Temporal organization in *Camponotus* ants:

endogenous clocks and zeitgebers responsible for synchronization of task-related circadian rhythms in foragers and nurses

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I hereby confirm that my thesis entitled "Temporal organization in *Camponotus* ants: endogenous clocks and zeitgebers responsible for synchronization of task-related circadian rhythms in foragers and nurses" is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

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Author contribution

The experiments reported in chapters I and V were performed in collaboration with project B5 (Claudia Groh, Annekathrin Lindenberg) within the Collaborative Research Center 1047 “Insect Timing“. The field work reported in chapter V was performed in collaboration with Oliver Geissler (Zoology II, University of Würzburg).

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Chapter I: **Age-dependent division of labor and the ontogeny of circadian rhythms in the ant *Camponotus rufipes***				
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Study Design	SM	AL	FR	CG
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I confirm that I have obtained permission from both the publishers and the co-authors for legal second publication.

I also confirm my primary supervisor's acceptance.

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Summary

The rotation of the earth around its axis causes recurring and predictable changes in the environment. To anticipate those changes and adapt their physiology and behavior accordingly, most organisms possess an endogenous clock. The presence of such a clock has been demonstrated for several ant species including *Camponotus* ants, but its involvement in the scheduling of daily activities within and outside the ant nest is fairly unknown. Timing of individual behaviors and synchronization among individuals is needed to generate a coordinated collective response and to maintain colony function. The aim of this thesis was to investigate the presence of a circadian clock in different worker castes, and to determine the daily timing of their behavioral tasks within the colonies of two nectar-collecting *Camponotus* species.

In **chapter I**, I describe the general temporal organization of work throughout the worker life in the species *Camponotus rufipes*. Continuous tracking of behavioral activity of individually- marked workers for up to 11 weeks in subcolonies revealed an age-dependent division of labor between interior and exterior workers. After eclosion, the fairly immobile young ants were frequently nurtured by older nurses, yet they started nursing the brood themselves within the first 48 hours of their life. Only 60% of workers switched to foraging at an age range of one to two weeks, likely because of the reduced needs within the small scale of the subcolonies. Not only the transition rates varied between subcolonies, but also the time courses of the task sequences between workers did, emphasizing the timed allocation of workers to different tasks in response to colony needs.

Most of the observed foragers were present outside the nest only during the night, indicating a distinct timing of this behavioral activity on a daily level as well. As food availability, humidity and temperature levels were kept constant throughout the day, the preference for nocturnal activity seems to be endogenous and characteristic for *C. rufipes*. The subsequent monitoring of locomotor activity of workers taken from the subcolonies revealed the presence of a functional endogenous clock already in one-day old ants. As some nurses displayed activity rhythms in phase with the foraging rhythm, a synchronization of these in-nest workers by social interactions with exterior workers can be hypothesized.

Do both castes use their endogenous clock to schedule their daily activities within the colony? In **chapter II**, I analyzed behavioral activity of *C. rufipes* foragers and nurses within the social context continuously for 24 hours. As time-restricted access to food sources may be one factor affecting daily activities of ants under natural conditions, I confronted subcolonies with either daily pulses of food availability or *ad libitum* feeding. Under nighttime and *ad libitum* feeding, behavioral activity of foragers outside the nest was predominantly nocturnal, confirming the results from the simple counting of exterior workers done in chapter I. Foragers switched to diurnality during daytime feeding, demonstrating the flexible and adaptive timing of a daily behavior. Because they synchronized their activity with the short times of food availability, these workers showed high levels of inactivity. Nurses, in contrast, were active all around the clock independent of the feeding regime, spending their active time largely with feeding and licking the brood. After the feeding pulses, however, a short bout of activity was observed in nurses. During this time period, both castes increasingly interacted via trophallaxis within the nest. With this form of social zeitgeber, exterior workers were able to entrain in-nest workers, a phenomenon observed already in chapter I. Under the subsequent monitoring of locomotor activity under LD conditions the rhythmic workers of both castes were uniformly nocturnal independent of the feeding regime. This endogenous activity pattern displayed by both worker castes in isolation was modified in the social context in adaption to task demands.

Chapter III focuses on the potential factors causing the observed plasticity of daily rhythms in the social context in the ant *C. rufipes*. As presence of brood and conspecifics are likely indicators of the social context, I tested the effect of these factors on the endogenous rhythms of otherwise isolated individuals. Even in foragers, the contact to brood triggered an arrhythmic activity pattern resembling the arrhythmic behavioral activity pattern seen in nurses within the social context. As indicated in chapter I and II, social interaction could be one crucial factor for the synchronization of in nest activities. When separate groups were entrained to phase-shifted light-dark-cycles and monitored afterwards under constant conditions in pairwise contact through a mesh partitioning, both individuals shifted parts of their activity towards the activity period of the conspecific. Both social cues modulated the endogenous rhythms of workers and contribute to the context dependent plasticity in ant colonies.

Although most nursing activities are executed arrhythmically throughout the day (chapter II), previous studies reported rhythmic translocation events of the brood in

Camponotus nurses. As this behavior favors brood development, the timing of the translocations within the dark nest seems to be crucial. In **chapter IV**, I tracked translocation activity of all nurses within subcolonies of *C. mus*. Under the confirmed synchronized conditions of a LD-cycle, the daily pattern of brood relocation was based on the rhythmic, alternating activity of subpopulations with preferred translocation direction either to the warm or to the cold part of the temperature gradient at certain times of the day. Although the social interaction after pulse feeding had noticeable effects on the in-nest activity in *C. rufipes* (chapter I and II), it was not sufficient to synchronize the brood translocation rhythm of *C. mus* under constant darkness (e.g. when other zeitgebers were absent). The free-running translocation activity in some nurses demonstrated nevertheless the involvement of an endogenous clock in this behavior, which could be entrained under natural conditions by other potential non-photic zeitgebers like temperature and humidity cycles.

Daily cycling of temperature and humidity could not only be relevant for in-nest activities, but also for the foraging activity outside the nest. **Chapter V** focuses on the monitoring of field foraging rhythms in the sympatric species *C. mus* and *C. rufipes* in relation to abiotic factors. Although both species had comparable critical thermal limits in the laboratory, foragers in *C. mus* were strictly diurnal and therefore foraged under higher temperatures than the predominant nocturnal foragers in *C. rufipes*. Marking experiments in *C. rufipes* colonies with higher levels of diurnal activity revealed the presence of temporally specialized forager subpopulations. These results suggest the presence of temporal niches not only between the two *Camponotus* species, but as well between workers within colonies of the same species.

In conclusion, the temporal organization in colonies of *Camponotus* ants involves not only the scheduling of tasks performed throughout the worker life, but also the precise timing of daily activities. The necessary endogenous clock is already functioning in all workers after eclosion. Whereas the light-dark cycle and food availability seem to be the prominent zeitgebers for foragers, nurses may rely more on non-photic zeitgeber like social interaction, temperature and humidity cycles.

Zusammenfassung

Die Drehung der Erde um ihre eigene Achse erzeugt wiederkehrende und vorhersehbare Umweltschwankungen. Um diese Schwankungen zu antizipieren und Physiologie sowie Verhalten entsprechend anzupassen, besitzen fast alle Organismen eine innere Uhr. Bei einigen Ameisenarten, *Camponotus* Ameisen eingenommen, wurde die Präsenz einer inneren Uhr bereits bestätigt. Wie diese Uhr allerdings zur zeitlichen Abstimmung der Tagesaktivitäten innerhalb und außerhalb des Ameisennestes genutzt wird, war bis jetzt weitestgehend unbekannt. Für die Koordination einer kollektiven Verhaltensantwort und die Aufrechterhaltung der Kolonie ist dabei nicht nur die zeitliche Steuerung vom Verhalten Einzelner notwendig, sondern auch eine Synchronisation zwischen den Arbeiterinnen. Das Ziel dieser Doktorarbeit war es, die mögliche Präsenz einer inneren Uhr in verschiedenen Arbeiterkasten zu untersuchen, und die zeitliche Koordination von Tagesaktivitäten dieser Kasten innerhalb der Kolonien zweier *Camponotus* Ameisenarten zu bestimmen.

In **Kapitel I** beschreibe ich die grundlegende zeitliche Organisation der Arbeitsteilung im Laufe des Arbeiterinnenlebens in der Art *Camponotus rufipes*. Mithilfe einer lückenlosen Verfolgung der Tagesaktivitäten von individuell markierten Tieren in Subkolonien über bis zu 11 Wochen konnte eine altersabhängige Arbeitsteilung zwischen Innen- und Außendienstarbeiterinnen nachgewiesen werden. Nach dem Schlüpfen wurden die eher unbeweglichen jungen Ameisen oft durch ältere Brutpflegerinnen versorgt, engagierten sich dann aber schon innerhalb der ersten 48 Stunden ihres Lebens selbst in der Brutpflege. Wahrscheinlich wegen der verminderten Notwendigkeit zur ausgedehnten Futtersuche innerhalb der kleinen Versuchskolonien wechselten nur 60% der Innendienstarbeiterinnen nach ein bis zwei Wochen zum Fouragieren außerhalb der Kolonie. Nicht nur variierte der Prozentsatz des Verhaltensübergangs von Brutpflegerin zur Sammlerin zwischen den Subkolonien, sondern auch innerhalb der Subkolonien unterschieden sich Arbeiterinnen im Zeitverlauf der Aufgabenfolge. Diese Ergebnisse betonen die gezielte, zeitliche Zuweisung von Arbeiterinnen zu einer bestimmten Arbeiterkaste je nach Bedarf der Kolonie.

In diesem Experiment waren die Sammlerinnen vorwiegend nur während der Nachtphase außerhalb der Kolonie aktiv, was wiederum eine genaue zeitliche

Koordination des Sammelverhaltens auf Tagesbasis zeigt. Da die Futterverfügbarkeit sowie Temperatur- und Luftfeuchte über den Tag hinweg konstant gehalten wurden, scheint die bevorzugte Nachtaktivität endogen und charakteristisch für *C. rufipes* zu sein. Durch das anschließende Monitoring der Lokomotoraktivität von Arbeiterinnen aus diesen Subkolonien konnte gezeigt werden, dass schon einen Tag alte Ameisen eine funktionierende innere Uhr besitzen. Der Aktivitätsrhythmus mancher Brutpflegerinnen war dabei in Phase mit dem Sammelrhythmus der Kolonie, weswegen man von einer Synchronisation dieser Inndienstarbeiterinnen durch soziale Interaktion mit Außendienstarbeiterinnen ausgehen kann.

Doch nutzen beide Kasten ihre innere Uhr auch, um ihre Tagesaktivitäten innerhalb der Kolonie zeitlich abzustimmen? In **Kapitel II** habe ich die Verhaltensaktivität von *C. rufipes* Futtersammlerinnen und Brutpflegerinnen in ihrem sozialen Umfeld kontinuierlich für 24 Stunden verfolgt. Da der beschränkte Zugriff zu Futterquellen einer der Faktoren sein könnte, der die Tagesaktivitäten von Ameisen in der Natur beeinflusst, wurden Subkolonien entweder nur pulsartig oder *ad libitum* gefüttert. Während der Nacht- und *ad libitum* Fütterung waren Sammlerinnen vorwiegend nachtaktiv, was die Ergebnisse der simplen Zählung von Außendiensttieren in Kapitel I bestätigt. Während der Tagesfütterung wurden die Sammlerinnen tagaktiv, was die flexible und adaptive zeitliche Anpassung dieses täglichen Verhaltens veranschaulicht. Unabhängig von der Fütterungszeit waren Brutpflegerinnen rund um die Uhr aktiv, wobei sie die größte Zeit mit Fütterung und Säuberung der Brut verbrachten. Jedoch konnte kurz nach den Fütterungspulsen ein kurzer Aktivitätsanstieg verzeichnet werden, welcher auf die erhöhte Interaktion durch Trophallaxis mit den Sammlerinnen zurückzuführen ist. Wie bereits schon in Kapitel I angedeutet, können Außendiensttiere mithilfe dieses sozialen Zeitgebers Arbeiterinnen im Nest synchronisieren. Im anschließenden Monitoring der Lokomotoraktivität unter Licht-Dunkel-Bedingungen waren alle rhythmischen Arbeiterinnen einheitlich nachtaktiv, unabhängig von der vorausgegangen Fütterungszeit. Damit werden die endogenen Aktivitätsmuster, die beide Kasten in Isolation zeigen, im sozialen Kontext in Anpassung an die speziellen Anforderungen an die Kasten modifiziert.

Schwerpunkt des **Kapitels III** ist die Untersuchung der potentiellen Faktoren, die die gezeigte Plastizität der Tagesrhythmen bei Ameisen der Art *C. rufipes* bedingen. Da unter anderem das Vorhandensein von Brut und Artgenossinnen sozialen Kontext signalisieren können, wurde der Effekt dieser Faktoren auf die endogenen Rhythmen von ansonsten isolierten Individuen untersucht. Selbst in Sammlerinnen verursachte der Kontakt zu Brut ein arrhythmisches Aktivitätsmuster, welches dem Verhaltensmuster von Brutpflegerinnen innerhalb der Kolonie gleicht. Wie schon in Kapitel I und II deutlich wurde, könnten soziale Interaktionen einen wesentlichen Beitrag zur Synchronisation der Nestaktivitäten leisten. Dazu wurden Gruppen getrennt voneinander mit phasenverschobenen Licht-Dunkel-Zyklen entraint, und Tiere anschließend in paarweisem Kontakt durch ein Netzgitter aufgezeichnet. Beide Individuen verschoben einen Teil ihrer Aktivität in die Aktivitätsperiode des Partners. Damit modulierten beide getesteten sozialen Faktoren die endogenen Rhythmen der Arbeiterinnen, was letztendlich zur kontextabhängigen Plastizität der Rhythmen in Ameisenkolonien beiträgt.

Obwohl Brutpflegerinnen die meisten Verhaltensweisen arrhythmisch während des ganzen Tages ausüben (Kapitel II), zeigten vorangegangene Studien rhythmische Brutverlagerungen bei Brutpflegerinnen der *Camponotus*-Arten. Da dieses Verhalten die Brutentwicklung fördert, scheint das Timing der Verlagerungen innerhalb des ansonsten dunklen Nestes essentiell zu sein. In **Kapitel IV** verfolgte ich die Verlagerungsaktivität von allen Brutpflegerinnen in Subkolonien der Art *C. mus*. Unter den gesichert synchronisierten Bedingungen eines LD-Zykluses basierte das Brutverlagerungsmuster auf der rhythmischen, abwechselnden Aktivität von zwei Subpopulationen mit bevorzugter Verlagerungsrichtung in entweder den warmen oder kalten Bereich des Temperaturgradienten zu bestimmten Tageszeiten. Obwohl die soziale Interaktion nach Pulsfütterung einen deutlichen Einfluss auf die Nestaktivität bei *C. rufipes* hatte (Kapitel I und II), reichte diese Interaktion nicht aus um den Brutverlagerungsrhythmus bei *C. mus* innerhalb des dunklen Nests (d.h. unter Abwesenheit sonstiger Zeitgeber) zu synchronisieren. Nichtsdestotrotz zeigte der Freilauf der Brutverlagerungsrhythmen in einigen Brutpflegerinnen die Beteiligung einer inneren Uhr, welche durch anderweitige nicht-photische Zeitgeber wie Temperatur- und Feuchtigkeitszyklen synchronisiert werden könnte.

Tageszyklen in Temperatur und Feuchtigkeit könnten nicht nur relevant sein für Aktivitäten innerhalb des Nests, sondern auch für die Fouragieraktivität außerhalb des Nests. In **Kapitel V** wurden Fouragierrhythmen im Freiland bei den sympatrisch vorkommenden Ameisenarten *C. mus* und *C. rufipes* in Abhängigkeit von abiotischen Faktoren betrachtet. Obwohl die beiden Arten unter Laborbedingungen ähnliche kritische Temperaturgrenzen aufzeigten, waren die Fourageure der Art *C. mus* strikt tagaktiv und sammelten deswegen unter höheren Temperaturen Futter als die vorwiegend nachtaktiven Fourageure der Art *C. rufipes*. Bei *C. rufipe*s Kolonien mit erhöhter Tagaktivität wiesen Markierexperimente das Vorkommen von zeitlich spezialisierten Fourageur-Subpopulationen nach. Damit deuten die Ergebnisse nicht nur das Vorkommen von unterschiedlichen zeitlichen Nischen innerhalb der beiden *Camponotus*-Arten an, sondern auch zwischen Arbeiterinnen von Kolonien derselben Art.

Zusammenfassend gesehen umspannt die zeitliche Organisation in Kolonien der *Camponotus*-Ameisen nicht nur die zeitliche Planung der Aufgaben, die über das Arbeiterinnenleben hinweg ausgeführt werden, sondern auch das genaue Terminierung von Tagesaktivitäten. Bereits nach dem Schlüpfen besitzen allen Arbeiterinnen eine funktionsfähige und für die zeitliche Organisation notwendige innere Uhr. Während der Licht-Dunkel-Zyklus und Futterverfügbarkeit die bedeutenden Zeitgeber für Fourageure zu sein scheinen, könnten Brutpflegerinnen eher auf nicht-photische Zeitgeber wie soziale Interaktion, Temperatur- und Feuchtigkeitszyklen angewiesen sein.

General Introduction

Endogenous clocks – characteristics and function

The rotation of the earth around its own axis takes around 24 hours and causes periodic changes in environmental conditions. The most prominent daily change is probably the light-dark-cycle, which is accompanied for instance by changes in temperature and relative humidity. Being able to anticipate and to prepare for regular daily events can have many advantages concerning various aspects of life of organisms. To do so, almost all organisms have evolved an internal time-keeping system called endogenous clock.

The presence of endogenous clocks in a variety of taxa, ranging from simplest organisms like cyanobacteria to mammals (e.g. Lakin-Thomas, 2000; Bell-Pedersen et al., 2005), suggests the adaptive significance of these clocks. The most obvious advantage is probably the suitable timing of behavioral activities to favorable phases of the day ("extrinsic advantage hypothesis", reviewed in Sharma, 2003a; Vaze & Sharma, 2013). By avoiding predation, competition and harmful environmental conditions (reviewed in Kronfeld-Schor & Dayan, 2003), insects can increase their survival probability and fitness. Alongside the extrinsic advantages, endogenous clocks are reported to be adaptive for internal programming of physiological processes within an organism ("intrinsic advantage hypothesis", reviewed in Pittendrigh, 1993; Sharma, 2003b; Johnson, 2005). The demonstration of functional clocks in cave living animals (reviewed in Beale et al., 2016) and in populations of *Drosophila* raised for 300 generations under constant conditions (Shindey et al., 2016) supports this hypothesis. The "circadian resonance hypothesis" describes another adaptive value of endogenous clocks under periodic environments. It states that organisms show increased fitness when the periodicity of the endogenous clock matches the periodicity of environmental cycles (reviewed in Sharma, 2003a; Paranjpe & Sharma, 2005). Indeed, *Drosophila* flies raised under 24 hour LD-cycles live significantly longer than flies raised under shorter or longer days (Pittendrigh & Minis, 1972). In *Camponotus* ants, larvae and pupae develop faster under 24 hour LD-cycles than under non-24 hour LD-cycles (Lone et al., 2010).

Although the particular molecular mechanisms of endogenous clocks differ among taxa (reviewed in Danks, 2003; Paranjpe & Sharma, 2005), their general structure seems to be conserved. The clock system can be divided in three basic components: an input pathway to the clock, a central oscillator, and an output pathway of the clock (Johnson & Hastings, 1986). The central oscillator serves as pacemaker and generates multiple rhythms in physiology and behavior based on positive and negative molecular feedback loops (Dunlap, 1999). In insects, the central clock is located within the optic lobes of the brain (reviewed in Helfrich-Förster et al., 1998). Under the absence of any environmental fluctuations, the oscillator produces free-running rhythms with periods (τ) of approximately 24 hours. Because of this endogenous deviation from 24 hours, clock systems and rhythms are called “circadian” (from Latin: *circa*=approximately; *dies*=day; Halberg et al., 1959). As the innate period of the clock does not equal 24 hours, it has to be reset each day by suitable environmental stimuli called zeitgeber (reviewed in Johnson et al., 2003; Roenneberg et al., 2003). The most dominant and reliable zeitgeber for the entrainment of the clock is the light-dark-cycle (Devlin, 2002). Under the absence of the light-dark-cycle, for example within the nest of ant colonies, non-photic zeitgebers like temperature cycles are able to synchronize the clock (reviewed in Aschoff, 1954; Rensing & Ruoff, 2002). Although temperature cycles are able to reset the phase of the clock, one of the fundamental properties of circadian clocks is its temperature compensation (Pittendrigh, 1954; Hastings & Sweeney, 1957). That means that the period of the clock is unaffected by temperature changes, i.e., the clock does not run faster at higher temperatures.

The locomotor rhythm is one of the clock outputs used to study the presence of the circadian clock in insects. To demonstrate the presence and state of a circadian clock, locomotor rhythms need to be recorded both under the presence and absence of zeitgebers. When entrained by a suitable zeitgeber, the period of the rhythm matches the period of the zeitgeber cycle and both rhythms establish a stable phase relationship. A zeitgeber is truly entraining the clock when the observed rhythm follows a phase-shift of the zeitgeber cycle or a change of zeitgeber period. Under constant conditions, insects’ rhythms will show their endogenous, species-specific period (Aschoff, 1979). Here, activity is expected to free-run from the entrained activity.

Timing aspects in ants

Due to the various molecular and genetic tools available, the solitary insect *Drosophila* is probably the most prominent model used in chronobiological studies. Yet, insect societies with their non-productive division of labor between workers offer the possibility to study the plasticity of daily rhythms in individuals from the same species, because they perform different tasks under varying environmental conditions.

Timing of activities within ant colonies occurs both on annual as well as on daily time scales, and it involves inter- and intraspecific timing. Annual rhythms are mainly linked to the seasonal production of sexuals and to colony growth. In temperate climates, environmental factors vary strongly with season. During colder months, queens in many ant species stop their oviposition and brood development is paused (reviewed in Kipyatkov, 1993). The occurrence of spontaneous rhythms of developmental and diapause phases in ant species under constant laboratory conditions (Hölldobler, 1961; Kipyatkov, 1995) imply the involvement of an endogenous clock. Diapause is induced by short days and cold temperatures, whereas temperature is the more important zeitgeber for this annual rhythm (Kipyatkov & Lopatina, 2008). Start and end of diapause should be tightly synchronized with the warm season to maximize brood production and facilitate colony growth. Possibly in association with seasonal brood production and colony growth, ant nests show annual variations in nest maintenance activity (Medeiros et al., 2016).

Another annual timing event is the reproduction of sexuals and nuptial flights. Gynes and the short-living males are mostly produced only once a year (Hölldobler & Wilson, 1990), and mating flights are timed in accordance to air humidity, temperature, rainfall and cloud coverage (Talbot, 1945; Hölldobler & Maschwitz, 1965; Boomsma & Leusink; 1981; Nene et al., 2016). These authors report that the nuptial flights are not only scheduled on an annual basis, but additionally timed precisely to a species-specific daily time window to reduce predator stress and facilitate exogamous mating. Both sexual castes have circadian clocks essential for the necessary synchronization of this once-in-a-lifetime event (McCluskey, 1958; 1967). Although virgin ant queens show clear rhythms before mating, they lose their rhythmicity after mating (McCluskey, 1992; Sharma et al., 2004a). The plasticity in daily rhythms in this caste is reported to be linked to egg-laying behavior.

Daily rhythms are not only found in the reproductive caste, but also in the sterile worker castes. The most described example for daily rhythm in ant colonies is probably the foraging activity. This behavioral activity outside the ant nest is temporally scheduled in accordance to abiotic factors like temperature, humidity, light intensity or food availability, and biotic factors like competition or predation (Kaschef & Sheata, 1962;

Gentry, 1974; Hansen, 1978; Dejean, 1990; Passera et al., 1994). Other activities outside the ant nest like patrolling and maintenance are carried out following a daily rhythm as well (Gordon, 1983; 1984; 1986). Activity rhythms inside the ant nest are less easy to study and so far, most studies investigated only locomotor rhythms during short time intervals and not over the course of the day. Synchronized peaks of locomotor activity in groups of ants nevertheless suggest the presence of temporally coordinated rhythms within the nest (Franks et al., 1990; Cole, 1991c). The only known true endogenous behavioral rhythm inside of ant nests is the daily brood translocation rhythm in *Camponotus mus* (Roces & Núñez, 1989; 1996; Roces, 1995). As this behavior facilitates brood development (Falibene et al., 2016), the timing of the translocations within the dark nest seems to be crucial.

Synchronization of daily activities outside and within the ant nest are expected to increase colony efficiency, and workers need an endogenous clock to schedule such activities. Nevertheless, only foragers exhibited strong locomotor rhythms as output of a circadian clock, whereas nest workers showed no distinct activity patterns in two ant species, *Camponotus compressus* and *Pogonomyrmex occidentalis* (Sharma et al., 2004b; Ingram et al., 2009). The authors suggest that activity patterns in isolated workers reflect their behavioral activity patterns inside the colony in correlation to an age-dependent division of labor. Within this age polyethism, young workers stay inside the nest and are expected to care all around the clock for the brood, whereas old workers leave the nest to collect food displaying a foraging rhythm. Even if a circadian clock might not be needed during the early life of these ants, workers need a functional clock to schedule their activity outside the nest later in life. So far, however, the link between the development of behavioral rhythms within an age-dependent division of labor and clock function has not been studied in ants.

Thesis outline

The present thesis aims at describing the temporal organization of tasks within and outside of ant colonies and to identify the involvement of endogenous clocks in the timing of daily activities. The conducted research is presented in five chapters which are organized according to scientific publication standards (i.e. including an abstract, introduction, material and methods, results and discussion sections). In chapter I, I describe the general temporal organization of work based on an age-dependent polyethism in the ant *Camponotus rufipes,* as well as the presence and state of circadian clocks in nurses and foragers of known age. Chapter II focuses on caste-specific behavioral rhythms of *C. rufipes* nurses and foragers within the social context of the colony. It is investigated how foraging activity is scheduled in accordance to food availability, and whether behavioral activities inside the nest follow a daily rhythms as well. Within this chapter, I describe the task-related plasticity of daily rhythms in *C. rufipes*. In chapter III, I evaluate the importance of two social cues on this context-dependent plasticity. Chapter IV addresses another in-nest activity, the brood translocation rhythm in *C. mus*. Here I describe how individual rhythms in brood translocation are coordinated to generate a colony rhythm and investigate whether social interactions act as potential zeitgeber for them within the dark nest. In chapter V, I study the timing of foraging activity in field colonies of the sympatric species *C. mus* and *C. rufipes* as correlated to various environmental factors. Foraging rhythms in the natural environment are compared to those seen in the laboratory. The results of these five chapters are summarized in a general discussion at the end of the thesis.

Chapter I

Age-dependent division of labor and the ontogeny of circadian rhythms in the ant *Camponotus rufipes*

Abstract

Ant societies usually exhibit an age-dependent division of labor, where young ants stay inside the nest performing tasks like brood care or nest digging, and older workers leave the nest for exterior tasks like foraging and nest defense. Even if timing of activity might not be needed for interior tasks, workers need to schedule their foraging activity outside the nest later in life in accordance to food availability, and would therefore need an endogenous clock. We examined age-dependent division of labor in the nectar-collecting ant species *Camponotus rufipes* by tracking behavioral activities of 188 newly hatched ants 24-hours for up to 11 weeks in subcolonies. Locomotor activity rhythms of 145 workers with known age and caste out of these subcolonies were monitored to determine the presence and functionality of an endogenous clock. An age-dependent division of labor was detected in all subcolonies, but interior-exterior transition occurred in only 60% of workers. Workers started to engage in nursing activities within 48 hours after hatching, and switched to exterior activity at an age ranging from one to two weeks. Before actively engaging in food collection, workers explored the foraging arena for several days. Variation between and within colonies in proportion of workers switching to exterior work and time course of task allocation indicates the flexible character of age-dependent division of labor. Foragers were predominantly night active, indicating a precise timing of foraging activity. Even 1-day-old ants exhibited locomotor activity rhythms when removed from the colony, demonstrating the presence of a functional endogenous clock in all workers. The strong differences in activity levels, period lengths and age-dependent decline in rhythmicity among workers and subcolonies highlights the variability of circadian rhythms within one species. Even some nurses exhibited activity patterns in phase with the foraging rhythm, suggesting a form of social synchronization between the castes.

Introduction

Division of labor is one key factor of the evolutionary success of social insects (Oster & Wilson, 1978). In the non-reproductive division of labor, specialized worker castes perform tasks within and outside the nest without any central control (Robinson, 1992; Beshers & Fewell, 2001). In many ant species, media-sized workers engage in tasks both inside and outside the nest, and further task allocation between media-sized workers is based on worker age (*Messor/Myrmica*: Ehrhardt, 1930; *Pheidole dentata*: Wilson, 1976; *Solenopsis invicta*: Mirendra & Winson, 1981; *Tapinoma erraticum*: Lenoir, 1979; *Trachymyrmex septentrionalis*: Beshers & Traniello, 1995). Within this age polyethism, young workers perform tasks within the nest whereas older workers engage in tasks outside the nest like foraging and nest defense. Transition between interior and exterior works can be based on a discretized caste system, where age cohorts have no overlapping tasks, or a continuous caste system, where age classes have overlapping tasks (Wilson, 1976)

In the nectar-collecting ant species *Camponotus rufipes*, large workers were reported to engage mainly in nest defense, whereas minor and media perform nursing and foraging activities (Jaffé & Sánchez, 1984). When young (<45 days) and old (>45 days) workers were compared, they did not differ in the frequency of performing nursing and foraging activities (Soares et al., 2008). As transitions between interior and exterior work typically take place within the first three weeks of the worker life (Hölldobler & Wilson, 1990), age-dependent division of labor might not be detected in that study because of the broad age classes used. The demonstration of an increase in opsin gene expression and volume of optic neuropils within the first two weeks of *C. rufipes* workers indicates a preparation of the visual system for outside activities (Yilmaz et al., 2016), suggesting an age-dependent transition within this age range.

Timing of activities could not only be important over the course of the ant life, but also on a daily timescale. Foraging activity in nectar-collecting ants should be precisely scheduled in dependence of food availability (Passera et al., 1994; Heil et al., 2010), environmental factors (Azcárate et al., 2007; Chong & Lee, 2009) and competitors (Blüthgen et al., 2004; Anjos et al., 2016). Even inside the ant nest, synchronized locomotor activity rhythms occur among workers (Franks et al., 1990; Cole, 1991c), which could reduce redundancy and consequently increase efficiency of collective behaviors such as nest building and brood care. Yet, behavioral rhythms within the nest have so far not been studied.

To schedule daily activities and anticipate the time of the day, workers would need an endogenous clock. Isolated workers of several ants species exhibit locomotor rhythms under constant conditions (McCluskey, 1965; North, 1987), demonstrating the presence of such an endogenous clock. This raises the question whether all worker castes possess a clock and use it for timing of their daily activities. In species with an age-dependent division of labor, workers switch from interior to exterior work. Even if timing of activity might not be needed for interior tasks, workers need to schedule their activity outside the nest later in life. Consequently, all workers would need an endogenous clock at some time point of their life. The role of endogenous clocks for timing of daily activities within an age-dependent polyethism was demonstrated already in honey bees. Newly hatched bees do not display locomotor rhythms within the first days of their life (Spangler, 1972; Toma et al., 2000), which matches their around-the-clock nursing activity within the hive (Moore et al., 1998). Although older nurse workers (> 7 days) exhibit locomotor rhythms in isolation from the hive like foragers, their rhythmicity is still repressed or masked in the social context (Shemesh et al., 2007). When bees switch to foraging at an age of about two weeks (Lindauer, 1952; Winston, 1987), their circadian clock is fully developed (Moore & Rankin, 1985; Moore et al., 1998; Toma et al., 2000; Bloch et al., 2001) and used for timing of foraging activity (Beling, 1929; Wahl, 1932) and for the time-compensate sun compass orientation during foraging flights (von Frisch & Lindauer, 1956; Lindauer, 1961; von Frisch, 1967).

Like in bees, forager and nurse workers of several ant species differ in their locomotor rhythms. In *Pogonomyrmex occidentalis* and *Camponotus compressus*, foragers exhibit strong locomotor rhythms in isolation, whereas nest workers display no distinct activity patterns (Sharma et al., 2004; Ingram et al., 2009). Both studies did not control for worker age and determined caste affiliation only by location of the ants within the nest or by their body size, so a correlation with an age-dependent polyethism is difficult. In *Diacamma* ants, locomotor activity rhythms were monitored in different age classes (Fuchikawa et al., 2014). Callow workers (<7 days old) and “young” workers (<1 month old) exhibit strong locomotor rhythms, whereas old workers (>1 month) are arrhythmic.

In our present study, we investigated a possible age-dependent division of labor in *C. rufipes* in a first experiment in order to explore the general temporal organization in ant colonies of this species. For that, behavioral activity of newly-hatched *C. rufipes* workers were tracked in subcolonies for several weeks. We additionally monitored foraging activity on group and individual levels in these subcolonies to examine the daily timing of foraging. In our second experiment, we monitored locomotor rhythms of ants with known caste and age out of these subcolonies to examine whether both interior and exterior workers possess a functional endogenous clock to time their daily activities within the colony.

Material and Methods

For the laboratory studies, we used three *C. rufipes* colonies. The queens were collected during their mating flights in December 2011 and 2014 in La Coronilla, Uruguay (33°53'25.2"S, 53°31'27.6"W) and were brought to the laboratory at the University of Würzburg, Germany. Colonies were raised in plaster nests under constant conditions (25°C, 50%rH) and a 12:12h LD-cycle (300 lux during the light phase). Queenright colonies consisted of several thousand workers and were fed *ad libitum* with water, diluted honey and pieces of cockroaches.

Worker age & division of labor

To study the age-dependent division of labor between forager and nurse castes, we built three subcolonies of 100 workers and 100 larvae from three *C. rufipes* colonies (named H, 14 and A). A subcolony refers to a queenless group of workers and brood settled in an artificial brood chamber (9.5 x 9.5 x 5 cm) with access to an outside foraging arena (9.5 x 9.5 x 5 cm) via one tunnel (diameter: 0.5 cm, length: 100 cm), thus representing a functional group with workers that displayed both foraging and nursing behaviors. An *ad libitum* supply of food (honey-water, chopped cockroaches and water) was provided on an elevated platform (diameter: 5.5 cm, height: 2.3 cm) in the foraging arena. Food was replaced three times a week at randomized time points. The use of the platform assured that foragers actively searched for food, allowing their distinction from other outside workers like guards.

The two parts of the nest were located inside different incubators (I-30BLL, CLF PlantClimatics GmbH), so that we could simulate the different lighting regimes under natural nesting conditions. The brood chamber was kept under constant darkness, whereas a 12:12h light-dark-cycle (4000 lux during the light phase) was applied in the foraging arena. Two junctions in the tunnel connecting the nest parts prevented light from entering into the brood chamber. Both nest parts were maintained under similar constant temperature and humidity conditions (25°C, 60%rH). To build the subcolonies and promote division of labor, we collected ants with varying body sizes both from the brood chamber as well as the foraging areas of the large queenright colonies, and marked them with white paint (Edding) on their thorax to distinguish them from newly hatched ants. In the subcolonies, freshly hatched ants were marked daily under dim red light with paper tags to enable individual identification. First ants hatched after three to four weeks and their caste affiliation was tracked for several weeks. In a first experimental series, the daily disturbance because of withdrawal of newly hatched ants from the brood pile caused permanent evacuation of ants and brood to the tunnel connecting to the foraging area. As a consequence, the first experimental series was terminated after seven weeks, so that newly hatched ants were all in all tracked for up to four weeks (n=2 subcolonies). In a second experimental series, a door system was installed at the entrance of the brood chamber and closed before removing ants for marking, to prevent evacuation behavior. Further, the ambient temperature of the connecting tunnel was cooled down to 21°C, a temperature which is not preferred for brood rearing in this species (Roces & Núñez, 1995), but otherwise does not affect worker traffic between the nest parts. In this experimental series, individuals were tracked for up to 11 weeks (n=1 subcolony). To keep brood to worker ratio fairly constant throughout the experiment, we added one larvae for one hatched worker in every subcolony once a week.

Caste affiliation of the focal ants was determined on a daily basis for several weeks with the aid of 24-hour video recordings of the nest (NVR video recorder with IPC cameras, Alomna GmbH), as workers aged. Under darkness, videos were recorded under dim infrared light. Workers were classified as nurses when they were seen feeding, licking or translocating the brood or assisting by eclosion of pupae at least once a day. We distinguished further between workers that entered the foraging arena and were actively collecting food at the feeding platform (foragers), and those that were present in the foraging arena, but not actively foraging (exterior workers). Furthermore, we noted in which light phase workers were active in the foraging arena each day and tested for differences between subcolonies (χ^2 tests). Besides the timing of foraging activity on individual level, we counted the numbers of workers present in the foraging arena at every half an hour in every subcolony, as a measure of group-level foraging activity. To illustrate transition rate between the castes on group level, we calculated the percentage of active nurses, exterior workers and foragers for every age class (ages 1 day – maximum 77 days) per subcolony. We further determined the age interval in which ants were active as nurses, exterior workers or foragers, and tested for differences between subcolonies (Kruskal-Wallis tests). Typical task sequences were described and their frequencies were compared between subcolonies (χ^2 tests). Worker age at onset and offset of the monitored behavioral activities was in turn compared between this typical task sequences (Kruska-Wallis tests). Workers that were taken from the subcolonies for assessment of their locomotor rhythm (see next paragraph) or that lost their individual tags during the course of the experiment were excluded from the analysis. To examine whether the transition between interior and exterior activity depended on body size, we compared thorax lengths between exterior workers and of nurses older than 14 days (an age at which transition typically would have been completed). Thorax length, defined as the distance between anterior edge of the pronotum and the petiole, was measured for every individual taken from the subcolonies for further analysis or after the end of the experiment (Leica LAS EZ).

Worker age & circadian rhythms

Circadian rhythms were studied in workers of different ages. Therefore we withdraw individually identified ants of the subcolonies in up to 6 age classes (1 day, 7 days, 14 days, 21 days, 28 days, > 2 months after hatching). As the first experimental series had to be terminated after 8 weeks and first ants hatched only after three to four weeks, only workers of up to 25 days of age could be monitored in subcolony H and 14. Per week, a maximum of 5 ants were removed per subcolony to not perturb the division of labor.

To study locomotor rhythms and their endogenous nature in workers of different ages, we employed a commercially available locomotor activity recording system (LAM 16; TriKinetics, Waltham, USA). This recording method is well established for *Drosophila* (Chui et al., 2010; Schlichting & Förster, 2015) and was recently adapted to monitor social insects like bees and wasps (Giannoni-Guzman et al., 2014; Beer et al., 2016). One monitor (33 x 12 x 20 cm) allows the simultaneous recording of 32 individuals in an automated way for infinite time. For that, insects are placed individually into glass tubes (length: 10 cm, diameter: 1.5 cm), where their locomotor activity is monitored by three infrared light beams in the center of each glass tube. Automatic counts of the interruptions of the light beams caused by the insect's locomotor activity accumulate for each individual in self-selected time windows (in our experiments in ten minute bins) and are then communicated to a computer equipped with the corresponding recording program. Raw data can be processed later on and be used for example to display activity rhythms in form of actograms (e.g. via ActogramJ; Schmid et al., 2011).

The monitors were modified in our study to enable the recording of isolated ants over weeks (Fig. 1A/B). As ants are prone to desiccation in isolation of the colony and need, besides nutrition, constant access to water, we supplied them with *ad libitum* access to low concentrated sugar solutions. We designed special feeding caps that sealed the glass tubes at both ends (Fig. 1B), so the insects would not stay at one end of the glass tube and display reduced overall activity. Plastic caps were filled with sponges soaked with 5% sugar water (w/w; 1.5 ml volume per cap) and separated from the insects with plastic nets to keep them within the recording section. The sugar water needed to be refilled every third day, which was accomplished through injections in a single perforation of the plastic cap at random time points without interfering the recordings. A mesh tunnel connected the feeding caps with the glass tubes to provide aeration. Workers were placed in the locomotor activity monitors under dim infrared light and recorded over 21 days under constant darkness to study endogenous activity patterns.

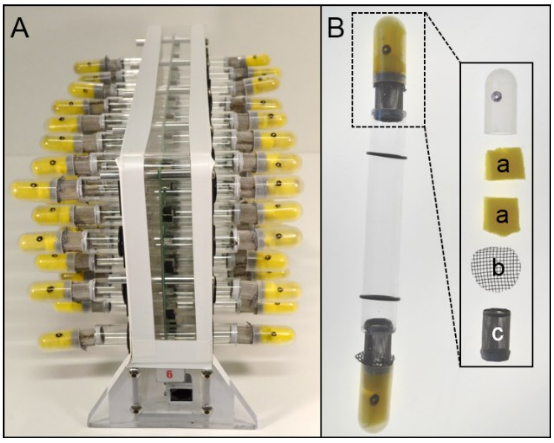

Fig. 1: Experimental setup for monitoring locomotor activities. (A): Modified locomotor activity monitors (TriKinetics). Activity of single individuals was recorded in isolation from the social context via crossings of infrared light beams in the center of the glass tubes (B). *Ad libitum* supply of 5% (w/w) sugar water was provided through sponges (a), separated from the glass tube by plastic nets (b) to allow fluid intake while avoiding chewing by the ant. A metal mesh tunnel segment (c) connected the glass tubes with the feeding devices to provide aeration. Glass tubes were fixed to the monitors by o-rings (d).

Locomotor activity was displayed in form of actograms via ActogramJ for all individuals. Based on the actograms, we determined survival rate and proportion of rhythmic individuals during the first week of recording (Lomb-Scargle method, ActogramJ). The effect of age on survival rate and rhythmicity was analyzed by χ^2 tests for every subcolony. We calculated the period and power of the individual activity during the first week of recording by periodogram analysis (Lomb-Scargle-method, ActogramJ), and tested for differences between the age classes within each subcolony (Kruskal-Wallis tests). For one day old ants, we calculated daily activity levels for 14 days of monitoring. As locomotor rhythms of individuals were either free-running from the subjective day or from the subjective night, we compared the proportion of workers with each free-running type between exterior and interior workers for every age class (χ^2 tests).

All statistical analysis were performed in STATISTICA (StatSoft, Inc., Version 10.0) after testing data sets for normal distribution via Shapiro-Wilks test.

Results

Worker age & division of labor

Behavioral transition from interior to exterior work was age-dependent in all three subcolonies, but transition rates and age ranges varied slightly (Fig. 2), so data sets were not pooled.

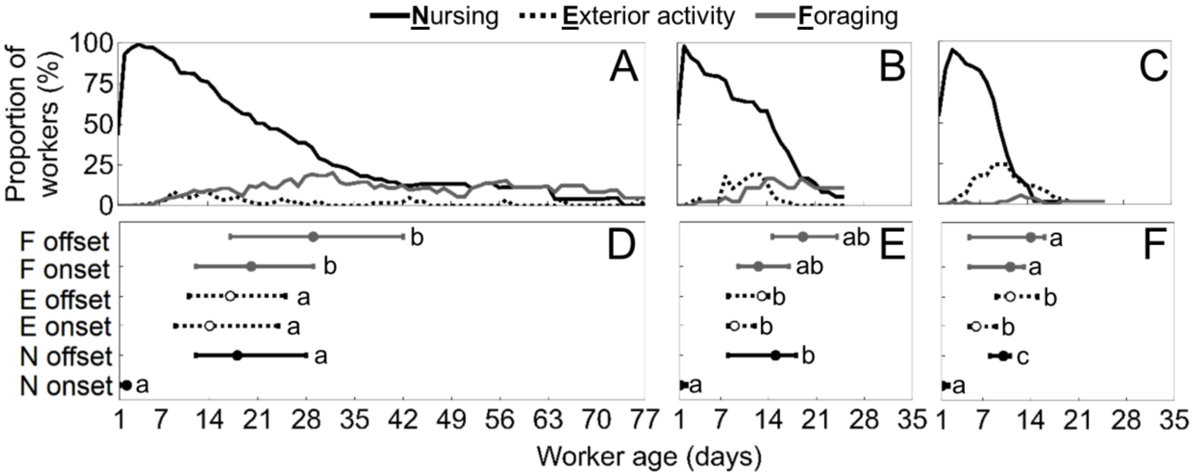

Fig. 2: Age-dependent transition in individuals from interior to exterior work in three subcolonies. Black: Nursing. Dashed: Exterior activity. Grey: Foraging. (A): Subcolony A. (B): Subcolony H. (C): Subcolony 14. Top: Proportion of workers engaged in nursing, exterior activity or foraging at different ages. Bottom: Average worker age (median±IQR) at both onset and offset of nursing, exterior activity and foraging.

Almost all newly-hatched individuals in the three subcolonies engaged in nursing activities (Fig. 2A-C). Independent of colony origin, workers started to engage in nursing activities one to two days after eclosion (Fig. 2D-F; Kruskal-Wallis test: H(2,242)=1.7, p=0.4). Workers of the three subcolonies performed nursing activities for different periods (Fig. 2D-F; Kruskal-Wallis tests: H(2,168)=39.2, p<0.0001), until an age of two to three weeks.

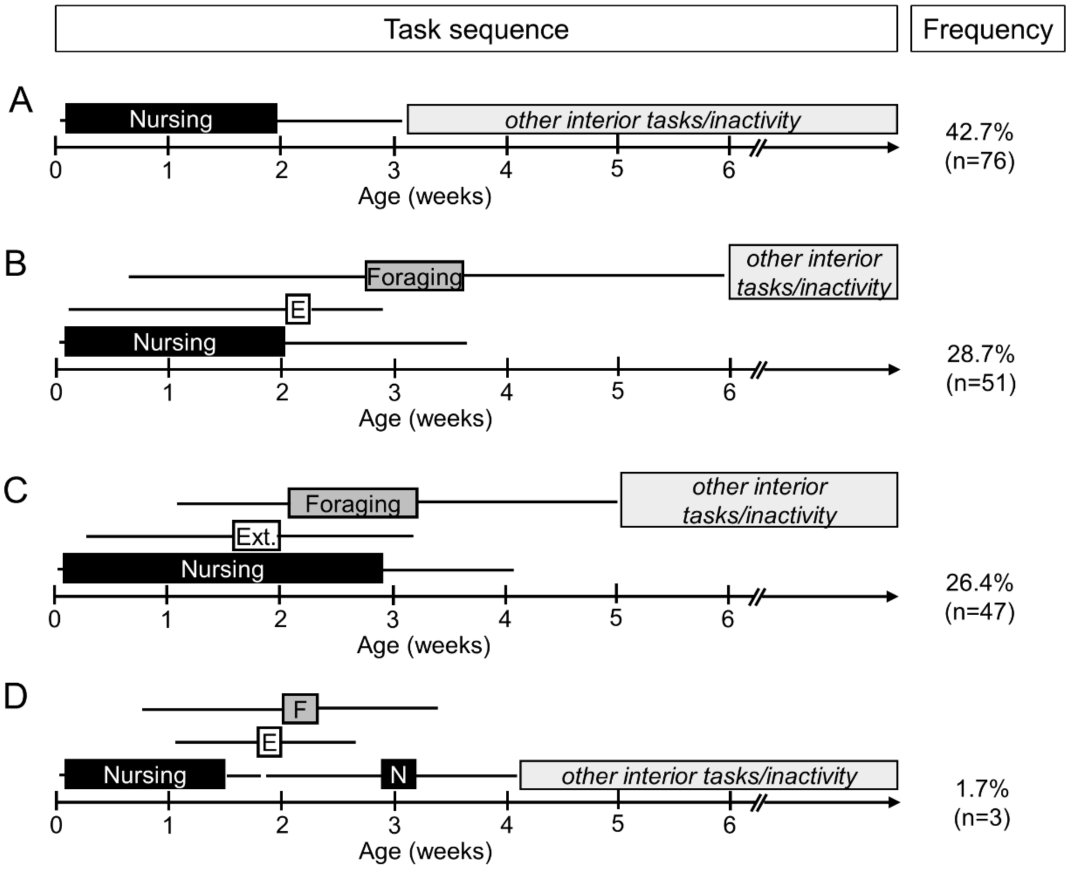

Fig. 3: Frequency distribution of age-dependent task allocation in *Camponotus* workers. Black: Nursing (N). White: Exterior activity (E). Dark Grey: Foraging (F). Light grey: undetermined other interior tasks (e.g. waste management) or inactivity within the nest chamber. Boxes indicate mean age at onset and offset of each task, whiskers indicate standard deviation (left: SD onset; right: SD offset). Datasets were pooled over the three subcolonies. (A): Workers remaining nurses for several weeks (n=76). (B): Workers with complete interior-exterior transition (n=51). (C): Workers switching to exterior work while continuing nursing activity (n=47). (D): Workers with an interior-exterior transition that switched back to interior activity (n=3). This task sequence was only seen in rare cases within the one subcolony were individuals were tracked for longer than four weeks. Due to the shortened observation period in two subcolonies, frequency of this task sequence may be underestimated.

Around 60% of newly-hatched individuals underwent the behavioral transition and became exterior workers (Fig. 3). Before engaging actively in the collection of food, all workers visited the foraging arena up to several days. Exterior activity took place during one and two weeks of age (Fig. 2), thus exterior activity and nursing activity were occasionally found to be performed in parallel for some periods. Workers started foraging at an age range of 11 to 20 days, and proceeded to forage until an age range of 14 to 29 days (Fig. 2). In subcolony A, switch to exterior and foraging activity was approximately one week later than in the other two subcolonies (Kruskal-Wallis tests; onset exterior activity: H(2,103)=29.0, $p<0.0001$; offset exterior activity: H(2,98)=12.8, p=0.002; onset foraging: H(2,61)=10.2, p=0.006; offset activity: H(2,61)=12.4, p=0.002; Fig. 2D-F), indicating flexibility in task allocation. In conclusion, three typical task sequences could be observed in *C. rufipes* (Fig. 3).

Frequencies of task sequences did not differ between the subcolonies (χ^2 test: $\chi^2=5.4$, p=0.5), so datasets were pooled. Most frequent, workers stayed nurses throughout several weeks (Fig. 3A). Other workers underwent the complete interior-exterior transition (Fig. 3B). In the next frequent task allocation pattern, workers switched to exterior activity, but proceeded to perform nursing activities (Fig. 3C). Intensity of activity in interior and exterior task could differ from each other, but was not quantified in the course of the experiment. Only during the long experimental phase of colony A, workers rarely switched back from exterior activity to interior activity (Fig. 3D).

Table 1: Worker age at onset and offset of behavioral activities in accordance to different task sequences. N: Workers remaining nurses for several weeks; N→N&F: Workers switching to exterior work while continuing nursing activity; N→F: Workers with complete interior-exterior transition; N→F→N: Workers with an interior-exterior transition that switched back to interior activity. Worker age is displayed as median±IQR. Different letters show significant differences between types of task sequences in offset or onset of a behavioral activity (Kruskal-Wallis tests).

Task sequence	Nursing		Exterior activity		Foraging		Nursing	
	Onset	Offset	Onset	Offset	Onset	Offset	Onset	Offset
N	1.5 ± 1.0^{a} (n=76)	12.0 ± 12.0^{a} (n=76)	-	-	-	-	-	-
N→N&F	2.0 ± 1.0^{a} (n=45)	16.0 ± 10.0^{bc} (n=45)	9.0 ± 7.0^{a} (n=45)	12.0 ± 9.0^{a} (n=45)	15.0 ± 12.0^{a} (n=19)	22.0 ± 17.0^{a} (n=19)	-	-
N→F	1.5 ± 1.0^{a} (n=52)	14.0 ± 11.0^{ac} (n=52)	11.5 ± 13.0^{a} (n=52)	10.0 ± 11.0^{a} (n=52)	19.0 ± 15.0^{a} (n=36)	24.0 ± 21.5^{a} (n=36)	-	-
N→F→N	1.0 ± 2.0^{a} (n=3)	10.0 ± 5.0^{ac} (n=3)	10.0 ± 13.0^{a} (n=3)	10.0 ± 13.0^{a} (n=3)	10.0 ± 17.0^{a} (n=3)	14.0 ± 14.0^{a} (n=3)	18.0±14.0 (n=3)	21.0±12.0 (n=3)

Between the four task allocation types, only the worker ages at offset of nursing differed significantly (Table 1; Kruskal-Wallis tests; nursing onset: H(3,176)=0.07, p=0.9;

nursing offset: H(3,176)=8.8, p=0.03; exterior onset/exterior offset: H(3,100)=0.0, p=1.0; foraging onset/foraging offset: H(3, 58)=0.0, p=1.0).

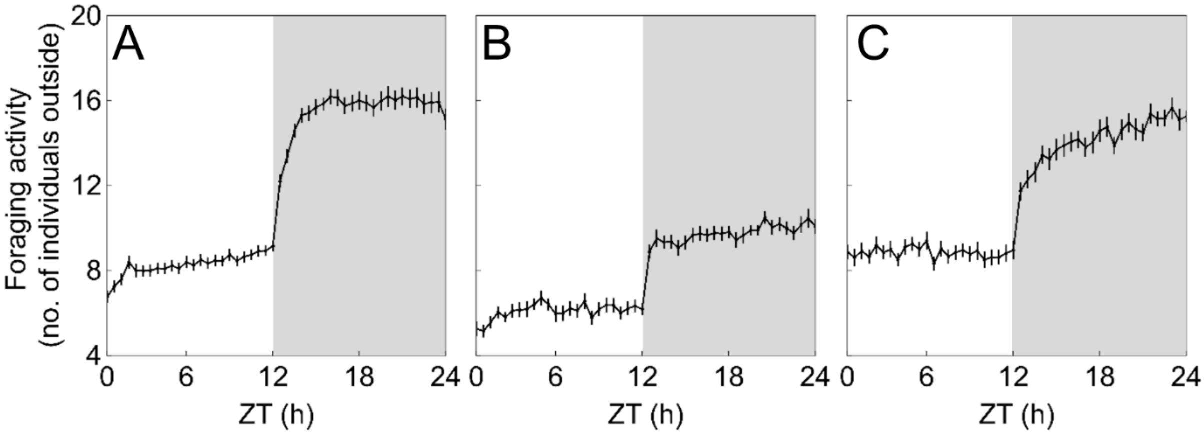

Fig. 4: Foraging activity in the course of 24 hours. (A): Subcolony A (n=77 days). (B): Subcolony H (n=28 days). (C): Subcolony 14 (n=28 days). Data points show mean±SE. The 12 hour dark phase is indicated in grey.

Foraging activity on subcolony level was predominant nocturnal, but a low proportion of workers was present in the foraging arena during the day (Fig. 4). On individual level, we found no differences in temporal preferences in foraging activity between the subcolonies (χ^2 test: χ^2=2.9, p=0.2), so datasets were pooled. The majority of foragers (67.0%) was present in the foraging arena strictly during nighttimes (Fig. 5). Only one individual in the three subcolonies was found to be strictly diurnal. Other 32% of individuals were active during both light regimes.

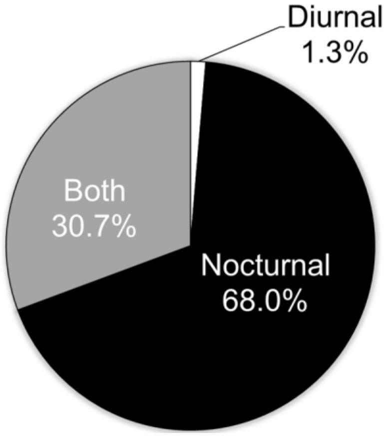

Fig. 5: Temporal preferences in foraging activity. Proportion of individuals that foraged either strictly diurnally (white; n=1), strictly nocturnally (black, n=75) or during both light regimes (grey, n=36). Data sets were pooled over the three subcolonies.

Not all individuals switched from interior to exterior activity, indicating that division of labor was not strictly age-dependent. As morphological differences can account for further task allocation, we compared body sizes between switchers and non-switchers. We found no differences between subcolonies (Kruskal-Wallis tests; switchers:

H(2,41)=0.04, p=0.9; non-switchers: H(2,33)=1.03, p=0.6), so datasets were pooled. Workers that did not undergo the transition showed higher variation in thorax lengths than workers switched to exterior activity (Fig. 6A). As a consequence, thorax lengths between these two groups differed significantly (Mann-Whitney U-test: U(41,33)=305.5, z=-4.0, p<0.0001; Fig. 6B).

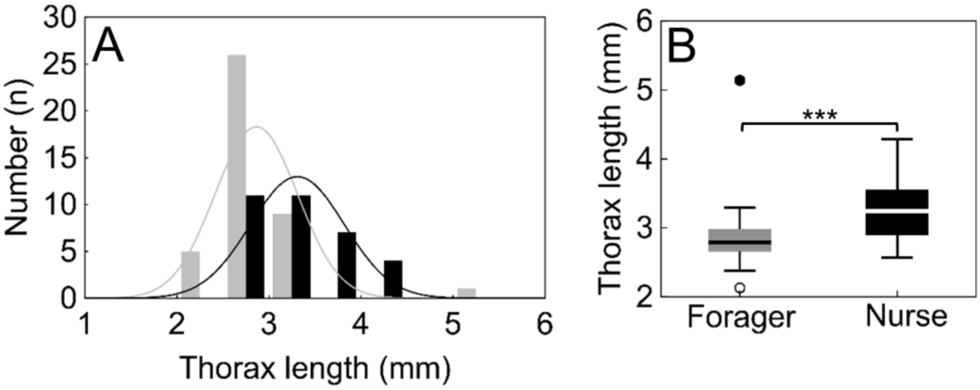

Fig. 6: Body size of individuals assigned to the forager and nurse caste. Thorax lengths were compared between foragers (grey, n=41) and nurses older than 14 days (black, n=33). Data sets were pooled over the three subcolonies. (A): Frequency distribution. (B): Boxplots. Boxes show medians (center lines) and interquartile ranges (boxes) for foragers (grey) and nurses (black). Whiskers indicate the minimum and maximum values, open circles show outliers. Asterisks indicate significant differences between groups (Mann-Whitney U-test).

Worker age & circadian rhythms

Colonies differed in several characteristics of their locomotor activity patterns, so datasets were not pooled. Exemplary actograms illustrate the effect of age on locomotor activity rhythms of individuals from the three subcolonies (Fig. 7). Rhythmic locomotor activity patterns were even detected in one-day old ants. Locomotor activity during the first days in the monitor was weak (Fig. 7), but strength of daily activity increased over the course of two weeks (Fig. 8). Initial activity levels differed strongly between subcolonies (Kruskal-Wallis test: H(2,51)=8.9, p=0.01; subcolony A: 1011.4±84.9 beam crosses/day, n=26; subcolony H: 903.0±113.3 beam crosses/day, n=13; subcolony 14: 1496.7±201.9 beam crosses/day, n=12). Because of high inter-individual differences within the subcolonies, activity levels after two weeks did not differ between the subcolonies anymore (Kruskal-Wallis test: H(2,49)=2.6, p=0.3; subcolony A: 2547.7±423.2 beam crosses/day, n=25; subcolony H: 3531.0±594.6 beam crosses/day, n=13; subcolony 14: 3489.4±702.2 beam crosses/day, n=11).

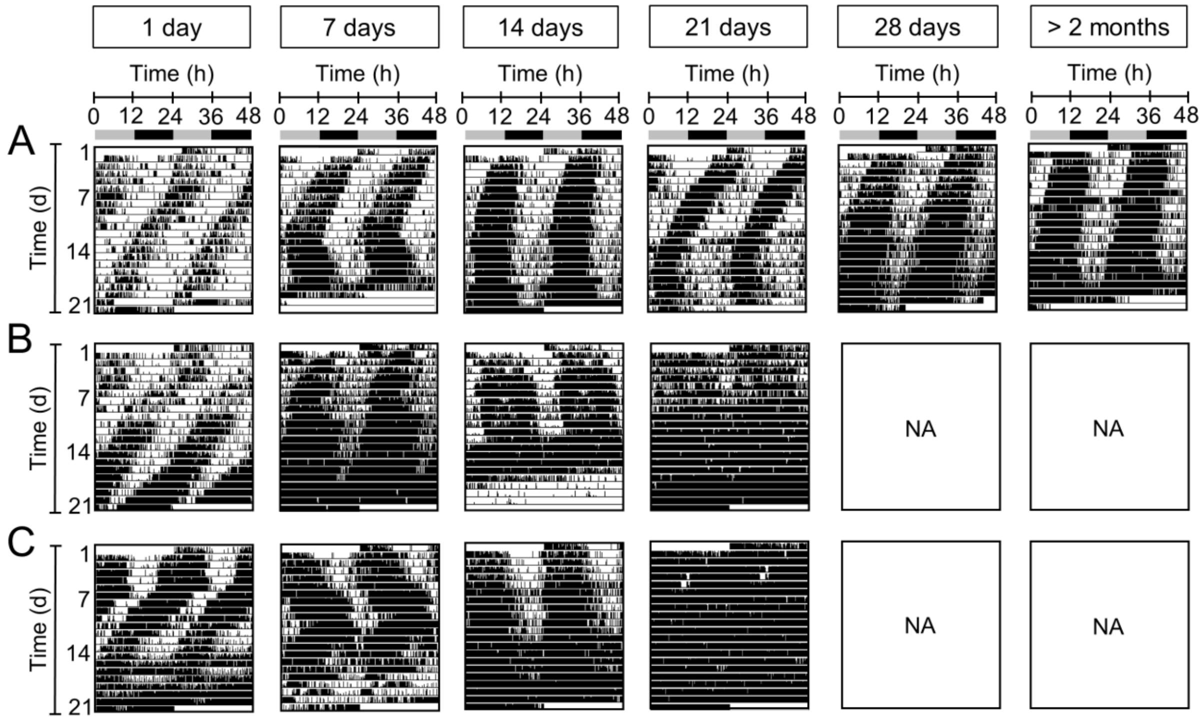

Fig. 7: Exemplary actograms of locomotor activity rhythms of individual workers of different age under constant darkness. Locomotor activity (indicated as black bars) under 21 days of constant darkness is shown as double plot. (A): Individuals from subcolony A. (B): Individuals from subcolony H. (C): Individuals from subcolony 14. As individuals in subcolonies H and 14 were only tracked until an age of 25 days, data within the age group of 28 days and > 2 months are lacking.

Age had no significant effect on survival of ants during the first week of isolation in the monitors (Table 2; χ^2 tests: subcolony A: χ^2=7.9, p=0.2; subcolony H: χ^2=0.8, p=0.8; subcolony 14: χ^2=3.7, p=0.3;). For rhythmicity of individuals, no consistent effect of age was detected. In subcolony H and 14 rhythmicity dropped already in 7-day old ants (χ^2 tests; subcolony H: χ^2=15.4, p=0.001; subcolony 14: χ^2=4.1, p=0.2), whereas rhythmicity levels in subcolony A remained constant until an age of two months (χ^2 test: χ^2=3.8, p=0.6). Period values varied around 24 hours and did not change with ongoing worker age (Kruskal-Wallis tests; subcolony A: H(5,70)=4.3, p=0.5; subcolony H: H(3,24)=5.1, p=0.2; subcolony 14: H(3,46)=1.6, p=0.6). As power of rhythm was calculated over one week, levels in 1-day-old ants that not differ from levels in other age classes (Kruskal-Wallis tests; subcolony A: H(5,74)=10.1, p=0.07; subcolony H: H(3,24)=4.3, p=0.2; subcolony 14: H(3,46)=1.6, p=0.7).

Table 2: Survival rate, proportion of rhythmic individuals and characteristics of the rhythms for different aged workers during the first week of monitoring. Differences in survival and rhythmicity rates were evaluated via X^2 tests; differences in activity periods and power of rhythms were evaluated via Kruskal-Wallis tests.

Subcolony A

Age class	Survival (%)	Rhythmicity (%)	Period (h) (median±IQR)	Power (rel. units) (median±IQR)
1 day	81.3 (n=26)	92.3 (n=24)	23.5± 0.95 (n=24)	51.6±56.5 (n=26)
7 days	91.7 (n=11)	90.9 (n=10)	23.2±1.6 (n=11)	76.6±75.5(n=11)
14 days	90.9 (n=10)	100.0 (n=10)	24.2±1.5 (n=10)	74.1±44.6 (n=10)
21 days	87.5 (n=7)	100.0 (n=7)	24.2±2.8 (n=7)	93.2±57.9 (n=7)
28 days	100.0 (n=8)	100.0 (n=8)	24.0±1.8 (n=8)	85.3±102.1 (n=8)
> 2 months	63.2 (n=12)	83.3 (n=10)	23.4±0.3 (n=10)	105.2±118.9 (n=12)
Statistical test	χ^2=7.9 p=0.2	χ^2=3.8 p=0.6	H(5,70)=4.3 p=0.5	H(5,74)=10.1 p=0.07

Subcolony H

Age class	Survival (%)	Rhythmicity (%)	Period (h) (median±IQR)	Power (rel. units) (median±IQR)
1 day	92.9 (n=13)	92.3 (n=12)	23.5±1.4 (n=13)	41.3±35.1 (n=13)
7 days	100.0 (n=3)	33.3 (n=1)	26.0±3.5(n=3)	55.0±52.9 (n=3)
14 days	100.0 (n=2)	50.0 (n=1)	24.2±1.0(n=2)	53.6±47.6 (n=2)
21 days	100.0 (n=6)	0.0 (n=0)	24.8±2.9 (n=6)	24.6±21.8 (n=6)
Statistical test	χ^2=0.8 p=0.8	χ^2=15.4 p=0.001	H(3,24)=5.1 p=0.2	H(3,24)=4.3 p=0.2

Subolony 14

Age class	Survival (%)	Rhythmicity (%)	Period (h) (median±IQR)	Power (rel. units) (median±IQR)
1 day	78.6 (n=11)	81.8 (n=9)	24.2±1.7 (n=11)	68.3±61.4 (n=11)
7 days	92.9 (n=13)	38.5 (n=5)	23.7±2.5 (n=13)	28.6±58.6 (n=13)
14 days	66.7 (n=20)	45.0 (n=9)	24.6±3.3 (n=20)	33.2±36.3 (n=20)
21 days	66.7 (n=2)	50.0 (n=1)	23.0±0.5 (n=2)	30.4±1.0 (n=2)
Statistical test	χ^2=3.7 p=0.3	χ^2=4.1 p=0.2	H(3,46)=1.6 p=0.6	H(3,46)=1.6 p=0.7

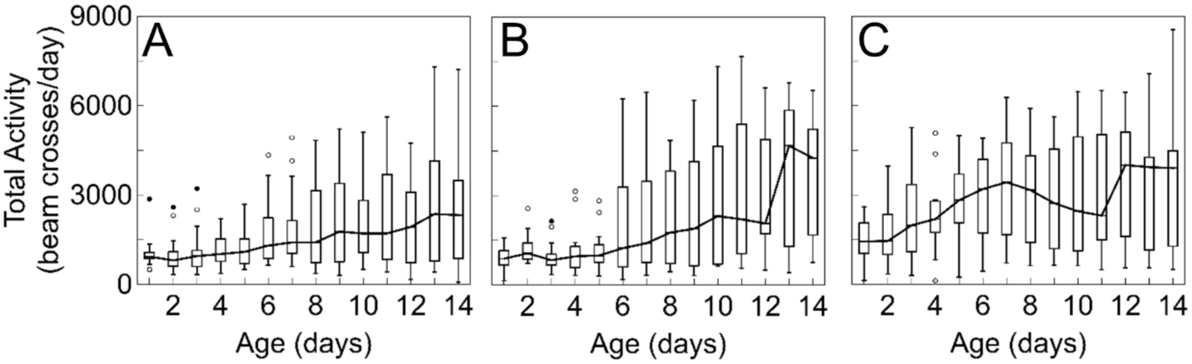

Fig. 8: Total levels of individual locomotor activity of 1-day old ants over two weeks of monitoring. Boxplots show medians (center lines) and interquartile ranges (boxes), whiskers show minimum and maximum values for the three subcolonies. Open circles show outliers. (A): Individuals (n=26) from subcolony A. (B): Individuals from subcolony H (n=13). (C): Individuals from subcolony 14 (n=11).

In both castes and in all age classes we found locomotor activity rhythms free-running from either the respective day (i.e. time of insertion in the monitors) or the respective night (i.e. peak of foraging activity in the subcolony; Fig. 9). In both nurses and foragers, the proportion of workers with rhythms free-running from the respective night did not increase with increasing time spent in the subcolonies (χ^2 tests with Bonferroni correction, α=0.025; nurses: χ^2=10.3, p=0.04; foragers: χ^2=0.3, p=0.9). When the proportion of workers with rhythms free-running from the subjective night was compared between nurses and foragers within every age class, we in turn found no significant differences (χ^2 tests with Bonferroni correction, α=0.025; 7 day old ants: χ^2=0.006, p=0.9; 14 day old ants: χ^2=3.7, p=0.05; 21 day old ants: χ^2=0.3, p=0.6; 28 day old ants: χ^2=0.9, p=0.3).

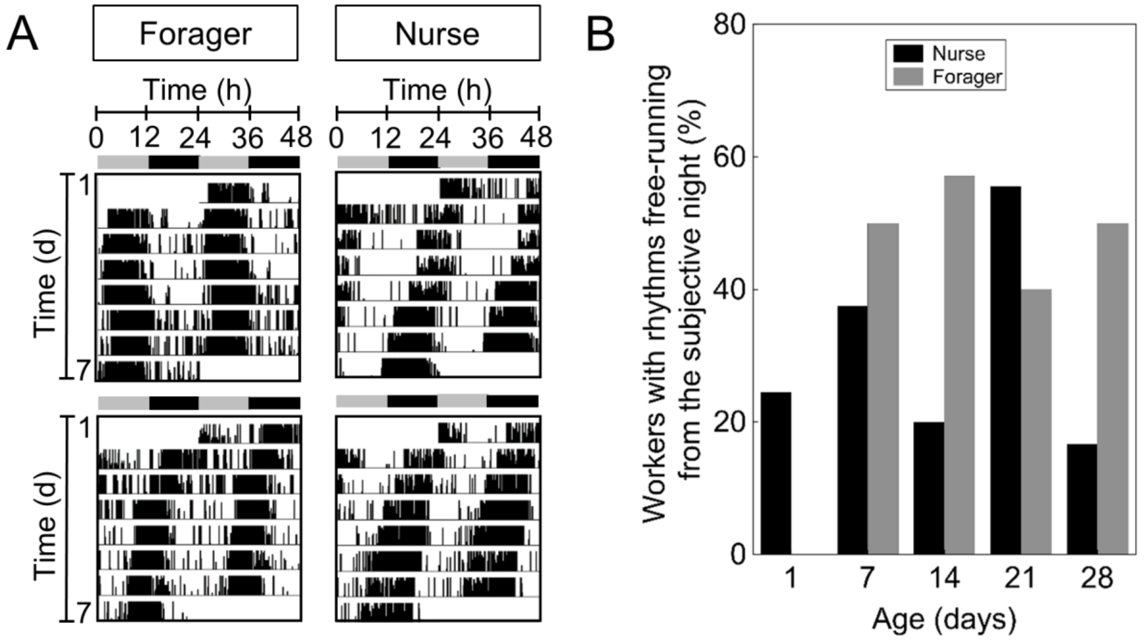

Fig. 9: Locomotor activity rhythms free-running from either the subjective day or the subjective night. (A): Exemplary actograms of individual locomotor rhythms. Locomotor activity (indicated as black bars) under first 7 days of constant darkness is shown as double plot for 14 day-old foragers (left) and nurses (right). Top: Locomotor rhythms free-running from the subjective day. Bottom: Locomotor rhythms free-running from the subjective night. (B): Proportion of workers with free-running rhythms from the subjective night in the different age groups and castes. Black: nurses (n_{1day}=49; n_{7days}=23; n_{14days}=23; n_{21days}=9; n_{28days}=6). Grey: foragers (n_{1day}=0; n_{7days}=4; n_{14days}=7; n_{21days}=5; n_{28days}=2).

Discussion

Worker age & division of labor

So far, only the presence of a size-dependent division of labor was reported for the nectar-collecting ant *C. rufipes* (Jaffé & Sánchez, 1984). Media-sized workers were shown to perform both interior and exterior work (Jaffé & Sánchez, 1984; Soares et al., 2004), so an additional mechanism of division of labor could refine task allocation especially in this size class. Our 24-hour tracking of behavioral activities of 188 newly hatched ants in three colonies for up to 11 weeks revealed the presence of an additional age-dependent polyethism. After eclosion, workers were fairly immobile and frequently fed by older workers, but started nursing themselves within the first 48 hours after hatching. After a transition period of few days exploring the foraging arena, workers began to forage actively at an age of two to three weeks. This subtle difference emphasizes that caste-affiliation can not be inferred by location alone, although being a good indicator.

Age range of workers performing interior and exterior tasks is comparable to that reported for various other ant species (*Pheidole dentata*: Wilson, 1976; *Solenopsis invicta*: Mirendra & Winson, 1981; *Tapinoma erraticum*: Lenoir, 1979; *Trachymyrmex septentrionalis*: Beshers & Traniello, 1995; *Messor/Myrmica*: Ehrhardt, 1930). The transition to exterior work at an age of one to two weeks in *C. rufipes* matches the previously reported age-dependent brain maturation in this species (Yilmaz et al., 2016). During the first two weeks of the worker life, volume of optic neuropiles and opsin gene expression increases in interior workers, suggesting a preparation of the visual system for the future demands associated with outdoor foraging. Age-dependent brain maturation as shown in this and other species (*C. floridanus*: Gronenberg et al., 2006; *Pheidole dentata*: Seid et al., 2005), as well as other physiological changes (Caemmerts-Tricot, 1974; Fénéron et al., 1996; Dolezal et al., 2012) could lead to altered thresholds associated with particular tasks and could therefore cause age-dependent division of labor.

Transition between interior and exterior work was not abrupt, so the same workers performed exterior and interior tasks simultaneously for some periods. Such overlapping activity suggests an age-dependent repertoire increase as seen in *Pheidole* ants (Wilson, 1976; Calabi et al., 1983). Frequency of interior activity is expected to decrease with age, while frequency of exterior activity is expected to increase (Seid & Traniello, 2006).

Transition rate and worker age at onset and offset of behavioral tasks varied between the colonies, emphasizing the flexible character of task allocation (Gordon, 1989; reviewed in Schmid-Hempel, 1992; Robinson et al., 2009; Waddington & Hughes, 2010). Not all workers switched from interior to exterior work, as shown for other species in the laboratory (*Lasius*: Lenoir, 1979; *Pogonomyrmex*: Gordon et al., 2005). This could result from the reduced needs within the small scale of the subcolonies, because foragers did not need to travel long distances or to recruit many individuals to exploit the food source in the arena. Another factor is the high survival rate and longevity of foragers in laboratory colonies. In *Pogonomyrmex* ants, the increased lifespan of foragers hinders the transition of new workers into the forager population (Kwapich & Tschinkel, 2015). In contrast to the laboratory, field colonies of this species experience a mortality of up to 5% of the forager population per day, leading to a complete turnover of this caste within four weeks. In our subcolonies, only few of the initial “old” foragers died and therefore did not need to be replaced, so transition rate was likely underestimated. Additionally, the ratio of young to old workers in colonies influences the transition rate (Lenoir, 1979). Hatching rate in our experiments varied from day to day and between subcolonies, ranging from one to eight eclosions per day. Although we kept worker to larvae ratio constant throughout the experiment, sizes of added larvae could have varied and consequently developmental times differed. When young workers hatched in higher frequencies, they replaced older nurses more quickly and advanced the transition in these ants.

As some individuals switched to exterior work as the aged, other individuals kept performing interior work. Same aged ants can differ in thresholds associated with these two task (foraging: Detrain & Pasteels, 1990; nursing: Weidenmüller et al., 2009), leading to differences in task allocation. As worker size could have added to this differentiation, we compared thorax lengths between 14-day old interior and exterior workers. At this age, transition to exterior activity would have taken place. We found a great variation in body sizes in nurse workers, but foragers were predominantly media-sized. This difference matches the previous records in *C. rufipes* (Jaffé & Sánchez, 1984; Soares et al., 2004). Even though these studies reported that large workers predominantly served as repletes, we could show their involvement in nursing activity.

Although we did not quantify frequency of nursing activity, large workers seemed to engage rather sparsely in brood care compared to other size classes.

Nocturnal foraging activity was previously shown for *C. rufipes* only on colony level (Jaffé & Sánchez, 1984). We confirmed not only the preference for nocturnal foraging on group level, but further on demonstrated nocturnal activity on individual level in the majority of foragers by continuous tracking of single workers in our experiment. Foragers being active during daytimes were rarer and were always active during nighttimes as well, suggesting a predominant nocturnality in all individuals. Field studies report the occasional diurnal activity in *C. rufipes* (Del-Claro & Oliviera, 1999; Fagundes et al., 2005), which could be an adaption to seasonal environmental changes. The presence of individuals with nocturnal and diurnal foraging preferences may enable the flexible shift of foraging period. As such timing of foraging activities would need an endogenous clock, we further determined its presence via locomotor activity recordings.

Worker age & circadian rhythms

We monitored endogenous locomotor rhythms in ants of known caste and age classes on a weekly base. Although their activity levels were low, even 1-day old ants exhibited endogenous locomotor activity rhythms with periods close to 24 hours. Activity levels increased within the course of one week, but the strong difference in final activity levels between the subcolonies highlights the variability of circadian rhythms within one species. Like *C. rufipes*, less than one-week old callow *Diacamma* ants already exhibit strong circadian rhythms (Fuchikawa et al., 2014). In contrast to ants, newly hatched honeybees develop a circadian rhythm not until one week of age (Toma et al., 2000; Bloch et al., 2002). Only newly hatched bees that experience the colony environment for at least two days display circadian rhythms in isolation (Eban-Rothschild et al., 2012; Fuchikawa et al., 2016). Although the young ants in our experiments experienced the colony environment for only 24 hours, they already displayed circadian locomotor rhythms. These results demonstrate fundamental differences in the development of rhythms between social insects. In contrast to nurse bees, which only care arrhythmically for the brood, nurses in *Camponotus* ants might need a functional circadian clock even in these early days for the rhythmic translocation of the brood (Roces & Núñez, 1989, 1996; Roces, 1995). Yet it is unknown, at which age young ants start to engage in brood translocation. Rhythmic locomotor rhythms were found in both nurse and forager ants, demonstrating the presence of a functional endogenous clock in the two worker castes. Tracking of behavioral activity of nurses in the colony would be needed to clarify whether other behavioral activities of the worker castes are displayed in a rhythmic manner as well (see chapter 2 & 4). In honey bees, nurses possess an endogenous clock as well, but their circadian rhythmicity is suppressed in the social context (Bloch et al., 2001; Shemesh et al., 2007; Meshi & Bloch, 2007). Consequently, locomotor rhythms of isolated individuals must not reflect behavioral activity patterns in the social context.

As the interior-exterior transition rate was low in our subcolonies, only low numbers of foragers were present across all age classes. Therefore, age effects were analyzed in general and not separate for the castes. The effect of age on circadian rhythm was intensively studied in solitary insects like *Drosophila* (reviewed in Giebultowicz & Long, 2015). Flies older than one to two months have weaker activity rhythms, accompanied with changes in period length and phase relationship (Joshi et al., 1999; Koh et al., 2006; Luo et al., 2012). In our experiments, age effects on circadian rhythms in ants differed between colonies. Whereas rhythmicity declined already in one-week old ants in two colonies, rhythmicity levels were constantly high in ants up to two months old in the other colony. Founding queens of the three colonies were collected in the same region, but could nevertheless differ genetically and therefore vary in their expression of circadian rhythms. This diversity is even present in individuals of the same colony, as seen in the variation of period and power of rhythms within same aged ants. Probably because of these variations, we did not see significant alterations of rhythms in older ants like described in *Drosophila*. In general, *Drosophila* flies and ants differ in their lifespan. Whereas mean lifespan in *Drosophila* is about four weeks (Luckenbrill & Clare, 1985; Curtsinger et al., 1992; Curtsinger & Khazaeli, 2002), ant workers can live up to several months (Calabi & Porter, 1989; Kramer & Schaible, 2013). Analysis of senescence in *Pheidole dentata* found no decrease in behavioral performance in 120-day old workers (Giraldo et al., 2016), so age-related alterations in rhythms could become only more present in way older ants.

Across all ages and castes, we found two types of free-running activity patterns in rhythmic workers. In some workers, activity was free-running from the subjective day, which was the time point when ants were placed in the monitors. Other workers displayed rhythms free-running from the subjective night, which was the peak time of foraging activity in the subcolonies. Not only foragers kept the phase of their activity in the social context, even some nurses were in phase with this colony activity. Twenty four hours in the colony were sufficient in 25% of young ants to synchronize their activity phase with the colony phase. These results suggest a form of social synchronization between nurses and foragers within the colony context, something that has already been demonstrated in bees as well (Frisch & Königer, 1994; Fuchikawa et al., 2016).

In this chapter we demonstrated the presence of an age-dependent division of labor in the ant species *C. rufipes*. Young workers stay inside the nest and engage in nursing, whereas older workers leave the nest to forage. All ants possess a functional endogenous clock, which is the foundation for temporal organization of activities within an ant colony.

Chapter II

Plasticity of daily behavioral rhythms in foragers and nurses of the ant *Camponotus rufipes*: influence of social context and feeding times

Abstract

Daily activities within an ant colony need precise temporal organization, and an endogenous clock appears to be essential for such timing processes. A clock drives locomotor rhythms in isolated workers in a number of ant species, but its involvement in activities displayed in the social context is unknown. We compared locomotor rhythms in isolated individuals and behavioral rhythms in the social context of workers of the ant *Camponotus rufipes*. Both forager and nurse workers exhibited circadian rhythms in locomotor activity under constant conditions, indicating the involvement of an endogenous clock. Activity was mostly nocturnal and synchronized with the 12:12h light-dark-cycle. To evaluate whether rhythmicity was maintained in the social context and could be synchronized with non-photic zeitgebers such as feeding times, daily behavioral activities of single workers inside and outside the nest were quantified continuously over 24 hours in 1656 hours of video recordings. Food availability was limited to a short time window either at day or at night, thus mimicking natural conditions of temporally restricted food access. Most foragers showed circadian foraging behavior synchronized with food availability, either at day or nighttime. When isolated thereafter in single locomotor activity monitors, foragers mainly displayed arrhythmicity. Here, high mortality suggested potential stressful effects of the former restriction of food availability. In contrast, nurse workers showed high overall activity levels in the social context and performed their tasks all around the clock with no circadian pattern, likely to meet the needs of the brood. In isolation, the same individuals exhibited in turn strong rhythmic activity and nocturnality. Thus, endogenous activity rhythms were inhibited in the social context, and timing of daily behaviors was flexibly adapted to cope with task demands. As a similar socially-mediated plasticity in circadian rhythms was already shown in honey bees, the temporal organization in *C. rufipes* and honey bees appear to share similar basic features.

This chapter is based on a pre-edited manuscript:

Mildner S, and Roces F (2017) Plasticity of daily behavioral rhythms in foragers and nurses of the ant *Camponotus rufipes*: influence of social context and feeding times. PLoS ONE 12:e0169244.

Introduction

Ant societies are well known for their decentralized structure and organization of activities through division of labor. Colony tasks are usually performed simultaneously inside and outside the nest by different worker groups. In general, task allocation in ants is based on both temporal and size polyethism (Hölldobler & Wilson, 1990). In the nectar-feeding ant *Camponotus rufipes*, for instance, media-sized workers engage in tasks both inside and outside the nest (Jaffé & Sánchez, 1984). Further task allocation is based on worker age. Young workers stay inside the nest engaging in nursing, whereas older workers leave the nest to forage (Soares et al., 2008). Beyond the observed plasticity in task allocation in social insects (Gordon, 1989; Robinson, 1992), daily activities should be synchronized among individuals inside and outside the nest to generate a coordinated colony response, especially under varying abiotic and biotic environmental factors.

Activity rhythms outside the nest

It is an open question the extent to which workers engaged in different nest tasks coordinate their activities within an ant colony. While some tasks like nest digging or brood care do not appear at first glance to underlie daily changes or to be synchronized with biotic or abiotic factors, other tasks such as foraging and feeding of brood are expected to be tightly synchronized in order to increase colony efficiency. In nectar-feeding ants of the genus *Camponotus*, which lack food stores inside their nests, workers repeatedly visit renewable nectar resources such as extrafloral nectaries or aphid colonies, and their foraging activity is expected to be synchronized with the availability of carbohydrate sources. As a consequence, even prey collection may be limited to a certain phase of the day. The access to food sources is likely to be temporally restricted by daily cycles in nectar production (Passera et al., 1994; Heil et al., 2000; Dattilo et al., 2014), changing environmental conditions (Kaschef & Sheata, 1963; Rosengren, 1977; Orr & Charles, 1994; Azcarate et al., 2007; Chong &

Lee, 2009) or competitors (Blüthgen et al., 2004; Lesssard et al., 2009). As such factors may have synergistic effects (Bestelmeyer, 2000; Barbieri et al., 2015), foraging activity needs to be scheduled to the right time of the day within a species-specific temporal niche. Studies on other nectar-feeding ant species focusing on the finding of food at a certain time of the day already provided further evidence for a robust time sense in these ants (Grabensberger, 1933; Harrison & Breed, 1987; Schatz et al., 1994; 1999; Fourcassié et al., 1999; Caemmerts, 2004). And in fact, *C. rufipes* workers have the ability to measure short time intervals while visiting nectar sources and to adjust their visiting times at food patches accordingly (Schilman & Roces, 2003). Although workers from this species predominantly forage during the nighttime (Jaffé & Sánchez, 1984), reports about occasional diurnal foraging (Oliveira et al., 1995; Del-Claro & Oliveira, 1999) highlight the flexible timing of activities. Activity shifts might represent a seasonal adaptation to changing environmental factors or food availability. Since such foraging rhythms were so far only demonstrated at the colony level, the questions whether colony-wide foraging rhythms reflect the rhythmical activity of single workers and are modulated by the temporal availability of food remained open. Therefore we investigated behavioral activity patterns of individual foragers under different feeding schedules.

Activity rhythms within the nest

There is also evidence that coordinated activity rhythms occur among workers inside an ant nest (Hatcher et al., 1992; Cole & Cheshire, 1996), which may reduce redundancy and therefore increase the efficiency of collective behaviors such as nest building and brood care. Efficient brood care seems to be of high importance because it is linked to colony growth and survival. Groups of *Leptothorax* ants, for instance, showed synchronized peaks of locomotor activity inside the nest (Franks et al., 1990; Cole, 1991c), with synchrony increasing with group size and presence of brood (Cole & Hoeg, 1996). It was hypothesized that synchronized locomotion resembles synchronized brood care (Hatcher et al., 1992; Cole & Chesire, 1996). These studies, however, quantified only locomotor activity inside the nest for short time intervals, so that the question whether specific nursing behaviors undergo a daily cycle remained unexplored. Studies focusing on brood relocation for temperature control in the ant *Camponotus mus*, however, provided unequivocal evidence about the occurrence of endogenous, self-sustained bimodal rhythms in thermopreference for relocation of the immobile brood inside the nest by nurse workers (Roces & Núñez, 1989; 1995; 1996; Roces, 1995). Rhythmic brood translocation of pupae optimizes brain development and could affect sensory processing and learning abilities in adult ants (Falibene et al., 2016), being therefore relevant for colony functioning. To what extent other nursing activities such as feeding of brood follow a daily rhythm, for instance locked-on to daily rhythms in food collection by foragers, is still unknown. Therefore, we quantified daily activity patterns in nurses within the nest as compared to the activity patterns of foragers.

Activity rhythms and endogenous clocks

Almost all organisms have an endogenous clock that enables them to schedule daily activities. Core element of such clock systems is an endogenous oscillator, which generates multiple rhythms in metabolism and behavior. Under suitable, predictable daily changes in the environment (´zeitgeber´, e.g. the light-dark cycle), the oscillator generates entrained rhythms with periodicities of 24 hours. Under the absence of zeitgebers, rhythms keep free running with periodicities close to 24 hours. In several ant species, single isolated workers exhibit strong circadian rhythms in locomotor activity, thus providing evidence about the existence of an endogenous clock (McCluskey, 1965; North, 1987; Sharma et al., 2004c). As shown for different ant species, worker castes differ in their locomotor activity patterns. In *Pogonomyrmex* and *Camponotus* ants, foragers exhibited rhythmic activity patterns, whereas nest workers showed no marked activity peaks (Sharma et al., 2004b; Ingram et al., 2009). Conversely, nurse workers in *Diacamma sp.* showed strong circadian rhythms, and foragers were arrhythmic with much higher activity levels (Fuchikawa et al., 2014). In both cases, locomotor rhythms are hypothesized to reflect the activity profiles of the castes within the colony. But as rhythms of specific behaviors of the castes within the nest have not been analyzed so far, those locomotor rhythms can not be linked to true behavioral rhythms in the social context. In a broader sense, we lack information about the extent to which endogenous rhythms are involved in the scheduling of daily behaviors in the social context and in the synchronization among workers performing different tasks in ants. In honey bees, nurses perform their work inside the hive around the clock, with no apparent rhythmicity (Lindauer, 1952; Crailsheim et al., 1996; Moore et al., 1998). In contrast, foraging bees exhibit robust diurnal patterns of activity in the social context, strongly linked to food availability (Beling, 1929; Wahl, 1932; Frisch &

Aschoff, 1987; Moore, 2001). As nurses show rhythmic locomotor activity like foragers when isolated from the colony, their endogenous rhythmicity is suppressed in the social context (Shemesh et al., 2007). Therefore, plasticity in daily activity rhythms is linked to division of labor and strongly depends on the social context (reviewed in Bloch, 2010). To evaluate the impact of social environment on daily rhythms in bees, locomotor activity of isolated bees has been recorded under contact to microclimate (including pheromones) of a small colony (Beer et al., 2016). Workers synchronized their activity with the activity of the hive and more workers exhibited rhythmic locomotor patterns than isolated bees without any contact, which emphasizes the modulating effects of the social environment. It is unknown whether socially-mediated plasticity in activity rhythms represents a conserved, adaptive trait in other social insects like ants as well. To provide evidence of socially-mediated plasticity in activity rhythms in ants, we compared the locomotor activity rhythms in both isolated forager and nurse workers with their behavioral rhythms in the social context of the colony.

Aim of the study

The present study was aimed at investigating the link between division of labor and temporal organization in the nectar-feeding ant *C. rufipes*. We studied activity patterns of foragers, as representatives for outside-nest activities, and nurses, which perform inside-nest behaviors. Both castes are closely linked through food availability, as foragers are responsible for food collection and nurses subsequently feed the brood with the collected food. Daily rhythms of locomotor activity in isolated workers of both castes were quantified under controlled conditions in a first experiment, to provide evidence about the presence of an endogenous clock. To highlight possible effects of the social context on activity rhythms, we monitored daily activity of individually-identified foragers and nurses in small colonies in a second experiment, and compared the patterns with those observed in isolation. As temporal changes in food availability may strongly influence daily activities, we presented colonies in different series with daily pulses of food availability as well as *ad libitum* feeding regimes. Therefore, we analyzed the potential link between temporal changes in resource availability, timing of foraging behavior, and synchronization with inside-nest nursing behavior in the ant *C. rufipes*.

Material and Methods

Study system

C. rufipes is a nectar-collecting ant species distributed over broad ranges in South America. For the laboratory studies, queens were collected during their mating flights in December 2011 and 2014 in La Coronilla, Uruguay (33°53'25.2"S, 53°31'27.6"W) and were brought to the laboratory at the University of Würzburg, Germany. The species *C. rufipes* is not endangered nor protected. Export permits were issued by the Departamento de Fauna de la Dirección General de Recursos Naturales Renovables, Ministerio de Ganadería, Agricultura y Pesca, Uruguay. Colonies were raised in plaster nests under constant conditions (25°C, 50%rH) and a 12:12h LD-cycle (300 lux during the light phase). Queenright colonies consisted of several thousand workers and were fed *ad libitum* with water, diluted honey and pieces of cockroaches. This study complies with the ethical guidelines of the country where the research was carried out.

Experiment 1: Locomotor rhythms of isolated foragers and nurses

To study daily patterns in locomotion and their endogenous nature in nurses and foragers in *C. rufipes,* we monitored isolated individuals in a locomotor activity recording system (LAM 16; TriKinetics, Waltham, USA; detailed methods in chapter I). To record locomotor activity of the two different worker castes, we initially built three "subcolonies" from three *C. rufipes* queenright colonies (named colony H, Q and 14). A subcolony was a queenless group of several workers and brood settled in an artificial brood chamber (diameter: 5.5 cm, height: 1.5 cm) with access to an outside foraging arena (diameter: 6.3 cm, height: 4.6 cm) via one tunnel (diameter: 0.6 cm, length: 3.5 cm), thus representing a functional group of workers that displayed both foraging and nursing behaviors. There is no evidence that the absence of the queen affects caste affiliation. It may reduce the total activity in both castes as compared to that of natural colonies, thus leading to an underestimation of activities in our experiments (Berton et al., 1992; Vienne et al., 1998). Subcolonies of 50 workers and 120 larvae were held under constant conditions (25°C, 50%rH) and a 12:12h LD-cycle (3000 lux during light phase) in transparent acrylic glass nest setups placed in incubators (I-30BLL, CLF PlantClimatics GmbH). In this way it was assured that all workers were exposed to the LD-cycle. To build the subcolonies and promote division of labor, we collected ants with varying body sizes both from the brood chamber as well as the foraging areas of the large queenright colonies. An *ad libitum* supply of food (honey-water, chopped cockroaches and water) was provided daily on an elevated platform (diameter: 2.6 cm, height: 5.0 cm) in the foraging arena. The use of the platform assured that foragers actively searched for food, allowing their distinction from other outside workers like guards. All workers were marked individually with printed paper tags glued to their gaster with acrylic paint (KÜNSTLER.FARBEN.FABRIK, Hallerndorf, Germany), thus affiliation of individuals to the forager or to the nurse caste could be determined based on their behavior and location. Foragers were considered as those ants present at the foraging platform collecting food, while nurses were those workers tending brood inside the brood chamber. Caste affiliation was determined visually on a daily basis throughout one week after the subcolonies were established, which simultaneously represented the 1-week-entrainment phase under the LD-cycle.

The small subcolonies allowed unequivocal recognition of tagged individuals, especially inside the brood chamber, but as a consequence of the reduced subcolony size, only few workers engaged as foragers. To obtain adequate sample sizes for locomotor activity monitoring of foragers, we additionally built larger subcolonies with 150 workers and greater numbers of brood items in a larger setup (brood chamber: 9x9x5.5 cm, foraging arena: 19x19x9 cm, feeding platform: diameter: 8.5 cm, height: 3.5 cm). Workers collecting food at the platform were considered as foragers and were marked daily throughout one week.

Thereafter, subcolonies were discontinued and single identified individuals of known caste were placed in the locomotor activity monitors and recorded over three weeks under different light regimes, each lasting one week. After one day of settling in, daily activity of single ants was recorded for seven days under the similar 12:12h LD-cycle used for the subcolonies. In the second week of recording, the LD-cycle was delayed for six hours, to test if individuals were able to resynchronize their activity after the shift. Finally, locomotor activity was recorded under constant darkness to provide evidence of endogenous activity patterns and to quantify free-running periods.

Activity measured over the whole recording period (22 days) was displayed in form of actograms via ActogramJ for all individuals. For the recording periods under the LD-cycle as well as the phase-shifted LD-cycle, we generated average activity patterns of all surviving ants based on seven days of each observation period (LD-cycle: $n_{Foragers}$=78, n_{Nurses}=46; phase-shifted LD-cycle: $n_{Forager}$=61, n_{Nurses}=37). Calculations of total activity levels as well as of the relative nocturnal activity (normalized for the total activity of every individual) on the second day of the respective recording period were selected to avoid pseudoreplications, and so to quantitatively compare activity patterns between the castes under the three light regimes (Mann-Whitney U-tests). For further description of the ants' endogenous clock we calculated the period values of the individual activity under the LD-cycles and constant darkness by periodogram analysis (Lomb-Scargle-method, significance level $\alpha=0.5$; ActogramJ), and tested for differences between the castes (Mann-Whitney U-tests). The effects of caste on survival rate and rhythmicity were analyzed by χ^2 tests. All statistical analysis were performed in STATISTICA (StatSoft, Inc., Version 10.0) after testing data sets for normal distribution via Shapiro-Wilks test.

Experiment 2: Caste-dependent plasticity in daily behavioral rhythms

Daily behavioral activity patterns of foragers and nurses in the social context

In order to investigate caste-specific daily activities, we built 32 subcolonies of individually marked ants as described for Experiment 1 (see previous paragraph for details) from four *C. rufipes* queenright colonies (named colony J, O, H and R).

To determine the synchronization abilities of workers with pulses of food availability, we carried out three experimental series with either time-restricted or *ad libitum* feeding regimes. In two series, food availability was restricted to a short time window either four hours after lights-on (**Z**eitgeber **T**ime: ZT 4; "daytime feeding") or four hours after lights-off (ZT 16; "nighttime feeding"). A supply of 10% sugar water and ten freshly killed *Drosophila* flies proved to be sufficient for subcolony maintenance. Depending on the collecting rate of the ants, food was present in the foraging arena only for up to two hours. In the third series, which served as a control, subcolonies were fed *ad libitum* throughout the day to identify endogenous foraging times. Here, pieces of cockroaches were fed instead of *Drosophila* flies to ensure permanent food availability. The amount of sugar water and cockroaches exceeded the subcolony's daily needs and were replaced daily at different times to avoid temporal cues.

Caste affiliation was determined on a daily basis throughout the first week after setting-up of the subcolony, which served as settling-in period and entrainment phase. In order to quantify all daily behaviors of the castes in a continuous way, we conducted 24-hour video recordings of the setups in the following week, i.e., after subcolonies had experienced the corresponding feeding regime for more than 7 but less than 14 days. Infrared cameras (CCD & CMOS, Conrad Electronic SE) and time-lapse analog video recorders (72-hour time-mode, Panasonic AG-6720/AG-6730) were used. Thereafter, an observer watched the videos and built ethograms for caste- and non-caste specific activities (Table 1) of randomly selected members of the two castes combined with their spatial location (TheObserver, Version 2.01, Noldus Information Technology).

Table 1: Activities displayed by single workers in the foraging arena (foragers) and in the brood chamber (nurses), monitored and continuously quantified over 24 h using time-lapse videos. Antennating: antennating objects, brood or conspecifics whilst standing. Walking: locomotor movement. Allo(grooming): self-grooming or grooming of conspecifics. Waste management: manipulating or carrying waste material (e.g. food remains, corpses). Trophallaxis: exchange of liquid food between workers. Food consumption: intake of food (foragers: ingestion of sugar water, water and feeding on *Drosophila* flies in the foraging arena; nurses: feeding on *Drosophila* flies in the brood chamber). Food transport: carrying *Drosophila.* Brood relocation: picking up and transporting larvae between the mandibles. Brood care: feeding liquid food to larvae or licking larvae.

Non-specific activities	Caste-specific activities	
	Foragers	Nurses
▪ Antennating	▪ Trophallaxis	▪ Brood relocation
▪ Walking	▪ Food consumption	▪ Food consumption
▪ (Allo)Grooming	▪ Food transport	▪ Brood care
▪ Waste management		

From this raw data, we calculated differences in total levels of inactivity per day between the castes for every feeding regime (Mann-Whitney U-tests), as well as within castes over the feeding regimes (Kruskal-Wallis tests) under Bonferroni correction ($\alpha=0.017$). Average time budgets for all behavioral activities were quantified. Daily patterns of caste-specific activity were calculated in addition by summing all activities belonging to this category. To enable data visualization, activities were pooled in 10 minute bins and therefore displayed as proportion per bin. We calculated period values (τ) of behavioral rhythms of both castes under the three feeding regimes using the cosinor method (significance level: $\alpha=0.05$; Cosinor program, Refinetti, version 3.1). Period values were categorized as ultradian (<20h), circadian (24±4h) or infradian (>28h). Foraging activity for every subcolony was quantified as the relative number of ants present in the foraging arena at every half an hour of the video recording, as well as total foraging activity per 12h-dark or light phase. Foraging activity was compared between the two 12h-light and dark phases within feeding regimes (Wilcoxon signed-rank test) and between feeding regimes (Kruskal-Wallis test) under Bonferroni correction ($\alpha=0.017$).

Daily locomotor activity of foragers and nurses in isolation from the social context

In order to evaluate the effects of the social context on the observed behavioral rhythms of foragers and nurses in the subcolonies, locomotor rhythms of all individually-identified workers were monitored over 8 days in isolation immediately after removal from the social context. The subsequent monitoring of locomotor activity served to verify the existence of a functional endogenous clock in the individuals. Experiments were performed using the modified locomotor activity monitors described above placed in incubators under constant conditions (25°C, 50%rH; Incubator: Rumed 1200, Rubarth Apparate GmbH; Incubator: I-30BLL, CLF PlantClimatics GmbH). A 12:12h LD-cycle was applied (4000-6000 lux during the light phase) to characterize daily locomotor rhythms. Data analysis was performed by calculating actograms (ActogramJ) and determining proportions of rhythmic individuals in each caste by periodogram analysis (Lomb-Scargle method, significance level $\alpha=0.05$), as well as the survival rate of castes after eight days of observation. The effect of caste and feeding regimes on survival rate and rhythmicity was analyzed by χ^2 tests under Bonferroni correction ($\alpha=0.017$). Representative actograms were chosen for every caste and feeding regime based on the most common activity patterns (rhythmic and arrhythmic).

Results

Experiment 1: Locomotor rhythms of isolated foragers and nurses

Although survival rates varied in both castes throughout the recordings, survival of nurses was only significantly lower than that of foragers during the last week of the recordings (Table 2; χ^2 tests; LD: χ^2=0.36, p=0.5; phase-shifted LD: χ^2=0.09, p=0.8; DD: χ^2=7.63, p=0.006). There were no differences between foragers and nurses in rhythmicity under both LD-regimes as well as under constant darkness (Table 2, χ^2 tests; LD: χ^2=0.00, p=0.9; phase-shifted LD: χ^2=0.72, p=0.4; DD: χ^2=1.31, p=0.2).

Table 2: Survival rate, proportion of rhythmic individuals and activity characteristics of foragers and nurses isolated in locomotor activity monitors. Different letters show significant differences between castes within the three light regimes (significance level α=0.05). Differences in survival and rhythmicity rates were evaluated via χ^2 tests; differences in activity periods (τ), total activity levels and proportion of night activity were evaluated via Mann-Whitney U-tests.

		Survival (%)	**Rhythmicity (%)**	**Period (h) [median±SE]**	**Total Activity (beam crosses/day) [median±SE]**	**Night activity (%) [median±SE]**
LD	**Forager**	55.7^a (n=78)	59.0^a (n=46)	24.1±0.1^a (n=74)	2040.5±139.2^a (n=78)	68.1±1.6^a (n=78)
	Nurse	80.7^a (n=46)	58.7^a (n=27)	24.0±0.12^a (n=45)	2868.7±218.0^b (n=46)	64.8±2.1^a (n=46)
LD+6h	**Forager**	78.3^a (n=61)	59.0^a (n=36)	24.6±0.2^a (n=60)	2616.1±229.3^a (n=61)	69.8±1.8^a (n=61)
	Nurse	64.9^a (n=37)	65.8^a (n=25)	23.9±0.3^a (n=36)	3172.7±283.0^a (n=37)	71.4±2.3^a (n=37)
DD	**Forager**	80.3^a (n=49)	55.1^a (n=27)	22.4±0.39^a (n=44)	2785.0±349.3^a (n=49)	-
	Nurse	54.0^b (n=20)	70.0^a (n=14)	23.1 ±0.76^a (n=18)	2634.0±621.8^a (n=20)	

Around 60% of individuals exhibited rhythmic activity patterns. In that case, foragers and nurses synchronized their activity rhythm strongly with the 12:12h LD-cycle (Fig. 1A/B, actograms on the left, Day 1-8). The average activity patterns in both castes revealed lower activity levels during day times (Fig. 1C/E) and sharp activity peaks after the two light transitions. After shifting the light regime by six hours, ants immediately resynchronized their activity (Fig. 1A/B, actograms on the left, Day 9-16) and therefore displayed an almost identical average activity pattern as before the phase shift (Fig. 1D/F). Total activity levels as well as relative levels in nocturnal activity were compared between castes to quantify differences in locomotor rhythms (Table 2).

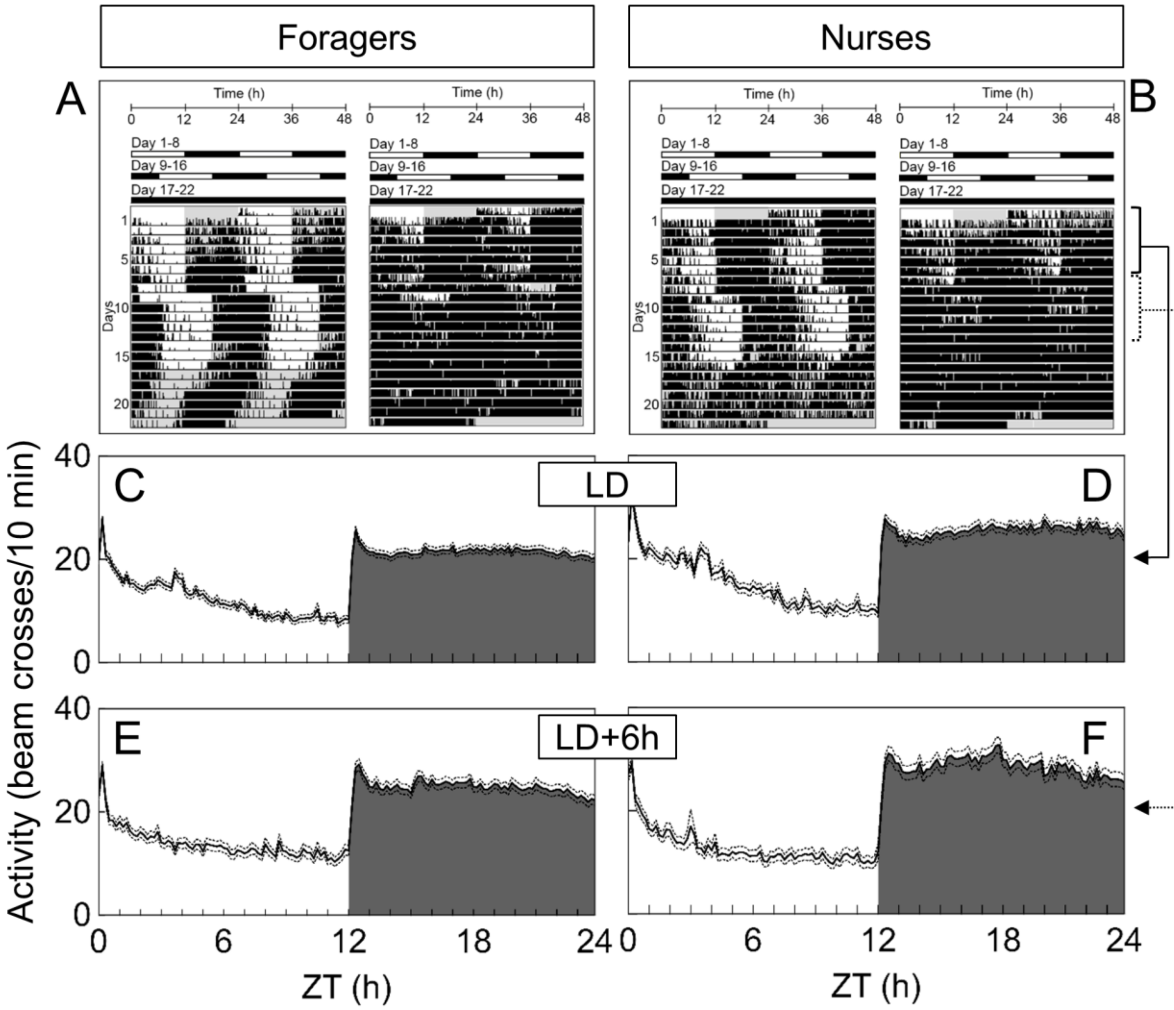

Fig. 1: Locomotor activity rhythms of foragers and nurses in isolation from the social context. Top: Examples of actograms of single workers of each caste. Locomotor activity (indicated as black bars) is shown as double plot under a 12:12h LD-cycle (day 1-8), after a 6 hour phase delay of the LD-cycle (day 9-15) and constant darkness (day 16-22). (A): Actograms of one rhythmic (left) and one arrhythmic (right) forager. (B): Actograms of one rhythmic (left) and one arrhythmic (right) nurse. Bottom: Average activity (mean: solid lines; mean±SE: dashed lines) over 7 days under the respective light regime. (C): Foragers under a 12:12h LD-cycle (n=78): (D): Nurses under a 12:12h LD-cycle (n=46); (E): Foragers after a 6 hour delay of the LD-cycle (n=61); (F): Nurses after a 6 hour delay of the LD-cycle (n=37).

Nurses exhibited total activity levels higher than foragers, but only during the first recording phase under the LD-cycle (Mann-Whitney U-tests; LD: U(78,46)=1203.5, z=-3.0, p=0.002; phase-shifted LD: U(61,37)=2060.0, z=-1.7, p=0.09; DD: U(49,20)=465.0, z=-0.3, p=0.7). There were no significant differences in levels of nocturnality between the castes under both light regimes (Mann-Whitney U-tests; LD: U(78,46)=1537.0, z=1.3, p=0.1; DD: U(61,37)=1204.0, z=-0.8, p=0.4). Here, foragers and nurses displayed similar period lengths close to 24 hours (Mann-Whitney U-tests; LD: U(74,45)=1357.0, z=-1.7, p=0.08; phase-shifted LD: U(60,36)=1582.5, z=1.2, p=0.2). During constant darkness, locomotor activity rhythms of both castes drifted with similar endogenous periods shorter than 24 hours (foragers: 22.4±0.39 h, nurses: 23.1±0.76 h; Mann-Whitney U-test: U(44,18)=1317.0, z=-1.1, p=0.3).

Experiment 2: Caste-dependent plasticity in daily behavioral rhythms

Daily behavioral activity patterns of foragers and nurses in the social context

With up to 50% of their time, foragers showed much higher levels of inactivity than nurses under the restricted feeding regimes (Fig. 2; Mann-Whitney U-tests under Bonferroni correction, α=0.017; daytime feeding: U(12,13)=17.0, z=3.3, p=0.001; nighttime feeding: U(12,12)=21.0, p=0.003). Under *ad libitum* feeding, however, only foragers tended to be more active (Kruskal-Wallis tests under Bonferroni correction, α=0.017; foragers: H(2, n=32)=4.23, p=0.12; *ad libitum* vs. daytime: p=0.13; *ad libitum* vs. nighttime: p=0.42; daytime vs. nighttime: p=1.0; nurses: H(2, n=37)=8.12, p=0.017; *ad libitum* vs. daytime: p=0.028; *ad libitum* vs. nighttime: p=1.0; daytime vs. nighttime: p=0.07) and therefore inactivity levels did not differ between castes (Mann-Whitney U-test, U(8,12)=40.0, z=0.58, p=0.6). In addition, foragers had consistently lower total caste-specific activity levels than nurses in each of the feeding regimes (Fig 2; Mann-Whitney U-tests; *ad libitum* feeding: U(8,12)=7.0, z=-3.12, p=0.0018; daytime feeding: U(12,13)=0.0, z=-4.2, p<0.001; nighttime feeding: U(12,12)=0.0, z=-4.1, p<0.001). The daily time budgets of the castes showed that in foragers, antennating (~25%) and walking (10-27%) were the most dominant activities (Fig. 2A/B/C). With 20 to 41% of time allocation, nurses spent their time predominantly with brood feeding (Fig. 2D/E/F). Both castes engaged with 5 to 7% of their time in exchange and distribution of food via trophallaxis.

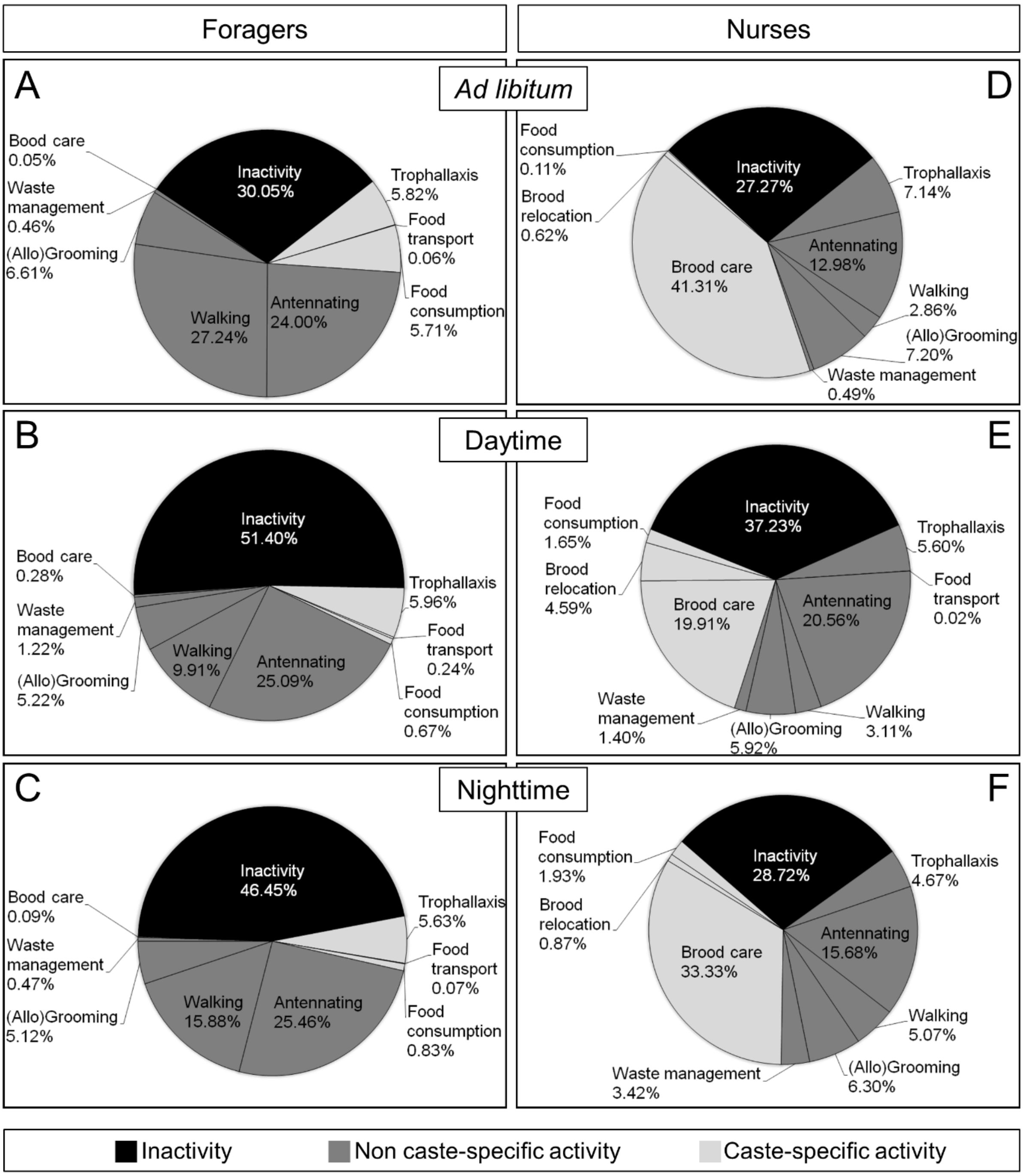

Fig. 2: Daily time budgets for activities displayed in the social context for both foragers and nurses under the three feeding regimes. (A): foragers (n=8) under *ad libitum* feeding; (B): foragers (n=12) under daytime feeding; (C): foragers (n=12) under nighttime feeding; (D): nurses (n=13) under *ad libitum* feeding; (E): nurses (n=12) under daytime feeding; (F): nurses (n=12) under nighttime feeding. Black: proportion of inactivity, light grey: proportion of caste-specific activity, dark grey: proportion of non caste-specific activity.

To characterize the rhythmicity of the behavioral patterns quantified in the social context, we calculated period values (τ) and summarized the percentage of foragers and nurses displaying either circadian, ultradian or infradian rhythms in their caste-specific activities (Fig. 3). Under *ad libitum* and daytime feeding, more than 60% of foragers displayed circadian activity patterns (Fig. 3A/B). Ultradian activity rhythms were detected in this caste under the three feeding regimes, but mostly under nighttime feeding (Fig. 3C). For all feeding regimes, the majority of nurses displayed ultradian activity rhythms (Fig. 3). Examples for both ultradian and circadian activity rhythms in the two castes are presented in Fig 4.

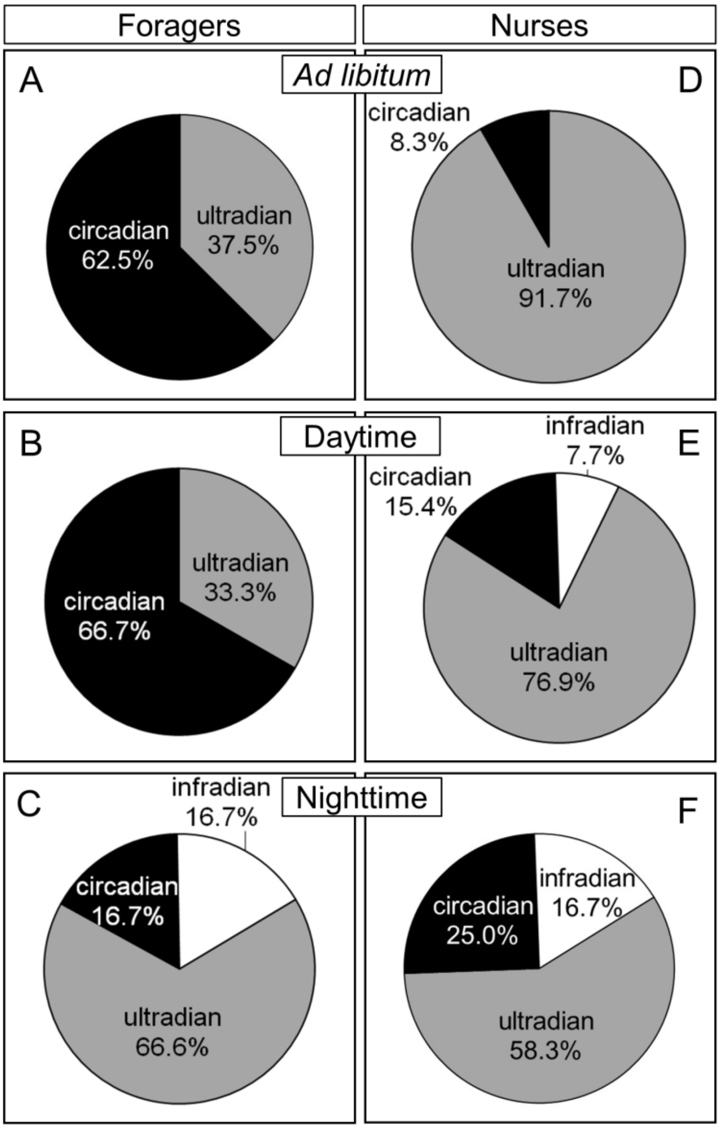

Fig. 3: Percentage of foragers and nurses displaying either circadian, ultradian or infradian rhythms of caste-specific activities in the social context, under the three feeding regimes. Black: circadian rhythms (period lengths between 20 and 28h). Grey: ultradian rhythms (period lengths <20h). White: infradian rhythms (period lengths >28h) (A): foragers (n=8) under *ad libitum* feeding; (B): foragers (n=12) under daytime feeding; (C): foragers (n=12) under nighttime feeding; (D): nurses (n=13) under *ad libitum* feeding; (E): nurses (n=12) under daytime feeding; (F): nurses (n=12) under nighttime feeding.

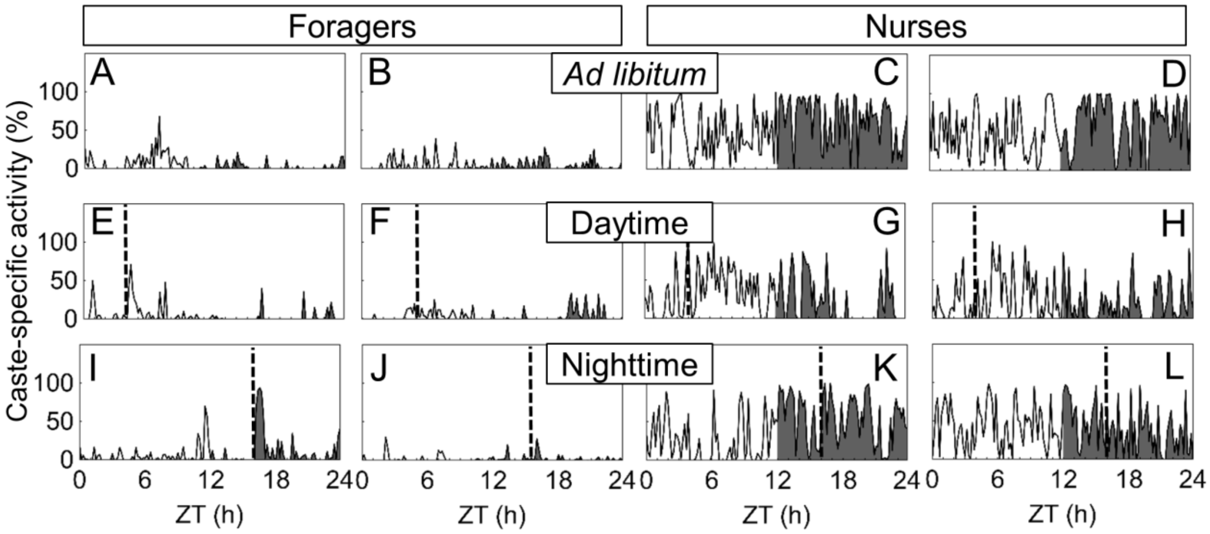

Fig. 4: Examples of caste-specific activity patterns of both foragers and nurses in the social context, under the three feeding regimes. Activity of single workers is shown for the day phase (white area) and night phase (black area) in relation to zeitgeber time (ZT). Feeding times (daytime feeding: ZT 4; nighttime feeding: ZT 16) are indicated as dashed red lines. For both castes and the three feeding regimes, one circadian (left) and one ultradian (right) activity pattern are shown as examples. (A): Forager under *ad libitum* feeding, τ=22.3h. (B): Forager under *ad libitum* feeding, τ=4.5h. (C): Nurse under *ad libitum* feeding, τ=21.3h. (D): Nurse under *ad libitum* feeding, τ=2.4h. (E): Forager under daytime feeding, τ=24.6. (F): Forager under daytime feeding, τ=13.4h. (G): Nurse under daytime feeding, τ=24.1h. (H): Nurse under daytime feeding, τ=3.1h. (I): Forager under nighttime feeding, τ=22.1. (J): Forager under nighttime feeding, τ=2.8. (K): Nurse under nighttime feeding, τ=22.4h. (L): Nurse under nighttime feeding, τ=3.6h.

Average patterns of caste-specific activities, as described in Table 1, were calculated to evaluate at which daytimes foragers and nurses were active in the social context (Fig. 5; see also Fig. S1 in the supplementary for examples of activity profiles of single foragers and nurses in the social context). Under *ad libitum* feeding of the subcolonies, foragers were similarly active during the light and dark phase (Fig. 5A). Under the restricted feeding regimes, foragers displayed prominent peaks of activity coupled to the food availability, either at the light or the dark phase (Fig. 5B-C, for further statistical analysis see Fig. S2 in the supplementary). In both cases, activity markedly increased after feeding and persisted high only for a short time thereafter. Independent of the feeding time, nurses were active both during the day and night time at constant high levels (Fig. 5D-F). Yet in both restricted feeding regimes, a trend of increased activity levels in the half-day period of actual feeding was observed, especially during nighttime feeding (see Fig. S2 in the supplementary).

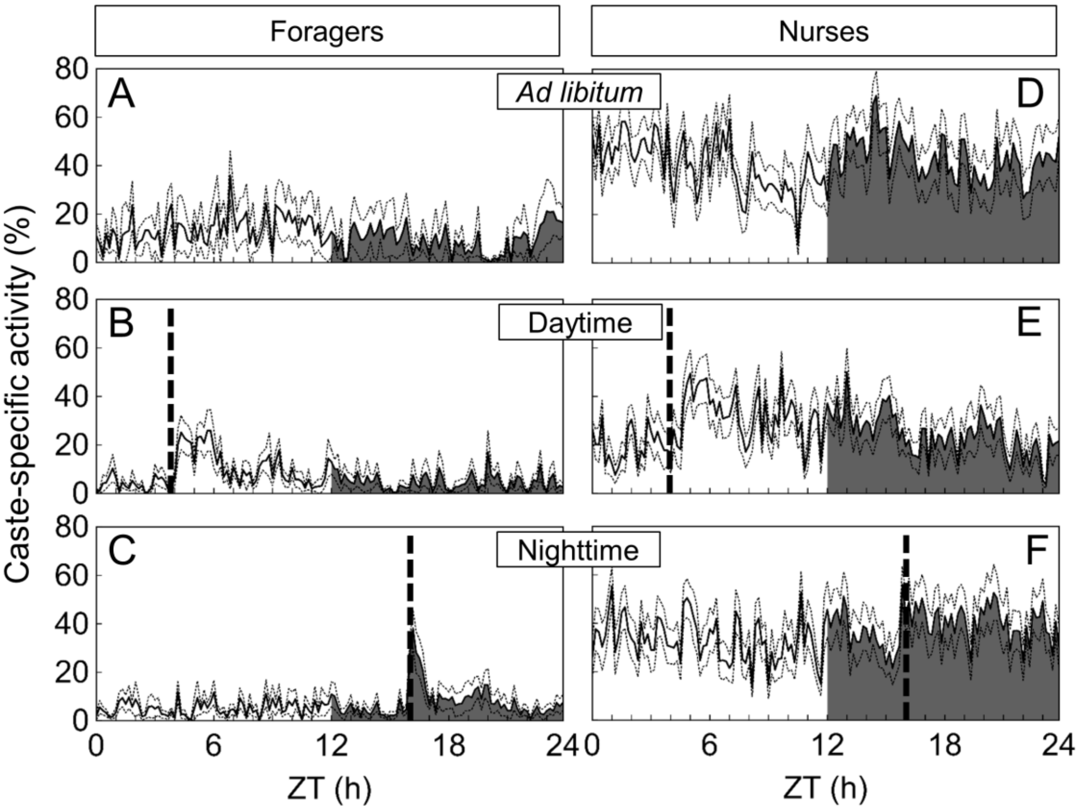

Fig. 5: Effect of feeding regime on caste-specific activity patterns in the social context, for both foragers and nurses. Mean activity (solid lines) and mean±SE (dashed lines) are shown for the day phase (white area) and the night phase (black area) in relation to zeitgeber time (ZT). Feeding times (daytime feeding: ZT 4; nighttime feeding: ZT 16) are indicated as dashed black lines. (A): foragers (n=8) under *ad libitum* feeding. (B): foragers (n=12) under daytime feeding. (C): foragers (n=12) under nighttime feeding. (D): nurses (n=13) under *ad libitum* feeding. (E): nurses (n=12) under daytime feeding. (F): nurses (n=12) under nighttime feeding.

At the subcolony level, foraging activity was higher during the dark phase under both *ad libitum* and nighttime feeding (Fig. 6, Wilcoxon signed-rank test with Bonferroni correction, α=0.017*; ad libitum* feeding: T(288)=157, z=10.02, p<0.001; nighttime feeding: T(576)=1033, z=13.71, p=0.001). Under daytime feeding, the daily activity pattern was not completely reversed, but activity levels in both phases changed, resulting in similar levels over the 24 h (Wilcoxon signed-rank test with Bonferroni correction, α=0.017*;* T(720)=21782, z=1.29, p=0.2). Thus, foraging activity during daytime feeding was significantly reduced in the dark phase and increased in the light phase (Kruskal-Wallis tests with Bonferroni-correction, α=0.017; light phase: H(2, n=792)=248.68, p=0.001; *ad libitum* vs. daytime: p<0.001 *ad libitum* vs. nighttime: p<0.001, daytime vs. nighttime: p<0.001; dark phase: H(792)=69.63, p<0.001; *ad libitum* vs. daytime: p=0.06, *ad libitum* vs. nighttime: p<0.001, daytime vs. nighttime: p=<0.001).

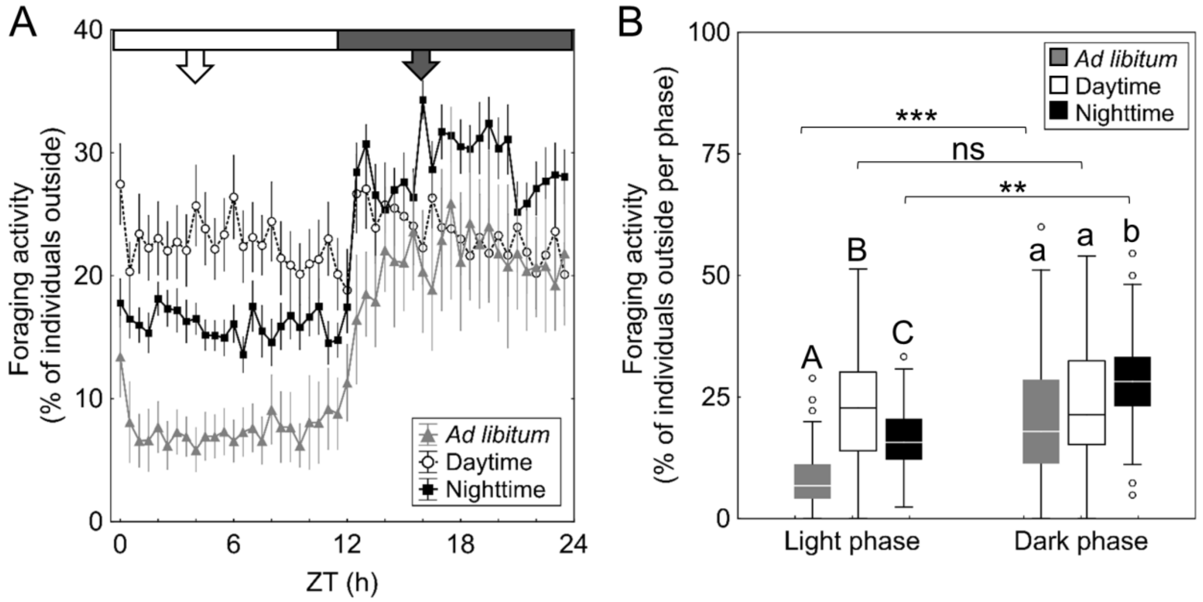

Fig. 6: Effect of feeding regime on foraging activity in subcolonies. (A): Foraging activity in the course of 24 hours (mean±SE) for each feeding regime (grey: *ad libitum* feeding, n=6 subcolonies; white: daytime feeding, n=15 subcolonies; black: nighttime feeding, n=12 subcolonies). (B): Foraging activity plotted separately for both the light and dark phases. Boxplots show medians (center lines) and interquartile ranges (boxes) for the three feeding regimes (grey: *ad libitum* feeding, n=6 subcolonies; white: daytime feeding, n=15 subcolonies; black: nighttime feeding, n=12 subcolonies). Whiskers indicate the minimum and maximum values and open circles show outliers. Different capital letters show differences during the day phase and small letters show significant differences during the night phase between the feeding regimes (Kruskal-Wallis tests with Bonferroni correction, α=0.017). Asterisks indicate differences between light phase and dark phase within every feeding regime (Wilcoxon signed-rank test with Bonferroni correction, α=0.017). **: $p<0.003$; ***: $p<0.0003$; ns: $p>0.017$.

Daily locomotor activity of foragers and nurses in isolation from the social context

Survival and rhythmicity of isolated workers strongly depended on caste affiliation (Table 3). Independent of the feeding regime, survival rate in nurses was roughly twice as high as in foragers. In both castes, rhythmic and arrhythmic individuals were present (Fig. 7), but their proportions varied depending on caste and feeding regimes (Table 3). Approximately 70% of the isolated foragers were rhythmic under *ad libitum* feeding of the subcolonies. Under the restricted feeding regimes, 20 to 30% of the foragers showed circadian rhythms. Most of the nurses (80 to 90%), in turn, were rhythmic in isolation, independent of the feeding regime, yet displayed arrhythmicity in the social context as described above. For further comparisons of the activity patterns, see Fig. S3 in the supplementary.

Table 3: Effect of feeding regime on rates of survival and rhythmic daily activity of isolated individuals of both castes in locomotor activity monitors. Differences between castes within a feeding regime and differences within castes between feeding regimes via χ^2 tests under Bonferroni correction (*: p<0.017; **: p<0.003; ***: p<0.0003; ns: p>0.017).

Feeding regime	**Survival rate (%)**			**Rhythmicity (%)**		
	Forager	**Nurse**	X^2 test	**Forager**	**Nurse**	X^2 test
Ad libitum feeding	34.6 (n=9)	74.4 (n=32)	** X^2 =8.8	66.7 (n=6)	90.6 (n=29)	ns X^2 =3.2
Daytime feeding	38.0 (n=30)	66.7 (n=88)	*** X^2 =16.5	33.3 (n=10)	76.1 (n=67)	*** X^2 =18.1
Nighttime feeding	41.1 (n=23)	84.3 (n=86)	*** X^2 =31.6	17.4 (n=4)	80.2 (n=69)	*** X^2 =32.4
X^2 test	ns X^2 =0.2	* X^2 =9.4		ns X^2 =7.2	ns X^2 =3.1	

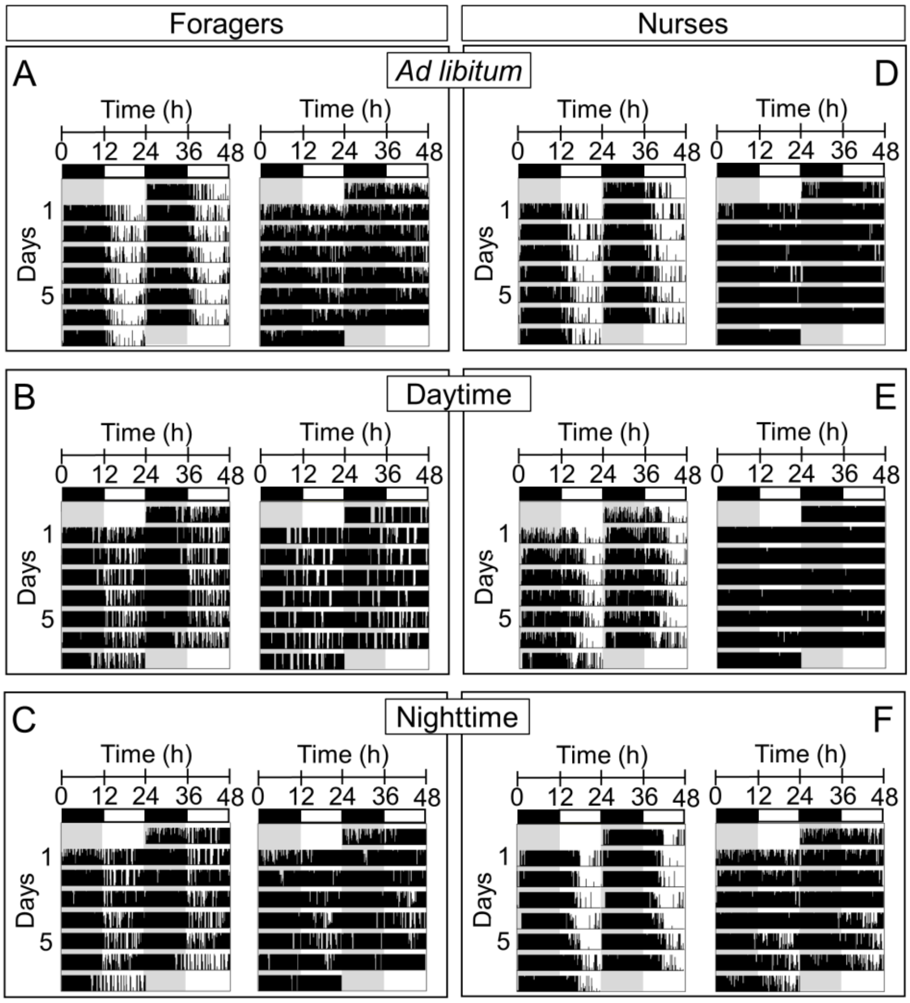

Fig. 7: Examples of actograms of workers in isolation from the social context. Locomotor activity (indicated as black bars) is shown as double plot for single workers of the nurse and forager caste under a 12:12h LD-cycle. For the three feeding regimes, actograms of one rhythmic (left) and of one arrhythmic individual (right) are shown. (A): Foragers after *ad libitum* feeding of the subcolony. (B): Foragers after daytime feeding of the subcolony. (C): Foragers after nighttime feeding of the subcolony. (D): Nurses after *ad libitum* feeding of the subcolony. (E): Nurses after daytime feeding of the subcolony. (F): Nurses after nighttime feeding of the subcolony.

Discussion

Locomotor rhythms of isolated C. rufipes workers

Based on the daily locomotor rhythms of isolated forager and nurse workers under a 12:12h LD-cycle, *C. rufipes* can be classified as a nocturnal species, thus complementing previous descriptions about the predominantly nocturnal foraging activity observed in both the laboratory and in the field (Jaffé & Sánchez, 1984; Oliveira et al., 1995; Del-Klaro & Oliveira, 1999). As individuals immediately shifted their activity following a six hour delay of the light phase, they were truly entrained by the LD-cycle

(Schlichting & Helfrich-Förster, 2015). Light can be claimed as a strong zeitgeber for the endogenous clock, since period lengths of the workers' locomotor activity strongly resembled the 24 hour period of the imposed light-dark cycle. The prominent activity peaks after the light transitions are most likely a result of stress reactions of the individuals to the step changes in illumination and likely represent a masking effect (Schlichting & Helfrich-Förster, 2015). To clarify this assumption, activity could be monitored in the future under increasing/decreasing light intensities simulating twilight conditions. Under constant darkness, the locomotor activity rhythms of both castes started to run free from the entrained activity, and drifted with period lengths close to 24 hours, i.e. the rhythm represents the output of a circadian, endogenous clock.

As we found no differences in levels of nocturnal activity, rhythmicity and period lengths in LD- and DD-regimes, we demonstrate the presence of a functional endogenous clock in both castes. The presence of some arrhythmic individuals in both castes might not be ascribed to a missing endogenous clock in these ants, but rather to their stressful reactions because of the prolonged social isolation.

In contrast to our results, other studies reported strong differences in locomotor rhythms between castes in other ant species. In *Diacamma* ants, young workers, likely nurses, exhibited strong rhythmic activity, whereas older workers, likely to behave as foragers, did not (Fuchikawa et al., 2014). The authors argued that this may reflect the arrhythmic foraging activity observed in the field. However, the illustrated arrhythmicity and increased activity levels in *Diacamma* foragers could also be the result of an aging clock, as previously shown in *Drosophila* (Koh et al., 2006; Luo et al., 2012). Conversely, major and media workers in *C. compressus*, likely foragers, were rhythmic, while minor workers, which likely perform inside-nest activities, showed no circadian rhythmicity (Sharma et al., 2004b). The authors hypothesized that the lack of rhythmicity in in-nest workers might reflect their all-around-the-clock working schedule within the colony, but no behavioral correlates of activity in the social context were provided. This is why we aimed at monitoring behavioral activity of foragers and nurses of *C. rufipes* in the social context of the colony, and extended the analysis of their rhythmic activity in subsequent isolation.

Caste-dependent plasticity of daily behavioral rhythms

As temporal changes in food availability may strongly influence the timing of activities, synchronization of daily activity patterns in both foragers and nurses was evaluated in subcolonies under restricted and *ad libitum* feeding regimes. For the first time in social insects, behavioral activities were continuously observed over 24 hours as ethograms for single nurses and foragers with the aid of detailed and enormously time-consuming manual tracking in long-term video recordings. One fundamental difference in activity profiles of the two castes was the variation in inactivity levels. Foragers remained inactive up to 50% of their time and always showed lower caste-specific activity levels than nurses, for all feeding regimes. This may in part result from the small size of the experimental setup, as foragers did not need to cover long distances to collect food as in the natural context. With 30% of their time, nurses showed considerable levels of inactivity as well. Such high inactivity levels were already described for workers of several ant species in the laboratory (Herbers, 1983; Herbers & Cunningham, 1983; Cole, 1986; Franks et al., 1990) as well as in the field (Charbonneau et al., 2004), and are therefore not a simple laboratory artifact. Even though ants in those studies were only observed for short time windows, our continuous observations confirm the presence of high inactivity levels over the whole day. Although inactivity levels of foragers were lower and similar to those observed in nurses under *ad libitum* feeding, they spent their active time with different behavioral activities. Foragers performed predominantly antennating and walking, behaviors classified as non-specific as they are performed by all workers. In a broader sense, however, these activities reflect the search for food in this caste. In addition, foragers showed always lower caste-specific activity levels than nurses because they synchronized their activity with food availability and the light-dark-cycle. Consequently, behavioral activity patterns of foragers were predominantly circadian. Only under nighttime feeding, activity levels in many foragers were markedly low and therefore only few circadian activity patterns could be detected under this feeding regime. Nurses were active all around the clock tending the brood, so mostly ultradian rhythmicities could be detected for their behavioral activity patterns.

Interestingly, although counts of foragers outside the nest under the *ad libitum* feeding regime indicated that the preferred foraging time at the group level occurred in the first hours of the night, continuous tracking of random individuals, however, showed similar foraging intensity in both phases of the day. Such flexibility may enable foragers to adjust their activity in correlation with temporal changes in food availability, an essential ability in ants that collect carbohydrate-rich food in renewable sources such as extrafloral nectaries or aphid colonies (Jaffé & Sánchez, 1984; Del-Claro & Oliveira, 1999). In this respect, *C. rufipes* was reported to forage during day and nighttimes

(Oliviera et al., 1995; Del-Claro & Oliveira, 1999; Fagundes et al., 1995) and field observations by one of the authors (FR) suggest that *C. rufipes* colonies may seasonally shift their activity from nocturnal to diurnal foraging, like many other ant species do (Retana et al., 1988; Cros et al., 1997).

In contrast to foragers, nurses were active in both phases of the daily cycle with high activity levels, mostly at the brood pile, likely to meet the needs of the brood. Consequently, feeding and licking of the brood was the most dominant activity in nurses, taking up to 40% of their time. A study on brood care in *Solenopsis invicta* showed that larvae are fed up to 50 times per hour and are patrolled 200-800 times per hour, which could lead to uniform levels of nourishment for all larvae (Cassil & Tschinkel, 1995). All-around-the-clock activity as observed in nurses appears to be a widely-occurring adaptive behavior in social insects, as it was also described for in-hive activities of honey bees (Crailsheim et al., 1996), and recently detected in intra-nest activities in *Temnothorax* ants (Charbonneau & Dornhaus, 2015). In our experiments, the feeding regimes had only negligible effects on the daily activity pattern of nurses. We observed a short increase of activity after feeding, which could be ascribed to stronger interactions with the returning foragers and suggests a form of social synchronization. Inter-caste interactions occurred predominantly via trophallaxis inside the brood chamber, where members of both castes spent comparable amounts of time. This emphasizes the temporal connection between the castes via food flow, as food is passed from returning foragers to nurses and afterwards to the brood. A study in *Leptothorax* ants already described such alteration of in-nest movement activity by returning foragers (Boi et al., 1999), analogous to the increase in caste-specific activities of nurses in our study.

As in honey bees, arrhythmicity at the colony level in *C. rufipes* was not based on rhythmic activity of individuals working in shifts or out of phase (Moore et al., 1996), but on the arrhythmic activities of single inside-nest workers (see supplementary). A shift system was proposed for ants because of spontaneous changes in period lengths and phase-shifts of free-running locomotor rhythms in isolated *C. compressus* workers (Sharma et al., 2004b;c). Based on our detailed study of in-nest activities, the existence of shifts to explain arrhythmicity can be ruled out in *C. rufipes*, as recently done in *Temnothorax rugatulus* ants as well (Charbonneau & Dornhaus, 2015).

Comparison between behavioral rhythms in the social context and locomotor activity rhythms in isolation

After having spent 14 days in the social context, individual foragers and nurses were monitored in isolation, thus allowing the comparison of daily behavioral activities with endogenous locomotor activity rhythms in the same workers. Irrespective of the preceding feeding regime, most nurses showed rhythmicity and night activity. This result again demonstrates that nurses possess a functional endogenous clock and are able to entrain to the LD-cycle, although they are behaviorally arrhythmic under this zeitgeber in the social context. Therefore, the output of the endogenous clock must be either masked or decoupled in the social context (Shemesh et al., 2007). As another output of their clock, *C. rufipes* nurses show circadian changes in temperature sensitivity for brood care (Roces & Núñez, 1996), and display a daily rhythm in brood translocation between different temperatures (Weidenmüller et al., 2009). Hence, some nursing behaviors seem to be performed in a rhythmic manner whilst others not. Since our experiments were done under constant temperature conditions, rhythmic brood translocation could not be observed. Future experiments are needed to clarify whether the same workers exhibit both rhythmic and arrhythmic components of nursing behavior and most notably, which zeitgebers synchronize rhythmic brood care inside the dark nest. Promising candidates are non-photic zeitgebers like cyclic social interaction (Southwick & Moritz, 1987; Lone & Sharma, 2011; Beer et al., 2016), as well as daily cycles in temperature and humidity.

Only after *ad libitum* feeding of the subcolonies, isolated foragers showed similar levels of rhythmicity and night activity like nurses, confirming the results obtained in Experiment 1. After the temporally restricted feeding regimes, the majority of foragers in contrast displayed arrhythmicity. This finding was unexpected because the same workers showed rhythmic foraging activity in the social context. This suggests that foragers may have experienced food shortages or suffered stress during the short feeding time-windows, as a result of the observed drastic increase of arousal and activity. The additional highly reduced survival of foragers indicates that they could be less robust than nurses under isolation and may have lost their rhythmicity. This effect could be a consequence of aging, since foragers are usually older than nurses (Mersch et al., 2013) and age negatively affects survival under social isolation in ants (Koto et al., 2014). Thus, not only caste affiliation but also previous rearing conditions significantly affect individual activity still in isolation. Further studies on ants of known age and caste affiliation are needed to analyze the performance of an aging clock in combination with an age-dependent polyethism. Although only the minority of foragers was rhythmic after the restricted feeding regimes, these workers were uniformly night active. Despite their previous synchronizing with a specific time point in the night or even their switch to day activity in the social context, they still displayed their endogenous rhythms in isolation and did not show any long-term effects of the previous entrainment.

Our study indicates that ants show flexible daily behavioral rhythms that are context-dependent in multiple ways. Endogenous daily activity patterns of individuals belonging to specific castes are modified in the social context based on specific task demands. Such a mechanism was previously described only in the honey bee (reviewed in Bloch, 2010), but ants seem to share the link between division of labor and socially-mediated plasticity in activity rhythms. In honey bees, too, all workers possess a circadian clock and show rhythmicity in isolation of the social context (Shemesh et al., 2007). Yet this endogenous rhythmicity is not observed in honey bee nurses in the social context (Crailsheim et al., 1996; Moore et al., 1998). Therefore, temporal organization in *C. rufipes* ants and honey bees appear to share similar basic features. In this regard, the molecular characterization of an ant clock in the species *Solenopsis invicta* showed that both bees and ants own a mammalian-like clock with similar mechanisms (Ingram et al., 2012). Expression patterns of clock genes hereby reflect task specific activity patterns and link division of labor with clock function in bees (Shemesh et al., 2010) as well as in ants (Ingram et al., 2009). Although their endogenous clock awaits molecular characterization, ants of the genus *Camponotus* appear to be a promising model system to further explore the link between chronobiology and sociobiology.

Supplementary

Activity profiles of foragers and nurses in both the social context and isolation

In Fig. S1 we show examples of activity profiles of single foragers and nurses in both the social context and in isolation, for the three assayed feeding regimes. In the social context, levels of total activity in foragers were lower than in nurses and showed no daily pattern (Fig. S1, column 1). As forager and nurses executed different behavioral activities, we calculated caste-specific activity profiles (Fig. S1, column 2) to determine differences in the temporal organization of foragers and nurses. Although locomotor activity of foragers in the social context rather reflected their caste-specific activity (compare columns 2 and 3), nurses showed hardly any locomotor activity in the social context (Fig. S1, column 3). Although mostly stationary, nurses were highly active displaying nursing behaviors. Thus, the locomotor activity profile failed to represent the characteristic, high level activity pattern of nurses.

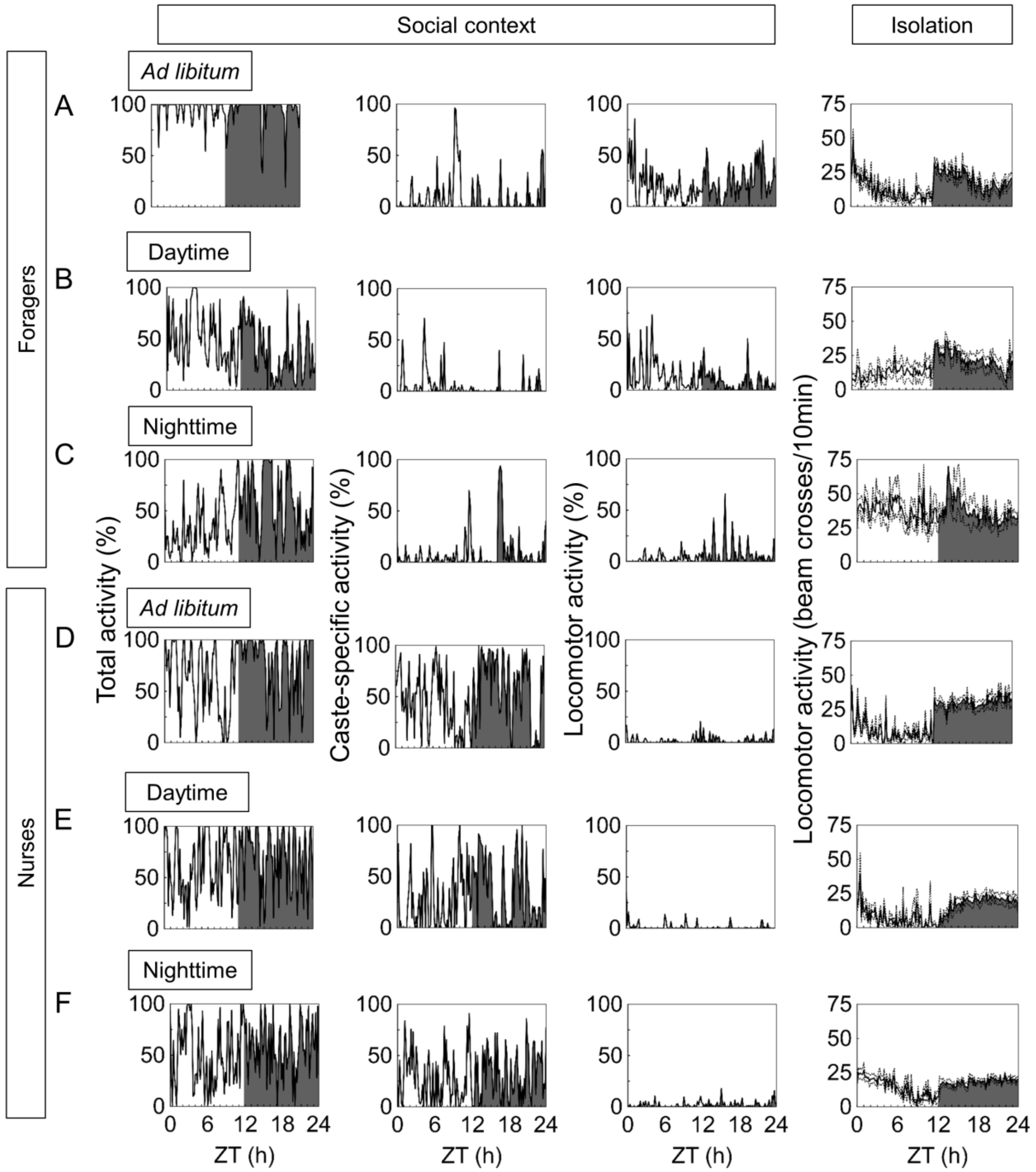

Fig. S1: Examples of activity profiles of foragers and nurses in both the social context and isolation, for the three assayed feeding regimes. (A): Forager under *ad libitum* feeding. (B): Forager under daytime feeding. (C): Forager under nighttime feeding. (E): Nurse under *ad libitum* feeding. (F): Nurse under daytime feeding. (F): Nurse under nighttime feeding. Column 1: Total activity levels in the social context. Column 2: Caste-specific activity levels in the social context. Column 3: Locomotor activity in the social context. Column 4: Locomotor activity in isolation. Caste-specific activities of foragers in these examples are circadian (*ad libitum*: τ= 20.0, daytime: τ=20.0h nighttime: τ=22.1h), and those of nurses are infradian (*ad libitum*: τ=12.9h, daytime: τ=7.4h, nighttime: τ=1.6h).

Experiment 2: Quantitative analysis of behavioral activity patterns in the social context

Relative levels of caste-specific activity during the night phase were determined by normalizing data sets of every individual for total activity of each ant (Fig. S2). This method provides comparison despite high inter-individual and inter-caste variances in activity levels. To determine day or night activity, levels of nocturnal caste-specific activity were tested against random activity in both the light and the dark phase for the two castes and the three assayed feeding regimes (one-sample t-test against 50% with Bonferroni correction, α=0.025). Within feeding regimes, we compared levels in nocturnal activity between castes (unpaired t-tests under Bonferroni-correction, α=0.025).

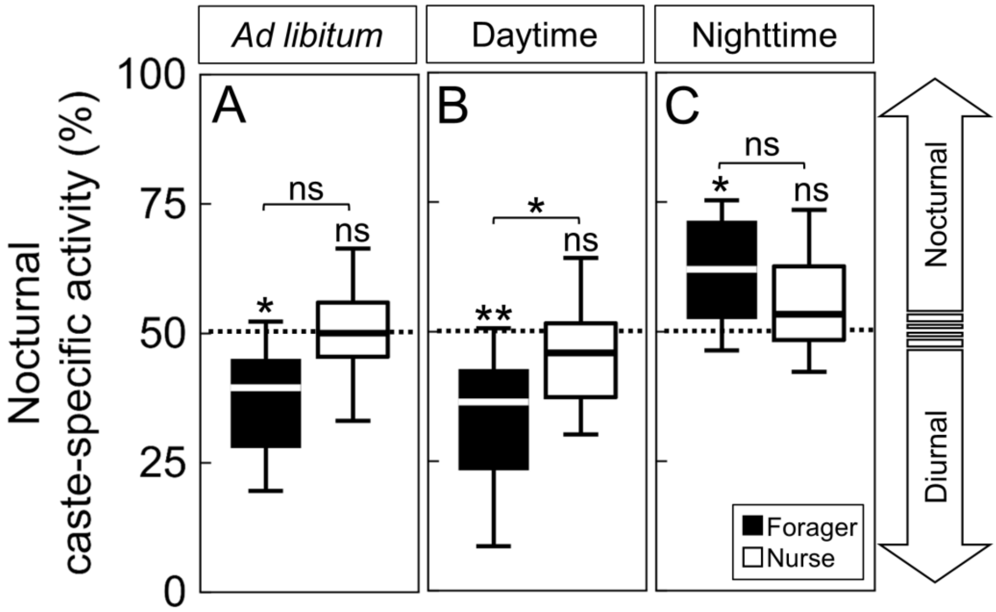

Fig. S2: Effect of feeding regime on nocturnal caste-specific activity of both foragers and nurses. Boxplots show medians (center lines) and interquartile ranges (boxes) for foragers (black) and nurses (white), for the three feeding regimes. (A): *Ad libitum* feeding. B: Daytime feeding. C: Nighttime feeding. Whiskers indicate the minimum and maximum values. Asterisks show significant differences from random distribution for every caste and feeding regime (one sample t-test against 50% with Bonferroni correction, α=0.025) and significant differences between castes for every feeding regime (unpaired t-test with Bonferroni correction, α=0.025). ns: p>0.025, *: p<0.025, **: p<0.005).

Experiment 3: Quantitative analysis of locomotor activity patterns in isolation

Average activity patterns of foragers and nurses were calculated separately for the rhythmic and arrhythmic individuals, and the most common pattern was used for further comparisons. The largest proportion of individuals was rhythmic under all three feeding regimes in nurses, and under *ad libitum* feeding in foragers. High proportions of arrythmic individuals were observed only under restricted feeding in foragers. Rhythmic foragers under ad libitum feeding (Fig. S3A) and rhythmic nurses under all feeding regimes (Fig. S3D-F) were mostly nocturnal. In contrast, the representative activity profiles of arrhythmic foragers after the restricted feeding depicted a clear reduction in night activity as well as damped overall activity levels compared to the other activity patterns (Fig. S3B-C).

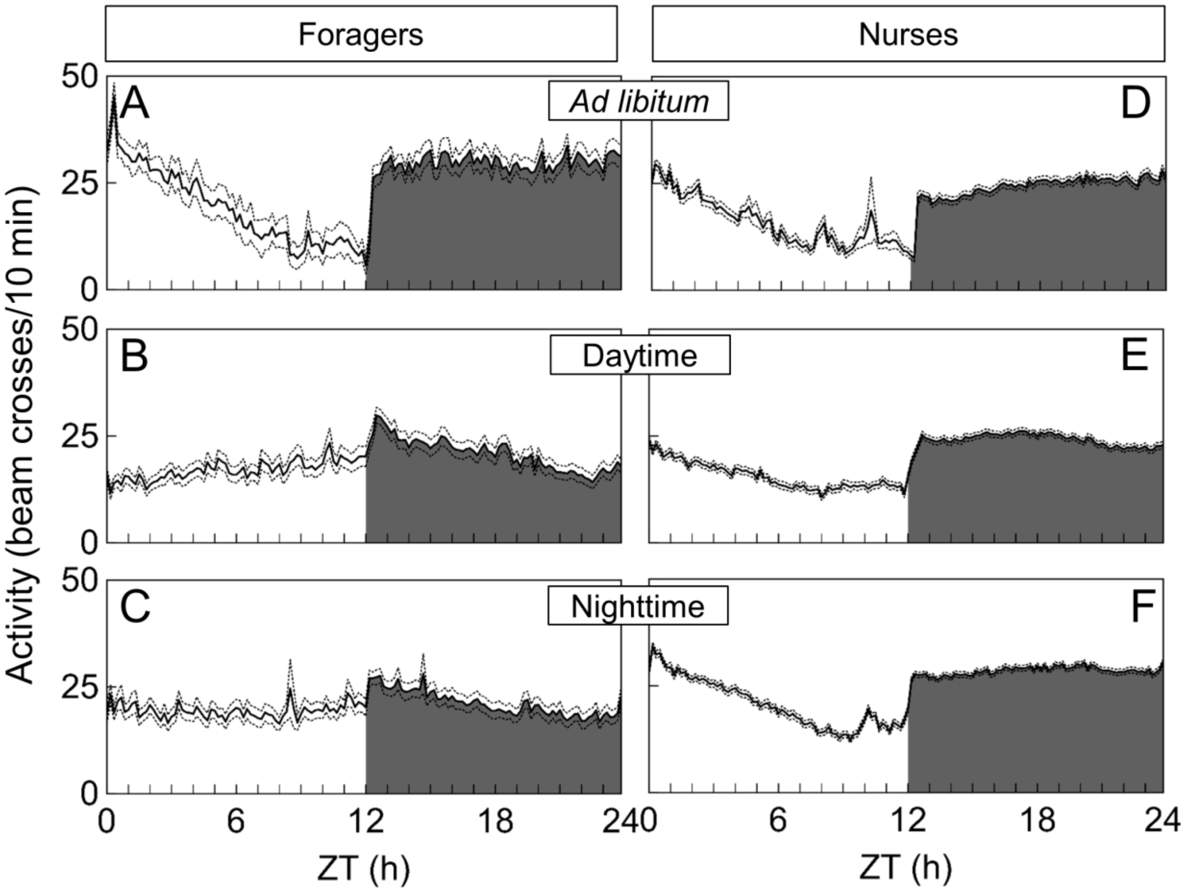

Fig. S3: Effect of feeding regime on diel locomotor activity of isolated nurses and foragers. Average activity (mean: solid lines; mean±SE: dashed lines; day phase: white area, ZT 0-12; night phase: grey area, ZT12-24). (A): Foragers (n=6) after *ad libitum* feeding of the subcolony. (B): Foragers (n=20) after daytime feeding of the subcolony. (C): Foragers (n=19) after nighttime feeding of the subcolony. (D): Nurses (n=29) after *ad libitum* feeding of the subcolony. (E): Nurses (n=67) after daytime feeding of the subcolony. (F): Nurses (n=69) after nighttime feeding of the subcolony.

Chapter III

Presence of brood and conspecifics leads to plasticity of daily locomotor rhythms in the ant *Camponotus rufipes*

Abstract

Division of labor in *Camponotus rufipes* ants is associated with task-related plasticity in daily rhythms. Although all workers possess an endogenous clock and are night active in isolation, only foragers show rhythmic activity synchronized with food availability in the social context, whereas nurses care for the brood around the clock. Although arrhythmic activity might be required for brood rearing, other collective behaviors like nest building might need temporal synchrony among workers. In the absence of the light-dark-cycle in the ant nest, social interactions could be an important zeitgeber to synchronize activity between individuals. To determine whether two prominent social cues influence the expression of endogenous rhythms in foragers and nurses, we monitored locomotor activity of isolated individuals in the presence of brood or when contact with a conspecific was possible. In both foragers and nurses, contact to brood led to arrhythmic locomotor activity similar to the arrhythmic behavioral activity displayed by nurses in the social context of the colony. This switch between rhythmic and arrhythmic activity even in foragers, which are actually not involved in brood care in the colony context, emphasizes the key role of brood presence in caste- and context-dependent temporal plasticity. Besides the contact to the brood, contact to conspecifics is one crucial factor of social life. We tested the importance of interactions with conspecifics on synchronization of daily locomotor rhythms by first entraining two separate groups of individuals to 12:12h light-dark cycles, which were phase-shifted by 6 hours. Afterwards, we monitored their locomotor activity rhythms under constant conditions in separate monitors, but with pairwise contact through a mesh partitioning. Workers with social contact did not show the strong free-running rhythms observed in isolated, control individuals, and shifted some of their activity towards the activity period of the conspecific. On the broader scale of the colony, such spread of activity may lead to synchronized activity bouts. It is concluded that the two assayed social cues shape individual rhythms context dependent, which might be a key mechanism for chronobiological plasticity in ant colonies.

Introduction

One of the major characteristics of social insects is the non-reproductive division of labor, where the various tasks within a colony are performed by specialized worker castes without any central control (Hölldobler & Wilson, 1990; Robinson, 1992; Bonabeau, 1998). Division of labor among individuals can be either based on size or age polymorphism of workers (Wilson, 1971; Oster & Wilson, 1978; Gordon, 1996). In the nectar-feeding ant *Camponotus rufipes*, media-sized workers engage in tasks both inside and outside the nest (Jaffé & Sánchez, 1984) and further task allocation between media-sized workers is based on worker age. Whereas young workers stay inside the nest engaging in nursing, older workers leave the nest to forage (Soares et al., 2008). This division of labor in ants and also honey bees is associated with task-related plasticity in daily rhythms. Although all individuals possess a functional molecular clock and display rhythmic activity patterns in isolation (bees: Bloch et al., 2001; Shemesh et al., 2007, 2010; ants: Ingram et al., 2009, 2012; own data, see chapter I and III), nurses care for the brood all around the clock in the colony context without any rhythm (bees: Crailsheim et al., 1996; Moore et al., 1998; ants: own data, see chapter II). In contrast, foragers exhibit strong rhythms even in the social context and synchronize their activity with food availability (bees: Beling, 1929; Wahl, 1932; Frisch & Aschoff; 1987; ants: Grabensberger, 1933; Harrison & Breed, 1987; own data, see chapter II). As this chronobiological plasticity was also demonstrated for bumble bees (Yerushalmi et al., 2006), it appears to be a widespread, adaptive trait in social insects.

Several authors proposed that such chronobiological plasticity could be elicited by social cues within the colony environment (Bloch, 2010; Eban-Rothschild et al., 2012; Beer et al., 2016; Fuchikawa et al., 2016), but the effect of direct interactions with brood and other colony members on daily rhythms has not been studied yet. In bumble bee queens, the presence of brood led to arrhythmic activity in former rhythmic individuals (Eban-Rothschild et al., 2011). As such profound changes in activity profiles were described as well in mated queens of honey bees (Harano et al., 2007; Johnson et al., 2010) and ants (McCluskey, 1967, Lone et al., 2012), interaction with brood is a likely cue that may modulate daily activity rhythms of workers as well. Therefore we confronted otherwise socially isolated nurses and foragers with the presence of brood, and determined the modification of their endogenous locomotor rhythms.

In insect societies, task like foraging and nursing are performed collectively by up to thousands of individuals and this might require temporal synchronization between the individuals (Bonabeau et al., 1998). For instance, being active at the same time could help to exploit food resources rapidly or enlarge the nest efficiently, thus synchrony among individuals might be needed inside and outside the nest. Foragers spend considerable amounts of time outside the nest and their activity is most likely entrained by the environmental light-dark-cycle, but interior workers do not experience this dominant zeitgeber in the dark nest. In general, these individuals could synchronize their activity in two different ways (reviewed in Bloch et al., 2013). First, exterior workers could indirectly transfer temporal information into the nest interior. Such a phenomenon was described for *Leptothorax* ants, where returning foragers trigger activity bouts in in-nest workers (Boi et al., 1999). But considering the occurrence of large, multi-chamber nests in many ant species, not all interior workers might get in direct contact to exterior workers. Here, single ants that get spontaneously active could activate ants close-by and therefore generate synchronized activity peaks at the group level. This type of self-organized synchrony was also demonstrated in *Lepthotorax* ants (Franks et al., 1990; Cole 1991a, b, c). As these studies focused either only on group-level activity or on short time intervals, we aimed at studying potential changes in the 24-hour activity patterns of isolated workers that had the possibility to interact with a conspecific, thus representing the most basal interaction form in ant colonies.

In this chapter, we modified the previously established automatic monitoring of locomotor activity to study the effect of two crucial social factors on the plasticity of endogenous rhythms of *C. rufipes* workers. In a first experiment, we compared daily rhythms of isolated nurses and foragers with their rhythms under brood presence to investigate whether the contact to brood alone causes a switch from rhythmicity to arrhythmicity. In a second experiment, we studied daily rhythms in workers that were allowed to contact a conspecific through a mesh, to explore a potential social synchronization of their locomotor rhythms.

Material and Methods

For the laboratory studies, queens were collected during their mating flights in December 2011 and 2014 in La Coronilla, Uruguay (33°53'25.2"S, 53°31'27.6"W) and were brought to the laboratory at the University of Würzburg, Germany. Colonies were raised in plaster nests under constant conditions (25°C, 50%rH) and a 12:12h LD-cycle (300 lux during the light phase) in climate chambers. Queenright colonies consisted of several thousand workers and were fed *ad libitum* with water, diluted honey and pieces of cockroaches.

Influence of brood presence on locomotor rhythms

To record locomotor activity of the two different worker castes under brood presence, we initially built subcolonies from three *C. rufipes* queenright colonies (named colony H, Q and R). Subcolonies consisted of 60 individually marked workers and 120 larvae settled in an artificial brood chamber (diameter: 5.5 cm, height: 1.5 cm) with access to an outside foraging arena (diameter: 6.3 cm, height: 4.6 cm) via one tunnel (diameter: 0.6 cm, length: 3.5 cm; see chapter 3). Caste affiliation was determined visually on a daily basis throughout one week after the subcolonies were established, which simultaneously represented the 1-week-entrainment phase under the LD-cycle. Foragers were considered as those ants present in the foraging arena actively collecting food, while nurses were those workers tending brood inside the brood chamber.

Thereafter, subcolonies were discontinued and single identified individuals of known caste were placed in the modified locomotor activity monitors (see chapter I & II) and recorded 8 days under the same 12:12h LD-cycle used for the subcolonies and otherwise constant environmental conditions (25°C, 50%rH; incubator I-30BLL, CLF PlantClimatics GmbH). Individuals were housed together with two larvae taken shortly before from the main colonies (brood present), or without brood (control). Usually, ants in the locomotor activity monitors are supplied with sugar water only, but rearing of larvae additionally requires protein food sources. Therefore one of the feeding caps was filled with Bathkar diet (Bathkar & Whitcomb, 1970) instead of a sponges soaked with sugar water.

Activity measured over the whole recording period was displayed in form of actograms via ActogramJ for all individuals. We generated representative average activity patterns over all surviving ants based on seven days of the observation period (first day of recording was excluded due to acclimatization; $n_{Foragers}=78$, $n_{Nurses}=46$, $n_{Foragers+brood}=24$, $n_{Nurses+brood}=32$). Calculations of total activity levels as well as of the relative nocturnal activity (normalized for the total activity of every individual) on the second day of the respective recording period were selected to avoid pseudoreplications, and so to quantitatively compare activity patterns between the castes. For further description of the activity rhythms we calculated the period values of the individual activity as well as the power of rhythms (periodogram analysis, Lomb-Scargle method; ActogramJ), and tested for differences between the castes (Mann-Whitney U-tests) and between treatments (brood vs no brood; Mann-Whitney U-tests) under Bonferroni correction ($\alpha=0.025$). The effects of caste and brood presence on survival rate and rhythmicity were analyzed by χ^2 tests under Bonferroni correction ($\alpha=0.025$). All statistical analysis were performed in STATISTICA (StatSoft, Inc., Version 10.0) after testing data sets for normal distribution via Shapiro-Wilks test.

Influence of presence of conspecifics on locomotor rhythms

To test if social zeitgebers are able to synchronize activity rhythms, we monitored locomotor rhythms of individuals in pairwise connection. Two monitors, each containing single ants, were coupled, so ants were in pairwise contact. Ants were separated by plastic nets to keep them within the recording section of their glass tube, so that we were able to record the individual locomotor rhythm. Through the wire mesh, ants could get in contact by antennating, trophallaxis and over the microclimate (treatment “contact”). As a control, ants of both groups were recorded in isolation in parallel (treatment “isolated”). Locomotor activity was recorded under constant darkness for seven days. We simulated a situation of desynchronized subpopulations within the nest by entraining the connected individuals to different light regimes. Therefore we initially built 8 subcolonies from two *C. rufipes* queenright colonies (named colony H and R). Subcolonies consisted of 100 workers and some brood, and were kept in plastic containers (19.5 x 19.5 x 5.5 cm) under constant environmental conditions (25 °C, 50 %rH; incubator I-30BLL, CLF PlantClimatics GmbH) and *ad libitum* feeding (honey-water, cockroaches, water). Two different light regimes were used to entrain the four subcolonies. Both light regimes consisted of a 12:12h LD-cycles (4000 lux during the light phase), but were phase-shifted by six hours towards each other (“early” LD-cycle: light phase 10 am - 10 pm local time; “late” LD-cycle: 4 pm – 4 am local time). After one week of entrainment, single ants were placed in the modified locomotor activity monitors (see chapter I & II) during the dark phase of both groups (9 am).

All statistical analysis were performed in STATISTICA (StatSoft, Inc., Version 10.0) after testing data sets for normal distribution via Shapiro-Wilks test. To compare activity rhythms we calculated total activity levels, as well as period and power (periodogram analysis, Lomb-Scargle method; ActogramJ). As no significant differences between early and late groups of each treatment or colony were found (Kruskal-Wallis tests), data could be pooled and simply compared between treatments (isolated vs. contact) via Mann-Whitney U-tests.

As a measure of phase relationship we calculated the acrophase (=peak of activity rhythm) for every individual and day in the monitors, and compared acrophase differences between early and late groups throughout the recording. The acrophases of the first day in the monitor were excluded from the analysis, as individuals showed stress reactions due to the sudden isolation. If ants would be able to synchronize their activity through their contacts, acrophases of both entrainment groups in the contact treatment should approach and turn to be similar over the days of the experiment. Without the possible synchronizing effect of the social interaction, acrophases in the control treatment were expected to drift in accordance to the free-running period according to their entrainment and to keep the six hour phase difference.

Results

Influence of brood presence on locomotor rhythms

Under isolation in the activity monitors, foragers and nurses did not show any differences in their locomotor behavior. With roughly 60% of individuals, both castes showed equal levels in rhythmicity (χ^2 test, χ^2=0.00, p=0.97; Table 1) and synchronized their activity rhythm strongly with the 12:12h LD-cycle (Fig. 1, top). As indicated in the average activity patterns (Fig. 1, bottom) and by comparing the levels of night activity (Fig. 3A), both castes were predominantly nocturnal (Mann-Whitney U-test, U(78, 46)=1537.0, z=1.3, p=0.18).

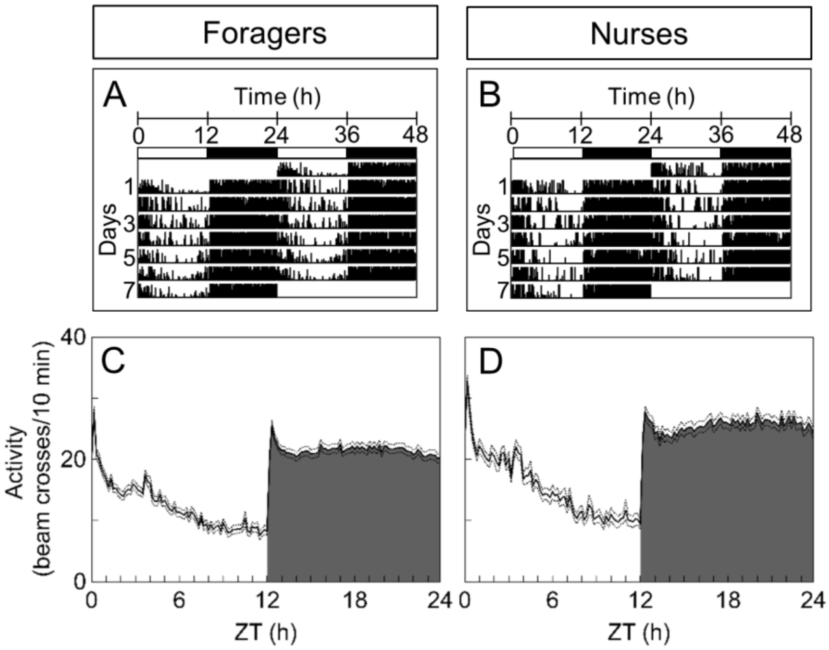

Fig. 1: Locomotor activity rhythms of forager and nurses in isolation from the social context. Top: Representative actograms of one worker of each caste. Locomotor activity (indicated as black bars) is shown as double plot under a 12:12h LD-cycle (A): Representative actogram of one forager. (B): Representative actogram of one nurse. Bottom: Average activity (mean: solid lines; mean±SE: dashed lines) over 7 days of the LD-cycle (C): Foragers (n=78). (D): Nurses (n=46).

Caste affiliation or presence of brood did not affect survival rate in the monitors (χ^2 tests; foragers vs nurses without brood: χ^2=0.36, p=0.5; foragers vs nurses with brood: χ^2=2.46, p=0.1; foragers without vs foragers with brood: χ^2=3.76, p=0.05; nurses without vs nurses with brood: χ^2=0.17, p=0.7; Table 1), but brood presence did affect rhythmicity. Under the presence of brood, the castes showed similar low rhythmicity levels (χ^2 test, χ^2=1.56, p=0.2; Table 1).

Table 1: Survival rate and rhythmicity of foragers and nurses under brood presence and absence in the locomotor activity monitors. Differences between castes within treatments and differences within castes between treatments were evaluated via X^2 tests after Bonferroni correction (α=0.025).

		Survival (%)			Rhythmicity (%)		
Without brood	Forager	55.7 (n=78)	ns		59.0 (n=46)	ns	
	Nurse	80.7 (n=46)		ns	58.7 (n=27)		***
With brood	Forager	37.5 (n=24)	ns	ns	16.7 (n=4)	ns	***
	Nurse	55.2 (n=32)			6.3 (n=25)		

Workers were arrhythmic and showed uniform activity levels throughout the day (Fig. 2). As a consequence, workers under brood presence had significantly reduced nocturnal activity levels compared to the castes without brood presence (Mann-Whitney U-tests; foragers vs nurses under brood presence: U(20, 32)=153.0, z=-3.1, p=0.001; foragers without vs foragers with brood: U(78, 20)=59.0, z=6.3, p<0.00001; nurses without vs nurses with brood: U(46, 32)=390.0, z=3.5, p=0.0003; Fig. 3A).

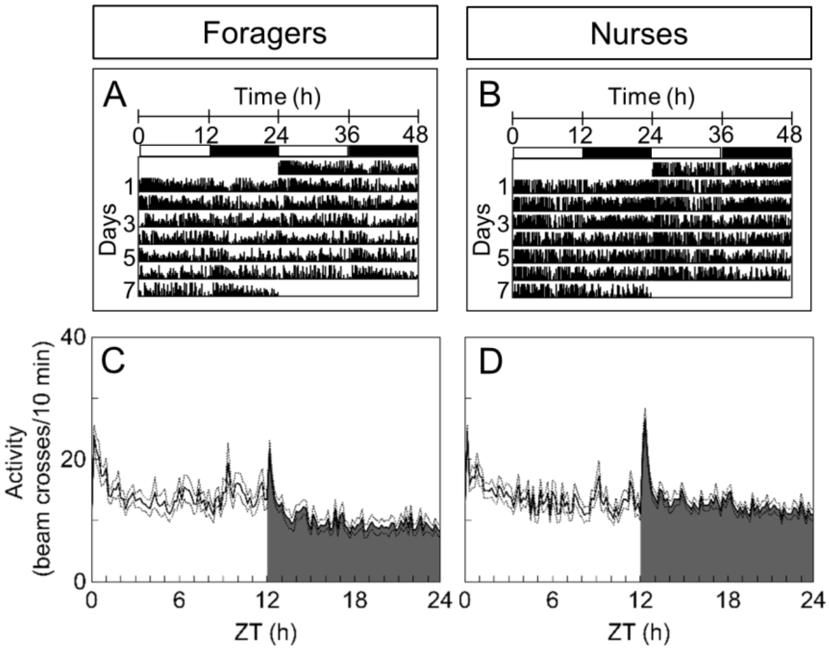

Fig. 2: Locomotor activity rhythms of forager and nurses under the presence of brood. Top: Representative actograms of one worker of each caste. Locomotor activity (indicated as black bars) is shown as double plot under a 12:12h LD-cycle (A): Representative actogram of one forager; (B): Representative actogram of one nurse. Bottom: Average activity (mean: solid lines; mean±SE: dashed lines) over 7 days of the LD-cycle (C): Foragers (n=20); (D): Nurses (n=32).

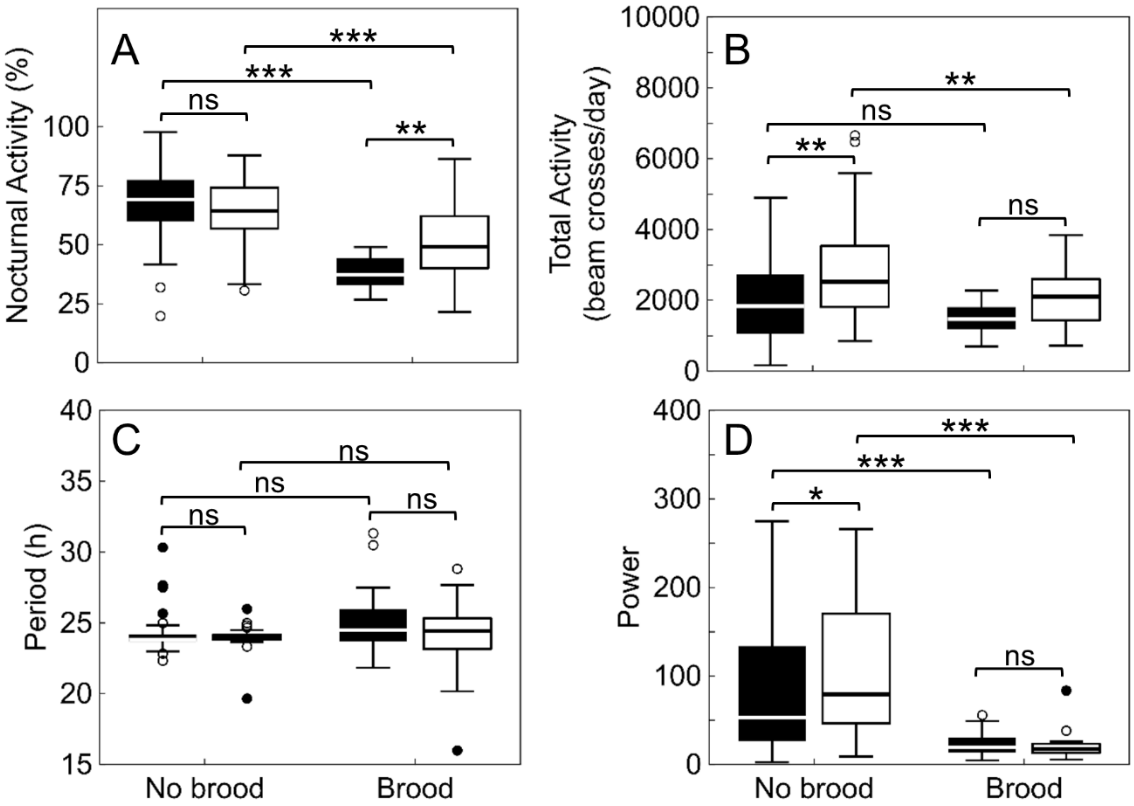

Fig. 3: Characteristics of locomotor activity rhythms of both forager and nurses under presence and absence of brood. Boxplots show medians (center lines) and interquartile ranges (boxes) for foragers (black) and nurses (white), whiskers indicate the minimum and maximum values. Open circles show outliers and filled circles extreme values. (A): Nocturnal activity. $n_{Foragers}$=78, n_{Nurses}=46, $n_{Foragers+brood}$=24, $n_{Nurses+brood}$=32; (B): Total activity. $n_{Foragers}$=78, n_{Nurses}=46, $n_{Foragers+brood}$=24, $n_{Nurses+brood}$=32; (C): Period. $n_{Foragers}$=74, n_{Nurses}=45, $n_{Foragers+brood}$=24, $n_{Nurses+brood}$=32; (D): Power. $n_{Foragers}$=78, n_{Nurses}=46, $n_{Foragers+brood}$=21, $n_{Nurses+brood}$=18; Asterisks indicate significant differences between castes within treatments (no brood/brood) or within caste between treatment (Mann-Whitney U-tests with Bonferroni correction, α=0.025).

Daily activity levels of both castes were reduced similarly in both castes under brood presence (Mann-Whitney U-tests; foragers without vs foragers with brood: U(78, 20)=604.5, z=-1.5, p=0.1; nurses without vs nurses with brood: U(46, 20)=451.0, z=-2.9, p=0.003; Fig. 3B). Nurses showed higher daily activity levels than foragers only in the absence of brood (Mann-Whitney U-tests; without brood: U(78, 46)=3465.0, z=-3.0, p=0.002; with brood: U(32, 20)=232.0; z=-1.6, p=0.09).

Although only few significant period values could be detected in both castes under brood presence, the average values were close to 24 hours and similar to those without brood (Mann-Whitney U-tests; foragers vs nurses without brood: U(74, 45)=1357.0, z=-1.7, p=0.09; foragers vs nurses with brood: U(20,18)=161.5, z=0.7, p=0.4; foragers without vs foragers with brood: U(74, 20)=519.5, z=-2.3, p=0.025; nurses without vs nurses with brood: U(45, 18)=350.5, z=-0.8, p=0.4; Fig. 3C). However, a wider distribution of period values could be noticed.

Without the presence of brood, nurses exhibit stronger activity rhythms than foragers (Mann-Whitney U-tests, U(78, 46)=1374.0, z=-2.3, p=0.02; Fig. 3D). This effect is not seen under the presence of brood (Mann-Whitney U-test, U(32, 20)=259.5, z=1.1, p=0.2). But in both castes, the power of rhythms is drastically lowered under brood presence (Mann-Whitney U-tests; foragers without vs foragers with brood: U(78, 20)=314.0, z=3.9, p<0.0001; nurses without vs nurses with brood: U(46, 32)=130.0, z=6.2, p<0.0001).

Influence of presence of conspecifics on locomotor rhythms

Colony origin and light regime had no effect on total activity (Kruskal-Wallis tests; contact: H(3, 72)=3.56, p=0.3; isolation: H(3, 89)=2.75, p=0.4), period (Kruskal-Wallis tests; contact: H(3, 52)=1.18, p=0.7; isolation: H(3, N=84)=1.15, p=0.1) and power of circadian rhythms (Kruskal-Wallis tests; contact: H(3, 72)=1.38, p=0.1; isolation: H(3, 89)=0.24, p=0.9), therefore data sets were pooled for treatments (isolation vs contact).

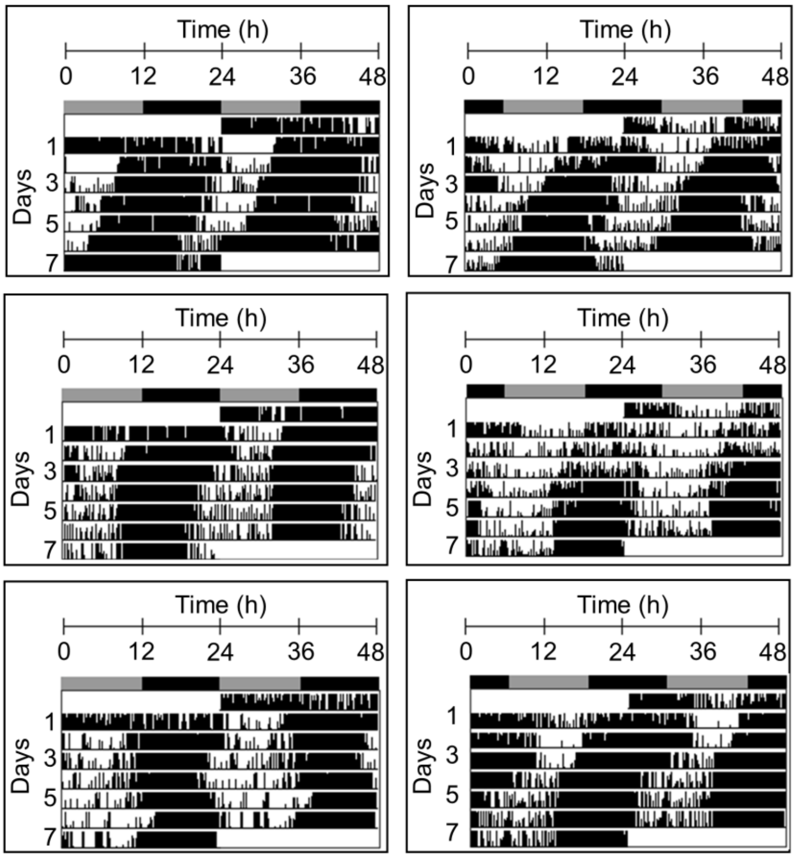

Fig. 4: Locomotor activity rhythms of worker castes in isolation from the social context. Exemplary actograms of workers with different free-running periods from both entrainment groups (left: “early” group; light phase 10 am - 10 pm local time; right: “late” LD-cycle: 4 pm – 4 am local time) are displayed. The most frequent activity patters ($\tau<24$ hours) is presented in the top row. Locomotor activity (indicated as black bars) is shown as double plot under constant darkness. Previous 12:12h LD-cycle during entrainment is indicated above each actogram**.**

Under constant conditions, activity rhythms of isolated individuals started free-running depending on the endogenous period of the individual (Fig. 4). As 65% of the individuals exhibited free-running rhythms with periods shorter than 24 hours, we chose actograms of this pattern for comparison with activity patterns of individuals under social contact (Fig. 5A). Ants that had pairwise contact displayed a modified activity pattern (Fig. 5B), which we quantified by different measures describing endogenous rhythms.

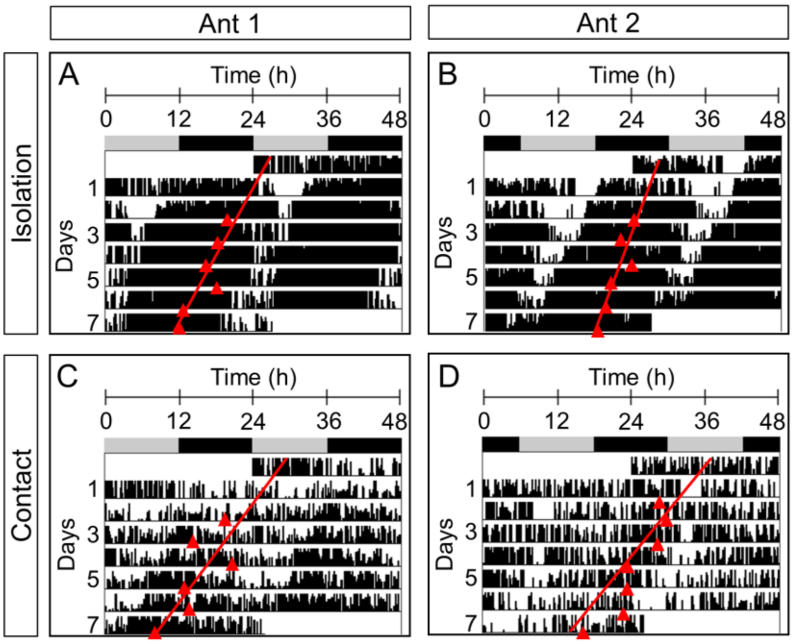

Fig. 5: Locomotor activity rhythms of worker castes in isolation from the social context (top) and under social contact (bottom). Locomotor activity (indicated as black bars) is shown as double plot under constant darkness. Previous 12:12h LD-cycle during entrainment is indicated above each actogram**.** Red triangles show acrophases for each day and red lines show the calculated regression lines for the daily acrophases. Exemplary actograms of one worker of each entrainment group (left: “early” group; light phase 10 am - 10 pm local time; right: “late” LD-cycle: 4 pm – 4 am local time) are displayed.

Individual ants that had pairwise contact in the locomotor activity monitors exhibited far lower daily activity levels than ants in complete isolation (Mann-Whitney U-test, U(89, 72)=1327.0, z=6.4, p<0.0001; Fig. 6A). With around 23.2 hours in the median, ants in contact and isolation did not differ in the period of rhythm (Mann-Whitney U-test, U(84, 52)=2026.0, z=0.7, p=0.4; Fig. 6B). However, the power of rhythm was strongly reduced in ants under pairwise contact (Mann-Whitney U-test, U(89,72)=1788.5, z=4.65, p<0.0001; Fig. 6C), indicating a weakening of the endogenous rhythm.

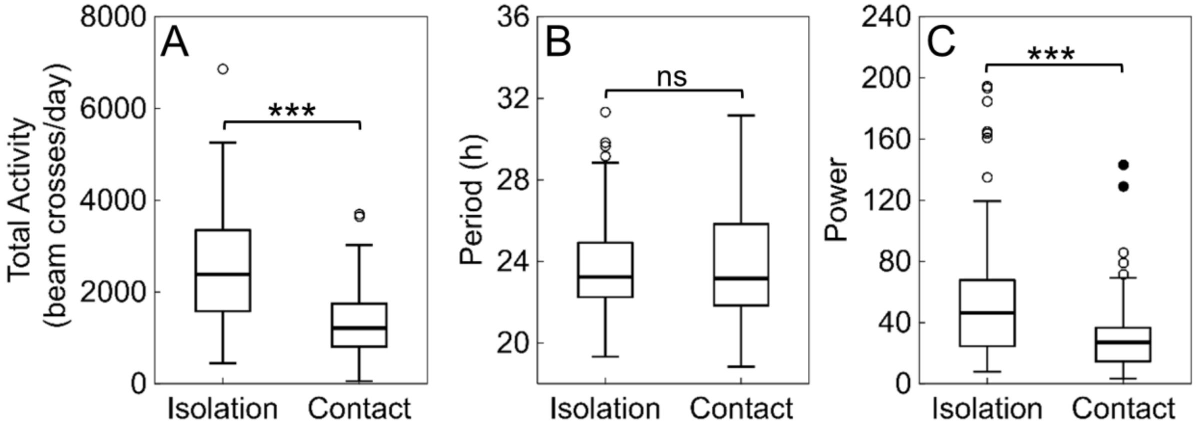

Fig. 6: Effect of social contact on locomotor activity rhythms. Boxplots show medians (center lines) and interquartile ranges (boxes), whiskers indicate the minimum and maximum values**.** Open circles show outliers and filled circles extreme values. (A): Total activity. $n_{isolation}$=89, $n_{contact}$=72; (B): Period. $n_{isolation}$=84, $n_{contact}$=52; (C): Power. $n_{isolation}$=89, $n_{contact}$=72; Asterisks indicate significant differences between treatments (Mann-Whitney U-tests).

As the groups were entrained to LD-cycles shifted by 6 hours, we expected that the isolated individuals display activity rhythms with an acrophase difference of approximately 6 hours. And as *C. rufipes* workers predominantly have free-running periods shorter than 24 hours (see chapter I and II), acrophases were expected to drift to earlier time points during each day of the monitoring. Due to the variation in endogenous periods across the individuals, we did not see these strong trends in the isolated individuals (Fig. 7, left). Consequently, the differentially entrained groups displayed not the six hour phase difference from the entrainment (Fig. 8A). Nevertheless, acrophases of early and late groups shifted in parallel with a fairly constant phase difference of three hours (median), indicating an independent drifting of activity rhythms. Acrophases of ants in social contact rather varied throughout the monitoring, but phase differences smaller than the three hour phase difference in isolated ants could be noticed throughout the monitoring (Fig. 7, right). Taken only the pairs of ants that had phase difference smaller than three hours at the least at the last day of the monitoring, we even found small acrophase differences throughout the whole monitoring (Fig. 8B). Ant pairs with larger phase differences at the last day of the monitoring even had larger acrophase differences than isolated ants (Fig. 8C).

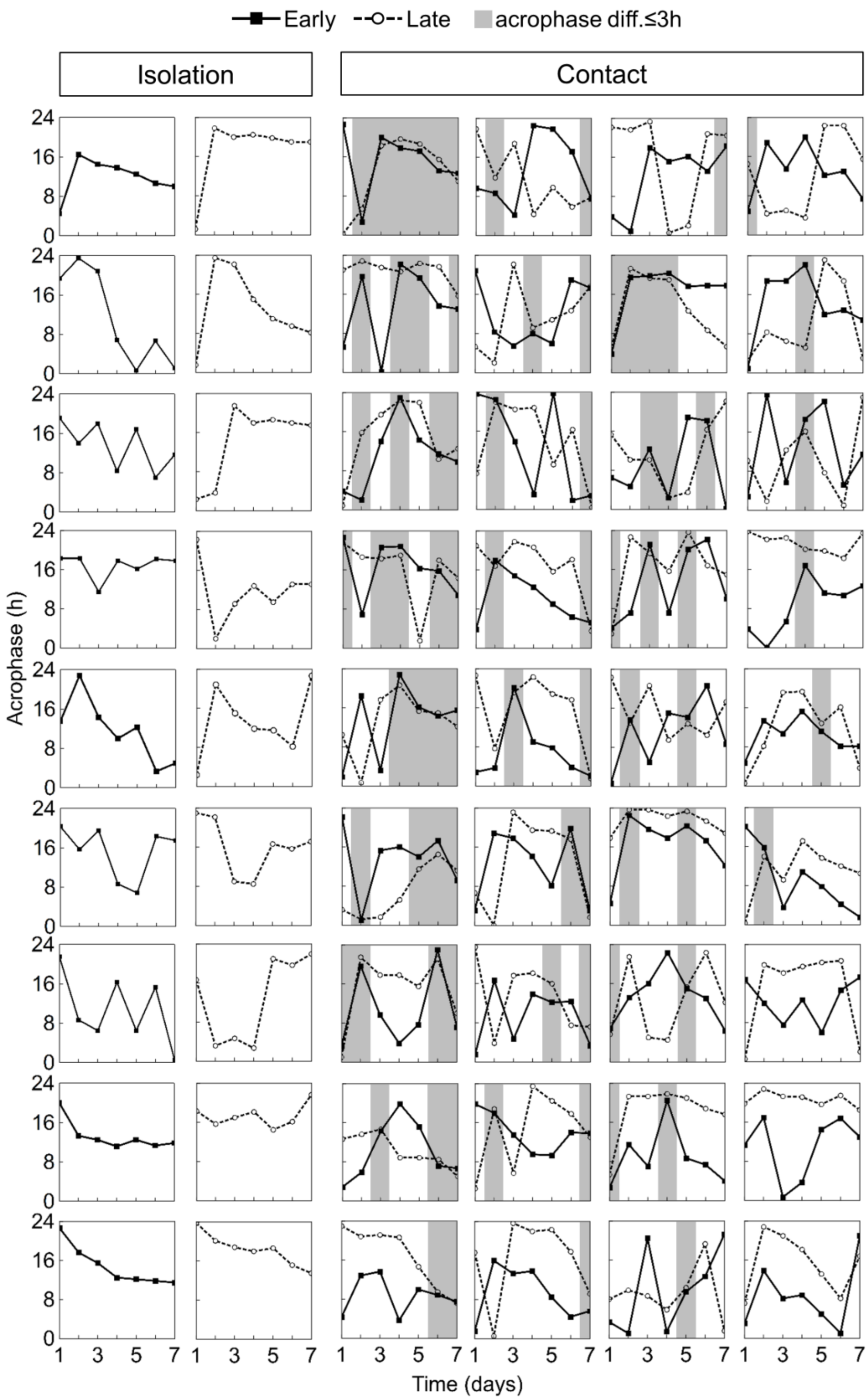

Fig. 7: Acrophases of locomotor activity rhythms of individuals under isolation and social contact. Acrophase of activity is shown for individuals from the early (black) and late (dashed) entrainment group during the seven days of monitoring. Left: Randomly chosen examples of individuals under isolation. Right: Acrophases of all monitored individuals under social contact. Each plot displays acrophases of two paired individuals. Days with acrophase differences of less than 3 hours are indicated in grey

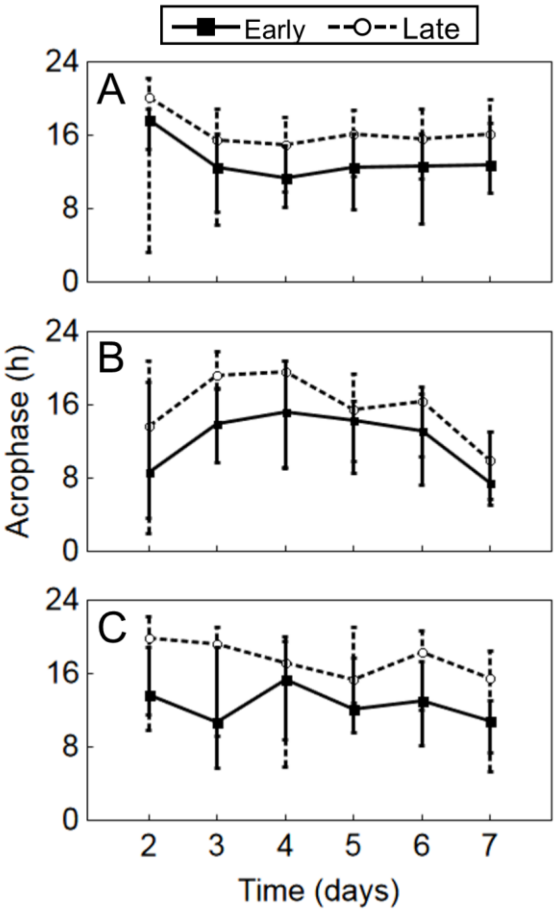

Fig. 8: Effect of social contact on the acrophase of locomotor activity rhythms. Acrophase of activity (median±IQR) is shown for the early (black) and late (dashed) entrainment groups for the last six days of monitoring (first day of monitoring was excluded from the analysis). (A): Acrophase under isolation. n_{early}=47; n_{late}=42; (B): Acrophase under social contact: individuals with synchronized activity at the last day of monitoring. n_{early}=19; n_{late}=19. (C): Acrophase under social contact: individuals with unsynchronized activity at the last day of monitoring. n_{early}=17; n_{late}=17.

Discussion

As shown in chapter I and II, all worker castes of the ant *Camponotus rufipes* possess an endogenous clock and are therefore able to exhibit circadian rhythms. Although foragers and nurses show almost identical activity rhythms when isolated in the locomotor activity monitors and are predominantly nocturnal, their endogenous rhythms are altered in the colony context in accordance to the task demands. In particular, nurses are behaviorally arrhythmic in the social context and tend the brood all-around the clock. Their endogenous rhythm is therefore either masked or depressed in the social context (Shemesh et al., 2010), yet the underlying mechanisms for that masking remained unexplored. Among other factors, the presence of brood and conspecifics are likely indicators of the colony context. We therefore tested if these two factors alone can signal colony context and are able to alter the endogenous rhythms of otherwise isolated individuals.

Under complete isolation, we identified few differences in activity rhythms of foragers and nurses. Nurses displayed stronger locomotor rhythms with higher activity levels than foragers, which results from age differences between these castes (see chapter I). As this distinction in activity patterns is completely lost under the presence of brood, daily activity rhythms of both castes are altered in a similar manner.

When isolated members of both castes were monitored together with brood, more than 80% of the individuals displayed arrhythmic activity. This activity pattern rather resembles the behavioral arrhythmicity of nurses in the social context (see chapter II) than the otherwise highly rhythmic locomotor activity of both castes under isolation. As a consequence of this arrhythmicity, we found uniform levels of activity throughout the day and not the strong night activity as under complete isolation. Observation of the ants´ behavior within the recording tubes indicated that the ants were predominantly either carrying around larvae or feeding and licking them. As a consequence of this constant interaction with the brood, the endogenous locomotor rhythm was lost and as a by-product, locomotor activity levels were clearly reduced. The analysis of daily behavior of brood tending ants in chapter II showed that even in the social context, walking activity is clearly reduced in nurses compared to foraging ants. But besides being more stationary, those ants are almost constantly active caring for the brood. A study on brood care in *Solenopsis invicta* showed that larvae are fed up to 50 times per hour and are patrolled 200-800 times per hour, which could lead to uniform levels of nourishment for all larvae (Cassil & Tschinkel, 1995). In addition, constant patrolling and frequent licking of the brood could be part of the hygienic repertoire to prevent infections (Tragust et al., 2013; reviewed in Cremer et al., 2007). Natural nests of *C. rufipes* ants undergo daily changes in temperature (Weidenmüller et al., 2009) and as temperature affects brood development (Roces & Núñez, 1989), nurses need to relocate the brood accordingly (Roces & Núñez, 1995). All this processes favor brood development (Falibene et al., 2016) and consequently colony growth, and might cause the all-around-the-clock activity detected in *C. rufipes* nurses.

Independent of the presence/absence of brood we found period values close to 24 hours in the two castes, which at first sight indicate strong synchronization with the 24-hour period of the light phase. However, under the presence of brood only few significant period values could be determined at all. The wide distribution of these few values as well as highly reduced power of rhythms rather reflect the poor synchronization with the light-dark-cycle in ants under brood presence.

We demonstrated that the presence of brood alone alters daily rhythms in both isolated nurses and foragers, which are actually not involved in brood care in the colony.

Without the presence of other colony members, foragers promptly engaged in brood care and therefore demonstrated the flexible character of task allocation in ants. As shown already in different ant species, task allocation is reversible and flexibly adapted to colony demands (Calabi & Traniello, 1988; Gordon, 1989; Robinson, 1992; Bernadou et al., 2015). Here we showed that the task switch from foraging to nursing is closely associated with alteration of the daily activity rhythm. As such a reversion of tasks and rhythms is seen in honey bees as well (Bloch & Robinson, 2001), these social insect might share principal mechanisms of chronobiological plasticity.

Apart from the presence of brood, the presence of other colony members is one of the crucial factors that characterizes social life. Interaction between colony members can stimulate or modulate behavioral activity and can therefore contribute to the temporal organization of activities in social insects. As tasks like nursing and foraging are performed simultaneously by several individuals, synchronization between workers may favor efficient collective behavior of the colony. Without the light-dark-cycle as prominent zeitgeber, social interaction could be even an important zeitgeber to synchronize activities within the dark ant nest. Exterior workers like foragers and guards, which experience the ambient light-dark-cycle, could indirectly transfer temporal information inside the nest. As illustrated in chapter II, *C. rufipes* foragers returning with food at a special time point can induce increased brood care activity in nurses. In harvester ants, increased interaction rates with returning foragers in the nest chamber stimulates foraging activity in inactive ants (Pless et al., 2015). And in the ant *Camponotus paria*, cyclic interaction with exterior workers synchronizes locomotor activity rhythms of nest workers kept in darkness (Lone & Sharma, 2011). Depending on colony size and nest structure, however, not all interior workers might get in contact with exterior workers. Are these nest workers nevertheless able to synchronize their activity with each other? Without any zeitgebers, activity rhythms would drift apart of each other depending on the free-running period of their endogenous clock and would therefore cause social desynchronization. We simulated this situation by entraining groups of ants to phase-shifted light-dark-cycles and monitored their synchronization abilities in pairwise interaction. As these ants spent more time interacting by antennating or trophallaxis, they exhibited far lower locomotor activity levels than completely isolated ants. Besides the distinctive contact between the ants, they did not completely synchronize their activity rhythms. Neither did one ant adopt the activity phase of the other, nor did the ants exhibit a merged intermediate activity rhythm.

Although workers with social contact showed free-running rhythms from the last dark phase like the completely isolated ants, rhythms lack distinct phases of inactivity and were of lesser amplitude. Thus, both interaction partners shifted some of their activity towards the respective activity period of the other. As a consequence, acrophases of ants under social contact varied over the course of the experiment, whereas isolated groups kept their phase difference. With that, ants exhibit different synchronization behavior than honey bees. Several studies showed that groups of bees entrained to different light-dark-cycles synchronize their metabolic activity rhythms under constant darkness to an intermediate rhythm (Southwick & Moritz, 1987; Moritz & Kryger; 1993). In addition, isolated bees that are in contact to the hive via a mesh show locomotor rhythms synchronized with the rhythm of the hive (Eban-Rothschild et al., 2012; Beer et al., 2016; Fuchikawa et al., 2016). All these authors suggested social cues like pheromones, microclimate and vibration as promising candidates for social entrainment. As increased locomotor activity in bees is accompanied with an increase in body temperature (Kaiser, 1988; Fuchikawa & Shimizu, 2007), cyclic temperature changes could add to the synchronizing factors. Contrary to bees, ants, which lack flight muscles, are not expected to produce large amounts of metabolic heat, although they show daily rhythms of respiratory rates, likely associated with locomotion rhythms (Takahashi-Del-Bianco et al., 1992). As we did not see such merged rhythms in locomotor activity in our experiment after pairwise interactions, such synchronization behavior could be only present in larger groups or it may require extended interactions within the colony context. However, the alteration of activity rhythms by the direct and indirect interaction between two workers observed in our experiments emphasizes the relative influence of social synchronization on daily rhythms. As shown in *Leptothorax* (Cole, 1991b) and *Diacamma* ants (Hayashi et al., 2012), physical contact between a pair of ants can cause phase shifts in individual ants. Depending on their endogenous clocks, ants are spontaneously active and could activate ants in their vicinity. Even without other synchronizing factors, this leads to synchronized bursts of activity within the nest, which have been described already in *Leptothorax* ants (Goss & Denebourg, 1988; Franks et al., 1990; Cole; 1991a, c; Cole & Cheshire, 1996).

In this chapter we revealed the modulating effects of two social factors on daily locomotor rhythms: brood presence and contact with conspecifics. Contact to brood triggers all-around-the-clock activity in otherwise strongly rhythmic individuals and can therefore be identified as major factor leading to chronobiological plasticity in both foraging and nursing ants. In contrast, indirect and direct contact between two workers lead to a weak form of synchronization between them in our experiments. This mechanism might be even more prominent in larger groups and in the behavioral context of the colony. In this study we showed that the two social cues shape individual rhythms context dependent and therefore contribute to the temporal organization of daily activities in ant colonies.

Chapter IV

Synchronization of brood translocation rhythms in the ant *Camponotus mus*

Abstract

Nurses of the nectar-collecting ant species *Camponotus mus* translocate the brood between two temperatures in the laboratory, mimicking the naturally occurring temperature fluctuations in the nest. As this rhythmic thermal exposition optimizes the brood's brain development, the timing of brood translocations seems of special importance. Daily translocations on colony level follow a circadian rhythm in temperature preference in nurses, but the contribution of individual rhythms and synchronization of these rhythms in the dark nest remained unexplored. We manually tracked individual brood-translocation activity of all individuals in the nest chambers of six subcolonies under both light-dark-conditions and constant darkness with the help of long-term video recordings (2000 hours). We used temporally restricted feeding regimes to analyze the effect of social synchronization between exterior workers, which experienced the LD-cycle in the foraging arena, and interior workers within the dark nest, as compared to control colonies under *ad libitum* feeding. An average of 60% of nurses per colony showed rhythmic translocation activity under the LD-cycle, but preferentially engaged in only one of the two daily translocation events. The daily pattern of brood translocation observed on colony level was generated by the alternating activity of these two subpopulation of workers, which could be mediated by inter-individual differences in temperature thresholds. Social interaction with exterior workers was not sufficient to synchronize translocation activity in nurses under constant darkness. Here, only a low proportion of nurses displayed rhythmic, yet free-running translocation activity, indicating the involvement of a circadian clock. Differences in both free-running periods of circadian activity and variation in temperature thresholds among nurses probably caused conflicting translocation activity, so individual rhythmicity could be masked in the colony context. Our experiments demonstrate the occurrence of rhythmic behavioral activity within the ant nest and give first insights in the integration of individual rhythms into a whole colony rhythm. The need of synchronization of activity within the dark nests makes *C. mus* as a qualified model organism for testing other non-photic zeitgebers like temperature and humidity cycles.

Introduction

Temperature is a crucial environmental factor in all life stages of ectothermic animals like ants. As temperature for brood rearing influences both development time and brood survival (Elmes & Wardlaw, 1983; reviewed in Ratte, 1985; Porter, 1988), it directly affects colony growth. Colonies of ant species differ in their preferences for thermal ranges (Kipyatkov & Lopatina, 2014), as well as in their preference for constant or fluctuating temperature regimes (Cokendolpher & Francke, 1984; Roces & Núñez, 1989, Porter & Tschinkel, 1993; Anderson & Munger, 2003). In contrast to other social insects like bees (Jones & Oldroyd, 2006), only few ant species are known to produce metabolic heat to control nest temperature (Rosengren et al., 1987). Yet, ants developed several strategies of social thermoregulation. By the selection of suitable nest sites and building material (Sanada-Morimura et al., 2006; Bollazzi & Roces, 2010), ants promote favorable conditions for optimal brood rearing. Depending on the nest structure, nest temperature nevertheless fluctuates more or less dependent on the daily and seasonal environmental temperature cycles (Steiner, 1928; Weidenmüller et al., 2009). To prevent exposure to extreme temperatures during these fluctuations, some ant species translocate the brood vertically within the nest (*Acromyrmex*: Bollazzi & Roces, 2002; *Formica*: Steiner, 1928; *Pogonomyrmex*: Anderson & Munger, 2003; *Solenopsis*: Porter & Tschinkel, 1993). Although brood translocation in some species seems to be a simple reaction to external temperature changes (Penick & Tschinkel, 2008), the anticipatory translocation activity in some ants indicates the involvement of an endogenous rhythm (Cole, 1994).

Several laboratory experiments verified the presence of a circadian rhythm of temperature preference for brood rearing in nurses of the nectar-collecting ant species *Camponotus mus* (Roces & Núñez, 1989, 1995, 1996; Roces, 1995). Under a light-dark-cycle, nurse workers translocate the brood twice a day, to 30.8°C during the middle of the light phase and to 27.5°C 8h later during the dark phase. Translocation activity follows a circadian rhythm in thermal tolerance of nurses (Roces & Núñez, 1995), demonstrating its endogenous nature in this species. Brood development and thermal preferences in adults are thought to be adapted to the naturally occurring temperature cycles. When colonies are exposed to thermal regimes mimicking these natural temperature fluctuations, no translocation activity occurs (Roces & Núñez, 1996).

The exposition of the brood to thermal fluctuations of 3.3°C amplitude optimizes brain development and could therefore be crucial for learning and memory formation in adults (Falibene et al., 2016). Consequently, the timing of the exposition of the brood to this particular fluctuating temperature regime seems of special importance. With the help of their circadian clock, nurses could be able to anticipate daily maximal and minimal daily temperatures and could relocate the brood accordingly within the nest. But as previous studies of brood translocation activity has only been studied on colony level, the contribution of individual rhythmic activity to the colony rhythm remained unexplored.

For timing of brood translocation activity, the circadian clock in workers has to be synchronized by suitable zeitgebers signaling the time of the day. The bimodal rhythm of temperature preference can be synchronized by a light-dark-cycle, but in its absence in the dark nest, the colony rhythm starts free-running (Roces, 1995). In that case, interior workers could synchronize their activity with the help of exterior workers, which experience the light-dark-cycle outside the nest. As described for *Leptothorax* ants, returning foragers trigger activity bouts in in-nest workers (Boi et al., 1999). Such an indirect transfer of temporal information into the nest interior was also documented in *C. rufipes* (chapter 2). With the help of temporally restricted feeding, foraging activity in laboratory subcolonies was constrained to a certain time of the day. Foragers returning to the nest shared the collected food rapidly with interior workers and triggered an increase in nursing activity. But as these experiments were performed under the presence of a light-dark-cycle and constant temperature conditions, the synchronizing ability of this social interaction for translocation activity was not investigated. Cyclic social interaction is sufficient to synchronize locomotor activity of interior workers in *Camponotus compressus* (Lone & Sharma, 2011), making it to a potential non-photic zeitgeber for behavioral activity as well. In addition, food availability itself could act as zeitgeber for in-nest workers as well (reviewed in Stephan, 2002).

The aim of this chapter was to first analyze how individual translocation rhythms contribute to generate a colony rhythm under the confirmed synchronized conditions of a light-dark-cycle. Therefore, we manually tracked brood-translocation activity of all members of six subcolonies under a light-dark-cycle for seven days with the help of long-term video recordings. Afterwards, nest chambers were transferred to constant darkness to observe translocating activity in the same workers under natural conditions. In three subcolonies, we used temporally restricted food availability to induce diurnal activity in foragers. We tested whether food availability and social interaction with returning foragers during a short time window is sufficient to synchronize in-nest brood-translocation activity. Translocation activity in these subcolonies was compared to activity in three control subcolonies under *ad libitum* feeding, where food availability could not be used as temporal cue.

Material and Methods

For the laboratory studies, queens were collected during their mating flights in December 2008 and 2015 in Sarandi del Yi, Uruguay (33°19'56.0"S 55°37'43.1"W) and were brought to the laboratory at the University of Würzburg, Germany. Colonies were raised in climate chambers under a 12:12h light-dark cycle (300 lux during the light phase), constant environmental conditions (25°C, 50%rH) and *ad libitum* food supply (honey-water, cockroaches, water).

To observe individual brood translocation activity in workers of *C. mus*, we built small subcolonies of 60 workers and 60 larvae of two queenright colonies (named 18 and A). Preliminary experiments had shown that subcolonies of this size and brood-to-worker ratio establish a stable division of labor between nurses and foragers, and allow continuous tracking of individual worker behavior. To promote division of labor in the subcolonies, ants of varying body sizes were collected from both the nest chamber and foraging arena of the main colonies. Subcolonies were housed in artificial nests with a closed brood chamber (40 x 5 cm) and exposed to a continuous thermal gradient between the two nest ends (20-40 °C; peltier elements). The brood was located mostly in one pile at the preferred temperature along the gradient. This brood chamber was connected to an open foraging arena (9.5 x 9.5 x 5 cm) with a feeding platform (diameter: 5.5 cm, height: 2.3 cm) under constant environmental conditions (25°C, 50%rH). The use of the platform assured that foragers actively searched for food, allowing their distinction from other outside workers like guards. As both parts of the nest were located in different incubators, lighting conditions in the foraging arena and brood chamber could be applied independently. Three junctions in the tunnel connecting the brood chamber and arena kept the nest parts light isolated.

We performed two two-weekly experimental series to test if social interactions between interior and exterior workers can synchronize activity rhythms within the nest. In the first week of both series, both parts of the experimental setup were kept under a 12:12h light-dark-cycle (LD; 1000 lux during the light phase). On one side, this phase served as an entrainment phase for the brood translocation rhythm and worker activities. In addition, this phase served to monitor the rhythm under assured synchronized conditions, since the LD-cycle acts as strong zeitgeber for the brood translocation rhythm (Roces & Nuñez, 1989; Roces, 1995). This is of special importance, because main colonies and subcolonies may differ in temperature preferences for brood relocation, as the individuals randomly picked from the main colony could differ in their temperature preferences (Weidenmüller et al., 2009). In the second week of the experiments, the light regime in the brood chamber was switched to constant darkness (DD), which should cause - without any other temporal cues - a desynchronization of the brood translocation rhythm. To test if the interaction with exterior workers is sufficient to synchronize the brood translocation rhythm under constant darkness, we used different feeding regimes in the two experimental series.

In the first experimental series (test series, n=3 subcolonies), we used temporally restricted feeding regimes to limit the interaction between interior and exterior workers to a certain time of the day. Food availability was restricted to a short time window two hours after lights-on (**Z**eitgeber **T**ime: ZT 2). A supply of 10% sugar water and ten freshly killed *Drosophila* flies proved to be sufficient for subcolony maintenance. Depending on the collecting rate of the ants, food was present in the foraging arena only for up to two hours and was shared rapidly with the interior workers. In the second experimental series (control series, n=3 subcolonies) subcolonies were fed *ad libitum* throughout the two weeks, so food availability could not be used as a temporal cue. Here, pieces of cockroaches were fed instead of *Drosophila* flies to ensure permanent food availability. The amount of sugar water and cockroaches exceeded the subcolony’s daily needs and were replaced daily at different times to avoid the generation of unwanted temporal cues. Under *ad libitum feeding*, foragers were seen to collect and transfer food throughout the day.

We used 24-hours video recordings (NVR video recorder with IPC cameras, Alomna GmbH) of both parts of the setup to continuously determine caste affiliation and translocation behavior of all ants (~4000 hours), which were individually marked with printed paper tags glued to their gaster with paint (Edding). Under the dark phases, videos were recorded under dim infrared light. Afterwards, caste affiliation was recorded on a daily basis based on the behavior and location of the ants. Workers that were feeding, licking or translocating the brood within the brood chamber were classified as nurses. Workers that entered the foraging arena and actively collected food at the feeding platform were classified as foragers. Workers were assigned to the nurse or forager caste of each light regime if they were foraging or nursing for more than two days. For the two weeks of the experiments, an observer recorded all brood translocation events in the brood chamber of every individual and the general direction of the brood relocation (“cold”: to the colder part of the temperature gradient; “warm”: to the warmer part of the gradient). A brood relocation event was defined as ants´ lifting and carrying pupae or larvae with their mandibles to a different location. Unintended displacements of brood items by ants crossing the brood pile or dragging brood items from the bottom of the brood pile were not classified as brood relocation event. As the floor temperature within the gradient was previously recorded (Voltcraft K204), the temperature preference on group level could be derived from the position of the brood pile along the gradient every 30 mins during the experiment (center of the brood pile was used as reference point). Under synchronized conditions, workers were expected to relocate the brood to two different temperatures, to 30.8°C at the middle of the light period and to 27.5°C eight hours later during the dark period (Roces & Nuñez, 1989; Roces, 1995).

Under constant darkness, the brood translocation rhythm could be synchronized in two ways by exterior workers experiencing the LD-cycle outside the nest. On the one hand, exterior workers could directly participate relocating the brood at the right time of the day. On the other hand, exterior workers could synchronize brood relocation activity indirectly by transferring a time signal to the interior workers. To determine the direct influence of exterior workers on the brood relocation rhythm, we compared the proportion of workers in the forager and nurse caste that participated in brood translocation between the LD- and DD-regime (χ^2 tests). Furthermore, we analyzed the contribution of nurses and foragers to total brood relocation activity in LD- and DD-regimes of each subcolony (χ^2 tests).

We determined ratios of workers translocating towards the colder or warmer part of the gradient, or translocating in both directions (χ^2 tests). Afterwards, ratios of these behavioral castes were compared between the LD- and DD-periods for every subcolony (χ^2 tests). We further compared the number of relocations events towards one part (warm or cold) or simultaneously to both parts (warm and cold) of the temperature gradient at every time point of the observation (χ^2 tests).

To further analyze how daily activity of individuals contributes to the colony rhythm, we plotted translocation activity of every worker in the subcolony during the two weeks of the experiment, as well as total translocation activity within the subcolony, in form of actograms (ActogramJ). This representation form allows the detection of rhythmic or arrhythmic activity patterns in individuals. Translocation activity to the warmer and colder part of the temperature gradient was analyzed separately to detect temporal differences. If individuals would exhibit rhythmic activity patterns, translocation activity to the warmer part of the temperature would be expected during the light phase and translocation to the colder part of the temperature would be expected during the dark phase only.

Proportion of rhythmic individuals was ascertained (Lomb-Scargle method, $p<0.05$) and compared between the LD- and DD-regime (χ^2 tests). We further calculated period values of individual translocation activity towards warmer and colder temperatures separately for the LD-and DD-regime (Lomb-Scargle-method, ActogramJ) and tested for differences between the light regimes.

Results

Independent of the feeding regime, all subcolonies showed daily rhythms in brood location along the thermal gradient under the LD-cycle (Fig. 1). The brood pile was located at higher temperatures during the light phase, and at colder temperatures during the dark phase, but the absolute temperatures strongly differed among subcolonies (*ad libitum feeding* subcolony 1: 28.2-34.1°C, Fig. 1A; subcolony 2: 29.7-34.9°C, Fig. 1B; subcolony 3: 31.5-34.1°C, Fig. 1C; pulse feeding subcolony 1: 27.3-35.8°; Fig. 1D; subcolony 2: 28.2-32.0°C, Fig. 1E; subcolony 3: 30.6-34.9°C, Fig. 1F) and varied slightly over the seven days of the LD-cycle. Relocation of the brood pile between higher and lower temperatures was performed gradually over several hours. During constant darkness, the majority of subcolonies exhibited weaker brood translocation rhythms. Under both *ad libitum* and pulse feeding, only one out of three subcolonies showed a sustainable brood translocation rhythm under constant conditions (Fig. 1A/D). The other two subcolonies of each feeding regime exhibited a strong dampening of the rhythm (Fig. 1B/C/E/F), indicating desynchronized translocation activity independent of the feeding regime.

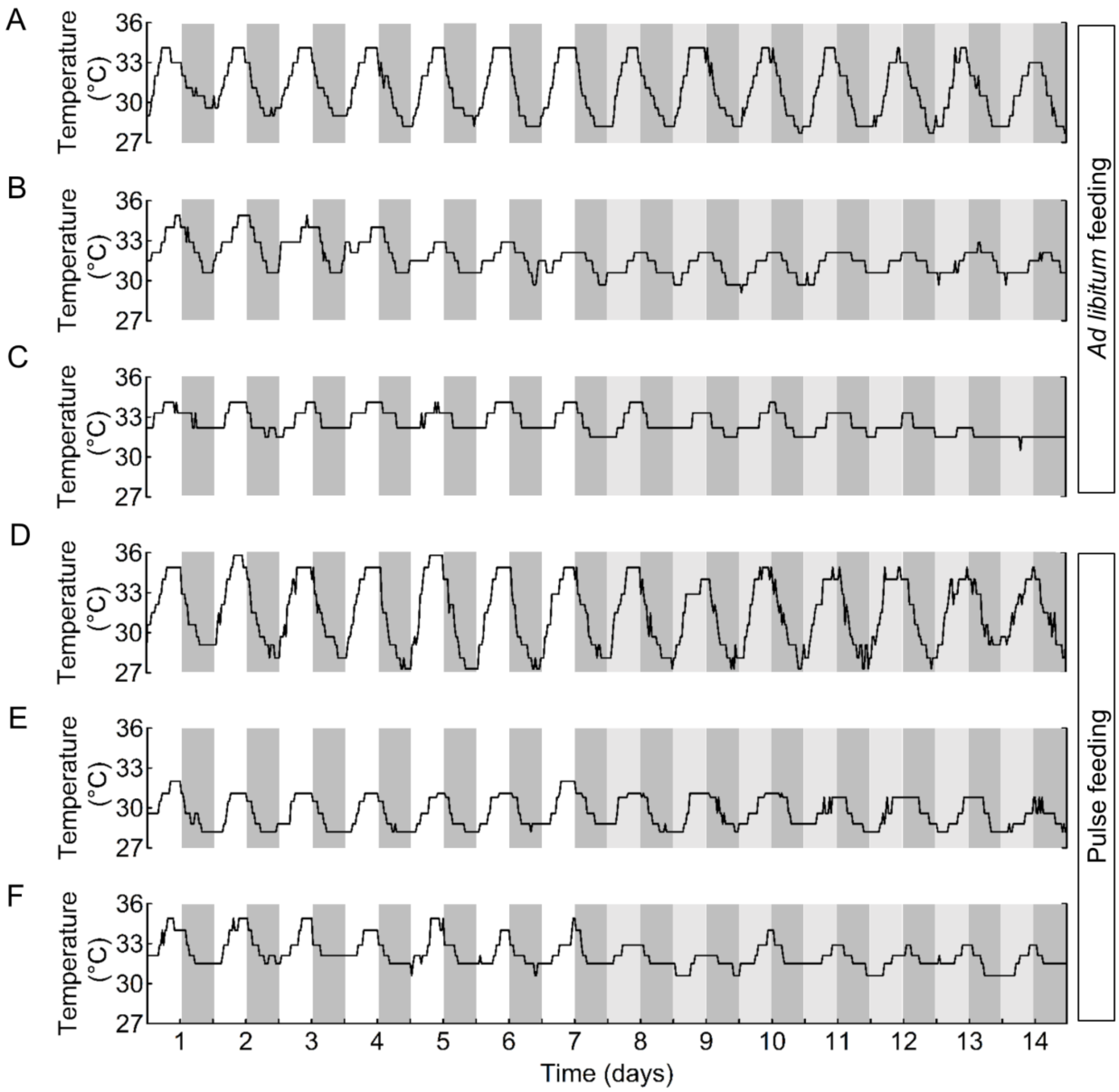

Fig. 1: Daily pattern of brood location along a thermal gradient. Position of the center of the brood pile was determined in six subcolonies every half an hour for the 14 days of the experiment. Day 1-7: thermal gradient under a 12:12h LD-cycle (dark phase indicated in dark grey). Day 8-15: thermal gradient under constant darkness (dark phase indicated in dark grey, respective light phase in light grey). (A): *ad libitum* feeding subcolony 1. (B): *ad libitum* feeding subcolony 2. (C): *ad libitum* feeding subcolony 2. (D): pulse feeding subcolony 1. (E): pulse feeding subcolony 2. (F): pulse feeding subcolony 3.

We first determined affiliation to discrete behavioral groups under both light regimes to determine if exterior workers contribute directly to a synchronized brood translocation rhythm. Affiliation to behavioral groups varied strongly between the six subcolonies (Table 1). From 60 individuals in the subcolony, 15 to 28 individuals engaged in nursing behavior and 16 to 30 individuals engaged in foraging behavior. Independent of both feeding and light regimes, a high proportion of nurses showed translocations of the brood to the different temperatures. Before translocating the brood, nurses were observed to spontaneously leave the brood pile and walk along the temperature gradient. During the two weeks of the experiment, nurses did not leave the nest chamber and stayed most of their time at the brood pile. In fewer cases, foragers were also observed to relocate the brood. But in contrast to nurses, they did not engage in other typical nursing behavior like feeding and licking the brood. With up to 35 workers, a high proportion of individuals was not engaged in one of these tasks. We found no differences in ratios of these behavioral castes between the LD- and DD conditions (χ^2 tests; *ad libitum* feeding subcolony 1: $\chi^2=5.6$, p=0.2; subcolony 2: $\chi^2=0.37$, p=0.9; subcolony 3: $\chi^2=1.47$, p=0.8; pulse feeding subcolony 1: $\chi^2=1.2$, p=0.9; subcolony 2: $\chi^2=2.7$, p=0.6; subcolony 3: $\chi^2=9.8$, p=0.05), indicating that the number of foragers that also relocate the brood does not increase under constant darkness to support synchronization. In the two subcolonies that showed a persistent relocation rhythm under constant darkness, a high number of foragers engaged in brood translocation under both light regimes (*ad libitum* feeding subcolony 1: 12-14 individuals, pulse feeding subcolony 1: 8-9 individuals).

Table 1: Number of ants per behavioral group in subcolonies during the two light regime. Foraging: entering the foraging arena and collecting food. Nursing: feeding and licking the brood. Translocating: picking up and relocating brood. Other: neither foraging, nursing nor translocating. Workers were assigned to the behavioral groups if they displayed behavioral activity on more than two days per light regime. Differences between light regimes within each subcolony were assessed per χ^2 tests.

Light regime	**Behavioral group**	***Ad libitum* feeding**			**Pulse feeding**		
		Subcolony 1	Subcolony 2	Subcolony 3	Subcolony 1	Subcolony 2	Subcolony 3
LD	Foraging	16	14	22	15	12	15
	Foraging + Translocating	14	11	2	8	4	8
	Nursing + Translocating	13	17	17	19	11	18
	Nursing	5	3	2	5	4	10
	Other	12	15	17	13	30	9
DD	Foraging	9	14	19	11	8	13
	Foraging + Translocating	12	9	2	9	1	2
	Nursing + Translocating	14	18	16	18	12	14
	Nursing	3	4	1	5	4	9
	Other	22	15	22	17	35	22
X^2 test		$X^2=5.6$ p=0.2	$X^2=0.37$ p=0.9	$X^2=1.47$ p=0.8	$X^2=1.2$ p=0.9	$X^2=2.7$ p=0.6	$X^2=9.8$ p=0.05

Although the number of translocating foragers did not increase during constant conditions, individual translocating activity by these workers could increase under DD to directly support synchronization. Therefore we further ascertained the contribution of foragers to the total translocation activity under the LD- and DD-regimes (Fig. 2A). In the two colonies that showed a sustained relocation rhythm under constant darkness, foragers increased their brood translocation activity under constant darkness (*ad libitum* feeding subcolony 1, pulse feeding subcolony 1). In both colonies, nurses also increased their relocation activity. In all other subcolonies, the proportion of relocation events by foragers was lower under constant darkness (*ad libitum* feeding subcolony 2/3, pulse feeding subcolony 1/2). Although foragers contributed to up to 50% of the relocation events in one subcolony of the *ad libitum* feeding regime (subcolony 2), the relocation rhythm under constant darkness was not synchronized. In two subcolonies, relocation activity under LD was low and even decreased under DD conditions (*ad libitum* feeding subcolony 3, pulse feeding subcolony 2), explaining the dampening of brood relocation rhythm under constant conditions in these subcolonies.

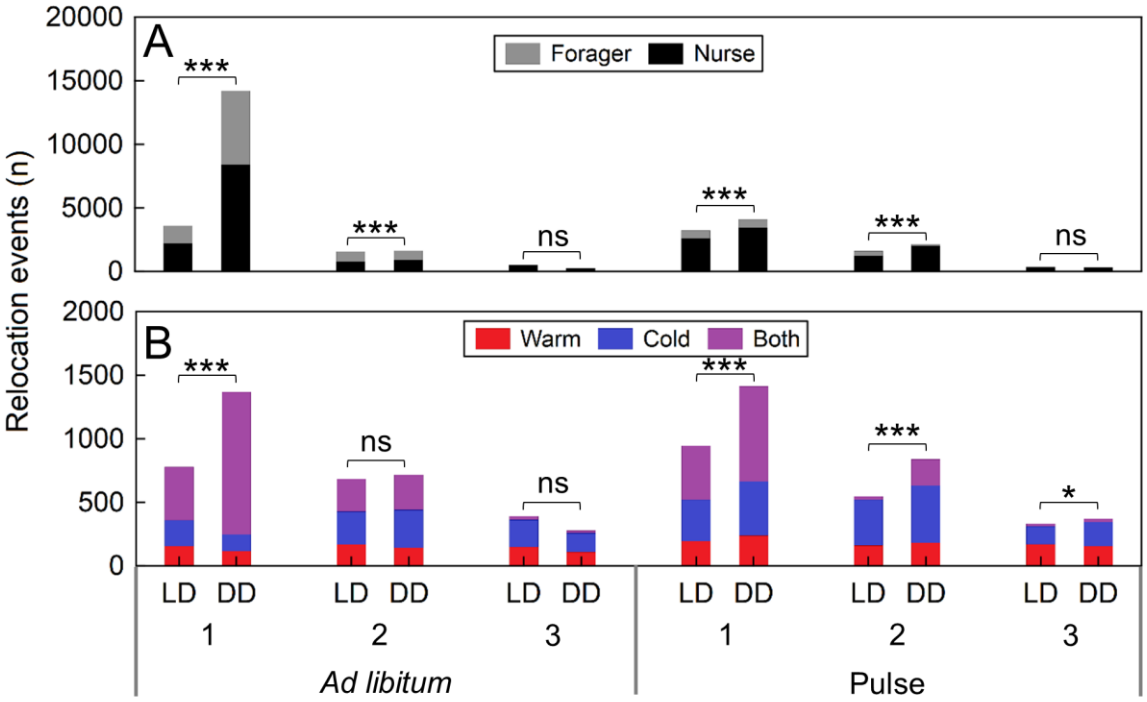

Fig. 2: Total relocation events in each subcolony. Relocation events are shown for the LD- and DD-period for the three subcolonies and the two feeding regimes. (A): Relocation events by nurses (black) and foragers (grey). (B): Number of relocations to solely the warm or the cold regimes of the temperature gradient, or contemporary to both warm and cold regimes of the temperature gradient at each time. Differences between light regimes within each subcolony was determined via χ^2 tests.

In conclusion, the majority of subcolonies exhibited altered levels of both total relocation activity and relocation activity of the castes under constant darkness (χ^2 tests; *ad libitum* feeding subcolony 1: χ^2=10.9, p=0.0009; subcolony 2: χ^2=12.5, p=0.0004; subcolony 3: χ^2=0.047, p=0.8; pulse feeding subcolony 1: χ^2=10.9, p=0.0009; subcolony 2: χ^2=238.4, p<0.0001; subcolony 3: χ^2=1.93, p=0.2).

We further determined for every time point of the observation, whether translocation occurred solely towards one temperature regime (warm or cold) or simultaneously towards both temperature regimes. Under constant darkness, levels of opposing translocation events increased in four subcolonies (Fig. 2B; χ^2 tests; *ad libitum* feeding subcolony 1: χ^2=193.6, p<0.001; pulse feeding subcolony 1: χ^2=15.2, p=0.005; pulse feeding subcolony 2: χ^2=93.5, p<0.0001; pulse feeding subcolony 3: χ^2=7.2, p=0.025), indicating a potential desynchronization between the workers under both feeding regimes. In the two subcolonies of the *ad libitum* feeding regime with general low translocation activity, relocation levels did not change across the light regimes (χ^2 tests; *ad libitum* subcolony 2: χ^2=4.8, p=0.09; *ad libitum* subcolony 3: χ^2=0.02, p=0.9)

Table 2: Number of ants that relocate brood either to the warmer values, to cooler values, or to both. The number of ants relocating primarily in one (cold or warm) or both (cold and warm) regimes of the temperature gradient is displayed for each colony and light regime. Differences between light regimes within each subcolony were assessed per χ^2 tests.

Light regime	**Temperature regime**	***Ad libitum* feeding**			**Pulse feeding**		
		Subcolony 1	Subcolony 2	Subcolony 3	Subcolony 1	Subcolony 2	Subcolony 3
LD	Warm	9	8	5	11	2	5
	Cold	6	7	8	4	5	1
	Warm & Cold	7	6	4	7	7	12
DD	Warm	6	9	7	10	1	3
	Cold	6	8	6	5	3	3
	Warm & Cold	13	5	3	12	7	8
X^2 test		X^2=2.22 p=0.3	X^2=0.19 p=0.9	X^2=0.73 p=0.7	X^2=0.97 p=0.4	X^2=0.48 p=0.8	X^2=1.83 p=0.4

We further analyzed how individual daily translocation activity contributes to the colony rhythm. In all subcolonies, we found subpopulations of workers that transferred the brood primarily to either warmer or to colder temperatures along the gradient (Table 2), suggesting that the translocation rhythm results from alternating activity of two subpopulations. Workers that transferred the brood towards both regimes were found in minor proportions. Ratios of these specialized subpopulations did not differ between

LD- and DD-conditions (χ^2 tests; *ad libitum* feeding subcolony 1: χ^2=2.22, p=0.3; subcolony 2: χ^2=0.19, p=0.9; subcolony 3: χ^2=0.73, p=0.7; pulse feeding subcolony 1: χ^2=0.97, p=0.4; subcolony 2: χ^2=0.48, p=0.8; subcolony 3: χ^2=1.83, p=0.4).

Colony level translocation activity was displayed in form of actograms (Fig. 3). Even under LD-conditions, relocation to warmer values was not strictly limited to the day phase (Fig. 3, left), and relocation to cooler values was not strictly limited to the night phase (Fig. 3, middle), leading to phases of opposing translocation movements at the subcolony level (Fig. 3, right). Subcolonies with broader ranges of temperature preferences (*ad libitum* feeding subcolony 1, pulse feeding subcolony 1) exhibited higher levels in translocation activity, as individuals had to cover longer distances within the temperature gradient. Under constant darkness, translocation activity started to free-run in subcolonies of both feeding regimes.

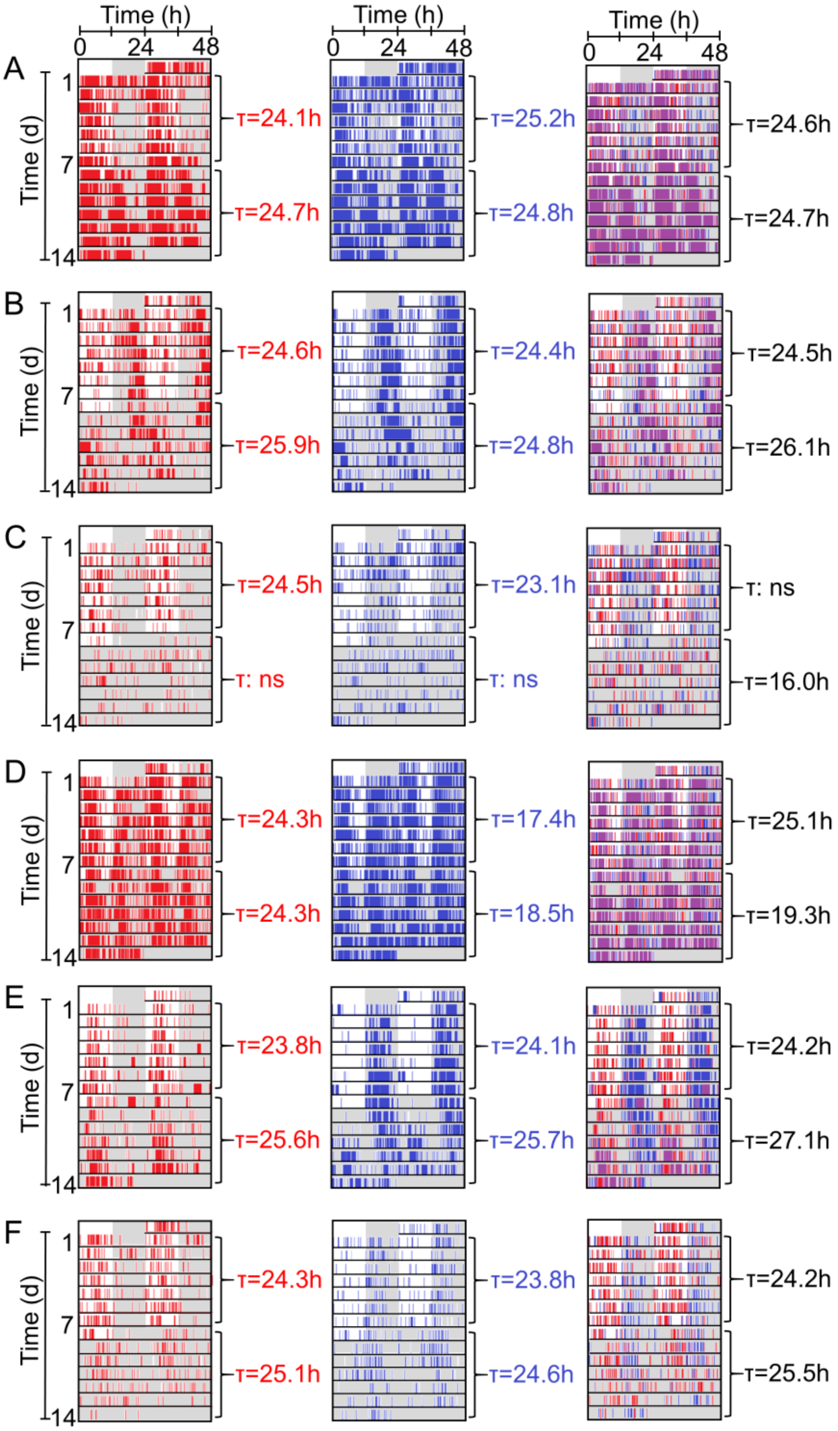

Fig. 3: Actograms of brood relocation activity on colony level. Actograms show brood relocation to the warm (left, red bars) and cold (middle, blue bars) area of the temperature gradient separately, as well as pooled relocation activity (right; red: translocations to warm only, blue: translocations to cold only, purple: simultaneous translocation to warm and cold). Relocation activity is shown for the LD-cycle (day 1-7) and constant darkness (day 8-15) as double-plot. Dark phases are highlighted in grey. Period values were calculated for each light regime. (A): Actograms of relocation activity in *ad libitum* feeding subcolony 1. (B): Actograms of relocation activity in *ad libitum* feeding subcolony 2. (C): Actograms of relocation activity in *ad libitum* feeding subcolony 3. (D): Actograms of relocation activity in pulse feeding subcolony 1. (E): Actograms of relocation activity in pulse feeding subcolony 2. (F): Actograms of relocation activity in pulse feeding subcolony 3.

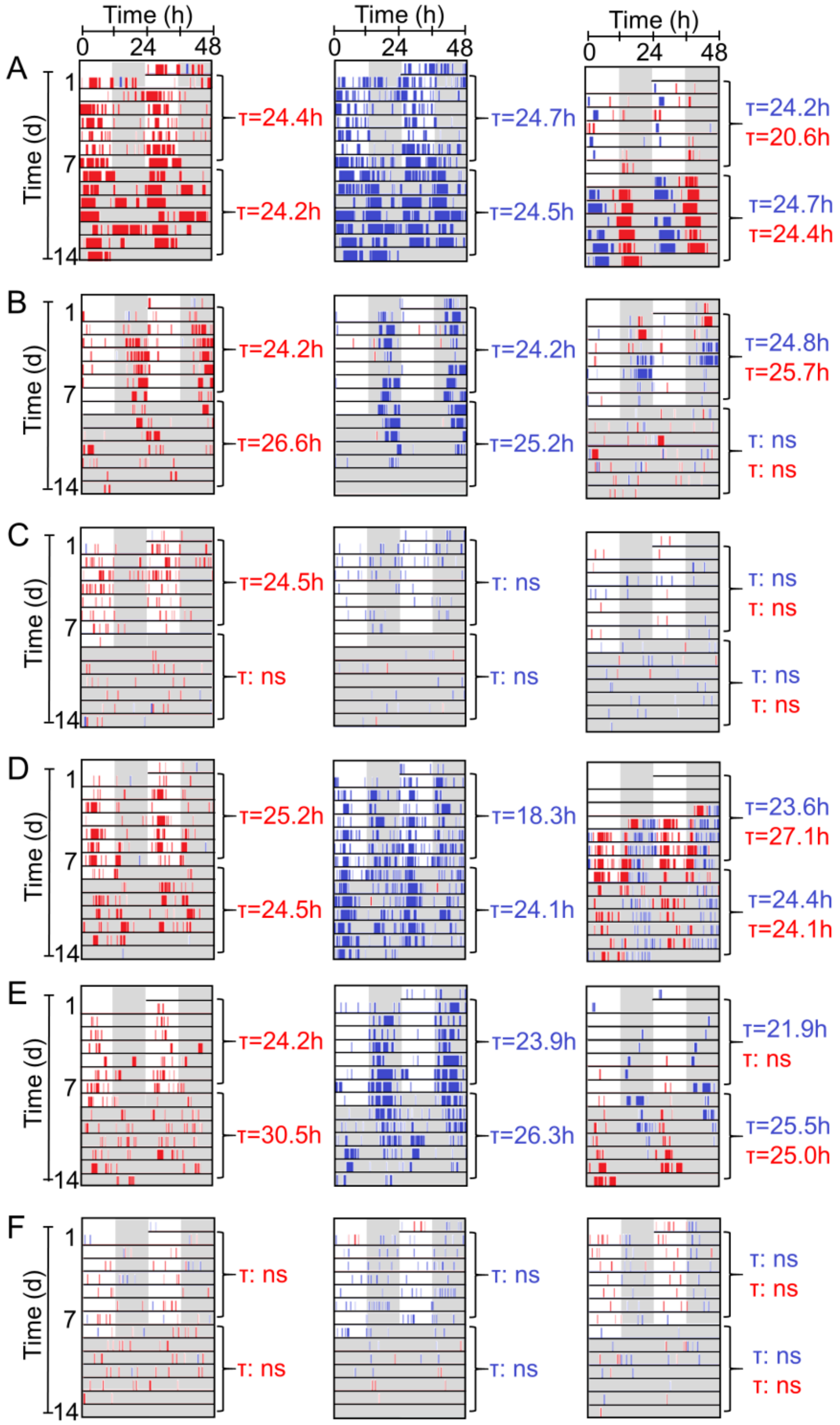

Fig. 4: Exemplary actograms of brood relocation activity by individuals. Actograms of individuals relocating predominantly to the warm (left), cold (middle) and both directions (right) of the temperature gradient. Relocation activity (red: relocation to warm; blue: relocation to cold) is shown for the LD-cycle (day 1-7) and constant darkness (day 8-15) as double-plot. Dark phases are highlighted in grey. Period values were calculated for each light regime. (A): Three representative actograms of individuals from *ad libitum* feeding subcolony 1. (B): Three representative actograms of individuals from *ad libitum* feeding subcolony 2. (C): Three representative actograms of individuals from *ad libitum* feeding subcolony 3. (D): Three representative actograms of individuals from pulse feeding subcolony 1. (E): Three representative actograms of individuals from pulse feeding subcolony 2. (F): Three representative actograms of individuals from pulse feeding subcolony 3.

Individual translocation activity was again displayed in form of actograms to determine how individual rhythms contribute to the rhythm seen on colony level (Fig. 4). Workers that relocated the brood predominantly only to the warm, cold or both directions were found to display rhythmic translocation activity under LD and DD conditions. Activity levels did not only differ between individuals (cf. Fig. 4C/4D), but also in the same individuals between the light regimes (e.g. Fig. 4A, right).

Table 3: Proportion of rhythmic individuals per subcolony and light regime. Differences between light regimes within each subcolony were determined via χ^2 tests.

Feeding regime	Subcolony	LD	DD	X^2 test
Ad libitum	1	61.5 (n=16)	44.4 (n=8)	X^2=1.2. p=0.2
	2	52.3 (n=11)	22.7 (n=5)	X^2=2.5, p=0.1
	3	25.0 (n=4)	6.2 (n=1)	X^2=3.4, p=0.06
Pulse	1	53.8 (n=14)	44.4 (n=12)	X^2=0.3, p=0.6
	2	85.7 (n=12)	45.4 (n=5)	X^2=4.6, p=0.03
	3	66.7 (n=10)	7.7 (n=1)	X^2=11.6, p=0.006

As some individuals displayed only low levels of translocation activity, or did not engage continuously in brood translocation over the two weeks of observation, rhythmic activity patterns were not detected in all workers (Table 3). Even under the LD-cycle, a high proportion of workers displayed arrhythmic translocation behavior (Table 3). All subcolonies showed the trend of increasing arrhythmicity under constant darkness (χ^2 tests; *ad libitum* feeding subcolony 1: χ^2=1.2, p=0.2; subcolony 2: χ^2=2.5, p=0.1; subcolony 3: χ^2=3.4, p=0.06; pulse feeding subcolony 1: χ^2=0.3, p=0.6; subcolony 2: χ^2=4.6, p=0.03; subcolony 3: χ^2=11.6, p=0.006), indicating a desynchronization of translocation activity independent of the feeding regime. Based on the dynamic of contradicting translocation activities, the brood pile was translocated nevertheless to different temperature regimes at different times of the LD-cycle.

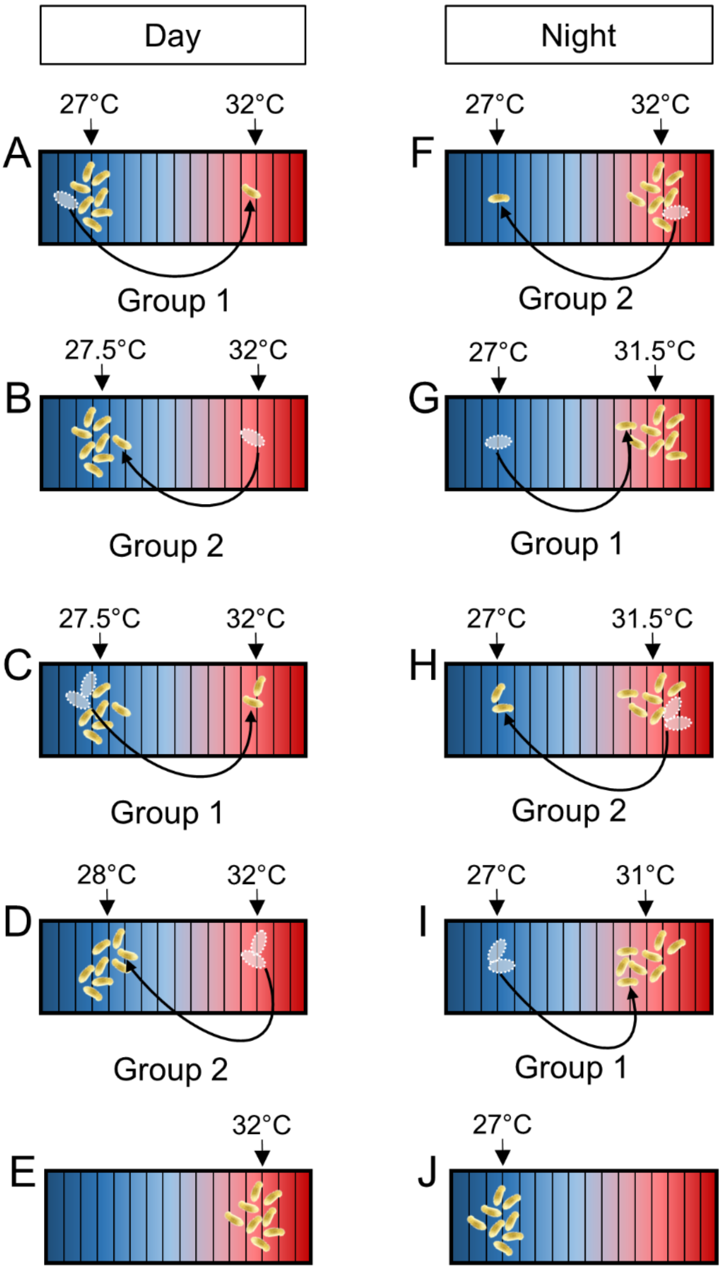

Fig. 5: Mechanism of the semi-daily brood translocation shift. During daytime, some ants (group 1) get spontaneously active and transfer brood items from the coldest part of the brood pile to the warmer part of the gradient (A). Subsequently, other ants (group 2) transfer these brood items back, but deposit them mainly at the warmest part of the brood pile (B). The repeated sequence of this opposing relocation movements leads to a gradual shift of the center of the brood pile to slightly warmer values at the colder part of the temperature gradient (C/D). In the end of this process, the brood pile is shifted completely to the warmer part of the gradient at a certain time of the day phase (E). During nighttime, the same ants from group 2 than again start relocating the brood from the warmest part of the brood pile to the colder part of the temperature gradient (F). Thereafter, ants from group 1 carry the brood back to the coldest part of the brood pile (G). This time, the repeated sequence of this opposing relocation movements leads to a gradual shift of the center of the brood pile to slightly cooler values at the warmer part of the temperature gradient (H/I). In the end of this process, the brood pile is shifted completely to the colder part of the temperature gradient at a certain time of the night phase.

The general mechanism of this process is described in Fig. 5. During the day phase, translocation of the brood pile to the warmer part of the gradient was initiated by individuals picking up brood items from the coldest area of the brood pile and transferring them to locations with temperatures of several degrees higher (Fig. 5A). Subsequently, other individuals relocated the same brood items again back to the brood pile, but deposited them mainly at the warmer region of the brood pile (Fig. 5B). At that, brood is deposited at the part of the brood pile with the shortest distance to the replaced brood items. The repeated sequence of this opposing relocation movements led to the observed gradual shift of the brood pile to the warmer part of the temperature gradient (Fig. 5C/D). The brood pile remains then at the warmer part of the temperature gradient for several hours (Fig. 5E). The same dynamic could be observed for translocation of the brood pile from the warmer to the colder part of the temperature gradient during the dark phase (Fig. 5F-J). Under constant darkness, the dynamic of the two subpopulations was unbalanced and brood items were relocated almost constantly back and forth along the temperature gradient.

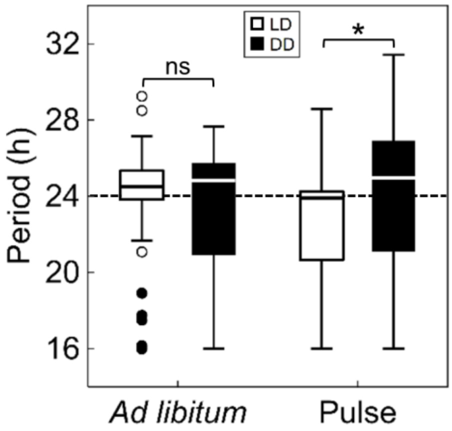

Fig. 6: Period values of translocation rhythms under both feeding regimes. Boxplots show medians (center lines) and interquartile ranges (boxes) for LD- (white) and DD-conditions (black). Whiskers show minimum and maximum values**.** Asterisks indicate significant differences between LD- and DD-conditions (Wilcoxon signed rank tests). *Ad libitum* feeding: n_{LD}=53, n_{DD}=62; pulse feeding: n_{LD}=55, n_{DD}=59.

Period values of individual translocation activity did not differ between subcolonies, so data sets were pooled for the two feeding regimes and the two lighting conditions (Fig. 6; Kruskal-Wallis tests; *ad libitum* feeding: warm translocators under LD: H(2,26)=0.7, p=0.7; warm translocators under DD: H(2,32)=6.7, p=0.05; cold translocators under LD: H(2,27)=1.4, p=0.5; cold translocators under DD: H(2,30)=0.0, p=1.0; pulse feeding: warm translocators under LD: H(2,31)=4.4, p=0.1; warm translocators under DD: H(2,31)=5.3, p=0.07; cold translocators under LD: H(2,26)=2.5, p=0.3; cold translocators under DD: H(2,26)=0.6, p=0.7) or cold and warm translocators (Mann-Whitney U-tests; *ad libitum* feeding: LD: U(26,27)=287.5, z=-1.1, p=0.3; DD: U(32,30)=414.5, z=0.9, p=0.3; pulse feeding: LD: U(31,24)=685.5, z=-0.22, p=0.8; DD: U(31,28)=786.5, z=0.8, p=0.4). Under LD-conditions, period values of individual translocation activity under both feeding regimes were close to 24 hours (*ad libitum* feeding: τ=24.5±1.5h, pulse feeding: τ=23.9±3.6h; median±IQR), but were widely distributed. Under DD-conditions, period values deviated more from 24 hours (*ad libitum* feeding: τ=24.8±4.8h, pulse feeding: τ =24.9±5.8h; median±IQR), but differed only significantly to LD-values under pulse feeding (Wilcoxon signed rank tests; *ad libitum* feeding: T(34)=297.0, z=0.008, p=0.9; pulse feeding: T(36)=183.0, z=2.3, p=0.02).

Discussion

Although activity rhythms of workers outside ant nests were well studied, daily activity rhythms within the ant nest are barely documented. One rare exception is the brood translocation rhythm in the ant species *C. mus*. In contrast to the selection of a constant temperature for the brood as in other ant species (Cokendolpher & Francke, 1984; Porter & Tschinkel, 1993), workers of *C. mus* select two different temperatures for brood location throughout the day in the laboratory (Roces & Núñez, 1989). The daily translocation of brood between different temperature values optimizes brain development (Falibene et al., 2016) and is suggested to be an adaptation to the naturally occurring daily temperature cycles in *C. mus* nests (see chapter V). The timing of these translocations seems therefore to be of special importance for optimal brood rearing. Previous studies showed that *C. mus* colonies exhibit daily rhythms in temperature thresholds even under constant darkness (Roces & Núñez, 1995), which suggests the involvement of an endogenous clock. But as rhythms in brood translocation and temperature thresholds were previously documented only on group level, the contribution of individual activity to this colony rhythm remained unexplored.

We tracked individual translocation activity in all workers of six subcolonies under a light-dark-cycle to describe the mechanism under confirmed synchronized conditions. Behavioral activities were continuously quantified over 7 days (~1000 hours) with the aid of detailed and enormously time-consuming manual tracking in long-term video recordings. Like previously reported (Roces & Núñez, 1989), brood was located at higher temperatures during the day time and at lower temperatures during nighttimes.

Maximal and minimal temperatures of brood location deviated from previous records and differed between subcolonies, probably as a result from differences in temperature preferences in workers engaging in brood translocation. Our frequent monitoring of the position of the brood pile via long-term video recordings revealed that the brood is shifted rather gradually over hours between the high and low temperatures. In contrast, previous studies described a step-like relocation of the brood twice a day (Roces & Núñez, 1989). This difference might be ascribed to the lower temporal resolution of the observations by camera snapshots in the earlier studies.

Most, but not all nurses engaged in translocating of the brood to the different temperature values. In low numbers, foragers engaged occasionally in relocation of the brood as well. This involvement in brood care in foragers might be an artefact of the small size of the subcolonies and might not occur in field colonies. However, the high proportion of inactive individuals indicates that group size of the subcolonies would have been sufficient for a more strict division of labor.

Based on the daily rhythms of temperature thresholds in *C. mus* colonies, we initially hypothesized that the same individuals translocate the brood to warmer temperatures during daytimes and to colder temperatures during nighttimes. Against our hypothesis, most individuals did not translocate the brood to both the warm and cold temperatures, but engaged preferentially in only one of these two events. As individuals of the two groups got spontaneously active at a certain time of the day, a daily rhythm in temperature thresholds could nevertheless trigger this behavior. As we tracked translocation activity of all individuals simultaneously, we could only record general direction of the translocation. Thus, individuals of the “warm” and “cold” translocation group could nonetheless show daily cycles of temperature preferences throughout the day.

Under the LD-cycle, only an average of 60% of the individuals displayed rhythmic translocation events. As a consequence, period values were close to 24 hours. Since some individuals displayed only inconsistent or low translocation activity throughout the seven days of the LD-regime, rhythmicity might not be detected in these ants and might therefore remain underestimated. However, the detection of rhythmic translocation activity represents the first record of behavioral rhythmicity of workers within an ant nest. Although nurses in *Camponotus* ants possess an endogenous clock, other nursing activities like feeding are performed arrhythmically (see chapter II).

All around the clock activity in this category could support uniform nourishment levels across all larvae (Cassil & Tschinkel, 1995) and therefore promote quantitative larval development. Rhythmic translocation activity favors brain development (Falibene et al., 2016), thus promoting in turn proper larval development.

Even in rhythmic individuals, translocations towards the warmer part of the gradient occurred not only during the daytime, and translocations to the colder part of the gradient occurred not only during the nighttime. When examining total translocation activity on subcolony level, we found surprisingly high levels of opposing brood translocation events. Almost every time when brood was transferred by one group to locations of temperatures several degrees higher or lower, another group carried the brood back closer to the brood pile. Dependent on the effort and number of ants translocating in a certain direction, the brood pile was nevertheless shifted gradually to the warmer or colder part of the gradient during certain times of the day. Workers could differ in their absolute thermal preferences (Grodzicki & Caputa, 2004; Weidenmüller et al., 2009; Oldroyd, 2009) and strength of their endogenous rhythms. Based on this inter-individual variability, individuals with preferences for lower temperatures at a certain time of the day are expected to start translocating the brood to a different temperature, whereas individuals with higher temperatures are expected to carry the brood back closer to the initial temperature. This mechanism seems uneconomic, but may prevent a relocation of the brood to too extreme temperatures and could lead to a more homogenous rhythm of brood location. Furthermore, the gradual change of temperature rather resembles the natural daily changes of temperature within the ant nest (see chapter V). As brood rearing is expected to be adapted to those environmental temperature cycles, this steady translocation might favor brood development.

Our experiments show that the daily pattern of brood location on the subcolony level is based on the alternating rhythmic activity of different subpopulations of individuals. So how could such rhythms be synchronized in the absence of the light-dark-cycle within the dark nest? One promising candidate zeitgeber for the synchronization of in-nest activity is via social interaction. Returning *Leptothorax* foragers trigger activity bouts in nest workers (Boi et al., 1999). In the strictly diurnal ant species *C. mus*, foragers leave the nest after sunrise and return frequently with food until sunset (see chapter V). Returning foragers could therefore indirectly signal daytime, whereas the presence of inactive foragers within the nest after sunset could signal nighttime. In this chapter we used temporally restricted feeding during the day phase to stimulate diurnal activity in foragers and tested if the resulting temporally restricted interaction between interior and exterior workers is sufficient to synchronize behavioral rhythms within the dark nest. As two out of three subcolonies under pulse feeding showed weaker rhythms and desynchronized translocation activity by nest workers like in control subcolonies under *ad libitum* feeding, social interaction did not act as an indirect zeitgeber for the relocation rhythm. Since nurse workers did not leave the nest to sample the light conditions, their activity was desynchronized. Although only few individuals exhibited rhythmic, but free-running translocation activity under constant darkness, this suggest the involvement of a circadian clock. Under the absence of zeitgebers, activity rhythms start free-running due to the individual endogenous period. As many individuals constantly reacted to the subjective “wrong” translocations of other individuals, their endogenous rhythm seems to be masked and therefore only evident in some individuals. To determine truly endogenous translocation rhythms, individuals should be studied in isolation.

Potentially due to strong differences in individual activity periods, contradictory translocations between the individuals increased under constant conditions. As a result, we did not see the previously reported free-running rhythm of brood location under constant darkness on colony level (Roces, 1995). Yet under both feeding regimes, only one subcolony displayed a sustainable rhythm of brood location under constant darkness. In these colonies, foragers engaged frequently in brood translocation and therefore affected the in-nest activity directly. In contrast to natural *C. mus* colonies, foragers left the nest occasionally during the night phase as well. Foraging activity in field colonies is strongly temporally restricted by environmental factors and biotic interactions (see chapter V), so interaction with returning foragers might therefore be a more solid temporal cue in the field.

The question arises whether such an indirect transfer of temporal information into the nest is really needed in field colonies. As *C. mus* nests are built in rotten wood and in empty spaces within stone walls, they are exposed to daily cycles in temperature, and brood development is expected to be adapted to this naturally occurring rhythm. When laboratory colonies are exposed to a daily temperature cycle as workers search for the brood, no brood translocations occur (Roces & Núñez, 1996). Consequently, the brood relocation activity in field colonies might be reduced to situations in which the temperature cycle at a certain part of the nest deviates from the expected values. Outer parts of the nest may heat and cool down faster than deeper parts of the nest (Steiner, 1928; Coenen-Staß et al., 1980; Cole, 1994), so brood could be transferred between chambers within the nest. In such cases, the environmental temperature cycle could act as reliable non-photic zeitgeber for the synchronization of activity within the dark nest. Field colonies of the sympatric ant species *C. rufipes* exhibit daily cycles in core temperature of up to 10°C amplitude (Weidenmüller et al., 2009) and temperature cycles of 1°C amplitude are already sufficient to synchronize locomotor rhythms in wood ants (Rosengren, 1977). Being entrained by the environmental temperature cycles, nurse ants are able to anticipate maximum and minimum temperature values and relocate the brood accordingly.

In this chapter we provided first insights in how individual translocation activity contributes to the observed colony rhythm. The rhythmic, alternating activity of worker groups translocating preferentially to warm or cold temperature regimes is integrated to a rhythmic pattern of brood location on colony level under a LD-cycle. This special form of task allocation may result from general differences in temperature preferences, as well as differences in circadian variation of thresholds between individuals. Task partitioning between translocating individuals could increase colony efficiency and contribute to a balanced pattern of brood location. Evaluation of daily variation in thermal preferences and thresholds of isolated individuals would be needed prior to observations of translocation activity in the group to fully understand the integration of individual endogenous rhythms into a colony rhythm. Under the absence of light, social interaction was not a sufficient zeitgeber for synchronization of the translocation rhythm. Naturally occurring cycles in temperature or humidity are expected to act more likely as zeitgeber for in-nest activities.

Chapter V

Temporal specialization of foragers from two sympatric *Camponotus* ant species in the field

Abstract

Foraging rhythms in ants are affected by several abiotic factors as well as by interactions between species. We studied daily foraging activity in field colonies of two sympatric *Camponotus* species as correlated to ambient temperature, humidity and light intensity levels. Foragers in *C. mus* nests were exclusively active during the day and did not show any activity below 15°C. In contrast, foraging activity in *C. rufipes* colonies was predominantly nocturnal. As both *Camponotus* species had overlapping dietary niches, temporal segregation may lead to reduced competition between the species. Both species had similar critical thermal limits in the laboratory, but were active under different thermal ranges in the field. Foraging activity in *C. mus* might be rather modulated by competition with the more dominant ant *C. rufipes*.

As we found elevated levels of diurnal foraging in some *C. rufipes* colonies, we tracked individually marked ants to study potential temporal specialization between foragers within the same colony. Almost 70% of foragers were re-caught in the same day phase as they were marked, which suggests that these workers showed temporal fidelity and did not switch between night and day activity. Subpopulations of day- and night caught foragers did not differ consistently in their body size, but could be specialized for nocturnal or diurnal foraging in other morphologic factors. As nocturnal and diurnal foraging might require different ways of orientation, workers could rather differ on a sensory level.

Our study reveals temporal specialization of foragers in co-occurring *Camponotus* species, as well among foragers within nests of one *Camponotus* species. The combined results of field and laboratory experiments suggest that temporal niches are shaped by environmental factors and interspecific competition.

Introduction

Many *Camponotus* ants show characteristic foraging activity rhythms, which can be documented both on a daily and a seasonal basis (Briese & Macauley, 1980; Cros et al., 1997; Cerdá et al., 1998; Santini et al., 2007). In general, foraging activity in ants is influenced by several biotic and abiotic factors, leading to the establishment of species-specific temporal niches. Activity in ectothermic ants is limited to the certain times of the day when environmental conditions are physiologically tolerable (Marsh, 1988; Cerda et al., 1998). Besides humidity (Whitford & Etthershank, 1975; Azcárate et al., 2007) and light intensity (Christian & Morton, 1992), temperature is the predominant abiotic factor constraining activity periods (Porter & Tschinkel, 1987; Drees et al., 2007; Ashikin & Hashim, 2015). Temperature directly influences walking speed (Shapley, 1920; Barnes & Kohn, 1932), respiration (Jensen & Nielsen, 1975) and desiccation (Lighton & Feener, 1989), so foraging activity is limited to a specific temperature range. Thermal tolerance varies between species (Cerda et al., 1998; Bestelmeyer, 2000) and can consequently lead to different temporal niches of coexisting ant species. In case of overlapping dietary niches and similar thermal tolerances, species abundance and dominance play a major role in temporal separation (Jayatilaka et al., 2001; Blüthgen & Fiedler, 2004; Huoadria et al., 2014; Barbieri et al., 2014; Anjos et al., 2016).

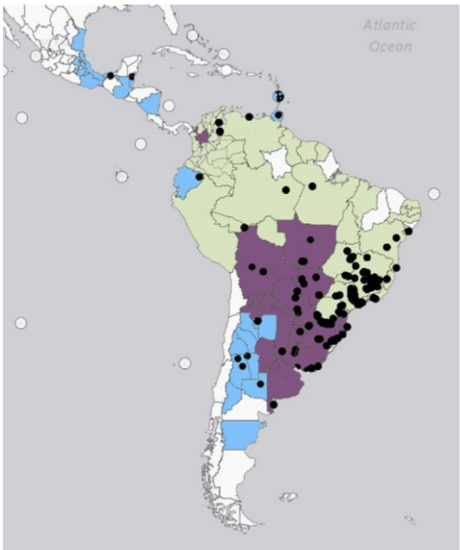

Fig. 1: Species distribution map. Distribution of *C. mus* (blue) and *C. rufipes* (green), as well as areas of co-distribution of both species (magenta) in South America (modified after antweb.org). Black dots refer to geographic coordinates of species collection. Remaining distribution is based in literature records.

We studied activity patterns of two sympatric nectar-collecting ants of the species *C. mus* and *C. rufipes*, which can be found widely distributed over broad ranges of South America (Fig. 1). Ants of the species *C. rufipes* forage at extrafloral nectaries or collect honeydew produced by treehoppers and aphids (Del-Claro & Oliviera, 1999; Fagundes et al., 2005). Although laboratory studies observed predominantly nocturnal foraging activity (Jaffé & Sánchez, 1984; own data, chapter I & II), trap experiments indicated nocturnal and diurnal activity of this species in the field (Del-Claro & Oliviera, 2000). The flexible switch of activity period might be correlated to food availability or environmental factors, but can also result from interspecies interactions within an ant community. Thus, daily activity patterns need to be studied under consideration of both abiotic and biotic factors. We studied the effect of temperature, humidity and light intensity on foraging activity of both *Camponotus* species. Critical thermal limits of worker activity were determined in the laboratory to discuss temperature range of foraging activity in the field and to reveal physiological constraints of foraging activity. We compared body sizes of diurnal and nocturnal foragers of *C. rufipes* to investigate whether temporal specialization is linked to morphological adaptation of worker subpopulations.

Material and Methods

Field observations of foraging activity of *C. mus* and *C. rufipes* colonies were done during spring (November - December 2015) in La Coronilla, Uruguay (33°53'25.2"S, 53°31'27.6"W). Nests of the observed ant species can occur in close proximity and a single colony can consist of several satellite nests. Therefore we tested every selected nest for its independency by marking and observing individuals, as well as by translocating ants between adjacent nests. Daily foraging activity of independent nests was determined by counting incoming and outgoing ants every half an hour during five minutes, for 24 hours. During the night, measurements were done under red light with low intensity. Simultaneously, we determined air temperature and humidity (EL-USB-2-LCD+, Lascar Electronics) as well as light intensity (light meter LM-100, Amprobe) every half an hour at the nest entrance. We evaluated the effect of each environmental factor on foraging activity separated for species, as well as correlation between the abiotic factors via linear regression and Spearman´s correlation.

Foraging activity in C. mus

We observed foraging activity of three *C. mus* colonies nesting several meters apart inside the same stone wall, whereby the height of nest entrances differed between colonies (colony 1: 10 cm, colony 2: 155 cm, and colony 3: 20 cm above the ground). Counts of incoming and outgoing ants were determined on a virtual circle around the single nest entrance of each colony (diameter: 5 cm).

Foraging activity in C. rufipes

C. rufipes ants build thatch nests in open grasslands and ants forage for nectar and insect prey in the surroundings bushes, using several foraging paths leaving the nest (Fig. 2A). As foraging activity can hardly be observed due to the vegetation, ant nests needed to be prepared for observation. Two days before the experiment, vegetation in 30 cm distance around the nest was removed and a plastic railing covered with paraffin oil was built around the nest to prevent ants from leaving the nest in all directions (Fig. 2B). Two to three cardboard bridges were installed in the main foraging pathways of the colony but were reduced to one bridge on the next day to channel foraging activity of the whole colony. After another day of familiarization with the experimental setup, foraging activity was measured as described above in the center of the cardboard bridge (Fig. 2C), for six independent colonies.

Fig. 2: Experimental arrangement for monitoring foraging activity in *C. rufipes*. Nests are usually built in open grasslands and are covered by vegetation (A), thus foraging activity is difficult to monitor. To facilitate observations, we removed vegetation in a 30 cm circle around the nest and installed a plastic barrier (B). Two to three cardboard bridges were installed in the main foraging trails of the colony, yet only one bridge was made available on the next day to channel all foraging activity of the colony (C). After a familiarization period of another day, foraging activity was recorded as the number of workers crossing a virtual line in the center of the cardboard bridge (indicated above with the red line). Photos: S. Mildner.

To describe the daily foraging process more closely, we additionally marked returning foragers of two nests with individual color codes (Edding) every hour for 24 hours. In the night phase, workers were marked under red light. At every time point, we determined if the individual forager was active during day- or nighttime. After one day, again returning marked foragers were collected every hour for 24 hours. We calculated the proportion of foragers being active in the same day phase at their recapture (day-day/night-night) or at different phases of the day (day-night/night-day) and tested for differences between nests (χ^2 test). To evaluate, if nocturnal and diurnal foragers differ morphologically, we collected foragers returning to two nest during the day (4 pm) and the night (4 am). The thorax length of every forager was determined as a measure of body size and compared between diurnal and nocturnal foragers (Mann-Whitney U-tests).

Critical temperature limits in C. rufipes and C. mus

We analyzed critical temperature limits of workers from both *Camponotus* species in the laboratory to test if species vary in their temperature tolerances. Water baths were used to regulate floor temperatures of small plastic containers (height: 5 cm, diameter: 3 cm), in which we placed single individuals (n=32) of four *C. mus* colonies (named 1, 18, 19, and 23) and three *C. rufipes* colonies (named H, R and Q; n=35). Colonies of both species were raised in climate chambers under a constant temperature of 25°C, therefore 25°C was set as initial temperature of the containers. After an acclimatization period of 10 min, we either increased the floor temperature by 1 °C every 10 mins to determine maximal critical temperature limits (CT_{max}), or decreased the temperature by 1°C every 10 mins to determine minimal critical temperature limits (CT_{min}) for every individual. At every temperature step, we tested the movement abilities of the ants by tapping them with forceps. A temperature value was considered as critical, if ants could not move anymore or could not return to their upright position. Critical maximal and minimal temperatures were compared between the species afterwards in relation to the ants´ body mass (ANCOVA, GraphPadPrism Version 5.00), which was determined prior to the experiments as a measure of the body size of the ants (Josens, 2002). Effect of the ants´ mass on critical temperature limits was determined via Spearman´s correlation. As the mass had no influence on the upper thermal limit, values were compared between species via an unpaired t-test.

Results

C. mus foragers were predominantly active during daytimes (Fig. 3). Foraging activity positively correlated with air temperature (linear regression: y=-26.4+1.92x, p<0.001, R^2=0.7; Spearman´s correlation: r_s=0.92, p<0.002; Fig. 4A, left) and light intensity (linear regression: y=9.4+0.0003x, p<0.0001, R^2=0.2; Spearman´s correlation: r_s=0.68, p<0.0001; Fig. 4A, middle), but negatively with air humidity (linear regression: y=76.3-0.86x, p<0.0001, R^2=0.7; Spearman´s correlation: r_s=-0.82, p<0.0001; Fig. 4A, right).

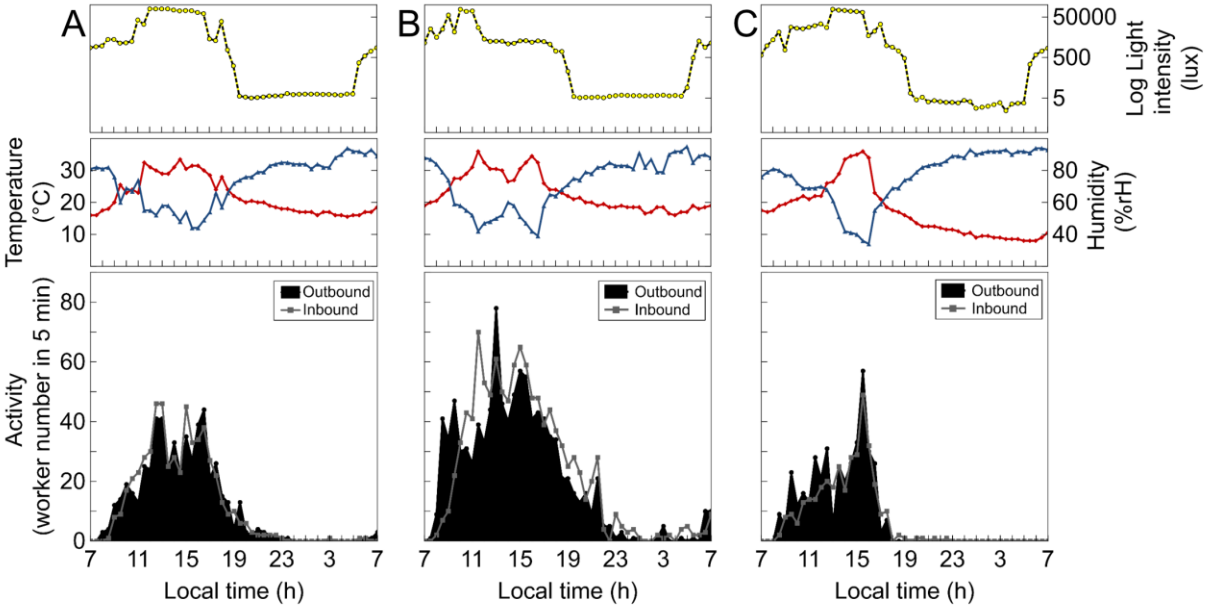

Fig. 3: Daily foraging activity in *C. mus* nests. Number of outbound (black) and inbound (grey) foragers was counted every half an hour for five minutes, in three different nests (A-C). Ambient temperature (red), humidity (blue) and light intensity (yellow) was recorded simultaneously at the nest entrance. Workers from all three nests displayed diurnal activity.

In *C. rufipes*, two different activity profiles were observed. Foragers in three nests were predominantly night active (Fig. 5A-C), whereas other three nests showed higher foraging activity during daytimes (Fig. 5D-F). Therefore we calculated effects of environmental factors separately for each three nests. In the predominantly night active nests, foraging activity was negatively correlated with temperature (linear regression: y=69.9-1.5x, p=0.0006, R^2=0.1; Spearman´s correlation: r_s=-0.31, p<0.0001; Fig. 4B, left) and light intensity (linear regression: y=49.7-0.0004x, p<0.0001, R^2=0.2; Spearman´s correlation: r_s=-0.58, p<0.0001; Fig. 4B, middle), but positively correlated with air humidity (linear regression: y=-16.2+0.7x, p<0.0001, R^2=0.1; Spearman´s correlation: r_s=0.24, p=0.003; Fig. 4B, right). In the nests with both nocturnal and diurnal activity an overall weaker correlation of foraging activity with the environmental factors was observed. Activity was negatively correlated with temperature (linear regression: y=153.9-2.4x, $p<0.0001$, $R^2=0.1$; Spearman´s correlation: $r_s=-0.29$, $p=0.003$; Fig. 4C, left) and light intensity (linear regression: y=114.9-0.004x, $p<0.0001$, $R^2=0.2$; Spearman´s correlation: $r_s=-0.49$, $p<0.0001$; Fig. 4C, middle). We could not find any significant correlation with air humidity (linear regression: y=63.6+0.56x, $p=0.01$, $R^2=0.04$; Spearman´s correlation: $r_s=0.15$, $p=0.06$; Fig. 4C, right).

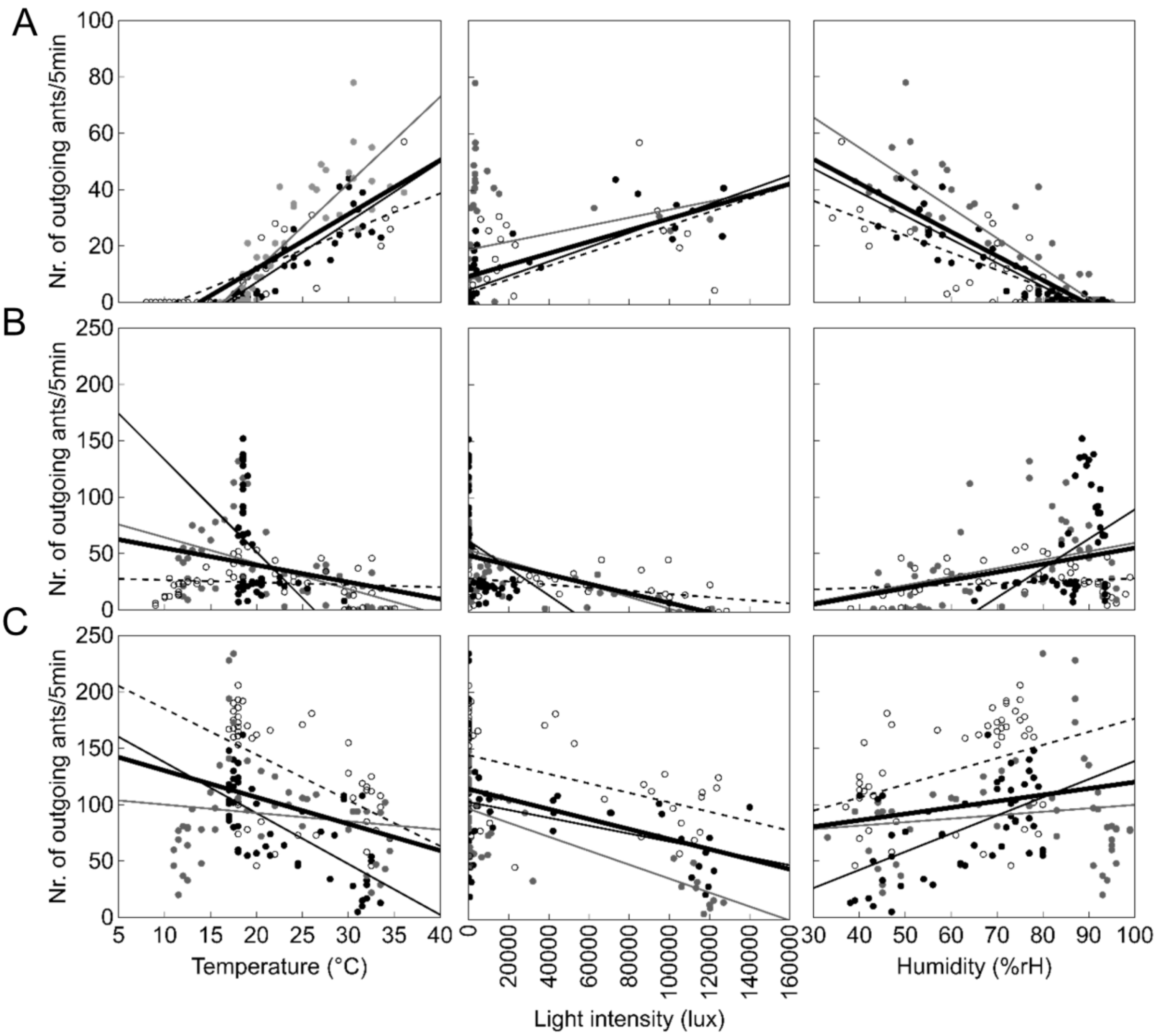

Fig. 4: Foraging activity in correlation to environmental factors. Number of outgoing ants in correlation to ambient temperature (left), light intensity (middle) and humidity (right). (A): Correlations for *C. mus*. (B): Correlations for nocturnal *C. rufipes* nests. (C): Correlations for “diurnal” *C. rufipes* nests. Correlations are based on data sets of three nests (nest 1: black, nest 2: grey, nest 3: white circles). Regression lines are shown for pooled nests (thick black line) and for each nest separately (nest 1: solid black, nest 2: solid grey, nest 3: dotted black).

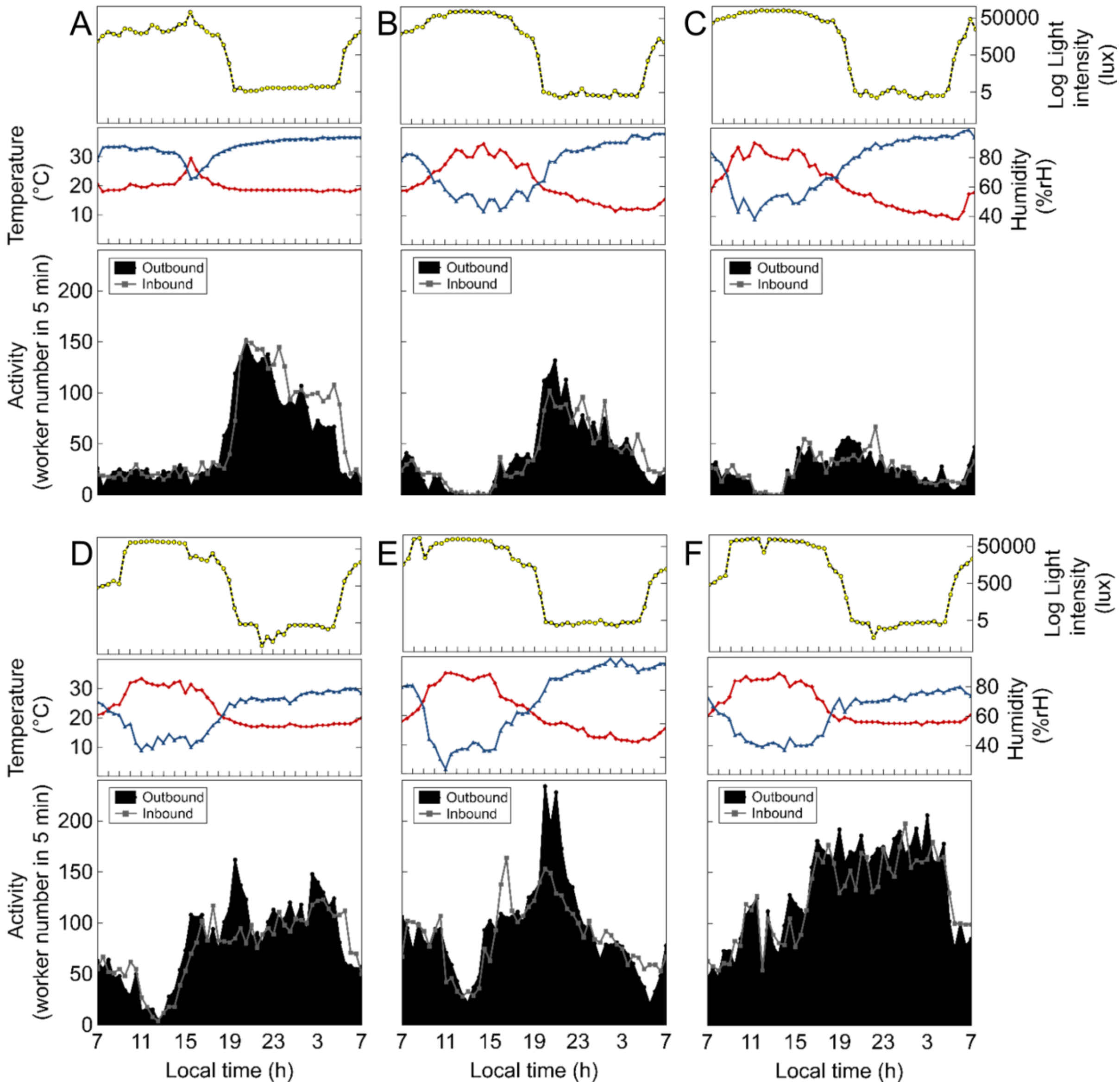

Fig. 5: Daily foraging activity in *C. rufipes* nests. Number of outbound (black) and inbound (grey) foragers was counted every half an hour for five minutes, in six different nests (A-F). Ambient temperature (red), humidity (blue) and light intensity (yellow) was recorded simultaneously at the nest entrance. Three nests exhibited predominantly nocturnal activity (A-C), whereas other three nests displayed increased diurnal activity levels.

Moreover, the measured environmental factors were interdependent. Light intensity correlated negatively with air humidity (linear regression: y=148517.0-1706.4x, R^2=0.5, p<0.0001; Spearman´s correlation: r_s=-0.66, p<0.0001; Fig. 6A) and positively with air temperature (linear regression: y=-72442.5+4951.1x, R^2=0.6, p<0.0001; Spearman´s correlation: r_s=0.83, p<0.0001; Fig. 6B). Air humidity was negatively correlated with air temperature (linear regression: y=121.5-2.3x, R^2=0.9, p<0.0001; Spearman´s correlation: r_s=-0.87, p<0.0001; Fig. 6C).

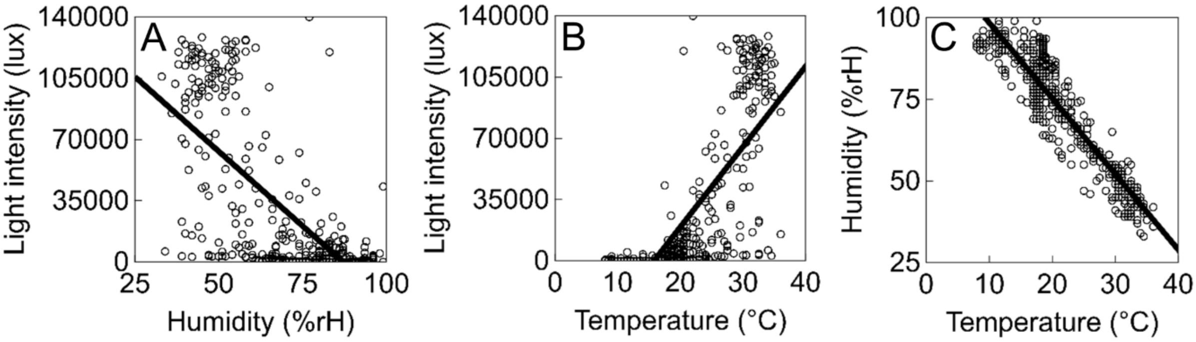

Fig. 6: Abiotic environmental factors in correlation to each other. (A): Light intensity in correlation to air humidity. (B): Air humidity in correlation to air temperature. (C): Light intensity in correlation to air humidity. Correlations (black lines) are shown for data sets pooled from all nests.

To test whether day active and night active foragers in *C. rufipes* belong to the same population or to separate, phase-shifted worker subpopulations, we marked and recaptured foragers returning to the nest. Of 480 ants out of two nests, only 18.5% were recaptured on the day after marking. As we saw no differences in foraging populations between the nests (χ^2 test: χ^2=1.1, p=0.8), data sets were pooled. With 68%, the majority of the foraging ants were caught in their former activity period (night/night: 22.2%, day/day: 45.6%; Fig. 7).

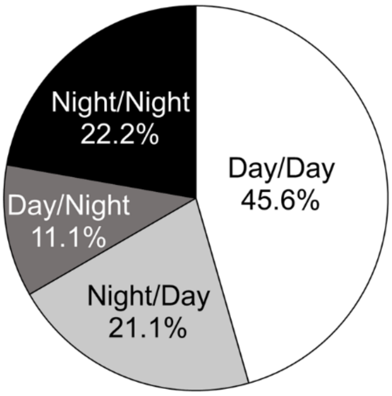

Fig. 7: Relative proportion of diurnal and nocturnal foragers. White: foragers marked and caught during the day (n=41). Black: foragers marked and caught during the night (n=20). Dark grey: foragers marked during the day and caught during the subsequent night (n=10). Light grey: foragers marked during the night and caught during the subsequent day (n=19).

Further, we compared thorax lengths between diurnal and nocturnal foragers. Although nocturnal foragers were significantly larger in one *C. rufipes* nest (diurnal: 4.3±0.7 mm, nocturnal: 4.7±0.7 mm; Mann-Whitney U-test: U(48,53)=913.5, z=-2.4, p=0.01; Fig. 8A), body sizes of foragers in the second nest did not differ between the day phases (diurnal: 4.5±0.7 mm, nocturnal: 4.4±0.6 mm; Mann-Whitney U-test: U(65,53)=1660.5, z=0.04, p=0.9; Fig. 8B).

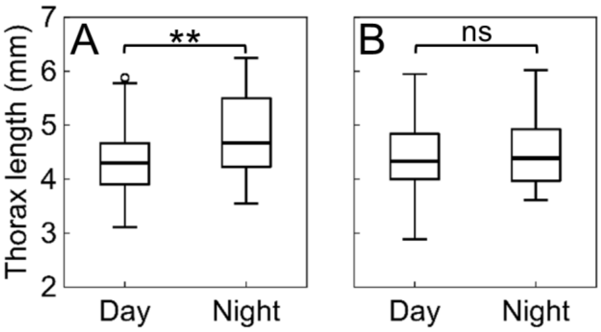

Fig. 8: Thorax lengths of *C. rufipes* foragers caught in the field. (A): Thorax lengths of diurnal and nocturnal foragers in nest 1. n_{day}=48, n_{night}=53. (B): Thorax lengths of diurnal and nocturnal foragers in nest 2. n_{day}=63, n_{night}=53. Boxplots show medians (center lines) and interquartile ranges (boxes), whiskers indicate the minimum and maximum values**.** Open circles show outliers and filled circles extreme values. Asterisks indicate significant differences between groups (Mann-Whitney U-tests; ns: p>0.05, **: p<0.01).

In the laboratory, critical maximal and minimal temperature limits were evaluated for both *Camponotus* species in relation to the worker mass (Fig. 9). Larger ants of both species were more prone to tolerate colder temperatures than smaller ants (*C. mus*: linear regression: y=8.3-0.187x, p=0.01, R^2=0.4; Spearman´s correlation: r_s=-0.75, p=0.0009; *C. rufipes*: linear regression: y=11.9-0.173x, p=0.008, R^2=0.4). In general, workers of *C. mus* exhibited lower minimal temperature limits than *C. rufipes* workers (ANCOVA; slopes of regression lines: $F_{1,29}$=0.02, p=0.9; intercepts of regression lines: $F_{1,30}$=9.2, p=0.004). In contrast to the minimal temperature limit, body size had no influence on the maximal temperature limits of both species (*C. mus*: linear regression: y=49.9+0.126x, p=0.2, R^2=0.1; Spearman´s correlation: r_s=0.33, p=0.2; *C. rufipes*: linear regression: y=47.7+0.086x, p=0.12, R^2=0.1; Spearman´s correlation: r_s=0.42, p=0.08). Independent of the body size, *C. mus* ants tolerated higher temperature values than *C. rufipes* ants (*C. mus*: 51.8±2.1°C, *C. rufipes*: 49.4±3.6°C, median±IQR; unpaired t-test: t(32)=-2.16, p=0.04).

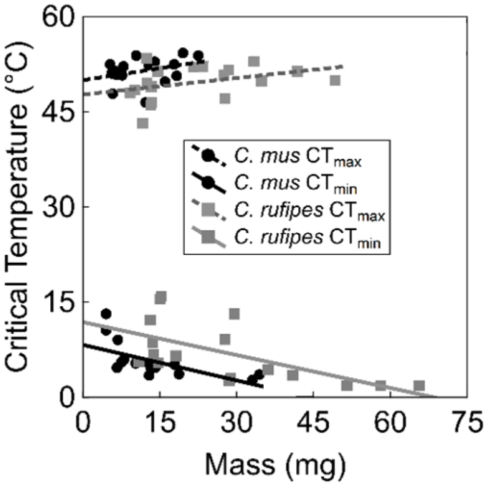

Fig. 9: Critical thermal limits of workers of both *Camponotus* species. Critical maximal and minimal temperatures of *C. mus* (black circles; n=32) and *C. rufipes* (grey squares; n=35) as a function of their body mass.

Discussion

Workers of both *Camponotus* species were found foraging at acacia trees, where they collected nectar at extrafloral nectaries (EFNs) and honeydew from treehoppers or aphids. As additional food sources, workers of both species collected acacia seeds and small insect prey. Likely due to their overlapping dietary niche, this two co-existing species occupy different temporal niches to avoid competition. Foragers in *C. mus* nest were exclusively active during the day and did not show any activity below 15°C, which is within the critical temperature range for foraging activity that has been described before (Falibene & Josens, 2014). In contrast to *C. mus*, *C. rufipes* foragers were predominantly active during the night. With that, we confirm the activity pattern of *C. rufipes* seen in the laboratory (Jaffé & Sánchez, 1984; own data, see chapter I and II), which probably reflects the endogenous foraging rhythm in this species. In contrast to the laboratory studies, several field studies demonstrated occasional diurnal activity in *C. rufipes* (Oliviera et al., 1995; Del-Claro & Oliviera, 1999; Fagundes et al., 2005). This switch might be a seasonal adaptation to the colder temperatures during winter, as seen in several *Camponotus* species (Briese & Macauley, 1980; Cros et al., 1997). As our field study took place in spring under moderate night temperatures, we observed both activity types. Half of the monitored nests showed the nocturnal "summer" activity type, whereas the other half exhibited the more diurnal "winter" activity type. As a consequence of the enlarged activity period, foragers were active over a broad temperature and humidity range, so that foraging activity was not strongly correlated with the measured environmental factors.

To test whether day active and night active foragers in *C. rufipes* belonged to the same population or to separate, phase-shifted subpopulations, we marked and recaptured individually marked ants in two nests. The major proportion of foragers was re-caught in the same day phase as they were marked, suggesting that they showed temporal fidelity and did not switch between day- and night activity. Such a form of shift-work was demonstrated in *C. gigas* as well (Orr & Charles, 1993) and can result from different physiological requirements towards diurnal and nocturnal foraging. As *C. rufipes* exhibits a size polymorphism (Diniz-Filho et al., 1994), subpopulations of foragers could differ in morphological traits to cope with these requirements. Foraging by larger workers could be advantageous during night times as these individuals cool down more slowly, whereas foraging by smaller workers could be advantageous during daytimes as they receive less solar radiation (Bernstein, 1976; Wetterer, 1990). We observed this exact pattern in one nest, but found no differences in body sizes between subpopulations in the other nest. Body size might be correlated rather weakly with environmental factors due to the small effect sizes on the small body size scale of ants. Of more importance might be a correlation of body size with the type of food source that is being collected. *C. rufipes* collects nectar at extrafloral nectaries mostly during night (Oliviera et al., 1995) as well as honeydew of aphids and treehoppers during day and night (Del-Claro & Oliviera, 1999; 2000; Fagundes et al., 2005). In other *Camponotus* species, honeydew collecting ants are smaller than other foragers (Gotwald, 1968; Fowler & Roberts, 1980), so small ants could be present day and night in *C. rufipes*. Nectar collection at EFNs might be associated with morphological diverse forager groups as well, as EFNs produce more nectar with higher sugar concentration during the day (Anjos et al., 2016). As larger workers are able to intake such high viscous more rapidly (Paul & Roces, 2003; Medan & Josens, 2005), larger workers could be active as well during the day. These points might explain the absent morphological differences between the putative diurnal and nocturnal foragers in one *C. rufipes* nest.

Foraging activity in ant species can be mediated by their thermal tolerances (Bishop et al., 2017). Heat tolerant species were reported to be active during daytimes throughout the seasons, whereas heat-averse species switch their activity period throughout the season (Cerdá et al., 1998). These species are nocturnal during summer to avoid heat, and switch to diurnal foraging during seasons with less extreme temperatures (spring and autumn). We determined critical thermal limits of both species to evaluate whether differences in these limits can explain their difference in temperature range for foraging. Within the same body size range, the diurnal species *C. mus* reached both lower and higher critical temperature values than *C. rufipes,* but differences in their thermal range were rather small. In general, thermal limits increased with body mass, whereas *C. rufipes* workers reached higher body masses. Consequently, *C. rufipes* reaches the same minimal and maximal critical thermal limits as *C. mus* and could therefore be active within the same thermal ranges in the field. Although *C. mus* reached critical minimal temperatures of less than 10 °C, we did not see any foraging activity below 15°C in the field. Critical temperature ranges for worker activity in the laboratory are usually wider than the actual critical temperature ranges of foraging activity in the field (Cerdá et al., 1998), because long-term effects of exposure on physiology and foraging efficiency become not evident. Although survival of ants is not affected by this temperature values, foraging efficiency is reduced to decreasing nectar intake rates (Falibene & Josens, 2014). But as *C. rufipes* proceeds foraging under this low temperature values, the absent nocturnal activity might be rather caused by the competition between the species than because of physiological constraints. Compared to other *Camponotus* species, *C. rufipes* is considered as dominant visitor of extrafloral nectaries (Del-Claro & Oliviera, 1999; 2000). Nocturnal foraging might be advantageous because of reduced thermal stress and predation risk, and therefore preferred by the dominant species *C. rufipes*. As a result of its dominance, submissive species are forced to forage daytimes under higher environmental stress (Anjos et al., 2016). Such subordinate species are generally active under wider temperature ranges (Bestelmeyer, 1997; Cerda et al., 1998; Lessard et al., 2009) and have higher maximal thermal limits (Jayatilaka et al., 2011). Moreover, workers of *C. mus* might be better adapted to diurnal foraging, as their body is less dark and covered more densely with hair, which might reflect solar radiation (Shi et al., 2015).

Our study suggests that *C. mus* and *C. rufipes* occupy the same dietary niche in the field, but differ in the temporal specialization of foraging workers. This coexistence might be shaped by the dominance of *C. rufipes* rather than by temperature, as laboratory experiments indicate comparable critical temperature ranges in the *Camponotus* species. Further studies of foraging activity in different seasons could give more insights on the relative effect of environmental factors and dominancy, as *C. rufipes* shifts to daytime foraging during winter (F. Roces, personal communication).

General Discussion

Temporal organization & division of labor

The studies presented in this thesis explored the temporal organization of behavioral activities in *Camponotus* ants, with special focus on two tasks within the ant colony: nursing and foraging. The laboratory experiments demonstrated that temporal scheduling involves not only the behavioral transition between nursing and foraging within the course of a worker life (chapter I), but also the timing and synchronization of activities on a daily basis (chapter II & IV).

On both time scales, timing of the examined collective behaviors is expected to be flexibly adapted to colony needs. During the course of the year or with increasing colony age, colony size increases and with that the demand for tasks like nursing, foraging or nest maintenance (Oster & Wilson, 1978; Thomas & Elgar, 2003; Mailleux et al., 2003). To meet the required needs, colonies could on the one hand alter the number of workers performing a certain task. In *Camponotus rufipes*, young workers stay inside the nest and engage in nursing activities, but up to 60% of workers switch to foraging at an age of one to two weeks (chapter I). In response to an increased foraging demand, relatively more workers could switch to foraging. This could lead to a modified sequence of task allocation in workers. Furthermore, behavioral transition to foraging could be accelerated, delayed, or even reversed, depending on the colony demands. Foragers are able to revert to nursing when the demand for brood care rises, for example when the brood mass increases or nurses are lacking (Ehrhardt, 1931; Weir, 1958; McDonald & Topoff, 1985; Symonowicz et al., 2015). Nurses in turn switch earlier to foraging under absence of older workers (Lenior, 1977; Sorensen et al, 1984; McDonald & Topoff, 1985). Although colony demands were maintained fairly constant in the conducted experiments, such plasticity was present within and between subcolonies. Further experiments with altered colony size, brood to worker ratios or caste perturbations could help to identify the specific factors regulating the timing of behavioral transitions.

In contrast to a change in the number of workers performing a given task, individual workers could increase or decrease their working effort in response to an altered task demand. This would rather affect the daily activity pattern of task performance within a behavioral caste than the general task sequence. In chapter II I report that *C. rufipes* foragers stay inactive for up to 50% of the day, but synchronize their foraging activity in response to food offered at certain time of the day. Foragers are expected to increase their activity when food is farther away from the nest or more dispersed. Moreover, foragers were reported to increase their activity in accordance to the higher food demand with increasing larval number (Ulrich et al., 2015). As shown in this thesis, nurses tend the brood frequently all-around-the clock, yet they also stay inactive for up to eight hours a day in total. Brood care activity is regulated by larval hunger as well as number of brood items (Cassil & Tschinkel, 1995; Brown & Traniello, 1998), so individual inactivity levels could be even more decreased under changing colony conditions.

Temporal organization & circadian clocks

In *Camponotus* ants, not only foragers show rhythmic activity outside the nest (chapter II), but also nurses display rhythmic activity inside the nest in the form of brood translocation behavior (chapter IV). As these ants undergo an age-dependent transition from nursing to foraging (chapter I), they need to schedule their daily behaviors flexibly in accordance to caste affiliation throughout their life. For that, even one-day old ants possess a functional circadian clock (chapter I). In this aspect of temporal organization, ants differ from another social insect, the honey bee. Only bees that experienced the colony environment for at least 48 hours exhibit circadian locomotor rhythms (Eban-Rothschild et al., 2012; Fuchikawa et al., 2016). The authors suggest that the clock system in these young bees is still immature and the colony surrounding (including pheromones and social interaction) contributes to the post-embryonic development of the circadian system. In comparison to *Camponotus* ants, nurse bees only display arrhythmic brood care behavior (Lindauer, 1952; Crailsheim et al., 1996; Moore et al., 1998) and a functional clock might not be needed for proper brood care during this early days of life. By contrast, *Camponotus* nurses could need a working circadian clock right after hatching, as they need to time the daily rhythmic translocation of brood as it favors brain development in larvae (Falibene et al., 2016). Yet to this point it is still unknown, at which age young *Camponotus* ants engage in brood relocations.

One aim of this thesis was to identify the environmental and social factors that are needed for proper timing of daily behaviors of nurses and foragers. For that, I quantified locomotor and foraging rhythms under controlled laboratory conditions. When housed under light-dark-conditions, isolated foragers and nurses of *C. rufipes* are strictly nocturnal in their locomotor activity (chapter II). Subcolonies kept under the same light-dark-cycle and under constant temperature, humidity and food availability throughout the day, display the same activity pattern in regard to the foraging activity (chapter I & II). Therefore, the nocturnal rhythms seen on individual and on group level seem to be endogenous and characteristic for this species. In this case, the light-dark-cycle serves as reliable zeitgeber for the timing of the foraging activity. Foraging activity in field colonies of *C. rufipes* is predominantly nocturnal as well, but the increased activity levels during the day suggest a modification of the endogenous rhythm in relation to relevant environmental factors (chapter V). In subsequent laboratory experiments, I identified food availability as one important factor influencing the foraging rhythm in individual workers. Yet on the colony level, daytime feeding alone does not lead to a complete suppression of the endogenous nocturnality (chapter II). It can be expected that variation in additional factors like temperature and humidity would lead to a more distinct activity shift. Further laboratory experiments with varying temperature, humidity and light intensity regimes could unravel the relevance of these abiotic zeitgebers for the timing of foraging in *Camponotus* ants.

In contrast to foragers, nurse workers do not leave the nest and can therefore not use the light-dark-cycle as zeitgeber to synchronize their daily activities. Consequently, these interior workers could partially rely on other daily environmental fluctuations like the temperature cycle present in the nest (Weidenmüller et al., 2009). As part of this thesis, I established a method for long-term recording of locomotor rhythms in ants, which is well suited to analyze the effect of various other zeitgebers on rhythms of individual workers. Former studies already demonstrated that temperature cycles are sufficient to entrain locomotor rhythms in wood ants (Rosengren, 1977; North, 1993), so it could be an appropriate zeitgeber for behavioral rhythms as well. As daily temperature cycles are accompanied with daily cycles in humidity, this could be another promising candidate zeitgeber. But as temperature and humidity fluctuate strongly from the day to day and throughout the year, these zeitgebers may not be as reliable as the light-dark cycle and be therefore rather weak zeitgebers. Several results of my experiments suggest that social interactions can serve as zeitgeber for in-nest activity. *Camponotus rufipes* nurses, which continuously stayed in the dark nest after eclosion, show locomotor rhythms in phase with the foraging rhythm of the subcolony when isolated (chapter I). Even isolated ants, which were in contact to a conspecific via a mesh partitioning, partly synchronize their locomotor activity (chapter III). A previous study already reported the synchronization of locomotor activity by cyclic social interaction in *C. pariah* (Lone et al., 2011), but the mechanism behind this synchronization was not identified. My subsequent analysis of behavioral activities of workers in the social context revealed that the increased interaction with foragers returning to the nest leads to an increase in activity in nurses (chapter II). Thus, the temporal information could be passively transferred into the nest not only by trophallactic interaction or antennation with exterior workers, but perhaps additionally over the food brought to the nest at certain times of the time. Consequently, food availability could be a zeitgeber for foragers as well as for nurses. Activity rhythms in several species are entrainable by feeding cycles (Aschoff, 1985; reviewed in Stephan, 2002; Mistelberger, 2009), and observation of locomotor activity of isolated individuals under feeding pulses would shed more light on its relevance for ants.

In conclusion, I experimentally demonstrated that endogenous rhythms of *Camponotus* ants are modified task-dependent in the social context of the colony. As division of labor in these ants is based on a temporal polyethism, workers need to re-schedule their daily activities as they switch from nursing to foraging with ongoing age. In the course of this age-dependent division of labor, individual behavioral rhythms are integrated to colony rhythms in nursing and foraging, highlighting the synchronization of daily behaviors across individuals. Environmental as well as social factors modulate these daily activity patterns. Within this thesis, the light-dark cycle and feeding cycles are identified as important zeitgebers for the timing of foraging activity outside the nest. Within the dark nest, non-photic zeitgebers like social interaction seem to be more relevant for the entrainment of daily activities.

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Publication list

Full paper (peer reviewed)

Mildner S, and Roces F (2017) Plasticity of daily behavioral rhythms in foragers and nurses of the ant *Camponotus rufipes*: influence of social context and feeding times. PLoS ONE 12:e0169244.

Conference abstracts

Mildner S, and Roces F (2013) The call of duty: light, temperature and humidity cycles as zeitgebers for circadian activity rhythms in *Camponotus* ants. 5th European Workshop of Myrmecology; Innsbruck, Austria.

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