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A Mutation With Major Effects on *Drosophila melanogaster* Sex Pheromones

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ABSTRACT

Sex pheromones are intraspecific chemical signals that are crucial for mate attraction and discrimination. In *Drosophila melanogaster*, the predominant hydrocarbons on the cuticle of mature female and male flies are radically different and tend to stimulate or inhibit male courtship, respectively. This sexual difference depends largely upon the number of double bonds (one in males and two in females) added by desaturase enzymes. A mutation was caused by a *PGal4* transposon inserted in the *desat1* gene that codes for the desaturase crucial for setting these double bonds. Homozygous mutant flies produced 70–90% fewer sex pheromones than control flies, and the pheromonal difference between the sexes was almost abolished. A total of 134 excision alleles were induced by pulling out all or a part of the transposon. The pheromonal profile was generally rescued in excision alleles with a completely or largely removed transposon whereas it remained mutant in alleles with a larger piece of the transposon. Five *desat1* transcripts were detected during larval-to-adult development. Their levels were precisely quantified in 24-hr-old adults, a critical period for the production of sex pheromones. Three transcripts significantly varied between control females and males; however, the predominant transcript showed no difference. In mutant flies, the predominant transcript was highly decreased with the two sexually dimorphic transcripts.These two transcripts were also absent in the sibling species *D. simulans*, which shows no sexually dimorphic hydrocarbons. We also induced a larval-lethal allele that lacked all transcripts and failed to complement the defective hydrocarbon phenotype of mutant alleles.

IN many animals, mate choice depends on species-specific signals that are exchanged during courtship (Bradbury and Vehrencamp 1998). Among the different signals involved in communication, chemical messages often play a major role in invertebrates (Wyatt 2003). In *Drosophila melanogaster*, sexual orientation relies largely upon the hydrocarbons present on the cuticle (cuticular hydrocarbons, or CHs; Jallon 1984; Ferveur *et al.* 1997). Predominant CHs are sexually dimorphic in both their occurrence and their effects: the male's 7-tricosene (7-T) is thought to stimulate females and to inhibit males while the female's 7,11-dienes (7,11-heptacosadiene, or 7,11-HD, and 7,11-nonacosadiene, or 7,11-ND) moderately stimulate conspecific male courtship (Ferveur and Sureau 1996) and strongly prevent mating by males of two closely related species, *D. simulans* and *D. mauritiana* (Coyne *et al.* 1994; Coyne and Oyama 1995; Savarit *et al.* 1999). The two latter species show no sexual dimorphism for predominant CHs and both sexes predominantly produce 7-T (Jallon and David 1987; Cobb and Jallon 1990).

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In *D. melanogaster*, the sexual difference for the production of CHs is largely based on the number of double bonds present on the carbon chains. Desaturase enzymes are thus crucial in transforming the fatty acids into mature CHs. In this species, six unique desaturase-encoding sequences have been deduced from the genomic sequence (Knipple *et al.* 2002). Among these sequences, the two clustered genes *desat1* and *desat2* code for two closely related desaturases with a different specificity for fatty acid precursors (palmitate and myristate, respectively; Wicker-Thomas *et al.* 1997; Dallerac *et al.* 2000). A survey of 24 geographic strains revealed that all strains, except those collected in Africa and in the Caribbean, have a similar mutation in the 5′ region of the *desat2* gene. The occurrence of this mutation has been correlated with a variable mating propensity shown by strains with unusual cuticular hydrocarbons and has been interpreted as a case of incipient speciation (Takahashi *et al.* 2001; Ting *et al.* 2001; Fang *et al.* 2002). Unlike *desat2*, which is predicted to produce only one transcript and which is known to be involved only in introducing a specific set of double bonds (on carbons 5 and 9), that is seen only in a minority of geographical strains, *desat1* is active in all *D. melanogaster* strains and has a complex structure, which suggests that it could play a variety of roles. Its first exon shows five promoting

sequences that could code for five transcripts whose translation would result in the same unique desaturase enzyme, perhaps expressed in different tissues (http://flybase.net/).

We carried out the genetic and molecular study of the *desat1* gene with a strain that carries a *PGal4* transposon inserted in its genome in which the production of sex pheromones was drastically reduced. The remobilization of the transposon allowed us to rescue the CH profile in 75% of alleles; the other alleles induced either a mutant-like or an intermediate profile. In most alleles, a relationship was found between the CH phenotype and the molecular structure of *desat1*. We detected five *desat1* transcripts and measured their variation in females and males of various genotypes and species. We also induced a "larval lethal" null allele that produced no transcript and failed to complement the mutant CH phenotype.

MATERIALS AND METHODS

Stocks and genetic procedure: All *D. melanogaster* and *D. simulans* strains were raised on yeast/cornmeal/agar medium and kept at 25° on a 12:12 hr light/dark cycle. Canton-S (Cs) was used as the control strain for the production of *D. melanogaster* male and female CHs (Figure 1). The *D. simulans* strain, collected in the Seychelles archipelago, has been for maintained >20 years in the lab (Ferveur 1991). Crosses were performed using standard techniques. A description of the chromosomes and genetic tools used in this study can be found in Lindsley and Zimm (1992). To generate derivative lines of $desat1^{1573\text{-}1}$, we used the scheme described by Cooley *et al.* (1988) to mobilize the *PGal4* transposon. Excision of the transposon was performed by crossing homozygous $desat1^{1573\text{-}1}$ females with males from a jump starter strain, which provided the *P*-element transposase (Robertson *et al.* 1988). Male progeny carrying the $desat1^{1573\text{-}1}$ *PGal4* transposon and the transposase-producing $\Delta 2\text{-}3$ chromosome were crossed to *w; +/TM3* females. To get flies without the *P* transposon, males of the next generation were scored for the loss of the $mini\text{-}white^{+}$ gene. Each excision event ($desat1^{1573\text{-}exc}$) was subsequently amplified in separate strains by crossing each w^{-}; $desat1^{1573\text{-}exc}/TM3$ male with several w^{-}; $PGal4\text{-}w^{+}/TM3$ virgins. Within each strain, white eye (w^{-}; $desat1^{1573\text{-}exc}/TM3$) males and females were mated together to produce a stable line.

Four Df(3R) deficiencies that theoretically partially or totally delete the *desat1* gene [*T32* (86_{E2}-87_{C7}), *P21* (86_{E19F1}-$87_{B11\text{-}15}$), *kar-H10* (87_{A7}-87_{D2}), and *ry615* ($87_{B11\text{-}15}$-$87_{E8\text{-}11}$); Gausz *et al.* 1981; Garcia-Bellido *et al.* 1983; Reuter *et al.* 1987] were available. All deficiencies were tested in reciprocal complementation crosses with the *C′1* null allele to measure their effect on the CH phenotype, developmental lethality, and *desat1* transcription profile. To assess the developmental stage during which *C′1/C′1* and *C′1/ry615* died, the proportion of first, second, and third instar surviving larvae was compared with that of heterozygotes (*C′1/TM3*; *ry615/TM3*) every 24 hr at 25°. The genotype of larvae was assessed by the presence/absence of the *TM3, Ser Act5-GFP* balancer chromosome that induces a characteristic fluorescent pattern (Ferrandon *et al.* 1998). The *l(3) S028813* allele with the *P(w)* transposon (Deak *et al.* 1997), which moderately affected the CH phenotype (Labeur *et al.* 2002), was also tested in complementation with the *C′1* allele.

Extraction and analyses of CHs: Male and female flies were sexed 0–2 hr after emergence under light CO_2 anesthesia and aged, in groups of five, in standard food vials. CHs were extracted from 5-day-old individual flies by gas chromatography following hexane extraction according to the method of Pechiné *et al.* (1985) and modified by Ferveur (1991). Analyses were performed with a Varian CP3380 chromatograph and equipped with a Cp-sil 25 mx 0.25-mm capillary column, using hydrogen as the carrier gas. The temperature program was: 120°–140°, 10°/min; 140°–300°, 4°/min, and then a constant temperature of 300° during 28 min. Peak detection was carried out using a flame ionization detector that yielded retention times and areas under each peak. All the main *D. melanogaster* CHs have been identified and characterized (Antony and Jallon 1982; Pechiné *et al.* 1985, 1988; Jallon and Pechiné 1989). Twenty-four CHs were systematically detected in female flies and 14 in male flies, both with chain lengths ranging from 23 to 29 carbons. Each CH was characterized by its percentage relative to the total amount of CHs. In addition, the area of each CH peak was compared with the area of an internal hexacosane standard to calculate its absolute amount (in nanograms). Our chemical analysis focused on 7,11-dienes (7,11-HD and 7,11-ND), which are the predominant CHs of mature wild-type *D. melanogaster* females, and on 7-monoenes [7-T and 7-pentacosene (7-P)], which are abundant in wild-type *D. melanogaster* males and in *D. simulans* flies. We also measured the sum of all detected unsaturated CH (ΣDesat) and each of the saturated CHs (23Lin, 25Lin, 27Lin, 29Lin), which are predominant in mutant flies, together with their sum (ΣLin). To compare the effect of *desat* alleles, we designed the desaturation index (DI) whose formula is: $DI = (\Sigma Desat - \Sigma Lin)/(\Sigma Desat + \Sigma Lin)$. DI can vary between +1 (for flies with high ΣDesat and low ΣLin) and −1 (for flies with low ΣDesat and high ΣLin). However, as in other studies, 5,9-HD was confounded with methyl-hexacosane (27Br; Coyne *et al.* 1999), and the sum of these two compounds seemed to not vary between mutant and control females.

Molecular characterization: To localize the *P*-element insertion, flanking DNA sequences were determined by sequencing inverse PCR products as described in detail at http://www.fruitfly.org/p_disrupt/inverse_pcr.html. In brief, genomic DNA was prepared from 50 adult flies, purified according to the method described in Sambrook *et al.* (1989), digested with *Sau*3A1 (Promega, Madison, WI), and then ligated under dilute conditions (1/40°) to increase intramolecular ligation. Four oligonucleotide primers internal to the transposon (P1: AGTCGGCAAATATCGCATGCTTGTTC; P2: TGCCGTCACA GATAGATTGGCTTCAG; P3: CCTTAGCATGTCCGTGGGG TTTGAAT; P4: CTTGCCGACGGGACCACCTTATGTTATT; Figure 2) were used with *Taq* polymerase (Eppendorf, Madison, WI). Thermal cycling was: 3 min at 94°; 40 cycles of 94° for 45 sec (denaturation), 60° for 45 sec (reannealing), 72° for 1 min/kb (extension); and a 10-min terminal extension at 72°. PCR products were purified and sequenced bidirectionally (MWG-Biotech, Ebersberg, Germany). To identify the insertion site of the *P* element in $desat1^{1573\text{-}1}$, BLASTN was used to align the sequences of PCR products and the available genomic DNA sequence on the BDGP database (http://flybase.net/blast/).

Characterization of ∼1 kb DNA flanking 5′ and 3′ the insertion of various $desat1^{1573\text{-}exc}$ alleles was carried out by PCR on genomic DNA with the primers P1/ds1.1 (CTTCTTTCG TGCATTTTAACTAAGC) and P4/ds1.2 (GGTTCTTCGTCGT CGGCAAT), respectively. Following amplification, one-fifth of the PCR product was analyzed by electrophoresis on a 1% (w/v) agarose gel containing ethidium bromide.

Total RNA was isolated from frozen adult flies or larvae using Trizol reagent (Invitrogen, San Diego). For RT-PCR, 1 μg of

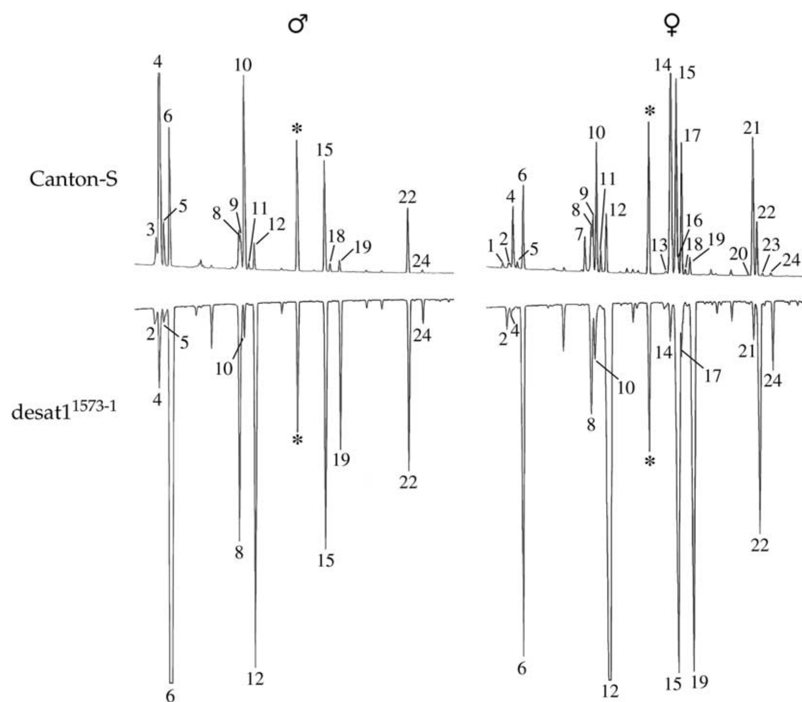

Figure 1.—Representative mirrored gas chromatograms of hexane extracts of individual male and female flies of the control (Canton-S) and mutant desat1$^{1573-1}$ strains. Each peak corresponds to a single hydrocarbon whose identity was previously revealed by mass spectrometry. All identified male and female CH peaks are numbered sequentially by increasing chain length (in italics); key to numbers is as follows. *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-P; (11) 5-P; (12) *n*-pentacosane (25Lin). *27 carbons*, (13) 9,13-HD; (14) 7,11-HD; (15) 2-methylhexacosane (27Br); (16) 5,9-HD; (17) 7-heptacosene (7-H); (18) 5-H; (19) *n*-heptacosane (27Lin). *29 carbons*, (20) 9,13-ND; (21) 7,11-ND; (22) 2-methyloctacosane (29Br); (23) 9-nonacosene (9-N); (24) *n*-nonacosane (29Lin). The chromatograms are to the same scale: they were aligned and calibrated by using an added standard of hexacosane (*).

total RNA was reverse transcribed in 20 μl using SuperScript II RNase H minus reverse transcriptase (from M-MLV of *Escherichia coli*; Invitrogen) in the presence of oligo(dT) primer according to the manufacturer's instructions. Then 1 μl of cDNA was amplified with 2 μm of a primer specific for each transcript (for RA, ds1.A: GCCATCACTAAACCAGGAGAATA; for RC, ds1.C: CGCCACTCCTACACTCAAAAATA; for RE, ds1.E: CAGATACAACATCCTAAACAAATCG; for RB, ds1.B: TAATGGCCCCATCCTGGT; for RD, ds1.D: CGAAACGGCTT GTTAATTTCTAGC) and a common primer in the *desat1* coding region (ds1.4: GGAGAGGGATGGCATAACTACCATC), following the procedure described above (Figure 2). Two primers corresponding to the *desat2* gene, as described in Dallerac *et al.* (2000), were used to detect the *desat2* transcript.

For the real-time PCR assay (Q-PCR), the procedure was similar to that described for RT-PCR except that the reactions were done on 1 μl of cDNA (made from 2 μg of total RNA) with the Quantitect SybrGreen kit (QIAGEN, Chatsworth, CA) with 2 μm of each specific primer (ds1.A-E) combined with ds1.3 primer (TTCGAGTGCGATGTGGAAACCACCG) in a final volume of 25 μl. These primers were designed to produce fragments that were similar in size (212–291 bp) to the *Actin5C* control (215 bp; see below) to quantify them accurately. Cycling conditions were as follows: 15 min at 95°, followed by 40 cycles of 94° for 45 sec, 60° for 30 sec, and 72° for 30 sec. For all reactions, each sample was duplicated on 96-well plates with optical sealing tape, and signals were measured with sequence detector system software (Applied Biosystems, Courtaboeuf, France). All signal thresholds to be compared were standardized with the *Actin 5C* mRNA, using an ABI prism 7700 detector system (Applied Biosystems). For both RT- and Q-PCR assays, the amplification of *Actin 5C* (Act5C.dir: CAGATCA TGTTCGAGACCTTCAA and Act 5C.rev: ATCTTCATCAGG TAGTCGGTCAA) served as a control.

Statistics: The hydrocarbon profiles of various *desat1*$^{1573\text{-}exc}$ excision alleles were compared with an ANOVA, using their DIs. *desat1*$^{1573\text{-}exc}$ alleles were considered as mutant, rescue, or intermediate according to the difference between their DI and the DIs of the mutant *1573-1* and/or of control Cs flies. The levels of *desat1* transcripts were compared between the sexes (with a Mann-Whitney test) and among genotypes (with a Kruskal-Wallis test). Only P-values <0.05 were considered to be statistically significant.

RESULTS

The production of sex pheromones is drastically decreased in mutant flies: We initially analyzed by gas chromatography the CHs in mature *D. melanogaster* female and male flies of >600 PGal4 strains obtained from several labs and found one strain (no. 1573) with defective CH profiles in both sexes. Homozygous mutant flies were perfectly viable and fertile but their production of unsaturated CHs with one and two double bonds (respectively, monoenes and dienes), including the known sex pheromones of control flies, was substantially smaller than that of controls (Figure 1; Table 1). For example, control Cs females produced 675 ng of 7,11-dienes [including the predominant 7,11-HD (peak 14) and 7,11-ND (peak 21)], representing 37.8% of the SumCH, while mutant females produced only 71 ng of 7,11-dienes (2.6% of the SumCH). Mutant females also decreased their levels of all other unsaturated CHs, including 7-T (peak 4), and 7-P (peak 10) if compared to control females (7-T + 7-P = 72 and 165 ng, respectively). The total fraction of unsaturated CHs dropped from 62.7% in control females to 6.2% in mutant females. Mutant males also showed a drastic decrease of their overall level of unsaturated CHs (7.2%) if compared to that of control males (62.7%). In particular, the absolute quantity of 7-monoenes (7-T + 7-P) in control males (512 ng = 52.4%) was reduced in mutant males (157 ng = 5.6%).

Conversely, mutant flies of both sexes showed much higher levels of *n*-alkanes than control flies (Figure 1;

TABLE 1

Amounts of CHs on 5-day-old *D. melanogaster* males and females of the control Cs and mutant (1573) strains

	Peak no.																
	2 23Br	4 7T	6 23Lin	7 7,11PD	8 25Br	9 9P	10 7P	12 25Lin	14 7,11HD	15 27Br	+	16 5,9HD	17 7H	19 27Lin	21 7,11ND	22 29Br	24 29Lin	SumCH
									Cs female									
ng	25 (9)	51 (9)	98 (11)	35 (5)	85 (11)	65 (9)	114 (12)	79 (9)	441 (43)		331 (34)		141 (20)	24 (4)	189 (36)	51 (10)	3 (1)	1788 (181)
%	1.4 (0.6)	3.0 (0.4)	5.5 (0.4)	2.0 (0.2)	4.7 (0.4)	3.6 (0.4)	6.5 (0.5)	4.3 (0.3)	25.2 (1.8)		18.9 (1.2)		7.9 (0.7)	1.3 (0.1)	10.0 (1.1)	2.7 (0.3)	0.2 (0.1)	
									1573 female									
ng	7 (2)	12 (2)	457 (29)	1 (0)	122 (9)	0 (0)	63 (5)	864 (41)	32 (4)		487 (25)		2 (1)	378 (19)	20 (3)	174 (8)	29 (2)	2681 (89)
%	0.3 (0.1)	0.5 (0.1)	17.0 (0.8)	0.0 (0)	4.5 (0.3)	0.0 (0)	2.3 (0.2)	32.1 (0.7)	1.2 (0.1)		18.3 (0.8)		0.1 (0)	14.2 (0.7)	0.8 (0.1)	6.6 (0.4)	1.1 (0.1)	

	3 9T	4 7T	5 5T	6 23Lin	8 25Br	9 9P	10 7P	11 5P	12 25Lin	15 27Br	17 7H	19 27Lin	22 29Br	24 29Lin	SumCH
									Cs male						
ng	33 (6)	376 (50)	33 (5)	136 (18)	47 (12)	27 (8)	136 (12)	4 (1)	23 (3)	113 (14)	4 (1)	8 (1)	39 (5)	2 (0)	982 (81)
%	3.2 (0.3)	37.4 (2.7)	3.3 (0.3)	13.7 (1.1)	4.4 (0.9)	3.0 (0.8)	15.0 (1.9)	0.4 (0.1)	2.4 (0.2)	11.7 (1.1)	0.4 (0.1)	0.9 (0.1)	4.1 (0.4)	0.2 (0.1)	
									1573 male						
ng	18 (3)	132 (13)	21 (2)	1536 (108)	300 (20)	5 (2)	39 (4)	1 (0)	410 (18)	294 (15)	0 (0)	123 (8)	139 (7)	16 (1)	3033 (165)
%	0.6 (0.1)	4.3 (0.4)	0.7 (0.1)	50.0 (1.1)	9.9 (0.4)	0.2 (0.1)	1.3 (0.1)	0.0 (0)	13.7 (0.5)	9.8 (0.4)	0.0 (0)	4.1 (0.3)	4.7 (0.2)	0.5 (0.1)	

Data shown are the mean absolute amount (given in nanograms ±SEM) and the percentage relative to the overall amount of detected CHs (SumCH) for $N = 20$ flies. For the sake of clarity, we show only the most abundant CHs. For the nomenclature and identity of all CHs, refer to Figure 1.

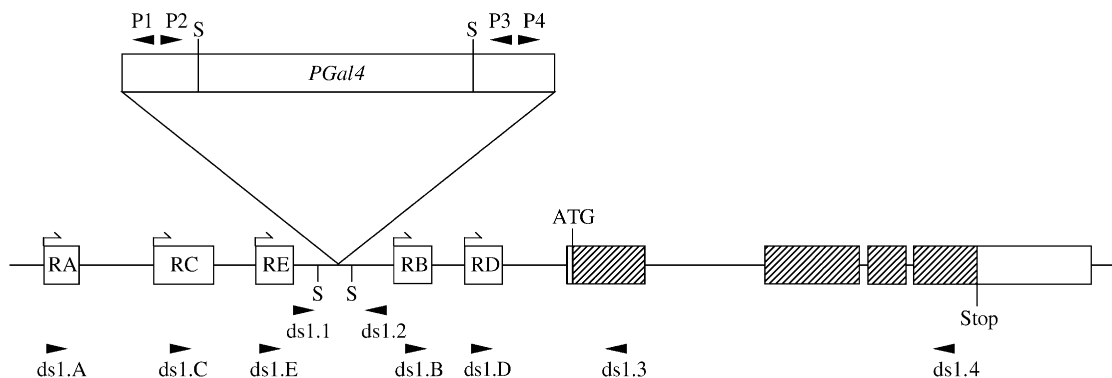

Figure 2.—Schematic of the *desat1*$^{1573\text{-}1}$ locus (chromosome III, $87_{B10\text{-}11}$). The triangle represents the *PGal4* (*PGawB*) transposon, which is inserted in the first intron of the *desat1* gene with its 3′ extremity mapped at −1691 bp from the ATG codon. The five open boxes below the triangle represent the five specific exons for each transcript (RA, RC, RE, RB, and RD), and the hatched boxes represent the translated *desat1* region. The primers used are indicated by solid arrowheads (see materials and methods). Briefly, P1/P2 and P3/P4, respectively, were used to amplify the 5′ and 3′ flanking DNA region after *Sau*3A1 restriction (sites are noted by "S"); ds1.1/P1 and P4/ds1.2 were used to obtain ∼1 kb of 5′ and 3′ flanking DNA; and ds1.A/ds1.3 and ds1.A/ds1.4, respectively, were used to detect the RA transcript in Q-PCR and RT-PCR (and similarly with the four other ds1.C, E, B, and D primers used to detect their respective RC, RE, RB, and RD transcripts).

Table 1). In mutant females and males, the sum of *n*-alkanes (23Lin + 25Lin + 27Lin + 29Lin = ΣLin) represented 60.5% and 67.7% of the detected CHs, respectively (*vs.* 11.1% and 17% in Cs females and males). 25Lin (*n*-pentacosane; peak 12) was the predominant CH of mutant females (31.6% = 837 ng), and 23Lin (*n*-tricosane; peak 6) was the predominant CH (50.2%) of mutant males and was 10 times more abundant (1387 ng) than in Cs males (136 ng). Also, the overall amount of CHs (SumCH) increased highly in mutant males (+176%) and females (+48%) if compared to same-sex control flies (Table 1). This increase was mainly caused by linear alkanes but not by methyl-alkanes, whose relative abundance increased only slightly in mutant females (+2%) and males (+3.9%). The DI (materials and methods), which can oscillate between +1 (for high production of unsaturated CHs) and −1 (for low production of unsaturated CHs) was very different between Cs flies (+0.688 in females and +0.566 in males) and mutant flies (−0.830 and −0.811, respectively).

The *PGal4* mutation induces a quasi-recessive effect on the CH profile because heterozygous mutant flies showed only a moderate variation. The percentage of alkenes decreased by 20–30%, and the percentage of alkanes increased in the same proportion in heterozygous flies, if compared to control flies. Their SumCH was similar to that of control flies (data not shown).

The transposon that decreases pheromonal production is inserted in the *desat1* gene: First, we verified with a Southern blot that only one *PGal4* transposon was inserted in the genome of the 1573-1 enhancer-trap strain (data not shown).

Then the gene altered by the transposon was mapped. After the digestion and circularization of the DNA of the mutant strain, primers were designed to amplify the genomic regions flanking the insertion point (Figure 2; see materials and methods). Two fragments, one in 5′ and the other in 3′ of the transposon, were cloned and sequenced. The comparison with the BDGP database revealed that both fragments share a complete identity with two contiguous sequences of the *desat1* gene, located on chromosome III at $87B_{10\text{-}11}$. *desat1* is a gene that codes for a Δ9 desaturase enzyme involved in setting a double bond on the carbon ω7 of fatty acids, leading to unsaturated CHs like 7-monoenes and 7,11-dienes (Wicker-Thomas *et al.* 1997). Therefore, the altered CH phenotypes shown by homozygous mutant female and male flies are probably the result of a mutation in *desat1*.

The transposon inserted in the *1573-PGal4* allele (now named *desat1*$^{1573\text{-}1}$, or *1573-1*, in this article) was pulled out to create 134 new excision alleles (*1573-exc*). All excision alleles produced homozygous viable female and male flies, with the exception of the *1573-C′1* and *-O7* alleles. We compared the DIs produced by homozygous *1573-exc* flies of both sexes with the DIs of Cs and 1573-1 strains (Table 2). According to this criterion, a phenotype similar to that of wild-type flies was rescued for 110 excision alleles in females (with +0.674 ≥ DI ≥ +0.217) and for 117 alleles in males (+0.730 ≥ DI ≥ +0.296). This rescue demonstrates that the *PGal4* transposon inserted in *desat1* causes the mutant CH phenotype. Fourteen other alleles induced a mutant CH profile in both females (−0.882 ≤ DI ≤ −0.542) and males (−0.894 ≤ DI ≤ −0.711). With DI values significantly different from both control and mutant profiles, 8 alleles were considered as intermediate in females (−0.389 ≤ DI ≤ +0.075), whereas only 1 allele was considered intermediate in males (*H6*; DI = +0.171).

TABLE 2

Principal CH parameters of 5-day-old male and female flies homozygous for various *desat1*[1573-1] alleles and the size of the transposon inserted into *desat1*

		Male				Female				Transposon length (kb)
		DI	ΣDesat (%)	ΣLin (%)	SumCH	DI	ΣDesat (%)	ΣLin (%)	SumCH	
Wild-type:	Cs	+0.566 (0.041)	62.7 (2.2)	17.2 (1.4)	982 (81)	+0.687 (0.017)	61.0 (1.0)	11.3 (0.6)	1788 (181)	
Mutant:	1573	−0.811 (0.010)	7.1 (0.4)	68.5 (0.8)	3033 (165)	−0.844 (0.010)	5.4 (0.3)	64.3 (1.1)	2681 (89)	11.3
	Excision-mutant									
	E1	−0.894 (0.010)	3.9 (0.4)	70.1 (0.6)	2921 (73)	−0.828 (0.020)	5.4 (0.6)	57.5 (1.2)	2023 (120)	6.2
	J2	−0.891 (0.011)	4.0 (0.4)	68.9 (1.0)	2366 (244)	−0.763 (0.013)	7.8 (0.4)	58.4 (0.9)	2282 (70)	8.7
	F3	−0.880 (0.008)	4.2 (0.2)	66.6 (1.4)	2471 (108)	−0.866 (0.003)	4.5 (0.1)	63.0 (0.4)	1804 (251)	9.6
	P3	−0.873 (0.029)	4.7 (1.1)	69.7 (1.7)	2481 (59)	−0.743 (0.012)	9.1 (0.5)	62.0 (0.9)	2029 (57)	10.6
	P′2	−0.872 (0.008)	4.7 (0.3)	69.1 (0.4)	1760 (219)	−0.752 (0.011)	8.4 (0.6)	58.8 (1.0)	2219 (62)	10.3
	C1	−0.863 (0.013)	5.2 (0.5)	70.7 (0.7)	2897 (176)	−0.872 (0.005)	4.6 (0.2)	67.3 (0.2)	2086 (190)	10.6
	C6	−0.862 (0.025)	5.1 (1.1)	68.5 (0.6)	2695 (194)	−0.660 (0.060)	11.6 (2.0)	56.6 (2.6)	2459 (37)	8.1
	C2	−0.855 (0.012)	5.8 (0.4)	73.7 (1.0)	2713 (248)	−0.882 (0.015)	4.2 (0.5)	67.8 (1.0)	1880 (36)	10.6
	C3	−0.849 (0.018)	5.9 (0.6)	72.0 (1.4)	2524 (50)	−0.696 (0.025)	10.3 (0.8)	57.5 (0.8)	2090 (147)	8.1
	A4	−0.844 (0.015)	6.1 (0.5)	72.6 (1.3)	2726 (170)	−0.821 (0.014)	5.9 (0.4)	60.4 (0.8)	2136 (27)	11.0
	D1	−0.824 (0.010)	7.0 (0.3)	72.4 (1.2)	1825 (174)	−0.823 (0.014)	6.3 (0.6)	64.9 (0.4)	2296 (105)	9.7
	C5	−0.817 (0.008)	7.0 (0.4)	69.2 (2.6)	2798 (245)	−0.542 (0.040)	15.9 (1.2)	53.7 (2.1)	2551 (119)	6.9
	P2	−0.779 (0.008)	8.4 (0.3)	68.2 (0.5)	3051 (202)	−0.819 (0.014)	6.3 (0.5)	63.5 (0.9)	2376 (210)	7.7
	Q′1	−0.711 (0.039)	10.9 (1.3)	64.5 (3.2)	2266 (240)	−0.605 (0.006)	13.1 (0.6)	52.0 (1.6)	2122 (135)	6.4
	Excision-intermediate									
	H6	+0.171 (0.030)	41.8 (1.3)	29.5 (1.1)	1278 (101)	−0.112 (0.055)	32.8 (2.0)	41.1 (2.1)	1873 (83)	8.6
	L6	+0.296 (0.026)	51.1 (1.1)	27.8 (1.2)	1508 (98)	+0.053 (0.045)	36.8 (1.6)	33.2 (1.6)	1969 (64)	5.1
	F7	+0.391 (0.070)	48.5 (2.7)	21.1 (2.4)	1467 (67)	−0.389 (0.087)	22.5 (3.2)	52.0 (3.7)	1561 (92)	0.1
	F4	+0.395 (0.032)	55.2 (1.3)	24.0 (1.4)	1327 (59)	−0.089 (0.051)	33.2 (1.6)	40.2 (2.3)	1884 (73)	0.1
	M3	+0.399 (0.097)	56.1 (3.0)	24.0 (2.7)	1452 (97)	−0.070 (0.038)	33.4 (1.1)	38.7 (1.8)	1636 (47)	6.8
	L2	+0.429 (0.018)	52.4 (1.1)	20.9 (0.6)	1373 (52)	+0.073 (0.041)	36.0 (1.3)	31.3 (1.5)	1496 (46)	5.8
	F6	+0.446 (0.066)	59.0 (2.6)	22.9 (2.8)	1690 (93)	−0.048 (0.070)	32.8 (2.2)	36.7 (2.7)	1925 (113)	0.1
	F2	+0.452 (0.025)	56.1 (1.3)	21.1 (0.9)	1434 (76)	+0.075 (0.048)	37.1 (1.7)	32.0 (1.9)	1812 (127)	0.1
	Excision-rescue									
	N2	+0.594 (0.025)	62.1 (0.9)	15.9 (1.1)	1284 (63)	+0.475 (0.047)	50.5 (1.6)	18.0 (1.7)	1604 (67)	0
	L3	+0.644 (0.026)	62.9 (1.0)	13.7 (1.1)	1070 (237)	+0.457 (0.022)	48.6 (1.0)	18.1 (0.8)	1475 (38)	0
	A′2	+0.676 (0.023)	61.8 (1.9)	11.9 (0.8)	1141 (201)	+0.539 (0.016)	52.7 (1.0)	15.8 (0.4)	1437 (57)	0

All CH data are mean (±SEM) for $N = 10$ flies. Excision alleles were classified into three categories according to their DI (see MATERIALS AND METHODS). Within each category, alleles were ranked from top to bottom according to male DIs. In mutant alleles (Excision-mutant) DIs were different from that of the control (Cs) and similar to that of mutant *1573-1*. DIs of rescued alleles (Excision-rescue) were similar to that of Cs and different from that of *1573-1*. DIs of intermediate alleles (Excision-intermediate) were different from the DIs of both Cs and *1573-1*, either for both sexes (*H6*) or for only females (*L6–F2*). ΣDesat (%) corresponds to the total percentage of all unsaturated CHs relative to the sum of detected CHs (SumCH, given in nanograms); ΣLin (%) corresponds to the total percentage of all saturated linear CHs. The transposon length (shown at the right) indicates the estimated size (in kilobases) of the fragment of the transposon that remains inserted in each allele. This estimation corresponds to the pooled size of three sequences detected by Southern blots.

The total amount of unsaturated CHs (ΣDesat) was much lower in mutant alleles (180 ng for females and 149 ng for males) than in rescued alleles (758 and 678 ng, respectively). Conversely, mutant alleles showed a much higher sum of linear saturated CHs (ΣLin; 1304 and 1778 ng in females and males) than rescued alleles (161 and 235 ng). The SumCH also increased in mutant alleles (2168 and 2535 ng) if compared to rescued alleles (1440 and 1116 ng). The intermediate alleles showed CH levels between those of the control Cs and the mutant 1573-1 strains.

Relationship between the molecular structure of *desat1* and the CH phenotype: We looked for a relationship between the molecular structure of the *desat1* locus and the CH profile variation. First, the flanking DNA 5′ and 3′ of the insertion site was amplified with primers designed within the transposon and at 1 kb of the insertion site. This study, carried out with all alleles, revealed no alteration of the *desat1* gene, with the exception of the *C′1* lethal allele (see below).

When a part of the transposon remained inserted, both the size and the nature of the fragment were determined. For all alleles, each of the three principal sequences (*Gal4*, *miniwhite*, and *pBSK*) forming the transposon was probed with Southern blotting and its size was evaluated by comparison with the complete

sequence detected in the 1573-PGal4 strain (data not shown). Then the overall size of the inserted fragment was estimated after pooling the size of the remaining sequences corresponding to the three fragments (Table 2; right column).

In mutant alleles, the size of transposon varied between 11 and 6.2 kb. In intermediate alleles, some fragments were relatively large (5.1–8.6 kb, the largest in *H6*), whereas others were as small as 155 bp in *F2*, *F4*, *F6*, and *F7* (the sequenced fragment was strictly identical in these 4 alleles and corresponded to the feet of the transposon). In most rescued alleles (91), no trace of the transposon was detected, and at least six alleles that were sequenced showed a precise excision. Among these precisely excised alleles, *N2* was used as a control in subsequent experiments. In other rescued alleles, a fragment of a significant size remained inserted and either was ≤2 kb (in 14 alleles) or varied between 3 and 6.3 kb (in 9 alleles). No relationship was found between the gravity of the CH phenotype and the nature of the molecular sequence(s) of the transposon fragment that remained in *desat1* (data not shown).

The level of *desat1* transcripts can vary between genotypes and species: On the basis of its sequence, *desat1* was predicted to code for five transcripts, each one with a specific region in the 5′-UTR of *desat1* and a common coding region (Figure 2). We first detected these five transcripts with RT-PCR and compared the transcription profile of females and males in the wild-type Cs strain, in homozygotes for the clean excision *1573-N2* allele, and in the mutant *1573-1* allele. The presence and the relative abundance of the five transcripts were measured at four developmental stages: in first and third instar larvae (L1, L3) and in 1-day- and 5-day-old adults (A1, A5; Figure 3).

In Cs and N2 flies of both sexes, four of the five transcripts ("RA," "RB," "RC," "RD") were present from larval to adult development whereas "RE" appeared during metamorphosis. "RE" seems to be more abundant in adult males than in females. The *1573-1* mutation drastically affected most transcripts in both sexes: "RA" and "RE" were never detected, whereas the signal corresponding to "RC" largely decreased. On the other hand, "RB" and "RD" showed no apparent change in larvae and adults of both sexes, if compared to both control strains.

Interestingly, "RA" and "RB" transcripts were never detected in *D. simulans* females and males whereas the pattern of the other three transcripts resembled that of control *D. melanogaster*: "RC" was apparently more abundant than "RE" and "RD"; "RE" was apparently higher in adult males than in females. The *desat2* transcript, which was never detected in *D. melanogaster*, was present in *D. simulans* adults of both sexes, with apparently higher levels in males.

Using Q-PCR, we precisely quantified and compared the level of the five *desat1* transcripts between 24-hr-old *D. melanogaster* adults (Table 3). This developmental period was chosen because it is critical for the differentiation of sex pheromones (Wicker and Jallon 1995; Ferveur *et al.* 1997; Savarit and Ferveur 2002a). "RD" showed the lowest and most constant level in all samples and was chosen as the reference (= 1) to estimate the variation of the four other transcripts. In the two control strains, "RC" was the most abundant transcript and represented 93–97% of the total amount detected, but showed no sexual difference. The quantity of "RC" was drastically reduced in *1573-1* flies and dropped to <1/2000th of its control value. "RA" and "RB" were more abundant in females than in sibling males, whereas "RE," which was rare, was only slightly more abundant in *N2* males. "RA," which represented 2–5% of the total transcript in the two control strains, was also drastically reduced in *1573-1* flies (22–59 times less abundant than "RD"). On the other hand, "RB," which dropped to only one-half to one-fourth of its control level, became the predominant transcript (82–87%) in mutant flies. "RE" was never detected in *1573-1* flies. It should be noted that the three most affected transcripts ("RA," "RC," and "RE") are located upstream of the transposon.

Characterization of *C′1*, a *desat1* null allele: Our data suggest that *1573-C′1* is a *desat1* null allele. *C′1* retained roughly the 5′ part of the transposon with its flanking region, but showed a complete deletion of the 3′ region of the DNA region encompassing all the coding region of *desat1*. Most *C′1/C′1* individuals died during their second larval instar stage (L2), remaining up to 5 days in L2 without being able to moult into L3 (the L2 stage normally lasts 24 hr). We chose to detect and compare the transcriptional pattern of L1 (with both sexes pooled) because the duration of the L1 stage, and the frequency of L1-to-L2 moult were similar in *C′1* and control genotypes. With RT-PCR, control L1 showed all transcripts (except "RE"), whereas no transcript was detected in *C′1* (Figure 3B). This result suggests that *C′1* is a null allele. This hypothesis is supported by the fact that *C′1* did not complement the defective CH phenotype induced by mutant and intermediate excision alleles (data not shown).

Four chromosomal deficiencies that theoretically partly or totally uncover the *desat1* gene were tested in complementation with the *C′1* null allele with regard to viability, CH phenotype, and transcription profile. These experiments yielded varying results: when combined with *C′1*, the three deficiencies *T32*, *KarH10*, and *P21*, but not *ry615*, could complement both the developmental viability and the CH phenotype. However, the *desat1* transcription pattern that was determined either in adults (for the three former genotypes) or in L1 (for *ry615/C′1* that induced lethality between late embryogenesis and L2 stage) was similar to that of the control strains. This indicates that none of these deficiencies completely delete the *desat1* gene and are therefore not appropriate to assess whether *desat1* is an essential gene required for larval development.

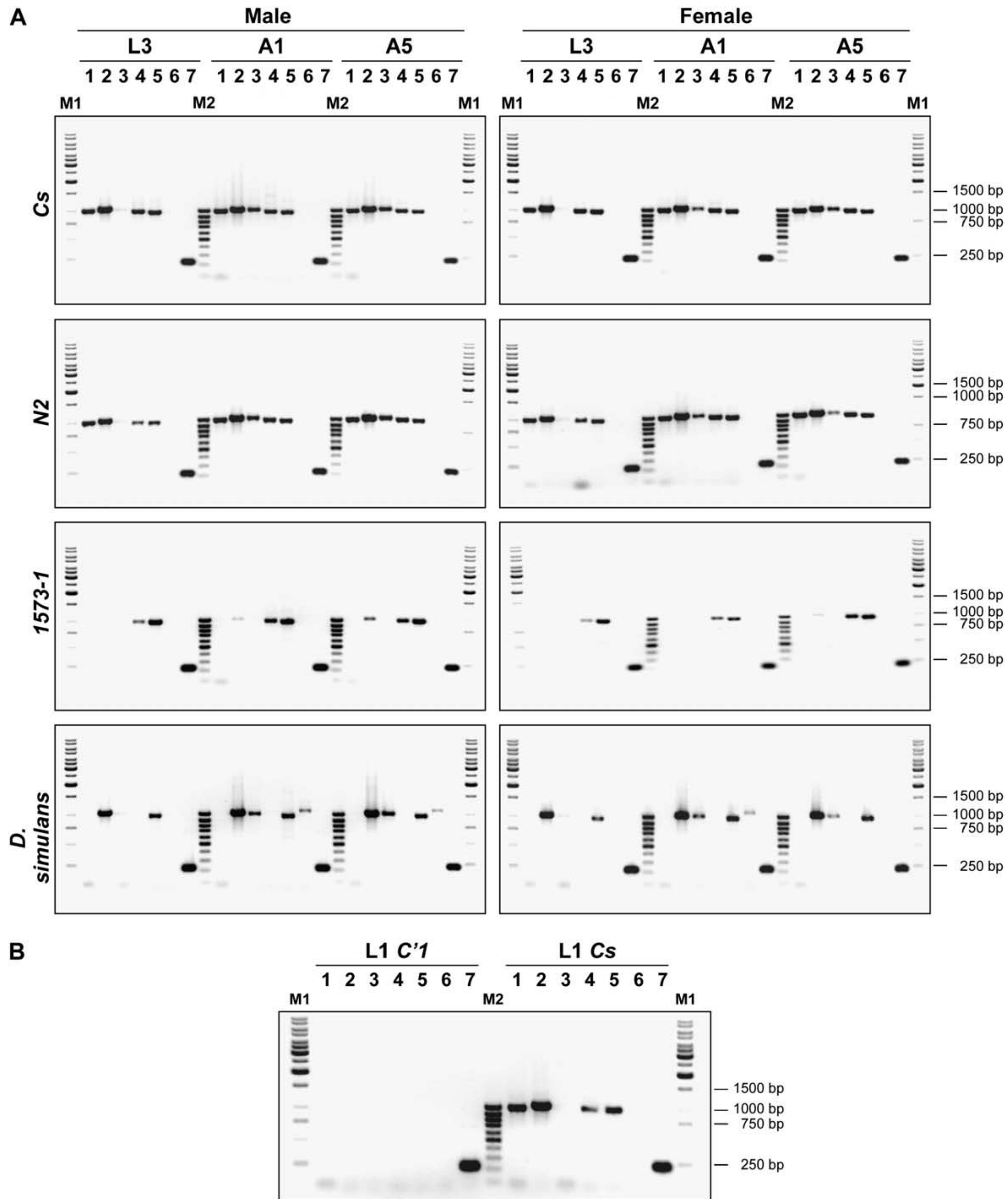

FIGURE 3.—Comparison of the *desat1* transcription pattern among sexes, genotypes, and species during development. Each transcript was detected by RT-PCR with the primers described in Figure 2. (A) The comparison was carried out between males and females at the following stages: third instar larva (L3), 24-hr-old adult (A1), and 5-day-old adult (A5). (B) Both sexes were pooled in first instar larva (L1). For *D. melanogaster*, the following genotypes were compared: wild-type Cs and homozygotes for the *1573-N2* rescued excision allele (*N2*), the *1573-1* mutant allele (*1573-1*), or the *C′1* null allele (C′1). *D. simulans* is a wild-type strain. Five *desat1* transcripts were detected: RA (1), RC (2), RE (3), RB (4), and RD (5). The *desat2* transcript (6) and the *Actin5C* transcript (7) were used as internal markers. M1 and M2 are the molecular weight markers (MBI, Fermentas; 1 kb and 100 bp, respectively). The approximate size of the fragments is indicated on the right.

C′1 could also complement adult viability and the CH phenotype of *P(w) l(3) S028813*, another lethal mutation associated with *desat1* (and inducing a mild effect on CHs; LABEUR *et al.* 2002), indicating that both the lethality and the pheromonal defect associated with this allele were perhaps not directly caused by *desat1* (data not shown). We did not study the second lethal allele (*1573-O7*) in detail because it complemented the CH phenotype of all mutant *desat1* excision alleles tested (data not shown).

TABLE 3

Comparison of the quantity of five *desat1* transcripts in 24-hr-old male and female flies of various genotypes

Transcript	Genotype	Female		Male		Quantity
		a.u.	%	a.u.	%	
	Canton-S	275 (41)	5.58	117 (5)	2.63	0.001
RA	N2	232 (27)	2.83	133 (10)	1.78	0.001
	1573	−22 (5)	0.17	−59 (11)	0.18	0.017
		0.001		0.005		
	Canton-S	4587 (82)	93.20	4292 (579)	96.36	0.248
RC	N2	8085 (637)	96.04	7289 (1226)	97.55	1
	1573	2.4 (0.2)	8.87	0.7 (0.6)	7.44	0.021
		0.007		0.012		
	Canton-S	8 (2)	0.16	12 (3)	0.28	0.248
RE	N2	9 (1)	0.11	16 (1)	0.22	0.021
	1573	0 (0)	0	0 (0)	0	1
		0.021		0.020		
	Canton-S	51 (7)	1.04	32 (4)	0.71	0.043
RB	N2	85 (7)	1.01	33 (1)	0.44	0.021
	1573	23 (2)	87.19	7.8 (0.8)	81.88	0.021
		0.007		0.023		
	Canton-S	1	0.02	1	0.02	
RD	N2	1	0.01	1	0.01	
	1573	1	3.77	1	10.51	

The quantity of each transcript was evaluated in arbitrary units (a.u.) with the amount of "RD" transcript chosen as the reference unit. Data shown correspond to the mean (±SEM) of a duplicated quantification carried out with two independent extractions ($N = 2 + 2$). Nonparametrical statistics allow us to compare the genotypes (with a Kruskal-Wallis test) and the sexes (with a Mann-Whitney test, except for "RD," which was constant). The percentage indicates the representativeness of each transcript relative to the total amount of detected transcripts in each sex and genotype. The negative value obtained in *1573-1* flies indicates that the quantity of "RA" was a fraction (1/22 in females, 1/59 in males) of 1 a.u

DISCUSSION

Our data indicate that the *PGal4* transposon inserted in the regulatory region of *desat1* acts recessively to strongly decrease the production of unsaturated CHs of mature Drosophila flies. Precise removal of the transposon rescued a wild-type CH profile. Incomplete excision of the transposon-induced new alleles with a CH phenotype whose gravity was generally related to the size of the fragment inserted in *desat1*. The *desat1* coding region was completely deleted in the *C′1* null allele.

Genetic and molecular basis of sexually dimorphic CHs: A set of sexually dimorphic characters was highly affected by the mutation. Does the dimorphism of *D. melanogaster* sex pheromones rely upon only a single desaturase enzyme? Figure 4 shows a simplified representation of the biosynthetic pathway leading to the production of CHs in Cs and Tai females and males together with the possible effect of the *1573-1* mutation in both sexes. With our data, we cannot assess whether the sexual difference for the number of double bonds can be induced by only a single desaturase (coded by *desat1*) or if the second double bond on carbon ω11, found only in females, is specifically processed by a second (as yet unknown) desaturase. The former hypothesis seems more probable: no CHs with a single double bond on carbon ω11 were detected in our mutant females. Furthermore, Desat1 can process two successive desaturation steps in the silkmoth *Bombyx mori* (Moto *et al.* 2004). In this case, *desat2*, which is active only in Tai-like females, would also create two double bonds successively on ω5 and on ω9 (Figure 4).

The moderate sexual dimorphism for CHs that persisted between mutant flies was mostly based on the variation of the ratio between *n*-tricosane (23Lin) and *n*-pentacosane (25Lin), which were predominant in mutant males and females, respectively. This quantitative difference suggests that a chain-lengthening enzyme, probably an elongase adding two carbons, is more active in mutant females. This situation contrasts with the sexual difference shown by Cs female flies where two elongation steps (2 + 2 carbons) occur together with two desaturation steps that are additively processed on C7 and on C11. Therefore, our data suggest that the activity of Desat1 is coupled with that of an elongase because both enzymes apparently decreased their activity in mutant *desat1* females. In *Musca domestica*, the sex specificity of pheromones depends largely upon the elongation of the carbon chain in male predominant CH (27C; Vaz *et al.* 1989). The analogous involvement

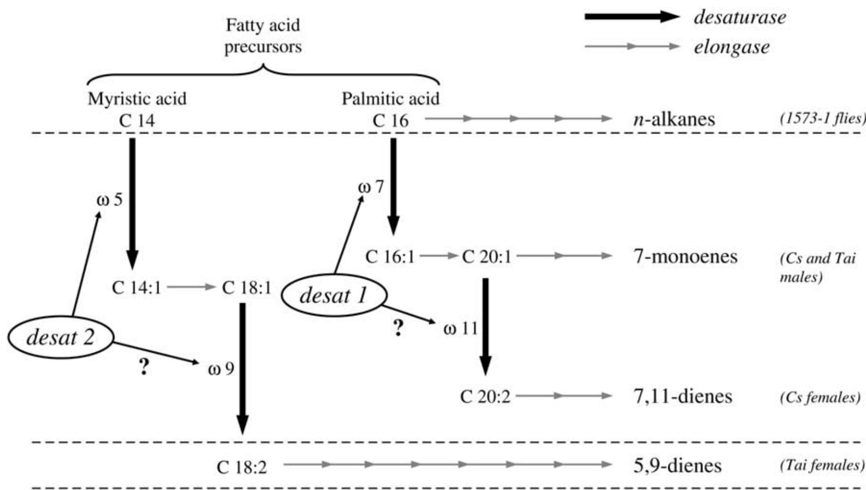

FIGURE 4.—Biosynthetic pathway of pheromones in *D. melanogaster* flies of different genotypes. The two genes *desat1* and *desat2* code for two desaturases with different substrate specificity (palmitate and myristate, respectively) that introduce a first double bond on the carbon ω7 (*desat1*) or on the carbon ω5 (*desat2*; adapted from JALLON and WICKER-THOMAS 2003). Hypothetically, the second double bond (on carbon ω11 for Cs females and on carbon ω9 for Tai females) could be processed by the same Desat enzyme on mono-unsaturated precursors (C20:1 for *desat1* and C18:1 for *desat2*). Subsequently, saturated and unsaturated fatty acid precursors are processed by several elongation steps coupled with a final decarboxylation step (not shown here), leading to the production of mature cuticular hydrocarbons. The *desat2* gene, which normally induces the production of 5,9 dienes in Tai-like females, is defective in Cs flies, leading to the predominant production of 7,11 dienes and of 7-monoenes by females and males, respectively. The $desat1^{1573\text{-}1}$ mutation largely reduces the levels of these unsaturated hydrocarbons and largely increases the levels of *n*-alkanes in homozygous mutant flies (1573-1). The solid and the shaded arrows represent the desaturation and the elongation steps, respectively (the number of shaded arrows is not indicative of the number of elongation reactions).

of an elongase on the sexual dimorphism of sex pheromones in the two dipteran species indicates a possible conservation of some of the biosynthetic mechanisms related to pheromonal communication.

Our data reveal two other sex differences: mutant alleles generally induced a higher increase of SumCH in males, whereas intermediate alleles more frequently affected the female's CH profile. We previously hypothesized that *desat1* interacts with sex determination genes because the ectopic expression of the dominant feminizing Tra^F factor, driven by the $desat1^{1573\text{-}1}$ *PGal4* enhancer-trap line, highly masculinized the predominant CHs of XX flies (SAVARIT and FERVEUR 2002b). It is possible that the overexpression of Tra^F in the oenocytes and in the fat body affects target genes that interact with *desat1*. One of the best Tra^F-dependent candidate genes is *doublesex*, the manipulation of which affects sex-specific CHs (WATERBURY *et al.* 1999).

Which *desat1* transcript(s) could be involved in the sex pheromone difference of control *D. melanogaster* flies? Among the five detected transcripts that were precisely quantified at the developmental period critical for the differentiation of sex pheromones (24-hr-old adults; WICKER and JALLON 1995; FERVEUR *et al.* 1997; SAVARIT and FERVEUR 2002a), only "RA" and "RB" showed a significant difference between the sexes. "RE" also showed a slight sexual quantitative difference during early adult development, but it is probably not involved because it was completely absent of mutant flies, which still produced a residual amount of sex-specific CHs. Conversely, "RA" and to a lesser extent "RB," were decreased in mutant flies, but both these transcripts still showed a significant quantitative sexual difference. This indicates that "RA" and/or "RB" could induce the sex specificity of *D. melanogaster* pheromones. This hypothesis is supported by our findings that neither "RA" nor "RB" was detected in the monomorphic species *D. simulans*. Nevertheless, our data cannot explain how transcripts could be significantly more abundant in 3-day-old females than in males (WICKER-THOMAS *et al.* 1997), given that "RC," which is by far the most abundant transcript (>93%) in 24-hr-old adults, showed no sex difference. This discrepancy suggests that "RC" could increase faster in females than in males in the first 3 days of adult life.

Pleiotropic functions of *desat1*: Although *desat1* and *desat2* genes show strong homology in their coding region (KNIPPLE *et al.* 2002), this does not mean that they have the same function. The fact that *desat2* is functional only in Tai-like females, whereas *desat1* is functional in all wild-type *D. melanogaster* flies, suggests that *desat1* is an essential gene for this species, whereas *desat2* is a variant found in some geographic strains, perhaps linked to a case of incipient speciation (TAKAHASHI *et al.* 2001; TING *et al.* 2001; FANG *et al.* 2002). The importance of *desat1* could be explained by its multiple functions (and transcripts), some of which could be indispensable, unlike *desat2* with its simpler regulation and only one predicted transcript.

The *desat2* gene has pleiotropic effects and can change reproductive characters and ecologically adaptative features, including resistance to desiccation (GREENBERG *et al.* 2003). Our study suggests that *desat1* also has a pleiotropic activity. Apart from the processing of sex pheromones, this gene could also be involved in other aspects of biosynthesis such as changing the overall level of CHs (SumCH), which may be involved in physiological or ecological characters. It is also possible that the *desat1* mutation increases the availability of fatty acid precursors or changes the turnover of alkanes that

would accumulate faster or remain longer on the fly cuticle than alkenes. The altered desaturase, or some side effects of the transposon, could also interfere with another, as yet unknown, metabolic pathway. Alternatively, the increased SumCH of mutant alleles could indicate that more fatty acid precursors are necessary to process alkenes than alkanes.

We do not know yet whether Desat1 is necessary for larval development. If this is the case, "RC" and/or "RD" should be necessary for larval survival because they were absent in lethal *C′1* larvae and present in viable *1573-1* mutants. On the contrary, "RA," "RB," and "RE" should not be required for viability because their absence in *1573-1* or in *D. simulans* flies had no visible consequence on viability. However, a precise chromosomal deficiency that exclusively and completely deletes *desat1* needs to be generated to verify that this gene codes for an essential function required for larval development.

Finally, the sexually dimorphic CHs that are altered by the $desat1^{1573\text{-}1}$ mutation are secondary sexual characters that are involved in mate choice and can affect the sex ratio of the progeny (Marcillac and Ferveur 2004). This indicates that the *desat1* gene could regulate some aspects of the conflict between the sexes (Chapman *et al.* 1995; Rice 1996). The fact that *D. melanogaster* female pheromones efficiently prevent interspecific courtship and mating (Coyne *et al.* 1994; Coyne and Oyama 1995; Savarit *et al.* 1999) emphasizes the role of *desat1* in reinforcing the mechanisms of sexual isolation. By combining molecular, genetic, biochemical, and behavioral approaches, we hope to better understand how *desat1* can be related to the evolution of pheromonal communication in Drosophila.

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