SEX determination in Drosophila is increasingly being studied with respect to relatively hidden phenotypes: those involving the insect's internal anatomy, biochemistry, and behavior (reviews: BURTIS and WOLFNER 1992; HALL 1994). In this regard, the genes acting with the fly's "sex-determination hierarchy" (SDH: reviewed, for example, by BAKER 1989; McKEOWN and MADIGAN 1992; BURTIS 1993) are found to influence certain internal phenotypes in a manner that parallels their effects on sex-specific aspects of external morphology. The development and functioning of the central nervous system may be a different story; for this, it has been hypothesized (e.g., TAYLOR et al. 1994) that elements of neural sex-specificity fall outside the purview of relatively "downstream" genes in the aforementioned hierarchy. In particular, the purported effects of doublesex (dsx) mutations on sex-specific behaviors began to be called into question because the formation of a male-specific muscle in the abdomen (the Muscle of Lawrence, or MOL)—which requires innervation by genetically male motor neurons emanating from the abdominal ganglion (LAWRENCE and JOHNSTON 1986)—was found to be immune to allelic variation at the dsx locus (TAYLOR 1992). In contrast, fruless (fru) mutations dramatically affect MOL formation (GAILEY et al. 1991; TAYLOR and KNITTEL 1995), as well as, by definition, courtship behavior (for a review, HALL 1994). This led to the idea that fru may help define a "new branch" of the SDH, which would be primarily concerned with the CNS. The elaboration of this hypothesis (TAYLOR et al. 1994) was accompanied by a preliminary re-examination and re-interpretation of the effects of one particular doublesex mutation (dsx') on courtship, as had been previously reported by McROBERT and TOPPKINS (1985) and JALLON et al. (1988).

The subsequent study of doublesex's effects on male-like courtship TAYLOR et al. (1994) showed that mutant (chromosomal) males do not tend to court females with extreme subnormalities. In addition, these mutants seemed qualitatively normal in most aspects of male courtship except for copulation (which is physically impossible because dsx flies are intersexual and lack external genitalia). Moreover, although externally they look like normal males (see above reviews), XX flies expressing a dsx allele that causes constitutive production of the male (alternative-splice) form of this gene's product exhibited no courtship whatsoever toward normal females (TAYLOR et al. 1994). From a few recordings of courtship wing vibrations, the results once again suggested normality for XY flies expressing a particular "dsx-null" allele (TAYLOR et al. 1994).

With regard to other aspects of these mutants' reproductive behavior, inbreeding problems that can accompany rendering a dsx mutation-bearing chromosome homozygous (cf. McROBERT and TOPPKINS 1985) were avoided by TAYLOR et al. (1994); however, performances of the relevant haplo-X dsx mutants was in part assessed in a "yes/no" manner. Whereas it did seem as if dsx mutants might court subnormally, the semi-quantitative nature of the data, along with the fact that the mutant males also expressed a severe eye-morphology marker.

Courtship Anomalies Caused by doublesex Mutations in Drosophila melanogaster

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Manuscript received October 31, 1995
Accepted for publication January 29, 1996

ABSTRACT

The role played by the sex-determining gene doublesex (dsx) and its influence on Drosophila courtship were examined. Against a background of subnormal male-like behavior that is reported to be an attribute of haplo-X flies homozygous for the original dsx mutation, and given that a sex-specific muscle is unaffected by genetic variation at this locus, analyses of several reproductive behaviors and control for genetic background effects indicated that XY dsx mutants are impaired in their willingness to court females. When they did court, certain behavioral actions were normal, including components of courtship song. However, these mutants never produced courtship humming sounds. Mature XY dsx flies elicited anomalously high levels of courtship; that this occurs merely because of a delay in imaginal development was experimentally discounted. The current analysis reconciled two ostensibly conflicting reports involving the courtship-stimulating qualities of this mutant type. Such experiments also uncovered a new behavioral anomaly: dsx mutations caused chromosomal males to court other males at abnormally high levels. These results are discussed from the perspective of doublesex's influence on internal tissues of adult Drosophila involved in the triggering and neural control of male- and female-like elements of courtship, reproductive pheromone production, or a combination of such factors.

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Genetics 143: 331–344 (May, 1996)
(TAYLOR et al. 1994), left this picture cloudy. Another recently unaddressed issue is the extent to which haplo-X, ddx1-mutant adults anomalously elicit courtship, which was previously found to be directed at them by wild-type males in one study (cf. MCROBERT and TOMPKINS 1985) but not another one (JALLON et al. 1988). The latter also reported that the haplo-X version of this genetic variant seemed male-like in terms of sex-specific pheromones.

In Drosophila, very young wild-type adult males elicit essentially as much courtship as does a female (for a review, TOMPKINS 1989). Immature males also court females barely or not at all (e.g., JALLON and HOTT A 1979; TOMPKINS et al. 1980). Thus, it was surmised by TAYLOR et al. (1994) that the ddx behavioral syndrome could be explained by a nonspecific developmental delay, in which a XY ddx mutant who is a few days old is in effect much younger; so that this fly would be aberrantly stimulating in its passive reproductive attributes and simultaneously unable actively to perform up to par. In general, then, it was inferred that ddx mutations do not “materially” affect Drosophila’s reproductive behavioral characteristics, in line with the hypothesis that fru and other (as yet unknown) genes are doing most of the sex-determination work in that area of male- and female-specific biology.

We have now delved deeper into the genetic, behavioral, and maturational issues just raised. The results of quantifying all the relevant features of courtship performance and elicitation, along with controlling for genetic background and age effects as well as manipulating the nature of the courting flies and the situations in which they find themselves, have led us to the conclusion that ddx flies are reproductively abnormal in ways that remain intriguing from the standpoint of sexual differentiation of the CNS, PNS, or both.

MATERIALS AND METHODS

Strains: Drosophila were reared at 25°, 50–70% humidity, on 12-hr:12-hr light-dark cycles and on a cornmeal-sucrose-yeast medium, containing the mold inhibitor Tegosept, and differentiating of the CNS, PNS, or both. The nature of the courting flies and the situations which a XY ddx mutant who is a few days old is in effect much younger; so that this fly would be aberrantly stimulating in its passive reproductive attributes and simultaneously unable actively to perform up to par. In general, then, it was inferred that ddx mutations do not “materially” affect Drosophila’s reproductive behavioral characteristics, in line with the hypothesis that fru and other (as yet unknown) genes are doing most of the sex-determination work in that area of male- and female-specific biology.

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Materials and Methods

Strains: Drosophila were reared at 25°, 50–70% humidity, on 12-hr:12-hr light-dark cycles and on a cornmeal-sucrose-yeast medium, containing the mold inhibitor Tegosept, and autoclaved further to minimize microbial growth. Flies were collected under light ether within 0–4 hr after eclosion and aged individually in autoclaved food vials (with no dry yeast added to the medium’s surface) for most behavioral tests; the exception involved observations made with wild-type (Canton-S) virgin females, in which those flies were aged in groups (≥10 vials) before testing. The wild-type males used in courtship “elicitation” experiments (see below) also came from a Canton-S strain.

The doublesex (dx) mutant stocks used were as follows: B°Y;In(3R)dx°/In(3R)TM6B, Th Hu, which carries a ddx-null breakpoint (see Discussion) (the ddx-bearing third chromosome could not be made homozygous in this stock); and three stocks carrying ddx (apparent) point mutations, y y Fdx°/TM6B, Th Hu (ddx = the original, spontaneous doublesex mutation; HILDRETH 1965); ddx°/TM3, Sb (no sex-chromosomal markers); and y dx°/TM6B, Th Hu (no Y-chromosomal marker) [the latter two ddx were EMS-induced and isolated by DUNCAN and KAUFMAN (1975) as ddxJ10J.41 and ddxJ10J.42, respectively]. A ddx-deletion (Df)-bearing stock was crossed to the above ddx mutants; the former was B°Y;Df(3R)dx°/TM6B, Th Hu. Males from the Df stock were crossed in turn to the four ddx mutants; XX or XY ddx-mutation/Df progeny were collected and aged individually (as above) before behavioral testing. The XX and XY ddx-mutation-expressing flies are intersexual in their adult appearance and thus were distinguished by virtue of the chromosomal males exhibiting Bar- Stone eyes. Also, homozygous ddx (chromosomal) males were taken from that stock (see above) as yellow (body-color) intersexes. Moreover, a series of elementary genetic crosses was performed to replace the sex chromosomes of the other ddx-variant-bearing stocks with y Y and y-marked X. This allowed for behavioral comparisons to be made between eye-depleted (B°) males and those with anatomically normal visual systems.

Recordings and analyses of courtship behaviors: Test flies were placed in a small plexiglass chamber with a nylon mesh bottom (1-cm diameter × 4-mm height). The chamber was then placed on top of a microphone inside an Insectavox (GORCZYCA and HALL 1987). A Sony Hi8 camera was used to make simultaneous video recordings of the flies' actions and audio recordings of courtship-song sounds produced by males or chromosomally male intersexes. The camera was connected to a television, permitting on-line monitoring of the flies’ behavior. The output from the microphone was processed through an equalizer, which removed signals ≥500 Hz. A sound spike was placed at the beginning and end of each courtship recording, by turning the Insectavox off and on; these sound markers framed the recording period (i.e., permitted its duration to be computed separately for each chamber’s worth of flies). Recordings were made at temperatures ranging between 22 and 25°.

After a recording was made, the video tape was played back, and the analog song signals on the VCR were digitized at 2 kHz into a Macintosh II computer using the software, LifeSong (BERNSTEIN et al. 1992). As the sound was being digitized, behavioral events were logged simultaneously, using a “logging event box.” This device is equipped with eight buttons, each generating a digitizable Δ5 V signal. A given button was assigned to a particular courtship behavior. When that behavior occurred, the corresponding button was depressed until the actions ceased.

Each of the outputs from the event-logging box was digitized by a GW Instruments (Somerville, MA) digital-to-digital daughterboard (GWI-DIO: 16 Bit Digital I/O). LifeSong then displays both the digitized audio signals and behavioral events that were logged. The digitized songs were stored on an optical disc ported from the Macintosh II. Such a file, containing each “pulse time” location, and the file containing all behavioral-event positions and durations (including the first and last spike) were transferred to a VAX/VMS system for analysis of behavior and several song parameters (cf. WHEELER et al. 1989; BERNSTEIN et al. 1992; also see below). These included interpulse intervals, whose boundaries were set at 15–80 msec (thus, a >80-msec interval of silence between pulse was defined as an interbout interval); numbers of cycles per pulse; and main intrapulse frequency, from Fast Fourier Transforms. Parameters associated with trains of pulses were computed and tabulated for singing bouts involving at least six pulses because such trains unequivocally consist of song sounds. Numbers of pulses per train were also calculated for more marginal bouts with at least four pulses, and the similarity among the mutant and control genotypes for this parameter (see last data column in Table 3) still obtained. To analyze reproductive behavioral actions on the VAX, a file was first created containing the file name and which buttons were used for logging a given kind of courtship action (also see next section). In processing the songs produced by...
d.shape of a specific frequency pattern produced by a male, and associated female mound formation, was independent of any song sounds (e.g., WHEELER et al. 1988, 1989), were indicated for each taped record by performing audio scans of the digitized flies (separate from those that generated the pulse logs). For songs produced by dsx'-expressing males, the scans were augmented by visual observations of the song records; this led to an estimate of the normal rate of sine-song production (bouts per minute).

A postlogging program was applied, that used the sound markers noted above to compute the recording time and also determine each time an events logging button was pressed and for how duration. The output from this program was handled by the final program in the sequence, which computed the total numbers of each behavior, average numbers of each behavior/seq, and average duration of each behavior.

**Courtship behavior performed by dsx-variant males:** XY flies expressing dsx', dsx', dsx', or dsx', owing to heterozygosity with Df(3R)dsx', were collected from cultures resulting from the crosses described above. The mutants were aged individually in autoclaved food vials for 4–6 days before testing. A given individual was then aspirated into a small recording chamber (described above) with three wild-type virgin females (1–3 days old); this number of females was used in an attempt to maximize the amount of courtship. Control wild-type males were paired with one virgin female each. The chamber was then placed on top of a microphone inside the insectavox, and a video and audio recording was made for 5–8 min. The percent of time that the test fly spent performing any and all courtships actions toward the other fly present (i.e., the courtship index or CI, e.g., TAYLOR et al. 1994) was determined for each observation. Wing extension (reviewed by HALL 1994) was logged when this behavior, as performed by the XY fly, was directed toward females. (Note: Bar-eyed males perform a fair amount of nontargeted wing extension). Re-observations of samples of these (video) data records were made, so that certain "micro" details of courtship could be tallied; this gave rough indications (owing to the difficulty of being certain, from observing a given playback, that a particular behavior did not occur) of the proportions of test flies that performed tapping, licking, and attempted copulation (cf. HALL 1994).

**Courtship elicited by dsx variants:** Wild-type adult males were aged as above. Then an individual such fly was placed in a recording chamber with either a homozygous dsx or heterozygote dsx/Df XX or YY adult; the only homozygous type used was dsx'; the other dsx variants (n = 4, including dsx'/Df) came from the outcrosses described above, in which the male parent carried Y'T (it was assumed that that marker should have relatively little effect on elicitation as opposed to active performance of male courtship actions). The 4–6-day-old wild-type males were tested with dsx variants that had been aged individually for 1, 4, 6, 8, or 12 days before testing; this was done to determine whether there is any 'maturation effect' of various dsx mutations, especially insofar as elicitation of copulation by haplo-X flies is concerned. Note that for one genotype homogeneity was difficult to age the chromosomal males for the full 12 days as >50% of these individual died by ~10 days posteclosion. For elicitation testing, each mutant/normal pair was recorded for 5–8 min. In controls, wild-type males were tested with one wild-type virgin female or one dsx mutation/Balancer male each (here, the females were aged in groups). The CIs and wing-extension time-percentages elicited in these experiments were logged (separately). In addition, any courtship actions that might have been performed by the (mutant) individuals who were formally designated as objects of courtship (courttesse) were quantified (see results).

**Abnormal courtship behavior:** To determine directly whether a male exhibits a courtship preference for one sex-chromosomal dsx type vs. the other, a series of 4–6-day-old wild-type males was presented simultaneously with an XX and XY dsx mutant (the later had also been aged individually for 4–6 days before testing). The trio's behavior was recorded for 6–8 min, and the orientation/looming and wing-extension performed by the wild-type male toward the XY dsx male and (separately) toward the XX dsx fly was logged. The heterozygous dsx/Ds variants tested (using all four alleles, but only one such within each trio) were generated from the y' and y'-containing stocks (see above); XY and XX dsx homoyzogotes were also tested.

To quantify how much rejection behavior is exhibited by dsx mutants, the number of times an XY or XX dsx individual exhibited wingflicks when courted by the wild-type male (e.g., PAULLETTE et al. 1991) was logged. This led to computations of 'rejection percentages,' which were based on wing-flick-associated courtship bouts/total bouts (cf. HALL 1978). By observing certain of the preference-test recordings (see below), rejection behaviors were thus logged for dsx' homozogotes and dsx'/Df heterozygotes.

To examine the possibility that an XY dsx mutant was not courted as much as a female (in certain experiments: Table 5), because the former might be responding to the (wild-type) male's courtships with more wing-flick rejection behavior than would an XX dsx fly, the wings of two dsx mutant types (XY and XX versions of each) were clipped with fine scissors when collected. These surgically flies were tested at 4 days old with one wild-type male each, as above.

To monitor general locomotor activity, XY dsx mutant or dsx'-bearing control males were collected (as above) and aged individually for 4–5 days. A series of single males were placed (one each) in cylindrical plastic chambers; a piece of filter paper, with a single line across its diameter, was placed on the floor of each chamber. After allowing the fly to acclimate to the chamber for 2 min, the number of times the male crossed the line in a 5-min observation period was counted. See KULKARNI and HALL (1987) for further details.

**Statistics:** Courtship indices were subjected to arcsine square root transformations (cf. VAN SWINDEREN and HALL 1995) for homogeneity of variance and to effect approximations of normal distributions (SOKAL and ROHLF 1981, p. 427). Analyses of variance (ANOVA) then were performed using JMP software (version 3.1 for the Macintosh: SAS Institute, Inc.). For planned pairwise comparisons, critical P values were adjusted for "experimentwise error" (cf. SOKAL and ROHLF 1981, p. 241) and are indicated below for each experiment.

**Courtship behavior of XY dsx variants with tiny vs. normal eyes:** Transformed Cls (for all courtship actions) from the tests of 11 groups [y'/Y; dsx'/Df (tiny), y'/Y; dsx'/Df (normal), y'/Y; dsx'/Df (tiny), y'/Y; dsx'/Df (normal), y'/Y; dsx'/Df (normal), y'/Y; dsx'/Df (tiny), y'/Y; dsx'/Df (normal), y'/Y; dsx'/Df (normal), y'/Y; dsx'/Df (normal), y'/Y; and wild type] were subjected to a one-way ANOVA with group [F(tiny,11) = 7.90, P < 0.0001] as the main effect. The six planned comparisons between tiny vs. normal eyes (dsx'/Df (tiny), dsx'/Df (normal), y'/Y; dsx'/Df (normal), y'/Y; and wild type) were deemed significant if P = 0.0085 (cf. SOKAL and ROHLF 1981), and no such differences were detected for any of the dsx variants (see Table 1). Consequently, the four pairs of tiny vs. normal dsx genotypes, were grouped together, and transformed Cls were subjected to a second one-way ANOVA with genotype [F(tiny,11) = 14.09, P < 0.001] as the main effect. The results of subsequent Tukey-
Kramer unplanned comparisons (α = 0.05) between particular genotypes are summarized in the legend to Table 1 (below). Transformed "wing-extension time-percentages" (always < the CI values, which refer to overall courtship actions) from the 11 groups [y/Y; dsx/DF (tiny), y'/y; dsx/DF (normal), y'/Y; dsx'/DF (tiny), y'/y; dsx'/DF (normal), y'/Y; dsx'/DF (tiny), y'/y; dsx'/DF (normal), B', y, and wild type] were subjected to a one-way ANOVA with genotype [F(5, 278) = 12.71, P < 0.001] as the main effect. The six planned comparisons involving the wing-extension performance of flies with tiny vs. normal eyes were deemed significant if P = 0.0085 (cf. Sokal and Rohlf 1981), and no such differences were detected for any of the dsx variants (see Table 1). Consequently, the two rows' worth of wing-extension—percent data for the four dsx-mutant genotypes (top section of Table 1) again could be collapsed to one (for as the CI s), to compare the effects of the different dsx alleles. For this, transformed CI s were subjected to a second one-way ANOVA with genotype [F(5,118) = 21.66, P < 0.0001] as the main effect; the results of subsequent Tukey-Kramer unplanned comparisons (α = 0.05) between particular genotypes are summarized in the legend to Table 1.

Acoustical details of courtship songs affected by dsx: All song parameters were distributed normally (hence were not subject to arcsine transformations). Cycles per pulse values from the songs of six genotypes (Y/Y; dsx/DF, y'/y; dsx/DF, y'/Y; dsx'/DF, y'/y; dsx'/DF, y'/Y; dsx'/DF, y'/y; dsx'/DF, y'/Y; dsx'/DF, y'/y; dsx'/DF, B', and wild type) were subjected to a one-way ANOVA with genotype [F(5, 210) = 1.88, P = 0.14] as the main effect. Intrapulse frequencies from six genotypes (Y/Y; dsx/DF, y'/y; dsx'/DF, y'/Y; dsx'/DF, y'/y; dsx'/DF, y'/Y; dsx'/DF, B', and wild type) were subjected to a one-way ANOVA with genotype [F(5, 210) = 1.88, P = 0.14] as the main effect. Train-length values from six genotypes (Y/Y; dsx/DF, y'/y; dsx'/DF, y'/Y; dsx'/DF, y'/y; dsx'/DF, y'/Y; dsx'/DF, B', and wild type) were subjected to a one-way ANOVA with genotype [F(5, 210) = 1.88, P = 0.14] as the main effect. Interpulse intervals from six genotypes (Y/Y; dsx/DF, y'/y; dsx'/DF, y'/Y; dsx'/DF, y'/y; dsx'/DF, y'/Y; dsx'/DF, B', and wild type) were subjected to a one-way ANOVA with genotype [F(5, 210) = 1.88, P = 0.14] as the main effect. The results of the five planned comparisons are summarized in the legend to Table 2.

Basic song performance of dsx males: Trains-per-minute values from six genotypes (Y/Y; dsx/DF, y'/y; dsx'/DF, y'/Y; dsx'/DF, y'/y; dsx'/DF, y'/Y; dsx'/DF, B', and wild type) were subjected to a one-way ANOVA with genotype [F(5, 210) = 24.16, P < 0.001] as the main effect. Five subsequent planned comparisons were deemed significant (α < 0.01), as is summarized in the legend to Table 3. Train-length values from six genotypes (Y/Y; dsx/DF, y'/y; dsx'/DF, y'/Y; dsx'/DF, y'/y; dsx'/DF, y'/Y; dsx'/DF, B', and wild type) were subjected to a one-way ANOVA with genotype [F(5, 210) = 1.31, P = 0.29] as the main effect. The CI s of the five planned comparisons were deemed significant at α of 0.01, as summarized in the legend to Table 3.

Courtship elicitation of XX and XY dsx mutants: Arcsin-transformed CI s from six genotypes [dsx'/dsx', dsx'/DF, dsx'/DF, dsx'/DF, dsx'/DF, and controls (dsx'/TM6B males and wild-type females)], two chromosomal sexes, and five ages (1-, 4-, 6-, 8- and 12-day-old adults) were subjected to a three-way ANOVA with (dsx-related) genotype [F(5, 270) = 3.75, P = 0.0027], chromosomal sex [F(1, 270) = 37.97, P < 0.0001] and age [F(3, 270) = 25.38, P < 0.0001] as main effects; with genotype × chromosomal sex [F(15, 810) = 19.35, P < 0.0001], age × genotype [F(20, 270) = 1.40, P = 0.12], and age × chromosomal sex [F(40, 270) = 1.24, P = 0.29] as two-way interaction terms; and with age × genotype × chromosomal sex [F(80, 270) = 1.05, P = 0.40] as the three-way interaction term. The subsequent planned comparisons, with a appropriately adjusted to 0.0026 (cf. Sokal and Rohlf 1981, p. 241), are summarized in the legend to Table 4.

For the "preference tests" (as whether a wild-type male would direct more courtship at an XX or XY version of a dsx mutant), t-tests were performed, as described in the legend to Table 5 (below).

RESULTS

da behavior with normal females: Would any doublesex mutations allow for robust male-like courtship behaviors when XY flies expressing such dsx alleles are in the presence of wild-type females? We quantified in more detail than in previous studies the male-like courtship behavior of various XY dsx mutants. This included replacing the Y chromosome of the dsx mutant types tested recently (Taylor et al. 1994) with one carrying Y instead of B'; thus we compared the courtship levels of these XY dsx mutants to those that have been hand-capped visually in previous studies (McRobert and Tompkins 1985; Taylor et al. 1994).

Table 1 summarizes the general courtship behavior of four heterozygous XY; dsx/DF types, after each such fly was placed with three wild-type virgin females. Overall, the courtship levels did not improve dramatically when the eye defect was corrected: two normal-eyed dsx mutant types (and the y' Y dsx' control) did court more vigorously in comparison with B'-bearing XY flies; but the opposite occurred when testing the other two dsx mutants (and none of these differences was significant: see legend to Table 1). Thus, the courtship sluggishness recorded in these tests is primarily due to the dsx mutations (however, see below). This includes the fact that all eight dsx-related tests led to significantly lower courtship levels than in the cases of normal-eyed control males; even the (straight) PY control males performed better than did three of the dsx mutant types. [Note: removing most of the eye tissue from dsx+ males did impair their courtship performance (Table 1).]

Homozygous haplo-X dsx flies were also tested with females and exhibited almost no interest in courting; the CI was 0 (n = 4 quartets of flies observed), which approaches the results for this mutant allele as reported previously (McRobert and Tompkins 1985; Taylor et al. 1994). This extreme subnormality may in large part be a genetic-background effect, given the better behavior exhibited by XY dsx/DF flies (Table 1). Overall, however, the relatively nonrobust male-like courtship
of the XY dsx mutants, and the differences among the four alleles, seems not to be solely a matter of genetic backgrounds: all stocks were outcrossed to the same (Df-bearing) one to generate these data; and infusing a similar background into three of them (i.e., by crossing the dsx stocks that carried B'/Y at the beginning of this study to a y/y Y strain), followed by the same outcrossing as just noted, did not radically change the levels of courtship recorded (see above).

In tests of general vigor (i.e., locomotion of solitary flies, hence unrelated to reproductive behavior), the numbers of lines crossed (per unit time) in the relevant arena (Kulkarni and Hall 1987) were quite subnormal for the XY dsx homoygotes (31% of wild type, n = 9 and 18, respectively), somewhat depressed when this mutation was heterozygous with the deletion (72%, n = 12), largely normal for dsx'/Df (114%, n = 8) and dsx'/Df (121%, n = 9) as well as another control: dsx'/dsx- (96%, n = 5); and rather elevated for dsx'/Df (175%, n = 11). Thus, the “worst courtier” among these mutants may have that property in part for nonspecific reasons, but this was a substantial effect only for the dsx- homoygote. The hyperactivity of the flies hemizygous for dsx- ran counter to the effects of this mutation on courtship vigor (Table 1).

Since most of the data pertaining to dsx-influenced courtship levels came from testing a given such mutation when it was heterozygous with the deletion, the results in Table 1 imply allele-specific decrements among the genetic variants at the doublesex locus; this is in agreement with data from Taylor et al. (1994). The rank orderings of subnormal courtship levels from that study compared with the current Table 1 are the same: dsx'/dsx- < dsx'/Df < dsx'/dsx- < dsx'/Df < dsx'/Df. All four of the XY dsx types exhibited qualitatively normal-appearing tapping and following of females, orientation toward them (if the latter were stationary for a moment), as well as wing extensions, licking bouts, and copulation attempts directed at them. Tapping, licking, and attempted copulation normally involve moments of contact between the courter and the courtee; these occurred routinely, but not relentlessly, in the mutant’s behavior (see legend to Table 1), except in the case of attempted copulation; for this, the XY dsx flies’ abdominal bends did not include contact between their nethermost regions and those of the female. Quantifications of orientation/following and wing extension appear in Table 1 (also see below). Regarding wing-extension quality, those of the mutants involved wild-type-like, ca. 90° pivotings of the left or right wing’s posture, implying, furthermore, that these wing extensions were unilateral (as in wild type) during a given courtship moment.

Aging of the XY dsx mutant individuals did not in general improve their courtship performances. One-day old flies (n = 14, distributed over the four dsx geno-

### Table 1

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Eyes</th>
<th>CI</th>
<th>Wing extension</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>y'/y; B'/Y; dsx-'/Df</td>
<td>Tiny</td>
<td>9 ± 4</td>
<td>3 ± 1</td>
<td>19</td>
</tr>
<tr>
<td>y'/y; Y; dsx-'/Df</td>
<td>Normal</td>
<td>19 ± 7</td>
<td>6 ± 3</td>
<td>15</td>
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<tr>
<td>y'/y; B'/Y; dsx-'/Df</td>
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<td>14 ± 1</td>
<td>4 ± 1</td>
<td>13</td>
</tr>
<tr>
<td>y'/y; Y; dsx-'/Df</td>
<td>Normal</td>
<td>25 ± 5</td>
<td>3 ± 1</td>
<td>17</td>
</tr>
<tr>
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<td>17 ± 3</td>
<td>6 ± 2</td>
<td>9</td>
</tr>
<tr>
<td>y'/y; Y; dsx-'/Df</td>
<td>Normal</td>
<td>11 ± 6</td>
<td>3 ± 2</td>
<td>14</td>
</tr>
<tr>
<td>y'/y; Y; dsx-'/Df</td>
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<td>30 ± 7</td>
<td>14 ± 6</td>
<td>10</td>
</tr>
<tr>
<td>y'/y; Y; dsx-'/Df</td>
<td>Normal</td>
<td>27 ± 7</td>
<td>7 ± 3</td>
<td>15</td>
</tr>
</tbody>
</table>

Controls:
- B'/Y Tiny 35 ± 4 17 ± 3 4
- y'/y Normal 67 ± 5 39 ± 7 4
- Wild type Normal 89 ± 2 59 ± 3 4

The dsx-'/Df chromosomal males with tiny eyes came from the cross: B'/Y; [Df(3R) dsx-'/TM6B × dsx-mutation/Balancer females (see MATERIALS AND METHODS). The dsx variant XY flies with normal eyes were generated from the cross: y'/y; [Df(3R) dsx-'/TM6B × y'/y; dsx-mutation/Balancer females. Test males (except for the wild types, which were tested with one virgin female each) were placed in a recording chamber with three wild-type virgin females (all wild-type flies came from a Canton-S strain). Observation periods were 5–8 min, or in some cases, until the male mated (ca. 2 min). The values tabulated are CI ± SEM (Courtship index: percent of observation period during which any/all courtship actions occurred) and the subset of that time during which wing-extension took place. Test males were 4–6 days old; wild-type virgin females were 1–3 days old. Planned comparisons after a one-way ANOVA on transformed CIs and the wing-extension time-percentages indicated no significant differences between the performance of tiny- vs. normal-eyed flies for any of the dsx variants, for B'/Y vs. y'/y, or y'/y vs. wild type (all P ≥ α = 0.05; see MATERIALS AND METHODS). The data from tiny- vs. normal XY flies were combined for each dsx allele, yielding six genotypes (dsx-/Df, dsx-'/Df, dsx-/Df, dsx'/Df, B'/Y; dsx-; and control males, which consisted of a merger between y'/y and wild type): these were subjected to Tukey-Kramer unplanned comparisons, after a second one-way ANOVA on transformed CIs, revealed significant differences between dsx-/Df vs. dsx'/Df, and between all four dsx variants and the controls (all P < 0.05). Similar Tukey-Kramer unplanned comparisons after a one-way ANOVA on the transformed wing extension percentages revealed no differences among the dsx mutant genotypes (all P > 0.05), whereas each dsx variant differed significantly from the controls (all P < 0.05). Observations involving qualitative features of male-like courtship (for dsx-mutation/Df flies carrying the y'/y) led to the following roughly appreciated tactics of one or more of the following events per courtship: For tapping: 60% of the dsx-'/expressing, half the dsx-'/expressing, two-thirds of the dsx-'/expressing, and all of the dsx-'/expressing XY flies touched the female’s abdomen with their forelegs early in the courtship sequence. For licking, none of the dsx-’, 10% of the dsx-’ and dsx-’, and none of dsx-’ extended their proboscis toward the female’s abdomen, relatively late in the courtship sequence. For attempted copulation, 30% of the dsx-’ and dsx-’, 40% of the dsx-’, and 20% of the dsx-’ curled their abdomens in the direction of the female. For the y'/y Y dsx- controls, all males tapped and attempted to copulate, and half of them licked.

### TABLE 1

**Courtship behavior of XY dsx mutants in the presence of females**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Eyes</th>
<th>CI</th>
<th>Wing extension</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>y'/B'/Y; dsx-'/Df</td>
<td>Tiny</td>
<td>9 ± 4</td>
<td>3 ± 1</td>
<td>19</td>
</tr>
<tr>
<td>y'/y; Y; dsx-'/Df</td>
<td>Normal</td>
<td>19 ± 7</td>
<td>6 ± 3</td>
<td>15</td>
</tr>
<tr>
<td>y'/Y; B'/Y; dsx-'/Df</td>
<td>Tiny</td>
<td>14 ± 1</td>
<td>4 ± 1</td>
<td>13</td>
</tr>
<tr>
<td>y'/y; Y; dsx-'/Df</td>
<td>Normal</td>
<td>25 ± 5</td>
<td>3 ± 1</td>
<td>17</td>
</tr>
<tr>
<td>y'/B'/Y; dsx-'/Df</td>
<td>Tiny</td>
<td>17 ± 3</td>
<td>6 ± 2</td>
<td>9</td>
</tr>
<tr>
<td>y'/y; Y; dsx-'/Df</td>
<td>Normal</td>
<td>11 ± 6</td>
<td>3 ± 2</td>
<td>14</td>
</tr>
<tr>
<td>y'/B'/Y; dsx-'/Df</td>
<td>Tiny</td>
<td>30 ± 7</td>
<td>14 ± 6</td>
<td>10</td>
</tr>
<tr>
<td>y'/y; Y; dsx-'/Df</td>
<td>Normal</td>
<td>27 ± 7</td>
<td>7 ± 3</td>
<td>15</td>
</tr>
</tbody>
</table>

Controls:
- B'/Y Tiny 35 ± 4 17 ± 3 4
- y'/y Normal 67 ± 5 39 ± 7 4
- Wild type Normal 89 ± 2 59 ± 3 4

The data from tiny- vs. normal XY flies were combined for each dsx allele, yielding six genotypes (dsx-'/Df, dsx-'/Df, dsx-'/Df, dsx-'/Df, B'/Y; dsx-; and control males, which consisted of a merger between y'/y and wild type): these were subjected to Tukey-Kramer unplanned comparisons, after a second one-way ANOVA on transformed CIs, revealed significant differences between dsx-'/Df vs. dsx-'/Df, and between all four dsx variants and the controls (all P < 0.05). Similar Tukey-Kramer unplanned comparisons after a one-way ANOVA on the transformed wing extension percentages revealed no differences among the dsx mutant genotypes (all P > 0.05), whereas each dsx variant differed significantly from the controls (all P < 0.05). Observations involving qualitative features of male-like courtship (for dsx-mutation/Df flies carrying the y'/y) led to the following roughly appreciated tactics of one or more of the following events per courtship: For tapping: 60% of the dsx-'/expressing, half the dsx-'/expressing, two-thirds of the dsx-'/expressing, and all of the dsx-'/expressing XY flies touched the female’s abdomen with their forelegs early in the courtship sequence. For licking, none of the dsx-’, 10% of the dsx-’ and dsx-’, and none of dsx-’ extended their proboscis toward the female’s abdomen, relatively late in the courtship sequence. For attempted copulation, 30% of the dsx-’ and dsx-’, 40% of the dsx-’, and 20% of the dsx-’ curled their abdomens in the direction of the female. For the y'/y Y dsx- controls, all males tapped and attempted to copulate, and half of them licked.
Heterozygous dsx/Df chromosomal males were collected from the cross: B"Y;Df[3R]dsx23/TM6B × dsx-mutation. Balancer females. Test males were aged 4–6 days then placed in a chamber with three wild-type virgin females (except for wild-type males: one female each) and recorded for 5–8 mins (or until the male mated in certain cases, ca. 2 mins from the start of recording. Song pulses were logged by correlating the relevant signals with visualization of wing extensions (see MATERIALS AND METHODS). The song sounds were analyzed for Interpulse interval (IPI; minimum-maximum values were specified as 15 and 80 msec, respectively); number of cycles per pulse (cycles/pulse), intrapulse frequency (Hz), number of peaks in Fast Fourier Transform plots resulting from FFT analysis to which each pulse was subjected (No. of peaks), and FFT peak width (Hz) (see MATERIALS AND METHODS). Each of these variables were distributed normally; thus, untransformed data were subjected to one-way ANOVAs, which revealed no significant differences among group means (dsx'/Df; dsx'~/Df; and dd3/Df flies and dsx'/Df courted with almost identical time-percentage values; and (2-day older) dsxz6/Df flies was aged for 8 days before testing (with ca. threefold less vigorously. An additional set of y'/y Y flies was aged for 8 days before testing (with normal females): the orientation and wing-extension percentages (cf. Table 1) were dsx23/Df: 9 ± 3/1 ± 1 (n = 8); dsx23/Df: 25 ± 10/7 ± 4 (n = 9); dsx10/Df: 36 ± 12/8 ± 3 (n = 8); and dsx16/Df: 8 ± 3/1 ± 0 (n = 9). Except for the twofold improvement exhibited by the oldest dsx'/Df flies (cf. Table 1), the tendency was for courtship vigor of doublesex mutants to remain the same or decline as the adults got older.

**Courtship song:** With respect to wing vibrations and singing behavior, if XY dsx- intersexes were somewhat “female-like” in the relevant portions of the CNS, then their songs would probably be strange sounding (cf. SCHILCHER and HALL 1979; TAYLOR et al. 1994). However, all the XY dsx flies tested in this study generated song pulses whose parameters were quite similar to that of dsx- bearing Drosophila melanogaster males (Table 2). For most song parameters (e.g., numbers of cycles per pulse, intrapulse carrier frequencies), all the dsx mutants yielded values within the ranges previously observed for normal D. melanogaster males (e.g., WHEELER et al. 1989; BERNSTEIN et al. 1992). The mutants’ “FFT peak width” values (a reflection of the “pulse envel-
Basic song performance of XY dsx mutants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Trains/min</th>
<th>Pulses/min</th>
<th>Train length (No. of pulses)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>y/y; B/Y; dsx/Df</td>
<td>2 ± 1</td>
<td>81 ± 13</td>
<td>9.8 ± 1.6</td>
<td>9</td>
</tr>
<tr>
<td>y/y; B/Y; dsx^17/Df</td>
<td>1 ± 0</td>
<td>13 ± 6</td>
<td>10.5 ± 1.1</td>
<td>7</td>
</tr>
<tr>
<td>y/y; B/Y; dsx^6/Df</td>
<td>5 ± 1</td>
<td>83 ± 17</td>
<td>15.1 ± 1.1</td>
<td>5</td>
</tr>
<tr>
<td>y/y; B/Y; dsx^23/Df</td>
<td>10 ± 3</td>
<td>138 ± 43</td>
<td>11.8 ± 0.3</td>
<td>4</td>
</tr>
<tr>
<td>Wild type</td>
<td>26 ± 3</td>
<td>338 ± 20</td>
<td>10.3 ± 0.3</td>
<td>3</td>
</tr>
<tr>
<td>B'Y^a</td>
<td>14 ± 3</td>
<td>210 ± 46</td>
<td>11.6 ± 0.5</td>
<td>4</td>
</tr>
</tbody>
</table>

dsx/Df chromosomal males came from the cross: B'Y;Df(3R) dsx^17/TM6B × dsx-mutation/Balancer females. The to-be-tested flies were aged 4–6 days then placed with three 1–3-day-old virgin females each (except for wild-type males: one female each). From the acoustical outputs from the ensuing courtships, the general song parameters [numbers of pulses per minute (pulses/minute), number of pulse-trains per minute (trains/minute), and train length (number of pulses per train)] were computed as described in MATERIALS AND METHODS. The resulting values are tabulated ± SEM, with the last column indicating the numbers of XY dsx's or control males recorded for each genotype. Each of the variables was distributed normally; thus, untransformed data were subjected to one-way ANOVAs (see MATERIALS AND METHODS), which revealed no significant differences among group means (dsx'/Df, dsx/17/Df, dsx/6/Df, dsx^23/Df, B'Y and wild type) for train length (P = 0.29), but significant group effects for pulses/minute (P < 0.001) and trains/minute (P < 0.001). For both pulses/minute and trains/minute, five subsequent planned comparisons revealed significantly fewer pulses or trains/minute for B'Y vs. wild-type males, and more pulses/minute and trains/minute for B'Y vs. dsx'/Df, dsx^17/Df or dsx^23/Df males (all Ps < 0.01).

* Data from TAYLOR et al. (1994).

(which, overall, were too low to be explained by visual-system defect or other genetic-background problems), the quality of their “pulse singing” tended to be normal. Yet, delving one step deeper into the songs recorded for these mutant males uncovered a striking deficit: this followed a previous indication (TAYLOR et al. 1994) that one dsx-mutant type (XY; dsx^23/Df) produced no “sine song” (from a small number of recorded individuals). This song component involves species-specific humming sounds, generated by courting Drosophila males (e.g., SCHILCHER 1977; WHEELER et al. 1988, 1989). Extending the earlier observation revealed that no sine-song bouts whatsoever were generated by any of the four dsx mutant types; for a grand total (for all genotypes of 450 min of recording time (cf. Tables 2 and 3). Sine-singing normally accompanies most bouts of pulse singing; whereby the <1-secduration hum proceeds into a train of pulses, or vice versa, and is not a rare event. For instance, in the contemporary control (dsx') songs, the first bout of singing (and most subsequent ones) included sine song, for each of the males (n = 4) examined in this manner; overall, these males generated 20 sine-sing bouts/min, from 26 min of total recording time.

**dsx-associated elicitation:** We examined courtship as elicited by dsx mutants, with respect to: (1) the effects of various dsx mutations in both XX and XY flies, the chromosomal sex comparison included direct “preference” testing in which a wild-type male was presented with both XX and XY dsx simultaneously, and (2) whether the age of XX and XY intersexes is a factor in terms how attractive the fly is; in particular, does elicitation for dsx mutants wane, eventually to the same extent as for wild-type males?

Table 4 summarizes elicitation levels associated with four heterozygous dsx/Df types and that of the original dsx' mutant (the only one previously tested, as a homozygote, for this courtship attribute: see above). Thus, are XX and XY dsx flies rather attractive to wild-type males, and equally so (cf. McROBERTS and TOMPKINS 1985) or is the wherewithal of mutant flies with either of these (sex-chromosomal) genotypes to elicit such behavior minimal, which would jibe with their apparent (wild-type male-like) lack of a key female “aphrodisiac” (cf. JALLON et al. 1988)? Both sets of investigators were measuring the levels of sex appeal in different ways; this issue was re-evaluated in the current study, along with our addressing of the other ones just noted.

Our results on dsx elicitation levels are actually consistent with those reported by both sets of authors just cited. The mutants elicit reasonable levels of courtship, in various circumstances, but rather little wing extension; the latter was the only “sex appeal” parameter assessed by JALLON et al. (1988). This can be misleading. For example, with 4-day-old flies (a similar adult age as the courteses in the earlier experiments), we found that both XX and XY dsx' homozygotes elicit the same levels of courtship; however, the percent of time wild-type males showed wing extensions toward dsx' was not only very low in absolute terms, but also relatively lower as well (as a proportion of the orientation/following percent), compared with the amount and kind of courtship...
Individual wild-type males were placed with either a XX or XY dsx fly that had been specifically aged, posteclosion: \( n \) values are in parentheses. The \( \text{Df}/\text{dsx} \) chromosomal males and females came from the cross: \( \text{Bf}/\text{Df}, \text{3R}/\text{Df}, \text{TM6B}/\text{X} \) male/female or \( \text{Bf}/\text{Df}, \text{TM6B}/\text{X} \) male/female. A single wild-type male (4-6 days old) was placed in a chamber with either a \( \text{dsx}^{-} \) or \( \text{dsx}^{+} \) female from normal males (Table 4). Because of the interaction between genotype and subadult \( \text{dsx}^{-} \) females were courted to a similar extent as were the \( \text{dsx}^{-} \) males and females (at the control level, see Table 4) and to a similar extent as were the control males and \( \text{dsx}^{-} \) females.
did elicit more courtship than did mutant chromosomal females; control males in the main elicited less courtship than did \(XY\) mutants (statistically, and by inspection for most of the mutant types), an exception being \(dsx^+\) chromosomal males (these nominally elicited more courtship than did \(dsx^+\) males, but the differences did not reach significance); and \(dsx\)-related genotypes led to different levels of elicitation, although this was mainly due to the effect of \(dsx^+\) vs. (any of) the mutant alleles (as can be discerned by a perusal of the CI values dispersed through the main portion of the table, i.e., except for "controls").

During the course of all these observations, we entertained the possibility that these \(dsx\) mutants might be fruitless-like in terms of courtships they would direct at their (genetically normal) male companion. This did not occur in dramatic fashion. In particular, all \(XY\) individuals homozygous or hemizygous for \(dsx^+\) (which in general leads to the feeblest courtship among these mutants) were courtees only. However, 4-6-day-old \(XY\) \(dsx^{2}/Df, \; dsx^{5}/Df\) and \(dsx^{2}/Df\) not only elicited high levels of courtship, but they also courted the normal male more than is usual (performance CIs for these three genotypes: \(8 \pm 4, 6 \pm 2, \text{and } 9 \pm 3\), respectively; wing-extension time: \(2 \pm 2, 1 \pm 1, \text{and } 4 \pm 2\)). Similar behavior was performed by 12-day-old \(dsx^{5}/Df\) and \(dsx^{5}/Df\) (chromosomal) males (CIs: \(17 \pm 5 \text{ and } 9 \pm 5\), respectively; wing extension: \(4 \pm 1 \text{ and } 2 \pm 2\)). These instances of mutant males courted other males were not as blatant or sustained as when fruitless males are tested for this behavior (e.g., HALL 1978; GAILEY and HALL 1989), but certain of the \(dsx\) types (especially the case of the relatively old \(XY\) \(dsx^{5}/Df\) flies) seemed distinctly fruitless-like in terms of this courtship anomaly—albeit perhaps only superficially so (see DISCUSSION). Moreover, fruitless-like courtship "chaining" behavior (cf. GAILEY and HALL 1989; HALL 1994) was never observed when genotypically uniform groups of \(XY\) \(dsx\) mutants were observed (no chains were formed during a 10-min observation of eight \(XY\) \(dsx^{2}/Df\) flies; and no chaining occurred over the course of three such observation periods, each involving eight \(dsx^{2}/Df\) flies).

Further elicitation tests were performed in which a wild-type male was placed with both an \(XX\) and an \(XY\) mutant simultaneously; the two chromosomal-sex types expressed the same \(dsx\) mutation in each such experiment. In these "preference tests," most \(XX\) \(dsx\)-mutant types were found to be courted more vigorously than the (nearby) \(XY\) versions of each mutant (Table 5; see its legend for statistics); whereas, in the single-pair tests, a given \(XY\) \(dsx\) mutant type was courted to a similar extent as the courtship elicited by the chromosomal (but similarly mutant) female (Table 4). The amounts of courtship elicited in the two kinds of (separate) single-pair tests involving a given doublesex mutation (Table 4) were merged (and normalized with respect to the varying observation periods in a given wild-type + \(XX\) \(dsx\) or wild-type males + \(XY\) \(dsx\) tests). When comparing such summed timed-percentages with the total amounts of courtship recorded in the preference tests (Table 5), more overall elicitation would appear to have occurred in the former experiments (data not shown). However, this is misleading, because, in the courting trios (Table 5), the mutant male was frequently observed to court the \(XX\) \(dsx\)-mutant individual; these courtship events interfered with the behavior that this chromosomal female might have (at that moment) elicited from the "test" (i.e., wild-type) male. Nevertheless, the \(XX\) \(dsx\) type still tended to elicit more courtship than did the \(XY\) type (i.e., in the preference tests, though not so much in the single-pair ones).

**Rejection responses of \(dsx\) mutants:** If the only fly available is a chromosomal male, albeit a \(dsx\) mutant, then perhaps the courtship that fly elicits would be boosted in comparison with the trio tests (Table 5)—if, in the latter conditions especially, the \(XY\) fly exhibits rejection behaviors that are much more vigorous than those performed by the \(XX\) \(dsx\) type who is present as well. Indeed, normal \(D.\; melanogaster\) males reject in what seems to human observers to be more blatant manner than do virgin females (e.g., cf. CONNOLLY and COOK 1973; HALL 1978; PAILETTE et al. 1991). Thus, the rejection behaviors performed by \(dsx^+/dsx^+\) and \(dsx^{2}/Df\) flies in the preference tests were logged; these two \(dsx\) mutant types were superficially the most different from each other (referring to Table 4: \(XY\) \(dsx^2\) ≈ \(XX\) \(dsx^+\), whereas \(XY\) \(dsx^{23}\) < \(XX\) \(dsx^{23}\)—although the chromosomal female was courted significantly more in both cases; see legend to Table 5). The rejection behaviors exhibited by \(XY\) and \(XX\) mutant individuals in the presence of a (courting) wild-type male are in Table 6. Both \(dsx^+\) and \(dsx^{23}\) haploid-X flies rejected more frequently than did the corresponding \(XX\) \(dsx\) flies. The rejection percentages (see MATERIALS and METHODS) were: \(XY\) \(dsx^+\), \(53 \pm 9 (n = 6)\); and for \(XY\) \(dsx^{23}\), \(52 \pm 12 (n = 7)\), similar to what is quantified when wild-type males are courted (HALL 1978). [In the current experiments, none of the six \(XX\) \(dsx^+\) individuals showed any such rejection behaviors (i.e., these mutant individuals behaved as do normal females); and only \(1/10\) of the \(XX\) \(dsx^{23}\) flies displayed some wing flicking toward the male (Table 6).] In summary, a relatively higher level of courtship that might be elicited by a particular \(XY\) \(dsx\)-mutant type does not seem to occur because it is feeble at fending off the courter’s advances.

Nevertheless, rejection behaviors could explain why most \(dsx\) mutant (chromosomal) males were courted less than the mutant \(XX\) flies (Table 5). To look further into this matter, elicitations associated with \(XX\) and \(XY\) flies—expressing three of the \(dsx\) mutations used in this study—were recorded in situations where wing-flicking was impossible: one wild-type male was placed with either one \(XY\) mutant whose wings had been clipped off, or with one wingless \(XX\) mutant individual. The CI val-
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A. Villella and J. C. Hall

TABLE 5
Preference tests: normal males in the presence of XX and XY dsx mutants

<table>
<thead>
<tr>
<th>Courtees</th>
<th>dsx²/dsx¹ (n = 6)</th>
<th>dsx²/Df (n = 14)</th>
<th>dsx²/dsx¹ (n = 13)</th>
<th>dsx²/Df (n = 8)</th>
<th>dsx²²/Df (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>y/y² Y</td>
<td>y/y</td>
<td>y/y² Y</td>
<td>y/y</td>
<td>y/y² Y</td>
</tr>
<tr>
<td>CI</td>
<td>16 ± 6</td>
<td>27 ± 6</td>
<td>5 ± 2</td>
<td>18 ± 4</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Wing extension</td>
<td>2 ± 1</td>
<td>2 ± 1</td>
<td>0 ± 0</td>
<td>3 ± 1</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

A single wild-type male was placed in a chamber with an XX and XY dsx mutant simultaneously. The percent of time the male spent courting each dsx intersex was recorded and expressed as CI ± SEM. That "total courtship" parameter and the amounts of wing extension elicited (also expressed as a time-percentage) were logged separately. The dsx heterozygotes were generated from the cross: y/y; Df(3R)dsx'/TM6B × dsx-mutation/Balancer females. Test flies were 4–6 days old. From the CI data collected, a further "index" was computed to express the wild-type males' courtship preference; hence the CPI is the time spent courting the female/total time spent courting both sexes. CPIs were arcsine transformed, and a t-test was performed comparing these values against 0.5 (which would indicate no preference). The resulting P values for the experiments using dsx'/Df, dsx¹/Df, and dsx²²/Df flies were <0.0001; for dsx²/dsx²¹, P = 0.01; and for dsx²²/Df, P = 0.12.

P values were similar to the values in Table 4, involving the 4-day-old (winged) courtees (data not shown); that is, no marked shifts in the extent to which the XX vs. XY mutants were courted occurred after the flies' wings had been removed; and the different (elicitation-related) effects of varying dsx-mutant alleles still obtained (35 wingless courtees were observed, distributed rather equally amongst the six genotypes: dsx¹ homozygotes, dsx²²/Df and dsx²²/Df heterozygotes, XX vs. XY within each). Moreover, there was no general tendency for the flicking-impaired flies to be courted more vigorously than in the case of the intact mutants (data not shown; cf. Table 4). In summary, the attractiveness of haplo-X dsx mutants cannot be explained by general enfeeblement, such as an inability to reject courtship advances.

DISCUSSION

doublesex and the nervous system: We conclude that dsx mutations affect the nervous system’s control of reproductive behavior in a manner that would be analogous to this gene’s influence on a certain sex-specific feature of neural development (Taylor and Truman 1992). The current courtship studies showed that XY dsx males court in a quantitatively subnormal manner, which could not be explained by inbreeding depression or the effects of an eye-reducing mutation (both problems accompanied the seminal behavioral study of a dsx mutant: McRobert and Tompkins 1985). Our genetic controls included the application of several dm mutations almost always tested after outcrossing (to a dsx-deletion). The results of such tests implied allele specificity in terms of the quantitative decrements in courtship levels that were recorded (Table 1). Only two of these dsx mutations—which led to different behavioral abnormalities in other contexts as well (Tables 4 and 5)—have been characterized molecularly: dsx²² carries

TABLE 6
Rejection behavior of XX and XY dsx¹ and dsx²¹ mutants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of bouts wild-type → XY dsx rejects</th>
<th>No. of bouts XY dsx rejects</th>
<th>Percentage XY dsx rejects</th>
<th>No. of bouts wild-type → XX dsx rejects</th>
<th>No. of bouts XX dsx rejects</th>
<th>Percentage XX dsx rejects</th>
</tr>
</thead>
<tbody>
<tr>
<td>dsx²²/Df</td>
<td>1</td>
<td>22</td>
<td>16</td>
<td>73</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>14</td>
<td>5</td>
<td>36</td>
<td>14</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>7</td>
<td>3</td>
<td>43</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>12</td>
<td>8</td>
<td>67</td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>100</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>16</td>
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</tr>
<tr>
<td></td>
<td>7</td>
<td>7</td>
<td>3</td>
<td>43</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>dsx¹/dsx²¹</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>25</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7</td>
<td>2</td>
<td>29</td>
<td>5</td>
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Rejection behavior was measured as the number of times XX and XY/dsx flies rejected when approached by a wild-type male, divided by the total number of bouts directed to the mutant fly, then expressed as a percentage (5th and 8th columns from the left). Each number represents a different observation. These data came from the same recordings as those of Table 5.
a breakpoint within the locus (Baker and Wolfner 1988) and appears to be a loss-of-function allele (R. N. Nagoshi, cited in Taylor et al. 1994) and dsex is at least part accounted for by an intra-ORF amino-acid substitution, which abolishes the DSX protein's DNA-binding activities (Erdman and Burtis 1993) and could also cause all dsex function to be lost. Incidentally, dsex-encoded mRNAs continue to be detectable in adult flies (Baker and Wolfner 1988; Burtis and Baker 1989), albeit at diminished abundances in comparison with metamorphosing animals (and not at all in dsex adults: see above). Whereas many aspects of Drosophila sex determination and differentiation revolve around the organism's development, it is conceivable that dsex is also involved in the ongoing operation of the imaginal nervous system (as opposed to playing a physiological role only in terms of, for instance, yolk protein and seminal fluids: see Burtis and Wolfner 1992). In situ expression of doublesex in adults has been monitored only indirectly with respect to genic targets of DSX action in certain tissues (e.g., Coschigano and Wensink 1993; An and Wensink 1995): whether dsex products are present and functioning in the imaginal nervous system is an open question.

Perhaps the most striking effect of (all four) dsex mutations on male-like behavior was elimination of sine song, the humming sounds that are normally generated as a part of the song. This is a true courtship character in that the frequencies associated with sine-songs are species specific (e.g., Cowling and Burns 1981; Wheeler et al. 1988) and not very close to the wingbeat rates recorded during flight (e.g., Schilcher 1977). The elimination of courtship hums by dsex mutations makes this the first song-abnormal variant that specifically lacks this element of the male's acoustical output.

In contrast, pulse-song production by the XY dsex flies was inherently normal within a particular moment when they vibrate their wings (Table 2); although such song bouts occurred in a distinctly infrequent manner (Table 3) and as was implied by the foregoing discussion of Table 1). If a nonspecific maturational problem underlies all this mediocre courtship performance, and the impressive levels of courtship elicited by XY dsex flies in separate tests (see below), then aging of such mutant adults should have improved their performance and led to a decrease in their chromosomally inappropriate sex appeal; neither was the case.

One way of viewing the mutants' subnormal male-like courtship is that the behaviors seem to be triggered with a lower than normal probability within a given minute during which they are in the presence of (normal) females (implicit in the data of Tables 1 and 3). Thus, XY mutant individuals failed to sustain courtship bouts once they were initiated, exhibiting instances of orientation and following that commonly did not progress to wing extension and courtship singing. The poor ability of dsex mutants to initiate and sustain courtship bouts suggests that the brains of these flies might not have differentiated in a thoroughly male manner. This could be connected with hypothetical "command" signals emanating from the brain that may normally be generated when a male encounters a female and might occur in a mediocre or anomalous manner in an anterior CNS ganglion of a XY dsex fly. When such command is given in this mutant, it would be able sometimes to cause that individual to proceed well into the courtship sequence (in terms of the number of subsequent steps performed and their normal-appearing qualities). Yet, the command-signal might be weak, since the mutants so frequently fail to proceed past the orientation/following stage of courtship.

Would a courtship-related command-center problem involve an influence of dsex solely on the anterior CNS? Consider the courtship actions of gynandromorphs in this regard: if such a XY/XO fruitfly is to court a female and extend its wings at her, portions of such a mosaic's brain must be genotypically male (for a review, see Stiegel et al. 1984). That relevant dorso-posterior brain tissue (diagrammatically summarized by Greenspan 1995) could be the key for understanding the effects of dsex mutations on the initiation and sustaining of courtship bouts. But at least a contributory effect could emanate from the imaginal discs and elements of the PNS that are elaborated from them. Indeed, certain of the adult's sensory appendages, which of course develop from those larval discs, are known to possess sexually dimorphic elements (for a review, see Hall 1994). It is possible that a hypothetically intersexual, or perhaps more generally defective, quality of sensory structures is responsible for the subnormal courtships exhibited by XY dsex mutants. The reception and initial inputting of reproductively relevant sensory stimuli could therefore be mediocre in these mutants; so the signals to—as opposed to from—the command center would be feeble or incorrect. In this regard, a high proportion of gynandromorphs that court females do so at quantitatively subnormal levels, for reasons that could not be explained solely by the fact that many such mosaics fail to progress to late stages of the courtship sequence (Hall 1979). By a loose analogy to the peripheral (PNS) problems with which dsex mutants may be burdened, many of the "male-courting" gynandromorphs had quite a lot of external tissue that was diplo-X even though the (haplo-X controlled) "focus" for a gynandromorph exhibiting any male courtship, as opposed to ignoring the female altogether, is thoroughly internal (see above). Thus, the focus for normal, overall male-like, behavior could be a rather diffuse one—located within a particular brain region and in certain PNS cells developing within anterior imaginal discs. Note, in this respect, that most sensory axons directly enter Drosophila's CNS and ramify there. So even if the primary focus of the courtship-performance deficits observed for dsex mutants

\[ \text{dsex Courtship Defects} \]
With regard to its sexual differentiation, owing to the complete absence of sine-song production by XY flies, "maps" solely to the imaginal discs, the CNS could also be defective in such XY flies, by way of an inductive effect. Another of the gynandromorph-related findings may be relevant here: the induction of abdominal-muscular maleness, which is controlled by the haplo-X genotype of innervating axons (Lawrence and Johnston 1986; also see Currie and Bate 1995).

How the dsx mutations may "partially transform" imaginal disc derivatives is unknown, below the level of gross observation—let alone whether there could also be an inductive influence of "intersexual" PNS elements on their central targets. Thus, for now it is warranted to entertain the possibility that part of the doublesex gene's neural effects are exerted directly on the CNS—in particular, within the anterior ganglia relevant to courtship control (Greenspan 1995). An analogy is provided by dsx mutations' effects on a sexually dimorphic element of the abdominal ganglion's development, as alluded to above (Taylor and Truman 1992).

This phenotypic defect, along with what is suggested by the current behavioral results, militates against a completely separate, CNS-related and dsx-independent branch within the sex-determination hierarchy. That "new" branch has been suggested to be controlled in part by the action of fruitless (Hall 1994; Taylor et al. 1994). A further hint that the two branches are partly intertwined would seem to stem from the fact that one XY dsx mutant type sang in a marginally female-like manner when it was placed with a female (dsx<sup>15</sup>'s pulse-production rate was rather slow, as is seen in the songs of fru1 mutant males: Wheeler et al. 1989); and three of these mutant types acted more obviously like a fruitless mutant does when they were placed with other males (described in conjunction with the presentation of Table 4). However, the courtships directed at wild-type males could have an elicitation etiology: chromosomal males expressing these mutations are courted vigorously (Tallon and Hotta 1979; Tallon and Hotta 1979; Szabad and Fajszl 1982; Tompkins and Hall 1983). In this sense, one aspect of femaleness involves the aphrodisiac pheromones that are a prominent aspect of conspecific signals. In any case, the assumption is that dsx is concerned with some component of sex-specific pheromone production, owing to the high degree of courtship elicited by XY flies expressing mutations at this locus.

It should also be kept in mind that signals emanating from the fly's head may participate in certain detailed features of pheromone production: decapitated young flies remain pheromonally immature (Wicker and Tallon 1995), an issue to be taken up below. Thus, the hypothetical brain effects of dsx mutations could include pheromonal ones. In any case, the assumption is that dsx is concerned with some component of sex-specific pheromone production, owing to the high degree of courtship elicited by XY flies expressing mutations at this locus.

Yet, what are we to make of the fact that a dsx mutation left XY mutant adults male-like in terms of their aphrodisiac quality (Jallon et al. 1988)? Accepting the results of this experiment (which has not been extended to test the effects of more than one mutant allele), then it could be that dsx causes a mysterious pheromonal substance to be produced by mutant XY flies. "Mysterious" would include the following possibilities: (1) XY dsx flies are not pheromonally female-like at all but do in fact retain the chemical qualities of immature males (see above); the new finding in this study, revealing that this courtship property never really "matures" (Table 4), does not rule out the possibility that young-male substances are produced by 1-day-old XY dsx flies (i.e., as in wild type) and are simply produced chronically thereafter; nor do the data of Jallon et al. (1988) rule this out: the particular long-chain hydrocarbons that are known (in general) to be associated with immature D. melanogaster adults (e.g., Jallon et al. 1986; Wicker and Tallon 1995) were not examined and, in any case, have not been definitively shown to be the reason that young wild-type males elicit court-
ship. (2) dsx mutant, chromosomal males could generate an aphrodisiac that is not male-like—and not necessarily female-like either; for example, these mutants might produce a rather volatile substance with courtship-stimulating attributes, which has not been previously detected and would not have been looked for by Jallon et al. (1988) because it is not produced by any type of normal fly. (5) A similar (but less labored) hypothesis would be based on the results of some old olfactometric experiments (Shorey and Bartell 1970; Averhoff and Richardson 1974); these have suggested that pheromones more volatile than most, or any, of the compounds routinely assayed in these kinds of experiments (including Jallon et al. 1988) are a normal feature of D. melanogaster female sex appeal; thus, it could be that XY dsx flies are partly female-like for these hypothetical substances, which have not been identified and to which little or no scrutiny is applied in studies of the fly’s ‘‘cuticular hydrocarbons’’ (Jallon 1984; Ferveur et al. 1994).

Prospects: One aspect of the current results’ heuristic value, then, could be to prompt a further examination of the chemically related sex appeal of Drosophila adults. Findings that could stem from a more thorough analysis of the pheromonal profile for XY dsx flies would simultaneously ask more general questions about the chemical differences between males and females.

By the same token, we suggest that delving deeper into matters of neuronal sexual dimorphisms—about which the surface has barely been scratched (Technau 1984; Taylor and Truman 1992; Wang et al. 1994 Heisenberg et al. 1995)—may uncover interesting dsx-related abnormalities. If any could be found in the nervous system, such anatomic data would probably have to be generated against a background of knowing much more than we now do about how a male’s CNS and PNS differ from those of the female. Also, it will be important to determine if the fruitless mutants exhibit abnormalities in sexually dimorphic regions of the fly’s anterior ganglia, as well as (by implication) in the abdominal ganglion (Gailey et al. 1991; Taylor and Knittle 1995; cf. Lawrence and Johnston 1986; Currie and Bate 1995). We suggest from the present behavior-genetic findings that there may be cross-talk between the newly hypothesized branch of the SDH, as it would be influenced by fru expression (Taylor et al. 1994), and the more classic one that runs through doublesex.

We thank Barbara Berwald and Michael Bialek for technical assistance; Bruce Baker, Kenneth Burtis, and David Wheeler for discussions. We appreciate comments on the manuscript from Barbara Taylor and Lisa Riner. We are especially indebted to Tim Tully for vast amounts of help with the statistics. This work was supported by grants from the U.S. Public Health Service, GM-21473 and NS-3352.

LITERATURE CITED


Hall, J. C., 1979 Control of male reproductive behavior by the


Communicating editor: R. E. Denell