Pleiotropic chemoreceptors facilitate the maintenance of signal-receptor coupling in pheromonal communication

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Abstract:

Sex pheromones are essential for the maintenance of prezygotic behavioral mating barriers between related animal species. Once established, quantitative or structural changes in either the pheromonal signal or pheromone receptors should carry a fitness cost, and therefore, are expected to be under purifying selection. However, because pheromone production and perception are two independent biological processes that typically reside in different tissues and cell types, it is puzzling how, once evolved, the functional coupling of pheromone-receptor pairs remains stable on an evolutionary timescale. Here we demonstrate that in *Drosophila*, one possible molecular solution to this conundrum is facilitated by the action of pleiotropic chemoreceptors, which play a role in both the perception of pheromones by the nervous system and their synthesis in oenocytes. Specifically, we demonstrate that the gustatory receptor *Gr8a* functions in both the chemosensory system and oenocytes. Genetic, behavioral, and chemical analyses reveal that the action of *Gr8a* is consistent with its dual role in the perception and production of an inhibitory mating chemical signal. Therefore, our studies provide an elegant, and a relatively simple molecular explanation to a long-standing evolutionary genetic conundrum.

One Sentence Summary: The *Drosophila* chemoreceptor *Gr8a* contributes to the maintenance of pheromonal signal-receptor coupling via its pleiotropic action in both the perception and production of mating pheromones.

Main Text:

In the *Drosophila* genus, cuticular hydrocarbons (CHCs) act as pheromones, which are essential for the integrity of reproductive boundaries barriers between closely related species by maintaining pre-zygotic behavioral barriers (*1-7*). In *Drosophila melanogaster* and closely related species, the function of few specific CHCs has been characterized. These include the female aphrodisiac pheromone 7-11-heptacosadiene (7,11-HD) and the male inhibitory pheromone *Z*-7-tricosene (7-T) (*2, 5, 8, 9*). Two additional male-specific mating inhibitory pheromones, CH503 and 11-cis-vaccenyl acetate (cVA), have been shown to be transferred from males to females during mating, which reduces the attractiveness of mated females to other males (*10-14*).

The perception of CHCs is mediated by specialized gustatory-like receptor neurons (GRNs) in appendages and the proboscis (*9, 15-18*). In contrast, the production of CHCs is localized to the fat body and oenocytes, which are large abdominal subcuticular cells (*2, 19, 20*). The striking developmental and physiological differences between GRNs and oenocytes suggest that the perception and production of CHCs are regulated via independent genetic networks. However, the assumption that the perception and production of mating pheromones are independent represents an evolutionary conundrum since any changes in either the signal or its perception could carry a fitness cost (*20, 21*). Therefore, it is expected that pheromone profiles across closely related species, especially in fast evolving clades, should be very similar due to stabilizing selection. However, several studies of various *Drosophila* phylogenetic clades indicate that mating pheromones can evolve rapidly (*1*), which present a puzzling evolutionary conundrum.

One simple genetic solution, which may explain how signal-receptor pairs retain robust functional coupling across populations and generations, is pleiotropy. Subsequently, we

hypothesized that some *Drosophila* pheromone receptors contribute to both the perception and production of mating pheromone signals via pleiotropic action in both the peripheral nervous system and pheromone-producing oenocytes.

To date, several members of the *Gustatory receptor* (*Gr*) gene family have been implicated in the detection of excitatory and inhibitory pheromones in *Drosophila* (*22-26*). We reasoned that if specific receptors play a dual pleiotropic role in the perception and production of mating pheromonal signals then they should be expressed in both the chemosensory system, which reside in appendages and mouthparts, and the pheromone producing oenocytes, which reside in the abdomen (*2*). Since the expression of most *Gr*'s in gustatory receptor neurons (GRNs) has already been established (*27, 28*), we used an RT-PCR screen to identify family members that are also expressed in the abdomen, which identified 24 such genes out of the 59 family members identified in the fly genome (**Table S1**).

One of the genes we have identified in our initial screen as male-enriched (**Table S1**), *Gr8a*, was recently shown to specifically detect the non-proteinogenic amino acid L-canavanine (*29*). However, the natural ligands and behavioral functions of *Gr8a* remain unknown. Because previous studies only looked at *Gr8a* in the context of sensory functions, we first investigated the spatial activity pattern of a *Gr8a* promoter-GAL4 construct across the whole body in both males and females. Our analysis revealed that, as was previously described, *Gr8a* is expressed in 14-16 GRNs in the proboscis of both males and females (**Fig. 1A**). We also identified *Gr8a* expression in two paired GRNs in the pretarsus of forelegs in both sexes (**Fig. 1B**). Outside the nervous system, our data indicate that *Gr8a* is broadly expressed in oenocyte-like, large subcuticular cells in males but not females (**Fig. 1C**). We confirmed the promoter activity patterns with mRNA expression analyses, which confirmed the sexually dimorphic expression pattern in abdomens

(**Fig. 1D**). To investigate the cellular identity of abdominal *Gr8a*-expressing cells, we generated flies that co-expressed a luciferase reporter under the control the promoter of the oenocyte marker gene *Desat1 (2),* and an EGFP reporter under the control of the *Gr8a* promoter. Co-staining for both reporters revealed that some *Gr8a*-positive cells are oenocytes (**Fig. 1E to G**). We also identified positive *Gr8a* signal in *Desat1*-negative cells with fat body-like morphology (**Fig. 1E to G**). These data indicate that in addition to its canonical chemosensory functions in both males and females, *Gr8a* also plays a physiological function in cellular components of the pheromone production system in males.

Phylogenetic analysis of *Gr8a* indicated that it is a highly conserved member of the *Gustatory receptor* gene family across *Drosophila* (**Fig. 2A**). In addition to protein sequence homology, we found that the sexually dimorphic *Gr8a* mRNA expression is also conserved across the genus (**Fig. 2B**). The GR8A protein alignment revealed that although the protein sequence is conserved across *Drosophila* species, at least one predicted extracellular domain is hypervariable, which suggests GR8A protein functions might vary across species (**Fig. 2C and D**). These data underlined *Gr8a* as an ideal candidate gene for specifically testing our primary hypothesis, which stipulates that some pleiotropic pheromone receptors contribute to both the perception and production of mating pheromones.

Therefore, we next investigated whether *Gr8a*, and the GRNs that express it, are required for sensory functions associated with normal mating behaviors in males and females. We found that blocking neuronal transmission in female *Gr8a*-expressing GRNs with the tetanus toxin (TNT) resulted in a shorter latency to mating with wild type males relative to control females (**Fig. 3A**). Similarly, homozygous (**Fig. 3B** and fig. S1) and hemizygous *Gr8a* mutant females exhibited shorter mating latency relative to wild type controls (**Fig. 3C**). The increased female copulation

receptivity phenotype in *Gr8a* mutant animals is also completely rescued by the expression of a *Gr8a* cDNA in all *Gr8a*-expressing cells, which further indicate this gene is required for normal mating decisions in females (**Fig. 3D**). However, blocking *Gr8a*-expressing neurons, or the *Gr8a* mutation, had no effect on male courtship behavior as measured by courtship latency or courtship index towards wild-type virgin females (fig. S2). Because mating decisions in flies involve both excitatory and inhibitory signals (*2, 30*), these data indicate that *Gr8a*, and the GRNs that express it, contribute to the perception of a male inhibitory chemical mating signal by females.

The enriched expression in male oenocytes suggest that, in addition to its role in the chemosensory system, *Gr8a* also contributes to the production of chemical mating signals in males. Indeed, we found that virgin wild type females exhibit higher mating receptivity towards *Gr8a* mutant males relative to wild type controls (**Fig. 3E**). However, under laboratory conditions, the *Gr8a* mutation does not have a significant negative impact on the fitness of males or females as measured by reproductive capacity (fig. S3). These data indicate that *Gr8a* contributes to the production of inhibitory chemical signal in males, which when perceived by *Gr8a*-expressing GRNs in virgin wild type females, inhibits their mating drive.

In addition to their role in regulating pre-mating behaviors, *Drosophila* cuticular pheromones are also important for regulating post-mating behaviors. For example, to increase their fitness, male *Drosophila* transfer inhibitory mating pheromones to females during copulation, which lowers the overall sexual attractiveness of mated females (*2, 12, 13, 31*). Therefore, we also tested the hypothesis that *Gr8a* contributes to the production and perception of copulation-transferred inhibitory mating pheromones (*32*). Consistent with a role for *Gr8a* in the production and perception of an inhibitory pheromone, we found that wild-type males fail to recognize the

mated status of wild-type females that previously mated with *Gr8a* mutant males (**Fig. 3F**). Similarly, we found that *Gr8a* mutant males are not able to recognize the mated status of wild type females (**Fig 3F**). These data indicate that *Gr8a* contributes to the regulation of pre- and post-mating decisions in males and females by regulating the perception and production of an unidentified inhibitory chemical mating signal.

To directly establish the contribution of *Gr8a* activity to pheromone production, we next examined the effects of the *Gr8a* mutation on the CHC profile of males and females. In agreement with our behavioral data, principle component analyses (PCA) revealed that the *Gr8a* mutation has a significant effect on the CHC signatures of males (**Fig. 4A**) and females (**Fig. 4B**). Analyses of individual CHC components revealed a significant effect of the *Gr8a* mutation on several specific CHCs in both males and females, including alkenes and methyl-branched CHCs (**Fig. 4C and D, Tables S2 and S3**), which have been implicated in mating decisions in several *Drosophila* species (*2, 3, 6*).

In our model, *Gr8a* contributes to the perception of pheromones via gustatory-like receptor neurons. In contrast, how *Gr8a* might affect pheromone production in oenocytes is not obvious. Although we do not understand the mechanism yet, we hypothesize that *Gr8a*, and probably other oenocyte-enriched receptors, regulate the synthesis and/or secretion rates of specific CHC via oenocyte-intrinsic cell signaling feedback loops.

We also do not know yet the chemical identity of the putative pheromone ligand of *Gr8a*. Previous studies have identified cVA and CH503 as inhibitory pheromones in *D. melanogaster* that are transferred from male to females during copulation. However, it is very unlikely that *Gr8a*, which is expressed in taste neurons, acts as a receptor for cVA because this volatile pheromone is primarily perceived by the olfactory system via the action of the olfactory receptor

Or67d (*10, 12, 31*). *Gr8a* is also not likely to serve as the receptor for CH503 because this ligand has already been shown to act via *Gr68a*-expressing cells, which do not overlap with the *Gr8a*-expressing neurons we describe here (*13, 14*). However, we and others have identified at least 19 additional CHCs expressed by males and females (tables S2 and S3), which are yet to be characterized in terms of their effects on behavior and physiology. Therefore, we speculate that *Gr8a* acts as a receptor for CHCs that act as inhibitory mating pheromones independently from the action of cVA and CH503.

In spite of a robust theoretical foundation for the possible role of signal-receptor co-evolution in determining and maintaining mating boundaries between closely related animal species, empirical data that support these models are rare. Here we provide evidence for a relatively simple genetic solution to the maintenance of coupled pheromone-receptor pairs, which depends on the pleiotropic action of genes that encode pheromones receptors in both the perception and production of chemical mating signals. Specifically, we demonstrate that *Gr8a*, a male-enriched member of the *Gustatory receptor* gene family in *D. melanogaster*, plays an important role in mating decisions in males and females via its pleiotropic action in both the perception and production of inhibitory mating pheromones. Data presented here deliver a robust experimental evidence for a genetic architecture that enables animals to maintain robust functional links between mating signals and their receptors, which is essential for maintaining robust behavioral mating species boundaries. Furthermore, pheromone chemoreceptor pleiotropy may explain how evolutionary changes in a few genes, such as insect pheromone receptors, would allow rapid species diversification via rapid evolution of pheromonal communication systems.

References and Notes:

1. J. A. Coyne, A. P. Crittenden, K. Mah, Genetics of a pheromonal difference contributing to reproductive isolation in Drosophila. *Science* **265**, 1461-1464 (1994).
2. J. C. Billeter, J. Atallah, J. J. Krupp, J. G. Millar, J. D. Levine, Specialized cells tag sexual and species identity in Drosophila melanogaster. *Nature* **461**, 987-991 (2009).
3. T. R. Shirangi, H. D. Dufour, T. M. Williams, S. B. Carroll, Rapid evolution of sex pheromone-producing enzyme expression in Drosophila. *Plos Biol* **7**, e1000168 (2009).
4. S. H. Ng *et al.*, Pheromone evolution and sexual behavior in Drosophila are shaped by male sensory exploitation of other males. *Proc Natl Acad Sci U S A* **111**, 3056-3061 (2014).
5. H. K. Dweck *et al.*, Pheromones mediating copulation and attraction in Drosophila. *Proc Natl Acad Sci U S A* **112**, E2829-2835 (2015).
6. H. Chung *et al.*, A single gene affects both ecological divergence and mate choice in Drosophila. *Science* **343**, 1148-1151 (2014).
7. H. Chung, S. B. Carroll, Wax, sex and the origin of species: Dual roles of insect cuticular hydrocarbons in adaptation and mating. *BioEssays : news and reviews in molecular, cellular and developmental biology* **37**, 822-830 (2015).
8. M. P. Fernandez *et al.*, Pheromonal and behavioral cues trigger male-to-female aggression in Drosophila. *Plos Biol* **8**, e1000541 (2010).
9. B. Lu, A. LaMora, Y. Sun, M. J. Welsh, Y. Ben-Shahar, ppk23-Dependent chemosensory functions contribute to courtship behavior in Drosophila melanogaster. *PLoS Genet* **8**, e1002587 (2012).
10. A. Kurtovic, A. Widmer, B. J. Dickson, A single class of olfactory neurons mediates behavioural responses to a Drosophila sex pheromone. *Nature* **446**, 542-546 (2007).
11. W. van der Goes van Naters, J. R. Carlson, Receptors and neurons for fly odors in Drosophila. *Curr Biol* **17**, 606-612 (2007).
12. S. R. Datta *et al.*, The Drosophila pheromone cVA activates a sexually dimorphic neural circuit. *Nature* **452**, 473-477 (2008).
13. J. Y. Yew *et al.*, A new male sex pheromone and novel cuticular cues for chemical communication in Drosophila. *Curr Biol* **19**, 1245-1254 (2009).
14. S. Shankar *et al.*, The neuropeptide tachykinin is essential for pheromone detection in a gustatory neural circuit. *eLife* **4**, (2015).
15. T. W. Koh *et al.*, The Drosophila IR20a clade of ionotropic receptors are candidate taste and pheromone receptors. *Neuron* **83**, 850-865 (2014).
16. R. Thistle, P. Cameron, A. Ghorayshi, L. Dennison, K. Scott, Contact Chemoreceptors Mediate Male-Male Repulsion and Male-Female Attraction during Drosophila Courtship. *Cell* **149**, 1140-1151 (2012).
17. H. Toda, X. Zhao, B. J. Dickson, The Drosophila Female Aphrodisiac Pheromone Activates ppk23+ Sensory Neurons to Elicit Male Courtship Behavior. *Cell reports* **doi:10.1016/j.celrep.2012.05.007**, 599-607 (2012).
18. B. Lu, K. M. Zelle, R. Seltzer, A. Hefetz, Y. Ben-Shahar, Feminization of pheromone-sensing neurons affects mating decisions in Drosophila males. *Biology Open* **In Press**, 152-160 (2014).
19. R. Makki, E. Cinnamon, A. P. Gould, The development and functions of oenocytes. *Annual review of entomology* **59**, 405-425 (2014).

20. J. J. Krupp *et al.*, Pigment-dispersing factor modulates pheromone production in clock cells that influence mating in drosophila. *Neuron* **79**, 54-68 (2013).
21. M. R. Symonds, M. A. Elgar, The evolution of pheromone diversity. *Trends Ecol Evol* **23**, 220-228 (2008).
22. S. Bray, H. Amrein, A putative Drosophila pheromone receptor expressed in male-specific taste neurons is required for efficient courtship. *Neuron* **39**, 1019-1029 (2003).
23. T. Miyamoto, H. Amrein, Suppression of male courtship by a Drosophila pheromone receptor. *Nat Neurosci* **11**, 874-876 (2008).
24. S. J. Moon, Y. Lee, Y. Jiao, C. Montell, A Drosophila gustatory receptor essential for aversive taste and inhibiting male-to-male courtship. *Curr Biol* **19**, 1623-1627 (2009).
25. K. Watanabe, G. Toba, M. Koganezawa, D. Yamamoto, *Gr39a*, a highly diversified gustatory receptor in *Drosophila*, has a role in sexual behavior. *Behav Genet* **41**, 746-753 (2011).
26. Y. Hu *et al.*, Gr33a modulates Drosophila male courtship preference. *Scientific reports* **5**, 7777 (2015).
27. L. Dunipace, S. Meister, C. McNealy, H. Amrein, Spatially restricted expression of candidate taste receptors in the Drosophila gustatory system. *Curr Biol* **11**, 822-835 (2001).
28. K. Scott *et al.*, A chemosensory gene family encoding candidate gustatory and olfactory receptors in Drosophila. *Cell* **104**, 661-673 (2001).
29. Y. Lee *et al.*, Gustatory receptors required for avoiding the insecticide L-canavanine. *J Neurosci* **32**, 1429-1435 (2012).
30. J. C. Billeter, J. D. Levine, Who is he and what is he to you? Recognition in Drosophila melanogaster. *Curr Opin Neurobiol* **23**, 17-23 (2013).
31. R. Benton, K. S. Vannice, L. B. Vosshall, An essential role for a CD36-related receptor in pheromone detection in Drosophila. *Nature* **450**, 289-293 (2007).
32. S. Zawistowski, R. C. Richmond, Inhibition of courtship and mating of Drosophila melanogaster by the male-produced lipid, cis-vaccenyl acetate. *Journal of Insect Physiology* **32**, 189-192 (1986).

Acknowledgments: We thank members of the Ben-Shahar lab for valuable comments on earlier versions of the manuscript. We thank the Millar laboratory (UC Riverside) for assistance with CHC analyses. We thank Nabeel Chowdhury for assistance with the RT-PCR screen, and Paula Kiefel for technical help with the generation of transgenic flies. This work was supported by NSF grant 1545778 and NIH grant NS089834 awarded to YB-S.

Supplementary Materials:

Materials and Methods

Figures S1-S3

Tables S1-S5
References References (*37-43*)

Figure legends:

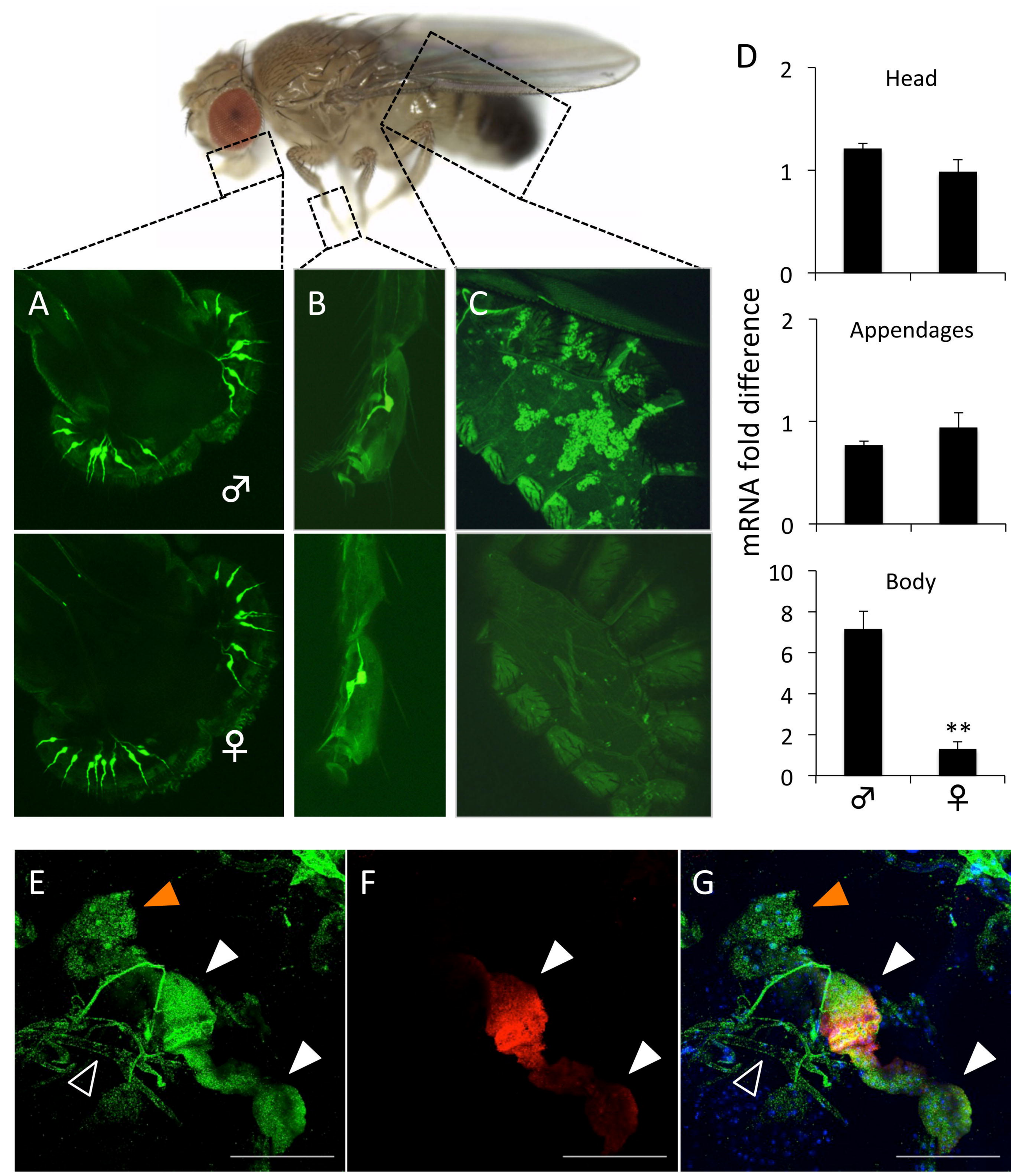
Fig. 1. ***Gr8a* is a sexually dimorphic chemosensory receptor enriched in male oenocytes**. (A) *Gr8a* promoter activity in proboscis, (B) forelegs, and (C) abdomens of males (top panels) and females (bottom panels). (D) *Gr8a* mRNA expression. Relative mRNA levels were measured by real-time quantitative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

Fig. 2. *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*. N=4, Black, males; white, females. *, $p<0.05$; **,$p<0.01$; Mann Whitney Rank Sum Test. Live *D. grimshawi* was not analyzed because live specimens are not currently available at the *Drosophila* Species Stock Center (DSSC). (C) Multiple aligned amino acid sequences of *Gr8a* protein sequences from 12 species across *Drosophila.* Box highlights a putative hypervariable protein domain, which is shown at a higher resolution in (D). Numbers on top of alignment indicate amino acid number. Black, 100% identical; Dark Gray, 80-100% similar; Light Gray, 60-80% similar, White, less than 60% similar (Blosum62 score matrix, threshold=1).

Fig. 3. *Gr8a* activity contributes to the perception and production of inhibitory signal associated with mating decision making in males and females. (A) Blocking neural activity in female *Gr8a*-expressing sensory neurons shortens copulation latency. Homozygous (B) or

hemizygous (C) *Gr8a* null females show shortened copulation latency relative to wild type controls. Df(1)BSC663 is a deficiency that covers the *Gr8a* locus. Df(1)BSC754 was used as a control. (D) Expression of *Gr8a* cDNA with *Gr8a* promoter rescues the copulation latency phenotype in *Gr8a* mutant females. (E) Wild type females exhibit shorter copulation latency when courted by *Gr8a* mutant relative to wild-type males. (F) *Gr8a* mutant males do not recognize mating status of females, and have a reduced transfer of inhibitory mating pheromones during copulations. Female, female genotype; Sperm donor, genotype of males mated 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, ANOVA $p<0.05$). *, $p<0.05$, Mann Whitney Rank Sum Test.

Fig. 4. The *Gr8a* mutation affects the pheromone profiles of males and females. (A) Principle component analyses (PCA) indicate that the overall cuticular pheromones profiles of *Gr8a* null males (A) and females (B) are different from the profiles of wild type animlas. *, $p<0.05$, MANOVA. The *Gr8a* mutation affects the abundance of specific alekenes and methyl-branched CHCs in males (C) and females (D). *, $p<0.05$, **, $p<0.001$, Mann Whitney Rank Sum Test.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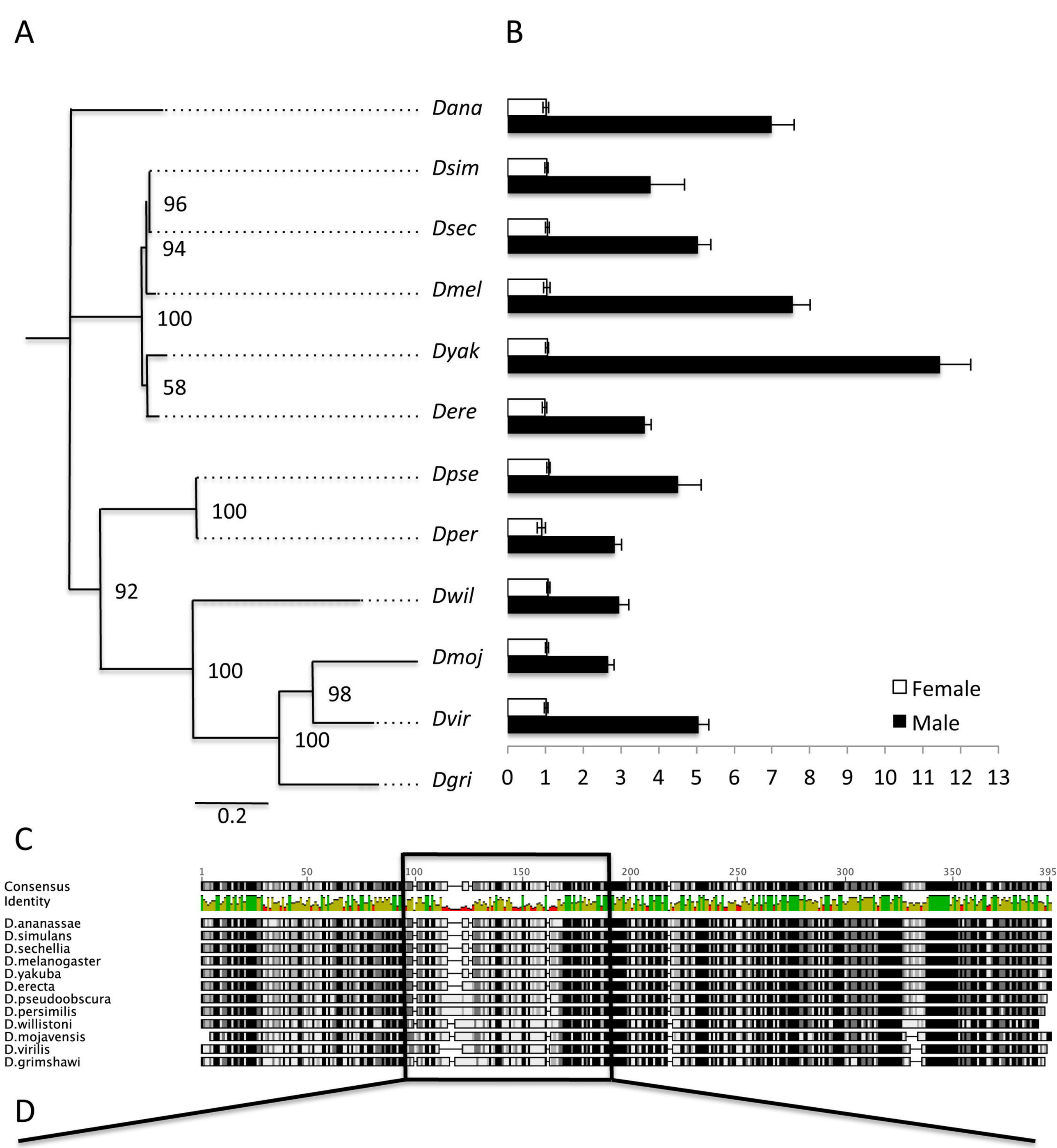
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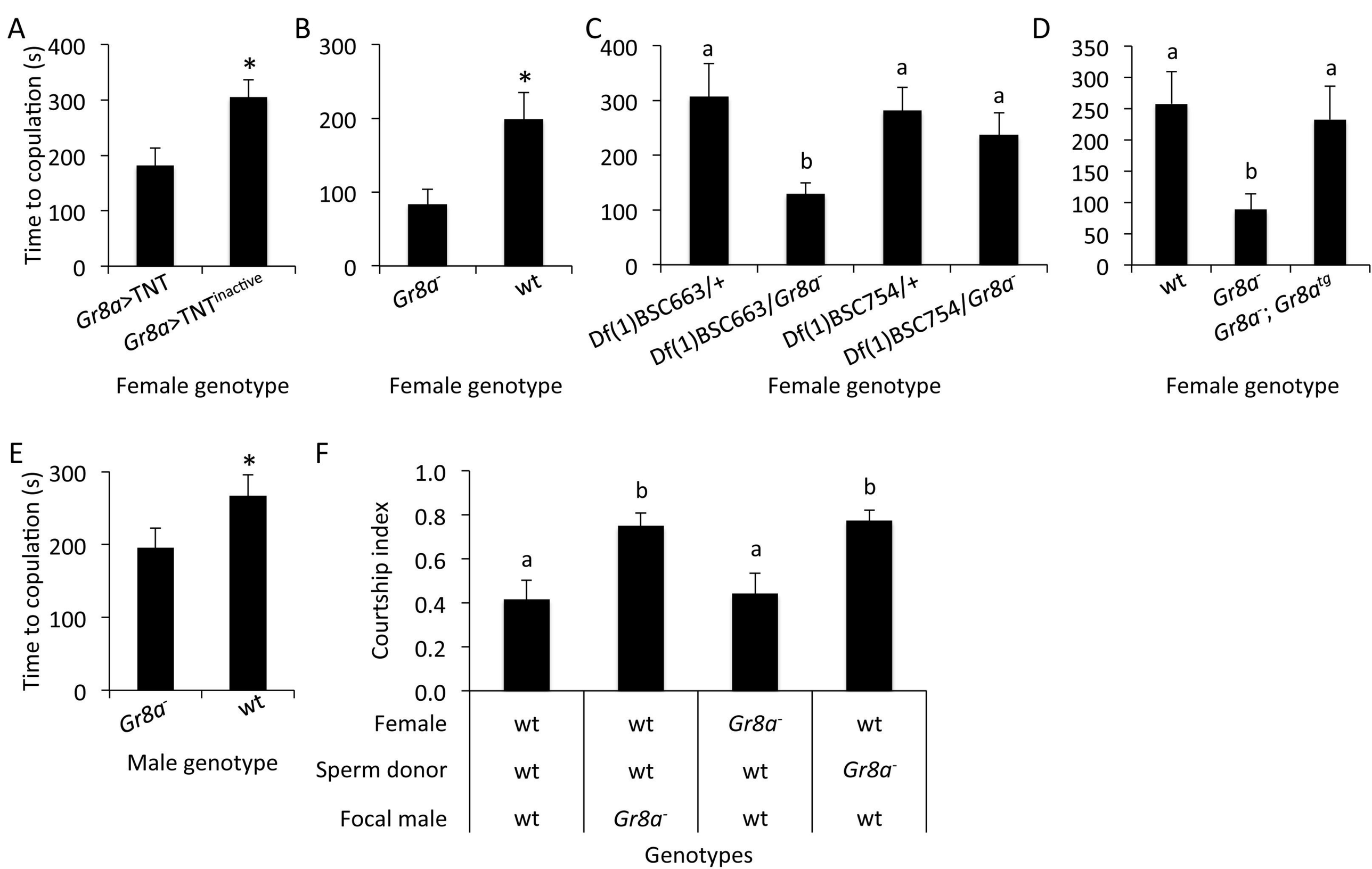
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Gr8a>TNTinactive
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Female genotype
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b
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a
Df(1)BSC663/+
Df(1)BSC663/Gr8a-
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Df(1)BSC754/Gr8a-
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Time to copulation (s)
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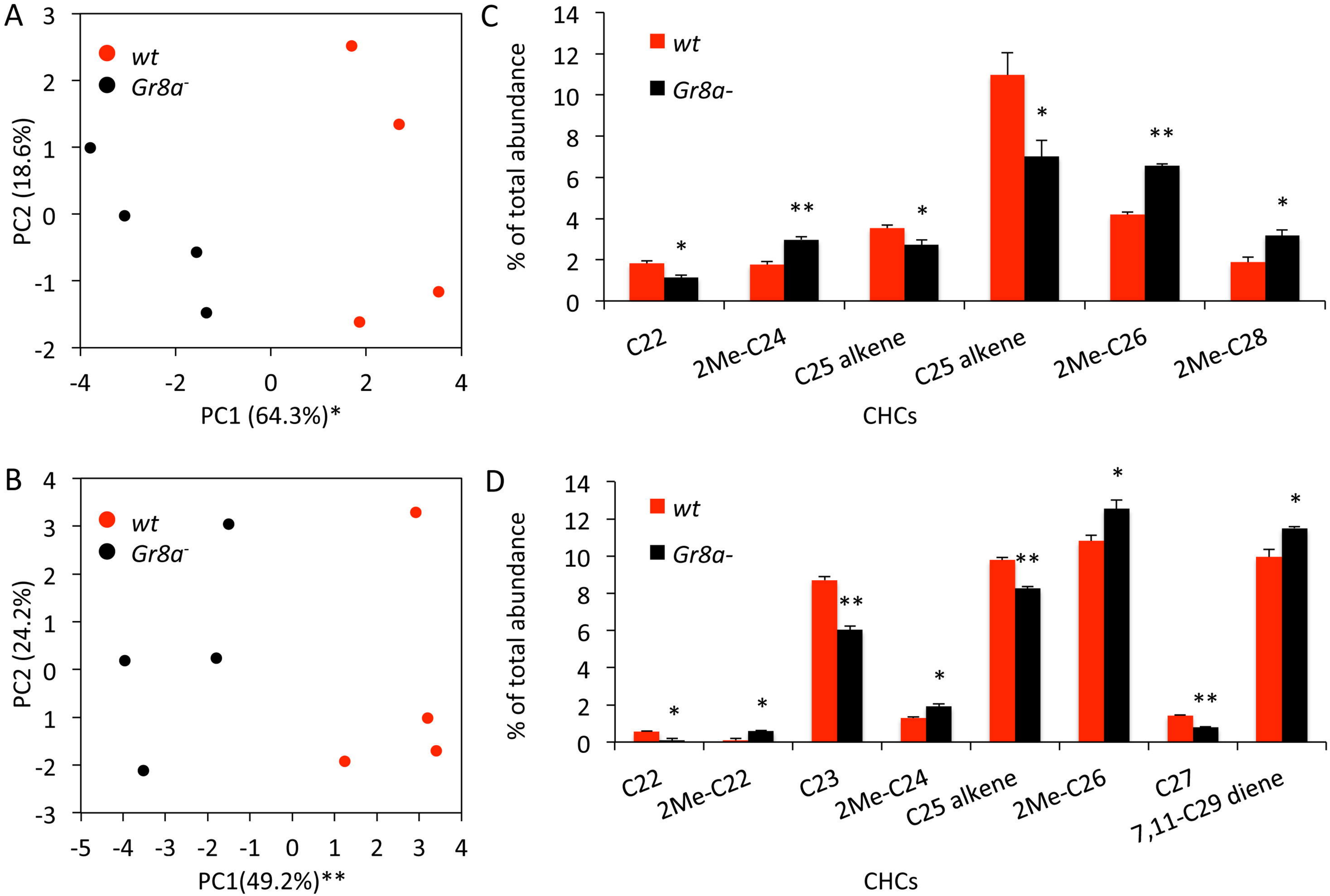
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C25 alkene
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2Me-C28
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C23
2Me-C24
C25 alkene
2Me-C26
C27
7,11-C29 diene
CHCs