Project Description – Project Proposals

Florian Menzel, Mainz
Barbara Feldmeyer, Frankfurt
Thomas Schmitt, Würzburg

Project Description

1 State of the art and preliminary work

1.1 Insect cuticular hydrocarbons - diversity, functions, and sources of intraspecific variation

The cuticle of essentially all insect species is covered with a layer of hydrocarbons (henceforth, CHC). The most common hydrocarbon substance classes found on insect cuticles are *n*-alkanes, monomethyl alkanes, dimethyl alkanes, or alkenes. Further, but less common substance classes include alkadienes, methyl-branched alkenes, trimethyl and tetramethyl alkanes (Martin and Drijfhout 2009). The chain-length of these hydrocarbons usually varies between C21 and C31, with chain-lengths up to C49 in few species (Blomquist 2010b; Menzel and Schmitt 2011). Individual CHC profiles consist of a complex blend of multiple compounds. In some ant species, the CHC profile consists of up to 200 different substances, but other species (ants and others) have profiles of merely 10 different compounds (Martin and Drijfhout 2009; FM & TS unpublished data). Many of these substances, however, occur in minute quantities.

The interest of biologists in insect CHC has dramatically increased over the past two decades. The reason for this is the diversity of functions, and their high variability within and between species. Firstly, they serve as barrier against desiccation and microorganisms, and secondly as info-chemicals. CHC are an important means to transport information on its bearer, such as its species membership and its sex (Ayasse et al. 2001; Howard and Blomquist 2005; Thomas and Simmons 2008).

In social insects, CHC are vital since they transmit a multitude of additional information, such as colony membership (Lahav et al. 1999; D'Ettorre and Lenoir 2010), tasks within the colony (i.e. behavioural caste) (Pamminger et al. 2014; Wagner et al. 2001), and reproductive status (Heinze et al 2002; Endler et al. 2004; Liebig et al. 2009). This allows other individuals to use the CHC profile as cue and adapt their behaviour accordingly (Greene and Gordon 2003).

The sources of intraspecific variation in cuticular hydrocarbon profiles have been studied extensively. Intraspecific variation is largely quantitative, i.e. members of the same species usually possess the same set of hydrocarbons, but the relative quantities differ (Blomquist 2010b; van Zweden and d'Ettorre 2010). The composition of CHC profiles is largely genetically driven (van Zweden et al. 2009). However, beside the CHC variation related to their function as infochemicals, climatic conditions also affect CHC profiles (Wagner et al. 2001; FM & BF unpublished data), which probably represents an acclimation response to different anti-desiccation requirements. Further environmental impacts on CHC profiles include food (Liang and Silverman 2000; Guerrieri et al. 2009) or nest site material (Heinze et al. 1996). Such CHC changes can result in altered inter-individual aggression (Heinze et al. 1996; Silverman and Liang 2001). However, not all hydrocarbons are equally relevant for nestmate recognition. For example, alteration of *n*-alkane composition did not change inter-individual aggression levels (Guerrieri et al. 2009; Martin et al. 2008a; Martin et al. 2008b), suggesting that they play a minor role for recognition. Most information seems to be encoded in unsaturated or methyl-branched hydrocarbons (Martin et al. 2008a; Martin et al. 2008b). However, the role of single compounds is difficult to elucidate due to the high number of substances, difficulties in chemical separation and minute substance quantities (Bello et al. 2015). Usually, CHC differentiation is thought to result in aggression, however, habituation to nearby colonies can result in lower intercolonial aggression despite differences in CHC profiles.

This has been shown for artificial mixed colonies (Errard et al. 2006) but also affects natural colonies through lower aggression towards familiar profiles (dear-neighbour phenomenon, Heinze et al. 1996; Knaden and Wehner 2003; Menzel et al. 2010). However, several colony-specific factors such as its size or its nutritional status can also affect intercolonial aggression (Grover et al. 2007; Stuart 1991).

1.2 Biosynthesis of CHC and its genetic basis

Cuticular hydrocarbons originate from the fatty acid metabolism, and are produced from fatty acids through decarboxylation (Blomquist 2010a). Insects synthesize long-chain fatty acids by elongating fatty acyl-CoAs with malonyl-CoA groups. Methyl-branched hydrocarbons (except 2-methylalkanes) arise from the incorporation of a propionyl-CoA group (as methylmalonyl-CoA) in place of an acetyl-CoA group (as malonyl-CoA) at specific points during chain elongation (Blomquist 2010a). This elongation process is mediated by elongases. Double bonds are inserted by desaturases (Chertemps et al. 2006), leading to the production of alkenes and alkadienes. According to current knowledge, the most important enzyme groups in CHC biosynthesis are elongases and desaturases, both of which are highly diverse (Chertemps et al. 2007; Falcón et al. 2014; Wicker-Thomas 2011). Within social hymenoptera, acyl-CoA desaturases are frequently lost and/or duplicated, which may reflect changes in response to ecological diversification and an increased demand for chemical signal variability (Helmkampf et al. 2014).Thus, investigating the genetic architecture of CHC biosynthesis will give insights into proximate mechanisms responsible for their diversification (Niehuis et al. 2011).

1.3 CHC variation between species

The CHC profiles of different species even within a genus can show great qualitative variation concerning the number of compounds, substance classes, and their relative abundances (Martin and Drijfhout 2009; Menzel and Schmitt 2011; FM & TS unpublished data). The enormous diversity raises the question of why such a multitude of profiles evolved. Although our insights in the identity, biosynthesis, and function of CHC profiles increased drastically in the last two decades, we know little about the factors responsible for the evolution and diversification of specific traits of these profiles.

To our knowledge, only few studies investigated the selective forces that cause CHC differentiation across species. For example, climate can impose different levels of waterproofing requirements to the cuticle (Gibbs et al. 2003; Rouault et al. 2004; van Wilgenburg et al. 2011). Since CHC might function as recognition cues for the opposite sex, they should differ between closely related species in sympatry as well as between sexes of the same species. The diversification of CHCs might lead to a more precise recognition of a potential mating partner. It has been shown that speciation causes rapid changes in CHC profiles of species which supposedly use CHCs as sex pheromones to avoid hybridizations or mating attempts with a “wrong” partner (Buellesbach et al. 2013; Coyne and Orr 2004; Mullen et al. 2007). Further insights on CHC evolution derives from interspecific associations (Menzel & Schmitt 2011). Most research on interspecific interaction via CHCs so far has focused on myrmecophiles, i.e. solitary species that depend on ants for their living (Lenoir et al. 2001), or social parasites (Brandt et al. 2005; Martin et al. 2010). The most common strategies involve chemical mimicry (closely mimicking the host’s profile) or chemical insignificance (reducing the own recognition cues, Lenoir et al. 2001).

1.4 Parabiotic associations

Several parabiotically associated ant species have an unusually high intraspecific cuticular hydrocarbon diversity, which by far exceeds the quantitative variation known from other species (Emery and Tsutsui 2013; Menzel et al. 2008a; Menzel et al. 2014; Fig. 1). This makes them ideal model systems to investigate the evolution of CHC profiles of ants. Parabioses are associations between two ant species who share a common nest but keep their brood separate (Kaufmann et al. 2003). This form of association is restricted to only few species pairs world-wide. Parabiotic associations between *Camponotus* and *Crematogaster* occur in the paleotropics (*Ca. rufifemur* and *Cr. modiglianii*) and the neotropics (*Ca. femoratus* and *Cr. levior*). Both associations are mutualistic (Menzel and Blüthgen 2010; Vantaux et al. 2007) and characterised by high tolerance towards the

partner (Menzel et al. 2008b; Menzel et al. 2014). *Crematogaster* benefits from the ability of *Camponotus* to defend the nest and to provide nest space (Davidson 1988; Menzel et al. 2012; Orivel and Dejean 1999). *Camponotus* benefits from following *Crematogaster* pheromone trails too food sources (Menzel et al. 2014; Menzel et al. 2010).

1.5 Chemical, behavioral and genetic evidence for the existence of multi-species complexes in paleotropical and neotropical parabiotic species

The paleotropical, parabiotic *Camponotus rufifemur* was previously thought to be a single species. However, our CHC and genetic studies show that they consist of at least three different, cryptic species (Menzel et al. 2008a; FM unpublished data). They have strongly different cuticular hydrocarbon profiles without any compounds in common. The chemical differentiation corresponds to a behavioural differentiation: *Ca. rufifemur* workers fiercely attack workers of a different *Ca. rufifemur* species, but show significantly less aggression against non-nestmates of the own species (Menzel et al. 2008a). Even the partner *Cr. modiglianii* can differentiate the *Camponotus* species and only tolerates the one it is associated to. However, *Cr. modiglianii* workers can also habituate to previously unfamiliar *Ca. rufifemur* chemotypes, and treat them peacefully after some time of habituation (FM pers.obs.). The chemical differentiation between the three *Ca. rufifemur* chemotypes corresponds to a genetic differentiation in two mitochondrial loci (FM unpublished data). *Crematogaster modiglianii*, in contrast, does not show any chemical or genetic differentiation into chemical or genetic types.

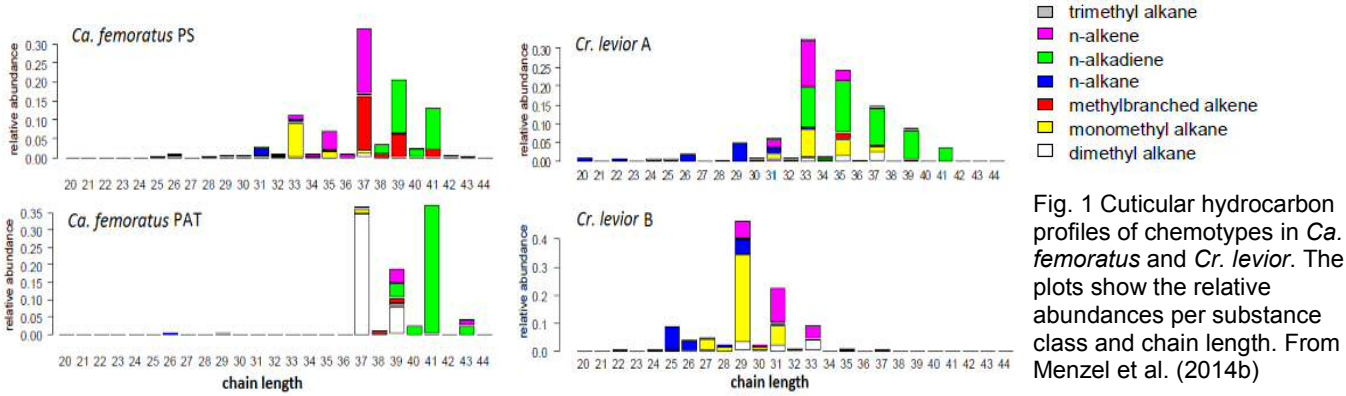

Fig. 1 Cuticular hydrocarbon profiles of chemotypes in *Ca. femoratus* and *Cr. levior*. The plots show the relative abundances per substance class and chain length. From Menzel et al. (2014b)

In the neotropics, *Crematogaster levior* lives in obligate parabiosis with *Camponotus femoratus* (Orivel et al. 1997). *Crematogaster carinata* has only been recently separated from *Cr. levior* (Longino 2003). It can live in parabiosis with *Odontomachus mayi* or nest on its own. Both *Ca. femoratus* and *Cr. levior* were originally thought to be single biological species, respectively. However, our chemical analyses suggest that *Cr. levior* and *Ca. femoratus* can be divided into two chemotypes (Fig. 1, 2; Table 1; Menzel et al. 2014).

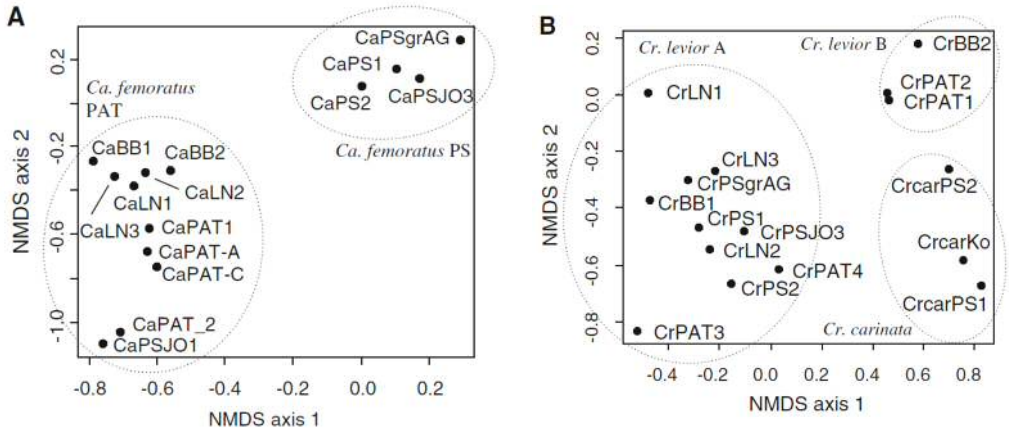

Fig. 2 Cuticular hydrocarbons of (A) *Ca. femoratus* and (B) *Cr. levior* and *carinata*. The graphs show NMDS ordinations of cuticular hydrocarbon profiles. Circles represent the different chemotypes. The colony codes indicate colony origin (PS Petit Saut, PAT Patawa, LN Les Nouragues, BB Brownsberg Nature Park, Ko Kourou). From Menzel et al. (2014b)

In *Camponotus femoratus*, one chemotype was confined to Petit Saut ('PS'), while the other one comprised samples from all four sites ('PAT', from Camp Patawa). In the PS type, methyl-branched alkenes and monomethyl alkanes were the most abundant substance classes, while dimethyl alkanes and alkadienes dominated in the PAT type. In *Crematogaster levior*, we found two sympatric chemotypes ('A' and 'B', see also Emery and Tsutsui 2013). Chemotype A had high amounts of long-chain alkenes and alkadienes (C33-C41), and colonies varied in the relative abundances of these compounds. In contrast, chemotype B was less variable and characterized by monomethyl alkanes and alkenes from C29-C33. *Crematogaster carinata* was chemically different from both *Cr. levior* types, but, similar to *Cr. levior* B, mainly possessed monomethyl alkanes and alkenes from C27-C29 (Menzel et al. 2014). The chemotypes often occur in sympatry (Fig. 4), and their nests resemble each other, suggesting similar microhabitats and microclimates. In many cases, nests of different chemotypes are spatially closer than nests with similar chemotypes. Hence, it is unlikely that the CHC differences between chemotypes are merely caused by environmental influences such as food, climate or nest material (van Zweden et al. 2009).

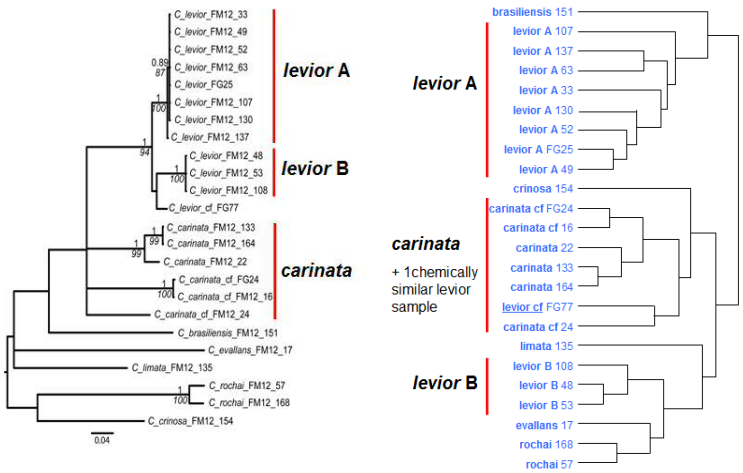

Fig. 3**:** a) (left) Preliminary phylogeny of the *Crematogaster levior* and *C. carinata* species complexes and closely related species, as estimated by Bayesian and ML analyses based on 658 bp of COI. Upper node support values are posterior probabilities, lower ones >4</number><dates><year>1988</year></dates><urls></urls></record></Cite></b) (right) b) (right) Cluster analysis of the same specimen, based on cuticular hydrocarbon profiles. Substances of the same compound group (e.g. monomethyl alkane) and chain length were pooled.

We performed genetic analyses of one nuclear and two mitochondrial loci on the two species (Fig. 3; unpublished work by Barbara Feldmeyer, Bonnie Blaimer and Florian Menzel). Our data tentatively suggest that the two different chemotypes of *Camponotus femoratus* are genetically different (based on mitochondrial DNA, COII). In *Crematogaster levior*, COI sequences similarly suggest a separation into two clades, which correspond to the two co-occurring chemotypes (*Cr. levior* A and B). Thus, our genetic data tentatively suggest sympatric speciation of *Cr. levior* in two cryptic sister species represented by the two chemotypes A and B. However, a more detailed population genetic analysis with more individuals from additional locations and more loci is necessary to determine the relation between chemotypes and genotypes. This study system is thus highly interesting from several perspectives. First, research on the two dynamic species complexes will provide new insights to speciation processes. In addition, the system is characterized by highly diverse CHC profiles within and between closely related species. Thus, it is a promising system to study evolution and divergence of CHC profiles, the genetic underpinning as well as its ecological relevance in inter- and intra-species recognition.

For the sake of simplicity, we conservatively use the term **'chemotype'** here to refer to chemically different varieties (including *Cr. carinata* as a chemotype of the *Cr. levior* complex), but we do not imply any statement about whether or not the concerning populations are reproductively isolated. The 'chemotypes' may turn out to be cryptic species, or even groups of more cryptic species (e.g. the chemically diverse chemotype *Cr. levior* A; Figs 2,3). However, we consider our knowledge about their species status to be tentative.

1.6 Project-related publications

1.6.1 Articles published by outlets with scientific quality assurance, book publications, and works accepted for publication but not yet published

Buellesbach J, Gadau J, Beukeboom LW, Echinger F, Raychoudhury R, Werren JH, **Schmitt T** (2013): Cuticular hydrocarbon divergence in the jewel wasp *Nasonia*: evolutionary shifts in chemical communication channels? ***Journal of Evolutionary Biology*** 26:2467-2478

Feldmeyer B, Elsner D, Foitzik S (2014)**:** Gene expression patterns associated with caste and reproductive status in the ant *Temnothorax longispinosus*: worker-specific genes are more derived than queen-specific ones. ***Molecular Ecology*** 23:151-161

Menzel F, Kriesell H, Witte V (2014): Parabiotic ants: the costs and benefits of symbiosis. ***Ecological Entomology*** DOI 10.1111/een.12116

Menzel F, Orivel J, Kaltenpoth M, **Schmitt T** (2014): What makes you a potential partner? Insights from convergently evolved ant-ant symbioses. ***Chemoecology*** DOI 10.1007/s00049-014-0149-2

Menzel F, Blüthgen N, Tolasch T, Conrad J, Beifuß U, Beuerle T, **Schmitt T** (2013): Crematoenones – a novel substance class exhibited by ants functions as appeasement signal. ***Frontiers in Zoology*** 10:32

Menzel F, **Schmitt T** (2011): Tolerance requires the right smell: first evidence for interspecific selection on chemical recognition cues***. Evolution*** 66-3: 896-904

Lang C, **Menzel F** (2011): *Lasius niger* ants discriminate aphids based on their cuticular hydrocarbons. ***Animal Behaviour*** 82: 1245-1254

Menzel F, Pokorny T, Blüthgen N, **Schmitt T** (2010): Trail-sharing among tropical ants: interspecific use of trail pheromones? ***Ecological Entomology*** 35: 495-503

Menzel F, Blüthgen N (2010): Parabiotic associations between tropical ants: equal partnership or parasitic exploitation? ***Journal of Animal Ecology*** 79: 71-81

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

1.6.2 Other publications

none

1.7 Patents

none (none issued, none pending)

2 Objectives and work programme

2.1 Anticipated total duration of the project

Three years

2.2 Objectives

2.2.1 Population-genetic and behavioural correlates of cuticular hydrocarbon diversification

In the proposed study, we plan to investigate genetic mechanisms and behavioural consequences of cuticular hydrocarbon diversification. The unusually high degree of chemical differentiation within *Cr. levior* and *Ca. femoratus* makes them ideal models to first investigate the relation of CHC differentiation to reproductive isolation (i.e. speciation) and its ecological consequences, i.e. its role for intra- and interspecific recognition (this chapter). Secondly, the system is well suited to study genetic mechanisms that underlie chemical diversification (2.2.2).

(i) Does the genetic population structure correspond to chemical and/or geographical differentiation? Are chemotypes reproductively isolated, or is there gene flow between them?

The *Crematogaster* population structure will be investigated using a GBS (genotyping by sequencing) approach, based on extensive collections across French Guiana and parts of Suriname. We will investigate chemical and genetic population structure to elucidate the relationship between geographic distance, chemical differentiation, genetic differentiation, and reproductive isolation. **(a) Chemical and genetic distance:** If chemotypes are reproductively isolated, we expect stabilising selection on recognition cues within the chemotype (to ensure recognition by conspecifics), but diversifying selection between chemotypes (to avoid interspecific matings). If this is the case, we expect (1) that chemical distance *within chemotypes* is low even for high genetic distance, and (2) that chemical distance *between chemotypes* is high even for relatively low genetic distance. No such differentiation is expected if we are dealing with a single, chemically diverse species. Knowledge about the degree of reproductive isolation within and between chemotypes will provide insights into how much chemical differentiation is possible without individuals being reproductively isolated. **(b) Effect of geographic distance on genetic and chemical distance:** In case of a single, chemically diverse species, we expect that genetic isolation increases with geographic distance (isolation-by-distance). The same is expected for chemical vs. geographic distance. Chemical distance can be related to geographic distance even if phylogenetic relatedness is accounted for, e.g. if geographic factors such as climate or varying food sources influence the CHC profile (Leonhardt et al. 2013). To our knowledge, however, the relation of chemical and geographic distance has only been studied in paper wasps (*Polistes*) so far (Bonelli et al. 2015), but not in any other taxa. On the other hand, if chemotypes are reproductively isolated, we expect isolation-by-distance patterns only *within* each chemotype. In contrast, no such pattern should exist across chemotypes.

(ii) Which chemical traits are relevant for intra- and interspecific recognition?

Cuticular hydrocarbon differentiation likely affects traits such as nestmate recognition and acceptance by the parabiotic partner. The degree of intercolonial aggression is often directly related to chemical differentiation (Drescher et al. 2010). Statistical analyses can identify compounds or substance classes that allow the researcher to discriminate between different colonies (D'Ettorre and Lenoir 2010), but it is largely unknown which traits of a CHC profile are actually used by the ants for this task. To our knowledge, experimental studies have only been conducted for *Formica exsecta* (Martin et al. 2008b). This species, however, is unusual in that it lacks methyl-branched alkanes, which are abundant in the vast majority of ant species (Martin and Drijfhout 2009).

We propose to investigate the relation of CHC traits and recognition on two levels. First, we will decompose the complex cuticular hydrocarbon profiles into one-dimensional traits based on substance class abundances and hydrocarbon chain length. By comparing such CHC traits to intercolonial aggression scores obtained in behavioural assays, we will pinpoint the traits related to intra- and interspecific aggression. This approach allows higher resolution than the multidimensional chemical data often used up to now. It will form the interpretational basis for experiments about the role of different substance classes for intra- and interspecific recognition. Subsequently, extracts will be fractionated into *n*-alkanes, methyl-branched alkanes, alkenes (including methyl-branched alkenes), and alkadienes for each chemotype. By comparing aggression towards unmanipulated CHC extracts and extracts where certain CHC fractions were altered, we will test whether the respective CHC fraction is perceived by the ants (Guerrieri et al. 2009). We will perform (1) intracolonial assays, where ants will be confronted with manipulated and unmanipulated extracts of nestmates, and (2) interspecific assays, where species will be confronted with extracts of their parabiotic partner. Since chemical profiles of ants consist of up to 150 different compounds, it is impossible to separate all of them in quantities large enough for bioassays, even if such an isolation can be successful for major compounds (Bello et al. 2015).

2.2.2 Genetic basis of CHC profile differentiation

The extensive chemical differences between closely related species suggest that small changes on the DNA and/or regulatory level suffice to induce large-scale changes in the CHC profile. However, little is known which, and how many genes are responsible for CHC biosynthesis, and which genes are expressed differently in different chemotypes. Using the *Crematogaster* and the *Camponotus* species complexes, we will identify differentially expressed and differentially selected genes. We

plan to conduct RNA-seq analyses to investigate gene expression differences between the three *Crematogaster* chemotypes, as well as the two *Camponotus femoratus* chemotypes, previously kept under standardized lab conditions for two weeks. Here, it is advantageous that *Cr. levior* A and B are closely related, but chemically distant. In contrast, *Cr. levior* B and *Cr. carinata* are less closely related but chemically more similar. Due to their close relatedness, overall genetic differences, as well as physiological or metabolic differences, between the three taxa are expected to be low. We thus expect that differences in expression profiles should largely be related to CHC synthesis. By analysing the transcriptome of *Ca. femoratus* chemotypes, we can take advantage of the already published genome of the congeneric *Camponotus floridanus* (Bonasio et al. 2010). Thus, reads of the sequenced RNA can be mapped against this genome, and a relatively large portion of the expressed sequences can be assigned to functional groups. Furthermore, expression patterns of elongases and desaturases can be compared between *Camponotus* and *Crematogaster*. We expect largely similar mechanisms to cause CHC differentiation in the two genera. However, *Camponotus* transcriptomes will also allow insights into the synthesis of methyl-branched alkenes. They are abundant in *Ca. femoratus*, but rare in *Cr. levior* and absent in most ant species studied to date, and may require a special biosynthetic pathway (Martin and Drijfhout 2009, Menzel et al. 2014, FM & TS unpublished data).

(i) Which genes are differentially expressed in different chemotypes?

We will focus on genes involved in CHC biosynthesis (e.g. elongases and especially desaturases, Chertemps et al. 2006, Chertemps et al. 2007, Wicker-Thomas 2011), and in olfactory receptors as candidate genes to be closely investigated (Koch et al. 2013; Zhou et al. 2012). We expect that desaturases and elongases are differentially expressed between different chemotypes. Comparing cuticular profiles, for example, desaturases should be little expressed in *Cr. levior* B or *Cr. carinata,* both of which lack alkenes, but highly expressed in *Cr. levior* A and the two *Ca. femoratus* chemotypes. In contrast, the expression of elongases and decarboxylases should be similar in all three *Crematogaster* chemotypes, but lower than in *Camponotus*. Candidate genes for both desaturases and elongases involved in CHC synthesis in the honeybee will be retrieved from Fálcon et al. (2014). Despite their diversity, elongases and desaturases both possess conserved domains (Helmkampf et al. 2014). Thus, their sequences can be identified even if referring specific gene has not yet been annotated.

(ii) Which genes are differentially selected between individuals of different chemotypes?

It is likely that CHC differences are not only due to gene expression levels, but also due to protein differences as a result of differences in the referring gene sequence. We will therefore investigate which of the transcribed genes are under selection using dN/dS ratios and the MK test (see below). We expect that CHC differences can be traced back partly to expression differences, and partly to sequence differences of the expressed CHC genes.

(iii) Does the experimental knockdown of specific enzymes alter CHC profiles and, in consequence, intercolonial aggression?

In order to verify the functional significance of the above identified candidate genes with respect to CHC synthesis as well as nestmate recognition, we will experimentally suppress expression using RNAi. We expect that knockdown of desaturases will decrease the quantities of unsaturated compounds in the CHC profile, while knockdown of elongases should result in shorter-chain CHC profiles. Both changes should strongly affect nestmate recognition, i.e. result in aggression from untreated nestmates.

2.3 Work programme incl. proposed research methods

2.3.1 Field sites & collection of samples

Two focal sites are well known to us and harbor many parabiotic colonies: The *Barrage de Petit Saut* (south of Sinnamary, ca. 2800mm annual precipitation) and *Camp Patawa* (east of Kaw, ca. 4200mm annual precipitation). In addition, further colonies will be sampled in *Kourou* (AgroParisTech campus), at the *Les Nouragues* research station and in *Brownsberg Nature Park*, Suriname. These sites are already known to us from previous sampling (Table 1).

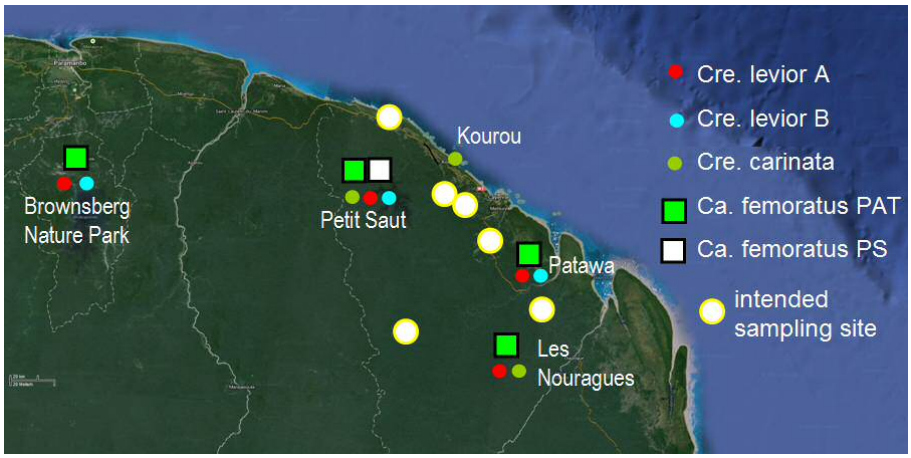

Fig. 4 Map of French Guiana and Suriname, including the five sites already sampled, and intended additional sampling sites (Table 1).

Since we aim to investigate regional chemical and genetic differentiation, we will search at further sites to look for additional chemotypes and get an overview of the geographic distribution of the chemotypes (Fig. 4). Sampling in French Guiana is mainly constrained by accessibility, since only few roads cross the forest. Therefore, we will mainly sample closer to the coast, but include an inland site reachable by plane (Saül). For *Crematogaster,* we will collect 15 replicates per site per each of 5 locations for each of the three chemotypes, i.e. *N* = 225 samples. For *Camponotus*, we will obtain at least 15 replicates per site and chemotype for each of 5 locations, i.e. 15 x 5 x 2 = 150 samples.

Table 1. Chemotypes according to sampling location of already obtained samples. All sites but Brownsberg Park are located in French Guiana.

	Cr. carinata	*Cr. levior A*	*Cr. levior B*	*Ca. femoratus PS*	*Ca. femoratus PAT*
Barrage de Petit Saut	x	x	x (1 sample)	x	x
Camp Patawa		x	x		x
Les Nouragues	x	x			x
Brownsberg Park, Suriname		x	x		x
Kourou	x				

We plan two field trips, one for sample collection and the second for behavioural and desiccation assays. The first one includes both PhD students and two PIs. Based on the samples obtained, we plan to analyse population genetics of *Cr. levior* A and B (using GBS and RAD tags), and produce transcriptomes of *Cr. levior* A, B, and *Cr. carinata*. However, we might find new chemotypes which are even better suited for our research questions. In a second field trip (one PhD student and one PI), we will do behavioural and RNAi experiments.

2.3.2 Cuticular hydrocarbon analysis

For all samples collected in the field, we will analyse the cuticular hydrocarbons using hexane extraction and a GC-MS device (Agilent) at the University of Mainz. We are experienced in the detection and identification of hydrocarbons up to a chain length of C50. To assess environmental impacts on CHC profiles such as food, nest site or climate, we will also compare CHC profiles of field colonies with those of colonies that have been kept under the same conditions in the lab for three weeks.

2.3.3 *Crematogaster* population genetics

To answer the question whether *Cr. levior* is a single species with extremely diverse chemical profiles, or whether chemotypes actually correspond to separate species, we will use a combination of mitochondrial and nuclear (SNP) markers. For *Cr. levior* A and B, we will collect 15 colonies from each of five locations, i.e. 2 x 5 x 15 = 150 individuals. DNA will be isolated using the standard protocol in our lab. COI amplification and sequencing will follow the protocol from Blaimer (2011). A GBS approach will be applied for large scale SNP discovery followed by a population genetic analysis. A combination of the restriction enzymes EcoRI and BfaI has already been used to create GBS data for a phylogenetic study including several *Crematogaster* species (J. Longino

pers. comm.). Using these two enzymes, our procedure will follow the protocol of Elshire et al. (2011). Libraries will be sequenced paired-end on an Illumina HiSeq 2000. Sequence filtering, quality trimming, filtering and individual genotyping will be conducted with Stacks (Catchen et al. 2013) and its implemented packages according to Hohenlohe et al. (2013) and Larson et al. (2014). Population genetic structure will be investigated first conducting a PCA (Jombart 2008) to assess similarity and divergence of each chemotype per population. Based on this analyses we will estimate pairwise F_{ST} values for each population using *Arlequin* (Excoffier and Lischer 2010). The same software will be used to detect loci under putative selection by F_{ST} outlier detection. This analysis will give insights into selected loci between the different populations and/or chemotypes and may thus indicate selection on the chemotypes that lead to population divergence. Since this analysis will be based on parts of the genome which are close the according enzyme restriction sites, these sequences can stem from a gene but also from intergenic regions. We will additionally include selection analyses based on the transcriptome obtained contigs which are exon-based (see below).

2.3.4 Identification of genes responsible for CHC profile differences

Sample collection: To determine the genetic basis of different chemotypes, we will sequence the transcriptomes (RNA-seq) of *Crematogaster* and *Camponotus* specimen. They will be collected at two locations (Patawa and Petit Saut) and kept under identical laboratory conditions for two weeks prior to RNA isolation to reduce environmental effects on the transcription level. We will only sample the gaster of individuals since CHCs are produced in fat bodies in the gaster. These will be grinded and stored in RNAlater (Life Technologies), which effectively conserves RNA under ambient temperatures. We will collect *Cr. carinata, Cr. levior* A and B, at Patawa and at Petit Saut (2 locations x 3 chemotypes x 3 biological replicates, total n = 18 transcriptomes). For *Camponotus*, we will collect two chemotypes from the same locations (2 locations x 2 chemotypes x 3 biological replicates, total n = 12 transcriptomes).

Transcriptome sequencing and expression analyses: Sequencing of the transcriptome, *de novo* assembly and data analysis will follow Feldmeyer et al. (2014). We will sequence ~40 Mio reads per replicate (pool of six individuals per colony) on an Illumina HiSeq 2000. Functional annotation and enrichment analyses will be conducted with Bingo, a plugin of Cytoscape (www.cytoscape.org). An additional network analysis (WGCNA package for R) will help to infer genes involved in CHC biosynthesis by means of correlative expression patterns with known CHC biosynthesis genes, such as elongases and desaturases. In a recent study on *Apis*, elongases and desaturases specifically relevant for CHC synthesis were identified (Falcón et al. 2014).

Identification of differentially selected genes: If we are dealing with different species, differentially selected CHC synthesis genes should indicate that the CHC profile is relevant for speciation. In addition to the above-mentioned F_{ST} outlier analysis, we will use the *de novo* assembled transcriptome contigs to infer selection based on dN/dS statistics (ratio of non-synonymous versus synonymous substitutions). First, we will generate orthologous gene clusters using the software OrthoMCL (Li et al. 2003). To determine whether selection acts on certain loci or sites, we will apply two different methods based on dN/dS statistics. First, we will use the CODEML from the PAML package (Yang 2007) which is a codon-based maximum likelihood method. Second, we will use the McDonald-Kreitman to detect selected loci. This tests not only investigates the ratio of non-synonymous to synonymous mutations, but also takes population polymorphisms into account (McDonald and Kreitman 1991). A locus is considered under positive selection if the ratio of fixed differences (D) to polymorphisms (P) is higher for non-synonymous ($_n$) than synonymous ($_s$) changes (i.e. $D_n/P_n > D_s/P_s$).

2.3.5 Behavioural experiments

A first set of assays with unmanipulated extracts on dummies will be combined with experimental manipulation of extracts in a second set of assays. We will only use colonies collected at least 100m apart from each other so that any bias due to inter-colony familiarity can be minimized.

2.3.5.1 Relation of CHC profile traits and intercolonial aggression

These experiments are to show which CHC traits (substance class proportions and chain length) are relevant for intra- and interspecific recognition. We will first conduct aggression experiments,

where entire extracts on dummies will be presented to nestmates and non-nestmates. The level of aggression will be analysed in respect to the difference in CHC traits (1) between the two participating colonies (intra-*Crematogaster* assays) or (2) between the tested extract and the parabiotic partner of the tested *Crematogaster* colony (inter-genus assays). This approach will allow to evaluate the relevance of CHC traits for intra- and interspecific recognition. Although tests on entire extracts are rather correlative, this test series is necessary since not all compounds can be experimentally separated (Bello et al. 2015). Moreover, other CHC profile traits such as median chain length cannot be experimentally manipulated. Apart from *n*-alkanes, the synthesis of single compounds is labour-intensive and costly, such that entire profiles are very difficult to produce artificially. However, we will fractionate cuticular extracts and separately test the behavioural effect of four different substance classes present in CHC profiles.

Intra-*Crematogaster* assays: Since we observed varying inter-colonial aggression in *Crematogaster levior* (Menzel et al. 2014b), but not in *Camponotus femoratus*, we will conduct these assays for *Cr. levior* only. Using *Cr. levior* A and B, we will assess aggression between different colonies of the same and of different chemotypes. The colonies will be kept in the lab for several weeks during the experimental assays. To determine their chemotypes, we will be able to use a GC-MS device in Cayenne (CNRS Cayenne, collaboration with Christophe Duplais).

We will test aggression in twelve *Crematogaster* colonies. Each colony will be confronted with dummies treated with extracts of its nestmates (as controls), with alien extracts of the same chemotype, and with alien extracts of a different chemotype. To avoid inter-dependence of the data, the alien colonies will be chosen such that no colony is part of more than one pairing. The high differentiation within some chemotypes, e.g. *Cr. levior* A, will increase the chemical variation and thus make it easier to detect relations between CHC trait variation and intercolonial aggression. Eight replicate assays will be performed for each treatment, resulting in a total of: 2 chemotypes x 12 colonies x (nestmate + non-nestmate A + non-nestmate B) x 8 = 576 replicates.

Inter-genus assays: Since *Camponotus* aggression towards *Crematogaster* is usually low (Menzel et al. 2014, Emery & Tsutsui 2013), we will focus on *Crematogaster* aggression towards *Camponotus*. We will use the same twelve colonies for each of *Cr. levior* A and B, and confront each with dummies treated with extracts of *Camponotus femoratus*. The *Camponotus* extracts will originate from its nestmate *Camponotus* and alien nests of *Camponotus* chemotypes PS and PAT, with eight replicates per treatment. Thus, the total number of assays will be 2 *Crematogaster* chemotypes x 12 colonies x 3 *Camponotus* colonies (nestmates, alien PS, alien PAT) x 8 replicates = 576. We expect that *Crematogaster* discriminates between the different chemotypes, and only tolerates those of their parabiotic partner (Menzel et al. 2008a; Menzel et al. 2008b).

2.3.5.2 Experimental manipulation of extracts

To assess which hydrocarbon classes affect nestmate recognition, we will use intraspecific extracts and artificially alter them by adding fractions of a specific substance class. These CHC fractions will stem from the same chemotype. In the laboratory in Würzburg, we can chemically separate saturated from mono- and di-unsaturated compounds using fractionation over $AgNO_3$-treated SiOH and elution with hexane and acetone (Bello et al. 2015). Subsequently, *n*-alkanes will be separated from methyl-branched alkanes using molecular sieves (O'Connor et al. 1962). Thus, we can create four fractions: (i) *n*-alkanes, (ii) methyl-branched alkanes, (iii) unbranched and methyl-branched alkenes, (iv) alkadienes. Based on pooled extracts, we will create these four fractions for each *Crematogaster* and *Camponotus* chemotype. The pooled extracts will be collected on our first field trip, then fractionated in Germany, and used for behavioural experiments on our second field trip (note that keeping parabiotic nests in the lab over longer time periods is not possible). We will use the same twelve x 2 *Cr. levior* colonies for the behavioural tests as above, and plan the following experimental setup:

Intra-*Crematogaster* assays: confrontations with nestmate extracts that have been manipulated by addition of four different CHC fractions, i.e. a total of 12 x 2 x 4 x 8 = 768 experiments.

Inter-genus assays: confrontations with nestmate *Camponotus* extracts, which have been altered in four different ways as above, i.e. a total of 768 experiments.

The total number of all behavioural assays is 2688, which should be doable in 3-4 months. Note, however, that this experimental plan will be open to change if we find additional chemotypes or unexpected aggression patterns in preliminary experiments.

2.4 RNAi experiments

In order to determine the function of specific elongases and desaturases in intra- and inter-species recognition, we plan to establish an RNA interference (RNAi) protocol to knock down specific candidate genes by feeding of double stranded (ds) RNA. Feeding dsRNA is a low cost, non-invasive technique, allowing high samples sizes. Previous studies successfully manipulated CHC profiles of termite queens using RNAi leading to a more worker-like CHC profile (Hoffmann et al. 2014). BF is currently involved in establishing an RNAi protocol in relation to another ant project, we thus expect to have sufficient expertise in establishing the RNAi protocol in the coming months. Candidate genes will be identified through the transcriptomic analyses described above. dsRNA will be obtained from Integrated DNA Technologies (IDT, San Diego, CA USA). Since the maintenance of large numbers of *Cr. levior* nests in the lab is not possible, we will bring only 3-4 nests to Europe to establish the RNAi protocol. We will feed ants in cohorts of ten, and four different concentrations of dsRNA over 1, 2, 4 and 6 days to establish a suitable protocol. Two controls will be performed; one will be fed with nonsense dsRNA to account for the uptake of dsRNA by itself, while the other control will receive food without additives. CHC profiles of all ants will be analysed by GC-MS as described above. Additionally, we will verify the gene specificity of our RNAi treatment via qPCR. Once the exact protocol is established, the PhD candidate will take large quantities of dsRNA to French Guiana, where he/she will perform the dsRNA feeding experiments. The experimental setup will follow as described in 2.3.5.1. We plan to conduct CHC analyses and behavioural experiments with 12 *Cr. levior* colonies of chemotype A, and B, i.e. a total of 24 colonies to test the effect of CHC change on nestmate recognition *in vivo*.

2.5 Time schedule

	2015 /4	2016 /1	2016 /2	2016 /3	2016 /4	2017 /1	2017 /2	2017 /3	2017 /4	2018 /1	2018 /2	2018 /3
Subproject A												
Collection of colonies	XX											
Preparation of extracts for behavioural experiments		XX										
Chemical analysis		XX	XX	XX								
Aggression experiments in the field								XX	XX			
Population genetics					XX	XX	XX					
Data analysis and write-up										XX	XX	XX
Subproject B												
Collection of colonies	XX											
Transcriptomic analysis		XX	XX	XX	XX	XX						
dN/dS analysis, MK test						XX						
RNAi establishment							XX	XX				
field trip for RNAi testing									XX			
Data analysis and write-up										XX	XX	XX

2.6 Data handling

All results will be published in international, peer-reviewed journals. Genetic data (sequences) will be published on GenBank. All other raw data will be published on internet databases such as dryad (www.datadryad.org).

2.7 Other information

Not available.

2.8 Descriptions of proposed investigations involving experiments on humans, human materials or animals

The planned experiments all follow the rules of the animal protection law (Tierschutzgesetz). Since French Guiana is part of the European Union, no import or export permits will be necessary.

2.9 Information on scientific and financial involvement of international cooperation partners

Jérôme Orivel (CNRS UMR, Kourou, French Guiana) agreed to function as local collaborator and will provide logistical help. He has not applied for funding specifically for this project.

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4 Requested modules/funds

4.1 Staff costs

We ask for funds to employ two doctoral researchers. PhD student 1 will mainly be based in Mainz, but spend part of the time in Würzburg for the preparation of chemical fractions. PhD student 2 will mainly be based in Frankfurt, but spend time in Mainz for the synthesis of all results. The funds requested are divided up among the three applicants as follows.

Florian Menzel

- A doctoral researcher with 65% TVL E13 salary for 24 months (the remaining 12 months will be under supervision of Thomas Schmitt).
- A doctoral researcher with 65% TVL E13 salary for 6 months (the remaining 30 months will be under supervision of Barbara Feldmeyer).

	1st year	2nd year	3rd year	total
PhD student 1	12 months 39.390€	12 months 39.390 €		78.780 €
PhD student 2			6 months 19.695€	19.695 €
Total				98.475 €

Barbara Feldmeyer

- A doctoral researcher with 65% TVL E13 salary for 30 months (the remaining 6 months will be under supervision of Florian Menzel), based at the BiK-F in Frankfurt.

	1st year	2nd year	3rd year	total
PhD student 2	12 months 39.390€	12 months 39.390€	6 months 19.695€	97.475 €

Thomas Schmitt

- A doctoral researcher with 65% TVL E13 salary for 12 months (the remaining 24 months will be under supervision of Florian Menzel).

	1st year	2nd year	3rd year	total
PhD student 1			12 months 39.390€	39.390 €

4.2 Consumables

Florian Menzel (total: 5,550 €)

Field and collecting equipment

GPS device suitable for dense forest	300 €
Forceps, bags to collect ants, exhaustors	500 €
RNA later, ethanol, hexane	450 €
GC-MS vials	600 €
Eppendorf cups	100 €
Total	**1,950 €**

Behavioural experiments

Experimental arenas	200 €
Fluon, acetone, hexane	300 €
Vials	200 €
Counters, stop watches, nest boxes, ant food	500 €
total (was 800€)	**1,200 €**

Chemical analysis

Hexane, dichloromethane	700 €
Glass pipettes	500 €
SiOH columns for purification	200 €
two GC-MS columns	1,000 €
total	**2,400 €**

Barbara Feldmeyer (total: 35,306 €)

RNA/DNA extraction

RNAlater	130 €
Trizol 100ml	310 €
RNase Away 250ml	55 €
RNAse free pipette tips (10, 200,1000µl): 151 € per set	455 €
RNA extraction 30 transcriptomes + 13 qPCR samples (Macherey-Nagel RNA extraction kit for 50 samples)	280 €
DNA extraction (1.97€) for 150 samples	300 €
total	**1530 €**

Population genetics: Genotyping by sequencing (GBS) of *Cr. levior*

for 150 individuals (5 sites x 15 colonies x 2 chemotypes)

	Package size	price (1x)	for 150 individuals	for 13 libraries of 12 individuals each
GBS wet lab				980 €
Bioanalyzer HS Chip	110	426 €		426 €
Pippin Prep Gel cassettes	40	390 €		390 €
Vulcanization		60		780 €
Sequencing	1 lane	2440 €		2440 €
total				**5016 €**

RNA-seq

We will produce a total of 30 transcriptomes (2 locations x 5 chemotypes x 3 biological replicates). The five chemotypes are *Ca. femoratus* PS and PAT*, Cr. levior* A and B, and *Cr. carinata.* The trancriptomes will be used for gene expression study on CHC genes and the development of marker genes (SNPs).

Library preparation per transcriptome		341 €
Sequencing 40Mio reads/library per transcriptome		~ 541 €
total	**30x**	**882 € = 26,460 €**

RNAi setup and qPCR validation

qPCR validation comprises two steps. First, primer and qPCR setup for 5 candidate plus 4 possible housekeeping genes and dilution series for three individuals. Second, the validation of loci specificity will be based on 5 candidate loci plus two housekeeping genes for 10 individuals)

	additional information	price
dsRNA for 5 candidate loci	~40€ per locus	200 €
Primer and qPCR establishment (9 loci x 3 indivs x 3 triplicates x 5 dilutions)	cDNA synthesis, reverse transcription and reactions	1300€
qPCR experiment for loci specificity validation (5 candidate loci x 2 housekeeping genes x 3 triplicates x 10 individuals)	cDNA synthesis, reverse transcription and reactions	800 €
total		**2.300 €**

Thomas Schmitt (total: 1,900 €)

Preparative fractionation of extracts

Hexane, dichloromethane	500 €
Molecular sieve equipment	400 €
Glass pipettes	200 €
SiOH columns for purification	300 €
one GC-MS column	500 €
total	**1,900 €**

4.2.1 Travel costs

Florian Menzel (total: 20,820 €)

Sampling trip

Participants: F. Menzel, B. Feldmeyer and 2 PhD students
FM and BF will stay 4 weeks, the 2 PhD students will stay 8 weeks.

Flight to Cayenne via Paris (1300 per person)	5,200 €
Rental car (8 weeks)	1,900 €
Fuel	300 €
Accomodation: 2 PIs for 4 weeks (40€ per day and person)	2,240 €
Accomodation: 2 PhD students for 8 weeks (40€ per day and person)	4,480 €
Transport to Les Nouragues via Helicopter	1,200 €
Transport to Saül: 150€ each for two persons	300 €
total	**15,620 €**

Participation in an international conference		**Participation in a national conference**	
Flight	1,000 €	Train	200 €
Conference fee	500 €	Conference Fee	300 €
Accomodation	300 €	Accomodation	300 €
subtotal	1,800 €	subtotal	800 €
once each for FM and PhD student 1		once each for FM and PhD student 1	
total	**3,600 €**	**total**	**1,600 €**

Barbara Feldmeyer (total: 5,200 €)

Participation in an international conference		**Participation in a national conference**	
Flight	1,000 €	Train	200 €
Conference fee	500 €	Conference Fee	300 €
Accomodation	300 €	Accomodation	300 €
subtotal	1,800 €	subtotal	800 €
once each for BF and PhD student 2		once each for BF and PhD student 2	
total	**3,600 €**	**total**	**1,600 €**

Thomas Schmitt (total: 12,640 €)

Field experiments trip

Participants: Th. Schmitt, F. Menzel, PhD student 1, PhD student 2.
TS and FM will stay 2 weeks, PhD student 1 will stay 10 weeks, PhD student 2 will stay 4 weeks.

Flight to Cayenne via Paris (1300 per person)	5,200 €
Rental car (2 weeks) (for collection)	500 €
Fuel	250 €
Accomodation at AgroParisTech, Kourou: (5€ per day and person)	
FM and TS for 2 weeks	140 €
PhD Student 1 for 10 weeks	350 €
PhD Student 2 for 4 weeks	100 €
total	**6,540 €**

Participation in an international conference		**Participation in a national conference**	
Flight	1,000 €	Train	200 €
Conference fee	500 €	Conference Fee	300 €
Accomodation	300 €	Accomodation	300 €
total (once for TS)	**1,800 €**	**total** (once for TS)	**800 €**

Project meetings

between Würzburg, Mainz, and Frankfurt every 4 months: 10 x 350€ **3500€**

4.3 Publication expenses

We wish to apply for publication funds in order to publish in open access journals, or to make our paper open-access in subscription journals. We apply for the maximal annual amount of 750€ per year, i.e. 2250€, to be equally distributed among the three applicants.

5 Project requirements

5.1 Employment status information

Florian Menzel, Dr. Akademischer Rat (A13), 31.12.2017, extension possible until 01/2020.
Barbara Feldmeyer, Dr., employed until 29.02.2016, to be tenured
Thomas Schmitt, Prof. Dr., W2, tenured

5.2 First-time proposal data

Not available.

5.3 Composition of the project group

DFG funding requested for:
- 2 PhD positions, which will be internationally advertised

Holding a position at the University of Mainz, Department of Evolutionary Biology:
- Dr. Florian Menzel, assistant professor
- Marion Kever, Stefanie Emmling, Heike Stypa, technical assistants

Holding a position at the BiK-F, Frankfurt.
- Dr. Barbara Feldmeyer, researcher

Holding a position at the University of Würzburg, Department of Animal Ecology and Tropical Biology:
- Prof. Dr. Thomas Schmitt, W2 professor
- Doris Waffler, technical assistant

5.4 Cooperation with other researchers

5.4.1 Researchers with whom you have agreed to cooperate on this project

- Dr. Jérôme Orivel (CNRS UMR, Kourou, French Guiana) has worked on ants in French Guiana for 17 years. He has ample experience on ant sampling and ant ecology in this region, will provide logistical help and function as local collaborator.
- Dr. Christophe Duplais (CNRS Cayenne, French Guiana) works on semiochemicals in insect-plant interactions. He will provide GC-MS facilities while in French Guiana, which will be necessary for us to identify the colonies suitable for experiments on our field experiments trip.

5.4.2 Researchers with whom you have collaborated scientifically within the past three years

- Prof. Dr. Volker Witte, University of München
- Prof. Dr. Nico Blüthgen, Ecological Networks, Technical University of Darmstadt
- Dr. Till Tolasch, University of Hohenheim
- Dr. Till Beuerle, Technical University of Braunschweig
- Dr. Martin Kaltenpoth, MPI for Chemical Ecology, Jena

- Prof. Dr. Markus Pfenninger, BiK-F, Frankfurt
- Prof. Dr. Ingo Ebersberger, University of Frankfurt
- Dr. Martin Plath, University of Frankfurt
- Dr. Stefan Merker, Staatliches Museum für Naturkunde, Stuttgart
- Prof. Dr. Heike Feldhaar, University of Bayreuth
- Prof. Dr. Anneli Hoikkala, University of Jyväskylä, Finland
- Dr. Oliver Niehuis, Zoologisches Forschungsmuseum König, Bonn
- Prof. Dr. Jürgen Gadau, Arizona State University, Phenix, AZ, USA
- Dr. Carlo Polidori, Museo Nacional de Ciencias Naturales, Madrid, Spain
- Prof. Dr. Joachim Ruther, University of Regensburg

5.5 Scientific equipment

A gas chromatograph-mass spectrometer (GC-MS) (Agilent Technologies, GC: Agilent 7890A; MS: Agilent 5975) and a fully equipped chemical lab is available at the department of Evolutionary Biology at the University of Mainz and at the Department of Animal Ecology and Tropical Biology at the University of Würzburg. Furthermore, there is a well-equipped genetic laboratory for RNA extraction as well as for DNA extraction and amplification available at the BiK-F in Frankfurt. Here we also have access to two high computational power clusters for the transcriptome analyses. Through the nucleic acids competence centre at the University of Mainz, we have access to an Illumina HiSeq 2000 machine as well as a fully equipped laboratory for library construction. Price calculations were based on this service unit.

5.6 Project-relevant interests in commercial enterprises

none