J Chem Ecol (2013) 39:579–590
DOI 10.1007/s10886-013-0284-1

Epicuticular Compounds of *Drosophila subquinaria* and *D. recens*: Identification, Quantification, and Their Role in Female Mate Choice

Sharon Curtis · Jacqueline L. Sztepanacz · Brooke E. White · Kelly A. Dyer · Howard D. Rundle · Paul Mayer

Received: 22 November 2012 / Revised: 4 February 2013 / Accepted: 24 March 2013 / Published online: 19 April 2013

Abstract The epicuticle of various *Drosophila* species consists of long-chain cuticular hydrocarbons (CHCs) and their derivatives that play a role in waterproofing and a dynamic means of chemical communication. Here, *via* gas chromatography and mass spectrometry, we identified and quantified the epicuticular composition of *D. recens* and *D. subquinaria*, two closely related species that show a pattern of reproductive character displacement in nature. Twenty-four compounds were identified with the most abundant, 11-*cis*-Vaccenyl acetate, present only in males of each species. Also exclusive to males were five tri-acylglycerides. The 18 remaining compounds were CHCs, all shared between the sexes and species. These CHCs were composed of odd carbon numbers (C_{29}, C_{31}, C_{33}, and C_{35}), with an increase in structural isomers in the C_{33} and C_{35} groups. Saturated hydrocarbons comprise only methyl-branched alkanes and were found only in the C_{29} and C_{31} groups. Alkenes were the least prevalent, with alkadienes dominating the chromatographic landscape in the longer chain lengths. Sexual dimorphism was extensive with 6/8 of the log-contrast CHCs differing significantly in relative concentration between males and females in *D. recens* and *D. subquinaria,* respectively. Males of the two species also differed significantly in relative concentration of six CHCs, while females differed in none. Female-choice mating trials revealed directional sexual selection on male CHCs in a population of each species, consistent with female mate preferences for these traits. The sexual selection vectors differed significantly in multivariate trait space, suggesting that different pheromone blends determine male attractiveness in each species.

Keywords *Cis*-vaccenyl acetate · Cuticular hydrocarbons · Tri-acylglycerides · Gas chromatography · Pheromones · Sexual selection

Electronic supplementary material The online version of this article (doi:10.1007/s10886-013-0284-1) contains supplementary material, which is available to authorized users.

S. Curtis · P. Mayer
Department of Chemistry, University of Ottawa, Ottawa, ON K1N 6N5, Canada

J. L. Sztepanacz · H. D. Rundle (✉)
Department of Biology, University of Ottawa, Ottawa, ON K1N 6N5, Canada
e-mail: hrundle@uottawa.ca

B. E. White · K. A. Dyer
Department of Genetics, University of Georgia, Athens, GA 30602, USA

Present Address:
J. L. Sztepanacz
School of Biological Sciences, University of Queensland, Brisbane QLD 4072, Australia

Introduction

The epicuticle of various insect species was first characterized as a waxy layer with waterproofing properties (Beament, 1945; Wigglesworth, 1945). In *Drosophila* in particular, a variety of studies have implicated epicuticular hydrocarbons (often called cuticular hydrocarbons, or CHCs) with variation in desiccation resistance (Toolson and Kuper-Simbron, 1989; Gibbs et al., 1997; Gibbs, 1998; Rouault et al., 2000; Howard and Blomquist, 2005; Kwan and Rundle, 2009). More recently, the role of the epicuticle as a dynamic means of chemical

communication was also recognized (Jallon, 1984; Markow and Toolson, 1990; Ferveur, 1997; Blows and Allan, 1998; Etges and Ahrens, 2001; Grillet et al., 2006). In a few species of *Drosophila*, epicuticular contact pheromones have been implicated in mate choice (Chenoweth and Blows, 2005; Grillet et al., 2006; Van Homrigh et al., 2007), sex recognition (e.g., Savarit and Ferveur, 2002; Billeter et al., 2009; Fernández et al., 2010), and intraspecific group recognition (e.g., Fang et al., 2002), as well as in species recognition/sexual isolation (e.g., Coyne et al., 1994; Billeter et al., 2009). Hydrocarbon expression has been shown to be plastic with respect to age, mating status, diet, and social environment (Petfield et al., 2005; Kent et al., 2008; Krupp et al., 2008; Everaerts et al., 2010; Yew et al., 2011), and also can rapidly evolve when selection is altered (e.g., Blows, 2002; Chenoweth et al., 2008; Kwan and Rundle, 2009; Sharma et al., 2012).

Here, we investigated the epicuticular composition of two closely related species: *Drosophila subquinaria* and *D. recens*. These species occur in western and eastern North America, respectively, and their ranges overlap for about 1,200 km in central Canada. Reproductive character displacement exists in *D. subquinaria*: while *D. subquinaria* females from populations where both species occur discriminate against *D. recens* males, *D. subquinaria* females from outside this region of sympatry do not (Jaenike et al., 2006). Moreover, these choosy *D. subquinaria* females from sympatry also discriminate, although to a lesser extent, against allopatric males of their own species, suggesting the existence of incipient stages of reproductive isolation within *D. subquinaria*. *Drosophila recens* shows neither of these patterns (Jaenike et al., 2006). A lack of genetic differentiation among sympatric and nearby allopatric *D. subquinaria* populations suggests these patterns of between and within-species mate discrimination are the result of a history of reinforcing selection on *D. subquinaria* females to avoid mating with *D. recens* males, which as a by-product may be causing a secondary speciation event between allopatric and sympatric *D. subquinaria* (Jaenike et al., 2006). This pattern is consistent with low offspring survival from *D. subquinaria* female × *D. recens* male crosses due to interspecific cytoplasmic incompatibility arising from the presence of a *Wolbachia* infection in *D. recens* but not *D. subquinaria* (Shoemaker et al., 1999).

Drosophila subquinaria and *D. recens* are morphologically indistinguishable with the exception of the male genitalia (Wheeler, 1960), and there are no sex-specific differences in pigmentation. There also are no known differences in their local ecology: both species exclusively utilize mushrooms as sites for mating and oviposition, and both species can be found together on the same mushrooms in the geographic areas of overlap. With the long-term goal of understanding the mechanisms by which signal traits and female preferences have diverged to generate the existing reproductive character displacement in mate discrimination, and how this is contributing to the early stages of behavioral isolation within *D. subquinaria,* it is necessary to identify the male traits underlying female mate discrimination within and between these species. Chemosensory signals are implicated by a recent study that ablated different sensory modalities and showed that in *D. subquinaria* females, the antennae, which are necessary for olfaction, are critical for mating, whereas causing these females to be deaf or blind had no effect (Giglio and Dyer, 2013). Here, we further investigated the potential role for chemosensory signals in species identification and mate discrimination. First, we identified the epicuticular compounds present in male and female *D. subquinaria* and *D. recens*, and then quantitatively compared their abundances between sexes and species. Finally, we quantified sexual selection on these traits in males of each species arising from female mate preferences in female-choice mating trials, and compared this selection between species.

Methods and Materials

Fly Strains and Culturing Flies for use in the chemical identification of the epicuticular compounds were collected during 2009 and 2010 from multiple populations from both sympatric and allopatric locations. Detailed analyses of approximately 30 individuals of each sex from each of 12 populations that were collected along a transect spanning the range of both of these species indicate that all compounds identified below are found in all populations (H. Rundle and K. Dyer, unpublished data). Individuals used in quantifying the abundances of the various compounds, and for use in the mating trials, were collected in 2008–2010 from a single allopatric population of each species: the Great Smoky Mountains, Tennessee (*D. recens*) and Deary, Idaho (*D. subquinaria*). These are repeat collections from sites described in Jaenike et al. (2006). Fly cultures were maintained on Instant *Drosophila* food (Carolina Biological, Burlington NC, USA) supplemented with commercial mushroom (*Agaricus bisporus*) and reared in an incubator set to 20 °C on a 14:10 l:D cycle and 60% relative humidity.

Chemical Identification Epicuticular compounds were extracted from single flies by placing individuals separately in 100 μl of hexane in a 500 μl insert (6 mm MicroSert from National Scientific, Rockwood, TN, USA) within a standard 2 ml autosampler vial. Individuals sat for 3 min and were then vortexed for 1 min before the fly was removed. Unless otherwise noted, all individuals were 7–9 day-old virgins. All extractions were completed in the morning within 3 h of incubator lights on. Extractions were stored at −20 °C and were subsequently shipped from Athens, GA, USA, to

Ottawa, ON, CA, for analysis. In addition to these individual extractions, mass spectrometry also was performed on some pooled samples in which 30 individuals were extracted together in a single vial containing 400 μl of hexane.

Gas chromatography with flame ionization detection (GC:FID) was performed on single-fly extractions only using a dual-channel Agilent Technologies 6890N gas chromatograph (GC) with a fast oven (198–231 V power supply), fitted with an HP-5 5% phenyl methyl siloxane column of 30 m×0.25 mm internal diam, pulsed splitless inlets (at 200 °C with a pulse of 206.8 kPa (30 psi) for the first 1.4 min). The flame ionization detectors were at 310 °C. Hydrogen was used as the carrier gas with a constant column flow of 2 ml/min The temperature program began by holding at 140 °C for 0.55 min, increasing to 230 °C at a rate of 120 °C/min, holding at 230 °C for 0 min, then increasing to 310 °C at a rate of 18 °C/min and finally holding at 310 °C for 2 min. While the run-time of this method is short, the parameters were selected *via* an extensive optimization procedure that sought to maximize efficiency while maintaining resolution (i.e., so-called 'fast GC'); slower temperature ramps and longer run-times did not improve resolution appreciably, likely because some of the compounds are very similar (see Results). From these data, individual epicuticular profiles were determined by integration of the area under 18 peaks in females and 24 peaks in males, representing all those that were reliably present (with the exception of FID peak #8, which was sometimes absent) and corresponding to those identified in the mass spectrometry analyses below.

Gas chromatography with mass spectrometry (GC/MS) was performed on both single-fly and pooled extractions using an Agilent 7820A GC with 5975 series mass selective detector, fitted with an HP-5 5% phenyl methyl siloxane column of 30 m×0.25 mm internal diam, and employing Helium as the carrier gas. To facilitate the comparison of retention times between the GC/FID and GC/MS chromatographic profiles, method parameters from the GC/FID analysis were converted for the GC/MS machine using the 'mxlator' v. 2.0a GC method translation software (Hewlett-Packard Company), available for free download from the Agilent Technologies (Santa Clara, CA, USA) website. Helium flow rate was 2 ml/min with a column pressure of 166.9 kPa (24.2 psi). The temperature program began at 140 °C and held for 1 min, then ramped to 230 °C over 2 min, followed by a slower ramp to 310 °C over 14.5 min. The sample inlet valve was set to splitless with an injection pulse pressure of 206.8 kPa (30 psi). The GC/MS detector remained off until 3 min into the run, thus avoiding the solvent peak. A 2 μl sample injection volume produced sufficient molecular ions for study of the pooled (30 fly) concentrated samples, and a 6 μl sample volume for the single fly extractions. In some cases in which GC:MS data were inconclusive due to low signal intensity, an additional 1 μl injection was used from single fly samples that had been evaporated and then reconstituted in 2 μl of hexane.

Equivalent chain length (ECL) values were calculated for all compounds on both the GC/FID and GC/MS instruments by comparison to a standard C_7-C_{40} saturated *n*-alkane mixture (Sigma Aldrich, product # 49452-U). ECL values were calculated by interpolation, assuming a linear relationship between the two *n*-alkane standards with retention times immediately before and after each sample peak (Miwa, 1963; Mjøs, 2006). These ECL numbers were cross referenced between the two instruments to ensure that the identification of all epicuticular compounds corresponded between them.

The identification of the class of epicuticular hydrocarbon was established from the presence of molecular ions in their chromatographic peaks. Individual compounds were identified by their mass spectrum fragmentation pattern and, when present in the NIST 11 mass spectral database (http://www.sisweb.com/software/ms/nist.htm) and/or the Wiley275 GC/MD database, by a direct comparison with these. When available in the literature, identifications also were confirmed by comparison of ECL data with previously published values for the given compound from other species (Howard et al., 1978, 2003; Carlson and Yocom, 1986; Everaerts et al., 2010) following Bartelt et al. (1986) and Carlson et al. (1998). Provisional identifications are given in certain cases in which the above methods provided a reasonable assignment but unequivocal confirmation was lacking, for example with respect to the positioning of the double bond within certain hydrocarbons, especially alkenes (denoted as *n*-hydrocarbons). In addition to the hydrocarbons, the presence of 11-*cis*-Vaccenyl acetate (cVa) and triacylglycerides (TAGs) within the male samples was achieved *via* comparison of mass spectrum fragmentation patterns with the Wiley275 GC:MD database, and *via* a glycerol trihexanoate standard (Sigma Aldrich T0888) used to provide comparative ECL data and MS fragmentation pattern for the TAGs.

Derivatization and Analysis of Free Fatty Acids Single-fly samples were evaporated and reconstituted in 20 μl *Bis(trimethylsilyl)trifluoroacetamide* and 1% *Trimethylchlorosilane* (Regis Technologies, Inc., Morton Grove, IL, USA), then heated to 70 °C for 5 min to derivatize the fatty acids. The resulting samples were analyzed on a Hewlett Packard 6880 series GC with a 5973 series mass selective detector, fitted with an HP1909A-102 column of 25 m×200 μm internal diam (0.33 μm capillary) using helium as the carrier gas. Two different oven gradients were employed. For elution of the smaller free fatty acids of >10 carbons, the lower temperature gradient ran from 40 °C to 300 °C at 10 °C/min,

with a helium flow rate of 0.9 ml/min, velocity of 37 cm/sec and 100.9 kPa (14.6 psi) pressure. The higher temperature gradient ran from 150 °C to 300 °C at 10 °C/min, with a helium flow rate of 0.5 ml/min, velocity 31cm/sec, and 100.9 kPa (14.6 psi) pressure.

Compound Abundances Abundances of the individual chemical compounds were obtained for males and females of each species from a separate GC/FID analysis of a total of 33/28 male and 32/29 female single-fly extractions for *D. recens* (Great Smoky Mountains, Tennessee) and *D. subquinaria* (Deary, Idaho), respectively. The areas under the same 24 (males) / 18 (females) peaks were integrated. To convert these areas to known abundances, and to correct for decreasing sensitivity of the GC/FID with increasing molecular mass of the compounds (caused primarily by differences in ionization potential), concentration gradients were derived for 0.5, 1, and 2 ng/μl samples of a C_7-C_{40} saturated alkane mixture. The abundance of each compound was then calculated using a linear calibration curve derived from the alkanes with retention times immediately before and after each sample peak. This method assumes that the ionization cross-sections of the two alkanes were similar to that of each sample compound. While this is reasonable for hydrocarbons, it is less accurate for the oxygen-containing compounds and the abundances of the tri-acylglycerides and cVA should be considered approximations. Final abundances were calculated as the average, among-individuals of a given sex and species, of these adjusted values (± standard error).

Total abundances quantified *via* gas chromatography can be subject to substantial technical error. To reduce such experimental error, abundances were expressed as proportions of the total concentration of all compounds for a given individual (i.e., as relative abundances), thereby removing among-fly variation in total CHC content. Individual proportions were then logcontrast transformed to remove the unit-sum constraint characteristic of such compositional data (Atchison, 1986), using the proportional area of FID compound #2 (2-methyl octacosane) as the common divisor, thereby permitting multivariate analyses to be performed. FID compound #8, a very low concentration CHC that was undetectable in many individuals, was excluded from these analyses because logcontrast values were undefined for individuals lacking this trait. Overall differences in relative abundances of all CHCs shared between the sexes were tested separately for each species using a multivariate analysis of variation (MANOVA), followed by individual tests of each compound using *Welch's t-test* for samples with unequal variance. Tests for differences between the species (separately by sex) for all compounds followed the same approach, employing a MANOVA first, and if significant, subsequent *Welch's t-test* of the individual compounds.

Mate Choice Trials To provide insight into the role of these epicuticular compounds as potential sexual display pheromones, we conducted a series of replicate binomial female choice mating trials separately for each species. We constructed a stock population of each species by pooling equal numbers of males and females from 10 isofemale lines from the Tennessee population of *D. recens*, and five isofemale lines from the Idaho population of *D. subquinaria*. These stocks were mass bred for three generations before being used in the mating trials in order to reduce any linkage disequilibrium that may have been present. In each mate choice trial, a single virgin female was allowed to choose between two virgin conspecific males from the same population and species. All flies were 7–9 day post-eclosion and were aspirated without anesthesia into the mating chamber. Individuals were observed and once copulation began, flies were anaesthetized using CO_2 and either the male that was chosen by the female (50% of the time, randomly determined) or the male that was rejected by the female (the remaining 50% of the time) was removed for subsequent extraction as described above. A total of 132 *D. recens* and 160 *D. subquinaria* males were extracted in this way.

Extractions from chosen and rejected males were analyzed *via* GC/FID as described above. For each individual male, the area under the same 24 peaks was integrated. However, previously published results from other species indicate that cVA and tri-acylglycerides can transfer from males to females during mating (see Discussion). Consistent with this, mated (i.e., chosen) males had lower concentrations of all six of these compounds compared to rejected males (unpublished data). Therefore, to avoid confounding such changes with female choice for these traits, we excluded these compounds from the sexual selection analyses. FID peak 8, a very low concentration CHC, which was undetectable in many individuals, also was excluded as subsequent data transformations (see below) were undefined for zero values.

Relative concentrations of the remaining 17 CHCs were determined by expressing each compound as a proportion of the total concentration for the given individual. Individual proportions again were logcontrast transformed as previously described, using the proportional area of FID compound #2 (2-methyl octacosane) as the common divisor. This yielded 16 logcontrast CHCs characterizing variation among individuals in relative concentrations. Prior to analysis, 11 multivariate outliers were identified and removed (6 *D. recens* and 5 *D. subquinaria* individuals) using the Mahalanobis distance technique described in Sall et al. (2005) and implemented in the multivariate package of JMP v. 10.0.1 (SAS Institute, Cary, NC, USA). Results do not change qualitatively if these values are included.

There was some multicolinearity among the 16 logcontrast CHCs, primarily in *D. subquinaria* (maximum variance

inflation factor, *D. recens*: 9.8; *D. subquinaria*: 34.9). While the presence of multicolinearity does not alter the overall significance of sexual selection, nor its explanatory ability in terms of male mating success (i.e., r^2 values), it does affect how this selection is apportioned among the analyzed traits, making the estimates of the individual gradients unreliable (Mitchell-Olds and Shaw, 1987). We, therefore, scored individuals for the principal components of the covariance matrix of logcontrast CHCs as calculated across all individuals (i.e., pooling the two species). Although performing a principal components analysis separately by species would entirely eliminate multicolinearity within each, our across-species approach sufficiently reduced it (maximum variance inflation factor, *D. recens*: 2.3; *D. subquinaria*: 8.2) while allowing selection to be estimated on the same set of traits within each species. Preserving the same traits across species is necessary if one wishes to compare and statistically test for differences in sexual selection between the species (see below).

Standardized sexual selection gradients (i.e., β vectors) were calculated *via* first-order multiple regression (Lande and Arnold, 1983) of relative mating success on the 16 principal components (each standardized to mean = 0, SD = 1). Because mating success is binomially distributed, significance testing was performed using logistic multiple regression, fit *via* maximum likelihood, using the Genmod procedure in SAS v. 9.3 (Fairbairn and Preziosi, 1996; Rundle et al., 2009). A difference in sexual selection between the species was tested using a sequential model-building approach, as described in Chenoweth et al. (2012). This approach determines whether estimating separate sexual selection gradients in the two species significantly improves the fit of a model compared to one that constrains sexual selection to be the same in each. The two logistic multiple regression models were fit *via* maximum likelihood, employing the Genmod procedure in SAS, with the full model including the 16 trait (i.e., CHC principal components) × species interactions to allow the separate partial regression coefficients on these traits in each species. A likelihood ratio test was used to compare this model to a reduced one lacking these interactions and thus constraining sexual selection to be the same in the two species.

Results

Chemical Identification of Epicuticular Compounds Eighteen/24 epicuticular compounds were identified in females and males, respectively, in both *D. recens* and *D. subquinaria* (Table 1; Figs. 1 and 2; see Online Resource 1 for structural elucidation). The most abundant of these, which was present only in males of each species, was identified as 11-*cis*-Vaccenyl acetate (cVa). Eighteen of the remaining compounds were hydrocarbons (CHCs), ranging from C_{28} to C_{35}, with each being present in males and females of both species. None of these CHCs co-eluted with the standard C_7-C_{40} saturated *n*-alkane mixture, demonstrating the absence of any straight-chain alkanes. Hydrocarbon peaks clustered around the odd carbon numbers, from C_{29}-C_{35}, with an increasing complexity of closely separated peaks at higher ECLs (i.e., C_{33} and C_{35}). The C_{29} and C_{31} clusters were similar in identity, with the respective 2-methyl alkane dominating in intensity. The C_{33} and C_{35} clusters peaks were also similar, dominated by a homologous series of 5–11 and 5-13-alkadienes, along with the 5-alkene.

Analyses also revealed five additional compounds, present only in males, provisionally identified as tri-acylglycerides. Derivatization by TMS revealed the presence of free fatty acids in both sexes and species. Lauric acid (C_{12}), myristic acid (C_{14}), palmitic acid (C_{16}), palmitoleic acid ($C_{16:1}$), oleic acid ($C_{18:1}$), and stearic acid (C_{18}) were found in all the samples, and capric acid (C_{10}), undecycllic acid (C_{11}), and undecyclenic acid ($C_{11:1}$) were present only in males. There was no evidence of smaller free fatty acids. The presence of the C_{10} and C_{11} free fatty acids only in males is consistent with the presence of tri-acylglycerides in this sex only. Samples from males also suggested odd carbon-number free fatty acids, C_{15}, C_{17}, and C_{19}, in small amounts. The presence of additional free fatty acids in both sexes suggests that these small tri-acylglycerides are likely accompanied by larger molecular weight tri-acylglycerides.

Differences in Abundance Between Sexes and Species Although all CHCs were shared between the sexes and species, there was substantial variation in absolute abundances (Table 2). Sexual dimorphism in CHC relative abundance also was extensive, with a significant difference between males and females overall in both *D. recens* (MANOVA: $F_{16,48}=12.3$, $P<0.001$) and *D. subquinaria* (MANOVA: $F_{16,40}=31.1$, $P<0.001$). In *D. recens,* five individual logcontrast CHCs differed significantly, and these were scattered across the range ECL values (Fig. 3). In *D. subquinaria*, eight logcontrast CHCs differed significantly between males and females, with the differences again being distributed across most of the range of ECL values (Fig. 3). The identity of the sexually dimorphic hydrocarbons did not necessarily correspond between species.

Between species, relative abundances of the suite of epicuticular compounds also varied overall in males (MANOVA: $F_{22,38}=135.2$, $P<0.001$), with significant differences detected in six of the individual logcontrast CHCs (Fig. 4). Again, the compounds differing between species were scattered across the range of ECL values.

Table 1 Epicuticular compounds of *Drosophila. recens* and *D. subquinaria*

FID #[a]	ECL[b]	Identification	Molecular formula	Method[c]	Notes
Acetates					
1	21.9	11-*cis*-Vaccenyl acetate (cVa)	$C_{20}H_{38}O_2$	L,MSL,MS	Males only
Hydrocarbons					
2	28.62	2-methyl octacosane	2-Me-C_{28}	L,MSL,MS	
3	28.78	(*Z*,*Z*)-5-9-nonacosadiene and 7-nonacosene	$C_{29:2}$, $C_{29:1}$	L,MS (P)	CHCs co-elute on GC: FID
4	28.85	5-nonacosene	$C_{29:1}$	L,MS	
5	30.66	2-methyl triacontane	2-Me-C_{30}	L,MS	
6	30.74	(*Z*,*Z*)-5-11-hentriacontadiene	$C_{31:2}$	MS (P)	
7	30.81	5-hentriacontene	$C_{31:1}$	L,MS	
8	30.88	hentria-*n*-*n*-contadiene	$C_{31:2}$	MS	
9	32.4	*n*-methyl dotriacontane	*n*-Me-C_{32}	L,MS	Methyl group is internal
10	32.47	*n*-tritriacontene	$C_{33:1}$	MS	Double bond is internal
11	32.56	*n*-tritriacontene	$C_{33:1}$	MS	Double bond is internal
12	32.66	(*Z*,*Z*)-5-13-tritriacontadiene	$C_{33:2}$	MS (P)	
13	32.74	(*Z*,*Z*)-5-11-tritriacontadiene	$C_{33:2}$	MS (P)	
14	32.83	(*Z*,*Z*)-*n*-*n*-tritriacontadiene	$C_{33:2}$	MS	
17	34.4	*n*-methyl tetatriacontane	*n*-Me-C_{34}	L	Methyl group is internal
18	34.47	*n*-pentatriacontene	$C_{35:1}$	MS	Double bond is internal
19	34.56	(*Z*,*Z*)-*n*-*n*-pentatriacontadiene	$C_{35:2}$	MS	
20	34.66	(*Z*,*Z*)-5-13-pentatriacontadiene	$C_{35:2}$	MS (P)	
21	34.74	(*Z*,*Z*)-5-11-pentatriacontadiene	$C_{35:2}$	MS (P)	
Tri-acylglycerides					
15	33.46	TAG		MS,S,L (P)	Males only
16	33.58	TAG		MS,S,L (P)	Males only
16a	33.72	TAG		MS,S,L (P)	Males only, not integrated on GC:FID
22	35.35	TAG		MS,S,L (P)	Males only
23	35.44	TAG		MS,S,L (P)	Males only
24	35.56	TAG		MS,S,L (P)	Males only

[a] As given in Figs. 1 and 2

[b] Equivalent chain length

[c] Methods of compound identification: comparison of ECL values with published literature (L; see Methods); comparison of mass spectrum fragmentation data with the NIST and/or Wiley275 GC:MS databases (MSL); mass spectral fragmentation patterns (MS); comparison to standards (S). Provisional identification (P), see Online Resource 1 for details

In contrast to males, in females CHC relative abundance did not differ overall between species (MANOVA: $F_{16,45}=1.45$, $P=0.161$).

Mate Choice Trials Sexual selection on the principal components (PCs) of CHCs in males was highly significant overall in both species (likelihood ratio test, *D. recens*: $\chi^2=53.5$, *d.f.* = 16, $P<0.001$; *D. subquinaria,* $\chi^2=39.4$, *d.f.*= 16, $P<0.001$), with variation among males in their CHCs explaining 25.6% and 13.5% (r^2_{adjusted}) of the variance mating success in *D. recens* and *D. subquinaria,* respectively. Individual selection gradients were significant on 8/5 principal components in *D. recens* and *D. subquinaria,* respectively (Table 3), and together with a complex pattern of loadings (Table S2) made the interpretation of this selection with respect to the individual logcontrast compounds difficult. However, the vectors of directional sexual selection (i.e., β's) were oriented 72.5° away from one another in the two species, suggesting substantial differences in how sexual selection acts on these CHCs that are consistent with strong differences in female mate preferences between the species. This difference in sexual selection is significant, as demonstrated by the better fit of a model that permits separate directional selection gradients to be estimated in each

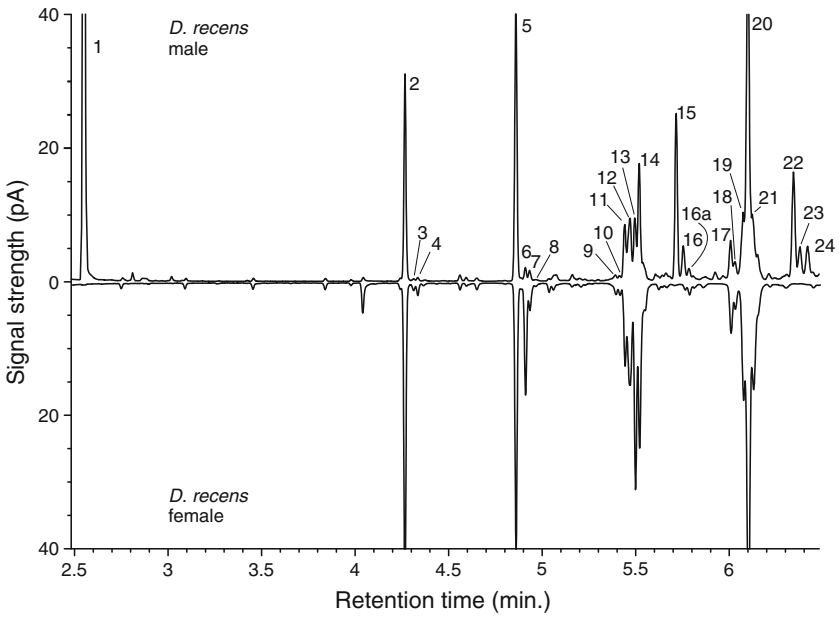

Fig. 1 Mirrored GC/FID chromatographic profile of a male (*above*) and female (*below*) *Drosophila recens*. *Numbers* indicate those compounds that were reliably present and are identified in Table 1

species compared to one that constrains selection to be the same (likelihood ratio test, χ^2=41.3, *d.f.*= 16, P<0.001).

Discussion

A critical first step in understanding the origins of behavioral isolation in general, and the evolution of an existing pattern of reproductive character displacement in particular, is to identify the signals that underlie species discrimination and mate choice. Here, we identified a suite of epicuticular compounds and their derivatives in the closely related *D. subquinaria* and *D. recens*, and characterized qualitative and quantitative differences in these compounds between the sexes and the species. We also showed that sexual selection on these compounds in males is strong within a population of each species, and that it differs between them. Our results, together with a recent manipulation of sensory systems that implicates olfaction in female mate choice (Giglio and Dyer, 2013), suggest that these epicuticular compounds may serve as important sexual display pheromones in males, and may, therefore, be targets of sexual selection arising from female mate preferences.

Our analyses revealed 18 cuticular hydrocarbons (CHCs) shared between both sexes and species, ranging in carbon chain length from C_{28} to C_{35}, and composed predominantly of alkadienes, followed by methyl alkanes, and then

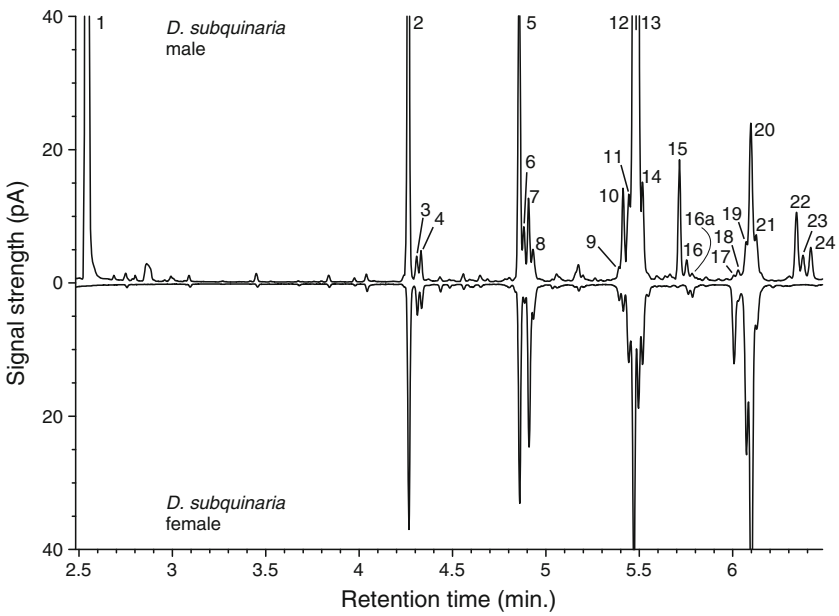

Fig. 2 Mirrored GC/FID chromatographic profile of a male (*above*) and female (*below*) *Drosophila subquinaria*. *Numbers* indicate those compounds that were reliably present and are identified in Table 1

Table 2 Absolute abundances (in ng) of epicuticular compounds (mean ± SE) from virgin male and female *Drosophila recens* and *D. subquinaria*

	D. recens		*D. subquinaria*	
FID #[a]	Female	Male	Female	Male
Acetates				
1	Not detected	1344.1±143.1	Not detected	2338.7±292.3
Hydrocarbons				
2	128.8±12.1	132.1±16.1	141.3±11.1	131.3±15.8
3	12.3±2.7	32.9±11.2	14.2±2.7	40.8±16.2
4	8.4±1.6	1.6±0.2	11.5±1.6	10.4±1.9
5	108.0±11.2	110.6±14.6	123.3±9.1	132.1±16.6
6	25.1±5.3	31.2±9.4	24.7±6.3	61.0±16.1
7	48.6±9.5	6.2±0.6	61.1±7.6	41.9±8.9
8	18.1±3.3	7.2±1.6	17.8±2.2	34.5±6.3
9	10.6±1.2	7.5±0.7	14.7±2.5	10.1±1.3
10	32.8±4.0	35.4±3.5	34.9±4.7	7.8±0.8
11	52.2±5.0	49.1±3.7	70.2±8.9	76.7±8.7
12	158.3±14.5	133.8±14.2	215.4±22.8	189.1±21.0
13	180.6±26.6	134.3±13.7	216.8±34.8	74.9±11.1
14	69.6±8.2	80.6±8.3	73.7±8.2	82.9±9.1
17	26.6±6.0	7.3±0.8	34.8±6.4	54.7±5.6
18	15.2±4.1	10.6±1.3	14.4±1.6	23.0±3.2
19	80.4±13.7	68.7±7.1	98.1±15.2	157.7±17.4
20	165.9±25.6	223.5±25.6	220.9±28.2	371.1±42.1
21	60.0±14.4	62.6±6.2	62.7±8.2	65.2±19.4
Tri-acylglycerides				
15	Not detected	143.3±19.9	Not detected	148.3±16.5
16	Not detected	34.9±4.7	Not detected	35.4±5.1
22	Not detected	69.5±9.7	Not detected	95.6±11.7
23	Not detected	27.6±4.1	Not detected	45.6±5.3
24	Not detected	28.9±3.3	Not detected	56.4±6.7

[a] As given in Figs. 1 and 2 and Table 1

alkenes. The number and variety of CHCs is not unusual relative to other *Drosophila* (Ferveur, 2005). The relative dominance of long chain hydrocarbons in these profiles is, however, somewhat unique. In several other *Drosophila* species in which CHCs have been identified, including members of the *affinis*, *pseudoobscura*, *montium*, and *melanogaster* subgroups, chain lengths generally range from C_{21} to C_{30} (Ferveur, 2005). In *D. recens* and *D. subquinaria* in comparison, eight of the 18 CHCs were positional isomers of tritriacontadiene (C_{33}) and pentatriacontadiene (C_{35}), respectively. Although less common in *Drosophila,* long chain C_{33} alkadienes previously have been described in *D. erecta* and *D. pallidosa* in the *ananassae* subgroup (Jallon and David, 1987; Nemoto et al., 1994; Jallon and Wicker-Thomas, 2003). They also have been found in several species of cactophilic *Drosophila* in the *repleta* group, including *D. mojavensis, D. navojoa*, and *D. arizonae*. Similar to *D. recens* and *D. subquinaria*, CHC profiles of these cactophilic species were composed of C_{29}-C_{40} compounds, with the C_{35} positional isomers of *n*-pentatriacontadiene accounting for 30–50% of all CHCs (Etges and Ahrens, 2001; Etges and Jackson, 2001; Oliveira et al., 2011).

In addition to the 18 CHCs identified in *D. subquinaria* and *D. recens*, the male epicuticle also contained 11-*cis*-Vaccenyl acetate (cVA). This is mildly volatile (at natural temperatures) and, with a chain-length of C_{20}, is the smallest compound identified in these species. Absent in females, cVA also was the most abundant compound in males of both species (Fig. 4). cVA also has been found exclusively on the male cuticle in *D. melanogaster,* located physically in and around the ejaculatory bulb (Yew et al., 2009), and has been the subject of much work investigating its role in social and sexual interactions in this species. The ligand and receptor of cVA also have been fully characterized (Mehren, 2007). cVA has been shown to promote social aggregation in both

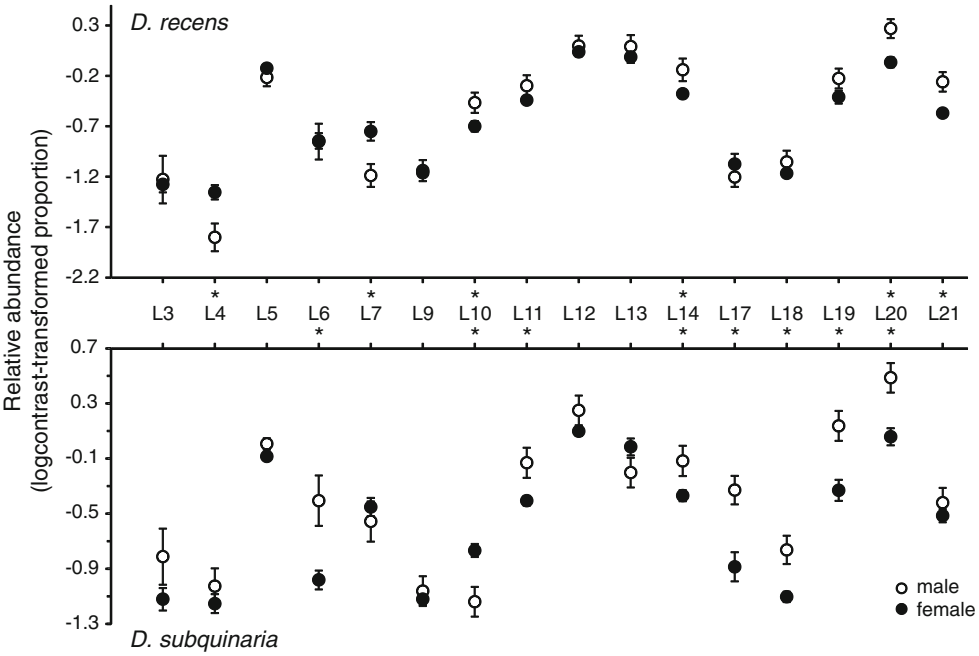

Fig. 3 Sexual dimorphism in the relative abundance (logcontrast-transformed proportions ± SE) of cuticular hydrocarbons shared between female (*filled circles*) and male (*open circles*) *Drosophila recens* (*above*) and *D. subquinaria* (*below*). Logcontrast CHCs are denoted by an 'L' followed by their FID number as given in Figs. 1 and 2. *Asterisks* above or below the labels indicate a significance difference in relative abundance of the given compound between the sexes in *D. recens* or *D. subquinaria* respectively (*$P<$ 0.05, *Welch's t-test*)

males and females (Bartelt et al., 1985), and also is transferred to females during copulation, where it subsequently acts as a courtship inhibitor to successive males (Bastock and Manning, 1955; Butterworth, 1969; Zawistowski and Richmond, 1986). Whether cVA mediates similar behaviors in *D. recens* and *D. subquinaria* is an interesting possibility for future research.

Found exclusively on the epicuticle of male *D. subquinaria* and *D. recens*, were also two groups of putative triacylglycerides (TAGs). Oxygen containing compounds, including TAGs, have been described on the epicuticle of

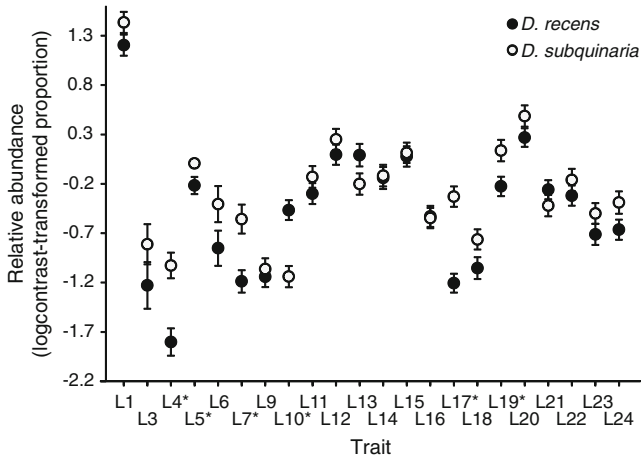

Fig. 4 Differences between *Drosophila recens* and *D. subquinaria* males in the relative abundance (logcontrast-transformed proportion ± SE) of different epicuticular compounds. Compounds are denoted by an 'L' followed by their FID number as given in Figs. 1 and 2. An *asterisk* next to a label indicates a significance difference between species in relative abundance of that compound (*$P<0.05$, *Welch's t-test*)

species from various insect groups (Lockey, 1979). In *Drosophila,* this class of compounds is not well characterized (but see Hammad et al., 2011), although there is some evidence that they may be involved in sexual signaling. In male *D. melanogaster,* for example, 3-O-acetyl-1,3-dihydroxy-octacosa-11,19-diene has been found to co-localize in the ejaculatory bulb with cVA, and during copulation it was transferred along with cVA to females, resulting in a long term depression of female sexual attractiveness following mating (Yew et al., 2009). Tri-acylglycerides specific to the male anogenital region also have been putatively identified in *D. mojavensis* and *D. arizonae.* During mating, these compounds were transferred to females, again suggesting a potential role in sexual signaling (Yew et al., 2011). As outlined above, the hydrocarbon profiles *D. subquinaria* and *D. recens* are dominated by high molecular weight alkadienes and methyl alkanes, similar to the profiles of the two species in which TAGs also have been reported and implicated as potential sexual signals (Yew et al., 2011).

The presence of fatty acids in epicuticular extracts, when standard extraction and GC/MS techniques are used, may be a product of contamination from internal lipids, where triglycerols are the most predominant compounds (Buckner, 1993). However, several lines of reasoning suggest that the fatty acids (i.e., putative TAGs) found in the current study are not an artifact of internal contamination. First, a short extraction time (4 min) with a non-polar solvent (hexane) was used. This is unlikely to have dissolved the epicuticle to a point where internal compounds were extracted. In addition, this method is identical to that used for CHC extraction in at least three other species (*D. serrata*, *D. birchii*, *D.*

Table 3 Vectors of standardized directional sexual selection gradients (β) on the principal components (PCs) of variation in 16 logcontrast CHCs for *Drosophila recens* and *D. subquinaria* males, and their comparison between these species

Male trait (PC)[a]	*D. recens*		*D. subquinaria*		Between-species difference (P^c)
	β	P	β	P	
1	0.048	0.906	0.113	0.296	0.670
2	−0.017	0.834	**0.236**	0.033	0.308
3	−0.020	0.641	−0.137	0.137	0.509
4	**0.138**	0.040	−0.322	0.115	**0.020**
5	**−0.206**	0.014	−0.306	0.145	0.895
6	**−0.188**	0.006	**−0.365**	0.009	0.170
7	0.108	0.231	−0.180	0.217	0.094
8	0.055	0.308	0.169	0.078	0.521
9	0.048	0.445	0.013	0.853	0.765
10	**0.247**	<0.001	0.049	0.676	0.069
11	**0.145**	0.035	0.217	0.083	0.716
12	0.107	0.436	0.075	0.365	0.910
13	**0.011**	0.690	**−0.179**	0.026	0.052
14	**0.149**	0.025	**−0.239**	0.003	**<0.001**
15	0.145	0.072	**0.211**	0.036	0.562
16	**−0.155**	0.038	−0.021	0.869	0.228

[a] See Table S2 (Online Resource 1) for principal component loadings

[b] Determined using logistic multiple regression (see text for details). Significant gradients ($P \leq 0.05$) are indicated in bold

[c] Significance of the trait×species interaction testing whether sexual selection on each trait differs between the two species. Significant differences ($P \leq 0.05$) are indicated in bold

bunnanda) in which TAGs have not been reported (Howard et al., 2003; Van Homrigh et al., 2007). Finally, extractions were performed in the same way for males and females, but TAGs were uniformly detected in all males yet were absent in all females. A more detailed investigation of these lipids, including confirmation of their identification, will require additional study *via* electrospray ionization and tandem mass spectrometry (e.g., Ogg and Stanley-Samuelson, 1992; Yew et al., 2008). Together with cVa, understanding the role, if any, of these compounds in mate choice in *D. subquinaria* and *D. recens* will require detailed investigation of their potential transfer during mating, as this would confound sexual selection analyses as normally performed (i.e., extracting chosen and rejected males after mating).

Female mate choice trials revealed significant directional sexual selection on CHCs in males of both *D. recens* and *D. subquinaria*, explaining 25.6% and 13.5%, respectively, of the variance in male mating success. These values are similar or higher than those commonly seen in other species (using the same methodology) in which the role of CHCs in sexual selection has been well studied (e.g., Chenoweth and Blows, 2005; Van Homrigh et al., 2007) and suggests that these compounds are important targets of female mate choice in both species. Extensive sexual dimorphism in CHC relative abundances in both *D. recens* and *D. subquinaria*, and species differences in relative abundances that were restricted entirely to males, are also consistent with these traits being targets of sexual selection in males, further suggesting their involvement in mate choice within populations (and hence species recognition). Finally, these traits are also strongly implicated as direct targets of sexual selection (as opposed to being correlated with one or more other traits that are actually targeted) by the results of a recent study that manipulated different sensory modalities, showing that olfaction, but not vision or hearing, were critical to mating by *D. subquinaria* females (Giglio and Dyer, 2013).

Sexual selection gradients calculated *via* female choice trials are representative of the population-level (i.e., average individual) female mate preference (Wagner, 1998; Chenoweth and Blows, 2006). Comparison of two selection (i.e., β) vectors, therefore, quantifies the extent to which mate preferences for these traits differ between the two groups. For *D. recens* and *D. subquinaria*, sexual selection gradients on CHCs differed significantly and the selection vectors were oriented quite differently in multivariate logcontrast traits space (72.5° away from one other), indicating substantial differences in how sexual selection acts in each population.

Sexual selection gradients were calculated from a single geographic population of each species, however, so additional assays with more geographic populations will be needed to evaluate this difference within the context of among-population variation within each species. Nevertheless, if based on epicuticular compounds, the observed reproductive character displacement in mate discrimination (Jaenike et al., 2006) suggests that between-species differences may be substantial.

Acknowledgments We thank J. Jaenike for sharing flies collected in Idaho and A. Ramakrishnan for help with statistical analyses and the preparation of figures. PMM and SC acknowledge the University of Ottawa for Core Facilities support of the John L. Holmes Mass Spectrometry Facility. Funding was provided by the National Science Foundation (KAD and HDR) and the Natural Sciences and Engineering Research Council of Canada (HDR).

References

ATCHISON, J. 1986. The Statistical Analysis of Compositional Data. Chapman and Hall, London.

BARTELT, R. J., SCHANER, A. M., and JACKSON, L. L. 1985. Cis-vaccenyl acetate as an aggregation pheromone in *Drosophila melanogaster*. *J. Chem. Ecol.* 11:1747–1756.

BARTELT, R. J., ARMOLD, M. T., SCHANER, A. M., and JACKSON, L. L. 1986. Comparative analysis of cuticular hydrocarbons in the *Drosophila virilis* species group. *Comp. Biochem. Physiol.* 83B:731–742.

BASTOCK, M. and MANNING, A. 1955. The courtship of *Drosophila melanogaster*. *Behaviour* 8:85–111.

BEAMENT, J. W. L. 1945. The cuticular lipoids of insects. *J. Exp. Biol.* 21:115–131.

BILLETER, J. C., ATALLAH, J., KRUPP, J. J., MILLAR, J. G., and LEVINE, J. D. 2009. Specialized cells tag sexual and species identity in *Drosophila melanogaster*. *Nature* 461:987–991.

BLOWS, M. W. 2002. Interaction between natural and sexual selection during the evolution of mate recognition. *Proc. R. Soc. Lond. B* 269:1113–1118.

BLOWS, M. W. and ALLAN, R. A. 1998. Levels of mate recognition within and between two *Drosophila* species and their hybrids. *Am. Nat.* 152:826–837.

BUCKNER, J. S. 1993. Cuticular polar lipids of insects, pp. 247–248, *in* D. W. Stanley-Samuelson and D. R. NELSON (EDS.), Insect Lipids: Chemistry, Biochemistry and Biology. University of Nebraska Press, Lincoln and London.

BUTTERWORTH, F. M. 1969. Lipids of *Drosophila*: A newly detected lipid in the male. *Science* 163:1356–1357.

CARLSON, D. A. and YOCOM, S. R. 1986. Cuticular hydrocarbons from six species of tephritid fruit flies. *Arch. Insect. Biochem. Physiol.* 3:397–412.

CARLSON, D. A., BERNIER, U. R., and SUTTON, B. D. 1998. Elution patterns from capillary GC for methyl-branched alkanes. *J. Chem. Ecol.* 24:1845–1865.

CHENOWETH, S. F. and BLOWS, M. W. 2005. contrasting mutual sexual selection on homologous signal traits in *Drosophila serrata*. *Am. Nat.* 165:281–289.

CHENOWETH, S. F. and BLOWS, M. W. 2006. Dissecting the complex genetic basis of mate choice. *Nat. Rev. Genet.* 7:681–692.

CHENOWETH, S. F., RUNDLE, H. D., and BLOWS, M. W. 2008. Genetic constraints and the evolution of display trait sexual dimorphism by natural and sexual selection. *Am. Nat.* 171:22–34.

CHENOWETH, S. F., HUNT, J., and RUNDLE, H. D. 2012. Analyzing and comparing the geometry of individual fitness surfaces, pp. 126–149, *in* E. I. Svensson and R. Calsbeek (eds.), The Adaptive Landscape in Evolutionary Biology. Oxford University Press, Oxford.

COYNE, J. A., CRITTENDEN, A. P., and MAH, K. 1994. Genetics of a pheromonal difference contributing to reproductive isolation in *Drosophila*. *Science* 265:1461–1464.

ETGES, W. J. and AHRENS, M. A. 2001. Premating isolation is determined by larval-rearing substrates in cactophilic *Drosophila mojavensis*. V. Deep geographic variation in epicuticular hydrocarbons among isolated populations. *Am. Nat.* 158:585–598.

ETGES, W. J. and JACKSON, L. L. 2001. Epicuticular hydrocarbon variation in *Drosophila mojavensis* cluster species. *J. Chem. Ecol.* 27:2125–2149.

EVERAERTS, C., FARINE, J.-P., COBB, M., and FERVEU, J.-F. 2010. *Drosophila* cuticular hydrocarbons revisited: mating status alters cuticular profiles. *PLoS One* 5:e9607.

FAIRBAIRN, D. J. and PREZIOSI, R. F. 1996. Sexual selection and the evolution of sexual size dimorphism in the water strider, *Aquarius remigis*. *Evolution* 50:1549–1559.

FANG, S., TAKAHASHI, A., and WU, C. 2002. A mutation in the promoter of desaturase 2 is correlated with sexual isolation between *Drosophila* behavioural races. *Genetics* 162:781–784.

FERNÁNDEZ, M. D. L. P., CHAN, Y.-B., YEW, J. Y., BILLETER, J.-C., DREISEWERD, K., LEVINE, J. D., and KRAVITZ, E. A. 2010. Pheromonal and behavioral cues trigger male-to-female aggression in *Drosophila*. *PLoS Biol.* 8:e100541.

FERVEUR, J. 1997. Genetic feminization of pheromones and its behavioral consequences in *Drosophila* males. *Science* 276:1555–1558.

FERVEUR, J.-F. 2005. Cuticular hydrocarbons: Their evolution and roles in *Drosophila* pheromonal communication. *Behav. Genet.* 35:279–295.

GIBBS, A. G. 1998. Water-proofing properties of cuticular lipids. *Am. Zool.* 38:471–482.

GIBBS, A. G., CHIPPINDALE, A. K., and ROSE, M. R. 1997. Physiological mechanisms of evolved desiccation resistance in *Drosophila melanogaster*. *J. Exp. Biol.* 200:1821–1832.

GIGLIO, E. M. and DYER, K. A. 2013. Divergence of premating behaviors in the closely related species *Drosophila subquinaria* and *D. recens*. *Ecol. Evol.* 3: In press.

GRILLET, M., DARTEVELLE, L., and FERVEUR, J.-F. 2006. A *Drosophila* male pheromone affects female sexual receptivity. *Proc. R. Soc. Biol.* 273:315–323.

HAMMAD, L. A., COOPER, B. S., FISHER, N. P., MONTOOTH, K. L., and KARTY, J. A. 2011. Profiling and quantification of *Drosophila melanogaster* lipids using liquid chromatography/mass spectrometry. *Rapid Commun. Mass Spectrom.* 25:2959–2968.

HOWARD, R. W. and BLOMQUIST, G. J. 2005. Ecological, behavioral, and biochemical aspects of insect hydrocarbons. *Annu. Rev. Entomol.* 50:371–393.

HOWARD, R. W., MCDANIEL, C. A., and BLOMQUIST, G. J. 1978. Cuticular hydrocarbons of the eastern subterranean termite, *Reticulitermes flavipes* (Kollar) (Isoptera: Rhinotermitidae). *J. Chem. Ecol.* 4:233–245.

HOWARD, R. W., JACKSON, L. L., BANSE, H., and BLOWS, M. W. 2003. Cuticular hydrocarbons of *Drosophila birchii* and *D. serrata*: Identification and role in mate choice in *D. serrata*. *J. Chem. Ecol.* 29:961–976.

JAENIKE, J., DYER, K. A., CORNISH, C., and MINHAS, M. S. 2006. Asymmetrical reinforcement and *Wolbachia* infection in *Drosophila*. *PLoS Biol.* 4:e325.

JALLON, J. M. 1984. A few chemical words exchanged by *Drosophila* during courtship and MATING. *BEHAV. GENET.* 14:441–478.

JALLON, J. M. and DAVID, J. R. 1987. Variation in cuticular hydrocarbons among the eight species of the *Drosophila melanogaster* subgroup. *Evolution* 41:294–302.

JALLON, J. M. and WICKER-THOMAS, C. 2003. Genetic studies on pheromone production in *Drosophila*, pp. 253–280, *in* G. J. Bloomquist and R. G. Vogt (eds.), Insect Pheromone Biochemistry and Molecular Biology: The Biosynthesis and Detection of Pheromones and Plant Volatiles. Elsevier Academic Press, Amsterdam, The Netherlands.

KENT, C., AZANCHI, R., SMITH, B., FORMOSA, A., and LEVINE, J. D. 2008. Social context influences chemical communication in *D. melanogaster* males. *Curr. Biol.* 18:1384–1389.

KRUPP, J. J., KENT, C., BILLETER, J.-C., AZANCHI, R., SO, A. K.-C., LUCAS, C., SMITH, B. P., SCHONFELD, J. A., and LEVINE, J. D. 2008. Social experience modifies pheromone expression and mating behavior in male *Drosophila melanogaster*. *Curr. Biol.* 18:1373–1383.

KWAN, L. and RUNDLE, H. D. 2009. Adaptation to desiccation fails to generate pre- and postmating isolation in replicate *Drosophila melanogaster* laboratory populations. *Evolution* 64:710–723.

LANDE, R. and ARNOLD, S. J. 1983. The measurement of selection on correlated characters. *Evolution* 37:1210–1226.

LOCKEY, K. H. 1979. Insect cuticular hydrocarbons. *Comp. Biochem. Physiol.* 65:457–462.

MARKOW, T. A. and TOOLSON, E. C. 1990. Temperature effects on epicuticular hydrocarbons and sexual isolation in *Drosophila mojavensis*, pp. 315–331, *in* S. F. Barker, W. T. Starmer, and R. J. McIntyre (eds.), Ecological and Evolutionary Genetics of *Drosophila*. Plenum Press, New York.

MEHREN, J. E. 2007. Mate recognition: Should fly stay or should fly go. *Curr. Biol.* 17:240–242.

MITCHELL-OLDS, A. T. and SHAW, R. G. 1987. Regression analysis of natural selection: Statistical inference and biological interpretation. *Evolution* 41:1149–1161.

MIWA, T. K. 1963. Identification of peaks in gas–liquid chromatography. *J. Am. Oil Chem. Soc.* 40:309–313.

MJØS, S. A. 2006. Prediction of equivalent chain lengths from two-dimensional fatty acid retention indices. *J. Chromatogr. A* 1122:249–254.

NEMOTO, T., DOI, M., OSHIO, K., MATSUBAYASHI, H., OGUMA, Y., SUZUKI, T., and KUVAHARA, Y. 1994. (Z, Z)-5,27-Tritriacontadiene: Major sex pheromone of Drosophila pallidosa (Diptera; Drosophilidae). *J. Chem. Ecol.* 20:3029–3037.

OGG, C. L. and STANLEY-SAMUELSON, D. W. 1992. Phospholipid and triacylglycerol fatty acid compositions of the major life stages and selected tissues of the tobacco hornworn *Manduca sexta*. *Comp. Biochem. Physiol.* 101:345–351.

OLIVEIRA, C. C., MANFRIN, M. H., SENE, F. D. M., JACKSON, L. L., and ETGES, W. J. 2011. Variations on a theme: diversification of cuticular hydrocarbons in a clade of cactophilic *Drosophila*. *BMC Evol. Biol.* 11:179.

PETFIELD, D., CHENOWETH, S. F., RUNDLE, H. D., and BLOWS, M. W. 2005. Genetic variance in female condition predicts indirect genetic variance in male sexual display traits. *Proc. Natl. Acad. Sci. U. S. A.* 102:6045–6050.

ROUAULT, J., CAPY, P., and JALLON, J. M. 2000. Variations of male cuticular hydrocarbons with geoclimatic variables: An adaptive mechanism in *Drosophila melanogaster*? *Genetica* 110:117–130.

RUNDLE, H. D., CHENOWETH, S. F., and BLOWS, M. W. 2009. The diversification of mate preferences by natural and sexual selection. *J. Evol. Biol.* 22:1608–1615.

SALL, J., CREIGHTON, L., and LEHMAN, A. 2005. JMP Start Statistics: A Guide to Statistics and Data Analysis Using JMP and JMP IN Software. Thomson Learning, Belmont, CA.

SAVARIT, F. and FERVEUR, J.-F. 2002. Genetic study of the production of sexually dimorphic cuticular hydrocarbons in relation with the sex-determination gene transformer in *Drosophila melanogaster*. *Genet. Res.* 79:23–40.

SHARMA, M. D., HUNT, J., and HOSKEN, D. J. 2012. Antagonistic responses to natural and sexual selection and the sex-specific evolution of cuticular hydrocarbons in *Drosophila simulans*. *Evolution* 66:665–677.

SHOEMAKER, D. D., KATJU, V., and JAENIKE, J. 1999. *Wolbachia* and the evolution of reproductive isolation between *Drosophila recens* and *Drosophila subquinaria*. *Evolution* 53:1157–1164.

TOOLSON, E. C. and KUPER-SIMBRON, R. 1989. Laboratory evolution of epicuticular hydrocarbon composition and cuticular permeability in *Drosophila pseudoobscura*: Effects on sexual dimorphism and thermal-acclimation ability. *Evolution* 43:468–473.

VAN HOMRIGH, A., HIGGIE, M., MCGUIGAN, K., and BLOWS, M. W. 2007. The depletion of genetic variance by sexual selection. *Curr. Biol.* 17:528–532.

WAGNER, W. 1998. Measuring female mating preferences. *Anim. Behav.* 55:1029–1042.

WHEELER, M. R. 1960. New species of the *quinaria* group of *Drosophila* (Diptera, Drosophilidae). *Southwest. Nat.* 5:160–164.

WIGGLESWORTH, V. B. 1945. Transpiration through the cuticle of insects. *J. Exp. Biol.* 21:97–114.

YEW, J. Y., CODY, R. B., and KRAVITZ, E. A. 2008. Cuticular hydrocarbon analysis of an awake behaving fly using direct analysis in real-time time-of-flight mass spectrometry. *Proc. Natl. Acad. Sci. U. S. A.* 105:7135–7140.

YEW, J. Y., DREISEWERD, K., LUFTMANN, H., MÜTHING, J., and KRAVITZ, E. A. 2009. A new male sex-pheromone and novel cuticular cues for chemical communication in *Drosophila*. *Curr. Biol.* 19:1245–1254.

YEW, J. Y., DREISEWERD, K., DE OLIVEIRA, C. C., AND ETGES, W. J. 2011. Male-specific transfer and fine scale spatial differences of newly identified cuticular hydrocarbons and triacylglycerides in a *Drosophila* species pair. *PLoS One* 6:e16898.

ZAWISTOWSKI, S. AND RICHMOND, R. C. 1986. Inhibition of courtship and mating of *Drosophila melanogaster* by the male-produced lipid, cis-vaccenyl acetate. *J. Insect Physiol.* 32:189–192.