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Female pheromones in *Drosophila melanogaster* are controlled by the doublesex locus

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Summary

XY and *XX doublesex D. melanogaster*, expressing variable intersexual phenotypes, were compared for their pheromone levels (e.g. 7, 11-heptacosadiene, the main excitatory pheromone of females, and vaccenyl acetate, an inhibitory compound produced only by males). Despite the intersexual phenotype and the presence of female traits, the pheromone patterns of the homozygous *dsx* mutants, *XY* as well as *XX*, were similar to those of heterozygous males. Female-specific dienes were never found in significant amounts in such flies, which often showed significant amounts of the male-specific acetate and triggered very reduced levels of male courtship wing vibration.

1. Introduction

The pheromonal system of mature *Drosphila melanogaster* flies consists of at least two sets of sex-specific molecules: (1) excitatory long chain dienes, especially 7, 11-heptacosadiene, produced by females and displayed on the surface of their cuticle; other excitatory molecules, chains with 27 ± 2 carbons, especially 7-pentacosene, are produced in lower amounts by both sexes (Antony & Jallon, 1982); (2) an inhibitory long-chain acetate, vaccenyl acetate, produced only by males (Jallon, Antony & Benamar, 1981).

7-tricosene, an aphrodisiac for males of the sibling species *D. simulans*, is found in much larger amounts in *D. melanogaster* males than in *D. melanogaster* females (Jallon, 1984).

The cuticular hydrocarbons of all adults about two days after eclosion replace a singular set of heavier hydrocarbons (31–37 carbons), including dienes, especially with 33 carbons, common to immature flies of either sex which moreover lack vaccenyl acetate (Antony & Jallon, 1981; Pechiné, Antony & Jallon, 1987).

In *Drosophila*, the sex of somatic and germ-line cells is determined by the *X* chromosome/autosome ratio (Bridges, 1925). This chromosomal signal seems to regulate a specific gene *sex-lethal* which itself controls a series of genes involved in somatic sexual differentiation (Cline, 1984): transformer (*tra* and *tra-2*), intersex (*ix*) and doublesex (*dsx*). While mutations in *tra-2* and *tra* transform females into phenotypic males, mutation at *dsx* results in intersexual development (Sturtevant, 1945; Watanabe, 1975; Hildreth, 1965; Kroeger, 1959; Baker & Belote, 1983). In individuals homozygous for *dsx*, with which this report is concerned, imago phenotypes of both chromosomal sexes are morphologically intermediate between those of males and females (Hildreth, 1965). The extreme classes (phenotypic males or females) were never found, but male-like phenotypes were more frequent in *XX*; *dsx*/*dsx* flies than female-like phenotypes in *XY*; *dsx*/*dsx* flies (Orssaud & Laugé, 1981). Except for a few clear cases of ovarial development the gonads were always small and most of the time ovoid. The 'atypical' gonads (Hildreth, 1965) were interpreted on histological grounds as either ovaries, testes, or gonads the sex of which could not be established (Orssaud & Laugé, 1982). A parallel investigation of external phenotypes, gonads and pheromones in such intersexes might reveal some relation between them. Further, we wished to understand how sex-controlling genes such as *dsx* affect the sex-specific pheromonal molecules and their control of male pre-copulatory behavior.

While the synthesis of vaccenyl acetate is clearly linked to the ejaculatory bulb (Butterworth, 1969), the site of production of cuticular hydrocarbons is less well known. In other Diptera they may be synthesized by epidermal cells or oenocytes (Dillwith & Blomquist, 1982; Ismail & Zacchary, 1984). Indirect evidence in *Drosophila* comes from sex mosaics whose attractivity has been studied by various authors (Nissani, 1977; Hall, 1978; Cook, 1978; Jallon & Hotta, 1979). Such

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data were used to localize an internal sex-appeal focus on the fate map in the ventroposterior region of the blastoderm (Jallon & Hotta, 1979). The behaviour of flies bearing mutant genes affecting the sex programme has been studied by McRobert & Tompkins (1985) and Jallon *et al.* (1986).

2. Material and Methods

A stock of y/B^s Y ;p^p *dsx*/TM6 was obtained from R. Nöthiger, Zoologisches Institut der Universität Zurich; the Canton S stock was from J.-M. Jallon's collection. All flies were raised at 24 °C on a corn-meal–agar medium.

A group of 76 intersexes, five days old, were selected under the dissecting microscope and classified according to their external phenotype (morphology of the last abdominal segments and external genitalia). Cuticular hydrocarbon composition was studied by making micro-extracts of each intersex according to the method described by Antony & Jallon (1982) with the following modification: each fly was bathed for 5 minutes in 50 μl hexane, then shaken for one minute. The solvent was evaporated under nitrogen and the residue dissolved in 20 μl hexane; 5 μl were injected into the gas chromatograph. Chromatographic conditions were identical to those described by Antony & Jallon (1982). Each chromatogram was analysed individually; as absolute numbers were very variable, propoprtions for every peak were calculated, then the data for all individuals belonging to the same category were compared and averaged. The gonads of each fly whose cuticle has been washed and analysed were then histologically examined.

For vaccenyl acetate analysis, 50 μl ether per fly was used instead of hexane for extraction: even for mature Canton S males, ether led to more reproducible and higher values. Apart from the change of solvent, each fly was treated in the same way as above. Student's *t* test was used to compare mean cuticular compositions.

The courtship behaviour of Canton S tester males was observed in the presence of *doublesex* homozygous or heterozygous flies in the conditions defined by Jallon & Hotta (1979). The sex-appeal parameter was calculated by measuring the cumulative wing vibration time of a tester male towards a courted object.

3. Results

We first investigated whether a relation exists between pheromones and morphological phenotype. *XY* ; *dsx*/*dsx* and *XX* ; *dsx*/*dsx* intersexes may be grouped into four categories: (1) *XY* flies with more male-like genital arches; (2) *XY* flies with less male-like genital arches; (3) *XX* flies more male-like; (4) *XX* flies less male-like. These categories correspond to Orssaud & Laugé's classes III, IV and V, VI, II and III, respectively (Orssaud & Laugé, 1981).

Extracts from intersexes and heterozygous *dsx*/+ males of the same age show a set of 11–12 peaks, which co-migrate with the largest peaks characterized as hydrocarbons in extracts of Canton S wild-type males. These substances are both alkanes and monoenes, with chain lengths of between 23 and 29 carbons (Antony & Jallon, 1982). We compared the cuticular hydrocarbon compositions of intersexes belonging to each category with those of heterozygous *dsx*/+ individuals, either males of females. There are no significant qualitative or quantitative differences between homozygous individuals belonging to the four categories: they are all similar to heterozygous males (Table 1). The largest peak is always 7-tricosene. The amount of 7-tricosene is relatively low and the amount of 7-pentacosene is relatively high in both intersexes and heterozygous males (35·0 % and 33·4 % respectively for 7-tricosene, 15·2 % and 20·3 % respectively for 7-pentacosene) in comparison with Canton S wild-type males (47·7 % for 7-tricosene and 10·8 % for 7-pentacosene, Jallon, 1984).

Cuticular extracts from intersexes are very different from those of heterozygous and Canton S wild-type females. Female-specific dienes were not present in significant amounts. We occasionally found traces of heptacosadiene, the major compound of female cuticle (on average 30·0 % in *dsx*/+, 23·0 % in

Table 1. *Comparison of main cuticular unsaturated hydrocarbon compositions in heterozygous and homozygous* doublesex *flies. Classification according to Orssaud & Laugé (1981) – see text – for intersexes, and Antony & Jallon (1982) for hydrocarbons.* ± *figures refer to standard deviations*

		XX; *dsx*/*dsx*		*XY* ; *dsx*/*dsx*		
Number of flies...	*XX* ; *dsx*/+	More ♂-like	Less ♂-like	More ♂-like	Less ♂-like	*XY* ; *dsx*/+
Total % hydrocarbons	10	9	24	10	33	10
7-tricosene	2·2 ± 0·4	40·1 ± 7·2	30·0 ± 4·3	41·8 ± 7·9	35·1 ± 3·0	33·4 ± 6·4
7, 11-pentacosadiene	4·5 ± 1·3	Trace	Trace	Trace	Trace	—
7-pentacosene	4·4 ± 0·6	14·4 ± 5·5	14·8 ± 3·0	13·6 ± 3·1	16·2 ± 2·3	20·3 ± 4·8
7, 11-heptacosadiene	30·2 ± 2·9	—	—	—	—	—
7-heptacosene	2·4 ± 0·4	—	—	—	—	1·7 ± 0·8
7, 11-nonacosadiene	6·5 ± 1·5	—	—	—	—	—

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Table 2. *Comparison of monoene and alkane contents of all* doublesex *intersexes with those having one or two ovaries and one or two testes*

Number of flies...	All intersexes	Intersexes with 1 or 2 ovaries	Intersexes with 1 or 2 testes
Total % hydrocarbons	76	15[a]	40[a]
7-pentacosene	15·2 ± 3·0	18·9 ± 7·7	15·2 ± 2·2
Total monoenes	61·7 ± 2·4	62·8 ± 4·8	64·1 ± 2·6
Alkanes	37·6 ± 2·4	36·1 ± 4·8	35·4 ± 2·5

[a] 25 flies, which had gonads whose sex could not be identified, were not included in these two columns. 4 flies were considered 2 times, see text.

Canton S females); traces of pentacosadiene were detected more frequently. Moreover, intersexes and males are richer than females in monoenes (61·7%, 64·7% and 15·0%, respectively). A male-like pattern of cuticular hydrocarbons is therefore present in both chromosomal intersexes. However, young emerging homozygous *dsx/dsx* flies of either chromosomal sex show a similar singular pattern of heavier chains with dienes to that observed in heterozygous *dsx/+* immature females and males and in Canton S young flies (Jallon, 1984).

We next investigated a possible relation between pheromones and gonads. After the histological analysis of the internal organs of the same set of intersexes, we studied only those which had one or two testes and one or two ovaries. The sex of the gonads were inferred from the structure of the mesodermal tissues (mainly sheaths of gonads and cyst cells (Orssaud & Laugé, 1982). Germ cells were either present or absent (agametic gonads). Four intersexes that had one ovary and one testis were considered in both classes.

Hydrocarbons were compared for each subset and for the whole set of intersexes. Table 2 presents only total compositions for either monoenes or alkanes. There is no significant correlation between any class of constituents and gonad type. There is a tendency for intersexes with one or two ovaries to have slightly less 7-tricosene and slightly more 7-pentacosene. However, even in intersexes with two ovaries, excitatory dienes were absent. These results are in good agreement with those of gonadic mutant studies reported by Jallon *et al.* (1986).

We then studied the male-specific inhibitory molecule, vaccenyl acetate, using a new set of flies of each genotype. As in the Canton S strain, vaccenyl acetate was observed in all mature heterozygous males (*XY*, *dsx/+*) but in no mature heterozygous female (*XX*, *dsx/+*) (Table 3). However it was less abundant than in Canton S mature males, suggesting a strain variability such as has recently been reported (Van der Meer *et al.* 1986). Vaccenyl acetate was also detected in many intersexes, more in *XX*; *dsx/dsx* than in *XY*; *dsx/dsx*. Two parameters–the percentage of flies which have vaccenyl acetate and the average extracted quantity–reflect this tendency (Table 3). Quantities extracted from *dsx* homozygous chomosomal males and females were significantly different ($P < 0{\cdot}02$), which surprisingly was not the case between *XX*; *dsx/dsx* and *XY*; *dsx/+*. Vaccenyl acetate, however, was absent in both heterozygous males and intersexes a few hours after eclosion. It should be remembered that most intersexes, *XX* as well as *XY*, show parts of an ejaculatory bulb, completely or incompletely developed (Orssaud, Laugé & Jallon, 1985). A precise histological analysis was, however, very difficult after the solvent washing necessary for the chemical analysis.

After investigating the cuticular composition of *doublesex* intersexes we tested their courtship-inducing abilities with a third group of flies. Whatever their

Table 3. *Comparison of vaccenyl-acetate levels after ether extraction between* dsx/dsx *intersexes of either chromosomal sex and* dsx/+ *flies* XX *or* XY

	dsx/+	*dsx/dsx* intersexes		*dsx/+*
	XY	*XY*	*XX*	*XX*
Number of flies	14	23	11	10
Percentage of flies with vaccenyl acetate	100	43	73	0
Extracted quantity[a]	4789 ± 1534	431 ± 376	3212 ± 3022	—

[a] In arbitrary units.

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Table 4. *Sex-appeal (SAP) of* dsx/dsx *intersexes and controls (heterozygous flies)*

Genotype	*N*	SAP (s)
dsx/+ XX	30	118·7±35·7
dsx/dsx XX	53	3·8±2·7
dsx/dsx XY	32	4·8±4·0
dxs/+ XY	13	0·2±0·3

chromosomal sex, many of these flies (40/85), which were rather sluggish, triggered some early courtship reactions from Canton S tester males, especially following orientation and brief wing vibrations. Sustained vibrations were observed in only 10 cases, and attempted copulation only 6 times during 85 5-minute tests. Sex-appeal parameter values (Jallon & Hotta, 1979) are compared in Table 4 for *doublesex* homozygous intersexes of each chromosomal sex with those of either heterozygous females or males. Among intersexes they were not significantly different for chromosomal males or for chromosomal females, but in both cases were signficantly higher than for heterozygous males. Moreover, they were only about 5% of the average value for heterozygous females.

4. Discussion

Before considering the genetic aspects of a possible control by the *dsx* locus of the biosynthesis of sex-specific molecules, we will relate the sexual behaviour of the mutants with the chemical data. It should be noted that we cannot exclude that *dsx* may exert its weak effect on elicitation of courtship through effects on cues other than chemical ones. Earlier studies have established that male wing vibration is mainly triggered by female cuticular compounds possessing certain structural characteristics (chain with 27±2 carbons, at least one double bond in position 7); among these substances 7, 11-heptacosadiene is the most efficient (Antony *et al.* 1985). This result is supported by data presented here: the sex-apeal of all intersexes which lack significant amounts of potentially aphrodisiac molecules (especially 7, 11-dienes) is low compared with that of heterozygous females. This result is somewhat different from that reported by McRobert & Tompkins (1985). Both groups observed that *XX dsx* heterozygotes elicit high levels of courtship, *XY* and *XX dsx* homozygotes elicit intermediate levels of courtship (with *XY* flies being slightly but not significantly more attractive than *XX* flies), and *XY dsx* heterozygotes elicit low levels of courtship. McRobert & Tompkins' *dsx* homozygotes seem to elicit somewhat more courtship than Jallon *et al*'s flies, but the comparison is difficult to make because the two assays for sex appeal are quite different: McRobert & Tompkins measured total courtship for 10 minutes in small chambers, while Jallon *et al.* measured wing vibration for 5 minutes under a watch glass. Moreover, we have observed that intersexes induced more elements characteristic of the beginning of courtship (orientation, following, brief vibrations) than elements characteristic of the bulk of male–female courtship (sustained vibrations, attempted copulation). Behaviours in the latter category were not observed with heterozygous males. Similar results were found for *D. melanogaster* Canton S males with *D. simulans* Seychelles mature females which lack 7, 11-heptacosadiene (Jallon, 1984). It is possible that such sibling *D. simulans* females, as well as *D. melanogaster dsx* intersexes, induce early courtship reactions because of the traces of dienes, especially pentacosadiene, that are sometimes detected. Another possibility is that early and later male courtship behaviours may correspond to different excitation states (Jallon & Hotta, 1981) and may therefore be induced by a different set of excitatory stimuli. A possible antagonistic effect of vaccenyl acetate which is present in intersexes must also be considered (Jallon *et al.* 1981). It is not known whether the acetate, which has been shown to be inhibitory for later steps of courtship such as sustained vibration (Jallon *et al.* 1981) and attempted copulation (Mane, Tompkins & Richmond, 1983), has any effect on early steps.

The *dsx* mutant used in this study is involved in sex determination. The *dsx+* locus is thought to express two alternative states: the dsx^{m+} function represses all female-specific genes in males, and the dsx^{f+} function represses all male-specific genes in females (Baker & Ridge, 1980; Baker & Belote, 1983; Nöthiger & Leuthold, 1987). However, even at the phenotypic level male characters seem to be more often expressed and female characters more often repressed. For example, 57·3% of *XX*; *dsx*, *dsx* intersexes have a male genital arch (male-like intersexes; class IV), while it is absent in only 24·9% of XY; *dsx/dsx* intersexes (female-like intersexes; classes IV, V) Orssaud & Laugé, 1981). One ovary may be found with one testis in *XX*; *dsx/dsx* intersexes, but never in *XY*; *dsx/dsx* (Orssaud & Laugé, 1982). Accessory glands, which are typical male organs, are present in nearly all *dsx* intersexes, whatever the chromosomal sex (Orssaud & Laugé, 1981). Many intersexes, *XX* as well as *XY*, show a complete or incomplete ejaculatory bulb in which the anti-aphrodisiac vaccenyl acetate is specifically found (Butterworth, 1969). The same tendency to a more pronounced maleness was observed for cuticular hydrocarbons: doublesex intersexes, independent of external or gonadal morphology, lack female-specific dienes. In mutant flies of either chromosomal sex, this pattern appears as the immature wild-type hydrocarbon pattern disappears; this suggest that the observed regulation by the *dsx* gene of pheromone synthesis occurs in a particular period of adult development.

The molecules with which we are dealing are not proteins and consequently not primary gene products.

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A biosynthetic pathway has been proposed which involves a limited number of steps and enzymes, some of which are common, some of which are sex-specific and species-specific (Jallon, 1984). Preliminary results suggest that palmitic acid, and not stearic acid, is a precursor of all monoenes and dienes (Chan Yong & Jallon, 1986, and unpublished results). The first desaturation, leading to 7 unsaturated hydrocarbons, after elongation and decarboxylation steps, might be introduced on palmitate by tissues of both sexes and does not seem to be sensitive to the action of *dsx*. To explain the introduction of the double bond in position 11, which is present in female-specific dienes, we can reasonably hypothesize the existence of a female-specific desaturase with a different specificity. Alternatively, there might be a female specificity in the desaturation–elongation coupling. On the other hand, the male-specific compound vaccenyl acetate, which also has a double bond in position 7 relative to the methyl end, could share part of the scheme before terminal male-specific steps. Such discrete steps might be under the direct control of *dsx* gene products, but more probably the *dsx* gene products might regulate other genes that, in turn, regulate a specific aspect of sex pheromone synthesis, since *dsx* regulates many aspects of sexual phenotype. As far as pheromones are concerned, all *dsx/dsx* intersexes are more male-like than female-like, but only the haplo-*X* *dsx/dsx* are able to court attractive flies, although at a low intensity, and thus seem to have a more male-like brain (McRobert & Tompkins, 1985).

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