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

Kathleen M. Zelle[1,†], Cassondra Vernier[1,†], Nicole Leitner[1], Xitong Liang[2], Sean Halloran[3], Jocelyn G. Millar[3], Yehuda Ben-Shahar[1,4,*]

[1]Department of Biology, Washington University in Saint Louis, 1 Brookings Drive, Saint Louis, MO 63130 USA.

[2]Department of Neuroscience, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110, USA

[3]Department of Entomology, University of California, Riverside, 900 University Avenue, Riverside, CA 92521, USA

[4]Lead Contact

[*]Correspondence: benshahary@wustl.edu (Y.B.)

[†]Denotes equal contribution

ABSTRACT

Optimal mating decisions depend on stable-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 and maintain their coupling on the evolutionary and physiological timescales remain unknown 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. Particularly, *Gr8a* acts as a pheromone chemoreceptor in the sensory system of males and females, and, independently regulates pheromone synthesis in the male fat body and 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 physiological 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 hypothesized that possible solutions for maintaining the physiological coupling of signal production and perception are through strong genetic linkage between the genes that regulate mating signal production and those that mediate its perception, or alternatively, via the action of pleiotropic genes that control both processes (Boake, 1991; Butlin and Ritchie, 1989; Shaw et al., 2011; Shaw and Lesnick, 2009). Both mechanisms could provide a robust molecular solution (Chebib and Guillaume, 2019; Kirkpatrick and Hall, 2004; Lande, 1984, 1981, 1980) for the problem of maintaining a stable and reliable population-level social communication system while still retaining the capacity for future signal diversification, as necessitated for speciation (Chebib and Guillaume, 2019; Hoy et al., 1977; Kirkpatrick and Hall, 2004; Lande, 1984, 1981, 1980; Shaw et al., 2011; Shaw and Lesnick, 2009; Wiley et al., 2012).

Although empirical data in support of the role of gene-linkage or pleiotropy in maintaining the physiological coupling between mating signal production and perception are rare, several studies have provided theoretical and experimental support that pleiotropy likely plays an important role in the evolution and maintenance of the physiological coupling between mating-specific signals and the behaviors they elicit at the population level (Chebib and Guillaume, 2019; Hoy et al., 1977; Shaw et al., 2011; Shaw and Lesnick, 2009; Wiley et al., 2012). However, the complex characteristics of mating behaviors, and the signals that mediate them, 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 remain stably coupled is particularly puzzling for mating pheromones because their perception is mediated by the peripheral sensory nervous system, while their production is often restricted to

66 specialized, non-neuronal pheromone producing cells (Chung and Carroll, 2015;
67 McKinney et al., 2015; Mucignat-Caretta, 2014). Therefore, whether pleiotropy could
68 function in both the production and perception of mating pheromones, a ubiquitous means
69 of signaling across animal mating systems (Mucignat-Caretta, 2014), remained unknown.

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

79

80 **RESULTS**

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

96 wings) and the proboscis (Koh et al., 2015; Lu et al., 2014, 2012; Thistle et al., 2012;
97 Toda et al., 2012). While some of the genes and pathways that contribute to CHC
98 synthesis in *Drosophila* are known, the molecular identities of most CHC receptors remain
99 unknown. Previous work suggests that the gene *Desat1*, which encodes a fatty acid
100 desaturase enriched in oenocytes, might also independently contribute to CHC
101 perception (Bousquet et al., 2011). However, due to the expression of *Desat1* in central
102 neurons (Billeter et al., 2009) and the broad impact its mutant alleles have on the CHC
103 profiles of both males and females (Labeur et al., 2002), whether *Desat1* directly
104 contributes to mating signal perception remains unresolved.

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

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

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

142 Together, the enriched expression of *Gr8a* in foreleg GRNs, a primary pheromone
143 chemosensory organ (Lu et al., 2014, 2012), and its sexually dimorphic expression in
144 oenocytes and fat body suggested that in addition to its role in L-Canavanine perception
145 (Lee et al., 2012; Shim et al., 2015), *Gr8a* contributes to mating behaviors. Since mating
146 choices of both male and female flies are determined by a complex blend of excitatory
147 and inhibitory pheromones (Billeter et al., 2009; Krupp et al., 2013), we anticipated that if
148 *Gr8a* is indeed a pleiotropic factor that independently contributes to the perception and
149 production of specific components of the male mating pheromone bouquet, then
150 disruptions of the signal production in males or its perception in females should carry
151 similar impacts on female mate choice behavior. To directly test this hypothesis, we first
152 investigated whether *Gr8a* and the GRNs that express it are required for sensory
153 functions associated with female mate choice by using a single-pair courtship paradigm
154 (Lu et al., 2014, 2012). We found that blocking neuronal transmission in *Gr8a*-expressing
155 GRNs with the transgenic expression of the tetanus toxin (TNT) in females resulted in a
156 shorter copulation latency relative to wild-type females when courted by wild-type males
157 (Figure 2A). Similarly, homozygous (Figure 2B) and hemizygous (Figure 2C) *Gr8a* mutant

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

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

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

185 Because *Gr8a* is involved in the perception of an inhibitory mating signal, we also tested
186 the hypothesis that *Gr8a* mutant males exhibit an abnormal capacity for identifying the
187 post-mating status of wild-type females. Indeed, we observed that *Gr8a* mutant males

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

196 Because our behavioral data indicate that the *Gr8a* mutation has a dramatic effect on the
197 production and perception of a putative inhibitory mating pheromonal signal, we next
198 examined whether the *Gr8a* mutation has a direct effect on qualitative and quantitative
199 aspects of male and mated-female CHC profiles. Analyses of male CHCs by gas
200 chromatography and mass spectroscopy revealed that the *Gr8a* mutation has a
201 significant impact on the overall qualitative characteristics of the CHC profile of males
202 (Figure 3A), as well as significant quantitative effects on the levels of several specific
203 CHCs in males (Figure 3B-3C and Table 2). In particular, the *Gr8a* mutation affects the
204 levels of several alkenes and methyl-branched alkanes, which have been implicated in
205 mate choice behaviors in diverse *Drosophila* species (Billeter et al., 2009; Chung et al.,
206 2014; Dyer et al., 2014; Shirangi et al., 2009). Similarly, the *Gr8a* mutation affects the
207 expression level of two desaturase enzymes in males, *desat1* and *CG8630* (Figure 3D),
208 which are involved in the biosynthesis of alkenes (Chung and Carroll, 2015). We also
209 found that the overall qualitative aspects of the CHC profiles of wildtype females were not
210 affected by mating with either *Gr8a* mutant or wildtype males (Figure 3E). However,
211 quantitative analyses of individual CHCs revealed that nonacosane (C_{29}) is higher in
212 females that mated with the *Gr8a* mutant relative to those that mated with wildtype males
213 (Figure 3F). Together, our behavioral and pheromonal data indicate that *Gr8a* action
214 contributes to mating decisions in females by co-regulating the perception of an inhibitory
215 mating pheromone by females, as well as its production by males, which is consistent
216 with a pleiotropic function for *Gr8a*.

217 Next, we sought to identify the specific mating inhibitory CHCs that require *Gr8a* for their
218 perception by testing the effect of perfuming males with specific CHCs on wild type female

219 copulation latency in a single-pair courtship paradigm (Lu et al., 2014, 2012). We found
220 that wild-type females did not copulate with *Gr8a* mutant males that were perfumed with
221 the alkenes 9-C_{25}, 7-C_{25}, and 7-C_{27} (Figure 4). Similarly, we found that wild-type males
222 exhibited a lower courtship index and longer courtship latency when wildtype females
223 were perfumed with 9-C_{25} (Figure 5A-C), and a longer copulation latency when wildtype
224 females were perfumed with 7-C_{25} (Figure 5D-5F). In contrast, perfuming wildtype
225 females with 7-C_{27} had no effect on male courtship or female mating latency (Figure 5G-
226 I). These data suggest that the alkenes 7-C_{25}, 9-C_{25}, and 7-C_{27} may contribute to an
227 inhibitory signal associated with *Gr8a*.

228

229 **DISCUSSION**

230 The data presented here demonstrate that *Gr8a* is a pleiotropic chemoreceptor that co-
231 regulates the perception and production of a specific pheromonal signal that plays an
232 important role in mating behaviors of both sexes. In contrast to its expected function in
233 the perception of chemical ligands, how *Gr8a*, a member of a canonical chemoreceptor
234 family might also contribute to the production of pheromonal signals is not obvious. In
235 some better understood secretory cell types, autoreceptors are essential for the
236 regulation of synthesis and secretion rates. For example, dopaminergic and serotonergic
237 cells regulate rates of synthesis and release of their respective neuromodulators by the
238 action of autoreceptors, which regulate synthesis rates via signaling feedbacks in
239 response to changes in the extracellular concentrations of the secreted molecule (Ford,
240 2014; Stagkourakis et al., 2016). Therefore, one possible explanation for how *Gr8a* might
241 regulate the synthesis and/or secretion of specific CHCs is by acting as an oenocyte-
242 intrinsic autoreceptor, which regulates CHC synthesis rates by providing feedback
243 information about extracellular levels of specific compounds (Figure 6).

244 Previous work indicates that *Gr8a* is expressed in “bitter” taste neurons in the proboscis
245 and are specifically required for the sensory perception of the feeding deterrent L-
246 canavanine (Lee et al., 2012; Shim et al., 2015), but not for the detection of other inhibitory
247 chemicals such as caffeine, strychnine, and umbelliferone (Lee et al., 2009; Poudel et al.,
248 2015). Our data indicate that *Gr8a* contributes to inhibitory functions associated with

249 mating decisions as well, as was previously reported for other 'bitter' receptors in
250 *Drosophila* (Lacaille et al., 2007; Moon et al., 2009). Together this indicates that 'bitter'
251 taste perception and the perception of inhibitory mating signals may share similar
252 pathways in this species.

253 *Gr8a*-dependent perception of L-canavanine seems to depend on its heterotrimeric
254 interaction with *Gr66a* and *Gr98b* in bitter sensing neurons in the proboscis (Shim et al.,
255 2015). Although both *Gr66a* and *Gr98b* were also identified in our initial screen for
256 receptors enriched in the abdomen, *Gr66a* was expressed in both males and females
257 while *Gr98b* was specifically enriched in females (Table 1). These data suggest that *Gr8a*-
258 dependent contributions to feeding and mating decisions are independently mediated via
259 interactions with different *Gr* genes in each sensory context, and further point to
260 combinatorial subunit composition as an essential aspect of the mechanism for ligand
261 specificity in insect gustatory receptors.

262 Although we do not yet know the specific chemical identity of the ligand of *Gr8a*, previous
263 studies indicated that at least two inhibitory mating pheromones, 11-cis-vaccenyl acetate
264 (cVA) and CH503, are transferred from males to females during copulations. While our
265 data suggest that the *Gr8a* mutation affects the level of cVA expressed by males, it is
266 unlikely that either cVA or CH503 are the putative *Gr8a* ligands because the volatile cVA
267 acts primarily via the olfactory receptor *Or67d* (Benton et al., 2007; Datta et al., 2008;
268 Kurtovic et al., 2007), and CH503 has been reported to signal via *Gr68a*-expressing
269 neurons, which are anatomically distinct from the *Gr8a* GRNs we describe here (Figure
270 1A-B) (Shankar et al., 2015; Yew et al., 2009). Instead, our CHC analyses in *Gr8a*
271 mutants (Figure 3), and the results of perfuming behavioral studies (Figures 4-5), suggest
272 that the alkenes 5-C_{25}, 7-C_{25}, and 7-C_{27} act as inhibitory mating signals as well.

273 Although the studies we present here do not directly address the possible contribution of
274 *Gr8a* to the rapid evolution of mating signals across *Drosophila*, phylogenetic analyses of
275 *Gr8a* orthologs indicate that this is a conserved, sexually dimorphic receptor across
276 *Drosophila* species (Figure 7A-B). Furthermore, analysis of the *Gr8a* protein alignment
277 revealed that in spite of the high overall sequence conservation, the *Gr8a* protein includes
278 at least one phylogenetically variable domain (Figure 7C-D). These data suggest that in

279 addition to the role of pleiotropic genes, such as *Gr8a*, in maintaining the physiological
280 coupling of the production and perception of mating pheromones, sequence
281 diversification of such genes might also drive the divergence of mating signaling systems,
282 such as the rapidly evolving pheromonal systems across the *Drosophila* species group.

283 Studies in other animal species suggest that receptor pleiotropy likely plays a role in
284 mating signaling via other sensory modalities including auditory communication in crickets
285 (Hoy et al., 1977; Wiley et al., 2012) and visual communication in fish (Fukamachi et al.,
286 2009). While the specific genes and signaling pathways that mediate mating systems in
287 these species are mostly unknown, these data suggest that the genetic coupling of signal-
288 receptor pairs in mating communication systems might be more common than previously
289 thought. Therefore, the genetic tractability of *D. melanogaster*, in combination with the
290 diversity of mating communication systems in this species-rich phylogenetic group,
291 provide a unique opportunity for understanding the evolution and mechanisms that drive
292 and maintain mating communication systems at the genetic, molecular, and cellular
293 levels.

294

295 **MATERIALS AND METHODS**

296 ***Animals.*** Flies were maintained on a standard cornmeal medium under a 12:12 light-dark
297 cycle at 25 Celsius. Unless specifically stated, the *D. melanogaster Canton-S (CS)* strain
298 served as wild-type control animals. UAS-mCD8::GFP, UAS-myr::GFP, UAS-TNT-E,
299 UAS-TNT-IMP-V1-A, *Gr8a-GAL4* and *Gr8a*^{1} fly lines were from the Bloomington Stock
300 center. Originally in the *w*^{1118} background, the *Gr8a*^{1} *null* allele was outcrossed for six
301 generations into the *CS* wild-type background. *PromE(800)-GAL4* and
302 *PromE(800)>Luciferase* were from Joel Levine at the University of Toronto. The following
303 *Drosophila* species were obtained from the San Diego Stock Center: *D. simulans* 14011-
304 0251.192, *D. sechellia* 14021-0248.03, *D. yakuba* 14021-0261.01, *D. erecta* 14021-
305 0224.00, *D. ananassae* 14024-0371.16, *D. pseudoobscura* 14011-0121.104, D.
306 *persimilis* 14011-0111.50, *D. willistoni* 14030-0811.35, *D. mojavensis* 15081-1352.23,
307 and *D. virilis* 15010-1051.118. The UAS-*Gr8a* transgenic line was generated by cloning
308 the *Gr8a* cDNA in the pUAST-attB vector using 5' EcoRI and 3' NotI restriction sites,

309 followed by *ΦC31* integrase-dependent transgene integration at a Chromosome 2 *att*P
310 landing site (2L:1476459) as previously described (Zheng et al., 2014). For genetic
311 rescue experiments, the UAS-*Gr8a* and *Gr8a*-GAL4 lines were transgressed into the
312 *Gr8a*^{1} CS genetic background.

313 The GFP-tagged allele of *Gr8a* was generated via CRISPR/*Cas9*-dependent editing
314 using a modified "scarless" strategy (www.flyCRISPR.molbio.wisc.edu) as described in
315 (Hill et al., 2019) using the sgRNA CGAGCAAGGCGGGAACGATT, and a donor plasmid
316 containing 1) A backbone with ampicillin resistance; 2) Eye-specific dsRed reporter driven
317 by the 3XP3 promoter, flanked by two *PiggyBac* transposase recognition sites; 3-4) The
318 left and right homology arms, which consisted of 1kb genomic DNA fragments upstream
319 and downstream of the sgRNA site respectively. Single base pair mutations were
320 introduced into the PAM sequence at each sgRNA binding site on the homology arms to
321 prevent Cas9-dependent cutting of the donor plasmid. The left homology arm included
322 the GFP coding sequence in the last intracellular loop of the Gr8a protein. pDCC6
323 plasmids containing sgRNA and cas9 sequences (100 ng/μL) and the donor plasmid (500
324 ng/μL) were co-injected into eggs of the *y*$^{-}$*w*$^{-}$ background. Subsequently, correct genomic
325 integration of the GFP tag was verified by screening for DsRed-positive animals, followed
326 by sequencing of genomic PCR fragments. Control lines with the same genetic
327 background were generated by selecting DsRed-negative animals. The final
328 tagged *Gr8a* allele was generated by removing the DsRed cassette via the introduction
329 of the *piggyBac* transposase (Bloomington #8285).

330

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

339 secondary antibodies (Both at 1:1000; Thermo Fisher Scientific). To visualize the GR8A
340 protein, abdomens of control flies and flies with CRISPR/Cas9 generated GFP-tagged
341 GR8A were dissected and immunostained as previously described (Lu et al., 2012; Zheng
342 et al., 2014) using a Rabbit anti-GFP antibody (1:1000; A-11122, Thermo Fisher
343 Scientific) followed by AlexaFluor 488 anti-rabbit secondary antibody (1:1000; Thermo
344 Fisher Scientific).

345

346 ***mRNA expression.*** Newly eclosed flies were separated by sex under CO_2 and aged for
347 5 days on standard cornmeal medium. On day 6, flies were placed in a -80°C freezer until
348 RNA extraction. To separate body parts, frozen flies were placed in 1.5ml microcentrifuge
349 tubes, dipped in liquid nitrogen, and then vortexed repeatedly until heads, appendages,
350 and bodies were clearly separated. Total RNA was extracted using the Trizol Reagent
351 (Thermo Fisher Scientific) separately from heads, bodies, and appendages for *Gr8a*
352 expression and from bodies for desaturase enzyme genes. cDNAs were synthesized
353 using SuperScript II reverse transcriptase (Thermo Fisher Scientific) with 500 ng total
354 RNA in a 20 uL reaction. Real-time quantitative RT-PCR was carried out as previously
355 described with *Rp49* as the loading control gene (Lu et al., 2014, 2012; Zheng et al.,
356 2014). Primer sequences are described in Tables 5, 6 and 7.

357

358 ***Courtship Behavior.*** Single-pair assays were performed as we have previously
359 published (Lu et al., 2014, 2012). In short, newly eclosed males were kept individually on
360 standard fly food in plastic vials (12 x 75mm). Newly eclosed virgin females were kept in
361 groups of 10 flies. All behaviors were done with 4-7 day-old animals, which were housed
362 under constant conditions of 25° C and a 12h:12h light-dark cycle. Courtship was video
363 recorded for 10 min for male courtship and 15 min for female mating receptivity. Male
364 courtship latency and index were measured as previously described (Lu et al., 2014,
365 2012). Female receptivity index was defined as the time from the initiation of male
366 courtship until copulation was observed. Unless otherwise indicated, assays were
367 performed under red light conditions.

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

372

373 ***Perfuming studies.*** Synthetic compounds were synthesized by J.G.M. Perfuming
374 studies were performed using a modified protocol from (Billeter et al., 2009). In short, 3
375 mg of each compound was dissolved in 6 mL hexane (Sigma-Aldrich #139386-500ML)
376 and 0.5 mL was pipetted into individual 2 mL glass vials fitted with 9mm PTFE lined caps
377 (Agilent Crosslab, Santa Clara, CA, USA). The hexane was evaporated under a nitrogen
378 gas flow, such that a residue of the compound was left around the bottom one-third of the
379 vial. Control vials were prepared using hexane without a spiked compound. Vials were
380 kept at -20°C until use. Flies used in these trials were collected as described above, kept
381 in single sex groups and aged for 4 days on standard cornmeal medium at 25°C. 24 hours
382 before perfuming, 20 flies of one or the other sex were placed in glass vials containing
383 standard cornmeal medium (12 x 75mm). To perfume the flies, these groups of 20 flies
384 were dumped without anesthesia into each 2 mL vial containing the compound of interest,
385 and were vortexed at medium-low speed for 3 pulses of 20 seconds punctuated by 20
386 second rest periods. Flies were transferred to new food vials and were allowed to recover
387 for one hour. Perfumed flies were then used in courtship behavior assays as described
388 above and the remaining flies were used in pheromone analyses to verify compound
389 transfer. The genotype of flies that were perfumed differed based upon the genotype with
390 the lower amount of each compound as determined in Figure 3 (B, C, F). In all cases,
391 compound transfer was verified by CHC extraction and GC/MS (Table 8).

392

393 ***Phylogenetic analysis.*** Protein sequences of GR8A orthologs from the 12 sequenced
394 *Drosophila* reference genomes were aligned by using the ClustalW algorithm in the
395 Omega package (Sievers et al., 2011), followed by ProtTest (v2.4) to determine the best
396 model of protein evolution (Abascal et al., 2005). Subsequently, Akaike and Bayesian
397 information criterion scores were used to select the appropriate substitution matrix. We

398 then used a maximum likelihood approach and rapid bootstrapping within RAxML v 7.2.8
399 Black Box on the Cipres web portal to make a phylogenetic tree (Miller et al., 2010).
400 Visualizations of the bipartition files were made using FigTree v1.3.1
401 (http://tree.bio.ed.ac.uk/software/figtree/).

402

403 ***Pheromone Analysis.*** Virgin flies were collected upon eclosion under a light CO^2
404 anesthesia and kept in single-sex vials in groups of 10 with 6 biological replications for
405 each genotype and sex. Virgin flies were aged for 5 days on standard cornmeal medium
406 at 25°C. To collect mated flies, both females and males were aged for 3 days before
407 single mating pairs were placed in a standard fly vial with standard cornmeal food for 24
408 hours. The pair was then separated for 24 hours before collection. Copulation was
409 confirmed by the presence of larvae in the vials of mated females several days later. On
410 the morning of day 5, flies were anesthetized under light CO^2 and groups of five flies were
411 placed in individual scintillation vials (VWR 74504-20). To extract CHCs, each group of
412 flies was covered by 100 uL hexane (Sigma-Aldrich #139386-500ML) containing 50µg/mL
413 hexacosane (Sigma-Aldrich #241687-5G) and was washed for ten minutes.
414 Subsequently, hexane washes were transferred into a new 2 ml glass vial containing a
415 350 uL insert (Thermo Scientific C4000-LV-1W) and were stored at -20°C until shipment
416 to the Millar laboratory.

417 Analyses of CHC profiles were done by gas chromatography and mass spectroscopy
418 (GC-MS) in the Millar laboratory at UC Riverside as previously described (Chung et al.,
419 2014). Peak areas were measured, and data was normalized to known quantity of internal
420 standard hexacosane (Sigma-Aldrich #241687-5G). The relative proportion of each
421 compound in each sample was calculated and used in further statistical analysis.
422 Qualitative data were analyzed through a permutation MANOVA using the ADONIS
423 function in the vegan package of R with Bray-Curtis dissimilarity measures (Oksanen et
424 al., 2017). Data were visualized using non-metric multidimensional scaling (vegdist
425 function in the vegan package of R followed by cmdscale function in the stats package
426 (Oksanen et al., 2017)) using Bray-Curtis dissimilarity, and either 2 or 3 dimensions in

427 order to minimize stress to < 0.1. Quantitative data were analyzed by using a t-test or
428 Mann-Whitney Rank Sum Test in R 3.3.2 (R Core Team, 2016).

429

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

432

433 **AUTHOR CONTRIBUTIONS**

434 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.,
435 J.G.M. and Y.B-S collected and analyzed data. K.M.Z., C.V. and Y.B-S wrote the
436 manuscript.

437

438 **ACKNOWLEDGMENTS**

439 We thank members of the Ben-Shahar lab for comments on earlier versions of the
440 manuscript. We thank the Millar laboratory (UC Riverside) for performing the CHC
441 analyses. We thank Joshua Krupp for assistance with perfuming studies, Nabeel
442 Chowdhury and Deanna Simon for assistance with qRT-PCR analysis, and Paula Kiefel
443 for technical help with generating transgenic flies. This work was supported by NSF grants
444 1545778 and 1754264, and NIH grant NS089834 awarded to Y.B. Stocks obtained from
445 the Bloomington Drosophila Stock Center (NIH P40OD018537) were used in this study.
446 Wild type *Drosophila* species were obtained from the National *Drosophila* Species Stock
447 Center at Cornell University.

448 **REFERENCES**

449 Abascal F, Zardoya R, Posada D. 2005. ProtTest: Selection of best-fit models of protein
450 evolution. *Bioinformatics* **21**:2104–2105. doi:10.1093/bioinformatics/bti263

451 Arnold SJ, Verrell PA, Tilley SG. 2006. The Evolution of Asymmetry in Sexual Isolation:
452 A Model and a Test Case. *Evolution (N Y)* **50**:1024. doi:10.2307/2410643

453 Benton R, Vannice KS, Vosshall LB. 2007. An essential role for a CD36-related receptor
454 in pheromone detection in Drosophila. *Nature* **450**:289–293.
455 doi:10.1038/nature06328

456 Billeter JC, Atallah J, Krupp JJ, Millar JG, Levine JD. 2009. Specialized cells tag sexual
457 and species identity in Drosophila melanogaster. *Nature* **461**:987–991.
458 doi:10.1038/nature08495

459 Blomquist GJ, Bagnères A-G. 2010. Insect Hydrocarbons: Biology, Biochemistry, and
460 Chemical Ecology. Cambridge: Cambridge University Press.

461 Boake CRB. 1991. Coevolution of senders and receivers of sexual signals: Genetic
462 coupling and genetic correlations. *Trends Ecol Evol* **6**:225–227. doi:10.1016/0169-
463 5347(91)90027-U

464 Bousquet F, Nojima T, Houot B, Chauvel I, Chaudy S, Dupas S, Yamamoto D, Ferveur
465 J-F. 2011. Expression of a desaturase gene, desat1, in neural and nonneural
466 tissues separately affects perception and emission of sex pheromones in
467 Drosophila. *Proc Natl Acad Sci* **109**:249–254. doi:10.1073/pnas.1109166108

468 Bray S, Amrein H. 2003. A putative Drosophila pheromone receptor expressed in male-
469 specific taste neurons is required for efficient courtship. *Neuron* **39**:1019–1029.
470 doi:10.1016/S0896-6273(03)00542-7

471 Brooks R, Hunt J, Blows MW, Smith MJ, Bussière LF, Jennions MD. 2005.
472 Experimental evidence for multivariate stabilizing sexual selection. *Evolution (N Y)*
473 **59**:871–880. doi:10.1111/j.0014-3820.2005.tb01760.x

474 Butlin RK, Ritchie MG. 1989. Genetic coupling in mate recognition systems: what is the
475 evidence? *Biol J Linn Soc* **37**:237–246. doi:10.1111/j.1095-8312.1989.tb01902.x

476 Chebib J, Guillaume F. 2019. Pleiotropy or linkage? Their relative contributions to the
477 genetic correlation of quantitative traits and detection by multi-trait GWA studies.
478 *bioRxiv* 656413. doi:10.1101/656413

479 Chenoweth SF, Blows MW. 2006. Dissecting the complex genetic basis of mate choice.
480 *Nat Rev Genet* **7**:681–692. doi:10.1038/nrg1924

481 Chung H, Carroll SB. 2015. Wax, sex and the origin of species: Dual roles of insect
482 cuticular hydrocarbons in adaptation and mating. *BioEssays* **37**:822–830.
483 doi:10.1002/bies.201500014

484 Chung H, Loehlin DW, Dufour HD, Vaccarro K, Millar JG, Carroll SB. 2014. A single
485 gene affects both ecological divergence and mate choice in Drosophila. *Science*
486 *(80-)* **343**:1148–1151.

487 Clyne PJ, Warr CG, Carlson JR. 2000. Candidate taste receptors in Drosophila.
488 *Science (80-)* **287**:1830–1834. doi:10.1126/science.287.5459.1830

489 Coyne JA, Crittenden AP, Mah K. 1994. Genetics of a pheromonal difference
490 contributing to reproductive isolation in Drosophila. *Science (80-)* **265**:1461–1464.
491 doi:10.1126/science.8073292

492 Datta SR, Vasconcelos ML, Ruta V, Luo S, Wong A, Demir E, Flores J, Balonze K,
493 Dickson BJ, Axel R. 2008. The Drosophila pheromone cVA activates a sexually
494 dimorphic neural circuit. *Nature* **452**:473–477. doi:10.1038/nature06808

495 Dunipace L, Meister S, Mcnealy C, Amrein H. 2001. Spatially restricted expression of
496 candidate taste receptors in the. *Curr Biol* 822–835.

497 Dweck HKM, Ebrahim SAM, Thoma M, Mohamed AAM, Keesey IW, Trona F, Lavista-
498 Llanos S, Svatoš A, Sachse S, Knaden M, Hansson BS. 2015. Pheromones
499 mediating copulation and attraction in Drosophila. *Proc Natl Acad Sci U S A*
500 **112**:E2829–E2835. doi:10.1073/pnas.1504527112

501 Dyer KA, White BE, Sztepanacz JL, Bewick ER, Rundle HD. 2014. Reproductive
502 character displacement of epicuticular compounds and their contribution to mate
503 choice in Drosophila subquinaria and Drosophila recens. *Evolution (N Y)* **68**:1163–
504 1175. doi:10.1111/evo.12335.REPRODUCTIVE

505 Ferveur JF. 2005. Cuticular hydrocarbons: Their evolution and roles in Drosophila
506 pheromonal communication. *Behav Genet* **35**:279–295. doi:10.1007/s10519-005-
507 3220-5

508 Ford CP. 2014. The role of D2-autoreceptors in regulating dopamine neuron activity and
509 transmission. *Neuroscience* **282**:13–22. doi:10.1016/j.neuroscience.2014.01.025

510 Fukamachi S, Kinoshita M, Aizawa K, Oda S, Meyer A, Mitani H. 2009. Dual control by
511 a single gene of secondary sexual characters and mating preferences in medaka.
512 *BMC Biol* **7**:64. doi:10.1186/1741-7007-7-64

513 Gutierrez E, Wiggins D, Fielding B, Gould AP. 2007. Specialized hepatocyte-like cells
514 regulate Drosophila lipid metabolism. *Nature* **445**:275–280.
515 doi:10.1038/nature05382

516 Hill AS, Jain P, Folan NE, Ben-Shahar YY. 2019. The Drosophila ERG channel seizure
517 plays a role in the neuronal homeostatic stress response. *PLoS Genet* **15**:1–24.
518 doi:10.1371/journal.pgen.1008288

519 Howard RW, Blomquist GJ. 2005. Ecological, Behavioral, and Biochemical Aspects of
520 Insect Hydrocarbons*. *Annu Rev Entomol* **50**:371–393.
521 doi:10.1146/annurev.ento.50.071803.130359

522 Hoy RR, Hahn J, Paul RC. 1977. Hybrid cricket auditory behavior: Evidence for genetic
523 coupling in animal communication. *Science (80-)* **195**:82–84.
524 doi:10.1126/science.831260

525 Hu Y, Han Y, Shao Y, Wang X, Ma Y, Ling E, Xue L. 2015. Gr33a modulates drosophila
526 male courtship preference. *Sci Rep* **5**:1–7. doi:10.1038/srep07777

527 Kallman BR, Kim H, Scott K. 2015. Excitation and inhibition onto central courtship
528 neurons biases drosophila mate choice. *Elife* **4**:1–18. doi:10.7554/eLife.11188

529 Kirkpatrick M, Hall DW. 2004. Sexual Selection and Sex Linkage. *Evolution (N Y)*
530 **58**:683–691.

531 Koh T, He Z, Gorur-shandilya S, Menuz K, Larter NK, Carlson JR. 2015. Candidate
532 Taste and Pheromone Receptors. *Neuron* **83**:850–865.

533 doi:10.1016/j.neuron.2014.07.012.The

534 Krupp JJ, Billeter JC, Wong A, Choi C, Nitabach MN, Levine JD. 2013. Pigment-
535 Dispersing Factor Modulates Pheromone Production in Clock Cells that Influence
536 Mating in Drosophila. *Neuron* **79**:54–68. doi:10.1016/j.neuron.2013.05.019

537 Kurtovic A, Widmer A, Dickson BJ. 2007. A single class of olfactory neurons mediates
538 behavioural responses to a Drosophila sex pheromone. *Nature* **446**:542–546.
539 doi:10.1038/nature05672

540 Labeur C, Dallerac R, Wicker-Thomas C. 2002. Involvement of desat1 gene in the
541 control of Drosophila melanogaster pheromone biosynthesis. *Genetica* **114**:269–
542 274. doi:10.1023/A:1016223000650

543 Lacaille F, Everaerts C, Ferveur JF. 2009. Feminization and alteration of Drosophila
544 taste neurons induce reciprocal effects on male avoidance behavior. *Behav Genet*
545 **39**:554–563. doi:10.1007/s10519-009-9286-8

546 Lacaille F, Hiroi M, Twele R, Inoshita T, Umemoto D, Manière G, Marion-Poll F, Ozaki
547 M, Francke W, Cobb M, Everaerts C, Tanimura T, Ferveur JF. 2007. An inhibitory
548 sex pheromone tastes bitter for Drosophila males. *PLoS One* **2**:1–7.
549 doi:10.1371/journal.pone.0000661

550 Lande R. 1984. The genetic correlation between characters maintained by selection,
551 linkage and inbreeding. *Genet Res* **44**:309–320. doi:10.1017/S0016672300026549

552 Lande R. 1981. Models of speciation by sexual selection on polygenic traits. *Proc Natl*
553 *Acad Sci* **78**:3721–3725.

554 Lande R. 1980. The genetic covariance between characters maintained by pleiotropic
555 mutations. *Genetics* **94**:203–215.

556 Lebreton S, Grabe V, Omondi AB, Ignell R, Becher PG, Hansson BS, Sachse S,
557 Witzgall P. 2014. Love makes smell blind: Mating suppresses pheromone attraction
558 in Drosophila females via Or65a olfactory neurons. *Sci Rep* **4**:1–6.
559 doi:10.1038/srep07119

560 Lee Y, Kang MJ, Shim J, Cheong CU, Moon SJ, Montell C. 2012. Gustatory receptors

561 required for avoiding the insecticide L-canavanine. *J Neurosci* **32**:1429–1435.
562 doi:10.1523/JNEUROSCI.4630-11.2012

563 Lee Y, Moon SJ, Montell C. 2009. Multiple gustatory receptors required for the caffeine
564 response in Drosophila. *Proc Natl Acad Sci U S A* **106**:4495–4500.
565 doi:10.1073/pnas.0811744106

566 Lofstedt C, Hansson BS, Roelofst W, Bengtsson B 0. 1989. No Linkage Between Genes
567 Controlling Female Pheromone Production and Male Pheromone Response in the
568 European Corn Borer, Ostm'nia nubilalis Hubner (Lepidoptera; Pyralidae).

569 Lu B, LaMora A, Sun Y, Welsh MJ, Ben-Shahar Y. 2012. ppk23-Dependent
570 chemosensory functions contribute to courtship behavior in Drosophila
571 melanogaster. *PLoS Genet* **8**:e1002587. doi:10.1371/journal.pgen.1002587

572 Lu B, Zelle KM, Seltzer R, Hefetz A, Ben-Shahar Y. 2014. Feminization of pheromone-
573 sensing neurons affects mating decisions in Drosophila males. *Biol Open* **3**:152–
574 160. doi:10.1242/bio.20147369

575 Makki R, Cinnamon E, Gould AP. 2014. The Development and Functions of Oenocytes.
576 *Annu Rev Entomol* **59**:405–425. doi:10.1146/annurev-ento-011613-162056

577 McKinney RM, Vernier C, Ben-Shahar Y. 2015. The neural basis for insect pheromonal
578 communication. *Curr Opin Insect Sci* **12**:86–92. doi:10.1016/j.cois.2015.09.010

579 Miller M, Pfeiffer WT, Schwartz T. 2010. Creating the CIPRES Science Gateway for
580 Inference of Large Phylogenetic Trees, Proceedings of the Gateway Computing
581 Environments Workshop: 14 Nov 2010. doi:10.1109/GCE.2010.5676129

582 Miyamoto T, Amrein H. 2008. Suppression of male courtship by a Drosophila
583 pheromone receptor. *Nat Neurosci* **11**:874–876. doi:10.1038/nn.2161

584 Moon SJ, Lee Y, Jiao Y, Montell C. 2009. A Drosophila gustatory receptor essential for
585 aversive taste and inhibiting male-to-male courtship. *Curr Biol* **19**:1623–1627.
586 doi:10.1038/jid.2014.371

587 Mucignat-Caretta C, editor. 2014. Neurobiology of Chemical Communication. CRC
588 Press.

589 Ng SH, Shankar S, Shikichi Y, Akasaka K, Mori K, Yew JY. 2014. Pheromone evolution
590 and sexual behavior in Drosophila are shaped by male sensory exploitation of other
591 males. *Proc Natl Acad Sci U S A* **111**:3056–3061. doi:10.1073/pnas.1313615111

592 Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, Minchin PR,
593 O'Hara RB, Simpson GL, Solymos P, Henry M, Stevens H, Szoecs E, Wagner H.
594 2017. vegan: Community Ecology Package.

595 Park JH, Kwon JY. 2011. A systematic analysis of drosophila gustatory receptor gene
596 expression in abdominal neurons which project to the central nervous system. *Mol*
597 *Cells* **32**:375–381. doi:10.1007/s10059-011-0128-1

598 Poudel S, Kim Y, Kim YT, Lee Y. 2015. Gustatory receptors required for sensing
599 umbelliferone in Drosophila melanogaster. *Insect Biochem Mol Biol* **66**:110–118.
600 doi:10.1016/j.ibmb.2015.10.010

601 Robertson HM, Warr CG, Carlson JR. 2003. Molecular evolution of the insect
602 chemoreceptor gene superfamily in Drosophila melanogaster. *Proc Natl Acad Sci U*
603 *S A* **100**:14537–14542. doi:10.1073/pnas.2335847100

604 Scott K, Brady R, Cravchik A, Morozov P, Rzhetsky A, Zuker C, Axel R. 2001. A
605 chemosensory gene family encoding candidate gustatory and olfactory receptors in
606 Drosophila. *Cell* **104**:661–673. doi:10.1016/S0092-8674(01)00263-X

607 Shankar S, Chua JY, Tan KJ, Calvert ME, Weng R, Ng WC, Mori K, Yew JY. 2015. The
608 neuropeptide tachykinin is essential for pheromone detection in a gustatory neural
609 circuit. *Elife* **4**:1–23. doi:10.7554/elife.06914

610 Shaw KL, Ellison CK, Oh KP, Wiley C. 2011. Pleiotropy, "sexy" traits, and speciation.
611 *Behav Ecol* **22**:1154–1155. doi:10.1093/beheco/arr136

612 Shaw KL, Lesnick SC. 2009. Genomic linkage of male song and female acoustic
613 preference QTL underlying a rapid species radiation. *Proc Natl Acad Sci U S A*
614 **106**:9737–9742. doi:10.1073/pnas.0900229106

615 Shim J, Lee Y, Jeong YT, Kim Y, Lee MG, Montell C, Moon SJ. 2015. The full repertoire
616 of Drosophila gustatory receptors for detecting an aversive compound. *Nat*
617 *Commun* **6**. doi:10.1038/ncomms9867

618 Shirangi TR, Dufour HD, Williams TM, Carroll SB. 2009. Rapid evolution of sex
619 pheromone-producing enzyme expression in Drosophila. *PLoS Biol* **7**.
620 doi:10.1371/journal.pbio.1000168

621 Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H,
622 Remmert M, Söding J, Thompson JD, Higgins DG. 2011. Fast, scalable generation
623 of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst*
624 *Biol* **7**. doi:10.1038/msb.2011.75

625 Singh ND, Shaw KL. 2012. On the scent of pleiotropy. *Proc Natl Acad Sci* **109**:5–6.
626 doi:10.1073/pnas.1118531109

627 Stagkourakis S, Kim H, Lyons DJ, Broberger C. 2016. Dopamine Autoreceptor
628 Regulation of a Hypothalamic Dopaminergic Network. *Cell Rep* **15**:735–747.
629 doi:10.1016/j.celrep.2016.03.062

630 Steiger S, Schmitt T, Martin Schaefer H. 2011. The origin and dynamic evolution of
631 chemical information transfer. *Proc R Soc B Biol Sci* **278**:970–979.
632 doi:10.1098/rspb.2010.2285

633 Svensson EI, Karlsson K, Friberg M, Eroukhmanoff F. 2007. Gender Differences in
634 Species Recognition and the Evolution of Asymmetric Sexual Isolation. *Curr Biol*
635 **17**:1943–1947. doi:10.1016/j.cub.2007.09.038

636 Sweigart AL. 2010. The genetics of postmating, prezygotic reproductive isolation
637 between Drosophila virilis and D. americana. *Genetics* **184**:401–410.
638 doi:10.1534/genetics.109.111245

639 Symonds MRE, Elgar MA. 2008. The evolution of pheromone diversity. *Trends Ecol*
640 *Evol* **23**:220–228. doi:10.1016/j.tree.2007.11.009

641 Team RC. 2016. R: A language and environment for statistical computing.

642 Thistle R, Cameron P, Ghorayshi A, Dennison L, Scott K. 2012. Contact
643 chemoreceptors mediate male-male repulsion and male-female attraction during
644 drosophila courtship. *Cell* **149**:1140–1151. doi:10.1016/j.cell.2012.03.045

645 Toda H, Zhao X, Dickson BJ. 2012. The Drosophila Female Aphrodisiac Pheromone

646 Activates ppk23+ Sensory Neurons to Elicit Male Courtship Behavior. *Cell Rep*
647 **1**:599–607. doi:10.1016/j.celrep.2012.05.007

648 van der Goes van Naters W, Carlson JRR. 2007. Receptors and Neurons for Fly Odors
649 in Drosophila. *Curr Biol* **17**:606–612. doi:10.1016/j.cub.2007.02.043

650 Wang Z, Singhvi A, Kong P, Scott K. 2004. Taste representations in the Drosophila
651 brain. *Cell* **117**:981–991. doi:10.1016/j.cell.2004.06.011

652 Watanabe K, Toba G, Koganezawa M, Yamamoto D. 2011. Gr39a, a Highly diversified
653 gustatory receptor in drosophila, has a role in sexual behavior. *Behav Genet*
654 **41**:746–753. doi:10.1007/s10519-011-9461-6

655 Wiley C, Ellison CK, Shaw KL. 2012. Widespread genetic linkage of mating signals and
656 preferences in the Hawaiian cricket Laupala. *Proc R Soc B Biol Sci* **279**:1203–
657 1209. doi:10.1098/rspb.2011.1740

658 Wyatt TD. 2014. Pheromones and Animal Behavior: Chemical Signals and Signatures,
659 2nd ed. Cambridge: Cambridge University Press.

660 Yew JY, Chung H. 2015. Insect pheromones: An overview of function, form, and
661 discovery. *Prog Lipid Res*. doi:10.1016/j.plipres.2015.06.001

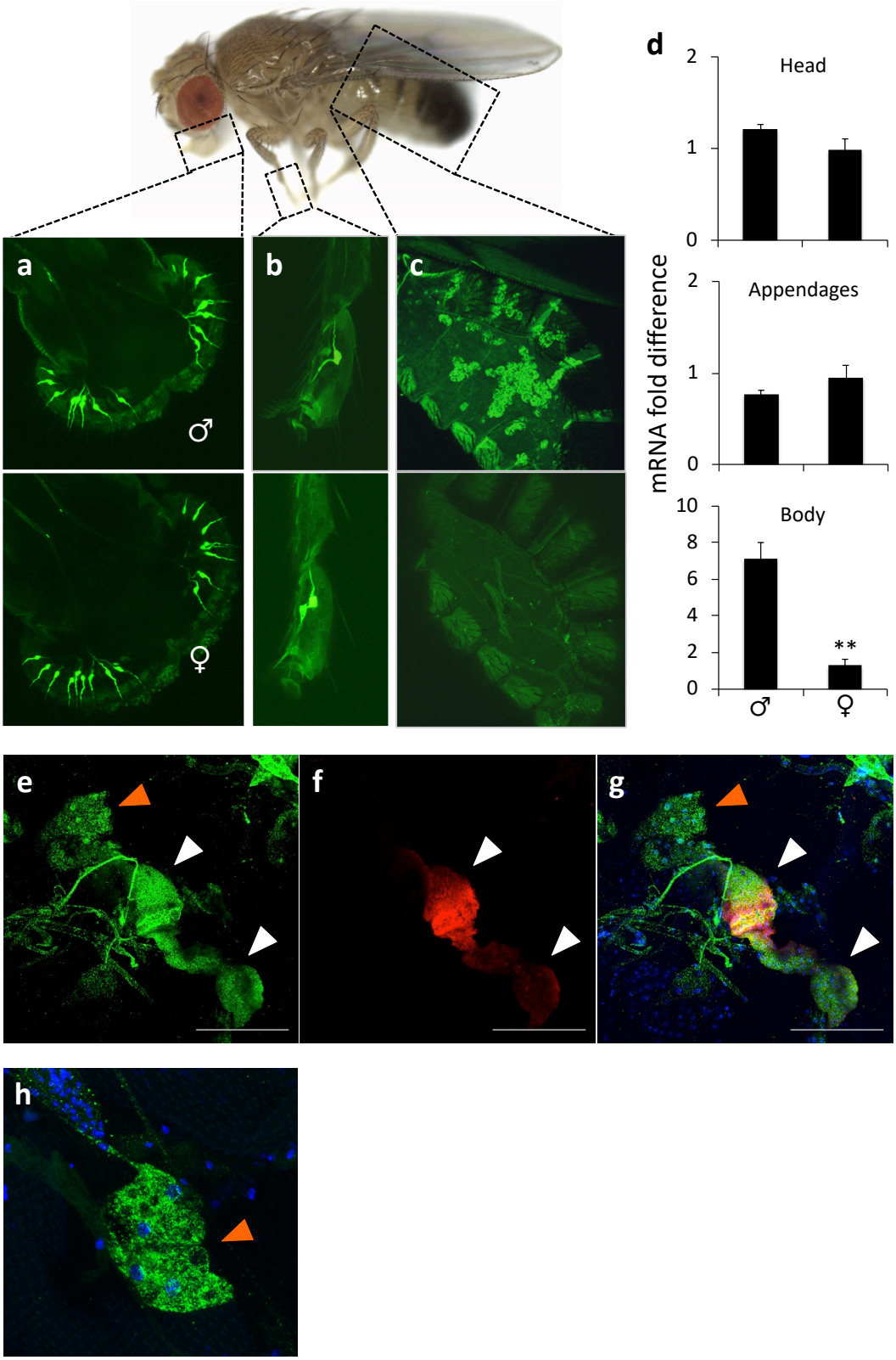
662 Yew JY, Dreisewerd K, Luftmann H, Müthing J, Pohlentz G, Kravitz EA. 2009. A New
663 Male Sex Pheromone and Novel Cuticular Cues for Chemical Communication in
664 Drosophila. *Curr Biol* **19**:1245–1254. doi:10.1016/j.cub.2009.06.037

665 Zheng X, Valakh V, DiAntonio A, Ben-Shahar Y. 2014. Natural antisense transcripts
666 regulate the neuronal stress response and excitability. *Elife* **3**.
667 doi:10.7554/elife.01849

668

669

670 **Figures and figure legends:**



671

672 **Figure 1**. ***Gr8a* is a sexually dimorphic chemosensory receptor enriched in male**
673 **oenocytes and fat body**. (A) *Gr8a* promoter activity in proboscis, (B) forelegs, and (C)
674 abdomens of males (top panels) and females (bottom panels). (D) *Gr8a* mRNA
675 expression. Relative mRNA levels were measured by real-time quantitative RT-PCR. **,
676 $p<0.01$ Mann Whitney Rank Sum Test, n=3/group. (E) Confocal z-stack image of
677 *Gr8a*>EGFP in abdominal cells. (F) Confocal z-stack image of *desat1*>Luciferase in
678 abdominal cells. (G) Co-expression of *Gr8a* and *desat1*. (H) Confocal z-stack image of
679 GFP-tagged GR8A protein in abdominal cells. Green, *Gr8a*; Red, *desat1*; Blue, nuclear
680 DAPI stain. Orange arrowhead, fat body cells; white arrowhead, oenocytes. Scale bar =
681 100μm.

682

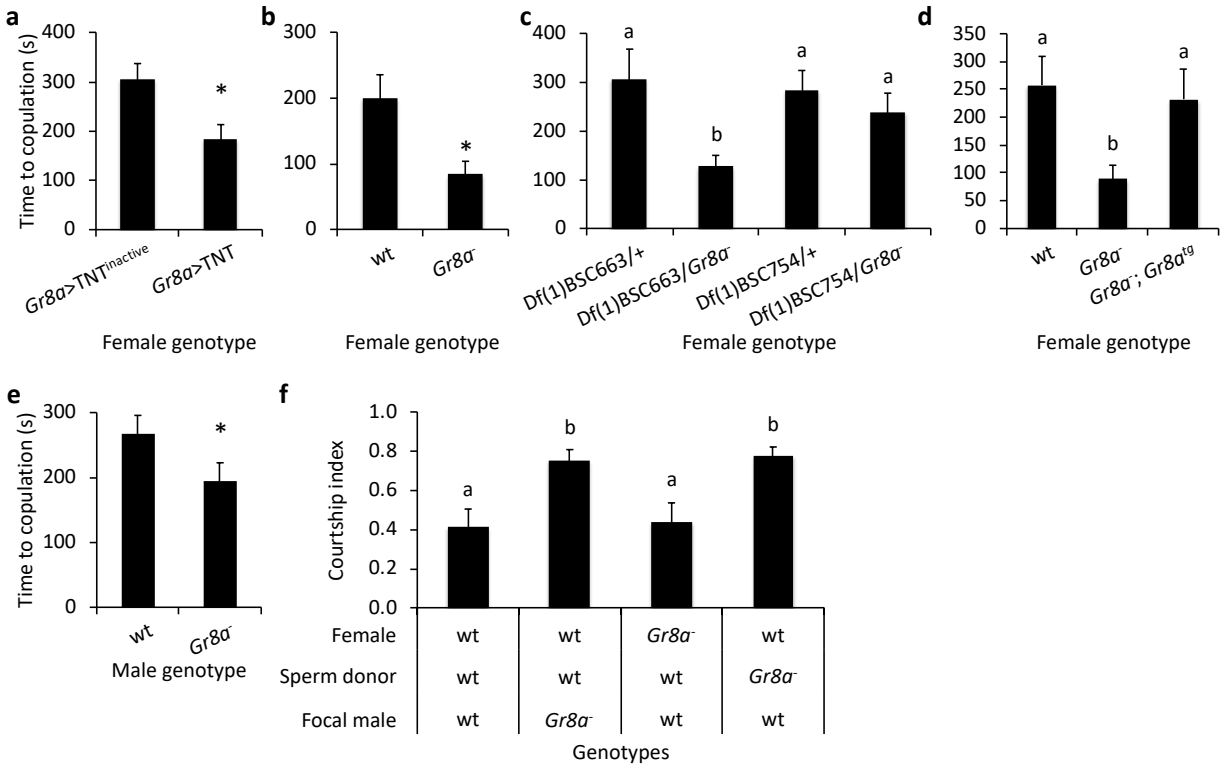


Figure 2. *Gr8a* activity contributes to the perception and production of an inhibitory signal associated with mating decisions in males and females. (A) Blocking neural activity in female *Gr8a*-expressing sensory neurons shortens copulation latency. Homozygous (B) or hemizygous (C) *Gr8a* null females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

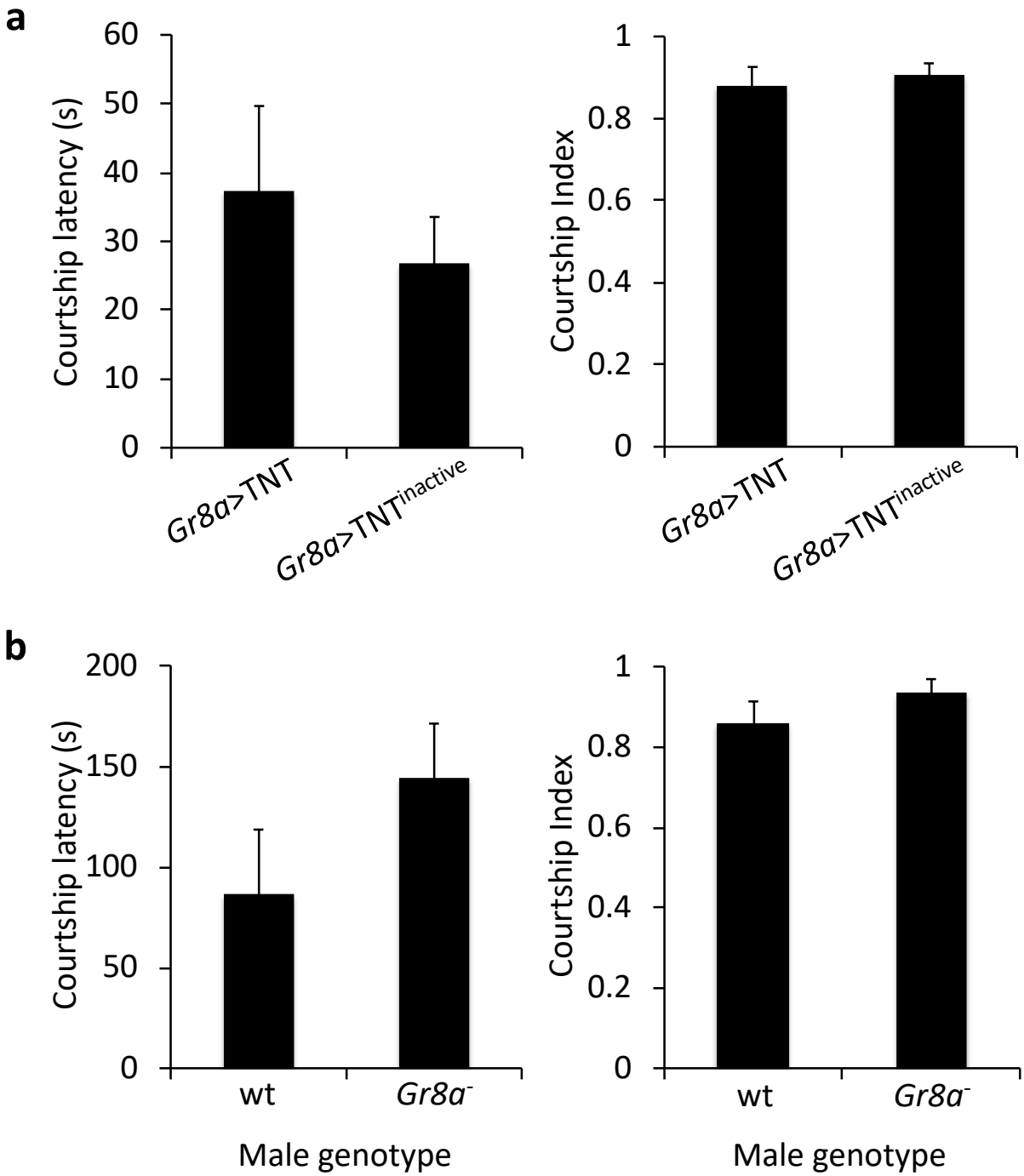
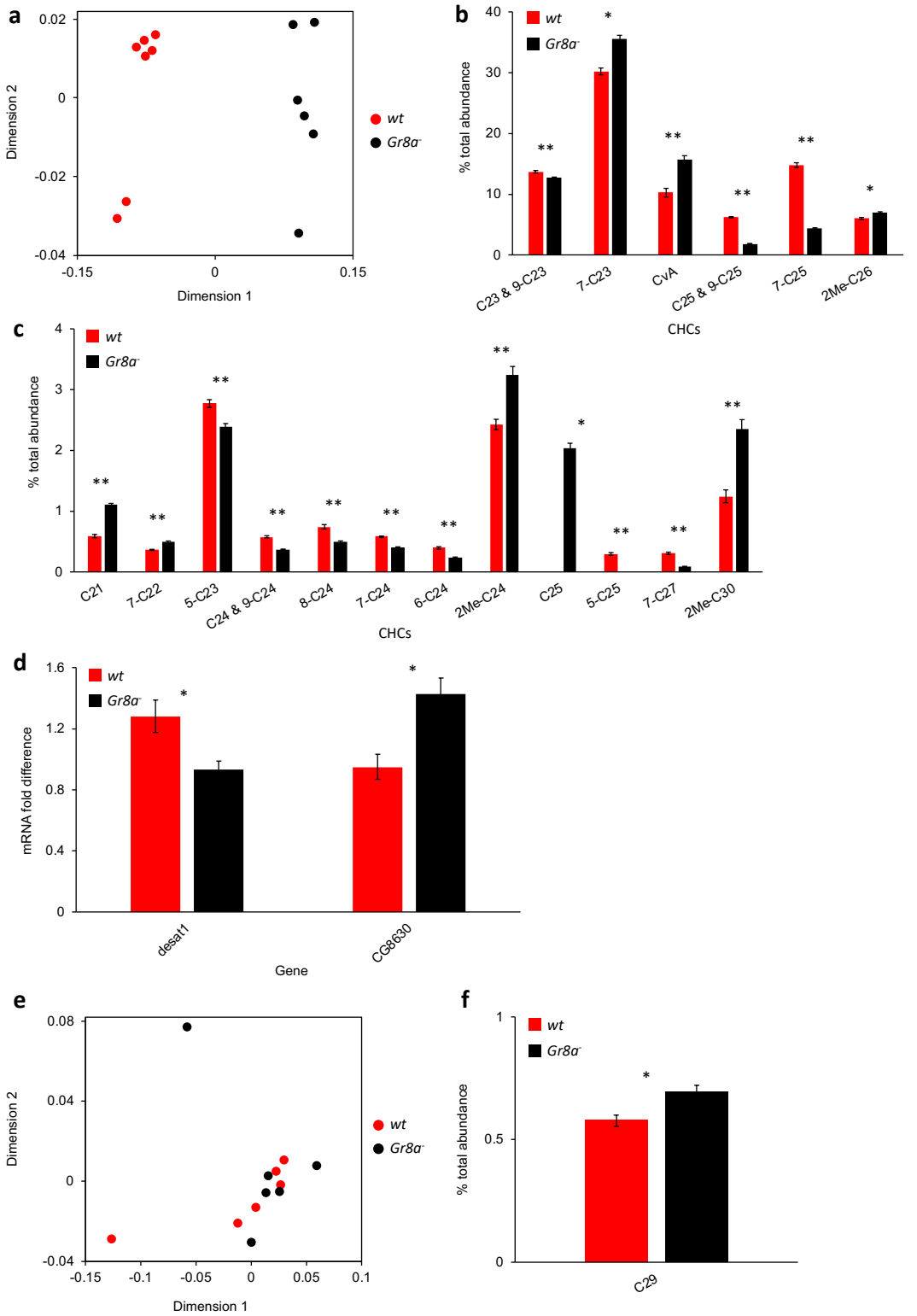


Figure 2—figure supplement 1. *Gr8a* has no effect on male Courtship Index or Latency toward wild-type females. (A) Courtship Index and Latency (s) of control *Gr8a-gal4/UAS-IMP-TNT- V1A* (*TNT*^{inactive}) and *Gr8a-gal4/UAS-TNT-E* mutant males towards wild-type females. (B) Courtship index and latency (s) of wild-type (*CS*) and

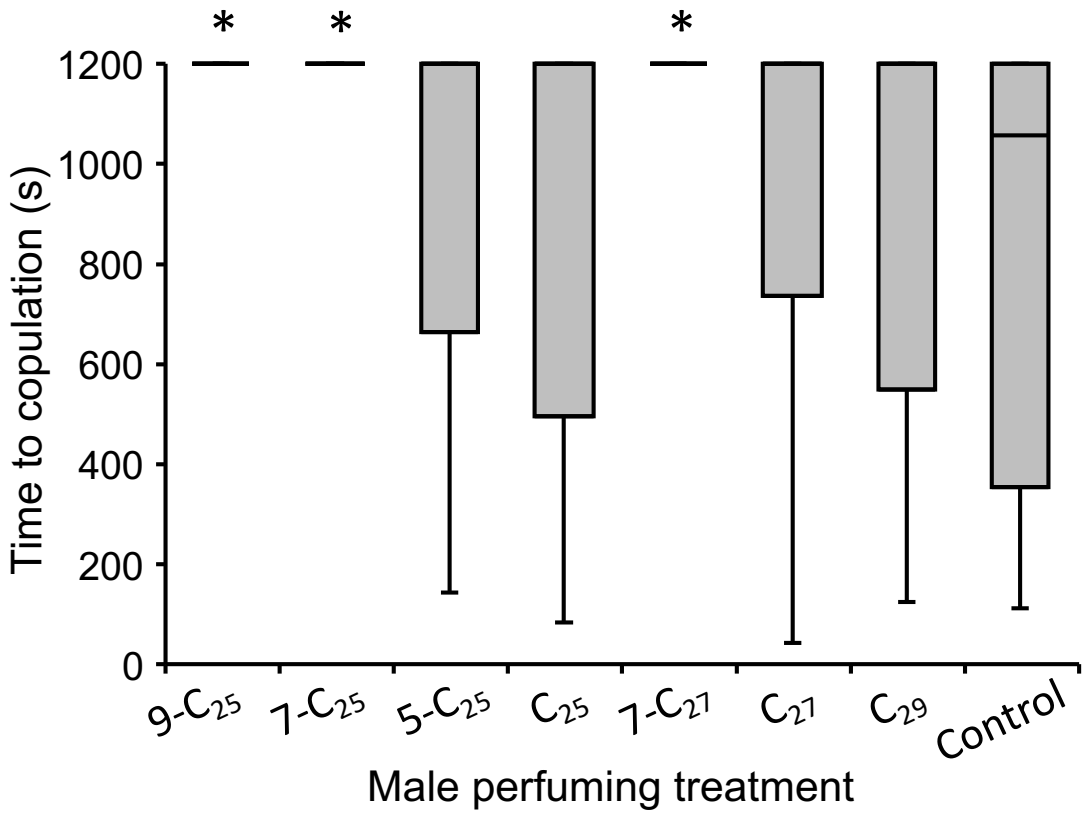
704 *Gr8a null* males toward wild-type decapitated females. Mann Whitney Rank Sum Test,
705 not significant ($p>0.05$), n=15/group.


a
Dimension 2
Dimension 1
wt
Gr8a-
b
% total abundance
C23 & 9-C23
7-C23
CvA
C25 & 9-C25
7-C25
2Me-C26
CHCs
c
% total abundance
C21
7-C22
5-C23
C24 & 9-C24
8-C24
7-C24
6-C24
2Me-C24
C25
5-C25
7-C27
2Me-C30
CHCs
d
mRNA fold difference
desat1
CG8630
Gene
e
Dimension 2
Dimension 1
f
% total abundance
C29

706

707 **Figure 3. The *Gr8a* mutation affects the pheromone profiles of males and mated**
708 **females. (A)** Nonmetric multidimensional scaling (NMDS) plot of CHC profiles of wild-
709 type and *Gr8a* mutant males. $p<0.001$, permutation MANOVA. **(B-C)** The effect of the
710 *Gr8a* mutation on levels of individual CHCs in males. (B) CHCs found at high proportions
711 in males. (C) CHCs found at low proportions in males. Only affected CHCs are shown.
712 See Table 2 for the complete list. *, $p<0.05$, **, $p<0.001$, Student's t-test or Mann Whitney
713 Rank Sum Test, n=6 (Gr8a-) or 7 (wt). **(D)** The effect of *Gr8a* mutation on the expression
714 level of several desaturase genes. Only affected genes are shown. See Table 3 for the
715 complete list. *, $p<0.05$, Student's t-test, n=4/group. **(E)** NMDS plot of CHC profiles of
716 females mated with wild-type or *Gr8a* mutant males. $p=0.570$, permutation MANOVA. **(F)**
717 Nonacosane (C_{29}) differs between females mated with wild-type and *Gr8a* mutant males.
718 See Table 4 for complete list of mated-female CHCs. *, $p<0.05$, Student's t-test,
719 n=6/group.

720



721

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

727

728

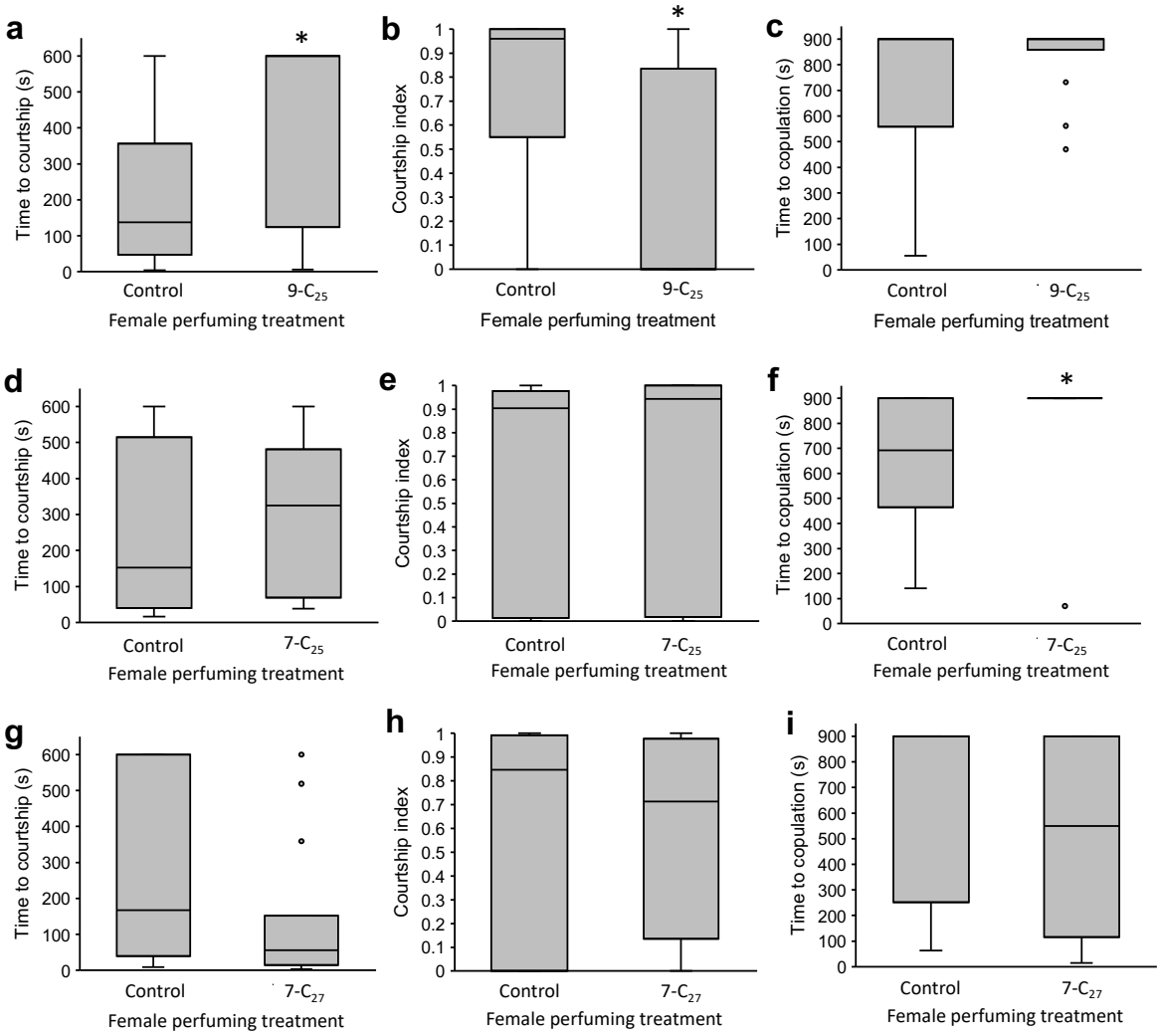


Figure 5. *Gr8a*-associated alkenes inhibit normal courtship behaviors when females are perfumed. Perfuming females with 9-C_{25} increases courtship latency (A), decreases courtship index (B), but does not affect copulation latency compared to control females. Perfuming females with 7-C_{25} does not affect courtship latency (D) or index (E), but increases copulation latency (F) compared to control females. Perfuming females with 7-C_{27} does not affect courtship latency (G), courtship index (H), or copulation latency (I) compared to control females. Asterisks above bars indicate statistically significant contrasts compared to control flies, $p<0.05$, Mann Whitney Rank Sum Test, n=15/group.

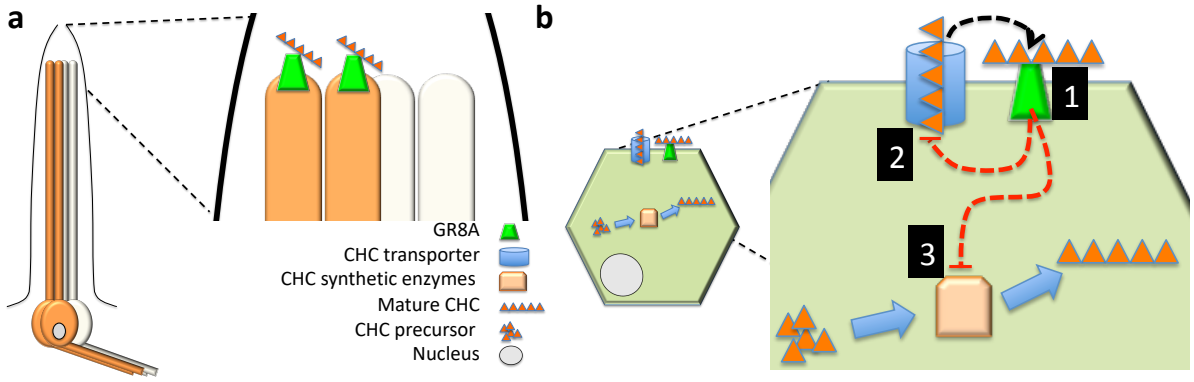
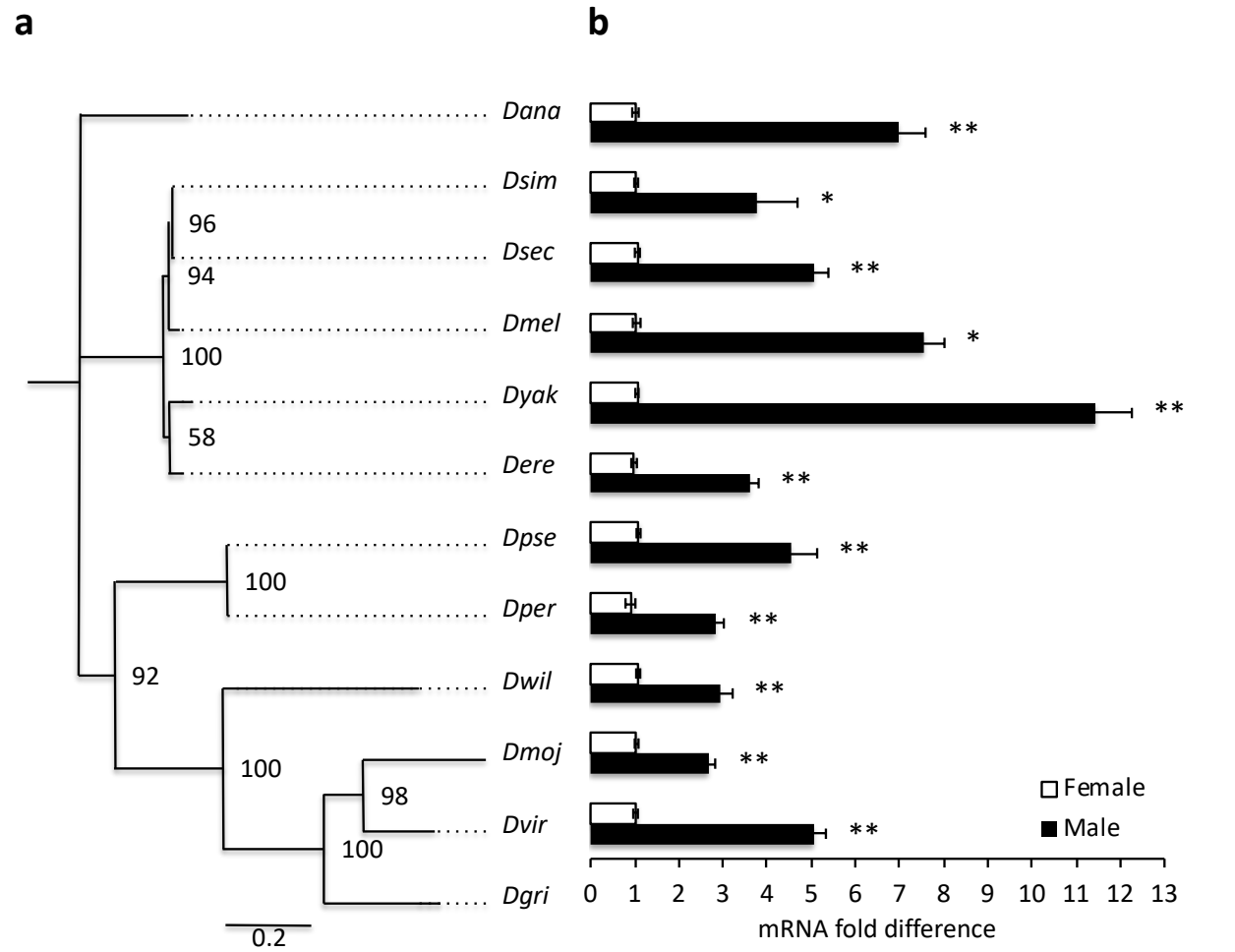
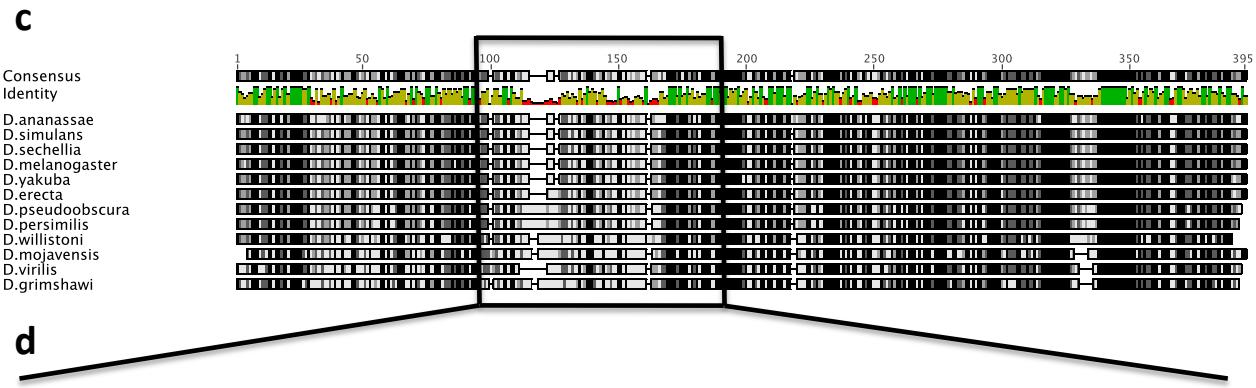
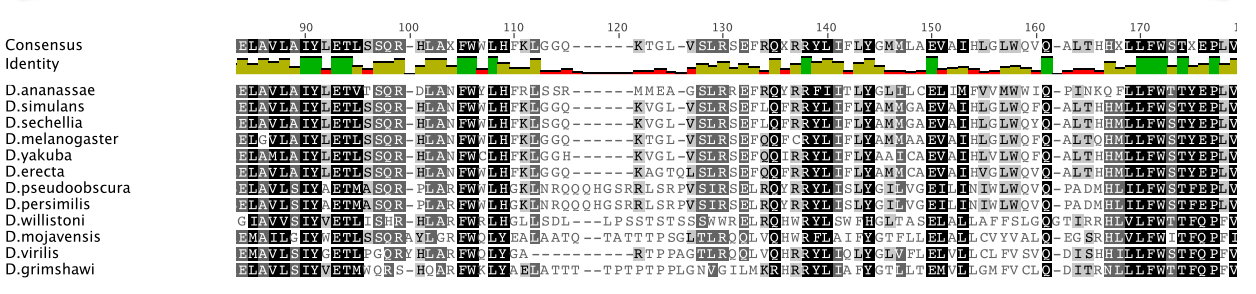


Figure 6. Model for the pleiotropic action of *Gr8a* in the perception and production of pheromones. (A) *Gr8a* functions as a chemoreceptor for an inhibitory signal in pheromone-sensing GRNs of males and females. (B) *Gr8a* also functions as a CHC autoreceptor in oenocytes, which regulates CHC secretion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


a
b
Dana
Dsim
Dsec
Dmel
Dyak
Dere
Dpse
Dper
Dwil
Dmoj
Dvir
Dgri
96
94
100
58
100
92
100
98
100
0.2
Female
Male
0 1 2 3 4 5 6 7 8 9 10 11 12 13
mRNA fold difference


c
Consensus
Identity
D.ananassae
D.simulans
D.sechellia
D.melanogaster
D.yakuba
D.erecta
D.pseudoobscura
D.persimilis
D.willistoni
D.mojavensis
D.virilis
D.grimshawi


d
Consensus
Identity
D.ananassae
D.simulans
D.sechellia
D.melanogaster
D.yakuba
D.erecta
D.pseudoobscura
D.persimilis
D.willistoni
D.mojavensis
D.virilis
D.grimshawi

746

747 **Figure 7. *Gr8a* expression is sexually dimorphic across the *Drosophila* genus.** (A)
748 Phylogenetic tree of *Drosophila Gr8a* proteins. Substitution rate = 0.2. (B) *Gr8a* mRNA
749 expression is enriched in males relative to females across *Drosophila*. Black, males;
750 white, females. *, $p<0.05$; **,$p<0.01$; Mann Whitney Rank Sum Test, n=4/group. Live *D.*
751 *grimshawi* was not analyzed because live specimens were not available at the
752 *Drosophila* Species Stock Center (DSSC). (C) Multiple aligned amino acid sequences of
753 *Gr8a* protein sequences from 12 species across *Drosophila.* Box highlights a putative
754 hypervariable protein domain, which is shown at a higher resolution in (D). Numbers on
755 top of alignment indicate amino acid number. Black, 100% identical; Dark Gray, 80-
756 100% similar; Light Gray, 60-80% similar; White, less than 60% similar (Blosum62 score
757 matrix, threshold=1). Bars below consensus represent overall level of amino acid
758 conservation.

759

760 **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	+	+

761

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

765

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

766

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

770

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

771

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

774

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

775

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

779

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	780
Gr64e	TGGCCACCACTTGCTCGCTG	ACGCCGCGTGTTAGCCGAAA	781
Gr64f	TCTGGCTAGGCAGTGGCCCC	GCATGGGCCACCGATGGCAT	
Gr66a	GAGGACCGCAGCCTAAAGGCC	CGCGGTCAACTGGTCGGAGAC	782
Gr77a	CGCAGTTCAGGCCGTCTGCTT	CCACAGGCGCTGACGGCAAATA	
Gr85a	CTCTCGCCTACATGGCACGC	ATAGTGACGTGCGTGTGCTGC	783
Gr89a	GCTGCATGGAGTTCCCCTGACC	ACATTGCCAGTGGGCGTGAGC	784
Gr8a	ACATACGCATCGCCGTGGACTG	ATGTAGGTGCCCACGGAACAGG	
Gr92a	TGTCTCCGTTGAGGGAGCGGT	TGGGTGACATCGAATAGGCCCA	785
Gr93a	GGTGAGGCAGTCAGAAGTGCC	CCTCGGACGCCTCAAAGCCAC	
Gr93b	ACCACGCCATTCTGCGAAGAC	GCCCAAAGGAAACACTCGCAGC	786
Gr93c	TGCGACGCCTGAGTCTGGAGA	CCCAAGGGTCTAACGCGAAACTCG	787
Gr93d	ACTCGGGAACGTGCTCTGGAT	AGAGGCCCAACAAGTTAACCCTGA	
Gr97a	TGCGTCGCTTCTGCGAGTTAGC	GCGTGCTGCAGATCGAATCGC	788
Gr98a	GTTGAGCACACAAGGGTGGCTG	GCTGTTGCTTGCTGCTGCTCTGA	789
Gr98b	TTGGAGGCTGGAGGGCGATGT	ATGGTGACAAGCAACCCGCCA	
Gr98c	GCCAGGATTCAGGAGCTGTGCG	CGCTGGTATTTTGTCTGTAGCCCA	790
Gr98d	CTGGCTGGACGCATTTGGGGC	ATGTCAAAGAGGCCACCGCATGT	
Gr9a	TGTCCTCTGTCTGGGCGACTGG	ACAAGGGACACGAACTGCAGGA	791

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

793

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

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

800

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

801

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

804