A pleiotropic chemoreceptor facilitates the coupling of pheromonal signal perception and production

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SUMMARY

Optimal mating decisions depend on reliable and precise species-specific coupling of the production and perception of mating communication signals because any functional changes in either the signal or its perception should carry a fitness cost (Boake, 1991; Brooks et al., 2005; Hoy et al., 1977b; Shaw et al., 2011; Shaw and Lesnick, 2009; Steiger et al., 2010; Sweigart, 2010; Symonds and Elgar, 2008; Wyatt and Cambridge University Press, 2014). Previously published theoretical models predict that genetic linkage between sensory-driven mate choices and specific communication signals could provide a robust mechanism for maintaining robust and stable signal production and perception at the level of populations, while still retaining the capacity for future signal diversification (Hoy et al., 1977b; Shaw et al., 2011; Shaw and Lesnick, 2009; Wiley et al., 2011). Several experimental studies have provided empirical evidence that pleiotropy is one possible genetic solution for maintaining functional coupling between specific communication signals and the mate choice behaviors they elicit at the population level (Hoy et al., 1977b; Shaw et al., 2011; Shaw and Lesnick, 2009; Wiley et al., 2011). However, the complex characteristics of mating signals and behaviors present a major barrier for identifying the actual pleiotropic genes and molecular pathways that provide genetic linkages between the production and perception of mating signals (Chenoweth and Blows, 2006; Singh and Shaw, 2012). Here we show that in the fruit fly *Drosophila melanogaster*, some pheromone-driven mating choices are coupled to the production of specific mating signals via pleiotropic chemoreceptors. Specifically, we demonstrate that *Gr8a*, a member of the gustatory receptor family, independently contributes to the perception of inhibitory mating signals in pheromone sensing neurons, as well as in regulating the production of inhibitory mating signals in oenocytes. Together, these data provide a relatively simple molecular explanation for how coupling of pheromone perception and production, two independent physiological processes, remains robust.

Keywords: *Drosophila melanogaster;* Vinegar fly; Fruit fly; Cuticular hydrocarbons; *Gr8a*; Oenocyte.

RESULTS AND DISCUSSION

Similar to other insect species, the fruit fly *Drosophila melanogaster* has evolved cuticular hydrocarbons (CHCs), synthesized by the fat body and oenocytes (Billeter et al., 2009; Gutierrez et al., 2006; Krupp et al., 2013; Makki et al., 2014), which primarily provide a hydrophobic desiccation barrier, as well as play an important role as pheromones that regulate diverse aspects of insect behavior (Blomquist and Bagnères, 2010; Chung and Carroll, 2015; Ferveur, 2005; Howard and Blomquist, 2005). Specifically, complex blends of CHCs are often utilized by insect species to communicate sex identity and female mating status, and well as defining the behavioral reproductive boundaries between closely related species (Billeter et al., 2009; Chung and Carroll, 2015; Chung et al., 2014; Coyne et al., 1994; Dweck et al., 2015; Ng et al., 2014; Shirangi et al., 2009; Yew and Chung, 2015). In *Drosophila*, the perception of volatile CHCs is mediated by olfactory sensory neurons located in the antennae and maxillary palps (Benton et al., 2007; Kurtovic et al., 2007; Lebreton et al., 2014; van der Goes van Naters and Carlson, 2007), while less volatile CHCs, which require contact, are sensed by specialized gustatory-like receptor neurons (GRNs) in the appendages (legs and wings) and the proboscis (Koh et al., 2014; Lu et al., 2012; Lu et al., 2014; Thistle et al., 2012; Toda et al., 2012). While some of the genes and pathways that contribute to CHC synthesis in *Drosophila* are known, the molecular identities of the majority of CHC receptors remain unknown. Previous work suggested that the gene *Desat1*, which encodes a fatty acid desaturase enriched in oenocytes, might also independently contribute to CHC perception (Bousquet et al., 2012). However, due to the expression of *Desat1* in central neurons (Billeter et al., 2009) and the broad impact its mutant alleles have on the CHC profiles of both males and females (Labeur et al., 2002), whether *Desat1* directly contributes to mating signal perception remains unresolved.

Consequently, we chose to examine members of the *Gustatory receptor* (*Gr*) gene family as candidates for pleiotropic factors that might contribute directly to both perception and production of pheromonal mating signals in *Drosophila*. Because several family members have already been implicated in the detection of specific excitatory and inhibitory pheromones (Bray and Amrein, 2003; Hu et al., 2015; Miyamoto and Amrein,

2008; Moon et al., 2009; Watanabe et al., 2011), and the majority of genes that encode family members are already known to be enriched in gustatory receptor neurons (GRNs) (Clyne et al., 2000; Dunipace et al., 2001; Scott et al., 2001; Wang et al., 2004), we reasoned that any pleiotropic *Gr* genes should also be expressed in abdominal oenocytes (Billeter et al., 2009). Subsequently, an RT-PCR screen revealed that 24 out of the 60 genes that encode *Gr* family members in the *Drosophila* genome (Clyne et al., 2000; Dunipace et al., 2001; Robertson et al., 2003; Scott et al., 2001) exhibit enriched expression in abdominal tissues (Table S1).

Although several members of the *Gr* receptor family, including *Gr68a, Gr32a, Gr66a, Gr39a*, and *Gr33a*, have been previously linked to the sensory perception of mating pheromones (Bray and Amrein, 2003; Lacaille et al., 2009; Miyamoto and Amrein, 2008; Moon et al., 2009; Watanabe et al., 2011), none of these candidate pheromone receptors were identified in our initial screen for *Gr* genes with enriched mRNA expression in abdominal tissues of either males or females (Table S1). However, *Gr8a*, which we have identified as a male-specific gene in our screen, is localized to a chromosomal region that was previously implicated in chemoreception with enriched expression in abdomens (Table S1) and was previously shown to specifically contribute to the sensory detection of the non-proteinogenic amino acid L-Canavanine (Lee et al., 2012; Shim et al., 2015). Because our initial expression screen was based on whole-abdomen RNAs, and it was previously reported that *Gr8a* is also expressed in some abdominal neurons (Park and Kwon, 2011), we next used a GAL4 transgenic driver to examine the spatial expression pattern of *Gr8a* in males and females. As was previously reported, we found that *Gr8a* is expressed in 14-16 GRNs in the proboscis (Figure 1A) (Lee et al., 2012) as well as in two paired GRNs in the foreleg pretarsus (Figure 1B) of males and females. In addition, the *Gr8a*-GAL4 reporter was also highly expressed in abdominal oenocyte-like cells in males but not females (Figure 1C). The male-specific abdominal expression pattern was further supported by qRT-PCR (Figure 1D). Using immunohistochemistry, we then demonstrated that *Gr8a* is co-expressed with the oenocyte marker *Desat1 (Billeter et al., 2009)*, as well as in *Desat1*-negative cells with fat-body-like morphology (Figure 1E-G).

Together, the enriched expression of *Gr8a* in foreleg GRNs, a primary pheromone chemosensory organ (Lu et al., 2012; Lu et al., 2014), and its sexually dimorphic expression in oenocytes suggested that in addition to its role in L-Canavanine perception, *Gr8a* also contributes to mating behaviors. Since mate choices of both male and female flies are determined by a complex blend of excitatory and inhibitory pheromones (Billeter et al., 2009; Krupp et al., 2013), we anticipated that if *Gr8a* is indeed a pleiotropic factor that independently contributes to the perception and production of specific components of the male mating pheromone bouquet, then disruptions of the signal production in males or its perception in females should carry similar impacts on female mate choice behavior. To directly test this hypothesis, we first investigated whether *Gr8a* and the GRNs that express it are required for sensory functions associated with female mate choice by using a single-pair courtship paradigm (Lu et al., 2012; Lu et al., 2014). We found that blocking neuronal transmission in *Gr8a*-expressing GRNs with the transgenic expression of the tetanus toxin (TNT) in females resulted in shorter copulation latency relative to wild-type females when courted by wild-type males (Figure 2A). Similarly, homozygous (Figure 2B) and hemizygous (Figure 2C) *Gr8a* mutant females exhibited shorter copulation latencies relative to wild-type controls, which could be rescued by the transgenic expression of a *Gr8a* cDNA (Figure 2D). In contrast, similar genetic manipulations of the *Gr8a* gene or *Gr8a*-expressing GRNs in males did not affect courtship behavior as measured by courtship latency and index towards wild-type females (Figure S1). These data indicate that *Gr8a* is required for female mate choice via the detection of male-borne inhibitory mating signals.

Because *Gr8a* expression is specifically enriched in male oenocytes, we next tested the hypothesis that *Gr8a* also plays a role in the production and/or release of inhibitory mating signals by males. Similar to the effect of the *Gr8a* mutation in females, we found that wild-type virgin females exhibited shorter copulation latencies towards *Gr8a* mutant males relative to wild-type controls (Figure 2E). These data indicate that *Gr8a* mutant males produce and/ or release abnormally low levels of inhibitory mating pheromones.

Previous studies showed that, in order to increase their fitness, *Drosophila* males transfer inhibitory mating pheromones to females during copulation, which subsequently lowers the overall attractiveness of mated females to other males and the probability of

females remating (Benton et al., 2007; Billeter et al., 2009; Datta et al., 2008; Yew et al., 2009). Because our data indicate that *Gr8a* plays a role in the production of an inhibitory signal in males, we next tested the hypothesis that *Gr8a* mutant males would have a reduced ability to produce and/ or transfer inhibitory pheromones to females during copulation. Accordingly, we found that wild-type males failed to recognize mating status of wild-type females that were previously mated with *Gr8a* mutant males (Figure 2F). These data indicate that *Gr8a* action is required for the production and/or transfer of inhibitory pheromones from males to females during copulation. Because *Gr8a* is involved in the perception of an inhibitory mating signal, we also tested the hypothesis that *Gr8a* mutant males will exhibit abnormal capacity for identifying the post-mating status of wild-type females. Indeed, we observed that *Gr8a* mutant males were more likely than wild type males to court a mated female, suggesting that in addition to its role in the production of inhibitory mating signals in males, *Gr8a* is also required in males for the sensory recognition of mating inhibitory signals that mark the post-mating status of females (Figure 2F). Together, these studies indicate that *Gr8a* is required in males for the production and perception of transferrable inhibitory mating signals that advertise post-mating status in females. These data further support the pleiotropic role of the *Gr8a* gene in the production of inhibitory mating signals in males and the perception of these signals in both males and females in sex-specific contexts.

Because our behavioral data indicate that the *Gr8a* mutation has a dramatic impact on the production and perception of a putative inhibitory mating pheromonal signal, we next examined whether the *Gr8a* mutation has a direct impact on qualitative and quantitative aspects of male and mated-female CHC profiles. As predicted by our behavioral data, analyses of male epicuticular pheromones by gas chromatography and mass spectroscopy revealed the *Gr8a* mutation has a significant impact on the overall qualitative characteristics of the CHC profile of males (Figure 3A). Additional analyses revealed significant quantitative effects of the *Gr8a* mutation on the cuticular levels of several specific components in males (Figure 3B,C and Table S2). In particular, the *Gr8a* mutation affects the levels of several alkenes and methyl-branched alkanes, which have been implicated in mate choice behaviors in diverse *Drosophila* species (Billeter et al., 2009; Chung et al., 2014; Dyer et al., 2014; Shirangi et al., 2009). Similarly, *Gr8a*

mutation affects the expression level of several desaturase enzymes in males (Figure 3D), which are involved in the biosynthesis of alkenes (Chung et al., 2014). Additionally, although the CHC profile of females mated with *Gr8a* mutant males did not qualitatively differ from those mated with wild-type males (Figure 3E), one specific component of the CHC profile, nonacosane (C_{29}), differed quantitatively between the two mated-female groups (Figure 3F). Together, our behavioral and pheromonal data indicate that *Gr8a* action contributes to mating decisions in females by co-regulating the female perception and male production and transfer of an inhibitory pheromone, which is consistent with a pleiotropic function for *Gr8a*.

The data presented here demonstrate that a pleiotropic chemoreceptor co-regulates the perception and production of a specific pheromonal signal that plays an important role in mate choice behaviors of both sexes. In contrast to its expected function in the perception of chemical ligands, how *Gr8a*, a member of a canonical chemoreceptor family might also contribute to the production of pheromonal signals is not as obvious. In some more well-understood secretory cell types, autoreceptors are essential for the regulation of synthesis and secretion rates. For example, dopaminergic and serotonergic cells regulate rates of synthesis and release of their respective neuromodulators by the action of autoreceptors, which are used as sentinels that regulate synthesis rates via specific molecular signaling feedbacks in response to level changes in the extracellular concentrations of the secreted molecule (Ford, 2014; Stagkourakis et al., 2016). Therefore, we hypothesize that one possible way *Gr8a* could regulate the synthesis and/or secretion of specific CHCs is by acting as an oenocyte-intrinsic autoreceptor, which integrates feedback information to the complex genetic network that regulates the epicuticular CHC profile of males (Fig. 4).

Previous work indicated that *Gr8a* is expressed in “bitter” taste neurons in the proboscis, and specifically required for the sensory perception of the feeding deterrent L-canavanine (Lee et al., 2012; Shim et al., 2015), but not for the detection of other inhibitory chemicals such as caffeine, strychnine, and umbelliferone (Lee et al., 2009; Poudel et al., 2015). Our data indicate that *Gr8a* contributes to inhibitory functions associated with both feeding and mating decisions, as was previously reported for other ‘bitter’ receptors in *Drosophila* (Lacaille and Hiroi, 2007; Moon et al., 2009). *Gr8a*-

depedent perception of L-canavanine seems to depend on its heterotrimeric interaction with *Gr66a* and *Gr98b* in bitter sensing neurons in the proboscis (Shim et al., 2015). Although both of these *Gr* genes were also identified in our initial screen for receptors enriched in the abdomen, *Gr66a* was expressed in both males and females while *Gr98b* was specifically enriched in females (Table S1). These data suggest that *Gr8a*-depedent contributions to feeding and mating decisions are independently mediated via interactions with different *Gr* genes in each sensory context, and further point to plastic subunit composition as an essential aspect of the mechanism for ligand specificity in insect gustatory receptors. Although we do not know yet the specific chemical identity of the ligand of *Gr8a*, previous studies indicated that at least two inhibitory mating pheromones, cVA and CH503, are transferred from males to females during copulations. Although our data suggest that the *Gr8a* mutation affects the level of cVA expressed by males, it is not likely that either cVA or CH503 are the putative *Gr8a* ligands because the volatile cVA acts primarily via the olfactory receptor *Or67d* (Benton et al., 2007; Datta et al., 2008; Kurtovic et al., 2007), and CH503 has been reported to signal via *Gr68a*-expressing neurons, which are anatomically distinct from the *Gr8a* GRNs we describe here (Figure 1A-B) (Shankar et al., 2015; Yew et al., 2009). Therefore, we anticipate that future studies will identify additional male CHCs that function as inhibitory mating signals.

As populations diversify, both pheromonal signals and their receptors have to co-evolve to maintain fitness via mate choice (Boake, 1991; Symonds and Elgar, 2008). However, because pheromone production and perception are anatomically and physiologically distinct biological processes, how they might be linked genetically at the molecular and physiological levels remains unknown for most studied mating communication systems. Several theoretical models have previously stipulated that one possible solution to this conundrum is a genetic linkage between signal production and associated mate choice behaviors, which supports the co-evolution of mating signals with their cognate receptors (Arnold et al., 1996; Lande, 1981; Svensson et al., 2007), *via* mechanisms such as pleiotropy and the co-segregation of linked alleles (Boake, 1991; Bousquet et al., 2012; Butlin and Ritchie, 1989; Lofstedt et al., 1989; Shaw et al., 2011; Singh and Shaw, 2012). The studies we present here do not directly address the possible

contribution of *Gr8a* to the rapid evolution of mating signals across *Drosophila*. Nevertheless, phylogenetic analyses of *Gr8a* orthologs indicate that this is a conserved, sexually dimorphic receptor across *Drosophila* (Figure S2A-B). Furthermore, analysis of the *Gr8a* protein alignment revealed that in spite of the high overall sequence conservation, the *Gr8a* protein includes at least one phylogenetically variable domain, (Figure S2C-D).

Studies in other animal species suggest that receptor pleiotropy might also play a role in mating communication via other sensory modalities including auditory communication in crickets (Hoy et al., 1977a; Wiley et al., 2011) and visual communication in fish (Fukamachi et al., 2009). While the specific genes and signaling pathways that mediate communication in these species are mostly unknown, these data suggest that the genetic coupling of signal-receptor pairs in mating communication systems might be more common than previously thought. Therefore, the genetic tractability of *D. melanogaster*, in combination with the diversity of mating communication systems in this species-rich phylogenetic group, provide a unique opportunity for understanding the evolution and mechanisms that drive and maintain mating communication systems at the genetic, molecular, and cellular levels.

AUTHOR CONTRIBUTIONS

K.M.Z., C.V., J.G.M. and Y.B-S designed experiments. K.M.Z., C.V., X.L., S.H., J.G.M. and Y.B-S collected and analyzed data. K.M.Z., C.V. and Y.B-S wrote the manuscript.

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Figures and figure legends:

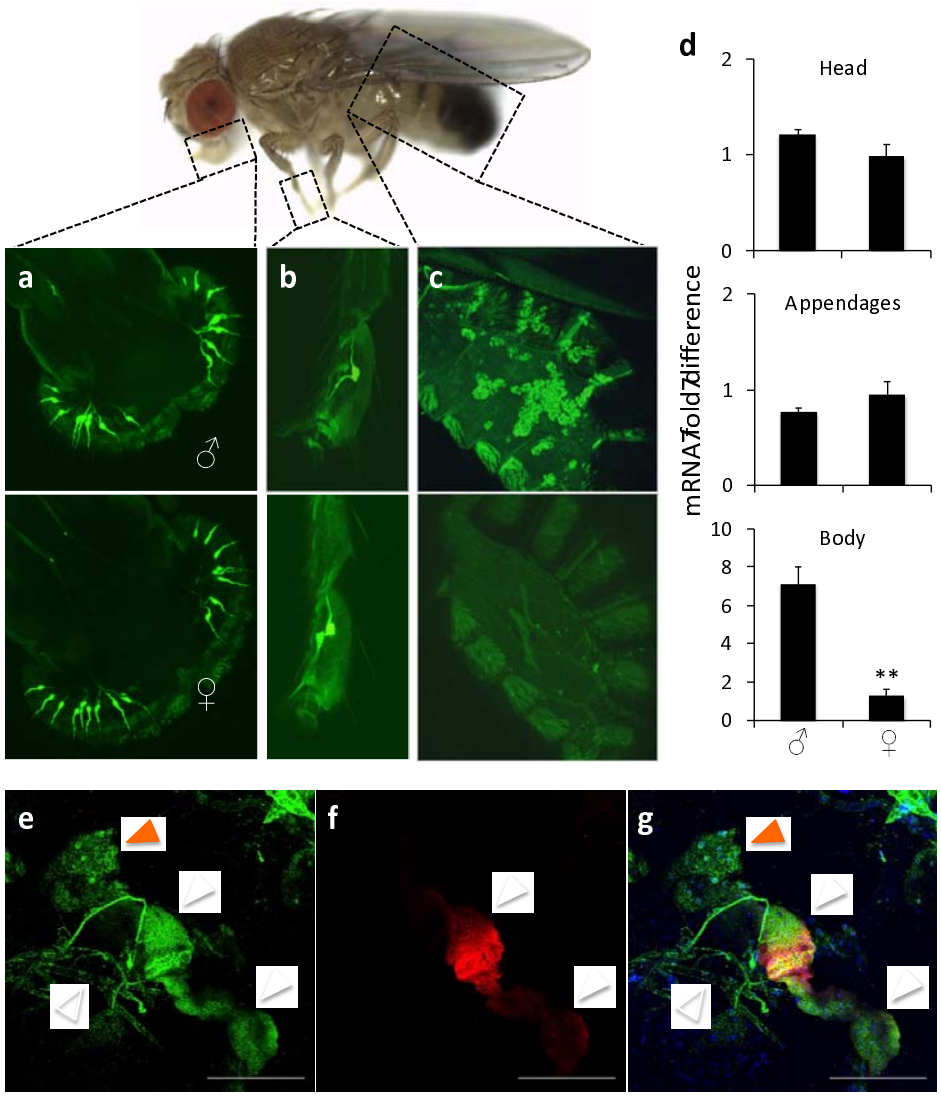

Figure 1. ***Gr8a* is a sexually dimorphic chemosensory receptor enriched in male oenocytes**. (A) *Gr8a* promoter activity in proboscis, (B) forelegs, and (C) abdomens of males (top panels) and females (bottom panels). (D) *Gr8a* mRNA expression. Relative mRNA levels were measured by real-time quantitative RT-PCR. **, p<0.01 Mann Whitney Rank Sum Test. (E) Confocal z-stack image of *Gr8a*>EGFP in abdominal cells. (F) Confocal z-stack image of *desat1*>Luciferase in abdominal cells. (g) Co-expression of *Gr8a* and *desat1*. Green, *Gr8a*; Red, *desat1*; Blue, nuclear DAPI stain. Orange arrowhead, fat body cells; white arrowhead, oenocytes. Scale bar = 100µm.

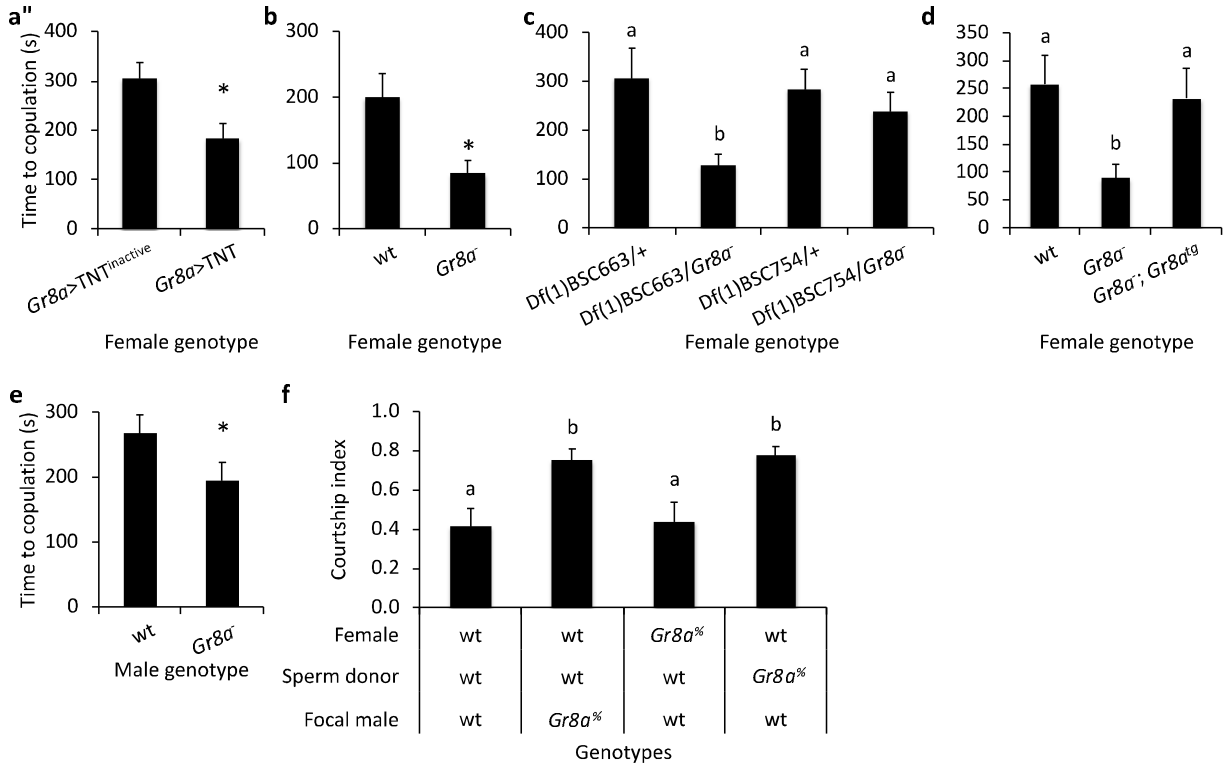

Figure 2. *Gr8a* activity contributes to the perception and production of inhibitory signal associated with mating decisions in males and females. (A) Blocking neural activity in female *Gr8a*-expressing sensory neurons shortens copulation latency. Homozygous (B) or hemizygous (C) *Gr8a* null females show shortened copulation latency relative to wild-type controls. Df(1)BSC663 is a deficiency that covers the *Gr8a* locus. Df(1)BSC754 was used as a control. (D) Expression of *Gr8a* cDNA with *Gr8a* promoter rescues the copulation latency phenotype in *Gr8a* mutant females. (E) Wild-type females exhibit shorter copulation latency when courted by *Gr8a* mutant relative to wild-type males. (F) *Gr8a* mutant males do not recognize mating status of females, and have a reduced transfer of inhibitory mating pheromones during copulations. Female, female genotype; Sperm donor, genotype of males mated first with focal females; Focal male, genotypes of experimental males presented with mated females. Different letters above bars indicate statistically significant *post hoc* contrasts between groups (panels C, D, and F; $p<0.05$ ANOVA, $n>15$/group). *, $p<0.05$, Mann Whitney Rank Sum Test, $n>15$/group)

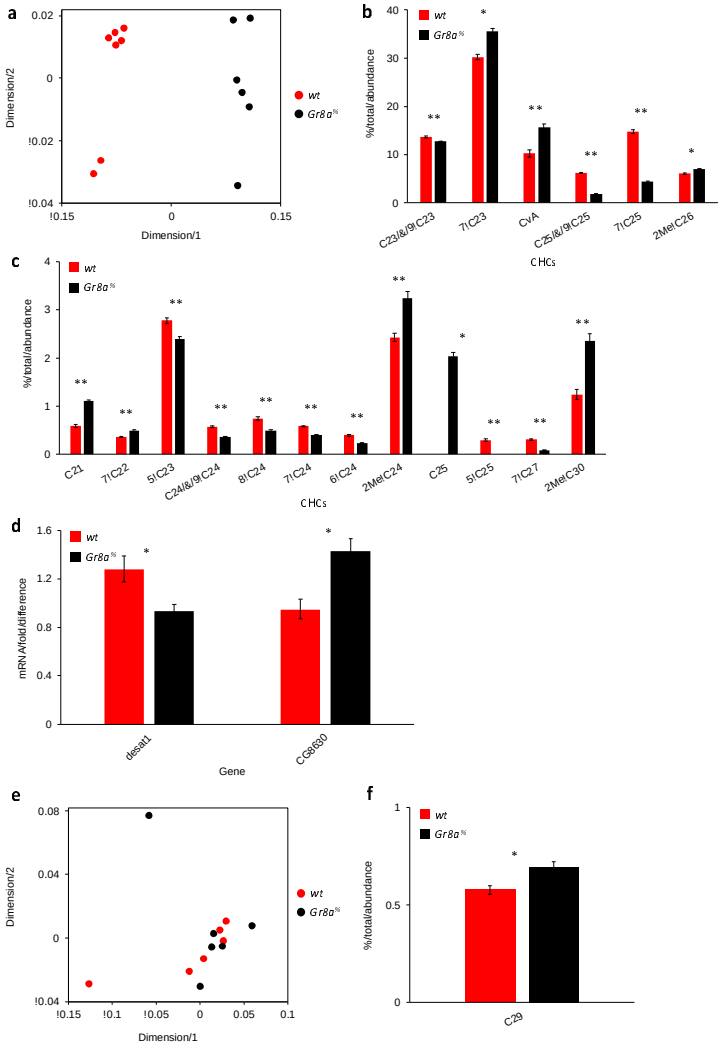

Figure 3. The *Gr8a* mutation affects the pheromone profiles of males and mated females. (A) Nonmetric multidimensional scaling (NMDS) plot of CHC profiles of wild-type and *Gr8a* mutant males. p<0.001, permutation MANOVA. (B-C) The effect of the *Gr8a* mutation on levels of individual CHCs in males. Only affected CHCs shown. See Table S2 for the complete list. *, p<0.05, **, p<0.001, Student's t-test or Mann Whitney Rank Sum Test. (D) The effect of Gr8a mutation on the expression level of several desaturase genes. Only affected genes shown. See Table S3 for the complete list. *, p<0.05, Student's t-test. (E) NMDS plot of CHC profiles of females mated with wild-type or *Gr8a* mutant males. p=0.570, permutation MANOVA. (F) Nonacosane (C_{29}) differs between females mated with wild-type and *Gr8a* mutant males. See Table S4 for complete list of mated-female CHCs. *, p<0.05, Student's t-test.

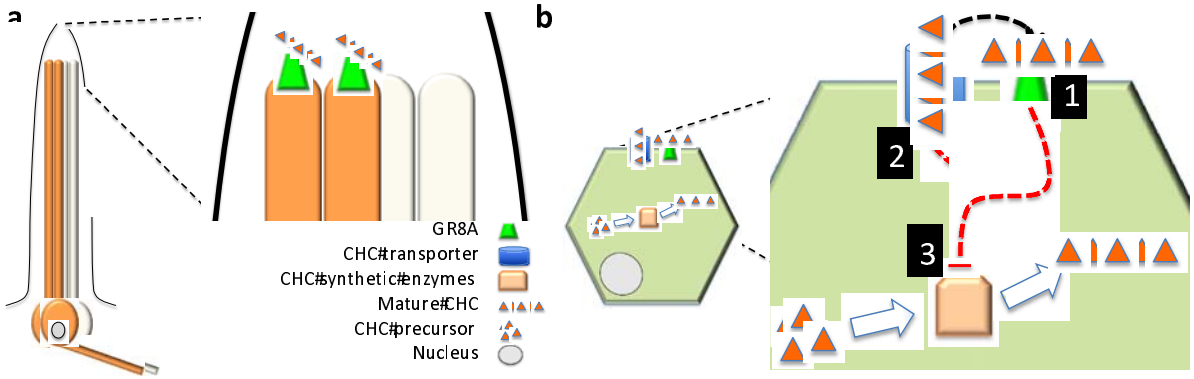

Figure 4. Model for the pleiotropic action of *Gr8a* in the perception and production of pheromones. (A) *Gr8a* functions as a chemoreceptor for an inhibitory signal in pheromone-sensing GRNs of males and females. (B) *Gr8a* also functions as a CHC autoreceptor in oenocytes, which regulates CHC secretion [1] or CHC synthesis [2] via signaling feedback loops [3].

Methods

Animals. Flies were maintained on a standard cornmeal medium under a 12:12 light-dark cycle at 25 Celsius. Unless specifically stated, the *D. melanogaster Canton-S (CS)* strain served as wild-type control animals. UAS-mCD8::GFP, UAS-myr::GFP*,* UAS-TNT-E, UAS-TNT-IMP-V1-A, *Gr8a-GAL4* and *Gr8a*^{1} fly lines were from the Bloomington Stock center. Originally in the *w*^{1118} background, the *Gr8a*^{1} *null* allele was outcrossed for six generations into the *CS* wild-type background. *PromE(800)-GAL4* and *PromE(800)>Luciferase* were from Joel Levine at the University of Toronto. The following *Drosophila* species were obtained from the San Diego Stock Center: *D. simulans* 14011-0251.192, *D. sechellia* 14021-0248.03, *D. yakuba* 14021-0261.01, *D. erecta* 14021-0224.00, *D. ananassae* 14024-0371.16, *D. pseudoobscura* 14011-0121.104, D. *persimilis* 14011-0111.50, *D. willistoni* 14030-0811.35, *D. mojavensis* 15081-1352.23, and *D. virilis* 15010-1051.118. The UAS-*Gr8a* transgenic line was generated by cloning the *Gr8a* cDNA in the pUAST-attB vector using 5' EcoRI and 3' NotI restriction sites, followed by *ΦC31* integrase-dependent transgene integration at a Chromosome 2 *att*P landing site (2L:1476459) as previously described (Zheng et al., 2014). For genetic rescue experiments, the UAS-*Gr8a* and *Gr8a*-GAL4 lines were transgressed into the *Gr8a*^{1} CS genetic background.

Immunohistochemistry. To visualize the expression pattern of *Gr8a* in males and females, *Gr8a-GAL4* flies (Lee et al., 2012) were crossed to UAS-CD8::EGFP and live-imaged at 5 days old using a Nikon-A1 confocal microscope. To demonstrate *Gr8a* expression in oenocytes, abdomens from *Gr8a*-GAL4/UAS-*myr::GFP ; PromE(800)>Luciferase* flies were dissected and immunostained as previously described (Lu et al., 2012; Zheng et al., 2014) by using a Rabbit anti-GFP (1:1000; A-11122, Thermo Fisher Scientific) and a mouse anti-luciferase (1:100; 35-6700, Thermo Fisher Scientific) antibodies followed by AlexaFluor 488 anti-rabbit and AlexaFluor 568 anti-mouse secondary antibodies (Both at 1:1000; Thermo Fisher Scientific).

mRNA expression. Newly eclosed flies were separated by sex under CO_2 and aged for 5 days on standard cornmeal medium. On day 6, flies were placed in a -80°C freezer until RNA extraction. To separate body parts, frozen flies were placed in 1.5ml microcentrifuge tubes, dipped in liquid nitrogen, and then vortexed repeatedly until heads, appendages, and bodies were clearly seperated. Total RNA was extracted using the Trizol Reagent (Thermo Fisher Scientific) separately from heads, bodies, and appendages for *Gr8a* expression and from bodies for desaturase enzyme genes. cDNAs were synthesized using SuperScript II reverse transcriptase (Thermo Fisher Scientific) with 500 ng total RNA in a 20 uL reaction. Real-time quantitative RT-PCR was carried out as previously described with *Rp49* as the loading control gene (Lu et al., 2012; Lu et al., 2014; Zheng et al., 2014). Primer sequences are described in Tables S5, S6 and S7.

Courtship Behavior. Single-pair assays were performed as we have previously published (Lu et al., 2012; Lu et al., 2014). In short, newly eclosed males were kept individually on standard fly food in plastic vials (12 x 75mm). Newly eclosed virgin females were kept in groups of 10 flies. All behaviors were done with 4-7 day-old animals, which were housed under constant conditions of 25° C and a 12h:12h light-dark cycle. Courtship was video recorded for 10 min for male courtship and 15 min for female mating receptivity. Male courtship latency and index were measured as previously described (Lu et al., 2012; Lu et al., 2014). Female receptivity index was defined as the time from the initiation of male courtship until copulation was observed. Unless otherwise indicated, assays were performed under red light conditions.

Fitness measures. Three-day old virgin male-female pairs were housed together for 24 hours after which males were discarded. Subsequently, groups of five females were allowed to lay eggs daily on fresh agar grape plates for five consecutive days. Fitness was measured as total number of eggs laid.

Phylogenetic analysis. Protein sequences of GR8A orthologs from the 12 sequenced *Drosophila* reference genomes were aligned by using the ClustalW algorithm in the Omega package (Sievers et al., 2011), followed by ProtTest (v2.4) to determine the best model of protein evolution (Abascal et al., 2005). Subsequently, Akaike and Bayesian information criterion scores were used to select the appropriate substitution matrix. We then used a maximum likelihood approach and rapid bootstrapping within RAxML v 7.2.8 Black Box on the Cipres web portal to make a phylogenetic tree (Miller et al., 2010). Visualizations of the bipartition files were made using FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

Pheromone Analysis. Virgin flies were collected upon eclosion under a light CO^2 anesthesia and kept in single-sex vials in groups of 10 with 6 biological replications for each genotype and sex. Virgin flies were aged for 5 days on standard cornmeal medium at 25°C. To collect mated flies, both females and males were aged for 3 days before single mating pairs were placed in a standard fly vial with standard cornmeal food for 24 hours. The pair was then separated for 24 hours before collection. Copulation was confirmed by the presence of larvae in the vials of mated females several days later. On the morning of day 5, flies were anesthetized under light CO^2 and groups of five flies were placed in individual scintillation vials (VWR 74504-20). To extract CHCs, each group of flies was covered by 100 uL hexane (Sigma-Aldrich #139386-500ML) containing 50µg/mL hexacosane (Sigma-Aldrich #241687-5G) and was washed for ten minutes. Subsequently, hexane washes were transferred into a new 2 ml glass vial containing a 350 uL insert (Thermo Scientific C4000-LV-1W) and were stored at -20°C until shipment to the Millar laboratory.

Analyses of CHC profiles were done by gas chromatography and mass spectroscopy (GC-MS) in the Millar laboratory at UC Riverside as previously described (Chung et al., 2014). Peak areas were measured, and data was normalized to known quantity of internal standard hexacosane (Sigma-Aldrich #241687-5G). The relative proportion of each compound in each sample was calculated and used in further statistical analysis. Qualitative data were analyzed through a permutation MANOVA using the ADONIS

function in the vegan package of R with Bray-Curtis dissimilarity measures (Oksanen et al., 2017). Data were visualized using non-metric multidimensional scaling (vegdist function in the vegan package of R followed by cmdscale function in the stats package (Oksanen et al., 2017)) using Bray-Curtis dissimilarity, and either 2 or 3 dimensions in order to minimize stress to < 0.1. Quantitative data were analyzed by using a t-test or Mann-Whitney Rank Sum Test in R 3.3.2 (R Core Team, 2016).

Data availability. All relevant data are available from the corresponding author upon request.

Supplemental Table Legends

Table S1. Candidate *Gr* genes in male and female abdomen. Plus sign indicates presence of PCR product and minus signs indicates PCR product not detected. Genes with no PCR product detected in male or female abdomens not shown.

Table S2. Male CHCs. Retention time (R.T.), compound, and percent total (% total) of each compound as part of the total pheromonal bouquet for wild-type (wt) and *Gr8a* mutant males.

Table S2. Male CHCs. Retention time (R.T.), compound, and percent total (% total) of each compound as part of the total pheromonal bouquet for wild-type (wt) and *Gr8a* mutant males.

Table S3. Desaturase gene expression. Relative mRNA expression of each desaturase gene for wild-type (wt) and *Gr8a* mutant males.

Table S4. Mated-female CHCs. Retention time (R.T.), compound, and percent total (% total) of each compound as part of the total pheromonal bouquet for females mated with wild-type (wt) or *Gr8a* mutant males.

Table S5. Nucleotide sequences for qRT-PCR primers for *D. melanogaster Gr* genes.

Table S6. Nucleotide sequences for qRT-PCR primers for *D. melanogaster Gr8a*, *Rp49* and orthologs.

Table S7. Nucleotide sequences for qRT-PCR primers for *D. melanogaster* desaturase enzyme genes