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Trapped in endoderm-1 Reveals a Novel Role for Fruitless in *Drosophila melanogaster* Courtship

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TRAPPED IN ENDODERM 1 REVEALS A NOVEL ROLE FOR FRUITLESS IN
DROSOPHILA MELANOGASTER COURTSHIP

A Thesis

Presented to

The Faculty of Department of Biological Sciences

San José State University

In Partial Fulfillment

of the Requirement for the Degree

Master of Science

by

Peter Luu

August 2016

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The Designated Thesis Committee Approves the Thesis Titled:

*TRAPPED IN ENDODERM 1 REVEALS A NOVEL ROLE FOR FRUITLESS IN
DROSOPHILA MELANOGASTER COURTSHIP*

by

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APPROVED FOR THE DEPARTMENT OF BIOLOGICAL SCIENCES

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August 2016

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ABSTRACT

TRAPPED IN ENDODERM 1 REVEALS A NOVEL ROLE FOR FRUITLESS IN *DROSOPHILA MELANOGASTER* COURTSHIP

By Peter Luu

Understanding how genes can direct behaviors has been a prevailing goal of neuroscience. Courtship in *D. melanogaster* is a complex yet stereotyped array of behaviors established by sex-specific genetic pathways mediated by sensory inputs by the nervous system. However, much remains to be discovered about the neurobiological and molecular mechanisms that regulate this complex set of behaviors. We have identified a group of cells expressing *Trapped in endoderm 1 (Tre1)* in which male Fruitless proteins are required to reduce the speed of courtship initiation. *Tre1* encodes a G-protein-coupled receptor required for establishment of cell polarity and cell migration and has previously not been shown to be involved in courtship behavior. By monitoring the latency to courtship initiation of male flies, we found the expression of female-specific transcription factors in *Tre1*-expressing neurons, or “feminization,” resulted in rapid courtship initiation. The *Tre1*-feminized males produced an increased number of offspring when challenged in a competitive fertility assay, suggesting that rapid courtship initiation led to a reproductive advantage. Interestingly, this did not hinder male flies’ ability to select an appropriate mate when they were confronted with a variety of mating targets. Using immunofluorescence, we showed that *Tre1* is expressed in a sexually dimorphic pattern in the central and peripheral nervous systems. Ultimately, we believe these cells may participate in an unforeseen “quality control” step that ensures the correct performance of the courtship ritual.

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Introduction

Genes and Behavior

Humans and other animals are born with a characteristic body plan and morphology, but we often overlook behavior as similarly heritable. Whether it is predator avoidance or a courtship pattern, certain behaviors have persisted through generations, suggesting a genetic component. This raises a long-standing question in behavioral neuroscience: do genes control behavior? How do genes direct neurodevelopment to integrate series of sensory inputs and motor outputs to orchestrate a complex behavior?

In a broad sense, behaviors can encompass anything from movement (courtship, nest building, and predation) to regulation (heart rate, breathing, and balance) (Lorenz, 1950). Instinctive or innate behaviors are stereotyped behaviors of an organism induced by specific sensory stimuli, also called a fixed action pattern. Innate behaviors are capable of being performed without training. The study of these behaviors offers the advantage of dedicated neurological pathways that are more amenable to genetic analyses.

Understanding how genes organize neuronal circuitry will help elucidate how the nervous system generates complex behaviors. However, delineating the mechanism by which genes control a behavior is challenging. For any innate behavior, there is a myriad of genetic effects working to integrate environmental (sensory) cues with hard-wired neuronal circuitry. To understand how genes, the brain, and behavior are associated, we require a tractable gene expression system, a well-characterized nervous system, and a

stereotyped set of responses. Male courtship behavior in *D. melanogaster* has emerged as an excellent model system for the study of genetic specification of behavior.

***D. melanogaster* Courtship**

D. melanogaster courtship involves a complex but stereotyped array of sexually dimorphic behaviors that have been studied for over 50 years (Shorey, 1962) (Figure 1). The male first recognizes olfactory and visual cues from the female and orients himself towards her. Next, he pursues her and taps the female to sample non-volatile chemicals present on her cuticle. He then extends and vibrates one wing towards the female to play a species-specific courtship song. If she is responsive, she stops movement, allowing the male to lick her genitalia to open the vaginal plates. The male then mounts the female and curls his abdomen to attempt copulation. A receptive female copulates with the male, allowing for the transfer of semen, which results in both fertilization and a reduction in female sexual receptivity (Tram & Wolfner, 1998). All of these steps are performed correctly and in the correct order by naive males (Hall, 1994), making *D. melanogaster* courtship an ideal model for the study of the genetic programming of complex innate behaviors.

Figure 1. The courtship ritual in *D. melanogaster*. (a) The male first orients towards and follows the female, (b) then taps her with his forelegs, (c) extends one wing towards the female, and vibrates it to generate a species-specific courtship song. (d) He then licks her genitalia and (e) curls his abdomen to attempt copulation. If the female is receptive (has not recently mated), (f) copulation will follow.

***D. melanogaster* Nervous System and Courtship**

Courtship initiation in *D. melanogaster* males relies on sensory information including visual, auditory, mechanosensory, and chemical cues which remains consistent within species (Boake, 1997; Greenspan & Ferveur, 2000). Because the process happens in a sex-specific fashion, integrations of these cues must rely on a sexually dimorphic

nervous system. The male isoform of Fruitless, Fru^M , generally mediates development of the nervous system by targeting neuronal morphogenetic genes (Neville et al., 2014). The expression of fru^M in females is sufficient to generate male courtship behavior, whereas the loss fru^M in males causes the absence of courtship behaviors (Demir & Dickson, 2005). Thus, to understand how genes control behavior, it is crucial to identify the differences between the male and female nervous systems.

The *D. melanogaster* nervous system has a volume of $\sim 0.07 \text{ mm}^3$ (Rein et al., 2002). Of roughly 135,000 neurons in the supraesophageal ganglion, about 2,000 express fru^M and are found in the olfactory, gustatory, auditory, and mechanosensory systems (Alivisatos et al., 2012; Datta et al., 2008). This suggests an integration of sensory information during courtship that is mediated by sex determination factors.

Visual cues. The optic lobes are located in the anterior and lateral position of the supraesophageal ganglion and process visual information (Yu et al., 2010). Despite the eye being dispensable for successful copulation (McRobert & Tompkins, 1987), fru^M expression in the optic lobe likely mediates male-specific visual cues. About 30 neurons in the male optic lobe express fru , compared with about five neurons in the female optic lobe (Kimura et al., 2005). Several neurons from the optic lobe integrate with the lateral horn, a known region with high levels of fru expression (Ruta et al., 2010). Knockout of fru^M in the optic lobe affects locomotive behavior, such as rates of following and turning, during courtship (Lee et al., 2000). During the early stages of the courtship ritual, the male follows the female, wherein his ability to see the female correlates with the amount of visibility (Sakai et al., 1997).

However, in the absence of light, the male *D. melanogaster* still performs the orientation and following stages of the courtship ritual, indicating that visual cues are less important than other forms of sensory input. Blind flies, as well as flies in dark environments, display delayed courtship initiation compared to seeing flies; this indicates that visual cues are important but not required for normal courtship initiation. Additionally, vision is not necessary for successful copulation in female flies (Markow, 1987).

Pheromonal and other chemosensory cues. A common feature among animals is the use of chemical cues, including pheromones, to modulate behavior. The two known brain regions that process pheromonal cues are the mushroom body and the lateral horn (Marin et al, 2002; Keene & Waddell, 2007). The effects of a few pheromones in *D. melanogaster* are listed in Table 1. In *D. melanogaster*, pheromones are synthesized by specialized cells called oenocytes (Makki et al., 2014). There are two general categories of pheromones: volatile and non-volatile.

Volatile pheromones, as their name implies, do not require physical contact for their effects; instead, they disperse to olfactory receptor neurons (ORNs) in the antenna and maxillary palp. The ORNs are housed in chemosensory hair-like structures called sensilla and project to the antennal lobe and subsequently to the mushroom body and the lateral horn. One example of such a cue is the volatile pheromone 11-cis-vaccenyl acetate (cVA). cVA is expressed by male fruit flies and influences courtship behavior of both male and female flies over the range of a few centimeters (Antony & Jallon, 1982; Kurtovic et al., 2007). cVA activates the Or67d olfactory receptor present in antennal

neurons in both sexes; however, it displays sexually dimorphic effects. Knockout of the Or67d receptor in males leads to indiscriminate courting of either sex, indicating a required function in mate selection in males (Kurtovic et al., 2007). On the other hand, when the Or67d receptor is knocked out in females, they become less receptive to male sexual advances (Kurtovic et al., 2007). Finally, high levels of cVA in conditions where there is a high density of males lead to aggressive behaviors (Liu et al., 2011). Based on the studies of Kurtovic et al. (2007) and Liu et al. (2011), it is clear that cVA functions to inhibit mating behavior in male while promoting mating in females.

Nonvolatile pheromones are exchanged through physical contact and act through gustatory receptors on the forelegs and proboscis during the licking and tapping steps of courtship. The male foreleg expresses specific gustatory receptors to detect inhibitory pheromones, such as the hydrocarbon 7-tricosene (7-T) (Ferveur & Sureau, 1996; Savarit et al., 1999). Similar to cVA, 7-T helps to inhibit male-male courtship while inducing aggressive behavior (Wang et al., 2011). Likewise, when males are perfumed with 7-T, females mate faster and more frequently (Grillet, Darteville, & Ferveur, 2006). Thus, like cVA, 7-T inhibits male-male mating behavior while promoting female receptivity.

Table 1. *The Effects of Common Courtship Pheromones*

Chemical Name	Abbr.	Effect on Courtship
5-tricosene or 5-C23:1	(5-T)	↯ Tapping
7-tricosene or 7-C23:1	(7-T)	↯ Wing song, attempted copulation, licking → Female receptivity
7-pentacosene or 7-C25:1	(7-P)	→ Male-male courtship
9-pentacosene or 9-C25:1	(9-P)	→ Attempted copulation
7, 11 heptacosadiene	(7, 11 HD)	→ Wing song, attempted copulation, licking
7, 11 heptacosene	(7, 11 ND)	→ Wing song, attempted copulation, licking
11-cis-vaccenyl acetate	(cVA)	→ Copulation

Notes. Adapted from Cobb & Jallon, 1990; Ferveur & Sureau, 1996; Liu et al., 2011. ↯ is inhibitory, → is stimulatory.

Auditory cues. During courtship, *D. melanogaster* males activate indirect flight muscles to vibrate their wings and produce an acoustic cue (Shirangi et al., 2013). This wing song consists of two phases, a “sine” phase and a “pulse” phase. The sine phase is species non-specific and thought to increase female receptivity, whereas the pulse phase is species-specific (Ewing & Bennet-Clark, 1968). The auditory circuit begins at the second segment of the antenna which houses auditory sensory neurons (Lai et al., 2012). These neurons project to antennal mechanosensory and motor centers (AMMC) in the brain (Lai et al., 2012).

The female hears the vibration and then becomes more receptive to copulation. Consequently, females with a deficit in hearing are less likely to copulate (Markow, 1987). For the male initiator of courtship, however, auditory deficiency does not affect copulation (Markow, 1987).

Fruitless and Behavioral Sex Determination

The *fruitless* (*fru*) gene is the master regulator of male courtship behavior. *fru* is both necessary and sufficient for every step of courtship and is required independently at each step (Demir & Dickson, 2005). The *fru* gene spans 120 kb of genomic DNA, contains four different promoters (*fruP1-fruP4*) and encodes 12 known isoforms of a *bric-a-brac/tramtrack/Broad* (BTB)/zinc finger transcription factor. *fruP1* is expressed in a sex-specific fashion, whereas *fruP2*, *P3*, and *P4* are expressed in both sexes and result in transcription of proteins that are essential for survival (Ryner et al., 1996).

Transcripts initiated from the *fru* P1 promoter are sex-specifically spliced under the control of the sex-determining pathway via the splicing factors Tra and Tra-2 (Figure 2). Male-specific splicing of *fru* P1 transcripts results in full-length Fru^M proteins, while female-specific splicing results in truncated Fru proteins due to the introduction of a premature stop codon (Anand et al., 2001). There are three protein isoforms of Fru^M (FruA, FruB, and FruC) that control neuronal arborization and courtship behavior (von Philipsborn et al., 2014).

fru^M mutant male behavior varies from courting male and female flies indiscriminately, to skipping steps, to complete lack of courtship behavior (Hall, 1994). Consistent with its role as a master regulator of courtship behavior, when *fru^M* expression is driven in female flies, they exhibit male courtship behaviors towards other females (Demir & Dickson, 2005; Manoli et al., 2005). When *fru^M* expression is knocked down in neurons in males, which “feminizes” the cells, the flies will appear morphologically male, but lack courtship behaviors that indicates a requirement for Fru^M proteins in sex

determination in the nervous system (Ryner et al., 1996). Finally, Fru^M transcription factors are expressed in ~2% of neurons in the male brain and ventral nerve cord (VNC) (Alivisatos et al., 2012).

Figure 2. Behavioral sex determination in *D. melanogaster*. Control of Fruitless splicing starts with the “measuring” of the autosome to X chromosome ratio, where a normal female has two X chromosomes to two sets of autosomes (2X:2A) and normal a male has one X chromosome to two sets of autosomes (1X:2A). A 2X:2A ratio results in increased expression of the x-linked gene *sisterless*, which inhibits expression of the autosomal gene *Deadpan*, allowing the expression of *sex-lethal* (*sxl*). *sxl* encodes a splicing factor, which drives female-specific splicing of *transformer* (*tra*). *tra* encodes yet another splicing factor (TraF) that directs female-specific splicing of *fru* transcripts. *fru^F* transcripts encode non-functional, truncated proteins. In males, the lack of TraF leads to male-specific splicing of *fru* P1 transcripts, resulting in full-length Fru^M proteins. TraF also regulates the splicing of another sex specific protein, Doublesex (Dsx), to a female isoform, Dsx^F, which mediates the feminization of somatic tissues.

Research Aims

Typically, disruption of *fru*^M expression leads to a delays in, or absence of, normal courtship behaviors. Here we have defined a set of neurons (*Tre1-GAL4* neurons) in which downregulation of *fru*^M causes unusually rapid initiation of courtship. Rapid courtship initiation would seem advantageous in nature by providing that leads to more offspring; however, there may be less apparent disadvantages. Males lacking *fru*^M expression in *Tre1-GAL4* neurons may be skipping a checkpoint during mate selection, which might allow rapid courtship initiation, but reduced reproductive success in the wild. Alternatively, the fate of the *Tre1-GAL4* cells may be altered by the absence of FruM, resulting in a previously uncharacterized gain-of-function effect on courtship initiation. This thesis aims to:

- Confirm previous results by feminizing 9-210 cells by expressing *Tra*^F
- Identify the *GAL4* line that causes rapid courtship initiation in strain 9-210
- Further characterize the courtship and mating phenotypes of males with rapid courtship initiation
- Identify the neurons that are important for the 9-210 courtship phenotype

Materials and Methods

Fly Strains and Genetics

The *P[GAWB]* enhancer trap line *GAL4⁹⁻²¹⁰* was a kind gift from Ulrike Heberlein (Meissner et al., 2011). Isolation of the three X-linked P-elements was accomplished by crossing *GAL4⁹⁻²¹⁰* females with *w¹¹¹⁸*; *Wild Type Berlin* males. Heterozygous females were selected in which recombination was free to occur, allowing the isolation of 50 recombinant lines. Identification of the individual P-element insertions present in recombinant lines was accomplished using PCR. *w¹¹¹⁸*; *Wild Type Berlin* is our laboratory control strain. *TreI^{EP496}* was obtained from the Bloomington *Drosophila* stock center (stock number: 10089). The *UAS-mCD8-GFP* and *UAS-Tra^F* lines were a kind gift from Bruce Baker. *D. simulans* and *D. mauritiana* were a kind gift from Theresa Logan-Garbisch.

Inverse PCR

Identification of the P-element insertion sites was performed using inverse PCR, according to the Berkeley *Drosophila* Genome Project (BDGP) protocol. Genomic DNA was extracted using ethanol precipitation, digested with *MspI* (New England Biolabs), ligated at low DNA concentration to favor circularization, then subjected to PCR with primers *GawB5'*out and *GawB5'*in. Resulting PCR products were cloned into the pCR4-TOPO-TA vector (Life Technologies) and sequenced with M13 for M13R primers.

Courtship Assays

Courtship assays were performed according to published protocols (Villella et al. 1997). Virgin males were kept in isolation for 2–3 days after eclosion. Each male was

then presented with a single 1-2 day-old w^{1118} ; *WTB* virgin female. Single male and female pairs were placed into custom plexiglass chambers 10 mm in diameter and 6 mm in height, separated by plastic transparencies. Contact between pairs was initiated by removal of the transparencies. Courtship behavior was recorded using Leica MZ8 in only infrared light for 20 min.

Fertility Assay

For the noncompetitive fertility assay, a single 2-3 day-old male fly was paired with a single 2-3 day-old virgin female fly. The flies were allowed to mate and lay eggs for 2 days, after which the adults were cleared from the vials. All flies eclosing from each vial were counted and averaged over all vials to calculate fertility of individual males. For the competitive fertility assay, two experimental males and two w^{1118} ; *WTB* males were placed in a vial with five w^{1118} ; *WTB* virgin females. All flies were 2–4 days old. The adults were allowed to mate and lay eggs for 2 days, after which the adults were cleared from the vials. The resulting progeny were counted and scored for their eye color phenotype.

Immunofluorescence

The CNS and peripheral tissue were prepared from three to six-day-old flies raised under standard conditions according to Wu & Luo (2006) with the following modifications: tissues were fixed for 1 h, incubated with NGS block for 24 h, stained in primary antibody for four days, and secondary for two days. NC82 mouse anti-Bruchpilot was used at 1:50 (Developmental Studies Hybridoma Bank AB 2314866). Rabbit anti-GFP was used at 1:750 (Life Technologies ref. number: A6455). Alexafluor 488

Goat anti-Rabbit was used at 1:1000 (Jackson ImmunoResearch ref: 111-545-144).

Alexafluor 594 Goat anti-Mouse was used at 1:1000 and provided by David Tran (Tran et al., 2014). Imaging was performed on LSM 700 using Zen blue imaging software and ImageJ with Fiji for post imaging analysis.

Quantitative RT-PCR

For quantitative reverse-transcriptase-mediated PCR (qRT-PCR), embryos, third instar larvae, pupae, and adult flies were snap-frozen on dry ice. Total RNA was extracted using Trizol reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions, resuspended in RNase-free water and stored at -80°C until use. Total RNA (2 µg) was reverse-transcribed using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems, Carlsbad, CA) according to the manufacturer's instructions. cDNA was analyzed by quantitative real-time PCR using the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems). Ribosomal protein 49 (*rp49*) transcript levels were used as an endogenous normalization control for RNA samples, and relative mRNA abundance was calculated using the comparative $\Delta\Delta C_t$ method (Schmittgen & Livak, 2008). Each sample was analyzed in triplicate. As a negative control, we used DNase-treated nonreverse-transcribed mRNA samples; no significant amplification was observed in these samples.

Statistics Analysis

For courtship assays, one-way ANOVA was conducted with a Tukey's HSD post-hoc analysis to determine significant differences in courtship log-transformed latency means (if $p < 0.05$). For graphs, latency was back-transformed.

For competitive and non-competitive fertility assays, χ^2 was used to determine significant differences (if $p < 0.05$). RStudio and Microsoft Excel 2013 were used for all statistical analyses.

Primer Sequences

Tre-1: left primer: 5'-TCGTTTCGTACTCGTGCATC-3'
 right primer: 5'-TGGAAGTTATCGTGGTTGCG-3'

CG42343: 5'-GTACTCCCT GTCCCACTCCA-3';

GawB5'in: 5'-CAATAATGGGTCTTTGGC GACGG-3'

GawB5'out: 5'-GCCGCACGTAAGGGTTAATG-3'

M13F: 5'-GTAAAACGACGGCCAG-3'

M13R: 5'-CAGGAAACA GCTATGAC-3'

Rp49: left primer: 5'-ACGTTGTGCAC CAGGAACTT-3'
 right primer: 5'-CCAGTCGGATCGAT ATGCTAA-3'

Results

Tissue-Specific Feminization Using the *GAL4* Line 9-210

As described above, knocking down Fru^M in the *P[GawB]9-210 (GAL4⁹⁻²¹⁰)* cells by driving two copies of a *fru^M-RNAi* construct, *GAL4⁹⁻²¹⁰/Y; UAS-fru^MIR/+; UAS-fru^MIR/+*, in males led to a reduction in latency to courtship initiation. Courtship latency is defined here as the time elapsed between the first introduction of a female to the first unilateral wing extension (indicative of wing song) by the male. Courtship latency in *GAL4⁹⁻²¹⁰/Y; UAS-fru^MIR/+; UAS-fru^MIR/+* males averaged 18 s, compared with 28–32 s in heterozygous genetic background controls (Tran et al., 2014).

We confirmed the results of our previous study (Tran et al., 2014) by driving the expression of the female-specific isoform of the splice factor Transformer (*Tra^F*), with *GAL⁹⁻²¹⁰*. As described in Figure 3, *Tra^F* directs the female-specific splicing of *fru* transcripts, which, in females, results in the absence of full-length Fru^M proteins. Thus expression of *Tra^F* in neurons that normally express Fru^M proteins resulted in the feminization of those neurons (Heinrichs, et al., 1998). On average, *GAL4⁹⁻²¹⁰; UAS-Tra^F* males initiated wing song within 19 s, while heterozygous controls averaged 29 and 37 s (Figure 3), similar to the effects seen with *UAS-fru^MIR*.

Figure 3. $GAL4^{9-210}/Y; UAS-Tra^F/+$ males display rapid courtship initiation. $GAL4^{9-210}/Y; UAS-Tra^F$ males have a wing song latency of 19 seconds, while control animals initiate courtship in an average of 28 to 37 seconds. The blue bar represents the “feminized” group, whereas the grey bars represent heterozygous controls for each component of the $GAL4/UAS$ system. * denotes $P < 0.05$, error bars indicate SD, mean latencies were log-transformed to obtain equal variance for the one-way ANOVA with Tukey’s HSD post hoc analysis, mean latencies were back-transformed for graphs, $N = 22 - 24$ males.

Isolation of P-Element Insertions from $GAL4^{9-210}$

The $GAL4^{9-210}$ line initially contained three independent X-linked P-element insertions at *Trapped in endoderm 1* (*Tre1*), CG42343 and *folded gastrulation* (*fog*) (David Tran and Rachael French, unpublished data). Between the initial characterization of $GAL4^{9-210}$ and our unpublished data, the transposon in *fog* was lost from the line, most likely due to it not being homozygous in the initial strain. The remaining two insertions were separated by meiotic recombination, generating 50 strains with a possible isolated

P-element insertion. The identities of the isolated P-element insertions were confirmed using PCR with primers recognizing the P-element and the individual loci.

Representative examples of PCR results are shown in Figure 4. PCR of genomic DNA from strains PL1, PL3, and the positive control 9-210 yielded products using primers from both *CG42343* (*CG*) and *Tre1*, while strain PL2 amplified only with *Tre1* primers, indicating that this strain contained a single P-element inserted in the *Tre1* locus.

Figure 4. Representative DNA gel to confirm successful isolation of *P[GawB]* transposons. *Tre1* and *CG42343* indicate the locus-specific primer used for that reaction. PL1-3 denotes the specific recombinant strain being assayed, 9-210 represents a positive control showing both *Tre1* and *CG* bands. Amplicon sizes: *Tre1* - 269 bp ; *CG42343* - 507 bp.

Feminization of *Tre-1*-Expressing Cells Causes Rapid Courtship Initiation

We next tested the isolated *Tre1-GAL4* and *CG42343-GAL4* insertions for their ability to cause rapid courtship initiation when used to drive *UAS-Tra^F*. We found that *Tre1-GAL4/Y; UAS-Tra^F/+* males initiated wing song significantly faster than control males (Figure 5). *Tre1-GAL4/Y; UAS-Tra^F/+* males have a courtship latency of 14 s,

while control animals initiated courtship on an average of 31 or 36 s (shown as grey columns). *CG42343-GAL4/Y; UAS-Tra^F/+* males averaged 53 s, an increase that was not significant due to a high degree of variability in the sample. These results demonstrated that the elimination of Fru^M proteins in *Tre1-GAL4* expressing cells was responsible for the significantly reduced latency to court seen in *GAL4⁹⁻²¹⁰/Y; UAS-fru^MIR/+* males.

Figure 5. *Tre1-GAL4/Y; UAS-Tra^F/+* males display rapid initiation of wing song. *Tre1-GAL4/Y; UAS-Tra^F* males have a wing song latency lower than control animals. *CG42343-GAL4/UAS-Tra^F/+* males averaged higher than *CG42343-GAL4/Y* control animals. * denotes $P < 0.05$, differing from all other means. Error bars indicate SD, mean latencies were log-transformed to obtain equal variance for the one-way ANOVA with Tukey's HSD post hoc analysis, mean latencies were back-transformed for graphs, $N = 22-24$ males.

***TreI-GAL4* is Expressed in a Sexually Dimorphic Pattern in Both the Central and Peripheral Nervous Systems**

In order to identify the cells that were being feminized in *TreI-GAL4/Y; UAS-Tra^F/+* males, we used *TreI-GAL4* to drive expression of *UAS-mCD8*, a mouse transmembrane glycoprotein fused to green fluorescent protein, under the control of the yeast UAS promoter. In the adult, *TreI-GAL4* is expressed in a sexually dimorphic pattern in the olfactory sensory neurons (OSNs). In males, approximately 15 neurons in the 3rd antennal segment (the primary *D. melanogaster* olfactory organ) express *TreI-GAL4*, while there is little to no expression in OSNs in females (Figure 6 A & C). Consistent with this observation, *TreI-GAL4* is also expressed in two to three antennal lobe glomeruli (Figure 6 B & D). Finally, there are extensive projections to the lateral horn (LH) of the brain in males and relatively few such projections seen in the female brain. This pattern is consistent with regions known to express *fru^M* (Alivisatos et al., 2012; Datta et al., 2008).

More importantly, the *TreI-GAL4* expression pattern is consistent with neurons that are involved in the detection and processing of olfactory cues. OSNs detect olfactory cues and project to specific glomeruli in the antennal lobe. Interneurons from the antennal lobe project to two major targets: neurons in the mushroom bodies of the brain (where neither *fru^M* nor *TreI-GAL4* are expressed) and neurons in the LH. The LH is divided into the anterior and posterior sections where the anterior is involved in the processing of innate pheromonal signals (Gupta & Stopfer, 2012; Jefferis et al., 2007). This suggests that the *TreI-GAL4* cells may be involved in the reception and/or

processing of a chemosensory signal involved in mate selection. In addition to the sexually dimorphic expression described above, *Tre1-GAL4* is also expressed in the auditory neurons of the second antennal segment and their projection target, the antennal mechanosensory and motor center (AMMC) (Figure 2.4 A & C) (Kamikouchi et al., 2006). Finally, projections are found in the subesophageal ganglion (SOG) in both sexes (Figure 6 B & D).

Figure 6. Sexually dimorphic *Tre1-GAL4* expression pattern in the male and female CNS. (A) Male 2nd and 3rd antennal segment. (B) Male CNS. (C) Female 2nd and 3rd antennal segment. (D) Female CNS. AS = antennal segment, SOG = subesophageal ganglion, AMMC = antennal mechanosensory and motor center, AL = antennal lobe, LH = lateral horn. Red channel shows background neuropil staining, green channel shows *Tre1-GAL4* expression, scale bars represent 50 μ m.

***Tre1-GAL4/Y; UAS-Tra^F* Males Have Increased Reproductive Fitness**

Given that feminizing the *Tre1-GAL4* neurons led to rapid courtship initiation, the phenomenon would seem to provide a potential mating advantage over wild type males. We tested the reproductive fitness or mating success of *Tre1-GAL4/Y; UAS-Tra^F* males in two ways. First, we tested their fertility in a noncompetitive assay with *w¹¹¹⁸; WTB* unmated females. *Tre1-GAL4/Y; UAS-Tra^F* males produce an average of 23 offspring per male, whereas the *UAS-Tra^{F/+}* and *Tre1-GAL4/Y* heterozygous background controls produced an average of 21 and 25 offspring, indicating that the fertility of *Tre1-GAL4/Y; UAS-Tra^F* males is normal (Figure 7).

We next tested *Tre1-GAL4/Y; UAS-Tra^{F/+}* males in competitive mating assays to test the hypothesis that rapid courtship initiation results in a competitive mating disadvantage. Five *w¹¹¹⁸; WTB* virgin females were presented with a choice between two unmated *Tre1-GAL4/Y; UAS-Tra^{F/+}* males and two *w¹¹¹⁸; WTB* and allowed to mate for 2 days. According to our pedigree, 75% of the progeny of *Tre1-GAL4/Y; UAS-Tra^{F/+}* males should have colored eyes, while none of the progeny of *w¹¹¹⁸; WTB* males will have colored eyes (Figure 8). Therefore, if mating success is equal between the two genotypes, the expected progeny eye color ratio is 37.5% colored eyed to 62.5% white eyed. Deviation from this ratio indicates altered fitness for a group.

We found that in 9 of 12 vials, *Tre1-GAL4/Y; UAS-Tra^{F/+}* males were at a competitive advantage compared with control males. Overall, 49% (304 of 619) of offspring were non-white, an overrepresentation of 31% relative to the expectation for equal success in mating (Table 2). Thus, under laboratory conditions, the rapid courtship

initiation seen in *Tre1-GAL4/Y; UAS-Tra^F/+* males provide a competitive advantage over wild type males.

Figure 7. Noncompetitive mating assay. *Tre1-GAL4/Y; UAS-TraF/+* males produce nearly identical numbers of offspring compared with heterozygous controls. *Tre1-GAL4/Y; UAS-Tra^F/+* males produce an average of 23 offspring per male, whereas the *UAS-Tra^F/+* and *Tre1-GAL4/Y* heterozygous background controls produced an average of 21 and 25 offspring (shown as grey columns). One-way ANOVA, n = 16 vials per genotype.

Figure 8. Expected progeny of *Tre1-GAL4/Y; UAS-TraF* males. We expected 75% of the offspring of *Tre1-GAL4/Y; UAS-TraF* males will have colored eyes. If mating success is equal with white-eyed control males, 37.5% of the resulting offspring will have colored eyes.

Table 2. *Competitive Mating Assay.*

Male Genotype	Competitor Male	Nonwhite Offspring (%)	Expected Nonwhite Offspring (%)	Total Offspring	χ^2 (P-value)
<i>Tre1-GAL4/Y;</i> <i>UAS-TraF/+</i>	<i>w¹¹¹⁸; WTB</i>	304 (49.1)	232 (37.5)	336	35.6 (P<0.0001)
<i>Tre1-GAL4/Y;+</i>	<i>w¹¹¹⁸; WTB</i>	141 (43.8)	161 (50)	302	4.7 (P = 0.030)

Notes. *Tre1-GAL4/Y; UAS-TraF/+* males have a competitive advantage compared with control males where 49% of offspring have colored eyes (expected 37.5%). *Tre1-GAL4/Y;+* serves as a heterozygous control. χ^2 test, n = 638.

***Tre1-GAL4* Neurons Are Not Required to Distinguish Mated from Unmated Females**

Female *D. melanogaster* are recalcitrant to a second mating; thus, male flies are less likely to initiate courtship with mated females. The identification of mated vs. unmated females is mediated by nonvolatile inhibitory cuticle hydrocarbons via gustatory receptors during the “tapping” step of courtship (Pavlou & Goodwin, 2013). In addition, males must also select for mates of the correct sex as well as species. The competitive fertility assay tested a specific scenario where only unmated conspecific females (the ideal mate) were presented to the males. Thus it is possible that *Tre1-GAL4/Y; UAS-Tra^F/+* males skip a mate-identification checkpoints during courtship and thus achieve a relative reproductive advantage under laboratory conditions. To test this hypothesis, we tested the latency to court in *Tre1-GAL4/Y; UAS-Tra^F/+* males with mated females.

When presented with mated *w¹¹¹⁸; WTB* females, *Tre1-GAL4/Y; UAS-Tra^F/+* males showed a normal delay in courtship initiation relative to unmated females. Courtship initiation in these males were delayed by 17.6 s compared to unmated *w¹¹¹⁸; WTB* females (Table 3). Furthermore there was no difference between *Tre1-GAL4/Y; UAS-Tra^F/+* males and control males in time to courtship initiation with mated females which all averaged 41 s. This uniformity suggests that the *Tre1-GAL4* expressing neurons are not required for males to discern mated from unmated females.

Table 3. *Courtship Latency of D. melanogaster Males with Mated Females*

Male Genotype	Unmated Female	Mated Female
<i>Tre1-GAL4/Y;UAS-Tra^F/+</i>	23.5 ± 4.4 s	41.09 ± 5.87 s (P<0.05)
<i>Tre1-GAL4/Y;+</i>	28.9 ± 4.6 s	40.85 ± 9.6 s (ns)
<i>+/UAS-Tra^F</i>	32 ± 4.5 s	41.2 ± 4.0 s 8.0 (ns)

Notes. *Tre1-GAL4/Y;UAS-Tra^F/+* males display the expected increase in latency to court when paired with mated females. Statistics were performed using two-way ANOVA on log-transformed courtship data to obtain equal variances. Tukey's HSD was used for pair-wise comparison. Data shown has outliers removed and without log transformation. ± SD, s denotes seconds, n = 18-23 males.

***Tre1-GAL4* Neurons Are Not Required to Distinguish Male Flies from Female Flies**

Among the most well-known effects of *fru* mutation in males is the indiscriminate courtship of both males and females (Hall 1994). Mutant males often court other male flies as vigorously as females, and when *fru* mutant males are placed in groups of males a snake-like chaining behavior occurs in which each male in the chain is simultaneously a courter and recipient. Male-male courtship is sometimes observed in wild type males, but the delay in courtship initiation compared with virgin females is significant. We therefore tested the ability of *Tre1-GAL4/Y; UAS-Tra^F/+* males to identify male flies as inappropriate courtship targets. We found courtship latency was drastically increased when male flies were presented with unmated males (Table 4). In fact, *Tre1-GAL4/Y;UAS-Tra^F/+* males showed a greater delay in courting unmated males, though this effect was not statistically significant. These results indicated that the *Tre1-GAL4* cells are not required to differentiate the sex of potential mates.

Table 4. *Courtship Latency of D. melanogaster Males with Unmated Males*

Male Genotype	Unmated Female	Unmated Male
<i>Tre1-GAL4/Y;UAS-Tra^F/+</i>	23.5 ± 4.4 s	386 ± 128 s (P<0.05)
<i>Tre1-GAL4/Y;+</i>	28.9 ± 4.6 s	205 ± 65 s (P<0.05)
<i>+/UAS-Tra^F</i>	32.0 ± 4.5 s	494 ± 291 s (P<0.05)
Wild type	63.9 ± 8.6 s	415 ± 97.6 s (P<0.05)

Notes. *Tre1-GAL4/Y;UAS-Tra^F/+* display a normal increase in courtship latency when presented with unmated males. Statistics were performed using two-way ANOVA on log-transformed courtship timing data. Tukey's HSD was used for pair-wise comparison (unmated female vs. unmated male). Data shown has outliers removed and without log transformation. ± SD, s denotes seconds, n = 3-24 males.

***Tre1-GAL4* Neurons Are Not Required for Identification of Conspecific Females**

In their natural environment, *D. melanogaster* encounter sibling species, with whom, if successful copulation occurred, the offspring would be sterile. Therefore, it is imperative for male flies to select conspecific partners. We therefore tested whether the *Tre1-GAL4* cells are required to distinguish conspecific females from females of two closely-related species, *D. simulans* and *D. mauritiana*. *Tre1-GAL4/Y;UAS-TraF/+* males, when presented with unmated *D. simulans* females, displayed a significantly delayed wing song initiation (57.7 seconds compared with 13.9 seconds with *D. melanogaster* females) (Table 5). The heterozygous controls, *Tre1-GAL4/Y;+* and *+/UAS-TraF*, each had nonsignificant increases in courtship latency with *D. simulans* females.

Similarly, when *Tre1-GAL4/Y;UAS-TraF/+* males were presented with unmated *D. mauritiana* females, all genotypes showed a significant increase in wing song initiation with an increase mean latency higher than *D. simulans*. The delay was likely

more profound due to the fact that *D. melanogaster* and *D. simulans* are more closely related than either with *D. mauritiana* (Cobb et al., 1988).

Table 5. Courtship Latency of *D. melanogaster* Males with Females of Sibling Species.

Male Genotype	<i>D. melanogaster</i>	<i>D. simulans</i>	<i>D. mauritiana</i>
<i>Tre1-GAL4/Y;UAS-TraF/+</i>	13.9 ± 6.2 s	57.7 ± 12.5 s *	87.2 ± 23.2 s *
<i>Tre1-GAL4/Y;+</i>	36.5 ± 5.0 s	50.2 ± 7.8 s (ns)	76.4 ± 16.7 s *
<i>+/UAS-TraF</i>	29.8 ± 2.7 s	38.7 ± 6.3 s (ns)	87.2 ± 23.3 s *

Notes. *Tre1-GAL4/Y;UAS-TraF/+* display the expected increase of courtship latency when presented with *D. simulans* and *D. mauritiana*. Statistics were performed on using two way-ANOVA on log-transformed courtship timing to obtain equal variances, Mean latency shown on the graph was back-transform. Tukey's HSD was used for paired-wise comparison. ± SD, s denotes seconds, * denotes P<0.05, n = 18-24 males.

Tre1-GAL4* May Be a Partial Loss of Function Allele of *Tre1

The *Tre1-GAL4* transposon is inserted into the coding region of the G protein-coupled receptor (GPCR) gene *Trapped in endoderm-1 (Tre1)*. The *Tre1-GAL4* transgene is greater than 11 kb in length, and is located on the sixth exon of all three *Tre1* transcripts; its size and location suggest that it disrupts the cytoplasmic domain between the sixth and seventh transmembrane domains of the protein (Figure 9). Therefore, we hypothesized that *Tre1-GAL4* is very likely a loss-of-function allele of *Tre1*. To test this hypothesis, we used quantitative reverse-transcriptase mediated PCR (qRT-PCR) to examine *Tre1* transcript levels in *Tre1-GAL4* animals. Because *Tre1* is known to regulate germ cell migration, we examined the transcriptional levels during the embryonic stage (Kunwar et al., 2008). As predicted, *Tre1-GAL4* disrupted embryonic expression of *Tre1*, but the gene's expression level during the pupal stage remained unchanged (Figure 10).

Figure 9. Schematic diagram of the *Tre1* gene. *Tre1-RA*, *Tre1-RB*, and *Tre1-RC* encode the same protein, but *Tre1-RA* is initiated ~3 kb further upstream. The *Tre1-GAL4* transposon is inserted into the sixth exon of all three transcripts.

Figure 10. Expression of *Tre1* RNA in *Tre1-GAL4* flies. Embryonic expression in *Tre1* was decreased by 37%, while expression in pupae was not significantly changed relative to control animals. Expression in *Tre1-GAL4* flies shown are relative to the *w¹¹¹⁸*; *WTB* control strain (represented by a transcript level of 1). Error bars denote high and low ranges of a single standard deviation, n = 3.

Discussion

In summary, feminization of *Tre1-GAL4* expressing cells resulted in rapid courtship initiation and increased mating success. The *Tre1-GAL4* neurons were not required for mate identification and were expressed in a sexually dimorphic pattern in both the CNS and PNS. Finally, *Tre1-GAL4* was a weak loss-of-function allele of *Tre1*, which may exert some of the phenotypes seen.

Courtship in *D. melanogaster* involves a complex but stereotyped array of sexually dimorphic behaviors mediated by sensory inputs via the peripheral nervous system and integrated in the central nervous system (Hall 1994; Baker et al., 2001; Demir and Dickson 2005; Dickson 2008; Pavlou and Goodwin 2013). The resulting behavioral phenotypes are dependent upon expression of the male-specific isoforms of the transcription factor Fru^M (Demir and Dickson 2005). Here we identified a unique role for Fru^M proteins. When cells expressing *Tre1-GAL4* were feminized via expression of the female splice form of Tra (Tra^F), which led to female-specific splicing of *fru* and therefore loss of *fru*^M transcripts, male flies initiated courtship much more quickly than wild type flies. Thus, in the *Tre1-GAL4* cells, Fru^M proteins were uniquely required to reduce the speed of courtship initiation. In addition, we found that *Tre1* expression under *GAL4* control was expressed in a sexually dimorphic pattern in the adult OSNs and central nervous system and that the *Tre1-GAL4* neurons were not required for distinguishing conspecific females, unmated females, or males from females.

***D. melanogaster* Mating Behavior and the Role of the *Tre1-GAL4* Neurons**

The identified *GAL4* transgene (*Tre1-GAL4*) is inserted into the coding sequence of the *Trapped in endoderm-1* gene. When this *GAL4* line was used to drive expression of either an RNAi targeting *fru^M* or the feminizing transgene *UAS-Tra^F*, it resulted in male flies that initiated courtship and achieved copulation much more quickly than control animals. Typically, loss of *fru^M* results in slowed, abnormal, or absent courtship (Demir and Dickson 2005), but the feminization of *Tre1* expressing-neurons does the opposite, resulting in a mating advantage for *Tre1-GAL4 /Y; UAS-Tra^F* males. Given these observations, it seems counterintuitive that neurons exist specifically for slowing down courtship. To address this conundrum, we tested several hypotheses about the function of the *Tre1-GAL4* neurons.

First, the cells may participate in “quality control,” ensuring the correct performance of the courtship ritual. This hypothesis predicts that courtship quality is reduced in animals displaying rapid courtship initiation, leading to reduced mating and reproductive success in competition with wild type males. On the contrary, *Tre1-GAL4 /Y; UAS-Tra^F* males appeared to perform all steps of the ritual correctly, leading to the reproductive advantage seen in the fertility assays.

Second, the *Tre1-GAL4* cells may function in correct mate identification, allowing males to distinguish an appropriate mate from a less appropriate mate. This theory predicts that rapid courtship reflects the skipping of the species-specific wing song, or failure to recognize visual cues. However, we tested three well-characterized aspects of mate selection in regard to recipient type: mated vs. unmated female, female vs. male,

and sibling species. In all three aspects, *Tre1-GAL4* /Y; *UAS-Tra^F*/+ males were normal. Our experiments indicated that the *Tre1-GAL4* cells were not involved in the ability of *D. melanogaster* males to identify conspecific females, nor did they appear to be required to distinguish virgin from non-virgin females or males from females.

The Tre1 Receptor and Courtship Behavior

Tre1-GAL4 is a transposon inserted into the coding sequence of the G protein-coupled receptor (GPCR) gene *Trapped in endoderm-1* (*Tre1*). The Tre1 GPCR has two known functions in *D. melanogaster*. First, it is necessary for transepithelial migration of maternal germ cells through the posterior midgut epithelium (Kunwar et al., 2008; Kunwar et al., 2003). Second, it is needed for the determination of neuroblast polarity during asymmetric stem cell division in the developing central nervous system (Fuse et al., 2003; Yoshiura et al., 2012). The ligand for Tre1 is not known, but based on sequence similarity, it belongs to a family of proteins related to the GPCR CXCR4 (Kunwar et al., 2003). *Tre1* is closely related to the vertebrate histamine and melatonin receptors and more distantly related to chemokine receptors (Kunwar et al., 2003).

Asymmetric cell division in neuroblasts requires modulation of the cytoskeleton (Fuse et al., 2003; Yoshiura et al., 2012), whereas germ cell migration requires polarized distribution of E-cadherin (Kunwar et al., 2008) (as well as reorganization of the cytoskeleton). In migrating germ cells, the role of Tre1 appears to be to change the polarity of the cells such that they are oriented toward their ultimate target, and to facilitate the loss of cell–cell adhesion between germ cells, thereby priming the cells to be receptive to further guidance signals (Kunwar et al. 2008). Based on these data, *Tre1* is

likely a neuropeptide or protein hormone receptor that, upon activation, leads to a polarization of the cell for asymmetric cell division or migration. This proposed function for *Tre1* is consistent with a role in axon outgrowth or synapse formation.

The *Tre1-GAL4* transgene is a large insertion (more than 11 kb) in the protein-coding sequence of *Tre1*, but how this affects the protein sequence is not known. It is possible that cryptic splice sites within the P-element result in the translation of a hybrid, nonfunctional protein, and this may contribute synergistically to the behavioral phenotype seen in *Tre1-GAL4/Y;UAS-Tra^F/+* males. This leaves open the possibility that some of the complexity of phenotype we are seeing is due to varying levels of *Tre1* function due the transgene insertion (Figure 9 and 10). Because we found that *Tre1-GAL4* is expressed in a sexually dimorphic pattern in the adult nervous system, one possible mechanism for *Tre1* in courtship behavior is that *Tre1* may mediate the development and targeting of sex-specific neurons. In support of this hypothesis, we have data indicating that loss-of-function mutations in *Tre1* display rapid courtship initiation as well as other courtship defects (Luu et al., 2016)

Currently, we are in the process of characterizing a mutant strain, *Tre1^{EP496}*, in courtship and fertility assays. We will also test downstream components of the known *Tre1* signaling pathway for courtship and mating phenotypes.

Future Directions

Feminization of the *Tre1-GAL4* neurons causes rapid courtship initiation, and this phenotype led to increased reproductive success in competition with normal males. This advantage indicates that *Tre1-GAL4/Y; UAS-Tra^F* males perform the courtship ritual

correctly and completely. *Tre1-GAL4* is expressed in a sexually dimorphic pattern, suggesting the existence of neural circuitry that is specific to male flies that regulates the speed of courtship initiation.

To further understand the mechanism of how *Tre1-GAL4/Y; UAS-Tra^F* affects neural activities, we will determine whether the *Tre1-GAL4/Y; UAS-Tra^F* courtship phenotype is due to loss or gain of neuronal activity. We will undertake a series of experiments to drive expression of a temperature-sensitive allele of *shibiri* (*shi^{ts}*), the gene encoding the fly homolog of dynamin (Gonzalez-Bellido, et al, 2009), as well as the heat-sensitive TrpA1 (Rosenzweig et al., 2005) channel in the *Tre1-GAL4* neurons. If rapid courtship results from inactivation of the *Tre1-GAL4* cells, expression of *shi^{ts}* should recapitulate the phenotype. On the other hand, if rapid courtship results from inappropriate neuronal activity, activation of TrpA1 in the *Tre1-GAL4* cells should phenocopy *Tre1-GAL4/Y; UAS-Tra^F*.

It is possible, even likely, that the *GAL4* expression does not accurately reflect the full *Tre1* expression pattern. Further characterization of the expression of Tre1 would require the generation of an antibody against the Tre1 receptor followed by immunofluorescence imaging.

Conclusions

In this thesis we identified rapid courtship initiation time as a unique role for the loss of *fru^M* expression. Male *D. melanogaster* with decreased Fru^M in *Tre1*-expressing neurons initiate courtship much faster than wild type males, resulting in a reproductive advantage. We localized this effect to the olfactory sensory neurons in the antennae which likely synapse with neurons in the lateral horn, an area known for integrating innate chemical signals. Future studies will explore the function of the *Tre1* signal transduction pathway in mating behavior as well as confirm the *Tre1* expression pattern.

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