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Evolution of Mechanisms that Control Mating in Drosophila Males

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Evolution of Mechanisms that Control Mating in Drosophila Males

by

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DISSERTATION

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I dedicate this body of work to the women who made it possible:

To my mother, *Ahlam*, whose name means *Dreams*;

To my sister, *Nuwar*, who is my twin in life;

To my partner, *Z*, who sees me always.

And to my brother, *Bobby Mozia*, who died way too young.

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CHAPTER 1:

Here I discuss the chemosensory control of courtship in *D. melanogaster* males, and highlight key studies of the evolution of courtship behaviors in closely related *Drosophila* species. I am thankful to Dev Manoli and Nirao Shah for edits on the abstract. I am also supremely grateful for Stephanie Redmond and Z. Yan Wang for their keen and critical feedback on this chapter.

CHAPTER 2:

This is a reprint of Fan et al., 2013. I helped with and conducted key experiments and behavioral analyses for this paper. Other authors on this study were: Pu Fan, Dev Manoli, Yi Chen, Neha Agarwal, Sara Kwong, Allen Cai, Jeffrey Neitz, Adam Rensio, Bruce Baker, and Nirao Shah. The studied was spearheaded by Pu Fan – see page x for permissions.

CHAPTER 3:

This chapter comprises the bulk of my independent research program at UCSF and was motivated by key discoveries highlighted in Chapter 2. I conducted experiments with help from Khin May Tun, Paula H. Serpa, and Justin Peng. Aram Avila-Herrera and

Katherine S. Pollard analyzed the Gr32a ~3.8 kb regulatory region. Graeme Davis provided invaluable advice, resources, and laboratory space for some of the experiments in this Chapter. Jon-Michael Knapp and David L. Stern provided reagents to generate transgenic *D. simulans* lines. Srinivas Parthasarathy helped with molecular analysis of some of the Gr33a and Ppk25 *D. simulans* mutants. I thank David Anderson for sharing the pJFRC2[UAS-ReaChR::Citrine] plasmid. I thank Z Yan Wang and Gabe McKinsey for helpful comments and revisions. Nirao M. Shah and I designed experiments and wrote the paper based on this chapter (see preprint here: Ahmed et al., 2017).

CHAPTER 4:

Here I summarize and discuss the major points of my thesis. I also conjecture a bit on how chemosensory pathways that control feeding may also control courtship behaviors. I provide unpublished, preliminary data to support this notion. In addition, I briefly discuss how genetic modularity can act as a substrate for evolution. I thank Stephanie Redmond for critical reading of this chapter.

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PERMISSIONS

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I give explicit permission to Osama M. Ahmed, co-author of Fan et al., 2013, to reprint the materials therein as part of his thesis dissertation. Osama contributed greatly to key experiments highlighted in the publication.

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ABSTRACT

Courtship in *Drosophila* comprises a complex series of species-specific behaviors controlled by genetic and neural substrates. These behaviors, as well as their underlying mechanisms, can evolve quickly to facilitate reproductive isolation – the reduction of gene flow between populations. This makes *Drosophila* courtship an excellent model for studying how genetic changes can generate divergent behavioral programs. Members of the chemosensory receptor gene families are required to regulate different aspects of *Drosophila* courtship. For instance, chemoreceptors allow male flies to discern potential mates in order to restrict courtship towards receptive, conspecific females. In Chapter 1, I review recent studies on the chemosensory control of courtship in *D. melanogaster*, and highlight key studies on the evolution of courtship behaviors.

In Chapter 2, we identify genetic and neural pathways that prevent *D. melanogaster* males from courting females of other fly species. The chemoreceptor Gr32a recognizes nonvolatile aversive cues present on heterospecific females and is required to inhibit interspecies courtship. In addition, activity of Gr32a neurons is necessary and sufficient to inhibit this behavior. We extended our work to non-model species of *Drosophila* to explore how chemosensory pathways that regulate courtship may evolve. In Chapter 3, we show that two closely related fly species use distinct mechanisms to inhibit interspecies mating. In both *D. simulans* and *D. melanogaster*, Gr32a is expressed in the male foreleg tarsi, and it is essential for sensing the bitter tastant quinine. However, Gr32a is not required for inhibiting interspecies courtship in *D. simulans* as it is in *D. melanogaster*. Although chemoreceptor mechanisms that

inhibit interspecies courtship have differentiated, we find that a similar chemosensory pathway promotes courtship in both species.

Many questions remain about the evolution of mechanisms that preclude interspecies courtship. In Chapter 4, I discuss experiments that address these questions. Preliminary results show that chemosensory pathways that detect bitter and sweet tastants may also control mating success, suggesting that such pathways may be appropriated to regulate seemingly disparate behaviors. In summary, comparative genetic and neural studies in closely related *Drosophila* species can contribute to a greater understanding of how courtship and other innate behaviors evolve.

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One could not be a calm, cool, and detached scientist

while Negroes were lynched, murdered, and starved.

//*W.E.B. Du Bois*

CHAPTER ONE

COURTSHIP IN *DROSOPHILA* AND THE EVOLUTION OF BEHAVIORAL REPRODUCTIVE ISOLATION

ABSTRACT

The genus *Drosophila* encompasses a tremendous number of fly species, with each species exhibiting a unique repertoire of innate behaviors, such as mating, feeding, and fighting. The behavioral complexity, genetic diversity, and availability of molecular tools in several *Drosophila* species makes this genus advantageous for studying key biological problems. In particular, what are the neural and genetic controls of innate behaviors, and how do these species-specific traits evolve. The male courtship routine – a complex species-specific "dance" performed towards conspecific females — stands out as an ideal behavioral model for addressing these questions. Courtship behaviors evolve quickly and can facilitate reproductive isolation, the reduction of gene flow between populations that can facilitate evolutionary divergence. Some of the critical molecular and neural substrates that regulate courtship in *D. melanogaster* have been identified. Yet, despite more than a century of genetic, ecological, and behavioral studies in different *Drosophila* species, some gaps in knowledge remain: What are the genetic and neural components that drive species-specific courtship? And, how do behavioral programs evolve? In this chapter, I review recent studies on the chemosensory control of courtship in *D. melanogaster* and I highlight key studies on the evolution of courtship behaviors. Comparative genetic and neural studies across closely related *Drosophila* species will provide a more comprehensive understanding of how courtship behaviors, and their genetic and neural substrates, evolve. These findings may also provide a useful framework for understanding how other innate behaviors evolve.

INTRODUCTION

All animals display species-specific courtship behaviors that enable the propagation of adaptive allele combinations and inhibit maladaptive hybridization. The recognition, reception, and generation of appropriate courtship behaviors can facilitate reproductive isolation, especially between closely related species that occupy the same habitats (Coyne and Orr, 1997; Spieth, 1949). The genus *Drosophila* comprises >1,500 documented fly species occupying practically all terrestrial habitats (Jezovit et al., 2017). These species demonstrate a diversity in morphology and behavior commensurate to their great numbers (Ringo, 1977; Singh, 2016). In particular, the courtship routines of various fly species have been vigorously studied for more than a century and are known to differ even between closely related species (Greenspan and Ferveur, 2000; Spieth, 1974; Sturtevant, 1915). Importantly, quantitative and qualitative differences in courtship routines can contribute to, or even drive, speciation (Coyne and Orr, 1997; Giglio and Dyer, 2013; Greenspan and Ferveur, 2000; Mendelson, 2003). Male flies produce a stereotyped series of species-specific courtship behaviors to attract conspecific females, and studies in *D. melanogaster* demonstrate that a suite of genetic and neural substrates mediate these behaviors (Bastock and Manning, 1955; Dickson, 2008). However, it remains unclear how these substrates have changed to give rise to divergent courtship routines.

PART I: CHEMOSENSORY CONTROL OF COURTSHIP IN *D. MELANOGASTER* MALES

Courtship in *D. melanogaster* has been an active area of research since pioneering studies by Sturtevant, Spieth, Manning, and later by Benzer and colleagues (Bastock and Manning, 1955; Hall, 1978; Konopka and Benzer, 1971; Spieth, 1952; Sturtevant, 1920).

Male flies perform a complex courtship routine toward receptive, conspecific females: males orient toward and tap females with the foreleg tarsi, extend one wing and vibrate it to produce a *D. melanogaster*-specific 'song', lick the female genitalia, and then attempt to copulate by bending the abdomen (Hall, 1994). A similar progression of behaviors has been documented in many *Drosophila* species and even socially- and sexually-naïve males perform these stereotyped displays in response to receptive, conspecific females (Capy and Gibert, 2004; Cobb et al., 1985; Spieth, 1974). Several genetic loci that are required to drive *D. melanogaster* male courtship have been identified (Auer and Benton, 2016; Dickson, 2008). For instance, the male-specific isoform of the transcription factor Fruitless (Fru^M) is a master regulator of the development and function of male courtship circuitry (Cande et al., 2014; reviewed in Douglas and Levine, 2006). In addition, many chemosensory pathways are required to regulate courtship and other innate *Drosophila* behaviors (Ziegler et al., 2013, and see below). These studies have provided strong evidence that genetically-hardwired neural circuitry controls both the sensory gating and execution of courtship.

Pheromonal control of male courtship

All sexually reproducing animals must correctly identify potential mates. Chemosensory cues are known to play a pivotal role in mate-recognition in a variety of animals, from mammals to insects (reviewed in Smadja and Butlin, 2008). Pheromones, secreted chemical factors used for social signaling between individuals, have been shown to inhibit or promote mating in numerous species (Buchinger et al., 2015; Cobb and Jallon, 1990; Liberles, 2014; Maex et al., 2016). In *Drosophila*, pheromonal signaling is mediated by cuticular hydrocarbons (CHCs), long hydrocarbon chains synthesized in

specialized abdominal cells called oenocytes and deposited on the cuticle (Billeter et al., 2009; Ferveur, 1997). These waxy CHCs protect flies from desiccation, and some have evolved roles as pheromones (reviewed in Chung and Carroll, 2015).

Many Drosophila sex pheromones were discovered in the 1980s by using hexane to strip flies of CHCs, and then analyzing the hexane/CHC mixture using gas chromatography and mass spectrometry (reviewed in Ferveur, 1997). Researchers identified key compounds that promoted or inhibited species-specific courtship behaviors by presenting male flies with dummy targets or immobilized flies perfumed with various CHC-extracts. Different species deposit a unique cocktail of pheromones on their cuticle, and they respond differently to these pheromones (Ferveur, 2005; Savarit et al., 1999). For instance, *D. melanogaster* females deposit 7,11-heptacosedeine, a pheromone that promotes courtship by *D. melanogaster* males and inhibits courtship by males of the closely related species, *D. simulans* (Billeter et al., 2009). These responses are observed in naive, socially isolated males, suggesting that a species-specific, genetically-hardwired pathway controls the response to 7,11-heptacosedeine, and presumably other pheromonal signals (Clowney et al., 2015).

Drosophila contains diverse chemoreceptor families — the pickpocket, gustatory, ionotropic, and olfactory receptor families — that are essential for sensing foods and potential mates (Dahanukar et al., 2005; Dethier and Chadwick, 1948). These families, collectively, contain more than 200 genes, the majority of which currently have unknown functions (Joseph and Carlson, 2015). Some of the receptors in these families allow males to detect and respond to pheromonal signals at close range and at greater

distances, and they are required for males to execute appropriate courtship behaviors towards suitable mates.

Contact-based chemosensory control of male courtship

D. melanogaster males initially "tap" potential mates with their foreleg tarsi, before choosing to initiate the courtship dance. In the case of conspecific females, males will pursue them vigorously and, during a later step of the courtship routine, will extend their mouthparts to directly contact the female's genitalia. Presumably, at the "tapping" and "licking" steps of courtship, chemosensory pathways detect pheromones deposited on the cuticle of potential mates (Ahmed et al., 2017; Fan et al., 2013; Yamamoto and Koganezawa, 2013). Indeed, many *Drosophila* pheromones are non-volatile and can only be detected at such close range. A subset of chemosensory genes in the pickpocket Degenerin/Epithelial Sodium Channel family are expressed in the tarsi and mouthparts and are essential for regulating pheromonal responses and controlling male courtship (Figure 1.1) (Ziegler et al., 2013). These pickpocket genes encode non-voltage-gated ion channels with diverse roles in neurophysiology and cell function (Zelle et al., 2013). One such gene, *Ppk23*, is required to control courtship towards conspecific males and females (Figure 1.1) (Lu et al., 2012; Thistle et al., 2012; Toda et al., 2012). Males mutant for this gene court conspecific females less than wildtype (WT) controls and they also show elevated levels of male-male courtship. *Ppk23* is expressed in two classes of sensory neurons in the foreleg tarsi of males: male-responsive (M) and female-responsive (F) cells, with each class responding to sex-specific pheromones (Kallman et al., 2015). M- and F-cells express Fru^{M} and inhibit or promote courtship behavior, respectively. Calcium imaging in P1, a collection of male-specific courtship command

neurons, revealed that this nucleus is inhibited or activated by stimulation of M- or F-cells, respectively (Clowney et al., 2015; Kallman et al., 2015; Kohatsu et al., 2011). These data show that *Ppk23*-expressing neurons modulate activity in central circuitry to regulate courtship behavior.

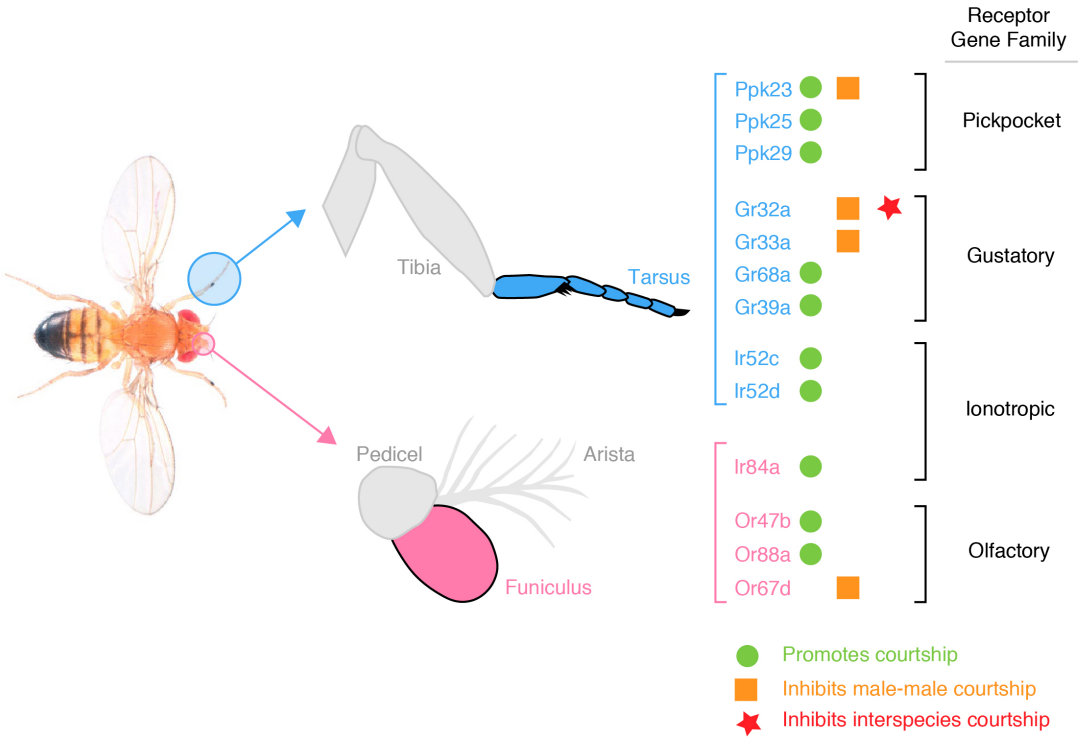

FIGURE 1.1: Chemosensory regulation of courtship in *D. melanogaster* males.

D. melanogaster sensory neurons in the male foreleg (tarsi: blue) and antennae (funiculus: pink) express subsets of genes from the pickpocket family and from the gustatory, ionotropic, and olfactory receptor families. These genes are required to promote conspecific courtship (green circles), to inhibit male-male courtship (orange squares), and to inhibit courtship towards other species (red star). Other structures of the foreleg (tibia) and antennae (pedicel, arista) are shown in gray but do not express the chemoreceptors shown above.

Some *Ppk23*-expressing neurons co-express the related pickpocket gene *Ppk29*, which also appears to be required for male-female courtship (Thistle et al., 2012). The decreased levels of courtship exhibited by males singly mutant for *Ppk23* or *Ppk29* cannot be reciprocally rescued by *Ppk29* or *Ppk23* respectively, suggesting that these two channel subunits operate non-redundantly and potentially as part of a single complex. In addition, males mutant for *Ppk29* do not show elevated levels of male-male courtship, suggesting that these males can still recognize and respond to male pheromones. It is possible that *Ppk29* is expressed in F-cells and not M-cells, and is thus required for regulating responses to female, but not male, pheromones.

In addition to *Ppk23*, F-cells express another member of the pickpocket family, *Ppk25,* which is required to promote courtship towards conspecific females (Lin et al., 2005; Starostina et al., 2012; Thistle et al., 2012; Vijayan et al., 2014). In line with this, Ppk25 is required for behavioral and cellular responses to the aphrodisiac pheromone 7'11-heptacosedeine. *Ppk25* is not expressed in M-cells and it is not required to inhibit male-male courtship, thus suggesting that Ppk25 has a more restricted role than Ppk23 in controlling courtship. Despite their importance for regulating courtship, how these channel subunits function to transmit pheromonal information is still unknown. These pickpocket proteins might interact with other unidentified proteins to form a functional pheromone-receptor, or they could be required to transduce a signal sensed by other co-expressed chemoreceptors (Depetris-Chauvin et al., 2015).

The gustatory receptor (Gr) family consists of ~70 proteins, many of which have been shown to control appetitive behaviors such as feeding and mating (Clyne et al.,

2000; Depetris-Chauvin et al., 2015; Dunipace et al., 2001; Montell, 2009; Robertson et al., 2003; Scott et al., 2001). For example, both Gr32a and Gr33a are required to inhibit male-male courtship and consuming bitter substances, and Gr32a also inhibits courtship towards different *Drosophila* species (Figure 1.1) (Fan et al., 2013; Lee et al., 2010; Moon et al., 2009). Although *Gr32a*-expressing neurons do not co-express Fru^M, they likely synapse directly onto Fru^M+ neurons in the subesophageal zone, a taste-processing center in the adult brain, and in the ventral nerve chord (Fan et al., 2013; Koganezawa et al., 2010). In turn, these Fru^M+ neurons are posed to inhibit P1 neurons and thereby prevent aberrant courtship (Clowney et al., 2015).

Another member of the Gr family, *Gr68a*, is widely expressed throughout the male foreleg tarsi (~10 neurons with 1-2 neurons per tarsal segment), and is not expressed in the foreleg tarsi of females (Bray and Amrein, 2003). Despite its protein sequence similarity to Gr32a, which inhibits courtship, Gr68a is required to promote courtship in *D. melanogaster* males (Figure 1.1) (Bray and Amrein, 2003; Ejima and Griffith, 2008). Additionally, tetanus-toxin induced inhibition of *Gr68a*-expressing neurons leads to decreased conspecific courtship. These findings suggest that *Gr68a*-expressing neurons respond to aphrodisiac pheromone signals exhibited by *D. melanogaster* females.

In the 17 years since the discovery of the *Drosophila* Gr family, only a few Grs have been implicated in the control of courtship (Figure 1.1). However, this large gene family likely contains other members that contribute to the generation, modulation, or inhibition of courtship behaviors (Depetris-Chauvin et al., 2015; Joseph and Carlson,

2015; Montell, 2009). Many questions still remain about how Grs function to regulate WT displays of courtship. For instance, males mutant for *Gr39a* show reduced courtship to conspecific females, yet how does Gr39a drive courtship behavior and where in the brain do *Gr39a*-expressing neurons project (Watanabe et al., 2011)? To complicate matters even more, while Gr68a and *Gr68a*-expressing neurons are required for WT levels of conspecific courtship, a recent study has shown that these neurons also respond to a courtship-suppressing male-specific pheromone (Shankar et al., 2015). Future studies will help determine how these receptors sense diverse chemical cues, and how Gr-expressing neurons ensure that courtship behavior is initiated and properly executed toward receptive, conspecific females.

Recently, a new family of ~35 gustatory ionotropic receptor (Ir) genes, called the *Ir20a* clade, has been described (Koh et al., 2014). These Irs genes are related to ionotropic glutamate receptor genes (Benton et al., 2009) and are co-expressed with known Grs in the foreleg tarsi of males. Two of these genes, *Ir52c* and *Ir52d*, are essential for WT levels of male courtship towards conspecific females (Figure 1.1) (Koh et al., 2014). In addition, *Ir52c*- and *Ir52d*-expressing neurons promote courtship behaviors in response to female pheromones. These neurons, like *Gr32a*-expressing neurons, do not express Fru^M but may synapse directly onto Fru^M circuitry (Koh et al., 2014).

Olfaction in courtship

Male and female drosophilids also exude volatile pheromones, and chemical communication can occur across greater distances that do not require direct contact

(reviewed in Ziegler et al., 2013). The fly's antennae and maxillary palps contain sensory neurons that express olfactory receptors (Ors) that detect airborne chemical cues (Figure 1.1) (Stocker, 1994; Vosshall et al., 1999). Antennal sensory neurons expressing *Or47b* or *Or88a* respond to conspecific male and female cuticular hydrocarbon extracts and these neurons potentially act as conspecific detectors, conveying signals such as proximity to another fly (van der Goes van Naters and Carlson, 2007). In this scenario, olfactory cues first signal the presence of and proximity to a potential mate, which then triggers contact-based (Gr-dependent) investigation to determine specific details of the potential mate, such as sex and species (Spieth, 1974). In addition, Or47b and Or88a contribute directly to courtship drive and success (Dweck et al., 2015; Lin et al., 2016; Lone et al., 2015). *Or47b*- and *Or88a*-expressing neurons detect methyl laurate, methyl myristate, and methyl palmitate — odorants exuded by male and females flies — and heterologous activation of *Or47b*-expressing neurons gives males a mating advantage when competing with control males (Dweck et al., 2015; Lin et al., 2016). As such, pheromone-responsive Or-expressing neurons can actively modulate male courtship performance.

Another Or expressed in the antennae, *Or67d*, is required to detect the pheromone 11-*cis*-vaccenyl acetate (cVA), a compound found on male flies and is often transferred to females during copulation (Ejima et al., 2007; Kurtovic et al., 2007). Unlike WT males, *D. melanogaster* males mutant for *Or67d* court conspecific males and mated females, presumably because they can no longer detect the aversive cVA. Strikingly, Or67d regulates courtship behavior in both sexes; female flies mutant for *Or67d* are less receptive to conspecific males. This difference between male and female

mutants is likely due to sexually dimorphic circuitry: *Or67d*-expressing project directly to the DA1 glomerulus before making sexually dimorphic connections with neurons in the protocerebrum (Datta et al., 2008). Investigating the development and modulation of sexually dimorphic neural circuitry will contribute to the understanding of how specific olfactory cues can trigger divergent behaviors, within and between species.

A subset of sensory neurons in the antennae also express Irs belonging to a family comprising ~60 genes (Benton et al., 2009); these Irs are distinct from the *Ir20a* clade described above (Koh et al., 2014). Antennal Ir+ neurons do not express Ors, yet many Irs are required for odor responses (Benton et al., 2009; Prieto-Godino et al., 2017; Rytz et al., 2013). Currently, only one gene in this family, *Ir84a*, has been shown to play a role in courtship (Figure 1.1). *Ir84a* is expressed in a subpopulation of Fru^M antennal sensory neurons and is required to detect the aromatic odors phenylacetic acid and phenylacetaldehyde (Grosjean et al., 2011). These volatile compounds are found on rotting fruits, where flies commonly feed and mate. Ir84a does not detect fly pheromones but is required for the high levels of male courtship exhibited towards conspecific females in the presence of phenylacetaldehyde. Given that flies often court and mate on their preferred food substrates, this discovery couples, at a molecular level, two seemingly disparate behaviors: feeding and mating.

Comparative study of chemosensory receptors that control courtship

Many *Drosophila* species deposit a unique mixture of pheromones on their cuticles, suggesting that the molecular and neural substrates that detect these pheromones may also be species-specific. Many of the genes that promote or inhibit courtship in *D.*

melanogaster are expressed in peripheral chemosensory neurons, and some are required to sense pheromones that signal sex and species (Figures 1.1). While these substrates are required for the initiation and execution of courtship in *D. melanogaster* males, their roles in controlling courtship in other drosophilids is not well understood. One strategy would be to test the role of these chemosensory pathways in species that are closely related to *D. melanogaster*. Such studies will provide a foundation for understanding how changes in chemosensory pathways give rise to divergent courtship behaviors.

PART II: EVOLUTION OF BEHAVIORAL REPRODUCTIVE ISOLATION

Interspecies hybrids are rarely found in nature; animals tend to mate with conspecifics and avoid mating with members of other, even closely related, species. Such reproductive isolation, the reduction of gene flow between species, is maintained by mechanisms that act before and after mating (Mayr, 1988; Noor, 1997). These mechanisms contribute to the diversity of life as they preserve allele combinations that ensure populations remain phenotypically and genetically distinct. Courtship behaviors are some of the most important contributors to reproductive isolation, as they act to restrict mating attempts to conspecifics (Fan et al., 2013; Mayr and Dobzhansky, 1945). Such behavioral reproductive isolation is even thought to evolve more quickly than mechanisms that act after mating, and is therefore a key contributor to the divergence of species (Coyne and Orr, 1997; Mendelson, 2003). Despite its important function in speciation and diversification of animal life, how courtship behaviors evolve remains an open question. Comparative studies of the courtship of multiple pairs of closely related

Drosophila species will generate a broader understanding of how behavioral reproductive isolation evolves (Figure 1.2).

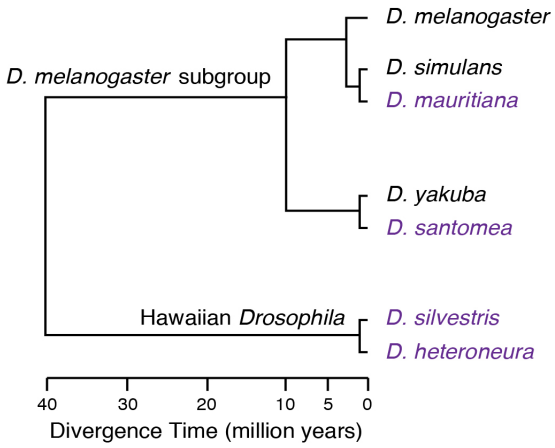

FIGURE 1.2: Phylogeny of closely related *Drosophila* species.

The pairs of closely related *Drosophila* species I discuss in this chapter have diverged ~0.25 — 5 million years ago. Here they are shown with their evolutionary relationship to each other. For instance, the *D. melanogaster* subgroup diverged from the Hawaiian *Drosophila* species ~40 million years ago. The island species are color-coded (purple).

Sensory control of courtship in *D. melanogaster* and *D. simulans*

A rich body of research exists comparing the courtship behaviors of *D. melanogaster* and *D. simulans,* which last shared a common ancestor ~3-5 million years ago (Figure 1.2) (Clark et al., 2007). These two species are found to coexist in habitats all around the world (Jezovit et al., 2017). They are so morphologically similar that, until 1919, they were thought to be a single species (Sturtevant, 1919). Since then, differences in their behavior and physiology, such as their courtship displays and pheromonal profiles, have been identified and documented (Barker, 1962; Capy and Gibert, 2004; Ellis and Carney, 2009; Manning, 1959).

By eye, the courtship displays of male *D. melanogaster* and *D. simulans* are practically indistinguishable. Males of each species produce a species-specific song that promotes mating; "muted" (wingless) males of either species are less successful at mating with conspecifics, and "deafened" (antennaeless) females are less willing to mate (Manning, 1967; Tomaru et al., 2000). Despite the similar importance of song for each species, the two songs are quantitatively different from each other (Ewing and Bennet-Clark, 1968, 1968; Tootoonian et al., 2012; Wheeler et al., 1988). Male song in both species consists of two components, a sine "hum" and a pulse train, and one key difference between the two species is the inter-pulse interval (IPI) (Bennet-Clark and Ewing, 1969; Kawanishi and Watanabe, 1981). On average, *D. simulans* males have an IPI that is ~50% longer than that of *D. melanogaster* (~70 ms vs ~35 ms, strain-dependent). Females respond more positively to the song features of conspecific males, which suggests that species-specific signals are important for mating success (Manning, 1967; Riabinina et al., 2011). There are likely many loci that control or shape species-

specific components of male song (Campesan et al., 2001; Cowling and Burnet, 1981; Ding et al., 2016; Turner and Miller, 2012).

The pheromone 7,11-heptacosedeine promotes courtship by *D. melanogaster* males and inhibits courtship by *D. simulans* males (Billeter et al., 2009). In *D. melanogaster*, Ppk25 is required for behavioral and pheromonal responses to 7,11-heptacosedeine and males mutant for *Ppk25* court conspecific females less than WT males (Lin et al., 2005; Starostina et al., 2012; Thistle et al., 2012; Vijayan et al., 2014). Surprisingly, *D. simulans* males mutant for *Ppk25* also court conspecific females less the WT *D. simulans* males (Ahmed et al., 2017). These results suggest that Ppk25 has a functionally conserved role in both species (to promote conspecific mating), although Ppk25 may not detect 7,11-heptacosedeine in *D. simulans* as it does in *D. melanogaster*. One possibility is that the pheromone tuning of Ppk25 has changed between the two species. Alternatively *Ppk25*-expressing cells may express species-specific combinations of other genes, or synapse unto species-specific downstream neural circuitry.

WT *D. melanogaster* and *D. simulans* males exhibit high levels of courtship towards conspecific females and low levels of courtship to females of other species (Ahmed et al., 2017; Fan et al., 2013; Manning, 1959). The foreleg tarsi, which are used for tapping potential mates early in the courtship routine, are required by males of both species to inhibit such interspecies courtship but are not essential for conspecific courtship (Ahmed et al., 2017; Fan et al., 2013; Manning, 1959). Thus, the sensory pathways that precludes interspecies courtship is conserved in both species. In *D. melanogaster*, the chemoreceptor Gr32a and *Gr32a*-expressing neurons are essential to

inhibit interspecies mating (Fan et al., 2013). In *D. simulans* however, Gr32a is not required for this inhibition, despite almost identical expression patterns between the two species (Ahmed et al., 2017). Divergence of Gr32a function in courtship suggests that *D. simulans* employ a different molecular mechanism to inhibit interspecies mating. Comparative studies of genes that control courtship (e.g. *Ppk23*, *Ppk25*, *Gr32a*, etc) will provide a better understanding of why *D. melanogaster* and *D. simulans* respond differently to similar courtship signals. Though it remains technically challenging to probe the function of genes and neural circuits in *D. simulans*, the recent development of genetic tools in this species will provide a way to test how mechanisms that control *D. melanogaster* courtship have diverged (Stern et al., 2017).

Song production in *D. simulans* and *D. mauritiana*

Courtship song is a highly divergent and complex behavioral trait in *Drosophila*, with some parameters of song differing even between closely related species (Hoy et al., 1988; Ritchie and Gleason, 1995; Saarikettu et al., 2005). This has motivated the search for the genetic substrates that underlie species-specific song components. One pair of species, *D. simulans* and *D. mauritiana*, last shared a common ancestor ~240,000 years ago and showcase subtle but significant differences in courtship song and other traits (Figure 1.2) (Ding et al., 2016; Kliman et al., 2000). Genetically, the two species are highly similar and, in lab conditions, can even produce fertile hybrids. While *D. simulans* is a global human-commensal, *D. mauritiana* is found almost exclusively on the island of Mauritius (Jezovit et al., 2017; Tascas and David, 1974). Their recent divergence from a common ancestor and their evolutionary proximity to *D.*

melanogaster makes these two species particularly interesting for studying how genetic changes can lead to divergent behavioral programs.

The courtship songs show of *D. mauritiana* and *D. simulans* are quantitatively different: *D. mauritiana* tends to have a sine "hum" carrier frequency that is ~10 Hz higher than *D. simulans* (~185 Hz vs. ~175 Hz) (Ding et al., 2016; Robertson, 1983a). Recently, the genetic substrate that confers this difference was identified using high-throughput song analysis, quantitative trait loci mapping, and introgression screening (Ding et al., 2016); the calcium-activated potassium channel *slopoke* causes these species-specific differences in sine song. Further, the lower sine song carrier frequency is caused by a retroelement insertion in the *slopoke* gene. This insertion likely causes alternative splicing of *slopoke* and decreases *slopoke* expression in subsets of neurons that control sine song structure. Retroelements tile the genomes of *D. simulans* and *D. mauritiana*, and may have profound effects in shaping behavioral repertoires across different strains and closely-related species of *Drosophila*.

Species discernment by *D. santomea* females

While male courtship behavior is important for subsequent reproductive behaviors, female mate choice has been demonstrated to be vital for species propagation (reviewed in Andersson and Simmons, 2006; Chenoweth and Blows, 2006). Thus, behavioral reproductive isolation is also instated by female behavior. For example, *D. santomea* females are courted by *D. santomea* and *D. yakuba* males but will only copulate with conspecific males (Mas and Jallon, 2005). Therefore, *D. santomea* females must have evolved mechanisms to discern species-specific differences in courtship parameters or

other sensory cues (Riabinina et al., 2011). This makes *D. santomea* and *D. yakuba* an attractive model for studying the evolution of behavioral reproductive isolation in females.

D. yakuba is found throughout sub-Saharan Africa and on some neighboring islands, while *D. santomea* is found on the island of São Tomé. The two species have only diverged about ~400,000 years ago and males of each species exhibit differences in courtship behaviors (Figure 1.2) (Cande et al., 2012; Lachaise et al., 2000; Llopart et al., 2002). Currently, it remains unclear which features of male courtship *D. santomea* females use to discriminate between species. Particular features of *D. yakuba* song may inhibit receptivity in *D. santomea* females, or conspecific song may act as a courtship-promoting signal. In another pair of closely related species, *D. montana* females reject *D. lummei* males only after the male starts to sing, but they are receptive to *D. lummei* males if *D. montana* male song is simultaneously played via a nearby speaker (Saarikettu et al., 2005). The development of new genetic tools in both *D. yakuba* and *D. santomea* makes these two species excellent models to determine which cues are important for D. santomea females to discern species, and the genetic and neural basis for female species recognition and mate choice (Stern et al., 2017).

Incipient reproductive isolation between Hawaiian *Drosophila* species

Approximately 1,000 *Drosophila* species are endemic to the islands of Hawaii, an area covering only 6,500 square miles (Kang et al., 2016). These charismatic species exhibit a tremendous diversity of morphological and behavioral traits, such as differences in size, wing pigmentation patterns, bristle formation, and complex courtship behaviors (Davis,

2000; Hoy et al., 1988; Ringo and Hodosh, 1978). Amazingly, this massive diversification of species likely originated from a single ancestor that colonized the island of Kauai only ~5 million years ago (Ringo, 1977). This rapid speciation is unlikely the result of natural selection alone, and could be better explained by sexual selection theory (Hoikkala and Kaneshiro, 1993; Ringo, 1977; Spieth, 1974). Despite these results, how these species have become reproductively isolated in a short amount of time remains unclear.

Two Hawaiian picture-winged species, *D. silvestris* and *D. heteroneura*, serve as compelling models for understanding incipient reproductive isolation. These two species are partially sympatric on the Big Island and have only diverged <0.5 million years ago, the approximate age of the island (Figure 1.2). Within this short time period, the two species have become only partially reproductively isolated, although they exhibit divergent behavioral and morphological traits. Unlike the males of the other species-pairs discussed in this chapter, male *D. silvestris* and *D. heteroneura* court females on carefully chosen mating arenas, called leks (Spieth, 1981). Females select mates at these locations, which are fiercely defended by individual males. These two species rarely interbreed in the wild, even though leks of both species may be in close physical proximity to each other and the male courtship behaviors are almost identical between these two species (Carson et al., 1989). It is thought that cues established early in the courtship routine are most important for establishing a reproductive barrier between these species (Price and Boake, 1995). For instance, although males produce species-specific songs, these song cues occur late in courtship and are not essential for copulation (Boake and Poulsen, 1997).

Matings between *D. silvestris* and *D. heteroneura*, although rare, can lead to fertile hybrid offspring (Ahearn et al., 1974). This suggests that these two species have yet to evolve reproductive barriers that act after fertilization, and therefore their incipient reproductive isolation must be facilitated by mechanisms that act prior to fertilization (i.e. courtship routines). Indeed, hybridization events are almost always the result of matings between *D. silvestris* females and *D. heteroneura* males; the reciprocal pairs rarely initiate courtship (Carson et al., 1989; Price and Boake, 1995). These results suggest that species recognition and preference in these species is evolving quickly and contributing to the rapid speciation of the Hawaiian drosophilids. This makes these species ideal for studying how genetic changes can lead to such major differences in morphology and behavior.

CONCLUDING REMARKS

Barriers preventing the exchange of genetic materials are paramount to maintaining the tremendous diversity of animal life on Earth. Courtship routines evolve quickly and facilitate behavioral reproductive isolation, yet how these routines diverge remains an unanswered question in biology. This question is particularly challenging to study in real time, since evolution tends to happen on timescales longer than the lifespan of any one biologist. However, all extant animal species represent snapshots of a particular moment in evolution and comparative behavioral, genetic, and neurobiological studies in closely related species will help us understand how courtship behavior, and reproductive isolation, evolves.

Flies in the genus *Drosophila* and their innate courtship displays are of particular interest to comparative neurobiology because even closely related species have evolved divergent mating patterns. Additionally, key components of these courtship routines likely facilitate reproductive isolation and directly contribute to speciation. Yet, for many of these species, the genetic and neural substrates that control courtship are unknown, for the most part. It is also challenging to understand how these substrates give rise to divergent behaviors. Remarkably, recent advances in molecular biology and genome technology has enabled the sequencing and manipulation the genomes in non-model organisms (Ding et al., 2016; Hammond et al., 2016; Kang et al., 2017; Koutroumpa et al., 2016; Stern et al., 2017; Wang et al., 2016). The decades of behavioral research on these diverse fly species can finally be matched by detailed analyses of the genes and neural circuits that control such behaviors.

By studying differences in courtship behaviors between multiple pairs of closely related species, we can potentially identify common substrates, or themes, for the evolution of divergent behaviors. Such behavioral studies must be accompanied by rigorous genetic and neurobiological approaches in numerous species. Recent advances in whole-genome sequencing, genetic engineering, and high-throughput behavioral analyses have given scientists unprecedented access to these questions. This will help uncover how genes and neural circuits change to produce species-specific patterns of innate behaviors.

CHAPTER TWO

GENETIC AND NEURAL MECHANISMS THAT INHIBIT DROSOPHILA FROM MATING WITH OTHER SPECIES

ABSTRACT

Genetically hard-wired neural mechanisms must enforce behavioral reproductive isolation because interspecies courtship is rare even in sexually naive 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 deadend 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 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 (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 2.1A) (Acebes et al., 2003; Greenspan and Ferveur, 2000; Kowalski et al., 2004; Krstic et al., 2009; Robertson,

1983b; 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 naive *D. melanogaster* males lacking specific sensory input (Figures 2.1B, 2.1G). *D. virilis* shared an ancestor with *D. melanogaster* ~40 million years ago (mya), and wildtype (WT) *D. melanogaster* males do not court *D. virilis* females (Figure 2.1H). 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 (Figures 2.1B-K). Gustatory cues are detected by neurons on mouthparts and on foreleg tarsi. Removal of all mouthparts led to desiccation and 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 (Figures 2.1B-K). Males usually tap other flies with their foreleg tarsi prior to proceeding with courtship (Figure 2.1A) (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* ~3-5 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 2.1C, 2.1H). *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 2.1H-K). Thus, foreleg tarsi are required to inhibit *D. melanogaster* males from courting *D. virilis*, a distant drosophilid.

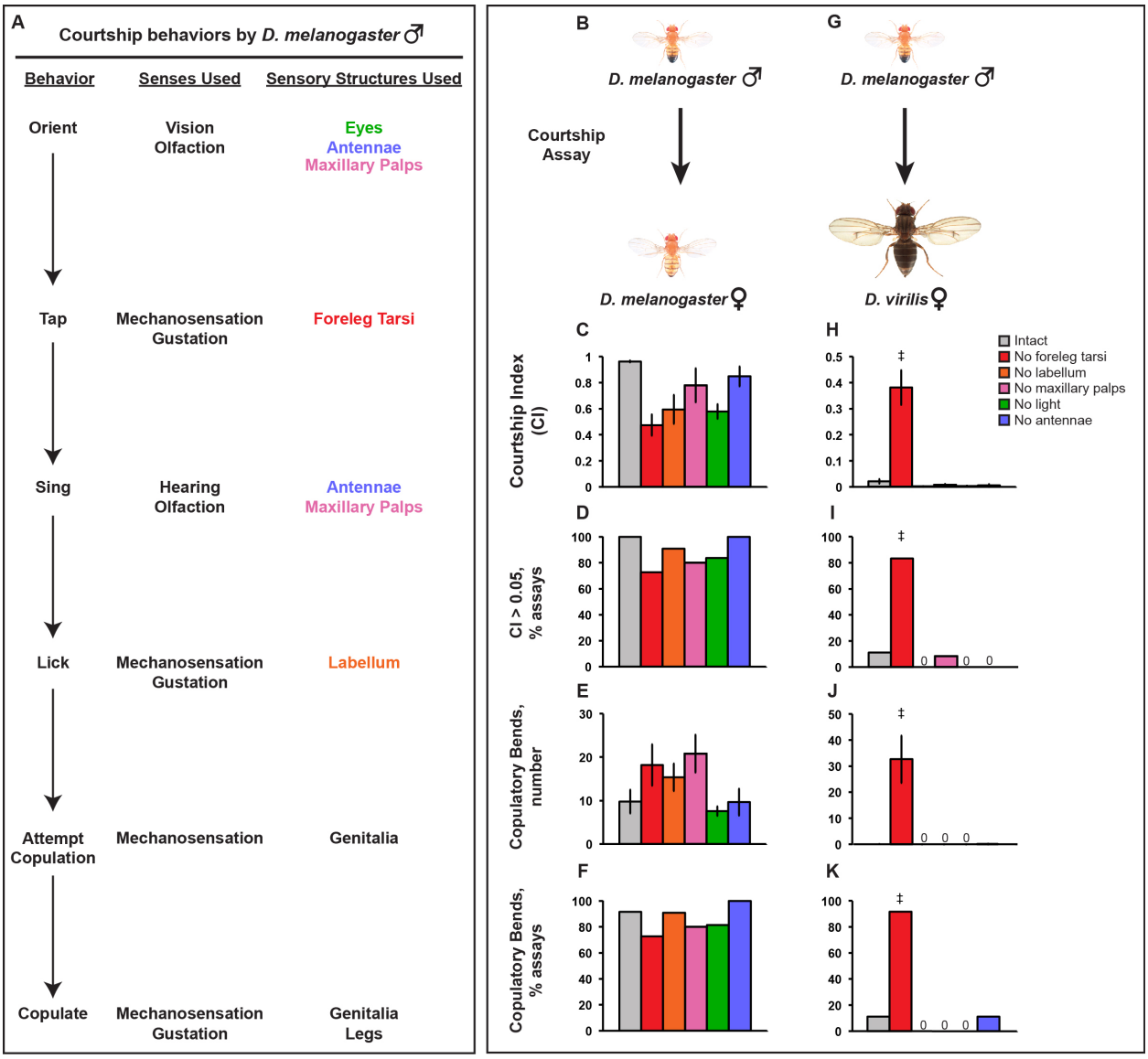

FIGURE 2.1: The foreleg tarsi inhibit courtship of other species.

(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 ≥ 11/experimental cohort; ‡$p < 0.001$.

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 2.2A-H, 2.3A-H, Table 2.1), 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 (Figures 2.2A-I). Strikingly, ablation of Gr32a or Gr33a neurons, but not other Gr neurons, allowed *D. melanogaster* males to court *D. virilis* females (Figures 2.2I, 2.4M-P). 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 (Figures 2.4A-D, 2.4M-P).

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 2.2A'-H', 2.3A'-H', Table 2.2). 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* ~3-5 and ~8 mya, respectively. We find that Gr32a:hid and Gr33a:hid males avidly courted conspecific as well as *D. simulans*, *virilis*, and *yakuba* females (Figures 2.4A-P). The vast majority of these assays had high levels of courtship, including attempted copulation by the experimental males (Figures 2.4A-P). 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* 3 - 40 mya.

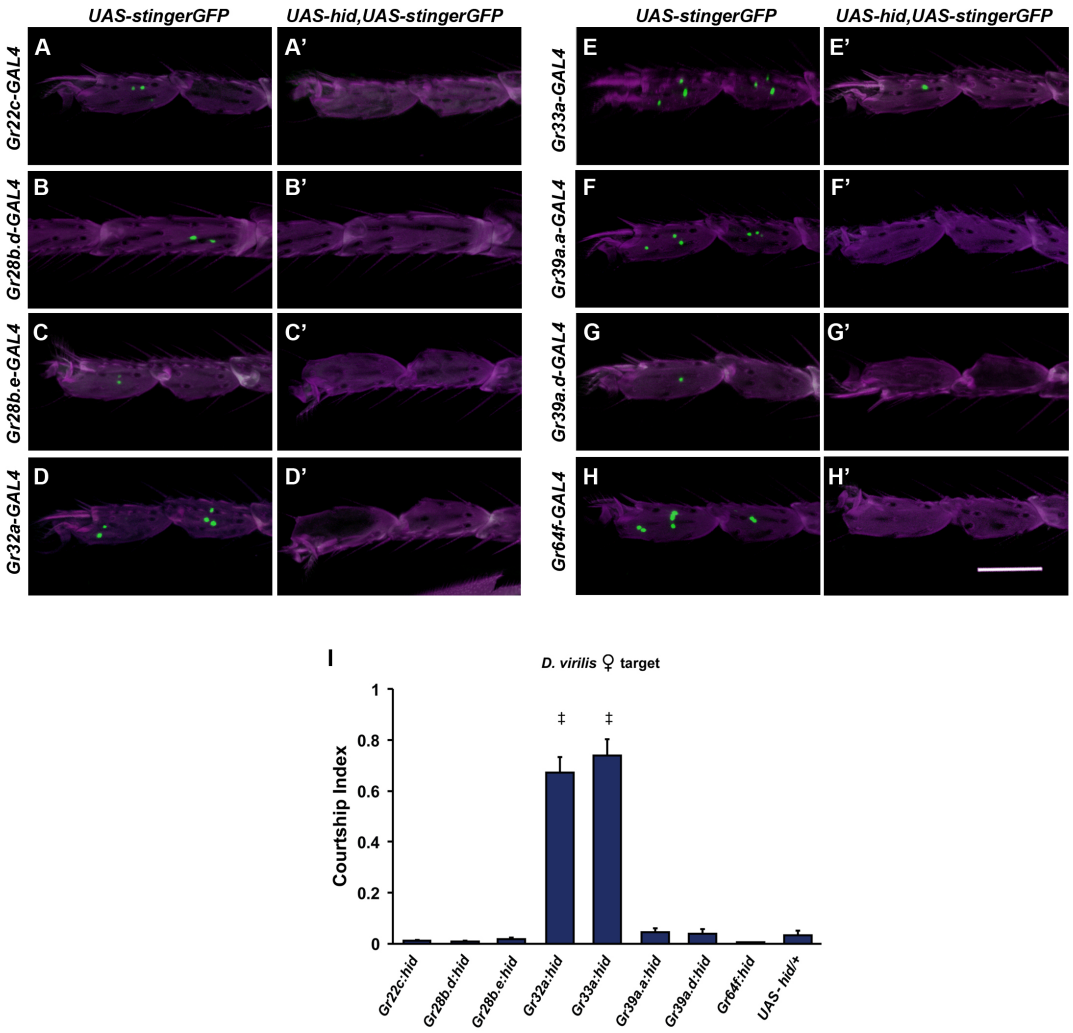

FIGURE 2.2: Identification of Gr neurons in the male foreleg that inhibit interspecies courting.

(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. Mean ± SEM; n = 5 - 10/genotype (A-H') and n = 8 - 12/genotype (I); ‡$p < 0.001$; scale bar = 50 μm. Please see Tables 2.1 and 2.2.

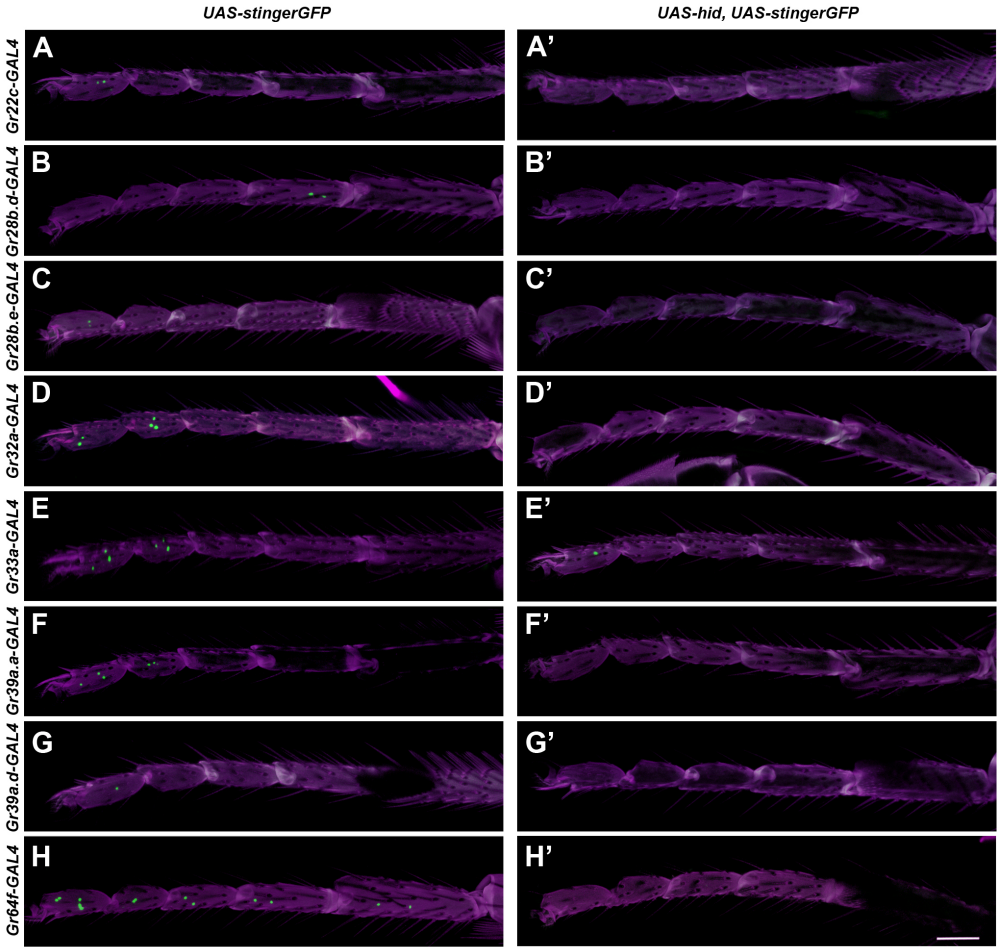

FIGURE 2.3: Identification and ablation of Gr neurons in the male foreleg.

(A-H') Expression (A-H) of different Grs (Gr:stingerGFP) and ablation (A'-H') of Gr neurons (Gr:stinger GFP, hid) in foreleg tarsi. Whole-mount preparation of all tarsi (t1–t5) shown, with more distal tarsi to the left. n= 5–10/genotype; scale bar = 50 μm. Please see Tables 2.1 and 2.2.

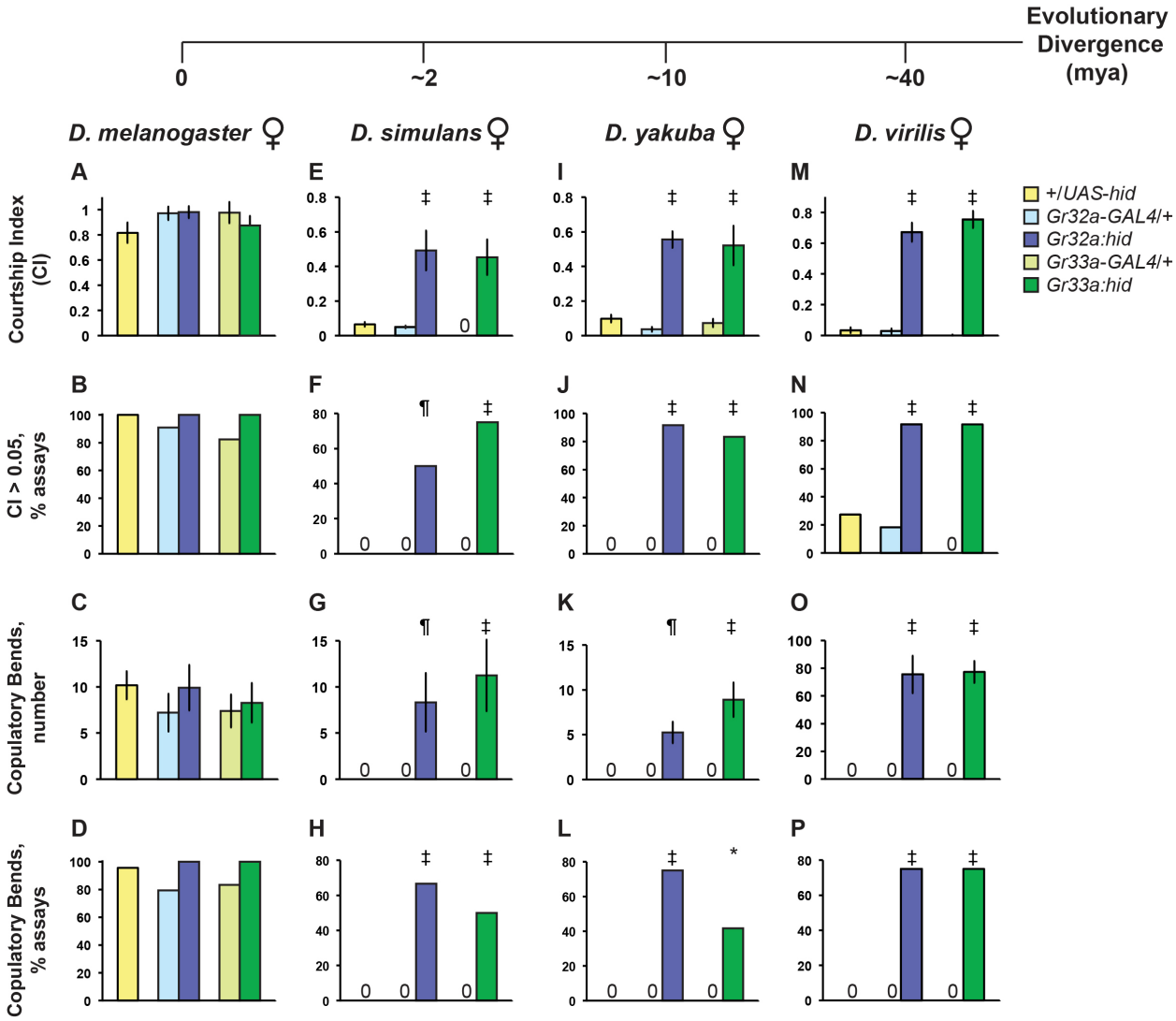

FIGURE 2.4: Ablation of Gr32a or Gr33a neurons permits courting of females of other species.

(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. Mean ± SEM; n = 10 - 24/genotype; *p < 0.05, ¶p < 0.01, ‡p < 0.001.

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 (Figures 2.5E-P). Gr32a null males displayed the entire range of courtship preceding copulation toward females of all species and copulated with conspecifics (Figures 2.5A-P).

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 (Figures 2.6A-P). 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 (Figures 2.6A-P). 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.

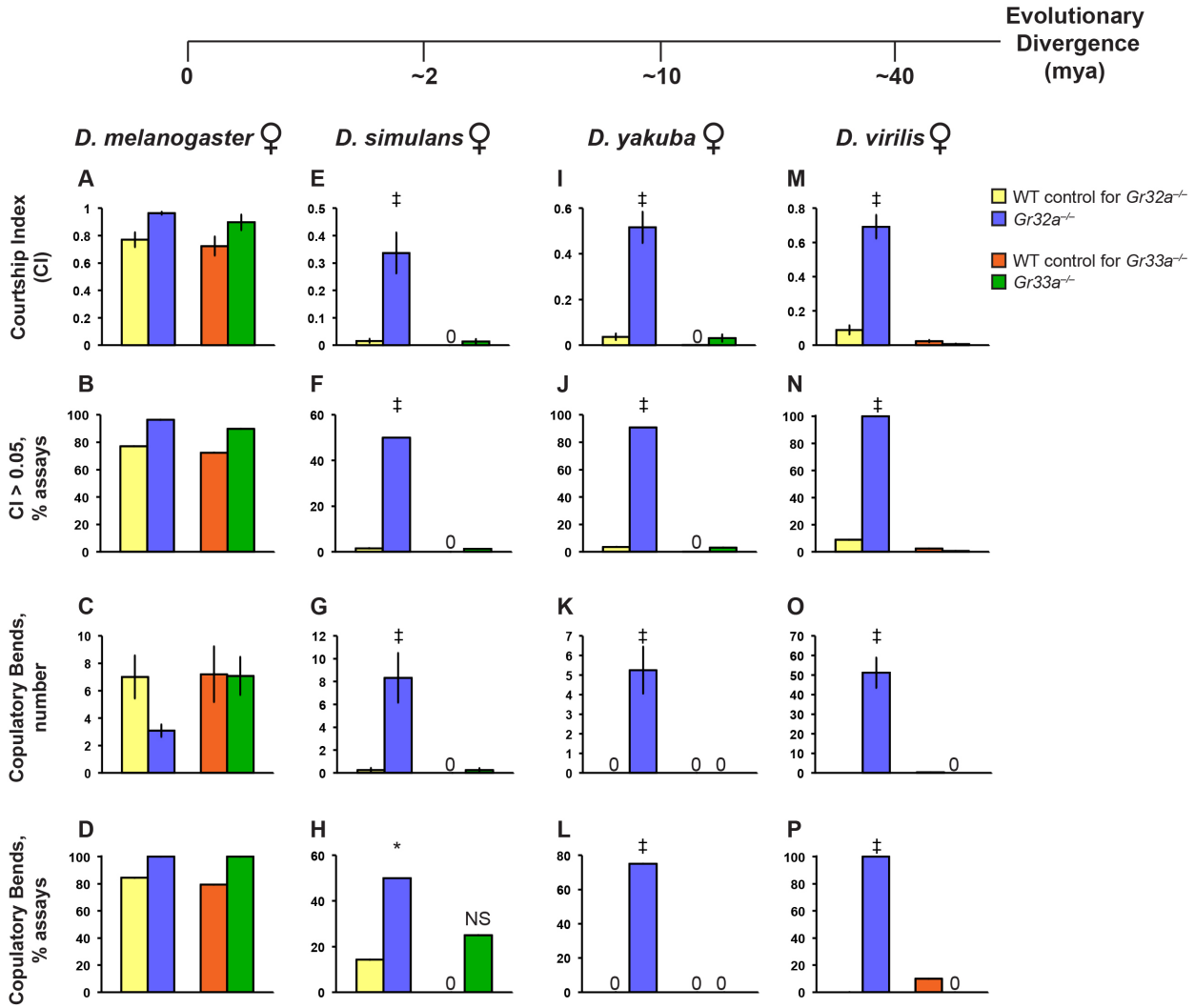

FIGURE 2.5: Gr32a inhibits interspecies courtship.

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. Mean ± SEM; n = 10–24/genotype; *p < 0.05, ‡p < 0.001; NS = not significant.

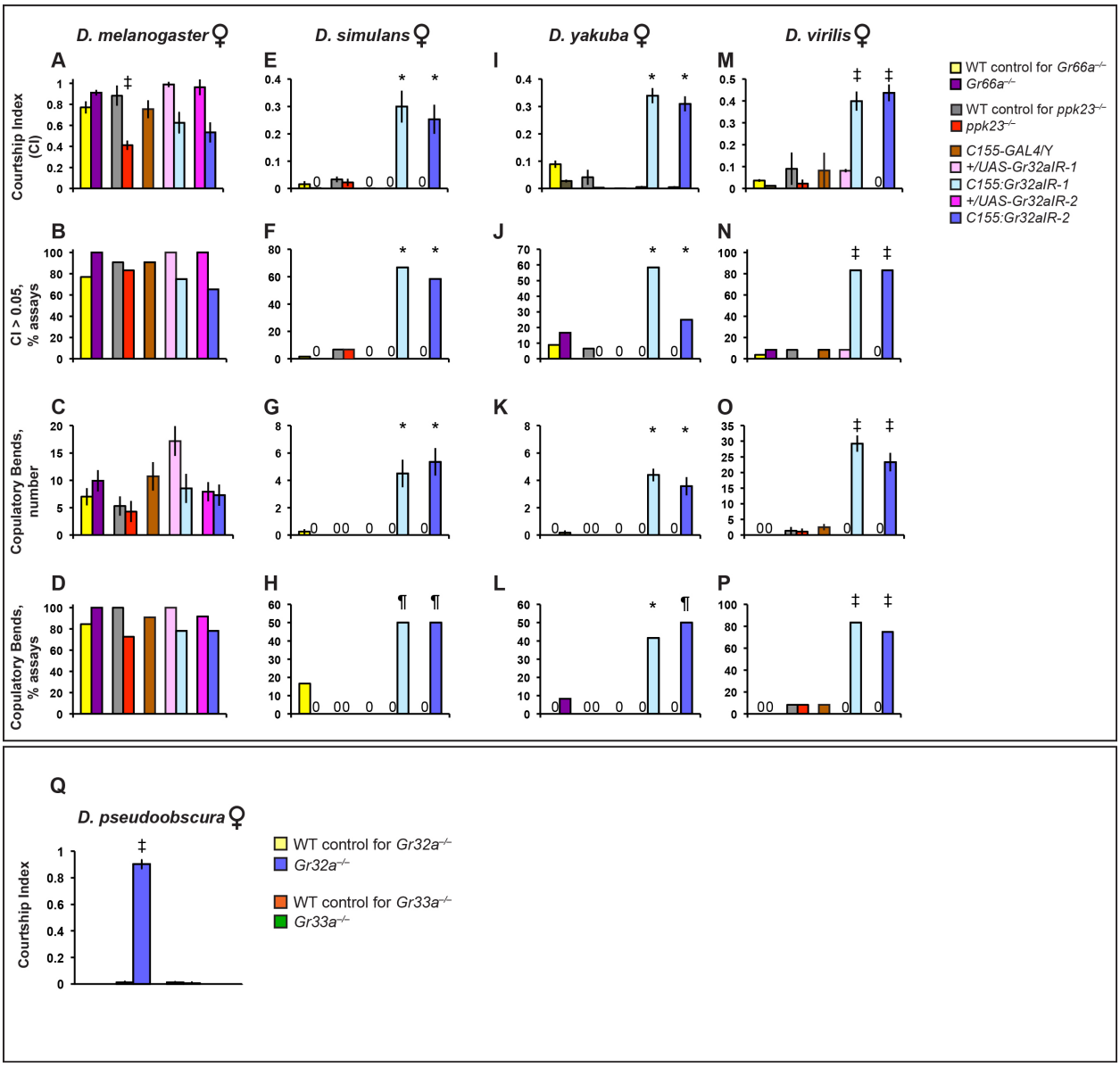

FIGURE 2.6: Gr32a, but not Gr66a or Ppk23, inhibits interspecies courtship.

Control and experimental males were tested for courtship with females of various species. **(A-D)** No difference in courting conspecific females between control, Gr66a null, and flies with knockdown of Gr32a (C155:Gr32aIR-1 or C155:Gr32aIR-2). As expected, Ppk23 null males show reduced courtship of conspecific females (Lu et al., 2012; Thistle et al., 2012; Toda et al., 2012). **(E-P)** Males with a knockdown of Gr32a, but not Gr66a or Ppk23 mutant males, court *D. simulans* (E-H), *D. yakuba* (I-L), and *D. virilis* (M-P) females. **(Q)** *Gr32a*$^{-/-}$ males court *D. pseudoobscura*. Mean ± SEM; n = 10 - 24/genotype; *p < 0.05, ¶p < 0.01, ‡p < 0.001.

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 2.7A, 2.7C). 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 2.7A, 2.7C). 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 2.7B, 2.7D). By contrast, at an elevated temperature that activates dTrpA1 these males courted conspecific but not *D. virilis* females (Figures 2.7B, 2.7D). Thus, activity of Gr32a neurons abrogates interspecies courtship but does not 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; Higgie 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 2.7E). Strikingly, *Gr32a*$^{-/-}$ males courted oe– flies regardless of the source of the cuticular extract (Figure 2.7E). 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 2.8) 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 that *Gr32a*$^{-/-}$, but not WT, males courted oe– targets coated with physiological concentrations of 7T similar to control oe– or WT *melanogaster* females (Figure 2.7E). 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 2.8) (Ferveur, 2005; Liimatainen and Jallon, 2007). *Gr32a*$^{-/-}$, but not WT, males courted 9T-coated oe– females vigorously (Figure 2.7E). 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 2.8), which appears restricted to *D. virilis* (Ferveur, 2005). We synthesized 11P (Figure 2.8) and tested whether 11P-coated oe– females elicited courtship. We found that *Gr32a*$^{-/-}$, but not WT, males courted such females vigorously (Figure 2.7E). Oe– females coated with both 9T and 11P did not elicit less courtship by WT males compared to 11P alone (Figure 2.7E), 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.

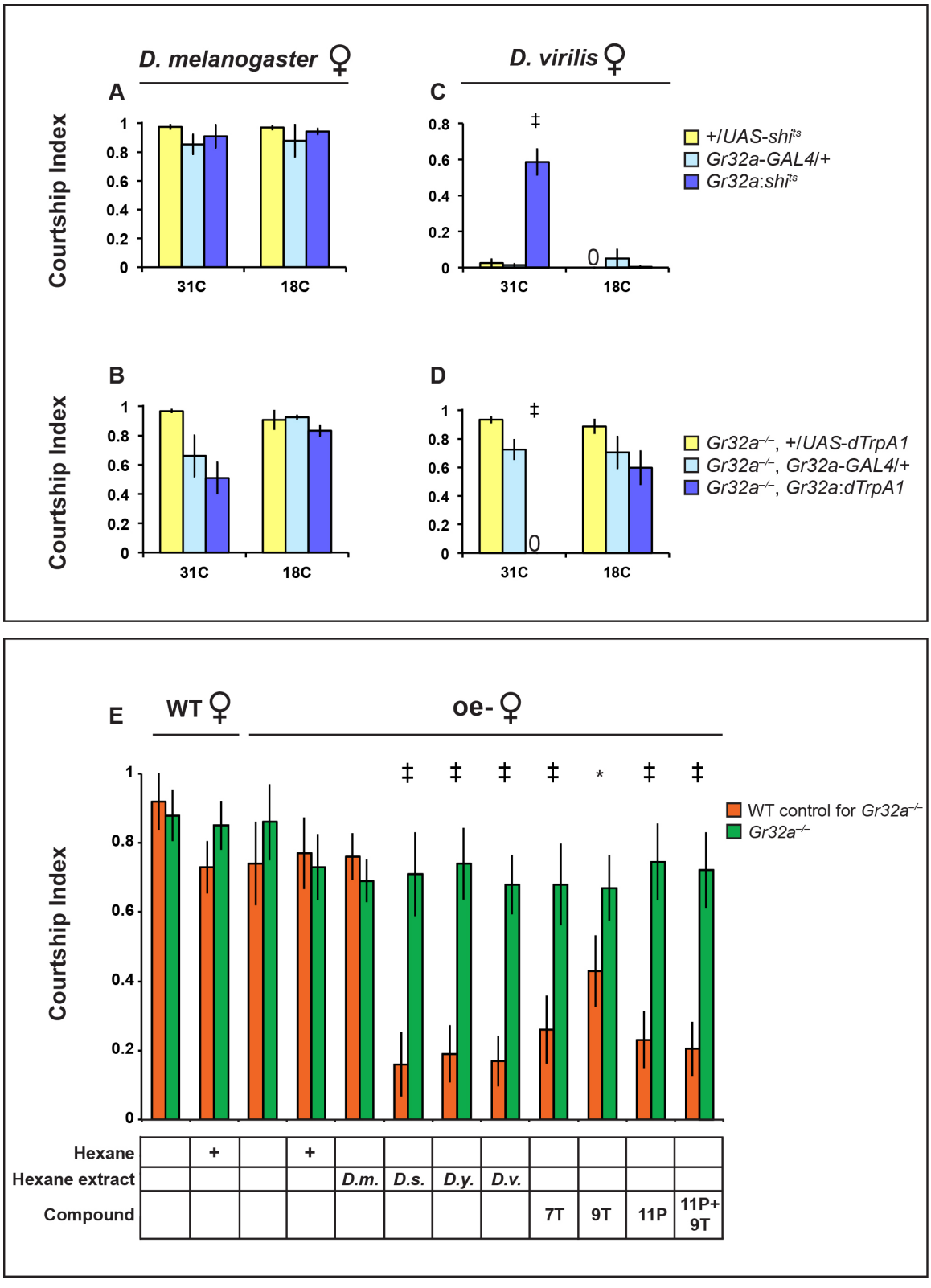

FIGURE 2.7: See next page for details.

FIGURE 2.7: Gr32a neurons inhibit interspecies courtship by recognizing cuticular hydrocarbons found on other species.

D. melanogaster males WT or mutant for Gr32a were tested for courtship with conspecific or *D. virilis* females. **(A)** Inactivation of synaptic release by Gr32a neurons (Gr32a:shi^{ts}) at the restrictive temperature (31^{o} 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:shi^{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. Mean ± SEM; n = 10 - 16/genotype; *p < 0.05, ‡p < 0.001.

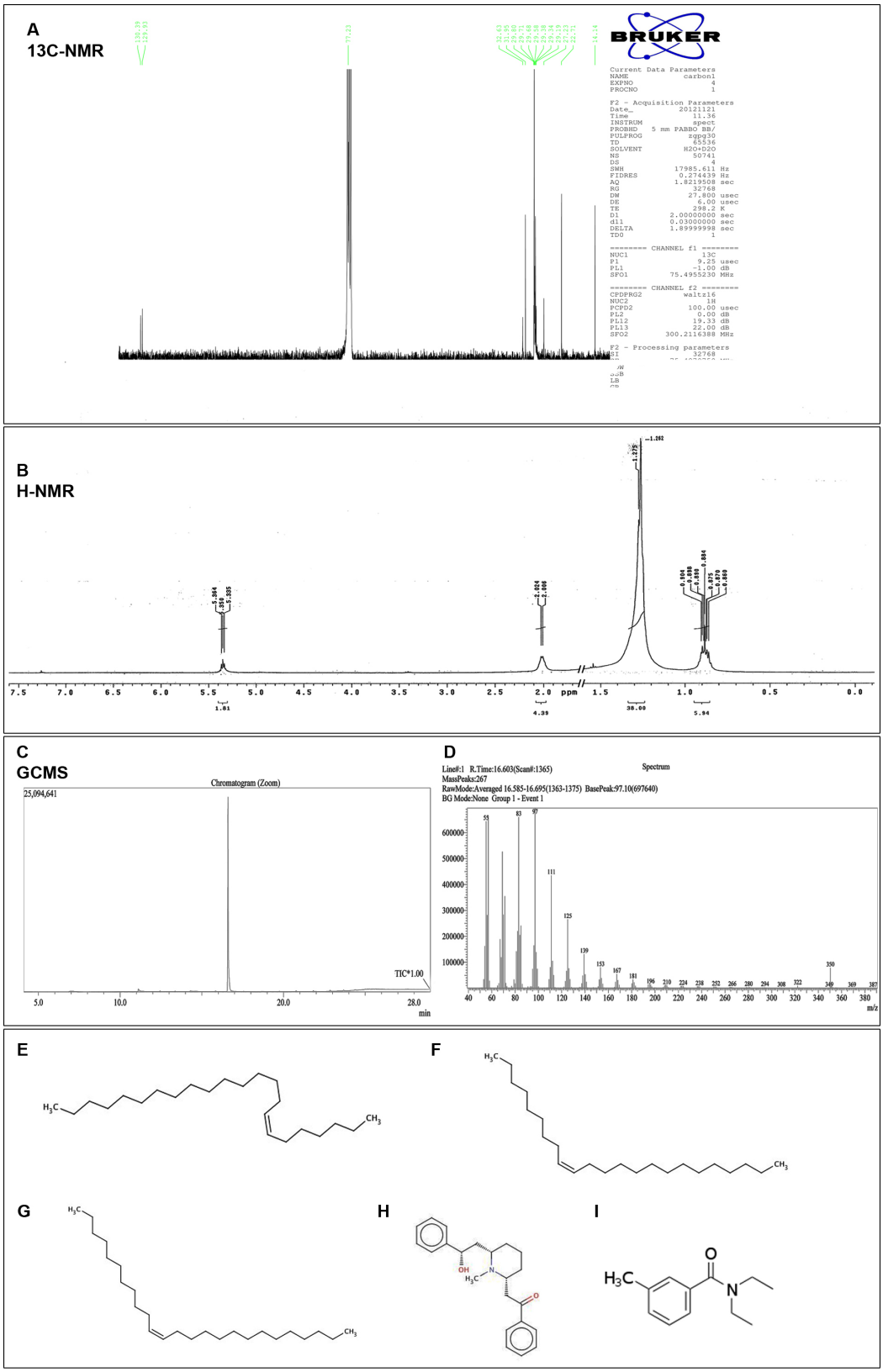

FIGURE 2.8: See next page for details.

FIGURE 2.8: Analytical data for z-11-pentacosene.

(A) ^{13}C NMR spectrum collected at 75 MHz. **(B)** ^{1}H NMR spectrum collected at 300 MHz. **(C)** GCMS of purified 11P, as detected by TIC (total ion current, mass spectrometric). **(D)** *m/z* composition of the dominant peak in the chromatogram shown in (C). **(E)** Chemical structure of z-7-tricosene (7T). **(F)** Chemical structure of z-9-tricosene (9T). **(G)** Chemical structure of z-11-pentacosene (11P). **(H)** Chemical structure of lobeline. **(I)** Chemical structure of N, N, diethyl-meta-toluamide (DEET).

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; 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$^{4\text{-}40}$/fru^{sat15}*) did not court any targets, including conspecific females, consistent with the requirement for Fru^M in male courtship (Figure 2.9A). However, males mutant, but not null, for Fru^M (*fru^1/fru$^{4\text{-}40}$*) courted conspecific females and those from other species (Figure 2.9A).

Fru^M and Gr32a both inhibit males from courting females of other species (Figures 2.5, 2.6, 2.9A) and conspecific males (Gill, 1963; Hall, 1978; Miyamoto and Amrein, 2008). We therefore tested whether *fru^1/fru$^{4\text{-}40}$* 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 D. *virilis* males (Figures 2.10F, 2.10I), 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, *Gr33a$^{-/-}$* males also court conspecific males (Figure 2.10G) (Moon et al., 2009), but they do not court other drosophilids (Figures 2.5, 2.10G). 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 of other species (Figures 2.6, 2.10H). 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 2.9B-D'', 2.10K-M''). 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 2.10A). 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 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

2.10N-Q). In our experimental flies, we observed native GFP fluorescence in the ventral nerve cord (VNC) and the subesophageal ganglion (SOG) (Figures 2.9E-G, 2.10R-T), 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 2.9H, 2.9I), 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 2.9J, 2.10J). In contrast to courtship of conspecific females, P52A:fru^MIR males sang only after tapping nonconspecific flies (Table 2.3). 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 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 2.10U-V''). The lack of GRASP signal does not reflect failure of expression of GRASP components because these could be visualized with immunolabeling (Figures 2.10W-W''). We also did not observe apposition of Gr32a and aDT6 processes within the SOG using the fly brainbow system (Figure 2.10X; 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.

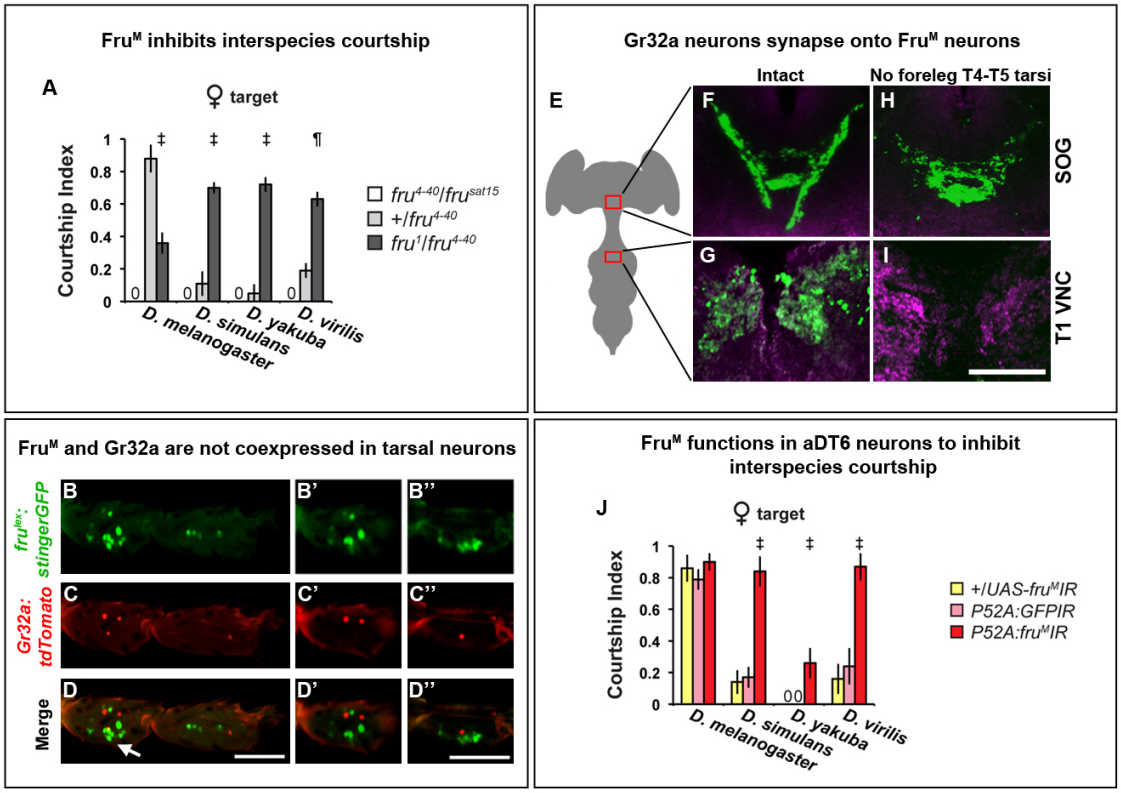

FIGURE 2.9: A cellular and molecular pathway that inhibits interspecies courtship.

(A) *fru*^{1}*/fru*$^{4\text{-}40}$ 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^{M}IR) permits courtship of conspecific females and females of other species. Mean ± SEM; n = 10 - 31/experimental cohort; ¶p < 0.01, ‡p < 0.001; scale bar = 20 µm. Please see Table 2.3.

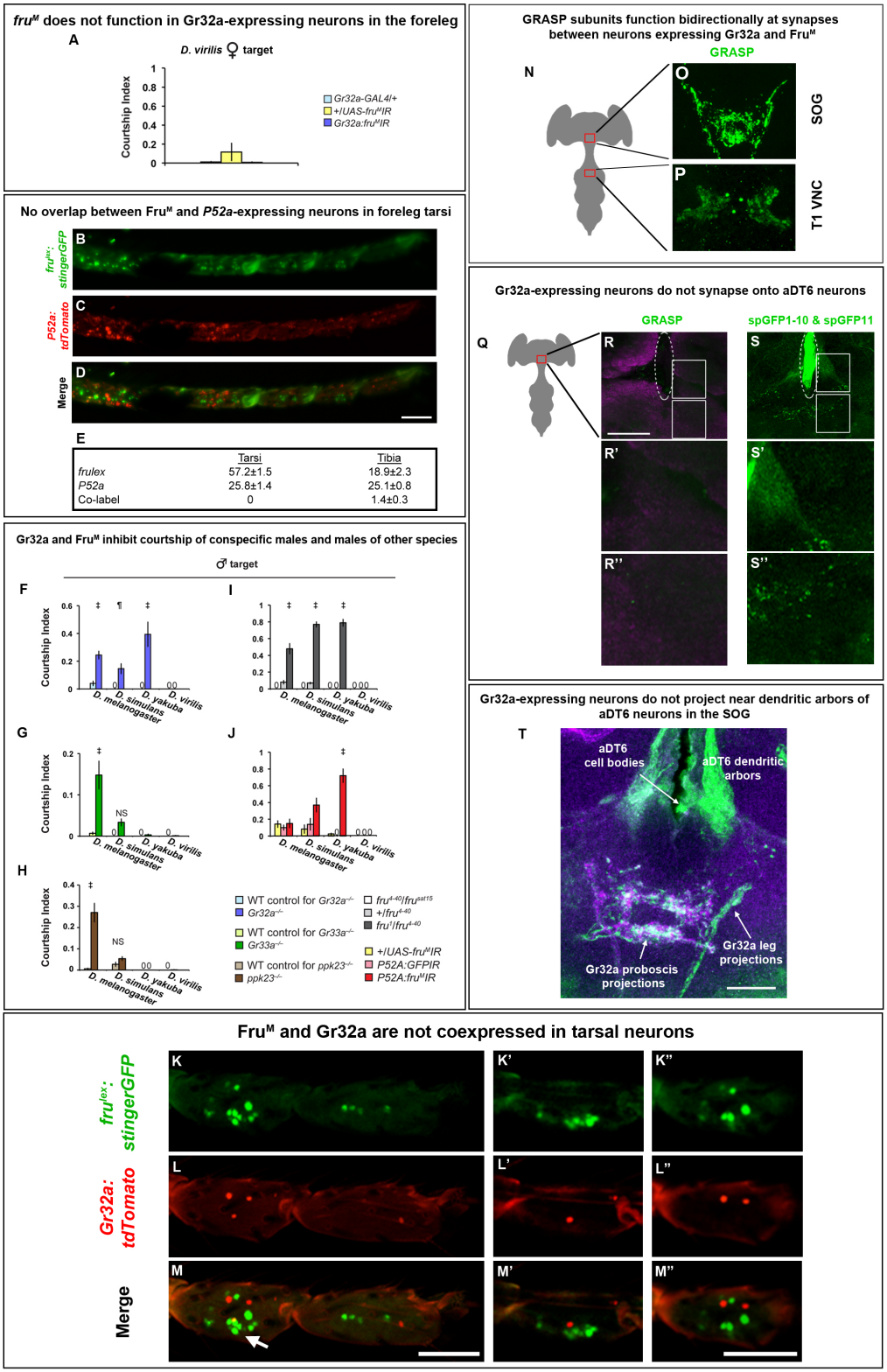

FIGURE 2.10: See next page for details.

FIGURE 2.10: Distinct mechanisms inhibit conspecific intermale and interspecies courtship.

(A) Consistent with the lack of Fru^M expression in Gr32a neurons, driving expression of a Fru^M RNAi in male Gr32a neurons does not lead to courtship of *D. virilis* females. **(B-D)** No overlap in Fru^M (fru^{lex}:stinger GFP) and P52A (P52A:tdTomato) cells in foreleg tarsi. P52A:tdTomato reveals previously unreported expression (Manoli and Baker, 2004) in the cuticle and other nonneural tissue in the foreleg, but these cells do not coexpress Fru^M in the tarsi. **(E)** Enumeration of Fru^M (fru^{lex}:stinger GFP) and P52A (P52A:tdTomato) cells in foreleg tarsi and tibia. **(F)** Gr32a null males court conspecific, *D. simulans* and *yakuba*, but not *virilis*, males. **(G)** Gr33a null males court conspecific males, but not males of other species. **(H)** Ppk23 null males court conspecific males, but not males of other species. **(I)** $fru^1/fru^{4\text{-}40}$ males court conspecific, *D. simulans* and *yakuba*, but not *virilis*, males. **(J)** Males with a knockdown of Fru^M in aDT6 neurons (P52A:fru^MIR) court *D. yakuba* males. As reported previously, these flies do not court conspecific males (Manoli and Baker, 2004). Taken together with the courtship phenotypes of Gr32a, Gr33a, and Ppk23 null males, our results suggest that it is possible to dissociate at a molecular and cellular level the atypical courtship of conspecific males from that of animals of other species. **(K-M")** No coexpression of Fru^M and Gr32a in foreleg tarsi of *D. melanogaster* males (K-M). Enlarged version of the panels shown in Figures 2.9B-D". A cell that appears colabeled for Fru^M and Gr32a in a Z projected image (arrow in M) in fact represents two distinct cells in different optical slices expressing either Fru^M (K"-M") or Gr32a (K'-M') but not both. **(N-Q**) Schematic of connectivity between L3 and Tm9 neurons in the M3 layer of the medulla (N); gray box represents the area of the histological images shown to the right (O-Q). Native GRASP fluorescence is visualized in the M3 layer (O, Q) but not in other regions of the medulla (visualized with nc82 immunolabeling in P, Q) although these regions do contain processes from L3 or Tm9 neurons. **(R-T)** Switching the expression of the GRASP components also labels contacts between Gr32a (Gr32a:LexA) and Fru^M (fru^M:GAL4) neurons in the SOG and T1 VNC of *D. melanogaster* males (*Gr32a:spGFP11::CD4, fru:spGFP1-10::Nrx*), as visualized by native GRASP fluorescence. **(U-V")** No contacts between Gr32a and aDT6 neurons in the SOG, as visualized by native GRASP fluorescence, in *D. melanogaster* males ($Gr32a^{lex}$:spGFP11::CD4, P52A:spGFP1-10::Nrx). The neuropil (magenta) is immunolabeled with nc82. **(W-W")** Immunolabeling confirms expression of the individual GRASP components in the SOG of *D. melanogaster* males (Gr32a:spGFP11::CD4, P52A:spGFP1- 10::Nrx). **(X)** dBrainbow-labeled clones in *D. melanogaster* males (hs:Cre, Gr32a:GAL4::VP16, P52A:GAL4, UAS-dBrainbow) show no overlap between axonal arbors of Gr32a neurons (green and magenta clones) in the SOG and aDT6 (green clones) soma and local dendritic arbors (Miyamoto and Amrein, 2008). aDT6 axon projections can be visualized as two thick green vertical bands exiting the top of the figure. Mean ± SEM; n = 10 - 31/experimental cohort for all panels except (W-W", n = 5); ¶$p < 0.01$, ‡$p < 0.001$; NS = not significant; scale bar = 50 μm (B-D), 10 μm (O-Q), and 20 μm (V-X).

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 2.11A-B). 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* ~26 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 *D. simulans* and *D. yakuba* males (Figures 2.11C-D). 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.

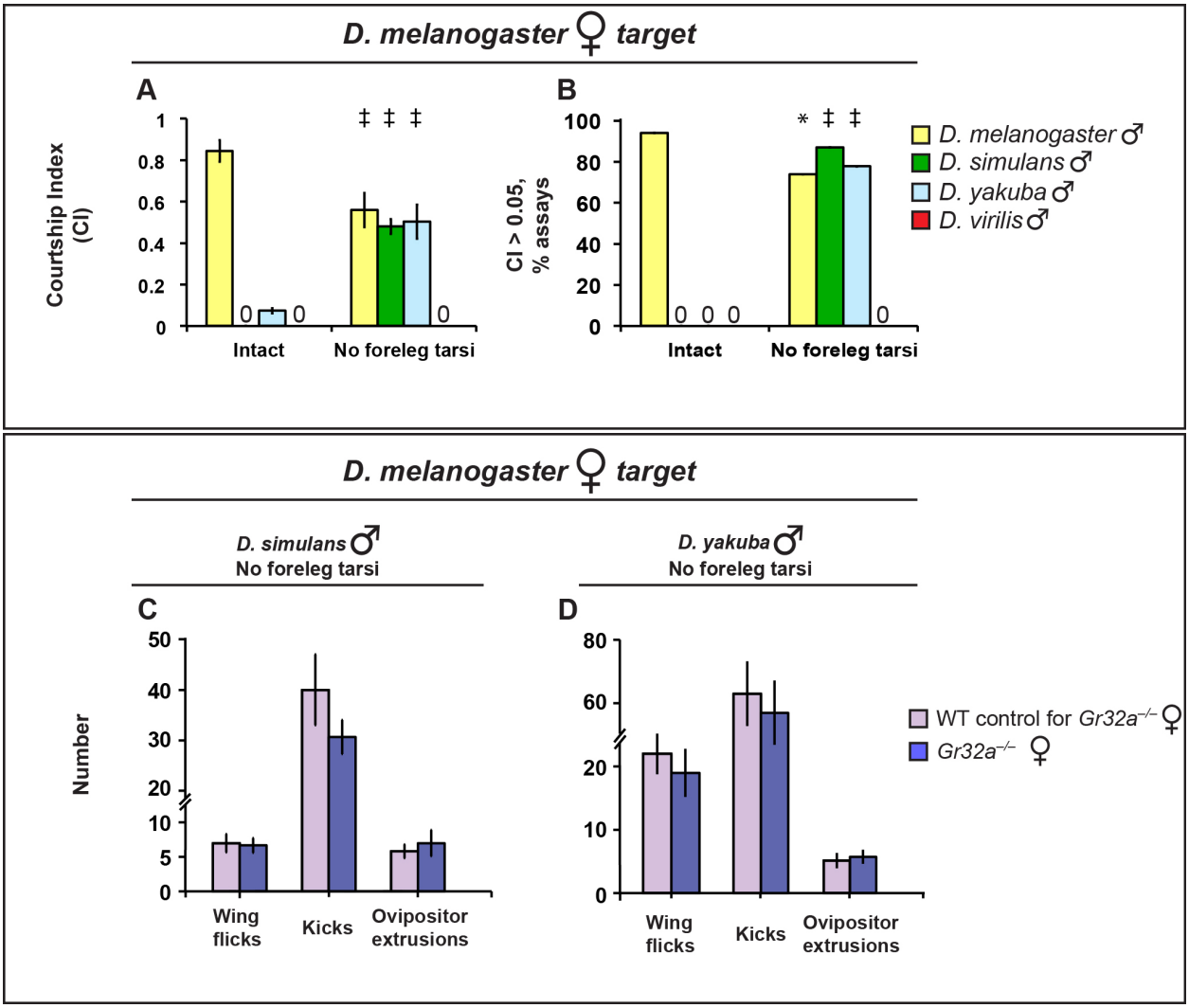

FIGURE 2.11: Sexually Dimorphic but Evolutionarily Conserved Regulation of Interspecies Courtship.

(A-B) Tarsiless *D. simulans* and *yakuba* males court *D. melanogaster* females similar to conspecific males. **(C)** *D. melanogaster* females reject courtship by *D. simulans* males with wing flicks, kicks, and ovipositor extrusions. **(D)** *D. melanogaster* females reject courtship by *D. yakuba* males with wing flicks, kicks, and ovipositor extrusions. Mean ± SEM; n = 11 - 18/experimental cohort; *p < 0.05; ‡p < 0.001.

DISCUSSION

Mythological assertions notwithstanding, animals rarely pick mates from other species (Ovid, 2009). The 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 2.8E-I). 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, 2012a, 2012b; 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, 2008; 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 males null for 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 intuits 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 sen- sory 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 court- ship 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.

Expressed in foreleg tarsi	Not expressed in foreleg tarsi
Gr22c	Gr2a
Gr28a	Gr9a
Gr28b.d	Gr22f
Gr28b.e	Gr36a
Gr32a	Gr36c
Gr33a	Gr47a
Gr39a.a	Gr57a
Gr39a.d	Gr58b
Gr64f	Gr77a
Gr68a	Gr92a

TABLE 2.1: Screen for Grs expressed in *D. melanogaster* male foreleg tarsi.

	UAS-stingerGFP	*UAS-hid, UAS-stingerGFP*
Gr22c-GAL4	3.1 ± 0.7	0.4 ± 0.3
Gr28b.d-GAL4	2.5 ± 0.2	0.3 ± 0.2
Gr28b.e-GAL4	1.5 ± 0.2	0.0 ± 0.0
Gr32a-GAL4	5.7 ± 0.2	1.5 ± 0.3
Gr33a-GAL4	5.4 ± 0.4	1.5 ± 0.3
Gr39a.a-GAL4	6.1 ± 0.3	0.6 ± 0.3
Gr39a.d-GAL4	1.0 ± 0.0	0.3 ± 0.2
Gr64f-GAL4	12.0 ± 0.3	1.0 ± 0.3

TABLE 2.2: Ablation of Gr-expressing cells in *D. melanogaster* male foreleg tarsi.

Target female	% *P52A:fru^{M}IR* males tapping prior to singing
D. melanogaster	7.7
D. simulans	100‡
D. virilis	100‡
D. yakuba	61.5‡

A higher percent of *P52A:fru^{M}IR* males tap prior to singing to *D. simulans, virilis*, and *yakuba* females compared to conspecific females.

n = 16-26/genotype; ‡ $p < 0.001$ compared to conspecific females.

TABLE 2.3: *P52A:fru^{M}IR* males tap females from other species prior to singing.

CHAPTER THREE

EVOLUTION OF CHEMOSENSORY MECHANISMS THAT CONTROL MATING IN *DROSOPHILA* MALES

ABSTRACT

Genetically wired neural mechanisms inhibit mating between species such that even naïve animals rarely mate with individuals of other species. These mechanisms must evolve rapidly in order to enforce behavioral reproductive isolation between closely related species that inhabit the same ecological niche. In order to inhibit such interspecies mating, evolutionary forces may modify peripheral recognitive mechanisms or more central neural circuits that process sensory inputs. Such selective pressures could be exerted on DNA sequences that regulate expression of critical genes or encode products of these genes. Here we have examined whether Gr32a, a chemoreceptor that inhibits interspecies courtship by *D. melanogaster* males, also underlies behavioral reproductive isolation in the closely related *D. simulans*. We show that similar to its *D. melanogaster* counterpart, *D. simulans* Gr32a is expressed in neurons of the foreleg tarsi, a sensory organ that is required to inhibit interspecies courtship by males of both species. Nevertheless, Gr32a is not required to inhibit *D. simulans* males from courting other species. In contrast to this divergence in function in courtship, we find that, similar to its role in *D. melanogaster*, Gr32a is required to sense the aversive tastant quinine in feeding assays. We further show that this evolution of Gr32a function in inhibiting interspecies mating does not reflect a complete reorganization of the genetic pathways underlying male courtship. Taken together, our findings reveal separable evolutionary mechanisms working on peripheral chemosensory pathways to promote conspecific and inhibit interspecific courtship.

INTRODUCTION

A species can be defined operationally as a set of organisms that share a gene pool and successfully breed with each other (Darwin, 1860; Dobzhansky, 1937; Mayr, 1988). An important tenet of evolutionary theory holds that species do not interbreed, thereby preserving the advantages conferred by allele combinations that occur in conspecific gene pools (Mayr, 1988; Mayr and Dobzhansky, 1945; Orr, 2005; Orr et al., 2004). This immediately suggests that mechanisms that preclude interbreeding must evolve so as to enforce reproductive isolation between species (Coyne and Orr, 1997; Mendelson, 2003). Given that even individuals from closely related species rarely attempt to mate with each other, the genetically-wired neural pathways underlying behavioral reproductive isolation must evolve rapidly. How such neural pathways evolve is poorly understood.

Drosophilids provide a facile model to understand how neural pathways that inhibit interspecies courtship have evolved. These flies are members of an old lineage consisting of multiple species, many of which co-exist in overlapping habitats (Jezovit et al., 2017; Markow, 2015). They engage in well-described, species-typical stereotyped courtship rituals, and many components of the genetic and neural networks that promote the courtship ritual of *D. melanogaster*, the most intensively studied drosophilid, are well defined (Bastock and Manning, 1955; Clowney et al., 2015; Demir and Dickson, 2005; Gill, 1963; Greenspan and Ferveur, 2000; Hall, 1978, 1994; Hotta and Benzer, 1976; Kallman et al., 2015; Kohatsu et al., 2011; Lin et al., 2016; Manoli et al., 2005; Pavlou and Goodwin, 2013; Ryner et al., 1996; Spieth, 1952; Thistle et al., 2012). Moreover, we have previously identified a sensory pathway expressing Gr32a

that is necessary to suppress interspecies courtship by *D. melanogaster* males (Fan et al., 2013). Gr32a, a gustatory chemoreceptor, is required to recognize cuticular hydrocarbons on non-*melanogaster* drosophilids and to inhibit interspecies mating. Strikingly, Gr32a is also necessary to inhibit courtship displays toward the closely related *D. simulans* that last shared an ancestor with *D. melanogaster* ~3-5 million years ago (mya). *D. simulans* and *melanogaster* are cosmopolitan species that co-exist in shared habitats around the world (reviewed in Jezovit et al., 2017). These two species are very similar in appearance and were only identified as distinct species upon close examination of male genitalia and the observation that they rarely courted each other (Sturtevant, 1919, 1920). Here we have examined how the Gr32a chemosensory pathway has evolved to inhibit interspecies courtship in *D. simulans*.

RESULTS

The chemosensory pathway that inhibits interspecies courtship is conserved

D. melanogaster males exhibit a highly ritualized courtship sequence such that, having oriented head first to a potential mate, they tap it with their foreleg tarsi. This tapping restricts subsequent steps of courtship to conspecific individuals because *D. melanogaster* males lacking foreleg tarsi continue to court conspecifics as well as individuals from other drosophilid species (Fan et al., 2013; Manning, 1959). *D. simulans* males also use foreleg tarsi to tap potential mates early in courtship, and we therefore tested whether these sensory appendages serve a similar function in *D. simulans* males (Figure 3.1A). We surgically ablated foreleg tarsi in adult *D. simulans* males and tested them for courtship toward conspecifics and individuals of other

species. We observed that male *D. simulans* lacking foreleg tarsi courted closely (*D. melanogaster*) and distantly (*D. pseudoobscura*, shared last common ancestor ~26 mya; *D. virilis*, shared last common ancestor ~40 mya) related drosophilids as well as male conspecifics (Figures 3.1B-E, 3.2A, 3.2D-E). Importantly, *D. simulans* males lacking foreleg tarsi, like their *D. melanogaster* counterparts (Fan et al., 2013), also courted conspecific females (Figures 3.1B, 3.1C). The lower courtship intensity exhibited by tarsiless males of either species toward conspecific females likely reflects a diminution in their ability to pursue the female effectively because of impaired locomotion or loss of neurons that detect attractive pheromones and promote courtship. Regardless, the behavior of tarsiless *D. simulans* males was in contrast to intact males who exhibited very low levels of courtship toward other species and conspecific males.

The hydrocarbon 7-tricosene is present on the cuticle of *D. simulans* but not *D. melanogaster* females, and it functions as an aphrodisiac and repulsive chemosensory cue for males of these two species, respectively (Billeter et al., 2009; Ferveur, 2005; Lacaille et al., 2007; Wang et al., 2011). Consistent with this notion, wildtype (WT) *D. melanogaster* males also do not court flies whose cuticle is experimentally or naturally enriched in 7-tricosene (Billeter et al., 2009; Fan et al., 2013). In addition, WT *D. simulans* courted *D. yakuba* females, whose cuticle is enriched in 7-tricosene, albeit with significantly lower intensity compared to conspecific females (Figures 3.1B, 3.2B; p-value < 0.001; n = 20 - 22 males/cohort). Removal of foreleg tarsi in the males did not further increase courtship toward *D. yakuba* females (Figures 3.2B, 3.2C). Our findings therefore suggest that multiple pathways exist in *D. simulans* to inhibit interspecies courtship toward other drosophilids. Nevertheless, and similar to *D.*

melanogaster males, severing foreleg tarsi of *D. simulans* males disinhibits courtship toward multiple reproductively futile targets without abolishing courtship with conspecific females.

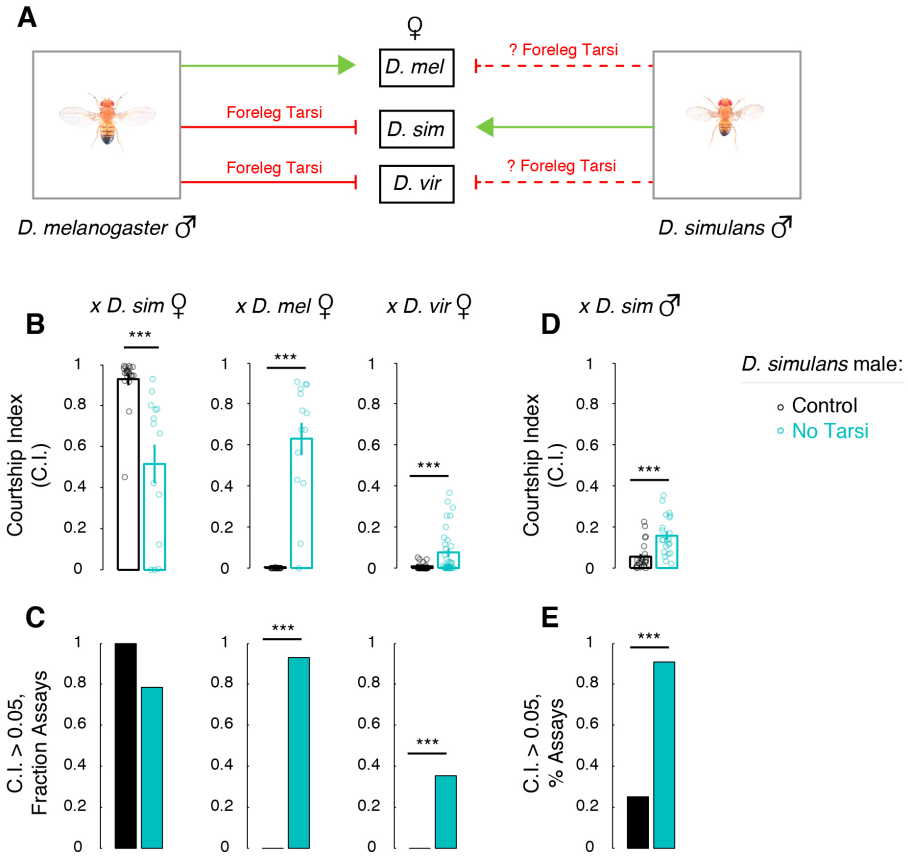

FIGURE 3.1: *D. simulans* male foreleg tarsi inhibit courtship of other species and are not essential for courtship of conspecific females.

(A) We tested whether, similar to *D. melanogaster* males, foreleg tarsi also inhibited interspecies courtship by *D. simulans* males. **(B)** *D. simulans* males lacking foreleg tarsi court conspecific, *D. melanogaster*, and *D. virilis* females. **(C)** *D. simulans* males lacking foreleg tarsi are more likely to show intense courtship toward *D. melanogaster* and *D. virilis* females. **(D)** *D. simulans* males lacking foreleg tarsi court conspecific males more intensely. **(E)** *D. simulans* males lacking foreleg tarsi are more likely to show intense courtship toward conspecific males. Mean ± SEM; CI = fraction time spent courting target fly; each circle denotes CI of one male; n = 14 - 41/cohort; ***p<0.001.

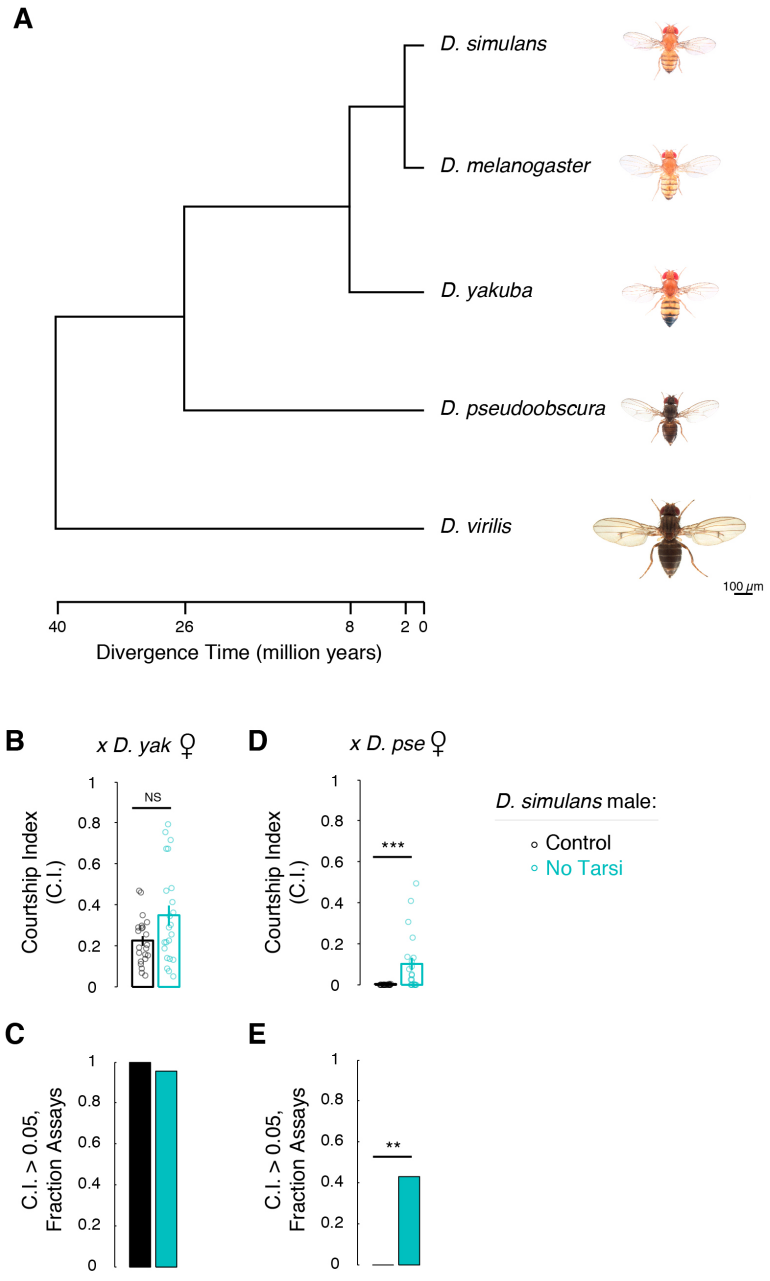

FIGURE 3.2: *D. simulans* foreleg tarsi inhibit courtship toward other drosophilids.

(A) Evolutionary relationship of the five *Drosophila* species used in this study. Females of each species shown to scale and with representative pigmentation pattern. **(B-E)** Foreleg tarsi inhibit *D. simulans* males from courting *D. pseudoobscura* but not *D. yakuba* females. Mean ± SEM; each circle denotes CI of a *D. simulans* male; n = 19 - 23/cohort; ***p<0.001.

Gr32a expression is conserved in *D. simulans* foreleg tarsi

Foreleg tarsi contain chemosensory neurons that presumably detect contact-dependent cues during tapping such that detection of non-conspecific cues inhibits subsequent steps in courtship. Indeed, the chemoreceptor-encoding gene *Gr32a* is expressed in specific sensory neurons in distal foreleg tarsi of *D. melanogaster* (Koganezawa et al., 2010; Miyamoto and Amrein, 2008; Moon et al., 2009; Scott et al., 2001; Thistle et al., 2012; Thorne et al., 2004), and it is required to inhibit interspecies courtship (Fan et al., 2013) (Figure 3.3A). Moreover, *Gr32a*-expressing neurons are functionally necessary and sufficient to inhibit interspecies courtship displays. Given that the genome of *D. simulans* also encodes *Gr32a* (Clark et al., 2007), we wondered whether this gene is expressed in foreleg tarsi of this species. The Gr gene family members are transcribed at very low levels (Clyne et al., 2000; Dunipace et al., 2001; Moon et al., 2009; Scott et al., 2001), and previous efforts have utilized cognate upstream promoter regions to visualize expression of Gr32a using the Gal4/UAS system (Park and Kwon, 2011; Scott et al., 2001; Wang et al., 2004; Weiss et al., 2011). These studies have shown that ~3.8 kb of *D. melanogaster* genomic DNA immediately upstream of the start codon is sufficient to drive reporter expression in subsets of neurons in chemosensory organs known to express Gr32a (Wang et al., 2004). Similar stretches of genomic DNA immediately upstream of the start codon are also sufficient to drive reporter expression of other Grs in various chemosensory neurons (Weiss et al., 2011), indicating a conserved regulatory logic of expression for this gene family in *D. melanogaster*. Accordingly, we subcloned ~3.8 kb genomic DNA upstream of the *D. simulans* Gr32a start codon and used it to drive GAL4 expression (Gr32a^{sim}-GAL4) in transgenic *D. simulans* and *D. melanogaster* flies (Pfeiffer et al., 2010; Stern et al., 2017) (Figure 3.3B). Transgene expression was

visualized in progeny bearing this allele as well as the fluorescent protein Citrine under control of UAS (Inagaki et al., 2014) (Figures 3.3C, 3.3D). We observed Citrine expression in a small subset of neurons in distal tarsal segments (T4, T5) of *D. simulans* and *D. melanogaster*, demonstrating therefore that regulatory sequences in the *D. simulans Gr23a* locus drive reporter expression in foreleg tarsi of both species (Figures 3.3C, 3.3D, 3.3G).

The expression pattern of Gr32a^{sim}-GAL4 was strikingly reminiscent of reporter expression for *D. melanogaster* Gr32a in *D. melanogaster* flies. We next wanted to test whether the ~3.8 kb regulatory DNA sequence from these two species drives expression in the same tarsal neurons. We therefore generated *D. melanogaster* flies harboring GAL4 under control of conspecific ~3.8 kb DNA sequence 5' of Gr32a such that this transgene (Gr32a^{mel}-GAL4) was inserted into the same landing site that we had utilized for Gr32a^{sim}-GAL4 (Figures 3.3B, 3.3D, 3.3F). This strategy ensured that transgenes from the two species were regulated by the identical genomic context flanking the insertion site. Importantly, Gr32a^{mel}-GAL4 regulated reporter expression in *D. melanogaster* foreleg tarsi (T4, T5) as described previously for other GAL4 alleles of Gr32a (Fan et al., 2013; Miyamoto and Amrein, 2008; Moon et al., 2009; Scott et al., 2001). In *D. melanogaster* flies bearing both Gr32a^{mel}-GAL4 and Gr32a^{sim}-GAL4, we observed a similar number of Citrine+ foreleg tarsal neurons compared to flies bearing each of these GAL4 drivers individually (Figure 3.4E). Together, these data are consistent with the notion that the upstream regulatory region of Gr32a in the two species is functionally conserved and sufficient to drive expression in the same foreleg tarsi neurons of *D. melanogaster*.

To test whether these regulatory features also function in *D. simulans*, we tested whether the ~3.8 kb genomic DNA upstream of *D. melanogaster* Gr32a would drive expression in foreleg tarsal neurons of *D. simulans*. Accordingly, we inserted Gr32a^{mel}-GAL4 into the identical landing site we used to generate *D. simulans* flies bearing Gr32a^{sim}-GAL4 (Figures 3.3B, 3.3E). As before, transgene expression was visualized in progeny bearing this allele and Citrine under control of UAS. We observed Citrine expression in 3-4 neurons restricted to distal tarsal segments (T4, T5) of *D. simulans* in a pattern mirroring that observed in *D. simulans* bearing Gr32a^{sim}-GAL4 (Figures 3.3C, 3.3E, 3.3G). Given that all transgenes we built in *D. simulans* were inserted into a single landing site that has afforded us reliable and non-leaky expression, we cannot directly test whether the same neurons were labeled by Gr32a^{sim}-GAL4 and Gr32a^{mel}-GAL4 in this species. Regardless, our findings strongly suggest that similar *cis* and *trans* regulatory features regulate Gr32a expression in foreleg tarsi of the two species. In agreement with this notion, comparison of this ~3.8 kb stretch of genomic DNA between these two species reveals >95% sequence identity (Figure 3.4A). More generally, this genomic DNA sequence is conserved across multiple drosophilids, and it also contains several phastCons elements, blocks of highly conserved sequence, across 27 insect species (Figure 3.4B). A test for non-neutral evolution of sequence in this stretch of DNA across multiple species also suggested that the vast majority of bases were conserved or indistinguishable from changes consequent to neutral evolution (>99% bases with phyloP score > -2) (Figures 3.4C, 3.4D). Taken together, our findings show that this ~3.8 kb region is functionally conserved in *D. melanogaster* and *D.*

simulans such that it is sufficient to drive expression in sensory neurons of foreleg tarsi, a structure that inhibits interspecies courtship by males of the two species.

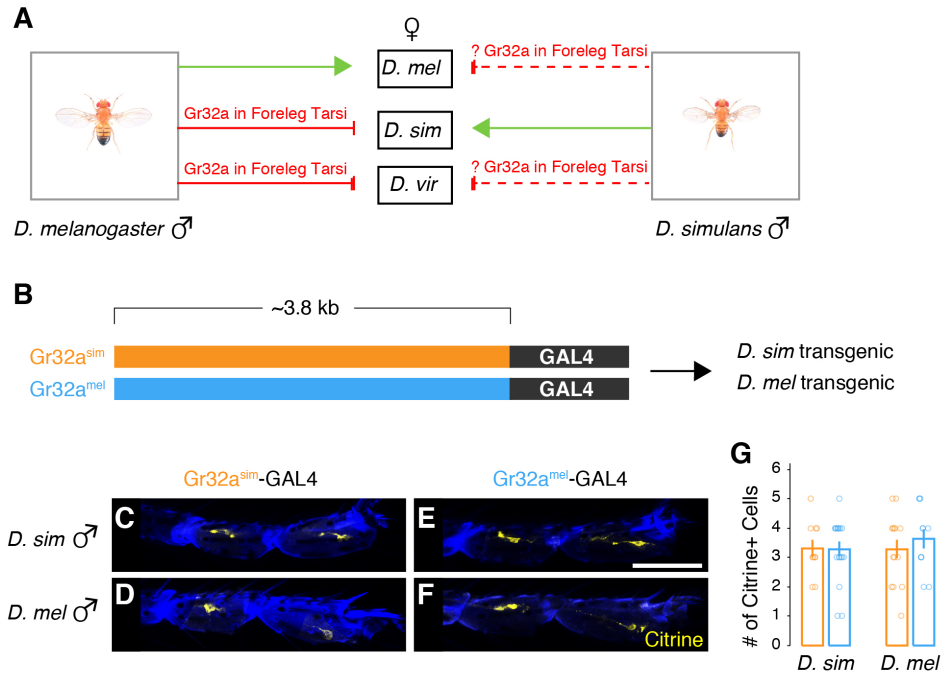

FIGURE 3.3: A regulatory region in the *Gr32a* locus is functionally conserved.

(A) We sought to determine whether, similar to *D. melanogaster*, Gr32a was expressed in *D. simulans* foreleg tarsi. **(B)** Schematic of transgenic constructs using a DNA sequence 5' of Gr32a start codon from *D. simulans* (orange) and *D. melanogaster* (blue) to drive GAL4 expression. **(C-F)** Gr32a^{sim}-GAL4 and Gr32a^{mel}-GAL4 each drive comparable citrine expression in distal tarsal segments T4 and T5 in both *D. simulans* and *D. melanogaster* male forelegs. **(G)** Quantification of data shown in histological panels (C-F). Mean ± SEM; each circle denotes number of Citrine+ cells per male foreleg tarsi per; n = 11 - 18/genotype; scale bar = 50 μm. See also Table 3.1.

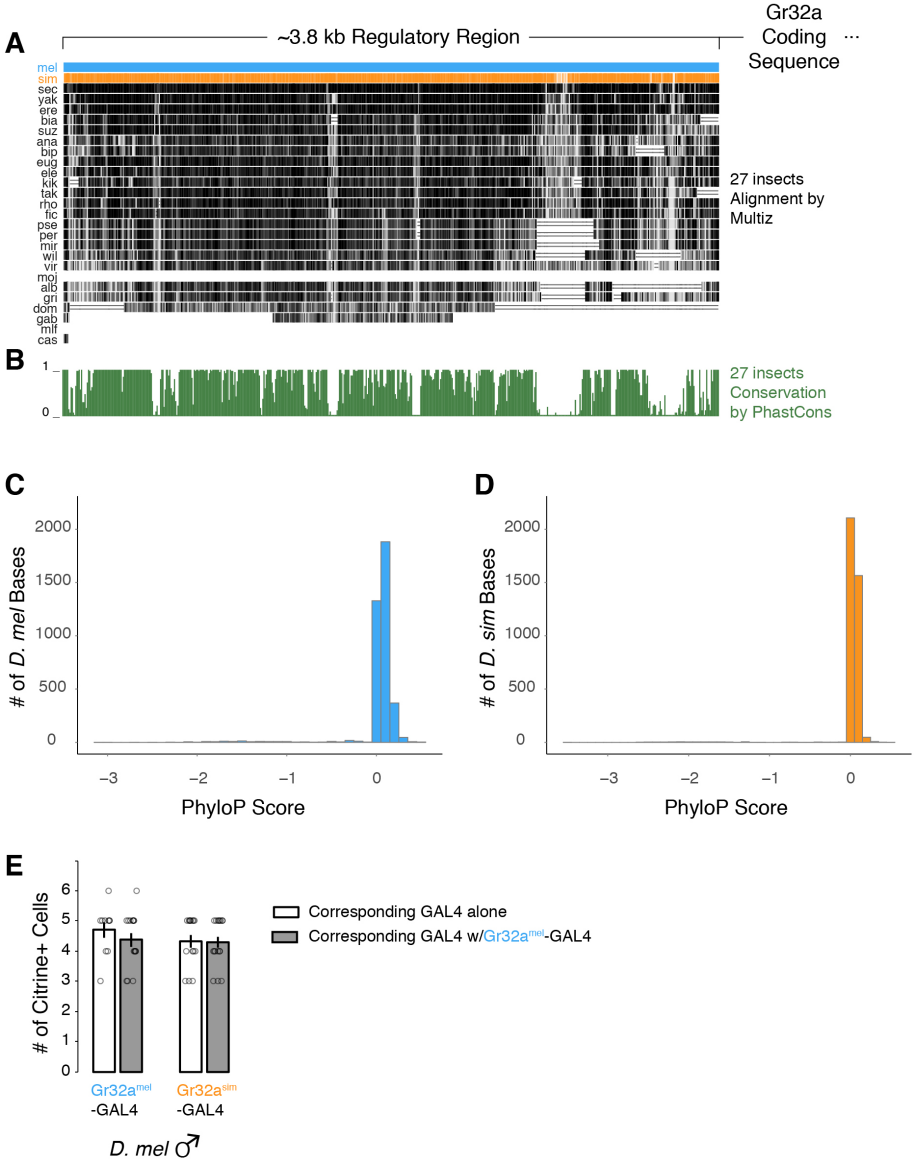

FIGURE 3.4: See next page for details.

FIGURE 3.4: A regulatory region upstream of *Gr32a* coding sequence is conserved across drosophilids.

(A) 27-insect alignment of the ~3.8 kb DNA element that drives *Gr32a* expression in *D. melanogaster* and *D. simulans*. mel, *D. melanogaster* (blue); sim, *D. simulans* (orange); sec, *D. sechellia*; yak, *D. yakuba*; ere, *D. erecta*; bia, *D. biarmipes*; suz, *D. suzukii*; ana, *D. ananassae*; bip, *D. bipectinata*; eug, *D. eugracilis*; ele, *D. elegans*; kik, *D. kikkawai*; tak, *D. takahashii*; rho, *D. rhopaloa*; fic, *D. ficusphila*; pse, *D. pseudoobscura*; per, *D. persimilis*; mir, *D. miranda*; wil, *D. willistoni*; vir, *D. virilis*; moj, *D. mojavensis*; alb, *D. albomicans*; gri, *D. grimshawi*; dom, *Musca domestica*; gab, *Anopheles gambiae*; mlf, *Apis mellifera*; cas, *Tribolium castaneum*. **(B)** PhastCons track showing conservation probabilities across the region in (A). Higher peaks indicate higher likelihood of bases being in a strongly conserved element. **(C, D)** Most bases in the ~3.8 kb regulatory region are likely conserved or neutrally evolving in *D. melanogaster* and *D. simulans*. PhyloP scores scale logarithmically in magnitude. Scores near 0 indicate neutral evolution. Large positive (> 2) or negative (< -2) phyloP scores indicate conservation or acceleration, respectively. **(E)** No difference in the number of Citrine+ cells in T4 and T5 foreleg segments of *D. melanogaster* males observed with either Gr32a^{mel}-GAL4 or Gr32a^{sim}-GAL4 alone or in combination. Mean ± SEM; each circle denotes Citrine+ cell count for a foreleg tarsum; n = 10 - 17/genotype.

Gr32a and Gr33a are not essential to inhibit interspecies courtship in *D. simulans* males

We next tested whether Gr32a was essential to inhibit interspecies courtship in *D. simulans* males (Figure 3.5A). We targeted the first coding exon of Gr32a in *D. simulans* via the CRISPR/Cas9 (Bassett and Liu, 2014; Bassett et al., 2013; Gokcezade et al., 2014) (Figure 3.5B, 3.6A). We used two guide RNAs targeting distinct sequences in this exon to generate three different *Gr32a* mutant alleles (Gokcezade et al., 2014) (Figure 3.5B, 3.6A). Two of the alleles ($Gr32a^{\Delta 10}$ and $Gr32a^{\Delta 26}$) are predicted to lead to small 10 and 26 bp deletions in the first coding exon that result in a frame-shift and premature stop codon; these likely encode a non-functional Gr32a chemoreceptor protein (Figures 3.5B, 3.5C). The third allele ($Gr32a^{\Delta 141}$) has a large 141 bp deletion that is predicted to eliminate 47 amino acids from the predicted N-terminal intracellular domain of this chemoreceptor (Figures 3.5B, 3.5C, 3.6A-D, 3.7A-B). We immediately back-crossed these mutant alleles into a WT background ≥5 times to remove any potential off-target mutations and subjected resulting progeny to further testing. We confirmed that these deletions were indeed present within the mRNAs transcribed from each of the three alleles *in vivo* in adult flies (Figure 3.6B). We next tested *D. simulans* males homozygous mutant for these three *Gr32a* alleles for courtship displays toward conspecifics and members of other species. We observed that each of these three mutants courted conspecific females at levels indistinguishable from WT controls (Figures 3.5D, 3.5E). Moreover, these mutants did not show significant increase in courtship toward conspecific males or *D. melanogaster, D. yakuba,* or *D. virilis* females (Figures 3.5D-G, 3.6E, 3.6F). The behavior of Gr32a mutant *D. simulans* males is in

sharp contrast to that of Gr32a null *D. melanogaster* males that court other species avidly (Fan et al., 2013).

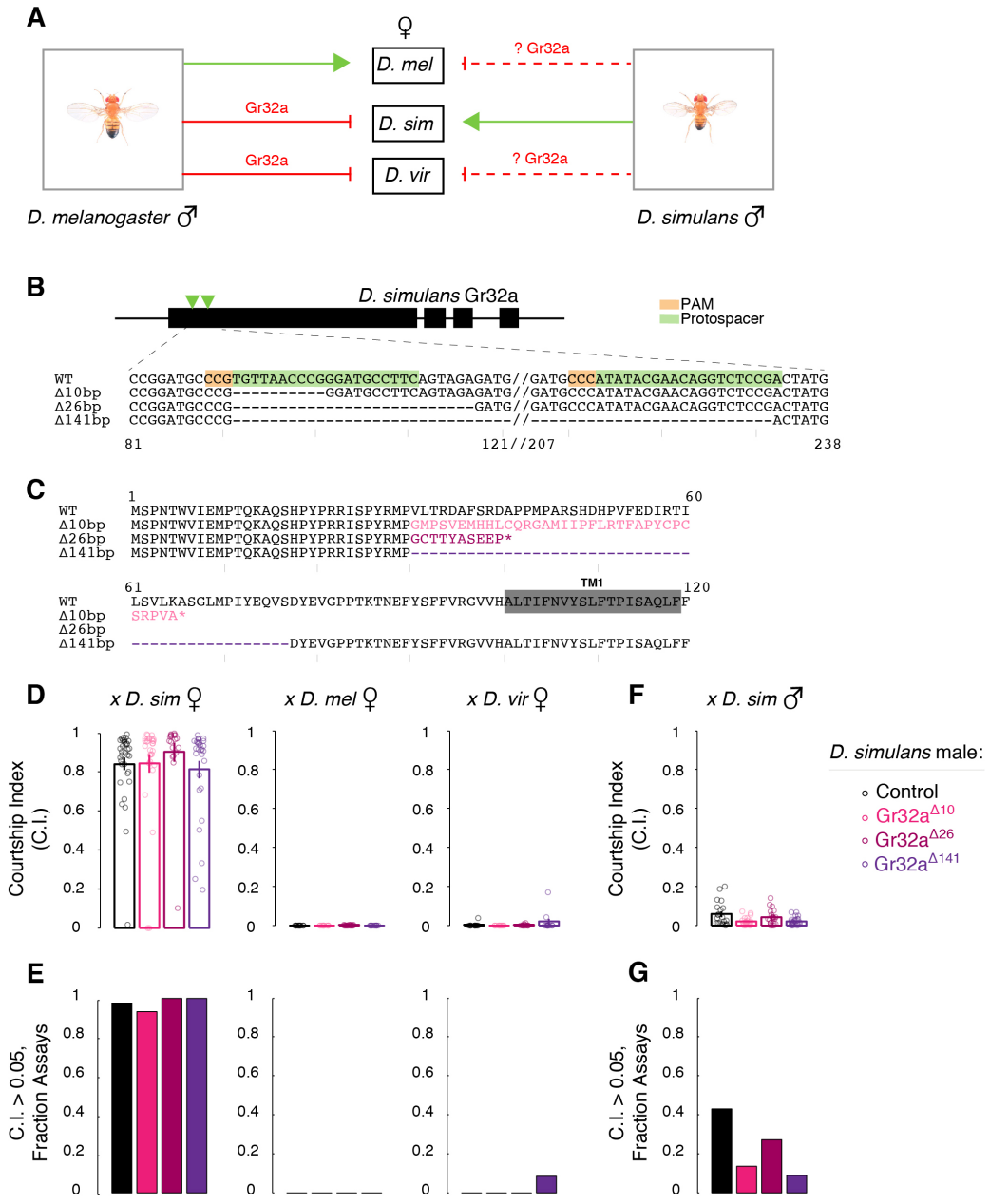

FIGURE 3.5: See next page for details.

FIGURE 3.5: Gr32a is not required to inhibit interspecies courtship behavior of *D. simulans* males.

(A) We tested whether, similar to *D. melanogaster* males, Gr32a inhibits interspecies courtship by *D. simulans* males. **(B)** Schematic of *D. simulans Gr32a* locus (top) and DNA sequence comparison of WT and mutant *Gr32a* alleles (bottom). Black rectangles, exons; green triangles, CRISPR target sites; PAM, Protospacer Adjacent Motif. **(C)** Predicted amino acid sequence of WT and mutant *D. simulans* Gr32a. The predicted first transmembrane domain (TM1) is highlighted in gray in the WT protein. *, premature stop codon. **(D, E)** WT and Gr32a mutant *D. simulans* males court conspecific but not *D. melanogaster* or *D. virilis* females. **(F, G)** WT and Gr32a mutant *D. simulans* males show similar low levels of courtship toward conspecific males. Mean ± SEM; each circle denotes CI of one male; n = 11 - 34/genotype. Please see Table 3.1.

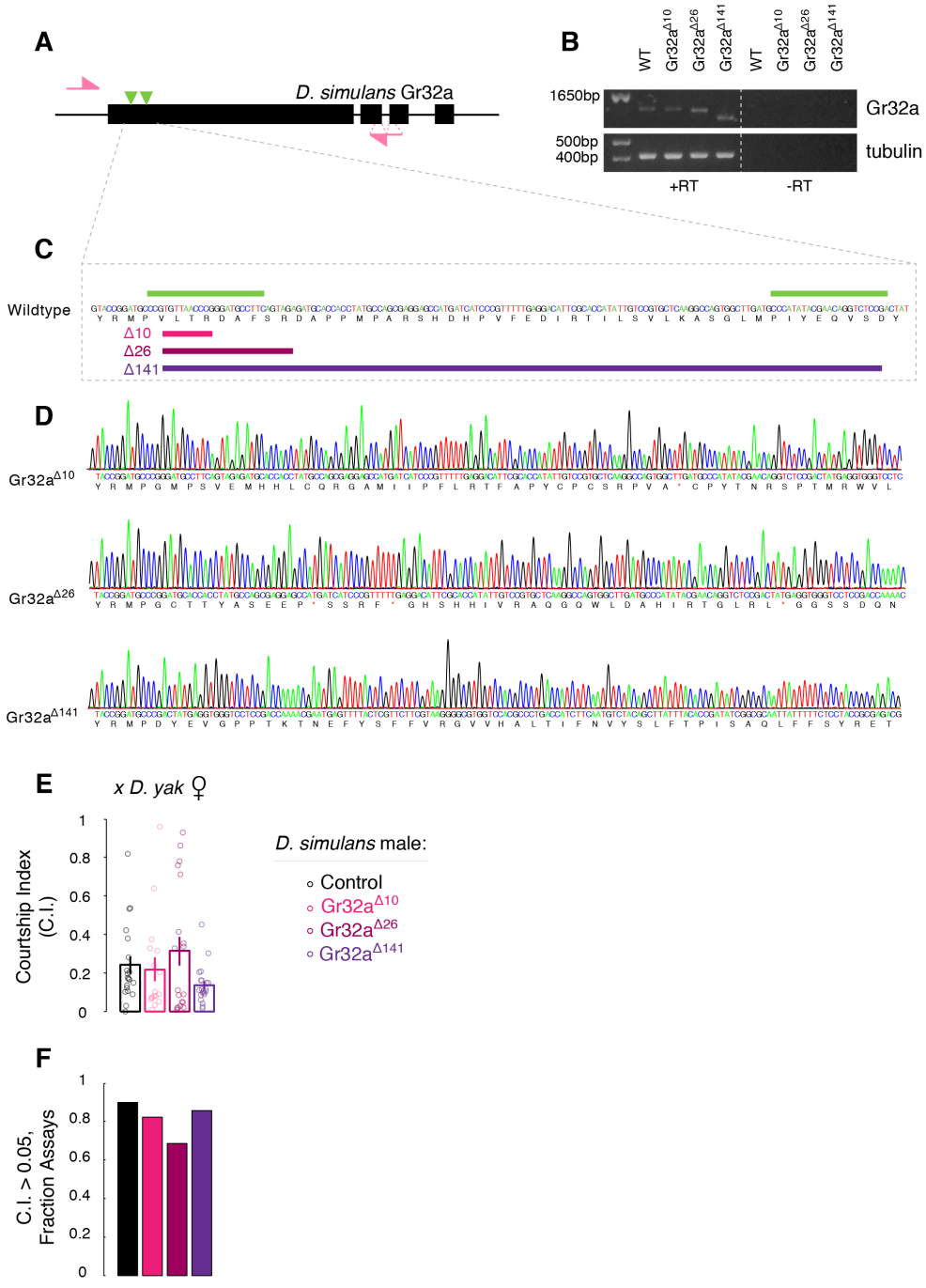

FIGURE 3.6: See next page for details.

FIGURE 3.6: Generating Gr32a mutant *D. simulans* via CRISPR/Cas9.

(A) Schematic of *D. simulans Gr32a* locus. Pink arrows, PCR primers; green triangles, CRISPR target sites; black rectangles, exons. **(B)** RT-PCR products for Gr32a and tubulin in WT and *Gr32a* mutant *D. simulans*, using PCR primers shown in (A). No DNA bands seen in –RT (No RT) control. DNA ladder shown in first lane (left panels). **(C)** WT sequence of *D. simulans Gr32a* encompassing CRISPR (gRNA) targeted sequences (green bar). The 10 bp, 26 bp, and 141 bp deletions of the three different Gr32a mutant alleles are depicted by colored bars below the WT sequence. **(D)** Electropherograms of DNA products of RT-PCR (as in B) from each of the three mutant alleles of *D. simulans Gr32a*. Only sequence immediately flanking the deletions is shown. **(E, F)** No difference in courtship of *D. yakuba* females by WT and *Gr32a* mutant *D. simulans* males. Mean ± SEM; each circle represents CI of a male; n = 17 - 21/genotype (E, F).

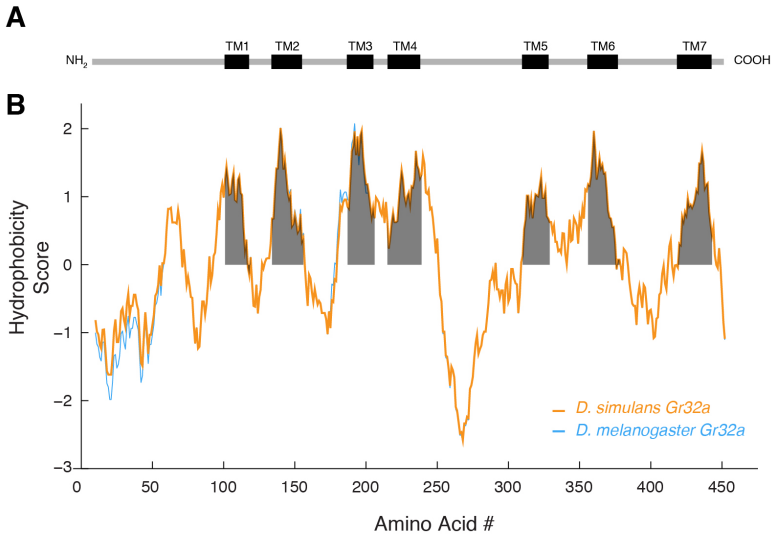

FIGURE 3.7: Hydrophobicity plot for Gr32a.

(A) Predicted location of the seven transmembrane domains (black rectangles) in Gr32a based on plot shown in (B). The NH_2 terminal is predicted to be intracellular. **(B)** Hydrophobicity plot of *D. simulans* and *D. melanogaster* Gr32a. Predicted transmembrane domains are shown by gray shading. Please see Table 3.2.

Gr33a is co-expressed with Gr32a in foreleg tarsi in *D. melanogaster*, and it is required to inhibit intermale but not interspecies courtship in males of this species (Fan et al., 2013; Moon et al., 2009). Gr33a is also encoded in the *D. simulans* genome (Clark et al., 2007), and we wondered if this chemoreceptor had evolved to inhibit interspecies courtship in this species. We tested this hypothesis by using CRISPR to generate two distinct alleles of *Gr33a*, one of which has a 10 bp deletion ($Gr33a^{\Delta 10}$) that leads to a frame-shift and premature stop codon whereas the second has a large in-frame deletion (96 bp, $Gr33a^{\Delta 96}$) (Figures 3.8A, 3.9A-D). As before, we also back-crossed these mutants ≥5 times into WT background to remove potential mutations resulting from off-target events. We confirmed the presence of these deletions within endogenously transcribed message in adult flies (Figure 3.9B). In behavioral testing, male *D. simulans* mutant for each of these alleles courted conspecific females similar to WT controls, but they did not display significant courtship toward conspecific males or *D. melanogaster, D. yakuba,* or *D. virilis* females (Figures 3.8B-E, 3.10A, 3.10B). Taken together, our results indicate that chemosensory receptor-mediated inhibition of courtship toward reproductively futile targets – conspecific males and members of other species – has diverged between the closely related *D. melanogaster* and *D. simulans*.

In light of these findings, we wondered whether genetic loci that promote courtship had also undergone similar evolutionary changes between these two species. Many distinct loci have previously been shown to promote courtship of *D. melanogaster* males toward conspecific females (Dickson, 2008; Yamamoto and Koganezawa, 2013). We chose to test the function of the Ppk25 Pickpocket ion channel that is expressed in

foreleg tarsi chemosensory neurons and appears to exclusively promote courtship in *D. melanogaster* (Kallman et al., 2015; Lin et al., 2005; Starostina et al., 2012; Vijayan et al., 2014). We generated two distinct alleles of Ppk25 in *D. simulans* via CRISPR/Cas9, one of which has a 2 bp insertion and the other has a 4 bp deletion in the first coding exon (Figures 3.8F, 3.11A-C). Both alleles are predicted to lead to frame-shifts and premature stop codons, and are likely therefore to encode null alleles of this gene (Figure 3.11D). Subsequent to ≥5 back-crosses to minimize CRISPR-generated off-target mutations in the background, we tested males mutant for each allele in courtship assays. *D. melanogaster* males, similar to *D. simulans* males, use multiple cues to initiate courtship with conspecifics, and *D. melanogaster* Ppk25 is required for male courtship in the dark (Boll and Noll, 2002; Jezovit et al., 2017; Kohatsu and Yamamoto, 2015; Krstic et al., 2009; Lin et al., 2005; Spieth, 1974). Unlike *D. melanogaster*, *D. simulans* males exhibit high levels of conspecific courtship only under bright illumination (Grossfield, 1971; Jezovit et al., 2017) (Figures 3.12A-F). We therefore tested whether Ppk25 modulated courtship by *D. simulans* males in bright light or dark conditions. In keeping with a role for this gene in promoting courtship, we observed that *D. simulans* males mutant for Ppk25 showed dramatically reduced courtship of conspecific females compared to WT control males in the dark (Figures 3.8G, 3.8H). These mutants also showed subtle, but significant, diminution in courtship under bright illumination, suggesting a more general requirement for Ppk25 in this behavior (Figures 3.8I, 3.8J). Together, our findings show that some but not all pathways that regulate courtship have functionally diverged between *D. melanogaster* and *D. simulans*.

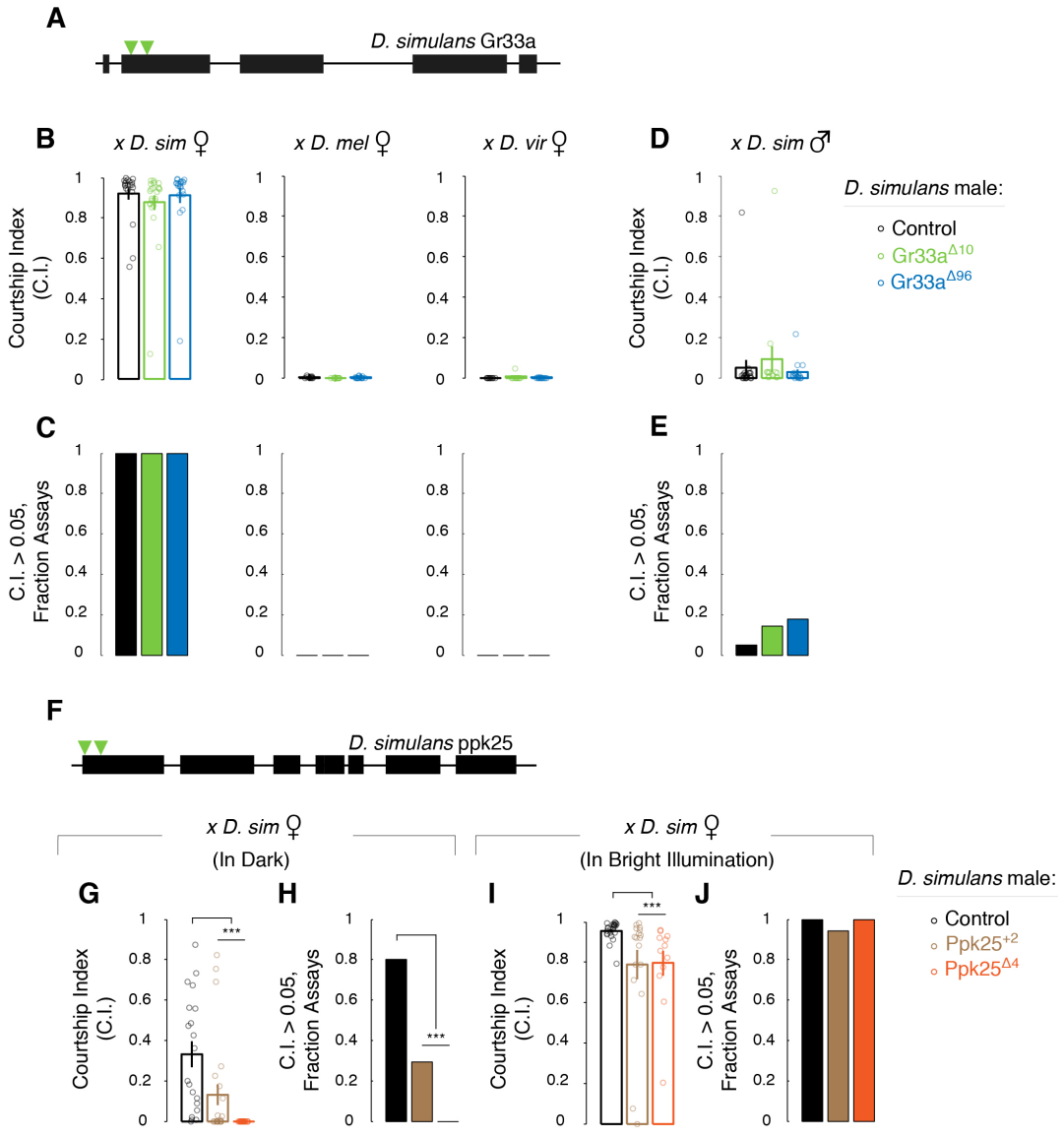

FIGURE 3.8: See next page for details.

FIGURE 3.8: *Ppk25* but not *Gr33a* regulates courtship in *D. simulans* males.

(A) Schematic of *D. simulans Gr33a* locus with CRISPR target sites (green triangles). Black rectangles, exons. **(B, C)** WT and *Gr33a* mutant *D. simulans* males court conspecific but not *D. melanogaster* or *D. virilis* females. **(D, E)** WT and Gr33a mutant *D. simulans* males show similar low levels of courtship toward conspecific males. **(F)** Schematic of *D. simulans Ppk25* locus with CRISPR target sites (green triangles). Black rectangles, exons. **(G-I)** *Ppk25* mutant *D. simulans* males show decreased courtship toward conspecific females. **(J)** No difference between WT and *Ppk25* mutant *D. simulans* males in percent assays with high levels of courtship of conspecific females. Mean ± SEM; each circle denotes CI for one male; n = 10 - 24/genotype; ***p<0.001. Please see Table 3.1.

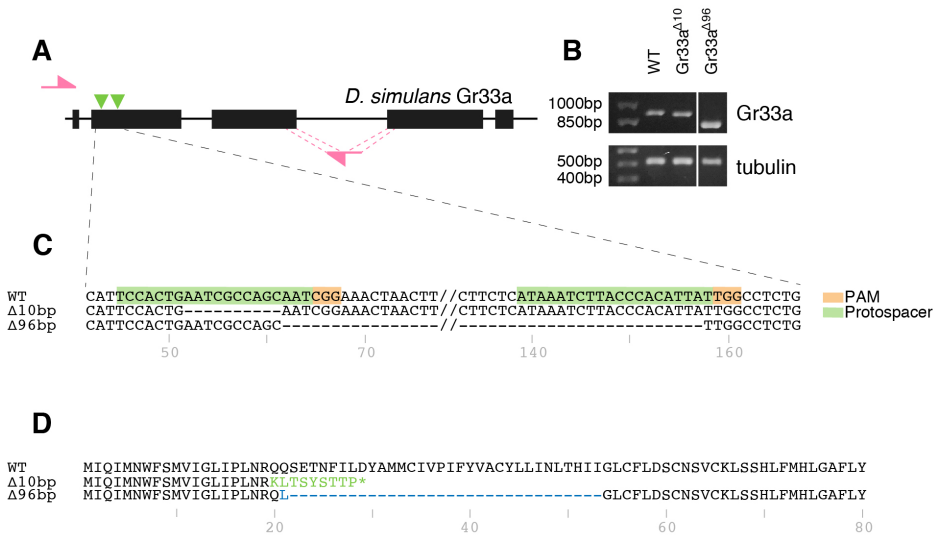

FIGURE 3.9: Generating Gr33a mutant *D. simulans* via CRISPR/Cas9.

(A) Schematic of *D. simulans Gr33a* locus. Pink arrows, PCR primers; green triangles, CRISPR target sites; black rectangles, exons. **(B)** RT-PCR products for Gr33a and tubulin in WT and *Gr33a* mutant *D. simulans*, using PCR primers shown in (A). No DNA bands seen in –RT (No RT) control. DNA ladder shown in first lane (left panels). **(C)** DNA sequence comparison of WT and mutant *Gr33a* alleles. **(D)** Predicted amino acid sequence of WT and mutant *D. simulans* Gr33a. *, premature stop codon.

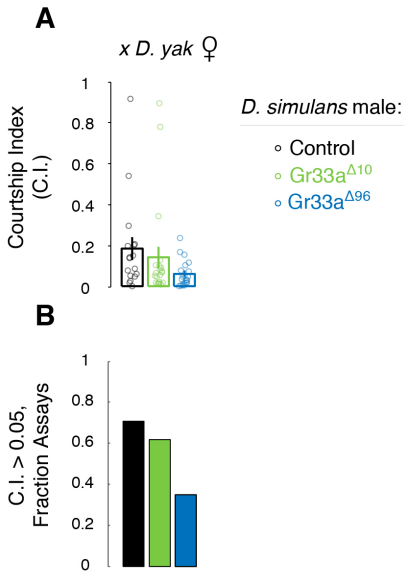

FIGURE 3.10: Gr33a does not control courtship toward *D. yakuba* females.

(A, B) WT and *Gr33a* mutant *D. simulans* males court *D. yakuba* females at comparable levels. Mean ± SEM; each circle denotes CI for one male; n = 17 – 22/genotype.

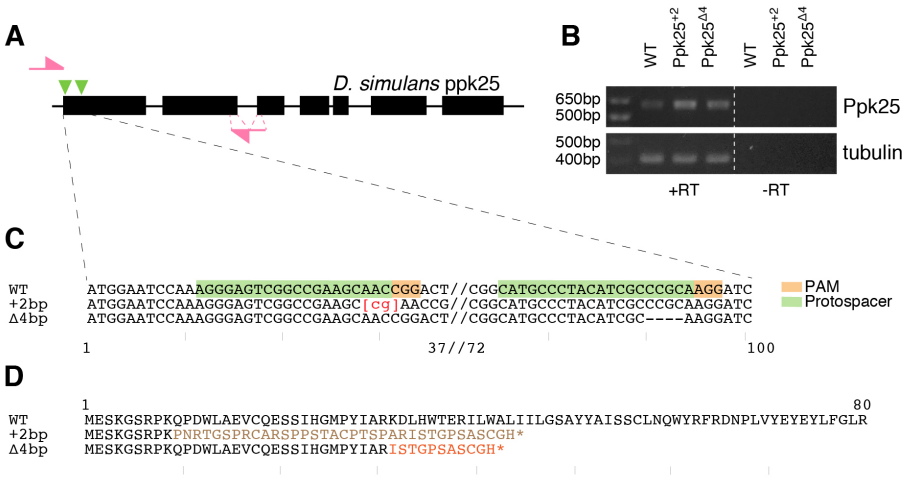

FIGURE 3.11: Generating *Ppk25* mutant *D. simulans* via CRISPR/Cas9.

(A) Schematic of *D. simulans Ppk25* locus. Pink arrows, PCR primers; green triangles, CRISPR target sites; black rectangles, exons. **(B)** RT-PCR products for Ppk25 and tubulin in WT and *Ppk25* mutant *D. simulans*, using PCR primers shown in (A). No DNA bands seen in –RT (No RT) control. DNA ladder shown in first lane (left panels). **(C)** DNA sequence comparison of WT and mutant *Ppk25* alleles. **(D)** Predicted amino acid sequence of WT and mutant *D. simulans* Ppk25. *, premature stop codon.

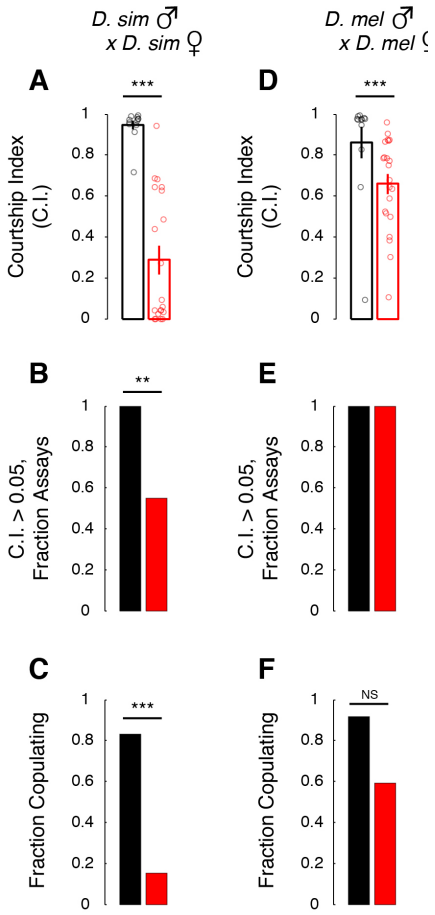

FIGURE 3.12: Light promotes conspecific courtship by *D. simulans* and *D. melanogaster* males.

(A, D) *D. simulans* and *D. melanogaster* males court conspecific females less intensely under red light-only illumination. **(B, E)** Fewer *D. simulans*, but not *D. melanogaster*, males court conspecific females intensely under red light-only illumination. **(C, F)** Fewer *D. simulans* males copulate under red light-only illumination whereas there is no difference in fraction *D. melanogaster* males copulating under red light-only or fluorescent illumination. Mean ± SEM; each circle denotes CI for one male; n = 12 - 22/cohort; ***p<0.01; ***p<0.001.

Both Gr32a and Gr33a are required functionally in *D. simulans* to detect the aversive tastant quinine

In addition to inhibiting courtship of reproductively futile targets by *D. melanogaster* males, Gr32a and Gr33a are essential for chemosensory neuronal responses to quinine as well as a behavioral aversion to this bitter tastant (Lee et al., 2010; Moon et al., 2009). Given that the *D. simulans* orthologs of these chemoreceptors do not appear to inhibit such courtship, we wondered if they were required for the aversive response to quinine (Figure 3.13A). We tested this by performing a feeding preference assay in which starved flies were offered the choice to feed on food containing low concentration of sugar (1 mM sucrose) or high concentration of sugar (5 mM sucrose) spiked with quinine (0.5 mM) (Montell, 2009; Tanimura et al., 1988) (Figure 3.13B). In contrast to WT *D. simulans* that strongly preferred feeding on the low concentration of sugar, flies mutant for either Gr32a or Gr33a showed no preference between these two food options (Figures 3.13.C, 3.13D). In fact, flies homozygous for the largest deletions ($Gr32a^{\Delta 141}$ and $Gr33a^{\Delta 96}$) even showed a slight preference for food containing higher concentration of sugar spiked with quinine. Our findings demonstrate that function of Gr32a and Gr33a in sensing quinine is conserved between *D. melanogaster* and *D. simulans*.

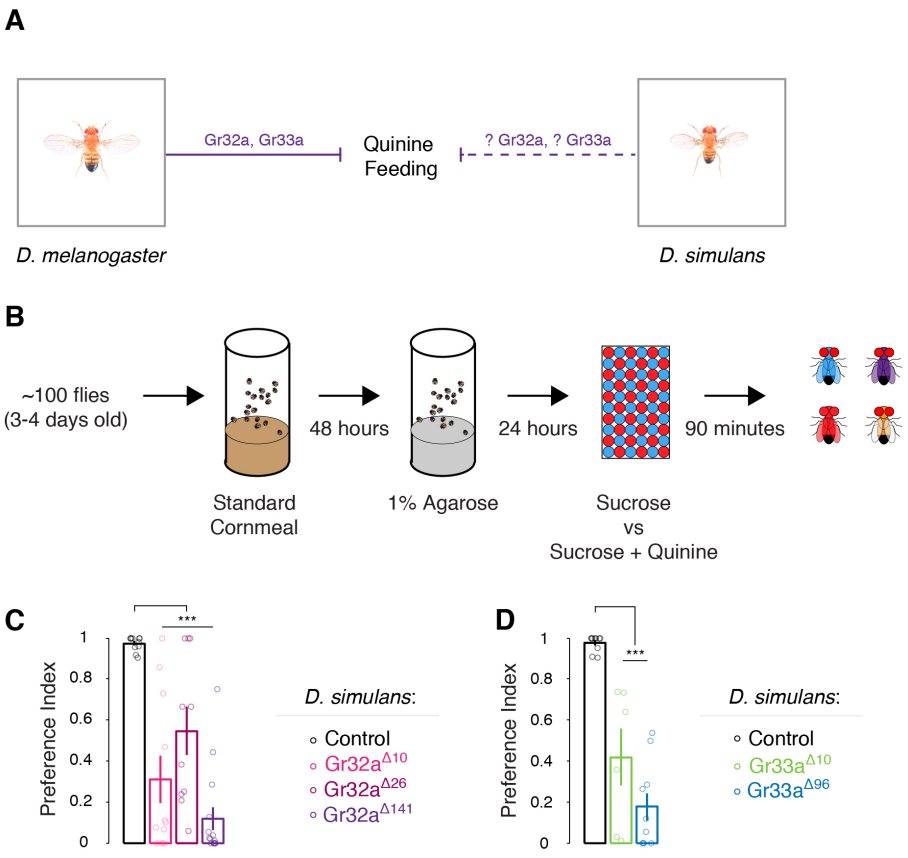

FIGURE 3.13: *Gr32a* and *Gr33a* inhibit *D. simulans* from feeding on quinine.

(A) We tested whether, similar to *D. melanogaster*, Gr32a and Gr33a inhibit feeding on quinine-containing food in *D. simulans*. **(B)** Schematic of feeding assay for starved *D. simulans* given choice of colored food containing sucrose or sucrose and quinine. Flies with blue, red, purple, or no food dye colored abdomens were enumerated after exposure to food for 90 min. **(C)** Significant decrease in preference by *Gr32a* mutant *D. simulans* for food containing only sucrose. **(D)** Significant decrease in preference by *Gr33a* mutant *D. simulans* for food containing only sucrose. Mean ± SEM; Preference Index = {(# flies that ate sucrose-only food + 0.5*(purple flies)}/(# flies that ate); each circle denotes Preference Index for one experiment; 99 ± 4 *D. simulans* of each genotype were used/experiment; n = 6 - 15 experiments/genotype; ***p<0.001.

DISCUSSION

The study of changes in morphological or other traits across evolution continues to be vigorously investigated (Carroll, 2008). We have examined how a pathway that inhibits interspecies courtship in *D. melanogaster* might perform a similar function in *D. simulans*, a sympatric species that diverged ~3-5 mya. Similar to *D. melanogaster*, we find that male foreleg tarsi in *D. simulans* are essential to inhibit this behavior but not for conspecific courtship. Gr32a, a chemoreceptor that is expressed in *D. melanogaster* foreleg tarsi neurons and required to inhibit interspecies courtship, is also expressed in foreleg tarsi neurons of *D. simulans*. Moreover, regulatory DNA sequence elements within the *Gr32a* locus of these species are likely functionally conserved as they appear sufficient to confer reporter expression in foreleg tarsi neurons of the cognate as well as the heterologous species. Remarkably however, Gr32a is not essential for inhibiting interspecies courtship in *D. simulans*. Thus, the peripheral neural pathway that inhibits interspecies courtship is conserved but chemoreceptor control of this behavior has diverged within ~3-5 million years.

Foreleg tarsi are required to inhibit interspecies courtship by both *D. simulans* and *D. melanogaster* males. Although Gr32a is not essential to suppress this behavior in *D. simulans*, it is possible that Gr32a neurons still function to inhibit this behavior. Accordingly, we attempted to functionally silence Gr32a+ neurons in an effort to determine if these neurons inhibit interspecies courtship by *D. simulans* males. However, it was technically challenging to generate and validate such reagents in this species, despite numerous attempts. *D. simulans* males sense aversive cues on *D. melanogaster* females, suggesting that they use a chemosensory pathway to avoid

mating with other species (Billeter et al., 2009; Fan et al., 2013). Given that Gr32a does not serve this function in *D. simulans*, what chemoreceptors might be employed to detect repellents that preclude interspecies courtship in this species? We find that the related chemoreceptor Gr33a, which is co-expressed in many Gr32a neurons in *D. melanogaster* foreleg tarsi, is not required to inhibit this behavior. The gustatory and ionotropic chemoreceptor families contain many members, any one (or more) of which may function to inhibit interspecies courtship by *D. simulans* males (Joseph and Carlson, 2015). Whether such a chemoreceptor functions in Gr32a+ or other neurons in foreleg tarsi also remains to be determined.

The divergence in chemoreceptor-mediated suppression of courtship between *D. melanogaster* and *D. simulans* does not reflect a global reorganization of molecular pathways that regulate courtship. We find that, similar to its role in *D. melanogaster*, Ppk25 is required to promote courtship toward conspecific females in *D. simulans*. Ppk25 is required to sense 7,11-heptacosadiene, an aphrodisiac cue, in *D. melanogaster* (Kallman et al., 2015; Starostina et al., 2012); however, 7,11-heptacosadiene is an aversive cue for *D. simulans* males such that they do not court targets coated with this pheromone (Billeter et al., 2009). Given these constraints, it will be interesting to understand how the chemosensory pathway expressing Ppk25 functions in both species to promote conspecific courtship.

The divergence in Gr32a function raises the possibility that this chemoreceptor serves an unrelated function in *D. simulans*. Consistent with this notion, Gr32a inhibits male courtship toward conspecific males in *D. melanogaster* but not *D. simulans*

(Miyamoto and Amrein, 2008). In fact, Gr33a, a chemoreceptor that inhibits intermale conspecific courtship in *D. melanogaster* is also not essential to suppress this behavior in *D. simulans* (Moon et al., 2009). Nevertheless, Gr32a as well as Gr33a are required for avoidance of the bitter tastant quinine in both *D. melanogaster* and *D. simulans* (Lee et al., 2010; Moon et al., 2009). These findings show that the function of Gr32a and Gr33a in avoiding quinine has been evolutionarily dissociated from their role in inhibiting courtship of reproductively futile targets. Our CRISPR-generated mutations in *D. simulans* Gr32a and Gr33a target N-terminal residues whereas the functions of these chemoreceptors in *D. melanogaster* were assessed via larger targeted N-terminal deletions or RNAi-mediated knockdown. We therefore cannot exclude the possibility that the mutations in these loci in *D. simulans* specifically disrupt quinine-sensing without altering control of male courtship. This seems unlikely given that two different alleles for each *Gr32a* and *Gr33a* are predicted null mutations of these loci. Our findings also demonstrate that the function of Gr32a has not entirely diverged between *D. melanogaster* and *D. simulans* because it is required for quinine avoidance in both species.

Figure Panels	Name	5' to 3' Sequence	Purpose
Fig 3.3B	sim32 Fw	GTCCCCTTGCGGTTGTTCT	Amplifying ~3.8kb Gr32a regulatory region from *D. sim* and *D. mel* to make Gr32a-GAL4s
	sim32 Rv	TTCAATTACCCAAGTGTTCG	
Fig 3.3B	mel32 Fw	AAGTGGTTGGTCTTGGAT	
	mel32 Rv	TTCAATTACCCAAGTGTTCG	
Fig 3.5A	CrisprGr32a A fwd	CTTCGGAAGGCATCCCGGGTTAACA	CRISPR oligos targetting *D. sim* Gr32a
	CrisprGr32a A rev	AAACTGTTAACCCGGGATGCCTTCC	
	CrisprGr32a B fwd	CTTCGTCGGAGACCTGTTCGTATAT	
	CrisprGr32a B rev	AAACATATACGAACAGGTCTCCGAC	
	CrisprGr32a C fwd	CTTCGTTTTACTCGTTCTTCGTAAG	
	CrisprGr32a C rev	AAACCTTACGAAGAACGAGTAAAAC	
Fig 3.8A	CrisprGr33a A fwd	CTTCGTCCACTGAATCGCCAGCAAT	CRISPR oligos targetting *D. sim* Gr33a
	CrisprGr33a A rev	AAACATTGCTGGCGATTCAGTGGAC	
	CrisprGr33a B fwd	CTTCGATAAATCTTACCCACATTAT	
	CrisprGr33a B rev	AAACATAATGTGGGTAAGATTTATC	
	CrisprGr33a C fwd	CTTCGGCTGAGTCTTTATCGCCGAA	
	CrisprGr33a C rev	AAACTTCGGCGATAAAGACTCAGCC	
Fig 3.8F	CrisprPpk25 A fwd	CTTCGAGGGAGTCGGCCGAAGCAAC	RISPR oligos targetting *D. sim* Ppk25
	CrisprPpk25 A rev	AAACGTTGCTTCGGCCGACTCCCTC	
	CrisprPpk25 B fwd	CTTCGCATGCCCTACATCGCCCGCA	
	CrisprPpk25 B rev	AAACTGCGGGCGATGTAGGGCATGC	
Figs 3.6A, 3.6B	Gr32a RTPCR fw	TAATCCACAATGCCAAGCAA	RT-PCR primers for *D. sim* Gr32a
	Gr32a RTPCR rev	AGGAACTTATCGATGATATTCTGAT	
Figs 3.9A, 3.9B	Gr33a RTPCR fw	CGGAGTAGCGAGTAAATTCCA	RT-PCR primers for *D. sim* Gr33a
	Gr33a RTPCR rev	TCGGATGTGTTTCCGGTATT	
Figs 3.11A, 3.11B	Ppk25 RTPCR fw	ACATCATGGAATCCAAAGG	RT-PCR primers for *D. sim* Ppk25
	Ppk25 RTPCR rev	ATCCAGTGTTTCTAGTTTGCC	
Figs 3.6B, 3.9B, 3.11B	tubulin RTPCR fw	CTTGTCGCGTGTGAAACACT	RT-PCR primers for *D. sim* tubulin
	tubulin RTPCR rev	GGATCCTGTCCAGAACCAGA	

TABLE 3.1: List of oligos used in this study.

	Gr32a Amino Acid Range	
	D. melanogaster	*D. simulans*
TM1	101-119	101-119
TM2	134-156	134-156
TM3	187-206	187-206
TM4	215-239	215-239
TM5	310-329	310-329
TM6	356-378	356-378
TM7	419-443	419-443

TABLE 3.2: HHMTOP-predicted transmembrane domains for *D. melanogaster* and *D. simulans* Gr32a.

CHAPTER FOUR

DISCUSSION AND FUTURE DIRECTIONS

"Ethological barriers to random mating constitute the largest and most important class of isolating mechanisms in animals"

//Ernst Mayr (1963)

Courtship in *Drosophila* is a complex, species-specific behavior that is dynamically regulated by key molecular and neural substrates. The mechanistic insights gained from studies of this behavior underline an important implication of Mayr's point: courtship itself is not random, but is precisely regulated by genetically built sensory pathways. The rich ecological and behavioral data available for pairs of closely related *Drosophila* species are an excellent starting point for generating a holistic understanding of the evolution of behavior (Nanda and Singh, 2012; Singh, 2016). Such pairs of fly species provide a unique snapshot in evolutionary history. The species-pairs I highlight in this thesis have diverged only recently from their last common ancestor (~0.5—5 million years ago), which guarantees that relatively few genetic changes have taken place. Therefore, differences in innate courtship behaviors could potentially be explained by few changes in DNA sequences that encode critical genes or control the expression of these genes. In addition, the decades of mechanistic studies in *D. melanogaster* will provide a strong foundation for such comparative studies. The major hurdle is, of course, gaining access to the genetic and neural substrates that control courtship in these diverse species. Aside from *D. melanogaster*, a comprehensive genetic toolkit is currently unavailable for the species discussed in this thesis. However, with the advent of CRISPR technologies, and as non-*melanogaster* transgenic fly stocks and whole genome sequencing become the norm, the future is bright (Stern et al., 2017; Turner and Miller, 2012).

Chemosensation and the evolution of courtship behaviors

Flies live in chemical world. They use gustatory and olfactory sensory systems to evaluate egg laying sites, potential mates, and appropriate foods (reviewed in Depetris-Chauvin et al., 2015; Joseph and Carlson, 2015). In addition, some chemoreceptor pathways appear to simultaneously control seemingly disparate behaviors. In *D. melanogaster*, the chemoreceptors Gr33a and Gr32a inhibit feeding on bitter substances and are also required to preclude aberrant courtship (Ahmed et al., 2017; Fan et al., 2013; Lee et al., 2010; Moon et al., 2009). These data suggest that these receptors might more broadly serve as 'no-go' signals in flies. How Gr32a and Gr33a function to inhibit two distinct behavioral programs, feeding and mating, is still an open question. These two are not the only examples of chemoreceptors that control distinct behaviors. Ir84a detects chemicals found on food substances and modulates courtship, but it does not respond directly to pheromones (Grosjean et al., 2011). In addition, the generic bitter receptor Gr66a mediates avoidance behaviors and promotes egg laying in female *D. melanogaster* (Joseph and Heberlein, 2012). These opposing behaviors are controlled by tissue-specific activation of *Gr66a*-expressing neurons, thus providing one mechanism by which heterogeneity of a single neuronal population can drive distinct behaviors. In sum, these results suggest that the control of feeding and mating could be more broadly coupled via chemosensory pathways.

Based on these data, I hypothesized that chemosensory pathways that control avoidance and approach could be appropriated or co-opted to control distinct behaviors. I therefore tested whether chemosensory pathways that promote (Gr5a, Gr64E) or

inhibit (Gr32a, Gr33a, Gr66a) feeding may have also evolved to promote or inhibit courtship. To test the role of these chemosensory pathways in conspecific courtship, I genetically ablated neurons expressing these particular Grs and screened for copulation success of *D. melanogaster* males (Figure 4.1). To generate Gr-ablated male flies, I crossed UAS-hid to flies carrying a Gr-GAL4 alleles that express in bitter receptor- or sweet receptor-expressing neurons and assayed how many males copulated within a 15-minute period (Clyne et al., 2000; Dahanukar et al., 2007; Fujii et al., 2015). I found that a higher fraction of males with ablated *Gr32a*-, *Gr33a*-, or *Gr66a*-expressing neurons copulated with females, while males with ablated *Gr5a*- or *Gr64E*-expressing neurons were less likely to copulate (Figure 4.1). These preliminary data suggest that bitter- and sweet-receptor neurons are required to inhibit or promote copulation success, respectively. How feeding and mating signals are integrated and translated by these neurons to regulate distinct behavioral programs is yet to be determined.

Insect chemoreceptors are mostly comprised of large families of gustatory, olfactory, and iontropic receptors (Grs, Ors, Irs; Benton et al., 2009; Clyne et al., 2000; Dahanukar et al., 2005; Koh et al., 2014; Scott et al., 2001; Vosshall et al., 1999) and many of the genes in these families are undergoing positive selection (Gardiner et al., 2008, 2009; Macharia et al., 2016). Many chemosensory pathways control courtship in *D. melanogaster* males (see Chapter 1). What function, if any, might these pathways play in other Drosophila species? We have shown that at least two genes that preclude aberrant courtship in *D. melanogaster* males (*Gr32a* and *Gr33a*) do not contribute to courtship behaviors in the related species *D. simulans*. Yet in both species, Gr32a and Gr33a are required for sensing the bitter substance quinine. Taken together, our results

indicate that the functions of these chemoreceptors are evolutionarily dissociable. It remains unclear whether Gr32a and Gr33a have evolved a novel function in *D. melanogaster* or whether this function was lost in the *D. simulans* lineage. Exploring the behavioral role of Gr32a and Gr33a in other species of the *D. melanogaster* species subgroup will help clarify these two possibilities.

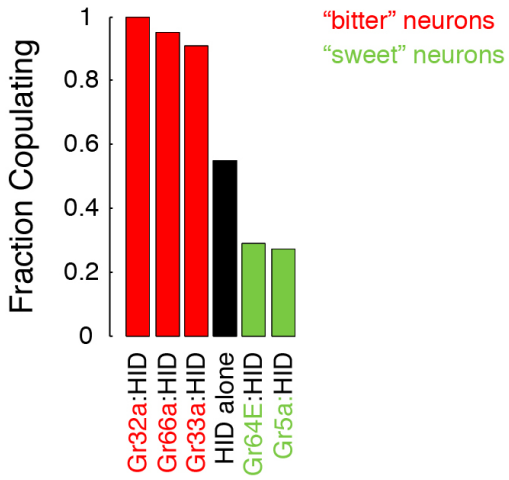

Figure 4.1: Bitter and sweet neurons are likely required for copulation success.

Ablation of bitter receptor-expressing neurons (red) increases copulation success while ablation of sweet receptor-expressing neurons (green) decreases copulation success. Control is UAS-hid/+ (black). n = 11 - 23/experimental genotype. n = 83 for HID alone control.

Furthermore, both *D. melanogaster* and *D. simulans* males use the same sensory pathways, the foreleg tarsi, to inhibit interspecies courtship. However, *D. melanogaster*, but not *D. simulans*, males require Gr32a for this behavior; in fact, it remains mysterious what chemoreceptor(s) *D. simulans* males use to inhibit interspecies mating. One possibility is that *D. simulans* males may employ species-specific chemoreceptor pathways to regulate courtship. If true, other Gr and/or Ir genes that are expressed in this sensorimotor organ underlie this behavior in *D. simulans*.

Modularity as fodder for evolution

The known *Drosophila* Ors, Grs, and Irs are expressed in subsets of sensory neurons. Such modular organization likely allows for divergent, species-specific chemosensory pathways to emerge. These chemoreceptors comprise a large family of potential controllers of *Drosophila* behavior, and changes in their expression or function can lead to novel adaptations (Prieto-Godino et al., 2017). Such changes in the regulation or structure of genes can gate entire behaviors, or lead to quantitative differences in behavioral components without altering the overall behavioral routine. In addition, recent studies have shown that networks of genes controls complex behaviors, and particular loci control particular aspects of these behaviors (Ding et al., 2016; Weber et al., 2013; Xu et al., 2012). Such modular genetic control could underlie the variability of behavior observed in natural and lab populations. As applied to male *Drosophila* courtship behaviors, changes in key genes can lead to quantitative differences in particular parameters of courtship displays (for example, Ding et al., 2016). Such differences, even if subtle, could facilitate reproductive isolation if mating success were sufficiently impacted. It will be interesting to identify the gene networks that control key

components of courtship displays in *Drosophila* species, and to understand how changes in these critical genes leads to variability in behavior.

Conclusion

Courtship behaviors evolve quickly to facilitate reproductive isolation between closely related species. In *Drosophila*, chemosensory pathways control many aspects of these routines and as such, these pathways have likely changed between species in order to give rise to divergent courtship routines. In this thesis, I have discussed divergent and shared chemosensory pathways that control courtship in two species, *D. melanogaster* and *D. simulans*. However, non-chemosensory pathways that control courtship have also likely diverged between *D. melanogaster* and *D. simulans*. For instance, visual cues appear to be more important for high levels of courtship in *D. simulans* but not in *D. melanogaster* (Ahmed et al., 2017; Allemand, 1982; Jezovit et al., 2017). Yet, how are diverse sensory signals processed to control species-specific courtship routines? Comparative neurobiological studies in closely related *Drosophila* species will uncover differences in how sensory signals are integrated and transformed to give rise to divergent behaviors. Such findings will form a strong foundation for understanding how other innate behaviors evolve.

EXPERIMENTAL PROTOCOLS

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); the R24C08-LexA (a gift from Gerry Rubin) was constructed as described previously (Pfeiffer et al., 2008, 2010). With the exception of the Gr32a-LexA and UAS-spGFP1-10::Nrx strains, the flies used in Chapter 2 have been described before (Anand et al., 2001; Barolo et al., 2004; Goodwin et al., 2000; Gordon and Scott, 2009; Hampel et al., 2011; Kitamoto, 2001; Lu et al., 2012; Manoli and Baker, 2004; Melnattur et al., 2002; Scott et al., 2001; Wang et al., 2004). The UAS-mCD8GFP and UAS-hid strains were obtained from the Bloomington Drosophila Stock Center. All *D. melanogaster* flies were in the Canton- S background except Gr32a (Oregon-R), and WT controls for the 3 mutant flies (Gr32a, Gr33a, Gr66a) were from the corresponding background strain.

D. simulans, w^{501} *D. simulans*, *D. yakuba*, *D. pseudoobscura*, and *D. virilis* were obtained from the Drosophila Species Stock Center at the University of California, San Diego. *D. melanogaster* UAS-ReaChR::Citrine.VK05 was obtained from the Bloomington Drosophila Stock Center (#53749). *D. simulans* transgenic flies were generated in this study by PhiC31 site-directed integration of plasmids ($Gr32a^{sim}$-GAL4, $Gr32a^{mel}$-GAL4, UAS-ReaChR::Citrine) into sim986 landing site background. *D. melanogaster* transgenic flies were generated similarly by integrating plasmids ($Gr32a^{sim}$-GAL4, $Gr32a^{mel}$-GAL4) into the attP2 landing site background. *D. simulans Gr32a*, *Gr33a*, and *Ppk25* mutant lines were generated by CRISPR/Cas9 (see section below).

Generating *D. simulans Gr32a*, *Gr33a*, or *Ppk25* mutants

CRISPR guides were chosen from a list generated by flyCRISPR Optimal Target Finder (flycrispr.molbio.wisc.edu/tools). We targeted exon 1 of *D. simulans Gr32a* and *Pk25*, and exon 2 of *Gr33a*. CRISPR oligos were annealed and ligated to plasmid pDCC6 {Addgene # 59985, (Gokcezade et al., 2014)} following restriction digest with *BbsI*. Sequences used to synthesize CRISPR oligos are provided in Table 3.1. Plasmids were injected at 100 ng/uL concentrations for each of 1 - 2 plasmids targeting a single gene. Animals were screened for mutations by PCR followed by 15% non-denaturing PAGE (Zhu et al., 2014) or directly by sequencing. All newly generated mutant strains were backcrossed at least 5 times to WT *D. simulans* before testing for behavior.

Molecular analysis of *Gr32a*, *Gr33a*, and *Ppk25* mutations in *D. simulans*

RNA was isolated from 10 WT or mutant *D. simulans* males (Trizol, ThermoFisher) and converted to cDNA using SuperScript III First-Strand Synthesis (Invitrogen, ThermoFisher). RT-PCR was performed using primers based on coding sequence that spanned exon-intron junctions to avoid amplifying products from genomic DNA (Table 3.1). RT-PCR products were then cloned and sequenced (Gr32a) or directly sequenced (Gr33a, Ppk25). RNA isolation and the subsequent RT-PCR and sequencing were performed on 2-3 independent cohorts of WT and mutant flies.

Generating *D. simulans* and *D. melanogaster* transgenic animals

To make Gr32a-GAL4 lines, we amplified the ~3.8 kb region upstream of the Gr32a start codon from *D. simulans* or *D. melanogaster* and subcloned it into pENTR/TOPO plasmid followed by Gateway-mediated subcloning into pBPGw. We then phiC31-

integrated each DNA construct into Chr III landing sites for each species, sim986 for *D. simulans* (generously provided by David Stern) and attp2 for *D. melanogaster*. pJFRC2(10xUAS-ReaChR::Citrine) plasmid (Inagaki et al., 2014) was provided by David Anderson, and it was used to generate the Citrine reporter in *D. simulans* using the landing site described above. Rainbow Transgenics and BestGene performed embryo injections.

The Gr32a-LexA DNA construct was generated by subcloning the ~3.8 kb Gr32a promoter region into the pBPLexA::p65Uw vector lacking DSCP (Pfeiffer et al., 2010; Weiss et al., 2011). To generate the UAS-spGFP1-10::Nrx DNA construct, the N-terminal signal peptide and spGFP1-10 was PCRed from constructs previously described (Feinberg et al., 2008) with 5' *Eco RI* site and 3' *Kpn I*-TAA- *Xba I* sites, and cloned into pUAST resulting in pUAST::spGFP1-10. Beginning with residue 107, the Nrx coding sequence was amplified and cloned in-frame into the 3' *Kpn I* site. Transgenic flies bearing these constructs were generated according to standard protocols.

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 3x with PBT (PBS [pH 7.4], + 0.3% Triton X-100) and then blocked with 10% normal donkey serum in PBT. These samples were mounted in Vectashield. Tarsi were dissected in ice-cold PBS, fixed in fresh 4% paraformaldehyde at 22°C, washed 3x in PBT, and then mounted in aquamount. All samples were imaged using a Zeiss LSM700 (Z-stacks) and processed in ImageJ.

Primary antibodies used were: 1) for visualization of GRASP components, mouse anti-GFP (1:1000; Invitrogen, #A11122; 1:100; Sigma, #G6539), rabbit anti-GFP (1:800; Abcam, ab290); 2) for *dBrainbow* imaging, rabbit anti-GFP (1:500; Invitrogen, #A11122), mouse anti-Myc (1:50; Developmental Studies Hybridoma Bank, clone 9E10), and rat-anti-HA (1:100; Roche, #11867423001). Secondary antibodies were AlexaFluor488 anti-rabbit (1:500, Molecular Probes, #A-11034), AlexaFluor488 anti-mouse (1:500, Molecular Probes, #A-11001) Cy3 anti-Rat (1:500, Jackson ImmunoResearch, #712-166-150), and Cy3 anti-mouse (1:500, Jackson ImmunoResearch, #715-166-150).

Tests for non-neutral evolution

Alignments of genomes from 27 insect species (23 drosophilids, housefly, mosquito, honeybee, and beetle) were generated for coordinates (dm6: chr2L:11,110,412-11,114,209) encompassing *D. melanogaster* Gr32a ~3.8 kb regulatory sequence, and this alignment was subsequently downloaded from the Table Browser (UCSC Genome Browser, 2015 update) (Blanchette et al., 2004; Karolchik et al., 2004; Rosenbloom et al., 2015). PhyloP scores were computed for this region across all branches as well as on the *D. melanogaster* branch and the *D. simulans* branch (Pollard et al., 2010). PhyloP scores scale logarithmically in magnitude. Scores near 0 indicate neutral evolution, while large positive (phyloP score > 2) or negative (phyloP score < -2) scores indicate conservation or acceleration, respectively. Nucleotides deemed to be undergoing acceleration were tallied inside and outside conserved elements for the tests on all branches, the *D. melanogaster* branch, and the *D. simulans* branch (Siepel et al., 2005).

P-values for non-neutral evolution of bases falling into four distinct sequence features (intron, CDS, UTR, and intergenic) within the ~3.8 kb Gr32a regulatory region were estimated by phyloP on the *D. melanogaster* branch, *D. simulans* branch, and across all branches. The phylogenetic model for neutral evolution was based on 4-fold degenerate sites in the 27-species alignment. The scores and R code are available for reproducible workflow, contact me or Aram Avila-Herrera (Allaire et al., 2017; Xie, 2017) (https://cran.r-project.org/doc/FAQ/R-FAQ.html#Citing-R).

Hydrophobicity plot

Hydrophobicity scores were generated with ProtScale (web.expasy.org/protscale) using *D. melanogaster* or *D. simulans* Gr32a amino acid sequences as input. We used the Kyte and Doolittle hydrophobicity scale with a window size of 19 amino acids and uniform weights across all residues. The seven transmembrane domains were identified using HMMTOP (Tusnády and Simon, 1998, 2001) to predict the topology of Gr32a for both *D. melanogaster* and *D. simulans*. See Table 3.2 for the amino acid coordinates of *D. melanogaster* and *D. simulans* Gr32a transmembrane domains.

11P synthesis and analysis

The alkyne precursor 11-pentacosyne was synthesized and reduced using hydrogen and Lindlar catalyst to generate the Z-alkene (Small Molecule Synthesis Facility at Duke University). ^{13}C NMR spectrum was recorded at 75 MHz. Chemical shifts were reported in parts per million (ppm) relative to deuterated solvent as the internal standard (δs: $CDCl_3$ 77 ppm): Z-11 ^{13}C 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.

For pentacose-11-yne, a solution of *n*-BuLi (1.6 M in hexanes, 1.0 ml, 1.6 mmol, 1.1 eq.) was added dropwise with stirring to a cooled (75° C, bath temperature) solution of 1-dodecyne (0.31 ml, 1.43 mmol) in dry THF (2 ml) containing HMPA (0.5 ml). The mixture was stirred for 2 hr then a solution of 1-bromotridecane (0.440 ml, 1.72 mmol, 1.2 eq.) in dry THF (0.5 ml) was added dropwise over 1-2 min. The mixture was allowed to warm to room temperature as the cooling bath melted. The reaction mixture was diluted with brine (10 ml) and extracted with hexane (2 x 10 ml). The combined extracts were dried (Na2SO4). The drying agent was removed by filtration. Silica gel (~1 g) was added and the filtrate was concentrated to dryness under reduced pressure. Flash column chromatography (RediSepR_f SiO_2 (12 g) 100% hexanes) gave the product (R_f = 0.40, 100% hexanes) as a clear, colorless liquid that slowly crystallized upon standing at room temperature (0.574 g).

For (11Z)-pentacos-11-ene, a suspension of the alkyne (0.5 g) and Lindlar's catalyst (300 mg) in quinoline:EtOAc (13 ml [1:1]) was stirred overnight under a balloon of H_2 after which time analysis of the reaction mixture by TLC (100% hexane) indicated complete consumption of starting material and the formation of a new species (R_f = 0.66). (A previous attempt at reduction of the alkyne using 5% Pd/CaCO3 without the quinoline additive resulted in a mixture of three products. Analysis by GC-MS indicated the mixture was comprised of the Z-alkene [*m/z* = 350.4, 56%], E-alkene [*m/z* = 350.4, 10%] and fully reduced pentacosene [*m/z* = 352.4, 33%].) The mixture was filtered through a pad of Celite. The pad was washed with EtOAc (2 x 20 ml). Silica gel (~10 g) was added to the filtrate and the mixture was concentrated to dryness under reduced

pressure. Flash column chromatography (RediSepR_f SiO_2 (40 g), 100% hexanes) gave a clear, colorless liquid that was shown by GC-MS to be the Z-alkene with no other detectable components (0.429 g, 85%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.35 (m, 2H), 2.01 (m, 4H) 1.26 (m, 38H), 0.88 (m, 6H).

Preparation and coating of oe– females

Cuticular extracts were prepared by washing 25-30 *D. melanogaster*, *D. simulans*, or *D. yakuba* flies with 50 mL of hexane, briefly and gently vortexing, and aspirating liquid. Extracts from *D. virilis* were prepared using 15-20 flies in a similar volume. For transfer of extracts to oe– flies, 50 μL of extract were pipetted onto filter paper in a 1.5 ml tube and solvent allowed to evaporate for 30 min. 3-5 flies were placed into the tube, and gently vortexed 3x for 20s each with 20s pauses. For individual compounds, previous work has demonstrated that ~3% of the total amount of a given compound that is placed onto a filter paper as above is transferred to each fly when 7 flies are prepared at a time using this technique (Wang et al., 2011). We therefore transferred 30x of the desired dose of 7T, 9T, and 11P on to a filter paper and coated seven target flies at a time as above. Target amounts of individual compounds were as follows: 7T, 1 mg/fly (Wang et al., 2011); 9T, 0.1 mg/fly (Butler et al., 2009); 11P, 2 mg/fly (Oguma et al., 1992).

Fly rearing and courtship assays

All flies and crosses were raised and maintained on standard cornmeal/molasses media at 25 C with 12:12 light:dark cycle and at 70% humidity. The only exceptions were for crosses using UAS-shi^{ts} (18^o C), UAS-dTrpA1 (18^o C), UAS-dBrainbow (18^o C), UAS-hid (25^o C or 29^o C), and those involving RNAi (29^o C). The Gr:hid behavioral screen to

identify chemoreceptors that inhibit inter- species mating was done with flies raised at 25° C. We subsequently observed a more robust cellular ablation and behavioral phenotype when the flies were raised at 29° C, and we therefore present data for Gr32a:hid and Gr33a:hid with *D. simulans* and *yakuba* from flies raised at 29° C. Even under these optimal culture conditions, we observed essentially no ablation of Gr28a and Gr68a neurons (Gr:stingerGFP, hid), and these flies were not analyzed further.

All courtship assays were performed at zeitgeber time 6-10 at 22° C, illuminated by a fluorescent ring lamp (22W) suspended 4 cm above the courtship chamber and recorded with a Sony camcorder (HDR-XR550V). Experiments performed under dark conditions were illuminated by red LEDs and recorded as above in a dark room. Virgin flies were collected at eclosion and light entrained (12 hours L/D, 25° C) for 5-7 days prior to testing. The only exception to these ages was that we used male and female *D. pseudoobscura* and *virilis* at 10-14 days and 7-10 days after eclosion, respectively. Experimental males were kept in isolation and tested with flies that were group-housed (~20 flies per vial) by species and sex. Flies were anesthetized by CO_2, 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). Tarsectomized *D. yakuba* males were introduced via mouth pipette into the courtship chamber without anesthesia. All courtship assays were performed at 22° C except in studies using UAS-shi^{ts} and UAS-dTrpA1 in which case the flies were tested at 18° C or 31° C. For tests performed at 31° C, the flies were warmed at 31° C for 20 min (UAS-shi^{ts} experiments) or 40 min (UAS-dTrpA1) prior to behavioral assays.

In instances where courtship assays were performed between males whose genotypes or species membership were not easily distinguishable, we trimmed the wings of target males bilaterally. The tarsi, antennae, and maxillary palps were surgically removed bilaterally under anesthesia 1–3 days prior to behavioral testing. The labellum was surgically removed under anesthesia 3–4 hr prior to behavioral testing. Oe– females and dBrainbow males were generated as described previously with the exception that dBrainbow males were raised at 18^{o} C and not subjected to heat shock (Billeter et al., 2009; Hampel et al., 2011).

Courtship assays were scored blind to genotype, using the MATLAB software ScoreVideo (Wu et al., 2009). We scored courtship as the period of time male flies spent chasing the stimulus fly, performing unilateral wing extension (courtship song), licking, abdominal bending (attempted copulation), or copulation. Courtship Index (CI) was calculated as the time spent by the male performing these behaviors, divided by the total assay time (15 minutes).

Taste assay

Preference assays were performed as described previously (Montell, 2009). Briefly, 60-well plates were prepared the day prior to experimentation and kept at 4^{o}C. Dyes were diluted from stock solutions (Brilliant blue FCF and Sulforhodamine B, 12.5 mg/ml each) and resuspended in agarose, to which sucrose or sucrose spiked with quinine-HCl were subsequently added. Final concentrations were: agarose (1%), Brilliant blue FCF (0.125 mg/mL; Wako Pure Chemical), Sulforhodamine B (0.125 mg/mL; SigmaAldrich),

sucrose (1 mM; JT Baker), and sucrose (5 mM) spiked with quinine (0.5 mM; SigmaAldrich). Substrate with sucrose or sucrose spiked with quinine were randomly colored blue or red and counterbalanced for all experiments. 3-4 day old male and female flies were flipped into fresh food for 2 days at 12-hour light/dark cycle at 25°C. Flies were then food deprived by flipping them into vials containing 1% agarose and placed in the dark for 24 hours. Flies were then briefly anesthetized with CO_2 and loaded onto the 60-well plates (zeitgeber time 2-3), which were put in a box and placed in the dark at 25°C for 90 min. Abdomens were scored as blue, red, purple (mixed eating), or no food coloring blind to genotype and color condition. A Preference Index was calculated for each 60-well plate as follows: $(N^B + 0.5*N^P)/(N^B + N^R + 0.5*N^P)$ or $(N^R + 0.5*N^P)/(N^B + N^R + N^P)$ where N^B, N^R, and N^P = total # flies with blue, red, and purple abdomens, respectively. Each genotype was tested ≥ 6 times.

Statistical Analyses for Behavioral Data

We used Fisher's exact test to analyze categorical data (e.g. percent assays with CI > 0.05) and we used the Bonferroni correction for multiple group comparisons as necessary. For other comparisons, we first tested whether data were normally distributed using a Lillefors' goodness-of-fit test using MATLAB. Data not violating this assumption were analyzed with parametric tests (Student's t-test for two groups or one-way ANOVA); otherwise, data were tested with a non-parametric test (Kolmogorov-Smirnov test for two groups or Kruskal-Wallis test). A Tukey's post hoc test following multiple group comparisons was used to determine which groups differed significantly.

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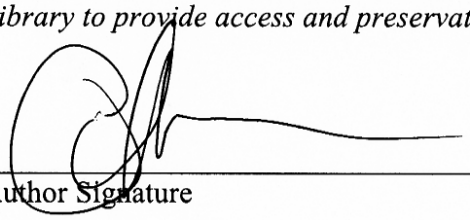

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