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Extended Reproductive Roles of the *fruitless* Gene in *Drosophila melanogaster* Revealed by Behavioral Analysis of New *fru* Mutants

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ABSTRACT

The *fruitless* mutants fru^3 and fru^4 were assessed for sex-specific reproductive-behavioral phenotypes and compared to the previously reported *fru* mutants. Among the several behavioral anomalies exhibited by males expressing these relatively new mutations, some are unique. fru^3 and fru^4 males are less stimulated to court females than fru^1 and fru^2. No courtship pulse song is generated by either fru^3 or fru^4 males, even though they perform brief wing extensions. fru^3 and fru^4 males display significantly less chaining behavior than do fru^1 males. The hierarchy of courtship responses by *fru* males directed toward females *vs.* males, when presented with both sexes simultaneously, is that fru^1 males perform vigorous and indiscriminant courtship directed at either sex; fru^4 males are similarly indiscriminant, but courtship levels were lower than fru^1; fru^2 males prefer females; fru^3 males show a courtship bias toward males. fru^3 and fru^4 males essentially lack the Muscle of Lawrence (MOL). On several reproductive criteria, there was no difference between *fru*-variant females and fru^+. The increases in phenotypic severity measured for the new mutants are discussed in the context of the emerging molecular genetics of *fru* and with regard to the gene's position within the sex-determination pathway.

THE *fruitless* locus in *Drosophila melanogaster* has long been associated with courtship abnormalities (Gill 1963; Hall 1978; Tompkins *et al.* 1980). The original fru^1-mutant (Gill 1963) was the result of an inversion breakpoint at cytogenetic map location 91B (Gailey and Hall 1989). The most salient of the fru^1 courtship defects are male behavioral sterility and high levels of courtship among males (Hall 1978; Gailey and Hall 1989). The fru^2 allele arose from the insertion of a tagged transposon at map location 91B [originally designated "ARO-1" (Moses *et al.* 1989)]. fru^2 males show a weaker expression of the *fru* syndrome in that they are often fertile with females (Gailey *et al.* 1991a). The genetic combination of two deletions, each with a breakpoint at 91B, also results in *fruitless* mutant phenotypes (Gailey and Hall 1989).

Two new *fru* mutants were reported, fru^3 and fru^4; they were isolated as male steriles mapping to 91B (Castrillon *et al.* 1993). When either of these transposon-tagged mutations is heterozygous with fru^1, the transheterozygous males are fertile. This suggestion of non-allelism suggests a complicated genetic locus, prompting a complete complementation analysis (this report).

A muscular abnormality within the mutant male abdomen (Gailey *et al.* 1991a) led to the hypothesis that the *fruitless* gene plays more than just a behavioral role in the sex-specific biology of Drosophila (Taylor 1992; Taylor *et al.* 1994). Thus *fru* has been hypothesized to act within the sex-determination hierarchy of gene actions and interactions (Taylor *et al.* 1994; Ito *et al.* 1996), and there is now good evidence that it does (Ryner *et al.* 1996). Furthermore, the gene seems primarily devoted to sexual differentiation within the fly's nervous system. Subnormal or otherwise anomalous *fru* gene action, therefore, can be rationalized as disrupting sex-specific behavior and also the formation of the aforementioned sex-specific structure, the Muscle of Lawrence (MOL), the development of which seems under the control of genetically male neurons projecting from the posterior-most ganglion of the central nervous system (Lawrence and Johnston 1986; *cf.* Currie and Bate 1995).

The infertility of fru^1 and some fru^2 males stems from the fact that their orientation to and following of females never progresses to attempts at copulation (*e.g.*, Gailey and Hall 1989). Males heterozygous for the aforementioned deletions are blocked at an even earlier stage of the courtship sequence (this report). With regard to attempted copulation, a structural impediment may exist in the mutant abdomen, although the MOL defect caused by the various *fru* mutations (including new data tabulated below) is not the etiology of behavioral sterility (Gailey *et al.* 1991a).

An additional courtship-behavioral defect reported for fru^1 is a rather mild abnormality in the male court-

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ship song (WHEELER *et al.* 1989). We surmised that a reexamination of this issue, including song recordings of the new mutants, might validate song defects as another feature of the *fru* syndrome.

Analysis of courtship and other sex-specific phenotypes of the new *fru* mutants has uncovered some striking behavioral differences among the mutant types. Some of our findings speak to the issue of whether individual courtship "steps" might be individually affected, or whether they can be altered as a nested set. Thus the findings presented here force us to modify and expand our view of the courtship-related roles played by this gene.

Cloning of *fru* (ITO *et al.* 1996; RYNER *et al.* 1996) may permit an understanding of the molecular and neurobiological etiologies of *fru*-mutant defects but will be crucial to bear the phenotypic complexities in mind. Another necessary connection between the molecular and the pheno-genetics comes from the fact that certain *fru* RNAs are expressed in the female CNS (RYNER *et al.* 1996). Thus we have now analyzed for the first time in detail elements of courtship and mating in fru^+ *vs.* *fru*-mutant females. Ultimately, the manner in which *fru* participates in programming flies of either sex to carry out their reproductive activities could be disclosed, given the fuller appreciation of *fru*'s phenotypic impact that is provided in part by the behavioral genetic findings we now report.

MATERIALS AND METHODS

Fly strains and crosses: Stocks of *D. melanogaster* were maintained at 25°, 70% relative humidity, on 12:12 hr light-dark (LD) cycles and grown in bottles on a sucrose-cornmeal-yeast medium containing the mold-inhibitor Tegosept. Cultures were cleared of adults after 5 days to avoid overcrowding, thus yielding test flies of uniform size. Males were collected 0–6 hr after eclosion under light ether anesthesia then aged individually in unyeasted food vials, except as specified. Females of the Canton-S strain were used in some behavioral tests; they were collected 0–6 hr after eclosion, grouped in food vials, then aged 1–5 days before testing with males. The $yellow^1$ body-color mutation was introduced into the genetic background for some tests, permitting males of fru^+ *vs.* *fru*-mutant genotypes to be discriminated visually. Flies from a *D. simulans* stock were used in some further behavioral observations involving fru^1 males.

The fru^1 stock used in this study was outcrossed by recombination for three generations, checked for *fru*-like behavior at each generation (see GAILEY *et al.* 1991a), and maintained with the balancer (Bal) *In(3LR)TM3, Stubble* (*Sb*). fru^2, also known as ARO-1, contains a w^+-marked *P*-element, inserted at 91B1-2 on chromosome *3* (MOSES *et al.* 1989); this strain had also been outcrossed by recombination to a "Cantonized" *white* stock for 11 generations (GAILEY *et al.* 1991a) and maintained as a homozygous stock.

The *P*-element-induced *fru* mutants fru^3 and fru^4 each contain a $P[lacZ, ry^+]$ transposon, which is inserted at the *fru* map site of 91B1-2; these two mutants were isolated by CASTRILLON *et al.* (1993) as male-steriles. Both insert-bearing lines were outcrossed to a $rosy^{506}$ stock for four generations; the third chromosomes were reextracted then maintained over the balancer *MKRS*. All observations in this study involved *fru* flies collected from these outcrossed stocks. The following deletions (*Dfs*) were used in combination with various *fru* mutations: $Df(3R)Cha^{M5}$ and *Df(3R)P14* (hereafter called Cha^{M5} and *P14*), as well as $Df(3R)Cha^{M7}$; these uncover the effects of the fru^1 and fru^2 mutations, whereas *Df(3R)148.5-1* does not (GAILEY and HALL 1989). The $Cha^{M5}/P14$ heterozygous male is phenotypically *fru* and is often called the "double-deletion" *fru* mutant type (below).

For viability tests individual female parents, homozygous for fru^3 or fru^4, were crossed to fru^3/Bal or fru^4/Bal males. Adults were cleared from the food vials after 1 week, and then homozygous *vs.* heterozygous adult progeny were counted for 4 consecutive days. The control counts came from crosses of wild-type females to either fru^3/Bal or fru^4/Bal males.

Analysis of *fru* variant male courtship behavior: *Audio-video recordings and song analyses:* Recordings were carried out as in BERNSTEIN *et al.* (1992), and VILLELLA and HALL (1996). Song pulses were logged by selecting trains consisting of more than three pulses; 15–20 trains were logged for the song of most males (there were only a few exceptions in which some of the songs had <15 trains for the recording period). For Interpulse Intervals (IPIs), a mean-of-means was calculated for each genotype (see Table 3); the cutoffs used were from 15–100 msec; an IPI > 100 msec was considered to be an intertrain interval. Median IPIs were calculated (for male-with-female recordings only: see Table 3) using Microsoft Excel. A mean-of-median IPIs was calculated for each genotype. Because the amplitude for some of the *fru*-mutant songs was relatively low, the background scaling level was reduced from 2 to 1 in this study (see WHEELER *et al.* 1989, and BERNSTEIN *et al.* 1992 for definitions and details). Other song parameters measured were as follows: cycles per pulse (CPP), intrapulse frequencies (determined from Fast-Fourier-Transform analysis of each pulse), and FFT width. Sine-song frequencies (*cf.* WHEELER *et al.* 1989) were estimated by counting the number of zero-line crossings for a given time interval, then dividing by the time (giving a frequency value). Between 10–25 of these measurements were taken for a given fly; a mean was then calculated for each male's song performance. This led to a mean-of-means for males of a given genotype (see legend to Table 3).

Fly-pair observations: An individual *fru* male (7–14 days post-eclosion) was placed in a small chamber (VILLELLA and HALL 1996) with either another male of the same genotype and similar age, or with a wild-type virgin female (1–5 days old). Males were aged longer for these observations since younger fru^1 males (3–5 days old) do not court as much as when older (WHEELER *et al.* 1989). The flies were video-recorded for 5–10 min. In male-pair recordings, the first male to exhibit a courtship bout of at least 20 sec became the male whose Courtship Index (CI) was determined, from viewing a playback of the tape and logging it with a digital event-recorder as in VILLELLA and HALL (1996). The CI is a cumulative score of all the courtship behaviors, including tapping, orientation, following, wing extension, and attempted copulation (*e.g.*, SIEGEL *et al.* 1984; TOMPKINS 1984, 1989; HALL 1994; COBB and FERVEUR 1996). For some of these observations the courtship-rejection behavior of wing flicking was quantified (*cf.* HALL 1978). In recordings of male-female pairs, the CI was determined as the percentage of time the male courted the female. A Wing Extension Index (WEI) was calculated as the fraction of time either wing was held angular to the body (~45–90 degrees). Some courtship testing of pairs to determine whether each "step" in the courtship sequence occurred, involved examination of the flies in small plastic chambers (*cf.* HALL 1979) containing food, under the microscope at

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25×. These pairs were observed for 1–2 hr per day over the course of 2–3 days.

Wild-type males with mutant males: In courtship-elicitation tests, a wild-type male (5–6 days post-eclosion) was placed in a depression well (see below) with an ether-anesthetized *fru*-variant male (4–6 days old). Four such pairs were observed simultaneously. A CI was determined as the amount of courtship directed toward the etherized fly (*cf.* GAILEY and HALL 1989) until it awakened or 10 min had elapsed. As a control, the CI of wild-type males with etherized virgin females was recorded. In another set of observations, the CI of *fru* males with y^1-marked fru^+ males was determined as above, but in this case the fru^+ males were not immobilized.

Ménages-à-trois observations: To determine if a male would exhibit a courtship preference for a partner of one sex or the other, a *fru* male (7–14 days old) was presented simultaneously with a male of the same genotype and a wild-type virgin female, and then the trios were videotaped for 5–10 min. As in fly-pair recordings, the first male to sustain a ~20-sec courtship bout became the male for whom two CIs were determined, one with the other male and one with the female. For fru^3 and fru^4 determinations, only one of the two males tended to be the domineering courter, with the other male performing very little courtship. In contrast, for fru^1 and fru^2, both males were generally active courters (see legend to Table 5). In addition, in each test two WEIs were recorded, one involving courtship directed at the other male and the other at the female.

Another series of tests was carried out to determine whether a fru^1 male could distinguish a wild-type male from either another *fru* male or a female. This was accomplished by comparing CIs of test fru^1 males in trios of two fru^1 males and a wild-type male, *vs.* trios consisting of a fru^1 male, a wild-type male, and a female. Only fru^1 males were tested in this latter type of preference tests, because fru^2 males court females more actively than males; also fru^4 and especially fru^3 are too "courtship sluggish" in these sorts of trios to generate meaningful data (see RESULTS).

A Courtship Preference Index (CPI) was calculated using each CI in Table 5 (*cf.* VILLELLA and HALL 1996), except that in this study we subtracted 0.5 (which would indicate no preference) from the ratio. These redefined CPIs represent the time spent courting a female/total time spent courting both sexes, minus 0.5 (except for trios involving two fru^1 males and a wild-type male; here, the second fru^1 male was used as the female portion in calculating the CPI). A CPI of zero means that there was no preference; a positive value indicates preference toward the female, and a negative one indicates a preference toward the male. If a fly did not court at all (CI = 0), then a CPI could not be calculated for that fly; therefore, the number of observations used in the statistical analysis (CPIs) is different from the CIs presented in Table 5.

Male behavioral sterility: fru^3 and fru^4 homozygous males were collected at eclosion, grouped (<10 males per food vial), and aged for 6–10 days. Single males were placed individually in food vials with three to four wild-type virgin females. The presence or absence of progeny was scored 10 days later. Vials with no progeny but in which the male was dead at the tenth day were excluded.

Courtship chains formed by mutant males: Observations of courtship chaining behavior were made at 25°, 70% relative humidity and in the late afternoon-early evening of their LD cycle (flies show relatively high levels of overall activity before lights off in a 12:12 hr LD cycle; *cf.* HAMBLEN-COYLE *et al.* 1992). A Chaining Index (ChI) was calculated as the percentage of time males spent in group courtships during a 10-min observation period. A chain was thus defined as an interacting group of at least three males, of the eight placed in the vial, courting one another (not necessarily in a linear manner: see RESULTS).

ChIs were measured for a series of 10-min observation periods on successive days, which became important with regard to distinguishing chaining behavior of the new mutants from the original one. Thus, a putative age effect on chaining was assessed by storing males individually in unyeasted food vials for 6–7 days before grouping. At this time (called day 1, with respect to the observations), eight males were placed in a food vial, and the ChI was determined from test day 1–5. On test day 1, the males were grouped for at least 2 hr before observations were made for that day. In a separate set of experiments, the males were put together in groups soon after eclosion, monitored for possible intermale courtship on that day, and on subsequent days as well (see RESULTS).

To test for the impact of wing behavior on chaining, males' wings were clipped with fine scissors at the time of collection. These flies were aged individually before grouping. Then a ChI for the wingless males was determined for consecutive days, as described above.

Interspecific pairings: fru males were observed with *D. simulans* males and females to determine how promiscuous the mutant might be in its courtship propensities. In 10-min observations, a fru^1 male (4–7 days old) was placed in a chamber with a *simulans* virgin female or *simulans* male. fru^1/Cha^{M5} males were also observed in these tests. To distinguish one male from the other, one of them was marked on one wing with a fine-tip Sharpie marker (this was accomplished under light ether anesthesia the day before testing). A CI was determined from these observations as the percentage of time the *fru*-variant male courted the *simulans* female or male. Control observations were cases in which both individuals were fru^1 or fru^1/Cha^{M5}.

Conditioning with immature males: These experiments were performed essentially as in GAILEY *et al.* 1991b (also see: GAILEY *et al.* 1982, 1986). These tests concentrated on fru^1 males, because they showed the highest courtship levels in most circumstances. Test males were aged individually for 5–8 days, then "conditioned" (as described below) in the presence of an immature male (<6-hr-old).

For these conditioning experiments, young males (<6 hr post-eclosion) were aspirated out of imago-containing culture vials and were used within 1–2 hr after collection. Conditioning experiments involved placing the test male and the immature male into a well (2 cm diameter) for 30 min; CIs were determined for successive 10-min intervals (*cf.* GAILEY *et al.* 1991b; GRIFFITH *et al.* 1993, 1994). The test male was then transferred into a new chamber with a naive, active, immature male, and another CI was determined for a 10-min test period. As a control, males were placed in empty chambers for 30 min (pseudo-training), then transferred into a new chamber for testing.

Locomotor activity of *fru*-variants: All *fru* homozygous types and the double-deletion males were tested for short-term and long-term activity.

Short-term activity: Males were collected at eclosion and aged individually for 7–8 days. In a given test, a single male was placed in a cylindrical plexiglass chamber (1 cm diameter × 6 mm height), containing on its floor a filter paper with a single bisecting line; the number of times the male crossed the line in a 3-min observation period was counted (*cf.* KULKARNI and HALL 1987).

Long-term activity: Locomotion was monitored for days as in HAMBLEN *et al.* (1986). Flies were entrained in a 12 hr light:12 hr dark cycle (LD) for 5–7 days, then kept in constant darkness (DD) for an additional 8–9 days. Data were recorded as average activity per half-hour, per fly (as in HAMBLEN-COYLE *et al.* 1989), during constant darkness (DD); the activity-event

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counts were separated into two groups: days 0–4 and 4–8 (or 4–9). For a given genotype, an average activity was calculated for both such time segments.

Flight tests: Males were tested in both tethered and free flight. To monitor tethered flight (method: BARNES and LAURIE-AHLBERG 1986) with a male under light ether anesthesia, a wire connected to a cork was glued (Permount: Fisher Scientific) to the dorsal side of the fly's thorax. On recovery from the ether, a gentle puff of air was directed at the anterior end of the fly, stimulating flight. The hanging fly, vibrating its wings, was placed into a temperature-controlled, water-jacketed chamber (outside dimensions: 10 cm long × 5.5 cm high × 5.5 cm deep) with a seal formed by the cork. The chambered fly had its wing-beat frequency recorded using a strobe light. For a given tethered fly, the strobe rate was adjusted until a single image of the wing appeared for at least 2–3 sec, then recorded (in cycles/sec). Two to three replicate readings were taken for a given fly. An average was then calculated for each fly followed by a mean for each genotype.

To test overall flight performance, the cylindrical flight tester described originally by BENZER (1973) was used (*cf.* KULKARNI and HALL 1987). This consisted of a 500-ml graduated cylinder in which the inside wall was coated with paraffin oil. Flies were dumped into the top of the cylinder. Genetically normal Drosophila tend to initiate flight immediately when introduced to the cylinder and hence get stuck on the oil as they strike the walls of the cylinder. The number of flies stuck at 5 cm intervals down the cylinder was counted and plotted (see RESULTS).

Genetics and anatomy of the MOL: Techniques for the dissection and visualization of the abdominal musculature were as described in GAILEY *et al.* (1991a). Briefly, the dorsal abdominal cuticle was isolated by dissection, then dehydrated, cleared in methyl salicylate, and permanently mounted. The muscular structure was visualized by its birefringence in polarized light. Given the range of *fru*-induced MOL abnormalities observed by GAILEY *et al.* (1991a), individual MOL scores were generated from these observations, based in the main more on refined criteria than in previous studies (see legend to Table 8 for details).

Female courtship and reproduction: Pairs consisting of wild-type males with homozygous *fru* females were observed until they either mated or until 8–20 min had elapsed (two different experiments). If a pair did not mate within 20 min, the flies were transferred to a food vial and checked 7–10 days later for progeny. The observation chambers were formed by covering the well of the plate with a microscope slide (see GAILEY *et al.* 1986, 1991a), with four test-pairs observed simultaneously.

Mating-initiation latencies were recorded in the same conditions as above for *fru*-variant and wild-type females. These were measured as the time elapsed between initially pairing the test female with a male and the beginning of copulation (20 min observation periods were used to measure the latencies). If a pair did not mate within 20 min, the fly was not used in calculating a mean latency value; however, the female-male pair was placed in a food vial to score for subsequent appearance of larvae.

A second set of experiments was performed to examine further the fertility of *fru* females. Single homozygous fru^3 and fru^4 virgin females were placed in a food vial, each with a wild-type male; the pair was observed until they mated (all mated within ~30 min); each mating male was then transferred into a new food vial with a single wild-type female (this served as a control for male fertility). The presence of progeny in all such cases was scored 7–10 days later.

Statistics: Courtship indices were subjected to arcsine square root transformations (*cf.* VILLELLA and HALL 1996), then the studentized residuals were tested for normal distribution approximations (SOKAL and ROHLF 1995). This was accomplished using JMP (version 3.1.5 for the Macintosh). In brief, application of that software involved saving the "studentized residuals" (SOKAL and ROHLF 1995); these represent the observed values (transformed ones, except for when specified), minus the mean value for that group (or genotype), divided by their standard error. For each major category of observation (referring, for example, to to all the data indicated in a given table within results), the studentized residuals were plotted together; the distributions (printed out via JMP) appeared to be normal, but normality was tested for explicitly using the Shapiro-Wilk W Test. From the seven relevant (large) data sets, two were normal (at an α of 0.05) and five were near normal (two with a P value that almost made the cut-off just stated). Nevertheless, we proceeded with the parametric statistics, given that that approach should not lead to erroneous statistical conclusions (compared to nonparametric methods) but is more sensitive in the detection of small intergroup differences; see Chapter 13 in SOKAL and ROHLF (1995) for a discussion of this issue, including the salutary consequences of underlying assumptions for application of parametrric methods not being met in the strictest sense, which they were not in the current experiments (as just described).

For the data from long-term activity monitorings, natural-log transformations were used (SOKAL and ROHLF 1995) and then tested for normality (as above). Analyses of variance (ANOVAs) were performed using JMP software. For planned comparisons, critical P values (α's) were adjusted for experiment-wise error (*cf.* SOKAL and ROHLF 1995) and are indicated in this section (below).

Courtship behavior of fru-variant males: Transformed CIs from 38 different groups (see Table 1, in which each row represents a different genotype, each of which involved two different courtship conditions: males courting males, and males courting females) were subjected to a one-way ANOVA with group [$F_{(37,603)} = 17.90$, $P < 0.0001$] as the main effect. Eight planned comparisons between wild type and other control groups [wild type, $fru^1/+$, $fru^2/+$, $fru^3/+$, and $fru^4/+$ (for males courting males and separately for males courting females) were deemed significant if $P < \alpha = 0.006$ (*cf.* SOKAL and ROHLF 1995); no significant differences were detected for any control groups. Therefore, control groups were combined for each courtship condition (male with male and male with female), and transformed CIs were subjected to a second one-way ANOVA with group (a total of 30 groups after combining controls) [$F_{(29,611)} = 23.00$, $P < 0.0001$] as the main effect. Fifteen subsequent planned comparisons were deemed significant if $P < \alpha = 0.003$ between *fru* variant males courting males *vs.* females, and Tukey-Kramer unplanned comparisons ($\alpha = 0.05$) between particular groups were made; such results are summarized in the legend to Table 1.

Eight planned pairwise comparisons between control groups (same genotypes as above) on transformed WEIs, following a one-way ANOVA with group [$F_{(37,603)} = 59.39$, $P < 0.0001$] as the main effect, indicated no differences in WEIs for *fru*-variant/+ *vs.* wild-type males (all Ps $> \alpha = 0.006$). Since there was no difference in WEI values among these fru^+-carrying control groups, all were combined into one of two groups to compare data from males courting males *vs.* males courting females. A second one-way ANOVA with the new groups (a total of 30 after combining) as the main effect was performed on transformed wing extension percentages [$F_{(29,611)} = 75.16$, $P < 0.0001$]. Sixteen planned comparisons between particular groups revealed significant differences (if Ps $< \alpha = 0.003$) in wing extension levels, as summarized in the legend to Table 1.

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Locomotor activity: A one-way ANOVA on untransformed short-term activity with genotype as the main effect revealed significant differences between genotypes [$F_{(5,90)} = 3.62$, $P = 0.005$]. Subsequent unplanned Tukey-Kramer ($\alpha = 0.05$) pairwise comparisons are summarized in the legend to Table 2A. Transformed long-term activity values (natural-log transformations) were subjected to a two-way ANOVA with fly nested within genotype × days [either days 0–4 or days 4–8 (or 4–9)] as the repeated measure [$F_{(82,67)} = 28.32$, $P < 0.0001$]; this revealed significant differences in activity among genotypes ($P = 0.0474$) but no difference in how active the flies were at days 0–4 compared to days 4–8 (or 4–9) ($P = 0.7017$); there was no interaction component between genotype × days ($P = 0.3057$). Ten subsequent planned pairwise comparisons ($\alpha = 0.005$) led to results indicated in the legend to Table 2B.

Preferences of fru-variant males toward one sex or the other: Untransformed CPIs (see MATERIALS AND METHODS) from nine genotypes (with each row in Table 5 representing a different one) were subjected to a one-way ANOVA with genotype [$F_{(8,114)} = 12.96$, $P < 0.0001$] as the main effect. *t*-test comparisons and *P* values associated with each mean CPI indicated whether there was a significant preference for one sex or another; these numerical findings are summarized in the legend to Table 5.

Chain formation of fru variant males: Transformed ChIs from homozygous and heterozygous *fru* variants (data in Figure 3 and Table 6) were subjected to a two-way ANOVA with fly nested within genotype × day as a repeated measure [$F_{(506,1060)} = 11.15$, $P < 0.0001$] as the main effects. There was a significant interaction component between genotype × day [$F_{(88,1060)} = 2.16$, $P < 0.0001$]. Twenty-six subsequent pairwise comparisons were made (these were deemed significant if Ps $< \alpha = 0.002$) between particular groups, and the outcome is summarized in the legend to Figure 3 (also see text of RESULTS).

Songs produced by fru variant males: Interpulse intervals (IPI), cycles per pulse (CPP), intrapulse frequencies, and FFT widths—from 10 genotypes [fru^1, $fru^1/P14$, fru^1/Cha^{M5}, fru^2, fru^2/Cha^{M5}, fru^1/fru^2, fru^1/fru^3, fru^1/fru^4, fru^2/fru^3, and fru^2/fru^4] and two differently sexed objects of courtship—were subjected to two-way ANOVAs, with genotype × object as the main effects (controls were excluded since only one sex object was analyzed); these results are summarized in the legend to Table 3. One-way ANOVAs on IPI, CPP, intrapulse frequencies, and FFT width (with 25 groups) revealed significant differences among groups: for IPI, [$F_{(24,99)} = 5.97$, $P < 0.0001$]; for CPP, [$F_{(24,99)} = 2.17$, $P = 0.0041$]; for intrapulse frequency [$F_{(24,99)} = 2.84$, $P = 0.0001$]; and for width of the FFT [$F_{(24,99)} = 3.47$, $P < 0.0001$]. Four subsequent planned comparisons ($\alpha = 0.013$) between *fru*-variant/+ males and wild-type males revealed no significant differences; therefore, the data from these fru^+-bearing controls were combined together into one new group. A second set of ANOVAs was performed on the same song parameters as above with the new groups as the main effect. Subsequent planned pairwise comparisons ($\alpha = 0.005$) are summarized in the legend to Table 3. Median IPIs were subjected to a one-way ANOVA [$F_{(14,58)} = 6.21$, $P < 0.0001$] with genotype as the main effect (for male-with-female observation only); four subsequent planned comparisons ($\alpha = 0.013$) revealed no differences in median IPI between wild-type and *fru*-variant/+ controls; therefore these fru^+-associated song values were combined into one control group. A second one-way ANOVA revealed differences in median IPI among genotypes [$F_{(10,62)} = 7.88$, $P < 0.0001$]; the results of the subsequent genotype-based comparisons are in the legend to Table 3.

Free flight: Transformed mean percentages for fru^1, fru^3, fru^4, *M5/P14*, *M5/+*, *P14/+*, *In(2LR)O*, *Cy* (*Curly* wing) (=*CyO*), and wild-type flies found at the first interval only (5 cm height; see Figure 1) were subjected to a one-way ANOVA with genotype [$F_{(7,29)} = 13.66$, $P < 0.0001$] as the main effect. Subsequent Tukey-Kramer unplanned pairwise comparisons ($\alpha = 0.05$) are summarized in the legend to Figure 1.

fru courtship toward D. simulans: Transformed CIs were subjected to a one-way ANOVA with group [$F_{(19,183)} = 8.11$, $P < 0.0001$] as the main effect. Subsequent planned comparisons (all Ps $> \alpha = 0.003$) between particular groups are in the legend to Table 7.

Conditioning with immature males: Transformed CI means for three 10-min periods of a 30-min training period, in which a test male was in the presence of an immature male, were subjected to a modified two-way ANOVA (as in TULLY and GOLD 1993), with fly nested within genotype and conditioning period (time 10 min; t20; and t30: see Figure 4A) as the main effects; these are summarized in the legend to Figure 4. Two-way ANOVAs were performed to determine the effects of conditioning with an immature male compared to controls; the latter males were left alone for 30 min before testing (see Figure 4B). The results of these statistical tests are in the legend to Figure 4.

Female behavior: Transformed CIs were subjected to a one-way ANOVA with genotype [$F_{(8,100)} = 1.56$, $P = 0.1473$] as the main effect. Natural log transformed latencies were subjected to a one-way ANOVA with genotype [$F_{(8,64)} = 2.84$, $P = 0.0093$] as the main effect. Eight subsequent planned comparisons for CIs and latencies were deemed significant if $P < \alpha = 0.006$; such results are summarized in the legend to Table 9.

RESULTS

Early stages and general levels of courtship exhibited by *fru*-mutant males: *Tapping:* Some of the more subtle courtship steps were not scrutinized in earlier studies of *fruitless* mutants. Thus, in microscope-aided observations, the new mutant-type males were first examined for their ability to initiate courtship, signaled by tapping of the female with the forelegs. Nine out of 38 fru^3 males (either homozygotes or in combination with Cha^{M5} or *P14*) clearly tapped the female. For fru^4, all 16 males (fru^4/Cha^{M5} and $fru^4/P14$) that exhibited any courtship (males that showed no interest were not included) exhibited tapping behavior. By comparison, all nine fru^1 males (homozygotes or $fru^1/P14$) tested exhibited tapping. Five of five fru^2 homozygotes performed this step. Predictably, all wild-type males tapped the female during the early moments of courtship ($n = 7$).

Wing extension: fru^3 and fru^4 showed relatively rare and unsustained wing extensions compared with the "pre-song" behavior of fru^1, fru^2, and various kinds of fru^+-bearing males (Table 1). The subnormal wing extensions associated with fru^3 and fru^4 were also observed when these mutations were heterozygous with Cha^{M5} and *P14*, or with each other (fru^3/fru^4).

Courting females vs. other males: These and other observations are summarized in Table 1. The behavioral tests involved determining the percentages of time *fru*-variant males spent courting, wing-extending, or both in the presence of other males (of the same genotype) or with wild-type females. Homozygous fru^1, fru^2, and fru^4 males courted other males of their respective types or

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TABLE 1

Courtship of *fru* variant males toward other mutant males or females

Genotype	Two same-genotype males: All courtship CI (%)	WEI (%)	n	Male + wild-type female: All courtship CI (%)	WEI (%)	n
fru^1	46 ± 3	19 ± 2	35	56 ± 5	31 ± 3	30
fru^2	35 ± 5	19 ± 4	24	54 ± 5	44 ± 4	22
fru^3	32 ± 5	1 ± 0	42	16 ± 5	0 ± 0	25
fru^4	41 ± 4	2 ± 1	37	31 ± 5	2 ± 1	29
fru^1/Cha^{M5}	42 ± 7	18 ± 5	12	39 ± 8	19 ± 5	13
$fru^1/P14$	44 ± 6	23 ± 6	7	53 ± 7	32 ± 8	9
fru^3/Cha^{M5}	20 ± 5	0 ± 0	18	15 ± 8	1 ± 1	13
$fru^3/P14$	47 ± 8	4 ± 2	15	19 ± 8	1 ± 0	15
fru^4/Cha^{M5}	29 ± 6	3 ± 1	23	24 ± 7	1 ± 1	20
$fru^4/P14$	22 ± 6	1 ± 0	17	9 ± 4	1 ± 1	16
fru^3/fru^4	47 ± 6	1 ± 0	20	25 ± 8	1 ± 0	15
fru^3/fru^1	48 ± 5	17 ± 4	17	78 ± 6	61 ± 6	14
fru^4/fru^1	53 ± 9	13 ± 5	10	88 ± 3	73 ± 3	8
$Cha^{M5}/P14$	8 ± 6	0 ± 0	8	0 ± 0	0 ± 0	8
Wild type	4 ± 1	0 ± 0	15	88 ± 2	50 ± 4	13
$fru^1/+$	4 ± 1	0 ± 0	11	81 ± 4	43 ± 5	13
$fru^2/+$	3 ± 1	0 ± 0	10	84 ± 3	49 ± 4	8
$fru^3/+$	2 ± 1	0 ± 0	15	88 ± 2	59 ± 3	16
$fru^4/+$	1 ± 0	0 ± 0	12	89 ± 2	45 ± 5	10

Test males were aged individually 7–14 days then placed in a small chamber with either another male of the same genotype or with a Canton-S wild-type virgin female (1–5 days old). Flies were recorded on a videotape for 5–10 min (or until the male mated in certain cases). The CI (courtship index) ± SEM represents the percentage of time that the test male spent courting another male or female, in a given observation period. The WEIs, or percentages of time during which wing extensions occurred, are indicated separately (second row of data for each genotypic entry). For male-male tests, each CI is the percentage of time male 1 (the first male to initiate courtship for more than 20 sec) courted male 2. For male-female tests, the CIs are the percentages of time the male spent courting the female. n = the total number of observations per genotype. The wild-type males were from a Canton-S strain, which was also the source of the + third chromosomes in the heterozygous control tests. A total of 19 genotypes were observed in two different sets of experimental conditions: males courting males and males courting females, giving a total of 38 groups (a subset of the data from the *fru*-mutant homozygotes, fru^3/fru^4 transheterozygote, and the wild-type control males are from Ryner *et al.* 1996). Planned comparisons of groups following a one-way ANOVA on transformed CIs (see materials and methods) indicated no significant differences between *fru*-variant/+ and wild-type controls in male-male, as well as male-female observations (all P's > α = 0.006; see materials and methods). Therefore, these controls were combined into one control group for each courtship condition: male-with-male and male-with-female observations (yielding a total of 15 genotypes). The new 30 groups were subjected to a second one-way ANOVA on transformed CIs (see materials and methods). Consequently, Tukey-Kramer unplanned pairwise comparisons revealed no significant differences among homozygotes fru^1, fru^2, fru^3, and fru^4 males courting other males (all P's > 0.05). Heterozygous combinations of fru^3 and fru^4 with either Cha^{M5} or *P14* were not significantly different from fru^1 over the same deletions in males courting other males (all P's > 0.05). The transheterozygote fru^3/fru^4 courted males no differently than fru^1, fru^3, and fru^4 (all P's > 0.05). All homozygous *fru* variant males courted other males at much higher levels than controls (all P's < 0.05). Tukey-Kramer unplanned pairwise comparisons revealed that fru^1 males courted wild-type females significantly more than fru^3 and fru^4 (all P's < 0.05), but not different from fru^2 males (all P's > 0.05); however, both fru^1 and fru^2 males courted females less than controls ($P < 0.05$). In addition, homozygous fru^3 and fru^4, heterozygous with Cha^{M5} or *P14*, and the transheterozygote type fru^3/fru^4 led to much less courtship of females than in the case of controls (all Ps < 0.05). Fifteen planned pairwise comparisons (deemed significant if $P < \alpha = 0.003$) revealed that homozygous fru^1, fru^2, and fru^4 courted other mutant males or wild-type females at similar levels (all P's > 0.003) compared to wild-type males, which are extremely biased to court females as opposed to males ($P < 0.003$). Homozygous fru^3 males courted other mutant males at higher levels compared to females ($P < 0.003$). Planned comparisons of groups (same as above) following a one-way ANOVA on transformed wing extension percentages indicated no differences in levels of wing extensions performed by *fru*-variant/+ controls and wild-type males (all P's > α = 0.006; see materials and methods). Therefore, as above, all wing-extension percentages for controls were combined into one group for each condition: males courting males and males courting females. A second set of planned comparisons of wing extension percentages revealed that fru^1 males displayed many more wing extensions than fru^3 and fru^4 when courting other mutant males (all P's < α = 0.003). fru^1 was the same as fru^2 ($P > 0.003$) in the levels of wing extensions displayed when courting other males, but exhibited fewer wing extensions toward females ($P < 0.003$). fru^1 and fru^2 exhibited less wing extension behavior in the presence of other males compared to females (all P's < α = 0.003), whereas fru^3 and fru^4 displayed few wing extensions toward either sex (P's > 0.003).

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wild-type females at the same levels (all P's $> \alpha = 0.003$); whereas, *fru*[3] males showed more interest toward males than females ($P = 0.0028$; see Table 1). *fru*[3] and *fru*[4] homozygotes gave low WEIs (Wing-Extension Indices) when these males were orienting toward or following flies of either sex (see second data column in Table 1).

Regardless of the allele, homozygous *fru*-variant males courted other males of the same respective genotype with similar intensities (from Tukey-Kramer unplanned comparisons: all P's ≥ 0.05). All the homozygous mutant-based CI values were substantially greater than those recorded for control *fru*[+] males courting other *fru*[+] males (Table 1). The phenotype involving *fru* males courting other males was found to be recessive in that all *fru*-variant/+ males behaved similarly to wild type in the presence of other males (Table 1).

Although *fru*[1] males courted females quite vigorously, these levels were significantly different from (lower than) the behavior of *fru*[+] males ($P < 0.05$ for Tukey-Kramer unplanned comparisons: see MATERIALS AND METHODS). *fru*[3] and *fru*[4] males courted females significantly less than did *fru*[1] or *fru*[+] controls (all P's < 0.05). *fru*[2] males courted females at a level similar to those for *fru*[1] and *fru*[4] but at a higher level than for *fru*[3]; however, *fru*[2] courtship directed at females was significantly lower than the behavior of *fru*[+] males courting females ($P < 0.05$).

Heterozygous combinations of *fru*[1]/*fru*[3] or *fru*[1]/*fru*[4] males courted each other as vigorously as in the case of homozygous *fru*[1]-male pairs (all P's > 0.05). These transheterozygous types courted females as vigorously as did *fru*[1] or control males (all P's > 0.05). The behavior of heterozygous *fru*[3]/*fru*[4] males was very similar to that of *fru*[3] or *fru*[4] homozygotes. When either *fru*[3] or *fru*[4] was heterozygous with either of the two *fru* deletions (Cha^{M5} or P^{14}), the heterozygotes courted other males or females in a similar manner to the performance of the homozygous *fru*[3] or *fru*[4] males (all P's > 0.05). Double-deletion males resulted in courtship-sluggishness (Table 1); these males exhibited little courtship toward each other or females (no interest towards the latter).

General activity and viability: Are the subnormal levels of courtship exhibited by certain *fruitless* mutants due to generalized debilitation? In tests of general locomotor-activity, the various *fru* mutants were not distinctly sluggish in either short-term or long-term counts and automated monitorings (Table 2: see its legend and MATERIALS AND METHODS for statistical details). However, the double-deletion males were less active in short-term activity observations ($P < 0.05$), locomoted normally in long-term activity tests (Table 2).

In viability tests, the relevant progeny counts (see MATERIALS AND METHODS) for *fru*[3] gave 111 *fru*[3] homozygotes compared to 128 balancer-bearing sibling heterozygotes; for *fru*[4], the corresponding numbers were 133 *vs.* 181. In control counts (from crosses in which one balancer-bearing parent carried a wild-type-derived third chromosome), the numbers were 82 *fru*[3]/*fru*[+] *vs.* 98 sibling balancer progeny, and 103 *fru*[4]/*fru*[+] *vs.* 107 sibling balancer progeny. Setting the non-balancer/bal-

TABLE 2

General activity of *fru* variants

Genotype	No. line crossings/3 min ± SEM[a]
A. Short-term activity	
fru[1]	64 ± 10 (7)
fru[2]	72 ± 5 (18)
fru[3]	64 ± 5 (18)
fru[4]	59 ± 7 (18)
Cha^{M5}/*P14*	50 ± 5 (17)
wild-type	81 ± 5 (18)

Genotype	Average activity ± SEM[a,b] 0–4 days	4–8 or 4–9 days
B. Long-term activity		
fru[1]	67 ± 8 (15)	68 ± 10 (15)
fru[2]	165 ± 22 (16)	180 ± 24 (16)
fru[3]	185 ± 42 (11)	172 ± 38 (11)
fru[4]	149 ± 28 (15)	154 ± 31 (15)
Cha^{M5}/*P14*	ND	120 ± 15 (11)
fru[1]/+	ND	119 ± 17 (8)
fru[1]/*TM3*	52 ± 14 (2)	67 ± 13 (2)
fru[3]/*MKRS*	101 ± 12 (3)	83 ± 8 (3)
fru[4]/*MKRS*	103 ± 26 (6)	101 ± 22 (6)
wild-type	143 ± 25 (7)	133 ± 18 (9)

(A) One-way ANOVA using the data from this test of general, short-term locomotion (lines crossed by flies moving in a small arena: see MATERIALS AND METHODS), with genotype as the main effect, revealed significant differences among genotypes [$F_{(5,90)} = 3.62$, $P = 0.005$]. Subsequent Tukey-Kramer unplanned pairwise comparisons ($\alpha = 0.05$) revealed that Cha^{M5}/*P14* were significantly less active than wild-type males ($P < 0.05$). All other other *fru* variant males were as active as wild-type (P's > 0.05). (B) The *fru*[+]-bearing balancers *TM3* and *MKRS* used in tests of three of the heterozygous types are described in MATERIALS AND METHODS; the first *fru*[1] heterozygous type carried a *fru*[+] chromosome from a Canton-S wild-type strain. A two-way ANOVA on long-term average activity, with this behavior occurring in constant darkness in devices designed to monitor Drosophila's circadian behavioral rhythms (see MATERIALS AND METHODS), was performed; data from individual flies were nested within genotype. The two successive time periods of locomotion-monitoring (0–4 days, and 4–8 or 4–9 days) was the repeated measure [$F_{(82,67)} = 28.32$, $P < 0.0001$] (Cha^{M5}/*P14* and *fru*[1]/+ not included, as there were data for only one of two time periods); the results revealed a slight genotype effect on activity ($P = 0.0474$). There was no time-period effect ($P = 0.7017$) and no interaction between genotype × day ($P = 0.3057$). Planned pairwise comparisons ($\alpha = 0.005$) among genotypes showed that all *fru*-variant males were as active as wild-type males (*fru*[1], $P = 0.0618$; *fru*[2], $P = 0.5398$; *fru*[3], $P = 0.6896$; and *fru*[4], $P = 0.8992$). ND, not determined.

[a] Number of males tested in parentheses.

[b] See MATERIALS AND METHODS for activity determinations.

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TABLE 3

Song summary for *fru* variants

Genotype	Fly-pair type	*n*	Intrapulse frequency	IPI Mean	IPI Median	CPP	FFT width
fru¹	*m + m*	7	246 ± 5	0.045		3.02 ± 0.21	137 ± 5
	m + f	11	232 ± 5	0.047	0.043	3.12 ± 0.14	117 ± 7
fru¹/P14	*m + m*	6	225 ± 9	0.049		2.97 ± 0.15	123 ± 4
	m + f	6	228 ± 6	0.048	0.045	3.23 ± 0.17	135 ± 7
fru¹/M5	*m + m*	5	236 ± 3	0.040		3.16 ± 0.07	130 ± 6
	m + f	5	231 ± 4	0.048	0.046	2.99 ± 0.11	136 ± 12
fru²	*m + m*	5	224 ± 4	0.044		2.61 ± 0.20	119 ± 7
	m + f	5	215 ± 3	0.043	0.041	2.96 ± 0.26	95 ± 5
fru²/M5	*m + m*	2	241 ± 3	0.047		2.95 ± 0.27	136 ± 13
	m + f	4	242 ± 7	0.048	0.046	2.97 ± 0.09	132 ± 5
fru³	*m + m*	31	—	—	—	—	
	m + f	20	—	—	—	—	
fru³/P14	*m + m*	8	—	—	—	—	
	m + f	8	—	—	—	—	
fru³/M5	*m + m*	7	—	—	—	—	
	m + f	5	—	—	—	—	
fru⁴	*m + m*	25	—	—	—	—	
	m + f	20	—	—	—	—	
fru⁴/P14	*m + m*	11	—	—	—	—	
	m + f	10	—	—	—	—	
fru⁴/M5	*m + m*	15	—	—	—	—	
	m + f	11	—	—	—	—	
Cha^M5/P14	*m + m*	8	—	—	—	—	
	m + f	4	—	—	—	—	
fru³/fru⁴	*m + m*	16	—	—	—	—	
	m + f	13	—	—	—	—	
fru¹/fru²	*m + m*	5	254 ± 9	0.041		2.70 ± 0.06	166 ± 16
	m + f	5	220 ± 7	0.041	0.039*	3.24 ± 0.09	122 ± 12
fru¹/fru³	*m + m*	5	216 ± 7	0.045		2.94 ± 0.06	129 ± 6
	m + f	5	230 ± 7	0.046	0.043	3.09 ± 0.10	141 ± 5
fru¹/fru⁴	*m + m*	5	224 ± 7	0.043		3.07 ± 0.11	141 ± 9
	m + f	5	223 ± 2	0.041	0.039*	3.42 ± 0.13	123 ± 7
fru²/fru³	*m + m*	5	242 ± 8	0.045		3.22 ± 0.10	126 ± 14
	m + f	5	238 ± 4	0.045	0.042	3.30 ± 0.17	135 ± 4
fru²/fru⁴	*m + m*	5	235 ± 9	0.046		3.30 ± 0.22	121 ± 14
	m + f	5	238 ± 6	0.046	0.043	3.24 ± 0.08	127 ± 6
fru¹/+	*m + f*	3	242 ± 11	0.031	0.030	2.81 ± 0.12	139 ± 6
fru²/+	*m + f*	3	234 ± 6	0.037	0.036	2.60 ± 0.14	147 ± 17
fru³/+	*m + f*	4	241 ± 3	0.033	0.032	2.69 ± 0.11	144 ± 5
fru⁴/+	*m + f*	3	237 ± 2	0.035	0.034	2.63 ± 0.08	149 ± 4
wild-type	*m + f*	5	258 ± 9	0.037	0.036	2.66 ± 0.19	180 ± 10

Song pulses were logged by taking trains that had at least three pulses per train (with the additional demand that intervals between pulses were <100 msec, see MATERIALS AND METHODS); 15–20 trains per singing male were analyzed (except for *fru¹/Cha^M5* that tended to sing feebly throughout a recording period). Songs were logged for males courting males (*m + m*) and for males courting females (*m + f*), except for *fru⁺*-bearing controls, because such males do not sing to one another. The second column represents the conditions (type of flies present) for each genotype. The third column gives the number of songs analyzed. The following song parameters were presented for each genotype courting either another male or a female: carrier frequencies (intrapulse frequency), IPIs (presented as both mean and median values), CPP, and the width of the FFT in Hz (cf. BERNSTEIN *et al.* 1992; VILLELLA and HALL 1996). The SEMs for mean and median IPIs are not tabulated; all such values were in the range of 0.001–0.003 sec. *fru³* and *fru⁴*, homozygous, heterozygous with *Cha^M5* and *P14*, and the transheterozygous *fru³/fru⁴* did not produce any song pulses (only rejection sounds and a few hum sounds were heard: see MATERIALS AND METHODS and RESULTS). Two-way ANOVAs were performed on the following: IPIs, CPP, intrapulse frequency, and FFT width, with genotype and courtship object (male or female) as the main effects (wild type was excluded, because these males courted essentially one kind of courtship object only). The results revealed a genotype effect on IPI ($P = 0.006$), FFT width ($P = 0.024$), and intrapulse frequency ($P = 0.003$). There was no effect of courtship object on IPIs ($P = 0.31$), FFT width ($P = 0.11$), and intrapulse frequency ($P = 0.12$); however the CPPs were slightly different depending on the sex of the courtee ($P = 0.03$), suggesting that there may be a difference in this one feature of song quality, in terms of the sounds directed toward females *vs.* males (however, in these

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TABLE 3

Continued

particular recordings, there may have been a relatively large number of rejection wing-flick sounds superimposed on some of the song pulses, in a situation where the test male was singing to the other one, with the latter rejecting at that moment). There were interaction effects between genotype × object for FFT width and for intrapulse frequency (P's = 0.02 and 0.04, respectively); yet there were no interactions between genotype × object for IPIs and CPP (P's = 0.36 and 0.56, respectively). Planned comparisons following one-way ANOVAs revealed no significant differences between wild-type and *fru*/+ males for IPI, CPP, intrapulse frequency, total number of pulses, total number of trains, and train length [P's $> \alpha = 0.013$] (not all of these song-related values are tabulated). Therefore, wild-type and *fru*/+ were grouped together into one control group. A second set of one-way ANOVAs with the new groups revealed significant differences among genotypes for IPI [$F_{(20,103)} = 6.84$, $P < 0.0001$], CPP [$F_{(20,103)} = 2.66$, $P = 0.0007$], and intrapulse frequency [$F_{(20,123)} = 2.94$, $P = 0.0002$] (see MATERIALS AND METHODS). Subsequent planned comparisons (these were deemed significant if P's $< \alpha = 0.005$) revealed that all *fru*-variant males had mean IPIs longer than those of control groups (see RESULTS). From the means of the median IPIs (see above and MATERIALS AND METHODS), planned comparisons of such values (untransformed), following a one-way ANOVA [$F_{(14,58)} = 6.21$, $P < 0.0001$], revealed no significant differences ($\alpha = 0.013$) among wild-type and the various *fru*/+ males. Therefore, the data from these males were combined into one control group. A second one-way ANOVA, after combining such control values, revealed significant differences in median IPIs among genotypes [$F_{(10,62)} = 7.88$, $P < 0.0001$]. Subsequent planned comparisons showed that all *fru* variant males, except for fru^1/fru^2 and fru^1/fru^4 (*), produced songs with longer-than-normal (median) IPIs values ($P < \alpha = 0.005$). The vigor of singing was assessed by computing overall rates of pulse-production per minute (hence, all pulses per recording were considered, not merely the train samples used to determine pulse-quality values): fru^1 with females, 176 ± 31; fru^1 with males, 162 ± 42; fru^2 with females, 281 ± 51; fru^2 with males, 79 ± 58; fru^1/+ with females, 185 ± 69; fru^2/+ with females, 381 ± 114; fru^3/+ with females, 302 ± 30; fru^4/+ with females, 414 ± 56; wild-type (Canton-S) males with females, 316 ± 61. Sine song bouts were logged (*cf.* VILLELLA and HALL 1996) for fru^1 and fru^2: the former generated 20 ± 15 (n = 2) sine song bouts/min; the latter 42 ± 13 (n = 4), compared to wild type 20 ± 9 (this control value is from VILLELLA and HALL 1996). Sine song frequencies (in Hz, see MATERIALS AND METHODS) were as follows: fru^1, 195 ± 9; fru^2, 209 ± 4; compared to wild type, 177 ± 11.

ancer ratios from the latter pair of counts to 1.0, the relative viabilities of fru^3 and fru^4 homozygotes are ~0.8 and 1.0, respectively (the slight decrement implied by the former value does not necessarily map to the *fru* locus).

Courtship song: Is the mild singing abnormality exhibited by fru^1 males (WHEELER *et al.* 1989) a fluke? Does this and other *fruitless* mutants sing differently when they court males instead of females? We thus recorded and analyzed the courtship songs of several *fru*-related genotypes in the two situations just indicated.

The song parameters for various *fru* mutant males singing one to another or to females are in Table 3. The legend to this table includes information on the quantity of song components generated by males that did produce such sounds. One difference involved fru^2 males courting other males: the number of pulses/min of wing extension value was about fivefold lower compared to the amount of acoustical output from fru^1 (220 *vs.* 1068, respectively), although this difference was not statistically significant ($P = 0.123$ from t-test comparison). Both fru^1 and fru^2 males generated the same number of pulse/min of wing extension when singing to either another male (see values above) or to a female (for fru^1, 1027; for fru^2, 460); in the latter situation, the quantitative pulse outputs from these two mutants were not significantly different from that of wild-type males (771 pulses/min of wing extension).

Songs produced by fru^1 and fru^2 mutants compared to those of wild-type had IPIs that were significantly longer than controls (Ps $< \alpha = 0.005$). In addition, the width of the FFT peak was narrower for both these mutants compared to controls (Ps < 0.005); there were slightly more CPP for fru^1 compared to controls (Ps < 0.005), and fru^2 males gave lower than normal intrapulse frequencies ($P < 0.005$), whereas fru^1's value was not different from the control ($P > 0.005$).

The quality of the song sounds were the same for both fru^1 and fru^2 males whether singing to a female or to another male. There was no interaction between genotype × courtship-object for IPI and CPP (Ps = 0.36 and 0.56, respectively). However, there was an interaction component for the intrapulse (carrier) frequencies ($P = 0.04$). In male-male recordings, many instances of wing-flick rejections were displayed by the male being courted (also see a later section). These flicking signals look similar to song pulses, but when analyzed for some of the song parameters, are different, at least in the sense that the average IPI is about twice as long in rejection sounds compared to love song (*cf.* PAILLETTE *et al.* 1991). To avoid logging wing-flick signals as song pulses, the latter were logged by only taking trains of pulses with at least three pulses for which the IPIs were <100 msec (see MATERIALS AND METHODS).

From attempts to record the songs of fru^3 or fru^4 males, none of them ever generated any pulse song (Table 3; this includes audio- and video-taped records from fru^3 or fru^4 heterozygous with either Cha^{M5} or *P14*, and the two mutations over each other). However, 8/305 fru^3 and fru^4 males (this denominator being the aggregate n from of all the genotypic combinations just indicated) produced one to two brief bouts of low-amplitude hums. These sounds were likely to have been bouts of "sine song" (*e.g.*, VILLELLA and HALL 1996),

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although they were almost indistinguishable from the background noise level.

The near-total inability of fru^3 and fru^4 to produce song sounds is another defect in their courtship wing usage. Thus, homozygotes, heterozygotes involving *P14* and Cha^{M5}, and fru^3/fru^4 males perform brief and infrequent wing extensions during courtship, as introduced above. In particular and in more of a song context, the WEI for fru^3 was only 1% (n = 128 males, homozygous or heterozygous for either of the two deficiencies, including both male-male and male-female recordings). Nevertheless, this meant 8.25 min wing extension in 673 min of recording time; in that amount of time, fru^+ males would generate ~2600 pulses. For fru^4 the WEI was 2% (n = 142 males, genotypes and courtship objects as in fru^3 above), *i.e.*, 15.5/783 min, fru^+ males would normally produce 4900 pulses given this number of minutes of wing extension. For fru^3/fru^4, WEI was 1% (n = 35 males recorded with either a female or another male), 1.3/194 min total, and expected number of pulses was 411.

The mutant male types expressing fru^3, fru^4, or both also showed brief vertical wing displays and wing scissoring; both wings were simultaneously extended ~45° to the body (*cf.* HALL 1978). Neither wing posture led to pulse song production (except for the brief hums noted above). By comparison, heterozygotes in which fru^3 or fru^4 were placed over fru^1 or fru^2 produced song-signals within the normal range (Table 3). Most of the *fru*-mutant types that sang gave mean and median IPI values that were longer than those of wild type (Ps < 0.005, except for fru^1/fru^2 and fru^1/fru^4: see Table 3). This confirms the findings of WHEELER *et al.* (1989), reported for the fru^1 mutant, and provides a significant extension of them: fru^2's slower than normal rate of pulse production may seem a subtle defect (Table 3), but it is the same kind of abnormality as exhibited by the other *fru* mutant that is able to sing (WHEELER *et al.* 1989).

The near songlessness of the new *fru* mutants is not the result of generalized wing debilitation. In tethered and free flight tests, both fru^3 and fru^4 males yielded the following wing-beat frequencies: for fru^3, 204 ± 10 Hz (n = 4); for fru^4, 205 ± 5 Hz (n = 4); and for fru^1 males, 161 ± 8 Hz (n = 4). These numbers are near or within the published wild-type frequency range of ~180–230 (*e.g.*, SCHILCHER 1977). Although fru^1 shows the lowest frequency, recall that its wing extension and song generation during courtship are largely normal. In free-flight tests, fru^3 and fru^4 males flew in a manner that was not significantly different from the performance of wild type (all Ps > 0.05; Tukey-Kramer pairwise comparisons: see MATERIALS AND METHODS), although fru^1 males did not fly as well (P value < 0.05: see Figure 1).

Thus it would appear that the anomalous wing usage exhibited by fru^3 and fru^4 males in courtship does not have a general thoracic etiology, such as a widespread defect in neuromuscular morphology or physiology. However, the double-deletion adults did not fly well (P < 0.05: see legend to Figure 1). Yet, the interpretation of that result is not straightforward: *P14*/+ heterozygotes were poor fliers (data not shown; see legend to Figure 1; it includes information on Cha^{M5}/+, which flew normally: P > 0.05). The *P14* deletion also causes what seems to be overall ill health; for instance, strains carrying this *Df* are difficult to maintain. Thus, the mediocre flight performance of double-deletion flies may not be a *fru*-specific problem, but rather the result of hemizygosity for a large number of genes (*P14* extends well to the centromere-proximal side of the *fru* locus, *e.g.*, GAILEY and HALL 1989; RYNER *et al.* 1996).

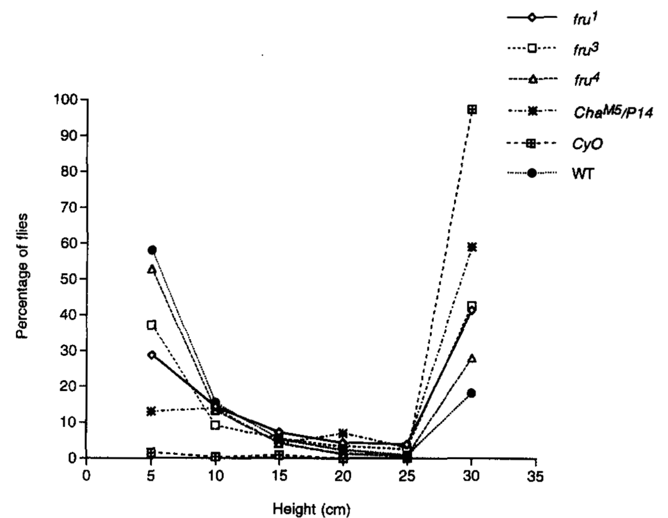

FIGURE 1.—Flight performance of *fruitless* mutants. Males were tested in the cylindrical flight assay as described in MATERIALS AND METHODS. The values on the ordinate represent mean percentages of flies that ended within a given segment along the long axis of a glass graduated cylinder (these determinations were made at 5-cm intervals). The means were calculated by taking the number of flies at each height/the total number of flies for each genotype, giving a percentage of the flies tested in a given trial that landed on successive segments. A mean of means was then calculated for each genotype. Numbers of trials (50–100 flies per trial, except for Cha^{M5}/*P14*, which was 25–75 flies per trial) were as follows: fru^1, six; fru^3, seven; fru^4, five; Cha^{M5}/*P14*, four; wild type (Canton-S), seven; *CyO*, two. Flies distributed near the top of the cylinder (*e.g.*, 5 cm) are good fliers, whereas those that end toward the bottom are either poor fliers or do not fly at all. *Curly*-winged flies (in particular, *In(2LR)O, Cy*/+: see LINDSLEY and ZIMM 1992) were used as controls for the latter phenotype. A one-way ANOVA on percentages of flies found at the first 5-cm interval (see MATERIALS AND METHODS) revealed differences among genotypes [$F_{(7,29)}$ = 13.66, P < 0.0001]. Subsequent Tukey-Kramer unplanned comparisons revealed that fru^3 and fru^4 were not significantly different from wild type (Ps > 0.05); whereas fru^1 and Cha^{M5}/*P14* were different from controls (Ps < 0.05). The mediocre flight performance of the double-deletion (Cha^{M5}/*P14*) fly was most likely due to the *P14* chromosome, since *P14*/+ (data not shown) flies did not perform well in this assay compared to wild type (P < 0.05), but Cha^{M5}/+ (data not shown) flies were not different from controls (P > 0.05).

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Later stages of courtship and mating: *Licking and attempted copulation:* Microscope-aided observations of the new mutants revealed, for fru^3 males (including homozygotes, and flies heterozygous for this mutation and either Cha^{M5} or *P14*), that of the five (of nine) individuals that had tapped the female, all eventually attempted licking of the female's genitalia; however, only one of these five males made genital contact with his proboscis. With regard to fru^4, 12/16 heterozygous fru^4/Cha^{M5} and $fru^4/P14$ males that tapped also attempted licking, and three of these males achieved genital contact with their probosces. All fru^1 males (whether homozygous or over *P14*: $n = 9$) exhibited licking attempts, and genital contact occurred in five of these observations. Similar numerical findings were made from high-magnification observations of fru^2 and wild-type males (3/5 and 4/7, respectively).

The new *fru* variants never attempted to copulate in these tests, and that behavior was also never observed in the more extensive observations of fru^3 and fru^4 that are reflected in Table 1. However in these high-magnification observations, 2/6 fru^3/Cha^{M5} and 2/10 fru^4/Cha^{M5} males displayed some abdominal bending. That fru^1 males did not attempt to copulate was reported long ago (HALL 1978), and none of the nine such males currently observed in this study performed that behavior. In this same series of observations, 3/5 of the fru^2 males tested (see above) attempted to copulate; recall that this *fruitless* type is fertile. All seven of the wild-type males attempted copulation.

Failure to attempt copulation in the short-term may not be an indicator of complete behavioral sterility. In this regard, long-term male-female pairings of all the mutant types were effected. Whether the genotype was the homozygotes fru^3 or fru^4, or the transheterozygote fru^3/fru^4, total male sterility resulted (Table 4). This replicates the sterility phenotype by which these new mutants were isolated by CASTRILLON *et al.* (1993), although those authors did not test whether fru^3/fru^4 males would mate. The *P14* and Cha^{M5} deletions led to total male sterility when either deletion was placed over fru^3 or fru^4 (Table 4). This pattern of results is the same as that previously reported for fru^1 (GAILEY and HALL 1989). However, strikingly high proportions fru^3/fru^1 or fru^4/fru^1 males were fertile (Table 4), confirming CASTRILLON *et al.* (1993). Although fru^2 allows for good fertility in long-term male-female pairings of this homozygous mutant type (GAILEY and HALL 1989), or when fru^2 is placed over fru^1 (Table 4), the fertility of fru^3/fru^2 or fru^4/fru^2 males was mediocre (~15% and 20%, respectively: Table 4).

Ménages-à-trois tests: The behavior of fly trios that included the *fru* mutant is quantified in Table 5. The *fru* individual being tested was given a choice between two males (a *fru* mutant of the same type or a wild-type male), or between a mutant male and a wild-type female. All *fru*-variant males, except for fru^2 ($P = 0.01$), courted the other male with no significant difference from the attention he directed toward the female (all *P*'s > 0.05: see legend to Table 5). Homozygous fru^1 and the heterozygotes involving fru^1 with either of the *fru* deletions courted males and females equally (fru^1/fru^1, $P = 0.43$; $fru^1/P14$, $P = 0.27$; and fru^1/Cha^{M5}, $P = 0.10$), although the CPI values were negative, nominally indicating a preference toward the male. fru^2 showed a strong preference for females over males, hence, a positive CPI value (Table 5). fru^3 and fru^4 courted the other male to the same extent that they did females ($P = 0.26$ for fru^3 and 0.17 for fru^4), the former showing a tendency to court males more than females. Both fru^3 and fru^4 mutant males tended to court flies of either sex, particularly females, less than did fru^1 males (Table 5). fru^1 and fru^2 males showed significantly higher courtship levels toward females than did fru^4 or, especially, fru^3 males. In these preference tests fru^3 and fru^4

TABLE 4

Fertility of *fru* variant males

Genotype	Fraction fertile
fru^1	0/54
fru^2	11/11
fru^3	0/49
fru^4	0/47
$fru^1/P14$	0/18
$fru^1/M5$	0/20
$fru^2/M5$	7/10
$fru^3/P14$	0/41
$fru^4/M5$	0/30
$fru^3/M7$	0/30
$fru^3/148.5\text{-}1$	16/16
$fru^4/P14$	0/33
$fru^4/M5$	0/28
$fru^4/M7$	0/17
$fru^4/148.5\text{-}1$	22/22
fru^2/fru^1	15/16
fru^3/fru^1	38/59
fru^4/fru^1	24/37
fru^3/fru^2	2/14
fru^4/fru^2	4/19
fru^3/fru^4	0/37
M5/P14	0/12
$fru^1/+$	13/13
$fru^2/+$	10/10
$fru^3/+$	21/21
$fru^4/+$	14/15
M5/+	10/10
Wild type	19/20

Fertility fractions were determined as described in MATERIALS AND METHODS. The numerator is the number of vials (each containing one test male and a few virgin females) that had progeny; the denominator the total number of vials screened. *P14, M5, M7,* and *148.5-1* are third-chromosomal deletions: *Df(3R)P14*, $Df(3R)Cha^{M5}$, $Df(3R)Cha^{M7}$, and *Df(3R)148.5-1* (*cf.* LINDSLEY and ZIMM 1992). The source of the + third chromosome, for tests of heterozygous controls, was a Canton-S wild-type strain. Also, the 20 males tested in the last row were from that strain.

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TABLE 5

Sex preference of *fru* variant males when given a choice of both sexes

Genotype	All courtship CI *vs.* WEI	Male + female	Male + male	CPI
2 fru^1/fru^1 males	CI	24 ± 3 (20)	26 ± 2 (20)	−0.03 ± 0.04 (20)
+ WT virgin female	WEI	4 ± 1 (16)	7 ± 2 (16)	
2 $fru^1/P14$ males	CI	21 ± 5 (12)	23 ± 3 (12)	−0.08 ± 0.07 (12)
+ WT virgin female	WEI	6 ± 2 (12)	6 ± 1 (12)	
2 $fru^1/M5$ males	CI	19 ± 3 (18)	23 ± 3 (18)	−0.07 ± 0.04 (18)
+ WT virgin female	WEI	5 ± 1 (18)	8 ± 1 (18)	
2 fru^2/fru^2 males	CI	30 ± 3 (13)	15 ± 4 (13)	0.18 ± 0.06 (13)[a]
+ WT virgin female	WEI	16 ± 2 (13)	9 ± 4 (13)	
2 fru^3/fru^3 males	CI	6 ± 2 (17)	15 ± 5 (17)	−0.11 ± 0.09 (11)
+ WT virgin female	WEI	0 ± 0 (17)	0 ± 0 (17)	
2 fru^4/fru^4 males	CI	14 ± 3 (18)	18 ± 4 (18)	−0.07 ± 0.05 (14)
+ WT virgin female	WEI	0 ± 0 (18)	1 ± 0 (18)	
2 WT males	CI	55 ± 3 (12)	3 ± 0 (12)	0.45 ± 0.00 (12)[b]
+ WT virgin female	WEI	15 ± 2 (12)	0 ± 0 (12)	
		fru + *fru* male	*fru* + WT male	
2 fru^1/fru^1 males	CI	13 ± 2 (13)	16 ± 2 (13)	−0.05 ± 0.03 (13)
+ WT male	WEI	3 ± 1 (13)	4 ± 1 (13)	
		fru + WT female	*fru* + WT male	
1 fru^1/fru^1 male + 1 WT virgin + WT	CI	11 ± 2 (10)	20 ± 4 (10)	−0.14 ± 0.04 (10)[c]
male	WEI	2 ± 1 (10)	6 ± 2 (10)	

Males were aged individually 7–14 days then placed in a small chamber with another male of the same genotype as well as a wild-type (WT) virgin female. Flies are recorded on videotape for 5–10 min. For a given recording, each male was observed separately, in terms of males courting one another and either one of them courting the female. All values are means ± SEM. CI values represent an average of all males for the percentage of time one male courted the other male (that is, only one male's behavior was logged: see MATERIALS AND METHODS) and courted the female (the exception involves all-male trios). Wing-extension percentages were logged separately for courtship directed at the other male *vs.* the female. Although most values for fru^3 and fru^4 wing extension were rounded off to 0 (see MATERIALS AND METHODS), some males did display brief wing extensions: 5/17 fru^3 males and 5/18 fru^4 ones. In parentheses are the numbers of males observed per genotype. In the current experiment, males can be the courters and courtees simultaneously (compared to Table 1); although with fru^3 and fru^4 (compared to fru^1 and fru^2), one male tends to be the predominant courter. In the ménages à trois experiments, all *fru* variant males showed higher levels of courtship toward the second male compared to the intermale courtship of wild-type male pairs when placed in the presence of a female (control tests). All the CIs in Table 5 are approximately one half those in Table 1. The reason is that in these three-fly experiments a given male spent approximately half his courtship time interacting with the other male and the rest of that time courting the female (in contrast to Table 1 observations in which a given male is courting only one target fly). In observations of three-male trios, the values were also much lower than those in Table 1; again this is because a given male spends about half the time courting one *vs.* the other male flies. *t*-test comparisons and *P* values associated with each CPI (see MATERIALS AND METHODS) asked whether there was a significant preference for one sex. The tabulated values represent a mean CPI ± SEM for the trios used to compute these indices; this was only a subset of them (compare *n*'s from the middle of the table to those toward the right), because the data from trios in which no courtship occurred were not used to compute the CPIs. Such a value of 0 (right column) means that there was no preference, a positive CPI indicates a preference toward the female, and a negative value indicates a preference toward the male.

[a] There was significantly more courtship directed toward the female than the male ($t = 3.05$, $P = 0.0101$).

[b] There was significantly more courtship directed toward the female than the male. ($t = 103.43$, $P < 0.00001$).

[c] There was significantly more courtship directed toward the male than the female ($t = -3.88$, $P = 0.0037$). In this trio, a fru^1 male was placed with a wild-type male and female, although there was significantly more courtship directed toward the male than the female, this was most likely due to the wild-type male being in the middle of the trio, vigorously courting the female in front; hence the *fru* male by default falling behind the wild-type male.

All the other genotypes in trio observations (consisting of two mutant males and a wild-type female) displayed no preferences for either sex: for fru^1, $t = -0.81$, $P = 0.43$; for $fru^1/P14$, $t = -1.15$, $P = 27$; for fru^1/Cha^{M5}, $t = -1.74$, $P = 0.10$; for fru^3, $t = -1.19$, $P = 0.26$; for fru^4, $t = -1.44$, $P = 0.17$. In trio observations of two fru^1 males and a wild-type male, fru^1 males showed no preference for either of the males ($t = -1.52$, $P = 0.15$).

courted at lower overall levels (Table 5) compared to results obtained from the single-male pair observations (Table 1).

Although fru^1 males did not discriminate between other fru^1 males and wild-type females (24 ± 3 and 26 ± 2, respectively), they did exhibit reduced levels of courtship toward each other in a test where two fru^1 males were placed with a wild-type male (Table 5). One reason for this significant reduction in fru^1 male-with-male courtship is that when two fru^1 males were placed

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with a wild-type male, the latter courted the *fru^1* males rather vigorously (homozygous *fru^1* males uniquely elicit courtship from any other male: see below). Therefore, the *fru^1* males might not have been able to interact with each other optimally, because the wild-type male was frequently interposed between the two mutant males.

When a *fru^1* male was placed simultaneously with a wild-type male and a female, the mutant individual courted the genetically normal male with more vigor than the attentions he directed at the wild-type female ($P = 0.004$). The major factor contributing to this difference in courtship levels was that the wild-type male generally was more aggressive in courting the female. It was as if the mutant could more readily court the wild-type male (the numbers are consistent with this idea), because he was more accessible than the female; in other words, the female was tied up by being courted by the wild-type male, who therefore was readily subject to the attentions of the mutant male.

fru^3 males courted females less than they did males in both single-pair tests (Table 1) and preference ones (Table 5), whereas *fru^1* and *fru^4* showed similar levels of courtship toward males *vs.* females in both tests. *fru^2* males exhibited no difference in courtship levels directed at either sex in the single-pair tests; yet in the trios these males courted females more vigorously than they did males ($P = 0.01$).

Courtship chaining behavior of the new *fru* variants: *A new courtship metric developed for behavior exhibited by groups of males:* To determine whether the *fru^3* and *fru^4* mutations would cause intermale courtship in groups, a ChI was developed; it was also applied to observations of the older *fru* mutants (Figure 3) and to various heterozygous combinations (Table 6). This behavioral anomaly for the new mutants can be striking (Figure 2), but not as vigorous as that exhibited by *fru^1* males when comparing ChIs among the mutants types (Figure 3).

These comparisons among the mutants necessarily included an aging component (it may also involve a social factor: see below). That is, the new mutants failed to exhibit any appreciable chaining when they were first grouped together on day 1 (see MATERIALS AND METHODS and the legend to Figure 3). When they began to chain, *fru^3* and *fru^4* males were consistently less vigorous chainers than in the case of the original *fru^1* mutant when the ChIs for day 1 or day 5 were compared (day 1: all *P*s $< 1 \times 10^{-37}$; day 5: *P*s $< 1 \times 10^{-10}$). At day 5, *fru^3* mutants chained more than *fru^2* ($P = 0.0016$, given $\alpha = 0.002$ for these tests), whereas *fru^4* gave ChIs that were similar to those for groups of *fru^2* males ($P = 0.050$). *fru^3* and *fru^4* groups behaved similarly to each in terms of the ChI values ($P = 0.177$, for day 5). Whereas both *fru^1* and *fru^2* yielded largely constant ChIs from day 1 to day 5 ($P = 0.850$ and $P = 0.014$, respectively, against a background of $\alpha = 0.003$), *fru^3* and *fru^4* showed significant increases in chain formation during this time span ($P = 6 \times 10^{-11}$ and $P = 2.1 \times 10^{-9}$ for day 1 and day 5, respectively).

Both *fru^3* and *fru^4* reached peak ChI values on day 3 (age post-eclosion: 8 days). Bear in mind that the ChIs for these new mutants were much lower than those of *fru^1* males, whose chaining intensities were already at maximal values as of the earliest observation day (Figure 3). In fact, *fru^1* males begin chaining within minutes of being grouped on the first observation day, whereas all the three transposon-tagged *fru* mutants gave not only lower ChIs (all *P*s $< 1 \times 10^{-34}$), but also showed mostly single male-to-male courtships. Rarely did a chain of three or more *fru^3* or *fru^4* males form on the first observation day. The difference in ChIs between the *P*-element *fru* mutants and those of *fru^1* most likely are not due to differences in genetic background. By comparing the behavior of males expressing these *fru* mutation over the two *fru* deletions (*Cha^{M5}* and *P14*), the chaining levels remain much higher for *fru^1* than *fru^3* and *fru^4*. For example, at day 5, *fru^1/Cha^{M5}* courted significantly more than both *fru^3/Cha^{M5}* and *fru^4/Cha^{M5}* (*P*s = 6.7×10^{-8} and 2.3×10^{-5}; see Table 5). ChIs for *fru^3/P14* and *fru^4/P14* were also significantly lower than those of *fru^1/P14* on day 5 (all *P*s $< 5 \times 10^{-9}$). On day 1, heterozygous types involving *fru^3* and *fru^4* (except *fru^4/Cha^{M5}*: $P = 0.006$, $\alpha = 0.002$) exhibited significantly less chaining than did the *fru^1* heterozygotes (all *P*s $< 2 \times 10^{-5}$). This control for genetic background is important in part because *fru^1* is a double mutant (GAILEY and HALL 1989; see DISCUSSION).

The high level of *fru^1*-induced chaining was also observed in a different kind of aging control in which males were stored as separate individuals until day 10–11 post-eclosion (this corresponds to day 5 for the experiment in which males were individually aged for only about half that amount of time before being grouped, as in Figure 3). These relatively old but inexperienced *fru^1* males nevertheless gave a high ChI: 66 ± 12 (n = 6 groups of the usual eight males each). In contrast, *fru^3* and *fru^4* males, aged for 10–11 days as individuals, gave low ChIs, respectively, 7 ± 4 ($n = 9$) and 8 ± 8 ($n = 9$), and are about threefold lower than the ChIs obtained for *fru^3* and *fru^4* when males had already been grouped for several days before tests. These results suggest a social component to the behavior.

In a separate kind of adult-maturation experiment, males homozygous for *fru^1*, *fru^3*, or *fru^4* were grouped on eclosion and behaviorally observed daily. All males yielded ChIs of zero on the day of eclosion, but *fru^1* males got up to speed rapidly (ChI for day 2: 26 ± 8, $n = 5$; day 3: 74 ± 3, $n = 5$) and courted with ChIs of ~70–80 through day 8. Thus, the behavior of these *fru^1* groups for days 3–8 was similar to the entire time course in Figure 3 (which began with several day-old flies). From the test in which flies were grouped on their first day of adult life, *fru^3* or *fru^4* males yielded ChIs of 0–3

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TABLE 6

Chaining behavior of heterozygous *fru* variant males

Genotype	ChI for intermale courtship (%) (n = 8 males/group) Day 1	Day 2	Day 3	Day 4	Day 5
fru[1]/*P14*	40 ± 5 (13)	56 ± 7 (10)	62 ± 8 (12)	70 ± 6 (10)	71 ± 10 (8)
fru[1]/*M5*	23 ± 5 (17)	45 ± 5 (21)	61 ± 5 (18)	60 ± 5 (16)	68 ± 5 (15)
fru[2]/*M5*	2 ± 1 (7)	7 ± 3 (11)	15 ± 4 (11)	7 ± 3 (7)	12 ± 7 (5)
fru[3]/*P14*	2 ± 1 (23)	16 ± 4 (24)	23 ± 5 (23)	21 ± 5 (22)	21 ± 5 (21)
fru[3]/*M5*	9 ± 3 (19)	20 ± 6 (19)	38 ± 7 (16)	40 ± 7 (16)	40 ± 7 (17)
fru[4]/*P14*	21 ± 9 (11)	13 ± 4 (16)	27 ± 5 (15)	27 ± 5 (14)	29 ± 5 (11)
fru[4]/*M5*	19 ± 7 (14)	20 ± 5 (19)	40 ± 6 (20)	43 ± 6 (19)	58 ± 6 (14)
fru[2]/*fru*[1]	13 ± 6 (16)	18 ± 6 (14)	27 ± 7 (10)	23 ± 6 (12)	21 ± 4 (7)
fru[3]*fru*[1]	25 ± 7 (14)	43 ± 8 (15)	57 ± 6 (14)	53 ± 4 (14)	64 ± 3 (11)
fru[4]*fru*[1]	37 ± 8 (15)	59 ± 4 (17)	57 ± 5 (17)	63 ± 4 (16)	67 ± 3 (14)
fru[3]*fru*[2]	22 ± 10 (12)	30 ± 9 (13)	43 ± 7 (12)	46 ± 8 (10)	51 ± 11 (8)
fru[4]*fru*[2]	30 ± 13 (10)	13 ± 8 (7)	36 ± 12 (8)	62 ± 6 (7)	40 ± 9 (8)
fru[3]*fru*[4]	5 ± 4 (13)	11 ± 3 (17)	17 ± 3 (18)	28 ± 8 (13)	36 ± 8 (11)
M5/P14	0 ± 0 (12)	2 ± 1 (13)	5 ± 1 (13)	5 ± 2 (15)	5 ± 2 (14)
fru[1]/+	0 ± 0 (7)	0 ± 0 (8)	0 ± 0 (8)	0 ± 0 (8)	0 ± 0 (7)
fru[2]/+	0 ± 0 (6)	0 ± 0 (6)	0 ± 0 (6)	0 ± 0 (5)	0 ± 0 (6)
fru[3]/+	0 ± 0 (6)	0 ± 0 (5)	0 ± 0 (5)	0 ± 0 (5)	0 ± 0 (5)
fru[4]/+	0 ± 0 (5)	0 ± 0 (5)	0 ± 0 (5)	0 ± 0 (5)	0 ± 0 (5)
Wild type	0 ± 0 (5)	0 ± 0 (5)	0 ± 0 (5)	0 ± 0 (5)	0 ± 0 (5)
Age of males (days)	~6–7	~7–8	~8–9	~9–10	~10–11

So-called chaining observations (strictly speaking, any kind of intragroup, intermale courtship activity, *cf.* Figure 2) were made by aging the males individually for 6–7 days and grouping them together, eight males per vial. The first day of grouping is referred to as day 1. Chaining indices (ChIs) were determined; these are the percentages of time that males spent courting one another (at least three males per courting group, during a given moment). Each observation period was 10 min and was performed repeatedly over the course of 5 consecutive days (the males were left grouped together between observation periods). Each value represents a mean ChI ± SEM; the number of observations for each day are in parentheses. The age of the males of each observation day is represented in the last row. To determine whether the age of the males is a factor in the percentage of chaining observed, older males (10–11 days old) were grouped together and a ChI was calculated on that day (see text). A two-way ANOVA with genotype × day as the main effects (see MATERIALS AND METHODS) revealed an interaction component between the two effects [$F_{(88,1060)} = 2.16$, $P < 0.0001$] (see legend to Figure 3 for further statistics).

through day 3; finally, by day 4 ChI values increased to ~10–20 and remained in that range through day 8.

All *fru*-variant males (including the many in Table 6) exhibited frequent head-to-head male-male interactions, as well as forming classical head-to-tail chains (Figure 2). A semi-qualitative difference among the mutants was also noted: the chaining behavior displayed by *fru*[3] and *fru*[4] males occurred mostly on the food surface (see MATERIALS AND METHODS), whereas *fru*[1] males chained on either the food or the walls of the glass vials.

Chaining behavior as a function of positive and negative courtship wing usages: Would males missing their wings chain more vigorously given their inability to perform wing-flick rejection behavior? Is courtship song production correlated with chain formation, given that song stimulation of groups of wingless wild-type males causes such flies to court one another vigorously (SCHILCHER 1976; KYRIACOU and HALL 1984), and that the weakly chaining *fru*[3] and *fru*[4] types are distinctly song-impoverished?

First, the courtship rejection behaviors of wing-flicking was quantified. For this, "flicking-failure" values were determined as the proportion of instances during which a male is courted but does not show rejection (*cf.* HALL 1978); this was determined as the number of bouts in which no wing flicking occurred/total number of courtship bouts. A higher flicking-failure ratio was reported by HALL (1978) for *fru*[1] (24%) compared to wild-type males (13%). Reexamination and extension of these observations showed that *fru*[1] and *fru*[3] flicking-failure values (31%, n = 8; 33%, n = 4, respectively) were greater than wild-type (14%, n = 8), whereas *fru*[2] and *fru*[4] yielded intermediate flicking-failure values (21%, n = 7; 23%, n = 4, respectively). The quantitative differences notwithstanding, all *fru* mutants are able to maneuver their wings in the performance of this courtship rejection behavior, by analogy to their solid wing usage in a non-courtship context (Figure 1 above).

With regard to courtship chaining, groups of wingless *fru*[1] males did not behave differently from that of *fru*[1] groups whose wings were intact. For example, the ChIs for wingless *fru*[1] ranged from ~60 to 80 for days 1–4 (n = at least five groups of eight males tested on a given day; aggregate n = 23 groups); this range of chaining values is similar to that plotted for intact males of this

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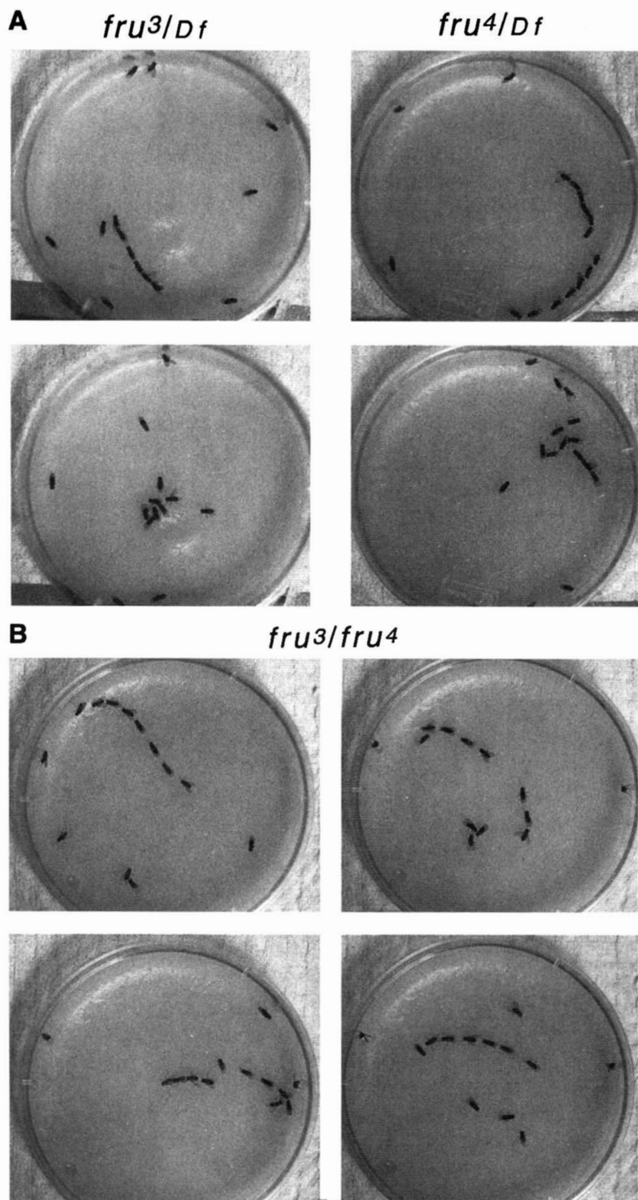

FIGURE 2.—Intermale courtships. Males expressing one (A) or both (B) of the new *fruitless* mutations were individually aged for 6–7 days then grouped together in food vials as was done for chaining observations (see MATERIALS AND METHODS and Figure 3). *Df* is the third-chromosomal deletion *Df(3R)P14*. Three or four days after grouping the males, eight to 14 of them were tranferred into a plastic petri dish (100 × 15 mm) containing food medium; the conditions were 25° and 70% relative humdity. Photographs of the behaviors exhibited by these males were taken the day after the males were transferred to the petri dish. The eight panels here represent the various kinds of group courtships that are (qualitatively) typical of *fruitless* males. The groups variously consist of males following other males in long chains, smaller groups of head-to-head flies, quasi-circles, and other indescribable geometries.

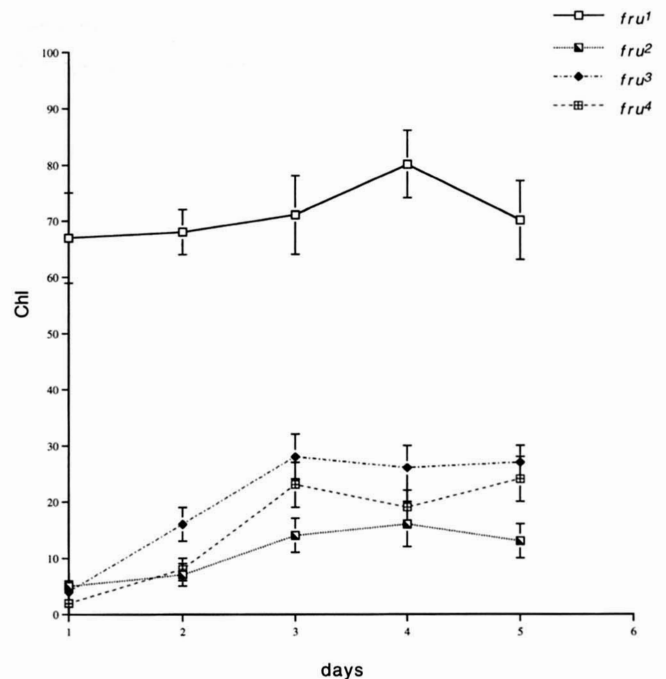

FIGURE 3.—Temporal dependence of courtship chaining behavior. Quantifications of intermale courtships (*cf.* Figure 2), generically termed "chaining" (hence, the chaining index), were effected as described in MATERIALS AND METHODS. Day 1 represents the first day of grouping the males after aging them as indicated in Table 6. ChIs were measured for the same group of flies on consecutive days. The mean ChI ± SEM was determined over the course of five consecutive days (for a given plotted point, the numbers of eight male groups ranged from 21 to 35 for all but fru^1, nine to 18 groups for that mutant); each of the four mutant types was homozygous for the *fru* allele indicated. A two-way ANOVA revealed an interaction component between genotype × day (see legend to Table 6). Subsequent planned pairwise comparisons (see MATERIALS AND METHODS: deemed significant if P's $< \alpha = 0.002$) revealed that fru^1 and fru^2 chained as much at day 1 as day 5 ($P = 0.85$ and $P = 0.013$, respectively). However, fru^3 and fru^4 showed significantly higher ChIs at day 5 compared to day 1 ($P = 6 \times 10^{-11}$ and $P = 2.1 \times 10^{-9}$, respectively). Both fru^3 and fru^4 were the same as each other in their levels of chain formation by day 5 ($P = 0.177$).

genotype (*cf.* Figure 3, including how it defines "days" on the ordinate). It may be difficult, however, to reveal an effect of removing the wings from fru^1 males, since these flies chain so much to begin with that there could be a "ceiling" effect. In this regard, wingless fru^2, fru^3, and fru^4 still chained reasonably well on days 1–4 but with ChIs lower than the corresponding plateau levels observed for the intact mutants (Figure 3). For fru^2, over the course of 4 days, the aggregate ChI was ~5 (a total of 21 groups of eight wingless males was monitored); for fru^3, ~10 ($n = 44$ groups); and for fru^4, ~18 ($n = 52$).

Thus, high-level intermale courtship are not exclusively the result of the subnormal courtship rejection capacities of *fruitless* males. In addition, the mediocre chaining exhibited by fru^3 or fru^4 groups cannot be explained by especially vigorous wing-flicking, because wingless mutant males of these types did not chain more than usual; this is consistent with the fact that the mutants generate wing-flick rejections reasonably well.

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The results obtained from observing groups of wingless *fru*[2], *fru*[3], and *fru*[4] males suggest that pulse-song production may accentuate chaining behavior although is not necessary for it; this would be relevant only for *fru*[2] among these three types. However, a connection between singing and chaining could not be inferred for *fru*[1] wingless males, because they chained to the same degree as intact-winged *fru*[1] males who sing well (see below). In general, song is not required for, nor is it particularly correlated with, chain formation: *fru*[3] and *fru*[4] males form courtship chains even though they are almost entirely mute. *fru*[2] males, who sing in a largely normal manner (see below), chained at similar or relatively low levels compared to *fru*[3] and *fru*[4]. Wingless males most likely showed reduced ChIs because the males seemed somewhat sluggish after their wings had been surgically removed (with the exception of *fru*[1]). Phototaxis associated with agitation of Drosophila is also sluggish for wingless males (BENZER 1967).

The new *fru* mutants in the presence of wild-type males: *Courtship elicitation tests:* One could infer that the higher levels of *fru*[1] chaining are in part dependent on this particular mutant's stimulating quality. Homozygous *fru*[1] males elicit high levels of courtship (as performed by wild-type males) because of a third-chromosomal breakpoint on the *fru*[1] chromosome separate from that at 91B (*fru*[1]: *In(3R)90C: 91B:* GAILEY and HALL 1989). To control for this, *fru*[1]'s active courtship defects (as opposed to the elicitation ones) were specifically uncovered by testing *fru*[1]/*Cha*[M5] heterozygotes for chaining (the *Cha*[M5] third chromosome includes an intact 90C region). These males chained vigorously (Table 6, row 2), although they do not elicit courtship as performed by wild-type males (GAILEY and HALL 1989). The transposon-induced *fru* mutations carry genetic defects only in 91B, and neither *fru*[3] nor *fru*[4] was anomalously attractive to wild-type males. The elicitation CIs (using mutant males immobilized by anesthesia: *cf.* GAILEY and HALL 1989) were only 4 ± 2 ($n = 16$) and 4 ± 1 ($n = 13$) for *fru*[3] and *fru*[4], respectively, barely above the values recorded for *fru*[2] (1 ± 1, $n = 4$). In contrast, homozygous *fru*[1] males elicited higher levels of courtship (16 ± 3, $n = 22$).

Mutant courtship directed at wild-type males: fru[1] males were reported to court genetically normal males (HALL 1978). Surprisingly, this matter has never been reexamined. To determine whether the new *fruitless* mutants would court wild-type males, observations were made by placing a series of single *fru*[3] or *fru*[4] males each with a *fru*[+] male marked by hemizygosity for the *yellow* mutation. These new mutant types, and *fru*[2] as well, yielded rather modest CIs (*fru*[2]: 17 ± 5, $n = 8$; *fru*[3]: 22 ± 9, $n = 8$; *fru*[4]: 19 ± 11, $n = 7$). *fru*[1] males showed the most interest toward the wild-type male (26 ± 5, $n = 12$). A Welch ANOVA (which was used since the variances were not equal in these observations: SOKAL and ROHLF 1995) on the above CIs (transformed) showed that there were differences among the mutant genotypes [$F_{(4,16)} = 6.49$, $P = 0.003$]. The control value for *fru*[+] males courting *yellow*-marked males was 3 ± 1 ($n = 5$).

How indiscriminate is the courtship behavior of *fru* males? *Courtship directed at males and females of another species:* The extent to which these mutants, notably *fru*[1], court other flies at such high levels prompted an examination of their behavior in the presence of *D. simulans* males. Would *fruitless* males court essentially any and all potential objects of courtship to the same extent? Table 7 summarizes the behavior of *fru*[1] males (homozygous *vs.* heterozygous with the *Cha*[M5] deletion) directed at *D. melanogaster* and *D. simulans* males and females. Whether homozygous or heterozygous with *Cha*[M5], *fru*[1] males courted *D. simulans*. *fru*[1] males courted *D. simulans* males and females equally ($P > 0.003$), with CI values similar to those recorded when the mutant was placed with *D. melanogaster* males and females (Table 5; α here was necessarily adjusted to 0.003: see MATERIALS AND METHODS). In contrast, wild-type *melanogaster* males courted *simulans* females significantly more than *simulans* males ($P < 0.003$) and courted females of both species to the same extent. *fru*[1] males courted *melanogaster* males no differently than they did *simulans* males ($P > 0.003$). Although *fru*[1]/*Cha*[M5] courted both *melanogaster* males and *simulans* males equally ($P > 0.003$), the CI values were approximately half of those for *fru*[1] males.

Courtship conditioning of fru males: This experience-dependent feature of courtship involves suppression of reproductive behavior, both during the "training" periods and when the "conditioned" male is subsequently placed with a potential courtship object (reviewed by HALL 1994; GREENSPAN 1995b). This led us to ask whether the apparent relentlessness of *fruitless* courtship would override the usual suppressive effects of previous experience. Indeed, the most relentlessly *fruitless* courter, *fru*[1] homozygous males, showed no significant reduction in courtship levels during 30-min conditioning periods with immature males (Figure 4). Wild-type males courted with decreasing intensity during such training periods, as has been extensively documented (see the reviews just cited). Similar results were obtained in conditioning experiments involving *fru*[1] placed over either of the deletions used throughout this study. Males heterozygous for *fru*[1] and either *fru*[3] or *fru*[4] also showed a reduction in courtship activity during conditioning with immature males (Figure 4). Moreover, these mutant males tended to exhibit higher levels of courtship than did *fru*[+] males during the first 10 min of their pairing with immature males. In this courtship-suppression paradigm, wild-type males tend to diminish their courtship with the first third of the training period (*e.g.*, GAILEY *et al.* 1982); in contrast, *fru*[1]/*fru*[3] or *fru*[1]/*fru*[4] males seemed in a courtship frenzy throughout this first 10 min of the time they were exposed to the conditioning stimulus.

fruitless does not appear to be recessive in the imma-

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TABLE 7

fru* males courting *D. simulans

Courter	Courtship indices Courtee				
	fru[1] male	*D. melanogaster* male	*D. simulans* male	*D. melanogaster* female	*D. simulans* female
D. melanogaster	1 ± 0 (10)[a]	1 ± 0 (9)	1 ± 0 (12)	62 ± 5 (7)	30 ± 6 (7)
fru[1]	47 ± 10 (10)	30 ± 7 (14)	28 ± 5 (25)	31 ± 10 (6)	13 ± 6 (17)
D. simulans	1 ± 0 (15)	8 ± 8 (5)	2 ± 1 (6)	0 ± 0 (7)	22 ± 11 (4)
	fru[1]/*Cha*[M5] male	*D. melanogaster* male	*D. simulans* male	*D. melanogaster* female	*D. simulans* female
fru[1]/*Cha*[M5]	25 ± 12 (6)	13 ± 5 (10)	11 ± 4 (12)	11 ± 4 (15)	21 ± 9 (7)

The CIs values represent the percentages of time the test male (the courter) spent courting a given kind of courtee during a 10-min observation period. In parentheses are the numbers of observations.

[a] The reason for no elicitation value being attached to *fru*[1] is that when a wild-type male was placed with such a mutant one, the latter tended to do most of the courting, therefore not allowing the wild-type male to court the mutant one (compared to a CI value of 16% when the *fru*[1] male was etherized (see RESULTS). Planned comparisons following a one-way ANOVA (see MATERIALS AND METHODS) revealed that both *fru*[1] and *fru*[1]/*Cha*[M5] males courted *D. simulans* males and females equally, not significantly differently compared to courtship directed toward *D. melanogaster* males and females (all P's $> \alpha = 0.003$: see RESULTS). Wild-type males also courted *melanogaster* and *simulans* females equally ($P > \alpha = 0.003$) but courted *simulans* males significantly less than *simulans* females ($P < \alpha = 0.003$).

ture male paradigm in that *fru*[1]/+ males exhibited unusually high levels of courtship (*i.e.*, did not show normal suppression) compared to wild type during the 30-min conditioning period (data not shown).

The MOL in males expressing the new *fru* mutations: Because the four mutant alleles of *fru* do not identify a single complementation group for all sex-related characters (recall the fertility of *fru*[1]/*fru*[3] and *fru*[1]/*fru*[4]), we examined the effects of the new mutations and other *fru*-variant combinations on the formation of the MOL. When homozygous, *fru*[1] and *fru*[2] usually result in intermediate forms of the male MOL (GAILEY *et al.* 1991a) given the similar anatomical effect observed in *fru*[1]/*fru*[2] males, *fru*[1] and *fru*[2] are allelic in this regard (GAILEY *et al.* 1991a). Table 8 presents a complementation matrix for all possible pairwise crosses of the four *fru* alleles, along with the results of our recently developed (more extensive) criteria for scoring dorsal abdominal anatomy. These results indicate a wide range of phenotypic expression, from nearly no MOL, exemplified by the *fru*[4] homozygous male, to the near morphological normality exhibited by *fru*[2] homozygotes. None of the transheterozygous mutant combinations led to a normal MOL in all individuals; thus all four *fru* alleles are in the same complementation group with respect to MOL determination.

***fru* mutations expressed in females:** Results of observing all four alleles worth of *fruitless* mutations for effects on female reproduction are in Table 9. The levels of wild-type male courtship directed at *fru*[1], *fru*[3], and *fru*[4] females (homozygous and heterozygous with *Cha*[M5] and/or *P*[14]), and latencies to mating initiation are tabulated. *fru*[3] and *fru*[4] females were courted by wild-type males at normal levels (all P's $> \alpha = 0.006$: planned pairwise comparisons; see legend to Table 9). All *fru*-variant females were not significantly different from normal with respect to courtship-time elapsed until mating began (all P's $> \alpha = 0.006$). In such latency tests (see Table 9), 1/15 *fru*[1] females took longer than 20 min to mate, 1/5 *fru*[3] females did not mate within 20 min, and one *fru*[3]/*P14* (out of 11) female took longer than the 20 min observation periods (see legend to Table 9). All other genotypes mated within the 20 min. The females that did not mate within such observation periods did so eventually (assessed as described in MATERIALS AND METHODS).

Additional female-fertility assessments that initially revealed an intriguing subnormality for the females expressing *fru*[3] were performed. In these tests only 11 of the 14 females that had been observed to mate (and for the usual ~20 min) produced progeny albeit in robust numbers, from examinations of the larval cultures (see section of general activity)]. For *fru*[4] and wild-type females, 5/5 and 3/3 mated and were fertile. These preliminary results prompted a second set of fertility experiments in which *fru*[3], *fru*[4], and wild-type females were mated to wild-type males then (as a control for male fertility) the same male was allowed to mate with a wild-type female: 1/21 homozygous *fru*[3] females produced no progeny compared to the control female, which were all fertile after matings with these self-same males. In retests of *fru*[4] females, 19/19 homozygotes produced progeny, and the subsequent control matings with the males all led to fertility as well. For retests of controls, 11/12 wild-type females were fertile, and when the same males were subsequently tested with a second female, 11/12 were fertile. For the latter control crosses, it was the same male (1/12) that was sterile in both sets of matings.

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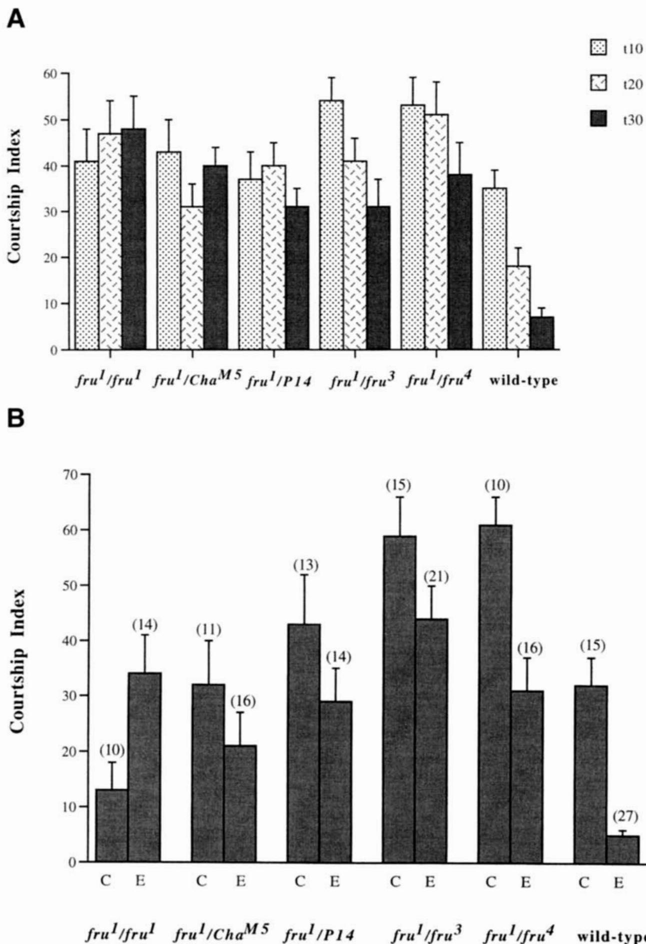

FIGURE 4.—Experience-dependent courtship of immature males. (A) Behavior during training. Males to be tested (meaning in the main to assess the effects of fru^1) were placed (one each) with very young Canton-S wild-type imagoes for 30 min. A CI was calculated for each 10 min of the 30-min conditioning period. Differently shaded bars represent the following: the first 10 min of conditioning (t10), 10–20 min (t20) and the last 10 min (t30). The ordinate values represent CIs ± SEM. A modified two-way ANOVA (see MATERIALS AND METHODS) revealed that there was a significant reduction in the CI for the behavior of wild-type (Canton-S) control males from t10 to t30 ($P = 0.0012$). fru^1 ($P < 0.0001$), fru^1/Cha^{M5} ($P < 0.0001$), $fru^1/P14$ ($P = 0.0003$), and fru^1/fru^4 ($P = 0.0128$) showed decays from t10 to t30 that were significantly less than those of wild type. Only fru^1/fru^3 showed a decrease in CIs that was similar to that of wild type ($P = 0.1481$). (B) Post-training behavior. Males were conditioned with an immature male for 30 min (see A) then these same males (that is, which led to the data plotted in A) were immediately tested with a second active immature wild-type male. The (former) test male is indicated as experienced (E). Controls (C) consisted of males placed in a chamber alone for 30 min and then tested with an immature male. The ordinate values are CIs ± SEM. Numbers in parentheses above the bars represent the total number of observations for each group. A two-way ANOVA with genotype and condition (pairing with an immature male *vs.* sham training) as the main effects [$F_{(11,181)} = 9.19$, $P < 0.0001$) revealed significant effects for genotype ($P < 0.0001$), condition ($P = 0.0006$), and an interaction component between genotype × condition ($P = 0.0009$). Planned pairwise comparisons ($\alpha = 0.009$) between controls and experienced males showed that fru^1 had CIs that were not significantly different ($P = 0.0260$). Thus, the putative training in the presence of an immature male did not lead to courtship suppression when this mutant was tested with a second immature male, compared to the courtship behavior of inexperienced fru^1 controls. Two heterozygous types, fru^1/Cha^{M5} ($P = 0.2383$) and $fru^1/P14$ ($P = 0.2004$), which did not exhibit signs of courtship suppression during training (see A), also did not show a difference in the levels of courtship exhibited by controls *vs.* experienced males. The fru^1/fru^3 heterozygote showed a significant decrease in courtship during conditioning (see A), but here there were no differences in CIs before and after having the experience ($P = 0.0818$). A final heterozygous type, fru^1/fru^4, gave CIs after the conditioning period that were less than its corresponding control ($P = 0.0026$). For the Canton-S wild-type controls, there was a highly significant reduction in CIs of experienced compared to that of controls ($P = 3 \times 10^{-5}$).

In aggregate of the two latter experiments, 4/35 (or 11%) mated fru^3 females were sterile; however, only the second set of experiments included a control for the tester males' fertility. All fru^4 females were fertile (a total of 24/24), and 1/15 mated wild-type females was sterile (not her fault). We are left with the meager observation that one fru^3 female (out of 21) whose tester male was controlled for (hence 5% of such test results) seems to have been sterile, owing to an egg-laying defect or some other problem with her reproductive system's internal workings. If this is a real defect, it is mild in the quantitative sense (very low penetrance).

Table 10 gives a summary of most of the reproductively related phenotypes for homozygous *fruitless* mutants and males expressing selected heterozygous genotypes (also see DISCUSSION).

DISCUSSION

The new *fru* mutants fru^3 and fru^4 are similar to the original fru^1 one in that all three of these mutants are sterile (Table 10); this was their isolation phenotype (GILL 1963; CASTRILLON *et al.* 1993). Yet, these three mutant types court females, albeit at lower than normal levels, with fru^3 being the most subnormal. We believe this should be kept in mind, lest one overemphasize the fact that *fruitless* mutants court other males much more than wild-type males do.

The sterility of fru^1, fru^3, and fru^4 males is behavioral. While courting females they make no attempt to copulate, as if they are blocked at this stage of the courtship sequence. It is now known that fru^1 males perform genital licking, the step before attempted copulation; the same is so for fru^3 and fru^4. But unlike fru^1, the fru^3 and fru^4 mutations are not simply blocked at attempted-copulation stage of courtship. These two mutants also failed to generate courtship song sounds, although humming sounds (possibly sine song) were heard from a few fru^3 and fru^4 individuals (see RESULTS). Near muteness was also the case for the fru^3/fru^4 transheterozygote type and for males in which fru^3 or fru^4 was uncovered by *fru* deletions. These mutant types were also distinctly

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TABLE 8

MOL in *fru* mutant males

Phenotypic class[a]	Percentage of dissected males in a given category, for genotypes									
	fru^1/fru^1 [b]	fru^1/fru^2 [b]	fru^1/fru^3	fru^1/fru^4	fru^2/fru^2 [c]	fru^2/fru^3	fru^2/fru^4	fru^3/fru^3	fru^3/fru^4	fru^4/fru^4
Abs|Abs	50	—	17	6	—	22	—	89	94	94
Abs|a-1	10	10	6	—	—	11	6	11	6	—
Abs|a-2	10	—	11	17	—	—	11	—	—	6
a-1|a-1	10	—	28	11	—	11	11	—	—	—
a-1|a-2	—	20	11	39	6	17	44	—	—	—
a-2|a-2	—	50	17	11	—	22	11	—	—	—
Abs|b	—	—	—	—	—	—	—	—	—	—
a-1|b	—	—	—	—	—	—	11	—	—	—
a-2|b	20	10	—	—	—	—	—	—	—	—
b|b	—	—	—	17	6	—	—	—	—	—
Abs|+	—	—	—	—	—	—	6	—	—	—
a-1|+	—	—	—	—	6	—	—	—	—	—
a-2|+	—	—	11	—	6	—	—	—	—	—
b|+	—	10	—	—	6	6	—	—	—	—
+|+	—	—	—	—	72	11	—	—	—	—

For each of these 10 genotypes, 18 males were dissected in recent observations; for the exception see [b]. Also, 20 wild-type (Canton-S) males were dissected; all 40 bilaterally symmetrical MOLs were normal. For the mutant, the percentages of normal or (to varying degrees) defective MOL morphologies as tabulated indicate that fru^3 and fru^4, compared to fru^1 and fru^2, lead to more defective phenotypes when the new alleles are either homozygous or heterozygous with each other: 50 of 54 fru^3 and fru^4 males developed with no trace of the MOL, three of the 36 homozygous males and one transheterozygous male showed only the weakest development of the MOL (last three columns). This is connected with the a-1 and a-2 categories (see below) being the weakest expressors of MOL-related *fru* function. The hierarchy of phenotypic expression with respect to MOL formation can be devised as fru^+ > b > a-2 > a-1 > absent; with the corresponding allele hierarchy being $fru^+ > fru^2 > fru^1 > fru^3 = fru^4$. For example, fru^2 is nearest to normal, based on the large fraction of males (72%) with a normal MOL (recall that this mutation allows for fertility). fru^3 and fru^4 each failed to complement fru^1 and fru^2 for MOL development (columns 3–4, 6–7). The first two columns show a reassessment of the same 10 fru^1 homozygous males and the same 10 fru^1/fru^2 males as in the first report involving *fruitless* and this male-specific abdominal muscle (GAILEY *et al.* 1991a); here the additional findings came from breaking down the "type a" of GAILEY *et al.* (1991a) into two subclasses, type a-1 and type a-2. Several elements of the older and the current results are congruent; however, the current fru^2 homozygous stock (whose analysis appears in column 5) led to a much higher frequency of normal MOLs: 72% *vs.* 0% (fru^2 in GAILEY *et al.* 1991, being the *P(w$^+$)ARO-1* homozygous type in Table 2 of that report). There is no explanation for this improvement in fru^2's MOL phenotype. An effect of that allele on this aspect of male anatomy has not reverted (or totally disappeared via "genetic background" effects), because the current version of fru^2 produced a normal MOL in only 11% of the males when heterozygous with fru^3 (column 6), and no MOLs among the fru^2/fru^4 males (column 7). Abs, absent (no distinguishable difference from longitudinal fibers in other segments); a-1, thickening and/or clustering of three or more fibers at the expected site of the MOL (fiber insertions at sites anticipated for normal longitudinal fibers); a-2, (as in a-1, but posterior insertion of fibers at MOL site more posteriorly into A6 as expected for a normal MOL; b, nearly normal, MOL-like fibers in A5 of wild-type density (thicker than in the a-2 designation) but no anterior lengthening of fibers as seen in wild-type; +, MOL indistinguishable from wild-type.

[a] The MOL appears normally as a bilaterally symmetrical structure in A5; since many males had MOLs whose "halves" did not fall into the same category, all such possible "mixed" phenotypes are listed. The vertical lines separate the phenotype observed in one side of the abdomen *vs.* the other. Only one mixed class was not observed: Abs|b. Collectively these mixed classes represent the "type c" MOLs as described in GAILEY *et al.* (1991a).

[b] Reassessment of the same 10 males as originally reported in GAILEY *et al.* (1991a).

[c] These results are from dissecting 18 fru^2/fru^2 males in a stock that has not been anatomically examined for 6 years (see above); the effects on MOL formation of this *fru* allele were originally reported in GAILEY *et al.* (1991a).

subnormal in wing extension, another early courtship stage. Thus, fru^3 and fru^4 can be viewed as skipping a courtship step, with singing excised out of the middle of the sequence. No reproductive behavioral variant of this type has previously been reported in Drosophila. This includes the fact that *XX//X0* gynandromorphs, many of which perform male behaviors only up to a certain stage of courtship, were almost never observed to fail in their performance of a courtship step but then resume the sequence at a later one (HALL 1979).

The fru^2 mutant has been analyzed in the present study in more detail than before. This mutant sings a near-normal song, with a slightly slower-than-normal generation of pulses. We believe that this *fru* song abnormality is biologically real. The IPI lengthening for fru^1 (WHEELER *et al.* 1989) has been confirmed, with fru^2 now shown to exhibit the same kind of subtle song defect (Table 3). fru^2 is not blocked at any courtship step.

It is important to note that fru^2 is the result of a transposon insertion at 91B (MOSES *et al.* 1989); this genetic variant was not originally identified as a behavioral mutant. That it is one is revealed by the fact that

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TABLE 9

Courtship elicitation of *fru* variant females and latencies to mating

Genotype	CI	Latencies (min)
fru^1	62 ± 5 (15)	6.8 ± 1.7 (14)[a]
fru^1/Cha^{M5}	53 ± 11 (6)	5.8 ± 2.3 (6)
fru^3	52 ± 5 (20)	5.5 ± 1.7 (4)[a]
$fru^3/P14$	51 ± 7 (11)	9.5 ± 1.6 (10)[a]
fru^3/Cha^{M5}	27 ± 11 (6)	4.1 ± 1.0 (6)
fru^4	60 ± 4 (22)	4.2 ± 1.6 (10)
$fru^4/P14$	56 ± 19 (4)	3.9 ± 2.5 (4)
fru^4/Cha^{M5}	48 ± 9 (5)	1.4 ± 0.4 (5)
Wild type	54 ± 5 (20)	4.3 ± 0.7 (14)

For each element of this series of observations, a *fru*-variant or control female (3–6 days old) was placed with an individual wild-type male (4–6 days old). A pair was observed for either 8 or 20 min (see MATERIALS AND METHODS). The percentage of time the male spent courting the female was determined and tabulated as a CI, the means and SEMs for which are in the first column. The average latencies to initiation of mating are in the second column; such latencies were calculated only for pairs that began to mate within 20 min (this is why the *n*'s are different from those of CIs). If the females did not mate within 20 min, the pair was nevertheless placed in a food vial and checked for the presence of progeny after 7–10 days.

[a] In one observation for each of these genotypes, the pair did not mate within 20 min. The total proportions of females that did not mate within the 20-min observation periods (including those homozygous for the *fru* mutation in question, and those carrying the mutation heterozygous with the Cha^{M5} or *P14* deletions) were as follows: for fru^1, 1/21; fru^3, 2/22; fru^4, 0/19; and for wild-type, 0/14. Planned comparisons (deemed significant if $P < \alpha = 0.006$) following a one-way ANOVA on transformed CIs (see MATERIALS AND METHODS) revealed that all *fru*-variant females (homozygous and heterozygous) were courted as vigorously as were wild-type females (all *P*'s > 0.006). Planned comparisons (deemed significant if $P < \alpha = 0.006$) on transformed latency values (see MATERIALS AND METHODS) showed that all *fru*-variant females were no different than controls (all *P*'s > 0.006) in terms of how readily they accepted male mating attempts.

fru^2 males form courtship chains (and other kinds of intermale clusterings: *cf.* Figure 2). Also fru^2 causes MOL defects (GAILEY *et al.* 1991a; confirmed here: Table 8). fru^2 fertility is unique among *fru* mutants (Table 4); an apparent corollary is that this mutant exhibits a preference for females (Table 5). A further feature of the fru^2 phenotype might seem connected: these males do not chain as vigorously as fru^1 (Figure 3, Table 6). However, this correlation breaks down in the overall sense, because neither fru^3 nor fru^4 males chain at the high levels recorded for fru^1 males, but they are completely sterile (Table 4).

The high degree of chaining behavior exhibited by fru^1 could be explained as a breakpoint effect operating exclusively in this particular *fruitless* mutant. Thus fru^1 has an inversion breakpoint at the *fruitless* locus (GAILEY and HALL 1989), whereas all other *fru* mutants are caused by transposon inserts (ITO *et al.* 1996; RYNER *et al.* 1996). The other breakpoint in fru^1 causes males to elicit courtship at abnormally high levels (GAILEY and HALL 1989). In this respect, it is important to bear in mind that fru^1/Dfs-91B still chained early and often (Table 6), as do fru^1 homozygotes (Figure 3). Thus, fru^1's uniquely vigorous chaining maps to 91B, not to the "elicitation locus" (90C).

This leads to two further points: (1) fru^3 and fru^4 do not elicit courtship (this report) and indeed are mutated only at the performance-deficit locus (by transposon insertions within region 91B of *3R*) and (2) those two new mutants are weak in their chaining behavior until they have been aged for some days as adults (Figure 3, Table 6). We have no explanation of this "maturation of chaining" phenomenon, but it helps further to distinguish the behavior of these new mutants from either of the first two alleles. We have also shown, by removing the wings of males put together in groups, that courtship song sounds are not a necessary stimulus for chaining behavior; this could have been the case (*cf.* SCHILCHER 1976; KYRIACOU and HALL 1984). Wing removal also eliminated the males' ability to perform wing-flick rejection behavior, but it did not lead to a breakdown of the hierarchy of chaining levels among *fruitless* mutants.

fru^3 and fru^4 are more similar to each other than to fru^1 or fru^2 (see above). However, fru^3 males court females less when compared with the performance of fru^4 (*e.g.*, Tables 1 and 5). In this respect, it is important to recall that these *fru* mutants were not subnormal in general phenotypes unrelated to courtship. Thus, the impaired courtship of females, the weaker overall chaining, and the absence of pulse song could not be accounted for by decrements in locomotor activity (Table 2), viability, or the inability or certain mutants (fru^3 or fru^4) to use their wings in nonreproductive circumstances (Figure 1).

These general behavioral tests should be regarded as more than mundane controls. The most recently induced *fru* mutations (chromosome breakpoints nearer to the *fru* locus ORF than fru^4's 91B breakpoint) are developmentally lethal mutations (RYNER *et al.* 1996). Thus, in retrospect, the possibility that fru^3 and fru^4 could have been generally sick and near-lethal in expression is not a matter of knocking down a wooden soldier. One of the earlier *fru*-variant types is, however, subnormal in its general health, the double-deletion type ($Cha^{M5}/P14$). This genotypic combination resulted in flies that were reasonably vigorous in assays of general locomotor-activity (Table 2), but they were poor fliers (Figure 1). Double-deletion males did not sing in the presence of a female or another male: an obvious consequence of the fact that they performed no wing extension (Table 1). But interpretation of this songlessness is problematical given the poor flight performance caused by this genotype. These two chromosome

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TABLE 10

Summary of sexually related phenotypes for homozygous *fru*-mutant males and for certain heterozygous mutant combinations

Phenotype	Genotypes							
	fru[1]	*fru*[2]	*fru*[3]	*fru*[4]	*fru*[3]/*fru*[4]	*fru*[3]/*fru*[1]	*fru*[4]/*fru*[1]	Controls
Fertility (%)	0	100	0	0	0	64	65	97
Male with female (CI)	56	54	16	31	25	78	88	86
Male with male (CI)	46	35	32	41	47	48	53	3
Chaining (ChI)	75	15	27	22	32	59	65	0
Song IPI (msec)	47	43	Mute	Mute	Mute	46	41	35
MOL (%; none, some, normal)	50, 50, 0	0, 30, 72	89, 11, 0	94, 6, 0	94, 6, 0	17, 84, 0	6, 95, 0	0, 0, 100

Fertility values are from Table 4 but are here represented as percentages. CI values from male-with-female and male-with-male observations are from Table 1. ChIs represent average ChI values for days 4 and 5 from Table 6. Pulse song IPIs are mean IPIs for male-with-female recordings only from Table 3. The numbers in the control column are unweighted averages computed using the data from the various *fru*$^+$-related controls for that phenotype, that is values from wild-type males and from other males that carried *fru* variants heterozygous with the normal allele. The MOL values for each genotype are classified by three percentages, indicating the proportions of males with no detectable muscle at all, with an intermediate MOL phenotype (see Table 8), and with a normal-appearing MOL; the three such values for some genotypes do not sum to 100% because of rounding (again, see Table 8). The control figures for MOL phenotypes did not involve any dissections of *fru*-variant/+ males (see above) but instead are derived from observations of the abdomens of wild-type *D. melanogaster* males (see legend to Table 8).

breakpoints could be causing molecular defects that approach those of the lethal breakpoints (*cf.* RYNER *et al.* 1996), or heterozygosity for the *P14* deletion may be the *fru*-unrelated culprit (see RESULTS). An additional and unique attribute of the double-deletion type is its near courtlessness in single-pair tests (Table 1); this correlates with these flies' low levels of chaining behavior (Table 6). Previously, chaining behavior of this genotype had been noted but not quantified (GAILEY and HALL 1989; GAILEY *et al.* 1991a).

Given the large size of the *fru* locus (~150 kb), the complexity of mRNAs produced by it, and the tissue distribution of gene products (RYNER *et al.* 1996), it is probably an oversimplification to formulate an expression hierarchy for the existing *fru* mutations relative to the various reproductive phenotypes. Nevertheless, certain consistencies can be inferred. For instance, the phenotypically similar *fru*^{3} and *fru*^{4} mutants are accounted for by *P*-element insertions roughly in the middle of the locus, albeit in a region that is still many kb 5′ to the coding region (RYNER *et al.* 1996). *fru*^{2} leads to seemingly less severe *fru* phenotypes and is caused by a separate *P*-element insertion (involving a different kind of *P* derivative); its insertion site is closer to the ORF (RYNER *et al.* 1996), notwithstanding its relatively mild phenotypic impairments. The two lethal breakpoints, which cause the most severe biological abnormalities, are in this same (*fru*^{2}-defined) region. These breakpoints were isolated after ionizing radiation treatments of *fru*^{2} and recovery of transposon-deficient marker-minus flies; however, the isolation strategy for these two newest mutations did not involve viability decrements. Pharate-adult lethality was discovered in subsequent testing (RYNER *et al.* 1996). Apparently, it is in part the nature of a given *fru* lesion that contributes to the resulting phenotypes as opposed to, for example, proximity to the protein-coding portion of the locus (which generates a collection of BTB/zinc-finger polypeptide isotypes: ITO *et al.* 1996; RYNER *et al.* 1996).

fru^1 causes one of the most dramatic departures from normal male courtship: off-scale chaining behavior. The quantification of this phenotype now sets *fru*^{1} apart from chaining associated with all other *fru* genotypes, including of course *fru*$^+$ (Figure 3, Table 6). However, *fru*^{1} is much nearer to normal in its courtship song than are most other *fru* mutants (Table 3), and the original mutation does not cause as severe MOL defects as do *fru*^{3} and *fru*^{4} (Table 8; also RYNER *et al.* 1996). Molecularly, *fru*^{1} is accounted for by a restriction fragment length polymorphism (RYNER *et al.* 1996) consistent with this mutant's courtship-performance breakpoint mapping to 91B (GAILEY and HALL 1989). The *fru*^{1} lesion is farther from the ORF in comparison to the intralocus sites of all other *fru* variants, including the two *Df* breakpoints noted above. These are roughly in the middle of the gene and well 5′ to the ORF, albeit ~25 kb apart, such that the double-deletion genotype does not lead to a homozygous deficiency of any portion of *fru*'s 5′-flanking region (RYNER *et al.* 1996).

That the *fru*^{1} lesion is not close to the new mutants may be loosely correlated with the fact that this *fru*^{1} males sing, whereas *fru*^{3} and *fru*^{4} do not. Yet, all three of these types are sterile. Because *fru*^{1} complements both *fru*^{3} and *fru*^{4} for fertility (CASTRILLON *et al.* 1993; and Table 4 of this report), it formally defines a different function compared to that associated with *fru*^{3}, *fru*^{4}, and the *Dfs*. But no simple explanation for this complementation falls out of the molecular mapping of these transposon inserts and chromosomal lesions. As if the potential for confusion were not enough already, consider that two mutations (*fru*^{1} and *fru*^{3}) whose sites are physically ~45 kb (RYNER *et al.* 1996) apart, lead to very

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similar song defects, the stretched out IPIs measured for both *fru*^{1} and *fru*^{2} (Table 3).

It may not be the case that all *fru* mutations are operating in the same molecular direction. Nevertheless, one can gingerly propose the following allelic series, in which < means farther from normality: *fru*^{w12} and *fru*^{w27} (the pharate-adult lethals of RYNER *et al.* 1996) < *M5/P14* (the double-deletion) < *fru*^{3} < *fru*^{4} < *fru*^{1} < *fru*^{2} < *fru*$^+$. We have perhaps incorrectly placed *fru*^{1} nearer to normal than *fru*^{3} or *fru*^{4} (see Table 10 for summary of phenotypes). This placement was based on *fru*^{1}'s courtship vigor, including the dramatic abnormalities recorded when this mutant is with one or more other males, and its relatively weak effects on the MOL (see Table 10). Perhaps this mutant is not hypomorphic at all. *fru*^{1} might be a hypermorph and in this way lead to its unique collection of *fru* phenotypes (Table 10). This hypothesis can be viewed as consistent with the fertility of *fru*^{1}/*fru*^{3} and *fru*^{1}/*fru*^{4} males (balancing effects of oppositely acting mutations?). Whether or not the particulars of this hypothesis and the hierarchy given above have any force, we believe that analysis of the molecular and eventually the tissue etiologies of the various *fru* mutants will help provide an understanding of the normal allele's function.

That function involves a clear participation of the *fru* gene-action among the functions of Drosophila sex-determination hierarchy (review BAKER 1989; MCKEOWN and MADIGAN 1992). In particular, sex-specific splicing of the *fru* primary transcript is controlled by *transformer* and *transformer-2* functions (RYNER *et al.* 1996). This scenario deepens our sense that the principal role of *fru* is to play a major role in controlling sexual differentiation of the fly's nervous system (*cf.* TAYLOR 1992; TAYLOR *et al.* 1994). In conjunction, with the identification of neural expression patterns of post-transcriptionally processed *fru* mRNA, an expanded courtship significance of *fru* function has been revealed, largely by the current series of studies (also see RYNER *et al.* 1996). Considering the wide array of *fru*-mutant defects, essentially all stages of male courtship are abnormal or absent.

The current connections between *fru*-related phenotypes and genotypes (including the molecular ones) enhance the interpretability of *fruitless* reproductive defects, along with raising further questions. For example, we buttress the notion that *fru* mutants with severe courtship defects are really reproductive variants by demonstrating that those behavioral abnormalities are distinctly separable from problems with general adult health and behavior (*e.g.*, Figure 1, Table 2). This is correlated with the almost certain fact that the near-courtless phenotypes caused by the new-breakpoint/partial-*fru*-locus-*Df* combinations are the result of lesions affecting specific subsets of *fru* translation products, whereby these genotypic defects leave the vital function of the locus intact (see DISCUSSION in RYNER *et al.* 1996).

A new question that suggests itself is the following: When one views the courtship effects of the several *fru* mutations, one finds that most but not all stages of the behavioral sequence are affected. The earliest step, orientation, involves recognizing that there is any kind of courtship object present, let alone the correct kind. This recognition stage is disrupted by the new breakpoint-over-deletion combinations just mentioned. It follows that all subsequent stages are severely subnormal or absent in these mutants (RYNER *et al.* 1996), but it is not clear if the ability of the male to perform a later stage is damaged as such or is absent because the sequence was never initiated or ground to a halt at a very early stage. In this respect, *fru*^{3} and *fru*^{4}'s behaviors are most interesting. These mutants are blocked at an early-to-middle stage (song) and at a quite late one (attempted copulation), but *fru*^{3} and *fru*^{4} are able to perform post-song licking behavior (this report). Therefore, no mutant, *fru* or otherwise, fails to perform this distinct courtship step (given that very early blockage of the entire sequence cannot be thought of as a licking defect) (see above).

In principle, and as is implied by certain of the current results, a licking mutant could exist: one that, for example, would sing, fail to lick, then attempt to copulate. The portions of the nervous system involved in these various steps of the courtship sequence have been tentatively identified (HALL 1979). The "licking focus" could be the neural target of some gedanken courtship mutant. In this regard, the behavioral defects exhibited by *fru* mutants, and the intra-brain expression pattern of the RNAs encoded by this gene, fit roughly with the previous conclusions based on behavioral analysis of gynandromorphs (summarized by GREENSPAN 1995a). It remains to be seen whether the mosaically determined licking focus will be interpretable in the context of *fru*'s molecular expression pattern if the supposition that *fru* may not affect this stage of the sequence is correct. Alternatively, a *fru* mutant may turn out to affect licking. Thus, examination of the brain regions in which *fru* products are found may sharpen our view as to which parts of the nervous system control this component of male courtship behavior.

The cloning of *fru* (ITO *et al.* 1996; RYNER *et al.* 1996) permits new *fru* mutants to be made by molecular manipulations as well as by *in vivo* mutagenesis (the source of all extant genetic variations at this locus). Manipulations of this kind have been effected for another sex-determination factor, *transformer*. This bears mentioning, because expression of the "female form" of the *tra*-encoded protein in various regions of the brain of *XY* flies led to intermale courtships (FERVEUR *et al.* 1995; O'DELL *et al.* 1995). Yet, the current study draws attention to a crucial difference between the different etiologies of courtship among males in Drosophila. In

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the cases just cited (also see ZHANG and ODENWALD 1995; HING and CARLSON 1996), it is not a genetic subnormality that leads to this kind of reproductive behavioral anomaly. In contrast, damage to a genetic locus, on which the entirety of the current study is based, is what causes *fru* males to court same-sex flies. Moreover, those subnormal levels of *fru* expression have simultaneously lead to defective courtship in the presence of females. None of the "gene-manipulation courtship variants" noted above have that property (see DE BELLE 1995 for a review of these studies).

Is female reproduction influenced by any *fruitless* gene action? The only solid finding in this respect is the lethality caused by the new chromosomal breakpoints, induced at the fru^2 site. Females as well as males die late in metamorphosis (RYNER *et al.* 1996). It will be important to ask whether female courtship is also affected by those breakpoints (when heterozygous with a *fru* lesion that allows for viability) or any of the previous homozygous-viable mutations. We could not reveal abnormalities of female reproduction in tests of either fru^3 or fru^4 (Table 9). However, it is interesting that homozygosity for the former of these mutations led to less-than-complete fertility for females that had mated. Thus, the possibility that an egg-laying (or intrafemale fertilization) defect could be caused by *fru* mutations should be investigated. If such a defect were a true feature of the overall *fru* syndrome, there would be at least four implications. (1) Expression of *fru* transcript within the female CNS (RYNER *et al.* 1996) could be rationalized, in that (2) the CNS regions in question could control the operation of posterior-abdominal tissues in a reproductive context, based on gynandromorph analysis of female behaviors occurring subsequent to the initial stage of just eliciting male courtship (COOK 1978; SZABAD and FJAZSI 1982). Although, (3) with certain *fru* products found in the female reproductive system (RYNER *et al.* 1996), a hypothetical effect on egg laying could have a more autonomous tissue etiology. Finally, (4) the position of *fru* in the sex-determination hierarchy (SDH), which molecularly is just downstream of the action of *tra* and *tra-2* (RYNER *et al.* 1996), could be rationalized from an additional perspective beyond the molecular one.

In this regard, it is perhaps puzzling that the positioning of *fru* in this hierarchy, not only at the downstream location just indicated but also in effect parallel to that of *doublesex*, was postulated in a situation whereby the latter gene, but not *fru*, affects female phenotypes when mutated (HILDRETH 1965; reviewed in MCKEOWN and MADIGAN 1992). In fact, this conceptual snag led to the squeezing in of a hypothetical gene into the SDH, variously called "*ambisex*" (TAYLOR *et al.* 1994) or "*gene-Z*" (HALL 1994), placed between the position of *tra* or *tra-2* and *fru* in the neural branch of this hierarchy. The current molecular picture belies any necessity for hypothesizing any such intercalating gene (RYNER *et al.* 1996). Elements of the present results, from behaviorally analyzing the *fruitless* mutants, suggest that female phenotypes will be one more way in which the extended reproductive roles played by *fru* must ultimately be appreciated.

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LITERATURE CITED

BAKER, B. S., 1989 Sex in flies: the splice of life. Nature **340:** 521–524.

BARNES, P. T., and C. C. LAURIE-AHLBERG, 1986 Genetic variability of flight metabolism in *Drosophila melanogaster*. III. Effects of *Gpdh* allozymes and environmental temperature on power output. Genetics **112:** 267–294.

BENZER, S., 1967 Behavioral mutants of *Drosophila* isolated by countercurrent distribution. Proc. Natl. Acad. Sci. USA **58:** 1112–1119.

BENZER, S., 1973 Genetic dissection of behavior. Sci. Am. **229 (6):** 24–27.

BERNSTEIN A. S., E. K. NEUMANN and J. C. HALL, 1992 Temporal analysis of tone pulses within the courtship songs of two sibling Drosophila species, their interspecific hybrid, and behavioral mutants of *D. melanogaster*. J. Insect Behav. **5:** 15–36.

CASTRILLON, D. H., P. GÖNCZY, S. ALEXANDER, R. RAWSON, C. G. EBERHART *et al.*, 1993 Toward a molecular genetic analysis of spermatogenesis in *Drosophila melanogaster*: characterization of male-sterile mutants generated by single *P* element mutagenesis. Genetics **135:** 489–505.

COBB, M., and J.-F. FERVEUR, 1996 Evolution and genetic control of mate recognition and stimulation in *Drosophila*. Behav. Process. **35:** 35–54.

COOK, R., 1978 The reproductive behaviour of gyandromorphic *Drosophila melanogaster*. Z. Naturforsch **33C:** 744–754.

CURRIE, D. A., and M. BATE, 1995 Innervation is essential for the development and differentiation of a sex-specific muscle in *Drosophila melanogaster*. Development **121:** 2549–2557.

CURTSINGER, J. W., and C. C. LAURIE-AHLBERG, 1981 Genetic variability of flight metabolism in *Drosophila melanogaster*. I. Characterization of power output during tethered flight. Genetics **98:** 549–564.

DE BELLE, J. S., 1995 *Drosophila* mushroom body subdomains: innate or learned representations of odor preference and sexual orientation? Neuron **15:** 245-247.

FERVEUR, J.-F., K. F. STÖRTKUHL, R. F. STOCKER and R. J. GREENSPAN, 1995 Genetic feminization of brain structures and changed sexual orientation in male *Drosophila melanogaster*. Science **267:** 902–905.

GAILEY, D. A., and J. C. HALL, 1989 Behavior and cytogenetics of *fruitless* in *Drosophila melanogaster*: different courtship defects caused by separate, closely linked lesions. Genetics **121:** 773–785.

GAILEY, D. A., F. R. JACKSON and R. W. SIEGEL, 1982 Male courtship in Drosophila: the conditioned response to immature males and its genetic control. Genetics **102:** 771–782.

GAILEY, D. A., R. C. LACAILLADE and J. C. HALL, 1986 Chemosensory elements of courtship in normal and mutant olfaction-deficient *Drosophila melanogaster*. Behav. Genet. **16:** 375–405.

GAILEY, D. A., B. J. TAYLOR and J. C. HALL, 1991a Elements of the *fruitless* locus regulate development of the muscle of Lawrence, a male-specific structure in the abdomen of *Drosophila melanogaster* adults. Development **113:** 879–890.

GAILEY, D. A., A. VILLELLA and T. TULLY, 1991b Reassessment of the

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effect of biological rhythm mutations on learning in *Drosophila melanogaster*. J. Comp. Physiol. **169:** 685–697.

Gailey, D. A., S. Ohshima, S. J.-M. Santiago, J. M. Montez, A. R. Arellano *et al.*, 1997 The Muscle of Lawrence in *Drosophila*: a case of repeated evolutionary loss. Proc. Natl. Acad. Sci. USA **94:** 4543–4547.

Gill, K. S., 1963 A mutation causing abnormal courtship and mating behavior in males of *Drosophila melanogaster* (abstr). Am. Zool. **3:** 507.

Gorczyca, M., and J. C. Hall, 1987 The Insectavox, an integrated device for recording and amplifying courtship songs of *Drosophila*. Drosophila Inform. Serv. **66:** 157–160.

Greenspan, R. J., 1995a Understanding the genetic construction of behavior. Sci. Am. **272(4):** 74–79.

Greenspan, R. J., 1995b Flies, genes, learning, and memory. Neuron **15:** 747-750.

Griffith, L. C., L. M. Versalis, K. M. Aitken, C. P. Kyriacou, W. Danho *et al.*, 1993 Inhibition of calcium-calmodulin-dependent protein kinase in Drosophila disrupts behavioral plasticity. Neuron **10:** 501–509.

Griffith, L. C., J. Wang, Y. Zhong, C.-F. Wu and R. J. Greenspan, 1994 Calcium/calmodulin-dependent protein kinase II and potassium channel subunit Eag similarly affect plasticity in *Drosophila*. Proc. Natl. Acad. Sci. USA **91:** 10044–10048.

Hall, J. C., 1978 Courtship among males due to a male-sterile mutation in *Drosophila melanogaster*. Behav. Genet. **8:** 125–141.

Hall, J. C., 1979 Control of male reproductive behavior by the central nervous system of Drosophila: dissection of a courtship pathway by genetic mosaics. Genetics **92:** 437–457.

Hall, J. C., 1994 The mating of a fly. Science **264:** 1702–1714.

Hamblen, M., W. A. Zehring, C. P. Kyriacou, P. Reddy, Q. Yu *et al.*, 1986 Germ-line transformation involving DNA from the *period* locus in *Drosophila melanogaster*: overlapping genomic fragments that restore circadian and ultradian rhythmicity to per^0 and per^- mutants. J. Neurogenet. **3:** 249–291.

Hamblen-Coyle, M. J., R. J. Konopka, L. J. Zwiebel, H. V. Colot, H. B. Dowse *et al.*, 1989 A new mutation at the *period* locus of *Drosophila melanogaster* with some novel effects on circadian rhythms. J. Neurogenet. **5:** 229–256.

Hamblen-Coyle, M. J., D. A. Wheeler, J. E. Rutila, M. Rosbash and J. C. Hall 1992 Behavior of period-altered rhythm mutants of Drosophila in light:dark cycles. J. Insect Behav. **5:** 417–446.

Hildreth, P. E., 1965 *doublesex*, a recessive gene that transforms both males and females of *Drosophila* into intersexes. Genetics **51:** 659–678.

Hing, A., and J. R. Carlson, 1996 Male-male courtship behavior induced by ectopic expression of the *Drosophila white* gene: role of sensory function and age. J. Neurobiol. **30:** 454–464.

Ito, H., K. Fujitani, K. Usui, K. Shimizu-Nishikawa, S. Tanaka *et al.*, 1996 Sexual orientation in Drosophila is altered by the *satori* mutation in the sex-determination gene *fruitless* that encodes a zinc finger protein with a BTB domain. Proc. Natl. Acad. Sci. USA **93:** 9687–9692.

Kyriacou, C. P., and J. C. Hall, 1984 Learning and memory mutations impair acoustic priming of mating behaviour in *Drosophila*. Nature **308:** 62–65.

Kulkarni, S. J., and J. C. Hall, 1987 Behavioral and cytogenetic analysis of the *cacophony* courtship song mutant and interacting genetic variants in *Drosophila melanogaster*. Genetics **115:** 461–475.

Lawrence, P. A., and P. Johnston, 1986 The muscle pattern of a segment of *Drosophila* may be determined by neurons and not by contributing myoblasts. Cell **45:** 505–513.

Lindsley, D. L., and G. G. Zimm, 1992 The Genome of *Drosophila melanogaster*. Academic Press, San Diego.

McKeown, M., and S. J. Madigan, 1992 Sex determination and differentiation in invertebrates: *Drosophila* and *Caenorhabditis elegans*. Curr. Opin. Cell Biol. **4:** 948–954.

Moses, K., 1989 The glass gene encodes a zinc-finger protein required by *Drosophila* photoreceptor cells. Nature **340:** 531–536.

O'Dell, K. M. C., J. D. Armstrong, M. Y. Yang and K. Kaiser, 1995 Functional dissection of the *Drosophila* mushroom bodies by selective feminization of genetically defined subcompartments. Neuron **15:** 55–61.

Paillette, M., H. Ikeda and J.-M. Jallon, 1991 A new acoustic signal of the fruit-flies *Drosophila simulans* and *D. melanogaster*. Bioacoustics **3:** 247–254.

Ryner, L. C., S. F. Goodwin, D. H. Castrillon, A. Anand, A. Villella *et al.*, 1996 Control of male sexual behavior and sexual orientation in Drosophila by the *fruitless* gene. Cell **87:** 1079–1089.

Schilcher, F. V., 1976 The role of auditory stimuli in the courtship of *Drosophila melanogaster*. Anim. Behav. **24:** 18–26.

Schilcher, F. V., 1977 A mutation which changes courtship song in *Drosophila melanogaster*. Behav. Genet. **7:** 251–259.

Siegel, R. W., J. C. Hall, D. A. Gailey and C. P. Kyriacou, 1984 Genetic elements of courtship in *Drosophila*: mosaics and learning mutants. Behav. Genet. **14:** 383–410.

Sokal, R. R., and F. J. Rohlf, 1995 *Biometry*, Ed. 3. W. H. Freeman & Co., New York.

Szabad, J., and C. Fajszi, 1982 Control of female reproduction in Drosophila: genetic dissection using gynandromorphs. Genetics **100:** 61–78.

Taylor, B. J., 1992 Differentiation of a male-specific muscle in *Drosophila melanogaster* does not require the sex-determining genes *doublesex* or *intersex*. Genetics **132:** 179–191.

Taylor, B. J., and L. Knittel, 1995 Sex-specific differentiation of a male-specific abdominal muscle, the Muscle of Lawrence, is abnormal in hydroxyurea-treated and in *fruitless* male flies. Development **121(9):** 3079–3088.

Taylor, B. J., A. Villella, L. C. Ryner, B. S. Baker and J. C. Hall, 1994 Behavioral and neurobiological implications of sex-determining factors in *Drosophila*. Dev. Genet. **15:** 275–296.

Tompkins, L., 1984 Genetic analysis of sex appeal in *Drosophila*. Behav. Genet. **14:** 411–440.

Tompkins, L., 1989 Homosexual courtship in *Drosophila*, pp. 229–248 in *Perspectives in Neural Systems and Behavior*, edited by T. J. Carew and D. B. Kelley. Alan R. Liss, New York.

Tompkins, L., J. C. Hall and L. M. Hall, 1980 Courtship-stimulating volatile compounds from normal and mutant *Drosophila*. J. Insect Physiol. **26:** 689–697.

Tully, T., and D. Gold, 1993 Differential effects of *dunce* mutations on associative learning and memory in *Drosophila*. J. Neurogenet. **9:** 55–71.

Villella, A., and J. C. Hall, 1996 Courtship anomalies caused by *doublesex* mutations in *Drosophila melanogaster*. Genetics **143:** 331–344.

Wheeler, D. A., S. J. Kulkarni, D. A. Gailey and J. C. Hall, 1989 Spectral analysis of courtship songs in behavioral mutants of *Drosophila melanogaster*. Behav. Genet. **19:** 503–528.

Zhang, S.-D., and W. F. Odenwald, 1995 Misexpression of the *white* gene triggers male-male courtship in *Drosophila*. Proc. Natl. Acad. Sci. USA **92:** 5525–5529.

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