Mechanisms and adaptive significance of interspecific associations between tropical ant species

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Hiermit erkläre ich, Florian Menzel, ehrenwörtlich, daß ich die vorliegende Dissertation selbständig angefertigt habe und keine weiteren als die angegebenen Quellen und Hilfsmittel verwendet habe. Die Dissertation wurde bisher weder vollständig noch teilweise einer anderen Hochschule mit dem Ziel der Erlangung eines akademischen Grades vorgelegt.

Am 1.2.2006 hat mir die Universität Würzburg den akademischen Grad des „Diplom-Biologen Univ.“ verliehen. Weitere akademische Grade habe ich weder erworben noch versucht zu erwerben.

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Table of contents

I. General introduction

This dissertation deals with interspecific associations between ant species. The focal type of association is a parabiosis between *Crematogaster modiglianii* and *Camponotus rufifemur*. 'Parabiosis' means that two ant species live together in a common nest and frequently interact, but keep their brood separate (Forel 1898). In my PhD project I tried to investigate this highly unusual association from several perspectives. I examined the proximate causes – behavioural and chemical mechanisms of interspecific nestmate recognition – which facilitate the high interspecific tolerance between these species. Moreover, since it has been largely unknown whether these associations are mutualistic, commensalistic, or parasitic, I conducted studies on possible ultimate causes of this association by trying to estimate the costs and benefits for both parabiotic partners. I included genetic analyses of the species in order to elucidate possible coevolutionary processes. This chapter will first cover the ecological background of associations between different species and their (co-) evolutionary implications. Secondly, we will deal with interspecific associations in ants, with a focus on interspecific ant-ant associations. The third subchapter will elaborate what is known about nestmate recognition in ants and how these mechanisms may apply to interspecific associations.

I.1 Associations among different species

Mutualistic interactions between different species of organisms have fascinated humans for a long time. For example, Herodotus discusses how plovers removed leeches from crocodiles' mouths ('The crocodile enjoys this, and never, in consequence, hurts the bird'). Aristoteles, Cicero and Plinius added more examples of mutualisms and argued that these 'friendships' helped to maintain nature's balance (Bronstein et al. 2006). The idea of harmony in nature persisted from ancient to the Middle Ages. Charles Darwin and Thomas Malthus were among the first who seriously perturbed this image. Darwin's idea that individual organisms struggled for life and competed against each other introduced the thought that an organism would only help another in exchange for something, and thus would never behave truly altruistically. This idea provided the basis for cost-benefit analyses and theoretical models on interspecific interactions in modern evolutionary ecology (Hoeksema and Bruna 2000). Depending on the cost/benefit ratio for each partner, associations or interactions between organisms can be defined as mutualistic, commensalistic, or parasitic. The most common mutualistic services are protection (against enemies or competitors), transport (of the mutualist itself or of its propagules), and nutrition (e.g. Bronstein and Barbosa 2002).

In any interaction between two species, the relation between costs and benefits determines the selection pressures on both partners. Each partner tries to draw the maximum benefit from its partner. Mutualistic interactions are hence prone to cheating (Yu 2001, Bronstein 2001b, Clement et al. 2008). In many taxa, parasites evolved in ancestrally mutualistic clades (Sachs and Simms 2006), such as Lycaenid butterflies (mutualists of ants, Pierce et al. 2002), mycorrhizae (Johnson et al. 1997), staphylinid-ant interactions (Hölldobler and Wilson 1990), and orchid-fungus interactions (Leake 1994). Over evolutionary time, a mutualism will only be maintained if both partners benefit and if cheaters are sanctioned (Edwards et al. 2006).

I.1.1 The parasitism-mutualism continuum and its implications for coevolution

The interaction between the very same two species can be mutualistic, commensalistic, or parasitic, depending on the biotic and abiotic environment (Bronstein 1994). For example, the magnitude of a benefit one partner receives can depend on the presence of a third species. If a mutualistic service is protection from an enemy, this benefit is absent in the absence of this enemy. A mutualism based on exchange of nutrients does not confer benefits if these nutrients are not a limiting factor (Bronstein 1994; Bronstein and Barbosa 2002). Thus, it depends on the environment whether an interaction is advantageous, neutral or harmful, and, the experienced net benefit of a mutualism can vary considerably within and across populations. If the mutualistic interaction is associated with a cost that is normally outweighed by the benefit, a species may be better off not associating with its partner when this benefit is absent.
A parasitic interaction will exert a selection pressure to evolve retaliation strategies in its host, e.g. protection mechanisms against the parasite. The parasite, in turn, is pressured to escape the host's strategy, which can result in a coevolutionary arms race (the 'red queen' effect; Brandt et al. 2005b; Strohm et al. 2008). This may lead to a close coevolution (Brandt et al. 2005a; Thompson 2005b). Within the ant tribe Tetramoriini, for example, parasitic ants show higher evolutionary rates at allozyme loci than do their non-parasitic relatives (Sanetra and Buschinger 2000). For the host, selection pressure to evolve retaliation strategies is only high in areas of high parasite abundance. If the parasite abundance varies across the host's range, the selection pressure on the host will spatially vary, resulting in a 'geographic mosaic of coevolution' (Gomulkiewicz et al. 2003; Thompson 2005a). Similarly, a mutualist may be pressured to evolve strategies against cheating (Edwards and Yu 2008). However, the damage caused by exploiters of mutualisms is usually low or even negligible, and thus may not exert high selection pressures (Bronstein 2001b). In contrast to parasitic interactions, it seems hence unlikely that a mutualistic interaction results in a coevolutionary arms race. Mutualists should thus evolve at a lower evolutionary rate than parasites and their hosts (Thompson 2005a). Finally, a commensalistic interaction is expected to exert selection pressures only on the party that benefits but not on the one that experiences neither costs nor benefits.

I.1.2 Determining costs and benefits

In order to determine the selection pressures exerted by interspecific relationships, one has to analyse the costs and benefits which either party incurs through its partner. They are usually quantified in terms of reproduction, survival, or growth. Many mutualistic systems allow experimental manipulation *in situ*, e.g. exclusion of one partner, to estimate its short-term impact on the other party. Using this approach, mutualistic benefits have been studied in various systems, e.g. between cleaner fish and their clients (Grutter 1999), plants and seed-dispersers (Levey et al. 2002), ants and trophobiotic aphids (Stadler and Dixon 2005), ants and myrmecophytes (Heil and McKey 2003), plants and pollinators (Kearns and Inoue 1993, Klein et al. 2003), and mycorrhizal fungi and their hosts (Johnson et al. 1997). However, many studies on mutualistic interactions focused on mutual benefits but neglected the – equally important – costs associated with the interaction (Bronstein 2001a).
Studies on benefits of interspecific associations are confronted with several methodological problems. Firstly, short-term benefits or costs may differ from the long-term perspective that encompasses each partner's life time. Benefits in terms of growth, reproduction and survival may be hard to quantify. In many species, these three parameters are positively or negatively correlated to each other, thus, they must be weighed up against each other in order to obtain

an estimate of whole-life reproductive success. This applies in particular to eusocial insects and to modular organisms such as plants. Secondly, long-term experimental manipulation - removing or excluding one of the partners without severely affecting the other is often practically impossible. Thirdly, mutualistic benefits are likely to differ strongly between individuals and populations, depending on the environment. Thus, it is debatable how many individual associations and which geographical range need to be studied for a reliable estimate of population-level selection pressures (Bronstein 2001a). Moreover, the variance of the selection pressures is as important as its mean, since may reflect the geographic mosaic of coevolution (Bronstein 2001a). Finally, it is unclear how different measures of costs and benefits can be translated into the same currency. In particular, if one or both partners cannot survive without the other one (e.g. in fig-fig wasp mutualisms), costs and benefits are difficult to interpret since the alternative of a non-associated individual does not exist (Bronstein 2001a). These problems also apply to the studied parabioses where one of the partners apparently cannot exist without its partner, and, since both partners are eusocial, long-term reproductive success is extremely difficlt to determine.

I.2 Social insect symbionts

At first glance, nests of eusocial insects seem to be closed societies, where access is only granted to colony members. However, they often represent whole ecosystems in themselves. Several thousand species of other arthropods or even molluscs manage to gain access into nests of e.g. ants or termites and make a living inside these colonies (Kistner 1979; Witte et al. 2002). Facultative or obligate ant guests (myrmecophiles) feed on ant brood, workers, or waste, or simply seek shelter and protection in the ant nest (Hölldobler and Wilson 1990). Several insect taxa include a wide range of obligate myrmecophilic species, such as lycaenid butterflies, staphylinid and carabid beetles, and crickets (Schultz and McGlynn 2000; Pierce et al. 2002; Geiselhardt et al. 2007). Ant guests may be commensals if they live from refuse or dead workers, or parasites if they feed on brood or workers or steal prey from the ants (Hölldobler and Wilson 1990).

I.2.1 Ant-ant associations

Intriguingly, even whole ant colonies can live inside nests of other ant species. Ant-ant associations range from loosely associated, facultative commensals or cleptoparasites to highly specialized social parasites which totally depend on their hosts (Kaufmann et al. 2003; Huang and Dornhaus 2008). Several attempts have been made to classify the wide range of ant-ant associations. Wasmann (1891) distinguished between ‘compound nests’, where “two or more species live very close to each other, in some cases even running their nest galleries together, but keep their brood separated”, and ‘mixed colonies’, where “the brood are intermingled and cared for communally” (Wilson 1971).

I.2.2 Mixed colonies: social parasitism

Mixed colonies generally represent cases of social parasitism. Hölldobler and Wilson (1990) distinguish three types of social parasitism in mixed colonies: temporary parasitism, dulosis, and inquilinism. These three types are not mutually exclusive, and some ant species perform two or all of these forms of parasitism (Hölldobler and Wilson 1990; Mori et al. 2001).

Temporary parasites depend on the host only for colony foundation, but later form independent, monospecific nests. Many of these species are only facultatively parasitic and can also found colonies independently (Hölldobler and Wilson 1990; Kronauer et al. 2003). Dulotic parasites are slave-makers. Freshly mated queens of dulotic species invade a host

nest, kill or drive out the workers, and take over the brood. From these pupae emerge the first slaves and subsequently rear workers of the slave-maker. In later stages of the colony, independent host colonies are raided to replenish the slave stock (Buschinger 1986). Individuals of obligatory dulotic species are often unable to forage, feed the larvae, or even to eat by themselves. They are usually adapted to fighting and possess saber-shaped, toothless mandibles, which they use to defeat their hosts' workers. Many of these species lack a worker caste (Buschinger 1986, Hölldobler and Wilson 1990). Inquilines spend their entire life cycle in their host's nest and often lack a worker caste as well. Their brood is intermingled with that of the host queen. Inquiline queens usually coexist with the host queen and are accepted and tolerated by the host workers (Hölldobler and Wilson 1990, Huang and Dornhaus 2008).
Various models and theories on the evolution on social parasitism have been developed (Hölldobler and Wilson 1990; Huang and Dornhaus 2008; Lowe et al. 2002). With one exception (Maschwitz et al. 2000), temporary parasitism and inquilinism occurs exclusively among close relatives, a relation which is known as Emery's rule. It seems likely that these forms of social parasitism evolved from intraspecific parasitism (Huang and Dornhaus 2008; Kronauer et al. 2003). Buschinger (1986) suggests that inquilinism evolved from colony multiplication through budding or adoption in polydomous and polygynous species. Dulosis, where Emery's rule often does not apply (Huang and Dornhaus 2008), may have evolved from predation on conspecific colonies or other ant species that subsequently survived in the ant nest.

I.2.3 Compound nests

In compound nests, two ant species share a nest but keep their brood separate. Compound nests range from loose, facultative to highly specialized, obligatory associations between two ant species. Wheeler (1910, as summarized by Wilson 1971 and Hölldobler and Wilson 1990) distinguished five types of compound nests: Plesiobiosis, xenobiosis, lestobiosis, cleptobiosis, and parabiosis.
In a plesiobiosis, two ant species nest closely together but do not otherwise interact. This form of association is mostly accidental and may be due to nest site scarcity (Czechowski 2004). Xenobiotic ants are 'guest ants' that live inside other ants' nests. Usually being much smaller than their hosts, they make a living from stealing food or inducing trophallaxis in their hosts. Hence, xenobiosis can be regarded as trophic parasitism. Only few xenobiotic genera are known, including *Formicoxenus* and *Megalomyrmex* (Errard et al. 1997; Hölldobler and Wilson 1990). A similar relationship is the lestobiosis (defined by Forel 1901), where a small ant (e.g. *Solenopsis*) lives in the nest wall of a larger species and steals food from the host or preys on host workers or brood (Wheeler 1910; Kaufmann et al. 2003). Cleptobiosis is characterized by a small ant that nests near larger species and feeds on refuse of its host or robbing host foragers. Thus, a cleptobiosis may be commensalistic if the small species only feeds on host refuse but does not rob or prey on host workers or brood (e.g. Yéo et al. 2006).
Being compiled from different authors (Forel 1898; Forel 1901; Wheeler 1901), Wheeler's original differentiation into the mentioned five types of compound nests is little satisfying since the definitions are often vague and partly overlapping. For example, cleptobiosis, lestobiosis and xenobiosis have in common that a small species lives in the nest of a larger one and either steals food, preys on the host or feeds on host refuse, while the latter differentiation is often not clearly defined among the three types (Kaufmann et al. 2003). In addition, these three types are differently defined by Lenoir et al. (2001b), who define cleptobiosis and lestobiosis as two ants occupying different nests whereas only xenobionts

live inside their host nests and strictly depend on them. Plesiobiosis, in contrast, appears to represent accidental nesting in close proximity and hence may not be a biologically meaningful association. Instead of a classification into these five types, Kaufmann et al. (2003) therefore suggest a detailed catalogue of criteria to describe ant-ant associations, which includes regularity of occurrence, spatial and temporal variation, interspecific communication, and the positive or negative effects on either species.

I.2.4 Parabiosis

The fifth type of compound nests is the so-called parabiosis. Parabioses are shared nests of two unrelated species that occur regularly between certain species pairs. In contrast to xenobiosis, lestobiosis, and cleptobiosis, the two partners can be of similar size, such that the association appears symmetric. Forel (1898) first described such an association (between *Dolichoderus* and *Crematogaster*) in the Colombian rainforest. By introducing the term 'parabiosis', he indicated that this was a new kind of association – it was not clear whether parabioses were mutualistic, commensalistic or parasitic. Parabioses are largely confined to associations between species of *Crematogaster* and either *Camponotus, Dolichoderus, Odontomachus* or *Pachycondyla* in South American and Southeast Asian rainforests (Menzel et al. 2008; Orivel et al. 1997). Often, these nests are inhabited by presumably mutualistic, epiphytic plants ('ant-gardens') and may include other associated species such as trophobionts and other insect guests (Corbara et al. 1999; Kaufmann and Maschwitz 2006). Since Forel's first report, the ecological character of neotropical ant-garden parabioses has been debated (e.g. Davidson 1988; Dejean et al. 2000; Swain 1980). It remains largely unresolved whether they represent a case of social parasitism, commensalism or mutualism.

I.3 Nestmate recognition in social insects

A eusocial insect colony can only function if all colony members can discriminate their nestmates from non-nestmates. This discrimination process is termed nestmate recognition (Vander Meer and Morel 1998). It possibly originated from kinship recognition, when kinship signals were replaced by nestmate recognition cues over evolutionary time (Lenoir et al. 1999). Nestmate recognition also plays an important role in interspecific associations among ants, since it determines whether heterospecific nestmates are tolerated or attacked.

I.3.1 Nestmate recognition cues

In ants, nestmate recognition is mediated by chemical cues on the insect cuticle (Howard and Blomquist 2005), while non-chemical (e.g. behavioural) features play a negligible role (Vander Meer and Morel 1998). The majority of these chemicals are hydrocarbons (Lenoir et al. 1997; Liu et al. 2000, Wagner et al. 2000). In various experiments, ants and termites distinguished between nestmate and non-nestmate hydrocarbon profiles, but not profiles of other cuticular lipids (Lahav et al. 1999, Wagner et al. 2000, D'Ettorre et al. 2002, Bagneres et al. 1991b, Kaib et al. 2004). The hydrocarbons are probably synthesized by oenocytes associated with either fat body or epidermal tissue and then transferred to the cuticle through the hemolymph (Soroker et al. 1995; Howard and Blomquist 2005; Lenoir et al. 1999). They originally evolved to prevent desiccation of the insect body (Gibbs 1998; Howard and Blomquist 2005) and presumably later acquired their function as nestmate recognition cues. Quantitative and qualitative hydrocarbon composition is mostly genetically determined (Carlin and Hölldobler 1986), but often heavily influenced by environmental factors such as diet or nest material (Heinze et al. 1996; Lenoir et al. 1999; Richard et al. 2004; Sorvari et al. 2008). Freshly eclosed workers (callows) initially lack cuticular hydrocarbons and are hence

'chemically insignificant'. In workers of *Manica rubida* and *Formica selysi,* their colony-specific hydrocarbon profile develops within few days, and the total amount of cuticular hydrocarbons gradually increases with age until it stabilizes at the age of one month (Lenoir et al. 1999 and references therein). Through allogrooming and trophallaxis, members of an ant colony constantly exchange hydrocarbons, thereby level out inter-individual profile differences and create a common colony odour. This common colony odour has been misleadingly termed '*Gestalt* odour' (Crozier and Dix 1979), probably to emphasize the complexity and the emergent properties in the nestmate recognition system (Leonhardt et al. 2007).

I.3.2 The role of the postpharyngeal gland

The postpharyngeal gland (PPG) plays a major role in the creation of a common colony odour. Located in the head, it opens out into the buccal cavity just in front of the esophagus (Soroker et al. 1995). Various studies have shown that the cuticular hydrocarbons are identical with those found in the PPG (Bagneres and Morgan 1991, Donascimento et al. 1993). Soroker et al. (1994) suggested that the PPG functions as a pool for both self-produced (endogenous) and exogenous hydrocarbons. While the former reach the PPG via self-grooming or directly through the hemolymph, exogenous hydrocarbons reach it through allogrooming or trophallaxis (Soroker et al. 1995; Vienne et al. 1995; Lenoir et al. 1999). The hydrocarbon exchange between PPG and cuticle is probably bidirectional (Hefetz et al. 1992). Since the PPG is located close to the mouth, PPG hydrocarbons can easily be smeared over the antennae during self-grooming and thus provide a constant supply of recognition cues. A regular 'update' of recognition cues is necessary since, due to environmental influences, the colony odour changes over time (Vander Meer and Morel 1998). Similar to cuticular extracts, ants can distinguish nestmate from non-nestmate PPG extracts (Hefetz et al. 1996; Soroker et al. 1994).

I.3.3 The nestmate recognition process and inter-colony tolerance

It is generally assumed that information on the common colony odour (i.e., on the colony's hydrocarbon profile) is represented as a neuronal template in the nervous system. The formation of this recognition template requires at least simple forms of learning (habituation), as well as a frequent update of this template through inter-individual contact (Dahbi et al. 1999; Soroker et al. 1994; Leonhardt et al. 2007). Ants perceive each other's cuticular hydrocarbon profile partly through volatile signals (Brandstaetter et al. 2008) and partly by touching each other with the antennae (Vander Meer and Morel 1998). The perceived chemical profile is then compared to the neuronal template (Crozier and Pamilo 1996; Errard et al. 2006). Generally, aggression results upon a mismatch of these two profiles, leading to high inter-colony aggression in most ant species. However, among invasive ant species, intraspecific aggression is often low or absent, which results in a unicolonial population structure and is a major cause for their ecologically devastating impact (Holway et al. 2002). Their high intraspecific tolerance is probably caused by a low genetic inter-colony differentiation, which translates into lower differentiation of the chemical recognition cues (Suarez et al. 2008; Tsutsui et al. 2000; 2003). A possibly similar mechanism has recently been described for non-invasive ants with unusually low intraspecific aggression (Foitzik et al. 2007).

I.3.4 Nestmate recognition in mixed-species colonies

As described above, mixed colonies of two ant species regularly occur in nature. Several studies report high interspecific tolerance in natural ant-ant associations, including lestobioses (*Camponotus* and either *Solenopsis* or *Brachymyrmex,* Errard et al. 1996; 2003) and South American parabioses (*Odontomachus* and *Crematogaster*, Orivel et al. 1997). In the two lestobiotic associations, tolerance seemed to be species-specific but not colony-specific (Errard et al. 1996; 2003), while the studied parabiotic species exhibited a high colony specificity and only tolerated their partner colony (Orivel et al. 1997).

Interspecific tolerance has been most extensively studied in social parasite-host systems. Various mechanisms have been discovered by which social parasites manage to get accepted in the host nest (Lenoir et al. 1997; 2001b):

1. chemical insignificance. The parasites are odorless at the time of usurpation, and are hence not recognized as enemy when they enter the host nest (D'Ettorre and Errard 1998; Lenoir et al. 2001b).
2. chemical mimicry and chemical camouflage. The parasite's hydrocarbon profile partly or completely overlaps with its hosts's. The parasite either biosynthesizes the host's chemical signature (mimicry) or acquires it through allospecific grooming, trophallaxis or other physical contact with the host or nest walls (camouflage) (Lenoir et al. 1997).
3. chemical weapons. The parasite uses chemical repellents to deter attacking host workers (D'Ettorre et al. 2000).
4. appeasement pheromones. The parasite secretes appeasement pheromones that make host workers amicable (Mori et al. 2000b).
5. learning. The host habituates to the parasite's chemical signature and subsequently tolerates the parasite. In order to achieve host habituation, the parasite usually has to employ one of the above strategies first (Lenoir et al. 2001b).

These mechanisms are not mutually exclusive, and several of them may be applied by the same social parasite in different stages of its life cycle. For example, *Polyergus rufescens* queens are chemically insignificant and use chemical weapons or appeasement pheromones when entering the host nest. Later, they acquire cuticular hydrocarbons from the host queen (Johnson et al. 2001; D'Ettorre et al. 2002). Due to the appeasement pheromones, the host workers may then reshape their neuronal template and habituate to the parasite's profile.

Both chemical mimicry and camouflage have been repeatedly found in myrmecophagous spiders, various myrmecophilous insects, and xenobiotic ants (Howard et al. 1990; Akino et al. 1996; Lenoir et al. 1997; 2001b; Howard et al. 2001; Akino 2002; Elgar and Allan 2004). For example, the butterfly *Maculinea rebeli*, a social parasite of *Myrmica*, performs both mimicry and camouflage: while earlier stages of the caterpillar synthesized many of its host's recognition signals, they later acquired missing hydrocarbons inside the ant nest (Akino et al. 1999).

The mechanisms of tolerance and habituation to foreign profiles in interspecific associations have been studied in artificial mixed colonies, where workers of two species were reared together from the callow stage (e.g. Errard and Hefetz 1997; Errard et al. 2006). Errard and colleagues (2006) reared workers of *Manica rubida* both in monospecific groups and together with either *Formica selysi, Myrmica rubra* or *Tetramorium bicarinatum*. They showed that workers from mixed-species groups do not become generally more tolerant. Instead, they acquire a broader template that includes heterospecific cuticular compounds, and habituate to two different surface profiles (Errard et al. 2006). Interestingly, the magnitude of the profile

difference plays a role in shaping the neuronal template: *M. rubida* reared with *Myrmica rubra* (which possesses a rather similar surface profile) exhibited less interspecific tolerance than those reared with either *F. selysi* or *T. bicarinatum* (which both possess profiles greatly different from *M. rubida*).
Similar to monospecific colonies, artificial mixed-species colonies may possess a common colony odour. To a certain degree, ants can acquire heterospecific compounds in artificial mixed-species colonies. In mixed colonies of *Formica selysi* and *Manica rubida*, both species changed their hydrocarbon profiles towards a higher similarity to the other species when reared together (Bagneres et al. 1991a; Vienne et al. 1995). Although the authors explain this by selective hydrocarbon synthesis on both sides, however, an active transfer of surface chemicals (i.e. reciprocal camouflage) also seems possible. Nevertheless, many other studies report that the chemical profiles of species in associations retained their respective hydrocarbon profile. For example, no chemical overlap could be detected among the profiles of *Camponotus morosus* and *Brachymyrmex giardii* (parabiotic association, Errard et al. 1996), *Polyergus samurai* and *Formica japonica* (slave-making ant and host, Liu et al. 2003), and the temporary parasitic ant *Lasius* sp. and its host *Lasius fuliginosus* (Liu et al. 2000).
Up to now, little is known interspecific tolerance in parabiotic associations. In a mutualism, there is a selective force towards tolerance in both partners whereas in a parasitic or commensalistic association, only one of the two has an interest in being tolerated. Hence, the ecological relationship between parabiotic ants has a great evolutionary influence on mutual tolerance and its underlying behavioural and chemical mechanisms.

I.3.5 Nestmate recognition assays: conceptual and methodical problems

The fundamental problem of studying nestmate recognition is that recognition is a cognitive process and cannot be observed as such. Whether an ant fails to recognize its counterpart or tolerates it upon recognition can hardly be distinguished based on behavioural observations. In interspecific associations, another complication is the lack of a plausible null hypothesis, i.e. the expected behaviour given recognition failure is unknown. Many studies inferred a recognition failure due to chemical mimicry when two associated species possessed similar chemical profiles (Bagneres et al. 1991a; Lenoir et al. 1997, 2001b), but assumed that the foreign profile was learned as an additional template when they differed (Errard 1994, Errard et al. 2003). Although this explanation is plausible, it is important to keep in mind that recognition failure cannot be shown in principle.
Similarly, long antennation phases during encounters of workers from different colonies may indicate either ‘recognition uncertainty’ or ‘recognition as foreign but tolerance’. In case of recognition uncertainty, i.e. if the workers are unable to categorize their opponent into ‘nestmate’ or ‘non-nestmate’, they may continue to acquire more recognition cues by antennating the opponent. Thus, continued antennation may indicate ‘recognition uncertainty’ but not necessarily ‘recognition as foreign but tolerance’, although this has sometimes implicitly been claimed in earlier studies (e.g. Steiner et al. 2007).
Laboratory assays are a popular method to study nestmate recognition since they are weather-independent and allow an exact experimental design as well as easy replication. Their reliability, however, remains questionable since aggression is often highly context-specific and can depend e.g. on the presence of brood or the queen, number of present nestmates, or the distance to the nest (Starks et al. 1998; Breed 2003; Knaden and Wehner 2003; Roulston et al. 2003; Velasquez et al. 2006). Moreover, laboratory-reared colonies often display lower intraspecific aggression than wild ones (Buczkowski et al. 2005; Buczkowski and Silverman

2005) and may not show differential behaviour towards different test ants. Thus, nestmate recognition assays directly at natural nests are generally preferable over laboratory assays. In either case, however, it is crucial to mimic the natural conditions and to avoid manipulation of the tested ants as much as possible.

I.4 Thesis outline

During my PhD research I covered various aspects of nestmate recognition and the ecological interactions of the two parabiotic species *Camponotus rufifemur* and *Crematogaster modiglianii*. My research is divided into the following parts:

I. Specificity of tolerance
   Using nestmate recognition bioassays, I determined the extent of intra- and interspecific tolerance of the two parabiotic species. In particular, I was interested whether the tolerance only extended to the partner colony, the partner species or even other species congeneric to the parabiotic partner.

II. Nestmate recognition cues in parabiotic species
   I chemically analyzed and identified the cuticular hydrocarbons of the two parabiotic species and related the profiles to interspecific recognition.

III. Function and molecular structure of unknown cuticular compounds
   Since I found that *Crematogaster modiglianii* possesses highly unusual, hereto unknown substances on its cuticle (in addition to the hydrocarbons), I performed experiments on the role of hydrocarbons versus the unknown compounds in the recognition process *between* the two species. The experiments were accompanied by analyses on the molecular structure of the unknown compounds.

IV. Intraspecific recognition in the two parabiotic ants
   Similar to the interactions *between* the two parabiotic species, I studied nestmate discrimination *within* parabiotic species. In particular, I investigated how differential inter-colony aggression is related to chemical differentiation. Moreover, I studied the function of hydrocarbons versus unknown compounds in the recognition process within *Crematogaster modiglianii*.

V. Trail-sharing associations and their underlying mechanisms
   A less intimate association than parabiosis is 'trail-sharing', where two species regularly use shared trails. In this study, I examined the behavioural and chemical mechanisms behind trail-sharing in both parabiotic and non-parabiotic, but trail-sharing ants.

VI. The ecological relationship between parabiotic ants
   It has long been debated whether parabioses are mutualistic, commensalistic or parasitic. However, investigations on mutual costs and benefits are impeded by the difficult experimental manipulation of parabiotic nests and the problem to estimate reproductive success in eusocial insects. I therefore conducted an in-depth analysis of different potential benefits between the two ant partners, combining observations with experimental evidence wherever possible.

VII. Population genetics of the two parabiotic ant species
   In order to evaluate the genetic evidence for coevolution between the two parabiotic partners, I analyzed mtDNA sequences (parts of the cytochrome oxidase gene) of parabiotic ants from different regions in Borneo.

II. Study area and focal species

II.1 Study area

Most of the studies were conducted at the Danum Valley Conservation Area (DVCA). This site is located approx. 70 km west of Lahad Datu in Sabah (Malaysian Borneo). The area (4°55'N 117°40'E, ca. 100 m asl) is one of the major remaining patches (438 km²) of lowland dipterocarp rainforest in Sabah. It has a typical equatorial rainforest climate with a mean annual temperature of 26.9 °C and a yearly rainfall of 2700 mm.

In addition, I sampled parabiotic ants at Kabili Sepilok Reserve, Mulu National Park, and Kuala Belalong Field Studies Center. Kabili Sepilok Reserve is located near Sandakan (Sabah) at 5°54' N, 118°04' E and 20-120 m asl. It comprises 4294 ha of coastal dipterocarp and mangrove forest. It is thus substantially smaller than DVCA and surrounded by oil palm plantations. The climatic conditions are similar to Danum Valley.

Gunung Mulu National Park covers 520 km² and is located at 4°57'N, 114°47'E, in northeastern Sarawak (Malaysian Borneo). The climate in this area is wet tropical with mean temperatures of about 26°C and 4000-5000 mm rain per year (Sarawak Weather Service, pers.comm.). Gunung Mulu comprises several forest types, including dipterocarp forest, limestone forest, *keranga*, and alluvial forest. Most of our sampling took place in the alluvial forest near the park headquarters and at Camp 5. This forest is flooded several times per year for 1-2 days after heavy rainfalls.

The Kuala Belalong Field Studies Center (KBFSC) is located at 4°33' 115°09, ca. 50m a.s.l, in Temburong District, Brunei. Kuala Belalong is surrounded by steep hills covered in undisturbed lowland rainforest. It is embedded in a large patch of primary forest, which, except for the partly logged Limbang area, extends to the ca. 45 km distant Mulu National Park. The climate is wet tropical with very high humidity (90% daytime, >99% at night, very little seasonal variation) and very high rainfall (4000-5000mm per year; Dykes 2000). An overview of all four study sites is given in Fig. 1.

II.2 Focal species

My focal species are *Camponotus (Myrmotarsus) rufifemur* Emery 1900 (Formicinae) and *Crematogaster (Paracrema) modiglianii* Emery 1900 (Myrmicinae). The two species live together in a parabiotic association, i.e. they share a common nest. These nests are usually hollow, living tree trunks but can also consist of dead logs or lianas. Besides, I studied *Camponotus (Myrmotarsus)* sp. 5 of Seiki Yamane's reference collection, which is most probably *Ca. irritabilis* (Smith 1857) (identification by Seiki Yamane) and also lives in parabiosis with *Cr. modiglianii*. While *Cr. modiglianii* can nest non-parabiotically, the mentioned *Camponotus* species have never been found without their partner species.

Apart from parabiotic associations, I also investigated trail-sharing associations between ants of the same habitat. The studied species include the formicines *Polyrhachis ypsilon* Emery 1887*, Polyrhachis bihamata* Drury 1773*, Camponotus (Colobopsis) saundersi* Emery 1889, two yet undescribed *Camponotus (Colobopsis) species, Dolichoderus cuspidatus* Smith 1857*,* and *Dolichoderus thoracicus* Smith 1860. All studied species have only been found in primary lowland rainforest so far. Voucher specimen of all species are deposited at the Forest Research Center in Sepilok, Sabah (Malaysia) and at the Department of Zoology, University of Würzburg.

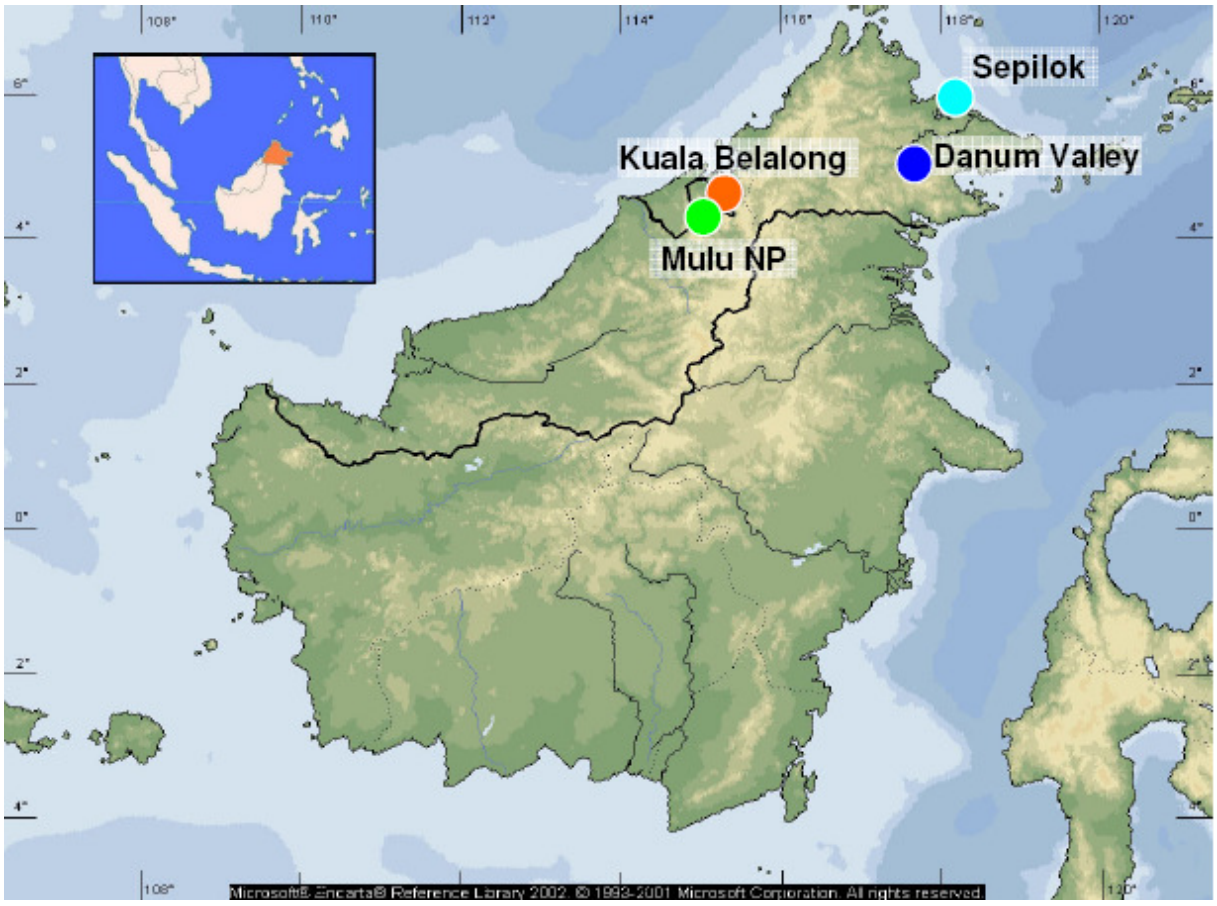

Fig. 1 Map of the study sites in Borneo. Inlet: Overview of Southeast Asia, with the province Sabah in red.

III. Interspecific tolerance in parabiotic associations

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III.1 Abstract

Associations between ants of the genera *Crematogaster* and *Camponotus* are found in many parts of the world. Associated species use common trails (trail-sharing) or even share a common nest (parabiosis). In a tropical lowland forest in Malaysian Borneo, we studied intraspecific and interspecific aggression among the parabiotic species *Crematogaster modiglianii* and *Camponotus rufifemur* using both field and laboratory assays. *Cr. modiglianii* tolerated *Ca. rufifemur* workers from certain foreign colonies, but fiercely attacked those of others. In contrast, *Ca. rufifemur* was tolerant even towards attacking allocolonial *Cr. modiglianii* workers but killed other *Crematogaster* species. By analogy, other *Camponotus* species mostly attacked and killed *Cr. modiglianii*. Intraspecific confrontations among *Ca. rufifemur* colonies yielded a gradient from allocolonial tolerance to strong aggression. The aggression patterns coincide with those of *Cr. modiglianii* towards *Ca. rufifemur* workers from the same colonies.

Our results suggest that *Ca. rufifemur* is either not able to recognize allocolonial *Cr. modiglianii* workers as foreign or that they are recognized but tolerated. The unilateral, species-specific but not colony-specific tolerance of *Ca. rufifemur* towards its partner species contrasts with highly colony-specific tolerance found among neotropical parabioses.

III.2 Introduction

Most social insects are highly aggressive towards individuals other than their nestmates. Especially nest entrances are fiercely defended against non-nestmates (Breed and Bennett 1987; Hölldobler and Wilson 1990; Roubik 1989). In ants, non-nestmates are often aggressively displaced from trails or food resources (Blüthgen and Fiedler 2004; Fellers 1987; Savolainen and Vepsäläinen 1988; Swain 1980). Discrimination between nestmate and non-nestmate thus plays a central role in encounters between individual ants. Ants recognize their nestmates through colony-specific chemical signals. Located on the body surface, they are perceived by other individuals through contact chemoreception (Vander Meer and Morel 1998) and compared to a cognitive reference template (Errard 1994; Lenoir et al. 1999). Most signalling substances are hydrocarbons (Lenoir et al. 1997; Lahav et al. 1999; Wagner et al. 2000, Suarez et al. 2002, but see Hernández et al. 2006). Ants continually exchange surface chemicals among nestmates through allogrooming and trophallaxis. Through this process, a mixed colony odor is created and continually redistributed among nestmates (Crozier and Dix 1979; Vander Meer and Morel 1998; Boulay et al. 2000) albeit chemical differences between certain groups, e.g. castes, may be maintained (Endler et al. 2004).

Social parasites (ants and other arthropods) overcome nestmate recognition and manage to get accepted in foreign ant colonies. In many associations, parasite and host possess similar chemical profiles. It is hence assumed that the hosts do not regard them as alien (chemical mimicry; Howard et al. 1990; Akino et al. 1996, 1999; Howard et al. 2001; Lenoir et al. 2001b; Akino 2002; Elgar and Allan 2004). Other social parasites, in contrast, possess distinct profiles that do not resemble their hosts. In these cases the host probably recognizes but tolerates the parasite since it has habituated to the parasite's profile (Errard et al. 1996; Liu et al. 2000, 2003).

However, there are other, presumably non-parasitic associations between ant species, mostly from different genera, that co-occur without aggression. Two types of association can be observed. The more frequent one is that of two ant species sharing a common trail, which has been reported from many parts of the world (Wilson 1965; Baroni Urbani 1969; Davidson 1988). Interspecific tolerance (i.e. absence of aggression) in trail-sharing species is often achieved through submissive avoidance behaviour of one species towards the other (Baroni Urbani 1969; Adams 1990). Similarly, appeasement behaviour also reduced aggression among competing ant species (Mercier and Dejean 1996).

Parabioses are associations where two ant species live together in the same nest. The most extensively studied parabioses are associations in neotropical ant gardens. These are inhabited by species of the *Crematogaster limata* complex (see Longino 2003) associated with *Camponotus* (most often *Ca. femoratus*), *Odontomachus, Pachycondyla* or *Dolichoderus* (Swain 1980; Davidson 1988; Orivel et al. 1997; Vantaux et al. 2007). The species keep their brood separate but otherwise share major or even all parts of the nest (Orivel et al. 1997). Few other parabiotic or parabiotic-like associations have been studied, including associations of *Brachymyrmex giardii* with *Camponotus morosus* and *Ca. chilensis* (Errard et al. 1996) and *Camponotus* nests inside *Iridomyrmex* mounds (Greaves and Hughes 1974; Hölldobler and Wilson 1990: p.467). A putatively parabiotic association between *Camponotus morosus* and *Solenopsis gayi* is probably a lestobiosis, i.e. *S. gayi* is probably a brood parasite that lives inside the *Ca. morosus* nest (Errard et al. 1996; Lenoir et al. 2001b; Errard et al. 2003).

Since parabiotic species need to tolerate heterospecific ants as nestmates, their nestmate recognition system might be adapted accordingly. Similar to social parasites, interspecific tolerance might be achieved either by chemical mimicry or habituation to the other's profile. The latter seems likely in the neotropical parabiotic ants *Odontomachus mayi* and *Cr. limata*, which possess completely different chemical profiles (Orivel et al. 1997). In artificially mixed colonies of *Formica selysi* and *Manica rubida*, both species, however, changed their hydrocarbon profiles towards a higher similarity to the other species when reared together, suggesting a mixed colony odor (Errard et al. 2006; Vienne et al. 1995). Chemical mimicry or habituation can also result in selective interspecific tolerance. In the neotropical parabioses of *Crematogaster limata* and *Odontomachus mayi*, interspecific tolerance was restricted to the respective partner colony, while both species were aggressive towards allocolonial workers of their partner species (Fig. 1a; Orivel et al. 1997). However, individuals of both *Solenopsis gayi* and *Camponotus morosus*, which can live together in a parasitic (lestobiotic) association, showed allocolonial interspecific tolerance when the involved *Ca. morosus* individuals were part of a lestobiotic association (Fig. 1b; Errard et al. 2003); similar patterns were found in associations between *Brachymyrmex giardii* and *Camponotus morosus* or *Ca. chilensis* (Errard et al. 1996).

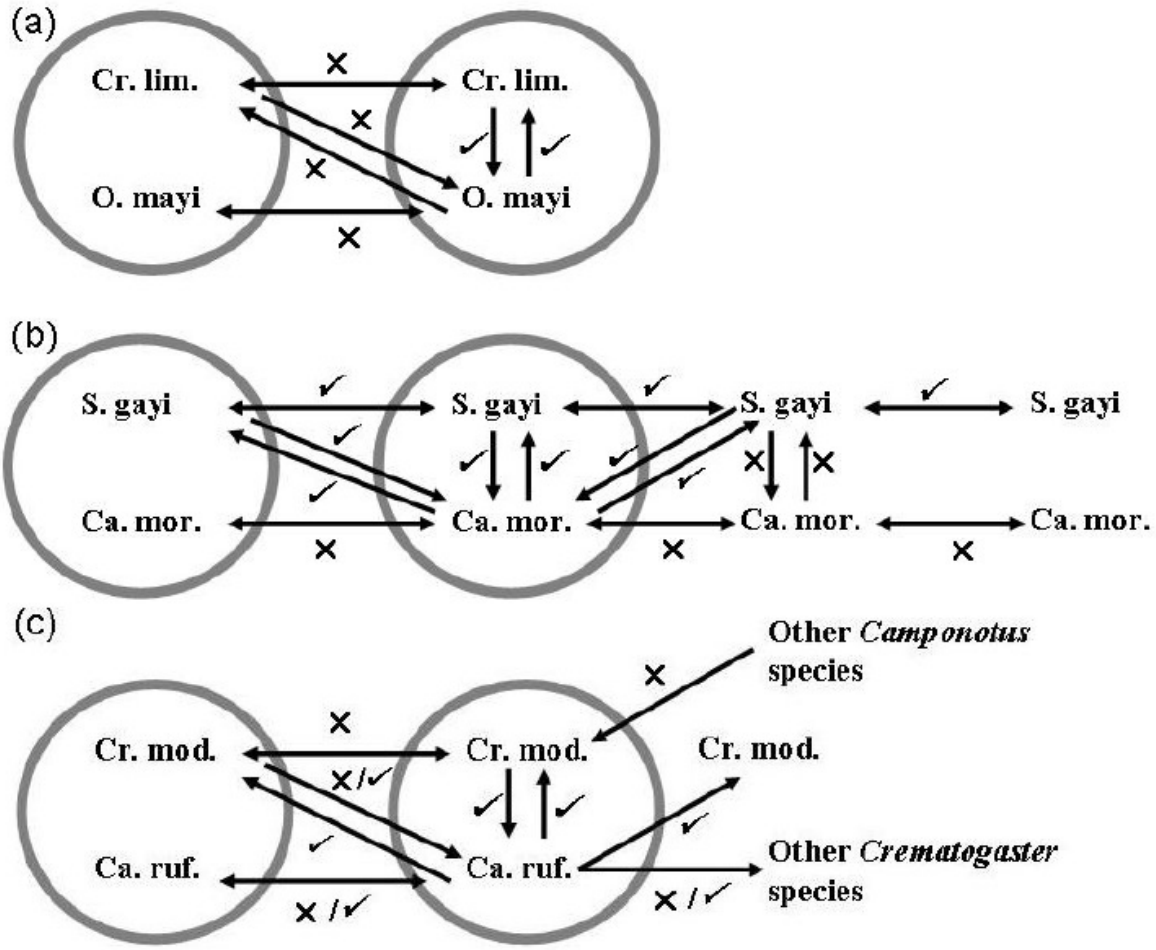

Fig. 1 Visualization of tolerance patterns between colonies in different associations. The arrows indicate mutual tolerance (✓) or mutual aggression (✕) between workers. Each circle represents a nest association; names outside circles represent non-associated colonies. (a) parabiotic association of *Crematogaster limata* and *Odontomachus mayi* (Orivel et al. 1997), (b) lestobiotic associations of *Solenopsis gayi* and *Camponotus morosus* (Errard et al. 2003), (c) parabiotic associations of *Crematogaster modiglianii* and *Camponotus rufifemur* (present study).

In the lowland rainforest of Borneo, parabiotic associations between *Crematogaster modiglianii* and *Camponotus rufifemur* are common. The aim of the present study was to investigate the extent of interspecific tolerance as well as the behavioural mechanisms of nestmate recognition and tolerance in these associations. Our research questions were:
(1) Do these parabiotic ant species discriminate between intra- and allocolonial workers of their partner species or between their partner species and other congeneric species?
(2) Do parabiotic species show an unusually high degree of intraspecific tolerance?
(3) Is interspecific tolerance mediated by behavioural mechanisms such as submissive or appeasement behaviour?

III.3 Materials and Methods

III.3.1 Study Site

The study was conducted in September to November, 2004, and September to November, 2006, at Danum Valley Conservation Area. The area is located at 5° N 117°50' E and approximately 100 m a.s.l. in Sabah (Malaysian Borneo). It represents one of the major

remaining patches of Sabah's primary lowland rainforest. Danum Valley has a typical equatorial rainforest climate, with a mean annual temperature of 26.7° C and a yearly rainfall of 2700 mm. Rainfall is usually minimal during March to April and highest from October to January (Walsh and Newbery 1999).

III.3.2 In Situ Assays

We studied four parabiotic colonies (A to D) of *Camponotus (Myrmotarsus) rufifemur* and *Crematogaster (Paracrema) modiglianii*. These parabioses occur in hollow, living trunks of various tree species, especially often in *Eugenia chrysantha* trunks. For the colonies A, B and C, we separately tested the behaviour of both parabiotic species towards an introduced individual. The introduced individuals were *Ca. rufifemur* workers (majors or minors) and *Cr. modiglianii* workers from the other two colonies or control individuals from the same colony that had been captured several hours earlier and kept in a small plastic container. In the following, these colony combinations are given with two letters, the first referring to the resident colony and the second to the introduced individual. Intraspecific recognition within *Ca. rufifemur* was additionally tested with the colony combinations (A-D) and (D-A). The colonies A to C were located at least 500 m from each other and separated by rivers; colony D was ca. 10 m from B. For each combination, 9 to 13 replicates were performed. The in situ assays were conducted in plastic arenas that were attached to the nest trunk. They consisted of a plastic platform covered with tissue paper. This platform carried the arena, a plastic ring (∅11.5 cm, height 5 cm) coated with paraffin oil so that the ants could not walk on it. The arena was connected to the nest tree with a small twig to allow the ants to walk into the arena, and a tuna bait was placed into it to attract ants. 30 to 60 min after placing the bait, the bridge was carefully removed so that the ants (at least 10 individuals) were captured in the arena with a minimum of disturbance. Since *Ca. rufifemur* is a nocturnal species, we brought the baits out shortly before dusk and then conducted the experiments at night under red light. Some of the tests with *Cr. modiglianii*, however, were performed during the day, since we did not find a difference in nocturnal and diurnal activity or behaviour in this species. The behaviour of the two species was recorded in separate assays although both species were present in the arena in 22% of the cases (n = 391).

All interactions of any resident individual towards the intruder were counted for three minutes. For each interaction, the behaviour was recorded separately and categorized into amicable (antennating or trophallaxis), weakly aggressive (antennating with open mandibles) and aggressive (biting or locked mandibles) for further analysis. An interaction was regarded as finished when the resident ant moved away from the intruder and counted as new interaction if it returned later. Temporal effects in the recognition process were recorded by dividing the three minutes into 18 steps of 10 s. For each interaction, we recorded the time step during which it was observed. If interactions lasted longer than 10 s ('continued interactions'), they were recorded again in the following time step.

III.3.3 Arena Confrontations

These confrontations were performed in order to estimate the reciprocal aggressiveness of *Camponotus* and *Crematogaster* colonies, measured by the numbers of workers killed. Of either colony, three individuals were placed into an arena with forceps almost simultaneously. The arena consisted of a fluon-covered plastic cylinder (Ø 7.5 cm, height 5 cm) on top of a paper sheet that was replaced after each experiment. The number of dead individuals was counted after 60 min, seriously injured individuals being regarded as half dead (i.e. a value of 0.5, which rarely occurred). Arena confrontations were performed for all pairwise

combinations of a set of six *Crematogaster* colonies (*Cr. (Paracrema) modiglianii*: four parabiotic colonies, *Cr. (Paracrema) coriaria* and *Cr. (Physocrema) inflata*: one colony each) and seven *Camponotus* colonies (*Ca. (Myrmotarsus) rufifemur*: four parabiotic colonies, *Ca. (Colobopsis) saundersi, Ca. (Colobopsis)* sp. 1, and *Ca. (Tanaemyrmex) arrogans*: one colony each) with six to eight replicates per combination. All studied ant colonies were scattered along the forest trails within 2 km distance to the Danum Valley Field Center. Voucher specimen of all colonies will be deposited both at the Department of Zoology III, University of Würzburg and the Forest Resarch Center in Sepilok, Sabah (Malaysia).

III.3.4 Statistical Analysis

We used generalized linear models (GLMs) to obtain a mathematical description of the different variables affecting aggression. For each replicate of the in situ assays, we calculated the sum of aggressive interactions (all time steps pooled; including weak aggression and both starting and continued interactions), versus the sum of all non-aggressive interactions. We used a quasibinomial error distribution with a logit link function since our response variable consisted of two respective proportions (of the total sum of interactions) and was overdispersed. The explanatory variables were 'species combination', 'IN-AL'(intracolonial versus allocolonial), 'colony combination' and the respective interactions. 'Species combination' refers to the respective species of the observed and the introduced ants and contains four categories. 'Colony combination' refers to the respective parabiotic nests of the observed and the test ants and is nested within 'IN-AL'. The number of present *Cr. modiglianii* and *Ca. rufifemur* workers were included as covariates. The impact of each variable was determined by likelihood ratio tests (*F* test). All computations were performed in R Version 2.4 (R Development Core Team 2008). We constructed a comprehensive model for all species combinations, excluding the test series A-D and D-A to achieve a balanced experimental design, and additionally analysed separate models for each species combination, whereby the dataset for species combination '*Ca. rufifemur – Ca. rufifemur*' also included the colony combinations A-D and D-A.

In addition, temporal aspects of the intraspecific recognition process were analysed using three derived variables. The 'average occurrence time' x provided information at which time step aggression (or amicable behaviour, respectively) was observed on average. For each replicate, the scalar product between 'time step number' t and 'number of aggressive interactions in this time step' a_t was divided by the total number of aggressive interactions in this replicate, viz.:

$$x = \sum_{t=1}^{18} t \cdot a_t \,/\, \sum_{t=1}^{18} a_t$$

In addition, aggression latency was determined as the first time step when strong aggression was observed. Aggression latency as well as proportion of continued antennation were then analysed using Kruskal-Wallis *H* test followed by Nemenyi's test (for allocolonial confrontations only).

The arena confrontations were analysed using generalized linear models with binomial error distribution and a logit link function. The response variable was the number of killed versus living *Crematogaster* workers in each trial. We used two different models to test the following effects:

(1) Differential aggression of *Ca. rufifemur* towards different *Crematogaster* species and towards intra- and allocolonial *Cr. modiglianii.*

(2) Differential aggression of different *Camponotus* species towards *Cr. modiglianii.*

III.4 Results

III.4.1 In Situ Assays: Interspecific Recognition

The in situ assays revealed high *Cr. modiglianii* aggression towards allocolonial *Ca. rufifemur* but low *Ca. rufifemur* aggression towards both intracolonial and allocolonial *Cr. modiglianii* (for an overview, see Fig. 1c). The comprehensive model explained 84.6% of the original deviance (Table 1), while the separate models for each of the four species combinations explained 69.1 to 87.0% of the original deviance, except for the behaviour of *Ca. rufifemur* towards *Cr. modiglianii* (15.5% explained deviance, Tables 2, 3).
Cr. modiglianii discriminated between intracolonial and allocolonial *Ca. rufifemur*. However, aggression in different allocolonial colony combinations differed significantly (Table 2). *Cr. modiglianii* from colonies A and B showed low aggression towards both *Ca. rufifemur* A and B but attacked *Ca. rufifemur* C whereas *Cr. modiglianii* C workers attacked *Ca. rufifemur* from both colonies A and B (Fig. 2a,c). The aggressive reaction of *Cr. modiglianii* did not depend on the number of *Ca. rufifemur* nestmates in the arena (Table 2); altogether, *Ca. rufifemur* was present in only 12% of the *Cr. modiglianii – Ca. rufifemur* assays. In turn, *Ca. rufifemur* showed a weak, but significant discrimination between intracolonial and allocolonial *Cr. modiglianii*, albeit most of the interactions even towards allocolonial workers were amicable (Fig. 2c, Table 3). We often observed that, although *Crematogaster* workers heavily attacked the allocolonial *Camponotus* worker by locking mandibles into its legs, the latter did not bite *Crematogaster* to defend itself. Attacked *Camponotus* workers ignored the attacking *Crematogaster* or antennated them so that they ceased biting. Generally, *Camponotus* sometimes antennated *Crematogaster* workers very intensely and moved their mouthparts towards them. This behaviour, which was regarded as amicable, was observed both in intracolonial and allocolonial confrontations.

III.4.2 In Situ Assays: Intraspecific Recognition

Crematogaster modiglianii heavily attacked allocolonial conspecifics. We did not detect differences between colony combinations (Fig. 2b, Table 2). *Camponotus rufifemur* also clearly discriminated between intra- and allocolonial conspecifics. However, in contrast to *Cr. modiglianii*, there was a highly significant influence of colony combination on intraspecific aggression in this species; several allocolonial *Ca. rufifemur* confrontations yielded only low aggression levels (Fig. 2d, Table 3). Intraspecific *Camponotus* confrontations triggered two different types of aggression: ‘biting’, which was a short interaction (< 1 s) whereby the intruder was quickly dismembered, and ‘locked mandibles’, which could last for several minutes but rarely resulted in cut limbs. While biting was strictly confined to combinations of *Ca. rufifemur* C with A or B, locked mandibles occurred in other combinations as well. The aggressive response towards introduced ants was not always symmetrical. Colony D intruders were tolerated in colony A, but A individuals were heavily attacked in colony D (Mann-Whitney $U = 6$, $N_1 = N_2 = 10$, $P = 0.0009$). In contrast to other aggressive colony combinations, D workers showed ‘locked mandibles’ but never ‘biting’ behaviour towards A intruders. A similar, albeit weaker, asymmetry was found between the colonies A and B ($U = 23$, $N_1 = N_2 = 10$, $P = 0.04$).

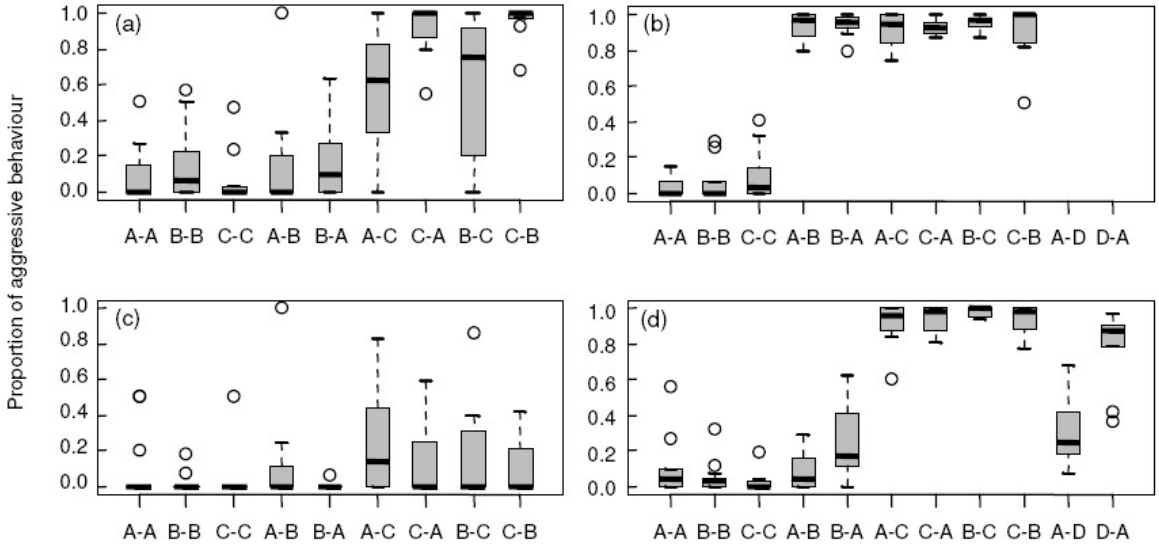

Fig. 2 Aggression (proportion of aggressive interactions of all interactions) among different colony combinations for in situ assays. Median, 1st and 3rd quantile and range are shown. The first letter refers to the resident colony, the second one to the intruder.
(a) *Crematogaster modiglianii* aggression towards *Camponotus rufifemur*; (b) *Cr. modiglianii* towards *Cr. modiglianii*, (c) *Ca. rufifemur* towards *Cr. modiglianii*; (d) *Ca. rufifemur* towards *Ca. rufifemur.*

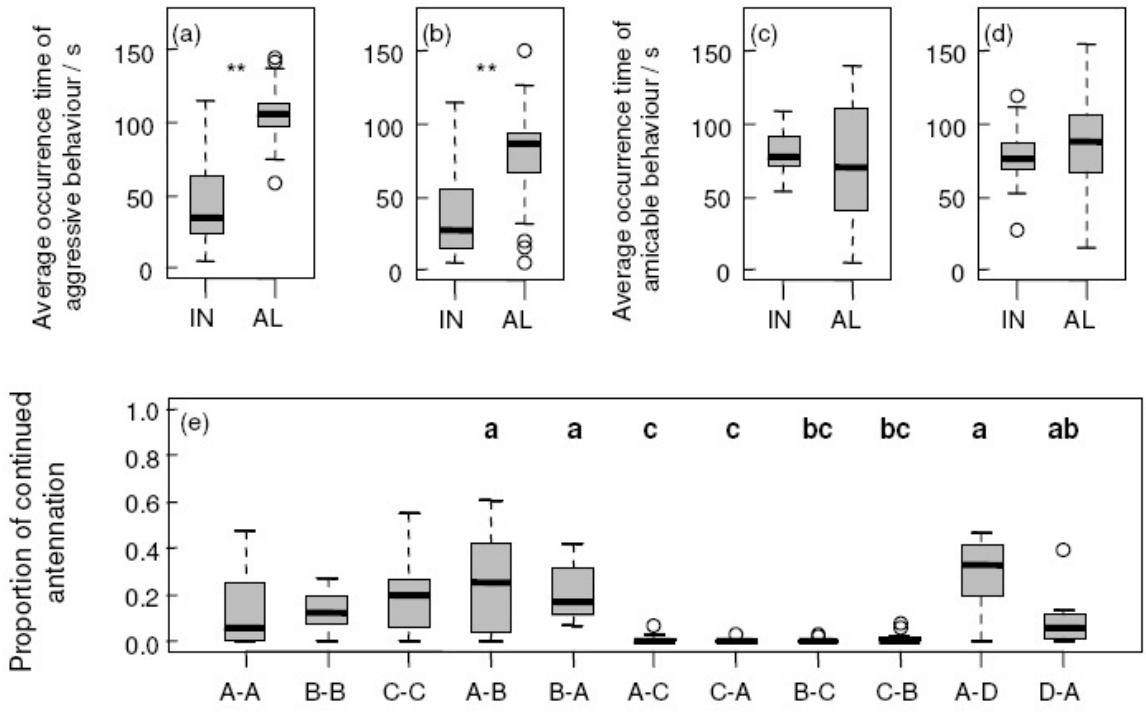

Fig. 3 Temporal aspects of the intraspecific recognition process in the in situ assays. Median, 1st and 3rd quantile and range are shown. The first letter refers to the resident colony, the second one to the intruder. (a), (b) Average occurrence time of aggressive behaviour in (a) intraspecific *Cr. modiglianii* and (b) intraspecific *Ca. rufifemur* assays. (c), (d) Average occurrence time of amicable behaviour in (c) intraspecific *Cr. modiglianii* and (d) intraspecific *Ca. rufifemur* assays. (e) Proportion of continued antennation in intraspecific *Ca. rufifemur* assays. **significant at $P = 0.001$. Bars with the same letters are not significantly different according to Nemenyi's test (allocolonial confrontations only).

Table 1 Generalized Linear Model (GLM) for aggression in the in situ assays with all species combinations included.

Parameter	Deviance	*df*	*F*	*P*
species combination	1788.5	3	33.61	<0.0001
IN-AL	3389.7	1	258.76	<0.0001
colony combination (nested in IN-AL)	1785.8	8	22.36	<0.0001
number *Cr. modiglianii*	2.5	1	0.54	0.46
number *Ca. rufifemur*	16.5	1	3.60	0.06
species combination:IN-AL	190.1	3	6.91	0.0002
species combination:colony combination	524.8	20	5.77	<0.0001
Residual error	1401.0	333		
total	9098.8	370		

Table 2 Generalized linear models for *Cr. modiglianii* aggression in the in situ assays.

	Cr. modiglianii towards *Ca. rufifemur*				*Cr. modiglianii* towards *Cr. modiglianii*			
Parameter	Deviance	*df*	*F*	*P*	Deviance	*df*	*F*	*P*
IN-AL	353.0	1	46.55	<0.0001	2199.7	1	436.28	<0.0001
colony combination (nested in IN-AL)	419.1	7	13.30	<0.0001	16.8	7	0.44	0.88
number *Cr. modiglianii*	2.4	1	0.53	0.47	6.8	1	1.22	0.28
number *Ca. rufifemur*	1.2	1	0.25	0.62	22.2	1	3.84	0.054
Error	346.0	82			335.8	73		
total	1121.5	92			2581.3	83		

Table 3 Generalized linear models for *Ca. rufifemur* aggression in the in situ assays.

	Ca. rufifemur towards *Cr. modiglianii*				*Ca. rufifemur* towards *Ca. rufifemur*			
Parameter	Deviance	*df*	*F*	*P*	Deviance	*df*	*F*	*P*
IN-AL	20.7	1	5.77	0.02	1306.3	1	61.00	<0.0001
colony combination (nested in IN-AL)	42.5	7	1.93	0.07	2064.6	9	32.51	<0.0001
number *Cr. modiglianii*	5.2	1	1.71	0.19	22.7	1	3.75	0.06
number *Ca. rufifemur*	0.0	1	0.001	0.98	85.1	1	13.14	0.0005
Error	260.5	89			573.1	101		
total	308.2	98			4052.0	113		

In most allocolonial confrontations between *Ca. rufifemur* individuals, aggression occurred already during the first 10 s. Aggression latency significantly differed between allocolonial colony combinations (H_7 = 18.52, P = 0.0098); it was highest in the less aggressive confrontation A-B, where first aggression occurred on average after 68.3 ±37.5 s, compared to the confrontations A-D (40.0 ±15.2 s), B-A (23.8 ± 12.2 s) and the remaining allocolonial confrontations (≤ 13.0 s). These three combinations also had high proportions of continued antennation, which were similar to intracolonial confrontations, but significantly differed from all other allocolonial confrontations except D-A (H_7 = 45.68, P < 0.0001, Fig. 3e). Intracolonial test ants elicited aggressive behaviour upon reintroduction as well. This

aggression, however, ceased soon whereas allocolonial intruders received constant or increasing aggression during the course of the observation period. Average occurrence time of aggression significantly differed in both intraspecific *Crematogaster* ($t_{90} = 5.56$, $P < 0.0001$; Fig. 3a) and intraspecific *Camponotus* confrontations ($t_{10.84} = 5.42$, $P = 0.0002$; Fig. 3b). In contrast, the average occurrence time of amicable behaviour did not differ between intra- and allocolonial confrontations in both species ($t_{49.5} = 1.1$, $P = 0.27$ and $t_{89.9} = 1.8$, $P = 0.07$; Figs 3c, d).

III.4.3 Arena Confrontations

The GLM for confrontations of *Camponotus rufifemur* with workers from *Cr. modiglianii* and other *Crematogaster* species explained 65.0% of the original deviance. *Ca. rufifemur* workers did not significantly differentiate between intracolonial and allocolonial *Crematogaster modiglianii* workers (Table 4). Although *Cr. modiglianii* frequently interacted with intra- and allocolonial *Ca. rufifemur* individuals, only 13 out of 288 *Cr. modiglianii* workers were killed. In a separate experiment, *Ca. rufifemur* workers also tolerated workers from a non-parabiotic *Cr. modiglianii* colony in spite of frequent interactions and killed no non-parabiotic *Cr. modiglianii* in a total of four arena confrontations. *Camponotus rufifemur* however significantly discriminated between different *Crematogaster* species, tolerating only *Cr. modiglianii* but mostly killing *Cr. coriaria* and *Cr. inflata* (Fig. 4a, Table 4). The *Ca. rufifemur* colonies strongly varied in their aggression towards the latter two *Crematogaster* species. Two *Ca. rufifemur* colonies killed on average 79% of the *Cr. coriaria* and 96% of the *Cr. inflata* workers while the other two colonies killed 12% and 15%, respectively. The significant interaction between *Ca. rufifemur* colony and *Crematogaster* species is due to these differences but equal tolerance of *Cr. modiglianii* by all *Ca. rufifemur* colonies.

All three studied non-parabiotic *Camponotus* species were highly aggressive against *Cr. modiglianii* (Fig. 4b-d, Table 5). Both *Ca. (Colobopsis)* sp. 1 and *Ca. (Colobopsis) saundersi* often killed *Cr. modiglianii* but mostly tolerated *Cr. coriaria* and *Cr. inflata* workers while *Ca. arrogans* was aggressive against all three *Crematogaster* species. The GLM, which explained 68.0% of the original deviance (number of killed *Cr. modiglianii*), also revealed highly significant differences between *Cr. modiglianii* colonies and a strong interaction term between *Cr. modiglianii* colony and *Camponotus* species. Possible effects of 'intracolonial' versus 'allocolonial' were not considered here since they had been shown to be not significant in the first GLM (Table 4). In all arena confrontations, *Camponotus* workers mostly survived the arena confrontations; in a total of 309 tests with 927 *Camponotus* individuals, only seven *Ca. (Colobopsis)* sp.1 and eight *Ca. (Colobopsis) saundersi* individuals were killed.

Table 4 Generalized linear model for the number of *Crematogaster* workers killed by workers from four *Ca. rufifemur* colonies in the arena confrontations.

Parameter	Deviance	*df*	*F*	*P*
IN-AL (nested within *Crematogaster* species: *modiglianii*)	2.8	1	2.84	0.09
Crematogaster species	159.0	2	79.49	<0.0001
Ca. rufifemur colony	93.6	3	31.20	<0.0001
IN-AL: *Ca.rufifemur* colony	1.9	3	0.62	0.61
Ca. rufifemur colony: *Crematogaster* species	23.5	6	3.92	0.0006
Residual error	151.3	156		
total	432.1	171		

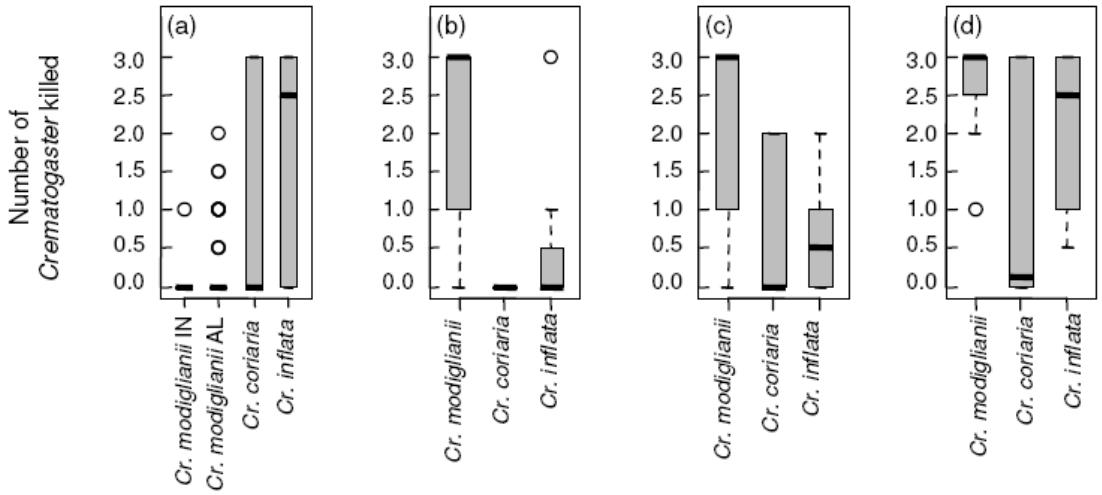

Fig. 4 Median number of killed *Crematogaster* individuals (with quantiles and range) in the arena confrontations. (a) *Ca. rufifemur* (intra- (IN) and allocolonial (AL) *Cr. modiglianii* are shown), (b) *Ca. (Colobopsis)* sp.1, (c) *Ca. (Colobopsis) saundersi,* (d) *Ca. arrogans.*

III.5 Discussion

III.5.1 Interspecific Recognition and Tolerance in Parabiotic Species

Both *Crematogaster modiglianii* and *Camponotus rufifemur* tolerated their respective parabiotic partner. *Cr. modiglianii* also tolerated *Ca. rufifemur* of certain foreign colonies, but strongly attacked *Ca. rufifemur* workers of others. These aggression patterns correspond to intraspecific aggression among the same *Ca. rufifemur* colonies (Fig. 2), suggesting that both species use similar recognition cues. Aggression of *Cr. modiglianii* also occurred when *Ca. rufifemur* nestmates were absent and thus did not depend on putative nestmate-*Camponotus* signals. *Cr. modiglianii* even differentiated between dead *Ca. rufifemur* workers, biting those from certain foreign colonies but only antennating those from others or the own colony (F. Menzel, unpublished data).

The attacked *Ca. rufifemur* individuals ignored the biting *Cr. modiglianii* ants or antennated them so that they ceased biting. This surprising unilateral tolerance accounts for the low number of killed allocolonial *Cr. modiglianii* in the arena confrontations. The fact that *Ca. rufifemur* tolerated allocolonial *Cr. modiglianii* workers but still showed slightly more aggression than towards intracolonial ones, either suggests that *Ca. rufifemur* received few nestmate recognition cues from *Cr. modiglianii* ('chemical insignificance' sensu Lenoir et al. 2001b) and was not able to recognize allocolonial *Cr. modiglianii* with certainty, or that allocolonial *Cr. modiglianii* were recognized as foreign but nevertheless tolerated. Notably, none of the amicable interactions between the two species involved any appeasement or otherwise submissive behaviour. However, the fact that attacking *Cr. modiglianii* ceased biting upon being antennated by *Ca. rufifemur* might be regarded as evidence of appeasement behaviour of *Ca. rufifemur* during aggressive interactions.

The tolerance of *Ca. rufifemur* towards *Cr. modiglianii* was species-specific and did not extend to other *Crematogaster* species. By analogy, *Cr. modiglianii* was only tolerated by its partner species but not by other *Camponotus* species. This species-specific high tolerance is in strong contrast to previous studies on other associated species. In parabioses of the

neotropical *Crematogaster limata* and *Odontomachus mayi*, both species tolerated their partner but were aggressive against allocolonial workers of the respective partner species in laboratory assays (Fig. 1a, Orivel et al. 1997). The tolerance patterns of the Southeast Asian parabioses rather resemble those of lestobiotic associations between *Solenopsis gayi* and *Camponotus morosus* (Fig. 1b,c, Errard et al. 2003).

Table 5 Generalized linear model for the number of *Cr. modiglianii* workers from four colonies killed by different *Camponotus* species in the arena confrontations.

Parameter	Deviance	*df*	*F*	*P*
Camponotus species	382.5	3	127.49	<0.0001
Cr. modiglianii colony	17.2	3	5.73	0.0006
Camponotus species:*Cr. modiglianii* colony	28.8	9	3.19	0.0007
Residual error	201.5	184		
total	629.9	199		

III.5.2 Intraspecific Recognition and Aggressiveness in Parabiotic Species

In contrast to a generally high aggression level between *Cr. modiglianii* colonies, intraspecific allocolonial confrontations in *Ca. rufifemur* yielded a gradient from complete tolerance to high aggression. Since mutual tolerance also occurred between distant colonies which were separated by rivers, they were unlikely to be part of a polydomous colony. In certain colony combinations, continued antennating and high aggression latencies provided evidence of recognition uncertainty. Recognition uncertainty has been found in previous studies in *Aphaenogaster senilis* (Lenoir et al. 2001a) and is probably caused by high chemical similarity between intruder and resident (Breed 2003). The low, but often observed aggression against intracolonial intruders may have been induced by keeping the test ant separate for several hours prior to the test, thereby altering the surface recognition cues. However, as evidenced by the average aggression times, this aggression quickly ceased whereas aggression against allocolonial intruders remained constant or increased over time. *Ca. rufifemur* colonies also differed in their overall aggressiveness, as was evident through both asymmetric intraspecific aggression and different aggression towards non-parabiotic *Crematogaster* species. The causes for mutual tolerance, as well as for differences in overall aggressiveness, are unknown. They might relate to different surface chemistry, different levels of intracolonial genetic diversity (Tsutsui et al. 2003) or different stages of the parabiotic association (e.g. colony size; Balas and Adams 1996).

III.5.3 The Experimental Setup of Nestmate Recognition Assays

The fundamental problem of detecting nestmate recognition is that recognition is a cognitive process and cannot be observed as such. Whether an ant fails to recognize its counterpart or tolerates it upon recognition can hardly be distinguished based on behavioural observations. In interspecific associations, another complication is the lack of a plausible null hypothesis, i.e. the expected behaviour given recognition failure is unknown. Many studies inferred a recognition failure due to chemical mimicry when two associated species possessed similar chemical profiles (Bagneres et al. 1991; Lenoir et al. 1997, 2001b), but assumed that the foreign profile was learned as an additional template when they differed (Errard 1994, Errard et al. 2003). Although this explanation is plausible, it is important to keep in mind that recognition failure cannot be shown in principle.

Laboratory assays are a popular method to estimate recognition since they are weather-independent and allow an exact experimental design as well as easy replication. Their reliability, however, remains questionable since aggression is often highly context-specific (Starks et al. 1998; Breed 2003; Velasquez et al. 2006); besides, laboratory-reared colonies often display lower intraspecific aggression than wild ones (Buczkowski et al. 2005; Buczkowski and Silverman 2005) and may not show differential behaviour towards different test ants. As mentioned, lack of differentiation cannot be assigned to lack of recognition. However, significant differentiations between test individuals observed in the laboratory are likely to have biological importance. Although rather coarse and conservative, carefully conducted laboratory assays can hence be meaningful tools to estimate intercolonial recognition and tolerance.

With the in situ assays we tried to mimic the natural conditions as much as possible, completely avoiding manipulating the resident ants or to immobilise the test ants during the experiment. However, we still did not detect significantly differential behaviour of *Ca. rufifemur* towards different *Cr. modiglianii.* Other ant associations in contrast showed strong differentiation between intra- and allocolonial allospecifics even in laboratory assays (Orivel et al. 1997). Especially given the high aggression detected in the other species combinations of our study, the lack of differentiation of *Ca. rufifemur* towards *Cr. modiglianii* is therefore unlikely to be an effect of experimental design or low sample size.

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IV. Cuticular substances of parabiotic ants

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IV.1 Abstract

Associations between animal species require that at least one of the species recognizes its partner. Parabioses are associations of two ant species which co-inhabit the same nest. Ants usually possess an elaborate nestmate recognition system, which is based on cuticular hydrocarbons and allows them to distinguish nestmates from non-nestmates through quantitative or qualitative differences in the hydrocarbon composition. Hence, living in a parabiotic association probably necessitates changes of the nestmate recognition system in both species, since heterospecific ants have to be accepted as nestmates.

In the present study we report highly unusual cuticular profiles in the parabiotic species *Crematogaster modiglianii* and *Camponotus rufifemur* from the tropical rainforest of Borneo. The cuticle of both species is covered by a set of steroids, which are highly unusual surface compounds. They also occur in the Dufour gland of *Crematogaster modiglianii* in high quantities. The composition of these steroids differed between colonies but was highly similar among the two species of a parabiotic nest. In contrast, hydrocarbon composition of *Cr. modiglianii* and *Ca. rufifemur* differed strongly and only overlapped in three regularly occurring and three trace compounds. The hydrocarbon profile of *Camponotus rufifemur* consisted almost exclusively of methyl-branched alkenes of unusually high chain lengths (up to C_{49}). This species occurred in two sympatric, chemically distinct varieties with almost no hydrocarbons in common. *Cr. modiglianii* discriminated between these two varieties. It only tolerated workers of the *Ca. rufifemur* variety it was associated with, but attacked the respective others. However, *Cr. modiglianii* did not distinguish its own *Ca. rufifemur* partner from allocolonial *Ca. rufifemur* workers of the same variety.

We conclude that there is a mutual substance transfer between *Cr. modiglianii* and *Ca. rufifemur*. *Ca. rufifemur* actively or passively acquires cuticular steroids from its *Cr. modiglianii* partner, while the latter acquires at least two cuticular hydrocarbons from *Ca. rufifemur*. The cuticular substances of both species are highly unusual regarding both substance classes and chain lengths, which may cause the apparent inability of *Cr. modiglianii* to discriminate *Ca. rufifemur* nestmates from allocolonial *Ca. rufifemur* workers of the same chemical variety.

IV.2 Introduction

Associations across different animal taxa require specific adaptations on one or both sides. In particular, recognizing the partner species is a crucial task to any form of association, albeit in host-parasite associations only the latter might need to recognize the partner (Lenoir et al. 2001b). Nestmate recognition mechanisms in associating species must therefore go beyond the own species and include the partner species.

In ants, one of the closest and most intriguing interspecific associations is parabioses, where two ant species live together in a common nest. This phenomenon is found in several parts of the world, including Southeast Asia (Menzel et al. 2008) and tropical South America (Orivel et al. 1997). Parabiotic ants have nestmates not only from their own colony, but also from a completely different species. Their nestmate recognition system therefore needs to include allospecific nestmates. In ants and other social hymenoptera, recognition is based on colony-specific chemical cues on the body surface that are perceived through olfactory or contact chemoreception (Hölldobler and Wilson 1990; Vander Meer and Morel 1998). Most of them are hydrocarbons (Lenoir et al. 1997, Lahav et al. 1999, Wagner et al. 2000). Via allogrooming and trophallaxis, the individuals continually take up their nestmates' surface compounds into the postpharyngeal gland (PPG), where they are mixed and redistributed. Through this process, a colony-specific odour is created (Boulay et al. 2000; Boulay et al. 2004; Soroker et al. 1994; Vander Meer and Morel 1998). This colony-specific odour is learned by the colony members and represented as a neuronal template in the nervous system (Errard et al. 2006). Nestmates are recognized by comparing the cuticular profile of the encountered individual to the neuronal template (phenotype matching), whereby a mismatch generally results in aggression (Vander Meer and Morel 1998).

Despite this complex nestmate recognition system, a considerable number of insect species manages to be accepted in Hymenoptera colonies, such as Lycaenid larvae, Staphylinidae, Ensifera, and Diptera (Akino et al. 1996; Akino et al. 1999; Akino 2002; Howard et al. 1990) as well as social parasites, such as the parasitic bumblebee *Psithyrus* (Dronnet et al. 2005) and inquiline ant species (Lenoir et al. 1997; Lenoir et al. 2001b]. In many of these associations, the parasite chemically resembles the host (chemical mimicry) (Akino et al. 1996; Akino et al. 1999; Akino 2002; Elgar and Allan 2004; Howard et al. 1990; Howard et al. 2001; Lenoir et al. 2001b). Another possible mechanism to remain incognito is chemical insignificance (Lenoir et al. 2001b). Several social parasite species are – like callows – chemically insignificant, i.e. they do not possess an individual surface profile and are hence not recognized as foreign by their hosts (D'Ettorre et al. 2002; Jeral et al. 1997; Lenoir et al. 2001b). Hydrocarbon profiles of very long chain lengths are difficult to perceive and hence may also promote chemical insignificance (Akino 2006; Lambardi et al. 2007). Still, numerous other social parasite species possess distinct profiles that do not resemble their hosts. Since these profiles neither show chemical mimicry nor insignificance, it has been supposed that the host species habituate to the parasites' profiles (Errard et al. 1996, Liu et al. 2000, 2003).

While the chemical mechanisms of tolerance between species have been studied in associations like social parasitism, little is known about parabiotic associations. It seems likely that parabiotic ants possess a nestmate recognition system that tolerates allospecific nestmates. In the present study we examined the relationship between interspecific tolerance and surface chemistry among the Southeast Asian parabiotic species *Crematogaster modiglianii* and *Camponotus rufifemur*. The two species tolerate ants from certain (but not

all) foreign parabiotic nests but attack non-parabiotic ant species (Menzel et al. 2008). We discovered that two morphological varieties of *Ca. rufifemur* (the 'red' and the 'black' variety, see Methods) also differ in their chemical profiles. This enabled us to study two different levels of chemical similarity – within and between the two varieties. Our research questions were:
(1) Do parabiotic species possess cuticular substances different from related, non-parabiotic species?
(2) Is there evidence for chemical mimicry, i.e., chemical overlap between parabiotic partners?
(3) Do chemical differences *within* species account for differences in interspecific allocolonial tolerance?

IV.3 Results

IV.3.1 Cuticular substances: Hydrocarbons and other aliphatic components

The cuticular profile of both *Camponotus rufifemur* and *Crematogaster modiglianii* highly differed from other, non-parabiotic *Camponotus* and *Crematogaster* species (Boulay et al. 2000; Boulay et al. 2003; Endler et al. 2004, Meskali et al. 1995; unpublished data). While there were only few aliphatic compounds with a chain length of C20-C33, both species possessed hydrocarbons of very high chain lengths (C35 up to C49, Fig. 1) as well as steroids, which have not previously been detected on insect cuticles. The aliphatic profile of *Crematogaster modiglianii* consisted of hydrocarbons between C33 and C40. Beside n-alkanes and methyl-branched alkanes, more than 68% of its aliphatic cuticular compounds were unsaturated (Fig. 1a, Tables 1, 2). Extracts of the body surface and postpharyngeal glands contained the same aliphatic substances in similar quantitative composition.
The *Camponotus rufifemur* surface profile mainly contained compounds beyond C_{38}, beside traces of lighter components. The two morphological varieties exhibit almost completely different surface profiles. The only substances in common were trace n-alkanes between C27 and C30 and C37-9-ene (Table 1). The red variety exhibited a highly unusual cuticular profile, 98% of the hydrocarbon quantities being methyl-branched alkenes. The main compounds, 27-MeC39-14-ene and 27-MeC39-16-ene, accounted for 88.7% of the total hydrocarbons. The other different methyl-branched alkenes were similar in respect to the positions of the methyl group and the double bond (Table 3). Chain lengths ranged from C38 to C41, with trace compounds between C24 and C37 (Fig. 1b, Tables 1,2).
The profile of the black *Ca. rufifemur* variety consisted of even larger molecules, with 92.8% of the surface compounds between C44 and C49 (Table 1). At least 80% of the compounds were unsaturated (Table 2). Methyl-branched alkenes were also present, albeit not as abundant as in the red *Ca. rufifemur* variety. Minor compounds included n-alkanes, methyl-branched alkanes and aldehydes (Table 2). In both *Ca. rufifemur* varieties, PPG and surface extracts contained the same aliphatic compounds in similar relative quantities.

IV.3.2 Cuticular substances: Steroid-like compounds

Besides aliphatic compounds, the surface profile of both ant species contained up to 24 components with a basic steroid structure (as inferred from mass spectra and diagnostic ions). Their mass spectra indicate a close chemical interrelatedness of the compounds (apart from one steroid mostly found in *Ca. rufifemur*). Due to the high substance quantities necessary for NMR analysis, their spatial molecular structure has not yet been resolved but is under investi-

Table 1 Aliphatic cuticular substances found in *Crematogaster modiglianii* and the two varieties of *Camponotus rufifemur.*
Relative peak areas (mean and standard error) for *Ca. rufifemur* and *Cr. modiglianii* are given based on FID data from n = 6 (red *Ca. rufifemur*), 3 (black *Ca. rufifemur*), and 8 (*Cr. modiglianii*) colonies. *found in less than 50% of the samples, +tentatively identified, ++position of double bond tentative, § number of substances and their exact structure could not be further determined. Retention indices beyond 44 are extrapolated.

	substance	substance class	retention index	red *Ca. rufifemur*	black *Ca. rufifemur*	*Cr. modiglianii*
1	C21	n-alkane	21		0.26 ± 0.01%	
2	C23:1	n-alkene	22.75		0.46 ± 0.03%	
3	unknown	unknown	22.9		0.20 ± 0.01%	
4	C23	n-alkane	23		0.16 ± 0.01%	
5	C24:1	n-alkene	23.78		0.49 ± 0.06%	
6	Docosenal+	aldehyde	24.07		0.40 ± 0.09%	
7	Docosenal+	aldehyde	24.12		0.20 ± 0.16%	
8	unknown	unknown	24.35		0.23 ± 0.06%	
9	12-MeC24	branched alkane	24.37	0.06 ± 0.05%		
10	11-MeC24	branched alkane	24.39		0.45 ± 0.02%	
11	C25	n-alkane	25			
12	Tricosenal+	aldehyde	25.11		0.11 ± 0.09%	
13	unknown	unknown	25.69	0.05 ± 0.02%		
14	unknown	unknown	25.7		0.67 ± 0.17%	
15	C26	n-alkane	26	0.02 ± 0.01%		
16	Tetracosenal+	aldehyde	26.09		0.72 ± 0.17%	
17	unknown	unknown	26.37		0.21 ± 0.03%	
18	unknown	unknown	26.72		0.08 ± 0.02%	
19	C27	n-alkane	27	0.13 ± 0.11%	0.13 ± 0.01%	
20	Pentacosenal+	aldehyde	27.15		0.47 ± 0.39%	
21	unknown	unknown	27.71	0.09 ± 0.04%		
22	unknown	unknown	27.73		0.52 ± 0.08%	
23	C28	n-alkane	28	0.09 ± 0.06%	0.14 ± 0.01%	
24	C29	n-alkane	29	0.21 ± 0.17%	0.27 ± 0.02%	
25	C30	n-alkane	30	0.01 ± 0.01%	0.20 ± 0.03%	
26	C31	n-alkane	31	0.15 ± 0.11%		
27	C32	n-alkane	32		0.08 ± 0.03%	
28	C35:1	n-alkene	34.85		0.26 ± 0.09%	0.15 ± 0.04%
29	C35	n-alkane	35.05		0.15 ± 0.06%*	0.76 ± 0.11%
30	17-MeC35, 15-MeC35, 13-MeC35	branched alkane	35.31			0.88 ± 0.25%
31	3-MeC35	branched alkane	35.74			0.3 ± 0.13%
32	C37:2	n-alkadiene	36.42			2.31 ± 0.28%
33	C37:2	n-alkadiene	36.51			1.56 ± 0.12%
34	C37:2	n-alkadiene	36.64			0.45 ± 0.07%
35	C37-13-ene, C37-14-ene, C37-15-ene, C37-16-ene	n-alkene	36.72			5.43 ± 0.49%
36	C37-9-ene	n-alkene	36.86		0.48 ± 0.15%	4.53 ± 0.4%

37	25-MeC37-14-ene, 25-MeC37-16-ene^{++}	branched alkene	36.96	0.44 ± 0.07%		
38	C37	n-alkane	37.05			0.52 ± 0.1%
39	19-MeC37, 17-MeC37, 15-MeC37, 13-MeC37, 11-MeC37	branched alkane	37.31			11.47 ± 0.28%
40	C38:2	n-alkadiene	37.45			0.18 ± 0.09%
41	11,27-DiMeC37, 11,25-DiMeC37	branched alkane	37.58			6.02 ± 0.33%
42	unknown	unknown	37.79			0.63 ± 0.1%
43	x(25,26,27)-MeC38-y(13,14,15,16)-ene$^{++\S}$	branched alkene	37.93	1.99 ± 0.11%		
44	C39:3	n-alkatriene	38.23			1.12 ± 0.13%
45	C39:3	n-alkatriene	38.3			1.46 ± 0.19%
46	C39:2	n-alkadiene	38.43			15.23 ± 0.76%
47	C39:2	n-alkadiene	38.53			13.7 ± 0.73%
48	C39-ene	n-alkene	38.73			3.66 ± 0.34%
49	unknown	unknown	38.79	0.55 ± 0.08%		
50	C39:1	n-alkene	38.79			7.62 ± 0.19%
51	C39:1	n-alkene	38.88			1.7 ± 0.08%
52	27-MeC39-14-ene, 27-MeC39-16-ene	branched alkene	39.02	88.66 ± 0.53%		3.15 ± 1.18%
53	19-MeC39, 17-MeC39, 15-MeC39, 13-MeC39, 11-MeC39	branched alkane	39.29	0.52 ± 0.24% (only 13-MeC39)		4.51 ± 0.2%
54	11,21-DiMeC39, 11,23-DiMeC39, 11,27-DiMeC39, 11,29-DimeC39	branched alkane	39.54			4.84 ± 0.52%
55	unknown	unknown	39.76	0.22 ± 0.01%		
56	27-MeC40-14-ene, 27-MeC40-15-ene, 27-MeC40-16-ene^{++}	branched alkene	39.97	3.41 ± 0.09%		
57	unknown	unknown	40.17			1.04 ± 0.18%
58	C40:3	n-alkatriene	40.35			0.36 ± 0.08%
59	C40:2	n-alkadiene	40.42			3.39 ± 0.24%
60	C40:2	n-alkadiene	40.57			3.01 ± 0.29%
61	x(27,29)-MeC41-y(14,16,18)-ene$^{++\S}$	branched alkene	40.94	3.35 ± 0.4%		
62	unknown	unknown	44.54		0.65 ± 0.12%	
63	unknown	unknown	44.68		0.45 ± 0.03%	
64	unknown	unknown	44.96		3.34 ± 0.19%	
65	C45:1	n-alkene	45.05		3.01 ± 0.04%	
66	36-MeC45:1	branched alkene	45.18		4.17 ± 0.06%	
67	unknown	unknown	45.49		1.09 ± 0.4%	
68	unknown	unknown	45.89		2.10 ± 0.16%	
69	unknown	unknown	45.97		0.98 ± 0.06%	
70	unknown	unknown	46.11		1.07 ± 0.06%	
71	C47:2	n-alkadiene	46.41		15.11 ± 0.52%	
72	C47:2	n-alkadiene	46.67		8.72 ± 0.37%	

73	C47:1	n-alkene	46.74	4.43 ± 0.18%
74	C48:1	n-alkene	46.88	22.95 ± 0.96%
75	C48:1	n-alkene	47.10	4.10 ± 0.07%
76	38-MeC47:1	branched alkene	47.16	9.20 ± 0.49%
77	unknown	unknown	47.42	1.49 ± 0.12%
78	unknown	unknown	47.46	1.29 ± 0.11%
79	unknown	unknown	47.81	1.91 ± 0.13%
80	unknown	unknown	48.01	0.54 ± 0.07%
81	C49:2	n-alkadiene	48.35	2.55 ± 1.7%
82	C49:2	n-alkadiene	48.45	2.40 ± 1.6%
83	C49:1	n-alkene	48.59	1.25 ± 0.11%

Table 2 Relative quantities of the different aliphatic substance classes in *Cr. modiglianii* and *Ca. rufifemur.*
Mean and standard error are given, based on FID data from n = 6 (red *Ca. rufifemur*), 3 (black *Ca. rufifemur*), and 8 (*Cr. modiglianii*) colonies.

substance class	**red *Ca. rufifemur***	**black *Ca. rufifemur***	***Cr. modiglianii***
n-alkane	0.64 ± 0.41%	1.25 ± 0.18%	1.29 ± 0.16%
n-alkene	0 ± 0%	37.44 ± 0.94%	23.06 ± 1.03%
n-alkadiene	0 ± 0%	28.77 ± 2.41%	39.83 ± 1.09%
n-alkatriene	0 ± 0%	0 ± 0%	2.8 ± 0.16%
branched alkane	0.58 ± 0.26%	0.45 ± 0.02%	28.07 ± 0.85%
branched alkene	98.1 ± 0.35%	13.37 ± 0.44%	3.15 ± 1.18%
aldehyde	0 ± 0%	1.9 ± 0.78%	0 ± 0%
unknown	0.91 ± 0.11%	16.82 ± 0.42%	1.77 ± 0.28%

Table 3 Diagnostic ions of the methyl-branched alkenes in the red *Ca. rufifemur* variety.
*diagnostic ion / molecule with respective double bond position at least twice as abundant as remaining ions / molecules; ** position of double bond was confirmed at the positions 14 and 16 via cleavage after ozonisation.

substance No.	**substance**	**diagnostic ions from hydration**	**diagnostic ions from DMDS derivatization**	**inferred double bond position**
37	25-MeC37-ene	196, 365	243*, 271, 355, 383*	14*, 16 or 21, 23*
43	25-MeC38-ene, 26-MeC37-ene, 27-MeC38-ene	182, 196, 210, 364, 379, 393	229, 243, 257, 271, 369, 383, 397, 411	13, 14, 15, 16 or 22, 23, 24, 25
52	27-Methyl-C39-ene	196, 393	243, 271, 383, 411	14**, 16** or 23, 25
56	27-Methyl-C40-ene	210, 393	243*, 257, 271, 397, 411, 425*	14*, 15, 16 or 24, 25, 26*
61	27-MeC41-ene, 29-MeC41-ene	196, 224, 392, 421	243, 271*, 299, 383, 411*, 439	14, 16*, 18 or 23, 25*, 27

gation. *Crematogaster modiglianii* possessed high amounts of steroids on the body surface (2.59 ± 0.58 µg/worker, n = 11 colonies, mean and SE) which by far exceeded the hydrocarbons (0.48 ± 0.05 µg/worker, n=11 colonies, mean and SE). In contrast, postpharyngeal gland extracts only contained minor amounts of steroids but high quantities of hydrocarbons. High steroid amounts of the same quantitative composition were also found in the Dufour gland, in separate alitrunk and gaster cuticular extracts and, albeit in lower amounts, in head cuticular extracts. They also occurred in cuticular extractions of living ants with SPME fibres, thus confirming that their presence in hexane extracts was not an artefact of concomitantly extracted glands. Altogether, *Cr. modiglianii* extracts contained 24 different steroid components with an abundance higher than 0.1% in at least one colony (percent of total steroid abundance). Their retention indices ranged between 20.38 and 25.77. Six of the 24 steroids were found in all *Cr. modiglianii* colonies in similar relative compositions. An additional eleven steroids were abundant in certain colonies but absent in others. The remaining seven steroids were irregularly found and never occurred in relative abundances higher than 1% (percent of total steroid abundance). *Camponotus rufifemur* extracts (both varieties) contained up to eight different steroids, all of which also occurred in *Cr. modiglianii*. The absolute steroid quantities in *Ca. rufifemur* were lower than the hydrocarbon quantities (black variety: 0.66 ± 0.22 µg steroids/worker and 1.79 ± 0.29 µg hydrocarbons/worker, n = 3 colonies; red variety: 0.41 ± 0.14 µg steroids/worker and 9.71 ± 3.79 µg hydrocarbons/worker, n = 4 colonies, mean and SE given).

IV.3.3 Chemical overlap among the parabiotic species

Six hydrocarbons were shared between both parabiotic species. The red *Ca. rufifemur* variety shared three hydrocarbons with *Cr. modiglianii*. These were the two methyl-branched alkenes, 27-MeC39-14-ene and 27-MeC39-16-ene, which are the main constituents of the red *Ca. rufifemur* surface profile, and its saturated derivative, 13-MeC39 (Table 1). All three are absent in the black *Ca. rufifemur* variety. *Cr. modiglianii* colonies living with the red *Ca. rufifemur* variety (henceforth, 'red' *Cr. modiglianii*) exhibited significantly more 27-MeC39-14-ene and 27-MeC39-16-ene than those associated with the black variety (henceforth, 'black' *Cr. modiglianii*) (Mann-Whitney W = 30, p = 0.0043; $N_1 = 5$, $N_2 = 6$ colonies, Figure 2). The quantities of 13-MeC39 were not compared since they could not be separated from other methyl-branched C39 alkanes in *Cr. modiglianii* (Table 1). Traces of three other hydrocarbons common in *Cr. modiglianii* were detected in the black *Ca. rufifemur* variety (C35:1, C35, C37-9-ene, Table 1). Albeit the associated *Cr. modiglianii* possessed slightly more C37-9-ene than those living with the red variety, no significant differences were found.
Eight of the steroids common in *Cr. modiglianii* were also frequently found in *Ca. rufifemur* (inclusion criterion: median abundance > 0% in 11 colonies of both species; Fig. 1d). Their relative abundances varied between parabiotic nests but were significantly correlated among the two species within a nest (Mantel test: r = 0.49, p = 0.041, N = 11; Bray-Curtis distances: 0.13 ± 0.08 (*Ca. rufifemur*), 0.43 ± 0.31 (*Cr. modiglianii*); mean and s.d.). A second Mantel test considered only three steroids with very similar mass spectra, which were present in all extracts (retention indices: 21.92, 22.24, 24.47; marked with asterisks in Fig. 1d). This test yielded a highly significant correlation of steroid abundance among the two species of each parabiotic nest (r = 0.620, p < 0.001, N = 11; Bray-Curtis distances: 0.06 ± 0.04 (*Ca. rufifemur*), 0.13 ± 0.07 (*Cr. modiglianii*)).

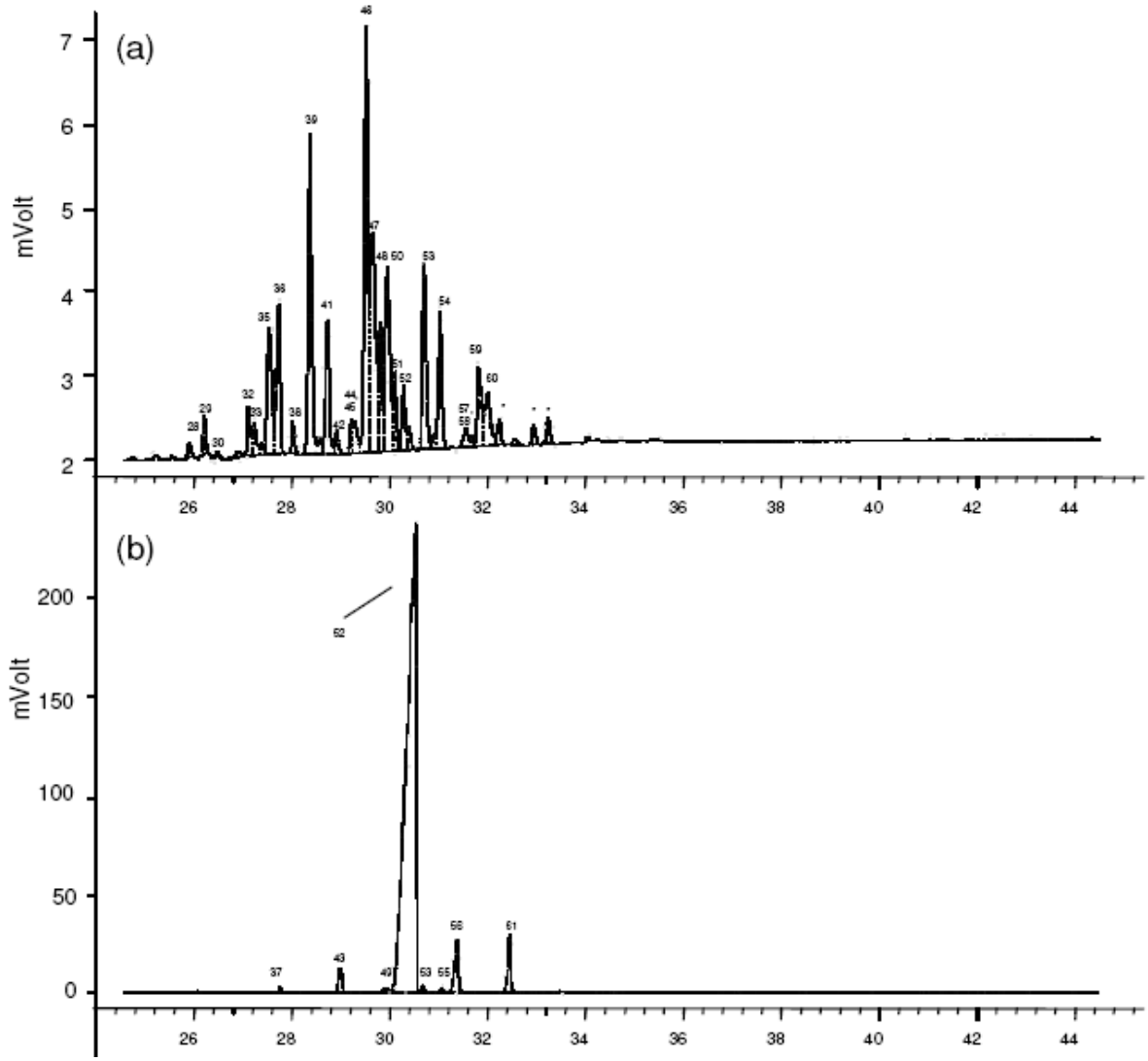

Fig. 1 a-b Gas chromatograms of cuticular hydrocarbons of the parabiotic ant species.
(a) *Crematogaster modiglianii* B2, (b) red *Camponotus rufifemur* R2, Graphs were acquired with a GC-FID. Only substances beyond a chain length of 34 are shown since shorter hydrocarbons make up less than 2% of the profile. Numbers refer to table 1. *unknown, irregularly occurring substance

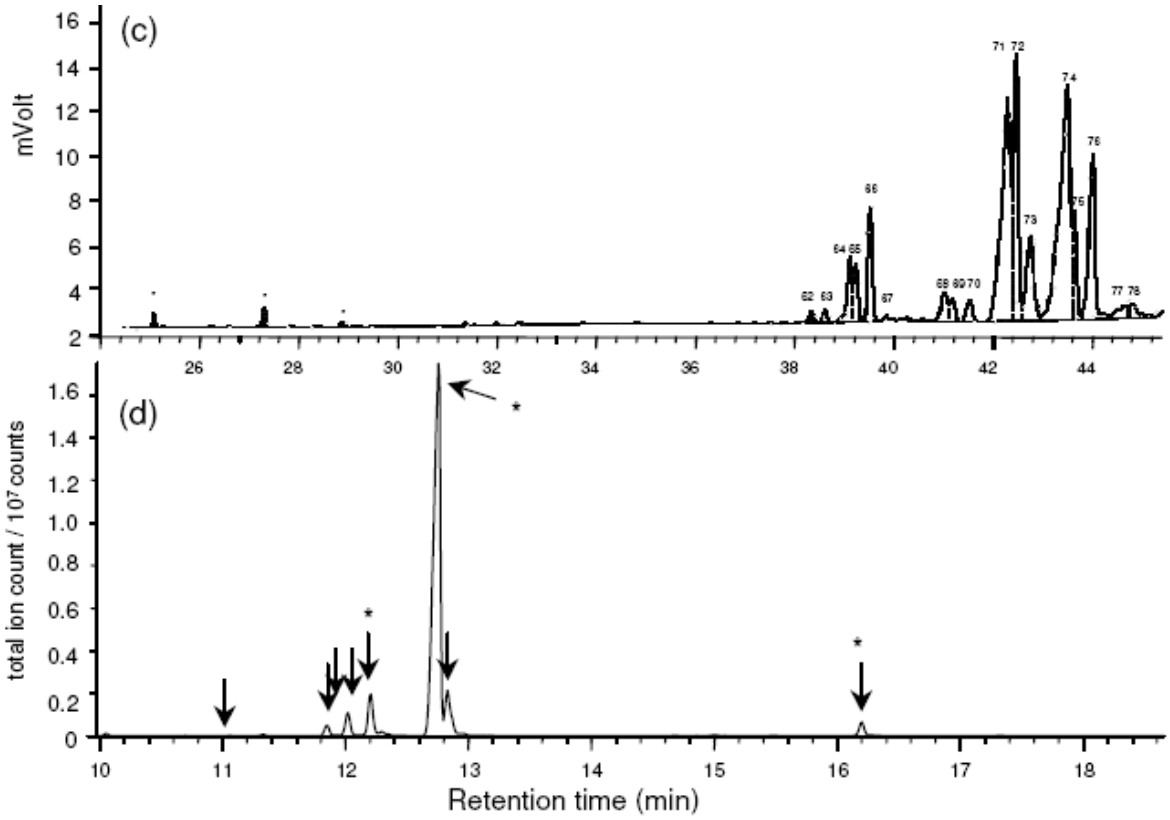

Fig. 1 c-d Gas chromatograms of cuticular hydrocarbons of the parabiotic ant species.
(c) black *Camponotus rufifemur* B4. Graphs were acquired with a GC-FID. Only substances beyond a chain length of 34 are shown since shorter hydrocarbons make up less than 2% of the profile. Numbers refer to table 1. *unknown, irregularly occurring substance
(d) Typical chromatogram of the cuticular steroids of *Cr. modiglianii*, acquired with GC-MS. Arrows indicate the steroid compounds common to both *Cr. modiglianii* and *Ca. rufifemur*. Asterisks indicate the three steroids with highly similar mass spectra used for the second Mantel test. No other steroids were present in the colony shown.

IV.3.4 Differences in allocolonial tolerance

Chemical differences between the two *Ca. rufifemur* varieties accounted for much of the variance in interspecific confrontations. In general, *Cr. modiglianii* workers tolerated only allocolonial *Ca. rufifemur* workers of the variety they were associated with. The focal *Crematogaster modiglianii* colony, which lived together with the red *Ca. rufifemur* variety, showed high aggression towards dead workers of the black *Ca. rufifemur* variety but not towards those of the red one (Fig. 3). The generalized linear model (GLM) for total aggression explained 65.6% of the total deviance and yielded a highly significant effect of the *Camponotus* variety (58.7% explained deviance, Table 4). The remaining deviance could in part be attributed to differences between *Camponotus* colonies ($p = 0.04$), whereas the difference between intracolonial and allocolonial *Camponotus* was not significant ($p = 0.12$, Table 4). The non-parabiotic *Camponotus (Tanaemyrmex) arrogans* was attacked to a similar degree as the black *Ca. rufifemur* variety (Fig. 3). When the analysis focused on the proportion of strong aggression only, the results were similar, with slightly stronger effects. *Cr. modiglianii* very rarely climbed onto the *Ca. rufifemur* bodies in this experimental series ('mounting behaviour').
In the arena confrontations, *Cr. modiglianii* was significantly more aggressive towards *Ca. rufifemur* from the respective other variety. The parameter 'within/across variety' explained 18.2% of the total deviance, followed by 'variety combination' (13.0% explained deviance), while 'intra-/allocolonial' did not explain a significant part of the deviance (Table 5). *Cr.*

modiglianii workers frequently climbed on *Ca. rufifemur* bodies and walked around on them for up to one minute. This 'mounting behaviour' represented on average 18.6% of all interactions (Fig. 4). The workers (especially in one of the two colonies) mounted *Ca. rufifemur* workers of their 'own' variety in significantly higher proportions (GLM for both colonies: $F_{df=1} = 6.85$, $p = 0.011$) but did not otherwise differentiate between intracolonial and allocolonial *Ca. rufifemur* workers ($F_{df=1} = 0.14$, $p = 0.71$).

In order to examine whether the differentiation between the colour varieties occurred in colonies *in situ* as well, we re-analyzed previous behavioural experiments reported in (Menzel et al. 2008). Allocolonial aggression of *Cr. modiglianii* towards *Ca. rufifemur* was highly variable in this dataset, and we confirmed a high impact of the two chemical varieties on allocolonial aggression. The variable 'within/across varieties' (colonies A and B: black variety, colony C: red variety) explained 60.1% of the total variance of the data and was a clearly more powerful predictor than the differentiation between intra- vs. allocolonial combination (0.03% deviance explained, Table 6). 'Red' *Cr. modiglianii* colonies only attacked black *Ca. rufifemur* intruders and vice versa (Fig. 5a). The highly significant impact of 'variety combination' (Table 6), however, showed that red *Cr. modiglianii* was more aggressive towards black *Ca. rufifemur* than black *Cr. modiglianii* towards red *Ca. rufifemur*.

In confrontations of *Ca. rufifemur* towards allocolonial *Cr. modiglianii*, Menzel et al. (Menzel et al. 2008) had found low levels of aggression albeit they were higher than against intracolonial *Cr. modiglianii*. Similar to above, *Ca. rufifemur* workers were more aggressive towards *Cr. modiglianii* from the respective other variety (Fig. 5b, Table 6).

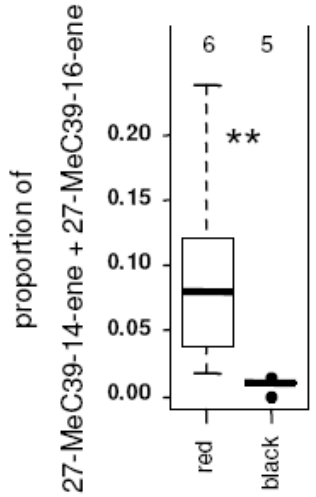

Fig. 2 Relative abundance of 27-MeC39-14-ene and 27-MeC39-16-ene in *Cr. modiglianii* workers living with the red vs. *Cr. modiglianii* workers living with the black *Ca. rufifemur* variety.
Median, quartiles, range, and outliers (i.e. all data points deviating from the box by more than 1.5 times the interquartile range) are shown in the present and the following figures. The number of analyzed colonies is given above each plot.
**highly significant (p=0.0043) according to U test.

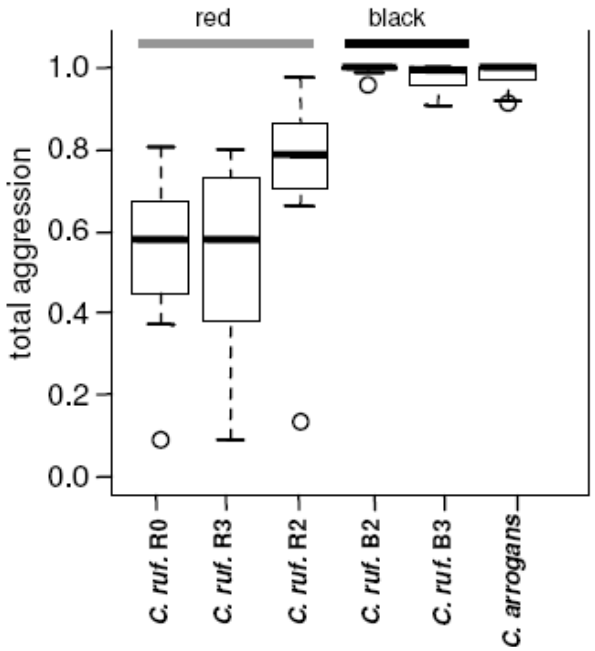

Fig. 3 Total aggression of *Crematogaster modiglianii* (colony R0) against different *Camponotus* colonies and species.
Data are given as proportions in relation to the total number of interactions. Each plot represents 10 replicates.

Table 4 GLM for total aggression of *Cr. modiglianii* towards dead *Ca. rufifemur* workers from different colonies.
Data from behavioural experiments with a *Cr. modiglianii* laboratory colony. '*Ca. rufifemur* colony' is nested within '*Ca. rufifemur* variety'.

Parameter	Deviance	*df*	*F*	*P*
Ca. rufifemur variety	735.3	1	74.16	<0.0001
Ca. rufifemur colony	62.8	2	3.45	0.040
intra-/allocolonial	24.7	1	2.53	0.12
residual error	430.0	46		
total	1252.9	50		

IV.4 Discussion

IV.4.1 Unusual features of the cuticular profiles in parabiotic ants

To our knowledge, steroids have not been found in surface extracts of other ant species up to now, and to our knowledge have been found on insect cuticles only in one halictid bee (Ayasse et al. 1999). However, various *Crematogaster* species are known to have highly efficient poisons (Heredia et al. 2005; Marlier et al. 2004). The genus *Crematogaster* has evolved a peculiar system of venom production which involves a cooperation of Dufour and poison gland. In several species the venom consists of precursors from the Dufour gland which are derivatized by enzymes from the poison gland (Leclerq et al. 1997; Pasteels et al. 1989). *Crematogaster* poisons – from Dufour and poison glands, but also from hypertrophied metapleural glands – belong to such different chemical classes as cyclohexan derivatives, crematofuranes (cembranoid diterpenes), coumarin derivatives, alkylphenols, alkylresorcinols, salicylic acids, resorcylic acids, and polyfunctionalized long-chain derivatives (Daloze et al. 1998; Jones et al. 2005; Laurent et al. 2003; Leclerq et al. 1997; Leclerq et al. 2000). Since extracts of *Cr. modiglianii* Dufour glands contained the same steroid composition as the body surface (but no other compounds), they are probably produced in this gland and then distributed onto the body surface. In *Cr. modiglianii,* steroid synthesis did not depend on biosynthetic precursors acquired from food. In two colonies kept in the laboratory for 15 and 6 months, respectively, the steroid profile did not change despite of an artificial diet of cockroaches, honey solution and Bhatkar diet (F.M. pers. obs.). Moreover, in one forest colony, the steroid profile remained relatively constant over three years, corroborating that the steroid composition is rather genetically determined than dependent on environmental factors.

It is notable that 98% of the entire hydrocarbon profile of the red *Ca. rufifemur* (and ≥ 13% of the black *Ca. rufifemur* hydrocarbon profile) were methyl-branched alkenes. This substance class seems to be generally very rare in insects and has been detected only in several Diptera and one Noctuid moth as pheromones (Carlson et al. 2005; Etges and Jackson 2001; Francke et al. 2000). Among ants, they have been found in traces in the ponerine ant *Pachycondyla villosa* and in two *Leptothorax* species (Lucas et al. 2002; Tentschert et al. 2002), but in higher abundances only in *Nothomyrmecia macrops* surface profiles, which is probably the most primitive existent ant species (Brown et al. 1990). That they make up almost the entire hydrocarbon profile is therefore highly unusual. Another unusual feature in both parabiotic species is the high hydrocarbon chain lengths. Although common in this study (Table 1), hydrocarbons beyond C_{37} have not been found in non-parabiotic *Camponotus* and

Crematogaster species (Boulay et al. 2000; Boulay et al. 2003; Endler et al. 2004; unpublished data). Other studies report small concentrations of heavier hydrocarbons in other ant genera, but always accompanied by high amounts of lighter ones (Dalecky et al. 2007; Lucas et al. 2004). It is possible that extremely long-chain hydrocarbons are difficult to perceive by receptors and thus promote interspecific tolerance (Gibbs and Pomonis 1995; Lambardi et al. 2007). In one case, we observed that a non-parabiotic *Cr. modiglianii* colony was initially very aggressive against (black) *Ca. rufifemur* workers but treated them amicably (and had hence become habituated) after less than 24h of exposure. Unsaturation in these long-chain hydrocarbons might be necessary to maintain a minimum fluidity of the cuticular profile (Gibbs and Pomonis 1995).

IV.4.2 Chemical overlap among parabiotic partners

Given the high allocolonial tolerance between parabiotic partners, the hydrocarbon overlap of the two species is surprisingly small. While the red *Ca. rufifemur* variety shared two compounds with its partner, the black variety only shared three trace compounds with *Cr. modiglianii* but otherwise possessed a completely different hydrocarbon profile. We tentatively suppose that *Cr. modiglianii* acquires 27-MeC39-14-ene 27-MeC39-16-ene from its red *Ca. rufifemur* partner although *Ca. rufifemur* generally tolerates *Cr. modiglianii* workers, including those lacking these substances (Menzel et al. 2008). In a *Cr. modiglianii* colony kept in the laboratory without its previous red *Camponotus* partner, the compound disappeared from the profile after eight months of separation (F.M. pers. obs.). It is possible that the other hydrocarbons of the red *Ca. rufifemur* are acquired by *Cr. modiglianii* as well but remain beyond detectability due to their low abundances. The hydrocarbons of the black *Ca. rufifemur*, in contrast, were never found on *Cr. modiglianii* surface extracts. This is probably due to their high chain lengths, which makes the cuticular profile more solid and do not allow chemical transfer (Gibbs and Pomonis 1995). In the light of the low overall hydrocarbon overlap among the two parabiotic ant species, chemical camouflage, a mechanism often found in social parasites (Akino et al. 1996, 1999, Akino 2002), must be dismissed as an explanation for mutual tolerance. However, the existence of only few substances common to both species might be a sufficient signal for tolerating the partner (D'Ettorre et al. 2004).

The steroid components, in contrast, showed high congruence among both species. We found that the relative composition of eight steroid compounds differs between colonies but is very similar among the two species of a parabiotic nest. Since it is highly improbable that *Ca. rufifemur* is able to synthetically copy the steroid profile of each respective partner colony, this result suggests that *Ca. rufifemur* acquires steroids from *Cr. modiglianii*. Notably, only a certain set of steroids is transferred to *Camponotus*, while others, despite of high abundance in *Cr. modiglianii*, were almost or completely absent from the *Ca. rufifemur* profile.

IV.4.3 Possible transfer mechanisms

Two mechanisms seem possible for the observed transfer of chemical cues, namely trophallaxis and direct physical contact. Via trophallaxis, individual ants exchange not only food but also the PPG content, i.e. hydrocarbons relevant for nestmate recognition (Lenoir et al. 1999). The PPG of *Cr. modiglianii* indeed contained steroids, albeit in much lower concentrations than on the body surface, thus making trophallaxis a possible pathway for chemical transfer. Interspecific trophallaxis has been observed between the two parabiotic species (F.M. and A. Endler, pers. obs.) and also shown via stained food only fed to *Cr. modiglianii* (F.M., pers. obs.).

Another possible transfer mechanism is direct physical contact. We frequently observed that *Cr. modiglianii* climbed on living or dead *Ca. rufifemur* individuals (workers and alates). The latter sometimes tried to shake them off but did not show aggression. Though almost never observed in the field, this 'mounting behaviour' could be easily induced in the laboratory by keeping the two species separate for one or two days. Mounting may therefore represent another possible mechanism for transfer of surface chemicals.

IV.4.4 Partner recognition is not colony-specific

The red and the black variety of *Camponotus rufifemur* are chemically distinct and – apart from trace compounds – do not share any hydrocarbons. The two dominant surface components of the red variety (substance #52, Table 1) are present in *Crematogaster modiglianii* colonies associated with this *Ca. rufifemur* variety but almost completely absent from those living with the black variety. Their abundance thus allows separating 'red' from 'black' *Cr. modiglianii* albeit the remaining surface profile is similar. The existence of two chemical *Ca. rufifemur* varieties accounts for most of the aggression variance in allocolonial encounters between the two species. *Cr. modiglianii* usually tolerated living or dead *Ca. rufifemur* workers of the same variety as their parabiotic partner but fiercely attacked those of the respective other variety (Figs 3, 4, 5, Tables 4, 5, 6). An analogous pattern was found in *Ca. rufifemur*. Despite of generally low aggression levels, black *Ca. rufifemur* workers were significantly more aggressive towards 'red' *Cr. modiglianii* workers than towards allocolonial 'black' *Cr. modiglianii* (Fig. 5b). However, we did not detect a corresponding difference in the red *Ca. rufifemur*.

While much of the interspecific aggression can be explained by chemical differences, however, the low interspecific aggression *within* chemical varieties is still surprising. Rather than recognizing heterospecific nestmates, the two species seemingly recognize only the chemical variety of their partner and do not discriminate within these varieties. Nestmate recognition rather depends on volatile substances than on substances only perceivable through antennal contact (Brandstaetter et al. 2008). Due to their low volatility (Gibbs and Pomonis 1995), very long-chain hydrocarbons are less detectable than short-chain molecules. Thus, olfactory receptors may additionally absorb traces of lighter hydrocarbons, thereby blur inter-colony profile differences and hampering inter-colony discrimination (Lambardi et al. 2007). The role of the steroids in the nestmate discrimination process is still unclear and under investigation.

The high interspecific tolerance strongly contrasts with the South American parabioses of *Crematogaster limata* and the ponerine ant *Odontomachus mayi*, where the ants never tolerated heterospecific workers from foreign parabioses (Orivel et al. 1997). In these associations, very low chemical overlap was found (no substance data given), suggesting that both species habituated to each other's colony-specific profiles. The associated Chilean species *Camponotus morosus* and *Solenopsis gayi* also showed distinct hydrocarbon profiles (Errard et al. 2003). In contrast to non-associated colonies, however, associated *Ca. morosus* had acquired small amounts of the *S. gayi* hydrocarbons. In both of these species, only individuals from associated colonies were tolerant towards allocolonial allospecifics (Errard et al. 2003), indicating that the acquisition of allospecific hydrocarbons promoted mutual tolerance.

IV.5 Conclusions

In this study we document the cuticular chemistry of the parabiotically associated ant species *Camponotus rufifemur* and *Crematogaster modiglianii*. In contrast to neotropical parabioses, these ant species did not show heterospecific nestmate recognition. In our experiments, *Cr. modiglianii* did not discriminate its partner *Ca. rufifemur* colony from other *Ca. rufifemur* colonies of the same chemical variety (nor vice versa). Rather, *Cr. modiglianii* distinguished only between the two *Ca. rufifemur* varieties, accepting the familiar one but attacking the respective other. This reduced discrimination of heterospecific nestmates may be caused by two unusual properties of the cuticular surface: Transfer of *Ca. rufifemur* hydrocarbons to the *Cr. modiglianii* profile (in one of the *Ca. rufifemur* varieties only), and the generally high chain hydrocarbon lengths in the two parabiotic species. As hypothesized elsewhere (Lambardi et al. 2007), extremely long-chain hydrocarbons may be difficult to perceive by receptors and hence promote chemical insignificance (sensu Lenoir et al. 2001b). It is currently investigated whether the cuticular steroids unique to these species play a role in nestmate or partner recognition.

IV.6 Materials and Methods

IV.6.1 Study site and ants

The studies were conducted at Danum Valley Conservation Area from September to November in the years 2004 and 2007. Danum Valley represents one of the major remaining patches of tropical lowland rainforest in Sabah (Malaysian Borneo). The site has a typical equatorial rainforest climate with a mean annual temperature of 26.9 °C and a yearly rainfall of 2700 mm. We studied parabiotic associations of *Camponotus (Myrmotarsus) rufifemur* Emery 1900 and *Crematogaster (Paracrema) modiglianii* Emery 1900. Their nests are commonly found in hollow, living tree trunks in the rainforest. Extracts of one parabiotic nest from the Kuala Belalong Field Studies Center (Brunei) were analyzed in addition.

Camponotus rufifemur occurs in two sympatric morphological varieties that have not previously been described (although Emery (1900) notes that specimen from Sarawak are darker in colour than those from Sumatra). While one variety (henceforth, 'red' variety) has a reddish alitrunk and light red-brown legs, the other one (henceforth, 'black' variety) possesses a black alitrunk and dark red-brown legs. The area between the frontal carinae of soldiers is dull in the red but shining in the black variety. Although the ratio head width/scape length (in frontal view) tends to be higher in the large soldier caste of the black variety than in that of the red variety, no significant morphometric differences were found. In the following, we will refer to the varieties as 'red' and 'black' *Ca. rufifemur*. In order to allow a differentiation, the respective associated *Cr. modiglianii* will be called 'red' and 'black' *Cr. modiglianii* although we did not find morphological distinctions within this species. Voucher specimen of *Cr. modiglianii* and both *Ca. rufifemur* varieties are deposited at the Department of Zoology III, University of Würzburg and at the Forest Resarch Center in Sepilok, Sabah (Malaysia).

IV.6.2 Preparation of extracts

Extracts were prepared from both body surface and postpharyngeal glands (PPGs). For body rinses, 10 to 90 ants were killed by freezing and immersed in hexane for ten minutes. Extracts from single individuals contained quantities too low for reliable substance identification. Eleven parabiotic nests were sampled with one to eight (mean: 3.5) replicates per colony and species (ten from Danum Valley, one from Kuala Belalong). PPG extracts were obtained from three to four freshly dissected PPGs per sample dissolved in hexane. Octadecane (n-C_{18}) was

used as internal standard in most samples. Cuticular substances were additionally obtained from living *Cr. modiglianii* workers brought into the laboratory in Würzburg with solid-phase microextraction (SPME). A SPME fibre (Supelco) coated with a 100 µm polydimethylsiloxan film was rubbed on the ant for 3 min and then directly injected into a ThermoQuest Trace GC.

IV.6.3 Chemical analysis

Substances were identified by coupled capillary gas chromatography-mass spectrometry (GC-MS) with a Hewlett Packard 6890 series gas chromatograph coupled to a HP 5973 Mass Selective Detector. The GC was equipped with a J&W Scientific DB-5 fused silica capillary column (30 m x 0.25 mm ID; df = 0.25µm). Temperature was kept at 60 °C for 2 min then increased by 60 °C/min up to 200°C and subsequently by 4 °C/min to 320 °C, where it remained constant for 10 min. Helium was used as carrier gas with a constant flow of 1 ml/min. A split/splitless injector was installed at 250°C in the splitless mode for 30 s. The electron impact mass spectra (EI-MS) were recorded with an ionisation voltage of 70 eV, a source temperature of 230°C and an interface temperature of 325°C. For analysis of hydrocarbons beyond C41, we used a DB-1 HT column (30m x 0.25 mm ID; df = 0,25µm). Temperature was raised from 60 °C by 5 °C/min up to 350 °C and then kept constant for 10 min. The interface had a temperature of 350°C. All other settings were as above. The software MSD ChemStation (Version A.03.00) for Windows was used for data acquisition. We restricted the analyses to substances with a retention time beyond that of C19 since compounds with shorter chain length are likely to be too volatile to be relevant for nestmate recognition (Lenoir et al. 1997; Vander Meer and Morel 1998). Substances present in less than 50% of the samples are given in Table 1 (marked with *) but were disregarded from further analysis.

For quantification of steroid-like compounds and aliphatics shorter than C_{33}, we used ion counts from the GC-MS data and analysed both substance classes separately. Heavier hydrocarbons (beyond C33) were quantified using a high-resolution ThermoQuest Trace GC-FID with H_2 as carrier gas in order to achieve a better separation of the substances. We used a nonpolar capillary column [DB1 (J&W Scientific, Folsom, CA), 20mx0.18mm, 0.18µm film thickness] and the first temperature program given above (split closed for 30 s for extracts and for 2 min when using SPME fibers). The split/splitless injector port was kept at 260°C and the flame ionization detector (FID) at 340°C. Peak areas were computed with Chrom-Card 1.19 (CE Instruments, Milan, Italy). Mean absolute substance quantities were estimated by comparing substance peak areas with that of the internal standard (acquired with GC-FID) and dividing by the number of extracted individuals.

Profile similarities between the two partner species were analyzed for eleven parabiotic nests (including one from Kuala Belalong Field Studies Center). The average proportions of the steroid components per colony and species were calculated. The distances between colonies were calculated for each species separately using Bray-Curtis index of similarity and then compared between species using a Mantel test (1000 permutations).

IV.6.4 Identification of cuticular hydrocarbons

Alkanes, methyl-branched alkanes and alkenes were characterized using diagnostic ions and retention indices calculated using Kovats' method (Carlson et al. 1998). Unsaturated methyl-branched hydrocarbons were hydrated under a H_2 atmosphere using Palladium on activated carbon as catalyst to determine the position of the methyl group. The position of the double bond in methyl-branched and n-alkenes was determined using DMDS derivatization following (Dunkelblum et al. 1985). For methyl-branched alkenes, DMDS derivatization was

insufficient for substance characterization since the position of the double bond relative to the methyl group remained unresolved and left two possible structures. Therefore, we cleaved the molecules in two parts at the position of the double bond via ozonisation. We diluted the sample in approx. 3ml hexane, applied a constant flow of O_3 (300mg/h) for ten minutes from a glass pipette (EO3G Ozone Generator, Easelec Technology Inc.) and directly injected the sample into the GC-MS. Ozonisation succeeded for substance 52 but not for the substances 37, 43, 56, and 61 (surface compounds of the red *Ca. rufifemur*, Table 1). However, it is highly probable that all methyl-branched alkenes are produced via the same biosynthetic pathway. We therefore tentatively inferred the position of the double bond from the structure of substances 52 (Table 1) and possibilities left from the DMDS results, which had succeeded for all of the above substances. Double bond positions in alkenes with chain lengths higher than C41 as well as in dienes and trienes could not be determined due to their low abundance and/or their high chain length, which resulted in derivatives which could not be detected using GC-MS. Aldehydes were identified by comparing their mass spectra to a commercial library (Wiley 275) and therefore remain tentative.
For the substances of the black *Ca. rufifemur* profile beyond C44, retention indices were calculated based on the retention times of an n-alkane standard (C21 to C40), C47 and C49, and therefore remain preliminary. These substances were identified based on mass spectra and hydrated samples. Unsaturation was further confirmed via fractionation using a SiOH column treated with $AgNO_3$. However, their characterization remains preliminary since the DMDS derivatized substances could not be detected using GC-MS.

IV.6.5 Behavioural experiments

We studied the reaction of *Crematogaster modiglianii* towards dead *Ca. rufifemur* workers from different colonies in Borneo. The reverse situation (*Ca. rufifemur* towards *Cr. modiglianii*) was not studied in this paper since *Ca. rufifemur* shows little discrimination between different *Cr. modiglianii* workers (Menzel et al. 2008). A *Cr. modiglianii* colony (R0) had been collected in the forest circa one week prior to the experiments and was kept together with its red *Ca. rufifemur* partner in its original nest (a small tree trunk) in an open plastic box. The dead ants were placed onto the nest trunk with forceps such that several ants could interact with it simultaneously. During three minutes, each observed interaction was classified as peaceful (antennating), weakly (open mandibles) or strongly aggressive (biting or locking mandibles). An additional behaviour classified as peaceful was ‘mounting’, where the smaller *Crematogaster* (body length approx. 2-3mm) climbed onto the *Camponotus* body (body length 5-13mm). Continued interactions were recorded again after 10 s (the same behavioural classification as used in (Menzel et al. 2008)).
The aggressiveness of two other *Cr. modiglianii* colonies was estimated in arena confrontations. The workers had been collected in the forest one day prior to the tests and were kept in a plastic box among nestmates (but separate from the partner species) over night. Five *Cr. modiglianii* individuals were placed into a fluon-covered plastic cylinder (Ø 7.5 cm, height 5 cm) on top of a paper sheet floor. After 1 min to calm down, a dead *Camponotus* specimen was introduced. For the following 100 s we recorded the behaviour of the ants as above. Each living or dead ant was used for one assay only. In all of the above assays, we performed ten replicates per treatment.
From each replicate we calculated the proportions of all aggressive versus all non-aggressive interactions. Both strong and total (including weak) aggression were analyzed using generalized linear models (GLM) with quasibinomial error distribution and logit link

function. In order to determine whether confrontations within and across chemical varieties differ, we used the according explanatory variable 'within/across variety' with two factor levels (which collapsed to '*Camponotus* variety' in the first dataset). The variable 'variety combination' (with the factor levels 'black→black', 'black→red', 'red→red', and 'red→black') was nested in the former one. Further explanatory variables were 'colony combination' (nested in 'variety combination'), which collapsed to '*Camponotus* colony' in the first dataset, and 'intra-/ allocolonial'. Due to their nested structure, no interactions between the variables were possible. The impact of each variable was determined by likelihood ratio tests (F tests). We also re-analyzed data from (Menzel et al. 2008) in a similar way, where we included the number of workers present in the experimental arena as explanatory variables (see (Menzel et al. 2008) for details on the experimental setup). Since the statistical results for total aggression and for strong aggression only were similar, only the former will be reported in the results section. All computations were performed in R Version 2.5.1 (R Development Core Team 2008).

Acknowledgements

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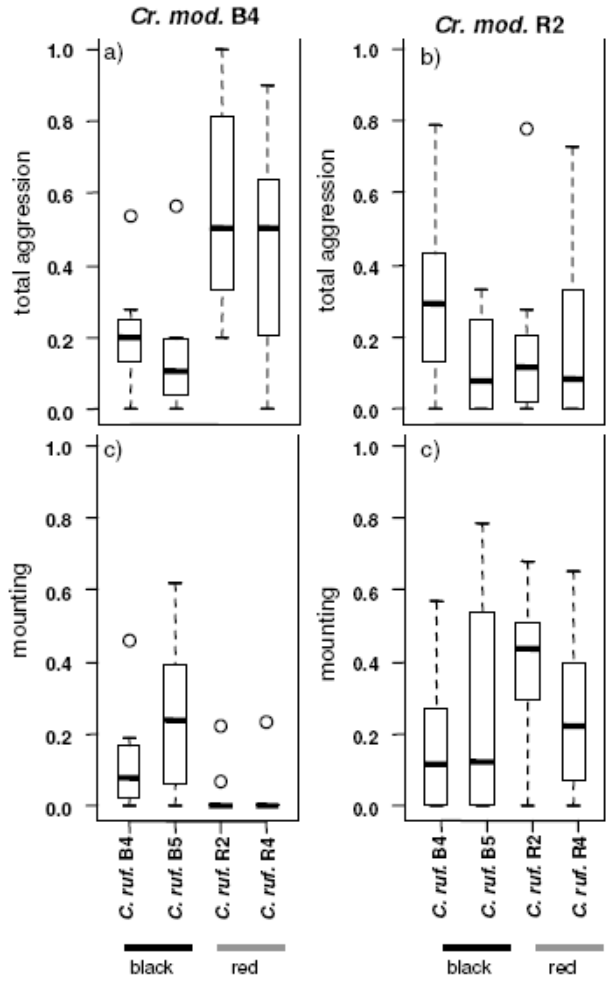

Fig. 4 Total aggression (a, b) and mounting behaviour (c, d) of *Cr. modiglianii* towards dead *Ca. rufifemur* from different colonies in arena assays.
Data are given as proportions in relation to the total number of interactions. Each plot represents 10-13 replicates. (a), (c) *Cr. modiglianii* B4, (b), (d) *Cr. modiglianii* R2.

Table 5 GLM for total aggression of *Cr. modiglianii* towards dead *Ca. rufifemur* from different colonies.
Data from arena confrontations with *Cr. modiglianii*. 'Variety combination' ist nested within the parameter 'within/across varieties'. 'Colony combination' is nested within 'variety combination'. Due to this nested structure, no interactions between the variables occur.

Parameter	Deviance	*df*	*F*	*P*
within/across varieties	107.8	1	20.64	<0.0001
variety combination	77.3	2	8.19	0.00056
colony combination	37.0	3	2.76	0.048
intra-/allocolonial	0.2	1	0.05	0.83
residual error	370.6	80		
total	592.9	87		

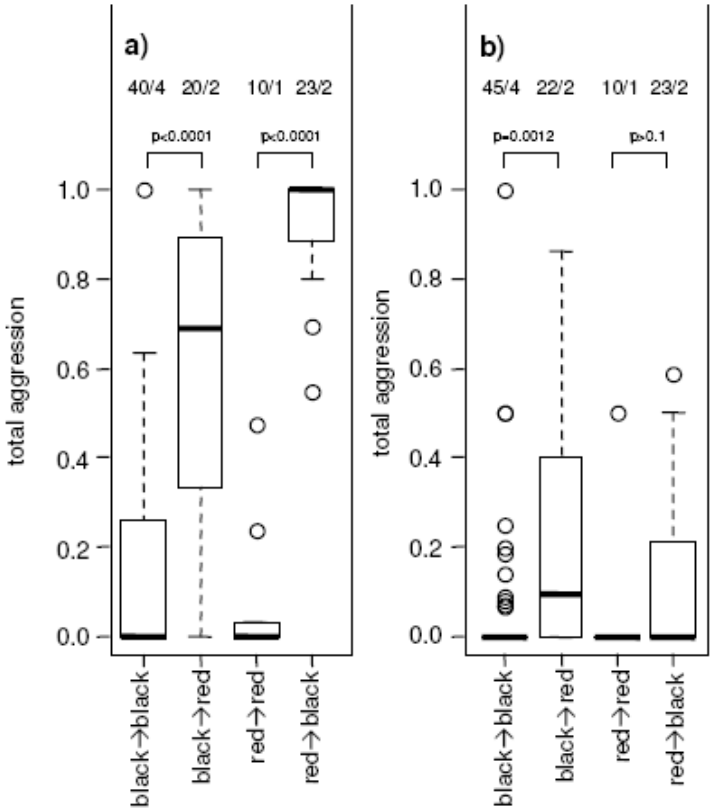

Fig. 5 Total aggression in allocolonial confrontations between parabiotic partners assays, pooled for variety combinations.
Data are from (Menzel et al. 2008), given as proportions in relation to the total number of interactions. The numbers above each plot indicate the overall number of replicates and the number of colony combinations tested. P values are given according to GLMs with binomial error distribution. (a) *Cr. modiglianii* towards *Ca. rufifemur*, (b) *Ca. rufifemur* towards *Cr. modiglianii*.

Table 6 GLM for total aggression in interspecific live confrontations.
Data from (Menzel et al. 2008). 'Colony combination' is nested within 'variety combination'. There are no interactions between the variables due to their nested structure.

	Cr. modiglianii → Ca. rufifemur				***Ca. rufifemur → Cr. modiglianii***			
	Deviance	***df***	***F***	***P***	**Deviance**	***df***	***F***	***P***
within/across varieties	674.3	1	124.56	<0.0001	45.2	1	13.09	0.00047
variety combination	79.4	2	9.19	0.0002	5.6	2	0.81	0.45
intra-/allocolonial	0.3	1	0.07	0.79	0.0	1	0.00	0.99
colony combination	18.0	4	1.01	0.41	12.4	4	0.99	0.42
No. *Camponotus*	1.4	1	0.32	0.57	0.0	1	0.00	0.96
No. *Crematogaster*	2.1	1	0.47	0.49	5.2	1	1.73	0.19
residual error	346.0	82			260.5	89		
total	1121.5	92			328.9	99		

V. Novel cuticular substances function as interspecific appeasement signals

This chapter has been submitted as:

Menzel F, Blüthgen N, Beuerle T, Schmitt T: Novel cuticular substances in parabiotic ants function as interspecific appeasement signals.

V.1 Abstract

The cuticular substances of the parabiotic ants *Crematogaster modiglianii* and *Camponotus rufifemur* differ from non-parabiotic ant species. Both species possess hydrocarbons of unusually high chain lengths. Since cuticular hydrocarbons generally function as nestmate recognition cues in ants, unusually heavy ones may substantially hamper nestmate recognition. Moreover, both species possess a set of hereto unknown cuticular substances. Their composition varies between parabiotic nests but is similar between the two species of a nest. Thus, they are a potential candidate for both intra- and interspecific nestmate recognition.
In the present study, we investigated the role of cuticular hydrocarbons versus the unknown cuticular compounds, accompanied by analyses of the latter's molecular structure. The main unknown compound ($C_{21}H_{32}O$) possesses three ring structures and three double bonds; the oxygen atom is probably linked to two alkyl groups. We divided cuticular extracts into hydrocarbons and the unknown compounds and studied recognition-related aggression towards dummies covered with the two substance classes. Our results show that both species use hydrocarbons as interspecific recognition cues. However, the unknown compounds strongly reduce aggression in *Ca. rufifemur* and thus may function as appeasement allomones. Thus, these substances seem to play an important role in facilitating interspecific tolerance among parabiotic partners.

V.2 Introduction

Interspecific associations among social insects require that both species refrain from hostile interactions. Social parasites often manage to get accepted through chemical mimicry, chemical camouflage or chemical insignificance (Lenoir et al. 2001b). In such cases, they cheat their host's nestmate recognition system. In other associations, however, such as parabiotic associations among ant species and certain social parasite-host associations (Liu et al. 2000, Menzel et al. 2008a, Errard et al. 2003), there is little overlap in the hydrocarbon profiles. Mutual tolerance in these cases is probably achieved by habituation to the respective other's profile, sometimes accompanied by appeasement allomones in social parasites (Mori et al. 2000b). Habituation to foreign profiles has often been observed within and across ant species (Errard and Vienne 1994; Leonhardt et al. 2007).

Parabioses are associations between different ant species which co-inhabit the same nest. Unlike social parasites, they may be mutualistic (Vantaux et al. 2007, Menzel and Blüthgen submitted), hence, both sender and receiver of recognition signals may have an interest in maintaining the association. Parabioses are thus associations between two eusocial partners, which generally possess elaborate nestmate recognition systems (Vander Meer and Morel 1998). Studying nestmate recognition in parabiotic ants can reveal how both species complement their recognition system to include heterospecific nestmates. The study of selective tolerance towards allospecific ants can provide insights into the more general mechanisms of nestmate recognition and allospecific aggression.

The parabiotically associated ants *Crematogaster modiglianii* and *Camponotus rufifemur*, which occur in the tropical rainforest of Borneo, display extraordinarily high tolerance between different parabiotic nests (Menzel et al. 2008b), coinciding with an unusual surface chemistry (Menzel et al. 2008a). Both species possess hydrocarbons of unusually high chain lengths. *Camponotus rufifemur* almost entirely lacks saturated hydrocarbons but possesses methylbranched alkenes instead, which have been very rarely described from insects in general (Menzel et al. 2008a). It has been hypothesized that very long-chain hydrocarbons are difficult to perceive due to their low volatility. In addition, they may 'absorb' traces of shorter, recognition-relevant hydrocarbons and hence substantially hamper nestmate recognition (Gibbs and Pomonis 1995; Lambardi et al. 2007). Furthermore and also highly unusual, surface profiles of both *Cr. modiglianii* and *Ca. rufifemur* contain a set of hereto unknown compounds, which are produced by *Cr. modiglianii* and probably transferred to *Ca. rufifemur*. Their composition varies across different colonies but is similar between the two partners of a parabiotic nest (Menzel et al. 2008a). These two properties make them a potential candidate for recognition cues between nestmates and non-nestmates from both the own and the partner species based on the same signal. Apart from two hydrocarbons that only certain colonies of both species have in common (Menzel et al. 2008a), the unusual surface chemistry of this parabiotic system provides the unique opportunity to experimentally separate potential recognition cues that both species have in common (unknown compounds) from those that almost completely differ (hydrocarbons). Thus, one can experimentally determine whether parabiotic ants recognize intra- and interspecific nestmates based on the same cues, or whether they habituate to the respective allospecific recognition signals and hence possess two distinct neuronal 'nestmate' templates.

Camponotus rufifemur often attacks other *Crematogaster* species, which lack the unknown compounds, but tolerates workers of *Crematogaster modiglianii* even when they attack *Camponotus* (Menzel et al. 2008b). It seems possible that the unknown compounds play a role in reducing interspecific aggression. Such an appeasement effect might promote habituation to the *Cr. modiglianii* profile by inhibiting aggression from its *Ca. rufifemur* partner. In the present study, we conducted chemical analyses of the unknown compounds as well as behavioural experiments to determine their role in the nestmate recognition process of parabiotic ants. Our aims were (a) to elucidate the molecular structure of the unknown compounds, (b) to identify the substance class that carries the nestmate recognition cues (hydrocarbons vs. unknown compounds) and (c) to determine whether the unknown compounds from *Cr. modiglianii* reduce aggression in its partner species *Ca. rufifemur*.

V.3 Materials and Methods

V.3.1 Study site and ants

Our studies were conducted at Danum Valley Conservation Area from September to December 2007. Danum Valley is located at approximately 100 m a.s.l. in Sabah (Malaysian Borneo) and represents one of the major remaining patches of Sabah's primary lowland rainforest. It has a typical equatorial rainforest climate with a mean annual temperature of 26.9 °C and a yearly rainfall of 2700 mm. We studied parabiotic nests of *Crematogaster modiglianii* and *Camponotus rufifemur*, which are commonly found in hollow, small tree trunks in the rainforest around Danum Valley Field Center (Menzel et al. 2008b). *Camponotus rufifemur* occurs in two chemically distinct varieties with no hydrocarbon overlap apart from n-alkane traces (Menzel et al. 2008a). They also differ in the colouring of alitrunk and legs (Menzel et al. 2008a) and will be referred to as 'black' and 'red' *Ca. rufifemur* in the following. The only detectable difference in the cuticular profile of their respective *Crematogaster modiglianii* partner is the abundance of two 27-MeC_{39}-alkenes, which represent the main surface component of the red *Ca. rufifemur* variety. The substances are common in *Cr. modiglianii* living with the red *Ca. rufifemur* variety (henceforth termed 'red' *Cr. modiglianii*) but nearly absent in those living with the black one (Menzel et al. 2008a). According to the *Ca. rufifemur* variety, the parabiotic nests used in this studies are labelled 'B' or 'R' plus a digit.

For the experiments, three nests (R0, R1, B1) were brought to the laboratory, where both species were kept together inside a section of their original nest trunk in a Fluon™-covered plastic box. An additional set of experiments was conducted with a *Ca. rufifemur* worker colony (B3) that was kept in a plastic box together with its *Cr. modiglianii* partner for a few days during the experiments.

V.3.2 Chemical Analysis

We analyzed the unknown compounds from *Cr. modiglianii* cuticular extracts using electron ionisation mass spectrometry, chemical ionisation mass spectrometry, and high resolution mass spectrometry. In addition, several derivatizations were performed. Electron ionisation mass spectra were aquired using capillary gas chromatography-mass spectrometry (GC-MS) with a Hewlett Packard 6890 series gas chromatograph coupled to a HP 5973 Mass Selective Detector. The GC was equipped with a J&W Scientific DB-5 fused silica capillary column (30 m x 0.25 mm ID; df = 0.25µm). The temperature was kept at 60 °C for 2 min, then increased by 60°C/min up to 200°C and subsequently by 4°C/min to 320°C, where it remained constant for 10 min. Helium was used as carrier gas with a constant flow of 1.0 ml/min. A split/splitless injector was installed at 250°C in the splitless mode for 30 s. The electron ionisation mass spectra (EI-MS) were recorded with an ionisation voltage of 70 eV, a source temperature of 230°C and an interface temperature of 325°C. The software MSD ChemStation (Version A.03.00) for Windows was used for data acquisition.

Chemical ionisation mass spectra were obtained with a Hewlett Packard 5890A gas chromatograph equipped with a 2 m fused silica guard column (deactivated, I.D. 0.32 mm) and a 30 m x 0.32 mm analytical column (ZB1 and ZB5, Phenomenex). The capillary column was directly coupled to a triple quadrupole mass spectrometer (TSQ 700, Finnigan). Injector and transfer line were kept at 280°C. Temperature was kept at 70°C for three min and then increased at 10°C/min up to 310°C, where it remained constant for five min. The CI mass spectra were recorded in the positive mode using ammonia as a reagent gas.

For high resolution mass spectrometry, an Agilent 6890 gas chromatograph was equipped with a 30 m analytical column (Phenomenex ZB5-MS, 30m x 0.25 mm ID, t_f=0.25 µm). A split injection port at 250°C was used for sample introduction with a split ratio of 3:1. The temperature program was the same as for chemical ionisation mass spectra. The helium carrier gas was set to 1.0 ml/min flow rate (constant flow mode). The transfer line was kept at 270°C. High resolution mass spectra were acquired using a JMS-T100GC (GCAccuTOF, JEOL, Japan) time of flight mass spectrometer in electron ionisation (EI) mode at 70eV and JEOL MassCenter™ workstation software. Source and transfer line temperature were 200°C and 270°C, respectively, and detector voltage was set at 2100 V. The acquisition range was m/z 41 to 600 with a spectrum recoding interval of 0.4 s. The system was tuned with PFK to achieve a resolution of 5,000 (FWHM) at m/z 292.9824.
The number of double bonds in the unknown compounds was determined by hydrogenating *Cr. modiglianii* extracts using hydrogen and palladium on carbon or rhodium on carbon as catalyst in methanol. In order to discriminate between primary and secondary alcohols, extracts were either treated with MSTFA to obtain trimethylsilyl derivatives or with acetic anhydride/pyridine to obtain acetyl esters using standard procedures. Carbonyl groups in the molecule were detected using methoximation at 60°C for 45 min (Miura et al. 2004).

V.3.3 Behavioural experiments

In order to disentangle recognition signals from different sources, we confronted the ants with freshly killed, untreated workers of other ant colonies, with their extracts, and with the unpolar (containing the hydrocarbons) or the polar fraction (containing the unknown compounds) of these extracts. Extracts and fractions were presented on dead, thoroughly extracted ants, henceforth termed 'dummies'. In each test series, we measured whether the observed ants distinguished between intra- and allocolonial allospecific ants, i.e. workers of the respective partner species from the same or a different parabiotic nest. Allocolonial confrontations were performed both within and across the two varieties. Ten replicates were carried out for each treatment.
A *Crematogaster modiglianii* colony (R0) was confronted with dead ants (killed by freezing) or dummies treated with extracts from their red *Ca. rufifemur* partner and two other *Ca. rufifemur* colonies (a black and a red one, i.e. B2 and R2). For surface extracts, 50 ants were killed by freezing and immersed in hexane for ten minutes. Unpolar and polar fractions of these extracts were eluted with distilled hexane, followed by chloroform, using conditioned SiOH columns (CHROMABOND, 100mg, Macherey-Nagel, Düren, Germany). GC-MS analyses confirmed that the hexane fractions contained the hydrocarbons while the chloroform fractions contained the unknown compounds. The chloroform of the polar fraction was evaporated, and the fraction was reconstituted in hexane. As dummies we used intracolonial *Camponotus rufifemur* bodies that had been extracted with hexane and chloroform for ten min twice each. Each dummy was treated with an extract quantity equivalent to five individuals. The dummy was held with forceps onto the nest trunk inside the plastic box so that several ants (up to nine) could interact with it simultaneously. During three minutes, each observed interaction was recorded and classified as peaceful (antennate), weakly (open mandibles) or strongly aggressive (bite or lock mandibles). Within these three minutes, continued interactions were recorded again after every 10 sec to provide more weight to long-lasting interactions. The classification is consistent with an earlier study (Menzel et al. 2008b). Different treatments were tested in haphazard order on different places of the nest trunk. Dummies with pure hexane were tested as controls.

Interspecific nestmate recognition of *Camponotus rufifemur* towards *Crematogaster modiglianii* was studied using a black and a red parabiotic nest (B1 and R1) and a parabiotic worker colony (B3). *Ca. rufifemur* from B1 and R1 were confronted with *Cr. modiglianii* from their own or the respective other colony, using freshly killed ants, dummies with their extracts, or fractions thereof. Thoroughly extracted *Cr. modiglianii* bodies were used as dummies. Since they were small compared to *Ca. rufifemur* workers, we successively held them in front of ten single workers and recorded their individual reactions as above. The red *Ca. rufifemur* colony (R1) was additionally confronted with whole extracts and hexane fractions from two further *Cr. modiglianii* colonies (R3 and B3). Black *Ca. rufifemur* from the worker colony (B3) were confronted with similar treatments of their partner (B3) and a red allocolonial *Cr. modiglianii* (R3). Dummies with pure hexane functioned as controls.
The effect of *Cr. modiglianii* unknown compounds on *Ca. rufifemur* (colony B1) aggression was additionally examined by comparing their aggression towards different extracts both with and without addition of the unknown compounds. We used total extracts of *Crematogaster coriaria* and hexane fractions of *Crematogaster difformis* and allocolonial *Cr. modiglianii* (colony R1). The *Cr. difformis* extracts were fractionated in order to remove the metapleural gland products peculiar to this species. We tested these three extracts both with and without addition of an allocolonial *Cr. modiglianii* (colony R1) polar fraction.
From each replicate, we calculated the proportion of all aggressive versus all non-aggressive interactions. We then performed pairwise comparisons between each nestmate and non-nestmate treatment for each test series and used generalized linear models (GLMs) with quasibinomial error distribution and logit link function. The effect of unknown compound addition was separately examined using similar GLMs with the explanatory variables 'extract species' and 'compound addition'. The influence of each variable was determined by likelihood ratio tests (F tests). We conducted separate analyses for both strong and total (including weak) aggression and report both results. All computations were performed in R Version 2.8.1 (R Development Core Team 2008).

V.4 Results

V.4.1 Chemical analysis

Overall, 24 unknown compounds with largely the same diagnostic ions were found on the cuticle of *Cr. modiglianii* from seven colonies (Fig. 1, table S1). The structures of three major compounds (no. 7, 10 and 18 in table S1), which possess highly similar mass spectra (Fig. 2), were further chemically characterized. An initial comparison of their electron ionisation mass spectra with mass spectra from commercial libraries showed high accordance with a basic steroid structure (Fig. 2; Menzel et al. 2008a). However, the results of the high resolution mass spectrometry as well as mass spectra of the hydrogenated compounds did not support these results (table 1). Despite their steroid-like mass spectra, the compounds 7, 10 and 18 consist of three ring structures unlike four ring structures expected in steroids. Treatment with MSTFA or with acetic acid, as well as methoximation, did not change their retention indices and mass spectra. Thus, the existence of primary or secondary hydroxyl groups as well as carbonyl groups in the molecule seems improbable.

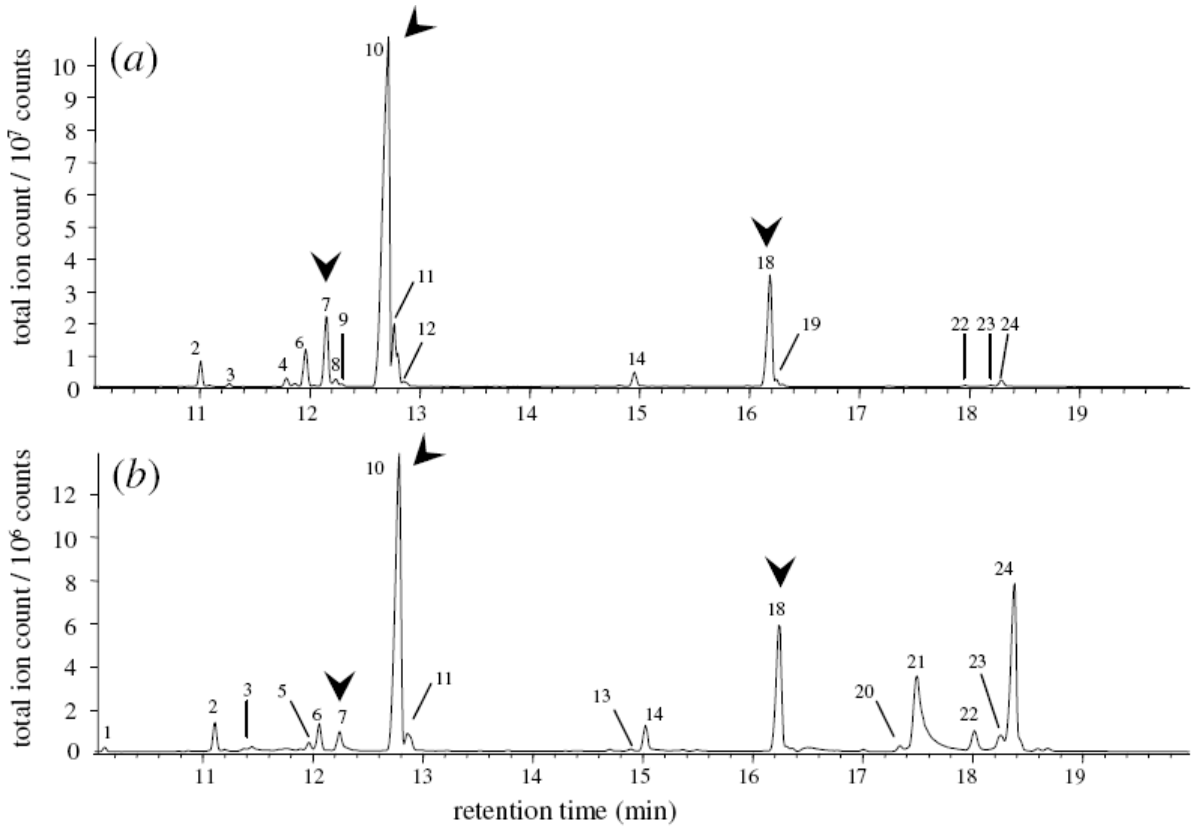

Fig. 1 Gas chromatograms of the unknown compounds in two different *Cr. modiglianii* colonies. a) colony B3, b) colony B4. Arrows indicate the three compounds depicted in Fig. 2.

V.4.2 Behavioural experiments

Crematogaster modiglianii (colony R0) clearly differentiated between intra- and allocolonial dead workers of *Ca. rufifemur* and attacked the latter significantly more than the former. The same, significant differentiation was found for both whole extracts and hydrocarbon (unpolar) fractions, presented on dummies. Both strong and total aggression against black *Ca. rufifemur* treatments were much higher than against those of red *Ca. rufifemur* (Fig. 3). In contrast, the polar fraction (which contained the unknown compounds) did not trigger a significant differentiation between intra- and allocolonial *Ca. rufifemur*.

Workers of the black *Camponotus rufifemur* colony (B1) did not differentiate between dead intracolonial and allocolonial *Crematogaster* workers. However, we observed a differentiation between total extracts of these workers. Extracts of allocolonial *Cr. modiglianii* elicited significantly more aggression than intracolonial ones. The hydrocarbon fractions of allocolonial red *Cr. modiglianii* triggered very high aggression, while hydrocarbon fractions of intracolonial *Cr. modiglianii* were treated amicably (Fig. 4a). This differentiation is highly significant. In contrast, the allocolonial polar fraction as well as a mixture of hydrocarbon and polar fractions of allocolonial *Cr. modiglianii* mainly provoked peaceful reactions (Fig. 4a), which corresponds to the weaker differentiation between the two total extracts compared to the two hydrocarbon fractions. Similarly, the black *Ca. rufifemur* worker colony (B3) significantly differentiated between hexane fractions of intra- and allocolonial *Cr. modiglianii* but not between their total extracts or their polar fractions (Fig. 4b). In contrast, workers of the red *Ca. rufifemur* colony (R1) seldom showed any aggression when confronted with dead ants, extracts, unpolar or polar fractions of other red or black *Cr. modiglianii* workers. This colony never discriminated between any intracolonial and allocolo-

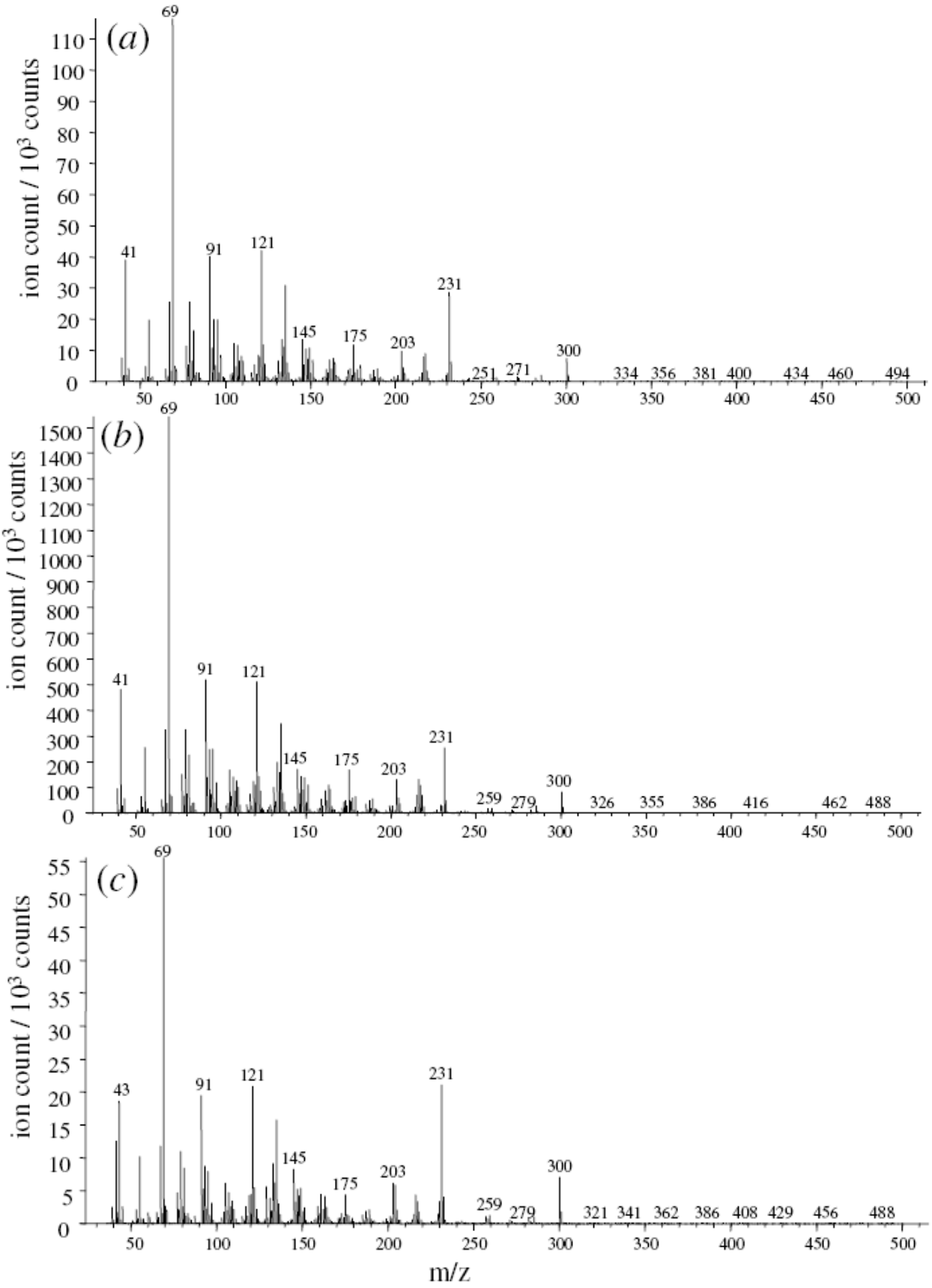

Fig. 2 Mass spectra of three unknown compounds which occur regularly in higher abundances. a) substance no. 7 (see table S1), b) substance no. 10, c) substance no. 18.

nial treatments (Fig. 4c). In both *Cr. modiglianii* and *Ca. rufifemur*, all intracolonial treatments elicited aggression levels comparable to or lower than hexane controls (Figs 3, 4a-c).

The addition of allocolonial *Cr. modiglianii* unknown compounds to different extracts significantly reduced aggressiveness of *Ca. rufifemur* workers. They strongly attacked dead bodies of *Crematogaster coriaria* and *Cr. inflata* (Fig. 5). Aggression towards extracts of *Crematogaster coriaria* as well as hydrocarbon fractions of *Crematogaster inflata* and allocolonial *Cr. modiglianii* was high, but the *Ca. rufifemur* workers reacted less aggressively to each of these treatments after addition of allocolonial *Cr. modiglianii* polar fractions (Fig. 5). The generalized linear model yielded a highly significant effect of addition of the unknown compounds. The effect was significantly higher in *Cr. modiglianii* extracts than in the other two species (Table 2).

V.5 Discussion

V.5.1 Nestmate recognition cues – hydrocarbons or unknown compounds?

The parabiotically associated ant species *Crematogaster modiglianii* and *Camponotus rufifemur* possess a set of hereto unknown cuticular compounds. To our knowledge, substances with similar mass spectra and diagnostic ions have not been found on insect cuticles up to now. The composition of these compounds varies between parabiotic nests but is similar among the two species within one nest (Menzel et al. 2008a). Based on these two properties, these compounds may hold nestmate recognition signals. They may enable both species to recognize both intra- and interspecific nestmates based on the same signal. On the other hand, more than 99% of the cuticular hydrocarbons of the two species are notably heavier (> C_{36}) than in related, non-parabiotic species (C_{20}-C_{33}) (Menzel et al. 2008a). It has been suggested that hydrocarbons of very high chain lengths evade perception due to their low volatility, thus camouflaging their carriers (Akino 2006; Lambardi et al. 2007). Thus, the hydrocarbon profiles alone may not provide enough cues to allow colony discrimination, whereas the unknown compounds are smaller and, being unique to each parabiotic nest, may function as recognition cues (Menzel et al. 2008a, Table S1). However, neither of the two species differentiated between intracolonial and allocolonial unknown compounds. In contrast, both species attacked certain allocolonial hydrocarbons of the respective partner species more fiercely than intracolonial ones. Thus, our experiments clearly show that interspecific nestmate recognition is mediated by cuticular hydrocarbons but not the unknown compounds.

Notably, interspecific tolerance between colonies was asymmetric. Black *Ca. rufifemur* attacked hexane fractions of red *Cr. modiglianii,* while red *Ca. rufifemur* tolerated hexane fractions of black *Cr. modiglianii*. The only difference of the chemical profiles in the two *Cr. modiglianii* types we detected were two 27-MeC_{39}-alkenes, which are abundant in the red *Cr. modiglianii* profile but nearly or completely absent in the black one (Menzel et al. 2008a). This might be merely due to inter-colony differences in aggressiveness (which we observed with other colonies, Menzel et al. 2008b). However, it is possible that black *Ca. rufifemur* detected the presence of these unfamiliar compounds whereas the red *Ca. rufifemur* failed to detect its absence. It has been shown previously that hosts can detect their parasite's unfamiliar surface cues, but hosts may fail to detect the absence of recognition cues (e.g. Akino et al. 1999).

V.5.2 The unknown compounds reduce aggressiveness

Though not as recognition cues, the unknown compounds seem to play a role in interspecific interactions by reducing *Ca. rufifemur* aggressiveness. While the black *Ca. rufifemur* variety showed low aggression towards allocolonial 'red' *Cr. modiglianii* (Menzel et al. 2008a; Menzel et al. 2008b) or its surface extracts, it fiercely attacked dummies carrying their hydrocarbons only (i.e. after removal of unknown compounds). Addition of these compounds to the hydrocarbon fractions resulted in low aggression comparable to an intracolonial level. A similar, albeit weaker effect of *Cr. modiglianii* unknown compounds was observed with extracts of two other *Crematogaster* species that were usually attacked by *Ca. rufifemur*. Thus, the unknown compounds seem to function as cuticular appeasement allomones. The use of appeasement allomones has been reported from the slave-making ant *Polyergus rufescens*. This species uses decyl butyrate from its Dufour gland to calm its host's aggression during host-colony usurpation (D'Ettorre et al. 2000; Mori et al. 2000a; Mori et al. 2000b). However, while certain other social parasites use 'propaganda' substances to elicit panic among their hosts (Brandt et al. 2006; Lenoir et al. 2001b), we are not aware of any case of appeasement allomones apart from the social parasite *Polyergus rufescens*.

Through this function, the compounds probably play an important role in the parabiotic association. *Cr. modiglianii* and *Ca. rufifemur* often share baits and other food sources. *Ca. rufifemur* tolerates the much smaller *Cr. modiglianii* but aggressively displaces other ant species. The appeasement effect of the unknown compounds may cause this tolerance, enabling *Cr. modiglianii* to forage together with *Ca. rufifemur* instead of being displaced. This effect also explains why *Ca. rufifemur* workers do not defend themselves against attacks of allocolonial *Cr. modiglianii* (Menzel et al. 2008b).

It is difficult to determine whether the unknown compounds reduce aggression at a neuronal level (i.e. tolerance despite of recognition as foreign) or whether they mask the recognition cues, i.e. hamper recognition itself at the receptor level. The former hypothesis implies that the unknown compounds act as behavioural modifiers, in analogy to e.g. honeybee queen pheromones in intraspecific signalling (Beggs et al. 2007; Vergoz et al. 2007). However, a definite distinction between these two possibilities will only be possible based on experiments that involve other behavioural answers than aggression, e.g. testing whether the ants can be conditioned on unknown compounds (Dupuy et al. 2006).

V.5.3 Characterization of the unknown compounds

Initially, the unknown compounds produced by *Cr. modiglianii* had been tentatively identified as steroid-like substances, based on comparing their mass spectra and diagnostic ions with commercial libraries (Menzel et al. 2008a). However, further chemical characterization as described above did not confirm our interpretation of the initial electron ionisation mass spectra data. A comparison of the number of unsaturations between three untreated and subsequently hydrogenated compounds suggests that the structure of these compounds consists of three instead of four rings, as one would expect for a steroid-like structure. The high resolution mass spectra data of two compounds (number 7 and 10 in table S1) give evidence for one oxygen atom in the molecule. The failed derivatisations of these compounds indicate that the oxygen atom is linked to two alkyl groups. Overall, our results suggest that the compounds have a terpene-like structure which might be elucidated using NMR technique.

Acknowledgements

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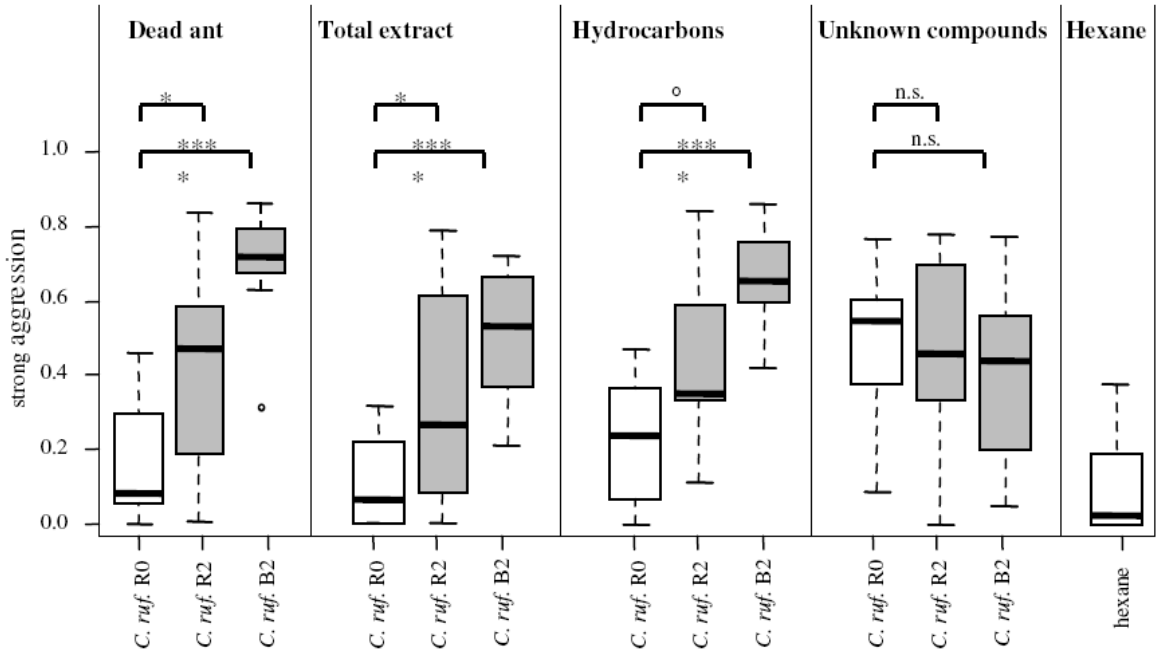

Fig. 3 Strong aggression of *Crematogaster modiglianii* (colony R0) against intracolonial *Ca. rufifemur* R0 (empty plots), allocolonial red *Ca. rufifemur* R2 and allocolonial black *Ca. rufifemur* B2. For each colony, dead ants, total extracts, hydrocarbons and unknown compounds were tested. Each plot represents 10 replicates. 'Hexane': Dummies treated with pure hexane. Asterisks denote significant differences according to GLMs with quasibinomial error distribution (df = 1 for each GLM). ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05, °p < 0.1, 'n.s.' p > 0.1. Analysis of total instead of strong aggression yielded the same results, except for (1) 'Dead ant': *C. ruf.* R0 - *C. ruf.* R2 (p = 0.08) and (2) 'Hydrocarbons': *C. ruf.* R0 - *C. ruf.* R2 (p > 0.1)

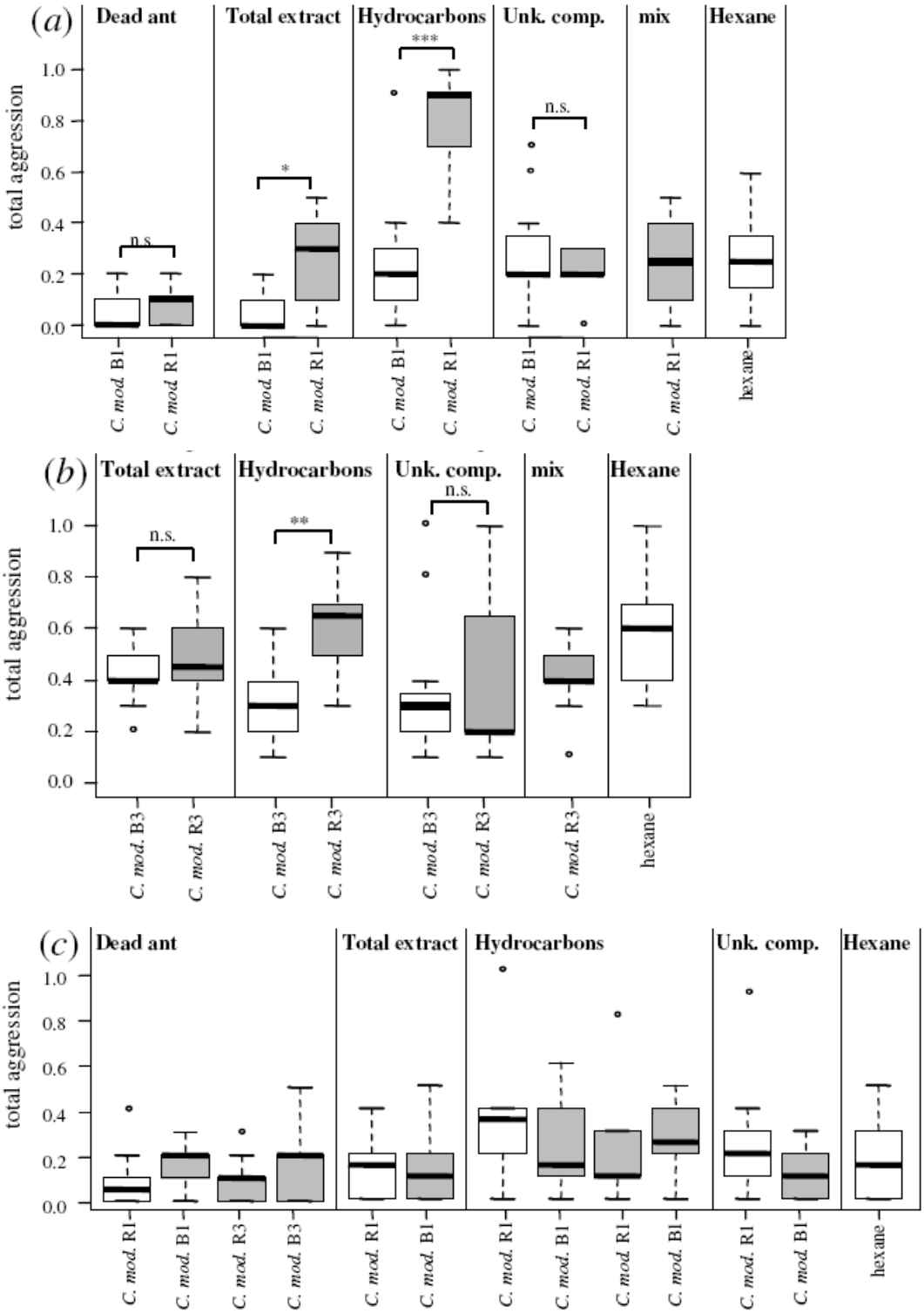

Fig. 4 Total aggression of *Camponotus rufifemur* towards intracolonial and allocolonial *Crematogaster modiglianii*. a) *Camponotus rufifemur* colony B1, b) colony B3, c) colony R1. For each colony, dead ants, total extracts, hydrocarbons and unknown compounds were tested. Each plot represents 10 replicates. 'mix': Hydrocarbons (unpolar fraction) and unknown compounds (polar fraction) mixed. 'Hexane': Dummies treated with pure hexane. Intracolonial treatments and hexane controls are represented by empty plots. Asterisks denote significant differences according to GLMs with quasibinomial error distribution (df = 1 for each GLM). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, 'n.s.' $p > 0.1$. Analysis of strong instead of total aggression yielded the same results except for (1) 'Total extract': *Cr. mod.* R1 - *Cr. mod.* B1 ($p > 0.1$, Fig. 4a) and (2) 'Hydrocarbons': *Cr. mod.* R3 – *Cr. mod.* B3 ($p > 0.1$, Fig. 4b).

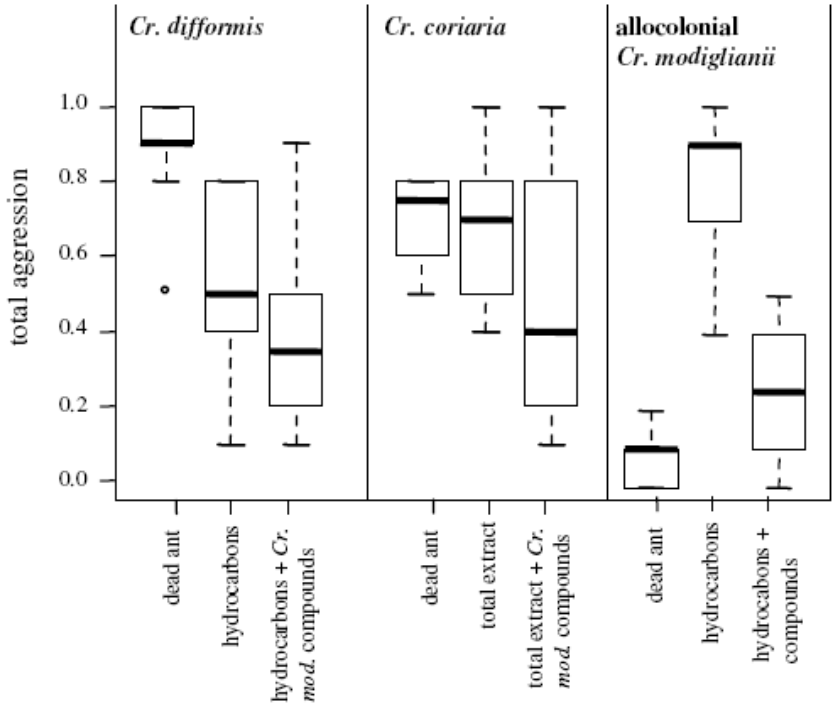

Fig. 5 Effect of addition of unknown compounds on total aggression of *Camponotus rufifemur* B1. The workers were confronted with dead ants, extracts (or hexane fractions, respectively) and extracts mixed with the polar fraction of *Cr. modiglianii* R1. Analysis of strong aggression only yielded the same results. The data partly overlap with those presented in Fig. 4a.

Table 1 Results of the GC-MS analysis of three major unknown compounds and their hydrogenated derivatives.

Peak	RT index	M^+ (EI)	M^+ (CI)	accurate mass	empirical formula	unsaturations
7	2192	302	302	302.2571	$C_{21}H_{34}O_1$	5 (2 double bonds)
10	2224	300	300	300.2470	$C_{21}H_{32}O_1$	6 (3 double bonds)
18	2447	360	360	360.2713	$C_{23}H_{36}O_3$	6 (3 double bonds)

Table 2 GLM results for the influence of addition of unknown compounds on total aggression of *Ca. rufifemur* B1.

Parameter	Deviance	*df*	*F*	*P*
compound addition	57.3	1	20.87	< 0.0001
species	5.4	2	0.98	0.38
species : compound addition	23.3	2	4.71	0.013
residual error	155.9	56		
total	242.0	61		

Table S1 List of unknown cuticular compounds with retention indices and relative abundance (excluding hydrocarbons) in the seven studied *Cr. modiglianii* colonies.

retention index		relative abundance in *Cr. modiglianii* cuticular extracts (%)						
		R0	R1	R2	R3	B1	B2	B3
1	2038	0.32	0.14	0.42	0.29	0.61	0.45	0.34
2	2113	0.11	2.92	14.84	0.11	7.10	0.04	0.46
3	2132	0.20	0.00	0.00	0.21	0.17	0.18	0.20
4	2168	0.21	0.00	0.00	1.22	0.22	0.71	1.30
5	2173	0.04	0.61	2.10	0.00	0.29	0.00	0.00
6	2180	2.09	0.24	0.30	5.04	1.16	4.55	3.96
7	2192	1.40	0.27	0.22	8.89	3.38	3.85	6.69
8	2199	0.07	0.00	0.00	0.78	0.18	0.14	0.53
9	2202	0.00	0.00	0.00	0.48	0.12	0.02	0.03
10	2224	83.49	6.19	2.98	60.73	44.90	81.20	73.20
11	2233	9.57	0.28	0.29	6.04	1.94	6.79	6.75
12	2239	0.38	0.35	0.01	5.67	2.47	1.08	0.17
13	2365	0.00	0.57	1.36	0.00	0.03	0.00	0.00
14	2375	0.17	1.34	34.70	0.25	0.38	0.37	0.39
15	2383	0.00	0.18	0.72	0.00	0.00	0.00	0.00
16	2391	0.00	0.17	0.44	0.00	0.00	0.00	0.00
17	2436	0.00	0.00	0.00	0.10	0.00	0.12	0.03
18	2447	1.52	0.98	0.41	9.78	25.40	0.15	5.62
19	2457	0.44	0.00	0.00	0.24	1.55	0.01	0.05
20	2516	0.00	2.19	2.59	0.00	0.00	0.00	0.00
21	2526	0.00	2.17	9.40	0.00	9.67	0.00	0.00
22	2558	0.00	4.54	4.55	0.04	0.06	0.01	0.05
23	2571	0.00	7.62	3.92	0.00	0.11	0.01	0.04
24	2577	0.00	69.25	20.74	0.13	0.27	0.25	0.18

VI. Intraspecific recognition in parabiotic ants

This chapter has been published as:

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VI.1 Abstract

Parabiotic ants – ants that share their nest with another ant species – need to tolerate not only conspecific nestmates, but also nestmates of a foreign species. The parabiotic ants *Camponotus rufifemur* and *Crematogaster modiglianii* display high interspecific tolerance, which exceeds their respective partner colony and extends to alien colonies of the partner species. The tolerance appears to be related to unusual cuticular substances in both species. Both species possess hydrocarbons of unusually high chain lengths. In addition, *Cr. modiglianii* carries high quantities of hereto unknown compounds on its cuticle. These unusual features of the cuticular profiles may affect nestmate recognition *within* both respective species as well. In the present study, we therefore examined inter-colony discrimination *within* the two parabiotic species in relation to chemical differentiation.

Cr. modiglianii was highly aggressive against workers from alien conspecific colonies in experimental confrontations. Despite high inter-colony variation in the unknown compounds, however, *Cr. modiglianii* failed to differentiate between intracolonial and allocolonial unknown compounds. Instead, the cuticular hydrocarbons functioned as recognition cues despite low variation across colonies. Moreover, inter-colony aggression within *Cr. modiglianii* was significantly influenced by the presence of two methylbranched alkenes acquired from its *Ca. rufifemur* partner.

Ca. rufifemur occurs in two varieties (‘red’ and ‘black’) with almost not overlap in their cuticular hydrocarbons. Workers of this species showed low aggression against conspecifics from foreign colonies of the same variety but attacked workers from the respective other variety. The low inter-colony discrimination within a variety may be related to low chemical differentiation between the colonies. *Ca. rufifemur* majors elicited significantly more inter-colony aggression than medium-sized workers. This may be explained by the density of recognition cues: majors carried significantly higher quantities of cuticular hydrocarbons per body surface.

VI.2 Introduction

Nestmate recognition is one of the key features that maintain integrity of insect societies and prevent or reduce the invasion of parasites, enemies or competitors (Hölldobler and Wilson 1990). Social insects discriminate between nestmates and alien conspecifics using olfactory signals provided by cuticular substances. These odour signals are in part genetically determined, but often heavily influenced by environmental factors such as diet or nest

material (Heinze et al. 1996; Lenoir et al. 1999; Liang and Silverman 2000; Richard et al. 2004; Sorvari et al. 2008). In ants, these signals are mostly hydrocarbons (Lahav et al. 1999; Wagner et al. 2000). Aggression between conspecific colonies is often directly correlated to the differentiation of cuticular hydrocarbons (e.g. Suarez et al. 2002). Most ant species show high aggression against members of foreign conspecific colonies. However, among invasive ant species, intraspecific aggression is low or even absent, which results in a unicolonial population structure and is a major cause for their ecologically devastating impact (Holway et al. 2002). Their high intraspecific tolerance is probably caused by a low genetic inter-colony differentiation, which translates into lower differentiation of the chemical recognition cues (Suarez et al. 2008; Tsutsui et al. 2000; Tsutsui et al. 2003). A possibly similar mechanism has recently been described for non-invasive ants with unusually low intraspecific aggression (Foitzik et al. 2007).

Parabiotic ants, however, who share their nest with another ant species, often tolerate their partner species despite completely different cuticular hydrocarbons (Menzel et al. 2008b; Orivel et al. 1997). In Southeast Asian parabioses of *Crematogaster modiglianii* and *Camponotus rufifemur*, the tolerance between species often goes beyond the parabiotically associated colony and extends to other colonies of the partner species. The high tolerance coincides with highly unusual cuticular profiles (Menzel et al. 2008a). The cuticle of *Cr. modiglianii* possesses a set of hereto unknown compounds, which can reduce aggressiveness of its *Ca. rufifemur* partner (Menzel et al. submitted). Moreover, both *Cr. modiglianii* and *Ca. rufifemur* possess hydrocarbons that are considerably heavier than in non-parabiotic species of the same respective genera (Menzel et al. 2008a; unpublished data). Hydrocarbons of high chain lengths are likely to hamper recognition between ant species due to their low volatility (Brandstaetter et al. 2008; Lambardi et al. 2007). The species *Ca. rufifemur* occurs in two sympatric but chemically distinct varieties (‘red’ and ‘black’ variety, Menzel et al. 2008a). They show almost no hydrocarbon overlap and may thus represent distinct, cryptic species. However, until genetical evidence exists we will more conservatively speak of two varieties.

These unusual features of the cuticular profiles are likely to affect nestmate recognition *within* both respective species as well. We therefore studied the role of cuticular hydrocarbons versus unknown compounds for intraspecific nestmate recognition in *Cr. modiglianii*. For both *Cr. modiglianii* and *Ca. rufifemur,* intraspecific inter-colony aggression was examined and related to their respective chemical variability. In the red *Ca. rufifemur* variety, we additionally investigated inter-individual hydrocarbon variation between colonies and worker castes in relation to inter-colony aggression.

VI.3 Methods

VI.3.1 Study site and ants

Experiments and sample collection were carried out at Danum Valley Conservation Area. The area is located at 5°N 117°50’E and approximately 100 m a.s.l. in Sabah (Malaysian Borneo) and represents one of the major remaining patches of Sabah’s primary lowland rainforest. Aggression bioassays were carried out at parabiotic nests of *Crematogaster modiglianii* and *Camponotus rufifemur*, which were located in hollow, living tree trunks. The experiments with cuticular extracts were performed with two parabiotic colonies (R0, R1) that were brought into the laboratory and kept in their original nests (small tree trunks) in open plastic boxes with fluon-coated walls.

VI.3.2 Bioassays with cuticular extracts

These assays were to determine the role of the unknown compounds in nestmate recognition. Two *Cr. modiglianii* laboratory colonies (R0, R1; kept together with their parabiotic partner) were confronted with corpses, extracts or extract fractions (hereafter, 'treatments') of both nestmates and non-nestmates. In each treatment, we measured whether the ants distinguished nestmates from non-nestmates. The four treatments were (1) dead ants from the same and alien conspecific colonies, (2) their respective cuticular extracts, (3) the hydrocarbon (i.e. unpolar) fractions, and (4) the polar fractions (which contained the unknown compounds) of nestmate and non-nestmate extracts. Extracts and fractions were applied onto a dead *Cr. modiglianii* nestmate that had been extracted with hexane and chloroform for ten minutes twice each (henceforth termed 'dummy'). For surface extracts, 50 ants were killed by freezing and immersed in hexane for ten minutes. Unpolar and polar fractions of these extracts were obtained using conditioned SiOH columns (CHROMABOND, 100mg, Macherey-Nagel, Düren, Germany) with distilled hexane and chloroform as respective eluents. The chloroform of the polar fraction was subsequently evaporated and the fraction was reconstituted in hexane. GC-MS analyses confirmed that the hexane fractions contained hydrocarbons while the chloroform fractions contained the unknown compounds. The amount of extract per dummy was adjusted such that each dummy carried the extract of five individuals.
In each bioassay, the dummy (or the dead ant) was held with forceps onto the nest trunk of the laboratory colony so that several ants (up to nine) could interact with it simultaneously. During three minutes, all observed interactions with the dummy were counted and classified as peaceful (antennate or perform trophallaxis), weakly (open mandibles) or strongly aggressive (bite or lock mandibles). To provide more weight to long-lasting interactions, continued interactions were recorded again after every 10 sec during the three minutes. This classification is consistent with an earlier study (Menzel et al. 2008b). Different treatments were tested in haphazard order on different places of the nest trunk. We carried out ten replicates for each treatment. Dummies with pure hexane were tested as controls. We conducted experimental series with two different alien colonies (R3, B4) as non-nestmates in the laboratory colony R1 and a series with a third alien colony (R5) for laboratory colony R0.

VI.3.3 In situ aggression bioassays

The *in situ* aggression bioassays estimated aggression against living 'intruder ants' from alien conspecific colonies. They were conducted in arenas directly at the parabiotic nests in the rainforest of the study site. The arenas consisted of plastic rings (∅11.5 cm, height 5 cm) coated with fluon (*Cr. modiglianii* assays) or paraffin oil (*Ca. rufifemur* assays). They were placed on a plastic platform with paper tissue as floor. For tests with *Cr. modiglianii,* ten resident workers were carefully caught with forceps and placed into the arena. After 5 min to calm down, a living intruder ant from another colony of the same site was carefully introduced with forceps. For tests with *Ca. rufifemur*, we used the same method as in Menzel et al. 2008b. An arena was provided with tuna bait and connected to the nest trunk with a twig such that the ants could walk into the arena. After 1-2 hours, the twig was carefully removed without disturbing the foraging workers, and the intruder ant (major or medium-sized worker) was introduced. The *Ca. rufifemur* assays were conducted at night, under red light, since this species is nocturnal. The number or workers in the arena was recorded as a covariate. All following interactions of the resident ants towards the intruder were then observed for three minutes as described above.

For *Cr. modiglianii*, we performed intraspecific *Cr. modiglianii* assays using 10 parabiotic and 3 non-parabiotic *Cr. modiglianii* colonies. The aggression assays comprised a total of 44 colony combinations, 31 between parabiotic nests and 13 between a parabiotic and a non-parabiotic *Cr. modiglianii* colony. Five to seven replicates were conducted per colony combination. Within the red *Ca. rufifemur* variety, three allocolonial colony combinations were studied with 12 replicates per combination, i.e. six major and six medium-sized workers as intruders. In addition, we re-analyzed data for eleven additional intra- and allocolonial colony combinations of *Ca. rufifemur* from Menzel et al. 2008b, and included only assays with majors as intruders. See Fig. 4a for number of replicates and colony combinations per *Ca. rufifemur* variety. All studied *Ca. rufifemur* colonies of either chemical variety were separated by rivers and by at least 500 m distance. Due to their much broader heads, *Ca. rufifemur* majors are allometrically distinct from smaller worker castes. Medium-sized workers were defined as non-major workers above six mm body length.

VI.3.4 Statistical analysis: bioassays

From each bioassay replicate we calculated the sum of all aggressive versus all non-aggressive interactions. For the bioassays with cuticular extracts, we used generalized linear models (GLMs) with quasibinomial error distribution and logit link function. Pairwise comparisons between nestmate and non-nestmate were performed for each test series. For the *Cr. modiglianii* aggression bioassays, we tested whether inter-colony aggression in *Cr. modiglianii* depended on the variety membership of their *Ca. rufifemur* partner. Proportions of strong and total aggression were analyzed using GLMs as above. In addition, the constant number of workers allowed analyzing the absolute numbers of interactions using a linear mixed-effect model.

The *Ca. rufifemur* aggression bioassays were analyzed using GLMs with quasibinomial error distribution and logit link function. The first model considered only assays with major workers as intruders. It included the parameters 'within/across variety' (intruder and resident from the same or different *Ca. rufifemur* varieties), 'intra-/allocolonial', as well as 'colony combination', and the number of workers present in the arena. A second model, which only considered only allocolonial confrontations within the red *Ca. rufifemur* variety, analyzed the effects of 'caste' (major/medium-sized worker) and 'colony combination' on aggression.

The influence of each parameter was determined by likelihood ratio tests (F tests). In all bioassays, strong and total (including weak) aggression were analyzed separately. Since the statistical results of both analyses were very similar, we will report the latter and mention the former only if different.

VI.3.5 Chemical analysis

Extracts for analysis were prepared by immersing 10 to 90 ants killed by freezing in hexane for ten minutes. Substance quantities in *Cr. modiglianii* were too low to allow individual extracts. All samples for analysis contained an internal standard of 2 µg octadecane. We studied the cuticular hydrocarbons of nine *Cr. modiglianii* colonies with 1-9 sample replicates per colony. All surface hydrocarbons had been identified in an earlier study (Menzel et al. 2008a). Quantification was carried out with a high-resolution ThermoQuest Trace GC-FID with H_2 as carrier gas in order to achieve a better separation of the substances. We used an unpolar capillary column [DB-1 (J&W Scientific, Folsom, CA), 20m x 0.18mm ID, 0.18µm film thickness]. Temperature was kept at 60°C for 2 min then increased by 60°C/min up to 200°C and subsequently by 4°C/min to 320°C, where it remained constant for 10 min. A split/splitless injector was installed at 260°C in the splitless mode for 30 s. The flame

ionization detector (FID) was kept at 340°C. Peak areas were computed with Chrom-Card 1.19 (CE Instruments, Milan, Italy).
Colony and caste differentiation (major or medium worker) within the red *Ca. rufifemur* variety was studied using analogous extracts of single individuals. We analyzed 4-6 individual extracts for each of two worker castes (major and medium workers) and the three colonies tested in aggression bioassays (total n = 29). Quantification of these hydrocarbons was carried out using a Hewlett Packard 5890 GC-FID with an unpolar capillary column [DB-1 (J&W Scientific, Folsom, CA), 30 m x 0.25 mm ID, 0.25 µm film thickness] and helium as carrier gas. A split/splitless injector was installed at 260°C in the splitless mode for 30 s. The flame ionization detector (FID) was kept at 340°C. The temperature program was as above. The acquired data were used for a discriminant analysis. Based on the internal standard, absolute hydrocarbon quantities per individual were compared among the two worker castes. The quantities were then related to two body size metrics (head width and hind tibia length, Hölldobler and Wilson 1990) acquired from 28 workers from two of the three focal colonies and one additional colony.

VI.3.6 Statistical analysis: surface hydrocarbons

In order to estimate inter-colony variation of cuticular profiles, we used data acquired with high-resolution GC-FID from multi-individual extracts. We calculated the mean relative abundances of cuticular substances for nine (*Cr. modiglianii*), nine (red *Ca. rufifemur*) or four (black *Ca. rufifemur*) colonies, respectively. For each substance with mean abundance > 3%, the coefficient of variation between colonies was calculated as CV = S.D. / mean relative abundance.
The correlation of chemical differentiation and inter-colony aggression in *Cr. modiglianii* was estimated using the relative abundances of 28 major hydrocarbon peaks in the *Cr. modiglianii* profile (Menzel et al. 2008a). We calculated the Bray-Curtis indices of dissimilarity for the profiles of nine different *Cr. modiglianii* colonies. The obtained distance matrix was compared to a matrix of inter-colony aggression (obtained from the *in situ* aggression bioassays) between the same nine colonies. Since not all possible colony combinations had been tested, we used a Mantel test adjusted for missing values. As aggression measures we used relative proportions as well as absolute numbers of strong or total (including weak) aggression. All computations were performed in R Version 2.7.0 (R Development Core Team 2008).

VI.3.7 Statistical analysis: discriminant analysis

We analyzed the relative abundance of seven substance peaks, which were the only ones detectable in single-individual extracts. These were five methylbranched alkene peaks and two not identifiable substances with retention indices 33.53 and 38.79 (see Table S1 and Menzel et al. 2008a for details). All peak areas were standardized according to $A_p' = \ln((A_p + 0.0001)/g(A_p))$, where A_p is the peak area and $g(A_p)$ is the geometric mean of all peak areas in the respective sample (Aitchinson 1986), in order to correct for the high interdependence of this type of data. The constant 0.0001 was added to provide non-detectable substances with a small non-zero value as recommended by Aitchinson (1986). The transformed data were entered into a step-wise forward discriminant analysis. We report Wilks' λ values and the percentage of correctly assigned samples (classification matrix). The discriminant analysis was performed using Statistica 7.0.

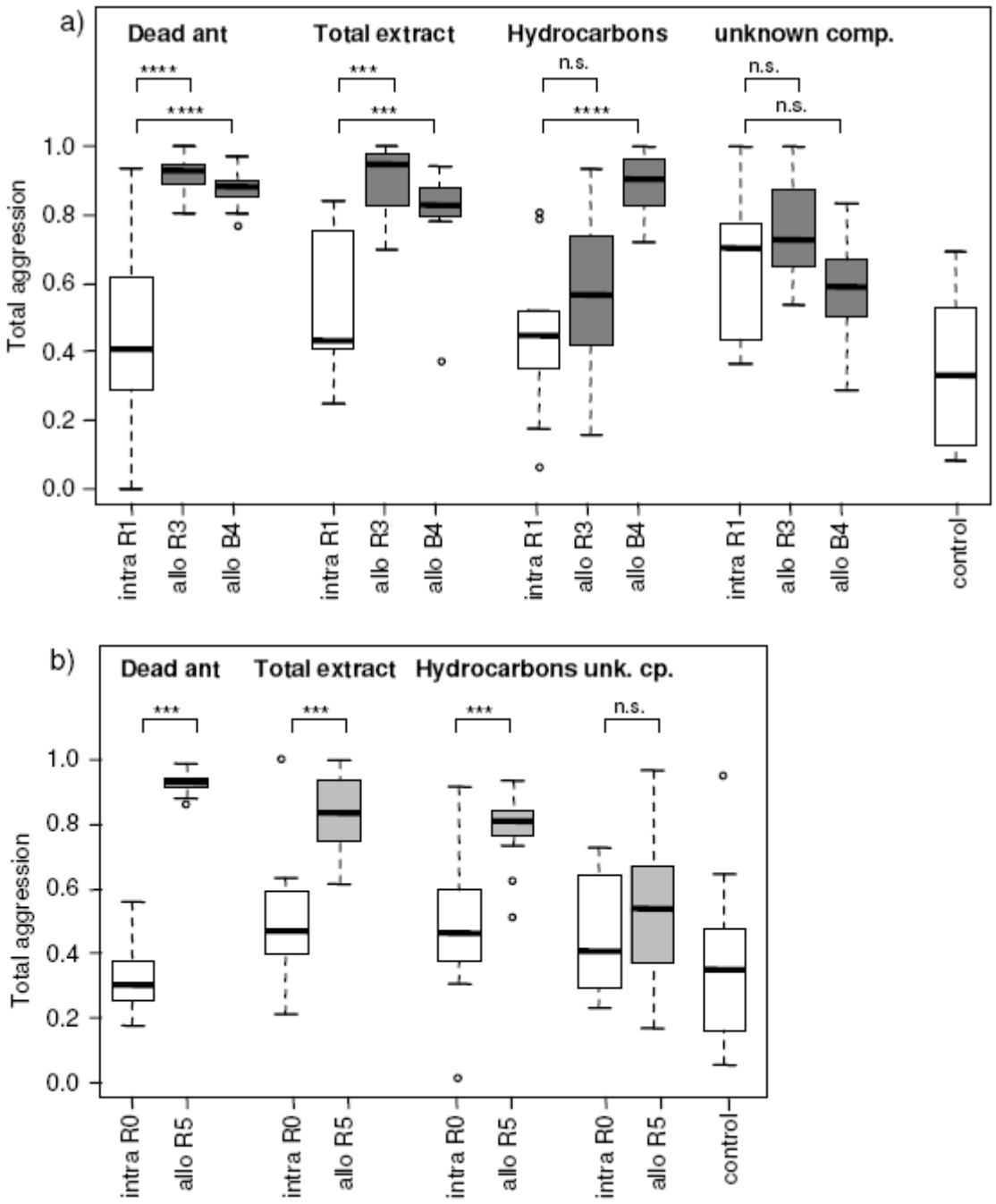

Fig. 1 Total aggression of *Crematogaster modiglianii* (a) colony R1 and (b) colony R4 against nestmate and non-nestmate extracts and fractions thereof. The plots shows median, quartiles and range of ten assay replicates each. 'unk.cp.': unknown compounds, 'intra': intracolonial treatments; 'allo': allocolonial treatments. 'R' and 'B' in the colony code refer to *Cr. modiglianii* colonies associated with red and black *Ca. rufifemur*, respectively. 'control': control bioassays with dummies covered in pure hexane. ****$P < 0.0001$; ***$P < 0.001$, 'n.s.' $P > 0.05$, according to GLM.

VI.4 Results

VI.4.1 Crematogaster modiglianii

Crematogaster modiglianii workers of both experimental colonies (R0 and R1) significantly differentiated between intra- and allocolonial dead *Cr. modiglianii* workers (Fig. 1). They attacked the latter but mainly antennated the former. The same, significant differentiation was

found for dummies that carried total extracts or hydrocarbon fractions, except for the hydrocarbon fraction of one foreign colony (Fig. 1). However, the workers never discriminated between intracolonial and allocolonial polar fractions, which contained the unknown compounds (Fig. 1), although their composition strongly varied among the used colonies (Bray-Curtis dissimilarities between unknown compounds of colony R1 and R3: 0.92; R1 and B4: 0.61).

Aggression between *Cr. modiglianii* workers from different parabiotic or non-parabiotic colonies was generally high. Out of 44 colony combinations, only three resulted in peaceful interactions. The Bray-Curtis dissimilarity between nine parabiotically associated colonies did not correlate with allocolonial aggression (adjusted Mantel test with proportions of total aggression: r = -0.19, P = 0.81, 10000 permutations, n = 36 colony combinations). Similar results were obtained for the absolute number of aggressive interactions. The three mentioned, peaceful colony combinations corresponded to intermediate Bray-Curtis dissimilarity values.

The quantitative hydrocarbon composition showed a relatively low variation between different *Cr. modiglianii* colonies (Fig. 2). The only exception were 27-MeC39-14-ene and 27-MeC39-16-ene (coefficient of variation: 1.39). These two substances, which were not separable by gas chromatography, represent the dominant cuticular hydrocarbons in the red *Ca. rufifemur* variety. They are present in *Cr. modiglianii* colonies associated with red *Ca. rufifemur* variety but absent in those associated with the black one (Menzel et al. 2008a). Composition of unknown compounds was highly variable between colonies (Fig. 2). While a certain set of these compounds was present in all colonies, others were dominant in some colonies but absent in others, leading to high coefficients of variation.

Cr. modiglianii workers were significantly less aggressive if the intruder ant was from a colony associated with the same *Ca. rufifemur* variety. Absolute numbers of aggressive interactions were significantly higher against intruders associated with the respective other *Ca. rufifemur* variety (linear mixed-effect model: $F_{1, 24}$ = 6.57, P = 0.017), which was not the case for peaceful interactions ($F_{1, 24}$ = 0.38, P = 0.55, Fig. 3). When regarding relative proportions of strong or total aggression, the effect was marginally significant (both P < 0.06).

VI.4.2 Camponotus rufifemur

Camponotus rufifemur workers significantly discriminated between intracolonial and allocolonial intruders and only attacked the latter (Table 1, Fig. 4a, regarding majors only). Aggression was especially high against intruders from the respective other variety but significantly lower against those from the same variety. The parameter 'within/across variety' alone explained 40.1% of the total variation in aggression (Table 1, Fig. 4a, majors only). Within the two respective varieties, allocolonial aggression was especially low against medium-sized intruders. Altogether, allocolonial medium workers received no strong aggression at all in 12 out of 22 assays within both respective varieties. Within the red *Ca. rufifemur* variety, they were significantly less attacked than majors (GLM for proportions of total and strong aggression: P = 0.047 and 0.019, respectively) (Table 2, Fig. 4b).

The variation in relative hydrocarbon quantities between nine colonies of the red *Ca. rufifemur* and between four colonies of the black variety was significantly lower than in nine *Cr. modiglianii* colonies (Wilcoxon rank sum test: *Ca. ru.* red-*Cr. mod.*: W = 35, P = 0.039; *Ca. ru.* black-*Cr. mod.*: W = 95, P = 0.0009; Fig. 2). Major and medium-sized workers in the red *Ca. rufifemur* could be significantly discriminated based on their quantitative cuticular composition. The discriminant model includes five substances (Wilk's λ = 0.379, $F_{7, 21}$ = 4.91,

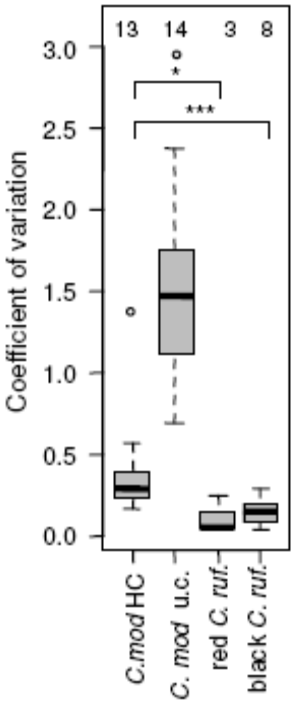

Fig. 2 Coefficients of variation of cuticular substances between colonies. For each substance with mean abundance > 3%, the relative proportion of this substance within the profile was compared among colonies by calculating variation coefficients as S.D. / mean. The numbers above each plot indicate number of considered cuticular substances. Data are given for hydrocarbons ('HC') and unknown compounds ('u.c.') of *Cr. modiglianii* (n = 9 colonies), and the hydrocarbons of red and black *Ca. rufifemur* (n = 9 and 4 colonies, respectively). The outlier in *Cr. modiglianii* hydrocarbons represents $27MeC_{39}$-14-ene and $27MeC_{39}$-16-ene. **P* < 0.05, *** *P* < 0.001, according to Wilcoxon rank sum test.

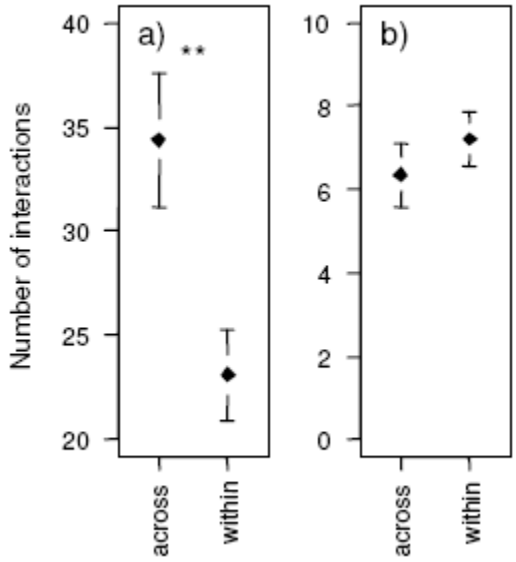

Fig. 3 Aggression bioassays in *Cr. modiglianii*, given as total number of (a) (strongly or weakly) aggressive and (b) peaceful interactions. The graphs show allocolonial confrontations of *Cr. modiglianii* workers associated with the same ('within', n = 78 assays) or different ('across', n = 61 assays) *Camponotus rufifemur* varieties (mean ± SE). **significant at *P* < 0.01 according to linear mixed-effect model.
b) Aggression of red *Camponotus rufifemur* workers against major and medium workers of allocolonial workers of the same variety. The plots show the proportion of strong aggression (median, quartiles and range), pooled for three colony combinations. Number of replicates is given above the plots. *significant *P* < 0.05 according to GLM with binomial error distribution.

$P = 0.0021$, $n = 29$). The model correctly classifies 89.7% of the samples into the two castes. In contrast, the discriminant analysis did not reveal any differentiation between the three colonies (Wilk's $\lambda = 0.91$, $F_{2, 26} = 1.21$, $P = 0.31$, $n = 29$).

Majors of the red *Ca. rufifemur* variety carried more than seven times more hydrocarbons (total quantities) than medium workers (Welch-corrected $t = 5.91$, $df = 14.44$, $P < 0.0001$) (Table 3). In contrast, the two morphometric measures differed by a much smaller factor between the two castes. Their squared major/medium ratios, which roughly reflect surface size ratios, were considerably lower (Table 3). This suggests that majors carry higher hydrocarbon quantities both in absolute terms and per body surface.

Table 1 GLM for the proportion of total aggression in *Camponotus rufifemur.*
Both varieties, but only majors as intruders are considered (n = 84 recognition assays)

	Deviance	*df*	*F*	*P*
Within/across variety	985.1	1	65.2	<0.0001
Intra-/allocolonial	426.3	1	37.38	<0.0001
Colony combination	469.1	11	5.296	<0.0001
No. *Camponotus*	44.7	1	6.03	0.017
No. *Crematogaster*	4.89	1	0.659	0.42
Residual error	528.9	68		
Total	2459.4	83		

VI.5 Discussion

VI.5.1 Crematogaster modiglianii seems to acquire recognition signals from its parabiotic partner

Cr. modiglianii workers were significantly less aggressive towards allocolonial conspecifics when they were associated with the same *Ca. rufifemur* variety. This coincides with the abundance of 27-MeC39-14-ene and 27-MeC39-16-ene in the *Cr. modiglianii* profile. The two methylbranched alkenes are abundant in the red *Ca. rufifemur* variety but absent in the black one. Consequently, they only occur in those *Cr. modiglianii* colonies associated with the red *Ca. rufifemur* variety (see Menzel et al. 2008a, Fig. 2 therein, for a comparison with largely the same colonies as used in this study), and are most probably acquired from its partner (comparable to artificial mixed colonies, Vienne et al. 1995). Regarding the hydrocarbon profile, this represents the only detectable difference between *Cr. modiglianii* associated with red and those associated with black *Ca. rufifemur*. Since nestmate recognition in *Cr. modiglianii* is mediated by hydrocarbons (Fig. 1), and *Cr. modiglianii* shows differential inter-colony aggression depending on the identity of its partner, it appears likely that *Cr. modiglianii* uses these methylbranched alkenes as a recognition signal provided by its parabiotic partner. Ants can adopt nestmate recognition cues from various environmental sources such as food (Sorvari et al. 2008, Richard et al. 2004) or nest material (Heinze et al. 1996). However, to our knowledge it has not been reported previously that ants also adopt intraspecific recognition cues from an associated species.

Except for these two substances, the detectable variation of the remaining cuticular hydrocarbons between nine colonies was low (Fig. 2). However, in contrast to *Ca. rufifemur, Cr. modiglianii* was highly aggressive against most alien workers, even against colonies only 2-3 meters away. Although the workers thus clearly differentiated between colonies, the chemical differentiation beside the substances acquired from *Ca. rufifemur* is probably too subtle for detection with our methods. This explains why we did not find a correlation between inter-colony aggression and chemical differentiation, although – as revealed by our extract bioassays – *Cr. modiglianii* does use cuticular hydrocarbons as nestmate recognition cues.

The unknown cuticular compounds, in contrast, do not function as nestmate recognition cues, although they are highly abundant and vary both quantitatively and qualitatively (Fig. 2).

Aggression against alien total extracts was similar to aggression against alien hydrocarbons in two out of three cases (Fig. 1). Hence, the unknown compounds most likely do not possess an aggression-reducing effect in intraspecific encounters, as has been shown for interspecific encounters with *Ca. rufifemur* (unpublished data).

VI.5.2 Camponotus rufifemur: low inter-colony aggression within chemical varieties

Albeit higher than against nestmates, inter-colony aggression between *Ca. rufifemur* workers of the same chemical variety was surprisingly low. A notable proportion of allocolonial intruders (especially medium workers) received no aggression at all. This was although the experimental setup – directly at the nest, minimized disturbance, one intruder only – should maximize aggression against alien ants. In contrast, many studies on other *Camponotus* species report high levels of inter-colony aggression, even if these species live in interspecific associations like lestobioses (Carlin and Hölldobler 1986; Errard et al. 2003, Boulay et al. 2000). However, *Ca. rufifemur* fiercely attacked workers of the respective other chemical variety and often dismembered them within two or three minutes.

The low intra-variety aggression cannot be explained by a dear-enemy phenomenon (e.g. Heinze et al. 1996) or a possible polydomous colony structure, since all colonies were distant from each other and separated by rivers. Its causes probably involve the high abundance of long-chain unsaturated cuticular hydrocarbons (C_{37}-C_{49}, Menzel et al. 2008a). Long-chain hydrocarbons are harder to perceive by olfactory receptors due to their low volatility (Gibbs and Pomonis 1995) and probably blur small differences in the recognition cues (Lambardi et al. 2007). Hence, the workers may be unable to detect small signal differences, e.g. between colonies of the same variety, but still recognize strongly (or qualitatively) different signals (e.g. between the two *Ca. rufifemur* varieties). It has been reported that long-chain hydrocarbons can hamper interspecific discrimination both in social parasites (Lambardi et al. 2007) and between *Cr. modiglianii* and *Ca. rufifemur* (Menzel et al. 2008a). However, to our knowledge it has not been shown previously that high interspecific tolerance also extends to intraspecific tolerance as reported here.

Moreover, there is little inter-colony hydrocarbon variation *within* the two respective *Ca. rufifemur* varieties. Inter-colony coefficients of variation were significantly lower than in *Cr. modiglianii* (Fig. 2). The discriminant analysis did not find colony differences based on individual cuticular extracts. We are aware that lack of detectable differentiation does not necessarily imply lack of differentiation (particularly in single-individual extracts with low substance quantities). For example, *Cr. modiglianii* displayed pronounced nestmate discrimination despite of low measureable differentiation. Nevertheless, the fact that hydrocarbon profiles of different colonies are often easily discernible in other ant species (Nielsen et al. 1999, Liu et al. 2001) argues for lower chemical differentiation in *Ca. rufifemur* than in other ant species. The long periods of antennation in within-variety allocolonial encounters (Menzel et al. 2008b) may indicate recognition uncertainty, probably caused by the low chemical differentiation between colonies. This behaviour can also be observed towards nestmates that were separated from the colony for hours or days (Menzel et al. 2008b). This corroborates that the continued antennation may be due to recognition uncertainty, which is different from 'tolerance despite of recognition as foreign'. As a matter of principle, however, one cannot infer recognition processes based on behavioural experiments, although this has sometimes implicitly been claimed in earlier studies (e.g. Steiner et al. 2007).

VI.5.3 Camponotus rufifemur: Inter-colony aggression depends on worker caste

An unexpected outcome of the aggression bioassays was that medium workers were significantly less attacked than majors. Majors carry on average more than seven times the amount of cuticular hydrocarbons as medium workers. Even when correcting for the larger body surface using two morphometric measures (Table 3), they possess higher hydrocarbon quantities per body surface. The higher availability of recognition cues in majors may make it easier for workers to recognize them as foreign, resulting in the observed higher aggression. The low discrimination between medium castes may thus be a consequence of the low chemical differentiation between *Ca. rufifemur* colonies of the same variety, coupled with low absolute quantities of recognition cues in medium-sized workers.
Notably, both *Ca. rufifemur* and their parabiotic partner *Cr. modiglianii* often fail to discriminate workers from different *Ca. rufifemur* colonies of the same variety (Menzel et al. 2008a). The unusual cuticular compounds found in this species (Menzel et al. 2008a) thus seem to influence both intraspecific and interspecific recognition.

Acknowledgements

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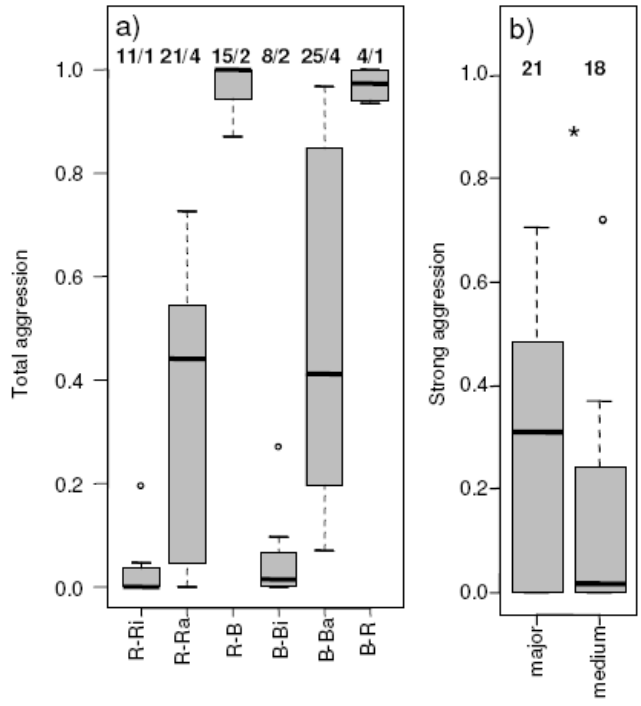

Fig. 4 a) Aggression of *Camponotus rufifemur* towards majors from different colonies, given as proportions of aggressive interactions. 'R-Ri' red *Ca. rufifemur* towards intracolonial red; 'R-Ra' red towards allocolonial red; 'R-B' red towards black; 'B-Bi' black towards intracolonial black; 'B-Ba' black towards allocolonial black; 'B-R' black towards red.

Table 2 GLM for the proportion of total aggression between colonies of the red *Camponotus rufifemur* variety (n = 39 recognition assays).

	Deviance	*df*	*F*	*P*
Caste	40.6	1	4.23	0.047
Colony combination	18.8	3	0.62	0.61
Caste:colony combination	29.4	2	1.49	0.24
Residual	372.6	32		
Total	461.5	38		

Table 3 Individual hydrocarbon quantities and morphometric measures in the red *Camponotus rufifemur* variety. Data given are mean and standard deviation.

	Hydrocarbon quantity (*n*=29)	Head width (*n*=28)	Hind tibia length (*n*=28)
Major	3.62 ± 2.05 µg	3.30 ± 0.34 mm	3.18 ± 0.26 mm
Medium	0.48 ± 0.22 µg	1.80 ± 0.28 mm	2.31 ± 0.20 mm
Ratio	7.54	1.83	1.39
Ratio²		3.31	1.93

Table S1 List of all substances detectable in individual surface extracts of the red *Ca. rufifemur* variety.
The reference number refers to Table 1 in Menzel et al. 2008a. The first compound had not been regularly detected in earlier studies. Relative abundance is given as relative peak area (mean and standard error). $^{+}$position of double bond tentative, §number of substances and their exact structure could not be further determined.

reference number	substance	relative abundance	retention index
-	unknown	0.27 ± 0.04 %	33.73
37	25-MeC37-14-ene, 25-MeC37-16-ene^{+}	0.29 ± 0.04 %	36.96
43	x(25,26,27)-MeC38-y(13,14,15,16)-ene$^{+\S}$	2.24 ± 0.68 %	37.93
49	unknown	0.14 ± 0.1 %	38.79
52	27-MeC39-14-ene, 27-MeC39-16-ene	90.79 ± 0.76 %	39.02
56	27-MeC40-14-ene, 27-MeC40-15-ene, 27-MeC40-16-ene^{+}	3.19 ± 0.13 %	39.97
61	x(27,29)-MeC41-y(14,16,18)-ene$^{+\S}$	3.08 ± 0.28 %	40.94 (extrapolated)

VII. Trail-sharing in parabiotic and non-parabiotic ants

VII.1 Abstract

1. Trail-sharing between different ant species is rare and restricted to certain pairs of species, but its underlying mechanisms are largely unknown.
2. We investigated two behavioural mechanisms that might cause or promote interspecific trail-sharing: interspecific trail-following, i.e. workers following another species' pheromone trail, and differential interspecific aggression.
3. In a tropical rainforest in Borneo, we studied a common trail-sharing association of *Polyrhachis, Camponotus* and *Dolichoderus* species and an association of *Camponotus* and *Crematogaster* species. Workers of each species were confronted with artificial pheromone trails of their associated species. Additionally, we studied interspecific aggression in the former associations.
4. In our assays, *Dolichoderus cuspidatus, Crematogaster modiglianii* and *Camponotus rufifemur* regularly followed heterospecific pheromone trails, the latter following trails of its parabiotic partner. Thus, these species parasitized on another species' information on food sources. However, in the remaining species, only few workers followed heterospecific pheromone trails.
5. Interspecific aggression among the trail-sharing species *P. ypsilon*, *Ca. saundersi* and *D. cuspidatus* was strongly asymmetric. All three species were significantly more aggressive (*P. ypsilon* and *D. cuspidatus*) or submissive (*Ca. saundersi*) towards heterospecific workers from a foreign vs. the same site.
6. Differential tolerance by dominant ant species may thus be mediated by selective habituation towards submissive species and this way determine the assembly of trail-sharing associations.

VII.2 Introduction

Ants are among the most important actors in terrestrial ecosystems, as measured both by their biomass and by their abundance (Hölldobler and Wilson 1990). Their world-wide success is partly due to their efficient exploitation of food sources. In the more advanced ant subfamilies, foraging workers use pheromone trails to quickly recruit nestmates to newly discovered food sources (Hölldobler and Wilson 1990). Pheromone trails hence convey important information about the location of both food sources and the nest. This information can be exploited by parasites, e.g. lycaenid caterpillars seeking the ant nest (Dejean and Beugnon 1996). Pheromone trails may also be used to exploit another species' information on food sources, which has been reported for various ants as well as stingless bees and was termed 'olfactory eavesdropping' or 'informational parasitism' (Nieh et al. 2004, Adams 1990, Gobin et al. 1998, Wilson 1965).
Interspecific exploitation of trail pheromones seems to be uncommon, and the vast majority of ant trails is used by only one species. However, certain pairs of species share trails frequently and regularly (e.g. Baroni Urbani 1969, Wilson 1965) so that their co-occurrence seems more common than expected by chance. A possible mechanism behind such trail-sharing associa-

Table 1 Overview of all conducted trail-following experiments.
The numbers indicate the number of individuals used for each extract. Letters indicate which body part extracts were tested in laboratory and field assays, respectively. h: head, t: thorax, g: gaster, d: digestive tract, p: poison and dufour gland, l: legs. 'LC' refers to experiments with a *Cr. modiglianii* laboratory colony with the setup used for field assays. Assays with a median of at least 20% (laboratory assays) or two individual (field assays) trail-following workers are indicated with asterisks, those with a median of at least 40% or four trail-following workers are indicated with two asterisks. Intraspecific combinations (workers and extracts of the same species) are shaded.

	tested species	extracted species	laboratory assays	field assays
System 1	*P. ypsilon*	*P. ypsilon* (4)	h t d* p	h t g*
		Ca. saundersi (8)	h t g	h t g
		D. cuspidatus (8)	h t g	
		P. olybria (4)		h t g
	Ca. saundersi	*P. ypsilon* (4)	h t g	h t g
		Ca. saundersi (8)	h* t* d** p*	h t g**
		D. cuspidatus (8)	h t g	
		P. olybria (4)		h t g
	D. cuspidatus	*P. ypsilon* (4)	h t g**	h t g
		Ca. saundersi (8)	h* t* g	h t g
		D. cuspidatus (8)	h t g**	
		P. olybria (4)		h t g
System 2	*Ca. rufifemur*	*Ca. rufifemur* (6)	h t d** p	h t g**
		Cr. modiglianii (20)	h t g l* LC: h t g l	l
	Cr. modiglianii	*Ca. rufifemur* (6)	h t d p	h t g
		Cr. modiglianii (20)	h t g l** LC: h t g l**	l**
System 3	*Ca. (Col.)* sp. 62	*Ca. (Col.)* sp. 62 (5)		h t g*
		Cr. modiglianii (15)		l
	Cr. modiglianii	*Ca. (Col.)* sp. 62 (5)		h* t* g**
		Cr. modiglianii (15)		l**

tions is the ability of one species to follow the other's pheromone trail. Henceforth, we will refer to this mechanism as 'interspecific trail-following', as opposed to the more general term 'interspecific trail-sharing', which we use for the phenomenon of two species using the same trail.

Whether several ant species share trails does not only depend on their ability to follow each other's trail pheromone, but also on reciprocal acceptance or aggression. In most ant communities, there is a clear dominance hierarchy, i.e. one species usually attacks the other upon encounter while the other either flees from the first or otherwise avoids contact (Adams 1990, Gobin et al. 1998, Wilson 1965, Baroni Urbani 1969). In few cases, however, no aggressive interactions were observed, even when one of the species seemed to be dominant (Dejean 1996; Starr 1981). Interspecific trail-sharing can thus not be understood without concomitant studies on mutual tolerance. Various ant species defend their foraging territories, trails, food sources, or only their nest (Hölldobler and Wilson 1990). It seems likely that these

two mechanisms – interspecific trail-following and interspecific aggression – essentially determine the structure of trail-sharing associations, and, thus, the distribution of ant species in a habitat.
In the present study, we investigated how interspecific trail-following and interspecific aggression structure trail-sharing associations. We studied three of the most common trail-sharing systems we found in the tropical lowland rainforest of Borneo. In each system, we determined whether there is interspecific trail-following, by confronting ants with artificial pheromone trails of other species. These assays were accompanied by experiments or observations on mutual tolerance between the trail-sharing species.

VII.3 Materials and methods

VII.3.1 Study site

The study was conducted from September to December 2007 in the Danum Valley Conservation Area, Sabah (Malaysian Borneo). The area covers 438 km² of primary lowland rainforest and has a typical rainforest climate with a mean annual temperature of 26.7 °C and an average rainfall of 2670 mm per year.

VII.3.2 Overview of the three study systems and experimental series

We studied interspecific trail-following in the three most common trail-sharing associations at the study site. Intraspecific trail-following assays served as validation of the experimental setup. An overview of all trail-following assays is given in table 1. Voucher specimens are held at the Forest Research Center (Sandakan, Sabah, Malaysia) and the Biocenter, University of Würzburg (Germany).
System (1). The most common trail-sharing association at the study site was between a *Polyrhachis (Polyrhachis)* species (either *P. ypsilon* Emery 1887 or *P. olybria* Forel 1912) (Formicinae) and a *Camponotus (Colobopsis)* species (either *Ca. saundersi* Emery 1889, sp. 1 or sp. 69 of Seiki Yamane's reference collection) (Formicinae). Two other *Polyrhachis* species, *P. (Myrmhopla) armata* (Le Guillou 1842) and *P. (Myrmhopla) abdominalis* Fr. Smith 1858, sometimes used these trails as well. We focused on a shared trail of *P. ypsilon* and *Ca. saundersi* but also included assays with extracts of *P. olybria*. At the study site, *Dolichoderus cuspidatus* Smith 1857 (Dolichoderinae) regularly used the same trail and was therefore included in the study.
System (2). *Camponotus rufifemur* Emery 1900 (Formicinae) and *Crematogaster modiglianii* Emery 1900 (Myrmicinae) live in a parabiotic association. They share a nest (usually in living, hollow trees), forage together at trophobioses and baits (Menzel and Blüthgen submitted), and also frequently share trails to food sources. Both are highly tolerant towards the respective other species (Menzel et al. 2008).
System (3). An undescribed *Camponotus (Colobopsis)* species (sp. 62 of Seiki Yamane's reference collection) regularly occurred on the nest trunk of parabioses; often ≥ 20 workers were sometimes observed simultaneously. They frequently shared baits with *Cr. modiglianii* but were aggressively displaced by *Ca. rufifemur*. We therefore conjectured that *Ca. (Colobopsis)* sp. 62 might follow *Cr. modiglianii* trails to reach parabiotic associations.
In all three systems, the ants were confronted with extracts of both their own and the respective other species (table 1). Both laboratory and field assays were conducted for systems 1 and 2, whereas system 3 was only tested in the field. In system 2, *Cr. modiglianii* extracts were additionally tested in a laboratory colony of *Cr. modiglianii* (which included few *Ca. rufifemur* workers) using the setup for field assays (Table 1). Interspecific aggression

tests were conducted for system 1. Additional observations on mutual tolerance at baits were performed for systems 1 and 3. All assays were conducted with workers and extracts from the same locations. However, due to low *P. ypsilon* and *Ca. saundersi* abundance at the site of system 1, we performed the laboratory assays of these two species using extracts and live individuals from a different trail-sharing site.

VII.3.3 Trail-following: preparation of extracts

The trail-following assays tested whether the ants followed artificial extract trails. Since the field conditions and the small size of species such as *Cr. modiglianii* did not allow preparation of the glands themselves, we made extracts of different body parts and tested whether the extracts containing the presumed trail pheromones elicited stronger trail-following behaviour than other body parts. The extracts were made of heads, thoraces (including legs) or gasters of freeze-killed ants that were immersed in chloroform for 1.5 hours. For intraspecific assays of *P. ypsilon, Ca. saundersi* and *Ca. rufifemur*, we separately tested extracts of digestive tract and poison/dufour gland (Table 1). For *Cr. modiglianii* we included separate leg extracts since trail pheromone glands in *Crematogaster* have repeatedly been reported from the hind tibiae (e.g.Fletcher and Brand 1968; Leuthold 1968; Morgan et al. 2004).
Corresponding to size differences between ant species, 4 to 20 individuals were used per extract (Table 1). Each extract was used for one experiment within an hour after preparation (systems 1,2) or on the same day (system 3). *Ca. saundersi* possesses hypertrophied mandibular glands that extend far into the gaster. For the gaster extracts of this species, the mandibular glands were carefully separated from the other tissue and discarded. In all species but *Cr. modiglianii* and *Ca.* sp. 62, the cuticle was removed from the gaster before immersion in chloroform.

VII.3.4 Trail-following: experimental setup and statistical analysis

In each trail-following assay, the ants had to choose between an artificial trail of pure solvent and one made with an extract. For each trial, a Y-shape was drawn on a sheet of paper with a pencil. Using pipettes, extract was applied retracing a bottom line (20 cm) and one arm of the Y-shape (15 cm) (extract trail), whereas pure chloroform was used for the second arm (solvent trail). For system 3, the sheets measured 7 cm (bottom line) and 5 cm (Y arms). The sides for solvent and extract were switched between trials to prevent a directional bias. Ants were allowed to follow the trail starting at the bottom line. An ant was regarded to perform trail-following behaviour when it followed the bottom line and at least 12 cm (system 3: 5 cm) of the extract trail.
For laboratory assays, we used worker colonies (40 to 80 individuals) that were kept in plastic boxes with fluon-coated walls in non-airconditioned rooms at the Danum Valley Field Center. They were fed with tuna, honey solution and water. Dry leaves and bark pieces were provided as shelter. At least two hours prior to an experiment, each colony was transferred into a prepared plastic box and provided with the same diet until the assay series was completed (two to six days). The box featured an exit hole on ground level that could be opened and closed in order to let ants out into the arena. It was placed in a tray (about 100 cm x 60 cm), which served as testing arena. For each assay, the sheet with the artificial trails was placed into the arena, with the starting point of the Y directly at the exit hole. We let out one ant at a time and recorded whether the ant followed the extract trail, the solvent trail, or neither. Each assay included ten workers.
For the field assays, each paper sheet was freshly prepared in the field. After evaporation of the solvent it was attached horizontally to a natural shared trail with the starting point directly

on the natural trail. This natural trail was located on a horizontal part of a liana (system 1), on the parabiotic nest trunk (System 2), or a log (system 3). Each paper sheet was left in place for 15 minutes after the first ant had followed one of the two artificial trails, or for 30 minutes if no ants followed the trail. During this time we recorded the number of ants following the extract or solvent trail.

For each species–extract combination, six (systems 1,2) or five to seven (system 3) replicates were performed. We directly compared the responses towards extract trails of different body parts, since the ants never followed the solvent trails (except for system 3). For each combination of living and extracted species, we used generalized linear models (GLMs) with a quasipoisson error distribution for field assays (count of trail-following workers) and a quasibinomial error distribution for the laboratory assays and the field assays in system 3 (proportion of trail-following workers). The impact of the parameter 'body part' was evaluated with F tests. For system 3, the dependent variable was the proportion of workers that followed the extract trail compared to all workers that had entered the paper sheet.

VII.3.5 Aggression tests

Aggression tests were conducted for system 1 only. On the shared trail, we recorded the behaviour of *P. ypsilon, Ca. saundersi* and *D. cuspidatus* workers towards various ant bodies. The ant bodies were freeze-killed workers of the same three species from the same site, and, for *P. ypsilon* and *Ca. saundersi*, additionally from a different trail-sharing site (ca. 1 km away). We also presented bodies of *Dolichoderus thoracicus,* which is a common species in the rainforest understory but not observed to be involved in any trail-sharing. The bodies were mounted on a wire (approx. 15 cm long) with a thin plastic thread. Each body was subsequently presented to 20 workers of each ant species on the trail. We recorded their reactions only if they had shortly antennated the body or at least held their antennae in close proximity (< 0.5 cm). Each reaction was categorized into 'bite', 'open mandibles', 'turn and flee', 'antennate', and 'ignore'. Ten replicates per colony combination were conducted. We compared reciprocal aggression (bite, open mandibles) and avoidance (turn and flee) between species of the same site. Moreover, we tested whether the ants discriminated between conspecific or heterospecific ant bodies from the same and an alien site. All of these pairwise comparisons were conducted using generalized linear models (GLMs) with binomial error distribution (all df = 1). The difference between the two groups of each model was estimated using a χ^2 test. All statistical computations were performed in R 2.8.1 (R Development Core Team 2008).

The aggression assays were supplemented by observations on interspecific aggression between *P. ypsilon* and *Ca. saundersi* at peanut butter or sugar baits. Interspecific interactions in system 3 were observed at honey or tuna baits between *Cr. modiglianii* and *Ca.* sp. 62 (n = 7), and *Ca. rufifemur* and *Ca.* sp. 62 (n = 3) during 3-5 min per bait.

VII.4 Results

VII.4.1 Intraspecific trail-following

In all species studied, trail-following could be triggered by conspecific extracts. In each case, one of the body parts elicited trail-following, whereas the other body parts triggered highly significantly less or no trail-following (except for *Camponotus (Colobopsis)* sp. 62; table 2, Figs. 1-3). Trail-following was elicited by digestive tract extracts and gaster extracts in *Polyrhachis ypsilon, Camponotus (Colobopsis) saundersi,* and *Camponotus (Myrmotarsus)*

rufifemur, by gaster extracts in *Dolichoderus cuspidatus* and *Camponotus (Colobopsis)* sp. 62 and by leg extracts in *Crematogaster modiglianii*.

VII.4.2 Interspecific trail-following

Three species displayed frequent interspecific trail-following. In the laboratory assays, *Dolichoderus cuspidatus* readily followed trails of *P. ypsilon* gaster extracts but no extracts of other body parts. Several *D. cuspidatus* workers also followed head and thorax extracts of *Ca. saundersi*, which also elicited intraspecific trail-following in *Ca. saundersi,* but did not follow its gaster extracts (Fig. 1a). *Camponotus rufifemur* regularly followed leg extracts of *Cr. modiglianii* but no extracts of other *Cr. modiglianii* body parts (Fig. 2a). The difference between extracts of different body extracts was highly significant in all above cases (table 2). However, interspecific trail-following in these species was not observed in the field (Figs. 1b, 2b). *Cr. modiglianii* did not follow *Ca. rufifemur* extracts but often followed gaster extracts of *Camponotus (Colobopsis)* sp. 62, and, to a lesser degree, head or thorax extracts of *Ca.* sp. 62 (Fig. 3). However, since *Ca.* sp. 62 gaster extracts left a grey trace on the paper sheet, it is possible that this optical token additionally reinforced trail-following behaviour.
Only occasional trail-following was observed in the remaining species-extract combinations. In *P. ypsilon, Ca. saundersi* and *Ca.* sp. 62, few workers followed heterospecific trails, and *Cr. modiglianii* did not follow any *Ca. rufifemur* trails (Figs. 1-3).

Table 2 Overview of the species combinations where trail-following was detected.
The statistical data were obtained through GLMs with quasibinomial error distribution (laboratory assays and system 3 assays) or poisson distribution (field assays). All n = 6 assays, except for: [2]n=10 and [3]n=5-7 assays. [1]tested in both systems 2 and 3. For further abbreviations see table 1.

	assay	tested body parts	F	df	p
Intraspecific trail-following					
P. ypsilon	laboratory	h t d* p	10.6	3	0.0002
	field	h t g*	5.4	2	0.02
Ca. saundersi	laboratory	h* t* d** p*	7.8	3	0.001
	field	h t g**	13.8	2	0.0004
D. cuspidatus	laboratory	h t g**	8.2	2	0.004
Ca. rufifemur	laboratory	h t d** p	10.0	3	0.0003
	field	h t g**	10.0	2	0.0017
Cr. modiglianii	laboratory	h t g l**	48.7	3	< 0.0001
	field[1]	l**	n/a	n/a	n/a
	lab colony	h t g l**	9.3	3	0.0004
Ca. (Col.) sp. 62	field	h t g*	3.2	2	0.073
Interspecific trail-following					
D. cuspidatus → P. ypsilon	laboratory	h t g**	12.1	2	0.0008
D. cuspidatus → Ca. saundersi	laboratory	h* t* g	8.8	2	0.003
Ca. rufifemur → Cr. modiglianii	laboratory[2]	h t g l*	17.1	3	< 0.0001
Cr. modiglianii → Ca. (Colobopsis) sp.62	field[3]	h* t* g**	5.5	2	0.019

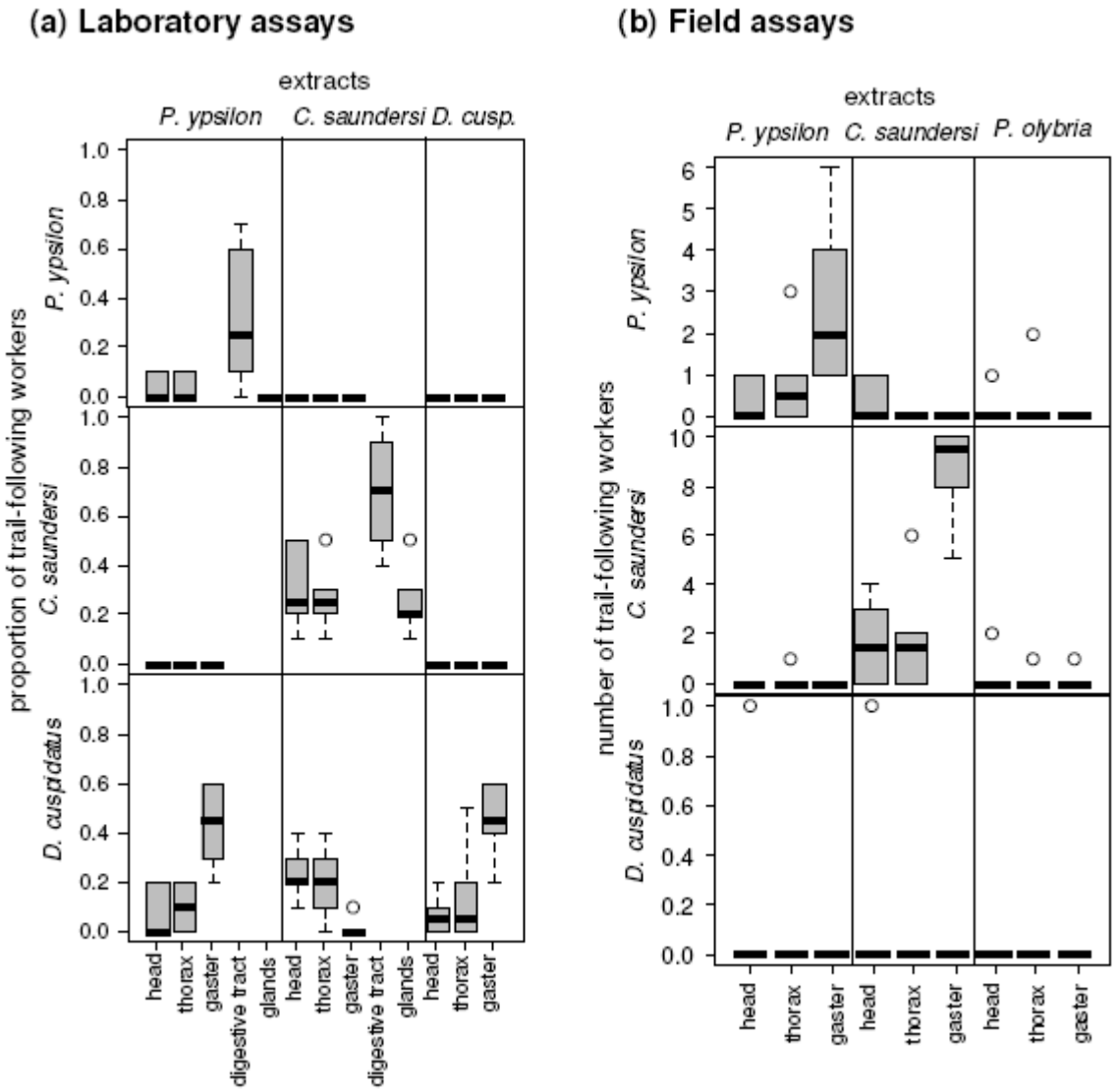

Fig. 1 Intra- and interspecific trail-following in system (1): *Polyrhachis ypsilon, Camponotus (Colobopsis) saundersi* and *Dolichoderus cuspidatus*. (a) proportion of trail-following workers in laboratory assays, (b) number of trail-following workers in the field assays. Each plot shows median, quartiles and range of six assay replicates. The laboratory assays were conducted with ten workers per replicate.

VII.4.3 Interspecific aggression

The aggression assays revealed strong differences in aggressive and submissive behaviour of *Polyrhachis ypsilon, Camponotus saundersi* and *Dolichoderus cuspidatus.* While *P. ypsilon* and *D. cuspidatus* exhibited high levels of aggression but rarely fled from the presented ant bodies, *Ca. saundersi* often fled but was seldom aggressive.

P. ypsilon attacked *Ca. saundersi* bodies significantly more often than vice versa (GLM: df = 1, $p < 0.0001$; Fig. 4). In contrast, *Ca. saundersi* significantly more often fled from *P. ypsilon* bodies than vice versa ($p < 0.0001$). A similar relation was found between *D. cuspidatus* and *Ca. saundersi* (Fig. 4). *D. cuspidatus* showed significantly more aggression towards *Ca. saundersi* than vice versa ($p = 0.003$), while the latter significantly more often fled from the former's bodies ($p < 0.0001$). *P. ypsilon* and *D. cuspidatus* exhibited similar aggression levels towards each other's bodies ($p = 0.67$), but *P. ypsilon* significantly more often fled from *D. cuspidatus* bodies than vice versa ($p = 0.014$).

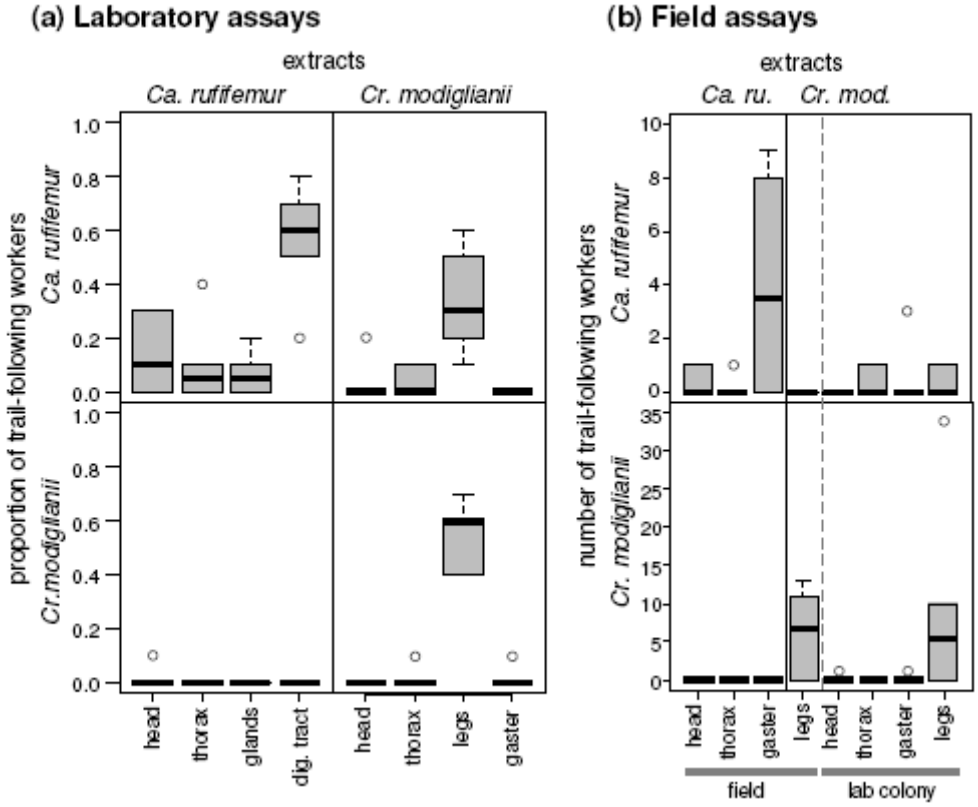

Fig. 2 Intra- and interspecific trail-following in system (2): *Camponotus rufifemur* and *Crematogaster modiglianii*. (a) proportion of trail-following workers in laboratory assays, (b) number of trail-following workers in the field assays and the *Cr. modiglianii* laboratory colony. The low response of *Ca. rufifemur* in the laboratory colony (b) is due to their low abundance in this nest. Each plot shows median, quartiles and range of six assay replicates. The laboratory assays were conducted with ten workers per replicate.

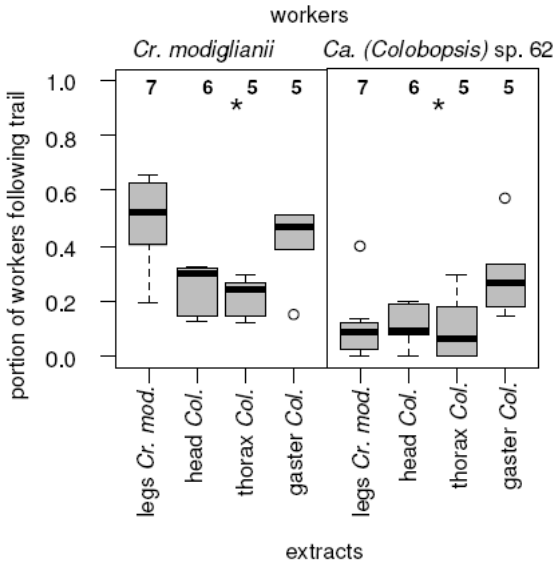

Fig. 3 Intra- and interspecific trail-following in field assays with system (3): *Crematogaster modiglianii* and *Camponotus (Colobopsis)* sp. 62 of SKY. The graphs show the proportion of workers that followed the extract trail related to the total number of workers on the paper sheet. The numbers above each graph indicate the number of replicates.

All three species strongly discriminated between ants from the same and a distant site. Both *P. ypsilon* and *D. cuspidatus* attacked foreign bodies of *P. ypsilon* and *Ca. saundersi* significantly more frequently than the respective ones from the same site (all $p < 0.0001$, Fig. 4). In contrast, *Ca. saundersi* fled from alien *P. ypsilon* and *Ca. saundersi* bodies significantly more often than from familiar ones (both $p < 0.0001$). This species was rarely aggressive; the highest aggression was observed against bodies of nestmates. However, this observation is most probably an artefact due to changes in cuticular chemistry after freezing the test ants. In all three species, antennating was almost exclusively observed towards bodies of nestmates, albeit *P. ypsilon* also antennated alien *P. ypsilon* bodies. Bodies of *Dolichoderus thoracicus* were often bitten by

P. ypsilon and *D. cuspidatus* but otherwise ignored.
On two other shared trails of *P. ypsilon* and *Ca. saundersi*, we observed 12-15 peaceful interactions but no aggression between the species (observation time: 15 min each). *Ca. saundersi* often avoided contact with *P. ypsilon* on the trail. However, *P. ypsilon* displaced *Ca. saundersi* from sugar or peanut butter baits, even when they were placed on the latter's nest (n = 5 baits at four different sites). In two cases, we observed 6-10 aggressive but only 1-3 peaceful interactions between the two species directly at a bait (observation time: 15 min each).
Ca. sp. 62 and *Cr. modiglianii* often antennated each other at artificial baits but never showed aggression. During seven assays, we observed 11±6 antennations of *Ca.* sp. 62 towards *Cr. modiglianii* but no biting or opened mandibles (mean ± SD, observation time: 3 or 5 min). *Cr. modiglianii* antennated *Ca.* sp. 62 3±2 times, and 0±1 times with opened mandibles, but never bit its opponent. In contrast, *Camponotus rufifemur* often bit *Ca.* sp. 62 but rarely tolerated it (9±7 bites, 1±1 antennation with opened mandibles, 1±1 short antennations with closed mandibles in three assays where *Ca. rufifemur* was present).

VII.5 Discussion

VII.5.1 Intraspecific trail-following

All studied species followed intraspecific extracts of those body parts that contain the trail pheromone glands in the respective genera, which validates the experimental setup we used (Keeling et al. 2004). These were the digestive tract (*P. ypsilon, Ca. saundersi, Ca. rufifemur*), the gaster (*D. cuspidatus, Ca. (Colobopsis)* sp. 62), or the legs (*Cr. modiglianii*). Thus, no motor display of the recruiting ant was necessary, and the extract alone was sufficient to evoke trail-following behaviour. In contrast to many congeneric species, *P. ypsilon* and *Ca. saundersi* seem to use mass-recruitment rather than group-recruitment (Hölldobler 1999, Traniello 1977). The lack of symmetric interspecific trail-following suggests that all studied species use different trail pheromones or pheromone mixtures.

VII.5.2 Interspecific trail-following as informational parasitism

Ca. rufifemur workers regularly followed artificial trails of its parabiotic partner *Cr. modiglianii* but not vice versa (Fig. 2, table 2). This trail-following represents a form of informational parasitism, where a species exploits another's information on food sources, i.e. its pheromone trails. Thus, *Ca. rufifemur* profits from the food-finding abilities of *Cr. modiglianii*, which is corroborated by the fact that *Cr. modiglianii* always arrived at baits before *Ca. rufifemur* (Menzel and Blüthgen submitted). Seidel (1994) also reports that, in a parabiosis of related species in West Malaysia, *Camponotus* did not find baits without *Crematogaster*. Similarly, *Dolichoderus cuspidatus* often followed artificial trails from *Polyrhachis ypsilon* gaster extracts. Interestingly, this species also regularly followed artificial trails of other *P. ypsilon* or *Ca. saundersi* extracts even if they did not contain trail pheromones (Fig. 1, table 2). *Cr. modiglianii* also followed artificial trails of *Ca.* sp. 62 gaster, head and thorax extracts (Fig. 3, table 2), suggesting that *D. cuspidatus* and *Cr. modiglianii* reacted to numerous chemical cues

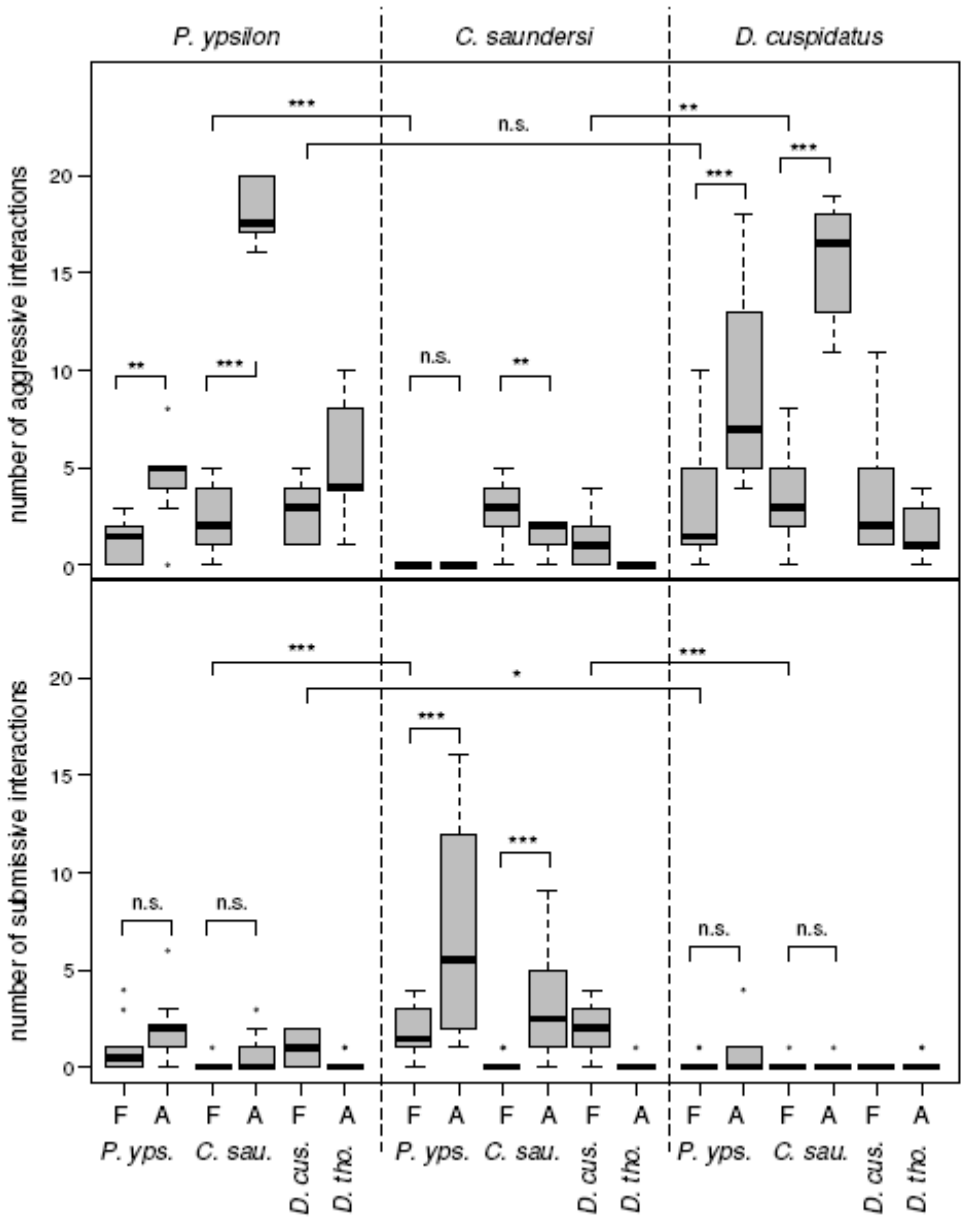

Fig. 4 Aggression and submissive behaviour of *P. ypsilon, Ca. saundersi,* and *D. cuspidatus* against different bodies of the same three species and *Dolichoderus thoracicus.* F: body of the same site as the tested workers (familiar); A: body from an alien site. Each plot shows median, quartiles and range of ten replicates. Asterisks indicate significance level according to pairwise GLMs (each df = 1) with binomial error distribution. n.s.: $p > 0.1$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Interspecific eavesdropping on pheromone trails or markings has been reported from several ant and stingless bee species (Adams 1990; Gobin et al. 1998; Nieh et al. 2004). Usually, it was asymmetric, and a submissive species followed trails of a dominant one but not vice versa. Gobin et al. (1998) describe a *Polyrhachis-Gnamptogenys* association where both species followed each other's trails albeit *Polyrhachis* seemed to be submissive to *Gnamptogenys*. However, the present study is the first to demonstrate that ants actually follow artificial pheromone trails of another species, and that interspecific trail-following can be independent of other tokens such as optical cues or superimposed con- and allospecific trail pheromones.

VII.5.3 Trail-sharing associations without interspecific trail-following?

Shared trails of *P. ypsilon* and *Ca. saundersi* are abundant in the studied rainforest habitat. Nevertheless, most workers of these species did not follow each other's extract trails. The frequent occurrence of shared trails thus cannot be explained by the hypothesis that all foragers on a trail follow heterospecific trail pheromones. A possible explanation, however, is based on differential behaviour of foragers and scout ants. In the trail-following assays in the field, some workers occasionally followed heterospecific extracts (including extracts that did not contain trail pheromones). Due to the length of the artificial trail, it is highly unlikely that they followed the entire length just by chance. While foragers are probably primed to follow their conspecific pheromone trails, scouts (or any small proportion of foraging ants, see Biesmeijer and de Vries 2001) are more explorative. They may occasionally follow a heterospecific pheromone trail (or possibly follow heterospecific foragers based on optical cues) and, if they lead to food sources, lay their own trail to recruit conspecific foragers. Thereby, scouts could initiate shared trails. Up to now, little is known about differential behaviour in scouts and foragers. Rather than being genetically determined, scouting seems to be a specialized behaviour that can be performed by every forager. In leaf-cutting ants, approx. 6% of the foragers show scout behaviour (N. Saverschek, O. Geißler, pers.comm). When studying ant behaviour, it is therefore important to consider not only the average but also the variance of behavioural reactions among colony members. Species like *D. cuspidatus* (system 1) and *Cr. modiglianii* (system 3), which often followed heterospecific extracts even when they did not contain trail pheromones, seem to be more explorative than others and thus more prone to following other scents. Interspecific trail-following thus may be more common than expected.

VII.5.4 Interspecific aggression, habituation and the assembly of ant mosaics

In some of the associations we studied, two ant species tolerated each others even at food sources. This was the case between *Cr. modiglianii* and *Ca. rufifemur* (Menzel et al. 2008) and between *Cr. modiglianii* and *Ca.* sp. 62. However, a clear dominance hierarchy was found in trail-sharing system (1). *Polyrhachis ypsilon* was both behaviourally and numerically dominant over *Camponotus saundersi*. It often attacked *Ca. saundersi* bodies in the aggression assays and quickly recruited to baits, where it displaced *Ca. saundersi* foragers. The latter, in contrast, often fled from *P. ypsilon* workers or its bodies. The third species of this system, *Dolichoderus cuspidatus,* was never numerically dominant but often attacked *P. ypsilon* or *Ca. saundersi* bodies.

All three species of this system discriminated between familiar and alien individuals of the respective other species. They showed significantly higher aggression (*P. ypsilon, D. cuspidatus*) or avoidance behaviour (*Ca. saundersi*) towards alien individuals. This strongly suggests that they had habituated to their partner's profiles. Habituation to nearby individuals and concomitant loss of aggressiveness has been termed 'dear-enemy' or 'dear-neighbour effect'. This phenomenon has been reported from a variety of taxa, including ants (Gordon 1989; Heinze et al. 1996; Jutsum et al. 1979; Knaden and Wehner 2003; Langen et al. 2000; Temeles 1994). The presumed underlying cause is that it is economic not to fight neighbours (or neighbouring colonies) which do not pose a threat to the own territory nor compete for food resources. In ant communities, dominant ant species tolerate only certain submissive species but strongly attack others (e.g. Hölldobler 1979; Hölldobler 1983), which leads to the formation of ant mosaics (Blüthgen and Stork 2007; Dejean and Corbara 2003). In an Australian ant mosaic, Blüthgen et al. (2004) indeed found that ant species that were tolerated

by dominants significantly differed in their nectar plant choices from those that rarely co-occurred with the dominants. It seems likely that the dominant species learn to selectively tolerate other species, depending on the latter's behaviour. Langen et al. (2000) showed that *Pheidole* workers habituated to alien workers of the same or different species when they had been exposed to them while prevented from fighting. In analogy, if a submissive ant species (such as *Ca. saundersi*) immediately retreats from a dominant (e.g. *P. ypsilon*) one upon encounter, the latter may habituate and eventually ignore them. However, if the other ant does not retreat, this may reinforce the dominant species to attack the other one. Accordingly, *P. ypsilon* often interacts amicably with *Ca. saundersi* on trails, but some of these encounters are mildly aggressive (e.g. opened mandibles, pers. obs. and Fig. 4), and there is overt aggression at food resources. Indeed, submissive species often avoid aggression by simply avoiding contact (Mercier et al. 1998, F.M. pers.obs.) or by appeasement behaviour (Gobin et al. 1998). Since some tropical ant workers can live at least up to several months (F.M. pers.obs., N. Saverschek, pers.comm.), habituation on a colony level seems possible. Habituation depending on the other's behaviour thus can result in a phenomenon similar to the dear-enemy effect and may be one of the main mechanisms structuring an ant mosaic.

VII.5.5 Conclusions

Our study revealed that two factors seem to have a major impact on the assembly of trail-sharing associations: interspecific trail-following (i.e. following a heterospecific pheromone trail) and interspecific aggression, which leads to a dominance hierarchy between ant species. The present study shows that certain species regularly follow heterospecific pheromone trails and thus exploit their information on food sources. However, other, frequently trail-sharing species only rarely follow each other's pheromone trails. In these cases, trail-sharing may have originated from scouts that followed a heterospecific pheromone trail to food sources and established their own one.

In certain trail-sharing associations, interspecific aggression is frequent and results in a dominance hierarchy. We suggest that differential tolerance by the dominant species is caused by differential habituation, depending on the other species' behaviour. This selective habituation to submissive species can result in the development of an ant mosaic.

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VIII. The ecological relationship between parabiotic ants

This chapter has been submitted as:

Menzel F and Blüthgen N: Parabiotic associations between tropical ants: equal partnership or parasitic exploitation?

VIII.1 Abstract

1. Parabiotic associations – two ant species sharing a nest – are known from only few places in the world. They occur in the Neotropical and Southeast Asian rainforests and are often associated with epiphytic plants ('ant-gardens'). It remains largely unknown whether parabioses are mutualistic, commensalistic or parasitic.
2. We studied potential costs and benefits among the two parabiotic ant species *Crematogaster modiglianii* and *Camponotus rufifemur* in the rainforest of Borneo. We experimentally investigated food competition (as one of the most probable costs), differentiation of foraging niches (which can reduce competition), and potential benefits, e.g. joint nest defence and mutual provision of nest space. Besides, we studied behavioural interactions between the two species, and their interactions with the associated hemiepiphyte *Poikilospermum cordifolium* (Cecropiaceae).
3. The two species never showed aggressive interactions and amicably shared food resources, which indicates low food competition. *Cr. modiglianii* had a wider temporal and spatial foraging range than *Ca. rufifemur*, suggesting different foraging niches. Moreover, *Cr. modiglianii* always found baits before *Ca. rufifemur* and recruited more efficiently, while *Ca. rufifemur* probably followed *Cr. modiglianii*'s pheromone trails. *Ca. rufifemur* was significantly more successful in defending the nest against alien ants. *Cr. modiglianii* hence may profit from its partner's defensive abilities.
4. *Cr. modiglianii* frequently nested without its partner, whereas we never found non-parabiotic *Ca. rufifemur* nests. As inferred from dissected nests, both species may profit from reciprocal provision of nest space, but this benefit seems to be of less importance.
5. *P. cordifolium* seedlings and saplings frequently grew in the entrances of parabiotic nests, obviously dispersed by the ants. In cafeteria experiments, both parabiotic ants carried its elaiosome-bearing seeds into the nest. However, *P. cordifolium* does not form ant-gardens and, thus, does not provide additional nest space, which strongly contrasts to Neotropical ant-garden parabioses.
6. The parabiotic association appears beneficial for both ant species, the main benefits being nest defence by *Ca. rufifemur* (for *Cr. modiglianii*) and interspecific trail-following (for *Ca. rufifemur*). However, *Ca. rufifemur* seems to be more dependent on its partner than vice versa.

VIII.2 Introduction

Colonies of social insects, especially ants, often represent whole ecosystems in themselves. Various species of invertebrates depend more or less strictly on ant colonies. Insects associated with ant colonies include parasites that prey on ant brood or workers, and commensals that feed on waste or simply seek shelter and protection in the ant nest (Geiselhardt et al. 2007; Pierce et al. 2002; Schultz and McGlynn 2000). Intriguingly, even whole ant colonies can live in nests of other ant species, ranging from loosely associated, facultative commensals or cleptoparasites to highly specialized social parasites (Kaufmann et al. 2003; Huang and Dornhaus 2008).

Very few cases worldwide, however, are known where two ant species live in an association that appears symmetric, without obvious parasitic or exploitative interactions. Forel (1898) first described such an association (between *Dolichoderus* and *Crematogaster*) in the Colombian rainforest and named it 'parabiosis'. By introducing this term, he indicated that this was a new kind of association – it was not clear whether parabioses were mutualistic, commensalistic or parasitic. However, since the two species shared a nest and tolerated each other, parabioses differed from 'compound nests' where two ant species nested closely together but did not share nests (see e.g. Czechowski 2004). Parabioses are largely confined to associations between species of *Crematogaster* and either *Camponotus, Dolichoderus, Odontomachus* or *Pachycondyla* in South American and Southeast Asian rainforests (Menzel et al. 2008b; Orivel et al. 1997). Often, epiphytic plants inhabit these nests ('ant-gardens'), which are important mutualists because their roots are crucial for the nest stability. The nests may include further associated species such as trophobionts and other insect guests (Corbara et al. 1999; Kaufmann and Maschwitz 2006). Since Forel's first report, the ecological character of neotropical ant-garden parabioses has been debated (e.g. Davidson 1988; Dejean et al. 2000; Swain 1980). It remains largely unresolved whether they represent a case of social parasitism, commensalism or mutualism, although a recent study tentatively suggested a mutualistic interaction (Vantaux et al. 2007). In Southeast Asia, parabioses have been poorly studied to date, so even less is known about reciprocal costs and benefits in these associations.

Mutualism is defined as an interaction between two species that conveys a net benefit to both partners. Thus, the benefits each species gains from its partner outweigh the costs of the interaction. The study of mutualistic relationships involves analysing the costs and benefits either party incurs through its partner. These are usually quantified in terms of reproduction, survival, or growth. Many mutualistic systems allow experimental manipulation *in situ*, e.g. exclusion of one partner, to estimate its impact on the other party. Using this approach, mutualistic benefits have been studied in various systems, e.g. between cleaner fish and their clients (Grutter 1999), plants and seed-dispersers (Levey et al. 2002), ants and trophobiotic aphids (Stadler and Dixon 2005), ants and myrmecophytes (Heil and McKey 2003), plants and pollinators (e.g. Kearns and Inoue 1993, Klein et al. 2003), and mycorrhizal fungi and their hosts (Johnson et al. 1997). In parabiotic associations, these costs and benefits are more difficult to estimate. Since both partners are eusocial and the colonies are long-lived, benefits in terms of growth, reproduction and survival are hard to quantify. In addition, removing or excluding one of the partners from a nest without severely affecting the other is practically impossible.

In the present study, we investigated parabioses of *Crematogaster modiglianii* and one of two *Camponotus (Myrmotarsus)* species in the lowland rainforest of Borneo. These parabiotic nests are often associated with the hemiepiphyte *Poikilospermum cordifolium*. Experimental

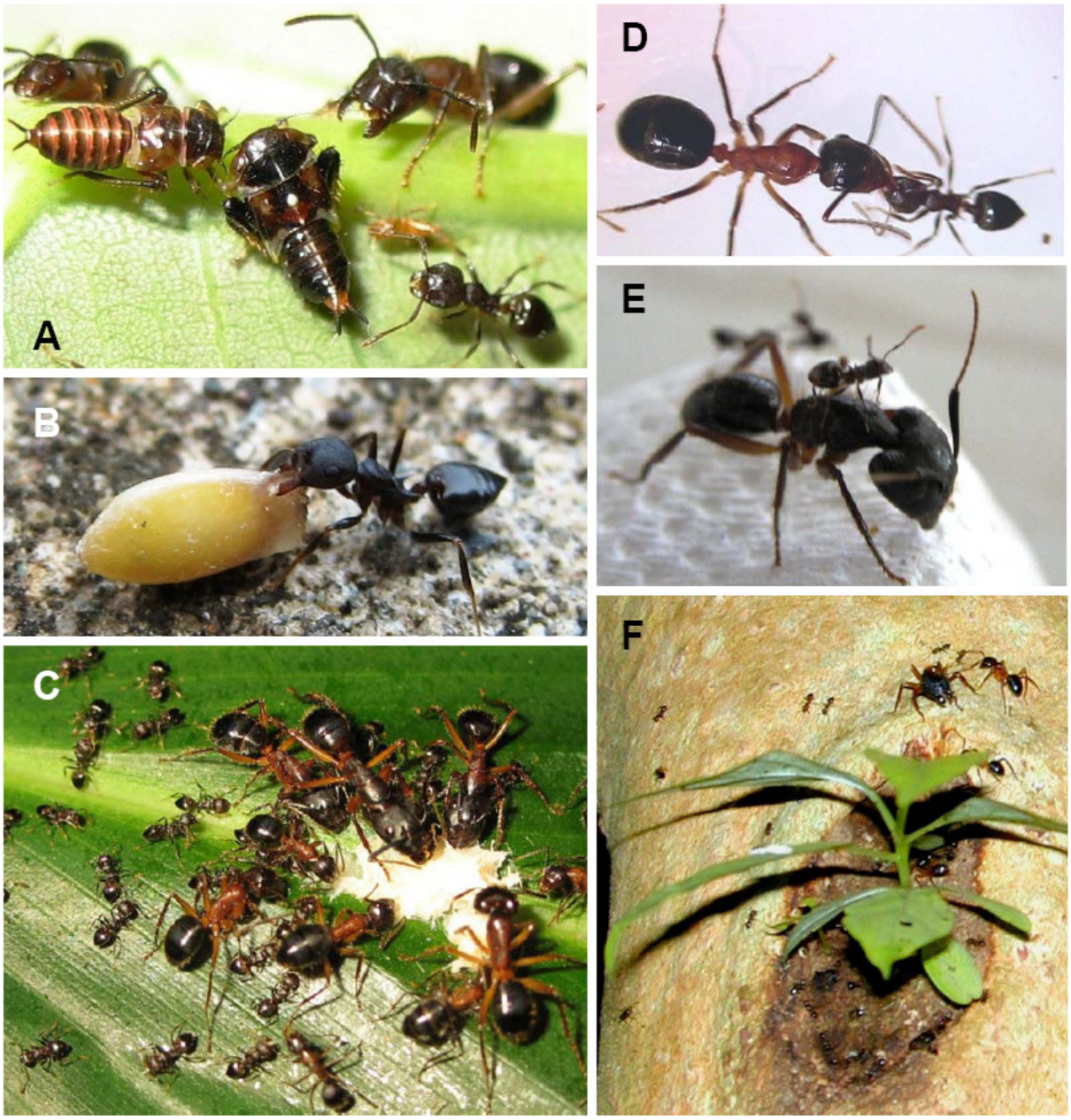

Fig. 1 (A) *Crematogaster modiglianii* and *Camponotus rufifemur* worker jointly tending trophpbionts. (B) *Cr. modiglianii* worker carrying a *Poikilospermum cordifolium* seed. Note the pronounced elaiosome. (C) The two parabiotic species amicably sharing a tuna bait. (D) *Cr. modiglianii* and *Ca. rufifemur* during trophallaxis. (E) Mounting behaviour of *Cr. modiglianii* towards a *Ca. rufifemur* soldier. (F) The two species at a common nest entrance on the nest tree; note the *P. cordifolium* sapling growing in the entrance hole. Photos (A)-(E) by F. Menzel, (F) by N. Blüthgen.

manipulation of the nests is additionally hampered since they are usually located in living trees in mature (and often protected) rainforests. We thus focused on potential costs and benefits that were experimentally accessible outside the nest and in laboratory experiments.

One of the most probable costs of being parabiotic is increased food competition, which is likely to result in aggressive resource monopolization. In turn, food competition may be reduced by foraging niche differentiation, e.g. spatial and temporal foraging range, different food preferences, or a dominance-discovery trade-off. Possible benefits that could be conveyed by the parabiotic lifestyle include joint nest defence, reciprocal provision of nest space (which may be mediated by associated epiphytes), food exchange via trophallaxis, and mutual brood care. Furthermore, parabiotic ants could directly benefit from the epiphytes

through provision of nest space or nutrition, while the plants themselves may benefit from seed dispersal and herbivore protection offered by parabiotic ants. We conducted several experimental series to evaluate these hypotheses. In addition, we dissected several parabiotic and non-parabiotic nests in order to obtain evidence about the ontogeny of parabiotic colonies.

VIII.3 Materials and methods

VIII.3.1 Study site and ants

Research was carried out at the Danum Valley Conservation Area between August and December in the years 2006 to 2008. The area (5° N, 117°50' E) is one of the major remaining patches of lowland dipterocarp rainforest in Sabah (Malaysian Borneo). It has a typical equatorial rainforest climate with a mean annual temperature of 26.9 °C and a yearly rainfall of 2700 mm.

We studied parabiotic associations between *Crematogaster (Paracrema) modiglianii* Emery 1900, which is monomorphic and measures approx. 2-3 mm, and two *Camponotus* species, both of which are polymorphic with body lengths between 5 and 13 mm. The *Camponotus* partner was chiefly *Ca. (Myrmotarsus) rufifemur* Emery 1900 (34/37 cases) or, in three cases, most probably *Ca. irritabilis* (Smith) 1857 (identification by Seiki Yamane). The 37 parabiotic nests were located in hollow, living tree trunks, lianas or (in three cases) in dead logs on the forest floor. Additional parabioses between the same species were studied in Mulu National Park (Sarawak) and the rainforest around Sepilok (Sandakan, Sabah). Voucher specimens of all ant species are deposited at the Forest Research Center in Sepilok, Sabah (Malaysia) and at the Department of Zoology, University of Würzburg.

VIII.3.2 Inventory of parabiotic nests

We recorded 37 parabiotic nests around Danum Valley Field Center (DVFC) and noted diameter at breast height, number of entrances, and the presence of epiphytes and carton nest material. Ten parabiotic nests were opened and thoroughly dissected, where we recorded nest architecture, presence of brood, and guest species. These nests were located in living trees (dbh 5-15 cm; n = 7), a liana, a dead tree, and a log. We surveyed 13 non-parabiotic *Cr. modiglianii* nests, eight of which were dissected (one in a living tree, seven in dead branches). Moreover, we surveyed trophobioses and extrafloral nectaries tended by one or both parabiotic species in the vicinity of two parabiotic nests.

VIII.3.3 Behavioural interactions

Behavioural interactions between the two species were studied in worker colonies. These were kept in the DVFC laboratory in fluon-coated plastic boxes on a tuna and honey diet for several days or weeks. We tested for interspecific trophallaxis by providing food dyed with Rose Bengale (Chroma, Münster/Germany) to a worker group of only one species (*Cr. modiglianii*: n = 3; *Ca. rufifemur*: n = 2). After two days, the other species was introduced. Two days later we dissected the gasters of the second species and checked whether the digestive tracts were stained with Rose Bengale. To estimate mutual brood care, worker groups of either species were put in a box together with brood of their own and the other species (n = 2 per species). The brood was uncovered, but we offered a shelter of small wooden pieces. After approx. one hour, we checked whether the workers had carried the brood under the shelter.

VIII.3.4 Nest defence

In order to estimate either parabiotic species' ability to defend the nest against intruders, we confronted nine *Ca. rufifemur – Cr. modiglianii* parabioses with living workers of three different ant species (*Myrmicaria* sp., *Crematogaster inflata* Smith 1857, and *Dolichoderus thoracicus* Smith 1860). All three are among the dominant ants in the rainforest around DVFC. While *Myrmicaria* sp. is strictly nocturnal, the other two species are active day and night, *Cr. inflata* being more active at night and *D. thoracicus* being more active during the day. In haphazard order, each of the three intruders was held with forceps at the nest entrance for three minutes or until it was killed. For each test, we recorded which species was present at the nest entrance, the number of bites of either parabiotic species towards the intruder, as well as which species killed the intruder. The tests were conducted at nine parabiotic nests with six replicates at night (after 6:30 pm) and during the day (before 6 pm), respectively.

VIII.3.5 Foraging behaviour

Food competition and potential foraging niche differentiation were estimated using bait experiments. We attached plastic platforms (ca. 8 × 8 cm) directly to the nest tree of seven parabiotic nests. Ten platforms per colony were provided with either honey or tuna baits and the workers present on the platform were counted 1-2 hours later. These surveys were conducted during the day (between 11:30am and 4:30pm) or at night (between 8pm and 11:30pm). We obtained data from 82 platforms (of a total of 170) where at least one parabiotic species had recruited. Using GLMs (with Gaussian error distribution), we calculated the effects of bait and time of day on the abundance of each species present at the platform. For *Cr. modiglianii*, the presence of *Ca. rufifemur* at the bait was included as an additional factor.

In addition, recruitment was studied in relation to nest distance. We spanned horizontal pairs of string (Ø 2 mm) from parabiotic nest trees (n = 7 nests) to surrounding trees in heights up to 4 m. Plastic platforms (ca. 6 × 6 cm, n = 95) were attached to these strings between 0 and 5.5 m from the nest tree. These platforms were provided with baits between 7:30pm and 10pm and checked for ants 30-70 min later. As baits we used either honey or tuna in equal frequencies. The experiment was carried out for 4-5 times per parabiosis. From these data we calculated the maximum foraging distance (per nest) for each species and recorded the number of baits tended by the two respective species in relation to nest distance.

Recruitment efficiency was studied by applying honey or tuna baits to leaves close to a parabiotic nest (1-3 m distance). We then recorded the number of workers at the baits every two minutes for 30-45 min. For two parabioses, a total of six diurnal and four nocturnal replicates were performed.

VIII.3.6 Interactions with Poikilospermum cordifolium epiphytes

The hemi-epiphyte *Poikilospermum cordifolium* (Barg-Petr.) Merr. (Cecropiaceae) often occurs together with parabiotic nests. Seedlings or larger individuals frequently grow in carton-covered or carton-free nest entrances. We estimated individual growth and turnover of these plants in several consecutive field seasons (March 2006 to October 2008). *P. cordifolium* epiphytes were surveyed up to a height of around 4 m at 15 parabioses. For all individuals with the largest leaf longer than 3 cm, we estimated percent herbivory damage to each leaf, and, for comparison, included five individuals that were not associated to parabiotic nests. In order to estimate the attractiveness of *P. cordifolium* compared to the congeneric, syntopic *P. suaveolens* to each parabiotic species, we conducted cafeteria experiments with

seeds and perianths of both *Poikilospermum* species. In *Poikilospermum*, the fleshy, bright blue perianths are persistent and cover the ripe seeds. On plastic platforms at the nest entrance, we offered both seeds (with their elaiosomes) and perianths of the two *Poikilospermum* species. Four pieces per item were provided, with four pieces of rice (grains cut into three pieces each) as controls (weight 5.9 ± 1.4 mg, mean ± SD, n = 40). For the following 30 minutes, we recorded the number of pieces carried into the nest by the two parabiotic ants as well as the number of workers at each item (as an alternative measure of attractiveness). The seeds used for the experiments weighed 3.1 ± 0.8 mg (*P. cordifolium*) and 5.3 ± 0.8 mg (*P. suaveolens*, both n = 36). Perianths weighed 7.3 ± 5.4 mg and 11.4 ± 4.7 mg, respectively (both n = 28). For three parabiotic nests, eight replicates each were carried out at night under red light. In order to lure out enough workers, tuna was offered prior to the experiment in a separate plastic cap (Ø 2.5 cm) that was completely removed at the onset of the experiment. The number of retrieved items (excluding controls) was analyzed using GLMs with binomial error distribution for each species. The impacts of the variables 'food item' and 'colony' within the GLM were estimated using χ^2 tests.
P. cordifolium leaves are often visited by *Cr. modiglianii* workers. The leaves produce small nectar droplets on their upper surface, which may be an important food source for ants. Using an amino acid analyzer (Biotronik LC3000), we therefore measured free amino acids in nectar droplets of three *P. cordifolium* individuals (see Appendix for further details)..

VIII.4 Results

VIII.4.1 Nest sites

Most (32/37) of the parabiotic nests of *Cr. modiglianii* and *Ca. rufifemur* occurred in hollow, living trees (Fig. 1f). Nest trees were 5-32.2 cm in dbh (median: 9.8 cm, n = 30) and belonged to ca. 24 species from 15 families. The commonest families were Euphorbiaceae (e.g. *Baccaurea* spp.) and Myrtaceae (exclusively *Syzygium* spp.), each represented by 7 out of 32 identified, living nest trees. Three further parabiotic nests were found in dead logs or branches on the forest floor and two were located in lianas (e.g. *Uncaria ferrea* DC, Rubiaceae). The nests had up to six entrances between 0 and 400 cm above the soil, which were partly covered with a carton-like material in 10/37 nests. Most of them were used by both species together.
We never found *Ca. rufifemur* nesting without its parabiotic partner. In contrast, 13 non-parabiotic *Cr. modiglianii* nests were found without *Ca. rufifemur*, but in some of these instances *Ca. rufifemur* could be lured to baits close to these nests. The non-parabiotic *Cr. modiglianii* nests were in small trees (dbh 4-6 cm; n = 6) or dead branches (n = 7). *Cr. modiglianii* worker groups were also found in carton shelters around small, living twigs or within dead branches.

VIII.4.2 Nest structure

Among the ten parabiotic nests that we opened, only two – located in living tree trunks – contained brood of both species. In one case, *Ca. rufifemur* workers and brood occupied the whole central cavity (which was compartmentalized with carton material), whereas *Cr. modiglianii* workers and brood only occurred in a small side compartment and in a bracket fungus close to a nest entrance. In the other nest, *Ca. rufifemur* and *Cr. modiglianii* brood (as well as *Cr. modiglianii* alates) was found in multiple separate, walnut-sized compartments within the trunk. *Cr. modiglianii* also settled in finely hollowed areas around openings (which were too narrow for *Ca. rufifemur*). Both species kept their brood separate but very close to

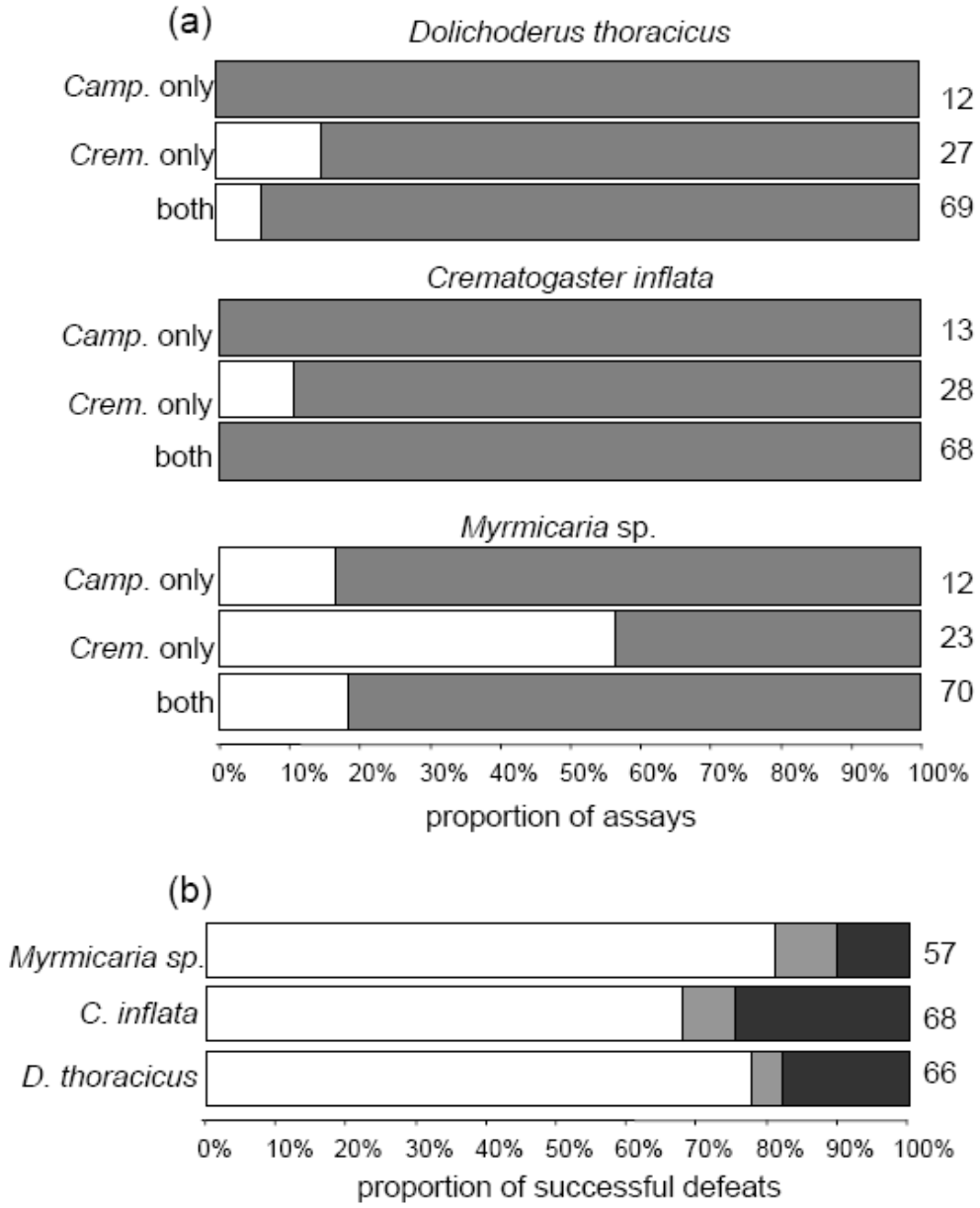

Fig. 2 (a) Proportion of successful defeats against different intruders in relation to the presence of either species at the nest. white: intruder not defeated; grey: intruder defeated.(b) Identity of attacking species when both are present, shown as proportion of successful attacks. white: *Camponotus* attacks, grey: both attack, black: *Crematogaster* attacks. Replicate numbers are given behind each bar.

each other, often separated by only a few centimeters. Likewise, the workers usually stayed among conspecifics albeit close to the partner species.

The remaining eight nests (also located in trees, lianas or logs) contained *Cr. modiglianii* workers, brood, and sometimes alate or dealate queens. *Ca. rufifemur* workers were always present inside the nest, but *Ca. rufifemur* brood was not. In three cases, fewer than 20 *Ca. rufifemur* workers but up to several thousand *Cr. modiglianii* workers were found in these nests. 'Shelters' with workers of *Cr. modiglianii* or both species, but no brood were frequently found in hollow logs, lianas, dead branches or on shrubs covered with carton. One very large non-parabiotic *Cr. modiglianii* nest in a small tree (*Diospyros* sp., Ebenaceae) consisted of several unconnected cavities in the trunk, which probably stemmed from activities of a wood-boring insect, and contained workers, brood, alates and dealate queens. The other dissected *Cr. modiglianii* nests were located in dead branches and contained

workers and sometimes brood. *Cr. modiglianii* workers, but not *Ca. rufifemur,* readily moved into hollow *Uncaria* stalks (inner diameter ca. 4 mm) offered close to existing nests and sometimes also stored brood in these stalks. Several newly eclosed imagines of the wood-boring beetle *Apriona flavescens* Kaup 1866 (Cerambycidae) were found inside parabiotic nests, suggesting that cavities made by their larvae may serve as starting points for *Cr. modiglianii* colony foundations. Further guest species of parabiotic nests included few (up to five) imagines of Tenebrionidae (cf. *Tetraphyllus* sp.) and Scarabaeidae, larvae of Scarabaeidae and Scirtidae, *Myrmecophilus* sp. (Myrmecophilidae, Grylloidea), Psychodidae larvae (Diptera), and less 100 ants of the genera *Leptogenys, Pristomyrmex* and an unidentified termite species.

VIII.4.3 Behavioural interactions between the species within the nest

In opened nests and laboratory colonies, the two species often antennated each other but never showed aggression (see also Menzel et al. 2008b). Trophallaxis between the two species, initiated via solicitation by *Ca. rufifemur,* was observed several times in laboratory colonies (Fig. 1d). Via stained food, food transfer from *Cr. modiglianii* to ten out of 17 *Ca. rufifemur* workers was detected in one out of three worker groups, but we could not detect food transfer in the opposite direction. *Cr. modiglianii* often climbed on *Ca. rufifemur* workers and walked around on their body and antennae (Fig. 1e). The latter sometimes tried to shake them off but did not show aggression. *Cr. modiglianii* also mounted *Ca. rufifemur* alates and dead *Ca. rufifemur* workers (Menzel et al. 2008a). Both trophallaxis and mounting behaviour mainly occurred after the two species had been kept separate for one or two days (Menzel et al. 2008a). Interspecific brood care was never observed. Each species only carried its own brood under the shelter and ignored brood or pupae of the respective partner.

VIII.4.4 Nest defence

In the nest defence assays, both *Ca. rufifemur* and *Cr. modiglianii* usually killed the intruder ants that were held at the nest entrances (88% of all assays, Fig. 2a-c). *Cr. modiglianii* repulsed intruders by spreadeagling them, i.e. several workers grabbed the intruder's legs or antennae with their mandibles and pulled backwards, leading to the intruder's death after some time. In successful cases, 7.5 ± 3.1 workers (mean and S.D.) had seized the intruder within three minutes. In contrast, most attacks by *Ca. rufifemur* only involved one or two *Ca. rufifemur* workers. They bit the intruder (3.9 ± 2.9 bites per successful repulse) and often killed it within less than 30 s.

The chances of a successful repulse were clearly higher when both species were present, compared to assays where only *Cr. modiglianii* was present (Fig. 2a-c). This effect was significant for the intruder species *Myrmicaria* sp. and *Crematogaster inflata* (Fisher's $p = 0.00096$ and 0.023, respectively), and marginally significant for *Dolichoderus thoracicus* (Fisher's $p = 0.095$). In contrast, *Ca. rufifemur* alone defended intruders as successfully as did both species together (Fisher's $p = 1$ for *Dolichoderus thoracicus* and *Myrmicaria* sp.; *Cr. inflata* was always killed when *Ca. rufifemur* was present, irrespective of presence or absence of *Cr. modiglianii*) (Fig. 2a-c).

In seven out of nine colonies, *Ca. rufifemur* majors were nearly always present at the nest entrances during both day and night. The probability of successful repulse of an intruder, as well as the presence of either species at the nest, did not differ between diurnal and nocturnal experiments (all three Fisher's $p = 1$). When both species were present (64% of all assays), it was usually *Ca. rufifemur* who attacked the intruder (75% of cases, Fig. 2d). Both *D. thoracicus* and *Cr. inflata* were observed to attack and kill single *Cr. modiglianii* workers, but

were always killed by *Ca. rufifemur*. *Myrmicaria* often sprayed venom and therefore was less frequently attacked by both parabiotic species.

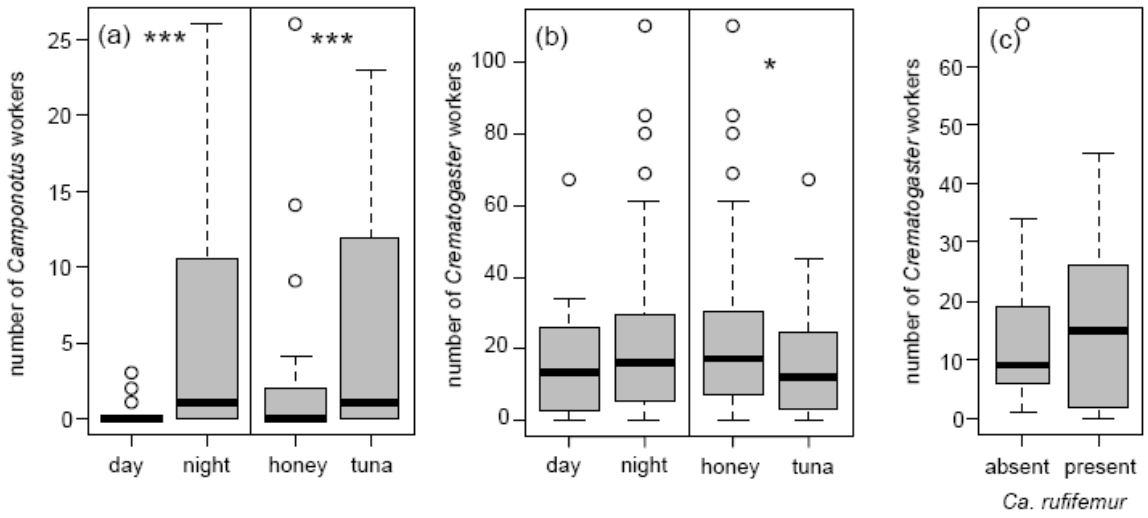

Fig. 3 Number of (a) *Ca. rufifemur* and (b) *Cr. modiglianii* workers recruiting to honey or tuna baits during day or night. (c) Number of *Crematogaster* workers at tuna baits depending on the presence or absence its parabiotic partner. Data for honey baits are similar. *** significant at $p < 0.001$.

VIII.4.5 Foraging and recruiting behaviour

Crematogaster modiglianii, often together with *Camponotus rufifemur*, regularly tended trophobionts. These included two Coccoidea species under carton shelters at parabiotic nests, various membracid and cicadellid nymphs (both Cicadelloidea) and a lycaenid caterpillar (Lepidoptera) (Fig. 1a). They also foraged together at carrion and extrafloral nectaries (e.g. of *Mallotus miquelianus* (Scheff.) Boerl., Euphorbiaceae, or *Diospyros toposioides* King and Gamble, Ebenaceae). Both ants were attracted to tuna and honey baits on platforms directly attached to the nest tree (Fig. 1c). In this experimental series, *Ca. rufifemur* workers were significantly more abundant at tuna baits than at honey baits (GLM: $F = 9.7$, $df = 1$, $p = 0.0025$, Fig. 3) but almost only recruited at night ($F = 16.2$, $df = 1$, $p = 0.0001$, Fig. 3). In contrast, *Cr. modiglianii* was slightly but significantly more abundant at honey baits ($F = 4.0$, $df = 1$, $p = 0.048$) and recruited during day and night ($F = 1.2$, $df = 1$, $p = 0.27$). The presence of *Ca. rufifemur* did not affect the number of *Cr. modiglianii* workers at the baits ($F = 0.75$, $df = 1$, $p = 0.39$, Fig. 3), thus, there was no evidence of resource monopolization. However, sometimes *Ca. rufifemur* workers non-aggressively replaced *Cr. modiglianii* foragers from the baits by their mere presence, although the latter always stayed close and later returned to the bait. Aggression between the two species was never observed at the baits. *Ca. rufifemur* largely ignored the much smaller *Cr. modiglianii*; however, sometimes single *Cr. modiglianii* workers were antennated very intensely.

As shown by the nest distance assays, *Cr. modiglianii* foraged on baits further distant from the nest than *Ca. rufifemur*. The maximal foraging distance of *Cr. modiglianii* (per parabiotic nest) was significantly higher than that of *Ca. rufifemur* (paired *t* test: $t = 7.02$, $df = 6$, $p = 0.0004$; Fig. 4a). Moreover, the proportion of baits attended by *Ca. rufifemur* or both species (as opposed to those attended by *Cr. modiglianii* only) decreased with nest distance (Fig. 4b). This relation was similar for both honey and tuna baits.

Cr. modiglianii was very effective in finding newly placed baits. The workers found them within 15 minutes in all of the ten recruitment surveys, with 22.8 ± 8.0 foragers at the baits after 15 min (mean ± S.E., Fig. 4c). In contrast, *Ca. rufifemur* only approached the baits in three cases at dusk or at night, and only reached them 3-40 min after *Cr. modiglianii*.

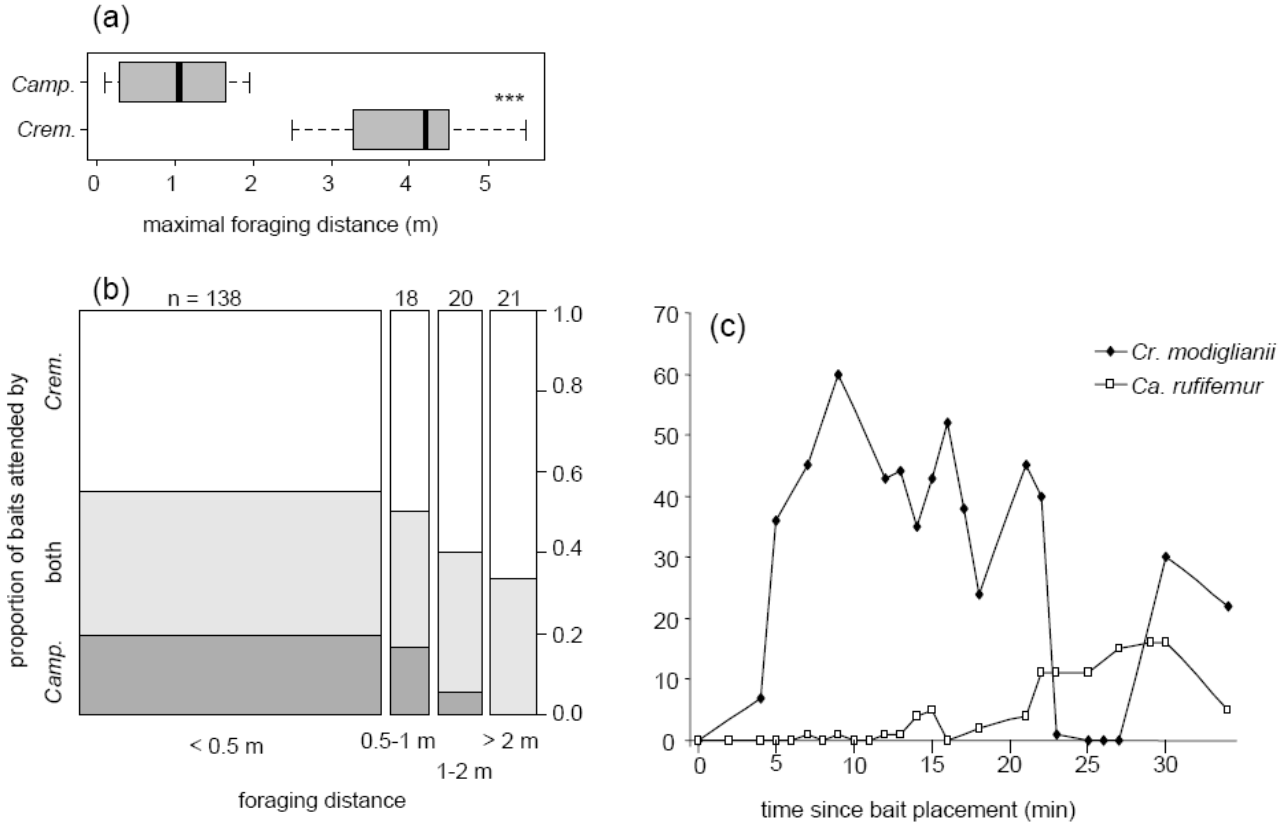

Fig. 4 (a) Maximal foraging distance per parabiotic colony for *Cr. modiglianii* and *Ca. rufifemur* (both n = 7 colonies) ***significant at $p < 0.001$. (b) Proportion of baits attended by *Cr. modiglianii*, *Ca. rufifemur*, or both, in relation to nest distance. (c) Example of recruitment progress of *Cr. modiglianii* and *Ca. rufifemur* to a tuna bait.

VIII.4.6 Epiphytes associated with parabiotic nests

The hemiepiphyte *Poikilospermum cordifolium* was found at 22 of the 37 parabiotic nests in Danum Valley (Fig. 1f). Individuals chiefly occurred as seedlings (two-cotyledon stage; 59.0%) or saplings (34.9%), with stalks up to 10 cm long and leaves up to 8 cm long, and grew in carton-covered (9/22 cases) or blank nest entrances (13/22 cases). Often, they grew in high densities, with small carton patches (ca. 5 × 3 cm) around a nest entrance being inhabited by one to six seedlings. At one nest, a carton patch of approx. 40 × 5 cm carried 68 *P. cordifolium* seedlings. Most of them, however, failed to establish over a longer period. Fifty-one of 77 individuals beyond the seedling stage had died or disappeared after one or two years. *P. cordifolium* seedlings or saplings also grew in four non-parabiotic *Cr. modiglianii* nests, including ones in dead logs or branches. Beside seedlings and saplings, several parabiotic nest trunks carried large *P. cordifolium* individuals with leaves of up to 60 cm length; at one nest, the whole tree was overgrown by a large *P. cordifolium* (>10 m high). *P. cordifolium* individuals on active parabiotic nests suffered slightly, but not significantly less herbivory than on presently unoccupied trees (Fig. 5c; Wilcoxon test, $W = 15$, $p = 0.36$, $N_1 = 9$, $N_2 = 5$).

P. cordifolium also grew at nest entrances of *Diacamma* sp. and *Crematogaster inflata*. The congeneric *P. suaveolens* sometimes grew at *Crematogaster difformis* nests but was never found at parabioses. In Mulu National Park, parabiotic nests were sometimes associated with *Poikilospermum oblongifolium*. Other epiphytes (e.g. Polypodiaceae, Piperaceae) were irregularly found growing on nest trees but never in the nest entrances.

In the cafeteria experiments, both *Ca. rufifemur* and *Cr. modiglianii* retrieved seeds and perianths of *Poikilospermum cordifolium* and *P. suaveolens* (Fig. 1b). We found no significant preference among these four items (GLM: $p > 0.8$ for both ant species, Fig. 5a, b). For *Ca. rufifemur,* the rate of retrieval differed strongly among the three parabiotic nests (GLM: $p < 0.0001$, Fig. 5a), which relates to unequal *Ca. rufifemur* abundance at these nests. *Cr. modiglianii* workers rarely carried the offered items to the nest, but all four items (excluding the rice controls) quickly attracted significant numbers of *Cr. modiglianii* foragers. On average, 6.1 ± 0.6 *Cr. modiglianii* workers (mean + SE, n = 96) were foraging at each item five minutes after start of the experiment. Their abundance significantly differed among the three colonies ($df = 2$, $F = 4.16$, $p = 0.019$) but not among the four food items ($df = 3$, $F = 1.45$, $p = 0.23$). After the experiments, *Poikilospermum* seeds were sometimes found in the carton material near the nest entrances.

Nectar droplets of *P. cordifolium* leaves regularly contained all essential amino acids except for methionine and tryptophane as well as up to twelve non-essential ones (see Appendix).

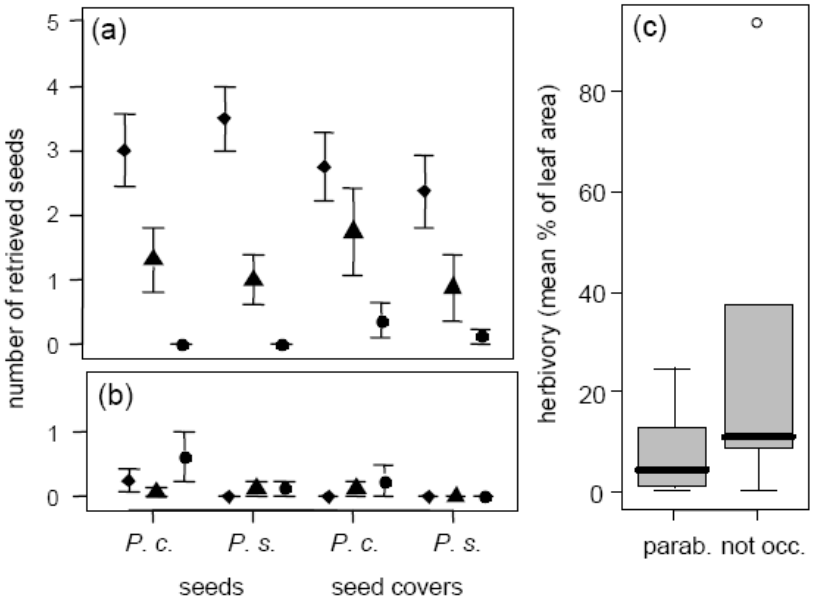

Fig. 5 Retrieval of seeds and seed covers of *Poikilospermum cordifolium* and *Poikilospermum suaveolens* by (a) *Ca. rufifemur* and (b) *Cr. modiglianii*. The three different symbols refer to three different parabiotic nests. (c) Mean % of leaf area (per tree) destroyed by herbivores of *P. cordifolium* epiphytes on parabiotic and non-parabiotic trees.

VIII.5 Discussion

The Southeast Asian parabiotic ant species *Crematogaster modiglianii* and *Camponotus rufifemur* share the same nest. They engage in mutual communication, including peaceful antennation and interspecific trophallaxis, and share food resources without aggression. The

tolerance between the two species is remarkably high and even extends to other parabiotic colonies (Menzel et al. 2008b). It is still unknown how parabiotic nests are founded. However, our observations that *Cr. modiglianii* nests often occur without *Ca. rufifemur* or with only few workers of this species, strongly suggest that *Cr. modiglianii* initiates the nest in hollow trunks, which are secondarily colonized by *Ca. rufifemur*. In contrast, neotropical parabioses are always initiated by *Camponotus femoratus* Fabr. 1804 (Davidson 1988), while its partner species *Crematogaster limata* agg. (including *Cr. levior,* Longino 2003) joins the ant-garden nest at a later stage.

Notably, both Bornean ant species seem to have certain adaptations that favor mutual tolerance. First, their nestmate recognition cues differ from related, non-parabiotic species. Both species possess cuticular hydrocarbons of unusually high chain lengths (Menzel et al. 2008a). This property might be a preadaptation that hampers interspecific recognition. Indeed, both species seem unable to discriminate their partner colony from alien colonies of the same species or variety (Menzel et al. 2008a; Menzel et al. 2008b). In addition, although *Cr. modiglianii* attacks *Ca. rufifemur* workers of an unfamiliar variety, it tolerates them after less than a day of habituation (Menzel et al. 2008a). Moreover, the cuticle of *Cr. modiglianii* is covered with highly unusual steroid-like compounds, which reduce aggression in *Ca. rufifemur* (Menzel et al. 2008a, unpublished data). Most probably, they additionally contribute to the high, but species-specific tolerance of *Ca. rufifemur* towards *Cr. modiglianii* (Menzel et al. 2008b). Unusual cuticular hydrocarbons and reduced interspecific nestmate recognition in both species, aggression-reducing cuticular substances in *Cr. modiglianii* and quick habituation of *Cr. modiglianii* to alien *Ca. rufifemur* thus may suggest a mutual interest in the association.

In contrast to various other mutualisms, studies on mutual costs and benefits in parabiotic associations are hampered by two main difficulties. First, experimental manipulation, e.g. removal or exclusion of one of the partners (e.g. Grutter 1999, Klein et al. 2003), is virtually impossible without severely affecting the other one. In addition, the reproductive success of an ant colony is difficult to quantify; any effects of experimental manipulations may be subtle and detectable only in long-term studies. In the following sections, we therefore discuss the experimental and observational evidence for possible mutual benefits in an attempt to evaluate their relative importance.

VIII.5.1 Provision of nesting space

Dry, suitable nest sites are limited in tropical rainforests (Wilson 1959). For the two parabiotic ants in this study, nest sites consist of hollow branches or trunks, while other parabiotic ants nest in free-hanging carton constructions (Davidson 1988; Weissflog 2001). Like other *Crematogaster* species (Longino 2003; Tschinkel 2002), *Cr. modiglianii* readily colonizes hollow structures like naturally hollow lianas (e.g. *Uncaria* sp.), but cavities made by wood-boring insects (e.g. *Apriona flavescens*). These pre-existing cavities may function as initial point for the foundation of a *Crematogaster modiglianii* colony. Since other *Crematogaster* species are capable of extensively hollowing out living or dead wood (e.g. *Cr. difformis,* N.B. pers. obs.), it is likely that *Cr. modiglianii* can expand the initial pre-formed cavities. This would explain why some *Cr. modiglianii* nests also consisted of large cavities in tree trunks even if few or no *Ca. rufifemur* workers were present. If *Ca. rufifemur* reaches established *Cr. modiglianii* colonies, it may profit from cavities built by *Cr. modiglianii,* but also provide additional nesting space when excavating wood itself. Thus, both ant species

may profit from the other's abilities to excavate wood, albeit probably at different stages of the parabiotic colony.
In neotropical parabioses, *Crematogaster limata* agg. and *Camponotus femoratus* live together in so-called ant-gardens. These are free-hanging carton nests around branches which are stabilized by epiphytes growing in the carton (e.g. Davidson 1988). Although both are attracted to epiphyte seeds, only *Ca. femoratus* is strong enough to carry them into the nest (Davidson 1988; Orivel and Dejean 1999). Thus, *Crematogaster* profits from its partner's ability to construct ant-gardens. A similar benefit has been suggested for the *Crematogaster* partner of an ant-garden-building *Camponotus (Myrmotarsus)* species from the Malay peninsula (Kaufmann 2002; Weissflog 2001). However, in nests of *Cr. modiglianii* and *Ca. rufifemur*, carton constructions at most occur at the nest entrances and thus, if at all, only represent a minor portion of the nest. Benefits to *Cr. modiglianii* from carton constructions or ant-gardens provided by *Ca. rufifemur* are thus unlikely.

VIII.5.2 Nest defence

Camponotus rufifemur majors are very aggressive and possess powerful mandibles. It seems likely that they effectively defend a nest against vertebrates and invertebrates. In the present study, their presence significantly raised the probability successfully repulsing ant intruders. Although *Ca. rufifemur* is mostly nocturnal, majors were usually also defending the nest entrances during the day. Hence, it seems likely that *Cr. modiglianii* profits from nest defence by its partner. However, *Cr. modiglianii* is an aggressive species as well, and successfully attacks humans and invertebrates. For example, we observed several raids of *Pheidole* and *Pheidologeton* against parabioses during late afternoon, where the nests were defended by *Crematogaster* but not by *Camponotus*. Moreover, the location of nests within trees (in contrast to free-hanging ant-gardens in the Neotropics) makes them less vulnerable to potential vertebrate predators. Thus, although *Cr. modiglianii* probably profits from *Ca. rufifemur*'s nest defence, it does not depend on it.

VIII.5.3 Joint exploitation of food sources

The joint exploitation of food sources seems to play a major role in the parabiotic association. *Camponotus rufifemur* and *Crematogaster modiglianii* share honey or tuna baits, but also carrion, trophobioses (see also Blüthgen et al. 2006) and extrafloral nectaries. Compared to other ants, this 'food-sharing' is highly unusual, given that most ant species aggressively monopolize high-quality food resources such as tuna, honey, or trophobioses (Blüthgen and Fiedler 2004; Blüthgen et al. 2006). In South American parabioses between *Crematogaster limata* agg. and *Camponotus femoratus*, food-sharing may not occur since the latter species aggressively monopolizes high-quality food sources against the former one (Swain 1980).
Cr. modiglianii is a very effective food scout and quickly recruits conspecific workers to newly discovered food. In contrast, *Ca. rufifemur* never reached baits before *Cr. modiglianii*. Experimental evidence confirmed that *Ca. rufifemur* follows trails of *Cr. modiglianii* but not vice versa (unpublished data). Similarly, Seidel (1994) reported that *Camponotus (Myrmotarsus) misturus* apparently followed trails of an unidentified, associated *Crematogaster* species in Western Malaysia. The exploitation of another species' pheromone trails is a clear example of 'olfactory eavesdropping' or 'informational parasitism' (Adams 1990; Nieh et al. 2004) and represents an important benefit *Ca. rufifemur* derives from its partner.
Cr. modiglianii, in turn, may benefit from *Ca. rufifemur*'s ability to aggressively monopolize food resources (e.g. trophobioses) against competitors. Other dominant ants in the same

habitat, e.g. *Dolichoderus thoracicus*, *Crematogaster inflata* and *Crematogaster difformis*, are individually stronger than *Cr. modiglianii*, but inferior to *Ca. rufifemur* (Menzel et al. 2008b, unpublished data). In the absence of the latter, these species may thus displace *Cr. modiglianii* from food sources. Interspecific trail-following also occurs among ants that live in different nests (Adams 1990; Baroni Urbani 1969). Therefore, food source-related interactions do not require that both species nest together and thus are not sufficient to explain the parabiotic phenomenon.

VIII.5.4 Interactions with Poikilospermum cordifolium

P. cordifolium grew in the nest entrances in 59% of the surveyed parabioses and also in several non-parabiotic *Cr. modiglianii* nests. As shown by the cafeteria experiments, both ant species are attracted to its seeds and perianths and carry them into the nest. Even the small *Cr. modiglianii* workers carried in *Poikilospermum* seeds, albeit in much smaller numbers than *Ca. rufifemur.* After the experiments, the seeds were sometimes found in the carton around nest entrances. Thus, *P. cordifolium* benefits from its seeds being dispersed and placed in a suitable site. However, the *P. cordifolium* saplings suffered a high mortality during our study; most of them had probably been removed by the ants. It is still unclear whether parabiotic ants, as a second benefit to *Poikilospermum*, deter herbivores as in other plant-ant associations (Heil and McKey 2003). *P. cordifolium* plants at parabioses did not suffer significantly less herbivory than those on trees without an ant nest (Fig. 6). In neotropical ant-gardens, epiphytes stabilize the ant nest with their roots (Yu 1994). In contrast, Bornean parabioses occur in hollow trunks and branches. *P. cordifolium* plants thus never provide additional nesting space. Thus, the ants probably benefit from nutrition through *P. cordifolium* elaiosomes, perianths and extrafloral nectar (Gammans et al. 2005), but benefits through provision of nest space seem doubtful.

The congeneric, syntopic *Poikilospermum suaveolens* was never found at parabiotic nests although its seeds were as attractive to ants as those of *P. cordifolium.* In contrast to the parabiotic ants, *P. suaveolens* seems to prefer open, sun-exposed sites such as gaps or the canopy layer (F.M. pers. obs.). We therefore suggest that the absence of *P. suaveolens* at parabiotic nests is not due to differential ant preferences but rather to different habitat requirements.

VIII.5.5 Conclusion: Southeast Asian parabioses – a mutualistic association?

Our studies suggest that both species derive a benefit from the parabiosis (Fig. 6). *Ca. rufifemur* exploits *Cr. modiglianii*'s trails to gain access to newly discovered food sources. Interspecific trail-following may also explain why *Ca. rufifemur* workers were sometimes found in non-parabiotic *Cr. modiglianii* nests or at nearby baits. As an additional benefit, *Ca. rufifemur* begs food from its partner by soliciting interspecific trophallaxis. Interspecific trophallaxis solicited by *Camponotus* was also observed by Seidel (1994) in a *Camponotus misturus – Crematogaster* sp. parabiosis in Western Malaysia. *Cr. modiglianii*, on the other hand, appears to profit from nest and resource defence by *Ca. rufifemur*. Hence, the mutual services in the parabiosis are essentially nutrition and protection. These two are among the most common services traded between mutualists, e.g. in ant-plant protection mutualisms or trophobiotic ant-aphid interactions (Bronstein and Barbosa 2002; Heil and McKey 2003; Stadler and Dixon 2005). However, the magnitudes of the respective benefits between the two ant partners are difficult to determine and may strongly vary in space and time. Whether there is a net benefit for both species or whether one species parasitizes the other may therefore strongly depend on local conditions, e.g. enemy pressure and availability of food and nest

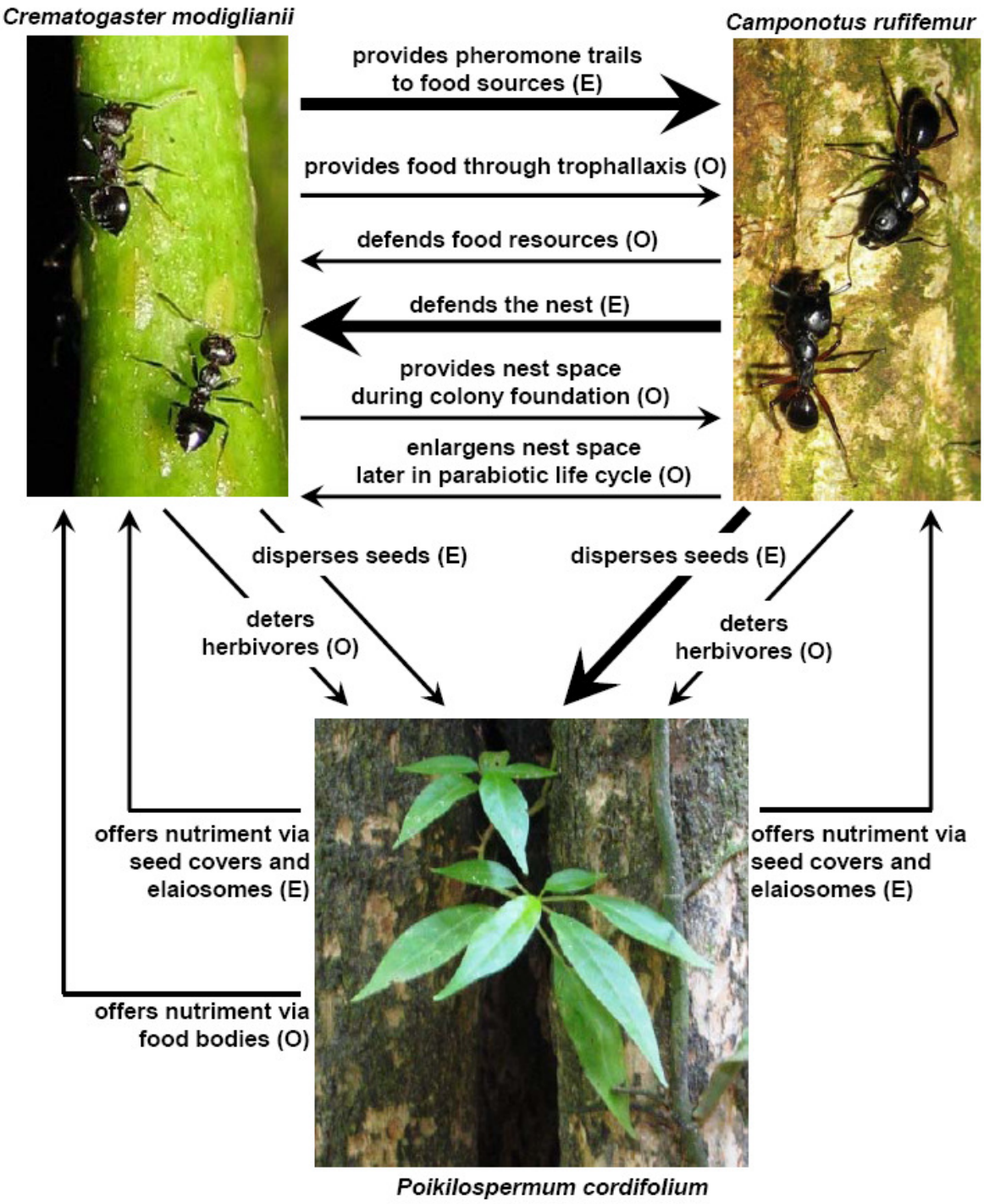

Fig. 6 Overview of the mutual benefits between *Crematogaster modiglianii, Camponotus rufifemur* and *Poikilospermum cordifolium*. Thick arrows highlight the alleged key benefits assumed by the authors (E) benefit experimentally shown; (O) benefit inferred from observations and therefore tentative.

sites (Bronstein 1994; Bronstein and Barbosa 2002; Johnson et al. 1997; Pontin 1978). It appears likely that *Ca. rufifemur* is more dependent on *Cr. modiglianii* than vice versa. Firstly, *Cr. modiglianii* can nest on its own in pre-formed or self-excavated cavities, while *Ca. rufifemur* was never found without its partner. Secondly, *Camponotus* never recruited to food sources on its own in our and Seidel's (1994) experiments and thus may depend on *Crematogaster*'s foraging abilities.

It has been suggested that neotropical parabioses of *Camponotus femoratus* and *Crematogaster limata* agg. are mutualistic as well, albeit for partly different reasons (Vantaux et al. 2007). Similar to Southeast Asian parabioses, *Ca. femoratus* seems to take advantage of its partner's resource-finding abilities by following its trails. The latter, however, is usually chased away from resources and thus suffers a cost from the informational parasitism of its partner (Davidson 1988; Swain 1980). In turn, the *Crematogaster limata* agg. profits from its partner's nest defence (Wheeler 1921), but primarily from its provision of suitable nest sites, i.e. ant-gardens (Vantaux et al. 2007). However, this benefit does not apply to the parabiotic association studied here since *Ca. rufifemur* does not build comparable ant-gardens.
Both species possess behavioural mechanisms to approach their partner. *Camponotus* follows *Crematogaster* trails, which may explain why we regularly found *Cr. modiglianii* nests with *Ca. rufifemur* workers, but without their brood. It seems probable that *Ca. rufifemur* finds and colonizes *Cr. modiglianii* nests, thereby forming parabiotic associations. *Cr. modiglianii* may tolerate *Ca. rufifemur* since it provides benefits as well. It remains to be explained why *Cr. modiglianii* approaches and mounts *Camponotus* workers and alate queens after a few days' separation (Menzel et al. 2008a). Like trail-following, this mounting behaviour may play an important role during the founding process of a parabiosis or its maintenance.

Acknowledgements

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VIII.6 Appendix

Analysis of amino acids in P. cordifolium nectar droplets

We measured free (water soluble) amino acids of nectar droplets from the upper leaf surface of three different *Poikilospermum cordifolium* individuals. Two of these plants were potted; one plant grew in the rainforest around Danum Valley Field Center. Using an ion exchange chromatograph (Biotronik, amino acid analyser LC 3000), the pooled nectar droplets of each plant (1.7, 1.9 and 14.7 mg) were extracted with 100 µl water for 30 min in an ultrasonic bath (EMAG, Emmi 20HC) and afterwards for 60 min in the refrigerator. After centrifugation (15000 g) and membrane filtration for 10 min, the supernatant was filled into a new microcentrifuge tube (Eppendorf), boiled for 2 min at 100 °C and cooled in ice to room temperature before a second centrifugation for 5 min. Afterwards 50 µl of the supernatant were extracted with 10 µl 12.5% 5-Sulfosalicylic acid in the refrigerator for 30 min for precipitation of proteins. 10 min of centrifugation followed and 50 µl of the supernatant plus 50 µl thinning buffer were filled into a fresh tube, mixed and pipetted in a membrane filter (Vecta Spin) before a last centrifugation for 5 min (10000 g) and measurement in the amino acid analyser.
The total abundance of free amino acids ranged from 2.7 – 64.1 µg / mg DW and 21.8 – 428.8 µMol / g DW. Methionine and tryptophane were not detected in all samples, but the remaining amino acids regarded as essential for most insects (Nation 2002) were regularly present (Table 1).

Table 1 Amino acid conentrations of *P. cordifolium* nectar droplets.
'Concentration range' refers to minimum and maximum concentrations in three plants. Essential amino acids (Nation 2002) are in bold face.

amino acid	concentration range	amino acid	concentration range
alanine	3.9 – 8.7%	**isoleucine**	**0.9 – 1.4%**
arginine	**4.4 – 13.2%**	**leucine**	**2.7 – 20.6%**
asparagic acid	0.6 – 1.7%	**lysine**	**0.1 – 2.6%**
asparagine	1.2 – 12.4%	**methionine**	**0 – 0.4%**
β alanine	0.1 – 16.1%	**phenylalanine**	**0.6 – 5.4%**
cysteine	0 – 0.1%	proline	0.8 – 3.5%
γ amino n-butyric acid	0.1 – 2%	serine	2.8 – 5.7%
glutamic acid	2 – 4.3%	**threonine**	**2.3 – 3.1%**
glutamine	4.6 – 25.5%	**tryptophane**	**0 – 3%**
glycine	2.8 – 5.3%	tyrosine	3 – 18.5%
histidine	**2.1 – 11.1%**	**valine**	**1.4 – 4%**
hydroxyproline	0 – 0%		

IX. Population genetics of parabiotic ants

IX.1 Materials and methods

We sampled parabiotic nests in Danum Valley (Sabah), Sepilok (near Sandakan, Sabah), Gunung Mulu National Park (Sarawak), and Kuala Belalong Field Studies Center (Temburong District, Brunei). An overview of the sites is given in Fig. II-1. The workers were collected directly from the nest entrances and immediately transferred into 100% EtOH.

From each nest, one worker was used for DNA extraction. DNA was extracted using Gentra Puregene Cell Kit (Qiagen, Hilden, Germany). For *Camponotus*, partial *cytochrome c oxidase subunit I* (COI) and *cytochrome b* sequences were amplified and sequenced using primers LCO/HCO (Folmer et al. 1994) and L3034/H3665 (Chiotis et al. 2000), respectively. For *Crematogaster modiglianii*, we obtained partial *cytochrome c oxidase subunit II* (COII) sequences. We performed a PCR with primers IPF/COII-Croz. and used the PCR product as template for a subsequent PCR with primers IPF/VARr (Crozier and Crozier 1993; Feldhaar et al. 2003; Kronauer et al. 2004). This nested PCR was necessary to obtain readable DNA sequences.

PCR reactions were performed in a final volume of 25 µl of purified water, which contained 2-3µl PCR buffer (Molzym or Genaxxon), 2µl $MgCl_2$ (25 mM; Molzym or Genaxxon), 2-3µl dNTP (25 mM; Genaxxon), 1-2µl of primers (10 µM), 1 U of *Taq* DNA polymerase (MolTaq/Molzym GmbH or Genaxxon), and 2 µl diluted genomic DNA. The respective concentrations were adjusted for each primer pair. The reactions were performed in an Eppendorf or Biometra thermocycler with the following parameters: 3 min at 94°C, followed by 30 cycles of 94°C, 45°, and 72°C, for 40-60 s each, and a final extension of 3 min at 72°C. PCR products were purified via ultra filtration through Montage™ filter devices (Millipore, Schwalbach, Germany) and sent to Seqlab (Göttingen, Germany) for sequencing.

The sequences were checked and edited using Chromas 2.31 (Technelysium Pty Ltd, www.technelysium.com.au), and assembled and aligned using ClustalW (Thompson et al. 1994) as implemented in BioEdit 7.0.9.0 (Hall 1999). Based on these alignments, haplotype networks were built using TCS 1.21 (Clement et al. 2000).

IX.2 Results

IX.2.1 Camponotus rufifemur and species 5 of SKY

Partial COI sequences were obtained from 28 individuals. The 503 bp fragment comprised 44 variable sites and 12 haplotypes. The haplotypes did not cluster according to geographic origin, and several sequences obtained from distant sites were identical (Fig. 1a).

However, the 12 haplotypes grouped into four genetic clusters that corresponded to chemically different varieties (Fig. 1b). Two of these could be assigned to the black and the red *Ca. rufifemur* variety (chapter IV). The third group contained samples identified as *Camponotus* sp. 5 of SKY (Seiki Yamane, pers. comm.). The fourth haplotype consisted of only one colony ('DTH') from Danum Valley, which was identified as *Ca. rufifemur* by Seiki Yamane but was chemically distinct from all other samples (data not shown). It differed from the nearest other haplotype by 27 base pair changes. Exclusion of this haplotype reduced the number of variable sites in the fragment to 28.

From 24 individuals, we obtained partial cytochrome b sequences. The 610 bp fragment comprised 21 variable sites and nine haplotypes. Similar to the partial COI sequence, the

haplotypes showed little genetic differentiation according to geographic origin (Fig. 2a), but a pronounced differentiation according to chemical varieties (Fig. 2b). The haplotypes grouped into three clusters representing red and black *Ca. rufifemur* and *Ca.* sp. 5. Unfortunately, the partial cytochrome b sequence could not be obtained for *Ca. rufifemur* 'DTH'.

IX.2.2 Crematogaster modiglianii

We obtained partial COII sequences from 21 individuals. The 566 bp fragment comprised 31 variable sites and 14 haplotypes. Thirteen of these only occurred in Danum Valley. The fourteenth haplotype corresponded to six samples from Gunung Mulu and Kuala Belalong Field Studies Center and was not found in Danum Valley (Fig. 3a). No further haplotype clusters were detected. The haplotypes did not group according to the associated *Camponotus* variety (Fig. 3b).

IX.3 Discussion

Our data suggest the existence of four sympatric varieties of the *Camponotus rufifemur/ Camponotus* sp. 5 complex. These varieties exhibited strong qualitative differences in their cuticular hydrocarbon profile (chapter IV, data not shown for *Ca.* sp. 5 and *Ca. rufifemur* 'DTH'), which was also supported by different mtDNA haplotypes. Within three of these varieties, genetic and chemical differentiation was comparatively low (chapter VI; Figs. 2, 3, chemical data not shown for *Ca.* sp. 5). Three of the four varieties occurred in both Danum Valley and Gunung Mulu. Their haplotypes showed little or no genetic differentiation between the two sites despite a distance of ca. 320 km. Moreover, we did not find any chemically or genetically intermediate forms, indicating that the varieties are reproductively isolated and, hence, may represent cryptic species.

Crematogaster modiglianii showed high genetic differentiation among the samples from Danum Valley, which had all been collected within approx. 2 km². In contrast, all samples from Gunung Mulu and the approx. 45 km distant Kuala Belalong Field Studies Center belonged to the same haplotype, which was not found at Danum Valley. Apart from this geographic differentiation, no haplotype clusters could be discerned, corresponding to the fact that we found only quantitative, but no qualitative differences in the cuticular hydrocarbons of *Cr. modiglianii* (chapter IV).

The genetic evidence hence indicates that one of the parabiotic species is differentiated into four varieties or possibly cryptic species, whereas its partner is genetically diverse, but not differentiated into distinguishable varieties. *Cr. modiglianii* colonies with identical haplotypes were associated with different *Ca. rufifemur* varieties. Thus, there is no cocladogenesis between *Cr. modiglianii* and the *Ca. rufifemur* varieties, although a less specific coevolution between the two partners seems likely. *Cr. modiglianii* workers can distinguish between different *Ca. rufifemur* varieties, but quickly habituate to workers of previously unfamiliar ones (chapter IV). Hence, the parabiotic association apparently does not require variety-specific adaptations in *Cr. modiglianii*.

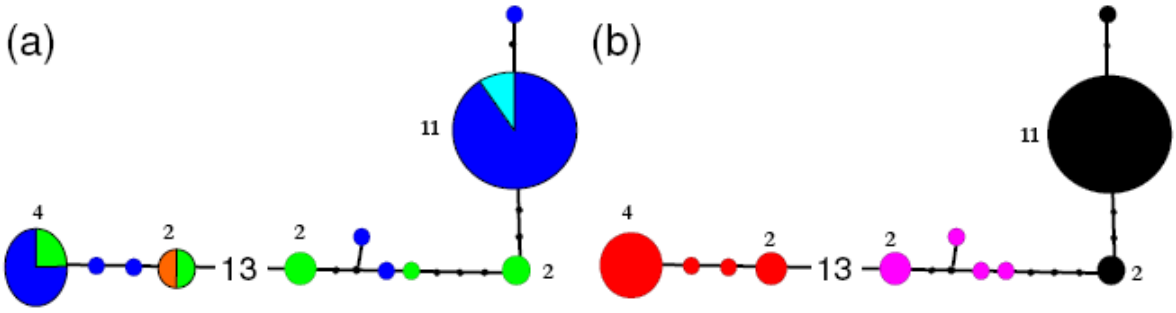

Fig. 1 Haplotype networks of the *Ca. rufifemur/Ca.* sp. 5 complex based on 503bp of COI (n = 27 sequences). Numbers next to the haplotype circles represent the number of sequences that share this haplotype; n = 1 sequence if no number is given. Small dots represent sequence steps; numbers within lines indicate the number of steps between two haplotypes.
(a) coloration according to geographical origin. Blue: Danum Valley, cyan: Kabili Sepilok Reserve, green: Gunung Mulu National Park, orange: Kuala Belalong Field Studies Center.
(b) coloration according to chemical variety. Red: red *Ca. rufifemur*; black: black *Ca. rufifemur*, magenta: *Ca.* sp. 5 of SKY. The fourth chemical variety (‚DTH') differs by 27 base changes from the nearest other sequence and was therefore not included in the depicted network.

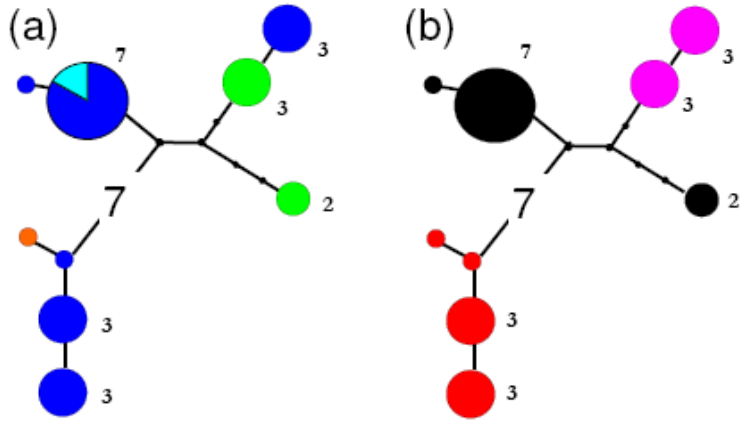

Fig. 2 Haplotype networks of the *Ca. rufifemur/Ca.* sp.5 complex based on 610bp of cytochrome b (n = 24 sequences).
(a) coloration according to geographical origin, (b) coloration according to chemical variety. See Fig. 1 for further information.

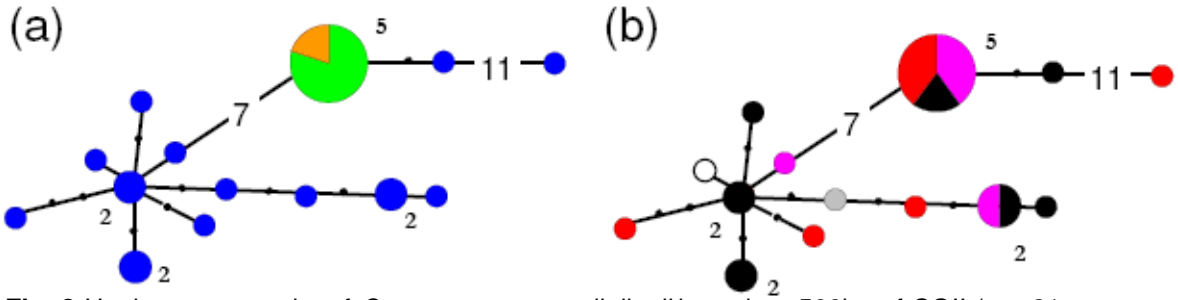

Fig. 3 Haplotype networks of *Crematogaster modiglianii* based on 566bp of COII (n = 21 sequences). Numbers next to the haplotype circles represent the number of sequences that share this haplotype; n = 1 sequence if no number is given. Small dots represent sequence steps; numbers within lines indicate the number of steps.
(a) coloration according to geographical origin. Blue: Danum Valley, green: Gunung Mulu National Park, orange: Kuala Belalong Field Studies Center.
(b) coloration according to the associated *Camponotus* variety. Red: red *Ca. rufifemur*; black: black *Ca. rufifemur*, magenta: *Ca.* sp. 5 of SKY, grey: *Ca. rufifemur* 'DTH', white: non-parabiotic.

X. General discussion

X.1 Parabiosis – a mutualistic association?

In order to estimate whether parabiosis is a mutualistic, commensalistic or parasitic association, we tested hypotheses on various benefits and costs that the parabiosis may convey to the two ant species. Our results indicate that both species may derive benefits from the parabiosis, including interspecific trail-following and joint nest defence (Fig. VIII-6). Moreover, food competition seems to be low since the foraging niches seem to differ, and we did not observe interference competition at baits. However, we do not know whether the presumably beneficial interactions between the two ants do translate into a higher reproductive success. Estimating any effects on reproductive success or long-term fitness would require long-term studies and experimental exclusion of one of the partners, which is hardly feasible in parabiotic nests within hollow trees. In addition, reproductive success is difficult to quantify in a long-lived eusocial insect colony.

Moreover, the magnitudes of the benefits between the two ant partners may vary in space and time and depend on local conditions, such as availability of food and nest sites or the presence of further interaction partners (Bronstein 1994; Bronstein and Barbosa 2002). For example, there are free-hanging ant-gardens on the Malay peninsula, which are parabiotically inhabited by *Camponotus* and *Crematogaster* (Kaufmann et al. 2006, Weißflog 2001). Based on morphological characters, these ants may be the same species as the Bornean ones (Seiki Yamane, pers. comm., and own identification). Parabiotic *Crematogaster* on the Malay peninsula seem to profit from ant-garden construction by *Camponotus*, whereas this benefit does not exist in Bornean parabioses. Thus, the benefits for *Crematogaster* may show a strong geographic variation, resulting in a dynamic mosaic of coevolution (Thompson 2005a). Altogether, our results hence allow the conclusion that the parabiotic association is probably beneficial to both partners, but the importance of these benefits remains unknown.

Both ant species possess certain properties that facilitate interspecific tolerance. The cuticular hydrocarbons of both species are unusually long compared to congeneric, non-parabiotic species, which may promote interspecific tolerance. Moreover, *Cr. modiglianii* produces large quantities of hereto unidentified surface compounds that reduce aggression in *Ca. rufifemur*. However, we do not know whether these properties are pre-adaptations, which facilitated the evolution of parabiotic associations, or actual adaptations to a parabiotic lifestyle, which are the results of selection pressures for both species to remain in the association. In this context, it is worth noting that *Ca. rufifemur* occurs in four sympatric, but chemically and genetically distinct varieties, whereas *Cr. modiglianii* is not differentiated into multiple varieties. *Cr. modiglianii* colonies with identical haplotypes can be associated with different *Ca. rufifemur* varieties. It remains to be resolved why there are four *Ca. rufifemur* varieties that apparently do not require specific adaptations in *Cr. modiglianii.* Further phylogenetic analyses, coupled with studies on cuticular hydrocarbons in non-associated *Camponotus* species, may elucidate this point.

X.2 Evolution of associations between *Camponotus* and *Crematogaster*

The evolution of the parabiotic way of life is largely unknown up to now. However, it seems likely that it evolved from other, less intimate associations, such as trail-sharing associations. We have shown earlier (chapter VII) that parabiotic *Camponotus rufifemur* follows artificial *Cr. modiglianii* trails. Interspecific trail-following and thus information on food sources seems to be the major advantage *Ca. rufifemur* gains from the association. By following *Cr. modiglianii* trails, *Ca. rufifemur* may not only reach food sources but also *Cr. modiglianii* nests. Provided that *Ca. rufifemur* queens also follow *Cr. modiglianii* trails, this may be the mechanism by which parabiotic nests are initiated. Since there is no cocladogenesis (chapter IX), which strongly argues against a joint foundation of parabiotic colonies, we suggest that parabiotic nests are formed when *Ca. rufifemur* joins existing *Cr. modiglianii* nests.

Interestingly, associations between various *Crematogaster* and *Camponotus* species can be found throughout the world. Beside the Southeast Asian parabioses, there are parabiotic associations between *Crematogaster limata* agg. and *Camponotus femoratus* in neotropical rainforests (e.g. Forel 1898, Wheeler 1921, Davidson 1988). Tolerance between *Camponotus* and *Crematogaster* at artificial food resources has been reported for *Crematogaster inflata* and an unidentified *Camponotus* species (Ito et al. 2004) on the Malay Peninsula, and *Crematogaster* cf. *polita* and *Camponotus vitreus* in Papua New Guinea (Milan Janda, pers. comm.). Trail-sharing associations, although rare among ants in general, frequently occur between species of *Crematogaster* and *Camponotus*. They include associations between *Crematogaster scutellaris* and either *Camponotus lateralis* or *Camponotus truncatus* (Mediterranean, Baroni Urbani 1969), *Crematogaster* cf. *polita* and *Camponotus vitreus* (Papua New Guinea, Milan Janda, pers. comm.), *Crematogaster coriaria* and *Ca. (Colobopsis)* sp. 1 of SKY (Borneo, FM pers. obs.), and *Crematogaster inflata* and a hereto undescribed *Camponotus* species (Malay Peninsula, Ito et al. 2004).

In all mentioned cases (except for South American parabioses), *Crematogaster* is numerically strongly dominant over *Camponotus* and either tolerates *Camponotus* or displaces it from baits (Ito et al. 2004; M. Janda, pers.comm; FM pers. obs.). The respective *Camponotus* species may profit from *Crematogaster* through either interspecific trail-following (*Cr. scutellaris* and *Ca. lateralis,* FM pers. obs.) or Batesian mimicry (Ito et al. 2004), such that these associations may be commensalistic. The only case where *Crematogaster* is aggressively displaced by *Camponotus* are South American parabioses (Swain 1980), which, however, seem to be mutualistic (Vantaux et al. 2007).

The wide array of associations between *Camponotus* and *Crematogaster* raises the question which properties enable these two genera in particular to associate with each other that often. It seems likely that these properties are related to those that facilitate interspecific trail-sharing, i.e. interspecific trail-following and interspecific tolerance. Thus, it seems plausible that preadaptations to facilitate interspecific associations in *Crematogaster* and *Camponotus* may include an ability to perceive and follow heterospecific trails as well as a nestmate recognition system that allows habituation to heterospecific cuticular profiles.

XI. References

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XII. Summary

Aggression between ants from different colonies or species is ubiquitous. Exceptions to this rule exist in the form of supercolonies (within a species) and interspecific associations (between species). One of the most intimate, yet little understood associations is the parabiosis. Parabiotically associated ants live together in a common nest. They keep their brood separate but frequently interact, e.g. perform interspecific trophallaxis, use common trails and often share food resources. Parabioses are restricted to associations between species of *Crematogaster* and either *Camponotus, Dolichoderus, Odontomachus,* or *Pachycondyla* and occur in South American and Southeast Asian rainforests. However, while the South American parabioses have been studied, albeit poorly, almost nothing is known about their Southeast Asian counterparts.
My PhD project focuses on Southeast Asian parabioses between the myrmicine ant *Crematogaster modiglianii* Emery 1900 and the considerably larger formicine *Camponotus rufifemur* Emery 1900. The two species frequently nest together in hollow trees in the tropical lowland rainforest of Borneo. The basic question of my PhD project is why these two species live together. I investigated both proximate and ultimate aspects of this question. For comparative purposes, I included studies on a trail-sharing association in the same habitat.

XII.1 Nestmate recognition in parabiotic ants

On the proximate level, I investigated which mechanisms facilitate tolerance towards heterospecific nestmates. Ants generally discriminate nestmates from non-nestmates via cuticular hydrocarbons that function as colony recognition cues. An individual ant perceives its opponent's hydrocarbons through olfaction and compares them to a neuronal template. The opponent is usually accepted as nestmate if its hydrocarbons match the template, but attacked if they do not.
I therefore studied nestmate recognition within and between the two parabiotic species. In particular, I was interested whether interspecific tolerance was colony-specific, species-specific, or genus-specific. I also analyzed the cuticular substances in both ant species in order to find potential differences to related, non-parabiotic species, and to estimate the substance overlap among the two species. A high substance overlap would suggest that interspecific tolerance is caused by chemical mimicry. Finally, bioassays were conducted to evaluate the function of different cuticular compounds.
Inter- and intraspecific nestmate recognition was studied using behavioural assays, where ants were confronted with an intruder ant (or a dummy) at their nest or in a laboratory colony. These assays were designed to minimize the disturbance effects. In each assay, the ants' behaviour towards the intruder was recorded for three minutes with a temporal resolution of 10 s. Cuticular compounds were analysed using gas chromatography coupled with mass spectrometry (GC-MS). The substances were identified based on their mass spectra, diagnostic ions and retention indices. In part, substances were derivatized for identification.
Interspecific tolerance in the two parabiotic species (chapter III) was species-specific but not colony-specific. *Ca. rufifemur* tolerated all *Cr. modiglianii* individuals, even those from foreign colonies, but strongly attacked workers of other *Crematogaster* species. *Cr. modiglianii*, in turn, tolerated *Ca. rufifemur* workers of certain foreign colonies but attacked those of others. Chemical analyses revealed that *Ca. rufifemur* occurred in two sympatric, chemically distinct varieties with almost no hydrocarbon overlap (which may represent

cryptic species). They were termed ‘red’ and ‘black’ variety, according to differences in coloration. I found that *Cr. modiglianii* only tolerated *Ca. rufifemur* workers from other colonies if they belonged to the same chemical variety as their own *Ca. rufifemur* partner, but fiercely attacked *Ca. rufifemur* workers of the other one. It also attacked three other, non-parabiotic *Camponotus* species. Thus, reciprocal interspecific tolerance was restricted to the species *Cr. modiglianii* and *Ca. rufifemur*. Probably, *Cr. modiglianii* habituated to the profile of their *Ca. rufifemur* partner and, due to low inter-colony differentiation, also tolerated foreign *Ca. rufifemur* workers of the same variety.

Ca. rufifemur frequently tolerated conspecific non-nestmates of the same chemical variety. Minor workers were more often tolerated than majors, possibly because they possess two to three times lower hydrocarbon quantities per body surface than majors. In contrast, *Cr. modiglianii* nearly always attacked conspecific non-nestmates (chapter VI).

The GC-MS analyses (chapter IV) revealed that the hydrocarbons of both species were of considerably higher chain lengths than in congeneric, non-parabiotic ant species. While cuticular hydrocarbons in other, non-parabiotic ant species range from C21 to C37, those of *Cr. modiglianii* ranged from C36 to C41 and those of *Ca. rufifemur* from C37 to C49. Long-chain hydrocarbons are less volatile than shorter ones. It has been hypothesized that they may promote interspecific tolerance by ‘absorbing’ shorter hydrocarbons and thus blurring the cuticular profile, while they are difficult to perceive themselves. The high chain lengths of cuticular hydrocarbons in both species may thus be an important factor that facilitates interspecific tolerance. Moreover, up to 98% of the cuticular hydrocarbons in *Ca. rufifemur* were methylbranched alkenes. This substance class is highly unusual among insect cuticular hydrocarbons, which generally consist of n-alkanes, n-alkenes, and methylbranched alkanes.

Cr. modiglianii and *Ca. rufifemur* had almost no hydrocarbons in common, refuting chemical mimicry as a possible cause of interspecific tolerance. The only major hydrocarbons both species had in common were the methylbranched alkenes 27-MeC39-14-ene and 27-MeC39-16-ene, which constituted 89% of the ‘red’ *Ca. rufifemur* hydrocarbon profile and also occurred in those *Cr. modiglianii* colonies that lived together with the ‘red’ *Ca. rufifemur* variety. Thus, *Cr. modiglianii* presumably acquired these two compounds from its red *Ca. rufifemur* partner (chapter IV). *Cr. modiglianii* was slightly, but significantly less aggressive towards foreign *Cr. modiglianii* workers that were associated with the same *Ca. rufifemur* variety than to those associated with the respective other one. Hence, this species seemed to use recognition cues acquired from its parabiotic partner (chapter VI). The remaining hydrocarbon overlap between *Cr. modiglianii* and *Ca. rufifemur* was restricted to one (red variety) and three (black variety) trace compounds.

Apart from hydrocarbons, both species possessed a set of hitherto unknown, but chemically interrelated substances on their cuticle. The main unknown compound ($C_{21}H_{32}O$) possessed three ring structures and three double bonds, its oxygen atom probably being linked to two alkyl groups (chapter V). However, its precise molecular structure has not yet been identified and will be elucidated using NMR. The quantitative composition of the unknown compounds varied between parabiotic nests but was similar among the two species of a nest. Since they also occurred in high quantities in the *Cr. modiglianii* Dufour gland, it is most likely that they were produced by *Cr. modiglianii* and transferred to their *Ca. rufifemur* partner. Possible transfer mechanisms include interspecific trophallaxis and ‘mounting behaviour’, where *Cr. modiglianii* climbed onto *Ca. rufifemur* workers without being displaced (chapter IV).

Although the composition of the unknown compounds greatly varied between nests, they did not function as nestmate recognition cues. In bioassays with dummies, both ant species

discriminated heterospecific nestmate from non-nestmate hydrocarbons, but did not differentiate between the unknown compounds of heterospecific nestmates and non-nestmates. This relation also held for intraspecific assays in *Cr. modiglianii*. Thus, both species used hydrocarbons for nestmate recognition. However, the unknown compounds significantly reduced aggression in *Ca. rufifemur* (chapter V). Workers of this species showed little to no aggression towards nestmate and non-nestmate *Cr. modigianii* workers or cuticular extracts. By contrast, they frequently attacked chemically separated non-nestmate *Cr. modiglianii* hydrocarbons (presented on dummies). This aggression could be greatly reduced by adding the unknown compounds to the non-nestmate hydrocarbons.

XII.2 Ecological interactions of associated ants

The ultimate, i.e. ecological and evolutionary aspects of my PhD research deal with potential costs and benefits that *Cr. modiglianii* and *Ca. rufifemur* may derive from the parabiotic association, their interactions with other species, and population genetic analyses. Additional studies on a trail-sharing association between three other ant species deal with two possible mechanisms that may cause or facilitate trail-sharing.

XII.2.1 Costs and benefits of living in a parabiotic association

Whether parabioses are parasitic, commensalistic, or mutualistic, is largely unknown and depends on the costs and benefits each party derives from the association. I therefore investigated food competition (as one of the most probable costs), differentiation of foraging niches (which can reduce competition), and several potential benefits of the parabiotic way of life. Besides, I studied interactions between the ant species and the hemiepiphyte *Poikilospermum cordifolium*.

Various bait experiments showed that the foraging niches of the two species differed regarding foraging range, daily activity pattern, and food preferences (chapter VIII). *Cr. modiglianii* was active during day and night and had a significantly wider foraging range than the mainly nocturnal *Ca. rufifemur*. This species always arrived at baits before *Ca. rufifemur* and thus seemed to be more effective in finding new food sources. Although both species often foraged together, their food preferences differed. *Ca. rufifemur* was significantly more abundant at tuna (which contain proteins and fats) than at honey baits (which contain chiefly carbohydrates). In contrast, *Cr. modiglianii* more abundantly foraged at honey baits, extrafloral nectaries, and tended trophobioses. None of the two species aggressively displaced its partner species from baits. Thus, interference competition for food seemed to be low or absent.

For both ant species, a number of benefits from the parabiotic lifestyle seem possible. They include interspecific trail-following, joint nest defence, provision of nest space by the partner species, food exchange via trophallaxis, and mutual brood care (chapters VII, VIII).

If an ant species follows another species' pheromone trails, it can reach food resources found by the other species. As shown by artificial extract trails, *Ca. rufifemur* workers indeed followed trails of *Cr. modiglianii* but not vice versa. Thus, *Ca. rufifemur* benefited from *Cr. modiglianii*'s knowledge on food sources (i.e. informational parasitism), and by doing so profited from its partner's foraging activities.

In turn, *Cr. modiglianii* seemed to profit from nest defence by *Ca. rufifemur*. *Ca. rufifemur* majors are substantially larger than *Cr. modiglianii* workers. In experimental tests, they were significantly more effective in killing 'intruder ants' that were presented at the nest entrances.

Although *Cr. modiglianii* often effectively defended the nest as well, it seemed likely that this species derived a benefit from its partner's defensive abilities.
For neotropical parabioses, it has been suggested that *Camponotus* benefits *Crematogaster* by providing nest space. Neotropical parabioses are so-called ant-gardens, i.e. they consist of free-hanging carton nests that are stabilized by the roots of epiphytic plants. The neotropical *Camponotus* species initiates these ant-gardens by actively planting epiphyte seeds into the carton material, whereas *Crematogaster* is too small to carry epiphyte seeds. Similarly, the Bornean parabioses often were inhabited by the hemiepiphyte *Poikilospermum cordifolium* (Barg.-Petr.) Merr (Cecropiaceae). *P. cordifolium* seedlings, saplings and sometimes larger individuals abundantly grew at the entrances of parabiotic nests. However, in contrast to neotropical parabioses, *P. cordifolium* did not provide additional nest space since the Bornean parabioses were not free-hanging but located within hollow trees. Thus, the ants did not benefit from nest stabilization by *P. cordifolium*, and consequently *Ca. rufifemur* did not benefit *Cr. modiglianii* by planting the seeds. *P. cordifolium* may provide another, albeit minor benefit to the ants through its nutritive elaiosomes and perianths (which attracted both ant species) and extrafloral nectar (which attracted *Cr. modiglianii*).
Interspecific trophallaxis between the two species was observed, but due to its rarity, its importance in the parabiotic association was difficult to judge. Both parabiotic species only cared for their own brood; thus, there was no benefit from mutual brood care.
In conclusion, the parabiosis is probably beneficial to both species. The main benefits seem to be nest defence (for *Cr. modiglianii*) and interspecific trail-following (for *Ca. rufifemur*), but it remains unknown how important these benefits actually are, and how they translate into reproductive success. However, *Ca. rufifemur* seems to be more dependent on its partner than vice versa. We never found non-parabiotic nests of this species, whereas *Cr. modiglianii* frequently nested without its partner.

XII.2.2 Trail-sharing associations and their underlying mechanisms

In Bornean rainforests, trail-sharing associations of *Polyrhachis (Polyrhachis) ypsilon* Emery 1887 and *Camponotus (Colobopsis) saundersi* Emery 1889 are common and often include further species such as *Dolichoderus cuspidatus* Smith 1857. I investigated a trail-sharing association between these three species and studied two mechanisms that may cause or facilitate these associations: interspecific trail-following, i.e. workers following another species' pheromone trail, and differential interspecific aggression (chapter VII). In trail-following assays, *D. cuspidatus* regularly followed extract trails of the other two species, thus probably parasitizing on their information on food sources. In contrast, only few *P. ypsilon* and *Ca. saundersi* workers followed heterospecific extract trails. Hence, the association between *P. ypsilon* and *Ca. saundersi* cannot be explained by foragers following heterospecific trails. In this case, trail-sharing may originate from few scout ants that do follow heterospecific pheromone trails and then lay their own trails.
Interspecific aggression among *P. ypsilon*, *Ca. saundersi* and *D. cuspidatus* was strongly asymmetric, *Ca. saundersi* being submissive to the other two species. All three species discriminated between heterospecific workers from the same and a distant trail-sharing site. They were significantly more aggressive (*P. ypsilon* and *D. cuspidatus*) or submissive (*Ca. saundersi*) towards heterospecific workers from the distant site than to those from their own site. Thus, it seems likely that the species of a given trail-sharing site habituate to one another. Differential tolerance by dominant ant species may be mediated by selective habituation towards submissive species, and thereby influence the assembly of trail-sharing associations.

XII.3 Population genetics of parabiotic ants

For both parabiotic species, I analyzed mitochondrial DNA (partial COI, COII and cytochrome b sequences) of ants from different regions in Borneo (chapter IX). My data suggest that there are four genetically and chemically distinct, but closely related varieties of *Camponotus rufifemur*. Beside the red and the black variety, two rarer ones exist. The four varieties showed little or no genetic differentiation between geographically distant populations, and probably represent cryptic species. In contrast, *Crematogaster modiglianii* showed high genetic differentiation between distant populations. However, this species was not differentiated into genetic or chemical varieties, and the *Cr. modiglianii* haplotypes did not group according to the variety membership of their *Ca. rufifemur* partners. This argues against variety-specific cocladogenesis between *Cr. modiglianii* and *Ca. rufifemur*, although a less specific coevolution of the two species is highly likely.

XIII. Zusammenfassung

Aggression zwischen Ameisen verschiedener Kolonien oder Arten ist allgegenwärtig. Ausnahmen von dieser Regel bilden Superkolonien (innerhalb einer Art) sowie interspezifische Assoziationen (zwischen Arten). Eine der engsten, aber bisher wenig verstandenen Assoziationen ist die Parabiose. Parabiotisch assoziierte Ameisenarten leben in einem gemeinsamen Nest. Sie trennen ihre Brut, interagieren jedoch oft miteinander, z. B. durch interspezifische Trophallaxis, die Nutzung gemeinsamer Pfade und die gemeinsame Ausbeutung von Nahrungsressourcen. Parabiosen sind auf Assoziationen zwischen *Crematogaster* und entweder *Camponotus, Dolichoderus, Odontomachus* oder *Pachycondyla* beschränkt und kommen nur in südamerikanischen und südostasiatischen Regenwäldern vor. Während jedoch die südamerikanischen Parabiosen bereits untersucht wurden – wenn auch spärlich –, ist fast nichts über ihre südostasiatischen Pendants bekannt.
Der Schwerpunkt meiner Doktorarbeit liegt auf südostasiatischen Parabiosen zwischen der myrmicinen Ameise *Crematogaster modiglianii* Emery 1900 und der deutlich größeren Formicine *Camponotus rufifemur* Emery 1900. Die beiden Arten nisten häufig gemeinsam in hohlen Bäumen im tropischen Tieflandregenwald Borneos. Die grundlegende Frage meiner Doktorarbeit ist, warum diese beiden Arten zusammenleben. Ich untersuchte sowohl proximate als auch ultimate Aspekte dieser Frage. Zu Vergleichszwecken führte ich Studien über eine *trail sharing*-Assoziation im selben Lebensraum durch.

XIII.1 Kolonieerkennung bei parabiotischen Ameisen

Auf proximater Ebene untersuchte ich, welche Mechanismen die Toleranz heterospezifischer Nestgenossinnen fördern. Im allgemeinen können Ameisen Nestgenossinnen von fremden Artgenossen mit Hilfe von kutikulären Kohlenwasserstoffen unterscheiden, die als Kolonie-Erkennungssignale dienen. Eine Ameise nimmt die Kohlenwasserstoffe ihres Gegenübers geruchlich wahr und vergleicht sie mit einer neuronalen Vorlage. Stimmen sie mit der Vorlage überein, wird das Gegenüber in der Regel als Nestgenossin akzeptiert, tun sie es nicht, wird es angegriffen.
Ich untersuchte Kolonieerkennung innerhalb und zwischen den beiden parabiotischen Arten. Insbesondere interessierte mich, ob interspezifische Toleranz kolonie-, art- oder gattungsspezifisch ist. Daneben analysierte ich die kutikulären Substanzen beider Ameisenarten, um etwaige Unterschiede zu verwandten, nichtparabiotischen Arten zu finden, und um abzuschätzen, wie stark die Substanzen beider Arten sich überschneiden. Eine starke Überschneidung (d.h. viele gemeinsame Substanzen) würde z.B. dafür sprechen, daß interspezifische Toleranz durch chemische Mimikry verursacht oder zumindest erleichtert wird. Außerdem untersuchte ich anhand von Biotests die Funktion zweier verschiedener kutikulärer Substanzklassen.
Inter- und intraspezifische Kolonieerkennung wurde mit Hilfe von Verhaltenstests untersucht, in denen Ameisen an ihrem Nest oder in einer Laborkolonie mit einer fremden Ameise (oder einem Dummy) konfrontiert wurden. Die Verhaltenstests waren so angelegt, daß Effekte durch Störung der Tiere minimiert wurden. In jedem Test wurde das Verhalten der Ameisen gegenüber der fremden Ameise 3 min lang mit einer zeitlichen Auflösung von 10 s aufgenommen. Kutikuläre Substanzen wurden mittels Gaschromatographie und Massenspektrometrie (GC-MS) analysiert und anhand ihrer Massenspektren, diagnostischer Ionen

und Retentionsindices identifiziert. Zur Identifikation mancher Substanzen wurden zusätzlich Derivatisierungen vorgenommen.
Die interspezifische Toleranz zwischen den beiden parabiotischen Arten (Kapitel III) war artspezifisch, aber nicht koloniespezifisch. *Ca. rufifemur* tolerierte alle *Cr. modiglianii*-Arbeiterinnen, auch von fremden Kolonien, attackierte aber Arbeiterinnen anderer *Crematogaster*-Arten. *Cr. modiglianii* dagegen duldete *Ca. rufifemur*-Arbeiterinnen von bestimmten fremden Kolonien, attackierte jedoch diejenigen bestimmter anderer Kolonien. Wie chemische Analysen ergaben, kommt *Ca. rufifemur* in zwei sympatrischen, chemisch verschiedenen Morphen vor, die praktisch keine Kohlenwasserstoffe gemeinsam haben und wahrscheinlich kryptische Arten darstellen. Aufgrund ihrer Färbung wurden sie als ‚rote' und ‚schwarze' Morphe bezeichnet. Demnach duldete *Cr. modiglianii* nur diejenigen *Ca. rufifemur*-Arbeiterinnen, die zur gleichen Morphe gehörten wie ihr eigener Partner, griff aber diejenigen an, die zur jeweils anderen Morphe gehörten. *Cr. modiglianii* attackierte auch drei weitere, nichtparabiotische *Camponotus*-Arten. Gegenseitige interspezifische Toleranz war also auf die Arten *Cr. modiglianii* und *Ca. rufifemur* beschränkt. Wahrscheinlich gewöhnte sich *Cr. modiglianii* an das Kohlenwasserstoffprofil seines *Ca. rufifemur*-Partners und tolerierte aufgrund der geringen chemischen Differenzierung zwischen Kolonien auch fremde *Ca. rufifemur*-Arbeiterinnen derselben Morphe.
Ca. rufifemur duldete häufig koloniefremde Artgenossen derselben Morphe. Die kleineren Arbeiterinnenkasten wurden eher geduldet als große Arbeiterinnen (Soldaten), möglicherweise weil sie 2-3-fach kleinere Kohlenwasserstoffmengen pro Körperoberfläche besitzen als letztere. Im Gegensatz dazu attackierte *Cr. modiglianii* fast stets koloniefremde Artgenossen (Kapitel VI).
Die GC-MS-Analysen (Kapitel IV) ergaben, daß die Kohlenwasserstoffe beider Arten beträchtlich länger waren als bei nichtparabiotischen Arten der gleichen Gattungen. Während kutikuläre Kohlenwasserstoffe bei anderen, nichtparabiotischen Arten Kettenlängen zwischen C21 und C37 aufweisen, lagen diejenigen von *Cr. modiglianii* zwischen C36 und C41 und die von *Ca. rufifemur* zwischen C37 und C49. Langkettige Kohlenwasserstoffe sind weniger flüchtig als kurzkettigere. In einigen Studien wurde bereits vermutet, daß extrem langkettige Kohlenwasserstoffe interspezifische Toleranz fördern könnten, indem sie kurzkettigere ‚absorbieren' und so das koloniespezifische Profil verwischen, selbst jedoch aufgrund ihrer geringen Flüchtigkeit nur schwer wahrnehmbar sind. Die hohen Kettenlängen bei beiden Ameisenarten könnten somit ein wichtiger Faktor sein, der interspezifische Toleranz fördert. Auffällig war weiterhin, daß die kutikulären Kohlenwasserstoffe bei *Ca. rufifemur* zu bis zu 98% aus Methylalkenen bestanden. Diese sind als kutikuläre Substanzen bei Insekten höchst ungewöhnlich, die in der Regel aus n-Alkanen, Methylalkanen und n-Alkenen bestehen.
Cr. modiglianii und *Ca. rufifemur* besaßen fast keine gemeinsamen Kohlenwasserstoffe, es lag also keine chemische Mimikry vor. Die einzigen gemeinsamen Kohlenwasserstoffe, die in größeren Mengen vorkamen, waren die Methylalkene 27-MeC39-14-en und 27-MeC39-16-en, die bei der roten *Ca. rufifemur*-Morphe ca. 89% des Kohlenwasserstoffprofils ausmachten und auch bei den *Cr. modiglianii*-Kolonien vorkam, die mit dieser Morphe zusammenlebten. Vermutlich übernahmen diese *Cr. modiglianii*-Kolonien die beiden Substanzen von ihren roten *Ca. rufifemur*-Partnern (Kapitel IV). *Cr. modiglianii*-Arbeiterinnen waren leicht, aber signifikant weniger aggressiv gegenüber fremden Artgenossinnen, wenn diese mit derselben *Ca. rufifemur*-Morphe assoziiert waren wie sie selbst. Diese Art schien demnach die Kohlenwasserstoffe, die sie von ihrem Parabiosepartner übernommen hatte, als Erkennungssignale zu nutzen (Kapitel VI). Die übrigen gemeinsamen Kohlenwasserstoffe

von *Cr. modiglianii* und *Ca. rufifemur* waren eine (rote Morphe) bzw. drei (schwarze Morphe) Verbindungen, die jeweils nur in Spuren vorlagen.
Neben den Kohlenwasserstoffen kam auf der Kutikula beider Ameisenarten eine Reihe bisher unbekannter, chemisch miteinander verwandter Stoffe vor. Die häufigste dieser Verbindungen ($C_{21}H_{32}O$) besaß drei Ringstrukturen und drei Doppelbindungen, und ihr Sauerstoffatom war vermutlich mit zwei Alkylgruppen verbunden (Kapitel V). Ihre genaue Molekularstruktur ist jedoch noch unbekannt und wird mittels NMR bestimmt werden. Die quantitative Zusammensetzung dieser Substanzen variierte zwischen parabiotischen Nestern, ähnelte sich aber jeweils zwischen den beiden Arten eines Nests. Da dieselben Substanzen in großen Mengen in der Dufourdrüse von *Cr. modiglianii* vorkamen, ist es wahrscheinlich, daß sie von dieser Art produziert und auf den *Ca. rufifemur*-Partner übertragen werden. Als mögliche Übertragungsmechanismen kommen interspezifische Trophallaxis sowie ‚Besteigeverhalten' in Betracht, bei dem *Cr. modiglianii* auf *Ca. rufifemur*-Arbeiterinnen klettert, ohne von diesen vertrieben zu werden (Kapitel IV).
Obwohl die Zusammensetzung der unbekannten Substanzen stark zwischen parabiotischen Nestern variierte, wurden sie nicht als Kolonieerkennungssignale verwendet. In Biotests mit Dummies diskriminierten beide Ameisenarten zwischen den Kohlenwasserstoffen von heterospezifischen Nestgenossen und Nestfremden, unterschieden aber nicht zwischen den unbekannten Substanzen von heterospezifischen Nestgenossen und Nestfremden. Dasselbe galt für intraspezifische Tests bei *Cr. modiglianii*. Folglich nutzten beide Arten die Kohlenwasserstoffe zur Kolonieerkennung. Die unbekannten Substanzen reduzierten jedoch signifikant die Aggressivität von *Ca. rufifemur* (Kapitel V). *Ca. rufifemur*-Arbeiterinnen waren wenig bis nicht aggressiv gegenüber nesteigenen oder nestfremden Arbeiterinnen oder Extrakten von *Cr. modigianii*. Sie attackierten jedoch häufig Kohlenwasserstoffe von nestfremden *Cr. modiglianii*, wenn diese chemisch von den unbekannten Substanzen getrennt und auf Dummies präsentiert wurden. Diese Aggression konnte durch erneute Zugabe der unbekannten Substanzen stark reduziert werden.

XIII.2 Ökologische Interaktionen assoziierter Ameisenarten

Die ultimaten, also ökologischen und evolutionären Aspekte meiner Doktorarbeit beschäftigen sich mit potentiellen Kosten und Nutzen, die *Cr. modiglianii* und *Ca. rufifemur* aus ihrer parabiotischen Lebensweise ziehen könnten, mit ihren Interaktionen zu weiteren Arten sowie populationsgenetischen Analysen. Meine Untersuchungen zu einer *trail sharing*-Assoziation zwischen drei anderen Ameisenarten beschäftigen sich mit zwei Mechanismen, die *trail sharing* verursachen oder fördern könnten.

XIII.2.1 Kosten und Nutzen einer parabiotischen Lebensweise

Ob Parabiosen parasitisch, kommensalistisch oder mutualistisch sind, ist weitgehend unbekannt und hängt von den Kosten und Nutzen ab, die beiden Partnern durch die Parabiose entstehen. Ich untersuchte deshalb Nahrungskonkurrenz (als eine der wahrscheinlichsten Kosten), Nischendifferenzierung in bezug auf die Nahrungssuche (was die Konkurrenz verringern könnte), sowie mehrere etwaige Nutzen aus der parabiotischen Lebensweise. Darüber hinaus untersuchte ich Interaktionen zwischen den Ameisen und dem Hemiepiphyten *Poikilospermum cordifolium*.
Wie diverse Köderversuche zeigten, unterschieden sich die Nischen der beiden Arten in bezug auf Fouragierdistanz vom Nest, tageszeitliche Aktivitätsspanne und Nahrungspräferenzen (Kapitel VIII). *Cr. modiglianii* war tag- und nachtaktiv und fouragierte in signifikant größerer

Entfernung vom Nest als die fast ausschließlich nachtaktive *Ca. rufifemur*. *Cr. modiglianii* entdeckte die Köder stets vor *Ca. rufifemur* und schien folglich effektiver im Auffinden von Nahrung zu sein. Obwohl die zwei Arten oft gemeinsam fouragierten, unterschieden sich ihre Nahrungspräferenzen. *Ca. rufifemur* war signifikant häufiger an (protein- und fetthaltigen) Thunfisch- als an (kohlehydrathaltigen) Honigködern. Hingegen fouragierte *Cr. modiglianii* stärker an Honigködern, extrafloralen Nektarien und betreute Trophobiosen. Keine der beiden Arten vertrieb die Partnerart gewaltsam von Ködern, so daß keine direkte Konkurrenz erkennbar war.

Für beide Ameisenarten sind eine Reihe von Vorteilen aus der parabiotischen Lebensweise denkbar. Darunter fallen interspezifisches Spurfolgeverhalten, gemeinsame Nestverteidigung, Bereitstellung von Nistraum durch die Partnerart, Nahrungsaustausch mittels Trophallaxis und gegenseitige Brutfürsorge (Kapitel VII, VIII). Wenn eine Ameisenart der Pheromonspur einer anderen Art folgt, erreicht sie Nahrungsressourcen, die die andere Art gefunden hat. Wie durch künstliche Pheromonspuren gezeigt wurde, folgte *Ca. rufifemur* tatsächlich Spuren von *Cr. modiglianii*, jedoch nicht umgekehrt. *Ca. rufifemur* profitierte damit vom Wissen ihrer Partnerart über Nahrungsressourcen (informationaler Parasitismus) und nutzte so deren Fouragieraktivität.

Cr. modiglianii wiederum schien von der Nestverteidigung durch *Ca. rufifemur* zu profitieren. *Ca. rufifemur*-Soldaten sind deutlich größer als *Cr. modiglianii*-Arbeiterinnen. Im Versuch waren sie signifikant effektiver darin, ‚Eindringlinge' anderer Ameisenarten zu töten, die am Nesteingang präsentiert wurden. Obwohl *Cr. modiglianii* oft ebenfalls effektiv das Nest verteidigte, erscheint es wahrscheinlich, daß diese Art einen Nutzen aus der Nestverteidigung durch *Ca. rufifemur* zieht.

Für neotropische Parabiosen wurde bereits vorgeschlagen, daß *Camponotus Crematogaster* durch die Bereitstellung von Nistraum nützt. Neotropische Parabiosen sind sogenannte Ameisengärten, d.h. sie bestehen aus freihängenden Kartonnestern, die durch die Wurzeln von epiphytischen Pflanzen stabilisiert werden. Die neotropische *Camponotus*-Art initiiert diese Ameisengärten, indem sie aktiv Epiphytensamen ins Kartonmaterial pflanzt, während *Crematogaster* zu klein ist, um Epiphytensamen zu tragen. Die Parabiosen Borneos waren ebenfall oft von dem Hemiepiphyten *Poikilospermum cordifolium* (Barg.-Petr.) Merr (Cecropiaceae) besiedelt. Keimlinge und größere Individuen von *P. cordifolium* wuchsen häufig an parabiotischen Nesteingängen. Im Gegensatz zu neotropischen Parabiosen stellt *P. cordifolium* jedoch keinen zusätzlichen Nistraum zur Verfügung, da die Parabiosen Borneos nicht freihängend sind, sondern sich in hohlen Bäumen befinden. Die Ameisen profitierten folglich nicht von Neststabilisierung durch *P. cordifolium*, und *Ca. rufifemur* nützte somit *Cr. modiglianii* auch nicht durch das Pflanzen von Samen. Durch seine nahrhaften Elaiosomen und Perianthe (die von beiden Ameisenarten eingetragen wurden) sowie durch extrafloralen Nektar (an dem *Cr. modiglianii* fouragierte) könnte *P. cordifolium* allerdings einen weiteren, wenn auch weniger bedeutenden Vorteil für die Ameisen darstellen.

Interspezifische Trophallaxis zwischen den beiden Arten wurde beobachtet, kam aber nur selten vor. Ihre Bedeutung für die parabiotische Assoziation ist daher schwer zu beurteilen. Die beiden Ameisenarten sorgten jeweils nur für ihre eigene Brut; ein Vorteil durch gegenseitige Brutfürsorge exisierte somit nicht.

Als Fazit scheint die Parabiose für beide Arten vorteilhaft zu sein. Die wichtigsten Vorteile sind Nestverteidigung (für *Cr. modiglianii*) und interspezifisches Spurfolgen (für *Ca. rufifemur*). Wie groß die Bedeutung dieser Vorteile ist und wie stark sie den reproduktiven Erfolg der beiden Arten beeinflusst, bleibt jedoch unbekannt. Allerdings scheint *Ca. rufifemur*

stärker von seinem Partner abzuhängig zu sein als umgekehrt, da diese Art nie ohne ihren Partner nistete, während nicht-parabiotische Nester von *Cr. modiglianii* häufig vorkamen.

XIII.2.2 Trail sharing-Assoziationen und ihre zugrundeliegenden Mechanismen

In den Regenwäldern Borneos sind *trail sharing*-Assoziationen zwischen *Polyrhachis (Polyrhachis) ypsilon* Emery 1887 und *Camponotus (Colobopsis) saundersi* Emery 1889 weit verbreitet und schließen oft weitere Arten wie *Dolichoderus cuspidatus* Smith 1857 ein. Ich untersuchte eine *trail sharing*-Assoziation zwischen diesen drei Arten und erforschte zwei Mechanismen, die eine solche Assoziation eventuell fördern könnten: interspezifisches Spurfolgeverhalten und differentielle interspezifische Aggression (Kapitel VII). In Spurfolgeversuchen folgte *D. cuspidatus* regelmäßig künstlichen Extraktpfaden der anderen beiden Arten. Auf diese Weise parasitierte *D. cuspidatus* wahrscheinlich auf deren Informationen über Nahrungsressourcen. Im Gegensatz dazu folgten nur wenige Arbeiterinnen von *P. ypsilon* und *Ca. saundersi* heterospezifischen Extraktpfaden. Die Assoziation zwischen *P. ypsilon* und *Ca. saundersi* kann folglich nicht dadurch erklärt werden, daß fouragierende Arbeiterinnen heterospezifischen Pheromonspuren folgen. In diesem Fall könnte *trail sharing* möglicherweise darauf beruhen, daß einige wenige *scouts* heterospezifischen Spuren folgen und anschließend ihre eigene Spur legen.
Die interspezifische Aggression zwischen *P. ypsilon*, *Ca. saundersi* und *D. cuspidatus* war stark asymmetrisch, denn *Ca. saundersi* war gegenüber den anderen beiden Arten stark submissiv. Alle drei Arten unterschieden heterospezifische Arbeiterinnen von ihrem eigenen und einem fremden Standort und waren gegenüber denen des fremden Standorts signifikant aggressiver (*P. ypsilon* und *D. cuspidatus*) bzw. submissiver (*Ca. saundersi*). Es erscheint daher wahrscheinlich, daß die Arten eines *trail sharing*-Standorts sich aneinander gewöhnen. Differentielle Toleranz durch dominante Ameisenarten könnte zustande kommen, indem sich diese selektiv an bestimmte submissive Arten gewöhnen, sie dulden und auf diese Weise die Zusammensetzung von *trail sharing*-Assoziationen beeinflussen.

XIII.3 Populationsgenetik der parabiotischen Arten

Von beiden parabiotischen Arten analysierte ich mitochondriale DNA (Sequenzabschnitte von COI, COII und Cytochrom b) von Ameisen aus verschiedenen Regionen Borneos (Kapitel IX). Nach meinen Ergebnissen existieren vermutlich vier genetisch und chemisch verschiedene, aber nah miteinander verwandte *Camponotus rufifemur*-Morphen. Neben der roten und der schwarzen Morphe kommen zwei seltenere vor. Die vier Morphen wiesen wenig bis keine genetische Differenzierung zwischen entfernten Populationen auf und stellen wahrscheinlich kryptische Arten dar. Im Gegensatz dazu zeigte *Crematogaster modiglianii* hohe genetische Differenzierung zwischen entfernten Populationen. Diese Art war jedoch nicht in genetische oder chemische Morphen differenziert, und die einzelnen *Cr. modiglianii*-Haplotypen ließen sich nicht nach der jeweiligen Morphe ihres *Ca. rufifemur*-Partners gruppieren. Dieses Ergebnis spricht gegen eine morphen-spezifische Cocladogenese zwischen *Cr. modiglianii* und *Ca. rufifemur*, obwohl eine weniger spezifische Coevolution der beiden Arten sehr wahrscheinlich ist.

XIV. Danksagung

Ohne die Hilfe und Unterstützung zahlreicher Menschen wäre ich nicht in der Lage gewesen, diese Dissertation zu erstellen. Ihnen allen möchte ich an dieser Stelle meinen Dank aussprechen.
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XV. Lebenslauf

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Geburtsdatum	11. Februar 1980
Geburtsort	Mutlangen

Schulbildung

1986-1990	Stauferschule Lorch
1990-1996	Progymnasium Lorch
1996-1999	Hans-Baldung-Gymnasium, Schwäbisch Gmünd
Juni 1999	Abitur mit Notenschnitt 1,0

Zivildienst

August 1999 bis July 2000	Landesbund für Vogelschutz in Bayern (LBV), Hilpoltstein

Akademische Ausbildung

Oktober 2000-Dezember 2005	Studium der Biologie an der Julius-Maximilians-Universität Würzburg
September 2002	Vordiplom mit Notenschnitt 2,0
Dezember 2005	Diplom mit Notenschnitt 1,0
seit Mai 2006	Doktorand an der Julius-Maximilians-Universität in Würzburg, Biozentrum, Lehrstuhl für Tierökologie und Tropenbiologie

Stipendien

Dezember 1998-September 2005, sowie seit Juli 2006	Studienstiftung des deutschen Volkes
August 2003-April 2004	Deutscher Akademischer Austauschdienst (DAAD)
seit Oktober 2006	Bayerische Eliteförderung (BayEFG)

Auslandsaufenthalte

März-Juli 2003	Forschungspraktikum an der Griffith University, Brisbane (Australia), über rindenbewohnende Käfergesellschaften
August 2003-April 2004	Studium an der Duke University, Department of Biology, Durham, NC (USA)
August-November 2004	Freilandarbeit für meine Diplomarbeit am Danum Valley Field Center in Sabah (malaysisches Borneo), über *Camponotus-Crematogaster*-Interaktionen in einem tropischen Regenwald
je 2-3 Monate in den Jahren 2006-2008	Freilandarbeit für meine Dissertation am Danum Valley Field Center und im Mulu National Park (Sarawak, malaysisches Borneo)
Fremdsprachen	englisch, spanisch, malaiisch
EDV-Kenntnisse	R, Turbo-Pascal, MS Office
Preise	1998 – Bundessieg bei „Jugend forscht“ in der Disziplin Biologie (Thema: Leitbündelevolution bei Farnen)
Hobbies und sonstige Aktivitäten	Organisation eines Workshops über globale ökologische Probleme und Lösungsmöglichkeiten (www.synagieren.de)
	Jazz- und Bluespiano
	Photographie

XVI. Publikationsliste

Zeitschriftenbeiträge

Menzel F, Blüthgen N, Beuerle T, Schmitt T (submitted): Novel cuticular substances in parabiotic ants function as interspecific appeasement signals.

Menzel F, Blüthgen N (in revision): Parabiotic associations between tropical ants: equal partnership or parasitic exploitation?

Menzel F, Schmitt T, Blüthgen N (2009): Intraspecific nestmate recognition in two parabiotic ant species: acquired recognition cues and low inter-colony discrimination. ***Insectes Sociaux*** (in press)

Menzel F, Blüthgen N, Schmitt T (2008): Tropical parabiotic ants: Highly unusual cuticular substances and low interspecific discrimination. ***Frontiers in Zoology*** 5: 16

Menzel F, Linsenmair KE, Blüthgen N (2008): Selective interspecific tolerance in tropical *Crematogaster-Camponotus* associations. ***Animal Behaviour*** 75: 837-846

Blüthgen N, Fründ J, Vázquez DP, Menzel F (2008): What do interaction network metrics tell us about specialization and biological traits? ***Ecology*** 89: 3387-3399

Blüthgen N, Menzel F, Hovestadt T, Fiala B, Blüthgen N (2007): Specialization, constraints, and conflicting interests in mutualistic networks. ***Current Biology*** 17: 341-346

Blüthgen N, Menzel F, Blüthgen N (2006): Measuring specialization in species interaction networks. ***BMC Ecology*** 6: 9

Zhou P, Menzel F, Shaw J (2007): Systematics and population genetics of *Sphagnum macrophyllum* and *S. cribrosum* (Sphagnaceae). ***Systematic Botany*** 32: 493-503

Beaulieu F, Walter DE, Proctor HC, Kitching RL & Menzel F (2006): Mesostigmatid mites (Acari: Mesostigmata) on rainforest tree trunks: arboreal specialists, but substrate generalists? ***Experimental and Applied Acarology*** 39: 25-40

Menzel F, Kitching RL, Boulter SL (2004): Host specificity or habitat structure? – The epicortical beetle assemblages in an Australian subtropical rainforest. ***European Journal of Entomology*** 101: 251-259

Menzel F (1999): Anatomie der Farnpflanzen: Artbestimmung und Evolution. ***Jahreshefte der Gesellschaft für Naturkunde Württemberg*** 155: 107-133

Menzel F (1999): Leitbündelevolution bei Farnen. ***junge wissenschaft*** 56: 34-39

Kongreßbeiträge

Menzel F, Schmitt T, Blüthgen N (2009): Tropical parabiotic ant: mutualistic partnership or parasitic exploitation? *14th Annual DZG Evolution PhD Meeting, München*

Menzel F (2007): High interspecific tolerance in tropical associated ant species. *1st Early Career Meeting of the British Ecological Society – Tropical Ecology Group, Leeds/England*

Menzel F, Blüthgen N (2006): *Crematogaster-Camponotus* Associations in a Tropical Rainforest: Mechanisms and Specificity of Interspecific Recognition. *19th Annual Conference of the Society for Tropical Ecology (GTÖ), Kaiserslautern.*

Blüthgen N, Menzel F (2006): Are tropical plant-animal networks more specialised? *19th Annual Conference of the Society for Tropical Ecology (GTÖ)*, Kaiserslautern.