A NEUROGENETIC AND COMPARATIVE ANALYSIS OF COURTSHIP SONG PRODUCTION IN *DROSOPHILA*

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Courtship behavior studies in the genus *Drosophila* have led to progress in understanding how innate behaviors are neurogenetically programmed, and strive to understand how these behaviors evolve. Courtship song in the *Drosophila* genus is extremely diverse and widespread, and plays an important role in courtship success.

Courtship behavior is believed to be sex-specifically programmed throughout central nervous system of male *D. melanogaster*. Courtship decisions in the brain indeed are sex-specifically programmed, but it was not clear if this is true for courtship motor patterns as well, such as courtship song. Evidence provided here suggests the song circuit itself is sex-specifically organized, requiring expression of the male-specific form of the sex-determination gene, *fruitless* (fru^M), in the song circuit itself. When lacking fru^M in the circuit, fewer males are able to sing, and those that do exhibit disrupted song structure. However, the critical aspect of song temporal structure remains intact.

Hawaiian *Drosophila* exhibit an extraordinarily diverse array of courtship song signals. However, it was not known if auditory receptors in these species were sensitive to these signals. Multiunit neurophysiology presented here suggests that *D. heteroneura* and *D. silvestris* can indeed hear conspecific

courtship song. Further, *D. heteroneura* courtship song signals have been generalized to be used in an aggressive context.

The foundations of an experiment extending the role fru^M in the song circuit is presented. This experiment is designed to identify neurons requiring fru^M for song production.

BIOGRAPHICAL SKETCH

Dustin Rubinstein was born in the Chicago suburbs in 1980, and grew up with this mother, Denise, father, Clifford, and younger sister, Ginger. Always interested in the natural sciences from a young age, a young Dustin could often be found flipping rocks, eagerly anticipating discovery of the critters beneath, or staring into ponds, hoping to catch a glimpse of the wildlife within. Throughout grammar school, Dustin was lucky to have his parents drive him to summer science camps and weekend science exploration courses. In 1998, Dustin began his undergraduate studies at the University of Illinois at Champaign-Urbana. Upon receiving a Hughes Undergraduate Research Fellowship, he began his science career in the laboratory of Gene Robinson at the University of Illinois, studying the role of honey bee division of labor on the regulation of the *period* gene. Dustin graduated in Honors Biology with Distinction in the Spring of 2002, and began graduate school in the Department of Neurobiology and Behavior at Cornell University in the following Fall. Dustin met Kristin Goble on board a United Airlines flight, both heading home to Chicago for Thanksgiving in 2003, and the two would eventually marry in the summer of 2007 in Chicago.

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CHAPTER 1

GENERAL INTRODUCTION

The ubiquity and bewildering diversity of courtship behavior in the genus *Drosophila* is only matched by its fruitfulness in the fields of evolution, heredity, neuroscience, and molecular biology of animal behavior. *Drosophila* courtship behavior provides an innate, complex, stereotyped behavior readily observed in the laboratory making controlled experiments simple, while its commonality throughout the genus make it ripe for studies ranging from phylogenetics to molecular biology.

The genus consists of about 1500 species and its members are found around the world. However, most attention is focused on a narrower number of species. *D. melanogaster* has proved to be an invaluable – arguably the most valuable – organism in understanding genetics and molecular biology, while species found in the Hawaiian islands have been a particularly useful clade in studies of evolution. Studies of the genetics of courtship behavior in *D. melanogaster* dating back to Sturtevant (1915) has provided a rich backdrop of genes important in courtship behavior and yielded answers regarding heredity of this innate behavior. Early phylogenetic mapping of the 500 or so Hawaiian species has provided a basis for comparative studies of the wide-ranging and diverse behaviors seen in this explosion of speciation.

***Courtship in* Drosophila**

Early studies of *D. melanogaster* courtship behavior revealed it to be a highly multimodal exchange of sensory information (Dickson 2008; Manning 1959; Sturtevant 1915), including acoustic signals generated by the male

(Shorey 1962), confirmed by mutant analysis of flies deficient in a particular sensory modality (Hall 1994; Villella & Hall 2008). No one particular modality is necessary for successful copulation, but when multiple modalities were perturbed, serious courtship deficits ensued.

Upon coming in near proximity to a female, a male will primarily orient towards her based on visual cues, with her increased movement drawing his attention (Dickson 2008; Hall 1994; Villella & Hall 2008). Chemical cues provided by the female also encourage male courtship by transmitting volatile signals and tactile signals by tapping of the female abdomen with the males prothoracic legs. The pheromone 7,11-heptacosadiene found on females activates male courtship, while the pheromone cis-vinylacetate found on males suppresses male courtship. The male then extends a single wing towards the female and vibrates it in a species-specific manner, producing a “love song” (Ewing 1978; Shorey 1962). The male will then ventrally curl his abdomen towards the female in an attempt to achieve copulation (Hall 1994).

There is tremendous diversity in the structure of love songs throughout *Drosophila* (Gleason 2005; Markow & O'Grady 2005), but the love song of *D. melanogaster* is composed of two phases (Hall 1994). Sine song, or "hum song"", consists of a sinusoidal vibration of the single wing in a pattern similar to that seen in flight, but slower. The male also produces pulse song, or "purr song", by producing a series of short pulses with strict temporal control. Each pulse consists of only or two cycles. The interpulse intervals (IPIs) are highly species-specific and repeatable across observations and across males, and are the most important aspect of the courtship song (Bennet-Clark & Ewing 1969). Further, IPIs themselves cycle over the course of about a minute, and this signal is also important to courtship success (Kyriacou & Hall 1982).

Genetics of courtship song

Single gene mutant analysis has helped reveal some important requirements to courtship song (Gleason 2005). Identification of courtship song mutants revealed many genes involved in membrane excitability (Peixoto & Hall 1998; von Schilcher 1977), suggesting precise timing is required in the nervous system to execute song properly. Also, mutants for sex-determination genes immediately downstream of the feminizing gene *transformer* (*tra*), *fruitless* (*fru*) and *doublesex* (*dsx*), were found to affect song (Taylor et al 1994; Villella et al 1997; Villella & Hall 1996), suggesting that courtship circuits are sex-specifically organized to produce proper song patterns.

Development of genetic techniques to create genetically mosaic flies in the nervous system drove *D. melanogaster* courtship studies into the next age of neurogenetics by ascribing particular parts of the nervous system functions relating to courtship. The first such technique provided the ability to create gynandromorphic sex mosaics (Hotta & Benzer 1970; 1976). This revealed that tissue in the dorsal brain is required to be male for a male to initiate courtship, while more posterior tissue is required for a male to attempt copulation (Hall 1977; 1979). In another experiment, it was shown that male tissue is required in the thoracic ganglia for proper courtship song to be executed (von Schilcher & Hall 1979). The next technique, the GAL4/UAS system (Brand & Perrimon 1993), allowed a gene to be expressed in subsets of neurons within the CNS. Driving *tra* in subcompartments of the antennal lobes and the mushroom bodies resulted in males that courted females as well as males, indicating sexual dimorphic states in these regions help control initiation of courtship behavior (Ferveur & Greenspan 1998; Ferveur et al 1995).

It had been hypothesized that splice isoforms of the *fru* gene found specifically in males (fru^M) are required to set up the male nervous system to court properly (Baker et al 2001; Ryner et al 1996). Using technologies allowing targeted mutations of *fru*, this hypothesis was confirmed in two independent studies (Demir & Dickson 2005; Manoli et al 2005). Males bereft of fru^M showed disrupted courtship, and females ectopically expressing fru^M exhibited courtship comparable to males. While fru^M and its regulation of downstream components are certainly critical in establishing a courtship-producing nervous system, the role of the other gene parallel to *fru* in the *tra*-dependent sex-determination hierarchy, *dsx*, is also very important for generating sex-specific differences in the nervous system required for courtship behavior (Rideout et al 2007; Taylor et al 1994). It has been suggested that fru has role in courtship behavior in other Drosophila species based on the conservation of this gene throughout the genus (Davis et al 2000a; Davis et al 2000b; Gailey et al 2000).

While the precise role of fru^M in the male nervous system is unclear, several mosaic studies have shown that fru^M acts during neural development to produce neurite projections only found in neurons expressing fru^M, or indirectly permitting these neurons to persist by blocking apoptosis (Kimura et al 2008; Kimura et al 2005). A single study investigating the role of fru^M on membrane excitability did not detect an effect (Datta et al 2008). Taken together, this suggests that fru^M sculpts the male nervous system by producing a limited number of neurons particularly suited to produce courtship behavior by integrating courtship related information into a circuit producing courtship, but that circuit itself is not necessarily dedicated to courtship.

Males mutant for *fru* show deficits in ability to produce courtship song (Rideout et al 2007; Ryner et al 1996; Villella et al 1997), suggesting that fru^M is required to establish the nervous system to produce courtship song. However, it is unclear whether fru^M is required in higher order brain areas to decide to sing or activate the song circuit or whether fru^M is required in the song circuit itself to properly execute the song motor pattern, or perhaps both. It is also known that *dsx*, another sex-specifically spliced gene, is required to establish the courtship song network (Rideout et al 2007) along with proper fru^M function.

In one study, fru^M-expressing neurons were artificially activated and courtship song signals were recorded (Clyne & Miesenböck 2008). Decapitated flies were used here, as courtship song was only produced if the flies lacked a head. Both males and females were found to generate song upon artificial excitation, but the song pattern was improved in females if they ectopically expressed fru^M. This study suggests that fru^M is sufficient to improve organization of the song circuit, artificial activation of neurons expressing fru^M is sufficient to produce song, and that artificial activity in either sex produces the behavior. It is still unclear whether intact behaving females never produce song because their song circuit is fully functional but never receives activation or whether the circuit is not complete in that endogenous excitation is not sufficient to drive the circuit to produce the song circuit rhythm, whereas their method of artificial excitation of all fru^M neurons is sufficient. Clearly, much remains to learned about the role of fru^M in the circuit controlling the production of this important courtship behavior.

Physiology of courtship song

The courtship circuit itself remains largely unidentified, and this is particularly true of the courtship song circuit. It is believed that the courtship song circuit has derived from a pre-existing circuit generating flight (Crossley 1990), but the only identified members of this circuit are the motor neurons innervating muscles believed to be controlling the behavior (Trimarchi & Schneiderman 1994). Most of what is known about courtship song production is informed by studies investigating flight, a much more well-worked out behavior (Kammer 1985). Flight is controlled by two groups of muscles: classic tubular muscles and fibrillar muscles unique to insects (Chapman 1998). Tubular muscles contractions are controlled directly by innervating motor neurons, electrophysiologically exciting these muscle membranes and inducing a contraction of the muscle via its actin and myosin machinery. In fibrillar muscle contraction, however, motor neuron input is required to excite these muscle membranes, but this excitation does not activate the actin and myosin machinery – it is instead induced by stretching of the muscles. The power required for flight is delivered by the indirect, fibrillar muscles, while the tubular muscles control wing position and angle, both during flight and rest. As each of the pair of antagonistic fibrillar muscle groups induce distortions of the thorax resulting in stretching of the other muscle group, the flight rhythm of upstrokes and downstrokes is directly controlled by the kinetic wing motion itself. In courtship song, where the most salient feature is believed to be its temporal rhythm, this is an important consideration (Bennet-Clark & Ewing 1969; Talyn & Dowse 2004).

Measurements of flight muscle activation during courtship song production have led to the most informative studies of the neurobiological

basis of these signals. During pulse song, both muscle groups receive excitation phase-locked to pulse generation (Ewing 1977; 1979). It is believed that while fibrillar muscles provide the power in each pulse, tubular muscles are required to stretch-activate the fibrillar muscles, dampen their contraction to produce single-cycle pulses, or both. During sine song, tubular muscles are phase-locked with the sinousoidal output, as are the fibrillar muscles, to a much lesser extent. The slow rate of inputs to these muscles during sine song (flight-like in structure, but much reduced in amplitude) lead to the conclusion that fibrillar muscles produce the wing rhythm, but minimal excitation state of these muscles ensures the amplitude remains small (Ewing 1977).

Courtship in Hawaiian **Drosophila**

While the courtship song of *D. melanogaster* has been studied in depth, the studies of Hawaiian *Drosophila* courtship song have helped inform the principles of evolution driving the massive radiation characteristic of this clade. It is believed that all Hawaiian *Drosophila* species evolved from a single continental *Drosophila* founder event, and lead to the clade of tremendous morphological and behavioral diversity (Boake 2005; Carson & Kaneshiro 1976). It is believed that the prevalent species-specific courtship behavior in these flies is a major isolating mechanism during the evolution of these species (Boake 2005; Carson & Kaneshiro 1976). As a boon to understanding these phylogenetic relationships, the Hawaiian archipelago is produced by a massive volcano spewing over a slowly gliding crust, thus the more recently diverged species are found on the newer islands.

The behavioral diversity of Hawaiian *Drosophila* is reflected in the variety of species-specific acoustic signals generating during courtship

(Hoikkala et al 1994; Hoy et al 1988), producing songs with a range of spectral and temporal characteristics, as well as a multitude of mechanisms (Hoikkala et al 1989; Hoy et al 1988). For example, high-frequency clicks at least an order of magnitude higher than flies are believed to be acoustically sensitive seem to generated directly by thoracic musculature or a novel cuticular structure at the wing base, and appear to transmitted through direct male-female contact rather than through the air (Hoikkala & Moro 2000; Hoy et al 1988). In another case, members of the planitibia subgroup, including *D. heteroneura* and *D. silvestris*, produce sound pulses with characteristics similar those found in classic wing-generated pulses, but are produced by contractions of abdominal muscles (Hoy et al 1988). While there is no concurrent direct male-female with production of these signals, it is currently unclear whether the female receives these signals seismically or acoustically.

As in most *Drosophila* species, species-specific properties of Hawaiian *Drosophila* song provide species identification signals resulting largely in stabilizing selection for courtship song (Boake 2005), except for one continental species (Hoikkala et al 1998).

Males of lekking *Drosophila*, such as *D. heteroneura* and *D. silvestris*, exhibit courtship directed at females, as well as aggression directed at other lekking males (Spieth 1981). Presumably, successful males hold their territory and gain greater access to females. The conspicuous sexual dimorphism seen in *D. heteroneura*, whereby males exhibit broad “hammer heads”, is believed to have arisen through destabilizing selection towards males with bigger heads, as males lock head-to-head during aggressive encounters (Spieth 1981). Additionally, it has been proposed that the *fru* gene, which underlies male-female courtship in *D. melanogaster*, may underly male-male encounters

seen in Hawaiian *Drosophila* based on the conservation of *fru*'s sequence and its sexually dimorphic expression (Davis et al 2000b).

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CHAPTER 2

ELIMINATION OF FRU^{M} EXPRESSION IN THE THORACIC COURTSHIP SONG CIRCUIT OF *DROSOPHILA MELANOGASTER* AFFECTS SONG PARAMETERS, BUT NOT INTERPULSE INTERVAL

Summary

Despite the growing body of research investigating the sex-specific organization of courtship behavior in *Drosophila melanogaster*, much remains to be understood about the sex-specific organization of the motor pattern generation of this behavior. To investigate the sex-specification of a tightly patterned component of courtship behavior, courtship song, we used the GAL4/UAS system to feminize the ventral ganglia in male Drosophila and analyzed the acoustic properties of courtship song. More specifically, we used the thoracic-specifying *teashirt* promoter (*tsh*^{GAL4}) to express feminizing constructs specifically in the ventral ganglia. When *tsh*^{GAL4} drove expression of *transformer* (*tra*), males were unable to produce prolonged wing extensions. Driving expression of an RNAi construct directed against male-specific fruitless (*fru*^{M}) transcripts resulted in normal wing extension, but highly defective courtship song, with 55% of males failing to generate detectable courtship song. Of those that did sing, pulse widths of individual pulses were significantly wider than controls, suggesting thoracic *fru*^{M} function serves to mediate proprioceptive-dependent wing vibrations dampening during pulse song. However, the most critical signal in the song, the interpulse interval, remained intact. The inability to phenocopy this effect by reducing *fru*^{M} expression in motor neurons and proprioceptive neurons suggests unidentified

interneurons require *fru*^{M} for proper pulse song execution and patterning of pulse structure, but not for pulse timing.

Introduction

A major challenge of neuroscience is to understand how complex, stereotyped behaviors are generated by the nervous system. The behaviorally reproducible and stereotyped behavior of male *Drosophila melanogaster* courtship along with amenability of its genetics make it a useful model in this regard (Dickson 2008; Villella & Hall 2008). A complete understanding of the neural basis of this complex behavior requires an understanding of how the nervous system integrates sensory information to generate behaviors in a stereotyped sequential pattern, as well as an understanding of how these motor behaviors are properly encoded and executed. While a history of ongoing research focuses on the initiation and activation of these behaviors (Datta et al 2008; Dickson 2008; Kimura et al 2008), progress in understanding the motor networks themselves lags behind, with notable exceptions (Clyne & Miesenböck 2008).

The male's courtship of female *Drosophila* is composed of a stereotyped sequence of behaviors including following, abdomen tapping, singing, and attempting copulation (Hall 1994). During courtship song, he extends one wing and vibrates it as he follows and circles the female. While it is generally accepted that *Drosophila* courtship song has derived from the flight system (Crossley 1990), it is unclear to what extent the components of courtship song (pulse song and sine song) versus flight are evoked by differential recruitment of shared but distributed networks or a common network with output differentiated by modulation, activation threshold, or

temporal pattern (Barnes et al 1998; Briggman & Kristan 2008; Dickinson 2006; Ewing 1977). The fact that some – but not all – flight mutants exhibit courtship song defects suggests an incomplete overlap of elements controlling flight and song (Barnes et al 1998; Villella et al 1997).

Rhythmic, sustained wing motion (flight and song) is controlled both by neurogenic muscles controlling wing posture acting directly on the wing base and myogenic muscles providing the bulk of flight power indirectly by resonant longitudinal and dorso-ventral distortion of the entire thorax “box”. Myogram recordings during song production revealed that both muscle groups receive phase-locked inputs during pulse and sine song, with more strict phase-locking during pulse song (Ewing 1977; 1979a). This phase-locking is due to proprioceptive wing feedback upon direct flight muscle motor neurons (Ewing 1979b; Tauber & Eberl 2001) and reciprocal inhibition among indirect flight muscle motor neurons (Ewing 1977; Harcombe & Wyman 1977; Levine 1973).

Although the genetic control of song has been extensively studied (Gleason 2005), the neural circuitry encoding this behavior remains elusive. Investigations of genetic gynandromorphs have identified several sexually dimorphic centers in the nervous system controlling courtship behavior: the dorsal brain must be male to initiate song and its prerequisite, wing extension (Hall 1977), while the thoracic ganglia must be male to generate the song motor pattern (von Schilcher & Hall 1979). The requirement of male thoracic ganglia (von Schilcher & Hall 1979) and the abnormal song produced by females artificially induced to sing (Clyne & Miesenböck 2008) suggests that some thoracic song components are sex-specifically organized. A thoracic circuit controlling song patterning is consistent with the cricket wing song pattern generator (Bentley 1977; Bentley & Hoy 1974; Huber et al 1989).

Specification of sexually dimorphic courtship behavior is dependent on the sex-determination genes *fruitless* (*fru*) and *doublesex* (*dsx*). Expression of a particular male-specific *fru* transcript (fru^M) has been shown to be both necessary and sufficient to initiate many of the courtship behaviors (Baker et al 2001; Demir & Dickson 2005; Manoli et al 2005), but the nervous system also requires sex-specific *dsx* function to establish the network properly (Kimura et al 2008; Rideout et al 2007; Villella & Hall 1996). Furthermore, genetic mosaic studies identified a small cluster of neurons in the dorsal brain region critical for courtship whose sexual dimorphism is mediated by fru^M and *dsx* (Kimura et al 2008). This small population of fru^M neurons in the brain appears to gate activation of a poorly understood circuit encoding the patterning of courtship song in the thoracic ganglia (Clyne & Miesenböck 2008; Konopka et al 1996; von Schilcher & Hall 1979). Alleles that eliminate fru^M function in the entire nervous system eliminate or drastically reduce courtship song production (Villella et al 1997), but fru^M itself is not sufficient for song initiation (Rideout et al 2007). Proper courtship song patterns are dependent on male tissue in the elusive song circuit (von Schilcher & Hall 1979), and ectopic fru^M expression in decaptitated females preparations artificially induced to sing resulted in pulse structure more like that of wild type males (Clyne & Miesenböck 2008). However, pulse timing was not affected in these fru^M-expressing female preparations.

Courtship song is an evolutionarily rapidly diverging trait nearly ubiquitous among species in the genus *Drosophila* (Markow & O'Grady 2005). In *D. melanogaster*, courtship song consists of a pulse component (“pulse song”), consisting of a train of discrete, single pulses, and a sinusoidal (125-200 Hz) component (“sine song”) as seen in flight, but slower. Each pulse is

composed of one to several cycles, separated by a species-specific interpulse interval (IPI) (Bennet-Clark 1971) which itself oscillates in a species-specific manner (Kyriacou & Hall 1980). In many species females use the patterning of IPIs as the primary species recognition signal in song (Kyriacou et al 1992; Kyriacou & Hall 1982; 1986) and is critical to maximizing a male's chance of copulation (Bennet-Clark & Ewing 1969; Kyriacou & Hall 1982; 1986).

Despite a growing knowledge of the neural network organizing courtship behavior (Dickson 2008; Manoli et al 2006), remarkably little is known about the song circuit itself (Villella & Hall 2008). As fru^M is expressed in neuronal populations from sensory neurons to motor neurons, investigations of its role in song using loss-of-function mutants perturb or alter fru^M function at all levels. This precludes direct analysis of the song network. It has been proposed that fru^M function is important at all levels of the nervous system related to courtship (Baker et al 2001), but there has been little evidence of this assertion to date (Clyne & Miesenböck 2008). We investigated the putative courtship song patterning circuit of the thorax selectively by using the *teashirt* (*tsh*) gene, which is responsible for specifying thoracic and abdominal segments (Röder et al 1992). The transcription factor *tsh* also functions in delineation of the thoracic-labial border (Dezulueta et al 1994), establishment of domains along the proximo-distal axis of the developing wing and leg via *wingless* and *nubbin* (Zirin & Mann 2007), as well as specification of the eye (Singh et al 2002). Because of its critical role in trunk development, *tsh* is widely expressed in the thoracic ganglia, and *tsh* expression persists into adulthood, but is only sparsely expressed in the brain. We used the tsh^{GAL4} allele to express GAL4 in a *tsh*-expressing cell-specific pattern (Brand & Perrimon 1993; Duffy 2002), which cell-specifically activates a UAS promoter.

We feminized the nervous system in this *tsh* pattern using transgenes to express *transformer* (*tra*), which controls *fru*'s sex-specific regulation (UAS-tra, Ferveur et al 1995), or to express a of fru^M-RNAi construct that eliminates fru^M expression (UAS-fruMIR, Manoli & Baker 2004). We sought to determine if sex-determination genes, such as *tra* and *fru*, underlie the sex-specific organization of the song circuit by specifically manipulating the thoracic song circuit in intact males.

Materials and Methods

Flies

Flies were maintained at 25° C in 12:12 LD conditions. All stocks used in tsh^{GAL4} (Calleja et al 1996) experiments were outcrossed into a common isogenic CS *w* background. All UAS-fru^M*IR* flies contain a UAS insert on the second and third chromosome (Manoli & Baker 2004). The $nsyb^{GAL80}$ line drives expression of GAL80, a GAL4 inhibitor, under the control of the *neuronal synaptobrevin* (*nsyb*) pan-neuronal promoter. The UAS-fru^M*IR* stocks were generously provided by Bruce Baker (Manoli & Baker 2004), and the $nsyb^{GAL80}$ stock and was a generous gift from Julie Simpson (Janelia Farm Research Campus). All stocks other than $nsyb^{GAL80}$ and UAS-fru^M*IR* were obtained from the Drosophila stock center (Bloomington, IN, USA). The motor neuron driver, D42 (Gustafson & Boulianne 1996), the chordotonal driver, ato^{GAL4} (Hassan et al 2000), and the proximal wing base driver, 30A (Brand & Perrimon 1993) have been previously described.

Behavioral assays and song analysis

Courtship observations consisted of a focal 5-6 day old male and a 3-5 day old CS virgin subject female, both isolated within 6 hours of eclosion.

Individual flies were aspirated into a 10 × 6 mm cylindrical chamber with a plexiglass ceiling and a fine mesh copper flooring mounted over a heat block held at 25° C. Acoustic recordings were made using a calibrated velocity sensitive microphone (Cator et al 2009; Tauber & Eberl 2001) beneath the chamber. Courtship and songs were recorded using digitally sampled audio (48 kHz) and video (standard NTSC). Recordings were made for five minutes or until successful copulation, whichever occurred first. The frequency response of the auditory apparatus was flat within 2 dB up to 4 kHz. Recordings were high-pass filtered at 50 Hz, anti-alias filtered, and resampled at 4 kHz, and passed through an integrating filter to compensate for the frequency-amplitude relationship of particle velocity measurements.

Courtship index (CI) and wing extension index (WEI), the proportion of time spent courting and extending a wing, respectively, were calculated from video. A wing extension (Figure 1) began with the promotion of the wing and ended with the retraction of the wing to resting position or initiation of another wing promotion. Pulses were initially detected through thresholding and confirmed through inspection of audio and video (wing extension) records, and pulse times were logged as time of midpoint of total energy within the pulse. Pulse trains were defined as at least 3 pulses separated by no more than 60 ms (Wheeler et al 1988). Pulse width was determined by finding the smallest window necessary to encompass 90% of the pulse energy. Sine song was detected as sinusoidal hums coinciding with unilateral wing extension (only hums longer than 100 ms were scored). Calculation of sound particle velocity levels (SPVL) used a standard reference of 50 nm s^{-1}.

Immunocytochemistry

The rat anti-Fru^M antibody, kindly provided by Bruce Baker (Janelia Farm Research Campus), targets the male-specific 101-amino acid sequence at the N-terminus of the peptide (Lee et al 2000). Within 6 hours of eclosion, adult w; tsh^{GAL4}, UAS-mCD8-GFP/ CyO male central nervous systems were dissected out and fixed in 3.5% paraformaldehyde, incubated in 1:300 α-Fru^M overnight, and incubated in 1:1000 TRITC-conjugated goat anti-rat (Jackson ImmunoResearch, West Grove, PA, USA) for two hours. Preparations were viewed with a TCS SP2 Leica confocal microscope system.

Flight assays

Flight ability was measured in an assay adapted from Drummond et al. (1991). Males were aged from 3 - 5 days and released on a platform in the center of an open-topped cylinder 45 cm wide and 54 cm high with a light source at the top. Flies were recorded as having landed on the bottom, landed on the side, or flown above the top of the cylinder. If no flight was initiated within 30 seconds, the fly was reapplied to the platform. Flies that never left platform after at least five trials were discounted.

Results

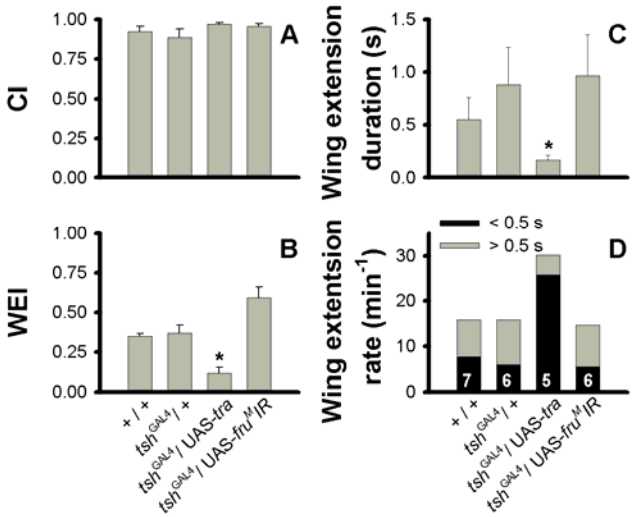
Wing extension

We utilized the trunk-specific expression of *teashirt* (*tsh*) to specifically manipulate gene expression in this area, allowing us to investigate the putative song patterning circuit. Driving expression of GAL4 in tsh^+ neurons in turn drove expression of genes downstream of a UAS sequence. We first investigated wing extension, a prerequisite step to producing courtship song. Video analysis of courtship trials showed that the feminizing construct UAS-*tra*

driven by tsh^{GAL4} had no detectable effect on courtship intensity measured by proportion of time spent courting (CI), compared to wild-type control males (+) or control males carrying *tsh*^{GAL4} alone (tsh^{GAL4}/+) (Figure 2.1A). During courtship, however, *tsh*^{GAL4}/UAS-*tra* males showed a significant decrement in (1) proportion of time spent extending a wing toward the female (wing extension index, WEI) as compared to tsh^{GAL4}/+ controls ($p < 0.05$, Figure 2.1B) as well as (2) median wing extension duration (ANOVA, $p< 0.0005$, t-test, $p<0.0005$; Figure 2.1C). Furthermore, these *tsh*^{GAL4}/UAS-*tra* males display an unusual wing extension profile consisting of many rapid wing extensions too fast to be measured with standard video. *tsh*^{GAL4}/UAS-*tra* males showed more rapid wing extensions and fewer sustained wing extensions (defined as being shorter or longer than 0.5 s, respectively) than *tsh*^{GAL4}/+ controls (Figure 2.1D).

Since the lack of wing extension exhibited by *tsh*^{GAL4}/UAS-*tra* males precluded courtship song production, we asked if elimination of *fru*^{M}, a downstream target of *tra* in the sex-determination hierarchy, would more selectively produce defective song that could be analyzed for its defects. We utilized an RNAi construct directed at *fru*^{M} transcripts (UAS-*fru*^{M}*IR*) to reduce *fru*^{M} expression (Manoli & Baker 2004) in a *tsh*-specific pattern. As observed in *tsh*^{GAL4}/UAS-*tra* males, males carrying both *tsh*^{GAL4} and UAS-*fru*^{M}*IR* showed no defect in CI (Figure 2.1A). However, unlike tsh^{GAL4}/UAS-*tra* males, measurements of wing extension revealed no differences between *tsh*^{GAL4}/UAS-*fru*^{M}*IR* males and controls (Figure 2.1B-D). We therefore continued our study of courtship song utilizing the UAS-*fru*^{M}*IR* transgene.

Figure 2.1: Measurements of wing extension behavior. (A) No differences in courtship index (CI) were observed among genotypes (mean ± s.e.m.). (B) *tsh*^{GAL4}/UAS-*tra* flies had a significantly decreased median wing extension duration compared to *tsh*^{GAL4}/+ controls ($p < 0.05$), while *tsh*^{GAL4}/UAS-*fru*^{M}*IR* males were no different than controls (mean ± s.e.m.). (C) Mean of a fly's median wing extension duration. *tsh*^{GAL4}/UAS-*tra* males display significantly shorter wing extensions than *tsh*^{GAL4}/+ controls (ANOVA, $p < 0.0005$, t-test, $p < 0.0005$, mean ± s.e.m.). (D) Wing extension frequency, separating extensions shorter and longer than 0.5 s. tsh^{GAL4}/UAS-*tra* flies are not different in frequency of total wing extensions (ANOVA, $p = 0.053$), but exhibit more frequent wing extensions shorter than 0.5 s, and less frequent wing extensions longer than 0.5 s compared to *tsh*^{GAL4}/+ controls (ANOVA, $p < 0.05$, t-test, $p < 0.05$). There is no difference between *tsh*^{GAL4}/UAS-*fru*^{M}*IR* males and *tsh*^{GAL4}/+ control males. Sample size indicated within bars in (D).


CI
1.00
0.75
0.50
0.25
0.00
A
WEI
B
Wing extension duration (s)
1.5
1.0
0.5
0.0
C
Wing extentsion rate (min-1)
30
20
10
0
< 0.5 s
> 0.5 s
D
7
6
5
6
+ / +
tsh GAL4 / +
tsh GAL4 / UAS-tra
tsh GAL4 / UAS-fruM IR

fru^{M} *RNAi reduces amount courtship song*

Utilization of the UAS-*fru^{M}IR* transgene to express RNAi directed at fru^{M} in tsh-expressing neurons produced a strong phenotype of reduced courtship song. All control flies included in this analysis exhibited pulse song, but 42% (5/12) of *tsh^{GAL4}*/UAS-*fru^{M}IR* males exhibited no detectable pulse song (Figure 2.2A) despite vigorous courtship (Figure 2.1A). Only those tsh^{GAL4}/UAS-*fru^{M}IR* males exhibiting song were included in further analysis. We found that *tsh^{GAL4}*/UAS-*fru^{M}IR* males sang at a significantly lower rate measured by pulse trains per minute (Figure 2.2B), as compared to *tsh^{GAL4}*/+ control males ($p < 0.001$, ANOVA: $p < 0.001$). *tsh^{GAL4}*/UAS-*fru^{M}IR* males also exhibited fewer pulses per pulse train than control flies ($p < 0.05$, ANOVA: $p < 0.0005$, Figure 2.2C). The high proportion of *tsh^{GAL4}*/UAS-*fru^{M}IR* males that did not produce song or sang at a decreased rate indicates that expression of *fru^{M}IR* in a tsh-specific pattern disrupts execution of pulse song.

To ensure that the reduction in courtship song had a neural basis rather than being due to broad *tsh* expression across thoracic tissues, we included another transgene expressing the GAL4 inhibitor GAL80 in a pan-neuronal, *n-synaptobrevin* (*nsyb*) pattern. Song deficits were fully rescued in *tsh^{GAL4}*/UAS-*fru^{M}IR* ; *nsyb^{GAL80}* males (Figure 2.2), confirming that the phenotype is indeed neuronal.

fru^{M} *RNAi disrupts song structure*

Representative data show that pulse song typically consists of a train of many pulses, each consisting of one or two cycles (Figure 2.3A-B). The *tsh^{GAL4}*/UAS-*fru^{M}IR* males are characterized by several defects, often exhibiting decreased amplitudes (Figure 2.3C) and a polycyclic phenotype (Figure 2.3D), in which extra cycles are present before and after the peak.

Figure 2.2: Expression of *fru*^{M}*IR* in *tsh*-specific pattern reduces amount of courtship song. Proportion of flies that produced audible output classified as pulse song (A). Only *tsh*^{GAL4}/UAS-*fru*^{M}*IR* males failed to produce courtship song. *tsh*^{GAL4}/UAS-*fru*^{M}*IR* also males exhibited fewer pulse trains per minute (B) and pulses per train (C) than *tsh*^{GAL4}/+ controls, while *tsh*^{GAL4}/UAS-*fru*^{M}*IR*;*nsyb*^{GAL80} males were no different than controls. n = 6 – 11.

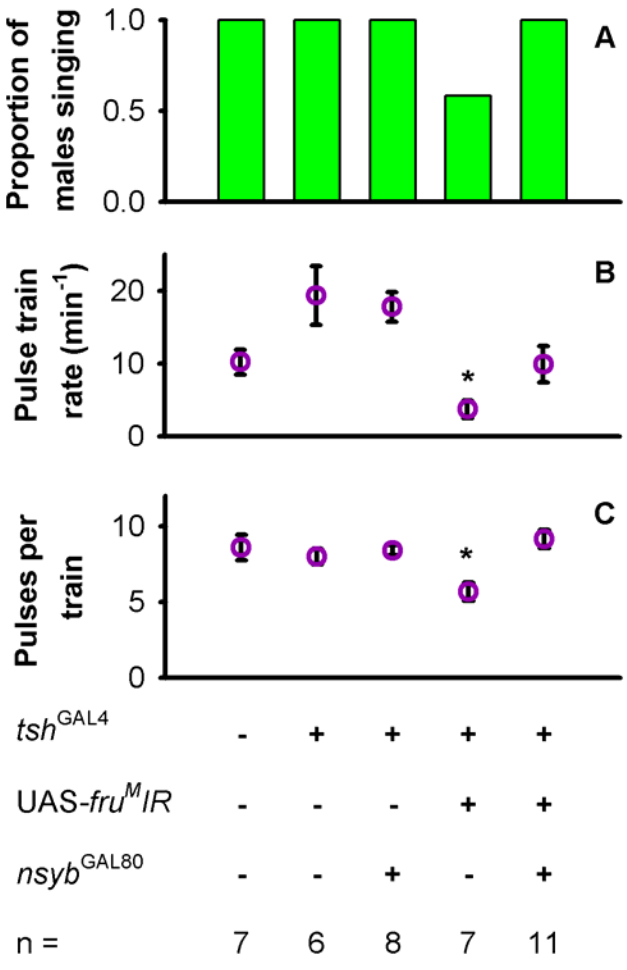
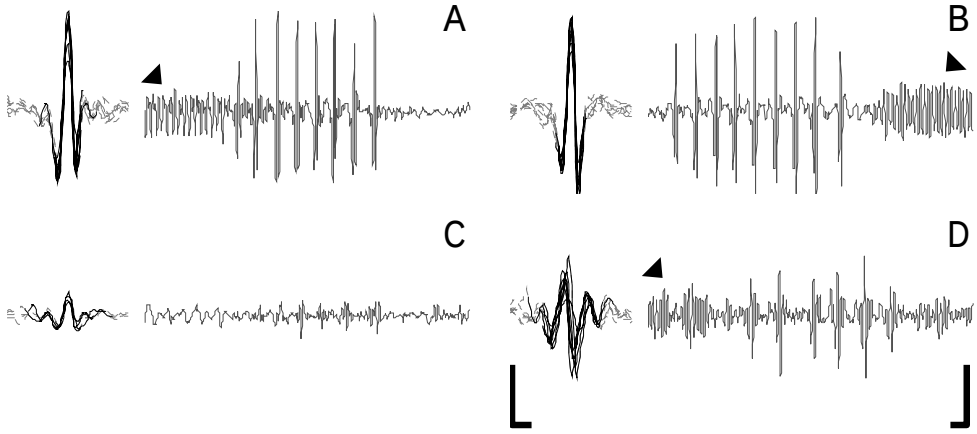
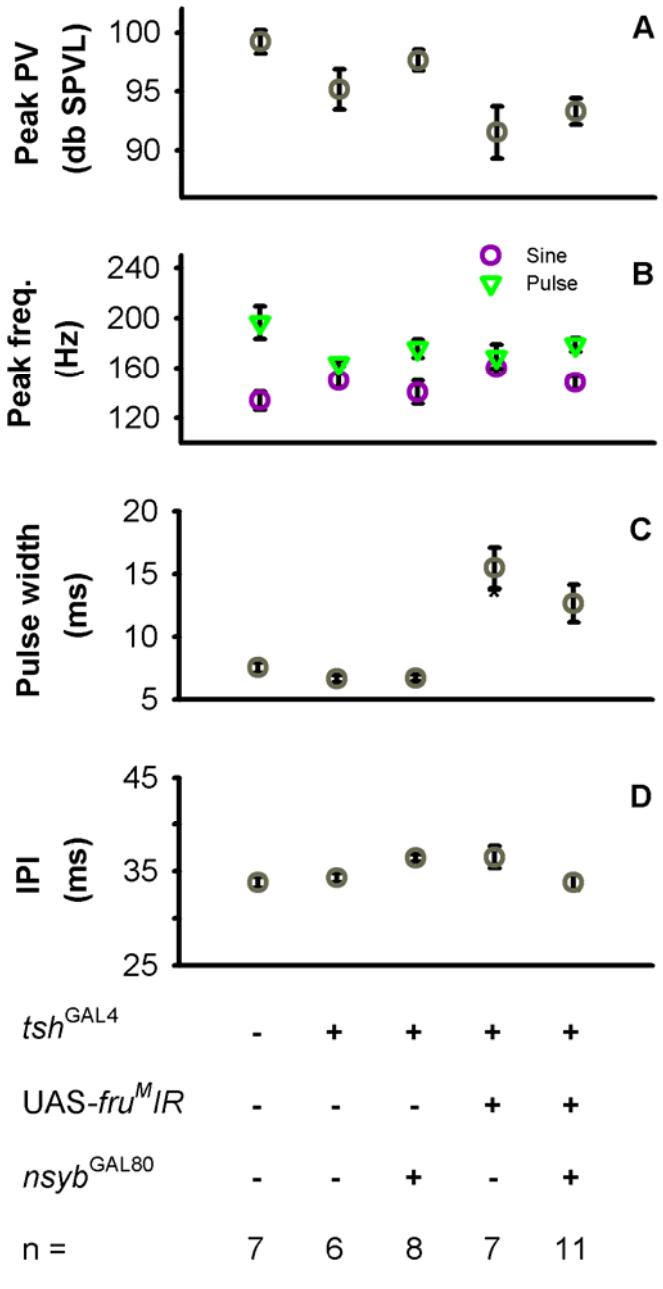

Proportion of males singing
1.0
0.5
0.0
A
Pulse train rate (min-1)
20
10
0
B
*
Pulses per train
10
5
0
C
*
tshGAL4 - + + + +
UAS-fruMIR - - - + +
nsybGAL80 - - + - +
n = 7 6 8 7 11

Figure 2.3: Representative traces of courtship song output. The left panel displays individual pulses within a pulse train aligned by midpoint of energy. Solid lines indicate amount of trace required to include 90% of the signal's energy (pulse width). Right panel displays the whole trace. Arrowheads indicate sine song. (A) tsh^{GAL4}/+ controls, (B) tsh^{GAL4}/UAS-$fru^{M}IR$; $nsyb^{GAL80}$ rescue flies, (C) representative small amplitude and (D) polycyclic nature of tsh^{GAL4}/UAS-$fru^{M}IR$ courtship song. Scale bars: Left panel, horizontal 5 ms, vertical 5 mm s^{-1}. Right panel, horizontal 25 ms, vertical 5 mm s^{-1}.


A
B
C
D

Also, some *tsh*^{GAL4}/UAS-*fru*^{M}*IR* males display wing generated output coinciding with unilateral wing extension that was not classifiable as either pulse or sine song (data not shown). Since these wing generated outputs are correlated with courtship behavior, yet do not share the tonal properties of sine song or the rapid amplitude modulation of pulse song, these were taken to be failed courtship song attempts, and were classified as neither pulse nor sine song. Wild-type control males exhibited a peak pulse particle velocity of 99.2 ± 1.0 db SPVL (re: 50 nm s^{-1}, mean ± SE). Pulse amplitudes from *tsh*^{GAL4}/UAS *fru*^{M}*IR* males showed a non-significant trend towards reduction of amplitude compared to tsh^{GAL4}/+ controls ($p = 0.0508$) and a significant decrease compared to wildtype controls ($p < 0.005$, ANOVA; $p < 0.005$; Figure 2.4A). Although *tsh*^{GAL4}/UAS-*fru*^{M}*IR* ; *nsyb*^{GAL80}/+ males showed a trend of increased amplitude compared to *tsh*^{GAL4}/UAS-*fru*^{M}*IR* males, these males also sang at a decreased amplitude compared to wild-type controls ($p < 0.05$), but not compared to tsh^{GAL4}/+ controls. Peak intrapulse frequencies and peak sine song frequencies were not found to differ among genotypes (Figure 2.4B). The similarity of *tsh*^{GAL4}/UAS-*fru*^{M}*IR* males mean pulse and sine peak frequencies should not be over-interpreted, as pulse and sine song frequency did not correlate in each fly examined, and intermale variation was quite high. Pulse widths were found to increase ($p < 0.005$) in *tsh*^{GAL4}/UAS-*fru*^{M}*IR* males as compared to *tsh*^{GAL4} and wildtype controls (Figure 2.4C). This phenotype was rescued in *tsh*^{GAL4}/UAS-*fru*^{M}*IR* ; *nsyb*^{GAL80}/+ males, which did not exhibit significantly increased pulse widths as compared to wildtype. Interestingly, males of *tsh*^{GAL4}/UAS-*fru*^{M}*IR* did not show a disrupted interpulse interval (IPI, Figure 2.4D), the most important feature of the courtship song.

Figure 2.4: Intrapulse data. (A) Peak particle velocity within a pulse is reported here as a mean of medians in dB SPVL, using 50 nm s^{-1} as a reference. There no significant differences were detected in dB SPVL, but there was a trend for a reduction in pulse amplitude for *tsh*^{GAL4}/UAS-*fru*^{M}*IR* males. (B) Peak frequency of sine songs (circle) and individual pulses (triangle). No significant differences were observed. (C) Pulse widths from *tsh*^{GAL4}/UAS-*fru*^{M}*IR* males were significantly wider compared to *tsh*^{GAL4}/+ controls. This increased pulse width is rescued in *tsh*^{GAL4}/UAS-*fru*^{M}*IR*; *nsyb*^{GAL80} males. (D) Mean interpusle interval (IPI) was unaffected by genotype.


Peak PV (db SPVL)
100
95
90
A
Peak freq. (Hz)
240
200
160
120
Sine
Pulse
B
Pulse width (ms)
20
15
10
5
C
IPI (ms)
45
35
25
D
tsh^GAL4 - + + + +
UAS-fru^M IR - - - + +
nsyb^GAL80 - - + - +
n = 7 6 8 7 11

Histology

To identify the specific regions of the nervous system affected by the *tsh*^{GAL4}/UAS-*fru*^{M}*IR* genotype, we analyzed the *tsh*^{GAL4} expression pattern in the CNS of recently emerged males using a membrane-bound GFP reporter gene (mCD8-GFP) and determined its corresponding overlap with Fru^{M} immunoreactivity. Expression of *tsh*^{GAL4}-driven mCD8-GFP was very limited in the brain (occasionally absent) with no observed overlap with Fru^{M} immunoreactivity (Figure 2.5). In contrast, *tsh*^{GAL4} driven mCD8-GFP was widely expressed throughout the somata and neuropils of the ventral ganglia, including all neuromeres (Figure 2.6A). No obvious sexual dimorphisms were detected in GFP labeled neurons (data not shown), but the extensive labeling by *tsh*^{GAL4} makes definitive analysis difficult. Colocalization of GFP and Fru^{M} immunoreactivity was observed in all five previously described (Lee et al 2000) groups of Fru^{M} ventral ganglia neurons (Figure 2.6B-F). The most extensive colocalization of Fru^{M} and *tsh*^{GAL4}-driven GFP expression was observed at the anterior margin of the ventral mesothoracic neuromere (Figure 2.6C). Although *tsh*^{GAL4} expression was observed in somata of widely varying size, Fru^{M} immunoreactivity was only detected in those *tsh*^{GAL4} expressing neurons with small somata (~ 5 μm in diameter). The somata of direct flight muscle motoneurons (DFMns) are known to be located in the anterior mesothoracic region of the ventral ganglion (Trimarchi & Schneiderman 1994), raising the possibility that DFMns require *fru*^{M} for proper courtship song functioning. However, we did not observe co-localization of GFP and Fru^{M} immunoreactivity in neurons with larger somata characteristic of motor neurons (≥ 10 μm).

Figure 2.5. Adult male w ; *tsh*^{GAL4}, UAS-mCD8-GFP/CyO (green) stained with an anti-Fru^{M} antibody (magenta). Little or no GFP expression is visible in the brain (br), while strong expression is visible in the ventral ganglia (vg). Anterior direction is indicated.

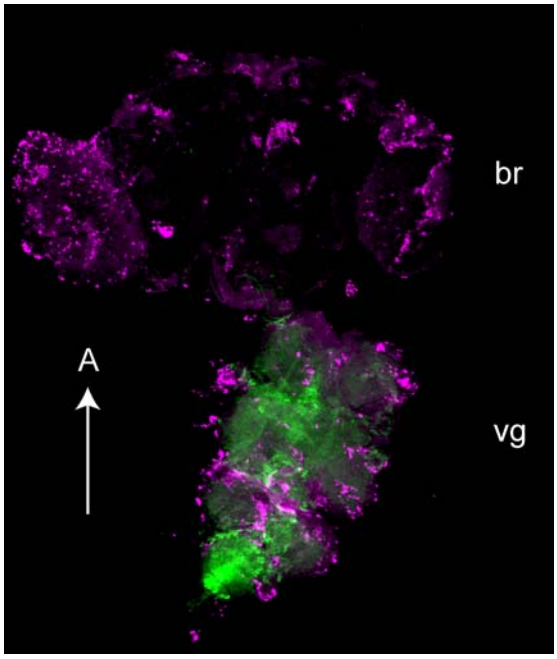
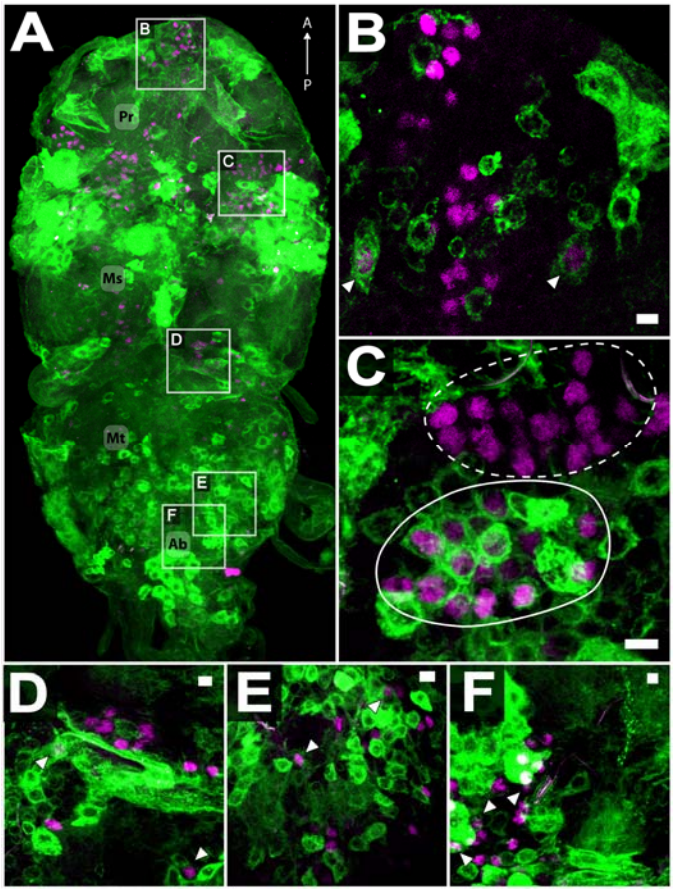

br
A
vg

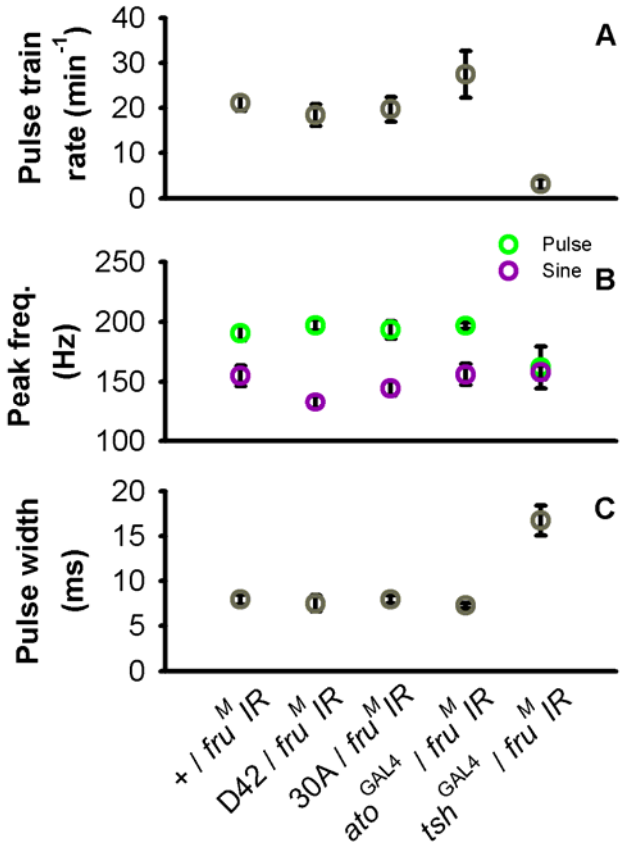
Figure 2.6: Immunocytochemistry of adult w ; *tsh*^{GAL4}, UAS-mCD8-GFP/CyO CNS, visualizing endogenous, membrane bound GFP (green) and Fru^{M} immunoreactivity (magenta). (A) Dorsal-ventral view of adult ventral ganglia. Extensive labeling is visible in prothoracic (Pr), mesothoracic (Ms), metathoracic (Mt), and abdominal (Ab) segments. Anterior-posterior axis is indicated. (B-F) 3 – 5 μm representative sections of the five groups of *fru*^{M} neurons in the ventral ganglia, according to (Lee et al 2000). Fru^{M} neural cluster 16 (B), 17 (C), 18 (D), 19 (E), and 20 (F). Arrowheads indicate examples of neurons coexpressing Fru^{M} and mCD8-GFP. In (C), a Fru^{M}-expressing cluster clearly coexpresses GFP (solid line), while an adjacent Fru^{M} cluster does not (dashed line). Scale bars (B-F) represent 5 μm.


A
B
A
P
Pr
C
Ms
D
Mt
E
F
Ab
B
C
D
E
F

Elimination of fru^{M} *in motor neurons and wing sensory neurons.*

Because motor neurons and proprioceptive sensory neurons are known to be critical members of pattern generating networks (Ewing 1979b; Harcombe & Wyman 1977; Levine 1973), we asked if the *tsh*^{GAL4}/*fru*^{M}*IR* phenotype could be phenocopied by expressing *fru*^{M}*IR* in one of these two groups. We first looked at motor neurons by driving *fru*^{M}*IR* with the motor neuron GAL4 driver D42 (Gustafson & Boulianne 1996). Since power and timing of sound pulses is provided by the direct and indirect flight muscles (Ewing 1977; 1979a), we hypothesized that *fru*^{M} activity in the motor neurons that innervate these muscles may be critical for proper song production. We eliminated expression of *fru*^{M} in motor neurons by driving *fru*^{M}*IR* with D42^{GAL4}, which is expressed in direct and indirect flight muscle motor neurons (Gustafson & Boulianne 1996; Usui-Aoki et al 2000). No differences between D42^{GAL4}/UAS-*fru*^{M}*IR* and UAS-*fru*^{M}*IR* controls were observed (Figure 2.7). This is consistent with another motor neuron GAL4 driver, P103.3 (Consoulas et al 2002), in that P103.3^{GAL4}/UAS-*fru*^{M}*IR* males also exhibited no detectable song defects (n = 4, data not shown). As motor neurons receive wing proprioceptive input entraining the song pattern and some sensory organs at base of the wing are known to express *fru*^{M} (Manoli et al 2005), we hypothesized that *fru*^{M} function in the sensory organs themselves may be necessary for proper courtship song production. The *ato*^{GAL4} construct drives expression in proprioceptive organs (Hassan et al 2000), but exhibited no detectable effect on courtship song when driving UAS-*fru*^{M}*IR* (Figure 2.7). Similarly, driving expression of UAS-*fru*^{M}*IR* with 30A, a GAL4 driver that is expressed at the

Figure 2.7: Courtship song is unaffected by driving *fru*^{M}*IR* in motor neurons and sensory neurons. No significant effects of genotype were found on (A) pulse rate, (B) pulse and sine song peak frequency, or (C) pulse width compared to UAS-*fru*^{M}*IR* controls. The *tsh*^{GAL4}/UAS-*fru*^{M}*IR* mutant phenotype is replotted from Fig. 2 and Fig. 4 for comparison. n = 6 - 8.


Pulse train rate (min-1)
40
30
20
10
0
A
Peak freq. (Hz)
250
200
150
100
Pulse
Sine
B
Pulse width (ms)
20
15
10
5
0
C
+ / fru M IR
D42 / fru M IR
30A / fru M IR
ato GAL4 / fru M IR
tsh GAL4 / fru M IR

presumptive wing base of the imaginal disc (including precursors for the wing proprioceptive organs) (Brand & Perrimon 1993) does not produce a detectable courtship song phenotype (Figure 2.7).

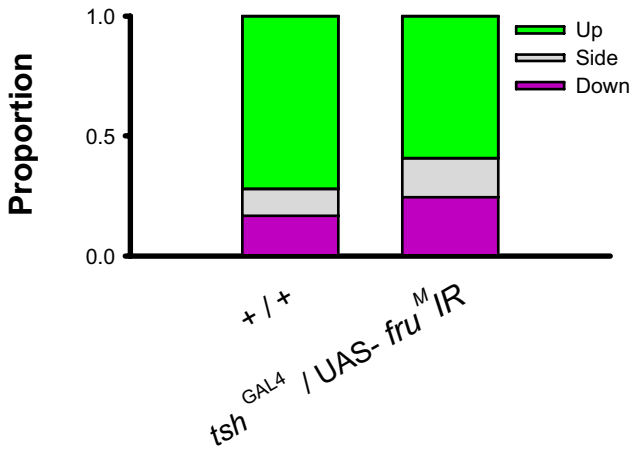
Flight assays

Because flight and courtship song use overlapping motor components but only courtship song behavior is sexually dimorphic, we tested the flight ability of males exhibiting courtship song defects. In an assay adapted from Drummond et al. (1991), males were released in the center of a large cylinder, allowed to fly freely, and their landing site was recorded. Flies that never left the platform after four 30 second trials were excluded from analysis (1 of 20 wild-type controls and 4 of 41 *tsh*^{GAL4}/UAS-*fru*^{M}*IR* males). In total, 73% of wild-type control males (n = 19) flew over the top of the cylinder, compared to 60% of *tsh*^{GAL4}/UAS-*fru*^{M}*IR* males (n = 37), where as 17% of wild-type control males landed on the bottom, compared to 24% of *tsh*^{GAL4}/UAS-*fru*^{M}*IR* males (Figure 2.8). No significant differences in flight ability were observed (Wilcoxon rank-sum).

Discussion

Although the role of sex-determination genes in the initiation of courtship behaviors is well studied, very little is known about what role these genes play in the neuronal circuits directly controlling these behaviors. Previous studies of *fru*^{M} mutants exhibiting aberrant courtship song (Ryner et al 1996; Villella et al 1997) were ambiguous as to whether *fru*^{M} is needed during the initiation of courtship song (i.e., the brain) or the execution of courtship song (i.e., the thoracic ganglia). Our results indicate *fru*^{M} function is

Figure 2.8: Flight ability of *tsh*^{GAL4}/UAS-*fru*^{M}*IR* males. Males were placed in the center of a cylinder and allowed to freely fly. Landing sites were recorded as the bottom (magenta), side (yellow), or out of the cylinder (green). Control + / + males (n = 19) were not significantly than *tsh*^{GAL4} / UAS-*fru*^{M}*IR* males (n = 37) in landing location (Wilcoxon rank-sum).


Proportion
1.0
0.5
0.0
Up
Side
Down
+ / +
tsh GAL4 / UAS- fru M IR

necessary in the song circuit itself for normal courtship song execution. To our knowledge, this direct perturbation of the song circuit is the first in an intact, freely behaving animal. Courtship motor circuits have been specifically manipulated in a decapitated preparation (Clyne & Miesenböck 2008). The inability of motor neuron and wing sensory neuron GAL4 drivers to phenocopy *tsh*^{GAL4}/UAS-*fru*^{M}*IR* suggests that the *fru*^{M} requirement is restricted to interneurons, perhaps along the anterior mesothoracic segment, where many neurons affected in this study are found.

Despite the strong disruption of courtship song by elimination of *fru*^{M} in *tsh*$^{+}$ neurons, the most critical song parameter, interpulse interval (IPI), is unaffected. The robustness of this attribute in song patterning reflects the importance it carries in species-recognition and female receptivity to copulation throughout *Drosophila* (Bennet-Clark et al 1976; Markow & O'Grady 2005; Talyn & Dowse 2004). Villella et al. (1997) have shown that males mutant for *fru*^{M} have an extended mean IPI. The data presented here provides the hypothesis that either this dependence of IPI on *fru*^{M} is not found in the thorax (rather, in the brain) or this dependence is conferred by other *fru*^{M}, non-tsh^{+} thoracic neurons. The finding that, in a decapitated fly induced to sing by photoexcitation of *fru*^{M} neurons, the presence of *fru*^{M} has no bearing on IPI (Clyne & Miesenböck 2008) provides evidence in favor of the former. Furthermore, the role of the circadian rhythm gene, *period*, on the cycling of IPIs is conferred in the thoracic ganglia (Konopka et al 1996). Thus, the mean IPI might be regulated by the brain, perhaps by tonic drive, while the thoracic ganglia might be responsible for instilling the oscillation of IPI (a stronger descending excitation resulting in shorter mean IPI). The long IPIs of all flies photoinduced to sing (Clyne & Miesenböck 2008) would then argue that this

effect is an artifact of the photoactivation assay, such that photoactivation provides less circuit activation than endogenous excitation.

The *fru*^{M} song phenotype could be exerting its affect in any one of the four conceivable song circuit components: descending inputs, motor neurons, proprioceptive neurons, and local interneurons. The selective expression of *tsh* in the thorax and abdomen eliminates the role of descending brain interneurons in this *fru*^{M}-dependent song phenotype. Invertebrate flight motor neurons integrate sensory afferents and related motor neuron efferents, thus are critical for flight pattern generation (Harcombe & Wyman 1977; Levine 1973). However, elimination of *fru*^{M} in motor neurons using D42^{GAL4} did not result in a detectable phenotype, consistent with *fru*^{M}'s lack of an effect on innervation patterns of direct flight muscles (Rideout et al 2007). Wing sensory neurons limit the number of cycles per pulse and ensure short pulse durations, as sensory information entrains elements dampening the wing vibration (Ewing 1979b; Tauber & Eberl 2001). However, eliminating *fru*^{M} in proprioceptive organs using *ato*^{GAL4} and at the developing wing base using 30A^{GAL4} had no detectable effect on courtship song, including pulse duration. Taken together, our results argue against a motor neuron, sensory neuron, or descending interneuron courtship song requirement for *fru*^{M}, suggesting local interneurons have a sex-specific *fru*^{M} requirement to properly assemble the song patterning circuit.

The wing extension phenotype observed upon feminization of *tsh*^{GAL4} neurons provides evidence that *tra*-dependent sex-specification of *tsh*-expressing neurons is critical for extending a wing long enough to sing. This thoracic execution requirement is in addition to the previously identified initiation requirement of male tissue in the dorsal brain (Hall 1977).

Alternatively, *tra* expression may result in a subtle wing cuticle or muscle phenotype in males, as *tsh* is important for determining the proximal wing domain (Zirin & Mann 2007). In either case, the normal wing extensions observed in *tsh*^{GAL4}/UAS-*fru*^{M}*IR* males suggest *fru*^{M}, which is downstream of *tra* in the sex-determination pathway, is not responsible for the wing extension phenotype, emphasizing the role that other downstream genes (e.g. *doublesex*) may have in the organization and function of the courtship network (Rideout et al 2007).

Mechanisms of fru^{M} *function in the song circuit*

Thoracic *fru*^{M} function may control wiring of the song circuit by controlling the song circuit's connection to the descending command system or by sculpting the connections among song circuit components themselves. In the former case, a *fru*^{M} "identity" signal may be required in the song circuit to receive projections from the *fru*^{M}-expressing song command system in the brain. Expression of *fru*^{M} has been demonstrated in several cases to induce particular neurons to produce a *fru*^{M}-specific projection absent when *fru*^{M} is eliminated (Datta et al 2008; Kimura et al 2008). Song circuits not expressing *fru*^{M} would not receive descending excitation (or insufficient descending excitation), resulting in aberrant courtship song. This is consistent with the fact that females lacking *fru*^{M} can be artificially induced to sing, but will not sing naturally (Clyne & Miesenböck 2008). In the latter case, *fru*^{M} would be required for the song patterning circuit itself to be wired properly. This would also explain the thoracic requirement of *fru*^{M} reported here, but would require an interpretation of the results of Clyne and Miesenböck (2008) that although females can be photoinduced to sing, the song circuit in females is not fully assembled, instead producing song output by tonically activating all elements

of the circuit via photoexcitation rather than through endogenous excitatory connections to a limited set of circuit elements. As pattern generating circuits rely on patterns emerging from communication within the circuit, the latter explanation seems unlikely. Although we have focused on fru^M's ability to mediate sex-specific differences in neuronal projection patterns (Datta et al 2008; Kimura et al 2008; Kimura et al 2005), it is important to note that studies investigating a neurophysiological role for fru^M neurons are extremely limited (Datta et al 2008), so should also be considered.

The neuromuscular mechanism producing pulse song is still unclear, but one hypothesis is that pulse timing is achieved by direct, timed inputs onto an unidentified direct flight muscle (see Introduction) that moves the wing and distorts the thorax to trigger a contraction of a power-delivering fibrillar muscle (Ewing 1977). Lagging sensory input via proprioceptive feedback (Ewing 1979b; Reddy et al 1997; Tauber & Eberl 2001) dampens the ensuing antagonistic indirect flight muscle contraction directly by activating opposing direct flight muscles or indirectly by releasing tension in the thoracic box to prevent its stretch-activation. In this model, the decreased amplitude of tsh^{GAL4}/UAS-fru^M*IR* pulse song suggests that indirect flight muscles may not be sufficiently recruited in the absence of fru^M. The increased pulse width suggests that dampening may not occur properly.

Although neurons co-expressing *tsh* and fru^M are widely distributed throughout the thoracic ganglia, a large cluster of co-expressing neurons is located ventrally along the anterior margin of the mesothoracic segment. This region has been speculated to be important in courtship song production (Rideout et al 2007; von Schilcher & Hall 1979), particularly since dsx^M and fru^M are highly co-expressed in this region, both of these factors are important

in proper song production (Rideout et al 2007), and this region is responsible for control of wing movement. These *fru*^{M}-dependent song circuit neurons may overlap with the pool of sexually dimorphic neurons previously identified (Rideout et al 2007).

Quantification of the rapid wing extension rate in *tsh*^{GAL4}/UAS-*tra* males (> 15Hz) may provide a useful tool in identification of muscles responsible for courtship song generation. The use of standard video here precluded quantification of the wing extension rate, but high-speed video may reveal the rapid wing extension rate corresponds to pulse song IPI, thus, they may be subserved by similar etiologies. As wing extensor motor units have been shown to be activated during the generation of each pulse (Ewing 1979a), the rapid wing extension rate observed here may be underlied by an aberrant attempt to produce pulse song in a fly without an extended wing, and, more generally, may be sufficient to drive rapid wing extension rate at a rate comparable to the IPI.

Evolution of courtship song

The use of IPI in *Drosophila* courtship song is quite conserved across the genus (Crossley 1990; Markow & O'Grady 2005; Ritchie & Gleason 1995). The sequence of the *fruitless* is also relatively well conserved throughout *Drosophila* (Davis et al 2000; Gailey et al 2000). We speculate that the ancestral age of the neural mechanism responsible for setting the IPI may rival that of *fru*'s role in the song circuit (Davis et al 2000; Gailey et al 2000; Hoy et al 1988). Perhaps *fru*^{M} elements sex-specifically controlling courtship song were overlaid upon a pre-existing, non-sex-specific flight apparatus, which already possessed the machinery to control IPI. If such a flight pre-adaptation is true, a possible explanation of the evolution of the song circuit begins with a

non-sex-specific flight circuit, containing command neurons pre-adapted to send information encoding IPI. Then, fru^M may have exploited the IPI preadaptation to produce the sex-specific secondary sexual characteristic of courtship song by recruiting new interneurons to the circuit as rapid radiation of the *Drosophila* genus ensued. The lack of an observable effect on flight ability is consistent with the subtle flight defect detected in only some fru^M mutants (Villella et al 1997).

Courtship song intensity

To our knowledge, our recordings are the first calibrated measure of acoustic output of a courting male fly reported in the literature. The mean sound particle velocity level (SPVL) of a wild-type male sound pulse as measured here is 99.2 ± 1.0 db SPVL (re: 50 nm s^{-1}, mean ± s.e.m.). As males were allowed to freely move around the chamber while singing at distances from 1 – 6 mm from -- and unrestricted angles to -- the microphone, a standardized output intensity is confounded by distance and directional effects, as well as complex near-field propagation physics. Nonetheless, this figure corresponds well to the predictive figure calculated by Bennet-Clark (1971) of 95 dB at 5 mm in front of the courting male. The near-field dipteran auditory organ, the arista, attenuates the vibration amplitude at 166 Hz by approximately only 2.5 dB (Gopfert & Robert 2002), thus it is obviously sensitive enough to detect sounds we measured.

This report provides evidence that fru^M function is critical in organizing the nervous system to perform highly patterned behaviors specific to courtship. However, fru^M does not affect the most important feature of that pattern, IPI. Identification of neurons critical to patterning the song in a fru^M-

dependent matter will greatly further our understanding of how complex behaviors are produced by the nervous system.

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CHAPTER 3

COURTSHIP SONG PRODUCTION AND AUDITORY RECEPTION IN HAWAIIAN *DROSOPHILA* OF THE PLANATIBIA SUBGROUP

Summary

Interspecific differences in courtship behavior are thought to be major drivers of speciation within Drosophila. These interspecific differences in courtship behavior extend to courtship song production, leading to tremendous variation in courtship song. In one species, *D. heteroneura*, acoustic signals are generated during male-female courtship as well as male-male aggression. Characteristics of these signals are very similar and are believed to be generated by a common mechanism which has been generalized to be used in both social contexts. Also, acoustic sensitivity of two Hawaiian planitibia species, *D. heteroneura* and *D. silvestris*, was assessed neurophysiologically. Despite the near ubiquity of courtship song, it was unknown whether Drosophila auditory receptors were sensitive to these signals. Acoustic sensitivity of the Johnston's organ extends to bandwidths including the carrier frequency of wing-generated courtship song.

Introduction

Acoustic signals are commonly found in courtship of *Drosophila* species (Markow & O'Grady 2005), and the species rich group of Hawaiian *Drosophila* are no exception (Hoy et al 1988). One particular clade of Hawaiian *Drosophila*, the planitibia group exhibit a diverse array of courtship songs (Hoikkala & Kaneshiro 1997; Hoikkala et al 1994). This group consists of approximately 100 species found throughout the islands, but a particularly

interesting pair of lekking sibling species are found in sympatry on the main island, Hawaii. The sympatric species *D. heteroneura* and *D. silvestris* exhibit strong premating behavioral isolation not seen in with other allopatric planitibia species (Ahearn et al 1974; Boake et al 2000; Kaneshiro 1976), suggesting sexual selection has acted to reduce copulations between these species that are likely to come in contact with one another in the wild. Although acoustic signals are important in Drosophila courtship, the full breadth of signals exchanged between males and females during courtship is highly multimodal.

In addition to male-female sexual selection, strong male-male sexual selection is found in these species (Hoikkala et al 1994), as the head-to-head interactions seen in *D. heteroneura* aggressive bouts drive selection for the “hammer head” anatomy characteristic of males of this species (Boake et al 1997).

Although wing generated courtship song is the most common mechanism of acoustic courtship signal production, evolutionary innovations using other sound-generating mechanisms have arisen in Hawaiian *Drosophila*. High-frequency clicks in *D. fasciculisetae* appear to be produced directly by thoracic flight muscles or a novel cuticular structure on the wing base (Hoikkala & Moro 2000; Hoy et al 1988).

Another Hawaiian innovation that will be treated further here is a variation on sound pulses common to those seen in continental *Drosophila*, but are produced through an alternative mechanism. In *D. silvestris* and *D. heteroneura*, abdominal vibrations originating at the dorsal anterior of the thorax produce these pulses rather than wing vibrations observed in other species (Hoikkala et al 1994; Hoy et al 1988). These abdominal pulses are in addition to wing-generated sound bursts during courtship.

The first portion of this chapter describes thoracically generated pulse trains of *D. heteroneura*, one of the two elements in the acoustic repertoire of this species, during bouts courtship and aggressive behavior. While only a limited data set is provided, these phenomena have not been previously reported. Unfortunately, populations of many Hawaiian *Drosophila* species, especially *D. heteroneura*, are undergoing massive crashes as a result of feral introductions of food competitors and predators (Boake 2005), so this report serves to ensure this data is available in some form.

Although a great variety of acoustic signals is found in Hawaiian *Drosophila*, it is not known whether these species can detect these airborne acoustic signals. Diptera transduce airborne acoustic vibrations, including courtship signals, with the antennal arista, which vibrates like a sail with the bulk movements of the air medium (Bennet-Clark 1971; Ewing 1978; Gopfert & Robert 2002). The arista in turn drives rotation of the third antennal segment with respect to the second to stimulate the Johnston's organ, a sensitive chordotonal organ. It is common among insects to match sensitivity of the acoustic receptor or its afferents to conspecific signals (Bennet-Clark 1971; Hoy & Robert 1996). However, it is unclear if the strong premating isolation exhibited between *D. heteroneura* and *D. silvestris* is due to differences in acoustic sensitivities correlating with the subtle differences in conspecific signals previously observed during wing-generated sound bursts. These differences are 232.5 Hz and 192.5 Hz for *D. heteroneura* and *D. silvestris*, respectively in one study (Hoikkala et al 1994) and 90-222 Hz and 203-250 Hz in another study (Hoikkala & Welbergen 1995). The second portion of this chapter examines the sensitivity of the primary auditory receptor in these species using multiunit electrophysiology.

Materials and methods

Two species of the planitibia subgroup of Hawaiian *Drosophila*, *D. silvestris* and *D. heteroneura*, were generously provided by Ken Kaneshiro (University of Hawaii, HI).

Acoustic and seismic behavior recording

Courtship behavior assays included a male and conspecific female in a 9 cm behavior arena. Aggressive behavior assays included two conspecfic males in a 2.5 cm arena. Records of *D. heteroneura* courtship and aggression behavior were made by recording video, auditory, and seismic (modified phonograph pickup) signals in separate channels. Auditory signals were recorded using a Brüel & Kjær (Nærum, Denmark) Type 4135 pressure microphone and a Type 5935 preamplifier. Seismic signals were recorded by removing a piezo transducer from a consumer-grade phonograph cartridge and placing it in contact with the behavior arena substrate. These signals were then amplified using a high impedance amplifier. Subsequent analysis of acoustic signals was performed with software described in the preceding chapter, which provided a temporal record of acoustic pulses. The interpulse intervals (IPIs) were recorded for further analysis. Data presented here consist of analysis of a single observation of courtship and aggression, but pulse seismic pulse song production was routinely detected in courtship and aggression (n = 6 and 5, respectively), however these other records are no longer available.

Sensory physiology

Johnston's organ auditory physiology was carried out in females of *D. heteroneura* and *D. silvestris*. Flies were mounted in a modified 200 μL pipette tip (Eberl et al 2000) and placed 1.25 cm away from a 2cm opening of a cone

mounted over a calibrated speaker. Acoustic stimuli were generated in Matlab with a National Instruments digital acquisitions interface, attenuated with a PA4 programmable attenuator (Tucker-Davis Technologies), and the amplified signal drove the calibrated speaker. The stimulus consisted of a 700 ms sine wave modulated with a 200 ms attack and decay. Carrier frequencies of stimuli ranged from 110 – 279 Hz, corresponding to the frequencies of wing vibrations during courtship and aggression in *D. heteroneura* and *D. silvestris* (Hoikkala et al 1994). Each frequency was presented in three to five trials, and over a 26 dB range of intensity levels. Stimulus amplitude is reported here as dB SPVL (standard particle velocity level), using a standard particle velocity reference of 5×10^{-8} m/s, calibrated with a Brüel & Kjær (Nærum, Denmark) Type 4135 pressure microphone. The tungsten recording electrode was placed at the base of the first antennal segment and the reference electrode was placed in the eye. The multiunit, auditory neurophysiological responses were high-pass filtered at 100 Hz, amplified in an A-M Systems AC filter, and acquired using a National Instruments data acquisitions system. Using a sacrificed preparation as a control for stimulus artifact resulted in no detectable signals. The response of the auditory afferents (rms_{resp}) was quantified by subtracting the root mean square of the activity immediately preceding the stimulus presentation (rms_{pre}) from the root mean square of the activity during the stimulus presentation (rms_{stim}). For the isointensity response analysis, rms_{resp} across frequencies was normalized to the maximal rms_{resp} within each fly. For the threshold response analysis, the lowest intensity stimulus yielding a response (rms_{resp}) significantly different from zero was recorded as the auditory threshold.

Results

Courtship and aggression signals in D. heteroneura

Pulse trains were detectable during courtship and aggression by measuring both acoustic and seismic vibration signals. Acoustic signals were processed for further analysis (Figure 3.1). The temporal structure of both courtship and aggression song are quite similar. Measuring interpulse intervals (IPIs) from a 25 s sample of courtship song and aggression song, IPIs for courtship were 58.0 ± 1.3 ms (mean ± s.e.m., n = 175), compared to 61.0 ± 1.1 ms (n = 249) for aggression. The variance of IPIs was also similar in courtship and aggression (variance: 0.263 ms and 0.264 ms, respectively). Medians of IPIs deviated more (54.1 ms for courtship and 61.2 ms for aggression), suggesting the distribution of IPIs might differ among behavioral contexts. IPI histograms show that IPIs produced during courtship are more clustered towards short IPI durations, where as IPIs produced during aggression are more normally distributed around the median (Figure 3.2).

The differences in pulse structure recorded in these single behavioral measurements of courtship song and aggression song are evident in Figure 3.3A and Figure 3.3B. Pulses recorded during courtship have a longer duration and routinely consist of multiple cycles, each cycle approximately 5 ms long. Pulses recorded during aggression are shorter and consist of a single cycle, approximately 2 ms long. Further, the frequency content of pulses produced during courtship and aggression is different (Figure 3.3C), although in both the spectral energy is concentrated over a relatively narrow band. The mean peak frequency of each pulse is significantly different ($p < 0.005$) and is 325.4 Hz for courtship and 550.6 Hz for aggression (Figure 3.3D) for the bout of courtship and aggression song measured here.

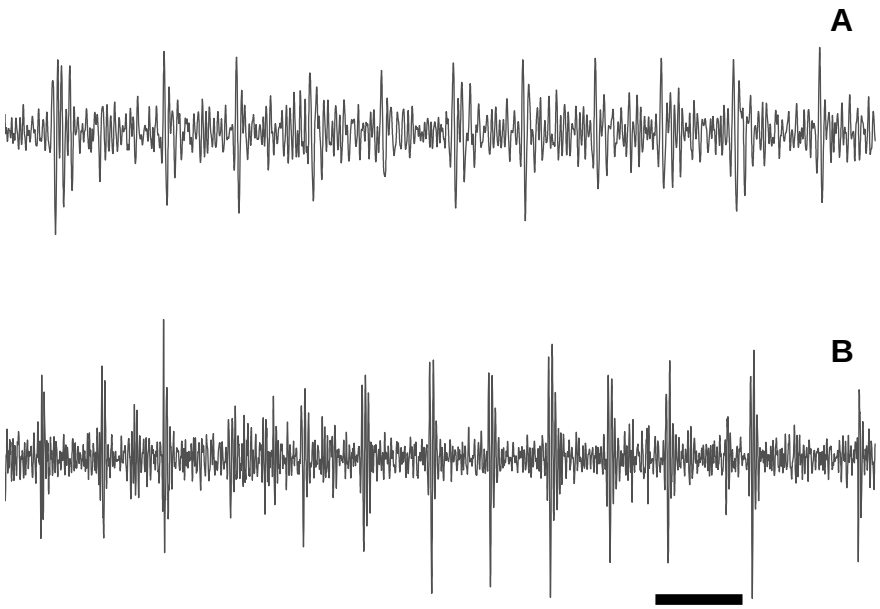


Figure 3.1: Sample traces of acoustic signals during *D. heteroneura* courtship (A) and aggression (B). Scale bar represents 50 ms.

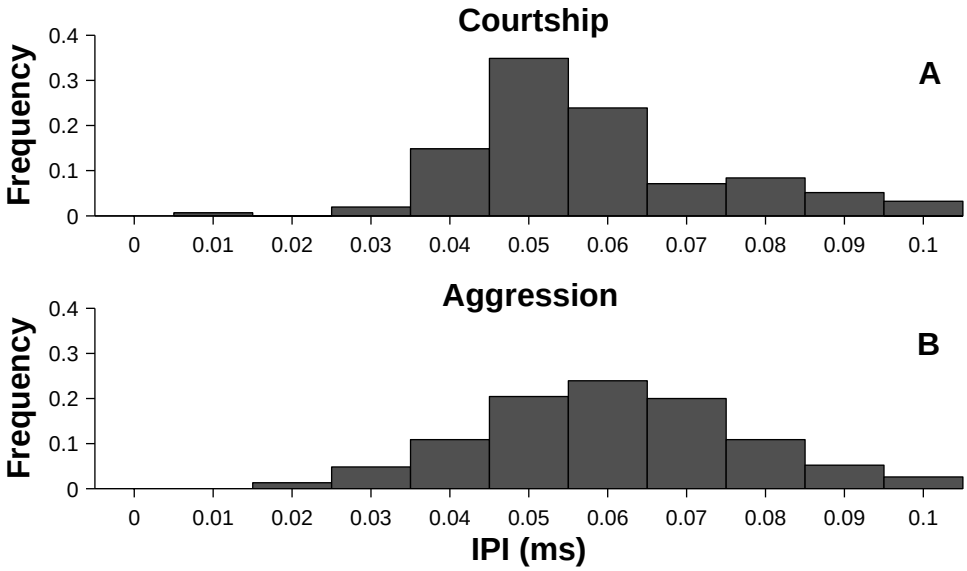
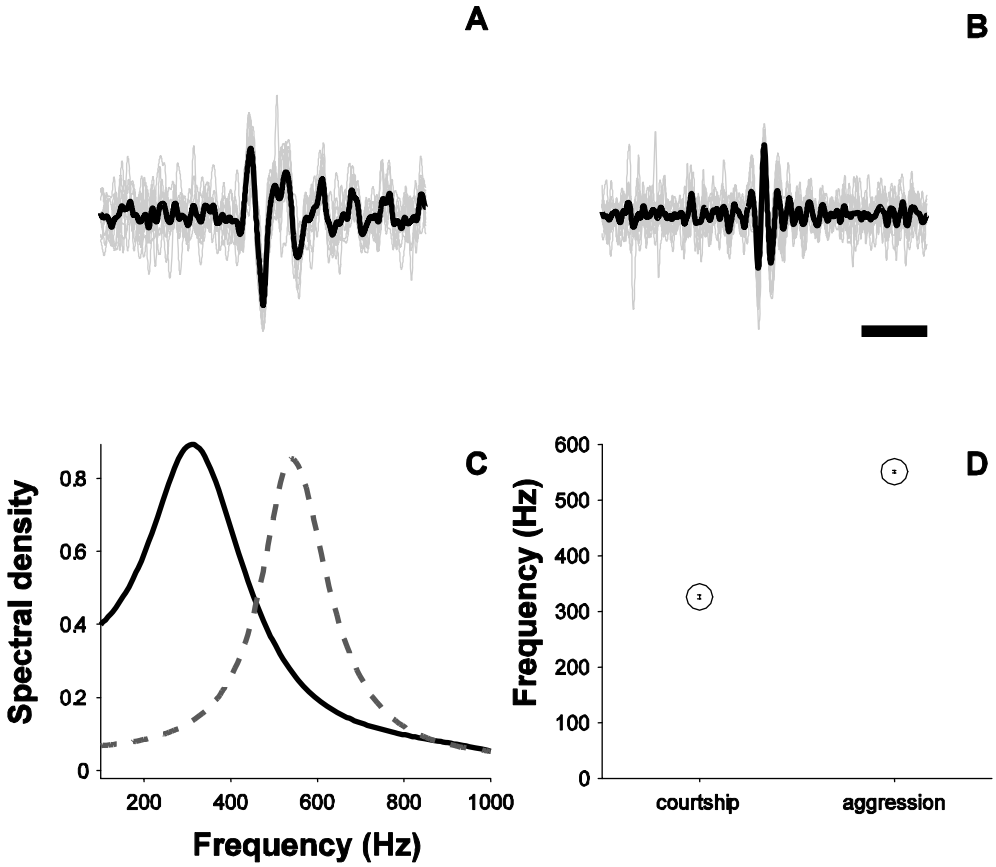


Figure 3.2: IPI histograms during D. heteroneura courtship (A) and aggression (B). IPIs produced during courtship show positive skew and are more tightly clustered near the mode, whereas IPIs produced during aggression are less skewed and more evenly dispersed on either side of the mode.

Figure 3.3: Pulse characteristics of *D. heteroneura* courtship and aggression song. Sample traces of individual pulses from courtship (A) and aggression (B) song. Panels (A) and (B) illustrate 10 consecutively produce sample pulses in gray and the mean pulse trace in black. Scale bar represents 10 ms. (C) Mean frequency content of pulses from courtship (solid black) and aggression (dashed gray) songs. (D) Frequency of maximal energy content for all pulses from the bout of courtship and aggression measured, mean ± s.e.m. (n = 192 and n = 149 for courtship and aggression, respectively).


A
B
C
D
Spectral density
0
0.2
0.4
0.6
0.8
200
400
600
800
1000
Frequency (Hz)
Frequency (Hz)
0
100
200
300
400
500
600
courtship
aggression

Auditory physiology of D. heteroneura *and* D. silvestris

Multiunit recordings from the antennal Johnston's organ were made for females of two Hawaiian planitibia species, *Drosophila heteroneura* and *D. silvestris*. Auditory sensitivities were assayed for a limited bandwidth containing wing-generated auditory signals produced during courtship and aggression (Hoikkala et al 1994). Auditory afferents responded to isointensity stimuli of 40 dB SPVL pure tones broadly across the bandwidth measured here (Figure 3.4). The maximal response magnitude appears around 200 Hz, with a gradual attenuation of the response on either side. Auditory responses of *D. heteroneura* and *D. silvestris* females are largely similar and exhibit no experiment-wide significant differences. Auditory threshold data show the peak sensitivity appears to be lower than 100 Hz, with a gradual roll-off of sensitivity as frequency increases (Figure 3.5). According to calibrations performed here, the auditory organ of *D. heteroneura* and *D. silvestris* is quite sensitive, down to 13 dB SPVL at 100 Hz and 27 dB SPVL at 280 Hz.

Discussion

Song production

D. heteroneura acoustic courtship song data presented here provide a glimpse of *D. heteroneura* courtship and aggressive behavior not yet reported in the literature. A more thorough study of *D. heteroneura* courtship and aggression signals would be welcomed, provided the endangered *D. heteroneura* species perseveres long enough for such an analysis.

The *D. heteroneura* courtship IPI values here are comparable to previously reported values (Hoikkala & Welbergen 1995). The difference in distribution of IPIs between courtship and aggressive contexts suggests

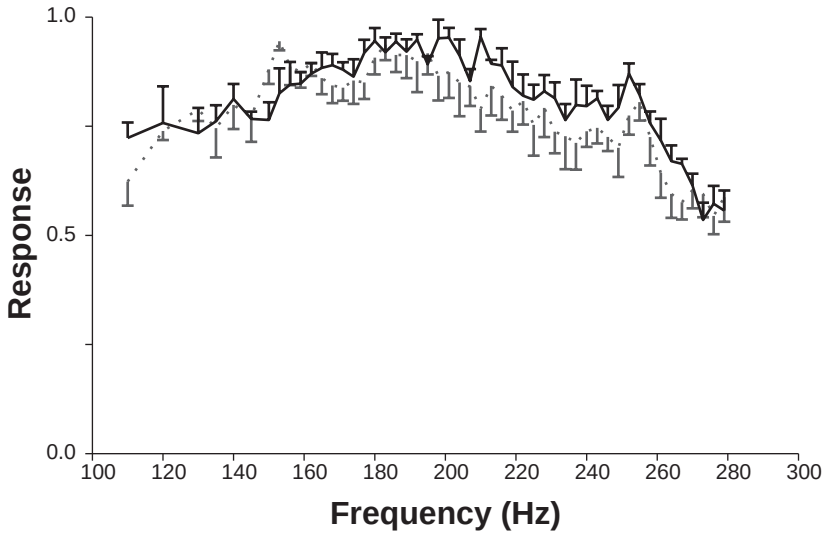
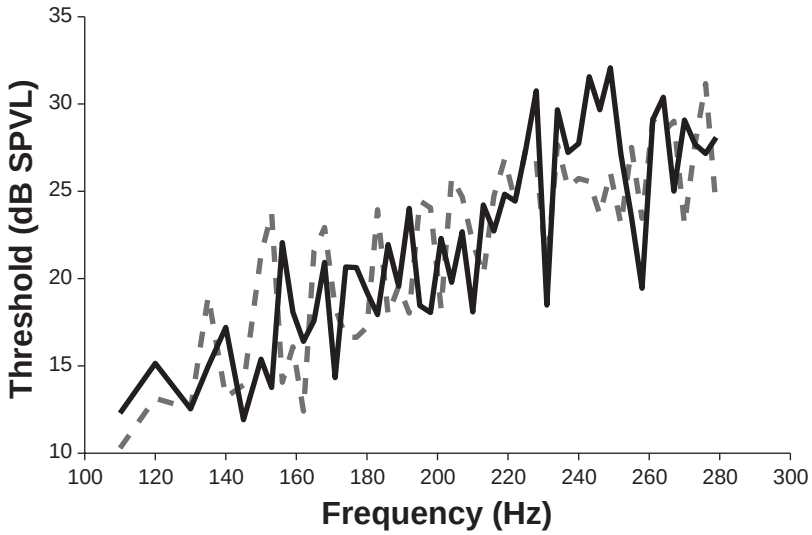


Figure 3.4: Auditory physiology responses of *D. heteroneura* (solid black) and *D. silvestris* (dotted gray) to an isointensity stimulus of 40 dB SPL. Normalized response magnitudes are very similar for both species. Auditory afferents are apparently maximally responsive at about 200 Hz and are sensitive to frequency bandwidths above and below those tested here (n = 4, mean ± s.e.m.).

Figure 3.5: Auditory afferent response thresholds of *D. heteroneura* (solid black) and *D. silvestris* (dashed gray). Both species exhibit similar response thresholds. The peak sensitivity appears to be lower than 110 Hz. The sensitivity of the female near-field auditory organ suggests sufficient sensitivity to detect courtship song signals.


Threshold (dB SPVL)
35
30
25
20
15
10
100
120
140
160
180
200
220
240
260
280
300
Frequency (Hz)

differing selective pressures may be exerted. It is common among Drosophila courtship song to IPI distributions like that seen in D. heteroneura. It is believed there is a selective pressure in many species towards shorter IPIs, as artificial selection acts more readily when selecting for longer IPIs and most isolated song mutants of *D. melanogaster* result in longer IPIs (Gleason 2005). Since, it is known that females exert stabilizing selection towards a species-specific template IPI, the selection for shorter IPIs may then be due to natural selection or some other aspect of courtship behavior. Since the same IPI distribution is not seen in aggressive bouts, it does suggest females may prefer shorter IPIs within a general species-specific IPI range, while other males do not respond to this signal during an aggressive context. Of course, the possibility of a false positive due to insufficient sample size cannot be excluded.

D. heteroneura pulses produced during courtship song were significantly lower in their carrier frequency than those produced during aggression. However, in *D. melanogaster*, the carrier frequency of courtship song pulses is not as important as temporal features like IPI (Bennet-Clark et al 1976; Bennet-Clark & Ewing 1969), so it is unclear if this spectral difference is functional or is merely an epiphenomenon. The existence of the spectral difference suggests that abdominal postural muscles are differentially activated during courtship and aggression. The higher frequency of abdominal pulses noted during aggression (and the apparent shorter pulse width) suggests more tension is present between abdominal sclerites during aggression song. This feature might reflect a more active posture required for other aspects of aggression behavior. Regardless, the common pulse shape and reasonable proximity of the carrier frequencies of courtship and

aggression song do not suggest these signals are produced by separate mechanisms.

In a classic lek, females observe displaying males. In Hawaiian *Drosophila*, females may be observing not only courtship displays, but may be eavesdropping on aggressive displays and their outcomes. In terms of the evolution pulse song in aggression, courtship behavior, including song, was a pre-existing sensory channel by which males communicated to females. It may have been generalized here to communicate among males (Stiles 1982), allowing females to eavesdrop on the conspicuous results of these competitions. In most *D. melanogaster* species, males try to prevent courtship song signals from reaching other males by optimizing wing angles and utilizing a low courtship song intensity, as it results in a corresponding increase in courtship from the eavesdropping male (Bennet-Clark 1971). However, in this case courtship song-like signals are directed towards males, albeit with a different function than that seen in *D. melanogaster*.

Auditory reception

Recordings from *D. heteroneura* and *D. silvestris* auditory receptor afferents suggest that they are suited to detect airborne signals generated during courtship, as opposed to detecting these signals seismically. This does not necessarily mean the airborne component is more salient than the seismic component. Auditory afferents are sensitive enough to detect both wing and abdominally generated courtship song based on measurements reported and by others (Hoikkala et al 1994; Hoy et al 1988). However, the limited bandwidth of acoustic sensitivity measured here makes it difficult to asses whether they are sensitive to aggression signals, as these frequencies were higher than the bandwidth measured. But recent approaches to study acoustic

sensitivity of the Johnston's organ in a mosquito (Cator et al 2009) reveal that Johnston's organs can be sensitive to frequencies far exceeding that seen in pulses during aggression song.

Future investigators who may wish to study Hawaiian auditory reception may choose a wider bandwidth, as the frequency of maximal sensitivity was not determined here. Further, the sensitivity of the Johnston's organs reported here is in line with previously published reports of mosquito behavior response thresholds (Wishart & Riordan 1959). The data presented here indicate that the sensitivity of the Johnston's organ decreases as the stimulus frequency increases from 110 Hz to 280 Hz. No detectable interspecies differences were observed in auditory sensitivity. This is not altogether unexpected, as species-specific differences in wing vibratory signals are quite small, approximately 190 Hz and 220 Hz for *D. silvestris* and *D. heteroneura*, respectively (Hoikkala et al 1994). Also, it has been argued that species identification decisions are made early in the courtship ritual among planitibia species (Price & Boake 1995), and thus species-specific song may not be an important factor in premating isolation.

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CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

The foundation of a MARCM study to identify D. melanogaster *courtship song neurons requiring* fru^M

Introduction

Male *Drosophila* courtship behavior has emerged as an intriguing, tractable model of complex social behavior, with the ultimate goal of a full neurogenetic understanding of the behavior and the neural substrates underlying it. Yet, the wealth of information gleaned from the genetic tractability of the organism still awaits identification of a number of neural substrates responsible for conferring this behavior. The results of a number of studies reveal that in D. melanogaster, the sex determination genes are necessary to sculpt the central nervous system to enable males to court females. However, very little is known about the regions of the brain that underlie these behaviors.

Chromosomal sex mosaics have shown that the sexual identity of compartments of the central nervous system is critical for proper courtship behavior, with courtship initiation and early courtship behaviors dependent on the dorsal brain (Hall 1977) and courtship song patterning dependent on the thoracic ganglion identity (von Schilcher & Hall 1979). Genetic studies have focused on a number of sex-determination genes that have been shown to be crucial in endowing the male nervous system with the potential to court. The transcription factor *transformer* (*tra*) is expressed only in females and controls sex-specific splicing of *fruitless* (*fru*) and *doublesex* (*dsx*). The *fru* locus expresses a male-specific transcript (fru^M) that has been shown to be

necessary (Ryner et al 1996) and sufficient (Demir & Dickson 2005; Manoli et al 2005) to provide the potential for courtship behavior. However, *fru*^{M} also works in conjunction with *dsx* to specify the male nervous system (Kimura et al 2008; Rideout et al 2007), both controlling the differentiation and maintenance of a limited set of neurons during adult development.

Males mutant for *fru*^{M} show strongly disrupted courtship song (Villella et al 1997), and this deficit has been traced to the song circuit itself (Rubinstein et al., in preparation). While the neural circuit responsible for patterning courtship song is found in both sexes (Clyne & Miesenböck 2008), expression of *fru*^{M} in headless females photoinduced to sing improves the song's characteristics and its reception by other males. While a role for *fru*^{M} in courtship song patterning is clearly evident, it isn't clear where in the CNS this effect takes place.

Using MARCM analysis, the sexual dimorphism of a well-defined cluster of *fru*^{M} neurons in the dorsal posterior brain was shown to be dependent on *fru*^{M} and *dsx*, and this dimorphism was strongly correlated with male courtship behavior (Kimura et al 2008), implicating this neuronal population in making the decision to court.

Although there is inevitably considerable overlap between the flight and song circuit (Crossley 1990; Ewing 1977; 1979), members of the song circuit remain elusive. Previously, a driver derived from the *teashirt* gene (*tsh*^{GAL4}) has been shown to label the thoracic and abdominal ganglia specifically, including the courtship song circuit (Rubinstein et al., in preparation). We aim to combine the selective *tsh*^{GAL4} labeling of the thoracic ganglia with the stochastic labeling of the MARCM technique to reduce *fru*^{M} expression in random clusters of *tsh* neurons courtship song in *Drosophila melanogaster*.

The labeled neurons will then be correlated with courtship song production ability. We are currently generating MARCM males, assessing singing ability, and assessing labeled CNS neurons to identify neurons requiring fru^M for proper song.

Materials and methods

Flies. All stocks used to make the MARCM genotype were backcrossed to a common isogenic genetic background for six generations. MARCM males were of the genotype w, hs^{FLP} / Y ; tsh^{GAL4} , UAS-fru^M*IR* / UAS-mCD8-GFP ; FRT > tub^{GAL80} / FRT > UAS-fru^M*IR*. The UAS-fru^M*IR* construct was a generous gift from Bruce Baker (Genalia Farms), and all other stocks are available at the Bloomington stock center (Bloomington, IN).

Generation of MARCM flies. Mated females will be protein-starved the day before embryo collection to maximize egg-laying when returned to embryo collection vials. For MARCM males generated thus far, embryos were heat-shocked for 20 – 40 minutes 2-5 hours after egg laying. Embryonic neuroblast delamination occurs in several waves whose temporal pattern has been previously described (Bossing et al 1996). To maximize the number of labeled neuroblasts, future heat shock regimes will consist of one half hour shock applied at 3.5 hours and another at 6 hours after egg laying.

Behavior analysis. Courtship song was recorded as has been done previously (Rubinstein et al., in preparation). Briefly, males were individually isolated for 4-5 days after emergence and paired with a virgin female. Video and acoustic recordings of the courtship behavior were made. Since the aberrant song phenotype of tshGAL4/UAS-fru^M*IR* males is very strong (Rubinstein et al., in preparation), the courtship song was noted as either normal or aberrant/absent. Since no differences in singing ability among left and right

wings has been observed, consistent with the gynandromorph study (von Schilcher & Hall 1979), normal song produced from either wing results in a "normal" categorization for that fly.

Histological analysis. Immediately after courtship assays, individual males were placed on ice, their CNS was dissected out, fixed in paraformaldehyde, and imaged for mCD8-GFP expression pattern using confocal imaging. The presence or absence of mCD8-GFP signal (hence, reduction of fru^M expression) throughout the CNS is being categorized by a system previously used to categorize neurons expressing fru^M (Lee et al 2000), dividing the nervous system into 20 clusters, 15 in the brain, 5 in the ventral ganglia.

Projected sample size

Informal calculations indicate a given neural cluster is labeled 14% of the time when a heat shock coincides with a neuroblast's initiation of proliferation. This was determined by noting the frequency of labeling a particular conspicuous cluster at the anterior midline of the prothoracic neuromere. The courtship song gynandromorphy study revealed that male tissue in either hemisegment is sufficient for normal singing, so it is expected that fru^M will need to be eliminated in both hemisegments to induce the singing mutant phenotype. Using the above rate of neuroblast labeling, bilateral labeling of a given neuroblast will occur in approximately 2% of specimens. Combining this with the more comprehensive heat shock regime, analyzing 50 flies will theoretically result in bilateral labeling of all neuroblasts, producing a detectable phenotype regardless of the neuroblast responsible for fru^M courtship song phenotype. This projected mutant phenotype rate of 2% is less than the observed mutant phenotype rate so far, 8%. If the average of these rates is used, 1 in 20 flies will produce a mutant phenotype. Since behavioral

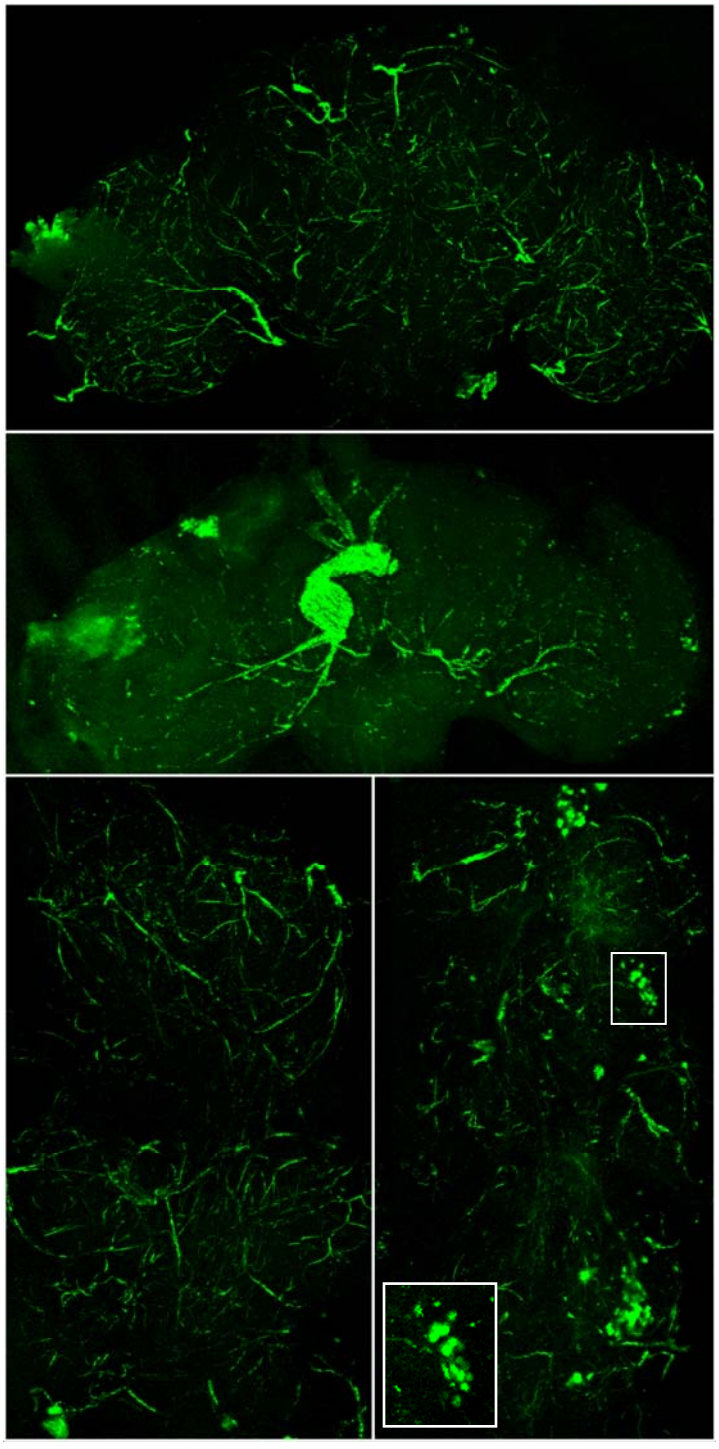
assays can be used to prescreen males for the necessity of their downstream, more time-consuming histological analysis, this will greatly aid in efficiency. If 100 males are screened for behavioral deficits (which can be done in 5 dedicated days), the five projected mutant singing males and thirty five normal singing males will be histologically processed.

Preliminary results

Since MARCM males carry *flp* under control of a heat-shock promoter, stochastic recombination and subsequent activation of GAL4 throughout embryonic development can be controlled with heat-shock regimes. Consistent with this temperature-dependent recombination, males carrying the full complement of MARCM constructs show no recombination and, subsequently, no GFP expression when embryos are not exposed to heat shock (Figure 1A,C). When heat-shocked, MARCM successfully labels only select lineages of *tsh* neurons (Figure 1B,D).

Song recordings will be combined with patterns of GFP expression to correlate neural regions responsible for the fru^{M} requirement in courtship song. Males not subjected to heat-shock exhibit wild-type courtship song (7/7) as expected. But males exposed to embryonic heat-shock varied in their courtship song. Thus far, 1 of 14 males exhibited abnormal courtship song. This is consistent with the 25% rate of aberrant song phenotype seen in a chromosomal gynandromorph study (von Schilcher & Hall 1979), considering labeling in either hemisegment was sufficient for a courtship song effect, unlike this study.

Figure 4.1: MARCM labeling of *tsh* neurons in the CNS of males not subjected to heat shock (A,C) and males subjected to heat shock (B,D). Trachea are highly visible in these preparations due to the age of the flies. No labeling is seen in the brain (A) or ventral ganglia (C) of males not subjected to heat shock. A representative sample of an embryonically heat shocked adult brain (B) and ventral ganglia (D). This animal exhibits limited expression in a neuronal cluster in the left anterior prothoracic segment (group 16), the left anterior mesothracic segment (group 17), and the left posterior margin of the metathoracic segment (group 19). The inset in (D) is an expansion of the boxed cluster of labeled neurons in the right anterior mesothoracic segment (group 17).



Utility of a pharmacological induction of courtship song

A pharmacological induction of courtship song would be a major advance in understanding the neurogenetics underlying the pattern produced by the song circuit pattern generator. Such a system would provide a means for mounting an intact male and activating the song circuit, while both measuring the acoustic output and monitoring neural activity, either electrophysiologically or optically (Datta et al 2008). Further, a pharmacological approach would likely not restrict experiments to *D. melanoaster*, as genetic and molecular studies of courtship song have done.

We have preliminary results inducing courtship song production using a pharmacological protocol to induce cricket calling song (Wenzel & Hedwig 1999). Application of the acetylcholine agonist, eserine, to the exposed *Drosophila* brain, elicited unilateral wing extension and vibration in one instance. This was not pursued, but remains a promising avenue. Another strategy, transgenic means of photoexcitation, has been successfully employed (Clyne & Miesenböck 2008) to elicit courtship song in headless *Drosophila*. This strategy could indeed be prove useful in a neurophysiological dissection of courtship song, especially using genetic tools to selectively limit the neurons receiving photoexcitation. However, this technique is confounded by the fact that, so far, the phenomenon only occurs in decapitated preparations, making studies of the intact nervous system difficult.

Hawaiian **Drosophila** *as a model for courtship initiation*

Much of the current research investigating the neurobiological basis behind courtship behavior focuses on the initiation of this behavior. For example, how are courtship-activating stimuli processed by sensory

processing and higher order brain regions to activate courtship behavior (Datta et al 2008; Kimura et al 2008)? More precisely, stimuli presented by the female are sufficient to induce courtship (most notably chemical stimuli) whereas stimuli presented by males are not sufficient, including the inhibitory male chemical profile (Ferveur et al 1997; Kurtovic et al 2007). How do these differences in social stimuli result in differential activation of motor patterns. For male *D. melanogaster,* the social responses to males and females are very distinct. In proper conditions, male-male social interactions result in aggressive encounters with an apparent territory defense function (Chen et al 2002). It is conceivable that networks controlling courtship and aggression in *D. melanogaster* are independent and are activated by their own respective stimuli, although may be organized by common principles of sex-determination genes (Lee & Hall 2000; Vrontou et al 2006). However, in some Hawaiian *Drosophila* species of the planitibia group, aggression and courtship behavioral repertoires are much more overlapping. Although head-to-head interactions characteristic of *D. heteroneura* aggression are not found in courtship, once a dominance hierarchy has been established, future encounters resemble courtship, with the dominant male "courting" the submissive male. The dominant male exhibits many of the behavioral characteristics of courtship, including double-wing extensions, production of abdominal clicks, and circling around the submissive male, taking on a head-under-wings posture while vibrating both wings. Since similar social motor patterns are activated by different stimuli in *D. heteroneura*, the neural network underlying courtship and aggression may show more considerable overlap than that seen in *D. melanogaster*, where stimuli from other males actively repress courtship behavior. Thus, differences in brain regions associated with

courtship behavior may reflect two different neural strategies for activating courtship behavior selectively over other behavioral programs, like aggression. Further, these phylogenetic differences in the establishment of the courtship and aggression network may be underlied by fru, as its sequence and sexual dimorphic expression patterns are well conserved throughout *Drosophila* (Davis et al 2000a; Davis et al 2000b; Gailey et al 2000). Since the scarcity of *D. heteroneura* specimens would impede techniques requiring sacrificing of the subjects, other species exhibiting similarities in courtship and aggression interactions could be utilized, likely *D. silvestris*.

General conclusions

The work presented here refocuses the attention motor circuits must receive in understanding how *Drosophila* courtship is produced. Sex-specific gene expression has been shown here to be important in crafting this circuit to produce some of the more idiosyncratic features of song in *D. melanogaster*, as it has been shown previously to craft courtship behavior in general. Identification of neuronal populations critical to patterning the song in a fru^M-dependent matter will greatly further our understanding of how complex behaviors are produced by the nervous system. An emerging role of fru^M in aggression (Lee & Hall 2000; Vrontou et al 2006) casts sex-specification genes (e.g. *fru* and *dsx*) as major role-players in establishing networks important for social behavior in general. Further, selection appears to have made more broad use of courtship song generating circuits in planitibia *Drosophila* by utilizing them in social contexts other than courtship, like aggression, and it can only be speculated that selection on sex-specification genes may underlie these divergent behavior patterns.

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