Chemoreceptor pleiotropy facilitates the functional coupling of mating pheromone synthesis and perception

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ABSTRACT

Optimal mating decisions depend on stable, coupled signaling systems because any independent changes in either the signal or its perception could carry a fitness cost. However, since the perception and production of specific mating signals are often mediated by different tissues and cell types, the genetic and cellular mechanisms that drive their coupling on the evolutionary and physiological timescales remain a mystery for most animal species. Here we show that in *Drosophila melanogaster,* sensory perception and synthesis of an inhibitory mating pheromone is regulated by the action of *Gr8a*, a member of the *Gustatory receptor* gene family. In particular, *Gr8a* acts as a neurosensory receptor for pheromones in males and females and independently regulates their synthesis in the male fat body and pheromone-producing oenocytes. These data provide a relatively simple molecular explanation for how genetic coupling allows for the robust and stable flow of social information at the population level.

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

INTRODUCTION

The majority of sexually-reproducing animals use intricate mating signaling systems, which rely on the robust functional coupling between the production and perception of signals since any independent changes in either the signal or the capacity to sense it would carry a fitness cost (Boake, 1991; Brooks et al., 2005; Hoy et al., 1977; Shaw et al., 2011; Shaw and Lesnick, 2009; Steiger et al., 2011; Sweigart, 2010; Symonds and Elgar, 2008; Wyatt, 2014). Previously published theoretical models have predicted that one possible solution for maintaining the functional coupling of signal production and perception is through genetic coupling, in which physically linked or pleiotropic genes control both mating signal production and perception (Boake, 1991; Butlin and Ritchie, 1989; Shaw et al., 2011; Shaw and Lesnick, 2009). Genetic coupling promotes a genetic correlation between signal and receptor (Chebib and Guillaume, 2019; Kirkpatrick and Hall, 2004; Lande, 1984, 1981, 1980), which provides a robust molecular mechanism for maintaining stable and reliable population-level communication systems while still retaining the capacity for future signal diversification, as necessitated for speciation (Hoy et al., 1977; Shaw et al., 2011; Shaw and Lesnick, 2009; Wiley et al., 2012). While previous models suggest these two genetic architectures should function similarly in maintaining signal-receptor genetic correlations (Lande, 1984), a recent model shows that pleiotropy, in which a single gene controls more than one phenotypic trait, maintains a higher signal-receptor genetic correlation than gene linkage in the face of evolutionary processes (Chebib and Guillaume, 2019). This suggests that pleiotropy may play a particularly robust and important role in maintaining stable signal-receptor coupling in animal mating systems.

Several experimental studies have provided empirical evidence that pleiotropy maintains functional coupling between mating-specific signals and the behaviors they elicit at the population level (Hoy et al., 1977; Shaw et al., 2011; Shaw and Lesnick, 2009; Wiley et al., 2012). However, the complex characteristics of mating signals and animal behaviors present a major barrier for identifying the actual pleiotropic genes and molecular pathways that provide genetic coupling between the production and perception of mating signals (Chenoweth and Blows, 2006; Singh and Shaw, 2012). Furthermore, how the perception and production of mating signals remains stably coupled is particularly

66 puzzling for mating pheromones, since the sensing of specific chemicals is mediated by
67 the action of the chemosensory peripheral nervous system, while their production is often
68 restricted to specialized, non-neuronal pheromone producing cells (Chung and Carroll,
69 2015; McKinney et al., 2015; Mucignat-Caretta, 2014). Therefore, whether pleiotropy
70 could function in both the production and perception of mating pheromones, a ubiquitous
71 means of signaling across animal mating systems (Mucignat-Caretta, 2014), remained
72 unknown.

73 Here we show that, in the vinegar fly *Drosophila melanogaster*, some pheromone-driven
74 mating decisions are coupled to the production of specific pheromones via pleiotropic
75 chemoreceptors. Specifically, we demonstrate that *Gr8a*, a member of the gustatory
76 receptor family, independently contributes to the perception of inhibitory mating signals in
77 pheromone-sensing neurons, as well as the production of inhibitory mating pheromones
78 in pheromone producing cells called oenocytes. Together, these data provide a relatively
79 simple molecular explanation for how the perception and production of specific mating
80 pheromones are functionally and genetically coupled.

81

82 **RESULTS**

83 Similar to other insect species, in *Drosophila,* cuticular hydrocarbons (CHCs), or long-
84 chain fatty acids synthesized by the fat body and oenocytes (Billeter et al., 2009; Gutierrez
85 et al., 2007; Krupp et al., 2013; Makki et al., 2014), provide a hydrophobic desiccation
86 barrier for the insect body and play an important role as pheromones in regulating diverse
87 behaviors, including those used in mating (Blomquist and Bagnères, 2010; Chung and
88 Carroll, 2015; Ferveur, 2005; Howard and Blomquist, 2005; McKinney et al., 2015).
89 Specifically, complex blends of CHCs are often utilized by insect species to communicate
90 sex identity and female mating status, as well as to define the behavioral reproductive
91 boundaries between closely related species (Billeter et al., 2009; Chung et al., 2014;
92 Chung and Carroll, 2015; Coyne et al., 1994; Dweck et al., 2015; Ng et al., 2014; Shirangi
93 et al., 2009; Yew and Chung, 2015). In *Drosophila*, the perception of volatile CHCs is
94 mediated by olfactory sensory neurons located in the antennae and maxillary palps
95 (Benton et al., 2007; Kurtovic et al., 2007; Lebreton et al., 2014; van der Goes van Naters

96 and Carlson, 2007), while less volatile CHCs, whose perception requires physical contact,
97 are sensed by specialized gustatory-like receptor neurons (GRNs) in the appendages
98 (legs and wings) and the proboscis (Koh et al., 2015; Lu et al., 2014, 2012; Thistle et al.,
99 2012; Toda et al., 2012). While some of the genes and pathways that contribute to CHC
100 synthesis in *Drosophila* are known, the molecular identities of most CHC receptors remain
101 unknown. Previous work suggested that the gene *Desat1*, which encodes a fatty acid
102 desaturase enriched in oenocytes, might also independently contribute to CHC
103 perception (Bousquet et al., 2011). However, due to the expression of *Desat1* in central
104 neurons (Billeter et al., 2009) and the broad impact its mutant alleles have on the CHC
105 profiles of both males and females (Labeur et al., 2002), whether *Desat1* directly
106 contributes to mating signal perception remains unresolved.

107 Consequently, we chose to examine members of the *Gustatory receptor* (*Gr*) gene family
108 as candidates for pleiotropic factors that might contribute directly to both the perception
109 and production of pheromonal mating signals in *Drosophila*. Because several family
110 members have already been implicated in the detection of specific excitatory and
111 inhibitory pheromones (Bray and Amrein, 2003; Hu et al., 2015; Miyamoto and Amrein,
112 2008; Moon et al., 2009; Watanabe et al., 2011), and the majority of genes that encode
113 family members are already known to be enriched in GRNs (Clyne et al., 2000; Dunipace
114 et al., 2001; Scott et al., 2001; Wang et al., 2004), we reasoned that any pleiotropic *Gr*
115 genes should also be expressed in abdominal oenocytes (Billeter et al., 2009).
116 Subsequently, an RT-PCR screen revealed that 24 out of the 60 genes that encode *Gr*
117 family members in the *Drosophila* genome (Clyne et al., 2000; Dunipace et al., 2001;
118 Robertson et al., 2003; Scott et al., 2001) exhibit enriched expression in abdominal
119 tissues (Table 1).

120 Although several members of the *Gr* receptor family, including *Gr68a, Gr32a, Gr66a,*
121 *Gr39a*, and *Gr33a*, were previously linked to the sensory perception of mating
122 pheromones (Bray and Amrein, 2003; Lacaille et al., 2009; Miyamoto and Amrein, 2008;
123 Moon et al., 2009; Watanabe et al., 2011), none of these candidate pheromone receptors
124 were identified in our initial screen for *Gr* genes with enriched mRNA expression in
125 abdominal tissues of either males or females (Table 1). However, *Gr8a*, which we
126 identified as having male-specific expression in the abdomen, is localized to a

127 chromosomal region with enriched expression in abdomens that was previously
128 implicated in chemoreception (Table 1) and was shown to contribute to the sensory
129 detection of the non-proteinogenic amino acid L-Canavanine (Lee et al., 2012; Shim et
130 al., 2015). Because our initial expression screen was based on whole-abdomen RNAs,
131 and it was previously reported that *Gr8a* is also expressed in some abdominal neurons
132 (Park and Kwon, 2011), we next used a GAL4 transgenic driver to examine the spatial
133 expression pattern of *Gr8a* in male and female flies. As was previously reported, we found
134 that *Gr8a* is expressed in 14-16 GRNs in the proboscis (Figure 1A) (Lee et al., 2012) as
135 well as in two paired GRNs in the foreleg pretarsus (Figure 1B) of male and female flies.
136 In addition, the *Gr8a*-GAL4 reporter was also highly expressed in abdominal oenocyte-
137 like cells in males but not females (Figure 1C). The male-specific abdominal expression
138 pattern was further supported by qRT-PCR (Figure 1D). Using immunohistochemistry, we
139 then demonstrated that *Gr8a* is co-expressed with the oenocyte marker *Desat1* (Billeter
140 et al., 2009), as well as in *Desat1*-negative cells with fat-body-like morphology (Figure
141 1E-G). Finally, by using immunohistochemistry on a CRISPR/Cas9 generated GFP
142 tagged allele of the *Gr8a* locus, we demonstrated that the GR8A protein localizes to the
143 fat body (Figure 1H).

144 Together, the enriched expression of *Gr8a* in foreleg GRNs, a primary pheromone
145 chemosensory organ (Lu et al., 2014, 2012), and its sexually dimorphic expression in
146 oenocytes and fat body suggested that in addition to its role in L-Canavanine perception
147 (Lee et al., 2012; Shim et al., 2015), *Gr8a* contributes to mating behaviors. Since mating
148 choices of both male and female flies are determined by a complex blend of excitatory
149 and inhibitory pheromones (Billeter et al., 2009; Krupp et al., 2013), we anticipated that if
150 *Gr8a* is indeed a pleiotropic factor that independently contributes to the perception and
151 production of specific components of the male mating pheromone bouquet, then
152 disruptions of the signal production in males or its perception in females should carry
153 similar impacts on female mate choice behavior. To directly test this hypothesis, we first
154 investigated whether *Gr8a* and the GRNs that express it are required for sensory
155 functions associated with female mate choice by using a single-pair courtship paradigm
156 (Lu et al., 2014, 2012). We found that blocking neuronal transmission in *Gr8a*-expressing
157 GRNs with the transgenic expression of the tetanus toxin (TNT) in females resulted in a

158 shorter copulation latency relative to wild-type females when courted by wild-type males
159 (Figure 2A). Similarly, homozygous (Figure 2B) and hemizygous (Figure 2C) *Gr8a* mutant
160 females exhibited shorter copulation latencies relative to wild-type controls, which could
161 be rescued by the transgenic expression of a *Gr8a* cDNA (Figure 2D). In contrast, similar
162 genetic manipulations of the *Gr8a* gene or *Gr8a*-expressing GRNs in males did not affect
163 courtship behavior as measured by courtship latency and index towards wild-type females
164 (Figure 2- figure supplement 1). These data indicate that *Gr8a* is required for female mate
165 choice via the detection of male-borne inhibitory mating signals.

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

172 Mating decisions in *D. melanogaster* rely on a balance of excitatory and inhibitory drives
173 (Kallman et al., 2015). Therefore, in this case, male-borne inhibitory signals may promote
174 female mate choice. Additionally, previous studies showed that, in order to increase their
175 fitness, *Drosophila* males transfer inhibitory mating pheromones to females during
176 copulation, which subsequently lowers the overall attractiveness of mated females to
177 other males and the probability of females remating (Benton et al., 2007; Billeter et al.,
178 2009; Datta et al., 2008; Yew et al., 2009). Because our data indicate that *Gr8a* plays a
179 role in the production of an inhibitory signal in males, we reasoned that the same inhibitory
180 signal may be transferred from males to females during copulation. Therefore, we next
181 tested the hypothesis that *Gr8a* mutant males would have a reduced ability to produce
182 and/or transfer inhibitory pheromones to females during copulation. Accordingly, we
183 found that wild-type males failed to recognize the mating status of wild-type females that
184 were previously mated with *Gr8a* mutant males (Figure 2F). These data indicate that *Gr8a*
185 action is required for the production and/or transfer of inhibitory pheromones from males
186 to females during copulation. Because *Gr8a* is involved in the perception of an inhibitory
187 mating signal, we also tested the hypothesis that *Gr8a* mutant males will exhibit an
188 abnormal capacity for identifying the post-mating status of wild-type females. Indeed, we

189 observed that *Gr8a* mutant males were more likely than wild type males to court a mated
190 female (Figure 2F), suggesting that in addition to its role in the production of inhibitory
191 mating signals, *Gr8a* is also required in males for the sensory recognition of mating
192 inhibitory signals that mark the post-mating status of females. Together, these studies
193 indicate that *Gr8a* is required in males for the production and perception of transferrable
194 inhibitory mating signals that advertise post-mating status in females. These data further
195 support the pleiotropic role of the *Gr8a* gene in the production of inhibitory mating signals
196 in males and the perception of these signals in both males and females in sex-specific
197 contexts.

198 Because our behavioral data indicate that the *Gr8a* mutation has a dramatic effect on the
199 production and perception of a putative inhibitory mating pheromonal signal, we next
200 examined whether the *Gr8a* mutation has a direct effect on qualitative and quantitative
201 aspects of male and mated-female CHC profiles. As predicted by our behavioral data,
202 analyses of male epicuticular pheromones by gas chromatography and mass
203 spectroscopy revealed that the *Gr8a* mutation has a significant impact on the overall
204 qualitative characteristics of the CHC profile of males (Figure 3A). Additional analyses
205 revealed significant quantitative effects of the *Gr8a* mutation on the cuticular levels of
206 several specific CHCs in males (Figure 3B-3C and Table 2). In particular, the *Gr8a*
207 mutation affects the levels of several alkenes and methyl-branched alkanes, which have
208 been implicated in mate choice behaviors in diverse *Drosophila* species (Billeter et al.,
209 2009; Chung et al., 2014; Dyer et al., 2014; Shirangi et al., 2009). Similarly, *Gr8a* mutation
210 affects the expression level of two desaturase enzymes in males (Figure 3D), which are
211 involved in the biosynthesis of alkenes (Chung and Carroll, 2015). Additionally, although
212 the CHC profile of females mated with *Gr8a* mutant males did not qualitatively differ from
213 those mated with wild-type males (Figure 3E), one specific, lowly-expressed component
214 of the CHC profile, nonacosane (C_{29}), differed quantitatively between the two mated-
215 female groups (Figure 3F). In addition to these identified CHCs, *Gr8a* may have a direct
216 effect on other pheromones that are unable to be measured. Together, our behavioral
217 and pheromonal data indicate that *Gr8a* action contributes to mating decisions in females
218 by co-regulating the perception of an inhibitory mating pheromone by females, as well as
219 its production by males, which is consistent with a pleiotropic function for *Gr8a*.

220 Next, we sought to identify the *Gr8a*-associated inhibitory component of the male
221 *Drosophila* CHC profile by testing the effect of the specific CHCs impacted by the *Gr8a*
222 mutation on courtship behaviors. By measuring female copulation latency in a single-pair
223 courtship paradigm (Lu et al., 2014, 2012), we found that wild-type females did not
224 copulate with *Gr8a* mutant males that were first perfumed with an exaggerated amount
225 of the alkenes 9-C25, 7-C25, and 7-C27 (Figure 4). Similarly, we found that wild-type
226 males exhibited a lower courtship index and longer courtship latency when females were
227 perfumed with an exaggerated amount of 9-C_{25} (Figure 5A-C), and a longer copulation
228 latency when females were perfumed with an exaggerated amount of 7-C_{25} (Figure 5D-
229 5F), but no differences when females were perfumed with an exaggerated amount of 7-
230 C_{27} (Figure 5G-I). These data suggest that the alkenes 7-C_{25}, 9-C_{25}, and 7-C_{27} may
231 contribute to an inhibitory signal associated with *Gr8a*.

232

233 **DISCUSSION**

234 The data presented here demonstrate that a pleiotropic chemoreceptor co-regulates the
235 perception and production of a specific pheromonal signal that plays an important role in
236 mate choice behaviors of both sexes. In contrast to its expected function in the perception
237 of chemical ligands, how *Gr8a*, a member of a canonical chemoreceptor family might also
238 contribute to the production of pheromonal signals is not obvious. In some of the better
239 understood scretory cell types, autoreceptors are essential for the regulation of synthesis
240 and secretion rates. For example, dopaminergic and serotonergic cells regulate rates of
241 synthesis and release of their respective neuromodulators. This is accomplished by the
242 action of autoreceptors, which are used as sentinels that regulate synthesis rates via
243 specific molecular signaling feedbacks in response to level changes in the extracellular
244 concentrations of the secreted molecule (Ford, 2014; Stagkourakis et al., 2016).
245 Therefore, we hypothesize that one possible way *Gr8a* might regulate the synthesis
246 and/or secretion of specific CHCs is by acting as an oenocyte-intrinsic autoreceptor,
247 which integrates feedback information to the complex genetic network that regulates the
248 epicuticular CHC profile of males (Figure 6).

249 Previous work indicated that *Gr8a* is expressed in "bitter" taste neurons in the proboscis
250 and are specifically required for the sensory perception of the feeding deterrent L-
251 canavanine (Lee et al., 2012; Shim et al., 2015), but not for the detection of other inhibitory
252 chemicals such as caffeine, strychnine, and umbelliferone (Lee et al., 2009; Poudel et al.,
253 2015). Our data indicate that *Gr8a* contributes to inhibitory functions associated with
254 mating decisions as well, as was previously reported for other 'bitter' receptors in
255 *Drosophila* (Lacaille et al., 2007; Moon et al., 2009). Together this indicates similar
256 function in 'bitter' taste perception and the perception of inhibitory signals in this species.

257 *Gr8a*-dependent perception of L-canavanine seems to depend on its heterotrimeric
258 interaction with *Gr66a* and *Gr98b* in bitter sensing neurons in the proboscis (Shim et al.,
259 2015). Although both of these *Gr* genes were also identified in our initial screen for
260 receptors enriched in the abdomen, *Gr66a* was expressed in both males and females
261 while *Gr98b* was specifically enriched in females (Table 1). These data suggest that *Gr8a*-
262 dependent contributions to feeding and mating decisions are independently mediated via
263 interactions with different *Gr* genes in each sensory context, and further point to plastic
264 subunit composition as an essential aspect of the mechanism for ligand specificity in
265 insect gustatory receptors. Although we do not yet know the specific chemical identity of
266 the ligand of *Gr8a*, previous studies indicated that at least two inhibitory mating
267 pheromones, cVA and CH503, are transferred from males to females during copulations.
268 Although our data suggest that the *Gr8a* mutation affects the level of cVA expressed by
269 males, it is unlikely that either cVA or CH503 are the putative *Gr8a* ligands because the
270 volatile cVA acts primarily via the olfactory receptor *Or67d* (Benton et al., 2007; Datta et
271 al., 2008; Kurtovic et al., 2007), and CH503 has been reported to signal via *Gr68a*-
272 expressing neurons, which are anatomically distinct from the *Gr8a* GRNs we describe
273 here (Figure 1A-B) (Shankar et al., 2015; Yew et al., 2009). Our data indicate that the
274 alkenes 5-C_{25}, 7-C_{25}, and 7-C_{27} differ in abundance between the CHC profiles of wild-type
275 and *Gr8a* mutant males and likely function as inhibitory mating signals. If Gr8a is acting
276 as an autoreceptor, then it is possible that these alkenes are ligands of *Gr8a*. However,
277 future studies are needed to assess the specific relationship of these alkenes with *Gr8a*.

278 As populations diversify, sexual communication systems within a species should be
279 evolutionarily constrained, since any mismatch between signal and its perception would

280 result in a decrease in fitness (Boake, 1991; Symonds and Elgar, 2008). However, sexual
281 isolation between closely related species often relies on distinct sexual signaling systems,
282 suggesting that these systems must undergo frequent and quick divergence (Shaw and
283 Lesnick, 2009). Therefore, how mating signals and their receptors are linked at the
284 molecular and physiological levels but retain the ability for future diversification remains
285 unknown for most studied mating communication systems. Several theoretical models
286 have previously stipulated that one possible solution to this conundrum is a genetic
287 coupling between signal production and associated mate choice behaviors, which
288 supports the co-evolution of mating signals with their cognate receptors in the face of
289 speciation (Arnold et al., 2006; Boake, 1991; Butlin and Ritchie, 1989; Lande, 1981;
290 Lofstedt et al., 1989; Shaw et al., 2011; Shaw and Lesnick, 2009; Singh and Shaw, 2012;
291 Svensson et al., 2007). The studies we present here do not directly address the possible
292 contribution of *Gr8a* to the rapid evolution of mating signals across *Drosophila*.
293 Nevertheless, phylogenetic analyses of *Gr8a* orthologs indicate that this is a conserved,
294 sexually dimorphic receptor across *Drosophila* species (Figure 7A-B). Furthermore,
295 analysis of the *Gr8a* protein alignment revealed that in spite of the high overall sequence
296 conservation, the *Gr8a* protein includes at least one phylogenetically variable domain
297 (Figure 7C-D). This suggests a possible role of *Gr8a* in the divergence of mating
298 pheromone signaling systems across *Drosophila* species, yet more studies are needed
299 to elucidate this.

300 Studies in other animal species suggest that receptor pleiotropy might also play a role in
301 mating communication via other sensory modalities including auditory communication in
302 crickets (Hoy et al., 1977; Wiley et al., 2012) and visual communication in fish (Fukamachi
303 et al., 2009). While the specific genes and signaling pathways that mediate
304 communication in these species are mostly unknown, these data suggest that the genetic
305 coupling of signal-receptor pairs in mating communication systems might be more
306 common than previously thought. Therefore, the genetic tractability of *D. melanogaster*,
307 in combination with the diversity of mating communication systems in this species-rich
308 phylogenetic group, provide a unique opportunity for understanding the evolution and
309 mechanisms that drive and maintain mating communication systems at the genetic,
310 molecular, cellular, and evolutionary levels.

311

312 **MATERIALS AND METHODS**

313 ***Animals.*** Flies were maintained on a standard cornmeal medium under a 12:12 light-dark
314 cycle at 25 Celsius. Unless specifically stated, the *D. melanogaster Canton-S (CS)* strain
315 served as wild-type control animals. UAS-mCD8::GFP, UAS-myr::GFP, UAS-TNT-E,
316 UAS-TNT-IMP-V1-A, *Gr8a-GAL4* and *Gr8a*^{1} fly lines were from the Bloomington Stock
317 center. Originally in the *w*^{1118} background, the *Gr8a*^{1} *null* allele was outcrossed for six
318 generations into the *CS* wild-type background. *PromE(800)-GAL4* and
319 *PromE(800)>Luciferase* were from Joel Levine at the University of Toronto. The following
320 *Drosophila* species were obtained from the San Diego Stock Center: *D. simulans* 14011-
321 0251.192, *D. sechellia* 14021-0248.03, *D. yakuba* 14021-0261.01, *D. erecta* 14021-
322 0224.00, *D. ananassae* 14024-0371.16, *D. pseudoobscura* 14011-0121.104, D.
323 *persimilis* 14011-0111.50, *D. willistoni* 14030-0811.35, *D. mojavensis* 15081-1352.23,
324 and *D. virilis* 15010-1051.118. The UAS-*Gr8a* transgenic line was generated by cloning
325 the *Gr8a* cDNA in the pUAST-attB vector using 5' EcoRI and 3' NotI restriction sites,
326 followed by *ΦC31* integrase-dependent transgene integration at a Chromosome 2 *att*P
327 landing site (2L:1476459) as previously described (Zheng et al., 2014). For genetic
328 rescue experiments, the UAS-*Gr8a* and *Gr8a*-GAL4 lines were transgressed into the
329 *Gr8a*^{1} CS genetic background.

330 The GFP-tagged allele of *Gr8a* was generated via CRISPR/*Cas9*-dependent editing
331 using a modified "scarless" strategy (www.flyCRISPR.molbio.wisc.edu) as described in
332 (Hill et al., 2019) using the sgRNA CGAGCAAGGCGGGAACGATT, and a donor plasmid
333 containing 1) A backbone with ampicillin resistance; 2) Eye-specific dsRed reporter driven
334 by the 3XP3 promoter, flanked by two *PiggyBac* transposase recognition sites; 3-4) The
335 left and right homology arms, which consisted of 1kb genomic DNA fragments upstream
336 and downstream of the sgRNA site respectively. Single base pair mutations were
337 introduced into the PAM sequence at each sgRNA binding site on the homology arms to
338 prevent Cas9-dependent cutting of the donor plasmid. The left homology arm included
339 the GFP coding sequence in the last intracellular loop of the Gr8a protein. pDCC6
340 plasmids containing sgRNA and cas9 sequences (100 ng/μL) and the donor plasmid (500

341 ng/μL) were co-injected into eggs of the *y w* background. Subsequently, correct genomic
342 integration of the GFP tag was verified by screening for DsRed-positive animals, followed
343 by sequencing of genomic PCR fragments. Control lines with the same genetic
344 background were generated by selecting DsRed-negative animals. The final
345 tagged *Gr8a* allele was generated by removing the DsRed cassette via the introduction
346 of the *piggyBac* transposase (Bloomington #8285).

347

348 ***Immunohistochemistry.*** To visualize the expression pattern of *Gr8a* in males and
349 females, *Gr8a-GAL4* flies (Lee et al., 2012) were crossed to UAS-CD8::EGFP and live-
350 imaged at 5 days old using a Nikon-A1 confocal microscope. To demonstrate *Gr8a*
351 expression in oenocytes, abdomens from *Gr8a*-GAL4/UAS-*myr::GFP ;*
352 *PromE(800)>Luciferase* flies were dissected and immunostained as previously described
353 (Lu et al., 2012; Zheng et al., 2014) by using a Rabbit anti-GFP (1:1000; A-11122, Thermo
354 Fisher Scientific) and a mouse anti-luciferase (1:100; 35-6700, Thermo Fisher Scientific)
355 antibodies followed by AlexaFluor 488 anti-rabbit and AlexaFluor 568 anti-mouse
356 secondary antibodies (Both at 1:1000; Thermo Fisher Scientific). To visualize the GR8A
357 protein, abdomens of control flies and flies with CRISPR/Cas9 generated GFP-tagged
358 GR8A were dissected and immunostained as previously described (Lu et al., 2012; Zheng
359 et al., 2014) using a Rabbit anti-GFP antibody (1:1000; A-11122, Thermo Fisher
360 Scientific) followed by AlexaFluor 488 anti-rabbit secondary antibody (1:1000; Thermo
361 Fisher Scientific).

362 ***mRNA expression.*** Newly eclosed flies were separated by sex under CO_2 and aged for
363 5 days on standard cornmeal medium. On day 6, flies were placed in a -80°C freezer until
364 RNA extraction. To separate body parts, frozen flies were placed in 1.5ml microcentrifuge
365 tubes, dipped in liquid nitrogen, and then vortexed repeatedly until heads, appendages,
366 and bodies were clearly separated. Total RNA was extracted using the Trizol Reagent
367 (Thermo Fisher Scientific) separately from heads, bodies, and appendages for *Gr8a*
368 expression and from bodies for desaturase enzyme genes. cDNAs were synthesized
369 using SuperScript II reverse transcriptase (Thermo Fisher Scientific) with 500 ng total
370 RNA in a 20 uL reaction. Real-time quantitative RT-PCR was carried out as previously

371 described with *Rp49* as the loading control gene (Lu et al., 2014, 2012; Zheng et al.,
372 2014). Primer sequences are described in Tables 5, 6 and 7.

373

374 ***Courtship Behavior.*** Single-pair assays were performed as we have previously
375 published (Lu et al., 2014, 2012). In short, newly eclosed males were kept individually on
376 standard fly food in plastic vials (12 x 75mm). Newly eclosed virgin females were kept in
377 groups of 10 flies. All behaviors were done with 4-7 day-old animals, which were housed
378 under constant conditions of 25° C and a 12h:12h light-dark cycle. Courtship was video
379 recorded for 10 min for male courtship and 15 min for female mating receptivity. Male
380 courtship latency and index were measured as previously described (Lu et al., 2014,
381 2012). Female receptivity index was defined as the time from the initiation of male
382 courtship until copulation was observed. Unless otherwise indicated, assays were
383 performed under red light conditions.

384

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

389

390 ***Perfuming studies.*** Synthetic compounds were synthesized by J.G.M. Perfuming
391 studies were performed using a modified protocol from (Billeter et al., 2009). In short, 3
392 mg of each compound was dissolved in 6 mL hexane (Sigma-Aldrich #139386-500ML)
393 and 0.5 mL was pipetted into individual 2 mL glass vials fitted with 9mm PTFE lined caps
394 (Agilent Crosslab, Santa Clara, CA, USA). The hexane was evaporated under a nitrogen
395 gas flow, such that a residue of the compound was left around the bottom one-third of the
396 vial. Control vials were prepared using hexane without a spiked compound. Vials were
397 kept at -20°C until use. Flies used in these trials were collected as described above, kept
398 in single sex groups and aged for 4 days on standard cornmeal medium at 25°C. 24 hours
399 before perfuming, 20 flies of one or the other sex were placed in glass vials containing

400 standard cornmeal medium (12 x 75mm). To perfume the flies, these groups of 20 flies
401 were dumped without anesthesia into each 2 mL vial containing the compound of interest,
402 and were vortexed at medium-low speed for 3 pulses of 20 seconds punctuated by 20
403 second rest periods. Flies were transferred to new food vials and were allowed to recover
404 for one hour. Perfumed flies were then used in courtship behavior assays as described
405 above and the remaining flies were used in pheromone analyses to verify compound
406 transfer. The genotype of flies that were perfumed differed based upon the genotype with
407 the lower amount of each compound as determined in Figure 3 (B, C, F). In all cases,
408 compound transfer was verified by CHC extraction and GC/MS (Table 8).

409

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

419

420 ***Pheromone Analysis.*** Virgin flies were collected upon eclosion under a light CO^2
421 anesthesia and kept in single-sex vials in groups of 10 with 6 biological replications for
422 each genotype and sex. Virgin flies were aged for 5 days on standard cornmeal medium
423 at 25°C. To collect mated flies, both females and males were aged for 3 days before
424 single mating pairs were placed in a standard fly vial with standard cornmeal food for 24
425 hours. The pair was then separated for 24 hours before collection. Copulation was
426 confirmed by the presence of larvae in the vials of mated females several days later. On
427 the morning of day 5, flies were anesthetized under light CO^2 and groups of five flies were
428 placed in individual scintillation vials (VWR 74504-20). To extract CHCs, each group of
429 flies was covered by 100 uL hexane (Sigma-Aldrich #139386-500ML) containing 50µg/mL

430 hexacosane (Sigma-Aldrich #241687-5G) and was washed for ten minutes.
431 Subsequently, hexane washes were transferred into a new 2 ml glass vial containing a
432 350 uL insert (Thermo Scientific C4000-LV-1W) and were stored at -20°C until shipment
433 to the Millar laboratory.

434 Analyses of CHC profiles were done by gas chromatography and mass spectroscopy
435 (GC-MS) in the Millar laboratory at UC Riverside as previously described (Chung et al.,
436 2014). Peak areas were measured, and data was normalized to known quantity of internal
437 standard hexacosane (Sigma-Aldrich #241687-5G). The relative proportion of each
438 compound in each sample was calculated and used in further statistical analysis.
439 Qualitative data were analyzed through a permutation MANOVA using the ADONIS
440 function in the vegan package of R with Bray-Curtis dissimilarity measures (Oksanen et
441 al., 2017). Data were visualized using non-metric multidimensional scaling (vegdist
442 function in the vegan package of R followed by cmdscale function in the stats package
443 (Oksanen et al., 2017)) using Bray-Curtis dissimilarity, and either 2 or 3 dimensions in
444 order to minimize stress to < 0.1. Quantitative data were analyzed by using a t-test or
445 Mann-Whitney Rank Sum Test in R 3.3.2 (R Core Team, 2016).

446

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

449

450 **AUTHOR CONTRIBUTIONS**

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

454

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462 Bloomington Drosophila Stock Center (NIH P40OD018537) were used in this study. Wild
463 type *Drosophila* species were obtained from the National *Drosophila* Species Stock
464 Center at Cornell University.

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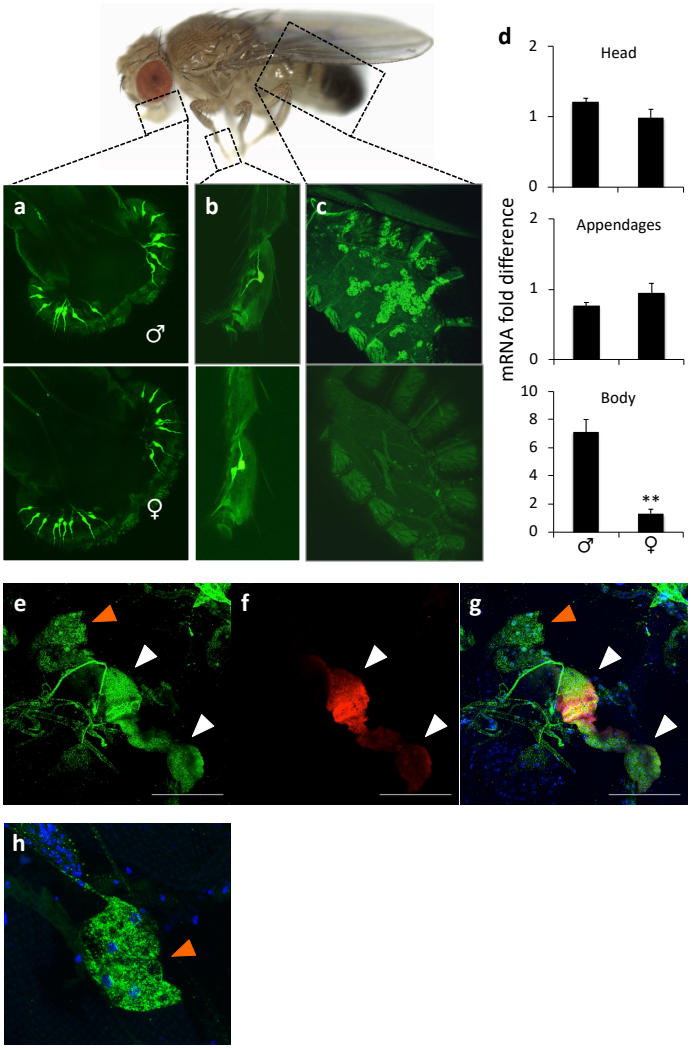
686

687 **Figures and figure legends:**



688

689 **Figure 1**. ***Gr8a* is a sexually dimorphic chemosensory receptor enriched in male**
690 **oenocytes and fat body**. (A) *Gr8a* promoter activity in proboscis, (B) forelegs, and (C)
691 abdomens of males (top panels) and females (bottom panels). (D) *Gr8a* mRNA
692 expression. Relative mRNA levels were measured by real-time quantitative RT-PCR. **,
693 p<0.01 Mann Whitney Rank Sum Test, n=4/group. (E) Confocal z-stack image of
694 *Gr8a*>EGFP in abdominal cells. (F) Confocal z-stack image of *desat1*>Luciferase in
695 abdominal cells. (G) Co-expression of *Gr8a* and *desat1*. (H) Confocal z-stack image of
696 GFP-tagged GR8A protein in abdominal cells. Green, *Gr8a*; Red, *desat1*; Blue, nuclear
697 DAPI stain. Orange arrowhead, fat body cells; white arrowhead, oenocytes. Scale bar =
698 100μm.

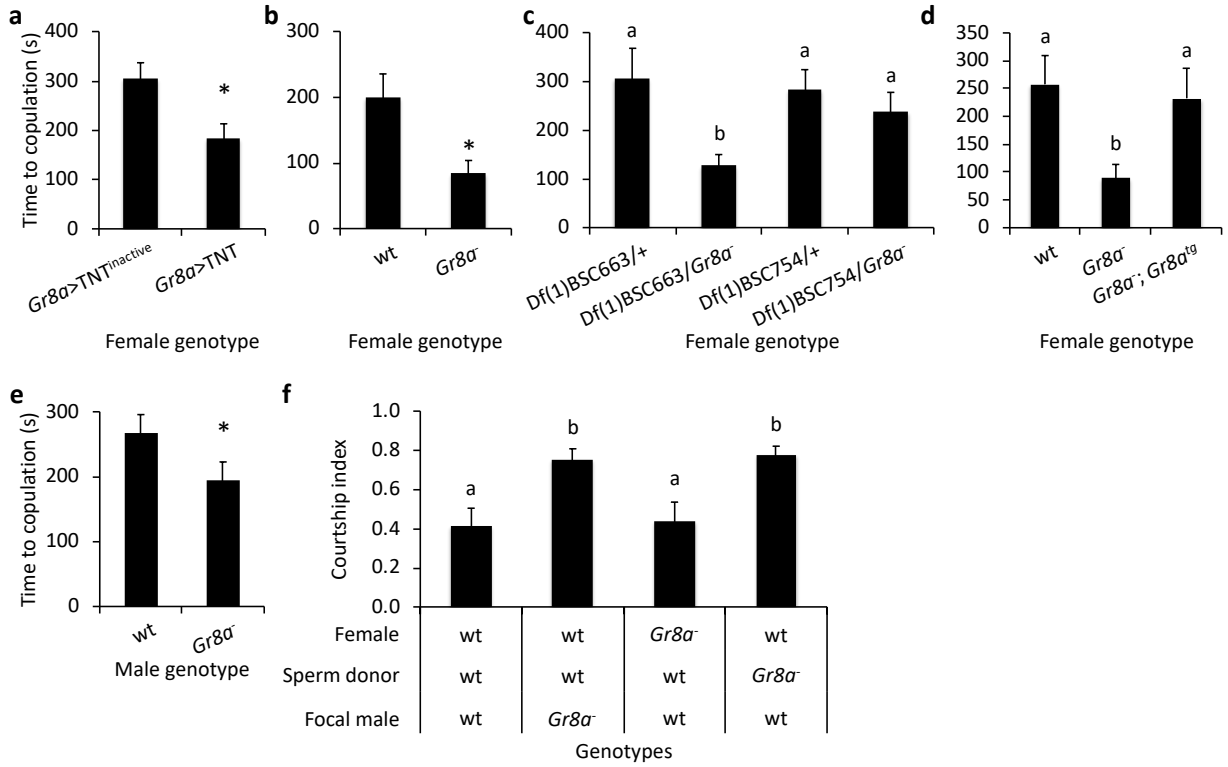
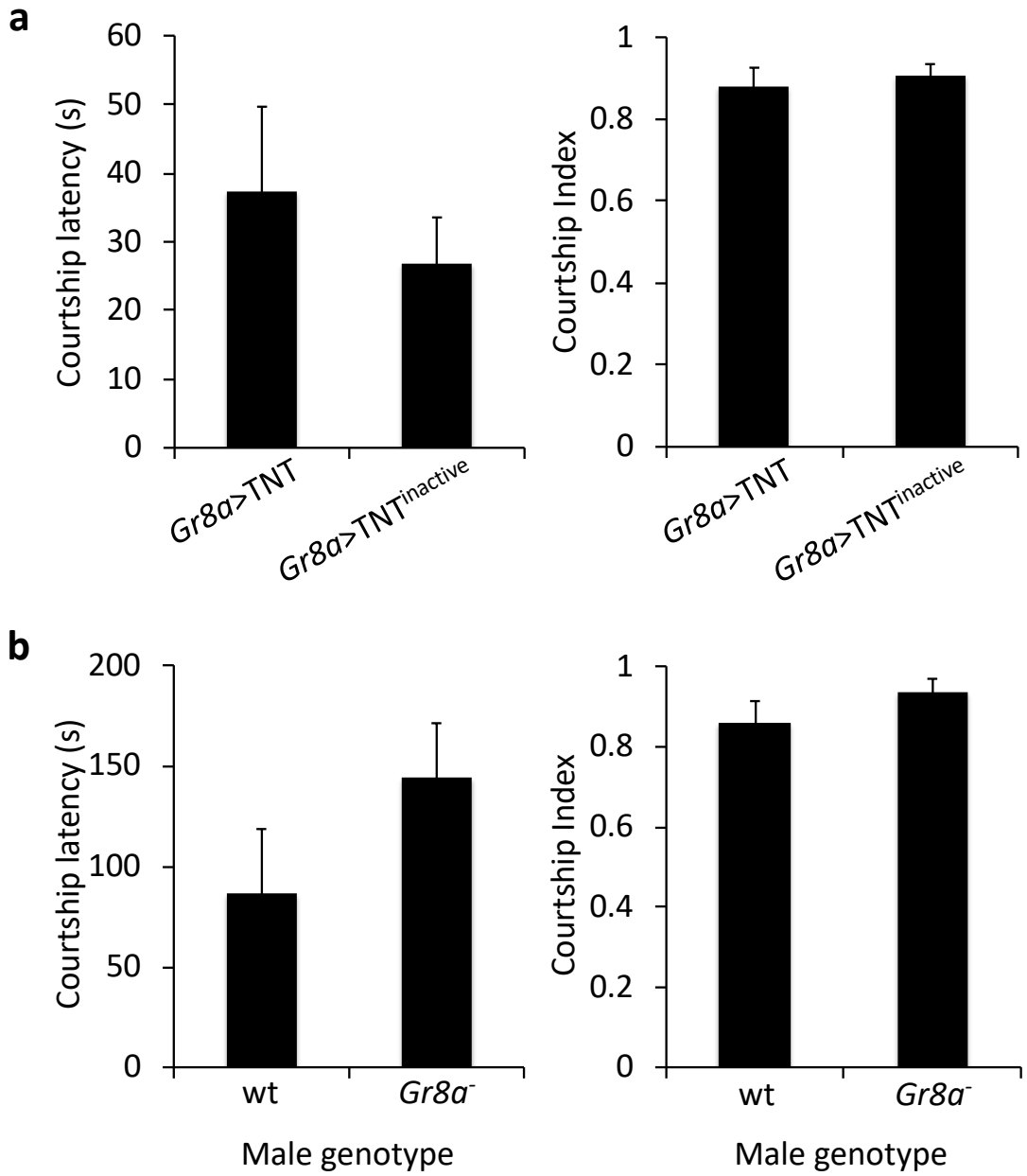


Figure 2. *Gr8a* activity contributes to the perception and production of an inhibitory signal associated with mating decisions in males and females. (A) Blocking neural activity in female *Gr8a*-expressing sensory neurons shortens copulation latency. Homozygous (B) or hemizygous (C) *Gr8a* null females 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 (Gr8a-;Gr8a^{tg}) 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).



716

717 **Figure 2—figure supplement 1.** *Gr8a* has no effect on male Courtship Index or
718 Latency toward wild-type females. (A) Courtship Index and Latency (s) of control *Gr8a-*
719 *gal4/UAS-IMP-TNT- V1A* (*TNT*^{inactive}) and *Gr8a-gal4/UAS-TNT-E* mutant males
720 towards wild-type females. (B) Courtship index and latency (s) of wild-type (*CS*) and
721 *Gr8a null* males toward wild-type decapitated females. Mann Whitney Rank Sum Test,
722 not significant (p>0.05), n=15/group.

723

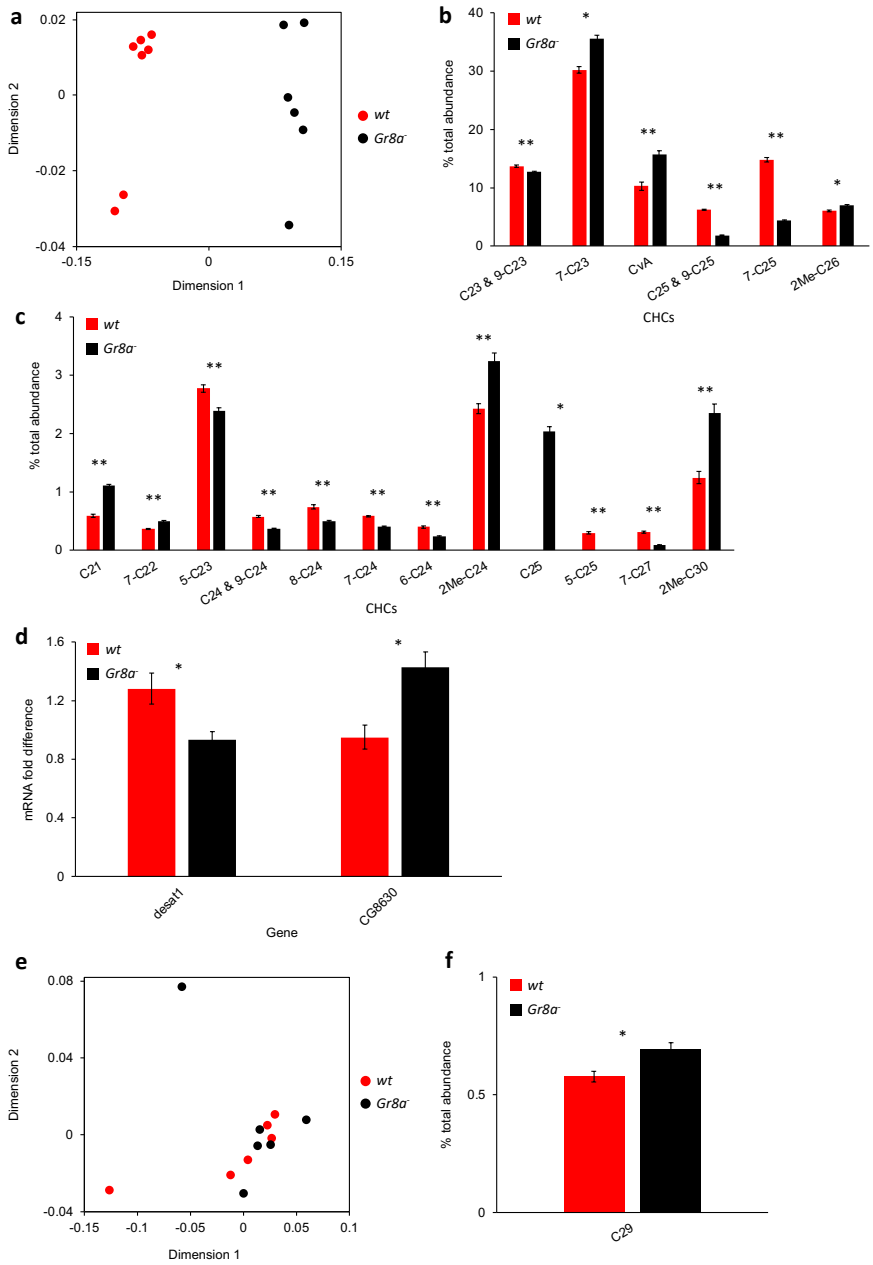
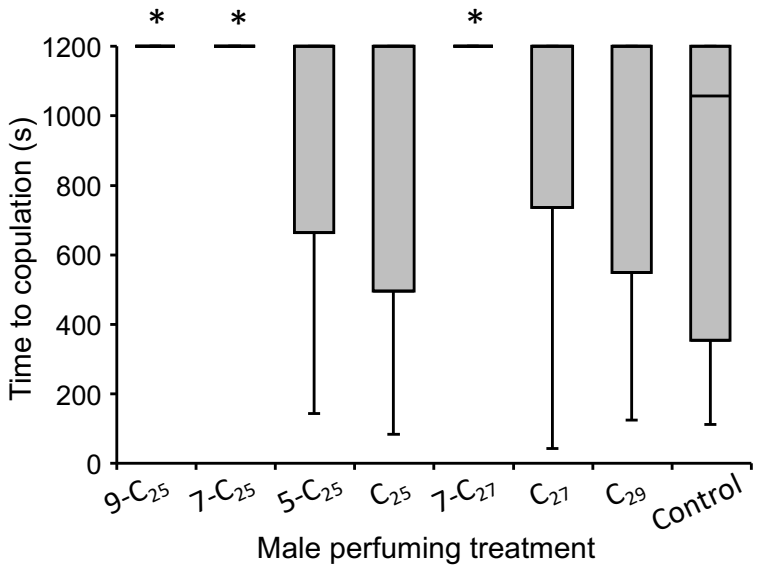


Figure 3. The *Gr8a* mutation affects the pheromone profiles of males and mated females. (A) Nonmetric multidimensional scaling (NMDS) plot of CHC profiles of wild-type and *Gr8a* mutant males. p<0.001, permutation MANOVA. **(B-C)** The effect of the *Gr8a* mutation on levels of individual CHCs in males. (B) CHCs found at high proportions in males. (C) CHCs found at low proportions in males. Only affected CHCs are shown.

731 See Table 2 for the complete list. *, $p<0.05$, **, $p<0.001$, Student's t-test or Mann Whitney
732 Rank Sum Test, n=6 (Gr8a-) or 7 (wt). **(D)** The effect of *Gr8a* mutation on the expression
733 level of several desaturase genes. Only affected genes are shown. See Table 3 for the
734 complete list. *, $p<0.05$, Student's t-test, n=4/group. **(E)** NMDS plot of CHC profiles of
735 females mated with wild-type or *Gr8a* mutant males. p=0.570, permutation MANOVA. **(F)**
736 Nonacosane (C_{29}) differs between females mated with wild-type and *Gr8a* mutant males.
737 See Table 4 for complete list of mated-female CHCs. *, $p<0.05$, Student's t-test,
738 n=6/group.

739

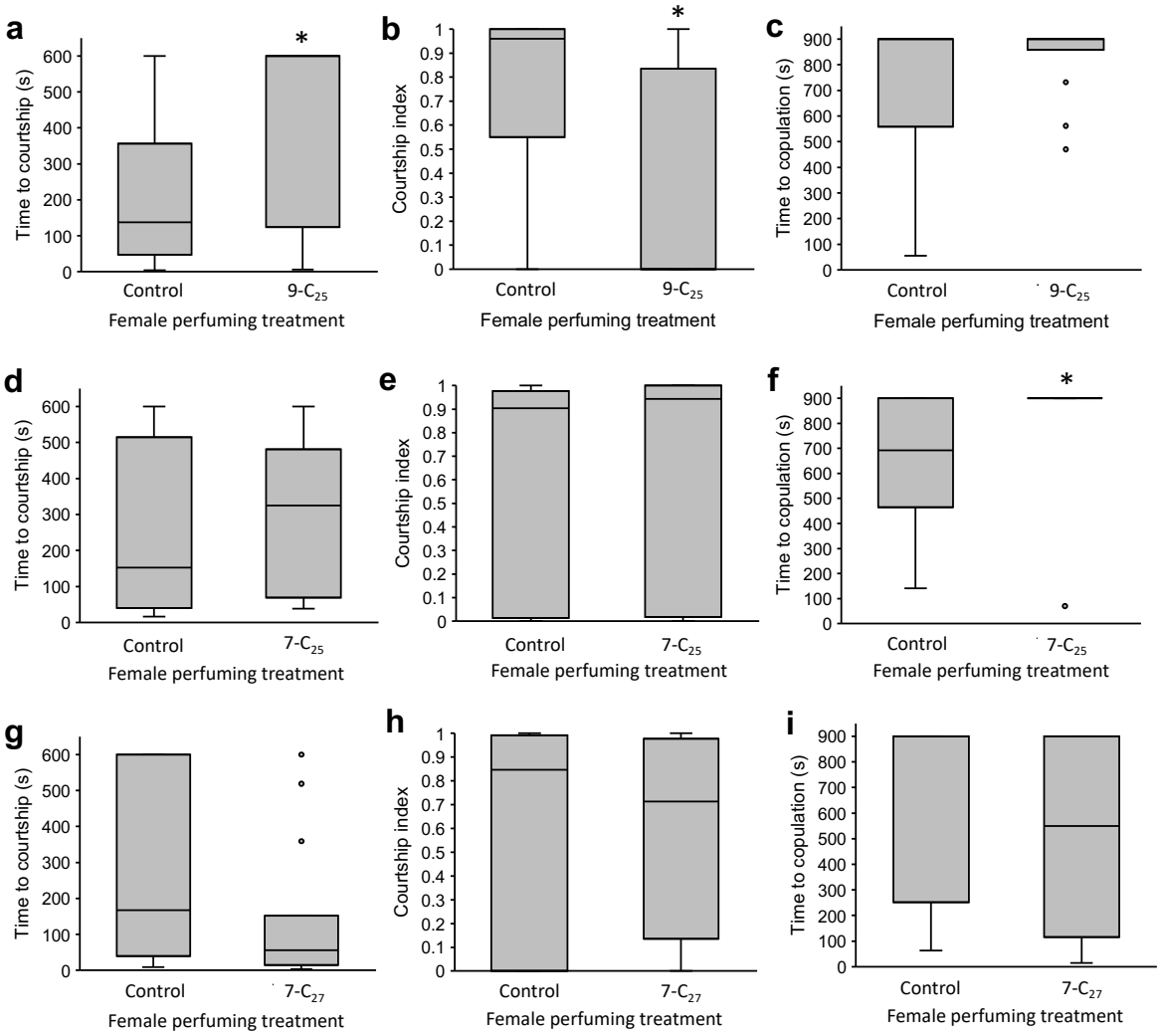


740

741 **Figure 4. *Gr8a*-associated alkenes inhibit normal courtship behaviors when males**
742 **are perfumed.** Perfuming males with exaggerated amounts of several alkenes increases
743 copulation latency compared to control males. Asterisks above bars indicate statistically
744 significant contrasts compared to control flies, $p<0.05$, Kruskal-Wallis Test followed by
745 Dunn's Test, n=15/group.

746

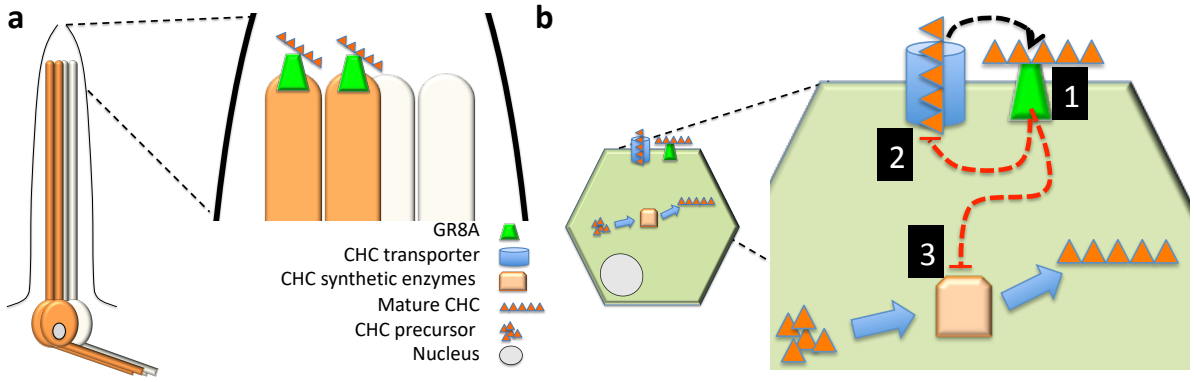
747



748

749 **Figure 5. *Gr8a*-associated alkenes inhibit normal courtship behaviors when**
750 **females are perfumed.** Perfuming females with 9-C_{25} increases courtship latency (A),
751 decreases courtship index (B), but does not affect copulation latency compared to control
752 females. Perfuming females with 7-C_{25} does not affect courtship latency (D) or index (E),
753 but increases copulation latency (F) compared to control females. Perfuming females with
754 7-C_{75} does not affect courtship latency (G), courtship index (H), or copulation latency (I)
755 compared to control females. Asterisks above bars indicate statistically significant
756 contrasts compared to control flies, $p<0.05$, Mann Whitney Rank Sum Test, n=15/group.

757



758

759 **Figure 6. Model for the pleiotropic action of *Gr8a* in the perception and production**
760 **of pheromones.** (A) *Gr8a* functions as a chemoreceptor for an inhibitory signal in
761 pheromone-sensing GRNs of males and females. (B) *Gr8a* also functions as a CHC
762 autoreceptor in oenocytes, which regulates CHC secretion [1] or CHC synthesis [2] via
763 signaling feedback loops [3].

764

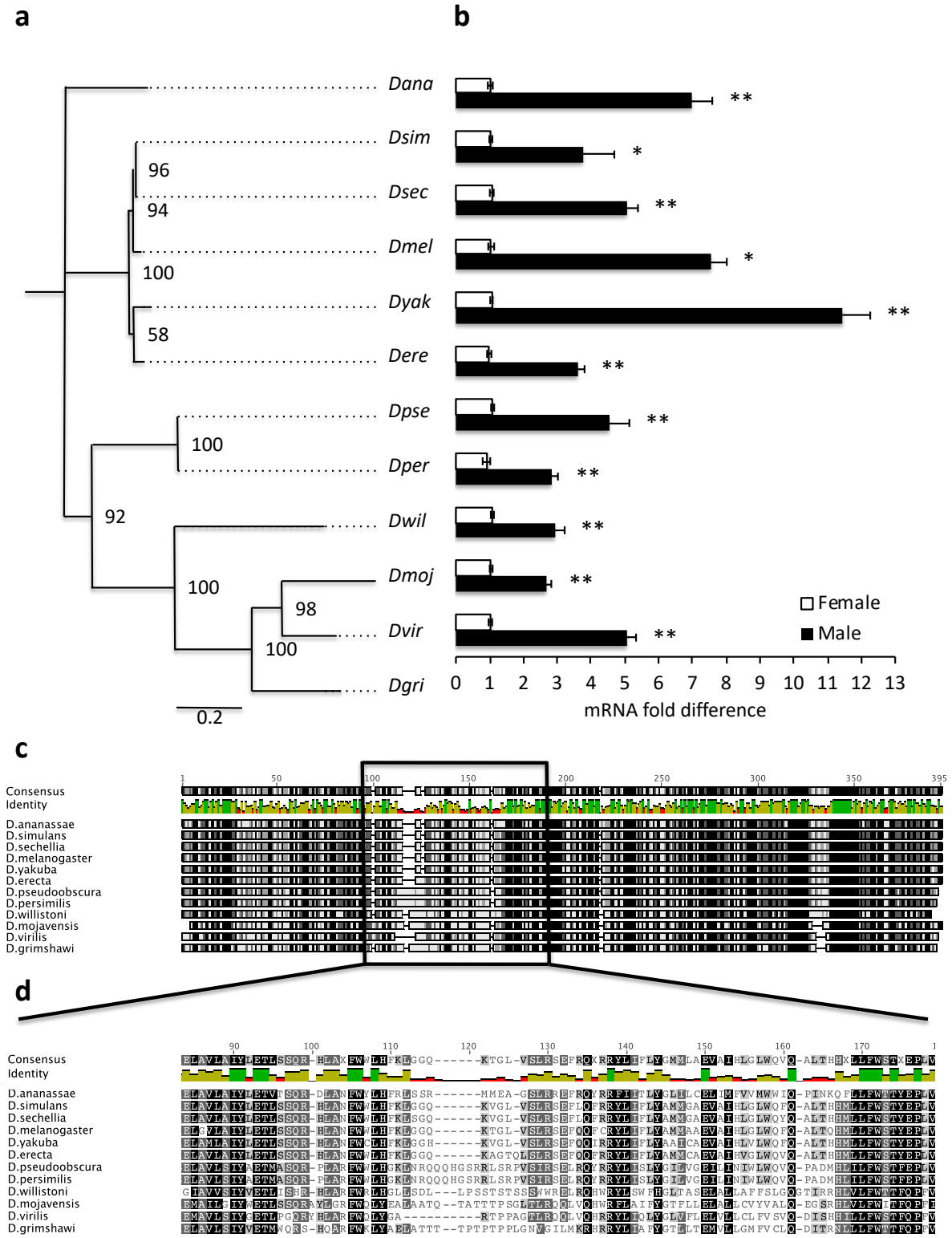


Figure 7. *Gr8a* expression is sexually dimorphic across the *Drosophila* genus. (A) Phylogenetic tree of *Drosophila Gr8a* proteins. Substitution rate = 0.2. (B) *Gr8a* mRNA expression is enriched in males relative to females across *Drosophila*. Black, males;

769 white, females. *, p<0.05; **,p<0.01; Mann Whitney Rank Sum Test, n=4/group. Live *D.*
770 *grimshawi* was not analyzed because live specimens were not available at the
771 *Drosophila* Species Stock Center (DSSC). (C) Multiple aligned amino acid sequences of
772 *Gr8a* protein sequences from 12 species across *Drosophila.* Box highlights a putative
773 hypervariable protein domain, which is shown at a higher resolution in (D). Numbers on
774 top of alignment indicate amino acid number. Black, 100% identical; Dark Gray, 80-
775 100% similar; Light Gray, 60-80% similar; White, less than 60% similar (Blosum62 score
776 matrix, threshold=1). Bars below consensus represent overall level of amino acid
777 conservation.

778

779 **Tables and table legends**

Gene	Male	Female
Gr2a	-	+
Gr8a	+	-
Gr10a	+	+
Gr21a	-	+
Gr22a	+	-
Gr22e	+	+
Gr36c	+	-
Gr58c	+	+
Gr59a	+	+
Gr59b	+	+
Gr63a	+	-
Gr64a	+	-
Gr64b	+	+
Gr64c	+	+
Gr64d	+	-
Gr66a	+	+
Gr89a	+	+
Gr93a	-	+
Gr93d	+	+
Gr97a	+	+
Gr98a	+	+
Gr98b	-	+
Gr98c	+	+
Gr98d	+	+

780

781 **Table 1.** Candidate *Gr* genes in male and female abdomen. Plus sign indicates presence
782 of PCR product and minus signs indicates PCR product not detected. Genes with no PCR
783 product detected in male or female abdomens not shown.

784

R.T.	Compound	wt % total	*Gr8a* % total	p value
12.31	C_{21}	0.589	1.102	<0.001
13.24	Unknown	0.071	0.191	<0.001
14.2	C_{22}	0.893	0.949	0.339
14.34	7-C_{22}	0.362	0.490	<0.001
15.25	Unknown	0.128	0.226	<0.001
16.22	C_{23} & 9-C_{23}	13.682	12.671	<0.001
16.4	7-C_{23}	30.201	35.478	0.002
16.53	5-C_{23}	2.772	2.389	<0.001
16.71	CvA	10.240	15.700	<0.001
18.03	C_{24} & 9-C_{24}	0.578	0.367	<0.001
18.19	8-C_{24}	0.742	0.493	<0.001
18.27	7-C_{24}	0.579	0.402	<0.001
18.37	6-C_{24}	0.395	0.233	<0.001
18.46	5-C_{24}	0.040	0.047	0.943
19.09	2Me-C_{24}	2.426	3.240	<0.001
19.95	C_{25}	0.000	2.038	0.001
20.02	C_{25} & 9-C_{25}	6.195	1.793	<0.001
20.18	7-C_{25}	14.781	4.344	<0.001
20.42	5-C_{25}	0.296	0.000	<0.001
22.89	2Me-C_{26}	6.007	6.933	0.002
23.7	C_{27}	1.127	0.719	0.052
23.94	7-C_{27}	0.308	0.083	<0.001
26.46	2Me-C_{28}	4.992	5.775	0.078
27.25	C_{29}	0.351	0.317	0.574
29.89	2Me-C_{30}	1.245	2.353	<0.001

785 **Table 2.** Male CHCs. Retention time (R.T.), compound, and percent total (% total) of each
786 compound as part of the total pheromonal bouquet for wild-type (wt) and *Gr8a* mutant
787 males.

788

Gene	wt mRNA fold difference	*Gr8a* mRNA fold difference	p value
desat1	1.282	0.931	0.037
desat2	1.413	1.270	0.506
CG8630	0.951	1.429	0.012
CG9747	0.838	0.525	0.343
CG9743	1.060	0.959	0.373
CG15331	0.774	1.000	0.21

789 **Table 3.** Desaturase gene expression. Relative mRNA expression of each desaturase
790 gene for wild-type (wt) and *Gr8a* mutant males.

791

R.T.	Compound	wt % total	*Gr8a* % total	p value
12.31	C_{21}	0.234	0.242	0.712
14.2	C_{22}	0.330	0.352	0.512
14.34	7-C_{22}	0.029	0.029	0.958
15.25	Unknown	0.238	0.200	0.128
16.09	C_{23}	6.481	6.580	0.748
16.4	7-C_{23}	2.454	2.729	0.149
16.39	7,11-C_{23}	0.473	0.426	0.208
16.53	5-C_{23}	0.254	0.235	0.416
16.71	CvA	1.349	1.135	0.233
17.99	C_{24}	0.660	0.732	0.209
18.19	8-C_{24}	0.156	0.174	0.334
19.09	2Me-C_{24}	1.214	1.104	0.155
19.95	C_{25}	5.404	5.683	0.336
20.02	C_{25} & 9-C_{25}	3.141	3.851	0.699
20.18	7-C_{25}	3.144	2.999	0.422
20.25	7,11-C_{25}	1.822	1.684	0.315
20.42	5-C_{25}	0.526	0.555	0.375
20.47	5,9-C_{25}	0.686	0.704	0.589
22.89	2Me-C_{26}	10.010	9.247	0.1554
23.7	C_{27}	3.616	3.393	0.24
23.8	9-C_{27}	2.498	2.739	0.937
23.94	7-C_{27}	3.819	4.885	0.24
24.1	7,11-C_{27}	21.464	19.770	0.18
24.28	5,9-C_{27}	2.557	2.438	0.12
25.85	7,11-C_{28}	0.658	0.645	0.573
26.46	2Me-C_{28}	4.779	5.022	0.306
27.25	C_{29}	0.577	0.693	0.01
27.7	7,11-C_{29}	19.411	19.589	0.824
29.89	2Me-C_{30}	1.359	1.465	0.17
31.03	7,11-C_{31}	0.562	0.606	1

792 **Table 4.** Mated-female CHCs. Retention time (R.T.), compound, and percent total (%
793 total) of each compound as part of the total pheromonal bouquet for females mated with
794 wild-type (wt) or *Gr8a* mutant males.

795

Gene	Forward Primer	Reverse Primer
Gr10a	AGCGCGAGAGCTTTCGGATGC	AAAGTCCACACAGCATGGGCGG
Gr10b	GCGACTGTCCAGAGGATGTGGC	CAGCTGATCAAAGCGAATGCGC
Gr21a	AACTACCGGCTCGCCGATCG	AGGTTGCCCCGTGGTCGATGA
Gr22a	TGGGACCTTTGGCAGGGAATCG	TGCGATGACTGCAGAAGAGGGT
Gr22b	GGTTGTGCATTGCCGCATGCG	CTGCAAAGCCAAGCGAACTCGT
Gr22c	GCCGCCAGCGATCTAGCTGAG	CAATGACCGCACAGCAATGCGA
Gr22e	TGGTCTTCGTTCAAGCGCTGGT	GCCGGCGATGGCTGCAGAATAAT
Gr22f	GATCGCCTGCTGAAGCTGAGTGA	GTGGGTGCACAGCCATGCGAAT
Gr23a	AGCGATTTGTGGTGACCGCCA	CATCCGCTGCCTCGCGTTGA
Gr28a	TCGCCCTGCCGCACATCAATA	GGGTGAGAGTTCCAAGGCGCT
Gr28b	ACTGGAGGCATAGTGCACTCCCT	AGTGGTCAAGGCCCCGCTGAT
Gr2a	TTTGCGACCGAACGGCCCAG	GTTGAAGAAGCCAGCGGCGC
Gr32a	ACACGGTCACCACCAACTGCT	ACCAAGTATGTTGTGACGGCCGAA
Gr33a	ATCGCCACGCACCCCTTACC	GCTCACCACAAAGCAGGCCG
Gr36a	GAGGTCGTGTCGTTGCAGCCC	GTGCTCGCTGACGCCATCTGT
Gr36b	TGGCATGCAAGGGCTTATGGCT	CCGCAGTTGAGCCACGCGTG
Gr36c	CGGCAGAGCAAACATGTTGCACG	ACTGCAAGCCCAGTCCCAGGT
Gr39a	TACCCCGAACCGGGACTGGG	CCAGAGCGAAAAATCCATAAGCGGT
Gr39b	GGGCGCCAACTGCACATTGG	TCCAATCGAGGGATGGCAGGACA
Gr43a	GCTGGCCCTGGCTCCATATGC	AACCCCTTGTTCTTGGCTGCGGC
Gr47a	GGTCGGGCCAGAGAAATCGGC	CCCGCTTGGGATGGAGGGCC
Gr47b	GCCCTGGGATCCCGAGTACG	TGCCCGATGTCAACTGGTGATCT
Gr57a	GGCAAGGAGATGCCTTCGCCT	GCCCATCTTCTGGGTCATGGCG
Gr58a	TGTGGATGACGTGACGCGGA	ACGGATGACAACGAAGGTGATCCGA
Gr58b	ACCTCCTGCAACAACACTGGCC	CCGATTCTGCCTCAGCTGCATGG
Gr58c	ACCGCTGTAAGTGGTTGGGACT	TCCAACGATGTGCAGACGTCTCA
Gr59a	GCCAAAGTTCTTCGCCCCCGA	TCCAATCGCGTGCTGCTCTCG
Gr59b	CAGCCAAGCACTCGTGGAGTGA	TGGCAAACCACATGCTGCGATT
Gr59c	CCGAGTGCTGGCGGATCGAAC	CTGGCCAGTTGCAGTTGGAAGT
Gr59d	CGTCGATTGCTGGATCAACGCG	TCGACCAAACTCGAAAAGGCCG
Gr59e	CCTCTCTGGGAGGAGTCCGTGC	AGGGCAACGCCCATTAGCGACT
Gr59f	GCCATGCACGTGGGAAAGGTGT	ACATTGCCCTGAACCGACTTGGA
Gr5a	GCCTGCAACGTGCTCGTCCT	TTGAGCGGGACAAGGCGCAG
Gr61a	AAAGTGCGGCGCCAGAAGCA	GTGCGACCAGGAGCGAGCAA
Gr63a	CGCAAGAAGGGTGACGCGGTG	AAGGGTCCACTCAGGGAGCGC
Gr64a	CGCGTGCAATTGGCTGCCTTG	GTCCGCCGCCGTGAGGAATAC
Gr64b	GCCCGCGGTCTTTTGGACAGA	CATGGCCAGGACGAGTGAGCG
Gr64c	ATGCACTGGCGCATCAGGCA	CCGCCCTCGCAGAAGCTCTT

Gr64d	ACCGCGCCTGGATGGTGTTC	TGCACCACGGCACCTTGCAT
Gr64e	TGGCCACCACTTGCTCGCTG	ACGCCGCGTGTTAGCCGAAA
Gr64f	TCTGGCTAGGCAGTGGCCCC	GCATGGGCCACCGATGGCAT
Gr66a	GAGGACCGCAGCCTAAAGGCC	CGCGGTCAACTGGTCGGAGAC
Gr77a	CGCAGTTCAGGCCGTCTGCTT	CCACAGGCGCTGACGGCAAATA
Gr85a	CTCTCGCCTACATGGCACGC	ATAGTGACGTGCGTGTGCTGC
Gr89a	GCTGCATGGAGTTCCCCTGACC	ACATTGCCAGTGGGCGTGAGC
Gr8a	ACATACGCATCGCCGTGGACTG	ATGTAGGTGCCCACGGAACAGG
Gr92a	TGTCTCCGTTGAGGGAGCGGT	TGGGTGACATCGAATAGGCCCA
Gr93a	GGTGAGGCAGTCAGAAGTGCC	CCTCGGACGCCTCAAAGCCAC
Gr93b	ACCACGCCATTCTGCGAAGAC	GCCCAAAGGAAACACTCGCAGC
Gr93c	TGCGACGCCTGAGTCTGGAGA	CCCAAGGGTCTAACGCGAAACTCG
Gr93d	ACTCGGGAACGTGCTCTGGAT	AGAGGCCCAACAAGTTAACCCTGA
Gr97a	TGCGTCGCTTCTGCGAGTTAGC	GCGTGCTGCAGATCGAATCGC
Gr98a	GTTGAGCACACAAGGGTGGCTG	GCTGTTGCTTGCTGCTGCTCTGA
Gr98b	TTGGAGGCTGGAGGGCGATGT	ATGGTGACAAGCAACCCGCCA
Gr98c	GCCAGGATTCAGGAGCTGTGCG	CGCTGGTATTTTGTCTGTAGCCGA
Gr98d	CTGGCTGGACGCATTTGGGGC	ATGTCAAAGAGGCCACCGCATGT
Gr9a	TGTCCTCTGTCTGGGCGACTGG	ACAAGGGACACGAACTGCAGGA

796 **Table 5.** Nucleotide sequences for qRT-PCR primers for *D. melanogaster Gr* genes.

797

Species	*Gr8a* Forward Primer	*Gr8a* Reverse Primer
D. melanogaster	TGACCATCAACATACGCATCG	CGTATATGAAGGCGGGAATCTC
D. simulans	GAACTTTTCGCTGCAACTCC	ACTTCGGTATAAACTGGATGGTG
D. sechellia	GAGATTCCCGCCTTCATATACG	GAGTTGCAGCGAAAAGTTCTG
D. erecta	CAGATTCAGAACTTTTCGCTGC	GGTGTAGATCATGTAGGTGCC
D. yakuba	TGCCTCGGACTAACAATTCTG	GTGTAGATCATGTAGGTGCCC
D. ananassae	AATGTACCGAAGTTTCCAGGG	GCGGGTATGATCAGGAAATAGTC
D. pseudoobscura	CCCGTTTCCGTGACAATATTG	ACCATCTACATATCCGTTGCC
D. persimilis	TTTCGCTTCTCCACACTGAC	AGGCGGGCAATATCAAAGAG
D. willistoni	GAAATGTTGCCCAGAATAGCC	CCCAAAGCATGTATAACCACTG
D. virilis	TCTTCAGATCCAAAACTTTTCGC	TTGGGCATCAGTTGTACGG
D. mojavensis	CATATACCCGCCTTTCTCTACAC	GTTCGTGCAGAATTTGTAGCG

Species	*Rp49* Forward Primer	*Rp49* Reverse Primer
D. melanogaster	ATCTTGGGCCTGTATGCTG	TGTGATGGGAATTCGTGGG
D. simulans	GTCGGATCGATATGCTAAGCTG	CAGATACTGTCCCTTGAAGCG
D. sechellia	CATACAGGCCCAAGATCGTG	CAGATACTGTCCCTTGAAGCG
D. erecta	GTCGGATCGATATGCTAAGCTG	CAGATACTGTCCCTTGAAGCG
D. yakuba	CATACAGGCCCAAGATCGTG	GGCATCAGATACTGTCCCTTG
D. ananassae	TACAGGCCCAAGATCGTTAAG	GTACTGACCCTTGAAGCGAC
D. pseudoobscura	CCAGCTCCAAAATGACGATTC	TCAATACCCTTAGGCTTGCG
D. persimilis	AAGCACTTCATCCGTCACC	TCAATACCCTTAGGCTTGCG
D. willistoni	AAGCACTTCATCCGTCACC	GTTGGGCATCAGATATTGGC
D. virilis	AGTCGGATCGTTATGCTAAGTTG	TGGAGGGTACGCTTGTTTG
D. mojavensis	ACCATTCGTCCAGCATACAG	TTGGCCCTTGAAGCGAC

Table 6. Nucleotide sequences for qRT-PCR primers for *D. melanogaster Gr8a*, *Rp49* and orthologs.

Gene	Forward Primer	Reverse Primer
desat1	TTCTGAACGTAACCTGGCTG	GGGAAGACGTGATGGTAGTTATG
desat2	TTTACTGGAATGAGACGCTGG	CTGGGTAGGATTCATGGTCTTG
Fad2	GTGCCTCAGTCTCAATCTCATC	GTGGTAGTTGTGGTATCCCTC
CG8630	ATGTGATGGCTAAGATCGGAC	TGGTCGGAATGGCACTATTG
CG9747	TCAAGTGGGACAAAGTCATCC	GCCGTAACTCCGAATCCG
CG9743	GGTACTTCTGGAACGAGGATC	TCAGTGGACATGAGGTTCTTG
CG15531	CTCAAGGATGTGGATATGTCGG	ATGGAGGTGGCTAGGGAG
rp49	CACCAAGCACTTCATCCG	TCGATCCGTAACCGATGT

801 **Table 7.** Nucleotide sequences for qRT-PCR primers for *D. melanogaster* desaturase
802 enzyme genes.

803

Compound	Genotype	Sex	ng/sample	ng/fly
C_{25}	wt	male	1350	270
C_{25}	wt	male	1170	234
C_{25}	wt	male	27730	5546
9-C_{25}	Gr8a-	male	67390	13478
9-C_{25}	Gr8a-	male	59380	11876
9-C_{25}	Gr8a-	male	1000	200
7-C_{25}	Gr8a-	male	43790	8758
7-C_{25}	Gr8a-	male	31330	6266
7-C_{25}	Gr8a-	male	24130	4826
5-C_{25}	Gr8a-	male	12050	2410
5-C_{25}	Gr8a-	male	14160	2832
5-C_{25}	Gr8a-	male	1870	374
C_{27}	Gr8a-	male	890	178
C_{27}	Gr8a-	male	720	144
C_{27}	Gr8a-	male	263	52.6
7-C_{27}	Gr8a-	male	36710	7342
7-C_{27}	Gr8a-	male	21250	4250
7-C_{27}	Gr8a-	male	15910	3182
C_{29}	wt	male	260	52
C_{29}	wt	male	340	68
C_{29}	wt	male	890	178
9-C_{25}	wt	female	14060	2812
9-C_{25}	wt	female	13830	2766
7-C_{25}	wt	female	23200	4640
7-C_{25}	wt	female	12780	2556
7-C_{27}	wt	female	1010	202
7-C_{27}	wt	female	1080	216

804 **Table 8.** Amount (ng) of each perfumed compound measured in each sample of perfumed
805 flies (5 flies per sample).

806