Title
Functional dissection of the neural substrates for sexual behaviors in Drosophila melanogaster.

Permalink
https://escholarship.org/uc/item/20q7v70s

Journal
Genetics, 189(1)

ISSN
0016-6731

Authors
Meissner, Geoffrey W
Manoli, Devanand S
Chavez, Jose F
et al.

Publication Date
2011-09-01

DOI
10.1534/genetics.111.129940

Peer reviewed
ABSTRACT The male-specific Fruitless proteins (FruM) act to establish the potential for male courtship behavior in Drosophila melanogaster and are expressed in small groups of neurons throughout the nervous system. We screened ~1000 GAL4 lines, using assays for general courtship, male–male interactions, and male fertility to determine the phenotypes resulting from the GAL4-driven inhibition of FruM expression in subsets of these neurons. A battery of secondary assays showed that the phenotypic classes of GAL4 lines could be divided into subgroups on the basis of additional neurobiological and behavioral criteria. For example, in some lines, restoration of FruM expression in cholinergic neurons restores fertility or reduces male–male courtship. Persistent chains of males courting each other in some lines results from males courting both sexes indiscriminately, whereas in other lines this phenotype results from apparent habituation deficits. Inhibition of ectopic FruM expression in females, in populations of neurons where FruM is necessary for male fertility, can rescue female infertility. To identify the neurons responsible for some of the observed behavioral alterations, we determined the overlap between the identified GAL4 lines and endogenous FruM expression in lines with fertility defects. The GAL4 lines causing fertility defects generally had widespread overlap with FruM expression in many regions of the nervous system, suggesting likely redundant FruM-expressing neuronal pathways capable of conferring male fertility. From associations between the screened behaviors, we propose a functional model for courtship initiation.

G ENETICS offers powerful approaches for (1) identifying the neural circuitry underlying complex behaviors, (2) elucidating how such neural circuits are organized during development or modified by experience, and (3) understanding how such circuits function in behaving animals. Indeed, distinct genetic elements may regulate the neural substrates of behaviors as diverse as courtship and mating, aggression and avoidance, speech, language, and social behavior (Juntti et al. 2008; Robinson et al. 2008; Fisher and Scharff 2009; Siwicki and Kravitz 2009; Wu et al. 2009; Juntti et al. 2010; Robinett et al. 2010).

As reproductive behaviors are often innate, they provide excellent systems for genetic approaches (Manoli et al. 2006; Kimchi et al. 2007; Portman 2007; Juntti et al. 2008; Villella and Hall 2008). Neuronal circuits mediating reproductive behaviors must act to discriminate relevant from nonrelevant stimuli, integrate information across multiple sensory modalities, and generate appropriate behavioral output. Understanding how sex-specific genetic functions organize this circuitry, as well as how that circuitry functions, will hopefully elucidate how complex behaviors are generated by the nervous system. Here, we use molecular tools derived from the Drosophila melanogaster sex determination gene fruitless to dissect the behavioral components of male courtship behavior.

The D. melanogaster male courtship ritual is an extensively studied, complex innate behavior that can be executed by males reared in isolation (Hall 1994; Greenspan and
Ferveur 2000). Information perceived via multiple sensory modalities is integrated to direct both the initiation and progression of courtship. The initial identification of appropriate female targets is via visual and olfactory cues (Sturtevant 1915; Hall 1994; Greenspan and Ferveur 2000; Stockinger et al. 2005; Kurtovic et al. 2007; Krstic et al. 2009). Subsequent steps are mediated via contact-mediated chemosensory or mechanosensory cues perceived during tapping, licking, and attempted copulation (Acebes et al. 2003; Bray and Amrein 2003; Lacaille et al. 2007; Moon et al. 2009; Koganezawa et al. 2010). In addition, auditory cues (song) generated by males enhance male courtship drive and stimulate female receptivity (Kowalski et al. 2004; Ejima et al. 2005). Although most steps of courtship are innate, some are experience dependent (Griffith and Ejima 2009). Thus studies of male courtship behavior can contribute to understanding sensory processing and integration, coordination of motor programs, and motor output, as well as experience-dependent behavioral modifications.

In Drosophila a regulatory gene hierarchy governs all aspects of somatic sexual differentiation, including the potential for male courtship behavior. This cascade directs the synthesis of the sex-specific transcription factors encoded by the fruitless (fru) and doublesex (dsx) genes (Baker et al. 2001; Manoli et al. 2006; Dickson 2008; Villella and Hall 2008; Yamamoto 2008; Siwicki and Kravitz 2009). Fru is the key effector through which the nervous system is sculpted for male behavior. Transcripts derived from the distalmost promoter (P1) of the fru locus (fruM) are sex-specifically spliced to produce mRNAs that encode Fru proteins (FruM) in males and are untranslated in females (Ryner et al. 1996; Heinrichs et al. 1998; Lee et al. 2000; Usui-Aoki et al. 2000). FruM transcription factors are expressed in ~2% of neurons in the brain and ventral nerve cord (VNC), as well as in sensory components of the PNS (Ryner et al. 1996; Lee et al. 2000; Manoli et al. 2005; Stockinger et al. 2005; Cachero et al. 2010; Yu et al. 2010). FruM expression in the appropriate neurons is necessary and sufficient to generate the potential for nearly all aspects of male courtship behavior (Manoli et al. 2005; Stockinger et al. 2005; Manoli et al. 2006). Although the fruM expression pattern is grossly similar between males and females (Manoli et al. 2005; Stockinger et al. 2005), visualization of subsets of fruM-expressing neurons revealed several fruM-dependent sexual dimorphisms (Kimura et al. 2005; Rideout et al. 2007; Datta et al. 2008; Kimura et al. 2008; Koganezawa et al. 2010; Mellert et al. 2010). More recent systematic anatomical characterizations of individual fruM neurons in males and females have revealed extensive sexual dimorphism in the fru circuitry (Cachero et al. 2010; Yu et al. 2010).

The presence or absence of fruM products also governs other sex-specific Drosophila behaviors, which include male- and female-specific aggression behaviors (Chen et al. 2002; Nilsen et al. 2004; Vrontou et al. 2006; Chan and Kravitz 2007), and at least some aspects of female reproductive behaviors (Kvitsiani and Dickson 2006; Yapici et al. 2008; Häsemeyer et al. 2009; Yang et al. 2009). fruM-expressing neurons appear to be dedicated to mediating these social interactions, since silencing these neurons via expression of the neural silencer shiM does not affect other general behaviors (Manoli et al. 2005; Stockinger et al. 2005), and activating them triggers courtship behavior (Kohatsu et al. 2011; Pan et al. 2011; von Philippsborn et al. 2011).

dsx plays an important role in the production of courtship song and the generation of sexually dimorphic numbers of neurons in parts of the central nervous system (CNS) and peripheral nervous system (PNS) (Demir and Dickson 2005; Manoli et al. 2005; Rideout et al. 2007; Kimura et al. 2008; Sanders and Arbeiteman 2008; Mellert et al. 2010; Rideout et al. 2010). However, the overall effects of dsx null mutants on courtship behavior are subtle compared to those of fru.

The broad pattern of fruM expression and the potent effects of impaired fruM function on sexual behavior raises the question of which neurons regulate particular aspects of the behavior. Mosaic mapping experiments using XO/XX mosaics identified regions of the fly that need to be male for different steps of courtship to occur (Hotta and Benzer 1972; Hall 1977, 1978, 1979). Although these studies have inherent limitations that restrict their resolution (Kankel and Hall 1976), these results are consistent with the proposition that subsets of the CNS appear to function in specific steps of sexual behaviors, a key premise of the current study.

More recently, many studies focused on select groups of fruM neurons have associated particular behavioral phenotypes with defined subsets of fruM-expressing neurons (Ferveur et al. 1995; O'Dell et al. 1995; Ferveur and Greenspan 1998; Lee and Hall 2001; Lee et al. 2001; Manoli and Baker 2004; Couto et al. 2005; Fishilevich and Vosshall 2005; Chan and Kravitz 2007; Kurtovic et al. 2007; Clyne and Miesenböck 2008; Datta et al. 2008; Kimura et al. 2008; Koganezawa et al. 2010; Kohatsu et al. 2011; von Philippsborn et al. 2011). These studies provide entry points for the further understanding of the circuitry underlying male sexual behavior.

To more systematically assess the functional roles of subsets of FruM-expressing neurons, we have used >1000 random GAL4 enhancer traps to target RNAi-mediated inhibition of FruM expression in reproducible, restricted subsets of FruM neurons (Figure 1). Our primary screen used assays for male fertility, elevated levels of male–male interactions, and perturbations in easily scored aspects of male–female courtship to dissect the functional components of Drosophila sexual behavior. From these assays, we identified lines producing altered latencies to courtship initiation, increased male chaining, male–male courtship or aggressive behaviors, and defects in male fertility. The GAL4 lines identified in our initial screen were subjected to secondary screens that sought to differentiate between various neural processes that may have contributed to similar behavioral phenotypes, as well as to assess the contribution of certain types of neurons to the phenotypes observed. Finally, as an initial attempt to correlate these functionally defined phenotypic classes with the manipulation of particular subsets of fru–PI-expressing neurons, we determined which
neurons express the GAL4 drivers that produced deficits in male fertility.

Materials and Methods

Drosophila stocks and cultures

Flies were raised on standard dextrose media. Crosses were performed at 29°C to optimize GAL4 and RNAi function. CO2 was used to anesthetize flies. The UAS-fruMIR, UAS-GFP-IR, and fru-P1-LexA stocks have been previously described (Manoli et al. 2005; Mellert et al. 2010). The GAL4 enhancer trap collection was generated in the laboratory of Ulrike Heberlein (personal communication; University of California, San Francisco) and was produced via the mobilization of the pGAWB element into a w Berlin strain. The Canton-S strain (CS) used was the CS-A strain obtained from the laboratory of J. Hall (Brandeis University, Boston). The UAS-fruMIR, Cha-GAL80 stock was made by recombining the Cha-GAL80 (Kitamoto 2001) transgene with the chromosome III UAS-fruMIR. The fruΔvre allele was obtained from the Dickson lab (Demir and Dickson 2005). We used a nuclear GFP reporter for fru-P1-LexA, LexAop-Stinger-GFP, made by replacing the UAS element of UAS-Stinger-GFP with four LexAop binding sites (Barolo et al. 2000; Lai and Lee 2006).

Primary assays

F1 males containing each GAL4 enhancer trap and two UAS-fruMIR (experimental) or two UAS-GFP-IR (control) transgenes were collected within 12 hr of eclosion from 29°C crosses and stored individually at 29°C for the times indicated. To verify behavioral phenotypes, genotypes displaying altered behavior were produced from new crosses and subject to identical retests.

Fertility: Three to 5 days after collection, 5–10 F1 males were crossed individually to a pair of ~1-week-old CS virgins. Crosses were carried out at 29°C and the number of pupae counted once the first pharates appeared (7–8 days). GAL4/UAS-GFP-IR males were used as controls and produced an average of 45.7 pupae (standard deviation of 12.9). Genotypes with an average fecundity of <25 pupae were retested.

General courtship: F1 males were entrained for 3 days in a 12-hr light:dark (12 l/d) cycle at 29°C. Following entrainment, typically four males and four 2-day-old CS virgin females were anesthetized with CO2 2–4 hr after lights on [zeitgeber time (ZT) 2–4] and placed into individual wells of a 24-well tissue culture plate (Falcon). Animals were allowed to recover for 3–5 hr at 25°C following anesthesia. For courtship assays, plates containing males were inverted onto those containing females, with a thin plastic barrier between the chambers at 25°C. The barrier was moved allowing a row of males to be simultaneously introduced into the female chambers below and the barrier replaced, giving each well one male/female pair. Latency to courtship initiation (first wing extension) was measured, and any gross aberrations in courtship behavior were noted. Retests were performed on genotypes in which two or more males initiated courtship in <60 sec or failed to initiate courtship within 2 min.

GAL4 lines showing defects in wing extension and vibration were reexamined in detail on video recordings. Pairs were observed in a standard Plexiglas mating wheel with cylindrical chambers (diameter 10 mm, height 6 mm) (Villega et al. 1997) at 25°C and videotaped for 10–15 min or until copulation. Observations were conducted between ZT 2 and ZT 7. At least four males from each line were videotaped and for some assays a program called LifesongX programmed by J. Reiffel was used to count the number of abnormal behaviors, as well as to record time spent courting. Both CS and GAL4/UAS-GFP-IR males were used as controls.

Male–male interaction: Following 3–5 days at 29°C, males were entrained at 29°C for 3–4 days in a 12 l/d cycle. A total of seven to eight males per line were then anesthetized and placed together into a 24-well tissue culture plate (Falcon)
that contained ~1 mL of standard dextrose media at 29°C. Males were observed for several minutes twice daily (ZT 2–3 and ZT 8–10) for 3 days and chaining behavior and male–male courtship and aggressive behaviors were scored qualitatively on a 0–3 scale, ranging from no aberrant behavior to very high levels of abnormal behavior. Chaining was defined as three or more males showing persistent courtship in a series for at least 5 sec, whereas male–male courtship was between pairs of males (supporting information, File S1). Aggressive behaviors were defined as previously described (File S2) (Chen et al. 2002; Nilsen et al. 2004).

**Secondary Assays**

**Male–male habituation**: Males from lines exhibiting chaining behavior or male–male courtship were isolated and entrained as in the male–male interaction assay. Typically 20 males were anesthetized using CO2 in the circadian morning (ZT 2–4) and placed individually in wells of a 48-well tissue culture plate (Falcon). Animals were allowed to recover for 4–5 hr following anesthesia at 25°C. Plates containing males were inverted onto those containing sibling males, with a thin plastic barrier between the chambers. For a given line (10 sibling pairs), the barrier was partially moved, the male from the upper chamber was introduced into the lower chamber, and the barrier replaced. Males were videotaped for 7 min immediately after pairing and for 5 min beginning 55 min after pairing, at 25°C. Courtship indexes (time courting/observation period) were determined for minutes 2–7 (initial) and 55–60 (final).

**Mate preference**: Males from lines exhibiting chaining behavior or male–male courtship were tested for their sexual preference in a triad assay in which one test male was placed together with a target female and a target (decoy) male. Decoy males were genotypically c155-GALA/ UAS-fruMIR and thus phenotypically male externally, but unable to court due to the expression of fru RNAi throughout their nervous system. For these assays, test males were isolated and entrained as in the male–male interaction assay. CS virgins were 4–6 days old at testing, not entrained, and kept at room temperature following collection. c155-GALA/UAS-fruMIR decoy males were 18 hr to 3 days old at the time of testing and were maintained at 29°C until testing. Test males were anesthetized with CO2 and allowed to recover for at least 3.5 hr at 25°C. All behavioral tests took place in the circadian afternoon (ZT 7–9). Chambers were 13 mm in diameter and 18 mm deep and split into three vertical sections by removable thin plastic dividers. Flies were separated until testing, then introduced together by removal of the dividers. Following divider removal and tapping down, trials were recorded for 5 min.

To calculate the courtship preference index (Cpi), test males were scored for 5 min or until copulation. The courtship index was calculated as follows: CI = (time spent courting)/(duration of trial); Clf and Clm represent the fraction of time courting the female and decoy male, respectively; Cpi = (Clf − 0.5)/(Clf + Clm). Mean Cpi for each line was compared to mean wild-type Cpi and to zero by a one-sample t test.

**Cha-GAL80 suppression**: Enhancer-trap lines exhibiting chaining behavior or reduced fertility were tested as to whether the fru neurons through which these phenotypes were generated were cholinergic. F1 GAL4/UAS-fruMIR males with and without a Cha-GAL80 transgene were collected and isolated within 12 hr of eclosion from parallel crosses raised at 29°C. Fertility and male–male interactions were assayed as in Primary Assays above.

**Female fertility rescue**: Enhancer-trap lines that had reduced male fertility in the primary assay were subsequently examined for their ability to rescue female infertility induced by expression of the fru-masculinizing fruAra allele (Demir and Dickson 2005). Males containing UAS-fruMIR and the fruAra allele were crossed to females from each GAL4 line, and their w; UAS-fruMIR/GAL4; UAS-fruMIR, fruAra/+ male (as a control for the fruAra) and female offspring were tested for fertility as previously. Males were crossed to two CS females, whereas females were crossed to two CS males.

**Expression**

To visualize fruM expression independently of GAL4, we inserted the LexA::VP16 transcripational activator into the fru locus (Mellert et al. 2010). This transactivation system makes use of the Escherichia coli LexA gene fused with the VP16 activation domain, which targets specific LexAop binding sites (Lai and Lee 2006).

We used a nuclear GFP reporter, LexAop-Sti-GFP with fruP1-LexA. As has been reported (Lai and Lee 2006), LexAop reporters often suffer from leaky expression. We used an insertion (E) with the least leakage for examination of fruM and GAL4 overlap in the CNS and genitalia, and that insertion in combination with a second one (F, which does leak in the CNS and genitalia) for examination of expression in the antenna, maxillary palp, proboscis, and legs, where the stronger expression provided by two insertions is useful. We used fru-P1-LexA, LexAop-Sti-GFP in conjunction with an optimized nuclear DsRed, UAS-Red-Sti (Barolo et al. 2004) to simultaneously visualize LexA and GAL4 expression.

For the visualization of FruM protein we used rat anti-FruM antibody at 1:300 dilution and Cy3 antirat secondary antibody from Jackson ImmunoResearch at a 1:800 dilution, as described (Lee et al. 2000). Samples were imaged at >20 magnification on one of the following confocal microscopes: BioRad MRC 1024, Zeiss 510 Meta, or Zeiss 710, and processed with ImageJ software.

**Statistical analysis**

Statistical analyses were performed with Apple Numbers 2009, Microsoft Excel 2008, and SAS JMP 7. Multivariate regression analyses were performed relating expression and...
infertility, including survival analysis and logistic regression. As quantitative fertility data were not collected for lines with high fertility, we used survival analysis to account for the right censored data, treating fertility as the time variable. In parallel, treating fertility as a binary result, we performed nominal logistic regression on the same dataset.

Results

Screen design

A total of 1057 independent GAL4 enhancer-trap lines were crossed to a stock containing both second and third chromosome inserts of a GAL4-responsive RNAi gene that targets male-specific Fru isoforms (UAS-fruMIR) (Manoli and Baker 2004), and their male progeny were screened for alterations in sexual behaviors. Given the specificity of the RNAi for fruM isoforms, only fruM-expressing neurons in which GAL4 is expressed should be affected by UAS-fruMIR expression in these males (Figure 1A). To minimize background effects the GAL4 lines were in a common Berlin genetic background and the UAS-fruMIR transgenes were introgressed into the same genetic background.

Three high-throughput behavioral assays were used to screen multiple GAL4; UAS-fruMIR male progeny from each GAL4 line for courtship defects (Figure 1B). Males were tested for (1) reduced fertility, (2) the occurrence of chaining behavior (in which multiple males form a chain, each male courting another male), male–male courtship or aggression between pairs of males, and (3) overt courtship defects and alterations in the time to initiation of courtship (courtship latency). Following initial testing, all putative positive lines were retested in the same assays using progeny from independent crosses. To control for nonspecific effects of RNAi expression, a randomly selected subset of GAL4 lines (see below) were crossed to an RNAi targeting GFP transcrips (UAS-GFP-IR). Behavioral defects seen with UAS-fruMIR were not observed or were at a much lower rate with UAS-GFP-IR.

Male fertility

Previous studies have demonstrated that FruM is required for fertility, and that FruM-dependent reductions in male fertility can result from overall decreases in courtship, inability to initiate or successfully complete copulation, defective innervation of male internal genitalia, as well as defects in the transfer of sperm and seminal contents (Villella et al. 1997; Lee et al. 2001; Manoli and Baker 2004). Thus, we postulated that perturbations of courtship behavior at multiple stages might manifest as male infertility.

To measure fertility 5–10 GAL4/UAS-fruMIR male progeny of each GAL4 line were crossed individually with two CS virgin females and the number of offspring measured as described (see Materials and Methods). A total of 235 lines showing fertility defects were initially identified and through retesting, 19 lines were verified as sterile and 90 as having substantially reduced fertility.

Male–male interactions

Social behaviors require that an animal correctly identify a conspecific and its sex, as well as respond appropriately to both sexes in different ethological contexts. Intrasexual interactions occur both in the context of reproduction, as with courtship, as well as in aggressive interactions. As wild-type male reproductive and aggressive interactions are both dependent on fru (Hall 1978; Ito et al. 1996; Ryner et al. 1996; Villella et al. 1997; Goodwin et al. 2000; Anand et al. 2001; Vrontou et al. 2006), we assayed for abnormalities in interactions within groups of GAL4/UAS-fruMIR males.

For male–male interaction assays, seven to eight GAL4/UAS-fruMIR males were grouped and interactions scored as described (see Materials and Methods). Wild-type males displayed little in the way of male–male courtship interactions, likely due to inhibitory cues from males and habituation to positive cues (Miyamoto and Amrein 2008; Lacaille et al. 2009). Similarly, control males showed little aggression, likely due to our chambers having a large available food surface, as limited and localized resources appear to evoke territorial/aggressive behaviors in wild type (Chen et al. 2002; Nilsen et al. 2004).

From 378 lines initially selected as exhibiting elevated levels of male–male interactions, retesting using the same experimental regime identified 120 lines showing chaining behavior, 53 lines showing high levels of male–male courtship (between pairs of males), and 112 lines showing aggression (File S1, File S2, and see Materials and Methods). Lines showing both male–male courtship and chaining at different times were scored as chaining lines. Interestingly, for the aggression lines, only a single male per chamber showed aggressive behavior at each observation, often controlling the entire food surface. Because males were unmarked and only periodically observed rather than constantly monitored, it was not possible to determine whether a single male was aggressive across all observations (which might suggest the establishment of dominance hierarchies) (Nilsen et al. 2004; Vrontou et al. 2006). At different times of observation, 33 lines showed chaining and aggressive behaviors, whereas 20 other lines showed both male–male courtship and aggression, often contemporaneously. The high incidence of lines displaying male–male interactions is notable: of the 1057 GAL4 lines tested, 288 (27%) had reproducibly elevated levels of one or more types of male–male interactions, with significant associations between these and other behavioral phenotypes (see Discussion).

Overt courtship defects

FruM function is required for all steps of courtship from its initiation through copulation and ejaculation (Villella et al. 1997; Lee et al. 2001; Manoli and Baker 2004). We thus assayed abnormalities in courtship latency (time to the first wing extension) and the more visually overt courtship behaviors (i.e., wing extension, wing vibration, and copulation) of individual GAL4/UAS-fruMIR males paired with a CS female.
Initial screening of four males per GAL4 line yielded 260 lines that showed either reduced or extended courtship latencies. After retests, 86 lines with a short courtship latency (fast courtship initiation, median latency <30 sec), and 24 lines with a long latency (slow courtship initiation, median latency >4 min) were selected. Control flies expressing UAS-GFP-IR under control of randomly selected GAL4 lines had a latency of 157 ± 97 sec. To determine whether the observed courtship latency phenotypes were due to changes in the overall activity of mutant males, we assayed locomotive activity of the fast and slow lines (using a Trikinetics DAM). We found that courtship latencies were due to changes in locomotive activity of the fast and slow lines (using a Trikinetics DAM). The strongest fast and slow lines did not dramatically differ in activity from GAL4/UAS-GFP-IR and CS controls (data not shown).

For ~40 lines, GAL4/UAS-fruMIR males exhibited defects in wing extension or vibration (on the basis of visual examination). In addition, one line exhibited defective copulatory behavior (File S3). These lines were examined in greater detail in smaller, standard courtship chambers and videotaped for 10 min or until copulation. Lines in which at least 75% of males tested demonstrated a given phenotype were selected for further analysis. Nine lines showed abnormal wing extension, in which the male rotates the wing upwards to ~30° above the horizontal plane, instead of the usual extension parallel to horizontal. While the wing is held at this upward angle, no vibration is observed. Seventeen lines showed simultaneous extension of both wings, varying between 30° and 80° in extent, instead of the usual unilateral wing extension (File S4). Seven lines showed scissoring behavior, in which both wings are rapidly extended somewhat outward and then retracted again (File S5). Five of the lines showing scissoring also showed double-wing extension, suggesting a relationship between the two behaviors. Eight lines showed permanent wing extension, in which one or both wings were permanently extended perpendicular to the body, and not visibly vibrated during courtship. This phenotype was exhibited even before the male was presented with the female, in contrast to the previous phenotypes. Control flies expressing GFP-IR under the control of a subset of these GAL4 drivers did not show these defects.

From these three parallel behavioral screens of 1057 GAL4 lines (2833 initial assays, as not every line was tested in all three screens, and ~6000 total assays with retests), we obtained 373 lines with robust behavioral changes, 83 of these with defects in more than one assay.

**Secondary assays**

We used a battery of secondary assays to examine whether each phenotypic class of GAL4 lines could be divided into subgroups on the basis of additional neurobiological and behavioral criteria. That the groups of lines with similar behaviors in our primary assays were divisible into different subgroups on the basis of secondary assays suggests that the phenotypic classes identified in the primary screens were generated by multiple mechanisms.

**Mechanisms underlying male–male courtship:** We sought to determine whether chaining behavior resulted from a shared disruption or whether subsets of the chaining lines showed the same overall phenotype as the consequence of perturbations in different neural processes. We therefore examined whether chaining behavior in our lines resulted from: (1) a defect in habituation, by which naïve *D. melanogaster* males learn not to court other males or (2) from a change in mate preference, such that males are competitive with or preferred over females as courtship targets (Gailey et al. 1986; Griffith and Ejima 2009). We examined habituation in the lines showing chaining or high levels of male–male courtship in a modified assay in which pairs of GAL4/UAS-fruMIR males from each line were placed together and the amount of courtship measured both immediately after they were placed together and again after they had been together for 1 hr. Although control GAL4/UAS-GFP-IR and CS males, as well as the majority of GAL4/UAS-fruMIR males showed robust decreases in courtship over 1 hr, we found that 15 of the 109 lines assayed initially had at least a moderate level of courtship (courtship index >0.05, Figure 2A), which was unchanged or increased after 1 hr. Thus, increasing attraction toward males or increasing indiscriminate levels of courtship drive may play a role in this behavior. As pairs of mutant males were used rather than one mutant and one wild type, it is also possible that the rejection behavior of the courted male has changed rather than that of the courting male. Thus, although we term the sustained courtship a failure to habituate, other mechanisms are not excluded.

To examine whether chaining behavior reflects a change in sexual orientation (Villella et al. 1997) we carried out a mate preference assay, in which a GAL4/UAS-fruMIR test male was put together with a wild-type virgin female and a noncourting male, and the fraction of time the GAL4/UAS-fruMIR test male spent courting each of the other two flies was measured. To create noncourting males that would not compete with the experimental male yet retained male somatic identity, we used the panneuronal C155(elav)-GAL4 driver to drive UAS-fruMIR, thus eliminating courtship by these males.

To quantify the data from these assays, a courtship preference index based on three parameters is calculated for each line from the analysis of video recordings (see Materials and Methods). The Cpi calculates the total relative courtship directed at each target and is defined as Cpi = (CIf – 0.5)/(CIf + CIm), where CIf and CIm represent the fraction of time courting the female and decoy male, respectively (Villella et al. 1997). Many lines exhibited a reduced preference for females, with ~30% of the 46 tested lines showing similar courtship of both males and females (Figure 2B). That many chaining lines also show reduced female preference suggests that some fruM neurons contribute to sexual preference by inhibiting inappropriate courtship of males. Most chaining males are willing to court males and females, but either retain a female preference or have no

---

G. W. Meissner et al.

---
preference for either sex, rather than a distinct bias for courtship toward males. Thus, it appears that inhibition of FruM expression is capable of reducing female preference while maintaining significant levels of courtship, supporting the separation of the mechanisms underlying arousal and courtship drive from those mediating the specificity of courtship targets.

Phenotypes involving cholinergic fruM-expressing neurons: To begin to elucidate the neural mechanisms underlying the behavioral phenotypes we observed, we examined the contribution of cholinergic FruM neurons to fertility defects and chaining behaviors. Inhibition of neural activity in a subset of primarily cholinergic neurons has previously been associated with chaining behavior (Kitamoto 2001). A role in fertility was suggested by the finding that male-specific cholinergic neurons in the abdominal ganglion regulate the release of sperm and seminal fluids (Acebes et al. 2004). Thus, we examined the effects of rescuing FruM expression in cholinergic neurons on courtship behavior and fertility. We used the GAL4 repressor GAL80 to selectively prevent the inhibition of FruM expression (by GAL4 driven UAS-fruM-IR) in the subset of neurons that express choline acetyltransferase (Cha) (Figure 3A), thus allowing us to determine the contribution of these neurons to the phenotypes we have identified. Thus we combined the cha-GAL80 transgene (Kitamoto 2001) with UAS-fruM-IR and each GAL4 line to observe the extent of rescue.

Of 99 retested lines originally showing fertility defects, 44 lines more than doubled their fecundity when inhibition of FruM expression was prevented with cha-GAL80 compared to simultaneous controls lacking the GAL80 (Figure 3B and data not shown). Thus perturbation of FruM expression in cha–GAL80-expressing neurons in these 44 lines substantially contributed to their decreased fertility. The failure to rescue the other infertile lines indicates that, consistent with previous studies, FruM function is also required in non-cholinergic neurons for full fertility (Lee et al. 2001).

Of the 123 retested lines showing high levels of chaining behavior because of GAL4-directed fruMIR expression, 8 lines showed a complete suppression of the aberrant behavior with cha-GAL80 and 17 showed a substantial reduction (Figure 3C). Thus, similar to the fertility rescue results, it appears that both cholinergic and noncholinergic neurons contribute to the regulation of male–male interactions.

Rescue of female fertility: The general similarity of FruM neuronal expression in males and females (Manoli et al. 2005; Stockinger et al. 2005) raised the question of whether the labeled neurons share homologous functions in both sexes. Masculinization of fruM neurons in a female, via the use of a dominant male fruM* allele, is sufficient to produce
sterility (Demir and Dickson 2005). Furthermore, UAS-shits-based silencing of the fruM neurons in females caused virgins to exhibit mated female behavior, including reduced courtship receptivity and increased egg laying, even though unmated (Kvitsiani and Dickson 2006). While these findings show that some fruM-expressing neurons are necessary for female fertility, they do not address whether these are the same subset of fruM-expressing neurons that are required for male fertility. As our screen identified a subset of GAL4 lines that produced male infertility when driving UAS-fruMIR, we asked whether the fruM-expressing neurons in these lines are also important for female fertility.

We assayed whether the female infertility produced by fruM expressing neurons can be suppressed by UAS-fruMIR expression driven by the GAL4 lines that caused male fertility defects in our screen. We examined the female fertility of 75 GAL4 lines carrying UAS-fruMIR and fruM expressing by crossing them individually to two CS males and counting their offspring (Figure 4). Eighteen of the lines showed significant rescue ($P < 0.05$, one-way ANOVA comparison to a negative control GAL4 line). Male siblings of the test females generally showed fertility levels similar to what was observed in previous tests of the lines without the fruM expressing, indicating that the UAS-fruMIR is able to overcome any dominant effects of the fruM expressing allele (data not shown). However, analysis revealed no significant correlation between the level of reduction of male fertility and rescue of female fertility in this assay (Figure 4), suggesting that although some neurons may be important for fertility in both sexes, there are likely also neurons that are more important for fertility in just one sex. More detailed examinations of fruM expression in males and females have also identified several sexually dimorphic patterns of cell survival and projections, which could also explain the lack of correlation (Kimura et al. 2005, 2008; Cachero et al. 2010; Yu et al. 2010).

Expression

As described, we used a large collection of GAL4 enhancer-trap lines to inhibit fruM expression in subsets of neurons in males, who were then screened for courtship defects. Beyond the immediate value of these behavioral genetic studies as discussed below, our larger goal has been to identify the particular neurons affected in each GAL4 line and thus delimit the neurons that contribute to different aspects of sexual behaviors. Our general anatomical approach has been to fluorescently label fruM and GAL4 neurons using separate reporters and determine the neurons in which the reporters’ expression overlapped. The neurons expressing both labels represent those affected by the RNAi against fruM and thus the maximal set of neurons implicated in...
the observed behavioral phenotypes, as the UAS-fruMIR has no known effect outside of fruM-expressing neurons (Manoli and Baker 2004).

When we initiated studies to identify the overlaps in expression between fruM and these GAL4’s we used anti-FruM antibodies and fluorescent-labeled secondary antibodies together with a UAS-driven nuclear GFP (UAS-Stinger) (Barolo et al. 2000) to detect fruM and GAL4 expression, respectively, in the brain and VNC, as fruM expression was believed at that time to be restricted to the CNS (Ryner et al. 1996; Lee et al. 2000). We examined 183 lines for the concordance of nuclear GFP and FruM expression in 22 regions of the CNS using the FruM clusters described by Lee et al. (2000), as the basis for this CNS analysis. At the 0–24 hr posteclosion time examined, cluster 11 was not reliably labeled by our antibody and was excluded. We additionally subdivided VNC clusters 17–19 into dorsal (D) and ventral (V) subclusters due to their separation along the D–V axis.

The discovery of fruM expression in extensive sets of peripheral sensory neurons (Manoli et al. 2005; Stockinger et al. 2005) significantly complicated initial attempts to associate the behavioral phenotypes identified in our screen with particular sets of neurons and substantially increased the scope of the project. Because of the tough cuticle surrounding peripheral sensory neurons, antibodies do not reliably penetrate to label these neurons, one of the reasons fruM expression had not been identified there previously. Furthermore, although fru-P1-GAL4 beautifully labels these PNS neurons, our screen of GAL4 lines uses the same driver system, preventing us from unambiguously identifying the overlap between the two expression patterns.

This discovery of peripheral fruM expression provided a potential explanation for our results up to this point, as our analyses had revealed only limited correlations of CNS expression and behavior (Figure S4 and see below). Primarily, lines with delayed courtship and/or chaining behavior tended to have broad CNS overlap with FruM. But from that dataset, we were unable to tie a behavior to expression in any particular FruM cluster.

To label fruM expression independently of GAL4 in both the CNS and PNS, we used homologous recombination to insert the LexA::VP16 transcriptional activator into the fruP1 locus (Lai and Lee 2006; Mellert et al. 2010), which allowed us to follow fruM expression via expression of Lex-Aop-GFP. To detect patterns of GAL4 expression, we used an optimized nuclear DsRed, UAS-Red-Stinger (Barolo et al. 2004).

As a test case for this approach, we examined the concordance of nuclear GFP and RFP expression in lines that had fertility defects. The examined set included 96 lines with fertility defects and 48 control GAL4 lines selected on the basis of normal behavior in our screen. Although we focused on lines with fertility defects, many lines had phenotypes in other assays as well, allowing a limited examination of other phenotypic classes. The results for these other assays may be biased by this selection, however.

We used the clusters described by Lee et al. (2000) as a basis for recording expression in the CNS, additionally noting Kenyon cell expression and subdividing the VNC clusters 16–19 into dorsal and ventral subsets. Cluster 15 could not be distinguished from cluster 14 with our reporter and the two were considered together as 14. We additionally scored expression in the following regions of the PNS: the second and third antennal segments, maxillary palp, and proboscis of the head; the five tarsal segments, tibia, and femoral chordotonal organ of the foreleg; and the anal plate, lateral plate, and claspers of the external genitalia. Together these observations identified the fruM neurons in which each GAL4 line is expressed.

Using this approach, we found that the vast majority of lines causing behavioral phenotypes had extensive, distributed overlap with different FruM clusters (Figure 5, Figure
S2, File S6, File S7). As a result, direct comparison of the GAL4 and fruM expression with observed behavioral defects has yet to indicate an individual cluster where expression is necessary or sufficient to cause fertility defects. Nonetheless, we did observe a general trend toward lines showing behavioral defects, and especially delayed courtship initiation or chaining, tending to have increased average expression compared to controls (Figure 5). Clustering by expression allowed for the visualization of the individual lines’ expression and phenotypes but again did not indicate an obvious association beyond the general trend seen in the averaged data (Figure S3).

We used multivariate regression to look for an expression:phenotype relationship that eluded direct comparison. As fertility was not counted for vials with >50 offspring, thus right censoring the data, we first treated fertility as a time variable in multivariate parametric survival analysis, examining the contribution of each expression cluster to the likelihood of the observed fertility (Table S1). The analysis identified expression in foreleg tarsal segments 4 and 5 and anterior brain cluster 6 as significantly likely to result in lowered fertility. We separately treated fertility as a binary result and analyzed the same dataset with logistic regression. This approach also identified tarsal segment 4 and brain cluster 6 (but not tarsal segment 5) as significant drivers of infertility, supporting the result from the survival analysis. We are nonetheless hesitant to draw strong conclusions about these clusters without further experimental data to directly test their behavioral effects, as basic assumptions of regression models—especially independence of the variables—are unlikely to be met in this complex biological system, but these neural populations remain potential targets for further study.

One explanation for the difficulty of relating expression and behavior from these results is that expression in some of the larger clusters was present in almost all of the lines, and such large clusters likely represent multiple functional classes of neurons, as suggested by their divergent projection patterns and lineage relationships (Cachero et al. 2010; Yu et al. 2010). For example 146 out of the 153 examined lines had overlap with fruM expression in region 20, the abdominal ganglion, including 54 of 58 lines with normal fertility. Similarly, 123 lines (and 20 out of 58 with normal fertility) overlapped with fruM in the third antennal segment, and 117 lines (and 24 out of 58 with normal fertility) overlapped with fruM in region 14 on the posterior brain. This breadth of expression precluded drawing a direct relationship between expression and phenotype, as effects of expression in other clusters cannot be ruled out with the existing dataset.

Discussion

The studies presented here use an intersectional approach to probe the functional organization of the neural substrates underlying male sexual behaviors in Drosophila. The results

![Figure 5](image)

**Figure 5** Average GAL4 overlap with fruM for each courtship phenotype. Phenotypes are sorted by total average expression, with control lines having lowest expression and lines with delayed courtship initiation having highest. Kenyon cell and leg chordotonal expression were scored on a qualitative 0–3 scale for strength of expression. R1–R20, regions 1–20 of the CNS as per Lee et al. (2000); Ant 2 and 3, antennal segments 2 and 3; Prob, proboscis; T1–T5, tarsal segments 1–5 of the forelimb; G_CL, genital claspers; G_LP, genital lateral plate; G_MAN anal plate; KC, Kenyon cells; LegChord, foreleg chordotonal neurons of the femur. See Figure S2 for expression scaled by cluster size, Figure S4 for similar analyses of antibody data, and File S6 for an animation stepping through each phenotype.
of our screens—considered both with respect to the phenotypes we recovered and their underlying causes, as well as the relative frequencies with which certain phenotypic classes were recovered—provide insights into the organization of the fruM-specified circuitry underlying courtship behaviors in D. melanogaster.

The frequency with which abnormalities in courtship behavior were detected in our three primary screens is at first glance surprisingly high. Of the GAL4 lines successfully tested in all three primary assays, 44% (345/777) showed reproducible courtship phenotypes in at least one screen. When considered at the level of individual primary tests, 18% (482/2704) revealed reproducible behavioral defects. There are several factors that likely contribute to this high incidence of courtship phenotypes. First, the progenitor cells that give rise to the CNS are derived from most if not all segments of the body, and fruM-expressing neurons comprise a widely dispersed set of ~2-3% of CNS neurons. Second, fruM neurons make up significant proportions of the primary neurons of the olfactory, gustatory, auditory, and (to a lesser degree) mechanosensory systems, which are derived from imaginal discs. Thus collectively, fruM neurons provide a broad set of targets across the fly for potential overlap with the expression of GAL4 enhancer traps. In this context, it is reasonable that the screen we conducted identified a high frequency of lines in which GAL4-driven UAS-fruMIR expression perturbed male courtship.

That the groups of lines with similar behaviors in our primary assays were divisible into subgroups on the basis of the secondary assays suggests that the phenotypic classes identified in the primary screens were generated via multiple mechanisms. Being able to make such subdivisions is important not only for specifying the functions perturbed in individual lines, but also for identifying subgroups of lines that are more likely to have a homogenous mechanism of action and thus a common set of affected fruM-expressing neurons.

Comparisons of the individual classes of courtship behavior defects detected in our screens and secondary tests reveal that there are substantial differences in the relative frequencies with which different phenotypic classes were detected. As the same set of GAL4 lines was used in all screens, the different frequencies with which particular courtship behavior defects are detected reflect properties of different parts of the courtship circuitry. Following discussion of the individual assays, we discuss associations between phenotypes and propose a simple functional model for courtship initiation that builds upon our observations.

**Sterility**

Reduced fertility was chosen as one of the diagnostic screen phenotypes because behavioral male sterility is a phenotype associated with many fru mutants and mutant combinations. Further, fru mutants defective at different stages of courtship were associated with male sterility (Hall 1978; Villella et al. 1997; Manoli and Baker 2004). We thus anticipated that male sterility would be associated with the GAL4/UAS-fruMIR silencing of FruM expression in multiple different sets of neurons and hence be found as a relatively common phenotype in our screens. Against this background, we were surprised that only 24 of the 911 lines tested produced sterile males. This relative refractoriness of those aspects of the fruM circuitry necessary for fertility to RNAi perturbations suggests the possibility that there is functional redundancy in these aspects of the circuitry or that fruM neurons essential for fertility are restricted with regard to gene expression and thus only rarely overlap with GAL4 expression patterns. From an evolutionary perspective, functional redundancy in the circuitry required for fertility makes sense. It is notable that this robustness of male fertility is not likely due to simple bilateral redundancy in the nervous system, since all the GAL4-expression patterns that we observed were bilaterally symmetrical. That many more lines showed perturbed behavior than sterility indicates that the observed insensitivity of fertility to GAL4/UAS-fruMIR manipulations is also not simply due to a general ineffectiveness of the RNAi, although it is nonetheless possible that the neurons important for fertility are particularly refractive to the RNAi. In addition, the result that many lines altered aspects or specificity of sexual behaviors but only partially impaired these males’ fecundity suggests many fruM neurons may play a modulatory role in these behaviors rather than being strictly necessary for fertility.

**Altered courtship latency**

Our general courtship assay revealed GAL4 lines with altered courtship initiation latencies toward females. More than three times as many lines were recovered with a shortened courtship latency (95 “rapid” lines), than lines with lengthened courtship latency (25 “delayed” lines). The isolation of rapid courtship lines per se suggests that the fruM-specified courtship circuitry contains components that actively inhibit the initiation of courtship. Additionally, the relatively high incidence of rapid courtship lines may suggest that the mechanisms inhibiting courtship in inappropriate contexts are less robust to disruption compared to mechanisms driving courtship, perhaps due to there being more of a cost to delaying courtship than to triggering it inappropriately. Encouragingly, one group of fruM neurons in the median bundle that appear to restrain courtship has been identified (Manoli and Baker 2004). That components of the fruM circuitry function to slow progression through courtship is consistent with arguments that ancestral Diteran courtship was rapid, similar to what is seen in solitary flies like *Musca* and *Calliphora*, where the strategy for courtship appears to be to identify an object of near optimal size and speed, grab it, then determine if it is an appropriate mating target (Spieth 1974; Collett and Land 1975). Under this view, during the course of evolution from rapid mating to expanded courtship, *D. melanogaster* would have acquired functions in its courtship circuitry that generate the relatively complex, ordered process seen today. Such a protracted courtship may have an evolutionary advantage in allowing the male to demonstrate his virility and both the female and male to receive sufficient
information to evaluate a potential mate. It remains to be determined whether lines that demonstrate rapid courtship onset also display specific deficits in discriminating appropriate courtship targets. It should be noted, however, that the previously characterized group of fruM-expressing neurons that function to restrain courtship (Manoli and Baker 2004) do not have a fruM-dependent role in the discrimination of females from males as appropriate courtship targets.

Male–male interactions

The most common phenotype detected in our screens was an elevated level of male–male interactions: 26% (232/908) of the GAL4 lines tested had reproducibly elevated levels of one or more types of male–male interaction. There are several elements of note with respect to the high incidence of lines producing a male–male interaction phenotype.

First, male–male interactions encompasses at least two classes of male behavior that likely have different etiologies. The category includes 117 lines showing aggressive male–male interactions, 121 lines showing chaining behavior (involving three or more males), and 49 lines showing high levels of male–male courtship (between pairs of males). It is currently unclear whether the latter two categories are distinct phenotypic classes or just differ in the level of male–male courtship, although some lines showed very high levels of male–male courtship without chaining, perhaps due to differences in rejection behavior.

Second, even with this subdivision of male–male interacting lines the incidence of lines showing male–male courtship is quite high. Contributing to the high incidence of male–male courtship lines in our screen is the fact that this phenotype itself can be potentially further subdivided into classes of lines having different etiologies. A priori these include: (1) a change in sexual preference so that males are the preferred courtship targets, (2) an inability to distinguish females from males as appropriate courtship targets resulting in both being courted, (3) a failure of males to reduce initial courtship of other males via habituation (i.e., learning not to court males; Gailey et al. 1982), or (4) an increase in sensitivity to general conspecific cues such that inhibitory cues that typically prevent male–male courtship are insufficient to do so. For example, there have been a number of reports of flies with altered gustatory perception showing male–male chaining behavior (Lacaille et al. 2007; Miyamoto and Amrein 2008; Moon et al. 2009). Our tests to investigate these possibilities revealed that in some lines increased male–male courtship may be attributed to a lack of male habituation or increased sensitivity to general cues. Many other lines showed reductions in their preference of females over males as courtship targets. However, none of the lines we examined showed a clear preference for males over females.

Cholinergic fruM-expressing neurons

For the GAL4 lines that produced chaining or fertility defects when driving UAS-fruM-IR we extended our intersectional strategy to examine the contribution of cholinergic fruM-expressing neurons to these chaining and fertility defects. In particular, we used Cha-GAL80 to selectively restore FruM expression in the subset of GAL4/UAS-fruM-IR-expressing neurons that also likely express choline acetyltransferase (Cha) (Figure 3A). The expression of Cha-GAL80 at least doubled the fecundity of ~40% (44/99) of lines with fertility defects, whereas it substantially or completely suppressed chaining in ~20% (25/123) of lines originally showing chaining. As cholinergic neurons are found in the CNS and also make up most if not all, olfactory, gustatory, auditory, and chordotonal sensory neurons (Salvaterra and Kitamoto 2001), the most informative aspect of these results are the findings of GAL4 lines with fruM phenotypes that are not suppressed by the restoration of FruM function in cholinergic neurons, as these likely identify cases where fruM CNS expression is important for the observed phenotype. It is worth noting that the nine chaining lines in which restoration of Frum function produced the greatest suppression of chaining (and 20 of the top 25 such lines) showed normal behavior in the original courtship and fertility assays and retests. This suggests that FruM function in specific cholinergic neurons may be necessary to prevent chaining or the courtship of other males but that these neurons do not play a role in the proper performance of other courtship behaviors. Thus it appears that some elements underlying the discrimination of a courtship target are distinct from those suberving arousal or the drive to display specific sexual behaviors and appear to be mediated by separable subsets of fruM-expressing neurons. That FruM expression in cholinergic neurons is important for selection of a courtship target is in part not surprising, as chemochemical cues contribute significantly to mate preference (Stockinger et al. 2005; Kurtovic et al. 2007; Datta et al. 2008; Krtic et al. 2009) and most if not all chemosensory neurons are cholinergic (Salvaterra and Kitamoto 2001). Similarly, that fertility in part was restored by rescue of expression in cholinergic neurons is consistent with the importance of cholinergic and noncholinergic neurons in copulation (Lee et al. 2001; Acebes et al. 2003; Acebes et al. 2004).

Behavioral associations

In one sense, our screens can be viewed as having isolated an allelic series of fruM mutants, in each of which only some subset of fruM-expressing neurons are mutant in phenotype. By examining the behavior of many lines with altered patterns of FruM expression, we can begin to gain insights into the functional units governing the interactions of male flies with other flies. For example, we observed that specific behavioral phenotypes tended to co-occur among sets of enhancer-trap lines (Figure 6), which could indicate that reduced FruM expression in neurons shared by the lines produces both behavioral outcomes. Alternatively, some enhancer traps may be expressed in several groups of neurons that independently give rise to subsets of the correlated phenotypes. Of the 343 lines showing robust behavioral changes, 84 lines showed changes for more than one type of behavior, providing a substantial group for comparison.
The two most striking correlations we found (1) that lines with longer courtship latencies tended to show chaining behavior, whereas (2) lines with shorter latencies tended to show aggression, are discussed below.

**Delayed courtship associates with chaining:** We initially hypothesized that FruM likely functions in some neurons to repress courtship, such that increased male–male courtship or chaining resulting from the suppression of FruM function might also be associated with rapid initiation of female courtship. We found in fact the opposite: a positive correlation between delayed courtship initiation toward females and chaining behavior. Whereas a minority of lines (120 out of 835, or 14.4%) showed chaining behavior, over half of the lines (14 of 24, or 58.3%) with slow courtship initiation also showed chaining ($P < 0.001$, Pearson’s chi square test; Figure 6). No lines showed delayed courtship and high male–male courtship without also exhibiting chaining behavior.

Four differences between the male–male interaction assay and the courtship assay may contribute to this association: (1) the target of courtship being male or female, thus changing the nature of the stimulus; (2) the number of flies in the chamber (two vs. eight), thus changing the overall level of general conspecific cues in the environment; (3) the time together being minutes vs. days, thus allowing for increased stimulation after longer, sustained interactions; and (4) the presence of food in the chamber, thus altering the environmental cues and perhaps arousal. Our examination of male habitation in the chaining lines also addresses whether lengthened exposure could stimulate courtship of males. Our results suggest that this may be the case in a subset of lines, as 15 of the 109 tested lines showed persistent or increasing male–male courtship over an hour. In general however, the lines showing both delayed courtship and chaining did not show defects in habituation. Similarly, the majority of lines that showed chaining behavior did not have a significant change in sexual orientation in the mate preference assay but, rather, appeared to have an overall decrease in the sex specificity of their courtship. Thus, while we cannot rule out a stimulatory role for the presence of food or overall increases in social behaviors or arousal due to cues from conspecifics, we hypothesize that these lines have a decreased sensitivity to stimulation, which results in courtship only in the presence of multiple conspecifics, rather than when a single female or male was present.

**Rapid courtship initiation associates with aggression:** Lines showing rapid courtship initiation of females were more likely to show aggressive behavior toward males (20 out of 86 lines or 23.2%) than expected, on the basis of the fraction of total lines showing aggression (112 of 835 lines, 13.4%; $P < 0.01$, Pearson’s chi square test; Figure 6). A supportive, but borderline significant trend was seen with lines with delayed courtship, none of which showed aggression ($0$ of $24$, $P = 0.0504$, Pearson’s chi square). Thus, it appears that the inhibition of FruM expression in regions sufficient to cause rapid courtship initiation toward females can be sufficient to increase other sexual behaviors in different contexts. This suggests that common mechanisms may control the stimulus thresholds necessary to elicit appropriate sexual behaviors in specific contexts, while others mediate the discrimination necessary to specify which behaviors occur in distinct ethological situations.

**Mechanisms underlying the specificity and sensitivity of courtship initiation:** On the basis of the assays described, the relative frequencies of lines producing distinct phenotypic classes, and the correlations described between different behaviors, we have developed a relatively simple functional model for the mechanisms underlying the initiation of sexual behaviors (Figure 7). Although other models can be arrived at from our data, this one is concise and has several attractive features that make it integrate well with our knowledge of fruM circuitry as well as observations made by many groups regarding various aspects of Drosophila sexual behaviors. Notably, the model does not distinguish between different sensory modalities. We hope that it will prove useful in interpreting our data and understanding functional elements from expression studies (see File S8, Supplemental Model Discussion).

**Expression**

**General considerations:** We have presented an unbiased approach to attempt to discern correlations between specific populations of fruM neurons and distinct aspects of sexually dimorphic behaviors. However, it has proven challenging to

---

**Figure 6** Associations between behavioral assays. (A) If chaining, aggression, and courtship behaviors were independent, the indicated number of GAL4 lines would show each phenotype or combination thereof. (B) The observed associations significantly ($P$ values indicate Pearson’s chi square significance) deviate from the expected values. In particular we observed an increase in lines with both long courtship latency and chaining behavior, as well as lines with both short courtship latency and aggressive behavior.

---
correlate common sets of fruM neurons with specific aspects of sexual behavior. Factors likely contributing to these difficulties include: the nature of the GAL4 enhancer-trap lines, functional diversity between neighboring populations of fruM neurons, and redundancy in neural circuitry underlying sexual behaviors.

**Functional considerations:** Several factors have directed our thinking as to how best to use this data to identify the fruM-expressing neurons responsible for the various courtship phenotypes we observed. Our initial observations with the FruM antibody, that GAL4 expression commonly overlapped many FruM clusters per line, agreed with our expectation that some overlapping neurons may not be involved in the specific behavioral phenotype(s) we observed. There are several ways in which we can envisage expressing UAS-fruMIR in a subset of fruM neurons might not elicit a behavioral phenotype in our assays. First, a set of fruM neurons might be functionally redundantly with some other subset of fruM neurons. Second, subtle phenotypes in sexual behaviors may not have been detected in our assays. Third, some fruM neurons may be involved in conveying information from the circuitry mediating sexual behaviors to parts of the nervous system involved in other behavioral processes. Taken together these thoughts suggest two approaches that might be used to associate particular behavioral phenotypes with individual groups of fruM neurons.

First, the neurons most likely to contribute to a given behavioral phenotype are those that are common to multiple drivers that produce that behavioral deficit. Thus, to narrow down the set of neurons to those most likely responsible for an observed behavior, we took advantage of the many lines that showed similar phenotypes. We assume that the neurons not responsible for the phenotype will be labeled in some but not other GAL4 lines producing the same phenotype, whereas the essential neurons will be more consistently labeled in multiple GAL4 lines producing the same phenotype. By examining the neurons affected in each such line and comparing them between the lines, one expects to find the shared subset responsible for the observed phenotype.

However, our studies revealed some important challenges to this initial theoretical framework. One is that, as we observed from subdividing the initial behavioral classes by our secondary assays, similar phenotypes can have multiple underlying causes. Thus, multiple independent sets of neurons may be able to cause these phenotypes, preventing isolation of a single group of neurons responsible for a given phenotype. We hope to have reduced this concern by increasing our behavioral resolution with the secondary assays, but likely have not eliminated it. Thus, our analysis must allow for multiple foci for a phenotype rather than seeking a single neuronal population. Second, as described above, while covering several ethological contexts, our assays by no means exhaust the myriad of conditions in which sexual dimorphisms in behaviors may occur. Thus, neuron populations that overlap with regard to phenotypic class in our assays may be too secondary to deficits in pathways that function in contexts outside of those tested, thus preventing their separation.

![Functional diagram](image)

**Figure 7** Functional model of mechanisms mediating sex specificity and stimulation during courtship initiation. (A) In a wild-type male, arousal cues common to both sexes act via FruM-independent mechanisms to increase the likelihood of either aggressive or courtship behaviors, but are insufficient to elicit behavioral programs themselves (black). Female-specific cues act and are enhanced via FruM-dependent mechanisms to drive the initiation of courtship and also likely maintain courtship drive until successful mating (blue). Furthermore, distinct mechanisms modulated by FruM regulate the threshold of stimulation necessary to elicit courtship. Thus, we propose that FruM functions to allow the detection of female-specific cues to increase the strength of signaling downstream of this perception to drive courtship and to modulate the activation threshold for initiation of courtship. The detection of male-specific cues via FruM-dependent mechanisms additionally provides stimulus toward both the initiation of aggression and the inhibition of courtship initiation (green). FruM-dependent mechanisms distinct from the detection of and response to male-specific cues act to modulate the threshold of stimulation necessary to elicit aggressive or courtship behaviors when such cues are perceived. Thus, in addition to regulating the processing of sex-specific cues, distinct FruM-dependent mechanisms modulate the activation thresholds for specific behavioral programs, enabling the alteration of thresholds for sexual behaviors without affecting the specific contexts within which these behaviors occur. (B) In males lacking FruM function, FruM-dependent mechanisms are either eliminated or exist in a default state (red). In the absence of FruM function, these pathways may represent female-like mechanisms of stimulus detection or processing.
An alternative to the approach just outlined picks out the GAL4 line(s) with a given phenotype that have the most restricted overlap with fruM expression. Fewer potential neurons responsible for a phenotype are naturally more straightforward to interpret. Both of these approaches have proved useful in delimiting groups of neurons important for particular behaviors (O’Dell et al. 1995; Broughton et al. 2004; Manoli and Baker 2004; Stockinger et al. 2005; Luan et al. 2006; Chan and Kravitz 2007; Kimura et al. 2008; Luo et al. 2008; Häsemeyer et al. 2009; Yang et al. 2009).

**Neuroanatomical considerations:** Perhaps unsurprisingly, screening for behavioral defects without regard to expression patterns of their drivers has yielded many GAL4 lines with interesting and informative behaviors but with complex expression patterns. The challenge then is to design constraints upon the manipulators either into the initial screen or efficient secondary tests. The primary constraint upon which we relied when we began the screen in 2003 was the restriction of GAL4/UAS-fruMIR effects to fruM-expressing neurons. Discovery of fruM expression in the peripheral nervous system substantially expanded the scope of this set of neurons and required new tools to efficiently label fruM expression (Manoli et al. 2005; Stockinger et al. 2005; Mellert et al. 2010). Additionally, we did not anticipate the breadth of typical GAL4-driver expression across the nervous system, resulting in simultaneously altering Frum levels in diverse sets of neurons. Finally, while the initial neuroanatomical analysis of fruM expression revealed likely lineage-related populations based on the proximity of nuclei, recent analyses of the morphology of fruM neurons suggests that even developmentally related populations appear functionally diverse on the basis of their projection patterns and presumed connectivity (Datta et al. 2008; Kimura et al. 2008; Cachero et al. 2010; Yu et al. 2010; and data not shown). These observations suggest more refined neuroanatomical parameters are likely also necessary to functionally subdivide populations of fruM neurons and establish behavioral correlations.

**Conclusion**

We anticipate that the set of lines we have isolated and the richness of behavioral data based on phenotypic classes that we have generated presents a resource that will advance our understanding not only of the neural substrates of sexual behavior in the fly but perhaps also the fundamental principles that underlie the specification and function of circuitry that subserves complex behaviors in general. Once identified, the characterization and manipulation of these distinct sets of neurons will allow us to begin to understand the representation of information relevant to specific behavioral states within the nervous system and how such information is processed to generate and execute innate behavioral programs.

Finally, it is also worth noting that the formal logic of our approach—carrying out a large-scale screen for genetic variants that have phenotypes perturbing a process of interest, validating those phenotypes by retests, and then sorting them into classes and subclasses on the basis of subsequent secondary assays—is the basic paradigm that has been used across all model organisms to dissect developmental and physiological processes of interest. Our experiments establish that this approach can be extended to the analysis of an innate behavior and provide guides for designing similar studies of other behaviors and their underlying genetic and neural substrates.

**Acknowledgments**

We thank E. Ochoa and W. Woo for their contributions to these studies. We thank U. Heberlein for generously sharing the GAL4 collection, and J. Hall, T. Kitamoto, and B. Dickson for fly strains. We thank G. Bohm for preparation of fly food and J. Reifel for LifesongX software. We also thank T. Claudinin, L. Luo, and members of the Baker lab for helpful discussions, comments on the manuscript, and thoughtful feedback. This work was funded by the National Institutes of Health, Howard Hughes Medical Institute, and Stanford Medical Scientist Training Program.

**Literature Cited**

Acebes, A., M. Cobb, and J.-F. Ferveur, 2003 Species-specific effects of single sensillum ablation on mating position in Drosophila. J. Exp. Biol. 206: 3095–3100.

Acebes, A., Y. Grosjean, C. Everaerts, and J.-F. Ferveur, 2004 Cholinergic control of synchronized seminal emissions in Drosophila. Curr. Biol. 14: 704–710.

Anand, A., A. Villella, L. C. Ryner, T. Carlo, S. F. Goodwin et al., 2001 Molecular genetic dissection of the sex-specific and vital functions of the Drosophila melanogaster sex determination gene fruitless. Genetics 158: 1569–1595.

Baker, B. S., B. J. Taylor, and J. C. Hall, 2001 Are complex behaviors specified by dedicated regulatory genes? Reasoning from Drosophila. Cell 105: 13–24.

Barolo, S., L. A. Carver, and J. W. Posakony, 2000 GFP and beta-galactosidase transformation vectors for promoter/enhancer analysis in Drosophila. Biotechniques 29: 726, 728, 730, 732.

Barolo, S., B. Castro, and J. W. Posakony, 2004 New Drosophila transgenic reporters: insulated P-element vectors expressing fast-maturing RFP. Biotechniques 36: 436–440, 442.

Bray, S., and H. Amrein, 2003 A putative Drosophila pheromone receptor expressed in male-specific taste neurons is required for efficient courtship. Neuron 39: 1019–1029.

Broughton, S. J., T. Kitamoto, and R. J. Greenspan, 2004 Excitatory and inhibitory switches for courtship in the brain of Drosophila melanogaster. Curr. Biol. 14: 538–547.

Cachero, S., A. D. Ostrovsky, J. Y. Yu, B. J. Dickson, and G. S. X. E. Jeffers, 2010 Sexual dimorphism in the fly brain. Curr. Biol. 20: 1589–1601.

Chan, Y.-B., and E. A. Kravitz, 2007 Specific subgroups of fruM neurons control sexually dimorphic patterns of aggression in Drosophila melanogaster. Proc. Natl. Acad. Sci. USA 104: 19577–19582.

Chen, S., A. Y. Lee, N. M. Bowens, R. Huber, and E. A. Kravitz, 2002 Fighting fruit flies: a model system for the study of aggression. Proc. Natl. Acad. Sci. USA 99: 5664–5668.
Manoli, D. S., M. Foss, A. Villella, B. J. Taylor, J. C. Hall et al., 2005 Male-specific fruitless specifies the neural substrates of Drosophila courtship behaviour. Nature 436: 395–400.

Manoli, D. S., G. W. Meissner, and B. S. Baker, 2006Blueprints for behavior: genetic specification of neural circuitry for innate behaviors. Trends Neurosci. 29: 444–451.

Mellert, D. J., J.-M. Knapp, D. S. Manoli, G. W. Meissner, and B. S. Baker, 2010Midline crossing by gustatory receptor neuron axons is regulated by fruitless, doublesex and the Roundabout receptors. Development 137: 323–332.

Miyamoto, T., and H. Amrein, 2008Suppression of male courtship by a Drosophila pheromone receptor. Nat. Neurosci. 11: 874–876.

Moon, S. J., Y. Lee, Y. Jiao, and C. Montell, 2009 A Drosophila gustatory receptor essential for aversive taste and inhibiting male-to-male courtship. Curr. Biol. 19: 1623–1627.

Nilsen, S. P., Y.-B. Chan, R. Huber, and E. A. Kravitz, 2004Gender-selective patterns of aggressive behavior in Drosophila melanogaster. Proc. Natl. Acad. Sci. USA 101: 12342–12347.

O’Dell, K. M., J. D. Armstrong, M. Y. Yang, and K. Kaiser, 1995Functional dissection of the Drosophila mushroom bodies by selective feminization of genetically defined subcompartments. Neuron 15: 55–64.

Pan, Y., C. C. Robinett, and B. S. Baker, 2011Turning males on: activation of male courtship behavior in Drosophila melanogaster. PLoS ONE 6: e21144.

Portman, D. S., 2007Genetic control of sex differences in C. elegans neurobiology and behavior. Adv. Genet. 59: 1–37.

Rideout, E. J., J.-C. Billete, and S. F. Goodwin, 2007The sex-determination genes fruitless and doublesex specify a neural substrate required for courtship song. Curr. Biol. 17: 1473–1478.

Rideout, E. J., A. J. Dornan, M. C. Neville, S. Eadie, and S. F. Goodwin, 2010Control of sexual differentiation and behavior by the doublesex gene in Drosophila melanogaster. Nat. Neurosci. 13: 458–466.

Robinett, C. C., A. G. Vaughan, J.-M. Knapp, and B. S. Baker, 2010Sex and the single cell. II. There is a time and place for sex. PLoS Biol. 8: e1000365.

Robinson, G. E., R. D. Fernald, and D. F. Clayton, 2008Genes and social behavior. Science 322: 896–900.

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

Salvaterra, P. M., and T. Kitamoto, 2001Drosophila cholinergic neurons and processes visualized with Gal4/UAS-GFP. Gene Exp. Patterns 1: 73–82.

Sanders, L. E., and M. N. Arbeitman, 2008Doublesex establishes sexual dimorphism in the Drosophila central nervous system in an isoform-dependent manner by directing cell number. Dev. Biol. 320: 378–390.

Siwicki, K. K., and E. A. Kravitz, 2009Fruitless, doublesex and the genetics of social behavior in Drosophila melanogaster. Curr. Opin. Neurobiol. 19: 200–206.

Spieth, H. T., 1974Courtship behavior in Drosophila. Annu. Rev. Entomol. 19: 385–405.

Stockinger, P., D. Kvitsiani, S. Rotkopf, L. Tirián, and B. J. Dickson, 2005Neural circuitry that governs Drosophila male courtship behavior. Cell 121: 795–807.

Sturtevant, A., 1915Experiments on sex recognition and the problem of sexual selection in Drosophila. J. Anim. Behav. 5: 351–366.

Usui-Aoki, K., H. Ito, K. Ui-Tei, K. Takahashi, T. Lukacsovich et al., 2000Formation of the male-specific muscle in female Drosophila by ectopic fruitless expression. Nat. Cell Biol. 2: 500–506.

Villella, A., D. A. Gailey, B. Berwald, S. Ohshima, P. T. Barnes et al., 1997Extended reproductive roles of the fruitless gene in Drosophila melanogaster revealed by behavioral analysis of new fruitless mutants. Genetics 147: 1107–1130.

Villella, A., and J. C. Hall, 2008Neurogenetics of courtship and mating in Drosophila. Adv. Genet. 62: 67–184.

von Philipsborn, A. C., T. Liu, J. Y. Yu, C. Masser, S. S. Bidaye et al., 2011Neuronal control of Drosophila courtship song. Neuron 69: 509–522.

Vrontou, E., S. P. Nilsen, E. Demir, E. A. Kravitz, and B. J. Dickson, 2006Fruitless regulates aggression and dominance in Drosophila. Nat. Neurosci. 9: 1469–1471.

Wu, M. V., D. S. Manoli, E. J. Fraser, J. K. Coats, J. Tollkuhn et al., 2009Estrogen masculinizes neural pathways and sex-specific behaviors. Cell 139: 61–72.

Yamamoto, D., 2008Brain sex differences and function of the fruitless gene in Drosophila. J. Neurogenet. 22: 309–322.

Yang, C.-H., S. Rumpf, Y. Xiang, M. D. Gordon, W. Song et al., 2009Control of the postmating behavioral switch in Drosophila females by internal sensory neurons. Neuron 61: 519–526.

Yapici, N., Y.-J. Kim, C. Ribeiro, and B. J. Dickson, 2008A receptor that mediates the post-mating switch in Drosophila reproductive behaviour. Nature 451: 33–37.

Yu, J. Y., M. I. Kanai, E. Demir, G. S. X. E. Jefferis, and B. J. Dickson, 2010Cellular organization of the neural circuit that drives Drosophila courtship behavior. Curr. Biol. 20: 1602–1614.

Communicating editor: R. Anholt
Functional Dissection of the Neural Substrates for Sexual Behaviors in Drosophila melanogaster

Geoffrey W. Meissner, Devanand S. Manoli, Jose F. Chavez, Jon-Michael Knapp, Tasha L. Lin, Robin J. Stevens, David J. Mellert, David H. Tran, and Bruce S. Baker
Figure S1  Male-male habituation and changes in mate preference in males that display chaining behavior; complete data. A) Courtship index decreases over time in most GAL4 lines, when initial (x axis) courtship is compared to that after one hour (y axis). A subset of lines, highlighted in Figure 2A, have substantial courtship and no decrease over the course of the assay. B) Mate preference data for complete set of lines tested, with a subset highlighted in Figure 2B.
Figure S2  Average GAL4 overlap with fru\textsuperscript{M} for each courtship phenotype, rescaled relative to cluster size. To compensate for size differences in the fru clusters presented in Figure 5, expression was scaled independently relative to each cluster. See File S7 for an animation of each phenotype in sequence.
Figure S3: Phenotypic and overlap data for each line. Gal4 lines were clustered by patterns of overlap with fru. Grey expression indicates stronger overlap. For phenotypic data, blue indicates an altered phenotype, red indicates wild type, and white indicates no data. Order of fru clusters and abbreviations follow Figure 5.
Figure S4  GAL4 overlap with Fru$^M$ antibody for each courtship phenotype, related to Figure 5 and S3. Analyses were performed as in A) Figure 5 and B) Figure S3, but limited to CNS clusters.
Files S1-S7

Supporting Movies

Files S1-S7 are available for download as .mov files at
http://www.genetics.org/content/suppl/2011/06/24/genetics.111.129940.DC1.

FILE S1. Line 7-73 expressing UAS-fruIR demonstrates high levels of chaining behavior, in which multiple males court each other in an extended chain.

FILE S2. Line 10-164 expressing UAS-fruIR demonstrates high levels of aggressive behavior, with one male chasing others on the food surface, pulling them and lunging at them.

FILE S3. Line 19-6 expressing UAS-fruIR appears to temporarily become stuck in a bent position after attempted copulation.

FILE S4. A male from line 9-178 expressing UAS-fruIR frequently extends both wings simultaneously during courtship of the female.

FILE S5. A male from line 5-118 expressing UAS-fruIR frequently displays scissoring behavior when courting the female. His wings are rapidly extended and retracted from their rest position to a roughly 15 degree angle.

FILE S6. Animated version of Figure 5.

FILE S7. Animated version of Figure S2.
File S8

Supplemental Model Discussion

We propose that the elicitation of sexual behaviors occurs via the perception, integration, and translation of sex-specific (Figure 7A, green and blue) as well as non-sex-specific (Figure 7A, black) cues to activate appropriate behavioral responses. Thus, in a wild-type male, arousal cues common to both sexes, perhaps signifying conspecifics, act via Fru<sup>M</sup>-independent mechanisms to increase the likelihood of either aggressive or courtship behaviors, but are insufficient to elicit behavioral programs themselves (black). In addition female-specific cues, acting via multiple sensory modalities, act and are enhanced via Fru<sup>M</sup>-dependent mechanisms to drive the initiation of courtship, and also maintain courtship drive until successful mating (blue). Finally, distinct mechanisms modulated by Fru<sup>M</sup> regulate the threshold of stimulation necessary to elicit courtship (black circle). Thus, we propose that Fru<sup>M</sup> functions to allow the detection of female-specific cues, to amplify signaling downstream of this perception to drive courtship, and to modulate the activation threshold for initiation of courtship.

We propose that in a wild-type male, in addition to Fru<sup>M</sup>-independent mechanisms that process general arousal cues as described above, the detection of male-specific cues via Fru<sup>M</sup>-dependent mechanisms provides stimulus towards both the initiation of aggression, and the inhibition of courtship initiation (Figure 7A, green). As in the response to female cues, Fru<sup>M</sup>-dependent mechanisms distinct from the detection of and response to male-specific cues act to modulate the threshold of stimulation necessary to elicit aggressive or courtship behaviors (black circle). Thus, for the responses of a wild-type male to males, we propose that Fru<sup>M</sup> functions to inhibit courtship initiation by processing signals downstream of the detection of male cues to inhibit courtship initiation and promote aggressive displays. Thus, when Fru<sup>M</sup> expression is perturbed, cues that normally inhibit male-male courtship become activating and cause chaining behavior with males rather than aggression, while female-specific cues are either equally or less attractive, resulting in increased courtship latency, and either non-specific or reversed sexual orientation.

As discussed for the response to males and females, Fru<sup>M</sup>-dependent mechanisms appear to modulate the activation thresholds for specific behavioral programs, enabling the alteration of stimulus thresholds for sexual behaviors without affecting the contexts within which these behaviors occur (Figure 7A, black circle). For example, we observe that lines that show a rapid onset of courtship towards females are more likely to show increased aggression towards males. We propose that males with such phenotypes have decreased Fru<sup>M</sup>-dependent inhibition of the initiation of sexual behaviors, but are likely to have no
disruption in the pathways that mediate the processing of cues dictating whether courtship or aggressive programs are appropriate. The frequency with which we observe rapid courtship initiation and aggression, either together or separately, suggests that the mechanisms regulating the threshold(s) for behavior initiation are less robust to perturbation than other Fru^M-dependent processing, perhaps indicating recent evolutionary changes from other species.

In contrast to an alteration of stimulus thresholds for the display of specific behaviors, we propose that multiple mechanisms involved in detection or processing of sex-specific cues may induce chaining behavior in grouped males. Although disruptions in both processes may result in chaining behavior, each mechanism can be distinguished by its distinct courtship latency phenotype (Figure 7B). For example, in males that display chaining with no changes in courtship latency, we propose a partial loss of the Fru^M-dependent processing of inhibitory signals from the perception of males to the stimulation of courtship, thus yielding increased male-male courtship in grouped males, with no changes affecting female courtship. In males that display chaining behavior along with increases in courtship latency, we propose a partial loss in the perception of female-specific cues in exchange for sensitivity to more general arousal cues, thus allowing for stimulation by males of courtship behaviors, but a decreased response to females.

Based on these correlations between phenotypic classes and the frequencies at which individual classes arise, we propose a model for Fru^M-dependent regulation of the initiation of sexual behaviors in specific contexts. We suggest that Fru^M function affects the perception or processing of sensory cues that stimulate the drive towards distinct programs for sexual behaviors. The processing affected by Fru^M function thus includes 1) the detection of sex-specific and general cues, 2) the strength of signaling downstream of such detection, 3) the stimulatory or inhibitory function of such signaling, and 4) the thresholds for the activation of distinct behavioral programs. From data in this and other studies, we conclude that the mechanisms that determine the contexts in which distinct behavioral programs are initiated in response to sensory cues, and the strength of these cues to elicit behavior, are largely separable from the mechanisms that set the thresholds for activation of these behavioral programs.

A number of general principles found within this model make it both concise and consistent with current knowledge of Fru^M function. First, we propose that Fru^M functions as a binary switch at specific points in the circuit mediating initiation, a feature consistent with the exclusively binary mechanisms underlying most sex-determination mechanisms as well as fruitless regulation itself. Second, in accordance with the existence of isolated examples of Fru^M-dependent changes in gross neuronal
morphology or specification of male-specific neural structures, we propose that Fru\textsuperscript{M} primarily functions to alter either fine synaptic structure or neuronal physiology (Kimura et al. 2005; Manoli et al. 2005; Stockinger et al. 2005; Datta et al. 2008; Kimura et al. 2008; Cachero et al. 2010; Yu et al. 2010). For example, changes in the strength of signaling downstream of sex-specific cues may be mediated by increased synaptic density at the termini of Fru\textsuperscript{M} neurons relaying such information, consistent with Fru\textsuperscript{M} dependent changes in the volume of antennal lobe glomeruli innervated by Fru\textsuperscript{M}-expressing neurons (Kondoh et al. 2003; Stockinger et al. 2005). As another example, Fru\textsuperscript{M} dependent changes in neural physiology may occur via changes in neurotransmitter expression, consistent with previously described Fru\textsuperscript{M}-dependent sexual dimorphisms in neural function (Lee and Hall, 2001). Via a different mechanism, but also consistent with Fru\textsuperscript{M}-dependent function as a binary switch, subsets of Fru\textsuperscript{M} neurons survive in a male-specific manner, thus potentially providing male-specific detection or processing of information (Kimura et al. 2005). Third, based on the phenotypic categories defined here, we have been able to show that only one change is required for all observed categories of behavior. Thus, we find, for example, that relatively few changes in a Fru\textsuperscript{M}-dependent pathway inhibiting stimulation of courtship by males can result in higher levels of male-male courtship while still preserving a female bias in mate preference.
Table S1  Multivariate statistical tests relating overlap to fertility data. Survival analysis was used for the first analysis, as vials with fecundity over 50 were not counted. As a complementary approach, a nominal logistic regression between expression and the presence or absence of a fertility defect was performed. A) Both analyses applied to fru<sup>M</sup> GAL4 overlap found a significant relationship for clusters T4, R06, and LegChord, with expression in the former two being associated with reduced fecundity and in the latter with increased fertility. B) Parallel analysis of GAL4 overlap with FruM antibody found a relationship for R08 only in the survival analysis.

### A

#### Parametric Survival

| Source    | Chi Square | P > ChiSq | Estimate | Std Error |
|-----------|------------|-----------|----------|-----------|
| T4        | 17.15      | <.0001*   | -0.614   | 0.135     |
| LegChord  | 8.54       | 0.0035*   | 0.497    | 0.180     |
| T5        | 8.30       | 0.0040*   | -0.610   | 0.205     |
| R02       | 7.88       | 0.0050*   | 0.707    | 0.263     |
| R06       | 6.32       | 0.0119*   | -0.126   | 0.050     |
| R07       | 3.80       | 0.0512    | -0.153   | 0.071     |
| R11       | 3.74       | 0.0532    | 0.178    | 0.093     |
| R04       | 3.47       | 0.0625    | 0.172    | 0.094     |
| R01       | 2.79       | 0.0946    | -0.636   | 0.374     |
| R18D      | 2.70       | 0.1005    | 0.751    | 0.454     |

#### Weibull Distribution

| Source | N | Degrees of Freedom | Chi Square | Prob > Chi Square |
|--------|---|--------------------|------------|------------------|
|        | 144 | 36               | 84.5       | <0.0001*         |

#### Logistic Regression

| Source    | Chi Square | P > ChiSq | Estimate | Std Error |
|-----------|------------|-----------|----------|-----------|
| LegChord  | 11.72      | 0.0006*   | 1.024    | 0.339     |
| R06       | 9.47       | 0.0021*   | -0.361   | 0.136     |
| T4        | 8.28       | 0.0040*   | -0.961   | 0.382     |
| R18D      | 3.21       | 0.0731    | 1.302    | 0.717     |
| R01       | 2.83       | 0.0925    | -1.008   | 0.620     |
| R17V      | 2.78       | 0.0954    | 0.258    | 0.165     |
| KC        | 1.54       | 0.2144    | -0.402   | 0.328     |
| T5        | 1.52       | 0.2176    | -0.647   | 0.559     |
| Tibia     | 1.49       | 0.2220    | 0.446    | 0.331     |
| R12       | 1.38       | 0.2405    | -0.854   | 0.741     |

| Source | N | Degrees of Freedom | Chi Square | Prob > Chi Square |
|--------|---|--------------------|------------|------------------|
|        | 144 | 36               | 67.0       | 0.0013*         |

### B

#### Parametric Survival

| Source    | Chi Square | P > ChiSq | Estimate | Std Error |
|-----------|------------|-----------|----------|-----------|
| R08       | 5.35       | 0.0207*   | -1.489   | 0.624     |
| R18V      | 3.80       | 0.0511    | -0.395   | 0.201     |
| R19D      | 3.49       | 0.0617    | -0.827   | 0.409     |
| R04       | 2.28       | 0.1308    | 0.549    | 0.393     |
| R03       | 2.16       | 0.1417    | -0.356   | 0.248     |
| R17V      | 2.15       | 0.1424    | 0.225    | 0.170     |
| R09       | 1.91       | 0.1671    | 6.517    | 1362.305  |
| R19V      | 1.53       | 0.2155    | -0.518   | 0.367     |
| R02       | 1.36       | 0.2428    | 0.516    | 0.469     |
| R13       | 1.21       | 0.2711    | -0.149   | 0.132     |

#### Weibull Distribution

| Source | N | Degrees of Freedom | Chi Square | Prob > Chi Square |
|--------|---|--------------------|------------|------------------|
|        | 182 | 22               | 30.4       | 0.1080         |

#### Logistic Regression

| Source    | Chi Square | P > ChiSq | Estimate | Std Error |
|-----------|------------|-----------|----------|-----------|
| R02       | 2.65       | 0.1033    | 0.477    | 0.320     |
| R03       | 2.46       | 0.1168    | -0.272   | 0.178     |
| R09       | 2.37       | 0.1239    | 1.903    | 10.703    |
| R19D      | 2.13       | 0.1449    | -0.447   | 0.314     |
| R08       | 1.54       | 0.2142    | -0.561   | 0.454     |
| R17V      | 1.44       | 0.2297    | 0.104    | 0.090     |
| R13       | 1.44       | 0.2304    | -0.127   | 0.106     |
| R18V      | 1.16       | 0.2824    | -0.151   | 0.138     |
| R19V      | 0.97       | 0.3247    | -0.311   | 0.295     |
| R12       | 0.84       | 0.3582    | -0.353   | 0.386     |

| Source | N | Degrees of Freedom | Chi Square | Prob > Chi Square |
|--------|---|--------------------|------------|------------------|
|        | 182 | 22               | 30.0       | 0.1182          |