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Adaptive dynamics of cuticular hydrocarbons in *Drosophila*

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Abstract

Cuticular hydrocarbons (CHCs) are hydrophobic compounds deposited on the arthropod cuticle that are of functional significance with respect to stress tolerance, social interactions and mating dynamics. We characterized CHC profiles in natural populations of *Drosophila melanogaster* at five levels: across a latitudinal transect in the eastern United States, as a function of developmental temperature during culture, across seasonal time in replicate years, and as a function of rapid evolution in experimental mesocosms in the field. Furthermore, we also characterized spatial and temporal changes in allele frequencies for SNPs in genes that are associated with the production and chemical profile of CHCs. Our data demonstrate a striking degree of parallelism for clinal and seasonal variation in CHCs in this taxon; CHC profiles also demonstrate significant plasticity in response to rearing temperature, and the observed patterns of plasticity parallel the spatiotemporal patterns observed in nature. We find that these congruent shifts in CHC profiles across time and space are also mirrored by predictable shifts in allele frequencies at SNPs associated with CHC chain length. Finally, we observed rapid and predictable evolution of CHC profiles in experimental mesocosms in the field. Together, these data strongly suggest that CHC profiles respond rapidly and adaptively to environmental parameters that covary with latitude and season, and that this response reflects the process of local adaptation in natural populations of *D. melanogaster*.

Introduction

The colonization of terrestrial environments necessitated evolution of mechanisms to minimize desiccation and preserve water balance, particularly for small animals with a high surface area to volume ratio (Edney & Nagy, 1976; Gibbs & Rajpurohit, 2010). Arthropods were among the first terrestrial animals and are particularly vulnerable to desiccation; one primary adaptation to desiccating terrestrial environments is the production and deposition of a variety of hydrophobic compounds (hydrocarbons) on the cuticle that function as a barrier to water transpiration (Jallon & David, 1987; Gibbs, 1998, 2002a, b). These cuticular hydrocarbons (CHCs) have been secondarily co-opted for a variety of other functions such as communication and the conveyance of information regarding species identity or mating status (Venard & Jallon, 1980; Jallon, 1984; Ferveur, 2005; Takahashi *et al.*, 2012). CHC profiles are genetically determined, exhibit extensive phenotypic variation within and among populations and covary with environmental parameters such as temperature and humidity; this suggests that the evolution of CHCs may be dynamic and rapid in natural populations. Despite the established functional links to fitness traits that vary in nature, the potential role of CHCs in adaptation to heterogeneous environments has not been comprehensively examined.

Temperature and humidity are perhaps the most pervasive abiotic stresses terrestrial organisms encounter, and often vary predictably across environmental

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gradients such as latitude and altitude. Such gradients in environmental stress can be associated with divergent selection pressures that both maintain genetic variation through various forms of overdominance (Hedrick *et al.*, 1976; Barton, 1989; Yeaman & Jarvis, 2006; Bergland *et al.*, 2014) and phenotypic variation through life history trade-offs (Stearns, 1992; Roff, 2002). This process of adaptation to local environments is often characterized by distinct selection regimes, which can result in the formation and maintenance of clines (Barton, 1999; Savolainen *et al.*, 2013). In *Drosophila melanogaster*, latitudinal clines in allele frequencies (Hoffmann *et al.*, 1995; McColl & McKechnie, 1999; Sezgin *et al.*, 2004; Kolaczkowski *et al.*, 2011; Fabian *et al.*, 2012) and fitness-associated phenotypes (Hoffmann & Harshman, 1999; Rajpurohit & Nedved, 2013) have been widely observed. Although such patterns may be affected by demography and colonization history (Bergland *et al.*, 2016), parallel clines on multiple continents (e.g. Knibb, 1982; Paaby *et al.*, 2010) and between latitudinal and seasonal patterns (Bergland *et al.*, 2014; Behrman *et al.*, 2015; Zhao *et al.*, 2015) strengthen the inference of selection and adaptation to climatic conditions that vary predictably over space and time. However, the mechanistic basis for the formation and maintenance of clines is largely unknown (Hoffmann & Harshman, 1999; Hoffmann *et al.*, 2004; Adrion *et al.*, 2015).

Many clinal patterns in *Drosophila* appear directly related to thermal and desiccation tolerance (Hoffmann & Harshman, 1999; Gilchrist *et al.*, 2008; Rajpurohit *et al.*, 2013). In terrestrial insects, the cuticular exoskeleton is the primary interface between the organism and the external biotic and abiotic environment, providing multiple protective functions (Wigglesworth, 1948; Chapman & Simpson, 2012). The outermost layer is composed of a mixture of lipids synthesized by oenocytes. These lipid molecules are then transported through the hemolymph to the cuticular surface through pore canals (Romer, 1991; Schal *et al.*, 1998; Blomquist & Bagnères, 2010). These compounds are commonly known as cuticular waxes. The most abundant components of cuticular waxes are CHCs and are thought to represent a primary adaptation to desiccation imposed by the transition to a terrestrial existence (Jallon *et al.*, 1997).

Although CHCs have been co-opted for a variety of secondary functions, such as the mediation of social interactions through chemical cues (Ferveur, 2005; Fedina *et al.*, 2012; Kuo *et al.*, 2012; Gershman *et al.*, 2014) as well as mimicry (Blomquist & Bagnères, 2010), their primary role is to regulate transpiration through the surface of the animal (see Gibbs & Rajpurohit, 2010). Even a brief treatment of the insect cuticle with organic solvents to remove surface lipids can result in water-loss rates increasing 10–100-fold, thus demonstrating the importance of CHCs in water conservation (Hadley, 1994). Resistance to desiccation is affected by specific properties of CHCs, notably a negative association between the length of the hydrocarbon chains and rates of water loss (Toolson, 1982; Gibbs & Pomonis, 1995; Gibbs, 2002a, b). In natural *Drosophila* populations, the role of CHCs in desiccation tolerance is well established (Toolson, 1982; Lockey, 1988; Rouault *et al.*, 2004; Gibbs & Rajpurohit, 2010; Rajpurohit & Nedved, 2013; Rajpurohit *et al.*, 2013); these observations are further supported by artificial selection studies, in which desiccation resistance is associated with CHC chemical composition (Gibbs *et al.*, 1997; Kwan & Rundle, 2009).

In addition to the predicted associations between CHC profiles and desiccation stress in natural habitats (Rourke, 2000; Rouault *et al.*, 2004; Howard & Blomquist, 2005; Parkash *et al.*, 2008), CHCs also affect tolerance to freezing (Nelson & Lee, 2004) and exhibit phenotypic plasticity in response to rearing temperature (Rouault *et al.*, 2004). Thus, CHCs are predicted to vary among natural populations that experience distinct climatic environments (Toolson & Kuper-Simbron, 1989; Rouault *et al.*, 2004). However, the extent to which CHC profiles of natural populations reflect plasticity in response to environmental variance vs. evolutionary divergence among populations is unknown. CHCs are produced from acetyl-CoA, and some of the molecular biosynthesis pathways are known (Wicker-Thomas, 2007; Pardy, 2012; Wicker-Thomas *et al.* 2015). CHC profiles demonstrate significant heritability (Sharma *et al.*, 2012), and mapping in recombinant inbred lines has identified a number of sex-specific quantitative trait loci (Foley *et al.*, 2007). Recent genomewide association (GWA) analyses in the *Drosophila* Genome Reference Panel identified 24 candidate genes associated with CHCs in *D. melanogaster*, and manipulation of their expression resulted in altered CHC profiles (Dembeck *et al.*, 2015).

Despite the established associations between CHCs, fitness and environmental conditions that vary predictably over spatial and temporal gradients, the potential role of CHCs in climatic adaptation has not been comprehensively examined. To explore how natural selection via abiotic factors affects patterns of CHC variation in natural populations of *D. melanogaster*, we examined the covariance between CHC profiles and environmental factors at five levels: (i) at the latitudinal spatial scale, that is geographical variation; (ii) under varying temperature conditions, that is thermal plasticity; (iii) at the temporal scale, that is seasonal variation; (iv) at the genetic level through examination of spatial and seasonal changes in allele frequency of SNPs in genes associated with CHC composition; and (v) the rapid evolution of CHC profiles over short timescales in field mesocosms, that is the evolutionary ecology of CHCs. Our results demonstrate significant and predictable variation in CHC profiles of natural *D. melanogaster* populations in eastern North America. The data demonstrate a striking degree of parallelism

across various levels of investigation in this study, suggesting that CHC profiles represent one component in a suite of physiological traits shaped by natural selection in a heterogeneous climatic environment.

Materials and methods

Stocks and maintenance

Gravid females were collected by direct aspiration on fallen fruit from orchards in Maine (Bowdoin 44.03°N, 73.20°W), Pennsylvania (Media 40.04°N, 76.30°W) and Florida (Jacksonville 30.33°N, 81.66°W) in September 2011 (Fig. 1a). Individual isofemale lines were established in the field on standard medium; species identity was confirmed by examining male progeny. Isofemale lines were maintained in narrow *Drosophila* culture vials on regular corn–agar–molasses medium at room temperature. For the seasonal collections, populations from a Pennsylvania (Media 40.04°N, 76.30°W) orchard were collected in spring (1–7 June) and fall (1–7 November) for two consecutive years (2013 and 2014; Bergland *et al.*, 2014; Behrman *et al.*, 2015). All the collections described above were maintained under common garden conditions for > 5 generations before experimental use in order to remove confounding environmental and maternal effects (Hercus & Hoffmann, 2000; Hoffmann & Weeks, 2007).

Spatiotemporal population cages

To examine the effects of geographical origin on CHC profiles, we established population cages using isofemale lines from the orchard populations in Florida and Maine. Each population cage was constructed by pooling 10 mated females from each of 25 isofemale lines; two replicate cages were constructed from each geographical source, using independent sets of lines.

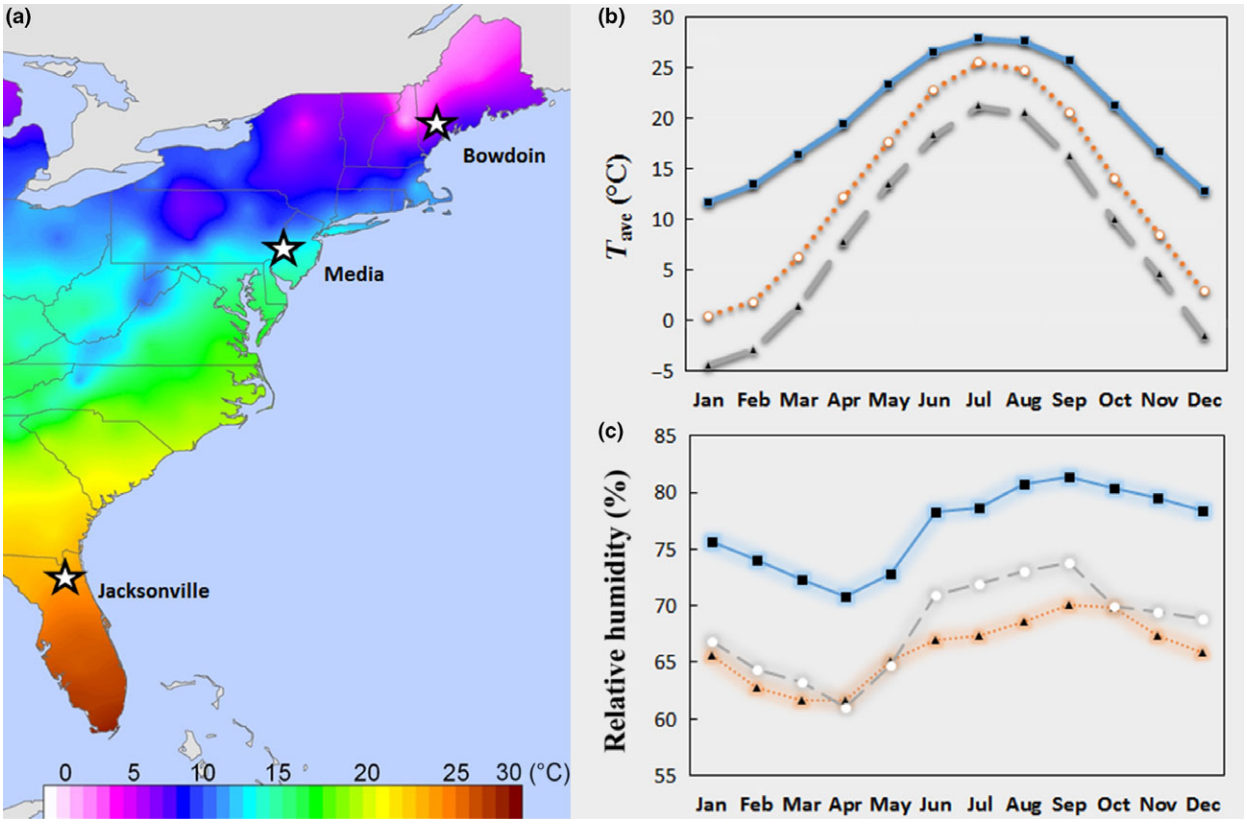

Fig. 1 Temperature map of the east coast of the United States of America (a). The three collection sites for natural *Drosophila melanogaster* populations are denoted by stars (Jacksonville, FL, 30.33°N, 81.66°W; Media, PA, 40.04°N, 76.30°W; Bowdoin, ME, 44.03°N, 73.20°W). Seasonal collections were made in the spring and fall in two successive years (2012 and 2013) from the Media, PA population. Thermal plasticity experiments were performed on FL and ME populations only. Twelve months' average temperature (b) and relative humidity (c) patterns for the sites of origin of populations. Jacksonville (30.33°N), Media (40.04°N) and Bowdoin (44.03°N) stations' T_{ave} and relative humidity are shown in solid (with rectangles), dotted (with circles) and dashed (with triangles) lines, respectively. Projections displayed here are the averages of 30 years (1980–2010). Climatic data were obtained from National Oceanic and Atmospheric Administration (NOAA; www.ncdc.noaa.gov).

Experimental populations were maintained in 0.3 × 0.3 × 0.3 m insect enclosures (Live Monarch Foundation, Boca Raton, FL, USA) on a 20-d generational cycle. After five generations of outcrossing, samples were collected for CHC characterization.

Seasonal cages were established as described above from orchard collections made in successive years in Media, PA, USA (Fig. 1a). We established one spring and one fall cage for each collection year; thus, there were a total of four experimental seasonal cages constructed. The two sampling years were treated as replicates for seasonality.

Thermal plasticity experiments

To study developmental temperature effects on CHC profiles, flies from the replicate Florida and Maine populations were cultured from egg to adult at three different temperature conditions (18 °C, 25 °C and 30 °C) in Percival I36VL incubators on a photoperiod of 12L : 12D. Eggs were collected in narrow *Drosophila* culture vials at 25 °C over a period of 5–6 h. To control density (30–50 eggs per vial), extra eggs were removed from each of the vials, which were then immediately transferred to the respective temperature treatment.

Experimental manipulation of CHC evolution

To examine how CHC profiles evolve under field conditions, we established 20 experimental mesocosms at a field site in Philadelphia, PA, USA. Each mesocosm was an 8-m^3 outdoor insect rearing enclosure (Bioquip Products, Gardena, CA, USA) that surrounded a peach tree on dwarfing root stock. Each mesocosm was seeded with 1000 individuals (500 males, 500 females) derived from a collection made in 2012 from the same PA orchard as described above. This progenitor population was created by pooling 86 independent inbred lines, allowing them to recombine and expand for 10 generations in the laboratory, and then maintained at large census size (~10^6) in the laboratory for the duration of the experiment (1 July to 1 November 2014). Each cage was randomly assigned to a treatment: evolving (E or seasonally evolving) or control (C or nonseasonally evolving). In the E treatment, populations were supplied with fresh food/oviposition sources (500 mL standard cornmeal–molasses medium) every 2d, and the populations were allowed to evolve and adapt to seasonal environmental conditions. In the F treatment, fresh food/oviposition sources were also supplied: however, all eggs laid by the experimental flies were counted, removed, and replaced with the same number of eggs from the progenitor laboratory population that was maintained under aseasonal, laboratory conditions. Thus, the F populations were maintained under normal demographic trajectories and flies were directly exposed to the field environment, but these populations were not allowed to evolve to the field conditions; the F treatment represents the progenitor laboratory population while including any potential epigenetic marks that may be elicited upon exposure to field conditions during direct development as well as any evolution in the aseasonal, laboratory environment. At the end of the experiment, a sample of approximately 2000 eggs was collected from each of the 20 cages and brought back to the laboratory. These collections were allowed to develop in the laboratory at 25 °C and subsequently passed through two generations of common garden, density controlled culture. In the F3 generation subsequent to the field collection, experimental animals were collected and processed for CHC profiles.

Sample collections

From all the populations described above (geographic, seasonal, plasticity & field mesocosms), freshly eclosed males and females were collected and kept in isolation until experimental flies were 4 days of age; for each experimental unit, 50 males and 50 females were collected in regular *Drosophila* culture vials. Three replicates were collected in total from each experimental unit. After flies were 4 days old, they were transferred to −80 °C. After a period of 24 h, experimental replicates (50 individuals per sex) were prepared by transferring flies to 20 × 150 mm borosilicate glass vials (Kimble Chase, Vineland, NJ, USA). These glass tubes were placed in an airtight container with silica gel for drying.

Isolation and purification of CHCs

Dried flies (50 males or 50 females) were vortexed three times with 200 μL of hexane for one min. The pooled lipid extracts were applied on small glass columns (3 mm i. d.) containing precleaned cotton wool and silica gel (particle size 50–100 μm; *ca.* 75 mg) at the bottom. CHCs were eluted with 3 × 100 μL of hexane. The resulting samples were concentrated under a stream of nitrogen to approximately 25 μL and stored at −18 °C.

GC-MS analyses of CHCs

The GC-MS analyses were performed using a 7890A gas chromatograph coupled to a 5975C mass spectrometer equipped with an electron ionization (EI) source and a quadrupole analyser (Agilent Technologies, Santa Clara, CA, USA). The samples (1 μL) were injected with a split ratio 20 : 1 at 320 °C. A DB-1 ms fused silica capillary column (30 m × 250 μm; a film thickness of 0.25 μm; J&W Scientific, Santa Clara, CA, USA) was used for separation using carrier gas helium at a constant flow rate of 1.0 mL min^{-1}. The temperature program was as follows: 50 °C (1 min), then 50 °C min^{-1}

to 150 °C, followed by 5 °C min^{-1} to 330 °C (10 min). The temperatures of the transfer line, ion source and quadrupole were 320 °C, 230 °C and 150 °C, respectively. The standard 70 eV EI spectra were recorded from 25 to 600 *m/z*.

Cuticular hydrocarbon data mining

The peak areas of the GC chromatograms corresponding to individual CHCs were converted into relative proportions of the sum of CHC peak areas. Only the variables (CHCs) representing at least 0.5 relative percentage in at least one individual of the given sample set were selected for further analysis. The relative proportions were then calculated again including only the selected peaks. The relative data were arcsine-transformed and the overall quantitative variability among samples and treatments visualized using principal component analysis (PCA) performed in Statistica 8.0. Subsequently, the same relative and arcsine-transformed data were used for Canonical Correspondence Analysis (CCA) in Canoco 4.5, with intersample distance being analysed using Hill's scaling followed by Monte Carlo permutation test with 999 permutations to assess the statistical significance of the differences among treatments. The reported *P* values for the Monte Carlo permutation tests were calculated from the formula $P = (n_x+1)/(N + 1)$ where n_x stands for the number of permutations with multivariate pseudo F-ratio value equal to or larger than that of the non-permuted real data set, and *N* represents the total number of permutations (Šmilauer & Lepš, 2014). The variables, fitting the best the calculated model of intertreatment differences, were then extracted from the CCA and listed using CanoDraw plugin under Canoco 4.5.

The basic characterization of CHC structures was based on interpreting their EI mass spectra. The spectra reliably revealed number of carbons, methyl branching in saturated chains and number of double bonds in unsaturated chains. The chemical structures (i.e. names) of CHCs described in this study were based on descriptions from the published literature (Everaerts *et al.*, 2010).

GWA analysis, genomic enrichment and parallelism tests

We examined how SNPs associated with CHC composition varied across the east coast of the United States and among seasons over 3 years (2009–2011) in the sampled Pennsylvanian orchard. To perform this analysis, we utilized previously reported line means for CHC abundance in the DGRP (Dembeck *et al.*, 2015). We performed PCA of scaled and centred CHC abundance using the *prcomp* function in R (R Core Team 2015) separately for male and females after normalizing for *Wolbachia* infection and presence/absence of the major, cosmopolitan inversions (*In(2L)t, In(2R)NS, In(3R)P, In (3R)Mo* and *In(3R)K*). We sought to identify which principal component was significantly associated with CHC chain length by classifying CHCs as short (< 24C for males, < 25C for females) or long (≥ 24C for males, ≥ 25C for females). We found that principal component 1 (PC1) was significantly differentiated between long and short CHCs for both males ($F_{1,37} = 34.8$, $P = 8.56e\text{-}7$) and females ($F_{1,49} = 42.5$, $P = 3.6e\text{-}8$). In this rotation of CHCs, a smaller value of PC1 was associated with shorter CHCs. We performed GWA on both male and female PC1 rotation scores for each DGRP line using *PLINK v1.07* (Purcell *et al.*, 2007).

Next, we examined whether SNPs associated with CHC PC1 were enriched for signals of clinality and seasonality as defined in Bergland *et al.* (2014). We defined SNPs significantly associated with CHC PC1 as those with a nominal *P*-value < 0.0005 (corresponding to a genomewide *q* value of ~0.25), clinal SNPs as those with nominal *P*-value < 0.01 ($q < 0.05$) and seasonal SNPs as those with nominal *P*-value less than 0.05 ($q < 0.75$). Our choice of *P*-value thresholds was intentionally liberal for both biological and statistical reasons. Population differentiation in quantitative traits often occurs at many loci each with small effects on phenotype (Turchin *et al.*, 2012) and the demographic history of North American *D. melanogaster* may mask signals population differentiation caused by selection (Bergland *et al.*, 2016). In addition to these biological considerations, we chose liberal *P*-value thresholds to allow for a sufficient number of SNPs to perform enrichment tests and also note that power to detect SNPs that are associated with CHC PC1, latitude or season is limited (Bergland *et al.*, 2014; Dembeck *et al.*, 2015), further justifying the use of liberal *P*-value thresholds. We identified 500 sets of control SNPs that were matched to SNPs significantly associated with CHC PC1 (males and females were considered separately). Control SNPs were identified based on chromosome, heterozygosity in the DGRP and whether they occurred in any of the large, cosmopolitan inversions that are known to vary clinally in North American populations. We then performed blocked bootstrap resampling of SNPs with block size of 10Kb and 500 bootstrap replicates to ensure that signals of overlap enrichment were not inflated by multiple, closely linked polymorphisms. Further details of our blocked bootstrap procedure can be found in Bergland *et al.* (2014).

Finally, we examined whether SNPs that were associated with CHC PC1 and varied across latitude or season varied in a congruent (parallel) fashion. Specifically, we sought to test whether alleles common at high latitudes or during the spring season were more likely to also be associated with smaller values of PC1 (i.e. shorter CHCs). For this analysis, we only examined SNPs below our GWA and clinality or seasonality *P*-value thresholds

or their respective controls. As in our overlap tests described above, tests of parallelism took into account the spatial distribution of SNPs along the chromosome through a block-bootstrap procedure.

Results

The CHC profiles of the characterized *Drosophila* populations exhibited saturated and unsaturated aliphatic hydrocarbons with up to 35 carbons. Both straight-chain, methyl-branched and unsaturated (up to two double bonds) hydrocarbons were present, the most abundant being those with odd number of carbon atoms (C21–C31). As an example, Fig. 2 shows male and female CHC profiles that were clearly differentiated. A shift towards longer retention times (CHC with longer chains) for flies developed at higher temperatures was observed in all sample sets. Repeatedly detected peaks of male and female CHCs were relatively quantified and used in subsequent PCA; the detected CHCs are summarized in Table 1.

Geographical variation in CHCs

First, we analysed the variation in CHC profiles of females and males originating from three distinct geographical locations (Florida, Pennsylvania and Maine) and cultured at the standard, laboratory temperature of 25 °C. For females, a total of 24 variables were over the 0.5% inclusion/exclusion threshold and were selected for further analysis. PCA clearly separated the populations (Fig. 3a, b), which also exhibited seasonal variability in the Pennsylvania samples (described in the seasonal variation section below). CCA using geographical origin as a categorical predictor also showed significant differences among the CHC chemical profiles of flies from Florida, Pennsylvania and Maine (Monte Carlo test, $P = 0.001$). Among the variables retrieved to

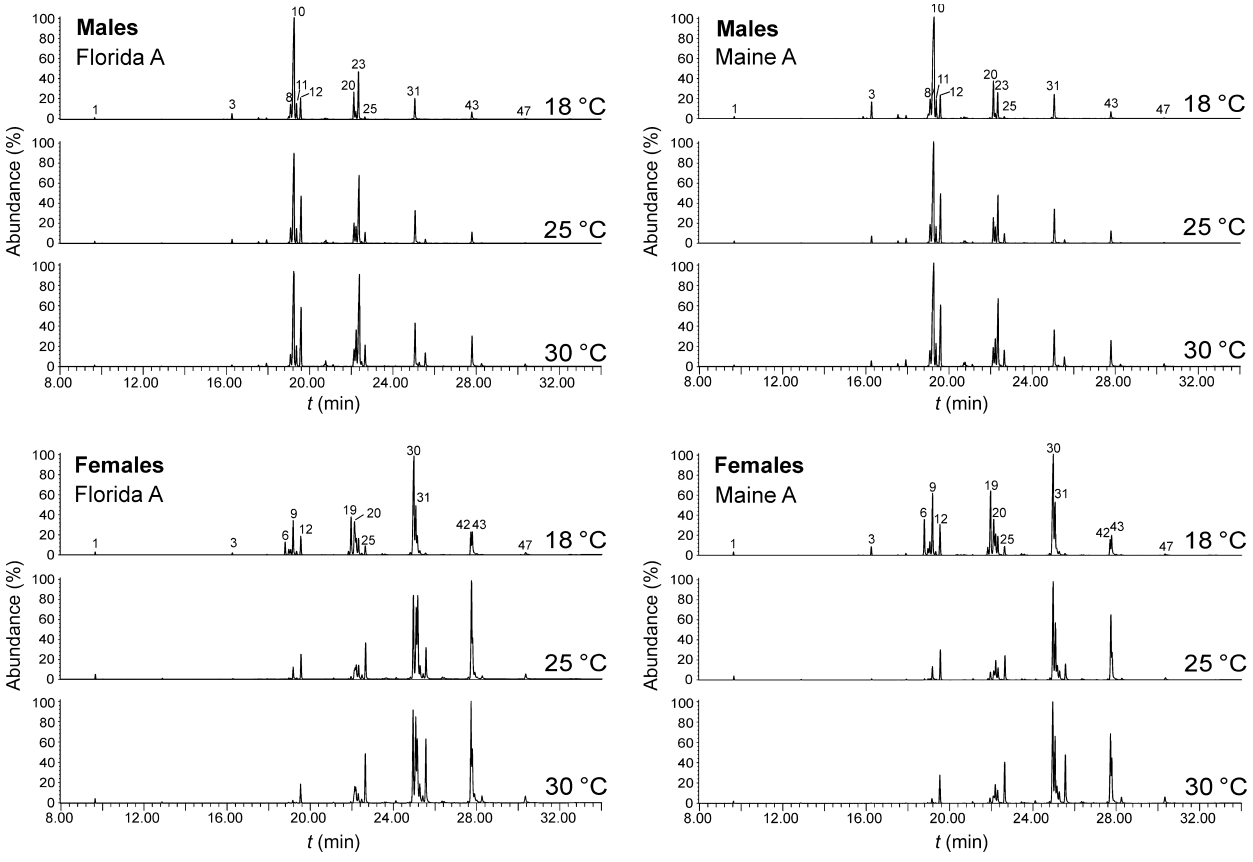

Fig. 2 Representative GC chromatograms showing cuticular hydrocarbons (CHCs) of males and females from two different localities (FL and ME) and cultured at three different temperatures. Note the shift towards longer-chain CHCs with the increasing temperature. Peak identifications: 1: *n*-heptacosane; 3: *n*-heneicosane; 6: (*Z*,*Z*)-7,11-tricosadiene; 8: (*Z*)-9-tricosene; 9: (*Z*)-7-tricosene; 10: (*Z*)-7-tricosene; 11: (*Z*)-5-tricosene; 12: *n*-tricosane; 19: (*Z*,*Z*)-7,11-pentacosadiene; 20: 2-methyltetracosane; 23: (*Z*)-7-pentacosene; 25: *n*-pentacosane; 30: (*Z*,*Z*)-7,11-heptacosadiene; 31: 2-methylhexacosane; 42: (*Z*,*Z*)-7,11-nonacosadiene; 43: 2-methyloctacosane; 47: 2-methyltriacontane + hentriacontadiene. For other peaks, see Table 1.

Table 1 *Drosophila melanogaster* cuticular hydrocarbons (CHCs) identified by GC/MS.

Peak number	Rt (min)	CHC	Sex	Peak number	Rt (min)	CHC	Sex
1	9.68	17 : 0	♀♂	25	22.67	25 : 0	♀♂
2	12.91	19 : 0	♀♂	26	23.49	26 : 2	♀
3	16.28	21 : 0	♀♂	27	23.62	26 : 0 (br)	♀♂
4	17.56	22 : 1	♂	28	24.15	26 : 0	♀
5	17.94	22 : 0	♀♂	29	24.83	27 : 2	♀
6	18.82	23 : 2	♀	30	24.94	27 : 2	♀♂
7	18.99	23 : 0 (br) + 23 : 2	♀♂	31	25.07	27 : 0 (br)	♀♂
8	19.09	23 : 1	♀♂	32	25.13	27 : 1	♂
9	19.20	23 : 1	♀	33	25.17	27 : 2	♀
10	19.26	23 : 1	♀♂	34	25.20	27 : 1	♀
11	19.38	23 : 1	♀♂	35	25.28	27 : 1	♀♂
12	19.58	23 : 0	♀♂	36	25.45	27 : 1	♀
13	20.58	24 : 0 (br)	♂	37	25.57	27 : 0	♀♂
14	20.73	24 : 1	♂	38	26.29	28 : 2	♀
15	20.79	24 : 1	♂	39	26.37	28 : 2	♀
16	20.87	24 : 1	♂	40	26.46	28 : 2	♀
17	21.14	24 : 0	♀♂	41	27.61	29 : 2	♀
18	21.87	25 : 2	♀	42	27.72	29 : 2	♀♂
19	21.98	25 : 2	♀	43	27.81	29 : 0 (br)	♀♂
20	22.14	25 : 0 (br)	♀♂	44	27.92	29 : 1 + 29 : 2	♀
21	22.17	25 : 2	♀	45	28.02	29 : 1	♀♂
22	22.23	25 : 1	♀♂	46	28.28	29 : 0	♀♂
23	22.35	25 : 1	♀♂	47	30.37	31 : 2 + 31 : 0 (br)	♀♂
24	22.50	25 : 1	♀♂	48	30.50	31 : 0 (br)	♀

CHCs are denoted as the number of carbons: number of double bonds; '(br)' stands for branched.

have the best fit with the model predictions, shorter-chain CHCs (*n*-tricosane; (*Z*,*Z*)-7,11-pentacosadiene; (*Z*)-9-pentacosene) were over-represented in the Maine and Pennsylvania populations, whereas CHCs with relatively longer chains ((*Z*,*Z*)-5,9-heptacosadiene; heptacosene; nonacosene + nonacosadiene) were more abundant in the populations originating from Florida.

For males, a total of 20 variables met the 0.5% threshold criterion and were selected for analysis. As was observed for females, PCA clearly separated the populations according to geography (Fig. 3c, d). CCA using geographical origin (Florida, Pennsylvania and Maine) as the categorical predictor again showed significant differences among the CHC chemical profiles of the various populations (Monte Carlo test, $P = 0.017$). Again, shorter-chain CHCs were over-represented in the Maine and Pennsylvania populations (*n*-heneicosane; (*Z*)-7-docosene; *n*-docosene), whereas CHCs with relatively longer chains were more abundant in the Florida populations ((*Z*)-7-pentacosene; (*Z*)-5-pentacosene; *n*-pentacosane; (*Z*)-7-heptacosene).

Thermal plasticity in CHCs

Second, we cultured the Maine and Florida populations, which are located at the ends of the latitudinal range of the U.S. east coast, at three different temperatures. For females, the quantitative pattern of 31 selected CHCs is visualized in Fig. 4a, showing the scores for the first two PCA factors. The clusters of samples are significantly distinct at several hierarchical levels. The two geographical regions were clearly separated across all temperatures, and within-population variability was lower than variability between the replicate populations from the same geographical region. Further, the distribution pattern revealed the separation of samples along a temperature gradient from 18 through 25 to 30 °C. Subsequent CCA with temperature selected as the categorical predictor confirmed temperature as a significant separation factor (Monte Carlo test, $P = 0.001$). Figure 4b shows the plot of variable correlations with the first two PCA factors. The visual inspection of the plot shows that longer-chain CHCs are correlated with the shift from lower to higher temperatures. The variables showing the highest correlation with the model prediction of CCA are highlighted in Fig. 4b and confirm the trend of increasing chain length as a function of increasing temperature.

A similar pattern of geographical and temperature-dependent variability was observed for males, based on 24 CHCs retrieved from the original 27 compounds quantified (Fig. 4c, d). The geographical shift in the relative CHC compositions was significant in the CCA and Monte Carlo test ($P = 0.001$); as was also seen in females, the relatively longer-chain CHCs were observed to increase in abundance with increasing temperature.

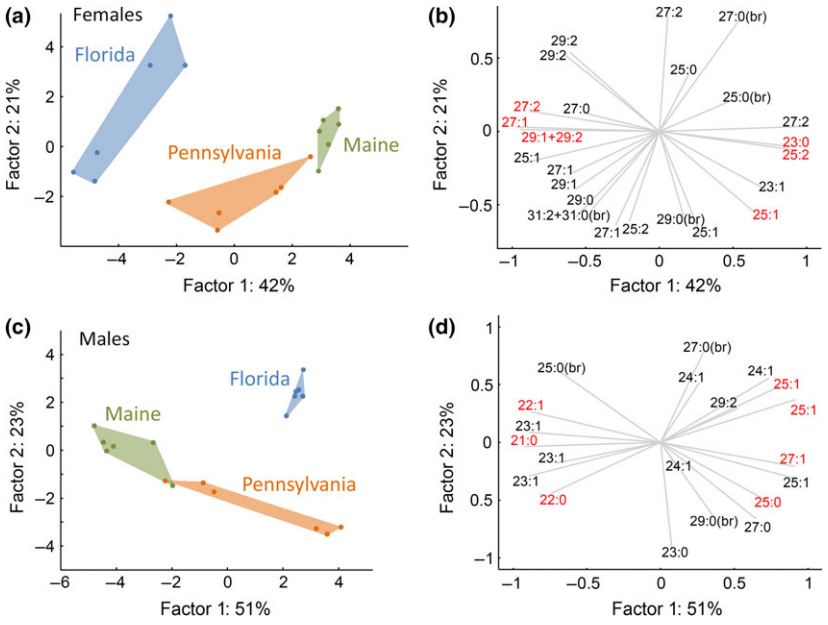

Fig. 3 Projection of principal component analysis (PCA) factor scores and variable correlations with the first two PCA factors of the quantitative patterns of cuticular hydrocarbons (CHCs) at 25 °C in females (a, b) and males (c, d) from the three geographically distinct populations. PCA clearly separated the populations (Florida, Pennsylvania and Maine) and for both sexes, the geographical differences were significant. In both sexes, the variables retrieved to have the best fit with the model predictions were longer-chain CHCs, over-represented in the Florida populations, whereas shorter-chain CHCs were more abundant in the remaining two populations (Pennsylvania and Maine). Variables in red were retrieved as among the most correlated with the Canonical Correspondence Analysis model using locality as a categorical predictor.

Seasonal variation in CHC profiles

Third, we evaluated the seasonal dynamics of male and female CHC patterns in populations derived from spring and fall collections from Media, PA, in two successive years (2013 and 2014). Figure 5a, c represents the projection of scores for the first two PCA factors in females, including 25 of the 42 quantified CHCs. Both year and season clearly influenced the clustering patterns of the samples. Accordingly, when the year of collection was set as a categorical predictor in the CCA, a significant difference between 2013 and 2014 samples was observed (Monte Carlo test, $P = 0.02$). Similarly, when season was selected as categorical predictor, significant differences were observed between early and late season samples: the CHCs with relatively longer chain lengths ((*Z*)-7-heptacosene; (*Z*)-7-nonacosene; *n*-nonacosane; hentriacontadiene + 2-methyltriacontane) were more represented in the fall populations.

For males, 17 of 29 quantified CHCs were included in the subsequent analyses. At first glance, the PCA plot of the factor scores for the first two PCA factors shows a general pattern similar to that of the female samples (Fig. 5b, d). However, the CHC patterns for autumnal samples were more heterogeneous and overlap with the clusters of samples from spring populations. Indeed, although the 2013 and 2014 samples were significantly distinct by CCA and a Monte Carlo test ($P = 0.001$), the effect of season was not significant (Monte Carlo test, $P = 0.197$).

CHC profiles of experimental evolution cages

Fourth, we used an experimental evolution design to test the hypothesis that CHC profiles would evolve and diverge between laboratory control and seasonal field environments. For females, 26 of the 36 quantified variables, corresponding to individual CHCs, were included in the analysis based on their relative abundance. The visual representation of the PCA factor scores for the first two factors (Fig. 6a, b) demonstrates differentiation between the experimental treatments, even though the two groups were slightly overlapping. In addition, the CHC profiles of the control, laboratory populations appeared to be more homogeneous when compared to the seasonally evolving populations. The subsequent CCA demonstrated that the treatments did differ significantly in the quantitative pattern of CHC abundance (Monte Carlo test, $P = 0.007$): the compounds with relatively shorter chain lengths (*n*-heneicosane; *n*-docosane; tricosadiene + 2-methyldocosane; (*Z*,*Z*)-9,13-pentacosadiene; (*Z*,*Z*)-7,11-pentacosadiene)

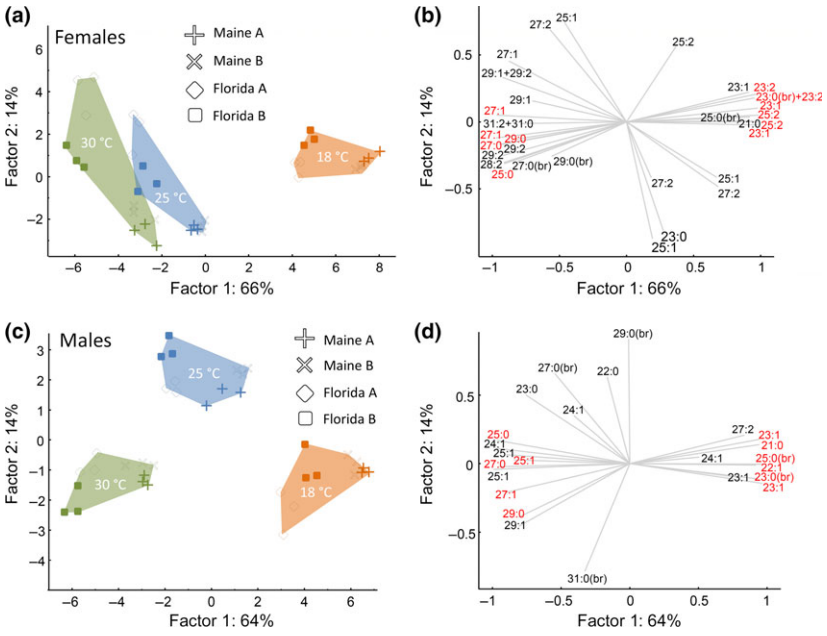

Fig. 4 Projection of principal component analysis (PCA) factor scores and variable correlations with the first two PCA factors, showing the quantitative patterns of cuticular hydrocarbons (CHCs) in female (a, b) and male (c, d) populations from two geographical regions (Florida and Maine) cultured at three different temperatures (18 °C, 25 °C and 30 °C). Variables in red were retrieved as among the most correlated with the Canonical Correspondence Analysis model using temperature as a categorical predictor. Longer-chain CHCs are strongly associated with the shift from lower to higher temperatures. For both sexes, longer-chain CHCs were observed to increase in abundance with increasing temperature.

were characteristic of the control (nonseasonally evolving) populations, whereas the compounds with relatively longer chain lengths ((*Z*)-5-pentacosene; (*Z*, *Z*)-7,11-heptacosadiene; 2-methylhexacosane; (*Z*,*Z*)-5,9-heptacosadiene; (Z)-9-heptacosene) were characteristic of the seasonally evolving populations.

In males, 18 of the 29 CHCs quantified passed the inclusion rules for further analysis. The PCA graphical representation indicated a large overlap in the quantitative diversity of CHCs in seasonally evolving and control populations (Fig. 6c, d). Indeed, the differences in the quantitative patterns were not significant in the subsequent CCA followed by Monte Carlo test (Monte Carlo test, $P = 0.07$). As was observed in the female samples, males from the control treatment appeared to be more homogeneous in the relative quantities of CHCs.

Enrichment and parallelism of SNPs associated CHC profile across time and space

In the last step, we examined whether SNPs associated with CHC chain length profile were enriched for signals of clinal or seasonal variation and whether CHC-associated SNPs varied through time and space in a predictable fashion. We find that SNPs associated with CHC chain length (PC1) were enriched for signals of clinal variation for males (Fig. 7; $\log_2$(odds-ratio) = 0.52 ± 0.11SD, $P < 0.002$) but not females ($\log_2$(odds-ratio) = −0.027 ± 0.14SD, $P = 0.4$). SNPs associated with CHC chain length (PC1) were not significantly enriched for signals of seasonality for either males ($\log_2$(odds-ratio) = −0.212 ± 0.15SD, $P = 0.08$) or females ($\log_2$(odds-ratio) = −0.17 ± 0.21SD, $P = 0.19$). Clinal SNPs associated with CHC PC1 are significantly more likely to vary across the cline in a congruent fashion (i.e. the northern allele tends to be associated with smaller values of CHC PC1 and thus shorter CHCs) for both males ($\log_2$(odds-ratio) = 3.24 ± 0.24SD, $P < 0.002$) and females ($\log_2$(odds-ratio) = 1.68 ± 0.28SD, $P < 0.002$). Seasonal SNPs associated with CHC PC1 showed a nonsignificant trend to vary among seasons in a congruent fashion (i.e. winter alleles tending to be associated with smaller values of CHC PC1) for both males ($\log_2$(odds-ratio) = 0.34 ± 0.29SD, $P = 0.112$) and females ($\log_2$(odds-ratio) = 0.23 ± 0.43SD, $P = 0.3$).

Discussion

CHCs play an important role in terrestrial arthropod ecophysiology (Blomquist & Bagnères, 2010) and affect stress tolerance and behaviour in a variety of taxa including various *Drosophila* species (Gibbs & Pomonis, 1995; Hine *et al.*, 2004; Rouault *et al.*, 2004; Liimatainen & Jallon, 2007; Chenoweth *et al.*, 2008; Alves *et al.*, 2010; Everaerts *et al.*, 2010; Frentiu & Chenoweth, 2010; Gibbs & Rajpurohit, 2010; Sharma *et al.*, 2012; Rajpurohit *et al.*, 2013; Etges & de Oliveira, 2014). Here, we used a variety of natural collections and experiments to examine variation in CHCs as a function of both latitudinal and seasonal origin of source populations, culture temperature and evolutionary change over seasonal time in the field. We observed significant spatiotemporal variation in CHCs, a strong response to growth temperature conditions, and rapid

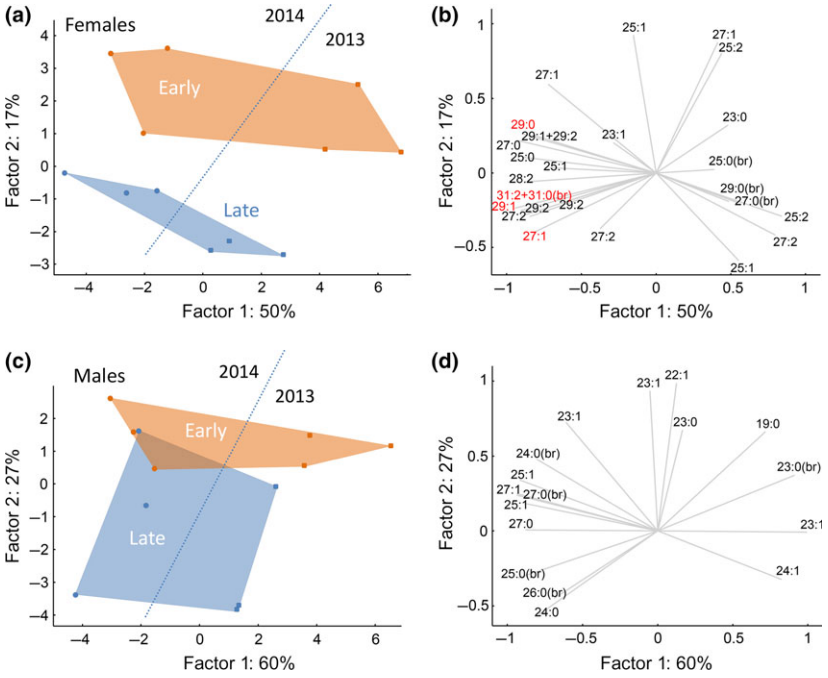

Fig. 5 Projection of principal component analysis (PCA) factor scores and variable correlations with the first two PCA factors, showing the quantitative patterns of cuticular hydrocarbons (CHCs) in female (a, b) and male (c, d) in populations from different years (2013 and 2014) and seasons (early (spring) and late (autumn)). When the year of collection was set as a categorical predictor in the Canonical Correspondence Analysis (CCA), a significant difference between 2013 and 2014 samples was observed. When the season was selected as a categorical predictor, significant differences were observed between early and late season samples in females, in which CHCs with relatively longer chain lengths were more represented in the late season collections. Variables in red were retrieved as among the most correlated with the CCA model using the season as a categorical predictor.

evolution of CHC profiles under natural conditions in *D. melanogaster*. Further, the patterns observed at the phenotypic levels were mirrored by patterns of allele frequency variation for SNPs previously shown to be associated with CHC profiles (Dembeck *et al.*, 2015).

Cuticular hydrocarbons contribute to desiccation resistance in insects by reducing water loss across the insect cuticle (Gibbs, 2002a, b). Previous experimental manipulations have shown that artificial selection for desiccation-resistant *D. melanogaster* results in CHC profiles with higher relative concentrations of long-chained compounds in comparison with unselected controls (Gibbs *et al.*, 1997; Kwan & Rundle, 2009). It has been hypothesized that observed shifts in CHC profiles may be associated with variation in desiccation tolerance in natural populations of *Drosophila* species (Karan *et al.*, 1998; Parkash *et al.*, 2010, 2009; Parkash & Ranga, 2013; but see Parkash, 2010). However, the total CHC profile consists of a blend of many independent compounds; the relative distribution and/or concentration of these compounds, as well as their potential role in desiccation tolerance, remains poorly understood (Hosken *et al.*, 2009; Kalra *et al.*, 2014).

Along the U.S. east coast, average temperature gradually decreases as a function of increasing latitude. In northern temperate habitats, the winter season is harsh and comparatively longer than that experienced in southern, subtropical habitats; seasonal variation in temperature and associated environmental variables is also more pronounced at higher latitudes (Fig. 1b, c). Temperature effects on CHC chain length are well established (Rouault *et al.*, 2004). We hypothesized that exposure of populations to distinct thermal regimes and associated selection pressures would result in predictable, genetically based differences among populations in CHC profiles. Specifically, we predicted longer-chain CHCs in populations exposed to elevated temperature growth conditions, those originating from lower latitudes, and populations collected after multiple generations of exposure to elevated temperature over seasonal time. We further predicted that CHC profiles would evolve and diverge between control, laboratory populations and experimental populations evolving to heterogeneous climatic conditions in the field. We also predicted that these patterns would be evident in genomic characterizations, indicating that spatially and temporally variable selection shapes patterns of allele frequency variation for SNPs in genes that are involved in CHC production.

The data show that there is a parallel shift in CHCs between populations derived from the spring and fall

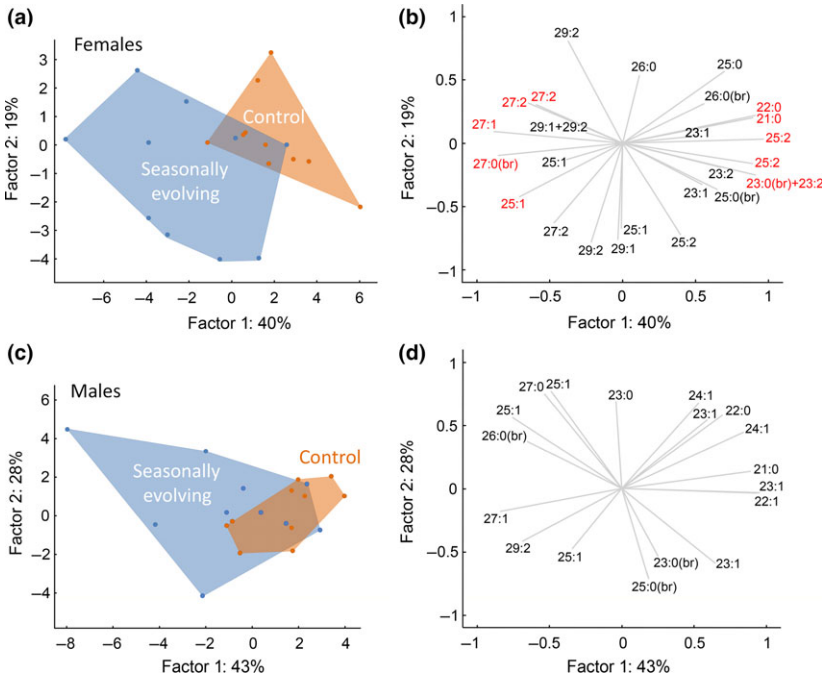

Fig. 6 Projection of principal component analysis (PCA) factor scores and variable correlations with the first two PCA factors of the quantitative patterns of cuticular hydrocarbons (CHCs) in control and evolving populations of females (a, b) and males (c, d). The visual representation suggested a differentiation between the experimental treatments, even though the two groups were slightly overlapping; the treatments differed significantly in CHC profiles for females only. The CHC profiles of the control, laboratory populations were more homogeneous in comparison with the seasonally evolving populations. Variables in red were retrieved as among the most correlated with the Canonical Correspondence Analysis model using treatment as a categorical predictor.

seasons as well as between northern and southern populations; these profiles are distinct and maintained after common garden culture for multiple generations, and thus represent parallel evolutionary divergence. The prediction of rapid evolutionary response in CHC profiles was also supported by the mesocosm field experiments, in which female CHCs diverged between field and laboratory populations cultured under distinct climatic regimes. These observed patterns of evolutionary change in latitudinal and seasonal populations are qualitatively identical to patterns of phenotypic plasticity elucidated under the exposure of experimental flies to different culture temperatures. The parallel responses we observed strongly suggest that the observed patterns of CHC variation in natural *D. melanogaster* populations are adaptive and not random. Furthermore, the common factor of temperature across the various levels of analysis in this study implicates temperature-based selection in generating the observed CHC profiles, although this was not directly tested.

Geographical variation in CHCs

Drosophila melanogaster populations from the east coast of the U.S. showed significant geographical variation where shorter carbon-chained compounds were more abundant in higher latitude Maine and Pennsylvania populations (i.e. temperate climates), whereas the opposite pattern of longer-chained CHCs was observed for the lower latitude, subtropical populations (Fig. 3a–d). Given the clear and contrasting patterns of CHCs, our data demonstrate a latitudinal cline for CHC profiles in *D. melanogaster* populations from the eastern U.S. We observed clinal variation for CHC profiles that is mirrored by patterns of allele frequency variation for SNPs in genes that have been shown to be associated with CHC profiles (Dembeck *et al.*, 2015). This supports the hypothesis that CHC profiles among populations are modulated by selection associated with temperature and desiccation stress in natural habitats. Our findings parallel those of Frentiu & Chenoweth (2010) with respect to CHC profiles and temperature along the environmental gradient in eastern Australia.

Our results are also consistent with earlier work by Rouault *et al.* (2000) where a clear geographical change in (*Z*)-7-pentacosene (males) and (*Z*)-7-tricosene (females) was observed. The ratio between these alkenes ((*Z*)-7-tricosene: (*Z*)-7-pentacosene) is known to decrease with latitude in *D. melanogaster* males (Rouault *et al.*, 2000). Many of these CHCs also serve as pheromones. A clear difference between (*Z*)-7-pentacosene and (*Z*)-7-tricosene in natural populations (Florida vs. Maine) of *D. melanogaster* clearly indicated varying pheromone composition (see results). (*Z*)-7-tricosene is a major male pheromone in *D. melanogaster*, and therefore natural selection effects on CHC ratios could affect male attractiveness, potentially leading to reproductive isolation in natural populations (Grillet *et al.*, 2012).

Thermal plasticity in CHCs

Cuticular lipid composition is influenced by a series of environmental factors including both temperature and diet (Ohtsu *et al.*, 1988; Howard, 1993; Howard *et al.*, 1995). In our experiments, culturing populations at three different temperatures (18 °C, 25 °C and 30 °C)

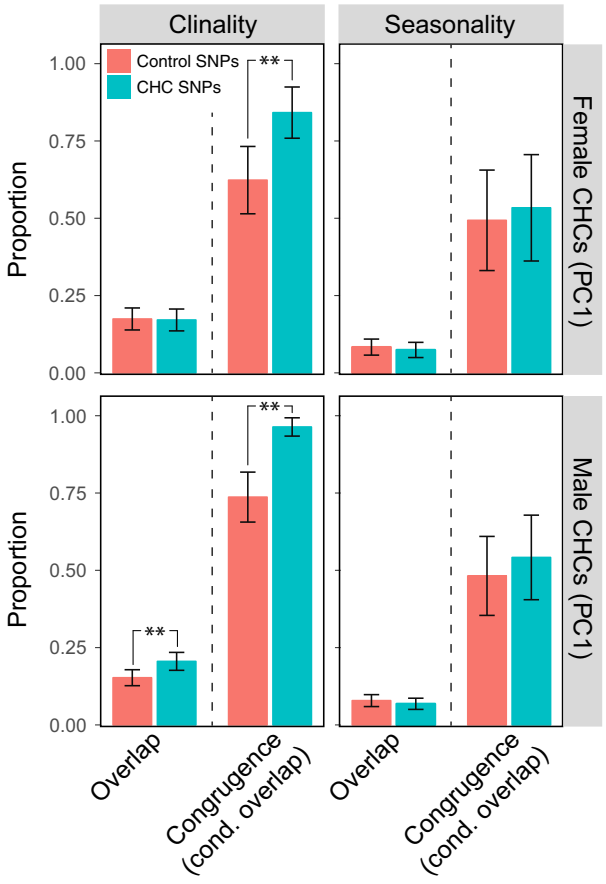

Fig. 7 Patterns of clinal and seasonal variation at SNPs associated with cuticular hydrocarbon (CHC) profiles and their genomic controls. Examination of overlap is based on co-occurrence of CHC SNPs among SNPs previously identified as clinal or seasonal (see Materials and Methods for details). Congruence of the direction of SNP effect in a genomewide association study was examined at SNPs that were both significantly associated with CHC PC1 and varied through time or space. ** corresponds to bootstrap *P*-value <0.01.

produced distinct CHC profiles (Fig. 4a, c). Specifically, exposure to higher temperatures during development was associated with higher concentrations of longer-chain CHCs. Our observations are consistent with previous results (Gibbs *et al.*, 1998) and further place these observations in a broad ecological context.

Seasonal variation in CHCs

In natural populations of insects, clines in fitness traits have been widely observed (Schmidt *et al.*, 2005; Hoffmann & Weeks, 2007; Rajpurohit & Nedved, 2013), but seasonal variation has received less attention (Hadley, 1977; Toolson & Hadley, 1979). Here, we observed significant and repeatable differences between early and late season populations: CHCs with relatively longer chain lengths were more represented in the late season collections and CHCs with relatively shorter chains were more characteristic of the early season collections (Fig. 5a–d). Interestingly, these patterns match the observed cline and associations with developmental temperature (Figs 3 and 4). In orchards of the north-eastern United States, *D. melanogaster* population density fluctuates seasonally, reaching peak abundance in late summer followed by a pronounced crash in fall (Ives, 1970). Recent work in these habitats has demonstrated that these populations experience rapid adaptive responses to seasonality in replicate years. This occurs over approximately 10–15 generations (June to November), and results in (i) a genomic signature of seasonal adaptation, in which a nonrandom set of molecular variants cycle in frequency with season across three replicate years (Cogni *et al.*, 2013; Bergland *et al.*, 2014; Lavington *et al.*, 2014); and (ii) predictable change in life histories (Schmidt & Conde, 2006; Behrman *et al.*, 2015). This rapid response to seasonality parallels previously observed patterns of geographical differentiation for fitness traits (e.g. Hoffmann *et al.*, 2003; Schmidt & Paaby, 2008) and allele frequencies (Kolaczkowski *et al.*, 2011; Fabian *et al.*, 2012; Bergland *et al.*, 2014) that have been attributed to climatic adaptation.

Rapid evolution of CHCs in the field

The genetic basis of CHCs (Pardy, 2012; Dembeck *et al.*, 2015) and their association with fitness suggests that CHC profiles may respond adaptively to environmental variation in natural habitats. This hypothesis is supported by observed latitudinal patterns, and the differentiation in CHC profiles between spring and fall populations further suggests that this evolutionary response may be rapid and dynamic. It had never been tested whether CHCs evolve over the short timescales when temperature and humidity change rapidly and predictably with seasonality. Our field-based experimental evolution study addressed this directly. We used an innovative egg replacement approach to experimentally prevent seasonal evolution in fly populations growing in replicate tree-scale mesocosms and then compared population dynamics in this treatment to dynamics in mesocosms where flies evolve over the season. We hypothesized that exposure to temporally heterogeneous field conditions would result in predictable patterns of evolutionary change in CHC profiles relative to those in a constant, nonseasonal environment. We observed that CHC profiles of control (nonseasonally evolving) populations were more homogeneous in composition and were characterized by compounds with relatively shorter chain lengths. In the field mesocosms in which flies evolved to environmental conditions that were highly variable both over diurnal and seasonal time, CHC profiles were more heterogeneous and demonstrated an evolutionary shift towards longer chain lengths. These findings

demonstrate the rapid, replicated and predictable evolution of CHC profiles in the field.

Conclusions

In this study, we documented significant differences in CHCs at all five levels investigated: spatial and temporal variation in CHC profiles as well as genomewide allele frequencies for SNPs associated with CHC profiles, thermal plasticity and experimental evolutionary response. Any of the five analyses, by itself, presents a weak picture of correlation between a specific factor and CHC profiles. However, examining associations between CHC profiles and environmental variables at multiple, independent levels provides a more powerful and robust analysis. We observed a striking degree of parallelism across these levels of analysis, in which CHC profiles varied predictably based on known associations between chain length, environmental variables and ecological function. Taken together, our results suggest that patterns of variation in CHC profiles in North American populations of *D. melanogaster* are generated by selection pressures that vary with climate and temperature, and represent one aspect of physiological adaptation to environmental heterogeneity.

Acknowledgments

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