Cell Reports
Article

Motor Control of *Drosophila* Courtship Song

Troy R. Shirangi,[1,*] David L. Stern,[1] and James W. Truman[1]
[1]Janelia Farm Research Campus, Howard Hughes Medical Institute, 19700 Helix Drive, Ashburn, VA 20147, USA
*Correspondence: shirangit@janelia.hhmi.org
http://dx.doi.org/10.1016/j.celrep.2013.09.039

SUMMARY

Many animals utilize acoustic signals—or songs—to attract mates. During courtship, *Drosophila melanogaster* males vibrate a wing to produce trains of pulses and extended tone, called pulse and sine song, respectively. Courtship songs in the genus *Drosophila* are exceedingly diverse, and different song features appear to have evolved independently of each other. How the nervous system allows such diversity to evolve is not understood. Here, we identify a wing muscle in *D. melanogaster* (hg1) that is uniquely male-enlarged. The hg1 motoneuron and the sexually dimorphic development of the hg1 muscle are required specifically for the sine component of the male song. In contrast, the motoneuron innervating a sexually monomorphic wing muscle, ps1, is required specifically for a feature of pulse song. Thus, individual wing motor pathways can control separate aspects of courtship song and may provide a "modular" anatomical substrate for the evolution of diverse songs.

INTRODUCTION

Animal courtship often involves some of the most elaborate behaviors ever observed. Courting males of many insect species, for example, utilize complex songs (Gerhardt and Huber, 2002) and dances (Spieth, 1974) to enhance their chance of mating. These courtship behaviors are exceedingly diverse between species, most likely as a result of sexual selection (Andersson, 1994). How the neural circuits encoding these complex behaviors allow such diversity to evolve is not known. The modularity of development is understood to have enabled the evolution of complex morphological forms (Schlosser and Wagner, 2004). Likewise, complex behaviors may also be built from modules that can be quickly modified and rearranged during evolution (Weber et al., 2013). The courtship songs of the genus *Drosophila* display enormous diversity, and individual features of the song appear to have evolved independently of each other (reviewed in Markow and O'Grady [2005]). This pattern of evolutionary change suggests that the neural control of courtship song may be modular, but where this modularity may lie in the neural circuit controlling song is not known.

Previous work has led to the identification of multiple classes of neurons active in the *Drosophila melanogaster* male courtship song circuit, from neurons in the brain that integrate social cues (Kimura et al., 2008; Kohatsu et al., 2011; Pan et al., 2012; von Philipsborn et al., 2011) to descending neurons that activate pulse song production (Kohatsu et al., 2011; von Philipsborn et al., 2011) and the thoracic interneurons that may contribute to the pattern generator(s) that compose the pulse song (Clyne and Miesenböck, 2008; von Philipsborn et al., 2011). Despite this progress, it is not understood how these circuits orchestrate the peripheral neuromuscular events that modulate the wing vibrations of courtship song. Over 30 years ago, Ewing showed that some wing muscles fire rhythmically during pulse, sine song, or both (Ewing, 1977, 1979). However, these studies lacked the resolution to determine the causal links between individual wing muscles and song components.

Here, we identify a thoracic wing muscle in *D. melanogaster*, hg1, which is uniquely enlarged in males. Courting males with inhibited hg1 motoneurons are unable to produce sine song, but sing pulse song normally. Feminization of the hg1 muscle reduces the size of hg1 in males and reduces the volume with which males sing sine song. In contrast, males with silenced motoneurons innervating a sexually monomorphic wing muscle, ps1, have normal sine song, but generate pulse song with a decreased carrier frequency and amplitude. These results demonstrate that the motor control of *Drosophila* courtship song is modular. Changes in individual motor pathways during evolution would thereby allow discrete components of the male song to change independently of others. Finally, we show that females are less willing to mate with males that either lack sine song or produce sine song with reduced volume, suggesting that a female may judge male quality in part by how loudly a male sings sine song.

RESULTS

A Sexually Dimorphic Control Wing Muscle in *Drosophila*

In *Drosophila*, contractions of the large indirect wing muscles deform the thorax to power the wings during flight (reviewed in Dickinson and Tu [1997]) and during courtship song (Ewing, 1977). Additional small muscles located adjacent to the lateral

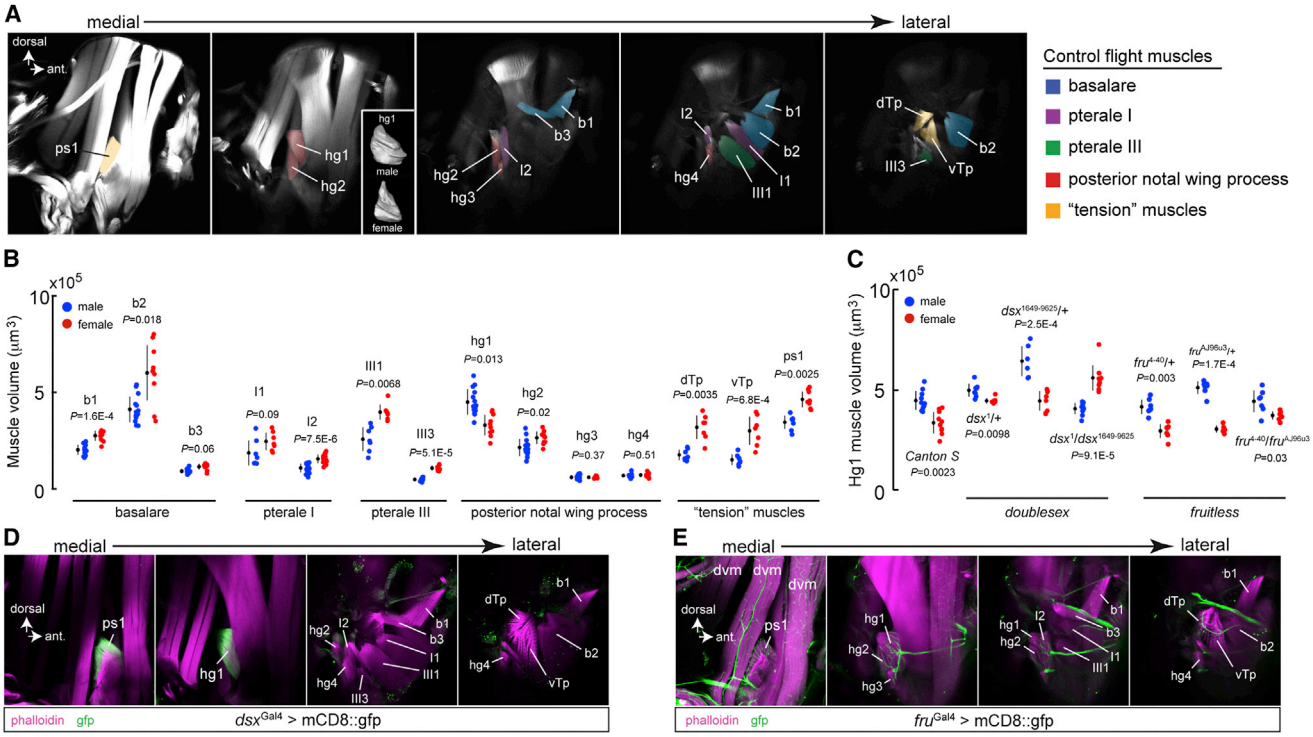

Figure 1. A Sexual Dimorphism in the Direct Wing Muscles of *Drosophila melanogaster*

(A) Single slices from a confocal stack of a hemithorax stained with phalloidin to show the locations of 13 of the 18 control wing muscles (Dickinson and Tu, 1997; Wisser and Nachtigall, 1984). The direct wing muscles are classified according to the sclerite in which they insert (basalare, first and third pterale, and the posterior notal wing process; Wisser and Nachtigall, 1984). "Tension muscles" (Wisser and Nachtigall, 1984) adjust wing movement indirectly by regulating the rigidity of the pleural wall. Inset shows the difference in size between the hg1 muscles of males versus females.

(B) The volumes of the control wing muscles in males and females. Individual points, the mean and SD are given for each. P values were obtained using a standard Student's t test.

(C) Sexually dimorphic development of hg1 is dependent upon *dsx* and not male-specific *fru*. Null allele combinations were used. Individual points, the mean and SD are given for each muscle. P values were obtained using a standard Student's t test.

(D) Confocal sections showing that dsx^{Gal4} (Robinett et al., 2010), which accurately recapitulates the endogenous expression of *dsx*, drives reporter expression only in the ps1 and hg1 muscles.

(E) Confocal sections showing that fru^{Gal4} (Stockinger et al., 2005) drives reporter expression in motoneurons that innervate seven control wing muscles (ps1, hg1-3, l2, and d-, v-Tp). fru^{Gal4} is also expressed in motoneurons that innervate the dorsal longitudinal muscle (not shown) and the dorsoventral muscles (dvms).

thoracic wall—the control wing muscles—modulate these wing movements (Dickinson and Tu, 1997; Ewing, 1979; Figure 1A). Most control muscles insert into sclerites at or near the wing hinge (Wisser and Nachtigall, 1984) and are thought to influence wing motion by altering the mechanical properties of the hinge (Dickinson and Tu, 1997). We observed that most wing muscles are larger in females than in males, consistent with the difference in overall body size between the sexes (Figures 1A and S1). In contrast, the control wing muscle hg1 is larger in males than in females (Figures 1A, second panel, inset, and 1B). Hg1 inserts into the posterior notal wing process (Wisser and Nachtigall, 1984) and, in *Calliphora*, hg1 activity is associated with changes in wing-stroke amplitude during a flight turn (Dickinson and Tu, 1997; Nachtigall and Wilson, 1967).

We tested whether sexually dimorphic development of hg1 requires the activity of the *Drosophila* sex differentiation genes *doublesex* (*dsx*) and *fruitless* (*fru*) (reviewed in Billeter et al. [2006] and Christiansen et al. [2002]). Among thoracic wing muscles, *dsx* is expressed only in muscles hg1 and ps1 (Figure 1D), whereas male-specific *fru* is not expressed in muscles but rather in several wing motoneurons (more than described previously [Rideout et al., 2007]), including a motoneuron that innervates hg1 (Figure 1E). Removal of *dsx* function abolished the dimorphism, reducing the size of hg1 in males relative to its size in females (Figure 1C). The loss of male-specific *fru*, however, did not alter sexually dimorphic development of hg1 (Figure 1C). Thus, the male- and female-specific isoforms of *dsx* promote and suppress hg1 muscle growth, respectively, whereas *fru* function does not influence the size dimorphism of hg1.

The hg1 Motoneuron Is Required for Sine Song but Not Pulse Song

To test whether the hg1 motoneuron contributes to courtship song, we identified two transgenic lines (Jenett et al., 2012; Pfeiffer et al., 2008), *R21A01-Gal4* and *R14B02-LexA::p65*, that each drove reporter expression in the motoneuron that innervates hg1 and in additional neurons in the nervous system (not

Cell PRESS
Open ACCESS

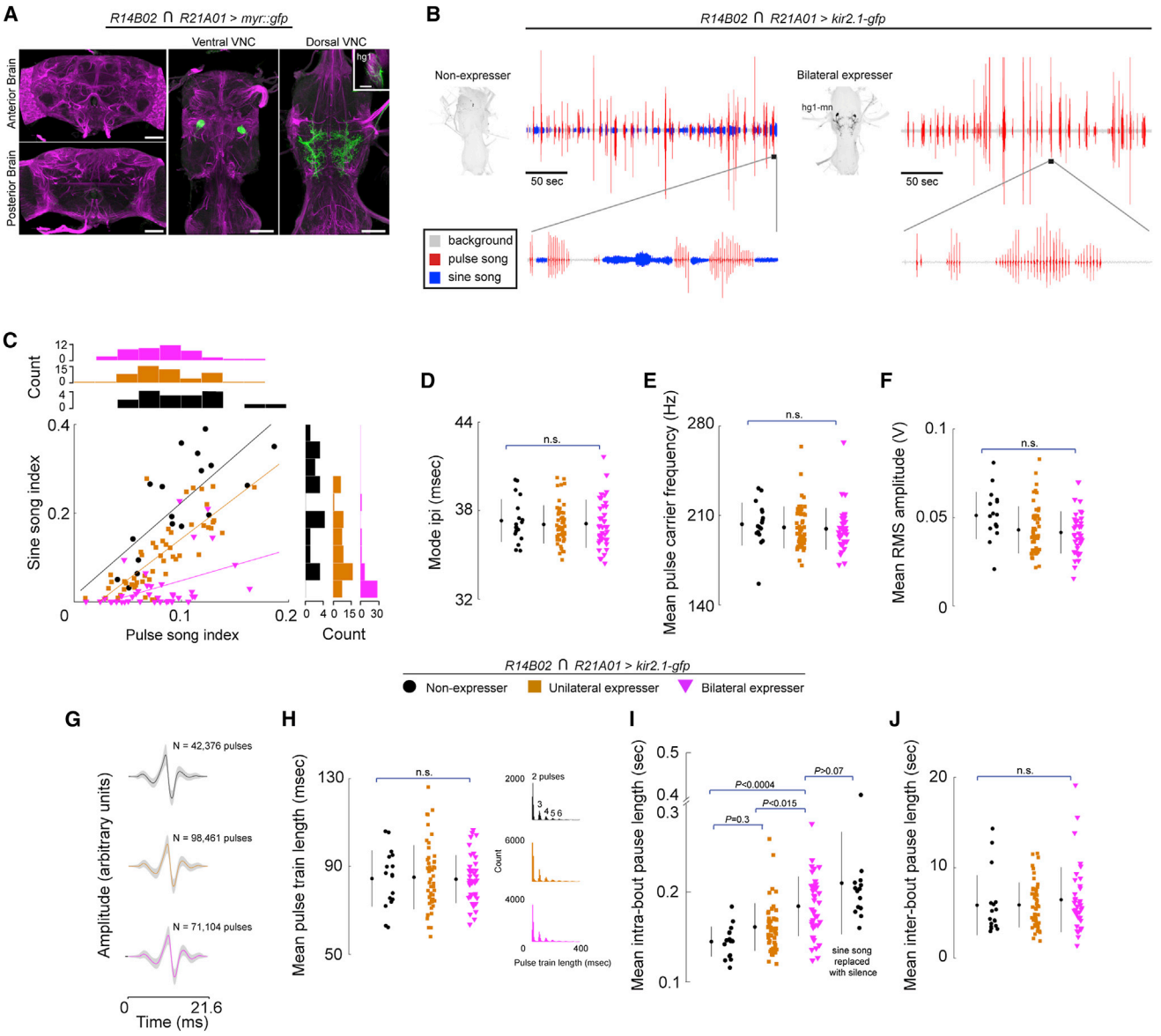

Figure 2. Silencing the hg1 Motoneuron in Males Specifically Impairs Their Ability to Generate Sine Song

(A) The intersection between *R14B02-LexA::p65* and *R21A01-Gal4* targets the hg1 motoneuron in adult males with perfect specificity. Magenta, neuroglian; green, GFP. The scale bar represents 50 μm. Inset: hg1 neuromuscular junction. Magenta, phalloidin; green, GFP.

(B) Expression of kir2.1-GFP in the hg1 motoneuron selectively ablates sine song during male courtship behavior. Pulse and sine song events were detected (Arthur et al., 2013) in a 5 min clip.

(C) The proportion of sine song amount relative to pulse song amount of nonexpressers and uni- and bilateral expressers. To quantify the amount of pulse or sine song that a courting male sings, we measured the pulse and sine song index, which is the fraction of time the male spends singing pulse or sine song. Histograms show the distribution of pulse and sine song indices among the three classes.

(D–J) Males with silenced hg1 motoneurons generate pulse song normally. (D) Mode interpulse interval, (E) mean pulse carrier frequency, (F) mean rms amplitude, (G) mean rescaled pulse shape, and (H) mean pulse train length are statistically equivalent between the three classes. Mean pulse shape ± SD for all pulses collected from individuals of the three classes is shown in (G). Histograms in (H) show the distribution of pulse train lengths among the three classes. (I) Mean intrabout pause length is greater in the bi- and unilateral males due to reduced sine song production. Replacing sine song with silence in the nonexpresser males increases their mean intrabout pause length to an amount comparable to bilateral expressers. (J) Mean interbout pause length is equivalent between the sets. (D)–(F) and (H)–(J) show individual points, the mean and SD. (n.s.) Significance measured using one-way ANOVA with Tukey-Kramer post hoc test for multiple comparisons. The P value between the bilateral or unilateral males and the nonexpresser males is greater than 0.7. n.s., not significant.

shown). To target the hg1 motoneuron specifically, *R14B02-LexA::p65* was used to drive a Flp recombinase, which excised a transcriptional stop cassette from an upstream activating sequence (UAS)-GFP transgene driven by *R21A01-Gal4*. When genetically intersected in adult males, these lines drove reporter gene expression in only the hg1 motoneuron (Figure 2A). This

motoneuron likely provides sole excitatory input to hg1, because all control muscles examined thus far in *Drosophila* are innervated by a single excitatory motoneuron (Trimarchi and Schneiderman, 1994). We used this intersection (*R14B02* ∩ *R21A01*) to suppress the activity of the hg1 motoneuron by driving a GFP-tagged version of the inwardly rectifying K^+ channel, Kir2.1 (Baines et al., 2001). *R14B02* ∩ *R21A01* drove kir2.1-GFP expression stochastically; males either lacked kir2.1-GFP expression ("nonexpressers") or expressed kir2.1-GFP in the hg1 motoneuron unilaterally or bilaterally (Figure S2A). This allowed us to compare the songs produced by genetically identical males that had functional hg1 motoneurons or hg1 motoneurons that were silenced on one or both sides. We recorded courtship song (Arthur et al., 2013) from *R14B02* ∩ *R21A01* > kir2.1-GFP males and then dissected the ventral nerve cords of these males to determine which hg1 motoneurons expressed kir2.1-GFP.

Males lacking kir2.1-GFP expression in both hg1 motoneurons generated abundant pulse and sine song (Figures 2B and 2C). In contrast, most males expressing kir2.1-GFP in both hg1 motoneurons displayed a complete absence of sine song (Figures 2B and 2C). Males with kir2.1-GFP expressed in only one hg1 motoneuron produced a quantity of sine song intermediate between the nonexpressers and bilateral expressers (Figure 2C). All three categories of males produced statistically indistinguishable quantities of pulse song (Figure 2C), including interpulse intervals, pulse carrier frequencies, pulse amplitudes, pulse shapes, and pulse train lengths (Figures 2D–2H). Similar results were obtained using tetanus neurotoxin light chain (Eisel et al., 1986; Pfeiffer et al., 2010; Sweeney et al., 1995) as an alternate means to silence the hg1 motoneuron (Figures S2B–S2F).

These data indicate that the hg1 motoneuron is required for sine song but is dispensable for pulse song. Nevertheless, it appears that the nervous system of a male with compromised hg1 motoneurons continues to send the command to produce sine song. Courtship song is normally arranged in bouts of song (i.e., concatenated trains of pulse or sine song separated by pauses of less than a second) separated by periods of silence. The duration of the silent periods between bouts of song was similar in males of the three classes (Figure 2J). The intrabout pauses, however, were lengthened in males that expressed kir2.1-GFP in the hg1 motoneurons bilaterally compared to nonexpressers (Figure 2I). This increased intrabout pause duration most likely reflects the dropping out of trains of the sine song during song bouts, because we can mimic this duration in normal males by computationally replacing their sine song trains with silence (Figure 2I). Simultaneous video and audio recording revealed that courting males with bilaterally silenced hg1 motoneurons extend a wing during periods of silence that often precede or follow trains of pulse song (Movie S1), which we interpret as putative sine song trains. Moreover, the intermediate reduction in sine song production among males with a unilaterally silenced hg1 motoneuron reflects their ability to sing sine song with the contralateral wing, but not the ipsilateral wing (Movie S1). Therefore, it appears that males with silenced hg1 motoneurons "think" they are singing sine song, despite being mechanically unable to do so.

Males with Feminized hg1 Muscles Sing Sine Song Quietly

To test the role of hg1's sexual size dimorphism in the production of courtship song, we feminized hg1 (thereby, reducing its size) in otherwise normal males by driving female-specific *transformer* (*tra*F) (McKeown et al., 1988) in the hg1 muscle. We identified a Gal4 line (Jenett et al., 2012; Pfeiffer et al., 2008; *R84G06-Gal4*) that targeted all fibers of several control wing muscles, including the two *dsx*-expressing muscles, hg1 and ps1 (Figures 1D, 3A, and 3B), and several neurons in the adult nervous system (Figure S3A; not shown). *R84G06-Gal4* driving *traF* (*R84G06-Gal4* > *tra*F) reduced the hg1 muscle to the size observed in females (Figure 3C). These males were otherwise phenotypically normal in gross appearance; they courted females vigorously and generated most aspects of song normally relative to controls (Figures 3D–3G). However, *R84G06-Gal4* > *tra*F males sang sine song with significantly reduced amplitude compared to controls, whereas pulse song amplitude was unaffected (Figure 3H). This phenotype was not due to *tra*F expression in the nervous system because *R84G06-Gal4* > *tra*F males carrying a transgene (*R57C10-Gal80-6* [Harris, 2012]) to suppress the neuronal expression of *tra*F also sang sine song with reduced volume relative to controls (Figures S3A–S3F). Moreover, the change in sine song amplitude in *R84G06-Gal4* > *tra*F males did not result from *tra*F expression in ps1. We identified a line, *R40D04-Gal4*, which targeted the ps1, but not the hg1, muscle (Figures S3G and S3H). *R40D04-Gal4* > *tra*F males generated song normally compared to controls (Figures S3I–S3N). Thus, the hg1 motoneuron is required to generate sine song, whereas the sexual dimorphism of hg1 is necessary for males to sing sine song at normal volume.

The ps1 Control Wing Motoneuron Is Required for a Discrete Feature of Pulse Song

It is noteworthy that manipulating hg1 function did not affect pulse song. This suggests that some wing motor pathways may influence courtship song in specific ways. To test this, we drove kir2.1-GFP specifically in the motoneuron innervating the ps1 control muscle by genetically intersecting two transgenic lines (*R48F07-LexA::p65* and *R73C03-Gal4*; Jenett et al., 2012; Pfeiffer et al., 2008; Figure 4A). Ps1, like hg1, expresses *dsx* (Figure 1D) and is innervated by a *fru*M-expressing motoneuron (Figure 1E) but is not enlarged in males (Figure 1B). Males with inhibited ps1 motoneurons displayed a reduction in their pulse carrier frequency and pulse amplitude relative to two controls but sang pulse and sine song otherwise normally (Figures 4B–4F). These data are consistent with the hypothesis that the motor control of *Drosophila* courtship song is, at least in part, modular.

Males that Lack Sine Song Are Ineffective Courters

Our ability to precisely manipulate sine song production allowed a test of the requirement for sine song during courtship. *R14B02* ∩ *R21A01* males were used to drive kir2.1-GFP or tetanus neurotoxin light chain in the hg1 motoneuron, and these males were tested for their ability to court, to sing pulse and sine song, and to mate with wild-type females relative to two control genotypes. Males with silenced hg1 motoneurons courted females (Figure 5A) and sang pulse song (Figures 5B and 5C) at levels statistically indistinguishable from controls but were

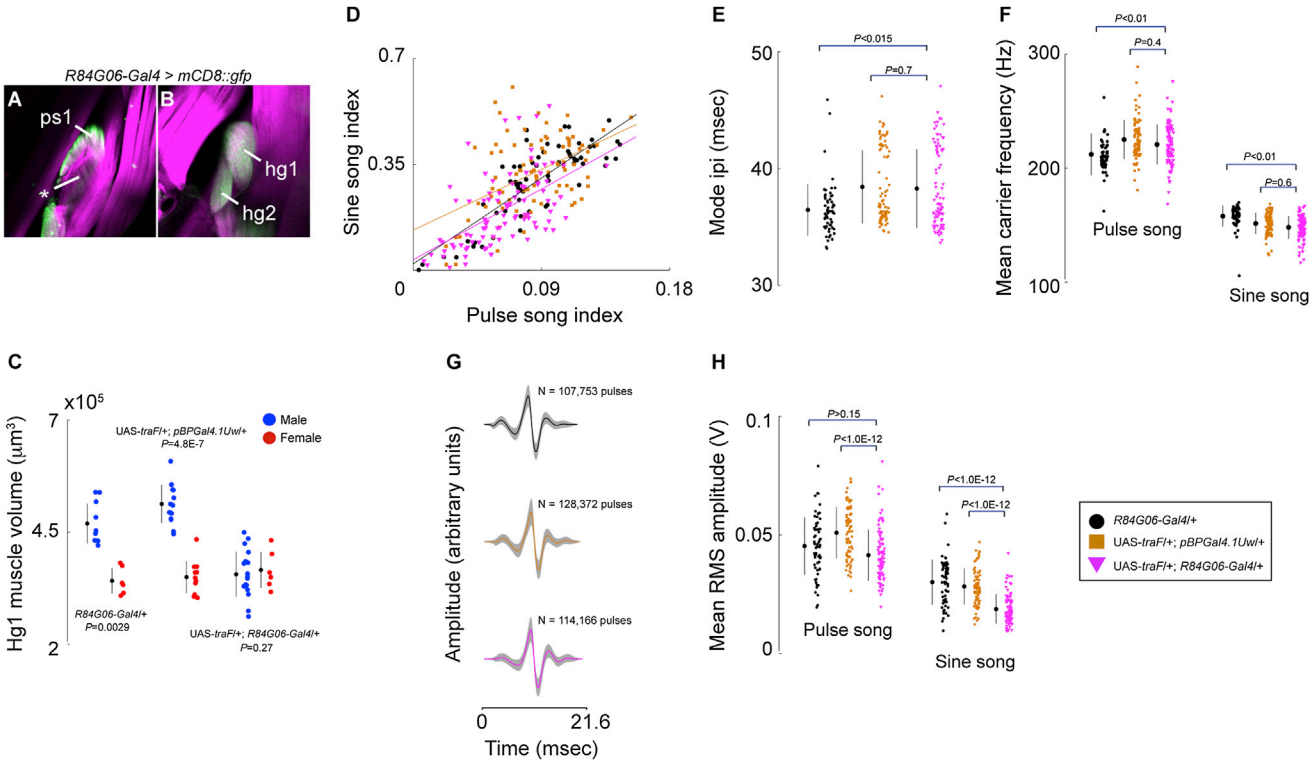

Figure 3. Sexually Dimorphic Development of hg1 Is Required for Maximal Sine Song Amplitude

(A and B) *R84G06-Gal4* drives reporter expression in all fibers that constitute the ps1, hg1, and hg2 wing muscles (and others not shown). The asterisk refers to an unidentified muscle associated with ps1. This muscle is not targeted by dsx^{Gal4} (Figure 1D). *R84G06-Gal4* was crossed to *pJFRC2-10XUAS-IVS-mCD8::GFP*. Magenta, phalloidin; green, GFP.

(C) *R84G06-Gal4*-driving *tra*F feminizes and reduces the size of the hg1 muscle in males. P values were obtained using a standard Student's t test.

(D–H) Feminization of the hg1 muscle in males does not affect (D) the relative amount of sine and pulse song produced, (E) the mode interpulse interval, (F) the mean pulse and sine song carrier frequencies, or (G) the mean rescaled pulse shape relative to control genotypes. (H) Mean rms amplitude of sine song, but not pulse song, is reduced in males with a feminized hg1 muscle relative to controls. (A), (C), (E), and (F) show individual points, the mean and SD. Significance in (E), (F), and (H) was measured using one-way ANOVA and Kruskal-Wallis test, respectively, with Tukey-Kramer post hoc test for multiple comparisons. *pBPGal4.1Uw* (Pfeiffer et al., 2008) strain carries the "empty vector" Gal4 inserted into attP2.

strongly impaired in their ability to sing sine song (Figures 5B and 5C). These males mated at a significantly lower rate than did control males (Figure 5D). These mating deficits are most parsimoniously attributed to either the large reduction in sine song production or the presence of larger gaps within the bouts of song. However, at present, we cannot exclude the possibility that some other defects unnoticed in our analyses account for the reduction in mating success.

We also tested if sexually dimorphic development of hg1—and thus production of relatively loud sine song—is important for female receptivity. *R84G06-Gal4* > *tra*F males courted females robustly (Figure 5E), produced pulse song normally (Figures 3B–3E), and generated sine song with lower amplitude compared to controls (Figure 3H). Females mated with *R84G06-Gal4* > *tra*F males at a lower rate than they mated with controls (Figure 5F). These mating deficits were not due to *tra*F expression in the ps1 muscle, because *R40D04-Gal4* > *tra*F males mated with females as efficiently as did controls (Figure S3O). These results support the hypothesis that sine song produced at wild-type volume contributes to the mating efficiency of *D. melanogaster* males. Females may use the volume of sine song as an indicator of male quality, as others have postulated (Rybak et al., 2002).

DISCUSSION

We have shown that the hg1 wing muscle and its sexually dimorphic development are required for the sine component of courtship song, whereas the ps1 wing muscle is required for a specific aspect of pulse song, but not sine song. The sexual size dimorphism in hg1 is analogous to the sexual differences in the size and physiology of the laryngeal muscles of singing *Xenopus laevis* frogs (Kelley and Tobias, 1999). Contraction of hg1 pulls the posterior notal wing process in an anterioventral direction (Dickinson and Tu, 1997; Wisser and Nachtigall, 1984), but how this event relates to the wing motions underlying sine song is not clear. Our observation that feminizing hg1 reduces the amplitude of sine song suggests that hg1 may provide power to the wing strokes that generate sine song. Although hg1 is essential for sine song, it obviously does not work alone and the performance of this song component involves the synergistic actions of other wing muscles (Ewing, 1977, 1979). Given the role

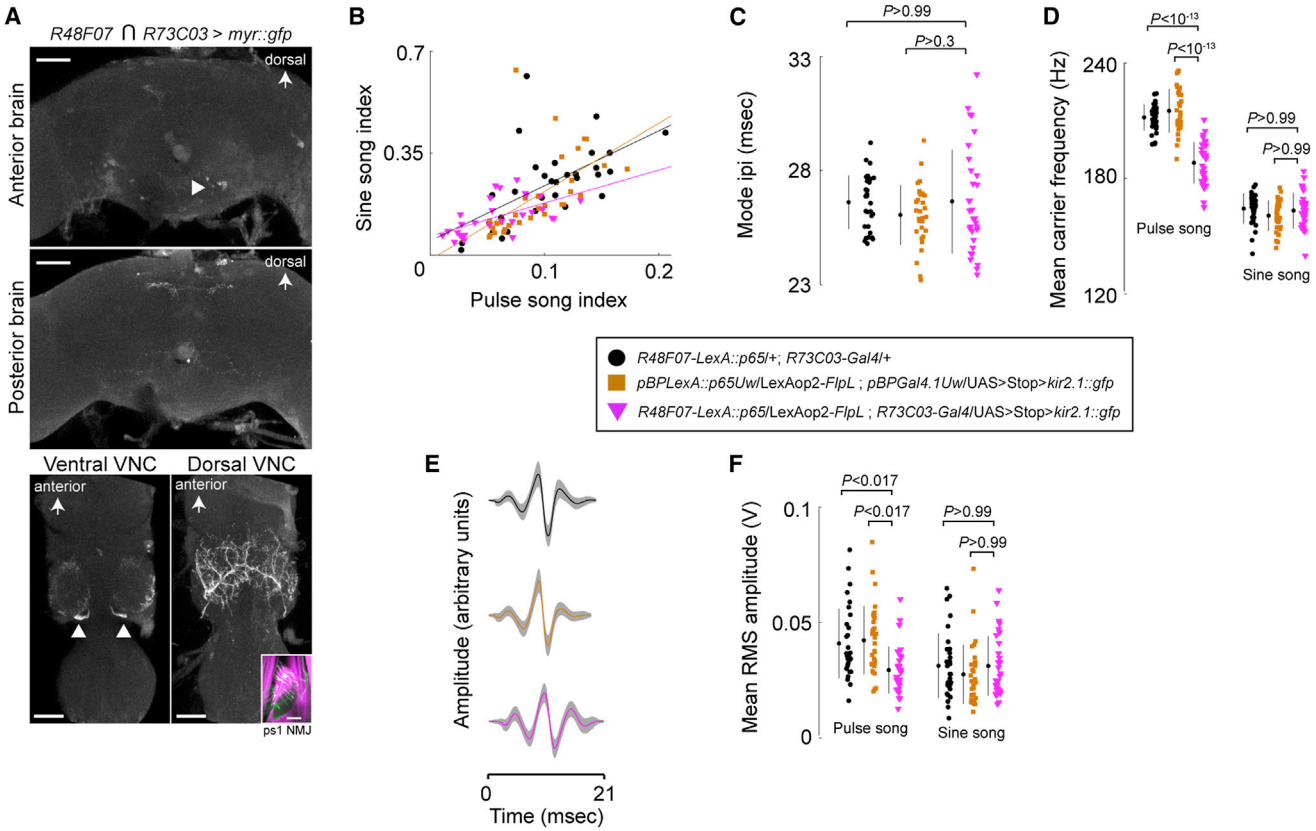

Figure 4. Inhibition of the ps1 Motoneuron Specifically Reduces Pulse Carrier Frequency and Amplitude

(A) The intersection between *R48F07-LexA::p65* and *R73C03-Gal4* targets the ps1 motoneuron in adult males. Arrowheads in "ventral VNC" point to the ps1 motoneuron cell body. We note that this intersection stochastically labels three or four interneurons in the mesothoracic ganglion that appear to innervate the leg neuropil (not present in this preparation) but consistently labels the ps1 motoneuron. This intersection also weakly targets four or five neurons in the anterior brain (arrowhead points to cell bodies in "anterior brain"). The scale bar represents 50 mm. Inset: ps1 neuromuscular junction. Magenta, phalloidin; green, GFP.

(B–F) The proportion of sine song relative to pulse song (B), the mode interpulse interval (C), and the mean rescaled pulse shape (E) are statistically equivalent between experimental (magenta triangles) and controls (orange squares and black circles). Mean pulse carrier frequency (D) and mean rms amplitude (F) of pulse song, but not sine song, is reduced in the experimental males relative to controls. (n.s.) The P value between the experimental class and each control is greater than 0.99.

of ps1 in linking the pleural and sternal apophyses (Dickinson and Tu, 1997; Pringle, 1957), our results further suggest that thoracic rigidity regulates pulse carrier frequency.

Our findings echo a burgeoning idea that complex behaviors are composed of "modules" that allow discrete aspects of a behavior to evolve independently of others (Weber et al., 2013). Our results demonstrate that pulse and sine song are produced in part by separate sets of wing muscles, suggesting that the wing periphery is to a certain extent modular. By "modular," we mean that discrete features of the behavior can be functionally mapped to morphologically discrete subunits in the motor periphery. Given that the wing periphery consists of a relatively small number of muscles, the modularity we observe may be due to the biomechanical constraints intrinsic to the wing musculoskeletal system. Species of the genus *Drosophila* display extensive diversity in courtship song, and different song features appear to evolve independently of each other (Hoikkala, 2005; Markow and O'Grady, 2005). The apparent specialization of wing motor pathways for different aspects of song may provide a modular anatomical template for the evolution of different components of courtship song.

EXPERIMENTAL PROCEDURES

Fly Stocks

Flies were reared on standard cornmeal and molasses food at 25°C. The stocks used in this paper included the following: *Canton S* (*CSA*), dsx^{GAL4} (Robinett et al., 2010), fru^{Gal4} (Stockinger et al., 2005), *pJFRC2-10XUAS-IVS-mCD8::GFP* (attP2) (Pfeiffer et al., 2010), *pBDPGAL4.1Uw* (attP2) (Pfeiffer et al., 2008), and *pBDPLexA::p65Uw* (attP40) (Pfeiffer et al., 2010). $dsx^{1649\text{-}9625}$ (i.e., *Df(3R)f01649-d09625*; Chatterjee et al., 2011) was a gift from C. Robinett (HHMI/JFRC). dsx^{1}, fru^{440}, and fru^{AJ96u3} were provided by B. Baker (HHMI/JFRC). *R21A01-Gal4*, *R84G06-Gal4*, *R40D04-Gal4*, and *R73C03-Gal4* are from the Rubin GAL4 collection (Jenett et al., 2012; Pfeiffer et al., 2008). *R14B02-LexA::p65* and *R48F07-LexA::p65* (attP40) were a gift from G. Rubin (HHMI/JFRC). *pJFRC79-8XLexAop2-FlpL* (attP40), *pJFRC41-10XUAS-FRT > STOP > myrGFP* (attP2), *pJFRC39-10X-FRT > STOP > FRT-E86tetLC* (attP2), *pJFRC56-10XUAS-FRT > STOP > FRT-kir2.1-gfp* (attP2), and *R57C10-Gal80-6* (su(Hw)attP8) (Harris, 2012) were gifts from B. Pfeiffer (HHMI/JFRC). A Flpd-OUT STOP cassette (Nern et al., 2011) was cloned in

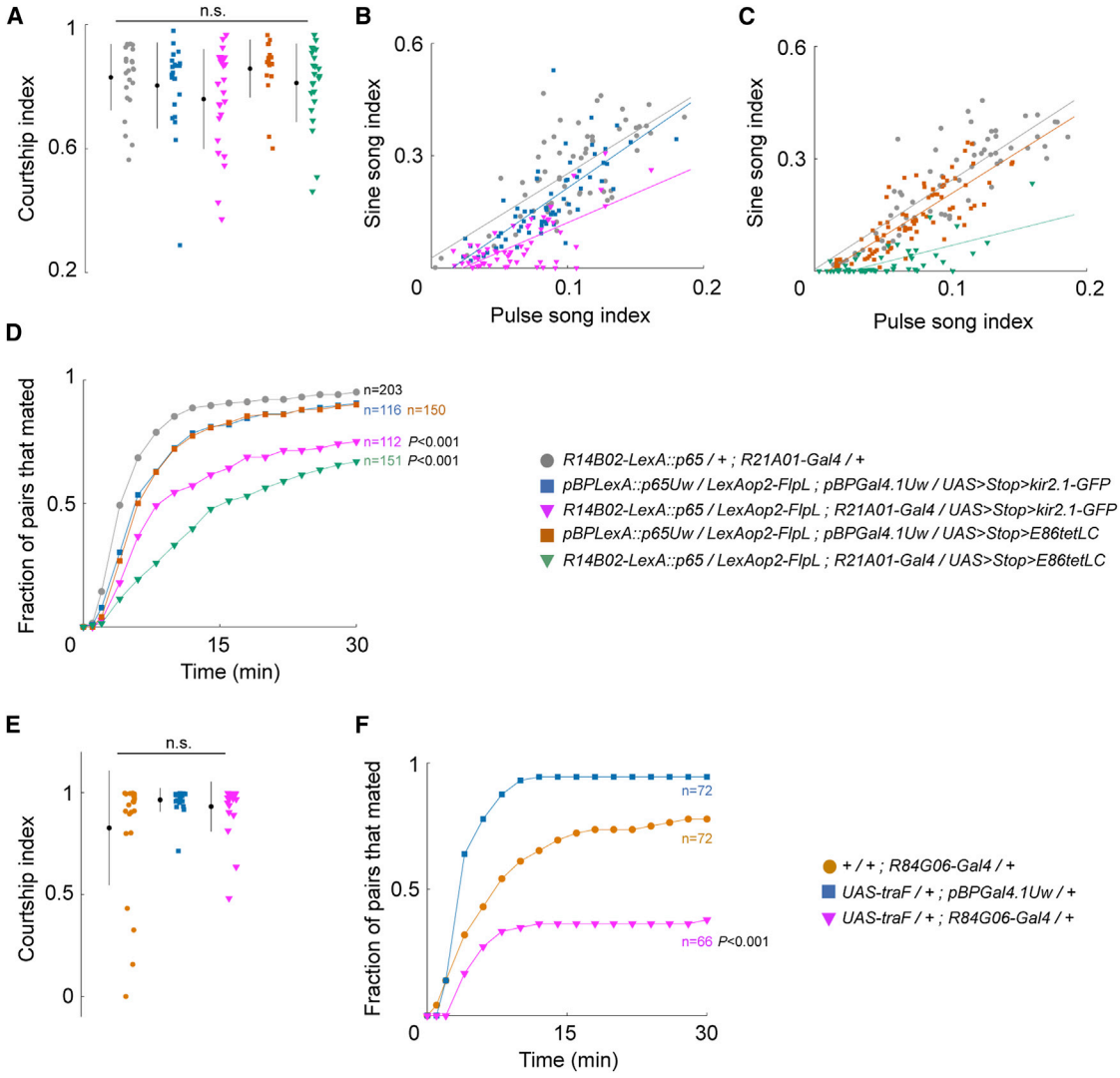

Figure 5. Reduced Production of Sine Song Is Associated with Lower Mating Success

(A) Males with silenced hg1 motoneurons court wild-type females as vigorously as do control males. Courtship index is the fraction of time a male spends engaged in any step of male courtship behavior during 10 min of observation. Individual points, the mean and SD are given for each. (n.s.) The P value between the experimental class and each control is greater than 0.99.

(B and C) Sine song indices of males with silenced hg1 motoneurons are reduced relative to controls, whereas their pulse song indices are comparable. The difference in mean song index between the experimental and controls was significant for sine song, but not significant for pulse song, at the 0.05 level.

(D) Females are less receptive to males with inhibited hg1 motoneurons. The fraction of male and female pairs that mated over 30 min is shown. P values were measured using a log rank test. *pBDPGal4.1Uw* and *pBDPLexA::p65Uw* strains carry the empty vector Gal4 and LexA::p65 inserted into attP2 and attP40, respectively.

(E) *R84G06-Gal4* males driving *tra*F court wild-type females as vigorously as controls. Courtship index measured as in (A).

(F) Females are less receptive to *R84G06-Gal4* > *tra*F males relative to controls. The fraction of male and female pairs that mated over 30 min is shown. P values were measured using a log rank test.

front of a 10XUAS vector (Pfeiffer et al., 2010) containing E86tetLC, the cloned bacterial tetanus toxin light chain gene as previously described (Eisel et al., 1986), containing a few minor base pair changes to the published sequence resulting in *pJFRC39-10X-FRT* > *STOP* > *FRT-E86tetLC* (B. Pfeiffer, personal communication). M. McKeown (Brown University) provided *UAS-tra*F (P-element).

Immunohistochemistry

To visualize GFP reporter expression in wing muscles or their neuromuscular junctions (NMJs), hemithoraces of adults aged for about 5 days were dissected in PBS and fixed in 4% formaldehyde (buffered in PBS) for approximately 50 min at room temperature. To effectively stain the control muscles that line the thoracic lateral wall, we removed the six large fibers of the dorsal longitudinal muscle after fixation. Fixed tissues were washed in PBS-TX (PBS with 1% Triton X-100) and incubated for 3 to 4 days at 4°C in PBS-TX containing rabbit anti-GFP (1:1,000; Invitrogen). Tissues were washed at room temperature for several hours in PBS-TX and incubated for 3 to 4 days at 4°C in PBS-TX containing AlexaFluor-488-conjugated donkey anti-rabbit (1:500; Invitrogen) and Texas Red-X phalloidin (1:50; Life Technologies). Tissues were washed all day in PBS-TX, placed onto poly-lysine-coated coverslips,

dehydrated through an ethanol series, cleared in xylenes, and mounted in DPX (Sigma-Aldrich). Nervous systems were prepared and stained as above, except mouse antineuroglian (1:40; Developmental Studies Hybridoma Bank) and AlexaFluor-649-conjugated donkey anti-mouse were included in the primary and secondary antibody incubations, respectively (and without the addition of phalloidin). Tissues were imaged on a Zeiss LSM 510 confocal microscope at 10× (hemithorax) or 40× (CNS) with optical sections at 1 μm (hemithorax) or 0.5 μm (CNS) intervals.

Measurement of Wing Muscle Volume

To visualize and measure the volumes of wing muscles, hemithoraces from males or females were dissected, fixed, and washed in PBS-TX as described above and placed in PBS-TX containing Texas Red-X phalloidin (1:50; Life Technologies) for 3 to 4 days at 4°C. Tissues were washed in PBS-TX all day at room temperature and cleared and mounted as described above. Confocal stacks of phalloidin-stained hemithoraces were imported into Amira (Visualization Sciences Group). Wing muscles were segmented and reconstructed by selecting and assigning pixels through the confocal series to labels of their respective wing muscle. Amira was used to measure muscle volume using the appropriate voxel dimensions (in μm).

LexA/Gal4 Intersectional Strategy

A subset of lines from the Rubin GAL4 collection (Jenett et al., 2012; Pfeiffer et al., 2008) was screened for reporter expression at the NMJ of wing muscles. *R21A01* and *R14B02* were found to target the hg1 motoneuron and additional nonoverlapping wing motoneurons and neurons in the CNS. *R48F07* and *R73C03* were found to target the ps1 motoneuron and additional neurons that were largely nonoverlapping. A LexA::p65 version of *R14B02* and *R48F07* (in attP40 on the second chromosome) was found to also target the hg1 and ps1 motoneurons, respectively. To intersect the Rubin LexA::p65 and Gal4 lines and specifically target the hg1 or ps1 motoneurons, *R14B02-* or *R48F07-LexA::p65* was used to drive a Flp recombinase, which excised a transcriptional stop cassette from a GFP-, kir2.1-GFP-, or E86tetLC-expressing transgene driven by *R21A01-* or *R73C03-Gal4*. The observed stochasticity was a useful feature of the cross. Males from a stock carrying *R14B02-LexA::p65* and *R21A01-Gal4* or *R48F07-LexA::p65* and *R73C03-Gal4* were crossed to virgin females from stocks carrying *pJFRC79-8XLexAop2-FlpL* with *pJFRC41-10XUAS-FRT > STOP > myrGFP*, *pJFRC39-10X-FRT > STOP > FRT-E86tetLC*, or *pJFRC56-10XUAS-FRT > STOP > FRT-kir2.1-gfp*.

Recording Courtship Song

Newly eclosed males were collected under CO_2 and individually housed for 4–7 days (unless otherwise noted) at 25°C and 30% humidity with a 12 hr light/dark cycle. Virgin *Canton S* females were group-housed and aged under similar conditions. Courtship song was recorded as described (Arthur et al., 2013) for 10–15 min at 25°C within 2 hr after the start of the subjective day using individual pairs of males and decapitated females. In experiments using *R14B02-LexA::p65* ∩ *R21A01-Gal4* and kir2.1-GFP, the ventral nerve cord (VNC) of each male was dissected immediately after recording and stained as described above to score kir2.1-GFP expression in the hg1 motoneurons. Individual song recordings were subsequently categorized according to expression (i.e., nonexpresser, unilateral, and bilateral expresser) and analyzed.

Recording Courtship Movies

We recorded audio and video simultaneously of courting pairs using a Flea3 (USB 3.0) color camera (FL3-U3-32S2C-CS from Point Grey) shutter-triggered by an output from the DAQ used to collect audio signals from the microphone placed directly beneath the courting flies (Arthur et al., 2013). Synchronized video and audio were captured using a custom Matlab program, called *omnivore*, written by B. Arthur (https://github.com/bjarthur/omnivore.git). Data were visualized and movies were exported from a custom Matlab program, called *tempo*, written by F. Midgley and B. Arthur (https://github.com/frank-midgley/tempo.git).

Courtship Song Analyses

Recordings of courtship song were segmented and analyzed using Matlab R2011b as described (Arthur et al., 2013). The pulse song index was calculated by dividing the sum of a male's interpulse intervals by the total recording time. The sine song index was calculated by dividing the sum of the lengths of a male's sine song trains by the total recording time. The interpulse interval, pulse and sine carrier frequencies, models of pulse shape, pulse train lengths, and bout pause lengths were measured as described (Arthur et al., 2013). Pulse and sine song amplitude was measured by calculating the square root of the mean of the squares (rms) of all pulses or trains of sine song. The interpulse intervals in Figure 5C were estimated independently of pulse carrier frequency by fitting an envelope to each pulse to estimate pulse duration and calculating the interpulse interval as the time from the end of one pulse to the center of the next pulse. We considered bouts of courtship song as concatenated trains of pulse or sine song separated by pauses of less than 1 s. Intrabout pauses are pauses of shorter than 1 s between trains of pulse and sine song.

Courtship and Mating Assays

Males and *Canton S* virgin females were collected, housed, and aged as described above. Males and females were aged for 4–10 and 5–8 days, respectively. Courtship and mating assays were done at 25°C under white light within 2 hr after the start of the subjective day using individual pairs of males and virgin *Canton S* females. Males and females were loaded into behavioral chambers (diameter: 1 cm; height: 2 mm) at room temperature, kept separated by a plastic sheet, and allowed to acclimate for 15 min in the behavioral incubator. The barrier was quickly removed, and the pairs were video recorded for 30 min. The courtship index was measured by dividing the total amount of time the male spent engaged in any courtship step by the total observation time.

Statistics

All statistics were calculated in Matlab. In most cases, a randomization test was performed to determine if the experimental and control classes displayed significant heterogeneity. For each data set, we performed ANOVA on 10,000 randomly permuted data sets and used the resultant distribution of F statistics to estimate the significance of the F statistic from the original data. For comparisons yielding significant heterogeneity, we performed a one-way ANOVA or a Kruskal-Wallis test, followed by a Tukey-Kramer post hoc comparisons test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.09.039.

ACKNOWLEDGMENTS

We thank B. Arthur, B. Baker, M. Dickinson, D. Mellert, and T. Morita for helpful discussions; B. Arthur for assembling the video-audio apparatus; B. Arthur and F. Midgley for providing data acquisition and visualization software; B. Pfeiffer for *pJFRC39*, *pJFRC56*, and *R57C10-Gal80-6* flies; G. Rubin for *R14B02-* and *R48F07-LexA::p65*; B. Baker and C. Robinett for fly stocks; J.-C. Kao and T. Laverty for fly husbandry; and A. Howard for administrative assistance. This work was supported by the Howard Hughes Medical Institute.

Received: May 9, 2013
Revised: September 6, 2013
Accepted: September 25, 2013
Published: October 31, 2013

REFERENCES

Andersson, M. (1994). Sexual Selection (Princeton: Princeton University Press).

Arthur, B.J., Sunayama-Morita, T., Coen, P., Murthy, M., and Stern, D.L. (2013). Multi-channel acoustic recording and automated analysis of Drosophila courtship songs. BMC Biol. *11*, 11.

Baines, R.A., Uhler, J.P., Thompson, A., Sweeney, S.T., and Bate, M. (2001). Altered electrical properties in Drosophila neurons developing without synaptic transmission. J. Neurosci. *21*, 1523–1531.

Billeter, J.C., Rideout, E.J., Dornan, A.J., and Goodwin, S.F. (2006). Control of male sexual behavior in Drosophila by the sex determination pathway. Curr. Biol. *16*, R766–R776.

Chatterjee, S.S., Uppendahl, L.D., Chowdhury, M.A., Ip, P.L., and Siegal, M.L. (2011). The female-specific doublesex isoform regulates pleiotropic transcription factors to pattern genital development in Drosophila. Development *138*, 1099–1109.

Christiansen, A.E., Keisman, E.L., Ahmad, S.M., and Baker, B.S. (2002). Sex comes in from the cold: the integration of sex and pattern. Trends Genet. *18*, 510–516.

Clyne, J.D., and Miesenböck, G. (2008). Sex-specific control and tuning of the pattern generator for courtship song in Drosophila. Cell *133*, 354–363.

Dickinson, M.H., and Tu, M.S. (1997). The function of dipteran flight muscle. Comp. Biochem. Physiol. A *116*, 223–238.

Eisel, U., Jarausch, W., Goretzki, K., Henschen, A., Engels, J., Weller, U., Hudel, M., Habermann, E., and Niemann, H. (1986). Tetanus toxin: primary structure, expression in E. coli, and homology with botulinum toxins. EMBO J. *5*, 2495–2502.

Ewing, A.W. (1977). The neuromuscular basis of courtship song in Drosophila: The role of the indirect flight muscles. J. Comp. Physiol. *119*, 249–265.

Ewing, A.W. (1979). The neuromuscular basis of courtship song in Drosophila: The role of the direct and axillary wing muscles. J. Comp. Physiol. *130*, 87–93.

Gerhardt, H.C., and Huber, F. (2002). Acoustic communication in insects and anurans: common problems and diverse solutions (Chicago: University of Chicago Press).

Harris, R.M. (2012). Form and function of the secondary hemilineages in the adult Drosophila thoracic nervous system (Seattle: University of Washington).

Hoikkala, A. (2005). Inheritance of male sound characteristics in Drosophila species (Boca Raton: Taylor & Francis).

Jenett, A., Rubin, G.M., Ngo, T.T., Shepherd, D., Murphy, C., Dionne, H., Pfeiffer, B.D., Cavallaro, A., Hall, D., Jeter, J., et al. (2012). A GAL4-driver line resource for Drosophila neurobiology. Cell Rep. *2*, 991–1001.

Kelley, D.B., and Tobias, M.L. (1999). The design of animal communication, M. Hauser and M. Konishi, eds. (Cambridge: MIT Press), pp. 9–35.

Kimura, K., Hachiya, T., Koganezawa, M., Tazawa, T., and Yamamoto, D. (2008). Fruitless and doublesex coordinate to generate male-specific neurons that can initiate courtship. Neuron *59*, 759–769.

Kohatsu, S., Koganezawa, M., and Yamamoto, D. (2011). Female contact activates male-specific interneurons that trigger stereotypic courtship behavior in Drosophila. Neuron *69*, 498–508.

Markow, T.A., and O'Grady, P.M. (2005). Evolutionary genetics of reproductive behavior in Drosophila: connecting the dots. Annu. Rev. Genet. *39*, 263–291.

McKeown, M., Belote, J.M., and Boggs, R.T. (1988). Ectopic expression of the female transformer gene product leads to female differentiation of chromosomally male Drosophila. Cell *53*, 887–895.

Nachtigall, W., and Wilson, D.M. (1967). Neuro-muscular control of dipteran flight. J. Exp. Biol. *47*, 77–97.

Nern, A., Pfeiffer, B.D., Svoboda, K., and Rubin, G.M. (2011). Multiple new site-specific recombinases for use in manipulating animal genomes. Proc. Natl. Acad. Sci. USA *108*, 14198–14203.

Pan, Y., Meissner, G.W., and Baker, B.S. (2012). Joint control of Drosophila male courtship behavior by motion cues and activation of male-specific P1 neurons. Proc. Natl. Acad. Sci. USA *109*, 10065–10070.

Pfeiffer, B.D., Jenett, A., Hammonds, A.S., Ngo, T.T., Misra, S., Murphy, C., Scully, A., Carlson, J.W., Wan, K.H., Laverty, T.R., et al. (2008). Tools for neuroanatomy and neurogenetics in Drosophila. Proc. Natl. Acad. Sci. USA *105*, 9715–9720.

Pfeiffer, B.D., Ngo, T.T., Hibbard, K.L., Murphy, C., Jenett, A., Truman, J.W., and Rubin, G.M. (2010). Refinement of tools for targeted gene expression in Drosophila. Genetics *186*, 735–755.

Pringle, J.W.S. (1957). Insect Flight (Cambridge: Cambridge University Press).

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

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

Rybak, F., Aubin, T., Moulin, B., and Jallon, J.-M. (2002). Acoustic communication in Drosophila melanogaster courtship: Are pulse- and sine-song frequencies important for courtship success? Can. J. Zool. *80*, 987–996.

Schlosser, G., and Wagner, G.P. (2004). Modularity in Development and Evolution (Chicago: University of Chicago Press).

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

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

Sweeney, S.T., Broadie, K., Keane, J., Niemann, H., and O'Kane, C.J. (1995). Targeted expression of tetanus toxin light chain in Drosophila specifically eliminates synaptic transmission and causes behavioral defects. Neuron *14*, 341–351.

Trimarchi, J.R., and Schneiderman, A.M. (1994). The motor neurons innervating the direct flight muscles of Drosophila melanogaster are morphologically specialized. J. Comp. Neurol. *340*, 427–443.

von Philipsborn, A.C., Liu, T., Yu, J.Y., Masser, C., Bidaye, S.S., and Dickson, B.J. (2011). Neuronal control of Drosophila courtship song. Neuron *69*, 509–522.

Weber, J.N., Peterson, B.K., and Hoekstra, H.E. (2013). Discrete genetic modules are responsible for complex burrow evolution in Peromyscus mice. Nature *493*, 402–405.

Wisser, A., and Nachtigall, W. (1984). Functional-morphological investigations on the flight muscles and their insertion points in the blowfly Calliphora erthrocephala (Insecta, Diptera). Zoomorph. *104*, 188–195.

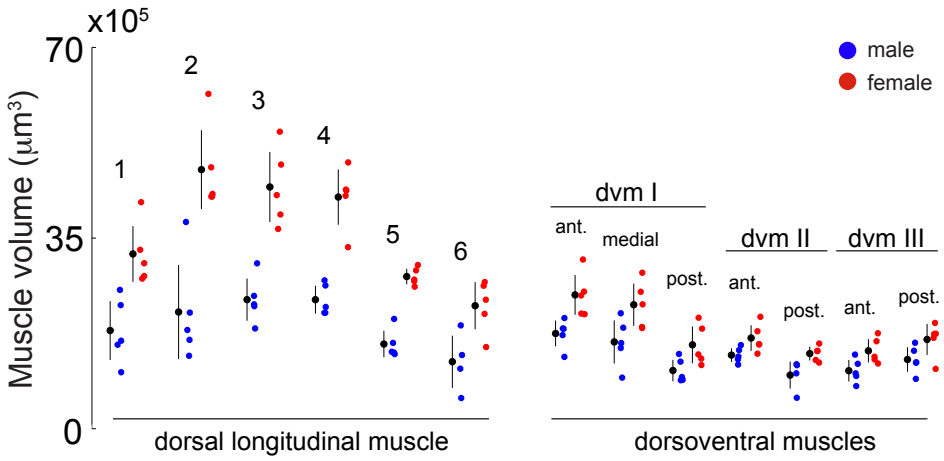

Figure S1 related to Figure 1.
The dorsal longitudinal (dlm) and dorsoventral (dvm) indirect power muscles are larger in females than in males, consistent with the difference in overall body size between the sexes. The volume of the each muscle fiber of the dlm and dvms was measured in males and females.

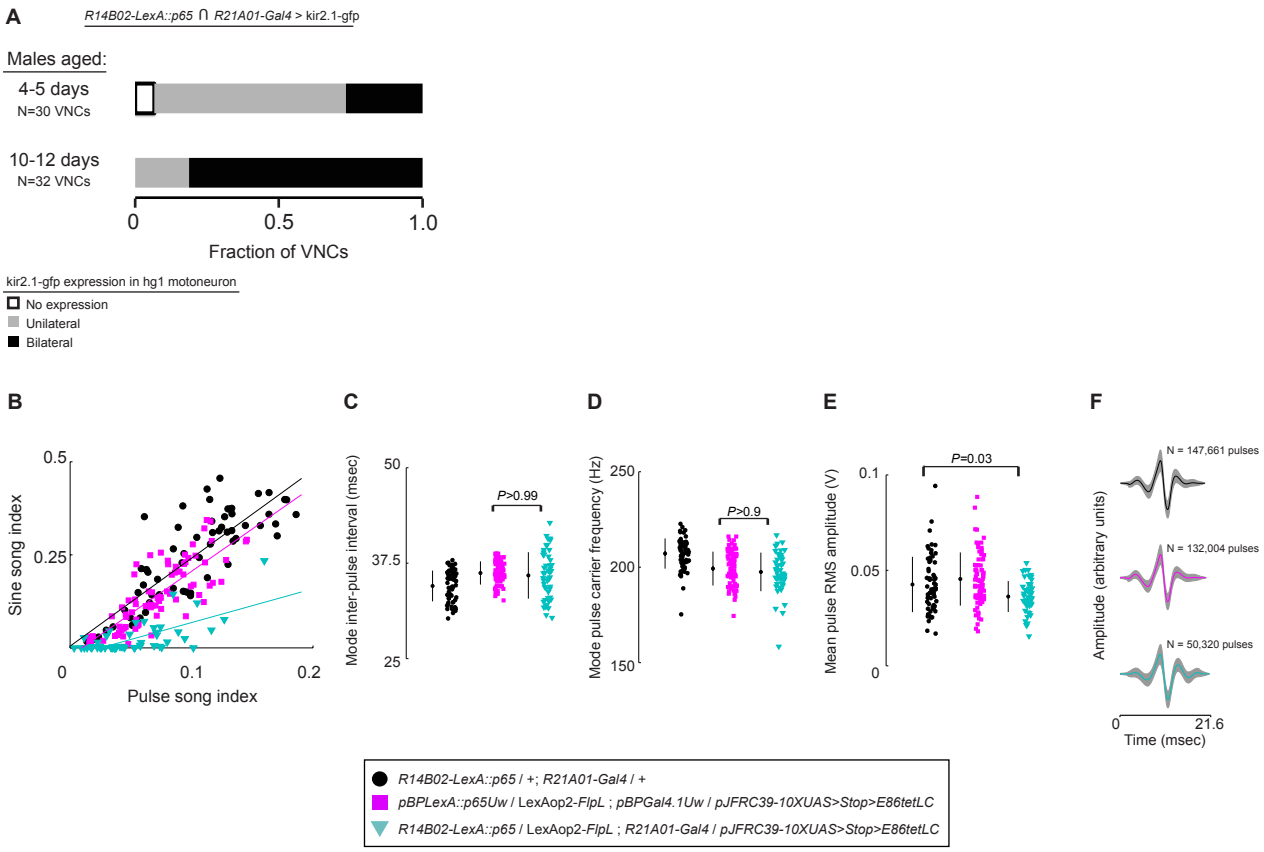

Figure S2, related to Figure 2.
(A) With the intersection between R14B02-LexA::p65 and R21A01-Gal4, the number of hg1 motoneurons that express kir2.1-GFP increases with the age of the fly. When males were aged for 4-5 days, most ventral nerve cords (VNCs) expressed the effector in only one of the paired hg1 motoneurons. After 10 days, however, both hg1 motoneurons expressed kir2.1-GFP in most VNCs. This is likely due to an increase in the number of cells that excise the transcriptional stop cassette in the kir2.1-GFP transgene over time. R14B02-LexA::p65 and R21A01-Gal4 individually drive expression in the hg1 motoneuron consistently across multiple individuals. Genotype of males: R14B02-LexA::p65 / pJFRC79-8XLexAop2-FlpL ; R21A01-Gal4 / pJFRC56-10XUAS-FRT>STOP>FRT-kir2.1-gfp. (B-F) Expression of tetanus neurotoxin light chain (E86tetLC) in the hg1 motoneuron inhibits the production of sine song, but leaves pulse song largely unaffected. (B) Males that express E86tetLC (Eisel et al., 1986; Sweeney et al., 1995; Pfeiffer et al, 2010) in the hg1 motoneuron display a strong reduction in their sine song index, but display pulse song indices comparable to controls. The difference in mean song index between the experimental (magenta triangles) and controls (black circles, orange squares) was significant for sine song, but not significant for pulse song, at the 0.05 level. (C-F) Expression of E86tetLC in the hg1 motoneuron fails to alter the (C) mode inter-pulse interval, (D) mode pulse carrier frequency, and (F) mean re-scaled pulse shape relative to males of control genotypes. (E) We note a difference in the mean pulse RMS amplitude between the experimental and controls. This is likely an artifact of genetic background. Significance was determined using a one-way ANOVA with a Tukey-Kramer post-hoc test for multiple comparisons.

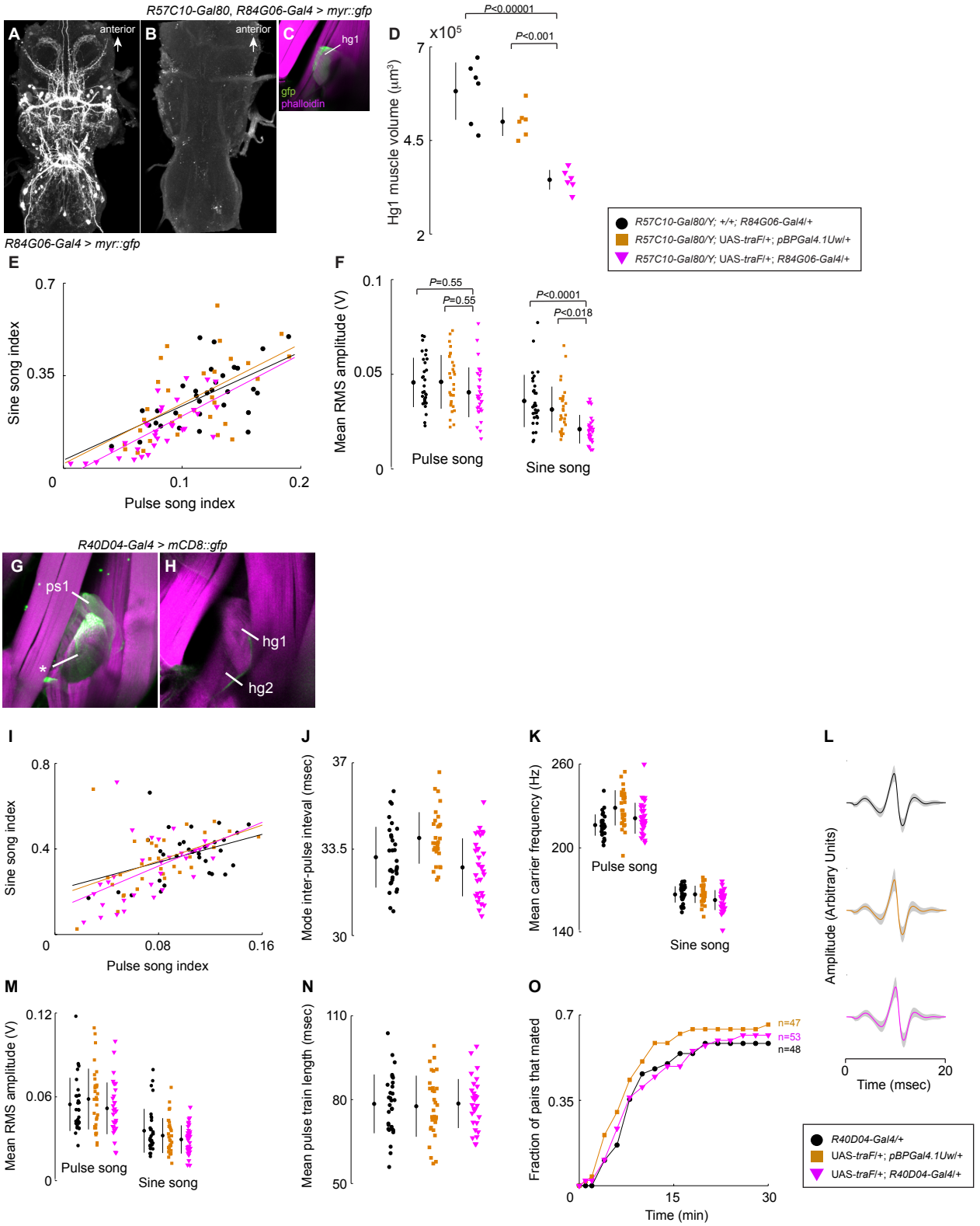
R57C10-Gal80, R84G06-Gal4 > myr::gfp
A
anterior
B
anterior
C
hg1
gfp
phalloidin
D
P<0.00001
P<0.001
x10^5
Hg1 muscle volume (µm³)
7
4.5
2
R57C10-Gal80/Y; +/+; R84G06-Gal4/+
R57C10-Gal80/Y; UAS-traF/+; pBPGal4.1Uw/+
R57C10-Gal80/Y; UAS-traF/+; R84G06-Gal4/+
R84G06-Gal4 > myr::gfp
E
Sine song index
0.7
0.35
0
0.1
0.2
Pulse song index
F
Mean RMS amplitude (V)
0.1
0.05
0
P=0.55
P=0.55
P<0.0001
P<0.018
Pulse song
Sine song
R40D04-Gal4 > mCD8::gfp
G
ps1
*
H
hg1
hg2
I
Sine song index
0.8
0.4
0
0.08
0.16
Pulse song index
J
Mode inter-pulse inteval (msec)
37
33.5
30
K
Mean carrier frequency (Hz)
260
200
140
Pulse song
Sine song
L
Amplitude (Arbitrary Units)
0
20
Time (msec)
M
Mean RMS amplitude (V)
0.12
0.06
0
Pulse song
Sine song
N
Mean pulse train length (msec)
110
80
50
O
Fraction of pairs that mated
0.7
0.35
0
15
30
Time (min)
n=47
n=53
n=48
R40D04-Gal4/+
UAS-traF/+; pBPGal4.1Uw/+
UAS-traF/+; R40D04-Gal4/+

Figure S3, related to Figure 3.
(A-F) Supressing traF expression the adult nervous system of R84G06-Gal4 > traF males using R57C10-Gal80 (nSyb-Gal80) (Harris, 2012). (A, B) R57C10-Gal80 fully suppressed reporter expression in the adult nervous system of R84G06-Gal4 > mCD8::GFP (VNC is shown only) but did not affect expression in the hg1 muscle (C). (D) The hg1 muscle of R57C10-Gal80, R84G06-Gal4, UAS-traF males is successfully feminized. P-values measured using student's t-test. (E, F) R57C10-Gal80, R84G06-Gal4, UAS-traF males sing normal amounts of pulse and sine song and display a significant reduction in sine song amplitude but not pulse song amplitude. P-values measured using a one-way ANOVA with Tukey-Kramer post-hoc test for multiple comparisons. (G,H) Reporter expression of R40D04-Gal4 in subsets of control flight muscles in the adult. R40D04-Gal4 drives reporter expression in all fibers of the ps1 flight muscle (and other flight muscles not shown), but not in the hg1 muscle. (*) refers to an unidentified muscle associated with ps1. This muscle is not targeted by dsxGAL4 (Figure 1D) or R84G06-Gal4 but is identified in R40D04-Gal4. Gal4 was crossed to pJFRC2-10XUAS-IVS-mCD8::GFP. Magenta = Phalloidin; Green = GFP. (I-N) Expression of traF in the ps1 muscle does not affect courtship song. The proportion of sine song amount relative to pulse song amount (I), mode inter-pulse interval (J), mean rescaled pulse shape (K), mean pulse and sine carrier frequency (L), mean RMS pulse and sine amplitude (M), and mean pulse train length (N) are similar between the experimental (magenta triangles) and controls (black circles and orange squares). (O) The fraction of male and female pairs that mated over 30 min.