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Investigation

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 Stökl 2014) and mediate mate choice in many solitary insects, *e.g.*, in *Drosophila* (Ferveur 2005; Rundle *et al.* 2005; Chung *et al.* 2014). Differences in the chemical profile can induce assortative mating that may even result in speciation (Schwander *et al.* 2013; Otte *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 (Billeter *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; Labeur *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 decarbonylized to hydrocarbons by cytochrome P450 enzymes

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(Chung et al. 2009, 2014; Qui et al. 2012). Members of the same gene families are involved in the biosynthesis of fatty acids and hydrocarbons (energy storage), which makes it difficult to disentangle these two biosynthetic pathways. Despite several studies in *Drosophila* (Dallerac et al. 2000; Labeur et al. 2002; Chertemps et al. 2006, 2007; Chung et al. 2009, 2014; Wicker-Thomas 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; Tupec 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.

Parabiosis is defined as two ant species sharing the same nest, but keeping their brood separate (Forel 1898). They tolerate each other, but still keep their own species-specific CHC profiles (Menzel et al. 2008a, b; Menzel 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, Sarstedt AG & Co. KG, Nürnbrecht, Germany). For *Camponotus*, we collected 15 individuals per group (castes: minor and media) close to the nest together with some dry 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 RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. In the final step, we dissolved the RNA in 30 µl of RNase-free water and stored the samples in a freezer at −80°C until sequencing. Library construction 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 assemblies.

De novo transcriptome assemblies

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

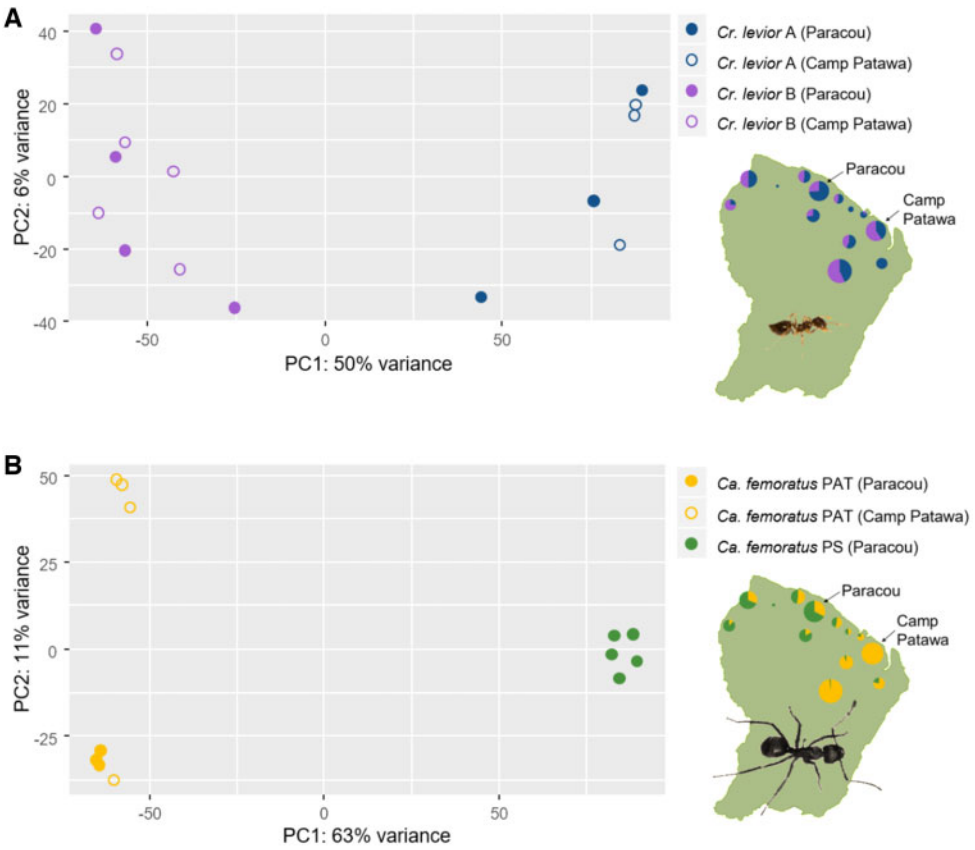

Figure 1 Principal component analysis of gene expression patterns of two cryptic species pairs of *Cr. levior* (A) and *Ca. femoratus* (B). Each dot in the ordination represents the gene expression pattern of a single individual. The cryptic species are indicated by different colors, the two sampling locations by either filled or open circles. The pie charts show frequency of the cryptic *Crematogaster* species (A) and cryptic *Camponotus* species (B). Sampling locations of this study were Paracou and Camp Patawa, which are marked with arrows. Cryptic species distribution data from Hartke *et al.* (2019b). Photos of the ants by B. Feldmeyer.

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 subassemblies, respectively, for *Crematogaster* and *Camponotus* with word size 35 and automatic bubble size (for the composition of samples for each subassembly see Supplementary Table S3). 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 300 bp 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 200 bp 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 *Crematogaster* 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 (ignoring the less likely possibility of qualitative 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-assembly for *Cr. levior* A and B resulted in a *de novo* transcriptome consisting of 60,185 transcripts, and the one for *Ca. femoratus* PAT and PS contained a total of 48,208 transcripts. 74.80% of the *Crematogaster* transcripts (45,016 transcripts) and 69.59% of the *Camponotus* transcripts (33,549 transcripts) could be BLAST annotated.

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-relevant candidate pathways privately

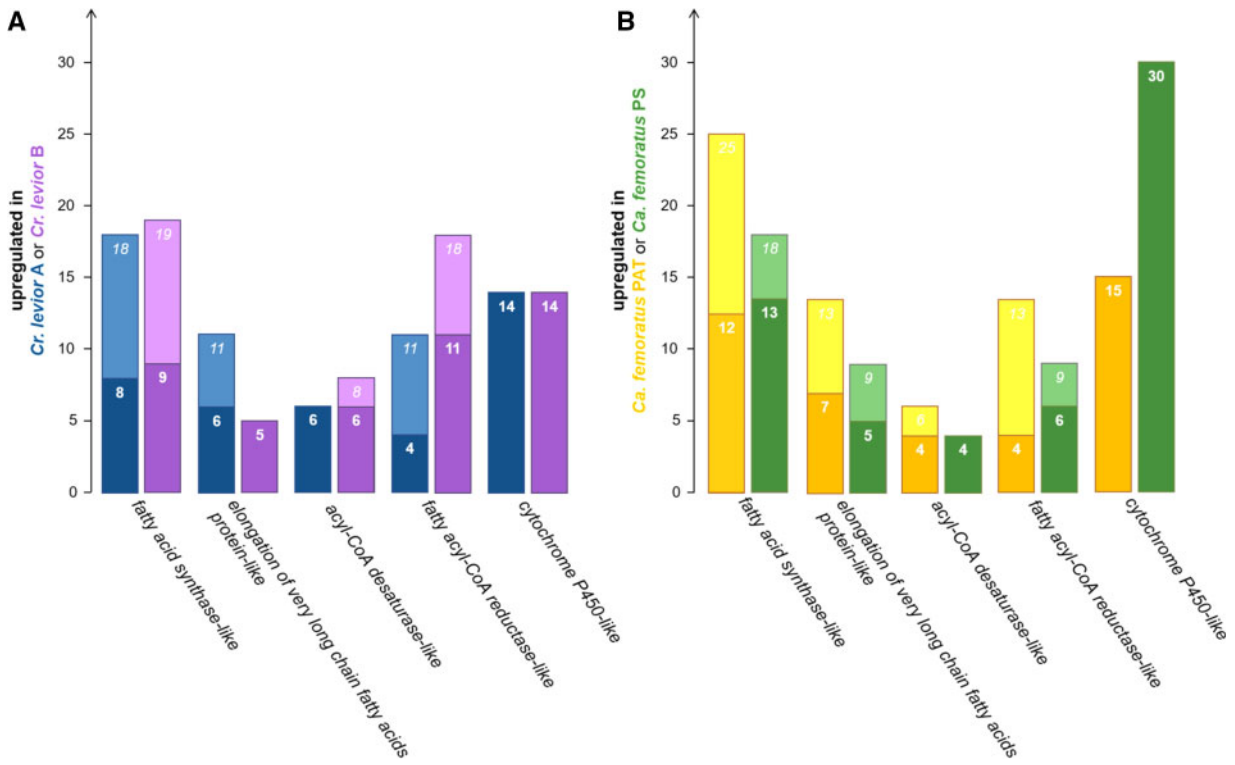

Figure 2 Differentially expressed transcripts of candidate gene families involved in CHC biosynthesis. The bars indicate the number of differentially expressed transcripts for five gene families (fatty acid synthases, very long-chain fatty acid elongases, acyl-CoA desaturases, fatty acid reductases, and cytochrome P450s) for (A) the two cryptic species of *Cr. levior* and (B) the two cryptic species of *Ca. femoratus*. Lightly colored bars with numbers in italics indicate BLAST hits against the 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).

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-$F_1 = 23.76$, $P = 0.003$; Figure 4A) and *Ca. femoratus* (pseudo-$F_1 = 57.50$, $P = 0.023$; 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-$F_1 = 7.06$, $P = 0.002$; *Ca. femoratus*: pseudo-$F_1 = 3.29$, $P = 0.029$). These effects of lab maintenance were species-specific (interaction cryptic species: maintenance: *Cr. levior*: pseudo-$F_1 = 3.48$, $P = 0.001$; *Ca. femoratus*: pseudo-$F_1 = 2.89$, $P = 0.033$). The lab condition CHC profile of colony 075-PAR (*Cr. levior* A) showed the strongest difference to the original profile of the colony (Figure 4A). In conclusion, we were able to show differences between original and lab CHC profile, but these changes were just quantitative, *i.e.*, differences in relative composition, while the CHCs were qualitatively still the same.

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

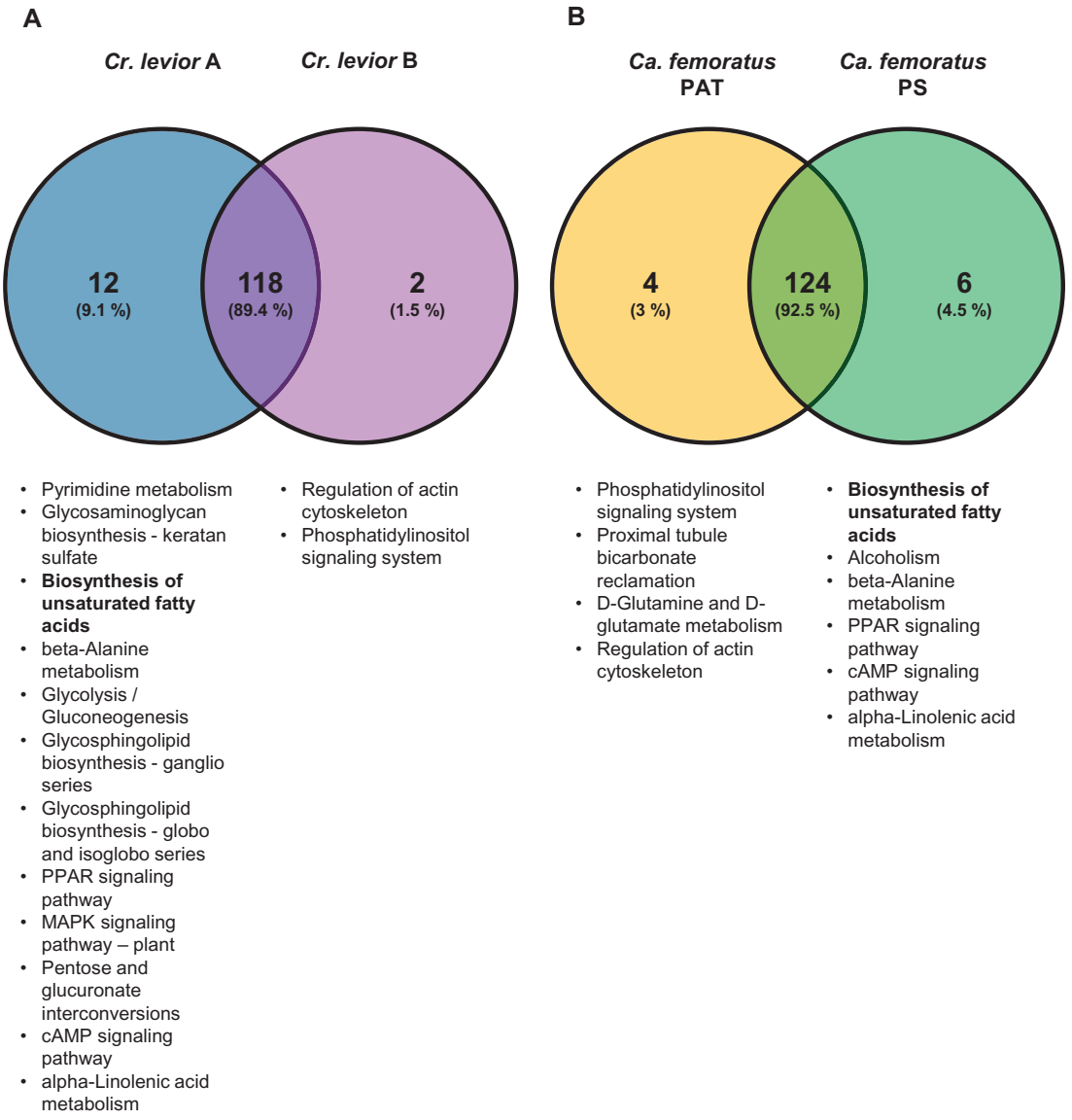

Figure 3 Venn diagrams of shared and privately expressed pathways. Two Venn diagrams representing the percentages of shared or privately expressed pathways between *Cr. levior* (A) and (B) and *Ca. femoratus* PAT and PS. Privately expressed pathways for each cryptic species are noted below the diagrams. Note: Shared pathways evidence for differentially expressed transcripts between cryptic species, which belong to the same pathway.

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).

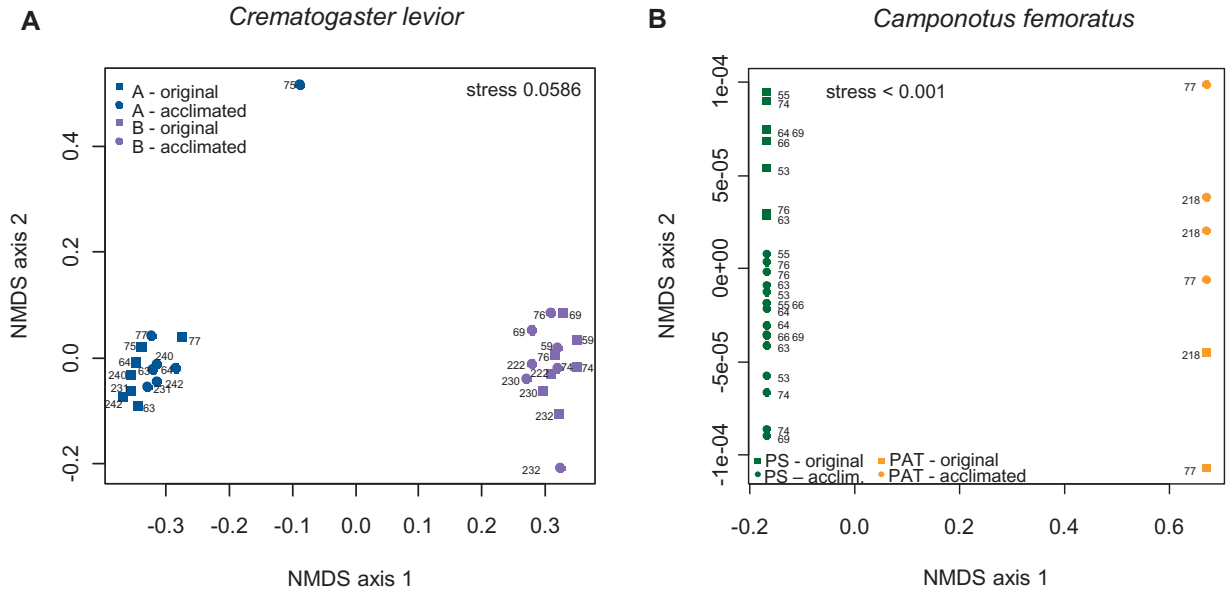

Figure 4 Nonmetric multidimensional scaling (NMDS) of the cuticular hydrocarbon profiles of acclimated and nonacclimated parabiotic ants. Here, we compare the CHC profiles of the cryptic species of (A) *Cr. levior* and (B) *Ca. femoratus* from their natural habitat and after 16 days of standardized lab conditions. Each dot represents the CHC profile of one colony. For *Ca. femoratus*, we had two acclimated individuals per colony but only one extract for the field-collected CHCs. Note that in (A) acclimated sample 075-PAR, albeit aberrant, is clearly *Cr. levior* A. *Cr. levior* A is characterized by high abundances of alkadienes with the chain lengths 33, 35, 37, and 39. The relative abundances of these four alkadienes vary across colonies. Here, sample 075-PAR differs from the others in having more C33-dienes and C35-dienes. Also, note that the nearly identical NMDS 1 scores in (B) within each cryptic species stem from the fact that most of the variation is explained by the dichotomous difference between *Ca. femoratus* PAT and PS.

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 desaturase-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 neofunctionalization in this gene (Helmkampf *et al.* 2015). In line with this, parabiotic ants produce unsaturated hydrocarbons with more variable double bond positions compared to *D. melanogaster* that mainly produces Z7-alkenes and Z7-Z11-alkadienes (Ferveur 2005; Sprenger *et al.* 2019).

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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