Aging-Related Variation of Cuticular Hydrocarbons in Wild Type and Variant Drosophila melanogaster

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Abstract
The cuticle of all insects is covered with hydrocarbons which have multiple functions. Cuticular hydrocarbons (CHCs) basically serve to protect insects against environmental harm and reduce dehydration. In many species, some CHCs also act as pheromones. CHCs have been intensively studied in Drosophila species and more especially in D. melanogaster. In this species, flies produce about 40 CHCs forming a complex sex- and species-specific bouquet. The quantitative and qualitative pattern of the CHC bouquet was characterized during the first days of adult life but remains unexplored in aging flies. Here, we characterized CHCs during the whole—or a large period of—adult life in males and females of several wild type and transgenic lines. Both types of lines included standard and variant CHC profiles. Some of the genotypes tested here showed very dramatic and unexpected aging-related variation based on their early days’ profile. This study provides a concrete dataset to better understand the mechanisms underlying the establishment and maintenance of CHCs on the fly cuticle. It could be useful to determine physiological parameters, including age and response to climate variation, in insects collected in the wild.

Keywords Cuticular profile · Ontogeny · Aging · Chemical communication

Introduction

While physiological changes in aging animals were already described by the Greek philosopher Aristotle (Woodcox 2018), in the 1930s researchers started to precisely measure quantitative changes occurring during aging (Crimm and Short 1934; Horst et al. 1934; McCay et al. 1939). Most of these studies dealt with rats, a model with an average 4–5 years lifespan. However, more recent research also made use of shorter-lifespan organisms such as yeast, worms, and insects. The model insect species Drosophila melanogaster became very popular in such research since it is amenable for the genetic dissection of biological and environmental factors involved in aging (Rose and Charlesworth 1981; Tower 2019). The most studied biological factors are genes, including those involved in sex determination, microbiota, and mitochondria (Clark and Walker 2018; Hur et al. 2014; Partridge and Tower 2008; Piper and Partridge 2016; Tower 2015; Tower 2017) whereas the most studied environmental factors are the temperature (Carvalho et al. 2017; Lamb 1968; Miquel et al. 1976; Moloñ et al. 2020), crowding conditions (Horváth and Kalinka 2016; Klepsatel et al. 2018; Lushchak et al. 2018; Miller and Thomas 1958), and diet during both preimaginal and adult development (Grangeteau et al. 2018; Murgier et al. 2019; Tatar et al. 2014). Typically, individuals of an outbred D. melanogaster strain raised in the laboratory, at 25 °C on a yeast-rich diet, and under uncrowded condition, show a median lifespan of approximately 50–60 days with very few individuals surviving over 90 days (Lee et al. 2008; Skorupa et al. 2008; Ziehm et al. 2013).

Insect cuticle is covered by a thin lipid layer, which largely consists of cuticular hydrocarbons (CHCs). CHCs primarily serve to limit water loss (Ferveur et al. 2018; Gibbs and Pomonis 1995; Hadley 1981; Locke 1965; Qiu et al. 2012) and also offer partial protection against entomopathogens (Howard and Blomquist 2005; Mannino et al. 2019). While the CHC profile has likely evolved to help insects adapt to the variation of environmental factors (Ferveur and Jallon 1996; Gosden and Chenoweth 2011; Menzel et al. 2018; Savarit and Ferveur 2002b), CHCs often serve also...
as contact pheromones and as an individual chemical signature (Blomquist and Bagnères 2010; Ginzel and Blomquist 2016) participating in the regulation of social behavior (Bagnères et al. 1991; Howard and Blomquist 2005; Le Conte and Hefetz 2008) and allowing the recognition of nestmates (Howard and Blomquist 2005; van Zweden and d’Ettorre 2010), castes (Bagnères et al. 1998; Kaib et al. 2000; Sevala et al. 2000; Smith and Taylor 1990), and polyethism (Rahman et al. 2016). CHC profile is under genetic control in both social and solitary insects (Bonavita-Cougourdan et al. 1996; Dronnet et al. 2006; Etges et al. 2009; Thomas and Simmonds 2008; Vauchot et al. 1996) but the chemical signature may vary according to endogenous factors, such as insect development, aging and endocrine system (Darrozet et al. 2014; Fan et al. 2004; Lengyel et al. 2007), and exogenous factors, such as climate (Bagnères et al. 1990), diet (Liang and Silverman 2000), parasitism (Bagnères et al. 1996; Le Conte et al. 2015; Lebreton et al. 2010), or time (Bagnères et al. 2011; Lebreton et al. 2009; Provost et al. 1993).

As in other insects, CHC profile is a highly complex and reliable physiological parameter in D. melanogaster and in phylogenetically related species since it depends on the exquisite equilibrium between many enzymes involved in CHC biosynthesis (Ferveur 1991; Jallon 1984). Given that mature D. melanogaster flies produce more than 40 CHCs (Everalerts et al. 2010), the quantitative variation of each CHC belonging to this profile can thus reveal very subtle physiological and/or developmental defects (Dembeck et al. 2015; Savarit and Ferveur 2002b). While the CHC profile has likely evolved to help insects cope with the environmental variations (Ferveur and Jallon 1996; Gosden and Chenoweth 2011; Menzel et al. 2018; Savarit and Ferveur 2002b), it may also result of the pheromonal effect of some of these compounds, in particular, those reinforcing interspecific barriers during incipient speciation (Dembeck et al. 1999; Grillet et al. 2012). For example, the two sibling species D. melanogaster and D. simulans show a great CHC difference: the former species—not the latter one—exhibits a clear qualitative sexual dimorphism for the principal groups of desaturated CHs: dienes (with two double bonds: only present in females) and monoenes (with one double bond: more abundant in males) which induce a reciprocal behavioural effect in males of the two species (Coyne 1996; Coyne and Oyama 1995; Seeholzer et al. 2018). In social insect species, CHC profile variation may be also related to social relationships, reproductive status, or mimicry between two species (Greene and Gordon 2003; Lenoir et al. 1997; Leonhardt et al. 2016; Liebig et al. 2000).

While the variation of CHCs profiles has been widely documented according to population, sex, caste, fertility, diet, and health state (Beani et al. 2019; Caputo et al. 2005; Mpuru et al. 2001; Nunes et al. 2009; Zhu et al. 2006), very few studies have investigated the aging effect on CHCs. The only exception concerns forensic studies dealing with necrophagous flies such as Diptera Calliphoridae and Sarcophagidae species and the study by Kuo and co-workers (Kuo et al. 2012). In the former species, CHC variation serves to determine the late post-mortem interval (Moore et al. 2014; Pechal et al. 2014; Pomonis 1989; Zhu et al. 2013; Zhu et al. 2006). The latter study (Kuo et al. 2012) reported CHCs variations at several adult ages between the 7th and the 65th day in a wild-type strain (Cs) and in one oenocyte-less line (corresponding to the #5 x #7 genotype tested in the present work).

Here, we precisely and systematically investigate, during the whole adult lifespan (80 days when possible), the aging effect on the CHCs in several D. melanogaster genotypes originating from natural populations and several transgenic strains known for CHC alteration during early adult ages.

### Material & Methods

#### Fly Strains and Husbandry

Four D. melanogaster natural strains were used: Canton-S (Cs), Oregon-R (Or-R), Dijon 2000 (Di2), and Zimbabwe30 (Z30). The Cs stock was originally collected in Canton, Ohio in 1916 (Bridges 1916). Due to its low mutation rate (Stern and Schaeffer 1943), the Cs strain was propagated in many research laboratories (Benzet 1967). Or-R stock originated from flies collected in Roseburg, Oregon around 1925 (Lindsley and Grell 1968). The Di2 stock was initiated with five pairs of flies caught in 2000 in Dijon, France (Svetec and Ferveur 2005). The Z30 line (Grillet et al. 2012; Grillet et al. 2018) was collected in 1990 in the Wildlife Reserve of Sengwa (Begun and Aquadro 1993) and provided by Prof. Jerry Coyne (Univ. of Chicago). The Cs, Di2, and Or-R strains belong to the cosmopolitan CHC type (= M strains) while the Z30 strain shows a variant CHC profile. In particular, M flies produce much more C7-mono- and di-unsaturated CHCs (7-monoenes and 7,11-diienes) than C5-desaturated CHCs (5-monoenes and 5,9-dienes) while Z flies produce a more balanced C7/C5 ratio (Cortot et al. 2019; Grillet et al. 2012). To generalize the data obtained with the Z30 line, we also tested two Z30-derived lines resulting from the introgression of the Z30 genome into a Di2 white genetic background (Cortot et al. 2019).

We also tested two transgenic lines: (1) the desat11573 Gal4 which is homozygous for a PGal4 transposable element inserted in the regulatory region of the desat1 gene produces a reduced quantity of desaturated CHCs (Marcillac et al. 2005a; Marcillac et al. 2005b); (2) the double transgenic line 5670-tra carrying the 5670-Gal4 transgene driving the dominant feminizing UAS-traF transgene (Ferveur...
et al. 1995). 5670-tra flies show early adult life alteration for their CHCs “gender” (Savarit and Ferveur 2002a).

We also tested “oenocyteless” flies (Oe”) genetically deprived of oenocytes, the tissue normally involved in CHC synthesis (Billeter et al. 2009; Ferveur et al. 1997). Oe” flies resulted of reciprocal crosses between flies carrying the temperature sensitive [+: PromE(800)-Gal4[2 M], Tub-Gal80fs] transgene (#5) and either the UAS-hid/CyO (hid = head involutive defective; #2) or UAS-hid, UAS-StingerII/CyO flies (#7) pro-apoptotic transgenes. We tested four oe” genotypes indicated by the cross between females x males: #2 x #5, #5 x #2, #7 x #5, #5 x #7 (the last genotype was also tested in Kuo et al. 2012). These strains were kindly provided by Prof. J-C Billeter (Univ of Gröningen). Oe” individuals were raised (i) during their complete preimaginal life at a permissive temperature (18 °C) allowing the Gal80” transgene to repress the effect of Gal4 activating the apoptotic UAS-hid transgene in the oenocytes then (ii) shifted at the restrictive 29° temperature just after adult emergence allowing Gal4-driven apoptosis of the oenocytes.

Stocks were maintained on alcohol-free standard corn-meal medium mixed with killed yeast in 30 ml glass vials, at 24 ± 0.5 °C (except for oe” genotypes) and 65 ± %5 humidity on a 12:12 dark:light cycle. One to 2-h old flies were sexed under light carbon dioxide anesthesia 2–4 h after lights on and were kept in fresh-food vials in small groups of 5–7 flies until the extraction of their CHCs. They were regularly transferred in a fresh-food vial every 7 days and due to death occurring during aging, flies from different vials were pooled to maintain the group size as constant as possible. We tested aging until 80 days in three key strains which are those most studied in the lab while the other natural variant and transgenic strains were tested until 24 days both for the sake of clarity and due to their high mortality.

Cuticular Hydrocarbon Extraction

Flies were individually plunged, at room temperature, for 5 min into vials containing 30 μl hexane with 3.33 ng/μl of n-C26 (n-hexacosane) and 3.33 ng/μl of n-C30 (n-triacontane) used as internal standards (ISs). After removing the fly, extracts were kept at −20 °C until analysis. CHCs were quantified by gas chromatography using a Varian CP3380 gas chromatograph fitted with a flame ionization detector, an apolar CP Sil 5CB column (25 m by 0.25 mm; internal diameter: 0.1 μm film thickness; Agilent) and a split–splitless injector (60 ml/min split-flow; valve opening 30 s after injection). Helium was used as the carrier gas (50 cm/s at 120 °C). The temperature program began at 120 °C, ramping at 10 °C/min to 140 °C, then ramping at 2 °C/min to 280 °C and holding for 10 min. The chemical identity of each peak was determined using according to (Everaerts et al. 2010). The amount (ng/insect) of each compound was calculated based on the readings obtained from the ISs. The overall sums of all CHCs (∑CHCs) and of desaturated CHCs (∑DesatCHCs) were noted, as well as the proportions of mono- and di-unsaturated CHCs (Monoenes% and Dienes%), of linear saturated CHCs (LinCHCs%) and of methyl branched CHCs (BrCHCs%).

For each age (until 80 days for Cs and 5670-tra, until 24 days for other lines), we individually extracted 14–30 Cs females and 8–30 Cs males; 30 5670-tra females and 11–30 5670-tra males; 14–20 Oe” females and 11–20 Oe” males; 20 Di2 flies of each sex; 7–20 Or-R females and 10–16 Or-R males; 6–20 Z30 females and 11–20 Z30 males; and for each Z30 derived line: 3–20 females and 8–20 males.

Statistics

All statistical tests were performed using XLSTAT 2021 (Addinsoft 2021). For each group of strains (natural strains, P-Gal4 transgenic lines, and double transgenic flies) and for each sex, we first carried out a Factorial Discriminant Analysis (FDA) using ∑CHCs, Monoenes% and/or Dienes%, where appropriate, and LinCHCs% and BrCHCs% as quantitative variables and the “strain/age” as qualitative variable. For the sake of clarity, the graphical representation of the results is simplified by using for each “strain/age” group the corresponding barycenter and equiprobable ellipse (p = 0.05) instead of individuals. Thereafter, for each sex we detailed the ontogenies of the overall amount of ∑CHCs and of ∑DesatCHCs as well as the % of the four CHCs classes of CHCs. These ontogenies were compared between strains using Kruskall-Wallis test followed by Conover-Iman multiple pairwise comparisons (p = 0.05, with a Bonferroni correction).

Results

CHC Variation during Complete Adult Life in Males and Females of Three Genotypes

We first compared CHC variation in aging male and female flies of the control Canton-S strain (Cs), and the two transgenic 5670-tra and desat1 lines (see Materials & Methods). CHC profiles were followed in flies aged 60 days (or more when they survived).

In these genotypes, aging male and female flies showed divergent CHC variation. This can be visualized on the Factorial Discriminant Analysis (FDA) separately performed for each sex (Supp Fig. 1). The three male flies showed very different FDA profiles. Cs males showed fast changes during the first three days of adult life followed by much smaller changes until 80 days. Differently, 5670-tra males showed
a regular variation during most lifespans whereas desat1 males showed a non-linear variation. Cs females also showed substantial FDA variations during the first 3 days followed by slighter changes while the most visible changes in 5670-tra and desat1 females occurred between 6 h and 2 days. During their early adult age, 5670-tra females showed a less drastic variation than males. In both FDAs, LinCHCs% was negatively related to Monoenes%.

To precisely determine the origin of the FDA variations, we analyzed several CHC parameters consisting of the major CHC classes (Fig. 1; see Material & Methods). First, ∑CHCs substantially increased in Cs males up to 20 days (2270 ng), followed by a slower increase until 80 days (2830 ng). Differently, 5670-tra males showed a strong and regular ∑CHCs increase until 72 days (7380 ng) while desat1 males showed an unstable variation between 1730 and 3100 ng with a final increase above 4400 ng (at 64 days). These inter-genotype variations are partly reflected by the variation of ∑DesatCHCs which was stabilized in Cs at 20 days (between 1700 and 2000 ng) while it continued to strongly increase in 5670-tra males (5670 ng at 72 days). ∑DesatCHCs remained low in desat1 males. Both Cs and 5670-tra males predominantly produced monoenes (64–72%) in a proportion that remained relatively stable after a few days. After 8 days, 5670-tra males produced 2% dienes. In desat1 males older than 16 days, LinCHCs% decreased (74 to 53%) while BrCHCs% increased (21 to 39%).

**Fig. 1** Age-related variation of cuticular hydrocarbons in male and female flies of three *D. melanogaster* lines. The variation of cuticular hydrocarbons (CHCs) was followed during the complete adult life (60 to 80 days; shown on x-axis) in males (left panels) and females (right panels) of three *D. melanogaster* lines: the wild type Canton (Cs; black colored lines), the double transgenic 5670-Gal4 > UAS-traF (5670-tra; green colored lines) and homozygous mutant desat1; Gal4 (desat1; purple colored lines). On the left side of each panel series, we show the sums of absolute amounts of all detected CHCs (∑CHCs) and of all detected desaturated CHCs (∑DesatCHCs) in ng, and of the right side the proportion (%) of the main CHC classes (from top to bottom): monoenes (Monoenes%), dienes (Dienes%), linear saturated CHCs (LinCHCs%) and methyl-branched CHCs (BrCHCs%). These % were calculated relatively to ∑CHCs. For Cs males, n = 10–30 (except for 45 days: n = 8, 50 days: n = 8, 60 days: n = 9, 70 days: n = 7, 75 days: n = 4); for Cs females, n = 25–30 (except for 60 days: n = 10). For 5670-tra males, n = 11–50; for 5670-tra females, n = 13–30. For desat1 males, n = 15; for desat1 females, n = 12 to 15 (except for 55 days, n = 7).
Females showed different age-dependent patterns, as compared to same-strain males. ∑CHCs regularly increased in both Cs and 5670-tra females until 8 days to reach relatively stable amounts (between 2070 and 3100 ng). Very differently, desat1 females showed an enormous ∑CHCs peak at 55 days (12,370 ng) before drastically decreasing until 80 days (4310 ng). More precisely, Cs females predominantly produced DesatCHCs (with a stable amount around 1500 ng) made of 40% dienes and 20% monoenes. Differently, ∑DesatCHCs showed a two-step increase in desat1 females: (1) a slow increase and a plateau until 40 days (380 ng) followed by (2) a steep increase up to 80 days (2280 ng). This effect was likely due to increased Dienes% (5 to 44%) during the same period. On the other hand, their large ∑CHCs decrease was likely due to the massive drop of LinCHCs% (45 to 15%) and also—but to a lesser extent—of BrCHCs% (50 to 35%), after 55 days.

In summary, male and female flies of these three genotypes showed very divergent CHC pattern variation during aging. While Cs flies showed a regular transformation of their CH pattern, 5670-tra males and desat1 females showed very dramatic variations which were unpredictable based on the first days’ pattern.

Age-Dependent CHC Variation in Flies of Four Wild-Type Strains and Two Z30 Derived Lines

We next investigated CHC variation in three more wild type strains: Di2, Or-R, and Z30 beside the Cs strain kept as a reference (the analysis was performed with different flies from those used above). The Cs, Di2, and Or-R strains belong to the cosmopolitan “M” CHC type while Z30 shows a variant CHC profile (Z-type; see Material & Methods). To generalize the data obtained with the Z30 wild type strain, we also tested two Z30-derived lines (1 W14 and 3 W1; (Cortot et al. 2019).

As above, we performed FDAs and measured the main CHC parameters during the first 24 days of adult life, in each sex. Both FDAs show a clear separation (on the F1 axis) between the three M strains and the three Z strains (Supp Fig. 2). Moreover, M—but not of Z—males and females showed a clear age-dependent variation.

Between 4 and 24 days, ∑CHCs increased in M males and slightly decreased in Z males (Fig. 2). This pattern largely followed the ∑DesatCHCs variation during the same period. However, Monoenes%, LinCHCs%, and BrCHCs% were very similar between all strains, and remained relatively constant between 4 and 24 days. Dienes were never detected in these males.

In females, CHCs parameters showed different age-dependent variation if compared to same-strain males. During aging, ∑CHCs showed a slight and parallel increase in Cs and Di2 females (+400 ng and +230 ng, respectively) and a stronger increase in Or-R females (+760 ng). Differently, ∑CHCs strongly decreased in Z females (−560 ng). In Or-R females, the ∑DesatCHCs increase paralleled the ∑CHCs increase. During aging, the two other M females showed relatively stable ∑DesatCHCs while it strongly decreased in Z females. Monoenes% (5–13%), Dienes% (50–70%), and proportions of the other CHC groups were generally similar between strains and did not show much age-related variation.

In summary, during aging M and Z flies showed a reciprocal variation—increase and decrease, respectively—of their ∑CHCs.

Age-Dependent CHC Variation in Oenocyteless Flies

Given the drastic effect resulting in the quasi-total absence of CHCs in oenocyteless flies during early adult life (oe−, (Billeter et al. 2009)), we tested CHCs in aging flies of four oe− genotypes (“2 × 5”, “5 × 2”, “7 × 5”, “5 × 7”; see Material and Methods). FDAs show a clear separation between oe− and Cs CHCs profiles (these Cs flies were already used in the previous experiments; Supp Fig. 3). This effect was expected given the dramatic CHC alteration reported in oe− flies. However, while no difference was observed between oe− females of the different genotypes, oe− males older than 4 days showed some differences: 7 × 5 and 5 × 7 males showed intermediate FDA profiles between those of Cs and of 2 × 5 and 5 × 2 males.

A closer examination of the age-dependent variations observed between oe− flies were also detected when comparing their CHC parameters (Fig. 3). While ∑CHCs was initially low in 2 × 5 and 5 × 2 males to slightly increase after 8 days (up to 200 ng), it substantially increased in 7 × 5 and 5 × 7 males between 8 and 24 days (900-1250 ng). These variations were largely paralleled to those of ∑DesatCHCs (only monoenes). Moreover, between 4 and 24 days, 2 × 5 and 5 × 2—but not 7 × 5 and 5 × 7—males showed a strong and reciprocal variation for Monoenes% (+35%) and BrCHCs% (−50%).

In oe− females, both ∑CHCs and ∑DesatCHCs strongly decreased between 4 and 8 days (from 300 to 700 ng to almost 0), and remained close to 0 until 24 days. Four days old oe− females produced 30–50% DesatCHCs (mostly dienes), 15–25% LinCHCs and 30–60% BrCHCs. The CHC proportions noted at the other ages may not be indicative given that they corresponded to very low absolute amounts.

In summary, oe− flies showed very unexpected CHC variation: in particular, between 4 and 8 days, ∑CHCs and ∑DesatCHCs substantially increased in 5 × 7 and 7 × 5 males while they strongly decreased in females to almost completely disappear.

Discussion

In the present study, we explored the variation of CHCs in D. melanogaster male and female flies of 12 genotypes during all—or a large part of—their adult life. Youngest (<6 h old) male and female flies of control and transgenic lines shared
very similar CHC global pattern, in support of previous studies (only reported for control strains; Antony and Jallon 1982; Cuccillo and Tompkins 1987; Tompkins 1984). When becoming sexually mature (≥2 days), CHC patterns in natural variants and transgenic flies started to diverge compared to well-known wild-type strains (Antony and Jallon 1982; Everaerts et al. 2010; Ferveur 2005; Jallon 1984). However, it must be stressed that the previous characterization of the CHC pattern of these wild type flies was only shown for the first days of adult life. Moreover, transgenic and natural variants showed unexpected CHC profiles, if referred to the literature (see below). After attempting to interpret each of the three main dataset (Figs. 1, 2, 3), and with all data gathered, we will propose some possible biological mechanisms underlying CHC biosynthesis during lifetime.

In the first dataset (Fig. 1), control Cs flies showed a biphasic pattern composed of (1) a regular CHC increase until 12 days, (2) followed by a much slower increase followed by a plateau interrupted by very small variations. Differently, during the second phase of their adult life, desat1 females and 5670-tra males showed very dramatic and irregular variations. For instance, ∑CHCs strongly and continuously increased, until the end of the life in 5670-tra males (72 days), or until 52-days before strongly decreasing in desat1 females. These massive variations were paralleled by those of ∑DesatCHCs (monoenes and dienes in 5670-tra males; only dienes in desat1 females). Both 5670-Gal4 and desat11573-Gal4 show a similar expression in the oenocytes (hepatocyte-like cells) and fat body, the two main tissues involved in CHC biosynthesis (Diehl 1973; Ferveur et al.

Fig. 2 Age-related variation of cuticular hydrocarbons in male and female flies of various wild type and derived lines. CHC variation was followed during the first 24 days (x-axis) of adult life in males (left panels) and females (right panels) of the following wild type strains: Cs (black colored lines), Dijon2000 (Di2; orange colored lines), Oregon-R (Or-R; pink colored lines) and Zimbabwe30 (Z30; green colored lines and circles). We also tested two Z30-derived lines: 1 W14 and 3 W1 (green colored lines and diamonds, or triangle, respectively; see Material and methods). Dienes were not detected in males. For CHCs information, see Fig. 1 legend. For all Cs and Di2 flies, n=20. For Or-R males, n=10–16; for Or-R females, n=7–14. For Z30 males, n=11; for Z30 females, n=16 except at 24d (n=6). For 3 W1 males, n=20; for 3 W1 females, n=12 to 20. For 1 W14 8d flies, n=20, and n=2 in 2d flies, n=8 for 4d males and n=3 for 4d females.
However, given that such tissue-specific expression was only observed in 2- and 4-day-old flies, we cannot rule out that Gal4 expression varies in older flies (Weaver et al. 2020). Also, both PGal4 transgenes were tested in a different genetic context: 5670-Gal4 was used to target the UAS-traF feminizing transgene whereas the desat1573-Gal4 transgene was tested in homozygous flies. Moreover, both transgenes (together with those tested in oe− flies) may also affect other gene(s) involved in developing tissues directly or indirectly involved in CHCs and other sex-specific characters (Houot et al. 2012; Wicker-Thomas and Jallon 2001). Moreover, expression of the PromE(800)-Gal4[2 M] line (#5 here) was shown to vary during early development (see Supplementary Fig. 1 in Billeter et al. 2009). The overall comparison between Cs and the two transgenic lines suggests that the biosynthetic mechanisms of the latter ones became seriously deregulated in aging flies. This interpretation is supported by the data obtained with the other—natural and transgenic—variant genotypes currently tested here (see Figs. 2 and 3).

Indeed, while flies of the three M-type strains (Cs, Di2, and Or-R; Fig. 2) showed a similar biphasic “increase + plateau” aging pattern for ∑CHCs, it was not the case in Z-type flies. Between 12 and 24 days, the ∑CHCs decrease observed in Z-type females, was paralleled by that of C9-DesatCHCs whereas C7-DesatCHCs increased.

Fig. 3 Age-related variation of cuticular hydrocarbons in male and female flies of oenocyteless lines. CHCs variation was followed during the first 24 days (x-axis) of adult life in male (left panels) and female (right panels) of four oenocyteless genotypes (oe−). Besides control Cs flies (black colored lines and empty circles; these flies were used in the two previous set of data), we analysed four oe− genotypes resulting of the following (females x males) crosses between two transgenic lines: UAS-hid/CyO x [+: PromE(800)-Gal4[2 M],Tub:Gal80ts] (2 × 5; light blue colored lines and empty circles), [+: PromE(800)-Gal4[2 M],Tub:Gal80ts] x UAS-hid/CyO (5 × 2; light blue colored lines and filled circles), UAS-hid, UAS-StingerII/CyO x [+: PromE(800)-Gal4[2 M],Tub:Gal80ts] (7 × 5; dark blue colored lines and empty circles) and [+: PromE(800)-Gal4[2 M],Tub:Gal80ts] x UAS-hid, UAS-StingerII/CyO (5 × 7; dark blue colored lines and filled circles). No dienes were detected in males. For CHCs information, see Fig. 1 legend. For Cs flies, n = 20. For all oe− males, n = 20, except for 2 × 5 at 12d (n = 17) and for 5 × 7 at 4d (n = 11). For all oe− females, n = 17–20, except for 2 × 5 at 12d (n = 17) and 24d (n = 6), for 5 × 2 at 12d (n = 17) and 7 × 5 at 24d (n = 6).
(Supp Fig. 4). Reciprocally, between 4 and 8 days, Z-type males showed increased C5-DesatCHCs and decreased C7-DesatCHCs. These effects were shared by the three Z-type lines indicating that they are coded by Zimbabwe-specific genes. Besides the desat2 gene involved in the C5-DesatCHCs synthesis (Coyne et al. 1999; Dallera et al. 2000; Houot et al. 2010), other interacting genes could affect CHCs biosynthesis during aging (Dembeck et al. 2015; Martins and Ramalho-Ortigão 2012; Michalak et al. 2007; Wicker-Thomas and Jallon 2001). Moreover, the sex specific variation of the C7/C5 ratio is coherent with its divergent genetic control between the sexes (Cortot et al. 2019).

During aging, oenocyteless flies (oe−; Fig. 3) and especially oe− males, reported to produce no or minute CHC amounts (Billeret et al. 2009), showed very unexpected CHC variation. While 4 days old oe− males effectively showed very low CHC levels, older oe− males (carrying the “#7” transgene) produced relatively high CHC levels. Reciprocally, between 4 and 8 days, oe− females showed decreased CHC levels. Given that the UAS-hid apoptotic transgene was conditionally silenced during preimaginal stage before being re-activated during early imaginal life, such manipulation may only have killed larval oenocytes which normally persist until 3 or 4 days of adult life (Evans 1967; Wigglesworth 1933). Their subsequent replacement by adult oenocytes, and their growth during aging could explain the partial CHC rescue after 4 days (Johnson and Butterworth 1985). Since this effect was not observed in oe− females, we believe that sex determination genes interfere with some the transgenes involved in oenocyte-targeted death.

How could the diversity of CHC variations observed during aging in the genotypes observed here help us to decipher some of the mechanisms underlying CHC biosynthesis? Sex specificity of the CHC variation is a prominent common feature in all our experiments. D. melanogaster is known for its qualitative CHC sexual dimorphism (Antony and Jallon 1982) which depends on the sex specific expression of several elongase- and desaturase-coding genes (Bousquet et al. 2012; Chertemps et al. 2007; Chertemps et al. 2005; Dallera et al. 2000). Moreover, the sex determination genes sex-lethal, transformer and doublesex can affect CHC production during early adult development (24 to 48 h; (Ferveur et al. 1997; Jallon et al. 1988; Tompkins and McRobert 1995). Also, the expression of the two desat1 and desat2 genes (and interacting genes) seems to be dissociated between the sexes: the C7/C5-DesatCHCs ratio (high in M strains) is low only in females of West African and Caribbean strains (Dembeck et al. 2015; Jallon and Pechine 1989), whereas it is low in both Zimbabwe males and females (Grillet et al. 2012). Such dissociation is also supported by the divergent genetic control of this C7/C5 ratio between Z females and Z males (Cortot et al. 2019). It is also possible that the tissue expression in some of the transgenics tested here changes during adult life depending on environmental variations (Cortot et al. 2019; Lazareva et al. 2007; Weaver et al. 2020). However, this possibility remains unexplored in the strains tested here.

The mechanisms underlying CHC release and accumulation on the cuticle together with their elimination remain poorly known. The CHC amount detected on the cuticle does not necessarily reflect the real amount synthesized and circulating inside the fly body, at a given time as evidenced in Musca domestica (Mpuru et al. 2001). In this species, there is a >24 h delay between the internal production and the external accumulation of CHCs, due to their transport from oenocytes onto the epicuticle (Mpuru et al. 2001). Indeed, the short duration of our current CHC extraction process (a few minutes in hexane) only reveals the presence of the most superficial CHCs. A longer duration extraction process with a polar solvent (24 h in dichloromethane) would reveal the real total CHC content (internal and external) as in non-Drosophilidae insects (Kühbandner et al. 2012; Rivault et al. 2002; Steiner et al. 2006; Würf et al. 2020). Subsequently to their biosynthesis, CHCs are bound to specific carrier proteins (apolipophorins) exclusively synthesized in the fat body (Parra-Peralbo and Culi 2011; Pho et al. 1996; Schal et al. 2001; Sevala et al. 2000). These proteins circulate in the hemolymph and they seem to release CHCs through the pore canals crossing the cuticle. The activity of pore canals depends on genes, some of which were recently identified (Wang et al. 2020). The CHC amount could also vary with the circadian activity, in particular, that of the desat1 gene in oenocytes (Krupp et al. 2008). However, such a potential factor of variation can be ruled out here since we always extracted CHCs at the same period of the day. The CHC amount present on the epicuticle can also be affected by physical interaction with other flies kept in the same vial (passive transfer; (Coyne et al. 1994; Everaerts et al. 2010; Savarit et al. 1999)). Therefore, CHC elimination may decrease in aging flies with decreased interaction frequency due to their lower behavioural activity. Therefore, the amount of CHCs detected on the cuticle could result of the A between CHCs accumulated on the cuticle and CHCs lost by passive transfer. Then, CHC amount on the cuticle would increase if release > transfer and decrease in the reciprocal case. This hypothesis suggests that CHC release is compensated by CHC abrasion in M wild type flies during their second aging phase, but not in transgenic 5670-tra females and desat1 males.

Given that the CHC variations observed are not only quantitative but also qualitative (specific CHCs can be affected during aging), we believe that the diversity of CHC pattern variation results of several factors combined (among those discussed above), with different influences on CHC profile according to the genotype considered. We illustrate this idea with desat1 females which showed...
a high CHC increase (up to 52 days) followed by a dramatic CHC decrease. Two biosynthetic mechanisms could be involved: until 52 days (phase #1), the LinCHCs amount strongly increased and stayed relatively high before (phase #2) strongly decreasing and be partly—but not completely—replaced by dienes which strongly increased during this time period. If it is true, it suggests that the desat1 gene activity can be restored, compensated, and even enhanced (after 64 days) in 1573 females compared to control Cs females. This hypothesis is quite puzzling given the quasi-irreversibility of gene mutation during lifetime (Láruson and Reed 2016). Given that the desat1 mutation is caused by the insertion of a transposable PGal4 element in the desat1 gene (Marcillac et al. 2005a; Marcillac et al. 2005b), we wonder whether this element could have jumped out, or lost its mutational effect, in aged female flies? More likely, other genes—normally interacting with desat1—could have compensated for the partial defection of this gene during aging (Greenspan 2001; Greenspan 2009). The fact this was not observed in males—taken together with the other data discussed above—suggests an ubiquitous and life-long involvement of sex determination factors in the mechanisms underlying CHC synthesis and release. The high production of monoenes by 5670-tra young females and of dienes by 5670-tra older males also indicates that the biosynthetic enzymes show different sensitivity to the dosage of sex determination (trans)genes products during the successive periods of adult life (Savarit and Ferveur 2002b).

In conclusion, our data reveal that the “classical” CHC profile reported in most Drosophila studies, only based on flies analyzed during their early adult life, can considerably change—sometimes very extremely—until the end of their life. The precise characterization of such age-related CHC variations indicates that the alteration of the exquisite balance between the expression and activity of biosynthetic enzymes can induce dramatic consequences on fly CHCs with potential implications on their fitness (reproduction and survival) which remained to be measured. Thus, the precise measurement of CHC variation during adult lifespan could represent a useful tool to accurately age individual flies found in nature and model their distribution relative to their reproductive and survival abilities in changing environments.

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Data Availability All data will be made available as supplementary material upon publication.

Code Availability Not applicable.

Declarations

Conflicts of Interest/Competing Interests The authors have no conflict of interest or competing interest to declare.

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