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Micro-climate correlations and conserved sexual dimorphism of cuticular hydrocarbons in European populations of the jewel wasp *Nasonia vitripennis*

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Abstract. 1. Protection against desiccation and chemical communication are two fundamental functions of cuticular hydrocarbons (CHCs) in insects. In the parasitoid jewel wasp *Nasonia vitripennis* (Walker), characterised by a cosmopolitan distribution through largely different environments, CHCs function as universally recognised female sex pheromones. However, CHC uniformity as basis for sexual recognition may conflict with the desiccation protection function, expected to display considerable flexibility through adaptation to different environmental conditions.

2. We compared male and female CHC profiles of *N. vitripennis* across a wide latitudinal gradient in Europe and correlated their CHC variation with climatic factors associated with desiccation. Additionally, we tested male mate discrimination behaviour between populations to detect potential variations in female sexual attractiveness.

3. Results did not conform to the general expectation that longer, straight-chain CHCs occur in higher proportions in warmer and drier climates. Instead, unexpected environmental correlations of intermediate chain-length CHCs (C31) were found exclusively in females, potentially reflecting the different life histories of the sexes in *N. vitripennis*.

4. Furthermore, we found no indication of population-specific male mate preference, confirming the stability of female sexual attractiveness, likely conveyed through their CHC profiles. C31 mono- and C33 di-methyl-branched alkanes were consistently and most strongly associated with sexual dimorphism, suggesting their potential role in encoding the female-specific sexual signalling function.

5. Our study sheds light on how both adaptive flexibility and conserved sexual attractiveness can potentially be integrated and encoded in CHC profiles of *N. vitripennis* females across a wide distribution range in Europe.

Key words. Chemical communication, climatic factors, desiccation resistance, sex pheromones, sexual dimorphism.

Introduction

Successful adaptation to ever-changing environmental conditions is of paramount importance in the survival of any species (Boulding & Hay, 2001; Reznick & Ghalambor, 2001).

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Varied interactions with the physical environment often have direct consequences for life history traits, and hence for the fitness of an organism (Burger & Lynch, 1995; Gomulkiewicz & Holt, 1995). In terrestrial environments, phenotypic traits related to the regulation of temperature and water balance have been hypothesised to be among the most important factors in determining successful colonisation and subsequent occupation of novel habitats (Chown *et al.*, 2011). In insects, cuticular hydrocarbons (CHCs) have been shown to be pivotal

phenotypic traits with the dual function of providing protection against water loss and forming the basis for a wide variety of chemical communication systems (Carlson *et al.*, 1971; Blomquist & Bagnères, 2010).

CHCs are long-chained lipids and major compounds of the outer layer located on the epicuticle of all insects (Lockey, 1980; Howard & Blomquist, 1982). After being produced in specialised cells, the oenocytes, they are transported through the haemolymph and exposed on the cuticular surface through specialised pore canals (Wigglesworth, 1933; Schal *et al.*, 1998). Linear *n*-alkanes, *n*-alkenes, and methyl-branched alkanes are the most common compound classes found in insect CHC profiles, typically ranging between 20 and 40 carbon atoms in chain length (Blomquist & Bagnères, 2010). However, compounds with carbon chain lengths of up to 60 carbon atoms have also been documented to occur, although their detection and structural characterisation usually eludes standard analytical methods (Akino, 2006; Cvacka *et al.*, 2006; Sutton *et al.*, 2013; Bien *et al.*, 2019).

The efficacy of CHC compounds to prevent desiccation is mostly determined by their individual melting temperature, which, in turn, is affected by two main factors. First, a positive correlation has been shown between melting temperature and carbon chain length, generally increasing 1–3 °C with each additional carbon atom (Toolson & Kupersimbron, 1989; Gibbs & Pomonis, 1995; Gibbs, 2002). Second, individual CHC compound classes display substantial differences in their melting temperature, which is generally highest for *n*-alkanes but substantially reduced when either double bounds or methyl-branches occur within the carbon backbone (Gibbs & Pomonis, 1995; Gibbs & Rajpurohit, 2010). Chain lengths as well as the proportion of *n*-alkanes are increased in numerous insect taxa occurring in warmer, drier climates, as shown for instance in beetles (e.g. Hadley, 1977), ants (e.g. Menzel *et al.*, 2018) and social wasps (e.g. Michelutti *et al.*, 2018).

Given the dual function of CHCs, surprisingly little is known about how potentially conflicting selection pressures for both adapting to different environmental conditions and maintaining uniformity in chemical signalling are resolved. To answer this question, tractable model organisms are required with varied ecological exposure to different climatic conditions as well as accessible CHC variation with a clearly determined signalling function. In the past decades, the parasitoid jewel wasp *Nasonia vitripennis* (Hymenoptera: Pteromalidae) has been established as an exceptionally well-suited insect model system to study complex adaptive traits on a genetic (e.g. Pegoraro *et al.*, 2016; Li *et al.*, 2017), behavioural (e.g. Cook *et al.*, 2015; Buellesbach *et al.*, 2017) and ecological (e.g. Koppik *et al.*, 2014; Boulton *et al.*, 2015) level.

The parasitoid *N. vitripennis* is characterised by a world-wide distribution. It parasitises and kills the pupae of several flesh- and blowfly species that occur at carcasses and in bird nests (Whiting, 1967; Darling & Werren, 1990). Due to its host range including economically relevant agricultural pest species, *N. vitripennis* is also being used as biological control agent against fly species causing damage in rural economies and livestock premises (Rutz & Scoles, 1989; Kaufman *et al.*, 2001; Machtinger & Geden, 2018). Concerning CHCs in *N. vitripennis*, it has been shown that female CHC extracts constitute sexual cues eliciting male courtship and copulation behaviour (Steiner *et al.*, 2006; Buellesbach *et al.*, 2013; Mair *et al.*, 2017). Moreover, *N. vitripennis* CHCs appear to be genetically fixed traits with a characteristic species-specific pattern (Niehuis *et al.*, 2011), as appears to be the case for other *Nasonia* species as well (Raychoudhury *et al.*, 2010; Buellesbach *et al.*, 2013; Mair *et al.*, 2017).

However, whether population-specific CHC variation occurs in *N. vitripennis* over its geographic distribution or how environmental factors and micro-climate impact CHC profiles in different locations has thus far not been assessed. Here, we investigate CHC profile variation between seven European *N. vitripennis* populations collected across a latitudinal gradient from Corsica to Finland. We hypothesise that population-specific CHC variation correlates with micro-climatic patterns associated with desiccation, *i.e.*, higher proportions of *n*-alkanes and compounds with longer carbon chains in warmer and drier habitats. We further predict to consistently detect a marked difference between male and female CHC profiles, reflecting the conserved sexual signalling function in female CHCs. To complement this, we assess potential differences in mate preference and sexual attractiveness with mating assays of a subset of the collected populations, comparing their courtship and copulation rates within and between populations.

Materials and methods

Field sampling

Natural populations of *N. vitripennis* were collected in the summer of 2009 from seven sites in Europe along a latitudinal gradient, from Corsica (42°N) to North Finland (65°N), with a distance of about 4–5 latitudinal degrees between each of them (Fig. 1 and Table 1). Wasps were collected from bird nests in nest boxes which were mainly used by great tits (*Parus major*), blue tits (*Parus caeruleus*), and flycatchers (*Ficedula hypoleuca*). Wasps were mainly collected by removal of the nests at least 5 days after the resident birds had fledged, and by subsequent dissection of the nests for potentially parasitised fly pupae. Also, baits were used consisting of mesh bags with approximately 25 laboratory-raised fly pupae (*Calliphora* spp.) that were placed in nest boxes for a few days to attract *N. vitripennis* wasps. The baits were checked later for parasitisation and wasp emergence. To account for most efficient sampling of all collection sites, adult wasps were also directly collected where possible from the nest material or on the baits. Iso-female lines were set up either from single adults directly collected from the field or from wasps that emerged from the pupae of bird nests or baits (for further details see Paolucci *et al.*, 2013). A single iso-female line was used for each site, except for the Corsica and Oulu sites, where three iso-female lines were used, respectively. So far, no chemical or ecological differences could be detected between *N. vitripennis* wasps sampled from different habitat patches (*e.g.* carcasses or bird nests, Buellesbach, Lammers & Pannebakker, unpublished data). Thus, we safely assumed having consistently

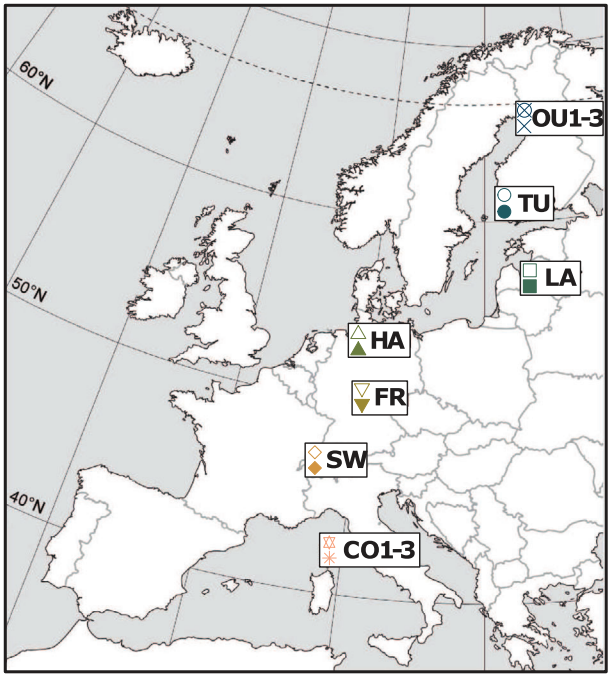

Fig. 1. Map of sampling locations of *Nasonia vitripennis* lines across Europe. Letters correspond to the general region or city closest to the sample location, three iso-female lines have been sampled as reference points at the southernmost and northernmost locations, respectively. CO1-3: Corsica, France; SW: Bois de la Croix, Switzerland; FR: Frankfurt, Germany; HA: Hamburg, Germany; LA: Mērsrags, Latvia; TU: Turku, Finnland; OU1-3: Oulu, Finnland. Colours and shapes indicate affiliation and sex of each iso-female line, respectively (see also Fig. 4-6).

sampled natural populations whose chemical and ecological diversity is most strongly determined by the different sample locations across Europe. Furthermore, inbreeding depression is negligible due to the purging of lethal mutations in the haploid males, and *Nasonia* laboratory strains derived from iso-female lines have the potential to be successfully maintained for decades (Werren, 1993; Henter, 2003; Tien *et al.*, 2015). Wasps analysed in this study were 48–72 h old adult males and females, from the 3rd to 4th generation of the iso-female lines established after their collection. They were cultured in plastic mass culture vials (height 75 mm, diameter 24 mm) and kept under standard laboratory conditions for *Nasonia* rearing enabling a generation time of about 14 days (25 °C, 45% relative humidity and a 16:8 light/dark cycle).

GC–MS analysis of CHC profiles

Wasps were freeze-killed and stored separately by sex in glass vials (height 30 mm, diameter 10 mm) at – 20 °C. For CHC extraction, 10 wasps of the same sex and iso-female line were placed for 10 min into 100 µl of hexane in fresh glass vials (height 30 mm, diameter 10 mm). After CHC extraction, wasps were removed and glass vials stored at −20 °C until further processing. For gas chromatography/mass spectrometry (GC–MS) analysis, CHC extracts where transferred to a conical 250 µl GC insert where the hexane was evaporated under a constant flow of nitrogen and concentrated to ∼1 µl. The entire sample was then injected into a HP 6890 gas chromatograph coupled with a HP 5973 mass selective detector (Hewlett Packard, Waldbronn, Germany) operating in electron impact ionisation mode at 70 eV and a source temperature of 230 °C. The split/splitless injector was operated in the splitless mode for 1 min at 250 °C. Separation of compounds was performed on a DB-5 fused silica capillary column (30 m × 0.25 mm ID × 0.25 µm, J&W Scientific, Folsom, California) with a temperature programme starting from 60 °C and increasing by 40 °C per min to 200 °C, followed by an increase of 5 °C per min to 300 °C, which was held for 10 min. Helium served as a carrier gas with a constant flow of 1 ml per minute.

CHC data acquisition

CHC peak detection, integration, quantification, and identification were all carried out with MassHunter Workstation Quantitative Analysis Software (Version B.09.00/Build

Table 1. Acronyms, collection sites, collection period, longitudinal, and latitudinal data as well as average summer temperatures (in °C), average maximum summer temperatures (in °C), total summer precipitation (in mm) and average summer humidity (in %) from the collection year at the respective collection sites of the European *N. vitripennis* iso-female lines.

Iso-female line	Collection site	Collection time	Longitude (E)	Latitude (N)	Average summer temp. (°C)	Average max. summer temp. (°C)	Total summer precipitation (mm)	Average summer humidity [%]
CO 1–3	Corsica, France	May–June 2009	8.7522	42.3778	22.92	28.57	4.4	70.33
SW	Ballens, Switzerland	June 2009	6.3967	46.5795	19.97	33.1	244.1	64.23
FR	Frankfurt (Schlüchtern), Germany	June 2009	9.5444	50.321	16.78	23.22	208.6	70.8
HA	Hamburg, Germany	June 2009	10.1716	53.6066	17.13	22.17	219.5	71.22
LA	Berzciems, Latvia	June 2009	23.1335	57.2678	15.66	20.21	309.1	79.59
TU	Turku, Finland	July 2009	22.2233	61.2613	15.33	20.75	150	77.14
OU 1–3	Oulu, Finland	July 2009	25.5152	65.0482	14.4	19.29	187.1	75.25

9.0.647.0, Agilent Technologies, Santa Clara, California). Data files were translated with the help of MassHunter GC/MS Translator tool (Version B.07.06 2704, Agilent Technologies) from their original format, initially acquired with HP Enhanced Chemstation (G1701AA, Version E.01.00.237, Hewlett-Packard Company, Palo Alto, California), to make them accessible for the MassHunter Workstation Software. Peaks were quantified using the most abundant diagnostic ion in their mass spectra as quantifiers ($m/z = 57$ for *n*-alkanes and methyl-branched alkanes, $m/z = 97$ for alkenes) and their characteristic diagnostic ions as qualifiers to allow for unambiguous detection by the quantification software. The pre-defined integrator Agile 2 was used as peak integration algorithm to allow for maximum flexibility. All peaks were then additionally checked for correct integration and quantification, and, where necessary, re-integrated manually. CHC compound identification was then carried out based on their characteristic diagnostic ions and by comparison to already published CHC profiles of *N. vitripennis* (Steiner *et al.*, 2006; Niehuis *et al.*, 2011; Buellesbach *et al.*, 2013; Mair *et al.*, 2017).

Explorative data and statistical analysis

All explorative data and subsequent statistical analyses were performed with the programme R, version 3.5.2 (R Core Team, 2018). To standardise the peak areas, the normalisation method 'decostand' of the community ecology R package 'vegan' was used (Oksanen *et al.*, 2008), based on the following formula:

$$T_{x,y} = P_{x,y} / \sqrt{\Sigma P_y^2}$$

$T_{x,y}$ refers to the transformed peak area x of individual y, $P_{x,y}$ to the absolute peak area x of individual y and ΣP_y^2 to the squared sums of all absolute peak areas of individual y. This standard method for normalising ecological data was chosen to make the peak areas comparable between the collected iso-female lines and sexes as well as to correct for size-dependent variations potentially affecting the extracted CHC quantities. Principal component analyses were performed with the R package 'stats' on CHC compounds grouped by their respective chain lengths, if the respective groups contained more than three compounds, leaving out C25 (two compounds), C27 (three compounds), and C32 (one compound). Average and maximum temperature as well as precipitation rates and average humidity for the collection season were considered the most relevant for desiccation and were obtained from the following sources (see Table 1): Corsica: NCEI (https://www.ncei.noaa.gov/data/global-summary-of-the-month/access/) and Météo France (https://www.historique-meteo.net/; https://donneespubliques.meteofrance.fr/); Switzerland: Federal Office of Meteorology and Climatology MeteoSwiss (https://www.meteoswiss.admin.ch/); Germany: DWD Climate Data Centre (https://cdc.dwd.de/portal/201912031600/index.html); Latvia: Latvian Environment, Geology and Meteorology Centre (https://www.meteo.lv/en/); Finland: Finnish Meteorological Institute (https://en.ilmatieteenlaitos.fi/).

Mating assays

Mating behaviour of wasps from the 5th generation after collection from their respective field sites was assessed in no-choice experiments with single pairs of 24–48 h old virgin males and females. After their eclosion, virgin males and females were kept individually in separate glass tubes (height 30 mm, diameter 10 mm). At the start of a mating assay, a single virgin male and female were combined by adjoining the openings of their respective tube. They were observed under a stereo binocular microscope until courtship and copulation had occurred, or for a maximum of 10 min. Courtship behaviour was scored as the male mounting the female and performing characteristic head-nod cycles and wing vibrations, copulation was scored as the male backing up and inserting his aedeagus into the female's genital orifice (Barrass, 1960; van den Assem & Vernel, 1979; van den Assem & Werren, 1994). Mate rejection was scored when the female did not become receptive after being mounted and courted by the male (Velthuis *et al.*, 2005). A subset of the collected *N. vitripennis* iso-female lines was used for the behavioural assays. Iso-female lines from the most northern (OU1-3 pooled together: OU) and the most southern (SW) iso-female line from mainland Europe were tested in all possible combinations with the other five mainland iso-female lines to gain an estimate of the levels of mate discrimination and potential divergence in female sexual attractiveness. Each intra- and interpopulation combination (20 in total) was replicated 60 times, resulting in 1200 mating assays in total (see Fig. 7). A generalised linear model (glm) was used to determine the effect of iso-female line (collection site). Chi-square ($\chi 2$) tests were used to assess significant differences in courtship and copulation rates between the different pairings.

Results

In total, 53 distinct CHC compounds were detected in our sampled *N. vitripennis* iso-female lines across a latitudinal gradient in Europe (Fig. 1). Their identifications or, in ambiguous cases, all potential configurations according to their diagnostic ions, as well as their relative quantities per sampled iso-female line are given in Table 2. All CHC compounds could be subdivided into the six major compound classes; *n*-alkanes, *n*-alkenes, mono-, di-, tri-, and tetra-methyl-branched alkanes. We grouped the identified CHC compounds into their respective compound classes and compared the average relative amounts between each of our sampled iso-female lines. (Fig. 2).

For the four climate factors most closely associated with desiccation, average summer temperature, average maximum summer temperature, average precipitation rates, and relative humidity, none of the singled-out compound class portions showed either positive or negative correlations (Tables S1 and S2). Therefore, we differentially grouped the detected CHC compounds according to their respective chain lengths (Fig. 3). Correlation analyses revealed significantly negative correlations of the total amounts of female C31 compounds with both average ($\text{rho} = -0.93$, $P < 0.001$, Fig. 4a) and maximum summer temperature ($\text{rho} = -0.96$, $P < 0.001$, Fig. 4c). The correlation with female C31 compounds and total summer precipitation was not

Table 2. CHC compound identifications and their respective relative quantities (in %) for representative male (m) and female (f) pools from all collected European *N. vitripennis* iso-female lines.

CHC identifications	CO1 m/f	CO2 m/f	CO3 m/f	SW m/f	FR m/f	HA m/f	LA m/f	TU m/f	OU1 m/f	OU2 m/f	OU3 m/f
n-C29	4.34/6.98	4.1/5.81	5.83/5.24	5.23/11.17	5.36/8.33	7.01/9.23	6.08/6.71	6.78/8.26	4.47/5.58	5.36/5.26	6.26/5.85
11-MeC29	0.16/1.11	0.02/0.88	0.43/0.61	3.73/0.56	0.14/0.7	0.07/2.06	0.1/1.33	0.09/1.09	0.19/1.56	0.12/0.87	0.24/1.39
7-MeC29	3.55/3.55	2.03/2.89	1.85/2.3	1.24/4.22	1.93/4.94	4.13/5.2	3.16/4.39	3.59/4.86	1.6/4.3	2.39/3.94	2.46/4.82
5-MeC29	0.51/0.52	0.3/0.45	0.35/0.33	0.49/0.62	0.44/0.63	0.44/0.66	0.52/0.4	0.55/0.54	0.28/0.48	0.36/0.4	0.36/0.4
7,11-; 7,15-DiMeC29	0.05/0.16	0.05/0.05	0.04/0.12	0.06/0.23	0.14/0.35	0.02/0.14	0.09/0.44	0.03/0.22	0.08/0.39	0.04/0.22	0.16/0.5
3-MeC29	0.24/0.19	0.04/0.95	0.12/1.01	0.62/0.45	0.12/0.38	0.18/0.6	0.43/0.93	0.17/0.33	0.05/0.4	0.21/0.56	0.18/0.48
7,9,11,15-TetraMeC29	0.39/0.55	0.1/0.38	0.33/0.13	0.89/0.53	0.42/0.66	0.32/0.39	0.32/0.47	0.24/0.42	0.1/0.73	0.28/0.58	0.35/0.88
n-C30	0.68/0.96	0.79/0.88	0.89/0.87	1.24/0.94	0.82/0.84	0.84/0.91	0.82/0.79	0.99/1.12	0.84/0.64	0.84/0.78	0.81/0.77
8-MeC30	0.07/0.18	0.06/0.12	0.07/0.07	0.11/0.13	0.06/0.16	0.07/0.17	0.06/0.18	0.08/0.14	0.06/0.22	0.06/0.17	0.06/0.18
7-MeC30	0.41/0.18	0.34/0.18	0.28/0.16	0.37/0.31	0.37/0.31	0.35/0.2	0.4/0.15	0.53/0.21	0.3/0.19	0.2/0.13	0.3/0.12
6-MeC30	0.32/0.28	0.35/0.26	0.32/0.27	0.58/0.32	0.5/0.39	0.4/0.25	0.44/0.29	0.47/0.38	0.26/0.24	0.33/0.26	0.3/0.32
9-C31ene	1.24/0.19	0.68/0.12	0.54/0.08	0.52/0.05	0.69/0.1	0.82/0.15	0.59/0.11	0.71/0.15	0.43/0.08	0.52/0.2	0.66/0.19
7-C31ene	0.19/0.07	0.12/0.06	0.13/0.05	0.08/0.07	0.16/0.09	0.31/0.12	0.17/0.07	0.31/0.12	0.19/0.03	0.12/0.09	0.24/0.09
n-C31	13.11/15.95	14.12/15.73	18.05/15.78	12.58/14.37	12.48/12.79	16.31/13.17	16.19/13.93	18.07/16.94	16.74/12.66	17.14/11.73	15.08/11.94
9-; 11-; 13-; 15-MeC31	2.61/9.42	3.08/8.07	3.01/8.84	3.94/10.56	4.09/12.71	2.63/6.91	4/9.31	4.79/12.71	3.26/8.23	4.58/10.26	4.53/10.94
7-MeC31	20.67/12.26	20.33/15.69	17.55/11.63	20.53/11.5	22.2/15.91	22.35/12.4	29.04/15.96	26.06/14.62	17.63/9.55	23.4/9.63	20.17/12.37
5-MeC31	5.55/3.2	5.21/3.33	4.95/2.85	5.35/3.03	5.38/2.94	5.64/3.94	6.35/3.45	5.98/4.26	4.45/2.46	5.47/2.74	4.52/2.33
11,15-; 11,21-DiMeC31	5.77/2.91	5.41/2.74	0/0	0/1	0/0.77	0/0.86	0/0.93	0/2.24	0/1.32	0/0.28	0/1.61
7,11-DiMeC31	1.07/1.36	0.77/1.01	1.34/1.24	1.81/2.08	2.09/1.66	1.13/2.25	1.21/2.15	1.48/1.65	1.35/1.67	1.12/1.61	1.96/2.23
7,23-DiMeC31	2.95/6.21	3.87/6.6	4.4/7.52	5.51/4.3	4.56/4.74	3.77/5.8	2.94/4.22	2.51/4.32	4.69/7.33	4.15/7.58	5.26/5.55
7,9-DiMeC31	2.85/1.69	3.23/2.27	3.94/2.43	2.64/2.17	5.96/3.19	3.52/2.63	1.3/2.73	2.84/1.51	7.75/9.93	2.9/12.47	3.36/10.73
7,21-DiMeC31	0.29/0.28	0.3/0.26	0.49/0.26	1.07/0.21	1.14/0.48	0.36/0.34	0.32/0.46	0.5/0.35	0.61/0.31	0.53/0.45	0.69/0.32
3,15-DiMeC31	0.23/0.6	0.3/0.57	0.43/0.57	0.19/0.59	0.37/0.59	0.25/0.79	0.31/0.59	0.44/0.59	0.42/0.84	0.4/0.43	0.39/0.66
3,7-DiMeC31	0.7/0.58	0.3/0.38	0.34/0.55	0.42/0.49	0.77/0.06	0.6/0.41	0.7/0.31	0.7/0.24	0.31/0.51	0.38/0.36	0.65/0.51
n-C32	0.62/0.54	0.48/0.55	0.13/0.8	0.5/0.79	0.58/0.94	0.52/0.77	0.52/0.7	0.27/0.66	0.2/0.65	0.46/0.71	0.49/0.65
6-MeC32	0.5/0.14	0.25/0.28	0.51/0.27	0.59/0.27	0.62/0.26	0.33/0.3	0.33/0.28	0.69/0.33	0.61/0.09	0.25/0.27	0.53/0.13
8,12,16-; 8,16,20-TriMeC32	0.23/0.69	0.22/0.49	0.39/0.72	0.44/1.21	0.36/1.12	0.24/1.04	0.24/0.96	0.1/0.6	0.45/1.56	0.35/0.82	0.38/1.01
9-C33ene	0.58/0.07	0.26/0.05	0.18/0.03	0.09/0.02	0.14/0.03	0.26/0.03	0.17/0.04	0.21/0.05	0.15/0	0.2/0.07	0.13/0.06
7-C33ene	0.49/0.07	0.32/0.1	0.19/0.06	0.3/0.04	0.29/0.09	0.31/0.07	0.2/0.05	0.37/0.12	0.26/0.06	0.26/0.1	0.31/0.1
n-C33	1.01/0.76	1.15/0.73	1.21/0.71	0.8/0.43	0.77/0.36	1.02/0.51	0.9/0.51	0.95/0.57	1.26/0.46	1.27/0.49	0.92/0.39
11-; 13-; 15-; 17-MeC33	3.89/5.54	4.39/6.24	2.05/6.52	2.85/4.83	3.03/4.61	3.09/4.29	3.62/5.22	3.16/5	3.15/4.55	3.18/5.25	2.36/5.08
7-MeC33	4.18/1.47	3.57/1.62	2.34/1.34	2.51/1.06	2.24/0.92	2.23/0.99	3.12/1.25	2.7/1.22	2.64/0.83	3.34/0.64	2.07/0.82
11,15-; 11,17-; 11,19-; 11,21-; 11,23-DiMeC33 (+ 5-MeC33)	1.48/2.45	1.78/1.76	2.56/3.86	0.84/4.13	0.96/4.06	1.53/3.35	1.87/4.55	0.85/3.98	1.61/4.4	1.69/2.7	2.5/2.19
13,15-; 13,17-; 13,19-; 13,21-; 13,23-DiMeC33	0.77/0.53	0/0.69	1.87/0.98	0.68/1.35	0.53/0.64	0.79/1.67	0/1.03	0.24/0.27	0.47/0.83	0.1/0.83	1.02/1.2
7,23-DiMeC33 (+ 7,11-DiMeC33)	3.44/2.22	3.46/1.19	3.51/3.7	7.01/2.96	6.04/3.13	3.31/1.51	3.32/2.93	3.57/1.77	4.6/2.22	4.42/2.59	5.03/1.8
5,9-; 5,23-DiMeC33	2.75/2.84	3.41/2.86	4.23/2.98	3.89/3.34	4.43/2.45	3.18/3.45	2.52/2.94	2.66/1.15	3.91/3.82	1.22/2.41	3.64/3.42
5,9,11-; 5,9,21-TriMeC33	0.79/1.27	0.94/1.07	1.52/1.12	1.32/1	1.27/0.81	0.9/1.37	0.59/0.88	0.63/0.64	1.23/1.21	1.23/1	1.29/1.04
3,9,11,15-; 3,9,11,17-TetraMeC33 (+ unknown compound)	0.35/0.28	0.37/0.28	0.38/0.49	0.22/0.3	0.15/0.21	0.19/0.16	0.18/0.16	0.16/0.25	0.39/0.27	0.25/0.27	0.31/0.33
3,7,11,15-TetraMeC33	0.46/1.25	0.56/1.11	0.79/1.42	0.29/0.81	0.35/0.59	0.28/0.85	0.22/0.67	0.2/0.46	0.47/1	0.34/0.95	0.38/0.78
11-; 13-; 15-; 17-MeC35	1.44/1.65	1.76/1.88	1.32/1.83	0.86/1	0.93/0.83	1.04/1.05	0.98/1.11	0.76/0.91	1.23/1.03	1.25/1.33	1.12/0.99
11,17-; 13,17-; 11,19-; 13,19-; 11,21-; 13,21-; 11,23-; 13,23-DiMeC35	1.54/4.49	2.13/4.47	2.35/5.82	0.56/2.88	0.19/2.19	1.74/3.98	0.91/2.55	0.84/2.13	1.91/3.49	1.63/4.05	1.76/1.18
7,19-; 7,21-; 7,23-DiMeC35	3.8/1.56	4.26/1.56	4.38/1.19	4.25/1.14	3.99/1	3.62/1.68	2.39/1.39	1.94/0.93	4.2/1.2	3.95/1.46	3.54/1.1
5,15-; 5,17-; 5,21-; 5,23-; 5,25-DiMeC35	1.16/1.59	1.53/1.48	1.96/1.6	1.18/1.13	1.36/0.9	1.64/1.73	1/1.27	0.75/0.89	2.11/1.72	1.68/1.34	1.66/1.43
13-; 15-; 17-; 19-MeC37	0.26/0.15	0.32/0.2	0.16/0.13	0.09/0.06	0.11/0.08	0.22/0.12	0.14/0.12	0.13/0.08	0.22/0.06	0.16/0.13	0.1/0.06
13,17-; 13,19-; 13,21-; 13,23-DiMeC37	0.4/0.29	0.64/0.81	0.53/0.85	0.19/0.62	0.26/0.52	0.72/0.52	0.26/0.26	0.14/0.29	0.73/0.31	0.42/0.78	0.19/0.5
7,21-; 7,23-; 7,25-DiMeC37	1.5/0.4	1.69/0.45	1.23/0.26	1.05/0.33	1.02/0.32	1.07/0.42	0.79/0.43	0.58/0.21	1.42/0.16	1.12/0.42	0.95/0.2
5,15-; 5,17-; 5,23-; 5,25-; 5,27-DiMeC37	0.43/0.37	0.58/0.43	0.53/0.38	0.29/0.39	0.19/0.26	0.28/0.53	0.19/0.38	0.18/0.22	0.72/0.43	0.34/0.42	0.37/0.36

Compounds in brackets indicate trace amounts detected in addition to the main identified compound(s). Where compound identifications were ambiguous due to multiple possible methyl-branch positions based on the detected diagnostic ion pairs, all possible compound configurations are given.

significant (rho = 0.7, $P = 0.13$, Fig. 4b), but a significantly positive correlation with average relative summer humidity could be detected (rho = 0.82, $P < 0.05$, Fig. 4d). There were additional positive correlations of female C29 CHCs with total summer precipitation rates (Table S3) and for male CHCs with a chain length of C30 with average summer temperature (Table S4).

For CHC chain length groups consisting of more than three individual CHC compounds, principal component analyses were performed to separately investigate their contributions to the sexual dimorphism in the collected *N. vitripennis* lines (Fig. 5). As the strongest sex-specific separation could be observed for compounds with chain lengths of C31 and C33, these groups were further subdivided by compound classes, *i.e.* mono- and di-methyl-branched alkanes as they constituted the majority of CHCs in the respective groups. Mono-methyl C31 and di-methyl C33 compound variation displayed the highest degree of sexual

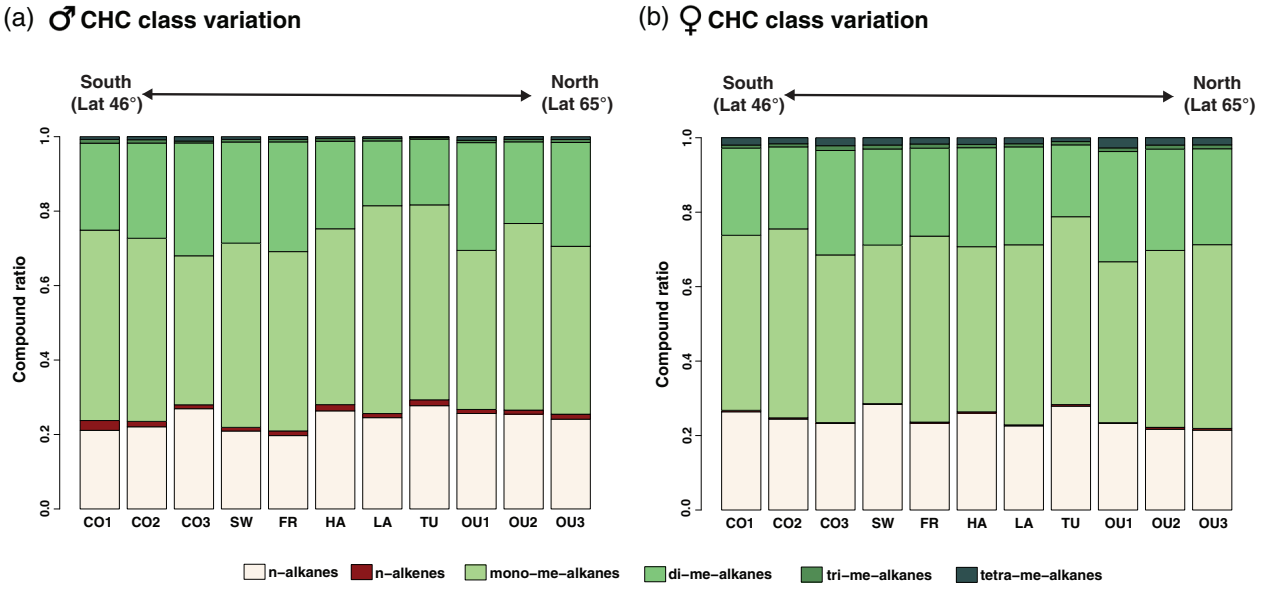

Fig. 2. Comparison of (a) male and (b) female average CHC ratios from the sampled *N. vitripennis* lines across Europe. The CHC compounds are categorised into six major compound classes, respectively: *n*-alkanes, *n*-alkenes, mono-, di-, tri, and tetra-methyl-branched alkanes. The Northern-Southern latitudinal gradient of the European sample locations is indicated by a two-sided arrow, which is also consistently shown in all following figures.

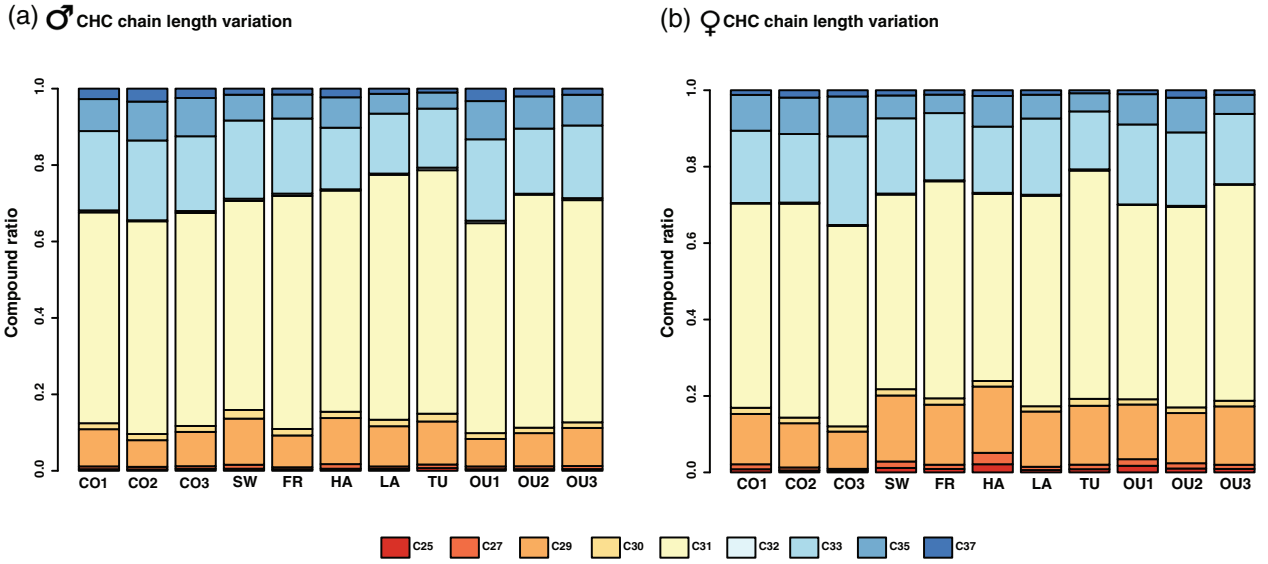

Fig. 3. Comparison of (a) male and (b) female average chain lengths of all identified CHC compounds from the sampled *N. vitripennis* lines across Europe. Relative portions (percentages) of identified chain lengths are shown, ranging from C25 to C37, chain length differences are indicated by a colour gradient (red to blue).

dimorphism with a clearly sex-specific separation (Fig. 6a,d), whereas sexual dimorphism was less obvious for the overall variation of di-methyl C31 and mono-methyl C33 compounds (Fig. 6b,c).

Concerning courtship and copulation rates between *N. vitripennis* lines from mainland Europe, we neither found any intra- (GLM: $\chi 2 = 0.15$, $P = 0.701$) nor inter- (GLM: $\chi 2 = 11.11$, $P = 0.851$) population variation, indicating no population-specific differences in mate preference and sexual attractiveness (Fig. 7).

Discussion

We investigated which components in CHC profiles of European *Nasonia vitripennis* populations are most closely associated

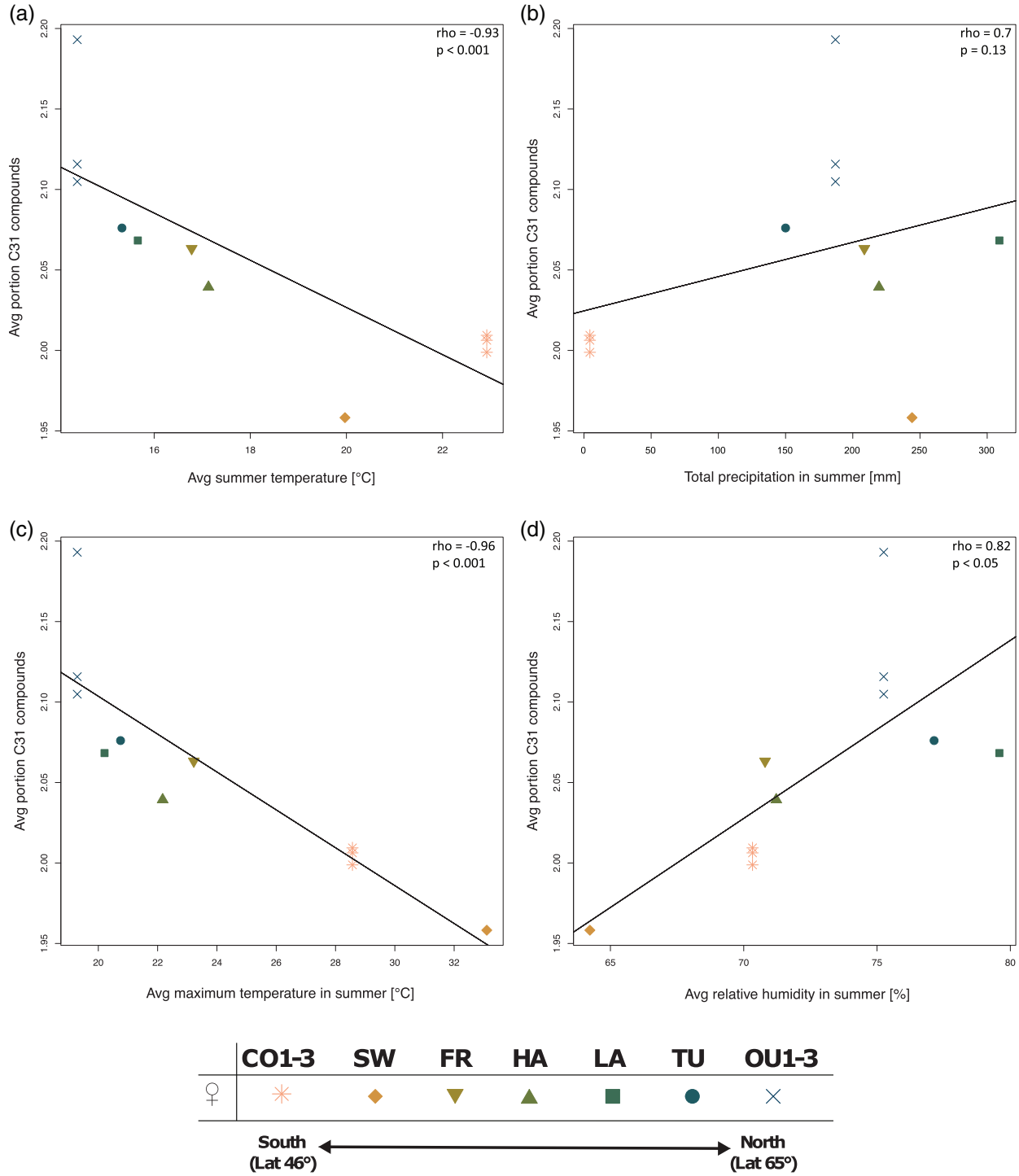

Fig. 4. Correlations of total average (avg) amounts of female CHC compounds with a chain length of C31 with (a) average summer temperature (b) total precipitation (c) average maximum summer temperature and (d) average relative humidity. Benjamini-Hochberg corrected Spearman rank correlation tests were performed to assess significance levels.

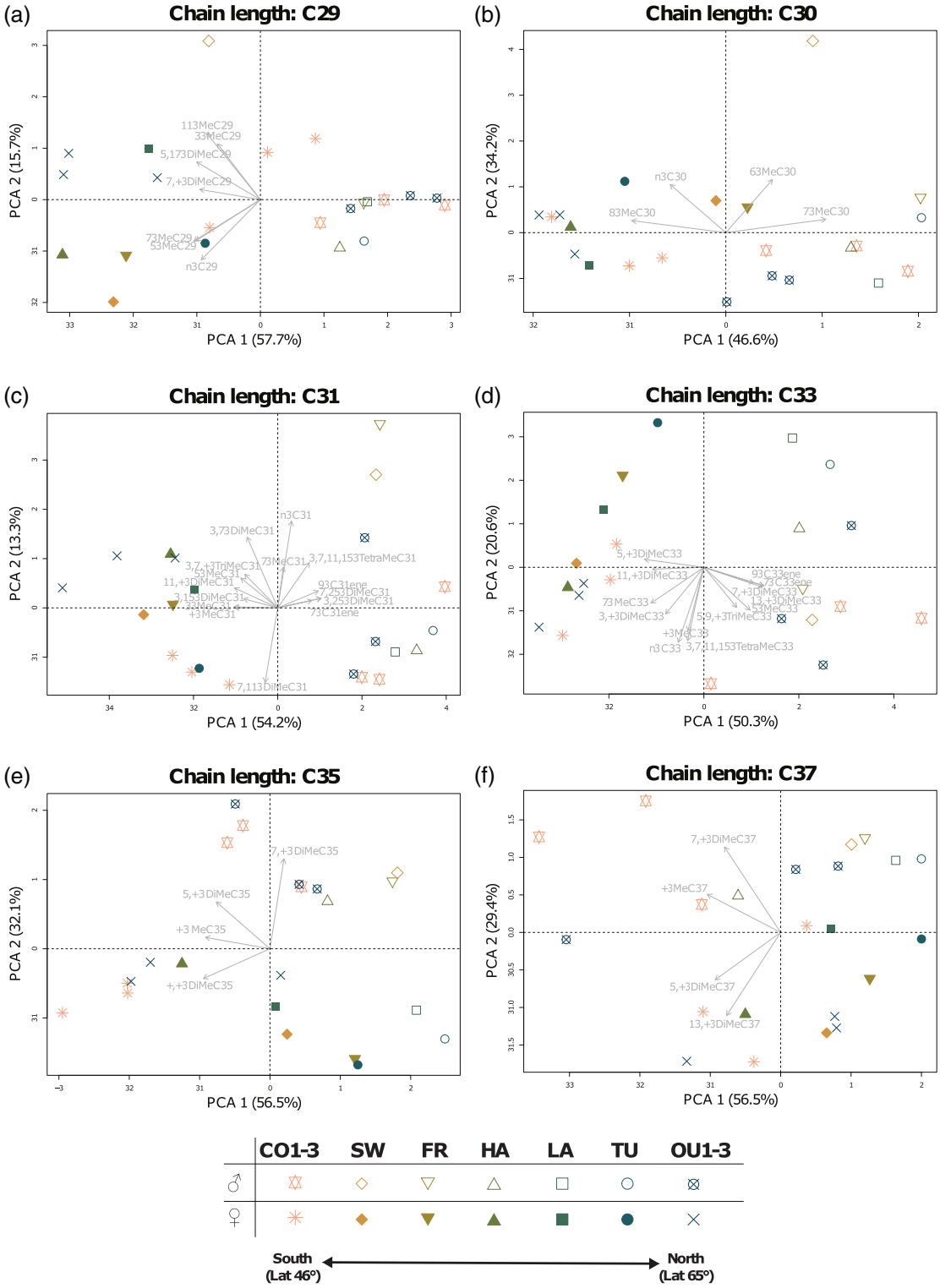

Fig. 5. Principal component analysis (PCA) plots of six CHC compound groups separated by the dominantly occurring chain lengths in profiles of the sampled European *N. vitripennis* iso-female lines: (a) C29, (b) C30, (c) C31, (d) C33, (e) C35, (f) C37. The first two principal components are plotted to show their respective contributions to the separation of male and female profiles, indicated as percentages in brackets. The dashed lines mark the zero lines for both principal components, and arrows indicate how each respective individual CHC compound per chain length group contributes to the separation originating from the crossing point of the two principal components' zero lines. Single data points correspond to the pooled samples from each iso-female line, shapes and colours indicate sex and population affiliation, respectively.

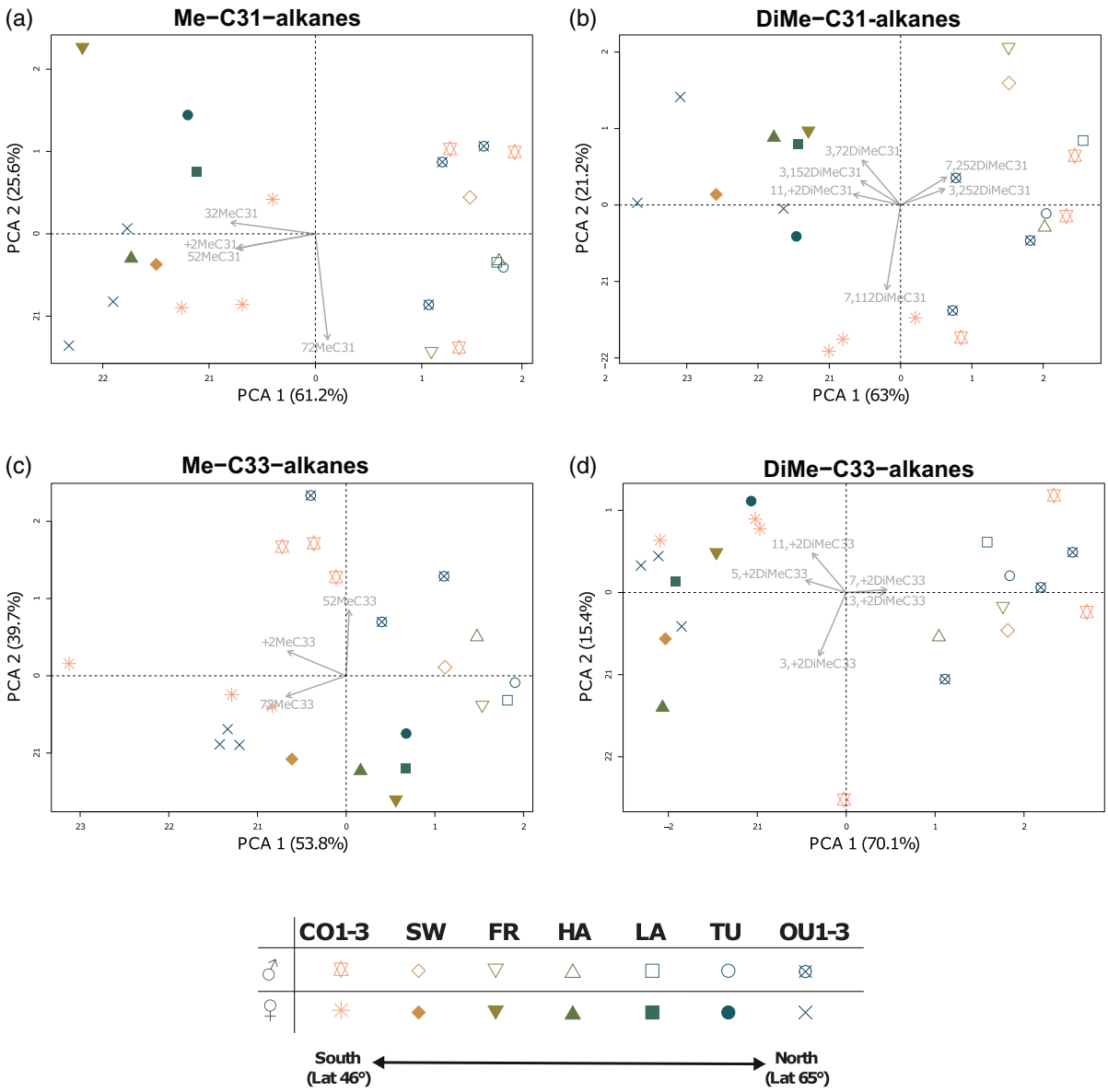

Fig. 6. Principal component analysis (PCA) plots of mono- and di-methyl-branched alkanes with chain lengths C31 (a,b) and C33 (c,d) from the CHC profiles of the sampled European *N. vitripennis* iso-female lines. The first two principal components are plotted to show their respective contributions to the separation of male and female profiles, indicated as percentages in brackets. The dashed lines mark the zero lines for both principal components, and arrows indicate how each respective individual CHC compound per group contributes to the separation originating from the crossing point of the two principal components' zero lines. Single data points correspond to the pooled samples from each iso-female line, shapes and colours indicate sex and population affiliation, respectively.

with climatic factors related to desiccation as well as which compound groups most strongly contribute to sexual dimorphism. We did not find positive correlations with either *n*-alkane proportions or higher CHC chain lengths in warmer and drier conditions, nor negative correlations with proportions of methyl-branched or unsaturated CHCs (Tables S1 and S2). This was surprising, as in numerous insect taxa, CHC profiles show either increased proportion of *n*-alkanes (Stinziano *et al.*, 2015; Buellesbach *et al.*, 2018; Sprenger *et al.*, 2018) increased proportion of longer-chained compounds (Menzel *et al.*, 2018; Duarte *et al.*, 2019) or both (Hadley, 1977; Toolson & Kupersimbron, 1989; Wagner *et al.*, 2001) in warmer and drier conditions. Methyl-branched and unsaturated CHC compounds, on the other hand, have been shown to decrease in overall proportion under these climatic conditions (Woodrow *et al.*, 2000; Michelutti *et al.*, 2018; Sprenger *et al.*, 2018). However, we found a significantly negative correlation of the proportion of CHCs with a chain length of C31 with both average and maximum summer temperature, albeit only in females (Fig. 4a,c). Moreover, the proportion of female C31

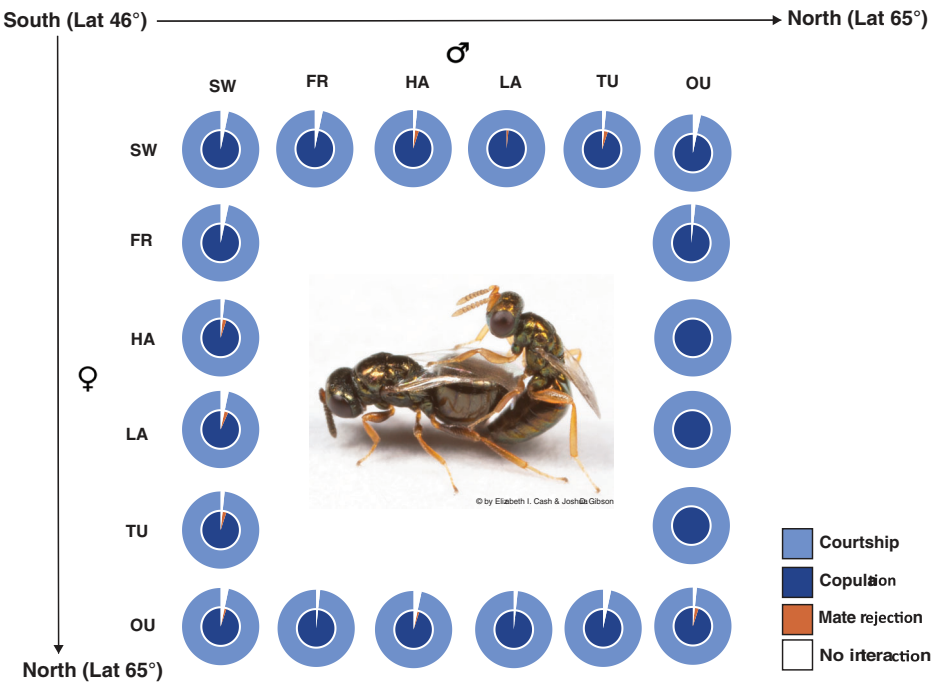

Fig. 7. Intra- and interpopulation courtship, copulation, and mate rejection rates for selected pairings of *N. vitripennis* lines from mainland Europe. The respective most Northern and Southern iso-female lines were the focal groups in cross-comparisons with all other mainland Europe iso-female lines. For each pairing, 60 replicates were tested. Outer circles (light blue) indicate courtship rates, inner circles (dark blue) copulation rates. Mate rejections are indicated in orange, and no interaction between the mating pair is indicated in white. The pairings are depicted with males from the respective iso-female lines on the upper side, and females on the left-hand side. No significant effect of iso-female line affiliation on courtship, copulation and mate rejection rates was found in either intra- ($\chi 2 = 0.15$, $P = 0.701$) or inter- ($\chi 2 = 11.11$, $P = 0.851$) line pairings. Picture in the middle depicts a male (right) and a female (bottom left) *N. vitripennis* wasp during copulation (credit: Elizabeth I. Cash and Joshua D. Gibson).

CHC compounds was also significantly positively correlated with relative summer humidity (Fig. 4d). The opposite would be expected for CHC compounds functionally recruited for water-proofing, namely, correlations simultaneously positive with temperature and negative with humidity (Wagner *et al.*, 2001; Gibbs & Rajpurohit, 2010). Although compounds with a chain length of C31 clearly dominate the CHC profiles of both males and females in our *N. vitripennis* lines overall (see Fig. 3), chain lengths can go up to C37 (see Table 2). Thus compounds with higher chain lengths do not seem to increase in European populations exposed to higher temperature and lower humidity as would be expected for maximum desiccation resistance efficiency (Gibbs & Pomonis, 1995; Gibbs & Rajpurohit, 2010).

Furthermore, the climatic correlations with C31 compounds have only been found in females, which may reflect the different life histories of males and females in this species. Whereas the flightless males are extremely restricted in their dispersal and confined to their site of emergence, females are fully capable of flight and typically disperse after mating to find new hosts for oviposition (Whiting, 1967; Werren & Loehlin, 2009). This would also predict higher exposure of the females to a broader range of climatic conditions, which might select for an increased protection against desiccation. Nevertheless, our findings in *N. vitripennis* females do not exactly match the predicted CHC compound properties coinciding with higher efficiency in desiccation prevention (Gibbs & Pomonis, 1995; Gibbs & Rajpurohit, 2010). Instead, we discovered an additional positive correlation of female C29 CHCs with average precipitation rates (Table S3), which would generally be expected for CHC compounds less important for desiccation resistance (Blomquist & Bagnères, 2010; Gibbs & Rajpurohit, 2010). Moreover, a single positive correlation was found for male CHCs, namely for average summer temperature with C30 CHC compounds (Table S4). However, these may be spurious correlations as no additional negative correlation with either precipitation rates or average humidity were found, which renders the recruitment of C30 CHCs as a desiccation barrier in males rather unlikely. These results illustrate the complex nature of insect CHCs and the difficulty to pinpoint the influences of varying environmental factors on them.

As our collections were all made in the summer, we did not account for potential seasonal variability of *N. vitripennis* CHC profiles throughout the year. Generally, the degree of phenotypic plasticity and adaptability in CHC profiles of different insect taxa appears to be highly variable and species-dependent (Liu *et al.*, 2001; Ingleby, 2015; Rajpurohit *et al.*, 2021), though several case studies managed to demonstrate plastic variability of CHC profiles with seasonality (e.g. Rouault *et al.*, 2004; Gefen *et al.*, 2015; Rajpurohit *et al.*, 2017). The exact extent of plasticity in *N. vitripennis* CHC profiles remains to be investigated, ideally under controlled laboratory conditions and

over several generations, to also get an estimate of the time frame necessary for a measurable change in CHC profiles according to different environmental conditions. As we reared our iso-female lines in the lab for three to four generations under constant conditions after collection, we attempted to minimise the influence of potentially direct effects on CHC profile variations in response to short-term ambient shifts. This was done to ultimately assess the more long-term effects of different local environmental factors across our whole collection range, the main focus of our study. However, it has to be stated that our sample size was limited and that we had to assess population-specific CHC variation through pooled samples, yielding representative CHC profiles per collected iso-female line. Future studies should also aim to incorporate individual CHC variations within populations and potentially take larger ranges of environmental gradients into account. Furthermore, it would be interesting to investigate correlations of environmental factors with very-long chain CHC compounds beyond C40, so far largely neglected as they are not detectable by traditional analytical methods (Akino, 2006; Cvacka *et al.*, 2006; Sutton *et al.*, 2013; Bien *et al.*, 2019).

A further aim of our study was to investigate which CHC compounds most strongly and consistently contribute to sexual dimorphism in *N. vitripennis*. Sexual dimorphism has previously been demonstrated in CHC profile comparisons in *N. vitripennis* (Carlson et al. 1999; Steiner *et al.*, 2006) and other *Nasonia* species (Raychoudhury *et al.*, 2010; Buellesbach *et al.*, 2013). However, all sex-specific differences in *Nasonia* have been found to be quantitative so far (Steiner *et al.*, 2006; Buellesbach *et al.*, 2013; Mair *et al.*, 2017), with no particular compounds appearing as exclusive candidates in female profiles for mediating the sexual signalling function. Nevertheless, numerous studies in other insect taxa hint at the importance of minute quantitative differences between CHC profiles as crucial determinants for functioning in sexual communication (e.g. Everaerts *et al.*, 2010; Berson & Simmons, 2019; Chen *et al.*, 2019). To shed more light on sex-specific CHC profile separation and the encoded female sexual signalling function, we subdivided all identified CHC compounds according to their chain length and separately analysed their respective contribution to the divergence between male and female CHC profiles (Fig. 5). Interestingly, only CHCs with chain lengths of C31 and C33 displayed a clear sexual dimorphism (Fig. 5c,d), which also constitute the majority of CHC compounds in both male and female profiles (see Fig. 3). Investigating C31 and C33 CHC compounds further, the vast majority of them consisted of mono- and di-methyl-branched alkanes, compounds with a particularly high potential for encoding chemical information due to their multiple possible methyl-branch positions (Blomquist & Bagnères, 2010; Chung & Carroll, 2015). Several studies in different insect taxa have already demonstrated the direct involvement and significance of methyl-branched alkanes in chemical communication (e.g. Lacey *et al.*, 2008; Holman *et al.*, 2010; Spikes *et al.*, 2010; Sakata *et al.*, 2017). However, isolating single CHC compounds out of complex profiles as the main carriers of chemical information has only rarely been achieved so far (e.g. Holman *et al.*, 2010; Smith *et al.*, 2015). Thus, an important first step in identifying the actual components conveying chemical information is to subdivide complex chemical profiles into compound groups sharing similar properties to narrow down the most likely candidate compounds. To this end, we subdivided the set of C31 and C33 methyl-branched CHCs further into mono- and di-methyl-branched alkanes and analysed their respective contribution to sex-specific variation (Fig. 6). The trend towards sexual dimorphism was most strongly displayed for mono-methyl-branched C31 and di-methyl-branched C33 alkanes (Fig. 6a,d), suggesting that variations in these specific compounds most strongly contribute to sex-specific separation in *N. vitripennis* CHC profiles.

Interestingly, our mating assays cross-comparing male mate acceptance rates between a subset of the different *N. vitripennis* lines from mainland Europe show no differences in male mate preference, neither within nor between each tested iso-female line (Fig. 7). As males are usually the mating initiators in *Nasonia* (Barrass, 1960; van den Assem & Vernel, 1979; van den Assem & Werren, 1994), this indicates no population-specific variations in female sexual attractiveness. By extension, this also indicates that the sexual cue encoded in the female CHC profiles remains consistent across our sampled iso-female lines. As we found C31 compounds to clearly correlate and vary with micro-climatic factors related to temperature and humidity in females, these are less likely to be involved in conveying the consistent sexual signalling function, thus their contribution can also not be excluded completely. Still, our findings argue for di-methyl-branched C33 alkanes being the more likely compound group governing female sexual attractiveness, but clearly, further research is necessary to unambiguously show which particular compounds encode this function.

In conclusion, we found unexpected correlations of intermediate chain length CHC compounds with environmental factors associated with desiccation across a latitudinal gradient of European *N. vitripennis* populations. Interestingly, these correlations were exclusive to female CHC profiles, hinting at a sex-specific difference in the degree of environmental influence on these particular CHC compounds, possibly related to the differential life histories of the sexes in *Nasonia*. Additionally, we narrowed down CHC compound groups most strongly associated with sexual dimorphism to mono-methyl-branched C31 alkanes and di-methyl-branched C33 alkanes while also showing consistently triggered male mating behaviour, and, by extension, female attractiveness across populations. Our study delivers intriguing first insights into how both conserved sexual signalling as well as adaptive environmental flexibility can be encoded among different CHC compound classes, constituting an important step towards deciphering how potentially conflicting functions can be unified in insect CHC profiles.

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Ecological Station, Germany), Ralph Peters (Hamburg University, Germany), Indrikis Krams and Mikus Abolins-Abols (Tartu University, Estonia), Tapio Eeva and Miia Koivula (Turku University, Finland), Markku Orell (Oulu University, Finland), Saleta Perez-Vila, Silvia Paolucci and Sjors Beukeboom (University of Groningen, the Netherlands). We especially thank Mareike Koppik and Shuqing Xu for valuable suggestions and comments on the statistical analysis and interpretation of the data, Louis van de Zande for his helpful comments on the first version of this manuscript, as well as Friederike Fröb for her valuable contribution to re-working the colour schemes of the figures. This work was funded by a Marie Curie Initial Training Network 'Understanding the evolutionary origin of biological diversity' (ITN-2008-213780 SPECIATION). We thank all members of the 'SPECIATION' Network for helpful and stimulating discussion. The authors declare no conflict of interest. Open access funding enabled and organized by Projekt DEAL.

Author contribution

Project design: TS, LWB; *methodology*: TS, LWB, WD, JB; *data collection*: WD, LWB; *data analysis*: JB; *writing - original draft preparation*: WD, JB; *writing - review and editing*: JB, LWB, TS; *visualisation*: JB, LWB; *supervision*: TS, LWB, JB; *funding acquisition*: LWB.

Ethical guidelines

There is no ethics committee overseeing experimental research on parasitoid wasps. However, all efforts have been made to treat the animals as humanely as possible.

Data availability statement

All chemical and behavioral raw data used in this manuscript are available online at dryad: https://doi.org/10.6078/D1Q41J.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Correlations of average female CHC portions divided by compound class with average summer temperature (in °C), average maximum summer temperature (in °C), total precipitation in summer (in mm), and average relative humidity in summer (in %) for *n*-alkanes, *n*-alkenes, mono-methyl-branched alkanes, di-methyl-branched alkanes, tri-methyl-branched alkanes, and tetra-methyl-branched alkanes. All measurements were taken during the year matching our collection season. Benjamini-Hochberg-corrected Spearman rank correlation tests were performed to assess significance levels.

Table S2. Correlations of average male CHC portions divided by compound class with average summer temperature (in °C), average maximum summer temperature (in °C), total precipitation in summer (in mm), and average relative humidity in summer (in %) for *n*-alkanes, *n*-alkenes, mono-methyl-branched alkanes, di-methyl-branched alkanes, tri-methyl-branched alkanes, and tetra-methyl-branched alkanes. All measurements were taken during the year matching our collection season. Benjamini-Hochberg-corrected Spearman rank correlation tests were performed to assess significance levels.

Table S3. Correlations of average female CHC portions divided by compound chain length with average summer temperature (in °C), average maximum summer temperature (in °C), total precipitation in summer (in mm), and average relative humidity in summer (in %). All measurements were taken during the year matching our collection season. Benjamini-Hochberg-corrected Spearman rank correlation tests were performed to assess significance levels. Significant correlations are indicated in bold.

Table S4. Correlations of average male CHC portions divided by compound chain length with average summer temperature (in °C), average maximum summer temperature (in °C), total precipitation in summer (in mm), and average relative humidity in summer (in %). All measurements were taken during the year matching our collection season. Benjamini-Hochberg-corrected Spearman rank correlation tests were performed to assess significance levels. Significant correlations are indicated in bold.

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