Annals of the Entomological Society of America, 113(1), 2020, 40–49
doi: 10.1093/aesa/saz056
Advance Access Publication Date: 24 November 2019
Research

Research

Epicuticular Compounds of *Protopiophila litigata* (Diptera: Piophilidae): Identification and Sexual Selection Across Two Years in the Wild

Christopher S. Angell,[1] Sharon Curtis,[2] Anaïs Ryckenbusch,[2] and Howard D. Rundle[1,3]

[1]Department of Biology, University of Ottawa, Ottawa, ON, K1N 6N5, Canada, [2]Department of Chemistry, University of Ottawa, Ottawa, ON, K1N 6N5, Canada, and[3]Corresponding author, e-mail: hrundle@uottawa.ca

Subject Editor: Charles Henry

Received 20 May 2019; Editorial decision 25 September 2019

Abstract

The epicuticular compounds (ECs) of insects serve both to waterproof the cuticle and, in many taxa, as pheromones that are important for various social interactions, including mate choice within populations. However, ECs have not been individually identified in many species and most studies of their role in mate choice have been performed in a laboratory setting. Here we newly identify and quantify the ECs of the antler fly, *Protopiophila litigata* Bonduriansky, and use a cross-sectional selection analysis to quantify their association with male mating success in the wild across two years (2013 and 2017). The ECs of antler flies include straight-chain and methylated alkanes, alkenes, and a family of branched wax esters. We find all ECs to be shared between males and females but also demonstrate sexual dimorphism in the abundance of several. Male EC relative abundances were significantly associated with mating success in both years, although the multivariate direction of selection differed significantly between the years. Surprisingly, only two of the 18 compounds (or groups of compounds) we identified were similarly associated with mating success across the sampling years. In 2017, we further partitioned sexual selection into intra- and intersexual components, revealing selection on ECs to be significant via female choice but not male–male competition. Our study is one of few to investigate the potential role of ECs in mating success in the wild and adds to a growing body of evidence demonstrating significant temporal variability in selection in natural populations.

Key words: cuticular hydrocarbon, gas chromatography, mate choice, pheromone, temporal variation

The insect epicuticle is made up of long-chain hydrocarbons and their derivatives collectively referred to as epicuticular compounds (ECs). ECs play an important role in desiccation resistance by waterproofing the cuticle with a waxy coating (Beament 1945, Wigglesworth 1945), but in multiple species, ECs also function as pheromones used as a means of chemical communication. ECs have variously been implicated in species recognition (Martin et al. 2008, Dyer et al. 2014), kin recognition (Lihoreau and Rivault 2009), group recognition (Fang et al. 2002, Vásquez et al. 2009), and mate choice (Howard et al. 2003, Steiger et al. 2013, Booksmythe et al. 2017) in diverse insect taxa. Extensive work characterizing ECs in several species of *Drosophila* has determined that they are plastic in response to aspects of the physical (Stinziano et al. 2015) and social environment (Petfield et al. 2005, Gershman et al. 2014, Gershman and Rundle 2017), and can evolve rapidly in response to altered natural and sexual selection (Blows 2002, Kwan and Rundle 2010, Sharma et al. 2012).

ECs have been specifically studied for their role as sexual signals used in mating in diverse insect taxa, including several species of *Drosophila* (Howard et al. 2003, Grillet et al. 2006, Van Homrigh et al. 2007, Curtis et al. 2013, Fedina et al. 2017), beetles (Ali and Tallamy 2010, Booksmythe et al. 2017), and crickets (Thomas and Simmons 2009, Steiger et al. 2013). Within a species, EC profiles have also been observed to differ in the presence versus absence of a closely related species in nature, generating a pattern of reproductive character displacement that implicates them in mate choice and species isolation (Higgie et al. 2000, Dyer et al. 2014). However, most studies of sexual selection on ECs have used laboratory mating trials, and investigations of the importance of ECs to mating success in nature are rare (but see Hine et al. 2004, Steiger et al. 2013, Gosden et al. 2016).

Here we identify and quantify the ECs of antler flies (*Protopiophila litigata* Bonduriansky), and investigate their role in both female mate choice and male territoriality in the wild. *Protopiophila litigata* are small (2 mm) necrophagous flies in the family Piophilidae. Unlike other piophilids, many of which are associated with carrion (Rochefort et al. 2015), *P. litigata* mate and oviposit exclusively on discarded cervid antlers (Bonduriansky 1995). From June to August,

both males and females congregate on antlers, which can host over 100 flies at a time (Mautz et al. 2019). Males, which have high site fidelity among antlers, aggressively defend territories on the antler surface for access to females, which disperse more widely but tend to congregate near the damaged areas of the antlers used for oviposition (Bonduriansky and Brooks 1999). Male density and body size are highest near oviposition sites, suggesting that these territories are highly contested and that territoriality is a major component of sexual selection on males in this species (Bonduriansky and Brooks 1999). *Protopiophila litigata* do not display substantial courtship behavior; males leap on to the backs of females, who may either accept or reject them (Bonduriansky 2003). Large-bodied males are more successful in agonistic contests (Bonduriansky and Brooks 1999) and are preferred by females as mates (Bonduriansky and Brooks 1998a), but little else is known about traits under inter- and intrasexual selection in this species.

We quantify sexual selection via a cross-sectional analysis that compares the EC profiles of wild-collected *P. litigata* males that were either mating or not at the time of collection. Our collections were performed in two different years (2013 and 2017), allowing us to compare the multivariate direction of sexual selection on ECs between these years. Selection in nature is often highly variable through time and its consistency (or, conversely, variability) has important implications for our understanding of evolutionary change (Siepielski et al. 2009). In 2017, we also further partition selection into intra-sexual (i.e., male–male competition over territories on the surface of an antler) and intersexual (female choice) components by distinguishing non-mating males that were either holding a territory or not when collected, providing more detailed insight into potential source(s) of sexual selection.

Materials and Methods

Chromatography

Gas chromatography with flame ionization detection (GC:FID) was performed on single-fly extractions using a dual-channel Agilent 6890N 'fast' (220V oven) gas chromatograph fitted with HP-5 phenylmethyl siloxane columns of 30 m length and 250 µm internal diameter (0.1 µm film thickness) and flame ionization detectors (at 310°C). The inlet was set to splitless (at 275°C) with a pulse pressure of 206.8 kPa (30 psi) for 1.4 min. The injection volume was 1 µl and hydrogen was used as the carrier gas with a constant flow of 2.7 ml/min. The temperature program began by holding at 150°C for 0.55 min, then increasing to 200°C at a rate of 120°C/min, then increasing to 265°C at a rate of 7°C/min, and finally increasing to 310°C at a rate of 120°C/min. These method parameters were selected via an optimization procedure that sought to minimize the total run time while maintaining peak resolution. The run time of a single sample was just less than 12 min.

Gas chromatography with mass spectrometry (GC:MS) was performed on both single and multi-fly 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 diameter and employing helium as the carrier gas. To facilitate the comparison of retention times between GC:FID and GC:MS chromatographic profiles, method parameters from the former instrument were converted for the latter using 'mxlator' v. 2.0a, a GC method translation software that is available for free download from the Agilent Technologies (Santa Clara, CA) website. Helium flow rate was set to 1.92 ml/min with a column pressure of 187.33 kPa (27.170 psi). The column temperature program began by holding at 150°C for 2.095 min, then ramped to 200°C at a rate of 31.5°C/min, followed by a slower ramp to 265°C at 1.838°C/min, and finally concluded by ramping to 310°C at 31.5°C/min. The sample inlet valve was set to splitless, with an injection pulse pressure of 206.8 kPa (30 psi). The detector remained off until 3 min into the run to avoid the solvent peak. A 5 µl injection volume was used for both single and multi-fly extractions.

Compound Identification and Quantification

For EC identification and quantification respectively, non-virgin adults (males and females) were sampled from two different laboratory stock populations that were founded in 2012 (2017) from greater than 500 (>200) individuals collected from moose antlers (*Alces alces* (Linnaeus) (Artiodactyla: Cervidae)) at the Algonquin Wildlife Research Station in Algonquin Provincial Park, Ontario, Canada (45.591373, −78.522472). These antlers were collected from throughout the park and relocated to the research station, so the sampled flies likely included individuals from the immediate local population and those which had grown as larvae within the relocated antlers. The flies were then maintained in the lab in mixed-sex cages at 23°C and 60% relative humidity with a 17:7h light–dark cycle following Oudin et al. (2015). Samples used for quantification were generated by immersing individual flies in 100 µl of hexane for 5 min, including one minute of vortexing. Pooled extractions used for GC:MS were similarly produced by immersing 20 flies in hexane for 5 min.

Equivalent chain length (ECL) values were calculated for all compounds on both instruments by comparison to a standard C_7-C_{40} saturated *n*-alkane mixture (Sigma Aldrich, product # 49452-U) following Curtis et al. (2013). These ECL numbers were cross-referenced between the two instruments to ensure that the identification of all ECs related to the same peaks. Compounds were identified by comparing their ECL and mass spectra to the alkane standard, MS databases, and published literature (see Supp Information [online only]). The presence of a homologous series of esters was inferred using single ion chromatography, mainly on mass 99 Da. Provisional identifications are given in certain cases in which these methods provided a reasonable assignment, but unequivocal confirmation was lacking, e.g., with respect to the positioning of the double bond within alkenes, which were not present in sufficient quantities for derivatization.

For descriptive purposes, absolute EC abundances were obtained separately for single-fly extractions of 20 males and 19 females by integrating the area under 18 peaks (i.e., ECs) in the GC:FID chromatograms using ChemStation software v. A.01.05 (Agilent Technologies, Santa Clara, CA). To convert integrated peak areas to known abundances, concentration gradients were derived using 0.0625, 0.125, 0.25, 0.5, 1, and 2 ng/µl samples of the C_7-C_{40} saturated *n*-alkane mixture and the abundance of each compound was calculated using a linear calibration curve derived from the alkanes with retention times immediately before and after each sample peak.

Following extraction, flies were dried and individually weighed to the nearest 0.01 mg on a MX5 Microbalance (Mettler Toledo, Columbus, OH). As total EC amount varied with dry mass (see Results), and males and females differ in mean body size (Bonduriansky 1995), we calculated size-corrected EC abundances by separately regressing the square root of each compound abundance onto the cube root of dry mass (as the former depends on surface area and the latter on volume; Lande and Arnold 1983, Kwan and Rundle 2010), and adding the residual values to the grand mean for that compound. A multivariate analysis of variance (MANOVA), implemented in the R package *car* (Fox and Weisberg 2011), was used to test for differences between males and females in

size-corrected concentration of the 18 ECs. Given significant sexual dimorphism overall, individual peaks were subsequently tested using separate Welch's *t*-tests for unequal variance.

Field Collection and Quantification of ECs

To quantify sexual selection on ECs in the field, a cross-sectional selection analysis was performed in which wild males were collected from the surface of several moose antlers located around the Algonquin Wildlife Research Station. Collections were performed in the early summer of 2013 and 2017 and, in both years, collected males were classified as either mating (i.e., copulating with a female at the time of collection or mate guarding a female they recently mated; n = 126 in 2013, n = 60 in 2017) or non-mating (n = 125 in 2013, n = 109 in 2017). In 2017, non-mating males were further subdivided into those that were observed to be actively defending a territory on the upper surface of the antler prior to collection (n = 62) versus those resting on the underside of the antler (n = 47) where males rarely defend territories (Bonduriansky and Brooks 1999). Shortly after collection, males were anesthetized with CO_2 and immersed individually in 100 µl of hexane for 5 min for EC extraction, after which they were discarded. Copulating pairs were gently separated under anesthesia prior to extraction. Voucher specimens were deposited in the Canadian National Collection of Insects, Arachnids and Nematodes, Ottawa, Ontario, Canada.

EC abundances were obtained separately for all samples via GC:FID and the area under the same 18 peaks was integrated for each. Due to the very small concentrations involved, minor variation in the extraction protocol can introduce substantial noise into GC:FID estimates of absolute compound abundances, even when using an internal standard (Blows and Allan 1998). Accordingly, past studies of sexual selection on ECs in insects have therefore focused on relative abundances calculated by dividing the area of each peak by the total area under all peaks for a given individual (Chenoweth and Blows 2005, Van Homrigh et al. 2007, Curtis et al. 2013), and we take this approach for the sexual selection analysis. However, relative abundances are a form of compositional data to which standard statistical methods should not be applied (Aitchison 1986, Egozcue and Pawlowsky-Glahn 2011). To address this, we calculated center-log-ratio (CLR) transformed values using the 'clr' function in the R package *Hotelling* (Curran 2017). Multicollinearity among CLR-transformed relative concentrations was high (maximum variance inflation factor = 52.3), so a principal component analysis (PCA) was performed on the covariance matrix of the combined data for both years following Bonduriansky et al. (2015). The first 17 principal components were used in the subsequent analyses because the 18th has an eigenvalue of zero due to the unit-sum constraint inherent in compositional data.

ECs are both plastic and capable of evolving in response to selection. To characterize temporal variability in ECs, we compared the epicuticular profiles of males collected in 2013 and 2017. The overall difference in ECs was assessed with a MANOVA performed on the principal components of EC variation. Given a significant difference between years, we subsequently performed Welch's *t*-tests on CLR-transformed relative abundances of each FID-quantified compound. Interannual variation in relative EC abundances was visualized using the R package *vioplot* (Adler and Kelly 2018).

Sexual Selection Analysis

Linear (i.e., directional) standardized sexual selection gradients on ECs were estimated using a standard multiple regression of relative mating success (0 = non-mating, 1 = mating) of males on the 17 principal components of EC variation (each standardized to a mean of 0 and unit standard deviation) separately by year (Lande and Arnold 1983). While these models were fit using standard least squares, because mating success is binomially distributed significance testing was performed using a generalized linear model (GLM) fit using maximum likelihood, specifying a binomial error distribution and a logit link function (Fairbairn and Preziosi 1996). For each year, the overall significance of sexual selection was tested by comparing the fit of this model with that of an intercept-only reduced model using a likelihood ratio test (LRT). Date of collection and antler identity were nonsignificant in both years and were thus excluded from all models.

Differences in the proportion of mated males collected between the sampling years (0.5 in 2013 vs. 0.35 in 2017) will generate differences in the apparent strength of selection when estimated separately by year. For this reason, comparison of the magnitude of selection gradients between the years is not appropriate, but the differences in the direction of selection in multivariate trait space remain meaningful. The multivariate direction of linear sexual selection was compared between years by calculating the correlation of the vectors of selection gradients when estimated separately by year. The vector correlation is the dot product of the column vectors of selection gradients normalized to unit length and ranges from 1 (selection in the identical direction in multivariate trait space) to –1 (selection in precisely opposing directions in multivariate trait space), with 0 representing orthogonal vectors (Rundle et al. 2008). All of our analyses focused on linear selection alone because our sample sizes were insufficient to reliably estimate the 153 quadratic and correlational gradients required to quantify non-linear selection on a suite of 17 traits.

To determine if sexual selection differed significantly between years, we used a sequential model-building approach (Chenoweth et al. 2012). Data were combined across years and a logistic GLM was fit that included an effect of year as well its interactions with each of the 17 traits (i.e., EC principal components). The fit of this model was compared to a reduced model lacking the year × trait interactions using a LRT. The main effect of year in these models accounts for differences in the proportion of mating males in 2013 versus 2017, meaning significance of the year × trait interactions would indicate differences in the relative strength and/or multivariate direction of sexual selection.

In 2017, the change in sampling design allowed sexual selection to be partitioned into intrasexual and intersexual selection components. Intrasexual selection (i.e., male–male competition, representing the probability of a single male holding a territory) was tested by comparing the EC profiles of non-mating males with and without territories. Intersexual selection (i.e., female choice, representing the likelihood of a territory-holding male achieving a mating) was tested by comparing the mating and territory-holding non-mating males. For each analysis, we used a binomial GLM for significance testing and ordinary least squares regression to estimate selection, as described above. All analyses were performed in R version 3.5.1 (R Core Team 2018).

Finally, to aid with the interpretation of our results, we projected a subset of the selection gradients back into the original trait space following Chong et al. (2018). Separately by year, we calculated selection gradients on CLR-transformed relative abundances of the eighteen EC compounds (or groups of compounds) using estimated selection gradients for the first nine principal components, which together explain 95.8% of the variation in male ECs (Supp Table S4 [online only]). We determined whether selection on each trait was significant in each year by performing *t*-tests on the projected selection

gradients using their associated standard errors (calculated from their variances, which were estimated following Lafi and Kaneene 1992).

Data Accessibility

GC:FID data used in this study have been archived in the Dryad Digital Repository (Angell et al. 2019).

Results

Identity and Abundance of ECs

Twenty-seven compounds were identified on the cuticles of both male and female antler flies via GC:MS (Table 1). Twenty-one of these compounds were hydrocarbons that ranged from C_{27} to C_{31} (excluding C_{28} and C_{30}) and included two alkanes (C_{27} and C_{29}) that co-eluted with their respective compounds in the C_7-C_{40} *n*-alkane standard, four alkenes, and fifteen mono- or dimethyl alkanes. Due to low absolute abundances, we were unable to positively determine the placement of the double bonds in the alkenes via derivatization. However, three of the alkene compounds (peaks 6, 8, and 16) had similar fractional ECL values (ca. x.75), which previous studies have reported as representing 9-alkenes (Vaníčková et al. 2012, Curtis et al. 2013). The hydrocarbons clustered around the odd carbon numbers, with C_{29} exhibiting the greatest variety of compounds (Fig. 1). Both the C_{27} and C_{29} groups were dominated by a single alkene and its corresponding alkane, while the C_{31} compounds were present at much lower concentrations (see below). The C_{27}, C_{29}, and C_{31} groups each contained a pair or trio of methyl alkanes that co-eluted in a single FID peak (peaks 6, 11, and 17), and a similar pair of co-eluting dimethyl alkanes was present in the C_{29} and C_{31} groups (peaks 13 and 18).

The remaining six compounds were provisionally identified as a family of C_{23}-C_{27} esters differing primarily by the addition of one or more CH_2 to the alkyl chain group. Derivatization of samples with TMS did not alter these peaks, revealing that they were not alcohols or carboxylic acids, and the NIST MS Search 2.0 database suggested

Table 1. Epicuticular compounds of *Protopiophila litigata*

FID peak no.	ECL[a]	Identification	Molecular formula	Mass (Da)	Diagnostic ions (m/z)	Method[b]	Notes
1a	22.92	*x*-heptadecyl hexanoate	$C_{23}H_{46}O_2$	354	99, 238	MS (P)	Not integrated on GC:FID
1b	23.08	*x*-nonadecyl butanoate	$C_{23}H_{46}O_2$	354	71, 266	MS (P)	Not integrated on GC:FID
1c	23.89	*x*-octadecyl hexanoate	$C_{24}H_{48}O_2$	368	99, 253	MS (P)	Not integrated on GC:FID
1	24.97	*x*-nonadecyl hexanoate	$C_{25}H_{50}O_2$	382	99, 266	MS (P)	
1d	25.83	*x*-icosyl hexanoate	$C_{26}H_{52}O_2$	394	99, 280	MS (P)	Not integrated on GC:FID
2	26.74	*x*-heptacosene[c,d]	$C_{27}H_{54}$	378	378	MS, L (P)	
3	26.81	*x*-heneicosyl hexanoate	$C_{27}H_{54}O_2$	410	99, 294	MS (P)	
4	26.89	unknown					Not detected via GC:MS
5	27.00	heptacosane	$C_{27}H_{56}$	380	380	ST	
6	27.32	11- and 13-methylheptacosane	$C_{28}H_{58}$	394	11-methyl: 141, 168, 224, 252, (M-15) 13-methyl: 168, 196, 224, (M-15)	MS, L	Co-eluted on GC:FID
7	27.73	3-methylheptacosane	$C_{28}H_{58}$	394	337, 365, 393	MS, L	
8	28.75	*x*-nonacosene[c,d]	$C_{29}H_{58}$	406	406	MS, L (P)	
9	28.83	*x*-nonacosene[c]	$C_{29}H_{58}$	406	406	MS (P)	
10	29.00	nonacosane	$C_{29}H_{60}$	408	408	ST	
11	29.32	11- and 13-methylnonacosane	$C_{30}H_{62}$	422	11-methyl: 141, 168, 253, 281, (M-15) 13-methyl: 168, 196, 224, 253, (M-15)	MS, L	Co-eluted on GC:FID
12	29.50	4-methylnonacosane	$C_{30}H_{62}$	422	337, 365, (M-15)	MS, L	
13	29.58	11,15- and 13,17-dimethylnonacosane	$C_{31}H_{64}$	436	11,15-dimethyl: 141, 169, 197, 211, 225, 239, 267, 295 13,17-dimethyl: 169, 197, 239, 267 (Symmetrical)	MS, L	Co-eluted on GC:FID
14	29.73	3-methylnonacosane	$C_{30}H_{62}$	422	365, 393 (M-15)	MS, L	
15	29.80	*n*-methylnonacosane	$C_{30}H_{62}$	422	379	MS, L	
16	30.77	*x*-hentriacontene[c,d]	$C_{31}H_{62}$	434	434	MS, L (P)	
17	31.34	11-, 13-, and 15-methylhentriacontane	$C_{32}H_{66}$	450	11-methyl: 141, 168, 218, 309, (M-15) 13-methyl: 168, 196, 252, 281, (M-15) 15-methyl: 196, 224, 252, (M-15)	MS, L	Co-eluted on GC:FID
18	31.64	11,15- and 13,17-dimethylhentriacontane	$C_{33}H_{68}$	464	11,15-dimethyl: 141, 169, 225, 239, 295, 323 13,17-dimethyl: 169, 197, 225, 239, 267, 295	MS, L	Co-eluted on GC:FID

[a]Equivalent chain lengths of all compounds calculated from GC:FID except 1a-d which were calculated from GC:MS.

[b]Techniques for authentication of compound: comparison of ECL values with published literature (L; see Methods); mass spectral fragmentation patterns (MS); comparison to standards (S). Provisional identification (P).

[c]*E-Z* configuration for these compounds is unknown.

[d]ECL values for these compounds suggest the double bond is at the 9-position.

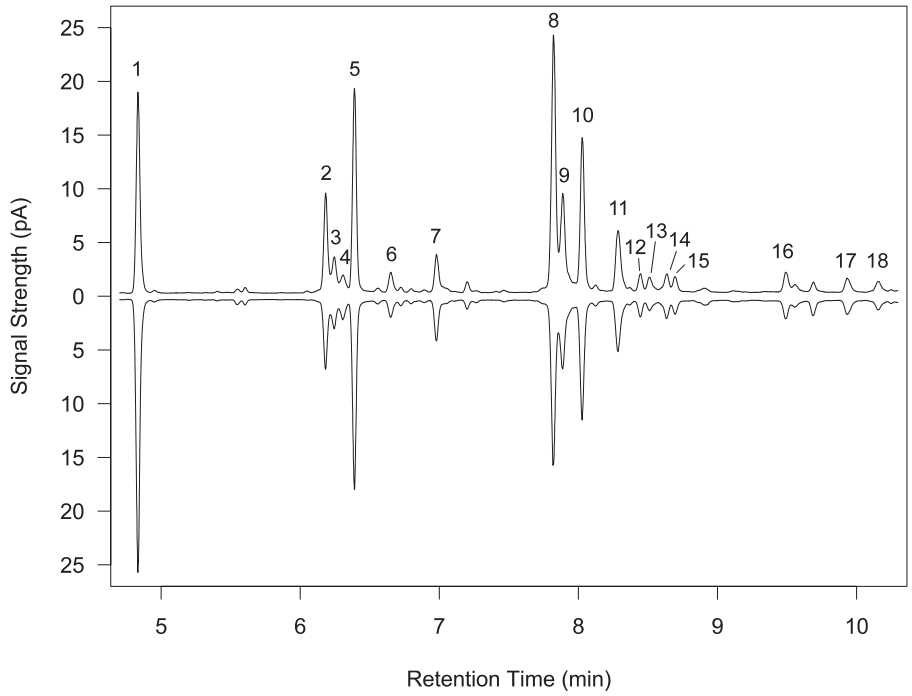

Fig. 1. Mirrored GC:FID chromatographic profiles of male (above) and female (below) *P. litigata*. Numbers correspond to compounds identified in Table 1. Esters 1a-d are not shown because they were not reliably detected via FID.

that they were C_6 branched wax esters of secondary alcohols based on the 99 Da head group shared among them (except 1b, which had a head group of 71 Da, smaller by two CH_2 groups). While MS fragmentation patterns were sufficient to determine the size of the ester head group and alkyl tail, evidence for the placement of the alcohol oxygen on the alkyl chain was equivocal. Thus, we denote these compounds as *x*-alkyl esters (Table 1). Finally, GC:FID revealed one additional low concentration compound that eluted just prior to heptacosane and that had an ECL of 26.89 (FID peak 4, Table 1; Fig. 1). This compound was not detected via GC:MS and therefore remains an unidentified member of the C_{27} cluster.

Concentrations of 18 of the ECs (or co-eluting groups of ECs) were determined via GC:FID, including all 21 hydrocarbons, the unidentified peak #4, and two of the six esters. (Signals for the other four esters, 1a-d, were weak and not reliably detected via GC:FID, suggesting low concentrations.) In both sexes, the highest concentration compounds, on average, included the ester nonadecyl hexanoate (FID peak 1), the C_{27} and C_{29} alkanes, and one of the C_{29} alkenes (Table 2). The square root of total EC amount increased significantly with the cube root of dry mass ($F_{1,37} = 9.16$, $P = 0.004$, $R^2 = 0.199$). While males and females did not differ in the identity of any EC (i.e., all detected compounds were present in both sexes), they did differ significantly in size-corrected EC concentrations overall (MANOVA, $F_{18,20} = 6.78$, $P < 0.001$), with six of the 18 individual compounds (or co-eluting methyl alkanes) exhibiting significant sexual dimorphism (Welch's *t*-tests: $P < 0.05$; Table 2). Overall, the EC profile composition of wild-caught males differed significantly between years (MANOVA: $F_{17, 406} = 154.6$, $P < 0.001$), indicating plastic and/or evolved differences. 13 of the 18 individual EC peaks differed in relative abundance between 2013 and 2017 (Welch's *t*-tests: $P < 0.05$; Fig. 2).

Sexual Selection

Male mating status was significantly associated overall with variation in the principal components of relative EC concentration in both years (LRT, 2013: $\chi^2 = 51.5$, df = 17, $P < 0.001$; 2017: $\chi^2 = 51.5$, df = 17, $P < 0.001$), consistent with linear sexual selection on these traits. EC phenotype explained 12% and 17% of the variance in male mating success in 2013 and 2017 respectively (R^2_{adj}). In 2013, sexual selection was significant on three principal components (PCs 5, 8, and 11), and in 2017 was significant on five principal components, two from 2013 (PCs 5 and 11) and three additional ones (PCs 7, 13, and 15; Supp Table S2 [online only]). Sexual selection also differed significantly between these two years overall (LRT: $\chi^2 = 37.9$, df = 17, $P < 0.003$), with selection on three specific principal components (PCs 7, 8, and 13) differing significantly between 2013 and 2017. The correlation of the normalized selection vectors between 2013 and 2017 was low ($r = 0.194$, equivalent to an angle of 79° between these two vectors), indicating substantial differences in the overall direction of sexual selection in multivariate trait space.

When sexual selection was partitioned into intra- and intersexual components using the 2017 data, we found contrasting patterns. The probability of a territory-holding male achieving a mating remained significantly associated with male EC profile (LRT: $\chi^2 = 42.1$, df = 17, $P < 0.001$), consistent with female choice based on these traits. Five principle components (PCs 5, 11, 13, 15, and 17) were associated with male mating success (Supp Table S3 [online only]). These include four of the five PCs for which significant overall selection was detected in that year (excluding PC7) as well as one additional principal component, PC17. In contrast, the probability of an unmated male holding a territory was not significantly associated with his EC profile (LRT: $\chi^2 = 23.4$, df = 17, $P = 0.14$), suggesting hydrocarbons are not important to success in male–male competition.

When selection was projected back into the original trait space, ten compounds (or groups of compounds) were significantly associated with male mating success in 2013 and six were in 2017 (Fig. 3). These compounds included all classes of molecules identified in this study except unbranched alkanes. Four peaks had a significant association in both years, but only two (peaks 9 and 16, representing *x*-nonacosene and *x*-hentriacontene, respectively) were in the same direction. In contrast, the selection gradients for

Table 2. Mean ± standard error (SE) of absolute and size-corrected abundances of epicuticular compounds in male and female *P. litigata*

	Absolute compound abundance (ng)		Size-corrected values (ng)		
FID peak no.[a]	Males (SE)	Females (SE)	Males (SE)	Females (SE)	Sexual dimorphism (P)[b]
1	87.2 (3.89)	134.3 (11.6)	**94.2 (5.53)**	**124.9 (8.56)**	**0.005**
2	39.5 (2.99)	36.2 (2.87)	40.6 (3.14)	34.9 (2.48)	0.161
3	20.0 (1.21)	19.2 (1.39)	20.4 (1.27)	18.6 (1.19)	0.270
4	14.2 (0.614)	16.7 (1.24)	14.8 (0.712)	16.0 (0.999)	0.331
5	91.8 (6.42)	120.4 (10.9)	97.1 (6.82)	113.4 (8.95)	0.158
6	13.5 (0.551)	13.3 (0.722)	13.7 (0.574)	13.0 (0.655)	0.450
7	19.0 (1.13)	27.4 (1.94)	**20.1 (1.28)**	**25.9 (1.51)**	**0.006**
8	103.0 (7.14)	93.7 (7.54)	106.1 (7.56)	90.0 (6.33)	0.112
9	55.7 (3.22)	50.0 (4.31)	57.2 (3.41)	48.2 (3.75)	0.083
10	75.7 (4.00)	74.0 (5.79)	77.7 (4.13)	71.6 (5.10)	0.358
11	36.5 (1.95)	37.6 (2.53)	37.4 (2.09)	36.5 (2.18)	0.761
12	12.1 (0.637)	12.7 (0.536)	12.4 (0.500)	13.1 (0.522)	0.296
13	11.9 (0.526)	13.5 (0.63)	12.1 (0.561)	12.5 (0.450)	0.616
14	12.7 (0.637)	19.2 (1.38)	**13.4 (0.740)**	**18.2 (1.14)**	**0.001**
15	11.2 (0.434)	14.0 (0.641)	**11.5 (0.482)**	**13.6 (0.524)**	**0.006**
16	13.0 (0.497)	15.9 (0.897)	13.4 (0.561)	15.3 (0.746)	0.054
17	14.6 (0.536)	16.6 (0.848)	**14.9 (0.588)**	**17.3 (0.764)**	**0.020**
18	12.1 (0.521)	14.9 (0.72)	**12.4 (0.558)**	**14.6 (0.646)**	**0.016**

Bold denotes significant sexual dimorphism in size-corrected abundances.

[a]FID peak numbers refer to compounds identified in Table 1.

[b]Significance from a Welch's *t*-test performed separately on the size-corrected abundance of each compound.

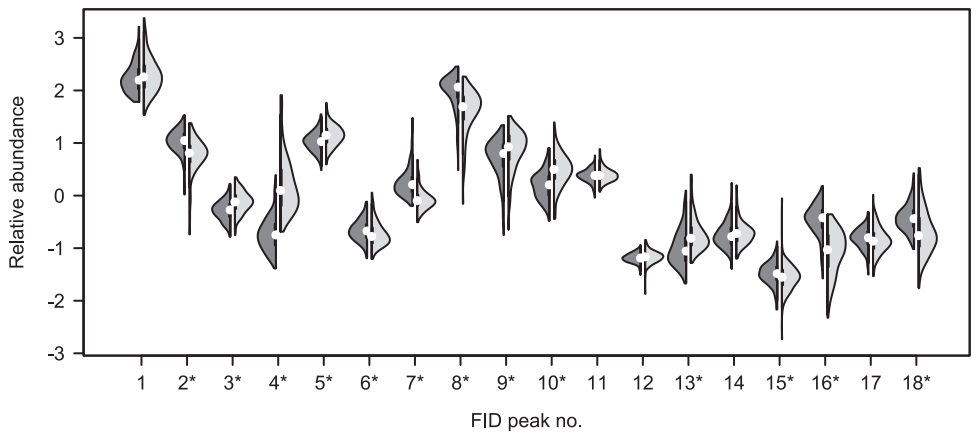

Fig. 2. Violin plot showing CLR-transformed relative abundances of male epicuticular compounds collected in the wild across two years. Dark gray violins are males collected in 2013 (n = 251) and light gray violins are males collected in 2017 (n = 173). White circles represent the median and the internal black boxes demark the first and third quartiles. Asterisks beside peak numbers indicate significant differences between years (Welch's *t*-tests: $P < 0.05$). See Table 1 for compound identifications.

peak 6 (11- and 13-methylheptacosane, co-eluted) and peak 14 (3-methylnonacosane) were significant in both years but opposite in sign. In general, the pattern of intersexual selection in 2017 was similar to the overall pattern of sexual selection in that year, although two peaks differed in their significance between the two analyses (Fig. 3B and C). Peak 18 (11,15-, and 13,17-dimethylhentriacontane, co-eluted) was significant only in the 'overall' selection analysis, while peak 2 (*x*-heptacosene) was significant in the intersexual analysis but not overall.

Discussion

Although insects' ECs have been implicated in both desiccation resistance (e.g., Beament 1945, Wigglesworth 1945) and female mate choice (e.g., Jallon and David 1987, Howard et al. 2003) for decades, they remain uncharacterized in many species and behavioral studies are often laboratory-based such that the importance of these traits as sexual signals has rarely been studied in nature (but see Hine et al. 2004, Steiger et al. 2013, Gosden et al. 2016). Here we identified (in some cases provisionally) the predominant ECs of the antler fly, *Protopiophila litigata*, quantified sexual dimorphism in these traits, and measured their association with mating success over two years in males in the wild. We also partitioned this association into intra- versus intersexual components to gain further insight into their potential role in mate competition. Due to our whole-fly extraction protocol, we do not know the fine-scale localization of ECs across the body of *P. litigata* (Niehoff et al. 2014), although this may also be important to sexual interactions (Yew et al. 2011, Booksmythe et al. 2017).

The ECs of *P. litigata* clustered primarily around odd carbon numbers, a pattern that has previously been observed to varying degrees in other insects (Blomquist et al. 1985, Howard et al. 2003,

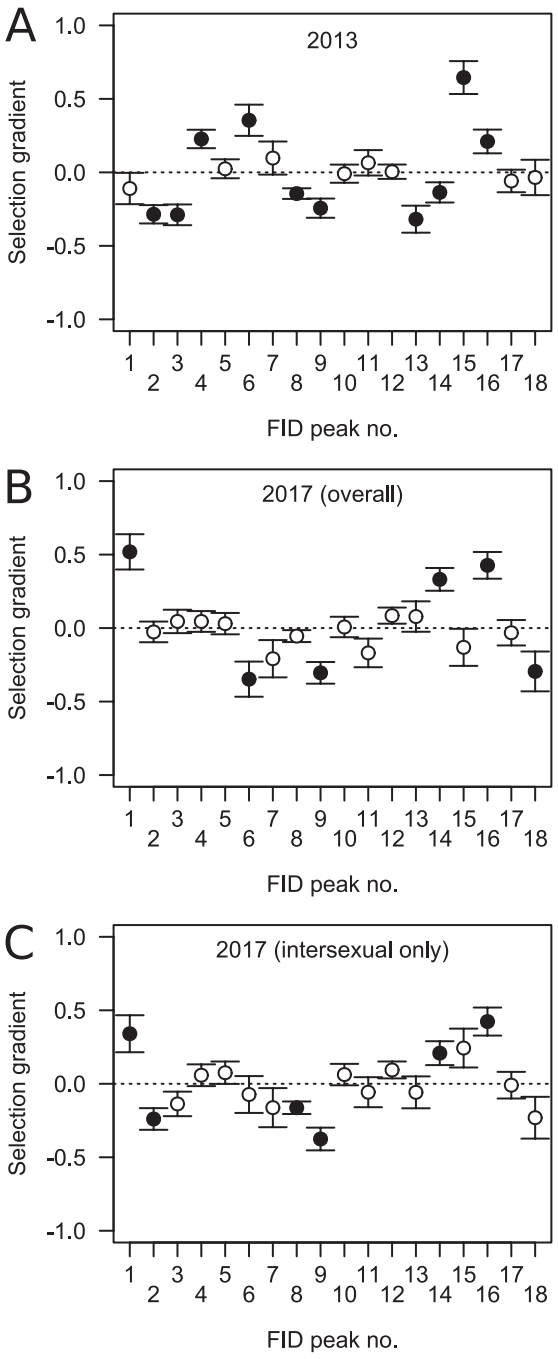

Fig. 3. Sexual selection gradients (±SE) on 18 epicuticular compounds (or groups of compounds) in wild *P. litigata* males in (A) 2013, (B) 2017, and (C) 2017 including intersexual selection only (see Methods). Filled symbols represent significant sexual selection gradients ($P < 0.05$, based on single-year *t*-tests), and open symbols represent nonsignificant estimates. See Table 1 for compound identifications.

Yew et al. 2011, Curtis et al. 2013). The majority of compounds present in the cuticle were hydrocarbons that ranged from C_{27} to C_{31} and included alkanes, alkenes, and several methyl-branched alkanes that collectively span the upper range of chain lengths commonly observed for hydrocarbons in *Drosophila* (Ferveur 2005). The number and variety of hydrocarbons we detected is not unusual for an insect (Ferveur 2005). Five of our 18 FID peaks were made up of multiple co-eluting methyl (peaks 6, 11, and 17) or dimethyl (peaks 13 and 18) alkanes, with methyl branch locations shared across peaks (Table 1). These hydrocarbons have previously been shown to co-elute at the ECL we report (Howard et al. 2001).

In addition to hydrocarbons, we also provisionally identified six homologous branched wax esters, two that are highly abundant and four that are less prevalent. Esters have been reported as cuticular pheromones, or potential pheromones, in flies (Chao et al. 2010, Dweck et al. 2015), bees (Buckner et al. 2009), ants (Nelson et al. 2001), wasps (Howard and Baker 2003), thrips (Krueger et al. 2016), beetles (Keppner et al. 2017), and spiders (Chinta et al. 2016). Wax esters of secondary alcohols, like those identified here, have occasionally been reported as ECs in various insects (Blomquist et al. 1972, Finidori-Logli et al. 1996, Howard and Baker 2003, Böröczky et al. 2008).

We detected no sex-specific ECs (i.e., all were shared by males and females). Although many insects display qualitative sexual dimorphism with respect to EC identity (e.g., Curtis et al. 2013, Dweck et al. 2015), not all do (e.g., Bartelt et al. 1986, Jallon and David 1987, Howard et al. 2003). We did detect quantitative differences in compound absolute abundances between males and females, however, even after accounting for sexual dimorphism in body size. Such quantitative sexual dimorphism in EC abundances is prevalent in insects (e.g., Bartelt et al. 1986, Jallon and David 1987, Howard et al. 2003, Curtis et al. 2013, Booksmythe et al. 2017).

Sexual dimorphism suggests a history of sex-specific selection, with sexual selection being a prime candidate. Consequently, one might expect the ECs under consistent sexual selection to exhibit greater sexual dimorphism. However, this did not appear to be the case. Six of the compounds (or groups of compounds) we quantified displayed significant sexual dimorphism in abundance, after correcting for differences in body size (Table 2), but none of them were under consistent selection across the sampling years. Three of them (peaks 1, 15, and 18) were associated with male mating success in only one year, and one (peak 14) was negatively associated with mating success in 2013 but positively associated in 2017. Two of the sexually dimorphic compounds, peaks 7 and 17, were not significantly associated with male mating success in either year (Fig. 3). Conversely, neither peak 9 nor peak 16, which were consistently associated with male mating success, differed between the sexes, although the dimorphism for peak 16 approached significance. Overall, these data suggest that sexual dimorphism in EC abundances do not reflect the strength of contemporary sexual selection on male traits. This is not surprising as current selection may differ from that experienced historically, and the evolution of sexual dimorphism depends also on genetic constraints between the sexes that arise from a shared genome (Ingleby et al. 2014).

We detected significant differences in male EC relative abundances in this population between our sampling years. These may partly represent evolved differences in the EC phenotype, but they could also result from plastic responses to differing environmental conditions. The summer of 2013 was hot and dry, whereas 2017 was unusually cold and wet. Insects can alter their EC profile to aid in desiccation resistance (Stinziano et al. 2015), so it is plausible that flies in each year adjusted their ECs to cope with different environmental conditions. Some laboratory studies suggest that an increase in the relative abundance of longer, more saturated, and less-branched hydrocarbons is associated with increased resistance to desiccation (Gibbs and Pomonis 1995, Gibbs et al. 1997, Stinziano et al. 2015). Evidence for the relative enrichment of such ECs in 2013 is mixed: while 2013 males had relatively higher levels of some longer-chain hydrocarbons (peaks 16 and 18), they also displayed high levels of relatively short-chained peaks 2 and 7 (Fig. 2). Furthermore, both unbranched, saturated alkanes were significantly higher in relative

abundance in 2017 (peaks 5 and 10; Fig. 2). There may be other ways to achieve desiccation resistance, a possibility consistent with the fact that desert-adapted *Drosophila mojavensis* display many branched and unsaturated hydrocarbons (Toolson et al. 1990).

A cross-sectional selection analysis revealed significant association between relative EC concentrations and mating success in males in the wild, consistent with directional sexual selection on these traits. Variation in male EC phenotype explained 12% and 17% of variation in mating success in 2013 and 2017, respectively. These values are similar to those seen in binomial analyses of mated versus nonmated males in other species in which the role of ECs as sexual displays has been well established, although primarily via lab studies (Chenoweth and Blows 2005, Van Homrigh et al. 2007, Curtis et al. 2013), and it suggests that ECs may be an important sexual signal in *P. litigata* as well. However, like all observational selection analyses, it is possible that variation in male mating success was causally associated with one or more other, unmeasured traits that are phenotypically correlated with EC variation in this population, such as male body size, behavior, or overall health and vigor. Confirmation of a role for ECs in antler flies will therefore require isolating these traits (e.g., assaying the effects of EC extracts alone), testing the effects of sensory manipulations (e.g., Giglio and Dyer 2013), or manipulating EC profile via perfuming assays or a selection experiment (e.g., Hine et al. 2011, Dyer et al. 2014). It is also possible that observed EC differences between mated and single males are a consequence of mating itself (e.g., if ECs highly abundant on females are transferred to males during mating). While this remains to be addressed in *P. litigata*, mating is known not to alter the sexually selected ECs of male *Drosophila serrata* (Gershman and Rundle 2016). Nevertheless, ECs are confirmed as sexual signals in diverse arthropod species, and the existing sexual dimorphism in EC abundances in antler flies implies a history of sexually antagonistic selection, consistent with these traits being targets of sexual selection in males.

When sexual selection was partitioned into its intra- and intersexual components, ECs were significantly associated with the mating success of territory-holding males, suggesting female mate choice for particular pheromone blends. In contrast, we did not detect any association between male EC profile and territory possession, suggesting ECs are not used in male–male interactions related to acquiring or holding a territory on the upper surface of an antler. Variation in ECs has been implicated in male–male competition in some insects (e.g., Moore and Moore 1999, Lane et al. 2016), although in *D. serrata*, similar to antler flies, ECs have been implicated as a target of female mate choice but do not affect male–male contests over territories (White and Rundle 2015).

In our case, non-territory-holding males were collected from the underside of moose antlers where males rarely defend territories. However, male *P. litigata* frequently rest underneath the antler for a period of time, ranging from minutes to hours, after mate guarding is completed following a successful mating (Bonduriansky and Brooks 1998b). For this reason, our males without territories may have included both individuals that had mated—and thus, likely had held a territory previously—and those that had not. This may have reduced the power to detect an association between ECs and territory success. Further insight into the role of ECs in intrasexual competition would require associating EC variation with the outcome of observed male–male contests.

In addition, territoriality is not the only form of intrasexual competition in *P. litigata*. Single males sometimes attempt to take over matings from copulating or mate guarding rivals (Bonduriansky and Brooks 1998b). These take-overs may represent an alternative intrasexual strategy or a common mating tactic that is not captured by data on territory holding alone. However, because male take-overs are rarely successful (Bonduriansky and Brooks 1998b), we consider territory defense to be the main manifestation of intrasexual selection in *P. litigata*.

Finally, the association between mating success and particular male ECs differed significantly between our two sampling years, suggesting temporal variation in sexual selection. This is consistent with many other multi-year studies of selection in the wild, which have often found temporal changes both in the magnitude and direction of selection on phenotypic traits (Siepielski et al. 2009). Seven compounds (or groups of compounds) were associated with mating success in only one of our sampling years, and two, peaks 6 (11- and 13-methylheptacosane, co-eluted) and 14 (3-methylnonacosane), were subject to opposing selection between the years (Fig. 3). Strikingly, only two of the 18 compounds, peak 9 (*x*-nonacosene) and peak 16 (*x*-hentriacontene), were concordantly associated with mating success across years. It is conceivable that these differences could result from changes in the mean EC phenotype between years despite a static fitness surface, if selection was nonlinear and trait distributions were located on different parts of the surface each year. However, although a few ECs show very different ranges of relative abundance between the sampling years (e.g., peaks 4, 16, and 18), many compounds that differed significantly still broadly overlap in relative abundance (Fig. 2). In these cases of high phenotypic overlap, it is likely that selection on ECs truly differed through time.

Environmental and demographic changes are known to drive temporal variation in sexual selection (Jann et al. 2000, Kasumovic et al. 2008, Miller and Svensson 2014) and could therefore underlie the between-year differences we observed. For example, both the strength and direction of sexual selection on males can vary with density and sex ratio (Jann et al. 2000, Kasumovic et al. 2008). Furthermore, mate preferences, like the sexual displays they target, are traits under selection, and this selection may change through time (Chaine and Lyon 2008), so temporal variation in sexual selection may also arise from changes in female mate preferences themselves, either evolved (Dodd 1989) or plastic (Chaine and Lyon 2008, Robinson et al. 2012). In addition, processes such as mate-choice copying (e.g., Mery et al. 2009) and preferences for rare phenotypes (e.g., Hughes et al. 2013) might lead to variable mate preferences. In addition, the genetic background of the 2013 and 2017 populations may have differed due to natural migration as well as experimental relocation of larva-containing antlers, potentially contributing to differences in both EC profiles and mate preferences.

Alternatively, healthier and more vigorous males often have greater success in reproductive competition, meaning sexual as well as natural selection may favor traits that increase male vigor. ECs play an important role in desiccation resistance and relative enrichment of longer, more saturated hydrocarbons is associated with decreased cuticular permeability in *Drosophila melanogaster* (Gibbs et al. 1997, Stinziano et al. 2015). Therefore, EC phenotypes that promoted male vigor, and hence male mating success, in hot, dry 2013 may not have had similar positive effects in colder, wetter 2017. Accordingly, changes in selection on ECs between our sampling years may have arisen from consistent female preference for healthier, more vigorous males rather than variation in their preferred pheromone blends. Understanding the underlying causes and consequences of temporal variation in sexual selection will be a challenge for evolutionary and behavioral ecologists going forward.

Supplementary Data

Supplementary data are available at *Annals of the Entomological Society of America* online.

Acknowledgments

We thank Olivia Cook, Corey Godfrey, and Brian Mautz for their assistance in data collection and the University of Ottawa for supporting the John L. Holmes Mass Spectrometry Facility. This work was also funded by a grant from the Natural Sciences and Engineering Research Council of Canada (HDR).

References Cited

Aitchison, J. 1986. The statistical analysis of compositional data. Monographs on statistics and applied probability. Chapman and Hall, London, United Kingdom.

Ali, J. G., and D. W. Tallamy. 2010. Female spotted cucumber beetles use own cuticular hydrocarbon signature to choose immunocompatible mates. Anim. Behav. 80: 9–12.

Adler, D., and S.T. Kelly. 2018. vioplot: violin plot. The Comprehensive R Archive Network. https://CRAN.R-project.org/package=vioplot

Angell, C., S. Curtis, A. Ryckenbusch, and H. Rundle, Howard. 2019. Data from: Epicuticular compounds of *Protopiophila litigata* (Diptera: Piophilidae): identification and sexual selection across two years in the wild. Dryad Digital Repository. doi:10.5061/dryad.2jm63xsjn

Bartelt, R. J., M. T. Armold, A. M. Schaner, and L. L. Jackson. 1986. Comparative analysis of cuticular hydrocarbons in the *Drosophila virilis* species group. Comp. Biochem. Physiol. Part B Comp. Biochem. 83: 731–742.

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

Blomquist, G. J., C. L. Soliday, B. A. Byers, J. W. Brakke, and L. L. Jackson. 1972. Cuticular lipids of insects: V. Cuticular wax esters of secondary alcohols from the grasshoppers *Melanoplus packardii* and *Melanoplus sanguinipes*. Lipids. 7: 356–362.

Blomquist, G. J., E. C. Toolson, and D. R. Nelson. 1985. Epicuticular hydrocarbons of *Drosophila pseudoobscura* (Diptera; Drosophilidae) Identification of unusual alkadiene and alkatriene positional isomers. Insect Biochem. 15: 25–34.

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

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

Bonduriansky, R. 1995. A new Nearctic species of *Protopiophila* Duda (Diptera: Piophilidae), with notes on its behaviour and comparison with *P. latipes* (Meigen). Can. Entomol. 127: 859–863.

Bonduriansky, R. 2003. Layered sexual selection: a comparative analysis of sexual behaviour within an assemblage of piophilid flies. Can. J. Zool. 81: 479–491.

Bonduriansky, R., and R. J. Brooks. 1998a. Male antler flies (*Protopiophila litigata*; Diptera: Piophilidae) are more selective than females in mate choice. Can. J. Zool. 76: 1277–1285.

Bonduriansky, R., and R. J. Brooks. 1998b. Copulation and oviposition behavior of *Protopiophila litigata* (Diptera: Piophilidae). Can. Entomol. 130: 399–405.

Bonduriansky, R., and R. J. Brooks. 1999. Why do male antler flies (*Protopiophila litigata*) fight? The role of male combat in the structure of mating aggregations on moose antlers. Ethol. Ecol. Evol. 11: 287–301.

Bonduriansky, R., M. A. Mallet, D. Arbuthnott, V. Pawlowsky-Glahn, J. J. Egozcue, and H. D. Rundle. 2015. Differential effects of genetic vs. environmental quality in *Drosophila melanogaster* suggest multiple forms of condition dependence. Ecol. Lett. 18: 317–326.

Booksmythe, I., H. D. Rundle, and G. Arnqvist. 2017. Sexual dimorphism in epicuticular compounds despite similar sexual selection in sex role-reversed seed beetles. J. Evol. Biol. 30: 2005–2016.

Böröczky, K., K. C. Park, R. D. Minard, T. H. Jones, T. C. Baker, and J. H. Tumlinson. 2008. Differences in cuticular lipid composition of the antennae of *Helicoverpa zea*, *Heliothis virescens*, and *Manduca sexta*. J. Insect Physiol. 54: 1385–1391.

Buckner, J. S., T. L. Pitts-Singer, C. Guédot, M. M. Hagen, C. L. Fatland, and W. P. Kemp. 2009. Cuticular lipids of female solitary bees, *Osmia lignaria* Say and *Megachile rotundata* (F.) (Hymenoptera: Megachilidae). Comp. Biochem. Physiol. B. Biochem. Mol. Biol. 153: 200–205.

Chaine, A. S., and B. E. Lyon. 2008. Adaptive plasticity in female mate choice dampens sexual selection on male ornaments in the lark bunting. Science. 319: 459–462.

Chao, T. H., L. Ehrman, A. Permaul, R. Vincent, L. Sattaur, and D. Brandt. 2010. Male-specific cuticular compounds of the six *Drosophila paulistorum* semispecies: structural identification and mating effect. J. Chem. Ecol. 36: 933–942.

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

Chenoweth, S. F., J. Hunt, and H. D. Rundle. 2012. Analyzing and comparing the geometry of individual fitness surfaces, pp. 126–149. *In* Svensson, E., Calsbeek, R. (eds.), The adaptive landscape in evolutionary biology. Oxford University Press, Oxford.

Chinta, S. P., S. Goller, G. Uhl, and S. Schulz. 2016. Identification and Synthesis of Branched Wax-type Esters, Novel Surface Lipids from the Spider *Argyrodes elevatus* (Araneae: Theridiidae). Chem. Biodivers. 13: 1202–1220.

Chong, V. K., H. F. Fung, and J. R. Stinchcombe. 2018. A note on measuring natural selection on principal component scores. Evol. Lett. 2: 272–280.

Curran, J. 2017. Hotelling: hotelling's T^2 Test and Variants. The Comprehensive R Archive Network. https://CRAN.R-project.org/package=Hotelling

Curtis, S., J. L. Sztepanacz, B. E. White, K. A. Dyer, H. D. Rundle, and P. Mayer. 2013. Epicuticular compounds of *Drosophila subquinaria* and *D. recens*: identification, quantification, and their role in female mate choice. J. Chem. Ecol. 39: 579–590.

Dodd, D. M. B. 1989. Reproductive isolation as a consequence of adaptive divergence in *Drosophila pseudoobscura*. Evolution. 43: 1308–1311.

Dweck, H. K., S. A. Ebrahim, M. Thoma, A. A. Mohamed, I. W. Keesey, F. Trona, S. Lavista-Llanos, A. Svatoš, S. Sachse, M. Knaden, et al. 2015. Pheromones mediating copulation and attraction in *Drosophila*. Proc. Natl. Acad. Sci. U. S. A. 112: E2829–E2835.

Dyer, K. A., B. E. White, J. L. Sztepanacz, E. R. Bewick, and H. D. Rundle. 2014. Reproductive character displacement of epicuticular compounds and their contribution to mate choice in *Drosophila subquinaria* and *Drosophila recens*. Evolution. 68: 1163–1175.

Egozcue, J. J., and V. Pawlowsky-Glahn. 2011. Basic concepts and procedures, pp. 12–27. *In* V. Pawlowsky-Glahn, A. Buccianti (eds.), Compositional data analysis: theory and applications. John Wiley & Sons, Hoboken, NJ.

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

Fang, S., A. Takahashi, and C. I. Wu. 2002. A mutation in the promoter of desaturase 2 is correlated with sexual isolation between *Drosophila* behavioral races. Genetics. 162: 781–784.

Fedina, T. Y., D. Arbuthnott, H. D. Rundle, D. E. L. Promislow, and S. D. Pletcher. 2017. Tissue-specific insulin signaling mediates female sexual attractiveness. PLoS Genet. 13: e1006935.

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

Finidori-Logli, V., A. G. Bagnères, D. Erdmann, W. Francke, and J. L. Clément. 1996. Sex recognition in *Diglyphus* isaea walker (Hymenoptera: Eulophidae): role of an uncommon family of behaviorally active compounds. J. Chem. Ecol. 22: 2063–2079.

Fox, J., and S. Weisberg. 2011. An R Companion to Applied Regression, 2nd ed. Sage, Thousand Oaks, CA.

Gershman, S. N., and H. D. Rundle. 2016. Level up: the expression of male sexually selected cuticular hydrocarbons is mediated by sexual experience. Anim. Behav. 112: 169–177.

Gershman, S. N., and H. D. Rundle. 2017. Crowd control: sex ratio affects sexually selected cuticular hydrocarbons in male *Drosophila serrata*. J. Evol. Biol. 30: 583–590.

Gershman, S. N., E. Toumishey, and H. D. Rundle. 2014. Time flies: time of day and social environment affect cuticular hydrocarbon sexual displays in *Drosophila serrata*. Proc. R. Soc. Lond. B Biol. Sci. 281: 20140821.

Gibbs, A., and J. G. Pomonis. 1995. Physical properties of insect cuticular hydrocarbons: the effects of chain length, methyl-branching and unsaturation. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 112: 243–249.

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

Giglio, E. M., and K. A. Dyer. 2013. Divergence of premating behaviors in the closely related species *Drosophila subquinaria* and *D. recens*. Ecol. Evol. 3: 365–374.

Gosden, T. P., J. R. Thomson, M. W. Blows, A. Schaul, and S. F. Chenoweth. 2016. Testing for a genetic response to sexual selection in a wild *Drosophila* population. J. Evol. Biol. 29: 1278–1283.

Grillet, M., L. Dartevelle, and J. F. Ferveur. 2006. A *Drosophila* male pheromone affects female sexual receptivity. Proc. R. Soc. Lond. B Biol. Sci. 273: 315–323.

Higgie, M., S. Chenoweth, and M. W. Blows. 2000. Natural selection and the reinforcement of mate recognition. Science. 290: 519–521.

Hine, E., K. McGuigan, and M. W. Blows. 2011. Natural selection stops the evolution of male attractiveness. Proc. Natl. Acad. Sci. U. S. A. 108: 3659–3664.

Hine, E., S. F. Chenoweth, and M. W. Blows. 2004. Multivariate quantitative genetics and the lek paradox: genetic variance in male sexually selected traits of *Drosophila serrata* under field conditions. Evolution. 58: 2754–2762.

Howard, R. W., and J. E. Baker. 2003. Cuticular hydrocarbons and wax esters of the ectoparasitoid *Habrobracon hebetor*: ontogenetic, reproductive, and nutritional effects. Arch. Insect Biochem. Physiol. 53: 1–18.

Howard, R. W., G. Pérez-Lachaud, and J.-P. Lachaud. 2001. Cuticular hydrocarbons of *Kapala sulcifacies* (Hymenoptera: Eucharitidae) and its host, the ponerine ant *Ectatomma ruidum* (Hymenoptera: Formicidae). Ann. Entomol. Soc. Am. 94: 707–716.

Howard, R. W., L. L. Jackson, H. Banse, and M. W. Blows. 2003. Cuticular hydrocarbons of *Drosophila birchii* and *D. serrata*: identification and role in mate choice in D. serrata. J. Chem. Ecol. 29: 961–976.

Hughes, K. A., A. E. Houde, A. C. Price, and F. H. Rodd. 2013. Mating advantage for rare males in wild guppy populations. Nature. 503: 108–110.

Ingleby, F. C., P. Innocenti, H. D. Rundle, and E. H. Morrow. 2014. Between-sex genetic covariance constrains the evolution of sexual dimorphism in *Drosophila melanogaster*. J. Evol. Biol. 27: 1721–1732.

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

Jann, P., W. U. Blanckenhorn, and P. I. Ward. 2000. Temporal and microspatial variation in the intensities of natural and sexual selection in the yellow dung fly *Scathophaga stercoraria*. J. Evol. Biol. 13: 927–938.

Kasumovic, M. M., M. J. Bruce, M. C. Andrade, and M. E. Herberstein. 2008. Spatial and temporal demographic variation drives within-season fluctuations in sexual selection. Evolution. 62: 2316–2325.

Keppner, E. M., M. Prang, K. C. Engel, M. Ayasse, J. Stökl, and S. Steiger. 2017. Beyond cuticular hydrocarbons: chemically mediated mate recognition in the subsocial burying beetle *Nicrophorus vespilloides*. J. Chem. Ecol. 43: 84–93.

Krueger, S., G. Moritz, P. Lindemann, D. Radisch, and G. Tschuch. 2016. Male pheromones influence the mating behavior of *Echinothrips americanus*. J. Chem. Ecol. 42: 294–299.

Kwan, L., and H. D. Rundle. 2010. Adaptation to desiccation fails to generate pre- and postmating isolation in replicate *Drosophila melanogaster* laboratory populations. Evolution. 64: 710–723.

Lafi, S. Q., and J. B. Kaneene. 1992. An explanation of the use of principal-components analysis to detect and correct for multicollinearity. Prev. Vet. Med. 13: 261–275.

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

Lane, S. M., A. W. Dickinson, T. Tregenza, and C. M. House. 2016. Sexual Selection on male cuticular hydrocarbons via male-male competition and female choice. J. Evol. Biol. 29: 1346–1355.

Lihoreau, M., and C. Rivault. 2009. Kin recognition via cuticular hydrocarbons shapes cockroach social life. Behav. Ecol. 20: 46–53.

Martin, S. J., H. Helanterä, and F. P. Drijfhout. 2008. Evolution of species-specific cuticular hydrocarbon patterns in *Formica* ants. Biol. J. Linn. Soc. 95: 131–140.

Mautz, B., S., N. O. Rode, R. Bonduriansky, and H. D. Rundle. 2019. Comparing ageing and the effects of diet supplementation in wild vs. captive antler flies, *Protopiophila litigata*. J. Anim. Ecol. In press.

Mery, F., S. A. Varela, E. Danchin, S. Blanchet, D. Parejo, I. Coolen, and R. H. Wagner. 2009. Public versus personal information for mate copying in an invertebrate. Curr. Biol. 19: 730–734.

Miller, C. W., and E. I. Svensson. 2014. Sexual selection in complex environments. Annu. Rev. Entomol. 59: 427–445.

Moore, A. J., and P. J. Moore. 1999. Balancing sexual selection through opposing mate choice and male competition. Proc. R. Soc. Lond. B Biol. Sci. 266: 711–716.

Nelson, D. R., M. Tissot, L. J. Nelson, C. L. Fatland, and D. M. Gordon. 2001. Novel wax esters and hydrocarbons in the cuticular surface lipids of the red harvester ant, *Pogonomyrmex barbatus*. Comp. Biochem. Physiol. B. Biochem. Mol. Biol. 128: 575–595.

Niehoff, A. C., H. Kettling, A. Pirkl, Y. N. Chiang, K. Dreisewerd, and J. Y. Yew. 2014. Analysis of *Drosophila* lipids by matrix-assisted laser desorption/ionization mass spectrometric imaging. Anal. Chem. 86: 11086–11092.

Oudin, M. J., R. Bonduriansky, and H. D. Rundle. 2015. Experimental evidence of condition-dependent sexual dimorphism in the weakly dimorphic antler fly *Protopiophila litigata* (Diptera: Piophilidae). Biol. J. Linn. Soc. 116: 211–220.

Petfield, D., S. F. Chenoweth, H. D. Rundle, and M. W. Blows. 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.

R Core Team. 2018. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Robinson, M. R., G. S. van Doorn, L. Gustafsson, and A. Qvarnström. 2012. Environment-dependent selection on mate choice in a natural population of birds. Ecol. Lett. 15: 611–618.

Rochefort, S., M. Giroux, J. Savage, and T. A. Wheeler. 2015. Key to forensically important Piophilidae (Diptera) in the Nearctic region. Can. J. Arthropod Identif. 27: 1–37.

Rundle, H. D., S. F. Chenoweth, and M. W. Blows. 2008. Comparing complex fitness surfaces: among-population variation in mutual sexual selection in *Drosophila serrata*. Am. Nat. 171: 443–454.

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

Siepielski, A. M., J. D. DiBattista, and S. M. Carlson. 2009. It's about time: the temporal dynamics of phenotypic selection in the wild. Ecol. Lett. 12: 1261–1276.

Steiger, S., G. D. Ower, J. Stökl, C. Mitchell, J. Hunt, and S. K. Sakaluk. 2013. Sexual selection on cuticular hydrocarbons of male sagebrush crickets in the wild. Proc. R. Soc. Lond. B Biol. Sci. 280: 20132353.

Stinziano, J. R., R. J. Sové, H. D. Rundle, and B. J. Sinclair. 2015. Rapid desiccation hardening changes the cuticular hydrocarbon profile of *Drosophila melanogaster*. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 180: 38–42.

Thomas, M. L., and L. W. Simmons. 2009. Sexual selection on cuticular hydrocarbons in the Australian field cricket, *Teleogryllus oceanicus*. BMC Evol. Biol. 9: 162.

Toolson, E. C., T. A. Markow, L. L. Jackson, and R. W. Howard. 1990. Epicuticular hydrocarbon composition of wild and laboratory-reared *Drosophila mojavensis* Patterson and Crow (Diptera: Drosophilidae). Ann. Entomol. Soc. Am. 83: 1165–1176.

Van Homrigh, A., M. Higgie, K. McGuigan, and M. W. Blows. 2007. The depletion of genetic variance by sexual selection. Curr. Biol. 17: 528–532.

Vaníčková, L., A. Svatoš, J. Kroiss, M. Kaltenpoth, R. R. do Nascimento, M. Hoskovec, R. Břízová, and B. Kalinová. 2012. Cuticular hydrocarbons of the South American fruit fly *Anastrepha fraterculus*: variability with sex and age. J. Chem. Ecol. 38: 1133–1142.

Vásquez, G. M., C. Schal, and J. Silverman. 2009. Colony fusion in Argentine ants is guided by worker and queen cuticular hydrocarbon profile similarity. J. Chem. Ecol. 35: 922–932.

White, A. J., and H. D. Rundle. 2015. Territory defense as a condition-dependent component of male reproductive success in *Drosophila serrata*. Evolution. 69: 407–418.

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

Yew, J. Y., K. Dreisewerd, C. C. de Oliveira, and W. J. Etges. 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.

Supplemental Information

for

Epicuticular compounds of *Protopiophila litigata* (Diptera: Piophilidae): identification and sexual selection across two years in the wild

Annals of the Entomological Society of America

Christopher S. Angell[1], Sharon Curtis[2], Anaïs Ryckenbusch[2], and Howard D. Rundle[1]*

[1]*Department of Biology, University of Ottawa; Ottawa ON, K1N 6N5, Canada*

[2]*Department of Chemistry, University of Ottawa; Ottawa ON, K1N 6N5, Canada*

*Corresponding author, e-mail: hrundle@uottawa.ca.

Compound Identification

Gas chromatography with mass spectrometry (GC:MS) was used to generate mass spectra for individual epicuticular compounds (ECs), as described in "Methods and Materials" of the main text. Equivalent chain lengths (ECL) were also calculated for each chromatogram peak using a C_7-C_{40} alkane standard (Sigma Aldrich, product # 49452-U), following (Curtis et al. 2013). In general, MS fragmentation patterns and ECL numbers were cross referenced with databases, i.e. the Wiley 275 GC:MS database, NIST MS search 2.0, NIST Chemistry WebBook SRD 69 (https://webbook.nist.gov/chemistry), and Pherobase (www.pherobase.com) and published literature sources (McLafferty 1980, Lockey 1988, Howard and Blomquist 2005). ECL values themselves can also be used to make inferences about compound identities, as hydrocarbons with similar structures (e.g. locations of methyl groups or double bonds) exhibit the same fractional values (i.e. x.75), while differences in the integer portion reflect differences in overall carbon length (Bartelt et al. 1986).

Alkanes

Linear alkanes were identified by direct comparison of ECL and mass spectrum with the C_7-C_{40} alkane standard. For example, the ECL of heptacosane occurs at exactly at 27.00 and the mass spectrum is marked by a characteristic pattern of fragment ions, as can be observed in Fig. S1 (inset). FID peak 5 (see Table 1) of the antler fly sample also has a peak at the same ECL and retention time, with a nearly identical mass spectrum. This peak is therefore identified as heptocosane ($C_{27}H_{56}$, 380, Da). Similarly, FID peak 10 (ECL 29.00) was identified as nonacosane ($C_{29}H_{60}$, 408 Da) by comparison of the mass spectrum and ECL to nonacosane from the standard.

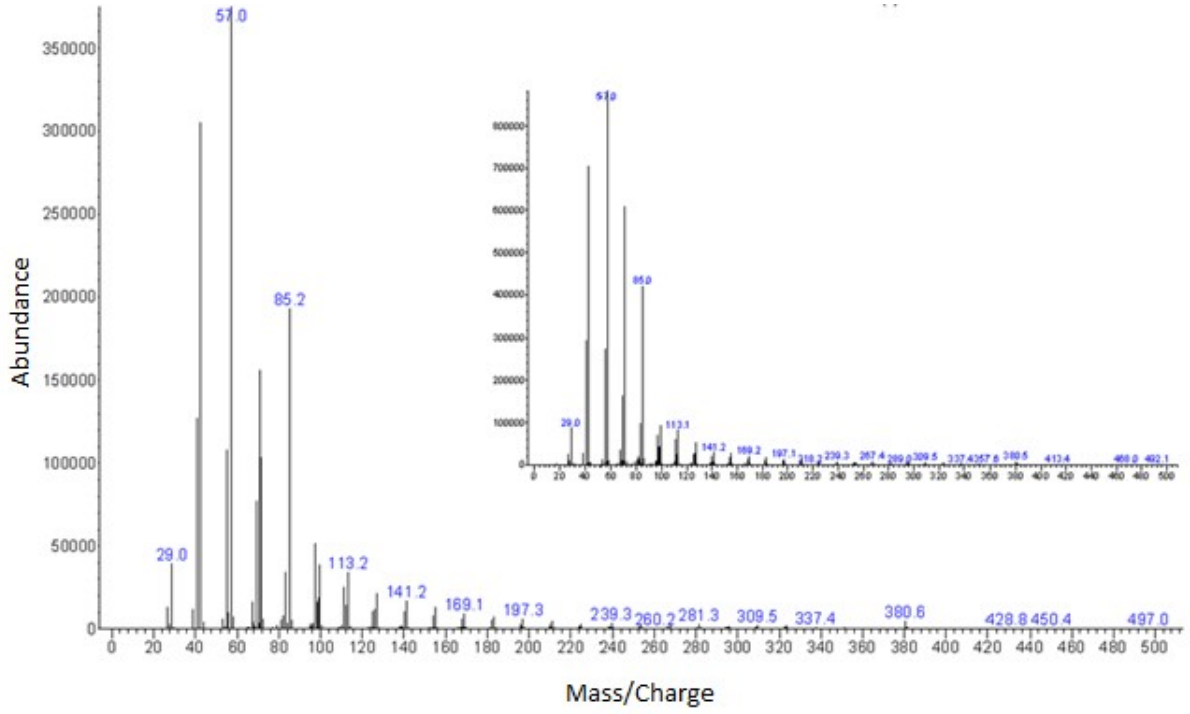

Fig. S1 Observed mass spectrum of FID peak 5 (heptacosane) from antler fly sample. The inset shows mass spectrum of heptacosane from the alkane standard.

Methyl Alkanes

In general, the mass spectra of methlyl alkanes are characterized by a stable secondary carbocation radical formed upon ionisation (McLafferty 1980). Fragmentation in these species tends to occur either before or after the methyl branch, and the charge is localised on the secondary cation, so these ions are represented in the mass spectrum. Therefore, the position of the side chain can be easily identified via the mass spectral fragmentation pattern. The identity of these compounds was also verified using the ECL and the Pherobase website (http://www.pherobase.com).

As an example, FID peak 14 has an ECL of 29.73, suggesting a hydrocarbon of length C_{29}-C_{30}. Fragmentation at the methyl side chain produced a stable secondary radical cation at mass 393.6 Da, corresponding to a C_{28} fragment (Fig. S2). Therefore, we identified the compound as 3-methylnonacosane ($C_{29}H_{60}$, 408 Da). Likewise, the mass spectrum of FID

peak 7 had an ECL of 27.73 and exhibited the presence of a secondary radical cation at mass 365.6, suggesting it is 3-methylheptacosane ($C_{28}H_{58}$, 394 Da). The fractional ECL value is the same as peak 14 (x.73), lending further support to the placement of the methyl group at the C_3 position. This same process was used to identify the remaining methyl alkanes.

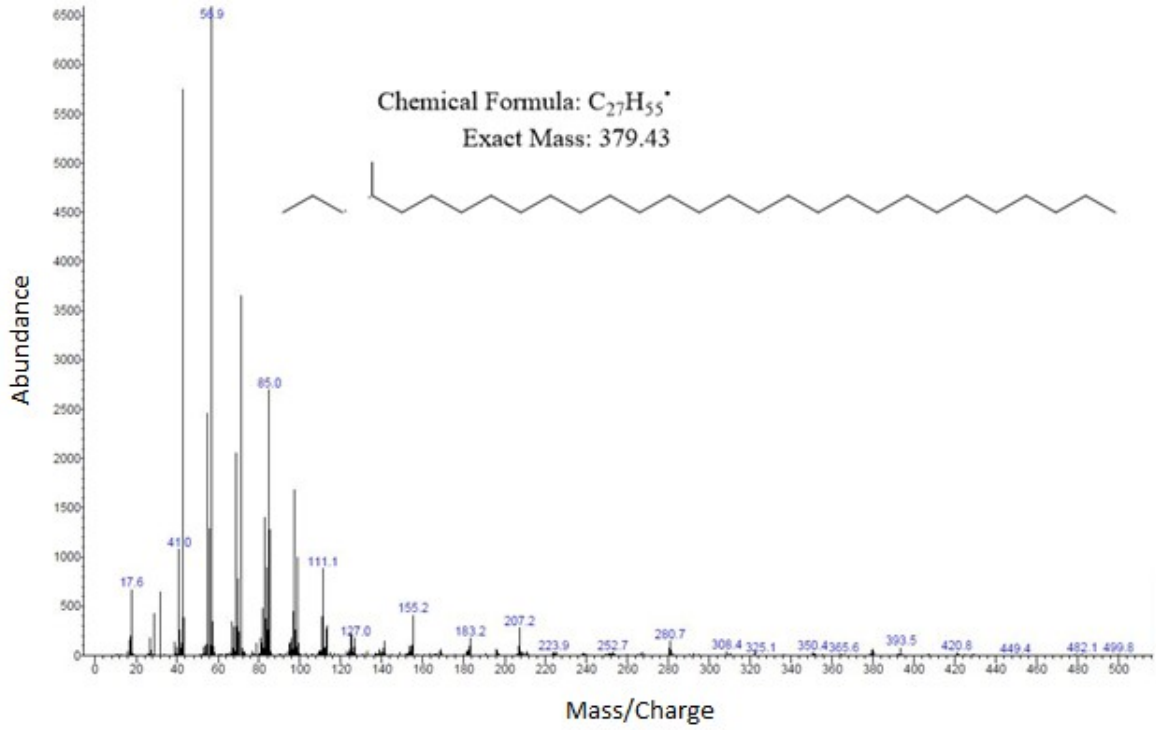

Fig. S2 Observed mass spectrum of FID peak 14 (3-methylnonacosane), including a stick diagram of the molecule to illustrate the fragmentation that produces the secondary radical cation.

Methyl Alkanes, coeluted

The spectra of peaks 6, 11 and 13 show many ions differing in mass by C_2H_4 due to the co-elution of two or three different methyl alkanes. The methyl branches are positioned near the centre of the molecule, so their precise location does not affect the boiling point, or the interaction with the column, and the compounds thus elute at the same time. The stability of the secondary radical cation produced by α cleavage at the branch allows for their

identification. The methyls are located near the centre of the chain, creating some symmetry by which the molecule produces two identical ions from each half of itself. Fig. S3 illustrates the origin of the ions visible in peak 6, a mixture of 11-methylheptacosane and 13-methylheptacosane. The other two peaks were identified in the same way.

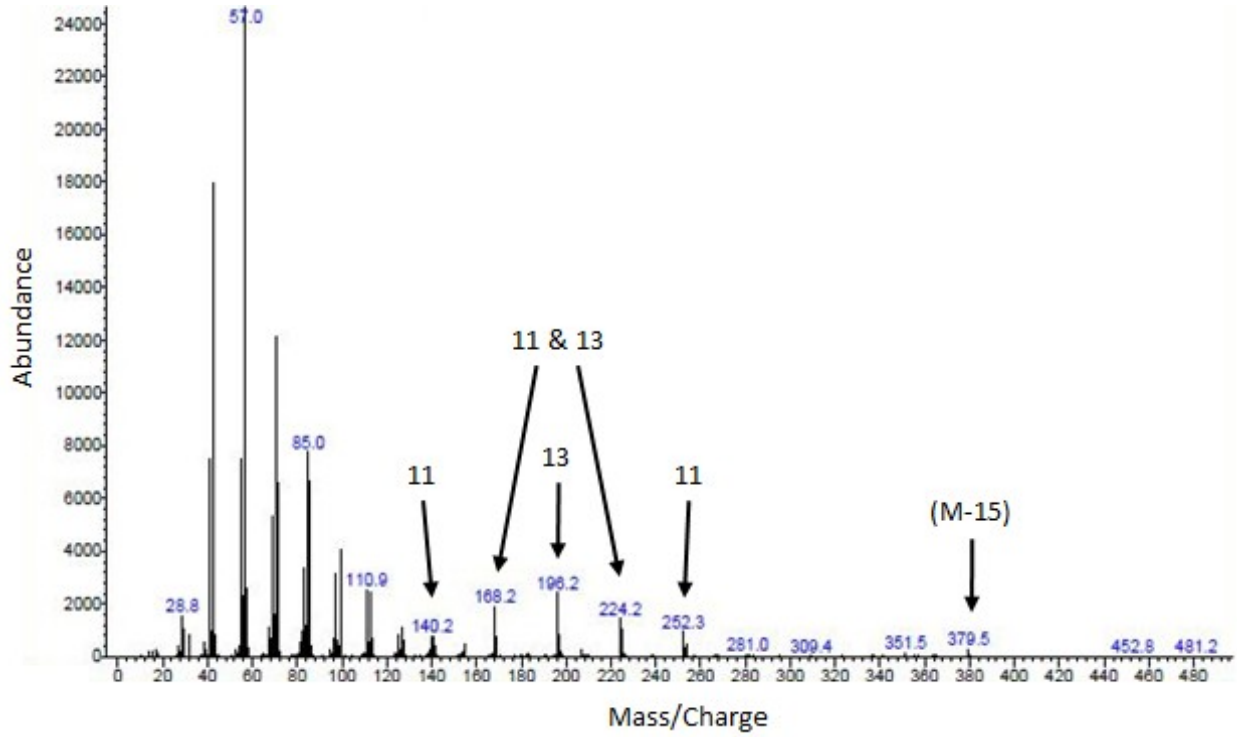

Fig. S3 Observed mass spectrum of FID peak 6, containing 11-methylheptocosane and 13-methylheptacosane, co-eluted. Numbers indicate which of the two species (or both) each diagnostic peak derives from.

Dimethyl Alkanes

Dimethyl alkanes can be identified in essentially the same way as methyl alkanes, as they also preferentially fragment adjacent to the methyl branches. Thus, for each methyl group there is a set of two peaks. The mass spectra are complicated by the fact that each of our dimethyl alkanes co-eluted with a second dimethyl species. Like many of the methyl alkanes above, the methyl branches are near the centre of the molecule, creating symmetry in

which the same size fragment can be produced in more than one way. In these cases, the ion often appears more intense on the mass spectrum. Using FID peak 13 (ECL 29.58) as an example, Table S1 demonstrates the masses produced by these cleavages and Fig. S4 illustrates the diagnostic ions used to confirm these chemical species.

Table S1 Diagnostic MS ion fragments caused by α cleavage in 11,15-dimethylnonacosane and 13,17-dimethylnonacosane, which co-elute as FID peak 13.

Location of cleavage	Ion fragments produced (m/z)
11,15-dimethylnonacosane	
11-methyl	141 (C_{10}) and 295 (C_{21}) 169 (C_{12}) and 267 (C_{19})
15-methyl	211 (C_{15}) and 225 (C_{16}) 197 (C_{14}) and 239 (C_{17})
13,17-dimethylnonacosane	
13-methyl	169 (C_{12}) and 267 (C_{19}) 197 (C_{14}) and 239 (C_{17})
17-methyl	239 (C_{17}) and 197 (C_{14}) 169 (C_{12}) and 267 (C_{19})

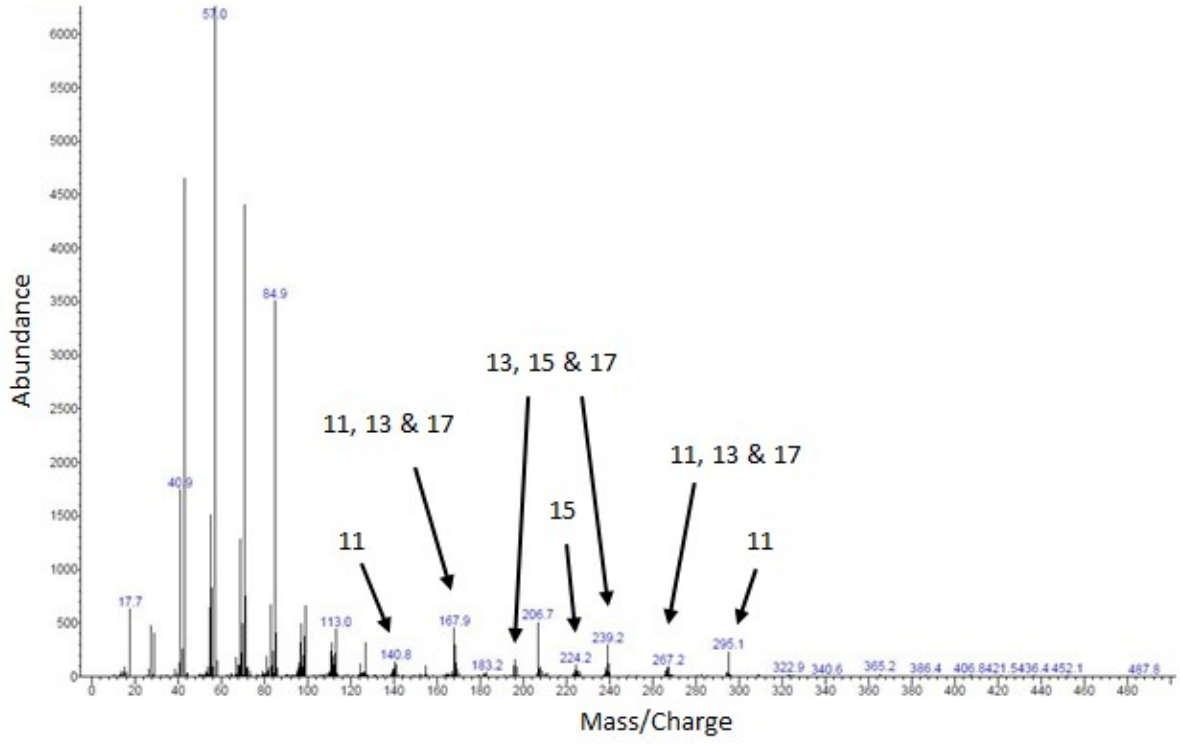

Fig. S4 Observed mass spectrum for peak 13, containing 11,15-dimethylnonacosane and 13,17-dimethylnonacosane. Numbers indicate locations of cleavage that would produce each diagnostic peak (see Table S1).

Alkenes

Most of the alkenes were identified from their mass spectrum and their corresponding molecular ion (M^+) peak. The positioning of the double bond cannot be determined from mass spectra alone because this does not produce an ion of significant stability. When possible, we confirmed double bond positions via comparison of spectra and ECL values with databases and other literature sources (Bartelt et al. 1984, 1986, Blomquist et al. 1985, Howard et al. 2001, Vaníčková et al. 2012, Curtis et al. 2013, Soares et al. 2017). For example, FID peak 2 (ECL 26.74, Fig. S5) was identified as heptacosene using these sources. The molecular ion is clear at 378 Da and the ECL is consistent with a C_{27} compound. Comparison of our observed ECL to the literature suggests that this compound contains a double bond at the 9-position

(Vaníčková et al. 2012, Curtis et al. 2013), although this cannot be definitively verified with the mass spectrum alone. Unfortunately, derivitization of these compounds was not possible due to their low absolute quantities. Peaks 8 (ECL 28.75) and 16 (ECL 30.77) was also be identified as an alkene, with the double bond also likely in the 9th position, from the mass spectrum and other literature sources. In one other case (FID peak 9, ECL 28.83), the structure of an alkene could not reasonably be inferred via ECL, as multiple isomers have similar values.

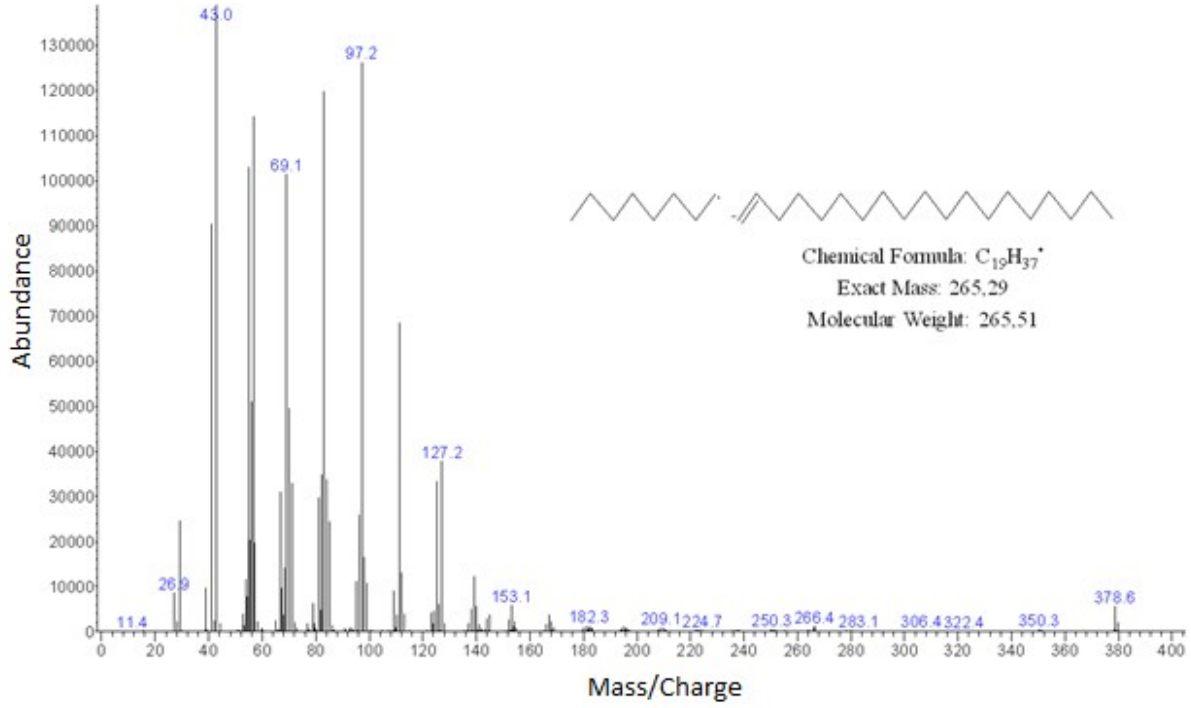

Fig S5 Observed mass spectrum of peak 2 (9-heptacosene), with stick diagram showing fragmentation pattern.

Esters

A homologous series of esters was discovered upon investigation of FID peaks 1 and 3. The mass spectrum of these two chromatographic peaks shared a base peak at 99 Da. When a single ion chromatogram (Fig. S6) was taken of this ion it revealed a family of compounds

differing in the addition of one or more CH_2 groups. Although some of these were not integrated on the FID, they were further investigated on the GC:MS. The structures of these compounds were verified by comparison to MS databases and published literature (Blomquist et al. 1972, Finidori-Logli et al. 1996, Patel et al. 2001, Howard and Baker 2003, Böröczky et al. 2008, Dweck et al. 2015, Chinta et al. 2016, Pitts-Singer et al. 2017, Merli et al. 2018).

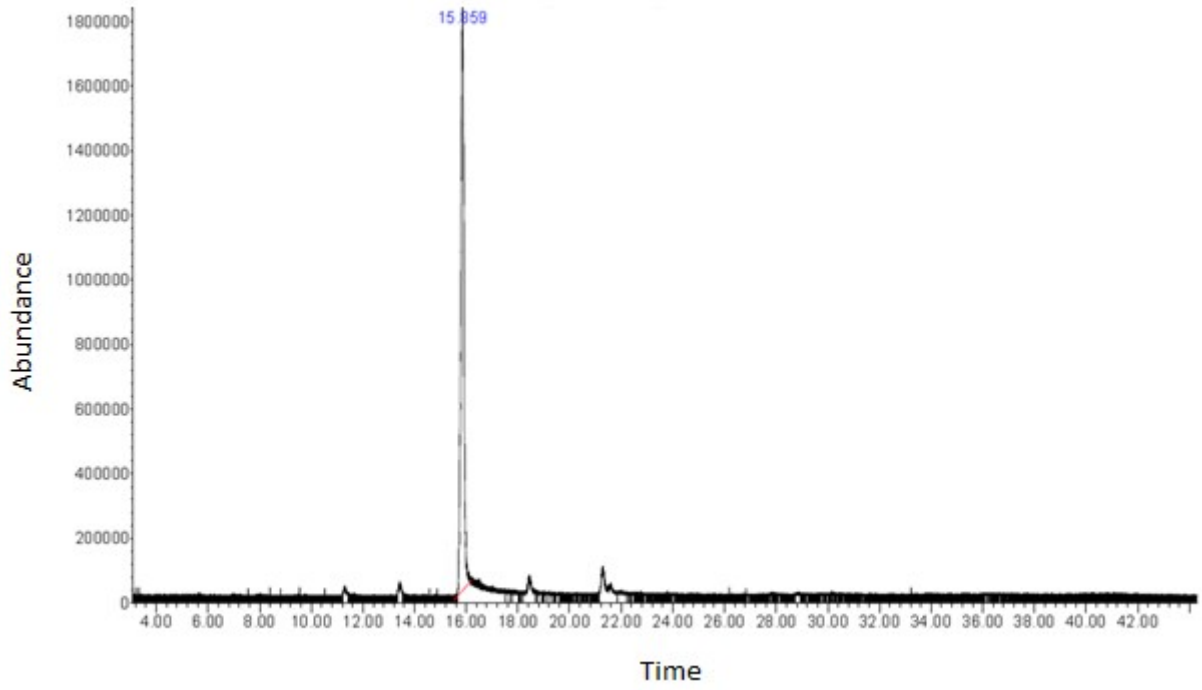

Fig. S6 Observed single ion chromatogram of mass 99 Da.

Derivatization of the sample with TMS did not alter these peaks, revealing they were neither alcohols nor carboxylic acids. The NIST MS search 2.0 GC:MS database identified these as branched esters. Using this information and a couple of diagnostic fragment ion peaks in each spectra, along with the ECL numbers, it is possible to deduce their formula, but without other sources of verification their inferred structure is not definitive. Consider FID Peak 1 as an example. Peak 1 has an ECL number of 24.97, which suggests a carbon number of 24 or 25. The M^+ ion of RCOOR′R″ esters generally becomes less prominent the longer the

R groups, and it is often not present in the mass spectrum, as in this case. The dominant peak at 99 Da seen in five of the six esters corresponds to a hexanoic acid head group produced by alpha cleavage at the carbonyl group (Fig. S7). This is characteristic of fragmentation of higher molecular weight esters. Cleavage adjacent to the alcohol oxygen produces the alkyl tail ion, which is also clearly seen in the mass spectrum of all six esters (Fig. S7). This corresponds to the other dominant ion at higher mass seen in Fig. S7 at 266 Da. It is not possible to determine exactly where the alcohol oxygen is along the alkyl chain, but the peak at 183 Da suggests it may be located at the C_6 position. This structure was also suggested by the NIST MS search database. Nevertheless, due to the lack of unequivocal evidence, we provisionally identify this compound as *x*-nonadecyl hexanoate ($C_{25}H_{50}O_2$, 382 Da).

Peaks 1a, 1c, 1d, and 3 were identified in the same way. All of these compounds produced the same 99 Da ion, representing a six-carbon ester head group, yet differed in the size of the alkyl tail ion, having more or fewer CH_2 groups. Peak 1b is an exception as it exhibited a dominant peak at 71 Da instead of 99 Da, indicating a two-carbon smaller head group. This suggests the ester has been synthesised from butyric as opposed to hexanoic acid, as illustrated in Fig. S8. Another dominant ion is present at 266 Da, indicating a C_{19} alkyl tail. Therefore, we provisionally identified this compound as *x*-nonadecyl butanoate ($C_{23}H_{46}O_2$, 354 Da).

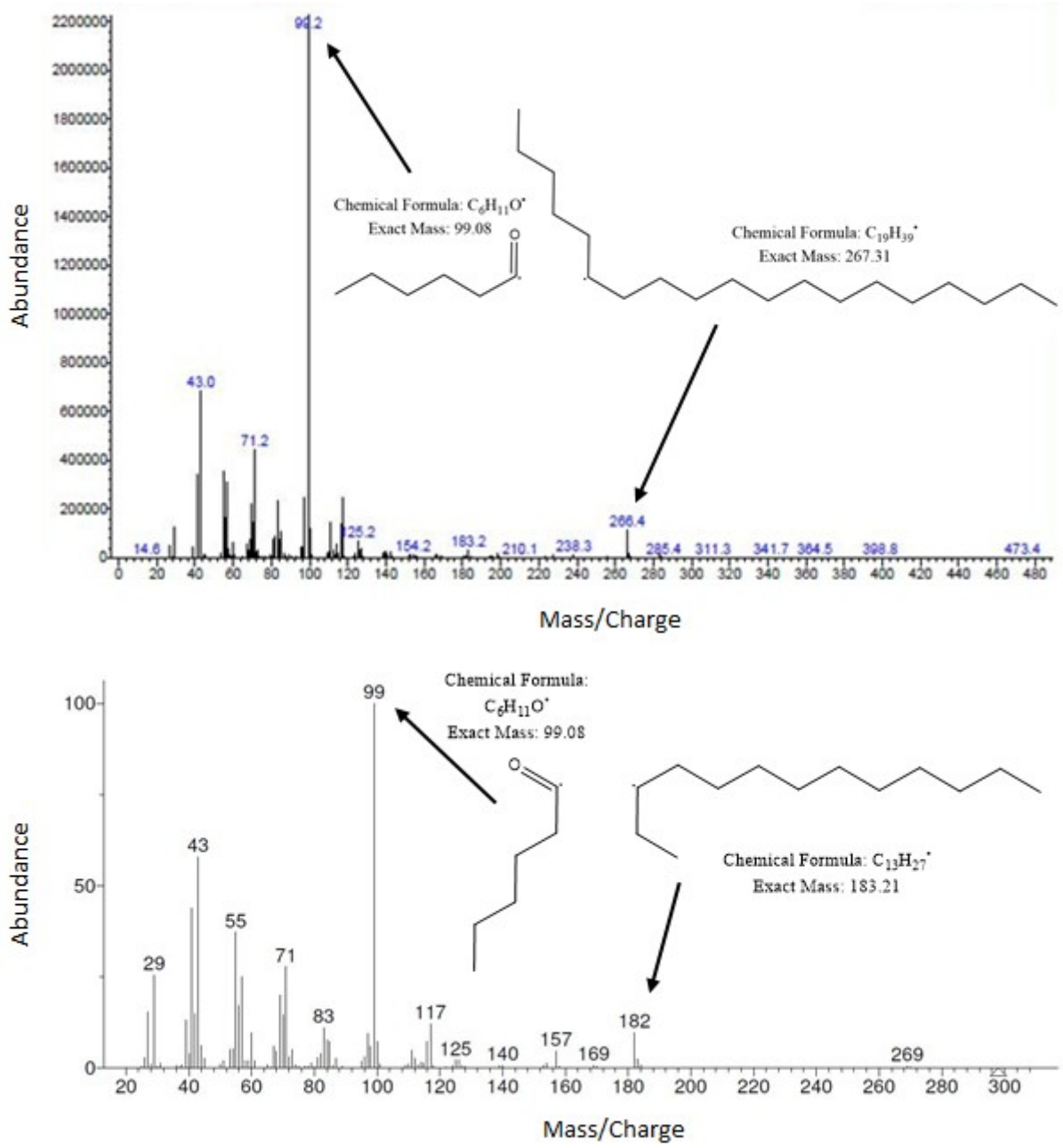

Fig. S7 Top panel, observed mass spectrum of FID peak 1 (*x*-nonadecyl hexanoate) with fragmentation pattern of proposed molecule (conjecturally drawn as 6-nonadecyl hexanoate). Bottom panel, database spectrum and fragmentation scheme of 3-tridecyl hexanoate for comparison.

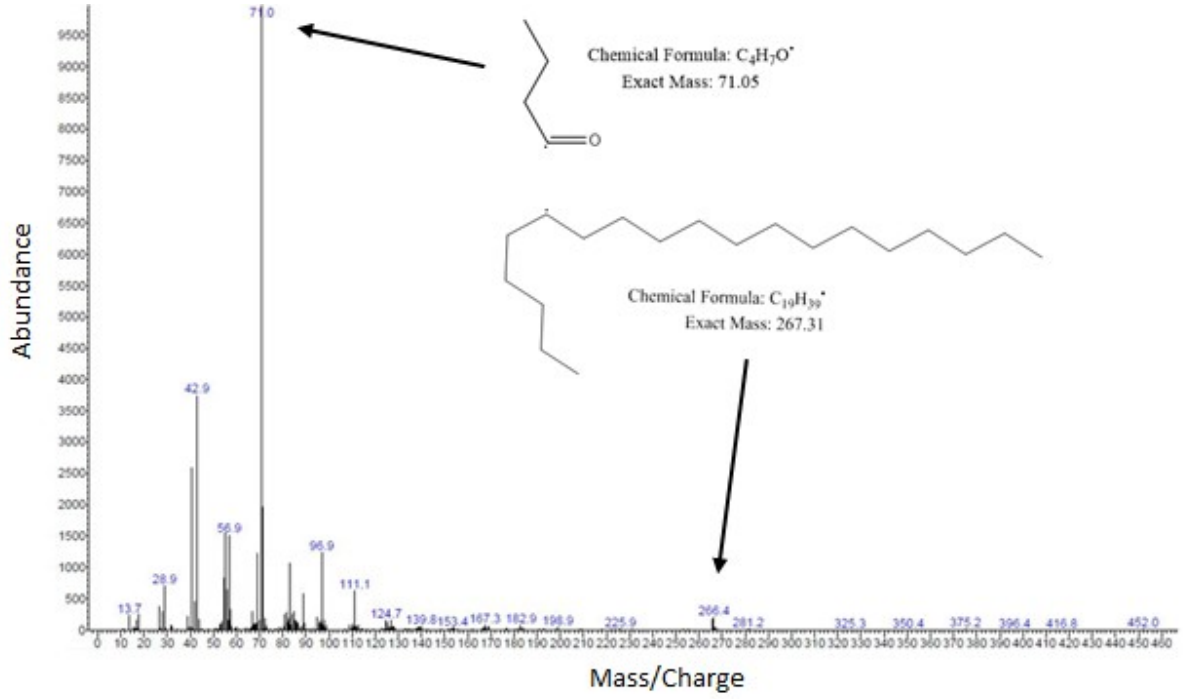

Fig. S8 Observed mass spectrum of peak 1b ester (*x*-nonadecyl butanoate), with fragmentation pattern of proposed molecule (drawn as 6-nonadecyl butanoate).

Sexual Selection Analysis

Table S2 Standardized sexual selection gradients on 17 PCs of male epicuticular compound variation across two years. Gradients and significance values for each year are based on single-year LMs and binomial GLMs, respectively, while between-year difference represents the significance of each year × PC interaction in a binomial GLM using combined data from both years. Significant effects (LRT: $P < 0.05$) are denoted in bold.

Male trait (PC)[a]	β (2013)	P (2013)	β (2017)	P (2017)	Between-year difference (P)
1	0.043	0.498	0.098	0.309	0.774
2	0.049	0.844	0.042	0.602	0.776
3	-0.130	0.126	-0.157	0.217	0.895
4	0.068	0.489	-0.035	0.898	0.494
5	**-0.419**	**< 0.001**	**-0.405**	**0.008**	0.793
6	0.209	0.084	0.098	0.787	0.690
7	0.292	0.074	**-0.835**	**0.002**	**< 0.001**
8	**0.808**	**< 0.001**	-0.219	0.339	**0.001**
9	-0.223	0.282	0.134	0.626	0.913
10	0.376	0.106	0.365	0.216	0.974
11	**-0.641**	**0.040**	**-0.620**	**0.022**	0.680
12	-0.361	0.293	-0.368	0.166	0.922
13	-0.185	0.553	**0.892**	**0.006**	**0.035**
14	-0.280	0.531	0.455	0.212	0.805
15	-0.092	0.608	**0.864**	**0.037**	0.117
16	0.062	0.952	0.027	0.965	0.777
17	-0.324	0.768	0.417	0.481	0.805

[a]See Table S4 for principal component loadings.

Table S3 Standardized intersexual selection (i.e. female mate choice) gradients on 17 PCs of male epicuticular compound variation in 2017. Estimates and significance values are based on LM and binomial GLM, respectively. Significant selection gradients (LRT: $P < 0.05$) are indicated with bold text.

Male trait (PC)[a]	β	P
1	0.125	0.277
2	0.111	0.449
3	-0.261	0.096
4	-0.113	0.372
5	**-0.474**	**0.010**
6	0.252	0.465
7	-0.486	0.166
8	0.244	0.465
9	-0.250	0.455
10	0.285	0.431
11	**-0.681**	**0.046**
12	0.379	0.359
13	**1.202**	**0.014**
14	-0.009	0.856
15	**1.590**	**0.006**
16	-0.240	0.703
17	**-2.067**	**0. 031**

[a]See Table S4 for principal component loadings

Table S4 Eigenvectors of 17 principle components of EC variation in wild male *Protopiophila litigata*. The PCA was performed on CLR-transformed relative compound abundances using the pooled 2013 and 2017 data. FID peak numbers refer to compounds identified in Table 1.

EC (FID #)	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10	PC11	PC12	PC13	PC14	PC15	PC16	PC17
% var	44.1	25.1	7.4	6.7	4.6	2.7	2.0	1.8	1.5	1.3	0.8	0.7	0.5	0.4	0.3	0.2	0.1
1	0.253	0.172	-0.028	-0.048	-0.087	-0.369	-0.544	0.021	-0.239	0.417	-0.004	-0.007	0.336	-0.054	-0.225	0.039	-0.007
2	-0.277	-0.015	0.412	0.222	0.178	-0.154	-0.204	-0.044	0.142	-0.220	-0.406	-0.174	-0.189	-0.156	-0.079	0.189	-0.174
3	0.002	-0.142	0.372	-0.125	0.180	-0.046	-0.256	-0.010	0.255	0.160	0.394	-0.321	-0.181	-0.049	0.338	-0.264	-0.321
4	0.471	-0.587	-0.082	0.527	-0.235	-0.038	0.034	0.124	0.053	-0.087	-0.046	-0.015	-0.026	0.069	0.030	-0.024	-0.015
5	0.045	-0.072	-0.154	-0.428	0.006	-0.213	-0.090	0.182	0.169	-0.094	-0.234	0.097	-0.367	0.566	-0.151	0.174	0.097
6	0.049	0.154	0.038	0.086	0.389	-0.081	0.072	0.605	-0.061	-0.283	0.277	0.350	0.072	-0.245	-0.075	0.117	0.350
7	0.004	0.285	0.172	-0.030	-0.475	-0.533	0.444	-0.143	-0.026	-0.231	0.194	-0.101	0.013	-0.035	0.044	-0.010	-0.101
8	-0.438	-0.090	0.040	0.217	0.022	-0.049	0.024	-0.158	-0.041	0.029	-0.344	0.211	0.317	0.022	-0.090	-0.192	0.211
9	-0.267	-0.378	0.009	-0.056	0.163	-0.070	0.288	-0.250	0.033	0.410	0.368	0.296	-0.019	0.054	-0.178	0.221	0.296
10	-0.061	-0.311	-0.375	-0.469	-0.015	0.030	0.034	0.016	0.192	-0.249	-0.077	-0.283	0.333	-0.405	-0.070	-0.080	-0.283
11	0.008	0.028	-0.088	-0.021	0.254	-0.004	0.164	0.019	-0.439	-0.027	-0.071	-0.068	-0.153	0.189	-0.049	-0.705	-0.068
12	-0.018	-0.017	0.043	-0.116	0.019	0.121	-0.051	-0.046	-0.244	-0.123	-0.068	0.138	0.340	0.275	0.729	0.239	0.138
13	0.334	0.118	0.243	-0.053	0.162	0.403	0.003	-0.424	-0.097	-0.314	0.152	-0.109	0.153	0.166	-0.392	0.159	-0.109
14	0.169	0.129	-0.005	-0.137	-0.253	0.204	-0.179	-0.249	0.165	-0.026	-0.096	0.603	-0.309	-0.352	0.112	-0.210	0.603
15	-0.048	0.017	0.333	-0.167	-0.363	0.445	0.236	0.435	-0.156	0.354	-0.179	-0.151	-0.027	-0.076	-0.105	0.077	-0.151
16	-0.443	0.155	-0.345	0.264	-0.329	0.257	-0.335	0.082	0.006	-0.162	0.382	-0.122	-0.099	0.174	-0.084	0.007	-0.122
17	0.064	0.137	-0.384	0.120	0.200	-0.002	0.122	-0.184	-0.318	0.173	-0.116	-0.260	-0.396	-0.303	0.168	0.346	-0.260
18	0.152	0.416	-0.201	0.213	0.184	0.099	0.240	0.024	0.606	0.273	-0.125	-0.084	0.203	0.161	0.075	-0.083	-0.084

References Cited

Bartelt, R. J., M. T. Armold, A. M. Schaner, and L. L. Jackson. **1986**. Comparative analysis of cuticular hydrocarbons in the *Drosophila virilis* species group. Comp. Biochem. Physiol. Part B Comp. Biochem. 83: 731–742.

Bartelt, R. J., T. P. Krick, and R. L. Jones. **1984**. Cuticular hydrocarbons of the yellowheaded spruce sawfly, *Pikonema alaskensis*. Insect Biochem. 14: 209–213.

Blomquist, G. J., C. L. Soliday, B. A. Byers, J. W. Brakke, and L. L. Jackson. **1972**. Cuticular lipids of insects: V. Cuticular wax esters of secondary alcohols from the grasshoppers *Melanoplus packardii* and *Melanoplus sanguinipes*. Lipids. 7: 356–362.

Blomquist, G. J., E. C. Toolson, and D. R. Nelson. **1985**. Epicuticular hydrocarbons of *Drosophila pseudoobscura* (Diptera; Drosophilidae) Identification of unusual alkadiene and alkatriene positional isomers. Insect Biochem. 15: 25–34.

Böröczky, K., K. C. Park, R. D. Minard, T. H. Jones, T. C. Baker, and J. H. Tumlinson. **2008**. Differences in cuticular lipid composition of the antennae of *Helicoverpa zea*, *Heliothis virescens*, and *Manduca sexta*. J. Insect Physiol. 54: 1385–1391.

Chinta, S. P., S. Goller, G. Uhl, and S. Schulz. **2016**. Identification and synthesis of branched wax-type esters, novel surface lipids from the spider *Argyrodes elevatus* (Araneae: Theridiidae). Chem. Biodivers. 13: 1202–1220.

Curtis, S., J. L. Sztepanacz, B. E. White, K. A. Dyer, H. D. Rundle, and P. Mayer. **2013**. Epicuticular compounds of *Drosophila subquinaria* and *D. recens*: identification, quantification, and their role in female mate choice. J. Chem. Ecol. 39: 579–590.

Dweck, H. K. M., S. A. M. Ebrahim, M. Thoma, A. A. M. Mohamed, I. W. Keesey, F. Trona, S. Lavista-Llanos, A. Svatoš, S. Sachse, M. Knaden, and B. S. Hansson. **2015**. Pheromones mediating copulation and attraction in *Drosophila*. Proc. Natl. Acad. Sci. 112: E2829–E2835.

Finidori-Logli, V., A.-G. Bagnères, D. Erdmann, W. Francke, and J.-L. Clément. **1996**. Sex recognition in *Diglyphus isaea* Walker (Hymenoptera: Eulophidae): role of an uncommon family of behaviorally active compounds. J. Chem. Ecol. 22: 2063–2079.

Howard, R. W., and J. E. Baker. **2003**. Cuticular hydrocarbons and wax esters of the ectoparasitoid *Habrobracon hebetor*: ontogenetic, reproductive, and nutritional effects. Arch. Insect Biochem. Physiol. 53: 1–18.

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

Howard, R. W., G. Pérez-Lachaud, and J.-P. Lachaud. **2001**. Cuticular hydrocarbons of *Kapala sulcifacies* (Hymenoptera: Eucharitidae) and its host, the ponerine ant *Ectatomma ruidum* (Hymenoptera: Formicidae). Ann. Entomol. Soc. Am. 94: 707–716.

Lockey, K. H. 1988. Lipids of the insect cuticle: origin, composition and function. Comp. Biochem. Physiol. Part B Comp. Biochem. 89: 595–645.

McLafferty, F. W. 1980. Interpretation of mass spectra, 3. ed. ed, Organic chemistry series. Univ. Science Books, Mill Valley, Calif.

Merli, D., B. Mannucci, F. Bassetti, F. Corana, M. Falchetto, A. R. Malacrida, G. Gasperi, and F. Scolari. **2018**. Larval diet affects male pheromone blend in a laboratory strain of the medfly, *Ceratitis capitata* (Diptera: Tephritidae). J. Chem. Ecol. 44: 339–353.

Patel, S., D. R. Nelson, and A. G. Gibbs. **2001**. Chemical and physical analyses of wax ester properties. J. Insect Sci. 1.

Pitts-Singer, T. L., M. M. Hagen, B. R. Helm, S. Highland, J. S. Buckner, and W. P. Kemp. **2017**. Comparison of the chemical compositions of the cuticle and dufour's

gland of two solitary bee species from laboratory and field conditions. J. Chem. Ecol. 43: 451–468.

Soares, E. R. P., N. R. Batista, R. da S. Souza, V. de O. Torres, C. A. L. Cardoso, F. S. Nascimento, and W. F. Antonialli-Junior. **2017**. Variation of cuticular chemical compounds in three species of *Mischocyttarus* (Hymenoptera: Vespidae) eusocial wasps. Rev. Bras. Entomol. 61: 224–231.

Vaníčková, L., A. Svatoš, J. Kroiss, M. Kaltenpoth, R. R. do Nascimento, M. Hoskovec, R. Břízová, and B. Kalinová. **2012**. Cuticular hydrocarbons of the South American fruit fly *Anastrepha fraterculus*: variability with sex and age. J. Chem. Ecol. 38: 1133–1142.