Causes and consequences of cuticular hydrocarbon divergence in parabiotic ants

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Zusammenfassung

Interaktionen zwischen Arten wie Konkurrenz, Antagonismus und Mutualismus werden oft als Gründe für die Diversifizierung phänotypischer Merkmale angeführt und tragen somit signifikant zur Artenvielfalt auf der Erde bei. Kutikuläre Kohlenwasserstoffe (engl. CHCs) sind die Hauptkomponenten der Wachsschicht, die fast alle terrestrischen Arthropoden besitzen. Die Profile dieser CHCs können als komplexes phänotypisches Merkmal betrachtet werden. Sie haben vielfältige Funktionen, insbesondere schützen sie Insekten vor Austrocknung, sind aber auch wichtig für die chemische Kommunikation. Diese Kommunikationsfunktion ist besonders wichtig für soziale Insekten wie Ameisen, Bienen, Wespen oder Termiten. Um den Erfolg einer Kolonie zu sichern ist es von größter Wichtigkeit Informationen wie die Kolonie- und Kastenzugehörigkeit, Anwesenheit und Fruchtbarkeit der Königin oder das Alter einzelner Individuen auszutauschen. Weiterhin benutzen Ameisen CHC Profile um mutualistische Partnerspezies zu identifizieren. Eine besondere Form eines solchen Mutualismus ist die Parabiose, sprich zwei Ameisenarten, die sich friedlich ein Nest teilen. In dieser Arbeit untersuche ich die Ursachen und Folgen der Divergenz von CHC Profilen in den parabiotischen Ameisenarten *Crematogaster levior* und *Camponotus femoratus* aus dem tropischen Regenwald Südamerikas.

In **Kapitel 1** untersuche ich den Artstatus chemisch verschiedener Formen beider oben erwähnter Gattungen und diskutiere welche Rolle CHCs in der Speziation spielen könnten. Ich demonstriere schlüssig, dass beide, *Cr. levior* und *Ca. femoratus*, eigentlich aus zwei kryptischen Arten bestehen, welche sich trotz nur geringer morphologischer Unterschiede stark in ihren CHC Profilen, dem genetischen Hintergrund und in *Cr. levior* auch in auf der Kutikula nachweisbaren Sekundärmetaboliten unterscheiden. Durch eine detaillierte Untersuchung der CHC Unterschiede innerhalb und zwischen den kryptischen Arten von *Cr. levior* und *Ca. femoratus*, identifiziere ich mehrere ultimate Ursachen für die Variation in den CHC Profilen in **Kapitel 2**. Besonders die parabiotische Lebensweise führt zu starken Veränderungen wie beispielweise der Verlängerung der Kohlenstoffketten der CHCs in beiden *Ca. femoratus* Arten, jedoch nur einer der *Cr. levior* Arten. Dies scheint innerhalb der *Crematogaster* Arten einen phylogenetisch fortschrittlichen Zustand darzustellen. Obwohl die kryptischen Arten nah verwandt sind, zeigen sie stark verschiedene CHC Profile. Daher untersuchte ich weiterhin in **Kapitel 3** ob Genexpressionsunterschiede am Ort der CHC Biosynthese diese Unterschiede erklären könnten. In diesem Kapitel identifiziere ich mehrere Kandidatengene und ihre Expressionsmuster als proximate Ursachen für die CHC Unterschiede. In vielen Fällen konnte ich hier zeigen, dass die Genexpressionsunterschiede sich in den Unterschieden im CHC Profil widerspiegeln.

Die starken Unterschiede in den CHC Profilen haben vermutlich Folgen für die Nestgenossenerkennung und das Aggressionsverhalten der Ameisen, weshalb ich die Erkennung innerhalb und zwischen den kryptischen Arten von *Cr. levior* in **Kapitel 4** untersuchte.

Hierbei versuchte ich herauszufinden, welche Substanzen oder strukturelle Substanzklassen für die Erkennung eine Rolle spielen. Übereinstimmend mit der Hypothese das die Verlängerung der Kohlenstoffketten die Kohlenwasserstoffmoleküle schwieriger wahrzunehmen macht, fand ich, dass CHC Extrakte der *Cr. levior* Art mit kürzeren Kohlenwasserstoffketten scheinbar besser wahrnehmbar waren und daher aggressiver behandelt wurden. Weil die Divergenz der CHC Profile und die Aufspaltung in zwei kryptische Arten übereinstimmen, scheint es wahrscheinlich, dass dies weitere ökologische Folgen haben könnte. In **Kapitel 5** untersuchte ich daher ob die Artaufspaltung von Partitionierung in der Nahrungsnische zwischen a) den mutualistischen Partnern und b) den kryptischen Arten von *Cr. levior* und *Ca. femoratus* begleitet wurde. Die Ergebnisse implizieren, dass die Mutualisten um Nahrung konkurrieren und die Konkurrenz möglicherweise durch einen Trade-off zwischen Nahrungsauffindung und Dominanz verringert wird. Die Unterschiede zwischen den kryptischen Arten jedoch waren sehr subtil, was darauf hindeutet, dass entweder Partitionierung in Nischendimensionen stattfindet, die wir bisher nicht untersucht haben oder dass die kryptischen Arten ein Beispiel für „neutrale Arten" sind.

In **Kapitel 6** verschaffe ich einen Überblick über die Ebenen und das Ausmaß der Variation von CHCs bei Ameisen aber auch anderen Insekten. Durch die Aufarbeitung aktueller Literatur biete ich einen detaillierten Einblick in Quellen für fixierte und plastische Variation auf individueller, kolonieweiter, populationsweiter und artspezifischer Ebene. Weiterhin diskutiere ich welche Faktoren zu adaptiven CHC Änderungen führen und wie diese durch biosynthetische und biophysikalische Mechanismen eingeschränkt werden.

Zusammenfassend zeigt diese Arbeit die Existenz kryptischer Arten mittels integrativer Taxonomie, bietet Einblicke in die komplexen Selektionsdrücke, die die Evolution von CHC Profilen beeinflussen und identifiziert mehrere Kandidatengene, die in die Divergenz der CHC Profile involviert sein könnten. Diese Divergenz in CHC Profilen könnte eine wichtige Rolle bei der Speziation einnehmen. Es ist jedoch bisher unklar ob die chemischen Unterschiede zur Partnerwahl und damit zu präzygotischer reproduktiver Isolation beitragen, oder ob die Profilunterschiede erst nach der Speziation verstärkt wurden. Während CHC Divergenz, wenn sie zu assortativer Paarung führte, sympatrische Artbildung erlauben würde, würden allopatrische Populationen durch Prozesse wie Isolation-by-distance, genetische Drift oder lokale Anpassung in ihren CHC Profilen divergieren. Weiterhin zeigt diese Arbeit, dass die kryptischen Arten der parabiotischen Ameisen ein interessantes Modell sein könnten um zu untersuchen wie ökologisch ähnliche Arten einen Konkurrenzausschluss vermeiden können und möglicherweise auch für die Untersuchung „neutraler Prozesse", die Artenkoexistenz in tropischen Ökosystemen ermöglichen.

Summary

Species interactions such as competition, antagonism and mutualism are thought to promote diversification in phenotypic traits and thus significantly contribute to species diversity on Earth. Cuticular hydrocarbons (CHCs) are the major components of the waxy layer covering basically all terrestrial arthropods. They can be seen as a complex phenotypic trait and have multiple functions, most importantly protecting insects from desiccation and acting as agents of chemical communication. The communication functions of CHCs are especially important in social insects, such as ants, bees, wasps or termites. To successfully run a colony, they need to exchange information such as colony or caste membership, queen presence and fertility or age of an individual. Furthermore, ants use CHC profiles to identify mutualistic partner species. A special form of such a mutualism is parabiosis, i.e. two ant species mutualistically sharing the same nest. In this thesis, I investigate the causes and consequences for the divergence of CHC profiles in the parabiotic ant species *Crematogaster levior* and *Camponotus femoratus* from the South American rainforest.

In **Chapter 1**, I elucidate the species status of chemically diverged morphs of the ants of both genera mentioned above and discuss which role CHCs could have mediating speciation. I conclusively demonstrate that both, *Cr. levior* and *Ca. femoratus*, in fact consist of two cryptic species that, despite only slight morphological differences, strongly differ in their CHC profiles, their genetic background and in *Cr. levior* also in secondary metabolites found on the cuticle. By in detail investigating CHC differences within and between the cryptic species of *Cr. levior* and *Ca. femoratus*, I identify several ultimate causes for variation of the CHC profiles in **Chapter 2**. Especially the parabiotic lifestyle led to strong changes such as elongations of the carbon backbone of the CHCs, in both cryptic species of *Ca. femoratus*, but only one of *Cr. levior*. This seems to be a phylogenetically derived state at least in *Crematogaster*. Although the cryptic species are closely related, they show vastly different CHC profiles, which is why I further investigated if gene expression differences at the site of CHC biosynthesis might explain this in **Chapter 3**. In this chapter, I identify several candidate genes and their expression patterns as proximate causes for the CHC variation. In many cases I am able to show that the gene expression differences between the cryptic species are mirrored in the differences observed in their CHC profiles.

The strong differences in CHC profiles are likely to have consequences for nestmate recognition and aggression behavior, which is why I investigated recognition within and between the cryptic species of *Cr. levior* in **Chapter 4**, trying to identify which substances or structural CHC classes are involved. In line with the hypothesis that elongations of the carbon backbone of CHCs make the molecules harder to perceive, I found that CHC extracts of the shorter-chained *Cr. levior* species were probably more perceivable and thus treated more aggressively. Because the divergence in CHC profiles coincided with the spilt into two

cryptic species each, this is likely to have further ecological consequences. In **Chapter 5**, I examine if the species divergence was accompanied by trophic niche partitioning a) between the mutualistic partners and b) between the cryptic species of *Cr. levior* and *Ca. femoratus*. Here, the results imply that competition between the mutualists is mediated by a discovery-dominance trade-off and differences in the trophic niche. The differences between the cryptic species, however, were very subtle suggesting that there is either niche differentiation in dimensions I did not investigate so far or that these cryptic species might be an example for 'neutral species'.

Finally, in **Chapter 6**, I provide an overview on the levels and magnitude of variation in CHC profiles in ants, but also other insects. By reviewing up-to-date literature, I provide detailed insights into sources of fixed and plastic variation on the levels of individuals, social insect colonies, populations and species. Furthermore, I discuss which factors may lead to adaptive CHC changes and how these could be constraint by biosynthetical and biophysical mechanisms.

In conclusion, this thesis unravels the existence of cryptic species using integrative taxonomy, provides important insights into the complexity of selection pressures shaping the evolution of CHC profiles and identifies several candidate genes that could be involved in divergence of such profiles. The divergence in CHC profiles could play an important role in mediating speciation. However, it is yet unclear if chemical differences mediated mate choice and led to prezygotic reproductive isolation or if the CHC profiles diverged through reinforcement after speciation. While CHC divergence enabling assortative mating would allow speciation even in sympatry, allopatric populations might diverge through isolation-by-distance, genetic drift or local adaptation reinforcing CHC differences. Furthermore, this thesis identifies the cryptic species of parabiotic ants as an interesting model system to examine how ecologically similar species might avoid competitive exclusion and potentially for the investigation of 'neutral processes' mediating species coexistence in tropical ecosystems.

General Introduction

Species interactions and their potential influence on cuticular hydrocarbon divergence

Philipp P. Sprenger

"The ant world is a tumult, a noisy world of pheromones being passed back and forth." –

Edward O. Wilson

Interspecific interactions as potential drivers of trait and species diversification

Understanding the origin of diversity of life on Earth has been one of the major questions in evolutionary biology ever since Darwin's conceptual work (Darwin 1859). Coevolution, i.e. the reciprocal adaptation of two or more organisms, has been widely discussed as a major source promoting diversification and divergence of phenotypic and ecological traits (Ehrlich & Raven 1964; Thompson 2005, 2009; but see Tobias *et al.* 2014). However, despite extensive research in this field, empirical evidence if and how coevolution influences trait divergence and species diversification and is still heavily discussed (Althoff, Segraves & Johnson 2014; Hembry, Yoder & Goodman 2014; Tobias *et al.* 2014). An essential aspect to note is that the type of interaction should strongly influence the importance of coevolution for diversification (Yoder & Nuismer 2010; Hembry *et al.* 2014).

Coevolving species interactions are usually classified into competitive, antagonistic or mutualistic interactions. Theoretical models predict that competitive and antagonistic interactions should promote diversification through divergent selection on phenotypic traits, while these models yielded variable results for mutualisms (Doebeli & Dieckmann 2000; Yoder & Nuismer 2010). The strongest empirical evidence for phenotypic diversification through coevolution are found in cases of competition for food sources that led to the famous examples of character displacement in beak morphology of Darwin's finches (Grant & Grant 2006) and oral jaws of cichlids in the great lakes of East Africa (Albertson, Streelman & Kocher 2003; Brawand *et al.* 2014) and their adaptive radiations. Also coevolving antagonistic interactions, such as predator-prey (Nosil & Crespi 2006), plant-herbivore (Wheat *et al.* 2007; Agrawal *et al.* 2009) or host-parasite arms races (Buckling & Rainey 2002; Summers *et al.* 2003) have been suggested to promote phenotypic diversification. For mutualisms though, it is unclear whether and especially how coevolution affects diversification rates or if they just promote the maintenance of existing diversity.

In contrast to competition and antagonism that intuitively pose sources of divergent selection, mutualisms could promote species diversification under two scenarios: a) if they allow organisms to use novel resources or enable occupation of novel niches and by that facilitate radiation or b) if coevolution promotes reproductive isolation of diverging populations (Hembry *et al.* 2014). The first case can be found in many endosymbioses for example in nutritional symbioses of plant-sucking insects (e.g. hemipterans) and bacteria in which the symbionts enable their host to utilize novel food sources such as plant saps

(Moran 2007). Another example are symbioses of beewolf wasps with antibiotic-producing bacteria that are used as pathogen defense for the wasp larvae, which show signs of diffuse codiversification (Kaltenpoth *et al.* 2014). Niche expansions allowing diversification have also been described in plants that evolved extrafloral nectaries (EFNs) recruiting arthropod mutualists which will defend the plants (Weber & Agrawal 2014) or in frugivorous primates that contributed to seed dispersal (Gómez & Verdú 2012). The second scenario probably applies in so-called brood pollination mutualisms as found in figs and fig wasps, yuccas and yucca moths, leafflowers and leafflower moths or saxifrages and *Greya* moths (reviewed in Hembry & Althoff 2016). In these highly specialized, obligate mutualisms insects pollinate flowers of their hosts while laying their eggs, which will develop into larvae that consume a subset of the plants' seeds. The taxa involved in such mutualisms without doubt codiversify in phenological and morphological traits and often show phylogenetic congruence on higher taxonomic scales, but the mechanisms by which these mutualisms diversify are largely unknown and evidence for a role of coevolution in speciation are very limited (Cruaud *et al.* 2012; Thompson *et al.* 2013; Hembry & Althoff 2016).

To promote species diversification, divergence in phenotypic traits between populations is required. Finally, this may result in reproductive isolation and the emergence of new species. Such divergence in phenotypic traits cannot only be caused by coevolution in biotic interactions, but also through adaptation to other (e.g. abiotic) ecological selection pressures. Ecologically based divergent selection between different environments might (depending on the phenotypic trait under selection) ultimately lead to reproductive isolation between populations. This process is called ecological speciation (Schluter 2001; Nosil 2012). The evolution of reproductive isolation preferably occurs if divergent ecological adaptations affect sexual selection either by changing mating opportunities temporally or spatially or by acting on mating preferences or sexually selected traits (Maan & Seehausen 2011; Hendry 2017). Traits under divergent selection that are directly involved in mate choice and lead to non-random mating are often referred to as 'magic traits' (Servedio *et al.* 2011; Nosil 2012; Thibert-Plante & Gavrilets 2013). The most conclusive example for such a trait is the wing coloration pattern in mimetic *Heliconius* butterflies, which on one hand are under selection as aposematic signals against predators but on the other hand are sexual signals that cause assortative mating (Jiggins 2008). As many insects communicate mainly chemically, also some insect pheromones have the potential to be sexually selected and thus could act as magic traits if they are under natural selection at the same time (Rundle, Chenoweth & Blows 2009; Steiger *et al.* 2013; Chung & Carroll 2015).

Cuticular hydrocarbons and their role in insect chemical ecology

The emission and perception of chemicals is considered a primordial form of communication and is widely used throughout the animal kingdom (Wyatt 2014). One type of chemicals present in nearly all terrestrial arthropods are cuticular hydrocarbons (CHCs), the major components of the waxy layer covering their body surfaces (Howard & Blomquist 1982, 2005). They fulfill multiple functions, the most important ones being protection against water loss and chemical communication (Blomquist & Bagnères 2010), but also defense against microbes (Herzner & Strohm 2007; Wurdack *et al.* 2017) and foot adhesion (Drechsler & Federle 2006). Usually CHCs are further divided into different structural classes: straight-chain *n*-alkanes, mono- or multiply methyl-branched hydrocarbons, mono- or poly-unsaturated hydrocarbons (mostly alkenes and alkadienes) and, albeit rather rare, methyl-branched alkenes (Martin & Drijfhout 2009c; Blomquist 2010a). Because of their biophysical properties such as melting behavior or viscosity, these different structural classes may serve different functions, i.e. more viscous or solid molecules are preferable for the waterproofing function of the CHC layer, while more fluid (less viscous) molecules are easier to perceive and thus are better for communication purposes (Sprenger *et al.* 2018; Menzel *et al.* 2019).

As CHCs fulfill this multitude of different functions, the evolution of their compositional profiles is shaped by several different selection pressures, trade-offs between them but also biosynthetic and biophysical constraints. The waterproofing function of CHCs is likely shaped by the climate in an insect's habitat: In wild *Drosophila melanogaster* populations local adaptation to climate was shown in CHC-associated single-nucleotide polymorphisms (SNPs) that varied with cline and season (Rajpurohit *et al.* 2017). Furthermore, ant species living in habitats with higher precipitation consistently show higher proportions of unsaturated CHCs probably because less desiccation protection is required (van Wilgenburg, Symonds & Elgar 2011; Menzel, Blaimer & Schmitt 2017a). Next to genetically fixed variation in a species' CHC profile, insects may also plastically change their CHC composition to react to daily or seasonal changes in temperature and humidity (reviewed in Otte, Hilker & Geiselhardt 2018). Such changes are adaptive, i.e. they enhance survival under stressful conditions, and can happen within short time periods like only few hours to days (Stinziano *et al.* 2015; Menzel, Zumbusch & Feldmeyer 2018; Sprenger *et al.* 2018).

Communication using CHCs is shaped by different levels of interacting individuals. Within species CHCs may be under sexual selection if they are used for mate choice such as for

example in different *Drosophila* or different Orthopteran species (Ferveur 2005; Steiger *et al.* 2013; Steiger & Stökl 2014). In social insects, CHCs also serve multiple communication functions within a colony. Amongst other functions, they inform about queen presence and fertility (Liebig 2010; Will *et al.* 2012; Van Oystaeyen *et al.* 2014), inbreeding status (Menzel, Radke & Foitzik 2016) or different tasks of workers (Wagner *et al.* 1998; Greene & Gordon 2003; Pamminger *et al.* 2014). Importantly, the CHC profiles are species-specific and differ between colonies. This enables social insects to discriminate between nestmates and non-nestmates resulting either in mutual tolerance or aggressive reactions towards enemies or competitors (Lahav *et al.* 1999; van Zweden & d'Ettorre 2010).

Cuticular hydrocarbon profiles are a complex phenotypic trait that is also influenced by biotic interactions between species, especially in organisms such as ants that often engage in mutualisms or are prone to parasitism due to their social organization (Lenoir, d'Ettorre & Errard 2001b; Ness, Mooney & Lach 2010). Ants have many social parasites that, in the first place, try to avoid aggression from their hosts. For avoiding detection by the ant hosts, social parasites (such as rove beetles, butterfly caterpillars or other ant species) evolved different strategies: Chemical mimicry (i.e. active biosynthesis of CHC profiles similar to those of the host), chemical camouflage (i.e. acquisition of host CHCs through physical contact with the host itself or nest material) or chemical insignificance (i.e. production of only few recognition cues) (Lenoir *et al.* 2001b; von Beeren *et al.* 2011; Kleeberg, Menzel & Foitzik 2017).

Similar to other antagonistic species interactions, such chemical adaptation strategies between hosts and parasites may result in coevolutionary arms races (Brandt *et al.* 2005; Nash *et al.* 2008). A well-studied system showing such a coevolutionary arms race in CHC profiles is slavemaking in *Temnothorax* ants: The slavemaker *T. americanus* uses chemical mimicry to parasitize colonies of *T. longispinosus*, as a preferred host, or *T. curvispinosus*, as an alternative host species. The degree of mimicry in the slavemaker differs according to the community composition of host species showing higher specialization if only the preferred host species is present (Brandt *et al.* 2005). Furthermore, the success of slavemaker colonies depends on the proportion of host recognition cues in the slavemaker CHC profile (Kaur, Stoldt *et al.* 2019). In the preferred host species, *T. longispinosus*, on the other hand, the presence of *T. americanus* in the habitat leads to a higher variability of recognition cues in the CHC profiles (Jongepier & Foitzik 2016). In addition, the host species show considerable behavioral plasticity and variability in acceptance thresholds towards the parasites depending on the parasite pressure and the season, i.e. if the slavemakers do or do not raid (Brandt *et al.* 2005; Jongepier *et al.* 2014; Grüter, Jongepier & Foitzik 2018).

Next to such antagonistic interactions, which are well studied, also mutualistic species interactions are often mediated by chemical communication via CHCs. Ants for example recognize mutualistic aphid species using their CHC profiles (Lang & Menzel 2011). An exceptional case of mutualism is parabiosis in ants, i.e. two different ant species mutualistically sharing the same nest.

Parabiosis and its influence on CHC profiles

Parabiosis was first described between *Crematogaster* cf. *carinata* (as *limata* var. *parabiotica*) and a *Dolichoderus* species in the rainforest of Colombia in the late 19th century. It was defined as two independent species that forage together and peacefully share the same nest, but keep their brood separate (Forel 1898). Later observations in Guiana (formerly British Guiana) indicated that the most frequent parabiotic association was between *Camponotus femoratus* (Fabricius 1804) and a *Crematogaster* species (back then also called *Cr. limata* var. *parabiotica*) (Wheeler 1921), that jointly inhabited so-called 'ant gardens' (Box I.1). Observations of these two species living together were later confirmed in Venezuela and Suriname (Weber 1943). The *Crematogaster* species most likely found back then by Wheeler (1921) and Weber (1943) nowadays is called *Cr. levior* Longino, 2003, which was separated from its sister species *Cr. carinata* in this very recent species description. Today, several cases of parabiosis have been described and it is broadly accepted that these associations are confined to tropical regions. In the Neotropics, many of them involve species of the genus *Crematogaster* associated with larger species of the genera *Camponotus*, *Dolichoderus*, *Odontomachus* or *Pachychondyla* (Orivel, Errard & Dejean 1997; Longino 2003; Menzel & Blüthgen 2010). Similarly, in the paleotropical rainforest of South East Asia, *Cr. modiglianii* lives in parabiosis with at least two different varieties of *Ca. rufifemur* (Menzel, Blüthgen & Schmitt 2008a; Menzel, Linsenmair & Blüthgen 2008b). Furthermore, a parabiotic association has been described between the small ant *Strumigenys maynei* and the much larger ponerine ant *Platythyrea conradti* in the afrotropical rainforest (Parmentier *et al.* 2017).

All these parabioses seem to be mutualistic associations. Between the paleotropical and neotropical *Crematogaster*-*Camponotus* associations, there are many similarities and they probably evolved convergently (Menzel *et al.* 2014): The smaller *Crematogaster* species are more numerous and (at least in the Paleotropics) more efficient in finding and recruiting to food sources (Vantaux *et al.* 2007; Menzel & Blüthgen 2010; Menzel *et al.* 2014). This resource discovery ability is probably an advantage to *Camponotus*, which in both cases, i.e. the

paleotropical and the neotropical parabiotic species, are able to follow the pheromone trails laid by the *Crematogaster* workers (Vantaux *et al.* 2007; Menzel & Blüthgen 2010; Menzel *et al.* 2014). The *Crematogaster* species, in turn, profit from *Camponotus*' protection abilities against large enemies such as vertebrates (Davidson 1988; Vantaux *et al.* 2007; Menzel & Blüthgen 2010). A difference between the paleotropical and neotropical parabioses is probably the initiation of the shared nest: While in the paleotropical system most likely *Cr. modiglianii* initiates the nest in hollow trees (Menzel & Blüthgen 2010), carton structures for the neotropical ant gardens are presumably built by *Ca. femoratus*, which also collects seeds of the ant garden plants (Orivel & Dejean 1999; Dejean *et al.* 2000; Youngsteadt *et al.* 2008; Box I.1). Thus, *Cr. levior* also profits from *Ca. femoratus* providing and protecting the nest and the associated epiphytes (Vantaux *et al.* 2007; Vicente, Dáttilo & Izzo 2014; Leal *et al.* 2017). Although the neotropical parabiosis is generally considered as mutualism providing net benefits to both partners (Vantaux *et al.* 2007), there is still the possibility of context-dependent costs for either of the partners. Furthermore, although not otherwise specified in this thesis, the interaction with ant garden epiphytes (Box I.1) makes the evaluation of costs and benefits in this mutualistic association even more complex.

Parabiotic associations are characterized by the high mutual tolerance between species. Although it was often hypothesized that nestmate recognition is based on neuronal comparison with a self-referent template, all parabiotic ants keep their own species-specific CHC profiles. However, they still tolerate their mutualistic partners suggesting that in this case CHC templates are rather learned (Orivel *et al.* 1997; Errard, Hefetz & Jaisson 2006). Despite that the afrotropical parabiotic ants *S. maynei* and *P. conradti* have about 50% of the compounds in their CHC profiles in common (Parmentier *et al.* 2017), such a big overlap in CHC profiles is rarely found in any other parabiotic species (Orivel *et al.* 1997; Menzel *et al.* 2008a; Emery & Tsutsui 2013). A similarity found in the paleotropical and neotropical parabiotic *Camponotus*-*Crematogaster* associations, is an elongation of the carbon backbone of CHC molecules that most likely makes perception of these CHCs more difficult (Menzel & Schmitt 2012; Menzel *et al.* 2017a). In the paleotropical parabiosis, *Cr. modiglianii* is tolerant towards workers of *Ca. rufifemur* of its own nest but not necessarily of other nests (Menzel *et al.* 2008b). This is reflected in *Ca. rufifemur* being present in two chemically different varieties (or chemotypes) that can be differentiated by *Cr. modiglianii* (Menzel *et al.* 2008a). *Camponotus rufifemur* on the other hand was not able to discriminate between *Cr. modiglianii* from its own or foreign nest (Menzel *et al.* 2008b). Furthermore, *Cr. modiglianii* uses a special substance

class ('crematoenones') as appeasement allomones probably preventing colony-specific recognition by *Ca. rufifemur* (Menzel *et al.* 2013).

In the neotropical parabiotic ants, *Ca. femoratus* and *Cr. levior*, it has been shown that *Cr. levior* was able to discriminate between nestmate and non-nestmate *Ca. femoratus* workers, while *Camponotus* could not differentiate *Crematogaster* workers (Emery & Tsutsui 2013). However, these observations could not be confirmed in another experiment due to overall low interspecific aggression (Menzel *et al.* 2014). Interestingly, two different chemical morphs (chemotypes) have been reported in both mutualistic partners. In *Cr. levior* these chemotypes were called A and B (Emery & Tsutsui 2013; Menzel *et al.* 2014) and in *Ca. femoratus* they were named after the location where they were found first: Camp Patawa ('PAT') and Petit Saut ('PS') (Menzel *et al.* 2014). Note that in *Cr. levior* the names ('A' and 'B') were exchanged between the two publications describing them (Emery & Tsutsui 2013; Menzel *et al.* 2014) and in this thesis I will refer to the classification of Menzel *et al.* (2014). The chemotypes of *Ca. femoratus* and *Cr. levior* are clearly different in their CHC profiles containing mostly different CHC classes. These two studies already hypothesized that the chemotypes might be cryptic species, but it remains unclear why these different chemotypes have evolved in these ant species that show no obvious morphological or ecological differences. Furthermore, it is unclear which consequences this CHC divergence has for the behavior and ecology of the ants. Finally, it remains to be tested if the emergence of the different chemotypes in both genera could be due to coevolution followed by a cospeciation event.

This thesis

In this thesis, I investigate causes and consequences of the divergence of cuticular hydrocarbon profiles in the neotropical parabiosis between *Crematogaster levior* Longino, 2003 and *Camponotus femoratus* (Fabricius 1804). As implied by the title, this includes three major topics: the identification and quantification of CHC diversity in the neotropical parabiotic ants, the investigation of proximate and ultimate causes of this CHC variation and the behavioral and ecological consequences of the divergence of chemical profiles. By exploring these three topics in this complex mutualistic system, I try to answer five main questions that similarly tackle the interaction between the *Cr. levior* and *Ca. femoratus* as parabiotic partners, but also the coexistence of two different chemical varieties in each genus:

Box I.1: Neotropical ant gardens – a complex mutualism

Ant gardens (AGs) were first described as carton ant nests overgrown with specialized epiphytes found in the Brazilian rainforest in the early 20th century (Ule 1901, 1905). Ule (1901) already hypothesized that the ants collect seeds of these epiphytes and incorporate them in their carton nests to stabilize them. Although criticized by Wheeler (1921), subsequent studies found that ants indeed initiate the AGs and cultivate the epiphytes (Davidson 1988; Orivel & Dejean 1999). AGs are often pioneer formations and typically found in sunny locations at forest edges, next to rivers or in treefall gaps inside the forest (Yu 1994; Dejean *et al*. 2000). It is worth mentioning that AGs were also found in the paleotropical rainforest of South-East Asia (Kaufmann & Maschwitz 2006), but I will focus on neotropical AGs here. Due to the variety of involved ant and plant species and the various different interactions, they are considered one of the most complex ant-plant associations (Orivel & Leroy 2011).

AGs are mutualisms of specialized epiphytes with certain ant species that 1) disperse their seeds, 2) protect the plants from herbivory and 3) provide a nutrient-rich substrate for them (Blüthgen *et al*. 2001; Vantaux *et al*. 2007; Orivel & Leroy 2011). Carton nests built and moistened by ants are richer in nitrogen, phosphorus and potassium than usual epiphyte substrates (Blüthgen *et al*. 2001; Schmit-Neuerburg & Blüthgen 2007). The epiphytes, in exchange, stabilize the carton structures with their roots, protect the nest from soaking during heavy rains and potentially provide food resources in form of extrafloral and floral nectar, elaiosomes or fruit pulp (Davidson 1988; Yu 1994). The latter, however, does not seem to be of major impact as such resources are seasonal as the plants do not flower throughout the year and the ants mostly rely on hemipteran trophobionts (Orivel & Leroy 2011).

True AG ant species were defined by two criteria: 1) They are capable to build arboreal carton nests rich in humus and 2) they retrieve the plant seeds and incorporate them into their nests (Corbara, Dejean & Orivel 1999). The inhabitants most frequently found in neotropical AGs are *Pachychondyla goeldii* and the parabiotic ant species *Camponotus femoratus* and *Crematogaster levior* (Orivel & Dejean 1999; Dejean *et al*. 2000). The plant species by far most frequent in neotropical AGs are from four plant families of six genera: Bromeliaceae (*Aechmea* and *Streptocalyx*), Areaceae (*Anthurium* and *Philodendron*), Gesneriaceae (*Codonanthe*) and Piperaceae (*Peperomia*) (Davidson 1988; Orivel & Dejean 1999; Orivel & Leroy 2011). Although the ant species can be associated with either of these plant species, *P. goeldii* is preferably associated with *Aechmea mertensii* and *Anthurium gracile*, while the AGs inhabited by parabiotic ants mainly consisted of *Codonanthe calcarata*, *Peperomia macrostachya* and *Philodendron spp*. (Orivel & Dejean 1999) (Fig. I.1).

Initiation of AGs was only in detail described for *P. goeldii* that builds carton structures and transports seeds of *Ae. mertensii* and *An. gracile* to these nests (Corbara & Dejean 1996). Nevertheless, also *Ca. femoratus* is frequently found in very small initial AGs, often with *P. macrostachya* as a pioneer plant (pers. obs.). *Ca. femoratus* is highly attracted to seeds of *P. macrostachya* probably mediated by the seed odor (Youngsteadt *et al*. 2008). Interestingly, all seeds of AG epiphytes share one volatile compound: methyl-6-methylsalicylate (6-MMS), that has never been reported from other plants, but is found in the mandibular glands of male *Ca. femoratus* (Seidel, Epstein & Davidson 1990). However, only the species-specific volatile blends of the seeds seem to elicit retrieval by the ants (Davidson, Seidel & Epstein 1990; Youngsteadt *et al*. 2010). Also *Odontomachus mayi* and *Azteca trailii* were found to be attracted to epiphyte seeds and thus potentially are capable to induce AGs (Orivel, Dejean & Errard 1998; Marini-Filho 1999). Finally, many other ants such as *Azteca spp*. or *Crematogaster spp*. can secondarily inhabit abandoned AGs (Orivel & Leroy 2011, pers. obs.).

Figure I.1: Ant gardens inhabited by *Ca. femoratus* and *Cr. levior* in French Guiana. (A) Young ant garden with the plant *Peperomia macrostachya*. (B, D) AGs with *Codonanthe calcarata* as dominant plant species. (C) AG containing *C. calcarata*, but also the bromeliad *Aechmea mertensii*. (E) Fully grown AG with *C. calcarata*, *P. macrostachya* and *Philodendron sp*. All photos were taken by Philipp Sprenger.

(1) Is the differentiation into chemotypes linked to genetic differentiation, i.e. are the chemotypes of *Cr. levior* and *Ca. femoratus* separate (cryptic) species? Are there other phenotypic traits diverging between the chemotypes? And does coevolution between the two genera contribute to species divergence, i.e. is there cospeciation? (**Chapter 1**)

Causes

(2) How do mutualisms, i.e. parabiotic associations, influence CHC composition and the profile divergence and which other abiotic or biotic factors influence the CHC profile? (**Chapter 2** and more generally in **Chapter 6**)

(3) What are the molecular mechanisms behind the biosynthesis of such largely different CHC profiles? (**Chapter 3**)

Consequences

(4) Which consequences does the CHC divergence have for chemical communication within species and between the cryptic species? (**Chapter 4**)

(5) Are there costs (in form of competition) between the mutualistic partners and how can the ecologically very similar chemotypes (or cryptic species) coexist and avoid competitive exclusion? (**Chapter 5**)

Although CHC profiles are usually species-specific and are often used for taxonomic delimitation (Seppä *et al.* 2011; Kather & Martin 2012), there can also be considerable qualitative and quantitative variation *within* the same species (Wurdack *et al.* 2015). As mentioned before, the two chemotypes of *Cr. levior* and *Ca. femoratus* were described before this work, but this was either without detailed identification of the CHCs or based on individuals from a limited number of sites (Emery & Tsutsui 2013; Menzel *et al.* 2014). Although already hypothesized before this study, it was unclear if the chemotypes in fact are different cryptic species. In **Chapter 1** of this thesis, we aimed to elucidate the species status of the chemically different varieties found within *Cr. levior* and *Ca. femoratus*. Also, we investigated how common each variety is and where they are present geographically. Here, we show that the different chemotypes in either genus indeed are cryptic species by multiple lines of evidence. In addition, we infer their frequencies and geographic distribution along a climatic gradient, but also investigate if there are preferences for a certain chemotype of the parabiotic partner as indication for coevolution. Furthermore, we explore if other ecological factors like canopy cover or the presence of particular epiphytes might explain presence or absence of a species. In this context, we discuss how the differentiation of the CHC profiles between the cryptic species might have contributed to species divergence.

After showing that the chemotypes are indeed cryptic species, in **Chapter 2** we wanted to characterize their CHC profiles in detail but also report cases of two additional presumable species that were found in parabiotic association. Due to their multiple functions many abiotic and biotic factors jointly influence the evolution of CHC profiles (Menzel *et al.* 2017a). Parabiosis has been shown to coincide with chain elongations in CHCs and high proportions of rather unusual substance classes such as alkadienes and methyl-branched alkenes probably making the CHC profiles less perceivable (Menzel & Schmitt 2012; Menzel *et al.* 2017a). Here, we investigated if the parabiotic lifestyle can be an ultimate cause of adaptive CHC variation by comparing the CHC profiles of our study species to other parabiotic and

non-parabiotic *Crematogaster* and *Camponotus* ants. Furthermore, we elucidate if adaptations to the parabiotic lifestyle derived from a non-parabiotic ancestral state in *Crematogaster*. In addition, we look at the influences of the species identity of the mutualistic partner (biotic factor) and climatic conditions (abiotic factor) as sources of plastic variation. Finally, we discuss how physiological constraints could have led to different adaptations to a similar selection pressure, i.e. the parabiotic lifestyle.

To get more insights into the proximate mechanisms how such very different CHC profiles are produced by otherwise very similar species, we used RNA sequencing to investigate gene expression patterns in the oenocytes as the location of CHC biosynthesis in **Chapter 3**. Here, we searched for differentially expressed genes of five candidate gene families (fatty acid synthases, elongases, desaturases, fatty acyl-CoA reductases and cytochrome P450s) between the two cryptic species of *Cr. levior* and *Ca. femoratus*. To infer if they could be involved in the CHC biosynthesis, we compared them to orthologs in *Drosophila* that have a known function (Wicker-Thomas & Chertemps 2010; Qui *et al.* 2012; Dembeck *et al.* 2015). Furthermore, we compared differentially enriched biological processes and privately expressed pathways between the cryptic species. Here, we discuss how gene expression differences might translate into the differentiation seen in the CHC profiles.

In **Chapter 4**, we try to identify the behavioral consequences of CHC divergence in the parabiotic ants. Ants usually treat competitors from other species or foreign colonies aggressively, which largely contributes to their ecological success because it enables them to effectively defend their nests and monopolize food resources. Nestmates and non-nestmates are recognized via their CHC profiles (Lahav *et al.* 1999; van Zweden & d'Ettorre 2010). Here, we investigate how the CHC adaptations to parabiotic lifestyle affect intra- and interspecific recognition in the two cryptic species of *Cr. levior*. To do so, we performed reciprocal observations of aggressive behavior towards CHC extracts of both species and tried to infer which substance classes are used for recognition by manipulating nestmate CHC profiles with fractions of different structural CHC classes. We discuss differential discrimination abilities between the cryptic species and how their CHC profiles could elicit variable aggression responses.

In **Chapter 5**, we investigate potential costs of the mutualism through competition for food sources, and ecological consequences of the species divergence, i.e. if there is partitioning between the cryptic species in their trophic niche. To answer our questions, we used an integrative approach combining food choice observations in cafeteria experiments, analysis

of neutral lipid fatty acid profiles and analysis of stable isotope signatures (Rosumek *et al.* 2018) to evaluate if there are differences in the trophic niche on the two levels (i.e. among mutualistic partners and among the cryptic species). In this context, we discuss potential conflicts in the mutualistic association and how the cryptic species might coexist in sympatry while avoiding competitive exclusion.

Lastly, in **Chapter 6**, we review on which organizational levels (individuals, colonies and species) CHC profiles can differ in ants and which magnitude those differences have. As various different biotic and abiotic factors can cause variation in CHC profiles, we give an overview on the current knowledge on sources of fixed and plastic CHC variation and their adaptive value. In this chapter, we review extrinsic factors affecting CHC profiles such as climate, diet, microbiome or pathogens and parasites as well as intrinsic factors on different levels related to physiology and genetic heritability, i.e. genetic drift or adaptation. Finally, we emphasize the need for a predictive framework investigating defined traits of a CHC profile and raise questions that are of major importance for future research avenues.

Chapter 1

Cuticular hydrocarbons as potential mediators of cryptic species divergence in a mutualistic ant association

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Abstract

Upon advances in sequencing techniques, more and more morphologically identical organisms are identified as cryptic species. Often, mutualistic interactions are proposed as drivers of diversification. Species of the neotropical parabiotic ant association between *Crematogaster levior* and *Camponotus femoratus* are known for highly diverse cuticular hydrocarbon (CHC) profiles, which in insects serve as desiccation barrier but also as communication cues. In the present study, we investigated the association of the ants' CHC profiles with genotypes and morphological traits, and discovered cryptic species pairs in both genera. To assess putative niche differentiation between the cryptic species, we conducted an environmental association study that included various climate variables, canopy cover, and mutualistic plant species. Although mostly sympatric, the two *Camponotus* species seem to prefer different climate niches. However, in the two *Crematogaster* species, we could not detect any differences in niche preference. The strong differentiation in the CHC profiles may thus suggest either a possible role during speciation itself by inducing assortative mating, or by reinforcing sexual selection after the speciation event. We did not detect any further niche differences in the environmental parameters tested. Thus, it remains open how the cryptic species avoid competitive exclusion, with scope for further investigations.

Keywords:

speciation, population structure, niche differentiation, environmental association, sexual selection, integrative taxonomy

1.1 Introduction

Diversity on Earth is reflected in the ongoing discovery of a large number of species every year. Among animals, insects are especially species-rich and, out of an estimated 5 million species, only about 1 million have been described (Stork 2018). Finding new species can be challenging due to remote and undiscovered habitats or a high morphological similarity to closely related species. The latter, so-called cryptic species, are defined as distinct, but morphologically similar species (Bickford *et al.* 2007). They are often identified coincidentally based on genetic data, chemical profiles or behavior. The lack of morphological differentiation between cryptic species can be due to recent divergence and too little time for distinct morphological features to evolve (Grundt *et al.* 2006; Gustafson *et al.* 2014), or by selection on morphological stasis (Bickford *et al.* 2007; Struck *et al.* 2018). It has also been postulated that taxa, which communicate mating signals via non-visual cues (e.g. chemicals, vibrations, sounds) are more likely to harbor cryptic species, as morphological differentiation in these taxa is less important than e.g. in some birds, which use visual signals as mating displays (Andersson 1982; Hudson & Price 2014).

Given that cryptic species are morphologically alike and often closely related, one would expect them to be ecologically very similar and to exhibit only slight niche differentiation (Violle *et al.* 2011). Already very subtle ecological divergence in traits like thermal niche or food preferences, as well as spatio-temporal heterogeneity (e.g. different availability of resources) could allow such species to share the same habitat and avoid competitive exclusion (Gause 1932; Hardin 1960; Scriven *et al.* 2016). In ants for example, cryptic species can occur sympatrically, if they inhabit distinct niches, e.g. by specializing on different symbiotic fungi (Schultz *et al.* 2002). Next to the question how cryptic species coexist, it is also often unclear how species boundaries can be maintained between closely related species sharing the same habitat. One proposed mechanism is the expression of phenotypic traits that lead to assortative mating, and thus reduce gene flow (Dieckmann & Doebeli 1999). In this context, phenotypic traits might favor speciation even in sympatry if they are shaped by ecological selection pressures and at the same time induce assortative mating (so-called 'magic traits'), such as color patterns or smell (Servedio *et al.* 2011; Nosil 2012; Thibert-Plante & Gavrilets 2013).

Species interactions can promote and speed up the emergence of novel phenotypic traits, lead to coevolution and diversification (Hoeksema & Bruna 2000; Guimarães, Jordano & Thompson 2011; Thompson *et al.* 2013). For mutualisms, adaptive dynamics models predict

that if in a population of a mutualistic species certain groups of one species become more attractive and are thus chosen as partners more often, evolutionary branching should occur (i.e. the split into two distinct phenotypic clusters; Doebeli & Dieckmann 2000). This dimorphism in one mutualistic partner can lead to disruptive selection in the other partner and therefore to a cospeciation event (Doebeli & Dieckmann 2000). Although strict cospeciation seems rather rare (de Vienne *et al.* 2013), in mutualisms it was described repeatedly e.g. between arthropods and their endosymbionts (Degnan *et al.* 2004; Hosokawa *et al.* 2006; Bolaños *et al.* 2019), in specialized ant-plant mutualisms (Chomicki, Ward & Renner 2015), and fig-pollinating wasps and figs (Jousselin *et al.* 2008; Cruaud *et al.* 2012). Alternatively, species diversification in mutualisms can also be facilitated by partner switches like in pollination mutualisms (Janz, Nyblom & Nylin 2001; Kawakita *et al.* 2004) or ant-plant associations (Quek *et al.* 2004).

A remarkable example of mutualism are parabioses, which are defined as interactions between two different ant species sharing a nest with separate brood chambers (Orivel *et al.* 1997; Menzel *et al.* 2008b). Here, we investigate the neotropical ant species *Crematogaster levior* and *Camponotus femoratus* that live parabiotically in so-called ant gardens and both profit from abilities of their partners (Davidson 1988; Vantaux *et al.* 2007). Although the two species share a common nest and show interspecific tolerance, they keep their own species-specific cuticular hydrocarbon (CHC) profiles (Emery & Tsutsui 2013). Previous studies revealed two substantially different chemical phenotypes (or chemotypes) in both *Cr. levior* and *Ca. femoratus*, that otherwise were morphologically and ecologically indistinguishable (Emery & Tsutsui 2013; Menzel *et al.* 2014). CHCs cover the cuticle of basically all terrestrial arthropods. They are the main component of the waxy epicuticular layer, whose primary role is to prevent desiccation (Blomquist & Bagnères 2010). However, CHCs secondarily evolved several important roles in chemical communication like mediating recognition of mating partners (Thomas & Simmons 2008), and (in social insects) of nestmates and castes (van Zweden & d'Ettorre 2010). A CHC profile usually consists of structurally different groups of hydrocarbons, namely straight-chained *n*-alkanes, mono- or poly-methyl-branched alkanes and mono- or poly-unsaturated alkenes, in different combinations (Blomquist 2010a). As CHC profiles are usually species-specific, but similar even between distant populations (Martin, Helanterä & Drijfhout 2008a), high diversity is unusual within a single species.

In this study we elucidate the species status of the different chemotypes of both, *Cr. levior* and *Ca. femoratus*, by multiple lines of evidence within the framework of integrative taxonomy (Heethoff *et al.* 2011; Steiner *et al.* 2018). We compared cuticular hydrocarbons,

secondary metabolites, morphological traits and genotypes between different colonies, and find clear evidence for two cryptic species in each of the two genera. Next, we asked whether these cryptic species differ ecologically, and conducted an environmental association study including local climate, mutualistic partners, ant garden plants and canopy cover. Finally, we tested for partner preferences among the mutualistic species.

1.2 Materials and methods

1.2.1 Sampling

We collected parabiotic ants of the species *Crematogaster levior* and *Camponotus femoratus* along an east-west gradient in French Guiana from August to October 2016. The east-west transect in French Guiana coincides with a climatic gradient (i.e. higher precipitation and lower temperatures in the east of the country and vice versa). We only collected ants foraging outside the nests, thereby leaving the colonies intact. To make sure we sampled different colonies of these polydomous species, we only collected ants from ant gardens which were at least 20 meters apart from each other. In total, we collected 333 colonies from 13 different locations (Table 1.1). If we could not reach the garden itself, we looked for shared trails or extrafloral nectaries attended by both species. In some of these cases (n = 20), we were not able to obtain individuals of *Ca. femoratus*. For each colony collected, we took a GPS point using a Garmin eTrex H personal navigator (Garmin Europe Ltd., Southampton, UK), noted plant genera present on the ant gardens (*Philodendron, Aechmea, Codonanthe, Peperomia* and *Anthurium*) and took a vertical photo of the canopy with a Nikon Coolpix W100 (Nikon GmbH, Düsseldorf, Germany). Samples for genetic and morphological analyses were stored in 99% ethanol.

Table 1.1: Sampling sites with details on sampled and analyzed colonies. Numbers (#) of sampling sites refer to numbers on the map in Figure 1.1.

Site	*Code*	*#*	*Latitude*	*Longitude*	*Elevation (m)*	*Number of colonies*	*Genetically analyzed samples (Cr | Ca)*	*Chemically analyzed samples (Cr | Ca)*
Apatou	AP	1	5.200783	-54.312017	28	16	16 | 16	16 | 16
Saint-Laurent-du-Maroni	SL	2	5.463902	-53.997322	63	36	33 | 29	36 | 32
Angoulême	AN	3	5.409200	-53.650933	64	1	01 | 01	01 | 01
Sinnamary	SI	4	5.352035	-53.077604	45	20	20 | 20	19 | 20
Petit Saut	PS	5	5.061213	-52.988772	93	21	19 | 17	18 | 18
Paracou	PAR	6	5.265905	-52.933605	41	53	50 | 47	50 | 49
Les Nouragues	LN	7	4.039650	-52.673933	63	74	72 | 60	72 | 61
Kourou	KO	8	5.083106	-52.643022	23	12	12 | 10	11 | 11
Montsinéry-Tonnegrande	MT	9	4.866000	-52.538483	26	4	04 | 04	04 | 04
Cacao	CA	10	4.557416	-52.463067	71	22	21 | 19	21 | 20
Cayenne	CAY	11	4.793831	-52.317594	20	6	06 | 05	06 | 05
Régina	RE	12	4.181286	-52.131963	82	16	16 | 13	16 | 14
Camp Patawa	PAT	13	4.546067	-52.130483	282	52	52 | 48	52 | 51

1.2.2 Chemical analyses

To analyze the CHC profiles, we immersed 10 freeze-killed *Cr. levior* or 5 *Ca. femoratus* workers per colony for 10 minutes in hexane. In *Cr. levior*, the cuticle contained polar secondary metabolites next to CHCs. These two substance groups were separated by fractionation using SiOH columns (Chromabond, 1mL/100mg, Macherey-Nagel, Düren, Germany). CHC fractions were eluted with hexane; the polar compounds with dichloromethane. The samples of polar compounds were dried under a gentle nitrogen stream and re-dissolved in approximately 50 µl hexane for analysis.

Cuticular hydrocarbons were analyzed using gas chromatography-mass spectrometry (GC-MS). The gas chromatograph (7890A, Agilent Technologies, Santa Clara, CA, USA) was equipped with a Zebron Inferno ZB5-MS capillary column (length 30 m, Ø 0.25 mm, 0.25 µm coating, Phenomenex, Aschaffenburg, Germany) and Helium was used as carrier gas with a flow rate of 1.2 mL per minute. The mass spectrometer (5975C, Agilent Technologies) was used with electron ionization (EI) at 70 eV.

For the *Cr. levior* CHC extracts, 4 µl were injected into the GC at 40°C using a PTV (Programmed Temperature Vaporizing) method and this temperature was held constant for 2 minutes. Thereafter, the oven heated up with 60°C per minute to 200°C and above this temperature with 4°C per minute to 320°C which were kept for 10 minutes. The PTV method allows a higher injection volume, which was needed because of the presumably lower quantity of the much smaller *Crematogaster* ants. In *Ca. femoratus* 2 µl of extract was injected at 60°C using the splitless method. The oven heated up with 60°C per minute to 200°C and then with 4°C per minute to 320°C which again were kept constant for 10 minutes. The same temperature program as for *Camponotus* CHCs was used to analyze the polar compounds of *Cr. levior*. The resulting chromatograms were integrated manually using *MSD ChemStation* (E.02.02.1431, Agilent Technologies).

CHCs were identified using Kovats indices and diagnostic ions (Carlson, Bernier & Sutton 1998). We excluded all substances which were not hydrocarbons as well as substances which had proportions less than 0.1% on average or were present in less than 20% of the samples (of the respective chemotype). Because the number of double bonds sometimes differed between colonies, we still included substances with multiple double bonds even if they occurred in less than 20% of the samples if other alkenes of the same chain length were present in other samples.

The polar substances produced by *Cr. levior* were likewise analyzed via GC-MS as described above. They were aligned based on their mass spectra using a custom database. To investigate the molecular formula of the polar substances, highly concentrated samples of the *Cr. levior* A and B (100 individuals per sample) were analyzed using GC-EI-HRMS (= gas chromatography coupled with high resolution mass spectrometry). The setup we used allows the generation of accurate masses to establish molecular formulae of molecular and fragment ions at Δm <3.0 mmu. For GC-EI-HRMS we used an Agilent 6890 gas chromatograph equipped with an analytical column (30 m × 0.25 mm i.d., film thickness 0.25 µm; ZB-1MS, Phenomenex, Aschaffenburg, Germany), helium as carrier gas (1.0 mL/min; constant flow mode) and a temperature program of 100 °C (3 min)–10 °C/min–320 °C (10 min). Injection volume was 1 µL in splitless mode. The gas chromatograph (GC) was coupled directly to a JMS-T100GC time-of-flight (TOF) mass spectrometer (GCAccuTOF, JEOL, Tokyo, Japan) in electron ionization (EI) mode at 70 eV. The source and transfer line temperatures were set at 200 and 310 °C, respectively. The detector voltage was set at 2050 V. The acquisition mass range was set from m/z 41 to m/z 650 with a spectrum recording interval of 0.4 s. The system was tuned with perfluorokerosene to achieve a resolution of

6000 (full width at half maximum) at m/z 292.9824. *JEOL MassCenterTM* workstation software was used for data acquisition and data evaluation.

1.2.3 Statistical analyses – chemical data

In total we analyzed 322 different *Cr. levior* and 302 *Ca. femoratus* colonies. The colonies were assigned to the CHC chemotypes described previously (Menzel *et al.* 2014) based on NMDS ordinations (Appendix Fig. S1.1).

To check for major differences in the CHC composition, we pooled substances according to their substance class (*n*-alkanes, mono-, di- and tri-methyl alkanes, mono-unsaturated alkenes, alkadienes, alkatrienes and methyl-branched alkenes). We tested whether their abundances (dependent variables) differed between the two chemotypes of either genus (fixed factor) using PERMANOVAs (command *adonis*, R package *vegan* (Oksanen *et al.* 2019)). If a certain substance class was absent from several samples, we added minute normally distributed random numbers (mean: $10^{-8} \pm 10^{-8}$) to the respective class for all samples, as PERMANOVA cannot manage samples with zero distance. This was only the case for alkadienes and methyl-branched alkenes in *Crematogaster*.

To quantitate the separation of the chemotypes, we adapted the concept of haplotype networks to CHC profiles. As compositional data is continuous, we categorized the profiles based on a principal component analysis (PCA). This method has the advantage that one can quantitate the separation between CHC profiles and display information of multiple PC axes (i.e. more than two dimensions) at the same time, and provide a clear visualization of the degree of variation between and within groups. To this end, we firstly performed a PCA based on our CHC data after centered log-ratio (clr) transformation (Aitchison 1982; Brückner & Heethoff 2017). Subsequently, we assigned a number of possible categories to each PC axis based on their eigenvalues (i.e. the number of categories per PC axis equaled its eigenvalue divided by 5 to obtain a 'handable' number of axes and distances between samples), and was rounded to two if the eigenvalue was between 10 and 5. PC axes with eigenvalues < 5 were not considered. In our case most of the CHC variation was explained by the first PCs, which is why we only used the first three PCs for the network of *Crematogaster* (explained variance: 58.75%) and the first two PCs for the network of *Camponotus* (explained variance: 73.75%; all other PCs having eigenvalues < 5).

Then, the PC loadings for each sample were transformed into distinct categories by dividing the distance of a certain PC loading to the minimum by the whole range of the PC loadings

and rounding this value to integer numbers. As a result, we obtained a sequence of categories for each sample, with the length of the character sequence being the number of PC axes used. We used the R package *pegas* (Paradis 2010) with the *haplotype* command to calculate different clusters (chemical types) based on the character sequences. Subsequently, we calculated the (integer) Euclidean distances between samples for each PC axis and summed them up. Networks were then constructed using *haploNet* (package *pegas*).

To find out if *Cr. levior* populations can be differentiated by their polar metabolites, we visualized ordinations based on Bray-Curtis distance matrices. Additionally, we performed random Forest analyses using the *randomForest* package (Liaw & Wiener 2002) to check if we could assign the samples to the CHC chemotype based on their polar substances. All statistics were conducted using *R* version 3.5.0 (R Core Team 2018).

1.2.4 Morphological measurements

After classification based on the CHC profiles, we measured 30-40 individuals per cryptic species of both genera from independent colonies that were randomly distributed over the different sampling locations (total N = 160). As *Ca. femoratus* workers are dimorphic, we took only minors (the smaller caste) for our analyses. All measurements were taken blindly in a random order (per genus) using a Keyence VHX-2000 digital microscope (Keyence International (Belgium) NV/SA, Urdorf, Switzerland). Thirty specimens of *Crematogaster* and *Camponotus* were photographed and measured twice to assess reliability (= 1 - measurement error, see Bartlett and Frost 2008). In the further analysis, we took the mean of both measurements for those specimens. Variables with reliability < 85% were omitted from the analyses (Appendix Table S1.1; Fig. S1.2). For calculating reliability we used the Intraclass Correlation Coefficient with the function *ICCest* as provided by the R package *ICC* (see also Wolak, Fairbairn & Paulsen 2012).

We measured 23 characters for *Crematogaster* and 20 characters for *Camponotus* (based on Seifert 2008; Csösz *et al.* 2014; and additional criteria). For *Crematogaster*, all measurements were taken under 200-fold magnification, while for *Camponotus* three different magnifications were used due to their larger body size. We used 100-fold to measure the mesosoma, 150-fold for head, legs and antennae and 200-fold magnification for all other characters of *Camponotus*. Measurements were taken using *ImageJ* (version 1.50e, National Institutes of Health, USA) and the *straight* measure tool. We used an in-house ImageJ script to convert pixels into µm for each measurement.

We used multivariate ratio analysis (MRA) to analyze our body measurements. MRA comprises a set of tools for analyzing size and shape separately in a multivariate framework (see e.g., Baur & Leuenberger 2011; Baur *et al.* 2014; Gebiola *et al.* 2017 for a detailed description of the application). One of these tools is the shape PCA, which in contrast to a conventional PCA, allows to compare body shape irrespective of *isometric* body size. The effect of allometric variation (e.g., allometric scaling, see Baur & Leuenberger 2011; Klingenberg 2016) may then be explored by plotting the first two shape PCs against isometric size. First, we ran a shape PCA for each genus separately. Next, the PCA ratio spectrum, another method of the MRA toolkit, allowed the interpretation of individual shape PCs in terms of ratios. Finally, isometric size was calculated as the geometric mean of all measurements per individual. For calculating the shape PCA, isometric size and the PCA ratio spectra we used a slightly modified version of the R-script published by Baur *et al.* (2014). Plots were generated using *ggplot2* (Wickham 2016).

To statistically test for morphological separation of the cryptic species, we calculated MANOVAs with the first two shape PCs as dependent variables and the species identity as well as sampling location as fixed factors. We used the first two PC axes since they explained 48% and 56.6% of the variance in *Crematogaster* and *Camponotus*, respectively (the cryptic species did not differ in PC3). To compare the isometric size between each species within a genus, we calculated Welch two sample t-tests. Calculation of these statistics was done with the basic functions *manova* and *t.test* provided by *R*.

1.2.5 COI barcoding

To test for genetic separation, one individual of *Cr. levior* and *Ca. femoratus* of every sampled colony was barcoded at the mitochondrial COI locus. DNA was extracted following the HotSHOT protocol (see Montero-Pau, Gomez & Muñoz 2008). For DNA extraction, two legs of each individual of *Cr. levior* and one leg for *Ca. femoratus* respectively were used and DNA fragments of the COI locus (primers: LCO1490, HCO2198) were amplified using the following PCR cycling protocol: 5 minutes of denaturation at 95° C, followed by 35 cycles of 30 seconds of denaturation at 95° C, 60 seconds annealing at 48° C and 90 seconds extension at 72° C. This was followed by a final extension step at 72° C for 10 minutes. For detailed PCR and sequencing reaction mix see Appendix Table S1.2. Thermocycler conditions for the sequencing reaction were: 1 minute of denaturation at 95° C, followed by 30 cycles of 10 seconds denaturation at 96° C, 10 seconds of annealing at 50° C and 2 minutes extension at

60° C. This was followed by 10 minutes of final extension at 72° C. Resulting DNA fragments were sequenced on an ABI PRISM 3700 (Thermo Fisher Scientific, Waltham, MA, USA). Sequences were trimmed and aligned in *GENEIOUS* v. 10.1.3 using the *ClustalW* (Thompson, Higgins & Gibson 1994) plugin. All sequences were manually checked and curated if necessary. The final alignment had a length of 449 bases.

1.2.6 COI – parsimony networks, phylogeny and population genetic parameters

Haplotype networks were created for *Cr. levior* and *Ca. femoratus* using the TCS algorithm in *PopART* v. 1.7 (Leigh & Bryant 2015). In addition, Bayesian phylogenies were created using *MrBayes* v. 3.2 (Ronquist *et al.* 2012) upon identification of the best substitution model (HKY+G for *Crematogaster* and *Camponotus*) with *MEGA7* (Kumar *et al.* 2018). Phylogenetic analyses for both species ran for 13,500,000 generations for *Cr. levior* and 9,020,500 for *Ca. femoratus* respectively with a burn-in of 25%; trees were sampled every 500 generations. Resulting trees were visualized in *Archaeopteryx* v. 0.992 beta (Han & Zmasek 2009). Based on networks and phylogenies, *Cr. levior* and *Ca. femoratus* were both separated into two distinct clusters each corresponding to the previously identified chemotypes. Thus, for the following analyses, we treated them as four separate cryptic species and call them *Cr. levior* A and B, as well as *Ca. femoratus* PAT and PS.

To investigate allele frequency differences between the different sampling sites, pairwise F_{ST} values were calculated between all population pairs separately for each of the two cryptic species pairs of *Cr. levior* and *Ca. femoratus*, using Arlequin v. 3.5. (Excoffier & Lischer 2010). In addition, Tajima's D (Tajima 1989) was calculated as a measure for potential selection.

1.2.7 Nuclear markers for *Camponotus*

Based on the small number of SNPs that separate the two cryptic species of *Ca. femoratus* at the COI locus, we sequenced four additional nuclear loci to obtain more details on the genetic population structure. For *Cr. levior* we plan to use a PoolSeq approach in a future study to obtain this information on a genome wide basis. In the following, we sequenced one individual per colony from locations with at least three PAT and three PS colonies (max. 12 colonies). In total 14 unannotated Exon-primed intron-crossing (EPIC) primers (Appendix Table S1.3; Ströher, Li & Pie 2013) were tested. Four primer pairs (ant.1FR, ant.389FR, ant.1087FR, ant.1401FR) that amplified and showed variability were sequenced and further analyzed. The PCR master mix was the same as for COI barcoding, except for 0.1µl of each

primer instead of 0.2µl. Thermocycler conditions were: 5 minutes of denaturation at 95° C followed by 35 cycles of 1 minute of denaturation at 92° C for primer pair 1087 and 40 cycles for the remaining primer pairs respectively, 1 minute of annealing at 59° C and 2 minutes extension at 70° C. This was followed by 6 minutes of final extension at 72° C. For details on the sequencing reaction see above in the COI section. Forward and reverse sequences were assembled and manually curated. Alignment lengths differed between all loci (ant.1: 137 bp, ant.389: 239 bp, ant.1087: 379 bp, ant.1401 399 bp = 1154 bp in total), and so did the number of sequence polymorphisms (ant.1: 4 SNPs, ant.389: 4 SNPs, ant.1087: 5 SNPs, ant.1401: 5 SNPs = 18 SNPs in total).

1.2.8 *Camponotus* nuclear markers – parsimony networks and phylogeny

As for COI, we calculated the TCS networks with *PopART* (Leigh & Bryant 2015). We furthermore used *BEAST* v. 2.5 (Bouckaert *et al.* 2014) to calculate a phylogeny based on all four nuclear markers and the previously obtained COI sequences, comprising all individuals for which each locus was successfully sequenced (n = 93). Each locus was tested for the best substitution model in *MEGA7* (Kumar *et al.* 2018). *BEAUTi*, implemented within the *BEAST* package, was used to set up specifications for BEAST using *StarBEAST*2. Based on the Akaike's Information Criterion (AIC), we chose JC69 as best substitution model for nuclear marker ant.1FR and HKY for all others. For all markers a relaxed log normal clock model was used. Remaining parameters were set to default. *BEAST* was started with a chain length of 100,000,000, sampling trees every 1000 generations. The resulting trees were summarized in *TreeAnnotator* (included in *BEAST*) with a burn-in of 20% that was previously established in *TRACER* v. 1.6 (Rambaut *et al.* 2018). The resulting tree was visualized in *Archeaopteryx* v. 0.992beta (Han & Zmasek 2009). In addition, we used *STRUCTURE* 2.3.4 on the same dataset. The admixture model was used for calculations with a Burnin Period of 10,000 and a number of MCMC repetitions of 1,000,000 for a set number of two populations (k = 2).

1.2.9 Ecological and environmental association

Based on chemical and genetic information we could unambiguously assign each colony to *Cr. levior* A or B, or *Ca. femoratus* PAT or PS. First, we tested for non-random associations between the two cryptic *Crematogaster* and *Camponotus* species using a χ^2-test.

Second, we obtained climate data from CHELSA bioclim variables (Karger *et al.* 2017), consisting of composed climate data for the years 1979-2013 for the GPS location of every

sampled colony. We performed a PCA with all 19 climate variables to reduce the number of variables. Most variance was explained by the first PC axis (76.47%), and was characterized by an inverse relationship of precipitation and temperature variables (i.e. higher precipitation correlates with colder temperatures). A high factor loading coincided with high annual precipitation (mean: 3137.08 mm; minimum: 1979 mm; maximum: 4873 mm) and a low annual mean temperature (mean: 25.6°C; minimum: 24.4°C; maximum: 26.3°C).

Third, the presence/absence of plant genera on the ant nest was coded as a binomial variable (1 = present, 0 = absent). Canopy cover was estimated in *ImageJ*: All pictures taken from the canopy above each ant nest were converted to black-and-white using the *Make binary* command; covered areas were measured using the *Histogram* function. The obtained data was transformed to relative proportions.

For each colony, we created binomial variables of the species for *Crematogaster* (A vs. B) and *Camponotus* (PAT vs. PS). These were used as dependent variables in two binomial generalized linear mixed models with logit link function. As explanatory variables, we used the loading of PC1 from the climate PCA described above, the percentage of canopy covered, the identity of the parabiotic partner and a binomial variable for the presence of each plant genus on the ant gardens. We allowed interactions for each of these variables with the climate PC1, because canopy cover or species distributions might be influenced by the climate. Both models were reduced in a stepwise manner until the AIC was lowest.

1.2.10 Statistical analyses – comparing data sets

To analyze associations between chemical profiles, genetic distance and geographical distance, we performed Mantel tests based on Pearson correlation with 9999 permutations. As measure for chemical distance (CHCs and polar substances separately), we used Bray-Curtis dissimilarities (command *vegdist*, package *vegan*, Oksanen *et al.* 2019). For each of the two haplotype pairs, Tamura-Nei (Tamura & Nei 1993) pairwise genetic distances were calculated with MEGA7 based on the COI sequences. Geographical distances were measured as Euclidean distances between the GPS coordinates. All tests were done using R v. 3.5.0.

1.3 Results

1.3.1 CHC differences between cryptic species

As described earlier (Emery & Tsutsui 2013; Menzel *et al.* 2014), we found two clearly distinct chemotypes in both *Crematogaster levior* and *Camponotus femoratus*.

For *Crematogaster* (Fig. 1.1 C), the chemical networks yielded two large clusters in *Cr. levior* A (cluster XII and VII) and one large cluster in *Cr. levior* B (cluster V). The profiles of *Cr. levior* A seemed more variable as we found 13 different chemical types (with two singletons), compared to only 8 in *Cr. levior* B (with one singleton). *Crematogaster* A and B were clearly separated in the network. However, there was one exception, with the colony forming the singleton type XVIII showing characteristics of both chemotypes. In the network it was closer connected to *Cr. levior* A, but clearly clustered with chemotype B in an NMDS ordination (Appendix Fig. S1.1). This colony had the same COI-haplotype as other B colonies.

The profile of *Cr. levior* A (n = 174) was dominated by several alkadienes of odd chain length ranging from C29 to C41 (total abundance: 27.44 ± 6.24%; Appendix Fig. S1.3 A). In contrast, the main peak in *Cr. levior* B (n = 148) was a mixture of 13- and 15-methyl nonacosane (17.91 ± 7.73 %; Appendix Fig. S1.3 B). The CHCs of both cryptic *Crematogaster* species were vastly different with substances most common in A (substances >5% abundance: 30.24 ± 10.50%) being rare in B (6.36 ± 1.99%) and vice versa for substances most common in B (in B: 40.23 ± 9.51%; in A: 8.62 ± 3.37%). In comparison, the profile of *Cr. levior* A had more alkadienes (PERMANOVA: pseudo-F_1 = 137.98, p = 0.001), alkenes (pseudo-F_1 = 73.09, p = 0.001), di-methyl alkanes (pseudo-F_1 = 57.33, p = 0.001) and methyl-branched alkenes (pseudo-F_1 = 155.24, p = 0.001; Fig. 1.2 A), while *Cr. levior* B had much higher proportions of mono-methyl alkanes (pseudo-F_1 = 637.39, p = 0.001) and *n*-alkanes (pseudo-F_1 = 191.56, p = 0.001; Fig. 1.2 B).

The two cryptic *Ca. femoratus* species were obviously distinct without any exceptions. *Ca. femoratus* PAT colonies were mostly assigned to a single cluster (cluster I) and few colonies to a second one (cluster II). In comparison, PS colonies were distributed among three chemical types (cluster III, IV and V; Fig. 1.1 D).In *Ca. femoratus* PAT (n = 195) the CHC profile was dominated by 13,23-dimethyl heptatriacontane (22.47 ± 10.21%), as well as several different C41 alkadienes (13.24 ± 4.42% and 11.61 ± 3.42% for the two most abundant ones; Appendix Fig. S1.3 C). In *Ca. femoratus* PS (n = 107), the most abundant substance was a 13-methyl

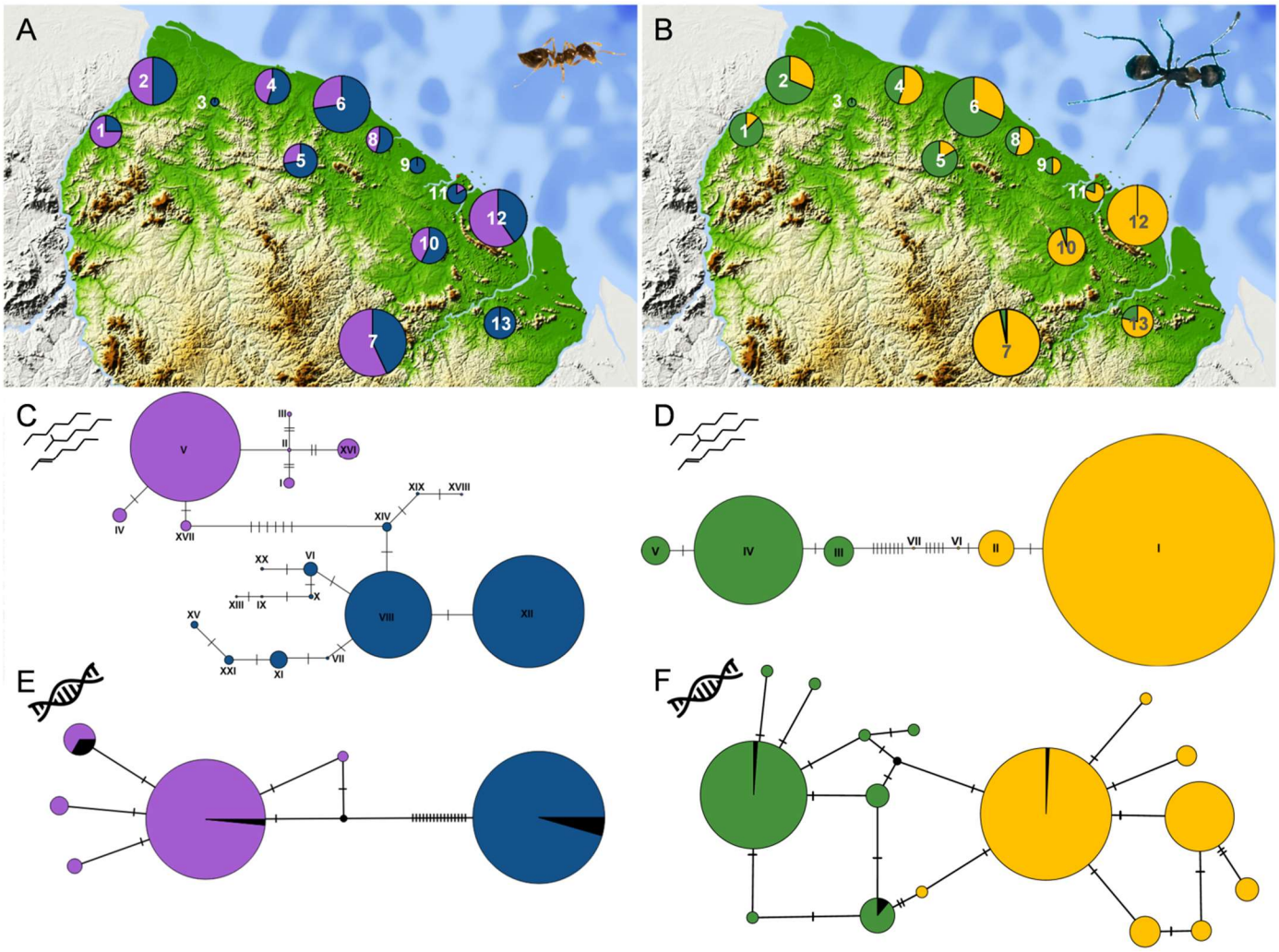

Figure 1.1: Chemotype and haplotype distribution across French Guiana and their differentiation. (A) Distribution of the cryptic *Cr. levior* species (*Cr levior A*: blue; *Cr. levior* B: purple). The size of the circles reflects the number of sampled colonies. (B) Distribution of cryptic *Ca. femoratus* species (*Ca femoratus* PAT: yellow; *Ca. femoratus* PS: green). Numbers in (A) and (B) refer to sampling locations in Table 1. (C) and (D) Chemical networks of *Cr. levior* and *Ca. femoratus*, using the same color code. (E) and (F) Haplotype networks (based on COI) using the same color code. Black coloration represents colonies without CHC information. Circles represent chemical types or haplotypes, respectively, and hatch marks indicate the number of character changes between them. Circle sizes reflect the number of colonies per chemical type or haplotype with singletons depicted slightly larger than according to their proportion. Pictures of *Cr. levior* (A) and *Ca. femoratus* (B) (© B. Feldmeyer).

heptatriacontene (13.49 ± 4.01%) followed by 13- and 15-methyl tritriacontane (9.60 ± 2.75%; Appendix Fig. S1.3 D). The profiles of the cryptic *Camponotus* species differed strongly with the most common CHCs of *Ca. femoratus* PAT (substances >5% abundance: 62.75 ± 7.24%) being less common in PS (9.67 ± 2.60%) and the other way around although less pronounced (in PS: 50.55 ± 8.23%; in PAT: 19.19 ± 5.19%). The PAT colonies had higher proportions of dimethyl alkanes (PERMANOVA: pseudo-F_1 = 629.70, p = 0.001), alkadienes (pseudo-F_1 =

202.82, p = 0.001) and *n*-alkanes (pseudo-F_1 = 16.87, p = 0.001, Fig. 1.2 C), while the PS ones had more mono-methyl alkanes (pseudo-F_1 = 1205.50, p = 0.001), methyl-branched alkenes (pseudo-F_1 = 1013.00, p = 0.001) and alkenes (pseudo-F_1 = 105.53, p = 0.001; Fig 1.2 D).

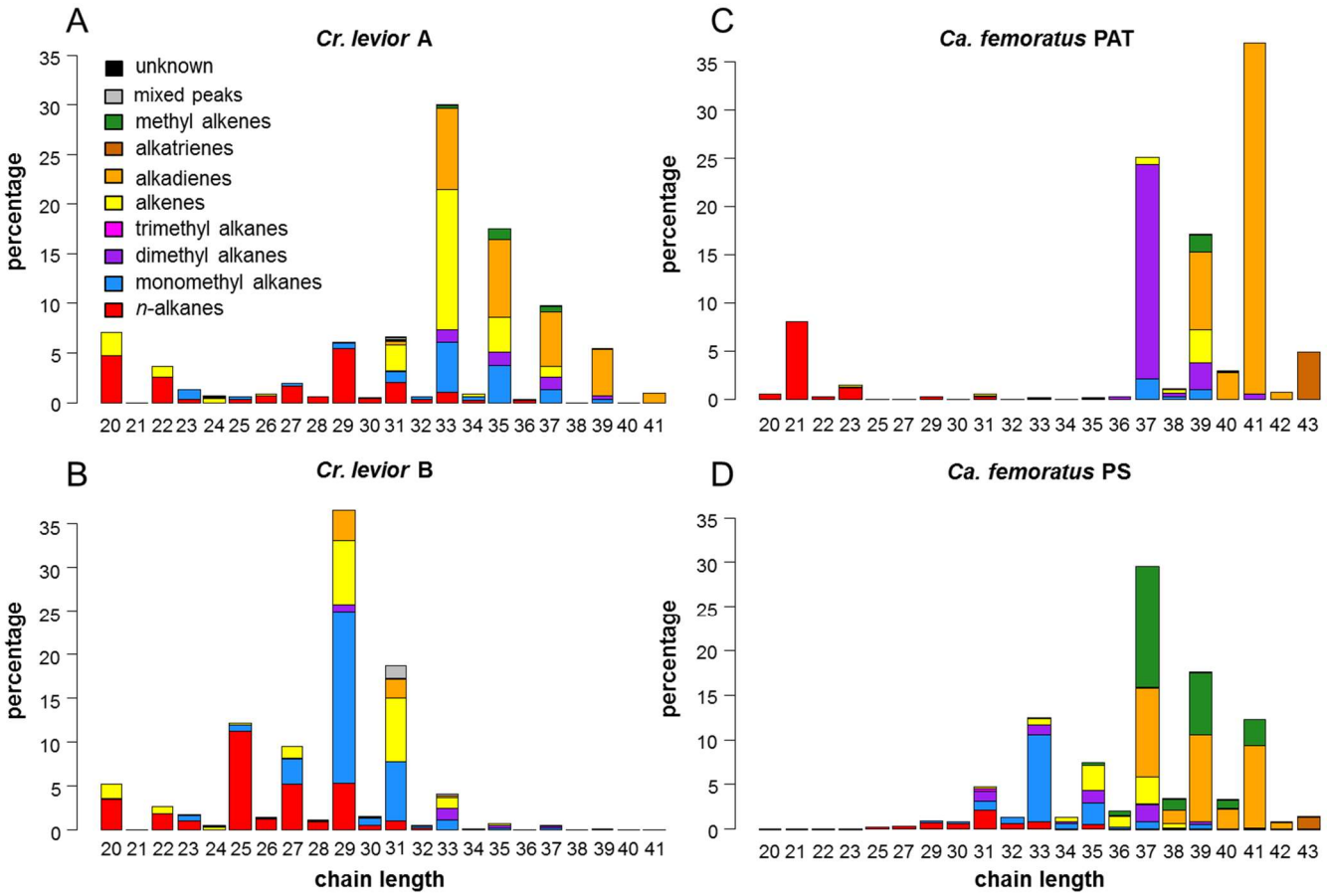

Figure 1.2: Differences between CHC profiles of the cryptic *Crematogaster levior* and *Camponotus femoratus* species. Plots show the mean proportion of different substance classes per chain length for all colonies of the respective species.

1.3.2 Differentiation by polar metabolites

In 254 out of 322 *Cr. levior* colonies, we found a total of 60 different polar compounds on the cuticle. In the remaining extracts, polar substances were either not detected or had too low concentrations for reliable quantification. Similar to the CHCs, the colonies could be differentiated into two different clusters (Fig. 1.3; Appendix Fig. S1.4 A, D). CHC chemotypes could be correctly identified based on polar chemistry using a random forest

algorithm which had a 1.18% OOB estimate of error rate. All 138 samples from A and 113 of 116 samples of B (error rate of 0.026%) were classified correctly.

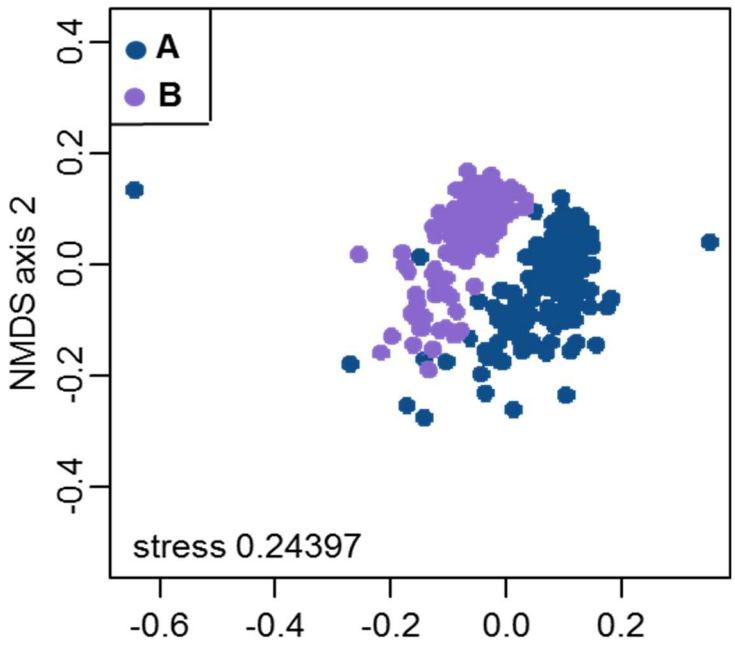

Figure 1.3: Differences in polar secondary metabolites of *Cr. levior*. NMDS ordination of the polar secondary metabolites produced by *Cr. levior*. Each dot represents the polar compound profile of one colony of *Cr. levior*.

The most common substances in *Cr. levior* A had abundances of 8.55 ± 8.12% (Retention time 24.10, Appendix Fig. S1.4 B), 8.74 ± 6.70% (RT 24.62) and 19.19 ± 10.32% (RT 26.24; Appendix Fig. S1.4 C), respectively, but lower abundances in B (3.45 ± 3.11%; 0.86 ± 1.07%; 3.82 ± 2.58%). In *Cr. levior* B, most abundant substances had proportions of 17.26 ± 10.81% (RT 20.20, Appendix Fig. S1.4 E), 13.55 ± 4.40% (RT 20.30, Appendix Fig. S1.4 F) and 5.45 ± 6.35% (RT 20.90), which were only 1.14 ± 1.13%, 3.35 ± 1.58% and 0.44 ± 0.67% (respectively) in A (all retention times given refer to the Zebron Inferno ZB5-MS capillary column). Using HR-MS the sum formulae of the major polar substances were derived as $C_{24}H_{36}O_4$ (polar substance at retention time 20.20, Appendix Fig. S1.4 E), $C_{24}H_{38}O_4$ (RT 20.30, Appendix Fig. S1.4 F), $C_{24}H_{36}O_4$ (RT 20.90), $C_{26}H_{38}O_4$ (RT 24.10, Appendix Fig. S1.4 B), $C_{26}H_{40}O_4$ (RT 24.62) and $C_{28}H_{44}O_4$ (RT 26.24, Appendix Fig. S1.4 C). The results showed a series of closely related compounds characterized by C24 to C28 carbon atoms containing four oxygen atoms, differing in the number of double bonds or rings from 6 to 8. In most cases there was a pair of compounds showing the same number of carbons only differing in the number of double bonds/rings. This pair-wise difference is also reflected in two series of fragment ions of m/z 237, 224, 209 and m/z 235, 222, 207 respectively, indicating an additional double bond isomer. However, to gain more insight into the underlying structures, higher quantities at higher purities are needed for NMR analysis.

1.3.3 Morphology

In shape, the two cryptic species of *Cr. levior* were largely overlapping. Nevertheless, the shape significantly differed between them (MANOVA based on shape-PCA: $F_1 = 18.07$, $p < 0.001$) but not between sampling locations ($F_{11} = 0.79$, $p = 0.73$). *Cr. levior* A and B differed in shape PC1 ($F_1 = 30.37$, $p < 0.001$; Fig. 1.4 A) but only insignificantly in shape PC2 ($F_1 = 3.18$, $p = 0.079$; Fig. 1.4 B). Shape PC1 was best described by the ratio between spine length and eye width (Fig. 1.4 A), while shape PC2 was largely explained by the maximal distance between the spines (Fig. 1.4 B). Moreover, *Cr. levior* B was larger than A (Welch t-test: $t_{74.94} = -3.61$, $p < 0.001$; Fig. 1.4 A, B).

The morphological traits of *Ca. femoratus* largely overlapped between cryptic species as well, despite significant differences (MANOVA: $F_1 = 16.67$, $p < 0.001$). Again, we found no effect of sampling location ($F_{11} = 0.43$, $p = 0.16$). While we detected differences in body shape (shape PC1: $F_1 = 17.08$, $p = 0.001$, Fig. 1.4 C; shape PC2: $F_1 = 10.04$, $p = 0.003$, Fig. 1.4 D), the cryptic species did not differ in isometric size ($t_{57} = -0.41$, $p = 0.68$; Fig. 1.4 C, D). While the first shape PC was characterized by multiple traits on different body parts (Fig. 1.4 C), shape PC2 was mainly explained by the ratio between petiole length to petiole width (Fig. 1.4 D).

1.3.4 Genotyping results and population structure

1.3.4.1 COI – parsimony networks and phylogeny

The TCS networks of the COI sequences show two distinct genotype clusters for both *Cr. levior* (Fig. 1.1 E) and *Ca. femoratus* (Fig. 1.1 F) with a 1:1 association of genotype to chemotype. The *Cr. levior* group that corresponds to A consisted of a single haplotype only. *Cr. levior* B showed more genetic variation with five haplotypes. The separation between both species was based on 16 SNPs (single nucleotide polymorphisms), indicating divergent clades. In *Ca. femoratus*, the resulting haplotype networks were more diverse. Both *Ca. femoratus* PS and PAT consisted of eight distinct groups. Here, the cryptic species were separated by two SNPs.

The phylogenies showed a similar pattern. In *Cr. levior* the separation between cryptic species was strongly supported with a posterior probability of 1 (Appendix Fig. S1.5). In *Ca. femoratus* the separation was not as clear, based solely on COI with a posterior probability of 0.61 and two subgroups per cryptic species (Appendix Fig. S1.6).

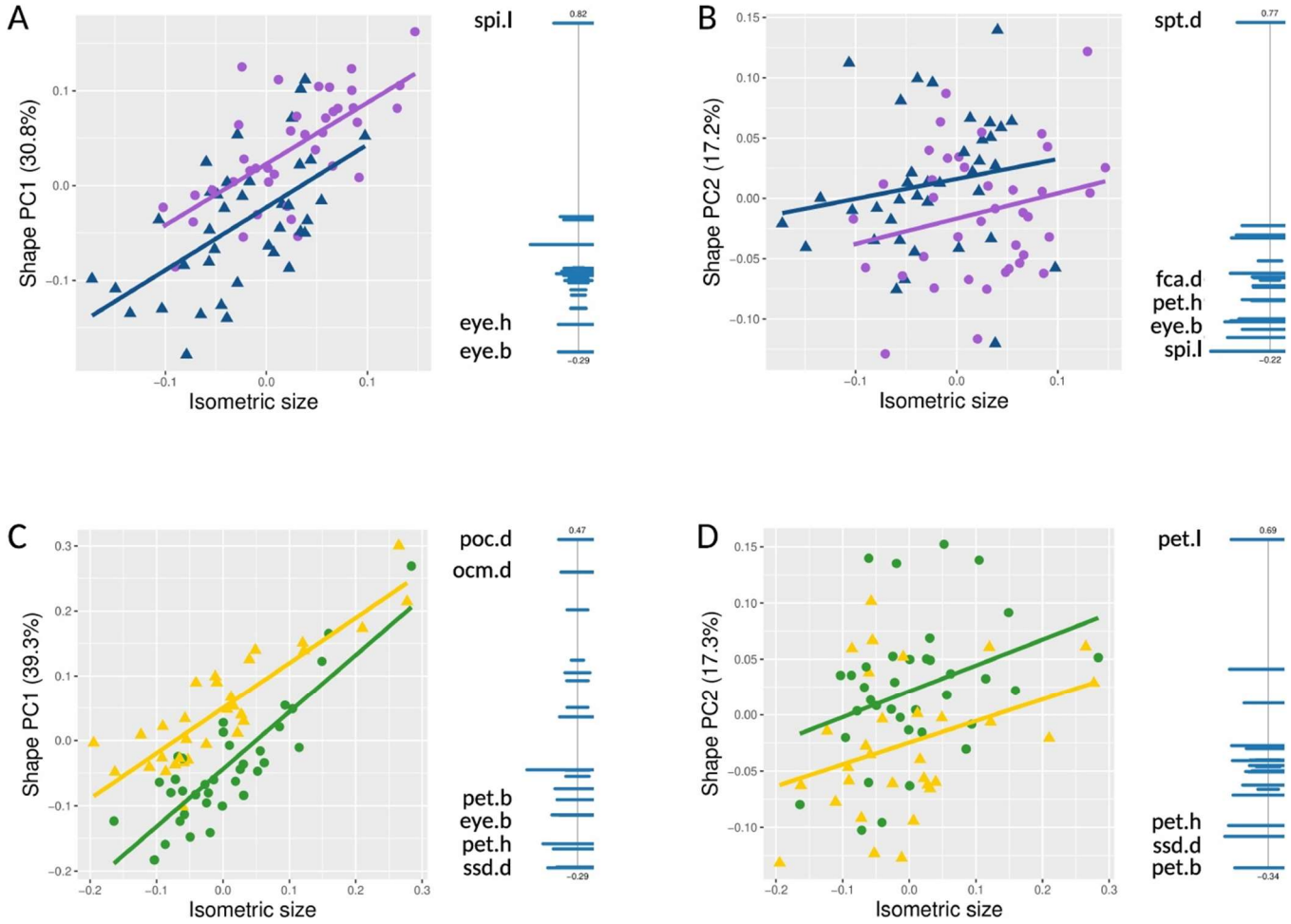

Figure 1.4: Morphological differentiation of the cryptic species of both ant genera. (A-D) Scatter plots depicting morphological differences of *Cr. levior* (A, B) and *Ca. femoratus* (C, D) and PCA ratio spectra. We plotted the first and second axis of a shape PCA (A, C and B, D, respectively) against isometric size. Each dot represents one individual of independent colonies. Symbols and colors correspond to cryptic species as follows: *Cr. levior*: blue triangle = A, purple dot = B; *Ca. femoratus*: yellow triangle = PAT, green dot = PS. To the right of the scatterplots, the ratio spectrum of the shape PC is shown. Up to four of the most relevant variables for calculating body ratios are indicated the ends of the spectra using the variable codes (see Appendix Table S1.1). Bars indicate the 68% confidence intervals based on 1000 bootstrap replicates (bars trimmed on right hand side due to the arrangement of figures).

1.3.4.2 COI – population genetic structure

As measure for population differentiation, we calculated pairwise F_{ST} values separately for all four cryptic species, between all sampled sites. In *Cr. levior* A, results are not shown due to a lack of population differentiation (F_{ST} = 0 in all population comparisons). For *Cr. levior* B (Table 1.2), only few populations were genetically different with significant differentiation found between Kourou & Les Nouragues (F_{ST} = 0.308, p = 0.036), Kourou & Saint-Laurent

(F_{ST} = 0.531, p = 0.045) and Saint-Laurent & Les Nouragues (F_{ST} = 0.127, p = 0.045). In *Ca. femoratus* we found greater differentiation between populations compared to *Cr. levior*, with six occurrences of fixed differences (F_{ST} = 1). In 42% of all pairwise comparisons, populations were significantly different in PS (Table 1.3), and 29% of all comparisons in PAT yielded significant differences (Table 1.4). We furthermore tested for potential selection using Tajima's D statistic (Appendix Table S1.4). Results for *Cr. levior* A are again not shown due to a lack of genetic differences. In *Cr. levior* B, Tajima's D was not significant in any population. In *Ca. femoratus* PS, Tajima's D was significantly smaller than zero in the Saint-Laurent population (TD = -1.513, p = 0.033) only. In *Ca. femoratus* PAT, Tajima's D was significantly smaller than zero in the populations of Paracou (TD = -2.072, p = 0.003), Les Nouragues (TD = -2.107, p = 0.002) and Saint-Laurent (TD = -1.486, p = 0.04).

Table 1.2: Population pairwise F_{ST} between 10 populations of *Cr. levior* B, based on the COI locus. Bold characters indicate statistical significance (p < 0.05) based on a permutation test.

	AP	*PAR*	*PS*	*LN*	*PAT*	*CAY*	*CA*	*KO*	*SI*	*SL*
AP	-									
PAR	-0.006	-								
PS	0.000	-0.130	-							
LN	0.108	0.112	0.010	-						
PAT	-0.012	-0.006	-0.117	0.070	-					
CAY	0.000	-0.096	0.000	0.037	-0.085	-				
CA	0.000	-0.031	0.000	0.088	-0.030	0.000	-			
KO	0.462	0.203	0.195	**0.308**	-0.009	0.250	0.392	-		
SI	0.034	0.007	-0.116	0.104	-0.025	-0.078	0.000	0.253	-	
SL	0.000	0.017	0.000	**0.127**	0.003	0.000	0.000	**0.532**	0.068	-

Table 1.3: Population pairwise F_{ST} between 9 populations of *Ca. femoratus* PS, based on the COI locus. Bold characters indicate statistical significance ($p < 0.05$) based on a permutation test.

	AP	*PAR*	*PS*	*LN*	*RE*	*MT*	*KO*	*SI*	*SL*
AP	-								
PAR	-0.037	-							
PS	0.156	**0.092**	-						
LN	**1.000**	0.778	**0.796**	-					
RE	**1.000**	**0.787**	**0.811**	0.000	-				
MT	1.000	**0.778**	**0.796**	0.000	0.000	-			
KO	0.189	0.001	-0.135	0.817	**0.847**	0.817	-		
SI	0.000	-0.054	0.120	**1.000**	**1.000**	**1.000**	0.126	-	
SL	0.014	0.009	0.016	**0.892**	**0.897**	**0.892**	-0.079	-0.008	-

Table 1.4: Population pairwise F_{ST} between 12 populations of *Ca. femoratus* PAT, based on the COI locus. Bold characters indicate statistical significance ($p < 0.05$) based on a permutation test.

	AP	*PAR*	*PS*	*LN*	*RE*	*PAT*	*CAY*	*MT*	*CA*	*KO*	*SI*	*SL*
AP	-											
PAR	**0.716**	-										
PS	0.500	-0.084	-									
LN	-0.153	**0.763**	**0.637**	-								
RE	0.248	**0.205**	-0.200	**0.437**	-							
PAT	-0.032	**0.545**	**0.273**	**0.065**	**0.130**	-						
CAY	0.250	0.173	-0.333	**0.451**	-0.209	**0.076**	-					
MT	0.000	0.694	0.368	-0.277	0.164	-0.133	0.111	-				
CA	-0.034	**0.521**	0.202	**0.080**	0.074	-0.034	0.010	-0.144	-			
KO	0.000	**0.732**	0.579	-0.099	0.296	0.013	0.333	0.000	0.017	-		
SI	0.516	0.001	-0.273	**0.648**	-0.020	**0.362**	-0.108	0.464	0.307	**0.551**	-	
SL	-0.167	**0.618**	0.325	-0.051	0.141	-0.063	0.101	-0.313	-0.083	-0.098	**0.400**	-

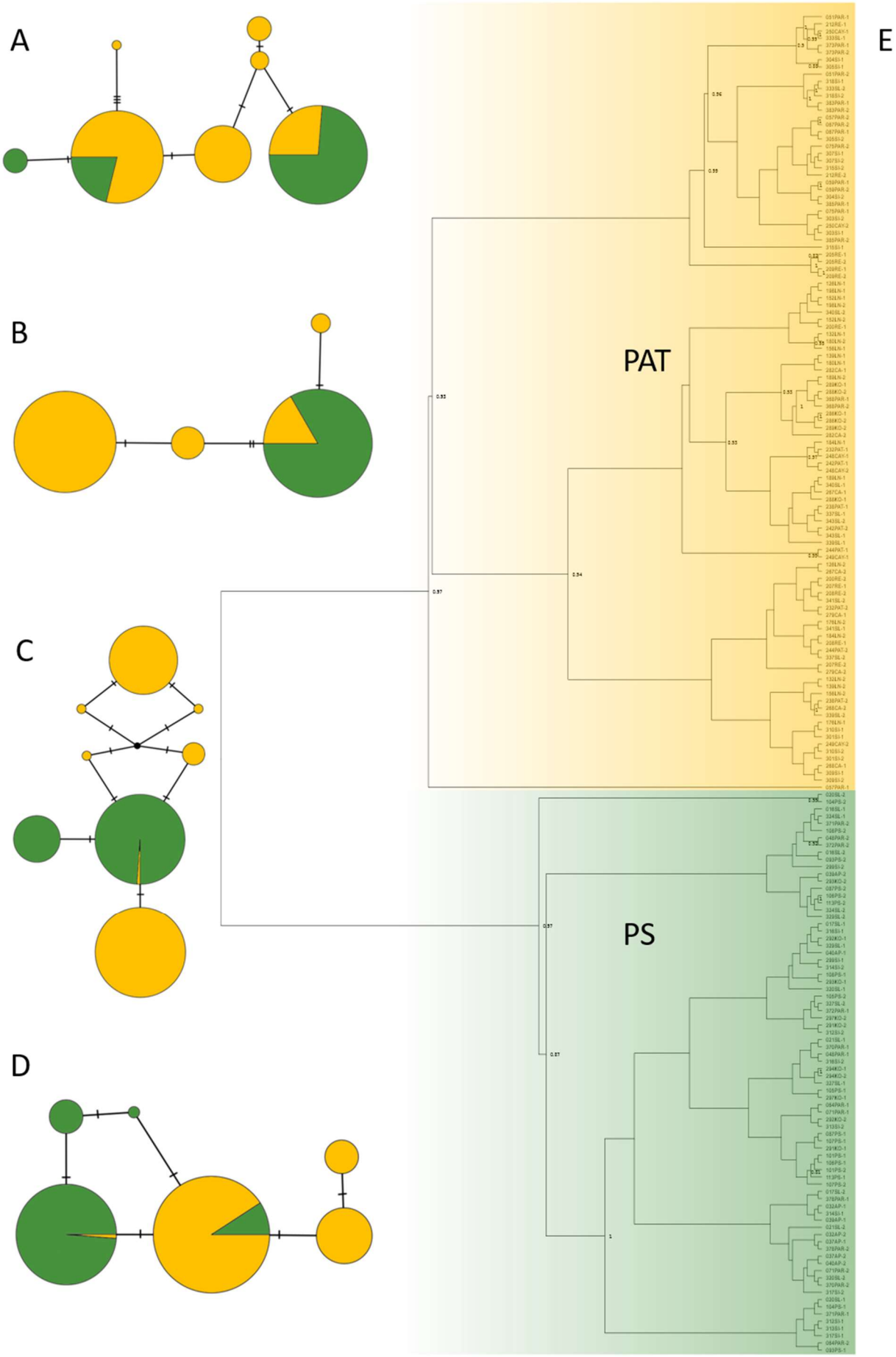
A
B
C
D
E
PAT
PS

Figure 1.5: Genetic differentiation of cryptic *Ca. femoratus* species. (A-D) TCS Haplotype networks of four nuclear markers of *Ca. femoratus*. (A) ant.1401FR, (B) ant.1FR, (C) ant.1087FR, (D) ant.389FR. Green color indicates *Ca. femoratus* PS and yellow colour indicates *Ca. femoratus* PAT respectively. Haplotypes are shown as circles, with size depending on the number of included colonies. Number of SNPs (single nucleotide polymorphisms) between the haplotypes are shown as hatch marks. (E) Phylogenetic tree based on all four nuclear markers and mitochondrial COI for *Ca. femoratus*. Posterior probabilities >0.8 are displayed. Yellow color corresponds to chemotype *Ca. femoratus* PAT, green indicates *Ca. femoratus* PS. Only individuals with all five loci sequenced were included (N = 93).

1.3.4.3 Camponotus *nuclear markers – parsimony networks and phylogeny*

As for COI sequences, we constructed TCS parsimony networks based on four additional nuclear markers (Fig. 1.5 A-D) (we sequenced additional nuclear loci for *Camponotus* only, since a population genomic study is on the way for *Crematogaster*). In contrast to the network based on COI mitochondrial sequences, the networks of nuclear markers showed less clear separation of cryptic species (Fig. 1.5 A-D). In contrast, a phylogenetic tree based on all five sequenced markers (Fig. 1.5 E) clearly separated *Ca. femoratus* PAT and PS into two clades. Also, the *STRUCTURE* analysis showed that all individuals could be assigned to one of the two chemotypes (Appendix Fig S1.7).

1.3.5 Partner preference and environmental association of cryptic species

There was no indication for a preferred association between either cryptic *Cr. levior* and *Ca. femoratus* species (Pearson's χ^2-test: $\chi^2_1 = 1.76$, $p = 0.18$). *Cr. levior* A nested with *Ca. femoratus* PAT in 100 and with PS in 65 cases, while *Cr. levior* B cohabited 96 times with *Camponotus* PAT and 44 times with PS.

The distribution of cryptic *Crematogaster* species was independent of PC1, i.e. precipitation and temperature (binomial GLM: $N = 292$, $\chi^2_1 = 1.12$, $p = 0.29$), indicating sympatric occurrence of the cryptic species which is also visible when looking at their distribution across the complete sampling range (Fig. 1.1 A). Neither canopy cover, nor the presence of any plant influenced the probability of species membership (A vs. B) in *Crematogaster* (all $p > 0.2$). However, species identity was influenced by an interaction of climate and *Camponotus* partner ($\chi^2_1 = 5.97$, $p = 0.015$). *Ca. femoratus* PS was less common in areas with high annual precipitation and lower annual mean temperature (i.e. the eastern part of French Guiana),

while *Ca. femoratus* PAT was present across the whole sampling area (binomial GLM: N = 279, climate PC1: χ^2_1 = 111.91, p < 0.001; Fig. 1.1 B). None of the other factors tested influenced the probability of the species' presence (all p > 0.15). However, there was a weak interaction between climate PC1 and *Crematogaster* partner (χ^2_1 = 5.06, p = 0.025) indicating slightly differing partner availability depending on climate.

1.3.6 Connecting chemical profiles, genetic background and geographic distance

The CHC distances in *Cr. levior* A slightly increased with geographic distance (Mantel test: r = 0.066, p = 0.011). However, this was not true for *Cr. levior* B (r = 0.044, p = 0.084). In *Camponotus*, CHC distances increased with geographical distances for PS (r = 0.182, p < 0.001), but not for PAT (r = 0.040, p = 0.15). The Bray-Curtis dissimilarities of CHCs and polar compounds of *Crematogaster* were highly correlated (N = 253, r = 0.42, p < 0.001), further indicating that the polar differentiation exactly matches the CHC differentiation. However, within each cryptic species, CHC distance and distance in polar compounds were not correlated (*Cr. levior* A: r = 0.04, p = 0.15; *Cr. levior* B: r = 0.02, p = 0.34).

Mantel tests between pairwise Tamura-Nei distances and geographic distances revealed no isolation-by-distance pattern for *C. levior* B (r = -0.065, p = 0.968), but for *Ca. femoratus* PS (r = 0.38, p < 0.001) and - albeit only weakly - *Ca. femoratus* PAT (r = 0.09, p = 0.038). *Cr. levior* A consisted of only one haplotype without any variation at the COI locus, which is why this analysis was not possible here.

In *Cr. levior* B, colonies that were genetically more distant also had more dissimilar CHC profiles (r = 0.15, p = 0.021). However, such an association was neither detectable within *Ca. femoratus* PAT (r = 0.05, p = 0.15) nor within *Ca. femoratus* PS (r = 0.03, p = 0.29).

1.4 Discussion

This study investigated the parabiotic ant species *Cr. levior* and *Ca. femoratus* whose shared nests (so called ant-gardens) are abundant in the neotropics (Davidson 1988). Both previously identified species occur in two distinct CHC chemotypes, which are morphologically highly similar. We show that within *Cr. levior* and within *Ca. femoratus,* these chemotypes form two distinct units that can be classified as cryptic species. This is

supported by multiple lines of evidence, all of which show conclusive results. First, the cuticular hydrocarbon analysis shows that both formerly classified species split into two clearly distinguishable chemotypes across our sampling range without intermediate profiles. For *Cr. levior*, we additionally show a clear separation in polar metabolites. Secondly, we morphometrically analyzed the different species. Although there is a large overlap in traits between groups, we found slight but significant differences in body shape between the two cryptic *Camponotus* and between the two cryptic *Crematogaster* species. Moreover, *Cr. levior* B is slightly larger than *Cr. levior* A. Lastly, we barcoded all sampled colonies and found a 1:1 association between the previously assigned CHC chemotypes and newly assigned genotypes. Phylogenies based on COI perfectly split *Cr. levior* into two clusters. The same holds true for *Ca. femoratus* based on COI and four additional nuclear markers, where again two distinct clusters are found. These results support our initial hypothesis that apparent CHC diversity is in fact a sign of distinct genetic lineages, i.e. cryptic species (in the sense of De Queiroz 2007). In the following sections, we first discuss the distribution and ecological niches of the cryptic species, then their population structures and possible scenarios explaining those, and lastly, the putative role of the vastly different cuticular hydrocarbon profiles during or after the speciation process.

Previous studies that looked at the distribution of cryptic species mostly found evidence for the competitive exclusion principle (García-Robledo *et al.* 2015; Leavitt *et al.* 2015; Vodă *et al.* 2015). In fig wasps for example, morphologically similar species are less likely to occur in sympatry than morphologically dissimilar sister species (Darwell & Cook 2017). Interestingly, in our case, the two *Crematogaster* and *Camponotus* sister species co-occur across the whole sampling range with only one case of niche differentiation within the factors tested here. *Camponotus femoratus* PS is more common in the drier, western half of the country, while PAT was more frequently found in the wetter and slightly cooler east of the country. The high proportions of alkadienes in the CHC profile of *Ca. femoratus* PAT are in line with this climatic difference. This corroborates other studies in which alkadienes were found to be present more frequently and in higher percentages (only in interaction with cooler temperature) in multiple different species from high precipitation areas (van Wilgenburg *et al.* 2011; Menzel *et al.* 2017a). In contrast, the two *Crematogaster* species occur in similar frequencies across the whole sampling range with no obvious signs for niche differentiation in the parameters we tested. However, other ecological parameters, such as dietary differences or niche partitioning concerning the time of foraging activity or mating flights may still be of importance. Alternatively, *Cr. levior* A and B may represent ecologically

neutral species (Hubbell 2001; Adler, HilleRisLambers & Levine 2007; Bell 2017). In this scenario diverse communities of functionally equivalent species coexist due to neutral dynamics (Hubbell 2005). We furthermore found no preferential association of either *Crematogaster* species for any of the two *Camponotus* species or vice versa, rendering co-speciation a more unlikely scenario. The lack in preference may not be too surprising given the distribution of the species. While the two *Crematogaster* species occur in similar frequencies throughout the sampling range, the two *Camponotus* species show the above mentioned east-west gradient. The choice of the mutualistic partner might therefore be a question of availability rather than preference.

Population structure, as well as haplotype diversity differed strongly between species. It was most extreme, with only a single haplotype and no population differentiation in *Cr. levior* A between all 12 sampled populations. We found five different haplotypes in *Cr. levior* B and eight in both *Ca. femoratus* species. In *Cr. levior* B population structure was very weak and there was no sign for isolation by distance. This result is surprising insofar, as other studies on the genus *Crematogaster* usually show strong geographical or ecological structure (Türke *et al.* 2010; Boyle *et al.* 2018). In *Ca. femoratus* PS and PAT respectively, the COI locus, as well as two nuclear markers showed clear signs for isolation by distance. Tajima's D analysis furthermore showed signs for sudden population expansions in several of the observed populations of *Ca. femoratus* PS and PAT. Genetic differences between the two *Camponotus* species were generally low and only a small part of the nuclear markers we tested were variable between species. Furthermore, the previously assigned CHC chemotypes did not perfectly match the haplotypes of any of the nuclear loci, which may be due to incomplete lineage sorting, a possible sign of recent speciation between *Ca. femoratus* PS and *Ca. femoratus* PAT.

The lack of any population differentiation in *Cr. levior* A, with only a single COI haplotype in all sampled populations, could be explained by two different scenarios. The first is a strong bottleneck event coupled with a recent population expansion. A second explanation could be a selective sweep in haplotype A together with a population expansion. In insects, this is often found in the context of an infection with the endosymbiont *Wolbachia* that can manipulate its hosts reproduction (through e.g. mate-discrimination, cytoplasmic incompatibilities; Hoffmann, Turelli & Simmons 1986; Schuler *et al.* 2016). However, the same signatures can be found after the spread of a beneficial mutation within a population, that will lead to reduced heterozygosity around the selected locus (Schlenke & Begun 2004). While we found only weak genetic differences between the cryptic *Camponotus* species,

chemical differences were pronounced. Also *Crematogaster* showed unusually high interspecific differences in their chemical profile, which has previously been discussed as a mechanism to reinforce species divergence (Menzel, Schmitt & Blaimer 2017b). The overlap in CHC composition between the two species of each genus was low, with peaks that were abundant in one species being low or absent in the other (see 1.3 Results). This means that the CHC profiles differ much more than one would expect between sister species sharing similar abiotic and biotic niches (Menzel *et al.* 2017b). Especially compared to other traits, e.g. morphology or behavior, chemical trait differences seem to be higher and less phylogenetically conserved (Blomberg, Garland & Ives 2003; Kamilar & Cooper 2013). Chemical distance and genetic distance were correlated in *Cr. levior* B – but not in A, or any of the cryptic *Ca. femoratus* species. Interestingly, in *Cr. levior* A, in which we only found a single COI haplotype, the chemical diversity was very large compared to the uniformity we observed in the COI locus. Taken together, this in our opinion suggests that the CHC divergence may have played a role in species divergence – either during or after speciation. The main role of cuticular hydrocarbons is to serve as desiccation barrier but, especially in social insects, additionally play a role in communication and as mating cues (Thomas & Simmons 2008). They therefore have been discussed as possible 'magic traits', i.e. traits that affect both ecological adaptation and mate signaling (Smadja & Butlin 2009; Chung & Carroll 2015), which can be mediated by a single gene only (Chung *et al.* 2014). Changes in such traits will often lead to assortative mating and ultimately to speciation (Chung & Carroll 2015). In *Timema* stick insects, speciation events were generally associated with a divergence in CHC profiles, however, it remained unclear whether speciation followed CHC divergence or if CHC profiles diverged due to selection during the evolution of reproductive isolation (Schwander *et al.* 2013). The same holds true for both cryptic species pairs in *Crematogaster* and *Camponotus*. The surprisingly high chemical divergence, combined with low genetic diversity (at least in *Camponotus*), might be indicative for a role of CHCs in species divergence. But it remains to be elucidated whether CHCs played a role in the speciation event itself by inducing assortative mating, by reinforcing sexual selection after the speciation event or by niche partitioning, i.e. adaptation to a yet unknown factor.

1.5 Conclusions

We could conclusively show that both *Crematogaster levior* and *Camponotus femoratus* split into two morphologically nearly indistinguishable cryptic species. It remains unclear how speciation took place in the two genera, but the strong separation in cuticular hydrocarbon profiles suggests that they are involved in mediating species divergence. Since *Crematogaster levior* and *Camponotus femoratus* are only found in mutualistic associations, we were rather surprised to find no partner preferences as indication for cospeciation in this mutualistic complex. Moreover, the highly different population structures between and within genera point to a rather loose relationship among the mutualists, whereas similar population structures would be expected if there was a strict partner specialization. Future studies should investigate partner choice and recognition, the evolution of the distinct chemotypes, the phylogeography of the species, as well as genome wide patterns of selection to shed further light on this highly interesting association and its players. This will help to deepen our knowledge on the effect of mutualistic interactions on species divergence.

1.6 Acknowledgements

Removed for privacy purposes.

1.7 Supplementary material

Table S1.1: Reliability of body measures. The table shows the variable codes and names, the reliability R as well as the lower and upper 95 % confidence intervals (LCI and UCI). For definitions of measures see our Dryad data (doi:10.5061/dryad.m7ks1g8).

variable code	variable name	R	LCI	UCI
Crematogaster				
spo.d	Min. spine distance	70.99	48.40	82.94
ppt.l	Postpetiole length	71.30	48.91	83.13
ocm.d	Ocular-malar-distance	77.16	58.77	86.67
pet.h	Petiole height	89.07	79.68	93.71
hea.l	Head length	91.81	84.68	95.31
eye.b	Eye breadth	93.64	88.05	96.36
eye.h	Eye length	94.19	89.07	96.68
pet.l	Petiole length	94.91	90.40	97.09
tb3.l	Tibia length	95.47	91.44	97.41
ppt.h	Postpetiole height	96.04	92.52	97.74
ppt.b	Postpetiole breadth	96.97	94.25	98.27
fca.d	Max. distance of frontal lobes	97.77	95.77	98.73
fci.d	Min. distance of frontal lobes	97.81	95.85	98.76
pet.b	Petiole breadth	97.91	96.04	98.81
fm3.l	Femur length	98.01	96.23	98.87
poc.d	Postocular-distance	98.11	96.41	98.93
mes.l	Mesosoma length	98.29	96.75	99.03
spt.d	Max. spine distance	98.33	96.83	99.05
sca.l	Scape length of the antennae	98.56	97.26	99.18
spi.l	Spine length	98.57	97.29	99.19
mes.b	Mesosoma breadth	98.97	98.03	99.41
eye.d	Min. head breadth	99.00	98.09	99.43
hea.b	Max. head breadth	99.16	98.40	99.52

Camponotus				
snp.d	Stigma-spine of petiole distance	73.32	52.22	84.55
pet.l	Petiole length	87.15	76.12	92.70
eye.b	Eye breadth	92.73	86.27	95.90
pet.h	Petiole height	96.45	93.23	98.01
hea.l	Head length	97.52	95.25	98.61
tb3.l	Tibia length	97.68	95.57	98.70
sca.l	Scape length of antennae	97.91	96.00	98.83
mes.l	Mesosoma length	98.36	96.86	99.08
eye.h	Eye length	98.69	97.48	99.27
fm3.l	Femur length	98.92	97.92	99.39
ssm.d	Stigma distance	99.23	98.52	99.57
pet.b	Petiole breadth	99.28	98.61	99.60
poc.d	Postocular-distance	99.28	98.62	99.60
fci.d	Min. distance frontal lobes	99.41	98.86	99.67
ocm.d	Ocular-malar-distance	99.45	98.94	99.69
mes.b	Mesosoma breadth	99.73	99.49	99.85
fca.d	Max. distance frontal lobes	99.74	99.50	99.85
ssd.d	Mesosoma stigma distance	99.76	99.54	99.87
eye.d	Min. head breadth	99.89	99.79	99.94
hea.b	Max. head breadth	99.96	99.92	99.98

Table S1.2: PCR mastermix and sequencing reaction.

PCR mastermix	Volume	Sequencing reaction	Volume
10x Buffer B (molegene)	1.0 µl	Big Dye Terminator Mix	0.16 µl
$MgCL_2$ 25mM (molegene)	1.0 µl	5x Buffer B (molegene)	1.84 µl
dNTP-Mix 2mM each (molegene)	0.1 µl	ddH_2O	6.50 µl
Primer 10 pmol/µl)	0.2 µl of each primer	Primer (10 pmol/µl)	0.5 µl of each primer
Taq-Polymerase (molegene)	0.1 µl	PCR product	1.0µl
ddH_2O	6.5 µl		

Table S1.3: Overview of all test primer pairs. (Ströher *et al.* 2013)

Primer	Primer length	Primer sequence	
ant. 1F	27	CCTTCGTGCCTAYGAGAATAGYGTTAC	Resulting sequences were variable enough for further analyses
ant. 1R	21	AACGACGTCSGACGGTTCCAT	
ant. 389F	21	ACGGACCCCACATTGAGAAGAAC	
ant. 389R	21	CYTTACCCACCTCCTCCACCA	
ant. 1087 F	21	ACCAGCAGAGGCTGGACGTGA	
ant. 1087 R	27	GCCAAGTTGATTGTGTACGAACTTTCT	
ant. 1401F	22	GYAGGAAGGACGCTCTTAATCT	
ant. 1401R	26	AAGCTTATCTCTAGGAAACTCCCATC	
ant. 1225 F	26	TAATACRACTGAAGAGAGACCAGGAG	Not used for further analyses as resulting sequences were either not variable or did not amplify in PCR
ant. 1225 R	27	GACTAGATCCTAAGCTAGAGAGRCTGG	
ant. 1281 F	23	GACGCAGGTTGYAACGAAATCAC	
ant. 1281 R	24	GCCRCTAATATCCAGCTTCACGAG	
ant. 384 F	27	TAGTAGTCGAAGGAGTCATACCAAAGG	
ant. 384 R	20	TGYGTGTTCGATGCCGTTGA	
ant. 965 F	24	AGTTCAAGGTTCACCGGTGCCTAA	
ant. 965 R	25	GAGAAGGYGAAYTTAAAGACTGATG	
ant. 1503F	21	GRTTYGCCTTCCAGGAGATCA	

ant. 1503R	23	AAGTAGTCCAGGCAGAACCACAC	
ant. 202F	26	CCYATCAACTCTGTTAATATCGAACG	
ant. 202R	22	GACACAATGTTGGAAGCCCTTG	
ant. 263F	27	GACTAGCTCAGAATCACACTCTTCCAC	
ant. 263R	24	GTTGTTTTGGWGGCAATATTGGAG	
ant. 346F	23	GTGGTCCACCATCCGTKGGATCT	
ant. 346R	26	GGATTGTTTTGTGTAATCTGCGTTCG	
ant. 505F	24	CCTCAGATGAAGTTYCGAGTTCC	
ant. 505R	26	TAAYCCGRACACCCTCACTTTATACG	
ant. 839F	25	CAATGGCGATTTACAACGAATTTCT	
ant. 839R	22	CAGGCANAGCAGCAATGTGACG	

Table S1.4: Tajima's D statistics. Given are values within all sampled populations of *Crematogaster levior* B and *Camponotus femoratus* PS and PAT. Bold characters indicate statistical significance ($p < 0.05$) based on a permutation test. *Crematogaster levior* A is not shown as there was only a single haplotype found at all locations.

	***Cr. levior* B**		***Ca. femoratus* PS**		***Ca. femoratus* PAT**	
	Tajima's D	**p**	**Tajima's D**	**p**	**Tajima's D**	**p**
AP	0	1	0	1	0	1
SL	0	1	**-1.513**	**0.033**	**-1.486**	**0.04**
SI	-1.088	0.189	0	1	1.284	0.888
PS	0	1	0.545	0.764	0	1
PAR	-1.149	0.161	-1.142	0.145	**-2.072**	**0.003**
LN	0.713	0.869	0	1	**-2.107**	**0.002**
KO	1.225	0.943	-0.817	0.321	0	1
MT	-	-	0	1	0	1
CA	0	1	-	-	0.944	0.843
CAY	0	1	-	-	2.125	0.986
RE	-	-	0	1	2.192	0.993
PAT	-0.774	0.218	-	-	-0.657	0.297

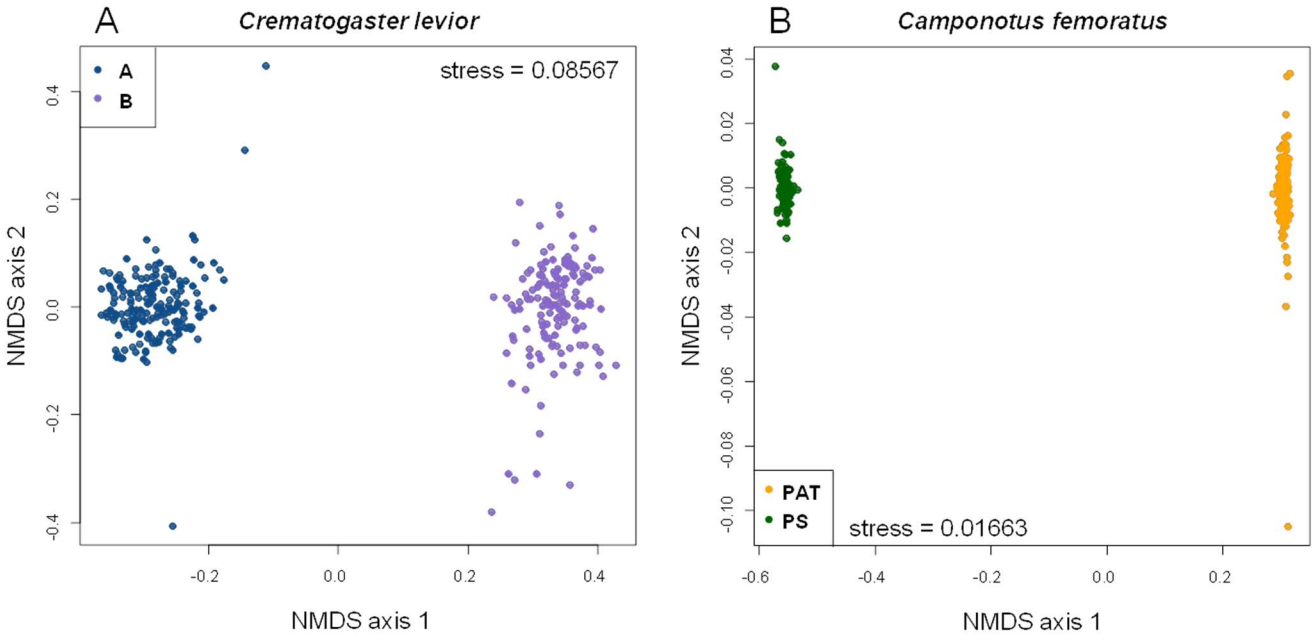

Figure S1.1: Non-metric multidimensional scaling (NMDS) ordinations of the CHC profiles of the cryptic *Cr. levior* and *Ca. femoratus* species. (A) Shows an NMDS ordination of *Cr. levior* CHC profiles (*Cr. levior* A: blue; *Cr. levior* B: purple). Each dot represents one colony. (B) Shows a similar ordination for *Ca. femoratus* CHCs (*Ca. femoratus* PAT: yellow; *Ca. femoratus* PS: green).

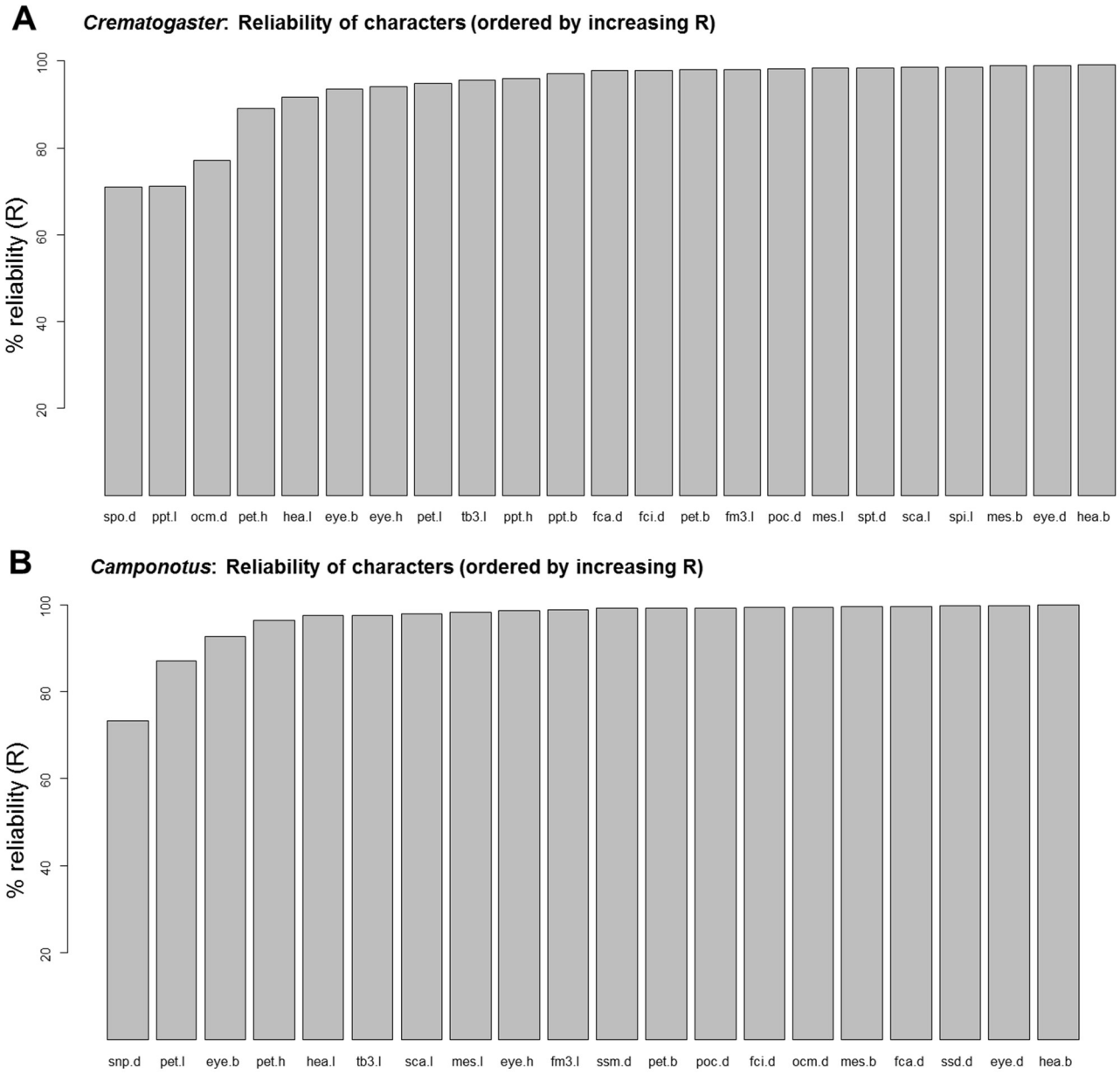

Figure S1.2: Reliability of morphological characters. The barplots show the reliability (R) of characters measured in *Crematogaster* (A) and *Camponotus* (B). Characters with reliability < 85% were not included into the multivariate ratio analysis.

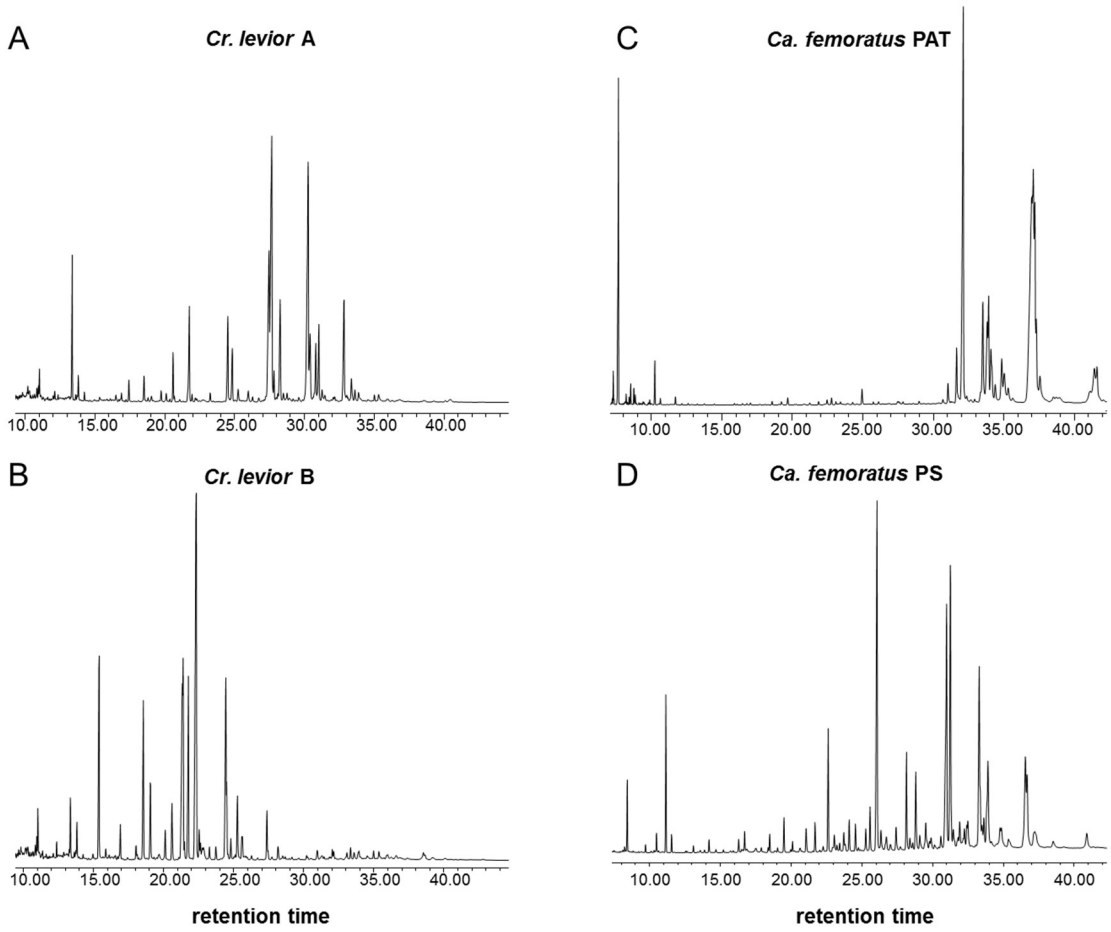

Figure S1.3: Representative chromatograms of cuticular hydrocarbon profiles of the cryptic species. (A+B) Show chromatograms of the CHC profiles of *Cr. levior* A and B. (C+D) Show chromatograms of the CHC profiles of *Ca. femoratus* PAT and PS.

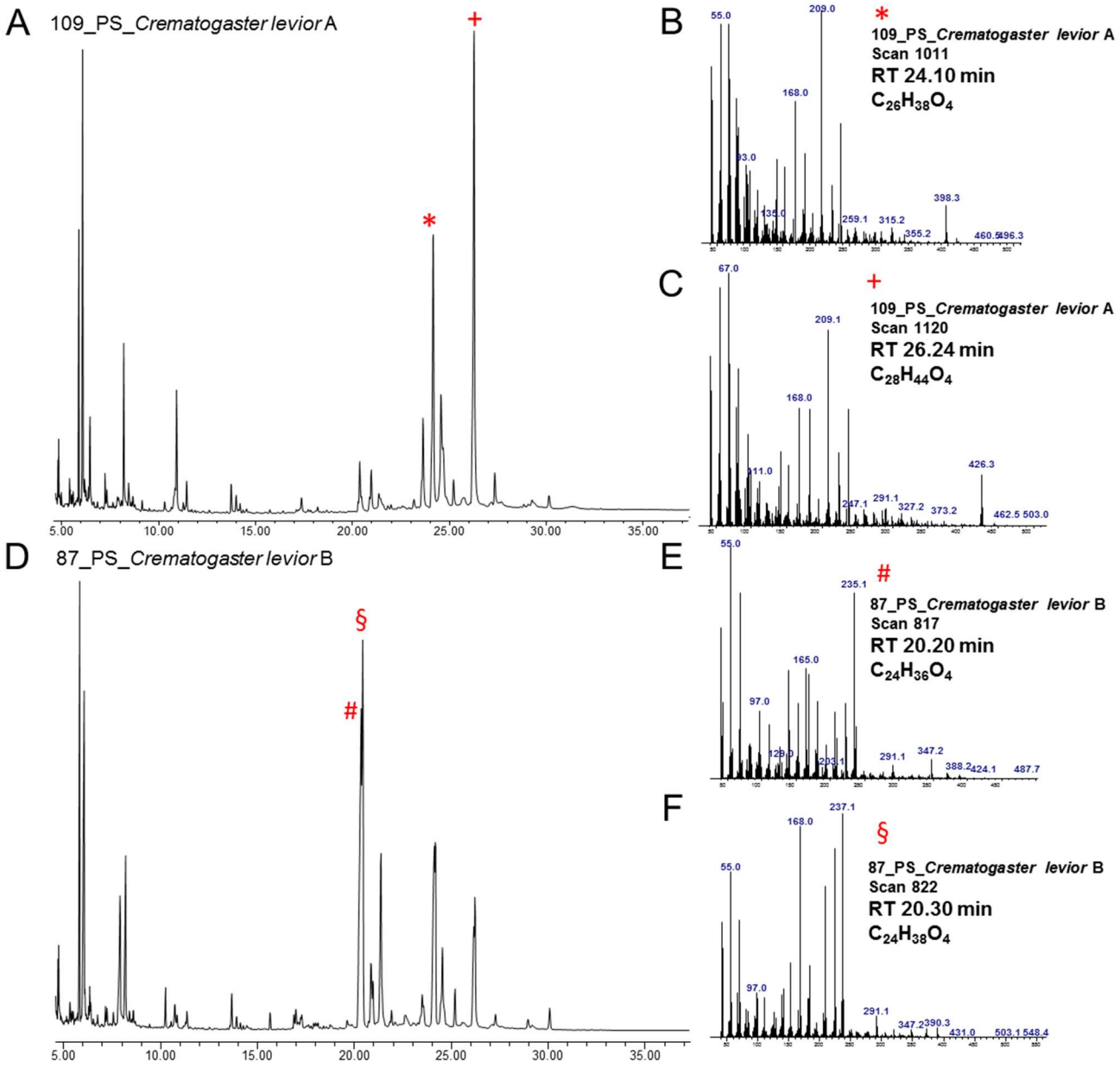

Figure S1.4: Representative chromatograms of the polar substances of *Cr. levior* A and B and mass spectra of the most abundant substances. (A-C) Show a representative chromatogram of the polar substances of *Cr. levior* A (A) and the mass spectra of the two most common substances in this species (B+C). (D-F) Show a representative chromatogram of the polar substances of *Cr. levior* B (D), and the mass spectra of the two most common substances in the species (E+F).

Chapter 2

Influence of mutualistic lifestyle, mutualistic partner, and climate on cuticular hydrocarbon profiles in parabiotic ants

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Abstract

A vital trait in insects is the cuticular hydrocarbon (CHC) profile, which protects the insect against desiccation and serves in chemical communication. Due to these functions, CHC profiles are shaped both by climatic conditions and biotic interactions. Here, we investigated CHC differentiation in the neotropical parabiotic ant species *Crematogaster levior* and *Camponotus femoratus*, which mutualistically share a nest. Both consist of two cryptic species each (*Cr. levior* A and B and *Ca. femoratus* PAT and PS) that differ genetically and possess strongly different CHC profiles. We characterized and compared CHC profiles of the four cryptic species in detail. Our results suggest that *Cr. levior* A, *Ca. femoratus* PAT and *Ca. femoratus* PS adapted their CHC profiles to the parabiotic lifestyle by producing longer-chain CHCs. At the same time, they changed their major CHC classes, and produce more alkadienes and methyl-branched alkenes compared to *Cr. levior* B or non-parabiotic species. The CHC profiles of *Cr. levior* B were more similar to related, non-parabiotic species of the *Orthocrema* clade than *Cr. levior* A, and the chain lengths of B were similar to the reconstructed ancestral state. Signals of both the parabiotic partner (biotic conditions) and climate (abiotic conditions) were found in the CHC profiles of all four cryptic species. Our data suggest that mutualisms shaped the CHC profiles of the studied species, in particular chain length and CHC class composition. Beside this, signals of the parabiotic partners indicate potential impacts of biotic interactions, via chemical mimicry or chemical camouflage.

Keywords:

adaptation, chemical communication, cryptic species, Formicidae, mimicry, mutualism, parabiosis

2.1 Introduction

Cuticular hydrocarbons (CHCs) cover the surface of nearly all terrestrial arthropods and function as barriers against water loss and as agents in chemical communication. They can be classified in three major groups: straight-chain *n*-alkanes, methyl-branched alkanes and unsaturated alkenes (Blomquist 2010a). The substances produced by an insect are usually species-specific, but can differ in their relative proportions (Martin *et al.* 2008a). The composition of CHC profiles is also shaped by abiotic selection pressures such as temperature or humidity, but also by communication requirements (Chung & Carroll 2015). Indeed, ant species from habitats with higher rainfall produce rather unusual substance classes like alkadienes and methyl-branched alkenes, which are less effective in preventing water loss (van Wilgenburg *et al.* 2011; Menzel *et al.* 2017a). However, several studies revealed that relative CHC profile composition is rather flexible and organisms are able to respond plastically to temperature and humidity variation on a rather short-term basis (Wagner, Tissot & Gordon 2001; Stinziano *et al.* 2015; Menzel *et al.* 2018; Sprenger *et al.* 2018) and can be influenced by biotic factors like e.g. diet, parasites or pathogens (Otte *et al.* 2018).

Well-known examples of how biotic interactions shape the evolution of CHC profiles come from antagonistic interactions. Many parasites and predators mimic the CHC profiles of their hosts (or prey) to avoid being detected. This phenomenon is especially common in parasites (myrmecophiles) or predators of ants. Some species use chemical mimicry, i.e. the active production of similar substances, to impede nestmate recognition by social insects (Lenoir *et al.* 2001b; Bagnères & Lorenzi 2010). As a second mechanism, the parasites can acquire their host's CHCs without producing them themselves either passively through the host or nesting material or actively like e.g. *Formicoxenus* ants that groom off their host's CHCs (chemical camouflage; Lenoir, Malosse & Yamaoka 1997; Lenoir *et al.* 2001). A third possible way to avoid recognition is chemical insignificance, in which the parasite possesses only few recognition cues (low quantity of CHC), substances which are hard to distinguish (e.g. *n*-alkanes) or additional chemical substances (e.g. alkaloids) to cover the recognition cues (Kleeberg *et al.* 2017; Neupert *et al.* 2018). Some species also use CHCs or different chemical compounds as appeasement allomones to get accepted in foreign colonies or reduce interspecific aggression (Mori *et al.* 2000; Menzel *et al.* 2013; Elia *et al.* 2018). Exploitation by parasites can also be a selection pressure on the host leading to diversification in CHC profiles as a counter-adaptation (Jongepier & Foitzik 2016).

Beside host-parasite interactions, mutualisms can also exert selection on CHC profiles (Menzel & Schmitt 2012). Mutualistic interactions are often drivers of the evolution of novel phenotypic traits and are expected to speed up their emergence (Herre *et al.* 1999; Hoeksema & Bruna 2000; Guimarães *et al.* 2011). An example of a remarkable mutualism is the so-called parabiosis in ants, in which two different species live in a common nest while keeping their brood separated (Orivel *et al.* 1997; Menzel *et al.* 2008b). Parabioses are restricted to tropical habitats and often include species of the genus *Crematogaster* (Swain 1980; Davidson 1988; Orivel *et al.* 1997; Vantaux *et al.* 2007; Menzel *et al.* 2008b; but see Parmentier *et al.* 2017). They are characterized by high interspecific tolerance (Menzel *et al.* 2008a; b), which is unusual for ants of different species or colonies. The interspecific tolerance in parabiotic ants is most likely achieved through adaptations of the CHC profile including increase in chain length and production of rare substance classes such as alkadienes or methyl-branched alkenes (Menzel *et al.* 2008a; Menzel & Schmitt 2012).

In a recent study, we presented strong evidence for cryptic speciation in the neotropical parabiotic species *Crematogaster levior* and *Camponotus femoratus* (Hartke, Sprenger *et al.* 2019, Chapter 1). Both exhibit two different chemical morphs, which also differ genetically and, albeit only slightly, in their morphology. In addition, *Cr. levior* was only quite recently taxonomically separated from *Cr. carinata*. Both belong to a species complex within the *Orthocrema* clade, one of three subgenera of *Crematogaster*, that is suspected to contain even more cryptic species (Longino 2003). The two species live in commonly shared nests, so-called ant gardens (Davidson 1988; Orivel & Leroy 2011). These consist of specialized epiphytic plants, whose seeds were carried in by *Ca. femoratus* (Youngsteadt *et al.* 2008) and whose root systems are coated with carton by the ants and serve as actual ant nest. *Camponotus femoratus* profits from the ability of *Cr. levior* to discover prey and was shown to follow their trail pheromones (Vantaux *et al.* 2007; Menzel *et al.* 2014). *Crematogaster levior* on the other hand benefits from *Ca. femoratus* building nest structures, planting epiphytic seeds, responding to damages on the ant garden plants and aggressively defending the nest against attacking vertebrates (Vantaux *et al.* 2007; Youngsteadt *et al.* 2008; Vicente *et al.* 2014; Leal *et al.* 2017) including field biologists (personal observations).

In this study, we characterized the chemical diversity of parabiotic *Camponotus* and *Crematogaster* species in French Guiana. We characterized the cryptic species in both genera and investigated in detail how they differed from each other. Furthermore, we report additional variants that did not fit into these cryptic species. By comparing the chemical profiles of the cryptic *Cr. levior* and *Ca. femoratus* species to other related species, we inferred

CHC changes related to the parabiotic lifestyle and reconstructed the ancestral state for CHC chain length in *Crematogaster*. Finally, we investigated how the CHC profiles were influenced by climatic conditions (due to the need for waterproofing) and by the parabiotic partner (due to mimicry or substance transfer).

2.2 Materials and methods

2.2.1 Sample collection

We collected workers of the arboreal ant species *Crematogaster levior* (N = 332 colonies) and *Camponotus femoratus* (N = 306 colonies) in 13 different locations in French Guiana from August to October 2016 (Table S2.1, Fig. S2.1). Furthermore, we also collected workers of *Crematogaster* found in parabiosis or trail-sharing with *Camponotus* species, as well as their partners to infer if there were more potentially cryptic species. We noted the GPS coordinates of the exact sampling locations for each colony. We freeze-killed the collected workers at -20°C and subsequently extracted the CHCs by immersing groups of 10 *Cr. levior* (small, dry mass: 0.074 ± 0.016 mg) or 5 *Ca. femoratus* workers (large, 0.922 ± 0.092 mg), in hexane. After 10 minutes the ants were removed from the extracts using freshly cleaned forceps.

2.2.2 Chemical analyses

Crematogaster CHC samples were purified using silica columns (Chromabond, SiOH 1mL/100mg, Macherey-Nagel, Düren, Germany) before analysis since they contained hereto unknown polar compounds which concealed the hydrocarbon profile. All samples were concentrated to approximately 20 µl under a gentle N_2 stream and were analyzed using gas chromatography-mass spectrometry (GC-MS). We used a Zebron Inferno DB5-MS capillary column (length 30 m, Ø 0.25 mm, 0.25 µm coating, Phenomenex Ltd., Aschaffenburg, Germany) in an Agilent gas chromatograph (7890A, Agilent Technologies, Santa Clara, CA, USA) with Helium as carrier gas at a flow rate of 1.2 mL per minute. The mass spectrometer (5975C, Agilent Technologies) ran with electron ionization (EI) at 70 eV.

We used a Programmed Temperature Vaporization (PTV) injection method for the *Cr. levior* CHC extracts. This injection method was used to inject higher extract quantities, which is necessary to achieve good CHC detection despite the low CHC quantities of this species.

With the PTV method, the sample is injected and the solvent is then rapidly vaporized. 4 µl of the extracts were injected at 40°C and this temperature was held constant for 2 minutes. Then, the oven was heated with 60°C per minute to 200°C and following this with 4°C per minute to 320°C. This temperature was then kept constant for 10 minutes. Similarly, 2 µl of *Ca. femoratus* extracts were injected at 60°C using the splitless method. The oven heated with 60°C per minute up to 200°C and then with 4°C per minute to 300°C, which were kept constant for 10 minutes.

We integrated the chromatograms manually using *MSD ChemStation* (E.02.02.1431, Agilent Technologies). Afterwards substances were aligned in Microsoft Excel 2010. We excluded all non-hydrocarbon substances as well as entire substances if their average abundance was below 0.1% or if they were present in less than 20% of the samples of the respective cryptic species. However, we included substances that had multiple double bonds if other alkenes of the same chain length were present, even if they were present in less than 20% of the samples (as they might be varieties coming from the same biosynthetic pathway). CHCs were identified using diagnostic ions and Kovats indices calculated based on a standard series of *n*-alkanes (Carlson *et al.* 1998, Table S2.2 and S2.3). To identify their positions in alkenes and alkadienes, we performed methylthiolation of the carbon-carbon double bonds using DMDS (dimethyl-disulfide, Sigma-Aldrich, St. Louis, MO, USA) (Attygalle 1998). Methyl-branched alkenes were identified by their mass spectra as previously described in other parabiotic ant species (Menzel *et al.* 2008a). Diagnostic ions for methyl-branched alkenes and exemplary mass spectra are shown in the supplementary material (Tab. S2.2, S2.3; Fig. S2.2).

2.2.3 Statistical analyses

We aimed to find out which cuticular hydrocarbons differentiated the two cryptic species within *Cr. levior* and *Ca. femoratus* (respectively) from each other, and if they differed in overall chain length or CHC class composition. Next, we compared the CHC profiles of the cryptic *Cr. levior* species to other species of the *Orthocrema* clade (including an ancestral state reconstruction for CHC chain length) and those of *Ca. femoratus* to other *Camponotus* species. Finally, we investigated the impact of biotic (parabiotic partner) or abiotic factors (climate) on the CHC profiles. All statistical tests were performed using *R* version 3.6.0 (R Core Team 2018), and conducted separately for *Cr. levior* and *Ca. femoratus* samples.

As a first step, we assigned the colonies according to their CHC profiles. To this end, we first did a non-metric multidimentional scaling ordination (NMDS) based on Bray-Curtis

dissimilarities using the R-package *vegan* (Oksanen *et al.* 2019) on the proportions of each cuticular hydrocarbon. In addition, we performed principal component analyses (PCA) on the centered log-ratio transformed compositional data (Aitchison 1982), and compared the clusters produced by each ordination method. We performed both methods since they are very different concerning their assumptions and the underlying algorithms (Brückner & Heethoff 2017); hence comparing both methods was a way to assess the robustness of the obtained results.

Afterwards, we tested whether the colonies could be correctly assigned to cryptic species using a random forest approach with 10,000 permutations (R-package *randomForest* (Liaw & Wiener 2002)). An advantage of this machine learning method is that it calculates the importance of the single substances for group assignment (Brückner & Heethoff 2017). In addition to this 'variable importance', we report the OOB (out-of-bag) error rate which is the classification error averaged across all training subsets. The training subsets (used to train the classification algorithm) consist of the full data set minus one of the samples; thus, the number of subsets equals the number of samples.

We created a list of CHCs ordered by importance for group assignment, and used the upper third of this list to perform an average-linkage hierarchical cluster analysis. This allowed us to identify which substances were more common in either of the cryptic species and thus to describe which compounds contributed most to the species differences (and hence defined species-specific profiles). Then, we used these CHCs to test whether certain hydrocarbon classes were enriched in one of the two cryptic species per genus, or whether the species-specific hydrocarbons differed systematically in chain length. To this end, for each substance class, we compared the number of species-specific CHCs between cryptic species using χ^2 tests. Further, we compared their chain lengths between cryptic species using Wilcoxon rank-sum tests. Beside these qualitative tests (that were based on the presence or absence of certain CHCs), we incorporated the abundances of the characteristic CHCs, and tested for differences between the cryptic species using Wilcoxon rank-sum tests.

In two additional analyses we compared the cryptic species to the profiles of other related species. *Crematogaster levior* was compared with 13 other species of its *Crematogaster* clade, *Orthocrema* (Blaimer 2012) using data from Menzel *et al.* (2017b). *Camponotus femoratus* was compared with 37 other *Camponotus* species using data from Menzel *et al.* (2017a) (to our knowledge, there is no robust phylogeny of *Camponotus* available, thus making it impossible to identify the species closely related to *Ca. femoratus*). We added 10 randomly chosen

colonies from each of our cryptic species (R command *sample*) to keep the analysis concise. We used an NMDS ordination based on Bray-Curtis dissimilarities to compare the cryptic species to the other related species and to investigate if our samples cluster together with their conspecifics from original dataset. Additionally, we conducted an average-linkage cluster analysis for each dataset (R-command *hclustCBI* from the *fpc* package; based on the same distance matrices). Afterwards, we assessed the cluster stability using the mean Jaccard similarity of 100 bootstrap iterations (R-command *clusterboot* from the *fpc* package). Clusters with a mean Jaccard coefficient larger than 0.75 are usually considered stable (Hennig 2007).

The most prominent difference between *Cr. levior* A and B is the average chain length. Since a phylogeny for the *Orthocrema* clade including *Cr. levior* is available (Bayesian phylogeny based on five nuclear genes; Menzel *et al.* (2017b)), we used it to reconstruct the ancestral state of this trait. Average chain length (weighed according to CHC proportion) was calculated for each species in the phylogeny based on data from this study or from Menzel *et al.* (2017b) (using species averages if applicable). The analysis was done using the commands *fastAnc* for estimating the ancestral states of the character at each node and its 95% confidence intervals as well as *contMap* to visualize them on the phylogeny (R-package *phytools;* Revell 2012).

To detect species-specific CHCs as signals of the parabiotic partner in their CHC profile, we determined the substances with the strongest differences according to their parabiotic partner. To this end, we ran univariate PERMANOVA analyses based on Euclidean distance for each compound separately with 999 permutations and added the partner identity as fixed factor. This analysis yields in pseudo-F values equivalent to univariate F-statistics as effect sizes (Anderson 2017). We identified the 10% compounds with the highest F-values, i.e. the highest differentiation according to the identity of the parabiotic partner. In the PERMANOVAs, minute random values with a normal distribution (mean ± SD: $10^{-8} \pm 10^{-8}$) were added to avoid samples with distances of zero in the distance matrix. The analysis was done for each of the four cryptic species separately.

Finally, we obtained from bioclim variables from CHELSA (Karger *et al.* 2017), which provides data with a resolution of about 1 km accuracy. Subsequently, we created subsets of the climatic variables according to the coordinates of colonies present for each species and performed a principal component analysis (PCA) to reduce the number of climate variables. Most of the variance was explained by annual precipitation and annual average temperature, which were negatively correlated (Fig. S2.3). Overall effects of partner, climate and their

interaction were analyzed using multivariate PERMANOVAs based on Bray-Curtis dissimilarities with 999 permutations on entire CHC profiles (separately for *Crematogaster* and *Camponotus*, but for the two cryptic species together in each genus) (R command *adonis*, package *vegan*). As fixed factors we used species identity, partner identity and the loadings of climate PC1. Similar to the analysis for signals of the parabiotic partners, we also performed univariate PERMANOVAs based on Euclidean distances on each single CHC for the climate effects.

2.3 Results

2.3.1 Differentiation between CHCs of the cryptic species

In *Crematogaster levior*, we differentiated three different groups. *Crematogaster levior* A and B were already known from previous studies (Emery & Tsutsui 2013; Menzel *et al.* 2014; Hartke, Sprenger *et al.* 2019, Chapter 1), and a third group of *Crematogaster* colonies, which in the following will be referred to as *Cr. levior* C (n = 10; Fig. 2.1 A, B). The CHC profile of *Cr. levior* C was rich in *n*-C27 (mean ± SD: 11.34 ± 8.84%), C27-alkenes (15.83 ± 14.07%) and C29-alkenes (13.36 ± 6.64%) (Table S2.2). These colonies were sometimes found in parabiosis with *Odontomachus mayi* or were sharing a trail with unidentified *Camponotus* species. Morphological examination (by B. Blaimer, Raleigh NC, USA) showed that most C individuals had the carinae (keel-like crests) typical for *Cr. carinata* on their pronotum. One colony showed characteristics of both groups, A and B. It clustered with A in the PCA (Fig. 2.1 C) but with B in an NMDS ordination (Fig. 2.1 A), making unambiguous assignment difficult.

In *Camponotus femoratus*, we could confirm the clear differentiation of the two cryptic species *Ca. femoratus* Patawa (PAT) and Petit Saut (PS) that were described in earlier studies (Menzel *et al.* 2014; Hartke, Sprenger *et al.* 2019, Chapter 1). However, four colonies found together with *Cr. levior* had profiles with several CHCs being not present in the cryptic *Ca. femoratus* species that formed a third cluster (Fig. 2.1 C, D). These colonies were tentatively identified as a different species as they differ morphologically and genetically (at the COI locus) from *Ca. femoratus* (data not shown). However, they were still found in association with *Cr. levior*, which is why we present these data. As these colonies were restricted to our sampling locations in Saint-Laurent-du-Maroni (in the west of French Guiana, Fig. S2.1), we will refer

to those as *Camponotus* sp. SL. The most common substances in their CHC profiles were *n*-C31 (31.33 ± 4.15%), 11-MeC31 (21.17 ± 4.41%), 2-;4-MeC30 (8.63 ± 1.47%) and an unknown unsaturated CHC (9.35 ± 0.69%) (Table S2.3).

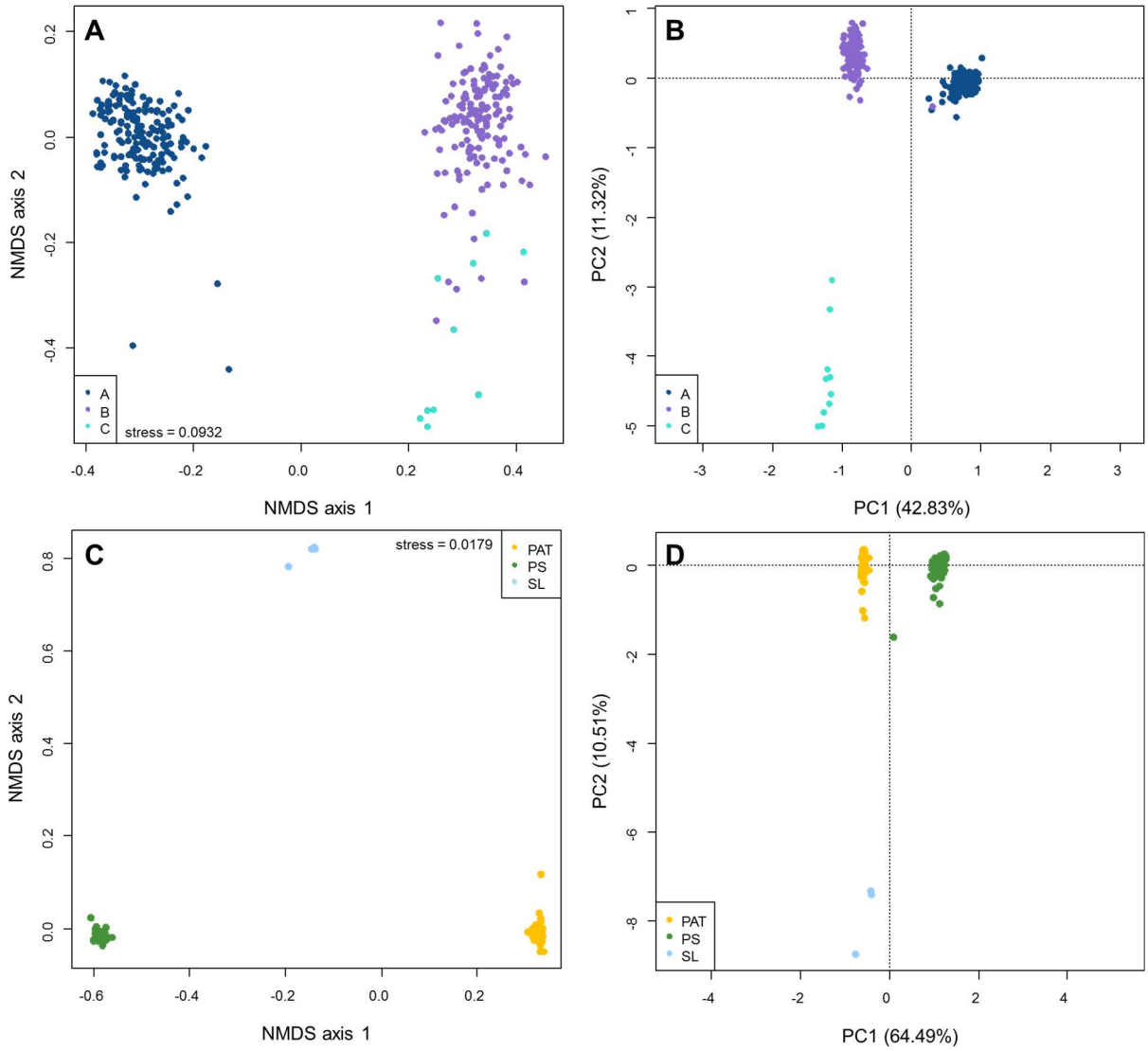

Figure 2.1: Differentiation of CHCs in *Cr. levior* and CHCs in *Ca. femoratus*. (A) & (C) Show non-metric multidimensional scaling (NMDS) ordinations based on Bray-Curtis dissimilarities of the CHC profile of *Cr. levior* and *Ca. femoratus*, respectively. Each data point represents the profile of one colony. (B) & (D) Show principal component analysis (PCA) ordinations of the CHC profiles of *Cr. levior* and *Ca. femoratus*. Similarly, each dot represents the profile of one colony.

2.3.2 Characterization of the CHC profiles

For the following analyses, we used a dataset excluding *Cr. levior* C (n = 10) and *Camponotus* sp. SL (n = 4), due to their low sample sizes. For *Crematogaster levior* A and B (total N = 322), the random forest algorithm classified all A individuals correctly (n = 174), but misclassified one out of 148 colonies of *Cr. levior* B as A (error rate 0.0068%). Based on the random forest, we identified 28 CHCs defining *Cr. levior* A and 17 substances defining *Cr. levior* B (Fig. 2.2 A). Among these, a C37-alkadiene (variable importance: 28.06), a C39-alkadiene (27.81), *n*-C25 (27.27), a C27-alkene (26.72) and 9-C35-alkene (27.58) were most important. The C37- and C39-alkadiene and 9-C35-alkene were characteristic for *Cr. levior* A, while *n*-C25 and C27-alkene were more common in B. The out-of-bag (OOB) estimate of error rate was low with 0.31%.

The 28 substances characterizing *Cr. levior* A had overall longer chains compared to the 17 substances from B (Wilcoxon rank sum test: $W = 20$, $p < 0.001$). The number of characteristic hydrocarbons per substance class did not differ between groups (χ^2-test: $\chi^2_7 = 11.96$, $p = 0.10$). However, when we included the relative abundance of substances, we found that *Cr. levior* A had higher proportions of alkadienes, alkenes, dimethyl alkanes and methyl-branched alkenes in the characterizing substances (Wilcoxon tests: all four $W > 20000$, $p < 0.001$). On the other hand, *Cr. levior* B possessed relatively more characteristic monomethyl alkanes ($W = 222$, $p < 0.001$) and *n*-alkanes ($W = 46$, $p < 0.001$).

For *Camponotus femoratus* PAT and PS (n = 195 and 107, respectively), the random forest algorithm classified all colonies correctly (OOB estimated error rate: 0%). For PS, 30 CHCs were characteristic, the most important ones being a C36-alkene (variable importance: 19.68), C34-alkene (19.59), C40-alkadiene (19.40), C33-alkene (19.27), 13-MeC37-alkene (18.86) and 13-MeC41-alkene (18.84). In contrast, a set of 9 CHCs sufficed to define *Ca. femoratus* PAT, with a C40-alkadiene (11.42) and cf. 9,33-C41-alkadiene (11.18) as most important ones (Fig. 2.2 B).

In contrast to *Cr. levior* A and B, the compounds that characterized *Ca. femoratus* PAT and PS (respectively) on average did not differ in chain lengths nor substance class membership (chain length: Wilcoxon test; $W = 163.5$, $p = 0.35$; CHC class: χ^2 test; $\chi^2_6 = 9.06$, $p = 0.17$). However, when we took their relative abundances into account, *Ca. femoratus* PAT had higher proportions of dimethyl alkanes (Wilcoxon test: $W = 20808$, $p < 0.001$) and *n*-alkanes ($W = 18220$, $p < 0.001$) among the characteristic substances. In contrast, *Ca. femoratus* PS had

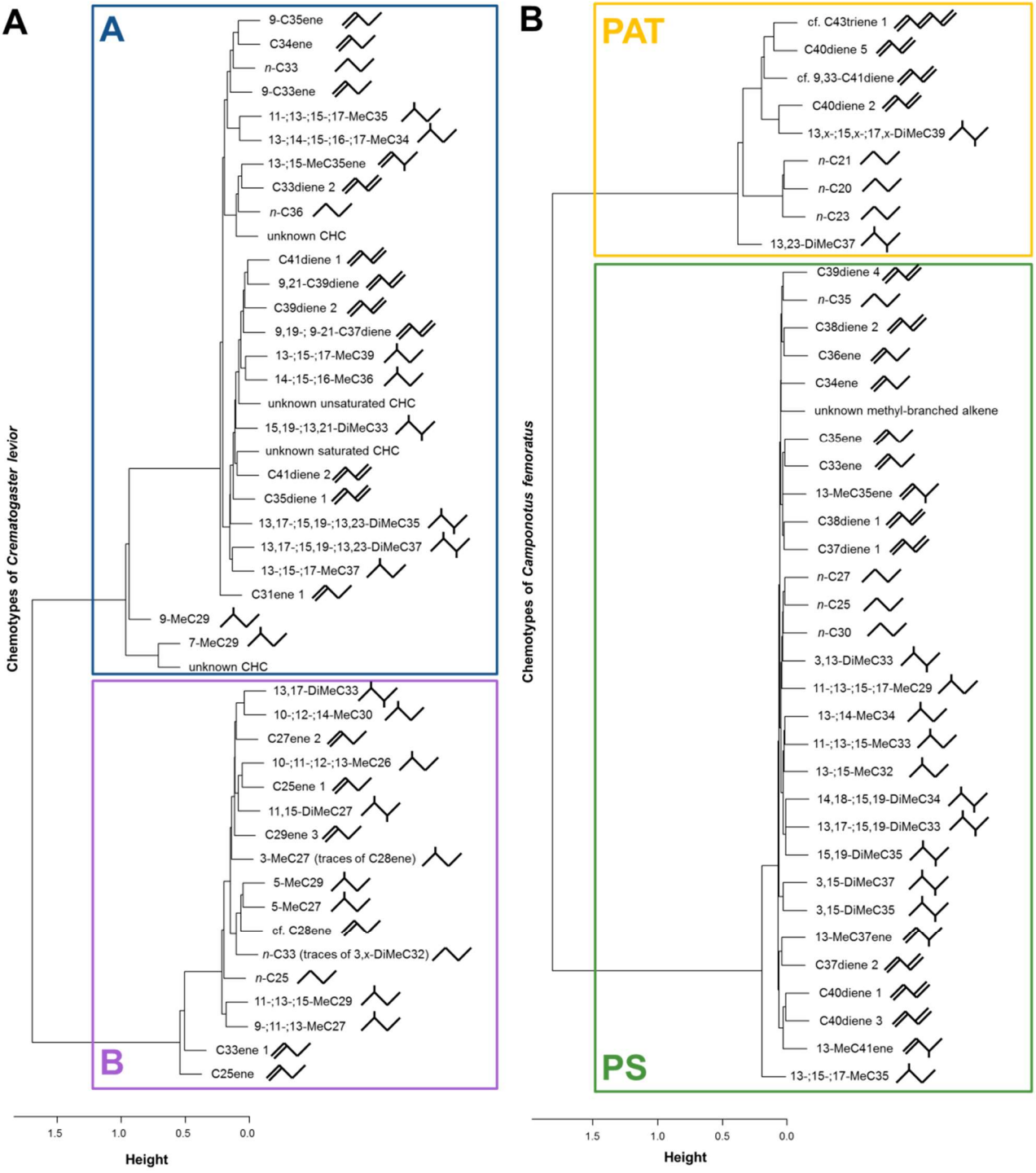

Figure 2.2: Hierarchical cluster analysis of substances characterizing the chemotypes of parabiotic ants. (A) Shows the different CHCs of the cryptic *Cr. levior* species with A in one cluster (top – blue) and B in one cluster (bottom – purple). (B) Shows the same for *Ca. femoratus* with PAT defined by fewer substances (top – yellow) compared to PS (bottom – green). Structures behind the substances indicate their substance class identities - note that these structures are just simplified symbols that do not reflect the actual structures (i.e. double bonds are most likely not conjugated and methyl groups usually do not occur on adjacent carbons in insect CHCs).

higher proportions of alkadienes ($W = 53$, $p < 0.001$) and also possessed alkenes ($W = 0$, $p < 0.001$), monomethyl alkanes ($W = 0$, $p < 0.001$) and methyl-branched alkenes ($W = 0$, $p < 0.001$), all three of which lacked in the characterizing substances of *Ca. femoratus* PAT.

2.3.3 Comparison of *Crematogaster levior* with other *Orthocrema* species

Crematogaster levior A was clearly separated from all other species in both the NMDS ordination (Fig. 3.3 A) and the cluster analysis (Fig. 3.3 B). The closest other species was *Cr. brasiliensis,* most likely caused by the high chain lengths of both species. In contrast, *Cr. levior* B grouped more closely to the related non-parabiotic species and the third chemotype *Cr. levior* C, and formed three groups rather than a single group within a bigger cluster (Fig. 3.3 B). The assignment of the third chemotype *Cr. levior* C was also ambiguous as they did not group evenly in the cluster.

The ancestral state reconstruction for the average CHC chain length in the *Orthocrema* clade revealed that the ancestral chain length was 27.91 ± 2.82 carbon atoms (reconstructed ancestral state ± SD; Table S3.4). The weighted average chain length of *Cr. levior* A was 31.91 and thus above this range, while the one of *Cr. levior* B was 27.19 (Fig. 3.3 C). This indicates that the chain length of *Cr. levior* A is a derived state, while for *Cr. levior* B, the trait does not differ from its ancestral state.

2.3.4 Comparison of *Camponotus femoratus* with other *Camponotus* species

In the ordination, colonies of *Ca. femoratus* PAT and PS were clearly separated, and located most closely to other parabiotic species (Fig. 4.4 A). The cluster analysis resulted in two stable parabiotic and one big stable non-parabiotic clusters (Fig. 4.4 B; Jaccard coefficient > 0.75). Three clusters were not stable and in total contained only four species (one parabiotic and three non-parabiotic; Jaccard coefficient < 0.75), leaving one species-rich parabiotic, one smaller parabiotic and one species-rich non-parabiotic as stable clusters. *Camponotus femoratus* PAT and PS were grouped next to each other in the species-rich parabiotic cluster together with three other parabiotic species (Menzel *et al*. 2017a).

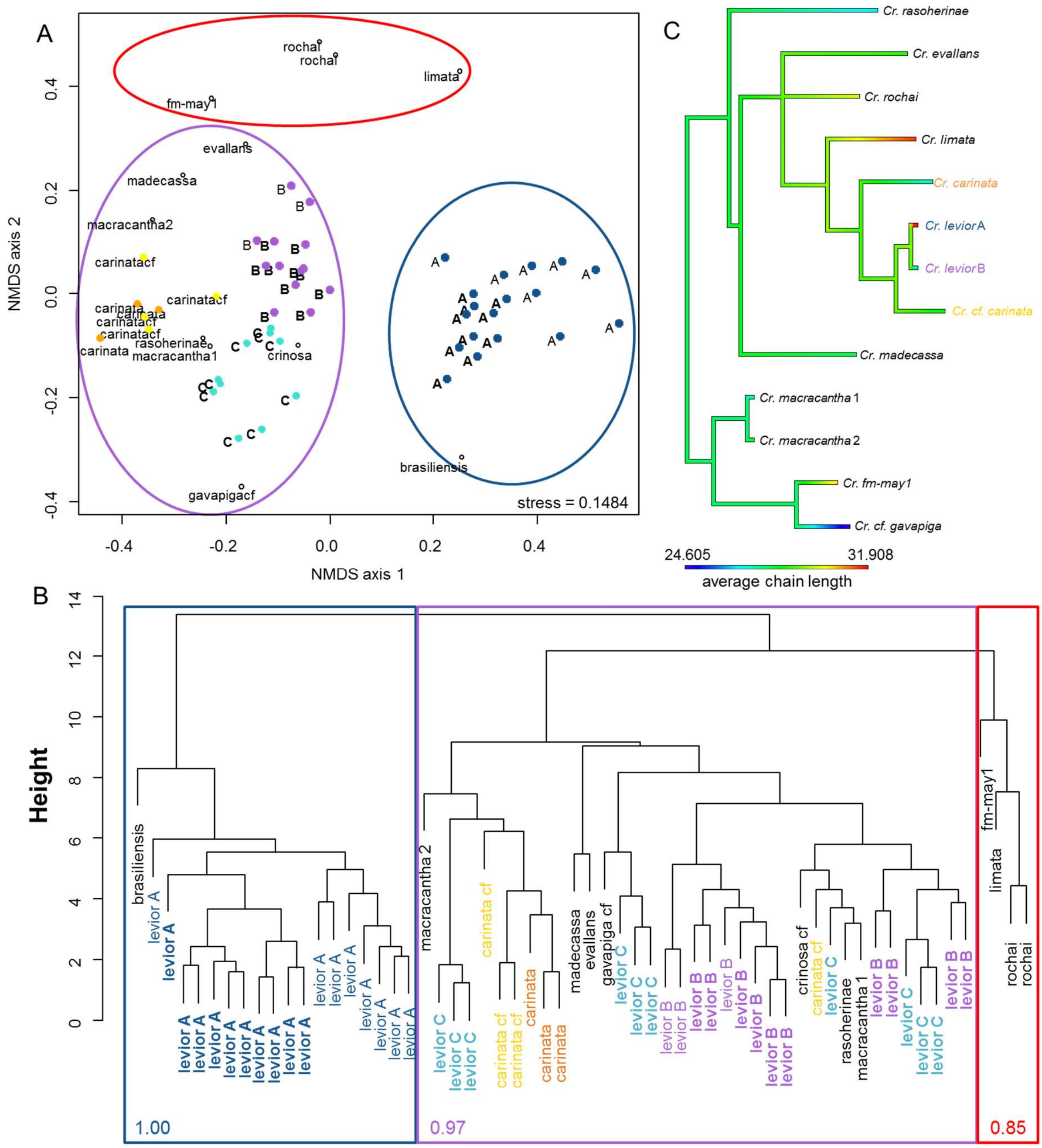

Figure 2.3: Ordination, cluster analysis and CHC chain length ancestral state of *Orthocrema* species. (A) Shows an NMDS ordination using Bray-Curtis dissimilarities of *Orthocrema* species based on the CHC data from (Menzel *et al.* 2017b) and ten colonies each of the three chemotypes we found (printed in bold). *Cr. levior* A clusters together with *Cr. brasiliensis* (blue circle), but is clearly separated from all other species, including *Cr. levior* B and C (purple circle). *Cr. limata* and *Cr. rochai* also form an own cluster (red circle). (B) Shows an average linkage hierarchical cluster analysis based on the same data. Numbers in the clusters show the mean Jaccard similarity between 100 bootstrap iterations. Values larger than 0.75 indicate that a cluster is stable. Again *Cr. levior* A as well as *Cr. limata* and *Cr. rochai* are clearly separated from all other *Orthocrema* species. Within the big group, *Cr. levior* B is in a single cluster, while *Cr. levior* C is distributed across two bigger branches. The *Cr. levior* A and B samples from this study group together with those of the previously published dataset in both analyses. (C) Shows an ancestral state reconstruction for the average CHC chain length based on the previously published phylogeny of the *Orthocrema* clade (Menzel *et al.* 2017b). The chain lengths of *Cr. levior* A and *Cr. limata* are strongly elongated compared to the ancestral state.

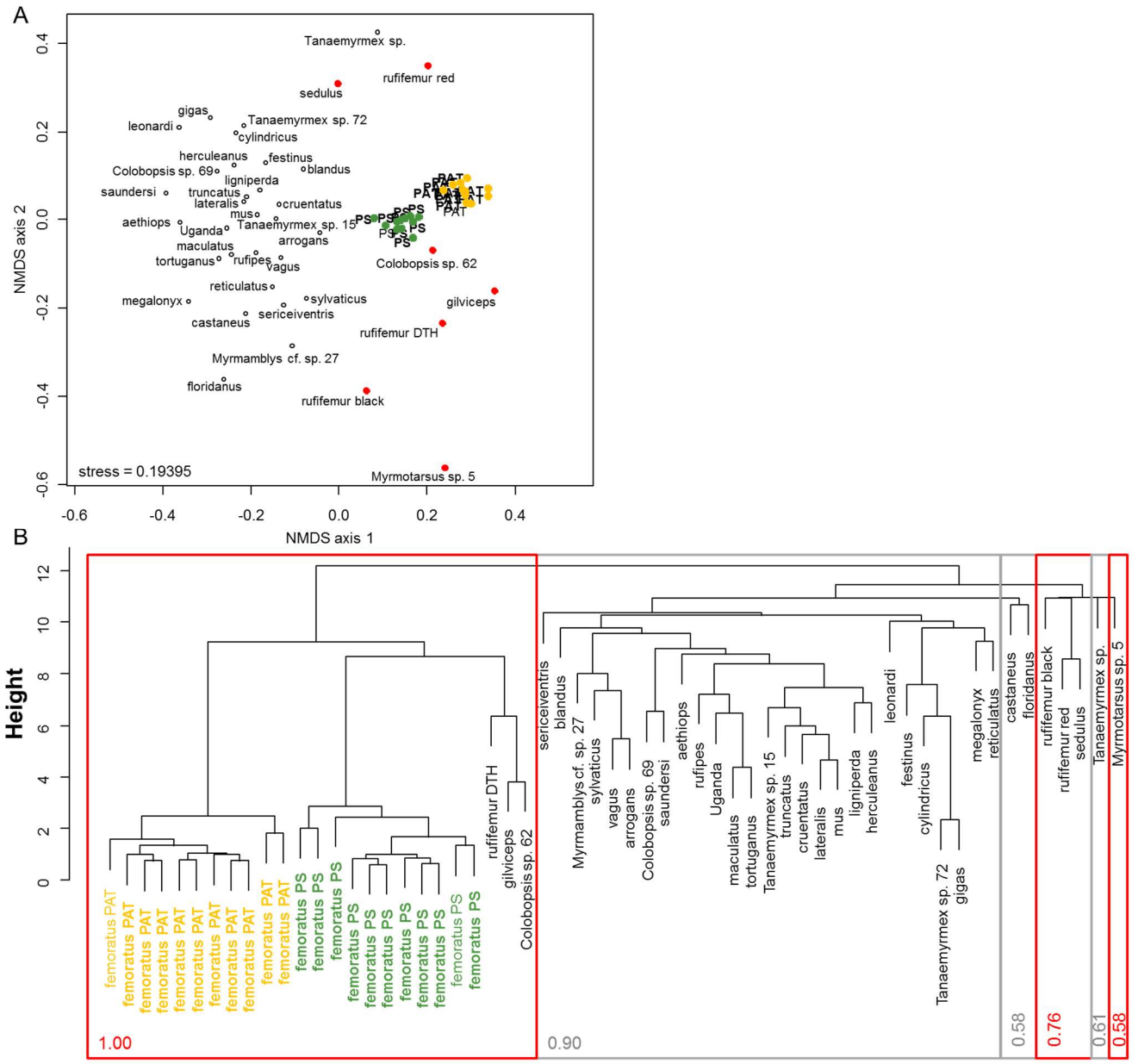

Figure 2.4: Ordination and cluster analysis of *Camponotus* species. (A) Shows an NMDS ordination using Bray-Curtis dissimilarities of *Camponotus* species based on the CHC data from (Menzel *et al.* 2017a) and each ten colonies of the *Ca. femoratus* species we found. *Ca. femoratus* PAT is depicted in orange, *Ca. femoratus* PS in green. Other parabiotic species are marked red. (B) Shows an average linkage hierarchical cluster analysis based on the same data. Numbers in the clusters show the mean Jaccard similarity between 100 bootstrap iterations. Values bigger than 0.75 indicate that a cluster is stable. Clusters with parabiotic species are framed in red, non-parabiotic ones in grey. New data points for *Ca. femoratus* PAT and PS reassemble the data from the previous study.

2.3.5 Signals of parabiotic partner

For each cryptic species separately, we searched in detail for substances which differed according to the identity of their parabiotic partner, based on pseudo-F values from multiple PERMANOVA analyses for each substance. Indeed, several of the identified CHCs were exclusively found or more abundant in the profiles of the respective mutualistic partners (Table 2.1).

In *Cr. levior* A, 9,19-; 9,21-C37-alkadiene was more abundant in colonies associated with *Ca. femoratus* PS (PERMANOVA: pseudo-F_1 = 15.57, p = 0.001). These substances were common in *Ca. femoratus* PS (mean abundance ± SD: 9.91 ± 3.65) but absent in *Ca. femoratus* PAT. In *Cr. levior* B, we only found substances more common when living together with PAT (i.e. no CHCs were more abundant in colonies living with PS). Here, 13,25-dimethyl C39 (pseudo-F_1 = 11.15, p = 0.003) was particularly interesting, because this substance was exclusively present in *Ca. femoratus* PAT (2.82 ± 1.08%).

For *Ca. femoratus* PAT, the substance which differed most depending on the parabiotic partner was a C40-alkadiene (pseudo-F_1 = 13.72, p = 0.001). However, this substance is neither present in *Cr. levior* A nor B. Under the aspect of signals of the mutualistic partner, *Ca. femoratus* PS was more interesting. The strongest effect was found in 11-;13-;15-monomethyl C29, which was more common in *Ca. femoratus* PS colonies associated with *Cr. levior* B (pseudo-F_1 = 19.02, p = 0.001), and which at the same time was one of the most abundant substances in this species compared to *Cr. levior* A (17.91 ± 7.73% vs. 0.36 ± 0.30%). In contrast, a C39-alkadiene was more common in *Camponotus* PS colonies living with *Cr. levior* A (pseudo-F_1 = 14.60, p = 0.001) than in PS colonies living with *Cr. levior* B. C39-alkadienes were common in *Cr. levior* A (4.85 ± 5.13%) while lacking in B.

2.3.6 Effects of species identity, partner and climate on the entire CHC profile

As expected, the CHC profile differed the strongest between *Cr. levior* A and B (PERMANOVA based on Bray-Curtis dissimilarities: pseudo-F_1 = 461.39, p = 0.001). Besides, we also found significant effects of their parabiotic partner (PAT vs PS; pseudo-F_1 = 4.27, p = 0.013) and of the climate (pseudo-F_1 = 4.20, p = 0.018). Interactions between species identity, partner and climate indicate that the cryptic species responded differently to climate and partner species, which is why they were analyzed separately below (interaction

species:partner: pseudo-F_1 = 3.33, p = 0.030; interaction species:climatePC1: pseudo-F_1 = 4.85, p = 0.010; interaction partner:climatePC1: pseudo-F_1 = 3.23, p = 0.029).

Ca. femoratus PAT and PS profiles likewise differed most strongly (pseudo-F_1 = 1233.83, p = 0.001). There was a trend towards an influence of climate (pseudo-F_1 = 2.99, p = 0.067), but the *Cr. levior* partner had no impact on the CHC profile in its entirety (pseudo-F_1 = 1.64, p = 0.174). We also found effects of the climate on single CHCs in both genera (Supplementary Results 2.1, Table S2.5).

Table 2.1: CHCs influenced by the identity of the parabiotic partner. The table lists the CHCs that differ among colonies of a single species with the two respective parabiotic partners, including the corresponding pseudo-F and p values from univariate PERMANOVAs based on Euclidean distance. For each substance, proportions in the focal species according to partner identity are given (relative proportion of CHC (%) of species living with partner; with higher proportion in bold), and the proportions in the respective partner species (relative proportion of CHC (%) in partner species), with substances printed bold if they fit the trend in the focal species. Numbers behind alkenes or alkadienes indicate different substances eluting at different retention times.

species	substance	pseudo-F	p	% of *Cr. levior* profile living with partner PAT	% of *Cr. levior* profile living with partner PS	% in *Ca. femoratus* PAT	% in *Ca. femoratus* PS
***Crematogaster levior* A**	13,17-;15,19-DiMeC35	21.042	0.001	1.16 ± 0.46	**1.55 ± 0.61**	0 ± 0	**0.88 ± 0.65** [3]
	15-MeC37ene 1	19.711	0.001	**0.57 ± 0.68**	0.17 ± 0.23	0 ± 0	13.44 ± 4.01 [4]
	C39diene 3	19.253	0.001	**0.17 ± 0.12**	0.09 ± 0.10	8.05 ± 2.24 [1]	9.75 ± 2.37 [1]
	unknown CHC (RT 11.44)	18.780	0.001	**0.22 ± 0.17**	0.12 ± 0.12	0 ± 0	0 ± 0
	13-;14-;15-;16-MeC32	15.694	0.001	0.23 ± 0.07	**0.28 ± 0.07**	0 ± 0	**0.70 ± 0.31** [2]
	9,19-; 9,21-C37diene (1)	15.570	0.001	3.24 ± 2.75	**5.26 ± 3.77**	0 ± 0	**9.91 ± 3.65** [1]
	15-MeC37ene 2	14.150	0.001	0.18 ± 0.25	**0.36 ± 0.35**	0 ± 0	**13.44 ± 4.01** [4]
	C33diene 2	14.025	0.001	**7.43 ± 7.04**	3.45 ± 5.90	0 ± 0	0 ± 0
	unknown CHC (RT 13.48)	12.367	0.002	**0.13 ± 0.09**	0.09 ± 0.05	0 ± 0	0 ± 0
	unknown CHC (RT 11.10)	12.358	0.001	**0.20 ± 0.18**	0.11 ± 0.12	0 ± 0	0 ± 0
	unknown CHC (RT 12.10)	12.077	0.002	**0.27 ± 0.22**	0.16 ± 0.14	0 ± 0	0 ± 0
***Crematogaster levior* B**	13,17-;15,19-DiMeC37	30.728	0.001	**0.33 ± 0.20**	0.15 ± 0.10	0 ± 0	0.27 ± 0.25 [3]
	unknown CHC (RT 11.10)	16.337	0.001	**0.01 ± 0.01**	0.01 ± 0.01	0 ± 0	0 ± 0
	13,25-DiMeC39	11.148	0.003	**0.13 ± 0.09**	0.08 ± 0.06	**2.82 ± 1.08** [5]	0 ± 0
	cf. 7-MeC23	10.152	0.005	**0.10 ± 0.09**	0.05 ± 0.07	0 ± 0	0 ± 0
	unknown CHC (RT 11.72)	9.557	0.002	**0.16 ± 0.10**	0.10 ± 0.09	0 ± 0	0 ± 0
	unknown CHC (RT 11.44)	9.555	0.002	**0.15 ± 0.11**	0.09 ± 0.09	0 ± 0	0 ± 0
	cf. 5-MeC23	9.392	0.002	**0.25 ± 0.13**	0.17 ± 0.14	0 ± 0	0 ± 0
	unknown CHC (RT 11.92)	8.727	0.002	**0.20 ± 0.17**	0.12 ± 0.10	0 ± 0	0 ± 0
	unknown CHC (RT 12.10)	8.006	0.007	**0.36 ± 0.21**	0.25 ± 0.18	0 ± 0	0 ± 0
	unknown CHC (RT 13.48)	7.960	0.007	**0.20 ± 0.09**	0.16 ± 0.06	0 ± 0	0 ± 0
	9-;11-MeC23	7.802	0.004	**0.41 ± 0.19**	0.32 ± 0.14	0 ± 0	0 ± 0
	unknown CHC (RT 11.60)	7.785	0.006	**0.17 ± 0.10**	0.12 ± 0.09	0 ± 0	0 ± 0
	unknown CHC (RT 12.48)	6.900	0.009	**0.25 ± 0.13**	0.20 ± 0.12	0 ± 0	0 ± 0

species	substance	pseudo-F	p	% of *Ca. femoratus* profile living with partner A	% of *Ca. femoratus* profile living with partner B	% in *Cr. levior* A	% in *Cr. levior* B
***Camponotus femoratus* PAT**	C40diene 4	13.721	0.001	**1.16 ± 0.40**	0.96 ± 0.36	0 ± 0	0 ± 0
	13,x-;15,x-;17,x-DiMeC39	6.473	0.010	**3.01 ± 1.00**	2.62 ± 1.14	**0.36 ± 0.20** [6]	0.11 ± 0.09 [6]
	n-C23	4.392	0.038	0.95 ± 1.23	**1.35 ± 1.42**	0.30 ± 0.20	**0.89 ± 0.66**
	C41diene 4	4.406	0.045	**3.00 ± 1.06**	2.71 ± 0.89	**0.93 ± 1.14** [1]	0 ± 0
	C41diene 5	5.699	0.015	**0.65 ± 0.45**	0.52 ± 0.28	**0.93 ± 1.14** [1]	0 ± 0
***Camponotus femoratus* PS**	11-;13-;15-MeC29	19.018	0.001	0.12 ± 0.13	**0.33 ± 0.36**	0.36 ± 0.30	**17.91 ± 7.73**
	n-C25	16.592	0.001	0.11 ± 0.09	**0.22 ± 0.18**	0.26 ± 0.20	**11.20 ± 5.69**
	C39diene 1	14.603	0.001	**0.09 ± 0.14**	0 ± 0	**4.84 ± 5.12** [1]	0 ± 0
	15,19-DiMeC41	5.886	0.016	0.08 ± 0.11	**0.15 ± 0.21**	0 ± 0	0 ± 0
	C33ene	5.598	0.003	**0.79 ± 0.84**	0.48 ± 0.27	**14.25 ± 4.13** [1]	1.14 ± 1.50 [1]
	unknown CHC (RT 30.40)	5.464	0.019	0.02 ± 0.04	**0.05 ± 0.07**	0 ± 0	0 ± 0
	C39diene 2	5.364	0.031	**6.17 ± 2.12**	5.24 ± 1.93	**4.84 ± 5.12** [1]	0 ± 0
	C37diene 1	5.302	0.011	**0.62 ± 0.35**	0.48 ± 0.22	**5.53 ± 4.08** [1]	0 ± 0
	unknown CHC (RT 30.60)	5.296	0.020	**0.12 ± 0.11**	0.08 ± 0.08	0 ± 0	0 ± 0
	C38diene 1	4.994	0.021	**1.01 ± 0.28**	0.89 ± 0.28	0 ± 0	0 ± 0

[1] combined percentages of all alkenes or alkadienes; [2] only 13-;15-MeC32; [3] only 15,19-DiMe; [4] 13-MeC37ene – different methyl-group position; [5] mixed with other dimethyl-alkanes; [6] 13,x-;13,25-DiMeC39

2.4 Discussion

With our large-scale sampling, we confirmed that both previously described chemotypes of *Cr. levior* and *Ca. femoratus* were stable and present over a wide geographical range (Emery & Tsutsui 2013; Menzel *et al.* 2014; Hartke, Sprenger *et al.* 2019, Chapter 1). In the following, we will discuss the chemical diversity between the parabiotic cryptic species and how they differ from related non-parabiotic species, the influence of biotic interactions (parabiotic partner) and the impact of climate on their CHC profiles.

2.4.1 Chemical diversity and influence of parabiotic lifestyle

The parabiotic lifestyle was shown to promote certain characteristics of the CHC profile like higher chain length or higher abundances of alkadienes and methyl-branched alkenes (Menzel & Schmitt 2012). This is probably the case because it requires a reduction of recognition cues that can be perceived by the mutualistic partner to facilitate interspecific tolerance. By increasing the chain length of hydrocarbons, their biophysical properties will change: Usually the melting point increases with chain length of structurally similar substance classes (Gibbs & Pomonis 1995). However, insects should need to maintain a

sufficiently high fluidity in their CHC layer for functions like communication, foot adhesion or lubrication (Sprenger *et al.* 2018), which is why chain elongation in hydrocarbons is constrained. To keep a more semi-liquid state of the CHC layer, the insertion of disrupting features, either methyl-branches or double bonds, that lower the melting temperature are required (Menzel *et al.* 2017a). At ambient temperatures, long-chained hydrocarbons with such disrupting features should be similarly liquid but less volatile (and thus less perceivable) than shorter-chained ones as the difference between melting and boiling point increases with chain length (Li, Higashi & Tamura 2006).

Although both *Cr. levior* species live in parabiosis, only the CHC profiles of *Cr. levior* A showed elongated carbon chains and high abundances of alkadienes. In contrast, *Cr. levior* B had CHCs of shorter chain length, which require fewer disrupting features (here, a high abundance of mono-methyl alkanes; Fig. 2.2 A). In our comparison with other species of the *Orthocrema* clade *Cr. levior* A was chemically most distant from all others (with only *Cr. brasiliensis* in the same cluster; Fig. 2.3 B). This separation is probably due to longer CHC chains in *Cr. levior* A. *Cr. levior* B on the other hand was more similar to most other *Orthocrema* species, and their chain lengths were similar to the ancestral state.

The lack of characteristic adaptations of the CHC profile in *Cr. levior* B despite living in parabiosis is surprising. It calls for additional studies to investigate whether *Cr. levior* A and B differ in how much they are tolerated by *Camponotus femoratus*. For example, due to their shorter chain lengths, *Cr. levior* B might be attacked more frequently. Alternatively, there might be other mechanisms than modification of the CHC profile to achieve interspecific tolerance. For example, beside CHCs, *Cr. levior* A and B also possess species-specific polar substances on their cuticles (Hartke, Sprenger *et al.* 2019, Chapter 1). Their function is currently unknown, but they might function as appeasement allomones, similarly to other parabiotic *Crematogaster* species in South-East Asia (Menzel *et al.* 2013). Additional behavioral tests using fractions of these polar compounds could give insights if they indeed function as appeasement allomones.

Interestingly, we found an additional chemotype in *Crematogaster* (*Cr. levior* C) that greater CHC similarity to *Cr. levior* B compared to A (see Fig. 2.1 A, 2.3 A). Morphological examination revealed that the individuals had carinae (keel-like crests on the pronotum) comparable to *Cr. carinata,* which is otherwise morphologically very similar to *Cr. levior*. Both were only quite recently divided into separate species (Longino 2003). Nevertheless, genetically, the *Cr. levior* C colonies were different from *Cr. carinata* and also from *Cr. levior* A

and B (concerning the COI locus; data not shown). They either lived in parabiosis with *Odontomachus mayi* or were found trail-sharing with unidentified *Camponotus* ants. Similarly, *Cr. carinata* is also facultatively parabiotic with different *Dolichoderus* and *Odontomachus* species (Longino 2003). This finding suggests that there might be an even higher chemical diversity within the *Cr. levior/Cr. carinata* complex, with possibly more cryptic species to be discovered.

In *Camponotus*, all parabiotic species in our comparison differed from non-parabiotic congeners (Fig. 2.4). The cryptic species *Ca. femoratus* PAT and PS possessed profiles that differed substantially from each other. *Ca. femoratus* PS had higher proportions of alkadienes and methyl-branched alkenes, which are generally more common in parabiotic species (Martin & Drijfhout 2009c; Menzel & Schmitt 2012; Menzel *et al.* 2017a). The mean chain length, however, was increased in both species as well as other parabiotic species, which could again indicate adaptations to the mutualism. Nevertheless, *Ca. femoratus* PAT and PS seem to use slightly different strategies to allow this chain elongation, i.e. to introduce disruptive features into the CHC chains: While both have characteristic alkadienes, *Ca. femoratus* PAT has high proportions of di-methyl alkanes, whereas *Ca. femoratus* PS produces methyl-branched alkenes, a quite rare substance group in ants (Martin & Drijfhout 2009c) (Fig. 2.2 B). The compounds with a high chain length thus differ in their chemical structure between the two cryptic species.

2.4.2 Biotic interactions influence CHC profiles

Although the two partner species had consistently different CHC profiles, we detected some signals of major compounds of one species in the respective parabiotic partner. In several cases, we found a major compound in one partner to be more abundant in those colonies of the other species that lived with this partner. However, only few CHCs showed this signal, indicating that there is no simple substance transfer between parabiotic partners. Rather, there might be either selective transfer of few compounds (via trophallaxis or via nest material), or partial chemical mimicry where one species produces higher quantities of a certain CHC only if it is associated to one of the two cryptic species. Although the relative abundances of these substances could still be high in one partner while they were only traces in the other partner, in both cases these shared CHCs might promote the interspecific tolerance of the parabiotic ants and make it easier to familiarize with the profile of the associated species (Orivel *et al.* 1997; Lenoir *et al.* 2001b).

Based on our data, we cannot conclusively distinguish between chemical mimicry (i.e. active production of similar CHCs) and chemical camouflage (i.e. active or passive acquisition of the partner CHCs). However, not only the most common substances were exchanged, which would be the most likely scenario if the mechanism was chemical camouflage. Only a subset of certain substances could be found and they were not always present in the parabiotic partner, which tentatively hints towards an active production of CHCs according to the associated partner. Interestingly, we found that *Cr. levior* B has higher proportions of 13,25-DiMeC39, a substance only common in *Ca. femoratus* PAT if associated with them. On the other hand, *Cr. levior* A has higher proportions of C41-dienes if they live with *Ca. femoratus* PAT colonies. This indicates that in *Cr. levior* B, itself richer in monomethyl alkanes, profiles change in abundance of a dimethyl alkane, while in the alkadienes-rich *Cr. levior* A alkadienes change most. In *Camponotus*, we found almost no signal of the *Cr. levior* partner in PAT colonies, while *Ca. femoratus* PS showed signals of very prominent CHCs of their respective parabiotic partners (with A: C39-dienes; with B: 11-;13-;15-methyl nonacosane; Table 2.1). According to these trends, it seems that *Crematogaster* needs to have changed profiles to co-habit with *Ca. femoratus* PAT (which itself remains unchanged), while *Ca. femoratus* PS shows signals of its respective *Crematogaster* partner.

In *Cr. levior* A one of the CHCs differing depending on the parabiotic partner was 15-MeC37ene. *Ca. femoratus* PAT however produced 13-MeC37ene, a similar substance with a different methyl-group position. Although we do not know the position of the double bond in both cases, this could indicate an incomplete chemical mimicry by *Cr. levior* A. Such imperfect mimicry with CHC profiles that do not entirely fit the host profile can be found in two parasitoid wasp species and the ant *Ectatomma parasiticum* that both parasitize the ant *E. tuberculatum* (Savarit & Fénéron 2014; Pérez-Lachaud *et al.* 2015).

The chemical interactions in this parabiotic association are likely to be even more complex since both species also interact with the ant garden plants. *Camponotus femoratus* was found to actively collect seeds of specific ant garden plants and recognize them based on chemical signals (Youngsteadt *et al.* 2008, 2009). However, the seeds of different plants did not share common signals eliciting this carrying behavior (Youngsteadt, Bustios & Schal 2010). Furthermore, at least *Ca. femoratus* recruits to damaged leaves of the ant garden plant, indicating that they actively defend the plants against herbivory (Vantaux *et al.* 2007; Vicente *et al.* 2014; Leal *et al.* 2017). Further studies should investigate if the chemically different cryptic species described here differ in their reactions to certain plant odors or show

differences in recruiting to leaf damage. Such experiments would give insight if certain plants coevolve with one or the other cryptic species of *Ca. femoratus* or *Cr. levior*.

2.4.3 Climate influences on CHC profiles

We found that the climate affected the whole CHC profile of both species of *Cr. levior*. Climate adaptations were found in *Drosophila melanogaster*, populations along the east coast of the United States (Rajpurohit *et al.* 2017) but also in several ant species (Menzel *et al.* 2017a). Furthermore, ants are able to acclimate their CHC profile on a short-term basis e.g. to higher temperatures (Menzel *et al.* 2018; Sprenger *et al.* 2018) which can lead to differences in populations between seasons (Buellesbach *et al.* 2018). In *Ca. femoratus*, we only found a non-significant trend when also accounting for species identity and partner in the PERMANOVA. Probably, this is because the distribution of the cryptic *Ca. femoratus* species was associated with the climatic gradient in French Guiana. *Ca. femoratus* PS was very rare in eastern French Guiana, where precipitation is higher and temperature slightly lower (Hartke, Sprenger *et al.* 2019, Chapter 1; Fig. S2.1), while both PS and PAT occur in the drier west. This difference in distribution could probably hint at niche partitioning according to different climate adaptations.

2.5 Conclusions

In this study, we demonstrate that the cryptic species of *Cr. levior* and *Ca. femoratus* strongly differ in their CHC profiles as well as in the proportions of certain substance classes. The parabiotic lifestyle led to hydrocarbon backbone elongations in *Cr. levior* A, *Ca. femoratus* PAT and *Ca. femoratus* PS. These results suggest that differences in certain substance classes might represent different strategies to allow CHC chain elongation towards the very high chain lengths present in parabiotic ants that promotes the highly unusual interspecific tolerance. In contrast, *Cr. levior* B has a CHC profile comparable to other non-parabiotic *Orthocrema* clade-species and thus is closer to the ancestral state concerning the average CHC chain length. It remains to be studied whether this CHC differences results in differences in interspecific tolerance that *Cr. levior* A and B experience from their parabiotic partner.

Importantly, we also demonstrate that the CHC profiles of parabiotic ants are influenced by their mutualistic partners as well as by the climate. Depending on the association, one or the

other partner seems to adjust its CHC profile either actively or passively, which could facilitate the co-habituation. Although we cannot determine the mechanism behind the shared CHCs among the parabiotic partners (i.e. mimicry or substance transfer) yet, the interspecific tolerance in this parabiotic system offers further chances to investigate the establishment of mutualistic interactions and the chemical mechanisms facilitating them.

2.6 Acknowledgements

Removed for privacy purposes.

2.7 Supplementary material

2.7.1 Supplementary results 2.1: Effects of climate on single CHCs

The CHC profiles of all four species were affected by climate, however with different consequences. In *Cr. levior* A climate affected the CHC profile (multivariate PERMANOVA based on Bray-Curtis dissimilarities: pseudo-F_1 = 6.13, p = 0.001) via an increase of three alkadienes and two methyl-branched alkenes at higher precipitation and lower temperature. On the other hand, one *n*-alkane, two monomethyl alkanes, one dimethyl alkane, one alkene and one alkadiene with higher chain length were more abundant at lower precipitation and higher temperatures (Table S2.5). Climate also affected the CHC profile of *Cr. levior* B (pseudo-F_1 = 5.01, p = 0.001). The relative proportion of two alkenes increased with higher precipitation and lower temperatures while two alkadienes decreased with increasing rainfall and cooler temperatures (Table S2.5).

In *Camponotus*, the CHCs of *Ca. femoratus* PAT were significantly influenced by climatic conditions (pseudo-F_1 = 3.21, p = 0.022). Here, one alkene, four alkadienes and one methyl-branched alkene were affected most strongly and all these hydrocarbons increased at higher temperature with less precipitation while two dimethyl alkanes decreased (Table S2.5). The effect of climate was even stronger in *Ca. femoratus* PS (pseudo-F_1 = 4.86, p = 0.001). One alkadiene increased with higher precipitation and lower temperature, whereas four methyl-branched alkenes decreased along this gradient (Table S2.5).

Table S2.1: Sampling locations. Note that the coordinates reflect the actual sampling locations, not the cities the locations are named after.

Location	**Latitude**	**Longitude**	**Elevation (m)**	**#** ***Crematogaster***	**#** ***Camponotus***
Apatou (AP)	5.200783	-54.312017	28	16	16
Saint-Laurent-du-Maroni (SL)	5.463902	-53.997322	63	37	36
Angoulême (AN)	5.409200	-53.650933	64	1	1
Sinnamary (SI)	5.352035	-53.077604	45	19	20
Petit Saut (PS)	5.061213	-52.988772	93	22	18
Paracou (PAR)	5.265905	-52.933605	41	51	49
Les Nouragues (LN)	4.039650	-52.673933	63	73	61
Kourou (KO)	5.083106	-52.643022	23	12	11
Montsinéry-Tonnegrande (MT)	4.866000	-52.538483	26	4	4
Cacao (CA)	4.557416	-52.463067	71	23	20
Cayenne (CAY)	4.793831	-52.317594	20	6	5
Régina (RE)	4.181286	-52.131963	82	16	14
Camp Patawa (PAT)	4.546067	-52.130483	282	52	51
Total				**332**	**306**

Table S2.2: Cuticular hydrocarbons of the cryptic *Crematogaster levior* species. In this table we present the identified CHCs, their mean abundances and the retention indices (RI) diagnostic ions for identification. Numbers in italics are the M^+ ions; diagnostic ions in brackets were obtained after DMDS derivatization of unsaturated hydrocarbons. Note that, for unsaturated hydrocarbons, position of double bonds could only be determined for *Cr. levior* A.

CHC	RI	mean ± SD in *Cr. levior* A	mean ± SD in *Cr. levior* B	mean ± SD in *Cr. levior* C	diagnostic ions and M^+
C20ene	20.00	2.36 ± 1.74	1.69 ± 0.97	2.17 ± 1.51	*280*
n-C20	20.06	4.72 ± 3.2	3.49 ± 1.86	3.53 ± 1.09	*282*
unknown CHC (RT 8.84)	20.14	0.57 ± 0.46	0.39 ± 0.23	0.48 ± 0.25	
C22ene	21.99	1.14 ± 0.77	0.82 ± 0.41	0.72 ± 0.21	*308*
n-C22	22.05	2.47 ± 1.6	1.84 ± 0.83	1.77 ± 0.55	*310*
unknown CHC (RT 11.10)	22.12	0.63 ± 0.45	0.44 ± 0.23	0.47 ± 0.17	
unknown CHC (RT 11.44)	22.38	0.18 ± 0.19	0.11 ± 0.08	0.11 ± 0.08	
unknown CHC (RT 11.48)	22.41	0.18 ± 0.16	0.13 ± 0.11	0.14 ± 0.07	
unknown CHC (RT 11.60)	22.50	0.37 ± 0.26	0.25 ± 0.14	0.26 ± 0.1	
unknown CHC (RT 11.72)	22.59	0.24 ± 0.17	0.16 ± 0.1	0.17 ± 0.07	
unknown CHC (RT 11.94)	22.76	0.25 ± 0.27	0.15 ± 0.1	0.1 ± 0.12	
unknown CHC (RT 12.10)	22.88	0.24 ± 0.21	0.17 ± 0.15	0.22 ± 0.09	
n-C23	23.05	0.3 ± 0.2	0.88 ± 0.66	3.28 ± 3.84	*324*
unknown CHC (RT 12.48)	23.15	0.4 ± 0.35	0.33 ± 0.21	0.33 ± 0.16	
unknown CHC (RT 12.74)	23.33	0.36 ± 0.24	0.24 ± 0.13	0.23 ± 0.12	
cf. 9-;11-MeC23	23.41	0.42 ± 0.28	0.39 ± 0.18	0.44 ± 0.17	140/224; 168/196
cf. 7-MeC23	23.47	0.12 ± 0.16	0.09 ± 0.09	0.14 ± 0.11	112/252
cf. 5-MeC23	23.54	0.35 ± 0.34	0.22 ± 0.14	0.24 ± 0.12	85/281
3-MeC23	23.77	0.08 ± 0.09	0.07 ± 0.07	0.15 ± 0.11	57/309
unknown CHC (RT 13.48)	23.84	0.13 ± 0.09	0.09 ± 0.05	0.08 ± 0.05	
C24ene	23.99	0.43 ± 0.29	0.31 ± 0.15	0.32 ± 0.09	*336*
C25ene 1	24.79	0 ± 0	0.03 ± 0.26	0.12 ± 0.11	*350*
C25ene 2	24.88	0 ± 0	0.12 ± 0.1	2.04 ± 2.65	*350*
C25ene 3	25.04	0 ± 0	0.08 ± 0.27	0.25 ± 0.29	*350*
n-C25	25.05	0.26 ± 0.2	11.2 ± 5.69	10.08 ± 7.58	*352*
unknown CHC (RT 15.64)	25.27	0.2 ± 0.13	0.19 ± 0.08	0.15 ± 0.04	
cf. 9-;11-;13-MeC25	25.41	0.35 ± 0.31	0.68 ± 0.36	0.86 ± 0.5	140/252; 168/224; 197/197
7-MeC25	25.46	0 ± 0	0 ± 0	0.34 ± 0.43	112/280
unknown CHC (RT 16.22)	25.64	0.25 ± 0.14	0.17 ± 0.09	0.19 ± 0.06	
C26ene	26.01	0.18 ± 0.11	0.12 ± 0.07	0.12 ± 0.08	*364*
n-C26	26.06	0.66 ± 0.3	1.14 ± 0.36	0.92 ± 0.28	366
unknown CHC (RT 16.98)	26.12	0.12 ± 0.09	0 ± 0	0 ± 0	
8-;9-;10-MeC26	26.37	0 ± 0	0 ± 0	0.25 ± 0.22	126/280; 140/266; 154/252
11-;12-;13-MeC26	26.40	0 ± 0	0.14 ± 0.05	0 ± 0	168/238; 182/224; 196/210

C27diene 1	26.58	0 ± 0	0 ± 0	0.06 ± 0.09	*376*
C27diene 2	26.62	0 ± 0	0 ± 0	0.07 ± 0.06	*376*
C27diene 3	26.75	0 ± 0	0 ± 0	0.12 ± 0.15	*376*
C27ene 1	26.78	0 ± 0	0.29 ± 0.58	10.17 ± 9.65	*378*
C27ene 2	26.81	0 ± 0	0.81 ± 1.64	4.71 ± 8.39	*378*
C27ene 3	27.02	0 ± 0	0.25 ± 0.83	0.95 ± 0.98	*378*
n-C27	27.06	1.69 ± 1.32	5.17 ± 2.01	11.43 ± 8.84	*380*
9-;11-;13-MeC27	27.39	0.25 ± 0.11	2.35 ± 1.57	0.64 ± 0.79	140/280; 168/252; 196/224
unknown CHC (RT 19.08)	27.44	0.17 ± 0.16	0.18 ± 0.37	4.85 ± 5.83	
7-MeC27	27.51	0 ± 0	0 ± 0	1.83 ± 2.72	112/308
5-MeC27	27.58	0 ± 0	0.17 ± 0.24	0.12 ± 0.07	85/336
11,15-DiMeC27	27.69	0 ± 0	0.12 ± 0.06	0.22 ± 0.21	168/266, 238/196
cf. C28ene	27.75	0 ± 0.01	0.19 ± 0.16	0.54 ± 0.61	*392*
3-MeC27 (with traces of C28ene)	27.80	0 ± 0	0.27 ± 0.17	0.02 ± 0.05	57/365
n-C28	28.07	0.58 ± 0.21	0.92 ± 0.22	0.52 ± 0.23	*394*
unknown CHC (RT 20.70)	28.46	0 ± 0	0 ± 0	0.26 ± 0.31	
C29diene 1	28.66	0 ± 0	0 ± 0	0.93 ± 0.9	*404*
C29ene 1	28.72	0 ± 0	0 ± 0	0.9 ± 1.61	*406*
C29ene 2	28.75	0 ± 0	0 ± 0	0.85 ± 1.08	*406*
C29diene2	28.81	0.02 ± 0.09	0.25 ± 1.5	0.63 ± 1.38	*404*
C29ene 3	28.82	0.11 ± 0.13	4.12 ± 5.75	7.55 ± 6.5	*406*
C29diene 3	28.89	0 ± 0	3.28 ± 4.06	0 ± 0	*404*
C29ene 4	28.9	0 ± 0	2.76 ± 4.06	3.73 ± 5.14	*406*
C29ene 5	28.92	0 ± 0	0.46 ± 0.32	0.05 ± 0.1	*406*
C29ene 6	29.04	0.06 ± 0.26	0 ± 0	0.28 ± 0.58	*406*
n-C29	29.08	5.46 ± 2.23	5.35 ± 1.85	3.19 ± 1.47	*408*
11-; 13-;15-MeC29 (11-MeC29 only in *Cr. levior* B)	29.38	0.36 ± 0.3	17.97 ± 7.73	1.72 ± 1.85	168/280; 196/252; 224/224
9-MeC29	29.43	0.01 ± 0.03	0.12 ± 0.51	2.25 ± 1.92	140/308
7-MeC29	29.47	0.06 ± 0.04	0.49 ± 1.01	1 ± 0.96	112/336
5-MeC29	29.57	0 ± 0	0.95 ± 1.26	0.08 ± 0.04	85/365
13,17-DiMeC29 (traces of 11,*x*)	29.65	0 ± 0	0.62 ± 0.77	0.85 ± 0.75	196/266, 266/196
11,*x*-DiMeC29	29.68	0 ± 0	0.21 ± 0.46	0 ± 0	168/294, x
C30diene (with traces of 3-MeC29)	29.86	0 ± 0	0.31 ± 0.26	0.01 ± 0.03	*418*
C30ene	29.88	0 ± 0	0.16 ± 0.26	0.3 ± 0.38	*420*
3-MeC29	29.89	0 ± 0	0 ± 0	0.33 ± 0.43	57/393
n-C30	30.06	0.4 ± 0.1	0.45 ± 0.16	0.14 ± 0.04	*422*
10-;12-;14-MeC30	30.38	0 ± 0	0.9 ± 0.54	0 ± 0	154/308; 182/280; 210/252
cf. 12,16-;13,17-DiMeC30	30.64	0 ± 0	0.05 ± 0.09	0 ± 0	182/294, 252/224; 196/280, 266/210
C31triene	30.66	0.12 ± 0.78	0.05 ± 0.27	0 ± 0	*430*
C31diene 1	30.68	0 ± 0	0.01 ± 0.07	2 ± 2.18	*432*
C31ene 1	30.69	0.12 ± 0.16	0 ± 0	0.12 ± 0.2	*434*
C31diene 2	30.80	0 ± 0	0.6 ± 1.61	0.86 ± 1.01	*432*
C31ene 2	30.81	0 ± 0	5.29 ± 6.04	2.52 ± 3.14	*434*

C31diene 3	30.85	0.27 ± 1.5	1.54 ± 2.07	0 ± 0	*432*
C31ene 3 (9-C31ene)	30.86	2.53 ± 1.32	1.71 ± 2.69	0.54 ± 0.66	*434* [173/355]
C31diene 4	30.91	0.07 ± 0.86	0.02 ± 0.14	0 ± 0	*432*
C31ene 4	30.93	0.09 ± 0.11	0.18 ± 0.28	0.09 ± 0.14	*434*
n-C31	31.07	2.01 ± 0.63	0.9 ± 0.4	0.43 ± 0.39	*436*
cf. 5-MeC31ene	31.29	0 ± 0	0.12 ± 0.2	0 ± 0	83/390, 97/406, *448*
11-;13-;15-MeC31 (11-MeC31 only in *Cr. levior* B and C)	31.37	1.11 ± 0.48	6.59 ± 4.32	0.54 ± 0.54	168/308; 196/280; 224/252
9-MeC31	31.48	0 ± 0.01	0.18 ± 0.45	0.2 ± 0.25	140/336
7-MeC31	31.50	0 ± 0	0.13 ± 0.25	0.26 ± 0.3	112/364
5-MeC31; 13,17-DiMeC31	31.58	0.27 ± 0.11	1.3 ± 0.85	0 ± 0	85/393; 196/294, 266/224
unknown CHC (RT 25.94)	31.87	0.44 ± 0.12	0.16 ± 0.12	0 ± 0	
n-C32	32.07	0.26 ± 0.12	0.17 ± 0.09	0 ± 0	*450*
13-;14-;15-;16-MeC32	32.37	0.25 ± 0.07	0.2 ± 0.13	0 ± 0	196/294; 210/280; 224/266; 238/252
C33diene 1	32.53	0.23 ± 0.26	0 ± 0	0 ± 0	*460*
12-,16-DiMeC32	32.61	0 ± 0	0.11 ± 0.17	0 ± 0	182/322, 252/252
C33diene 2	32.63	5.69 ± 6.86	0 ± 0	0 ± 0	*460*
C33triene 1	32.64	0 ± 0	0.01 ± 0.06	0 ± 0	*458*
C33diene 3	32.71	0.47 ± 0.54	0.18 ± 0.62	0.03 ± 0.08	*460*
C33ene 1	32.74	0.16 ± 0.3	1.04 ± 1.45	0.05 ± 0.1	*462*
C33triene 2	32.79	0.04 ± 0.56	0 ± 0	0 ± 0	*458*
C33diene 4	32.81	1.69 ± 2.61	0.06 ± 0.63	0 ± 0	*460*
C33ene 2	32.82	0 ± 0	0.07 ± 0.22	0.07 ± 0.19	*462*
C33diene 5	32.87	0.06 ± 0.78	0.08 ± 0.1	0 ± 0	*460*
C33ene 3 (9-C33ene)	32.89	14.09 ± 4.08	0.03 ± 0.13	0 ± 0	*462* [173/383]
n-C33 (traces of 3,x-DiMeC32)	33.05	0 ± 0	0.16 ± 0.09	0 ± 0	*464*
n-C33	33.07	1.08 ± 0.4	0 ± 0	0 ± 0	*464*
15-MeC33ene	33.14	0.35 ± 0.38	0 ± 0	0 ± 0	223/278, 238/294, *476*
9-;11-;13-;15-;17-MeC33	33.37	5 ± 1.62	0.99 ± 0.97	0.15 ± 0.46	140/364; 168/336; 196/308; 224/280; 252/252
C34diene 1	33.54	0.03 ± 0.06	0 ± 0	0 ± 0	*474*
13,17-DiMeC33	33.6	0 ± 0	1.43 ± 1.95	0 ± 0	196/322, 266/252
15,19-; 13,21-DiMeC33	33.61	1.18 ± 0.5	0.02 ± 0.21	0 ± 0	224/294, 294/224; 196/322, 322/196
C34diene 2	33.88	0 ± 0.04	0 ± 0	0 ± 0	*474*
C34ene	33.89	0.28 ± 0.09	0 ± 0	0 ± 0	*476*
n-C34	34.08	0.2 ± 0.07	0.12 ± 0.07	0.05 ± 0.02	*478*
13-;14-;15-;16-;17-MeC34	34.37	0.32 ± 0.11	0 ± 0	0 ± 0	196/322; 210/308; 224/294; 238/280; 252/266
C35diene 1	34.58	3.22 ± 2	0 ± 0	0.01 ± 0.03	*488*
C35diene 2	34.67	0.87 ± 0.81	0 ± 0	0 ± 0	*488*
C35triene	34.73	0.01 ± 0.14	0 ± 0	0 ± 0	*486*
C35diene 3 (cf. 9,19-;9,21-C35diene) [1]	34.74	3.65 ± 4.46	0 ± 0	0 ± 0	*488* [173/409, 271/311; 243/339]

C35ene 1 (14-;16-;17-C35ene)[2]	34.76	1.02 ± 1.18	0.24 ± 0.53	0.02 ± 0.05	*490* [243/341; 271/313; 285/299]
C35diene 4	34.87	0.02 ± 0.16	0 ± 0	0 ± 0	*488*
C35ene 2 (9-C35ene)	34.88	2.53 ± 0.82	0 ± 0	0 ± 0	*490* [173/411]
13-;15-MeC35ene 1	35.07	0.61 ± 0.56	0 ± 0	0 ± 0	223/306, 238/322, *504*
13-;15-MeC35ene 2	35.16	0.09 ± 0.1	0 ± 0	0 ± 0	223/306, 238/322, *504*
13-;15-MeC35ene 3	35.21	0.45 ± 0.82	0 ± 0	0 ± 0	223/306, 238/322, *504*
11-;13-;15-;17-MeC35	35.37	3.78 ± 1.32	0.25 ± 0.24	0.03 ± 0.09	168/364; 197/336; 224/308; 252/280
cf. 13,17-;15,19-; 13,23-DiMeC35	35.59	1.33 ± 0.57	0.22 ± 0.21	0 ± 0	196/350, 266/280; 224/322, 294/252; 196/350, 350/196
n-C36	36.08	0.14 ± 0.08	0 ± 0	0 ± 0	*506*
14-;15-;16-MeC36	36.37	0.19 ± 0.11	0 ± 0	0 ± 0	210/336; 224/322; 238/308
C37diene 1 (cf. 9,19-; 9,21-C37diene)	36.60	4.09 ± 3.43	0 ± 0	0 ± 0	*516* [173/437, 299/311; 271/339]
C37diene 2	36.73	1.38 ± 2.02	0 ± 0	0 ± 0	*516*
C37ene 1 (16-;18-C37ene)	36.75	0.83 ± 1.15	0 ± 0	0 ± 0	*518* [271/341; 299;313]
C37diene 3	36.90	0.06 ± 0.14	0 ± 0	0 ± 0	*516*
C37ene 2	36.91	0.27 ± 0.22	0 ± 0	0 ± 0	*518*
15-MeC37ene 1	37.07	0.4 ± 0.56	0 ± 0	0 ± 0	223/334, 238/350, *532*
15-MeC37ene 2	37.16	0.24 ± 0.3	0 ± 0	0 ± 0	223/334, 238/350, *532*
13-;15-;17-MeC37	37.36	1.26 ± 0.82	0.18 ± 0.25	0 ± 0	196/364; 224/336; 252/308
13,17-;15,19-; 13,23-DiMeC37	37.58	1.21 ± 0.64	0.27 ± 0.19	0 ± 0	196/378, 266/308; 224/350, 294/280; 196/378/350/224
C39diene 1 (cf. 9,21-C39diene)	38.58	4.12 ± 4.55	0 ± 0	0 ± 0	*544* [173/465, 299/339]
C39diene 2	38.72	0.58 ± 0.65	0 ± 0	0 ± 0	*544*
C39ene 1	38.73	0.01 ± 0.14	0 ± 0	0 ± 0	*546*
C39diene 3	39.01	0.14 ± 0.12	0 ± 0	0 ± 0	*544*
unknown CHC (RT 35.94)	39.15	0.09 ± 0.24	0 ± 0	0 ± 0	
13-;15-;17-MeC39	39.33	0.28 ± 0.21	0 ± 0	0 ± 0	196/392; 224/364; 252/336
13,25-; 13,x-DiMeC39	39.57	0.36 ± 0.2	0.11 ± 0.09	0 ± 0	196/406, 378/224
C41diene 1	40.58	0.71 ± 0.97	0 ± 0	0 ± 0	*572*
C41diene 2	40.71	0.22 ± 0.26	0 ± 0	0 ± 0	*572*
unknown saturated CHC (RT 40.02)	41.7	0.16 ± 0.09	0 ± 0	0 ± 0	
unknown unsaturated CHC (RT 42.36)	43.29	0.15 ± 0.18	0 ± 0	0 ± 0	

[1] assignment to peak is tentative

[2] identical to 17-; 19-; 21-C35ene (counted from the other end of the molecule)

Table S2.3: Cuticular hydrocarbons of the cryptic *Camponotus femoratus* species and *Camponotus spec*. SL. In this table we present the identified CHCs, their mean abundances and the retention indices (RI) diagnostic ions for identification. Numbers in italics are the M^+ ions; diagnostic ions in brackets were obtained after DMDS derivatization of unsaturated hydrocarbons. Note that, for unsaturated hydrocarbons, position of double bonds could only be determined for *Ca. femoratus* PAT.

CHC	RI	mean ± SD in *Ca. femoratus* PAT	mean ± SD in *Ca. femoratus* PS	mean ± SD in *Ca. spec.* SL	diagnostic ions and M^+
n-C20	20.07	0.39 ± 0.41	0 ± 0	0.22 ± 0.12	*282*
n-C21	21.03	7.97 ± 8.02	0 ± 0	4.47 ± 2.63	*296*
n-C22	22.07	0.18 ± 0.19	0 ± 0	0 ± 0	*310*
C23ene	22.83	0.23 ± 0.25	0 ± 0	0 ± 0	*322*
n-C23	23.00	1.14 ± 1.33	0 ± 0	0 ± 0	*324*
C25ene	25.01	0 ± 0	0 ± 0.03	0 ± 0	*350*
n-C25	25.06	0 ± 0	0.16 ± 0.15	0 ± 0	*352*
unknown unsaturated CHC (RT 13.22)	25.31	0 ± 0	0 ± 0.35	0 ± 0	
9-MeC25	25.35	0 ± 0	0 ± 0.24	0 ± 0	140/252
7-MeC25	25.41	0 ± 0	0 ± 0.09	0 ± 0	112/280
2-;4-MeC25	25.61	0 ± 0	0 ± 0.02	0 ± 0	43/351; 71/323
C26ene	25.94	0 ± 0	0 ± 0.02	0 ± 0	364
n-C26	26.00	0 ± 0	0 ± 0.09	0 ± 0	366
10-MeC26	26.34	0 ± 0	0 ± 0.09	0 ± 0	154/252
8-MeC26	26.37	0 ± 0	0 ± 0.06	0 ± 0	126/280
4-MeC26	26.63	0 ± 0	0 ± 0.52	0 ± 0	71/337
C27en	26.94	0 ± 0	0 ± 0.28	0 ± 0	*378*
n-C27	27.00	0 ± 0	0.31 ± 0.25	0.21 ± 0.12	*380*
9-MeC27en	27.36	0 ± 0	0 ± 2.37	0 ± 0	138/278, 153/293, *392*
n-C28	28.00	0 ± 0	0 ± 0.02	0 ± 0	*394*
4-MeC28	28.70	0 ± 0	0 ± 0	0.2 ± 0.16	71/365
n-C29	29.01	0.23 ± 0.19	0.75 ± 0.53	0.55 ± 0.14	*408*
11-; 13-;15- MeC29	29.39	0 ± 0	0.21 ± 0.27	2.19 ± 0.77	168/280; 196/252; 224/224
7-MeC29	29.43	0 ± 0	0 ± 0	0.28 ± 0.04	112/336
11,15-DiMeC29	29.61	0 ± 0	0 ± 0	2.66 ± 1.65	168/294, 238/224
7,11-DiMeC29	29.70	0 ± 0	0 ± 0	0.12 ± 0.12	112/350, 182/280
3-MeC29 (traces of C30ene)	29.81	0 ± 0	0 ± 0	0.39 ± 0.15	57/392
n-C30	30.00	0 ± 0	0.6 ± 0.33	0.57 ± 0.34	*422*
10-MeC30 (traces of 11-;12-MeC30)	30.35	0 ± 0	0.05 ± 0.09	0.52 ± 0.9	154/308; 168/294; 182/280
2-; 4-MeC30	30.64	0 ± 0	0.19 ± 0.56	8.63 ± 1.47	43/421; 71/393
C31ene 1	30.86	0.16 ± 0.11	0.24 ± 0.18	2.94 ± 1.63	*434*
C31ene 2	30.93	0 ± 0	0 ± 0	0.14 ± 0.11	*434*
C31ene 3	31.01	0 ± 0	0 ± 0	1.49 ± 0.48	*434*

n-C31	31.11	0.19 ± 0.17	2.13 ± 1.27	1.34 ± 0.17	*436*
9-;11-;13-;15-MeC31 (in *Ca. spec.* SL only 11-MeC31)	31.42	0.07 ± 0.66	1.04 ± 1.31	31.33 ± 4.15	140/336; 168/308; 196/280; 224/252
11,15-DiMeC31	31.64	0 ± 0	0 ± 0	21.17 ± 4.41	168/322, 238/252
11,15-;13,17-;13,21-;13,25-DiMeC31	31.66	0.03 ± 0.31	0.46 ± 0.74	0 ± 0	168/322, 238/252; 196/294, 266/224; 196/294, 322/168; 196/294, 238/252
cf. DiMeC31	31.73	0 ± 0	0.09 ± 0.19	0 ± 0	
3-MeC31;15,21-DiMeC31	31.83	0.01 ± 0.16	0.47 ± 0.34	0.8 ± 1.38	57/421; 224/266, 322/168
C32ene	31.89	0 ± 0	0.01 ± 0.07	0.1 ± 0.17	*448*
11,15,19-TriMeC31	31.93	0 ± 0	0.32 ± 0.23	0 ± 0	168/336, 238/266, 308/196
unknown unsaturated CHC (RT 24.30)	32.12	0 ± 0	0.06 ± 0.38	9.35 ± 0.69	
n-C32	32.13	0 ± 0	0.58 ± 0.34	0 ± 0	*450*
10-;11-MeC32	32.34	0 ± 0	0 ± 0	0.78 ± 0.19	154/336; 168/322
13-;15-MeC32	32.42	0 ± 0	0.7 ± 0.31	0 ± 0	196/294; 224/266
C33diene	32.79	0 ± 0	0 ± 0.01	0.63 ± 0.27	*460*
C33ene	32.91	0 ± 0	0.67 ± 0.68	0.22 ± 0.37	*462*
n-C33	33.12	0.07 ± 0.06	0.83 ± 0.45	0.17 ± 0.29	*464*
11-;13-;15-MeC33 (in *Ca. spec.* SL mainly 11-MeC33)	33.44	0 ± 0	9.55 ± 2.75	1.71 ± 0.66	168/336; 196/308; 224/280
13,17-;15,19-DiMeC33	33.66	0 ± 0	0.87 ± 0.48	0.34 ± 0.58	196/322, 266/252; 224/294, 294/224
unknown saturated CHC (RT 26.62)	33.72	0 ± 0	0.15 ± 0.22	0.08 ± 0.14	
3-MeC33	33.89	0 ± 0	0.11 ± 0.14	0 ± 0	57/449
C34ene	33.94	0 ± 0	0.53 ± 0.23	0.09 ± 0.16	*476*
3,13-DiMeC33	34.14	0 ± 0	0.31 ± 0.15	0.19 ± 0.33	57/463, 210/308
13-;14-MeC34	34.42	0 ± 0	0.61 ± 0.15	0.04 ± 0.06	196/322; 210/308
cf. 16-MeC34	34.43	0 ± 0	0 ± 0	1.35 ± 2.33	238/280
14,18-;15,19-DiMeC34	34.63	0 ± 0	0.21 ± 0.13	0 ± 0	210/322, 280/252; 224/308, 294/238
C35ene	34.95	0 ± 0	2.75 ± 1.22	0.05 ± 0.09	*490*
n-C35	35.13	0 ± 0	0.45 ± 0.32	0 ± 0	*492*
13-MeC35ene	35.28	0 ± 0	0.28 ± 0.11	0 ± 0	195/334, 210/350, *504*
13-;15-;17-MeC35	35.44	0.12 ± 0.08	2.31 ± 0.62	0.66 ± 0.9	196/336; 224/308; 252/280
15,19-DiMeC35	35.65	0 ± 0	0.88 ± 0.65	0 ± 0	224/322, 294/252
13,x-DiMeC35	35.71	0 ± 0	0.13 ± 0.12	0.06 ± 0.1	196/350, x
3-MeC35	35.90	0 ± 0	0.12 ± 0.11	0 ± 0	57/477
C36ene	35.96	0 ± 0	1.12 ± 0.43	0.04 ± 0.07	*504*
C36diene	36.02	0 ± 0	0.16 ± 0.15	0 ± 0	*502*
3,15-DiMeC35	36.17	0 ± 0	0.45 ± 0.22	0 ± 0	57/491, 238/308
13-MeC36ene	36.24	0 ± 0	0.53 ± 0.15	0 ± 0	195/348, 210/364, *518*
15-;16-;17-;18-MeC36	36.39	0 ± 0	0 ± 0	0.29 ± 0.5	224/322; 238/308; 252/294; 266/280

12-;13-;14-MeC36	36.42	0 ± 0	0.24 ± 0.11	0 ± 0	182/364; 196/350; 210/336
unknown CHC (RT 30.40)	36.48	0 ± 0	0.03 ± 0.06	0 ± 0	
unknown CHC (RT 30.60)	36.63	0 ± 0	0.11 ± 0.1	0 ± 0	
12,x; 13,x; 14,x; 15,x-DiMeC36 (tentative x = 22-25)	36.68	0.24 ± 0.13	0 ± 0	0 ± 0	182/378, x; 196/364, x; 210/350, x; 224/336, x
C37diene 1	36.76	0 ± 0	0.57 ± 0.31	0 ± 0	*516*
C37ene 1 (9-C37ene; traces of C37diene)	37.04	0.7 ± 0.31	3.09 ± 2.05	0.11 ± 0.18	*518* [173/439]
C37diene 2	37.09	0 ± 0	9.34 ± 3.65	0.22 ± 0.37	*516*
unknown CHC (RT 31.26)	37.14	0.06 ± 0.05	0 ± 0	0 ± 0	
unknown unsaturated CHC (RT 31.38)	37.23	0.11 ± 0.12	0 ± 0	0 ± 0	
13-MeC37ene	37.29	0 ± 0	13.44 ± 4.01	0.58 ± 1	195/362, 210/378, *532*
13-; 15-; 17-MeC37	37.45	1.97 ± 0.88	0.8 ± 0.18	0.05 ± 0.09	196/364; 224/336; 252/308
unknown unsaturated CHC (RT 31.70)	37.48	0.11 ± 0.16	0 ± 0	0 ± 0	
unknown methyl-branched alkene (RT 31.72)	37.49	0 ± 0	0.25 ± 0.1	0 ± 0	
15,21-; cf. 15,19-DiMeC37	37.63	0 ± 0	0.27 ± 0.25	0 ± 0	224/350, 322/252; 224/350, 294/280
13,23-DiMeC37	37.77	22.47 ± 10.21	0.43 ± 0.25	0.06 ± 0.1	196/378, 350/224
C38diene 1	37.80	0 ± 0	0.96 ± 0.28	0.03 ± 0.06	*530*
C38ene 1 (9-; 10-C38ene)	37.99	0.28 ± 0.15	0.42 ± 0.19	0 ± 0	*532* [173/453; 187/439]
C38diene 2	38.05	0 ± 0	0.61 ± 0.16	0.03 ± 0.06	*530*
3,15-DiMeC37	38.18	0 ± 0	1.22 ± 0.65	0.06 ± 0.11	57/518, 238/336
13-MeC38ene	38.24	0.12 ± 0.08	1.21 ± 0.39	0.08 ± 0.13	195/376, 210/392, *546*
13-; 15-; 17-MeC38 (in *Ca. femoratus* PS only 13-MeC38)	38.40	0.17 ± 0.07	0.13 ± 0.11	0 ± 0	196/378; 224/350; 252/322
8-MeC38	38.45	0 ± 0	0 ± 0	0.08 ± 0.14	126/448
C39diene 1	38.63	0 ± 0	0.06 ± 0.12	0 ± 0	*544*
12,x-;13,x-;14,x-;15,x-DiMeC38	38.71	0.44 ± 0.26	0 ± 0	0 ± 0	182/406, x; 196/392, x; 210/378, x; 224/364, x
C39diene 2 (cf. 9,29-C39diene)	38.81	4.11 ± 1.2	5.79 ± 2.08	0.16 ± 0.27	*544* [173/465, 187/451]
C39diene 3	38.85	0.04 ± 0.18	1.64 ± 0.71	0.05 ± 0.09	*544*
C39diene 4	38.96	0 ± 0	1.18 ± 0.48	0.05 ± 0.08	*544*
C39ene 1 (9-C39ene; traces of C39diene)	39.01	3.47 ± 1.89	0 ± 0	0 ± 0	*546* [173/467]
C39diene 5	39.09	3.9 ± 1.3	1.07 ± 0.42	0.38 ± 0.39	*544*
13-MeC39ene	39.27	1.85 ± 1.1	6.89 ± 1.92	0.41 ± 0.71	195/418, 210/434, *588*
9-MeC39	39.34	0 ± 0	0 ± 0	0.1 ± 0.17	140/448
7-MeC39	39.37	0 ± 0	0 ± 0	0.06 ± 0.1	112/476

13-; 15-; 17-MeC39	39.38	0.9 ± 0.38	0.39 ± 0.18	0.03 ± 0.05	196/392; 224/364; 252/336
cf. DiMeC39	39.47	0 ± 0	0.03 ± 0.08	0 ± 0	
13,x-;15,x-DiMeC39	39.52	0 ± 0	0 ± 0	0.04 ± 0.06	196/406, x; 224/378, x
15,19-; 13,23-DiMeC39	39.60	0 ± 0	0.35 ± 0.27	0.04 ± 0.06	224/378, 294/308; 196/406, 350/252
13,x-;15,x-;17,x-DiMeC39 (tentative x = 23; 25; 27)	39.70	2.82 ± 1.08	0 ± 0	0 ± 0	196/406, x; 224/378, x; 252/350, x
C40diene 1	39.77	0 ± 0	1.04 ± 0.38	0.06 ± 0.1	*558*
C40diene 2	39.82	1.69 ± 0.53	0 ± 0	0 ± 0	*558*
C40diene 3	39.84	0 ± 0	1.09 ± 0.37	0.06 ± 0.11	*558*
C40diene 4	39.88	0 ± 0	0.14 ± 0.12	0 ± 0	*558*
unknown CHC (RT 35.16)	39.91	0.02 ± 0.07	0 ± 0	0 ± 0	
C40diene 5	40.00	1.06 ± 0.39	0 ± 0	0 ± 0	*558*
unknown CHC (RT 35.42)	40.09	0.02 ± 0.04	0 ± 0	0 ± 0	
13-MeC40ene	40.25	0.17 ± 0.11	0.94 ± 0.35	0.08 ± 0.14	195/404, 210/420, *574*
8-MeC40	40.53	0 ± 0	0 ± 0	0.04 ± 0.07	126/476
C41diene 1	41.16	13.24 ± 4.42	5.53 ± 1.83	0.29 ± 0.51	*572*
C41diene 2 (cf. 9,29-; 9,31-C41diene)[1]	41.25	11.61 ± 3.42	3.71 ± 0.95	0.2 ± 0.35	*572* [173/493, 215/451; 187/479]
C41diene 3 (cf. 9,33-C41diene)[1]	41.32	7.46 ± 1.81	0 ± 0	0 ± 0	*572* [173/493, 159/507]
C41diene 4 (cf. 6,32-C41diene)[1,2]	41.41	2.86 ± 0.99	0 ± 0	0 ± 0	*572*
C41diene 5	41.51	0.59 ± 0.38	0 ± 0	0 ± 0	*572*
C41diene 6	41.63	0.73 ± 0.65	0 ± 0	0 ± 0	*572*
13-MeC41ene	41.70	0 ± 0	2.82 ± 1.22	0.19 ± 0.32	195/418, 210/434, *588*
C41diene 7	41.79	0.1 ± 0.12	0 ± 0	0 ± 0	*572*
13,29-; x,y-DiMeC41	42.29	0.45 ± 0.31	0 ± 0	0 ± 0	196/434, 434/196
15,19-DiMeC41	42.34	0 ± 0	0.11 ± 0.16	0 ± 0	224/406, 294/336
C42diene 1	42.40	0.23 ± 0.11	0 ± 0	0 ± 0	*586*
unknown CHC (RT 38.70)	42.54	0 ± 0	0.02 ± 0.06	0 ± 0	
C42diene 2	42.61	0.42 ± 0.21	0.67 ± 0.36	0.06 ± 0.11	*586*
13-MeC42ene	43.08	0 ± 0	0.13 ± 0.14	0 ± 0	195/432, 210/448, *644*
cf. C43triene 1	44.27	0.84 ± 0.47	0 ± 0	0 ± 0	*598*
cf. C43triene 2	44.46	2.16 ± 0.99	1.28 ± 0.74	0.08 ± 0.13	*598*
cf. C43triene 3	44.61	1.8 ± 0.86	0 ± 0	0 ± 0	*598*
unknown unsaturated CHC (RT 41.78)	44.78	0 ± 0	0.09 ± 0.11	0 ± 0	
13-MeC43ene	44.88	0 ± 0	0.08 ± 0.14	0 ± 0	195/446, 210/462, *658*

[1] assignment to peaks is tentative

[2] identical to 9,35-C41diene (counted from the other end of the molecule)

Table S2.4: Reconstructed ancestral states of average CHC chain length in the *Orthocrema* clade. The table shows the average CHC chain length reconstructed for each node. Nodes are numbered from the most ancestral to the most recent one.

Node	Ancestral average chain length	95% Confidence intervals
Node 1	27.91	[22.39; 33.43]
Node 2	27.79	[23.31; 32.26]
Node 3	27.81	[23.64; 31.99]
Node 4	27.63	[26.13; 29.14]
Node 5	28.10	[22.95; 33.24]
Node 6	28.26	[23.25; 33.28]
Node 7	29.02	[24.50; 33.53]
Node 8	29.02	[24.50; 33.53]
Node 9	29.38	[24.62; 34.14]
Node 10	28.83	[24.46; 33.20]
Node 11	29.11	[25.93; 32.28]
Node 12	29.49	[28.01; 30.97]

Table S2.5: CHCs influenced by the climate. The table lists the 20% of substances differing most according to climate and being abundant more than 0.5% for each of the cryptic species. Shown are pseudo-F and p-values from univariate PERMANOVAs based on Euclidean distances, plus the direction of the effect.

species	substance	pseudo-F	p	increasing/decreasing with higher precipitation (+/-)
***Crematogaster levior* A**	13,17-;15,19-DiMeC35	25.445	0.001	-
	13-;15-MeC35ene	24.500	0.001	+
	C33diene 2	21.180	0.001	+
	15-MeC37ene	22.933	0.001	+
	C35ene 2 (9-C35ene)	10.524	0.001	-
	C37diene 1 (cf. 9,19-; 9,21-C37diene)	14.176	0.002	-
	13-;15-;17-MeC37	7.995	0.005	-
	C35diene 2	19.269	0.001	+
	11-;13-;15-MeC31	5.885	0.014	-
	C33diene 3	15.573	0.001	+
	n-C27	8.084	0.011	-
***Crematogaster levior* B**	C29diene 2	17.803	0.001	-
	C31ene 3	14.508	0.002	+
	C33ene 1	11.520	0.002	+
	C31diene 2	8.264	0.004	-
***Camponotus femoratus* PAT**	C39diene 5	19.210	0.001	-
	13-MeC39ene	12.457	0.001	-
	12,x-;13,x-;14,x;15,x-DiMeC37	8.485	0.001	+
	C41diene 4 (cf. 6,32-C41diene)	8.171	0.008	-
	C41diene 6	7.380	0.009	-
	C39ene 1	7.224	0.009	-
	C41diene 5	6.926	0.007	-
	13,23-DiMeC37	5.341	0.025	+
***Camponotus femoratus* PS**	13-MeC38ene	21.718	0.001	-
	13-MeC41ene	19.905	0.001	-
	13-MeC40ene	17.999	0.001	-
	C39diene 2	16.773	0.001	+
	13-MeC39ene	13.236	0.002	-

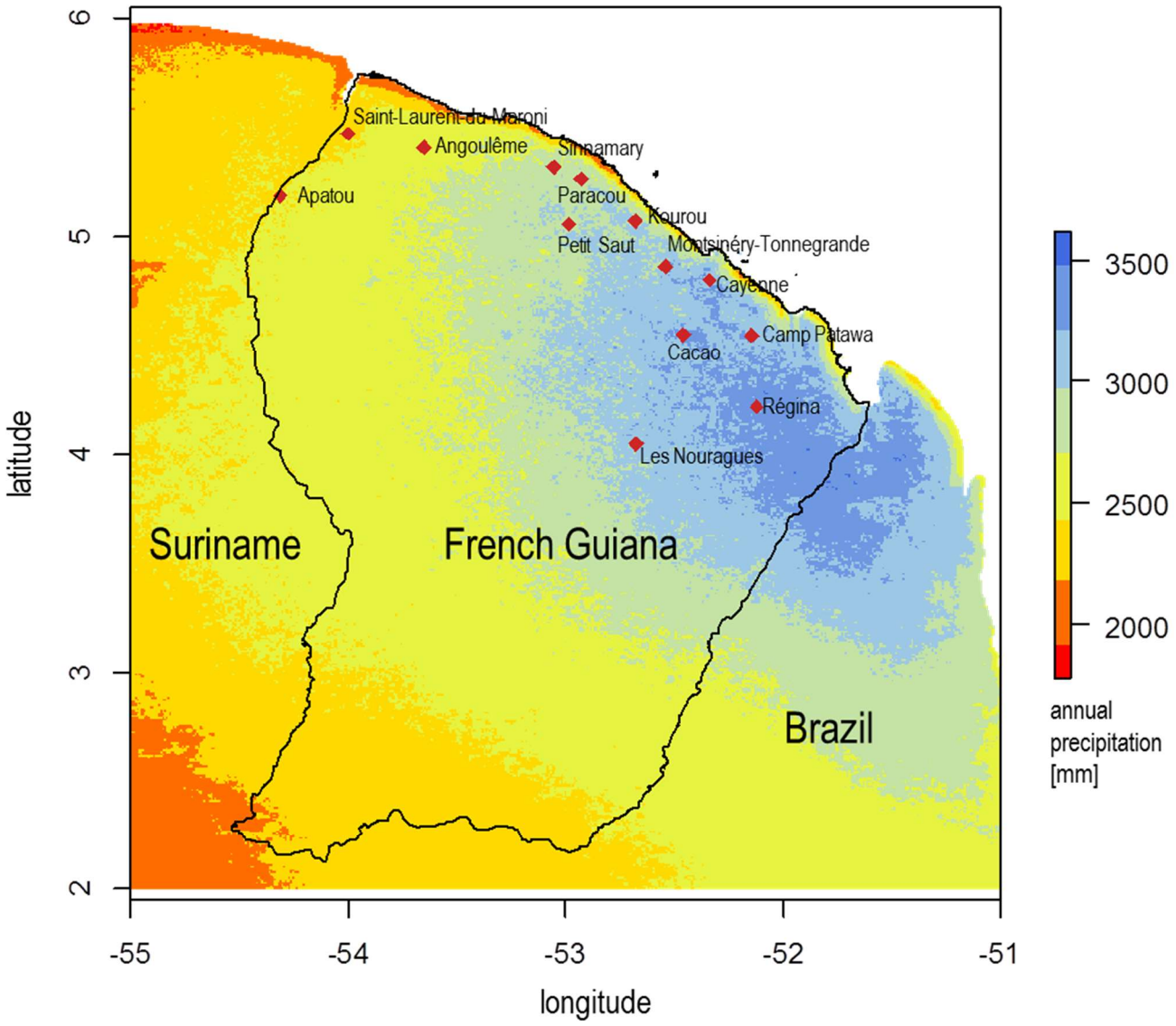

Figure S2.1: Map of our sampling locations in French Guiana. The sampling locations are indicated by red diamonds. Annual precipitation in French Guiana was downloaded from WorldClim and is indicated by colors. For coordinates and numbers of samples see Table S2.1.

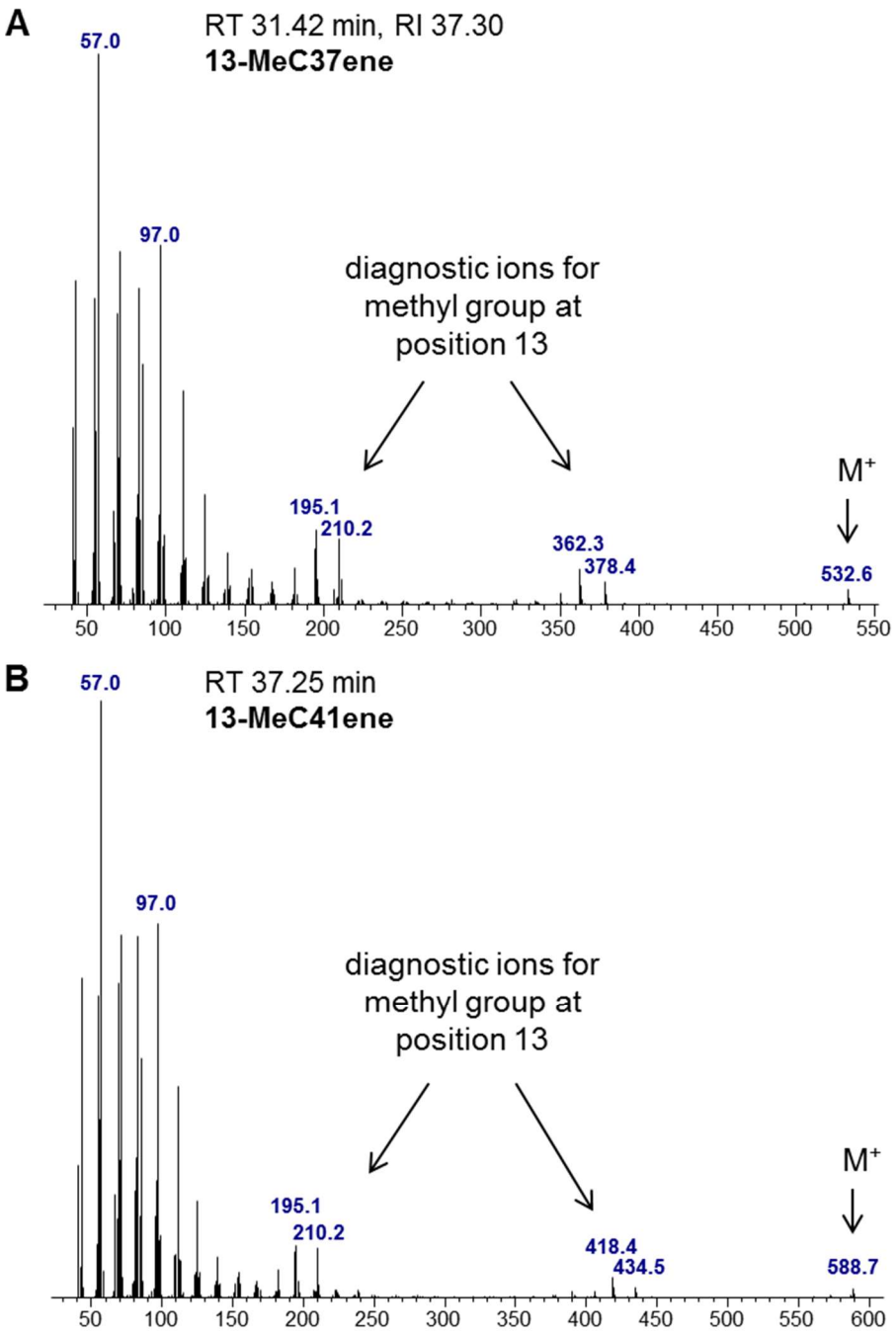

Figure S2.2: Representative mass spectra for two methyl-branched alkenes of *Ca. femoratus* PS. Methyl-branched alkenes eluted shortly before the saturated methyl-branched alkanes with the same methyl-branch position. We identified them based on the molecular peak (M^+) that is two amu below that expected for monomethyl alkanes of the same chain length. Because of the double bond, molecules break in two different ways leading to four diagnostic ions instead of two. The first pair of ions is similar to those expected for the saturated molecule minus two amu (here 195/362 m/z in (A) and 195/418 m/z in (B)) and the second pair is 15 to 16 amu heavier than the first ones (here 210/378 and 210/434 m/z). For comparison, the diagnostic ions of (saturated) 13-MeC37 are 196 and 365 m/z; those of 13-MeC41 are 196 and 420 m/z.

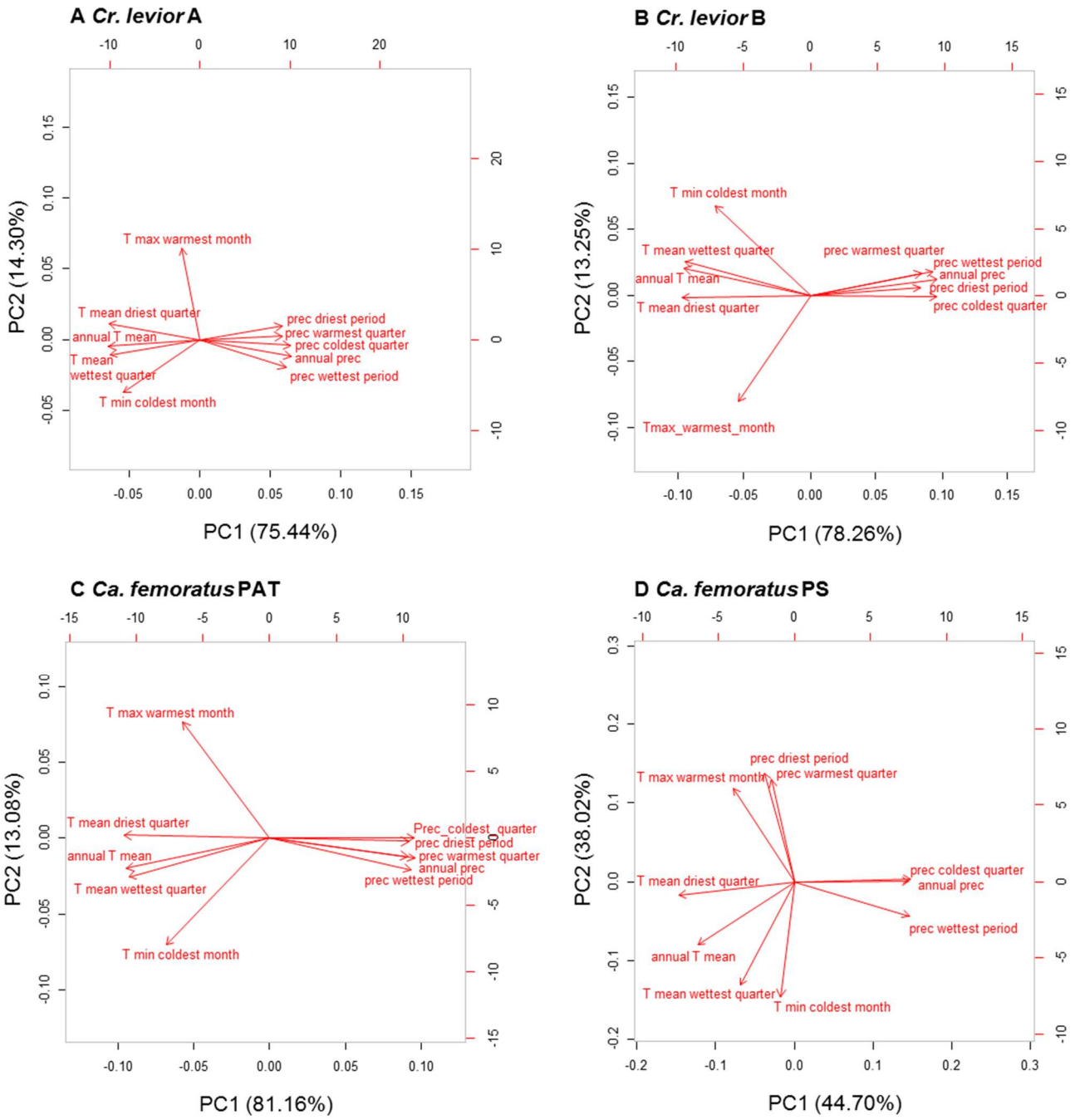

Figure S2.3: Factor loadings of the climate variables on CHC profiles. The arrows indicate factor loadings of each of the 10 climate variables concerning principal components 1 and 2. Temperature (annual mean temperature, minimum temperature in the coldest month, maximum temperature in the warmest month, mean temperature in the driest quarter and mean temperature in the wettest quarter) is abbreviated with "T" and precipitation (annual precipitation, precipitation in the coldest quarter, precipitation in the warmest quarter, precipitation in the driest period and precipitation in the wettest period) with "prec".

Chapter 3

Candidate genes involved in cuticular hydrocarbon differentiation between cryptic, parabiotic ant species

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Abstract

Insect cuticular hydrocarbons (CHCs) have multiple functions, the two most important ones being communication (especially important in ants and other social insects) and protection against desiccation. CHC profiles are complex and highly variable across species. However, with the exception of *Drosophila*, information on genes involved in CHC biosynthesis is scarce. The neotropical parabiotic ants *Crematogaster levior* and *Camponotus femoratus* live together in a mutualistic association. Both taxa consist of two cryptic species each. The respective sister species are closely related and highly similar in ecology and morphology, but express largely different CHC profiles. This makes them ideal models to study the genetic basis of CHC differentiation. Therefore, we investigated their gene expression patterns to identify candidate genes involved in CHC biosynthesis using individuals that had been kept under the same lab conditions for a minimum of 10 days.

We found several thousand differentially expressed transcripts within each species pair. As reflected by more dissimilar CHC profiles, we found more differentially expressed transcripts in *Camponotus* compared to *Crematogaster*. Many of them were related to metabolic processes, probably accounting for physiological differences. Moreover, we identified several candidate genes from five gene families involved in CHC biosynthesis, namely fatty acid synthases, elongases, desaturases, fatty acyl-CoA reductases and cytochrome P450s, plus candidate CHC-orthologs from *Drosophila*. The CHC differences between the species remained qualitatively stable even when kept under the same lab conditions, implying that species differences indeed have a genetic basis.

By assigning candidate transcripts to orthologs in *Drosophila*, we are able to infer which compounds are putatively influenced in their synthesis by differential gene expression. In many cases the expression of these candidates is mirrored in the composition of the CHC profiles: Most interestingly, the expression patterns of elongases and fatty acyl-CoA reductases in the cryptic *Cr. levior* species fit the observed CHC chain length differences between these species. This is one of the first studies to identify CHC candidate genes in ants and will provide a basis for further research on the genetic basis of CHC biosynthesis.

Keywords:

CHC biosynthesis, cryptic species, mutualism differential gene expression, social insects, speciation

3.1 Introduction

Chemical communication is widespread in insects (Symonds & Elgar 2008; Hansson & Stensmyr 2011; Leonhardt *et al.* 2016). One group of substances found in nearly all terrestrial arthropods and frequently used as chemical signal or cue are cuticular hydrocarbons (CHC) (Howard & Blomquist 2005; Blomquist & Bagnères 2010; Leonhardt *et al.* 2016). They are widely used as sex pheromones (Carlson *et al.* 1971; Steiger & Stökl 2014) and mediate mate choice in many solitary insects, e.g. in *Drosophila* (Ferveur 2005; Rundle *et al.* 2005; Chung *et al.* 2014). Differences in the chemical profile can induce assortative mating that may even result in speciation (Schwander *et al.* 2013; Otte, Hilker & Geiselhardt 2015). In ants, CHC profiles are used to inform about fertility, caste membership and tasks within the colony, but most importantly to discriminate nestmates from non-nestmates (Lahav *et al.* 1999; Greene & Gordon 2003; Leonhardt *et al.* 2016). However, CHCs have multiple functions, because they are not only important agents of chemical communication, but also serve as a barrier to water-loss preventing insects from desiccation and as barriers to microbes (Gibbs & Rajpurohit 2010; Chung & Carroll 2015). Although the qualitative CHC composition is genetically determined (Ferveur 2005; van Zweden, Dreier & d'Ettorre 2009), short-term exposures to warm temperatures and drought have been shown to activate quantitative acclimation responses in the CHC profile (i.e. increases in the proportion of more viscous CHC classes at the expense of more liquid CHCs) enhancing the survival of the insects (Stinziano *et al.* 2015; Menzel *et al.* 2018; Sprenger *et al.* 2018).

Long-chain cuticular hydrocarbons are synthesized de novo in the oenocytes, which are specialized cells associated with the peripheral fat body and the epidermal layer (Billeter *et al.* 2009; Wicker-Thomas, Guenachi & Keita 2009; Blomquist 2010b). The synthesis of CHCs is associated to the fatty acid metabolism: In a first step fatty acid synthases (FAS) produce fatty acyl-CoA from acetyl-CoA, which is then elongated by the FAS and by very long-chain fatty acid elongases (Blomquist 2010b; Chung & Carroll 2015). During this process acyl-CoA desaturases can introduce double bonds to the molecule (which will then result in alkenes or alkadienes) (Dallerac *et al.* 2000; Labeur, Dallerac & Wicker-Thomas 2002; Chertemps *et al.* 2006; Chung & Carroll 2015). Finally, fatty acyl-CoA reductases convert the acyl-CoA side chain to aldehydes that subsequently get decarbonylized to hydrocarbons by cytochrome P450 enzymes (Chung *et al.* 2009, 2014; Qui *et al.* 2012). Members of the same gene families are involved in the biosynthesis of fatty acids and hydrocarbons (energy storage), which makes it difficult to disentangle these two biosynthetic pathways. Despite several studies in

Drosophila (Dallerac *et al.* 2000; Labeur *et al.* 2002; Chertemps *et al.* 2006, 2007; Chung *et al.* 2009, 2014; Wicker-Thomas & Chertemps 2010; Qui *et al.* 2012; Dembeck *et al.* 2015; Ng *et al.* 2015), genes that are particularly involved in CHC biosynthesis are still largely unknown, especially in insect families in which the involved gene families underwent large gene expansions (Hartke *et al.* 2019a; Tupec *et al.* 2019). In social insects, and especially ants, CHC profiles are often very complex and can comprise of hundreds of different molecules (Martin & Drijfhout 2009c; Sprenger & Menzel 2020). This is reflected by high numbers of elongases (Hartke *et al.* 2019a) and desaturases (Helmkampf, Cash & Gadau 2015) found in ants compared to other insect taxa.

Parabiosis is defined as two ant species sharing the same nest, but keeping their brood separate (Forel 1898). They tolerate each other, but still keep their own species-specific CHC profiles (Menzel *et al.* 2008a; b; Menzel & Schmitt 2012). The parabiotic ant species known as *Crematogaster levior* and *Camponotus femoratus* from the Amazonian rainforest each consist of two cryptic species that differ genetically and have largely different CHC profiles: *Cr. levior* A and B and *Ca. femoratus* PAT and PS (Hartke, Sprenger *et al.* 2019, Chapter 1; Sprenger *et al.* 2019, Chapter 2). While both cryptic species of *Cr. levior* were sympatric across an east-west transect in French Guiana, *Ca. femoratus* PS was rarely found in the east, in contrast to *Ca. femoratus* PAT, which was common over the whole sampling area. The distribution of both species, as well as some of their CHCs might be determined by climatic conditions (higher annual precipitation and a slightly colder annual mean temperature from east to west) (Hartke, Sprenger *et al.* 2019, Chapter 1; Sprenger *et al.* 2019, Chapter 2). *Crematogaster levior* A has an unusually high average chain length of the carbon backbone of its CHCs and more unsaturated hydrocarbons, while the CHC profile of *Cr. levior* B has shorter chained CHCs and more monomethyl alkanes (Sprenger *et al.* 2019, Chapter 2). The two cryptic species of *Ca. femoratus* do not differ in mean chain length, but in the composition of substance classes with PAT mainly having more dimethyl alkanes and PS having more alkenes and methyl-branched alkenes (Sprenger *et al.* 2019, Chapter 2).

In this study, we used these two pairs because they are closely related sister species that also are ecologically very similar, but differ strongly in their CHC profiles. This makes them an ideal model system to contrast gene expression patterns with the aim to identify candidate genes putatively involved in CHC biosynthesis. To ensure that CHC differences are genetically determined and do not represent an acclimation response to climate in their natural habitats, we kept the ants under standardized lab conditions and compared these acclimated CHC profiles to ones obtained in natural habitat.

3.2 Methods

3.2.1 Study species and sampling

We collected ants of each of the four parabiotic cryptic species at two different locations in French Guiana in October 2016 (N = 16 *Crematogaster* and N = 16 *Camponotus* worker groups; see Supplementary Table S3.1). We sampled the *Crematogaster* ants in their shelters (dry leaves or hollow sticks) close to the actual ant garden and put them into 50 mL plastic tubes (115 x 28 mm, Sarstedt AG & Co. KG, Nürnbrecht, Germany). For *Camponotus*, we collected 15 individuals per group (castes: minor and media) close to the nest together with some dry leaves as shelter, and put them in 500 mL PET bottles. Tubes and bottles were closed using a lid with a wire mesh (diameter 15 mm; mesh 0.2 mm) to ensure air exchange. For transport the ants were fed with small pieces of sausage and sweet cookies and a moistened paper tissue.

3.2.2 Acclimation

The living ants were brought back to the lab in Mainz, Germany, and put into plastic boxes (95×95×60 mm, Westmark GmbH, Lennestadt-Elspe, Germany) with some of the original nesting material and moistened plaster floor. *Crematogaster* nests were sealed with closed lids (~100% RH); *Camponotus* nests had lids with a wire mesh window (70×70 mm; mesh 0.2 mm; ~70% RH) to avoid self-poisoning with formic acid. To rule out effects of acclimatory CHC changes, all ants were kept under standardized conditions in a climate chamber at 25°C. They were fed with water, honey and crickets *ad libitum*. After 10 to 13 days, we dissected the fat body of each of two individuals per colony (one for RNA extraction, and one as backup). After 16 days, we additionally took two individual *Camponotus* ants and ten pooled *Crematogaster* ants for CHC extraction to check if the species differences would be stable even under similar environmental conditions.

3.2.3 Fat body dissections

We excised the cuticle of the second and third segment of the gaster for *Crematogaster*, and the second segment only for *Camponotus*, together with the attached fat body that contains the oenocytes, and crushed the tissue in 50 µL of TRIzol reagent (Invitrogen AG, Carlsbad,

CA, USA). The samples were incubated in TRIzol at room temperature for five to seven minutes and then frozen at -80 °C until RNA extraction.

3.2.4 RNA extraction and sequencing

For RNA extraction, we slowly thawed the samples buffered in TRIzol and precipitated the RNA using 50 µL of chloroform. Subsequently, we used the QIAGEN RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. In the final step, we dissolved the RNA in 30 µL of RNase-free water and stored the samples in a freezer at -80°C until sequencing. Library construction and paired-end sequencing was conducted at the BGI NGS Lab (Hong Kong) on an Illumina HiSeq 4000 platform.

We subsequently checked the read quality using the program *FastQC* (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) together with *MultiQC* (Ewels *et al.* 2016) before and after adapter trimming with *Trimmomatic* (Bolger, Lohse & Usadel 2014) using standard settings. Due to failure of library preparation for three samples and the removal of one sample with skewed GC content we used the remaining biological replicates of *Cr. levior* A (n = 6) and *Cr. levior* B (n = 9), as well as *Ca. femoratus* PAT (n = 7) and *Ca. femoratus* PS (n = 5) for the genus specific *de novo* transcriptome assemblies.

3.2.5 De novo transcriptome assemblies

As the main goal of this study was to compare gene expression patterns between the two closely related species of each genus, we decided to use a co-assembly approach, as previously successfully done in closely related *Drosophila* species (Lopez-Maestre *et al.* 2017). We thus pooled sequences of the two cryptic species for each assembly. As *CLC Main Workbench* v. 7.9.1 (QIAGEN) is sensitive to the number of input reads, we first generated five and four subassemblies, respectively, for *Crematogaster* and *Camponotus* with word size 35 and automatic bubble size (for the composition of samples for each subassembly see Supplementary Table S3.2). Subsequently, we used *MIRA* (Chevreux, Wetter & Suhai 1999) for meta-assemblies for each of the two transcriptomes (settings: job = de novo, genome, accurate, sanger). After the meta-assembly, we re-included reads from the 'debris' that were not assembled by *MIRA*, as those most likely represent group specific CLC-transcripts, and removed all transcripts below 300 bp length to obtain our final reference transcriptome. A *BlastX* (Altschul *et al.* 1990) search against the non-redundant arthropod protein database

(NCBI, state January 2018) was used to obtain annotations for the transcripts. The raw reads can be accessed at NCBI under BioProject ID PRJNA540400.

3.2.6 Differential gene expression and functional enrichment analyses

To quantify read numbers per transcript for each sample, we used *kallisto* (Bray *et al.* 2016) and subsequently performed the differential gene expression analysis using *DESeq2* (Love, Huber & Anders 2014). Here, we compared differentially expressed genes between the cryptic species of each genus separately using linear models. All p-values were adjusted by false discovery rate (FDR) correction as implemented in *DESeq2*. To identify CHC candidate genes in the DEG list, we followed two approaches: a) we searched for DEGs with BLAST annotation as one of the candidate gene families fatty acid synthases, very long chain fatty acid elongases, acyl-CoA desaturases, fatty acyl-CoA reductases and cytochrome P450s, and b) conducted a *BlastX* search to 18 genes of these gene families with known functions in the CHC biosynthesis of *Drosophila* obtained from FlyBase (https://www.flybase.org/) (Supplementary Table S3.3). Here we first filtered for hits with e-values < 0.00001, then excluded those shorter than 200bp and only took those with the best match to one of the candidate gene families.

Additionally, we translated the nucleotide sequences into amino acid sequences with *Transdecoder* (Haas *et al.* 2013) and subsequently used *InterProScan* (Jones *et al.* 2014) to extract Gene Ontology (GO) terms and KEGG pathway (KO) IDs for our list of differentially expressed genes and conducted a GO enrichment analysis for biological processes (BP) in *topGO* (Alexa & Rahnenfuhrer 2018) using the 'weight01' algorithm and Fisher test. We visualized the results with *REVIGO* (Supek *et al.* 2011).

In a further analysis, we scanned for shared or privately expressed molecular pathways using *KEGG Mapper* (https://www.genome.jp/kegg/tool/map_pathway1.html).

3.2.7 CHC analysis

For CHC extraction, the ants were freeze-killed at -20 °C. CHCs were extracted by immersing the ants in chromatography-grade hexane for 10 minutes. The samples were stored at -20 °C until analysis using gas chromatography coupled to mass spectrometry (GC-MS). As the samples of *Crematogaster* contained also polar secondary metabolites (Hartke, Sprenger *et al.* 2019, Chapter 1), the CHCs were purified using silica columns (Chromabond, SiOH

3mL/100mg, Macherey-Nagel, Düren, Germany). Hydrocarbon fractions were eluted with hexane. We used an Agilent 7890A gas chromatograph coupled to a 5975C mass selective detector (Agilent Technologies, Santa Clara, CA, USA) equipped with a Zebron Inferno DB5-MS column (Phenomenex Ltd., Aschaffenburg, Germany). Areas under chromatogram peaks were integrated manually in *MSD ChemStation* (Agilent Technologies) and afterwards transformed to relative proportions. For further details on the GC-MS analysis see (Sprenger *et al.* 2019, Chapter 2). To compare CHC profiles from constant lab conditions (25°C, 70% and 100% RH, respectively) tropical natural habitat conditions, we added the CHC data for each colony from a previous dataset (Sprenger *et al.* 2019, Chapter 2). Mean temperatures in the quarter of collection (dry season; based on the long-term database CHELSA, Karger *et al.* 2017) were 27.0°C (Paracou) and 25.5°C (Camp Patawa). Precipitation in the driest quarter was 48.8 mm (Paracou; mean annual precipitation 2588.7 mm) and 108.4 mm (Camp Patawa; mean annual precipitation 4756.7 mm). For *Ca. femoratus* the number of extracted individuals differed between these two datasets. It is thus possible that lower concentrations in the acclimated samples prevented detection of certain CHCs. For this reason, we only included substances that were present in both datasets and only investigated quantitative changes (ignoring the less likely possibility of qualitative changes; Sprenger & Menzel 2020, Chapter 6).

Subsequently, we tested for differences between the cryptic species (A vs. B or PAT vs. PS), the acclimation status ('natural' vs. 'acclimated' profile) and their interaction with a PERMANOVA based on Bray-Curtis dissimilarity in the program *PRIMER 6* with *PERMANOVA+* (Anderson, Gorley & Clarke 2008). We also included the colony ID nested in cryptic species as random effect. Subsequently, we visualized the comparison with a non-metric multidimensional scaling (NMDS) ordination (command *metaMDS*, R-package *vegan*, Oksanen *et al.* 2019). Finally, we calculated the mean per substance for the acclimated profiles and the Bray-Curtis dissimilarity between the cryptic species.

3.3 Results

3.3.1 De novo transcriptome assembly

The co-assembly for *Cr. levior* A and B resulted in a *de novo* transcriptome consisting of 60,185 transcripts, and the one for *Ca. femoratus* PAT and PS contained a total of 48,208 transcripts.

74.80% of the *Crematogaster* transcripts (45,016 transcripts) and 69.59% of the *Camponotus* transcripts (33,549 transcripts) could be BLAST annotated.

3.3.2 Differential gene expression analysis

The gene expression analyses revealed a total of 5,317 differentially expressed transcripts between the cryptic *Cr. levior* species (3,006 upregulated in *Cr. levior* A and 2,311 in B; Fig. 3.1 A), and 6,153 between the two cryptic *Ca. femoratus* species (3,269 upregulated in *Ca. femoratus* PAT and 2,884 in PS; Fig. 3.1 B).

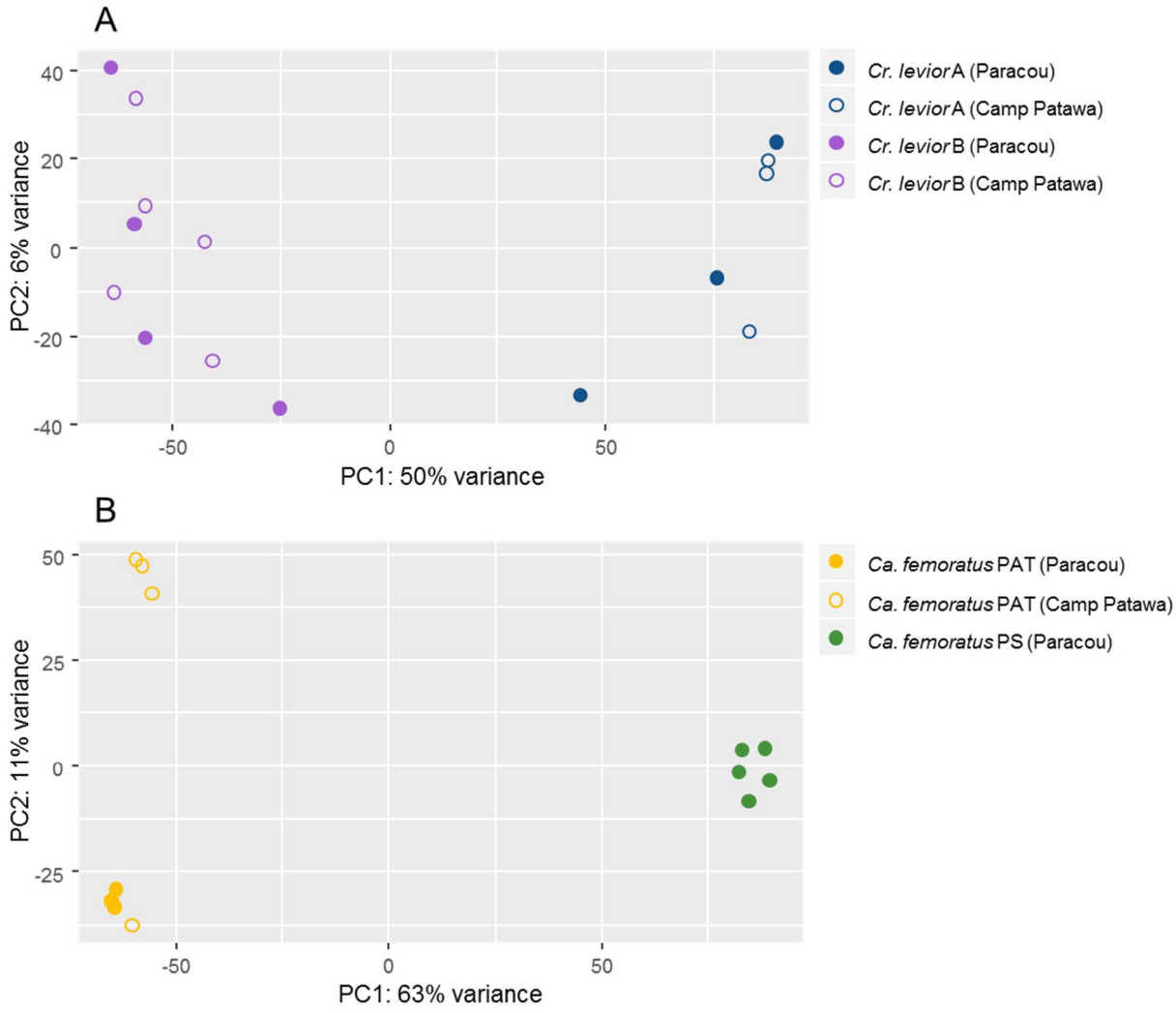

Figure 3.1: Principal component analysis of gene expression patterns of two cryptic species pairs of *Cr. levior* (A) and *Ca. femoratus* (B). Each dot in the ordination represents the gene expression pattern of a single individual. The cryptic species are indicated by different colors, the two sampling locations by either filled or open circles.

We identified differentially expressed transcripts that had BLAST annotations to genes potentially involved in CHC biosynthesis. In *Cr. levior*, we found 37 *fatty acid synthase-like* (17 of these had hits in *Drosophila* genes, i.e. they played a role in CHC biosynthesis in *Drosophila*, Supplementary Table S3.3), 14 *acyl-CoA Delta(11) desaturase-like* (12 hits in *Drosophila*), 16 *elongation of very long chain fatty acids protein-like* (11 hits in *Drosophila*) and 29 *fatty acyl-CoA reductase-like* transcripts (15 hits in *Drosophila*) to be differentially expressed (Fig. 3.2 A; Electronic Supplementary Table S3.4 A). We also identified 28 *cytochrome P450-like* differentially expressed transcripts (Fig. 3.2 A; Electronic Supplementary Table S3.4 A) that were similar to *Drosophila* cytochrome P450 genes known to be involved in CHC biosynthesis (Supplementary Table S3.3).

In the two species of *Ca. femoratus*, we found 43 *fatty acid synthase-like* (25 hits in *Drosophila* genes), 10 *acyl-CoA Delta(11) desaturase-like* (8 hits in *Drosophila*), 22 *elongation of very long chain fatty acids protein-like* (12 hits in *Drosophila*) and 22 *fatty acyl-CoA reductase-like* (10 hits in *Drosophila*) differentially expressed transcripts (Fig. 3.2 B; Electronic Supplementary Table S3.4 B). Looking at the genes similar to *Drosophila*, we identified 45 *cytochrome P450-like* candidate transcripts that were differentially expressed between the two cryptic species (Fig. 3.2 B; Electronic Supplementary Table S3.4 B).

3.3.3 Enrichment analysis

The GO enrichment analyses for upregulated genes in *Cr. levior* A and B respectively revealed that both sets contained significantly more metabolism-related genes than expected by chance (GO:0008152 – metabolic process; Fisher-test: $p = 0.0004$ in A, $p = 0.0003$ in B; Supplementary Figs S3.1, S3.2; Electronic Supplementary Table S3.5). In *Cr. levior* B the functions 'carbohydrate metabolic process' (GO:0005975; $p = 0.014$) and 'superoxide metabolic process' (GO:0006801; $p = 0.030$) were also significantly enriched. The molecular pathways that involved differentially expressed genes were mostly shared between the two cryptic species, but there were some putatively CHC biosynthesis-relevant candidate pathways privately expressed by *Cr. levior* A. These included 'biosynthesis of unsaturated fatty acids', 'alpha-Linolenic acid metabolism' and other metabolic processes as well as signaling pathways (Fig. 3.3 A).

In *Ca. femoratus*, we found lipid metabolism-associated genes significantly enriched in the set of transcripts upregulated in PAT compared to PS (GO:0006629 – lipid metabolic process; Fisher test: $p = 0.0074$; Supplementary Fig. S3.3; Electronic Supplementary Table S3.5).

Although again most pathways were shared between the cryptic species, indicating the use of different genes for similar functions, similarly to *Cr. levior* A, we found the pathways 'biosynthesis of unsaturated fatty acids' and 'alpha-Linolenic acid metabolism' to be exclusively upregulated in *Ca. femoratus* PS (Fig. 3.3 B).

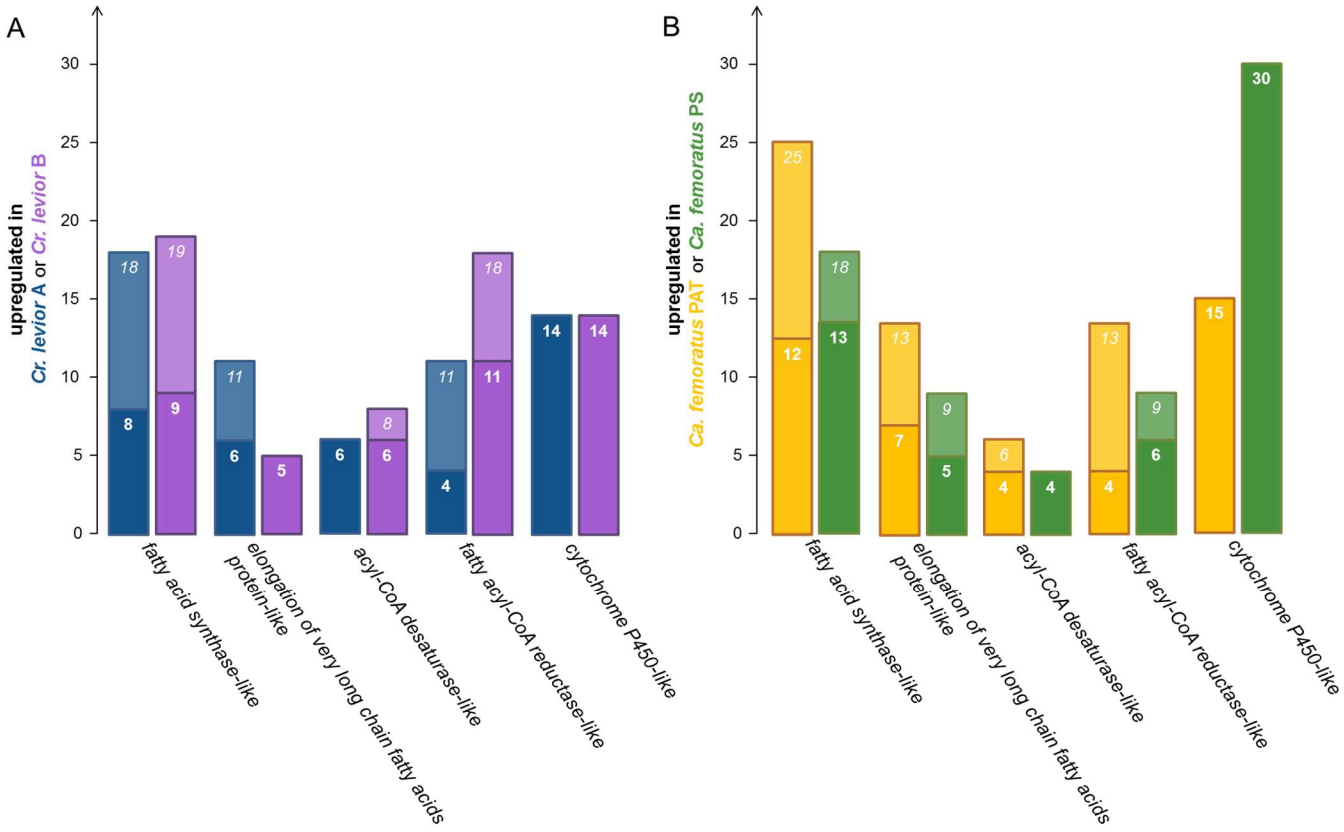

Figure 3.2: Differentially expressed transcripts of candidate gene families involved in CHC biosynthesis. The bars indicate the number of differentially expressed transcripts for five gene families (fatty acid synthases, very long chain fatty acid elongases, acyl-CoA desaturases, fatty acid reductases and cytochrome P450s) for (A) the two cryptic species of *Cr. levior* and (B) the two cryptic species of *Ca. femoratus*. Lightly colored bars with numbers in italics indicate BLAST hits against the non-redundant arthropod protein database (NCBI, state January 2018), dark colored bars with bold numbers indicate transcripts that additionally had a BLAST hit against genes of *Drosophila* that were shown to play a role in CHC biosynthesis (Supplementary Table S3.3).

3.3.4 Acclimatory changes of the CHC profile

Since the environment, climatic conditions and/or food sources might affect the CHC profile, we studied how the chemical profile of lab-maintained ants differed from those caught in the wild. After acclimating the ants to identical lab conditions, each species still

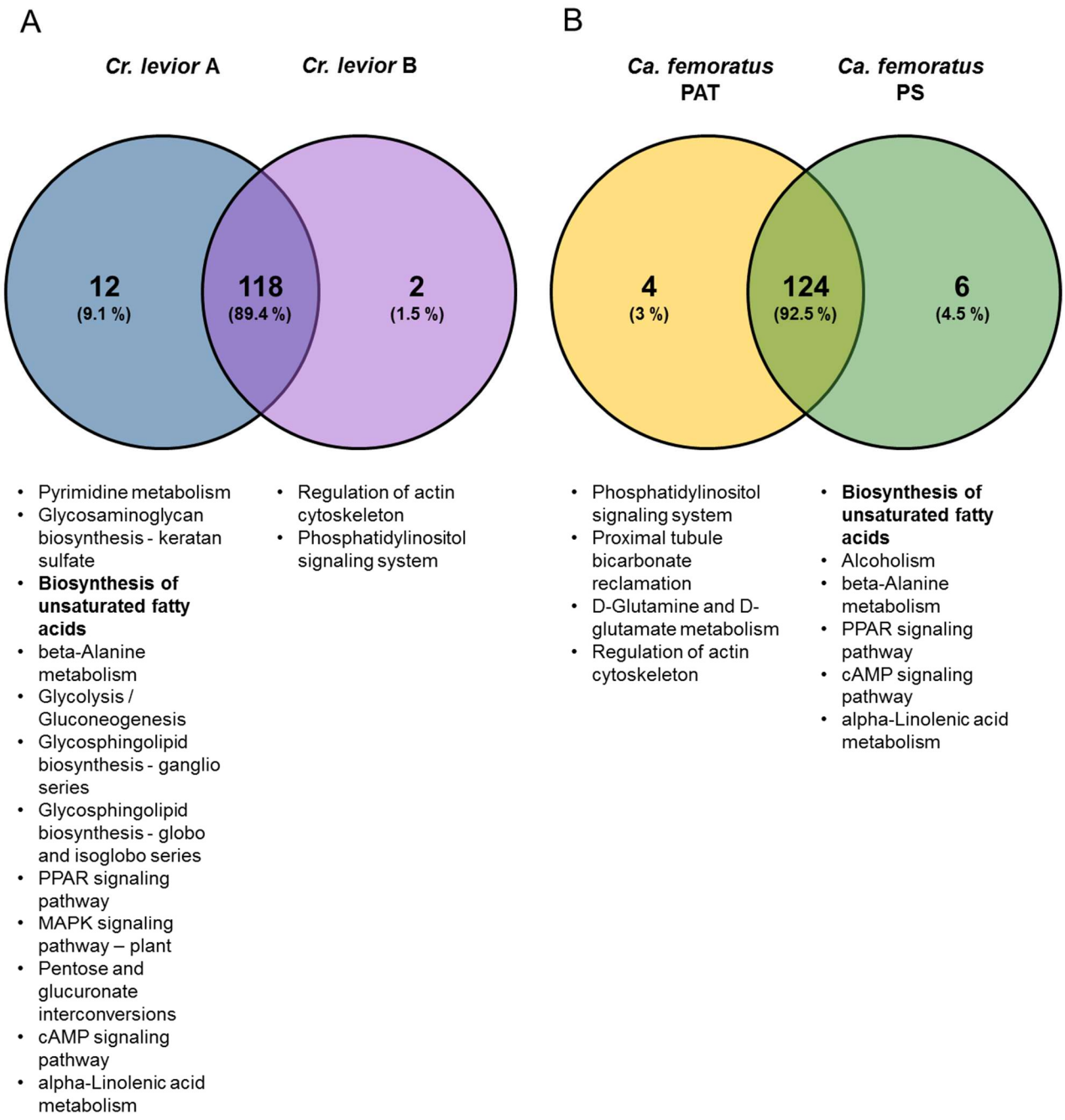

Figure 3.3: Venn diagrams of shared and privately expressed pathways. Two Venn diagrams representing the percentages of shared or privately expressed pathways between *Cr. levior* A and B and *Ca. femoratus* PAT and PS. Privately expressed pathways for each cryptic species are noted below the diagrams. Note: Shared pathways evidence for differentially expressed transcripts between species, which belong to the same pathway.

had a distinct species-specific profile, i.e. we were still able to clearly distinguish the two cryptic species for both genera; in *Cr. levior* (PERMANOVA: pseudo-F_1 = 23.76, p = 0.003; Fig. 3.4 A) and *Ca. femoratus* (pseudo-F_1 = 57.50, p = 0.023; Fig. 3.4 B). The Bray-Curtis dissimilarity between the cryptic species of *Camponotus* was higher than between the *Crematogaster* species (*Ca.*: 0.72 vs. *Cr.*: 0.61).

However, we were also able to detect an effect of lab conditions on the CHC profiles, which slightly differed from the original ones (*Cr. levior*: pseudo-F_1 = 7.06, p = 0.002; *Ca. femoratus*: pseudo-F_1 = 3.29, p = 0.029). These effects of lab maintenance were species-specific (interaction cryptic species:mainenance: *Cr. levior*: pseudo-F_1 = 3.48, p = 0.001; *Ca. femoratus*: pseudo-F_1 = 2.89, p = 0.033). The lab condition CHC profile of colony 075-PAR (*Cr. levior* A) showed the strongest difference to the original profile of the colony (Fig. 3.4 A). In conclusion, we were able to show differences between original and lab CHC profile, but these changes were just quantitative, i.e. differences in relative composition, while the CHCs were qualitatively still the same.

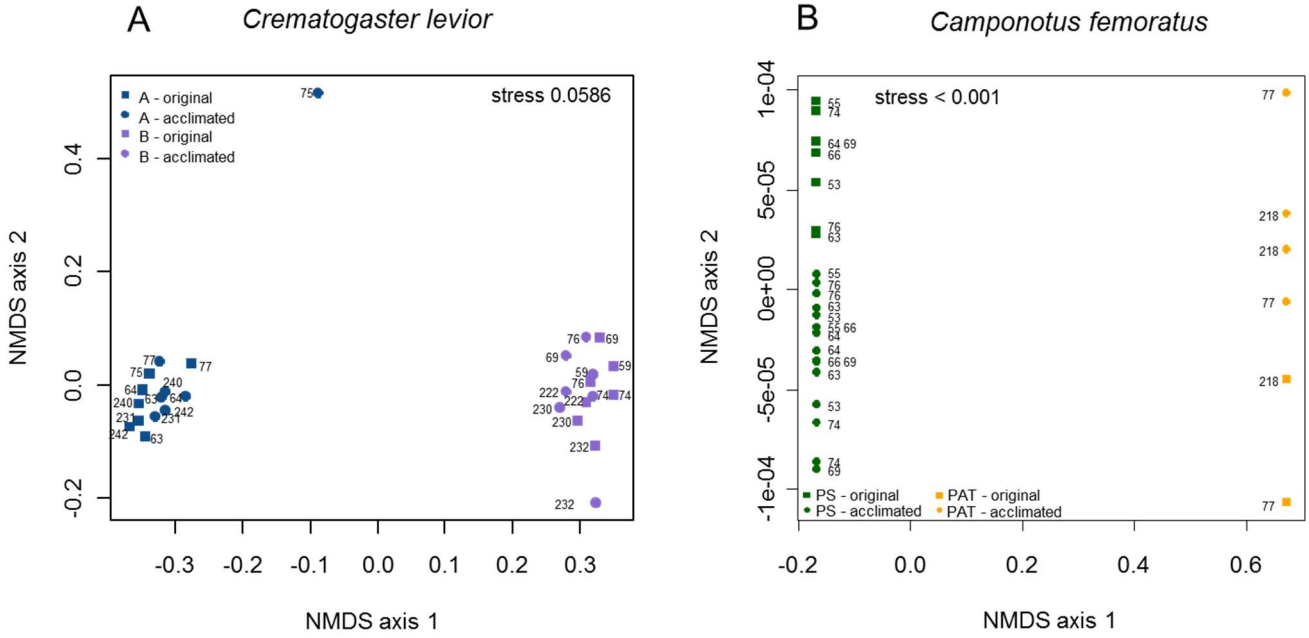

Figure 3.4: Non-metric multidimensional scaling of the cuticular hydrocarbon profiles of acclimated and non-acclimated parabiotic ants. Here we compare the CHC profiles of the cryptic species of (A) *Cr. levior* and (B) *Ca. femoratus* from their natural habitat and after 16 days of standardized lab conditions. Each dot represents the CHC profile of one colony. For *Ca. femoratus*, we had two acclimated individuals per colony but only one extract for the field-collected CHCs.

3.4 Discussion

Both of the parabiotic cryptic species of *Crematogaster levior* and *Camponotus femoratus* are closely related but express largely different CHC profiles despite similar ecological niches (Hartke, Sprenger *et al.* 2019, Chapter 1; Sprenger *et al.* 2019, Chapter 2). Here we compared gene expression patterns between the cryptic species and identified a number of candidate genes that may be involved in the synthesis of their species-specific CHC profiles.

3.4.1 General differences in gene expression

In both cryptic species pairs, we found very different gene expression patterns with over 5,000 differentially expressed genes (Fig. 3.1). This high number might not be surprising given that we compared different species: In *Crematogaster* 8.83% of the transcriptome was differentially expressed, which is comparable to 8% reported for the comparison of *Drosophila mojavensis* and *D. arizoniae* (Lopez-Maestre *et al.* 2017). The percentage of differentially expressed transcripts in *Camponotus*, however, was slightly higher with 12.76%.

Stronger differences in gene expression coincide with the higher Bray-Curtis dissimilarity in the CHC profiles between the two species of *Ca. femoratus*. Interestingly, the latter two cryptic species however, seem to be more closely related than the cryptic species pair in *Cr. levior* (Hartke, Sprenger *et al.* 2019, Chapter 1). The stronger genetic differentiation between *Cr. levior* A and B but higher CHC dissimilarity may indicate that few differentially expressed genes are sufficient to synthesize such highly different CHC profiles. We consistently found enriched gene functions related to metabolism in the upregulated transcripts of three out of four species, and in *Ca. femoratus* PAT we found functions related to lipid metabolism. Cuticular hydrocarbons, which are lipids in the broader sense, are derived from the fatty acid biosynthetic pathway (Blomquist 2010b). Therefore, the enrichment of 'lipid metabolism' might account for higher number of differentially expressed CHC genes.

Interestingly, we found 'biosynthesis of unsaturated fatty acids' as a privately expressed pathway in *Cr. levior* A and in *Ca. femoratus* PS (Fig. 3.3). This strikingly fits the composition of substance classes in the CHC profiles of these species. *Crematogaster levior* A possesses more mono- as well as di-unsaturated hydrocarbons in its CHC profile than its cryptic sister species (Hartke, Sprenger *et al.* 2019, Chapter 1; Sprenger *et al.* 2019, Chapter 2). Similarly, *Ca. femoratus* PS has more unsaturated CHCs such as alkenes and methyl-branched alkenes than the other species, although PAT itself has proportionally more alkadienes (which is driven by several different C41 alkadienes) (Hartke, Sprenger *et al.* 2019, Chapter 1; Sprenger *et al.* 2019, Chapter 2). Thus, this pathway is likely important for the biosynthesis of unsaturated CHCs. In a similar line, the metabolism of alpha-linolenic acid, a poly-unsaturated fatty acid, was also exclusively expressed in *Cr. levior* A and *Ca. femoratus* PS. Although speculative, this is consistent with the idea that poly-unsaturated fatty acids can serve as precursors of unsaturated CHCs in these species.

3.4.2 Candidate genes putatively involved in the biosynthesis of different CHC profiles

We looked for differentially expressed candidate genes of five different gene families that are known to be involved in CHC biosynthesis: Fatty acid synthases (FAS), very long-chain fatty acid elongases, acyl-CoA desaturases, fatty acyl-CoA reductases and cytochrome P450s (Blomquist 2010b; Chung & Carroll 2015).

Fatty acid synthases (FAS) produce and elongate fatty acyl-CoA (Chung & Carroll 2015). They can be distinguished in cytosolic FAS responsible for non-branched CHCs (*n*-alkanes and alkenes) and microsomal FAS, that produce methylmalonyl-CoA as precursor for methyl-branched CHCs (Juárez, Chase & Blomquist 1992; Gu *et al.* 1997). For *fatty acid synthase-like* transcripts, we found 37 and 43 differentially expressed transcripts in *Crematogaster* and *Camponotus*, respectively, three of which could be assigned to *Drosophila* CHC FAS candidates. *FASN2* was shown to be a microsomal FAS that specifically synthesizes the precursors for methyl-branched CHCs in *Drosophila* (Chung *et al.* 2014). In addition, previous studies found that complex methyl-branched CHC profiles are likely linked to gene expansions in FAS (Finck *et al.* 2016). The high number of *fatty acid synthase-like* transcripts without a BLAST hit in *Drosophila* tentatively suggests a higher number of FAS in ants, which could account for the higher diversity of methyl-branched hydrocarbons.

Very long-chain fatty acid elongases add two additional carbons stepwise to the fatty acyl-CoAs. Their activity is sequence-specific, which determines the length of the final fatty acids or CHCs (Denic & Weissman 2007). Interestingly, in line with the CHC profiles, where *Cr. levior* A shows strong chain elongations, we found 11 transcripts upregulated in A and only 5 in B. The lower number of upregulated elongase-like transcripts in *Cr. levior* B reflects probably the lack of overall chain elongation typical for parabiotic ants, as e.g. in *Cr. levior* A or the two *Ca. femoratus* species (Sprenger *et al.* 2019, Chapter 2). We found BlastX similarity for three out of six upregulated elongase-like transcripts of *Cr. levior* A to a putative elongase (*CG9458*), whose knock-down led to decreases in the proportion of *n*-alkanes and alkenes in *D. melanogaster* females (Dembeck *et al.* 2015). This gene may thus also be responsible for the high proportion of long-chained alkenes found in *Cr. levior* A (Sprenger *et al.* 2019, Chapter 2). Similar to *Cr. levior* A, we found several upregulated elongase-like transcripts in each of the two cryptic species of *Ca. femoratus* that also possess many CHCs of high chain length (Sprenger *et al.* 2019, Chapter 2).

During elongation, acyl-CoA desaturases insert one or more double bonds into the acyl-CoA chain (Chung & Carroll 2015). From *Drosophila* three desaturases are known to be involved in CHC biosynthesis: *desat1*, *desat2* and *desatF* (Dallerac *et al.* 2000; Chertemps *et al.* 2006). We found most differentially expressed desaturase-like transcripts in our four ant species to be most similar to *desat1*, which was also associated with CHC biosynthesis in seaweed flies (Berdan *et al.* 2019). Finding so many *desat1* hits in ants could indicate duplications and subsequent neofunctionalization in this gene (Helmkampf *et al.* 2015). In line with this, parabiotic ants produce unsaturated hydrocarbons with more variable double bond positions compared to *D. melanogaster* that mainly produces Z7-alkenes and Z7-Z11-alkadienes (Ferveur 2005; Sprenger *et al.* 2019, Chapter 2).

Fatty acyl-CoA reductases convert the acyl-CoA chain to an aldehyde (Chung & Carroll 2015). In *Drosophila* a knockdown of two putative fatty acid acyl-CoA reductases led to an increased production of longer-chain CHCs (Dembeck *et al.* 2015). In *Cr. levior* B 11 (out of 18) upregulated transcripts gave Blast hits to these two genes, while it were only 4 (in total 11) in *Cr. levior* A. Similar to the elongases, this is in line with the observation that the CHC profile of *Cr. levior* B consists of shorter compounds (lower mean chain length) compared to A and the two *Ca. femoratus* species (Sprenger *et al.* 2019, Chapter 2).

The final step of the CHC biosynthesis is the conversion of aldehydes to hydrocarbons by cytochrome P450s (Qui *et al.* 2012; Chung & Carroll 2015). Previous studies suggested that these enzymes could be specific to certain subsets of CHCs, although the function of many cytochrome P450s is unknown so far (Chung *et al.* 2009; Dembeck *et al.* 2015). While we found the same number (14 each) of these enzymes upregulated in *Cr. levior* A and B, we identified 30 putatively involved cytochrome P450s in *Ca. femoratus* PS and only 15 in PAT. Interestingly, *Ca. femoratus* PS produces nearly twice as many different CHCs compared to PAT (Sprenger *et al.* 2019, Chapter 2), which could explain this higher number of upregulated transcripts, if cytochrome P450s are indeed specific to a subset of CHCs.

3.4.3 CHC acclimation

Although the profiles of the lab-acclimated ants differed from those sampled at natural conditions, we could show that the species-specific CHC profiles were relatively stable despite environmental conditions and the acclimation responses were also species-specific. These results are thus consistent with CHC profiles of the ants being heritable (van Zweden *et al.* 2009; Walsh *et al.* 2019). The differences between acclimation and natural profile may be

the result of various differences between natural and lab conditions such as climate (Menzel *et al.* 2018; Sprenger *et al.* 2018), different food (Liang & Silverman 2000; Sorvari *et al.* 2008), different nest materials (Crosland 1989; Heinze *et al.* 1996) or isolation from either their parabiotic partners (Sprenger *et al.* 2019, Chapter 2) or their queen and subsequent changes in fertility (Liebig *et al.* 2000; Dietemann *et al.* 2003).

3.5 Conclusions

The regulation of CHC biosynthesis is complex due to direct and pleiotropic gene interactions affecting the CHC metabolism (Dembeck *et al.* 2015; Wicker-Thomas *et al.* 2015; Chiang *et al.* 2016; Massey *et al.* 2019). Interestingly, the gene expression differences identified here were higher between the *Camponotus* species pair than between the *Crematogaster* species. This matches the fact that the *Camponotus* species are also more different concerning their CHC profile, although they are more closely related than the two *Crematogaster* species (Hartke, Sprenger *et al.* 2019, Chapter 1). With this study, we identified candidate genes in two cryptic species pairs of parabiotic ants that are putatively involved in CHC biosynthesis. Although the functional validation of the candidate genes remains open, the parallelism between the differentially expressed transcripts, their known function in *Drosophila*, and the CHC profiles strongly suggest that most of the presented candidates contribute to the largely different CHC profiles of these ants (Hartke, Sprenger *et al.* 2019, Chapter 1; Sprenger *et al.* 2019, Chapter 2). By identifying candidate genes, we provide a basis for further studies on CHC biosynthesis and their evolution in a highly interesting model system.

3.6 Acknowledgements

Removed for privacy purposes.

3.7 Supplementary material

Table S3.1: List of colonies sampled. The table includes the colony ID, species, sampling location and GPS coordinates of the parabiotic colonies collected initially and their experimental usage (RNA = used for RNAseq and transcriptome assembly; CHC = used for chemical analysis).

Colony ID	Species	Location	GPS	Usage
063-PAR	*Cr. levior* A	Paracou	N 05°15.574 W 52°55.815	RNA + CHC
064-PAR	*Cr. levior* A	Paracou	N 05°15.552 W 52°55.852	RNA + CHC
075-PAR	*Cr. levior* A	Paracou	N 05°15.639 W52°56.097	RNA + CHC
077-PAR	*Cr. levior* A	Paracou	N 05°15.698 W 52°55.913	CHC only; library preparation failed
231-PAT	*Cr. levior* A	Camp Patawa	N 04°31.945 W 52°07.391	RNA only
240-PAT	*Cr. levior* A	Camp Patawa	N 04°33.241 W 52°09.040	RNA + CHC
242-PAT	*Cr. levior* A	Camp Patawa	N 04°33.281 W 52°09.065	RNA + CHC
059-PAR	*Cr. levior* B	Paracou	N 05°15.344 W 52°55.617	RNA + CHC
069-PAR	*Cr. levior* B	Paracou	N 05°15.503 W 52°55.982	RNA + CHC
074-PAR	*Cr. levior* B	Paracou	N 05°15.578 W 52°56.133	RNA + CHC
076-PAR	*Cr. levior* B	Paracou	N 05°15.698 W 52°55.913	RNA + CHC
218-PAT	*Cr. levior* B	Camp Patawa	N 04°32.703 W 52°07.991	RNA only
219-PAT	*Cr. levior* B	Camp Patawa	N 04°32.702 W 52°07.946	RNA only
222-PAT	*Cr. levior* B	Camp Patawa	N 04°32.687 W 52°07.872	RNA + CHC
230-PAT	*Cr. levior* B	Camp Patawa	N 04°31.572 W 52°07.107	RNA + CHC
232-PAT	*Cr. levior* B	Camp Patawa	N 04°32.269 W 52°07.923	RNA + CHC
045-PAR	*Ca. femoratus* PAT	Paracou	N 05°16.671 W 52°55.110	group dead upon arrival
051-PAR	*Ca. femoratus* PAT	Paracou	N 05°16.097 W 52°55.611	RNA only
059-PAR	*Ca. femoratus* PAT	Paracou	N 05°15.344 W 52°55.617	RNA only
077-PAR	*Ca. femoratus* PAT	Paracou	N 05°15.698 W 52°55.913	RNA + CHC
218-PAT	*Ca. femoratus* PAT	Patawa	N 04°32.703 W 52°07.991	RNA + CHC
232-PAT	*Ca. femoratus* PAT	Camp Patawa	N 04°32.269 W 52°07.923	RNA only

240-PAT	*Ca. femoratus* PAT	Camp Patawa	N 04°33.241 W 52°09.040	RNA only
242-PAT	*Ca. femoratus* PAT	Camp Patawa	N 04°33.281 W 52°09.065	RNA only
053-PAR	*Ca. femoratus* PS	Paracou	N 05°16.163 W 52°55.447	RNA + CHC
055-PAR	*Ca. femoratus* PS	Paracou	N 05°15.702 W52°55.443	RNA + CHC
063-PAR	*Ca. femoratus* PS	Paracou	N 05°15.574 W 52°55.815	RNA + CHC
064-PAR	*Ca. femoratus* PS	Paracou	N 05°15.552 W 52°55.852	CHC only; library preparation failed
066-PAR	*Ca. femoratus* PS	Paracou	N 05°15.865 W 52°55.500	CHC only; removed due to skewed GC content
069-PAR	*Ca. femoratus* PS	Paracou	N 05°15.503 W 52°55.982	CHC only; library preparation failed
074-PAR	*Ca. femoratus* PS	Paracou	N 05°15.578 W 52°56.133	RNA + CHC
076-PAR	*Ca. femoratus* PS	Paracou	N 05°15.698 W 52°55.913	RNA + CHC

Table S3.2: Sample combination for subassemblies. The table shows the CLC subassemblies and the samples used for those. Subassemblies were afterwards meta-assembled with *MIRA*.

Subassembly	Sample IDs
***Cr. levior* A #1**	63-PAR-Cr-FB2 64-PAR-Cr-FB2 75-PAR-Cr-FB1
***Cr. levior* A #2**	231-PAT-Cr-FB1 240-PAT-Cr-FB2 242-PAT-Cr-FB2
***Cr. levior* B #1**	59-PAR-Cr-FB2 69-PAR-Cr-FB2 74-PAR-Cr-FB2
***Cr. levior* B #2**	222-PAT-Cr-FB2 230-PAT-Cr-FB2 232-PAT-Cr-FB1
***Cr. levior* B #3**	76-PAR-Cr-FB2 218-PAT-Cr-FB1 219-PAT-Cr-FB1
***Ca. femoratus* PAT #1**	51-PAR-Ca-FB1 59-PAR-Ca-FB1 218-PAT-Ca-FB1 240-PAT-Ca-FB2
***Ca. femoratus* PAT #2**	77-PAR-Ca-FB2 232-PAT-Ca-FB2 242-PAT-Ca-FB1
***Ca. femoratus* PS #1**	53-PAR-Ca-FB1 55-PAR-Ca-FB1
***Ca. femoratus* PS #2**	63-PAR-Ca-FB2 74-PAR-Ca-FB2 76-PAR-Ca-FB1

Table S3: List of genes known to be involved in CHC biosynthesis in *Drosophila*. We list the gene IDs, gene names, (putative) functions and source literature supporting its role in CHC biosynthesis.

Annotation ID in *Drosophila*	Gene name	Function	Literature
CG3524	*Fatty acid synthase 2*	Fatty acid synthase	Chung *et al.* 2014
CG17374	*Fatty acid synthase 3*	Fatty acid synthase	Wicker-Thomas *et al.* 2015
CG16905	*Elongase F*	Very long-chain fatty acid elongase	Chertemps *et al.* 2007
CG2781	*ELOVL fatty acid elongase*	Very long-chain fatty acid elongase	Chiang *et al.* 2016
CG6921	*bond*	Very long-chain fatty acid elongase of the Elov1 family	Ng *et al.* 2015
CG9458	*uncharacterized protein CG9458*	Putative very long-chain fatty acid elongase	Dembeck *et al.* 2015
CG18609	*uncharacterized protein CG18609*	Putative very long-chain fatty acid elongase	Dembeck *et al.* 2015
CG30008	*uncharacterized protein CG30008*	Putative very long-chain fatty acid elongase	Dembeck *et al.* 2015
CG5887	*Desaturase 1*	Delta(9)-Desaturase	Dallerac *et al.* 2000; Labeur *et al.* 2002
CG5925	*Desaturase 2*	Delta(9)-Desaturase	Dallerac *et al.* 2000
CG7923	*Desaturase F*	Delta(9)-Desaturase	Chertemps *et al.* 2006
CG13091	*uncharacterized protein CG13091*	Putative acetyl-CoA reductase	Dembeck *et al.* 2015
CG10097	*uncharacterized protein CG10097*	Putative acetyl-CoA reductase	Dembeck *et al.* 2015
CG17562	*uncharacterized protein CG17562*	Putative acetyl-CoA reductase	Chiang *et al.* 2016
CG18377	*Cyp49a1*	Putative cytochrome P450	Dembeck *et al.* 2015
CG11466	*Cyp9f2*	Putative cytochrome P450	Dembeck *et al.* 2015
CG9081	*Cyp4s3*	Putative cytochrome P450	Dembeck *et al.* 2015
CG3972	*Cyp4g1*	Cytochrome P450	Qui *et al.* 2012

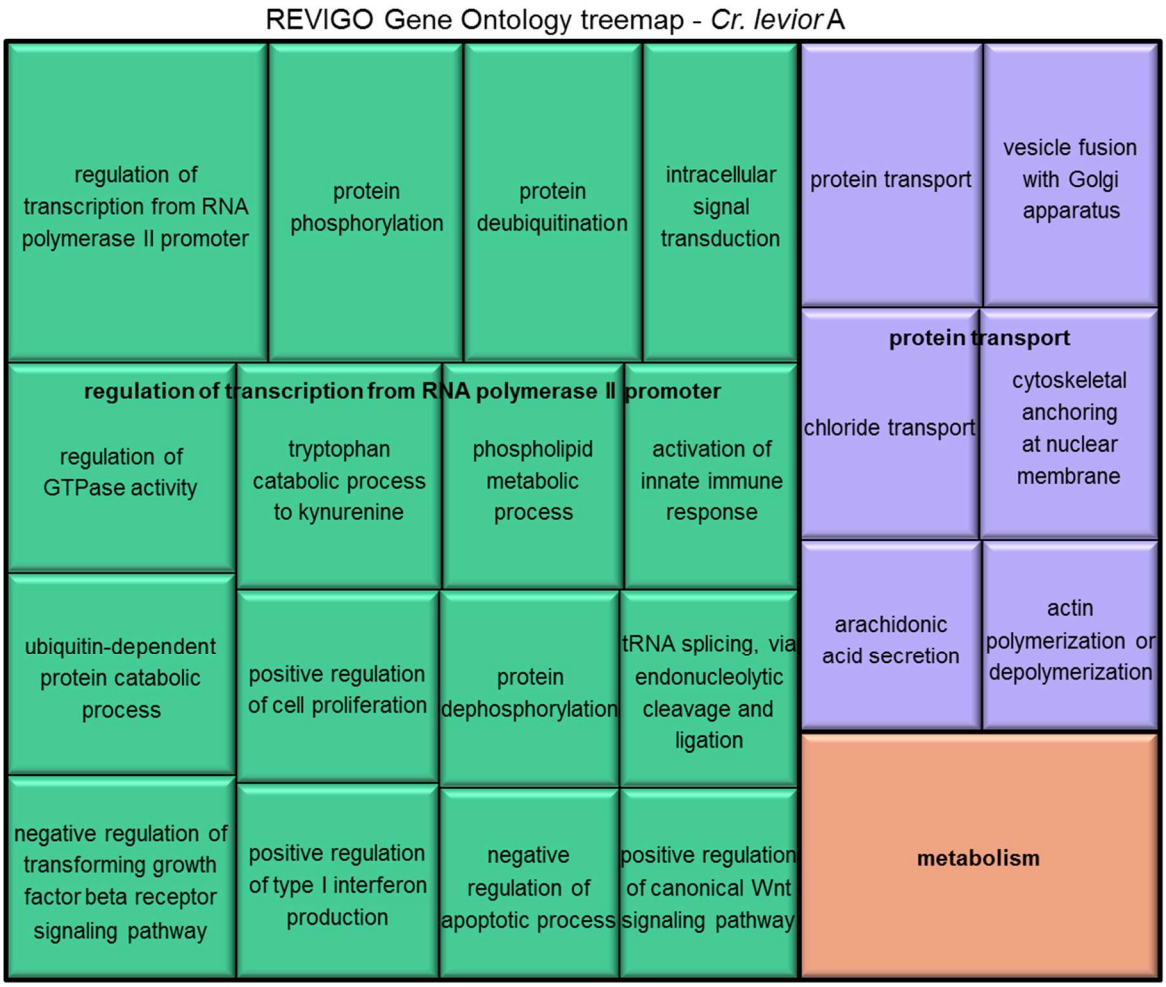

Figure S3.1: REVIGO treemap illustrating significantly enriched GO terms in *Crematogaster levior* A. Significantly enriched GO terms (Fisher-test: $p < 0.05$) of genes upregulated in *Cr. levior* A compared to B. Relative square sizes are scaled to match the degree of overexpression (Supek *et al.* 2011).

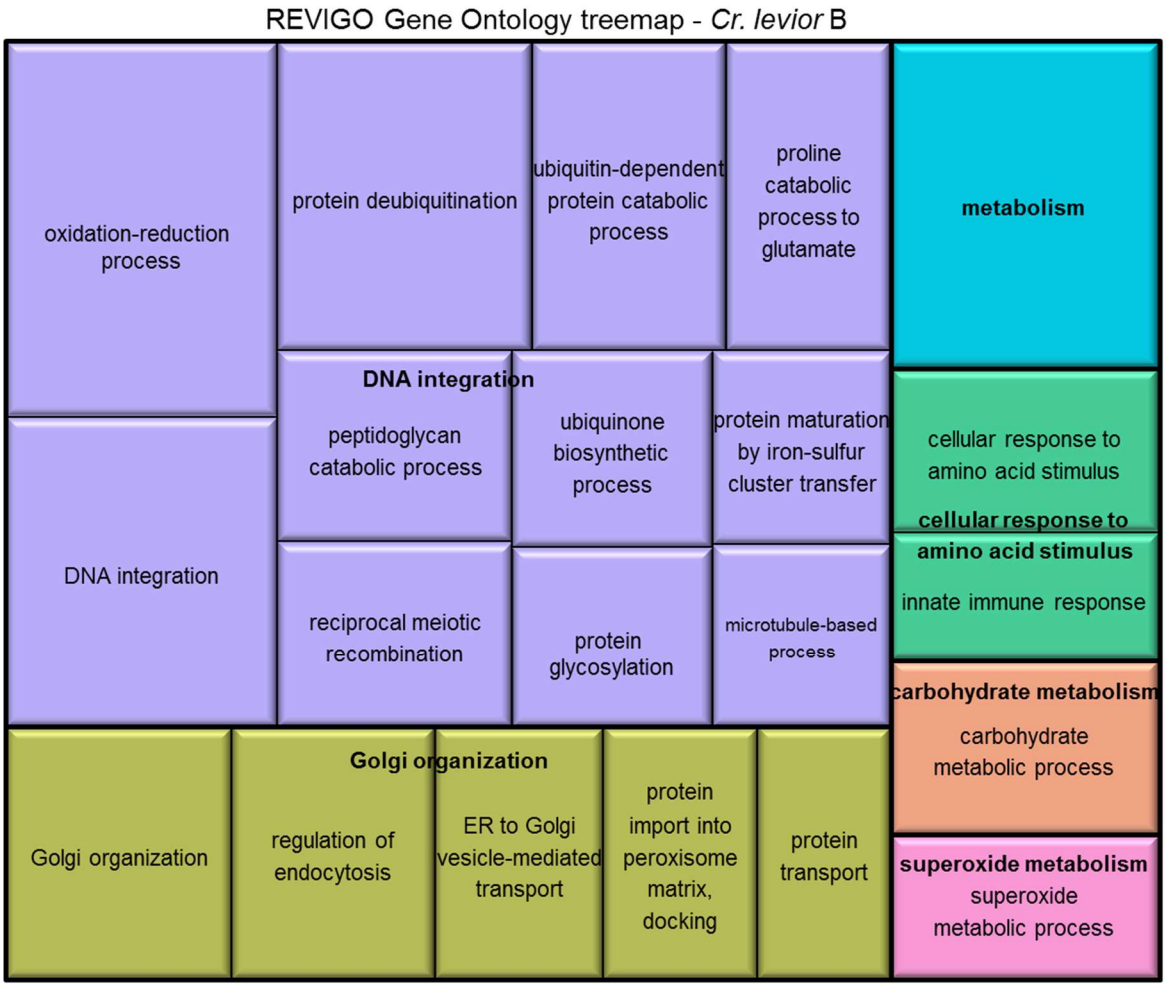

Figure S3.2: REVIGO treemap illustrating significantly enriched GO terms in Crematogaster levior B. Significantly enriched GO terms (Fisher-test: $p < 0.05$) of genes upregulated in Cr. levior B compared to A. Relative square sizes are scaled to match the degree of overexpression (Supek *et al.* 2011).

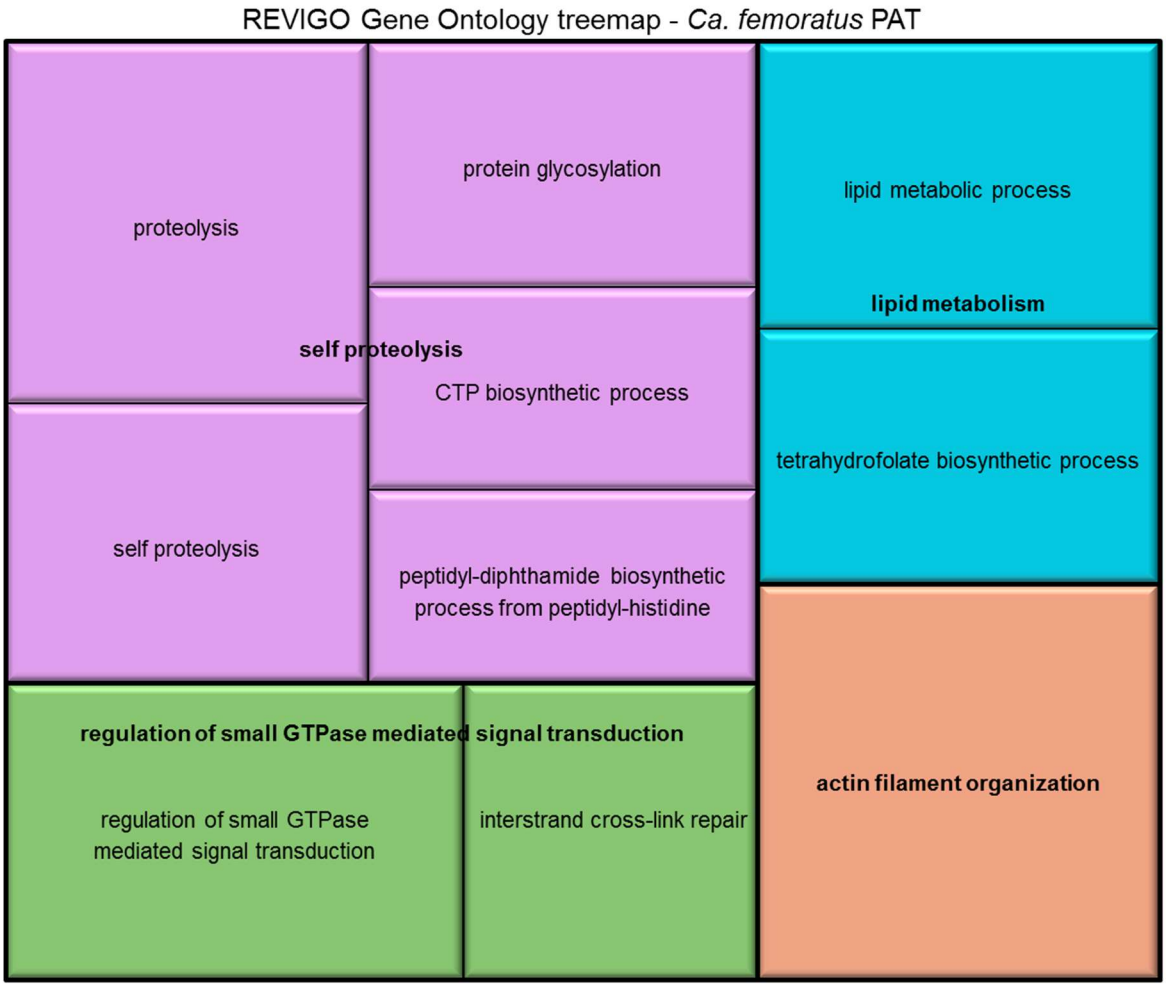

Figure S3.3: REVIGO treemap illustrating significantly enriched GO terms in *Camponotus femoratus* PAT. Significantly enriched GO terms (Fisher-test: $p < 0.05$) of genes upregulated in *Ca. femoratus* PAT compared to PS. Relative square sizes are scaled to match the degree of overexpression (Supek *et al.* 2011).

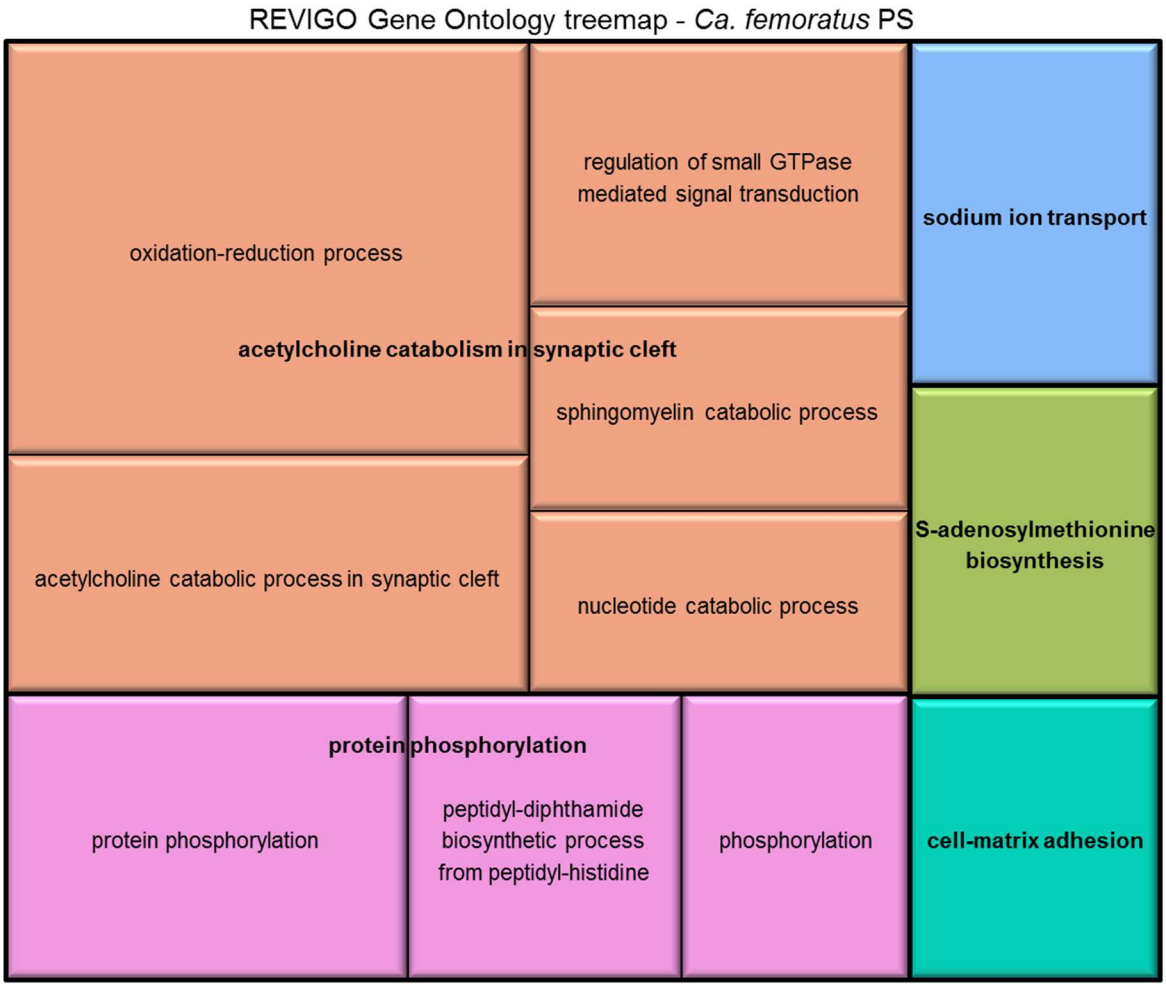

Figure S3.4: REVIGO treemap illustrating significantly enriched GO terms in *Camponotus femoratus* PS. Significantly enriched GO terms (Fisher-test: $p < 0.05$) of genes upregulated in *Ca. femoratus* PS compared to PAT. Relative square sizes are scaled to match the degree of overexpression (Supek *et al.* 2011).

Chapter 4

Nestmate recognition in the cryptic species of *Crematogaster levior*

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Abstract

Ant colonies discriminate nestmates from foreigners by their cuticular hydrocarbon (CHC) profiles. Most often it is assumed that hydrocarbons with methyl branches or double bonds can be distinguished more easily and thus, have higher information content. The mutualistically associated ant *Crematogaster levior* is present in two chemically different varieties that in fact represent cryptic species (*Cr. levior* A and B). Which CHCs or CHC ratios are used for nestmate recognition is most likely species-specific, which is why we here aimed to investigate which CHCs are used for nestmate recognition in *Cr. levior* A and B. In experiment 1, we used a full-factorial design to test if ants of either species react more or less aggressively towards dummies with CHCs of either conspecifics or the other species and if this correlates with the chemical distance between the reacting colony and the presented odor. Although we found that workers of *Cr. levior* A were more aggressive and CHC extracts of *Cr. levior* B were treated more aggressively, we did not find any evidence for nestmate recognition or correlations with chemical distance. In experiment 2, we aimed to manipulate nestmate extracts with different fractions of foreign CHC profiles, but could not find any differences between the treatments. The finding that we could not demonstrate nestmate recognition in our experiments is surprising. It is interesting because of the low genetic diversity found at least in *Cr. levior* A, but could also be an artefact of the usage of dummies with CHC extracts. Furthermore, possibly similar acclimations to our maintenance conditions could have led to reduction of nestmate recognition cues and thus loss of the discrimination ability.

4.1 Introduction

Among all insect families, ants are among the most diverse and ecologically most successful ones (Hölldobler & Wilson 1990). They occur worldwide and it was assumed that their biomass equals or even exceeds that of humans on Earth (Hölldobler & Wilson 1990, 1994). Cooperation between colony members is thought to be a key feature for the ecological success of ants because it enables them to e.g. effectively defend their brood and their nest against intruders or to monopolize food sources by expelling conspecific and allospecific competitors. To only cooperate with individuals of the same colony, it is necessary for ant workers to reliably distinguish nestmates from non-nestmates. Nestmate recognition in ants is possible through colony-specific cuticular hydrocarbon (CHC) profiles (Morel, Vander Meer & Lavine 1988; Lahav *et al.* 1999). Cuticular hydrocarbons are important agents of chemical communication, but at the same time protect insects against desiccation (Blomquist & Bagnères 2010). A CHC profile usually consists of a mixture of *n*-alkanes, methyl-branched alkanes and/or unsaturated hydrocarbons like alkenes and alkadienes (Sprenger & Menzel 2020, Chapter 6; Blomquist 2010a).

According to the 'Gestalt model' the colony odor in ants is achieved through constant exchange of CHCs between nestmates that compare similarity between a 'neuronal template' and the opponent's chemical label (Crozier & Dix 1979). It is assumed that CHC profiles are homogenized in the postpharyngeal glands of the workers and applied on the colony members via allogrooming (Soroker & Hefetz 2000; Soroker *et al.* 2003). However, not all workers of a colony have the same CHC profile as it usually differs between reproductive and behavioral castes and informs colony members about their counterpart's tasks (Wagner *et al.* 1998; Liebig *et al.* 2000; Greene & Gordon 2003). The neuronal template can be established via two mechanisms: 'self-referent phenotype matching', i.e. an individual compares its own odor to the opponent, or 'prior association', i.e. an individual learns the odor of other individuals encountered before (d'Ettorre & Lenoir 2010). In colonies that contain different species, ants learn CHC profiles that are very different from their own ones (Errard *et al.* 2006; Menzel *et al.* 2008b) and can remember this template even over long time periods like one year (Errard 1994). However, the recognition template is plastic and reformed constantly to allow for rapid behavioral changes (Leonhardt, Brandstaetter & Kleineidam 2007).

Although CHCs enable nestmate discrimination, it seems rather species-specific which hydrocarbons or ratios of hydrocarbons actually contain the information on nestmate identity: In some *Formica* species nestmate recognition was found to be based on either the ratio of Z9-alkenes to *n*-alkanes or even Z9-alkenes alone (Akino *et al.* 2004; Martin *et al.* 2008c). Some other studies found that only dimethyl alkanes elicited aggression in *Camponotus herculeanus* (Guerrieri *et al.* 2009), while *Tetramorium caespitum* workers supplemented with monomethyl alkanes or alkenes were both treated aggressively by nestmates (Sano, Bannon & Greene 2018). Also supplementation of alkenes that are not produced by an ant species itself usually trigger aggression (Meskali *et al.* 1995). Argentine ants were shown to rather perceive differences methyl-branch positions than the chain lengths of homologous series of hydrocarbons (van Wilgenburg *et al.* 2010) and it seems that ants perceive information of functional group and chain length independently (Bos *et al.* 2012). Further on, aggression towards conspecifics was found to correlate with chemical distance between colonies in *Temnothorax unifasciatus* and *Anoplolepis gracilipes* (Foitzik *et al.* 2007; Drescher *et al.* 2010).

Despite ants usually treating individuals of different colonies or species aggressively, parabiotic ants of the genera *Crematogaster* and *Camponotus* live in the same nest and tolerate the other species (Menzel *et al.* 2008b, 2014). The parabiotic ants keep their own species-specific profiles that usually have elongated hydrocarbons and higher proportions of rare substance classes as adaptations to this mutualistic lifestyle (Menzel & Schmitt 2012; Sprenger *et al.* 2019, Chapter 2). In the Neotropics, the two cryptic ant species *Crematogaster levior* A and B are present in parabiosis with two other cryptic species of *Camponotus femoratus* (Hartke, Sprenger *et al.* 2019, Chapter 1). Interestingly, both have strongly different CHC profiles: *Crematogaster levior* A shows typical chain elongations and possesses mainly alkenes and alkadienes, while *Cr. levior* B carries more mono-methyl alkanes (Sprenger *et al.* 2019, Chapter 2). Because the CHC profiles of parabiotic ants are highly unusual, we here aimed to find out which CHCs are used as nestmate recognition signals and if the adaptations found in *Cr. levior* A affect intra- and interspecific recognition. In the first experiment, we asked whether aggression correlates with chemical distance, e.g. whether colonies of the respective other cryptic species are treated more aggressively. Further on, in experiment 2, we manipulated nestmate odor by supplementing different CHC classes to the profile to infer which of them could play a role in nestmate recognition.

4.2 Materials and methods

4.2.1 Colony collection and maintenance

We collected 18 ant gardens habituated by the parabiotic ant species *Crematogaster levior* and *Camponotus femoratus* at four different locations in French Guiana during September and October 2018 (Table S4.1) and kept them at the Paracou Research Station (5°14.04 N, 52°54.28 W). The colonies were maintained in large plastic boxes (Carrefour Home, 20 L) covered with linen sheets. To prevent the ants from escaping, the walls of the boxes were covered with Fluon® (Whitford GmbH, Diez, Germany). We fed the ants every two days with honey and luncheon meat (Zwan, Almelo, The Netherlands); water was provided *ad libitum* in cotton pads. To maintain humidity, the ant gardens were moistened with every feeding. Species identity of the *Cr. levior* colonies was determined by the species-specific CHC profile analyzed using gas-chromatography mass-spectrometry (GC-MS) at the University of Würzburg, Germany.

4.2.2 Experiment 1: Correlation of chemical distance and aggression

In experiment 1, we tested the aggression between twelve colonies of *Cr. levior* (*Cr. levior* A: n = 6; *Cr. levior* B: n = 6; Table S4.1) in a full-factorial design with every possible combination. To ensure that the CHCs elicited the aggressive behaviors, we prepared odorless dummies and applied CHC extracts on them. Afterwards, we tested for correlation of the chemical distance with the aggression between the colonies.

4.2.2.1 Extract and dummy preparation

For the following aggression tests, we prepared CHC extracts of the *Cr. levior* colonies. In experiment 1, we used equivalents of the CHCs of five workers per dummy. To this end, we collected 250 ant workers outside the nests (i.e. foragers) from each of the twelve colonies used here and freeze-killed them at -20°C. Subsequently, we extracted the CHCs by immersing the ants in *n*-hexane for ten minutes. To purify the extracts, we fractionated them with SiOH columns (3 mL, Chromabond, Macherey-Nagel, Düren, Germany). The CHC fraction was eluted with *n*-hexane, while any more polar compounds that are present on the cuticle of *Cr. levior* (Hartke, Sprenger *et al.* 2019, Chapter 1) remained on the column. Finally, we evaporated the hexane and resolved the CHCs in a standardized quantity (here 550 µL – enough for all behavioral tests and GC-MS analysis). However, due to strong evaporation of

the solvent during the aggression tests, we had to re-extract ants of some colonies for the last round of replicates according to the tests already conducted (Table S4.2). After the aggression tests, the extracts were analyzed using GC-MS as described in (Sprenger *et al.* 2019, Chapter 2).

Dummies were washed for one hour in *n*-hexane, one hour in dichloromethane and again one hour in *n*-hexane to remove all CHCs and more polar chemical substances from their cuticle. Afterwards, they were dried and kept at -20°C until usage.

4.2.2.2 Aggression tests

To test the aggression, we applied 10 µl of the CHC extract of a colony on a dummy and waited for at least one minute for evaporation of the solvent. The dummy was then presented to four foragers collected from the stock colonies in a round plastic arena (Ø 7 cm) using straight tweezers (Dumont, Montignez, Switzerland). The walls of the plastic arenas were covered with Fluon® to prevent the ants from escaping and blank paper was used as subsurface. In the following, we observed the ants for three minutes and recorded six different behaviors: short antennation (2 or less contacts), long antennation, mandible spreading, gaster lifting, biting and mandible locking. Antennations were considered amicable, while all other behaviors were judged as aggression.

We used a full-factorial design testing dummies with CHCs of every of the twelve colonies against living ants from each of these colonies and did four replicates per each combination of colony and CHC extracts (N = 12 x 12 x 4 = 576 tests). All aggression tests were conducted blindly in regard of the colony extracts tested.

4.2.2.3 Statistical analyses

To test if the aggression differed between *Cr. levior* A and *Cr. levior* B and the response towards their CHC profiles, we first calculated an aggression index (AI) using the formula

$$\mathrm{AI} = \frac{0.5\times\mathrm{MS}+0.5\times\mathrm{GL}+\mathrm{B}+\mathrm{LM}}{\text{interactions total}} \qquad (4.1)$$

with mandible spreading (MS) and gaster lifting (GL) scored as medium aggressive and biting (B) and locking mandibles (LM) as aggressive behaviors.

The AI was subsequently used as dependent variable in an univariate PERMANOVA based on Euclidean distance, which is similar to an ANOVA with 999 permutations (Anderson 2017, command *adonis*, R-package *vegan*) as the data neither followed a Gaussian distribution nor any other distribution we could fit. We used the species identity of the focal colony (A vs. B), the species identity of the presented extract, the nestmate identity (nestmate vs. non-nestmate), colony ID (nested in species identity) and the trial round (1 to 4) as fixed effects for the PERMANOVA. To avoid values with zero distance, we added minute normally distributed random values (mean ± SD: $10^{-8} \pm 10^{-8}$) to every AI.

To compare if the aggression between colonies correlated with their chemical dissimilarity, we performed Mantel tests based on Spearman's rank correlation. We used triangular distance matrices of the aggression indices (1 vs. 2, 2 vs. 1 and a mean of both) to compare them to Bray-Curtis-dissimilarity matrices containing chemical distances. The distances were calculated based on the relative abundances of all CHCs, or only based on the abundances of the compounds within a certain substance class (*n*-alkanes, alkenes, alkadienes, monomethyl alkanes, dimethyl alkanes and unknown CHCs).

4.2.3 Experiment 2: Interference of nestmate recognition

In experiment 2, we aimed to manipulate the ratios of certain substance classes in a colony CHC extract to examine which substances would hamper nestmate recognition. We presented dummies with distorted nestmate CHC profiles to foragers from a focal colony and observed if manipulation of the colony odor would result in aggression. Here, we used ten colonies of *Cr. levior* A and eight colonies of *Cr. levior* B (Table S4.1).

4.2.3.1 Extract preparation

We prepared nestmate CHC extracts that were equivalent to five workers per dummy (here 120 ants per nestmate extract). Furthermore, we prepared extracts from foreign colonies for manipulations with equivalents of three workers per dummy (Table S4.3). One of these extracts was used as positive control for manipulation (contained all substance classes), while the second one was fractionated using $AgNO_3$-treated silica columns to obtain one fraction with saturated and one with unsaturated CHCs (Bello, McElfresh & Millar 2015; Sano *et al.* 2018). Saturated CHCs were eluted with *n*-hexane, unsaturated ones with dichloromethane (DCM).

For the aggression tests, we prepared six different manipulation treatments: nestmate CHC extracts plus A) synthetic *n*-nonacosane (*n*-C29; Sigma-Aldrich, St. Louis, MO, USA), B) the hexane-fraction of the $AgNO_3$ fractionation (saturated CHCs of a foreign colony), C) the DCM-fraction of the $AgNO_3$ fractionation (unsaturated CHCs of a foreign colony), D) *n*-hexane that was filtered through an $AgNO_3$ column (negative control 1), E) DCM that was filtered through an $AgNO_3$ column, evaporated and re-eluted with *n*-hexane (negative control 2) and F) the complete CHC extract of a foreign colony (positive control). For each of the six treatments we had one extract per colony (60 treatment extracts for *Cr. levior* A, 48 for *Cr. levior* B). All extracts used in the aggression tests were composed of 20 µL nestmate CHC extract, 20 µL of the manipulation extracts (A-F) and 20 µL (in A) or 30µL (in B) of *n*-hexane. In the *n*-nonacosane treatment, we needed to strongly dilute the extracts (by adding additional 60 µL of *n*-hexane) since the hydrocarbons formed crystals in the extracts. In addition, due to evaporation some extracts had to be slightly diluted.

4.2.3.2 Aggression tests

We performed four aggression tests per treatment per colony (*Cr. levior* A: 6 treatments x 10 colonies x 4 replicates = 240 tests; *Cr. levior* B: 6 x 8 x 4 = 192 tests). The protocol was similar as in experiment 1. All tests were conducted blindly in regard of the treatment (except for treatment A with *n*-nonacosane, since it was clearly visible).

4.2.3.3 Statistical analyses

Similar to experiment 1, we compared the aggression indices between the treatments. To do so, we used linear mixed-effects models (LMM; command *lme*; R-package *lmer*) with the log+1-transformed aggression index as dependent variables and the treatment and colony ID as fixed factors. The trial round (1 to 4) was implemented as random effect. We did two different models for *Cr. levior* A and *Cr. levior* B. All statistical analyses were conducted in *R* v. 3.6.0 (R Core Team 2018).

4.2.4 Control experiment: Aggression against dead workers

In an additional control experiment, we performed aggression tests with *Cr. levior* B as described above with freshly killed workers instead of dummies. Here, we wanted to observe if *Cr. levior* B is capable of differentiating nestmates from non-nestmates and if the number of interactions performed differs from the one in experiment 2 (N = 32 tests). We

again compared aggression indices between allo- and intracolonial opponents with a LMM using colony ID as random factor. For comparing if the numbers of interactions differed between dummies with extracts and freeze-killed workers, we used Welch t-tests.

4.3 Results

4.3.1 Experiment 1: Correlation of chemical distance and aggression

In the first experiment, we found that colonies of *Cr. levior* A were more aggressive than *Cr. levior* B (univariate PERMANOVA: $F_1 = 46.65$, $p = 0.001$; Fig. 4.1 A). Furthermore, dummies covered with CHCs of *Cr. levior* B were treated more aggressively ($F_1 = 5.90$, $p = 0.016$; Fig. 4.1 B). To our surprise, the ants did not discriminate between nestmates and non-nestmates in this experiment ($F_1 = 0.34$, $p = 0.57$; Fig. 4.1 C). In addition to that, we found that the ants differed in their aggressive behavior between the different trial rounds ($F_3 = 13.64$, $p = 0.001$; Fig. 4.1 D) and between the colonies ($F_{10} = 10.67$, $p = 0.001$; Fig. 4.1 E). Further on, the effect of test run differed between colonies and between the cryptic species (interaction colony ID:test run: $F_{30} = 1.79$, $p = 0.007$; interaction species ID:test run: $F_3 = 0.02$, $p = 0.011$).

The CHC profiles of the two cryptic species *Cr. levior* A and B differed considerably between each other and between the tested colonies (Fig. 4.1 F). However, the chemical distance between colonies did not correlate with the mean aggression between the colonies (Mantel test based on Spearman's rank correlation: $\rho = 0.018$, $p = 0.44$). Similarly, aggression in both directions (i.e. colony A against B and B against A) did not correlate with the chemical distance (both Spearman's $\rho < 0.025$, $p > 0.44$). Finally, also no chemical distance of any substance class (i.e. *n*-alkanes, mono- and dimethyl alkanes, alkenes and alkadienes) correlated with aggression (all $p > 0.05$).

4.3.2 Experiment 2: Interference of nestmate recognition

The ants showed neither detectable differences in aggressive reactions towards manipulated nestmate CHC extracts of *Cr. levior* A (LMM: $X^2_5 = 4.04$, $p = 0.54$; Fig. 4.2 A) nor *Cr. levior* B (LMM: $X^2_5 = 1.82$, $p = 0.87$; Fig. 4.2 C). However, in both experiments, the colonies strongly differed in aggression indices (*Cr. levior* A: $X^2_9 = 110.6$, $p < 0.0001$; Fig. 4.2 B; *Cr. levior* B: $X^2_7 = 28.78$, $p = 0.00016$; Fig. 4.2 D).

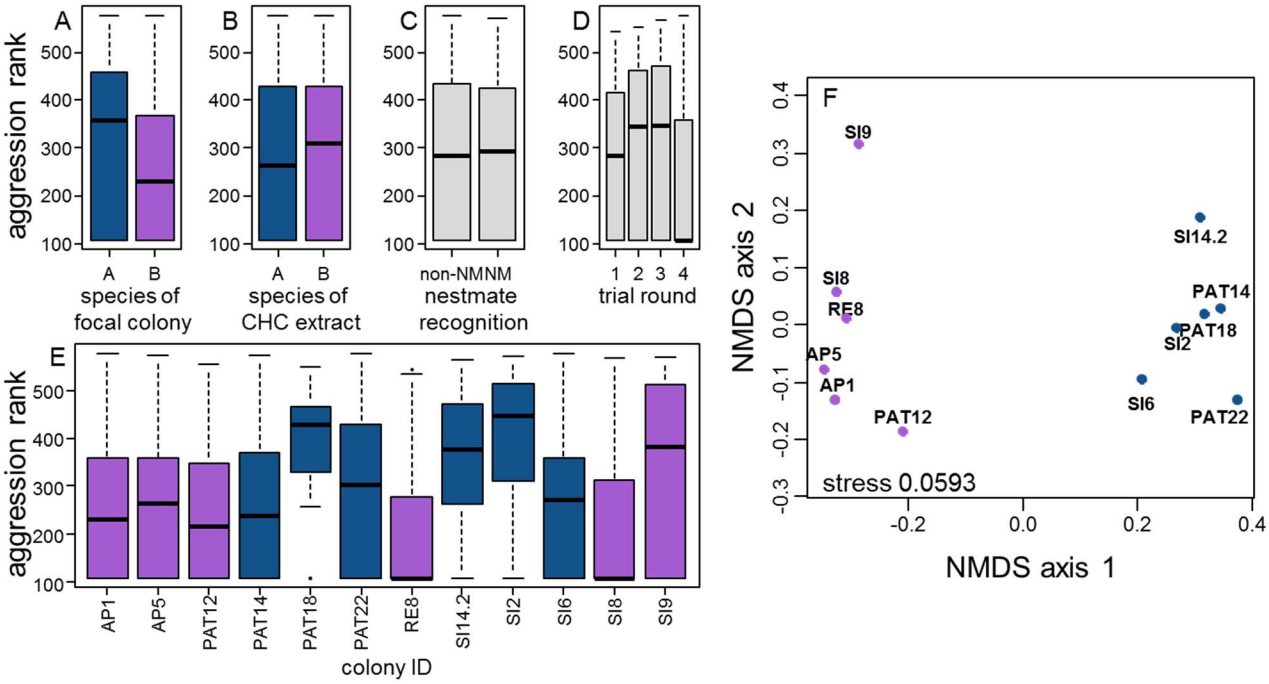

Figure 4.1: Aggression and CHC differences between colonies of *Cr. levior* A and B. (A-E) Show the differences in rank-transformed aggression indices between the focal colonies (A), towards the CHC extracts (B), between nestmates (NM) and non-nestmates (non-NM) (C), between the trial rounds (D) and between colonies (E). For comparison with means ± s.e.m. see Fig. S4.1. (F) Shows an NMDS ordination of the CHC profiles of each colony used in experiment 1. Each dot represents one colony. *Cr. levior* A is represented by blue dots, *Cr. levior* B by purple dots.

4.3.3 Control experiment

Workers of *Cr. levior* B were able to discriminate between freshly freeze-killed nestmate vs. non-nestmate workers of *Cr. levior* B (LMM: $X^2_1 = 28.01$, $p < 0.0001$; Fig. 4.3 A). Furthermore, we found that the number of interactions did not differ between dummies in experiment 2 (all experiments pooled) and freshly killed workers in the control experiment (Welch t-test: t = -1.87, df = 53.25, p = 0.067; Fig. 4.3 B). This was even more apparent if we only included reactions towards nestmates that is more comparable to the conditions in experiment 2 (dummies vs. freshly killed nestmates: t = -0.62, df = 20.16, p = 0.54; Fig. 4.3 B).

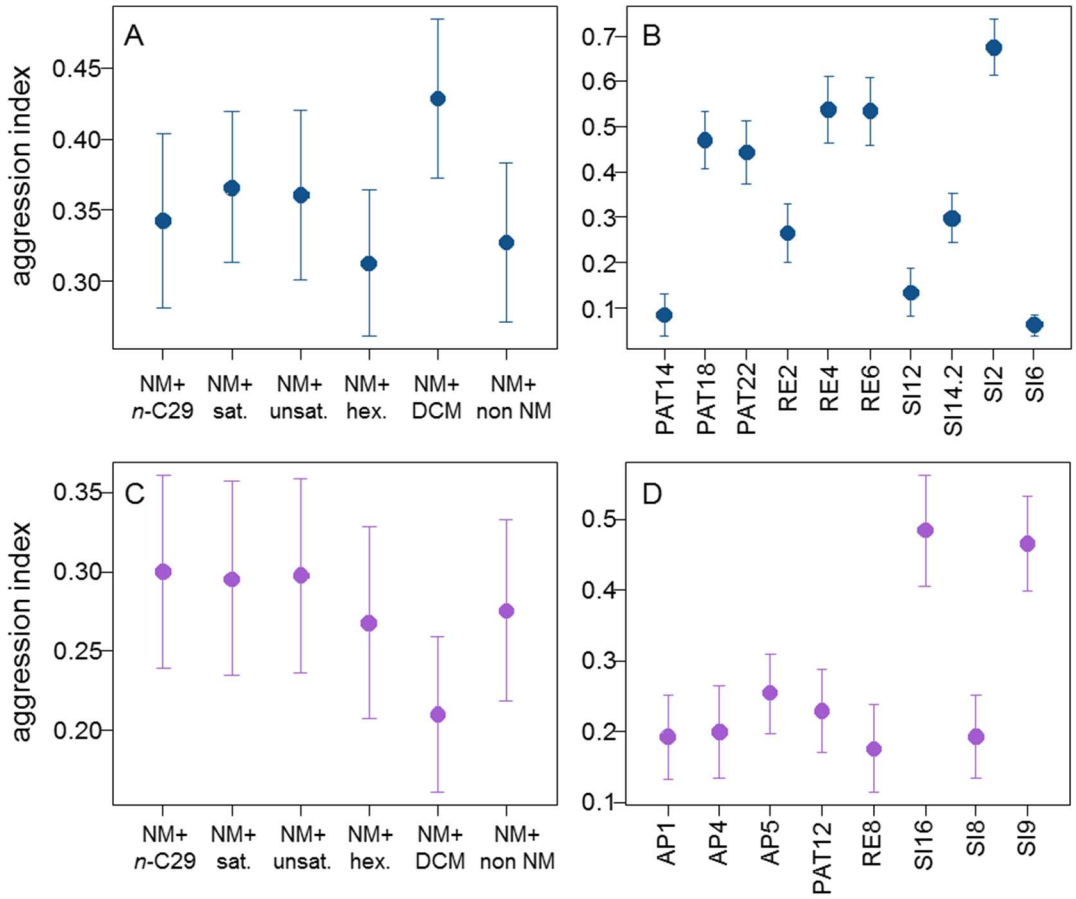

Figure 4.2: Aggression towards manipulated nestmate CHC extracts. The plots show mean ± s.e.m. of the aggression indices. (A, B) Show the aggression of *Cr. levior* A, (C, D) of *Cr. levior* B. (A, C) Describe the aggression towards the differently manipulated nestmate extracts (NM). (B, D) Describe colony differences in aggressive behaviors.

4.4 Discussion

The aggression tests with *Cr. levior* A and B yielded ambiguous results. While in experiment 1, we could show that workers of *Cr. levior* A were more aggressive and *Cr. levior* B extracts were treated more aggressively, we failed to demonstrate nestmate recognition using extract-treated dummies in both cryptic species (although at least *Cr. levior* B discriminated non-nestmates in the control experiment). Moreover, in experiment 2 none of our manipulation attempts led to discrimination between the differential supplementation of CHC classes. In both experiments it was apparent that there were strong inter-colonial differences in aggressive behavior. In the following, we will discuss possible reasons for the results, especially the lack of nestmate recognition and the relatively low aggression at least in the extract-based experiments.

4.4.1 Biological explanations for the lack of nestmate recognition and low aggression

Ants usually show strong discrimination between CHC extracts of nestmates and non-nestmates (Lahav *et al.* 1999; Martin, Helanterä & Drijfhout 2008b; Guerrieri *et al.* 2009; Menzel *et al.* 2013). Surprisingly, we neither found any evidence for nestmate recognition in *Cr. levior* A nor B in experiment 1. As CHC profiles are genetically heritable (van Zweden *et al.* 2009; Walsh *et al.* 2019), this could provide a possible explanation for the results seen here: At least *Cr. levior* A shows a very low genetic diversity at the mitochondrial COI-locus (Hartke, Sprenger *et al.* 2019, Chapter 1), which could reduce the ability for nestmate recognition due to high relatedness. Similarly, low haplotype diversity in *Temnothorax nylanderi* and *T. crassispinus* resulted in a high overlap of colony CHC profiles and less efficient nestmate recognition that allows colony take-overs (Foitzik *et al.* 2007). Furthermore, high relatedness is known to reduce aggression in supercolony-forming invasive species like *Linepithema humile* (Jaquiéry, Vogel & Keller 2005). Alternatively, the adaptations to parabiosis in the CHC profiles of *Cr. levior* A could possibly hamper intraspecific recognition next to interspecific recognition.

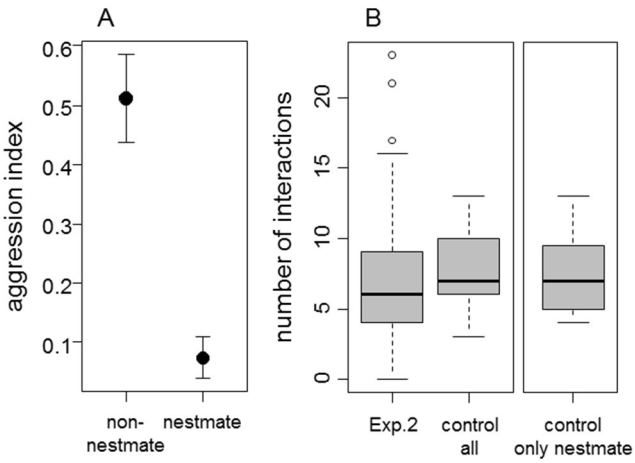

Figure 4.3: Results of the control experiment. (A) Shows mean ± s.e.m. of the aggression index towards non-nestmates and nestmates. (B) Shows boxplots of the total number of interactions with dummies (Exp. 2) vs. freshly killed workers (control).

However, both cryptic species, *Cr. levior* A and B, were able to discriminate nestmates from non-nestmates if they were presented freeze-killed workers in earlier studies (Emery & Tsutsui 2013; Menzel *et al.* 2014) or our control experiment, respectively (this study). Both of these experiments, however, were limited in sample size. Although many studies demonstrated the importance of CHCs in nestmate recognition (Lahav *et al.* 1999; Menzel *et al.* 2013), it could be possible that CHCs alone are not sufficient for nestmate recognition in *Cr. levior*. Next to the CHCs, both cryptic species also carry species-specific polar compounds of not entirely clarified chemical structure (Hartke, Sprenger *et al.* 2019, Chapter 1), which

potentially could be used for recognition as well. In the parabiotic *Cr. modiglianii* ants the novel polar compound class of 'crematoenones' did not play any role in nestmate recognition though, but was used as appeasement allomones to ensure interspecific tolerance of its parabiotic partner *Camponotus rufifemur* (Menzel *et al.* 2013). In comparison, intercolonial aggression in *Cr. modiglianii* was higher than in *Cr. levior* based on CHC extracts (Menzel, Schmitt & Blüthgen 2009).

In experiment 1 *Cr. levior* A was generally more aggressive, while the CHC extracts of *Cr. levior* B were treated more aggressively (c.f. Fig. 4.1 A, B; Fig. S4.1 B). In contrast to *Cr. levior* B, *Cr. levior* A shows the elongation of carbon backbones in its CHCs (Menzel *et al.* 2014; Sprenger *et al.* 2019, Chapter 2). Such long-chain CHCs should be harder to perceive as shorter-chained ones because the difference between melting and boiling point increases with chain length (Li *et al.* 2006). Firstly, this might explain why the CHC extracts of *Cr. levior* B (with shorter-chained CHCs) were treated more aggressively and secondly, one can hypothesize that *Cr. levior* A is able to distinguish its conspecifics CHC profiles while it was potentially harder for *Cr. levior* B to perceive the elongated CHCs of *Cr. levior* A.

Another possible explanation why we could not demonstrate nestmate recognition could be due to our animal maintenance: The microclimate in the plastic boxes was likely different to an intact ant garden in the forest. Although we moistened the ant gardens in the plastic boxes, the microclimate could have been drier than in nature, since in our setup the nests could not get water from dew or rain. Different ant species are known to acclimate to warm and/or dry conditions by producing more *n*-alkanes (Wagner *et al.* 2001; Menzel *et al.* 2018; Sprenger *et al.* 2018). In our chemical analyses, we found that the workers used in experiment 1 had much higher proportions of *n*-alkanes than ones collected in nature: workers of *Cr. levior* had extremely high proportions of *n*-alkanes in this experiment (*Cr. levior* A: 64.43 ± 15.50%; *Cr. levior* B: 52.95 ± 12.05%; mean ± SD), while these proportions were much lower in workers collected directly from their natural habitat (*Cr. levior* A: 20.24 ± 6.20%; *Cr. levior* B: 31.60 ± 8.09%; Sprenger *et al.* 2019, Chapter 2). This could reflect acclimation to the microclimate and/or substrate in the plastic boxes. In line with these high proportions of *n*-alkanes (i.e. fewer recognition cues) and assuming that the ants use their current colony odor as template for recognition (Vander Meer, Saliwanchik & Lavine 1989), this could explain the drop of aggressiveness in round 4 of experiment 1 (Fig. 4.1 D). For the last experimental round, we used newly extracted CHC profiles that then should be (similarly to the workers used in the experiment) more acclimated to the microclimate in the plastic boxes (Table S4.2).

4.4.2 Potential bias from experimental setup

Both cryptic species seemed to be able to distinguish freshly frozen nestmates from non-nestmates. This implies a potential problem in our experimental design. Many studies before used natural or artificial glass or Teflon dummies and applied CHCs to them (e.g. Greene & Gordon 2003; Martin *et al.* 2008b; Funaro *et al.* 2018). Similar to Funaro *et al.* (2018), who used equivalents of six termite workers per dummy, we used equivalents of five workers per dummy in our experiments 1 and 2. However, before our experiments, we purified the CHC extracts by fractionating them over silica gel (Bello *et al.* 2015); a step in which we probably lost considerable quantities of CHCs. Smaller quantities of CHCs might be more difficult to detect for the ants. In experiment 2, we separated saturated from unsaturated hydrocarbon fractions using $AgNO_3$-treated silica gel. In some cases, it seems that we did not condition these columns enough before fractionation and this could have contaminated the extracts used to distort the nestmate extracts probably causing similar aggression in all treatments. Although the fractionation worked well, some of the supplementation-treatment extracts and controls contained contaminations (especially the DCM control where we found contaminations in 3 of 5 supplementation extracts used; Fig. S4.2). Another potential source of aggression in the ants was maybe the smell of the solvent (*n*-hexane) itself. If our waiting time before the start of the aggression tests was too short for the solvent to fully evaporate, this could have biased our results as well. In addition to these points, testing only four ant workers in an arena is a very artificial situation, which could yield in less aggression compared to natural habitats as well although workers in arenas often react aggressively.

4.5 Conclusions

Although large parts of our results were ambiguous and we cannot exclude some biases from our experimental procedures, we can still conclude on some interesting findings: Generally, aggression in both species of *Cr. levior* was lower than in the parabiotic *Cr. modiglianii* from South-East Asia. As presented in Chapter 1, there is neither specialization of *Cr. levior* A nor B on either of the two *Camponotus* partner species (Hartke, Sprenger *et al.* 2019, Chapter 1). This could either indicate a broader acceptance of other CHC profiles in the neuronal template for nestmate recognition or simply that these templates are not innate (compared against an individual's own odor), but rather learned (Errard *et al.* 2006; Leonhardt *et al.* 2007), which already had been suggested for parabiotic ants before (Orivel *et*

al. 1997). Furthermore, it looks like *Cr. levior* A is generally more aggressive than *Cr. levior* B. This finding is difficult to interpret, but it could be due to better recognition abilities that allow discriminating of CHCs of both cryptic species, while *Cr. levior* B might not be able to fully perceive CHCs of *Cr. levior* A. Finally, it is highly interesting that CHC extracts of *Cr. levior* B, which do not show obvious adaptations to the parabiotic lifestyle (Sprenger *et al.* 2019, Chapter 2), were treated more aggressively in experiment 1. This clearly demonstrates that *Cr. levior* A and B respond aggressively towards other *Cr. levior* B colonies. However, additional experiments are needed to understand the consequences of this apparent lack of chemical adaptations of *Cr. levior* B for the mutualistic association with *Ca. femoratus*.

4.6 Acknowledgements

Removed for privacy purposes.

4.7 Supplementary material

Table S4.1: Origin and usage of ant colonies. Colonies marked in the columns Exp. 1 and Exp. 2 were used for the respective experiments.

Colony ID	Species	Sampling site	Latitude	Longitude	Exp.1	Exp. 2
SI 2	*Cr. levior* A	Sinnamary	05° 20.121 N	53° 02.198 W	X	X
SI 6		Sinnamary	05° 19.613 N	53° 02.313 W	X	X
SI 12		Sinnamary	05° 17.767 N	53° 03.084 W		X
SI 14.2		Sinnamary	05° 23.495 N	53° 04.883 W	X	X
PAT 14		Camp Patawa	04° 32.875 N	52° 08.689 W	X	X
PAT 18		Camp Patawa	04° 32.773 N	52° 08.539 W	X	X
PAT 22		Camp Patawa	04° 32.474 N	52° 08.109 W	X	X
RE 2		Régina	04° 13.616 N	52° 07.319 W		X
RE 4		Régina	04° 13.926 N	52° 07.006 W		X
RE 6		Régina	04° 14.975 N	52° 07.057 W		X
SI 8	*Cr. levior* B	Sinnamary	05° 18.252 N	53° 02.800 W	X	X
SI 9		Sinnamary	05° 17.954 N	53° 02.994 W	X	X
SI 16		Sinnamary	05° 22.886 N	53° 05.294 W		X
PAT 12		Camp Patawa	04° 33.198 N	52° 08.675 W	X	X
RE 8		Régina	04° 15.068 N	52° 07.173 W	X	X
AP 1		Apatou	05° 09.914 N	54° 20.099 W	X	X
AP 4		Apatou	05° 14.251 N	54° 17.083 W		X
AP 5		Apatou	05° 14.353 N	54° 17.052 W	X	X

Table S4.2: Additional extractions of CHCs in experiment 1.

Colony ID	Number of ants	*n*-hexane added [µL]
SI 2	90	180
SI 6	90	180
SI 14.2	100	200
PAT 14	105	210
PAT 18	100	200
PAT 22	90	180
SI 8	90	180
SI 9	100	200
PAT 12	90	180
RE 8	95	190
AP 1	95	190
AP 5	105	210

Table S4.3: Composition of manipulation extracts for experiment 2. The top part describes manipulation extracts for experiment 2 with *Cr. levior* A, the bottom part with *Cr. levior* B. GPS information on place of finding for the colony fragments used in the experiment with *Cr. levior* B is lacking unfortunately.

Colonies used in aggression tests	**Colony fragments for manipulation extract (+ place of finding)**	**Number of workers for fractionation (Treatments B and C)**	**Number of workers for positive control (Treatment F)**
SI 6 PAT 18 RE 4 SI 12	001 PAT N 04° 33.037 W 52° 08.853	48	48
SI 2 PAT 14 RE 2	008 PAT N 04° 32.911 W 52° 08.729	36	36
PAT 22 RE 6 SI 14.2	010 PAT N 04° 32.949 W 52° 08.712	36	36
SI 8 AP 1 RE 8 SI 9	023 PAT 4th road into forest from D6 behind Camp Patawa towards Kaw	48	48
AP 4 PAT 12 SI 16 AP 5	024 PAT 4th road into forest from D6 behind Camp Patawa towards Kaw	48	48

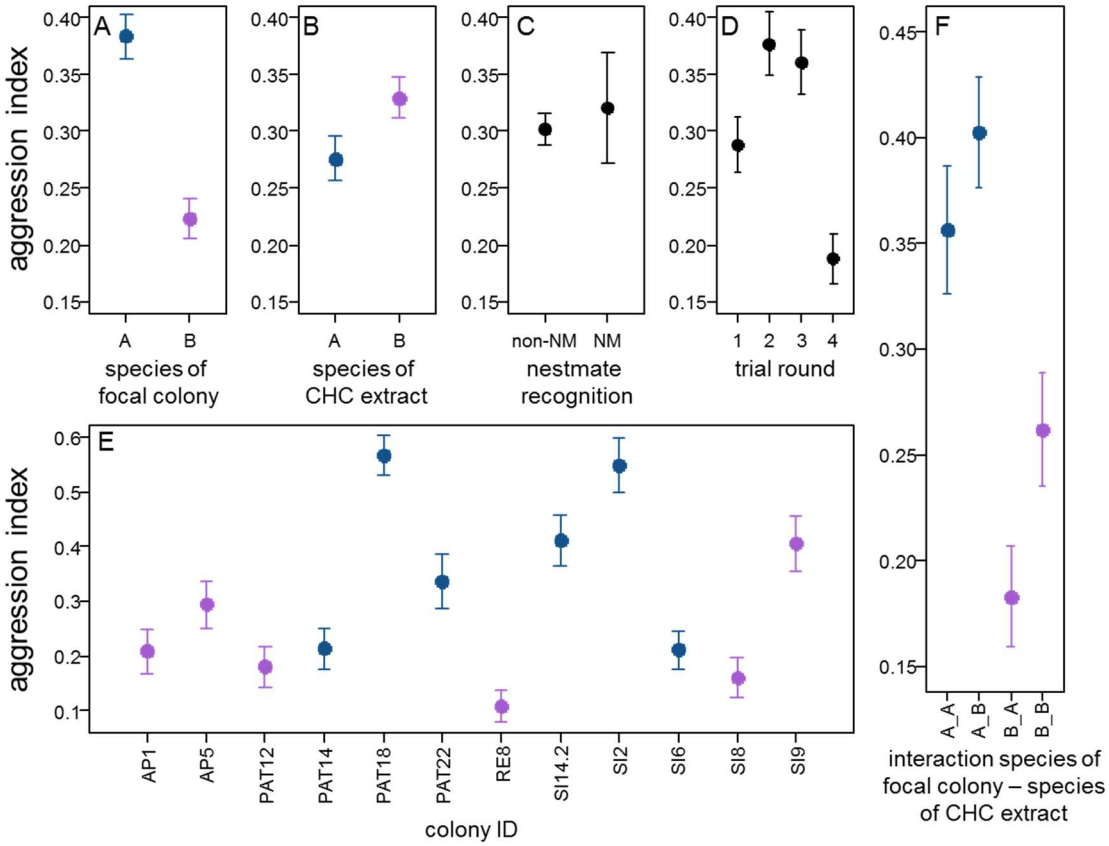

Figure S4.1: Aggression differences between colonies of *Cr. levior* A and B. The plots each show mean ± s.e.m. of aggression indices measured in experiment 1. We show differences between the focal colonies (A), towards the CHC extracts (B), between nestmates (NM) and non-nestmates (non-NM) (C), between the trial rounds (D), between colonies (E) and for visualization the interaction between species of the focal colony and the CHC extracts (for non-nestmates only) (F).

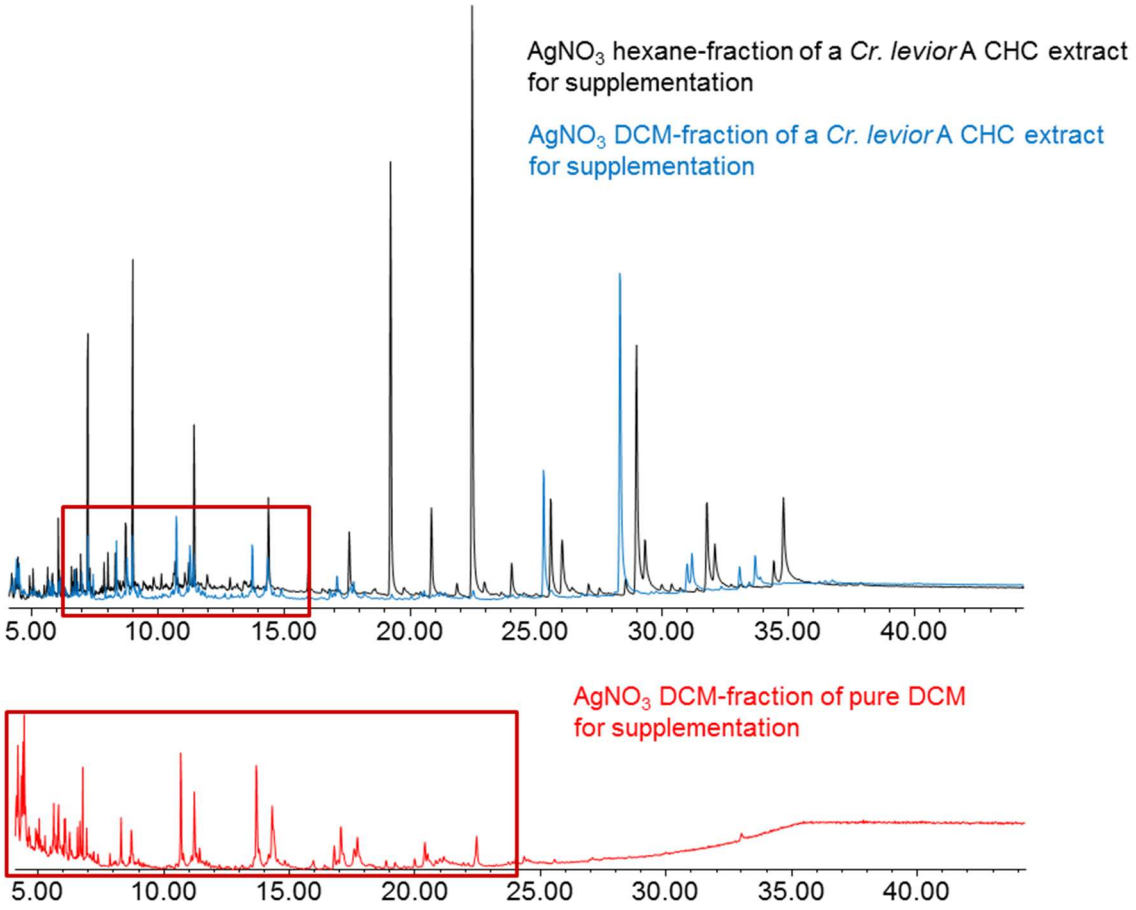

Figure S4.2: Examples for supplementation-treatment extracts used in experiment 2 after $AgNO_3$ fractionation. The chromatograms on top compare the fractions diluted with hexane (black; saturated CHCs) and dichloromethane (blue; unsaturated CHCs). The chromatogram on the bottom exemplifies possible contaminations found in 3 of 5 DCM controls (red). The red squares indicate contaminations in both DCM fractions

Chapter 5

Dinner with the roommates: Trophic niche differentiation and competition in a mutualistic ant-ant association

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Abstract

Despite net benefits for both parties, mutualisms can involve costs, such as competition for food. The two neotropical ants *Camponotus femoratus* and *Crematogaster levior* live in a mutualistic association (parabiosis), and share so-called ant gardens as common nests. While each parabiotic nest involves one *Crematogaster* and one *Camponotus* partner, both taxa were recently found to comprise two cryptic species that show no partner preferences and seem ecologically similar. Since these cryptic species often occur in close sympatry, they might need to partition their niches to avoid competitive exclusion. Therefore, we investigated two questions here: First, is there competition between mutualistically associated *Camponotus* and *Crematogaster*, and do they prefer different food sources? And second, is there trophic niche partitioning between the cryptic species of either genus? Using cafeteria experiments, neutral lipid fatty acid and stable isotope analyses, we found evidence for interference competition, but also trophic niche partitioning between *Camponotus* and *Crematogaster*. Both preferred protein- and carbohydrate-rich baits over uric acid, seeds and oleic acid, but at protein-rich baits *Ca. femoratus* displaced *Cr. levior* over time, suggesting a potential discovery-dominance trade-off between parabiotic partners. Only limited evidence was found for trophic differentiation between the cryptic species of each genus. Although we cannot exclude differentiation in other niche dimensions, we argue that neutral dynamics might mediate the coexistence of cryptic species. This model system is highly suitable for further studies of the maintenance of species diversity and the role of mutualisms in promoting species coexistence.

Keywords:

Cryptic species, neutral theory, niche partitioning, nutrition, parabiosis, trade-offs

5.1 Introduction

All organisms preferably occur in environments most suited for their physiological needs, i.e. their fundamental niches (Hutchinson 1957). However, as most resources are limited, organisms with similar ecological requirements have to compete for them. If one species is a stronger competitor, this should lead to competitive exclusion of the other species (Gause 1932; Hardin 1960). One mechanism to avoid competitive exclusion is niche partitioning. It can occur in various different dimensions, like spatial, temporal or dietary differentiation (Tanaka, Yamane & Itioka 2010; Stuble *et al.* 2013; Houadria *et al.* 2015; Grevé *et al.* 2019). Trophic niche partitioning is thought to be one of the key mechanisms allowing species coexistence (Rosumek *et al.* 2018; Grevé *et al.* 2019). Ants are among the most abundant and species-rich terrestrial arthropods especially in tropic rainforests, and high competition between locally co-occurring species shapes ant communities worldwide, often via direct behavioral interactions (Savolainen & Vepsäläinen 1988; Hölldobler & Wilson 1990). Because of this expected high competition, the need for niche partitioning should also be high. Nest sites or food sources containing the currently needed nutrients are often limiting resources for ants (Blüthgen & Feldhaar 2010). The food choice of a species may strongly depend on its competitive abilities, since dominant species will often monopolize and aggressively defend suitable food sources (Hölldobler 1983; Dejean *et al.* 2005). Strong competition for food sources between dominant and submissive species could result in behavioral or physiological trade-offs, e.g. the discovery-dominance trade-off (Fellers 1987; Sarty, Abbott & Lester 2006) or in the thermal vulnerability-dominance trade-off (Cerdá, Retana & Manzaneda 1998).

Mutualisms are close associations of coexisting species that provide net benefits to the partners. However, mutualisms also have the potential for certain costs, e.g. in form of competition (Bronstein 2001). An exceptional form of mutualism, in which the partners should have rather similar nutrient requirements, is parabiosis in ants. Parabiosis is defined by two ant species commonly sharing the same nest and tolerating each other, while keeping their brood separate (Orivel *et al.* 1997; Menzel *et al.* 2008b). In the Neotropics the parabiotic ants *Camponotus femoratus* and *Crematogaster levior*, that share so-called ant gardens as their nests, are among the ecologically most dominant arboreal species (Davidson 1988). While *Camponotus* probably profits from the resource discovery abilities of *Crematogaster* and follows interspecific pheromone trails, *Crematogaster* has the benefit of *Camponotus* building and stabilizing the nest and aggressively defending it against predators or intruders (Orivel

& Dejean 1999; Vantaux *et al.* 2007; Youngsteadt *et al.* 2008; Vicente *et al.* 2014). Although the association between *Ca. femoratus* and *Cr. levior* is considered mutualistic, previous observations also indicated that these species may compete for certain food sources: While *Cr. levior* often arrives at a food source first, it is often displaced by *Ca. femoratus* at least from protein-rich food sources like dead insects (Swain 1980; Vantaux *et al.* 2007; Menzel *et al.* 2014).

In addition to the mutualistic association, the neotropical parabiotic ants consist of two ecologically similar cryptic species each (*Ca. femoratus* PAT and PS; *Cr. levior* A and B). They differ genetically, in their cuticular hydrocarbon profiles and slightly in their morphology, but largely overlap in their geographic distribution (Hartke, Sprenger *et al*. 2019, Chapter 1). Interestingly, both *Camponotus* PAT and PS live in association with both *Cr. levior* A and B, without evidence for mutual specialization (Hartke, Sprenger *et al*. 2019, Chapter 1). Colonies of the two cryptic species in both *Cr. levior* and *Ca. femoratus* frequently co-occur in close sympatry. Hence, there should be competition between sister taxa, which may be ameliorated if there is niche differentiation between cryptic species.

Trophic niches are most easily assessed by observations of feeding behavior in a natural environment. However, behavioral observations of feeding choices actually represent 'temporal snap-shots' of the current food preferences or choices of best option in the presences of a competitor. In addition, the attractiveness of baits can be influenced by their rarity and current needs of the ant colony (Kay 2004), i.e. if the colony needs protein and protein sources are rare, ants will prefer this bait. This is why indirect measurements, such as analysis of fatty acids or stable isotope signatures, can be helpful to get insights into the trophic ecology of terrestrial arthropods such as ants. Neutral lipid fatty acids (NLFAs; saturated and mono-, di- and polyunsaturated fatty acids) are stored in the fat bodies of arthropods and are a major source of energy (Stanley-Samuelson *et al*. 1988). Unsaturated fatty acids are preferable to saturated ones because they are easier to mobilize and metabolize, thus providing a better way of energy storage (Price 2010; Guglielmo 2018). As it requires less energy to directly incorporate dietary fatty acids without modifications, they can be used to infer trophic transfer between consumer and diet (i.e. dietary routing) (Ruess & Chamberlain 2010). Specific fatty acids can be used as biomarkers when they are specific to certain food sources. But also more widespread fatty acids can accumulate in a consumer, thus indicating its dietary origin (Ruess & Chamberlain 2010; Rosumek *et al.* 2017). Hence, fatty acid profiles can be highly useful to study trophic niche differentiation. A third powerful tool commonly used to infer the trophic position of an organism in a food web or

to detect trophic niche partitioning is the analysis of stable isotopes. Heavy isotopes of nitrogen ($\delta^{15}N$) accumulate in the food chain due to differential digestion or fractionation during metabolic processes by the consumer (Post 2002; Heethoff & Scheu 2016). For example, predators usually have higher $\delta^{15}N$ than primary consumers, or producers (Blüthgen, Gebauer & Fiedler 2003; Davidson *et al.* 2003; Feldhaar, Gebauer & Blüthgen 2009). Carbon isotopes ($\delta^{13}C$) may additionally inform about the carbon sources used by arthropods in a food web; in particular plants (e.g. C_3 and C_4 plants) can strongly differ in their $\delta^{13}C$ signature and accordingly in an organism consuming these plants or their nectar (McCutchan Jr *et al.* 2003; Swap *et al.* 2004; Blüthgen & Feldhaar 2010).

In this study, we followed the integrative framework of Rosumek *et al.* (2018) to investigate trophic niche differences among cryptic species of the parabiotic ants *Ca. femoratus* and *Cr. levior*. By comparing field data from a cafeteria experiment, neutral lipid fatty acid and stable isotope analyses, we aimed to answer two questions: First, is there competition between the mutualists and if so, which food sources do they compete for? And second, is there trophic niche partitioning between the cryptic species of *Ca. femoratus* and *Cr. levior*?

5.2 Materials and methods

5.2.1 Study sites and species identification

Diet experiments and sample collection took place in three different sites in French Guiana in October 2018. These were the Paracou Research Station (n = 17 colonies; 5°14.04 N, 52°54.28 W), next to the Route de Saint-Èlie near Sinnamary (n = 13 colonies; 5°17.49 N, 53°14.46 W) and close to the village of Kaw next to the D6 road at Camp Patawa (n = 14 colonies; 4°32.56 N, 52°09.45 W).

The cryptic species identity of the tested colonies was identified using CHC extracts of five *Cr. levior* or one *Ca. femoratus* worker taken from ant gardens prior to the experiment and analyzed using gas-chromatography mass-spectrometry (GC-MS, see Sprenger *et al.* (2019), Chapter 2, for details on the method).

5.2.2 Cafeteria experiments

We conducted cafeteria experiments by offering five different food sources on a PVC platform (16.5 cm x 14 cm with a v-shaped notch for the trunk) attached to the vegetation 1

to 3 m away from the ant garden (N = 44 nests). The food sources offered were 1) a protein source resembling vertebrate carcasses (chicken luncheon meat, Zwan, Almelo, The Netherlands), 2) a sugar source resembling natural sugar sources like extra floral nectaria (20% v/v sugar solution), 3) a fat source resembling plant oleosomes (10% v/v oleic acid solution), 4) a nitrogen source resembling bird feces (10% v/v uric acid solution) and 5) crushed plant seeds as a starch source (Sittich Perle®, Vitakraft, Bremen, Germany). The food sources were placed in a circular way on the platform in a randomized order. Pictures were taken after 15, 60 and 120 minutes to document the number of ants at each time point the number of foragers at each food source was counted in a 1 cm diameter around each food source.

5.2.3 Statistical analysis of cafeteria experiments

All statistical tests were conducted in *R* v. 3.6.0. First, we analyzed whether any food sources were visited more intensely by either *Camponotus* or *Crematogaster*. To this end, we performed a 'hotlink' analysis (Junker, Höcherl & Blüthgen 2010; Grevé *et al.* 2019), which compares the relative food preferences of the two genera while competing (i.e. the realized trophic niche). To exclude random encounters at a food source, we only included observations that had at least 5 ants at the bait and those represented at least 10% of total workers observed. Secondly, we tested for overall differences in food choice between genera and cryptic species with a PERMANOVA, and whether the level of inter-colony variation of food choices differed between genera or cryptic species using PERMDISP based on Bray-Curtis dissimilarities (commands *adonis* and *betadisper* + *permutest*, R package *vegan*, Oksanen *et al*. 2019). In the PERMANOVA, we furthermore included the test site as well as the time point as fixed factors. Thirdly, we separately analyzed the numbers of either *Camponotus* or *Crematogaster* ants at the different baits, as well as the summed numbers of workers of either species. Each of these variables was used as dependent variable in a linear mixed effects model (LMM), with the fixed factors 'time point' (1 to 3), 'cryptic species identity', 'cryptic species identity of the partner', and 'number of workers of the partner species at the respective bait', and 'colony ID' as random factor (R package *lme4*, command *lmer*, Bates *et al*. 2015). To avoid over-parametrization, we allowed two-way and three-way interactions, but not higher-level interactions. For the same reason, we determined beforehand (using Akaike's Information Criterion, AIC) whether the 'site' (Patawa, Paracou or Sinnamary) was to be included as fixed or random effect, or not at all; the respective model with the lowest AIC was then chosen for further analysis. All total numbers were log+1-transformed in each

of the models. We chose to analyze absolute numbers rather than *Camponotus*-*Crematogaster* ratios since we deemed them more informative and since at some baits, only one species was present. As the number of foragers at some baits was very low, we had to transform the data to binomial variables (present or absent) for seeds and uric acid in *Camponotus* as well as oleic acid in *Crematogaster* and calculated generalized linear mixed effects models (command *glmer* with family binomial) with similar fixed and random factors. In each model, non-significant interactions were removed in a stepwise fashion, until only significant interactions remained.

5.2.4 Neutral lipid fatty acid (NLFA) analysis

Before starting each cafeteria experiment, we collected two *Crematogaster* and two *Camponotus* workers (one backup sample each) that were freeze-killed and kept at -20°C until fatty acid extraction. The extraction protocol followed the steps described in Rosumek *et al*. (2017). In brief, fatty acids were extracted from individual workers by immersing them in a 2:1 chloroform-methanol (v/v) mixture for 24 hours. Neutral lipid fatty acids (NLFA) were separated from phospholipid fatty acids (PLFA) using chloroform- and hexane-conditioned SiOH-columns (Chromabond, 1mL/100mg, Macherey-Nagel, Düren, Germany). The NLFAs were eluted with chloroform, while PLFAs remained in the column. We let the solvent evaporate under a gentle nitrogen stream and re-dissolved the NLFAs in a dichloromethane-methanol (2:1 v/v) solution. For quantification, we added 10 µl of nonadecanoic acid (C19:0, solved in dichloromethane-methanol 2:1 v/v, 0.2 mg/mL) as an internal standard. For analysis, the fatty acids were derivatized to fatty acid methyl esters (FAME) with 20 µl trimethylsulfonium hydroxide (TMSH; Sigma-Aldrich, Munich, Germany).

In total, we analyzed 86 FAME samples with gas-chromatography mass-spectrometry (GC-MS). 2 µl of the samples were injected into the GC (7890A, Agilent Technologies, Santa Clara, CA, USA) that was equipped with a Zebron Inferno ZB5-MS capillary column (length 30 m, Ø 0.25 mm, 0.25 µm coating, Phenomenex, Aschaffenburg, Germany) in the splitless mode. As carrier gas, we used helium at a flow rate of 1.2 mL per minute. Initially, the oven had a temperature of 60°C and heated up with 15°C per minute until it reached 150°C. In the following, the temperature increased with 3°C/minute up to 200°C and then with 10°C/minute up to 320°C. This temperature was held constant for additional 10 minutes. The separated FAMEs were transferred to the MS (5975C, Agilent Technologies) and fragmented by an electron beam at 70 eV. We identified them via their fragmentation patterns (i.e.

molecular and diagnostic ions). Resulting chromatogram peaks were integrated manually using the software *MSD ChemStation* (E.02.02.1431, Agilent Technologies).

We compared the fatty acid profiles between genera, cryptic species (21 *Cr. levior* A, 18 *Cr. levior* B, 24 *Ca. femoratus* PAT, 15 *Ca. femoratus* PS and 8 of unknown cryptic species identity) and sites using a PERMANOVA based on Bray-Curtis dissimilarities and tested if they differed in their variances using a PERMDISP (commands *adonis* and *betadisper* + *permutest*, R package *vegan*, Oksanen *et al*. 2019). Additionally, we tested if certain traits of the fatty acid profiles, i.e. absolute quantity of fatty acids as well as proportions of saturated, mono-unsaturated and poly-unsaturated fatty acids, differed with linear regression models. If necessary, the values were transformed to meet the model assumptions. Finally, we used a Mantel test based on Pearson's product moment correlation to test if the fatty acid profiles of *Crematogaster* and *Camponotus* workers from the same nests correlated.

5.2.5 Stable isotope analyses

In total, we analyzed 72 samples belonging to 38 different ant gardens (22 *Cr. levior* A, 12 *Cr. levior* B, 21 *Ca. femoratus* PAT, 12 *Ca. femoratus* PS and 5 of unknown cryptic species identity; 11-15 per location). All samples were collected before the cafeteria experiments and stored in absolute ethanol. We measured the isotope composition of nitrogen (N) and carbon (C) using standard gases (N_2 and CO_2) in a coupled elemental analyzer – isotope ratio mass spectrometer (EA-IRMS). For the analysis, we used a Carlo Erba 1108 elemental analyzer (Carlo Erba, Milano, Italy) coupled to a delta S isotope ratio mass spectrometer (Finnigan MAT, Bremen, Germany) via a ConFlo III open-split interface (Thermo Fisher Scientific, Bremen, Germany) in a dual element analysis mode. The standard gases were calibrated against international standards (N_2 in air and V-PDB) using reference substances (N1 and N2 for the nitrogen isotopes; CH6, CO8 and NBS18 for carbon isotopes; standards from the International Atomic Energy Agency, Vienna, Austria).

We compared the $\delta^{15}N$ and $\delta^{13}C$ signatures (= (R_{sample} / $R_{standard}$ -1) x 1000 [‰]; with R being the ratio of heavy to light isotopes) between genera, cryptic species and sites using linear models (LM). For the models, we each used the cryptic species nested in genus and the sampling site as fixed effects. Further on, we separately tested if the $\delta^{15}N$ and $\delta^{13}C$ signatures of *Camponotus* and *Crematogaster* of the same nest depended on each other using linear models with site as additional fixed factor.

5.3 Results

5.3.1 Cafeteria experiments

In general, the number of *Crematogaster levior* workers (mean ± s.e.m.: 25.20 ± 3.34) was higher than the number of *Camponotus femoratus* workers (16.95 ± 1.46) in our experiments. Both numbers increased over time (LMM: *Camponotus*: $\chi^2_2 = 27.44$, $p < 0.001$; *Crematogaster*: $\chi^2_2 = 18.55$, $p < 0.001$; increase from 15 to 60 minutes, but not from 60 to 120 minutes in both genera). By reciprocally testing the effect of the worker numbers on each other, we found that the number of *Camponotus* workers was negatively affected by the number of *Crematogaster* workers ($\chi^2_1 = 7.29$, $p = 0.0069$) but not the other way around ($\chi^2_1 = 0.05$, $p = 0.82$). This is most likely due to the stronger effect of the test site in *Crematogaster* ($\chi^2_2 = 9.59$, $p = 0.008$; fewer workers at Camp Patawa compared to Sinnamary: post-hoc Tukey test: $t_4 = -3.09$, $p = 0.011$; Fig. S5.1). Worker numbers did not differ between cryptic species (all $p > 0.17$).

Although both genera foraged most on sausage and sugar (Fig. 5.1 A, B), *Camponotus* workers foraged more intensely than *Crematogaster* at sausage at all three time points (Hotlink analysis: all $p \leq 0.046$). *Crematogaster,* in turn, foraged more intensely than *Camponotus* at uric acid after 60 and 120 minutes (both $p \leq 0.022$), while the two genera did not differ in relative foraging activity at sugar (all $p \geq 0.17$; Tab. 5.1). Taken together, the food choices (i.e. the number of workers foraging at the five baits) differed between the two genera (PERMANOVA based on Bray-Curtis dissimilarities: pseudo-$F_1 = 21.29$, $p = 0.001$) with *Crematogaster* showing a more variable food choice (PERMDISP: $F_1 = 18.07$, $p = 0.001$). Food choices also differed between time points (PERMANOVA: pseudo-$F_2 = 7.03$, $p = 0.001$), but not between the cryptic species within each genus (pseudo-$F_2 = 1.03$, $p = 0.40$). Furthermore, interactions indicated that genus effects differed between sites (interaction genus:site: pseudo-$F_2 = 2.45$, $p = 0.003$) and between time points (interaction genus:time: pseudo-$F_2 = 2.10$, $p = 0.016$).

At the sausage baits, the numbers of *Camponotus* and *Crematogaster* workers negatively influenced each other (LMM: *Camponotus*: $\chi^2_1 = 19.87$, $p < 0.0001$; *Crematogaster*: $\chi^2_2 = 20.25$, $p < 0.0001$) and were higher after 60 and 120 minutes than after 15 minutes in both genera (*Camponotus*: $\chi^2_2 = 51.74$, $p < 0.0001$; *Crematogaster*: $\chi^2_2 = 21.79$, $p < 0.0001$; Fig. 5.1 A). After 60 minutes, the number of *Ca. femoratus* PS workers influenced the number of *Crematogaster*

workers more negatively than PAT workers, (3-way interaction between number of *Camponotus* workers, *Camponotus* species and time: $\chi^2_2 = 10.65$, p = 0.0049; Fig. 5.2 A).

Table 5.1: Results of the hotlink network analysis. Significant p-values (printed in bold) indicate that colonies used one of the baits more frequently than expected by their total number of *Crematogaster* and *Camponotus* workers at the five baits. The baits are ordered according to attractiveness (i.e. total/mean number of attracted foragers).

genus	sausage	sugar	uric acid	seeds	oleic acid
after 15 minutes					
Camponotus	**0.046**	0.86	1	1	1
Crematogaster	0.99	0.46	0.46	0.11	1
after 60 minutes					
Camponotus	**< 0.001**	0.92	1	1	1
Crematogaster	1	0.17	**0.022**	0.14	0.53
after 120 minutes					
Camponotus	**< 0.001**	0.85	1	1	1
Crematogaster	1	0.28	**< 0.001**	0.08	1

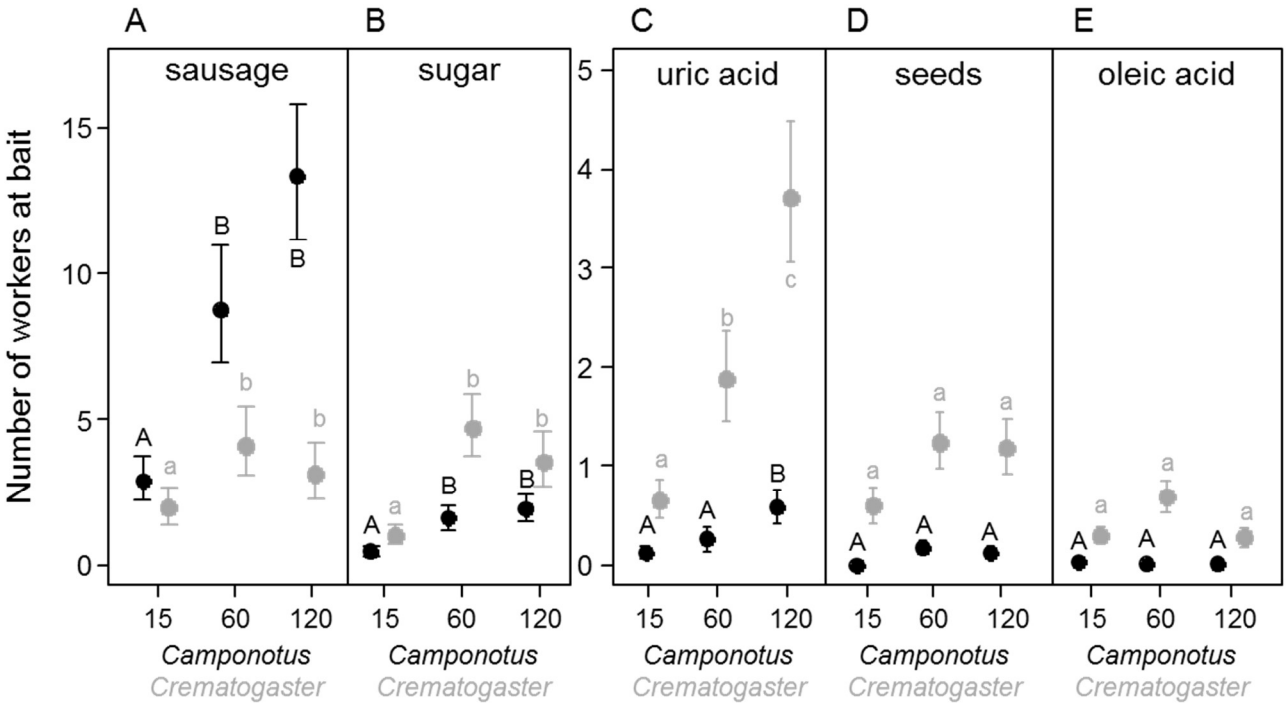

Figure 5.1: Food choice and competition between *Camponotus femoratus* and *Crematogaster levior*. The plots show back-transformed means ± s.e.m. of the worker numbers of *Ca. femoratus* (black) and *Cr. levior* (grey) at each 3 time points (after 15, 60 and 120 minutes) for five different baits: sausage (A), sugar (B), uric acid (C), seeds (D) and oleic acid (E). Letters indicate statistical differences between time points in *Camponotus* (capital letters) and *Crematogaster* (lower letters).

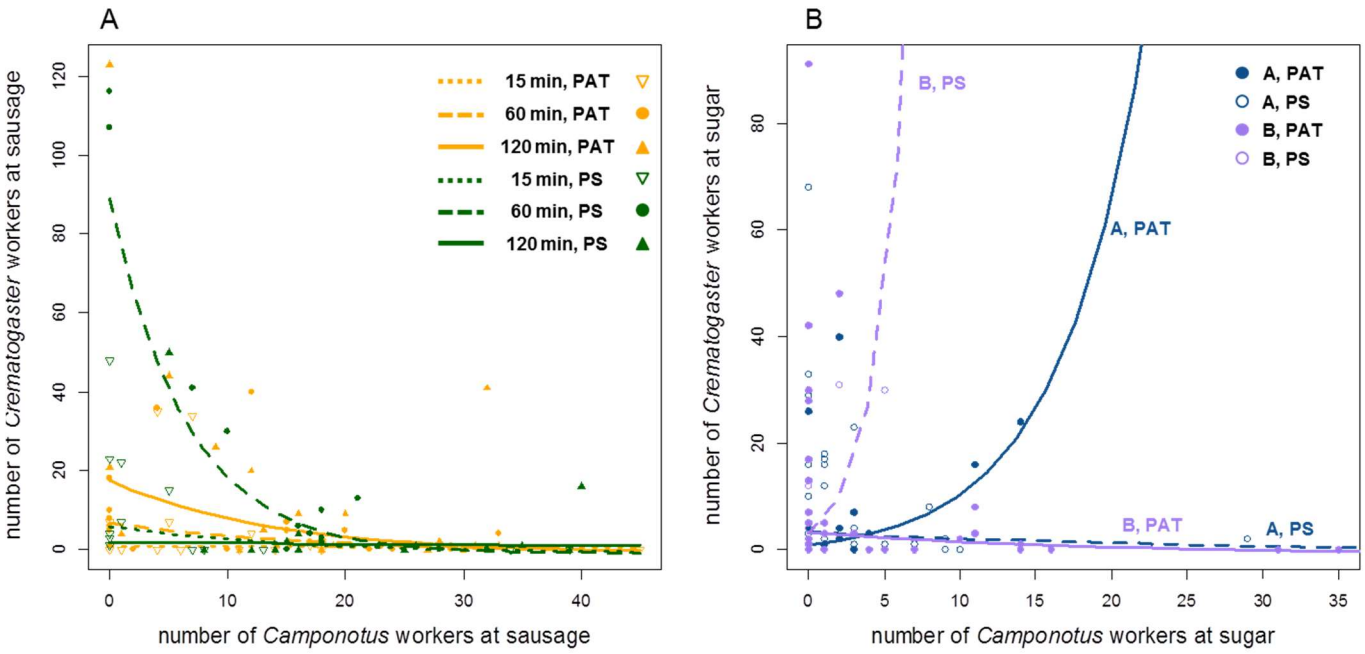

Figure 5.2: Cryptic species interactions in competition at two different baits (sausage and sugar). (A) Plot of the interaction between number of *Camponotus* workers, their cryptic species identity and the time point at the sausage bait. We plotted log-regression lines for each cryptic species (*Ca. femoratus* PAT: orange; PS: green) at each time point (15 min: dotted line; 60 min: dashed line; 120 min: solid line). The data points for each regression are represented by different symbols. (B) Plot of the interaction between number of *Camponotus* workers and cryptic species identities of *Ca. femoratus* and *Cr. levior* at the sugar bait. We plotted log-regression lines for each combination of cryptic species (*Cr. levior* A, *Ca. femoratus* PAT: blue, solid line; A, PS: blue, dashed line; B, PAT: purple, solid line; B; PS: purple, dashed line). The data points for each regression are represented by open or closed circles in the colors described before.

At sugar, the numbers of workers increased over time with lower abundances at the first time step in both genera (*Camponotus*: $\chi^2_2 = 16.75$, $p = 0.0002$; *Crematogaster*: $\chi^2_2 = 25.96$, $p < 0.0001$; Fig. 5.1 B). Additionally, the abundance of *Crematogaster* workers was lower at Camp Patawa than at Sinnamary ($\chi^2_2 = 11.31$, $p = 0.0035$; post-hoc Tukey test: $t_8 = -3.24$, $p = 0.008$). Interestingly, the worker number on sugar baits was affected by the composition of the species pair: *Camponotus* and *Crematogaster* numbers increased in parallel in pairs of either *Ca. femoratus* PAT with *Cr. levior* A (n = 33 observations) or *Ca. femoratus* PS with *Cr. levior* B (n = 6 observations), but not in the other two combinations (3-way interaction between numbers of either *Ca.* or *Cr.* workers, *Camponotus* species and *Crematogaster* species: *Ca.*: $\chi^2_1 = 3.98$, $p = 0.046$; *Cr.*: $\chi^2_1 = 6.88$, $p = 0.0087$; Fig. 5.2 B).

Uric acid baits were more often visited by *Camponotus* colonies if they were associated with *Cr. levior* B (binomial LMM: $\chi^2_1 = 4.98$, $p = 0.026$). Since *Camponotus* numbers were generally low at uric acid, we analyzed the frequencies of foraging rather than worker numbers. Foraging frequencies were higher after 120 minutes than after 15 or 60 minutes ($\chi^2_2 = 7.88$, $p = 0.019$; Fig. 5.1 C). Colonies in Camp Patawa foraged more frequently at uric acid than the ones in Sinnamary ($\chi^2_2 = 6.95$, $p = 0.031$, post-hoc Tukey test: $t = 2.60$, $p = 0.026$). *Crematogaster* worker numbers continuously increased over time (LMM: $\chi^2_2 = 34.62$, $p < 0.0001$; Fig. 5.1 C), but did not differ between sites or cryptic species.

Seeds and oleic acid were only rarely visited by both genera, and nearly not at all by *Camponotus* (Fig. 5.1 D, E). *Crematogaster* workers foraged on seeds more often in Sinnamary than Camp Patawa (LMM: $\chi^2_2 = 8.26$, $p = 0.016$, post-hoc Tukey test: $t_3 = 2.85$, $p = 0.020$). Also, the probability of *Crematogaster* to forage on oleic acid was slightly influenced by time (binomial LMM: $\chi^2_2 = 6.43$, $p = 0.040$, post-hoc Tukey test 15 vs. 60 min: $t = -2.33$, $p = 0.052$, other comparisons $p > 0.14$).

5.3.2 Neutral lipid fatty acid (NLFA) analysis

The fatty acid profiles strongly differed between *Camponotus* and *Crematogaster* (PERMANOVA based on Bray-Curtis dissimilarities: pseudo-$F_1 = 14.65$, $p = 0.001$). Furthermore, *Ca. femoratus* PAT had a different fatty acid profile than PS (species nested in genus: pseudo-$F_2 = 3.76$, $p = 0.003$; PERMANOVA with *Camponotus* subset: pseudo-$F_1 = 9.49$, $p = 0.001$), while *Cr. levior* A and B did not differ (PERMANOVA with *Crematogaster* subset: pseudo-$F_1 = 0.59$, $p = 0.66$). In addition, we found a trend for *Ca. femoratus* PS to have a more variable fatty acid profile than PAT (PERMDISP: $F_1 = 3.49$, $p = 0.069$), while there was no such difference between the cryptic *Cr. levior* species (PERMDISP: $F_1 = 0.11$, $p = 0.75$).

Unsurprisingly, the absolute amount of fatty acids was higher in *Camponotus* compared to the much smaller *Crematogaster* (LM: $F_1 = 45.97$, $p < 0.0001$; Fig. 5.3 A). The cryptic species of either genus did not differ in their absolute fatty acid quantity (cryptic species identity nested in genus: $F_2 = 0.76$, $p = 0.47$; Fig. 5.3 A). Furthermore, ants from Paracou contained more fat than ones from Camp Patawa ($F_2 = 4.16$, $p = 0.020$, post-hoc Tukey test: $t_{68} = 2.81$, $p = 0.017$).

In the composition of the fatty acid profiles, we found quite some differences between *Camponotus* and *Crematogaster*: While *Crematogaster* had more saturated fatty acids (LM: F_1 = 18.78, p < 0.0001; Fig. 5.3 B), *Camponotus* had higher proportions of mono-unsaturated fatty acids (F_1 = 11.98, p = 0.0009; Fig. 5.3 C). The difference between the genera however, was driven by *Ca. femoratus* PAT, which had higher proportions of mono-unsaturated fatty acids than *Ca. femoratus* PS, which had similar saturated fatty acid levels as *Crematogaster* (cryptic species identity nested in genus: F_2 = 4.45, p = 0.015, post-hoc Tukey test PAT vs. PS: t_{72} = 2.94, p = 0.023; Fig. 5.3 C). On the other hand, *Crematogaster* had more di-unsaturated fatty acids than *Camponotus* (F_1 = 6.29, p = 0.014; Fig. 5.3 D), while the cryptic species within each genus did not differ (F_2 = 0.82, p = 0.44; Fig. 5.3 D).

We did not find evidence that fatty acid profiles between *Camponotus* and *Crematogaster* from the same ant garden were correlated (Mantel test: R^2 < 0.0001, p = 0.43).

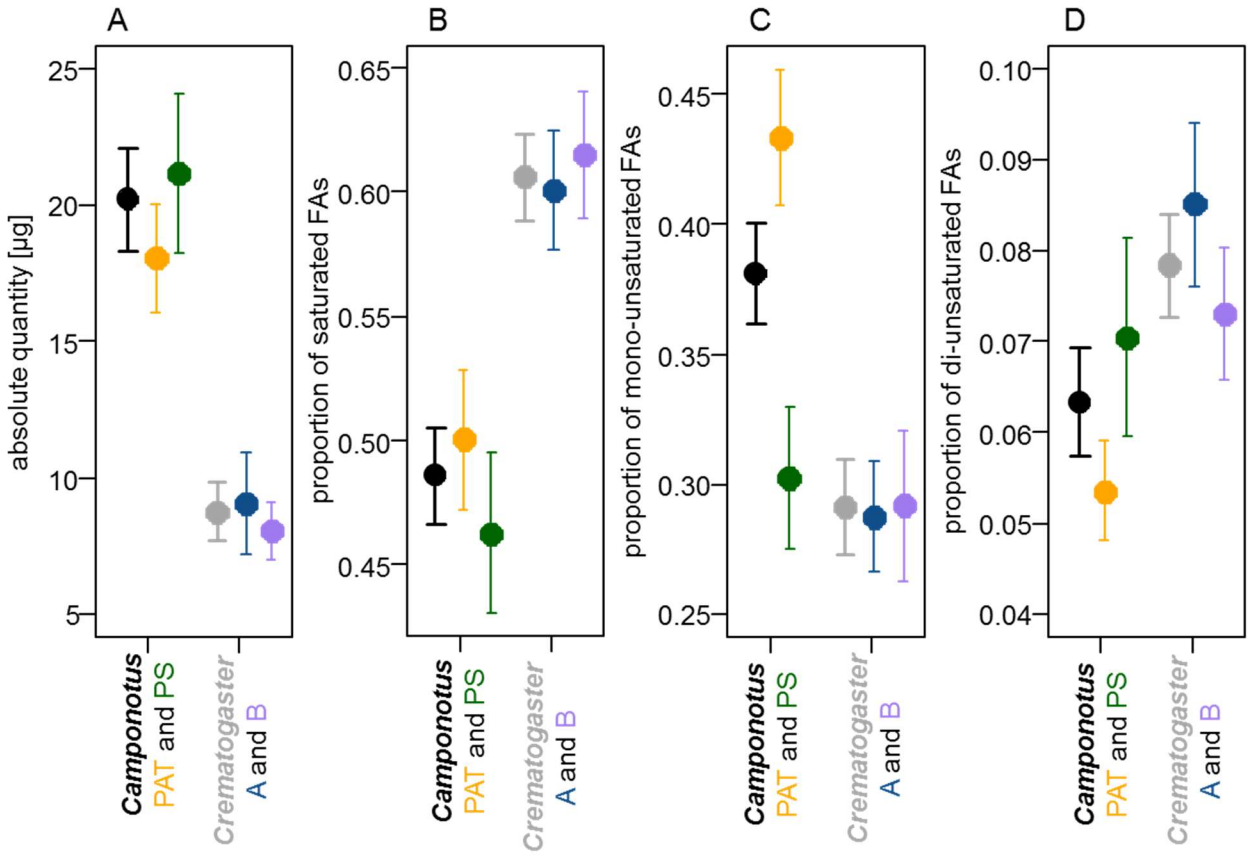

Figure 5.3: Absolute quantity and composition of neutral lipid fatty acids in the cryptic species of *Ca. femoratus* and *Cr. levior*. The plots show back-transformed means ± s.e.m. of the absolute quantity (A) or the relative proportion of structural classes of fatty acids (B-D). *Camponotus femoratus* PAT is represented in orange, PS in green and *Cr. levior* A in blue, B in purple. The bold plots in black (*Camponotus*) and grey (*Crematogaster*) represent the means including samples of both cryptic species each.

5.3.3 Stable isotope analyses

Stable isotope signatures of nitrogen were lower in *Camponotus* than in *Crematogaster* (LM: F_1 = 6.19, p = 0.016; Fig. 5.4). The $\delta^{15}N$ differed between sites (F_2 = 4.18, p = 0.020), being lowest at Paracou (z = -2.54, p = 0.030 in comparisons to either Camp Patawa or Sinnamary) but not between cryptic species (cryptic species identity nested in genus: F_2 = 0.05, p = 0.95; Fig. 5.4).

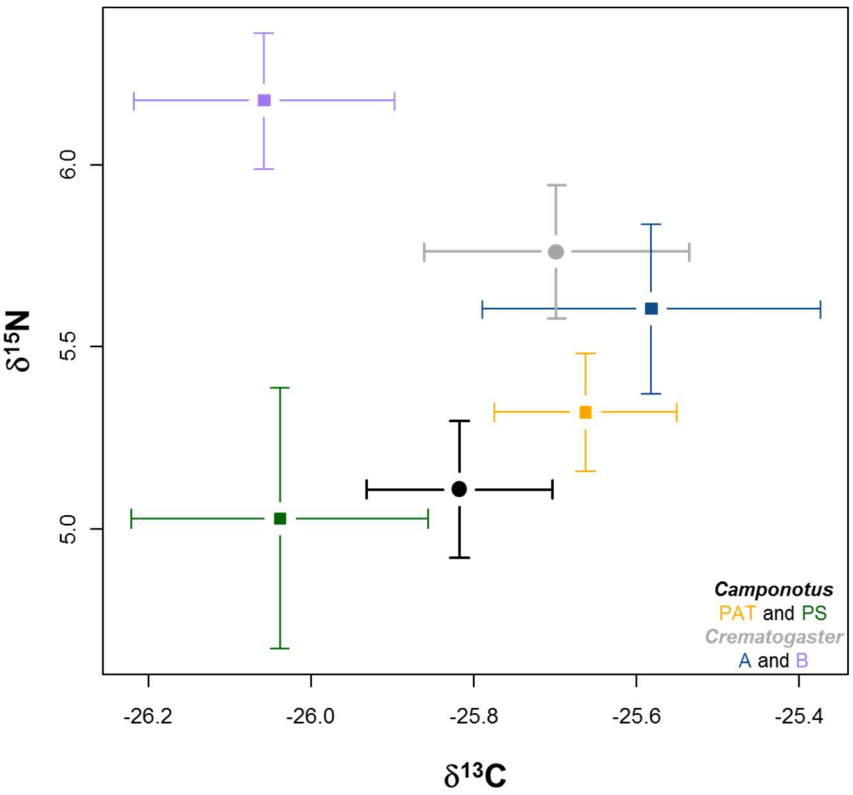

Figure 5.4: Stable isotope signatures of $\delta^{15}N$ and $\delta^{13}C$ in the cryptic species of *Ca. femoratus* and *Cr. levior*. The plots with squares show means ± s.e.m. of the $\delta^{15}N$ signature (y-axis) and the $\delta^{13}C$ signature (x-axis) of *Ca. femoratus* PAT (orange), PS (green), *Cr. levior* A (blue) and B (purple). The bold plots with dots in black (*Camponotus*) and grey (*Crematogaster*) represent the means including samples of both cryptic species each.

The $\delta^{13}C$ signature neither differed between *Camponotus* and *Crematogaster* (F_1 = 0.10, p = 0.75) nor sampling sites (F_2 = 1.94, p = 0.15). However, *Cr. levior* A had higher $\delta^{13}C$ signatures than B (cryptic species identity nested in genus: F_2 = 3.27, p = 0.045; A vs. B: t = -2.30, p = 0.025; Fig. 5.4), while there was no difference between the cryptic *Ca. femoratus* species (PAT vs. PS: t = 0.88, p = 0.38).

The $\delta^{15}N$ signatures of *Camponotus* and *Crematogaster* ants of the same ant garden co-varied (LM: F_1 = 6.73, p = 0.015), but the $\delta^{13}C$ signatures did not (F_1 = 0.25, p = 0.62). Site did not influence this covariation in both isotope signatures ($\delta^{15}N$: F_2 = 1.03, p = 0.37; $\delta^{13}C$: F_2 = 1.17, p = 0.32).

5.4 Discussion

In the present study, we aimed at answering two questions: (1) Do *Camponotus femoratus* and *Crematogaster levior,* despite their mutualistic relationship, compete for similar food sources and do they differ in their trophic niches? Costs due to the mutualistic partner should reduce the net benefit from the interaction and hence mutualism stability. (2) Is there any trophic niche partitioning between the two cryptic species of *Ca. femoratus* (PAT and PS) and/or *Cr. levior* (A and B)? Differences among the cryptic sister taxa may be relevant in the light of competitive exclusion when both occur in sympatry, but may also matter for coevolution with the parabiotic partner, e.g. if one of the cryptic species shows less interference competition against the partner than the other. In the following, we will discuss how our findings help us to understand competition and feeding ecology within the parabiosis and how the cryptic species of *Ca. femoratus* and *Cr. levior* might coexist without excluding each other.

5.4.1 Competition between parabiotic ants

Our cafeteria experiments indicate that there is competition between *Camponotus* and *Crematogaster* for certain food sources although their realized food niches differed (cf. PERMANOVA results) and *Crematogaster* was more variable in food choice (cf. PERMDISP result). *Camponotus* foraged at a higher frequency at sausage as indicated by the 'hotlink' analysis (Tab. 5.1; Fig. 5.1 A) and their number negatively affected the number of *Crematogaster* workers at sausage, suggesting competition and displacement from this bait (Figs 5.1 A, 5.2 A). These findings imply that *Ca. femoratus* probably displaced *Cr. levior* from sausage baits. The displacement is consistent with earlier experiments that used wasp larvae, termites, locusts or crushed insects, respectively as protein-rich baits (Swain 1980; Vantaux *et al.* 2007; Grevé *et al.* 2019). Interestingly, displacement by *Ca. femoratus* PS was stronger than by *Ca. femoratus* PAT after 60 minutes, but not anymore after 120 minutes. This pattern might be explained by *Ca. femoratus* PS workers having already displaced all *Crematogaster* workers from the sausage bait at this time point, tentatively suggesting a higher tendency in *Ca. femoratus* PS to exclude its partner than in PAT (Fig. 5.2 A). Other studies found that *Crematogaster* was able to find protein-rich food sources before *Camponotus* and retrieve food pieces before getting displaced (Vantaux *et al.* 2007; Menzel *et al.* 2014), suggesting that a discovery-dominance trade-off among the partners reduced competition (Fellers 1987; Parr &

Gibb 2010). However, such a trade-off seems to be rather rare in most ant communities (Parr & Gibb 2012).

At sugar baits we found that the numbers of *Camponotus* and *Crematogaster* increased in parallel (albeit only in two pairs combinations of cryptic species: *Ca. femoratus* PAT and *Cr. levior* A or *Ca. femoratus* PS and *Cr. levior* B; Figs 5.1 B, 5.2 B). This lack of competition for carbohydrates confirms experiments in which *Ca. femoratus* and *Cr. levior* were found simultaneously feeding on sugar baits (Swain 1980). Since both species forage arboreally, it seems likely that sugar sources such as trophobionts or EFNs are less limited than prey items or other protein sources (Davidson *et al.* 2003). Early observations of the parabiosis between *Cr. levior* and *Ca. femoratus* even reported interspecific trophallaxis (Wheeler 1921), which we, however, never observed. Despite this seemingly peaceful relationship, Davidson (1988) found *Camponotus* to monopolize higher quality honey baits even if it did not exclude *Crematogaster* from lower quality baits.

Crematogaster workers fed significantly more often on uric acid (resembling bird feces) after 60 and 120 minutes and their worker numbers increased constantly over time (Tab. 5.1; Fig. 5.1 C). In line with that, recent observations of dietary differentiation in neotropical ant communities showed that *Cr. levior*, but also *Ca. femoratus*, foraged more frequently on bird feces than expected by chance (Grevé *et al.* 2019). Interestingly, here *Cr. levior* was more frequently found on uric acid. *Camponotus femoratus* hosts endosymbiotic *Blochmannia* bacteria, which enables them to additionally utilize urea (from mammal urine) (Sauer *et al.* 2000; Feldhaar *et al.* 2007) in comparison to *Crematogaster*, which may be restricted to bird feces.

5.4.2 Trophic niche partitioning between mutualists

In the cafeteria experiments, both *Camponotus* and *Crematogaster* were most numerous at sausage and sugar baits. This is consistent with many other ant species mainly foraging on protein and carbohydrate sources (Houadria *et al.* 2015). After 60 minutes however, it seems that *Camponotus* started displacing *Crematogaster* from the sausage bait which then started to recruit to the uric acid baits instead.

The analysis of neutral lipid fatty acids from one individual per genus of 43 independent ant gardens indicated differences in the trophic niches between *Camponotus* and *Crematogaster* with the former having more mono-unsaturated fatty acids and the latter having more saturated and di-unsaturated fatty acids (Fig. 5.3). Such strong differences are unlikely to be

exclusively caused by differences in fatty acid metabolism and could instead be caused by different diets (Budge, Iverson & Koopman 2006). In ants, the enrichment of dietary fatty acids seems to be similar among species kept on the same diet and it significantly contributes to overall changes in the NFLA profiles over time (Rosumek *et al.* 2017). Similarly, quantitative fatty acid composition correlates with the amount of dietary precursors fed to herring gulls (Käkelä *et al.* 2009). The surprisingly strong consistent difference in unsaturated fatty acid proportions between the closely related *Ca. femoratus* PAT and PS is in our opinion more likely due to nutrition than differences in metabolism.

Signatures of $\delta^{15}N$ and $\delta^{13}C$ found for *Ca. femoratus* and *Cr. levior* in our study resembled those of an earlier study on community level (Davidson *et al.* 2003). There was no difference between *Camponotus* and *Crematogaster* in $\delta^{13}C$ signatures, but consistent with earlier findings, *Camponotus* had lower $\delta^{15}N$ than *Crematogaster* (Davidson et al. 2003; Fig. 5.4). Formicines (like *Camponotus*) usually are found to forage on lower trophic levels (i.e. being less predatory and/or more engaged in trophobiotic associations) than Myrmicines (like *Crematogaster*) (Blüthgen *et al.* 2003; Csata & Dussutour 2019). Although both, *Ca. femoratus* and *Cr. levior*, are found tending trophobionts (Davidson 1997; Davidson *et al.* 2003), such associations are more often found in *Camponotus* and other Formicines (Davidson 1997; Blüthgen & Feldhaar 2010; Zhang, Zhang & Ma 2012; Menzel *et al.* 2014). Our results also coincide with data from a paleotropical parabiosis, where *Ca. rufifemur* had lower $\delta^{15}N$ than its parabiotic partner *Cr. modiglianii*. Possibly, like for *Ca. rufifemur,* the $\delta^{15}N$ signature of *Ca. femoratus* might also be lowered by its consumption of ^{15}N-depleted nitrogen sources like mammal urine (containing urea, which cannot be utilized by *Crematogaster*, Feldhaar et al. 2007) or bird feces (containing uric acid) (Menzel *et al.* 2012).

The correlation in $\delta^{15}N$ signatures of *Camponotus* and *Crematogaster* from the same nest was independent of the site, which suggests trophic niche overlap between the mutualistic partners, potentially causing competition. Beside the trophic niche, both *Ca. femoratus* and *Cr. levior* tend to forage during the day (whereas most other *Camponotus* species are nocturnal). This overlap in temporal niche may additionally increase competition (Grevé *et al.* 2019).

5.4.3 Niche partitioning vs. neutral processes in cryptic species

In our previous study, we found that *Ca. femoratus* PS was less common in the East of French Guiana, which is characterized by high precipitation and slightly cooler temperatures compared to the West of the country, while *Ca. femoratus* PAT was present in all areas

(Hartke, Sprenger *et al*. 2019, Chapter 1). Apart from that, we found no ecological factors (e.g. canopy cover or presence of certain ant garden plants in their nests) that differed between the cryptic sister species of either *Ca. femoratus* or *Cr. levior* species (Hartke, Sprenger *et al*. 2019, Chapter 1). Therefore, we investigated here if there is trophic niche partitioning between these cryptic species as a potential explanation for their co-occurrence.

In the cafeteria experiments, the food choice did not differ between the cryptic species of *Ca. femoratus* and *Cr. levior* (cf. PERMANOVA results). Nevertheless, the cryptic species of *Ca. femoratus* differed in their neutral lipid fatty acid composition, with PS having more variable fatty acid profiles and PAT containing way more mono-unsaturated fatty acids. This suggests different food resources, e.g. different prey species (Rosumek *et al.* 2018), although we cannot pinpoint the precise resources so far. In *Crematogaster levior*, we found that species A had a significantly higher $\delta^{13}C$ signature compared to B, while they did not differ in their fatty acid profiles. Differences in the $\delta^{13}C$ signature between the cryptic *Cr. levior* species could be affected by differential use of plant extrafloral nectaries or trophobionts on different plants, since C_3 and C_4 plants differ in ^{13}C-abundance (Swap *et al.* 2004; Blüthgen & Feldhaar 2010). All these differences are subtle, but could indicate that despite the large overlap, there is at least weak niche partitioning between the cryptic species of both genera which may prevent competitive exclusion in the long term.

Usually it is assumed that niche partitioning allows cryptic species to escape competitive exclusion, e.g. spatial partitioning as shown in butterflies or fig wasps (Vodă *et al.* 2015; Darwell & Cook 2017) or very different trophic niches as shown for bats (Siemers *et al.* 2011) and dolphins (Owen, Charlton-Robb & Thompson 2011). In *Ca. femoratus*, we found evidence for climatic segregation between the cryptic species although both still occur sympatrically at many sites (Hartke, Sprenger *et al*. 2019, Chapter 1). Here, we found that the trophic niches of the co-occurring cryptic species of both genera are largely overlapping. This is similar to findings in a cryptic species complex of freshwater amphipods that showed overlapping stable isotope signatures if they co-occurred (Dionne, Dufresne & Nozais 2017). Although niche partitioning often can explain species coexistence, ecological differences between co-occurring species can sometimes be very subtle, especially in closely related and/or cryptic species. If species entirely overlap in their niche and fulfill similar functions in an ecosystem, they are considered as 'neutral species' within (but not beyond) their functional group (McPeek 2017). Such neutral species can persist in a community, for example via random processes like ecological drift, or if competitive superiority is context-dependent (Leibold & McPeek 2006; Andersen 2008; Dionne *et al.* 2017; Gilbert & Levine 2017). Hence, *Ca. femoratus*

PAT and PS, and *Cr. levior* A and B may represent two pairs of neutral species. Beside colony size, context-dependent competitive outcomes might mean here that colony fitness varies with the identity of the parabiotic partner.

5.5 Conclusions

Despite their mutualistic relationship, *Ca. femoratus* and *Cr. levior* compete for certain food sources. Often *Cr. levior* is the first at protein sources, but gets displaced by *Ca. femoratus* over time, which suggests a discovery-dominance trade-off between the mutualistic partners (Fellers 1987; Sarty *et al.* 2006). The realized food niches between the mutualists, nevertheless, seem to differ as indicated by differences in their neutral lipid fatty acid profiles and stable isotope signatures.

The cryptic species of both genera showed only very subtle differences in their trophic niches and possibly feed on largely similar food sources. Since so far, climatic partitioning was only found for *Ca. femoratus* (Hartke, Sprenger *et al*. 2019, Chapter 1), it remains open at least for *Cr. levior* A and B how the cryptic species avoid competitive exclusion. If sympatric coexistence of the cryptic species studied here is stable, this can be either mediated by through partitioning in niche dimensions unknown so far, but also via neutral processes (Hubbell 2005; Adler *et al.* 2007). These neutral processes could be highly environment-dependent competitive outcomes, hence limiting competitive exclusion of one species by another (Andersen 2008), or competitive outcomes dependent on the parabiotic partner species, e.g. if a partner species displaces one or the other cryptic species faster from baits, or takes part in competitive encounters with neighboring colonies. The cryptic *Ca. femoratus* and *Cr. levior* species complexes offer the chance to further investigate how mutualistic interactions might affect sympatric coexistence of cryptic species despite no obvious ecological differences.

5.6 Acknowledgements

Removed for privacy purposes.

5.7 Supplementary material

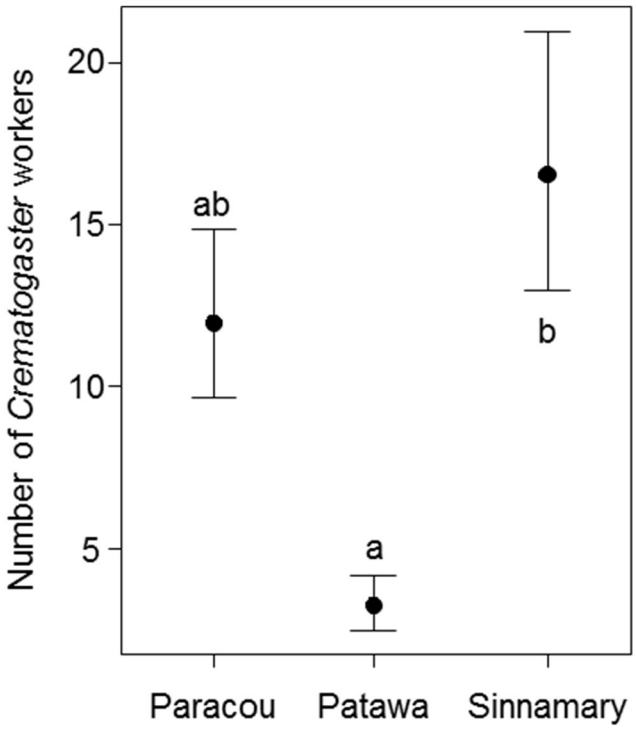

Figure S5.1: Total number of *Crematogaster levior* workers at the baits at different sites. The plots show back-transformed means ± s.e.m. of the number of *Cr. levior* workers at Paracou, Camp Patawa and Sinnamary. Different letters in the plots indicate statistically different comparisons.

Chapter 6

Cuticular hydrocarbons in ants and other insects: How and why they differ among individuals, colonies and species

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Abstract

The body surface of nearly all insects, including ants, is covered with a lipid layer that largely consists of cuticular hydrocarbons (CHC). They fulfill several functions, the two best-studied ones being communication and protection against water loss. CHC profiles are astonishingly diverse as even a single individual can possess more than 100 different hydrocarbon molecules. Species vastly differ in their CHC composition. But also within species, CHC profiles vary among individuals of different sex, caste, fertility, age, health state, etc. This variation has been intensely studied especially in eusocial insects like ants, where differences are likely to have a signaling function. However, with so many sources of variation in CHC profiles, it is easy to lose track of which factors are more important than others, which patterns can be generalized and which are idiosyncratic. Thus, we need a deeper understanding of how precisely different factors influence CHC variation. In this review, we aim to provide an overview of what is known to date about fixed and plastic CHC variation, and discuss sources of variation on the level of individuals, social insect colonies, populations, and species. We focus on abiotic and biotic environmental factors, social structure and the genetic background as sources of CHC variation. Finally, we discuss how variation can be adaptive and how it can be constrained by biophysical and biosynthetic mechanisms. Focusing on clearly defined CHC traits will help us to build a predictive framework in order to understand how CHC profiles are shaped by multiple selection pressures, to identify how different sources affect fixed and plastic CHC variation, and determine the adaptive value of CHC traits.

Keywords:

Acclimation, adaptation, communication, nestmate recognition, queen pheromone, review, waterproofing

6.1 Introduction

6.1.1 Functions of cuticular hydrocarbons

The body surface of nearly every insect is covered with a layer of cuticular hydrocarbons (CHCs). They fulfill several functions vital for the insect, the two best studied ones being protection against water loss (waterproofing) and communication. The waterproofing function was already recognized almost 85 years ago, when Ramsay (1935) noted that water droplets on the wings of cockroaches evaporated slower than those on artificial surfaces. Later studies following this discovery centered on the role of CHCs in preventing water loss (Beament 1945; Edney 1957; Locke 1965). Only in the 1960s, with the advent of gas-chromatography mass-spectrometry (GC-MS), biologists started to grasp the immense diversity of CHCs on insects (Blomquist & Bagnères 2010). Even a single insect can possess up to ca. 100 different hydrocarbons (Blomquist 2010a). They vary in chain length (mostly between C20 and C45), number and position of methyl branches, and number and position of double bonds (Fig. 6.1). Nearly all ant species contain *n*-alkanes, and most species also possess monomethyl alkanes (which can make up 50% of the CHC profile; F. Menzel unpubl. data). Further common substance classes include dimethyl alkanes, alkenes, and (less commonly) alkadienes, tri- and tetramethyl alkanes. Even rarer are alkatrienes and methyl alkenes (with a double bond *and* a methyl group). A few studies also detected very long-chain compounds (up to C60) in ants and other insects, some of which are hydrocarbons (Akino 2006; Cvačka *et al.* 2006; Sutton *et al.* 2013; Bien *et al.* 2019). So far, they have been studied in relatively few species, such that more research is needed to understand their variability and their biological function.

Within a species, CHC variation is mostly quantitative, i.e. individuals possess the same set of hydrocarbons, but in different relative quantities (Fig. 6.2, 6.3). Notably, many species possess homologous series, i.e. hydrocarbons with the same methyl group and/or double bond positions, but different chain lengths (Martin & Drijfhout 2009a). Across species, in contrast, we find an enormous *qualitative* diversity of cuticular hydrocarbons, i.e. insects of different species can possess entirely differently sets of hydrocarbons (Figs 6.1, 6.4). The profiles are usually so specific that one can easily identify species based on their cuticular hydrocarbon profile alone (Kather & Martin 2012) (Box 6.1). The complex composition of CHC profiles allows to store a lot of information. Indeed, their important role for chemical communication was discovered in the early 1970s (Carlson *et al.* 1971; Blomquist & Bagnères

2010), and since then there has been a plethora of studies on this function. In many solitary insects, they serve as contact sex pheromones (Thomas & Simmons 2008; Niehuis *et al.* 2011; Thomas 2011; Buellesbach *et al.* 2013) and can indicate breeding status in burying beetles (Steiger *et al.* 2007; Steiger, Peschke & Müller 2008; Scott, Madjid & Orians 2008).

Cuticular hydrocarbons are particularly important in social insects, where much more information needs to be exchanged to run the colony. Social insects, like ants, bees or termites, use them to tell apart nestmates from non-nestmates (Lahav *et al.* 1999; Soroker & Hefetz 2000). Within the colony, they provide information whether an individual is a queen or a worker, and (for workers) whether it is a forager or a nurse (Leonhardt *et al.* 2016). Hence, CHC variation in social insects is especially important concerning intraspecific and intra-colonial variation, and the resulting selection pressures will be discussed below (section 6.3).

Less well-studied functions of CHC include their role as a barrier against microbes (Wurdack *et al.* 2017), lubrication of the cuticle (Cooper *et al.* 2009), and the enhancement of foot adhesion via CHC droplets left as footprints when an insect walks (Drechsler & Federle 2006; Wüst & Menzel 2017). Furthermore, CHCs mediate interspecific recognition between host and parasite (Lenoir *et al.* 2001b) and between mutualists (Menzel *et al.* 2008a, 2014; Lang & Menzel 2011, Fig. 6.5). Naturally, many functions mean many, and possibly conflicting, requirements. The complex interplay of all these different functions makes the evolution of CHC highly complex and intriguing.

6.1.2 Linking composition and function

A plethora of studies shows how CHC profiles vary within a species, e.g. across different seasons, diets, ages, nest sites, or climates, which makes it challenging to see general patterns. Even more stunning is the enormous variation of CHC profiles across species (see section 6.6) – stunning because up to now, we are far from understanding the causes of this diversity. Interestingly though, CHC variation has been mostly investigated in the context of intraspecific communication, while the variation among species received considerably less attention.

So how does CHC variation influence the functionality of the CHC layer? Answering this question is essential if we want to understand how CHC profiles evolve and why they are so diverse. Hence, we need to understand in what ways CHC variation can be adaptive, and which factors cause non-adaptive variation.

Firstly, all CHC functions are likely to be influenced by the biophysical properties of the CHC layer. Most of them, like waterproofing, protection against microbes, lubrication and foot adhesion, depend even solely on these physical properties (chemical effects such as toxicity, polarity or chemical interactions with the insect cuticle or the surface of microbes are unlikely since hydrocarbons lack functional groups). For example, the waterproofing ability of a CHC layer is higher if it is viscous and/or contains solid parts (Sprenger *et al.* 2018; Menzel *et al.* 2019). *n*-alkanes and terminally branched monomethyl alkanes aggregate most tightly and are thus more viscous than other CHCs or even solid. Hence, they should be most beneficial in preventing water loss (Gibbs 1998; Gibbs & Rajpurohit 2010; Brooks *et al.* 2015). The waterproofing ability correlates with the melting temperature (T_m), and thus increases with chain length in homologous series of hydrocarbons (Gibbs & Pomonis 1995; Gibbs 2002). Methyl branches (mainly in di-, tri- and tetramethyl alkanes) and unsaturation introduce 'disorder' into the layer, hindering the molecules to aggregate tightly. This reduces T_m, which is why these 'disruptive' substance classes provide less protection against water loss (Gibbs & Pomonis 1995; Gibbs 1998). Thus, the composition of CHC profiles directly influences its viscosity and melting range. Acclimatory CHC changes (Fig. 6.2) are predictable based on these physical properties (Menzel *et al.* 2018; Sprenger *et al.* 2018), which confirms that they are relevant for biological functionality. Beside acclimation, they should also matter for footprint adhesion. Foot adhesion might be enhanced if hydrocarbons are less viscous, such that larger hydrocarbon droplets are left as footprints when the insect walks.

For communication, biophysical properties are relevant as well, because they influence the perceptibility of the communication signal. Here, the vapor pressure of a hydrocarbon (which, in liquid compounds, is directly related to its viscosity, Othmer & Conwell 1945) should be especially important: A high vapor pressure means that more molecules enter the gas phase and hence are easier to perceive. All hydrocarbons beyond C20 are liquid or solid at room temperature, and hence little to non-volatile. However, they still differ in perceptibility – based on vapor pressure, liquid CHCs with a low viscosity should be easier to perceive than highly viscous liquid CHCs or solid CHCs (Menzel *et al.* 2019).

CHC-based information is encoded via compositional differences, be they quantitative or qualitative (see Box 6.2 for perception and neural processing). For example, CHC profiles can encode the queen signal, which regulates worker reproduction in a colony (see section 6.3). In this context, it is important to distinguish between *signals*, which were selected for communication (i.e. intended exchange of information that benefits both parties), and *cues*,

which unintentionally display information that is used by a receiving individual but not necessarily beneficial for the emitter (Dusenbery 1992). Generally, the information content of a signal depends on its evolutionary history (Leonhardt *et al.* 2016). Signals can be highly species-specific, reflecting contingent evolution, or phylogenetically conserved (see section 6.3). As discussed below, selection pressures on CHCs as communication signal can be complex, and there is much theoretical and empirical work on these issues.

CHC profiles are multidimensional. Hydrocarbon differentiation among colonies, castes, sexes, etc. is frequently analyzed by quantifying all hydrocarbons, followed by multivariate statistics. While useful, this approach emphasizes differences among groups, but neglects what they actually consist in. Thus, one might lose sight of the actual magnitude of these differences relative to the entire profile. For example, compare the differences between 20°C- and 28°C-acclimated workers (Fig. 6.2) or between nurses and foragers (Fig. 6.3) to those between queens and workers in *Myrmica* (Fig. 6.3) or between species (Figs 6.1, 6.4). All of them are highly significant, but the latter concern a much larger proportion of the entire profile than the former – however, in separate multivariate analyses, this would not be obvious. In our opinion, we need to study what differences among groups actually consist in if we want to fully understand causes and consequences of CHC variation. To this end, it is helpful to use clearly defined unidimensional traits, such as the proportion of a certain CHC class, average chain length, or the number of homologous series, and treat them as functional traits *sensu* McGill *et al.* (2006). This approach allows clear and testable predictions how each trait affects CHC functionality, for example based on biophysical properties or biosynthetic pathways.

Due to their multiple functions, it is likely that CHCs influence not only interactions among conspecifics (e.g. by encoding information), but also contribute to a species' ecological niche (e.g. if it protects against reducing water loss, but only to a certain degree or only for a certain temperature range). However, the complexity of CHC profiles makes it challenging to understand which chemical trait serves which function, and whether there are conflicts or trade-offs between different functions. Furthermore, we have to understand how biophysical mechanisms and biosynthetic pathways constrain CHC variation as we will outline below. Finally, we have to distinguish plastic and genetically fixed variation, both of which may or may not be adaptive.

In this review, we aim to provide an overview of the extrinsic sources of plastic CHC variation (section 6.2), the intrinsic sources of CHC variation within a colony (section 6.3),

among conspecific colonies (section 6.4), between sexes (section 6.5) and across species (section 6.6) as well as potential constraints on CHC variation (section 6.7). In Box 6.1, we explore how different sources of variation act on the same CHC profile, and to which degree this variation allows to classify individuals. We will summarize what is known on certain sources of variation, discuss which general patterns can be derived from this and whether this variation is likely to be adaptive. Although we largely focus on ants, most effects we describe either have been shown or are likely to occur in other insect taxa as well.

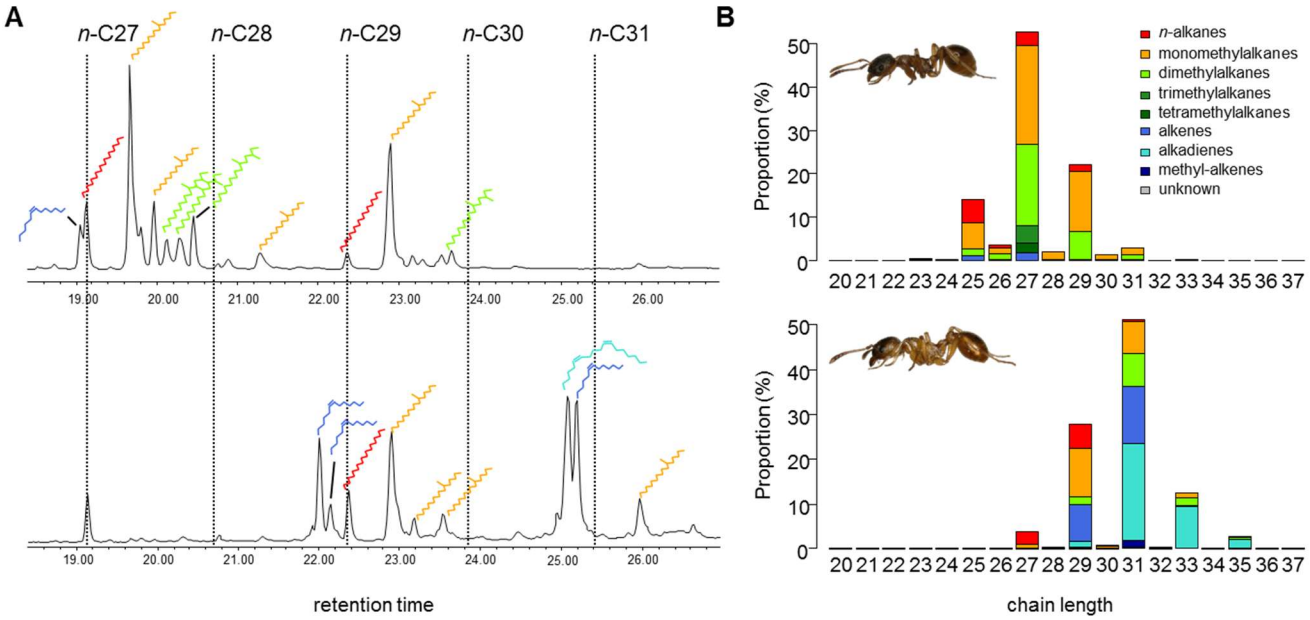

Figure 6.1: Cuticular hydrocarbon profiles of the ants *Myrmica rubra* (above) and *Myrmica ruginodis* (below). The graphs in (A) show the peaks as the output of the GC-MS analysis. Vertical dotted lines indicate the retention times of *n*-alkanes. The substance class of the major peaks is indicated by small symbols with a color code as. Within a single chain length, methyl-branched alkanes appear after the corresponding *n*-alkane, while unsaturated hydrocarbons usually appear before the corresponding *n*-alkane. Note that, for better visibility, the graphs only show the peaks between C27 and C31. These GC-MS graphs are then transformed to barplots (B), where hydrocarbons are pooled according to CHC class and chain length. Using these plots, you can see that most CHCs have odd-numbered chain length (i.e. number of carbon atoms in the backbone of the molecule). Furthermore, > 80% of all CHC belong to only three chain lengths (*M. rubra*: C25, C27, C29; *M. ruginodis*: C29, C31, C33). This method of visualization allows a quick overview of the CHC composition – the idea is to visualize overall CHC composition, and variation thereof. This way, variation between profiles (or treatments) can be seen in relation to overall CHC variation. Note however, that different methyl group and/or double bond positions *within* the same CHC class and chain length are not resolved. Photos of the ants were taken by Philipp Sprenger.

Box 6.1: Different sources of CHC variation and their relative contributions

Based on two previously published datasets on acclimated individuals of the ant species *Temnothorax longispinosus*, *T. ambiguus*, *Myrmica rubra* and *M. ruginodis* (Menzel *et al.* 2018; Sprenger *et al.* 2018), we quantify different sources of CHC variation. We used a random forest algorithm (Liaw & Wiener 2002) to determine differences among groups. As a measure of differentiation, we used the error rate in cross-validation of the results. An error rate of 0 means that the CHC profile allows classifying all individuals unambiguously. Note, for example, that species classifications are possible without error. In contrast, acclimatory changes and forager/nurse differences, while highly significant, do not allow to unambiguously assigning workers to the respective categories. Finally, worker/queen differences are in between, and allow assignment of the reproductive caste in most (but not all) cases.

The random forest method additionally allows to infer the importance of single hydrocarbons for the classification and thus shows which substances differ most strongly. For each classification, we report the five CHCs (or CHC blends) most important for the classification. Note that their substance classes often differ among sources of variation: For example, the most important species and genus differences concern mostly trimethyl, dimethyl and monomethyl alkanes. In contrast, temperature differences concern mostly *n*-alkanes and monomethyl alkanes. Different substance classes are indicated as different colors (see Fig. 6.1).

Comparison	**Classification error rates**			
	T. ambiguus	*T. longispinosus*	*M. rubra*	*M. ruginodis*
genus (workers only)	 *0 %* *n*-C28 unknown CHC unknown TriMe	13,17- ; 13,19-DiMe C33 15,19- and other DiMe C35		
species (workers only)	 *0 %* 7,15,21-TriMe C31 11,15,19-TriMe C31 3,7,13-TriMe C29 9,15,21-TriMe C31 3,7,15-TriMe C33		 *0 %* 9-; 11-; 13-; 15-Me C29 9-C29ene 10-; 12-; 13-; 14-Me C28 5,9,13-; 5,9,15-TriMe C29 C31diene	
reproductive caste (queen/worker) (data available for *Temnothorax* only)	 *3.45 %* 3 unknown CHCs 5,9-; 5,17-DiMe C27 7-Me C27	 *2.39 %* 2 unknown CHCs 5-Me C29 12,16,20-TriMe C36 7,11,15,19-TetraMe C35		
behavioral caste (forager/nurse)	 *57.50 %* *n*-C27 11-; 12-; 13-; 14-Me C30 3 unknown CHCs	 *40.00 %* 5-Me C29 7-Me C29 3 unknown CHCs	 *37.92 %* 11,15-DiMe C27 5-Me C29 C25ene *n*-C25 3,7,11,15-TetraMe C27	 *36.67 %* *n*-C26 C31diene *n*-C25 *n*-C27 9,13-DiMe C27
temperature (20°C/28°C) (workers only)	 *14.58 %* *n*-C31 *n*-C29 13-; 15-Me C31 unknown mixture of methylbranched CHC *n*-C30	 *17.50 %* *n*-C31 *n*-C30 3-Me C27 *n*-C29 11-; 12-; 13-; 14-Me C30	 *7.92 %* 5-Me C23 5-Me C25 9-; 11-; 13-; 15-Me C31 *n*-C29 C27ene	 *1.67 %* 5-Me C27 *n*-C29 5-Me C29 9-; 11-; 13-Me C27 *n*-C31
humidity (50% /100% rh) (workers only)	 *20.83 %* *n*-C29 13-; 15-Me C31 13,17-; 15,19-DiMe C33 *n*-C28 *n*-C27	 *20.00 %* 7-Me C27 C29ene *n*-C29 13,17-; 15,19-DiMe C33 *n*-C28	 *24.17 %* 2 unknown CHCs *n*-C19 15,x-DiMe C35 2-; 4-Me C30	 *26.25 %* *n*-C24 9,13-; 7,11-DiMe C29 10-; 12-; 13-; 14-Me C30 15,19-DiMe C35 13,17- ; 13,19-DiMe C33

Box 6.2: Perception of cuticular hydrocarbons in ants

Insects perceive chemicals using three families of chemosensory receptors: odorant receptors (ORs), gustatory receptors (GRs) and ionotropic receptors (IRs) (Hansson & Stensmyr 2011). Although GRs can perceive pheromones in *Drosophila*, they are not expressed on ant antennae, and thus presumably do not contribute to CHC perception (Fleischer *et al.* 2018). Odors are perceived via olfactory sensillae on the antenna: One form, the basiconical sensillae, seem to be responsible for CHC perception (Nakanishi *et al.* 2009; Sharma *et al.* 2015). Each of them contains multiple odorant receptor neurons (ORN), the membranes of which contain olfactory receptors (ORs) next to their obligate *orco* co-receptor proteins (Ozaki & Wada-Katsumata 2010; Sharma *et al.* 2015; Trible *et al.* 2017; Yan *et al.* 2017). After CHCs diffuse into the sensillum lymph, they initially bind to an odorant binding protein (OBP) in the lymph and then are transported to the ORs (Fleischer & Krieger 2018; Fleischer *et al.* 2018). Neural processing of the perceived CHCs happens in glomeruli in the antennal lobe (Nakanishi *et al.* 2010; Trible *et al.* 2017). The morphology of the sensillae and that of antennal lobes in ants is plastic, but also sex-specific (Nakanishi *et al.* 2010; Ghaninia *et al.* 2018). Interestingly, eusocial insects possess higher numbers of both, receptors and glomeruli, increase from non-social to eusocial compared to solitary insects (Zhou *et al.* 2012; Tsutsui 2013).
The ability of ants to perceive the variety of different CHCs is reflected gene expansions in the OR family (Zhou *et al.* 2012; McKenzie *et al.* 2016). Recent studies found that individual ORs are narrowly tuned and might respond to only few compounds (albeit not single CHC), such that they collectively generate an integrated odor perception (Pask *et al.* 2017; Slone *et al.* 2017). The olfactory system of ants shows very similar stimulation through nestmate and non-nestmate hydrocarbons (Brandstaetter & Kleineidam 2011; Brandstaetter, Rössler & Kleineidam 2011; Sharma *et al.* 2015), suggesting that nestmate recognition involves learning mechanisms.

6.2 Extrinsic causes for reversible plasticity

6.2.1 Abiotic condition

A growing body of literature reveals that cuticular hydrocarbon profiles are rather plastic in respect to the insects' environment. This includes climate variables of their habitat like temperature, humidity or UV radiation, but also nesting material like different types of soil or wood. While climate-related CHC changes apparently represent adaptive plastic responses of the insect, the effect of nest material is probably non-adaptive although the precise mechanism and its adaptive value have, to our knowledge, not yet been investigated. In the following, we will first discuss what is known about acclimatory CHC changes in response to temperature, then turn to humidity-induced changes and finally report the current knowledge about the influence of nesting material.

6.2.1.1 Temperature

A CHC layer should protect against water loss, requiring a viscous and (ideally) partly solid layer, but simultaneously needs to be fluid enough to ensure functions like the transfer of communication signals, foot adhesion and lubrication (Drechsler & Federle 2006; Cooper *et al.* 2009; Dirks, Clemente & Federle 2010; Gibbs & Rajpurohit 2010). However, the need for these two rather opposing properties varies with temperature: Under warm conditions, the vapor pressure of water is higher, which means a higher risk of desiccation. To counteract this, CHCs with higher melting points are needed, which are more viscous or even solid (Gibbs 2002). However, in a cool climate the molecules will most likely be solid, impeding functions that require fluidity of the CHC layer. Thus, maintaining a sufficiently high fluidity (i.e. low viscosity) of the CHC profile is also an important part of temperature acclimation (Sprenger *et al.* 2018; Menzel *et al.* 2019). Nevertheless, very low temperatures can also cause desiccation stress and result in acclimatory responses similar to warm temperatures in *Drosophila* (Ala-Honkola *et al.* 2018).

Already in the late 1970s studies on desert-dwelling scorpions and beetles revealed that CHC profiles underwent seasonal changes, with more long-chained alkanes during summer (Hadley 1977; Toolson & Hadley 1979). Continuing the work on desert species, studies on the harvester ant, *Pogonomyrmex barbatus*, showed that the exposure to warm, dry climate likewise resulted in increased abundances of *n*-alkanes in workers performing their tasks outside the nest (Wagner *et al.* 1998, 2001).

The chemical strategies to cope with high ambient temperatures differ among species: Firstly, insects can enhance waterproofing by adjusting the composition of CHC classes in their profile. Here, the largest acclimatory effects often concern strongly aggregating (e.g. *n*-alkanes) and strongly disruptive (e.g. multiply methyl-branched alkanes, alkadienes) compounds, while intermediate classes like monomethyl alkanes show weaker changes although they constitute 20-50% of the CHC profile in some cases (Menzel *et al.* 2018; Sprenger *et al.* 2018). With higher temperatures, linear *n*-alkanes increase in abundance, while multiply methyl-branched alkanes and alkadienes decrease (grasshoppers: Gibbs & Mousseau 1994; termites: Woodrow *et al.* 2000; ants: Buellesbach *et al.* 2018; wasps: Michelutti *et al.* 2018; ants: Sprenger *et al.* 2018) (Fig. 6.2). For alkenes, the response is less clear up to now – although one would expect them to reduce overall waterproofing, they often co-vary tightly with *n*-alkanes, i.e. they also increase with temperature (Buellesbach *et al.* 2018; Sprenger *et al.* 2018). The reason for this is still unclear. Beside these changes in relative

abundance of substance classes, some insects and other arthropods can also change the average chain length, i.e. they increase the ratio of longer-chained to short-chained CHCs in the profile (beetles: Hadley 1977; scorpions: Toolson & Hadley 1979; ants: Menzel *et al.* 2018; P. Sprenger & F. Menzel unpubl. data; Duarte *et al.* 2019) (Fig. 6.2).

Depending on the species-specific CHC composition, acclimation to fluctuating temperatures poses a challenge that differs from acclimation to constant temperature regimes (Sprenger *et al.* 2018). However, to our knowledge there are no further studies comparing acclimation to constant vs. fluctuating conditions, such that more evidence is needed to evaluate how responses to these challenges might differ among species.

6.2.1.2 Humidity

Similar to high temperatures, low humidity increases the risk of desiccation. That given, it is not surprising that acclimatory changes in the CHC composition during warm acclimation were also found during drought acclimation. For example, dry-acclimated workers of *Temnothorax* ants increased the proportion of *n*-alkanes at the expense of dimethyl alkanes (Menzel *et al.* 2018), and *Anopheles* flies increased *n*-alkanes while decreasing proportions of methyl-branched and unsaturated hydrocarbons (Reidenbach *et al.* 2014). In females of *Drosophila melanogaster,* even few hours of exposure to drought ('rapid desiccation hardening') led to higher proportions of saturated versus unsaturated CHCs and a higher desiccation resistance (Bazinet *et al.* 2010; Stinziano *et al.* 2015).

Another strategy against drought stress is increasing the overall CHC quantity. Examples include the desert beetle *Eleodes armata* (although only at high temperatures) (Hadley 1977), *Musca domestica* flies (Noorman & Den Otter 2002), the desert scorpion *Buthus occitanus* (Gefen *et al.* 2015), aposymbiotic *Oryzaephilus surinamensis* beetles (Engl *et al.* 2018a) and two *Myrmica* ant species (Sprenger *et al.* 2018). Other studies, however, report no effects of drought acclimation on total CHC quantities (Kalra, Parkash & Aggarwal 2014; Menzel *et al.* 2018).

Both CHC changes in response to temperature and to humidity probably represent beneficial acclimatory responses (Leroi, Bennett & Lenski 1994), and are consistent with predictions based on their biophysical properties: Warm and dry conditions both lead to higher proportions of more aggregating substances like *n*-alkanes, while the more fluid methyl-branched and unsaturated hydrocarbons decrease.

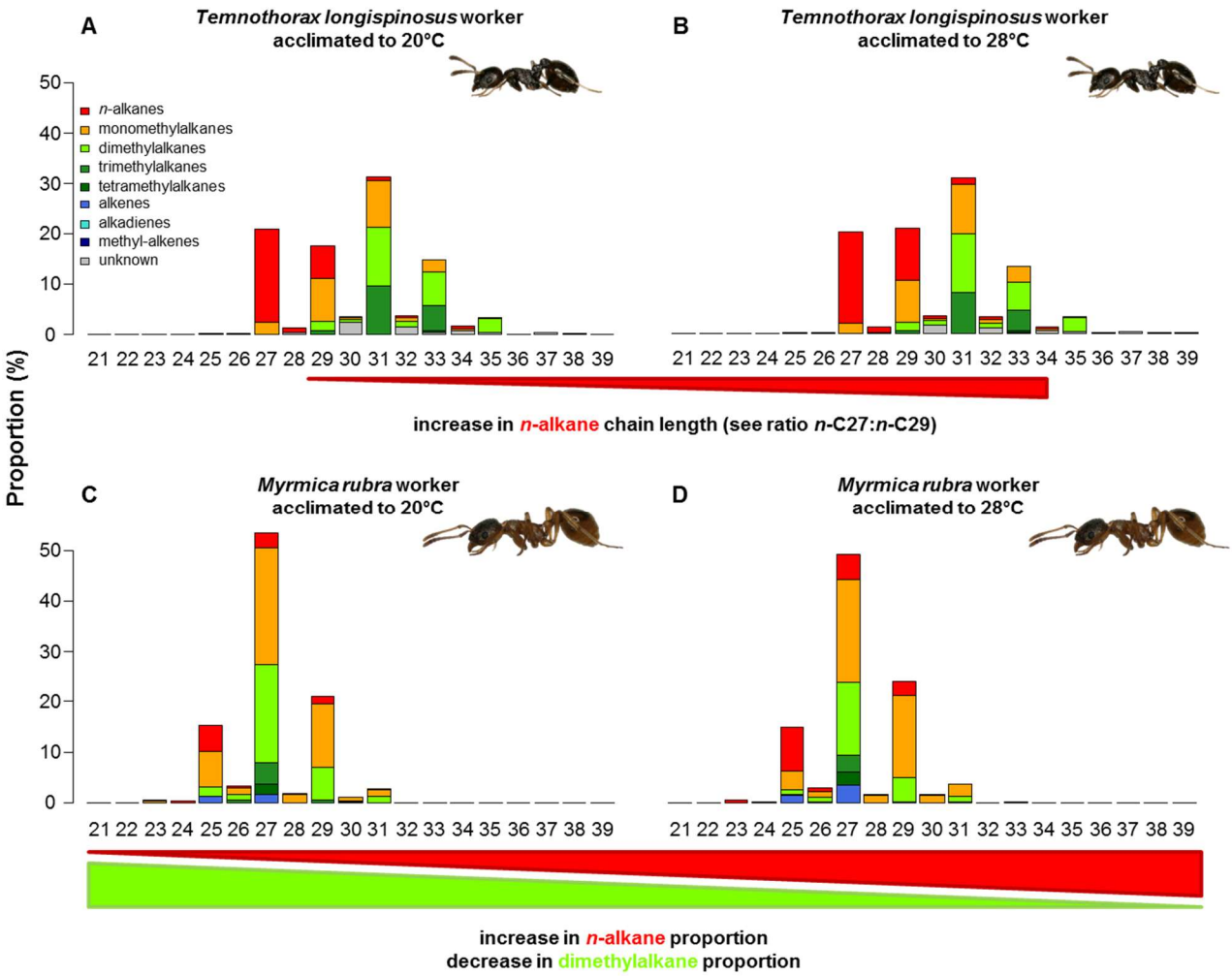

Figure 6.2: Cuticular hydrocarbon temperature acclimation in the two ants *Temnothorax longispinosus* and *Myrmica rubra*. Bar graphs of CHC plots (see Fig. 6.1 for details) for *Temnothorax longispinosus* (A, B) and *Myrmica rubra* (C, D) workers acclimated to either 20°C (A, C) or 28°C (B, D). The two species represent two (not mutually exclusive) acclimation strategies: *T. longispinosus* workers increase the average chain length of *n*-alkanes (e.g. more *n*-C29 compared to *n*-C27), while *M. rubra* workers increase the proportion of *n*-alkanes under high temperature, while both species reduce the proportion of dimethyl alkanes. All plots are based on mean proportions of 120 workers each. Photos of the ants were taken by Philipp Sprenger. Data from Menzel *et al.* (2018) and Sprenger *et al.* (2018).

6.2.1.3 Nest material

Behavioral experiments indicate that the nest material can influence the colony odor and thus interfere with nestmate recognition in ants (Crosland 1989; Heinze *et al.* 1996). Some studies demonstrated that the smell of the nest material modulates the template responsible for nestmate recognition, i.e. the ants habituate to the presence of additional compounds, but do not necessarily change their CHC profile (because additional non-CHC compounds can

be present on the cuticle) (Pickett, McHenry & Wenzel 2000; Katzav-Gozansky *et al.* 2004). The nest material can take up ant CHCs, thus supporting CHC exchange among nestmates to unify the colony odor (Bos, Grinsted & Holman 2011). Similarly, honey bees acquire hydrocarbons from wax combs during physical contact, which are colony-specific and affect nestmate recognition (Breed *et al.* 1995a; b, 1998). CHC deposition in nest material or on the soil around the nest can also be useful for home-range marking (Lenoir *et al.* 2009). However, also social parasites can acquire their host's CHCs from the nest material and this way facilitate their integration into the colony (Lenoir *et al.* 2001b; Emery & Tsutsui 2016).

The exact mechanism of how nest material influences nestmate recognition is not entirely clear. As described above, ant CHCs may be deposited in the nest soil, and taken up by other individuals again. However, workers might also acquire other compounds like resin from the nest material, which may elicit aggression. Furthermore, different kinds of nest material could cause different microclimates or different microbial communities, such that CHC profiles change via acclimatory or microbiome-induced changes.

6.2.2 Biotic conditions

Next to abiotic factors, diet, microbes, and parasites or pathogens were shown to affect CHC profiles, which will be discussed here. As diet can affect the gut microbiome, their effects on CHCs may often be interconnected. To our knowledge, little is known about the adaptive value of these changes. In our opinion, CHC changes linked to diet or microbiomes may often be non-adaptive side effects. In contrast, parasite- or pathogen-induced changes might be adaptive if they function as a signal to elicit care by nestmates (but this remains to be shown). However, diet-induced differences can lead to changes in mate choice via assortative mating (Rundle *et al.* 2009; Schwander *et al.* 2013; Otte *et al.* 2015). This way, specialization on a certain food source or host plant could lead to reproductive isolation and speciation over time.

6.2.2.1 Diet

CHCs originate from the fatty acid metabolism and are produced by elongation of fatty acyl-CoA via malonyl-CoA to very long-chain fatty acids that are reduced to aldehydes, and then decarbonylized to hydrocarbons (Blomquist 2010b; Chung & Carroll 2015). Methyl-branched hydrocarbons arise from incorporation of methylmalonyl-CoA instead of malonyl-CoA during chain elongation (Blomquist 2010b; Chung & Carroll 2015).

There are two possible ways the diet of an insect could influence its CHC profile: Firstly, the diet can contain precursors for CHC biosynthesis like fatty acids or amino acids (which are precursors for malonyl-CoA or methylmalonyl-CoA). Effects of fatty acids were shown in *Phaedon* leaf beetles (Otte *et al.* 2015) and *Drosophila melanogaster* (Pennanec'h *et al.* 1997). In *D. melanogaster* CHC changes in response to fat-enriched diet interfered with mate choice and sexual attractiveness, since CHCs serve as sex pheromones in fruit flies (Sharon *et al.* 2010; Fedina *et al.* 2012; Schultzhaus *et al.* 2017, 2018). In social insects, dietary differences can affect colony-specific CHC profiles, and thus affect nestmate recognition and intercolonial aggression, as has been shown for several ant species (Liang & Silverman 2000; Buczkowski *et al.* 2005; Buczkowski & Silverman 2006; Sorvari *et al.* 2008; Ichinose *et al.* 2009; but see Mothapo & Wossler 2016).

A second way is direct incorporation of dietary CHCs into the profile. This was found in many different insect taxa (reviewed in Otte *et al.* 2018). In ants, direct incorporation of CHCs was shown by supplementing synthetic hydrocarbons to honey or sugar solution (Guerrieri *et al.* 2009, J. Friedel & F. Menzel, unpubl. data) and by identifying CHCs derived from prey insects (Liang & Silverman 2000; Silverman & Liang 2001; Buczkowski *et al.* 2005; Vonshak *et al.* 2009). Possibly, ingested hydrocarbons might be transported from the digestive tract to the hemolymph, and from there via lipophorin to the epicuticle (Schal *et al.* 1998; Fan *et al.* 2004), or to the postpharyngeal gland and from there spread on the cuticle via grooming. However, to our knowledge the precise mechanism of CHC transport is still unknown.

To our knowledge, incorporation of dietary hydrocarbons, as well as dietary effects on the CHC profile and on nestmate recognition were only shown under artificial lab conditions with low food diversity. Hence, accumulation of certain substances from the diet could easily be tracked, and strong dietary differences in the lab led to traceable CHC changes. Thus, it is unclear how strongly diet effects on CHC profiles contribute to intercolonial CHC variation under natural conditions, with multiple different food sources available. It seems likely that multiple different food sources will blur diet-induced CHC differences among colonies. In our view, this makes dietary differences unlikely to contribute to nestmate recognition in nature.

6.2.2.2 Microbiome

As diet can also affect the gut microbiome, dietary effects on CHCs may be linked to microbial changes in many cases. The microbiome, i.e. bacterial endosymbionts or –parasites,

was in some cases shown to influence the CHC production and profile. The role of microbial symbionts on insect pheromone communication was recently reviewed in detail (Engl & Kaltenpoth 2018), which is why we will focus on few interesting studies presenting effects of the microbiome on CHC profiles specifically. In the last paragraph, we focus on how microbe-induced CHC changes could affect the behavior and ecology of ants.

An early, but very conclusive, study demonstrated a direct role of the microbiome in CHC production: Gut microorganisms in the termite *Zootermopsis nevadensis* converted radioactively labelled succinate into propionate and methylmalonate, which the termites used as precursors of two methyl-branched alkanes (Guo *et al.* 1991). Further studies were often driven by the question how microbes influence mate choice and, ultimately, reproductive isolation and speciation: In *Drosophila melanogaster*, *Lactobacillus* bacteria were shown to affect CHC profiles and, thereby, mate choice (Sharon *et al.* 2010). However, this effect could not be replicated in another *D. melanogaster* strain (Leftwich *et al.* 2017). In *Drosophila paulistorum*, *Wolbachia* is present in the oenocytes and directly or indirectly affects the male pheromone blend (Schneider *et al.* 2019). Although *Wolbachia* is known to manipulate mate choice, physiology and reproductive biology in a wide range of invertebrates (reviewed in Werren, Baldo & Clark 2008; Engelstädter & Hurst 2009), this is, to our knowledge, the only study that investigated the direct link between CHC profiles and *Wolbachia* presence.

A knock-down of the obligate endosymbiont *Wigglesworthia* in tsetse flies led to abundance changes of 15,19,23-trimethyl-heptatriacontane, which functions as contact sex pheromone, leading to changes in mate choice of both sexes (Engl *et al.* 2018b). In the grain beetle *Oryzaephilus surinamensis,* endosymbionts support the cuticle synthesis. Beetles with experimentally removed symbionts had thinner cuticles and compensated this by changes in CHC composition and an overall higher CHC production under drought stress (Engl *et al.* 2018a). This is an example for endosymbionts influencing different aspects of development and life-history, which can lead to CHC variation as a side-effect.

In ants, differences in the microbiome might also influence behavior and ecology: In *Pogonomyrmex barbatus* harvester ants, application of microbes onto the cuticle triggered aggression towards nestmates, indicating that either the microbes directly affect the CHC profile or that ants can perceive the microbes themselves, with the aggression representing a form of social immunity (Dosmann, Bahet & Gordon 2016). However, antibiotic removal of cuticular bacteria in the leaf-cutting ant *Acromyrmex subterraneus subterraneus* did not result

in CHC changes (De Souza *et al.* 2013). In *Acromyrmex echinatior*, treatment with antibiotics changed the gut microbiome, which correlated with a decrease of two *n*-alkanes, as well as two acids from metapleural gland secretions (Teseo *et al.* 2019). Although the treatment triggered aggression, there was no association between microbiome composition and chemical distances among CHC profiles, suggesting that here, the microbiome had a limited effect on the CHC profile (Teseo *et al.* 2019).

The effects of the microbiome on CHC profiles are still scarcely understood. More research is needed to determine how bacterial endosymbionts can influence CHC biosynthesis. While some bacteria might produce hydrocarbons or their precursors for their host, others might influence other aspects of their host's physiology or life-history and thus indirectly cause CHC variation.

6.2.2.3 Pathogens and parasites

Especially ground-dwelling insects are exposed to entomopathogenic fungi and bacteria. Cuticular hydrocarbons presumably function as mechanical barrier against such pathogens, especially bacteria and viruses, while some fungi can penetrate the CHC layer (Howard & Blomquist 2005; Mannino *et al.* 2019). Entomopathogenic fungi, such as *Beauveria bassiana*, degrade insect CHCs by terminally oxidizing them to alcohols using fungal cytochrome P450 monooxygenases (Pedrini, Crespo & Juárez 2007; Pedrini *et al.* 2013). Thus, infection with such fungi can directly change the CHC profiles of infected insects. However, papers reporting effects of entomopathogenic fungi on e.g. the mating behavior of insects are rare, thus the magnitude and biological impact of these changes remains unclear (Hansen & De Fine Licht 2019). One example is the cockroach *Blatta orientalis*, which produced higher quantities of hydrocarbons and other surface compounds after exposure to a fungal pathogen (Paszkiewicz *et al.* 2016). In social insects, pathogen-induced CHC changes ('sickness cues') can have signaling function: *Lasius neglectus* pupae infected with the fungus *Metarhizium brunneum* had an aberrant CHC profile, which triggered hygienic behaviors in the tending workers (Pull *et al.* 2018). In honeybees, individuals infected with the pathogens *Nosema apis* and *N. ceranae* possessed an altered *n*-alkane profile, but this did not trigger any behavioral responses in their nestmates (Murray *et al.* 2016). Thus, CHC changes upon infection with entomopathogenic fungi can be 1) adaptive for the host if they impede the infection or signal the disease to nestmates, 2) adaptive to the fungi, e.g. via the degradation of the CHC layer or 3) by-products, which benefit neither of them. However, inferring benefits for either side can be challenging.

The same is true for CHC changes induced by parasites. Infections by strepsipteran endoparasites altered the CHC profiles of their paper wasp hosts in the European species pair *Xenos vesparum* and *Polistes dominulus*, but also the South American *Xenos* endoparasite and its *Polistes ferreri* host (Dapporto *et al.* 2007; De Oliveira Torres *et al.* 2016). In the ant *Temnothorax nylanderi*, the CHC profile of workers infected with the tapeworm *Anomotaenia brevis* differs from their healthy nestmates, which coincides with increased care for infected workers (Trabalon *et al.* 2000; Beros, Foitzik & Menzel 2017). Workers infected by *A. brevis* usually stay inside the nest close to the brood. Interestingly, infected workers have a profile that resembles nurses (younger workers) more than foragers (older workers, Kohlmeier, Feldmeyer & Foitzik 2018), although they turned out to be even older than most foragers (Beros *et al.* 2017). This suggests that age-related CHC changes may be less important than CHC changes linked to a worker's task or position in the nest.

Also ectoparasites cause changes in their hosts' cuticular profiles as shown in honeybees infected by *Varroa* mites (Salvy *et al.* 2001; Cappa *et al.* 2016). Infected bees had higher relative abundances of methyl-branched CHCs and were treated more antagonistically by guard bees of different colonies (Cappa *et al.* 2016). Thus, such higher abundances of methyl-branched CHCs in infected workers could be advantageous for the hosts as they allow better discrimination (Cappa *et al.* 2016; Beros *et al.* 2017). In turn, the parasite might have an evolutionary interest in modifying the recognition abilities of their hosts by broadening the nestmate recognition template, thereby increasing their chance to be tolerated (Csata *et al.* 2017).

Changes in the CHC profile after being infected or parasitized could be adaptive for the host in a social context: If an individual can signal that it is infected, it could help the colony to isolate this individual and prevent the spread through the colony, or alternatively ask for more care by its nestmates. In this case, the parasite/pathogen should be selected to counteract CHC changes to remain unrecognized. However, such host manipulation is hard to demonstrate. Thus, CHC changes due to infection might as well be physiological side-effects that do not benefit either side.

6.3 Intrinsic variation within social insect colonies

A eusocial insect colony can only function if groups of individuals can be recognized as different. For example, reproductive division of labor requires that all colony members can recognize which individuals are allowed to reproduce and which are not. This information is

usually encoded in the CHC profile. To understand how eusocial insect colonies function, it is hence crucial to understand intra-colonial CHC variation. This section focuses on intrinsic variation, which is related to the individual's physiology and caste membership. Here, differences are less likely caused by genetic (allelic) differences since colony members are more or less closely related, but rather by differences in gene expression or further epigenetic effects. In many cases, as outlined below, this differentiation is adaptive and necessary to communicate information about its bearer. In contrast to other selection pressures, however, here in most cases it seems to matter only that individuals differ, but not necessarily how they differ. It is important to note that intra-colonial variation (e.g. between queens and workers, Fig. 6.3) is much smaller than differences among species (Fig. 6.4) (Brunner *et al.* 2011; Menzel *et al.* 2018).

Probably the best-studied CHC differentiation within ant colonies is the difference between queens and workers, i.e. the queen signal. The queen signal informs the workers about her presence, and inhibits worker reproduction (Keller & Nonacs 1993). In most ants, queen pheromones are encoded in the CHC profile (Kocher & Grozinger 2011; Van Oystaeyen *et al.* 2014). They can be quantitative (concerning ratios of certain compounds; Van Oystaeyen *et al.* 2014: supplement) or qualitative (certain CHCs only present in queens; Liebig 2010). In several cases, also non-hydrocarbon compounds were found to function as queen or fertility signal. This includes ants like *Solenopsis* and *Odontomachus*, termites, bumblebees and honeybees (reviewed in Smith, Millar & Suarez 2016; Eliyahu *et al.* 2011; Kocher & Grozinger 2011). It remains open, and hard to judge, whether the prominence of hydrocarbons as queen signals reflects a biological reality or a research bias. Possibly, CHC-based queen signals are additionally enhanced by non-hydrocarbon compounds at least in some species.

Queen-worker differences are often species-specific (Brunner *et al.* 2011; Leonhardt *et al.* 2016; Smith *et al.* 2016), which suggests contingent evolutionary trajectories. They may have evolved from signals of fertility or mating status (Leonhardt *et al.* 2016), which might originally have been by-product CHC changes that came along with physiological (e.g. hormonal) changes due to mating or ovary development. Indeed, CHCs can vary with ovary development (Foitzik *et al.* 2011), fertility (Monnin 2006; Will *et al.* 2012), or mating status (Johnson & Gibbs 2004; Oppelt & Heinze 2009), which makes such a trajectory plausible. Moreover, reproductive and sterile workers can possess different CHC profiles (Liebig *et al.* 2000; Cuvillier-Hot *et al.* 2001; Dietemann *et al.* 2003; Van Oystaeyen *et al.* 2014). Signals of mating status have also been shown for solitary insects like *Drosophila* (Everaerts *et al.* 2010).

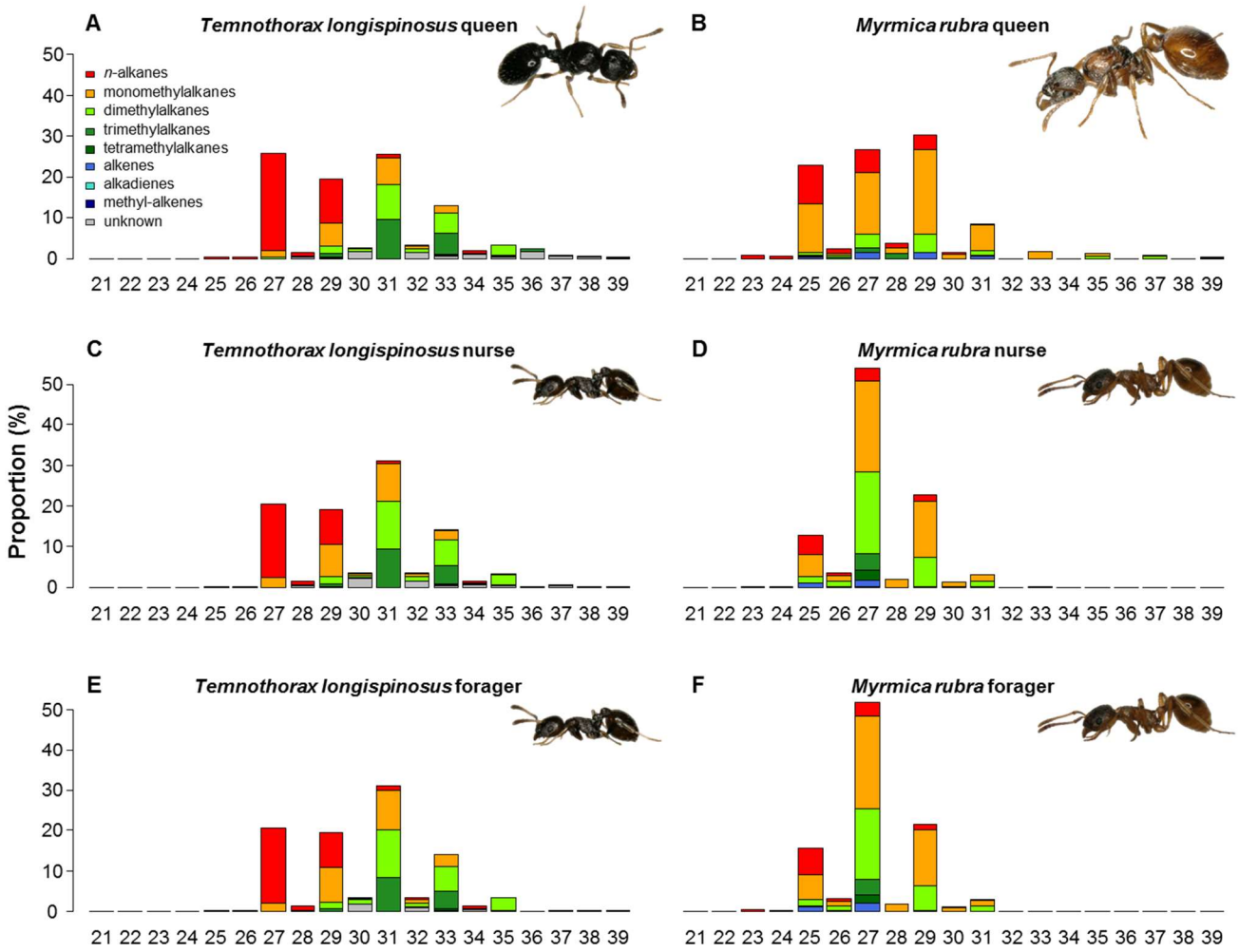

Figure 6.3: Cuticular hydrocarbon caste differences in the two ants *Temnothorax longispinosus* and *Myrmica rubra*. Barplots of CHC profiles of fertile egg-laying queens, nurses and foragers (from top to bottom) for *Temnothorax longispinosus* (A, C, E) and *Myrmica rubra* (B, D, F) are shown. While the profiles of queens are clearly distinct, the differences between the behavioral castes are minute and barely visible. Photos of the ants were taken by Philipp Sprenger.

Using bioassays, Holman and colleagues identified a queen signal (3-MeC31) that is highly abundant in queens and queen-laid eggs, reduces ovarian activity and aggressive behavior in workers (Holman *et al.* 2010; Holman, Hanley & Millar 2016). Being downregulated upon immune challenge, it may reflect an honest signal of queen fitness (Holman *et al.* 2010) – note here that honest signals need not be costly as has been shown in theoretical models (Holman 2012). Interestingly, 3-monomethyl alkanes seem to be an evolutionarily conserved queen pheromone, and are more abundant in queens than workers across numerous *Lasius* species (Holman, Lanfear & d'Ettorre 2013a). A further study suggested that wasps, ants, and some bees all use structurally related hydrocarbons as queen signal (Van Oystaeyen *et al.* 2014), but here, in our view more bioassays are needed to empirically test their effect on worker reproduction.

To reconcile these two seemingly opposing results – species-specific vs. conserved queen signals – a recent review suggested that in species with smaller colonies, queen signals might have evolved from species-specific signals that are learned by the workers (in species with smaller colonies) to conserved signals with an innate response (in species with large colonies) (Smith & Liebig 2017). Whether queen signals actually represent an honest signal of queen dominance and fertility, or a chemical manipulation, by which workers are deterred from reproducing, has been intensely debated. However, most recent studies support the view that queen pheromones are an honest signal, and we refer to several reviews on this issue (Oi *et al.* 2015; Grüter & Keller 2016; Leonhardt *et al.* 2016; Smith & Liebig 2017).

Next to queen-worker differences, there is variation among different behavioral castes among workers. Foragers (including scouts) and nurses often have different CHC profiles, and these differences help to organize tasks within the colony. For example, *Pogonomyrmex* foragers wait for the return of scouts to the nest before they leave to harvest seeds. This behavior could be elicited by scout CHCs, but not nurse CHCs (Greene & Gordon 2003), indicating that chemical differences among worker castes have signaling function. The forager-nurse differentiation is consistent with their different environments: being exposed to the sun and outside the humid nest, a forager may experience more drought stress than a nurse. In several species, foragers indeed have more and/or longer *n*-alkanes than nurses, which can increase desiccation resistance (Wagner *et al.* 2001; Martin & Drijfhout 2009b; Pamminger *et al.* 2014; Menzel *et al.* 2018).

Age can also affect CHC profiles, although in ants this is often confounded with behavioral caste. Callows often, but not always have fewer CHCs (Ichinose & Lenoir 2009; Johnson & Sundström 2012; Teseo *et al.* 2014). Depending on the sensitivity of the analyzing device, this can produce apparent (but false) differences in composition, because at lower concentrations, small peaks will not be detected. Finally, developmental stages like larvae and pupae possess CHC profiles that differ from adults (Lok, Cupp & Blomquist 1975; Cotoneschi *et al.* 2007; Richard *et al.* 2007). Interestingly, in many social insect species, identifying brood-specific CHCs turned out difficult (reviewed in Penick & Liebig 2017). This contrasts to the clear ability of workers to identify larval CHCs (Kohlmeier *et al.* 2018), indicating that more research is needed to characterize CHC variation between life stages.

CHCs often vary with an ant's social environment. The presence of alien individuals in the nest can trigger an increase in the relative abundance of di- and trimethyl alkanes, but also higher overall CHC quantities (Beros *et al.* 2017). This might be due to the need to express

more recognition cues to facilitate nestmate recognition in the presence of non-nestmates. On the other side of the gradient, isolation of single individuals can lead to differences in overall CHC quantity (Ichinose & Lenoir 2009), but also in compositional differences (Lenoir, Cuisset & Hefetz 2001a). For example, isolated ants can carry lower proportions of methyl-branched CHCs, which are relevant for recognition, and possess more *n*-alkanes instead (Kleeberg *et al.* 2017). In isolated workers, inter-individual differences (e.g. between patrilines) may become apparent, which are otherwise concealed by the 'Gestalt' odor (Martin *et al.* 2012). However, the precise reasons for changes due to isolation are hard to uncover, since isolation means various changes - social stress, lack of CHC exchange with nestmates, queen absence, and further factors.

Another interesting, genetically based effect is that CHCs vary with the inbreeding status of an individual. Due to their unusual mating system, ants of the species *Hypoponera opacior* can be highly inbred without suffering from inbreeding depression. In this species, CHC profiles of inbred individuals are less diverse than those of more heterozygous individuals (Menzel *et al.* 2016), which might be used by colony members to assess the overall inbreeding status of a colony.

Finally, CHCs can differ among workers of different matrilines or patrilines within a colony. These differences are relevant as they could enable nepotism, i.e. workers could prefer their own kin over less related nestmates (Boomsma & d'Ettorre 2013). In some species, CHCs indeed bear information on kinship (Nehring *et al.* 2011; Helanterä & d'Ettorre 2015). In the primitively eusocial ant *Pachycondyla*, this leads to workers preferentially associating with kin (Helanterä *et al.* 2013). In contrast, no kinship information was detectable in facultatively polygynous ant species (Martin *et al.* 2009; Helanterä *et al.* 2011) although the profiles of their queens differ between monogynous and polygynous colonies (Eliyahu *et al.* 2011; Johnson & Sundström 2012). Often, differences among patrilines or matrilines are apparently too weak to allow within-colony kin recognition, and may be lost in the colony environment (Boomsma *et al.* 2003; Nehring *et al.* 2011; van Zweden *et al.* 2011; Martin *et al.* 2012). Overall, nepotism seems to be rarer than expected (Keller 1997; Leonhardt *et al.* 2016), possibly because nepotism would lead to intracolonial conflicts, such that they would be less fit and hence selected against.

Thus, cuticular hydrocarbon variation among members of the same colony is influenced by physiological (age, fertility/ovary development, mating status, isolation stress) and genetic effects. Many of these differences are likely to be species-specific and thus hard to generalize.

In some cases they might be due to pleiotropic effects, if e.g. fertility, aging, mating status, and ovarian development activate genes that also influence CHC profiles. Other effects may be consistent across species, including conserved queen signals, and forager-nurse differences, which may be due to different waterproofing requirements.

6.4 Variation among colonies

The CHC profile of social insects is usually colony-specific, and this is true for both sexes (Martin *et al.* 2008b; Oppelt *et al.* 2008). These differences are maintained in common garden experiments (van Zweden *et al.* 2009), and CHC differences among lineages are usually related to their genetic distance (Blight *et al.* 2012; Fürst, Durey & Nash 2012; Teseo *et al.* 2014; but see Frizzi *et al.* 2015). Genetically more diverse populations are also chemically more diverse, e.g. in the invasive ant *Linepithema humile* (Brandt, van Wilgenburg & Tsutsui 2009). Overall, this indicates that the majority of among-colony variation is genetically determined.

For ants, among-colony variation matters for two reasons: it allows nestmate recognition and it may be the result of local adaptation. Nestmate recognition is mediated by cuticular hydrocarbon differences among colonies (Lahav *et al.* 1999; Sturgis & Gordon 2012). The chemical distance between two opponents is usually correlated to their aggression against each other (Foitzik *et al.* 2007; Drescher *et al.* 2010; Blight *et al.* 2012; Smith *et al.* 2013), and intercolonial aggression is higher in chemically more diverse populations (Errard *et al.* 2005). Here, the *Gestalt* model assumes that within colonies, ants exchange hydrocarbons. This way, they can achieve a rather uniform colony-specific odor, which allows discrimination between nestmates (with a similar signature as oneself) and non-nestmates (with a different signature) (Crozier & Dix 1979). Hydrocarbon exchange among workers is mostly mediated by the postpharyngeal gland, where CHCs are stored, mixed and redistributed (Soroker *et al.* 1994; Soroker, Vienne & Hefetz 1995). Interestingly, this relation holds true even in non-social insects: gregarious cockroaches use CHC similarity for kin recognition (Lihoreau, Rivault & van Zweden 2016), suggesting CHC-mediated kin recognition as a potential precursor of nestmate recognition (Leonhardt *et al.* 2016). Next to adults, also pupae can carry nestmate recognition cues, which influences how they are treated by nurses (e.g. in brood retrieval rates, Pulliainen *et al.* 2018).

To allow nestmate recognition, CHC profiles must differ among colonies, but it is less relevant which CHCs differ as long as the difference is detectable. However, because aggression increases with chemical distance between the opponents, nestmate recognition

should in theory select *against* polymorphic cues, which would make nestmate recognition impossible ('Crozier's paradox', Crozier 1986). Crozier concluded that they are selected for something else. Later models suggested that cue diversity could persist in a population under disassortative mating or if colonies with rare odors have a higher fitness (because they are more effective in identifying non-nestmates; negative frequency-dependent selection, Ratnieks 1991; Holman *et al.* 2013b). Indeed, *Linepithema* ants from genetically less diverse colonies are less tolerant than those from more diverse colonies, which may select for a reduction in overall genetic (and cue) diversity (Tsutsui, Suarez & Grosberg 2003).

The need to recognize and reject non-nestmates or parasites can result in selection for character displacement in the colony signature. This was detected in *Temnothorax longispinosus*, where the level of cue variation differs among populations. Here, the presence of social parasites increases the need for efficient nestmate recognition, which results in a higher among-colony differentiation compared to populations without social parasites (Jongepier & Foitzik 2016).

Beside character displacement, CHC differences among colonies or populations (e.g. Smith *et al.* 2013) may arise from drift or from local adaptation. Drift should lead to isolation-by-distance patterns not only for genetic markers, but also for CHC profiles. Indeed, this pattern has been found in *Polistes* wasps (Dapporto, Palagi & Turillazzi 2004; Bonelli *et al.* 2014) and in parabiotic ants (Hartke, Sprenger *et al.* 2019, Chapter 1). However, in other species, CHC profiles can be remarkably stable even across large parts of their distribution range (Martin *et al.* 2008a; Guillem, Drijfhout & Martin 2016). Local adaptation of CHC profiles can concern adaptation to social parasites as described above, but also to the local climate or microclimate. However, demonstrations of local adaptations in CHC profiles are scarce because environmental conditions often change over space, such that drift and local adaptation may create similar patterns. Moreover, common garden experiments are necessary to disentangle fixed and plastic variation. Nevertheless, in *Drosophila melanogaster* local adaptation in natural populations was shown: CHC chain length showed parallel changes among clinal variation and among seasons. The clinal variation was mirrored in shifts in allele frequencies at SNPs associated with CHC chain length (Rajpurohit *et al.* 2017). Moreover, parallel changes in CHC profiles of two *Drosophila* species along a latitudinal gradient suggest local adaptation to abiotic factors (Frentiu & Chenoweth 2010).

6.5 Sex differences and sexual selection

Most studies on sex differences in CHC profiles so far were on solitary insects. Quantitative and/or qualitative sex differences were reported e.g. for crickets (Tregenza & Wedell 1997; Thomas & Simmons 2008), beetles (Steiger *et al.* 2009; Ginzel 2010 and references therein), flies (reviewed in Ferveur 2005; Ferveur & Cobb 2010), but also non-social Hymenoptera like jewel wasps (Buellesbach *et al.* 2013; Bien *et al.* 2019), mason wasps (Wurdack *et al.* 2015) or solitary bees (Ayasse, Paxton & Tengö 2001; Conrad, Stöcker & Ayasse 2017). Sexual CHC dimorphism suggests that CHCs function as sex pheromones (Thomas & Simmons 2008), and may be sexually selected (see below). In ants, surprisingly few studies deal with male CHC profiles. Here, sex differences are mostly quantitative (Antonialli-Junior *et al.* 2007; Beibl, d'Ettorre & Heinze 2007; Oppelt *et al.* 2008; Chernenko *et al.* 2012; Kleeberg *et al.* 2017). However, there are sex-specific hydrocarbons in ponerine ants of the genera *Diacamma* (Cuvillier-Hot *et al.* 2001) and *Odontomachus* (Smith *et al.* 2014, 2016). Due to the relative scarcity of studies on sexual CHC dimorphism in ants, it is difficult to draw general conclusions here.

In many solitary insects like *Drosophila*, cuticular hydrocarbons are also under sexual selection. They are important for courtship and mating (Ferveur & Cobb 2010), and female preferences for certain CHC profiles can differ among populations (Rundle *et al.* 2005). In *Drosophila* both male CHCs and female preferences can depend on social environment (Gershman, Toumishey & Rundle 2014; Gershman & Rundle 2017). Sexual selection on CHCs seems to be widespread (reviewed in Steiger & Stökl 2014). Although most studies in this regard were lab studies, it has also been shown in the field for a cricket species (Steiger *et al.* 2013). Unfortunately, little is known about sexual selection on CHC profiles in social insects, because mating flights are usually difficult to observe or manipulate. If it occurs, sexual selection on CHC profiles should lead to variation among founding queens, and hence among colonies as well. In solitary insects, CHC differences can also lead to assortative mating (Otte *et al.* 2015), which may ultimately lead to speciation (Rundle *et al.* 2009; Schwander *et al.* 2013). Although speculative, such a scenario might apply in ants as well, and there might even be selection for character displacement among newly diverged populations if hybrids have reduced fitness. Thus, it remains to be studied whether sex differences show patterns generalizable across species, and how much CHC profiles are shaped by sexual selection or character displacement among sister taxa.

6.6 CHC variation among species

As mentioned, CHC profiles are highly species-specific (Box 6.1; Fig. 6.4) and to large parts genetically heritable (Martin *et al.* 2008a; van Zweden *et al.* 2009; Guillem *et al.* 2016); which is why they can be used as taxonomic tools for species delimitation (Seppä *et al.* 2011; Kather & Martin 2012; Berville *et al.* 2013). This chemical diversity leaves many questions open: How do CHC profiles evolve, and how fast can evolutionary CHC changes happen? Why do CHC profiles diversify? And how are CHC differences linked to speciation? Firstly, CHC variation among species can be due to drift, which should be detectable as a phylogenetic signal. Secondly, CHC variation may be due to character displacement after speciation events. Finally, CHC traits can represent adaptations to different abiotic and biotic selection pressures.

6.6.1 Non-adaptive variation: phylogenetic signal and genetic drift

Whether CHC differences show a phylogenetic signal depends on the taxonomic level, but also the kind of data investigated. When considering presence or absence of homologous series, van Wilgenburg and colleagues found evidence for gradual evolution of this trait (van Wilgenburg *et al.* 2011), possibly because it reflects the availability of the biosynthetic pathways to produce certain substances. This is in stark contrast to the strong qualitative differences (especially concerning CHC class composition) between species in many pairs of closely related species, which are sympatric in at least part of their range (Elmes *et al.* 2002; Morrison & Witte 2011; Seppä *et al.* 2011; Pokorny *et al.* 2013; Sprenger *et al.* 2018; Hartke, Sprenger *et al.* 2019, Chapter 1)

. Many other quantitative traits like the proportion of specific CHC classes or average chain lengths did not show phylogenetic signal, indicating that they can evolve faster than expected under a Brownian Motion model (Menzel *et al.* 2017b), which may be reinforced by plastic variation. Qualitative traits like the presence or absence of CHC classes may or may not show a phylogenetic signal (van Wilgenburg *et al.* 2011; Menzel *et al.* 2017b). Not unexpectedly, this suggests that quantitative traits evolve faster than qualitative ones, probably because quantitative changes presumably happen via gene regulatory changes rather than via changes in gene sequence. Since most CHC classes are also found in primitive hymenopteran species, it is likely that the required biosynthetic pathways were already present in the common ancestor of ants, bees and wasps (Kather & Martin 2015). CHC

diversification in a population often coincides with speciation, suggesting that most CHC traits may evolve in a rather 'saltational' mode (Mullen *et al.* 2007; Schwander *et al.* 2013; Menzel *et al.* 2017b).

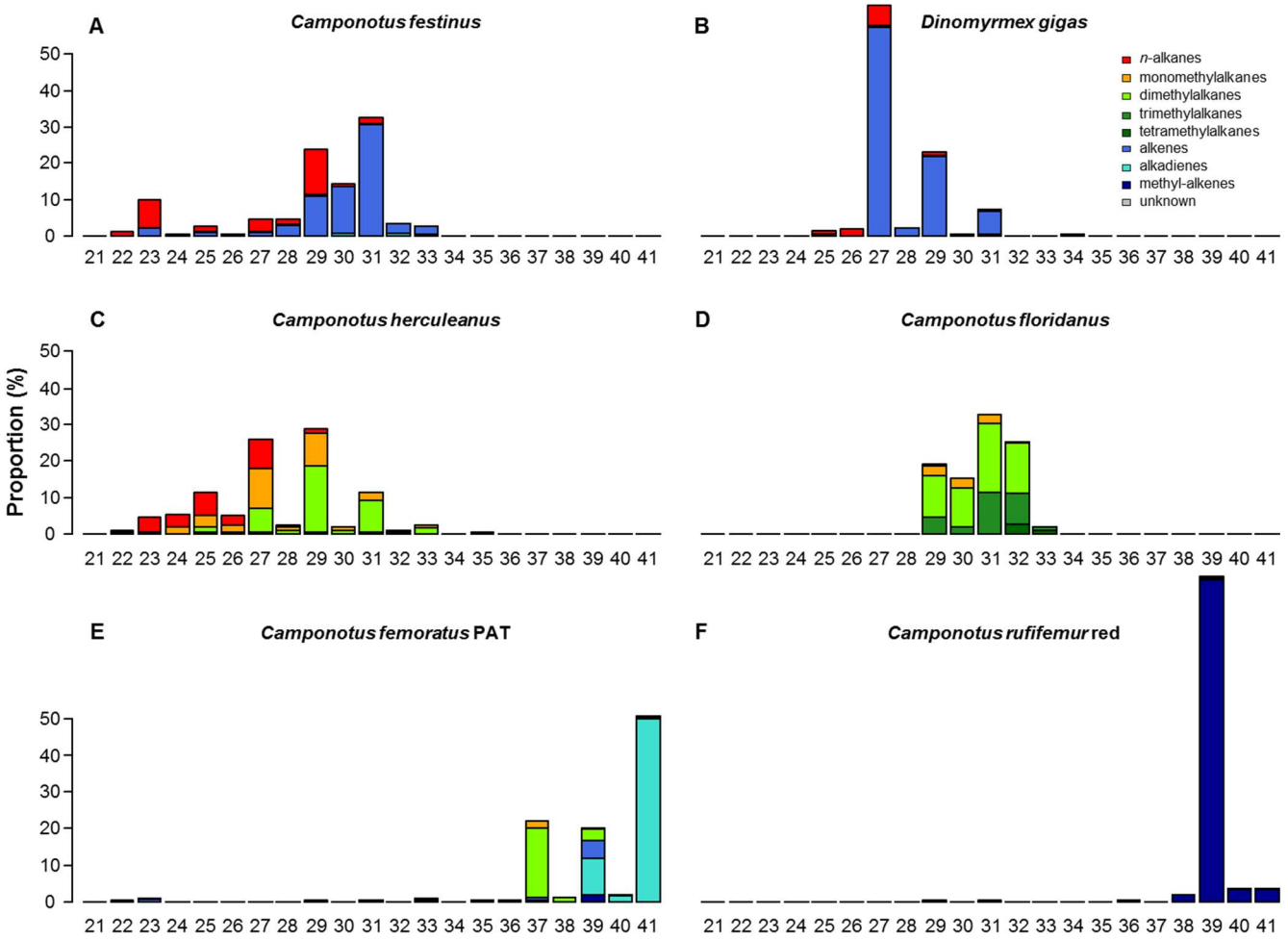

Figure 6.4: Cuticular hydrocarbon profiles of selected Camponotini species. The species were selected to exemplify the astonishing chemical variability even within a single tribe of ants. The profiles of *Camponotus festinus* (A) and *Dinomyrmex* (formerly *Camponotus*, Ward, Blaimer & Fisher 2016) *gigas* (B) are dominated by alkenes and *n*-alkanes, while *C. herculeanus* (C) and *C. floridanus* (D) possess mainly methyl-branched compounds next to *n*-alkanes. *Camponotus femoratus* PAT (E) and *C. rufifemur* red (F) represent examples of parabiotic species showing characteristic chain elongations and high abundances of alkadienes and methylbranched alkenes, which are rather unusual in non-parabiotic species. Data from Menzel *et al.* (2017a).

6.6.2 Character displacement and speciation

The coincidence between speciation events and CHC diversification is striking, but it remains unclear whether CHC divergence causes speciation via assortative mating, or whether CHC profiles change via sexual character displacement. Assortative mating

according to the CHC profile is well known from *Drosophila* (Rundle *et al.* 2009; Chung & Carroll 2015) and has also been shown in herbivorous leaf beetles (Otte *et al.* 2015) and *Nasonia* wasps (Buellesbach *et al.* 2013). Character displacement of CHCs has been observed in sympatric species pairs of *Drosophila* (Higgie, Chenoweth & Blows 2010; Dyer *et al.* 2013) and beetles (Peterson *et al.* 2007; Zhang *et al.* 2014). Due to their dual functions in desiccation resistance and (sexual) communication, ecologically driven CHC changes might lead to assortative mating and speciation (Rundle *et al.* 2005; Chung & Carroll 2015). In social insects, the observation that sympatric sister species often have strongly different CHC profiles suggests that the differentiation might at least partly be due to character displacement – either to reinforce assortative mating, or as 'ecological speciation' (Nosil 2012) if CHC differences allow partitioning of microclimatic or microhabitat niches. Since queen-worker differences are usually lower than interspecific differences, sexually selected profiles of queens should also be reflected in worker profiles.

6.6.3 Adaptive variation among species

6.6.3.1 Climate adaptation

The CHC profile, most apparently, should be adapted to the climate as the epicuticular layer prevents desiccation. However, there are only few conclusive comparisons of species differences regarding their habitats' climate. Studying presence and absence of certain substance classes only yielded limited and contradictory effects of climate adaptation on the CHC profile: While the annual mean temperature did not affect the presence of particular substance classes, alkadienes were more common in ant species living in high precipitation areas (van Wilgenburg *et al.* 2011). In a worldwide comparison of *Camponotus* and *Crematogaster* ant species, an increase in alkene proportion coincided with increasing annual precipitation in the ant's habitat, while the opposite was found for dimethyl alkanes (Menzel *et al.* 2017a). Interestingly, the proportions of other compounds like *n*-alkanes or monomethyl alkanes were not affected by precipitation, and none of the proportions were influenced by annual mean temperature. Here, more studies are necessary to corroborate climate effects on CHC composition. In particular, the adaptive value of different CHC classes remain unclear: while it seems plausible that certain CHC classes provide better waterproofing than others, the selective advantage of having apparently non-optimal waterproofing agents (alkenes or alkadienes) in wet habitats remains to be studied.

6.6.3.2 Adaptation to biotic interactions

Ants interact with many arthropods, and these interactions are often mediated by CHCs (Lenoir *et al.* 2001b; Ness, Mooney & Lach 2010, Fig. 6.5). Firstly, a multitude of 'ant guests' (myrmecophiles) exploits ant colonies. Various rove beetles (Coleoptera: Staphylinidae), caterpillars (Lepidoptera: Lycaenidae), crickets, cockroaches, springtails, spiders or mites (Witte *et al.* 2008; Parmentier, Dekoninck & Wenseleers 2014) live in ant nests. They manage to get fed by the ants, steal food from them or eat food remainders, but some of them also eat ant brood – thus, they are commensals or parasites.

Figure 6.5: Cuticular hydrocarbons play important roles in intraspecific and interspecific interactions. (A) A virgin queen of *Camponotus ligniperda* shortly after leaving its nest, Germany. CHC profiles of virgin queens differ from worker profiles, but also from those of mated queens. (B) A *Camponotus cruentatus* worker antennating an aphid, Southern France. (C) *Formica sanguinea* workers tending aphids, Northern Spain. Although these trophobiotic interactions are driven by aphid honeydew, ants recognize their aphid partner based on CHC profiles. All photos by Florian Menzel.

For these species, the most important thing is to avoid ant aggression. Many species do this by carrying similar recognition cues as their hosts, either via chemical mimicry (i.e. biosynthesis of host CHC) or chemical camouflage (i.e. active acquisition of host CHC through physical contact). Chemical mimicry is employed by socially parasitic ants as well as by non-ant myrmecophiles (Lenoir *et al.* 2001b; von Beeren, Pohl & Witte 2012b; Guillem, Drijfhout & Martin 2014). For example, *Maculinea* caterpillars (Lycaenidae) mimic the profiles of their *Myrmica* hosts (Akino *et al.* 1999; Elmes *et al.* 2002; Nash *et al.* 2008). CHC profiles of parasites can even show local adaptation to better mimic local hosts (Ruano *et al.* 2011). Camouflage, i.e. active acquisition of host cues, has been shown for various taxa, including spiders (von Beeren, Hashim & Witte 2012a), silverfish (von Beeren *et al.* 2011), and *Formicoxenus* guest ants (Lenoir *et al.* 1997). Some species, including social parasites and Lycaenid caterpillars, employ mimicry and camouflage at the same time (Akino *et al.* 1999; Bauer *et al.* 2010). While many ant guests are rather harmless, social parasites can be devastating and essentially kill a colony, and thus exert a strong selection on their hosts. The host species counteract chemical mimicry by diversifying their recognition cues, making it more difficult for parasites to mimic their profiles. Indeed, host populations where slave-making ants are present show higher CHC diversity (within and among colonies) compared to unparasitized populations (Martin, Helanterä & Drijfhout 2011; Jongepier & Foitzik 2016).

Chemical mimicry works best if parasites use only one host species. Parasites exploiting multiple hosts are chemically in between their host species. They seem to synthesize cues of each host, which selectively disappear after the adoption by the host such that only those specific to the actual host remain (Schlick-Steiner *et al.* 2004). A similar 'aggregate-odor multi-host mimicry' was found for *Temnothorax* slavemakers (Brandt *et al.* 2005; Bauer *et al.* 2010). Often, however, the parasite is less successful in populations with two host species, since the mimicry of two hosts is necessarily imperfect. Here, parasites often prefer one host species even if both are equally susceptible (Brandt & Foitzik 2004), suggesting that parasites may benefit from specialization. Host specialization, however, causes a faster arms race between parasite and favored host, resulting in negative frequency-dependent selection (Brandt & Foitzik 2004).

In the long run, however, multi-species systems should favor a second strategy to avoid recognition, which is to express generally few recognition cues (Kleeberg *et al.* 2017; von Beeren *et al.* 2018). This so-called 'chemical insignificance' was shown e.g. for *Brachymyrmex* (a lestobiotic ant), which produces only few cuticular hydrocarbons (Lenoir *et al.* 2001b). Similarly, queens of the slavemaker *Polyergus* exhibit few CHC before they enter host nests

for the first time, which may facilitate their acceptance (Lenoir *et al.* 2001b). In other species, chemical insignificance is not achieved by generally less cuticular hydrocarbons, but by providing fewer informative hydrocarbons. *n*-alkanes are generally thought to have little value for nestmate recognition. A high proportion of *n*-alkanes can thus ensure waterproofing and simultaneously expose few recognition cues. This 'chemical transparency' is employed by the social parasite *Acromyrmex insinuator* (Nehring *et al.* 2015) and evolved several times convergently among *Temnothorax* slavemakers (Kleeberg *et al.* 2017). Whether chemical mimicry or insignificance are employed can also depend on the parasite's life history, i.e. whether it lives in the host nest or only sneaks in temporarily (Uboni *et al.* 2012).

Next to parasites, also mutualists shape CHC profiles. One remarkable example are parabiotic associations, in which two ant species share a common nest in amity (Orivel *et al.* 1997; Menzel *et al.* 2008b). These associations often involve species of the ant genera *Camponotus* and *Crematogaster* (Menzel & Blüthgen 2010). Both parabiotic partners usually keep their (strongly different) species-specific CHC profile and attack non-parabiotic species, but tolerate their partner species (Menzel *et al.* 2008a; Parmentier *et al.* 2017). This unusually high tolerance is probably linked to very high chain lengths, which are characteristic for the profiles of parabiotic species, and evolved convergently in several *Camponotus* and *Crematogaster* clades (Menzel *et al.* 2017a; b). A second characteristic of parabiotic CHC profiles is their high proportions of unsaturated CHCs, which might arise from biophysical constraints and the need to ensure a partly liquid CHC layer despite high chain lengths (Menzel & Schmitt 2012; Menzel *et al.* 2014; Sprenger *et al.* 2019, Chapter 2) (Fig. 6.4 E, F). Thus, living in a parabiotic association exerts predictable selection pressures on CHC traits and represents a striking example of biotic interactions shaping CHC evolution (Menzel & Schmitt 2012; Menzel *et al.* 2017a).

6.7 Constraints on variation

Upon comparing cuticular hydrocarbon profiles among species, one notices that by far not all possible combinations of cuticular hydrocarbons are realized – thus, there are constraints on CHC variation. Firstly, most species produce only a limited range of chain lengths. Among CHC profiles of 85 *Camponotus* and *Crematogaster* species (Menzel *et al.* 2017a), three different chain lengths (e.g. C27, C28, C29) already accounted for more than 50 % of all CHCs in 49 species. In 10 species, more than 50 % of all CHCs even belonged to a single chain length. Furthermore, most CHCs have odd-numbered chain lengths – 86.3 ± 1.6 % SE in the

same dataset (F. Menzel unpubl. data). Finally, insects (not only ants) often produce homologous series of hydrocarbons over several chain lengths, such that the number of homologous series is substantially lower than the actual number of different CHCs on an insect (Martin & Drijfhout 2009a, F. Menzel unpubl. data). These observations may not be surprising, but they indicate that CHC composition in insects is constrained. These constraints probably arise from their biosynthesis. The preponderance of odd-chain CHCs stems from their origin from fatty acids, which are step-wise elongated by C2 units, until the terminal carboxyl moiety is reduced to a carbonyl moiety and then removed (Blomquist 2010b). The presence of homologous series, and at the same time a limited range of chain lengths, might originate from biosynthetic pathways if enzymes involved in CHC elongation produce a normal distribution of homologous CHC series rather than a single CHC type, e.g. if enzymes that stop chain elongation via reduction are not substrate-specific but accept substrates of different chain lengths. If this is the case, producing a homologous series of CHCs might require fewer different enzymes and thus be cheaper than producing CHCs of different homologous series. However, up to now this is speculation because the specificity of enzymes involved in CHC biosynthesis is scarcely known.

In contrast, the costs of CHC synthesis itself (given the enzymes are present) probably do not constrain CHC production or diversification. For a cockroach species, the cost of CHC production was estimated as only 0.97 % of its resting metabolic rate (Dirks & Federle 2011). Most importantly however, costs are unlikely to differ among different compounds, such that the costs of producing a methyl-branched hydrocarbon should not differ much from those of an *n*-alkane or an alkene (the more so as only one methylmalonyl-CoA is required to insert a methyl branch during chain elongation, compared to > 12 malonyl-CoA molecules for the rest of the molecule). However, a limited supply of methyl-branched amino acids (valine, isoleucine, and methionine; Blomquist 2010b) might constrain the production of methyl malonate, and hence methyl-branched hydrocarbons, such that availability of precursors rather than actual metabolic costs might be limiting. However, to our knowledge the effects of such limitation on CHC profiles have not yet been shown empirically.

The second type of constraints acting on CHC profiles are functional constraints due to their material properties. The CHC layer must be liquid to enable a homogenous coating of the CHC layer. A sufficiently low viscosity is also required for communication: recognition cues must be volatile enough to be perceived by other species (Menzel *et al.* 2019), and the need for sufficiently low viscosity accounts for several constraints found in CHC profiles. For example, the proportion of *n*-alkanes and monomethyl alkanes decreases strongly with the

average chain length of a CHC profile, while the proportion of multiply branched or unsaturated compounds is higher in profiles with high average chain length (Menzel *et al.* 2017a). This can be explained from the above considerations (see section 6.1), namely that an increase in chain length leads to higher viscosity and too great parts of the CHC layer being solid; hence insects must introduce methyl groups and/or unsaturations in these compounds to maintain the fluidity of the CHC layer. Different requirements for viscosity (e.g. in more or less flexible parts of the body), or different requirements for waterproofing may even apply *within* the same individual: Recently, two studies suggested that CHC profiles vary between body parts of the same individual (Wang *et al.* 2016a; b).

Further constraints of variation concern co-variation of substance classes. For example, alkadienes are usually confined to species with alkenes, and trimethyl alkanes only occur in species with dimethyl alkanes (Kather & Martin 2015; Menzel *et al.* 2017a), which is presumably because these CHC classes originate from the same biosynthetic pathways (respectively). Beside this positive co-variation of CHC classes, there is an interesting negative co-variation: surprisingly few species produce both dimethyl alkanes and alkenes in quantities >5% (Kather & Martin 2015; Menzel *et al.* 2017a). Up to now it remains open whether this negative covariation is due to biosynthetic or functional (biophysical) constraints.

Next to covariation among different CHCs, pleiotropic effects can cause effects of physiological changes on CHC profiles. In *Drosophila*, the expression of multiple genes that directly or indirectly influence CHC biosynthesis was often interrelated (Dembeck *et al.* 2015). The TOR pathway and insulin signaling can affect pheromone production, and hence possibly CHC profiles (Kuo *et al.* 2012; Lin *et al.* 2018). Juvenile hormone influences sex pheromones (i.e. CHCs) in *Drosophila* (Wicker & Jallon 1995) and fertility signals in honeybees (Malka, Katzav-Gozansky & Hefetz 2009). In *Lasius niger* ants, it can simultaneously affect ovarian activity, reproduction, and the CHC profile (Holman 2012). Further pleiotropic effects on CHC profiles have been found for processes related to cuticle sclerotization and melanization (Flaven-Pouchon *et al.* 2016; Massey *et al.* 2019). It seems likely that such effects are responsible for the link between CHC profile and physiological processes such as ovarian development, fertility, or aging, or the link between CHC profile and division of labor in workers (Koto *et al.* 2019) – hence, many of the CHC differences among colony members discussed above. How they constrain CHC variation still awaits further research.

6.8 Conclusions

A huge number of factors influence CHC profiles, and disentangling them is challenging. While multivariate analyses of the entire CHC composition are useful to quantify different sources of variation, they yield few insights as to *how* CHC profiles vary, and how the studied variation scales to overall variability of the profile. Here, the most promising approach in our opinion is to investigate univariate CHC traits such as proportions of different CHC classes, average chain lengths (per CHC class), and number of homologous series. In addition, analysis of variation within specific subsets of the CHC profile (like certain CHC classes) may be useful to test specific hypotheses, e.g. on the role of different CHC classes for waterproofing or communication (Martin *et al.* 2013). Depending on the research question, quantitative (based on the compound abundances) or qualitative (based on their presence/absence) traits should be chosen. This way, we can account for different biophysical properties or biosynthetic pathways of different CHCs, and formulate specific predictions. For example, certain traits, like the abundance of *n*-alkanes or their composition, should be more affected by the climate (and, via climate, by geographic location or season), while others, like the abundance of alkenes or their composition, should be less affected by climate but show a stronger signal of colony identity (Martin *et al.* 2013). Such approaches will help to identify which traits vary independently from each other, and where there is co-variation.

To understand how the fascinating diversity of insect CHCs evolved, we need to determine the adaptive value of single CHC traits for each function of a CHC layer. To get there, we also need to identify co-variation of traits and understand its biophysical or biosynthetic underpinnings. Only then can we find out how insects can modify their CHC profiles – via adaptation or acclimation – such that they fulfill their multiple functions. In our opinion, the following research areas seem particularly promising:

1) The link between physical properties and chemical composition. Here, future research can develop precise predictions how physical behavior varies with CHC composition, and this will help to elucidate the adaptive value of CHC composition and its link to an insect's ecological niche.
2) The genomic basis of CHC variation: the genes involved in CHC biosynthesis and the biosynthetic pathways necessary to synthesize an entire profile. Which genomic changes are responsible for profile differences between sister taxa? How many allelic changes are needed to cause quantitative or qualitative profile

differences? How many pathways are up- or downregulated during CHC acclimation? Here, it will be important to understand how many genes underlie the synthesis of a homologous series or an entire profile. This way, we can understand which compounds are biosynthetically linked (leading to pleiotropic effects, constraining CHC variation), and which are decoupled (Martin & Drijfhout 2009a).

3) The evolution of communication signals. Which components of a signal (e.g. fertility signals, forager-nurse differences, colony signatures) are conserved, and which are species-specific? Is colony identity encoded in ways such that the signal is less affected by acclimatory changes? Here, further research can identify the evolutionary trajectories of such differences, and link them to physiological differences.
4) The perception of CHCs: How specific are olfactory receptors and pheromone-binding proteins? To understand CHC-based communication, we also need to consider receptor variation among species, and account for potential differences in receptivity among species or among castes within a colony.
5) Which role do CHCs play in speciation? How does CHC differentiation evolve within a population, and in which cases is it followed by assortative mating? Since CHCs often function as sex pheromones, they might be drivers of speciation, and thus, the evolution of biodiversity.

6.9 Acknowledgements

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PERSPECTIVES

How and why cuticular hydrocarbons could be important in species divergence

Philipp P. Sprenger

"It is our mission to seek out life, in all forms. We are privileged to have been present at the emergence of a new species." – Jean-Luc Picard

Star Trek: The Next Generation, Season 3, Episode 25

The evolution of cuticular hydrocarbon profiles is shaped by a variety of biotic and abiotic factors, biophysical and biosynthetic constraints as well as the interplay of natural and sexual selection (Menzel *et al*. 2017a, 2019; Berson, Zuk & Simmons 2019; Chapter 6). In this thesis, I investigated the complex mutualism between the parabiotic ants *Crematogaster levior* and *Camponotus femoratus*. In the following, I will try to revisit the findings presented before, draw conclusions and discuss their impact for further research avenues that can shed light on the role of CHCs in speciation, the evolution and biosynthesis of CHC profiles, the coexistence of ecologically similar species and conflict in mutualistic interactions.

Cuticular hydrocarbons: Mediators of speciation?

In Chapter 1, I answered the question if the previously described chemical morphs (or chemotypes) of *Cr. levior* and *Ca. femoratus* in fact are different species. We convincingly demonstrate that the chemotypes in both genera consistently diverged in phenotypic traits: in their CHC profiles, in their morphology (albeit only slightly) and for *Cr. levior* also in secondary metabolites they produce. In addition, their genetic background and their gene expression patterns diverged in parallel (Chapter 3). Taken together, this strongly suggests that they indeed are different (cryptic) species according to the concept of integrative taxonomy (De Queiroz 2007; Heethoff *et al.* 2011; Steiner *et al.* 2018). However, the divergence between the species in all these traits is more difficult to explain: Although the link between chemical, genetic and morphological divergence is very clear, we cannot conclusively elucidate the role of CHCs in this process. Particularly challenging is the question if the CHC divergence itself led to speciation by mediating mate choice and enabling assortative mating or if the cryptic species were first reproductively isolated and the phenotypic divergence happened via reinforcement, i.e. if speciation is the cause for, or the consequence of the divergence in CHC profiles. A similar problem has been described in an earlier study on stick insects that found rapid CHC profile changes to be associated with speciation events and to be correlated with reproductive isolation (Schwander *et al.* 2013).

Finding out under which geographical and ecological circumstances the speciation happened would help to infer the potential role of CHCs as mediators. Divergence in the CHC profiles can have different causes and consequences under different speciation scenarios and adaptations can depend on the degree of geographical isolation during speciation. The cryptic species of both, *Cr. levior* and *Ca. femoratus*, are recently present in sympatry in large parts of our sampling range. However, we do not know their distribution during their

evolutionary history, i.e. it is unclear if the speciation happened in sympatry or if population expansions led to a secondary contact of previously allopatric species. From our current data we are far from knowing the full biogeographic distribution of the cryptic species as our sampling was confined to the Northern part of French Guiana. The parabiotic ants have also very frequently been found in Mitaraka in the South-West of French Guiana close to the Brazilian and Surinamese border (Leponce *et al.* 2019). Additionally, *Cr. levior* and *Ca. femoratus* are present in a much wider area of the neotropical rainforest, i.e. Amazonian portions of Brazil, Guianas (including Suriname), Venezuela, Colombia, Ecuador, Peru and Bolivia (Longino 2003). Examining the cryptic species identities and genotypes of the ants in other areas would give additional information on their distribution and probably their evolutionary history.

Another intriguing question is if and how coevolution between the mutualists might affect the divergence into cryptic species (either alone or in interplay with other selection pressures) or in particular if the parabiosis promoted species diversification. Most intuitively, coevolution would contribute to phenotypic divergence within populations if the interactions exert a disruptive selection pressure on the coevolving species (Schluter 2000; Yoder & Nuismer 2010; Althoff *et al.* 2014; Hembry *et al.* 2014). As mutualisms by definition provide net benefits to both interacting partners, they should however rather exert stabilizing selection, which would not lead to divergence within a population. In the system studied here, it is thus unclear how the two chemotypes might have emerged in the first place. Nevertheless, if mutualisms are seen as reciprocal exploitation arms races between the partners (Herre *et al.* 1999), there could be selection on both species to maximize their own benefit and minimize the costs from the interaction.

In the following, I will present three (not necessarily mutually exclusive) speciation scenarios in which I discuss how the phenotypic differentiation could have happened and which role the CHC profiles and the mutualistic species interaction could have played for species divergence:

Scenario 1: Allopatric speciation

In a scenario of allopatric speciation, with the populations of the cryptic species now being in secondary contact, the divergence in CHC profiles between species could be explained by two mechanisms: Either the species divergence could happen through stochastic processes such as genetic drift or mutation-order speciation (i.e. the fixation of incompatible mutations

in separate populations adapting to similar selection regimes; Schluter 2009), or through ecological speciation with local adaptations to different abiotic and/or biotic selection pressures. Adaptations could be morphological, physiological, life history and/or behavioral traits responding to factors such as local climate, food sources, partner availability or parasite pressure in isolated habitats (Reznick & Ghalambor 2001; Chapters 2, 5 and 6). For CHC profiles, it could be similar to experimental evolution experiments in *Drosophila serrata* kept on different diets without gene flow between the populations: Accumulating changes in the CHC profile could in the long term be accompanied by changes in mating preferences consistent with speciation happening as a by-product (Rundle *et al.* 2005). In *Cr. levior* A and B respectively, we indeed found signatures of population differentiation associated to the biosynthesis and perception of CHCs (Hartke *et al.*, submitted). At the same time, we found that individual and population-specific CHC profiles correlated with local climate parameters (Chapter 2; Hartke et al., submitted). The signatures of selection however were species-specific and not parallel between the cryptic species (Hartke *et al.*, submitted). Thus, differential adaptations and accompanying changes in the CHC profile to probably very similar local and climatic selection pressures might have led to mutation-order speciation. Alternatively, the different signals of selection could be a sign of genomic redundancy, i.e. mutations in different genes can lead to similarly adaptive CHC phenotypes (Hartke *et al.*, submitted).

Coevolution could be indirectly involved in allopatric speciation: The parabiotic association and the concomitant increase in competitive abilities of the mutualists against other species could have facilitated niche or range expansions in these ants (Lankau & Strauss 2008; Althoff *et al.* 2014). Parabiotic ants are often among the behaviorally dominant species in arboreal ant communities, which, next to the aggressiveness of *Ca. femoratus*, probably also is enabled by mutualistic cooperation (Dejean *et al.* 2018). Range expansions have the potential to lead to isolation between populations if a new habitat gets separated from the focal one and gene flow gets reduced. Thus, range expansions could facilitate mechanisms of allopatric speciation such as isolation-by-distance, genetic drift and local adaptation (Althoff *et al.* 2014). If one of these mechanisms applies in the parabiotic species is difficult to evaluate without having more detailed information on their biogeography. While we found species-specific signatures of local adaptation in *Cr. levior* A and B (Hartke *et al.*, submitted), we found isolation-by-distance patterns in *Ca. femoratus* PAT and PS (Chapter 1). As similarly found in yucca-yucca moth mutualisms, such biogeographic factors can be more important for the diversification of phenotypic traits than coevolution itself (Althoff *et al.* 2012).

Scenario 2: Sympatric or parapatric speciation

If subpopulations could even diverge if they occur in sympatry or parapatry, i.e. if there are no or weak barriers to gene flow, speciation would strongly depend on mechanisms such as assortative mating based on a trait that is either correlated with reproductive traits or directly influences mate choice (Dieckmann & Doebeli 1999; Althoff *et al.* 2014). As CHCs are not only important in chemical communication, but are also used to prevent water-loss, they are likely to be shaped by natural and sexual selection at the same time and thus are potential 'magic traits' (Maan & Seehausen 2011; Steiger & Stökl 2014; Chung & Carroll 2015; cf. Chapter 6). If one assumes that the CHC profiles of the cryptic species diverged upon natural selection by one or multiple biotic and/or abiotic factors (cf. Chapter 2), differential adaptive CHC changes could have altered mate choice and led to assortative mating restricting gene flow between diverging subpopulations (Rundle *et al.* 2009; Otte *et al.* 2015). If the parabiotic lifestyle or the mutualistic partner exerts a selection pressure on the CHC profiles of the ants (Chapter 2), this would additionally reflect the special situation of coevolution shaping the divergence in the CHC composition. Consequently, self-referent assortative mating based on the CHCs would potentially enable speciation even in sympatric populations if there is selection against hybrids, i.e. if hybrids are less attractive mating partners due to intermediate CHC profiles (Nosil 2012). Although it is not known if there is sexual selection on CHC profiles in ants (cf. Chapter 6), it could play a crucial role in the scenario of sympatric speciation. If a particular substance or CHC trait (e.g. the occurrence of a novel structural CHC class) is under directional selection in a subpopulation through either male or female choice, this may rapidly result in divergence in CHC profiles and character displacement between the cryptic species (Dyer *et al.* 2013; Zhang *et al.* 2014). If the CHC trait under selection would be genetically correlated to the preference for this trait, also mechanisms such as runaway selection might apply here. So far, we mostly investigated CHC profiles of workers, which makes conclusive predictions difficult. To elucidate the role of sexual selection for the divergence of the cryptic species, investigation of CHC profiles and mate choice of reproductive individuals is needed in future research.

Scenario 3: Mutualistic coevolution as main driver of speciation

Next to these classical scenarios of speciation, also multiple geographic modes of divergence could characterize a single speciation event, i.e. speciation could begin in allopatry with initial divergence in a phenotypic trait and be completed via reinforcement after secondary contact in sympatry (Schluter 2001; Nosil 2012). Also, divergence in the CHC profiles and

other traits could be mediated by a geographic mosaic of coevolution combined with the other processes described above (Thompson 2005; Nash *et al.* 2008; Althoff *et al.* 2014). In the parabiotic ants *Ca. femoratus* provides and defends the nest and in turn profits from the resource discovery abilities of *Cr. levior* (Vantaux *et al.* 2007; Orivel & Leroy 2011). These mutualism services could involve certain cost in form of competition for resources such as certain nutrients or nesting space. In line with this, I show that the parabiotic ants may compete for food sources in Chapter 5, suggesting that the parabiosis could indeed occasion costs. Depending on the ecological context (such as food availability, presence of competitors or parasites) the cost-benefit ratios might differ between certain combinations of parabiotic ants. As tentatively indicated in the recruitment to sugar in our cafeteria experiments (Fig. 5.2 B), the benefits gained from the parabiosis might differ between particular combinations of partners. If the partner identity changes the cost-benefit ratio of the association, there should be selection towards preferring a certain partner species. This would have allowed for closer coevolution and possibly cospeciation. However, in our case strict cospeciation does not seem very likely, because we did not find any signs of partner specialization in the sampled populations (Chapter 1). The diversity of ant species in the Neotropics is very high. On macroevolutionary scale several lineages diversified in this biogeographical region through factors such as the emergence of angiosperms and their association with sap-feeding hemipterans, or endosymbiotic bacteria (Moreau *et al.* 2006; Russell *et al.* 2009; Moreau & Bell 2013). Thus, it remains open if indeed the parabiotic lifestyle promotes species diversification and if this factor is as important as the species interactions mentioned above. Nevertheless, the convergent shifts towards higher CHC chain length in three of the four species could still be indicative for diffuse coevolution facilitating mutual tolerance between the interacting species (Chapter 2).

Further implications of genetic divergence and population structure

Based on a mitochondrial molecular clock estimation for insects (2.3% - 4% sequence divergence per million years; Papadopoulou, Anastasiou & Vogler, 2010; Norman *et al.* 2016), we could infer when the cryptic species in the two genera probably diverged: In *Cr. levior*, the two species were already separated by 3.79% of the mitochondrial cytochrome *c* oxidase subunit 1 (COI) gene (Chapter 1), which according to the estimation would indicate a split between 946 kya and 1.65 mya (J. Hartke, pers. comm.). In *Ca. femoratus* PAT and PS, this differentiation at the COI locus was detectable, but not very strong with only 0.39% sequence divergence (cf. Chapter 1). Accordingly, the split between *Ca. femoratus* PAT and PS would

have been very recently between 97 kya and 168 kya (J. Hartke, pers. comm.). In the nuclear loci investigated in *Ca. femoratus* PAT and PS, we found evidence for incomplete lineage sorting, which could be an additional indication for recent speciation (Chapter 1).

Having a closer look at the population structure of the parabiotic ants can be helpful to infer possible biogeographic patterns, such as isolation leading to reduced gene flow, or possible range expansions. Although some of our sampling locations were separated by several hundreds of kilometers and large rivers crossing French Guiana, we found high levels of gene flow and low population differentiation in both species of *Cr. levior* (A and B; Hartke *et al.*, submitted). Contrary to that, we found at least weak isolation-by-distance patterns in both species of *Ca. femoratus* (PAT and PS; Chapter 1). In addition, all colonies of *Ca. femoratus* PS found East of the Cayenne river had the same haplotype at the COI locus, which might indicate that they probably originate from the same range expansion event. Based on these findings on the population structure, it seems that at least in French Guiana there are only little barriers to gene flow between the populations. Thus, if the cryptic species have allopatric origins, they are probably found outside of our sampling region. More information on the dispersal abilities of the parabiotic ant species could help to evaluate population connectivity and opportunities for range expansions.

To draw more solid conclusions on the origin of the cryptic species of *Cr. levior* and *Ca. femoratus*, the mode of speciation and macroevolutionary patterns, it is necessary to get more information on their whole biogeographic distribution range, their phylogeny and evolutionary age, and their population structures. To test if coevolution played a role in trait and species diversification, one should first test if there is evidence for ongoing coevolution between the cryptic species of both genera, e.g. by testing if there are reciprocal fitness effects between the interacting species depending on the identity of the parabiotic partner (Althoff *et al.* 2014). In this line, it could be useful to thoroughly test for differences in CHC-based interspecific recognition between the cryptic *Cr. levior* and *Ca. femoratus* species. If one partner is tolerated more frequently, this could indicate coevolutionary selection by the mutualism. Furthermore, if the changes in the CHC profiles influence mate choice and thus could restrict gene flow, this could also indicate an influence of coevolution on species diversification (Althoff *et al.* 2014). In future research, this should be tested using mate choice experiments with CHC profiles of virgin queens and males of both cryptic species reciprocally presented to the other sex.

Proximate and ultimate causes for CHC profile divergence

As discussed earlier, there can be many ultimate sources of variation of CHC profiles and it can be difficult to disentangle them (cf. Chapter 6). It could be that the elongated CHC backbones only enabled the establishment of the mutualism in the first place (i.e. it could be a pre-adaptation). However, as argued in Chapter 2, the parabiotic lifestyle could also be a selection pressure promoting the elongation of CHC chains. We show that the elongated carbon backbones of CHCs are (at least in *Crematogaster*) a phylogenetic state that derived from ancestral shorter-chained CHC profiles. Interestingly, we only found this adaptation to be present in *Cr. levior* A, but not in B. Thus, it does not seem unlikely that the mutualism was already established in the last common ancestor of *Cr. levior* A and B. It seems that *Cr. levior* B ensures mutual tolerance of its *Camponotus* partner only by habituation similar to looser *Crematogaster-Camponotus* associations that do not show carbon chain elongations (Menzel *et al.* 2010). Elucidating if habituation as mechanism applies here as well would require more interspecific recognition assays testing if *Camponotus* (similarly to *Crematogaster*; Chapter 4) reacts more aggressively to *Cr. levior* B than to A and if recognition depends on the identity of the partner (i.e. if the CHC profile of the partner is learned during co-habitation). Preliminary experiments indicate only small effects of habituation and generally low aggression of the *Ca. femoratus* species towards both *Cr. levior* species (F. Menzel, unpubl. data).

In Chapter 2, we also show that the identity of the parabiotic partner and the climate have an influence on the CHC profile. The changes described in Chapter 2 could either be plastic or represent a genetically fixed adaptation. As we found only rather small effects each and plasticity seems to be high (cf. Chapters 3 and 4), the CHC changes observed are probably rather plastic. The identity of the cohabiting partner could influence the CHC profile by transferring substances either actively or passively through the nest material (i.e. chemical camouflage, cf. Chapter 6) or through active biosynthesis of some similar substances ensuring easier habituation. Investigating the mechanism how parabiotic ants partially share CHCs is challenging and would require experiments isolating the species and/or exchanging the partners to see if they transfer the CHCs or synthesize them depending on the partner that is present. Furthermore, radioactive marking of CHC precursors could be useful to confirm either biosynthesis or exchange between the species.

Similar to many other examples (Chapter 6), climate influenced the CHC profiles of the parabiotic ants (Chapter 2). Although we found climate adaptations in genes that could be

involved in CHC biosynthesis and perception in both species of *Cr. levior* (Hartke *et al.*, submitted), we cannot conclusively distinguish which of the chemical differences we found are due to genetically fixed adaptations to climate (Menzel *et al.* 2017a; Rajpurohit *et al.* 2017) or plastic acclimation responses as seen in the ants we kept under lab conditions (Menzel *et al.* 2018; Sprenger *et al.* 2018; Chapter 3). By comparing populations from a bigger geographical range, future projects could explore more patterns of local adaptation. Also, the knock-down of genes under selection might help to understand which genes indeed have an influence on the expressed CHC profiles. Furthermore, investigating acclimation to different temperature or humidity treatments could help to infer which parts of the CHC profile are plastic. It would be interesting to elucidate whether, analogous to species-specific adaptation patterns (Hartke *et al.*, submitted), each species would show species-specific CHC acclimation responses or if they would use different strategies to cope with similar climatic conditions (Sprenger *et al.* 2018; cf. Chapters 2, 3 and 6).

With analyzing gene expression differences between the cryptic species, we identified several interesting candidate genes that could be the proximate causes for the CHC differentiation (Chapter 3). An interesting aspect is that we found stronger gene expression differences in the species pair of *Ca. femoratus* which also differs stronger in the CHC profiles. If, as discussed earlier, the species divergence of *Ca. femoratus* PAT and PS was rather recent, this could be a good example for how strong gene expression differences could rapidly result in 'saltational shifts' in the CHC profile despite close relatedness of the species. Thus, expression differences in the candidate genes presented in Chapter 3 might strongly contribute to divergence in the CHC profiles and probably in last consequence to species separation as similarly found in two sister species of grasshoppers (Finck *et al.* 2016).

Although we found stronger transcriptomic differences in *Camponotus*, we also identified a multitude of differentially expressed genes between the two cryptic species of *Cr. levior*. A first approach for functional validation of a subset of these candidate genes using RNAi knock-downs resulted in some promising effects in the CHC profiles and in the behavioral responses towards treated ants (J. Hartke, P. Sprenger, N. Goß, T. Schmitt, F. Menzel & B. Feldmeyer, unpubl. data). Future studies should concentrate on the most promising genes to more closely investigate their expression and their effects on the CHC profiles. Furthermore, we now have the genome of *Cr. levior* A (Hartke *et al.* 2019) and it would be interesting to also sequence the genome of *Cr. levior* B for comparison. Interestingly, we found very high copy numbers of elongase and desaturase genes in the genome of *Cr. levior* A (Hartke *et al.* 2019), which is in line with the observed CHC profile that consists of many alkenes and

alkadienes of high chain length (Chapter 2). Investigating if *Cr. levior* B have fewer elongases, since its CHC profile is more similar to the shorter-chain ancestral state in the *Orthocrema* clade (Chapter 2), would be an interesting approach for future genome-wide comparisons.

Behavioral and ecological consequences of phenotypic divergence

In the Chapters 4 and 5 we were interested in the consequences of CHC divergence and speciation. Changes in the CHC profiles are likely to influence nestmate recognition, which is probably one of the most important functions for the ecological success of ants (van Zweden & d'Ettorre 2010; Leonhardt *et al.* 2016). The strong differentiation between cryptic species and, in particular, the highly unusual CHC composition found in parabiotic ants could thus have a large impact on intra- and interspecific recognition abilities. In Chapter 4, we focused on nestmate and non-nestmate discrimination abilities within and between *Cr. levior* A and B. Also, the divergence into separated species could be associated to segregation into different ecological niches, which is why we investigated possible climatic niche partitioning (in Chapter 1) and trophic niche partitioning (in Chapter 5). Furthermore, we also examined if the parabiotic partners of the same nest would compete for certain food sources.

Nestmate recognition

Although the conclusions we can draw from the experiments presented in Chapter 4 are limited, there was still the interesting observation that the workers of both, *Cr. levior* A and B, generally treated CHC extracts of *Cr. levior* B more aggressively than those of A. We can interpret this as an evidence supporting the hypothesis that CHC profiles with longer-chained CHCs are more difficult to perceive (Menzel & Schmitt 2012; Menzel *et al.* 2014) and that it probably requires an adapted set of odorant receptors to do so (cf. Box 6.2). Although nestmate recognition cues have been identified in several ant species (Akino *et al.* 2004; Martin *et al.* 2008c; Guerrieri *et al.* 2009; Sano *et al.* 2018), they are still likely to be species-specific (cf. Section 6.4), which makes it an interesting topic for ant species with such unusual CHC profiles like parabiotic ants. The experiments presented here may inspire additional recognition assays that could a) repeat the experiments under more standardized conditions to investigate which CHC classes might mediate nestmate recognition in the different species of *Cr. levior*, b) elucidate the role of the polar secondary metabolites found in *Cr. levior* (Chapter 1), that may, similarly to the polar substances produced by the paleotropical parabiotic ant *Cr. modiglianii*, function as an appeasement allomone towards the parabiotic partner species (Menzel *et al.* 2013) and c) reciprocally test for interspecific recognition

between the parabiotic ant species. Experiments on interspecific recognition conducted so far show that *Ca. femoratus* was unable to discriminate between *Cr. levior* A and B (Emery & Tsutsui 2013), although there was a slightly non-significant tendency of *Camponotus* workers to be more aggressive towards *Cr. levior* A if they lived together with *Cr. levior* B (F. Menzel, unpubl. data). Although Emery & Tsutsui (2013) stated that *Cr. levior* was able to differentiate between *Ca. femoratus* PAT workers from their own or a foreign nest, this observation could not be confirmed later on (Menzel *et al*. 2014) and it remains open if they can differentiate between the two cryptic species of *Ca. femoratus*. The ability of the ants to discriminate between the cryptic partner species would only enable an active choice of the parabiotic partner and thus bear the potential to influence the cost-benefit ratio. If the species would not be able to discriminate the partners, this probably could explain the apparent lack of partner specialization.

Ecological divergence of cryptic species?

We were interested to investigate if the divergence of species was accompanied by segregation into different ecological niches. To do so, we examined if there was climatic and/or trophic niche partitioning between the cryptic species. Although, we found that *Ca. femoratus* PS was nearly lacking in the Eastern part of French Guiana, all species were still sympatrically occurring in large parts of the sampling range (Chapter 1). By investigating trophic niche partitioning, we only found few and sometimes subtle differences: the cryptic species of *Ca. femoratus* differed in the proportion of mono-unsaturated fatty acids and those of *Cr. levior* in the $\delta^{13}C$ signature. Although from this we may conclude that they slightly differ in their trophic niche, one can be in doubt if this differentiation is enough to entirely avoid competition. Competition for food sources, but also mutualistic partners, nest sites, etc. between the cryptic species could also be an additional source of coevolutionary selection pressures. In future experiments, it would be interesting to investigate if there is partitioning in other niche dimensions such as life history, daily activity or (micro-)habitat choice etc., or to elucidate if the cryptic species of *Cr. levior* and *Ca. femoratus* indeed reflect an example for 'neutral species' (Hubbell 2001; McPeek 2017; cf. Chapter 5).

Costs of mutualism

In our cafeteria experiments, we investigated if the mutualistic partners (i.e. *Camponotus* and *Crematogaster*) compete for food sources and found potential indications for a discovery-dominance trade-off between *Camponotus* and *Crematogaster* (Chapter 5). Older studies that

investigated potential costs and benefits of the mutualism between *Cr. levior* and *Ca. femoratus* were not aware of the cryptic species in both genera (Swain 1980; Davidson 1988; Vantaux *et al.* 2007; Vicente *et al.* 2014; Leal *et al.* 2017). Thus, future research would need to reconfirm some of those findings under consideration of the cryptic species identities of the participating partners. Although we did not detect any partner preferences so far, the cost-benefit ratio might differ between the four possible combinations of parabiotic partners, as possibly indicated in recruitment to sugar sources in our cafeteria experiments (Fig. 5.2 B). Further studies on ecological interactions in all combinations of parabiotic partners may shed light on possible diffuse coevolution in this complex mutualistic system and allow more general conclusions about complex multi-species mutualisms and the influence of different cost-benefit ratios between partners on divergent selection and species diversification (see above).

Conclusions

In this thesis, I unraveled two pairs of cryptic species by integrative taxonomy in the neotropical parabiotic ants *Cr. levior* and *Ca. femoratus*. Using different approaches to measure genetic and phenotypic differences allowed us to gain more knowledge on species diversity in the Neotropics that are known as diversity hotspots. Also, I shed light on the complex evolution of cuticular hydrocarbon profiles and how they can be influenced by a variety of selection pressures such as biotic species interactions (i.e. parabiosis) or abiotic factors like climate. The parabiotic association seems to be a strong selection pressure facilitating the elongation of carbon backbones in the cuticular hydrocarbons and through this most likely promotes the mutual tolerance seen in these ants. Furthermore, through the identification of candidate genes involved in the biosynthesis of very different cuticular hydrocarbon profiles, we gained a deeper understanding on the molecular basis of cuticular hydrocarbon divergence between species. In future, this will allow us to investigate many processes that involve chemical communication, desiccation resistance and other functions of cuticular hydrocarbons. Finally, I show that the cryptic species in the neotropical parabiotic ants are ecologically very similar and occur in sympatry. Thus, they can be an interesting model system to study species coexistence and the avoidance of competitive exclusion. Next to the findings summarized above, this thesis also provides a fruitful starting point for follow-up studies on recent speciation events, selection pressures on cuticular hydrocarbon profiles, the transcriptomic basis of CHC biosynthesis and phenotypic divergence, and for ecological studies on mutualistic species interactions as well as cryptic species complexes.

Author contributions

Chapter 1: Juliane Hartke*, **Philipp P. Sprenger***, Jacqueline Sahm, Helena Winterberg, Jérôme Orivel, Hannes Baur, Till Beuerle, Thomas Schmitt, Barbara Feldmeyer*, Florian Menzel* (2019), "Cuticular hydrocarbons as potential mediators of cryptic species divergence in a mutualistic ant association", *Ecology and Evolution* 9: 9160-9176. (* first and last authors each contributed equally)

FM, BF and TS conceived the study. JH, PPS, JO, BF and FM collected the specimens and field data. PPS, JS, TB, TS and FM did the chemical analyses and respective data analyses. JS and HB did the morphological measurements and corresponding statistical analysis. JH, HW and BF performed sequencing and genetic analyses. JH, PPS, BF and FM wrote the first version of the manuscript, HB and TB added to the methods and results sections. All authors contributed to writing this version and approved the submission.

Chapter 2: **Philipp P. Sprenger,** Juliane Hartke, Barbara Feldmeyer, Jérôme Orivel, Thomas Schmitt, Florian Menzel (2019) "Influence of Mutualistic Lifestyle, Mutualistic Partner, and Climate on Cuticular Hydrocarbon Profiles in Parabiotic Ants", *Journal of Chemical Ecology* 45(9): 741-754.

FM, BF and TS designed the research. PPS, JH, BF, JO and FM collected the animals. PPS performed the chemical analyses and collected the data. PPS and FM analyzed the data. PPS and FM wrote the first version of the manuscript. All authors revised and approved the final manuscript.

Chapter 3: **Philipp P. Sprenger**, Juliane Hartke, Thomas Schmitt, Florian Menzel*, Barbara Feldmeyer* (submitted) "Candidate genes involved in cuticular hydrocarbon differentiation between cryptic, parabiotic ant species", *BMC Evolutionary Biology* (* last authors contributed equally)

FM, BF and TS designed the research. PPS and JH collected the specimens. PPS performed the RNA and CHC extractions. PPS and BF performed the bioinformatics analyses. PPS and FM analyzed the CHC data. PPS, FM and BF wrote the first version of the manuscript. All authors revised and approved the final manuscript.

Chapter 4: **Philipp P. Sprenger**, Freya Kristin Zäpernick, Florian Menzel: "Nestmate recognition in the cryptic species of *Crematogaster levior*"

PPS and FM designed the experiments. FKZ performed experiment 1, PPS performed experiment 2. PPS, FKZ and FM analyzed the data. PPS wrote the manuscript, FKZ and FM commented and approved the final version.

Chapter 5: **Philipp P. Sprenger***, Christian Müsse*, Juliane Hartke, Barbara Feldmeyer, Thomas Schmitt, Gerhard Gebauer, Florian Menzel (submitted) "Dinner with the roommates: Trophic niche differentiation and competition in a mutualistic ant-ant association", *Oecologia* (* authors contributed equally)

PPS, JH, BF, TS and FM designed the experiments. CM conducted the experiments in the field and the fatty acid analysis, GG performed the stable isotope analysis. PPS, CM and FM analyzed the data. PPS and FM wrote the first version of the manuscript, which was then commented and corrected by all authors.

Chapter 6: **Philipp P. Sprenger,** Florian Menzel (2020) "Cuticular hydrocarbons in ants (Hymenoptera: Formicidae) and other insects: How and why they differ among individuals, colonies and species", *Myrmecological News* 30: 1-26.

PPS and FM performed literature research, wrote and revised the review.

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Curriculum vitae

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