



Genetic and Neural Mechanisms that Inhibit *Drosophila* from Mating with Other Species

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SUMMARY

Genetically hard-wired neural mechanisms must enforce behavioral reproductive isolation because interspecies courtship is rare even in sexually naïve animals of most species. We find that the chemoreceptor Gr32a inhibits male *D. melanogaster* from courting diverse fruit fly species. Gr32a recognizes nonvolatile aversive cues present on these reproductively dead-end targets, and activity of Gr32a neurons is necessary and sufficient to inhibit interspecies courtship. Male-specific Fruitless (Fru^M), a master regulator of courtship, also inhibits interspecies courtship. Gr32a and Fru^M are not coexpressed, but Fru^M neurons contact Gr32a neurons, suggesting that these genes influence a shared neural circuit that inhibits interspecies courtship. Gr32a and Fru^M also suppress within-species intermale courtship, but we show that distinct mechanisms preclude sexual displays toward conspecific males and other species. Although this chemosensory pathway does not inhibit interspecies mating in *D. melanogaster* females, similar mechanisms appear to inhibit this behavior in many other male drosophilids.

INTRODUCTION

A species can be defined as a set of organisms that share a gene pool and breed with each other (Darwin, 1860; Dobzhansky, 1937; Mayr, 1988). The lack of interspecies breeding results from mechanisms that promote breeding with conspecifics and those that interpose a reproductive barrier between species. Reproductive barriers can occur prior to or after fertilization. If

fertilization is successful, there exist genetic pathways that lead to sterile or inviable interspecies hybrids (Coyne and Orr, 1998; Orr et al., 2004; Wu and Ting, 2004). Anatomy, physiology, and geographical isolation impose prefertilization barriers to interspecies breeding. Mechanisms that inhibit sexual displays toward other species are also important prefertilization barriers because such courtship increases predation risk and is energetically and reproductively wasteful. Recognition of conspecifics prior to mating is critical in habitats where many species coexist. Indeed, closely related species of fish, amphibians, and birds do not interbreed despite sharing territory (Blair, 1964; Dobzhansky and Mayr, 1944; Konishi, 1985; Seehausen and van Alphen, 1998). Despite the prevalence of behavioral reproductive isolation and its importance to evolution, the neural pathways that suppress interspecies courtship are poorly understood.

D. melanogaster offers a powerful model to study behavioral reproductive isolation. Many drosophilids coexist in nature and the mechanisms that influence courtship in *D. melanogaster* are well studied (Billeter et al., 2006; Dahanukar and Ray, 2011; Siwicki and Kravitz, 2009; Spieth, 1952). Behavioral reproductive isolation appears to operate in *D. melanogaster* because interspecies hybrids are rarely found in nature (Barbash, 2010; Spieth, 1974). The absence of such hybrids does not simply reflect their inability to mature or survive in nature, and previous work suggests that neural pathways that inhibit interspecies courtship in *D. melanogaster* are important for reproductive isolation (Dukas, 2004; Sturtevant, 1920).

We employed behavioral and genetic screens to identify mechanisms that inhibit courtship of *D. melanogaster* males toward other species. We find that Gr32a is required to detect aversive cues on such atypical mating targets and that Gr32a sensory neurons are necessary and sufficient to inhibit courtship of other drosophilids. Fru^M, a master regulator of male courtship

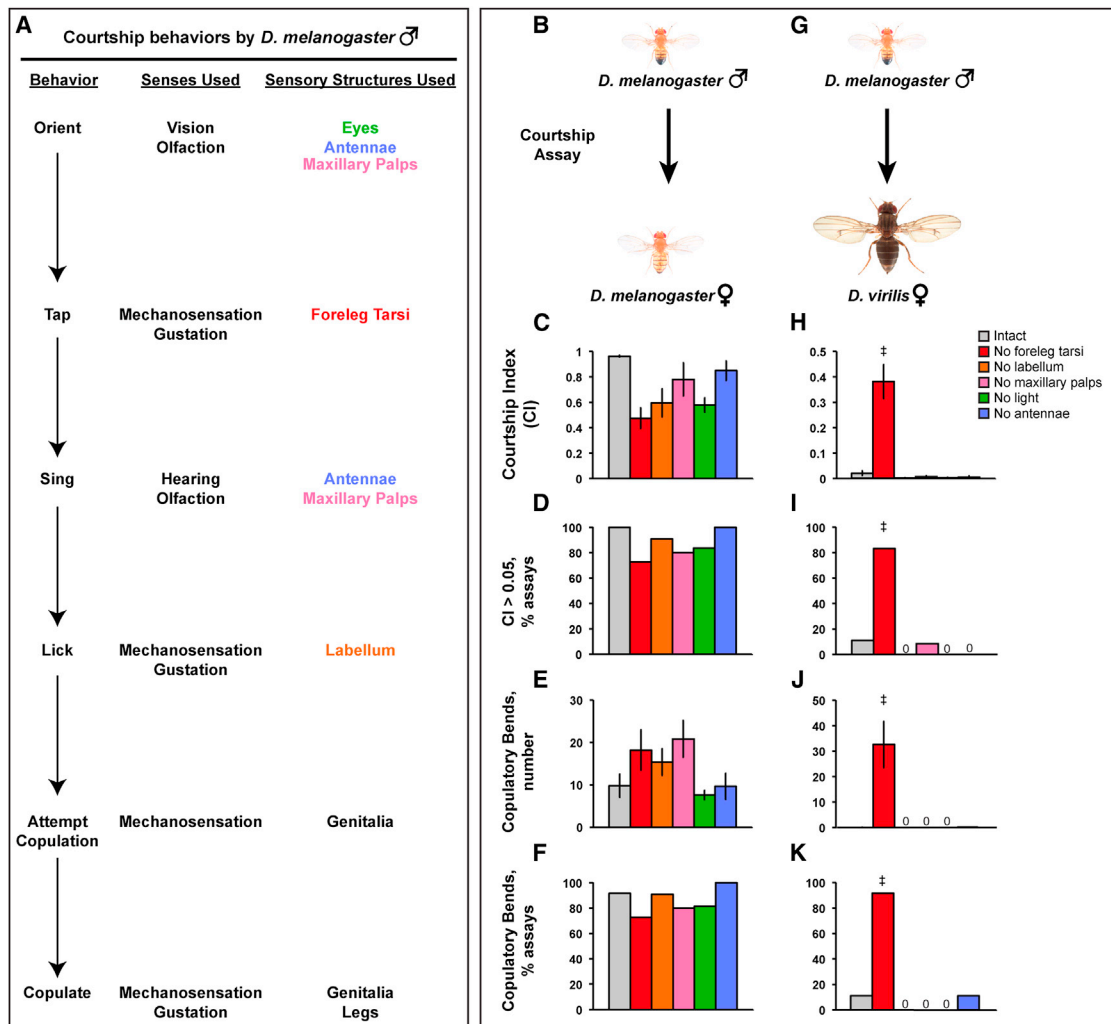


Figure 1. The Foreleg Tarsi Inhibit Courtship of Other Species

(A) Overview of *D. melanogaster* male courtship behaviors and their likely sensory control.

(B and G) WT *D. melanogaster* males were provided with either conspecific or *D. virilis* females.

(C and H) Males lacking labellum, maxillary palps, antennae, or visible light court conspecific, but not *D. virilis*, females. Males lacking foreleg tarsi court conspecific and *D. virilis* females.

(D and I) Males lacking foreleg tarsi show high levels of courtship toward conspecific and *D. virilis* females in the majority of assays.

(E and J) Males lacking foreleg tarsi attempt to copulate with conspecific and *D. virilis* females.

(F and K) Males lacking foreleg tarsi attempt copulation with conspecific and *D. virilis* females in most assays.

Error bars represent SEM; $n \geq 11$ /experimental cohort; † $p < 0.001$.

(Demir and Dickson, 2005; Manoli et al., 2005; Ryner et al., 1996; Stockinger et al., 2005), also suppresses interspecies courtship. Gr32a and Fru^M are not coexpressed, but Gr32a neurons appear to contact Fru^M neurons, suggesting that these genes function in the same neural circuit to inhibit courtship of other species. Gr32a and Fru^M also suppress conspecific intermale courtship (Manoli et al., 2006; Miyamoto and Amrein, 2008). However, we show that distinct mechanisms inhibit courtship of conspecific males and flies of other species. In addition, our observations suggest that other drosophilids employ similar pathways to enforce behavioral reproductive isolation.

RESULTS

The Foreleg Is Essential to Inhibit Interspecies Courtship by Males

We wished to identify male *D. melanogaster* sensory structures that inhibit courtship with other drosophilids. *D. melanogaster* males utilize vision, hearing, mechanosensation, smell, and taste during courtship (Figure 1A) (Acebes et al., 2003; Greenspan and Ferveur, 2000; Kowalski et al., 2004; Krstic et al., 2009; Robertson, 1983; Spieth, 1974; Tompkins et al., 1980, 1982). Accordingly, we asked whether these modalities inhibited interspecies

courtship. We used conspecific or *D. virilis* females as mating partners of socially naïve *D. melanogaster* males lacking specific sensory input (Figures 1B and 1G). *D. virilis* shared an ancestor with *D. melanogaster* ~40 million years ago (mya), and wild-type (WT) *D. melanogaster* males do not court *D. virilis* females (Figure 1H). Males lacking olfactory (antennae or maxillary palps) or auditory (antennae) structures as well as males tested in the dark courted conspecific but not *D. virilis* females (Figure 1). Gustatory cues are detected by neurons on mouthparts and on foreleg tarsi. Removal of all mouthparts led to desiccation and a deterioration in general health and mating performance (data not shown). We therefore extirpated only the male labellum, the mouthpart that likely contacts the female. Such males courted conspecific, but not *D. virilis*, females (Figure 1). Males usually tap other flies with their foreleg tarsi prior to proceeding with courtship (Figure 1A) (Bastock and Manning, 1955). The foreleg is required to inhibit *D. melanogaster* males from courting *D. simulans* females, a species that diverged from *D. melanogaster* ~2 mya (Manning, 1959). Males lacking both foreleg tarsi courted conspecific and *D. virilis* females with a similar courtship index (CI), the fraction of time spent courting (Figures 1C and 1H). *D. virilis* females were not receptive to *D. melanogaster* males as evidenced by repeated kicking and walking away (data not shown). Nevertheless, tarsiless males reliably displayed sustained courtship, including courtship songs and copulation attempts, toward *D. virilis* females (Figures 1H–1K). Thus, foreleg tarsi are required to inhibit *D. melanogaster* males from courting *D. virilis*, a distant drosophilid.

Identification of Chemosensory Neurons that Inhibit Interspecies Courtship

We sought to identify the foreleg neurons that inhibit interspecies courtship by males. The tarsi contain chemosensory neurons that detect contact-based chemical cues (Dethier and Chadwick, 1948; Dunipace et al., 2001; Frings and Frings, 1949; Scott et al., 2001). The fly genome encodes a gene family of gustatory receptors (Grs) that are expressed in chemosensory neurons (Clyne et al., 2000; Dunipace et al., 2001; Hallem et al., 2006; Scott, 2005; Scott et al., 2001). To identify Grs expressed in foreleg tarsal neurons, we used 20 published *Gr-GAL4* lines to express nuclear EGFP (stinger GFP; *UAS-stingerGFP*). We identified eight Grs expressed in male foreleg tarsi (Figures 2A–2H, S1, available online, and Table S1; see also Extended Experimental Procedures), some of whose expression patterns have been described (Bray and Amrein, 2003; Moon et al., 2009; Scott et al., 2001; Thorne and Amrein, 2008; Weiss et al., 2011).

We used these eight *Gr-GAL4* lines to ablate chemosensory neurons with *UAS-head involution defective* (*UAS-hid*) and assess their role in inhibiting interspecies courtship (Figure 2). Strikingly, ablation of Gr32a or Gr33a neurons, but not other Gr neurons, allowed *D. melanogaster* males to court *D. virilis* females (Figures 2I and 3). The extent and quality of courtship toward *D. virilis* females displayed by males lacking Gr32a or Gr33a neurons resembled that seen with conspecific females despite rejection by *D. virilis* females (Figure 3 and data not shown).

The specificity of the phenotype observed with *Gr32a:hid* and *Gr33a:hid* could reflect the possibility that only these GAL4 and

HID pairings ablated the corresponding sensory neurons. We tested this directly by driving stingerGFP and HID in Gr neurons (*Gr:stingerGFP, hid*) to visualize their loss. We find comparable reduction of sensory neurons with these eight Gr lines, with only an occasional escapee (Figures 2A'–2H', S1, Table S1). Thus, the other Gr neurons we tested are not required to inhibit interspecies courtship. Although Gr32a and Gr33a are expressed in the foreleg and labellum, removal of the former but not the latter permits interspecies courting. Thus, our findings indicate that Gr32a or Gr33a foreleg neurons inhibit courtship toward *D. virilis* females.

We tested whether Gr32a and Gr33a neurons also inhibited males from courting females of *D. simulans* and *D. yakuba*, species that diverged from *D. melanogaster* ~2 and ~10 mya, respectively. We find that *Gr32a:hid* and *Gr33a:hid* males avidly courted conspecific as well as *D. simulans*, *virilis*, and *yakuba* females (Figure 3). The vast majority of these assays had high levels of courtship, including attempted copulation by the experimental males (Figure 3). Males displayed attempted copulation most toward *D. virilis* females. In fact, *D. virilis* females move less and more slowly compared to the other females we tested, and this may allow males to attempt copulation more frequently. *D. virilis* females may also provide other cues (or lack chemorepellents) that elicit courtship in the absence of Gr32a or Gr33a neurons. In summary, Gr32a and Gr33a neurons inhibit courtship toward females of diverse species that last shared an ancestor with *D. melanogaster* 2–40 mya.

Gr32a Inhibits Interspecies Courtship

In the foreleg, most Gr32a neurons also express Gr33a (Moon et al., 2009). Thus, one or both of these Grs could be required to inhibit interspecies courtship. We tested *D. melanogaster* males null for Gr32a (*Gr32a^{-/-}*) or Gr33a (*Gr33a^{-/-}*) for courtship toward females of other species (Miyamoto and Amrein, 2008; Moon et al., 2009). *Gr32a^{-/-}*, but not *Gr33a^{-/-}*, males courted *D. simulans*, *virilis*, and *yakuba* females (Figure 4, Movies S1, S2, and S3). *Gr32a^{-/-}* males displayed the entire range of courtship preceding copulation toward females of all species and copulated with conspecifics (Figure 4 and data not shown).

Two Grs, Gr5a and Gr66a, that detect sugars and bitter tastants, respectively, are broadly expressed in tarsal neurons (Chyb et al., 2003; Koganezawa et al., 2010; Thorne et al., 2004; Wang et al., 2004). Ablating Gr5a neurons (*Gr5a:hid*) did not permit courtship of other species (data not shown). *Gr66a^{-/-}* males also do not court nonconspecific females (Figure S2). Thus, inhibition of interspecies courtship may not be a general function of chemoreceptors that detect aversive tastants. Rather, we have uncovered a role of Gr32a in restricting *D. melanogaster* males to courting conspecific females.

We further confirmed the role of Gr32a in inhibiting interspecies courtship by using RNAi to knockdown Gr32a. We used the pan-neuronal *C155-GAL4* to drive two separate RNAi constructs targeting Gr32a (Dietzl et al., 2007). Male flies expressing each of these transgenes courted conspecific females and females of other species (Figure S2). Thus, disruption of Gr32a function, either by a null mutation or by RNAi, permits *D. melanogaster* males to court females of many other drosophilids without disrupting courtship of conspecific females.

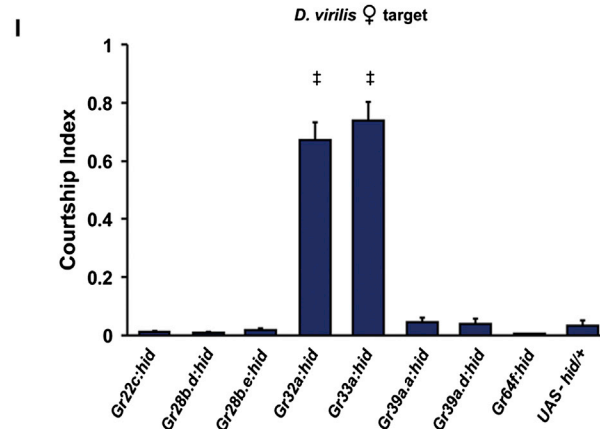
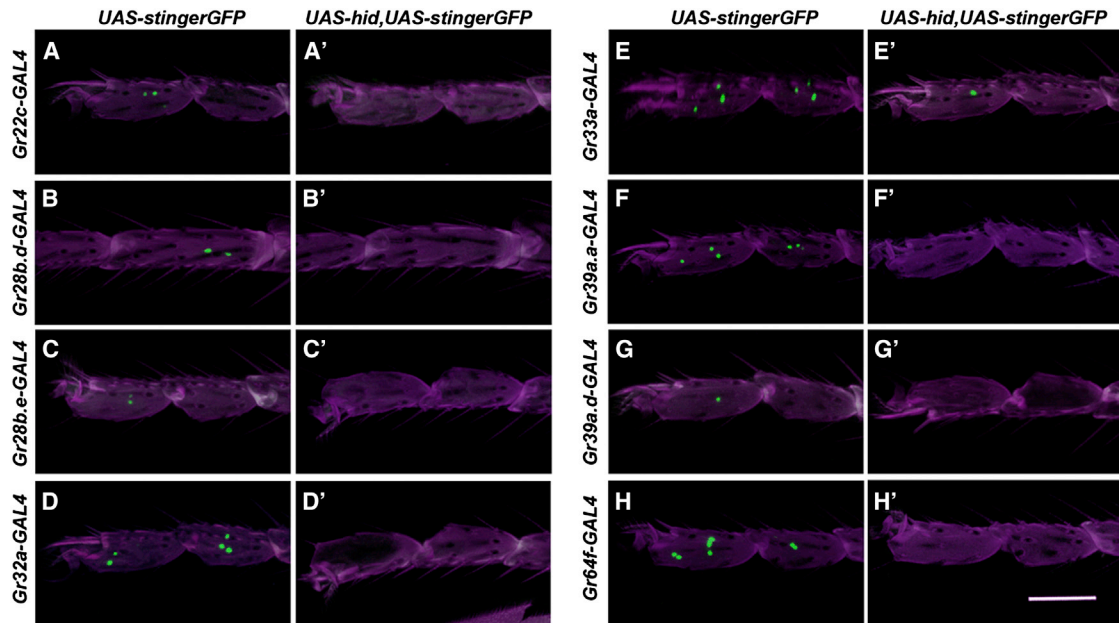


Figure 2. Identification of Gr Neurons in the Male Foreleg that Inhibit Interspecies Courting

(A–H') Expression of different Grs (A–H) and ablation of Gr neurons (A'–H') in foreleg tarsi. Whole-mount preparation of tarsal segments 4 and 5 (t4, t5) (A, A', and C–H') and t2 (B and B') shown. More distal tarsal segments are on the left.

(I) Ablation of Gr32a or Gr33a neurons in *D. melanogaster* males permits courting of *D. virilis* females.

All statistical comparisons in this and subsequent figures were performed between experimental and the corresponding control genotypes. Error bars represent SEM; $n = 5\text{--}10/\text{genotype}$ (A–H') and $n = 8\text{--}12/\text{genotype}$ (I); ‡ $p < 0.001$; scale bar, 50 μm .

Please see Figure S1 and Table S1.

Gr32a Neurons Function Acutely to Inhibit Interspecies Courtship

Our findings so far suggest that activity of Gr32a neurons suppresses sexual displays toward nonconspecific females. We tested this possibility by expressing the temperature-sensitive dominant negative dynamin mutant, *shibire^{ts}* (*UAS-shi^{ts}*), in Gr32a neurons (Kitamoto, 2001). At permissive temperatures, *Gr32a:shi^{ts}* males courted conspecific, but not *D. virilis*, females (Figures 5A and 5C). However, at restrictive temperatures, when synaptic vesicle recycling is inhibited by *Shi^{ts}*, these males courted *D. virilis* females as avidly as conspecific females (Figures 5A and 5C). Thus, functional silencing of Gr32a neurons

permits interspecies courtship even though these neurons express WT Gr32a.

We tested whether heterologous excitation of Gr32a neurons inhibits interspecies courtship in *Gr32a^{-/-}* males. We therefore generated males that expressed the heat-activatable cation channel, dTrpA1 (*UAS-dTrpA1*) (Pulver et al., 2009), in neurons that would normally express Gr32a (*Gr32a^{-/-}, Gr32a:dTrpA1*). As expected, these flies courted *D. virilis* females at the permissive temperature (Figures 5B and 5D). By contrast, at an elevated temperature that activates dTrpA1 these males courted conspecific but not *D. virilis* females (Figures 5B and 5D). Thus, activity of Gr32a neurons abrogates interspecies courtship but does not

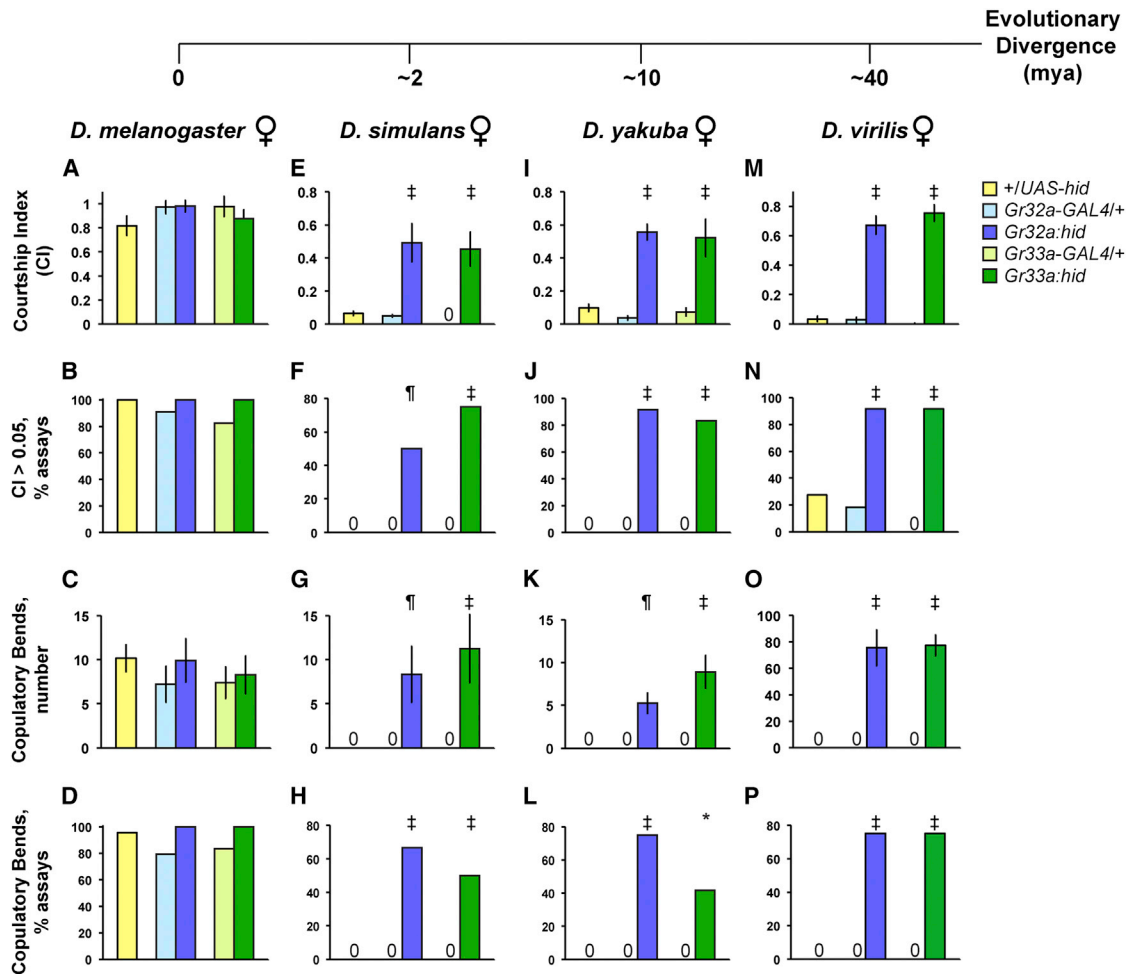


Figure 3. Ablation of Gr32a or Gr33a Neurons Permits Courting of Females of Other Species

(A–P) *D. melanogaster* males with ablation of Gr32a or Gr33a neurons (*Gr:hid*) were tested for courtship with females. Last common ancestor (evolutionary divergence) shared with *D. melanogaster* shown as mya (not to scale) above the bar graphs.

(A–D) Ablation of Gr32a or Gr33a neurons does not alter courtship of conspecific females.

(E–P) Ablation of Gr32a or Gr33a neurons permits courtship of *D. simulans* (E–H), *D. yakuba* (I–L), and *D. virilis* (M–P) females.

Error bars represent SEM; n = 10–24/genotype; *p < 0.05, ¶p < 0.01, †p < 0.001.

appear to significantly inhibit courtship of conspecific females. In summary, functional activation of Gr32a neurons is necessary and sufficient to inhibit courtship specifically toward reproductively futile targets such as females of other species.

Gr32a Is Required to Detect Aversive Ligands Secreted by Other Species

We sought to determine the cues recognized by Gr32a that restrict courtship to conspecific females. Chemosensory cues encoded by cuticular hydrocarbons (CHs) profoundly influence social behavior in flies (Antony and Jallon, 1982; Billeter et al., 2009; Coyne et al., 1994; Ferveur, 2005; Grillet et al., 2012; Higgin et al., 2000; Jallon and David, 1987; Savarit et al., 1999). We asked whether cuticular extracts from *D. simulans*, *virilis*, and *yakuba* females inhibited courtship by *D. melanogaster* males. We applied these extracts to conspecific females lacking oenocytes, the cells that secrete CHs. WT males courted

oenocyteless (*oe-*) females (Billeter et al., 2009), including when *oe-* females were coated with conspecific cuticular extract, but they showed minimal courtship of *oe-* females coated with cuticular extracts from other species (Figure 5E). Strikingly, *Gr32a*^{-/-} males courted *oe-* flies regardless of the source of the cuticular extract (Figure 5E). Thus, cuticular extracts from other drosophilids inhibit sexual displays by WT *melanogaster* males in a Gr32a-dependent manner.

We wished to identify the cuticular compounds that inhibit interspecies mating. The CH z-7-tricosene (7T; Figure S3) is secreted by *D. melanogaster* males and to ≥ 10 -fold lesser extent by females (Jallon and David, 1987), and it inhibits intermale courtship (Ferveur, 2005; Lacaille et al., 2007). Moreover, Gr32a is required to detect 7T (Wang et al., 2011). Both sexes of *D. simulans* and *D. yakuba* secrete 7T in copious amounts (Jallon and David, 1987), and we asked whether 7T-coated *oe-* females would be courted by *D. melanogaster* males. We found

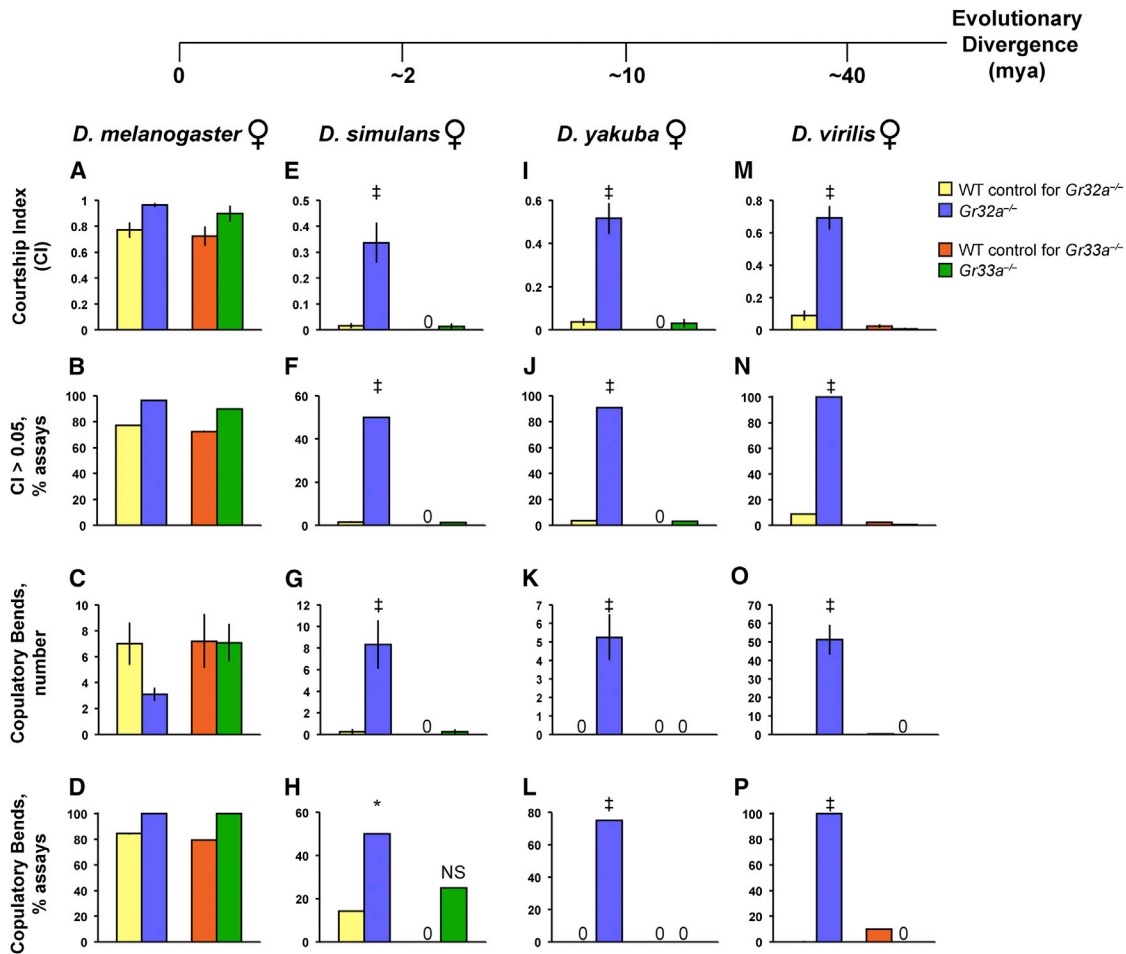


Figure 4. Gr32a Inhibits Interspecies Courtship

(A–P) Gr32a and Gr33a mutant and control *D. melanogaster* males were tested for courtship with females.

(A–D) No difference in courting conspecific females between control and Gr32a or Gr33a mutants.

(E–P) Gr32a, but not Gr33a, mutants court *D. simulans* (E–H), *D. yakuba* (I–L), and *D. virilis* (M–P) females.

Error bars represent SEM; $n = 10\text{--}24/\text{genotype}$; * $p < 0.05$, † $p < 0.001$; NS = not significant. Please see Figure S2 and Movies S1, S2, and S3.

that Gr32a^{-/-}, but not WT, males courted oe⁻ targets coated with physiological concentrations of 7T similar to control oe⁻ or WT *melanogaster* females (Figure 5E). Although 7T is secreted by many drosophilids, it is essentially undetectable on the *D. virilis* cuticle. *D. virilis*, but not *melanogaster*, *simulans*, or *yakuba*, secrete the related CH z-9-tricosene (9T; Figure S3) (Ferveur, 2005; Liimatainen and Jallon, 2007). Gr32a^{-/-}, but not WT, males courted 9T-coated oe⁻ females vigorously (Figure 5E). Cuticular extracts from *D. virilis* appeared more effective than 9T alone in suppressing courtship of oe⁻ females, suggesting the presence of other CHs on *D. virilis* that inhibit courtship. One such CH may be z-11-pentacosene (11P; Figure S3), which appears restricted to *D. virilis* (Ferveur, 2005). We synthesized 11P (Figure S3) and tested whether 11P-coated oe⁻ females elicited courtship. We found that Gr32a^{-/-}, but not WT, males courted such females vigorously (Figure 5E). Oe⁻ females coated with both 9T and 11P did not elicit less courtship by WT males compared to 11P alone (Figure 5E), consistent with the notion that both cues are recognized by Gr32a. In summary, Gr32a is required to detect

at least three CHs, 7T, 9T, and 11P, secreted by conspecific males or flies of other species but not by conspecific females, and this recognition inhibits courtship of such reproductively dead-end targets.

A Distinct Cellular Pathway Inhibits Interspecies Courtship

Fru^M isoforms are necessary and sufficient for most components of male courtship (Demir and Dickson, 2005; Gill, 1963; Hall, 1978; Ito et al., 1996; Manoli et al., 2005; Ryner et al., 1996; Stockinger et al., 2005). We tested whether Fru^M also restricts courtship to conspecifics. Males null for Fru^M (*fru*⁴⁻⁴⁰/*fru*^{sat15}) did not court any targets, including conspecific females, consistent with the requirement for Fru^M in male courtship (Figure 6A). However, males mutant, but not null, for Fru^M (*fru*¹/*fru*⁴⁻⁴⁰) courted conspecific females and those from other species (Figure 6A).

Fru^M and Gr32a both inhibit males from courting females of other species (Figures 4, 6A, and S2) and conspecific males

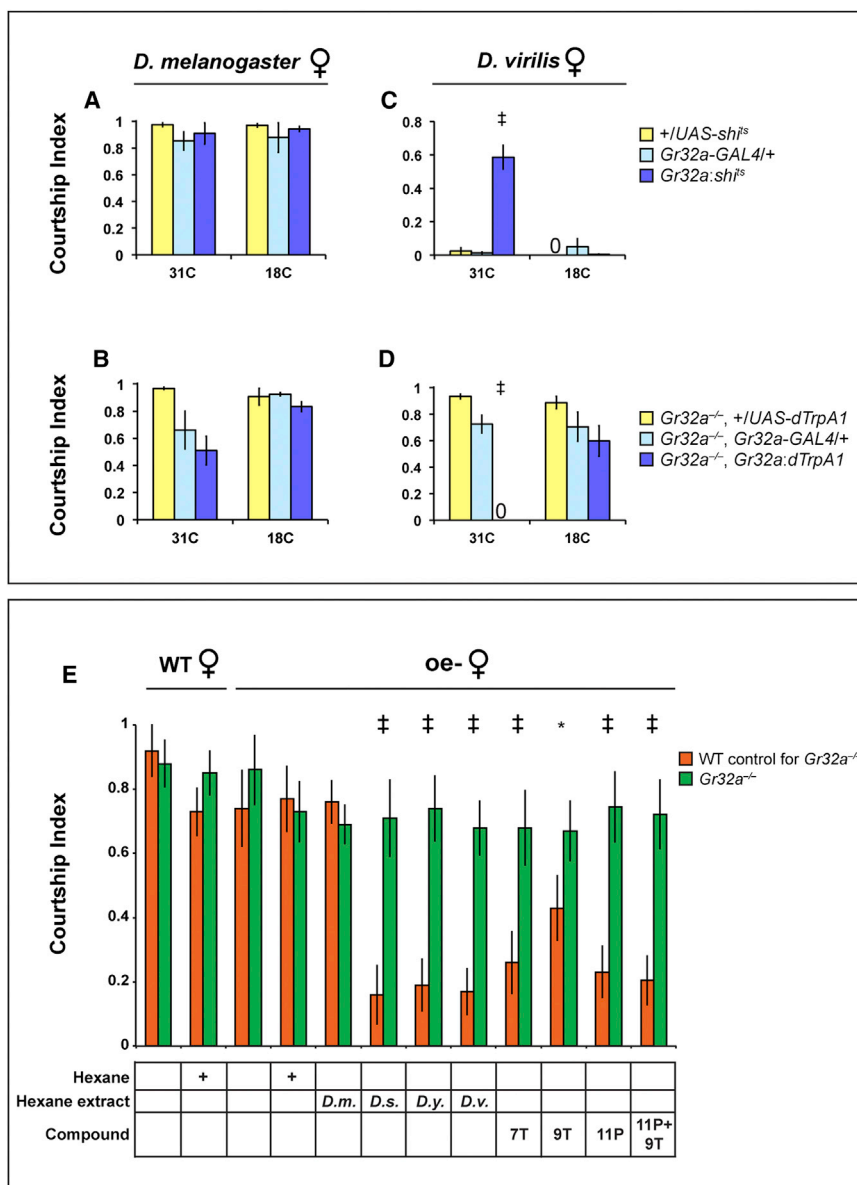


Figure 5. Gr32a Neurons Inhibit Interspecies Courtship by Recognizing Cuticular Hydrocarbons Found on Other Species

(A–D) *D. melanogaster* males WT (A and C) or mutant for Gr32a (B and D) were tested for courtship with conspecific or *D. virilis* females.

(A) Inactivation of synaptic release by Gr32a neurons (*Gr32a:sh^{TS}*) at the restrictive temperature (31°C) does not alter courtship of conspecific females.

(B) Increase in electrical activity in Gr32a neurons (*Gr32a:dTrpA1*) at 31°C does not alter courtship of conspecific females.

(C) Inactivation of synaptic release by Gr32a neurons permits courtship of *D. virilis* females by *Gr32a:sh^{TS}* males.

(D) Increase in electrical activity in Gr32a neurons abrogates courtship of *D. virilis* females by *Gr32a^{-/-}, Gr32a:dTrpA1* males.

(E) *Gr32a^{-/-}* males court oe- conspecific females coated with cuticular extracts from *D. melanogaster* (*D.m.*), *simulans* (*D.s.*), *yakuba* (*D.y.*), and *virilis* (*D.v.*), as well as with specific CHS present on these species.

Error bars represent SEM; n = 10–16/genotype; *p < 0.05, †p < 0.001. Please see Figure S3.

of other species (Figures S2 and S4H). Thus, a loss of sex recognition is not sufficient to permit courtship of other species, and different molecular and cellular pathways regulate courtship of conspecific males and other drosophilids.

We wondered whether Fru^M functioned in Gr32a neurons to inhibit interspecies courtship. Gr32a neurons in adult foreleg tarsi and labellum do not express Fru^M (Figures 6B–6D", S4K–S4M", and data not shown). To preclude transient or weak, undetectable, Fru^M expression in Gr32a neurons, we utilized a validated RNAi strain (*UAS-fru^MIR*) (Manoli and Baker, 2004) to knockdown Fru^M in Gr32a cells. However, *Gr32a:fru^MIR*

flies also did not court *D. virilis* females (Figure S4A). We cannot exclude the possibility that Fru^M regulates differentiation of Gr32a neurons prior to Gr32a expression to regulate interspecies courtship. Nevertheless, our findings indicate that Fru^M is not required in Gr32a neurons to inhibit interspecies courtship.

We tested whether Gr32a neurons might contact Fru^M neurons. We employed an enhanced variant of GFP reconstitution across synaptic partners (GRASP) (Feinberg et al., 2008) in which one component of GRASP is targeted to synapses, thereby restricting GFP reconstitution to synapses. Briefly, spGFP1–10 was targeted to synapses by fusing it to Neurexin (*UAS-spGFP1–10::Nrx*), a transmembrane protein involved in synapse formation and maturation (Knight et al., 2011), and spGFP11 was fused to CD4 (*LexO-spGFP11::CD4*) (Gordon and Scott, 2009) to permit cell-surface expression. Our strategy labeled a known

(Gill, 1963; Hall, 1978; Miyamoto and Amrein, 2008). We therefore tested whether *fru¹/fru⁴⁻⁴⁰* or *Gr32a^{-/-}* males courted males of other species. We find that Fru^M or Gr32a mutant males court conspecific, *D. simulans* and *yakuba* males, but not *virilis* males (Figures S4F and S4I), thereby revealing a broad, but not comprehensive, deficit in sex and species recognition. It is unlikely that a loss of sex recognition in Fru^M or Gr32a mutant males would permit them to court same-sex conspecifics as well as other drosophilids (Grosjean et al., 2008). Indeed, *Gr32a^{-/-}* males also court conspecific males (Figure S4G) (Moon et al., 2009), but they do not court other drosophilids (Figures 4 and S4G). Moreover, males mutant for Ppk23, a Degenerin/Epithelial sodium channel expressed in Fru^M neurons in foreleg tarsi, court conspecifics of both sexes (Lu et al., 2012; Thistle et al., 2012; Toda et al., 2012), but these mutants did not court individuals

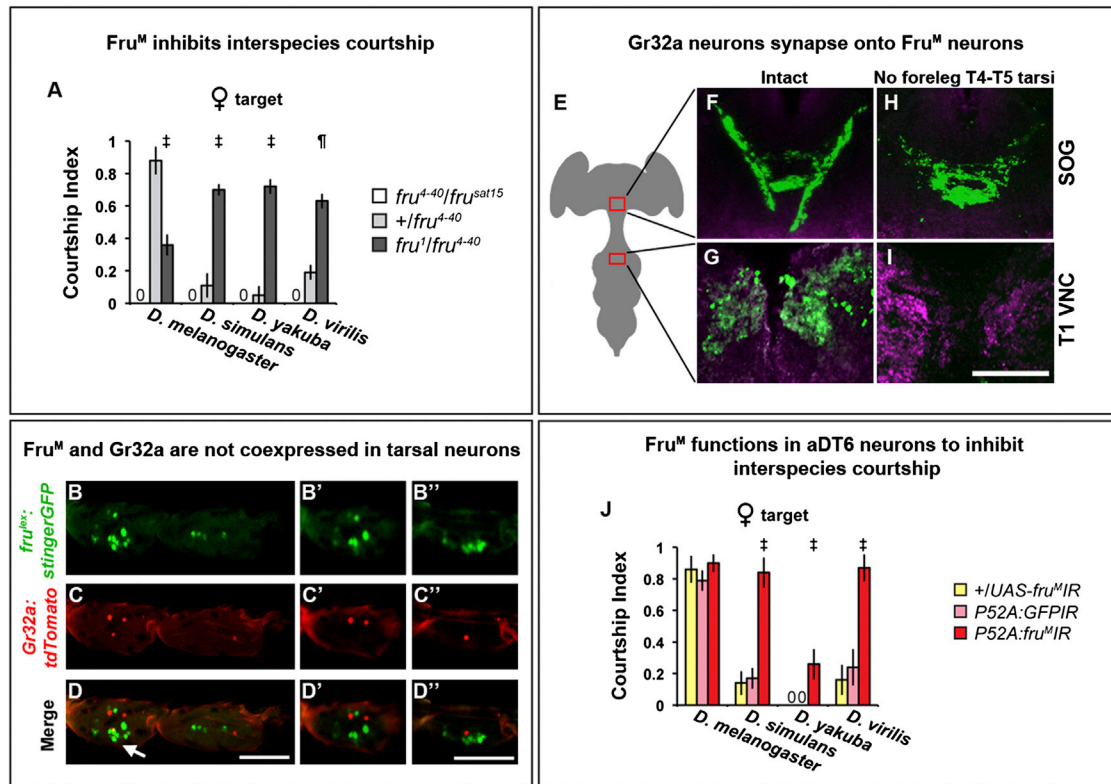


Figure 6. A Cellular and Molecular Pathway that Inhibits Interspecies Courtship

(A) fru^1/fru^{4-40} males court conspecific females and females of other species.

(B–D'') No coexpression of Fru^M and Gr32a in foreleg tarsi of *D. melanogaster* males (D). A cell that appears colabeled for Fru^M and Gr32a in a Z projected image (arrow in D) in fact represents two distinct cells in different optical slices expressing either Fru^M (B'–D'') or Gr32a (B'–D'), but not both (lines used: fru^{lex} , $lexO$ -*stingerGFP* (line E, F) and $Gr32a$ -*GAL4*, *UAS*-*tdTomato*; abbreviated to $fru^{lex}:stingerGFP$, $Gr32a:tdTomato$).

(E) Schematic of the fly central nervous system shows the location of the SOG and first thoracic segment (T1) VNC (red boxes).

(F–I) Native GRASP fluorescence (green) in the vertical limb of the SOG and the T1 VNC in *D. melanogaster* males ($Gr32a:spGFP1-10::NrX$, $fru^{lex}:spGFP11::CD4$) is lost upon T1 tarsectomy. The neuropil (magenta) is immunolabeled with nc82.

(J) Knockdown of Fru^M in male aDT6 neurons ($P52A:fru^{MIR}$) permits courtship of conspecific females and females of other species.

Error bars represent SEM; n = 10–31/experimental cohort; ¶p < 0.01, ‡p < 0.001; scale bar, 20 μm. See Figure S4, Table S2, and Movie S4.

synapse but not neighboring pre- or postsynaptic processes. L3 and Tm9 neurons have processes outside the M3 medullary layer, but only synapse within M3 (Gao et al., 2008; Yamaguchi and Heisenberg, 2011); correspondingly, we observed native GFP fluorescence only in M3 but not in L3 or Tm9 processes (Figures S4N–S4Q). In our experimental flies, we observed native GFP fluorescence in the ventral nerve cord (VNC) and the subesophageal ganglion (SOG) (Figures 6E–6G, see also Figures S4R–S4T), locations at which tarsal sensory neurons synapse with central neurons (Dunipace et al., 2001; Scott et al., 2001; Stocker, 1994). Such GRASP signal suggests synaptic contact between Gr32a and Fru^M neurons that will have to be verified with electron microscopy or electrophysiology. Removal of foreleg tarsi eliminated native GFP fluorescence in the VNC and the vertical limb of innervation in the SOG (Figures 6H and 6I), demonstrating that these contacts with Fru^M neurons emanated from foreleg Gr32a neurons (Wang et al., 2004). The residual GRASP fluorescence in the SOG is consistent with projections of proboscis Gr32a neurons. Our results are consistent with

the notion that Gr32a and Fru^M function within a shared neural circuit to inhibit interspecies courtship.

The enhancer trap $P52A$ -*GAL4* labels a bilateral set of ~60 Fru^M neurons (aDT6 neurons) within the SOG (Cachero et al., 2010; Manoli and Baker, 2004; Yu et al., 2010). Knockdown of Fru^M in aDT6 cells ($P52A:fru^{MIR}$) permits males to sing and copulate without tapping a conspecific female (Manoli and Baker, 2004). Importantly, $P52A:fru^{MIR}$ males court conspecific females but not males, suggesting that sex recognition and mating can occur without tapping (Manoli and Baker, 2004). We wondered whether these males would court other species. Strikingly, $P52A:fru^{MIR}$ males courted *D. simulans*, *virilis*, and *yakuba* females and *yakuba* males (Figures 6J and S4J). In contrast to courtship of conspecific females, $P52A:fru^{MIR}$ males sang only after tapping nonconspecific flies (Table S2). Our findings suggest that males can recognize conspecific females as mating targets prior to tapping, which may be used to determine species membership before proceeding with courtship. In any event, aDT6 cells define a central neuronal

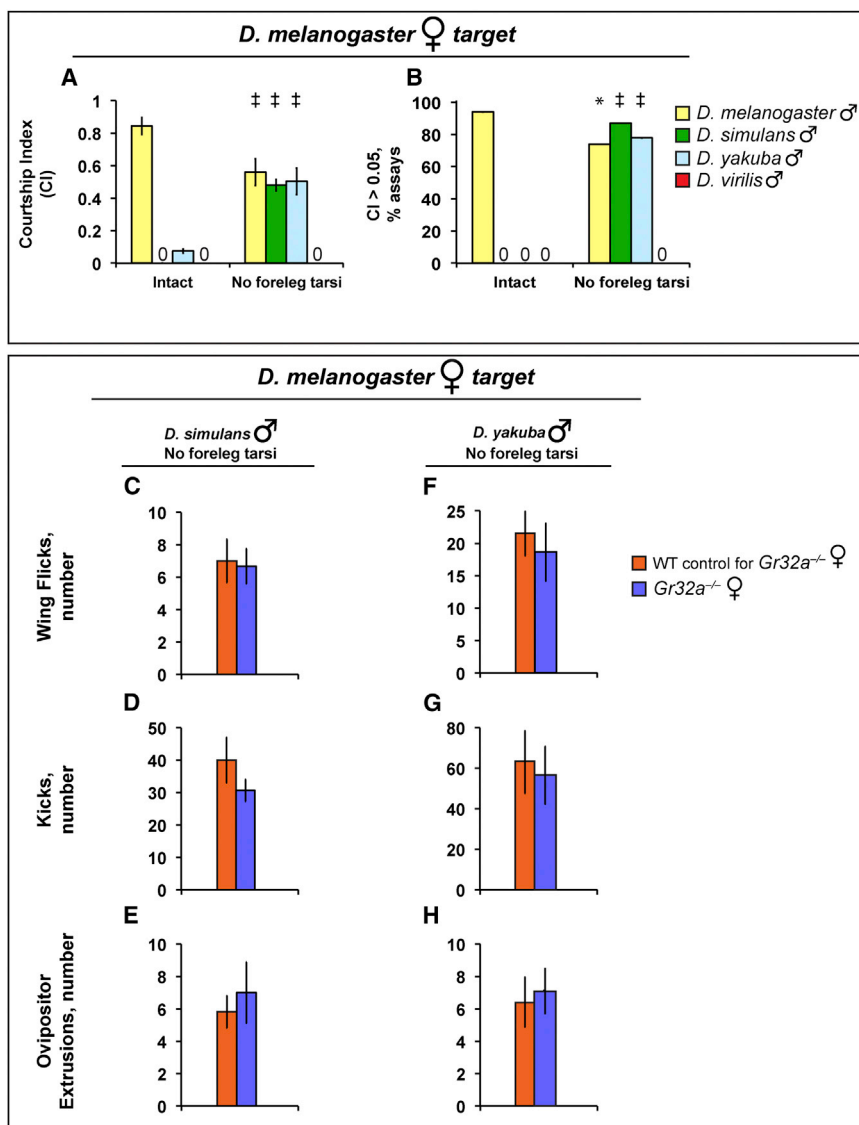


Figure 7. Sexually Dimorphic but Evolutionarily Conserved Regulation of Interspecies Courtship

(A and B) Tarsiless *D. simulans* and *yakuba* males court *D. melanogaster* females similar to conspecific males.

(C–E) *D. melanogaster* females reject courtship by *D. simulans* males with wing flicks, kicks, and ovipositor extrusions.

(F–H) *D. melanogaster* females reject courtship by *D. yakuba* males with wing flicks, kicks, and ovipositor extrusions.

Error bars represent SEM; n = 11–18/experimental cohort; *p < 0.05; ‡p < 0.001. See Movies S5, S6, and S7.

Sex and Species-Specific Regulation of Interspecies Courtship

We tested whether other drosophilid males use foreleg tarsi to reject nonconspecifics as mates. Tarsiless *D. simulans* and *yakuba*, but not *virilis*, males courted *melanogaster* females vigorously (Figures 7A and 7B, Movies S5, S6). Tarsiless males of *D. mauritiana*, a species closely related to *D. simulans*, also courted *melanogaster* females (data not shown). The role of foreleg tarsi in *D. pseudoobscura*, a species that diverged from *melanogaster* ~25 mya, could not be ascertained because such tarsiless males were very unhealthy (data not shown). In summary, the function of foreleg tarsi in rejecting potential mates from other species is conserved across many drosophilids.

Single genes such as *period* influence reproductive isolation in both sexes by modulating various behaviors (Ritchie et al., 1999; Tauber et al., 2003; Wheeler et al., 1991). *Gr32a* is expressed equivalently in both sexes in mouthparts, tarsi, and in the abdominal wall (Park and Kwon, 2011), which is contacted by males when they tap females. We therefore tested whether *D. melanogaster* females utilize *Gr32a* to reject other drosophilid males. Using wing flicks, kicks, and ovipositor extrusion, both WT and *Gr32a*^{-/-} females rejected courtship attempts of tarsiless *simulans* and *yakuba* males (Figures 7C–7H, and Movies S6 and S7). As expected, *Gr32a*^{-/-} females mated successfully with conspecific males (data not shown) (Miyamoto and Amrein, 2008). Thus, the control of interspecies courtship by *Gr32a* is sexually dimorphic such that males but not females utilize *Gr32a*-based signaling to restrict courtship to conspecifics.

DISCUSSION

Mythological assertions notwithstanding, animals rarely pick mates from other species (Ovid, *Metamorphoses*). The

population that inhibits interspecies, but not conspecific intermale, courtship in a *Fru*^M-dependent manner. These findings provide further evidence showing that distinct cellular and molecular mechanisms inhibit intermale conspecific and interspecies courtship.

We tested whether aDT6 neurons are postsynaptic to *Gr32a* SOG projections using our enhanced GRASP variant. Despite the widespread expression of the *P52A-GAL4* driver (Manoli and Baker, 2004), we did not observe native GFP fluorescence in the SOG (Figures S4U–S4V''). The lack of GRASP signal does not reflect failure of expression of GRASP components because these could be visualized with immunolabeling (Figures S4W–S4W''). We also did not observe apposition of *Gr32a* and aDT6 processes within the SOG using the fly brainbow system (Figure S4X and Movie S4; n = 11) (Hampel et al., 2011). Thus, if *Gr32a* and *Fru*^M aDT6 neurons inhibit interspecies courtship via a shared circuit, they are synaptically linked via one or more interposed neurons.

reproductive isolation imposed by inhibiting interspecies mating affords a powerful barrier to the admixing of gene pools. We have uncovered genes and neural pathways in *D. melanogaster* males that inhibit interspecies courtship. Although *D. melanogaster* females utilize unrelated mechanisms to reject males of other species, remarkably, many other drosophilid males may employ a similar pathway to *D. melanogaster* males to reject nonconspecific females.

Chemical Control of Interspecies Courtship

Gr32a belongs to a family of contact-based chemoreceptors, whose putative ligands, tastants, and pheromones elicit robust spiking in sensory neurons (Hallem et al., 2006; Scott, 2005). Gr32a is required for the response to many aversive, bitter-tasting compounds, including alkaloids such as lobeline and the insect repellent N, N, diethyl-meta-toluamide (DEET). The Grs coexpressed with Gr32a, Gr33a, and Gr66a, also respond to these or other bitter, aversive tastants (Lee et al., 2010; Moon et al., 2006, 2009; Weiss et al., 2011). Here, we show that Gr32a is required for *D. melanogaster* males to detect diverse CHs found on other drosophilids and *D. melanogaster* males but not females. These CHs appear to serve as semiochemicals such that their presence on potential sexual partners permits *D. melanogaster* males to reject them as mates. These findings suggest a model wherein activation of Gr32a neurons by diverse cues may lead to avoidance of a potential food source or mate.

It is surprising that Gr32a is required for the recognition of diverse compounds such as alkaloids, the dialkylamide DEET, and CHs. It is unknown whether Grs detect such ligands in the absence of additional coreceptors or cofactors. It is possible, therefore, that Gr32a partners with different coreceptors to detect these distinct cues (Figures S3E–S3I). Even though Gr32a, Gr33a, and Gr66a recognize alkaloids, only Gr32a is required to recognize CHs on flies. Although we have tested diverse drosophilids, Gr33a and Gr66a may recognize CHs that were not tested in this study. CH detection by these Grs may also be redundant to recognition by Gr32a. In any event, Gr32a is required for the detection of aversive CHs on nonconspecifics and for inhibiting interspecies courtship.

A Molecular and Neural Pathway that Inhibits Interspecies Courtship

Despite pioneering efforts (Coyne et al., 1994; Hollocher et al., 1997; Laturney and Moehring, 2012; Manning, 1959; Mayr and Dobzhansky, 1945; Moehring et al., 2006; Nanda and Singh, 2012; Ritchie et al., 1999; Shirangi et al., 2009; Smadja and Butlin, 2009; Spieth, 1949; Sturtevant, 1920), little is known about the neural pathways that inhibit interspecies mating. Gr32a appears to function in foreleg neurons to inhibit interspecies courtship, consistent with the observation that *D. melanogaster* males tap potential mates early during courtship. Labellar Gr32a neurons may be redundant to Gr32a foreleg neurons, they may lack a coreceptor essential for recognizing CHs, or their distinct central projections may not activate circuits that inhibit interspecies mating (Park and Kwon, 2011; Wang et al., 2004). Labellar Gr32a neurons are also likely activated during licking, a step by which males may be unable to disengage from mating.

Indeed, courtship is thought to proceed via steps whose initiations depend on progressive sensory input (Manoli and Baker, 2004). Regardless, Gr32a foreleg neurons appear to inhibit interspecies courtship, and this foreleg inhibitory pathway is conserved across many drosophilids.

Heterologous activation of Gr32a neurons suppresses interspecies courtship by *Gr32a*^{-/-} males. Such activation does not significantly inhibit courtship of conspecific females. In fact, distinct genes, chemosensory neurons, and pheromones are important for courting conspecific females (Bray and Amrein, 2003; Ejima and Griffith, 2008; Grosjean et al., 2011; Kurtovic et al., 2007; Lin et al., 2005; Lu et al., 2012; Thistle et al., 2012; Watanabe et al., 2011). Thus, neural pathways that elicit courting of conspecific females may override courtship-inhibiting signaling by Gr32a neurons. Our findings also suggest that, in addition to courtship-promoting neural circuits, evolutionary constraints can select for pathways such as Gr32a and Fru^M neurons that suppress courtship of reproductively futile targets.

Several observations show that Gr32a mutant males are not simply hypersexual. They court conspecific females in a WT manner (Miyamoto and Amrein, 2008) and spend less time courting conspecific males than females. Gr32a mutants also court *D. virilis* females but not males, nor do they court ants and houseflies (data not shown), observations that suggest the existence of other pathways to inhibit such courtship. Thus, loss of Gr32a function does not lead to a release of sexual behavior toward all similarly-sized moving objects.

Gr32a also regulates intermale aggression (Wang et al., 2011). *Gr32a*^{-/-} males may court target flies of other species or conspecific males because they cannot fight with them. However, WT males did not attack *D. virilis* targets of either sex, and *Gr32a*^{-/-} males courted *D. virilis* females vigorously. Rather than modulate aggression, functional activation or inactivation of Gr32a neurons regulated interspecies courtship with *D. virilis* females. It is possible that Gr32a first mediates species recognition, and if the fly is a male conspecific then Gr32a may activate aggression. Regardless, Gr32a inhibits interspecies courtship, and Gr32a neurons acutely inhibit courtship of reproductively futile targets such as members of other species.

Separable genetic and neural mechanisms in *D. melanogaster* males inhibit courtship of conspecific males and other species. Gr33a and Ppk23 inhibit courting of conspecific males but not other species. The few Gr33a foreleg neurons that do not express Gr32a may specifically preclude mating with conspecific males (Moon et al., 2009). Fru^M function in aDT6 neurons inhibits courtship of other species but not conspecific males. Thus, the mechanisms that inhibit interspecies and same-sex conspecific courtship are doubly dissociable.

Molecular Mechanisms of Speciation

One intuit that multiple sensory pathways recognize conspecifics as well as nonconspecifics. Strikingly, however, Gr32a sensory pathways alone are necessary and sufficient to inhibit courtship toward nonconspecifics of diverse drosophilids. Although sensory pathway evolution underlies many behavioral adaptations, Gr32a is, to the best of our knowledge, the first sensory receptor found to inhibit interspecies courtship behavior (Gracheva et al., 2010, 2011; Jiang et al., 2012; Jordt and Julius,

2002; McGrath et al., 2011; Nathans, 1999; Wisotsky et al., 2011). Gr32a could influence speciation by imposing behavioral reproductive isolation between drosophilids. It will be important to test whether Gr32a or other Grs inhibit interspecies courtship in other male drosophilids. Gr32a regulates interspecies courtship in male but not female *D. melanogaster*, and this sexual dimorphism may permit differential control of mate selection in the two sexes. Chemoreceptors in the mouse nose recognize other species (Dewan et al., 2013; Ferrero et al., 2011; Isogai et al., 2011; Papes et al., 2010), and it is also possible that they inhibit interspecies mating. In fact, yeast employ pheromone signaling for conspecific recognition and sexual reproduction (Julius et al., 1983; McCullough and Herskowitz, 1979), suggesting that chemosensory inhibition of interspecies mating occurs in unicellular as well as metazoan lineages. Our findings suggest that Fru^M inhibits interspecies courtship via central neural pathways. Fru^M neurons appear dedicated to courtship and aggression and are not required for other behaviors in males (Manoli et al., 2005; Stockinger et al., 2005). Thus, polymorphisms in *fru^M* potentially provide a mechanism to specifically link changes in social behavior with reproductive isolation.

In summary, we have identified genes and neurons that inhibit interspecies courtship in *D. melanogaster* males, but not females. Moreover, these pathways may be conserved in many other drosophilid males. Our study therefore provides a model system to characterize the neural circuits underlying behaviorally mediated reproductive isolation and to understand how such circuits have diverged between the sexes.

EXPERIMENTAL PROCEDURES

Drosophila Stocks

The *L3-GAL4 (R14B07-GAL4)* and *Tm9-LexA (R24C08-LexA)* driver lines were identified by screening the Janelia *GAL4* collection (Jenett et al., 2012) (A. Nern, personal communication); the *R24C08-LexA* (a gift from Gerry Rubin) was constructed as described previously (Pfeiffer et al., 2010). Fly husbandry was performed as described earlier (Manoli and Baker, 2004; Manoli et al., 2005) with some modifications (Extended Experimental Procedures).

Histology

To visualize native GRASP fluorescence, CNS structures were dissected in ice-cold PBL (0.075 M lysine, 0.1 M sodium phosphate buffer [pH 7.4]), fixed for 30 min in 4% paraformaldehyde in PBL at 22°C, washed 3× with PBT (PBS [pH 7.4], + 0.3% Triton X-100) and then blocked with 10% normal donkey serum in PBT. Samples were mounted in Vectashield.

¹³C Synthesis and Analysis

The alkene precursor 11-pentacosyne was synthesized and reduced using hydrogen and Lindlar catalyst to generate the Z-alkene (Small Molecule Synthesis Facility at Duke University). ¹³C NMR spectrum was recorded at 75 MHz. Chemical shifts were reported in parts per million (ppm) relative to deuterated solvent as the internal standard (δ: CDCl₃ 77 ppm): Z-11 ¹³C NMR (CD₃Cl) δ 129.9, 31.9, 29.8, 29.7, 29.6, 29.5, 29.4, 29.3, 29.2, 22.7, 14.1.

Drosophila Behavioral Assays

Flies were anesthetized by CO₂, introduced into a humidified courtship chamber divided by a plastic film to separate experimental from target flies, and allowed to recover at rearing temperature for 3–4 hr prior to testing, as described before (Manoli et al., 2005; Meissner et al., 2011).

Details regarding animals, data analyses, and the procedures described above can be found in the Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures four figures, two tables, and seven movies and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2013.06.008>.

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REFERENCES

- Acebes, A., Cobb, M., and Ferveur, J.-F. (2003). Species-specific effects of single sensillum ablation on mating position in *Drosophila*. *J. Exp. Biol.* 206, 3095–3100.
- Antony, C., and Jallon, J.-M. (1982). The chemical basis for sex recognition in *Drosophila melanogaster*. *J. Insect Physiol.* 28, 873–880.
- Barbash, D.A. (2010). Ninety years of *Drosophila melanogaster* hybrids. *Genetics* 186, 1–8.
- Bastock, M., and Manning, A. (1955). The Courtship of *Drosophila melanogaster*. *Behaviour* 8, 85–111.
- Billeter, J.-C., Rideout, E.J., Dorman, A.J., and Goodwin, S.F. (2006). Control of male sexual behavior in *Drosophila* by the sex determination pathway. *Curr. Biol.* 16, R766–R776.
- Billeter, J.-C., Atallah, J., Krupp, J.J., Millar, J.G., and Levine, J.D. (2009). Specialized cells tag sexual and species identity in *Drosophila melanogaster*. *Nature* 461, 987–991.
- Blair, W.F. (1964). Isolating Mechanisms and Interspecies Interactions in Anuran Amphibians. *Q. Rev. Biol.* 39, 334–344.
- Bray, S., and Amrein, H. (2003). A putative *Drosophila* pheromone receptor expressed in male-specific taste neurons is required for efficient courtship. *Neuron* 39, 1019–1029.
- Cachero, S., Ostrovsky, A.D., Yu, J.Y., Dickson, B.J., and Jefferis, G.S.X.E. (2010). Sexual dimorphism in the fly brain. *Curr. Biol.* 20, 1589–1601.
- Chyb, S., Dahanukar, A., Wickens, A., and Carlson, J.R. (2003). *Drosophila* Gr5a encodes a taste receptor tuned to trehalose. *Proc. Natl. Acad. Sci. USA* 100(Suppl 2), 14526–14530.
- Clyne, P.J., Warr, C.G., and Carlson, J.R. (2000). Candidate taste receptors in *Drosophila*. *Science* 287, 1830–1834.
- Coyne, J.A., and Orr, H.A. (1998). The evolutionary genetics of speciation. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 353, 287–305.
- Coyne, J.A., Crittenden, A.P., and Mah, K. (1994). Genetics of a pheromonal difference contributing to reproductive isolation in *Drosophila*. *Science* 265, 1461–1464.

- Dahanukar, A., and Ray, A. (2011). Courtship, aggression and avoidance: pheromones, receptors and neurons for social behaviors in *Drosophila*. *Fly (Austin)* 5, 58–63.
- Darwin, C. (1860). *On the Origin of Species by Means of Natural Selection, or, The Preservation of Favoured Races in the Struggle for Life* (New York: Appleton).
- Demir, E., and Dickson, B.J. (2005). fruitless splicing specifies male courtship behavior in *Drosophila*. *Cell* 121, 785–794.
- Dethier, V.G., and Chadwick, L.E. (1948). Chemoreception in insects. *Physiol. Rev.* 28, 220–254.
- Dewan, A., Pacifico, R., Zhan, R., Rinberg, D., and Bozza, T. (2013). Non-redundant coding of aversive odours in the main olfactory pathway. *Nature* 497, 486–489.
- Dietzl, G., Chen, D., Schnorrer, F., Su, K.-C., Barinova, Y., Fellner, M., Gasser, B., Kinsey, K., Oettel, S., Scheiblaue, S., et al. (2007). A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*. *Nature* 448, 151–156.
- Dobzhansky, T. (1937). Genetic Nature of Species Differences. *Am. Nat.* 71, 404–420.
- Dobzhansky, T., and Mayr, E. (1944). Experiments on Sexual Isolation in *Drosophila*: I. Geographic Strains of *Drosophila Willistonii*. *Proc. Natl. Acad. Sci. USA* 30, 238–244.
- Dukas, R. (2004). Male Fruit Flies Learn to Avoid Interspecific Courtship. *Behav. Ecol.* 15, 695–698.
- Dunipace, L., Meister, S., McNealy, C., and Amrein, H. (2001). Spatially restricted expression of candidate taste receptors in the *Drosophila* gustatory system. *Curr. Biol.* 11, 822–835.
- Ejima, A., and Griffith, L.C. (2008). Courtship initiation is stimulated by acoustic signals in *Drosophila melanogaster*. *PLoS ONE* 3, e3246.
- Feinberg, E.H., Vanhoven, M.K., Bendesky, A., Wang, G., Fetter, R.D., Shen, K., and Bargmann, C.I. (2008). GFP Reconstitution Across Synaptic Partners (GRASP) defines cell contacts and synapses in living nervous systems. *Neuron* 57, 353–363.
- Ferrero, D.M., Lemon, J.K., Fluegge, D., Pashkovski, S.L., Korzan, W.J., Datta, S.R., Spehr, M., Fendt, M., and Liberles, S.D. (2011). Detection and avoidance of a carnivore odor by prey. *Proc. Natl. Acad. Sci. USA* 108, 11235–11240.
- Ferveur, J.-F. (2005). Cuticular hydrocarbons: their evolution and roles in *Drosophila* pheromonal communication. *Behav. Genet.* 35, 279–295.
- Frings, H., and Frings, M. (1949). The Loci of Contact Chemoreceptors in Insects. A Review with New Evidence. *Am. Midl. Nat.* 41, 602–658.
- Gao, S., Takemura, S.Y., Ting, C.-Y., Huang, S., Lu, Z., Luan, H., Rister, J., Thum, A.S., Yang, M., Hong, S.-T., et al. (2008). The neural substrate of spectral preference in *Drosophila*. *Neuron* 60, 328–342.
- Gill, K.S. (1963). A mutation causing abnormal courtship and mating behavior in males of *Drosophila melanogaster*. *Am. Zool.* 3, 507.
- Gordon, M.D., and Scott, K. (2009). Motor control in a *Drosophila* taste circuit. *Neuron* 61, 373–384.
- Gracheva, E.O., Ingolia, N.T., Kelly, Y.M., Cordero-Morales, J.F., Hollopeter, G., Chesler, A.T., Sánchez, E.E., Perez, J.C., Weissman, J.S., and Julius, D. (2010). Molecular basis of infrared detection by snakes. *Nature* 464, 1006–1011.
- Gracheva, E.O., Cordero-Morales, J.F., González-Carcacia, J.A., Ingolia, N.T., Manno, C., Aranguren, C.I., Weissman, J.S., and Julius, D. (2011). Ganglion-specific splicing of TRPV1 underlies infrared sensation in vampire bats. *Nature* 476, 88–91.
- Greenspan, R.J., and Ferveur, J.F. (2000). Courtship in *Drosophila*. *Annu. Rev. Genet.* 34, 205–232.
- Grillet, M., Everaerts, C., Houot, B., Ritchie, M.G., Cobb, M., and Ferveur, J.-F. (2012). Incipient speciation in *Drosophila melanogaster* involves chemical signals. *Sci Rep* 2, 224.
- Grosjean, Y., Grillet, M., Augustin, H., Ferveur, J.-F., and Featherstone, D.E. (2008). A glial amino-acid transporter controls synapse strength and courtship in *Drosophila*. *Nat. Neurosci.* 11, 54–61.
- Grosjean, Y., Rytz, R., Farine, J.-P., Abuin, L., Cortot, J., Jefferis, G.S.X.E., and Benton, R. (2011). An olfactory receptor for food-derived odours promotes male courtship in *Drosophila*. *Nature* 478, 236–240.
- Hall, J.C. (1978). Courtship among males due to a male-sterile mutation in *Drosophila melanogaster*. *Behav. Genet.* 8, 125–141.
- Hallem, E.A., Dahanukar, A., and Carlson, J.R. (2006). Insect odor and taste receptors. *Annu. Rev. Entomol.* 51, 113–135.
- Hampel, S., Chung, P., McKellar, C.E., Hall, D., Looger, L.L., and Simpson, J.H. (2011). *Drosophila* Brainbow: a recombinase-based fluorescence labeling technique to subdivide neural expression patterns. *Nat. Methods* 8, 253–259.
- Higgin, M., Chenoweth, S., and Blows, M.W. (2000). Natural selection and the reinforcement of mate recognition. *Science* 290, 519–521.
- Hollocher, H., Ting, C.T., Wu, M.L., and Wu, C.I. (1997). Incipient speciation by sexual isolation in *Drosophila melanogaster*: extensive genetic divergence without reinforcement. *Genetics* 147, 1191–1201.
- Isogai, Y., Si, S., Pont-Lezica, L., Tan, T., Kapoor, V., Murthy, V.N., and Dulac, C. (2011). Molecular organization of vomeronasal chemoreception. *Nature* 478, 241–245.
- Ito, H., Fujitani, K., Usui, K., Shimizu-Nishikawa, K., Tanaka, S., and Yamamoto, D. (1996). Sexual orientation in *Drosophila* is altered by the satori mutation in the sex-determination gene fruitless that encodes a zinc finger protein with a BTB domain. *Proc. Natl. Acad. Sci. USA* 93, 9687–9692.
- Jallon, J.-M., and David, J.R. (1987). Variation in Cuticular Hydrocarbons Among the Eight Species of the *Drosophila melanogaster* Subgroup. *Evolution* 41, 294–302.
- Janet, A., Rubin, G.M., Ngo, T.-T.B., 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.
- Jiang, P., Josue, J., Li, X., Glaser, D., Li, W., Brand, J.G., Margolskee, R.F., Reed, D.R., and Beauchamp, G.K. (2012). Major taste loss in carnivorous mammals. *Proc. Natl. Acad. Sci. USA* 109, 4956–4961.
- Jordt, S.-E., and Julius, D. (2002). Molecular basis for species-specific sensitivity to “hot” chili peppers. *Cell* 108, 421–430.
- Julius, D., Blair, L., Brake, A., Sprague, G., and Thorne, J. (1983). Yeast alpha factor is processed from a larger precursor polypeptide: the essential role of a membrane-bound dipeptidyl aminopeptidase. *Cell* 32, 839–852.
- Kitamoto, T. (2001). Conditional modification of behavior in *Drosophila* by targeted expression of a temperature-sensitive shibire allele in defined neurons. *J. Neurobiol.* 47, 81–92.
- Knight, D., Xie, W., and Boulianne, G.L. (2011). Neurexins and neuroligins: recent insights from invertebrates. *Mol. Neurobiol.* 44, 426–440.
- Koganezawa, M., Haba, D., Matsuo, T., and Yamamoto, D. (2010). The shaping of male courtship posture by lateralized gustatory inputs to male-specific interneurons. *Curr. Biol.* 20, 1–8.
- Konishi, M. (1985). Birdsong: from behavior to neuron. *Annu. Rev. Neurosci.* 8, 125–170.
- Kowalski, S., Aubin, T., and Martin, J.-R. (2004). Courtship song in *Drosophila melanogaster*: a differential effect on male–female locomotor activity. *Can. J. Zool.* 82, 1258–1266.
- Krstic, D., Boll, W., and Noll, M. (2009). Sensory integration regulating male courtship behavior in *Drosophila*. *PLoS ONE* 4, e4457.
- Kurtovic, A., Widmer, A., and Dickson, B.J. (2007). A single class of olfactory neurons mediates behavioural responses to a *Drosophila* sex pheromone. *Nature* 446, 542–546.
- Lacaille, F., Hiroi, M., Twele, R., Inoshita, T., Umemoto, D., Manière, G., Marion-Poll, F., Ozaki, M., Francke, W., Cobb, M., et al. (2007). An inhibitory sex pheromone tastes bitter for *Drosophila* males. *PLoS ONE* 2, e661.
- Laturney, M., and Moehring, A.J. (2012). The genetic basis of female mate preference and species isolation in *Drosophila*. *Int. J. Evol. Biol.* 2012, 328392.

- Lee, Y., Kim, S.H., and Montell, C. (2010). Avoiding DEET through insect gustatory receptors. *Neuron* 67, 555–561.
- Liimatainen, J.O., and Jallon, J.-M. (2007). Genetic analysis of cuticular hydrocarbons and their effect on courtship in *Drosophila virilis* and *D. lummei*. *Behav. Genet.* 37, 713–725.
- Lin, H., Mann, K.J., Starostina, E., Kinser, R.D., and Pikielny, C.W. (2005). A *Drosophila* DEG/ENaC channel subunit is required for male response to female pheromones. *Proc. Natl. Acad. Sci. USA* 102, 12831–12836.
- Lu, B., LaMora, A., Sun, Y., Welsh, M.J., and Ben-Shahar, Y. (2012). ppk23-Dependent chemosensory functions contribute to courtship behavior in *Drosophila melanogaster*. *PLoS Genet.* 8, e1002587.
- Manning, A. (1959). The sexual isolation between *Drosophila melanogaster* and *Drosophila simulans*. *Anim. Behav.* 7, 60–65.
- Manoli, D.S., and Baker, B.S. (2004). Median bundle neurons coordinate behaviours during *Drosophila* male courtship. *Nature* 430, 564–569.
- Manoli, D.S., Foss, M., Vilella, A., Taylor, B.J., Hall, J.C., and Baker, B.S. (2005). Male-specific fruitless specifies the neural substrates of *Drosophila* courtship behaviour. *Nature* 436, 395–400.
- Manoli, D.S., Meissner, G.W., and Baker, B.S. (2006). Blueprints for behavior: genetic specification of neural circuitry for innate behaviors. *Trends Neurosci.* 29, 444–451.
- Mayr, E. (1988). The Why and How of Species. *Biol. Philos.* 3, 431–441.
- Mayr, E., and Dobzhansky, T. (1945). Experiments on Sexual Isolation in *Drosophila*: IV. Modification of the Degree of Isolation Between *Drosophila Pseudoobscura* and *Drosophila Persimilis* and Sexual Preferences in *Drosophila Prosoltans*. *Proc. Natl. Acad. Sci. USA* 31, 75–82.
- McCullough, J., and Herskowitz, I. (1979). Mating pheromones of *Saccharomyces kluyveri*: pheromone interactions between *Saccharomyces kluyveri* and *Saccharomyces cerevisiae*. *J. Bacteriol.* 138, 146–154.
- McGrath, P.T., Xu, Y., Ailion, M., Garrison, J.L., Butcher, R.A., and Bargmann, C.I. (2011). Parallel evolution of domesticated *Caenorhabditis* species targets pheromone receptor genes. *Nature* 477, 321–325.
- Meissner, G.W., Manoli, D.S., Chavez, J.F., Knapp, J.-M., Lin, T.L., Stevens, R.J., Mellert, D.J., Tran, D.H., and Baker, B.S. (2011). Functional dissection of the neural substrates for sexual behaviors in *Drosophila melanogaster*. *Genetics* 189, 195–211.
- Miyamoto, T., and Amrein, H. (2008). Suppression of male courtship by a *Drosophila* pheromone receptor. *Nat. Neurosci.* 11, 874–876.
- Moehring, A.J., Llopart, A., Elwyn, S., Coyne, J.A., and Mackay, T.F.C. (2006). The genetic basis of prezygotic reproductive isolation between *Drosophila santomea* and *D. yakuba* due to mating preference. *Genetics* 173, 215–223.
- Moon, S.J., Köttgen, M., Jiao, Y., Xu, H., and Montell, C. (2006). A taste receptor required for the caffeine response in vivo. *Curr. Biol.* 16, 1812–1817.
- Moon, S.J., Lee, Y., Jiao, Y., and Montell, C. (2009). A *Drosophila* gustatory receptor essential for aversive taste and inhibiting male-to-male courtship. *Curr. Biol.* 19, 1623–1627.
- Nanda, P., and Singh, B.N. (2012). Behavioural reproductive isolation and speciation in *Drosophila*. *J. Biosci.* 37, 359–374.
- Nathans, J. (1999). The evolution and physiology of human color vision: insights from molecular genetic studies of visual pigments. *Neuron* 24, 299–312.
- Orr, H.A., Masly, J.P., and Presgraves, D.C. (2004). Speciation genes. *Curr. Opin. Genet. Dev.* 14, 675–679.
- Ovid (2009). *Metamorphoses*. A.D. Melville, tr. (New York: Oxford University Press).
- Papes, F., Logan, D.W., and Stowers, L. (2010). The vomeronasal organ mediates interspecies defensive behaviors through detection of protein pheromone homologs. *Cell* 141, 692–703.
- Park, J.-H., and Kwon, J.Y. (2011). A systematic analysis of *Drosophila* gustatory receptor gene expression in abdominal neurons which project to the central nervous system. *Mol. Cells* 32, 375–381.
- Pfeiffer, B.D., Ngo, T.-T.B., 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.
- Pulver, S.R., Pashkovski, S.L., Hornstein, N.J., Garrity, P.A., and Griffith, L.C. (2009). Temporal dynamics of neuronal activation by Channelrhodopsin-2 and TRPA1 determine behavioral output in *Drosophila* larvae. *J. Neurophysiol.* 101, 3075–3088.
- Ritchie, M.G., Halsey, E.J., and Gleason, J.M. (1999). *Drosophila* song as a species-specific mating signal and the behavioural importance of Kyriacou & Hall cycles in *D. melanogaster* song. *Anim. Behav.* 58, 649–657.
- Robertson, H.M. (1983). Chemical stimuli eliciting courtship by males in *Drosophila melanogaster*. *Experientia* 39, 333–335.
- Ryner, L.C., Goodwin, S.F., Castrillon, D.H., Anand, A., Vilella, A., Baker, B.S., Hall, J.C., Taylor, B.J., and Wasserman, S.A. (1996). Control of male sexual behavior and sexual orientation in *Drosophila* by the fruitless gene. *Cell* 87, 1079–1089.
- Savarit, F., Sureau, G., Cobb, M., and Ferveur, J.F. (1999). Genetic elimination of known pheromones reveals the fundamental chemical bases of mating and isolation in *Drosophila*. *Proc. Natl. Acad. Sci. USA* 96, 9015–9020.
- Scott, K. (2005). Taste recognition: food for thought. *Neuron* 48, 455–464.
- Scott, K., Brady, R., Jr., Cravchik, A., Morozov, P., Rzhetsky, A., Zuker, C., and Axel, R. (2001). A chemosensory gene family encoding candidate gustatory and olfactory receptors in *Drosophila*. *Cell* 104, 661–673.
- Seehausen, O., and van Alphen, J.J.M. (1998). The effect of male coloration on female mate choice in closely related Lake Victoria cichlids (*Haplochromis nyererei* complex). *Behav. Ecol. Sociobiol.* 42, 1–8.
- Shirangi, T.R., Dufour, H.D., Williams, T.M., and Carroll, S.B. (2009). Rapid evolution of sex pheromone-producing enzyme expression in *Drosophila*. *PLoS Biol.* 7, e1000168.
- Siwicki, K.K., and Kravitz, E.A. (2009). Fruitless, doublesex and the genetics of social behavior in *Drosophila melanogaster*. *Curr. Opin. Neurobiol.* 19, 200–206.
- Smadja, C., and Butlin, R.K. (2009). On the scent of speciation: the chemosensory system and its role in premating isolation. *Heredity (Edinb)* 102, 77–97.
- Spieth, H.T. (1949). Sexual behavior and isolation in *Drosophila*; the interspecific mating behavior of species of the willistoni group. *Evolution* 3, 67–81.
- Spieth, H.T. (1952). Mating behavior within the genus *Drosophila* (Diptera). *Bulletin of the AMNH* vol. 99, article 7. New York: American Museum of Natural History.
- Spieth, H.T. (1974). Courtship behavior in *Drosophila*. *Annu. Rev. Entomol.* 19, 385–405.
- Stocker, R.F. (1994). The organization of the chemosensory system in *Drosophila melanogaster*: a review. *Cell Tissue Res.* 275, 3–26.
- 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.
- Sturtevant, A.H. (1920). Genetic Studies on DROSOPHILA SIMULANS. I. Introduction. Hybrids with DROSOPHILA MELANOGASTER. *Genetics* 5, 488–500.
- Tauber, E., Roe, H., Costa, R., Hennessy, J.M., and Kyriacou, C.P. (2003). Temporal mating isolation driven by a behavioral gene in *Drosophila*. *Curr. Biol.* 13, 140–145.
- Thistle, R., Cameron, P., Ghorayshi, A., Dennison, L., and Scott, K. (2012). Contact chemoreceptors mediate male-male repulsion and male-female attraction during *Drosophila* courtship. *Cell* 149, 1140–1151.
- Thorne, N., and Amrein, H. (2008). Atypical expression of *Drosophila* gustatory receptor genes in sensory and central neurons. *J. Comp. Neurol.* 506, 548–568.
- Thorne, N., Chromey, C., Bray, S., and Amrein, H. (2004). Taste perception and coding in *Drosophila*. *Curr. Biol.* 14, 1065–1079.
- Toda, H., Zhao, X., and Dickson, B.J. (2012). The *Drosophila* female aphrodisiac pheromone activates ppk23(+) sensory neurons to elicit male courtship behavior. *Cell Rep* 1, 599–607.

- Tompkins, L., Hall, J.C., and Hall, L.M. (1980). Courtship-stimulating volatile compounds from normal and mutant *Drosophila*. *J. Insect Physiol.* *26*, 689–697.
- Tompkins, L., Gross, A.C., Hall, J.C., Gailey, D.A., and Siegel, R.W. (1982). The role of female movement in the sexual behavior of *Drosophila melanogaster*. *Behav. Genet.* *12*, 295–307.
- Wang, Z., Singhvi, A., Kong, P., and Scott, K. (2004). Taste representations in the *Drosophila* brain. *Cell* *117*, 981–991.
- Wang, L., Han, X., Mehren, J., Hiroi, M., Billeter, J.-C., Miyamoto, T., Amrein, H., Levine, J.D., and Anderson, D.J. (2011). Hierarchical chemosensory regulation of male-male social interactions in *Drosophila*. *Nat. Neurosci.* *14*, 757–762.
- Watanabe, K., Toba, G., Koganezawa, M., and Yamamoto, D. (2011). Gr39a, a highly diversified gustatory receptor in *Drosophila*, has a role in sexual behavior. *Behav. Genet.* *41*, 746–753.
- Weiss, L.A., Dahanukar, A., Kwon, J.Y., Banerjee, D., and Carlson, J.R. (2011). The molecular and cellular basis of bitter taste in *Drosophila*. *Neuron* *69*, 258–272.
- Wheeler, D.A., Kyriacou, C.P., Greenacre, M.L., Yu, Q., Rutila, J.E., Rosbash, M., and Hall, J.C. (1991). Molecular transfer of a species-specific behavior from *Drosophila simulans* to *Drosophila melanogaster*. *Science* *251*, 1082–1085.
- Wisotsky, Z., Medina, A., Freeman, E., and Dahanukar, A. (2011). Evolutionary differences in food preference rely on Gr64e, a receptor for glycerol. *Nat. Neurosci.* *14*, 1534–1541.
- Wu, C.-I., and Ting, C.-T. (2004). Genes and speciation. *Nat. Rev. Genet.* *5*, 114–122.
- Yamaguchi, S., and Heisenberg, M. (2011). Photoreceptors and neural circuitry underlying phototaxis in insects. *Fly (Austin)* *5*, 333–336.
- Yu, J.Y., Kanai, M.I., Demir, E., Jefferis, G.S.X.E., and Dickson, B.J. (2010). Cellular organization of the neural circuit that drives *Drosophila* courtship behavior. *Curr. Biol.* *20*, 1602–1614.