Burkhardt 1991; Challet et al. 2005; Hangartner 1969; Jost et al. 2007), the question of whether these responses contribute to the control of nest climate remains elusive. The present experimental results, and those recently obtained in *A. ambiguus* (Bollazzi and Roces 2007) (see also chapter 6), strongly suggest that ants use climatic variables, such as temperature and humidity, to decide whether or not nest modifications aimed at regulating nest climate should be performed.

In builder organisms, such as leaf-cutting ants, building behaviour could lead to improve the organism's survival in a given habitat, if it brings about a significant advance in their abilities to control the internal nest climate (Hansell 1993; Hansell 1996; Hansell 2005), providing that a building response for controlling a given parameter, such as temperature or humidity, do no interfere with the control of one another. In the leaf cutting-ants genus *Acromyrmex,* the building of a thatched nest allowed some *Acromyrmex* species to extend their distribution range more in the southern temperate regions, compared to those inhabiting subterranean nests (Farji-Brener 2000) (see also chapter 4). Results reported here suggest that thermoregulation in thatching *Acromyrmex* species is not only achieved through the long-term response of constructing a thatch (chapter 4), yet by short-term responses as well, as the use of nest temperature for triggering thermoregulatory building behaviour. Besides their occurrence in temperate regions, thatch-builder *Acromyrmex* species also colonized some South American semi-arid and hot regions. The two *Acromyrmex* species that are thatch-builders per excellence, *A. heyeri and A. lobicornis,* also inhabit the Argentinean and Paraguayan dry Chaco (Bucher and Montenegro 1974; Farji-Brener and Ruggiero 1994; Fowler 1985), which should be, a priori, considered as unsuitable for the highly-exposed fungus gardens of superficial thatched nests. It is argued that thatching *Acromyrmex* colonies are able to survive in these regions because of the existence of regulatory building responses that trade off thermoregulation for humidity control, allowing the immobile brood and the symbiotic fungus to be effectively protected against harmful water losses.

6. To build or not to build: circulating dry air organizes collective building for climate control in the leaf-cutting ant *Acromyrmex ambiguus*

Summary To investigate to what extent climatic variables are used by ant workers to decide whether and where nest building should be started, the effect of humidity content of the circulating air on building behaviour was studied in the leaf-cutting ant *Acromyrmex ambiguus*. In the laboratory, a colony was confronted with an experimentally-induced circulation of either dry or wet air through its nest chambers, and the plugging of nest tunnels with leaf fragments was recorded as indicative building response. Results showed that circulating dry air triggered building responses. Tunnel plugs were constructed mostly in the inflows of dry air and to a much lesser extent in the outflows of dry air, indicating that the direction of the air movement inside tunnels spatially guided the workers' building behaviour. However, circulating humid air, or a passive loss of humidity from the nest, did not suffice to trigger tunnel plugging. Taken together, results showed that *A. ambiguus* workers use the direction of the airflow as an environmental cue for spatially guiding their building behaviour, yet workers only responded if the humidity content of the circulating air was low. These building responses seem to result from a compromise between the need of air renewal and the avoidance of unsuitable climatic conditions. These results are the first in demonstrating that ant workers respond to a specific climatic variable to decide whether and where a nest structure, related to the control of nest climate, should be built.

6.1. Introduction

In social insects, the control of internal nest climate is not only achieved through particular workers' behaviours, such as wing fanning in honey bees (Jones et al. 2004) and bumblebees (Weidenmüller et al. 2002), active brood heating in honey bees (Kleinhenz et al. 2003), or brood-carrying to suitable nest environments in ants (Roces and Núñez 1995), but also by some features of the nest the workers have built, as material composition and shape (Turner 2000b). For instance, nests can act as an insulating barrier retaining the internal nest heat (chapter 3) (Coenen-Stass et al. 1980; Frouz 2000; Horstmann 1990), or promote ventilation and therefore gas exchange with the environment (Kleineidam et al. 2001; Korb 2003; Lüscher 1961; Turner 2001). Although nest architecture may have a long-term effect on the stabilization of nest climate, short-term variations of the internal nest environment, as caused by nest ventilation, are expected to be controlled by the colony members. Nest ventilation has to be promoted for allowing air and heat exchanges with the environment, but at the same time controlled in order to avoid adverse consequences such as colony cooling or desiccation of internal air.

This trade-off is expected to be particularly relevant for leaf-cutting ants, which rear large fungus gardens inside their nests. This symbiotic fungus represents the sole food source for the colony, and it has strict demands of high humidity and temperatures between 25 and 30°C for proper growth (Powell and Stradling 1986). Workers seem to maximize the growth of the fungus by relocating the fungus gardens to nest areas where temperature and humidity are more suitable for their survival (Bollazzi and Roces 2002; Lapointe et al. 1998; Navarro and Jaffé 1985; Roces and Kleineidam 2000; Weber 1957) (see also chapter 2). In addition, workers are also able to counteract the unfavourable climatic conditions inside nest by modifying the nest architecture (Jonkman 1980), as also observed in *Formica* ants (Horstmann and Schmid 1986), thus influencing nest ventilation.

Most nests built by colonies of *Acromyrmex* species are located superficially (Fowler and Claver 1991), so that they are highly exposed to external climatic conditions. As a consequence, *Acromyrmex* species are expected to have developed specific mechanisms for controlling the nest climate. For instance, in the leaf-cutting ant *Acromyrmex ambiguus,* which inhabits superficial nests excavated in sandy soils of Uruguay, Argentina and south Brazil (Farji Brener 1994; Gonçalves 1961; Zolessi et al. 1989), workers collect leaf fragments not only as substrate for the fungus, but also as building material, using them to plug the tunnels that connect the fungus chambers with the outside (Bonetto 1959). The idea that such leaf plugs may reduce nest ventilation and protect the colony against desiccation of internal nest air is intuitive, although their function, and the variables that trigger their construction, are unknown.

Although the idea that climatic variables inside social insect nests may act as organizers of building behaviour is commonly accepted (Bonabeau et al. 1997; Cox and Blanchard 2000; Theraulaz et al. 1998), almost no attempt has been made to quantify their effects under controlled experimental conditions. Up to date, studies on building behaviour in social insects concentrated on two major topics. First, on the description and modelling of organizing processes once nest building has been started, without considering the variables that trigger workers' responses in the absence of a preceding structure or stimulation through nestmates (see reviews in: Camazine et al. 2001; Hansell 2005). Second, on the consequences of the whole building effort, i.e., the resulting nest, for the internal nest climate (Heinrich 1993a; Korb 2003; Sudd 1982; Turner 2000b; Turner 2001). Worker building responses are usually seen as the way through which the colony achieves an accurate control of nest climate (Hansell 2005; Turner 2000b). However, that control can only be achieved if workers are able to properly decide where and when a structure has to be built or modified. Interestingly, this question has not been experimentally addressed so far. The present study was aimed at elucidating whether, and which, climatic variables are used by *A. ambiguus* leaf-cutting ant workers to start building and to spatially guide their responses.

Colonies of *Acromyrmex ambiguus* appear to be, for three main reasons, particularly suited for investigating the occurrence of collective building responses and the environmental variables responsible for their organization. First, colonies are small and the spatial arrangement of the nest chambers can be simulated in the laboratory. Second, colonies show a conspicuous and quantifiable building behaviour, i.e., workers relocate leaf fragments usually used to feed the fungus for plugging the nest tunnels. Third, the relocation of leaf fragments to build plugs, as a consequence of experimentally-induced changes in climatic variables, can be used as indicative response to investigate what variables trigger workers' building behaviour.

In the present study, a colony of *Acromyrmex ambiguus* was placed in a laboratory nest that simulated the spatial arrangement of chambers found in nature, and it was confronted with an experimentally-induced circulation of either dry or humid air through its nest chambers. The questions whether the humidity content of circulating air and/or the direction of air flow trigger tunnel plugging were experimentally addressed, by quantifying the percentage of harvested leaf fragments relocated to close nest tunnels.

6.2. Methods

A one year old *Acromyrmex ambiguus* colony was collected in Bello Horizonte, Uruguay (34°45’32’’ S, 55°40’57’’ W), and transported to the Department of Behavioural Physiology and Sociobiology at the University of Würzburg, Germany. At its arrival the colony was placed inside the artificial nest where the experiments were carried out. Since colonies vary greatly in size, worker size distribution, and harvesting motivation, a standardization of colony responses would be very difficult to achieve, and pooling of data from different colonies may mask the effects of the variables studied. In order to avoid these sources of variability, only one colony was used to perform the laboratory experiments.

6.2.1. Experimental setup

The experimental arrangement of the laboratory nest reproduced a natural nest of *A. ambiguus* as described by Bonetto (1959) (Fig. 6.1a), with chambers located in the lower level and foraging boxes in the upper level (Fig. 6.1b). The lower level consisted of three interconnected chambers placed inside a thermostatic box (Tb) maintained at 27°C and 98% RH (Fig. 6.1b). The central chamber contained the colony with a single, well-developed fungus garden (F) (volume: 300 cm^3), while the two lateral chambers, Ca and Cb, were empty (Fig. 6.1b, c). The upper level consisted of two pairs of foraging boxes (A1-A2 and B1-B2) placed above the thermostatic box (Fig. 6.1b,c), and maintained at room conditions (25°C, 40% RH). The floor of each foraging box was connected downwards via a single tube (10 mm in diameter) to one of the empty chambers (Fig. 6.1b,c,d). As a consequence, the nest was divided in two lateral areas located at opposite sides of the fungus chamber, each one composed by an empty chamber and two foraging boxes (Fig. 6.1c). Due to this spatial arrangement, foraging workers coming into the fungus chamber from the foraging boxes needed to pass through one of the empty chambers.

The nest arrangement described above not only reproduced the shape of a natural nest, but also allowed a controlled air circulation, as it may occur during nest ventilation, to be experimentally simulated. For that, air was pumped in one of the foraging boxes (for instance B1), forced to circulate through the empty chamber

(Cb), and it left the nest through the paired foraging box (B2, Fig. 6.1d). In addition, the temperature difference between the thermostatic box (27°C) and the room air (25°C) passively promoted convective air movements between the three chambers and the outside, when no forced air circulation was induced. Through these convective air movements, the colony experienced a passive air exchange with the outside and a loss of humidity from the fungus garden, saturated with water vapour, into the dry room air.

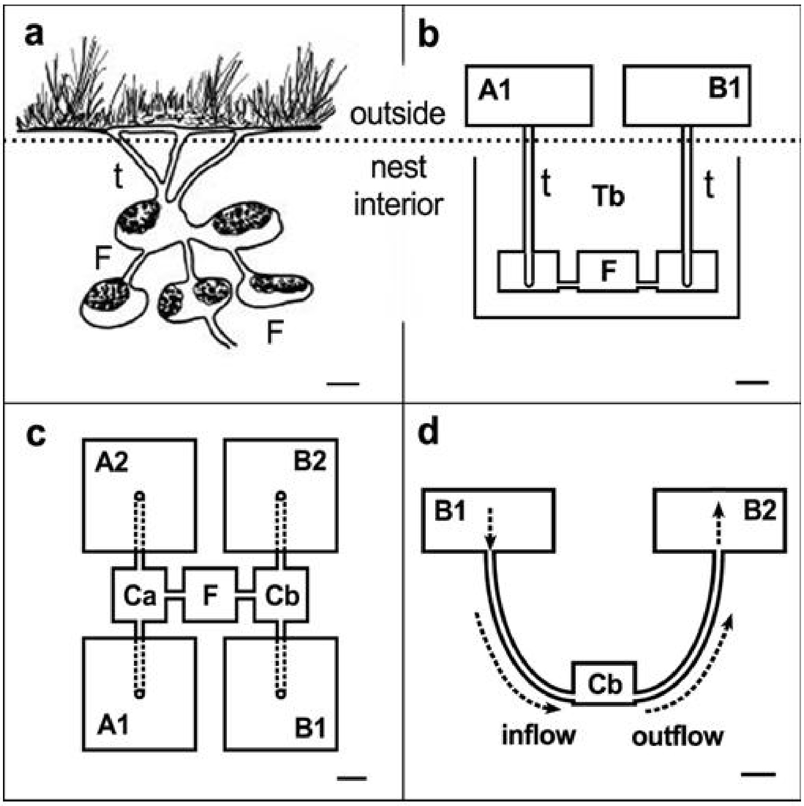

Figure 6.1: ***a)*** *Acromyrmex ambiguus* nest, from Bonetto (1959). ***b)*** frontal view of the experimental nest. The horizontal dotted line crossing through the figures a and b indicates the boundary line between the nest interior and the outside. ***c)*** top view of the experimental nest showing the two lateral areas located at each side of the fungus chamber (A1-Ca-A2 and B1-Cb-B2). ***d)*** lateral view of the experimental nest showing one nest side (B1-Cb-B2); the dashed arrows indicate the air circulation inside the tunnels. The tunnel entrances that provided the inflow and outflow of air are indicated. t = tunnels, F =fungus chamber, Ca and Cb = empty chambers, Tb = thermostatic box, A1 to B2 = foraging boxes. In all figures the scale bar represents 5 cm.

To give an idea of the humidity and temperature conditions experienced by field colonies of *A. ambiguus,* the relative humidity and the temperature of both the outside air and the internal nest air were recorded in a field nest located in Uruguay (for more details see caption of Fig. 6.2). Even though the conditions inside the nest were stable, the outside air, which might enter the fungus chamber during nest ventilation, reached extreme temperatures and a relative humidity as low as 25% (Fig. 6.2).

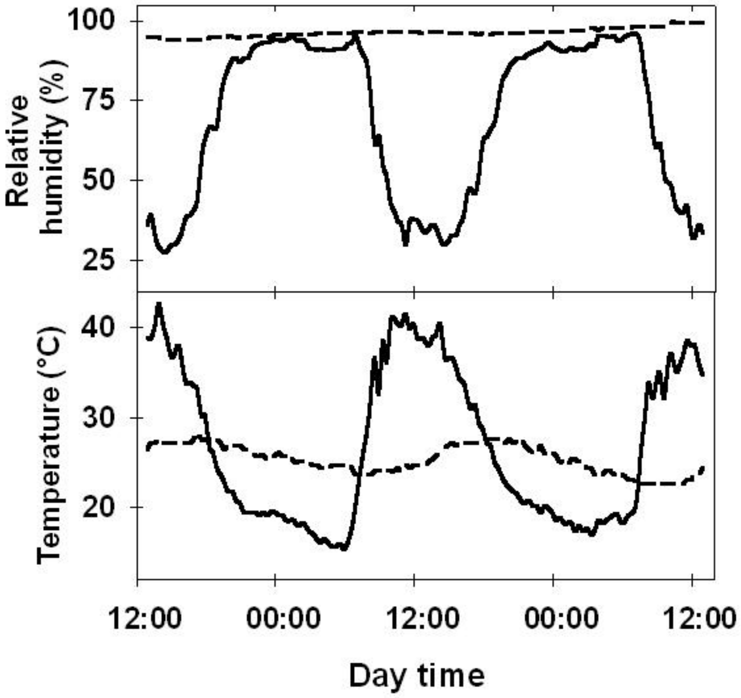

Figure 6.2: Variation of relative humidity and temperature inside the fungus chamber of an *Acromyrmex ambiguus* field nest (dashed lines) and in the environment (continuous lines) as a function of day time (21st to 23rd January 2003). The nest was located at the Aguas Dulces beach in Uruguay (34°9'24'' S, 53°40'24'' W), and was composed of a small fungus chamber placed at 15-25 cm depth, connected to the outside through three tunnels with a diameter of approximately 1 cm.

6.2.2. Experimental series

The experimental nest arrangement was aimed at exploring whether the humidity content of circulating air would trigger collective building responses. For that, workers were confronted with the circulation of either dry (below 25 % RH) or humid air (over 90% RH) through the empty chambers, while they foraged in the upper boxes and transported leaf fragments into the fungus garden. Depending on the sensed humidity in the air circulating through the empty chambers (Ca and Cb, Fig. 6.1c,d), workers might decide about the destination of the carried leaf fragment. As a response, they could carry the fragments into the fungus garden, or accumulate them inside the tunnels ending in the empty chamber. As described above, each empty chamber (either Ca or Cb) is connected with three locations: two foraging boxes that delivered either an inflow or outflow of air, and the fungus chamber (Fig. 6.1c,d). The accumulation of leaf fragments inside any of these three nest tunnels was recorded as indicative response of whether circulating dry or humid air triggered tunnel plugging. The characteristics of the experimental nest, with a central fungus chamber and two nest sides with independent control of air circulation, allowed us to present the colony simultaneously with different regimes of air circulation at the opposite sides of the fungus garden, following the experimental schedule presented in Table 6.1. The experimental series A and B were done to elucidate whether the circulation of either dry or humid air suffices to trigger the deposition of leaf fragments inside tunnels. The series C was aimed at investigating whether circulation of humid air in the whole nest triggers tunnel plugging by workers. The series D was done to elucidate whether workers react to airflow through a generalized response by plugging all nest tunnels through which air circulates, or whether the humidity content of the air, independent of the airflow, triggers building behaviour.

Experimental series	Airflow condition at one nest side:	Airflow condition at the other nest side:
A	Passive air exchange (76.8 ± 13.1 %)	Forced dry air (12.9 ± 10.1 %)
B	Passive air exchange (78.0 ± 14.2 %)	Forced humid air (95.6 ± 4.4 %)
C	Forced humid air (98.5 ± 0.6 %)	Forced humid air (98.5 ± 0.6 %)
D	Forced humid air (98.1 ± 0.9 %)	Forced dry air (12.4 ± 6.2 %)

Table 6.1: Air condition inside each lateral area of the nest during the experimental series A to D. The values in brackets represent the measured relative humidity (Mean ± SD, N=10 each).

6.2.3. Experimental procedure

All assays began with a completely humidified nest and no forced circulation of air. Every day at 17:00 h the colony was fed with a similar amount of leaves (*Medicago* sp.) at each foraging box (approx. 1 g). The experimentally-induced circulation of air was turned-on, and the colony was left undisturbed over 16 hours. Air inside tunnels was adjusted to circulate at 6 –7 cm/s (measured with an anemometer, Testo® 405-V1), in the range of natural airflows measured in tunnels of *Atta* leaf-cutting ant nests (Kleineidam et al. 2001). At 09:00 h of the next day, the air humidity inside the empty chambers was measured by introducing termohygrometers (Testo® 605 H-1) through small holes located in theirs covers. After that, the empty chambers were opened, the material deposited by the ants in each tunnel was collected and weighted, and the amount found inside the tunnels was expressed as a percentage of the total offered to forage. Between 0900 hours, end of the experimental series, and 17:00 h, beginning of a new one, the colony was not fed, the air circulation was interrupted, and the chambers were left open to humidify them via the saturated air of the thermostatic box. The experiments A, B, C and D were daily alternated, and the conditions at each lateral area were randomly assigned. Ten replicates were done for each experiment. Throughout the experiments, and irrespective of the airflow conditions imposed, the relative humidity inside the fungus garden box remained constant over 98 %.

6.3. Results

When workers experienced the circulation of dry air within the nest (experiment A), they deposited leaf fragments inside the tunnels through which dry air circulated. However, no accumulation of material inside tunnels with passive air movements was observed (Fig. 6.3, experiment A).

With forced circulation of humid air, no accumulation of leaf fragments was observed inside tunnels of the nest side where humid air circulated or in the side with passive air movements (Fig. 6.3, experiment B). In addition, forced circulation of humid air at both sides of the fungus chamber did not trigger accumulation of leaf material inside tunnels at all (Fig. 6.3, experiment C).

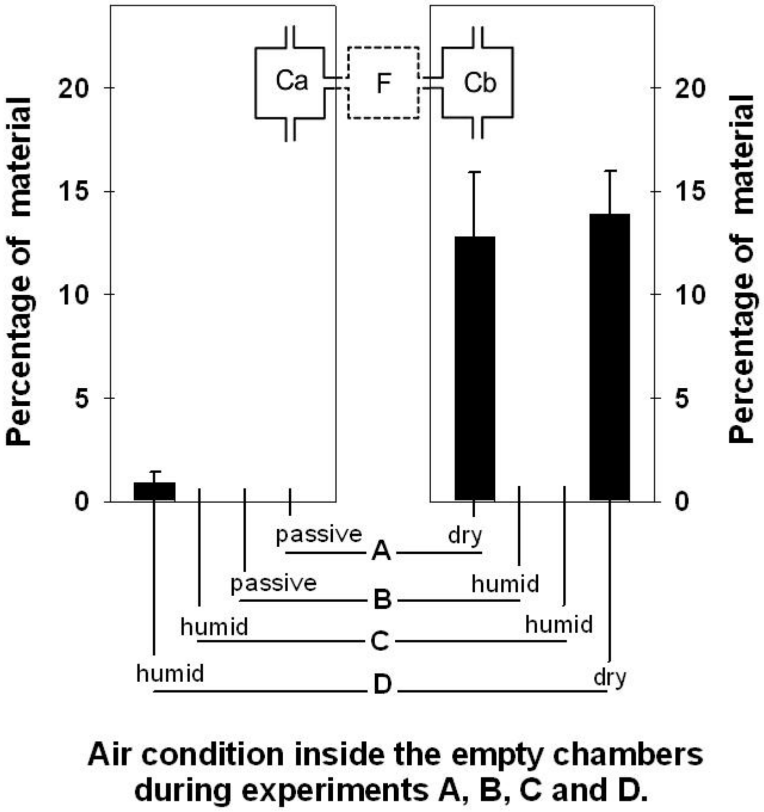

Figure 6.3: Bars indicate the percentage (± SE) of material found inside the tunnels of each empty chamber, from the total of leaf material collected by workers during foraging, for the four experiments. The airflow conditions simultaneously imposed at the two nest sides during the experiments A, B, C and D are indicated on the abscissa, and correspond to the conditions presented in Table 6.1. The scheme inserted at the upper part corresponds to figure 1c and shows the connecting tunnels of the empty chambers where workers could deposit leaf material.

In the experiment with simultaneous forced circulation of dry and humid air (Fig. 6.3, experiment D), workers deposited leaf fragments in the tunnels of the nest side through which dry air circulated, and to a much lesser extent inside the nest side where humid air circulated (paired *t* test: $t_9 = 6.73$, $p<0.001$).

When presented with circulating dry air (experiments A and D), workers distributed the leaf fragments among the three tunnels of the empty chamber through which dry air circulated. However, the material was mostly deposited inside the tunnel that delivered the inflow of dry air (Fig. 6.4, one way ANOVA: $F_{48,2} = 30.34$, $p<0.001$), forming what was defined as a leaf plug. Such leaf plugs looked like an accumulation of leaf fragments that tightly fit into the tunnel diameter, although workers were still able to walk through, and covered 3-4 cm of tunnel length (see photograph in Fig. 6.4, on the left). To give an idea of how many fragments are needed to build a tunnel plug, the average mass of a plug, 0.66 g, SD=0.21, N=17, was divided by the average mass of a harvested leaf fragment, 0.0078 g, SD=0.0053 g, N=36. Approximately 84 fragments are needed to plug a tunnel of 10 mm in diameter, as the tunnels used in the experimental nest.

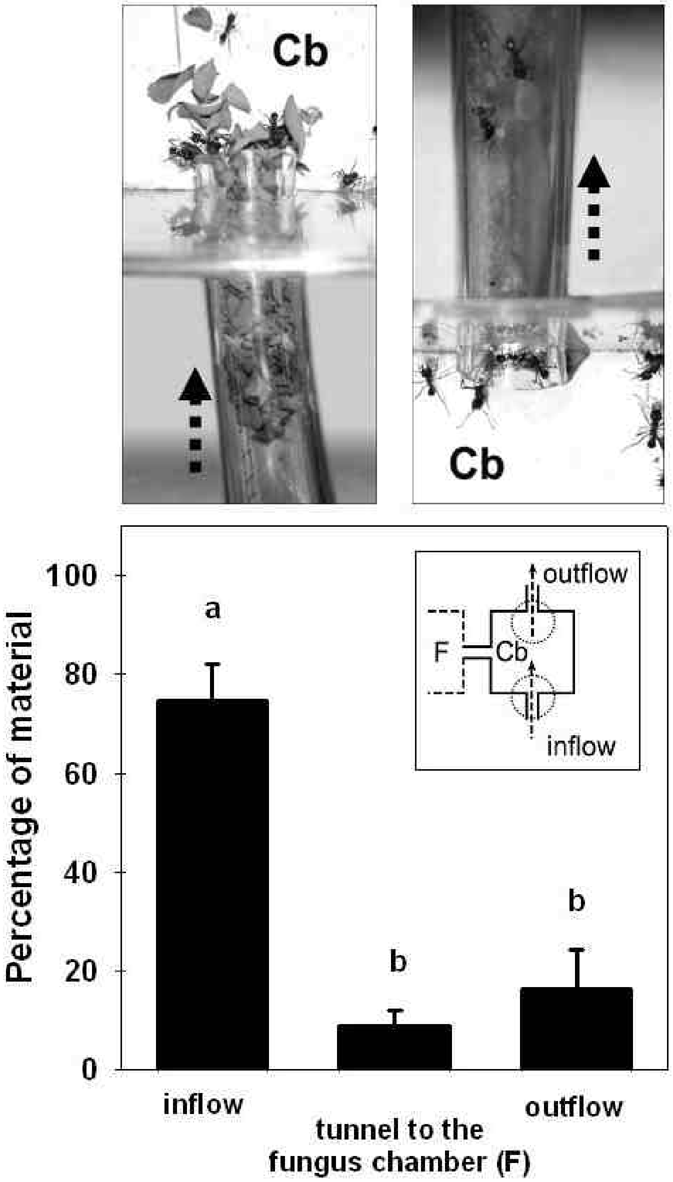

Figure 6.4: Percentage (± SE) of material inside the three tunnels of the empty chamber where dry air circulated. The percentages are expressed as a function of the total amount found in the three tunnels. Results of experiments A and D were pooled. The inset, extracted from figure 6.1c, shows the locations of the three tunnels and the direction of circulating air: the inflow tunnel, the outflow tunnel, and the tunnel that led into the fungus chamber. The pictures placed above the graph show an inflow tunnel with a leaf plug (left) and an outflow tunnel without accumulated material (right), the arrows represent the direction of the air flow inside the tunnels. The dotted circles in the inset indicate the places where the pictures were taken. The columns sharing the same letter are not significantly different, Tukey HSD and Scheffé Post-hoc test, $p<0.001$.

6.4. Discussion

The results reported here indicated that specific climatic variables are used by workers of the leaf-cutting ant *Acromyrmex ambiguus* to decide whether, and where, a particular nest structure should be built. The low humidity content of the air circulating through the nest chambers triggered building responses. However, airflow with high humidity, or a passive loss of humidity from the nest, did not suffice to trigger the building of a tunnel plug. The observed building responses consisted in the relocation of leaf fragments, harvested and carried into the nest to feed the fungus, to the construction of leaf plugs inside tunnels through which dry air circulated. Workers relocated in average 12-13 % of the leaf fragments harvested as forage. The fact that workers mostly accumulated the leaf material in the tunnel that delivered the inflow of dry air indicates that the direction of air movements inside the nest is a cue that spatially guides their building behaviour. Responses to air movements have already been investigated in solitary insects (Bell and Kramer 1979; Böhm 1995), and during building activities in termites (Howse 1966), but the present results on *A. ambiguus* emphasize their importance for the collective control of nest climate.

What are the consequences of the described building responses of *Acromyrmex ambiguus* workers for the maintenance of an adequate nest microclimate? Due to the superficial location of *A. ambiguus* nests (Fowler and Claver 1991), movements of the external air at the soil surface can passively promote air exchanges between the nest and the outside (Kleineidam et al. 2001; Vogel 1978), and workers are expected to show regulatory responses in order to avoid changes of the internal nest climate. For that, workers should be able to sense the relevant environmental variables, such as CO_2, air temperature and humidity (Kleineidam et al. 2000; Kleineidam and Tautz 1996), and use them as cues for guiding their building responses. Results suggest that *Acromyrmex ambiguus* workers sense the humidity content of the circulating air, and that low air humidity releases tunnel plugging to avoid nest desiccation. However, nest plugging to inhibit the inflow of dry air cannot be an all-or-non response, since complete nest-closing may lead to poor nest ventilation and therefore to increased CO_2 concentrations inside the nest, as reported for fungus-growing ants and termites (Kleineidam and Roces 2000; Korb 2003). If high CO_2 concentrations have to be avoided, air exchanges with the outside have to be allowed, as much as the humidity content of the circulating air does not drop beyond a value harmful for the symbiotic fungus. From this point of view, it seems reasonable that the circulation of humid air does not trigger building responses, as observed in the experiments. Only when the circulating air becomes extremely dry, regulatory building responses are triggered, so that the inflow of dry air would be avoided via the construction of a leaf plug. Thus, the observed workers' responses can be considered as a trade-off between the colony necessities for air exchange, to get rid of the CO_2-enriched air, and the maintenance of high humidity levels by avoiding the circulation dry air.

It is important to indicate that plugging of nest entrances has been already described for ants in different contexts, such as colony defence and protection during colony overwintering, (Brandão et al. 2001; Eidmann 1942; Wilson 1974). In leaf-cutting ants, it has been reported that workers of *Atta vollenweideri* close the nest entrances during heavy rains so as to prevent nest flooding (Kleineidam and Roces 2000), and also in winter, probably to avoid the inflow of cold air (Jonkman 1980). In laboratory colonies of *Atta sexdens*, workers were observed to form leaf caches at nest entrances, or to put leaf fragments on holes drilled in the nest boxes (Leite 1995). Field colonies of *Atta texana* close their nest entrances during the day, with dry twigs and sand pellets, and reopen them at night to resume foraging (McCook 1879). Although the cues triggering nest closing in *Atta* workers were not explored, workers responses appear to be similar to those analyzed in the present paper for *Acromyrmex ambiguus* in two ways. First, workers plugged nest tunnels at the boundary with the outside air, where step gradients in climatic variables such as air humidity, temperature or even air pressure are expected to occur. Second, nest plugs seem to be an adaptation aimed at protecting the colony against adverse external conditions.

Nests of social insects are considered as adaptive structures, providing colony members with protection and a stable climatic environment (Emerson 1956; Hansell 2005; Sudd 1982; Turner 2000b). As a consequence, the idea that workers control nest climate through their building activities is intuitive and commonly accepted (Stuart 1972; Turner 2000a), although the question of what climatic variables trigger and spatially guide local building responses remained experimentally unexplored. A collective control of nest climate can only be achieved if workers are able to properly decide where and when a structure has to be built for the control of nest ventilation.

In ants, although digging behaviour, workers' activity as well as the dynamics of corpse aggregation are known to be influenced by climatic variables (Burkhardt 1991; Challet et al. 2005; Hangartner 1969; Jost et al. 2006), the question whether these responses contribute to the control of nest climate remains elusive.

Understanding how workers decide to start building, and where, is critical if nest modifications are considered as adaptations aimed at providing the colony with a suitable nest environment. These results are probably the first in demonstrating that ant workers respond to a specific climatic variable to decide whether and where a nest structure, related to the control of nest climate, should be built. As stated by Hansell (2005), "Natural selection should operate on builders through the success of the structures that they build and also the success of the building process compared with rival methods of construction". In this sense, collective building responses triggered and spatially organized by environmental variables are expected to influence nest climate in social insects, and hence improve colony fitness by achieving a suitable environment for the colony members.

7. General discussion

In insects, long-term adaptations to adverse or changing climatic conditions may adopt the form of diapause, aestivation or some form of dormancy, yet in the short-term, mostly the form of behavioural responses (Chown and Nicolson 2004). Since fungus-growing ants originated in South America during the early Cenozoic, 45-65 million of years ago (Mueller et al. 2001), they had to adapt to the climatic changes that occurred in the continent over evolutionary time. At the beginning of the Cenozoic, the South American climate was warm, wet and non-seasonal (Ortiz-Jaureguizar and Cladera 2006), conditions that can be considered as suitable for fungus growing in general (Griffin 1994). While these conditions remained unchanged in northern South America, the climate became dryer and seasonal in regions southern than 15°S, and the biomes changed from tropical forests to steppes (Ortiz-Jaureguizar and Cladera 2006). Given that the species richness of leaf-cutting ants of the genera *Atta* and *Acromyrmex* is much higher southern of the 15°S, and that colonies depend on a symbiotic fungus with strict demands for humidity and temperature for proper growth, leaf-cutting ants are expected to have developed, over evolutionary time, behavioural adaptations to maintain proper climatic conditions for fungus growing inside their nests.

In social insects, the outcome of their collective building activities, i.e., the nest, has been considered the main way through which colonies gain some degree of control over the environment (Hansell 1993; Hansell 1996). For instance, nest architecture influences nest ventilation, and account for the maintenance of suitable nest temperatures and humidity (Frouz 2000; Kleineidam et al. 2001; Korb 2003; Rosengren et al. 1987; Turner 1994; Turner 2001). Up to date, theoretical models that consider stigmergic mechanisms, self-organizing mechanisms and templates, emphasized that the structure of social insect nests results from a succession of stimulus-response steps involving the workers, the environment, and the by-product of their activities (Bonabeau 1998; Deneubourg and Franks 1995; Theraulaz and Bonabeau 1999; Theraulaz et al. 1998). However, such models have so far not considered the question about the adaptive value of the observed structures, i.e., to what extent distinct nest designs might result from adaptations to local climatic conditions. As stated by Bonabeau (1998), self-organizing mechanisms that only consider the interactions among workers, and with the structure they are building, might not explain how colonies adapt to changing environmental conditions by building different nest designs. For that, organizing forces outside the system, i.e., which do not result from the interactions among workers and the structure, have to be considered as well, in particular climatic factors such as temperature and humidity (Bonabeau 1998). In the African termite *Macrotermes bellicosus* it has been shown that different temperature regimes in savannas and gallery forests lead to distinct nest architectures, which positively influence thermal homeostasis within the nest in both habitats (Korb 2003). In ants, it remained experimentally unexplored up to date whether building behaviour organized by climatic factors results in distinct nest designs adapted to different local climatic conditions.

7.1. Climatic variables as cues for building behaviour: the maintenance of a proper nest climate

In *Acromyrmex,* climatic variables, particularly temperature and humidity, were shown to act as cues for workers' building behaviour, which may account for the maintenance of nest climate in a proper range for the inhabiting colony. For instance, workers respond to soil temperature, as well as its changes and decide accordingly where to start, or whether to stop digging (chapter 3). In addition, results strongly suggest that this use of soil temperature as a cue for digging behaviour may result in the occurrence of different nest designs that maintain nest climate in a proper range for colonies. The observed nest depth of *Acromyrmex* nests decreases from the South American tropics towards the temperate regions, resulting in subterranean and superficial nesting habits, respectively, and in the maintenance of proper temperatures for the fungus gardens (chapter 3). Results furthermore indicate that *Acromyrmex* colonies achieve benefits through the determination of nest depth based on thermoregulatory needs. For instance, in South American temperate areas, temperature inside superficial thatched nests was higher, and more suitable for fungus and colony growth, than that inside subterranean nests (chapter 4).

The hypothetical interactions between soil temperature and temperature-sensitive digging in determining nest depth in South American *Acromyrmex* species is summarized in Figure 7.1 (upper and middle portion). Although highly advantageous for colonies in terms of nest temperature, the determination of nest depth based on thermoregulatory needs may compromise humidity maintenance or nest ventilation depending on the nest type (Fig. 7.1, lower portion). First, nest humidity would be particularly compromised in the more exposed fungus gardens of those colonies exhibiting a superficial nesting habit, i.e., inhabiting thatched nests or changing between subterranean and thatched nest. This view is supported by the demonstration that short-term building responses triggered and spatially organized by nest temperature, humidity and the direction of circulating air in fact occur (chapters 6 and 7), which may counteract undesired changes of nest humidity in species with superficial nesting habit. Second, nest ventilation would be particularly compromised in the fungus gardens of those colonies exhibiting a subterranean nesting habit, i.e. inhabiting only subterranean nests, since nest depth is known to negatively affect the exchange of respiratory gases between the nest and the atmosphere (Wilson and Kilgore, 1978). Such deficient nest ventilation is unlikely to be counteracted by short-term building responses of workers. Long-term physiological adaptations of fungus, workers, and brood, such as an increased tolerance to high CO_2 concentrations, are therefore expected to have taken place, as known for several soil-dwelling insects (Kupka and Schaerffenberg 1947).

7.2. Use of climatic factors as cues for building: its adaptive value

Although leaf-cutting ants are commonly considered to be characteristic of tropical forests, members of both leaf cutting-ants genera, *Atta* and *Acromyrmex,* are more abundant and diverse in the South American subtropics (Cherrett 1989; Fowler 1983b). For *Acromyrmex*, species richness increases from the tropics towards northern Argentina and southern Brazil, and Paraguay and Uruguay are particularly rich for their sizes (Cherrett 1989). Two different hypotheses have been advanced to account for this pattern of species richness. First, the higher species richness with increasing latitudes was considered the consequence of the greater diversity of vegetation types found in subtropical regions compared to the tropical ones, which allowed the *Acromyrmex* genus to radiate in several leaf- and grass-cutters species (Cherrett 1989). Second, it was considered as the consequence of the pattern of decreasing levels of plant allelochemicals from the tropical to the temperate regions (Fowler 1983b). They are expected to significantly compromise fungal growth and survival, and therefore to have stronger limited radiation in the tropics than in the subtropics. Both hypotheses will therefore explain why the genus *Acromyrmex* presents its major taxonomic richness in the temperate and subtropical South American regions, from 20 to 30°S (Fig 7.2). However, the observed south-biased distribution exclusively results from an increase in the number of species that have been recorded showing a superficial nesting habit, i.e., species that are either exclusively thatch builders or that may change between subterranean and thatched nests (Fig 7.2).

As discussed above, nest depth determination based on thermoregulatory needs leads to a subterranean nesting habit in tropical regions and a superficial nesting habit in subtropical regions, which compromises either nest ventilation or nest humidity, respectively (Fig 7.1, lower portion). It is suggested that one important reason why species richness in *Acromyrmex* is higher in temperate and subtropical South America is the existence of short-term building responses for the control of humidity losses in those species showing a superficial nesting habit (Fig. 7.1, lower portion, right side). By the contrary, colonies showing a subterranean nesting habit in the tropics would not be, a priori, able to counteract deficient nest ventilation by short-term building responses, which may negatively impact on fungus and colony growth (Fig. 7.1, lower portion, left side). Species richness in *Acromyrmex* would therefore be higher in the South American subtropics because of the occurrence of behavioural adaptations to counteract the negative effects of nest-depth determination based on thermoregulatory needs, which may have not occurred in the tropics. In builder organisms, such as ants, natural selection will favour those building behaviours that allow colonies to breed early, have more offspring and survive better, but only if the resulting nest does not create trade-offs between life traits that negatively impact on colony life (Hansell, 2005). Thus, building behavior for the control of nest humidity would be a key behavioural adaptation that improves colony’s survival in temperate and subtropical regions, because it counteract the compromised nest humidity imposed by nest depth determination based on thermoregulatory needs. Although for builder organisms, improved environmental control through building behaviour would not necessarily be linked to speciation, the increased chances of survival in a given habitat might subsequently facilitate radiation (Hansell 2005). Therefore, besides the great diversity of vegetation types and the low concentration of plant allelochemicals in the subtropics (Cherrett 1989; Fowler 1983b), the use of climatic variables as cues for nest building, aimed at controlling both temperature and humidity losses, should be considered one additional factor that explains the higher richness of *Acromyrmex* species in the subtropical areas of the South American continent.

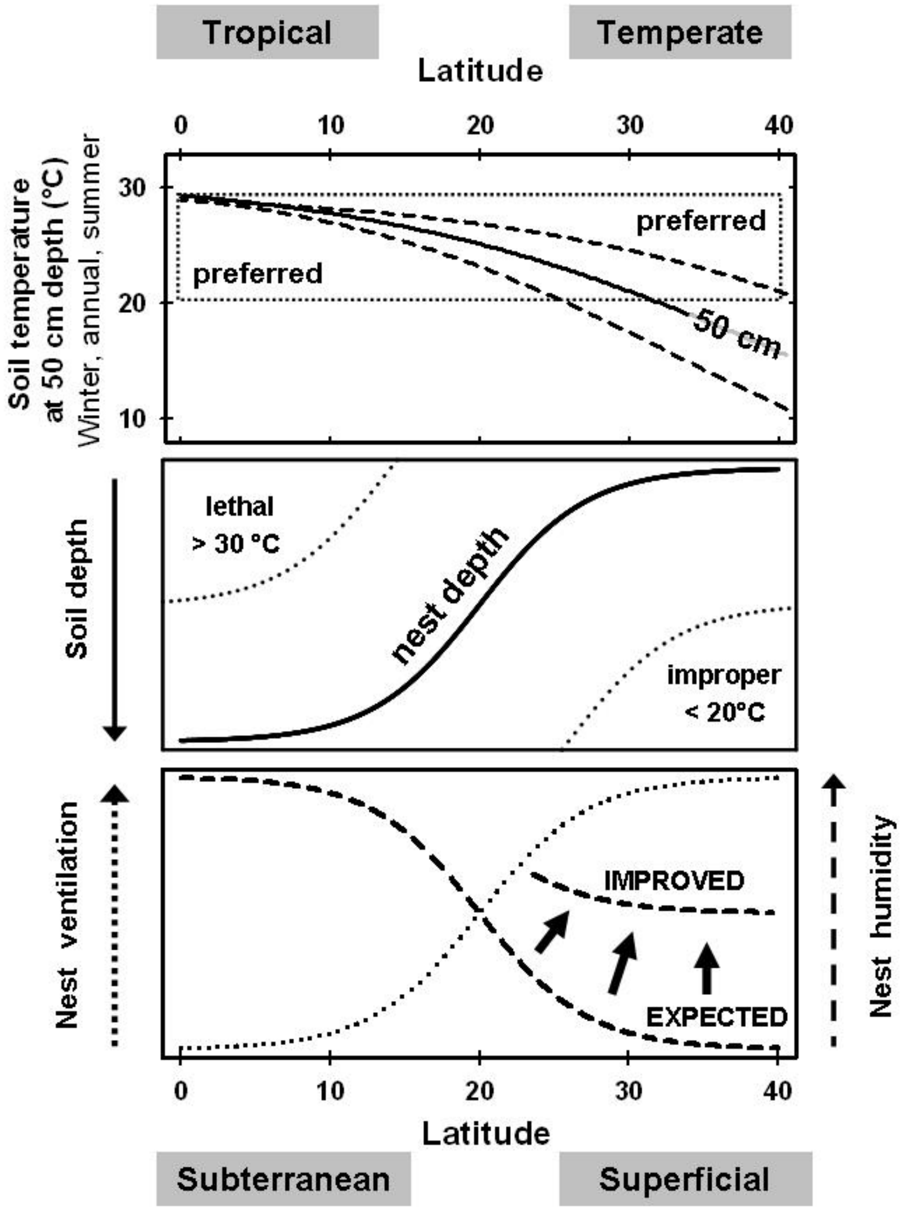

Figure 7.1: Suggested model for the interaction between soil temperature and temperature-sensitive digging in determining nest depth in South American *Acromyrmex* leaf-cutting ants, and how the different nest types affect nest humidity and ventilation. **Upper portion:** in South America, average soil temperature at 50 cm depth decreases from 30°C in tropical regions, to less than 20°C in temperate ones (upper portion, based on Van Wambeke (1981)). Independently of latitude, soil temperature decrease with increasing depth. Thus, the range of soil temperatures preferred by workers to establish the fungus chambers, from 20°C to maximally 30°C (dotted area), will be located above 50 cm in temperate regions, and bellow 50 cm in tropical regions. **Middle portion**: nest depth is

expected to decrease with increasing latitude (continuous line), since *Acromyrmex* colonies should excavate their fungus chambers so as avoid soil temperatures higher than 30°C, located above 50 cm depth in the tropics (lethal for fungus, dotted line, left upper corner), as well as improper temperatures lower than 20°C, to be found below 50 cm depth in temperate regions (dotted line, right down corner). Thus, the interaction between temperature-sensitive digging and how soil temperature varies with latitude and depth would result in a superficial nesting habit in temperate and subtropical regions, and a subterranean nesting habit in the tropical ones. **Lower portion:** The nest type is expected to differentially affect nest ventilation (left Y-axis) and humidity (right Y-axis) depending on how nest exposition influences the exchange of inside air with the (mostly dry) outside air. On the one hand, colonies inhabiting the non-exposed subterranean nests (left side) might benefit from high humidity inside their nests, but face long-term deficient nest ventilation. On the other hand, colonies inhabiting the highly exposed superficial nests (right side) might benefit from improved nest ventilation and, a priori, low nest humidity, due to humidity losses ("expected"). However, this desiccation risk could be counteracted by the workers' building behaviour, so that a higher nest humidity can be maintained inside the nests ("improved").

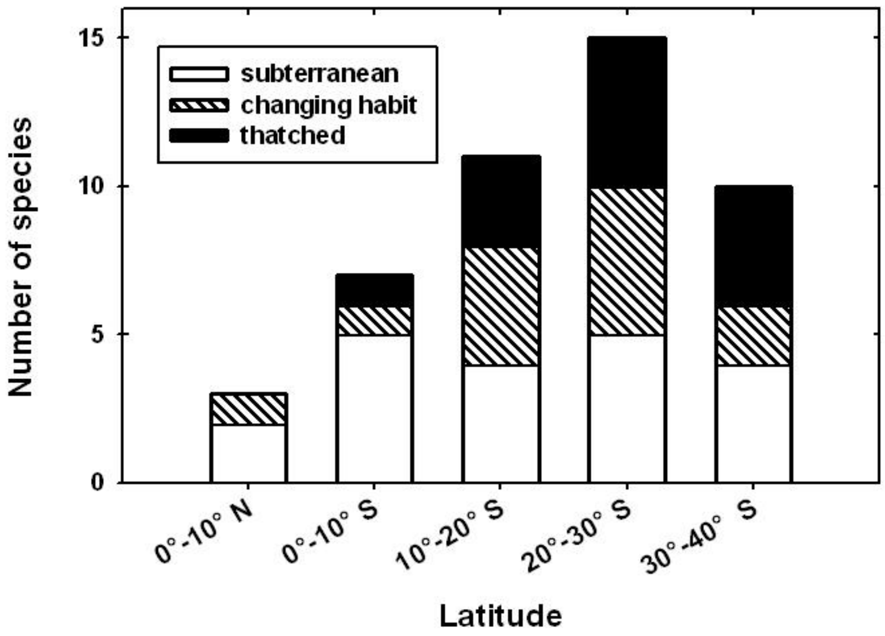

Figure 7.2: Number of *Acromyrmex* species presenting only subterranean nests (white portion), only thatched nests (black portion), or that change between subterranean and thatched nests (striped portion) in relation to the latitudinal range of the South American continent. Based on Farji Brener (2000), Fowler and Claver (1991), Gonçalves (1961), Bonetto (1959), and Fowler (1985).

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Erklärung

Erklärung gemäß § 4 Abs. 3 S. 3, 5 und 8 der Promotionsordnung der Fakultät für Biologie der Bayerischen Julius-Maximilians-Universität zu Würzburg vom 15 März 1999.

Hiermit erkläre ich, dass ich die die vorlegende Dissertation „Building behaviour and the control of nest climate in *Acromyrmex* leaf-cutting ants“ selbständig angefertigt zu haben und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben. Alle aus der Literatur genommenen stellen sind als solche kenntlich gemacht. Das Weiteren erkläre ich, dass die vorliegende Arbeit weder in gleicher noch in ähnlicher Form bereits in anderen Prüfungsverfahren vorgelegen hat. Zuvor habe ich keine akademischen Grade erworben oder zu erwerben versucht.

Würzburg, den 19. 03. 2008

Leonardo Martin Bollazzi Sosa

Lebenslauf

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Würzburg, 19.03.08 Martin Bollazzi

Publikationsliste

Full Papers:

Bollazzi M, Roces F (2002) Thermal preference for fungus culturing and brood location by workers of the thatching grass-cutting ant *Acromyrmex heyeri*. Insectes Soc. 49:153-157

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Bollazzi M, Kronenbitter J, Roces F (2008) Soil temperature, digging behaviour, and the determination of nest depth in South American species of *Acromyrmex* leaf-cutting ants. Submitted to Oecologia

In preparation:

Bollazzi M, Roces F. Adapting to temperate climate through building behaviour: comparative thermal biology in thatched and subterranean grass-cutting ant nests (*Acromyrmex heyeri*)

Bollazzi M, Roces F. Building behaviour for climate control in leaf-cutting ants: *Acromyrmex heyeri* workers trade off thermoregulation for humidity control

Conference article:

Bollazzi, M. and Roces, F. 2001 "The control of foraging decisions in leaf-cutting ants: individual vs. collective aspects". European Congress of IUSSI (International Union for the Study of Social Insects), Berlin, Germany.

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