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Review Article

Cuticular hydrocarbons in ants (Hymenoptera: Formicidae) and other insects: how and why they differ among individuals, colonies, and species

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Abstract

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

Key words: Acclimation, adaptation, communication, nestmate recognition, queen pheromone, review, waterproofing.

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Introduction

Functions of cuticular hydrocarbons: The body surface of nearly every insect is covered with a layer of cuticular hydrocarbons (CHCs). They fulfil several functions vital for the insect, the two best studied ones being protection against water loss (waterproofing) and communication. The waterproofing function was already recognised almost 85 years ago, when Ramsay (1935) noted that water droplets on the wings of cockroaches evaporated slower than those on artificial surfaces. Later studies following this discovery centred on the role of CHCs in preventing water loss (Beament 1945, Edney 1957, Locke 1965). Only in the 1960s, with the advent of gas-chromatography mass-spectrometry (GC-MS), biologists started to grasp the immense diversity of CHCs on insects (Blomquist & Bagnères 2010). Even a single insect can possess up to ca. 100 different hydrocarbons (Blomquist 2010b). They vary in chain length (mostly between C20 and C45), number and position of methyl branches, and number and position of double bonds (Fig. 1). Nearly all ant species contain *n*-alkanes, and most species also possess monomethyl alkanes (which can make up 50% of the CHC profile; F. Menzel, unpubl.). Further common substance classes include dimethyl alkanes, alkenes, and (less commonly) alkadienes, tri- and tetramethyl alkanes. Even rarer are alkatrienes and methyl alkenes (with a double bond *and* a methyl group). A few studies also detected very long-chain compounds (up to C60) in ants and other insects, some of which are hydrocarbons (Akino 2006, Cvačka & al. 2006, Sutton & al. 2013, Bien & al. 2019). So far, they have been studied in relatively few species, such that more research is needed to understand their variability and their biological function.

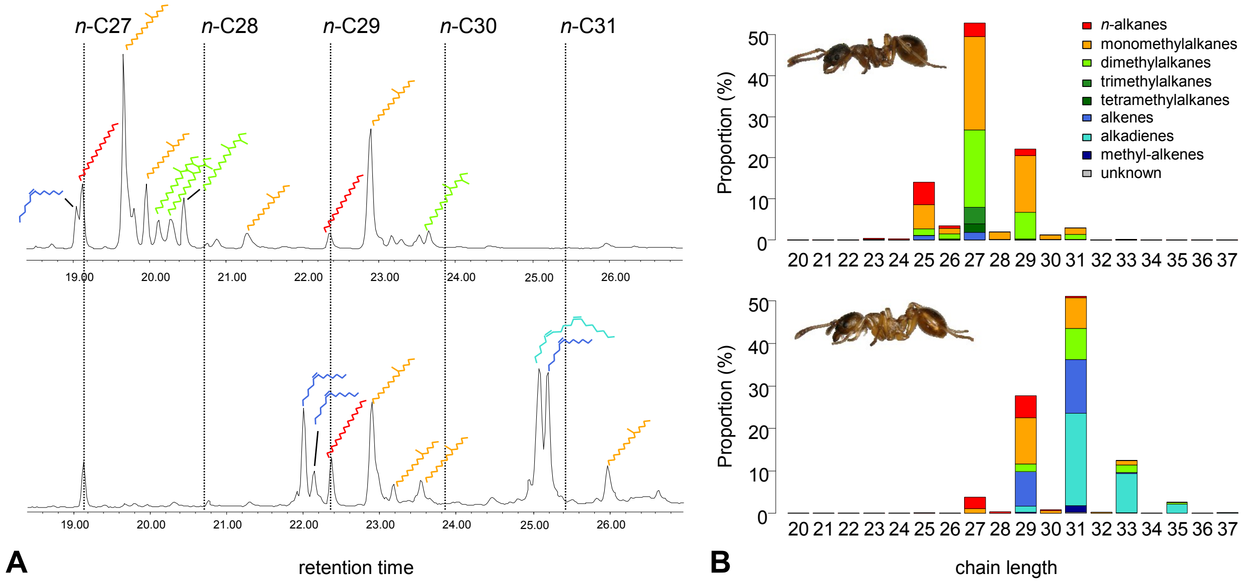

Fig. 1: Cuticular hydrocarbon profiles of the ants *Myrmica rubra* (above) and *Myrmica ruginodis* (below). The graphs in (A) show the peaks as the output of the GC-MS analysis. Vertical dotted lines indicate the retention times of *n*-alkanes. The substance class of the major peaks is indicated by small symbols with the same colour code as Fig. 1B. Within a single chain length, methyl-branched alkanes appear after the corresponding *n*-alkane, while unsaturated hydrocarbons usually appear before the corresponding *n*-alkane. Note that, for better visibility, the graphs only show the peaks between C27 and C31. These GC-MS graphs are then transformed to barplots (B), where hydrocarbons are pooled according to CHC class and chain length. Using these plots, you can see that most CHCs have odd-numbered chain length (i.e., number of carbon atoms in the backbone of the molecule). Furthermore, > 80% of all CHC belong to only three chain lengths (*M. rubra*: C25, C27, C29; *M. ruginodis*: C29, C31, C33). This method of visualisation allows a quick overview of the CHC composition – the idea is to visualise overall CHC composition, and variation thereof. This way, variation among profiles (or treatments) can be seen in relation to overall CHC variation. Note however, that different methyl group and / or double bond positions *within* the same CHC class and chain length are not resolved. Photos of the ants were taken by Philipp Sprenger.

Within a species, CHC variation is mostly quantitative, that is, individuals possess the same set of hydrocarbons, but in different relative quantities (Figs. 2, 3). Notably, many species possess homologous series, that is, hydrocarbons with the same methyl group and / or double bond positions, but different chain lengths (Martin & Drijfhout 2009a). Across species, in contrast, we find an enormous qualitative diversity of cuticular hydrocarbons, that is, insects of different species can possess entirely differently sets of hydrocarbons (Figs. 1, 4). The profiles are usually so specific that one can easily identify species based on their cuticular hydrocarbon profile alone (Kather & Martin 2012) (Box 1). The complex composition of CHC profiles allows to store a lot of information. Indeed, their important role for chemical communication was discovered in the early 1970s (Carlson & al. 1971, Blomquist & Bagnères 2010), and since then there has been a plethora of studies on this function. In many solitary insects, they serve as contact sex pheromones (Thomas & Simmons 2008, Niehuis & al. 2011, Thomas 2011, Buellesbach & al. 2013) and can indicate breeding status in burying beetles (Steiger & al. 2007, Scott & al. 2008, Steiger & al. 2008).

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

Less well-studied functions of CHCs include their role as a barrier against microbes (Wurdack & al. 2017), lubrication of the cuticle (Cooper & al. 2009), and the enhancement of foot adhesion via CHC droplets left as footprints when an insect walks (Drechsler & Federle 2006, Wüst & Menzel 2017). Furthermore, CHCs mediate interspecific recognition between host and parasite (Lenoir & al. 2001b) and between mutualists (Menzel & al. 2008a, Lang & Menzel 2011, Menzel & al. 2014) (Fig. 5). Naturally, many functions mean many, and possibly conflicting, requirements. The complex interplay of all these different functions makes the evolution of CHC highly complex and intriguing.

Linking composition and function: A plethora of studies shows how CHC profiles vary within a species, for

Box 1: Various sources of CHC variation and their relative contributions.

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

The random forest method additionally allows to infer the importance of single hydrocarbons for the classification and thus shows which substances differ most strongly. For each classification, we report the five CHCs (or CHC blends) most important for the classification. Note that their substance classes often differ among sources of variation: For example, the most important species and genus differences concern mostly trimethyl, dimethyl and monomethyl alkanes. In contrast, temperature differences concern mostly *n*-alkanes and monomethyl alkanes. Different substance classes are indicated as different colours (see Fig. 1).

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

Comparison	Classification error rates			
	T. ambiguus	*T. longispinosus*	*M. rubra*	*M. ruginodis*
humidity (50% / 100% rh) (workers only)	*20.83%* *n*-C29 13-; 15-Me C31 13,17-; 15,19-DiMe C33 *n*-C28 *n*-C27	*20.00%* 7-Me C27 C29ene *n*-C29 13,17-; 15,19-DiMe C33 *n*-C28	*24.17%* 2 unknown CHCs *n*-C19 15,x-DiMe C35 2-; 4-Me C30	*26.25%* *n*-C24 9,13-; 7,11-DiMe C29 10-; 12-; 13-; 14-Me C30 15,19-DiMe C35 13,17- ; 13,19-DiMe C33

Box 2: Perception of cuticular hydrocarbons in ants.

Insects perceive chemicals using three families of chemosensory receptors: odorant receptors (ORs), gustatory receptors (GRs) and ionotropic receptors (IRs) (Hansson & Stensmyr 2011). Although GRs can perceive pheromones in *Drosophila*, they are not expressed on ant antennae, and thus presumably do not contribute to cuticular hydrocarbon (CHC) perception (Fleischer & al. 2018). Odours are perceived via olfactory sensillae on the antenna: One form, the basiconical sensillae, seem to be responsible for CHC perception (Nakanishi & al. 2009, Sharma & al. 2015). Each of them contains multiple odorant receptor neurons (ORN), the membranes of which contain olfactory receptors (ORs) next to their obligate *orco* co-receptor proteins (Ozaki & Wada-Katsumata 2010, Sharma & al. 2015, Trible & al. 2017, Yan & al. 2017). After CHCs diffuse into the sensillum lymph, they initially bind to an odorant binding protein (OBP) in the lymph and then are transported to the ORs (Fleischer & al. 2018, Fleischer & Krieger 2018). Neural processing of the perceived CHCs happens in glomeruli in the antennal lobe (Nakanashi & al. 2010, Trible & al. 2017). The morphology of the sensillae and that of antennal lobes in ants is plastic, but also sex-specific (Nakanashi & al. 2010, Ghaninia & al. 2018). Interestingly, eusocial insects possess higher numbers of both, receptors and glomeruli, compared to solitary insects (Zhou & al. 2012, Tsutsui 2013).

The ability of ants to perceive the variety of different CHCs is reflected in gene expansions in the OR family (Zhou & al. 2012, McKenzie & al. 2016). Recent studies found that individual ORs are narrowly tuned and might respond to only few compounds (albeit not single CHC), such that they collectively generate an integrated odour perception (Pask & al. 2017, Slone & al. 2017). The olfactory system of ants shows very similar stimulation through nestmate and non-nestmate hydrocarbons (Brandstaetter & al. 2011, Brandstaetter & Kleineidam 2011, Sharma & al. 2015), suggesting that nestmate recognition involves learning mechanisms.

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

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

Firstly, all CHC functions are likely to be influenced by the biophysical properties of the CHC layer. Most of them, like waterproofing, protection against microbes, lubrication and foot adhesion, depend even solely on these physical properties (chemical effects such as toxicity, polarity or chemical interactions with the insect cuticle or the surface of microbes are unlikely since hydrocarbons lack functional groups). For example, the waterproofing ability of a CHC layer is higher if it is viscous and / or contains solid parts (Sprenger & al. 2018, Menzel & al. 2019). *n*-alkanes and terminally branched monomethyl alkanes aggregate most tightly and are thus more viscous than other CHCs or even solid. Hence, they should be most beneficial in preventing water loss (Gibbs 1998, Gibbs & Rajpurohit 2010, Brooks & al. 2015). The waterproofing ability correlates with the melting temperature (T_m), and thus increases with chain length in homologous series of hydrocarbons (Gibbs & Pomonis 1995, Gibbs 2002). Methyl branches (mainly in di-, tri- and tetramethyl alkanes) and unsaturation introduce "disorder" into the

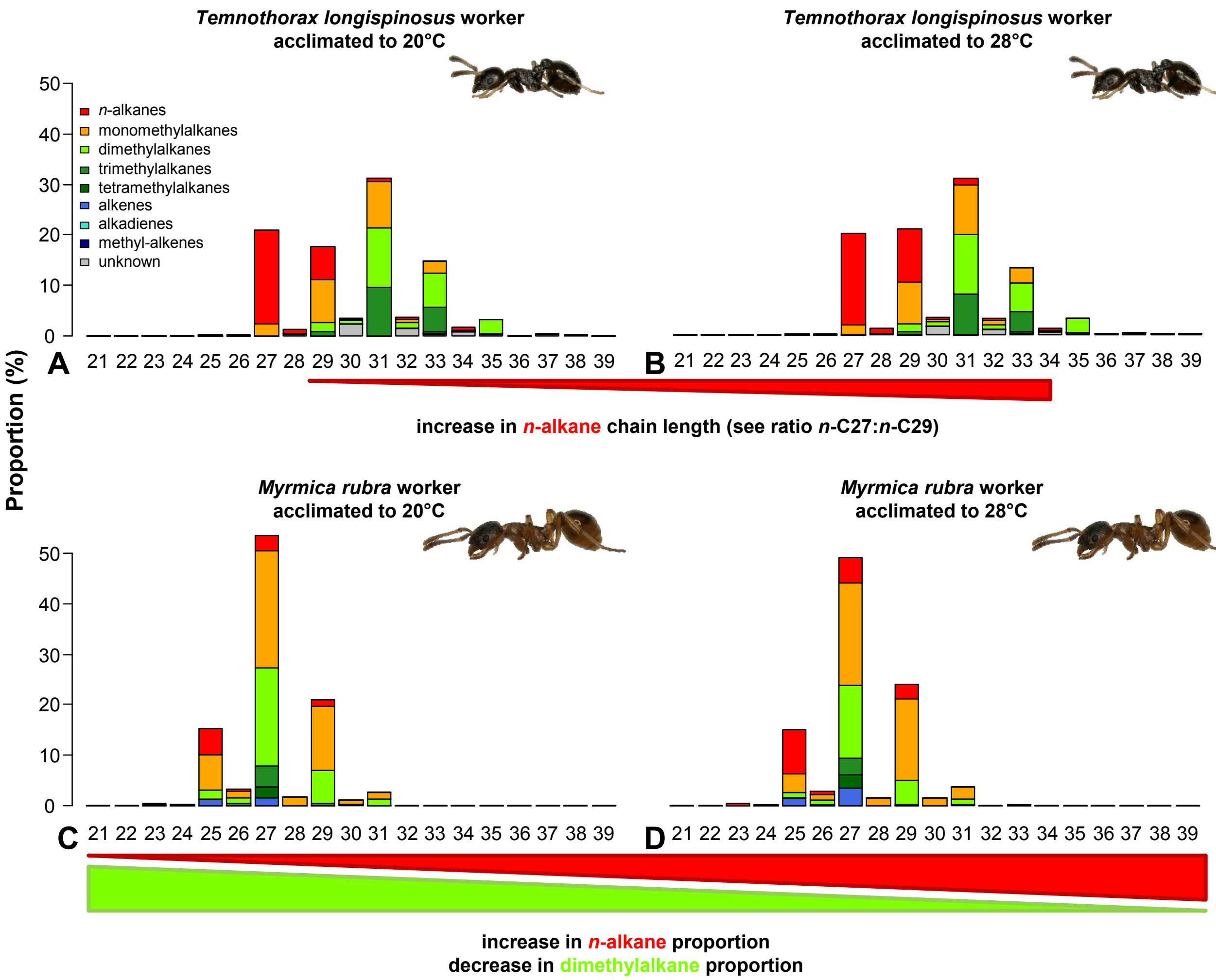

Fig. 2: Cuticular hydrocarbon temperature acclimation in the two ants *Temnothorax longispinosus* and *Myrmica rubra*. Bar graphs of CHC plots (see Fig. 1 for details) for *Temnothorax longispinosus* (A, B) and *Myrmica rubra* (C, D) workers acclimated to either 20 °C (A, C) or 28 °C (B, D). The two species represent two (not mutually exclusive) acclimation strategies: *T. longispinosus* workers increase the average chain length of *n*-alkanes (e.g., more *n*-C29 compared to *n*-C27), while *M. rubra* workers increase the proportion of *n*-alkanes under high temperature, while both species reduce the proportion of dimethyl alkanes. All plots are based on mean proportions of 120 workers each. Photos of the ants were taken by Philipp Sprenger. Data from Menzel & al. (2018) and Sprenger & al. (2018).

layer, hindering the molecules to aggregate tightly. This reduces T_m, which is why these "disruptive" substance classes provide less protection against water loss (Gibbs & Pomonis 1995, Gibbs 1998). Thus, the composition of CHC profiles directly influences its viscosity and melting range. Acclimatory CHC changes (Fig. 2) are predictable based on these physical properties (Menzel & al. 2018, Sprenger & al. 2018), which confirms that they are relevant for biological functionality. Beside acclimation, they should also matter for footprint adhesion. Foot adhesion might be enhanced if hydrocarbons are less viscous, such that larger hydrocarbon droplets are left as footprints when the insect walks.

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

CHC-based information is encoded via compositional differences, be they quantitative or qualitative (see Box 2 for perception and neural processing). For example, CHC profiles can encode the queen signal, which regulates worker reproduction in a colony (see section "Intrinsic variation within social insect colonies"). In this context, it is important to distinguish between signals, which were selected for communication (i.e., intended exchange of in-

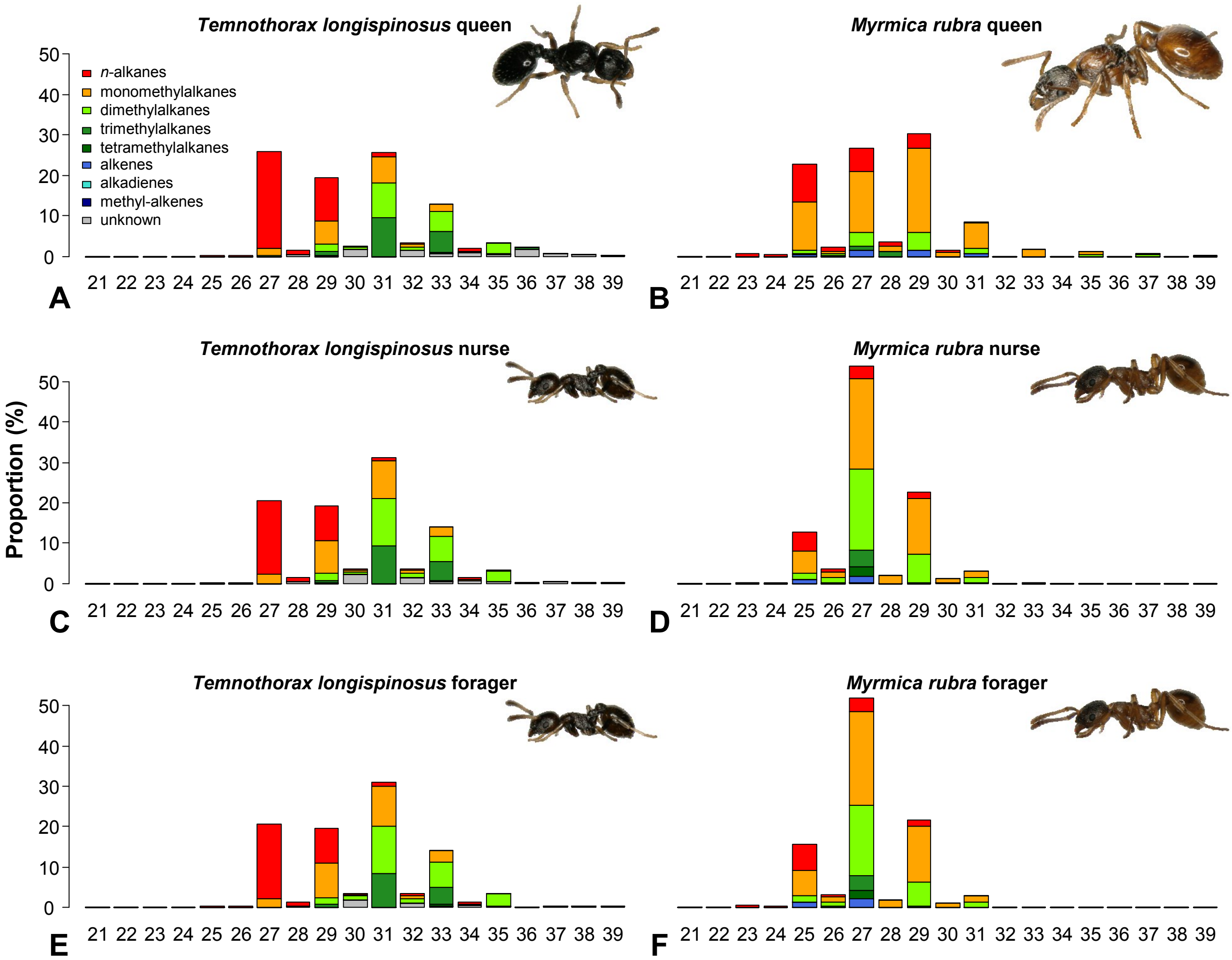

Fig. 3: Cuticular hydrocarbon caste differences in the two ants *Temnothorax longispinosus* and *Myrmica rubra*. Barplots of CHC profiles of fertile dealate queens, nurses and foragers (from top to bottom) for *T. longispinosus* (A, C, E) and *M. rubra* (B, D, F) are shown. While the profiles of queens are clearly distinct, the differences between the behavioural castes are minute and barely visible. Photos of the ants were taken by Philipp Sprenger.

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

CHC profiles are multidimensional. Hydrocarbon differentiation among colonies, castes, sexes, etc. is frequently analysed by quantifying all hydrocarbons, followed by multivariate statistics. While useful, this approach emphasises differences among groups, but neglects what they actually consist in. Thus, one might lose sight of the actual magnitude of these differences relative to the entire profile. For example, compare the differences between 20 °C - and 28 °C - acclimated workers (Fig. 2) or between nurses and foragers (Fig. 3) to those between queens and workers in *Myrmica* (Fig. 3) or between species (Figs. 1, 4). All of them are highly significant, but the latter concern a much larger proportion of the entire profile than the former – however, in separate multivariate analyses, this would not be obvious. In our opinion, we need to study what differences among groups actually consist in if we want to fully understand causes and consequences of CHC variation. To this end, it is helpful to use clearly defined unidimensional traits, such as the proportion of a certain CHC class, average chain length, or the number of homologous series, and treat them as functional traits sensu McGill & al. (2006). This approach allows clear and testable predictions how each trait affects CHC functionality, for example based on biophysical properties or biosynthetic pathways.

Due to their multiple functions, it is likely that CHCs influence not only interactions among conspecifics (e.g., by encoding information), but also contribute to a species' ecological niche (e.g., if it protects against reducing water

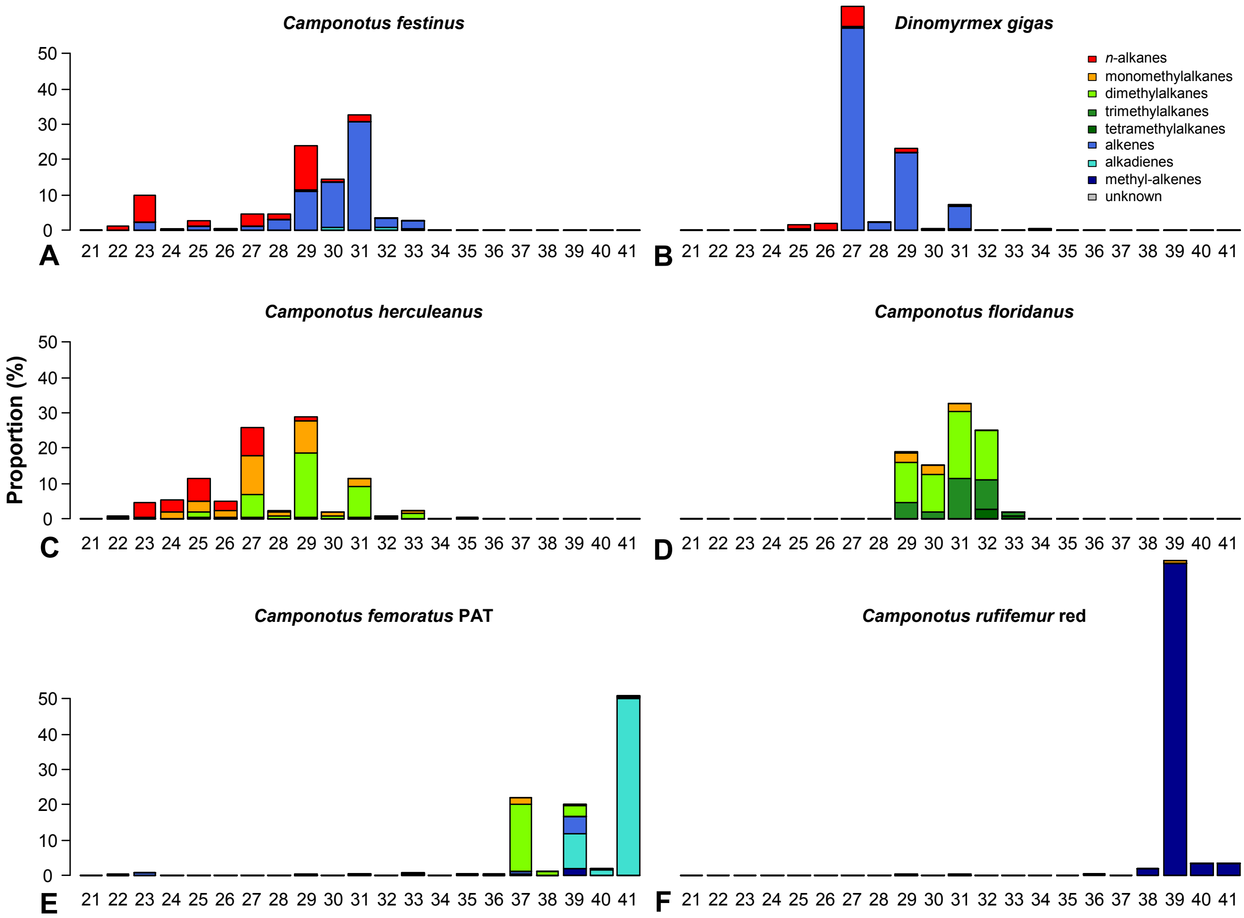

Fig. 4: Cuticular hydrocarbon profiles of selected Camponotini Formicidae: Formicinae species. The species were selected to exemplify the astonishing chemical variability even within a single tribe of ants. The profiles of *Camponotus festinus* (A) and *Dinomyrmex* (formerly *Camponotus*, Ward & al. 2016) *gigas* (B) are dominated by alkenes and *n*-alkanes, while *C. herculeanus* (C) and *C. floridanus* (D) possess mainly methyl-branched compounds next to *n*-alkanes. *Camponotus femoratus* PAT (E) and *C. rufifemur* red (F) represent examples of parabiotic species showing characteristic chain elongations and high abundances of alkadienes and methylbranched alkenes, which are rather unusual in non-parabiotic species. Data from Menzel & al. (2017).

loss, but only to a certain degree or only for a certain temperature range). However, the complexity of CHC profiles makes it challenging to understand which chemical trait serves which function, and whether there are conflicts or trade-offs between different functions. Furthermore, we have to understand how biophysical mechanisms and biosynthetic pathways constrain CHC variation as we will outline below. Finally, we have to distinguish plastic and genetically fixed variation, both of which may or may not be adaptive.

In this review, we aim to provide an overview of the extrinsic sources of plastic CHC variation, the intrinsic sources of CHC variation within a colony, among conspecific colonies, between sexes and across species, as well as potential constraints on CHC variation. In Box 1, we explore how different sources of variation act on the same CHC profile, and to which degree this variation allows to classify individuals. We will summarize what is known on certain sources of variation and discuss which general patterns can be derived and whether the variation is likely to be adaptive and/or predictable. Although we largely focus on ants, most effects we describe either have been shown or are likely to occur in other insect taxa as well.

Extrinsic causes for reversible plasticity

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

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

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

The chemical strategies to cope with high ambient temperatures differ among species: Firstly, insects can enhance waterproofing by adjusting the composition of CHC classes in their profile. Here, the largest acclimatory effects often concern strongly aggregating (e.g., *n*-alkanes) and strongly disruptive (e.g., multiply methyl-branched alkanes, alkadienes) compounds, while intermediate classes like monomethyl alkanes show weaker changes although they constitute 20 - 50% of the CHC profile in some cases (Menzel & al. 2018, Sprenger & al. 2018). With higher temperatures, linear *n*-alkanes increase in abundance, while multiply methyl-branched alkanes and alkadienes decrease (grasshoppers: Gibbs & Mousseau 1994, termites: Woodrow & al. 2000, ants: Buellesbach & al. 2018, wasps: Michelutti & al. 2018, ants: Sprenger & al. 2018) (Fig. 2). For alkenes, the response is less clear up to now – although one would expect them to reduce overall waterproofing, they often co-vary tightly with *n*-alkanes, that is, they also increase with temperature (Buellesbach & al. 2018, Sprenger & al. 2018). The reason for this is still unclear. Beside these changes in relative abundance of substance classes, some insects and other arthropods can also change the average chain length, that is, they increase the ratio of longer-chained to short-chained CHCs in the profile (beetles: Hadley 1977, scorpions: Toolson & Hadley 1979, ants: Menzel & al. 2018, Duarte & al. 2019; P. Sprenger & F. Menzel, unpubl.) (Fig. 2).

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

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

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

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

N e s t m a t e r i a l. Behavioural experiments indicate that the nest material can influence the colony odour and thus interfere with nestmate recognition in ants (Crosland 1989, Heinze & al. 1996). Some studies demonstrated that the smell of the nest material modulates the template responsible for nestmate recognition, that is, the ants habituate to the presence of additional compounds, but do not necessarily change their CHC profile (because additional non-CHC compounds can be present on the cuticle) (Pickett & al. 2000, Katzav-Gozansky & al. 2004). The nest material can take up ant CHCs, thus supporting CHC exchange among nestmates to unify the colony odour (Bos & al. 2011). Similarly, honey bees acquire hydrocarbons from wax combs during physical contact, which are colony-specific and affect nestmate recognition (Breed & al. 1995a, b, Breed & al. 1998). CHC deposition in nest material or on the soil around the nest can also be useful for home-range marking (Lenoir & al. 2009). However, also social parasites can acquire their host's CHCs from the nest material and this way facilitate their integration into the colony (Lenoir & al. 2001b, Emery & Tsutsui 2016).

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

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

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

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

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

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

Microbiome. As diet can also affect the gut microbiome, dietary effects on CHCs may be linked to microbial changes in many cases. The microbiome, that is, bacterial endosymbionts or parasites, was in some cases shown to influence the CHC production and profile. The role of microbial symbionts on insect pheromone communication was recently reviewed in detail (Engl & Kaltenpoth 2018), which is why we will focus on few interesting studies presenting effects of the microbiome on CHC profiles specifically. After that, we focus on how microbe-induced CHC changes could affect the behaviour and ecology of ants.

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

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

In ants, differences in the microbiome might also influence behaviour and ecology: In *Pogonomyrmex barbatus* harvester ants, application of microbes onto the cuticle triggered aggression towards nestmates, indicating that either the microbes directly affect the CHC profile or that ants can perceive the microbes themselves, with the aggression representing a form of social immunity (Dosmann & al. 2016). However, antibiotic removal of cuticular bacteria in the leaf-cutting ant *Acromyrmex subterraneus subterraneus* did not result in CHC changes (De Souza & al. 2013). In *Acromyrmex echinatior*, treatment with antibiotics changed the gut microbiome, which correlated with a decrease of two *n*-alkanes, as well as two acids from metapleural gland secretions (Teseo & al. 2019). Although the treatment triggered aggression, there was no association between microbiome composition and chemical distances among CHC profiles, suggesting that here, the microbiome had a limited effect on the CHC profile (Teseo & al. 2019).

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

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

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

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

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

Intrinsic variation within social insect colonies

A eusocial insect colony can only function if groups of individuals can be recognized as different. For example, reproductive division of labour requires that all colony members can recognize which individuals are allowed to reproduce and which are not. This information is usually encoded in the CHC profile. To understand how eusocial insect colonies function, it is hence crucial to understand intra-colonial CHC variation. This section focuses on intrinsic variation, which is related to the individual's physiology and caste membership. Here, differences are less likely caused by genetic (allelic) differences since colony members are more or less closely related, but rather by differences in gene expression or further epigenetic effects. In many cases, as outlined below, this differentiation is adaptive and necessary to communicate information about its bearer. In contrast to other selection pressures, however, here in most cases it seems to matter only that individuals differ, but not necessarily how they differ. It is important to note that intra-colonial variation (e.g., between queens and workers, Fig. 3) is much smaller than differences among species (Fig. 4) (Brunner & al. 2011, Menzel & al. 2018).

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

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

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

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

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

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

CHCs often vary with an ant's social environment. The presence of alien individuals in the nest can trigger an increase in the relative abundance of di- and trimethyl alkanes, but also higher overall CHC quantities (Beros & al. 2017). This might be due to the need to express more recognition cues to facilitate nestmate recognition in the presence of non-nestmates. On the other side of the gradient, isolation of single individuals can lead to differences in overall CHC quantity (Ichinose & Lenoir 2009), but also in compositional differences (Lenoir & al. 2001a). For example, isolated ants can carry lower proportions of methyl-branched CHCs, which are relevant for recognition, and possess more *n*-alkanes instead (Kleeberg & al. 2017). In isolated workers, inter-individual differences (e.g., among patrilines) may become apparent, which are otherwise concealed by the "Gestalt" odour (Martin & al. 2012). However, the precise reasons for changes due to isolation are hard to uncover, since isolation means various changes - social stress, lack of CHC exchange with nestmates, queen absence, and further factors.

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

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

Thus, cuticular hydrocarbon variation among members of the same colony is influenced by physiological (age, fertility / ovary development, mating status, isolation stress) and genetic effects. Many of these differences are likely to be species-specific and thus hard to generalise. In some cases they might be due to pleiotropic effects if, for example, fertility, aging, mating status, and ovarian development activate genes that also influence CHC profiles. Other effects may be consistent across species, including conserved queen signals, and forager-nurse differences, which may be due to different waterproofing requirements.

Variation among colonies

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

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

To allow nestmate recognition, CHC profiles must differ among colonies, but it is less relevant which CHCs differ as long as the difference is detectable. However, because aggression increases with chemical distance between the opponents, nestmate recognition should in theory select against polymorphic cues, which would make nestmate recognition impossible ("Crozier's paradox", CROZIER 1986). CROZIER concluded that they are selected for something else. Later models suggested that cue diversity could persist in a population under disassortative mating or if colonies with rare odours have a higher fitness (because they are more effective in identifying non-nestmates; negative frequency-dependent selection, RATNIEKS 1991, HOLMAN & al. 2013b). Indeed, *Linepithema* ants from genetically less diverse colonies are less tolerant than those from more diverse colonies, which may select for a reduction in overall genetic (and cue) diversity (TSUTSUI & al. 2003).

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

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

Sex differences and sexual selection

Most studies on sex differences in CHC profiles so far were on solitary insects. Quantitative and / or qualitative sex differences were reported, for example, for crickets (TREGENZA & WEDELL 1997, THOMAS & SIMMONS 2008), beetles (STEIGER & al. 2009, GINZEL 2010 and references therein), flies (reviewed in FERVEUR 2005, FERVEUR & COBB 2010), but also non-social Hymenoptera like jewel wasps (BUELLESBACH & al. 2013, BIEN & al. 2019), mason wasps (WURDACK & al. 2015) or solitary bees (AYASSE & al. 2001, CONRAD & al. 2017). Sexual CHC dimorphism suggests that CHCs function as sex pheromones (THOMAS & SIMMONS 2008), and may be sexually selected (see below). In ants, surprisingly few studies deal with male CHC profiles. Here, sex differences are mostly quantitative (ANTONIALLI-JUNIOR & al. 2007, BEIBL & al. 2007, OPPELT & al. 2008, CHERNENKO & al. 2012, KLEEBERG & al. 2017). However, there are sex-specific hydrocarbons in ponerine ants of the genera *Diacamma* (CUVILLIER-HOT & al. 2001) and *Odontomachus* (SMITH & al. 2014, 2016). Due to the relative scarcity of studies on sexual CHC dimorphism in ants, it is difficult to draw general conclusions here.

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

CHC variation among species

As mentioned, CHC profiles are highly species-specific (Box 1; Fig. 4) and to large parts genetically heritable (MARTIN & al. 2008b, VAN ZWEDEN & al. 2009, GUILLEM & al. 2016); which is why they can be used as taxonomic tools for species delimitation (SEPPÄ & al. 2011, KATHER & MARTIN 2012, BERVILLE & al. 2013). This chemical diversity leaves many questions open: How do CHC profiles evolve, and how fast can evolutionary CHC changes happen? Why do CHC profiles diversify? And how are CHC differences linked to speciation? Firstly, CHC variation among species can be due to drift, which should be detectable as a phylogenetic signal. Secondly, CHC variation may be due to character displacement after speciation events. Finally, CHC traits can represent adaptations to different abiotic and biotic selection pressures.

Non-adaptive variation: phylogenetic signal and genetic drift: Whether CHC differences show a phylogenetic signal depends on the taxonomic level, but also on the kind of data investigated. When considering presence or absence of homologous series, van Wilgenburg and colleagues found evidence for gradual evolution of this trait (van Wilgenburg & al. 2011), possibly because it reflects the availability of the biosynthetic pathways to produce certain substances. This is in stark contrast to the strong qualitative differences (especially concerning CHC class composition) between species in many pairs of closely related species, which are sympatric in at least part of their range (Elmes & al. 2002, Morrison & Witte 2011, Seppä & al. 2011, Pokorny & al. 2013, Sprenger & al. 2018, Hartke & al. 2019). Many other quantitative traits like the proportion of specific CHC classes or average chain lengths did not show phylogenetic signal, indicating that they can evolve faster than expected under a Brownian Motion model (Menzel & al. 2017b), which may be reinforced by plastic variation. Qualitative traits like the presence or absence of CHC classes may or may not show a phylogenetic signal (van Wilgenburg & al. 2011, Menzel & al. 2017b). Not unexpectedly, this suggests that quantitative traits evolve faster than qualitative ones, probably because quantitative changes presumably happen via gene regulatory changes rather than via changes in gene sequence. Since most CHC classes are also found in primitive hymenopteran species, it is likely that the required biosynthetic pathways were already present in the common ancestor of ants, bees and wasps (Kather & Martin 2015). CHC diversification in a population often coincides with speciation, suggesting that most CHC traits may evolve in a rather "saltational" mode (Mullen & al. 2007, Schwander & al. 2013, Menzel & al. 2017b).

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

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

Adaptations to biotic interactions. Ants interact with many arthropods, and these interactions are often mediated by CHCs (Lenoir & al. 2001b, Ness & al. 2009) (Fig. 5). Firstly, a multitude of "ant guests" (myrmecophiles) exploits ant colonies. Various rove beetles (Coleoptera: Staphylinidae), caterpillars (Lepidoptera: Lycaenidae), crickets, cockroaches, springtails, spiders or mites (Witte & al. 2008, Parmentier & al. 2014) live in ant nests. They manage to get fed by the ants, steal food from them or eat food remainders, but some of them also eat ant brood – thus, they are commensals or parasites.

For these species, the most important thing is to avoid ant aggression. Many species do this by carrying similar recognition cues as their hosts, either via chemical mimicry (i.e., biosynthesis of host CHC) or chemical camouflage (i.e., active acquisition of host CHC through physical contact). Chemical mimicry is employed by socially parasitic ants as well as by non-ant myrmecophiles (Lenoir & al. 2001b, von Beeren & al. 2012a, Guillem & al. 2014). For example, *Maculinea* caterpillars (Lycaenidae) mimic the profiles of their *Myrmica* hosts (Akino & al. 1999, Elmes & al. 2002, Nash & al. 2008). CHC profiles of parasites can even show local adaptation to better mimic local hosts (Ruano & al. 2011). Camouflage, that is, active acquisition of host cues, has been shown for various taxa, including spiders (von Beeren, Hashim, & al. 2012), silverfish (von Beeren & al. 2011), and *Formicoxenus* guest ants (Lenoir & al. 1997). Some species, including social parasites and Lycaenid caterpillars, employ mimicry and camouflage at the same time (Akino & al. 1999, Bauer & al. 2010). While many ant guests are rather harmless, social parasites can

Fig. 5: Cuticular hydrocarbons play important roles in intraspecific and interspecific interactions. (A) A virgin queen of *Camponotus ligniperda* shortly after leaving its nest, Germany. CHC profiles of virgin queens differ from worker profiles, but also from those of mated queens. (B) A *Camponotus cruentatus* worker antennating an aphid, Southern France. (C) *Formica* workers tending aphids, Northern Spain. Although these trophobiotic interactions are driven by aphid honeydew, ants recognize their aphid partner based on CHC profiles. All photos by Florian Menzel.

be devastating and essentially kill a colony, and thus exert a strong selection on their hosts. The host species counteract chemical mimicry by diversifying their recognition cues, making it more difficult for parasites to mimic their profiles. Indeed, host populations where slave-making ants are present show higher CHC diversity (within and among colonies) compared to unparasitized populations (Martin & al. 2011, Jongepier & Foitzik 2016).

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

In the long run, however, multi-species systems should favour a second strategy to avoid recognition, which is to express generally few recognition cues (Kleeberg & al. 2017, von Beeren & al. 2018). This so-called "chemical insignificance" was shown, for example, for *Brachymyrmex* (a lestobiotic ant), which produces only few cuticular hydrocarbons (Lenoir & al. 2001b). Similarly, queens of the slavemaker *Polyergus* exhibit few CHC before they enter host nests for the first time, which may facilitate their acceptance (Lenoir & al. 2001b). In other species, chemical insignificance is not achieved by generally less cuticular hydrocarbons, but by providing fewer informative hydrocarbons. *n*-alkanes are generally thought to have little value for nestmate recognition. A high proportion of *n*-alkanes can thus ensure waterproofing and simultaneously expose few recognition cues. This "chemical transparency" is employed by the social parasite *Acromyrmex insinuator* (Nehring & al. 2015) and evolved several times convergently among *Temnothorax* slavemakers (Kleeberg & al. 2017). Whether chemical mimicry or insignificance are employed can also depend on the parasite's life history, that is, whether it lives in the host nest or only sneaks in temporarily (Uboni & al. 2012).

Next to parasites, also mutualists shape CHC profiles. One remarkable example are parabiotic associations, in which two ant species share a common nest in amity (Orivel & al. 1997, Menzel & al. 2008b). These associations often involve species of the ant genera *Camponotus* and *Crematogaster* (Menzel & Blüthgen 2010). Both parabiotic partners usually keep their (strongly different) species-specific CHC profile and attack non-parabiotic species, but tolerate their partner species (Menzel & al. 2008a, b, Parmentier & al. 2017). This unusually high tolerance is probably linked to very high chain lengths, which are characteristic for the profiles of parabiotic species, and evolved convergently in several *Camponotus* and *Crematogaster* clades (Menzel & al. 2017a, b). A second characteristic of parabiotic CHC profiles is their high proportions of unsaturated CHCs, which might arise from biophysical constraints and the need to ensure a partly liquid CHC layer despite high chain lengths (Menzel & Schmitt 2012, Menzel & al. 2014, Sprenger & al. 2019) (Fig. 4 E, F). Thus, living in a parabiotic association exerts predictable selection pressures on CHC traits and represents a striking example of biotic interactions shaping CHC evolution (Menzel & Schmitt 2012, Menzel & al. 2017a).

Constraints on variation

Upon comparing cuticular hydrocarbon profiles among species, one notices that by far not all possible combinations of cuticular hydrocarbons are realised – thus, there are constraints on CHC variation. Firstly, most species produce only a limited range of chain lengths. Among CHC profiles of 85 *Camponotus* and *Crematogaster* species (Menzel & al. 2017a), three different chain lengths (e.g., C27, C28, C29) already accounted for more than 50 % of all CHCs in 49 species. In 10 species, more than 50 % of all CHCs even belonged to a single chain length. Furthermore, most CHCs have odd-numbered chain lengths – 86.3 ± 1.6 % SE in the same dataset (F. Menzel, unpubl.). Finally, insects (not only ants) often produce homologous series of hydrocarbons over several chain lengths, such that the number of homologous series is substantially lower than the actual number of different CHCs on an insect (Martin & Drijfhout 2009a; F. Menzel, unpubl.). These observations may not be surprising, but they indicate that CHC composition in insects is constrained. These constraints probably arise from their biosynthesis. The preponderance of odd-chain CHCs stems from their origin from fatty acids, which are step-wise elongated by C2 units, until the terminal carboxyl moiety is reduced to a carbonyl moiety and then removed (Blomquist 2010a). The presence of homologous series, and at the same time a limited range of chain lengths, might originate from biosynthetic pathways if enzymes involved in CHC elongation produce a normal distribution of homologous CHC series rather than a single CHC type, for example, if enzymes that stop chain elongation via reduction are not substrate-specific but accept substrates of different chain lengths. If this is the case, producing a homologous series of CHCs might require fewer different enzymes and thus be cheaper than producing CHCs of different homologous series. However, up to now this is speculation because the specificity of enzymes involved in CHC biosynthesis is scarcely known.

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

The second type of constraints acting on CHC profiles are functional constraints due to their material properties. The CHC layer must be partly liquid to enable a homogenous coating of the CHC layer. A sufficiently low viscosity is also required for communication: recognition cues must be volatile enough to be perceived by other species (Menzel & al. 2019), and the need for sufficiently low viscosity accounts for several constraints found in CHC profiles. For example, the proportion of *n*-alkanes and monomethyl alkanes decreases strongly with the average chain length of a CHC profile, while the proportion of multiply branched or unsaturated compounds is higher in profiles with high average chain length (Menzel & al. 2017a). This can be explained from the above considerations (see introduction), namely that an increase in chain length leads to higher viscosity and too great parts of the CHC layer being solid; hence insects must introduce methyl groups and/or unsaturations in these compounds to maintain the fluidity of the CHC layer. Different requirements for viscosity (e.g., in more or less flexible parts of the body), or different requirements for waterproofing may even apply *within* the same individual: Recently, two studies suggested that CHC profiles vary among body parts of the same individual (Wang & al. 2016a, b).

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

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

Conclusions

A huge number of factors influence CHC profiles, and disentangling them is challenging. While multivariate analyses of the entire CHC composition are useful to quantify different sources of variation, they yield few insights as to *how* CHC profiles vary, and how the studied variation scales to overall variability of the profile. Here, the most promising approach in our opinion is to investigate univariate CHC traits such as proportions of different CHC classes, average chain lengths (per CHC class), and number of homologous series. In addition, analysis of variation within specific subsets of the CHC profile (like certain CHC classes) may be useful to test specific hypotheses, for example, on the role of different

CHC classes for waterproofing or communication (Martin & al. 2013). Depending on the research question, quantitative (based on the compound abundances) or qualitative (based on their presence / absence) traits should be chosen. This way, we can account for different biophysical properties or biosynthetic pathways of different CHCs, and formulate specific predictions. For example, certain traits, like the abundance of *n*-alkanes or their composition, should be more affected by the climate (and, via climate, by geographic location or season), while others, like the abundance of alkenes or their composition, should be less affected by climate but show a stronger signal of colony identity (Martin & al. 2013). Such approaches will help to identify which traits vary independently from each other, and where there is co-variation.

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

1. The link between physical properties and chemical composition. Here, future research can develop precise predictions how physical behaviour varies with CHC composition, and this will help to elucidate the adaptive value of CHC composition and its link to an insect's ecological niche.
2. The genomic basis of CHC variation: the genes involved in CHC biosynthesis and the biosynthetic pathways necessary to synthesize an entire profile. Which genomic changes are responsible for profile differences between sister taxa? How many allelic changes are needed to cause quantitative or qualitative profile differences? How many pathways are up- or downregulated during CHC acclimation? Here, it will be important to understand how many genes underlie the synthesis of a homologous series or an entire profile. This way, we can understand which compounds are biosynthetically linked (leading to pleiotropic effects, constraining CHC variation), and which are decoupled (Martin & Drijfhout 2009a).
3. The evolution of communication signals. Which components of a signal (e.g., fertility signals, forager-nurse differences, colony signatures) are conserved, and which are species-specific? Is colony identity encoded in ways such that the signal is less affected by acclimatory changes? Here, further research can identify the evolutionary trajectories of such differences, and link them to physiological differences.
4. The perception of CHCs: How specific are olfactory receptors and pheromone-binding proteins? To understand CHC-based communication, we also need to consider receptor variation among species, and account for potential differences in receptivity among species or among castes within a colony.
5. Which role do CHCs play in speciation? How does CHC differentiation evolve within a population, and in which cases is it followed by assortative mating? Since CHCs often function as sex pheromones, they might be drivers of speciation, and thus, the evolution of biodiversity.

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