RESEARCH ARTICLE

Aging modulates cuticular hydrocarbons and sexual attractiveness in *Drosophila melanogaster*

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SUMMARY

Attractiveness is a major component of sexual selection that is dependent on sexual characteristics, such as pheromone production, which often reflect an individual's fitness and reproductive potential. Aging is a process that results in a steady decline in survival and reproductive output, yet little is known about its effect on specific aspects of attractiveness. In this report we asked how aging impacts pheromone production and sexual attractiveness in *Drosophila melanogaster*. Evidence suggests that key pheromones in *Drosophila* are produced as cuticular hydrocarbons (CHC), whose functions in attracting mates and influencing behavior have been widely studied. We employed gas chromatography/mass spectrometry and laser desorption/ionization mass spectrometry to show that the composition of *D. melanogaster* CHC is significantly affected by aging in both sexes and that these changes are robust to different genetic backgrounds. Aging affected the relative levels of many individual CHC, and it shifted overall hydrocarbon profiles to favor compounds with longer chain lengths. We also show that the observed aging-related changes in CHC profiles are responsible for a significant reduction in sexual attractiveness. These studies illuminate causal links among pheromones, aging and attractiveness and suggest that CHC production may be an honest indicator of animal health and fertility.

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Key words: aging, attractiveness, pheromone.

INTRODUCTION

Aging is a process that results in a broad functional decline in health, increasing vulnerability to diseases, and eventual death. It is normally accompanied by declining reproductive output, implying that older individuals have reduced reproductive potential when compared with younger animals of similar genetic constitution. An accurate assessment of the reproduction potential of a prospective mate is crucial to maximize an individual's reproductive success, and various chemical sensory systems have evolved that effectively discern the fitness of individuals based on certain phenotypic characteristics (Dickson, 2008; Keller et al., 2009). These considerations suggest the possibility that chemical cues can reveal the age of an individual and that they may serve as key attractive characteristics, perhaps because they are costly to produce/maintain and therefore difficult for older individuals to fake (Zahavi, 1975) or because they are subject to direct physiological constraints in old animals (Maynard-Smith and Harper, 2003). Regardless of their nature, these traits may be direct or indirect indicators of animal condition (Hill, 2011).

In insects, cuticular hydrocarbons (CHC) have been suggested to play important roles in short-distance and contact male–female communication (Ferveur, 2005). In *Drosophila melanogaster*,

several studies based on gas chromatography/mass spectrometry (GC/MS) demonstrate sexual dimorphism in CHC and support their function as pheromonal compounds (Coyne and Oyama, 1995; Ferveur et al., 1997). Female flies produce sex-specific dienes, such as 7,11-hexacosadiene (7,11-HD), which can stimulate male courtship (Antony et al., 1985; Antony and Jallon, 1982; Jallon, 1984). The compounds 7-pentacosene (7-P) and 7,11-nonacosadiene (7,11-ND) induce courtship progression in males, albeit with a much lower efficiency than 7,11-HD (Antony et al., 1985). In contrast, male CHC profiles are dominated by monoenes, such as 7-tricosene (7-T) and 7-P, the former of which has been shown to inhibit male–male courtship and increase female receptivity (Ferveur and Sureau, 1996; Grillet et al., 2006). Males also produce the volatile pheromone 11-cis-vaccenyl acetate (cVA), which is produced in the male ejaculatory bulb and has multiple roles in social interaction including increasing female receptivity (Kurtovic et al., 2007). Recent advances have allowed for the identification of new CHC, whose functions have yet to be determined (Everaerts et al., 2010; Yew et al., 2008). More polar CHC, for example, may not be amenable to traditional GC/MS but can be analyzed by laser desorption/ionization orthogonal time-of-flight mass spectrometry (LDI-MS) (Yew et al., 2009). When used in combination, GC/MS

and LDI-MS provide a more complete representation of cuticular pheromone profiles than has previously been possible (Fernandez et al., 2011).

Despite the complexity of CHC profiles, only a few details are known about their regulation in *Drosophila*. CHC composition in the fly has been shown to respond to external influences, including diet and temperature during development, social interactions and light cycles (Etges et al., 2006; Kent et al., 2008; Krupp et al., 2008; Savarit and Ferveur, 2002). Their levels also fluctuate in a circadian manner, suggesting high rates of turnover (Krupp et al., 2008). Development and aging have been shown to influence CHC profiles in several insect species, including the honey bee, ant and mosquito (Hugo et al., 2006; Ichinose and Lenoir, 2009; Nunes et al., 2009). In *D. melanogaster*, however, CHC profiles have only been studied in young flies, ranging from newly eclosed to 8 days of adult age (Arienti et al., 2011). Importantly, the role of CHC in mediating sexual attractiveness of aging flies has not been established.

Here, we investigated the links among aging, CHC production and sexual attractiveness in *D. melanogaster*. By measuring CHC levels at several ages throughout the lifespan using GC/MS together with LDI-MS techniques, we show that the composition of CHC changes with age consistently in male and female flies. These changes are highly reproducible among individuals, and they are robust to variability in genetic background. Moreover, aging leads to reduced sexual attractiveness, which, in females, can be attributed to changes in CHC profiles. We suggest that the effects of aging on fly pheromones and CHC profiles may accurately reflect changes in the animal's health and reproductive status and be perceived by potential mates as reduced sexual attractiveness.

MATERIALS AND METHODS
Fly stocks and husbandry

Canton-S flies were obtained from the Bloomington Stock Center. Fv wild-type flies were provided by D. Promislow (University of Georgia) from a stock originating in the University of Georgia Horticultural Farm in Watkinsville, GA, USA. Oenocyte-less flies were created from the progeny of the cross of '+: *PromE(800)-Gal4, tubP-Gal80^{ts}*;+' to '+: *UAS-StingerII, UAS-hid/CyO*;+'; both strains were provided by J. Levine (Billeter et al., 2009).

For all experiments, larvae were cultured in cornmeal–sugar–yeast 'larval' media. Virgin adults were collected shortly after eclosion and kept in 10% sugar/yeast food. All flies were maintained at 25°C and 60% relative humidity on a 12h:12h light:dark cycle. Fresh food was provided every 2 or 3 days. Details on media recipes can be found elsewhere (Poon et al., 2010).

CHC detection
GC/MS analysis

A large cohort of each genotype was established by collecting virgin males or females following eclosion and placing them into *Drosophila* laboratory vials containing 10% sugar/yeast media. Flies were sampled from these cohorts every 2–3weeks, and CHC samples were immediately extracted. Three replicate CHC samples were prepared for each age and each genotype. For each sample, 5 flies from a single vial were placed in 100µl of hexane, which contained 10µgml^{-1} of the hydrocarbon hexacosane (Sigma-Aldrich, St Louis, MO, USA) as an internal standard. Following incubation at room temperature for 30min, the cuticular extract was removed and placed in a clean glass vial. The solvent was then evaporated under a chemical hood. Extracts were stored at –80°C and re-dissolved in 30µl of heptane prior to GC/MS analysis (for additional details, see supplementary material Fig. S1).

The GC/MS analysis was performed with a Quattro micro-GC/MS (Waters, Milford, MA, USA) equipped with an HP-5 column (5% phenyl-methylpolysiloxane column; 30m length, 0.32mm i.d., 0.25µm film thickness; Agilent, Santa Clara, CA, USA). Ionization was achieved by electron ionization (EI) at 70eV. One microliter of the sample was injected using a splitless injector. The helium flow was set at 1.3mlmin^{-1}. The column temperature program began at 50°C for 2min, and increased to 300°C at a rate of 15°Cmin^{-1}. The quadrupole mass spectrometer was set to unit mass resolution and 3scanss^{-1}, from m/z 37 to 700. Chromatograms and mass spectra were analyzed using MassLynx (Waters).

Compounds were identified on the basis of retention time and EI mass spectra. The signal intensity of each compound was calculated as the area under its corresponding peak. To obtain a measure for the total amount of CHC, the sum of the signal intensities of all identified compounds was divided by that of the internal standard (10µgml^{-1} hexane). Relative CHC profiles were derived by dividing individual peak intensities by the sum of the intensities of all identified hydrocarbon signals.

LDI-MS analysis

In contrast to the GC/MS analysis, multiple, independent cohorts were established every 2–3weeks, and all flies were sampled on the same day for CHC analysis (for additional details, see supplementary material Fig. S1).

We closely followed the method outlined by Yew and colleagues (Yew et al., 2009; Yew et al., 2011b) for detecting CHC by LDI-MS. To account for biological variability, quantitative data were derived by averaging the signal intensities obtained from 3–5 individual flies for each age and genotype. Flies were anesthetized and mounted with fine forceps onto adhesive tape (G304, Plano GmbH, Wetzlar, Germany), which was attached to a glass coverslip. The coverslip was attached to a milled-out, custom-built sample plate with adhesive tabs. The fly body remained intact during analysis in the mass spectrometer. However, flies are presumably rapidly killed after transfer into the fine vacuum of the mass spectrometer ion source. During transfer they stay anesthetized. The orthogonal mass spectrometer is equipped with an N$_2$ laser emitting 3ns long pulses at a wavelength of 337nm and a repetition rate of 30Hz. The laser beam spot size on the sample is ~200µm in diameter and has an approximately flat-top intensity profile. Ions were generated in a buffer gas environment using 2mbar (1bar≈100kPa) of Argon gas. For acquisition of a mass spectrum, 900 laser pulses were applied to one spot (or small area of body parts) over 30s. Laser fluence (light energy per pulse and area) was adjusted to values moderately above the ion detection threshold, corresponding to values between 100 and 200Jm^{-2}. All data were acquired in positive ion mode, and mass spectra were processed using MoverZ software (v. 2001.02.13, Genomic Solutions, bioinformatics.genomicsolutions.com). Potassiated molecules [M+K]$^+$ formed the dominant hydrocarbon ion signals in all LDI-MS mass spectra. Elemental composition assignments are based on the assumption that the observed and theoretical mass values agree within ±0.02Da. Relative quantification was expressed using a normalized intensity, derived by dividing individual signal intensities by the sum of the intensities of all identified hydrocarbon signals.

Behavior
Two-choice courtship assay

A standard courtship assay was used to measure male preference (i.e. female attractiveness) between young and old females. For each measure, two subject females for comparison (young/old) were

decapitated and placed on the opposite sides of a single well of a 24-well cell culture plate containing standard 10% sugar/yeast fly medium. One 6–8 day old Canton-S virgin male was subsequently aspirated into the cell, and the duration of courtship behaviors directed toward each female (including orientation, wing vibration and attempted copulation) was recorded for 10 min. The assay was conducted between 10:00 h and 13:00 h and at 25°C. To minimize potential measurement error, only courtship bouts lasting longer than 5 s were included in the analysis. To control for individual variability in total courtship behavior, male preference is presented as the percentage of time courting young females divided by the total courtship time [i.e. young/(young+old)].

Two-choice video analysis

To simplify the behavior analysis and to more accurately quantify the extent of preference, we also employed video recording followed by video analysis using fly tracking software that was developed in our laboratory. In this assay, two-choice subject females (i.e. young/old) were decapitated and embedded in agar 15–20 mm apart and 7–10 mm away from the side of the dish. After the agar solidified, a single, 4–8 day old virgin Canton-S male was released in the arena and given 15–20 min to acclimate to the new environment. Video recording was then started and continued for 30 min.

Videos were recorded at 2 frames s^{-1} and converted to AVI file format, which was analyzed with our VideoFly software. The software was written in C and CSharp and was built around the OpenCV image analysis library (http://opencv.willowgarage.com/wiki/). The source code and compiled executable (Windows 7 compatible) are freely available from our laboratory website (http://sitemaker.umich.edu/pletcherlab/data). VideoFly calculates the amount of time spent by a focal fly inside a circle of 3 mm radius centered on each decapitated subject fly. Instances where the total time was less than 50 s (2.8% of the total time of observation) were removed from further analysis to standardize the denominator for proportional data analysis. As with our courtship assay, male preference was calculated as the percentage of time males spent in the circles centered on young females divided by the total time spent in both circles [i.e. young/(young+old)].

Female preference for males of different ages was assessed using the same setup and is presented as the percentage of time females spent in the circles centered on young males divided by the total time spent in both circles [i.e. young/(young+old)], with the exception that female behaviors were recorded for 60 min. For female preference tests, periods of quiescence (defined as ≥5 min of no movement) were interpreted as sleep and excluded from the analysis.

CHC re-application experiments

To determine whether differences in CHC profiles were directly responsible for the observed preferences, video analysis was used to examine the attractiveness of oenocyte-less flies, which are largely devoid of CHC, that had been covered with CHC extracts of young or old flies. The generation of oenocyte-less flies followed published protocols (Billeter et al., 2009). Briefly, the progeny of the cross of +: *PromE(800)-Gal4, tubP-Gal80^{ts};+* to +: *UAS-StingerII, UAS-hid/CyO;+* were maintained at 18°C until eclosion. Following emergence, flies were kept at 25°C for at least 24 h and then subjected to three overnight heat treatments at 30°C (on days 2, 3 and 4). Adults were left to recover for at least 24 h and checked for the GFP fluorescence to confirm oenocyte ablation.

To prepare the hexane extracts for re-application, large groups of flies were incubated for 10 min at room temperature (with gentle shaking) in volumes corresponding to 10 μl hexane per fly. Each extract was then transferred into a new glass vial, evaporated under the chemical hood and stored at –80°C. Extracts were resuspended in volumes corresponding to 1 μl hexane per fly, resulting in a 10-fold increase of CHC concentration compared with the original extract. Two microliters of each CHC extract of interest (e.g. old *vs* young flies) were pipetted onto oenocyte-less flies, which were decapitated and embedded in agar as described above.

Quantitative real-time PCR

Total RNA was extracted from 10 virgin females at 10–15 days of age by Trizol (Invitrogen, Carlsbad, CA, USA). After DNAse I treatment (Invitrogen), the extracted RNA was reverse transcribed into cDNA by Superscript III First-Strand Synthesis (Invitrogen) using oligo-dT primers. Five replicate RT-PCR reactions were performed using an ABI Prism 7000 (Foster City, CA, USA) and RT^2 SYBR Green/Rox PCR Master Mix (SABiosciences, Frederick, MD, USA). The quantitative levels were normalized to an endogenous control *rp49* and calculated by the ΔΔCT method. The following primers were used (F, forward; R, reverse): *desaturase1*F (TGCCGATTGCTTGCTTCAT), *desaturase1*R (TTCACCCC-AGGCGTACATG), *desaturaseF*F (TCCGTGTGGGTGAGG-GATA), *desaturaseF*R (AGCTCGGCGCTCTTGTAGTC), *elongaseF*F (CCATTATTCTGCTCCACTGTACCA), *elongaseF*R (GTCTGTTGACCGCGCAGTT), *RP49*F (ACTCAATGGAT-ACTGCCAG) and *RP49*R (CAAGGTGTCCCACTAATGCAT).

Statistics

For both GC/MS and LDI-MS data, statistically significant changes in the levels of individual compounds or in the total amount of CHC with age were determined by standard (or weighted when appropriate) regression analysis and ANOVA. Regression analysis was also used to determine the correlation between carbon chain lengths and the percentage change of normalized signal intensity – calculated by the difference between the oldest and the youngest animals [i.e. (65 days–7 days)/7 days old] and to determine the correlation of CHC between anal–genital and foreleg regions from the LDI-MS data. For both courtship assay and video analysis, Wilcoxon signed rank test was applied to test the null hypothesis of no preference (i.e. no difference from 50%).

RESULTS AND DISCUSSION
Aging robustly alters CHC profiles in *Drosophila*

Aging is accompanied by broad declines in physiological functions and reproductive output, and we asked how these physiological changes influence CHC profiles. The average lifespan of *D. melanogaster* is normally 2–3 months in our standard laboratory conditions, which include housing at 25°C and 60% relative humidity in laboratory vials containing a 10% sugar/yeast fly medium (Skorupa et al., 2008). Male and female flies from two genetic backgrounds, Canton-S (a traditional laboratory stock) and Fv (a recently wild-caught strain), were aged in the laboratory and were collected at different ages throughout their lifespan for analysis of CHC profiles. Multiple, independent replicates were collected at each age, and all ages were analyzed simultaneously using regression analysis and ANOVA to identify statistically significant changes.

The two different analysis techniques, GC/MS and LDI-MS, differ with respect to the way they are executed as well as the types of compounds they identify. GC/MS was applied to CHC samples that were extracted from dead flies, while LDI-MS was used to
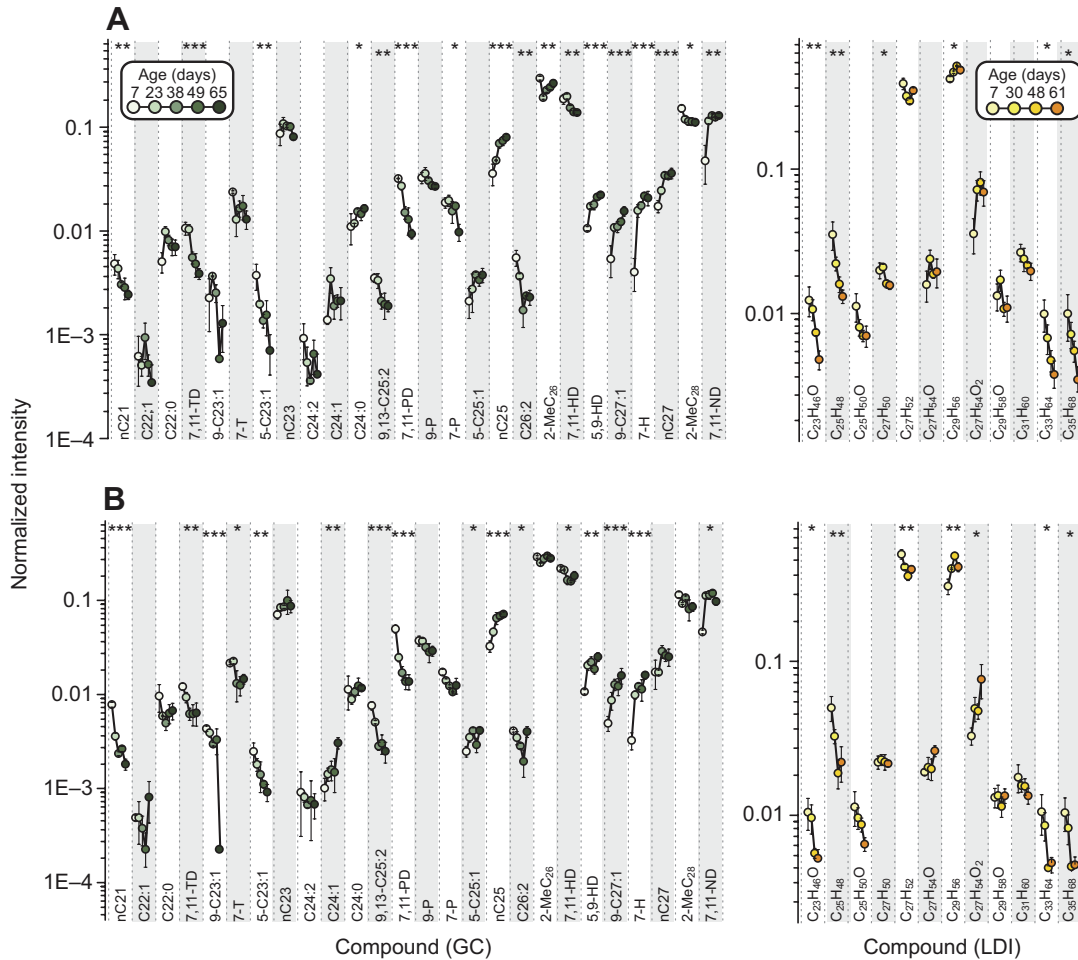
Fig. 1. Aging robustly alters female cuticular hydrocarbon (CHC) profiles in *Drosophila*. Using gas chromatography (GC/MS) and laser desorption/ionization orthogonal time-of-flight mass spectrometry (LDI-MS), we found that the representations of many female cuticular hydrocarbons of (A) Canton-S and (B) Fv wild-type strain were affected by aging. Flies were sampled at five different ages for GC/MS and four different ages for LDI-MS analyses. Age-dependent measurements from the same compound are connected by lines. For GC/MS, compound names indicate the number of carbon atoms followed by the number of double bonds. In some cases, the common chemical name preceded by the position of the double bond(s) is used (TD, tricosadiene; T, tricosene; PD, pentacosadiene; P, pentacosene; HD, heptacosadiene; H, heptacosene; ND, nonacosadiene). Branched compounds are indicated by Me followed by the position of the branched methyl group. Some compounds are identified by both methods: $C_{25}H_{48}$ (LDI-MS) corresponds to 9,13-C25:2 and 7,11-PD (GC/MS); $C_{27}H_{52}$ (LDI-MS) corresponds to 7,11-HD and 5,9-HD (GC/MS); $C_{29}H_{56}$ (LDI-MS) corresponds to 7,11-ND (GC/MS). Statistically significant changes in ages were determined using regression and ANOVA applied to individual compounds. *$P<0.05$, **$P<0.01$, ***$P<0.001$. Note the logarithmic scale.

determine CHC composition directly from the cuticle of intact flies. Accordingly, while several different ages were examined using each method, the sampling procedures were different. All flies that were used for GC/MS originated from a single cohort (sampled at 7, 23, 38, 49 and 65 days of age), while flies used for LDI-MS were obtained from a series of four staggered cohorts (leading to measurements at 7, 30, 48 and 61 days of age; supplementary material Fig. S1). GC/MS has been widely used for decades to study CHC in insects (Howard and Blomquist, 2005). It provides quantitative estimates of the levels of many compounds, as well as some structural elucidation for less polar hydrocarbons. LDI-MS more efficiently detects polar CHC (e.g. oxygen-containing compounds), many of which are not detected by GC/MS (supplementary material Fig. S2) (Yew et al., 2009), as well as particular long-chained hydrocarbons (Yew et al., 2011a), but it does not detect alkanes. Because only intact molecular ions are detected and thus no structural information is obtained, the stereochemistry

of a compound cannot be elucidated. Therefore, for the LDI-MS data, chemical identities are given as the chemical composition. An individual LDI-MS signal may thus represent more than one compound. For example, $C_{23}H_{46}$ (LDI-MS) could correspond to 9-C23:1, 7-T and 5-C23:1. LDI-MS technology allows analysis of CHC profiles from specific body parts with a resolution of roughly 100 µm (Yew et al., 2009). We acquired mass spectra from both the foreleg and anal–genital region of virgin flies. These two body part measures were highly correlated (supplementary material Fig. S3); for simplicity, we focused on data obtained from the foreleg.

By combining GC/MS and LDI-MS, we detected all of the previously identified classes of CHC, including alkanes, alkenes, dienes and oxygen-containing compounds. Several compounds exhibited a significant change with age in female flies (Fig. 1). Moreover, the aging effects were remarkably consistent across replicate samples in the two genetic backgrounds, suggesting that they result from strongly regulated changes in CHC composition
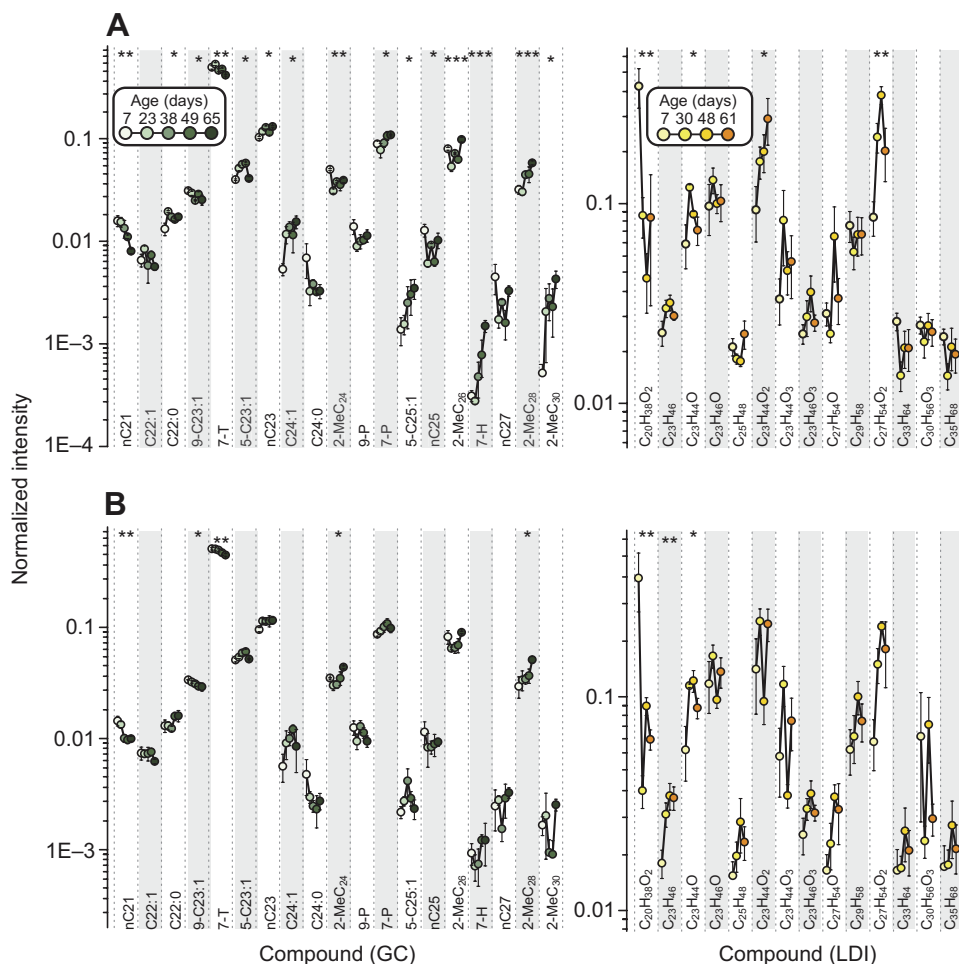
Fig. 2. Aging robustly alters male CHC profiles in *Drosophila*. Several male hydrocarbons in both (A) Canton-S and (B) Fv strains were affected by aging. $C_{23}H_{46}$ (LDI-MS) corresponds to 9-C23:1, 7-T and 5-C23:1 (GC/MS). The data analysis and presentation are as described in Fig. 1. Note the logarithmic scale.

rather than stochastic variation with age (Fig. 1). We observed changes in CHC profiles beginning as early as 23 days of age, with most of the early trends continuing through to 65 days of age. Changes in cohort density do not appear to be a major influence on our data because significant mortality does not occur until more than 40 days of age, which is well after most age-dependent patterns have been established. Two major unsaturated compounds that have been documented as pheromones, 7,11-HD ($C_{27}H_{52}$) and 7,11-ND ($C_{29}H_{56}$), were detected by both GC/MS and LDI-MS (Antony and Jallon, 1982; Antony et al., 1985; Jallon, 1984). Levels of 7,11-HD decreased with age in both strains, while levels of 7,11-ND increased with age. Other compounds that were detected only by either GC/MS or LDI-MS also showed consistent aging patterns, although little is known about the functions of most of these compounds (Fig. 1). Total amounts of CHC per female fly (as assessed by GC/MS analysis) showed on average an increase with age, particularly very late in life (supplementary material Fig. S4).

Male CHC profiles were also significantly affected by aging. We found similar age-dependent trends in many CHC from Canton-S and Fv males, and seven compounds reached statistical significance in both strains [nC21, 9-C23:1, 7-T, 2-MeC$_{24}$ and 2-MeC$_{28}$ from GC/MS; $C_{20}H_{38}O_2$ (cVA) and $C_{23}H_{44}O$ from LDI-MS] (Fig. 2). The compound 7-T is a known pheromone, and we found it to decrease significantly with age (Ferveur and Sureau, 1996; Grillet et al., 2006). Another prevalent compound, 7-P, exhibited a significant increase with age. CHC analysis of the male foreleg region by LDI-MS revealed that the volatile pheromone cVA ($C_{20}H_{38}O_2$) (Bartelt

et al., 1985) exhibited a dramatic decrease in level from 7 days of age to 23 days of age with little change thereafter, suggesting that, in our conditions, only young males release large amounts of this compound. In contrast, levels of a newly identified pheromone, CH503 ($C_{30}H_{56}O_3$) (Yew et al., 2009), which is only detected by LDI-MS, were unaffected by aging. Unlike in females, we did not detect significant changes in total male CHC levels with age (supplementary material Fig. S4).

Aging leads to an increase in the proportion of longer chain CHC

The effects of aging on individual CHC levels were distributed across the profile in a non-random manner such that older females tended to have relatively more CHC with longer carbon chains and fewer CHC with shorter chain lengths (Fig. 1). Consistent with this observation, we found a significant positive correlation between the percentage change in normalized CHC intensity with age and carbon chain length (Fig. 3A). The same pattern was observed in male profiles (Fig. 2 and Fig. 3B), suggesting that similar molecular mechanisms may be responsible for CHC changes in the two sexes.

Because of the observed increases in the proportion of longer chain CHC, we predicted that the expression level of enzymes whose primary function is to lengthen the carbon chain would be increased with age. The only known elongase involved in *Drosophila* CHC formation, *elongaseF*, is female specific and catalyzes the addition of carbon atoms to the carboxylic ends of CHC (Chertemps et al.,
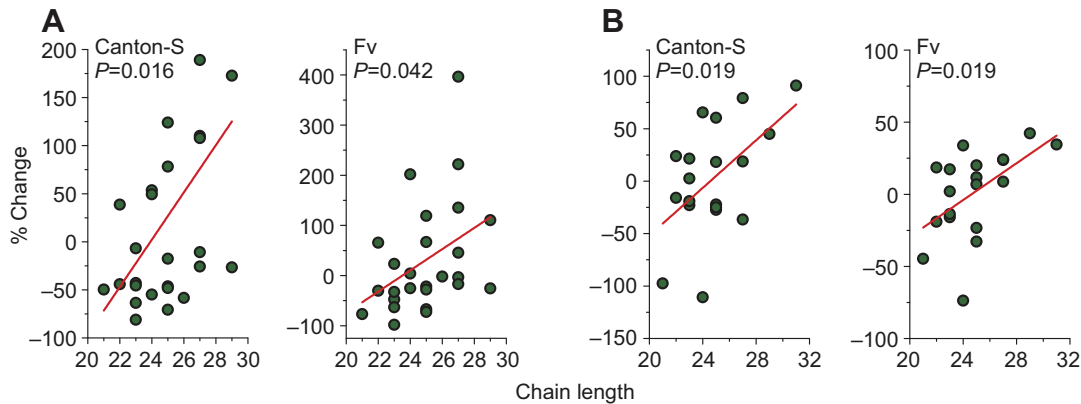
Fig. 3. Aging results in an increased proportion of longer chain CHC. Aging resulted in a change in the distribution of CHC leading to lesser amounts of CHC with a shorter chain length and greater levels of compounds with a longer chain length in old (A) female and (B) male flies. Each data point represents a single compound from GC/MS measurements. Data are presented as percentage change of normalized CHC intensity in the oldest animal from that in the youngest animal [(65 days–7 days)/7 days old]. *P*-values are determined by regression analysis.

2007). Surprisingly, quantitative PCR analysis showed that aging had no effect on the expression of this gene (supplementary material Fig. S5). Therefore, the observed increases in the representation of CHC with longer chain length are likely due to other unidentified genes or mechanisms.

The increase in prevalence of CHC with greater chain length with age prompted us to examine whether compounds that share other general chemical properties were affected by aging in a similar way. We noted that aging resulted in significant changes in the levels of specific dienes, such as 7,11-HD and 7,11-ND, and asked whether aging impacted the overall levels of unsaturated compounds, i.e. alkenes and dienes. We found, however, that aging did not consistently influence the overall levels of these subgroups (data not presented). In addition, the mRNA levels of two identified desaturases involved in double bond formation, *desaturase1* and *desaturaseF* (Chertemps et al., 2006; Dallerac et al., 2000), were also unchanged with age (supplementary material Fig. S5). Again, it is likely that other, unknown genes that function in CHC synthesis are the target of aging or that modulation of CHC composition occurs post-transcriptionally.

Aging-related changes in CHC influence sexual attractiveness
Given their putative importance in sexual communication, we next asked whether the aging-related changes that we observed in CHC profiles influence animal attractiveness. We first examined female attractiveness by assessing male preference for young (8 days old) *vs* old (52 days old) decapitated females in a two-choice courtship assay. We found that males devoted significantly more than 50% of their courtship activity to young females, which suggests that they are more attractive to males than are old females (Fig. 4A). Video analysis (see Materials and methods), which precisely quantifies the percentage of time individual males spend in the proximity of each test female, confirmed the males' preference for young females (Fig. 4A).

Several lines of evidence implicate aging-related changes in CHC composition in the observed differences in attractiveness. First, male discrimination between young and old females disappeared following a hexane wash, which effectively removes all CHC from the females (Fig. 4A). Second, we repeated the courtship assay under dim red light, which is undetectable to the flies, and found that the

preference for young females remained, which rules out visual cues as a major sensory input responsible for the observed differences (Fig. 4A). Finally, we applied CHC extracts from young or old flies onto same-age, oenocyte-less flies, which do not produce CHC (Billeter et al., 2009), and tested male preference. We found that males preferred oenocyte-less females covered with CHC from young animals over those covered with CHC from old animals (Fig. 4A).

Changes in the levels of known pheromones may be responsible for the decrease in female attractiveness with age. Although it may not be sufficient to induce male courtship alone (Billeter et al., 2009), a pheromonal role for 7,11-HD has been suggested from several studies (Antony et al., 1985; Antony and Jallon, 1982). We found that levels of 7,11-HD decreased with age. A second compound, 7,11-ND, may also function to stimulate male courtship (Antony et al., 1985). However, our data show that older females, who are significantly less attractive, exhibit relatively high 7,11-ND levels. It seems likely, therefore, that female attractiveness in our study results from synergistic effects induced by changes in multiple compounds rather than additive contributions from individual compounds.

In males, the observed decreases of 7-T and cVA, both of which are known pheromones that increase female receptivity, suggested that aging males may also lose their attractiveness. To determine whether this was indeed the case, we used video analysis to assess male attractiveness by quantifying the amount of time individual females spent in the immediate proximity of test males. This method avoids potential confounding effects from differences in courtship behaviors between young and old males. Indeed, we found that even in the absence of courtship from males, females spent significantly more time near decapitated, immobilized young males than they did with old males (Fig. 4B). This effect was small (~5% preference) but significant (*P*=0.02). However, differences did not persist in the dark (Fig. 4B), suggesting that visual cues are important for female preference. These results do not, however, rule out a possible effect of male CHC profiles in female choice. It may be that females rely on multiple stimuli and sensory modalities to make choices. Vision, for example, may be necessary for females to remain in the proximity of a specific male to detect the CHC. Analysis of preference in mutant females with deficits in specific aspects of sensory perception would help disentangle such influences.
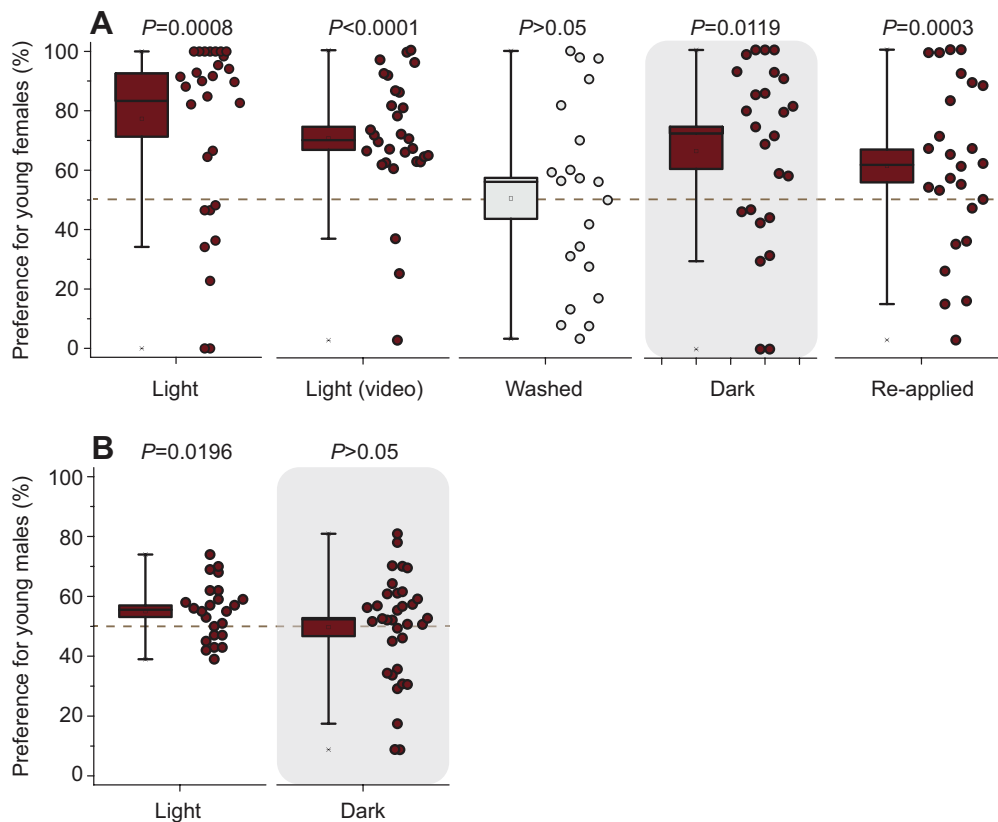
Fig. 4. Aging significantly reduces sexual attractiveness in *Drosophila*. (A) Female attractiveness: males exhibited a strong and highly significant preference for young females when measured either by direct observation in the light using a live courtship assay (N=29) or by automated video analysis (N=30). This preference was lost when females were exposed to a hexane wash, which removed CHC (video analysis; N=21). Preferences persisted in the dark (live observation; N=25) and when oenocyte-less females were covered with CHC extracted from young or old females (video analysis; N=26). (B) Male attractiveness: females exhibited modest preference for young males (video analysis; N=24), but the preference was lost in the dark (video analysis; N=34). For all behavior assays, preference is presented as the percentage of time the choosing animals spend either actively courting (live analysis) or in the vicinity (video analysis) of the young test fly divided by the total time attributed to both test flies [young/(young+old)]. All experiments used Canton-S flies. *P*-values represent the results of a Wilcoxon signed-rank test applied to test the null hypothesis of no preference (i.e. the time spent courting either fly=50%).

Together, these data show that aging significantly reduces attractiveness in both male and female *D. melanogaster*. While the effect of aging on female attractiveness can be ascribed to changes in CHC profiles, its influence on male attractiveness is more subtle and likely due to alterations in multiple sensory cues.

CHC as honest indicators of reproductive potential

Why are CHC profiles altered by aging? We believe that it is unlikely that these changes reflect a general deterioration of the capacity to produce individual CHC. General deterioration would be expected to manifest in reduced synthesis, increased variance and inconsistent results from strain to strain. In contrast, the age-related changes that we observed were highly repeatable and, if anything, total CHC levels increased with age. It is plausible that the observed changes in CHC result from mechanisms unrelated to CHC synthesis. Long-chain compounds, for example, have lower vapor pressure, which may result in a slow accumulation over time. Alternatively, the consistent changes across different genetic backgrounds and the age-dependent increases of long-chain length species in both sexes is consistent with a model whereby CHC synthesis is strongly regulated throughout the lifespan. Although we found no evidence that this regulation was occurring at the level of gene transcription (mRNA levels of genes involved in CHC synthesis were unchanged in this study), CHC regulation

could occur post-transcriptionally or through changes in the expression of other unidentified genes. CHC analysis using especially long- or short-lived strains of flies would provide insight into the influence of each of these possibilities.

Aging-related changes in CHC profiles may be adaptive and may promote specific aspects of stress resistance. Many researchers have suggested that the ancestral function of CHC in insects involves the prevention of water loss (Foley and Telonis-Scott, 2011; Gibbs et al., 1997). Older flies exhibit a higher water loss rate and are less resistant to desiccation (Gibbs and Markow, 2001). Therefore, it may be that increases in total CHC or changes in the CHC profile, specifically increases in long-chain CHC, are initiated to protect aging flies against increased susceptibility to water loss (Gibbs, 2002).

If changes in CHC composition are driven by challenges associated with aging physiology, it may be that flies evolved the ability to recognize them as honest indicators of organism health. Our data show that aging reduces organism attractiveness and that CHC composition, at least in females, is a major determinant of this change. Aging is a process that affects key fitness characteristics, with most animals experiencing a decline in general health and a reduction in reproductive output. Therefore, aging-related changes in CHC could have originated as an adaptive response and have since evolved to be used by potential mates as indicators of an

individual's age or condition. Similar ornamental indicator traits have been suggested in other systems. Carotenoid and melanin-based pigments, for example, have been shown to reflect immunity and the ability to gather resources in birds and fishes (Searcy and Nowicki, 2005). Most of the evidence, however, is based on correlations, and molecular connections between the indicator traits and underlying fitness characteristics have not been established (Hill, 2011). Our data suggest the intriguing hypothesis that molecular mechanisms that affect animal longevity or reproduction might also directly affect sexual characteristics and that this may be a common phenomenon. It will therefore be of interest to determine whether genetic or environmental manipulations that modulate lifespan or reproduction influence CHC or other sexually attractive characteristics in the fly and whether these relationships are conserved in other species.

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