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Genetic study of the production of sexually dimorphic cuticular hydrocarbons in relation with the sex-determination gene *transformer* in *Drosophila melanogaster*

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Summary

In *Drosophila melanogaster*, the main cuticular hydrocarbons (HCs) are some of the pheromones involved in mate discrimination. These are sexually dimorphic in both their occurrence and their effects. The production of predominant HCs has been measured in male and female progeny of 220 PGal4 lines mated with the feminising UAS-*transformer* transgenic strain. In 45 lines, XY flies were substantially or totally feminised for their HCs. Surprisingly, XX flies of 14 strains were partially masculinised. Several of the PGal4 enhancer-trap variants screened here seem to interact with sex determination mechanisms involved in the control of sexually dimorphic characters. We also found a good relationship between the degree of HC transformation and GAL4 expression in oenocytes. The fat body was also involved in the switch of sexually dimorphic cuticular hydrocarbons but its effect was different between the sexes.

1. Introduction

Cuticular hydrocarbons are some of the crucial molecules involved in mate recognition during courtship of different species of the *Drosophila melanogaster* complex (Antony & Jallon, 1982; Cobb & Jallon, 1990; Coyne *et al*., 1994; Cobb & Ferveur, 1996; Ferveur, 1997; Savarit *et al*., 1999; Greenspan & Ferveur, 2000). In the model species *D. melanogaster*, the predominant cuticular hydrocarbons (HCs) are sexually dimorphic (Fig. 1): only females produce dienes (HCs with two double bonds) including predominant *cis*, *cis* 7,11-heptacosadiene (7,11-HD; 27 carbons) and *cis*, *cis* 7,11-nonacosadiene (7,11-ND; 29 carbons), whereas males produce large amounts of monoenes (monounsaturated HCs) principally represented by *cis* 7-tricosene (7-T; 23 carbons) and *cis* 7-pentacosene (7-P; 25 carbons). *D. melanogaster* females also produce 7-T and 7-P but in much smaller amounts than do males. The predominant HCs of both sexes have different effects on male courtship. The 7,11-dienes strongly prevent interspecific courtship and mating, and slightly reinforces intraspecific precopulatory stimulation, whereas 7-T tends to decrease *D. melanogaster* homosexual courtship (Scott & Jackson, 1988; Ferveur & Sureau, 1996; Savarit *et al*., 1999). Interestingly, 7-T elicits a strong excitation in males of the sibling species *D. simulans*, in which there is no qualitative sexual dimorphism for cuticular hydrocarbons (Jallon, 1984; Coyne *et al*., 1994; Coyne & Oyama, 1995; Savarit *et al*., 1999).

Sexual dimorphism of the predominant HCs has been explored with several mutations that affect the genetic pathway controlling sexual differentiation during development. Mutations in the *Sex-lethal* (*Sxl*), *transformer* (*tra*), *transformer2* and *doublesex* (*dsx*) genes can switch the gender of the predominant HCs (Tompkins & McRobert, 1989; Tompkins & McRobert, 1995; Jallon *et al*., 1986; Jallon *et al*., 1988). In general, the alteration of these genes led to an increased production of male 7-monoenes together with a decrease of 7,11-dienes in XX flies. The fact that these mutations had no detectable pheromonal effect on XY flies suggests that mutant sex-determination genes specify the production of male predominant HCs by default. Conversely, the ectopic expression of the dominant Sxl, Tra (with the UAS-*tra* transgene) or Dsx female products feminised the predominant HCs of XY flies (Tompkins & McRobert, 1989; Ferveur *et al*., 1997; Waterbury *et al*., 1999). Targeting of adult oenocytes (i.e. clusters

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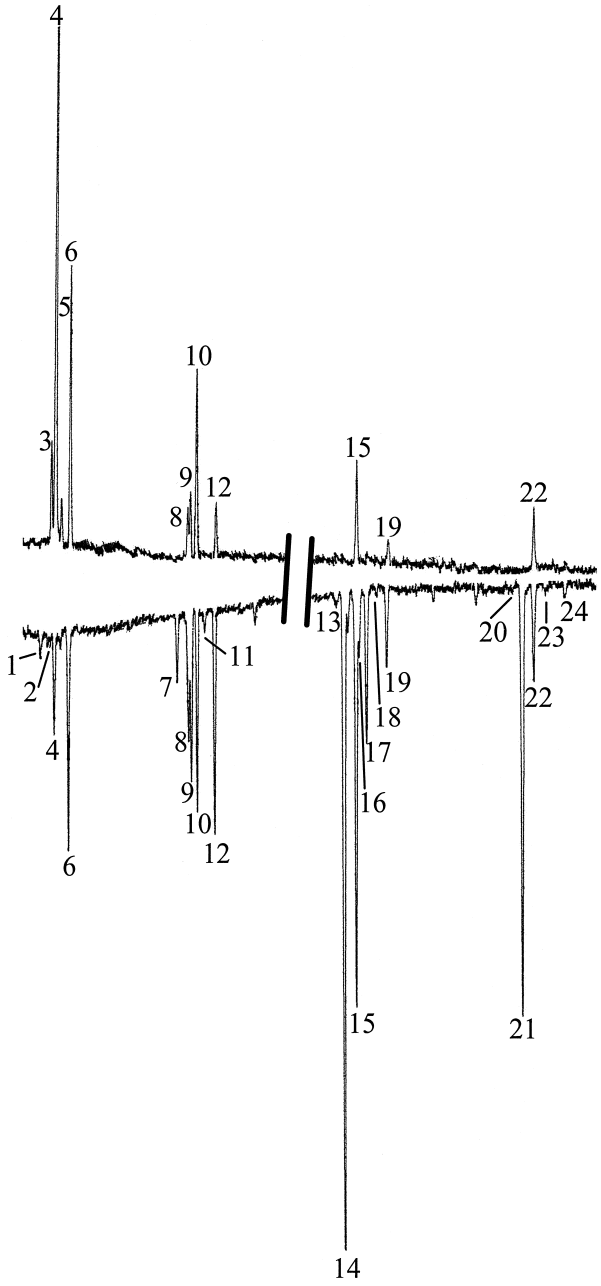

Fig. 1. Representative mirrored gas chromatograms of hexane extracts of two individual flies, a male (top) and a female (bottom) of the Cs strain. Each numbered peak corresponds to a single hydrocarbon (HC) whose identity was previously revealed by mass-spectrometry. The two chromatograms are to the same scale: they were aligned and calibrated by using an added standard of hexacosane on each chromatogram (removed for the sake of clarity, and indicated by two vertical lines). All identified male and female HCs are listed below according to their chain-length (*in italics*) and are given with their abbreviation (in brackets). *23 carbons* – 1: 7,11-tricosadiene (7,11-TD); 2: 2-methyldocosane (23Br); 3: 9-tricosene (9-T); 4: 7-tricosene (7-T); 5: 5-tricosene (5-T); 6: *n*-tricosane (23Lin). *25 carbons* – 7: 7,11-pentacosadiene (7,11-PD); 8: 2-methyltetracosane (25Br); 9: 9-pentacosene (9-P); 10: 7-pentacosene (7-P); 11: 5-pentacosene (5-P); 12: *n*-pentacosane (25Lin). *27 carbons* – 13: 9,13-heptacosadiene (9,13-HD); 14: 7,11-heptacosadiene (7,11-HD); 15: 2-methylhexacosane (27Br); 16: 9-heptacosene (9-H); 17: 7-heptacosene (7-H); 18: 5-heptacosene (5-H); 19: *n*-heptacosane (27Lin). *29 carbons* – 20: 9,13-nonacosadiene (9,13-ND); 21: 7,11-nonacosadiene (7,11-ND); 22: 2-methyloctacosane (29Br); 23: 7-nonacosene (7-N); 24: *n*-nonacosane (29Lin).

of big subepidermal cells in the abdomen) by UAS-*tra* completely feminised HCs of XY flies, suggesting that these structures control the complete sexual differentiation of HCs (Ferveur *et al*., 1997).

However, the manipulation of UAS-*tra* has also revealed that sexual transformation was not always an 'all-or-none' process because XY flies of many PGal4 UAS-*tra* strains showed intermediate degrees of feminisation (e.g. a mixture of male and female predominant HCs) (Ferveur & Sureau, 1996). In these strains, the fact that the increase in female-specific HCs was related to the decrease of predominant male-specific HCs and *vice versa* indicates that male and female hydrocarbons share common biosynthetic pathways.

Here, we have studied the effect on HC production of the genetic interaction between 220 PGal4 enhancer-trap strains and the dominant feminising UAS-*tra* transgene. The production of all measurable hydrocarbons was assessed in male and female flies of all PGal4 UAS-*tra* strains. XY flies of 45 strains were largely or totally feminised for their HCs, and XX flies of 14 strains showed a partial masculinisation of their HCs. All transformed strains were grouped according to their degree of feminisation and/or masculinisation. Then, β-galactosidase production was also compared between groups to map the tissues involved in the production and in the sexual differentiation of sex-specific HCs.

2. Materials and methods

(i) Drosophila *strains and crosses*

All strains of *D. melanogaster* were kept on standard cornmeal and yeast medium under a 12–12 h light–dark cycle at $25 \pm 0{\cdot}5$ °C. A Canton S (Cs) strain was used as the reference for the production of male and female HCs (Fig. 1). All the other strains were derived from the cross between a PGal4 enhancer-trap strain (Brand & Perrimon, 1993) and either the feminising UAS-*tra* (Ferveur *et al*., 1995) strain or the UAS-*lacZ* strain necessary to visualize β-galactosidase production (Brand & Perrimon, 1993); the UAS-*lacZ* strain was provided by E. Giniger (Harvard, USA).

PGal4 strains were provided by different laboratories: 7B, 8B, 10B, 13AX, 28A, 30B and 32B PGal4 strains were generated by A. Brand and N. Perrimon (Harvard, USA). The initial 28A strain was split into three lines based on eye colour: 28AR (red eyes), 28AO (orange eyes) and 28AP (purple eyes). Both 65 and 321 PGal4 strains were generated in the lab-

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oratory of R. F. Stocker (Fribourg, Switzerland). 59Y, c62 and c133 PGal4 strains were generated in the laboratory of K. Kaiser (Glasgow, UK). 023, 090, 095, 101, 215 and 358 PGal4 strains were produced in the laboratory of C. O'Kane (Cambridge, UK). The MZ PGal4 strains (18, 19, 97, 142, 801 and 1172) were generated by J. Urban and K. Ito (Mainz, Germany). All 45 Tp PGal4 strains were provided by T. Préat (Gif-sur-Yvette, France). All the strains provided by A. Brand, K. Kaiser and R. F. Stocker (with the exception of strain 321) have been described previously (Ferveur & Sureau, 1996). With the exception of C. O'Kane's strains, which were selected on the basis of a patchy expression in the abdomen, PGal4 strains were primarily screened for restricted or absent expression in the adult nervous system (Ferveur *et al.*, 1995; Ferveur & Greenspan, 1998).

In order to ensure that F1 progeny carried both PGal4 and UAS-reporter transgenes (because we did not know which chromosome was carrying the PGal4 insert), all crosses were performed between PGal4 females and UAS males. To make sure that the expression of UAS-*tra* in tissues was responsible for changes in HC profiles, controls were obtained from PGal4 UAS-*lacZ* flies of each PGal4 line.

(ii) *Extraction and analysis of cuticular hydrocarbons*

Male and female flies were sexed 0–2 h after emergence under light CO_2 anaesthesia and aged in standard food vials in groups of ten. Hydrocarbon extraction was performed following standard procedure (Ferveur, 1991). Briefly, individual four-day-old flies were soaked in a microtube containing 50 μl hexane for 10 min. After removal of the flies, 20 μl of hexane containing 800 ng of hexacosane (C26; used as an internal standard) was added to each microtube. Then, 5 μl of each sample was injected in a Perkin-Elmer Autosystem gas phase chromatograph (GPC) equipped with a (25 QC2/BP1 0,1) 25 m capillary column with hydrogen as the carrier gas. During chromatography, 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) that yielded retention times and areas under each peak. All the *D. melanogaster* predominant HCs have already been identified and characterized (Antony & Jallon, 1982; Pechiné *et al.*, 1985; Pechiné *et al.*, 1988; Jallon & Pechiné, 1989).

For each fly, HC production was calculated with the following procedure. First, we pooled the total amount of HCs (SumHCs) calculated with a maximum of 24 compounds with a chain length ranging from 23 to 29 carbons. Each HC was characterized by its percentage relative to SumHCs. In addition, the area of each HC peak was compared with the area of the internal hexacosane standard in order to calculate its absolute amount (in ng).

(iii) *Hydrocarbon parameters and sexual dimorphism of HCs*

Results are reported as percentages rather than absolute amounts because percentages are less sensitive to experimental variation. However, we are aware that the variation of a percentage for a predominant molecule can result from the variation of another major compound. In the previous feminisation experiments, Ferveur & Sureau (1996) showed that the decrease of male predominant 7-T was correlated with the increase of female 7,11-dienes. In the present study, the ratio between predominant female and male HCs was used to map the tissues involved in their sexually dimorphic production.

With our chromatographic procedure, amounts of 22–24 HCs were estimated in individual male and female flies (Fig. 1). Control Cs flies showed clear sexual dimorphism for the major compounds: 7-T and 7-P were predominant in males whereas high levels of 7,11-HD and 7,11-ND were produced exclusively by females. The amount of 7-T and 7-P represents $\sim 65\%$ of the total detected hydrocarbons (SumHCs) in control males (and $< 10\%$ in control females), whereas the total 7,11-dienes (7,11-HD + 7,11-ND) represents $\sim 45\%$ of SumHCs in control females.

(iv) *Histological techniques and treatment*

Patterns of *GAL4* expression were visualized with the UAS-*lacZ* reporter gene, producing a blue stain after histochemical reaction with X-Gal. Of all PGal4 strains discussed here, 55 were selected mainly on the basis of their effects on HC transformation: the *GAL4* expression pattern was analysed for all the strains that showed substantial HC effects, whereas only a fraction of the strains with milder effects were so analysed. For all strains, at least six PGal4 UAS-*lacZ* flies of each sex were observed. Flies were anaesthetised, their wings were removed and they were placed in Tissue-Tek® compound. Two or three flies were simultaneously placed in a small drop of Tissue-Tek that was held just above liquid nitrogen for 10 sec to harden. Frozen blocks were trimmed and immediately cut into ribbons of 10 μm sections with a Leica-Jung 3000 cryostat (at -20 °C). Sections were fixed for 10 min in 1·5% (v/v) glutaraldehyde, 2·0% (w/v) formaldehyde (from paraformaldehyde), 30% (w/v) sucrose, 1% (w/v) calcium chloride, 1% (w/v) gum arabic and 0·05 M cacodylic acid at 4 °C. After two rinses (10 min) in distilled water, β-galactosidase activity was revealed with a solution made with 25 μl of a diluted solution of X-gal (8% (v/v) X-gal in dimethylformamide) mixed with 1 ml Fe/Na phos-

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phate buffer at 37 °C, in a dark room saturated with humidity. In the strains studied here, GAL4 was generally revealed after 15 min to 3 h of exposure. Slides were rinsed twice in PBS for 10 min. Preparations were then immersed in 90% glycerol, 10% PBS 1 × and covered with a cover glass sealed with colourless polish. All slides were observed on a Leica DMRB microscope equipped with a microphotographic system.

We chose to focus our study on adult expression because it was previously shown that the formation of sexually dimorphic HCs occurs between 12 h and 48 h of imaginal life (Ferveur *et al*., 1997). Accordingly, detailed GAL4 expression was followed in two- and four-day-old flies of the 55 PGal4 strains. Expression was surveyed in all adult tissues with the help of Miller's anatomical handbook (Miller, 1950). In particular, oenocytes, fat body, five parts of the digestive tract and the reproductive apparatus of both sexes were examined carefully for X-Gal staining. In the case of the corpus allatum/cardiacum complex (CA/CC) and of other structures in the central nervous system, the presence or absence of staining was noted without further detail. The expression in some parts of the gut and salivary glands was found in all strains, as previously noted (Brand *et al*., 1994; Timmons *et al*., 1997); the expression in these tissues was used as an internal standard to evaluate the strength of expression in tissues of interest.

(v) *Statistical tests*

We have designed a procedure to simplify the characterization of the hydrocarbon phenotype because of the large number of moecules (up to 24) that was characterized in the 88 (or 90) strains. A principal components analysis (PCA) was carried out to compare the overall variation of each HC relative to the total variation of all compounds. The PCA was performed with software written by J. Rouault (Orsay, France) and allowed measurement of the level of correlation between HCs.

3. Results

We surveyed the hydrocarbon production of 4-day-old XY and XX F1 flies resulting from the cross of virgin females from 220 PGal4 strains with males carrying the feminising UAS-*tra* transgene (Ferveur *et al*., 1995; Ferveur *et al*., 1997; O'Dell *et al*., 1995; Ferveur & Sureau, 1996; Ferveur & Greenspan, 1998; Balakireva *et al*., 1998; Savarit *et al*., 1999).

(i) *Feminisation of HCs in XY flies is not an 'all-or-none' process*

The feminisation of HCs occured to varying degrees in XY flies of different PGal4 UAS-*tra* strains (as mentioned above in Ferveur & Sureau, 1996). In order to compare the degree of feminisation between strains, we carried out a PCA that partitioned the variation of all detected hydrocarbons in XY PGal4 UAS-*tra* flies of 88 strains, including all the strains producing feminised flies (Fig. 2). According to this PCA, strains were distributed in three groups, each exhibiting a different dispersion pattern along both factor axes. Strains of group 1 were dispersed along PC 1 and strains of group 3 were dispersed along PC 2, whereas strains of group 2 showed a wider dispersion along both axes.

The levels of male and female HCs found on the transformed males were highly and positively correlated within each 'gender group' of HCs, whereas they were highly and negatively correlated between male and female groups of HCs (data not shown). This marked gender bipolarity allowed us to select a unique female-specific HC (7,11-HD) and a unique male-predominant HC (7-T) to simplify our approach. The 7,11-HD : 7-T ratio was defined as the 'feminisation index' (Fi) providing a single parameter to characterize the HC phenotype and to compare the degree of feminisation in XY PGal4 UAS-*tra* flies of different PGal4 strains.

The HC profile of males from the 88 strains that were analysed with the PCA is shown in Fig. 3 (with the reference profile of control male and female flies shown on top). In group 1 (19 strains), the Fi of XY PGal4 UAS-*tra* flies ranged between 86·7 (for Tp3502 strain) and 2·19 (for 10B strain). This high variation of Fi was mainly caused by the variation of 7-T (peak 4), whose percentage varied between 0·3% and 11·1% of SumHCs. The mean absolute amounts of 7-T ranged between 5 ng and 158 ng. By comparison, control Cs females produced 59 ± 5 ng of 7-T (3·3%). The percentage of 7,11-HD (peak 14) varied between 19·5% and 32·4%, with absolute amounts of between 263 ng and 509 ng (for Cs females, 466 ± 33 ng, which is 25·4%). Males of group 1 generally produced more 7-P (peak 10) than 7-T. The absolute level of 7-P ranged between 41 ng and 220 ng (3–11·6%). By comparison, control females produced 97 ± 7 ng of 7-P (5·3%). Feminised males of group 1 always produced less 7,11-ND (peak 21; 72–236 ng, which is 5·1–14·8%) than control Cs females (328 ± 21 ng, which is 18%). In these feminised males, the SumHCs varied between 1118 ng and 1945 ng (1827 ± 117 ng in control females).

XY flies of group 2 (26 strains) showed an intermediate degree of feminisation ($1{\cdot}90 < Fi < 0{\cdot}30$). Their mean levels of 7-T were 8–24·2% (127–476 ng) and mean levels of 7,11-HD were 7·2–19·5% (95–404 ng). These flies also produced more 7-P (148–468 ng, which is 12·6–21·2%) and less 7,11-ND (6–57 ng, which is 0·5–4·3%) than flies of group 1. Their SumHCs ranged between 1101 ng and 2256 ng.

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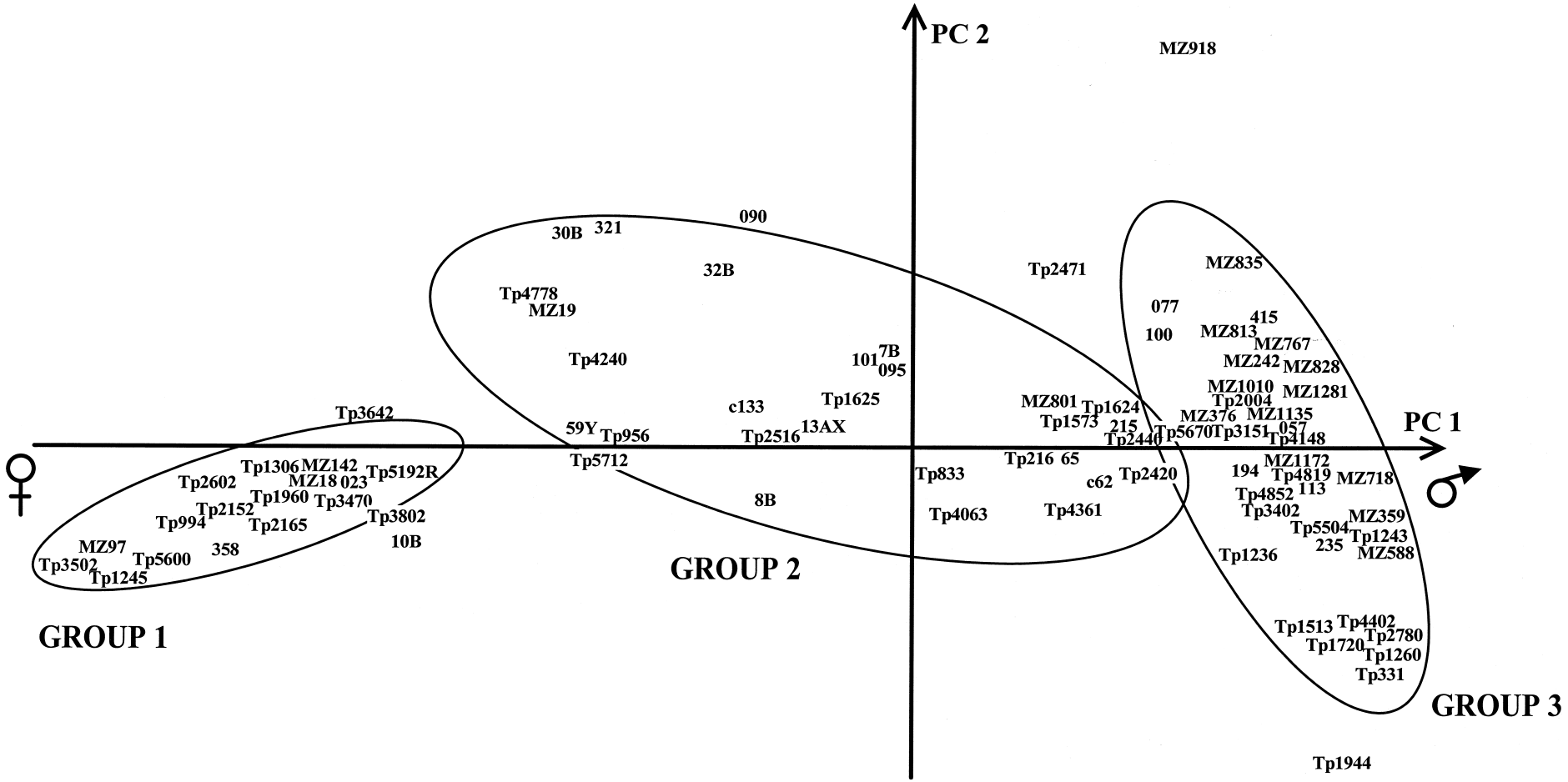
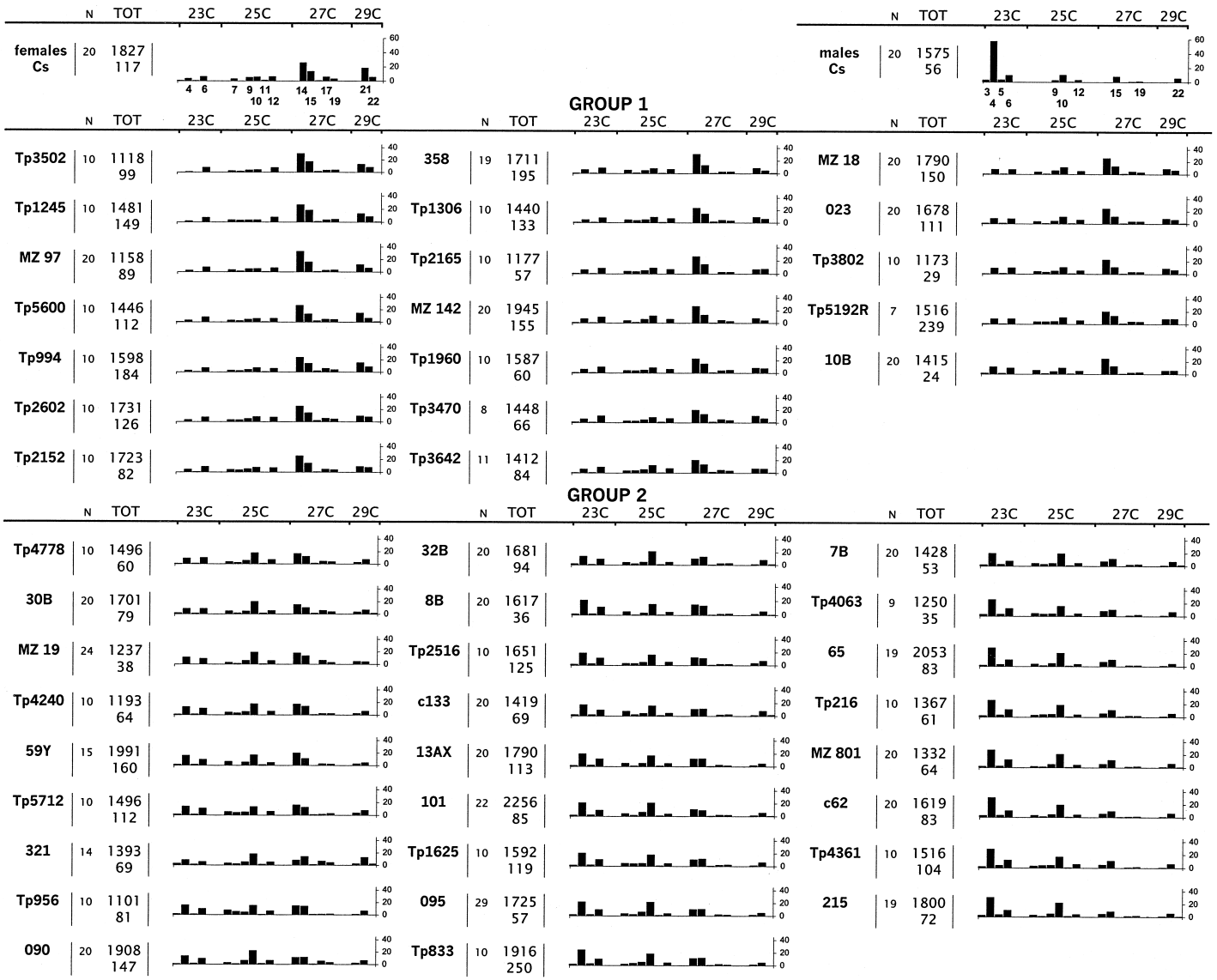


Fig. 2. The degree of HC feminisation varies between XY flies of different PGal4 UAS-*tra* lines. The degree of feminisation of XY flies was compared between 88 PGal4 UAS-*tra* strains with a PCA. Principal components 1 and 2 represent 88·4% and 6·3%, respectively, of the HC variability. Ellipses delineate the three groups of strains, corresponding to various degrees of feminisation (decreasing from group 1 to group 3).



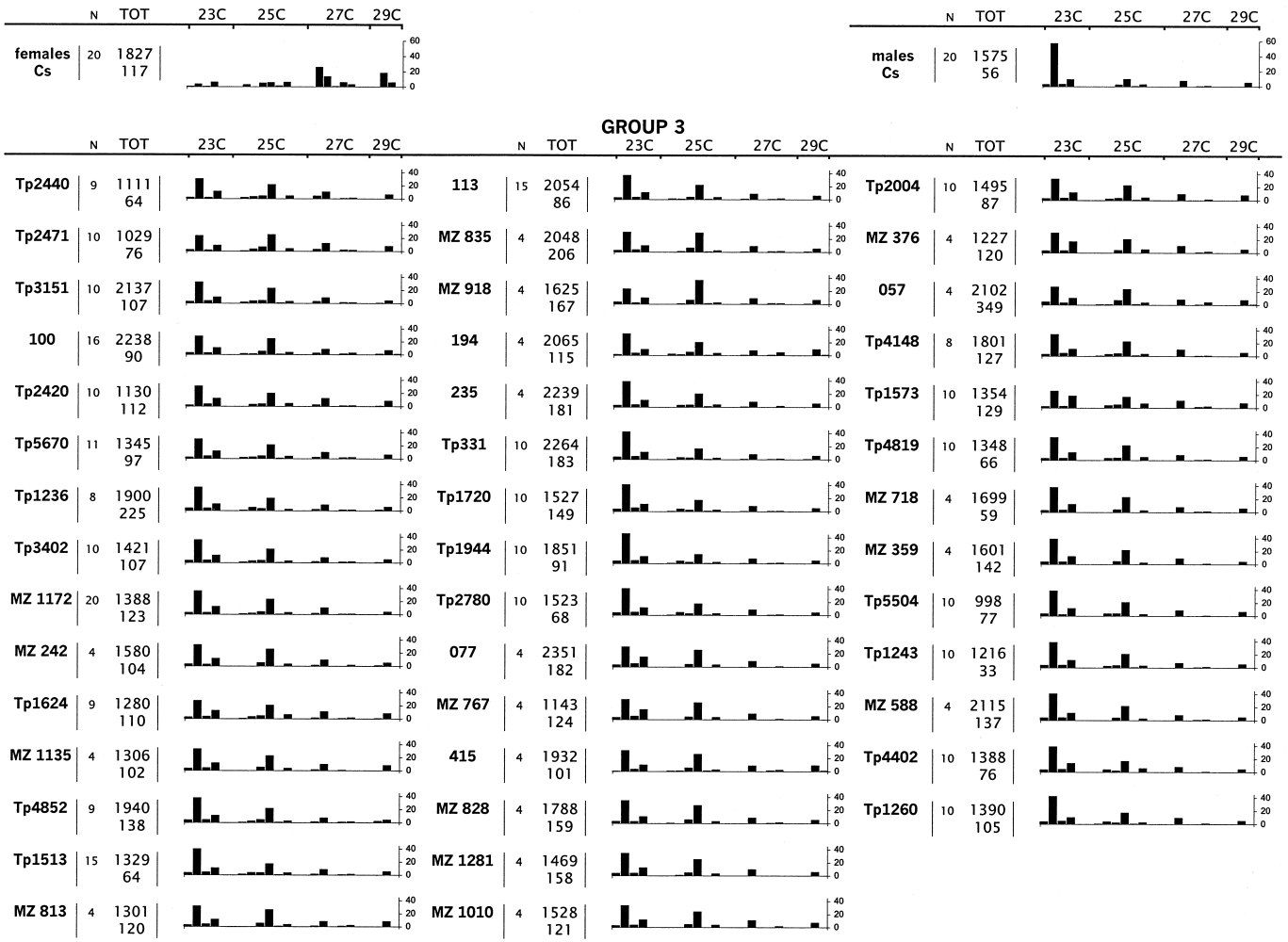


Fig. 3. Histograms of complete HC profiles in XY flies of 88 PGal4 UAS-*tra* strains. Strains were separated into three groups following the PCA shown on Fig. 2. Control female and male (Cs) flies are shown on the top (left and right, respectively). The numbers below both control histograms correspond to the HCs identified on Fig. 1. Each histogram shows the mean percentage of all detected HC grouped by chain length (23–29 carbons). The strain number is on the extreme left. The sample size (N), the mean sum of HCs (TOT) and its standard error (below the mean) are shown between the two vertical bars. Within each group, strains are ranked by decreasing Fi values, from top to bottom and from left to right. The profiles produced by all PGal4 × UAS-*lacZ* XY flies were similar to those of Cs males and, for the sake of clarity, are not shown here.

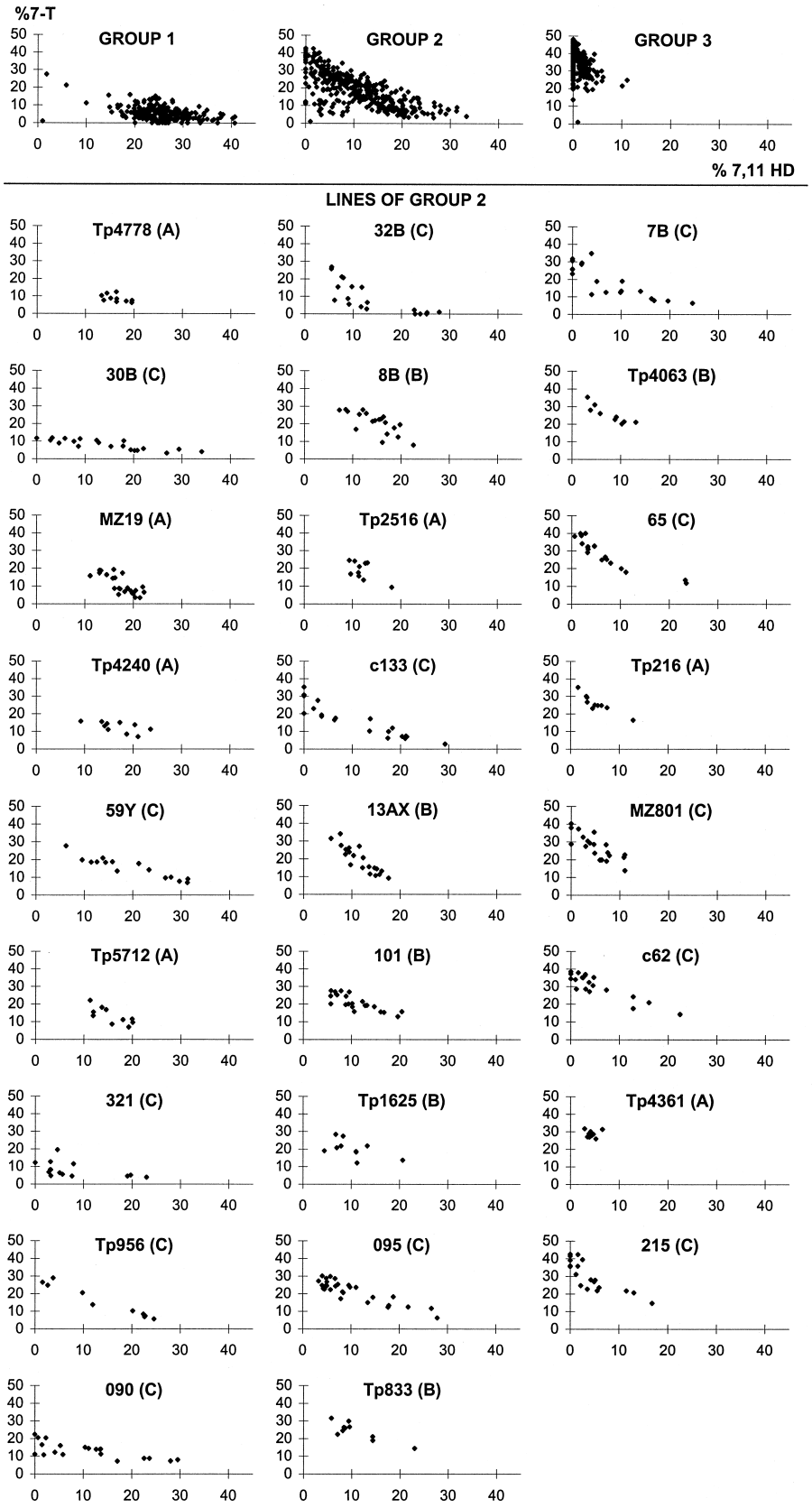


Fig. 4. Intrastrain variability for the production of predominant female and male HCs in XY flies of various PGal4 UAS-*tra* strains. Each individual fly was represented according to its proportion of 7,11-HD (*x* axis) and 7-T (*y* axis). On the top, the distribution of individuals is shown for all flies of each of the three groups: group 2 shows the highest variability. The distribution of individuals for each strain of group 2 are shown below. According to their range of proportions of 7,11-HD and 7-T variability, they were assigned to subgroups A, B or C (between brackets).

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In group 3 (43 strains), XY flies produced high levels of 7-T (241–957 ng, which is 23·1–42·1 %), and generally very low amounts of (or no) 7,11-HD (< 30 ng, which is < 6·8 %). Although the Fi of all these strains was < 0·30, males of some strains (Tp3151, Tp1236, Tp4852, 113, 194, 235 and Tp331) still produced a substantial amount of 7,11-HD (maximum of 136 ng) that was compensated (in the Fi ratio) by an high level of 7-T (> 650 ng). 7-P varied between 16·3 % and 36·1 % (222–594 ng), whereas 7,11-ND was either very reduced (23 ng, which is < 1·1 %) or absent in group 3 males. The SumHCs varied between 998 ng and 2351 ng. The 'non-feminised' males of group 3 included some strains with 'slightly feminised' males because, in most of the latter strains, the results were biased by a few individuals (or even by a single male), such that the degree of feminisation was not predictable in these strains. Male progeny from 132 other PGal4 UAS-*tra* lines yielded HC profiles that were very similar to the control male pattern (data not shown).

(ii) *Intrastrain variation in the degree of feminisation*

We previously noted that the strains composing groups 1 and 3 exhibited much less dispersion along the PCA axes than strains of group 2. Graphs of 7-T and 7,11-HD percentages of individual flies (top of Fig. 4) indicate that there was also less variability in these HCs within strains of groups 1 and 3 than in those of group 2. The overall dispersion of group 2 individuals was very wide and overlapped extensively with the distributions of groups 1 and 3. In group 2, the intrastrain variability revealed three subgroups of strains according to the dispersion of individual Fi values. Fig. 4 shows that the type of dispersion seems to be independent of the Fi value (the strains with higher Fis are ranked on the left from the top; strain Tp4778 had the highest Fi, and strain 215 had the lowest Fi).

Strains in subgroup 2A (Tp4778, MZ19, Tp4240, Tp5712, Tp2516, Tp216 and Tp4361 strains) exhibited less dispersion than other subgroups. The range of variability in the proportion of 7,11-HD did not exceed 10–12 %, whereas the range of variability in the proportion of 7-T did not exceed 15–18 %. In strains of subgroup 2B (8B, 13AX, 101, Tp1625, Tp833 and Tp4063), the dispersion of variability in the proportions of both 7,11-HD and 7-T among flies of each strain was wider than among flies of subgroup A strains, but their range of variability rarely exceeded 18 % and 15 %, respectively. Moreover, these six strains had very similar Fi values (0·71–0·30). In the 13 strains forming subgroup 2C, the range of variability in the proportions of 7,11-HD and 7-T generally exceeded 30 %. In three strains of subgroup 2C (30B, 090 and, to a lesser extent, 321), the variability in the proportion of 7-T, but not of 7,11-HD, was very reduced: the value of their regression slope (−0·26 to −0·33) was much smaller than that of all the other strains (−0·44 to −1·98, except for strain Tp4361 (0·08), which had a very narrow distribution). Therefore, the variation of percentages for male- and female-specific HCs was not correlated in these three strains. In the ten other strains of subgroup 2C, the regression slope indicates that the variability in the proportions of both 7,11-HD and 7-T were negatively related. We also observed a similar negative relationship between female and male predominant HCs in most strains of groups 1 and 3.

(iii) *Masculinisation of hydrocarbons in genetically feminised females*

We obtained an unexpected hydrocarbon phenotype in females of 14 PGal4 UAS-*tra* lines (out of 220) because these XX flies showed a decreased proportion of 7,11-HD together with an increased proportion of 7-T, corresponding to a substantial masculinisation of HCs (Fig. 5). Masculinisation of HCs in XX flies was studied with a procedure similar to the one used to study feminisation in XY flies (see above). The variation of all detected HCs in XX flies of 90 PGal4 UAS-*tra* strains (87 of the 88 strains shown above plus three 28A strains that yielded only XX progeny) was analysed by PCA (Fig. 5). The sum of the two principal components represents almost 90 % of the total variation (74 % for the first component and 15·8 % for the second). The masculinised strains were clearly separated from non-masculinised strains along PC 1. However, no obvious difference of dispersion along the PC 2 was noted between masculinised and non-masculinised strains.

Unlike the feminisation experiment, the amounts of 7-T and 7-P, on one hand, and those of 7,11-HD and of 7,11-ND, on the other, were not highly correlated (data not shown). For this reason, the levels of these four substances were taken in account to calculate the masculinisation index (Mi).

$$\mathrm{Mi} = \frac{([\text{7-T} + \text{7-P}] - [\text{7,11-HD} + \text{7,11-ND}])}{([\text{7-T} + \text{7-P}] + [\text{7,11-HD} + \text{7,11-ND}])}$$

The Mi was used empirically to separate PGal4 strains into two groups according to the degree of masculinisation of their XX PGal4 UAS-*tra* flies. A cut-off Mi value of −0·45 allowed the separation of strains with masculinised females from strains with non-masculinised females (Fig. 6). 14 PGal4 strains (including two 'intermediate' strains, 28AR and 023) were masculinised and formed the M group. If we exclude the two intermediate strains and consider only 12 strains, the Mi values of XX flies varied between +0·69 (for the highest degree of masculinisation) and −0·19. They produced between 7·4 % and 34·3 % of

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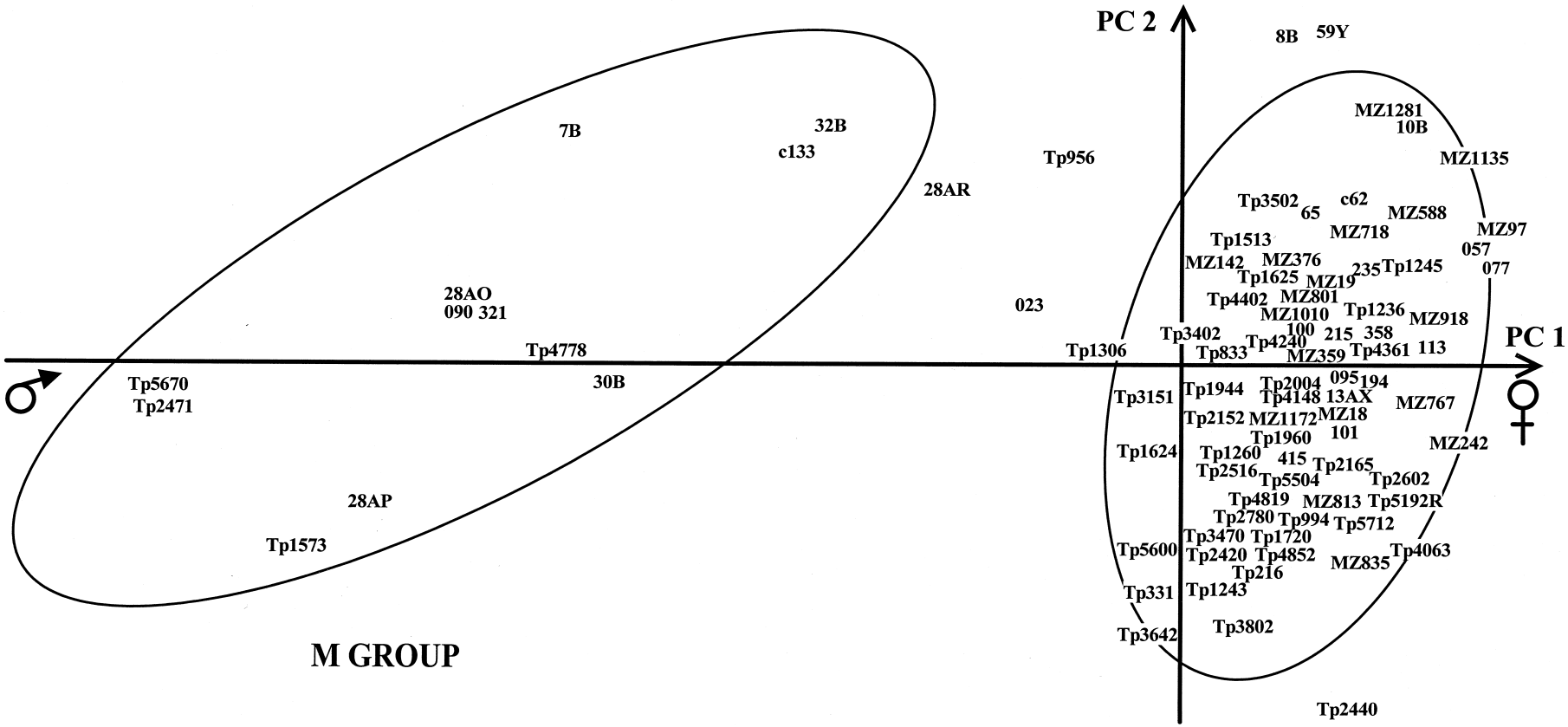

Fig. 5. The degree of HC masculinisation varies between XX flies of different PGal4 UAS-*tra* lines. The degree of masculinisation of XX flies was compared between 90 PGal4 UAS-*tra* strains with a PCA. Principal components 1 and 2 represent 74% and 15·8%, respectively, of the HC variability. Ellipses were drawn to delineate most strains belonging either to the group with masculinised females (M group) or to the group without masculinised females.

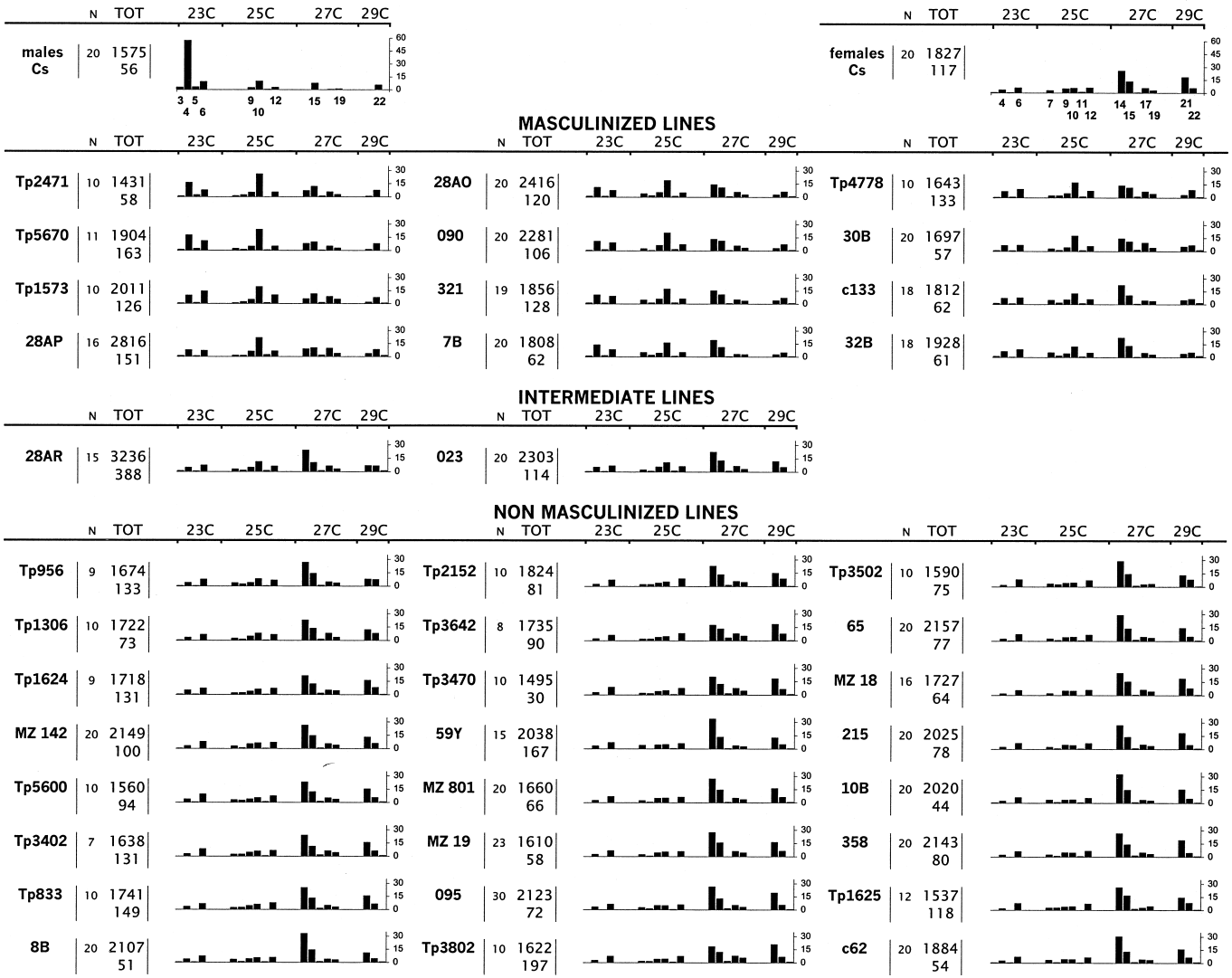

Fig. 6. Histograms of complete HC profiles in XX flies of 38 PGal4 UAS-*tra* strains. Strains were separated into two groups following the PCA shown in Fig. 5. However, strains with masculinised females (M group) were split into two subgroups depending upon their masculinisation index (Mi): 12 masculinised lines and two intermediate lines. We decided not to show the profile for all 77 strains without masculinised females but only a sample including most of the strains with feminised XY flies. Mi values determined for PGal4 × UAS-*lacZ* XX flies were similar to those for Cs females. For further detail, see Fig. 3.

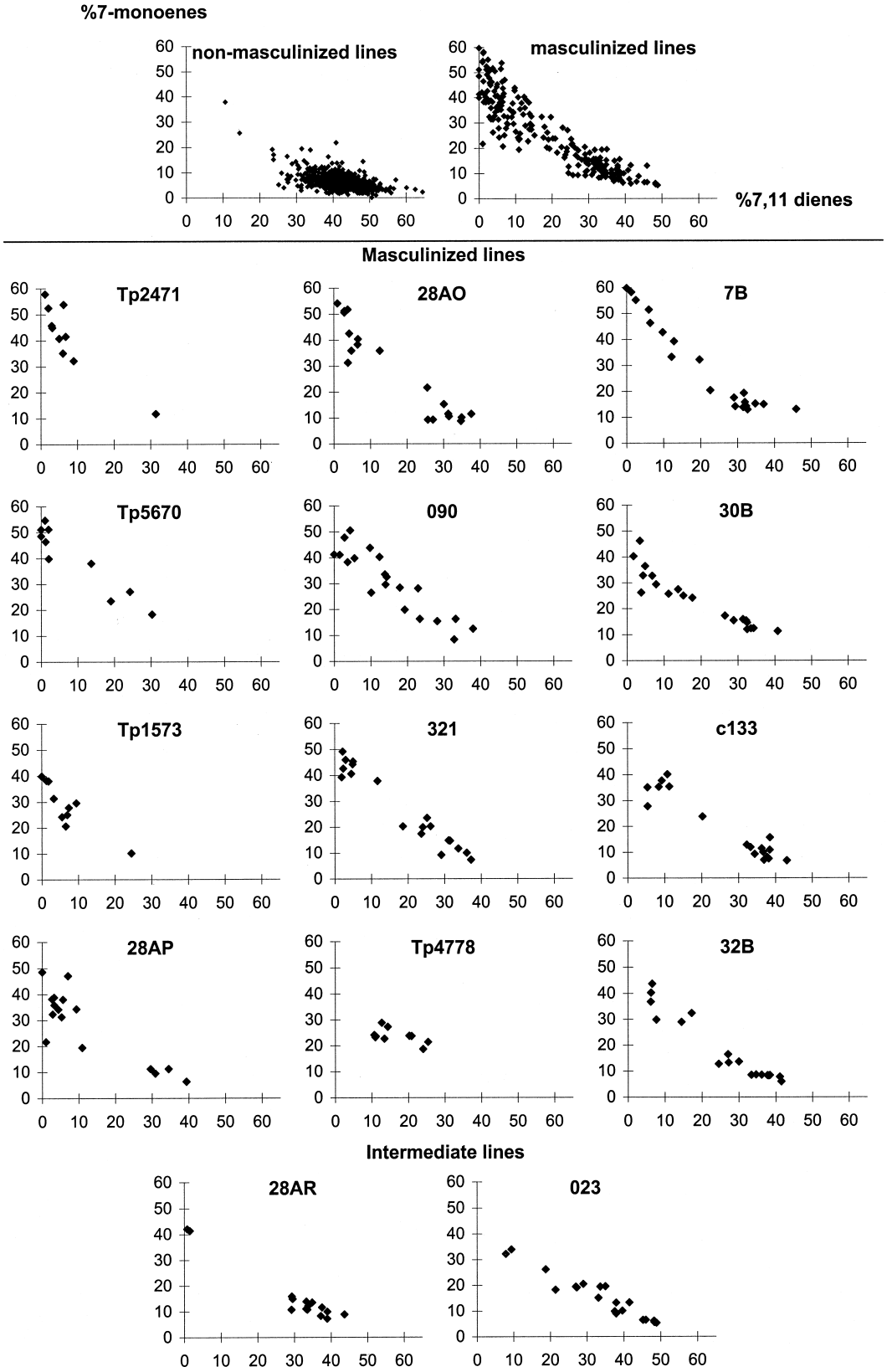

Fig. 7. Intrastrain variability for production of predominant female and male HCs in XX flies of various PGal4 UAS-*tra* strains. Each individual fly was represented according to its proportion of 7,11 dienes (*x* axis) and 7-monoenes (*y* axis). On the top, individual variability is shown for each of the two groups: the group with non-masculinised females with a unimodal distribution and the group with masculinised females (M group), which exhibits the highest variability. Intrastrain distribution for all masculinised strains is shown below. The two intermediate strains (with the mildest masculinisation effect) are shown on the bottom.

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7,11-dienes (7,11-HD + 7,11-ND), corresponding to 108–997 ng. Therefore, female-specific molecules never completely disappeared even in the most extreme cases of masculinisation (as in Tp5670 and Tp2471 strains). In the same XX flies, the proportion of 7-T varied between 3·1 % and 17·2 % (113–333 ng), whereas the proportion of 7-P varied between 7·9 % and 25·7 % (224–608 ng). SumHCs varied between 1431 ng and 2281 ng, except for 28AO- and 28AP-*tra* females (2416 ng and 2816 ng, respectively). Both intermediate strains (023 and 28AR) had very low Mi values (−0·41 and −0·36). However, they were included in the M group because their XX flies produced very high amounts of male 7-monoenes (7-T + 7-P = 337 ng and 467 ng, respectively). The high amount of 7,11-dienes (798 ng and 997 ng) produced by these flies explain the relatively low Mi.

XX progeny from the 76 other PGal4 UAS-*tra* lines produced HCs in amounts very similar to those of control Cs females. In that group of strains, the proportion of 7,11-dienes varied between 30·3 % and 45·8 % (536–1136 ng), the proportion of 7-T varied between 0·7 % and 5·1 %, and the proportion of 7-P varied between 2 % and 6·1 %. The total amount of 7-T + 7-P was generally < 200 ng and SumHCs varied between 1400 ng and 2562 ng.

(iv) *Intrastrain variation for the degree of masculinisation*

The wide dispersion of the strains of the M group on both PCA axes (Fig. 5) indicates that the degree of masculinisation varied. Similarly to the approach carried out to study feminisation of male HCs (see above; Fig. 4), we measured the variability of masculinised HCs by examining intrastrain distributions in graphs of the proportion of 7-monoenes against the proportion of 7,11-dienes. The dispersion of individuals clearly differs between 'masculinised' and 'non-masculinised' groups (top of Fig. 7): the HC phenotype of 'non-masculinised' females is rather homogenous whereas individuals of strains producing 'masculinised' females can exhibit a wide variety of HC phenotypes, ranging from normal female to normal male profiles.

The comparison of the 14 'M' strains revealed different distribution patterns (as for feminised males of group 2): Tp4778-, Tp2471- and Tp1573-*tra* females showed relatively unimodal distributions (with a single individual being the exception in the two latter strains). Tp4778-*tra* females produced an intersexual HC mixture (23·7 % 7-T + 7-P and 16·4 % 7,11-dienes), whereas most Tp2471- and Tp1573-*tra* females were very masculinised for their predominant HCs (41·8 % and 28·5 % of 7-monoenes for only 7·4 % and 6·7 % of 7,11-dienes, respectively).

The other M strains showed widely dispersed distributions, with phenotypes that stretched between male and female HC phenotypes. Although the apparent difference between bimodal and continuous distributions might result from a sampling artefact, the phenotypic heterogeneity within most of these M strains was very marked: some females exhibited male-like HCs and others showed a female-like profile. Furthermore, the extreme amplitude of masculinisation varied: for example, females of Tp4778-, c133- and 32B-*tra* strains never showed a strong male-like phenotype. In conclusion, the UAS-*tra* transgene masculinised female HCs in some PGal4 lines, but with varying degrees of sexual transformation.

(v) *Correlation between the sexual transformation of HCs and GAL4 expression*

In order to map the different tissues involved in the process of sexual maturation of HCs, we studied the correlation between the degree of HC transformation (feminisation in males/masculinisation in females) and *GAL4* expression. *GAL4* expression (characterized with *lacZ* staining) was compared in 2- and 4-day-old male and female flies; we report results for 4-day-old flies (Table 1) because no significant age effects were detected (except in males of the 215 strain). However, sexually dimorphic expression patterns were observed in many strains (Table 1).

Expression was compared between four groups of PGal4 strains: the group producing masculinised females (the M group; Fig. 5) and the three groups defined by their effects on the feminisation of male HCs (Fig. 2). All the strains that belonged to the M group were excluded from the three feminising groups (which were thus noted as groups 1^{-M}, 2^{-M} and 3^{-M}). Careful attention was specifically paid to all structures known to be relevant to HC biosynthesis and/or maturation (see Material and Methods). A strain was counted 'positive' for a particular structure when *GAL4* expression was repeatedly detected in at least one sex.

The data in Table 1 clearly support the finding that adult oenocytes are involved in the sexual transformation of HCs under the control of the sex-determination gene *transformer* (Ferveur *et al*., 1997). All strains with substantially feminised PGal4-*tra* males (groups 1^{-M} and 2^{-M}) and with masculinised PGal4-*tra* females (M group) showed *GAL4* expression in the oenocytes of 4-day-old flies. The only exception to this rule was the Tp4361 strain (of group 2^{-M}), in which no oenocyte expression was detected. Moreover, no qualitative difference of expression was detected between dorsal and ventral oenocytes, or between oenocytes of different segments. Conversely, oenocyte expression was detected only in one strain of group 3^{-M} (Tp1236), and only in males of this strain.

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Table 1. *Strain correlation between* GAL4 *expression and the degree of HC transformation*

			Fat body			Digestive tract				Male reproductive apparatus			Female reproductive apparatus						CNS		
Lines	Group (subgroup)[a]	Oeno-cytes	Head	Thorax	Abdomen	Oes.	Mal. t.	Ant. int.	Rectum	Testis	Acc. gl.	Ejac. b.	Uterus	Foll. c.	Sperm	S. r.	CA	CC	Brain	Thor. gg.	Thor. musc.
Tp2471	M(3)	+	+( )	−( )	+	−(+)	+	−	+	−			+		+				+	+	
Tp5670	M(3)	+	+	+	+	+		−( )	−	−		+	−	−							+
Tp1573	M(3)	+	+	+	+	+	+	+	+(−)				−						+(−)		
28AP	M(−)	+	+	+	+	+	+	+(−)	+	+		+		+					−	−	+
090	M(2C)	+	+(−)	+(−)	+(−)	+	+	+	+	−	−	−	+	+	+	+			−		+
28AO	M(−)	+	+	+	+	+	+(−)	+	+	+	−	+	−						+( )		+
321	M(2C)	+	+	+	+	+	+(−)	−	+(−)			−							+	−	+
Tp4778	M(2A)	+	−		+				+				+			+					
7B	M(2C)	+			−( )	+		−(+)	+	−			−						+(−)	−	−
30B	M(2C)	+	+	+	+	+	+(−)	+(−)	+	−			−	+	+						+
c133	M(2C)	+											−						+	+	
32B	M(2C)	+	+	+	+	+		+	+				−		+				−	−(+)	−
28AR	M(−)	+	+	+	+	+	+	+	+	−		+									+
023	M(1)	−(+)			−		+	−(+)	+	−			−		−						(−)
Tp3502	1-M	+				+	+	+	−(+)	+	−		+		+				−	(−)	+
Tp1245	1-M	+				−	+	+	+	−			+					(+)	−(+)	−(+)	−
MZ97	1-M	+	−( )	−( )	+(−)	+		+(−)	−			−	+						+(−)		−
Tp5600	1-M	+	−( )		+(−)	+			+	+	+		−	+					+	+	+
Tp994	1-M	+	+	+	+	+	+	+	+	−			−	−					+	+	(−)
Tp2602	1-M	−(+)			+( )	−( )			+				+					(+)	+(−)	−(+)	+
Tp2152	1-M	+			+( )	−( )			+(−)	−			−						+	+(−)	+
358	1-M	+	+	+	(+)	(−)			+( )				−						−		
Tp1306	1-M	+	+	+	+	+		−	+	−	−		−		−						
Tp2165	1-M	+	+( )	+( )	+(−)	−( )	+( )			+	+		+						+( )	−	−
MZ142	1-M	+	−	−	+	+		+(−)	−	−	−		+		−		+		+	+	−
Tp1960	1-M	(+)			+( )	+( )		(+)	+				−		−				−(+)	+	
Tp3470	1-M	+	+	+	+	+	+	+(−)	+	+	+		−	+	+	+			+	+	−
Tp3642	1-M	+							−( )	−			+						−	−	
MZ18	1-M	+			−( )	+							+						+	+	
Tp5192R	1-M	−( )	+( )		+	+		+	−	+	−		+						+(−)		−
10B	1-M	+										−	+					+	−(+)	−	+

Table 1 (*cont.*)

MZ19	2^{-M}(A)	+	+	+	+	−	−	−	−	−			−		+			−		−
Tp4240	2^{-M}(A)	+				−( )	+		+				+	+	+			+		
Tp5712	2^{-M}(A)	+	+	+	+		−	−	−	−			−				−(+)			+
Tp2516	2^{-M}(A)	(+)			+	+		+	+	+			+			+		−	−(+)	
Tp4361	2^{-M}(A)												+							
8B	2^{-M}(B)	(+)											+	+	+		+	+	+	+(−)
13AX	2^{-M}(B)	−( )			−( )	+	−( )	+	+	+			+		−			+	+	+
101	2^{-M}(B)	+	−( )		−	−		+	−(+)	−	−		+					+	+	+
Tp1625	2^{-M}(B)	−				−			+				−				+	+		−
Tp833	2^{-M}(B)	+( )	+( )	+( )	+	+		+	+	−		−	+					+	+	+
59Y	2^{-M}(C)	+( )			+				+( )	+		−	+		−			+	+	+
Tp956	2^{-M}(C)	+	+( )	−( )	+( )	−( )	+( )		+	−			−	−				−( )	−( )	+( )
095	2^{-M}(C)	−	−	−( )	−(+)	(−)	−( )	(−)	−	+	−		−		−			+	+	+(−)
65	2^{-M}(C)	+	+( )	+( )	+( )	+	+	+( )	+( )	−			+					+	+( )	−
MZ801	2^{-M}(C)	−			+( )	+	−	+	+	−			+		+			−(+)	+	
c62	2^{-M}(C)	(+)	(+)	(+)	(+)	+( )					−		+		−		+	+(−)	+	
215	2^{-M}(C)	−			−( )								+	−						
100	3^{-M}				(+)	−( )	−( )	(+)	+(−)	+	−		+								+
Tp1236	3^{-M}	−( )			+( )	(−)				+	+		−	+				+	(−)	+
MZ1172	3^{-M}				+(−)	+		+	+	+			+				(−)	+	+	+
113	3^{-M}				−	−			+	+	+	−	+		+			−	+	
Tp4402	3^{-M}				+( )	+	−( )	−( )	+(−)		−		+	+						+(−)
Tp4819	3^{-M}				+	−(+)			+(−)		−		+					+		
Tp5504	3^{-M}					+		−					+					−	−	

[a] Within groups (or subgroups), strains were ranked from top to bottom according to their Fi or Mi. For strains of the M group, the feminisation group is indicated between brackets; (−) indicates that no male progeny was produced. Groups 1^{-M}, 2^{-M} and 3^{-M} correspond to the groups 1, 2 and 3 (with diversely feminised XY flies, see Fig. 3) from which all strains belonging to group M (with masculinised XX flies; see Fig. 6) were excluded. Strains of group 2 were ranked by subgroup order (A, B and C, as indicated between brackets).

The strength of *GAL4* expression visualized with the UAS-*lacZ* histological marker transgene in each main anatomical structure of XY, PGal4 UAS-*lacZ* flies is indicated as follows: ' + ', strong; ' − ', weak; blank, no expression detected. In case of sexual dimorphism, expression in XX, PGal4 UAS-*lacZ* flies is indicated between brackets. A peculiar attention was brought to the sites potentially involved in HC processing.

Abbreviations: Abd., abdomen; Ac. gl., accessory glands; Ant. int., anterior intestine; CA, corpus allatum; CC, corpus cardiacum; CNS, central nervous system; Ejac. b., ejaculatory bulb; Foll. c., follicular cells; Mal. t., malpighian tubules; Oes., oesophagus; Sper., spermathecae; S. r., seminal receptacle; Thor., thorax; Thor. gg., thoracic ganglion; Thor. musc., thoracic muscles.

GAL4 expression was never observed in the oenocytes of any strain with an untransformed HC profile (130 strains examined; data not shown).

A lower frequency of *GAL4* expression was seen in both the head and the thoracic fat body (FB) of strains with transformed flies: 40% and 48%, respectively, of strains in groups 1^{-M} and 2^{-M}, and 73–82% of M-group strains. Expression was generally correlated in both FB parts. Conversely, no strain in group 3^{-M} showed *GAL4* expression in the head and thoracic FB. However, in these strains, *GAL4* expression was also infrequently detected in the oenocytes. *GAL4* expression was frequently detected (76–91%) in the abdominal FB of all groups, suggesting that these abdominal structures do not strongly influence HC phenotype.

We did not observe any significant relationship between the transformation of HCs and *GAL4* expression in the muscular, nervous or digestive systems, nor in the main structures composing the male and female reproductive systems. The high frequency (71%) of staining in male accessory glands in group 3^{-M} strains is probably the result of a sampling artefact (5/7 positive strains). Finally, the fact that very few strains expressed *GAL4* in the CA/CC complex allows us to eliminate a possible role of *tra* in this structure, which is *a priori* potentially related to the sexual maturation of HCs.

Among the enhancer-trap strains that revealed sexually dimorphic expression (see brackets in Table 1), several strains showed sex-specific expression in the adult oenocytes: in group 1^{-M}, one strain was female specific (Tp1960) and another male specific (Tp5192R); three male-specific (13AX, Tp833, 59Y) and three female-specific (Tp2516, 8B, c62) strains were found in group 2^{-M}; and one male-specific (Tp 1236) strain was found in group 3^{-M}. If none was found in group M, the 023 strain showed a quantitative difference. Several strains showed sex-specific expression in the FB: the c62 strain was the only female-specific strain expressing *GAL4* in the complete FB (in this strain, expression was also female specific in the oenocytes and male specific in the oesophagus). The Tp956 and 65 strains expressed *GAL4* specifically in males in the three parts of the FB and in other various structures. Four other strains (MZ 97, Tp833, Tp2165 and Tp2471) showed male-specific expression in head and thoracic (but not in abdominal) FBs. Several other PGal4 strains showed sex-specific expression in other various structures (Table 1).

4. Discussion

Here, we have shown that sexually dimorphic hydrocarbons (HCs), which play a crucial role in the mechanism of mate recognition and sexual isolation, are not controlled in an 'all-or-none manner' in flies of both sexes. We believe that the degree of HC transformation depends upon the level of – or the number of crucial cells that produce – the Tra female product. Indeed, *GAL4* expression was strongly detected in the oenocytes of all masculinised strains (14 strains of group M) and of 16 out of 17 highly feminised strains (group 1^{-M}), whereas it was weaker and less frequently detected in the oenocytes of the intermediate group of feminisation (group 2^{-M}) than in the former two groups. XY flies with no *GAL4* expression in adult oenocytes were poorly or not feminised. However, the quantitative level of *GAL4* expression in the oenocytes remains difficult to measure. The correlation between oenocytes and HC feminisation in XY flies was very high (there was only a single exception), but the correlation between HC masculinisation and *GAL4* expression in both oenocytes and the FB had several exceptions: c133 (in the M group) had no expression in the FB, whereas the Tp994, Tp1306 and Tp3470 strains (in group 1) expressed *GAL4* in both the oenocytes and the FB. This discrepancy could be caused by a variation in the level or pattern of *GAL4* expression in both structures. We are as yet unable to explain why the head and thoracic FBs, but not the abdominal FB, can affect the hydrocarbon profile.

If a very low variability of HC amounts was detected in highly feminised XY flies (group 1 strains), strains of group 2 showed a wider variability. In the latter group, strains of subgroups A and B showed relatively homogeneous HC patterns that could be caused by an incomplete but constant *GAL4* expression in some of the tissue(s) that control the HC maturation. Conversely, the extreme dispersion of the HC phenotypes characterizing the strains of subgroup C could result from subtle variations (in a tissue or during development), allowing the level of Tra expression to fluctuate around a crucial threshold, and would be sufficient to switch the gender of the HC profile.

Why do the dominant feminising UAS-*tra* transgenes masculinise the production of predominant hydrocarbons in XX flies? It is known that both the excess and the default amount of product of major sex determination genes induce a similar mutational effect on sex-specific characters (Parkhurst & Meneely, 1994; Jinks *et al*., 2000). The HC masculinisation in XX flies described here results from an excess of female Tra product and resembles that induced by the absence of the female product in XX flies (where several sex determination mutants produced masculinised HCs) (Tompkins & McRobert, 1989; Tompkins & McRobert, 1995; Jallon *et al*., 1986; Jallon *et al*., 1988). Furthermore, it is possible that the modification of *tra* expression deregulates the activity of target genes like *doublesex* that control hydrocarbon biosynthesis (Waterbury *et al*., 1999). Therefore, the

overexpression of the female Tra product in crucial cells (of the oenocytes and/or of the FB) could deregulate the enzymatic balance that controls the production of female-specific HCs in XX flies. Unfortunately, there is no available tool to differentiate *in situ* female- and male-specific Tra products (Tian & Maniatis, 1993).

Another finding also concerns sexual determination. The strains with masculinised XX flies also showed an effect on feminisation of their XY flies, but there was a negative relationship between the degree of HC feminisation and the degree of masculinisation (in the same strains). Among the 11 M-*tra* strains that produced male and female progeny (the three 28A-*tra* lines were excluded), the three strains that exhibited the most extreme masculinised HC profiles (Tp2471, Tp5670 and Tp1573) had the least feminised males (group 3), whereas the only M strain with an intermediate Mi (023) had highly feminised males. It is possible that the feminised HC phenotype results from the conjugated effects of feminisation and masculinisation in XY flies. In other words, the amount of female HCs in XY flies would be greatly decreased if a high level of masculinisation simultaneously occurred, implying that masculinisation and feminisation can act additively in the same fly. The sexual difference in the degree of HC transformation could also be explained by the fact that feminisation in XY flies depends only on the expression of the UAS-*tra* transgene in the adult oenocytes (Ferveur *et al*., 1997), whereas masculinisation in XX flies requires the simultaneous expression of UAS-*tra* in the FB and in the oenocytes.

Finally, our results suggest that the HC phenotype could be sensitive enough to detect alteration of the sex determination pathway. Therefore, the precise characterisation of this sexually dimorphic phenotype after ectopic expression of UAS-*tra* would be an easy and powerful approach to screen new enhancer-trap strains that interact with the sex determination pathway. If most PGal4 × UAS-*tra* strains with feminising effects are not useful for such a screen (because they only use the potential of the enhancer-trap to misexpress Tra in crucial tissues), we suspect that the genes of the 13 enhancer-trap strains composing subgroup 2C interfere with some of the sexually dimorphic mechanisms involved in sex pheromone production: the six strains labelled 'M(2C)' (top of Table 1) produced a marked effect in the HC transformation in both sexes, whereas the other seven strains (labelled 2^{-M}(C)) revealed a sexually dimorphic expression in their oenocytes and/or in their FB, structures that are likely to be involved in the sexual maturation of HCs. We believe that, in the M(2C) strains, the targeted genes and/or tissues interact with *tra*, because they alter the production of a sexually dimorphic character (HCs) in both sexes. In the 2^{-M}(C) strains, the sexually dimorphic expression occurs in the oenocytes and/or FB, which are the crucial tissues for sex-specific HCs. Therefore, the intermediate mixtures of HCs could be related to the partial alteration of sex-specific mechanisms in the oenocytes, FB and, possibly, other tissues. It is likely that the sexual maturation of the HC phenotype depends on complex regulatory interactions similar to those controlling sexually dimorphic abdominal pigmentation (Kopp *et al*., 2000).

Our future work will aim to identify the genetic and molecular mechanisms intrinsic to the oenocytes and to the FB that switch the production of sex-specific hydrocarbons.

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