Chemical and transcriptomic diversity do not correlate with ascending levels of social complexity in the insect order Blattodea

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Abstract

Eusocial insects, such as ants and termites, are characterized by high levels of coordinated social organization. This is contrasted by solitary insects, which display more limited forms of collective behavior. It has been hypothesized that this gradient in socio-behavioral sophistication is positively correlated with chemical profile complexity, due to a potentially increased demand for diversity in chemical communication mechanisms in insects with higher levels of social complexity. However, this claim has rarely been assessed empirically. Here, we compare different levels of chemical and transcriptomic complexity in selected species of the order Blattodea that represent different levels of social organization, from solitary to eusocial. We primarily focus on cuticular hydrocarbon (CHC) complexity, since it has repeatedly been demonstrated that CHCs are key signaling molecules conveying a wide variety of chemical information in solitary as well as eusocial insect species. We assessed CHC complexity and divergence between our studied species of different social complexity levels as well as the differentiation of their respective repertoires of CHC biosynthesis gene transcripts. Surprisingly, we did not find any consistent pattern of chemical complexity correlating with the degree of social complexity, nor did the overall chemical divergence or transcriptomic repertoire of CHC biosynthesis genes reflect on the levels of social complexity. Our results challenge the assumption that increasing social complexity is generally reflected in more complex chemical profiles and point towards the need for a more cautious and differentiated view on correlating complexity on a chemical, genetic, and social level.

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Abstract

Eusocial insects, such as ants and termites, are characterized by high levels of coordinated social organization. This is contrasted by solitary insects, which display more limited forms of collective behavior. It has been hypothesized that this gradient in socio-behavioral sophistication is positively correlated with chemical profile complexity, due to a potentially increased demand for diversity in chemical communication mechanisms in insects with higher levels of social complexity. However, this claim has rarely been assessed empirically. Here, we compare different levels of chemical and transcriptomic complexity in selected species of the order Blattodea that represent different levels of social organization, from solitary to eusocial. We primarily focus on cuticular hydrocarbon (CHC) complexity, since it has repeatedly been demonstrated that CHCs are key signaling molecules conveying a wide variety of chemical information in solitary as well as eusocial insect species. We assessed CHC complexity and divergence between our studied species of different social complexity levels as well as the differentiation of their respective repertoires of CHC biosynthesis gene transcripts. Surprisingly, we did not find any consistent pattern of chemical complexity correlating with the degree of social complexity, nor did the overall chemical divergence or transcriptomic repertoire of CHC biosynthesis genes reflect on the levels of social complexity. Our results challenge the assumption that increasing social complexity is generally reflected in more complex chemical profiles and point towards the need for a more cautious and differentiated view on correlating complexity on a chemical, genetic, and social level.

Introduction

Insects have exploited chemical signaling as their primary communication mode (Greenfield 2002; Missbach et al. 2014). Particularly cuticular hydrocarbons (CHCs), nonpolar lipids coating the epicuticle of terrestrial insects, have consistently been demonstrated as pivotal signals and cues in a wide variety of chemical communication systems (Blomquist and Bagnères 2010; Blomquist and Ginzel 2021). Predominantly, CHCs have been shown to be major signaling molecules for nestmate recognition in eusocial taxa (e.g. Leonhardt et al. 2016; Sprenger and Menzel 2020) and for sexual and species-specific signaling mechanisms in solitary taxa (e.g. Chung and Carroll 2015; Shahandeh et al. 2018). It has been suggested that chemical profiles in eusocial insect societies, with their multiple castes, task allocations and collective processes, display a higher degree of complexity than in solitary species (Linksvayer 2015; Korb and Thorne 2017; Kronauer and Libbrecht 2018; Holland and Bloch 2020). However, there is no consensus as to how to assess, quantify, and compare the degree of CHC profile complexity across different species (Friedman et al. 2020; Holland and Bloch 2020). Chemical complexity in CHC profiles has previously been assessed as the total number of compounds of a given type, or total ratio of structurally more complex CHC compounds (*i.e.,* unsaturated and methyl-branched CHCs) versus less complex compounds (*i.e.,*straight-chain CHCs) (Martin and Drijfhout 2009; Kather and Martin 2015). Taking this approach, Kather and Martin (2015) did not find any correlation between CHC diversity and social complexity in a meta-study comparing chemical profiles in eusocial and solitary Hymenopteran species.

Like the Hymenoptera (ants, bees, wasps, sawflies), the order Blattodea encompasses all known levels of social complexity, from solitary cockroaches to obligately eusocial termites. Particularly in termites, which generally lack well-developed eyes, chemical signaling has been repeatedly demonstrated to be a wide-spread and dominant form of communication (Van der Meer et al. 1999; Bagnères and Hanus 2015). In this context, CHCs have been particularly well investigated as fundamental signaling cues for caste differentiation, nestmate recognition and reproductive status conveyance in termites (Liebig et al. 2009; Weil et al. 2009; Hoffmann et al. 2014). But in solitary cockroaches as well, CHCs appear to carry out diverse signaling functions, such as kin recognition and aggregation (Rivault et al. 1998; Lihoreau and Rivault 2008; Hamilton et al. 2019). To the best of our knowledge, no studies have yet attempted to directly compare CHC diversity across different levels of social complexity within the order Blattodea. In the present study, we compare levels of complexity between CHC profiles of representative solitary and social species within the order Blattodea. Through the availability of whole-genome transcriptomes for our selected study species, we additionally explored CHC biosynthesis gene transcript diversity and correlate it with the different levels of social complexity as well as

the respective CHC compound classes they are predominantly associated with.

Our cockroach study species are *Blatta orientalis* (Blattodea: Blattidae) and *Blattella germanica* (Blattodea: Ectobiidae). The former is known as one of the most common cockroach pest species in temperate regions around the world (Thoms and Robinson 1986, 1987; Edwards and Short 1993), whereas the latter is well-established in CHC-based chemical communication research (Gu et al. 1995; Rivault et al. 1998; Fan et al. 2003; Pei et al. 2019). Within the termites, although all species are considered eusocial, the level of organization and social complexity varies across different termite societies in terms of colony size, worker sterility and morphological differentiation of castes (Abe 1987; Thorne 1997; Korb and Hartfelder 2008; Korb et al. 2015). One piece life type (OPT) or single-site termite species have a low social complexity with small colonies and totipotent workers, spending their entire lives nesting and feeding within the same enclosed, wood-based habitat (Noirot 1970; Shellman-Reeve 1997; Korb and Thorne 2017). They display an exceptionally flexible caste development, with larval offspring retaining the capability to differentiate into reproductives, alates or soldiers well into their late instar stages (Noirot 1985b; Korb and Hartfelder 2008). This pattern is widely considered to be the ancestral form and is characterized by a low to intermediate form of social complexity (Noirot and Pasteels 1987, 1988; Legendre et al. 2008). Low social complexity OPT termites are represented in our study by the two species *Kalotermes flavicollis* and *Neotermes castaneus*(Blattodea: Kalotermitidae). Separate life type (ST) or central-site termite species divide their nesting place from their multiple food sources and are thus characterized by foraging (Noirot 1970; Abe 1987; Shellman-Reeve 1997). As opposed to OPT termites, ST termites are more constrained in their development due to an early instar separation into a wingless (apterous) line that can further differentiate into permanently sterile soldiers and workers and a nymphal line that eventually develops into sexual alates (Noirot 1985a; Korb and Hartfelder 2008; Roisin and Korb 2010). This pattern characterizes the most socially complex termite species which can reach much larger and more differentiated colonies than OPT termites (Noirot and Pasteels 1987, 1988; Legendre et al. 2008). *Reticulitermes flavipes* and*Coptotermes formosanus* (Blattodea: Rhinotermitidae) as well as*Mastotermes darwiniensis* (Blattodea: Mastotermitidae) represent ST termites in our study, whereas the latter constitutes a particularly interesting case: The species *M. darwiniensis* is the only extant member of the family Mastotermitidae and phylogenetically represents the most basal termite lineage. However, this species displays all characteristics of ST termites with large colonies, constrained developmental pathways and a true worker caste (Inward et al. 2007b; Krishna et al. 2013). Since the low social complexity OPT has been widely hypothesized to be the ancestral termite state, the clear ST pattern of *M. darwiniensis* as basal and most ancient extant termite lineage represents an unresolved and frequently debated conundrum (Inward et al. 2007a; Korb and Thorne 2017; Chouvenc et al. 2021).

We tested the central hypothesis that chemical and social complexity are correlated, and that, concordantly, the genetic repertoire for CHC biosynthesis gene transcripts increases with the level of social complexity. We focused on structurally complex CHC compounds (unsaturated and methyl-branched) and the candidate genes that potentially play a role in their biosynthesis and variation (mostly desaturases and microsomal fatty acid synthases, see Fig. 1 and Holze et al., 2021). Moreover, we constructed a chemical dendrogram based on CHC divergence, compared it to the molecular phylogeny of our study species, and correlated CHC biosynthesis gene transcript counts with the respective CHC compound counts per analyzed species.

Materials & Methods

Tested termite and cockroach species

In a previous study, chemical profiles analyzed among the same taxa where found to be solely discriminable on the species level, rather than on the colony (termites) or population (cockroaches) level (Golian et al. 2022). Therefore, we restricted ourselves to two respective laboratory colonies per termite species and two respective laboratory populations per cockroach species. All species used in this study were maintained in the Federal Institute of Materials Research and Testing (BAM), Berlin. Termite colonies of *R. flavipes, C.*

formosanus, K. flavicollis and *N. castaneus* were kept in a darkened room at 26°C and 84% humidity, and colonies of *M. darwiniensis* were maintained at 28°C and 83% humidity. All colonies were fed regularly with pre-decayed birch wood. The cockroaches *B. germanica* and *B. orientalis* were maintained in mixed open rearing boxes in 12-hour light/dark cycles at 26 °C and 50 % humidity, from the day of egg-laying until disposal of older adults. Cockroaches were reared on a mixture of 77.0 % dog biscuit powder, 19.2 % oat flakes, 3.8 % brewer's yeast and supplied with water ad libitum and weekly with apple and carrot slices. All cockroaches and termites were freeze-killed and stored at -20 °C until further analysis.

CHC extraction and analysis

To yield comparable amounts of extracts between our cockroaches and termites that vary largely in size, we had to adjust extraction volumes and pool smaller individuals. For this, we used 300 and 3000 µl MS pure hexane (UniSolv, Darmstadt, Germany) on single *B. germanica* and*B. orientalis* individuals, respectively, and 100 µl on pools of 3 individuals per termite species for extraction. Extraction procedures were then equalized to ensure comparability. Extractions were performed in glass vials (20 ml for cockroaches and 2 ml for termites, Agilent, Santa Clara, California, USA) on an orbital shaker (IKA KS 130 Basic, Staufen, Germany) for 10 minutes. Afterwards, the extract was evaporated under a constant stream of gaseous carbon dioxide. Then, it was resuspended in 5 µl in a hexane solution containing 7,5 ng/µl dodecane (C12) as an internal standard. 3 µl of the resuspended extract were then injected into a gas-chromatograph coupled with a tandem mass spectrometer (GC-MS/MS) (GC: 7890B, Triple Quad: 7010B; Agilent Technologies, Waldbronn, Germany) equipped with a fused silica column (DB-5MS ultra inert; 30 m x 250 µm x 0.25 µm; Agilent J&W GC columns, Santa Clara, California, USA) in splitless mode at a temperature of 300 °C with helium used as a carrier gas under constant flow of 2.25 ml/min. The temperature program started at 60 °C held for 5 min, increasing 20 °C per minute up to 200 °C and then increasing 3 °C per minute to the final temperature of 325 °C, held for 5 min.

CHC peak detection, integration, quantification and identification were all carried out with Quantitative Analysis MassHunter Workstation Software (Version B.09.00 / Build 9.0.647.0, Agilent Technologies, Santa Clara, California, USA). The pre-defined integrator Agile 2 was used for the peak integration algorithm to allow for maximum flexibility, quantification was carried out over total ion chromatograms (TIC). All peaks were then additionally checked for correct integration and quantification, and, where necessary, re-integrated manually. CHC compound identification was then carried out based on their characteristic diagnostic ions and retention indices. Analysis was focused exclusively on non-polar CHC compounds due to their repeatedly demonstrated involvement in chemical signaling in both solitary and eusocial Blattodea (e.g. Lihoreau and Rivault 2008; Hoffmann et al. 2014; Hamilton et al. 2019). The obtained values for the absolute peak area integrals were standardized by dividing them through the total of all CHC peak area integrals per sample, generating relative proportions for all CHC compounds. These proportions were then summarized for the individual CHC compound classes. Sample sizes for our individual species were *B. germanica* : 9, *B. orientalis* : 11, *C. formosanus* : 5, *K. flavicollis* : 4, *M. darwiniensis* : 4,*N. castaneus* : 5 and *R. flavipes* : 5. We focused on termite workers to obtain the general colony-specific chemical profiles as the vast majority of individuals constituting the respective colonies are workers (Korb 2007; Roisin and Korb 2010; Korb and Thorne 2017). Moreover, we attempted to render our study comparable to other studies on chemical complexity in eusocial species also focusing exclusively on profiles obtained from the worker caste (Martin and Drijfhout 2009; Kather and Martin 2015). Similarly, we pooled the sexes of both respective cockroach species to focus on representative species-specific chemical profiles, with less emphasis on the more subtle sex-specific differences (Pei et al. 2021).

Comparison of chemical and phylogenetic divergence

To standardize the absolute peak area values for chemical clustering, the normalization method of the function "decostand" of the community ecology R package "vegan" was used (Oksanen et al. 2008), based

on the following formula:

$$T_{x,y} = \frac{P_{x,y}}{\sqrt{\sum P_{y^2}}}$$

$T_{x,y}$ refers to the transformed peak area x of individual y, $P_{x,y}$ to the absolute peak area x of individual y and $\Sigma\ P_y{}^2$ to the squared sums of all absolute peak areas of individual y. This widely applied method for normalizing ecological data was chosen to make the peak areas comparable between our groups, to highlight the relative peak area differences and to correct for size-dependent variation. Agglomerative hierarchical cluster analysis ("**U** nweighted **P** air-**G** roup**M** ethod with **A** rithmetic means", *i.e.* UPGMA) was performed with the R package "ape" (Paradis et al. 2004), based on average chemical Manhattan distances reflecting the median CHC divergence separating the different cockroach and termite species. The formula for calculating Manhattan distances is as follows:

$$\sum [Y_j - Y_k]$$

The actual difference between two data points, in this case Y_j and Y_k, is used based on the total amount of CHC variation between the species. In contrast with Euclidean distance where squared differences are used, the Manhattan distance is less prone to be dominated by single large differences between the compared component. It has thus been suggested that for multidimensional phenotypes such as CHC profiles, the Manhattan distance metric is the most ecologically meaningful (Oksanen 2009). The molecular phylogeny was obtained and adapted from the latest published Blattodea phylogeny in He et al. (2021). A Mantel test (Mantel 1967), conducted with the R package "ade4" (Dray and Dufour 2007), compared the molecular distances based on the published Blattodea phylogeny with the average Manhattan CHC divergence. The Mantel test was performed five times with 9999 permutations for each single test, the average probability is presented.

Analysis of transcriptomic gene counts

We retrieved translated whole genome transcriptome sequences based on whole-body RNA extractions for each of the seven investigated Blattodea species as described in He et al. (2021). Total RNA was isolated from individuals for all species. Due to the large body size, adult cockroaches were cut into 4-6 parts for separate extraction, followed by re-pooling. For the extraction itself, samples were suspended in pre-cooled Trizol (Thermo Fisher Scientific) and homogenized twice at 10 s at 2 M/s with a 5-mm steal bead (Qiagen) using a tissue homogenizer (MP Biomedicals). Total RNA was isolated with a chloroform extraction, followed by isopropanol precipitation, according to instructions from Trizol. Extracted total RNA was dissolved in RNA storage solution (Ambion) and then incubated with 2 units of TurboDNase (Ambion) for 30 min at 37 °C, followed by purification with an RNAeasy Mini kit (Qiagen) according to manufacturer's instructions. Quantity and quality of RNA were determined by Qubit and Bioanalyzer 2100, respectively. Following pooling described in sample collection part, total RNA was used to construct barcoded cDNA libraries using a NEXTflex Rapid Directional mRNA-seq kit (Bioo Scientific). Briefly, mRNA was enriched using poly-A beads from total RNA and subsequently fragmentated. First and second-strand cDNA was synthesized and barcoded with NEXTflexRNA-seq Barcode Adapters. The libraries were sequenced on an Illumina NextSeq500/550 platform at Berlin Center for Genomics in Biodiversity Research (BeGenDiv). We obtained orthologous sequences of enzymes which have been selected according to a demonstrated function in CHC biosynthesis via targeted knockdown studies (summarized in Holze et al. 2021) from NCBI. In order to estimate numbers of transcripts orthologous to these enzymes, we first created Hidden Markov Models (HMMs) for each of their protein sequences. For this, each of the query sequences was blasted against a database of proteomes from 17 insect genomes (*Acyrthosiphon pisum, Bemisia tabaci, Blattella germanica, Clitarchus hookeri, Coptotermes formosanus, Cryptotermes secundus, Diploptera punctata, Drosophila melanogaster,*

Glossina morsitans, Locusta migratoria, Macrotermes natalensis, Medauroidea extradentata, Musca domestica, Periplaneta americana, Rhopalosiphum maidis, Stomoxys calcitrans, Zootermopsis nevadensis) with blastp (version 2.7.1+, Camacho et al. 2009). Blast output was filtered to contain only hits with an e-value $< 1e^{-10}$ and a minimum sequence identity of 50%. For each query gene, protein sequences of all significant hits were retrieved from the protein database and aligned with PRANK (version v.170427; Loytynoja 2014) at default settings. Hidden Markov Models (HMM) were created for each alignment using hmmbuild (version 3.1b2; Wheeler and Eddy 2013) at default settings. These HMMs were then used to search the proteomes of our seven focal Blattodea species using hmmsearch with a maximum e-value of $1e^{-5}$. Output of these hmm-searches were then filtered to contain only hits with a score of at least 100. If a transcript appeared in multiple lists, it was attributed to the query HMM for which it received the highest score. Finally, to verify these results, we blasted all transcript sequences against the swissprot database (accessed November 2020) with blastp (version 2.7.1+; Altschul et al. 1990). Any transcripts without clear orthology to the gene of interest were then excluded from the transcript counts. We performed used a generalized linear model (GLM) with Poisson family distribution to compare the variation in transcript counts among the levels of social complexity in our tested species and visualized the results with a heatmap, utilizing the function "heatmap" provided by the R package "stats". Furthermore, we compared total gene transcript counts with the total number of detected CHC compounds per species with a χ^2 (chi-square) test.

Results

We identified 134 CHC compounds in total from our representative termite and cockroach species (Tab. S1). The six major CHC compound classes detected were *n* -alkanes, *n* -alkenes, alkadienes as well as mono-, di-, and tri-methyl-branched alkanes (Fig. 2). The different compound classes greatly vary in relative amounts across all species and not all classes were observed in each species. On average,*n* -alkanes show higher relative abundancies in termites (29.1 %) than in cockroaches (15.43 %), whereas di-methyl-branched alkanes show the reversed pattern with much higher average abundancies in cockroaches (25.96 %) than in termites (0.027 %). Tri-methyl alkanes, on the other hand, only occur in detectable quantities in *B. germanica* . Alkadienes were only found in Rf, *M. darwiniensis* and *N. castaneus* , with minimal trace occurrences also in *B. orientalis* . Generally, mono-methyl-branched alkanes were the most abundantly detected compound class across the tested cockroach and termite species, however, they occurred in comparably low quantities in *M. darwiniensis* and *N. castaneus* . Unsaturated compounds, generally considered to be among the structurally more complex CHCs indicating higher chemical complexity together with methyl-branched alkanes (Martin and Drijfhout 2009; Kather and Martin 2015), occur inconsistently in two high (ST) and one low (OPT) social complexity termite species, but only in traces in the cockroaches. However, the most structurally complex methyl-branched alkanes with three methyl branches occur exclusively in just the cockroach species *B. germanica* . Moreover, the structurally most simple CHC profile, consisting almost exclusively of*n* -alkanes and mono-methyl-branched alkanes, was found in the high social complexity termite *C. formosanus* .

The molecular phylogeny of our tested termite and cockroach species mostly mirrors their respective levels of social complexity except for*M. darwiniensis* and *C. formosanus* , which represents the most basal termite group despite displaying a high level of social complexity (Fig. 3). This is not at all reflected in their chemical phylogeny based on the average CHC divergence between the species. Namely, the most highly supported cluster (99 Bootstrap) encompasses a solitary (*B. orientalis*), highly social (*C. formosanus*) and lowly social (*K. flavicollis*) species. Moreover, all levels of social complexity that cluster together in the molecular phylogeny are basically broken off in the chemical phylogeny, with no recognizable pattern. Unsurprisingly, a Mantel test found no significant correlation between the molecular and chemical phylogeny (r = 0.3971, P = 0.1291).

Comparing overall CHC biosynthesis gene transcript counts with the total number of CHC compounds detected in each species, we found no significant correlation between the quantities (χ^2 – test, r=0.12, p=0.79;

Fig. 4). *R. flavipes* has the highest transcript counts (191) but the third lowest CHC compound count (37), conversely,*M. darwiniensis* has the lowest transcript count (111) but the second highest CHC compound count (59). Again, no trend towards the different levels of social complexity could be detected, which is best exemplified in the three high social complexity termite species, where both the highest (*R. flavipes*) and the lowest (*M. darwiniensis*) number of transcripts as well as the second highest (*M. darwiniensis*) and the lowest number (*C. formosanus*) of CHC compounds was detected. Across all investigated CHC biosynthesis gene transcripts (Tab. 1), counts did not vary systematically by social complexity level (χ^2= 1.11, df = 1, p = 0.29), which is also apparent in a heat map representing the individual transcript counts per species normalized by their average relative abundancies (Fig. 5).

Discussion

CHC divergence and variation in relation to social complexity

We compared chemical and associated transcriptomic complexity between Blattodea species with increasing levels of social organization. Overall, we did not find a consistent correlative pattern of CHC-based chemical complexity paralleling the different levels of social complexity in our representative Blattodea species. These results point to large categorical differences in CHC profiles across species, which appear to be independent from their level of social organization.

CHC divergence based on average chemical distances between the species neither reflects the different levels of complexity nor the phylogenetic divergence within the Blattodea (Fig. 3). *M. darwiniensis* , the most basal termite lineage (Inward et al. 2007b; Krishna et al. 2013) chemically clusters together with both a high (*R. flavipes*) and a low (*N. castaneus*) social complexity termite, and the solitary cockroach *B. orientalis* clusters with both a high (*C. formosanus*) and a low (*K. flavicollis*) social complexity termite. It has been hypothesized that chemical divergence clearly differing from an established molecular phylogeny indicates selection on chemical profiles for different functions overriding their phylogenetic information (Marten et al. 2009; Buellesbach et al. 2013). However, our chemical divergence does not display any pattern congruent with the social hierarchy of our study species, rendering any assumptions on selection for CHC functions reflective of the species' respective social complexity highly unlikely. Concerning counts of gene transcripts stemming from orthologs of CHC biosynthesis genes from other insect species, these do neither quantitatively correlate with higher levels of social complexity nor with the total number of CHC compounds detected in each of our study species.

Future work could include more species as transcriptomes, genomes and chemical profiles become available, and utilize scalable frameworks such as phylogenetically-contrasted regression and Bayesian ancestral state reconstruction models (Simon et al. 2019). Additionally, caste-specific CHC variation in eusocial taxa could be taken into account in future studies as well, potentially adding another layer of complexity, despite the accompanying issues for direct comparability with solitary taxa.

CHC biosynthesis gene transcript variation across the studied Blattodea species

Acetyl-CoA carboxylase (ACC) catalyzes the first and rate-limiting step in CHC biosynthesis (Barber et al. 2005, see Fig. 1). In each of our analyzed cockroach and termite species, we found several distinct ACC transcripts (ranging from 3 in *C. formosanus* to 8 in *B. germanica*) based on orthology to the *Drosophila* gene (Tab. 1). This rich abundance of ACC transcripts strengthens the argument for the universality of ACC as fundamental catalyst for the first step in CHC biosynthesis (see also Fig. 5).

For FAS genes, seven of them had already been identified in*Blatella germanica* , with five showing a significant effect on CHC compound quantities upon knockdown (Pei et al. 2019). In our analyzed transcriptomes,

we were able to detect transcripts with strong orthology to two of these five FAS genes (BgFas 4 and 6). Transcripts with orthology to *BgFas4* were detectable across all seven species, whereas *BgFas6* transcripts were most abundant in the two cockroaches and only in three of the five analyzed termites (Tab. 1). Generally, it has been hypothesized that two types of FAS, cytosolic and microsomal, differentially impact CHC biosynthesis, with the former mainly governing non-methylated, straight-chain CHCs, and the latter being more specific for methylated CHCs (Chung et al. 2014; Wicker-Thomas et al. 2015, Fig. 1). Since we could not detect any transcript copies of *BgFas6* in both *M. darwiniensis* and*N. castaneus* , the two species with the lowest amounts of methyl-branched alkanes (Fig. 2), it is possible that the *BgFas6*transcripts stem from a microsomal FAS associated with the production of methyl-branched alkanes. Intriguingly, for *N. castaneus* , in addition to showing both the lowest number and proportion of methyl-branched alkanes, it also shows the lowest transcript copy number of *FASN2* orthologs, an oenocyte-specifically expressed*Drosophila* gene with a strong effect on methyl-branched CHCs and thus speculated to be microsomal (Chung et al. 2014; Wicker-Thomas et al. 2015).

Unsaturated compounds, whose biosynthesis is crucially dependent on desaturases (Wicker-Thomas et al. 1997; Coyne et al. 1999; Dallerac et al. 2000), occur in each of our studied Blattodea species, most abundantly in *R. flavipes* , *M. darwiniensis* and *N. castaneus* , intermediately in *B. orientalis* and *B. germanica* , but only in traces in *K. flavicollis* and *C. formosanus* (Fig. 2). This partially reflects the respective numbers of transcripts orthologous to the genes *desat1* and *desat2* , with the former being most abundantly represented in *M. darwiniensis* and *N. castaneus* , and the latter in *R. flavipes* (Tab. 1).

Genes coding for P450 Decarbonylases of the gene subfamily CYP4G have been shown to govern the final steps in CHC biosynthesis and have thus been suggested to be stable, highly conserved and particularly vital elements in this pathway (Feyereisen 2020; Holze et al. 2021, Fig. 1). Concordantly, at least one *Cyp4g* gene could be identified in all insect genomes screened to date (Qiu et al. 2012; Feyereisen 2020). We found transcripts orthologous to the *Drosophila* gene*Cyp4g1* as well as the migratory locust *Locusta migratoria*gene *LmCYP4G102* in all our tested species (Tab. 1). However, their numbers vary largely with no apparent consistent pattern, hinting at more transcriptomic variety for these vital CHC biosynthesis elements than previously assumed.

The assessment of transcript counts as approximation of the actual genomic repertoire for CHC biosynthesis genes naturally has its limits and will remain speculative until targeted knockdown studies confirm the actual functions of the transcripts as well as their underlying genes. However, analyzing the abundance of unique transcripts per ortholog allows valuable insights into functional diversity potentially exceeding the information contained within whole-genome sequences (He et al. 2021; Sprenger et al. 2021). Correlating transcriptomic diversity directly with CHC profile variation has been attempted surprisingly rarely despite its potential to approximate the genetic control of CHC variation more accurately, as it has repeatedly been shown that the same genotype can produce different CHC profiles (Holze et al. 2021; Sprenger et al. 2021). Thus, our findings constitute promising first steps for future studies with the potential to corroborate our transcriptomic assessment with target gene counts once further genomic resources become available.

CHC-based communication mechanisms and outlook for future studies

It has long been hypothesized that the complexity of CHC profiles reflects the complexity of chemically encoded communication mechanisms necessary to maintain more socially complex insect societies (Korb and Thorne 2017; Kronauer and Libbrecht 2018; Holland and Bloch 2020). However, how CHC profiles actually encode biologically relevant information such as nestmate affiliation or task allocation has remained largely elusive so far (Buellesbach et al. 2018; Menzel et al. 2019; Heggeseth et al. 2020). Further elucidation on the exact encoding mechanisms in CHC profiles and which compound combinations actually convey chemical information will be instrumental in gaining a better understanding on how insect populations and societies are maintained at different levels of social complexity. Furthermore, although insects generally synthesize the majority of the components in their CHC profiles themselves (Nelson and Blomquist, 1995; Blomquist and Bagnères, 2010), several studies have demonstrated the impact of several biotic and abiotic factors such

as diet, habitat and microbiome on CHC profiles as well (Fedina et al. 2012; Rajpurohit et al. 2017; Teseo et al. 2019). Thus, disentangling these factors from the conserved CHC profile functionalities represents an additional challenge in future studies that will nevertheless be indispensable to fully comprehend and explore CHC-mediated communication mechanisms.

Future studies should also consider the occurrence of very-long chained CHC compounds of up to C58 in Blattodea surface profiles as recently demonstrated with non-standard analytical methods (Golian et al. 2022). However, neither exact compound quantifications nor identifications (*e.g.,* discrimination between n -alkanes and methyl-branch alkanes) are so far possible in this higher chain length range extending beyond the CHC compounds traditionally accessed and identified through gas-chromatographic separation (Schnapp et al. 2016; Bien et al. 2019). Therefore, to include very long-chain CHCs in future analyses, novel methods are necessary to reliably assess their exact quantities and compound classes. Moreover, despite CHCs clearly constituting the dominant, most investigated compounds in chemical signaling (Blomquist and Bagnères 2010; Chung and Carroll 2015) and have been implied to have convergently evolved, unifying communication modalities in eusocial insects (Leonhardt et al. 2016; Funaro et al. 2018), we cannot exclude the additional potential of non-CHC compounds also used in signaling which might reflect social complexity (e.g. Hanus et al. 2010; Smith et al. 2016; Steitz et al. 2019). Lastly, CHC metabolic networks could be amenable to various kinds of more elaborate complexity analyses such as metabolic network pathfinding (Kim et al. 2017), identification of critical connectors (Kim et al. 2019), determination of topological characteristics (Goryashko et al. 2019), and flux balance analysis (Beguerisse-Díaz et al. 2018). These types of complexity analyses have, to our knowledge, not been attempted so far in this context at all and might largely aid in obtaining a more holistic correlative view on complexity on a chemical, genetic and social level.

Conclusions

Overall, we did not find any consistent patterns linking CHC profile variation, CHC biosynthesis transcriptome diversity, and social complexity across the seven Blattodea species included in our study. This is partially reflective of the results Kather and Martin (2015) obtained in their meta-analysis of solitary and eusocial Hymenopteran CHC diversity, although this study was lacking the genetic component. This implies that, at least for our representative species spanning different levels of social complexity within the order Blattodea, neither their CHC profiles nor their repertoire of CHC biosynthesis gene transcripts does reflect any social hierarchy or correlate with their social complexity. Concerning the genetic background of CHC biosynthesis, however, it must be taken into account that our general knowledge remains limited and mostly biased towards *Drosophila*(Holze et al. 2021), so it is quite possible that more Blattodea-specific CHC biosynthesis genes exist which have not been functionally characterized yet and have therefore eluded our comprehensive gene transcript investigation. Nevertheless, our study challenges the long-standing assumption of a general correlation between increasing social complexity and chemical profile sophistication for our Blattodea study species. Therefore, we strongly suggest more cautious approaches for assessing, comparing, and interpreting chemical complexity in insects with different levels of social organization.

Tables

Tab 1 : List of 21 genes with a demonstrated function in the CHC biosynthesis pathway for which we could detect transcripts in at least one of our tested cockroach and termite species. The numbers correspond to the position of the respective gene product in the biosynthesis pathway (compare to Fig. 1). Gene acronyms, (putative) functions, NCBI (or Genbank when not available in NCBI) IDs and the taxon where the gene was originally described are indicated along with the detected copy numbers in our tested species. The CHC biosynthesis genes were retrieved from Holze et al. (2021).

#	Gene acronym / (putative) function	NCBI or Gen-bank ID	Original taxon
1	*ACC* / Acetyl-CoA carboxylase	35761	*Drosophila melanogaster* (fru
2	*FASN2* / Fatty acid synthase 2	117361	*Drosophila melanogaster*
3	*FASN3* / Fatty acid synthase 3	3355111	*Drosophila melanogaster*
4	*BgFas4* / Fatty acid synthase	MK605591.1	*Blatella germanica* (German
5	*BgFas6* / Fatty acid synthase	MK605593.1	*Blatella germanica*
6	*CG5599* / putative NADH dehydrogenase with LaAt activity	32441	*Drosophila melanogaster*
7	*CG8680* / putative NADH dehydrogenase with LaAt activity	33744	*Drosophila melanogaster*
8	*Desat1* / Desaturase 1	117369	*Drosophila melanogaster*
9	*Desat2* / Desaturase 2	41536	*Drosophila melanogaster*
10	*Fad2* / Desaturase F	44006	*Drosophila melanogaster*
11	*CG18609* / putative Elongase (ELO)	37158	*Drosophila melanogaster*
12	*CG9458* / putative Elongase (ELO)	41214	*Drosophila melanogaster*
13	*spidey* / 3-keto-acyl-CoA-reductase (KAR)	31703	*Drosophila melanogaster*
14	*Hacd1* / 3-hydroxy-acyl-CoA-dehydratase (HADC)	34614	*Drosophila melanogaster*
15	*Hacd2* / 3-hydroxy-acyl-CoA-dehydratase (HADC)	34762	*Drosophila melanogaster*
16	*Sc2* / trans-enoyl-CoA-reductase (TER)	38457	*Drosophila melanogaster*
17	*NlFAR7* / fatty acyl-CoA reductase (FAR)	MG573162.1	*Nilaparvata lugens* (brown pl
18	*NlFAR9* / fatty acyl-CoA reductase (FAR)	MG573164.1	*Nilaparvata lugens*
19	*CG10097* / putative fatty acyl-CoA reductase (FAR)	3771756	*Drosophila melanogaster*
20	*Cyp4g1* / Cytochrome P450-4g1	30986	*Drosophila melanogaster*
21	*LmCYP4G102* / Cytochrome P450-4g1	ANW46746.1	*Locusta migratoria* (grass hop

Figure legends

Figure 1 : Simplified overview of CHC biosynthesis. The biosynthesis pathway branches at different stages into different CHC compound classes. The main CHC compound classes are methyl-branched alkanes, straight-chain alkanes, alkenes and dienes. Enzyme abbreviations: ACC: Acetyl-CoA carboxylase, FAS: Fatty acid synthase (m: microsomal, c: cytosolic), LaAT: Lipoamide acyltransferase, ELO: Elongase, KAR: 3-keto acyl-CoA-reductase, HADC: 3-hydroxy-acyl-CoA-dehydratase, TER: Trans-enoyl-CoA-reductase, FAR: Fatty acyl-CoA reductase. CYP4G: Cytochrome P450 Decarbonylase. Numbers next to the enzymes correspond to the associated gene transcripts we detected in our tested Blattodea species (compare to Tab. 1). Adapted from Holze, Schrader, and Buellesbach (2020).

Figure 2 : Comparison of average CHC ratios (relative percentages) from the studied representative termite and cockroach species, categorized according to their levels of social complexity. The six major CHC compound classes detected in these species were n-alkanes, n-alkenes, as well as mono-, di-, tri and tetramethyl branched alkanes and are indicated by different colors. Acronyms for the investigated species are used here and in all subsequent figures as follows: *R. flavipes* (Rf), *C. formosanus* (Cf), *K.flavicollis* (Kf), *N. castaneus* (Nc),*M. darwiniensis* (Md), *B. germanica* (Bg) and*B. orientalis* (Bo). Insect images have been obtained from the Darmstadt Insect Scanner DISC3D (Ströbel et al. 2018) and have been kindly provided by Sebastian Schmelzle.

Figure 3 : Comparison of molecular phylogeny (left) and a chemical dendrogram (right) of our Blattodea study species. The molecular phylogeny is adapted from He et al. (2021) and the chemical dendrogram is based on average chemical Manhattan distances reflecting the median CHC divergence separating the different cockroach and termite species.

Figure 4 : Comparison between counts of CHC biosynthesis gene transcripts (indicated in blue) and counts of total individual CHC compounds (indicated in orange) detected in our representative cockroach and termite species. Correlations between these two metrics were assessed with a 2 (chisquare) test (r=0.12, p=0.79). Their respective levels of social complexity are indicated in both cases. Insect images have been obtained from the Darmstadt Insect Scanner DISC3D (Ströbel et al. 2018) and have been kindly provided by Sebastian Schmelzle.

Figure 5 : Heatmap normalized by average relative abundances of CHC biosynthesis gene transcript counts (columns) from high (darker colors) to low (lighter colors) grouped by species and their respective level of social complexity. The numbers indicated at the gene transcripts correspond to their respective position in the CHC biosynthesis pathway (see Fig. 1 and Tab. 1).

Data Accessibility Statement

All data underlying the presented study will be made available at the dryad data repository under https://doi.org/10.5061/dryad.cc2fqz6dh.

Competing Interests Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions section

Conceptualization: J. B.; methodology: M.G., J.B., M.H.; validation: J. B., M.G., M.H.; formal analysis: M.G., J.B., M.H.; investigation: J. B., M.G., M.H.; resources: D.P.M.; M.H.; data curation: J. B., M.G., M.H.; writing—original draft preparation: J. B., D.A.F.; writing—review and editing: J. B., D.A.F., M.H., D.P.M., M.G.; visualization: J. B., D.A.F. supervision: J.B., D.P.M.; project administration: J. B., D.P.M.; funding acquisition: D.P.M., J.B.

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Appendix

Tab. S1 : Retention indices (RI), CHC compound identifications and the respective relative quantities as well as standard deviations (in %) for all tested cockroach and termite species. Non-detectable amounts of the respective compounds are indicated by hyphens. Where compound identifications were ambiguous due to multiple possible methyl branch positions based on the detected ion pairs, all possible compound configurations are given. Retention indices we calculated according to the position of detected n -alkanes in our samples and, where not available, with a C21-40 n -alkane standard run under the same conditions. Please note that for compounds beyond C40, the$n$ -alkane positions had to be extrapolated according to the average distances between n -alkanes.

RI	Compound ID	Diagnostic ions
1500	n-C15	212
1700	n-C17	240
1738	3-MeC17	239; 56; 224
2107	n-C21	296
2207	n-C22	310
2270	4-MeC22	309; 70; 280
2280	C23-ene1	322
2287	C23-ene2	322
2310	n-C23	324
2343	9-; 11-;13-MeC23	323; 140; 224; 168; 196;
2349	7-MeC23	323; 112; 252
2371	4-MeC23	323; 70; 294
2379	7,11-;7,13-DiMeC23	309; 112; 266; 182; 196; 210; 168
2380	3-MeC23	323; 56; 308
2380	C24-ene1	336
2386	C24-ene2	336
2394	C24-ene3	336
2409	n-C24	338
2442	10-;12-;14-MeC24	337; 154; 224; 182; 196; 210; 168
2463	C25-ene1	350
2473	4-MeC24	337; 70; 308
2483	C25-ene2	350
2483	C25-diene1	348
2480	3-MeC24	337; 56; 322
2484	C25-ene3	350
2492	C25-ene4	350
2509	n-C25	352
2540	C25-diene2	348
2542	9-;11-;13-;15-MeC25 (+ unsaturated non-CHC compound)	351; 140; 252; 168; 224; 196; 224, 168
2551	7-MeC25	351; 112; 280
2555	C25diene3	348
2562	C25diene4	348
2557	5-MeC25	351; 84; 308
2577	4-MeC25	351; 70; 322
2575	C25diene5	348
2581	3-MeC25	351; 56; 336
2586	5,13-DiMeC25	337; 84; 322; 210; 196
2593	C26-ene1	364
2600	C26-ene2	364
2608	n-C26	366
2613	3,7-; 3,9-DiMeC25	337; 56; 350; 126; 280; 154; 252
2641	12-; 14; 16-MeC26	365; 182; 224; 210; 196; 238; 168
2640	C26diene	362
2645	9-MeC26	365; 140; 266
2651	7-MeC26	365; 112; 294
2651	6-MeC26	365; 98; 308
2656	5-MeC26	365; 84; 322
2669	4-MeC26 (+ unsaturated non-CHC compound)	365; 70; 336
2681	3-MeC26	365; 56; 350
2680	C27-ene1	378

RI	Compound ID	Diagnostic ions
2684	C27-ene2	378
2689	C27diene1	376
2694	C27diene2	376
2699	C27-ene3	378
2703	C27-ene4	378
2711	n-C27	380
2744	9-; 11-; 13-; 15-MeC27	379; 140; 280; 168; 252; 196; 224
2742	C27diene3	376
2752	C28diene1	390
2756	C27diene4	376
2756	7-MeC27	379; 112; 308
2761	5-MeC27	379; 84; 336
2760	C27diene5	376
2770	11,13-; 11,15-; 11,17-; 13,15-; 13,17-; 15,17-DiMeC27	365; 168; 266; 210; 224; 238; 196
2775	4-MeC27	379; 70; 350
2775	9,15-: 9.17-DiMeC27	365; 140; 294; 238; 196; 266; 168
2779	C27diene6	376
2786	3-MeC27	379; 56; 364
2784	C28diene2	390
2792	5,17-DiMeC27	365; 84; 350; 266; 168
2790	5,15-DiMeC27	365; 84; 350; 238; 196
2791	5,9-; 5,11-; 5,13-DiMeC27	365; 84; 350; 154; 280; 182; 252; 210; 224
2802	C28-ene	392
2813	n-C28	394
2814	3,9-; 3,11-DiMeC27	365; 56; 378; 154, 280; 282; 252
2816	3,7-DiMeC27	365; 56; 378; 126; 308
2841	11-; 13-MeC28	393; 168; 266; 196; 238
2840	12-; 14-MeC28	393; 182; 252; 210; 224
2843	3,9,11-; 3,9,13-; 3,9,15-; 3,9,17-TriMeC27	393; 56; 392; 154; 294; 196; 252; 224; 280;
2853	6-MeC28	393; 98; 336
2858	5-MeC28	393; 84; 350
2864	C29diene1	404
2868	4-MeC28	393; 70; 364
2874	C29-ene1	406
2881	C29-ene2	406
2880	C29diene2	404
2883	C29diene3	404
2882	3-MeC28	393; 56; 378
2887	5,9-; 5,11-; 5,13-; 5,15-; 5,17-DiMeC28	379; 84; 364; 154; 294; 182; 266; 210; 238
2894	C29-ene3	406
2897	4,10-; 4,12-; 4,14-; 4,16-; 4,18-; 4,20-DiMeC28	379; 70; 378; 168; 280; 196; 252; 224; 308;
2909	n-C29	408
2920	3,7-; 3,9-; 3,11-DiMeC28	479; 56; 392; 126; 322; 154; 294; 182; 266
2940	11-; 13-; 15-MeC29	407; 168; 280; 196; 252; 224
2953	7-; 9-MeC29	407; 112; 336; 140; 308
2964	5-MeC29	407; 84; 364
2979	11,17-; 11,19-; 11,21-; 13,17-; 13,19-DiMeC29	393; 168; 294; 266; 196; 322; 140;
2983	7,11-DiMeC29	393; 112; 350; 182; 280
2985	7,17-DiMeC29	393; 266; 196
2986	3-MeC29	407; 56; 392

RI	Compound ID	Diagnostic ions
2994	5,9-; 5,11-; 5,13-DiMeC29	393; 84; 378; 154; 308; 182; 280; 210; 252
3013	3,7-DiMeC29	393; 56; 406; 126; 336
3019	3,9-; 3,11-DiMeC29	393; 56; 406; 154; 308; 182; 280
3031	3,7-DiMeC29	393; 56; 406; 126; 336
3041	3,7,11-; 3,7,13-; 3,7,15-; 3,7,17-; 3,9,11-; 3,9,13-; 3,9,15-; 3,9,17-TriMeC29	421; 56; 420; 126; 350; 196; 280; 224; 252;
3061	14,18-; 14-22-DiMeC30	407; 210; 266; 280; 196; 336; 140;
3063	4-MeC30	421; 70; 392
3075	C31ene	434
3076	C31diene	432
3080	5,9-; 5,11-; 5,13-; 5,15-DiMeC30	407; 84; 392; 154; 322; 182; 294; 210; 266;
3088	4,8-; 4,10-; 4,12-DiMeC30	407; 70; 406; 140; 336; 168; 308; 196; 280
3102	3,9-; 3,11-DiMeC30	407; 56; 420; 154; 322; 182; 294
3112	4,8,12-; 4,8,14-; 4,8,16-TriMeC30 (+ acetic acid)	435; 70; 420; 140, 350; 210; 280; 238, 252;
3130	11-; 13-; 15-MeC31	435; 168; 308; 196; 280; 224; 252
3154	13,17-; 15,17-DiMeC31	421; 196; 294; 266; 224
3178	5,9-;5,11-; 5,13-; 5,15-;5,17-DiMeC31	421; 84; 406; 154; 336; 182; 308; 210; 280;
3203	3,9-; 3,11-; 3,13-; 3,15-DiMeC31	421; 56; 434; 154; 336; 182; 308; 210; 280;
3477	C35-ene	490
3531	11-MeC35	491; 168; 364
3629	12-MeC36	505; 182; 364
3729	11-; 13-MeC37	519; 168; 392; 196; 364
3751	11,15-DiMeC37	505; 168; 406; 238; 336
3774	5,15-; 5,17-DiMeC37	505; 84; 490; 236; 336; 266; 308
3840	C39diene	544
3876	C39-ene	546
3927	11-;13-;15-MeC39	547; 168; 420; 168; 392; 224; 364
3949	11,15-DiMeC39	533; 168; 434; 238; 364
3953	13,17-DiMeC39	533; 196; 406; 266; 336
3973	5,15-; 5,17-DiMeC39	533; 84, 518; 238; 364; 266; 336
4054	C41diene	572
4074	C41-ene	574
4084	C41-ene	574
4143	11-; 13-; 15-; 17-MeC41	575; 168; 448; 196; 420; 224, 392; 252; 36
4173	13,15-; 13,17-DiMeC41	561; 196; 434; 238; 392; 266; 364

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