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

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Abstract:

Signal diversity in communication systems plays an essential role in maintaining mating boundaries between closely related species. To preserve fitness, it has been hypothesized that signal-receptor coupling is maintained via strong purifying selection. However, because strong negative selection antagonizes diversity, how communication systems retain their potential for diversification is puzzling. We propose that one possible solution to this conundrum is receptor pleiotropy. Specifically, we demonstrate that *Gr8a,* a member of the gustatory receptor family in *Drosophila*, is a pleiotropic receptor that contributes to both the perception and production of inhibitory mating pheromones in the peripheral nervous system and pheromone producing oenocytes, respectively. Together, our data provide an elegant genetic solution to a long-standing evolutionary 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:

Communication systems are essential for determining species mating boundaries via functionally coupled signal-receptor pairs (*1-4*). Because qualitative or quantitative changes in either signals or receptors could carry fitness costs, the coupling of signal-receptor pairs should be maintained via purifying selection (*4-6*). Yet, closely related species often utilize distinct communication signals (*7-10*). Therefore, it is puzzling how coupled pheromone production and perception can

be maintained by purifying selection; yet, retain the potential for signal diversification (*4, 5, 11*). Here we demonstrate that the perception and production of mating pheromones in *Drosophila*, two independent biological processes that reside in different tissues, are genetically coupled via the pleiotropic action of pheromone receptors in the peripheral nervous system and pheromone-producing oenocytes.

In *Drosophila*, cuticular hydrocarbons (CHCs) act as mating pheromones, which are essential for the integrity of reproductive boundaries between closely related species (*12-18*). The perception of CHCs, which are produced in the fat body and abdominal oenocytes (*11, 13, 19*), is mediated by specialized gustatory-like receptor neurons (GRNs) in appendages and the proboscis (*20-24*). We chose members of the *Gustatory receptor* (*Gr*) gene family as candidates for testing our hypothesis because several family members have already been implicated in the detection of excitatory and inhibitory pheromones (*25-29*). Since the expression of most *Gr*'s in gustatory receptor neurons (GRNs) has already been established (*30, 31*), we reasoned that candidate pleiotropic pheromone receptors should be also expressed in abdominal oenocytes (*13*). An RT-PCR screen identified 24, out of the 59 *Gr* family members in the *Drosophila* genome, as chemoreceptors with abdominal-enriched expression (**Table S1**).

Next, we focused our analyses on *Gr8a*, which was previously shown to contribute to the detection of the non-proteinogenic amino acid L-Canavanine (*32*). We found that *Gr8a* is expressed in 14-16 GRNs in the proboscis (**Fig. 1A**), and two paired GRNs in the foreleg pretarsus (**Fig. 1B**) of males and females. It is also expressed in abdominal oenocyte-like cells in males but not females (**Fig. 1C**). The sexually dimorphic expression pattern of *Gr8a* was further supported by qRT-PCR (**Fig. 1D**). We also found that *Gr8a* is co-expressed with the oenocyte marker *Desat1 (13)*, as well as *Desat1*-negative cells with fat body-like morphology (**Fig. 1E to**

G). These data suggest that in addition to its chemosensory function in males and females, *Gr8a* also functions in pheromone production system in males.

Therefore, we next investigated whether *Gr8a*, and the GRNs that express it, are required for sensory functions associated with mating decisions in males and females. We found that blocking neuronal transmission in *Gr8a*-expressing GRNs with the tetanus toxin (TNT) in females resulted in shorter copulation latency when courted by wild-type males (**Fig. 2A**). Similarly, homozygous (**Fig. 2B**) and hemizygous (**Fig. 2C**) *Gr8a* mutant females exhibited shorter copulation latency relative to wild-type controls, which could be rescued by the transgenic expression of a *Gr8a* cDNA in all *Gr8a*-expressing cells (**Fig. 2D**). In contrast, *Gr8a* and the neurons that express do not seem to contribute to male courtship latency or index towards wild-type virgin females (fig. S1). Since mating decisions in flies involve both excitatory and inhibitory signals (*13, 33*), a simple interpretation of these data is that in females, *Gr8a* contributes to the perception of male-emitted inhibitory mating signals.

Because *Gr8a* expression is enriched in male oenocytes, and *Gr8a* mutant females seem to be unable to sense a copulation inhibitory signal emitted by males, we next tested the hypothesis that *Gr8a* mutant males are unable to produce or release the putative copulation inhibitory signal detected by virgin females. Indeed, we found that wild-type virgin females exhibited shorter copulation latency towards *Gr8a* mutant males, which suggest these males did not produce/release the inhibitory signal important for the copulation decision of virgin females (**Fig. 2E**).

As predicted by our behavioral data, the *Gr8a* mutation also has a significant effect on the overall CHC profile of males (**Fig. 3A**). Analyses of individual pheromonal components revealed a significant contribution of *Gr8a* to levels of specific components in males, including alkenes

and methyl-branched CHCs (**Fig. 3B and Table S2**), which have been implicated in mating decisions in several *Drosophila* species (*13, 14, 17*). Together, behavioral and pheromonal data indicate that *Gr8a* action contributes to mating decisions in females by co-regulating the female perception and male production of an inhibitory pheromone, which is consistent with *Gr8a* pleiotropy.

Previous studies showed that male *Drosophila* increase their fitness by transferring inhibitory mating pheromones to females during copulation, which lowers their overall attractiveness (*13, 34-36*). Therefore, we hypothesized that *Gr8a* mutant males would have less ability to produce/transfer inhibitory pheromones during copulation, and would not be able to detect inhibitory signals in mated females. Accordingly, we found that wild-type males fail to recognize mated status of wild-type females that previously mated with *Gr8a* mutant males, and *Gr8a* mutant males are not able to recognize the mated status of wild-type females that previously mated with wild-type males (**Fig 3F**). Together, these data indicate that *Gr8a* is required in males for the production/transfer, and subsequent detection, of an inhibitory mating signal in females. Therefore, *Gr8a* contributes to the regulation of both pre- and post-mating decisions in males and females by regulating the perception and production/release/transfer of inhibitory chemical mating signals.

Here we demonstrated that a pleiotropic gene that encodes a putative pheromone receptor can simultaneously regulate the perception and production of pheromones important for mating decisions in *Drosophila*. Nevertheless, we still do not understand the exact mechanism by which *Gr8a* exerts its pleiotropic action. However, how a chemoreceptor like *Gr8a* contributes to CHC production in oenocytes is not obvious. We speculate that *Gr8a* could regulate the synthesis and/or secretion of specific CHCs by acting as an oenocyte-intrinsic receptor, which integrates

feedback information to the complex genetic network that regulates CHC synthesis (**Fig 4**). We also do not know yet the chemical identity of the ligand of *Gr8a*. Previous studies indicated cVA and CH503 as inhibitory mating pheromones that are transferred from male to females during copulation. However, these chemicals are not likely to function as *Gr8a* ligands because the volatile cVA acts primarily via the olfactory receptor *Or67d* (*34, 35, 37*), and CH503 has been reported to signal via *Gr68a*-expressing neurons, which are anatomically distinct from the *Gr8a* GRNs we describe here (*36, 38*).

Although we do not know yet whether the pleiotropic action of *Gr8a* supported the rapid species diversification in *Drosophila*, phylogenetic analysis of *Gr8a* indicated that its protein sequence and sexually dimorphic expression pattern are conserved across *Drosophila* species (fig. S2A), and alignment of orthologous sequences revealed that at least one predicted extracellular region is hypervariable (fig. S2C and D). These data suggest that pleiotropic pheromone receptors may have played a role in maintaining the functional coupling of the production and perception of mating pheromones while still retaining the capacity for species diversification.

Whether genetic coupling serves as an important mechanism for signal-receptor co-evolution in mating systems remains an open question (*39, 40*). Here we provide experimental data, which indicate that pleiotropic receptors can maintain signal-receptor coupling in a mating communication systems. We do not know yet whether pleiotropic chemoreceptor genes also contribute to pheromone-receptor coupling in other species or to communication systems that depend on other sensory modalities. Nevertheless, population genetics studies in crickets suggest that pleiotropy might be playing a role in auditory signal-receptor coupling as well (*40, 41*). While specific identities of the pleiotropic genes in these systems are mostly unknown, these

data suggest that the genetic coupling of signal-receptor pairs in communication systems might be more common than previously thought.

References and Notes:

1. T. D. Wyatt, Cambridge University Press. (Cambridge University Press,, Cambridge, 2014), pp. 1 online resource (426 pages).
2. R. R. Hoy, J. Hahn, R. C. Paul, Hybrid cricket auditory behavior: evidence for genetic coupling in animal communication. *Science* **195**, 82 (1977).
3. K. L. Shaw, S. C. Lesnick, Genomic linkage of male song and female acoustic preference QTL underlying a rapid species radiation. *Proceedings of the National Academy of Sciences* **106**, 9737-9742 (2009).
4. C. R. B. Boake, Coevolution of senders and receivers of sexual signals: Genetic coupling and genetic correlations. *Trends in Ecology & Evolution* **6**, 225-227 (1991).
5. M. R. Symonds, M. A. Elgar, The evolution of pheromone diversity. *Trends Ecol Evol* **23**, 220-228 (2008).
6. S. Steiger, T. Schmitt, H. M. Schaefer, The origin and dynamic evolution of chemical information transfer. *Proceedings of the Royal Society B: Biological Sciences*, (2010).
7. W. L. Roelofs, A. P. Rooney, Molecular genetics and evolution of pheromone biosynthesis in Lepidoptera. *Proceedings of the National Academy of Sciences* **100**, 9179-9184 (2003).
8. S. Janssenswillen *et al.*, Origin and Diversification of a Salamander Sex Pheromone System. *Molecular biology and evolution* **32**, 472-480 (2015).
9. O. Niehuis *et al.*, Behavioural and genetic analyses of Nasonia shed light on the evolution of sex pheromones. *Nature* **494**, 345-348 (2013).
10. 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).
11. J. J. Krupp *et al.*, Pigment-dispersing factor modulates pheromone production in clock cells that influence mating in drosophila. *Neuron* **79**, 54-68 (2013).
12. J. A. Coyne, A. P. Crittenden, K. Mah, Genetics of a pheromonal difference contributing to reproductive isolation in Drosophila. *Science* **265**, 1461-1464 (1994).
13. 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).
14. 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).
15. 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).
16. H. K. Dweck *et al.*, Pheromones mediating copulation and attraction in Drosophila. *Proc Natl Acad Sci U S A* **112**, E2829-2835 (2015).
17. H. Chung *et al.*, A single gene affects both ecological divergence and mate choice in Drosophila. *Science* **343**, 1148-1151 (2014).
18. 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).

19. R. Makki, E. Cinnamon, A. P. Gould, The development and functions of oenocytes. *Annual review of entomology* **59**, 405-425 (2014).
20. T. W. Koh *et al.*, The Drosophila IR20a clade of ionotropic receptors are candidate taste and pheromone receptors. *Neuron* **83**, 850-865 (2014).
21. 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).
22. 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).
23. 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).
24. 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).
25. 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).
26. T. Miyamoto, H. Amrein, Suppression of male courtship by a Drosophila pheromone receptor. *Nat Neurosci* **11**, 874-876 (2008).
27. 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).
28. 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).
29. Y. Hu *et al.*, Gr33a modulates Drosophila male courtship preference. *Scientific reports* **5**, 7777 (2015).
30. 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).
31. K. Scott *et al.*, A chemosensory gene family encoding candidate gustatory and olfactory receptors in Drosophila. *Cell* **104**, 661-673 (2001).
32. Y. Lee *et al.*, Gustatory receptors required for avoiding the insecticide L-canavanine. *J Neurosci* **32**, 1429-1435 (2012).
33. 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).
34. 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).
35. S. R. Datta *et al.*, The Drosophila pheromone cVA activates a sexually dimorphic neural circuit. *Nature* **452**, 473-477 (2008).
36. 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).
37. 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).
38. S. Shankar *et al.*, The neuropeptide tachykinin is essential for pheromone detection in a gustatory neural circuit. *eLife* **4**, (2015).

39. R. K. Butlin, M. G. Ritchie, Genetic coupling in mate recognition systems: what is the evidence? *Biological Journal of the Linnean Society* **37**, 237-246 (1989).
40. C. Wiley, C. K. Ellison, K. L. Shaw, Widespread genetic linkage of mating signals and preferences in the Hawaiian cricket Laupala. *Proceedings of the Royal Society B: Biological Sciences*, (2011).
41. R. Hoy, J. Hahn, R. Paul, Hybrid cricket auditory behavior: evidence for genetic coupling in animal communication. *Science* **195**, 82-84 (1977).

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Supplementary Materials:

Materials and Methods

Figures S1-S3

Tables S1-S4
References (*36-42*)

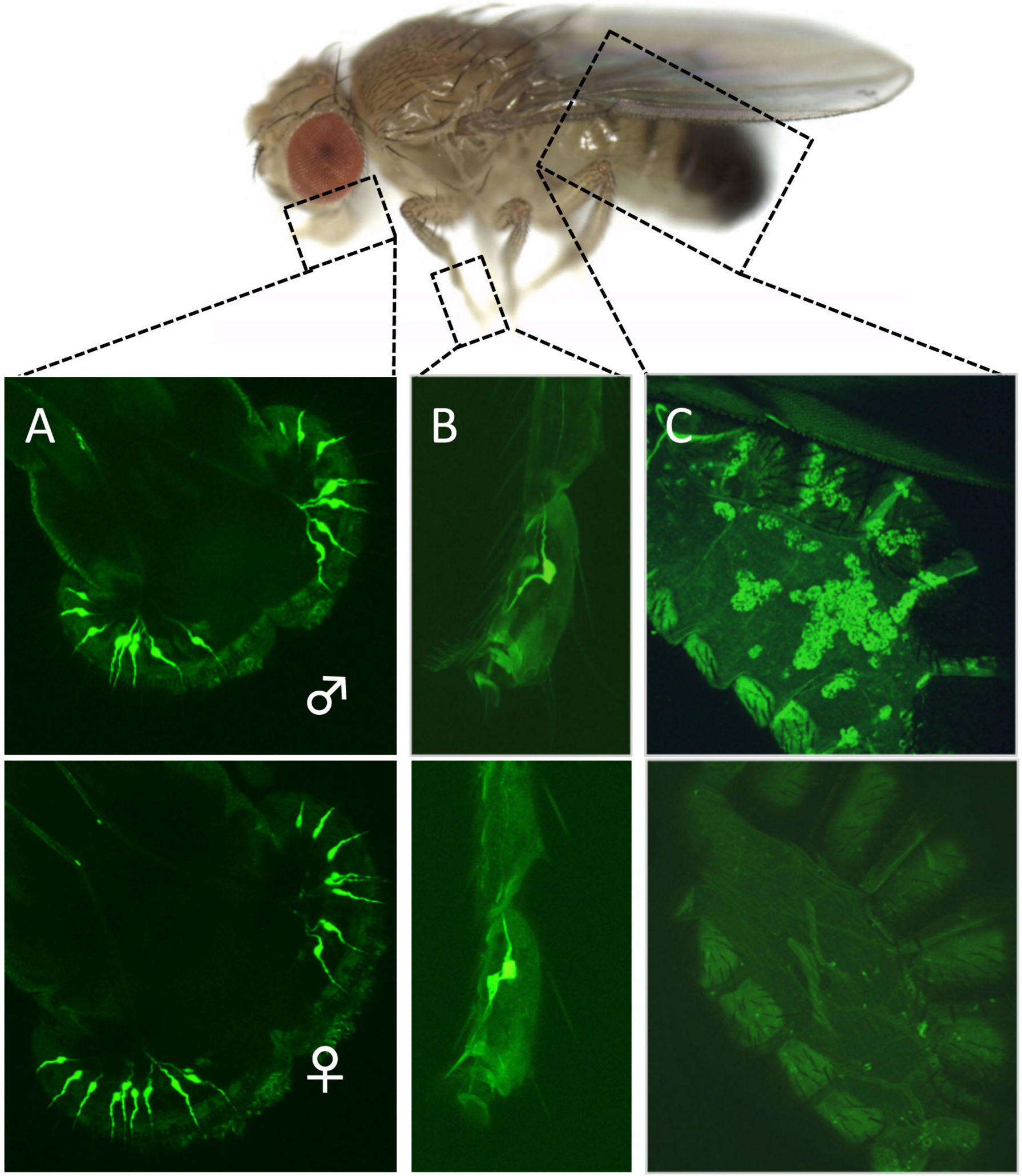
Figure legends:

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. Orange arrowhead, fat body cells; white arrowhead, oenocytes. Scale bar = 100μm.

Fig. 2. *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 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, ANOVA $p<0.05$). *, $p<0.05$, Mann Whitney Rank Sum Test.

Fig. 3. The *Gr8a* mutation affects the pheromone profiles of males and females. (A) Principle component analyses (PCA) of CHC profiles of wild-type and *Gr8a* mutant males. *, $p<0.05$, MANOVA. (B) The effect of the *Gr8a* mutation on levels of individual CHCs in males. Only affected CHCs shown. See Table S2 for the complete list. *, $p<0.05$, **, $p<0.001$, Mann Whitney Rank Sum Test.

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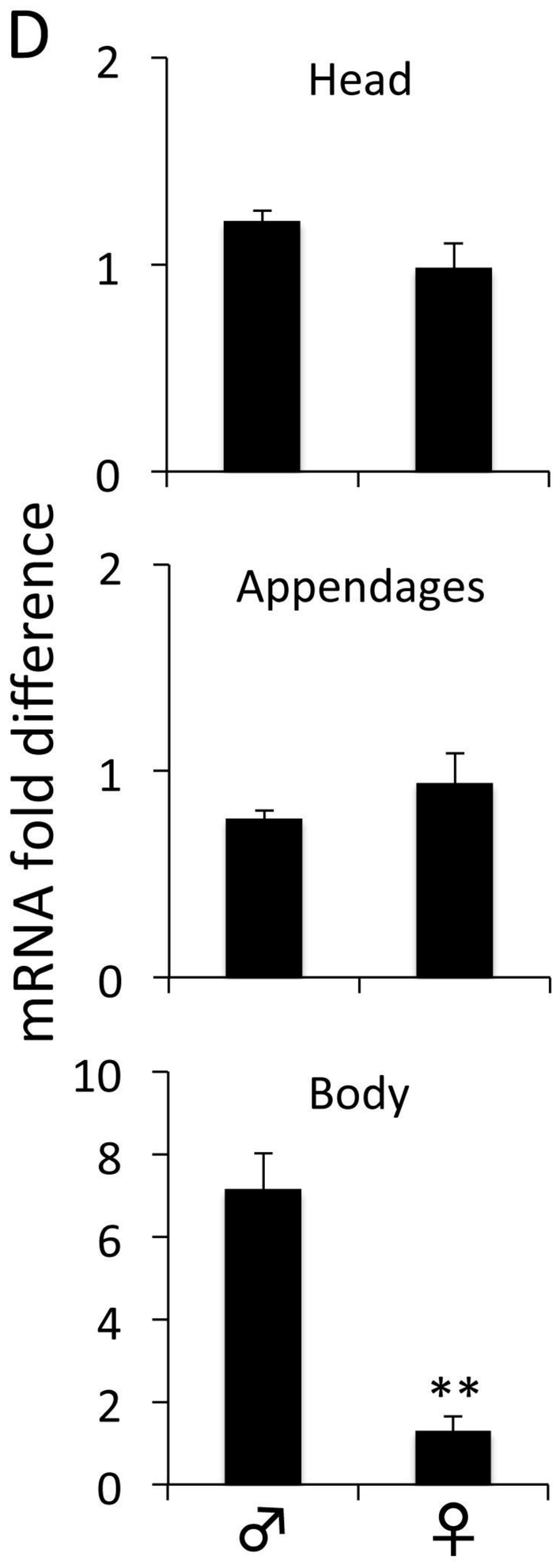
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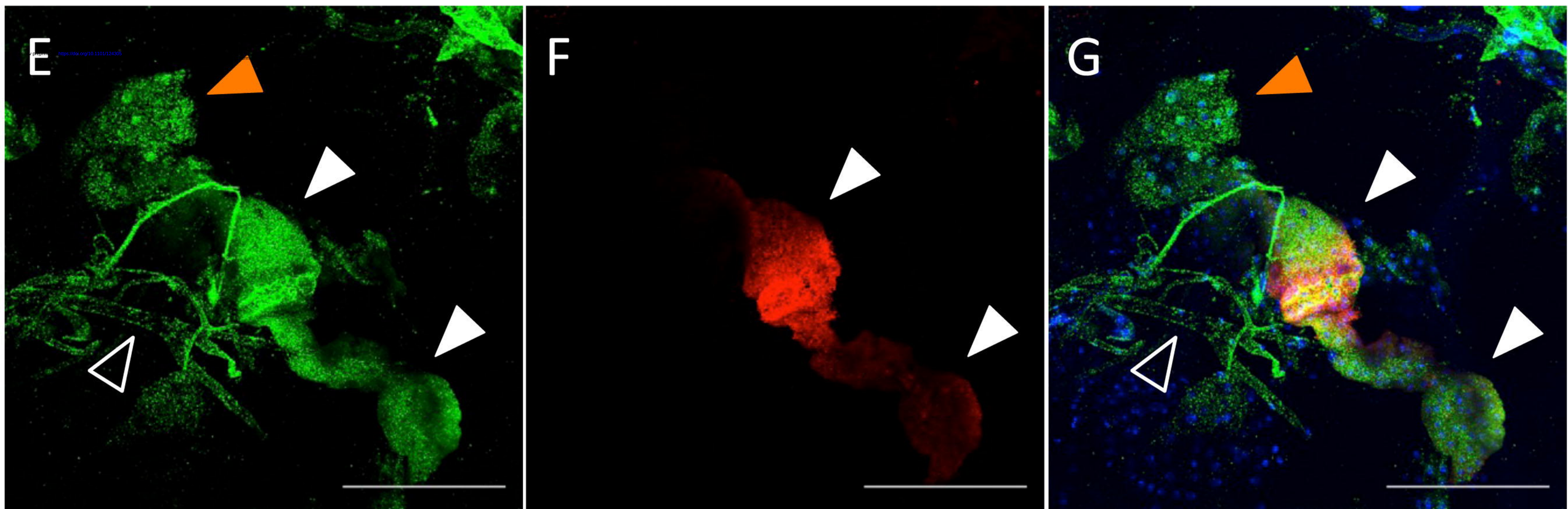
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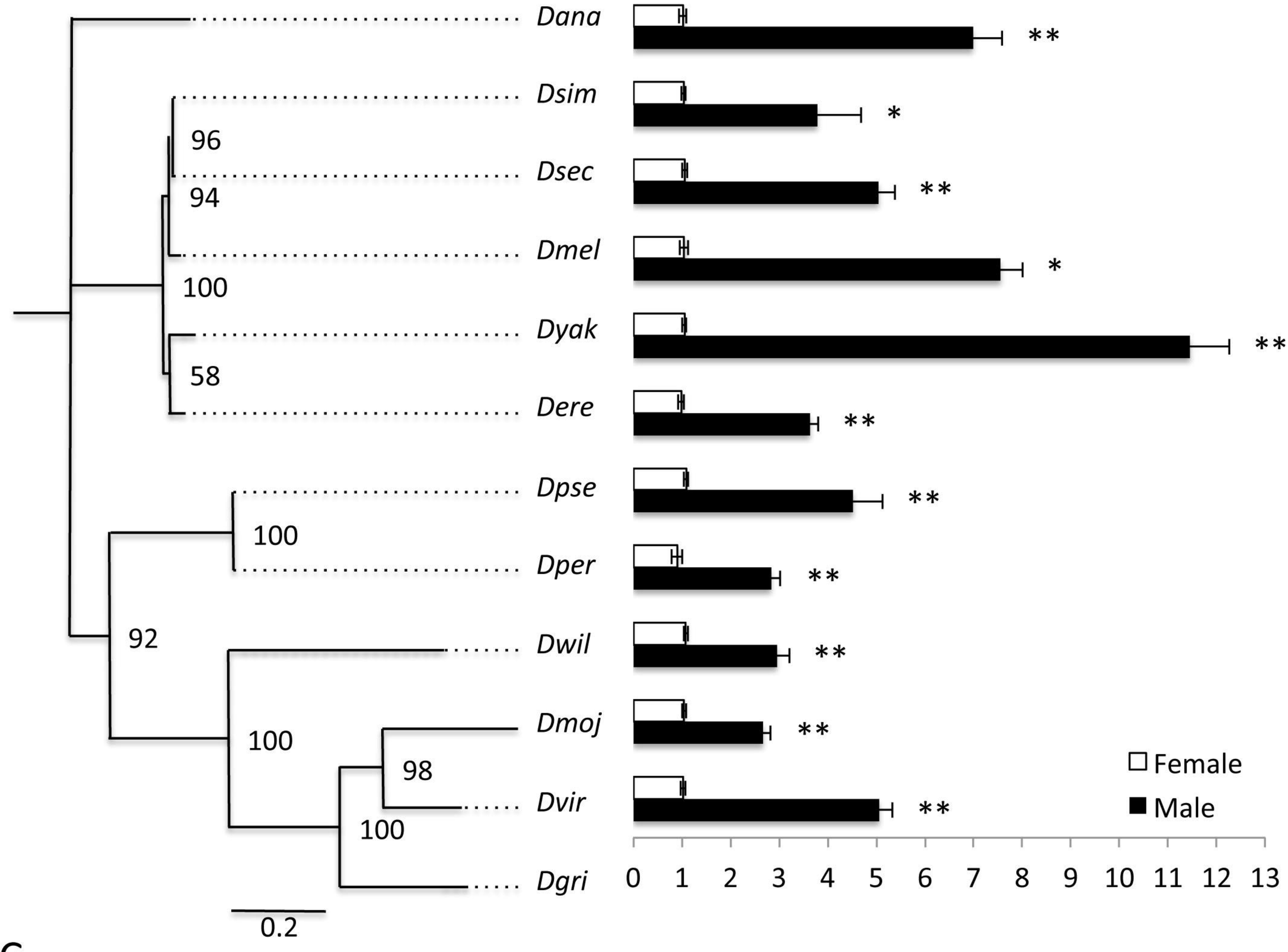

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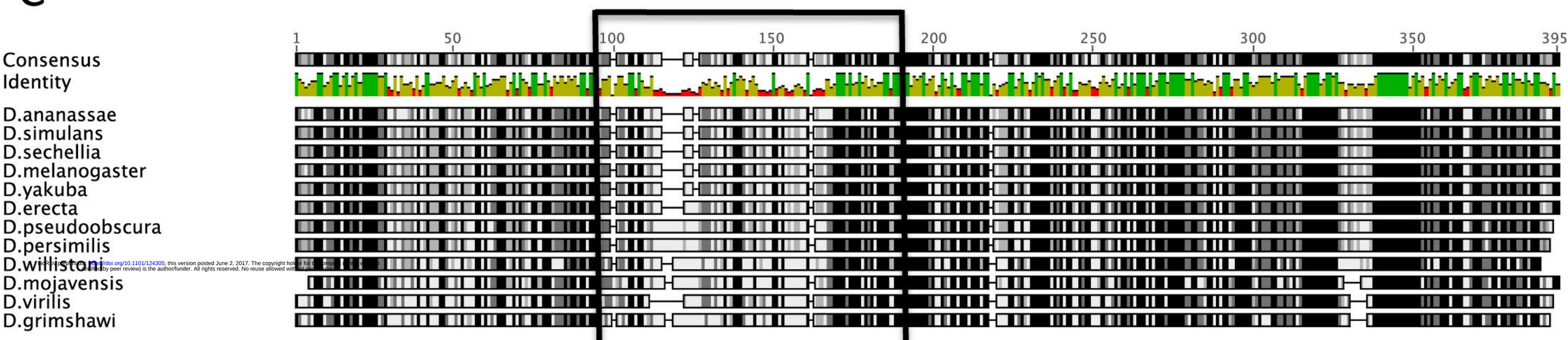

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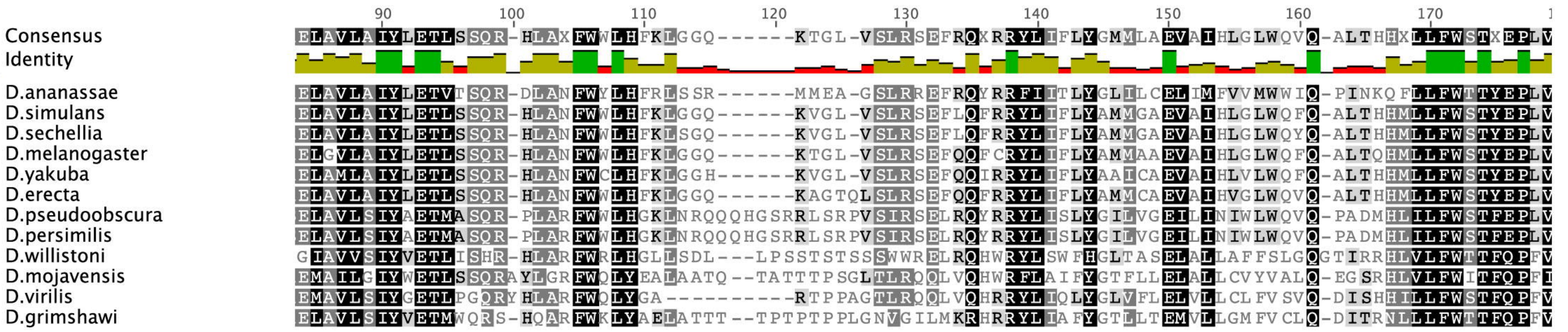

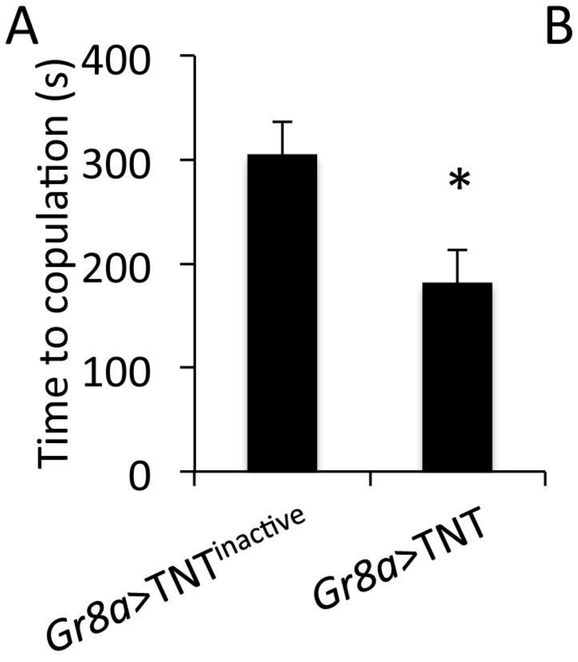

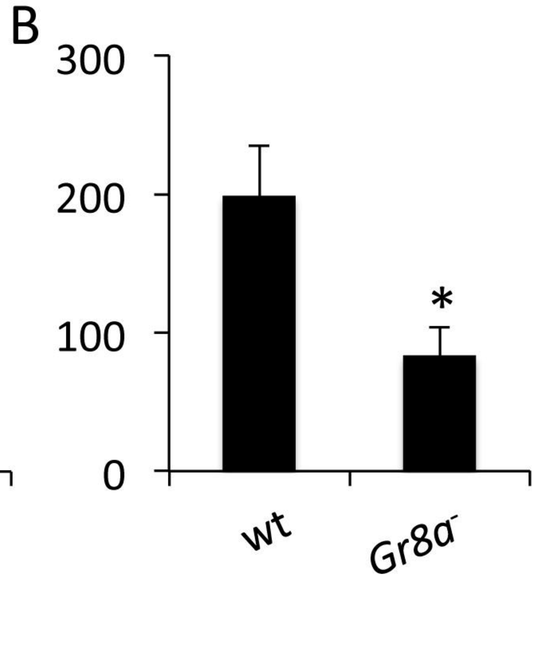

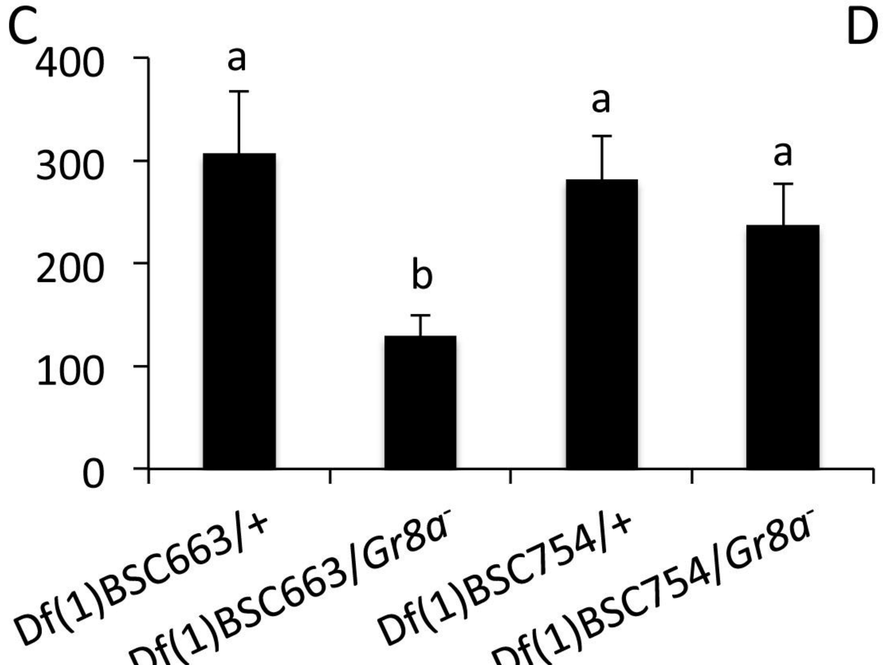

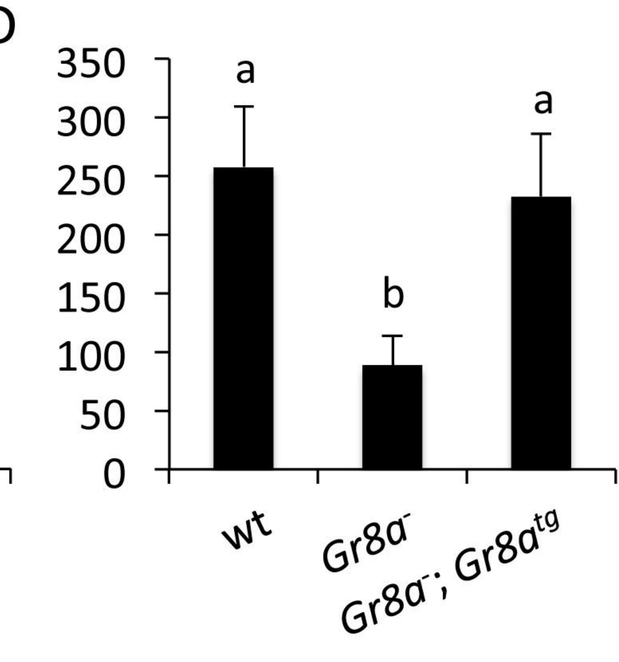

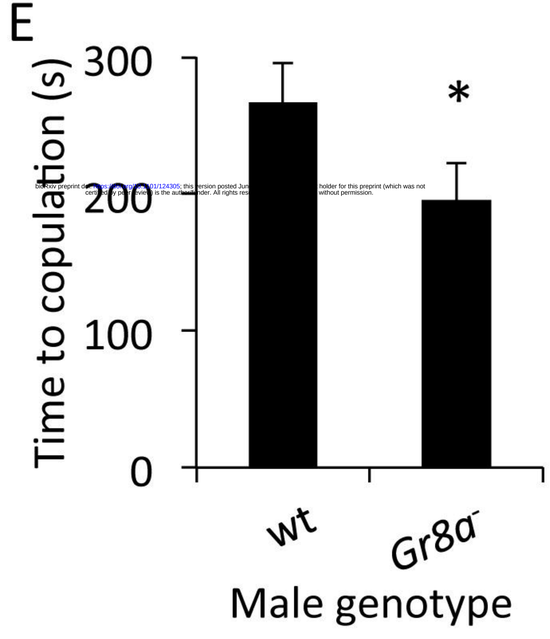

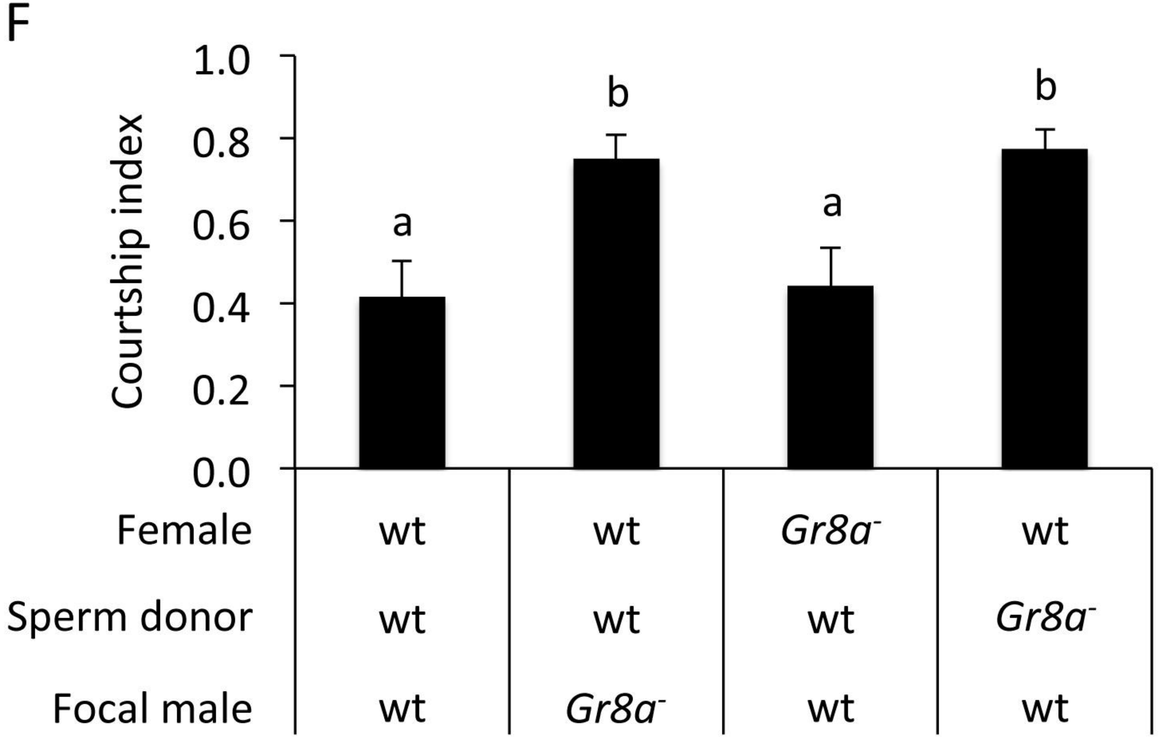

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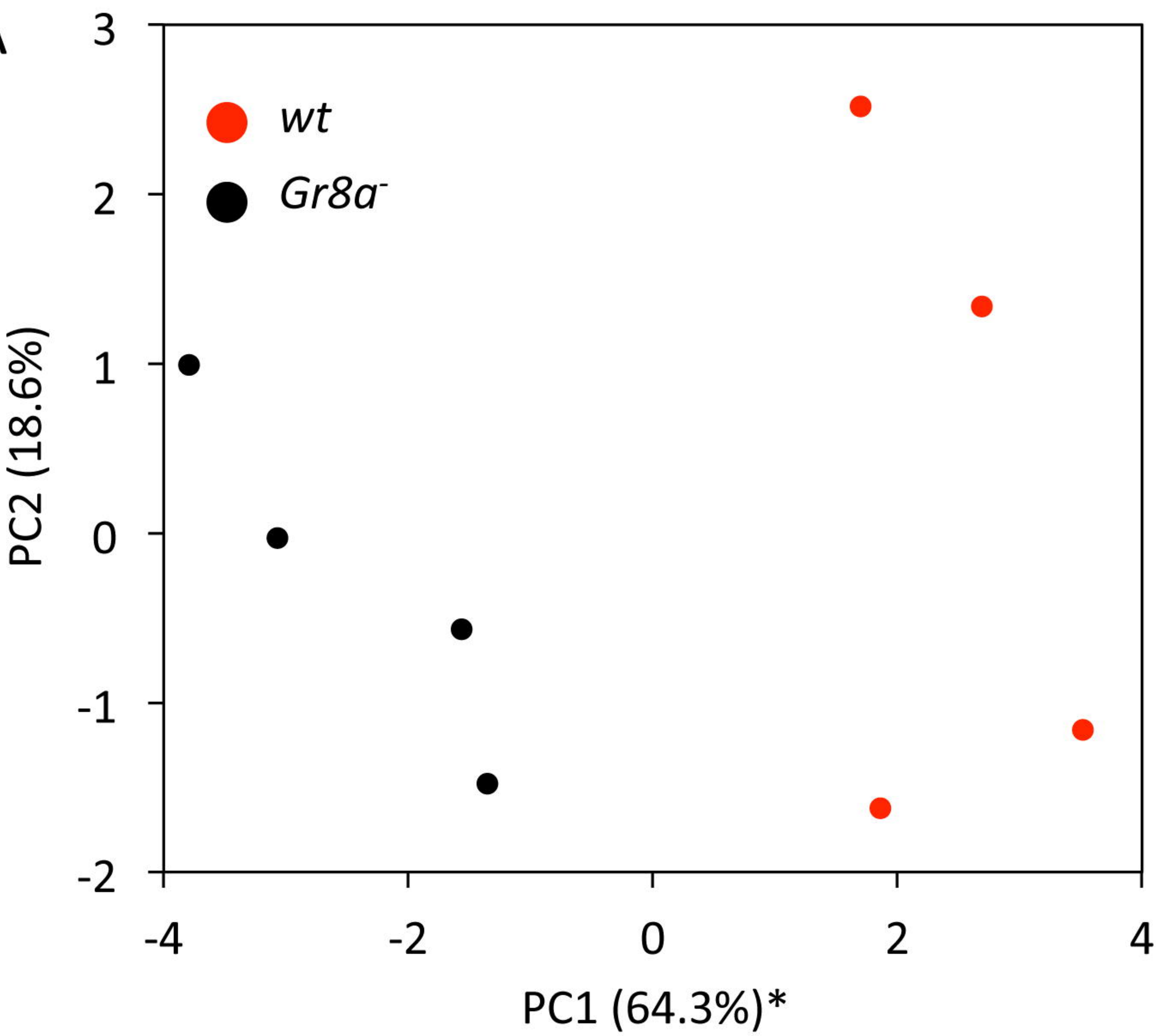

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