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Supplemental Information

Evolution of Mechanisms that Control Mating in *Drosophila* Males

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Figure S1, related to Figures 1 and 2

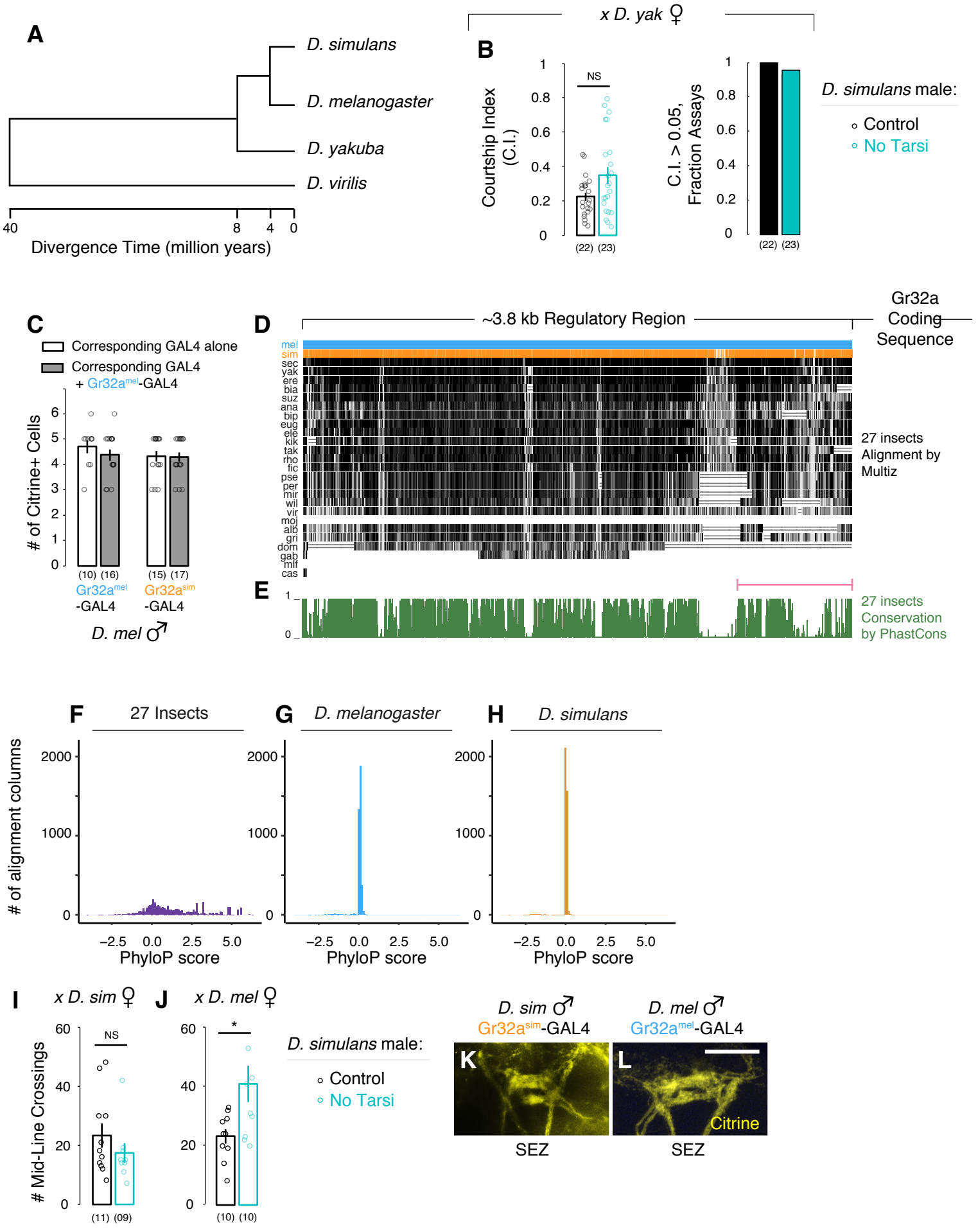

Figure S1: A regulatory region upstream of *Gr32a* coding sequence is conserved across drosophilids. Related to Figures 1 and 2.

(A) Evolutionary relationship of the four *Drosophila* species used in this study.

(B) Foreleg tarsi do not inhibit *D. simulans* males from courting *D. yakuba* females. Mean ± SEM; each circle denotes CI of a *D. simulans* male; n = 22 - 23/cohort; ***p<0.001.

(C) No difference in the number of Citrine+ cells in T4 and T5 foreleg segments of *D. melanogaster* males observed with either $Gr32a^{mel}$-GAL4 or $Gr32a^{sim}$-GAL4 alone or in combination. These findings indicate that the upstream regulatory sequence in Gr32a is functionally conserved between *D. melanogaster* and *D. simulans*; however, it is formally possible that the similarity in number of Citrine+ cells in *D. melanogaster* carrying one or both *GAL4* alleles reflects effects of transvection in the presence of both *GAL4* alleles rather than functional conservation. Mean ± SEM; each circle denotes Citrine+ cell count for a foreleg tarsum; n = 10 - 17/genotype.

(D) 27-insect alignment of the ~3.8 kb DNA element that drives *Gr32a* expression in *D. melanogaster* and *D. simulans*. mel, *D. melanogaster* (blue); sim, *D. simulans* (orange); sec, *D. sechellia*; yak, *D. yakuba*; ere, *D. erecta*; bia, *D. biarmipes*; suz, *D. suzukii*; ana, *D. ananassae*; bip, *D. bipectinata*; eug, *D. eugracilis*; ele, *D. elegans*; kik, *D. kikkawai*; tak, *D. takahashii*; rho, *D. rhopaloa*; fic, *D. ficusphila*; pse, *D. pseudoobscura*; per, *D. persimilis*; mir, *D. miranda*; wil, *D. willistoni*; vir, *D. virilis*; moj, *D. mojavensis*; alb, *D. albomicans*; gri, *D. grimshawi*; dom, *Musca domestica*; gab, *Anopheles gambiae*; mlf, *Apis mellifera*; cas, *Tribolium castaneum*.

(E) Track showing PhastCons conservation score across the region in (D). Pink bar indicates the intergenic region directly 5' of the Gr32a start codon and 5'UTR, which contains several blocks of highly conserved sequence. Higher peaks indicate higher likelihood of bases being in a strongly conserved element.

(F) Distribution of nucleotide resolution phyloP conservation scores for the region shown in (D). Most bases in the region are evolving at the same or slower rate than 4-fold degenerate (4D) sites in the multiple sequence alignment of 27 insects. Large positive (> 2) or negative (< -2) scores indicate conservation or acceleration, respectively. Scores near "0" indicate a similar substitution rate to 4D sites.

(G, H) Distribution of phyloP scores for branch-specific evolutionary tests. Most bases in the ~3.8 kb region are likely evolving more slowly or as slowly as expected in *D. melanogaster* (G) and *D. simulans* (H) compared to the other 26 insects in the tree. Scores near "0" indicate a similar rate of DNA evolution in the designated species relative to all other species in the tree.

(I, J) Removing the foreleg tarsi of *D. simulans* males does not diminish locomotor activity during courtship assays. Mean ± SEM; each circle denotes # of midline crossings of a *D. simulans* male; n = 9 - 11/cohort; *$p<0.05$.

(K, L) Projection pattern of Gr32a neurons in the SEZ is similar between *D. melanogaster* and *D. simulans* males. n = 3 - 4 brains/genotype; scale bar = 50 μm.

Figure S2, related to Figure 3

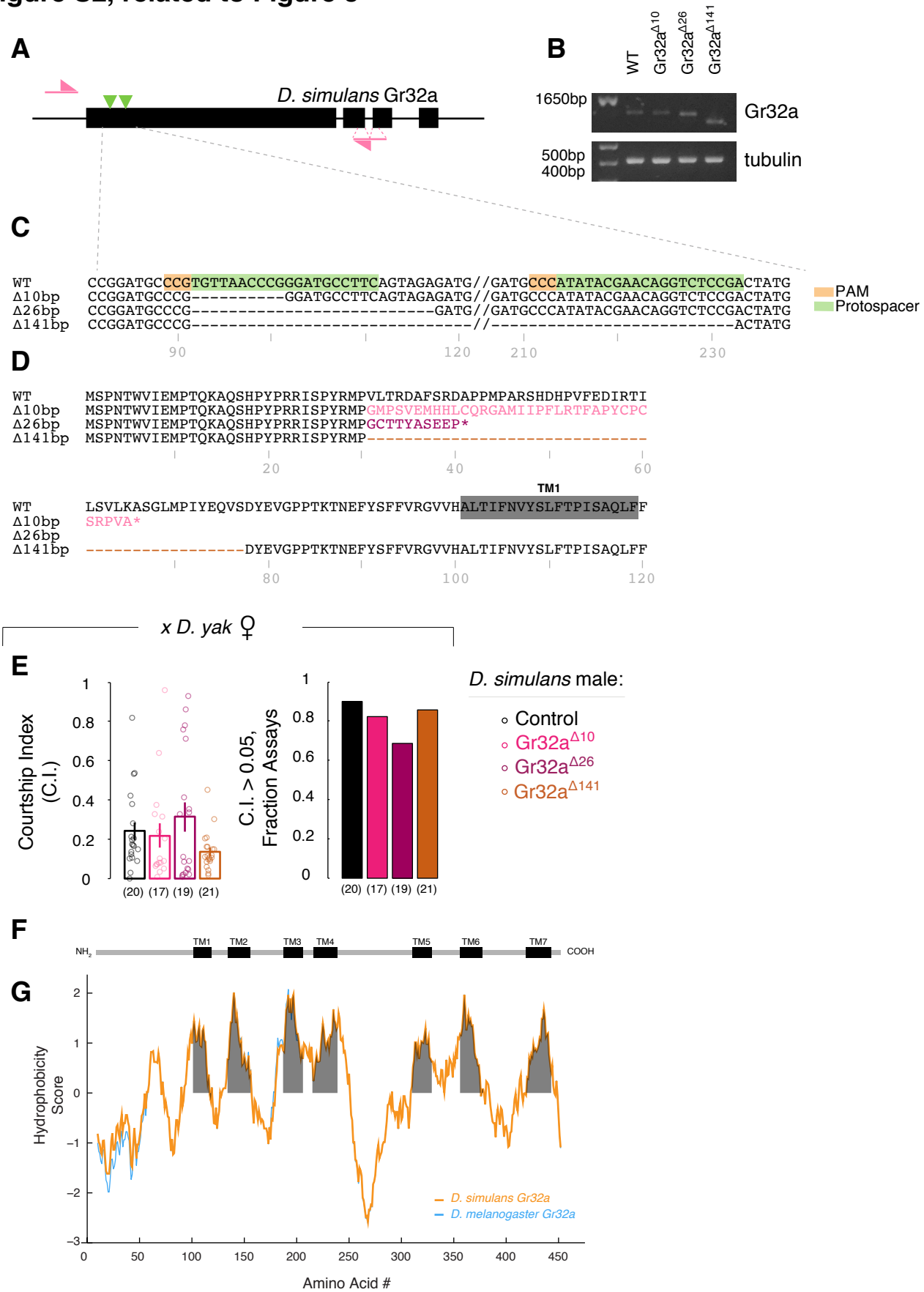

Figure S2: Generating Gr32a mutant *D. simulans* via CRISPR/Cas9. Related to Figure 3.

(A) Schematic of *D. simulans Gr32a* locus. Pink arrows, PCR primers; green triangles, CRISPR target sites; black rectangles, exons.

(B) RT-PCR products for Gr32a and tubulin in WT and *Gr32a* mutant *D. simulans*, using PCR primers shown in (A). DNA ladder shown in first lane.

(C) DNA sequence comparison of WT and mutant *Gr32a* alleles. PAM, Protospacer Adjacent Motif.

(D) Predicted amino acid sequence of WT and mutant *D. simulans* Gr32a. The predicted first transmembrane domain (TM1) is highlighted in gray in the WT protein. *, premature stop codon.

(E) No difference in courtship of *D. yakuba* females by WT and *Gr32a* mutant *D. simulans* males. Mean ± SEM; each circle represents CI of a male; n = 17 - 21/genotype.

(F) Predicted location of the seven transmembrane domains (black rectangles) in Gr32a based on plot shown in (G). The NH_2 terminal is predicted to be intracellular.

(G) Hydrophobicity plot of *D. simulans* and *D. melanogaster* Gr32a. Predicted transmembrane domains are shown by gray shading.

Please see Table S2.

Figure S3, related to Figure 3

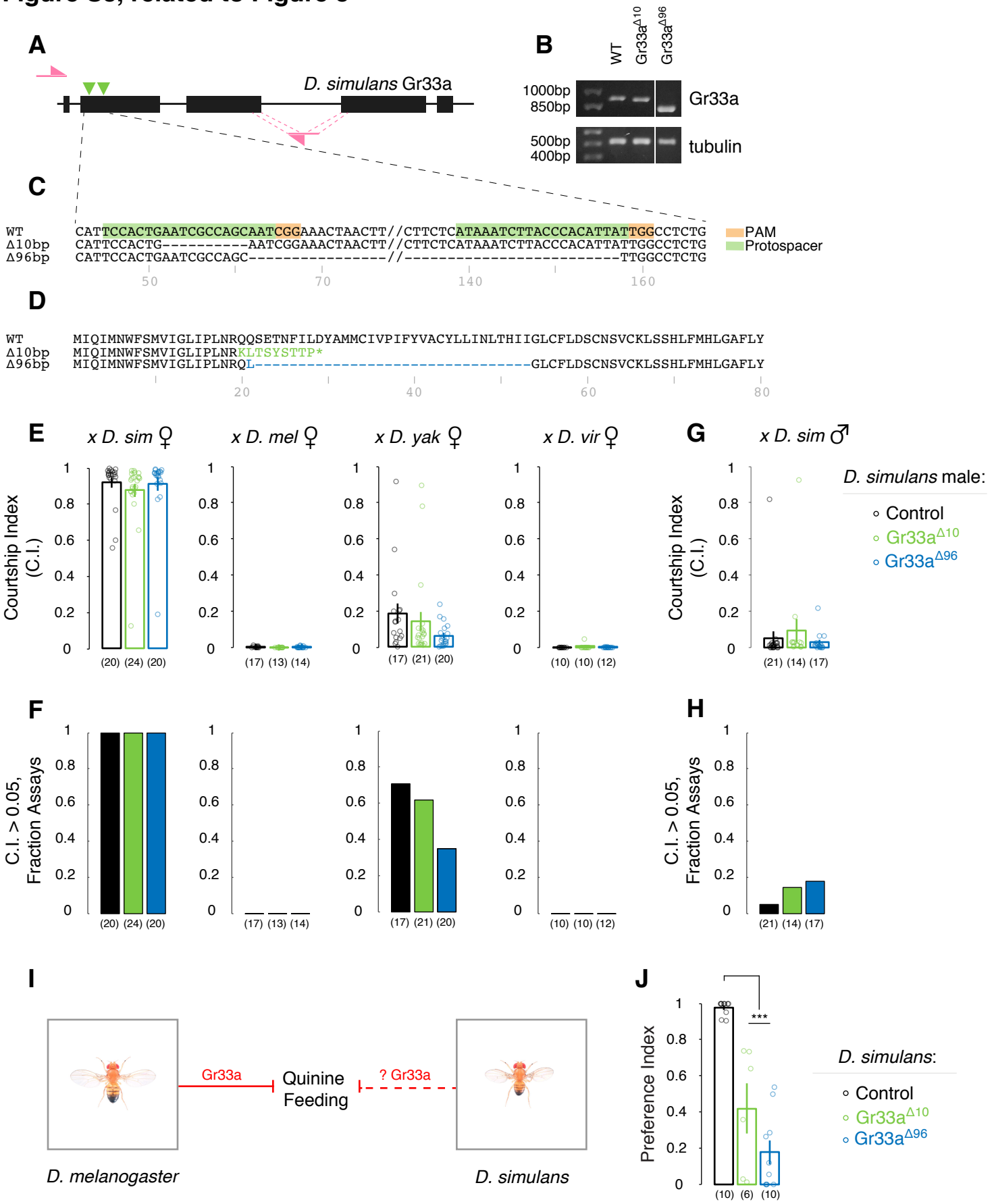

Figure S3: Gr33a is not required to inhibit interspecies courtship behavior of *D. simulans* males but does inhibit *D. simulans* from feeding on quinine. Related to Figure 3.

(A) Schematic of *D. simulans Gr33a* locus. Pink arrows, PCR primers; green triangles, CRISPR target sites; black rectangles, exons.

(B) RT-PCR products for Gr33a and tubulin in WT and *Gr33a* mutant *D. simulans*, using PCR primers shown in (A). Note that products from WT, $Gr33a^{\Delta 10}$ and $Gr33a^{\Delta 96}$ flies were run on the same gel, and lane between $Gr33a^{\Delta 10}$ and $Gr33a^{\Delta 96}$ has been cropped out for clarity of comparison. DNA ladder shown in first lane.

(C) DNA sequence comparison of WT and mutant *Gr33a* alleles.

(D) Predicted amino acid sequence of WT and mutant *D. simulans* Gr33a. *, premature stop codon.

(E, F) WT and *Gr33a* mutant *D. simulans* males court conspecifics at high levels and show similar low (*D. yakuba*) to minimal (*D. melanogaster* and *D. virilis*) levels of courtship toward females of other species.

(G, H) WT and Gr33a mutant *D. simulans* males show similar low levels of courtship toward conspecific males.

Mean ± SEM; each circle denotes CI of one male; n = 10 - 24/genotype.

(I) We tested whether, similar to *D. melanogaster*, Gr33a inhibits feeding on quinine-containing food in *D. simulans*.

(J) Significant decrease in preference by *Gr33a* mutant *D. simulans* for food containing only sucrose.

Mean ± SEM; each circle denotes Preference Index for one experiment; 90 ± 4 *D. simulans* of each genotype were used/experiment; n = 6 - 10 experiments/genotype; ***$p<0.001$.

Figure S4, related to Figure 4

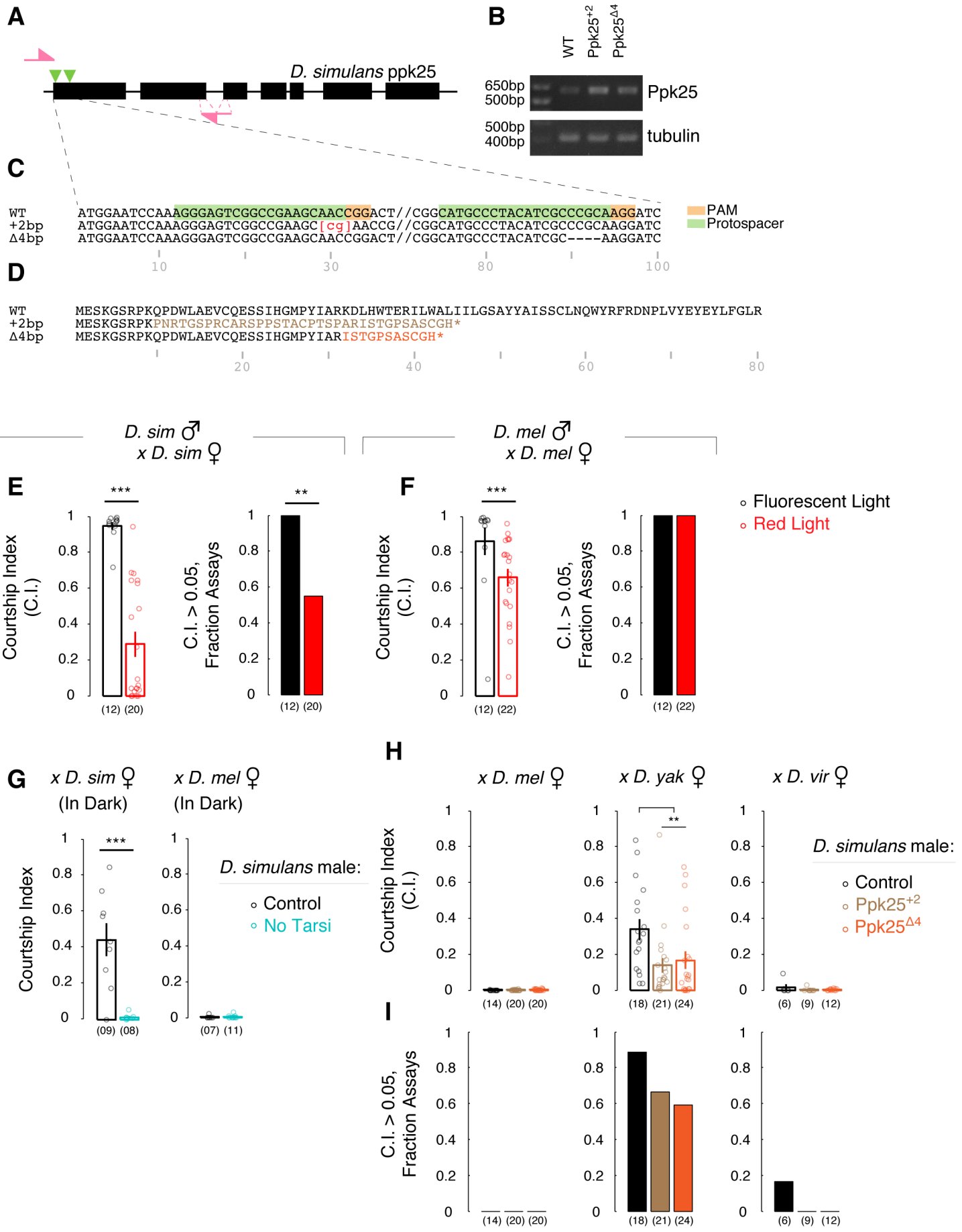

Figure S4: Ppk25 is not essential to inhibit interspecies courtship by *D. simulans* males. Related to Figure 4.

(A) Schematic of *D. simulans Ppk25* locus. Pink arrows, PCR primers; green triangles, CRISPR target sites; black rectangles, exons.

(B) RT-PCR products for Ppk25 and tubulin in WT and *Ppk25* mutant *D. simulans*, using PCR primers shown in (A). DNA ladder shown in first lane.

(C) DNA sequence comparison of WT and mutant *Ppk25* alleles.

(D) Predicted amino acid sequence of WT and mutant *D. simulans* Ppk25. *, premature stop codon.

(E, F) *D. simulans* and *D. melanogaster* males court conspecific females less under red light-only illumination.

(G) *D. simulans* males require foreleg tarsi for courtship under dark conditions.

(H, I) No difference between *D. simulans* males WT or mutant for Ppk25 in courtship of *D. melanogaster* and *D. virilis* females. Mutant males court *D. yakuba* females less than WT (G). Mean ± SEM; each circle denotes CI for one male; n = 6-24/cohort; **p<0.01; ***p<0.001.

Table S1. Related to Figures 2, S2, S3, and S4

Name	Experiment	5' to 3' Sequence
sim32 fwd	Amplifying ~3.8kb Gr32a regulatory region from *D. simulans* to generate $Gr32a^{sim}$-GAL4	GTCCCCTTGCGGTTGTTCT
sim32 rev		TTCAATTACCCAAGTGTTCG
mel32 fwd	Amplifying ~3.8kb Gr32a regulatory region from *D. melanogaster* to generate $Gr32a^{mel}$-GAL4	AAGTGGTTGGTCTTGGAT
mel32 rev		TTCAATTACCCAAGTGTTCG
CrisprGr32a A fwd	CRISPR oligos targeting *D. simulans* Gr32a	CTTCGGAAGGCATCCCGGGTTAACA
CrisprGr32a A rev		AAACTGTTAACCCGGGATGCCTTCC
CrisprGr32a B fwd		CTTCGTCGGAGACCTGTTCGTATAT
CrisprGr32a B rev		AAACATATACGAACAGGTCTCCGAC
CrisprGr32a C fwd		CTTCGTTTTACTCGTTCTTCGTAAG
CrisprGr32a C rev		AAACCTTACGAAGAACGAGTAAAAC
CrisprGr33a A fwd	CRISPR oligos targeting *D. simulans* Gr33a	CTTCGTCCACTGAATCGCCAGCAAT
CrisprGr33a A rev		AAACATTGCTGGCGATTCAGTGGAC
CrisprGr33a B fwd		CTTCGATAAATCTTACCCACATTAT
CrisprGr33a B rev		AAACATAATGTGGGTAAGATTTATC
CrisprGr33a C fwd		CTTCGGCTGAGTCTTTATCGCCGAA
CrisprGr33a C rev		AAACTTCGGCGATAAAGACTCAGCC
CrisprPpk25 A fwd	CRISPR oligos targeting *D. simulans* Ppk25	CTTCGAGGGAGTCGGCCGAAGCAAC
CrisprPpk25 A rev		AAACGTTGCTTCGGCCGACTCCCTC
CrisprPpk25 B fwd		CTTCGCATGCCCTACATCGCCCGCA
CrisprPpk25 B rev		AAACTGCGGGCGATGTAGGGCATGC
Gr32a RTPCR fwd	primers for RT-PCR of *D. simulans* Gr32a	TAATCCACAATGCCAAGCAA
Gr32a RTPCR rev		AGGAACTTATCGATGATATTCTGAT
Gr33a RTPCR fwd	primers for RT-PCR of *D. simulans* Gr33a	CGGAGTAGCGAGTAAATTCCA
Gr33a RTPCR rev		TCGGATGTGTTTCCGGTATT
Ppk25 RTPCR fwd	primers for RT-PCR of *D. simulans* Ppk25	ACATCATGGAATCCAAAGG
Ppk25 RTPCR rev		ATCCAGTGTTTCTAGTTTGCC

tubulin RTPCR fwd	primers for RT-PCR of *D. simulans* tubulin	CTTGTCGCGTGTGAAACACT
tubulin RTPCR rev		GGATCCTGTCCAGAACCAGA

Table S1: List of oligos and primers used in this study. Related to Figures 2, S2, S3, and S4.

Table S2. Related to STAR Methods and Figure S2

Predicted TM domains	Gr32a Amino Acid Range	
	D. melanogaster	*D. simulans*
TM1	101-119	101-120
TM2	134-156	131-149
TM3	187-206	187-206
TM4	215-239	215-239
TM5	310-329	310-327
TM6	356-378	354-378
TM7	419-443	419-443
TM, transmembrane		

Table S2: Predicted transmembrane domains of Gr32a. Related to STAR Methods and Figure S2.

Table S3. Related to STAR Methods and Figures S2, S3, and S4.

Locus	# CRISPR Guides Injected	# injected embryos	# larvae	# G0 flies	# G0 flies that yielded progeny (F1)	# G0 flies that yielded mutant F1 flies	# F1 flies bearing distinct indel mutations	Homozygous Stocks used in study
Gr32a	3	300	40	15	7	3	7	3
	2	300	15	10	5	1	3	0
Gr33a	3	519	298	78	26	3	7	2
Ppk25	2	510	185	86	44	2	4	2

Table S3: *D. simulans* pDCC6 CRISPR injection counts. Related to STAR Methods and Figures S2, S3, and S4.