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

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Abstract:

Diversity in pheromonal systems plays an essential role in maintaining mating boundaries between closely related species. To preserve fitness, it has been hypothesized that pheromone-receptor coupling is maintained via strong purifying selection. However, because strong negative selection antagonizes diversity, how pheromonal systems retain potential for plasticity is puzzling. Here we propose that receptor pleiotropy could represent one possible mechanism for retaining the capacity of pheromone-receptor pairs to diversify. Specifically, we demonstrate that *Gr8a,* a member of the gustatory receptor family in *Drosophila*, is a pleiotropic gene that contributes to both the perception and production of specific mating signals 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:

Mating pheromones play an essential role in determining behavioral mating boundaries betweeen animal species. Pheromone bouquets are typically comprised of a complex chemical blend, which conveys specific information from the emitter to the receiver via functionally coupled signal-receptor pairs (*1*). Because qualitative or quantitative changes in either pheromones or their receptors could carry fitness costs, it has been hypothesized that once formed, the

robustness of pheromone-receptor pairs is maintained via purifying selection (*2, 3*). Yet, closely related animal species often exhibit dramatically different mating pheromone profiles (*4-7*). Therefore, from a genetic perspective, it is puzzling how the robust coupling of pheromone production and perception, which are two independent biological processes that typically reside in different cell types, can both be maintained under purifying selection, yet, retain the capacity for diversification without major fitness costs due to the molecular uncoupling of pheromone-receptor pairs (*2, 8*).

Here we provide evidence that one possible solution to this genetic conundrum is pleiotropy. In the *Drosophila* genus, cuticular hydrocarbons (CHCs) act as mating pheromones, which are essential for the integrity of reproductive boundaries between closely related species (*9-15*). In *D. 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) (*10, 13, 16, 17*).. 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 (*18-22*).

In *Drosophila*, the perception of CHCs is mediated by specialized gustatory-like receptor neurons (GRNs) in appendages and the proboscis (*17, 23-26*).. In contrast, the production of CHCs is localized to the fat body and oenocytes, which are large abdominal, subcuticular cells (*8, 10, 27*). The striking developmental and physiological differences between GRNs and oenocytes suggest that the perception and production of CHCs are regulated via independent genetic networks, which can rapidly evolve (*9*). Therefore, we hypothesized that in *Drosophila*, the capacity to maintain the coupling of pheromone perception and production while retaining

the capacity for rapid pheromone diversification is mediated through the pleiotropic action of pheromone receotors in both the peripheral nervous system and pheromone-producing oenocytes. We reasoned that if specific genes 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 (*10*).

We chose members of the *Gustatory receptor* (*Gr*) gene family as candidate genes for testing our hypothesis because several family members have already been implicated in the detection of excitatory and inhibitory pheromones in *Drosophila* (*28-32*). Since the expression of most *Gr*'s in gustatory receptor neurons (GRNs) has already been established (*33, 34*),we reasoned that candidate pleiotropic pheromone receptors should be also expressed in abdominal oenocytes. Therefore, we next used an RT-PCR screen, which identified 24 out of the 59 *Gr* family members in the fly genome as genes with abdominal-enriched expression (**Table S1**).

Gr8a, one of the genes we have identified in our initial abdominal expression screen as male-enriched (**Table S1**), was previously shown to contribute to the detection of the non-proteinogenic amino acid L-Canavanine (*35*). However, the natural ligand and behavioral functions of *Gr8a* remain unknown. Because previous studies only investigated the chemosensory function of *Gr8a*, we next investigated the spatial expression 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**). In addition, we identified *Gr8a* expression in two paired GRNs in the foreleg pretarsus in both sexes (**Fig. 1B**). Outside the nervous system, we found that *Gr8a* is broadly expressed in large, oenocyte-like subcuticular cells in males but not females

(**Fig. 1C**). We confirmed the *Gr8a* promoter activity patterns with mRNA expression analyses, which further supported a sexually dimorphic expression pattern in abdomens (**Fig. 1D**). We also found that *Gr8a* expression co-localizes with the oenocyte marker gene *Desat1 (10)*, further indicating that at least some *Gr8a*-expressing cells in male abdomens 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 suggest that, in addition to its canonical chemosensory functions in both males and females, *Gr8a* also functions in cellular components of the pheromone production system in males.

Phylogenetic analysis of *Gr8a* indicated that its protein sequence and sexually dimorphic expression pattern are conserved across the *Drosophila* genus (**Fig. 2A and B**). Alignment of protein sequences of GR8A orthologs revealed that although the protein sequence is conserved across *Drosophila* species, at least one predicted extracellular region is hypervariable, suggesting that its receptor functions might vary across species (**Fig. 2C and D**). Together, these data signified *Gr8a* as an ideal candidate gene for testing our primary hypothesis, which stipulates that some pleiotropic pheromone receptors contribute to both the perception and production of mating pheromones in *Drosophila*.

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 specifically in females, blocking neuronal transmission in *Gr8a*-expressing GRNs with the tetanus toxin (TNT) resulted in shorter copulation latency when courted by wild-type males (**Fig. 3A**). Similarly, homozygous (**Fig. 3B**) and hemizygous (**Fig. 3C**) *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. 3D**). In contrast, the

Gr8a mutation, or blocking neuronal transmission in *Gr8a*-expressing neurons, did not affect male courtship latency or index towards wild-type virgin females (fig. S1). Because mating decisions in flies involve both excitatory and inhibitory signals (*10, 36*), the simplest interpretation of these data is that in females *Gr8a* contributes to the chemosensory detection of an inhibitory chemical signal in males that is associated with the copulation decisions of females, but is not required for the initiation or maintenance of male courtship behavior towards virgin females.

Our primary hypothesis stipulates that *Gr8a* acts as a pleiotropic factor that affects both perception and production of some pheromone. Thus, 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 inhibitory signal important for the copulation decision of virgin females (**Fig. 3E**).

To directly establish the contribution of *Gr8a* to pheromone production, we next examined the effects of the *Gr8a* mutation on the overall CHC profile of males. Principle component analyses (PCA) revealed that the *Gr8a* mutation has a significant effect on the overall CHC profile of males (**Fig. 4A**). Analyses of individual CHCs revealed a significant effect of the *Gr8a* mutation on several specific components in males, including alkenes and methyl-branched CHCs (**Fig. 4B and Table S2**), which have been implicated in mating decisions in several *Drosophila* species (*10, 11, 14*). Together, our behavioral and pheromonal data suggest that *Gr8a* action contributes to copulation decision-making in virgin females via the production and/or release of a putative

inhibitory pheromone in males, and chemosensory perception in virgin females, and are consistent with a pleiotropic gene function.

In addition to their role in regulating pre-mating behaviors, *Drosophila* cuticular pheromones are also important for regulating post-mating behaviors in females. 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 (*10, 20, 21, 37*). Therefore, we hypothesized that *Gr8a* might also contribute to the production and perception of inhibitory mating pheromones that are transferred from males to females at copulation (*38*). If true, we anticipated that *Gr8a* mutant males would have lower ability to produce/ transfer inhibitory pheromones during copulation, and lowered capacity to detect such a signal in already mated females. Accordingly, 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 that previously mated with wild-type males (**Fig 3F**). Together, these data indicate that *Gr8a* contributes to the production and/or transfer of an inhibitory signal from males to females during copulation, and for the recognition of this signal in mated females by males. Therefore, *Gr8a* contributes to the regulation of both pre- and post-mating decisions in males and females by regulating the perception and production of inhibitory chemical mating signals.

Based on our initial hypothesis, we predicted that the fly genome harbors pleiotropic genes that contribute to both the perception and production of pheromones that play a role in mating decision-making. All data we present here support such a role for *Gr8a* because this gene is required for the production and perception of a putative male inhibitory pheromones, which

affect the willingness of virgin females to copulate with males, and reduce the drive of males to court mated females.

We still do not understand the exact mechanism by which *Gr8a* exerts its pleiotropic action. While the function of *Gr8a* as a chemoreceptor in GRNs is conceptually well understood, how *Gr8a* might affect CHC production or release by oenocytes is not obvious. While still hypothetical, we propose that *Gr8a* might be regulating the synthesis and/or secretion of specific CHCs by acting via oenocyte-intrinsic 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* (*18, 20, 37*). *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 (*21, 22*). Therefore, we speculate that *Gr8a* acts as a receptor for ligands that act as inhibitory mating pheromones independently from the action of cVA and CH503.

Data presented here provide experimental support for the possible role of pleiotropic chemoreceptors in maintaining pheromone-receptor coupling while still retaining the capacity for evolutionary divergence with minimal fitness costs that would be expected from signal-receptor uncoupling. Specifically, we demonstrate that *Gr8a*, a male-enriched member of the *Gustatory receptor* gene family in *D. melanogaster*, plays an important role in the perception and production of a putative inhibitory mating signal in males and females via its pleiotropic action in both the peripheral chemosensory system and oenocytes.

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

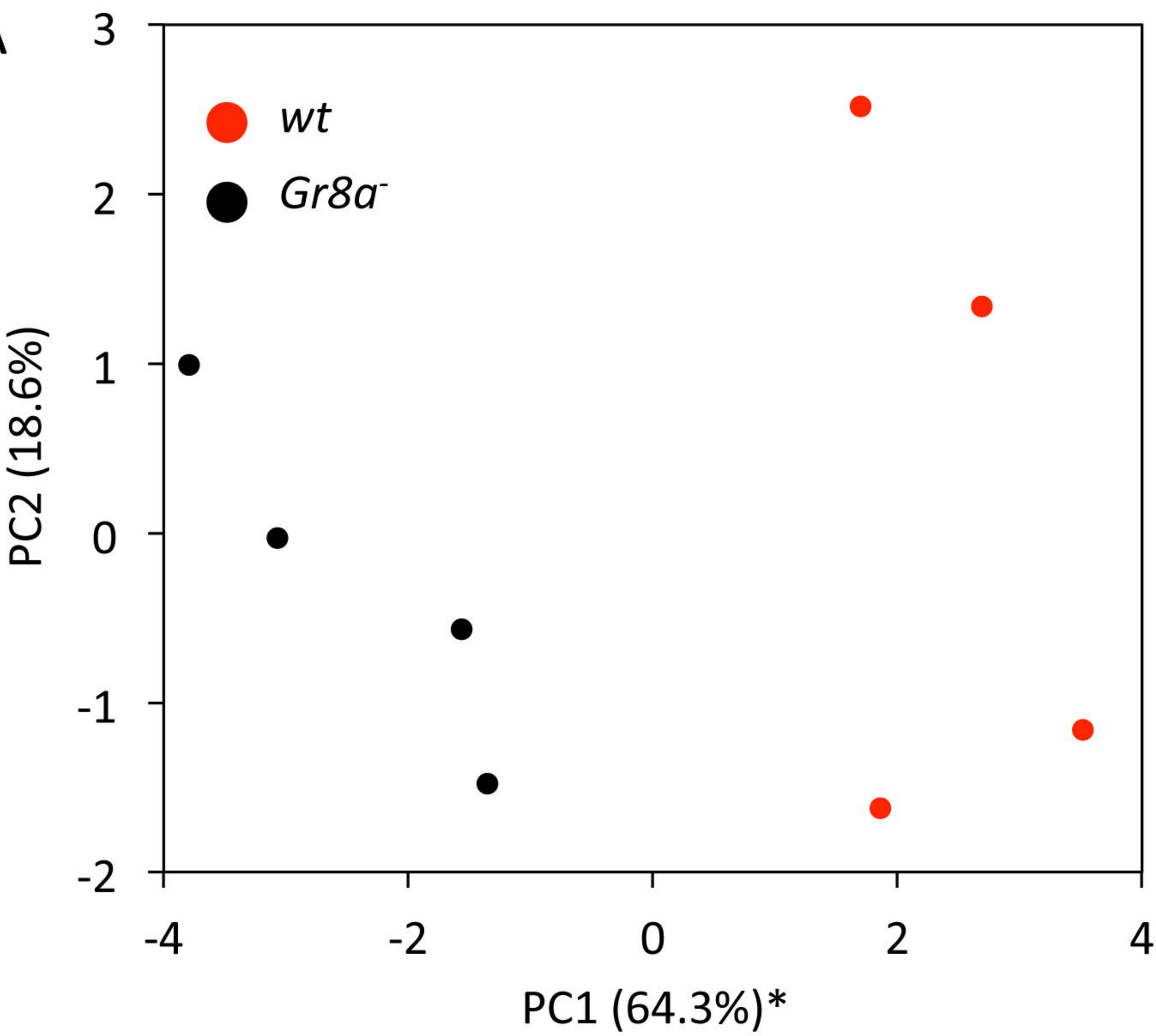
Fig. 2. *Gr8a* expression is sexually dimorphic across the *Drosophila* genus. (A) Phylogenetic tree of *Drosophila Gr8a* proteins. Substitution rate = 0.2. (B) *Gr8a* mRNA expression is enriched in males relative to females across *Drosophila*. N=4, Black, males; white, females. *, $p<0.05$; **,$p<0.01$; 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). Bars below consensus represent overall level of amino acid conservation.

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 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. 4. 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.

A



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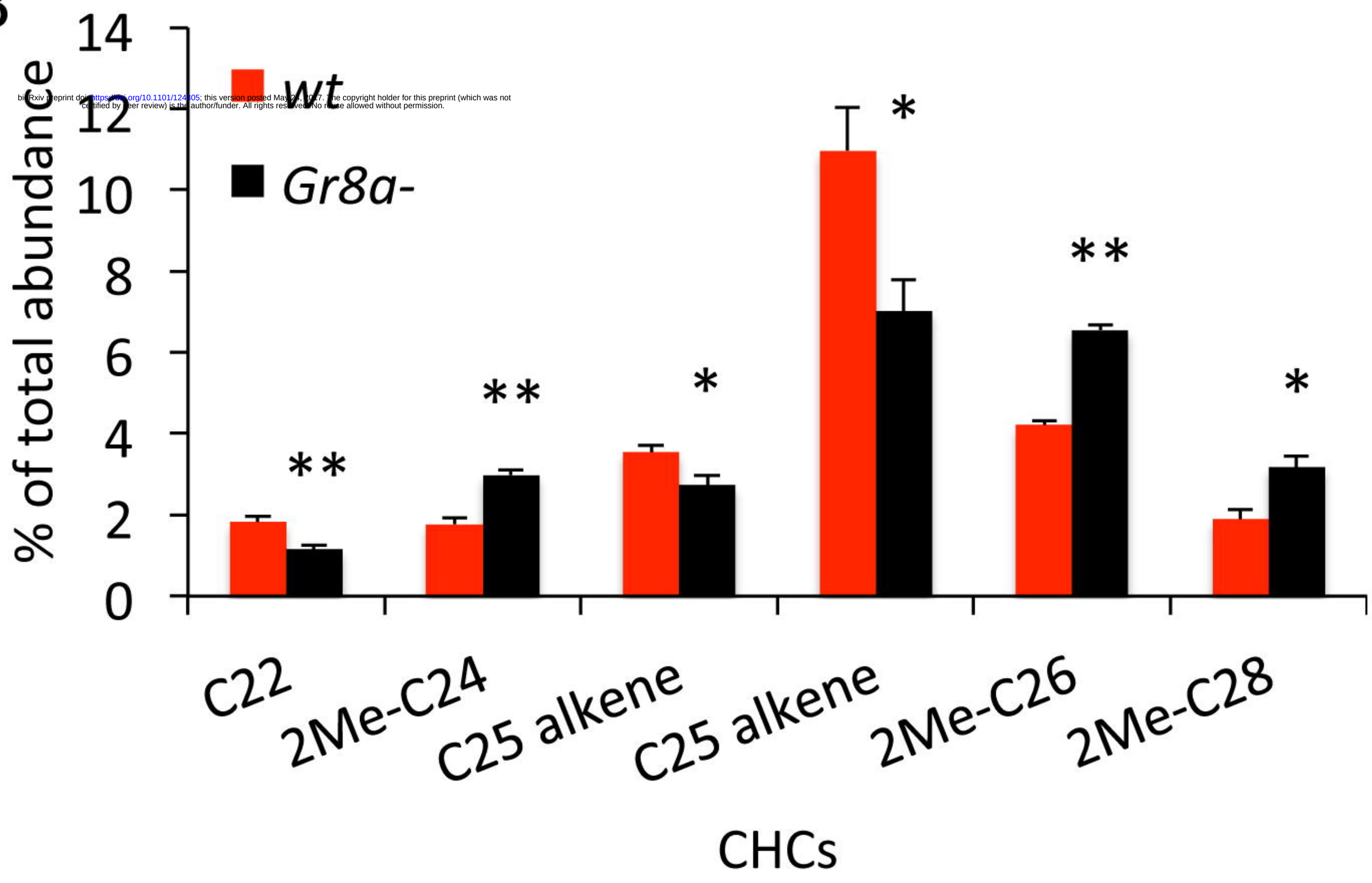


A

Time to copulation (s)

400
300
200
100
0

*

Gr8a>TNT^{inactive} *Gr8a>TNT*

Female genotype

B

300
200
100
0

*

wt *Gr8a*$^{-}$

Female genotype

C

400
300
200
100
0

a b a a

Df(1)BSC663/+ Df(1)BSC663/*Gr8a*$^{-}$ Df(1)BSC754/+ Df(1)BSC754/*Gr8a*$^{-}$

Female genotype

D

350
300
250
200
150
100
50
0

a b a

wt *Gr8a*$^{-}$ *Gr8a*$^{-}$; *Gr8a*^{tg}

Female genotype

E

Time to copulation (s)

300
200
100
0

*

wt *Gr8a*$^{-}$

Male genotype

F

Courtship index

1.0
0.8
0.6
0.4
0.2
0.0

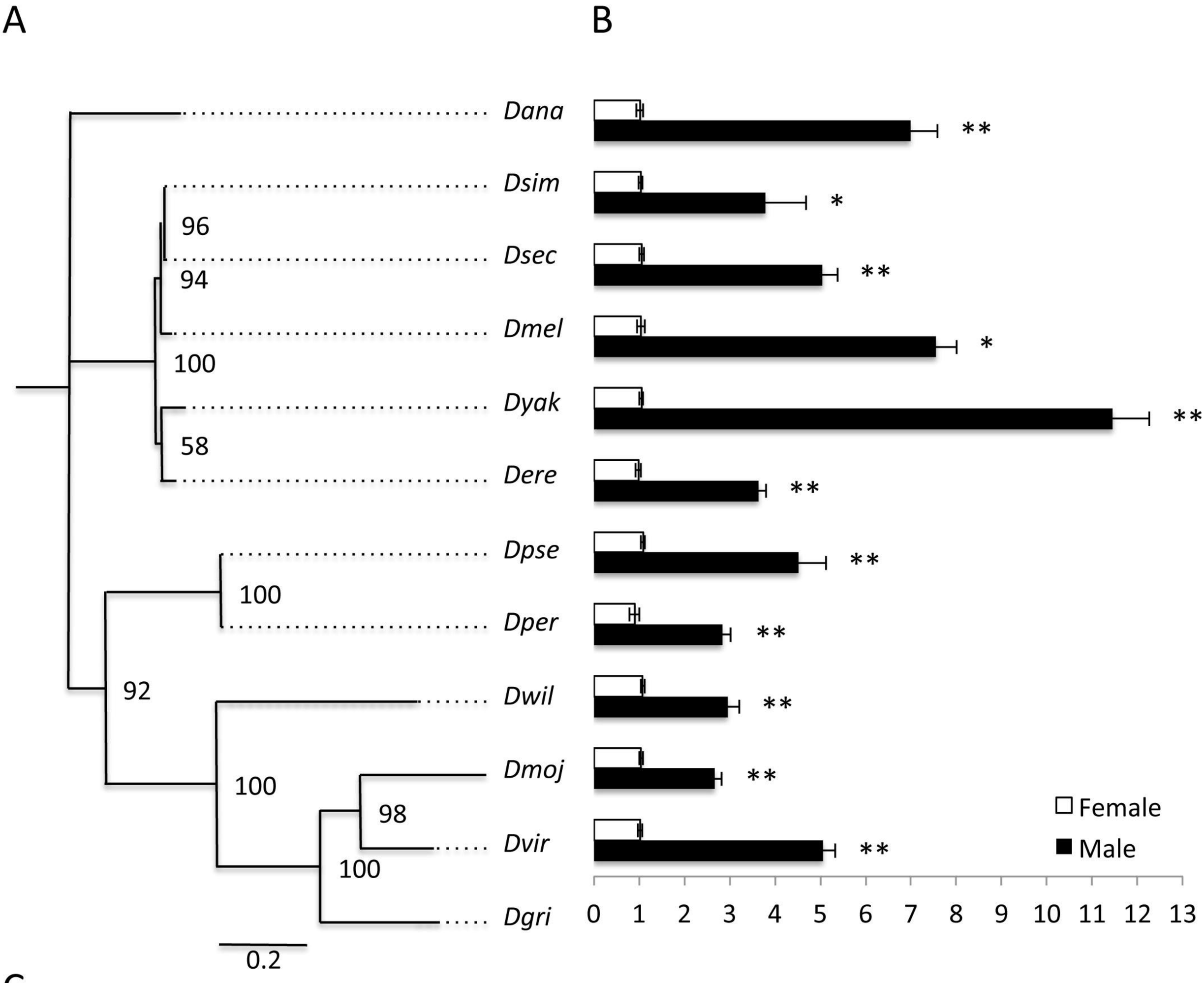
a b a b

Female	wt	wt	*Gr8a*$^{-}$	wt
Sperm donor	wt	wt	wt	*Gr8a*$^{-}$
Focal male	wt	*Gr8a*$^{-}$	wt	wt

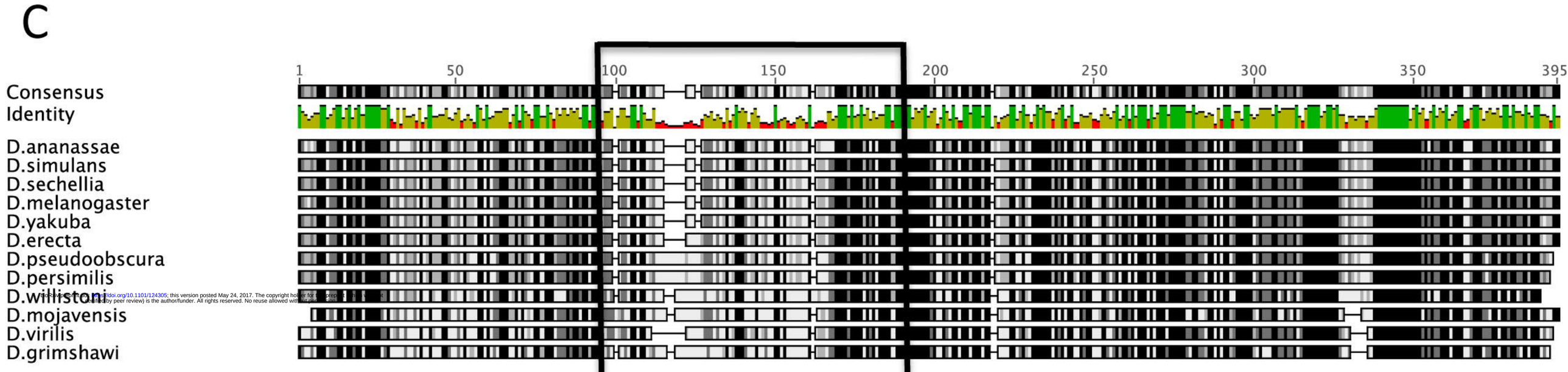
Genotypes

A

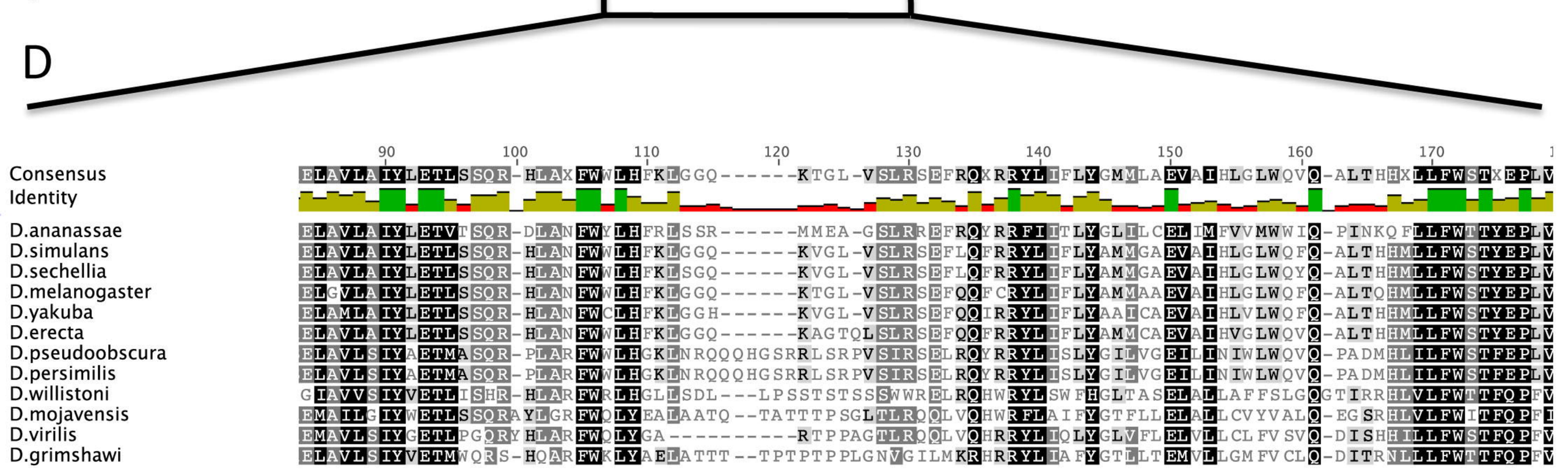
B

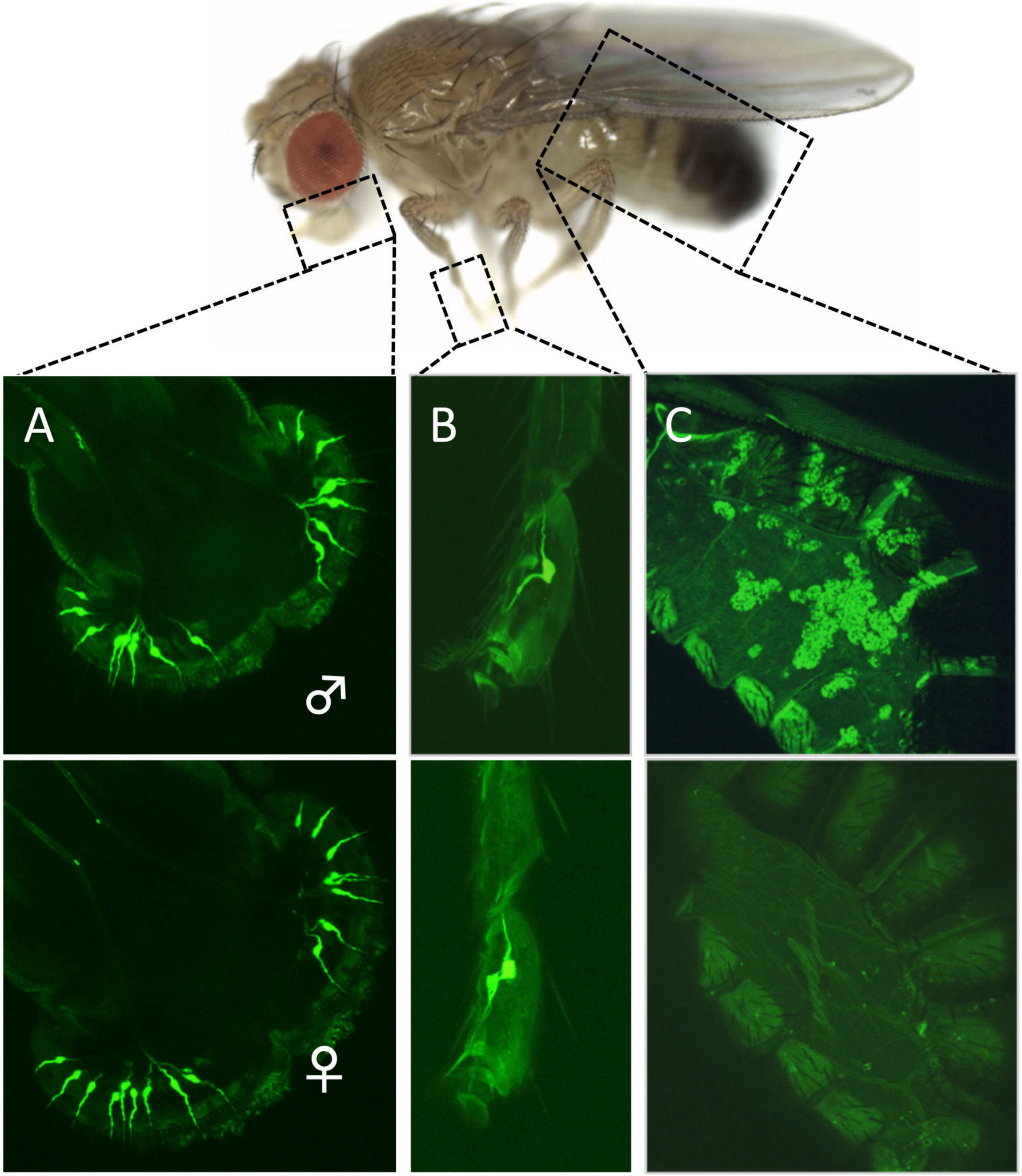


C



D

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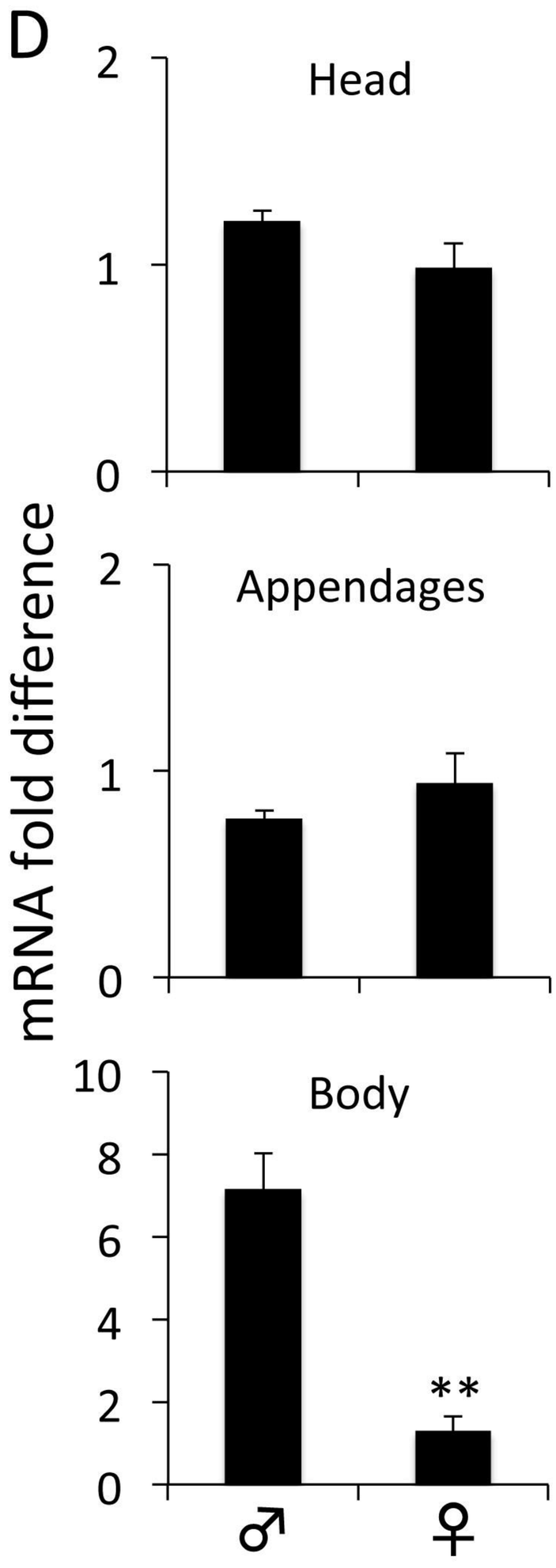
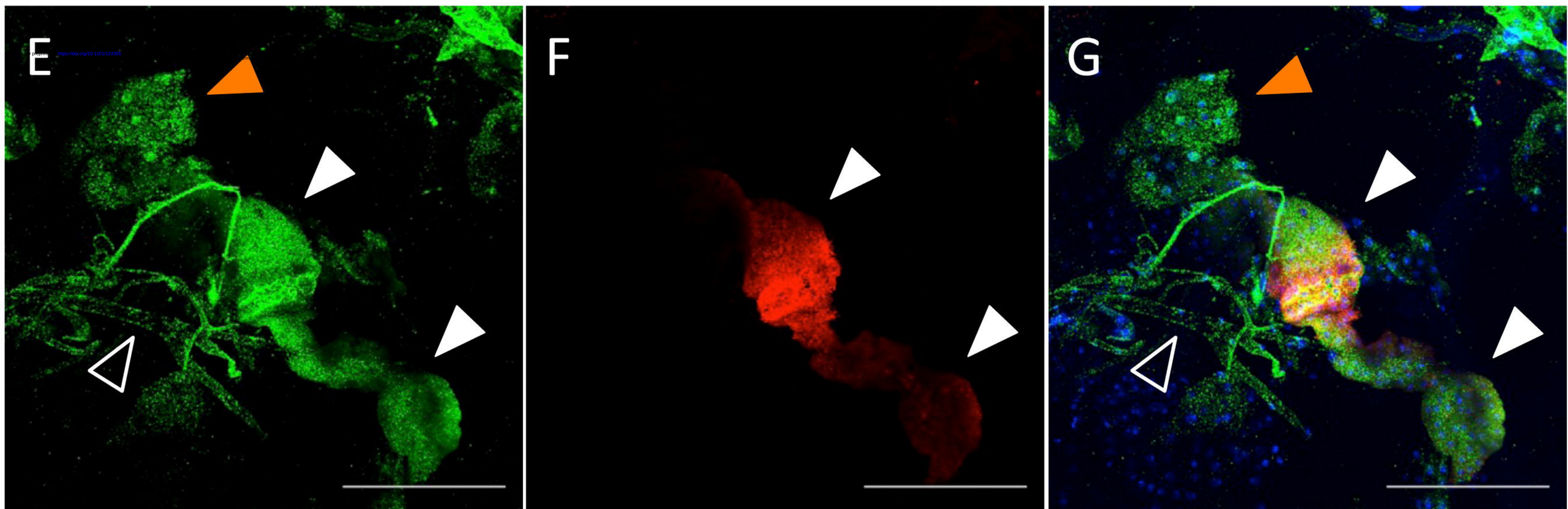

D
Head
Appendages
Body
mRNA fold difference
2
1
0
10
8
6
4
2
0
**
♂
♀


E
F
G