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RESEARCH ARTICLE

Coping with the climate: cuticular hydrocarbon acclimation of ants under constant and fluctuating conditions

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ABSTRACT

Terrestrial arthropods achieve waterproofing by a layer of cuticular hydrocarbons (CHCs). At the same time, CHCs also serve as communication signals. To maintain waterproofing under different climate conditions, insects adjust the chemical composition of their CHC layer, but this may affect the communication via CHCs. The detailed acclimatory changes of CHCs and how these influence their physical properties are still unknown. Here, we studied acclimation in two closely related ant species with distinct CHC profiles, *Myrmica rubra* and *Myrmica ruginodis*, in response to constant or fluctuating temperature and humidity regimes. We measured how acclimation affected CHC composition and viscosity, and the ants' drought survival. In both species, CHC composition showed strong, predictable responses to temperature regimes. Warm-acclimated individuals had higher proportions of linear alkanes, and less methyl-branched or unsaturated CHCs. These changes coincided with higher solid content and viscosity of CHCs in warm-acclimated ants. Temperature fluctuation caused effects similar to those observed under constant-cool conditions in *M. rubra*, but led to entirely different profiles in *M. ruginodis*, suggesting that fluctuating and constant conditions pose very different challenges. Acclimation to dry conditions led to higher absolute amounts of CHCs, which increased the ants' drought survival, whereas temperature acclimation did not. Hence, the temperature-induced CHC changes cannot be explained by the need for waterproofing alone. Although these changes could be non-adaptive, we propose that they serve to maintain a constant CHC viscosity, which may be essential for communication and other functions.

KEY WORDS: CHCs, Desiccation resistance, Drought survival, Phenotypic plasticity, Microrheology, Viscosity

INTRODUCTION

Climate change is predicted to raise the global mean temperature, increase temperature fluctuations, make weather events such as heat waves or drought events more common, and change the global distribution of water availability and precipitation (Coumou and Rahmstorf, 2012; Fung et al., 2011). Therefore, the effects of climatic factors such as temperature and humidity on animals have attracted increasing scientific interest. As a short-term mechanism to cope with altered conditions, animals can acclimate to climate conditions by modifying their behaviour, morphology and physiology via phenotypic plasticity (Angilletta, 2009). Phenotypic plasticity of physiological traits can increase the resistance of ectothermic animals to climate change (Seebacher et al., 2015).

Acclimation to different temperatures has been shown for a variety of animals (Angilletta, 2009), but responses differ between constant and fluctuating temperatures (Colinet et al., 2015). In addition to temperature, humidity and its interaction with temperature may affect the fitness of ectothermic animals (Chown et al., 2011; Terblanche and Kleynhans, 2009). Terrestrial arthropods should be highly susceptible to climatic changes, as their metabolic rate is directly affected by the environmental temperature and their large surface area-to-volume ratio makes them vulnerable to water loss. Most water loss under ambient temperatures occurs through the cuticle (Edney, 1977; Hadley, 1994). Therefore, it is important to understand the mechanisms insects use to reduce cuticular water loss.

In insects, waterproofing is achieved by a hydrocarbon layer on the cuticle (Blomquist and Bagnères, 2010). Cuticular hydrocarbons (CHCs) comprise a complex mixture of *n*-alkanes, methyl-branched alkanes and unsaturated hydrocarbons (alkenes) (Blomquist, 2010). These compounds differ in the carbon chain length and the number of methyl groups and/or double bonds. In addition to waterproofing, CHCs also serve several other functions, most importantly as communication signals in many insect 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). For example, behavioural castes among ant workers (such as scouts, foragers and nurses) possess quantitatively different CHC profiles, which can trigger different behavioural responses in their nestmates (Greene and Gordon, 2003; Pamminger et al., 2014; Wagner et al., 2001).

Variation in cuticular waterproofing depends both on the absolute amount and quantitative composition of the CHC layer (Chown et al., 2011; Edney, 1977). The strong temperature dependence of the waterproofing function of insect cuticle is thought to be caused by a 'melting' of the CHCs, which is influenced by their chemical composition (Gibbs, 1998; Gibbs and Pomonis, 1995). Early studies on several insect species showed that evaporation of water through a CHC layer increases drastically if the ambient temperature is raised above a critical temperature T_c (Beament, 1945, 1958, 1959; Gibbs, 2002; Ramsay, 1935; Rourke and Gibbs, 1999; Wigglesworth, 1945). The melting point of the CHC layer increases when CHC molecules align and aggregate. Generally, *n*-alkane molecules aggregate most tightly because of van der Waals forces, and this aggregation increases with chain length. Hydrocarbons with strong van der Waals bonds (e.g. *n*-alkanes, monomethyl alkanes) can even crystallize (Brooks et al., 2015). Thus, *n*-alkanes should provide the best waterproofing to the insect, especially if

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they have high chain lengths. In contrast, methyl groups and double bonds disrupt the linear geometry of *n*-alkanes and therefore hinder a tight molecular aggregation. The melting temperature T_m of a hydrocarbon therefore decreases from *n*-alkanes to monomethyl alkanes, dimethyl alkanes, alkenes and alkadienes (in that order) (Gibbs, 1998; Gibbs and Pomonis, 1995). However, hydrocarbon mixtures do not have a sharp melting point. Rather, melting occurs over a wider temperature range (Gibbs, 1995). Moreover, tight molecular cohesion of CHCs may increase not only their melting point, but also their viscosity. The higher the viscosity of the CHC layer, the lower the diffusion rate of CHC molecules across the body surface (the diffusion coefficient is inversely proportional to viscosity according to the Stokes–Einstein relation; Einstein, 1905). However, the ability of CHC molecules to move across the cuticle is essential for many biological functions. For example, CHCs can only serve as communication signals if they are sufficiently liquid to diffuse and spread across the cuticle, or onto sensory sensilla of other insects that act as communication partners (Blomquist and Bagnères, 2010; Maitani et al., 2010). Low CHC viscosity may also be essential for the repair of scratches (Wigglesworth, 1945), for the lubrication of joints (Cooper et al., 2009; Gorb, 2001) and for the rapid attachment and detachment of sticky footpads (Labonte and Federle, 2015). Hence, improving waterproofing via the production of CHCs with a tighter molecular cohesion and higher melting points may increase CHC viscosity and thereby compromise several other functions of the CHC layer. Nevertheless, previous studies on arthropod CHC have not considered CHC viscosity and the potential trade-offs of waterproofing with other functions. As a first step, in this study we have quantified CHC viscosity using microrheology, a technique that allows measurements for extremely small volumes of material (~30 pl).

Because molecular aggregation and viscosity depend on temperature, these physical effects should matter for an insect that experiences fluctuations of environmental temperature and/or humidity over time. Indeed, insects can adjust their CHC profile to climatic conditions (Gibbs and Mousseau, 1994; Hadley, 1977). Notably, it has been reported that physiological acclimation can differ between constant and fluctuating temperatures, e.g. in the expression of heat shock proteins and immune activity (Fischer et al., 2011) or fatty acid composition (van Dooremalen et al., 2011). Probably because of the nonlinear relationship between temperature and metabolic processes, fluctuating temperatures can lead to effects which differ from those reached at constant temperatures (Colinet et al., 2015).

CHC profiles of insects differ considerably within and between species. Even closely related species often possess entirely different CHC profiles, which may differ not only in their waterproofing function and viscosity at a given (constant) temperature, but also in their ability to function across a temperature range with daily fluctuations. Little is known about how insects adjust their CHC profile to constant versus fluctuating climate regimes, and how the different hydrocarbon classes contribute to waterproofing. In particular, it is unknown whether species with different abundance of unsaturated and methyl-branched hydrocarbons can acclimate to the same extent, and survive challenging environmental conditions.

Here, we measured acclimatory CHC changes and survival of acclimated workers in two closely related ant species with strongly different CHC profiles, as well as the concomitant changes in CHC viscosity. Alkadienes dominate the profile of *Myrmica ruginodis* Nylander 1846, but are absent in its sister species *Myrmica rubra* (Linnaeus 1758), whose profile is rich in dimethyl, trimethyl and tetramethyl alkanes (Fig. 1). The latter substances, in turn, are rare or

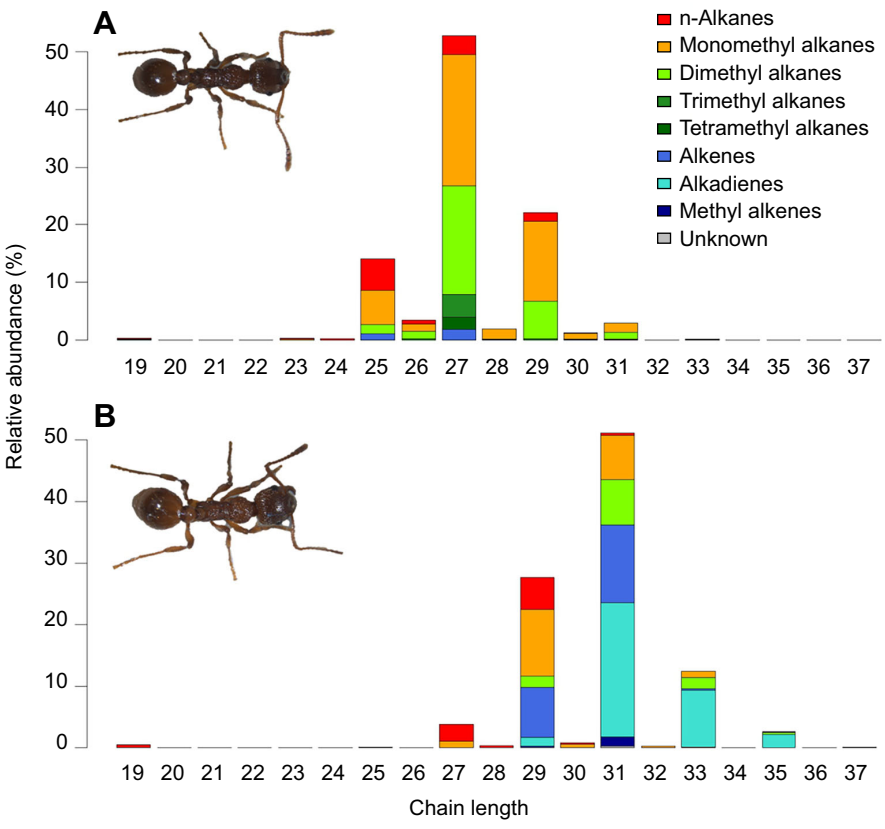

Fig. 1. Composition of cuticular hydrocarbon (CHC) profiles according to substance classes ordered by chain length for the two study species. (A) *Myrmica rubra* and (B) *M. ruginodis*. Plotted is the mean relative abundance of substance classes ordered by chain length from C19 to C37. As shown here, the CHCs of each species are confined to a small range of chain lengths only. Beside differences in CHC classes (visualized by colour codes), *M. ruginodis* has longer hydrocarbons than *M. rubra*, the most common chain length being C31 (versus C27 in *M. rubra*). Relationships between CHC composition and chain length have been found across a wide range of species and may relate to their viscosity (Menzel et al., 2017). For the sake of clarity, we did not separate different CHCs of the same chain length and substance class. For detailed composition, see Table S1.

Journal of Experimental Biology (2018) 221, jeb171488. doi:10.1242/jeb.171488

absent in *M. ruginodis*. This reflects two frequent CHC types in ants, where species either possess multi-methyl alkanes or unsaturated compounds, but rarely both substance classes in higher quantities (Menzel et al., 2017). The CHC profile may reflect different climatic niches (Menzel et al., 2017), with *M. ruginodis* inhabiting cooler and/or damper habitats than *M. rubra*, and representing the *Myrmica* species most adapted to cool temperatures among all Old World species (Radchenko and Elmes, 2010). However, *M. ruginodis* and *M. rubra* are ecologically similar and frequently co-occur in the same sites.

We expected that, at higher temperatures, both species would produce CHC profiles with more saturated and unbranched hydrocarbon classes to enhance waterproofing. Because of the division of labour in ants, workers foraging outside the nest should need stronger protection against environmental conditions than nurses, which remain inside. By disentangling temperature and humidity acclimation, we compared the effects of both factors on CHC composition and ant survival. Lastly, we used microrheology as a novel tool to quantify the viscosity of CHC profiles of ants acclimated to different temperatures.

MATERIALS AND METHODS

Study organisms

Both of our study species, *M. rubra* and *M. ruginodis*, are widely distributed across Europe, have similar life histories and can be found in the same habitats (Radchenko and Elmes, 2010). We collected 15 colonies of each species in the region around Freiburg (Germany) in April and June 2015. Colonies were collected at the following specific locations: Vogelsangpass (48.087°N, 7.697°E – both species), Eichelspitze (48.090°N, 7.694°E – *M. ruginodis* only), Burkheim/Rheinauen (48.099°N, 7.582°E – both species), Mooswald/Opfinger See (48.004°N, 7.752°E – *M. ruginodis* only) and Eichberg (47.975°N, 7.893°E – *M. rubra* only). They were kept in their original nesting material in plastic boxes (235×175×90 mm, Lifeca GmbH & Co. KG, Bad Salzulfen, Germany) with plastered ground and walls coated with Fluon® (Whitford GmbH, Diez, Germany) at 20°C and under a 12 h:12 h light:dark cycle. Relative humidity (RH) inside these stock colonies ranged from 85.66±3.61% to 99.69±1.01% between the boxes. Honey, dead crickets and water [in Eppendorf cups (Eppendorf AG, Hamburg, Germany) with a cotton plug] were provided once a week *ad libitum*.

Acclimation treatments

After keeping the colonies under laboratory conditions for at least 2 weeks (see above), we set up four temperature treatments: constant temperature at 12°C, 20°C and 28°C, and a fluctuating treatment with 12°C at night and 28°C during the day (with 3 h ramps between the two temperatures). For each of these treatments, we established two humidity conditions, dry (∼50% RH) and humid (∼100% RH). From each of the ant colonies, we created eight worker groups, which were distributed among the eight treatments. They consisted of six foragers (collected outside the nest), 18 nurses (collected inside the nest, if possible directly from the brood) and 10 brood items. Foragers and nurses represent distinct behavioural castes in many ant species and are known to possess different CHC profiles (Greene and Gordon, 2003; Pamminger et al., 2014). We kept the worker groups in plastic boxes (95×95×60 mm, Westmark GmbH, Lennestadt-Elspe, Germany) with a plaster ground and a cavity (ca. 50×30×3 mm) covered with glass plates and red foil.

All climate treatments were established in climate cabinets (RUMED 3101 and 3201, Rubarth Apparate GmbH, Laatzen, Germany) equipped with two 1000-g air dehumidifiers ($CaCl_2$; UHU GmbH & Co. KG, Bühl/Baden, Germany) to keep the air in the cabinets as dry as possible. For the humid treatment, the boxes were covered with lids, whereas the lids for the dry treatment were prepared with a window closed with wire mesh (70×70 mm; mesh 0.2 mm) to ensure continuous airflow in the nest boxes. The worker groups were kept at the different acclimation treatments for 3 weeks. We provided food (honey and dead crickets) *ad libitum*. Water was provided in an Eppendorf cup with a piece of cotton. To maintain specific humidity levels, additional water was applied to the plaster in quantities adjusted to each treatment. Food and water were added depending on the temperature and humidity treatment: in the 12°C dry treatment, only the Eppendorf cups were refilled every fourth day; in the 20°C dry treatment, 1 ml of water was additionally applied on the plaster near the nest entrance every second day; and in the fluctuating temperature and 28°C dry treatments, we similarly placed 1 or 2 ml of water, respectively, on the plaster. In the humid treatments, ants were fed similarly and water was added until the plaster was saturated with every feeding. Climatic conditions were surveyed using data loggers in additional, empty nest boxes (testo 174H, Testo AG, Lenzkirch, Germany), and we made sure that humidity levels were consistent across temperature regimes (Table 1). After 3 weeks of acclimation, workers were taken for chemical analyses and survival tests.

Chemical analyses

We sampled two outside workers (foragers) and two inside workers (nurses) from each worker group (N=480 per species). To correct for any potential daily fluctuations in CHC profile in the changing temperature treatments, we took one worker per caste at 08:00 h (end of the 12°C period) and the other one at 20:00 h (end of the 28°C period). Each single worker was put into a glass vial and frozen at −20°C until the extraction.

We analyzed the CHCs using gas chromatography–mass spectrometry (GC-MS). They were extracted by immersing single ants into *n*-hexane for 10 min. During the extraction, we added 100 ng *n*-octadecane (solved in 10 µl *n*-heptane) as internal standard for quantification of the absolute CHC amount. The extracts were transferred to a micro insert and concentrated under a gentle nitrogen stream to approximately 20 µl. For each sample, we then injected 2 µl into the GC (7890A, Agilent Technologies, Santa Clara, CA, USA) at a temperature of 250°C in splitless mode. As a carrier gas, we used helium (He) with a flow rate of 1.2 ml min^{-1} and a Zebron Inferno DB5-MS capillary column (length 30 m, Ø 0.25 mm, 0.25 µm coating, Phenomenex Ltd, Aschaffenburg, Germany) as stationary phase. The temperature programme started at 60°C. After 2 min, the oven heated at a rate of 60°C min^{-1} up to 200°C and afterwards at a constant rate of 4°C min^{-1} up to 320°C. This temperature was held constant for 10 min. The analytes then entered the MS (5975C, Agilent Technologies) and were accelerated

Table 1. Mean temperature and humidity of the treatments

Temperature (°C)	Dry	Humid
12	12.42±0.39°C	12.50±0.40°C
	61.84±9.95% RH	99.72±1.03% RH
20	19.66±0.15°C	19.99±0.18°C
	49.58±8.28% RH	99.97±0.97% RH
28	26.72±0.32°C	27.27±0.41°C
	50.99±12.88% RH	99.78±0.79% RH
Fluctuating temperature	19.25±7.23°C	19.58±7.31°C
	58.79±12.43% RH	99.74±0.79% RH

Data are means±s.d. RH, relative humidity.

Journal of Experimental Biology (2018) 221, jeb171488. doi:10.1242/jeb.171488

with an ionization voltage of 70 eV. The detector scanned for molecular fragments in a range of 40–550 *m*/*z*. Data were acquired using the software MSD ChemStation (E.02.02.1431, Agilent Technologies; Fig. 2). Hydrocarbons were identified based on a retention index based on a standard series of *n*-alkanes (Carlson et al., 1998) and diagnostic ions (Table S1). We excluded substances that were not hydrocarbons (<10% of the total extract), substances with a maximum below 0.5% and substances that occurred in less than 20% of the samples of either species.

Statistical analyses: CHC profiles

We conducted all statistical analyses using *R* version 3.2.2 (https://www.r-project.org/) and analyzed the following CHC traits: relative abundance of *n*-alkanes, monomethyl, dimethyl, trimethyl and tetramethyl alkanes, alkenes and alkadienes, and the absolute CHC quantity. For each chemical trait and for both species separately, we constructed linear mixed-effects (LME) models (command lme, package nlme; https://cran.r-project.org/web/packages/nlme/index.html), with temperature, humidity and caste as explanatory variables and colony ID and sampling location (nested in colony ID) as random effects. We reduced each model stepwise by removing the least significant interaction until Akaike's information criterion (AIC) was minimal. If necessary, data were transformed and/or, in few cases, outliers were excluded (see Table 2) to fulfil the model assumptions. Pairwise comparisons were executed using a Tukey test from the R package lsmeans (Lenth, 2016). In the Results section, results for temperature, humidity and caste originated from the same models, but will be reported in separate sections (see Table 2). Here, we focus on the CHC classes which were abundant and showed the strongest coefficient of variation (CV) in response to our experimental treatments, i.e. *n*-alkanes (*M. rubra*: CV=0.615; *M. ruginodis*: CV=0.705), dimethyl alkanes (*M. rubra*: CV=0.306; *M. ruginodis*: CV=0.425), alkenes (*M. rubra*: CV=0.700; *M. ruginodis*: CV=0.438) and alkadienes (CV=0.349, *M. ruginodis* only). Results for monomethyl (*M. rubra*: CV=0.128; *M. ruginodis*: CV=0.237), trimethyl and tetramethyl alkanes (CV=0.490 and 0.358, *M. rubra* only) are shown in Fig. S1 and Table S2. Methyl alkenes were not analyzed because their overall abundance was low (always <2%), and zero in most cases.

Finally, we performed three comprehensive analyses to compare effect sizes between species and treatments, assess overall CHC changes and analyze covariation of substance classes. We compared overall CHC composition between climate regimes using PERMANOVA in the program PRIMER (Primer-E Ltd, Auckland,

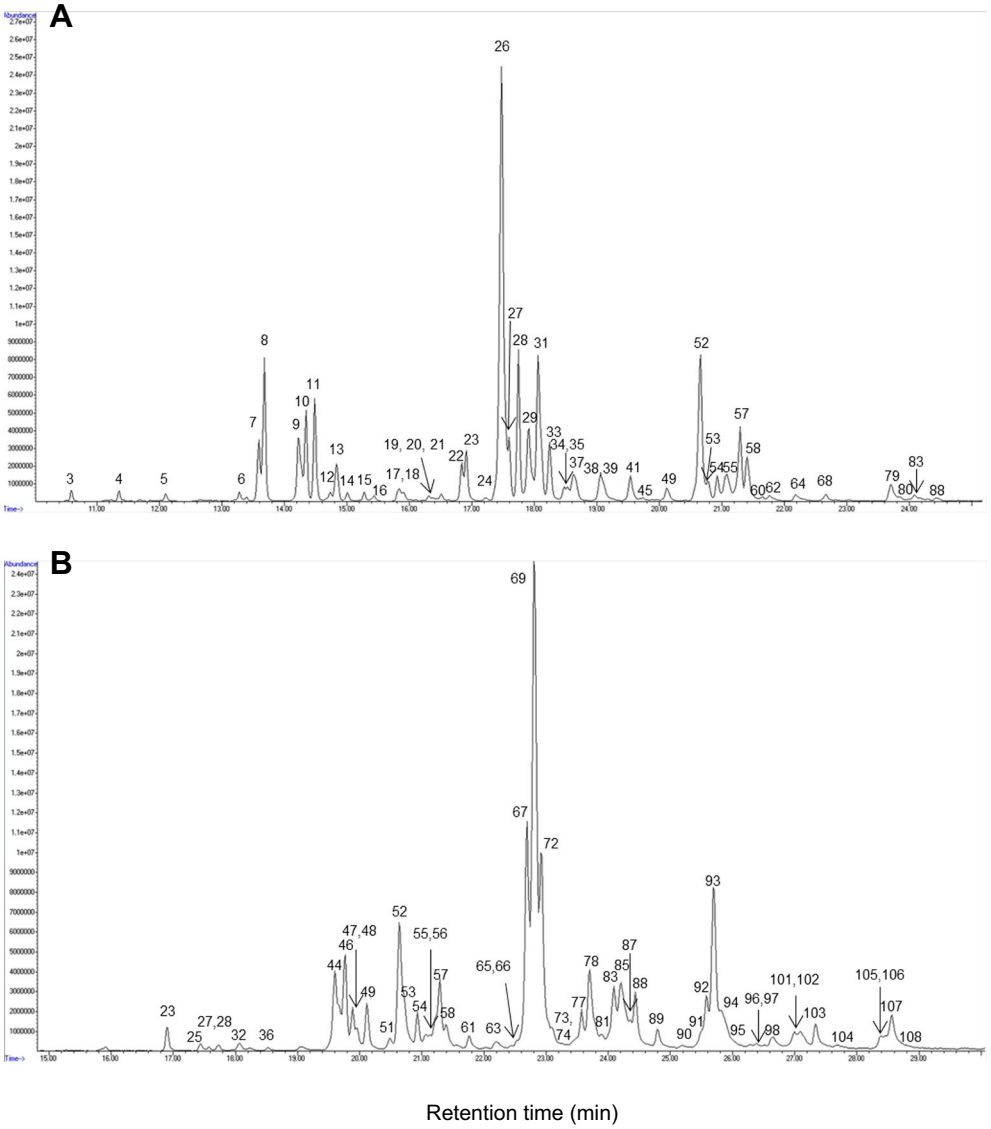

Fig. 2. Representative profiles of the two study species. (A) *Myrmica rubra* and (B) *M. ruginodis*. Each peak represents one substance (or mixture which could not be separated by gas chromatography). Numbers refer to the substances listed in Table S1. Note that we show only retention times with relevant peaks (i.e. minutes 10–25 for *M. rubra* and minutes 15–30 for *M. ruginodis*).

Journal of Experimental Biology (2018) 221, jeb171488. doi:10.1242/jeb.171488

Table 2. Model results for chemical traits of *Myrmica rubra* and *M. ruginodis*

Dependent variable	Fixed effect	*N*	d.f.	χ^2	*P*
M. rubra					
n-Alkanes[b]	Temperature	480	3	200.51	<0.0001
	Humidity		1	1.65	0.19
	Caste		1	27.03	<0.0001
Dimethyl alkanes[d]	Temperature	476	3	197.54	<0.0001
	Humidity		1	1.84	0.17
	Caste		1	52.94	<0.0001
Alkenes[c]	Temperature	473	3	240.80	<0.0001
	Humidity		1	14.67	0.0001
	Caste		1	45.41	<0.0001
	Temperature×Caste		3	11.04	0.011
Absolute quantity[a]	Temperature	472	3	199.33	<0.0001
	Humidity		1	106.36	<0.0001
	Caste		1	3.88	0.049
M. ruginodis					
n-Alkanes[b]	Temperature	480	3	388.21	<0.0001
	Humidity		1	8.88	0.0029
	Caste		1	51.28	<0.0001
	Temperature×Humidity		3	14.02	0.0029
	Temperature×Caste		3	9.60	0.022
Dimethyl alkanes[d]	Temperature	480	3	253.49	<0.0001
	Humidity		1	19.89	<0.0001
	Caste		1	42.00	<0.0001
Alkenes[b]	Temperature	480	3	208.71	<0.0001
	Humidity		1	31.40	<0.0001
	Caste		1	22.02	<0.0001
	Humidity×Caste		1	3.97	0.046
Alkadienes[d]	Temperature	480	3	172.97	<0.0001
	Humidity		1	2.66	0.10
	Caste		1	38.91	<0.0001
Absolute quantity[a]	Temperature	473	3	35.24	<0.0001
	Humidity		1	50.11	<0.0001
	Caste		1	17.33	<0.0001
	Humidity×Caste		1	6.64	0.010

All results from linear mixed-effects models (LME) including colony ID and sampling location as random factors. Superscript letters denote whether data were (a) log transformed, (b) logit transformed, (c) arcsine-square root transformed or (d) not transformed. In some cases, outliers were removed to fulfil model assumptions (see *N*). All dependent variables except for absolute quantity are relative abundances.

New Zealand) with the same fixed and random factors as above, and visualized it using non-metric multidimensional scaling (NMDS) ordination. Hierarchical cluster analyses were performed (hclust, R package vegan; https://cran.r-project.org/web/packages/vegan/index.html) with the complete linkage method to determine which treatment groups were most similar to each other (Fig. S2A,B). Covariation among substance classes was analyzed using a principal component analysis (PCA) on the abundances of CHC classes (Fig. S2C,D).

Survival experiment

We placed one nurse and one forager from each of the constant temperature treatments (*N*=360) in a polystyrole vial (Ø 28 mm, height 64 mm, volume 30 ml, K-TK e.K., Retzstadt, Germany) into which we plugged a piece of foam (1.04±0.09 cm thick) to approximately one-third of the height. We then filled it with 10.20±0.96 g silica gel (2–5 mm, Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany) and sealed it airtight with Parafilm® (Bemis Flexible Packaging, Neetah, WI, USA). The silica gel was used to absorb the humidity within the vial (Bazinet et al., 2010; Stinziano et al., 2015). The experiment was performed in a climate chamber at 20°C. We checked survival first after 6 h and afterwards once every hour until the 24th hour of the experiment. Ants that had died (defined here as the lack of any movement even after shaking the vial) in the first 6 h (68 cases) were excluded from the data set because the exact time of death was uncertain. These 68 cases were distributed largely evenly across the treatments, with marginally more ants from the 28°C treatments (χ^2-test: χ^2_2=5.69, *P*=0.058). All observations were conducted blindly.

The data were analyzed with a Cox mixed-effects model (command coxme, R package coxme; https://cran.r-project.org/web/packages/coxme/index.html) with species, temperature, humidity and caste as explanatory variables. Colony ID and test day were implemented as random effects as this combination yielded the best AIC. Variables were tested with a type-II ANOVA (command Anova, package car version 2.1-1, adjusted by J. Fox 2015; Fox and Weisberg, 2011). We removed non-significant interactions stepwise until AIC was lowest.

Microrheology of CHCs

To quantify the viscosity of CHC profiles, we used microrheology. This new approach allows measurements on small biological samples and cells that were previously impossible owing to the minute amounts of material available (for a review, see Waigh, 2005). In passive microrheology (or particle tracking microrheology), the viscosity of a fluid is derived from the Brownian motion of microscopic probe particles embedded in the fluid. In complex fluids, microrheology also allows the investigation of viscoelastic properties and phase heterogeneity. Microrheology experiments are usually performed on small volumes of the order of 1 µl. We have developed a fluid collection procedure for micrometric droplets, allowing microrheology measurements on extremely small volumes of the order of 10 to 100 pl (Abou et al., 2010).

Particle-tracking microrheology experiments were performed on several extracts of *M. rubra* workers acclimated to constant, dry conditions at either 12°C or 28°C. The CHC extract of an ant worker was dissolved in 20 µl pentane and placed on a glass slide to evaporate the solvent. To collect the CHC extracts from the glass slide (on an inverted Leica DM IRB microscope, Leica Microsystems GmbH, Wetzlar, Germany), we used a fine glass micropipette connected to a pneumatic microinjector (CellTram Air, Eppendorf AG, Hamburg, Germany) mounted on a three-axis micro-manipulator (Burleigh, Thorlabs SAS, Maisons-Laffitte, France). The fine tips (2–3 µm diameter) of the capillaries were prepared by pulling from borosilicate glass micropipettes with 1 mm outer diameter and 0.78 mm inner diameter (Harvard Apparatus S.A.R.L., Les Ulis Cedex, France) with a P-1000 micropipette puller (Sutter Instrument, Novato, CA, USA). The micropipette tip was moved onto the surface in order to collect the largest possible amount of CHC extracts. Owing to capillary effects, the CHC extracts spontaneously rose in the micropipette as soon as the tip touched the liquid.

Dry melamine beads (Acil, France; bead diameter: 0.740±0.005 µm) were deposited on a clean glass slide. The collected CHC extract was then ejected onto the beads by applying positive pressure to the capillary via the microinjector. After ejection, the capillary tip was moved along the glass slide to detach beads that were stuck to the glass surface. The CHC fluid was then drawn up and ejected again several times in order to mix the beads with the CHC extract (Abou et al., 2010).

The samples were observed using 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 was recorded for 20 s at 100 Hz with a fast sCMOS camera (OrcaFlash4.0 v2+, Hamamatsu Photonics France S.A.R.L., Massy, France) mounted on the inverted microscope. For reliable analysis of the Brownian motion, particular attention was paid to record only beads far from the surface of the droplet or the glass slide. A self-written image analysis software allowed us to track the x and y positions of any beads close to the focus plane of the objective. For each tracer bead, the time-averaged mean squared displacement (MSD) was calculated as: $<\Delta r^2(t)>_{t'} = ((x(t'+t)-x(t'))^2 + (y(t'+t)-y(t')^2)_{t'})$.

For Brownian motion of tracers in a Newtonian fluid, the ensemble-averaged MSD increases linearly with the lag time, as $<\Delta r^2(t)>=4Dt$ (in two dimensions), where D is the diffusion coefficient. In this case, the viscosity η can be 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).

We compared the viscosity of CHC extracts of *M. rubra* ants acclimated to 12°C and 28°C using a Wilcoxon rank sum test. Some samples were extremely viscous and could not be collected with micropipettes for measurement; these were assigned the highest viscosity ranks for statistical analysis. In addition, we tested whether the viscosity of a sample was linked to its chemical composition. Using Spearman's rank correlation, we tested whether viscosity was associated with the percentage of *n*-alkanes or dimethyl alkanes. These are the two substance classes that were both highly abundant and changed strongly during temperature acclimation.

RESULTS

Chemical analyses

The climate treatments strongly affected the CHC profiles in both ant species (Fig. 3). Overall, temperature regime had by far the strongest impact on CHC composition (PERMANOVA, *M. rubra*: pseudo-F_3=69.5, *M. ruginodis*: pseudo-F_3=76.7, both P=0.001; Table S2). In contrast, humidity-induced effects were considerably smaller, especially in *M. rubra* (*M. rubra*: pseudo-F_1=4.0, P=0.007; *M. ruginodis*: pseudo-F_1=18.2, P=0.001; Table S2). Furthermore, nurses and foragers had strongly different profiles in both species (both pseudo-F_1>20.0, P=0.001; Table S1).

Effects of different constant temperatures

In spite of their strongly different CHC composition (Figs 1, 2; Table S1), both *M. rubra* and *M. ruginodis* showed similar responses to the three constant temperature regimes: in both species, *n*-alkanes and alkenes increased with acclimation temperature. The increase was especially high between 20°C and 28°C, but weaker or, for alkenes in *M. ruginodis*, not detectable between 12°C and 20°C (Table 2, Fig. 4A–D). The relative amounts as well as the absolute quantities of *n*-alkanes increased with acclimation temperature (Fig. S3). In contrast, dimethyl alkanes decreased with temperature, again with a particularly high shift between 20°C and 28°C in both species (Table 2, Fig. 4E,F). Alkadienes, which were the dominant CHC class in *M. ruginodis* (34.68±12.08%) but absent in *M. rubra*, similarly decreased at 28°C compared with ants kept at 12°C or 20°C (Table 2, Fig. 4G). Similar effects were found for trimethyl alkanes, whereas the tetramethyl alkanes showed opposite changes (both of which occurred in *M. rubra* only; Table S2). Interestingly, monomethyl alkanes were the most abundant CHC class in *M. rubra* (48.39±6.78%) and were highly abundant in *M. ruginodis* (21.13±5.00%), but showed only weak responses to climatic conditions (Table S2, Fig. S1).

Absolute CHC quantities decreased with acclimation temperature in *M. ruginodis*. In *M. rubra*, they were also lowest at 28°C, but higher at intermediate temperatures than at 12°C (Table 2; Fig. S1A,B).

Constant versus fluctuating temperature

Compared with constant temperatures, the fluctuating regime resulted in yet another different CHC composition, and had different effects on the two ant species. In *M. rubra*, profiles from fluctuating temperatures were relatively similar to those of 12°C and

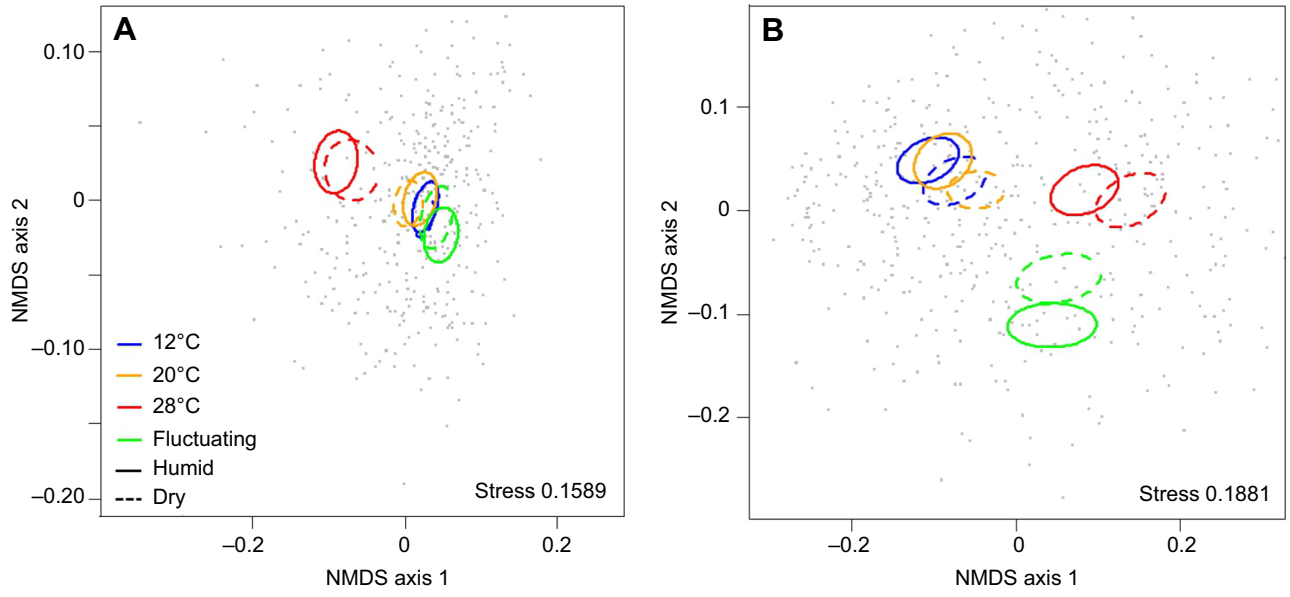

Fig. 3. Non-metric multidimensional scaling (NMDS) ordinations of the chemical profiles of the two study species, calculated for two dimensions. (A) *Myrmica rubra* and (B) *M. ruginodis*. The ellipses show the 95% confidence areas around the centroids for each climate regime (temperature: 12°C, 20°C, 28°C and fluctuating; humidity: dry and humid). Each dot represents one sample (note that some dots lie outside of the plotted range).

Journal of Experimental Biology (2018) 221, jeb171488. doi:10.1242/jeb.171488

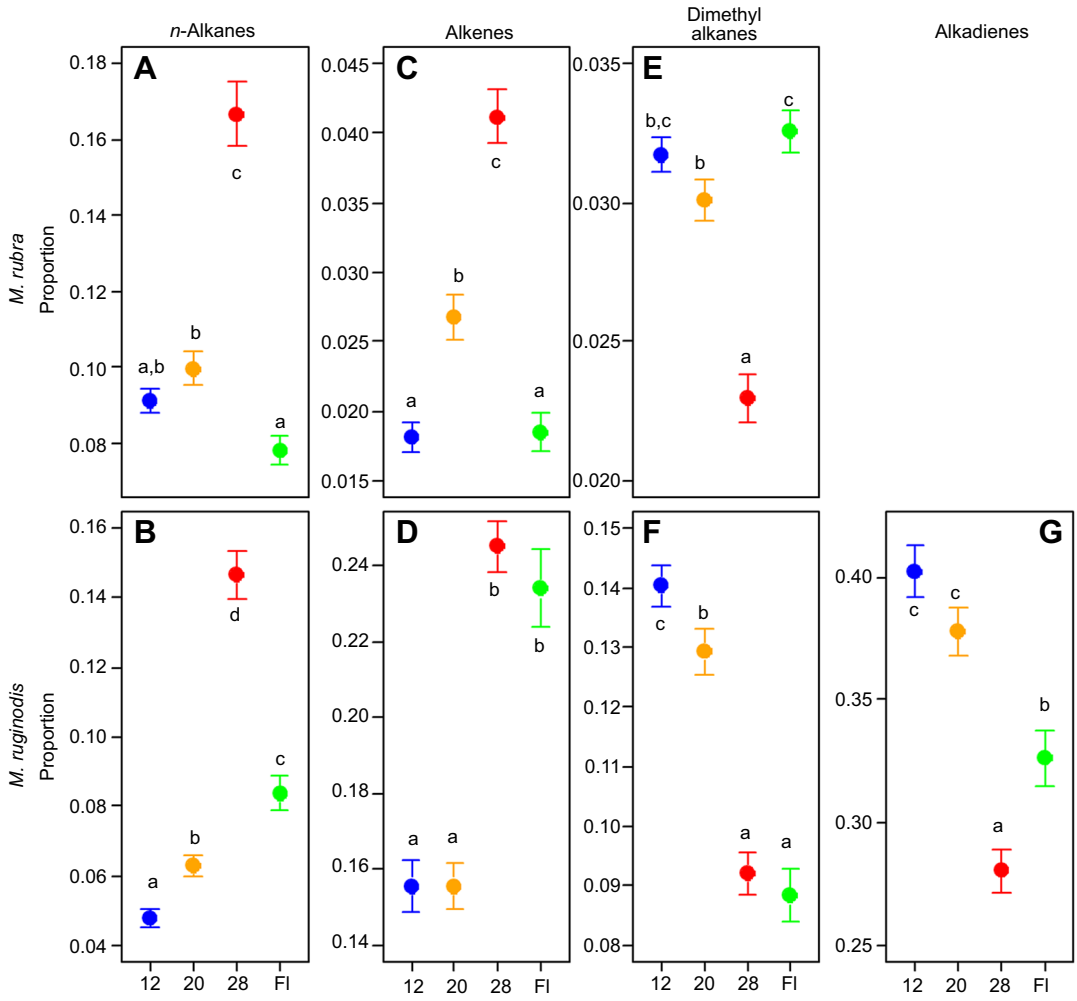

Fig. 4. Effects of treatment temperature on the ants' CHC profiles. The plots visualize the effects of four different temperature treatments (12°C, 20°C, 28°C and fluctuating, each represented by similar colour code as in Fig. 2 for easier comparison) on the proportions of (A,B) *n*-alkanes, (C,D) alkenes, (E,F) dimethyl alkanes and (G) alkadienes. All graphs show back-transformed means±s.e.m. Different letters indicate statistically significant differences according to pairwise Tukey tests on the LME data (P<0.05).

20°C. In contrast, *M. ruginodis* profiles from the fluctuating treatment showed stronger differences to all three constant temperature regimes, but resembled more closely those of 28°C. This could be confirmed for overall CHC composition with NMDS ordination (Fig. 3) and cluster analysis (Fig. S2A,B). Similar patterns were found for the proportions of substance classes: at fluctuating temperature, proportions of *n*-alkanes, alkenes and dimethyl alkanes in *M. rubra* were similar to those in the 12°C treatment (Fig. 4A,C,E). In *M. ruginodis*, fluctuating temperatures led to alkene and dimethyl alkane proportions similar to those of the 28°C treatment (Fig. 4D,F), whereas *n*-alkane and alkadiene proportions were in between those of the 20°C and the 28°C treatments (Fig. 4B,G). Absolute CHC quantities were highest at fluctuating temperatures in *M. rubra*, but had a low level comparable to the 28°C treatment in *M. ruginodis* (Fig. S1A,B).

Effects of humidity

In both species, dry conditions led to a massive increase in absolute CHC quantity (Table 2, Fig. 5A,B). However, CHC composition was less affected. In *M. rubra*, the only effect was an increase in alkenes under dry conditions. *Myrmica ruginodis* showed more changes under dry conditions, producing more alkenes (particularly in foragers) and less dimethyl alkanes (Table 2B, Fig. S4D,F). Moreover, dry conditions led to an increase in *n*-alkanes at 28°C but not at 20°C or 12°C (Table 2).

Differences between foragers and nurses

Foragers and nurses showed strong chemical differences, which were consistent across the two species (Fig. S5). Foragers had higher proportions of *n*-alkanes and alkenes, but lower proportions of dimethyl alkanes (Table 2; Fig. S5A–F). Moreover, *M. ruginodis* foragers had lower proportions of alkadienes than nurses. In both species, nurses possessed more CHC (in µg) than foragers, except for dry-acclimated *M. rubra* workers (humidity×caste interaction, Table 2; Fig. S5H,I). Contrary to our expectation, the two behavioural castes showed largely similar responses to the climatic treatments (i.e. no significant interactions of caste with temperature or humidity), with few exceptions: forager–nurse differences in the proportion of *n*-alkanes in *M. ruginodis* and alkenes in *M. rubra* (Table 2) were only significant at 20°C and at fluctuating temperature (temperature×caste interaction). Foragers kept under dry conditions possessed higher proportions of alkenes, whereas those kept under humid conditions did not differ from nurses (humidity×caste interaction; Table 2).

Journal of Experimental Biology (2018) 221, jeb171488. doi:10.1242/jeb.171488

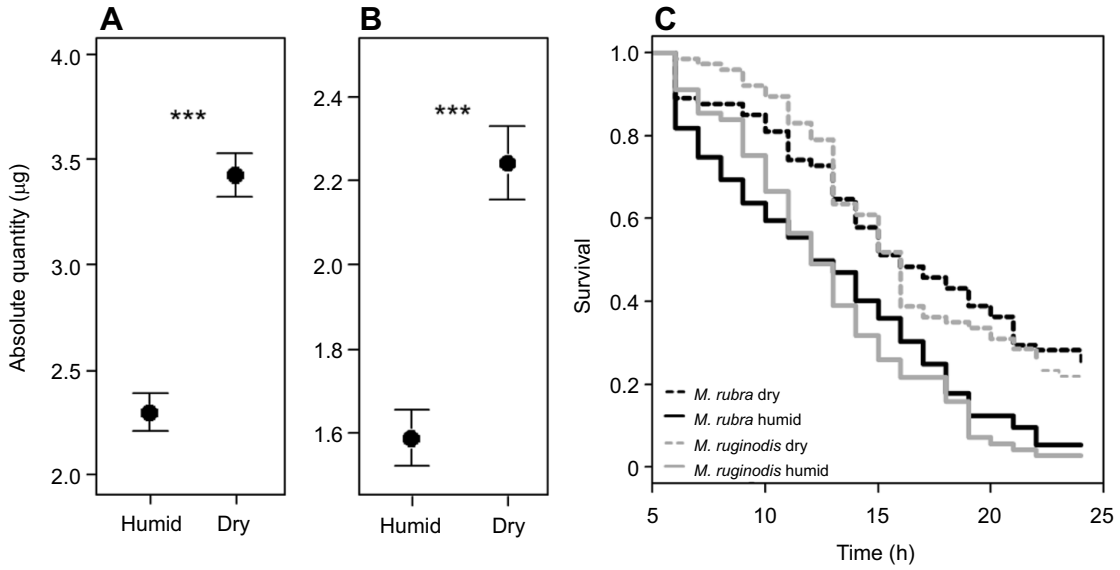

Fig. 5. Effects of humidity on the ants' absolute CHC amount and survival. (A,B) Absolute amount of CHCs (µg) of (A) *M. rubra* and (B) *M. ruginodis* workers acclimated to humid and dry conditions (back-transformed means±s.e.m. of log-transformed data). Significant differences are indicated by asterisks (***P<0.001). (C) Worker survival of dry conditions over time. The Kaplan–Meier plot shows that the two species do not differ in survival, but that there is an effect of acclimation to dry versus humid conditions on survival rate.

Survival experiment

Ants of both species survived drought stress longer if they had acclimated to dry conditions compared with humid conditions (Cox mixed-effects model: χ^2_1=33.07, P<0.0001; n=292 ants; 40/292 still alive after 24 h; Fig. 5C). Additionally, nurses survived better than foragers (χ^2_1=4.17, P=0.041). However, there was no difference between *M. rubra* and *M. ruginodis* workers (χ^2_1=0.16, P=0.69; Fig. 5C). Surprisingly, survival was not influenced by the acclimation temperature (χ^2_2=1.50, P=0.47) or any interaction of worker group, humidity and temperature (all non-significant after model reduction).

Phase characteristics and microrheology of CHC extracts

All CHC extracts were highly heterogeneous, with solid and liquid phases co-occurring even at ambient temperatures (Fig. 6A,B). Microrheology measurements on the liquid fraction of the CHCs were conducted for *M. rubra*. For several *M. rubra* colonies, extracts of cool-acclimated ants contained visible hydrocarbon crystals at temperatures below 20°C, which completely liquefied at 25°C (Fig. 6C–F), suggesting a broad phase transition.

CHC viscosity was strongly correlated with the chemical composition of the CHC extracts. The viscosity increased with higher percentage of *n*-alkanes (Spearman's rank correlation: N=17, ρ=0.65, P=0.005), but decreased with higher proportions of dimethyl alkanes (N=17, ρ=−0.67, P=0.003). This suggests that warm-acclimated ants, because of their higher amounts of *n*-alkanes and lower amounts of dimethyl alkanes, also should have more viscous CHC profiles. Indeed, extracts of ants acclimated to 28°C had a higher viscosity compared with those from 12°C-acclimated ants (Wilcoxon rank sum test: N=17, W=12, P=0.027).

Fig. 7 shows one example of the measured mean squared displacement (MSD) of the tracers measured at 22°C for two extracts of ants from the same colony, one warm-acclimated and one cold-acclimated. Here, the extract from the 28°C-acclimated *M. rubra* was ca. 70 times as viscous as the one from the 12°C-acclimated worker of the same colony (7540±250 mPa s versus 110 ±5 mPa s). The very large viscosity (~7000 times that of water) found in warm-acclimated *M. rubra* ants is consistent with the fact that four other extracts investigated were largely solid at 25°C, and too viscous to be collected with the micropipette (high *n*-alkanes contents up to 65%). These results suggest that the ants' acclimation to higher temperatures resulted not only in a higher proportion of CHCs that were solid at ambient temperature, but also in a higher viscosity of the liquid CHC.

DISCUSSION

Insects use a superficial layer of CHCs for waterproofing their body (Hadley, 1994), and for a variety of other biological functions including, in particular, communication (Blomquist and Bagnères, 2010; Leonhardt et al., 2016). The chemical composition of CHCs strongly influences these functions (Edney, 1977; Hadley, 1994), but the biophysical drivers of CHC variation are still poorly understood (Menzel et al., 2017). As insects have to survive under a variety of climatic conditions, the composition of their CHC layer may need to be adjusted to maintain its functions. The present study is one of the first to disentangle the effects of humidity and temperature on CHC profiles, and to compare constant and fluctuating temperature regimes.

Effects of constant temperature regimes

The different constant temperature regimes resulted in largely parallel changes in both species. After acclimation to higher temperatures, CHCs promoting tight molecular alignment (*n*-alkanes) increased in relative abundance, while substances disrupting molecular alignment (dimethyl and trimethyl alkanes, alkadienes) decreased. Such changes have been reported previously to provide a better waterproofing of the cuticle (Gibbs and Mousseau, 1994; Menzel et al., 2018; Wagner et al., 2001). However, our data also show that acclimation to different temperatures did not have any effect on drought survival. This indicates that the observed temperature-induced changes of the CHC profile are not related to waterproofing. Although we cannot exclude that the changes are non-adaptive, e.g. owing to shifts in physiological pathways, we hypothesize that they serve to adjust the

Journal of Experimental Biology (2018) 221, jeb171488. doi:10.1242/jeb.171488

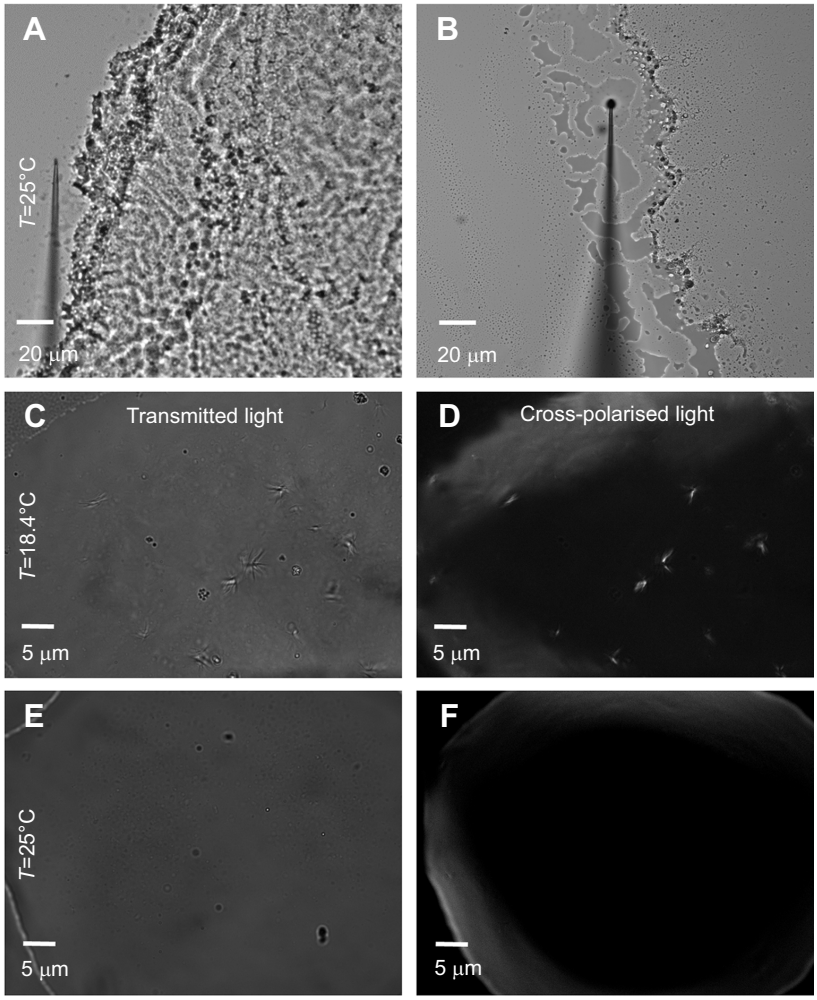

Fig. 6. Solid and liquid phases of CHC extracts of *M. rubra*. The CHC extract from a 28°C-acclimated ant (A) contains much more solid phase compared to the mixture of solid and liquid phases in CHC extracts of a 12°C-acclimated ant (B) (photos taken at 25°C). (C–F) Transmitted (C,E) and crossed-polarized light (D,F) photographs of CHC extracts from *M. rubra* ants acclimated to 12°C. Solid crystals are visible at 18.4°C (C,D) but not at 25°C (E,F), indicating a broad transition range and the existence of solid and liquid phases at ambient temperatures.

viscosity of the CHC layer. A sufficiently low viscosity may be critical for maintaining many other essential biological functions of the CHC layer, including the transfer of communication cues, diffusion of recognition cues across the body surface, healing of scratches, lubrication and adhesion (Cooper et al., 2009; Dirks et al., 2010; Drechsler and Federle, 2006; Gorb, 2001; Wigglesworth, 1945). All these functions depend to some extent on the viscosity of the CHC layer. Generally, the viscosity of liquids increases when cooled to lower temperatures, until they solidify at the melting point. Consistent with the observed shift of the CHC composition towards compounds that disrupt molecular aggregation, acclimation to lower temperatures resulted in lower proportions (and lower absolute amounts) of *n*-alkanes, but higher proportions of dimethyl alkanes, which resulted in a reduced CHC viscosity. Clearly, these changes cannot be explained by waterproofing requirements, as tightly aggregating CHC could provide efficient waterproofing at both warm and cold temperatures. Instead, we hypothesize that a selection pressure could act to prevent complete CHC solidification at low temperatures, because this would impede communication and other essential functions. One mechanism to prevent solidification would be to maintain CHC viscosity at a constant low level by adjusting the chemical composition of the CHC profile. A previous study on membrane lipids in *Drosophila* reported comparable chemical changes during acclimation, supporting the idea that acclimatory changes serve to maintain CHC viscosity (Overgaard et al., 2006). Our data are consistent with a homeostatic control of viscosity. However, the detailed adaptive benefits of low CHC viscosity have been largely ignored in previous work. Future research should test whether low CHC viscosity is indeed maintained in a homeostatic way, and study how changes in CHC composition and viscosity affect communication and other functions. Our study shows that microrheology is a powerful method to address these questions.

The strongest chemical changes, as measured by coefficients of variation, were found at the opposite ends of the aggregative–disruptive gradient (*n*-alkanes, dimethyl alkanes, alkadienes). Generally, higher concentrations (relative abundances) of *n*-alkanes (which increase viscosity) coincided with lower concentrations of dimethyl alkanes, trimethyl alkanes, methyl alkenes and alkadienes (all of which decrease viscosity). In contrast, only weak acclimation effects were found for monomethyl alkanes, which may have intermediate effects on viscosity, but were highly abundant in both species. This indicates that certain CHC classes can vary relatively independently of each other (Fig. S2C,D). The different effect sizes show that it was mainly the most aggregating and the most disruptive compounds that changed rather than the monomethyl alkanes. Surprisingly, the relative abundance of alkenes increased with temperature, in parallel with the *n*-alkanes. The significance of this finding is still unclear. Alkenes should reduce CHC viscosity and melting points to a similar (or even higher) degree as dimethyl alkanes (Gibbs, 2002; Gibbs and Pomonis, 1995). However, as alkenes and *n*-alkanes can crystallize

Journal of Experimental Biology (2018) 221, jeb171488. doi:10.1242/jeb.171488

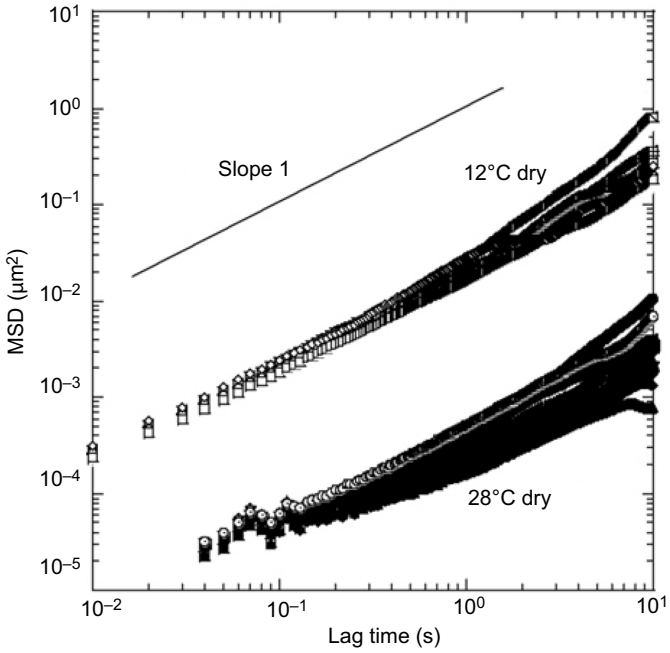

Fig. 7. Microrheology of CHC in *M. rubra*. Time-averaged mean-squared displacement (MSD) of 0.74 µm-diameter melamine tracer beads undergoing Brownian motion within the CHC sample. *Myrmica rubra* ants acclimated to 28°C (bottom) had CHC that exhibited lower MSD (indicating a higher CHC viscosity) than ants acclimated to 12°C (top).

separately (Gibbs, 2002), a mixture of both might promote the persistence of liquid parts in the CHC layer even when *n*-alkanes are abundant.

A further unexpected result was that the absolute CHC quantity decreased with temperature. CHC quantities decreased in *M. ruginodis* from 12°C to 28°C, while in *M. rubra*, they were highest under fluctuating temperatures, but also lowest at 28°C. This result differs from previous studies where CHC quantity was unaffected by (Gibbs et al., 1998) or even increased with temperature (Gefen et al., 2015). A possible explanation for this pattern is that, at higher temperatures, CHCs are lost faster via mechanical abrasion (when the ants are more active) or via footprints secreted from the tarsi during walking (Geiselhardt et al., 2010; Wüst and Menzel, 2017). However, our results show that the absolute amount of individual compounds such as *n*-alkanes increased at the highest temperatures, demonstrating that evaporation alone cannot explain the observed patterns.

Effects of fluctuating temperature

Determining the difference between constant and fluctuating temperature regimes is crucial to understanding how insects can cope with changing microclimate and weather conditions (Colinet et al., 2015). In many habitats, daily temperatures vary quickly, and probably faster than insects can acclimate. Interestingly, the CHC composition of ants from fluctuating regimes was not intermediate between the two corresponding constant temperatures: in *M. rubra*, the fluctuating regime led to CHC profiles very similar to those of the coolest, constant regime. In contrast, the CHC profiles of *M. ruginodis* differed strongly from all constant temperature regimes (Fig. 3). Thus, temperature fluctuations had a stronger effect on *M. ruginodis* profiles than on *M. rubra* profiles, indicating that the two species differ in CHC changes during acclimation.

Effects of humidity

Both *Myrmica* species showed increased absolute CHC quantities under dry conditions. Similar increases were shown for scorpions (Gefen et al., 2015) and (albeit at high temperatures only) in desert beetles (Hadley, 1977). As expected, dry-acclimated individuals were more resistant to drought stress, consistent with previous studies, showing the adaptive value of this drought acclimation (Bazinet et al., 2010; Terblanche and Kleynhans, 2009). It is possible that reports of higher CHC quantities in insects acclimated to warm conditions are the result of stronger drought stress and not of the higher temperatures themselves (Hadley, 1977). In contrast to previous work, our experiments disentangled these two factors, and showed that higher temperatures even led to reduced overall CHC quantities once humidity was controlled for. Notably, temperature acclimation did not affect drought survival. Our results indicate that at least for *Myrmica*, CHC quantity is more important for desiccation resistance than CHC composition.

Drought stress may partly explain the differences between nurses and foragers: being more exposed to the sun, foragers may face higher desiccation stresses than nurses, consistent with their increase in *n*-alkane concentration and reduction in dimethyl alkanes. The smaller absolute quantity of CHCs in the foragers is surprising, as foraging workers may be more exposed to drought than nurses staying inside the relatively humid nest. In our opinion, the smaller CHC quantity in foragers is not adaptive but the result of higher CHC evaporation or abrasion during their outside activity (Johnson, 2000; Johnson and Gibbs, 2004), or of their older age. This idea is corroborated by the lower desiccation survival of foragers than nurses in our assays.

Microrheology of CHCs

All CHC extracts contained both solid and liquid fractions, at least at temperatures below 30°C. Early work established that water loss rates in insects are minimal at low temperatures, but increase suddenly once the temperature exceeds a ‘critical’ temperature (Ramsay, 1935; Wigglesworth, 1945). This sudden increase of water loss was shown in many insects to coincide with measured melting points of CHC extracts (Gibbs, 1998, 2002), suggesting that the increase in water permeability is explained by a melting of the lipid layer at this temperature. Although CHC melting temperatures measured using capillary melting techniques, differential scanning calorimetry or infrared spectroscopy ranged from 27°C to ca. 100°C (Gibbs, 2002), our observations show that liquid CHCs are already present well below these temperatures, and that complex CHC profiles have a broad-phase transition range rather than a single sharp melting point. This is evidenced by the observed coexistence of liquid and solid parts in CHC extracts. Moreover, the microrheology experiments show that even the liquid fraction itself is heterogeneous and exhibits locally varying viscosity.

Our microrheology measurements confirm that the ants’ acclimation response modified the physical properties of the CHCs. The differences in viscosity can be explained by the observed changes in chemical composition: warm-acclimated ants showed higher amounts of solid *n*-alkanes and lower proportions of dimethyl alkanes, consistent with their more viscous CHC layers. Extracts from cold-acclimated ants had fewer *n*-alkanes, were less viscous and lacked any visible solid parts at the 25°C measurement temperature. Our measurements show that the viscosity of CHC extracts provides a good proxy to assess lipid mobility on the cuticle surface, opening up avenues for future research.

Conclusions

Insect CHC profiles are astonishingly diverse. The different functions of CHCs, particularly waterproofing and communication, depend on CHC composition and are affected by temperature and humidity. CHC profiles are therefore linked to the insects' climatic niche, and their ability to acclimate or cope with short-term weather fluctuations. We have shown that, despite strong chemical differences, *M. rubra* and *M. ruginodis* changed their profiles in similar and predictable ways during acclimation to constant temperatures (*n*-alkanes increased at higher temperatures, whereas dimethyl alkanes and alkadienes decreased) and humidity, and both species survived drought stress equally well. However, both species acclimated to fluctuating temperature regimes in a different way. Whereas the profiles of *M. rubra* acclimated to fluctuating temperature were similar to those of conspecifics acclimated to constant 12°C or 20°C, the profiles of *M. ruginodis* acclimated to fluctuating temperature differed from those of conspecifics acclimated to any constant temperature regime. Both ant species responded to dry conditions by producing larger amounts of CHCs, but only *M. ruginodis* also changed the composition of its profile. In summary, compared with *M. rubra*, *M. ruginodis* showed stronger CHC changes in response both to fluctuating temperature and drier conditions. Therefore, it is possible that CHC differences give rise to differences in position and width of microclimate niches, which may result in different microhabitats and geographic ranges.

CHC acclimation may be constrained by the need to maintain sufficiently low CHC viscosity for communication and other functions at low temperatures, and by the need to provide sufficient waterproofing at higher temperatures. In social insects in particular, CHC communication signals and recognition cues are exchanged between individuals. Future studies should investigate the biological effects of CHC viscosity, and in particular address the effects of CHC acclimation on nestmate recognition and other functions. The trade-off between waterproofing and communication requirements makes the evolution and plasticity of CHC profiles an intriguing field of research with many open questions.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: P.P.S., F.M.; Methodology: B.A., W.F.; Formal analysis: P.P.S., F.M.; Investigation: P.P.S., L.H.B., B.A., W.F.; Writing - original draft: P.P.S., F.M.; Writing - review & editing: P.P.S., B.A., W.F., F.M.; Visualization: P.P.S., B.A., W.F.; Supervision: F.M.

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Data availability

Data are available from the Dryad Digital Repository (Sprenger et al., 2018): https://doi.org/10.5061/dryad.891vt38

Supplementary information

Supplementary information available online at http://jeb.biologists.org/lookup/doi/10.1242/jeb.171488.supplemental

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