名古屋大学
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Auditory experience modulates song preference and sexual choice in *Drosophila*

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Abstract

In birds and higher mammals, auditory experience during development is critical to discriminate sound patterns in adulthood. However, the neural and molecular nature of this acquired ability remains elusive. In fruit flies, acoustic perception has been thought to be innate. Here I find, surprisingly, that auditory experience of a species-specific courtship song in developing *Drosophila* shapes adult song perception and resultant sexual behavior. Preferences in the song-response behaviors of both males and females were tuned by social acoustic exposure during development. I examined the molecular and cellular determinants of this social acoustic learning and found that GABA signaling acting on the $GABA_A$ receptor Rdl in the pC1 neurons, the integration node for courtship stimuli, regulated auditory tuning and sexual behavior. These findings demonstrate that maturation of auditory perception in flies is unexpectedly plastic and is acquired socially, providing a model to investigate how song learning regulates mating preference in insects.

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Introduction

Auditory learning studies in humans and songbirds

Vocal learning in infants or juvenile birds relies heavily on the early experience of the adult conspecific sounds. In humans, early language input in the phase of auditory learning is necessary to stably form the ability of phonetic distinction and pattern detection in adulthood (Doupe & Kuhl, 1999; Kuhl, 2004). Similar to the language acquisition process of infants, the song learning process of songbirds has two phases, the auditory learning phase and the sensorimotor learning phase. In the first stage, the young songbirds listen to the tutor's song, usually from his father, and memorize it; in the second stage, the juvenile songbirds practice vocalization to imitate the memorized song, until the produced song is crystalized. This process resembles the language acquisition process of human beings. Because of the strong parallels between speech acquisition of humans and song learning of songbirds, and the difficulties to investigate the neural mechanisms of human language acquisition at cellular resolution, songbirds have been used as a predominant model to study how the early experience sculpts the auditory perception and sensorimotor output (Marler, 1970; Doupe & Kuhl, 1999; Bolhuis & Moorman, 2015). However, it has been challenging to address where the memory for auditory learning is stored and how the auditory memory constitutes the basis for auditory distinction.

A recent study shows that in juvenile songbirds, a small subset of neurons in the higher-order auditory cortex responded selectively to a song experienced in the early exposure, and thus were thought to be the neuronal substrate for song memory formation (Yanagihara & Yazaki-Sugiyama, 2016). However, it remains unclear how the neurons that represent the sound memory are incorporated into the higher-order integration center to direct the sensorimotor output.

The advantages of using fruit fly in auditory neuroscience

The auditory system of *Drosophila melanogaster* has attracted increasing attention in recent years, for the huge progress in understanding its underlying neural mechanisms (Clemens *et al.*, 2015; Kamikouchi *et al.*, 2009; Zhou *et al.*, 2015). These rapid progress largely benefits from the tremendous advantages that fly has for biological research for over one hundred years. Firstly, although seemingly small and simple, *Drosophila* can perform a rich repertoire of behaviors, including sleeping, temperature sensing, food-seeking, flight control, fighting, courtship, learning and memory *etc.*, many of which resemble that of mammals. Surprisingly and importantly, these complex behaviors are mediated by only about 100, 000 neurons in the adult fly brain (Ito *et al.*, 2013), whose composition is much simpler than that of mammals and humans. Secondly, over the long history of using *Drosophila* in research, many powerful tools have been created to track the structural and functional characteristics of specific cells. The most commonly used tool is the binary expression system, GAL4/UAS, which enables scalable expression of genes of interest in genetically defined subpopulations of cells. Several independent collections of GAL4 lines have been developed, including the GMR collection (Pfeiffer *et al.*, 2008; Jenett *et al.*, 2012) and the VT collection (Tirian & Dickson, 2017) *etc.*, which facilitates the rapid and economical screening of specific neurons. Thirdly, one distinct advantage of fly is its short reproduction cycle, which greatly accelerates scientific progress. Last but not the least, the significance of using fruit flies for neuroscience research lies not only on the shared basic principles that govern the resemble of the nervous system and the execution of its functions, but also on the genetic conservation that over 75 percent of genes involved in human diseases have equivalent in flies (Reiter *et al.*, 2001). Taken together, these traits endow fly unique advantages to be a powerful model organism to investigate the systems neuroscience, including the auditory perception.

The courtship song and its perception in *Drosophila*

Among all the sophisticated behavioral sequences flies exhibited, the courtship behavior is probably one of the most thoroughly studied behaviors. The courtship behavior of male flies consists of several steps: orientation, chasing, tapping, wing vibration, and licking (**Figure 1A**, Ishikawa & Kamikouchi, 2016).

The wing vibration of males during the courtship ritual produces a species-specific communication sound so-called the "courtship song", which has been studied most among the communication sounds in flies (Laturney & Billeter, 2014). The courtship song, thought to be one of the primary cue affecting the female's choice of the courting male (Crossley *et al.*, 1995; Villella & Hall, 2008), comprises two components: trains of pulses called pulse song and sequences of humming called sine song (von Schilcher, 1976a; **Figure 1B**, Eberl et al., 2016).

Although the function of the sine song is not well understood, sound playback experiments have demonstrated that the pulse song promotes copulation in paired flies (Kyriacou & Hall, 1982; Ritchie *et al.*, 1999). Playback of an artificial pulse song improves the receptivity of females, by reducing female rejection responses and shortening the latency to copulation (Bennet-Clark & Ewing, 1969; Rybak *et al.*, 2002; von Schilcher, 1976a, 1976b). An artificial pulse song also increases sexual behavior in males, and even without the presence of females, stimulates the "chaining behavior", in which males chase each other and form male-male chains (Crossley *et al.*, 1995; Yoon *et al.*, 2013). This chaining behavior presumably arises from the increase of sexual arousal, which induces a male to join the courtship strived by other nearby males (Eberl *et al.*, 1997). Intriguingly, the quality of the pulse song affects sexual arousal. The temporal gap between the pulses in the pulse song, namely the inter-pulse interval (IPI), differs among sibling *Drosophila* species (Cobb *et al.*, 1989; Ewing & Bennet-Clark, 1968; Ewing & Manning, 1967; **Figure 1B**,

Eberl et al., 2016) and is thought to be the crucial parameters for sexual arousal and species recognition. Indeed, *D. melanogaster* males prefer the pulse song with a certain range of IPIs including 35 ms, the mean IPI of this species (Yoon *et al.*, 2013; Zhou *et al.*, 2015). This bias towards the conspecific pulse song raises a question of how IPI selectivity is formed, the investigation of which would make the fruit fly a simple model to investigate the mechanism underlying sound perception.

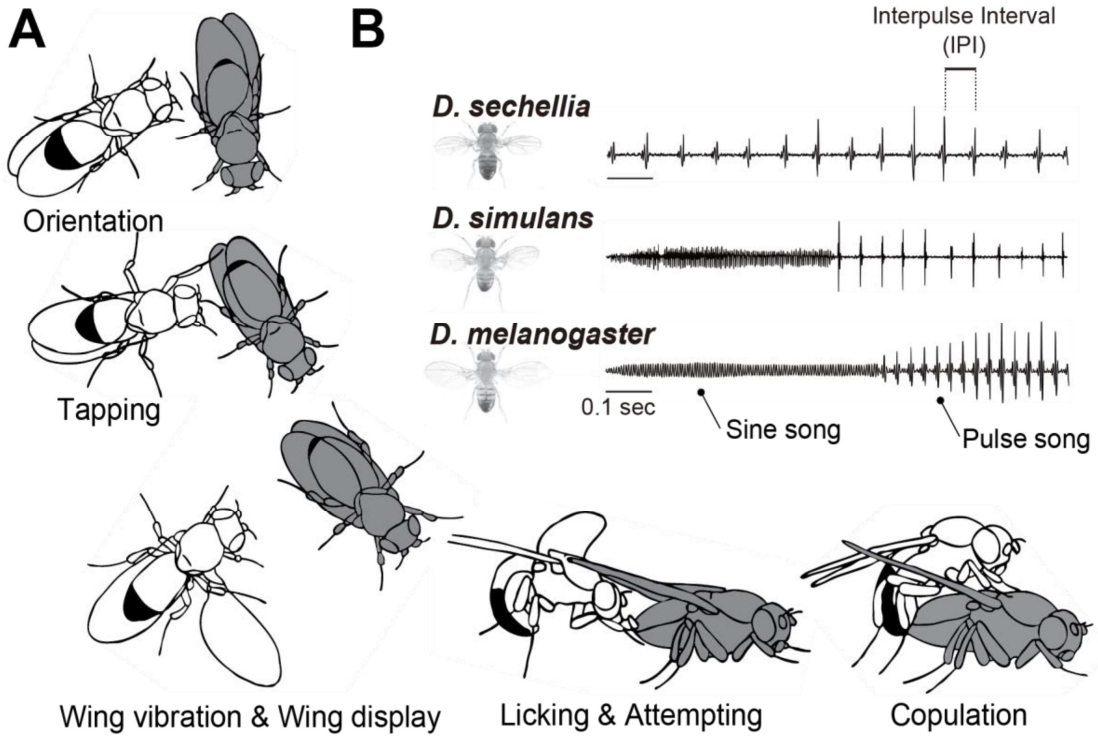


Figure 1. Courtship behavior and courtship song of *Drosophila*. (A) Stereotyped behavioral elements in courtship sequences of *D. melanogaster* (adapted from Ishikawa & Kamikouchi, 2017). White and gray colors represent males and females, respectively. (B) Composition of courtship song in three species (adopted from Eberl et al., 2016). Sine song and pulse song are denoted. Inter-pulse interval is shortened as IPI.

Fruit flies detect sound with antennal ears and, specifically, with mechanosensory neurons in Johnston's organ (JO) (Kamikouchi *et al.*, 2009). Regarding the two key features of *Drosophila* pulse song, intra-pulse frequency (IPF) and IPI, the antennal ear is mechanically tuned to detect the conspecific IPF, and the brain is hypothesized to process the conspecific IPI (Riabinina *et al.*, 2011). Recently an auditory pathway to perceive the pulse song that underlies the mating decision was delineated in *Drosophila* males. This pathway includes mechanosensory neurons in JO (JO neurons), aPN1 neurons (also known as AMMC-B1 neurons), vPN1 neurons, and pC1 neurons (Kamikouchi *et al.*, 2009; Vaughan *et al.*, 2014; Zhou *et al.*, 2015). In males, the pC1 cluster includes the courtship command-like P1 neurons. Multi-stage transformations by neurons in this auditory pathway refine the perception of IPIs until the response of the pC1 neurons matches the behavioral response to songs with different IPIs. These studies illustrate how the tuning towards the conspecific song with 35-ms IPI is achieved, and raise the question of how this IPI preference emerges.

Pioneering studies on zebra finches (Chen *et al.*, 2017; Cousillas *et al.*, 2006; Woolley *et al.*, 2010; Yanagihara & Yazaki-Sugiyama, 2016) and bats (Razak *et al.*, 2008) suggest that auditory selectivity in these animals develops in an experience-dependent manner. In accordance with this idea, I hypothesized that in young flies, IPI preference might also be refined by the experience of songs from nearby males, which might modulate the partner selection in sexual behaviors.

The influence of early experience on behaviors

Courtship involves multimodal sensations, motivation and decision-making, learning and memory, motor control, and more amazingly it takes into account the environmental information

and the individual experience (Greenspan, 2000; Pavlou & Goodwin, 2013). Although it is traditionally believed that the courtship behavior of flies is innate (Auer & Benten, 2016; Baker *et al.*, 2001; Hall, 1994), the programmed courtship machinery is susceptible to variables in development such as sleep deprivation (Kayser *et al.*, 2014), social isolation (Kim & Ehrman, 1998; Pan & Baker, 2014) and juvenile social experience (McRobert & Tompkins, 1988). The courtship behavior of flies is also affected by previous courtship outcomes, which has been used as a courtship conditioning assay to evaluate learning and memory in male flies (Keleman *et al.*, 2012; Koemans *et al.*, 2017). Moreover, by combining a sensitive system to record and analyze the courtship songs (Arthur *et al.*, 2013) with computational modeling, Coen *et al.* (2014) found that male courtship song can be modulated by dynamic sensory experience acutely. However, to what extent social experience and developmental plasticity contribute to the perception of the courtship song is not well understood.

Social interaction in group life influences circadian rhythms, fighting, courtship and collective behavior in fruit fly (Levine *et al.*, 2002; Wang *et al.*, 2008; Griffith & Ejima, 2009; Ramdya *et al.*, 2015). However, few people have investigated how group life experience affects behaviors induced by individual sensory cue.

In this study, I examined whether the auditory experience tuned the IPI selectivity in *Drosophila*. Based on the sexual behaviors of males and females upon song playback, I established a new behavioral paradigm in which the flies were exposed to specific sound patterns for long periods before their IPI preference was evaluated. Surprisingly, I found that the experience of hearing conspecific song, but not heterospecific song, tuned IPI perception in both males and female flies. Furthermore, I found that this experience-dependent IPI tuning relied on GABA synthesis, and that the ionotropic $GABA_A$ receptor of pC1 neurons gated IPI tuning in females. Our discovery

establishes a new and simple system to study how the experience-dependent auditory plasticity is incorporated into higher-order integration center to modulate sexual behaviors at the molecular and cellular levels.

Materials and methods

Key resources table				
Reagent type (species) or resource	**Designation**	**Source or reference**	**Identifiers**	**Additional information**
gene (*Drosophila melanogaster*)	*Rdl*	NA	FLYB: FBgn0004244	
gene (*Drosophila melanogaster*)	*GAD1*	NA	FLYB: FBgn0004516	
strain, strain background (*Drosophila melanogaster*)	*Canton-S*	other		gift from K. Ito
strain, strain background (*Drosophila melanogaster*)	*Gad1-GAL4*	PMID: 12408848		gift from K. Ito
strain, strain background (*Drosophila melanogaster*)	*UAS-Gad1 RNAi*	Vienna *Drosophila* Resource Center	VDRC ID: 32344; RRID: FlyBase_FBst0459538	
strain, strain background (*Drosophila melanogaster*)	*w1118*	Vienna *Drosophila* Resource Center	VDRC ID: 60000	
strain, strain background (*Drosophila melanogaster*)	*UAS-Rdl RNAi*	Bloomington *Drosophila* Stock Center	BDRC: 52903; RRID: BDSC_52903	
strain, strain background (*Drosophila melanogaster*)	*TRiP RNAi*	Bloomington *Drosophila* Stock Center	BDRC: 36304; RRID: BDSC_36304	
strain, strain background (*Drosophila melanogaster*)	*tubP>GAL80>; NP2631-GAL4/CyO; dsxFLP/TM2*	PMID: 27185554		gift from D. Yamamoto
software, algorithm	ChaIN (ver. 3)	PMID: 28701929		

Experimental animals

D. melanogaster was raised on standard yeast-based media at 25ºC and in 40% to 60% relative humidity on a 12-hour light/dark cycle. *Canton-S* (Hotta-lab strain, a gift from K. Ito) was used as a wild-type strain. For knockdown experiments, the following transgenic flies were used: *w; Gad1-GAL4* (Ng *et al.*, 2002) (a gift from K. Ito), *UAS-Gad1 RNAi* (GD line; RRID: FlyBase_FBst0459538) and its control line w^{1118} (VDRC ID: 60000) (Vienna *Drosophila* Resource Center), *UAS-Rdl RNAi* (VALIUM20; RRID: BDSC_52903) and its control line *TRiP RNAi* (RRID: BDSC_36304) (Bloomington *Drosophila* Stock Center), and *tubP>GAL80>; NP2631-GAL4/CyO;* dsx^{FLP}*/TM2* (Koganezawa *et al.*, 2016) (a gift from D. Yamamoto). Genotypes of flies used for each experiment are listed in **Supplementary file 1**. Flies that were 6 to 7-day after eclosion were used for behavioral tests. The wings of males were clipped on the day of eclosion, unless otherwise noted.

The neurons labeled by *Gad1-GAL4* show essentially consistent distributions with those identified by *in situ* hybridization against *Gad1* mRNA (Okada *et al.*, 2009). Silencing these *Gad1-GAL4* positive neurons in the adult stage did not affect fly survival (Muthukumar *et al.*, 2014). The *Gad1* RNAi used in this study was reported to knock down the *Gad1* mRNA level to approximately 60% of wild type (Jeong *et al.*, 2016). In our study, no obvious behavioral defects were observed in *Gad1* knockdown flies, and male *Gad1* knockdown flies still responded normally to conspecific courtship song when tested at 7 days after eclosion (**Supplementary figure 1**). The efficacy of *UAS-Rdl RNAi* has been demonstrated (Franco *et al.*, 2017; Koganezawa *et al.*, 2016).

Male-male chaining test

For males, the sound-evoked chaining test was performed as described (Yoon *et al.*, 2013) (**Figure 2**). Six flies were loaded into one lane (**Figure 2A**) of an acoustic behavior chamber (Inagaki *et al.*, 2010) and placed in front of a loudspeaker with a distance of about 11 cm (**Figure 2B**). As the test song, the artificial pulse song with 35-ms IPI or 75-ms IPI as used in the training session was delivered from a loudspeaker with an amplifier (Lepai LP-2020A+NFJ Edition, Bukang Electrics, Jieyang, China). Mean baseline-to peak amplitude of its particle velocity was 9.2 mm/s (Ishikawa *et al.*, 2017). The flies' contour was outlined by a backlit LED light box (ComicMaster Tracer, Too Marker Products, Tokyo, Japan), and captured by a monochrome camera (Himawari GE60, Library, Tokyo, Japan) with a zoom lens (Lametar 2.8/25 mm, Jenoptik GmbH, Jena, Germany) (**Figure 2C**). Flies were not exposed to sound for 5 min, and then exposed to an acoustic stimulus that lasted for 6.5 min. The recorded video was then down-sampled to 1 Hz and analyzed off-line using ChaIN method (Yoon *et al.*, 2013). I measured the number of only the follower flies in chains as the chain index using ChaIN version 3 (Ishikawa *et al.*, 2017), which is available at http://www.bio.nagoya- u.ac.jp/~NC_home/chain_E.html. The chain index between 5 min and the end of the sound playback were summed for comparison (summed chain index).

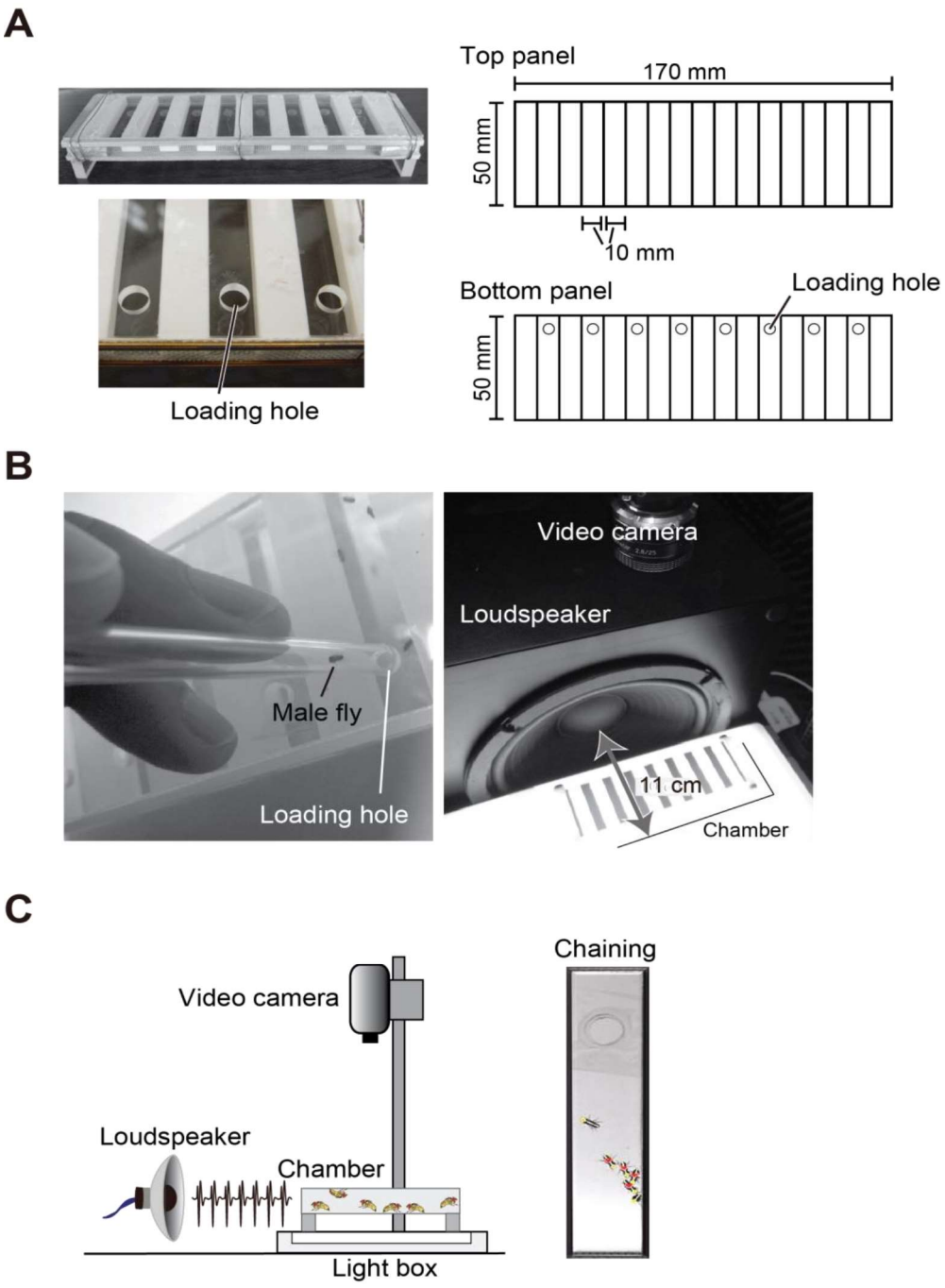


Figure 2. Setup for chaining behavior. (A) Parameters of the chambers for chaining assays. (B) Six males are loaded into a chamber by using an aspirator. The chamber is placed in appropriate place in front of a loudspeaker to adjust the sound volume received by the flies. (C) Recording of chaining behavior. The chaining chamber was illuminated by the backlight. Song was delivered from a loudspeaker. Appropriate song typically drove the male flies to

form male-male chains (chaining). One captured image is given to show the detected flies in a chain in the analysis. Males in a chain are marked with red dots.

Male experiment without the training session

In this experiment, male flies were subjected to chaining test without given sound exposure as training. Virgin males were collected within 10 hours after eclosion, and then housed in three different conditions: (1) grouped without wings, (2) grouped with intact wings, and (3) single-reared with intact wings. Flies housed in the first condition (grouped without wings) were prepared as described previously (Yoon *et al.*, 2013). In brief, their wings were clipped with forceps during brief anesthesia on ice soon after eclosion and the males were kept in a male-only group of 6 to 8 flies. Flies housed in the second (grouped with intact wings) and third (single with intact wings) conditions were kept with intact wings for 5 to 6 days, either in a group of 6 to 8 male flies or singly. Only one day before the test, the wings of flies housed in the second and third conditions were also clipped. The chaining behavior of all the males housed in three conditions was tested 6 to 7 days after eclosion.

Training

Training session started on the day of eclosion (**Figure 3A**). Adult virgin males and females were collected within 8 hours after eclosion under anesthesia on ice, and the wings of males were clipped. Each fly, whether a male or a female, was introduced gently to a training capsule and placed in front of a loudspeaker (FF225WK, FOSTEX, Foster Electric Company, Tokyo, Japan). As experienced group, flies were continuously exposed to one particular training song for 6 days of training (**Figure 3B**). Training song was an artificial pulse song comprised of the repetition of

1-s pulse burst and a subsequent 2-s pause, in which the pulses in the pulse burst had an IPI of 35 ms (“conspecific song”) or an IPI of 75 ms (“heterospecific song”) (Yoon *et al.*, 2013). Intrapulse frequency (IPF) of both IPI songs was set to be 167 Hz. As naïve group, flies were placed in front of the loudspeaker for 6 days after eclosion but not given any sound exposure.

During the training session, each fly was accommodated singly in a training capsule. A training capsule was made of a glass tube cut out from a Pasteur pipette, two pipette tips, mesh and mending tape (**Figure 3C**). Pipette tips, whose volumes are 1 ml, were cut to make the larger ends about 20 mm long. Two of these 20-mm pieces were hooked to a glass tube at its both ends. The size of a glass tube was about 27 mm long, with the internal diameter of 5.2 mm and the external diameter of 6.5 mm. Both exits of the glass tube were sealed with a piece of mesh stocking (made of nylon and polyurethane), which allowed free passage of air but not the fly. A thin layer of fly food, standard *Drosophila* yeast-based medium, was paved at the bottom of the glass tube. The food in each capsule was renewed every 36 hours.

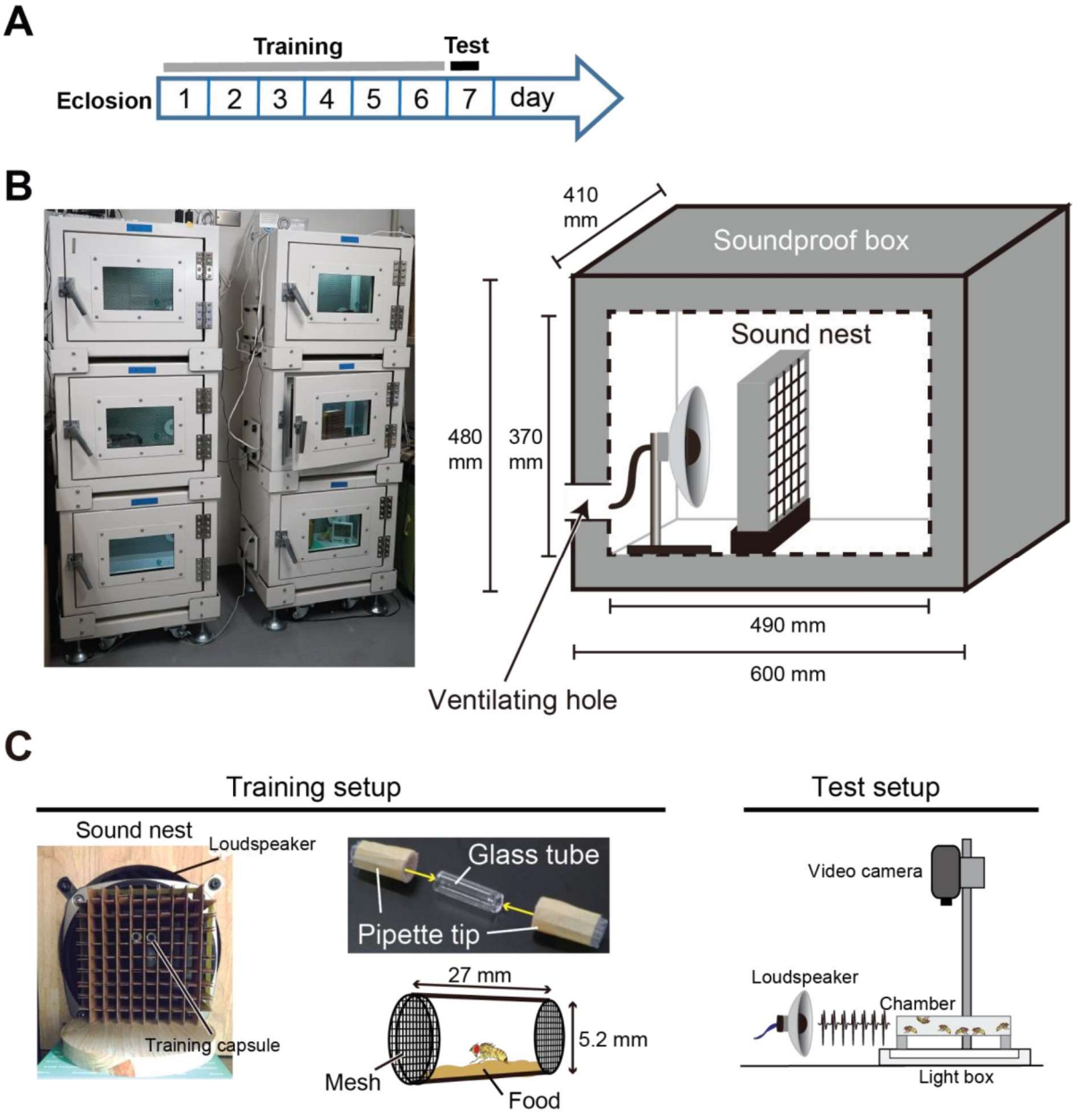


Figure 3. Experimental scheme and setup for the training. (A) Scheme for the training and test sessions. (B) Soundproof boxes for the training (left), each of which contains a loud-speaker and sound nest (right). (C) Setup for the training and chaining test. In the training session, single-housed flies were exposed to a training song for the first 6 days after eclosion. Training capsules that contain one fly each is placed in the sound nest. The training capsule consists of two pipette tips sealed with stocking mesh and a glass tube. The chaining test setup is as the same as the one used in Figure 2.

Training capsules were placed within latticework of a container, named a “sound nest” (**Figure 3B and 3C**). One of the mesh-ends of each training capsule faced the loudspeaker, so that sound could be delivered to each chamber with minimal disturbance. The distance between loudspeaker and the near end of the training capsules was 24 mm. All the setups for the training were placed into a soundproof box (W450mm×L450mm×H450mm, **Figure 3B**).

Sound playback was controlled by the Windows Media Player on a tablet PC (Windows 8.1, Diginnos DG-D08IWB, Dospara, Tokyo, Japan), and delivered by a loudspeaker with a digital power amplifier (Lepai LP-2020A+NFJ Edition, Bukang Electrics, Jieyang, China). The mean baseline-to peak amplitude of sound particle velocity was 8.6 mm/s when measured at the near end of the training capsules, and 6.6 mm/s at the far end of the training capsule. The sound particle velocity was identical for all training sounds.

After the 6-day training, male flies were collected into a group of seven without anesthesia, and transferred to a vial containing fly food. Female flies were still kept in the training capsules singly without sound playback until the copulation test. After one-night rest without any sound playback, all flies (7 days after eclosion) were subjected to the behavioral tests in the next morning (ZT 0-3).

Female copulation test

For females, their receptivity was evaluated by the time course of cumulative copulation and the latency to accept copulation. To monitor the training effect on females, I paired naïve or trained females with the naïve wild-type males (7-day old, wings clipped) in the copulation test. The test chamber, made of plexiglass, was made up by eight circular chambers (15 mm diameter, 3 mm depth) with their bottom covered with mesh for sound penetration (**Figure 4A**). A pair of female and male flies was gently aspirated into one of the eight chambers without anesthesia (**Figure 4B**). A pulse song was delivered to flies by a loudspeaker (Daito Voice AR-10N, Tokyo Cone Paper MFG. Co. Ltd. Saitama, Japan) placed 3.9 cm underneath the chambers. The sound particle velocity was 9.2 mm/s. Song playback was started at the same time as video recording was started. Behaviors of flies were recorded for 30 min with a web camera (Logicool® HD Webcam C270, Tokyo, Japan) (**Figure 4C**). Copulation timing was analyzed manually from the video playback. Inhibition index = (copulation ratio^{Naïve} - copulation ratio^{Experienced}) / copulation ratio^{Naïve}.

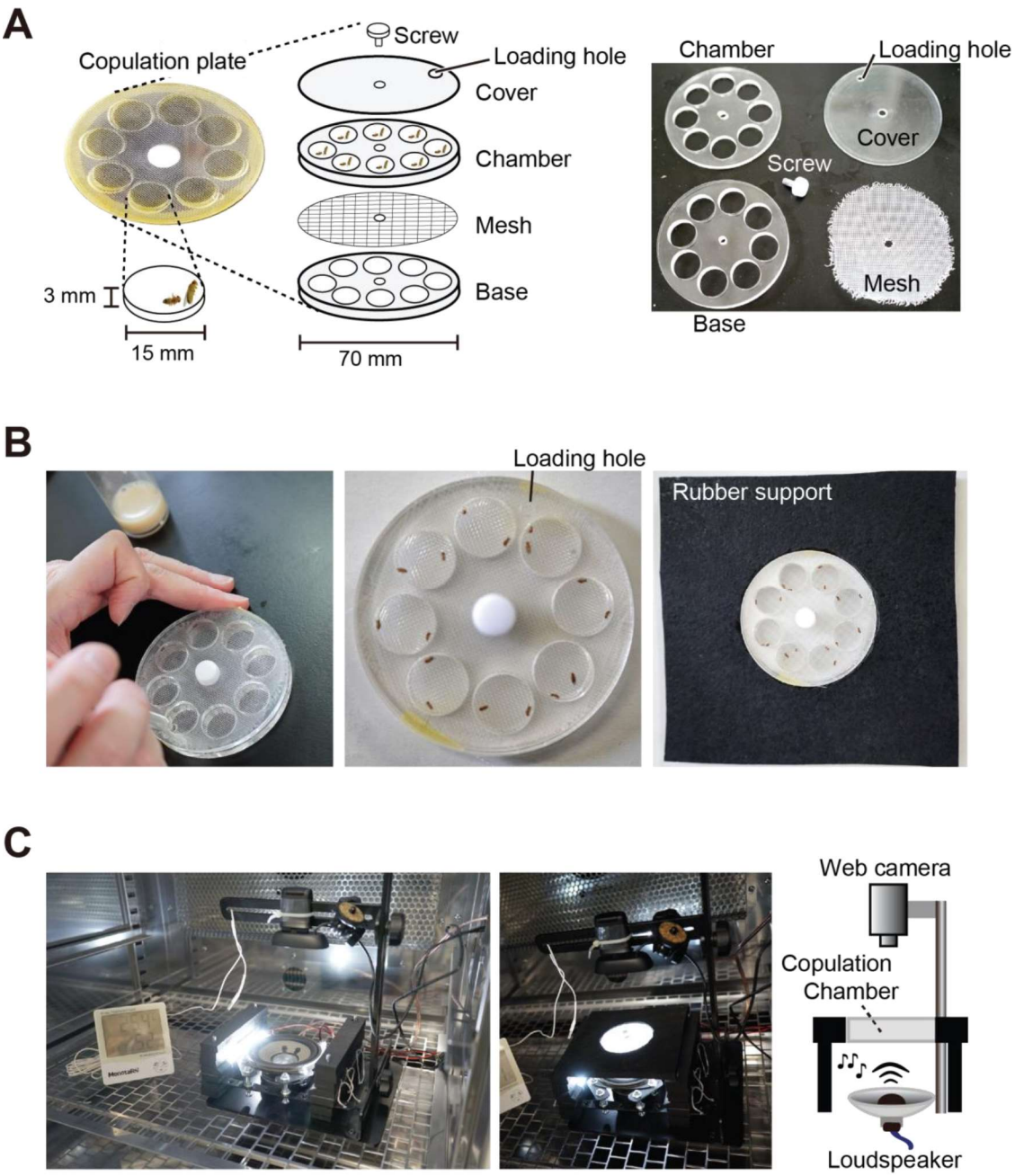


Figure 4. Setup for the copulation test. (A) Copulation plate for the female copulation assay. (B) Loading flies into a chamber. A fly pair is loaded into one of eight copulation chambers by using an aspirator. Each chamber has a test female and a partner male. The copulation plate is inserted into a hole of the rubber support. (C) Assay setup. Rubber support holding the copulation plate is placed over a loudspeaker with appropriate distance.

Statistical analysis

Statistical analysis was performed with R (version 3.0.3). Mann-Whitney U test (two-tailed) was used to compare two groups of samples in the chaining behavior. Kaplan-Meier curves were generated using R and Log rank test was performed to compare females' accumulative copulation rate between two groups in the copulation tests. The Kruskal–Wallis test (two-tailed) followed by Scheffe's test was used to compare the copulation latency. The detailed statistical results are shown in **Supplementary file 2**. The boxplot was drawn with ggplot2 package of R. Boxplots display the median of each group with the 25th and 75th percentiles and whiskers denote 1.5x the inter-quartile range.

Results

Experience-dependent tuning of IPI preference in male fruit flies

Previous study suggests that in *Drosophila melanogaster*, IPIs ranging from 35-ms to 75-ms induce the sexual behavior of males vigorously (Yoon *et al.*, 2013). Since the mean IPI of the courtship song in *D. melanogaster* is about 35 ms (Cowling & Burnet, 1981), it seems noteworthy that 75-ms IPI, which is out of the *melanogaster* IPI range (Arthur *et al.*, 2013) and likely comes from another *Drosophila* species (for example, an evolutionarily far species *Drosophila rosinae* in *fasciola* subgroup) (Costa & Sene, 2002), induces sexual behavior as strongly as 35-ms IPI. In searching for the cause why 75-ms IPI induced such significant response in *D. melanogaster*, I noticed that male flies that showed similar levels of response to both 35-ms and 75-ms IPI songs had been wing-clipped soon after eclosion and thus lacked experiences of wing-emitted sound (Yoon *et al.*, 2013). Because *Drosophilid*s gather in groups in feeding sites (Powell, 1997), I reasoned that flies probably had experiences of the courtship songs of other males in social interactions. How courtship song experience affects the song response is never investigated in *D. melanogaster* and it is plausible that male flies that were clipped of wings soon after eclosion showed abnormally strong response to 75-ms IPI. To test this hypothesis, I tested how the auditory experience affected the IPI selectivity.

First, to evaluate how the experience of wing-emitted sound from other males affects later acoustic preference, I measured the chaining behavior of males (**Figure 2**) that were reared for five to six days in the following three conditions: (1) grouped flies without wings, (2) grouped flies with intact wings, and (3) single-reared flies with intact wings (**Figure 5**). The wings of males in the latter two groups were clipped only one day before the chaining test. In the chaining tests

all the male flies had no wings, so that the sounds only come from the speaker. For the chaining test, I used two types of artificial pulse songs: 35-ms IPI and 75-ms IPI songs to represent conspecific and heterospecific songs, respectively. Consistent with our previous report (Yoon *et al.*, 2013), flies grouped without wings responded strongly to both conspecific and heterospecific songs (**Figure 5A**). In contrast, flies grouped with wings preferred conspecific over heterospecific song (**Figure 5B**). This selective response was not observed in single-reared flies with wings (**Figure 5C**). Together, these results indicate that the presence of other males with wings is required to shape the IPI preference in males.

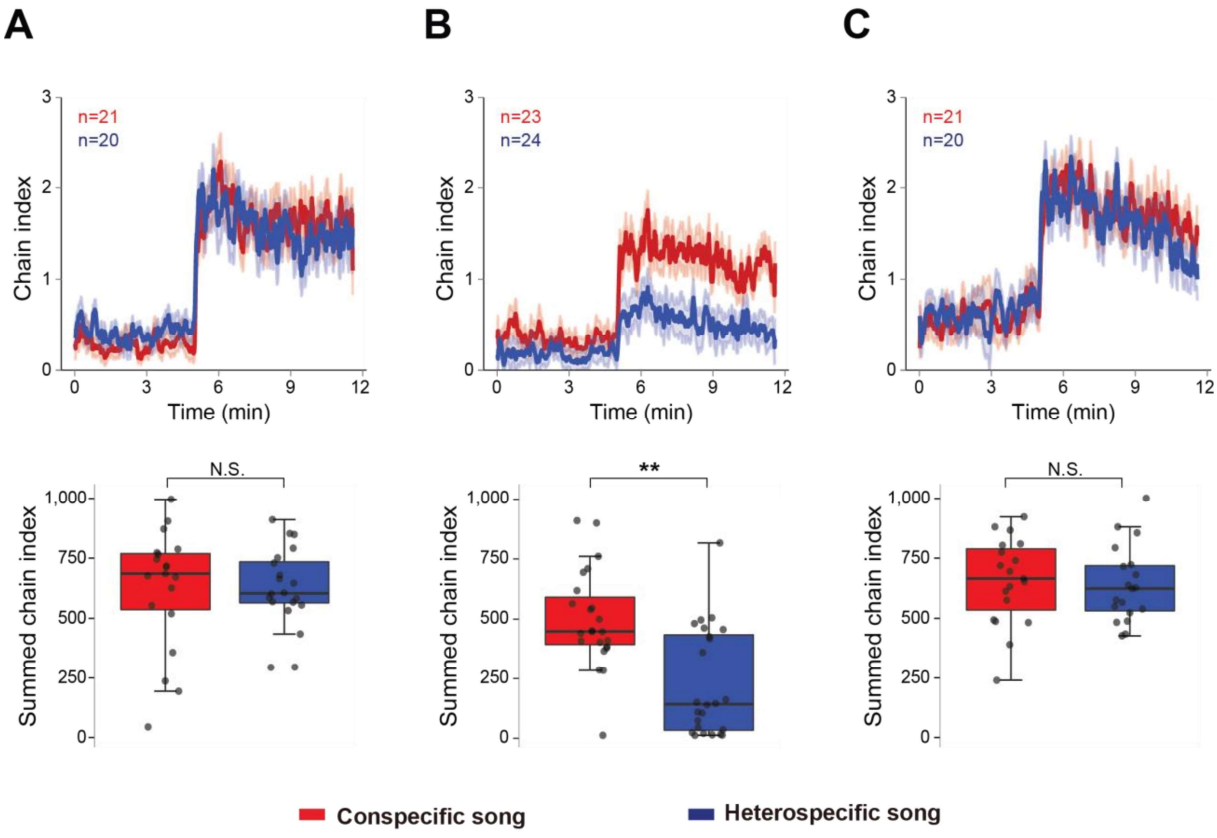


Figure 5. Social interaction shapes the preference to the song. Chaining response of naïve male flies that were housed in different experimental conditions, grouped without wings (A), grouped with intact wings (B), and single-reared with intact wings (C). The time-courses of the chain index in response to playback of conspecific song (red) and heterospecific song

(blue) are shown. Sound playback starts at 5 min. The bold line and ribbon represent the average value and standard error, respectively. The box plot shows the summed chain index between 5-min and 11.5-min. Boxplots display the median of each group with the 25th and 75th percentiles and whiskers denote 1.5x the inter-quartile range. N.S., not significant, $P>0.05$; $^{**}P<0.01$; Mann-Whitney U test. n, number of behavioral chambers examined.

Second, to investigate whether the prior sound experience modifies the IPI selectivity, I established a training procedure containing a training session and a subsequent test session (**Figure 3**). In the training session, I exposed wing-clipped single males to conspecific or heterospecific artificial song for 6 days after eclosion, which served as "auditory experience" to flies. Naïve flies were also prepared in the same manner as experienced flies except for the exposure to the training sound. In the test session, I monitored their behavioral performance using chaining test. Conspecific song induced a strong chaining behavior of males in both naïve and experienced groups, irrespective of the training sound (**Figure 6A**). In contrast, heterospecific song induced a strong chaining behavior in naïve but not in experienced flies when flies were trained with conspecific song (**Figure 6B, red line**). Flies trained with heterospecific song retained their response to the heterospecific song (**Figure 6B, orange line**). These results indicate that male flies selectively diminish the response to heterospecific song only after having experienced conspecific song.

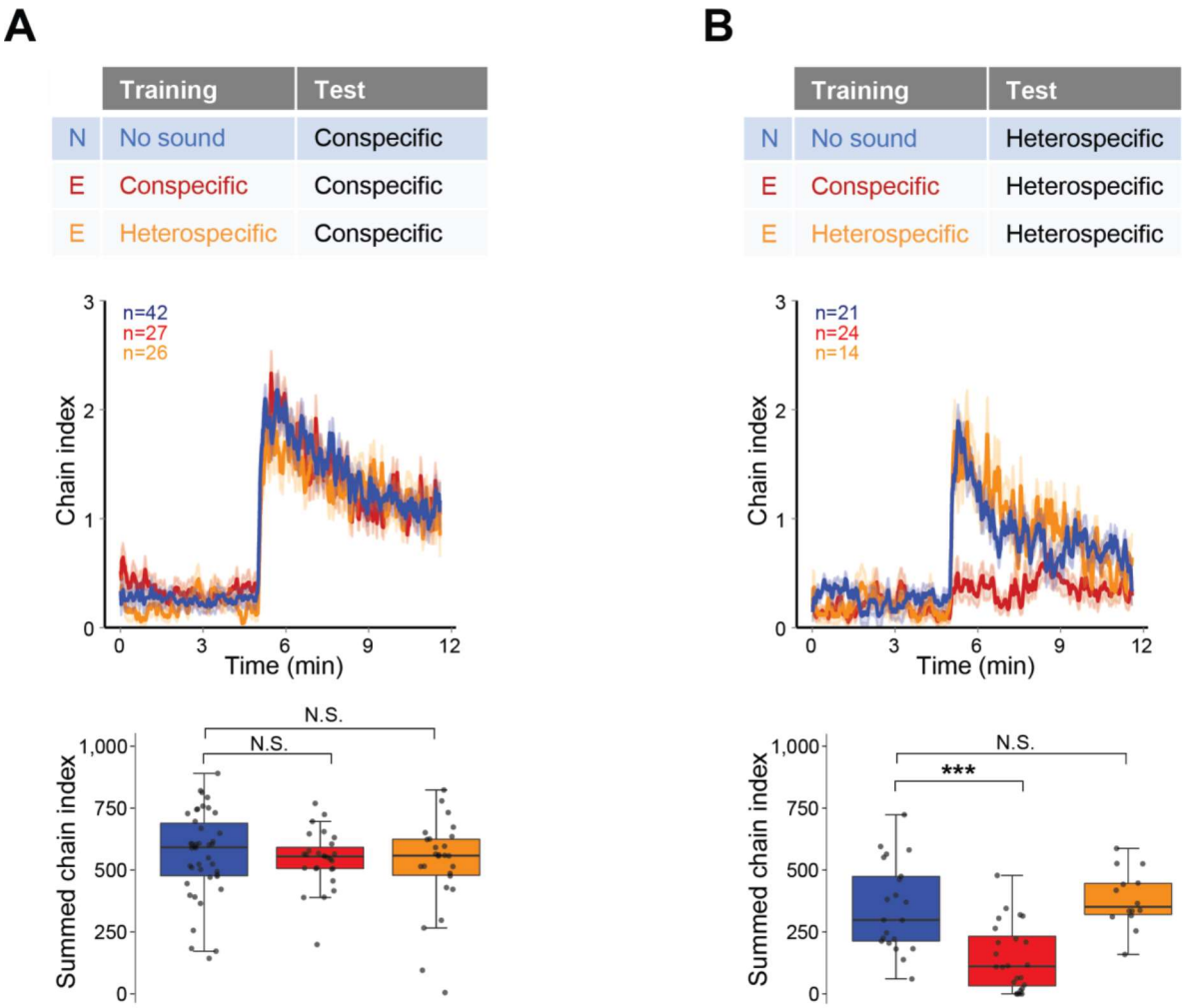


Figure 6. Fine-tuned song response of males after the training. (A, B) Chaining response to the conspecific song (A) or heterospecific song (B) after training. N, naïve group with no sound training (blue); E, experienced group with conspecific song training (red) or heterospecific song training (orange). The way to show the time courses of chaining behavior and the boxplot is similar to that depicted in Figure 5. N.S., not significant, P>0.05; ***P<0.001; Mann-Whitney U test versus naïve group.

Experience-dependent tuning of IPI preference in female fruit flies

Females decide whether to mate with courting males (Dickson, 2008). To test whether the mating decision of females could also be tuned by a prior auditory experience, I probed song effects on copulation behavior (**Figure 4**). First, I examined the copulation behavior of paired flies by pairing naïve females with naïve wing-clipped males, to confirm the IPI selectivity in promoting copulation as reported (Bennet-Clark & Ewing, 1969). Compared with the test condition without sound playback, either conspecific (35 ms) or heterospecific (75 ms) song playback promoted copulation significantly (**Figure 7**). Both songs promoted copulation equally, showing that naïve females had no selectivity between these two songs. In contrast, playback of songs with shorter (15 ms) or longer (105 ms) IPIs did not promote copulation. These results are consistent with previous findings that only songs with certain IPIs in a specific range promoted copulation(Bennet-Clark & Ewing, 1969).

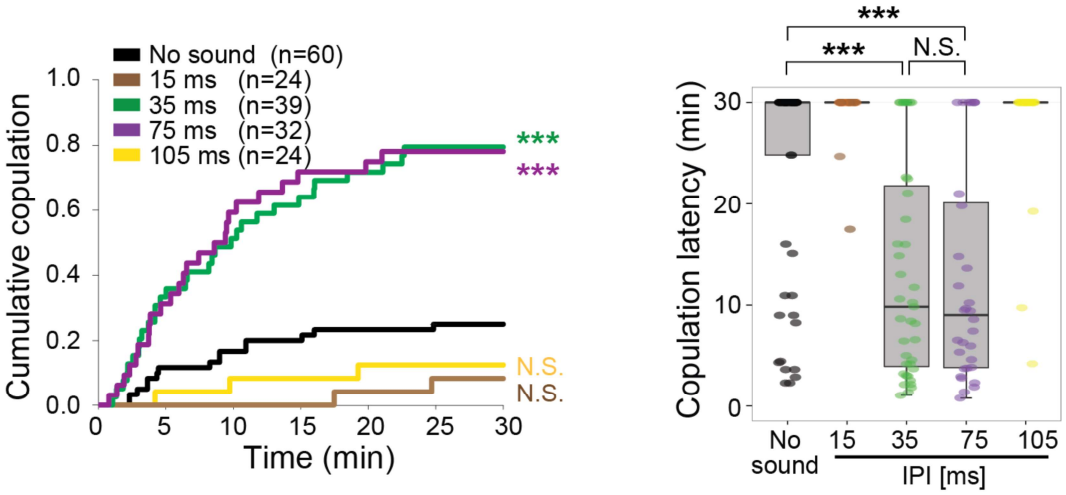


Figure 7. Playback of pulse song promotes copulation in wild-type fly pairs. Cumulative copulation rate and copulation latency with playback of artificial pulse songs of different inter-pulse interval (IPI) are shown. Copulation latency represents the latency to accept cop-

ulation in the 30-min observation period. The box plot shows the summed chain index between 5-min and 11.5-min. Boxplots display the median of each group with the 25th and 75th percentiles and whiskers denote 1.5x the inter-quartile range. N.S., not significant, $P>0.05$; ***$P<0.001$; Log rank test versus no sound group (left panel); Kruskal–Wallis test followed by Scheffe's test (right panel). n, number of fly pairs examined.

Then I tested whether previous sound experience affects female copulation behavior. I trained the females with conspecific or heterospecific song, in the same way as for males (**Figure 3**), and then tested the female receptivity to a mute male with song playback (**Figure 4**). To examine the song training effect on females, naïve or trained females were paired with naïve wild-type males that were wing-clipped for copulation test. With playback of conspecific song, females accepted mating with mute males regardless of the song experience during the training session (**Figure 8A**). In contrast, with heterospecific song playback, the copulation rate dramatically decreased in females trained with the conspecific song (**Figure 8B, red line**). Training with heterospecific song did not affect the receptivity to the heterospecific song (**Figure 8B, orange line**). Both the significant increase of the copulation latency (**Figure 8C**) and the highest inhibition index of the copulation rate (**Figure 8D**) supported the conclusion that training of the conspecific song reduced female acceptance during the heterospecific song test.

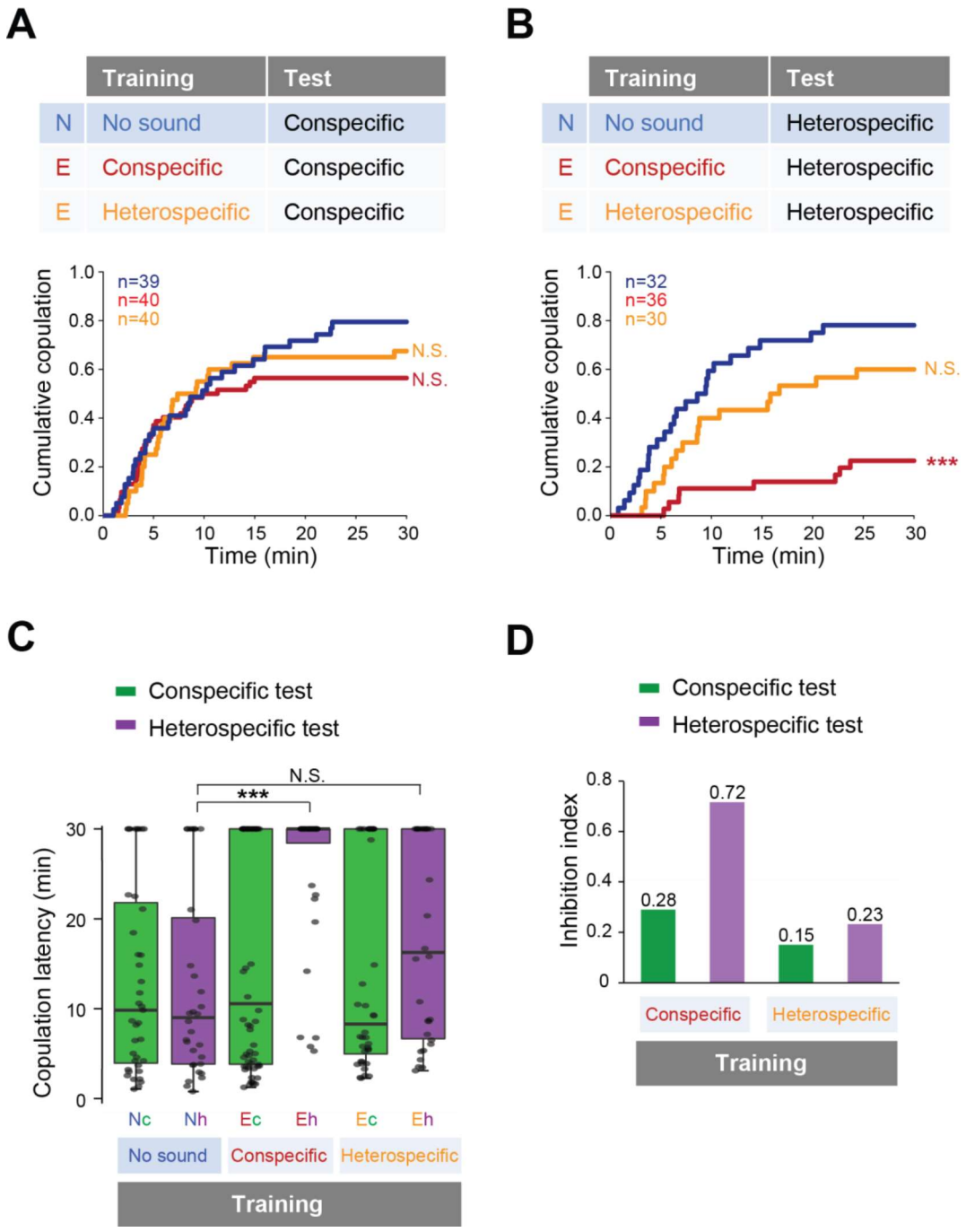


Figure 8. Fine-tuned song response of females after the training. (A, B) Cumulative copulation rate in the conspecific song test (A) or heterospecific song test (B) after training females. Naïve group (no sound during training) and experienced groups (trained with conspecific song or heterospecific song) are shown. The color code is the same with that in Figure

5. N, naïve; E, experienced. (C) Copulation latencies of females under playback of conspecific song (green bars) or heterospecific song (purple bars). Nc and Nh, naïve flies tested with conspecific and heterospecific songs, respectively. Ec and Eh, experienced flies tested with conspecific and heterospecific songs, respectively. (D) Inhibition index under playback of conspecific song (green bars) or heterospecific song (purple bars) after training of conspecific song or heterospecific song. Inhibition index = (copulation ratio^{Naïve} - copulation ratio^{Experienced})/ copulation ratio^{Naive}. N.S., not significant, $P>0.05$; ***$P<0.001$; Log rank test versus naïve group (A, B); Kruskal–Wallis test versus naïve group (C).

Taken together, previous experience of the conspecific song renders females more selective about the song when deciding to accept mating. Apparently, prior experience of the conspecific song fine-tunes the selectivity of the sound-evoked behavioral responses of both males and females, while prior experience of the heterospecific song does not.

Experience-dependent IPI tuning requires GABA synthesis

I next sought to identify the mechanism of this experience-dependent tuning of auditory behavior. In mammals, auditory experience governs the maturation of GABAergic inhibition that tunes the perception of sound in the auditory cortex (Dorrn *et al.*, 2010). Thus I asked whether GABA signaling was involved in the auditory plasticity that I found, by testing the receptivity of female flies with reduced GABA synthesis. I knocked down *Glutamic acid decarboxylase 1* (*Gad1*), a gene encoding the major GABA synthesis enzyme, in putative GABAergic neurons (*Gad1-GAL4>UAS-Gad1 RNAi*; see Materials and methods for fly strains) in females, and trained them with conspecific or heterospecific song. The copulation tests with conspecific song playback revealed that both *Gad1* knockdown and control (*Gad1-GAL4>RNAi* background w^{1118}) females in experienced groups responded to conspecific song as strongly as naïve females, irrespective of training experience (**Figure 9A**).

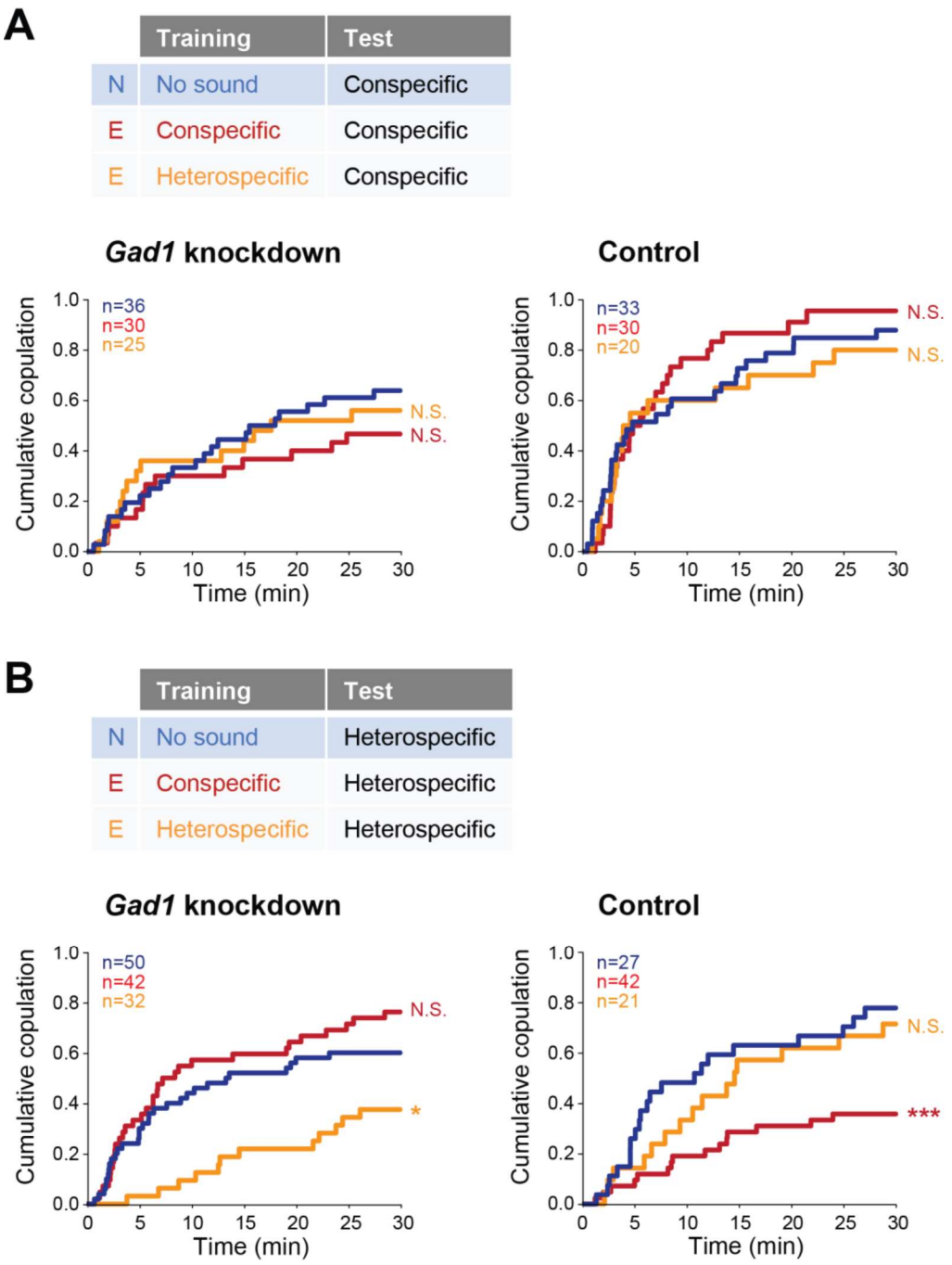


Figure 9. Involvement of *Gad1* in the experience-dependent song preference in females. (A, B) Cumulative copulation rate in the conspecific song test (A) or heterospecific song test (B) after training *Gad1* knockdown (left) and control (right) females. Naïve group (no sound training) and experienced groups (conspecific song training and heterospecific song training)

are shown. The color code is the same with that in Figure 5. N, naïve; E, experienced. N.S., not significant, $P>0.05$; *$P<0.05$; ***$P<0.001$; Log rank test versus naïve group.

In contrast, when I used heterospecific song in the tests, *Gad1* knockdown females showed two phenotypes different from the control group (**Figure 9B**). The first phenotype came after training of conspecific song (**Figure 9B, red lines**); while control females reduced receptivity like wild-type females (**Figure 9B, right**), receptivity of *Gad1* knockdown females stayed at the same level as in naïve females (**Figure 9B, left**). This result suggests the necessity of GABA in this experience-dependent IPI tuning. The second phenotype appeared after heterospecific song training (**Figure 9B, orange lines**); *Gad1* knockdown flies decreased their copulation rate dramatically when compared with naïve flies (**Figure 9B, left**), whereas control flies (**Figure 9B, right**) and wild-type flies (**Figure 8B**) did not. These results demonstrate that although the response to the conspecific song in females was neither interrupted by *Gad1* knockdown nor by training (**Figure 9A**), the response to heterospecific song was vulnerable to *Gad1* knockdown and training (**Figure 9B**). Training with both conspecific song and heterospecific song might have modified properties of the neural circuit for the processing of heterospecific song. GABA synthesis is necessary to show the plasticity induced by conspecific song training, and to defend against the modulation induced by heterospecific song training as well.

Together, these results prove that GABA synthesis is necessary for the IPI tuning induced by conspecific song training, which is reminiscent of the involvement of GABA in auditory plasticity exhibited in mammals and songbirds (Dorrn *et al.*, 2010; Kotak *et al.*, 2008; Yanagihara & Yazagi-sugiyama, 2016).

GABA mediates the experience-dependent plasticity via Rdl receptors in pC1 neurons

P1 neurons, a male-specific subset of pC1 neurons, are the mating command-like neurons that receive multimodal input from olfactory, gustatory, and auditory systems (Auer & Benton, 2016). Multimodal sensory information is transmitted to P1 neurons through excitatory and inhibitory pathways to achieve a stringent control of courtship decision-making in males (Clowney *et al.*, 2015; Koganezawa *et al.*, 2016).In these pathways, GABA transmits inhibitory signals to P1 neurons via $GABA_A$-type Rdl receptors (Kallman *et al.*, 2015; Koganezawa *et al.*, 2016). Similarly, female pC1 neurons, the counterpart of male pC1 neurons (Koganezawa *et al.*, 2016), regulate female receptivity by evaluating sexual signals from males including the courtship song and the male-specific pheromone cVA (Zhou *et al.*, 2014). Under the hypothesis that GABA signaling via Rdl receptors might also regulate female pC1 neurons, I asked whether pC1 neurons in females were the target neurons of GABA that mediates the experience-dependent IPI tuning. I knocked down the expression of *Rdl* by driving *Rdl* RNAi specifically in female pC1 neurons, defined by the intersection of an enhancer trap line *NP2631* and dsx^{FLP} (Koganezawa *et al.*, 2016). Consistent with the aforementioned results, in the conspecific song test both *Rdl* knockdown and control females in experienced groups responded similarly as naïve females did, irrespective of training experiences (**Figure 10A**). In the heterospecific song test, however, *Rdl* knockdown females, but not control and wild-type ones, kept the receptivity to the heterospecific song even after training with the conspecific song (**Figure 10B**). Accordingly, knockdown of *Rdl* in pC1 neurons abolishes the experience-dependent tuning of the IPI, indicating that GABA mediates this IPI tuning via $GABA_A$ receptors in pC1 neurons of females.

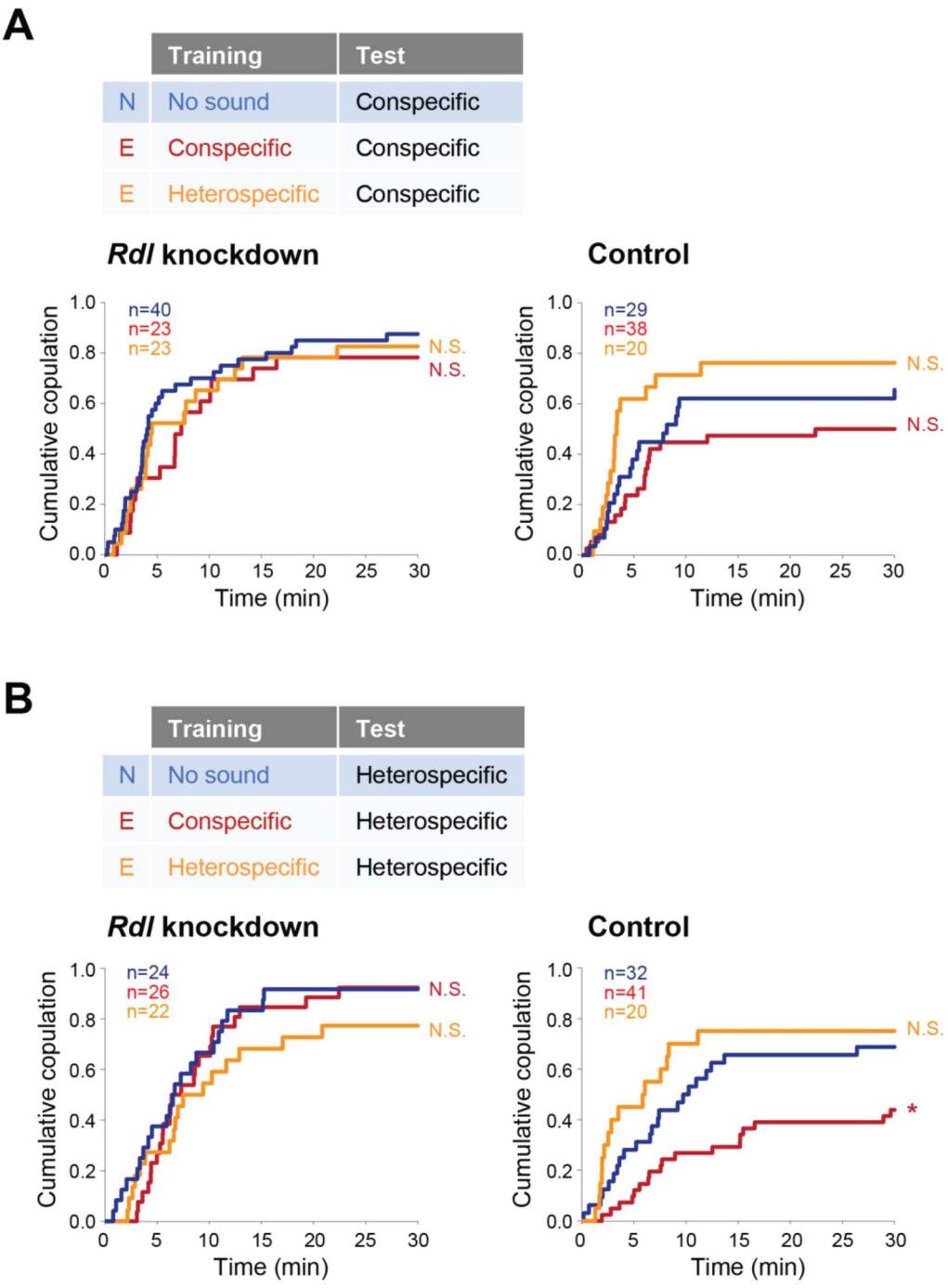


Figure 10. Rdl receptors in pC1 neurons modulate the experience-dependent song preference in females. (A, B) Cumulative copulation rate in the conspecific song test (A) or heterospecific song test (B) after training *Rdl* knockdown (left) and control (right) females.

Naïve group (no sound training) and experienced groups (conspecific song training or heterospecific song training) are shown. The color code is the same with that in Figure 5. N, naïve; E, experienced. N.S., not significant, $P>0.05$; *$P<0.05$; Log rank test versus naïve group.

Interestingly, training with heterospecific song induced no changes in both *Rdl* knockdown and control groups (**Figures 10A and 10B**). This result contrasts with that in *Gad1* knockdown flies, in which the experience of heterospecific song reduced the female receptivity upon exposure to heterospecific song (**Figure 9B**). Rdl receptors in female pC1 neurons are thus unlikely to be the direct target of GABA signaling to defend against the modulation induced by the training of heterospecific song.

Discussion

The courtship behavior of *Drosophila melanogaster* provides a simple model to understand how the innate perception of sensory signals is configured to direct the higher cognitive functions. Especially, the perception of auditory signals has attracted much attention from researchers because acoustic communication plays important roles in species identification and reproductive isolation of not only fruit flies, but also other animals such as birds (Catchpole, 1987), fishes (Amorim *et al.*, 2015), frogs (Backwell & Jennions, 1993), and crickets (Hedwig, 2006). Here I identify a novel phenomenon revealing the experience-dependent auditory plasticity that shapes sexual preference in fruit flies (*D. melanogaster*). Analogous to the regulatory role of GABA in shaping auditory circuits of zebra finch (Yanagihara & Yazaki-Sugiyama, 2016), I demonstrate that GABA signaling also shapes auditory selectivity in flies. I further identify the receptors responsible for this signaling on a small subset of central neurons that mediate the tuning of IPI perception. For the first time, our findings document how the experience-dependent mechanism is incorporated with an innate auditory system to shape the sexual behavior and accordingly establish the fruit fly, with its abundant molecular-genetic tools, as a powerful model to investigate the mechanisms of auditory plasticity on the molecular and cellular levels.

Song experience shapes the IPI preference

Temporal pattern of sound is a crucial feature in the communication signals of many animals, such as in bird songs, frog calls, cricket chirps, and human speech (Pollack, 2001). Particularly in lower-vertebrates and insects, understanding the simple patterns of sounds used in communication, such as the specific pulse rate, is important in deciphering the meanings of these signals (Alexander, 1962; Bass & McKibben, 2003; Doherty & Huber, 1983; Schöneich *et al.*, 2015). Fruit flies

use the pulse songs with a species-specific IPI during courtship (Ewing & Bennet-Clark, 1968). IPI information in the courtship song is processed by a central auditory circuitry via multi-stage transformations and affects the mating decisions in males (Zhou *et al.*, 2015). This pathway functions as a band-pass filter to achieve selective response of flies to specific range of IPIs. In this study, we found that the flies' behavioral response to the pulse song was tuned by sound experience in both males and females, suggesting experience-dependent plasticity of previously found band-pass filter, whose physiological evidences awaits to be addressed. Analogous to the report that naïve male flies showed a strong behavioral response to IPIs ranging between 35 ms and 75 ms (Yoon *et al.*, 2013), here in naïve pairs we also find the similar IPI preference in boosting the copulation success (**Figure 7**). Considering that mainly the female decides the copulation success in *D. melanogaster* (Dickson, 2008), this result suggests that fruit flies have no sexual dimorphism of innate IPI perception on the behavioral level.

The preference towards the conspecific IPI (35 ms) is likely to be tuned by the auditory experience during the social interaction in early life. Since the IPI distribution in the recorded natural courtship song is particularly enriched at around 35 ms (Arthur *et al.*, 2013), young adult flies are highly likely to be exposed to this conspecific IPI emitted by other males. This experience might tune the IPI preference and predispose partner selection in sexual behavior later in life. Indeed, our results prove that social interaction during early adulthood tunes the IPI preference towards the conspecific IPI (35 ms) (**Figure 5**). This beautiful coordination between innate preference and experience-dependent refinement allows enough flexibility in mating, and reduces the risk of crossbreeding between species, which contributes to species isolation.

Although the direct evidence that flies sing to each other in the young adulthood is lacking, an experience of staying with other intact young males was enough to tune the IPI preference

(**Figure 5**). The speculation that flies socially interact to achieve their sexual maturation is supported by the analogous experience-dependent courtship acquisition in *fruitless* (fru^M) mutant flies (Pan *et al.*, 2014). fru^M null males typically exhibit male-male courtship, but not male-female courtship. The acquisition of such male-male courtship requires the experience of group-housing with other individuals, regardless of sex or species, and *doublesex* (*dsx*), a gene involved in the sex determination system (Rideout *et al.*, 2010), is both necessary and sufficient for this acquisition. Thus it seems that the experience of social interaction not only specifies the potential of a mutant strain for sexual behavior, but also could reinforce the selectivity of IPI perception in wild-type flies in the definite context of audition.

Another point worth noting is that only the experience of conspecific song tunes the auditory preference, while the experience of heterospecific song does not. This asymmetric learning of conspecific and heterospecific songs suggests that naïve flies can already distinguish conspecific song from heterospecific song, since only the former is capable of modifying their later preference behavior.

It was previously reported that male *D. melanogaster* showed equal behavioral preference towards IPIs between 35 ms and 75 ms (Yoon *et al.*, 2013), which were used as conspecific and heterospecific songs in the present study. However, another report showed that male *D. melanogaster* preferentially responded to 35-ms over all other IPIs (Zhou *et al.*, 2015). This discrepancy can now be explained by the experimental difference between these two studies, whether the male flies kept in a group have the experience of carrying wings (Zhou *et al.*, 2015) or not (Yoon *et al.*, 2013). As for how long the necessary experience is, and whether a critical period exists, further study is needed to answer these questions.

Experience-dependent learning refines the mating preference

Whether nature or nurture plays dominant roles in the formation of animal behavior has been debated for a long time, yet the courtship behavior of *D. melanogaster*, including its underlying sensory perception, has long been recognized to be innate. Numerous empirical evidences have supported the capability of single-reared flies to perform all the courtship steps spontaneously and completely (Auer & Benton, 2016; Baker et al., 2001; Hall, 1994). However, my results reveal that the specific sound experience is necessary to refine the auditory preference in sexual behavior, which for the first time suggests a mechanism of learning in the song discrimination of flies.

In fact, animals in many species learn their mating preferences. One notable example is sexual imprinting, the process whereby mating preferences are affected by learning the species-specific characteristics at a very young age (Irwin & Price, 1999). As observed in birds (Ten Cate & Vos, 1999), fishes (Kozak *et al.*, 2011), and sheep and goats (Owens *et al.*, 1999), an early period of social interaction with parents or siblings helps the learner discriminate sex and species by learned phenotypic traits, and affects mating preference in the future (Verzijden *et al.*, 2012). Here I provide evidence that fruit flies refine the IPI preference by sexual imprinting, which would reinforce reproductive isolation together with innate auditory perception. This sexual imprinting of courtship song is apparently different from the lessons learned from the successful courtship experience (Saleem *et al*., 2014) or unsuccessful courtship attempts (Griffith & Ejima, 2009), by which male flies become more competitive over other males, or learn to avoid either mated or heterospecific females. Previous behavioral studies also indicated that social experience in juvenile stage affected adult courtship behaviors of insects. In crickets, juvenile experience of acoustic sexual signals influenced the development of three traits in adult: reproductive tactics, reproductive investment, and body condition (Bailey *et al.*, 2010). In fruit flies, young males courted by mature males with

intact wings mated significantly faster than those that had been stored alone, suggesting auditory experience in immature stage might affect later courtship (McRobert & Tompkins, 1988). Consistent with these observations and going deeper, our study directly demonstrate, with the underlying mechanisms, that auditory experience during the immature stage shaped perception of courtship song, and directed the sexual behavior at the adult stage.

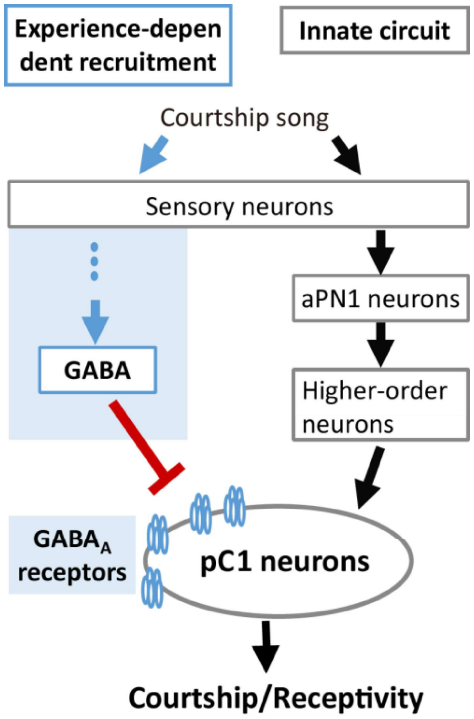


Figure 11. A model for experience-dependent tuning of IPI perception in *Drosophila*.

A new model to study auditory plasticity

Our findings greatly expand the understanding of the experience-dependent auditory plasticity in insects, whose mechanism is consistent with that of mammals and finches. In these vertebrates, acoustic input during the "critical period", during which a brain has enhanced plasticity, is

necessary for the adequate function of auditory neural circuits. Especially, in a variety of animals, maturation of excitation-inhibition balance that governs sound perception requires acoustic experience. In rats, developmental sensory experience balances the excitation and inhibition in the primary auditory cortex (A1) (Dorrn *et al.*, 2010), whose stereotyped sequential occurrence sharpens spike timing (Wehr & Zador, 2003). Hearing loss hinders the maturation of GABAergic transmission mediated by $GABA_A$ receptors in the auditory cortex of gerbils (Kotak *et al.*, 2008). In zebra finch, experience-dependent recruitment of GABAergic inhibition in the auditory cortex is necessary to form the memory template of the tutor song (Yanagihara & Yazaki-Sugiyama, 2016). In flies, our results also suggest that song experience recruits GABAergic inhibition on the auditory pathway, and the coordination of excitation and inhibition controls auditory responses and behavioral output (**Figure 11**). Interestingly, the phenotypes of *Gad1* knockdown in GABAergic neurons and *Rdl* knockdown in pC1 neurons were different when females were tested with heterospecific song (**Figures 9 and 10**). This finding suggests that there are at least two distinct GABAergic pathways to control the experience-dependent auditory plasticity. How these GABAergic pathways are organized cooperatively to shape the IPI preference awaits further analysis.

Interestingly, the combination of excitation and inhibition that modulates the mating decision in flies is not restricted to the auditory system, but also conserved in olfactory and gustatory systems (Auer & Benton, 2016; Clowney *et al.*, 2015; Kallman *et al.*, 2015). The difference is that the sexual circuitry in the chemosensory modalities is thought to be hard-wired (Auer & Benton, 2016; Hall, 1994; Pan & Baker, 2014), while the inhibition I find in the auditory system matures with experience. Intriguingly, all these inhibitions found in olfactory, gustatory, and auditory pathways function directly on the pC1 neurons, strengthening the role of pC1 neurons as a crucial

neural circuit node for multimodal integration (Auer & Benton, 2016; Clowney *et al.*, 2015; Kallman *et al.*, 2015).

The discovery that only the training of conspecific song refines the IPI preference of wild-type flies is reminiscent of vocal learning in zebra finches, which preferentially learn the courtship song of their own species (Brenowitz & Woolley, 2004; Doupe & Kuhl, 1999). Young zebra finches fostered by other species only learn syllable morphology, while keep the temporal gaps between syllables as innate, and such dedication to the recognition of innate temporal pattern is mediated by a specific set of neurons in the auditory cortex (Araki *et al.*, 2016). The IPI in the courtship song of *D. melanogaster* resembles the temporal gap between syllables in the finch song, and might also serve as a "barcode" for song identity. The IPI representation is tuned towards conspecific song along the ascending aPN1-vPN1-pC1 auditory pathways (Zhou *et al.*, 2015). pC1 neurons respond particularly strongly to IPIs between 35 ms and 75 ms, and this selectivity correlates closely with the chaining behavior of males, suggesting the dedicated role of pC1 neurons to detect conspecific song. Our results suggest in flies separate neural circuits might process the IPI information of conspecific song and heterospecific song (**Figures 9 and 10**). While how the GABAergic networks are organized to discriminate conspecific IPI and heterospecific IPI is unclear and the physiological response property after training remains to be addressed, our study suggests the involvement of GABA signaling in shaping the selective response of pC1 neurons to conspecific IPI (**Figure 11**). In addition, the courtship song preferences in female zebra finch are reportedly shaped by the developmental auditory experience (Chen *et al.*, 2017), sharing great similarity with our findings here. Unlike zebra finch, fruit flies rarely see their parents. My results demonstrate that learning from adolescent peers is sufficient to modulate the perception of IPIs (**Figure 5**). In the natural environment, young flies possibly learn from young flies as well as mature flies.

Thus the auditory study of fruit fly and zebra finch might complement and enlighten each other in exploring the mechanism of conspecific song identification and experience-dependent plasticity.

One major purpose to study the auditory plasticity in the model animals is to facilitate the understanding of the secret how humans develop the unique ability to acquire language. Infants are born with initial perceptual abilities that are necessary for language acquisition. While, what is amazing is that infants rapidly learn pattern detection from exposure to language and remodel the neural connections, which are reported only in very limited species, such as humans and songbirds. In humans, early auditory experience is critical to form the ability of phonetic discrimination in language acquisition. This study for the first time reports the necessity of auditory experience in refinement of song perception in fruit flies and reveals the similar mechanism of auditory modulation as in songbirds, suggesting a potential to facilitate the mechanism study of language acquisition. Taken together, our findings open a new research field to use the fruit fly, with its abundant molecular-genetic tools and simple neural circuits, to study the experience-dependent auditory information processing and sensorimotor output, which are challenging to examine at the molecular and cellular levels in zebra finches and vertebrates including humans.

References

Alexander, R. D. (1962). Evolutionary change in cricket acoustical communication. *Evolution, 16*(4), 443-467.

Amorim, M. C. P., Vasconcelos, R. O., & Fonseca, P. J. (2015). Fish sounds and mate choice. In F. Ladich (Ed.), *Sound Communication in Fishes* (pp. 1-33). Vienna: Springer Vienna.

Araki, M., Bandi, M., & Yazaki-Sugiyama, Y. (2016). Mind the gap: Neural coding of species identity in birdsong prosody. *Science, 354*(6317), 1282-1287.

Arthur, B. J., Sunayama-Morita, T., Coen, P., Murthy, M., & Stern, D. L. (2013). Multi-channel acoustic recording and automated analysis of *Drosophila* courtship songs. *BMC Biology, 11*(1), 11.

Auer, T. O., & Benton, R. (2016). Sexual circuitry in *Drosophila*. *Current Opinion in Neurobiology, 38*, 18-26.

Backwell, P. R. Y., & Jennions, M. D. (1993). Mate choice in the Neotropical frog, *Hyla ebraccata*: sexual selection, mate recognition and signal selection. *Animal Behaviour, 45*(6), 1248-1250.

Bailey, N. W., Gray, B., & Zuk, M. (2010). Acoustic experience shapes alternative mating tactics and reproductive investment in male field crickets. *Current Biology, 20*(9), 845-849.

Baker, B. S., Taylor, B. J., & Hall, J. C. (2001). Are complex behaviors specified by dedicated regulatory genes? Reasoning from *Drosophila*. *Cell, 105*(1), 13-24.

Bass, A. H., & McKibben, J. R. (2003). Neural mechanisms and behaviors for acoustic communication in teleost fish. *Progress in Neurobiology, 69*(1), 1-26.

Bennet-Clark, H. C., & Ewing, A. W. (1969). Pulse interval as a critical parameter in the courtship song of *Drosophila melanogaster*. *Animal Behaviour, 17*(4), 755-759.

Bolhuis, J. J., & Moorman, S. (2015). Birdsong memory and the brain: In search of the template. *Neuroscience and Biobehavioral Reviews, 50*, 41-55.

Brenowitz, E. A., & Woolley, S. M. N. (2004). The avian song control system: A model for understanding changes in neural structure and function. In T. N. Parks, E. W. Rubel, A. N. Popper & R. R. Fay (Eds.), *Plasticity of the Auditory System* (pp. 228-284). New York: Springer New York.

Catchpole, C. K. (1987). Bird song, sexual selection and female choice. *Trends in Ecology & Evolution, 2*(4), 94-97.

Chen, Y., Clark, O., & Woolley, S. C. (2017). Courtship song preferences in female zebra finches are shaped by developmental auditory experience. *Proc Biol Sci*, 284(1855).

Clemens, J., Girardin, C. C., Coen, P., Guan, X.-J., Dickson, B. J., & Murthy, M. (2015). Connecting neural codes with behavior in the auditory system of *Drosophila*. *Neuron, 87*(6), 1332-1343.

Clowney, E. J., Iguchi, S., Bussell, J. J., Scheer, E., & Ruta, V. (2015). Multimodal chemosensory circuits controlling male courtship in *Drosophila*. *Neuron, 87*(5), 1036-1049.

Cobb, M., Burnet, B., Blizard, R., & Jallon, J.-M. (1989). Courtship in *Drosophila sechellia*: Its structure, functional aspects, and relationship to those of other members of the *Drosophila melanogaster* species subgroup. *J. Insect. Behav., 2*(1), 63-89.

Coen, P., Clemens, J., Weinstein, A. J., Pacheco, D. A., Deng, Y., & Murthy, M. (2014). Dynamic sensory cues shape song structure in *Drosophila*. *Nature, 507*(7491), 233-237.

Costa, C., & Sene, F. (2002). Characterization of courtship sounds of species of the subgroup *fasciola* (Diptera, Drosophilidae, *Drosophila repleta* group): interspecific and interpopulational analyses. *Braz. J. Biol., 62*(4A), 573-583.

Cousillas, H., George, I., Mathelier, M., Richard, J.-P., Henry, L., & Hausberger, M. (2006). Social experience influences the development of a central auditory area. *Naturwissenschaften, 93*(12), 588-596.

Cowling, D., & Burnet, B. (1981). Courtship songs and genetic control of their acoustic characteristics in sibling species of the *Drosophila melanogaster* subgroup. *Animal Behaviour, 29*(3), 924-935.

Crossley, S. A., Bennet-Clark, H., & Evert, H. T. (1995). Courtship song components affect male and female *Drosophila* differently. *Animal Behaviour, 50*(3), 827-839.

Dickson, B. J. (2008). Wired for sex: the neurobiology of *Drosophila* mating decisions. *Science, 322*(5903), 904-909.

Doherty, J., & Huber, F. (1983). Temperature effects on acoustic communication in the cricket *Gryllus bimaculatus DeGeer*. *Verh. Dtsch. Zool. Ges., 188*, 188.

Dorrn, A. L., Yuan, K., Barker, A. J., Schreiner, C. E., & Froemke, R. C. (2010). Developmental sensory experience balances cortical excitation and inhibition. *Nature, 465*(7300), 932.

Doupe, A. J., & Kuhl, P. K. (1999). Birdsong and human speech: Common themes and mechanisms. *Annual Review of Neuroscience, 22*(1), 567-631.

Eberl, D. F., Duyk, G. M., & Perrimon, N. (1997). A genetic screen for mutations that disrupt an auditory response in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences, 94*(26), 14837-14842.

Eberl, D. F., Kamikouchi, A., Albert, J. T. (2016). Auditory Transduction. In: Pollack, G., Mason, A., Popper, A., Fay, R. (eds) Insect Hearing. Springer Handbook of Auditory Research, vol 55. Springer, Cham.

Ewing, A. W., & Bennet-Clark, H. (1968). The courtship songs of *Drosophila*. *Behaviour, 31*(3), 288-301.

Ewing, A. W., & Manning, A. (1967). The Evolution and Genetics of Insect Behaviour. *Annual Review of Entomology, 12*(1), 471-494.

Franco, L. M., Okray, Z., Linneweber, G. A., Hassan, B. A., & Yaksi, E. (2017). Reduced lateral inhibition impairs olfactory computations and behaviors in a *Drosophila* model of Fragile X Syndrome. *Current Biology, 27*(8), 1111-1123.

Greenspan, R. J., & Ferveur, J.-F. (2000). Courtship in *Drosophila*. *Annual Review of Genetics, 34*(1), 205-232.

Griffith, L. C., & Ejima, A. (2009). Courtship learning in *Drosophila melanogaster:* diverse plasticity of a reproductive behavior. *Learning and Memory, 16*(12), 743-750.

Hall, J. (1994). The mating of a fly. *Science, 264*(5166), 1702-1714.

Hedwig, B. (2006). Pulses, patterns and paths: neurobiology of acoustic behaviour in crickets. *J. Comp. Physiol., 192*(7), 677-689.

Inagaki, H. K., Kamikouchi, A., & Ito, K. (2010). Protocol for quantifying sound-sensing ability of *Drosophila melanogaster*. *Nature Protocols, 5*(1), 26-30.

Irwin, D. E., & Price, T. (1999). Sexual imprinting, learning and speciation. *Heredity, 82*(4), 347-354.

Ishikawa, Y., & Kamikouchi, A. (2016). Auditory system of fruit flies. *Hearing Research, 338*, 1-8.

Ishikawa, Y., Okamoto, N., Nakamura, M., Kim, H., & Kamikouchi, A. (2017). Anatomic and physiologic heterogeneity of subgroup-A auditory sensory neurons in fruit flies. *Front. Neural. Circuits., 11*, 46.

Ito, M., Masuda, N., Shinomiya, K., Endo, K., & Ito, K. (2013). Systematic Analysis of Neural Projections Reveals Clonal Composition of the *Drosophila* Brain. *Current Biology, 23*(8), 644-655.

Jenett, A., Rubin, Gerald M., Ngo, T.-T. B., Shepherd, D., Murphy, C., Dionne, H., . . . Zugates, Christopher T. (2012). A GAL4-Driver Line Resource for *Drosophila* Neurobiology. *Cell Reports, 2*(4), 991-1001.

Jeong, Y. T., Oh, S. M., Shim, J., Seo, J. T., Kwon, J. Y., & Moon, S. J. (2016). Mechanosensory neurons control sweet sensing in *Drosophila*. *Nat. Commun., 7*, 12872.

Kallman, B. R., Kim, H., & Scott, K. (2015). Excitation and inhibition onto central courtship neurons biases *Drosophila* mate choice. *Elife, 4*, e11188.

Kamikouchi, A., Inagaki, H. K., Effertz, T., Hendrich, O., Fiala, A., Gopfert, M. C., & Ito, K. (2009). The neural basis of *Drosophila* gravity-sensing and hearing. *Nature, 458*(7235), 165-171.

Kayser, M. S., Yue, Z., & Sehgal, A. (2014). A critical period of sleep for development of courtship circuitry and behavior in *Drosophila*. *Science, 344*(6181), 269-274.

Keleman, K., Vrontou, E., Krüttner, S., Yu, J. Y., Kurtovic-Kozaric, A., & Dickson, B. J. (2012). Dopamine neurons modulate pheromone responses in *Drosophila* courtship learning. Nature, 489, 145.

Kim, Y.-K., & Ehrman, L. (1998). Developmental isolation and subsequent adult behavior of *Drosophila paulistorum*. IV. Courtship. *Behavior Genetics, 28*(1), 57.

Koemans, T. S., Oppitz, C., Donders, R. A. T., van Bokhoven, H., Schenck, A., Keleman, K., & Kramer, J. M. (2017). *Drosophila* Courtship Conditioning As a Measure of Learning and Memory. *JoVE*(124), e55808.

Koganezawa, M., Kimura, K., & Yamamoto, D. (2016). The neural circuitry that functions as a switch for courtship versus aggression in *Drosophila* males. *Current Biology, 26*(11), 1395-1403.

Kotak, V. C., Takesian, A. E., & Sanes, D. H. (2008). Hearing loss prevents the maturation of GABAergic transmission in the auditory cortex. *Cerebral Cortex, 18*(9), 2098-2108.

Kozak, G. M., Head, M. L., & Boughman, J. W. (2011). Sexual imprinting on ecologically divergent traits leads to sexual isolation in sticklebacks. *Proc. R. Soc. Lond., B, Biol. Sci., 278*(1718), 2604-2610.

Kuhl, P. K. (2004). Early language acquisition: cracking the speech code. *Nature Reviews: Neuroscience, 5*(11), 831-843.

Kyriacou, C., & Hall, J. C. (1982). The function of courtship song rhythms in *Drosophila*. *Animal Behaviour, 30*(3), 794-801.

Laturney, M., & Billeter, J.-C. (2014). Neurogenetics of female reproductive behaviors in *Drosophila melanogaster*. *Advances in Genetics, 85*, 1-108.

Levine, J. D., Funes, P., Dowse, H. B., & Hall, J. C. (2002). Resetting the Circadian Clock by Social Experience in *Drosophila melanogaste*r. *Science, 298*(5600), 2010-2012.

Marler, P. (1970) "Birdsong and speech development: could there be parallels?" *American Scientist*. 58 (6) 669-673. JSTOR 27829317.

McRobert, S. P., & Tompkins, L. (1988). Two consequences of homosexual courtship performed by *Drosophila melanogaster* and *Drosophila affinis* males. *Evolution, 42*(5), 1093-1097.

Muthukumar, A. K., Stork, T., & Freeman, M. R. (2014). Activity-dependent regulation of astrocyte GAT levels during synaptogenesis. *Nature Neuroscience, 17*(10), 1340-1350.
Ng, M., Roorda, R. D., Lima, S. Q., Zemelman, B. V., Morcillo, P., & Miesenböck, G. (2002). Transmission of olfactory information between three populations of neurons in the antennal lobe of the fly. *Neuron, 36*(3), 463-474.
Okada, R., Awasaki, T., & Ito, K. (2009). Gamma-aminobutyric acid (GABA)-mediated neural connections in the *Drosophila* antennal lobe. *Journal of Comparative Neurology, 514*(1), 74-91.
Owens, I. P., Rowe, C., & Thomas, A. L. (1999). Sexual selection, speciation and imprinting: separating the sheep from the goats. *Trends in Ecology & Evolution, 14*(4), 131-132.
Pan, Y., & Baker, B. S. (2014). Genetic identification and separation of innate and experience-dependent courtship behaviors in *Drosophila*. *Cell, 156*(1), 236-248.
Pavlou, H. J., & Goodwin, S. F. (2013). Courtship behavior in *Drosophila melanogaster*: towards a 'courtship connectome'. *Current Opinion in Neurobiology, 23*(1), 76-83.
Pfeiffer, B. D., Jenett, A., Hammonds, A. S., Ngo, T.-T. B., Misra, S., Murphy, C., . . . Rubin, G. M. (2008). Tools for neuroanatomy and neurogenetics in *Drosophila*. *Proceedings of the National Academy of Sciences, 105*(28), 9715-9720.
Pollack, G. S. (2001). Analysis of temporal patterns of communication signals. *Current Opinion in Neurobiology, 11*(6), 734-738.
Powell, J. R. (1997). *Progress and prospects in evolutionary biology: the Drosophila model*: Oxford University Press, Oxford.
Ramdya, P., Lichocki, P., Cruchet, S., Frisch, L., Tse, W., Floreano, D., & Benton, R. (2014). Mechanosensory interactions drive collective behaviour in *Drosophila*. *Nature, 519*, 233.
Razak, K. A., Richardson, M. D., & Fuzessery, Z. M. (2008). Experience is required for the maintenance and refinement of FM sweep selectivity in the developing auditory cortex. *Proceedings of the National Academy of Sciences, 105*(11), 4465-4470.
Reiter, L. T., Potocki, L., Chien, S., Gribskov, M., & Bier, E. (2001). A Systematic Analysis of Human Disease-Associated Gene Sequences In *Drosophila melanogaster*. *Genome Research, 11*(6), 1114-1125.
Riabinina, O., Dai, M., Duke, T., & Albert, J. T. (2011). Active process mediates species-specific tuning of *Drosophila* ears. *Current Biology, 21*(8), 658-664.
Rideout, E. J., Dornan, A. J., Neville, M. C., Eadie, S., & Goodwin, S. F. (2010). Control of sexual differentiation and behavior by the doublesex gene in *Drosophila melanogaster*. *Nature Neuroscience, 13*(4), 458-466.
Ritchie, M. G., Halsey, E. J., & Gleason, J. M. (1999). *Drosophila* song as a species-specific mating signal and the behavioural importance of Kyriacou & Hall cycles in *D. melanogaster* song. *Animal Behaviour, 58*(3), 649-657.
Rybak, F., Aubin, T., Moulin, B., & Jallon, J.-M. (2002). Acoustic communication in *Drosophila melanogaster* courtship: Are pulse-and sine-song frequencies important for courtship success? *Canadian Journal of Zoology, 80*(6), 987-996.
Saleem, S., Ruggles, P. H., Abbott, W. K., & Carney, G. E. (2014). Sexual experience enhances *Drosophila melanogaster* male mating behavior and success. *PloS One, 9*(5), e96639.
Schöneich, S., Kostarakos, K., & Hedwig, B. (2015). An auditory feature detection circuit for sound pattern recognition. *Sci. Adv., 1*(8).
Ten Cate, C., & Vos, D. R. (1999). Sexual Imprinting and Evolutionary Processes in in birds: a reassessment. *Adv. Study. Behav., 28*, 1.

Tirian, L., & Dickson, B. (2017). The VT GAL4, LexA, and split-GAL4 driver line collections for targeted expression in the *Drosophila* nervous system. *bioRxiv*.

Vaughan, A. G., Zhou, C., Manoli, D. S., & Baker, B. S. (2014). Neural pathways for the detection and discrimination of conspecific song in *D. melanogaster*. *Current Biology, 24*(10), 1039-1049.

Verzijden, M. N., Ten Cate, C., Servedio, M. R., Kozak, G. M., Boughman, J. W., & Svensson, E. I. (2012). The impact of learning on sexual selection and speciation. *Trends in Ecology & Evolution, 27*(9), 511-519.

Villella, A., & Hall, J. C. (2008). Chapter 3 Neurogenetics of Courtship and Mating in *Drosophila Advances in Genetics* (Vol. 62, pp. 67-184): Academic Press, Cambridge.

von Schilcher, F. (1976a). The role of auditory stimuli in the courtship of *Drosophila melanogaster*. *Animal Behaviour, 24*(1), 18-26.

von Schilcher, F. (1976b). The function of pulse song and sine song in the courtship of *Drosophila melanogaster*. *Animal Behaviour, 24*(3), 622-625.

Wang, L., Dankert, H., Perona, P., & Anderson, D. J. (2008). A common genetic target for environmental and heritable influences on aggressiveness in *Drosophila*. *Proceedings of the National Academy of Sciences, 105*(15), 5657-5663.

Wehr, M., & Zador, A. M. (2003). Balanced inhibition underlies tuning and sharpens spike timing in auditory cortex. *Nature, 426*(6965), 442.

Woolley, S., Hauber, M. E., & Theunissen, F. E. (2010). Developmental experience alters information coding in auditory midbrain and forebrain neurons. *Developmental Neurobiology, 70*(4), 235-252.

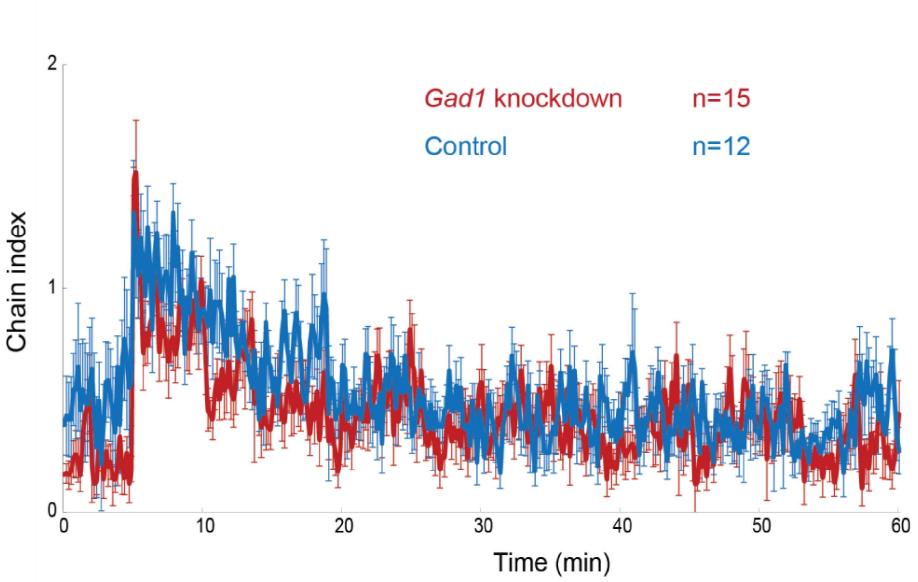
Yanagihara, S., & Yazaki-Sugiyama, Y. (2016). Auditory experience-dependent cortical circuit shaping for memory formation in bird song learning. *Nat. Commun., 7*, 11946.

Yoon, J., Matsuo, E., Yamada, D., Mizuno, H., Morimoto, T., Miyakawa, H., . . . Kamikouchi, A. (2013). Selectivity and plasticity in a sound-evoked male-male interaction in *Drosophila*. *PloS One, 8*(9), e74289.

Zhou, C., Franconville, R., Vaughan, A. G., Robinett, C. C., Jayaraman, V., & Baker, B. S. (2015). Central neural circuitry mediating courtship song perception in male *Drosophila*. *Elife, 4*, e08477.

Zhou, C., Pan, Y., Robinett, C. C., Meissner, G. W., & Baker, B. S. (2014). Central brain neurons expressing doublesex regulate female receptivity in *Drosophila*. *Neuron, 83*(1), 149-163.

Supplementary files



Supplementary figure 1. Male *Gad1* knockdown flies responded normally to conspecific courtship song. The time-courses of the chain index in response to playback of conspecific song in *Gad1* knockdown group (red, *Gad1-GAL4*/+; *UAS-Gad1 RNAi*/+) and control group (blue, *Gad1-GAL4*/+; +/+) are shown. The way to perform chaining test is similar to that described in the Method part. Sound playback starts at 5 min and lasts until 60 min. Error bars denotes s.e.m. n, number of behavioral chambers examined.

Supplementary file 1: Genotypes

The genotypes used in figures are as follows. In Figures 9 and 10, the genotypes of females are listed, while the paired males are always wild type.

Supplementary file 2: Statistical results

The detailed statistical results in each figure are listed. N.S., not significant, P>0.05; *P<0.05; **P<0.01; ***P<0.001.

Supplementary file 1: Genotypes

The genotypes used in figures are as follows. In Figures 9 and 10, the genotypes of females are listed, while the paired males are always wild type.

	Animals	**Genotype**
Figure 5	Wild type	*Canton S*
Figure 6	Wild type	*Canton S*
Figure 7	Wild type	*Canton S*
Figure 8	Wild type	*Canton S*
Figure 9	*Gad1* knockdown	*Gad1-GAL4/+; UAS-Gad1 RNAi/+*
	Control	*Gad1-GAL4/+;+/+*
Figure 10	*Rdl* knockdown	*tubP>GAL80>/+; NP2631-GAL4/UAS-Rdl-RNAi; dsx^{FLP}/+*
	Control	*tubP>GAL80>/+; NP2631-GAL4/+; dsx^{FLP}/+*

Supplementary file 2 **Statistical results**

The detailed statistical results in each figure are listed. N.S., not significant, P>0.05; *P<0.05; **P<0.01 ; ***P<0.001.

	Data	Statistical method	Animals	Panel		U/chi-sq value	Degrees of freedom	*p* -value	
Figure 5	Chaining response	Mann-Whitney U test	Wild type	a		U = 249		0.318	N.S.
				b		U = 429.5		0.0011	**
				c		U = 242.5		0.404	N.S.
Figure 6	Chaining response	Mann-Whitney U test	Wild type	a	N *vs* E	U = 633		0.421	N.S.
					N *vs* E	U = 610.5		0.488	N.S.
				b	N *vs* E	U = 410		0.0003	***
					N *vs* E	U = 124		0.448	N.S.
Figure 7	Copulation rate	Log rank test	Wild type	left	15ms *vs* no sound	chi-sq= 3.1	df = 1	0.081	N.S.
					35ms *vs* no sound	chi-sq= 31.7	df = 1	1.83E-08	***
					75ms *vs* no sound	chi-sq= 28.5	df = 1	9.16E-08	***
					105ms *vs* no sound	chi-sq= 1.6	df = 1	0.205	N.S.
					35ms *vs* 75ms	chi-sq= 0	df = 1	0.873	N.S.
	Copulation latency	Kruskal–Wallis test		right	15ms *vs* no sound	chi-sq= 4.1	df = 1	0.043	*
					35ms *vs* no sound	chi-sq= 23.9	df = 1	1.01E-06	***
					75ms *vs* no sound	chi-sq= 21.2	df = 1	4.22E-06	***
					105ms *vs* no sound	chi-sq= 2.19	df = 1	0.139	N.S.
					35ms *vs* 75ms	chi-sq= 0.05	df = 1	0.825	N.S.
Figure 8	Copulation rate	Log rank test	Wild type	a	N *vs* E	chi-sq= 2.7	df = 1	0.101	N.S.
					N *vs* E	chi-sq= 0.6	df = 1	0.423	N.S.
				b	N *vs* E	chi-sq= 26.3	df = 1	2.97E-07	***
					N *vs* E	chi-sq= 3.3	df = 1	0.069	N.S.
	Copulation latency	Kruskal–Wallis test		c	Nc *vs* Ec	chi-sq= 1.0	df = 1	0.313	N.S.
					Nc *vs* Ec	chi-sq= 0.4	df = 1	0.539	N.S.
					Nh *vs* Eh	chi-sq= 22.7	df = 1	1.92E-06	***
					Nh *vs* Eh	chi-sq= 3.6	df = 1	0.057	N.S.
Figure 9	Copulation rate	Log rank test	*Gad1* knockdown	a, left	N *vs* E	chi-sq= 1.6	df = 1	0.203	N.S.
					N *vs* E	chi-sq= 0.1	df = 1	0.704	N.S.
			Control	a, right	N *vs* E	chi-sq= 0.9	df = 1	0.351	N.S.
					N *vs* E	chi-sq= 0.4	df = 1	0.549	N.S.
			Gad1 knockdown	b, left	N *vs* E	chi-sq= 1.8	df = 1	0.178	N.S.
					N *vs* E	chi-sq= 6.3	df = 1	0.0122	*
			Control	b, right	N *vs* E	chi-sq= 13.2	df = 1	0.0003	***
					N *vs* E	chi-sq= 0.6	df = 1	0.428	N.S.
Figure 10	Copulation rate	Log rank test	*Rdl* knockdown	a, left	N *vs* E	chi-sq= 1.3	df = 1	0.258	N.S.
					N *vs* E	chi-sq= 0.4	df = 1	0.510	N.S.
			Control	a, right	N *vs* E	chi-sq= 1.5	df = 1	0.220	N.S.
					N *vs* E	chi-sq= 1.8	df = 1	0.180	N.S.
			Rdl knockdown	b, left	N *vs* E	chi-sq= 0.1	df = 1	0.765	N.S.
					N *vs* E	chi-sq= 2.1	df = 1	0.145	N.S.
			Control	b, right	N *vs* E	chi-sq= 6.2	df = 1	0.0128	*
					N *vs* E	chi-sq= 1.2	df = 1	0.272	N.S.