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Temperature affects the ontogeny of sexually dimorphic cuticular hydrocarbons in *Drosophila melanogaster*

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Summary

Hydrocarbons on the cuticle of mature *Drosophila melanogaster* flies play a crucial role in mate recognition, and protect against dehydration. We measured the effect of temperature on mature cuticular hydrocarbons (CHs) by (i) rearing two control strains at different temperatures, (ii) shifting the temperature after metamorphosis and (iii) inducing a single heat-shock pulse in control and heat-sensitive transgenic strains, over a period of 3 days following adult eclosion. This study describes the time course of the events involved in the production of male- and female-predominant CHs. We also found that 'immature' CHs, sexually monomorphic CHs on younger flies, were not affected by these treatments.

Key words: mate recognition, temperature, cuticle, cuticular hydrocarbon, *Drosophila melanogaster*, ontogeny, heat shock, biosynthesis, sex difference.

Introduction

One main function of cuticular hydrocarbons (CHs) is to protect the insect from dessiccation by reducing cuticle permeability to water (Edney, 1967). The viscosity and composition of CHs change with temperature because water loss is positively correlated with the number of unsaturated bonds and negatively correlated with the carbon-chain length (Gibbs, 1998). At least part of the pattern of CHs may be adaptative, because in several *Drosophila* species (*D. pseudoobscura*, *D. mojavensis*) they have been shown to vary with environmental conditions, particularly temperature and humidity (Toolson and Kuper-Simbron, 1989; Markow and Toolson, 1990). However, using the strict definition of adaptation as implying differential reproductive success or survival, none of these studies provided a clear relationship between fitness and water loss, although the existence of such a relationship is intuitive. In *D. melanogaster*, and in the sibling species *D. simulans*, the proportion of mature CHs appears to be strictly genetically controlled in laboratory strains. These two species show intraspecific variation for production of the predominant CHs (Luyten, 1982; Jallon, 1984; Sureau and Ferveur, 1999), but the adaptative basis of this polymorphism is not well understood.

Predominant CHs of mature *Drosophila melanogaster* flies are sexually dimorphic, both in their occurrence and in their effect on male courtship behaviour (Antony and Jallon, 1982; Ferveur, 1997). Male flies synthesize monoenes (containing one double bond) with 23 and 25 carbons [*cis*7-tricosene (7-T) and *cis*7-pentacosene (7-P)]. 7-T tends to inhibit intraspecific male excitation (Ferveur and Sureau, 1996). Female flies produce dienes (containing two double bonds) with 27 and 29 carbons [*cis*,*cis*7,11-heptacosadiene (7,11HD) and *cis*,*cis*7,11-nonacosadiene (7,11ND)]. Both dienes reduce interspecific male excitation but only slightly reinforce intraspecific male excitation, the main stimulation/inhibition signal being provided by 7-T and, eventually, by 7-P (Savarit et al., 1999; Sureau and Ferveur, 1999). Other CHs are found on the cuticle of immature imagoes but these are not sexually dimorphic; these substances generally have longer carbon chains and more double bonds than mature CHs (Pechiné et al., 1988). In *D. melanogaster*, these immature CHs strongly stimulate the courtship of mature homospecific males (Antony and Jallon, 1981; Cobb and Jallon, 1990).

Several experiments suggest that mature CHs are synthesised during early imaginal life. Immature CHs are almost completely replaced by shorter chain CHs after 20 h (Antony and Jallon, 1981). Misexpression of the sex determination gene *transformer* suggests that the ontogeny of mature *Drosophila* CHs occurs in at least two distinct steps: (i) the elongation of fatty acids into long or very long alkane chains, which occurs 6 h after adult eclosion (AE) (Savarit et al., 1999), in contrast to (ii) the sexual differentiation of elongated CHs, which occurs slightly later (12–48 h after AE) (Ferveur et al., 1997).

We measured the influence of temperature on the production of predominant and sexually dimorphic CHs in *D. melanogaster*. Two laboratory strains, Canton-S (Cs) and Tai,

with different CH morphs, were either raised at a constant temperature (20 or 25°C), or shifted between the two temperatures immediately after adult eclosion. The influence of temperature on CH maturation was studied during the first 3 days of imaginal life. During this period, males and females of control and transgenic strains carrying the heat-inducible *hsp70*-GAL4 transgene were subjected to a single 1 h heat-shock pulse. The production of immature long chain CHs was also measured in heat-shocked flies.

Materials and methods

D. melanogaster *strains*

All strains were kept on standard cornmeal and yeast medium under a 12 h:12 h light:dark cycle at 25°C, except where otherwise mentioned.

Two wild-type strains were used: (i) the Canton-S (Cs) strain, originating from North America; Cs males produce mainly 7-T (Antony and Jallon, 1982), and (ii) the Tai strain, originating from Ivory Coast; Tai males produce mainly 7-P (Jallon, 1984). Females of both strains show different principal hydrocarbons: 7,11HD and 7,11ND in Cs females; *cis*,*cis*5,9 heptacosadiene (5,9HD) and *cis*,*cis*5,9 nonacosadiene (5,9ND) in Tai females (Jallon and Péchiné, 1989).

In the transgenic *hsp70*-GAL4 strain (Brand et al., 1994), the Gal4 sequence is under the control of the *hsp70* gene promoter. The production of GAL4 protein can be induced ubiquitously subsequently to a heat shock at 37°C. In turn, GAL4 can activate a second transgene carrying a UAS-reporter sequence linked to the gene of interest. Therefore, the heat shock ubiquitously activates the transgene shortly (less than 1 h) after application (Greig and Akam, 1993). Here we used the UAS-*transformer* (UAS-*tra*) strain, which dominantly expresses the female form of the TRA protein (Ferveur et al., 1995), and the UAS-*lacZ* strain, which expresses the β-galactosidase (Brand and Perrimon, 1993). The transgenic strain combining *hsp70*-GAL4 with UAS-*tra* was chosen because we noted that the overall amount of CHs was very reduced after heat shock in young flies of this strain (Savarit et al., 1999; Ferveur and Savarit, 2000). In order to study the effect on CHs of both reporter transgenes, the effect of the UAS-*lacZ* transgene (also driven by *hsp70*-GAL4) was compared to that of UAS-*tra*. The heat-shock effect was also measured on Cs flies carrying one or no copies of the *hsp70*-GAL4 transgene. The comparison of 'Cs' and '*hsp70*-GAL4; Cs' genotypes allowed us to distinguish between the general effect of the heat shock and that of the activation of the *hsp70*-GAL4 transgene.

Manipulation of temperature and heat-shock procedure

In order to test the influence of temperature on CH production, flies were raised at a constant temperature of either 20°C or 25°C throughout their life (these flies being the offspring of flies kept at that same temperature), or shifted between the two temperatures (20→25°C or 25→20°C) less than 1 h after imaginal eclosion. Given that developmental time is a function of temperature, the age of flies was standardized for CH extraction: 4 days old for flies raised at 25°C, and 6 days old for flies raised at 20°C.

Flies were lightly anaesthetised with CO_2 soon after eclosion, sexed and placed in groups of ten in small polypropylene vials (11 cm^3) containing food. Heat-shock pulses were applied by submerging the vials in a water bath at 37°C for 1 h during the period between adult eclosion (AE) and 72 h later. We have shown elsewhere that variations in the tube volume and food alter heat shock efficiency (Ferveur and Savarit, 2000). Immediately after heat shock, flies were returned to 25°C, in standard food vials to await CH extraction.

Hydrocarbon extraction

CH extraction was performed on individual flies following the standard procedure (Ferveur, 1991). We give the absolute amount of each CH because (1) all experiments were simultaneously performed and (2) absolute amounts provide more complete information than relative quantities, the variation of which can indirectly depend upon other compounds (for a complete discussion, see Savarit and Ferveur, 2002).

Extraction was performed on mature flies (4 or 6 days old, see above), and also on immature flies (6–48 h after AE); at this age flies still produce longer carbon chain, immature CHs. For the latter experiment, 3 h imagoes were heat shocked and immature CHs were extracted at least 3 h later (i.e. at 6 h after AE), thus allowing the heat shock to affect CH biosynthesis. Individual flies were soaked in a microtube containing 50 µl hexane for 10 min. This short time was sufficient to extract most of the external but not the internal CHs. After removal of the flies, 20 µl of hexane containing 800 ng of hexacosane (C26; used as an internal standard) were added to each microtube. 5 µl of each sample were then injected into a Perkin-Elmer Autosystem gas-phase chromatograph (GPC) equipped with a 25 m capillary column (25 QC2/BP1 0,1), using hydrogen as the carrier gas. During chromatography the temperature was programmed to increase from 180°C to 270°C at a rate of 3°C min^{-1}. Peak detection was carried out using a Flame Ionization Detector coupled with a *Chromjet* integrator (Thermo Separation Product Inc.) that yielded retention times and areas under each peak. All the predominant CHs of *D. melanogaster* have already been identified and characterized (Antony and Jallon, 1982; Pechiné et al., 1985, 1988; Jallon and Pechiné, 1989).

Statistical analysis

The CHs of strains raised at constant or shifted temperatures were compared using non-parametric statistical tests. First, the level of each major CH was compared between flies raised at the four temperature conditions using a Kruskal–Wallis test. When a significant difference was detected, temperature conditions were compared two-by-two using a Mann–Whitney *U*-test. We used the sequential protocol of Bonferroni (Holm, 1979; Rice, 1989) to correct the significance levels, depending on the number of comparisons. Briefly, the levels of

significance for each comparison between conditions were ranked from lowest to highest value. The lowest value was then compared with the threshold divided by the total number of comparisons (*n*). Only where the first value was significant did we compare the second with the threshold divided by (*n*–1), and so on. This procedure was performed until a value showed a non-significant difference.

Results

The temperature during imaginal life influences the production of hydrocarbons

We compared the amounts of CHs in flies raised at a constant 20°C or 25°C temperature. We also measured the effect of reciprocal shifts between these temperatures, immediately after adult eclosion. Tai and Cs strains were chosen because there are major quantitative differences in the predominant CHs on mature flies from these two strains (Jallon 1984; Ferveur et al., 1996). The effect of temperature was measured in 4- or 6-day-old flies of both sexes (see Materials and methods).

Cs males showed significant differences in their production of 7-P (but not 7-T) when raised at a constant 20°C or 25°C (Table 1). Indeed, when raised at 25°C, Cs males produced 45% more 7-P (+48 ng) and 16% less 7-T (–165 ng) than sibling males raised at 20°C. When males were shifted from 20°C to 25°C, the levels of both 7-monoenes were significantly different from those measured in Cs males raised at constant 20°C. Furthermore, in shifted males, production of 7-P (but not 7-T) was significantly different from that in males raised at constant 25°C. Conversely, the levels of both 7-monoenes were the same in Cs males shifted from 25°C to 20°C and males raised at constant 20°C. There was no significant difference in the total amount of CHs (Sum CHs) in the four different conditions.

At 25°C, Cs females produced significantly less 7-T (–65%; –103 ng), less 7-P (–47%; –80 ng) and more 7,11ND (+41%; + 89 ng) than females raised at 20°C. At 25°C, the level of 7,11HD also decreased, although this was not significant (–22%; –117 ng) (Table 1). After a 20→25°C shift, the levels of the four unsaturated CHs were not significantly different from those measured in females raised at constant 25°C, but were all different from the levels in females raised at constant 20°C. With the reciprocal temperature shift (25→20°C), CH levels were similar to those in females raised at constant 20°C, but levels of 7-monoenes were different from those of females raised at constant 25°C or shifted from 20°C to 25°C. Although the SumCHs showed no difference between females

Table 1. *Production of predominant hydrocarbons in mature* D. melanogaster *flies raised at various temperatures*

		Hydrocarbons produced (ng)				
Fly	Hydrocarbon	Constant 20°C	25→20°C	20→25°C	Constant 25°C	K–W test
Cs males	7-T	1043±45^{a}	1052±82^{a}	846±37^{b}	878±60^{a,b}	P<0.05
	7-P	106±9^{a}	112±9^{a}	225±15^{b}	154±10^{b}	P<0.001
	SumCHs	1584±58	1628±105	1600±65	1550±87	N.S.
Cs females	7-T	159±14^{a}	149±8^{a}	71±6^{b}	56±6^{b}	P<0.001
	7-P	172±12^{a}	151±8^{a}	116±7^{b}	92±7^{c}	P<0.001
	7,11HD	534±36^{a}	563±40^{a}	373±17^{b}	417±30^{a,b}	P<0.01
	7,11ND	217±16^{a}	227±28^{a,b}	295±17^{b}	306±17^{b}	P<0.05
	SumCHs	1986±92^{a,c}	2049±97^{a}	1724±78^{b}	1702±104^{b,c}	P<0.05
Tai males	7-T	264±14^{a}	290±24^{a}	74±8^{a,b}	102±7^{b}	P<0.001
	7-P	1002±54	1027±86	908±62	848±70	N.S.
	SumCHs	1885±88^{a}	2014±151^{a}	1639±102^{a,b}	1540±121^{b}	P<0.05
Tai females	7,11HD	134±7^{a}	180±18^{a}	75±6^{b}	92±10^{b}	P<0.001
	5,9HD	997±44	975±69	817±71	870±41	N.S.
	7,11ND	168±11	173±17	149±15	154±15	N.S.
	SumCHs	1661±69	1748±127	1450±106	1542±92	N.S.

Values are means of absolute amounts (ng) ± S.E.M.

Flies of two wild-type strains, Canton-S (Cs) and Tai, were either raised at a constant temperature (20°C or 25°C) throughout development, or shifted immediately after adult eclosion from 25°C to 20°C (25→20°C) or from 20°C to 25°C (20→25°C).

Hydrocarbons were extracted from 4- or 6-day-old flies (see Materials and methods). Hydrocarbons shown are *cis* 7-tricosene (7-T; 23C); *cis* 7-pentacosene (7-P; 25C); *cis*,*cis*7,11-heptacosadiene (7,11HD); *cis*,*cis*5,9-heptacosadiene (5,9HD); *cis*,*cis*7,11-nonacosadiene (7,11ND), and the sum of all detected hydrocarbons (SumCHs). 5,9HD was not used for the tests because we could not separate 5,9HD and 27BR in our gas chromatograph conditions.

For each genotype (sex and strain), the result of Kruskall–Wallis (KW) tests that were used to compare experimental groups are shown on the right. When the test was significant, a *post-hoc* Mann–Whitney *U* test was used to compare groups in pairs. Five comparisons were performed: between constant temperatures and between each constant temperature and the shift condition. (Only the two shifting conditions were not compared). Superscript letters indicate significant differences between conditions (N=8–10); N.S., not significant.

raised at either constant temperature, slight differences were detected between flies raised at 20→25°C and 20°C, and between flies raised at 25→20°C and 25°C. In conclusion, in Cs flies of both sexes, the levels of the principal CHs seem to be related to the temperature experienced by the flies during early imaginal life: a temperature increase resulted in decreased amounts of shorter CH chains and more longer CH chains.

In Tai males, the effect of temperature was different to that observed in Cs males (Table 1). Tai males raised at constant 25°C produced less 7-T than those raised at constant 20°C (–61%; –162 ng). However, we noted no significant effect on 7-P production, although it had a tendency to decrease as temperature increased (–15%; –154 ng). Tai males shifted from 25→20°C significantly increased their levels of 7-T in comparison to homotypic flies raised at a constant 25°C. Comparison of the sumCHs over the four conditions suggests that Tai males have a tendency to produce fewer CHs when they experience a higher temperature during imaginal development.

In Tai females kept at 25°C after adult eclosion, the levels of 7,11HD decreased compared with those in adults held at 20°C (–31%; –42 ng for constant temperature; –58%; –105 ng, for shifted temperature). However, no changes in the levels of other CHs (including 5,9HD and 7,11ND) or in the SumCHs were detected (Table 1).

For the Tai strain, temperature variation thus only had a marked effect on 7-T in males and on 7,11HD in females. The effect of the temperature increase was different from that observed in Cs flies: the level of all predominant CHs seems to decrease in Tai flies with increased developmental temperature.

Precise characterization of the critical period for the maturation of male-predominant CHs

The previous experiment indicates that the temperature after adult eclosion largely determines the CH profile in male and female flies of two wild-type strains. In order to characterize the critical period for the processing of mature CHs precisely, we subjected flies to a single 1 h heat-shock pulse (at 37°) during the period between adult eclosion (AE) and 72 h after AE, by which time control flies show a mature CH profile. CH levels were measured in 4-day-old flies that had or had not been heat shocked. Four genotypes were compared: Cs flies, Cs flies

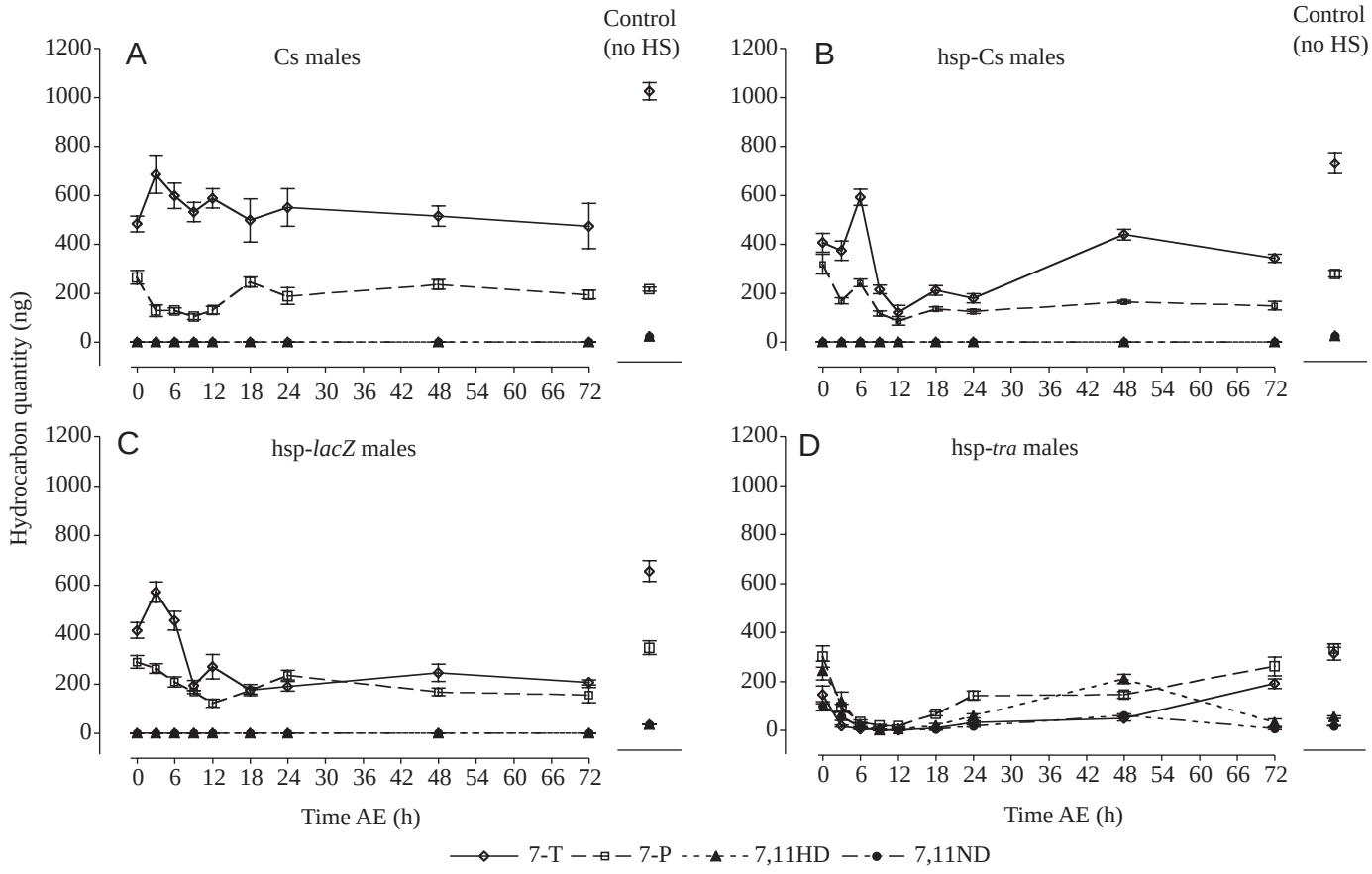

Fig. 1. Production of predominant cuticular hydrocarbons in 4-day-old males as a function of the temporal induction of heat shock. Values are means of absolute amounts (ng) ± S.E.M. A single 1 h heat-shock pulse was induced between adult eclosion (AE) and 72 h AE. Four genotypes were used: (A) Cs; (B) *hsp70*-GAL4; Cs (hsp-Cs); (C) *hsp70*-GAL4 × UAS-*lacZ* (hsp-*lacZ*); (D) *hsp70*-GAL4 × UAS-*tra* (hsp-*tra*). For each genotype, data from non-heat-shocked flies (no HS) are shown on the right of each graph. For each data point, *N*=9–29 (except for hsp-Cs at 24 h; *N*=4). For abbreviations of hydrocarbons, see legend to Fig. 3.

carrying a single copy of the *hsp70*-GAL4 transgene (hsp-Cs), and the two double transgenic strains *hsp70*-GAL4 × UAS-*lacZ* (hsp-*lacZ*) and *hsp70*-GAL4 × UAS-*tra* flies (hsp-*tra*; see Materials and methods). These four genotypes allowed us to measure the heat-shock effect (in Cs flies), the effect of the *hsp70*-GAL4 transgene, and that of two UAS-reporter transgenes driven by *hsp70*-GAL4, after heat shock.

In males (Fig. 1), there was a marked effect on predominant CHs when the heat shock was induced at different times between 0 and 72 h after AE, with further differences between the genotypes.

Cs males subjected to a single heat shock at any time between 0 and 72 h after AE showed a decrease in 7-T compared with the levels in non-heat-shocked flies (no heat shock). For example, at 3 h after AE, 7-T was significantly reduced (from 969±34 ng to 685±77 ng; z=28; t=2.86; P=0.004). By contrast, production of 7-P was affected only between 3 h and 12 h after AE; at 3 h after AE, levels of 7-P decreased from 186±8 ng to 129±24 ng (z=2.46; P=0.014). At 3 h after AE, no other CH levels were altered, but the total amount of CHs (SumCHs) was slightly reduced in heat-shocked Cs males (from 1724±52 ng to 1355±150 ng; z=2.07; P=0.039).

Heat-shocked hsp-Cs males also showed a substantial and lasting decrease in 7-T levels (with a short refractory period approximately 6 h after AE). 7-P showed a smaller decrease when heat shock was induced between 9 and 24 h after AE, but not between eclosion and 6 h after AE.

Heat-shocked hsp-*lacZ* males showed a substantial decrease in both 7-monoenes. As in heat-shocked hsp-Cs females, 7-T levels were less affected when the heat shock was induced between eclosion and 6 h after AE. The level of 7-P decreased at all times.

The situation was more complex in heat-shocked hsp-*tra* males because, as expected, they produced a mixture of male- and female-predominant CHs. Although the levels of 7-monoenes were relatively low in control flies, the heat-shock effect was very strong in this genotype and induced two distinct phenotypes: (i) the feminization of CHs (between AE and 6 h after AE, and between 24 and 48 h AE, and (ii) a drastic reduction of male- and female-predominant CHs between 6 and 12 h after AE. The effect of heat shock on 7-T production lasted longer (up to 72 h after AE), than on 7-P production (up to 24 h after AE).

Precise characterization of the critical period for the maturation of female predominant CHs

The heat-shock effect in transgenic females was similar to that observed in males of the corresponding genotypes. Females of the three strains carrying the *hsp70* sequence showed a marked reduction in the production of 7,11HD and

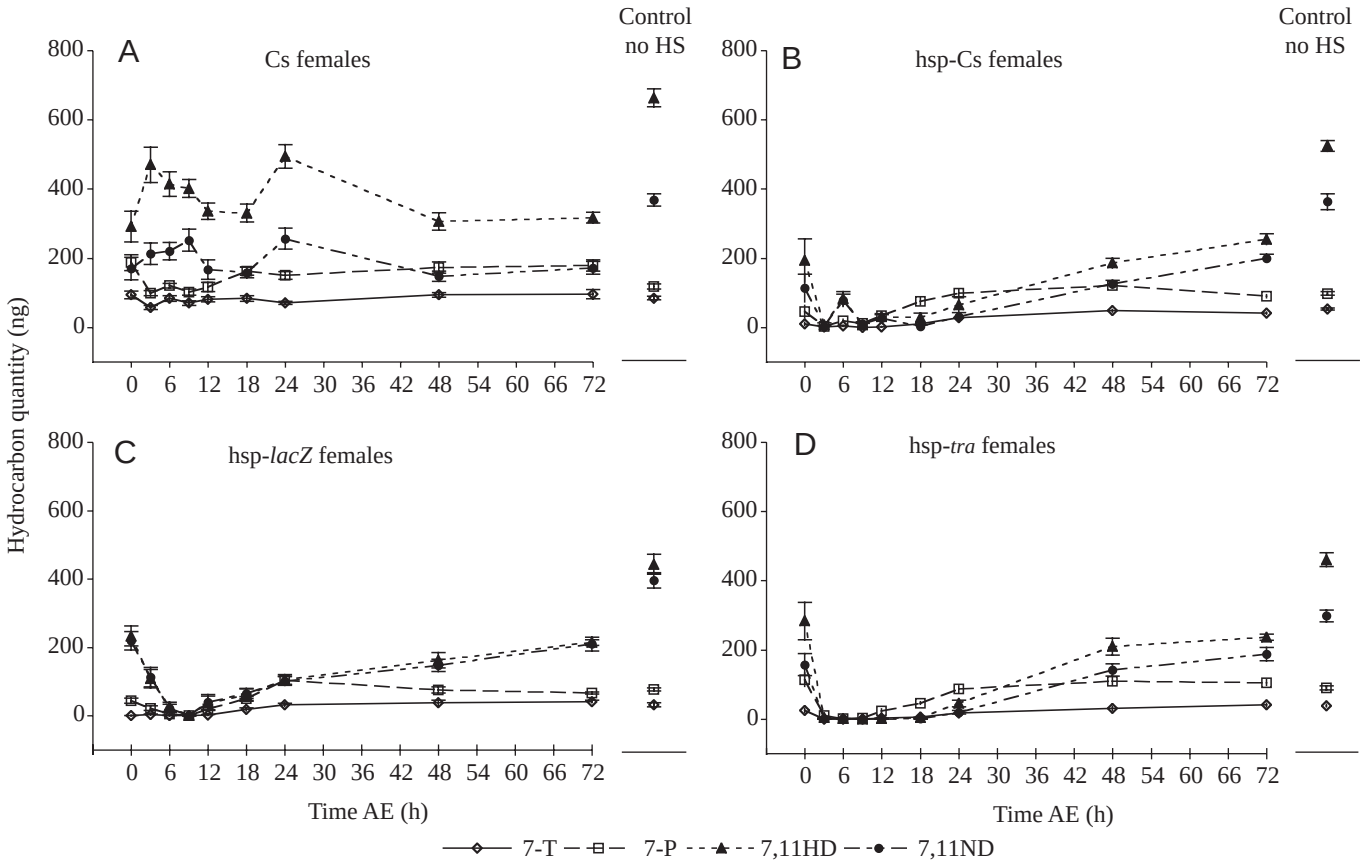

Fig. 2. Production of predominant cuticular hydrocarbons in 4-day-old females as a function of temporal induction of heat shock. For procedure and genotypes, see Fig. 1. For each data point, N=9–36. For abbreviations of hydrocarbons, see legend to Fig. 3.

7,11ND when the heat shock was induced between 0 and 72 h after AE (Fig. 2). When the heat shock was induced between 3 and 18 h after AE, the production of 7,11 dienes and of 7-monoenes was drastically reduced, or was absent. Between 18 and 72 h AE, the heat-shock effect was undetectable for 7-monoenes and a milder, but still substantial, decrease in the production of 7,11 dienes was induced.

Cs females that were subjected to a single heat-shock pulse at 9 h after AE showed a slight but significant decrease of 7,11HD when compared to 'no HS' flies (626±25 ng and 469±51 ng, respectively; d.f.=28; z=2.64; P=0.008). 7,11ND was similarly affected (343±18 ng and 212±31 ng, respectively; z=3.12; P=0.0017).

Heat-shock effect on all detected CHs

We also measured the levels of all CHs in flies of the four genotypes following heat shock at 9 h after AE (Fig. 3). We selected 9 h after AE because it was generally at this time that the heat-shock effect on the predominant CHs was the strongest (Figs 1, 2). In Cs males, heat induction mainly caused a decrease in 7-T and 7-P levels, with a smaller effect on 23C and 25C saturated linear alkanes (23LIN and 25LIN). The activation of the *hsp70*-GAL4 transgene (in both hsp-Cs and in hsp-*lacZ* genotypes) decreased levels of most linear CHs (with the exception of 9-P), but not those of the two branched CHs with 27C and 29C (27BR and 29BR). In hsp-*tra* males, heat shock also affected the level of

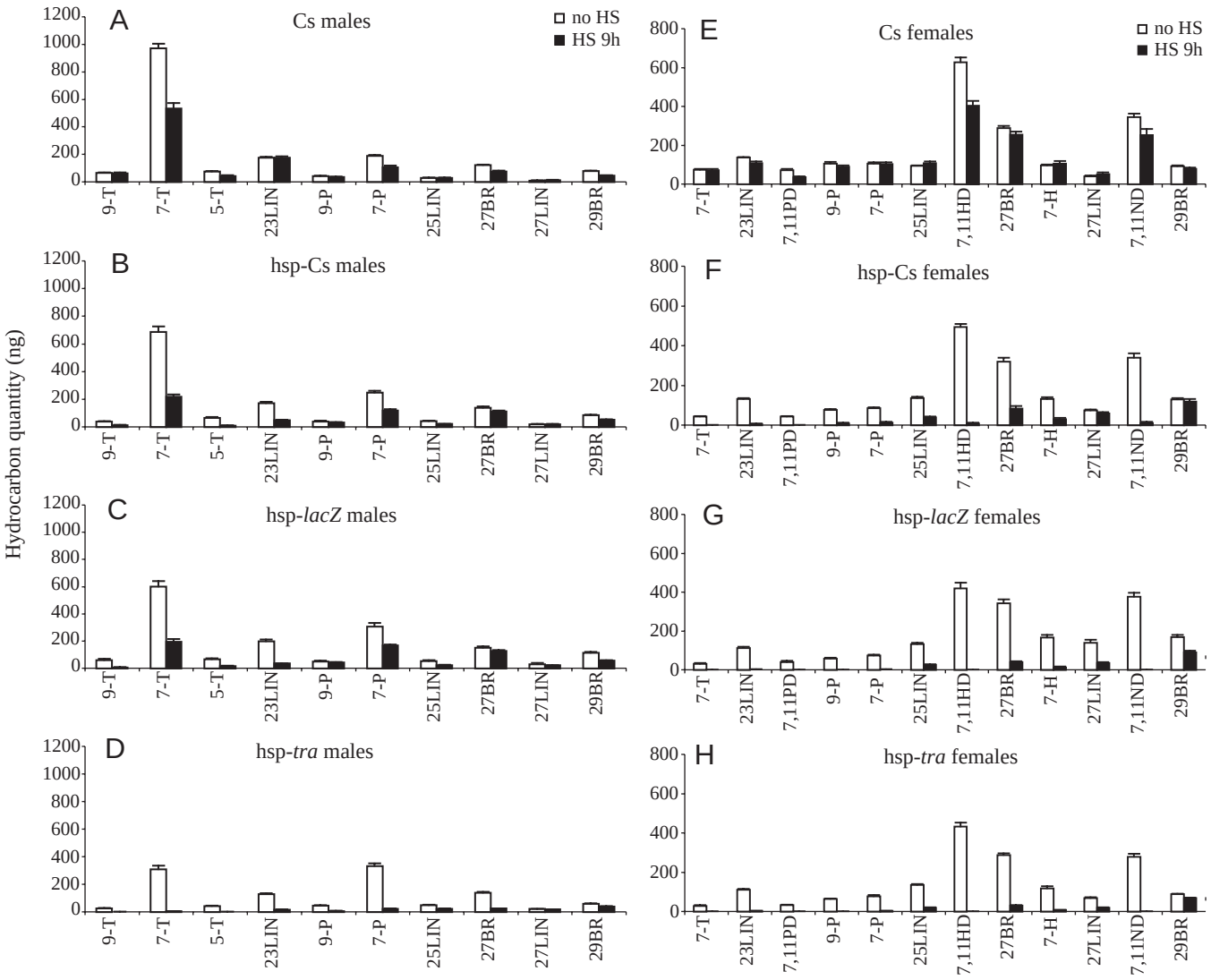

Fig. 3. Production of all detected cuticular hydrocarbons in 4- day-old flies after a single heat shock induced in adults 9 h old. Values are means of absolute amounts (ng) ± S.E.M. for heat-shocked (HS 9h; black bars) and non-heat-shocked (no HS; white bars) males (A–D) and females (E–F) of the *hsp70*-GAL4 × UAS-*tra* (hsp-*tra*) genotype. For genotypes, see Fig. 1. Hydrocarbon nomenclature: 9-T, *cis* 9-tricosene; 7-T, *cis*7-tricosene; 5-T, *cis* 5-tricosene; 23LIN, n-tricosane; 7,11PD, *cis*,*cis*7,11-pentacosadiene; 9-P, *cis* 9-pentacosene; 7-P, *cis* 7-pentacosene; 25LIN, n-pentacosane; 7,11HD, *cis*,*cis*7,11-heptacosadiene; 27BR, 2-methylhexacosane; 7-H, *cis*7-heptacosene; 27LIN, n-heptacosane; 7,11ND, *cis*,*cis*7,11-nonacosadiene; 29BR, 2-methyl-octacosane. The total of CHs shown here represents 88% of all detected CHs. For each genotype, N=10–20.

the predominant branched compound 27BR. In Cs females, heat shock decreased only 7,11HD and 7,11ND levels, whereas the three other genotypes (with the *hsp70*-GAL4 transgene) all showed a drastic reduction in their CHs levels. In these females, 29BR was always the least affected compound.

The levels of immature and mature CHs are independent

Two generations of CHs are present on the cuticle of *D. melanogaster* flies. The long-chain CHs (29C–33C; non-sexually dimorphic) are normally present on the cuticle of young imagoes (until 36–48 h after AE, at 25°C). During this period, they are gradually replaced by shorter chain molecules (23C–29C), which form the definitive sex-specific profile of mature flies. To investigate the relationship between the two types of CHs, we induced a single heat shock at 3 h after AE and measured the levels of both immature and mature CHs in 6–48 h-old hsp-*tra* flies.

In hsp-*tra* males, heat shock had no detectable effect on the production of immature CHs (27C–31C; Fig. 4A). Strikingly, heat shock altered the levels of mature CHs by increasing the quantity of 27C CHs at the expense of shorter-chain CHs (those with 23C). In females, the heat shock induced at 3 h after AE mainly decreased levels of 25C and 27C CHs, with a small effect on 23C CHs (Fig. 4B). Although the level of 29C CHs was not affected by heat shock, the levels of longer chain compounds (31C–33C) remained slightly higher after heat shock.

We cannot exclude the possibility that we missed slight quantitative differences in CHs because we were frequently unable to detect position isomers using gas chromatography.

Discussion

Our data indicate that the temperature during the first days of imaginal life influences the pattern of the predominant CHs present on *Drosophila melanogaster* flies. An increased temperature during early imaginal life (25°C during adulthood, or 1 h at 37°C) generally caused a decrease in levels of shorter chain alkanes (23C in all males, 23C–27C in all females) and an increase in the levels of longer chain molecules (25C in Cs males, 27C in immature males, 29C in females and 31C–33C

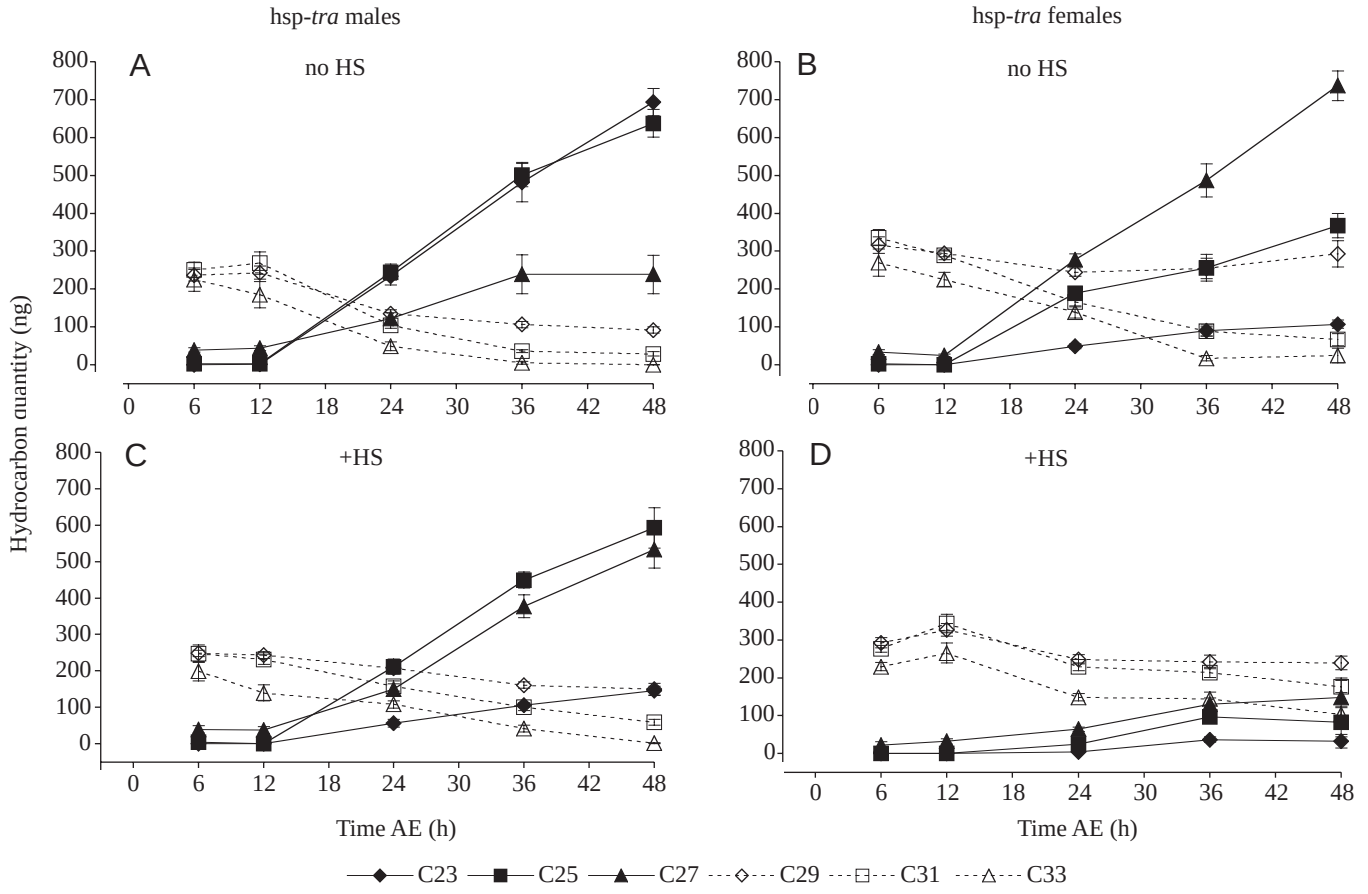

Fig. 4. Ontogeny of cuticular hydrocarbons of various chain lengths during the first 2 days of imaginal life following a heat shock 3 h after AE. Hydrocarbons were pooled according to the length of their carbon chain (23C–33C). The hydrocarbons usually secreted by immature flies range from 29C–33C (broken lines), whereas the CHs produced by mature flies mostly have 23C–29C (solid lines). Experimental (+HS) and control (no HS) flies are males (A) and females (B) from the *hsp70*-GAL4 × UAS-*tra* (hsp-*tra*) genotype. Values are means of absolute amounts (ng) ± S.E.M. of pooled CHs after extraction in 6–48 h-old flies (age is given as h after AE). For each data point, N=8–10.

in immature females). This observation agrees with results obtained from other *Drosophila* species, which suggest that species from warmer, drier habitats contain more longer CHs than those from temperate climates (for a review, see Gibbs, 1998). Our study shows that temperature has its greatest effect during a relatively brief time window. This finding could have important ecological implications for the timing of eclosion, in terms of ensuring that the adult fly has the correct species-specific and, where appropriate, sex-specific hydrocarbon profile.

We found intraspecific variation in the response to temperature that can be compared with the variations in physiological mechanisms observed in *D. melanogaster* strains selected for desiccation resistance (Gibbs et al., 1997). Mature Tai and Cs flies were affected differently by the temperature increase: in Cs flies, levels of shorter carbon chains decreased and those of longer chains increased, whereas in Tai flies the levels of all CHs decreased. While both strains responded differently to temperature variation, we do not know whether or not this difference is adaptative. Originally, Tai flies were collected in a humid, warm habitat (Ivory Coast), whereas the Cs strain comes from a drier and colder environment (northern USA). Strikingly, both strains, which have been maintained under constant laboratory conditions for at least several hundred generations, still retain different genetic potentials to react to temperature variation. However, it should be noted that the plastic response within species is not comparable to the differences in reaction to temperature noted between species. For example, we have found that *D. melanogaster* strains maintain their original CH profile over many generations, whereas other *Drosophila* species (*D. mojavensis*, *D. pseudoobscura*) can rapidly change as a response to laboratory conditions (Toolson and Kuper-Simbron, 1989; Markow and Toolson, 1990). We do not know whether the differential plasticity observed between *Drosophila* species reflects a greater capacity of some species to adapt their CH profile in response to changing environmental conditions. An alternative argument is that some species keep their CH profile more constant than others because of the role played by some CHs in sexual communication (Cobb and Jallon, 1990; Savarit et al., 1999). Also, in *D. melanogaster* and in other insects, internal hydrocarbons occur in the same composition and proportion as cuticular hydrocarbons (for a review, see Tillman et al., 1999). Therefore, it is possible that the high stability of the CH profile in *D. melanogaster* reflects the involvement of internal hydrocarbons in key physiological processes (such as reproduction). The balance between internal and cuticular hydrocarbons can change, as in *Blattella germanica* nymphs, where food intake affects the allocation of both hydrocarbon types (Young et al., 1999). In *Musca domestica*, quantitative variations of CHs depend upon age and sex. For example, in 6-day-old females, increased Z-9-tricosene is compensated by a decrease of Z-9-heptacosene (Mpuru et al., 2001).

No relationship was found between immature and mature CHs in heat-shocked flies. However, it seems probable that both generations of CHs share biosynthetic mechanisms. In this case, the biosynthesis of immature CHs would occur some time before imaginal life and would not be sensitive to the misexpression of the sex-determination gene *transformer* (Ferveur et al., 1997). This observation is consistent with the fact that immature CHs are not sexually dimorphic.

The present data confirm that mature CHs are processed in at least two steps that occur during early imaginal life: (i) elongation of relatively long saturated carbon chain alkanes and (ii) sexual differentiation after desaturation (Ferveur et al., 1997; Savarit et al., 1999), and that biosynthesis is still active in 3-day-old flies (Chan Yong and Jallon, 1986). They also provide two new insights into CH biosynthesis. Firstly, there is a short period of time, between adult eclosion (AE) and 6 h after AE in most genotypes, during which the heat-shock effect on CH production seems to be less dramatic. This refractory period could correspond to a heat-shock-induced protective mechanism equivalent to the neuroprotection at synapses observed with elevated HSP70 levels in *Drosophila* (Karunanithi et al., 1999). Selection at different temperatures has been shown to change the transcription of the HSP factor (Lerman and Feder, 2001). Secondly, a sex difference was found for sensitivity to heat shock: *D. melanogaster* females carrying at least one copy of the *hsp70*-GAL4 transgene showed a much higher CH decrease than homotypic males, specially when the heat shock was induced 6–12 h after AE. CHs in females are probably much more sensitive to stress than CHs in males. To explain the difference between transgenic strains, we propose that the heat induction of *hsp70*-GAL4 induces a toxic effect in those tissues, such as the oenocytes, that are involved in CH processing. The sexual difference in reaction to heat shock could indicate sex differences in tissues involved in the sexual maturation of CHs (oenocytes in males; oenocytes and fat body in females) (Savarit and Ferveur, 2002), or sex differences in the sensitivity of these tissues to heat shock. On the other hand, it is possible that CHs in *D. melanogaster* female are more sensitive to heat shock because, being characterized by dienes, they depend upon a more complex biosynthetic pathway than male monoenes.

In summary, the production of CHs in mature flies depends upon a series of biosynthetic mechanisms that occur during the first day of imaginal life. We propose that a non-sex-specific enzyme, acting like a fatty acid synthetase (FAS), controls the amount of most linear CHs between 6 and 12 h after AE. At 12 h after AE, the sexual differentiation of CHs precursor (including 16C palmitate) occurs in the oenocytes and/or in the fat body (Wicker-Thomas et al., 1997; Ferveur et al., 1997; Savarit and Ferveur, 2002). This sex-specific process could involve the coupling of one or several elongase and desaturase enzymes. The process of sexual differentiation would be under the control of a brain factor released before 24 h after AE (Wicker and Jallon, 1995) and would remain sensitive to the action of the *transformer* gene until 48 h after AE. The biosynthesis of CHs would be less active in 3 day-old flies, although it would still be present. Tests of these hypotheses will involve interspecific studies, in particular of *D. simulans*,

which has no qualitative sexual dimorphism, but has a similar ecology to *D. melanogaster*. Studies of other insect genera involving temperature shifts – even without the advantage of the genetic technology available for *D. melanogaster* – could provide valuable insights into the generality of our findings, and help deepen our understanding of the evolution of insect cuticular hydrocarbons and their multiple roles in ecology, physiology and communication.

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