# Supporting Information

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### SI Experimental Procedures

**Fly Lines and Husbandry.** The *don juan::*green fluorescent protein (*dj::GFP*) line was described previously (1). Wild-type flies, Oregon R (OR), were descendants of the Oregon R line of John Palka at the University of Washington that was used for juvenile hormone (JH) application experiments as described in Riddiford and Ashburner (2).

Over 50% of the females that had their corpora allata (CA) genetically ablated died at eclosion, either trapped in the puparial case or failed to expand their wings. The remaining allatectomized females (CAX) flies had similar viability as the parental controls during the first 8 d of adult life.

**Staining and Imaging of the CA.** After ice anesthetization, we dissected the anterior thorax containing the CA attached to the aorta in phosphate-buffered saline (PBS), then fixed in 4% (vol/vol) formaldehyde for 30 min at room temperature. After rinses, the tissue was incubated with 1:500 rabbit anti-GFP (Life Technologies) overnight followed by 1:500 Alexa Fluor 488 anti-rabbit immunoglobulin G and 1:50 Texas Red-phalloidin (Life Technologies) to label actin. For details, see ref. 3. The tissue was mounted in Vectashield (Vector Laboratories) and imaged on a Zeiss LSM510 laser scanning microscope.

**Application of Methoprene to Adult Females.** Methoprene (Wellmark), a juvenile hormone mimic (JHM), was serially diluted in acetone (Fisher Scientific, HPLC grade), and 0.2 μL was applied to the ventral abdomen of $CO_2$-anesthetized females within 2 h of eclosion using a 10 μL Hamilton Gastight syringe 1701 and a Hamilton repeating dispenser (Sigma-Aldrich). Controls received 0.2 μL acetone.

**Group-Mating Assay and Analysis of Mating.** For the group-mating assay, *dj::GFP* males, which produce GFP-labeled sperm (1) were collected daily after eclosion and housed in vials with food in groups of 12. Virgin females of selected genotype were housed in groups of 12, then at various ages (18, 24, 30, 36, 48, 72, or 96 h old) were placed together with 12 3- to 4-d-old *dj::GFP* males in a food vial for 1 h at 25 °C. At the end of this period, the percentage of mated females was determined based on abdominal fluorescence corresponding to GFP-labeled sperm in the seminal receptacle and spermatheca, as detected with a fluorescent dissecting microscope (Leica M205 FA). Flies used for the group assays involving six, three, or one pair of flies per vial were treated similarly before the assay.

**Statistical Analysis for Behaviors and Cuticular Hydrocarbons.** The Kolmogorov Smirnov test was used to check the significant difference of the cumulative distribution curves of fraction of females mated versus time. ANOVA followed by the Tukey multiple comparison test was used to find the significance for the percent mating in the group assay, onset of copulation in the single pair-mating assay, and the CHC data. The binomial probability and ANOVA followed by the Tukey multiple comparison test was used to check the significance of percent mating in the single pair mating assay data.


1. Santel A, Winhauer T, Blümer N, Renkawitz-Pohl R (1997) The *Drosophila don juan* (*dj*) gene encodes a novel sperm specific protein component characterized by an unusual domain of a repetitive amino acid motif. *Mech Dev* 64(1–2):19–30.
2. Riddiford LM, Ashburner MA (1991) Effects of juvenile hormone mimics on larval development and metamorphosis of *Drosophila melanogaster*. *Gen Comp Endocrinol* 82(2):172–183.
3. Riddiford LM, Truman JW, Mirth CK, Shen YC (2010) A role for juvenile hormone in the prepupal development of *Drosophila melanogaster*. *Development* 137(7):1117–1126.

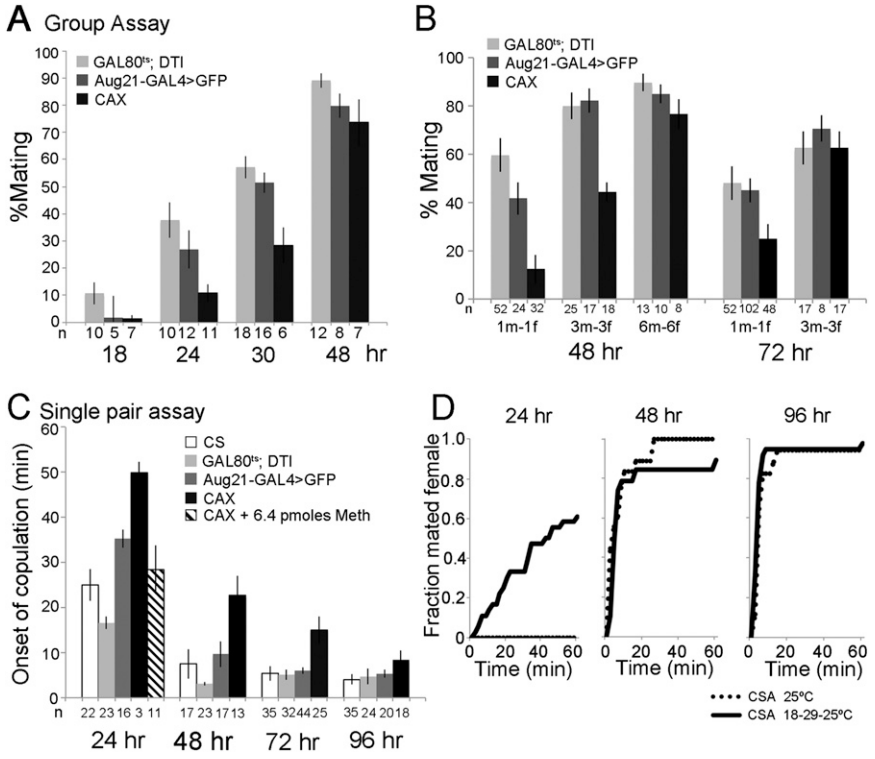


**Fig. S1.** Mating of the CAX and control females in group and single-pair assays. (*A*) Ablation of the corpora allata (CA) delays onset of mating of females in a group-mating assay. At the indicated time after eclosion, females treated as described in Fig. 1*A* were grouped with 12 *dj::GFP* males in food vials, and mating was assessed by presence of GFP in the receptacle and spermatheca in the female abdomen. *n* indicates the number of groups tested for each genotype. Error bars represent SEM. At 24 h, $P = 0.01$ and 0.17 for the CAX versus the *GAL80$^{ts}$; DTI* and the *Aug21-galactosidase4* (*GAL4*) > *EGFP* parental controls, respectively; at 30 h, $P = 0.0015$ and 0.014 for the CAX versus the *GAL80$^{ts}$; DTI* and the *Aug21-GAL4* > *EGFP* parental controls, respectively. There are no significant differences at 18 and 48 h. (*B*) An increased number of males and females in the group assay causes increased mating of CAX females at 48 and 72 h after eclosion. Error bars represent SEM. At 48 h, $P = 0.00004$ and 0.01 for CAX versus the *GAL80$^{ts}$; DTI* and the *Aug21-GAL4* > *GFP* parental controls, respectively, in the one-pair assay; $P = 0.00001$ and 0.00002 for CAX versus the GAL80$^{ts}$; DTI and the *Aug21-GAL4* > *GFP* parental controls, respectively, in the three-pair assay; $P = 0.11$ and 0.43 for CAX versus the *GAL80$^{ts}$; DTI* and the *Aug21-GAL4* > *GFP* parental controls, respectively, in the six-pair assay. At 72 h, $P = 0.049$ and 0.05 for CAX versus the *GAL80$^{ts}$; DTI* and the *Aug21-GAL4* > *GFP* parental controls, respectively, in the one-pair assay; $P = 0.66$ and 0.99 for CAX versus the *GAL80$^{ts}$; DTI* and the *Aug21-GAL4* > *GFP* parental controls, respectively, in the three-pair assay. (*C*) Ablation of the CA delays the onset of copulation in the single pair-mating assay. *n* indicates the number of females copulated in a 1-h assay at times after eclosion. Error bars represent SEM. At 24 h, $P = 0.01$, 0.0005, 0.38, and 0.08 for CAX versus Canton S (CS), the *GAL80$^{ts}$; DTI* and the *Aug21-GAL4* > *GFP* parental controls, and CAX+Meth, respectively; $P = 0.19$, 0.10, and 0.94 for CS versus the parental controls *GAL80$^{ts}$; DTI* and *Aug21-GAL4* > *GFP* and the CAX+Meth, respectively. At 48 h, $P = 0.0006$, $P < 0.0001$ and $P = 0.0032$ for CAX versus CS and the *GAL80$^{ts}$; DTI* and the *Aug21-GAL4* > *GFP* parental controls, respectively; $P = 0.66$ and $P = 0.95$ for CS versus the *GAL80$^{ts}$; DTI* and the *Aug21-GAL4* > *GFP* parental controls, respectively. At 72 h, $P = 0.0009$, 0.0003, and 0.003 for CAX versus CS and the *GAL80$^{ts}$; DTI* and the *Aug21-GAL4* > *GFP* parental controls, respectively; $P = 0.99$ and 0.99 for CS versus the *GAL80$^{ts}$; DTI* and the *Aug21-GAL4* > *GFP* parental controls, respectively. At 96 h, $P = 0.15$, 0.35, and 0.55 for CAX versus CS and the *GAL80$^{ts}$; DTI* and the *Aug21-GAL4* > *GFP* parental controls, respectively; $P = 0.98$ and 0.91 for CS versus the *GAL80$^{ts}$; DTI* and the *Aug21-GAL4* > *GFP* parental controls. All statistical comparisons in Fig. 1 *A*–*C* used ANOVA followed by the Tukey multiple comparison test. Genotypes are same as Fig. 1 *C* and *D*. (*D*) Fraction of wild-type CS females that were raised either continuously at 25 °C or in the temperature regime used for the CA ablation experiments (18-29-25 °C) which mated in the single pair-mating assay during 1 h. At 24 h after eclosion, none raised continuously at 25 °C mated during the hour in contrast to those raised in the fluctuating regime. At 48 and 72 h after eclosion, both sets of females mated rapidly. At 24 h, CS 25 °C ($n = 32$), CS (18-29-25 °C) ($n = 36$); at 48 h, CS 25 °C ($n = 18$), CS (18-29-25 °C) ($n = 19$); at 96 h, CS 25 °C ($n = 17$), CS (18-29-25 °C) ($n = 36$).

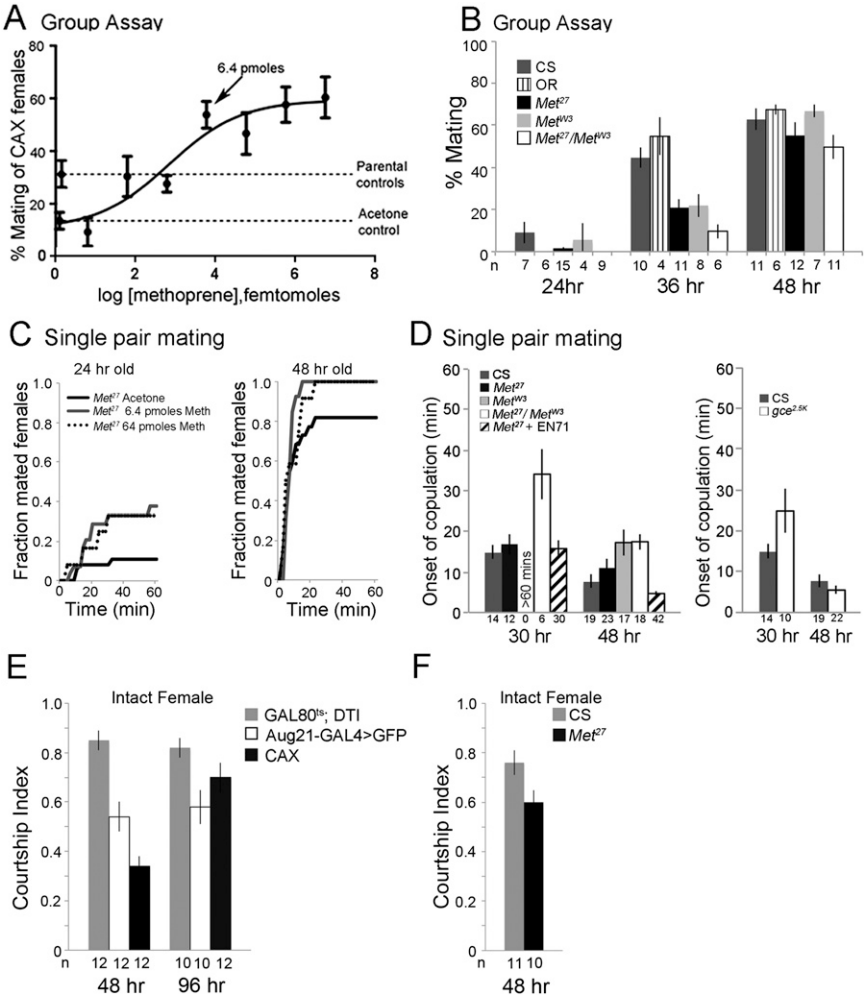


**Fig. S2.** Mating of CAX females treated with the JH mimic, methoprene, and mating of the *Met* and *gce* mutant females. (*A*) Dose–response curve for percentage mating of CAX females treated with increasing concentrations of methoprene just after eclosion and then tested in the group-mating assay at 24 h after eclosion. The black circles represent the average values of the multiple group mating ± SEM. Nine to eleven groups were assayed for each dose, except only six to seven groups for 0.0064, 0.064, and 64 pmoles. The best fit nonlinear dose–response curve was made using Prism (GraphPad). The average percent mating of acetone-treated controls ($n = 11$ groups) was given an arbitrary log value of 0.1 for purposes of curve-fitting. The base of the curve was found to be 10.4% mating, and the top of the curve was found to be 59.4% with a 50% effective dose ($ED_{50}$) of 0.72 pmoles. The average percent mating of the untreated parental genotype controls [*GAL80$^{ts}$; DTI* ($n = 10$ groups) and *Aug21-GAL4 > GFP* ($n = 12$ groups)] ± SEM at 24 h, respectively, is indicated. (*B*) Percent mating of females homozygous and heterozygous for *Met* alleles and wild type, CS, and Oregon R in the group-mating assay as a function of age. The number below each column represents the number of groups tested. Percent mating is the average ± SEM for each genotype. ANOVA followed by the Tukey multiple comparison test was used to test statistical significance. At 36 h, $P = 0.0021$, 0.0048, and <0.001 for OR versus *Met$^{27}$*, *Met$^{W3}$*, and *Met$^{27}$/Met$^{W3}$*, respectively. $P =$ 0.0047, 0.016, and < 0.001for CS versus *Met$^{27}$*, *Met$^{W3}$*, and *Met$^{27}$/Met$^{W3}$*. Genotypes: CS, OR, *wv Met$^{W3}$/wv Met$^{W3}$ (Met$^{W3}$)*, *wv Met$^{27}$/ wvMet$^{27}$ (Met$^{27}$)*, and *wvMet$^{W3}$/wvMet$^{27}$ (Met$^{W3}$/Met$^{27}$)*. (*C*) Mating of homozygous *Met$^{27}$* females treated with methoprene or acetone at eclosion, then assayed at 24 and 48 h after eclosion in the single pair-mating assay for 1 h. $P < 0.0001$ for *Met$^{27}$* treated with 6.4 ($n = 24$) or 64 pmoles ($n = 12$) methoprene versus *Met$^{27}$* treated with acetone ($n = 36$) at 24 h and $P < 0.0001$ for *Met$^{27}$* treated with 6.4 ($n = 13$) or 64 pmoles ($n = 12$) methoprene versus *Met$^{27}$* treated with acetone ($n = 22$) at 48 h using the Kolmogorov Smirnov test. (*D*) (*Left*) Time to copulation of the *Met* allele females with and without *Met* rescue transgene EN71 in the single pair-mating assay compared with that of CS females at 30 and 48 h after eclosion. *n* indicates the number of females that copulated. Error bars represent SEM. $P =$ 0.0038 and 0.018 for CS versus *Met$^{W3}$/Met$^{27}$* at 24 and 48 h, respectively, and $P = 0.014$ for CS versus *Met$^{W3}$* at 48 h. $P = 0.0004$ for the *Met$^{27}$* versus the *Met$^{27}$* + *Met* transgene at 48 h using ANOVA followed by the Tukey multiple comparison test. Genotypes are the same as in Fig. 2*A*. (*Right*) Time to copulation of *gce$^{2.5k}$* homozygous females and the CS control at 30 and 48 h after eclosion. *n* indicates the number of females that copulated. Error bars represent SEM. $P =$ 0.08 and $P = 0.31$ for *gce$^{2.5k}$* versus CS at 30 and at 48 h, respectively, using ANOVA followed by the Tukey multiple comparison test. Genotypes are same as Fig. 2*B*. (*E*) Courtship indices (CI) of wild-type males paired with intact CAX females and parental controls at 48 and 96 h after eclosion. CI was calculated from the behavior videos used for Fig. 1*D* as outlined in *Experimental Procedures*. Numbers below the columns represent the number of females tested. Error bars represent SEM. $P < 10^{-6}$ and $P = 0.011$ for CAX versus the *GAL80$^{ts}$; DTI* and the *Aug21-GAL4 > GFP* parental controls, respectively, at 48 h; $P = 0.32$ and $P = 0.09$ for the CAX versus the *GAL80$^{ts}$; DTI* and *Aug21-GAL4 > GFP* controls at 96 h using ANOVA followed by the Tukey multiple comparison test. Genotypes are the same as in Fig. 1 *C* and *D*. (*F*) Courtship indices of wild-type males paired with intact homozygous *Met$^{27}$* females and wild-type CS females at 48 h after eclosion. CI was calculated from the behavior videos used for Fig. 3*B*. Numbers below the columns represent the number of females tested. Error bars represent SEM. $P =$ 0.03 for the *Met$^{27}$* versus CS using ANOVA followed by the Tukey multiple comparison test. Genotypes are same as in Fig. 2*A*.

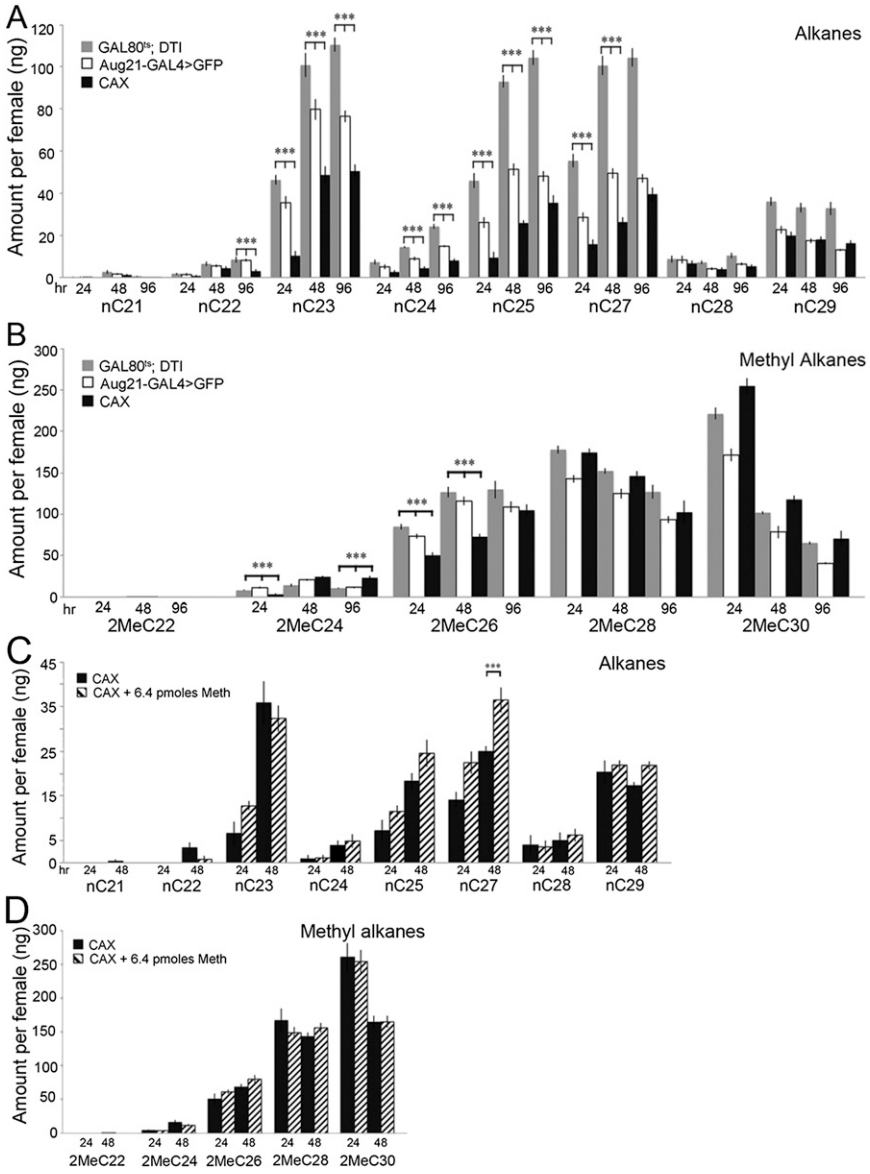


**Fig. S3.** Developmental time course of alkane and methyl alkane profiles of the CAX and the methoprene-treated CAX females. Cuticular hydrocarbons (CHCs) of CAX females and the parental controls were isolated and analyzed by gas chromatography with flame ionization detector (GC-FID) at 24, 48, and 96 h after eclosion. The amounts of alkanes and methyl alkanes are relative to the standards included in the hexane washes. (*A*) Straight -chain (*n*, *normal*) alkanes of the CAX and control females. C21, C22, etc. refer to the number of carbons. (*B*) Methyl alkanes (2MeC) of the CAX and control females. (*C*) Straight-chain (*n*) alkanes of the methoprene-treated CAX females at 24 and 48 h after eclosion. (*D*) Methyl alkanes (2MeC) of the methoprene-treated CAX females at 24 and 48 h after eclosion. The value for each CHC is represented as the average of 18 hexane-washed females ± SEM from two independent experiments (*A* and *B*). The value for each CHC is the average value from nine females ± SEM (*C* and *D*). Statistically significant values compared with both parental controls are shown with ***$P \leq 0.001$.

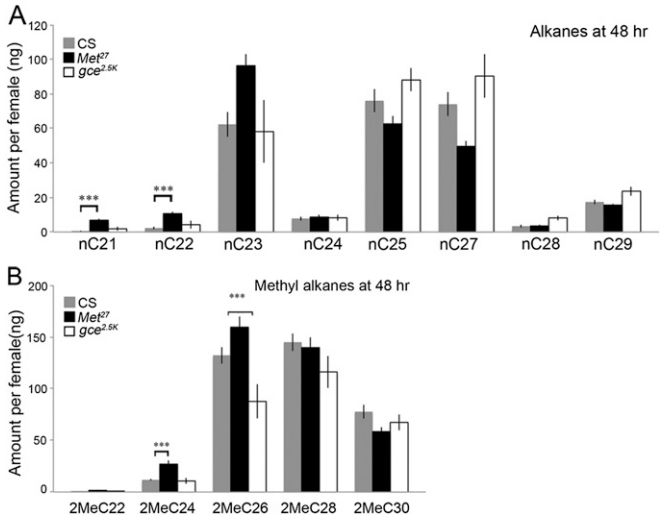


**Fig. S4.** The alkane and methyl alkane profiles of JH receptor mutant females, *Met*$^{27}$ and *gce*$^{2.5k}$. CHC profiles of *Met*$^{27}$ and *gce*$^{2.5k}$ homozygous females and CSA females were analyzed by GC-FID at 48 h after eclosion, and the amounts of alkanes and methyl alkanes were determined as in Fig. S3. (*A*) Straight-chain (*n*) alkanes. C21, C22, etc. refer to the number of carbons. (*B*) Methyl alkanes (2MeC). The value for each CHC is the average value from nine females ± SEM. Statistically significant values compared with wild-type females are shown with ***$P \leq 0.001$.