Aging modulates cuticular hydrocarbons and sexual attractiveness in Drosophila melanogaster

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Accepted 23 November 2011

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.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/215/5/814/DC1

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 ‘+’: PromE(800)–Gal4, tubP-Gal80P;+ to ‘+’: UAS-StingerII, UAS-hid/CyO;+; both strains were provided by J. Levine (Billette 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 2–3 weeks, 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 100 μl of hexane, which contained 10 μg ml⁻¹ of the hydrocarbon hexacosane (Sigma-Aldrich, St Louis, MO, USA) as an internal standard. Following incubation at room temperature for 30 min, 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 30 μl 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-methy polymersiloxane column; 30 m length, 0.32 mm i.d., 0.25 μm film thickness; Agilent, Santa Clara, CA, USA). Ionization was achieved by electron ionization (EI) at 70 eV. One microliter of the sample was injected using a splitless injector. The helium flow was set at 1.3 ml min⁻¹. The column temperature program began at 50°C for 2 min, and increased to 300°C at a rate of 15°C min⁻¹. The quadrupole mass spectrometer was set to unit mass resolution and 3 scans s⁻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 (10 μg ml⁻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 2–3 weeks, 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 N₂ laser emitting 3 ns long pulses at a wavelength of 337 nm and a repetition rate of 30 Hz. The laser beam spot size on the sample is ~200 μm in diameter and has an approximately flat-top intensity profile. Ions were generated in a buffer gas environment using 2 mbar (1 bar=100 kPa) of Argon gas. For acquisition of a mass spectrum, 900 laser pulses were applied to one spot (or small area of body parts) over 30 s. Laser fluence (light energy per pulse and area) was adjusted to values moderately above the ion detection threshold, corresponding to values between 100 and 200 J m⁻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 ±0.02 Da. 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 side 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 C# 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 (Billette et al., 2009). Briefly, the progeny of the cross of +: PromE(800)-Gal4, tubP-Gal80;+ 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 DNaseI 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 RT² SYBR Green/ Rox PCR Master Mix (SABiosciences, Frederick, MD, USA). The quantitative levels were normalized to an endogenous control probe and calculated by the ΔΔCt method. The following primers were used (F, forward; R, reverse): desaturase1F (TGCCGATTGCTGTGTCATG), desaturase1R (TTCACCCCCAGGGTGATACG), desaturase2F (TTCGTGTGGGTTGAGGGATA), desaturase2R (AGCTCGCGCTCTTTGTAGT), elongaseF (CCATTATTCTGCTCCACTGTACCA), elongaseR (GTCTTGTTAGCCAAGGCTAGTT), RP49F (ACTCAATGGACTGCCAG) and RP49R (CAAGGTGTCCCACTAATGCAT).

**Statistics**

For both GC/MS and LDMS-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 LDMS-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 LDMS-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 LDMS-MS was used to
Aging affects fly pheromones and attractiveness

Aging affects fly pheromones and attractiveness. 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_{25}H_{48} (LDI-MS) could correspond to 9,13-C_{25}: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.

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-C_{25}: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).
Changes in cohort density do not appear to be a major influence on most of the early trends continuing through to 65 days of age. 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. 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). 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.

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

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In 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. C20H46 (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.
Aging affects fly pheromones and attractiveness

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.

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.
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 (Seary 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.

ACKNOWLEDGMENTS
We thank all members of the Pletcher laboratory for help with Drosophila husbandry and comments on the experimental design, especially Katie Marney for video analysis and Azra Dervisefendic and Lisa Johnson for fly food preparation. We also thank D. Promislow and members of the Dierick laboratory for advice. We thank H. Luftmann (University of Münster) for help with GC/MS analysis and interpretation. E. A. Kravitz (Harvard Medical School, Boston) provided laboratory resources.

FUNDING
This research was supported by US National Institutes of Health [R01AG030593 and R01AG023166], the Glenn Foundation, the American Federation for Aging Research, the Ellison Medical Foundation (to S.D.P.), the Alexander von Humboldt foundation, Singapore National Research Foundation [RF001-363 to E. A. Kravitz], the National Institute of General Medical Sciences [GM074675 and GM0676450] and the National Science Foundation [IDS-075165 to E. A. Kravitz]. This research was supported by US National Institutes of Health [R01AG-013283]. Deposited in PMC for release after 12 months.

REFERENCES

The Journal of Experimental Biology
Figure S1
A. LDI Profile

B. Region Comparison

Figure S3
Figure S4
Figure S5

A. *elongaseF*

B. *desaturase1*

C. *desaturaseF*