DOI: 10.1534/genetics.108.089177

A Male-Specific Fatty Acid ω-Hydroxylase, SXE1, Is Necessary for Efficient Male Mating in *Drosophila melanogaster*

Shinsuke Fujii, Akemi Toyama and Hubert Amrein[1]

Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, North Carolina 27710

Manuscript received March 20, 2008
Accepted for publication June 17, 2008

ABSTRACT

In Drosophila, sexual differentiation, physiology, and behavior are thought to be mediated by numerous male- and female-specific effector genes whose expression is controlled by sex-specifically expressed transcriptional regulators. One such downstream effector gene, *sex-specific enzyme 1* (*sxe1*, *cyp4d21*), has been identified in a screen for genes with sex-biased expression in the head. *Sxe1* was also identified in another screen as a circadian regulated gene. Here, we analyzed the spatial and temporal regulation of *sxe1* and identified a function for this gene in male courtship. We show that male-specific transcriptional regulator DSX^M and the clock genes are necessary for cycling of *sxe1* mRNA during the diurnal cycle. Similar to *sxe1* mRNA, expression of SXE1 protein oscillates in a diurnal fashion, with highest protein levels occurring around midnight. SXE1 protein expression is restricted to nonneuronal cells associated with diverse sensory bristles of both the chemo- and mechanosensory systems. Suppression or knockout of *sxe1* significantly reduces mating success throughout the diurnal cycle. Finally, the metabolomic profile of wild-type and *sxe1* mutant males revealed that *sxe1* likely functions as a fatty acid ω-hydroxylase, suggesting that male courtship and mating success is mediated by small compounds generated by this enzyme.

DEVELOPMENT and differentiation of sex-specific structures and organs, and the establishment of sex-specific physiological/neuroanatomical traits, are essential processes common to virtually all animals. In *Drosophila melanogaster*, somatic differentiation of male and female flies is regulated by sex-specific splicing regulators including SEX-LETHAL (SXL) and TRANSFORMER (TRA) that lead to the expression of male- and female-specific spliced isoforms of the transcription factors, *FRUITLESS* (FRU^M) and *DOUBLESEX* (DSX^M and DSX^F). DSX^F, DSX^M, and FRU^M are thought to control the expression of numerous downstream effector genes both in the nervous and other organ systems, and ultimately establish the sex-specific properties and features of the adult fly (Burtis and Baker 1989; Coschigano and Wensink 1993; Ito *et al.* 1996; Ryner *et al.* 1996; Demir and Dickson 2005). These effector genes are thought to fall into two broad categories: (1) genes expressed during critical stages of development (pupae stage) and required for the differentiation of sex-specific phenotypical traits (male and female genitalia, male sex-comb, coloration of abdominal cuticle) and wiring of sex-specific neural circuits (male courtship, female egg laying, and mating/rejection behavior) and (2) genes required after development to assure sex-specific physiological properties and maintenance of sex-specific behaviors. While microarray and other large scale expression screens have led to the identification of hundreds of putative effector genes that are differentially expressed in males and females (Arbeitman *et al.* 2002, 2004; Dauwalder *et al.* 2002; Fujii and Amrein 2002; Parisi *et al.* 2004), the specific functions of virtually all of them remain unknown.

Courtship is an innate behavior that is characterized by manifestation of several behavioral displays executed in a characteristic sequential fashion by a male when encountering a female (Hall 1994). The appropriate execution of the male courtship sequence is genetically controlled mainly by FRU^M, and to a lesser degree DSX^M (Demir and Dickson 2005; Shirangi *et al.* 2006), and by inference, the effector genes regulated by these transcription factors.

We have previously identified 57 sex-biased and sex-specific candidate genes in adult heads using serial analysis of gene expression (SAGE) (Fujii and Amrein 2002). Nine of these genes were virtually sex specific, including all 5 genes known at the time to be expressed in the head in a sex-specific manner: the 3 female-specific *yolk protein* (*yp1*, *2*, and *3*) genes and the male-specific RNAs *roX1* and *roX2* (RNA on the *X 1* and *2*). The four novel genes included 3 male-biased genes *sex-specific enzyme 1* and *2* (*sxe1*, *sxe2*) and *turn on sex-specificity* (*tsx*) and a female-specific gene *female independent of transformer* (*fit*). The common feature of these 4 novel sex-biased genes is that their expression is nonneuronal, yet is largely restricted to the head, and expression (sex-biased expression in the case of *tsx*) is restricted to the

[1]*Corresponding author:* 252 CARL Bldg., Research Dr., Box 3509, Durham, NC 27710. E-mail: hoa1@duke.edu

adult stage (Fujii and Amrein 2002). This suggests that the main function of these genes is necessary in the adult physiology and/or behavior of male or female flies.

Sxe1 encodes one of >80 cytochrome P450 enzymes, which are crucial components in many biochemical pathways (Simpson 1997). We have previously shown that *sxe1* is a downstream target of *dsx* (Fujii and Amrein 2002). Specifically, *XX; dsx^{D}/Df(dsx)* flies, which only express DSX^{M} protein, show robust expression of *sxe1*, as do *XX; tra/tra* flies, which express both DSX^{M} and FRU^{M} (Fujii and Amrein 2002). Expression of *sxe1* mRNA was detected in the head and at reduced levels in the thorax, but not the abdomen. Within the head, *sxe1* mRNA is mostly found in carcasses (cuticle) and possibly fat cells, but is absent in the brain. *Sxe1* has also been identified in a screen for genes expressed in the head of adult flies that oscillate in a circadian fashion using microarray analysis (Claridge-Chang *et al.* 2001; McDonald and Rosbash 2001; Lin *et al.* 2002), suggesting that this gene may also be a downstream effector of the clock genes. Indeed, oscillation of *sxe1* is dependent on Clock (Clk), period (per), and timeless (tim), the main regulators required for oscillation of downstream effectors (Hardin 2005). Interestingly, *takeout* (*to*), which encodes a pheromone binding protein and is male-biased expressed in the fat body (Dauwalder *et al.* 2002), also oscillates with a 24-hr period (Sarov-Blat *et al.* 2000).

Circadian locomotor behavior was recently found to exhibit sex-specific features (Fujii *et al.* 2007), albeit both males and females are largely active during the day and rest during the night. Specifically, the activity of females, after an initial steep rise after dawn, continues to increase throughout the day, ending in a single peak before dusk, while males show two distinct activity peaks, one after dawn and a second one at dusk (coinciding with the single female peak). Surprisingly, circadian activity is reset when a male and a female cohabitate the same arena, with a drastic increase in nocturnal activity (Fujii *et al.* 2007). This activity shift is driven by male courtship and is dependent on the male's circadian system. Together, these observations suggest that the circadian clock of the two sexes may be regulated by distinct sex-specific factors.

Here, we determined *SXE1* expression using *sxe1-Gal4* drivers as well as anti-SXE1 antiserum and found that *SXE1* is expressed in nonneuronal tissues of the chemo- and mechanosensory systems. Molecular genetic analyses revealed that circadian regulation for *sxe1* is dependent on the male-specific transcription factor *DSX^{M}*. We employed RNA interference and gene targeting to show that *sxe1* is required for high mating success in males. Finally, we investigated the molecular function of SXE1 as a fatty acid ω-hydroxylase by analyzing the metabolomic profile of wild-type and *sxe1* mutant males and we show that this protein is essential for the production of many small molecules in the male, which may function as male- and species-specific neuromodulators.

MATERIALS AND METHODS

Fly strains: The following fly strains were used: Ore-R, *w^{1118}*, *per^{01}*, *yw; tim^{01}*, *Clk^{JRK}*, *cyc^{01} ry*, *B^{s}Y; dsx^{1}/TM3*, *BsY; hs->tra/ Df[3L] st tra p^{p} dsx^{D} Sb e/TM6*, *B^{s}Y; dsx^{Df}/TM3*, *p[ELAV]GAL4^{C155}*, *UAS-y_ds* (R. Costa), *UAS-mCD8-GFP*, *w^{1118}; 70FLP*, *70I-SceI*, *Sco/CyO*, and *w^{1118}; 70FLP* (K. Golic). All stocks were maintained under a 12-hr light:12-hr dark cycle at 25°.

Over 80% of homozygous *dsx^{1}* flies die <4 days after eclosion with standard food. To elongate their longevity and entrain them for LD cycles, we collected flies within 8 hr after eclosion and kept them in vials with food lacking yeast extract for 4 days. *Oregon-R*, *Clk^{jrk}*, *cyc^{01}*, *per^{01}*, and *tim^{01}* males were entrained for 7–10 days, whereas all flies carrying the *dsx^{1}* allele were entrained for 4 days.

Immunochemistry: Whole mount *LacZ* and antibody staining were carried out as described (Bray and Amrein 2003), with some modifications. A rabbit polyclonal antibody was generated against synthetic peptide STGNNVGLKPRTRVK, corresponding to amino acid 497 to 511 of SXE1 and affinity purified (AnaSpec, San Diego). The antiserum was used at 1:800 dilution for Western blotting and whole mount antibody staining. For Western blotting, ECL anti-rabbit IgG, HRP-linked F(ab′)$_2$ fragment and ECL Plus detection kit (Amersham Biosciences) were used. Western blots were repeated between five and nine times using freshly prepared protein extract for each genotype/time point.

Monoclonal anti-GFP (Molecular Probes), anti-α-tubulin (Sigma-Aldrich), anti-ELAV antibody and TOTO3 (Molecular Probes) were used at 1:1000, 1:50,000, 1:10, and 1:1000, respectively. Immunofluorescence imaging was performed using a Leica TCS5 confocal microscope as described previously (Thorne *et al.* 2004).

Northern blot analysis: Total RNA from heads was isolated for all Northern blot analysis using a TRIsol (Life Technologies). The coding region of *sxe1* cDNA was labeled with [^{32}P]-dCTP using Random Primer labeling kit (Stratagene). Northern blotting and hybridization was performed as previously described (Fujii and Amrein 2002). A Typhoon Imager (Amersham Biosciences) was used for measurement of signal strength. Northern blots in Figure 2A were repeated four to five times, each time with a new RNA sample for each genotype/time point, except for Ore-R females (performed once). Northern blots in Figure 2C were repeated twice, each time with a new RNA sample for each genotype/time point.

Transgenic fly construction: For the *p[sxe1]GAL4* driver, a 3.0-kb fragment upstream of the start codon was amplified from genomic DNA, sequenced, and cloned into the GAL4 transformation vector SM1 (Dunipace *et al.* 2001). Transgenic flies were generated as described previously (Dunipace *et al.* 2001).

For the *UAS-sxe1_ds* reporter, the entire coding region of *sxe1* was amplified from cDNA, cloned into pZero1 vector (Invitrogen), and sequenced. Two copies of this fragment were ligated sense and antisense (head-to-head) orientation, separated by a spacer (partial EGFP cDNA), and subsequently cloned into the pUAST vector (Brand and Perrimon 1993).

For the *sxe1* targeting construct, a 4.873-kb fragment (corresponding to nucleotides 7,600,804–7,605,677 on chromosome arm 2L; release 5.3 in FlyBase) was amplified to generate the 5′ arm (see Figure 4) using primers CCGGCGC GCCCACTTCGAGTGCTGATCGCAATTGG and GGCCTAG GTATTCTTACGGATAAATCGGCTTTACG, and a 3.619-kb fragment (nucleotides 7,605,736–7,609,355) was amplified to generate the 3′ arm using primers CCCGGTCCGAGTCG CAAACAATGGAGAGTTAACAC and CCGCTAGCGCCTTAA AAAAGATCTCAGAATGGTTC. Fragments were subcloned into CMC105 vector (C.-M. Chen and G. Struhl, personal

communication). The resulting targeting construct created a *sxe1* mutation in which the 58-bp DNA region starting at the −5 position of the translational start was replaced by the white minigene, creating a null allele for *sxe1*. Virgin female flies carrying a single targeting construct on the third chromosome (donor) were crossed to w^{1118}*;70FLP, 70I-SceI/TM3*, and 3- to 4-day-old progeny were heat-shocked at 38° for 60 min. Approximately 3000 white-eyed adult virgin females were crossed to w^{1118}*;70FLP* males and progeny were heat-shocked as described above. Four red-eyed flies were obtained in which the targeting construct relocated to the second chromosome, and a homozygous line for each of them was established. Genomic DNA of these lines was digested with *Nco*I, analyzed by Southern blotting using a DNA probe corresponding to the 5′ region of *sxe1* (see Figure 4). One line, $sxe1^{67}$, representing a precise recombination event, was backcrossed five times to *Oregon-R* and used for further investigation. A genomic rescue fragment was amplified with the most 5′ and the most 3′ primers used for the targeting construct, sequenced, cloned into pCsper4 transformation vector, and used to generate three independent transgenic rescue lines. Initial rescue experiments revealed varying degrees of rescue in mating assays; line 3, which conferred the strongest rescue, was backcrossed five times to *OreR* and used for further investigations as a rescue line.

Behavioral analysis: All males used in behavioral assays were of w^{+} background to eliminate any effects due to impaired visual perception caused by different levels of eye pigmentation provided by the various *w* minigene-containing transgenes. w^{1118} virgin females were used as mating targets in all assays. CI and frequency of nonmaters were measured as described (Bray and Amrein 2003). Males were kept in isolation for 14 days, whereas ~20 virgin females were kept in a vial together for 5–7 days.

Mating frequency assays were performed as described (Sakai and Ishida 2001), with the following modifications. Virgin flies were collected and sexed within 6 hr after eclosion, and animals of the same sex were kept for 1–2 weeks (~20/vial). One day before the experiment, animals of the same sex were transferred into new vials in batches of 5/vial; the following day, they were placed in a light-protected box for 30 min before ZT 4, 8, 16, and 20. After letting all the flies adjust to darkness, 5 males and 5 females were combined into one vial and kept for 15 min under darkness (dim red light, <1 lux); they were then knocked out using CO_2 to score actively mating (physically connected) pairs. Females not actively copulating were transferred into new vials individually, which were examined for larvae after 10 days to determine the female's mating status.

Locomotor activity: Virgin males were kept individually for 14 days. Flies were transferred with a "fly aspirator" into a small Plexiglas mating chamber (4 × 10 × 30 mm), left to adjust to the new environment for 2 min, and then monitored for crossing the midline of the chamber for 4 min at ZT 2–4.

Taste preference assay was performed as described previously (Thorne *et al.* 2004). Preference of males for 25 mm trehalose *vs.* water was measured. Three independent experiments were performed with ~40 flies per genotype per experiment.

Longevity of flies was measured as follows: Virgin males were collected within 6 hr after eclosion and kept at 10 flies per vial at 25°. Flies were transferred into new vials every 2 days, and dead flies were scored until all flies were dead. Three independent experiments were performed with 100 flies per genotype per experiment.

Fertility test: A single male and single w^{1118} virgin female were kept for 4 days in a vial at 25°. Parents were discarded and the vial was kept until adults emerged. The number of adult flies was scored.

Quantification and analysis of fatty acids: Roughly 1000 heads of $sxe1^{67}/sxe1^{67}$*; Rescue3/+* and $sxe1^{67}/sxe1^{67}$ males of 5–10 days of age were collected, weighed, and provided to Lipomics Technologies for lipid analysis using the TrueMass protocol. All lipid species were quantified as nanomoles per gram of tissue. A molecular description of fatty acid species is provided at http://www.lipomics.com/fatty_acids.

Statistical analysis: All analyses were performed using the JMP6 software suite (SAS).

RESULTS

***Sxe1* is expressed in nonneuronal cells associated with chemo- and mechanosensory sensilla:** We previously analyzed the expression of *sxe1* mRNA using Northern analysis, which revealed that the gene is abundantly expressed in the head and the thorax, but not in the abdomen. Dissection of various head parts further indicated that *sxe1* is absent from the brain, but is mostly expressed in tissues associated with the head carcass (Fujii and Amrein 2002). To determine the cellular expression profile of *sxe1*, we generated flies containing a *sxe1-Gal4* driver and a *UAS-lacZ* reporter (*p[sxe1]GAL4/UAS-LacZ*) and performed β-Gal staining. *lacZ* activity was observed in numerous sites, most notably the second antennal segment, at the base of the arista, in the labial palps, at the tip of maxillary palps, the vibrissae (ventral outside edge of compound eyes), and at the base of macrochaetae in the head and thorax (Figure 1, A–C). *sxe1* is also expressed in the base of bristles of the male forelegs (data not shown). In females, expression is absent in most of these sites, with the exception of the labial and maxillary palps; there, expression is much weaker than in the corresponding male structures (Figure 1D). These findings are consistent with our previous Northern analyses, which revealed male-specific *sxe1* expression in the head and thorax (Fujii and Amrein 2002).

To determine protein expression of SXE1, we generated antisera against this protein (see materials and methods) and performed Western analysis, which revealed a distinct band in wild-type but not *sxe1* mutant flies of the expected molecular weight of 58 kDa (supplemental Figure 1). We then performed whole-mount antibody staining of *p[elav]GAL4/UAS-mCD8-GFP* flies using anti-*SXE1*, anti-*ELAV*, and anti-*GFP* antibodies, which allowed us to determine whether SXE1 was present in any neuronal cell compartment. SXE1 immunoreactivity was observed in the same tissues where *lacZ* activity was localized in *sxe1-GAL4;UAS-lacZ* flies (compare Figure 1, A–C, with Figure 1, E–I). Confocal microscopy revealed that SXE1 is expressed in the cytoplasm of nonneuronal cells associated largely with sensory bristles (Figure 1, G, H, and J). SXE1 immunoreactivity was also observed in the female labial palps, but expression is weaker and restricted to a smaller area when compared to males (compare Figure 1, I, K, and L). Taken together, these studies indicate

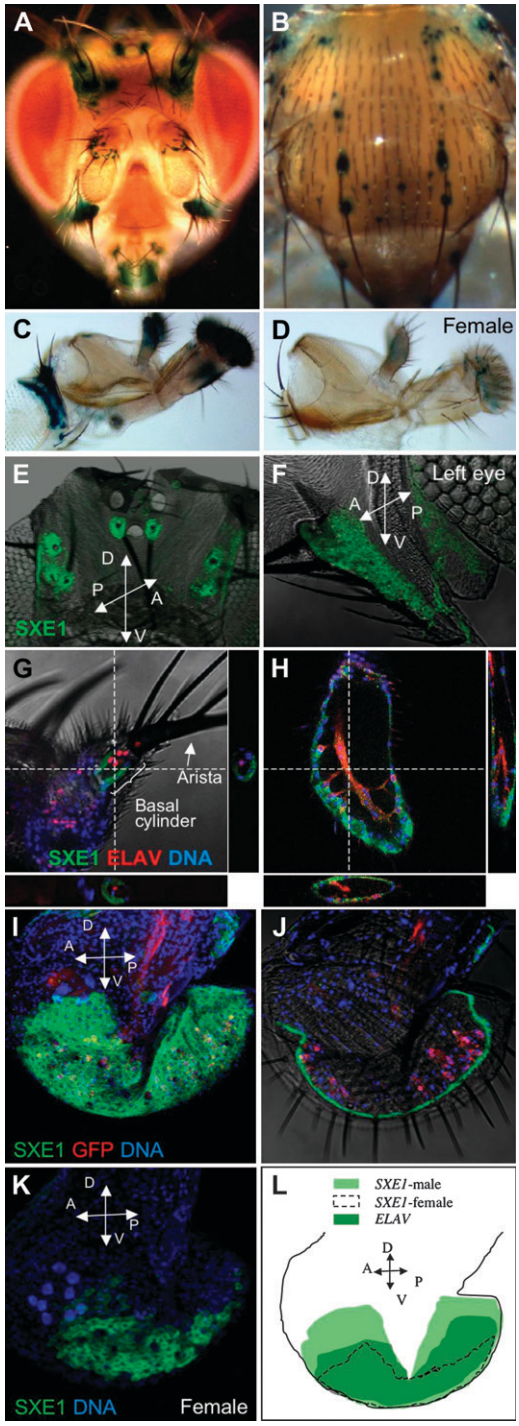


Figure 1.—*Sxe1* expression in the head and thorax of adult males and females (A–D) Whole-mount LacZ staining of *p[sxe1]GAL4/UAS-LacZ* in a male (A–C) and a female (D). In the head and thorax, *sxe1* is mainly expressed in cells associated with long mechanosensory bristles of males (macrochaetae, A and B), but absent in females (not shown). *Sxe1* expression is also observed in cells associated with taste (labial palps) and olfactory sensilla (maxillary palps) of males (C); in females weak expression in the later is also observed. (E–K) Whole-mount antibody staining of *p[elav]GAL4/UAS-mCD8GFP* in a male (E–J) and a female (K) using anti-SXE1, anti-ELAV (only G), or anti-GFP primary (H, I, and J) antibodies. SXE1 antigen (visualized in green) is present in cells surrounding the base of the long bristles on the head and the vibrissae (E and F). SXE1 is clearly nonneuronal as no colocalization is observed with ELAV (red, labels nuclei of neurons in G) or GFP (red, labels entire neural network in H, I, and J) in the third antennal segment/arista (G), the maxillary (H), and the labial palp (I), but is confined to a cell sheet just underneath the cuticle (H and J). Only very weak staining is observed in the labial palps of the female (K). (L) Schematic map of sexually dimorphic SXE1 expression in labial palps.

that SXE1 is localized in cells mostly associated with sensory bristles and, while not entirely male specific, it is expressed with a strong male bias.

Clock and sex determination genes coregulate *sxe1* expression: Claridge-Chang *et al.* (2001) showed significant changes in *sxe1* expression in the heads in *Clk*, *per*, and *tim* mutant flies using microarray analyses and suggested that the clock genes regulate *sxe1* expression (McDonald and Rosbash 2001; Lin *et al.* 2002). To obtain a definite view of how expression of *sxe1* is regulated, we performed a series of Northern and Western analyses with RNA and protein extracts from adult heads isolated at various time points during the day from flies mutant for individual clock and sex determination genes (Figure 2). First, we confirmed that the amount of *sxe1* mRNA in wild-type males oscillates (one-way ANOVA, $P > 0.0001$), with a peak at Zeitgeber time (ZT) 19. The strongest Zeitgeber for most animals is light, and zeitgeber time refers to a specific time point during the 12-hr light:12-hr dark cycle (ZT 0/24, light on; ZT 6, midday; ZT 12, light off ; ZT 18, midnight). Mutations in all four major clock regulators abolish *sxe1* oscillation in males, but the expression levels are still higher than those of wild-type females. To investigate whether the cycling of *sxe1* was also observed at the protein level, we performed Western analyses of protein extracts from heads at different circadian times. Indeed, the abundance of SXE1 protein followed an oscillating pattern throughout the day (one-way ANOVA, $P > 0.0001$; Figure 2B) with peak time at ZT 23, corresponding to a 4-hr time shift of the mRNA peak.

We have previously shown that male-biased *sxe1* expression is regulated by *dsx* (Fujii and Amrein 2002). We therefore determined the effects of the two DSX proteins on cycling of *sxe1* mRNA at ZT 3 and ZT 19, the times of trough and peak, respectively, in XX and XY flies with various *dsx* alleles. Homo- and hemizygous dsx^1 mutant flies (dsx^1/dsx^1 and dsx^1/dsx^D), which show an intersexual (male and female characteristics) phenotype (Erdman and Burtis 1993), generate no functional *DSX* protein, regardless of their sex chromosomes; in contrast, the dsx^D allele leads to TRA-independent splicing of dsx^m mRNA (and production of DSX^M protein), and therefore, *X/X;* dsx^1/dsx^D and *X/Y;* dsx^1/dsx^D flies are phenotypic male and express only DSX^M, but not DSX^F (Nagoshi and Baker 1990). As

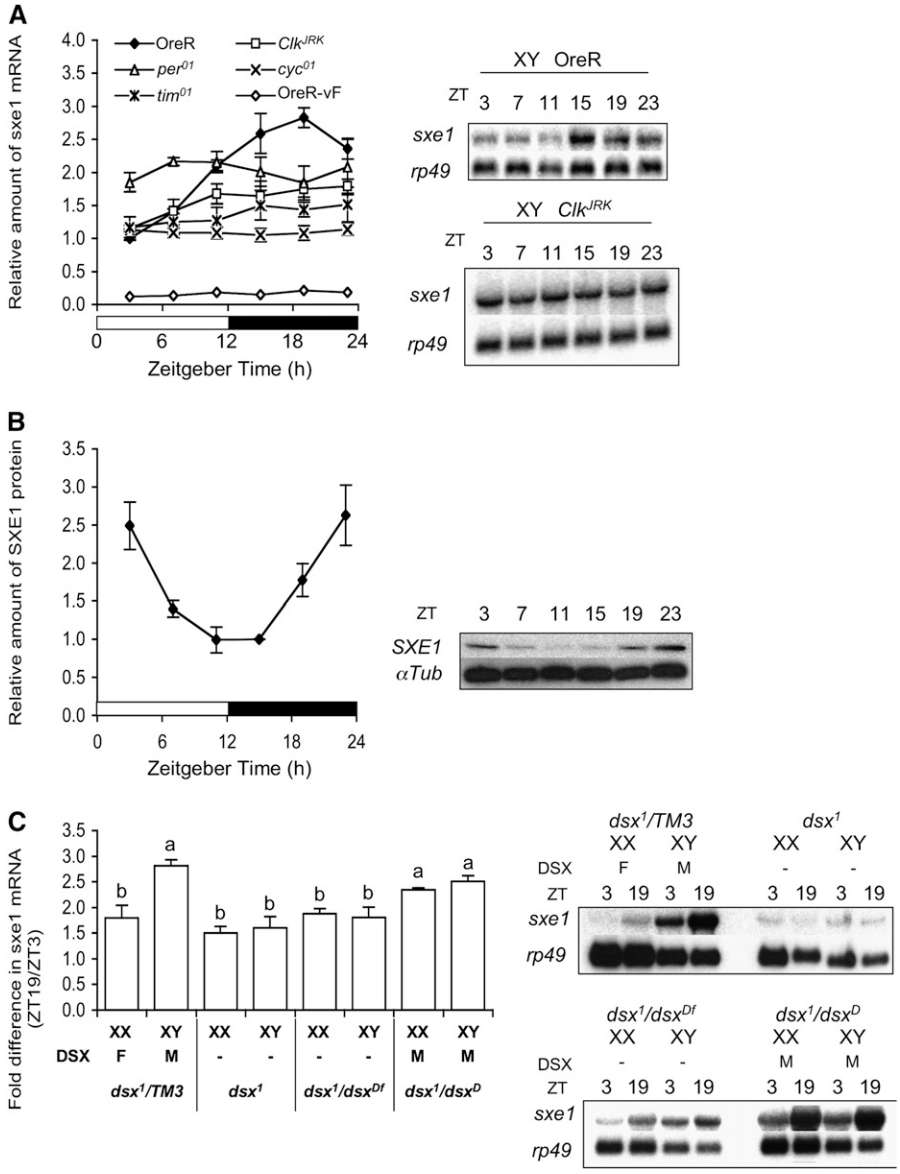


FIGURE 2.—Daily oscillation of *sxe1* depends on DSX^M. (A) Relative amount of *sxe1* expression in wild-type males and virgin females (OreR-vF) and various clock mutant males is plotted at various Zeitgeber times (ZT; left). Signal intensity of Northern blot for *sxe1* was normalized by that of *rp49*, and then all data were converted to relative amount to *OreR* males signal at ZT 3 (value 1). (n = 4–5 for each genotype and time point. n = 1 for OreR-vF). Only *OreR* showed a significant difference ($P < 0.0001$) in time by one-way ANOVA. Error bar represents SEM. Two representative Northern blots of *OreR* and *Clk^{JRK}* mutant males are shown on the right. An *rp49* probe was used as a control for RNA loading. (B) SXE1 protein expression in the head of *OreR* males (n = 9–10 for each time point) is plotted at various ZTs. Signal intensity of Western blot for *SXE1* at ZT 15 was converted to 1. Difference in time is significant ($P < 0.0001$) by one-way ANOVA. A Western blot is shown on the right. An α-Tub was visualized with a specific antibody monitor sample loading. (C) The relative amount of *sxe1* mRNA at ZT 19 as fold increase of levels at ZT 3 is shown for various genotypes. The genotypes expressing DSX^M show a significant higher elevation of *sxe1* mRNA at ZT 19 (a, $P < 0.05$), as compared to genotypes lacking DSX^M (b), as shown by one-way ANOVA followed by *post hoc* each pair Student's *t*-test. The Northern blots are shown on the right. An *rp49* probe was used as a control for RNA loading.

controls, we included XX and XY flies that contained one intact *dsx* allele (*TM3/dsx^1*), which produce DSX^F and DSX^M and develop as normal females and males, respectively. RNA from all flies showed an increase of *sxe1* mRNA from ZT 3 to ZT 19 in all genotypes, even in the absence of any DSX protein; however, only DSX^M, but not DSX^F leads to a significant increase in *sxe1* mRNA at ZT 19 (Figure 2C; $P < 0.05$). Taken together, these experiments establish that the clock genes are necessary for cycling of *sxe1* mRNA, and that DSX^M is required for amplifying the increase of *sxe1* expression at ZT 19.

Suppression of *sxe1* decreases male courtship and mating success: Male-specific expression of *sxe1* in the adult suggests a role for this gene in male reproductive physiology and/or behavior. We therefore employed RNA interference (RNAi) to suppress SXE1 activity. We generated flies expressing double-stranded *sxe1* mRNA under the control of the *sxe1* promoter (*sxe1*-RNAi flies: *p[sxe1]GAL4/UAS-sxe1_ds*; for details see MATERIALS AND METHODS) and performed both Northern and Western analyses. These experiments showed that both mRNA and protein expression levels were drastically reduced in *sxe1*-RNAi males, when compared to control males containing only the driver or the reporter (Figure 3A and data not shown). These flies are viable, healthy, and morphologically indistinguishable from control flies. Moreover, suppression of *sxe1* in male flies did not affect locomotor activity (Table 1). To investigate whether *sxe1* is involved in sexual behavior, we performed behavioral assays and determined the courtship index (CI) and mating success (see MATERIALS AND METHODS). Indeed, both the CI ($P < 0.001$) and mating success ($P < 0.05$) was significantly reduced in *sxe1*-RNAi males compared to control males (Figure 3, B and C). Because SXE1 expression oscillates during the diurnal cycle (Figure 2,

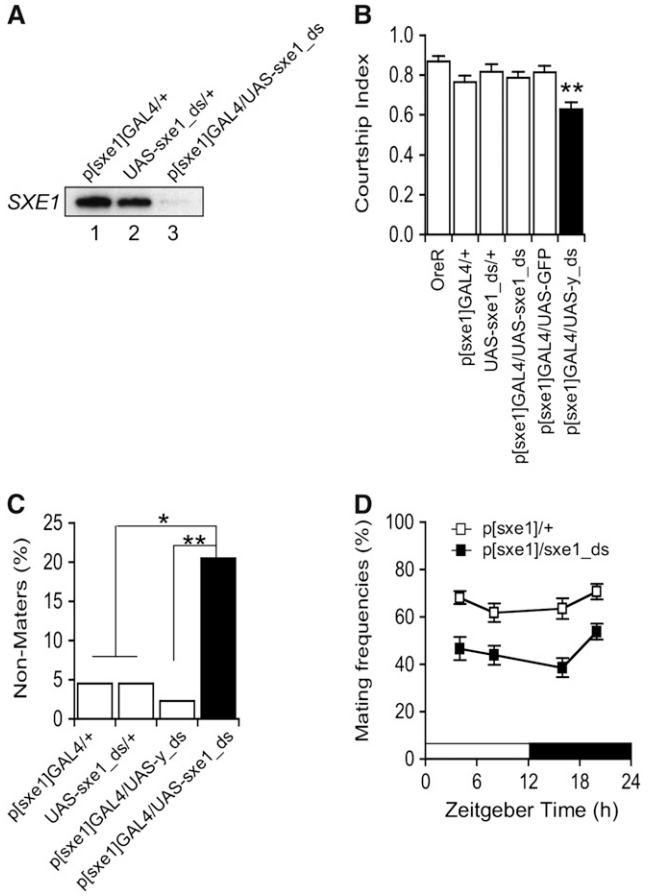


FIGURE 3.—Suppression of *sxe1* by RNAi decreases courtship and mating performance. (A) Western blot with anti-*SXE1* of protein extracts from heads of two types of control males (lanes 1 and 2) and *p[sxe1]GAL4/UAS-sxe1_ds* males (RNAi males; lane 3). (B) Courtship index (CI) for control and RNAi males ($n > 20$ for *OreR*, *UAS- p[sxe1]GAL4/UAS-y_ds*, and *p[sxe1]GAL4/UAS-GFP*; $n > 50$ for *p[sxe1]GAL4/+* and *p[sxe1]GAL4/UAS-sxe1_ds*). Error bars represent SEM. $^{**}P < 0.001$ (Wilcoxon/Kruskal-Wallis tests, the value of *p[sxe1]GAL4/UAS-sxe1_ds* was compared with that of *p[sxe1]-GAL4/+*). (C) Frequencies of nonmaters in single pair mating assay ($n = 44$ for each genotype; 30-min observation period). $^{*}P < 0.05$ or $^{**}P < 0.01$ (chi-square test) for values compared with *p[sxe1]GAL4/UAS-sxe1_ds*. (D) Mating frequencies at different ZT points. Five virgin males and five virgin females were placed in a vial and left for 15 min to copulate in the dark (<1 lux; $n = 39$–50 for each genotype and time point). Error bars represent SEM. Differences in genotype ($P < 0.0001$), time ($P = 0.0223$), and interaction of time and genotype ($P = 0.7249$) were analyzed by two-way ANOVA.

A and B) and because male courtship is also under circadian control (HARDELAND 1972; SAKAI and ISHIDA 2001; TAUBER *et al.* 2003; FUJII *et al.* 2007), we wondered whether mating success was affected differently at various time points during the day and night. Thus, we measured the frequency of successful matings at ZT 4, 7, 16, and 19 among sexually naive males and females. Because visual cues may override subtle deficiencies in other *sxe1* dependent sensory modalities, we performed these experiments under dim red light (<1 lux). Mating frequency of *sxe1*-RNAi males was significantly lower at all time points (Figure 3D). Two-way ANOVA revealed that there are significant differences between *sxe1*-RNAi males and control males (*p[sxe1]GAL/+*) in genotype ($P < 0.0001$) and time ($P = 0.022$), but not in the interaction of genotype and time ($P = 0.72$). Thus, our observations indicate that *sxe1* is required throughout the day for high mating success and show that *sxe1* is necessary in male courtship behavior and mating.

***Sxe1* mutant males show reduced mating efficiency:** Although RNAi is an effective tool for investigating gene function, downregulation is often incomplete; indeed SXE1 protein is not entirely depleted in *sxe1* RNAi males (Figure 3A). Conversely, RNAi can cause off target effects, especially if the target gene belongs to a large gene family that has extensive DNA homology, as do many *cyp* genes. Thus, we generated a null mutation for *sxe1* to verify the phenotypes observed with RNAi using homologous recombination (GONG and GOLIC 2003). Mapping and PCR analysis indicated that two potential *sxe1* mutations were obtained (Figure 4A and data not shown), and Southern analysis indicated that one of these two alleles ($sxe1^{67}$) represented a precise recombination event in which the first 18 codons of the *sxe1* gene were replaced by the *mini-white* gene (Figure 4B and MATERIALS AND METHODS). To investigate whether $sxe1^{67}$ was a true null allele, we performed Northern and Western analyses, which revealed that neither RNA nor protein is produced in homozygous mutant males (Figure 4, C and D). We then performed the same behavioral experiments on wild-type, $sxe1^{67}$ homozygous and heterozygous males as we did on *sxe1*-RNAi males. While no effect on the courtship index of $sxe1^{67}$ homozygous mutant males compared to control males was apparent (data not shown), we observed a significant increase in the fraction $sxe1^{67}$ homozygous mutant nonmaters, similar to *sxe1*-RNAi males (compare Figures 4E to 3C). Moreover, this phenotype was displayed throughout the diurnal cycle as in *sxe1*-RNAi males (compare Figures 4F and 3D). Significantly, these phenotypes were rescued when a *sxe1* transgene was introduced into a $sxe1^{67}$ homozygous mutant background (Figure 4, E and F). Two-way ANOVA indicates that there are significant differences in genotype ($P < 0.0001$) and time ($P = 0.0036$), but not in the interaction of genotype and time ($P = 0.9068$), between homozygous ($sxe1^{67}/sxe1^{67}$) and heterozygous ($sxe1^{67}/+$) males. Genotype ($P = 0.0003$) and time ($P = 0.0008$), but not the interaction of genotype and time ($P = 0.5158$) are also significantly different between homozygous ($sxe1^{67}/sxe1^{67}$) males and the same males carrying a rescue transgene ($sxe1^{67}/sxe1^{67}$;*Rescue3*/+). We tested several other behaviors and properties, such as locomotion, taste preference, longevity, and fertility; none of them were affected in $sxe1^{67}$ homozygous mutant males (Table 1). In summary, the genetic analysis of males

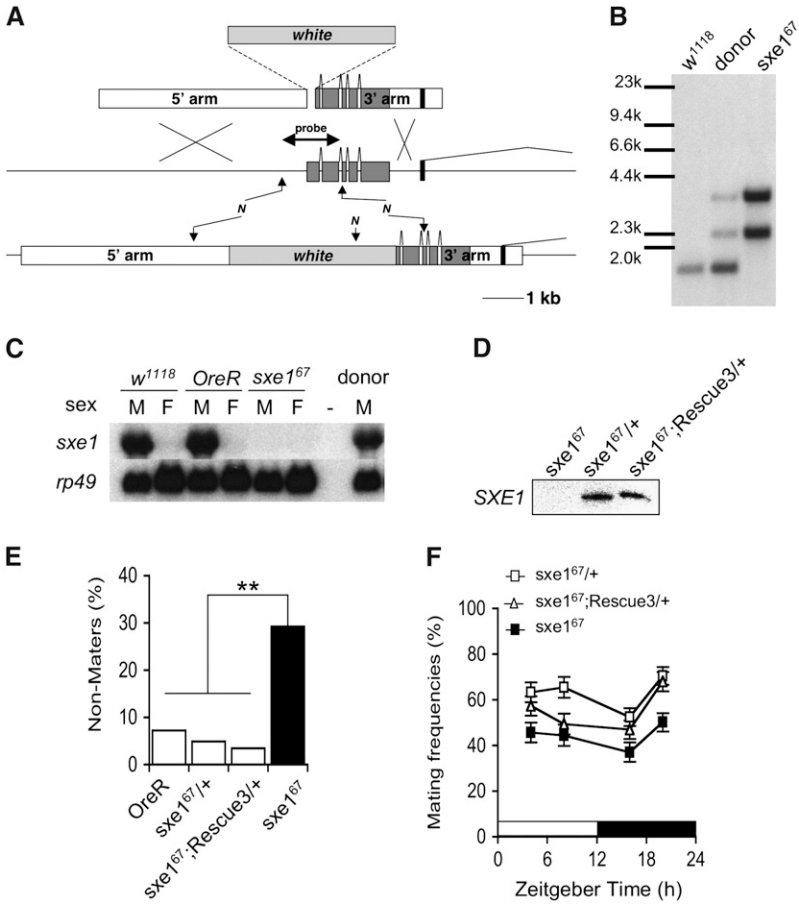


FIGURE 4.—*Sxe1* mutant males exhibit reduced mating success. (A) Diagram of the *sxe1* genomic region prior to (middle) and after (bottom) homologous recombination of the *sxe1* targeting construct (top). The mutant *sxe1* gene lacks the 5′ part (58 bp) of the *sxe1* gene including the start codon. N's indicate *Nco*I sites. (B) Genomic Southern analysis of DNA from control flies, flies carrying the targeting construct, and flies homozygous for the mutant $sxe1^{67}$ allele. The 1.5-kb *Nco*I fragment present in the *sxe1* gene, which also served as a probe in Figure 4A, is replaced by the white minigene and adjacent *sxe1* sequence, represented by two fragments of 3.3 and 2.3 kb in the $sxe1^{67}$ mutant allele. (C and D) Expression of *sxe1* mRNA (C) and SXE1 protein (D) is completely abolished in homozygous $sxe1^{67}$ mutant flies. (E) Frequencies of nonmaters in single pair mating assays of $sxe1^{67}$ mutant and control males ($n = 55$, 41, 29, or 89 for *OreR*, $sxe1^{67}/+$, $sxe1^{67}$;Rescue3/+ or $sxe1^{67}$, respectively). $^{**}P < 0.01$ (chi-square test) for values compared with $sxe1^{67}$. (F) Mating frequencies at different ZT points. Five virgin males and five virgin females were placed in a vial and left for 15 min to copulate in the dark (<1 lux; $n = 38$–44 for each genotype and time point). Error bars represent SEM. Differences were analyzed by two-way ANOVA and were as follows for $sxe1^{67}/+$ vs $sxe1^{67}$: $P < 0.0001$ (genotype), $P = 0.0036$ (time), and $P = 0.9068$ (interaction of time and genotype). For $sxe1^{67}/sxe1^{67}$;*Rescue3/+ vs.* $sxe1^{67}/sxe1^{67}$, they were $P = 0.0003$ (genotype), $P = 0.0008$ (time) and $P = 0.5158$ (interaction of time and genotype).

lacking a functional *sxe1* gene confirmed the mating phenotypes observed with *sxe1* RNAi, and demonstrate that *sxe1* is necessary in males for efficient mating.

Quantitative analysis of lipids and fatty acids in the head of *sxe1* mutant male: The cytochrome p450 enzymes can be subdivided into several protein subfamilies. In Drosophila, the CYP4 subfamily is one of the largest and includes 22 members, and several insect and mammalian CYP4 enzymes were shown to function as ω-hydroxylases. For example, human CYP4A11 and CYP4F2 are fatty acid ω-hydroxylases for lauric acids (POWELL *et al.* 1996) and leukotriene B4, a potent mediator of inflammation (KIKUTA *et al.* 1993), respectively. In Drosophila, CYP4G1 functions as an ω-hydroxylase for regulating triacylglycerol (TAG) levels in oenocytes (GUTIERREZ *et al.* 2007). To test whether SXE1/CYP4D21 may have a role in fatty acid metabolism, we analyzed and quantified lipids in the head of flies in the presence and absence of a functional *sxe1* gene (Figure 5 and supplemental Table 1). Overall, the concentration of all lipid classes is similar, regardless of whether a functional *sxe1* gene is present or not, with the possible exception of TAGs (Figure 5A). Interestingly, however, the concentration of plasmalogen 18:1n7, plasmalogen 18:1n9, adrenic acid (22:4n6), docosapentaenoic acid (22:5n6), docosahexaenoic acid (DHA, 22:6n3) of phosphatidylethanolamine (PE) are dramatically elevated in the head of *sxe1* mutant males (Figure 5B). In addition, the concentrations of γ-linolenic acid (18:3n6) of cholesterol ester (CE), plasmalogen 16:0 of lysophosphatidylcholine (LyPC), mead acid (20:3n9) of phosphatidylcholine (PC), and vaccenic acid (18:1n7) of TAG are elevated, whereas the concentrations of two saturated fatty acids, behenic acid (22:0) and lignoceric acid (24:0) of cardiolipin (CL), γ-linolenic acid (18:3n6), adrenic acid (22:4n6), and DHA (22:6n3) of phosphatidylcholine (PC), and vaccenic acid (18:1n7) of sphingomyelin (SM) are reduced in the head of *sxe1* mutant males (Figure 5B and supplemental Table 1). These results strongly suggest that SXE1 is involved in fatty acid metabolism of a substantial number of lipids, presumably as an ω-hydroxylase.

DISCUSSION

Hundreds of putative, sex-specific and sex-biased effector genes thought to be collectively responsible for sex-specific differentiation during development and sex-specific physiological processes in males and females have been identified in several large-scale expression studies (ARBEITMAN *et al.* 2002, 2004; DAUWALDER *et al.* 2002; FUJII and AMREIN 2002; PARISI *et al.* 2004).

TABLE 1

Suppression of *sxe1* affected neither locomotor activity nor fertility

	Locomotion[a] (no. of events/4 min)	Taste[b]	Life span[c] (days)	Fertility[d]
Oregon-R	—	—	—	69.3 ± 3.4
p[sxe1]GAL4/+	39 ± 3.7	—	—	—
UAS-sxe1_ds/+	37 ± 3.3	—	—	—
p[sxe1]GAL4/UAS-y_ds	34 ± 3.5	—	—	—
p[sxe1]GAL4/UAS-sxe1_ds	37 ± 3.2	—	—	—
$sxe1^{67}$/+	38 ± 3.6	0.85 ± 0.06	48 ± 1.2	72.1 ± 3.3
$sxe1^{67}$; *rescue3/+*	43 ± 2.6	0.86 ± 0.01	49 ± 1.9	—
$sxe1^{67}$	40 ± 2.9	0.86 ± 0.01	48 ± 2.6	75.2 ± 2.7

Sxe1 is not required for locomotion, taste (sugar) perception, normal life span, or fertility.

[a] Line crossing events were counted for 4 min ($n = 14$ for each genotype).

[b] Taste preference was determined by using the two-choice preference assay for 25 mM trehalose *vs.* water (Thorne *et al.* 2004). Three independent experiments ($n = 40$/experiment) for each genotype were performed.

[c] Life span indicates at which point 50% of flies have died. Three independent experiments per genotype were performed ($n = 100$/experiment).

[d] A single male and a single w^{1118} virgin female were kept in a vial. After 4 days, they were removed and flies were counted after all progeny had hatched. The number indicates the average progeny per vial. The number of single crosses for each genotype was between 20 and 26.

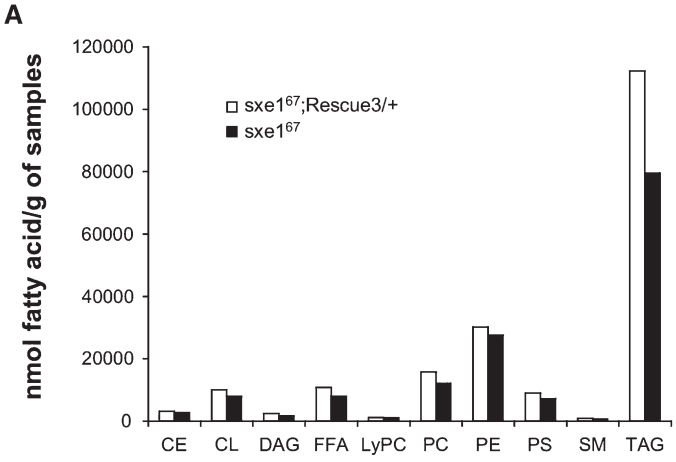
The main criteria of a sex-specific effector gene are (i) sex-specific or -biased expression outside the germ line, (ii) regulation by the sex determination genes and (iii) functional requirement in a sex-specific process in males or females. Despite the relatively large number of putative effector genes, virtually none of these genes have been studied in any detail. A main difficulty in uncovering their function is probably the subtle phenotypes that animals mutant for any of these genes display. One effector gene that has been functionally characterized is *to*, which is expressed in fat cells of the head in males and which was shown to be necessary for male sexual behavior (Lazareva *et al.* 2007). Fat cells are known to have endocrine roles, and TO, which is a member of the odorant/pheromone binding protein family is thought to be secreted from these cells and may function as a neuromodulator (Lazareva *et al.* 2007). Interestingly, fat cells in the adult fly are known to express several other putative sex-specific/biased effector genes, including the *yolk protein* (*yp*) genes and *female-specific independent of transformer* (*fit*) in females and *turn on sex-specificity* (*tsx*) in males, which also encodes a odorant/pheromone binding protein (Fujii and Amrein 2002).

Sxe1 is only the second male-specific effector gene with a role in male sexual behavior. Expression studies revealed that SXE1 protein is found in nonneuronal cells associated with chemo- and mechanosensory bristles of the head and thorax in males. Albeit SXE1 is also detected in the labial palps of females, both Northern and Western analysis revealed that the RNA/protein levels are severely reduced when compared to males. We also showed that both *sxe1* mRNA and protein oscillate in males during the day, with peak values at night and separated by ~4 hr.

While *sxe1* is necessary for efficient reproductive behavior of the male, demonstrated both in our RNAi studies as well as with the *sxe1* mutant fly strain, only *sxe1* RNAi males, but not *sxe1* homozygous mutant males, exhibited a significant decrease of the CI. The most likely explanation for this difference is that RNAi of *sxe1* causes off-target effects and downregulates other members of the many and highly conserved cytochrome p450 genes. The observation that *sxe1* mutant males show relatively normal levels of courtship, yet copulate less efficiently than control males suggests that they are slow maters. For example, it is possible that *sxe1* is necessary for aggressive pursuit of the female, when she exhibits coyness toward the end of courtship. However, once *sxe1* mutant males have copulated, they produce similar numbers of progeny as control males (Table 1), further supporting the notion that *sxe1* is indeed a behavioral, but not a fertility gene.

How does SXE1 function in male mating success, given its nonneural expression in sensory bristles? To address this, we reflected on the biochemical role of this enzyme. While the specific chemical reaction carried out by SXE1 remains to be characterized, the lack of SXE1 activity has severe consequences on the abundance of various metabolites in the male's head (Figure 5B and supplemental Table 1). Like other CYP4 enzymes, SXE1

Figure 5.—The concentration of all lipid classes in the head of *sxe1* mutant and control males is similar. Quantitative lipid concentration in the head of files with ($sxe1^{67}/sxe1^{67}$;*Rescue3/+*) and without ($sxe1^{67}/sxe1^{67}$) a functional *sxe1* gene. (A) No large differences were observed for any of the various lipid classes, with the possible exception of triacylglycerol. CE, cholesterol ester;



B

Increase in *sxe1* mutant males (candidate substrates for SXE1)

R=cholesterol ester (CE)

γ-linolenic acid (18:3n6) [4.8/249.4]

R=Lysophosphatidylcholine (LyPC)

plasmalogen 16:0 [0.2/8.0]

R=Phosphatidylcholine (PC)

mead acid (20:3n9) [1.7/18.2]

R=Phosphatidylethanolamine (PE)

plasmalogen 18:1n7 [2.5/29.1]

plasmalogen 18:1n9 [0.7/15.4]

adrenic acid (22:4n6) [1.3/41.6]

docosapentaenoic acid (22:5n6) [0.0/12.0]

docosahexaenoic acid (22:6n3, DHA) [12.7/220.3]

R=triacylglycerol (TAG)

vaccenic acid (18:1n7) [0.0/104.5]

Decrease in *sxe1* mutant males

R=Cardiolipin (CL)

behenic acid (22:0) [44.2/0.0]

lignoceric acid (24:0) [11.1/0.0]

R=Phosphatidylcholine (PC)

γ-linolenic acid (18:3n6) [0.7/0.1]

arachidonic acid (20:4n6) [72.0/3.9]

docosahexaenoic acid (22:6n3, DHA) [14.0/0.6]

R=sphingomyelin (SM)

vaccenic acid (18:1n7) [18.9/1.5]

CL, cardiolipin; DAG, diacylglycerol; FFA, free fatty acid; LyPC, lysophosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; and TAG, triacylglycerol. (B) Some specific compounds are drastically increased or reduced between the two genotypes (for detail, see supplemental Table 1). All chemical structures are referred from http://www.lipomics.com/fatty_acids. Values in brackets indicate nanomoles of fatty acid per gram of sample in the heads of *sxe1^{67}/sxe1^{67}; Rescue 3/+* and *sxe1^{67}/sxe1^{67}*mutant males, respectively.

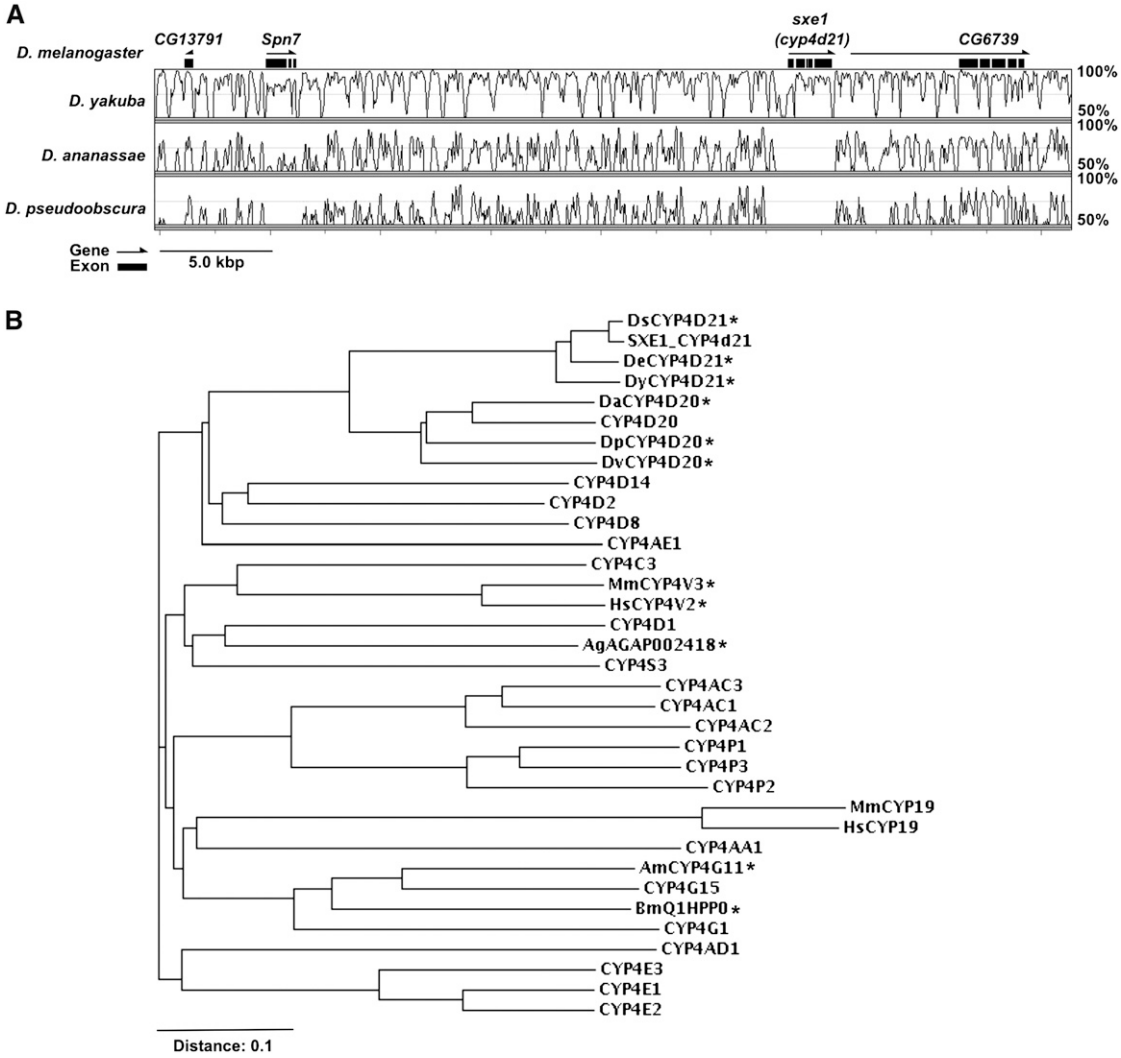


FIGURE 6.—The *sxe1* gene is conserved only in the *melanogaster* subgroup, but not in other Drosophila species. (A) The *sxe1* region (chr2L: 7,576,849–7,618,455) on the *D. melanogaster* genome (April 2004, the BDGP, Release 4), *D. yakuba* genome (April 2004, the Washington University School of Medicine, St. Louis, Release 1.0, SLAGAN), *D. ananassae* genome (July 2004, the Institute for Genomic Research, SLAGAN), and *D. pseudoobscura* genome (July 2003, the Human Genome Sequencing Center at Baylor College of Medicine, Freeze 1, SLAGAN) displayed by VISTA Browser (version 2.2.31 at http://pipeline.lbl.gov/cgi-bin/gateway2). Plots for *D. yakuba/D. melanogaster* (top), *D. ananassae/D. melanogaster* (middle), and *D. pseudoobscura/D. melanogaster* (bottom) are displayed on the scale of *D. melanogaster* sequence. Conserved regions above the level of 50% in a 100-bp window are plotted by the curve. (B) Neighbor-joining phylogenetic tree of CYP4-type chytochrome P450 in *D. melanogaster* with some SXE1 homologs in other Drosophila species. Proteins were aligned using ClustalW (EMBL-EBI, http://www.ebi.ac.uk) with default parameters. Prefixes Ds, De, Dy, Da, Dp, Dv, Mm, Hs, Ag, Am, and Bm indicate *D. simulans*, *D. erecta*, *D. yakuba*, *D. ananassae*, *D. pseudoobscura*, *D. virilis*, *Mus musculus* (mouse), *Homo sapiens* (human), *Anopheles gambiae* (mosquito), *Apis mellifera* (honey bee), and *Bombyx mori* (silkworm), respectively. *D. melanogaster*, *D. simulans*, *D. erecta*, and *D. yakuba* are *melanogaster* subgroup species of the genus Drosophila. Asterisks indicate the most similar protein of *SXE1* in each species determined by tblastn (FlyBase and NCBI).

may modify arachidonic acid derivatives such as leukotriene B4 and prostaglandins. Strikingly, *sxe1* mutant males accumulate plasmalogens (fatty acids with the dm prefix in supplemental Table 1) in their heads. Plasmalogens serve as a starting point for the release of arachidonic acid by phospholipase A2 (PLA2) and the synthesis of signaling molecules derived from arachidonic acid such as prostaglandins in mammals (FAROOQUI *et al.* 1995). In *sxe1* mutant males, accumulation of a SXE1 substrate may decrease PLA2 activity and prevent release of arachidonic acid from plasmalogens, thereby increasing the concentration of plasmalogens. Given the SXE1-dependent metabolites are small and soluble, they can easily reach other cells, not only within the vicinity of their production, but possibly in remote regions in the head and beyond via transport through the hemolymph. Thus, we propose that SXE1 expressed in sensory bristles acts in an endocrine fashion via a diffusible

metabolite on cells within the sensilla or possibly on neurons located elsewhere, including the brain.

Expression of SXE1 (and TO; see above) in nonneuronal cells of tissues not associated with sex-specific behaviors is intriguing, especially in light of the fact that several other sex-specific/biased genes have been found to be expressed in the same tissues. For example, the three *yp* genes and *fit* were all shown to be expressed in the fat cells in the female head, while *tsx* was found to be expressed in the same cells of males, and yet another gene, *sxe2* is expressed in nonneuronal cells in the head of males in a fashion similar to *sxe1* (Fujii and Amrein 2002) (S. Fujii and H. Amrein, unpublished data). A common feature of all these genes is that they either encode secreted proteins (TO, TSX, YP1, 2, and 3) or enzymes associated with the production of small metabolites (SXE1 and SXE2); hence, we propose that these tissues have endocrine properties and may be the source of various, hormone-like molecules involved in sexual behavior and other sex-specific physiological processes by acting on remote cells or neurons in the CNS.

Interestingly, *sxe1* and a few other sex-specific/biased genes (*to* and *tsx*), are regulated by the clock genes (Dauwalder *et al.* 2002; Fujii and Amrein 2002), and both *to* and *sxe1* expression oscillate in diurnal fashion. Therefore, one may expect that mating success in *sxe1* mutant males may be affected differently at various Zeitgeber times; instead, we observed a reduction throughout the day. Similarly, no circadian-dependent courtship phenotype has been reported in *to* mutant males, albeit a second function associated with this gene, food intake, has been reported to exhibit a circadian phenotype in *to* mutants. Regardless, the functional relevance of *sxe1* oscillation remains to be investigated in more detail in the future.

At last, we note that *sxe1* is a gene that emerged recently in evolution. Comparison of the genome sequence of various Drosophila species revealed that *sxe1* orthologs are only found in members of the *melanogaster* subgroup (Figure 6, A and B), which include *D. simulans*, *D. melanogaster*, *D. erecta*, *D. sechellia*, and *D. yakuba*. Drosophila species of the *melanogaster* group (*D. ananassae*) or the obscura group (*D. pseudoobscura*) lack *sxe1* in the syntenic region or elsewhere in the genome (Figure 6, A and B), as do more distant diptera such as the housefly or mosquitos. This suggests that the function of *sxe1* may be limited to a few, closely related Drosophila species, perhaps as a gene involved in speciation. However, many other sex-specific/biased effector genes such as *tsx* (*obp99b*), *fit*, *sxe2*, *to*, and the *yp genes* are conserved in all Drosophila species for which sequence data are available (data not shown).

We thank Jeff Hall for providing Clk^{JRK}, cyc^{01}, per^{01}, tim^{01}, Rodolfo Costa for *UAS-y_ds*, and Kent Golic for w^{1118}; *70FLP, 70I-SceI, Sco/CyO* and w^{1118}*;70FLP*. This work was supported by grants from National Institutes of Health (DC-005606) and National Science Foundation (IBN-03-49671) to H.A.

LITERATURE CITED

Arbeitman, M. N., A. A. Fleming, M. L. Siegal, B. H. Null and B. S. Baker, 2004 A genomic analysis of Drosophila somatic sexual differentiation and its regulation. Development **131:** 2007–2021.

Arbeitman, M. N., E. E. Furlong, F. Imam, E. Johnson, B. H. Null *et al.*, 2002 Gene expression during the life cycle of Drosophila melanogaster. Science **297:** 2270–2275.

Brand, A. H., and N. Perrimon, 1993 Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development **118:** 401–415.

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.

Burtis, K. C., and B. S. Baker, 1989 Drosophila doublesex gene controls somatic sexual differentiation by producing alternatively spliced mRNAs encoding related sex-specific polypeptides. Cell **56:** 997–1010.

Claridge-Chang, A., H. Wijnen, F. Naef, C. Boothroyd, N. Rajewsky *et al.*, 2001 Circadian regulation of gene expression systems in the Drosophila head. Neuron **32:** 657–671.

Coschigano, K. T., and P. C. Wensink, 1993 Sex-specific transcriptional regulation by the male and female doublesex proteins of Drosophila. Genes Dev. **7:** 42–54.

Dauwalder, B., S. Tsujimoto, J. Moss and W. Mattox, 2002 The Drosophila takeout gene is regulated by the somatic sex-determination pathway and affects male courtship behavior. Genes Dev. **16:** 2879–2892.

Demir, E., and B. J. Dickson, 2005 fruitless splicing specifies male courtship behavior in Drosophila. Cell **121:** 785–794.

Dunipace, L., S. Meister, C. McNealy and H. Amrein, 2001 Spatially restricted expression of candidate taste receptors in the Drosophila gustatory system. Curr. Biol. **11:** 822–835.

Erdman, S. E., and K. C. Burtis, 1993 The Drosophila doublesex proteins share a novel zinc finger related DNA binding domain. EMBO J. **12:** 527–535.

Farooqui, A. A., H. C. Yang and L. A. Horrocks, 1995 Plasmalogens, phospholipases A2 and signal transduction. Brain Res. Brain Res. Rev. **21:** 152–161.

Fujii, S., and H. Amrein, 2002 Genes expressed in the Drosophila head reveal a role for fat cells in sex-specific physiology. EMBO J. **21:** 5353–5363.

Fujii, S., P. Krishnan, P. Hardin and H. Amrein, 2007 Nocturnal male sex drive in Drosophila. Curr. Biol. **17:** 244–251.

Gong, W. J., and K. G. Golic, 2003 Ends-out, or replacement, gene targeting in Drosophila. Proc. Natl. Acad. Sci. USA **100:** 2556–2561.

Gutierrez, E., D. Wiggins, B. Fielding and A. P. Gould, 2007 Specialized hepatocyte-like cells regulate Drosophila lipid metabolism. Nature **445:** 275–280.

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

Hardeland, R., 1972 Species differences in the diurnal rhythmicity of courtship behaviour within the melanogaster group of the genus Drosophila. Anim. Behav. **20:** 170–174.

Hardin, P. E., 2005 The circadian timekeeping system of Drosophila. Curr. Biol. **15:** R714–R722.

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

Kikuta, Y., E. Kusunose, K. Endo, S. Yamamoto, K. Sogawa *et al.*, 1993 A novel form of cytochrome P-450 family 4 in human polymorphonuclear leukocytes. cDNA cloning and expression of leukotriene B4 omega-hydroxylase. J. Biol. Chem. **268:** 9376–9380.

Lazareva, A. A., G. Roman, W. Mattox, P. E. Hardin and B. Dauwalder, 2007 A role for the adult fat body in Drosophila male courtship behavior. PLoS Genet. **3:** e16.

Lin, Y., M. Han, B. Shimada, L. Wang, T. M. Gibler *et al.*, 2002 Influence of the period-dependent circadian clock on diurnal, circadian, and aperiodic gene expression in Drosophila melanogaster. Proc. Natl. Acad. Sci. USA **99:** 9562–9567.

McDonald, M. J., and M. Rosbash, 2001 Microarray analysis and organization of circadian gene expression in Drosophila. Cell **107:** 567–578.

Nagoshi, R. N., and B. S. Baker, 1990 Regulation of sex-specific RNA splicing at the Drosophila doublesex gene: cis-acting mutations in exon sequences alter sex-specific RNA splicing patterns. Genes Dev. **4:** 89–97.

Parisi, M., R. Nuttall, P. Edwards, J. Minor, D. Naiman *et al.*, 2004 A survey of ovary-, testis-, and soma-biased gene expression in Drosophila melanogaster adults. Genome Biol. **5:** R40.

Powell, P. K., I. Wolf and J. M. Lasker, 1996 Identification of CYP4A11 as the major lauric acid omega-hydroxylase in human liver microsomes. Arch. Biochem. Biophys. **335:** 219–226.

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

Sakai, T., and N. Ishida, 2001 Circadian rhythms of female mating activity governed by clock genes in Drosophila. Proc. Natl. Acad. Sci. USA **98:** 9221–9225.

Sarov-Blat, L., W. V. So, L. Liu and M. Rosbash, 2000 The Drosophila takeout gene is a novel molecular link between circadian rhythms and feeding behavior. Cell **101:** 647–656.

Shirangi, T. R., B. J. Taylor and M. McKeown, 2006 A double-switch system regulates male courtship behavior in male and female Drosophila melanogaster. Nat. Genet. **38:** 1435–1439.

Simpson, A. E., 1997 The cytochrome P450 4 (CYP4) family. Gen. Pharmacol. **28:** 351–359.

Tauber, E., H. Roe, R. Costa, J. M. Hennessy and C. P. Kyriacou, 2003 Temporal mating isolation driven by a behavioral gene in Drosophila. Curr. Biol. **13:** 140–145.

Thorne, N., C. Chromey, S. Bray and H. Amrein, 2004 Taste perception and coding in Drosophila. Curr. Biol. **14:** 1065–1079.

Communicating editor: G. Gibson