

1 **Coping with the climate: Cuticular hydrocarbon acclimation of ants under**  
2 **constant and fluctuating conditions**

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11 **Running headline:**

12 Ant cuticular hydrocarbon acclimation

13

14 **Summary statement:**

15 Ants adjust their cuticular hydrocarbon layer to humidity and temperature, thereby  
16 maintaining its functionality for waterproofing and communication. Varying and constant  
17 temperature regimes had different effects on hydrocarbon composition.

18 **Abstract**

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

38

39 **Key words**

40 Cuticular hydrocarbon acclimation, desiccation resistance, drought survival, phenotypic  
41 plasticity, microrheology, viscosity

42

43 **Introduction**

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

53 Acclimation to different temperatures has been shown for a variety of animals (Angilletta Jr.,  
54 2009), but responses differ between constant and fluctuating temperatures (Colinet et al.,  
55 2015). In addition to temperature, humidity and its interaction with temperature may affect  
56 the fitness of ectothermic animals (Chown et al., 2011; Terblanche and Kleyhans, 2009).  
57 Terrestrial arthropods should be highly susceptible to climatic changes, as their metabolic  
58 rate is directly affected by the environmental temperature and their large surface-to-volume  
59 ratio makes them vulnerable to water loss. Most water loss under ambient temperatures  
60 occurs through the cuticle (Edney, 1977; Hadley, 1994). Therefore, it is important to  
61 understand the mechanisms insects use to reduce cuticular water loss.

62 In insects, waterproofing is achieved by a hydrocarbon layer on the cuticle (Blomquist and  
63 Bagnères, 2010). Cuticular hydrocarbons (CHC) comprise a complex mixture of *n*-alkanes,  
64 methyl-branched alkanes and unsaturated hydrocarbons (alkenes) (Blomquist, 2010). These  
65 compounds differ in the carbon chain length and the number of methyl groups and/or double  
66 bonds. In addition to waterproofing, CHCs also serve several other functions, most  
67 importantly as communication signals in many insect species (Blomquist and Bagnères,

68 2010). Especially in social insects, cuticular hydrocarbons encode a plethora of information,  
69 regulating (amongst other things) nestmate recognition and the division of labour within a  
70 colony (Leonhardt et al., 2016). For example, behavioural castes among ant workers (such as  
71 scouts, foragers and nurses) possess quantitatively different CHC profiles, which can trigger  
72 different behavioural responses in their nestmates (Greene and Gordon, 2003; Pamminger et  
73 al., 2014; Wagner et al., 2001).

74 Variation in cuticular waterproofing depends both on the absolute amount and quantitative  
75 composition of the CHC layer (Chown et al., 2011; Edney, 1977). The strong temperature  
76 dependence of the waterproofing function of insect cuticle is thought to be caused by a  
77 ‘melting’ of the CHCs, which is influenced by their chemical composition (Gibbs, 1998;  
78 Gibbs and Pomonis, 1995). Early studies on several insect species showed that evaporation of  
79 water through a CHC layer increases drastically if the ambient temperature is raised above a  
80 critical temperature  $T_c$  (Beament, 1945; Beament, 1958; Beament, 1959; Gibbs, 2002;  
81 Ramsay, 1935; Rourke and Gibbs, 1999; Wigglesworth, 1945). The melting point of the CHC  
82 layer increases when CHC molecules align and aggregate. Generally, *n*-alkane molecules  
83 aggregate most tightly due to van der Waals forces, and this aggregation increases with chain  
84 length. Hydrocarbons with strong van der Waals bonds (e.g. *n*-alkanes, monomethyl alkanes)  
85 can even crystallize (Brooks et al., 2015). Thus, *n*-alkanes should provide the best  
86 waterproofing to the insect, especially so if they have high chain lengths. In contrast, methyl  
87 groups and double bonds disrupt the linear geometry of *n*-alkanes and therefore hinder a tight  
88 molecular aggregation. The melting temperature  $T_m$  of a hydrocarbon therefore decreases  
89 from *n*-alkanes to monomethyl alkanes, dimethyl alkanes, alkenes, and alkadienes (in this  
90 order) (Gibbs, 1998; Gibbs and Pomonis, 1995). However, hydrocarbon mixtures do not have  
91 a sharp melting point. Rather, melting occurs over a wider temperature range (Gibbs, 1995).  
92 Moreover, tight molecular cohesion of cuticular hydrocarbons may not only increase their

93 melting point, but also increase their viscosity. The higher the viscosity of the CHC layer, the  
94 lower is the diffusion rate of CHC molecules across the body surface (the diffusion  
95 coefficient is inversely proportional to viscosity according to the Stokes-Einstein relation,  
96 Einstein, 1905). However, the ability of CHC molecules to move across the cuticle is  
97 essential for many biological functions. For example, CHCs can only serve as communication  
98 signals if they are sufficiently liquid to diffuse and spread across the cuticle, or onto sensory  
99 sensilla of other insects that act as communication partners (Blomquist and Bagnères, 2010;  
100 Maitani et al., 2010). Low CHC viscosity may also be essential for the repair of scratches  
101 (Wigglesworth, 1945), for the lubrication of joints (Cooper et al., 2009; Gorb, 2001) and for  
102 the rapid attachment and detachment of sticky footpads (Labonte and Federle, 2015). Hence,  
103 improving waterproofing via the production of CHCs with a tighter molecular cohesion and  
104 higher melting points may increase CHC viscosity and thereby compromise several other  
105 functions of the CHC layer. Nevertheless, previous studies on arthropod CHC have not  
106 considered CHC viscosity and the potential trade-offs of waterproofing with other functions.  
107 As a first step, in this study we have quantified CHC viscosity by using microrheology, a  
108 technique that allows measurements for extremely small volumes of material (~30 picolitres).  
109 Since molecular aggregation and viscosity depend on temperature, these physical effects  
110 should matter for an insect that experiences fluctuations of environmental temperature and/or  
111 humidity over time. Indeed, insects can adjust their CHC profile to climatic conditions (Gibbs  
112 and Mousseau, 1994; Hadley, 1977). Notably, it has been reported that physiological  
113 acclimation can differ between constant and fluctuating temperatures, e.g. in the expression  
114 of heat shock proteins and immune activity (Fischer et al., 2011) or fatty acid composition  
115 (van Dooremalen et al., 2011). Probably because of the nonlinear relationship between  
116 temperature and metabolic processes, fluctuating temperatures can lead to effects which  
117 differ from those reached at constant temperatures (Colinet et al., 2015).

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

127 Here, we measured acclimatory CHC changes and survival of acclimated workers in two  
128 closely related ant species with strongly different CHC profiles, as well as the concomitant  
129 changes in CHC viscosity. Alkadienes dominate the profile of *Myrmica ruginodis*  
130 NYLANDER 1846, but are absent in its sister species *Myrmica rubra* (LINNAEUS 1758),  
131 whose profile is rich in di-, tri- and tetramethyl alkanes (Fig. 1). The latter substances, in turn,  
132 are rare or absent in *M. ruginodis*. This reflects two frequent CHC types in ants, where  
133 species either possess multi-methyl alkanes or unsaturated compounds, but rarely both  
134 substance classes in higher quantities (Menzel et al., 2017). The CHC profile may reflect  
135 different climatic niches (Menzel et al., 2017), with *M. ruginodis* inhabiting cooler and/or  
136 damper habitats than *M. rubra*, and representing the *Myrmica* species most adapted to cool  
137 temperatures among all old-world species (Radchenko and Elmes, 2010). However, *M.*  
138 *ruginodis* and *M. rubra* are ecologically similar and frequently co-occur in the same sites.

139 We expected that, at higher temperatures, both species produce CHC profiles with more  
140 saturated and unbranched hydrocarbon classes to enhance waterproofing. Because of the  
141 division of labour in ants, workers foraging outside the nest should need stronger protection  
142 against environmental conditions than nurses, which remain inside. By disentangling

143 temperature and humidity acclimation, we compared the effects of both factors on CHC  
144 composition and ant survival. Lastly, we used microrheology as a novel tool to quantify the  
145 viscosity of CHC profiles of ants acclimated to different temperatures.

146

## 147 **Materials and Methods**

### 148 STUDY ORGANISMS

149 Both of our study species, *M. rubra* and *M. ruginodis* are widely distributed across Europe,  
150 have similar life-histories and can be found in the same habitats (Radchenko and Elmes,  
151 2010). We collected 15 colonies of each species in the region around Freiburg (Germany) in  
152 April and June 2015. Colonies were collected at the following specific locations:  
153 Vogelsangpass (48.087 N, 7.697 E – both species), Eichelspitze (48.090 N, 7.694 E – *M.*  
154 *ruginodis* only), Burkheim/Rheinauen (48.099 N, 7.582 E – both species),  
155 Mooswald/Opfinger See (48.004 N, 7.752 E – *M. ruginodis* only) and Eichberg (47.975 N,  
156 7.893 E – *M. rubra* only). They were kept in their original nesting material in plastic boxes  
157 (235 x 175 x 90 mm, Lifeca GmbH & Co. KG, Bad Salzulfen, Germany) with plastered  
158 ground and walls coated with Fluon<sup>®</sup> (Whitford GmbH, Diez, Germany) at 20°C and a  
159 12h:12h light-dark cycle. Relative humidity inside these stock colonies ranged from 85.66 ±  
160 3.61% RH to 99.69 ± 1.01% RH between the boxes. Honey, dead crickets and water (in  
161 Eppendorf cups with a cotton plug) were provided once a week *ad libitum*.

162

### 163 ACCLIMATION TREATMENTS

164 After keeping the colonies under lab conditions for at least two weeks (see above), we set up  
165 four temperature treatments: constant temperature at 12°C, 20°C and 28°C, respectively, and  
166 a fluctuating treatment with 12°C at night and 28°C during the day (with 3 h ramps between  
167 the two temperatures). For each of these treatments, we established two humidity conditions,

168 dry (~ 50% RH) and humid (~ 100% RH). From each of the ant colonies, we created eight  
169 worker groups, which were distributed among the eight treatments. They consisted of 6  
170 foragers (collected outside the nest), 18 nurses (collected inside the nest, if possible directly  
171 from the brood) and 10 brood items. Foragers and nurses represent distinct behavioural castes  
172 in many ant species and are known to possess different CHC profiles (Greene and Gordon,  
173 2003; Pamminger et al., 2014). We kept the worker groups in plastic boxes (95 x 95 x 60  
174 mm, Westmark GmbH, Lennestadt-Elspe, Germany) with a plaster ground and a cavity (ca.  
175 50 x 30 x 3 mm) covered with glass plates and red foil.

176 All climate treatments were established in climate cabinets (RUMED 3101 and 3201,  
177 Rubarth Apparate GmbH, Laatzen, Germany) equipped with two 1000-g air dehumidifiers  
178 (CaCl<sub>2</sub>; UHU GmbH & Co. KG, Bühl/Baden, Germany) to keep the air in the cabinets as dry  
179 as possible. For the humid treatment, the boxes were covered with lids, while the lids for the  
180 dry treatment were prepared with a window closed with wire mesh (70 x 70 mm; mesh 0.2  
181 mm) to ensure continuous airflow in the nest boxes. The worker groups were kept at the  
182 different acclimation treatments for three weeks. We provided food (honey and dead crickets)  
183 *ad libitum*. Water was provided in an Eppendorf cup (Eppendorf AG, Hamburg, Germany)  
184 with a piece of cotton. To maintain specific humidity levels, additional water was provided to  
185 the plaster in quantities adjusted to each treatment. Food and water were added depending on  
186 the temperature and humidity treatment: in 12°C *dry* only the Eppendorf cups were refilled  
187 every fourth day, in 20°C *dry* 1 ml of water was additionally applied on the plaster near the  
188 nest entrance every second day, while in the fluctuating temperature and 28°C *dry* treatments  
189 we similarly placed 1 or 2 ml (respectively) on the plaster. Humid kept ants were fed  
190 similarly and water added until the plaster was saturated with every feeding. Climatic  
191 conditions were surveyed using data loggers in additional, empty nest boxes (testo 174H,  
192 Testo AG, Lenzkirch, Germany), and we made sure that humidity levels were consistent



193 across temperature regimes (Table 1). After three weeks of acclimation, workers were taken  
194 for chemical analyses and survival tests.

195

## 196 CHEMICAL ANALYSES

197 We sampled two outside workers (foragers) and two inside workers (nurses) from each  
198 worker group (N = 480 per species). To correct for any potential daily fluctuations in CHC  
199 profile in the changing temperature treatments, we took one worker per caste at 8 am (end of  
200 the 12°C period) and the other one at 8 pm (end of the 28°C period). Each single worker was  
201 put into a glass vial and frozen at -20°C until the extraction.

202 We analyzed the cuticular hydrocarbons using gas chromatography – mass spectrometry  
203 (GC-MS). They were extracted by immersing single ants into *n*-hexane for ten minutes.  
204 During the extraction we added 100 ng *n*-octadecane (solved in 10 µl *n*-heptane) as internal  
205 standard for quantification of the absolute CHC amount. The extracts were transferred to a  
206 micro insert and concentrated under a gentle nitrogen stream to approximately 20 µl. Per  
207 sample, we then injected 2 µl into the GC (7890A, Agilent Technologies, Santa Clara, CA,  
208 USA) at a temperature of 250°C in splitless mode. As a carrier gas we used helium (He) with  
209 a flow rate of 1.2 ml per minute and a Zebron Inferno DB5-MS capillary column (length 30  
210 m, Ø 0.25 mm, 0.25 µm coating, Phenomenex Ltd., Aschaffenburg, Germany) as stationary  
211 phase. The temperature program started at 60°C. After two minutes the oven heated with a  
212 rate of 60°C per minute up to 200°C and afterwards with a constant rate of 4°C per minute up  
213 to 320°C. This temperature was held constant for ten minutes. The analytes then entered the  
214 MS (5975C, Agilent Technologies) and were accelerated with an ionization voltage of 70 eV.  
215 The detector scanned for molecular fragments in a range of 40 – 550 m/z. Data was acquired  
216 using the software *MSD ChemStation* (E.02.02.1431; Agilent Technologies; Fig. 2).  
217 Hydrocarbons were identified based on retention index based on a standard series of *n*-

218 alkanes (Carlson et al., 1998) and diagnostic ions (Table A1). We excluded substances that  
219 were not hydrocarbons (< 10% of the total extract), substances with a maximum below 0.5 %  
220 and substances which occurred in less than 20 % of the samples of either species.

221

## 222 STATISTICAL ANALYSES: CHC PROFILES

223 We conducted all statistical analyses using *R* version 3.2.2 (R Core Team, 2017) and  
224 analyzed the following CHC traits: relative abundance of *n*-alkanes, mono-, di-, tri- and  
225 tetramethyl alkanes, alkenes, and alkadienes and the absolute CHC quantity. For each  
226 chemical trait and for both species separately, we constructed linear mixed-effects models  
227 (LME) (command *lme*, package *nlme*; Pinheiro et al., 2016), with temperature, humidity and  
228 caste as explanatory variables and colony ID and sampling location (nested in colony ID) as  
229 random effects. We reduced each model stepwise by removing the least significant  
230 interaction until AIC was minimal. If necessary, data was transformed and/or, in few cases,  
231 outliers were excluded (see Table 2A,B) to fulfil the model assumptions. Pairwise  
232 comparisons were executed using a Tukey test from the R package *lsmeans* (Lenth, 2016). In  
233 the results section, results for temperature, humidity and caste originated from the same  
234 models, but will be reported in separate chapters (see Table 2A,B). Here we focus on the  
235 CHC classes which were abundant and showed the strongest coefficient of variation in  
236 response to our experimental treatments, i.e. *n*-alkanes (*M. rubra*:  $c_v = 0.615$ ; *M. ruginodis*:  $c_v$   
237  $= 0.705$ ), dimethyl alkanes (*M. rubra*:  $c_v = 0.306$ ; *M. ruginodis*:  $c_v = 0.425$ ), alkenes (*M.*  
238 *rubra*:  $c_v = 0.700$ ; *M. ruginodis*:  $c_v = 0.438$ ) and alkadienes ( $c_v = 0.349$ , *M. ruginodis* only).  
239 Results for mono- (*M. rubra*:  $c_v = 0.128$ ; *M. ruginodis*:  $c_v = 0.237$ ), tri- and tetramethyl  
240 alkanes ( $c_v = 0.490$  and  $c_v = 0.358$ , *M. rubra* only) are shown in Supplementary Fig. S1 and  
241 Supplementary Table S1. Methyl alkenes were not analyzed since their abundance was  
242 overall low (always < 2%) and zero in most cases.

243 Finally, we performed three comprehensive analyses to compare effect sizes between species  
244 and treatments, to assess overall CHC changes, and to analyze co-variation of substance  
245 classes. We compared overall CHC composition between climate regimes using  
246 PERMANOVA in the program PRIMER (Primer-E Ltd., Lutton, United Kingdom) with the  
247 same fixed and random factors as above, and visualized it using non-metric multidimensional  
248 scaling (NMDS) ordination. Hierarchical cluster analyses were performed (*hclust*, R package  
249 *vegan*; Oksanen et al., 2016) with the complete linkage method to determine which treatment  
250 groups were most similar to each other (Supplementary Fig. S2A,B). Co-variation among  
251 substance classes was analyzed using a principal component analysis (PCA) on the  
252 abundances of CHC classes (Supplementary Fig. S2C,D).

253

#### 254 SURVIVAL EXPERIMENT

255 We placed one nurse and one forager from each of the constant temperature treatments (N =  
256 360) in a polystyrene vial (Ø 28 mm, height 64 mm, volume 30 ml, K-TK e.K., Retzstadt,  
257 Germany) and plugged a piece of foam (1.04 ± 0.09 cm thick) in to about 1/3 of the height. We  
258 then filled it with 10.20 ± 0.96 g silica gel (2-5 mm, Sigma-Aldrich Laborchemikalien  
259 GmbH, Seelze, Germany) and sealed it airtight with Parafilm® (Bemis Flexible Packaging,  
260 Neetah, WI, USA). The silica gel was used to absorb the humidity within the vial (Bazin et  
261 al., 2010; Stinziano et al., 2015). The experiment was performed in a climate chamber at  
262 20°C. We checked survival first after six hours and afterwards once every hour until the 24<sup>th</sup>  
263 hour of the experiment. Ants that had died (defined here as the lack of any movement even  
264 after shaking the vial) in the first six hours (68 cases) were excluded from the data set since  
265 the exact time of death was uncertain. These 68 cases were distributed largely evenly across  
266 the treatments, with marginally more ants from the 28°C treatments ( $\chi^2$ -test:  $\chi^2_2 = 5.69$ ,  $p =$   
267 0.058). All observations were conducted blindly.

268 The data was analysed with a Cox mixed-effects model (command *coxme*; R package *coxme*;  
269 Therneau, 2015) comprising species, temperature, humidity and caste as explanatory  
270 variables. Colony ID and test day were implemented as random effects as this combination  
271 yielded the best AIC. Variables were tested with a type-II ANOVA (command *Anova*;  
272 package *car* version 2.1-1, adjusted by J. Fox 2015; Fox & Weisberg, 2011). We removed  
273 non-significant interactions stepwise until AIC was lowest.

274

## 275 MICRORHEOLOGY OF CUTICULAR HYDROCARBONS

276 To quantify the viscosity of CHC profiles, we used microrheology. This new approach allows  
277 measurements on small biological samples and cells, which were previously impossible due  
278 to the minute amounts of material available (for a review: Waigh, 2005). In passive  
279 microrheology (or particle tracking microrheology), the viscosity of a fluid is derived from  
280 the Brownian motion of microscopic probe particles embedded in the fluid. In complex  
281 fluids, microrheology also allows the investigation of viscoelastic properties and phase  
282 heterogeneity. Microrheology experiments are usually performed on small volumes of the  
283 order of 1 microlitre. We have developed a fluid collection procedure for micrometric  
284 droplets, allowing microrheology measurements on extremely small volumes of the order of  
285 10 to 100 picolitres (Abou et al. 2010).

286 Particle-tracking microrheology experiments were performed on several extracts of *M. rubra*  
287 workers acclimated to constant, dry conditions at either 12°C or 28°C. The CHC extract of an  
288 ant worker was dissolved in 20 µl pentane and placed on a glass slide to evaporate the  
289 solvent. To collect the CHC extracts from the glass slide (on an inverted Leica DM IRB  
290 microscope, Leica Microsystems GmbH, Wetzlar, Germany), we used a fine glass  
291 micropipette connected to a pneumatic microinjector (CellTram Air, Eppendorf AG,  
292 Hamburg, Germany) mounted on a three-axis micro-manipulator (Burleigh, Thorlabs SAS,

293 Maisons-Laffitte, France). The fine tips (2-3  $\mu\text{m}$  diameter) of the capillaries were prepared by  
294 pulling from borosilicate glass micropipettes with 1 mm outer diameter and 0.78 mm inner  
295 diameter (Harvard Apparatus S.A.R.L, Les Ulis Cedex, France) with a P-1000 Micropipette  
296 puller (Sutter Instrument, Novato, CA, USA). The micropipette tip was moved onto the  
297 surface in order to collect the largest possible amount of CHC extracts. Due to capillary  
298 effects, the CHC extracts spontaneously rose in the micropipette as soon as the tip touched  
299 the liquid.

300 Dry melamine beads (Acil, France; bead diameter:  $0.740 \pm 0.005 \mu\text{m}$ ) were deposited on a  
301 clean glass slide. The collected CHC extract was then ejected onto the beads by applying  
302 positive pressure to the capillary via the microinjector. After ejection, the capillary tip was  
303 moved along the glass slide to detach beads that were stuck to the glass surface. The CHC  
304 fluid was then drawn up and ejected again several times in order to mix the beads with the  
305 CHC extract (Abou et al., 2010).

306 The samples were observed using bright field microscopy at 100x magnification (oil  
307 immersion objective, NA = 1.3, depth of focus:  $\sim 200 \text{ nm}$ ). The sample temperature was  
308 controlled by adjusting the objective temperature with an objective heater (Bioptechs Inc.,  
309 Butler, PA ,USA) to within  $\pm 0.1 \text{ }^\circ\text{C}$ .

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

318 Newtonian fluid, the ensemble-averaged MSD increases linearly with the lag time, as  
319  $\langle \Delta r^2(t) \rangle = 4Dt$  (in two dimensions), where  $D$  is the diffusion coefficient. In this case, the  
320 viscosity  $\eta$  can be estimated using the Stokes-Einstein relation,  $\eta = kT/6\pi RD$ , where  $R$  is  
321 the bead diameter and  $kT$  the thermal energy (Einstein, 1905).

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

330

## 331 **Results**

### 332 CHEMICAL ANALYSES

333 The climate treatments strongly affected the CHC profiles in both ant species (Fig. 3).  
334 Overall, temperature regime had by far the strongest impact on CHC composition  
335 (PERMANOVA, *M. rubra*: pseudo- $F_3 = 69.5$ , *M. ruginodis*: pseudo- $F_3 = 76.7$ , both  $p =$   
336  $0.001$ ; Supplementary Table S1). In contrast, humidity-induced effects were considerably  
337 smaller, especially so in *M. rubra* (*M. rubra*: pseudo- $F_1 = 4.0$ ,  $p = 0.007$ ; *M. ruginodis*:  
338 pseudo- $F_1 = 18.2$ ,  $p = 0.001$ ; Supplementary Table S1). Furthermore, nurses and foragers had  
339 strongly different profiles in both species (both pseudo- $F_1 > 20.0$ ;  $p = 0.001$ ; Supplementary  
340 Table S1).

341

342 *Effects of different constant temperatures*

343 In spite of their strongly different CHC composition (Fig. 1, Fig. 2, Table A1), both *M. rubra*  
344 and *M. ruginodis* showed similar responses to the three *constant* temperature regimes: in both  
345 species, *n*-alkanes and alkenes increased with acclimation temperature. The increase was  
346 especially high between 20°C and 28°C, but weaker or, for alkenes in *M. ruginodis*, not  
347 detectable between 12°C and 20°C (Table 2A,B; Fig. 4A-D). Not only the relative amounts,  
348 but also the absolute quantities of *n*-alkanes increased with acclimation temperature  
349 (Supplementary Fig. S3). In contrast, dimethyl alkanes decreased with temperature, again  
350 with a particularly high shift between 20°C and 28°C in both species (Table 2A,B; Fig. 4E,F).  
351 Alkadienes, which were the dominant CHC class in *M. ruginodis* ( $34.68 \pm 12.08\%$ ) but  
352 absent in *M. rubra*, similarly decreased at 28°C compared to 12°C or 20°C (Table 2B; Fig.  
353 4G). Similar effects were found for tri-methyl alkanes, while the tetra-methyl alkanes showed  
354 opposite changes (both of which occurred in *M. rubra* only; Supplementary Table S1).  
355 Interestingly, monomethyl alkanes were the most abundant CHC class in *M. rubra* ( $48.39 \pm$   
356  $6.78\%$ ) and highly abundant in *M. ruginodis* ( $21.13 \pm 5.00\%$ ), but showed only weak  
357 responses to climatic conditions (Supplementary Table S1, Supplementary Fig. S1).  
358 Absolute CHC quantities decreased with acclimation temperature in *M. ruginodis*. In *M.*  
359 *rubra*, it was lowest at 28°C as well, but higher at intermediate temperatures than at 12°C  
360 (Table 2A,B; Supplementary Fig. S1A,B).

361

### 362 *Constant vs. fluctuating temperature*

363 Compared to constant temperatures, the fluctuating regime resulted in yet different CHC  
364 composition, and had different effects on the two ant species. In *Myrmica rubra*, profiles  
365 from fluctuating temperatures were relatively similar to those of 12°C and 20°C. In contrast,  
366 *M. ruginodis* profiles from the fluctuating treatment showed stronger differences to all three  
367 constant temperature regimes, but resembled more closely those of 28°C. This could be

368 confirmed for overall CHC composition with NMDS ordination (Fig. 3) and cluster analysis  
369 (Fig. S2A,B). Similar patterns were found for the percentages of substance classes: at  
370 fluctuating temperature, percentages of *n*-alkanes, dimethyl alkanes, and alkenes in *M. rubra*  
371 were similar to the 12°C treatment (Fig. 4A,C,E). In *M. ruginodis*, fluctuating temperatures  
372 led to alkene and dimethyl alkane percentages similar to the 28°C treatment, while *n*-alkanes  
373 and alkadienes were in between the 20°C and the 28°C treatments (Fig. 4D,F; Fig. 4B,G).  
374 Absolute CHC quantities were highest at fluctuating temperatures in *M. rubra*, but had a low  
375 level comparable to the 28°C treatment in *M. ruginodis* (Supplementary Fig. S1A,B).

376

#### 377 *Effects of humidity*

378 In both species, dry conditions led to a massive increase in absolute CHC quantity (Table  
379 2A,B; Fig. 5A,B). However, CHC composition was less affected. In *M. rubra*, the only effect  
380 was an increase in alkenes under dry conditions. *Myrmica ruginodis* showed more changes  
381 under dry conditions, producing more alkenes (particularly in foragers) and less dimethyl  
382 alkanes (Table 2B, Supplementary Fig. S4D,F). Moreover, dry conditions led to an increase  
383 in *n*-alkanes at 28°C but not at 20°C or 12°C (Table 2B).

384

#### 385 *Differences between foragers and nurses*

386 Foragers and nurses showed strong chemical differences, which were consistent across the  
387 two species (Supplementary Fig. S5). Foragers had more *n*-alkanes and alkenes, but less  
388 dimethyl alkanes (Table 2A,B; Supplementary Fig. S5A-F). Moreover, foragers had less  
389 alkadienes than nurses in *M. ruginodis*. In both species, nurses possessed more CHC (in µg)  
390 than foragers, except for dry-acclimated workers in *M. rubra* (interaction humidity x caste,  
391 Table 2A,B; Supplementary Fig. S5H,I). Contrary to our expectation, the two behavioural  
392 castes showed largely similar responses to the climatic treatments (i.e. no significant



393 interactions of caste with temperature or humidity), with few exceptions: forager-nurse  
394 differences in the percentage of *n*-alkanes in *M. ruginodis* (Table 2B) and alkenes in *M. rubra*  
395 (Table 2A) were only significant at 20°C and at fluctuating temperature (interaction  
396 temperature x caste). Foragers kept at dry conditions possessed more alkenes while they did  
397 not differ from nurses under humid conditions (interaction humidity x caste, Table 2B).

398

### 399 SURVIVAL EXPERIMENT

400 Ants of both species survived drought stress longer if they had acclimated to dry conditions  
401 compared to acclimation to humid conditions (Cox mixed-effects model:  $\chi^2_1 = 33.07$ ,  $p <$   
402  $0.0001$ ;  $n = 292$  ants; 40/292 still alive after 24h; Fig. 5C). Additionally, nurses survived  
403 better than foragers ( $\chi^2_1 = 4.17$ ,  $p = 0.041$ ). However, there was no difference between *M.*  
404 *rubra* and *M. ruginodis* workers ( $\chi^2_1 = 0.16$ ,  $p = 0.69$ ; Fig. 5C). Surprisingly, survival was  
405 neither influenced by the acclimation temperature ( $\chi^2_2 = 1.50$ ,  $p = 0.47$ ) nor any interaction of  
406 worker group, humidity and temperature (all non-significant after model reduction).

407

### 408 PHASE CHARACTERISTICS AND MICRORHEOLOGY OF CHC EXTRACTS

409 All CHC extracts were highly heterogeneous, with solid and liquid phases co-occurring even  
410 at ambient temperatures (Fig. 6A,B). Microrheology measurements on the liquid fraction of  
411 the CHC were conducted for *Myrmica rubra*. For several *M. rubra* colonies, extracts of cool-  
412 acclimated ants contained visible hydrocarbon crystals at temperatures below 20°C, which  
413 completely liquefied at  $T=25^\circ\text{C}$  (Fig. 6C-F), suggesting a broad phase transition.

414 CHC viscosity was strongly correlated with the chemical composition of the CHC extracts.  
415 The viscosity increased with higher percentage of *n*-alkanes (Spearman's rank correlation:  $N =$   
416  $17$ ,  $\rho = 0.65$ ,  $p = 0.005$ ), but decreased with higher proportions of dimethyl-alkanes ( $N =$   
417  $17$ ,  $\rho = -0.67$ ,  $p = 0.003$ ). This suggests that warm-acclimated ants, due to their higher

418 amounts of *n*-alkanes and lower amounts of dimethyl alkanes, also should have more viscous  
419 CHC profiles. Indeed, extracts of ants acclimated to 28°C had a higher viscosity compared to  
420 those from 12°C-acclimated ants (Wilcoxon rank sum test:  $N = 17$ ,  $W = 12$ ,  $p = 0.027$ ).

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

431

## 432 **Discussion**

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

442

443 EFFECTS OF CONSTANT TEMPERATURE REGIMES

444 The different constant temperature regimes resulted in largely parallel changes in both  
445 species. After acclimation to higher temperatures, CHCs promoting tight molecular alignment  
446 (*n*-alkanes) increased in relative abundance, while substances disrupting molecular alignment  
447 (di- and trimethyl alkanes, alkadienes) decreased. Such changes have been reported  
448 previously to provide a better waterproofing of the cuticle (Gibbs and Mousseau, 1994;  
449 Menzel et al., 2018; Wagner et al., 2001). However, our data also show that acclimation to  
450 different temperatures did not have any effect on drought survival. This indicates that the  
451 observed temperature-induced changes of the CHC profile are not related to waterproofing.  
452 Although we cannot exclude that the changes are non-adaptive, e.g. due to shifts in  
453 physiological pathways, we hypothesise that they serve to adjust the viscosity of the CHC  
454 layer. A sufficiently low viscosity may be critical for maintaining many other functions  
455 essential biological functions of the CHC layer, including the transfer of communication  
456 cues, diffusion of recognition cues across the body surface, healing of scratches, lubrication  
457 and adhesion (Cooper et al., 2009; Dirks et al., 2010; Drechsler and Federle, 2006; Gorb,  
458 2001; Wigglesworth, 1945). All these functions depend to some extent on the viscosity of the  
459 CHC layer. Generally, the viscosity of liquids increases when cooled to lower temperatures,  
460 until they solidify at the melting point. Consistent with the observed shift of the CHC  
461 composition towards compounds that disrupt molecular aggregation, acclimation to lower  
462 temperatures resulted in lower proportions (and lower absolute amounts) of *n*-alkanes, but  
463 higher proportions of dimethyl alkanes, which resulted in a reduced CHC viscosity. Clearly,  
464 these changes cannot be explained by waterproofing requirements, as tightly aggregating  
465 CHC could provide efficient waterproofing at both warm and cold temperatures. Instead, we  
466 hypothesise that a selection pressure could act to prevent complete CHC solidification at low

467 temperatures, because this would impede communication and other essential functions. One  
468 mechanism to prevent solidification would be to maintain CHC viscosity at a constant low  
469 level by adjusting the chemical composition of the CHC profile. A previous study on  
470 membrane lipids in *Drosophila* reported comparable chemical changes during acclimation,  
471 supporting the idea that acclimatory changes serve to maintain CHC viscosity (Overgaard et  
472 al., 2006). Our data are consistent with a homeostatic control of viscosity. However, the  
473 detailed adaptive benefits of low CHC viscosity have been largely ignored in previous work.  
474 Future research should test whether low CHC viscosity is indeed maintained in a homeostatic  
475 way, and study how changes in CHC composition and viscosity affect communication and  
476 other functions. Our study shows that microrheology is a powerful method to address these  
477 questions.

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

492 a mixture of both might promote the persistence of liquid parts in the CHC layer even when  
493 *n*-alkanes are abundant.

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

504

#### 505 EFFECTS OF FLUCTUATING TEMPERATURE

506 The difference between constant and fluctuating temperature regimes is crucial to understand  
507 how insects can cope with changing microclimate and weather conditions (Colinet et al.,  
508 2015). In many habitats, daily temperatures vary quickly, and probably faster than insects can  
509 acclimate. Interestingly, the CHC composition of ants from fluctuating regimes was not  
510 intermediate between the two corresponding constant temperatures: in *Myrmica rubra*, the  
511 fluctuating regime led to CHC profiles very similar to those of the coolest, constant regime.  
512 In contrast, the CHC profiles of *M. ruginodis* differed strongly from all constant temperature  
513 regimes (Fig. 3). Thus, temperature fluctuations had a stronger effect on *M. ruginodis* profiles  
514 than on *M. rubra* profiles, indicating that the two species differ in CHC changes during  
515 acclimation.

516

517 EFFECTS OF HUMIDITY

518 Both *Myrmica* species showed increased absolute CHC quantities under dry conditions.  
519 Similar increases were shown for scorpions (Gefen et al., 2015) and (albeit at high  
520 temperatures only) in desert beetles (Hadley, 1977). As expected, dry-acclimated individuals  
521 were more resistant to drought stress, consistent with previous studies, showing the adaptive  
522 value of this drought acclimation (Bazinet et al., 2010; Terblanche and Kleynhans, 2009). It  
523 is possible that reports of higher CHC quantities in insects acclimated to warm conditions are  
524 the result of stronger drought stress and not of the higher temperatures themselves (Hadley,  
525 1977). In contrast to previous work, our experiments disentangled these two factors, and  
526 showed that higher temperatures even led to reduced overall CHC quantities once humidity  
527 was controlled for. Notably, temperature acclimation did not affect drought survival. Our  
528 results indicate that at least for *Myrmica*, CHC quantity is more important for desiccation  
529 resistance than CHC composition.

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

539

540 MICRORHEOLOGY OF CUTICULAR HYDROCARBONS

541 All CHC extracts contained both solid and liquid fractions, at least at temperatures below  
542 30°C. Early work established that water loss rates in insects are minimal at low temperatures,  
543 but increase suddenly once the temperature exceeds a ‘critical’ temperature (Ramsay, 1935;  
544 Wigglesworth, 1945). This sudden increase of water loss was shown in many insects to  
545 coincide with measured melting points of CHC extracts (Gibbs, 1998; Gibbs, 2002),  
546 suggesting that the increase in water permeability is explained by a melting of the lipid layer  
547 at this temperature. While CHC melting temperatures measured using capillary melting  
548 techniques, differential scanning calorimetry or infrared spectroscopy ranged from 27° to ca.  
549 100°C (Gibbs, 2002), our observations show that liquid CHC are already present well below  
550 these temperatures, and that complex CHC profiles have a broad phase transition range rather  
551 than a single sharp melting point. This is evidenced by the observed coexistence of liquid and  
552 solid parts in CHC extracts. Moreover, the microrheology experiments show that even the  
553 liquid fraction itself is heterogeneous and exhibits locally varying viscosity.  
554 Our microrheology measurements confirm that the ants’ acclimation response modified the  
555 physical properties of the CHCs. The differences in viscosity can be explained by the  
556 observed changes in chemical composition: warm-acclimated ants showed higher amounts of  
557 solid *n*-alkanes and lower proportions of dimethyl alkanes, consistent with their more viscous  
558 CHC layers. Extracts from cold-acclimated ants had fewer *n*-alkanes, were less viscous and  
559 lacked any visible solid parts at 25°C measurement temperature. Our measurements show  
560 that the viscosity of CHC extracts provides a good proxy to assess lipid mobility on the  
561 cuticle surface, opening up avenues for future research.

562

## 563 **Conclusions**

564 Insect cuticular hydrocarbon profiles are astonishingly diverse. The different functions of  
565 CHC, particularly waterproofing and communication, depend on CHC composition and are

566 affected by temperature and humidity. CHC profiles are therefore linked to the insects'  
567 climatic niche, and their ability to acclimate or cope with short-term weather fluctuations. We  
568 have shown that, despite strong chemical differences, *Myrmica rubra* and *M. ruginodis*  
569 changed their profiles in similar and predictable ways during acclimation to constant  
570 temperatures (*n*-alkanes increased at higher temperatures, whereas dimethyl alkanes and  
571 alkadienes decreased) and humidity, and both species survived drought stress equally well.  
572 However, both species acclimated to fluctuating temperature regimes in a different way.  
573 While the profiles of *M. rubra* acclimated to fluctuating temperature was similar to those  
574 acclimated to constant 12°C or 20°C, profiles of *M. ruginodis* acclimated to fluctuating  
575 temperature differed from all constant temperature regimes. Both ant species responded to  
576 dry conditions by producing larger amounts of CHC, but only *M. ruginodis* also changed the  
577 composition of its profile. In sum, compared to *M. rubra*, *M. ruginodis* showed stronger CHC  
578 changes in response both to fluctuating temperature and drier conditions. Therefore, it is  
579 possible that CHC differences give rise to differences in position and width of microclimate  
580 niches, which may result in different microhabitats and geographic ranges.

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

589

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596

### 597 **Competing interests**

598 The authors declare no competing interests.

599

### 600 **Author contributions**

601 FM and PS conceived the study and developed the experimental setup. PS and LB performed  
602 the acclimation treatments and the chemical analyses. PS performed the survival tests. PS and  
603 FM performed the statistical analyses. BA and WF conducted the microrheological  
604 measurements. FM, PS, WF and BA wrote the manuscript. All authors approved the final  
605 version of the manuscript.

606

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### 610 **Data Availability**

611 All raw data will be published on Dryad upon acceptance of the manuscript.

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## Tables

**Table 1.** Mean temperature and humidity of the treatments. Shown is the mean  $\pm$  standard deviation of temperature [ $^{\circ}\text{C}$ ] and relative humidity [% RH].

<b>Temperature</b>	<b>dry</b>	<b>humid</b>
<b>12<math>^{\circ}\text{C}</math></b>	12.42 $\pm$ 0.39 $^{\circ}\text{C}$	12.50 $\pm$ 0.40 $^{\circ}\text{C}$
	61.84 $\pm$ 9.95% RH	99.72 $\pm$ 1.03% RH
<b>20<math>^{\circ}\text{C}</math></b>	19.66 $\pm$ 0.15 $^{\circ}\text{C}$	19.99 $\pm$ 0.18 $^{\circ}\text{C}$
	49.58 $\pm$ 8.28% RH	99.97 $\pm$ 0.97% RH
<b>28<math>^{\circ}\text{C}</math></b>	26.72 $\pm$ 0.32 $^{\circ}\text{C}$	27.27 $\pm$ 0.41 $^{\circ}\text{C}$
	50.99 $\pm$ 12.88% RH	99.78 $\pm$ 0.79% RH
<b>fluctuating</b>	19.25 $\pm$ 7.23 $^{\circ}\text{C}$	19.58 $\pm$ 7.31 $^{\circ}\text{C}$
<b>temperature</b>	58.79 $\pm$ 12.43% RH	99.74 $\pm$ 0.79% RH

**Table 2.** Model results for chemical traits of A) *M. rubra* and B) *M. ruginodis*. 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-, b) logit-, c) arcsine-square root- transformed, 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.

<b>A) <i>M. rubra</i></b>					
<b>Dependent variable</b>	<b>Fixed effect</b>	<b>N</b>	<b>df</b>	<b><math>\chi^2</math></b>	<b>p</b>
<b><i>n</i>-alkanes<sup>b</sup></b>	Temperature	480	3	200.51	< 0.0001
	Humidity		1	1.65	0.19
	Caste		1	27.03	< 0.0001
<b>dimethyl alkanes<sup>d</sup></b>	Temperature	476	3	197.54	< 0.0001
	Humidity		1	1.84	0.17
	Caste		1	52.94	< 0.0001
<b>alkenes<sup>c</sup></b>	Temperature	473	3	240.80	< 0.0001
	Humidity		1	14.67	0.0001
	Caste		1	45.41	< 0.0001
	Temperature x Caste		3	11.04	0.011
<b>absolute quantity<sup>a</sup></b>	Temperature	472	3	199.33	< 0.0001
	Humidity		1	106.36	< 0.0001
	Caste		1	3.88	0.049
<b>B) <i>M. ruginodis</i></b>					
<b><i>n</i>-alkanes<sup>b</sup></b>	Temperature	480	3	388.21	< 0.0001
	Humidity		1	8.88	0.0029
	Caste		1	51.28	< 0.0001
	Temp. x Humidity		3	14.02	0.0029
	Temperature x Caste		3	9.60	0.022
<b>dimethyl alkanes<sup>d</sup></b>	Temperature	480	3	253.49	< 0.0001
	Humidity		1	19.89	< 0.0001
	Caste		1	42.00	< 0.0001
<b>alkenes<sup>b</sup></b>	Temperature	480	3	208.71	< 0.0001
	Humidity		1	31.40	< 0.0001
	Caste		1	22.02	< 0.0001
	Humidity x Caste		1	3.97	0.046
<b>alkadienes<sup>d</sup></b>	Temperature	480	3	172.97	< 0.0001
	Humidity		1	2.66	0.10
	Caste		1	38.91	< 0.0001
<b>absolute quantity<sup>a</sup></b>	Temperature	473	3	35.24	< 0.0001
	Humidity		1	50.11	< 0.0001
	Caste		1	17.33	< 0.0001
	Humidity x Caste		1	6.64	0.010

**Table A1.** Cuticular hydrocarbon profiles of *M. rubra* and *M. ruginodis*. Overall, we detected and identified 64 hydrocarbon peaks in *M. rubra* and 69 peaks in *M. ruginodis*. The table shows the retention index (Kovats index; RI) (Carlson et al., 1998), substance name, diagnostic ions (mass peaks are printed in italics) and its mean percentage  $\pm$  SD. Double bond positions for most abundant substances were determined by DMDS derivatisation (see diagnostic ions in brackets). Tentative identifications are marked by asterisks.

#	RI	Substance	Diagnostic ions (m/z)	<i>M. rubra</i>	<i>M. ruginodis</i>
				Mean $\pm$ SD	Mean $\pm$ SD
1	19.01	n-C19	268	0.29 $\pm$ 0.27	0.51 $\pm$ 0.68
2	19.59	unknown CHC	-	0.16 $\pm$ 0.13	-
3	23.02	n-C23	324	0.28 $\pm$ 0.37	-
4	23.53	5-MeC23	85/281	0.13 $\pm$ 0.12	-
5	24.02	n-C24	338	0.29 $\pm$ 0.19	0.05 $\pm$ 0.12
6	24.77	C25-ene	350	0.06 $\pm$ 0.1	-
7	24.96	C25-ene	350	1.1 $\pm$ 0.92	-
8	25.03	n-C25	352	5.51 $\pm$ 4.41	0.12 $\pm$ 0.16
9	25.36	9-,11-,13-MeC25	140/252; 168/224; 196	1.7 $\pm$ 0.75	-
10	25.43	7-MeC25	112/280	1.5 $\pm$ 0.97	-
11	25.52	5-MeC25	84/308	1.41 $\pm$ 0.8	-
12	25.67	8,12-DiMeC25	126/280, 196/210	0.47 $\pm$ 0.24	-
13	25.74	3-MeC25	56/336	1.26 $\pm$ 0.61	-
14	25.84	5,9-,5,11-DiMeC25	85/323, 154/252, 182/224	0.74 $\pm$ 0.41	-
15	26.01	n-C26	366	0.61 $\pm$ 0.26	0.05 $\pm$ 0.07
16	26.11	3,x-DiMeC25	56/364	0.37 $\pm$ 0.16	-
17	26.36	10-MeC26*	154/252	0.47 $\pm$ 0.39	-
18	26.4	8-MeC26*	126/280	0.82 $\pm$ 0.56	-
19	26.64	10,14-DiMeC26	154/266, 224/196	0.34 $\pm$ 0.3	-
20	26.68	8,12-DiMeC26	126/294, 196/224	0.5 $\pm$ 2.04	-
21	26.77	6,10-, 6.12-DiMeC26	98/322, 168/252 196/224	0.34 $\pm$ 0.24	-
22	26.98	C27-ene	378	1.83 $\pm$ 1.56	-
23	27.01	n-C27	380	3.37 $\pm$ 1.98	2.72 $\pm$ 2.26
24	27.2	4,8,12-TriMeC26	70/378, 140/308, 210/238	0.32 $\pm$ 0.33	-
25	27.34	11-,13-MeC27	168/252; 196/224	-	0.18 $\pm$ 0.15
26	27.36	9-,11-MeC27	140/280; 168/252	15.47 $\pm$ 4.89	-
27	27.43	7-MeC27	112/308	2.18 $\pm$ 1.58	0.21 $\pm$ 0.27
28	27.53	5-MeC27	85/337	4.93 $\pm$ 2.2	0.21 $\pm$ 0.23
29	27.63	11,15-DiMeC27	168/266, 238/196	5.39 $\pm$ 2.47	-
30	27.66	9,13-DiMeC27	140/294, 210/224	-	0.02 $\pm$ 0.05
31	27.72	7,11-DiMeC27	112/323, 183/252	8.15 $\pm$ 3.77	-
32	27.74	3-MeC27	57/365	0.15 $\pm$ 0.54	0.44 $\pm$ 0.49
33	27.84	5,11-DiMeC27	85/351, 183/253	5.23 $\pm$ 2.35	-

34	27.98	7,11,15-TriMeC27	112/337, 183/267, 253/197	1.35 ± 1.32	-
35	28.01	7,11,21-TriMeC27	112/337, 183/267, 337/112	0.68 ± 0.56	-
36	28.01	n-C28	394	-	0.33 ± 0.32
37	28.1	5,9,13-;5,9,15-TriMeC27	85/364, 154/294, 225, 253/197	1.6 ± 0.59	-
38	28.35	7-;10-;12-;13-;14-MeC28	112/323; 155/281; 181/253; 197/238; 210/225	1.87 ± 0.78	-
39	28.38	3,7,11-TriMeC27	56/392, 126/322, 196/252	0.33 ± 0.5	-
40	28.46	unknown CHC	-	0.12 ± 0.11	-
41	28.64	3,7,11,15-TetraMeC27	57/407, 127/337, 197/267, 267/197	1.8 ± 0.67	-
42	28.64	C29diene	404	-	0.42 ± 0.61
43	28.68	C29diene	404	-	0.92 ± 0.74
44	28.73	C29ene	406	-	1.23 ± 0.56
45	28.76	3,x,y,z-TetraMeC27	56/406	0.36 ± 0.29	-
46	28.79	C29-9-ene	406, [173, 327, 500]	-	4.18 ± 1.52
47	28.86	C29-7-ene	406, [145, 355, 500]	-	2.29 ± 1.1
48	28.9	C29ene	406	-	0.5 ± 0.42
49	29.01	n-C29	408	1.62 ± 1.12	5.22 ± 4.25
50	29.18	4,8,16-TriMeC28 (position of 3rd methyl group tentative)	71/393, 141/323, 267/196	0.04 ± 0.08	-
51	29.24	x-MeC29-diene	-	-	0.3 ± 0.24
52	29.33	9-;11-;13-;15-MeC29	141/308; 167/281; 197/252; 224	8.32 ± 2.85	7 ± 2
53	29.42	7-MeC29	112/336	1.06 ± 0.64	0.16 ± 0.44
54	29.52	5-MeC29	84/364	1.83 ± 0.97	1.5 ± 0.9
55	29.6	11,15-;13,17-DiMeC29	168/295, 239/224; 196/267, 267/196	3.27 ± 1.56	0.68 ± 0.81
56	29.7	9,13-; 7,11-DiMeC29	140/322, 210/252; 112/350, 182/280	-	0.49 ± 0.74
57	29.74	3-MeC29	57/393	2.51 ± 0.86	2.24 ± 0.85
58	29.82	5,17-DiMeC29	85/379, 267/196	2.64 ± 0.93	0.63 ± 0.37
59	29.87	5-21-DiMeC29*	85/379, 323/140	0.05 ± 0.11	-
60	29.98	7,11,17-TriMeC29	112/364, 183/296, 281/196	0.29 ± 0.31	-
61	30.05	n-C30	422	-	0.21 ± 0.21
62	30.07	3,11*-DiMeC29	56/406, 182/280	0.53 ± 0.37	-
63	30.31	10-;12-;13-;14-MeC30	154/308; 182/280; 196/266; 210/252	0.46 ± 0.25	0.59 ± 0.31
64	30.36	unknown CHC	-	0.12 ± 0.14	-
65	30.49	C31diene	432	-	0.11 ± 0.14
66	30.55	C31diene	432	-	0.42 ± 0.53
67	30.65	C31diene	432	-	4.76 ± 1.94
68	30.65	2-;4-MeC30	43/420; 71/393	0.62 ± 0.5	-
69	30.7	C31diene	432	-	14.35 ± 6.61
70	30.7	C31-11-ene	434, [201, 327, 528]	-	2.44 ± 3.61
71	30.8	C31diene	432	-	1.67 ± 2.54
72	30.8	C31-9-ene	434, [173, 355, 528]	-	8.63 ± 5.28
73	30.87	C31ene	434	-	0.68 ± 0.98
74	30.91	C31ene	434	-	0.92 ± 0.77

75	31	n-C31	436	-	0.46 ± 0.63
76	31.16	unknown CHC	-	-	0.32 ± 0.39
77	31.21	13-;14-MeC31ene	448, 194/278, 210/266	-	1.51 ± 0.82
78	31.3	9-;11-;13-;15-MeC31	140/336; 168/308; 196/280; 224/252	-	6.33 ± 2.37
79	31.32	13-;15-MeC31	196/280; 224/252	1.35 ± 0.94	-
80	31.41	7-MeC31	112/364	0.19 ± 0.18	-
81	31.44	cf. Methyltrien (unknown unsaturated)	-	-	0.42 ± 0.45
82	31.52	5-MeC31	85/392	0.04 ± 0.12	-
83	31.56	13,17-DiMeC31	196/295, 267/224	0.98 ± 0.81	3.46 ± 2.49
84	31.62	13,21-DiMeC31*	196/294, 322/168	0.07 ± 0.12	-
85	31.65	9,17-;9,19-;9,21-DiMeC31	140/350, 266/224, 294/196, 322/168	-	2.48 ± 2.17
86	31.7	unknown CHC	-	0.13 ± 0.15	-
87	31.74	3-MeC31	57/420	-	0.83 ± 0.67
88	31.8	5,15-;5,17-DiMeC31	85/407, 239/252, 267/224	0.19 ± 0.14	1.08 ± 0.64
89	32.04	3,15-DiMeC31	57/435, 239/252	-	0.3 ± 0.28
90	32.3	12-;13-;14-;15-;16-MeC32	182/308; 196/294; 210/280; 224/267; 238/252	0.04 ± 0.08	0.22 ± 0.22
91	32.51	C33diene	460	-	0.54 ± 0.52
92	32.57	C33diene	460	-	1.78 ± 0.98
93	32.65	C33diene	460	-	4.63 ± 1.89
94	32.73	C33diene	460	-	1.46 ± 1.14
95	32.79	C33diene	460	-	0.87 ± 1.11
96	32.87	C33ene	462	-	0.14 ± 0.2
97	33.13	unknown CHC	-	-	0.1 ± 0.16
98	33.3	11-;13-;15-;17-MeC33	169/336; 196/309; 224/280; 252	0.07 ± 0.16	1.11 ± 0.89
99	33.46	unknown CHC	-	0.05 ± 0.13	-
100	33.54	13,17-;13,19-DiMeC33	196/323, 267/252, 295/224	-	0.62 ± 0.59
101	33.61	9,x-;11,x-DiMeC33 [x=17 or 19]	140/379, 168/350, 267/252, 294/224	-	0.59 ± 0.6
102	33.67	unknown CHC	-	-	0.05 ± 0.11
103	33.78	5,21-;5,23-DiMeC33	84/434, 322/196, 350/168	-	0.43 ± 0.35
104	34.04	3,15-DiMeC33	56/462, 238/280	-	0.12 ± 0.24
105	34.45	C35diene	488	-	0.31 ± 0.39
106	34.49	C35diene	488	-	0.34 ± 0.38
107	34.55	C35diene	488	-	1.33 ± 1.06
108	34.65	C35diene	488	-	0.22 ± 0.35
109	35.24	13-;15-;17-MeC35	196/336; 224/338; 252/280	-	0.09 ± 0.18
110	35.4	15,x-DiMeC35	224/322	0.02 ± 0.07	-
111	35.45	15,19-DiMeC35	224/322, 294/252	-	0.23 ± 0.37
112	35.51	11/13/15/17,19/21/23-DiMeC35 (combination of methyl group positions unknown)	168/378, 196/350, 224/322, 252/294, 294/252, 322/224, 350/196	-	0.12 ± 0.16
113	36.41	C37diene	516	-	0.13 ± 0.22

## Figure legends

**Fig. 1. Composition of CHC profiles according to substance classes ordered by chain length for (A) *M. 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 (visualised by colour codes), *M. ruginodis* has longer hydrocarbons than *M. rubra*, the most common chain length being C31 vs. C27, respectively. Relations 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 A1.

**Fig. 2. Representative profiles of (A) *M. 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 A1. Note that we show only retention times with relevant peaks (i.e. minute 10-25 for *M. rubra* and minute 15-30 for *M. ruginodis*).

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

**Fig. 4. Effects of treatment temperature on the ants' CHC profiles.** The upper row shows *M. rubra*, the bottom row *M. ruginodis*. 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 *n*-alkanes (A,B), dimethyl alkanes (C,D), alkenes (E,F) and alkadienes (G). All graphs show back-transformed means  $\pm$  SE. Different letters indicate statistically significant differences according to pairwise Tukey-tests on the LME data ( $p < 0.05$ ).

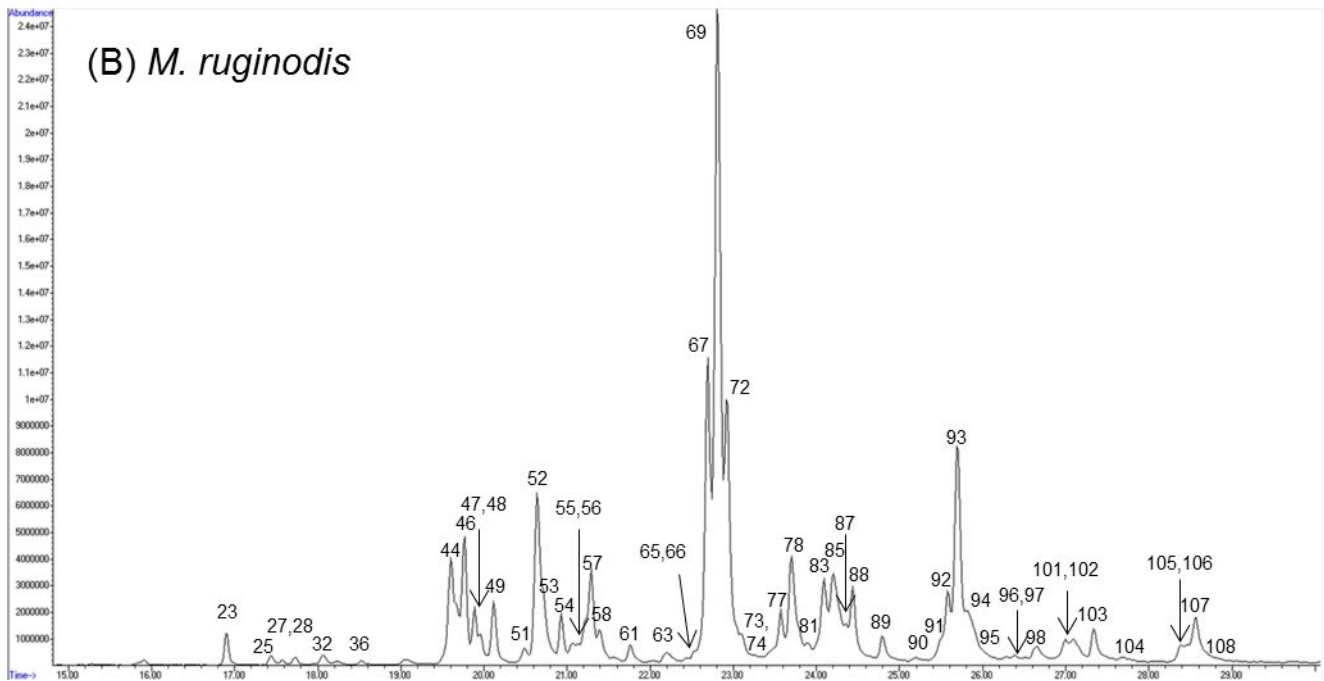
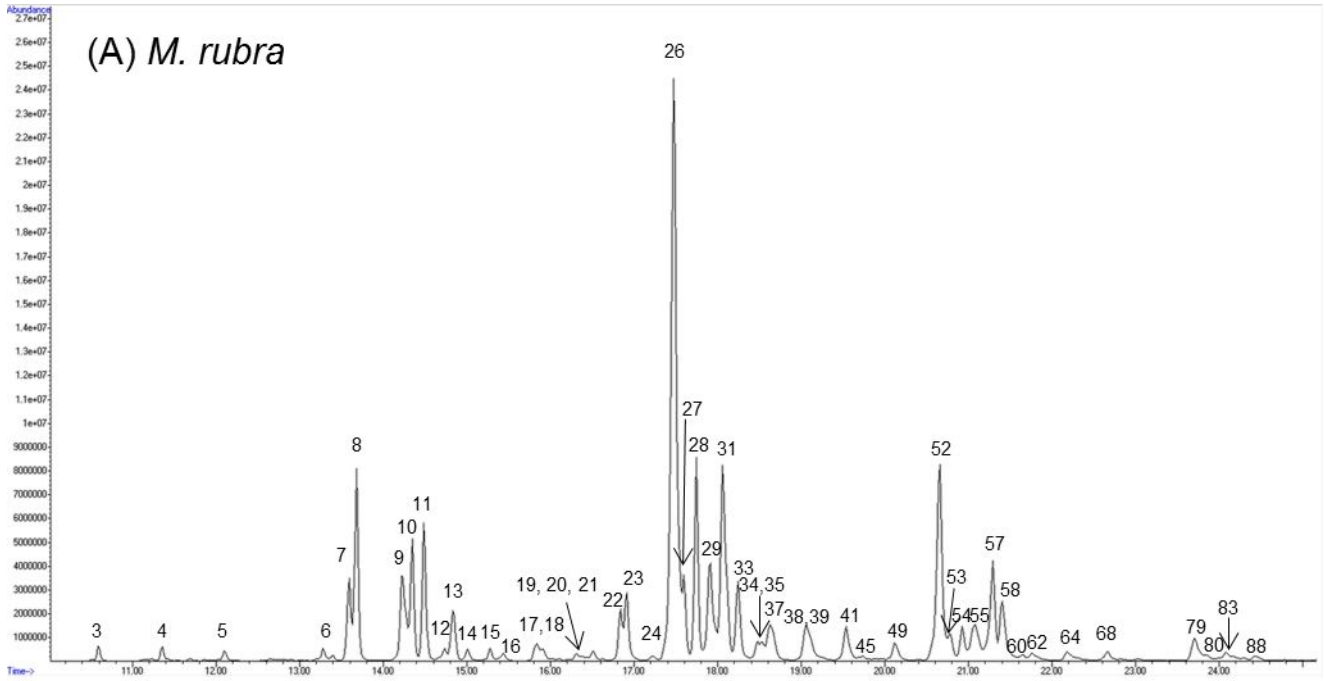
**Fig. 5. Effects of humidity on the ants' absolute CHC amount and survival.** (A) and (B) show the absolute amount of CHCs [ $\mu\text{g}$ ] of *M. rubra* and *M. ruginodis* workers acclimated to humid and dry conditions (back-transformed means of log-transformed data  $\pm$  SE). Significant differences 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 vs. humid conditions on survival rate.

**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). The photos in (C-F) show transmitted (C, E) and crossed-polarized light (D, F) photos 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.

**Fig. 7. Microrheology of CHC in *M. rubra* ants.** The plot shows the time-averaged mean-squared displacement (MSD) of 0.74 $\mu\text{m}$ -diameter melamine tracer beads undergoing Brownian motion within the CHC sample. *M. 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).

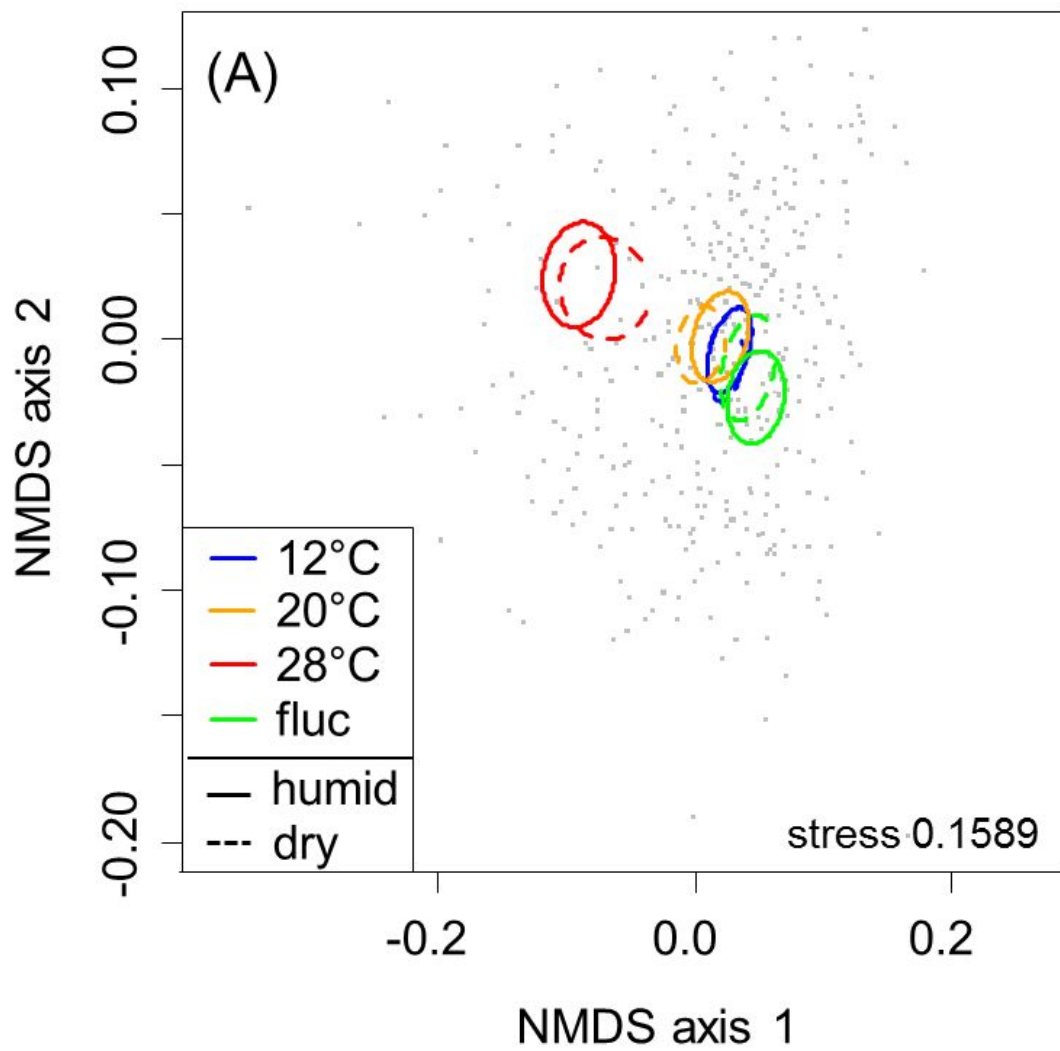






*M. rubra*

(A)



*M. ruginodis*

(B)

