# Two cytochrome P450 epoxidases mediate juvenile hormone biosynthesis in *Drosophila melanogaster*

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**Abstract**

Juvenile hormones (JHs) mediate various biological processes such as development and reproduction in insects. Although pleiotropic functions of JHs are well investigated in the fruit fly *Drosophila melanogaster*, their biosynthetic mechanisms are less well understood, partly because many JH biosynthetic enzymes still remain unidentified in this important model species. Here we report that two cytochrome P450 (CYP) epoxidases mediate JH biosynthesis in *D. melanogaster*. In addition to previously reported Cyp6g2, a second epoxidase, Cyp6a13, also functions in the corpus allatum, the major JH biosynthetic endocrine gland. Combined mutations of the genes encoding these enzymes cause developmental and reproductive defects, which can be rescued by JH application. JH biosynthetic functions of these genes were further confirmed by using a heterologous expression system and *ex vivo* tissue culture. Collectively, our results indicate that these two CYP epoxidases function cooperatively to mediate JH biosynthesis in *D. melanogaster*.

37 **Highlights**

38 - *Drosophila* Cyp6g2 and Cyp6a13 are highly expressed in the corpus allatum.

39 - Combined knockout mutants of the CYP genes exhibit developmental and reproductive defects.

40 - Mutant phenotypes were rescued by feeding juvenile hormones.

41 - Both Cyp6g2 and Cyp6a13 catalyze epoxidation of juvenile hormone precursors.

42

43 Keywords

44 Juvenile hormone; *Drosophila melanogaster*; Metamorphosis; Cytochrome P450; Mass

45 spectrometry

46 **1. Introduction**

47 Juvenile hormones (JHs) are aliphatic sesquiterpenoids that regulate diverse biological
48 processes in insects, including development and reproduction (Jindra et al., 2013; Jindra and
49 Noriega, 2026). JH acts as a "status quo" hormone that maintains the juvenile form across many
50 insect orders during early postembryonic development (Riddiford, 2020), and a decline in the
51 hemolymph JH titer triggers metamorphosis (Jindra, 2019). Ablation of the corpora allata (CA),
52 the JH-producing glands, causes precocious metamorphosis, whereas ectopic application of JH
53 analogs such as methoprene induces supernumerary larval or nymphal molts in various species
54 (Konopova et al., 2011; Kayukawa et al., 2012). JH is thus considered as a key regulator that
55 prevents metamorphosis (Jindra et al., 2013). Intriguingly, JH is also produced at the onset of
56 metamorphosis in holometabolous insects (Jindra, 2019). Disruption of JH receptors causes
57 pupation defects (Konopova and Jindra, 2007; Abdou et al., 2011), suggesting that JH
58 contributes to the proper initiation of metamorphosis, potentially by preventing premature adult
59 differentiation (Martín et al., 2026).

60 The biosynthetic pathway of JH is well characterized and divided into early and late steps
61 (Bellés et al., 2005). In the early steps, known collectively as the mevalonate pathway, farnesyl
62 pyrophosphate (FPP) is synthesized. In late steps, FPP is first converted into farnesoic acid (FA)
63 by a phosphatase and reductases (Noriega, 2014). The sequence of the final JH biosynthetic steps
64 differs among insect taxa. In Lepidoptera, FA is epoxidized to JH acid (JHA) by a cytochrome
65 P450 (CYP) epoxidase and then methylated by JHA methyltransferase (Jhamt) to produce JH
66 (Daimon et al., 2012; Shinoda et al., 2003). In contrast, in most other insect orders, FA is first
67 methylated by Jhamt to form methyl farnesoate (MF), which is subsequently epoxidized to yield
68 JH (Helvig et al., 2004; Defelipe et al., 2011). Once synthesized, JH regulates gene expression in

69 peripheral tissues by interacting with its intracellular receptor Methoprene-tolerant (Met). In the
70 fruit fly *Drosophila melanogaster*, this function is shared by its paralog, germ-cell expressed
71 (gce) (Konopova et al., 2007; Jindra et al., 2015).

72 The molecular mechanisms of JH-mediated developmental regulation have been extensively
73 investigated in *D. melanogaster* using genetic tools such as the Gal4-UAS system. Although
74 genetically allatectomized *Drosophila* larvae (also referred to as CA-ablated or CAX larvae) do
75 not undergo precocious metamorphosis, they exhibit premature differentiation of some tissues,
76 such as the eye disks and fat body (Liu et al., 2009; Riddiford et al., 2010; Zhang et al., 2022).
77 Both CAX and JH receptor knockout larvae remain viable until pupariation, but they typically
78 die around the time of head eversion, failing to complete pupation (Riddiford et al., 2010; Abdou
79 et al., 2011). In adults, JH functions as a pleiotropic hormone. It acts as a gonadotropin by
80 promoting vitellogenesis in females and accessory gland protein synthesis in males (Wilson et
81 al., 2003; Santos et al., 2019). Recent studies further implicate JH in the regulation of courtship
82 behavior, sleep, and aging (Wijesekera et al., 2016; Wu et al., 2018; Yamamoto et al., 2013).

83 In contrast to the well-characterized downstream effects of JH signaling, the regulation of JH
84 synthesis remains relatively understudied in *D. melanogaster*. Notably, several enzymes
85 catalyzing reactions in the late steps have yet to be fully identified (Fig. 1). For example, mutants
86 lacking *jhamt* do not exhibit the pupal lethal phenotype observed in *Met* and *gce* double mutants
87 (Abdou et al., 2011; Wen et al., 2015), suggesting the existence of other methyltransferase(s) that
88 compensate for the loss of Jhamt. Moreover, a canonical CYP epoxidase is not conserved in
89 higher dipterans. A recent study identified Cyp6g2 as an epoxidase in *D. melanogaster* (Jia et al.,
90 2024). This enzyme synthesizes not only JH III, the predominant form of JH in insects, but also
91 JH III bisepoxide (JHB3), a higher dipteran-specific JH (Richard et al., 1989). However, the

existence of additional epoxidases remains plausible, as *Cyp6g2* disruption does not cause severe developmental defects as compared to JH receptor knockouts (Abdou et al. 2011; Jia et al. 2024).

In the present study, we investigated CYP genes that are highly expressed in the CA in *D. melanogaster* and identified a second epoxidase, Cyp6a13. Combined knockouts of *jhamt*, *Cyp6g2*, and *Cyp6a13* caused severe developmental defects similar to those observed in *Met* and *gce* double mutants. These mutants also showed reduced egg production, a phenotype commonly associated with JH deficiency. Furthermore, we confirmed the enzymatic functions of these genes using a heterologous expression system and *ex vivo* tissue culture. Collectively, our results indicate that Cyp6g2 and Cyp6a13 function cooperatively to mediate JH biosynthesis in *D. melanogaster*.

## 2. Materials and methods

### 2.1. Flies

Flies were raised at 25°C under a 12 h-light and 12 h-dark photocycle on a standard cornmeal diet (Fujinaga et al., 2025). The control strain $w^{1118}$ and transgenic flies were obtained from the Bloomington *Drosophila* Stock Center (BDSC), Vienna *Drosophila* Resource Center (VDRC), Tzumin Lee, Michael E. Adams, and Michael B. O'Connor, as listed in Table S1. Complementary DNA (cDNA) fragments of *jhamt*, *Cyp6g2*, and *Cyp6a13* were obtained from cDNA clones AT13581, IP03155, and LD25139, respectively, from the *Drosophila* Genomic Resource Center (DGRC) and cloned into the *pUAST* vector (Table S2) to generate UAS constructs. Transgenic flies were generated by BestGene Inc. Null mutant flies were generated by deleting most or all of the coding sequences of the target genes using the CRISPR-Cas9 system. Mutagenesis of *Met*, *gce*, and *Cyp6a13* was performed following the protocol developed by Kondo and Ueda (2013), with slight modifications as described in our previous study (Fujinaga et al., 2025). Briefly, two guide RNA (gRNA) target sequences for each genomic locus were inserted into *pBFv-U6.2* vectors using oligonucleotide pairs listed in Table S3, and a plasmid with two gRNA sequences was constructed for each gene. For the *Met* and *gce* mutant strains, embryonic injection of the plasmids and generation of G1 mutants were performed by BestGene Inc. For the *Cyp6a13* mutant strain, a transgenic strain carrying the plasmid at the *attP2* site was generated by BestGene Inc. The obtained *U6-gRNA* flies were crossed with *nos-Cas9* flies to induce mutagenesis in germ cells. Mutations were confirmed using primers designed outside the target sites (Table S3). Gene disruption mutants of *jhamt* and *Cyp6g2* were generated by WellGenetics Inc., using CRISPR-based homologous recombination to insert

125 fluorescent protein-coding sequences. All mutants were backcrossed with the $w^{1118}$ control strain
126 for at least four generations to minimize off-target effects and standardize genetic backgrounds.
127
128 **2.2. Cell lines**
129 Human embryonic kidney (HEK) 293 cells were obtained from Michael E. Adams. Cells
130 were maintained in 75 $cm^2$ flasks (VWR) in a humidified incubator at 37°C and 5% $CO_2$ in
131 Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 mg/mL glucose, L-glutamine, and
132 sodium pyruvate (Corning, #10-013-CV) containing 10% fetal bovine serum (Corning, #10082-
133 139) and 1% penicillin-streptomycin (Thermo Fisher Scientific, #15-140-122).
134
135 **2.3. Transcriptome analysis using public RNA sequencing data**
136 Public RNA sequencing data (GSE229077) were obtained from the Gene Expression
137 Omnibus (Roger et al., 2023). Reads were aligned to the *Drosophila* genome (BDGP6.46,
138 Ensembl) using Hisat2 (Kim et al., 2019). Aligned reads per gene were counted using SAMtools
139 (Danecek et al., 2021) and featureCount (Liao et al., 2014). Reads per kilobase per million
140 (RPKM) were calculated based on total reads and are listed in Table S4. Genes with expression
141 levels above 20 RPKM were considered to be highly expressed.
142
143 **2.4. Visualization of *jhamt-LexA* expression patterns**
144 Wandering larvae and three-day post-eclosion adult males expressing *jhamt-LexA*-driven
145 *LexAop-mCD8::GFP* were chilled on ice for 10 min. GFP expression patterns in the whole body
146 were observed using a SteREO Discovery.V12 microscope (Zeiss). Detailed expression patterns
147 of *jhamt-LexA* were visualized by immunostaining. Tissues were dissected in phosphate-buffered

148 saline (PBS) (Fisher Scientific, #BP399-1), fixed with 4% paraformaldehyde (PFA) (Electron
149 Microscopy Sciences, #157-8) in PBS containing 0.1% TritonX-100 (4% PFA/PBSTx) for 20
150 minutes at room temperature (RT), and washed multiple times with PBSTx. Tissues were
151 blocked with 5% normal goat serum (NGS) (Sigma-Aldrich, G9023) in PBSTx for at least one
152 hour at RT, incubated overnight at 4°C with chicken anti-GFP antibody (1:500, Abcam,
153 #ab13970) in PBSTx with 5% NGS, and then washed multiple times with PBSTx. They were
154 then incubated for two hours at RT with goat anti-chicken IgG conjugated to Alexa Fluor 488
155 (Life Technologies, #A-11039) in PBSTx with 5% NGS, followed by additional PBSTx washes.
156 DNA was stained with Hoechst 33342 (1:2,000, Life Technologies, #H3570) for 30 min at RT.
157 After washing, tissues were mounted in Vectashield H-1000 (Vector Laboratories, #H-1000-10)
158 and observed using a Zeiss Axio Imager M2 equipped with ApoTome.2 (Zeiss).
159

160 **2.5. Total RNA extraction and quantitative reverse transcription PCR**

161 Total RNA was extracted from tissues using TRIzol reagent (Thermo Fisher Scientific,
162 #15596018), and purified with the RNeasy Mini Kit (Qiagen, #74106) following the
163 manufacturers' instructions. cDNA was synthesized from purified RNA using PrimeScript RT
164 Master Mix (Takara Bio, #RR036A). Quantitative reverse transcription (qRT)-PCR was
165 performed on a CFX Connect Real-Time PCR Detection System (Bio-Rad) using AzuraView™
166 GreenFast qPCR Blue Mix LR (Azura Genomics, AZ-2320). Serial dilutions of plasmids
167 containing target sequences were used as standards for absolute quantification. Transcript levels
168 of mRNA were normalized to the internal control gene, *rp49*, as in Okamoto et al., 2018. Primers
169 are listed in Table S3.
170

171 **2.6. RNA probe synthesis**

172 Coding sequences of the target genes were PCR-amplified using T7- and SP6-tagged primers
173 listed in Table S3. Digoxigenin (DIG)-labelled RNA was synthesized using the DIG RNA
174 Labeling Kit (SP6/T7, Roche, #11175025910) following the manufacturer's protocol. Following
175 DNase treatment at 37°C for 15 min, labelled RNA was purified using the RNeasy Mini Kit.
176 RNA was hydrolyzed at 60°C for 45 min in alkaline solution (80 mM sodium bicarbonate, 120
177 mM sodium carbonate, and 10 mM DTT), and the reaction was terminated with neutralization
178 buffer (300 mM sodium acetate, 1% acetic acid, and 10 mM DTT). RNA probes were purified
179 by ethanol precipitation and stored at -20°C.

180

181 **2.7. Whole mount *in situ* hybridization**

182 Wandering larvae of $w^{1118}$ were cut in half in PBS, and posterior segments were flipped to
183 expose internal organs. Tissues were fixed in 4% PFA in PBS for 20 min, then washed three
184 times with PBS containing 0.2% Tween 20 (PBST). Tissues were then rinsed three times with
185 methanol. After washing with 90% methanol containing 50 mM EGTA, half the volume was
186 replaced to 4% PFA three times, followed by fixation in 4% PFA in PBS for 20 min. Tissues
187 were treated with 50 µg/mL Proteinase K (Roche, #3115828001) in PBST for 5 min, followed by
188 fixation in 4% PFA in PBS for 20 min. After washing with PBST, tissues were incubated in the
189 pre-hybridization buffer (50% formamide, saline sodium citrate, 100 µg/mL heparin, 100 µg/mL
190 yeast RNA, 10 mM DTT, and 0.1% Tween 20) at 60°C for one hour. RNA probes (200 ng/mL
191 for *jhamt* and *Cyp6g2*, 1,000 ng/mL for *Cyp6a13*) were denatured in the hybridization solution
192 (10% dextran sulfate-containing pre-hybridization solution) at 80°C for 15 min, followed by
193 chilling on ice for 5 min. The RNA probe solution was then applied to tissues for overnight

194 incubation at 60°C. Tissues were washed six times for 30 min with the washing solution (50%
195 formamide, saline sodium citrate, and 0.1% Tween 20), then blocked with the blocking solution
196 (2% bovine serum albumin in PBST) for 30 min. Alkaline phosphatase-conjugated anti-DIG
197 antibody (Roche, #11093274910) diluted in the blocking solution (1:3,000) was applied
198 overnight at 4°C. Following three 20-min PBST washes, tissues were incubated in the alkaline
199 phosphatase buffer (100 mM Tris-hydrochloride, 100 mM sodium chloride, 50 mM magnesium
200 chloride, and 0.1% Tween 20, pH 9.5), followed by colorimetric development with nitro blue
201 tetrazolium (Roche, #11383213001) and 5-bromo-4-chloro-3-indolyl phosphate (Roche,
202 #11383221001) for one hour. After washing, tissues were permeated with glycerol, and the
203 central nervous system-ring gland (RG) complexes were dissected and imaged using a SteREO
204 Discovery.V12 microscope (Zeiss) and an Olympus BX53 microscope with a DP26 CCD
205 camera.
206
207 **2.8. Scoring of lethal stages**
208 Eggs of each genotype were collected on a grape juice plate with yeast powder. Larvae were
209 collected within two hours after hatching and reared on the standard cornmeal diet until 48 hours
210 after hatching. Homozygous mutants were selected based on the absence of the balancer
211 chromosome carrying GFP or RFP, followed by separation of males and females. Larvae were
212 then transferred to new vials (8-25 larvae/vial) containing the standard cornmeal diet with or
213 without specific concentrations of FA (Echeron Biosciences, #S-0151), MF (Echeron
214 Biosciences, #S-0153), JH III (Echeron Biosciences, #S-0155), or JHB3 (Echeron Biosciences,
215 #S-0157). Dead larvae and prepupae were counted six days after hatching, after which the size of
216 the pupal cases was measured. Animals that underwent head eversion were scored as pupae.

217 Dead pupae, early lethal adults, and normal adults were scored 10 days after hatching. The
218 developmental lethal phenotype was scored as follows: larval lethal = 0, prepupal lethal = 1,
219 pupal lethal = 2, early adult lethal = 3, and normal development = 4. The average score of each
220 genotype was calculated as the developmental score, and it was plotted along with the *Kruppel*
221 *homolog 1* (*Kr-h1*) expression level or the pupal case size for various genotypes using R. The
222 correlation coefficient and *p*-value (Pearson's test) for each plot were calculated using R.
223

224 **2.9. Fecundity assay**

225 Newly hatched larvae of selected genotypes were raised on the cornmeal diet until 48 hours
226 after hatching. Larvae were then transferred to vials at a density of fewer than 40 larvae per 3 g
227 of the diet and kept until eclosion. Five virgin females of each genotype were collected within
228 six hours after eclosion and kept in the vials containing the cornmeal diet with or without 0.5
229 ppm MF or JH III. Twenty-four hours later, three $w^{1118}$ males were added for mating. After
230 another 24 hours, flies were transferred to vials containing agarose-solidified grape juice with
231 yeast powder. Flies were transferred into fresh vials every 24 hours, and the number of eggs in
232 each vial was counted under a stereomicroscope.
233

234 **2.10. Enzymatic conversion assay in HEK293 cells**

235 Coding sequences of *jhamt*, *Cyp6g2*, and *Cyp6a13* for heterologous expression in HEK293
236 cells were obtained from cDNA clones AT13581, IP03155, and LD25139, respectively, as listed
237 in Table S2, and cloned into the *pcDNA3.1(+)* vector. HEK293 cells at a density of 2 x $10^5$ cells
238 in 2 mL of the culture medium were seeded on a 6-well clear flat bottom microplate (Bioland
239 Scientific, #703003). After preincubation for 24 hours, 0.3 µg of the *jhamt*-encoding plasmid and

240 0.9 μg of CYP enzyme-encoding plasmids were transfected using Attractene transfection reagent
241 (Qiagen, #301005) according to the manufacture's instruction. The empty vector was transfected
242 for control experiments. After 48 hours of incubation, $5 \times 10^5$ cells were transferred into glass
243 vials (Agilent, #5183-2068) containing S-adenosyl methionine (Millipore, #A7007; final
244 concentration: 50 μM) and the esterase inhibitor Paraoxon (Fisher Scientific, #NC0811417; final
245 concentration: 1 μM) in 720 μL of the FBS-free medium. Three hours after incubation, 7.2 μL of
246 1 mM FA was added, and the cells were further incubated for 18 hours. After mixing with 80 μL
247 of acetonitrile, vials were stored at -20°C until extraction.
248

249 **2.11. *Ex vivo* ring gland culture**

250 Ten RGs of selected genotypes were dissected from wandering larvae and preincubated in
251 720 μL of Schneider's *Drosophila* medium (Gibco, #21-720-024) in a glass vial for 30 min.
252 After adding 7.2 μL of 1 mM FA to the culture medium, the RGs were incubated for four hours
253 at 25°C. After incubation, 80 μL of acetonitrile was added, and the samples were stored at -20°C
254 until extraction.
255

256 **2.12. Hemolymph collection**

257 Wandering third instar larvae were rinsed in PBS and dried on tissue paper. The cuticle was
258 carefully torn to release the hemolymph onto a parafilm membrane. Ten μL of the hemolymph
259 was collected and mixed with extraction solution (90 μl of 0.9% sodium chloride solution and 10
260 μL of acetonitrile) in a glass insert vial (Agilent, #5181-1270).
261

262 **2.13. Sesquiterpenoid extraction**

263 Extraction of sesquiterpenoids was performed based on the previous study (Sarro et al., 2021,
264 adapted from Kai et al., 2018) with modifications. For hemolymph samples, 2 ng of citronellol
265 (Millipore, #W230915) or 4 ng of deuterium-labelled JH III (JH III-d3) (LGC Ltd., #E589402)
266 was added as an internal standard for gas chromatography-mass spectrometry (GC-MS) analysis
267 or liquid chromatography-mass spectrometry (LC-MS) analysis, respectively. After 100 µL of
268 hexane was added, the samples were vigorously mixed for 30 seconds. The samples were then
269 allowed to sit for 5 min, followed by centrifugation at 1,000 x g for 5 min. The hexane layer was
270 transferred to a new glass vial using a Hamilton syringe (Hamilton, #80600), and the extraction
271 was repeated. For LC-MS analysis, the pooled hexane extract was concentrated by CentriVap
272 concentrator (Labconco) to 100 µL. After adding 100 µL of methanol, the remaining hexane was
273 evaporated. For extraction from the culture medium, 16 ng of citronellol or 4 ng of JH III-d3 was
274 added. After adding 800 µL of hexane, the samples were vigorously mixed for 30 seconds. The
275 hexane layer was then collected as described above.
276
277 **2.14. Sesquiterpenoid determination**
278 For developing the simultaneous extraction method, FA, MF, JH III, and JHB3 (100 ng each)
279 were added to the extraction solution (400 or 720 µl of 0.9% sodium chloride solution and 400 or
280 80 µl of acetonitrile) in the glass vial. Compounds were extracted as described above, and the
281 hexane extract was concentrated to 100 µl using CentriVap concentrator (Labconco). After
282 adding 100 µl of methanol, the remaining hexane was evaporated. Extracted sesquiterpenoids
283 were analyzed using a 1220 Infinity LC system (Agilent Technologies) with a Vydac 218TP C18
284 HPLC column (100 mm x 4.6 mm, 5 µm particle size, Grace). HPLC-grade acetonitrile and
285 water were used as the liquid phase in a binary gradient flow (Pump A: 20% acetonitrile/80%

286 water, Pump B: 100% acetonitrile). Ten µl of the extracted sample or authentic standards was
287 injected, and absorbance at 217 nm was recorded for peak area analyses.
288 For biological samples, MF and JH III were detected and quantified using a Thermo
289 Scientific Trace 1310 GC-MS equipped with an AI 1310 autosampler and a TSQ Duo triple
290 quadrupole mass spectrometer (Thermo Scientific), following the method described previously
291 (Sarro et al., 2021) with slight modifications. Detailed parameters for selected reaction
292 monitoring were listed in Table S5. Authentic standards of MF and JH III were used to generate
293 standard curves. Peak areas of the target compounds were normalized by the peak area of
294 citronellol (10 ng/mL). JHB3 levels were determined using LC-MS at the UC Riverside
295 Metabolomics Core based on the previous study (Ramirez et al., 2020) with modifications.
296 Liquid chromatography separation was performed by an ultra-performance liquid
297 chromatography system (ACQUITY UPLC I-Class PLUS, Waters) equipped with an ACQUITY
298 UPLC BEH C18 column (2.1 × 100 mm, 1.7 µm, Waters). The column temperature was
299 maintained at 35°C. Gradient separation was performed between 0.1% formic acid in water
300 (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) at a flow rate of 0.3
301 mL/min. The gradient program was as follows: 4% B at 0 min; increase to 10% B in 2 min;
302 increase to 80% B in 12 min and hold for 0.1 min; increase again to 100% B in 0.9 min and hold
303 for another 0.1 min; then return to 4% B in 1.4 min; for a total run time of 16.5 min. Detection
304 was performed using a Xevo TQ-XS mass spectrometry system (Waters) equipped with an
305 electrospray ionization interface. The instrument was operated in multiple reaction monitoring
306 mode as described in Table S6. Authentic standards of FA, JH III, and JHB3 were used to
307 generate standard curves. Peak areas were normalized to the peak area of JH III-d3 (40 ng/mL).
308

309 **2.15. Phylogenetic analysis**

310 The unrooted maximum-likelihood phylogenetic tree was generated using MEGAX (Kumar
311 et al., 2018). Amino acid sequences of CYP 3 clan in *D. melanogaster* were selected using
312 Flybase (Öztürk-Çolak et al., 2024). Entire amino acid sequences of the enzymes in the housefly
313 (*Musca domestica*), yellow fever mosquito (*Aedes aegypti*), silkmoth (*Bombyx mori*), western
314 honey bee (*Apis mellifera*), red flour beetle (*Tribolium castaneum*), pea aphid (*Acyrthosiphon*
315 *pisum*), and Nevada termite (*Zootermopsis nevadensis*) were obtained from the National Center
316 for Biotechnology Information database (Sayers et al., 2022) using full length amino acid
317 sequences of *D. melanogaster* proteins as queries. The protein names and GenBank accession
318 numbers are listed in Table S7.

319

320 **3. Results**

321 **3.1. Identification of CYPs highly expressed in the CA during early metamorphosis**

322 We first investigated CYP enzymes that are highly expressed in the CA. In *D. melanogaster*,
323 the CA is located within the RG, a composite endocrine organ (Zhang et al., 2022). We therefore
324 analyzed previously published RNA sequencing data of the RGs from larvae at the wandering
325 stage (Roger et al., 2023), when JH titers are known to be high (Sliter et al., 1987). We identified
326 12 CYP genes highly expressed in the RG (Table S4), including four ecdysteroid biosynthetic
327 enzymes that are highly expressed in the prothoracic gland (Niwa and Niwa, 2026), which also
328 constitutes the RG.

329 We next compared the expression levels of these CYP genes in the RG using qRT-PCR
330 between control and CAX larvae. As the *Gal4* drivers previously used to induce CAX are also
331 expressed in the salivary glands (Liu et al., 2009; Riddiford et al., 2010), we used a more CA-
332 specific driver, *jhamt-LexA* (Ohhara et al., 2018; Fig. S1A), to ablate the CA by expressing pro-
333 apoptotic genes (Ren et al., 2018; Fig. S1B). Among these genes, *Cyp6g2* and *Cyp6a13* showed
334 high expression in the RG of control larvae as compared to CAX larvae (Fig. 2A), indicating that
335 these genes are highly expressed in the CA, similar to *jhamt*. Absolute quantification of the gene
336 expression revealed that *Cyp6g2* is expressed at a level more than 20 times higher than that of
337 *Cyp6a13* in the control RG (Fig. S2). *In situ* hybridization further confirmed the expression of
338 these genes in the CA, although the signal of the *Cyp6a13* antisense probe was weaker than that
339 of *jhamt* or *Cyp6g2* due to its lower expression in the CA (Fig. 2B).

340

341 **3.2. Combined mutations of the enzymes cause metamorphic defects similar to JH receptor**
342 **mutants**

343 We next generated null mutant alleles (Fig. S3) and analyzed the mutant flies to investigate
344 contributions of these enzymes to *Drosophila* development. We also used our newly generated
345 *Met*, *gce* double mutant line ($Met^{45B}$, $gce^{2Cl}$) as a JH signaling-deficient control throughout this
346 study, in order to exclude the potential effects of the intact coding sequence of the $Met^{27}$ allele
347 (Wilson and Ashok, 1998) used in the previously reported *Met*, *gce* double mutant (Abdou et al.,
348 2011).

349 Consistent with previous studies on JH signaling-deficient flies (Liu et al., 2009; Riddiford et
350 al., 2010; Abdou et al., 2011), our new CAX flies died at different stages of metamorphosis (Fig.
351 3A). Based on this, we observed and classified developmental lethality of the mutant flies into
352 the following five categories: larval lethal, prepupal lethal (PPL; characterized by incomplete air
353 bubble translocation and/or head eversion), pupal lethal (PL), early adult lethal (EAL; defined as
354 death within 24 hours after eclosion without wing expansion), and normal development. While
355 almost all control flies ($w^{1118}$) developed normally (Fig. 3B), most *Met*, *gce* double mutant flies
356 died during pupation, with some showing the pupal lethality. Many CAX flies reached the pupal
357 stage but eventually died during adult development or shortly after eclosion. *jhamt* single mutant
358 flies showed mild phenotypes, as previously reported (Wen et al., 2015; Jia et al., 2024); most of
359 these flies became adults, although about half of them died shortly after eclosion. Single mutants
360 of *Cyp6g2* or *Cyp6a13* did not exhibit significant developmental defects, whereas combining
361 them with the *jhamt* mutation showed much more severe phenotype, similar to *Met*, *gce* double
362 mutants. Most *jhamt*, *Cyp6g2* double mutant flies died during or after pupation. *jhamt*, *Cyp6a13*
363 double mutants showed slightly milder phenotypes than *jhamt*, *Cyp6g2* double mutants, although
364 they were still significantly more severe than *jhamt* single mutant flies. *Cyp6g2*, *Cyp6a13* double
365 mutants also showed mild lethal phenotypes. *jhamt*, *Cyp6g2*, *Cyp6a13* triple mutants showed

366 severe phenotypes similar to *jhamt*, *Cyp6g2* double mutants. Altogether, these findings suggest
367 that Jhamt, Cyp6g2, and Cyp6a13 function as JH synthetic enzymes and may have overlapping
368 functions, as single mutants exhibited only mild phenotypes. Moreover, tissue specific
369 knockdown using a *Gal4* driver expressed in the CA also suggested the JH synthetic activity of
370 these enzymes in the CA (Fig. 3C). Single knockdowns of the CYPs did not cause significant
371 developmental lethality. However, combined knockdown of *jhamt* and *Cyp6g2* or *jhamt* and
372 *Cyp6a13* induced severe developmental defects, similar to the corresponding mutant
373 combinations. Furthermore, the severity of developmental phenotypes of CAX and the mutant
374 flies correlated with expression levels of a JH response gene, *Kr-h1* (Fig. 3D). Flies with mild
375 developmental phenotypes (CAX; $jhamt^{-}$; and $Cyp6g2^{-}$, $Cyp6a13^{-}$) exhibited moderate reduction
376 of *Kr-h1* expression compared to $w^{1118}$ control, while those with more severe phenotypes
377 ($Met^{45B}$, $gce^{2C1}$; $jhamt^{-}$, $Cyp6g2^{-}$; $jhamt^{-}$, $Cyp6a13^{-}$; and $jhamt^{-}$, $Cyp6g2^{-}$, $Cyp6a13^{-}$) showed
378 further reduction of *Kr-h1* expression levels. This strong correlation was confirmed by the
379 correlation analysis (Fig. 3E), further suggesting the involvement of these enzymes in JH
380 biosynthesis.

381 We also analyzed the pupal body size of CAX and the mutant flies (Fig. S4A). Previous
382 studies have shown that JH-deficient flies exhibit a smaller body size along with slight
383 developmental delay (Riddiford et al., 2010; Mirth et al., 2014). Consistent with this, *Met*, *gce*
384 double mutant, CAX, *jhamt*, *Cyp6g2* double mutant, and *jhamt*, *Cyp6g2*, *Cyp6a13* triple mutant
385 flies were significantly smaller than $w^{1118}$ control flies in both sexes. Indeed, correlation analyses
386 confirmed a strong correlation between developmental lethality and pupal body size reduction
387 (Fig. S4B and C). In contrast, pupariation timing was not significantly altered, although mutants
388 with high lethality tended to exhibit slightly delayed pupariation timing (Fig. S4D).

389

390 **3.3. Sesquiterpenoid feeding rescues developmental defects of the enzyme mutants**

391 In order to confirm that developmental defects of CAX flies and the mutants were caused by
392 impaired JH biosynthesis, we applied JHs and their precursors to the third instar larvae (Fig. 4A-
393 H). Control flies showed no developmental defects when treated with the sesquiterpenoids (Fig.
394 4A). As expected, no rescue was observed in *Met*, *gce* double mutants (Fig. 4B), as they lack the
395 molecular machinery to respond to JH. In contrast, developmental defects of CAX flies were
396 rescued by all terpenoids tested, particularly MF, JH III, and JHB3 (Fig. 4C). These control
397 experiments demonstrate that exogenous sesquiterpenoid application can successfully
398 compensate for endogenous JH functions without any harmful side effects during development.
399 It should be noted that MF, like JH III and JHB3, can also activate JH receptors, although its
400 activity is weaker than JH III and JHB3 (Jindra et al., 2015). The enzyme mutants were also
401 rescued by MF, JH III, and JHB3 in a dose-dependent manner (Fig. 4D-H), suggesting that the
402 lethality observed in the mutant flies was indeed due to reduced JH production. Conversely,
403 application of the JH precursor, FA, hardly rescued the enzyme mutants, suggesting that these
404 enzymes function downstream of FA in the JH biosynthetic pathway (Fig. 1). Interestingly,
405 significant rescue of the developmental lethality in CAX flies was observed when the higher
406 concentration (5 ppm) of FA was applied, potentially suggesting the existence of an alternative
407 JH biosynthetic machinery outside the CA.

408 We also performed rescue experiments of *jhamt*, *Cyp6g2* and *jhamt*, *Cyp6a13* double
409 mutants by overexpressing each enzyme in the CA (Fig. 4I and J). Consistent with their
410 functions in the CA, overexpression of the enzymes in their corresponding mutants significantly
411 rescued the developmental defects. Importantly, *Cyp6g2* overexpression significantly rescued

412 *jhamt*, *Cyp6a13* double mutants, whereas *Cyp6a13* overexpression did not rescue *jhamt*, *Cyp6g2*
413 double mutants. These results suggest that Cyp6g2 can compensate for Cyp6a13 in JH
414 biosynthesis in the CA, whereas Cyp6a13 plays a more limited role.
415
416 **3.4. Contribution of the enzymes to reproduction**
417 During metamorphosis, the CA and corpora cardiaca (CC) complex migrates toward the
418 ventral ganglion and eventually settles on the esophagus in *D. melanogaster* (Dai and Gilbert
419 1991; Fig. S1A). Although *Cyp6a13* expression levels were significantly lower than the other
420 enzymes (~1.5% compared to *jhamt* expression), transcripts of all the three enzyme-encoding
421 genes were detectable in the adult CA/CC complex (Fig. 5A-C). Among the three enzyme-
422 encoding genes, *jhamt* expression increased within 24 hours after eclosion, potentially reflecting
423 the elevated JH III titer after eclosion (Reiff et al., 2015; Sugime et al., 2017).
424 Given the functions of JHs as gonadotropic factors in adult insects including *D.*
425 *melanogaster* (Marchal et al., 2014; Roy et al., 2018; Santos et al., 2019; Smykal et al., 2014;
426 Song et al., 2014), we next measured the number of eggs laid by the mutant flies to assess the
427 contributions of these enzymes to reproduction (Fig. 5D). *jhamt* single mutant and *Cyp6g2*,
428 *Cyp6a13* double mutant females laid significantly fewer eggs compared to $w^{1118}$ control (Fig.
429 5E). On the other hand, neither *Cyp6g2* nor *Cyp6a13* single mutants displayed statistically
430 significant reduction of the number of laid eggs. These results suggest that Cyp6g2 and Cyp6a13
431 may compensate for each other in JH production in adult females. Treatment with MF or JH III
432 after eclosion significantly increased the fecundity of females of all genotypes, further
433 suggesting the importance of JH signaling in reproduction in female flies.
434

435 **3.5. Cyp6g2 and Cyp6a13 are responsible for epoxidation of JH precursors**

436 To further investigate enzymatic activities *in vitro*, we modified the sesquiterpenoid
437 extraction described in Sarro et al. (2021) to enable simultaneous extraction of MF, JH III, and
438 JHB3 for GC-MS and LC-MS analyses. The modified hexane extraction method recovered
439 nearly 100% of FA, MF, JH III, and JHB3 (Fig. S5). Using this method, we conducted an
440 enzymatic conversion assay in HEK293 cells (Fig. 6A). Overexpression of *jhamt* induced
441 methylation of FA as expected from previous studies (Niwa et al., 2008), while its co-expression
442 with either *Cyp6g2* or *Cyp6a13* did not enhance MF production (Fig. 6B). In contrast, co-
443 expression of *Cyp6g2* with *jhamt* enhanced conversion of FA into JH III beyond the basal level
444 of JH III production observed in *jhamt* single overexpression (Fig. 6C), consistent with the
445 reported epoxidase activity of Cyp6g2 (Jia et al., 2024). Importantly, co-expression of *Cyp6a13*
446 with *jhamt* also promoted conversion of FA into JH III (Fig. 6C), suggesting that it encodes an
447 epoxidase similar to Cyp6g2.

448 Contrary to our expectations, JHB3 was not detected under any condition in the enzymatic
449 conversion assay in HEK293 cells. We speculate that an unknown cofactor present in the CA
450 may be necessary for JHB3 biosynthesis *in vivo*. We therefore conducted *ex vivo* culture of the
451 larval RGs to assess enzymatic activity of CYPs in the CA (Fig. 6D). JH III and JHB3 were
452 detected in the RGs from control larvae cultured with FA (Fig. 6E and F), confirming the
453 methylation and epoxidation activities in the cultured RGs. In the *Cyp6g2* mutant RG, JH III
454 production was significantly decreased (Fig. 6E), and JHB3 production was below the detection
455 limit (Fig. 6F). These results indicate that Cyp6g2 acts as an epoxidase that can produce both JH
456 III and JHB3. Although the RGs from the *Cyp6a13* single mutant larvae did not show
457 statistically significant reduction of JH III or JHB3 production, JH III was not produced in the

458 cultured RGs from the *Cyp6g2*, *Cyp6a13* double mutant larvae (Fig. 6E). This further suggests
459 that Cyp6a13 epoxidizes JH precursors to synthesize JH III.
460 Lastly, hemolymph JH levels were analyzed to confirm the JH biosynthetic activities of the
461 enzymes *in vivo*. In the hemolymph of wandering larvae, we could only detect JHB3, as the
462 reported amounts of larval JH III are far below our detection limit (~5 pg/μL hemolymph; Jones
463 et al., 2013; Wen et al., 2015; Barton et al., 2024). Compared to control, the hemolymph level of
464 JHB3 was significantly reduced in the *Cyp6a13* single mutant larvae, and it was not detected in
465 CAX larvae and the other mutants tested (Fig. 6G). Overall, our results indicate that both
466 Cyp6g2 and Cyp6a13 function as epoxidases in the CA to produce JHs *in vivo*.
467

468 **4. Discussion**

469 In the present study, we showed that two CYP enzymes, Cyp6g2 and Cyp6a13, likely
470 function as epoxidases for JH biosynthesis in the *Drosophila* CA. Higher dipteran insects, known
471 as *Cyclorrhapha*, have lost the canonical sesquiterpenoid epoxidase CYP15 in their genomes
472 (Dermauw et al., 2020; Smykal and Dolezel, 2023). Instead, they employ CYP6 enzymes as
473 epoxidases: for example, Cyp6a1 has been identified as an MF epoxidase in the housefly *Musca*
474 *domestica* (Andersen et al., 1997). A recent study showed that Cyp6g2 is a major epoxidase for
475 JH production in *D. melanogaster* (Jia et al., 2024). The present study now adds *Drosophila*
476 Cyp6a13 to the list of CYP6 epoxidases. Considering that the RGs isolated from wandering
477 *Cyp6g2*, *Cyp6a13* double mutant larvae did not produce detectable levels of JHs (Fig. 6E and F),
478 it is likely that Cyp6g2 and Cyp6a13 are the only epoxidases that contribute to JH production in
479 the *Drosophila* CA, at least during the wandering stage.

480 The relatively mild developmental defects seen in *Cyp6g2*, *Cyp6a13* double mutants are
481 likely due to the weak but significant JH receptor-activating capacity of MF (Jindra et al., 2015;
482 Wen et al., 2015). Consistent with this, previous reports suggest that MF is circulating in the
483 *Drosophila* hemolymph during development (Jones et al., 2013; Wen et al., 2015). Further
484 studies on this JH-like function of MF are required to evaluate the significance of epoxidation in
485 JH biosynthesis in *Drosophila*.

486 JHB3 is a unique JH species in higher dipteran insects that have lost CYP15 epoxidases
487 (Smykal and Dolezel, 2023). It is therefore likely that JHB3 is a specific product of CYP6
488 epoxidases. Although we failed to detect JHB3 production by the CYP6 enzymes in HEK293
489 cells, this result must be interpreted with caution, as human cells may lack specific components
490 (such as cofactors) required for full CYP6 activities. Consistent with this, although we detected

491 JH III biosynthetic activities of the CYP6 enzymes in this assay, less than 1% of MF produced
492 by JHAMT was converted to JH III (Fig. 6B and C), further highlighting the limitation of this
493 heterologous expression system.

494 Given that the *Cyp6g2* mutant RGs do not synthesize detectable amounts of JHB3 *ex vivo*,
495 we infer that Cyp6g2 is the only epoxidase that can synthesize JHB3 in *D. melanogaster*.
496 Although the hemolymph level of JHB3 was drastically reduced in *Cyp6a13* single mutant
497 larvae, this may reflect reduced production of JH III, the precursor of JHB3. Indeed, the small
498 amount of JH III produced by the *Cyp6g2* mutant RGs disappears in *Cyp6g2*, *Cyp6a13* double
499 mutant RG cultures, suggesting that Cyp6a13 is at least involved in JH III production in the RG.
500 The inferred functions of the CYP6 enzymes are summarized in Fig. 6H.

501 In *D. melanogaster*, it is known that the hemolymph JHB3 titer is much higher than that of
502 JH III (Wen et al., 2015; Ramirez et al., 2020). This is consistent with our hemolymph JH
503 analysis, in which we only detected JHB3. It is therefore interesting that comparable amounts of
504 JH III and JHB3 were produced in the RGs cultured *ex vivo*. A potential explanation for this is
505 that JH-degrading enzymes such as JH esterases and JH epoxide hydrolases (Kamita and
506 Hammock, 2010; Siegel et al., 2025) may preferentially degrade JH III over JHB3. Although a
507 previous *in vitro* study showed that *Drosophila* JH esterase has similar affinity for both JH III
508 and JHB3 (Campbell et al., 1998), another study suggested that high concentrations of JH
509 esterase preferentially degrades JH III when bound to its binding proteins (Touhara et al., 1995).
510 Since JHB3 does not bind to lipophorin, a major JH-binding protein (Trowell et al., 1994), JH
511 esterase may be less effective at degrading JHB3 as compared to JH III. Elucidating molecular
512 mechanisms behind the high titer of JHB3 in the *Drosophila* hemolymph may help us better
513 understand the physiological significance of this higher dipteran-specific JH species.

514 *D. melanogaster* Cyp6g2 and Cyp6a13, as well as *M. domestica* Cyp6a1, are distributed
515 within the insect CYP3 clan. Although *Drosophila* Cyp6a13 and *Musca* Cyp6a1 are located
516 within the same cluster, CYP3 enzymes of these two *Cyclorrhapha* species are greatly expanded
517 within this subclade (Fig. S6). Taken together, these observations suggest that their JH
518 biosynthetic functions evolved independently. Enzymes in the CYP3 clan (such as Cyp6 and
519 Cyp9 enzymes) are highly divergent in insects, and many are known to have xenobiotic-
520 detoxifying activities (Nauen et al., 2022). *Drosophila* Cyp6g2 and *Musca* Cyp6a1 are also
521 known for their detoxification roles (Feyereisen, 1999; Giang et al., 2025). Many other CYP3
522 clan enzymes catalyze epoxidation of their target compounds as well (Feyereisen, 1999). It is
523 therefore possible that other enzymes in this clan are also capable of epoxidizing
524 sesquiterpenoids, but only those expressed in the CA have obtained JH biosynthetic functions.
525 We could not find any other CYP enzymes specifically expressed in the CA, suggesting that
526 other *Drosophila* enzymes do not contribute to JH biosynthesis *in vivo*, at least during the
527 wandering stage. In contrast, it remains possible that other CYPs function as JH synthases in
528 peripheral tissues, as some studies suggest that JH biosynthesis occurs in other organs, such as
529 the adult gut (Rahman et al., 2017; Shianiou et al., 2023). These studies showed that *jhamt*
530 expression in the gut is responsible for maintaining cellular growth. Notably, *Cyp6a13* is
531 expressed not only in the CA, but also in peripheral tissues, according to Flybase expression data
532 (Öztürk-Çolak et al., 2024). It is therefore possible that Cyp6a13 acts with Jhamt for JH
533 biosynthesis in peripheral tissues.
534 In summary, our current work revealed that two CYP6 enzymes, Cyp6g2 and Cyp6a13,
535 function cooperatively to synthesize JH in the *Drosophila* CA. Further investigations of their
536 differential functions, along with identification of additional JH biosynthetic enzymes, are

537 expected to help us better understand both species-specific and conserved features of JH

538 signaling in insects.

539

540 **Conflict of interest**

541 The authors declare no competing interest.

542

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554

555 **Author contributions**

556 DF: conceptualization, methodology, investigation, formal analysis, writing – original draft,
557 writing – review and editing. YO: methodology, investigation, funding acquisition, writing –
558 review and editing. NO: investigation, funding acquisition, writing – review and editing. HC:
559 generation of *Met*, *gce* mutant flies, writing – review and editing. KM: methodology for
560 sesquiterpenoid determination, writing – review and editing. NY: conceptualization, funding
561 acquisition, project administration, supervision, writing – review and editing.

562

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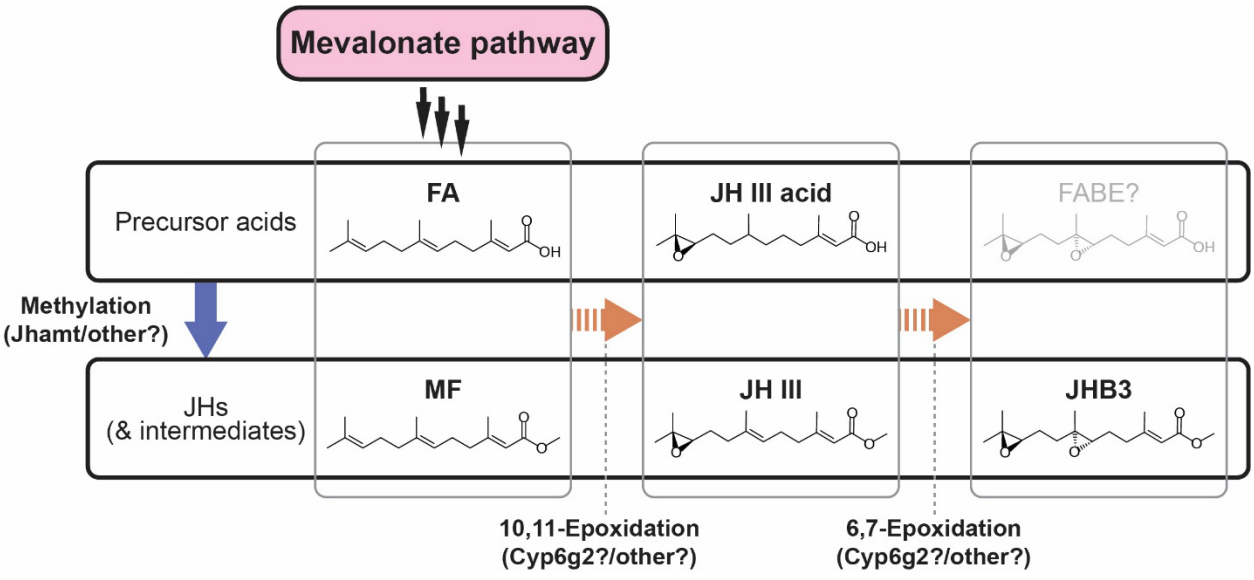


**Figure 1. Last steps of the JH biosynthetic pathway in *Drosophila melanogaster*.** Farnesoic acid (FA) is converted into juvenile hormone III (JH III) and JH-bisepoxide (JHB3) in the corpus allatum. Cyp6g2 predominantly epoxidizes FA into JH III acid, rather than methyl farnesoate (MF) into JH III (Jia et al., 2024). Jhamt converts both FA and JH III acid into MF and JH III, respectively (Niwa et al., 2008). Although FA bisepoxide (FABE) has been hypothesized as an intermediate of JHB3 synthesis (Moshitzky and Applebaum, 1995), it has not been detected in *D. melanogaster*.

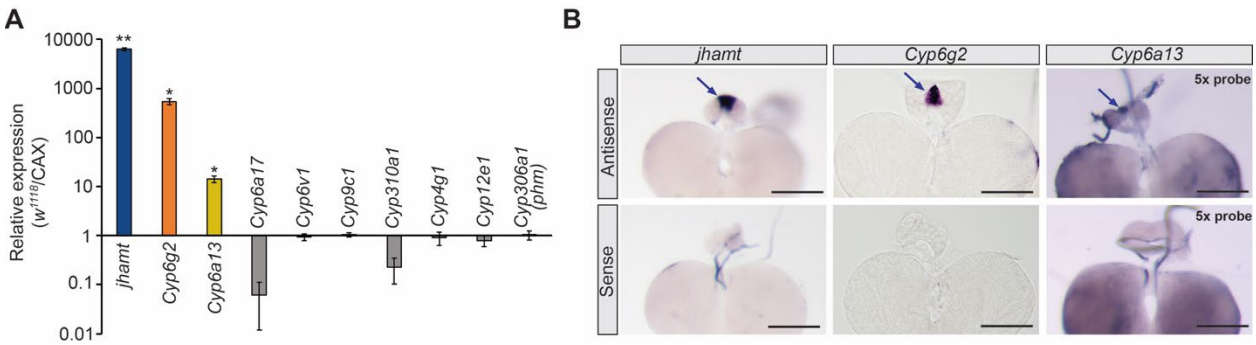


**Figure 2. Cytochrome P450 genes highly expressed in the larval CA.**

(**A**) Relative gene expression levels in the ring gland (RG) of control ($w^{1118}$) as compared to corpus allatum (CA)-ablated (CAX) larvae. *jhamt* and cytochrome P450 genes highly expressed in the RG of wandering larvae were quantified. High relative expression levels indicate CA-specific expression. Expression levels were normalized to a reference gene, *rp49*, in the same cDNA samples and shown on a log scale. Values are the means ± standard error ($n$ = 3). *$p$ < 0.05, **$p$ < 0.01 (Student's $t$-test). (**B**) *In situ* hybridization of *jhamt*, *Cyp6g2*, and *Cyp6a13* in the larval RG. Due to the lower expression of *Cyp6a13*, five times more RNA probe was used. The CA is indicated by arrows. Scale bars: 100 µm.

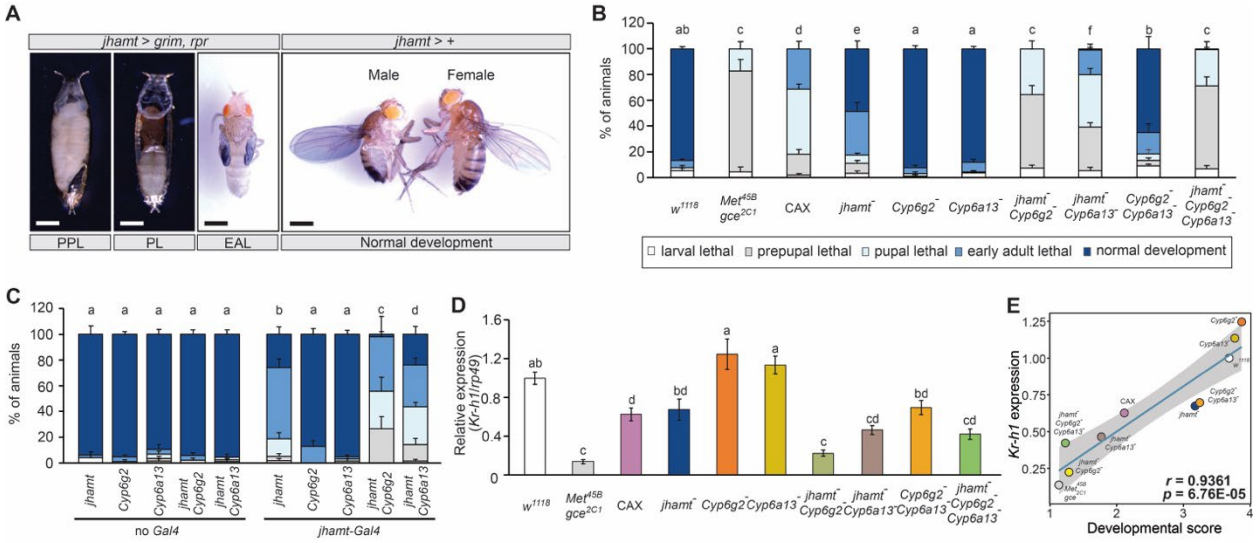


**Figure 3. Lethal phenotypes of CA-ablated flies and JH-related mutants.**

(**A**) Representative images of the lethal phenotypes of corpus allatum (CA)-ablated (CAX) flies induced by *jhamt-LexA*-driven expression of *grim* and *reaper* (*rpr*). CAX flies died at different stages of metamorphosis. (**B**) Developmental lethality observed in CAX flies and homozygous mutants of JH-related genes. Receptor double mutants and combined mutants of *jhamt* with *Cyp6g2* or *Cyp6a13* exhibited severe lethality. Values are the means ± standard error. $n = 3$ ($Met^{45B}$, $gce^{2Cl}$) or $n = 6$ (other mutants). Different letters above the bars indicate statistically significant differences ($p < 0.05$) between groups (Chi-square test with Bonferroni correction). (**C**) CA-specific knockdown of enzymes induced by *jhamt-Gal4*. Combined knockdown of *jhamt* with *Cyp6g2* or *Cyp6a13* exhibited severe lethality. Values are the means ± standard error ($n$ = 3–4). Different letters above the bars indicate statistically significant differences ($p < 0.05$, Chi-square test with Bonferroni correction). (**D**) Relative expression levels of *Kruppel homolog 1* (*Kr-h1*) in CAX and mutant flies at 0 h after puparium formation. Expression levels were normalized to a reference gene, *rp49*, in the same cDNA samples. Flies with severe lethality tend to show lower expression levels. Values are the means ± standard error ($n$ = 4–7). Different letters above the bars indicate statistically significant differences ($p < 0.05$, Tukey's honestly

826 significant difference test). (**E**) Correlation between developmental lethality and *Kr-h1*

827 expression levels at 0 hour after puparium formation. Developmental scores were calculated

828 based on lethal stages. A strong correlation ($r = 0.9361$) was observed between lethality and *Kr-*

829 *h1* expression levels across various genotypes.

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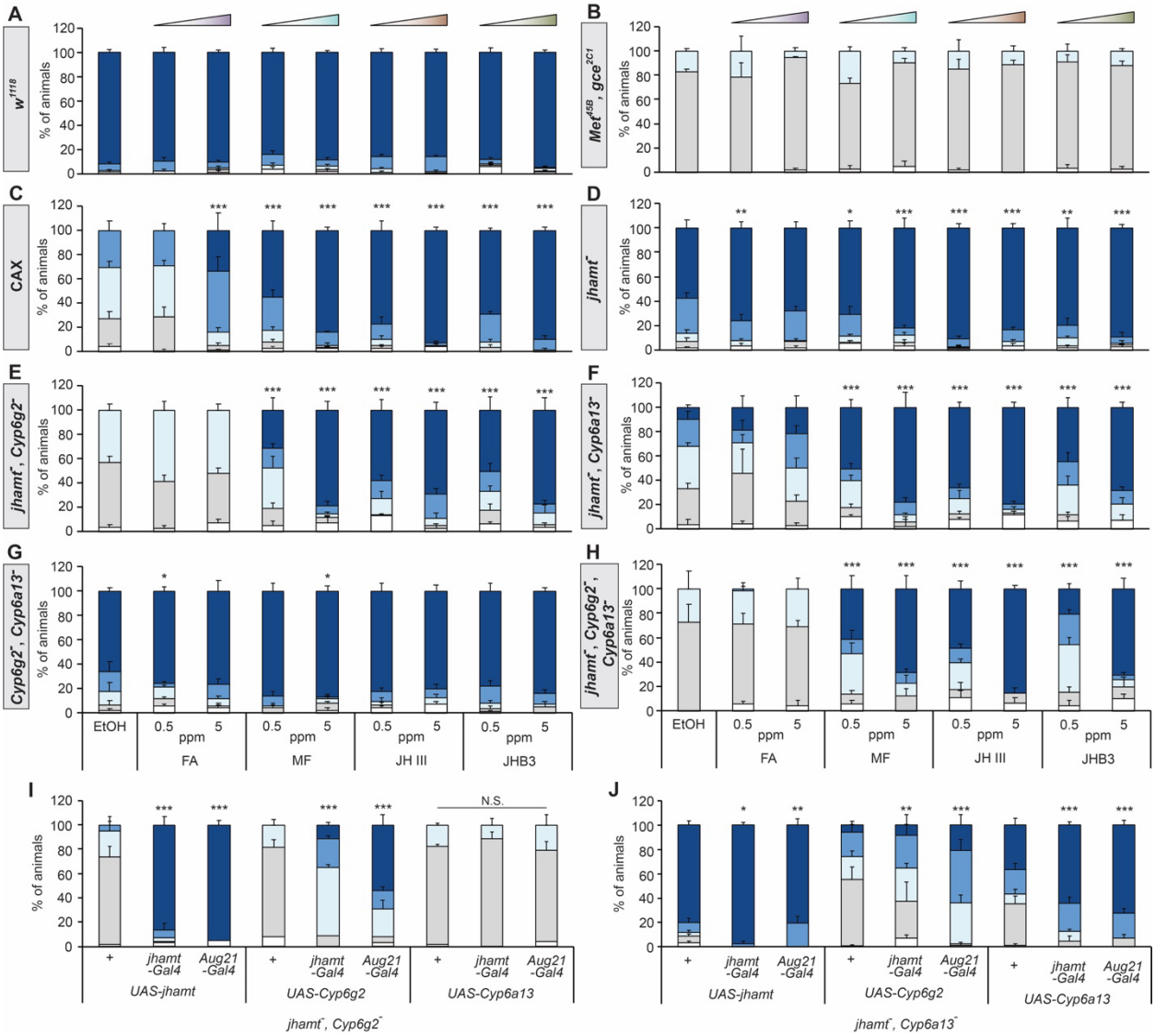


**Figure 4. Rescue of CA-ablated flies and JH-related mutants.**

(**A-H**) Rescue of developmental defects observed in control ($w^{1118}$), corpus allatum (CA)-ablated (CAX) flies, and homozygous mutants of JH-related genes by application of farnesoic acid (FA), methyl farnesoate (MF), juvenile hormone III (JH III), or juvenile hormone III bisepoxide (JHB3). Larvae were transferred onto the diet containing 0.5 or 5 ppm of one of the sesquiterpenoids at 48 hours after hatching and raised until eclosion. MF, JH III, and JHB3 rescued lethality of most genotypes, whereas FA had little effect. Values are the means ± standard error ($n$ = 3–6). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Chi-square test with Bonferroni

840 correction *vs* application of the solvent (EtOH) alone). (**I, J**) Rescue of *jhamt*, *Cyp6g2* (I) or

841 *jhamt*, *Cyp6a13* (J) double mutants by CA-specific overexpression of *jhamt*, *Cyp6g2*, or

842 *Cyp6a13*. *Cyp6g2* overexpression partially rescued *jhamt*, *Cyp6a13* double mutants, but

843 *Cyp6a13* overexpression did not rescue *jhamt*, *Cyp6g2* double mutants. Values are the means ±

844 standard error ($n$ = 3–6). **$p < 0.01$, ***$p < 0.001$ (Chi-square test with Bonferroni correction *vs*

845 control flies without a *Gal4* driver). N.S., not significant ($p > 0.05$).

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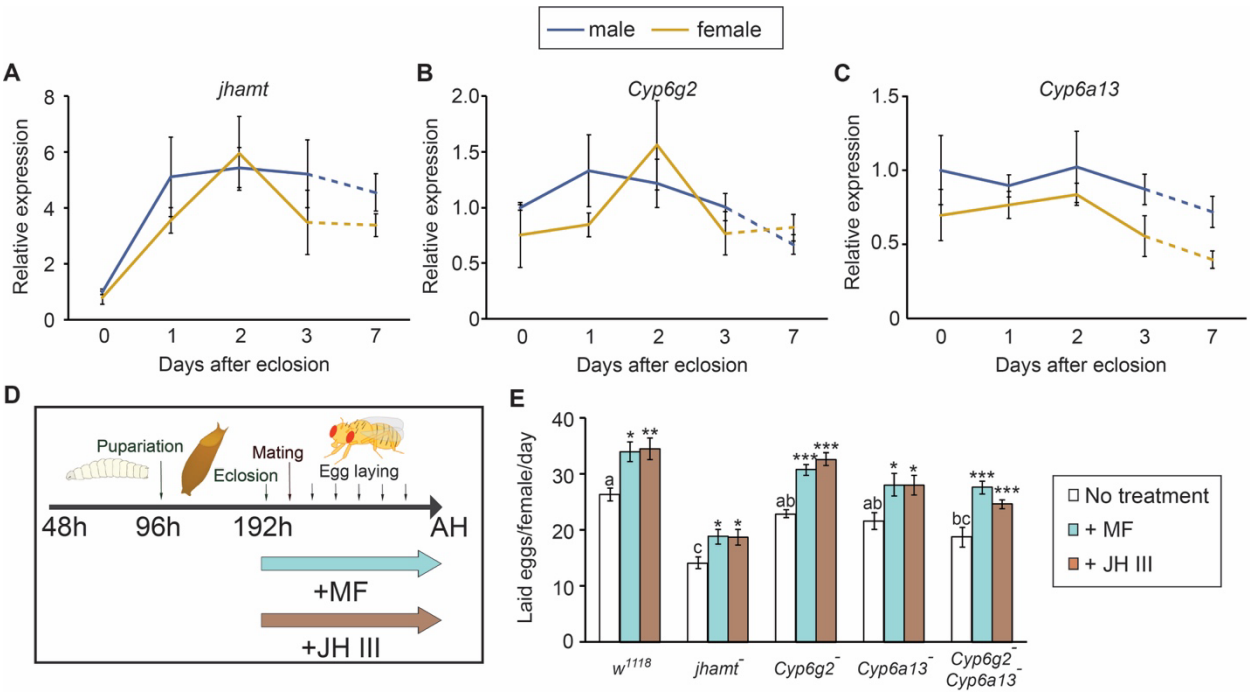


**Figure 5. Contributions of the CA-expressed enzymes to reproduction.**

(**A**–**C**) Changes in gene expression of *jhamt* (A), *Cyp6g2* (B), and *Cyp6a13* (C) in the corpus allatum/corpora cardiaca complex of the adults expressing *jhamt-LexA*-driven *LexAop-mCD8::GFP*. Expression levels were normalized to levels at day 0 after eclosion (within 6 hours). Values are the means ± standard error ($n$ = 3–4). (**D**) Schematic diagram of the fecundity assay. Mutants were reared normally until eclosion. Eclosed adults were then maintained with or without methyl farnesoate (MF) or juvenile hormone III (JH III). Mutant females were mated with $w^{1118}$ males 24 hours after eclosion. The number of eggs laid was counted every 24 hours from 3 to 7 days after eclosion. *Drosophila* illustrations were obtained from TogoTV (© 2016 DBCLS TogoTV, CC-BY-4.0 https://creativecommons.org/licenses/by/4.0/deed.ja). (**E**) Number of eggs laid by the mutant females. The numbers of eggs laid by untreated females were compared across genotypes using Tukey's honestly significant difference test. Different letters above the bars indicate statistically significant differences among different genotypes ($p < 0.05$). The numbers of eggs laid by MF- or JH III-treated females were compared to that of untreated

862 females of the same genotype using Dunnett's test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). *jhamt*

863 mutant and *Cyp6g2*, *Cyp6a13* double mutant females laid significantly fewer eggs than $w^{1118}$

864 control. Sesquiterpenoid treatment enhanced egg production in all genotypes tested. Values are

865 the means ± standard error ($n$ = 6–12).

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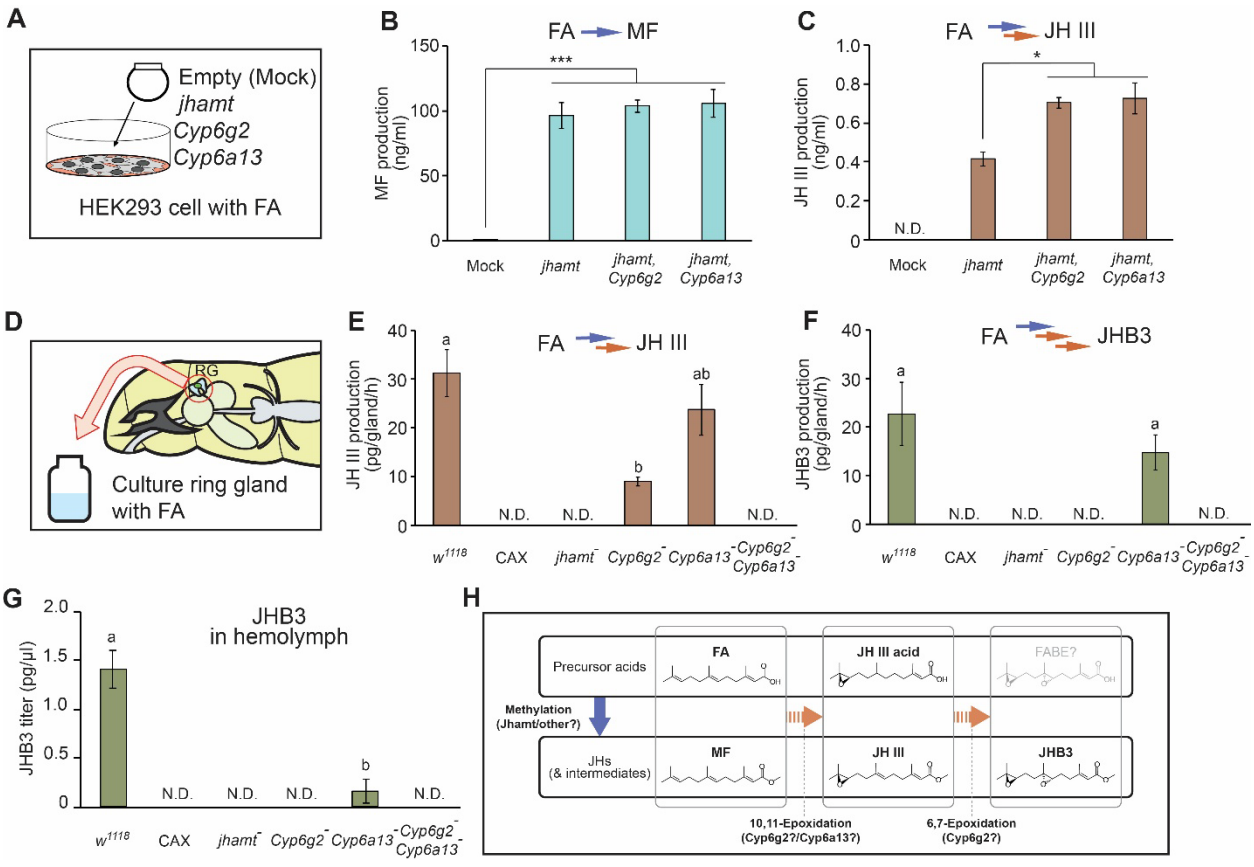


**Figure 6. Functional analyses of the CA-expressed enzymes.**

(**A**) Schematic diagram of the enzymatic conversion assay in HEK293 cells. (**B, C**) Conversion of farnesoic acid (FA) into methyl farnesoate (MF) (B) and juvenile hormone III (JH III) (C) in HEK293 cells ectopically expressing the CA-expressed enzymes. Jhamt catalyzes methylation, while Cyp6g2 and Cyp6a13 catalyze epoxidation to synthesize JH III. Values are the means ± standard error ($n$ = 3–4). *$p < 0.05$, ***$p < 0.001$ (Tukey's honestly significant difference test). N.D., not detected. (**D**) Schematic diagram of *ex vivo* culture of the ring glands from wandering larvae. (**E, F**) Conversion of FA into JH III (E) and juvenile hormone III bisepoxide (JHB3) (F) in cultured ring glands. JH production in the *jhamt* mutant ring glands was drastically reduced. The *Cyp6g2* mutant ring glands produced only a small amount of JH III. While JH production in the *Cyp6a13* mutant ring glands was not significantly reduced, it was abolished in the *Cyp6g2*,

879 *Cyp6a13* double mutant ring glands. Different letters above the bars indicate significant
880 differences ($p < 0.05$, Tukey's honestly significant difference test). Values are the means ±
881 standard error ($n = 4$–12). N.D., not detected. (**G**) JHB3 titers in the hemolymph of wandering
882 larvae of control ($w^{1118}$), corpus allatum (CA)-ablated (CAX) flies, and the enzyme mutants. The
883 hemolymph JHB3 titer was significantly reduced in *Cyp6a13* mutant larvae, and it was
884 undetectable in CAX, *jhamt* mutant, *Cyp6g2* mutant, and *Cyp6g2*, *Cyp6a13* double mutant
885 larvae. MF and JH III were below detection limits. Different letters above the bars indicate
886 significant differences ($p < 0.05$, Tukey's honestly significant difference test). Values are the
887 means ± standard error ($n = 4$–7). N.D., not detected. (**H**) Proposed model of the JH biosynthetic
888 pathway in the *Drosophila* CA.