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

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Abstract

Insect cuticular hydrocarbons (CHCs) are highly diverse and have multiple functions, including communication and waterproofing. CHC profiles form species-specific, complex blends of up to 150 compounds. Especially in ants, even closely related species can have largely different profiles, raising the question how CHC differences are mirrored in the regulation of biosynthetic pathways. The neotropical ants Crematogaster levior and Camponotus femoratus both consist of two cryptic species each that are morphologically similar, but express strongly different CHC profiles. This is ideal to study the molecular basis of CHC differences. We thus investigated gene expression differences in fat-body transcriptomes of these ants. Despite common garden conditions, we found several thousand differentially expressed transcripts within each cryptic species pair. Many of these were related to metabolic processes, probably accounting for physiological differences. Moreover, we identified candidate genes from five gene families involved in CHC biosynthesis. By assigning candidate transcripts to orthologs in Drosophila, we inferred which CHCs might be influenced by differential gene expression. Expression of these candidate genes was often mirrored in the CHC profiles. For example, Cr. levior A, which has longer CHCs than its cryptic sister species, had a higher expression of elongases and a lower expression of fatty acyl-CoA reductases. This study is one of the first 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

Introduction

Chemical communication is widespread in insects (Symonds and Elgar 2008; Hansson and 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 (CHCs; Howard and Blomquist 2005; Blomquist and Bagnères 2010; Leonhardt et al. 2016). They are widely used as sex pheromones (Carlson et al. 1971; Steiger and Stöki 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 et al. 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 nonnestmates (Lahav et al. 1999; Greene and 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 and Rajpurohit 2010; Chung and Carroll 2015). Although the qualitative CHC composition is relatively stable across environments (Ferveur 2005; van Zweden et al. 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 CHCs are synthesized de novo in the oenocytes, which are specialized cells associated with the peripheral fat body and the epidermal layer (Billette et al. 2009; Wicker-Thomas et al. 2009; Blomquist 2010). The synthesis of CHCs is associated with 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 2010; Chung and 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; Laburé et al. 2002; Chertemps et al. 2006, Chung and Carroll 2015). Finally, fatty acyl-CoA reductases convert the acyl-CoA side chain to aldehydes that subsequently get decarbonylated 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. Several studies in Drosophila (Dallerac et al. 2000; Labbeur et al. 2002; Chertemps et al. 2006, 2007; Chung et al. 2009, 2014; Wicker-Thomas and 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; Tüpec et al. 2019). In social insects, and especially ants, CHC profiles are often very complex and can comprise hundreds of different molecules (Martin and Drijfhout 2009; Sprenger and Menzel 2020). This is reflected by high numbers of elongases (Hartke et al. 2019a) and desaturases (Helmkampf et al. 2015) found in ants compared to other insect taxa. However, to our knowledge, in ants, there are no functionally validated genes shown to be directly involved in CHC biosynthesis.

Parabiogenesis 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 and 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 et al. 2019b; Sprenger et al. 2019). 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 (Figure 1). The distribution of both cryptic 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 et al. 2019b; Sprenger et al. 2019). Cr. 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). 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).

In this study, we used these two pairs of cryptic species because they are closely related and also 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 the ones obtained in their natural habitat.

Materials and methods
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 (16 worker groups each Crematogaster and Camponotus: N = 7 Cr. levior A; N = 9 Cr. levior B; N = 8 Ca. femoratus PAT and PS; see Supplementary Table S1 and Figure 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 × 28 mm, Sanstedt AG & Co. KG, Nürenbrecht, Germany). For Camponotus, we collected 15 individuals per group (castes: minor and media) close to the nest together with some dry leaf’s 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.

Lab maintenance

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 10 pooled Crematogaster ants for CHC extraction to check if the differences between cryptic species would be stable even under similar environmental conditions.

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 5–7 min and then frozen at −80°C until RNA extraction.

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 RNasy 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 from total RNA and 100 bp paired-end sequencing was conducted at the BGI NGS Lab (Hong Kong) on an Illumina HiSeq 4000 platform. An overview of all libraries, raw and trimmed read summary statistics is provided in Supplementary Table S2.

We subsequently checked the read quality using the program FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/, last accessed 03/18/2021) together with MultiQC (Ewels et al. 2016) before and after adapter trimming with Trimmomatic (Bolger et al. 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 assemblages.

De novo transcriptome assemblages

As the main goal of this study was to compare gene expression patterns between the two closely related cryptic 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. Using this method in comparison to single-species-transcriptome-ortholog clusters, had the advantages that we could (1) obtain transcripts even for genes with low expression levels, (2) we did not need to construct ortholog clusters (which could have only very few clusters as a result), and (3) genes which are not expressed in one species but in the other will turn up as differentially expressed genes, while we could not analyze those with single-species-transcriptomes due to missing orthologs. A disadvantage of our approach however is, that DEGs might stem from similar orthologs, transcript fragments, or isoforms.

We compared three different assembly strategies using (1) Trinity (Grabherr et al. 2011), (2) CLC Main Workbench v. 7.9.1. (QIAGEN), and (3) a meta-assembly with CLC Main Workbench combined with MIRA. As CLC Main Workbench is sensitive to the number of input reads, we first generated five and four sub-assemblies, 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). Subsequently, we used MIRA (Chevreux et al. 1999) for meta-assemblies for each of the two transcriptomes (settings: job = de novo, genome, accurate, sanger). Based on TransRate (Smith-Unna et al. 2016) summary stats (Supplementary Table S4), we decided to continue with the CLC Workbench + MIRA meta-assembly. After the meta-assembly, we reincluded reads from the “debris” that were not assembled by MIRA, as those most likely represent group-specific CLC-transcripts, and removed all transcripts below 300bp length to obtain our final reference transcriptome. A BlastX (Altschul et al. 1990) search against the nonredundant arthropod protein database (NCBI, state January 2018) was used to obtain annotations for the transcripts.

Differential gene expression and functional enrichment analyses

To quantify read numbers per transcript for each sample, we used kallisto (Bray et al. 2016) with standard settings and subsequently performed the differential gene expression analysis using DESeq2 (Love et al. 2014). Here, we compared differentially expressed genes between the cryptic species of each genus separately as pairwise contrast using the Wald test. 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: (1) we searched for DEGs with BLAST annotation as one of the candidate gene families FAS, very long-chain fatty acid elongases, acyl-CoA desaturases, fatty acyl-CoA reductases, and cytochrome P450s, and (2) conducted a BlastX search to 18 genes of these gene families with known functions in the CHC biosynthesis of Drosophila (as no ant CHC genes are functionally validated so far) obtained from FlyBase (https://www.flybase.org/, last accessed 04/18/2018; Supplementary Table S5). 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.

In addition, 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 transcripts and conducted a GO enrichment analysis for biological processes (BP) in topGO (Alexa and Rahnenfuhrer 2018) using the “weight01” algorithm and Fisher test. As background, we used the full list of transcripts (for each species pair respectively), and then tested for enriched functions in the transcripts upregulated in either of the cryptic species. 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, last accessed 03/18/2021).

CHC analysis

To rule out that CHC differences observed between wild populations are not a result of phenotypic plasticity in respect to local environmental conditions, and neither due to the uptake of compounds from food or nesting material, we analyzed the CHC profiles of ants kept under identical conditions to the ants for the expression analysis. Thereby, ants used for the expression analysis as well as CHC profile experienced the same lab conditions and could be directly matched, excluding any environmental bias. For CHC extraction, the ants were freeze-killed at −20°C. CHCs were extracted by immersing the ants in chromatography-grade hexane for 10 min. The samples were stored at −20°C until the analysis using gas chromatography coupled to mass spectrometry (GC-MS). As the samples of cryptic species contained also polar secondary metabolites (Hartke et al. 2019b), the CHCs were purified using silica columns (Chromabond, SiOH 3 mL/100 mg, Macherey-Nagel, Düren, Germany). Hydrocarbon fractions were eluted with hexane. We used an Agilent 7,890 A gas chromatograph coupled to a 5,975 C 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 afterward transformed to relative proportions. For further details on the GC-MS analysis see (Sprenger et al. 2019). 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 data set (Sprenger et al. 2019). 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 data sets. 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 data sets and only investigated quantitative changes; Sprenger and Menzel 2020.

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 et al. 2008). We also included the colony ID nested in cryptic species as random effect. Subsequently, we visualized the comparison with a nonmetric 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.

Data availability

The raw reads can be accessed at NCBI; BioProject ID PRJNA540400. CHC data is available in the online supplemental material (Supplementary Tables S8 and S9). Read counts and BLAST results are available as Supplementary Table S10. All supplementary tables are available at figshare: https://doi.org/10.25387/g3.14179745.

Results

De novo transcriptome assembly

The co-assemble 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.

Differential gene expression analysis

The gene expression analyses revealed a total of 5,317 differentially expressed transcripts between the cryptic Crematogaster species (3,006 upregulated in Cr. levior A and 2,311 in B, Figure 1A), and 6,153 between the two cryptic Camponotus species (3,269 upregulated in Ca. femoratus PAT and 2,884 in PS, Figure 1B).

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 S5), 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 (Figure 2A; Supplementary Table S6A). We also identified 28 cytochrome P450-like differentially expressed transcripts (Figure 2A, Supplementary Table S6A) that were similar to Drosophila cytochrome P450 genes known to be involved in CHC biosynthesis (Supplementary Table S5).

In the two cryptic 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 (Figure 2B, Supplementary Table S6B). Looking at the genes similar to Drosophila, we identified 45 cytochrome P450-like candidate transcripts that were differentially expressed between the two cryptic species (Figure 2B, Supplementary Table S6B).

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 Figures S1 and S2; Supplementary Table S7). 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-related 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 (Figure 3A).

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 Figure S3; Supplementary Table S7). Although again most pathways were shared between the cryptic species, i.e., differentially expressed genes not necessarily have different 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 (Figure 3B).

**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 cryptic species still 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-F₁ = 23.76, P = 0.003; Figure 4A) and Ca. femoratus (pseudo-F₁ = 57.50, P = 0.023; Figure 4B). The Bray-Curtis dissimilarity between the cryptic species of Camponotus was higher than between the cryptic 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-F₁ = 7.06, P = 0.002; Ca. femoratus: pseudo-F₁ = 3.29, P = 0.029). These effects of lab maintenance were species-specific (interaction cryptic species: maintenance:

![Figure 2](image_url) 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 nonredundant 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 S5).

**Discussion**

Both of the parabiotic cryptic species of Cr. levior and Ca. femoratus are closely related but express largely different CHC profiles despite similar ecological niches (Hartke et al. 2019b; Sprenger et al. 2019). 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.

**General differences in gene expression**

In both cryptic species pairs, we found very different gene expression patterns with over 5,000 differentially expressed genes (Figure 1). This high number might not be surprising given that we compared different (cryptic) 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 cryptic 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 et al. 2019b). 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 cryptic species, and in *Ca. femoratus* PAT we found functions related to lipid metabolism. CHCs, which are lipids in the broader sense, are derived from the fatty acid biosynthetic pathway (Blomquist 2010). Therefore, the enrichment of “lipid metabolism” might account for higher number of differentially expressed CHC genes.

We identified “biosynthesis of unsaturated fatty acids” as a privately expressed pathway in *Cr. levior* A and in *Ca. femoratus* PS (Figure 3). This strikingly fits the composition of substance classes in the CHC profiles of these cryptic species. *Cr. levior* A possesses more mono- as well as di-unsaturated hydrocarbons in its CHC profile than its cryptic sister species (Hartke et al. 2019b; Sprenger et al. 2019). Similarly, *Ca. femoratus* PS has more unsaturated CHCs such as alkenes and methyl-branched alkenes than the other cryptic species, although PAT itself has proportionally more alkadienes (which is driven by several different C41 alkadienes; Hartke et al. 2019b; Sprenger et al. 2019). 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 cryptic species.

**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: FAS, very long-chain fatty acid elongases, acyl-CoA desaturases, fatty acyl-CoA reductases, and cytochrome P450s (Blomquist 2010; Chung and Carroll 2015).
FAS produce and elongate fatty acyl-CoAs (Chung and Carroll 2015). They can be distinguished in cytosolic FAS responsible for nonbranched CHCs (n-alkanes and alkenes) and microsomal FAS, that produce methylmalonyl-CoA as precursor for methyl-branched CHCs (Juárez et al. 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 and 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 cryptic Camponotus species (Sprenger et al. 2019). We found BlastX similarity for three out of six upregulated elongase-like transcripts of Cr. levior A to a putative elongase (CG9458), whose knockdown 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). 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).

During elongation, acyl-CoA desaturases insert one or more double bonds into the acyl-CoA chain (Chung and 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 desaturation-like transcripts in our four cryptic 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 neo-functionalization 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).

Fatty acyl-CoA reductases convert the acyl-CoA chain to an aldehyde (Chung and 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 cryptic Camponotus species (Sprenger et al. 2019).

The final step of the CHC biosynthesis is the conversion of aldehydes to hydrocarbons by cytochrome P450s (Qui et al. 2012; Chung and 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), which could explain this higher number of
upregulated transcripts, if cytochrome P450s are indeed specific to a subset of CHCs.

**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. These results are thus consistent with CHC profiles of the ants being heritable and species-specific (van Zweden et al. 2009; Walsh et al. 2020), instead of resulting from phenotypic plasticity induced by environmental factors in the natural habitat. 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 and 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) or their queen and subsequent changes in fertility (Liebig et al. 2000; Dietemann et al. 2003).

**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 cryptic Camponotus species pair than between the cryptic Crematogaster species. This matches the fact that the cryptic Camponotus species are also more different concerning their CHC profile, although they are more closely related than the two cryptic Crematogaster species (Hartke et al. 2019b). 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 et al. 2019b; Sprenger et al. 2019). By identifying candidate genes, we provide a basis for further studies on CHC biosynthesis and their evolution in a highly interesting model system.

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F.M., B.F., and T.S. designed the research. P.P.S. and J.H. collected the specimens. P.P.S. performed the RNA and CHC extractions. P.P.S. and B.F. performed the bioinformatics analyses. P.P.S. and F.M. analyzed the CHC data. P.P.S., F.M., and B.F. wrote the first version of the manuscript. All authors revised and approved the final manuscript.

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**Literature cited**

Alexa A, Rahnenfuhrer J. 2018. topGO: enrichment analysis for gene ontology. https://www.bioconductor.org/packages/release/bioc/html/topGO.html (Accessed: 2018 February 22).

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. J Mol Biol. 215:403–410.

Anderson MJ, Gorley RN, Clarke K. 2008. PERMANOVA+ for PRIMER: guide to software and statistical methods. Plymouth: PRIMER-E Ltd.

Berdan E, Enge S, Nylund GM, Wellenreuther M, Martens GA, et al. 2019. Genetic divergence and phenotypic plasticity contribute to variation in cuticular hydrocarbons in the seaweed fly Coelopa frigida. Ecol Evol. 9:12156–12170.

Billetter JC, Atallah J, Krupp JJ, Millar JG, Levine JD. 2009. Specialized cells tag sexual and species identity in Drosophila melanogaster. Nature 461:987–991.

Blomquist GJ. 2010. Biosynthesis of cuticular hydrocarbons. In: GJ Bagnéres, A-G Blomquist, editors. Insect Hydrocarbons: Biology, Biochemistry, and Chemical Ecology. New York, NY: Cambridge University Press. p. 35–52.

Blomquist GJ, Bagnères A-G. 2010. Introduction: history and overview of insect hydrocarbons. In: GJ Blomquist A-G, Bagnères, editors. Insect Hydrocarbons: Biology, Biochemistry, and Chemical Ecology. New York, NY: Cambridge University Press. p. 3–18.

Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30:2114–2120.

Bray NL, Pimentel H, Melsted P, Pachter L. 2016. Near-optimal probabilistic RNA-seq quantification. Nat Biotechnol. 34:525–527.

Carlson DA, Mayer MS, Sillacek DL, James JD, Beroza M, et al. 1971. Sex attractant pheromone of the house fly: isolation, identification and synthesis. Science 174:76–78.

Chertemps T, Duportets L, Labeur C, Ueda R, Takahashi K, et al. 2007. A female-biased expressed elongase involved in long-chain hydrocarbon biosynthesis and courtship behavior in Drosophila melanogaster. Proc Natl Acad Sci USA. 104:4273–4278.

Chertemps T, Duportets L, Labeur C, Ueyama M, Wicker-Thomas C. 2006. A female-specific desaturase gene responsible for diene hydrocarbon biosynthesis and courtship behavior in Drosophila melanogaster. Insect Mol Biol. 15:465–473.

Chevreux B, Wetter T, Suhai S. 1999. Genome sequence assembly using trace signals and additional sequence information. In: Computer Science and Biology: Proceedings of the German Conference on Bioinformatics. p. 45–56. https://www.semanticscholar.org/paper/Genome-Sequence-Assembly-Using-Trace-Signals-and-Chevreux-Wetter/3254bccc3fc592f4056eb76f9ce8f1b1b79a930bccc.

Chiang YN, Tan KJ, Chung H, Lavrynenko O, Shevchenko A, et al. 2016. Steroid hormone signaling is essential for pheromone production and oenocyte survival. PLoS Genet. 12:e1006126.

Chung H, Carroll SB. 2015. Wax, sex and the origin of species: dual roles of insect cuticular hydrocarbons in adaptation and mating. Bioessays 37:822–830.
Chung H, Loehlin DW, Dufour HD, Vaccaro K, Millar JG, et al. 2014. A single gene affects both ecological divergence and mate choice in Drosophila. Science 343:1148–1151.

Chung H, Sztal T, Pasricha S, Srithar M, Batterham P, et al. 2009. Characterization of Drosophila melanogaster cytochrome P450 genes. Proc Natl Acad Sci USA. 106:5731–5736.

Crosland MJ. 1989. Kin recognition in the ant Rhytidoponera confusa. I. Environmental odour. Anim Behav. 37:912–919.

Dallerac R, Labeur C, Jailon JM, Knipple DC, Roelofs WL, et al. 2000. A Δ9 desaturase gene with a different substrate specificity is responsible for the cuticular diene hydrocarbon polymorphism in Drosophila melanogaster. Proc Natl Acad Sci USA. 97:9449–9454.

Dembeck LM, Böröczky K, Huang W, Schal C, Anholt RRH, et al. 2016. MultiQC: summary and nonreproductives in the ant Myrmecia gulosa. Proc Natl Acad Sci USA. 103:10341–10346.

Dallerac R, Labeur C, Jailon JM, Knipple DC, Roelofs WL, et al. 2000. ΑΔ9 desaturase gene with a different substrate specificity is responsible for the cuticular diene hydrocarbon polymorphism in Drosophila melanogaster. Proc Natl Acad Sci USA. 97:9449–9454.

Dembeck LM, Böröczky K, Huang W, Schal C, Anholt RRH, et al. 2015. Genetic architecture of natural variation in cuticular hydrocarbon composition in Drosophila melanogaster. Elife 4:e09861.

Denic V, Weissman JS. 2007. A molecular caliper mechanism for determining very long-chain fatty acid length. Cell 130:663–677.

Dyer EW, Magnusson M, Lundin S, Käller M. 2016. MultiQC: summary analysis results for multiple tools and samples in a single report. Bioinformatics 32:3047–3048.

Ferveur J-F. 2005. Cuticular hydrocarbons: their evolution and roles in Drosophila pheromonal communication. Behav Genet. 35:279–295.

Finck J, Berdan EL, Mayer F, Ronacher B, Geiselhardt S. 2016. Divergence of cuticular hydrocarbons in two sympatric grasshopper species and the evolution of fatty acid synthases and elongases across insects. Sci Rep. 6:33695.

Forel A. 1898. La parabiose chez les fourmis. Bull la Socie´te´ Vaudoise Des Sci Nat. 34:380–384.

Gibbs AG, Rajpurohit S. 2010. Cuticular lipids and water balance. In: Gibbs AG, Blomquist GJ, editors. Insect Hydrocarbons: Biology, Biochemistry, and Chemical Ecology. New York, NY: Cambridge University Press. p. 100–120.

Grabherr MG, Haas BJ, Levin JZ, Thompson DA, et al. 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat Biotechnol. 29:644–652.

Greene MJ, Gordon DM. 2003. Cuticular hydrocarbons inform task decisions. Nature 423:32.

Gu P, Welch WH, Guo L, Schegg KM, Blomquist GJ. 1997. Characterization of a novel microsomal fatty acid synthetase (FAS) compared to a cytosolic FAS in the housefly, Musca domestica. Comp Biochem Physiol B Biochem Mol Biol. 118:447–456.

Haas BJ, Fapaniwalou A, Yassour M, Haas, J, Thompson TD, et al. 2011. Full-length transcript assembly from RNA-Seq data without a reference genome. Nat Biotechnol. 29:644–652.

Greene MJ, Gordon DM. 2003. Cuticular hydrocarbons inform task decisions. Nature 423:32.

Gu P, Welch WH, Guo L, Schegg KM, Blomquist GJ. 1997. Characterization of a novel microsomal fatty acid synthetase (FAS) compared to a cytosolic FAS in the housefly, Musca domestica. Comp Biochem Physiol B Biochem Mol Biol. 118:447–456.

Haas BJ, Fapaniwalou A, Yassour M, Grabherr M, Blood PD, et al. 2013. De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. Nat Protoc. 8:1494–1512.

Hansson BS, Stensmyr MC. 2011. Evolution of insect olfaction. Neuron 72:698–711.

Hartke J, Schell T, Jongepier E, Schmidt H, Sprenger PF, et al. 2019a. Hybrid genome assembly of a neotropical mutualistic ant. Genome Biol Evol. 11:2306–2311.

Hartke J, Sprenger PF, Sahm J, Winterberg H, Orivel J, et al. 2019b. Cuticular hydrocarbons as potential mediators of cryptic species divergence in a mutualistic ant association. Ecol Evol. 9:9160–9176.

Heinze J, Foitzik S, Hippert A, Hölldobler B. 1996. Apparent near-e­nemy phenomenon and environment-based recognition cues in the ant Leptothorax nylanderi. Ethology 102:510–522.

Helmkampf M, Cash E, Gadju J. 2015. Evolution of the insect desaturase gene family with an emphasis on social Hymenoptera. Mol Biol Evol. 32:456–471.

Howard RW, Blomquist GJ. 2005. Ecological, behavioral, and biochemical aspects of insect hydrocarbons. Annu Rev Entomol. 50:371–393.

Jones P, Binns D, Chang H-Y, Fraser M, Li W, et al. 2014. InterProScan 5: genome-scale protein function classification. Bioinformatics. 30:1236–1240.

Juárez P, Chase J, Blomquist GJ. 1992. A microsomal fatty acid synthetase from the integument of Blattella germanica synthesizes methyl-branched fatty acids, precursors to hydrocarbon and contact sex pheromone. Arch Biochem Biophys. 293:333–341.

Karger DN, Conrad O, Böhmer J, Kowoli T, Kreft H, et al. 2017. Climatologies at high resolution for the earth’s land surface areas. Sci Data. 4:170122. [http://doi.org/10.1038/sdata.2017.122]

Labeur C, Dallerac R, Wicker-Thomas C. 2002. Involvement of des1 gene in the control of Drosophila melanogaster pheromone biosynthesis. Genetics. 114:269–274.

Lahav S, Soroker V, Hefetz A, Vander Meer RK. 1999. Direct behavioral evidence for hydrocarbons as ant recognition discriminators. Naturwissenschaften. 86:246–249.

Leonhardt SD, Menzel F, Nehring V, Schmitt T. 2016. Ecology and evolution of communication in social insects. Cell. 164:1277–1287.

Liang D, Silverman J. 2000. “You are what you eat”. Diet modifies cuticular hydrocarbons and nestmate recognition in the Argentine ant, Linepithema humile. Naturwissenschaften. 87:412–416.

Liebig J, Peeters C, Oldham NJ, Markstädter C, Hölldobler B. 2000. Are variations in cuticular hydrocarbons of queens and workers a reliable signal of fertility in the ant Harpegnathos saltator? Proc Natl Acad Sci USA. 97:4124–4131.

Lopez-Maestre H, Carmelossi EAG, Lacroix V, Burlet N, Mugat B, et al. 2017. Identification of misexpressed genetic elements in hybrids between Drosophila-related species. Sci Rep. 7:40618.

Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15:550.

Martin SJ, Drifhout FP. 2009. A review of ant cuticular hydrocarbons. J Chem Ecol. 35:1151–1161.

Massey JH, Akiyama N, Bien T, Dreiseverd K, Wittkopf PJ, et al. 2019. Pleiotropic effects of ebony and tan on pigmentation and cuticular hydrocarbon composition in Drosophila melanogaster. Front Physiol. 10:518.

Menzel F, Blüthgen N, Schmitt T. 2008a. Tropical parabiotic ants: highly unusual cuticular substances and low interspecific discrimination. Front Zool. 5:16.

Menzel F, Linsenmair KE, Blüthgen N. 2008b. Selective interspecific tolerance in tropical Crematogaster-Camponotus associations. Anim Behav. 75:837–846.

Menzel F, Schmitt T. 2012. Tolerance requires the right smell: first evidence for interspecific selection on chemical recognition cues. Evolution 66:896–904.

Menzel F, Zumbusch M, Feldmeyer B. 2018. How ants acclimate: impact of climatic conditions on the cuticular hydrocarbon profile. Funct Ecol. 32:657–666.

Ng WC, Chin JSR, Tan KJ, Yew JY. 2015. The fatty acid elongase bond is essential for Drosophila sex pheromone synthesis and male fertility. Nat Commun. 6:8263.

Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, et al. 2019. vegan: Community Ecology Package. https://cran.r-project.org/package=vegan (last accessed 03/18/2021)
Otte T, Hilker M, Geiselhardt S. 2015. The effect of dietary fatty acids on the cuticular hydrocarbon phenotype of an herbivorous insect and consequences for mate recognition. J Chem Ecol. 41:32–43.

Qui Y, Tittiger C, Wicker-Thomas C, Goff GL, Young S, et al. 2012. An insect-specific P450 oxidative decarbonylase for cuticular hydrocarbon biosynthesis. Proc Natl Acad Sci USA. 109:14858–14863.

Rundle HD, Chenoweth SF, Doughty P, Blows MW. 2005. Divergent selection and the evolution of signal traits and mating preferences. PLoS Biol. 3:e368.

Schwander T, Arbuthnott D, Gries R, Gries G, Nosil P, et al. 2013. Hydrocarbon divergence and reproductive isolation in Timema stick insects. BMC Evol Biol. 13:151.

Smith-Unna R, Boursnell C, Patro R, Hibberd JM, Kelly S. 2016. TransRate: reference-free quality assessment of de novo transcriptome assemblies. Genome Res. 26:1134–1144.

Sorvari J, Theodora P, Turillazzi S, Hakkarainen H, Sundström L. 2008. Food resources, chemical signaling, and nest mate recognition in the ant Formica aquilonia. Behav Ecol. 19:441–447.

Sprenger PP, Burkert LH, Abou B, Federle W, Menzel F. 2018. Coping with climate: cuticular hydrocarbon acclimation of ants under constant and fluctuating conditions. J Exp Biol. 221:jeb171488.

Sprenger PP, Hartke J, Feldmeyer B, Orivel J, Schmitt T, et al. 2019. Influence of mutalistic lifestyle, mutalistic partner, and climate on cuticular hydrocarbon profiles in parabiotic ants. J Chem Ecol. 45:741–754.

Sprenger PP, Menzel F. 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.

Steiger S, Stökl J. 2014. The role of sexual selection in the evolution of chemical signals in insects. Insects 5:423–438.

Stinziano JR, Sové RJ, Rundle HD, Sinclair BJ. 2015. Rapid desiccation hardening changes the cuticular hydrocarbon profile of Drosophila melanogaster. Comp Biochem Physiol A Mol Integr Physiol. 180:38–42.

Supek F, Bošnjak M, Škunca N, Šmuc T. 2011. REVIGO summarizes and visualizes long lists of gene ontology terms. PLoS ONE 6:e21800.

Symonds MRE, Elgar MA. 2008. The evolution of pheromone diversity. Trends Ecol Evol. 23:220–228.

Tupec M, Buček A, Janoušek V, Vogel H, Prchalová D, et al. 2019. Expansion of the fatty acyl reductase gene family shaped pheromone communication in Hymenoptera. Elife 8:e39231.

Walsh J, Pontieri L, d’Ettorre P, Linksvayer TA. 2020. Ant cuticular hydrocarbons are heritable and associated with variation in colony productivity. Proc Biol Sci. 287:20201029.

Wicker-Thomas C, Chertemps T. 2010. Molecular biology and genetics of hydrocarbon production. In: GJ, Blomquist A-G Bagne `res, editors. Insect Hydrocarbons: Biology, Biochemistry, and Chemical Ecology. New York, NY: Cambridge University Press. p. 53–74.

Wicker-Thomas C, Garrido D, Bontonou G, Napal L, Mazuras N, et al. 2015. Flexible origin of hydrocarbon/pheromone precursors in Drosophila melanogaster. J Lipid Res. 56:2094–2101.

Wicker-Thomas C, Guenachi I, Keita YF. 2009. Contribution of oenocytes and pheromones to courtship behaviour in Drosophila. BMC Biochem. 10:21.

van Zweden JS, Dreier S, d’Ettorre P. 2009. Disentangling environmental and heritable nestmate recognition cues in a carpenter ant. J Insect Physiol. 55:159–164.

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