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 to reduce dehydration. In many species, some CHCs also act as pheromones. CHCs have been intensively studied in Drosophila species and more specially 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.

Introduction

While physiological changes in aging animals were already described by the Greek philosopher Aristotle (Woodcox 2018), in the 1930's 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; Mołoń 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).

Physiologically-induced variations in life span can be related to many fitness alterations including (*i*) behaviours (such as reduced feeding, courtship, flying, walking and exploration, negative geotaxis, learning and memory), (*ii*) stress resistance, (*iii*) reproductive capacity (reduced egg laying and hatching success, sperm and accessory fluid production and sperm competitive success), (*iv*) immune capacity and barrier function in the gut, and (*v)* cardiac activity (Gargano et al. 2005; Grotewiel et al. 2005; Iliadi et al. 2012; Tamura et al. 2003). The profile of cuticular hydrocarbons (CHCs) 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 (Everaerts et al. 2010), the quantitative variation of each CHC belonging to this profile can thus reveal very subtle physiological and/or developmental defect (Dembeck et al. 2015; Savarit and Ferveur 2002b). Most, if not all, insects are covered with CHCs which primarly serve to limit water loss (Ferveur et al. 2018; Gibbs and Pomonis 1995; Hadley 1981; Locke 1965; Qiu et al. 2012) and also offer a 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 (temperature, humidity, food quality (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 (Grillet et al. 2012; Savarit et al. 1999). For example, the two sibling species *D. melanogaster* and *D. simulans* show a great CHC difference: the former species—but 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 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 relationship, reproductive status, or mimicry between two species (Greene and Gordon 2003; Lenoir et al. 1997; Leonhardt et al. 2016; Liebig et al. 2000).

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). However, very few studies have investigated aging effect on CHCs. The only exception concerns forensic studies dealing with necrophagous flies such as Diptera Calliphoridae and Sarcophagidae species. In these 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).

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

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), Cs strain was propagated in many research laboratories (Benzer 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-dienes) 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 of the introgression of the Z30 genome into a Di2 *white* genetic background (Cortot et al. 2019).

We also tested two transgenic lines: (1) the *desat1*^{1573}-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-tra*^{F} 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[2M],Tub:Gal80^{ts}] 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. 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°) allowing the Gal80^{ts} 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 cornmeal 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-hour old flies were sexed under light carbon dioxide anaesthesia 2–4 hours 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.

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 sec after injection). Helium was used as the carrier gas (50 cm/sec 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%).

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 Llife 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 of the two transgenic *5670-tra* and *desat1* lines (see Materials & Methods). CHC profiles were followed in flies aged of 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 (SuppFig.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 lifespan 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 hours 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 in the major CHC classes (Fig. 1; see Material & Methods). First, ∑CHCs substantially increased in Cs males up to 20 days (2270ng), followed by a slower increase until 80 days (2830ng). Differently, *5670-tra* males showed a strong and regular ∑CHCs increase until 72 days (7380ng) while *desat1* males showed a unstable variation between 1730 and 3100ng with a final increase above 4400ng (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 2000ng) while it continued to strongly increase in *5670-tra* males (5670ng at 72 days). ∑DesatCHCs remained low in *desat1* males. Both Cs and *5670-tra* males predominantly produced monoenes (64–72%) in a proportion which remained relatively stable after 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%).

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 3100ng). Very differently, *desat1* females showed a enormous ∑CHCs peak at 55 days (12370ng) before drastically decreasing until 80 days (4310ng). More precisely, Cs females predominantly produced DesatCHCs (with a stable amount around 1500ng) 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 (380ng) followed by (2) a steep increase up to 80 days (2280ng). 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 females flies of these three genotypes show 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 (1W14 and 3W1; (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 (SuppFig.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 (+ 400ng and +

230ng, respectively) and a stronger increase in Or-R females (+ 760ng). Differently, ∑CHCs strongly decreased in Z females (-560ng). In Or-R females, the ∑DesatCHCs increase paralleled the ∑CHCs increase. During aging, the two other M females showed a 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 ("2x5", "5x2", "7x5", "5x7"; 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; SuppFig.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: 7x5 and 5x7 males showed intermediate FDA profiles between those of Cs and of 2x5 and 5x2 males.

A closer examination of the age-dependent variations observed between *oe*$^{-}$ flies was also detected when comparing their CHC parameters. While ∑CHCs was initially low in 2x5 and 5x2 males to slightly increase after 8 days (up to 200ng), it substantially increased in 7x5 and 5x7 males between 8 and 24 days (900-1250ng). These variations were largely paralleled to those of ∑DesatCHCs (only monoenes). Moreover, between 4 and 24 days, 2x5 and 5x2—but not 7x5 and 5x7—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 300–700 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 5x7 and 7x5 males while they strongly decreased in females to almost completely disappear.

Discussion

In the present study, we explored the variation of CHCs in *Drosophila melanogaster* male and female flies of 12 genotypes during all—or a large part of—their adult life. Youngest (< 6 hours 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; Curcillo 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. 1–3), and with all data gathered, we will propose some possible biological mechanisms underlying CHC biosynthesis during life time.

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 desat1^{1573}-Gal4 show a similar expression in the oenocytes (hepatocyte-like cells) and in fat body, the two main tissues involved in CHC biosynthesis (Diehl 1973; Ferveur et al. 1997; Gutierrez et al. 2007; Savarit and Ferveur 2002a; Wigglesworth 1942; Wigglesworth 1988). 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-tra^{F}* feminizing transgene whereas the *desat1^{1573}-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 in other sex specific characters (Houot et al. 2012; Wicker-Thomas and Jallon 2001). 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 & 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 (SuppFig.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. Beside the *desat2* gene involved in the C5-DesatCHCs synthesis (Coyne et al. 1999; Dallerac 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 specially *oe*$^{-}$ males, reported to produce no or minute CHC amounts (Billeter 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 to 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; Dallerac et al. 2000). Moreover, the sex determination genes *sex-lethal*, *transformer* and *doublesex* can affect CHC production during early adult development (24 to 48 hours; (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 tissular 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 synthetized and circulating inside the fly body, at a given time. Indeed, the short duration of our current CHC extraction process (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). 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 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 Δ 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 influence 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 life time (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 females 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 involvment 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 implication 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 modelize their distribution relatively to their reproductive and survival abilities in changing environments.

Declarations

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CONFLICTS OF INTEREST/COMPETING INTERESTS

The authors have no conflict of interest or competing interest to declare.

CODE AVAILABILITY

Not applicable

DATA AVAILABILITY

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

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Figures

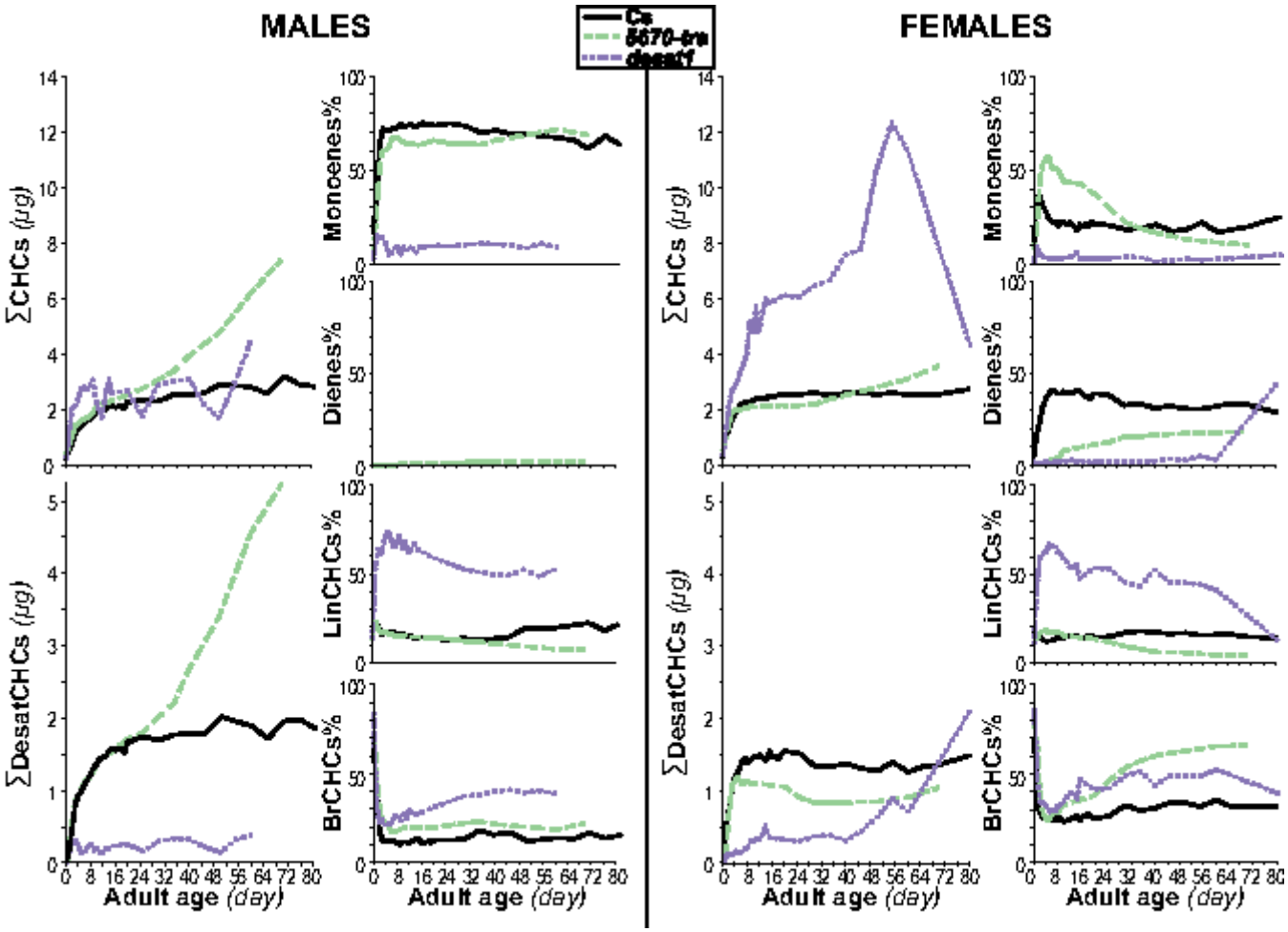

Figure 1

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 desat11573-Gal4 (desat1; purple colored lines). On the left side of each panel series, we show the 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).

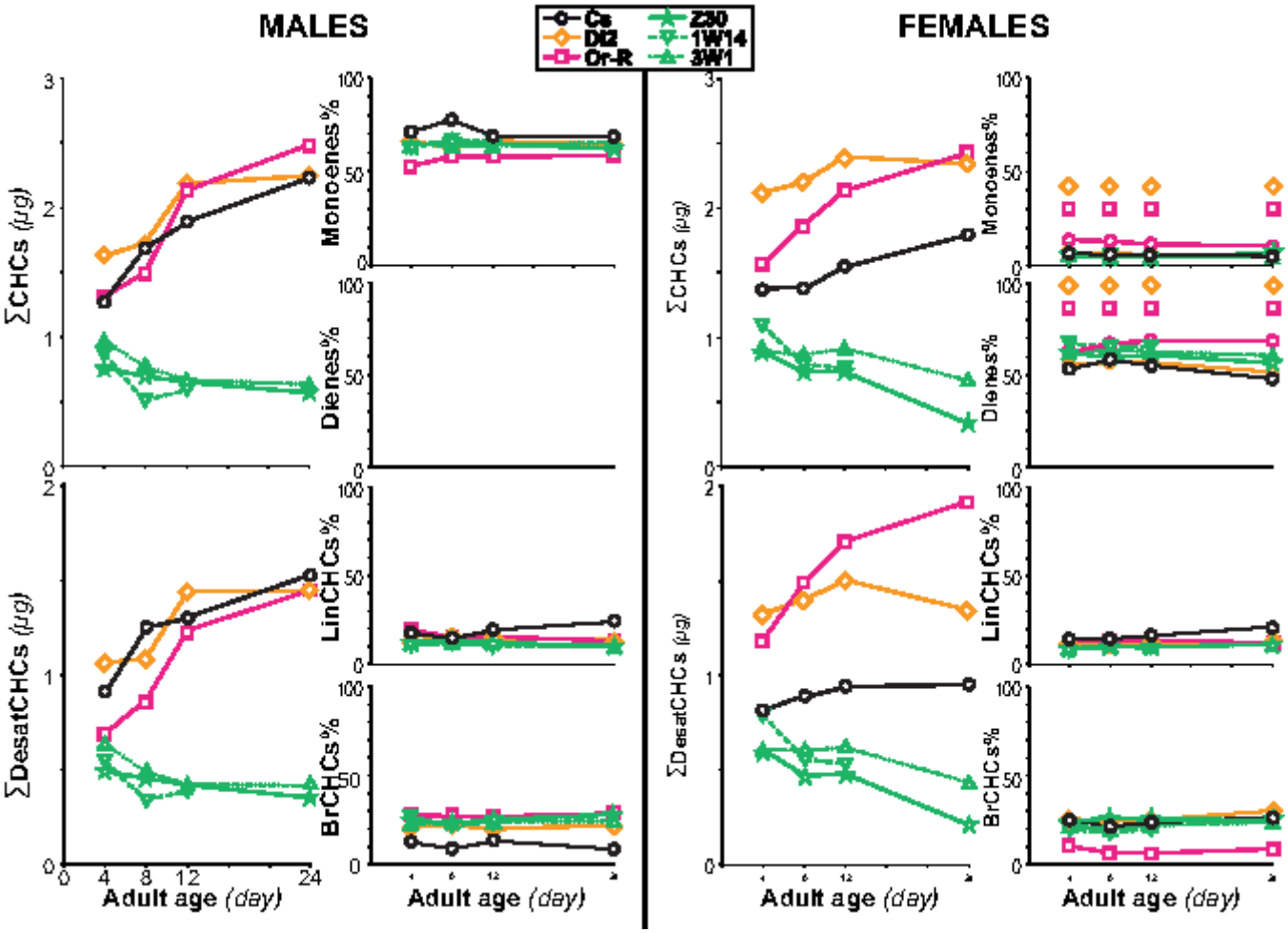

Figure 2

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: 1W14 and 3W1 (green colored lines and diamonds, or triangle, respectively; see Material and methods). Dienes were not detected in males. For CHCs information, see Figure 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 3W1 males, n=20; for 3W1 females, ns=12 to 20. For 1W14 8d flies, n=20, and n=2 in 2d flies, n=8 for 4d males and n=3 for 4d females.

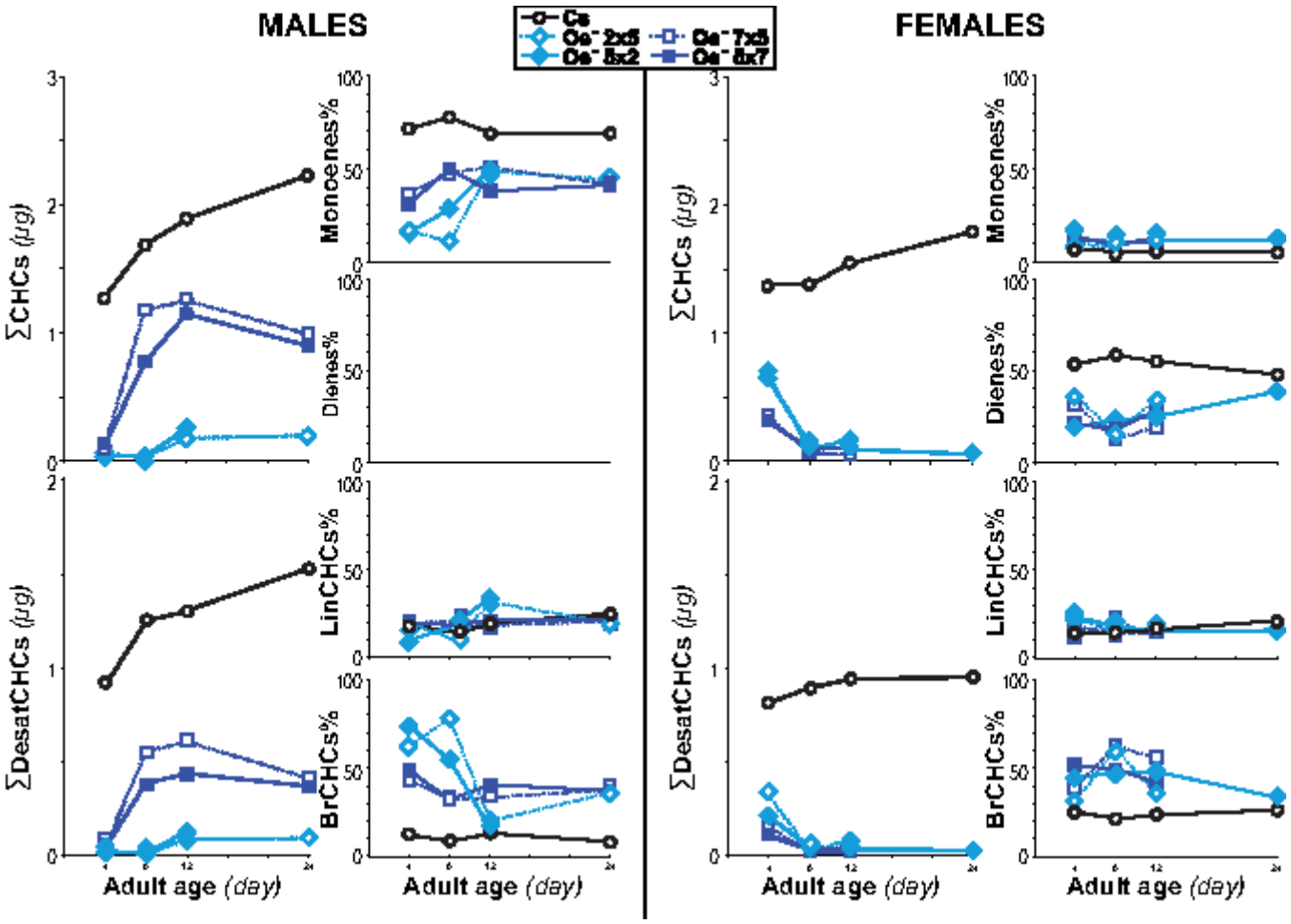

Figure 3

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 (righ panels) of four oenocyteless genotypes (oe-). Beside 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: #2 x #5 (2x5; light blue colored lines and empty circles), #5 x #2 (5x2; light blue colored lines and filled circles), #7 x #5 (7x5; dark blue colored lines and empty circles) and #5 x #7 (5x7; dark blue colored lines and filled circles). No dienes were detected in males. For CHCs information, see Figure 1 legend. For Cs flies, n=20. For all oe- males, n=20, except for 2x5 at 12d (n=17) and for 5x7 at 4d (n=11). For all oe- females, n=17-20, except for 2x5 at 12d (n=17) and 24d (n=6), for 5x2 at 12d (n=17) and 7x5 at 24d (n=6).

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