© 2019. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2019) 222, jeb210807. doi:10.1242/jeb.210807

RESEARCH ARTICLE

Communication versus waterproofing: the physics of insect cuticular hydrocarbons

Florian Menzel[1,*], Svenja Morsbach[2], Jiska H. Martens[1,3], Petra Räder[2], Simon Hadjaje[3], Marine Poizat[3] and Bérengère Abou[3]

ABSTRACT

Understanding the evolution of complex traits is among the major challenges in biology. One such trait is the cuticular hydrocarbon (CHC) layer in insects. It protects against desiccation and provides communication signals, especially in social insects. CHC composition is highly diverse within and across species. To understand the adaptive value of this chemical diversity, we must understand how it affects biological functionality. So far, CHCs have received ample research attention, but their physical properties were little studied. We argue that these properties determine their biological functionality, and are vital to understanding how CHC composition affects their adaptive value. We investigated melting behaviour and viscosity of CHCs from 11 ant species using differential scanning calorimetry and a novel microrheological technique. CHCs began melting below –45°C, and often were entirely liquid only above 30°C. Thus, they formed a solid–liquid mixture under ambient conditions, which contrasts to previous assumptions of entirely solid layers in many species. This may be adaptive as only biphasic CHC layers ensure uniform coating of the insect body, which is necessary for waterproofing. CHC viscosity was mostly between 0.1 and 0.2 Pa s^{-1}, thus similar to motor oils. Surprisingly, chemically different CHC profiles had similar viscosities, suggesting that a certain viscosity level is adaptive and ensures that communication signals can be perceived. With this study, we draw attention to the importance of studying the physics of CHC layers. Only by understanding how chemical and physical mechanisms enable CHC functionality can we understand the causes and consequences of CHC diversification.

KEY WORDS: Ants, Viscosity, Melting point, Lipid layer, Material properties, Phase behaviour, Ecophysiology

INTRODUCTION

Understanding the evolution of multifunctional traits is one of the major challenges in evolutionary biology. Traits – be they morphological, behavioural or physiological – are shaped by selection pressures arising from their function. Trait differences between species can often be traced back to ecological niche differences (Lamichhaney et al., 2014). However, many traits fulfil multiple functions at the same time. For example, mouth shapes in cichlid fishes have been selected by the species-specific feeding habits, but are also important for brood care (mouth breeding) (Brawand et al., 2014). The colour patterns in *Heliconius* butterflies serve as an aposematic signal against predators, but also attract mating partners (Jiggins, 2008). Such multifunctionality leads to multiple, potentially conflicting selection pressures, which makes the evolution of such traits especially complex and intriguing.

One multifunctional trait of paramount importance in virtually all insects is the cuticular hydrocarbon (CHC) layer. CHC layers are highly complex, comprising mixtures of up to 100 different compounds on a single insect. They cover the whole body surface and fulfil a variety of functions: they protect the insect against desiccation and ensure waterproofing (Ramsay, 1935), but also serve as communication signals in many species (Blomquist and Bagnères, 2010). Especially in social insects, CHCs encode a plethora of information, regulating (amongst other things) nestmate recognition and the division of labour within a colony (Leonhardt et al., 2016). Less well-studied functions of CHC include foot adhesion via CHC droplets (Drechsler and Federle, 2006), lubrication of the cuticle (Cooper et al., 2009), and presumably the formation of a barrier against microorganisms (Howard and Blomquist, 2005). The complexity of CHC profiles makes it challenging to understand how CHC layers can serve all functions at the same time, especially because different functions may pose conflicting requirements.

CHC profiles pose a puzzle to evolutionary biologists: even congeneric species show an enormous diversity, and sister species can have radically different profiles (Kather and Martin, 2012; Martin and Drijfhout, 2009; Menzel et al., 2017; Otte et al., 2018). However, the causes of this diversity are still largely unknown. In particular, we know surprisingly little about the selection pressures acting on CHC profiles and the causes and consequences of CHC diversification across species. Owing to their multiple functions, it is likely that CHCs influence not only interactions between conspecifics (e.g. in social insects), but also contribute to a species' abiotic and biotic ecological niche, thus mediating niche partitioning among sister taxa.

To understand the selection pressures on CHC layers that caused their diversification, we must understand how CHC composition affects biological functioning. For many of the functions mentioned above, the physical properties of CHC layers – their viscosity, phase behaviour and melting behaviour – are of crucial importance, as we will outline below. However, they have received little attention in studies on the biology of insect CHCs so far (but see Gibbs, 2002), which has hampered our understanding of CHC evolution in insects.

CHCs are not completely solid at room temperature, but can rather be pictured as a wax-like layer that covers the insect body. Like wax, CHCs can melt, i.e. they might be solid, liquid or a solid–liquid mixture. This is relevant for waterproofing: insects lose water when water molecules diffuse through the CHC layer, which is

[1]Institute of Organismic and Molecular Evolution, Faculty of Biology, University of Mainz, Hanns-Dieter-Hüsch-Weg 15, 55128 Mainz, Germany. [2]Max Planck Institute for Polymer Research, Ackermannweg 10, 55128 Mainz, Germany. [3]Matière et Systèmes Complexes (MSC), UMR CNRS 7057, Université de Paris, 75205 Paris Cedex 13, France.

*Author for correspondence (menzelf@uni-mainz.de)

F.M., 0000-0002-9673-3668

Received 17 July 2019; Accepted 28 October 2019

facilitated by lower viscosity of the liquid compounds and/or a lower abundance of the solid ones (Gibbs and Rajpurohit, 2010). Note that viscosity, which is related to the diffusion rate of molecules (Einstein, 1905), is not defined for solid matter. Indeed, melting seems to influence cuticular permeability: water permeability increases rapidly above a certain temperature (critical temperature, T_c). Previous studies related T_c to the melting of the CHC profile (Rourke and Gibbs, 1999), suggesting that permeability increases when all hydrocarbons are melted. However, this model is a simplification, because a CHC layer melts over a temperature range rather than at a specific temperature (Gibbs, 1995), making it difficult to pinpoint T_c from the melting behaviour alone.

The melting behaviour of a CHC layer is determined by its chemical composition. However, a single insect can possess up to 100 different hydrocarbons, including *n*-alkanes, monomethyl alkanes, alkenes, dimethyl alkanes, alkadienes and other CHC classes, with chain lengths mostly between C25 and C35 (Martin and Drijfhout, 2009). These CHC classes differ in their packing ability, which results in different melting points. *n*-Alkanes can pack (i.e. aggregate) most tightly owing to van der Waals bonds. Because they lack branches or kinks in their molecular structure, they can form crystals (Maroncelli et al., 1982). Pure *n*-alkanes of the chain lengths common in insects have melting points between 40 and 60°C (Lide, 2008). Monomethyl alkanes have lower melting points, because the methyl group hinders tight packing, but can crystallise nonetheless (Brooks et al., 2015). Hydrocarbons with methyl groups near the centre of the molecule (e.g. 11-MeC27) melt earlier than those with methyl groups located towards the chain ends (e.g. 3-MeC27) (Gibbs and Pomonis, 1995). This effect supposedly weighs heavier than differences in chain length (Gibbs and Rajpurohit, 2010), which lead to an increase in melting temperature by ∼2°C per additional carbon atom in the chain. Dimethyl alkanes usually melt earlier than monomethyl alkanes (Gibbs, 2002). Finally, alkenes have even lower melting points, usually being liquid at ambient temperature. In insects, only alkenes in the Z configuration have been found so far (Blomquist, 2010). Their low melting point is due to the kink in Z-alkenes, which hinders tight packing (Gibbs and Pomonis, 1995). Note that melting dynamics of multi-component mixtures can be hard to predict, e.g. due to melting point depression. Hence, melting behaviour is influenced non-additively by the presence of other components, and therefore differs between pure substances and the same substances when mixed with others (Lira-Galeana et al., 1996; Rim and Runt, 1984).

The high complexity of CHC blends should lead to broad melting ranges that include the melting points of different pure hydrocarbons. Although the existence of melting ranges was acknowledged earlier (Gibbs, 1995), they have not been thoroughly characterised or experimentally demonstrated to date. To visualise the melting range, Gibbs (2002) introduced the 'phase separation model', proposing that CHC layers are biphasic at ecologically relevant temperatures, with crystals of saturated hydrocarbons in a liquid matrix of unsaturated ones. Despite the vital importance of melting behaviour for biological functions, however, the biphasic state of CHCs under an insect's living conditions has not been empirically confirmed (nor rejected) up to now.

Not only waterproofing, but also CHC-based recognition is likely to be affected by the physical properties of the CHC layer. When individuals touch each other with their antennae, they perceive each other's CHC profile via olfactory sensillae (Ozaki and Wada-Katsumata, 2010). Hydrocarbons presumably bind to odorant-binding proteins (OBPs) and are then transported through the sensillum lymph to odorant receptors in the membrane of the odorant receptor neuron (ORN) (Ozaki and Wada-Katsumata, 2010; Sharma et al., 2015). CHC-based nestmate recognition in ants is possible without physical contact (Brandstaetter et al., 2008), indicating that some CHCs evaporate or sublimate in sufficiently high quantities to be detectable. Nestmate recognition also frequently happens via contact chemoreception, which is also mediated by ORNs (Sharma et al., 2015; Pask et al., 2017). Overall, CHC perception is likely enhanced by (for contact chemoreception), or even requires (for reception without contact), a certain level of volatility because volatile CHCs can reach the OBPs via diffusion through the air. Consequently, communication should benefit from a high vapour pressure of recognition cues. Given that vapour pressure of pure liquids decreases with their viscosity (Othmer and Conwell, 1945), CHC viscosity should indicate how well liquid CHCs can be perceived, and thus, how well information can be transmitted. Solid hydrocarbons might be perceivable by olfaction if they enter the gas phase directly from the solid state via sublimation, but the vapour pressure of solids is usually much lower than that of liquids.

Up to now, it was unclear how chemical CHC differences translate into biologically relevant properties. The only knowledge on the precise relation of chemical composition and physicochemical behaviour comes from pure substances or artificial mixtures of two compounds (Gibbs, 1995; Gibbs and Pomonis, 1995). From the above considerations, waterproofing and communication may pose conflicting requirements, and thus conflicting selection pressures on the physical properties of the CHC layer. For example, various arthropods adjust their CHC profiles to current climatic conditions to reduce water loss (Gefen et al., 2015; Menzel et al., 2018; Sprenger et al., 2018; Wagner et al., 2001). This seems counterintuitive – why produce less waterproof CHCs under cool or humid conditions (Menzel et al., 2018; Sprenger et al., 2018) instead of having optimal waterproofing all the time? The costs of CHC production are unlikely to matter here – they are low compared with the resting metabolic rate (Dirks and Federle, 2011) and, more importantly, unlikely to differ much between profiles with good or worse waterproofing abilities. This suggests that there is a trade-off between different CHC functions: a high viscosity enhances waterproofing, but may interfere with the inter-individual exchange of recognition cues encoded in the CHC profile and their diffusion across the insect body.

Research questions

Here, we studied melting behaviour and viscosity of insect CHCs. Our aim was to assess variation of these traits within and across species, and to study how they are linked to their chemical composition. Until recently, they were hardly measurable owing to the minute quantities of insect hydrocarbons. Here we applied a novel technique to measure viscosity in quantities of less than 100 pl, and performed highly sensitive differential scanning calorimetry measurements to determine melting ranges. We analysed melting behaviour, phase behaviour and viscosity of CHC extracts of eight to 11 ant species with highly different chemical profiles, and related these traits to their chemical composition. Based on these data, we discuss how chemical and physical properties may influence the balance between the different functions of CHC layers in insects.

MATERIALS AND METHODS

Samples

In ants, conspecific individuals usually possess the same set of hydrocarbons, while different species differ in qualitative hydrocarbon composition (Kather and Martin, 2015; Menzel et al., 2017). Since we wanted to analyse chemically different

CHC profiles, we chose 11 different ant species of the genera *Lasius*, *Formica* and *Myrmica* from Central Europe (Table S1). Species were selected to obtain high variation in CHC class composition. For example, we included species with large proportions of alkenes (*Lasius fuliginosus*), alkadienes (*Myrmica ruginodis*), as well as species that possessed neither of these classes (*Lasius niger*, *Formica rufibarbis*) (Table S1, Fig. 1A). For all species, we collected foraging workers directly in the field.

CHCs were extracted by immersing freeze-killed ants into hexane for 10 min. For viscosity samples, we extracted single workers from the same colony, with two samples each for 11 species. In addition, viscosity and CHC data were obtained from previously sampled ants, for nine *Myrmica rubra* (viscosity data for 25°C only) and two *M. ruginodis* (viscosity for 20, 25 and 28°C) samples from seven and one colony, respectively. All extracts were fractionated over SiOH columns (Chromabond, 100 mg, Macherey-Nagel) and eluted with hexane to remove potential polar contaminations or gland secretions, and retain only hydrocarbons in the extracts.

Melting ranges were obtained using differential scanning calorimetry (DSC). Since larger substance quantities are required here (low mg range), we used extracts of 400–1000 foragers from one colony per sample. For this reason, samples for DSC could only be obtained for eight species. However, where enough samples were available, reproducibility was checked by collecting two samples from different colonies (biological replicates from *L. niger*, *L. neglectus* and *M. ruginodis*). Hexane extracts were directly transferred into DSC sample aluminium pans (100 µl volume, Mettler-Toledo GmbH, Gießen, Germany) and the solvent was evaporated slowly under ambient conditions. DSC pans were covered with aluminium lids.

Chemical analysis

Chemical analysis was performed for those samples that were then used to measure viscosity. Extracts of individual ants were concentrated to a volume of ∼20 µl, and we injected 2 µl into a gas chromatograph coupled to a mass selective detector (GC-MSD) (Agilent Technologies, GC: Agilent 7890A, MSD: Agilent 5975C) equipped with a DB5-HT column (Zebron Inferno, 30 m×0.25 mm; coating: 0.25 µm). Injection was performed in the split-less mode at 250°C, using helium as carrier gas with a constant flow of 1.2 ml min^{-1}. The oven temperature stayed at 60°C for 2 min, then heated up at a rate of 60 K min^{-1} to 200°C and afterwards with a constant rate of 4 K min^{-1} up to 320°C. This temperature was held constant for 10 min. Masses were scanned in the range 40–500 amu at an ionization voltage of 70 eV. Data were acquired using the software MSD ChemStation (E.02.02.1431; Agilent Technologies). Hydrocarbons were identified according to a retention index based on a standard series of *n*-alkanes (Carlson et al., 1998) and diagnostic ions. We excluded substances that were not hydrocarbons (<10% of the total extract) and substances with maximum areas below 0.5% per species. To reduce the dimensionality of our dataset, we categorised each hydrocarbon into one of the following substance classes – *n*-alkanes, monomethyl alkanes, dimethyl alkanes, trimethyl alkanes, alkenes, alkadienes, alkatrienes, methylbranched alkenes and putative CHCs of unknown class – and calculated the proportional abundance of each class. Furthermore, we calculated the average chain length of the CHC profile, weighing each substance by its proportional abundance.

Differential scanning calorimetry

DSC measurements were performed on a DSC 823 instrument (Mettler-Toledo GmbH, Gießen, Germany). Heating–cooling–heating cycles were recorded with a heating/cooling rate of 10 K min^{-1} between −100 and +100°C. The measurements were performed under nitrogen atmosphere with a flow rate of 30 ml min^{-1}. For determination of melting ranges, the second heating cycle was evaluated to avoid effects of sample history. Heating curves were baseline-corrected and integrated in 15 K intervals between −75 and +60°C. The resulting areas under the curves correspond to the energy needed to melt the sample compounds in the respective temperature interval, and were therefore termed ‘melting heats’. To validate our measurements, we measured melting of *n*-C25 and *n*-C28 (Fig. S1). The melting points obtained agree with literature data (Srivastava et al., 1993). The higher the melting heat, the more substances are melting in the corresponding temperature interval. Previous studies measured CHC melting only above 10°C, and thus could not detect whether some portions of the CHC extract were liquid at this temperature already. In contrast, our measurements started at −100°C, thus enabling us to detect phase transitions in a much wider temperature range.

Viscosity

A single ant possesses 1–5 µg of CHCs (Wüst and Menzel, 2017). This is far too little for performing standard rheology measurements, which require at least a few millilitres of material. Microrheology, by contrast, typically requires less than 1 µl of sample and is thus suitable for rheological measurements even with tiny material quantities, which is common in the study of insects. However, even 1 µl of liquid insect CHCs can be difficult to obtain. Here, we used a novel technique to collect minute CHC droplets, hence gathering a sufficiently large amount for microrheological measurements that are possible even with only 10 to 100 pl of collected volume (Abou et al., 2010). The technique is innovative in two respects: the collection of the fluid dispersed into tiny droplets, and microrheology in a tiny volume using dry beads. This breakthrough opened up novel research avenues, as up to now, very little was known about the liquid behaviour of CHC layers. Owing to the elaborate procedures described below, however, measuring a single sample can take 1 day.

The CHC extract of an ant worker was dissolved in 20 µl pentane and placed on a glass slide to evaporate the solvent. The CHC droplets on the glass slide were collected with a micropipette equipped with a small tip of 2–3 µm in diameter. Micropipettes were fabricated from borosilicate glass capillaries (1 mm outer diameter and 0.78 mm inner diameter, Harvard Apparatus S.A.R.L., Les Ulis Cedex, France), using a P-1000 micropipette puller (Sutter Instrument, Novato, CA, USA). The micropipette was connected to a pneumatic microinjector (CellTram Air, Eppendorf AG, Hamburg, Germany) and mounted on a three-axis micro-manipulator (Burleigh, Thorlabs SAS, Maisons-Laffitte, France) coupled to an inverted Leica DM IRB microscope (Leica Microsystems GmbH, Wetzlar, Germany). The micropipette tip was moved onto the surface to collect the largest possible amount of CHC extracts, using capillary effects or the pneumatic microinjector with positive pressure when necessary.

After being collected, the CHC extract was ejected on dry melamine beads (Acil, France; bead diameter: 0.740±0.005 µm) deposited on a glass slide using the pneumatic microinjector with a negative pressure. The samples were observed with bright-field microscopy at 100× magnification (oil immersion objective, NA=1.3, depth of focus: ∼200 nm). The sample temperature was controlled by adjusting the objective temperature with an objective heater (Bioptechs Inc., Butler, PA, USA) to within ±0.1°C. The Brownian motion of the tracer beads immersed in the CHC extract

Journal of Experimental Biology (2019) 222, jeb210807. doi:10.1242/jeb.210807

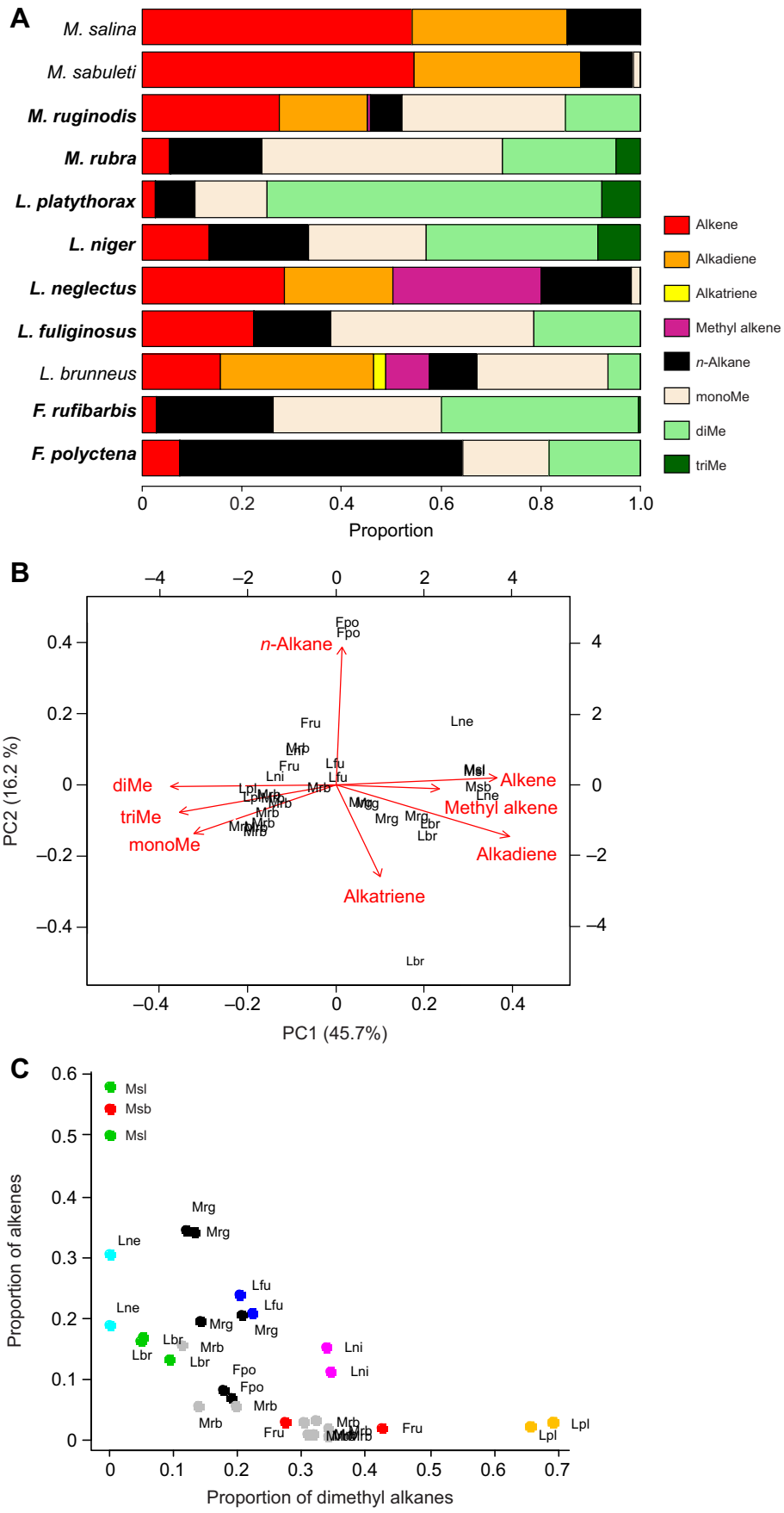

Fig. 1. Chemical composition of ant cuticular hydrocarbon (CHC) profiles. (A) Bar plots showing the relative abundance (%) of each substance class. The species for which differential scanning calorimetry (DSC) measurements were taken are printed in bold. The plots show species averages based on the samples used for viscosity measurements. (B) Chemical composition of ant CHCs. The plot shows the first two principal components of a PCA on the CHC extracts used for this study. The first PC axis (PC1) is positively correlated with the abundance of unsaturated compounds and negatively correlated with the abundance of methyl-branched hydrocarbons. The second PC axis (PC2) is positively correlated with the abundance of *n*-alkanes and negatively with those of alkatrienes. (C) Scatterplot showing the proportion of alkenes versus the proportion of dimethyl alkanes in different ant species. Fpo, *Formica polyctena*; Fru, *Formica rufibarbis*, Lbr, *Lasius brunneus*; Lfu, *Lasius fuliginosus*; Lne, *Lasius neglectus*; Lni, *Lasius niger*; Lpl, *Lasius platythorax*; Mrb, *Myrmica rubra*; Mrg, *Myrmica ruginodis*; Msb, *Myrmica sabuleti*; Msl, *Myrmica salina*; monoMe, monomethyl alkane; diMe, dimethyl alkane; triMe, trimethyl alkane.

Journal of Experimental Biology (2019) 222, jeb210807. doi:10.1242/jeb.210807

was recorded for 20 s at 100 Hz with an sCMOS fast camera (OrcaFlash4.0 v2+, Hamamatsu Photonics France S.A.R.L., Massy, France) mounted on the inverted microscope.

Self-written image analysis software allowed us to track the $x(t)$ and $y(t)$ positions in time t of any beads close to the focus plane of the objective. For each tracer bead, the time-averaged mean-squared displacement (MSD) was calculated according to the formula: $\langle \Delta r^2(t) \rangle_{t'} = \langle [x(t+t')-x(t)]^2 + [y(t+t')-y(t)]^2 \rangle_{t'}$.

For Brownian motion of tracers in a purely viscous fluid, the ensemble-averaged MSD linearly increases with the lag time, as $\langle \Delta r^2(t) \rangle = 4Dt$ (in two dimensions), where D is the diffusion coefficient. In this case, the viscosity η is estimated using the Stokes–Einstein relation, $\eta = kT/6\pi RD$, where R is the bead diameter and kT is the thermal energy (Einstein, 1905). Viscosities were measured at three different temperatures, which are in the range of living conditions for insects (20, 25 and 28°C). Each measurement was performed once temperature reached an equilibrium state. For some samples, especially at 20°C, viscosity could not be measured if too large proportions of the CHCs were solidified, thus making it impossible to collect CHCs with the micropipette. Full data on viscosity and CHC classes are available in the supplementary dataset.

Statistical analysis

The relation of chemical composition to viscosity was analysed for each measurement temperature (20, 25 and 28°C) separately. To reduce the number of variables in chemical composition, we first conducted a principal component analysis (PCA) with the relative abundances of substance classes entering the analysis. The data for these eight substance classes (*n*-alkanes, monomethyl, dimethyl and trimethyl alkanes, alkenes, alkadienes, alkatrienes and methylbranched alkenes) were Aitchison-transformed (Aitchison, 1986) before entering the PCA. Then, the first two principal components (with interactions allowed) and the average chain length were used as explanatory variables in three linear models, which used viscosity at 20, 25 and 28°C (respectively) as dependent variables. Here, untransformed viscosity data yielded the best distribution of model residuals; species identity was not included because this would have drastically increased the Akaike information criterion (AIC) values of the models. The impact of each factor was evaluated with a type II ANOVA (command Anova, package car; Fox and Weisberg, 2011).

In addition, viscosities were compared between measurement temperatures and species using a single linear mixed-effects model (LMM) with species and temperature as fixed effects (with interactions allowed) and sample ID as a random effect (command lmer, package lme4; Bates et al., 2015). For this model, viscosity data were fourth-root transformed to obtain normally distributed model residuals. All analyses were performed in R version 3.5.1 (https://www.r-project.org/).

RESULTS

Chemical composition

CHC extracts were highly diverse among the 11 species investigated. The content of unsaturated hydrocarbons ranged from 2.5±0.3% and 2.5±0.2% (*F. rufibarbis* and *L. platythorax*, respectively; means±s.e.m.) to 85.4±0.8% (*M. salina*). In contrast, the proportion of methylbranched alkanes ranged from 0±0% (*M. salina*) to 72.0±2.9% (*L. niger*) (Fig. 1A).

In the PCA, the first principal component (eigenvalue 3.7) explained 46% of the variation in substance class composition. It was positively correlated to the proportion of alkenes and alkadienes (Pearson correlation: both $r>0.8$, $P<0.0001$), methyl-branched alkenes ($r=0.52$, $P=0.0023$), negatively correlated to the proportions of monomethyl, dimethyl and trimethyl alkanes (all $r<-0.7$, $P<0.0001$), and not correlated to the proportion of *n*-alkanes ($r=0.031$, $P=0.87$). The second principal component (eigenvalue 1.29) explained 16% of the variance and was positively correlated to the proportion of *n*-alkanes ($r=0.86$, $P<0.0001$) and negatively to the proportion of alkatrienes ($r=-0.57$, $P=0.00064$), but not correlated to any other substance class ($-0.33<r<0.05$; $P>0.07$; Fig. 1B). Thus, most species were rich in either methylbranched or unsaturated hydrocarbons, but not both (Fig. 1C), while *n*-alkanes varied independently from this axis.

Melting and phase behaviour

DSC curves from the second heating cycle displayed only endothermic heat signals, which correspond to melting. This means that energy from the outside (i.e. melting heat) is needed to melt the compounds in the sample. Other phase transitions (e.g. metastable rearrangements) were not observed, but might have been covered by the melting effects. The CHC layers of all ant species had a large melting range with several distinct melting peaks visible. In all samples, substances started to melt between −60 and −40°C, and the last parts melted between 30 and 45°C (Figs 2 and 3). An

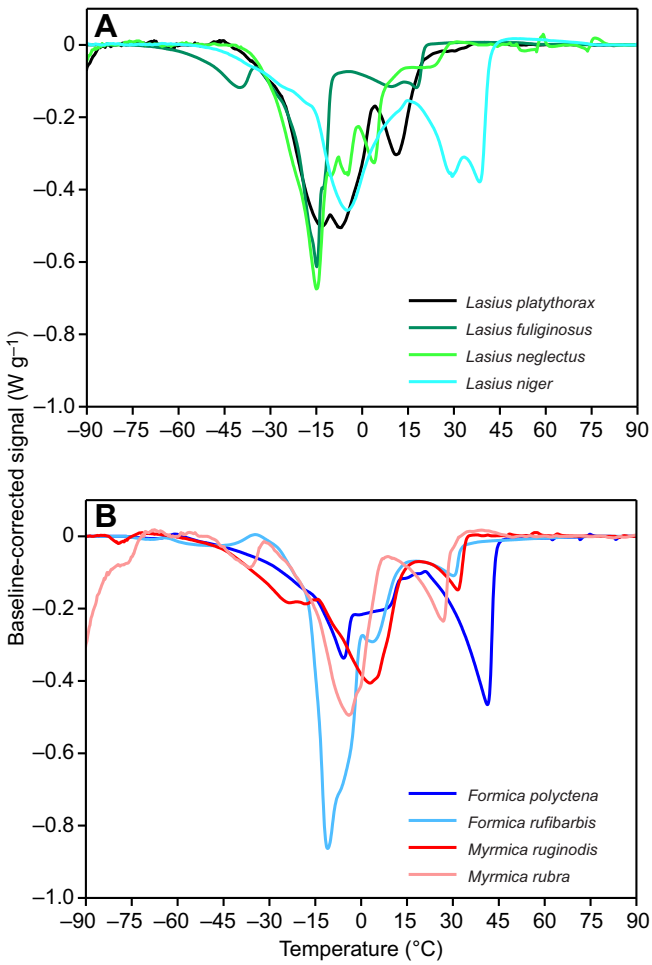

Fig. 2. DSC melting curves of extracted CHCs. All curves display the second heating cycle (heat rate of 10 K min^{-1}), are baseline corrected and normalised to the sample mass. (A) Melting curves of *Lasius* species; (B) melting curves of *Formica* and *Myrmica* species.

Journal of Experimental Biology (2019) 222, jeb210807. doi:10.1242/jeb.210807

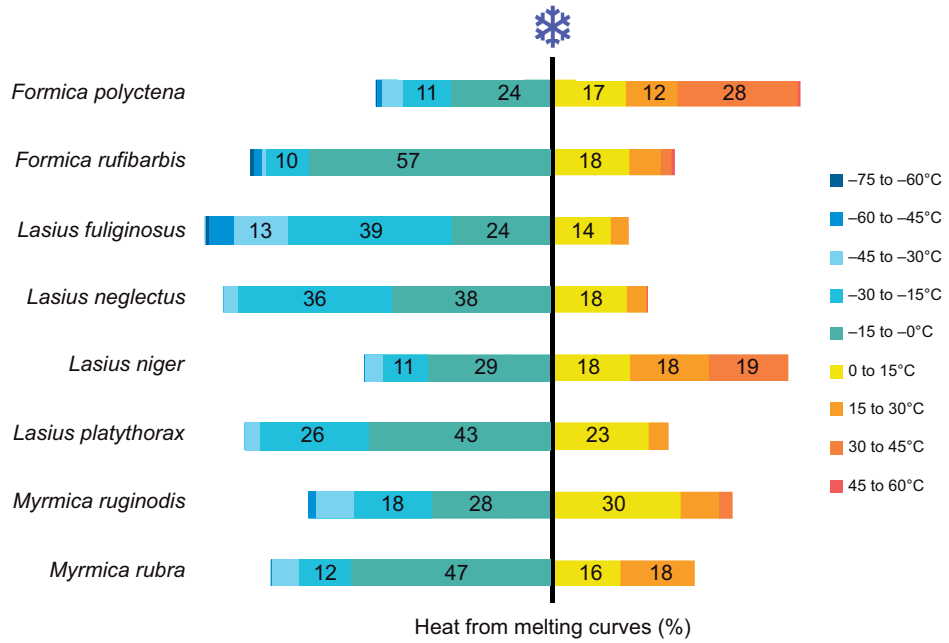

Fig. 3. Amount of heat converted for melting in regions from −75 to +60°C extracted from DSC melting curves. Values for each temperature region (15°C steps) were determined via integration of the baseline-corrected signals and are relative to the total area under each curve (each bar of the diagram represents the total heat corresponding to 100%). To make differences clearer, bars were aligned along the 0°C transition, indicated by the black line. Yellow–red colors refer to melting >0°C while green–blue colors refer to melting <0°C. Heat values >10% are written inside integration regions.

exception was *L. fuliginosus*, whose CHCs were entirely liquid at 23°C already. Replicated samples from other ant colonies of the same species, collected at similar climatic conditions, yielded similar melting curves (Fig. S2). Hence, at almost any temperature these species are likely to experience, the cuticular layer was always biphasic, and never all liquid nor all solid.

CHC layers differed in the quantities that melted at different temperature ranges, which corresponded to their chemical composition (Fig. 3). For *L. fuliginosus*, 58% of all hydrocarbons were already liquid at −15°C, compared with less than 40% in all other species. This coincides with high proportions of alkenes in this species, which have low melting points. In contrast, *Formica polyctena* CHCs, which are rich in *n*-alkanes, had the lowest proportion of melted hydrocarbons among all investigated species at 15°C (71% in *F. polyctena* versus 81% in *L. niger* and >96% in all other species; Table S2).

Viscosity

Although the CHC composition was highly variable among species, viscosities of the CHC extracts were rather similar (Fig. 4), ranging from 100 to 400 mPa s^{-1} for most samples, with the exception of some *M. rubra* samples at up to 7500 mPa s^{-1}. Surprisingly, we found only weak effects of chemical composition on viscosity. The only significant one was a negative effect of principal component 1 on viscosity at 25°C (F_1=6.24, P=0.024; Table S3), indicating that viscosity increased when samples were richer in methyl-branched alkanes and poorer in unsaturated hydrocarbons. No further effects of the principal components nor of chain length on viscosity were found (Table S3).

There were no obvious viscosity differences between species (LMM: χ^2_{10}=15.8, P=0.11). Overall, viscosity decreased with temperature (χ^2_1=41.7, P<0.0001), but this effect differed between species (species×temperature: χ^2_{10}=18.3, P=0.050). According to the model summary, temperature effects on viscosity were particularly strong in *L. neglectus*, *M. rubra* and *M. ruginodis*.

All samples were viscous and homogeneous at 28 and at 25°C, as indicated by the MSD curves increasing linearly with lag time. However, with decreasing temperature, many samples (13 out of 23) developed a biphasic behaviour below 23°C, with a liquid and a gelified phase clearly visible under the microscope. Upon decreasing temperature, gelified structures developed and spread into the liquid parts of the sample (Fig. 5). Eight out of 23 samples remained liquid when decreasing temperature down to 20°C, without the evidence of gel-forming structures, while two other samples were macroscopically gelified at 20°C, without visible liquid parts. Viscosity of the samples was higher at 20°C compared with 25°C (paired *t*-test: t_{16}=2.77, P=0.014), but did not measurably differ between 25 and 28°C (t_{21}=0.93, P=0.36).

DISCUSSION

Phase behaviour: CHCs form a solid–liquid mixture in all species

In all species we investigated, CHCs started to melt between −60 and −45°C. The last solid parts melted between +30 and +45°C in most species, and were entirely liquid in one species at 25°C already. Thus, for all investigated species, the CHC layer was biphasic (i.e. a solid–liquid mixture) throughout almost the whole temperature range they may experience in their environment. This is the first experimental evidence of the ‘phase separation model’, which has been suggested by Gibbs (2002) but, to our knowledge, not yet been empirically shown for temperatures below 30°C. Gibbs (2002) had proposed a solid–liquid mixture for species with alkenes, but hypothesised a solid, homogeneous (hence monophasic) CHC layer for species lacking alkenes, suggesting that lipid melting (or liquid CHCs, respectively) is ecologically irrelevant for many species. Our data reject this idea, because solid–liquid mixtures were also detected in species such as *F. rufibarbis*, *L. platythorax* and *L. niger*, which contain mostly methylbranched alkanes and *n*-alkanes, but only few alkenes. Note that the species we analysed were all from the temperate zone (and, being collected during summer, acclimated to summer conditions), and thus adapted to similar climatic conditions. Hence, we cannot exclude that the melting behaviour of CHCs from insects of other habitats, e.g. deserts or tropical rainforests, is fundamentally different. However, since many desert and rainforest ants possess CHCs of similar chain lengths and substance classes (Dahbi et al., 2008; Menzel et al., 2017), their melting ranges may differ quantitatively, but should not be in an entirely different temperature range. More importantly, a fundamental

Journal of Experimental Biology (2019) 222, jeb210807. doi:10.1242/jeb.210807

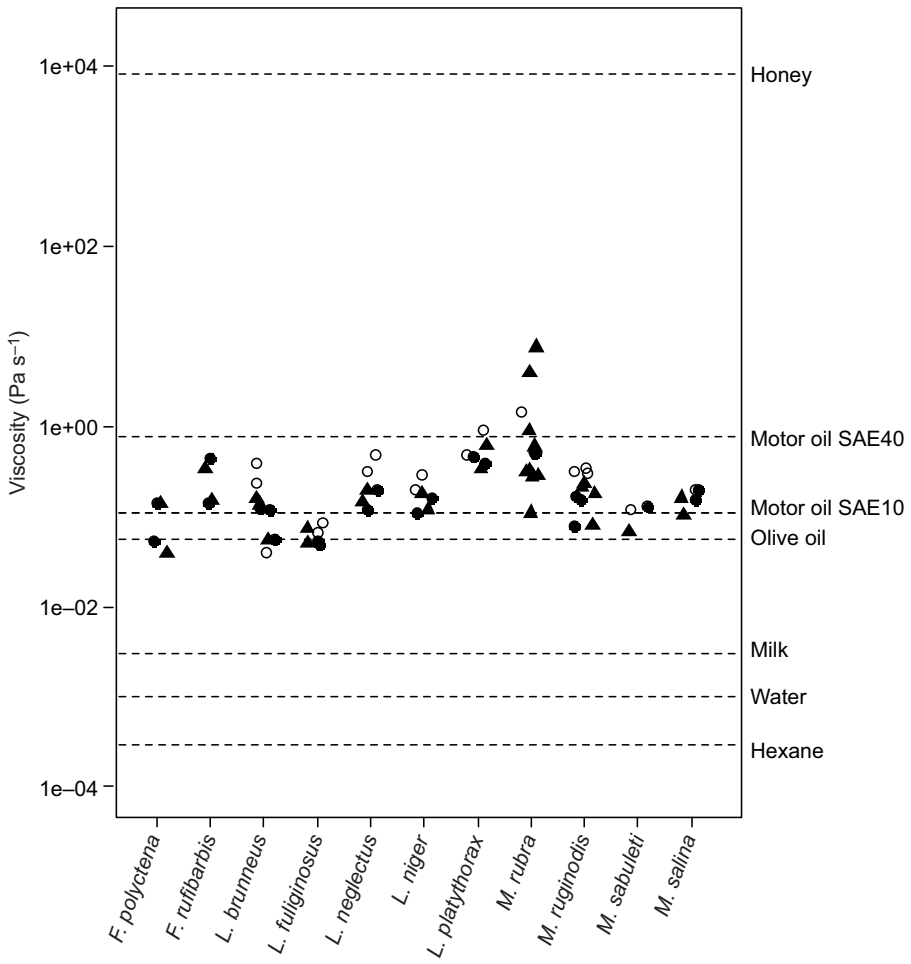

Fig. 4. Viscosities of CHCs (liquid phase only) of different ant species. Open circles, viscosity at 20°C; triangles, 25°C; full circles, 28°C. For comparison, viscosities of hexane, water, milk, olive oil, motor oil and honey are given (see Table S4 for details and references). Note the log scale of the *y*-axis.

difference in melting behaviour of alkene-rich versus methyl alkane-rich profiles (i.e. the first principal component axis in our analysis) should have been detectable also in species from similar climates, but was not confirmed here.

Adaptive significance of solid and liquid phases

Hence, the CHC layer is not solid at ambient temperature, as has been implicitly or explicitly assumed before (Gibbs, 2002; Rourke and Gibbs, 1999). Here, it is important to note that melting can only be detected if substances were solid at the lowest measured temperature. If measurements start at 30°C (e.g. Rourke and Gibbs, 1999), substances that are already liquid at this temperature will not be detected, which underestimates the overall melting range. To our knowledge, our study is the first to measure melting of insect CHCs below 0°C (starting at −100°C), which may explain why the wide melting ranges have not been detected earlier.

In our opinion, entirely solid CHC layers are highly unlikely and would not be adaptive biologically. Liquid–solid mixtures enable solid structures (gelified parts, crystals or polycrystals) to be surrounded by a liquid matrix. In contrast, solid CHCs alone would not be able to seal the insect body, because any movement of the insect would lead to cracks in the CHC layer. Indeed, pure long-chain *n*-alkanes form solid flakes and cannot cover a surface tightly. This is true for *n*-C24 (Rourke and Gibbs, 1999; F.M. and B.A., personal observations), which is short-chained compared with most insect CHCs and melts at 50°C (Lide, 2008). Longer *n*-alkanes, which are common in ants, melt at yet higher temperatures (Lide, 2008). Although mixtures of long-chain *n*-alkanes should be less brittle than pure ones, they are still solid and thus cannot seal the cuticle. That is probably why insects upregulate their alkene production concomitantly with the *n*-alkanes during warm acclimation, such that alkene and *n*-alkane proportions are tightly linked (Sprenger et al., 2018). This may seem counterintuitive at first glance, but is in agreement with the hypothesis that a liquid matrix may be necessary for solid inclusions (even on a microscopic scale) – to avoid cracks in the cuticular layer when the insect moves, and to smoothly cover the entire body surface. As a result of their very low melting points (Gibbs, 2002), alkenes should be the most suitable substance class for this purpose. However, in species with few (e.g. *F. rufibarbis* or *L. platythorax*) or no alkenes (e.g. *Temnothorax* species; Menzel et al., 2018), trimethyl or dimethyl alkanes may fulfil the same function.

In turn, the solid parts of a biphasic layer should be more capable than liquid parts of blocking the diffusion of water molecules through the whole layer. Water loss through the cuticle happens via ‘defects’ in lipid packing (Gibbs and Rajpurohit, 2010). These defects increase as lipids melt, and because of this more water molecules can diffuse into, and through, the CHC layer. The importance of solid phases in blocking desiccation may be reflected by T_c, beyond which water loss increased drastically. In a study on grasshoppers (Rourke and Gibbs, 1999), T_c was between 30 and 55°C, and correlated with a ‘hydrocarbon melting point’ inferred via Fourier-transform infrared (FTIR) spectroscopy (defined as the midpoint of the two asymptotes of a fitted sigmoid function). Possibly, this temperature reflects the melting of *n*-alkanes as the last solid parts of the CHC layer.

Journal of Experimental Biology (2019) 222, jeb210807. doi:10.1242/jeb.210807

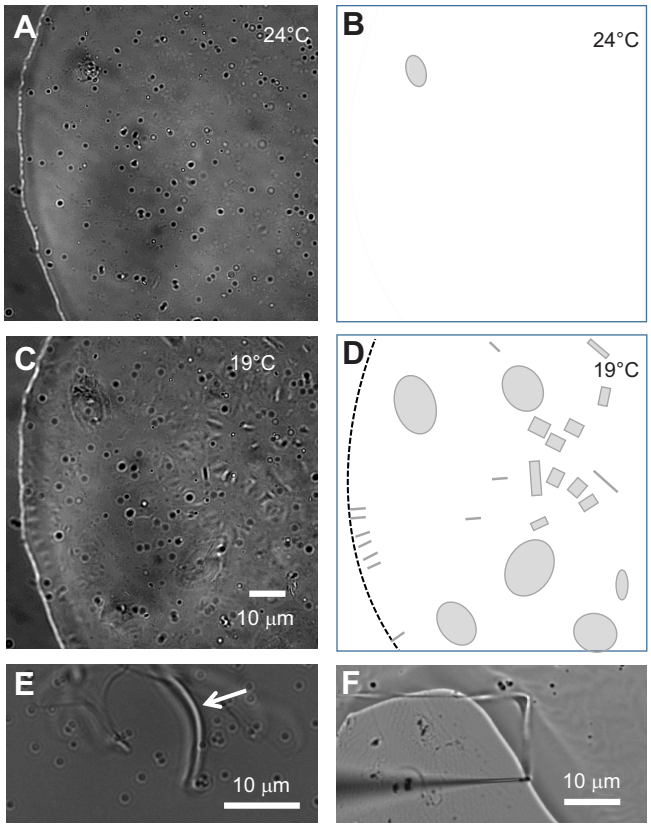

Fig. 5. Microscopic photos of CHC extracts, taken at 100× magnification. (A) *Myrmica rubra* extract at 24°C. The black dots are melamine beads (0.74 µm diameter). (B) Sketch of the photo in A, with gelified structures indicated as grey lines. (C,D) The same extract at 19°C. (E) Gelified structure in an extract of *Lasius fuliginosus* at 21°C, indicated by the arrow. (F) Trace left by the micropipette in liquid CHCs (*T*=23°C) of *Myrmica rubra*.

Adaptive value of wide melting ranges

That CHC blends have a melting range rather than a melting point was known before (Gibbs and Pomonis, 1995), but it was not clear how wide this range was. Previous studies on *Drosophila* flies (whose CHC mostly consist of alkenes and alkadienes) and *Melanoplus* grasshoppers reported melting ranges of 20–50°C and 30–50°C (respectively), reflecting a much narrower range than those reported here (Gibbs et al., 1997; Rourke and Gibbs, 1999). Given that alkenes have very low melting points, and that entirely solid CHC layers could pose problems for the insect, these ranges are unexpected. It remains to be determined whether the differences are due to taxonomic differences or to methodological issues as discussed above.

CHC layers with wide melting ranges should be adaptive because they ensure that the insect always carries a solid–liquid mixture of CHCs on its body surface. Thus, the CHC layer remains functional even in the case of sudden temperature changes. A foraging insect may experience sunny and shady microhabitats within short time intervals. Owing to its small body size, the insect may heat up quickly when exposed to sun, but cool down again if in the shade. Thus, the cuticular layer must be able to cope with such sudden changes, without entirely solidifying or evaporating.

Although we had too few DSC samples for a statistical analysis, we can point out some relationships between chemical composition and melting behaviour. For example, *F. polyctena* has a particularly large CHC proportion melting above 30°C (Fig. 2A), which coincides with its high content of *n*-alkanes (which have the highest melting point of all CHCs). In turn, the high alkene and alkadiene content in *L. fuliginosus* and *L. neglectus* may account for their CHC melting at low temperatures, with high proportions of CHC liquid at −15°C already, and the entire profile liquid at ca. 20 and 28°C, respectively. This fits our prediction that profiles rich in alkenes or alkadienes should be largely liquid at ambient temperatures. The complex CHC profiles, and the high variability of melting points even within substance classes (e.g. between different dimethyl alkanes of the same chain length (Gibbs and Pomonis, 1995), make it impossible at this point to infer more general relationships between CHC composition and melting profile, such that more research is needed here.

The need for a solid–liquid mixture may account for constraints on CHC variation across species. For example, a comparison across species showed that, with increasing average chain length, the abundance of *n*-alkanes and monomethyl alkanes (with high melting points) decreased while the abundance of CHC classes with low melting points (alkenes, dimethyl alkanes, alkadienes) increased (Menzel et al., 2017). This can be explained by the need to retain a liquid CHC phase, because melting points increase with chain length (Li et al., 2006).

The solidification of formerly liquid hydrocarbons with decreasing temperature leads to sample heterogeneity, i.e. the CHC extract becomes biphasic, with gelified structures surrounded by a liquid phase. In the microrheology measurements, we often observed these gelified structures at 20°C, but never at 28°C, although the DSC measurements indicated solid parts of the CHC profiles even at 28°C. This is due to the fact that, for microrheological measurements with the micropipette, we only collected the fraction of CHC that was liquid at room temperature (ca. 23°C). Because of this, our microrheology experiments and microscopic observations were performed without compounds that were solid at room temperature. Although this means that viscosity was measured only for a (liquid) subset of the CHC profile, they are biologically meaningful, because the presence of solid compounds does not alter viscosity. Hence, if we had collected the CHCs at a higher temperature (such that all are liquid), but measured at temperatures between 20 and 30°C, we would still measure the viscosity of only those hydrocarbons that are liquid at the temperature of measurement. However, the viscosities measured at 25 and 28°C may be somewhat underestimated because they do not include the CHCs solid at 23°C but liquid at 25 or 28°C.

Viscosity: similar viscosities across species despite variable CHC composition

Viscosity is equivalent to the diffusion rate of molecules, i.e. the diffusion coefficient of molecules is inversely proportional to viscosity according to the Stokes–Einstein relation (Einstein, 1905). As we will outline below, hydrocarbon diffusion over the insect cuticle is vital for several biological functions. Note that viscosity only applies to liquid phases; hence, the viscosity of liquid CHCs is not necessarily affected by certain CHCs solidifying as temperature decreases.

Although the CHC layers of different ant species differed greatly in chemical composition, their viscosities were relatively similar. For most samples, viscosity was between 0.1 and 0.4 Pa s^{-1}. These values are similar to motor (lubricant) oils, and slightly higher than various vegetable oils (Fig. 4; Table S4) (Diamante and Lan, 2014). Only some samples of *M. rubra* had much higher viscosities. Melting behaviour differs greatly between pure substances, even if they have similar chain lengths; for example, methyl-branched hydrocarbons melt at temperatures 10–30 K lower than *n*-alkanes of

the same chain length, and alkenes melt at temperatures more than 50 K lower (Gibbs and Pomonis, 1995; Gibbs and Rajpurohit, 2010). For example, C25-7-ene melts below −90°C (S.M., personal observation), while the corresponding *n*-alkane melts at 54°C (Lide, 2008). Given these differences in melting point between substances, one would expect that their viscosities should be equally variable. Additionally, viscosity increases with chain length even among hydrocarbons of the same homologous series (Cai et al., 2018). This makes it all the more surprising that the viscosities of our ant samples did not show larger variation. In particular, it was unexpected that CHC profiles rich in methyl-branched alkanes and those rich in unsaturated hydrocarbons (low versus high values on the PC1 axis; Fig. 1B) did not systematically differ in viscosity, with only a weak effect of PC1 at one of the three temperatures. In our view, it seems unlikely that random mixtures of hydrocarbons would yield similar viscosities as well. Thus, our results are consistent with stabilizing selection for a specific CHC viscosity, but further research is needed to confirm or reject this idea.

The strong chemical differences across species should still influence viscosity. However, these effects may be hard to detect if the CHC profiles were optimised for a similar viscosity, leading to small effect sizes. Our samples were all from ants acclimated to Central European summer temperatures. Acclimation to different temperatures can lead to acclimatory changes in CHC composition, which result in a changed CHC viscosity (Sprenger et al., 2018). These acclimatory changes concern the relative abundances of the different hydrocarbons, but the identity of the hydrocarbons in a profile stays constant. Hence, a higher number of replicates might have enabled us to detect differences between species (although this was not the focus of this study). Nevertheless, our choice of samples should have allowed us to detect general viscosity differences in alkene-rich versus methyl alkane-rich profiles.

What are the biological implications of this result? A certain viscosity range of the liquid CHCs may be adaptive to ensure a defined diffusion rate of hydrocarbons, and thus achieve a balance between perceptibility of hydrocarbon signals and a CHC layer that is sufficiently waterproof at the temperature the insect experiences.

In our view, diffusion of hydrocarbons across the cuticle is vital for several biological functions. Firstly, solid parts must diffuse in a liquid matrix so that they evenly coat the insect body and ensure waterproofing. Solid hydrocarbons prevent the diffusion of water molecules better than liquid phases (Gibbs and Rajpurohit, 2010). However, the insect is only protected if no part of the surface remains uncoated. Uniform coating with CHCs can only be achieved when CHCs can diffuse. When the insect moves, its cuticle can bend (especially in joints), and this would probably cause cracks in the CHC layer if it was entirely solid. Hence, a liquid matrix is necessary to 'seal' the cuticle, and to allow solid CHCs to diffuse. Indeed, diffusion of both solid and liquid CHCs across the insect body has been shown in potato beetles (Geiselhardt et al., 2010). Liquid parts of the CHC layer may also be essential for the repair of scratches (Wigglesworth, 1945) and the lubrication of joints (Cooper et al., 2009).

Secondly, hydrocarbon diffusion should be important for nestmate recognition. CHCs can only serve as communication signals if they are sufficiently liquid to diffuse across the cuticle to ensure an equal distribution of recognition cues on the body surface. Furthermore, perception requires that CHCs bind to olfactory binding proteins (OBPs) on the olfactory sensillae of the perceiving insect (Blomquist and Bagnères, 2010; Hansson and Stensmyr, 2011; Maitani et al., 2010). This is true for contact and distant chemoreception (Sharma et al., 2015; Pask et al., 2017), such that perceptibility requires, or is strongly enhanced by, CHCs entering the gas phase. Indeed, long-chain hydrocarbons can be detected in the headspace of an insect (Schmitt et al., 2007), and nestmate recognition is possible without tactile interaction. This indicates that recognition cues are volatile enough to be detected via olfaction (Brandstaetter et al., 2008), and the same is true for CHCs that signal fertility (D'Ettorre et al., 2004). Hence, hydrocarbons relevant for communication should be volatile to some degree, i.e. have a certain vapour pressure. Volatility increases with decreasing viscosity, at least for pure liquids (Othmer and Conwell, 1945). This suggests that to enable communication, CHC viscosity must not become too high.

Thirdly, a defined viscosity can be important for foot adhesion. Insects leave tiny hydrocarbon droplets when they walk. These 'footprints' are important for surface adhesion and the rapid attachment and detachment of footpads (Labonte and Federle, 2015), but also for the recognition of nestmates or other conspecifics (Lenoir et al., 2009; Wüst and Menzel, 2017). A constant viscosity should ensure that a specific quantity of footprints is excreted and attached to the ground when the insect walks. Too high a viscosity may prevent the insect from leaving footprints (Abou et al., 2010), whereas too low a viscosity would result in a higher loss of CHCs as footprints and a stronger abrasion.

Besides the current temperature, the CHC layer has to remain functional under temperature fluctuations, e.g. between day and night or between the inside of the nest and the outside environment. Hence, biophysical behaviour across large temperature gradients is of paramount importance for biological functionality. Many insect species possess a high number of different CHCs, much higher than one would in theory need to encode information. Often, they produce homologous series, i.e. CHCs of a similar structure that only differ in chain length. Even though this may be a by-product of CHC biosynthesis, it probably benefits the insect during temperature fluctuations. With many hydrocarbons solidifying at different temperatures (finely tuned via different chain lengths), the insect can ensure that viscosity is similar across different temperatures, and that there is always a liquid phase.

Conclusions

With this study, we bring attention to the importance of physical properties for the biological functioning of CHC layers. The CHCs of the species we studied consist of solid and liquid phases over most of the temperatures they are likely to experience. Such a melting behaviour is most likely adaptive, as we argue above. Likewise, we argue that the viscosity of liquid CHCs is biologically relevant because it reflects their molecular diffusion rate and their vapour pressure, which are necessary for the exchange of communication signals. This implies that, in order to explain CHC differences between species, but also between individuals of a social insect colony, we need to consider that hydrocarbons possess different material properties, which will impact their suitability for signalling purposes. Just like volatility matters for volatile signals, vapour pressure, melting and viscosity should matter for signals encoded in the CHC layer. Our results suggest that CHC variation may be constrained much more by such biophysical effects than has been appreciated up to now.

Insects may have to trade off waterproofing and perceptibility of communication signals at any given temperature. This does not mean that the two functions are traded off against each other, but rather that at each temperature, the CHC composition has to be adjusted in order to fulfil both functions at the same time. This can explain why many species exhibit temperature-dependent CHC

changes, even if they result in profiles that seem non-optimal for waterproofing (Sprenger et al., 2018).

Understanding the physical behaviour of complex chemical mixtures may become relevant for industrial applications where fluids (such as lubricants, inks or paints) need a defined viscosity that is robust to temperature fluctuations. Here, knowledge about insect CHCs may inspire biomimetic design of additives to these products. Future research on the physics of insect CHCs should compare species from habitats with different levels of temperature fluctuation. Adopting this ecophysiological perspective will shed light on the evolutionary mechanisms that cause CHC diversification, and help to predict how different species will respond to climatic changes.

Acknowledgements
We thank Heike Stypa, Marion Kever and Stefanie Emmling for help in the preparation of the DSC samples. We are also grateful to Thomas Eltz, Romain Libbrecht and an anonymous referee for their helpful comments, which significantly improved the manuscript.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: F.M., S.M., B.A.; Methodology: F.M., S.M., B.A.; Software: B.A.; Formal analysis: F.M., S.M., J.H.M., P.R., S.H., M.P., B.A.; Investigation: F.M., S.M., J.H.M., P.R., S.H., M.P., B.A.; Data curation: P.R., B.A.; Writing - original draft: F.M., S.M., B.A.; Writing - review & editing: F.M., S.M., B.A.; Visualization: F.M.; Supervision: F.M., S.M., B.A.; Project administration: F.M., B.A.; Funding acquisition: F.M.

Funding
This work was kindly supported by the Deutscher Akademischer Austauschdienst. F.M. was supported by PPP Procope France (project ID: 57388961) with funds from the Federal Ministry of Education and Research (Bundesministerium für Bildung und Forschung). B.A. was supported by PHC Procope 2018 (project ID: 40427NM) with funds from MEAE (Ministère de l'Europe et des Affaires étrangères) and MESRI (Ministère de l'Enseignement Supérieur, de la Recherche et de l'Innovation). FM is also grateful for support by a Heisenberg fellowship (ME3842/6-1) from the Deutsche Forschungsgemeinschaft (DFG).

Supplementary information
Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.210807.supplemental

References
Abou, B., Gay, C., Laurent, B., Cardoso, O., Voigt, D., Peisker, H. and Gorb, S. (2010). Extensive collection of femtolitre pad secretion droplets in the beetle *Leptinotarsa decemlineata* allows nanolitre microrheology. *J. R. Soc. Interface* **7**, 1745. doi:10.1098/rsif.2010.0075

Aitchison, J. (1986). *The Statistical Analysis of Compositional Data: Monographs in Statistics and Applied Probability*. London: Chapman and Hall.

Bates, D., Mächler, M., Bolker, B. and Walker, S. (2015). Fitting linear mixed-effects models using lme4. *J. Stat. Software* **67**, 1-48. doi:10.18637/jss.v067.i01

Blomquist, G. J. (2010). Structure and analysis of insect hydrocarbons. In *Insect Hydrocarbons. Biology, Biochemistry, and Chemical Ecology* (ed. G. J. Blomquist and A.-G. Bagnères), pp. 19-34. Cambridge: Cambridge University Press.

Blomquist, G. J. and Bagnères, A.-G. (2010). Introduction: history and overview of insect hydrocarbons. In *Insect Hydrocarbons. Biology, Biochemistry, and Chemical Ecology* (ed. G. J. Blomquist and A.-G. Bagnères), pp. 3-18. Cambridge: Cambridge University Press.

Brandstaetter, A. S., Endler, A. and Kleineidam, C. J. (2008). Nestmate recognition in ants is possible without tactile interaction. *Naturwissenschaften* **95**, 601-608. doi:10.1007/s00114-008-0360-5

Brawand, D., Wagner, C. E., Li, Y. I., Malinsky, M., Keller, I., Fan, S., Simakov, O., Ng, A. Y., Lim, Z. W. Bezault, E. et al. (2014). The genomic substrate for adaptive radiation in African cichlid fish. *Nature* **513**, 375-381. doi:10.1038/nature13726

Brooks, L., Brunelli, M., Pattison, P., Jones, G. R. and Fitch, A. (2015). Crystal structures of eight mono-methyl alkanes (C26–C32) *via* single-crystal and powder diffraction and DFT-D optimization. *IUCrJ* **2**, 490-497. doi:10.1107/S2052252515010271

Cai, G., Liu, Z., Zhang, L., Zhao, S. and Xu, C. (2018). Quantitative structure-property relationship model for hydrocarbon liquid viscosity prediction. *Energy Fuels* **32**, 3290-3298. doi:10.1021/acs.energyfuels.7b04075

Carlson, D. A., Bernier, U. R. and Sutton, B. D. (1998). Elution patterns from capillary GC for methyl-branched alkanes. *J. Chem. Ecol.* **24**, 1845-1865. doi:10.1023/A:1022311701355

Cooper, R., Lee, H., González, J. M., Butler, J., Vinson, S. B. and Liang, H. (2009). Lubrication and surface properties of roach cuticle. *J. Tribol.* **131**, 014502. doi:10.1115/1.3002327

Dahbi, A., Hefetz, A. and Lenoir, A. (2008). Chemotaxonomy of some *Cataglyphis* ants from Morocco and Burkina Faso. *Biochem. Syst. Ecol.* **36**, 564-572. doi:10.1016/j.bse.2008.03.004

D'Ettorre, P., Heinze, J., Schulz, C., Francke, W. and Ayasse, M. (2004). Does she smell like a queen? Chemoreception of a cuticular hydrocarbon signal in the ant *Pachycondyla inversa*. *J. Exp. Biol.* **207**, 1085-1091. doi:10.1242/jeb.00865

Diamante, L. M. and Lan, T. (2014). Absolute viscosities of vegetable oils at different temperatures and shear rate range of 64.5 to 4835 s^{-1}. *J. Food Process* **2014**, 1-6. doi:10.1155/2014/234583

Dirks, J.-H. and Federle, W. (2011). Mechanisms of fluid production in smooth adhesive pads of insects. *J. R. Soc. Interface* **8**, 952-960. doi:10.1098/rsif.2010.0575

Drechsler, P. and Federle, W. (2006). Biomechanics of smooth adhesive pads in insects: influence of tarsal secretion on attachment performance. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* **192**, 1213-1222. doi:10.1007/s00359-006-0150-5

Einstein, A. (1905). Über die von der molekularkinetischen Theorie der Wärme geforderte Bewegung von in ruhenden Flüssigkeiten suspendierten Teilchen. *Ann. Phys.* **322**, 549-560. doi:10.1002/andp.19053220806

Fox, J. and Weisberg, S. (2011). *An R Companion to Applied Regression*. Thousand Oaks, CA: SAGE Publications.

Gefen, E., Talal, S., Brendzel, O., Dror, A. and Fishman, A. (2015). Variation in quantity and composition of cuticular hydrocarbons in the scorpion *Buthus occitanus* (Buthidae) in response to acute exposure to desiccation stress. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **182**, 58-63. doi:10.1016/j.cbpa.2014.12.004

Geiselhardt, S. F., Lamm, S., Gack, C. and Peschke, K. (2010). Interaction of liquid epicuticular hydrocarbons and tarsal adhesive secretion in *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae). *J. Comp. Physiol.* **196**, 369-378. doi:10.1007/s00359-010-0522-8

Gibbs, A. G. (1995). Physical properties of insect cuticular hydrocarbons: model mixtures and lipid interactions. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **112**, 667-672. doi:10.1016/0305-0491(95)00119-0

Gibbs, A. G. (2002). Lipid melting and cuticular permeability: new insights into an old problem. *J. Insect Physiol.* **48**, 391-400. doi:10.1016/S0022-1910(02)00059-8

Gibbs, A. G. and Pomonis, J. G. (1995). Physical properties of insect cuticular hydrocarbons: the effects of chain lengths, methyl branching and unsaturation. *Comp. Biochem. Physiol.* **112B**, 243-249. doi:10.1016/0305-0491(95)00081-X

Gibbs, A. G. and Rajpurohit, S. (2010). Cuticular lipids and water balance. In *Insect Hydrocarbons. Biology, Biochemistry, and Chemical Ecology* (ed. G. J. Blomquist and A.-G. Bagnères), pp. 100-120. Cambridge: Cambridge University Press.

Gibbs, A. G., Chippindale, A. K. and Rose, M. R. (1997). Physiological mechanisms of evolved desiccation resistance in Drosophila melanogaster. *J. Exp. Biol.* **200**, 1821-1832.

Hansson, B. S. and Stensmyr, M. C. (2011). Evolution of insect olfaction. *Neuron* **72**, 698-711. doi:10.1016/j.neuron.2011.11.003

Howard, R. W. and Blomquist, G. J. (2005). Ecological, behavioral, and biochemical aspects of insect hydrocarbons. *Annu. Rev. Entomol.* **50**, 371-393. doi:10.1146/annurev.ento.50.071803.130359

Jiggins, C. D. (2008). Ecological speciation in mimetic butterflies. *Bioscience* **58**, 541-548. doi:10.1641/B580610

Kather, R. and Martin, S. J. (2012). Cuticular hydrocarbon profiles as a taxonomic tool: advantages, limitations and technical aspects. *Physiol. Entomol.* **37**, 25-32. doi:10.1111/j.1365-3032.2011.00826.x

Kather, R. and Martin, S. J. (2015). Evolution of cuticular hydrocarbons in the Hymenoptera: a meta-analysis. *J. Chem. Ecol.* **41**, 871-883. doi:10.1007/s10886-015-0631-5

Labonte, D. and Federle, W. (2015). Rate-dependence of 'wet' biological adhesives and the function of the pad secretion in insects. *Soft Matter* **11**, 8661-8673. doi:10.1039/C5SM01496D

Lamichhaney, S., Berglund, J., Almén, M. S., Maqbool, K., Grabherr, M. and Martinez-Barrio, A. (2014). Evolution of Darwin's finches and their beaks revealed by genome sequencing. *Nature* **518**, 371-375. doi:10.1038/nature14181

Lenoir, A., Depickère, S., Devers, S., Christidès, J.-P. and Detrain, C. (2009). Hydrocarbons in the ant *Lasius niger*: from the cuticle to the nest and home range marking. *J. Chem. Ecol.* **35**, 913-921. doi:10.1007/s10886-009-9669-6

Leonhardt, S. D., Menzel, F., Nehring, V. and Schmitt, T. (2016). Ecology and evolution of communication in social insects. *Cell* **164**, 1277-1287. doi:10.1016/j.cell.2016.01.035

Li, H., Higashi, H. and Tamura, K. (2006). Estimation of boiling and melting points of light, heavy and complex hydrocarbons by means of a modified group

vector space method. *Fluid Phase Equilib.* **239**, 213-222. doi:10.1016/j.fluid.2005.11.004
Lide, D. R. (2008). *CRC Handbook of Chemistry and Physics*. Boca Raton, FL: CRC Press.
Lira-Galeana, C., Firoozabadi, A. and Prausnitz, J. M. (1996). Thermodynamics of wax precipitation in petroleum mixtures. *AIChE J.* **42**, 239-248. doi:10.1002/aic.690420120
Maitani, M. M., Allara, D. L., Park, K. C., Lee, S. G. and Baker, T. C. (2010). Moth olfactory trichoid sensilla exhibit nanoscale-level heterogeneity in surface lipid properties. *Arthropod Struct. Dev.* **39**, 1-16. doi:10.1016/j.asd.2009.08.004
Maroncelli, M., Qi, S. P., Strauss, H. L. and Snyder, R. G. (1982). Nonplanar conformers and the phase behavior of solid *n*-alkanes. *J. Am. Chem. Soc.* **104**, 6237-6247. doi:10.1021/ja00387a013
Martin, S. and Drijfhout, F. (2009). A review of ant cuticular hydrocarbons. *J. Chem. Ecol.* **35**, 1151-1161. doi:10.1007/s10886-009-9695-4
Menzel, F., Blaimer, B. B. and Schmitt, T. (2017). How do cuticular hydrocarbons evolve? physiological constraints and climatic and biotic selection pressures act on a complex functional trait. *Proc. R. Soc. B* **284**, 20161727. doi:10.1098/rspb.2016.1727
Menzel, F., Zumbusch, M. and Feldmeyer, B. (2018). How ants acclimate: impact of climatic conditions on the cuticular hydrocarbon profile. *Funct. Ecol.* **32**, 657-666. doi:10.1111/1365-2435.13008
Othmer, D. F. and Conwell, J. W. (1945). Correlating viscosity and vapor pressure of liquids. *Ind. Eng. Chem.* **37**, 1112-1115. doi:10.1021/ie50431a027
Otte, T., Hilker, M. and Geiselhardt, S. (2018). Phenotypic plasticity of cuticular hydrocarbon profiles in insects. *J. Chem. Ecol.* **44**, 235-247. doi:10.1007/s10886-018-0934-4
Ozaki, M. and Wada-Katsumata, A. (2010). Perception and olfaction of cuticular compounds. In *Insect Hydrocarbons. Biology, Biochemistry, and Chemical Ecology* (ed. G. J. Blomquist and A.-G. Bagnères), pp. 207-221. Cambridge: Cambridge University Press.
Pask, G. M., Slone, J. D., Millar, J. G., Das, P., Moreira, J. A., Zhou, X., Bello, J., Berger, S. L., Bonasio, R., Desplan, C. et al. (2017). Specialized odorant receptors in social insects that detect cuticular hydrocarbon cues and candidate pheromones. *Nat. Commun.* **8**, 297. doi:10.1038/s41467-017-00099-1
Ramsay, A. (1935). The evaporation of water from the cockroach. *J. Exp. Biol.* **12**, 373-383.
Rim, P. B. and Runt, J. P. (1984). Melting point depression in crystalline/compatible polymer blends. *Macromolecules* **17**, 1520-1526. doi:10.1021/ma00138a017
Rourke, B. and Gibbs, A. G. (1999). Effects of lipid phase transitions on cuticular permeability: model membrane and in situ studies. *J. Exp. Biol.* **202**, 3255-3262.
Schmitt, T., Herzner, G., Weckerle, B., Schreier, P. and Strohm, E. (2007). Volatiles of foraging honeybees *Apis mellifera* (Hymenoptera: Apidae) and their potential role as semiochemicals. *Apidologie* **28**, 164-170. doi:10.1051/apido:2006067
Sharma, K. R., Enzmann, B. L., Schmidt, Y., Moore, D., Jones, G. R., Parker, J., Berger, S. L., Reinberg, D., Zwiebel, L. J., Breit, B. et al. (2015). Cuticular hydrocarbon pheromones for social behavior and their coding in the ant antenna. *Cell Rep.* **12**, 1261-1271. doi:10.1016/j.celrep.2015.07.031
Sprenger, P. P., Burkert, L. H., Abou, B., Federle, W. and Menzel, F. (2018). Coping with the climate: cuticular hydrocarbon acclimation of ants under constant and fluctuating conditions. *J. Exp. Biol.* **221**, jeb171488. doi:10.1242/jeb.171488
Srivastava, S. P., Handoo, J., Agrawal, K. M. and Joshi, G. C. (1993). Phase-transition studies in n-alkanes and petroleum-related waxes—a review. *J. Phys. Chem. Solids* **54**, 639-670. doi:10.1016/0022-3697(93)90126-C
Wagner, D., Tissot, M. and Gordon, D. (2001). Task-related environment alters the cuticular hydrocarbon composition of harvester ants. *J. Chem. Ecol.* **27**, 1805-1819. doi:10.1023/A:1010408725464
Wigglesworth, V. B. (1945). Transpiration through the cuticle of insects. *J. Exp. Biol.* **21**, 97-114.
Wüst, M. and Menzel, F. (2017). I smell where you walked – how chemical cues influence movement decisions in ants. *Oikos* **126**, 149-160. doi:10.1111/oik.03332