Graphical abstract

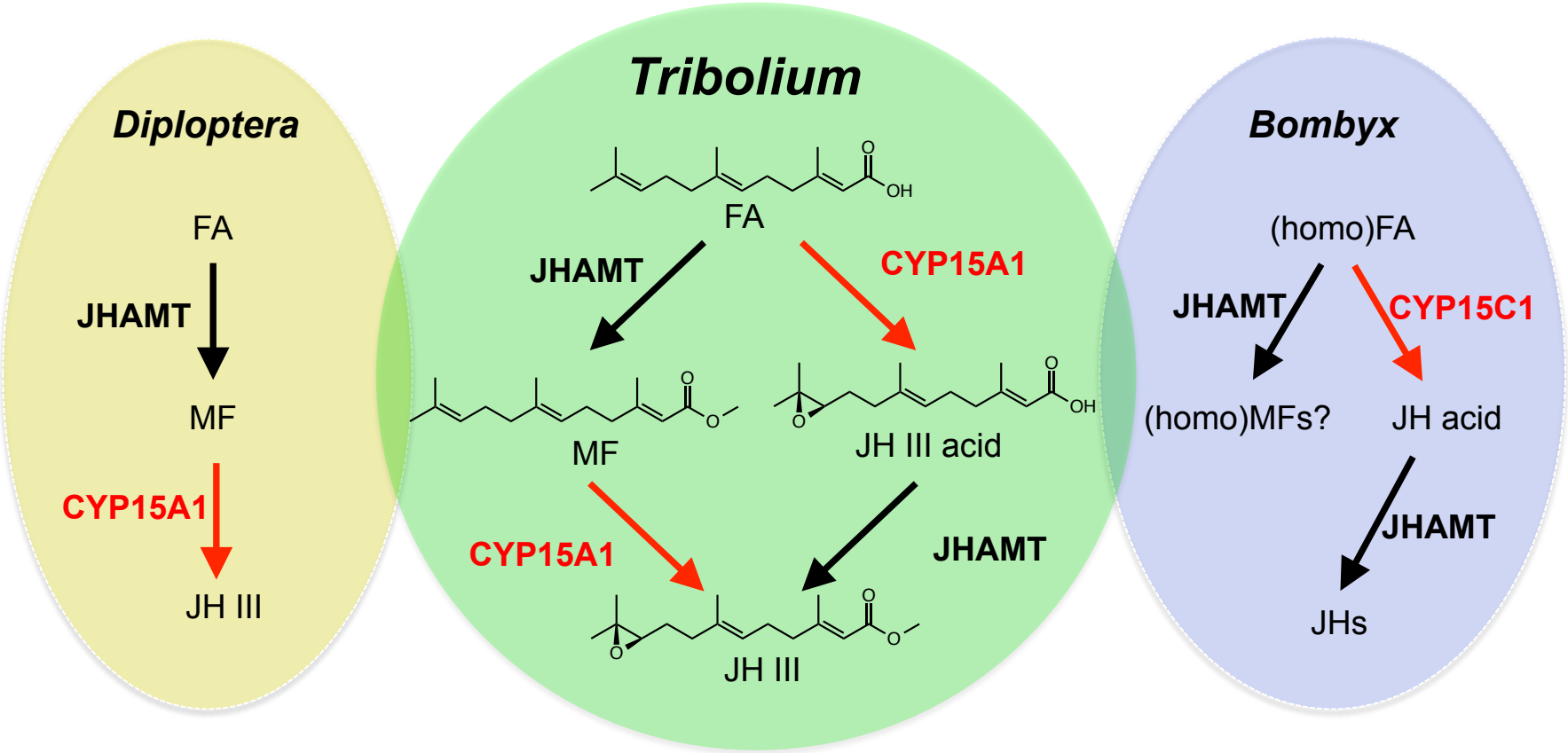


Figure 1

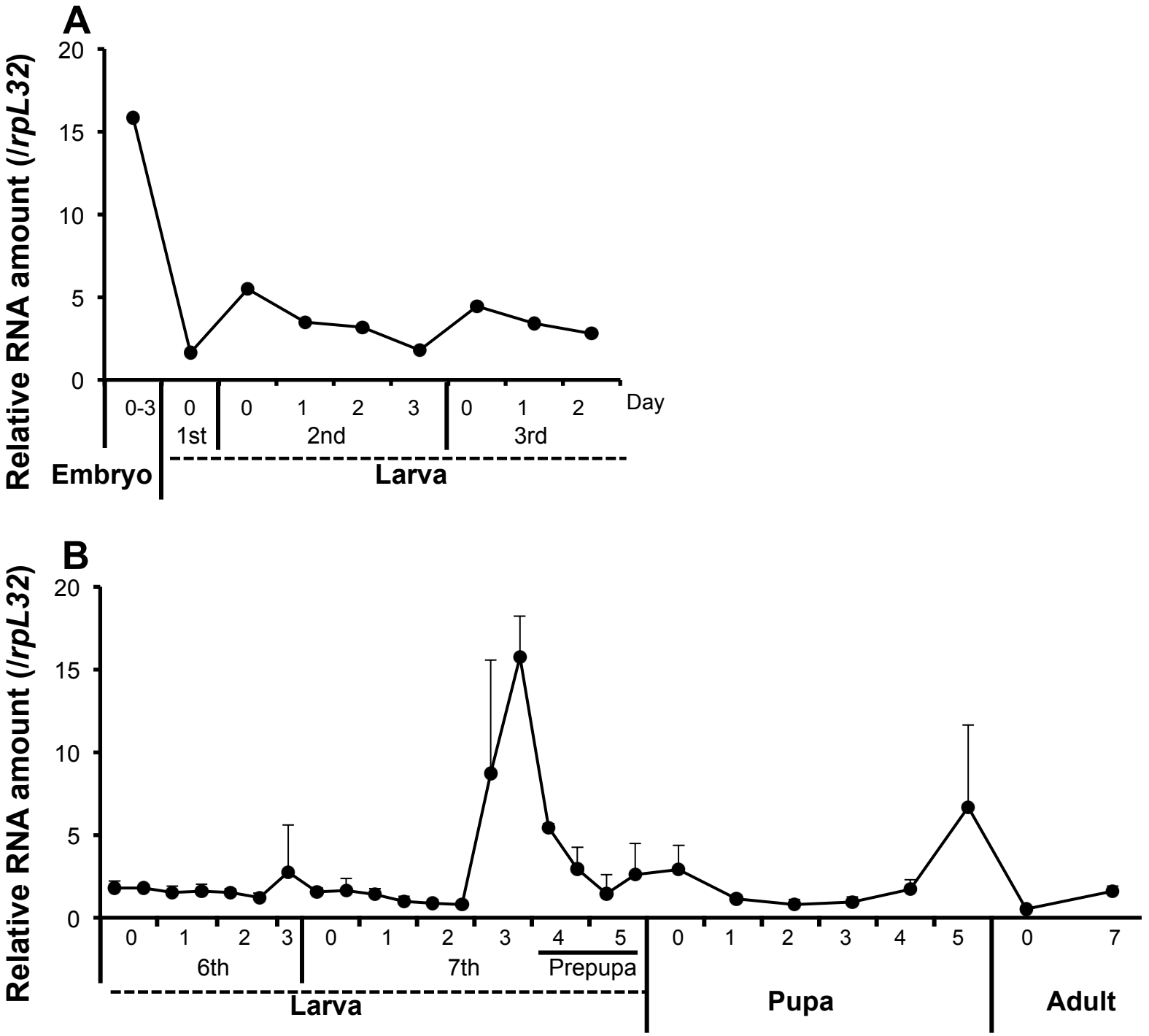

A
Relative RNA amount (/rpL32)
20
15
10
5
0
0-3
0
1st
0
1
2
3
2nd
0
1
2
3rd
Day
Embryo
Larva
B
Relative RNA amount (/rpL32)
20
15
10
5
0
0
1
2
3
6th
0
1
2
3
4
5
7th
Prepupa
0
1
2
3
4
5
0
7
Larva
Pupa
Adult

Figure 2

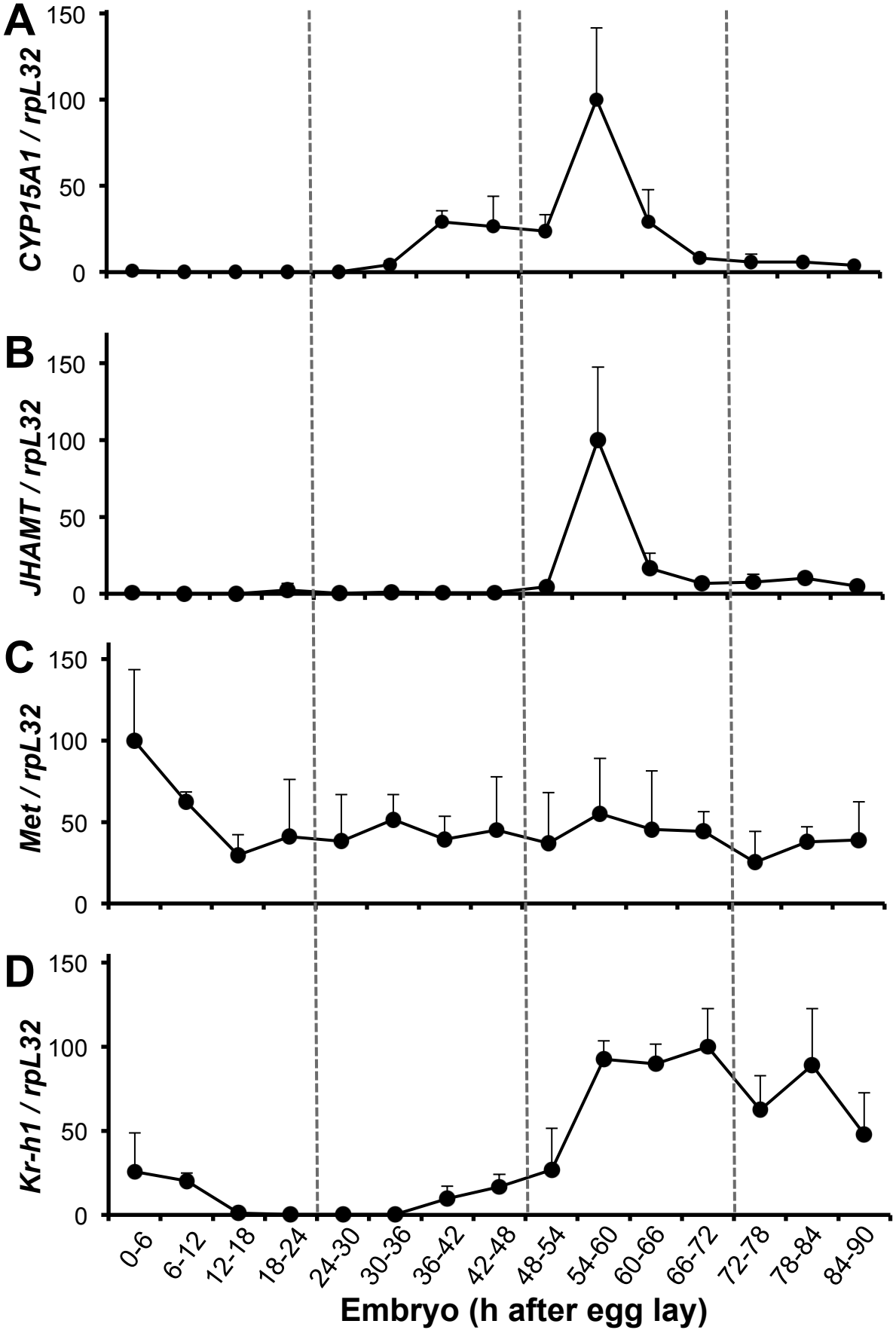

A
CYP15A1 / rpL32
150
100
50
0
B
JHAMT / rpL32
150
100
50
0
C
Met / rpL32
150
100
50
0
D
Kr-h1 / rpL32
150
100
50
0
0-6
6-12
12-18
18-24
24-30
30-36
36-42
42-48
48-54
54-60
60-66
66-72
72-78
78-84
84-90
Embryo (h after egg lay)

Figure 3

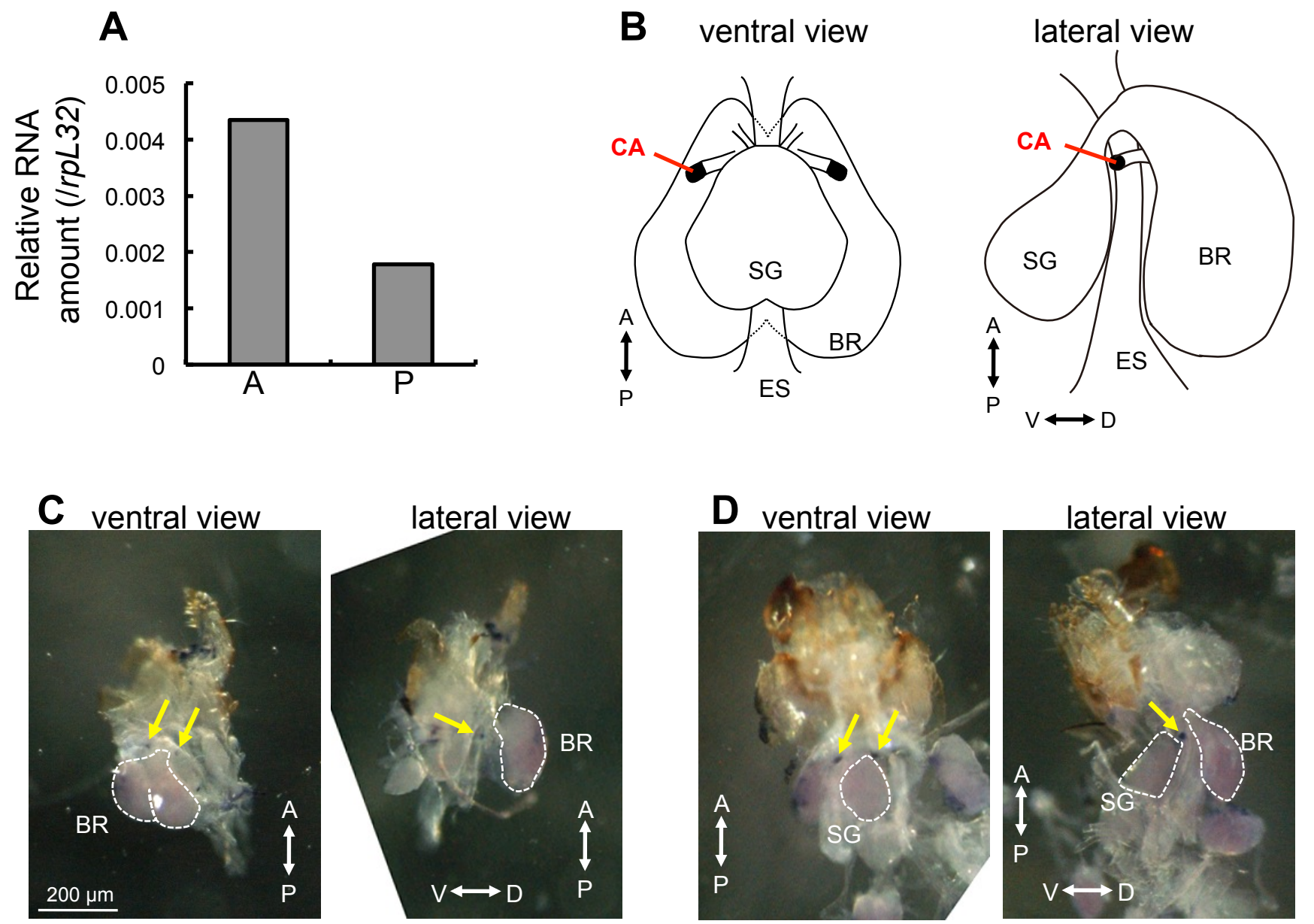

A
Relative RNA amount (/rpL32)
0.005
0.004
0.003
0.002
0.001
0
A
P
B
ventral view
lateral view
CA
SG
BR
ES
A
P
V
D
C
ventral view
lateral view
BR
200 μm
D
ventral view
lateral view
SG
BR

Figure 4

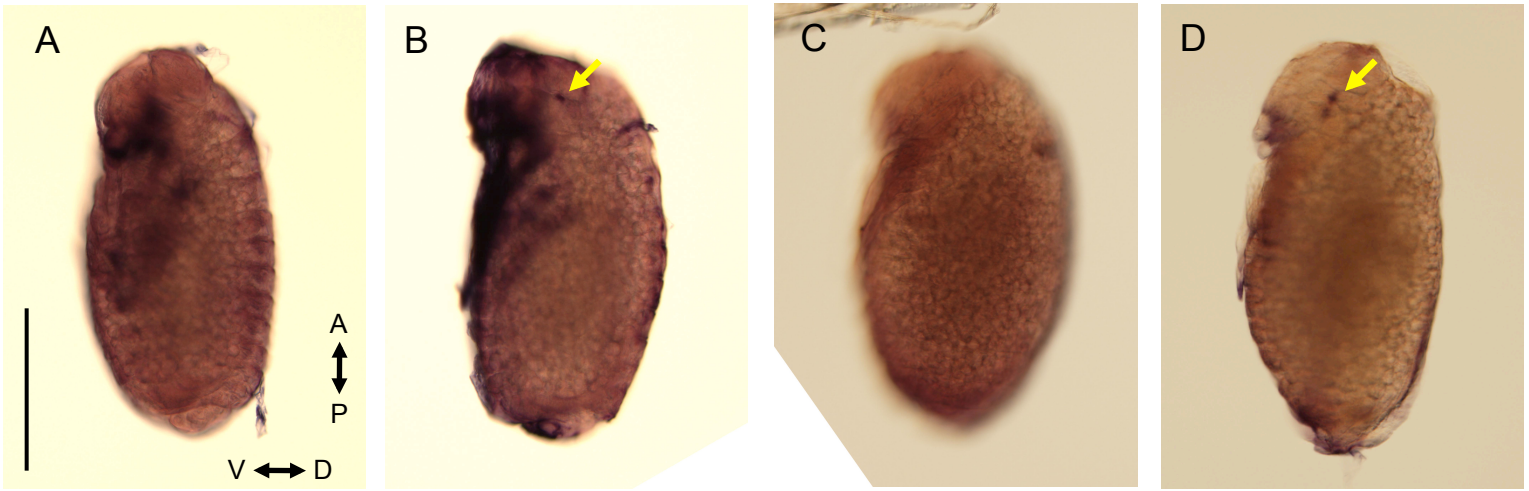


Figure 5

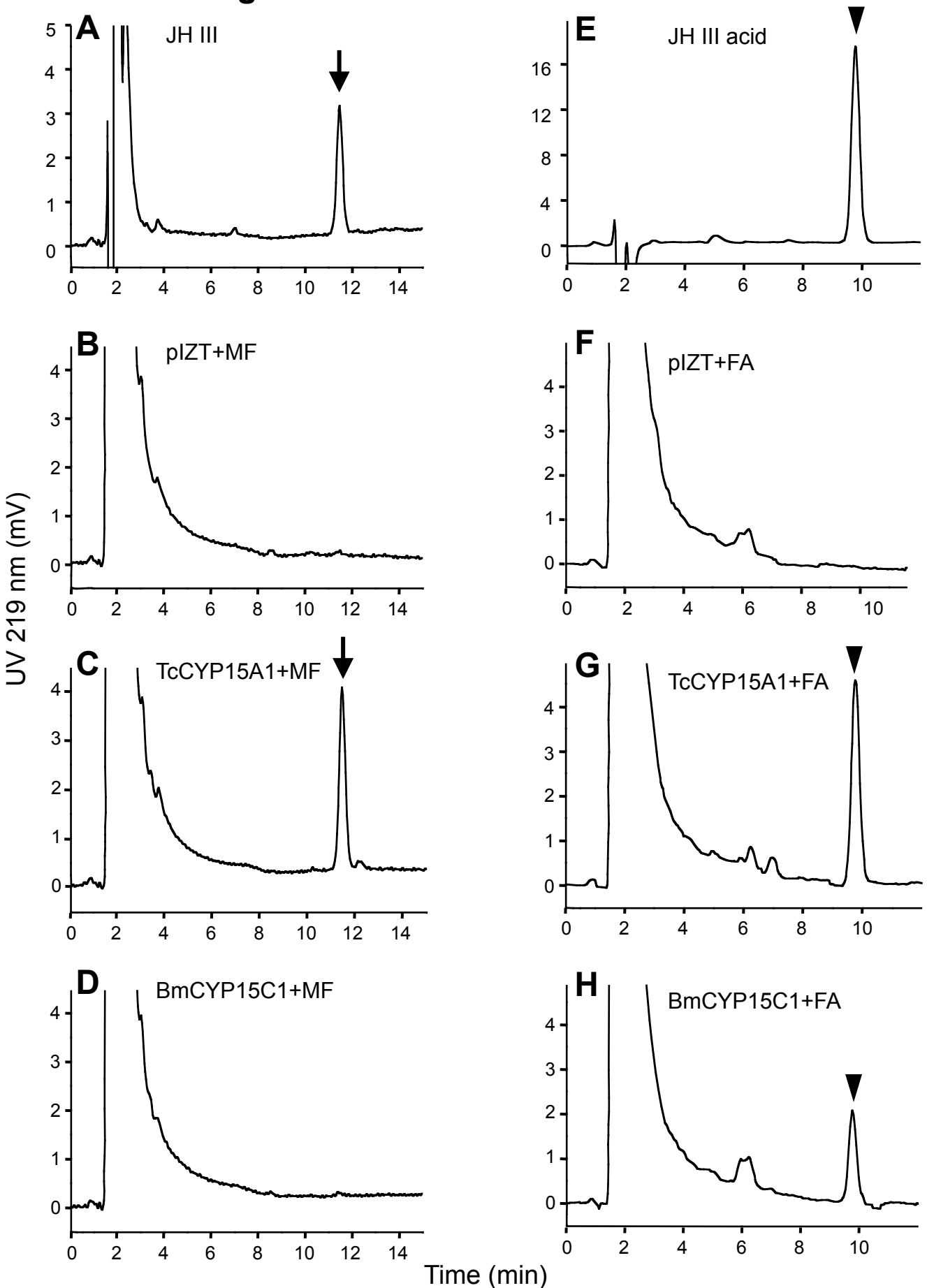

A
JH III
E
JH III acid
B
pIZT+MF
F
pIZT+FA
C
TcCYP15A1+MF
G
TcCYP15A1+FA
D
BmCYP15C1+MF
H
BmCYP15C1+FA
UV 219 nm (mV)
Time (min)

Figure 6

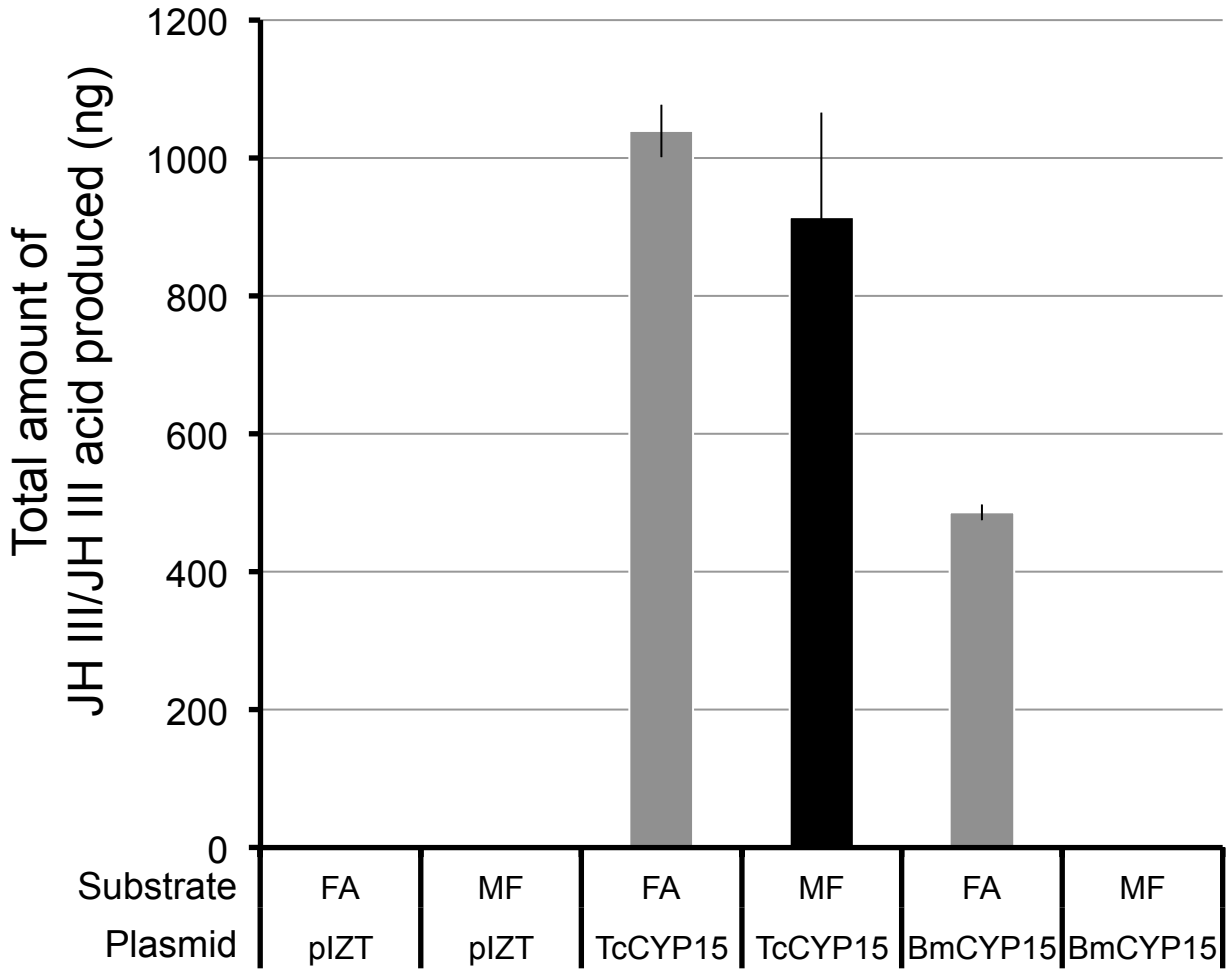

Total amount of JH III/JH III acid produced (ng)
1200
1000
800
600
400
200
0
Substrate
Plasmid
FA
MF
FA
MF
FA
MF
pIZT
pIZT
TcCYP15
TcCYP15
BmCYP15
BmCYP15

Figure 7

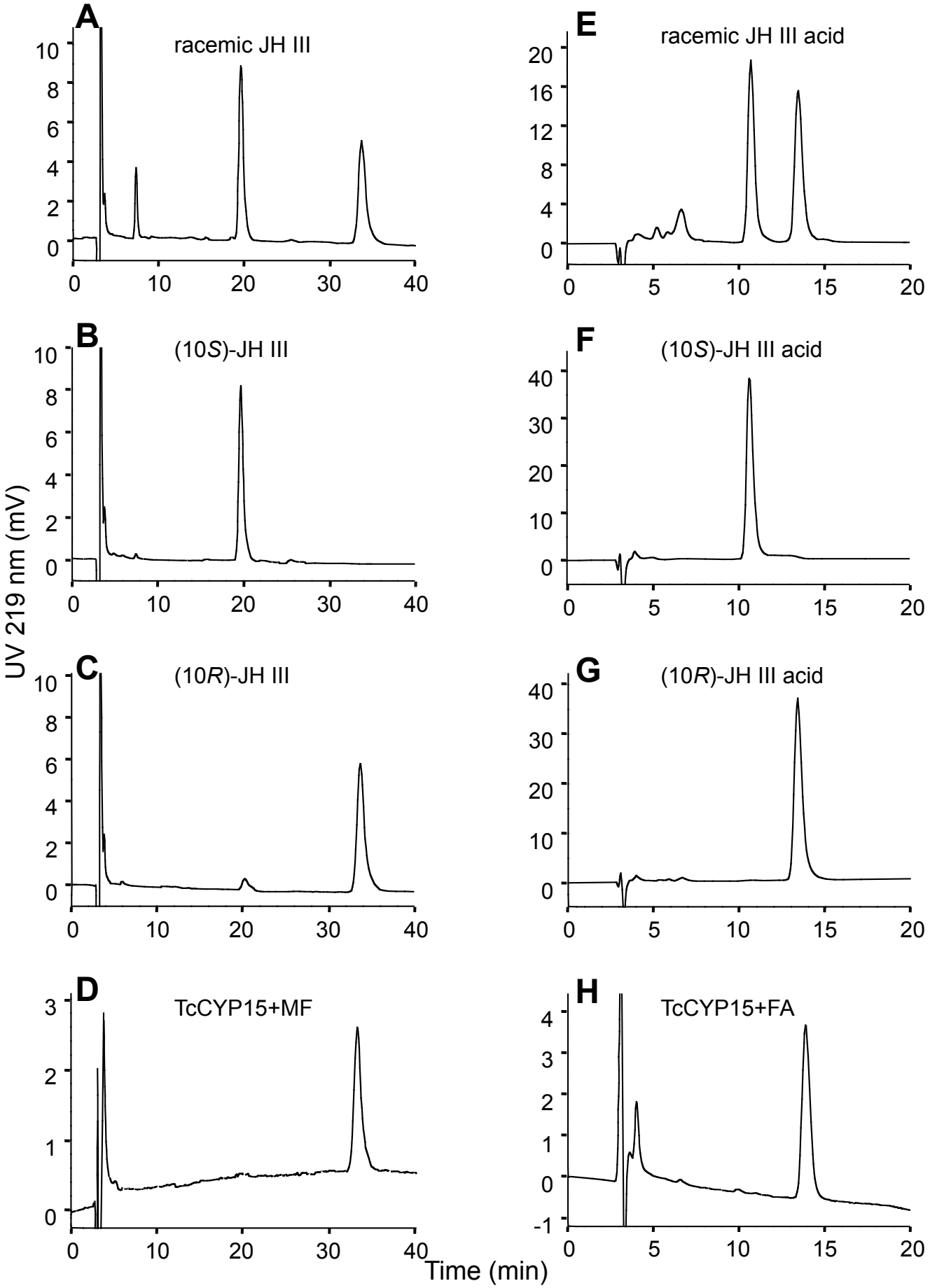
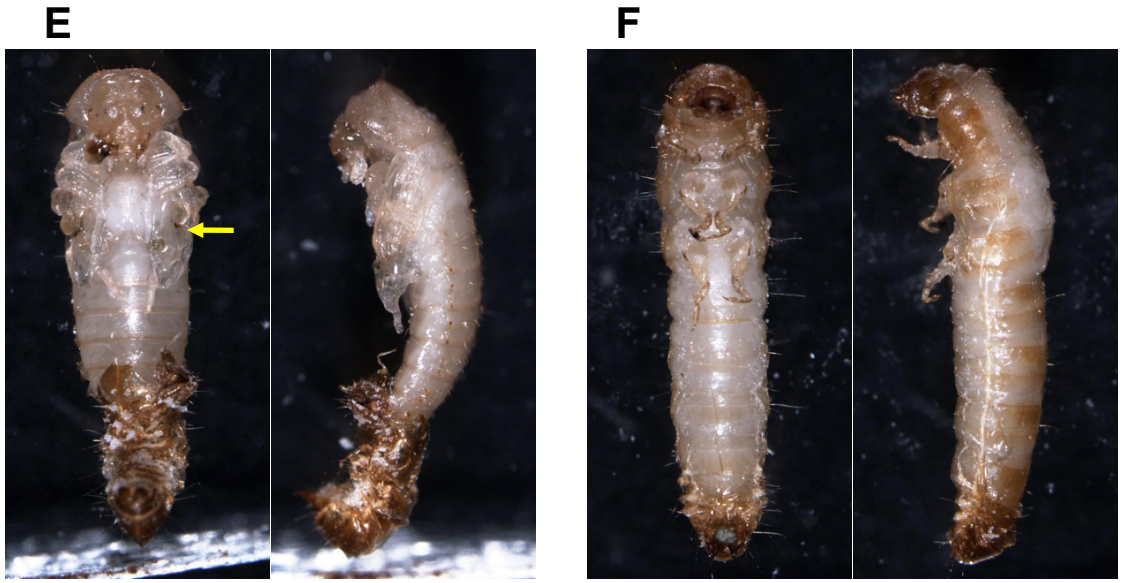

A
racemic JH III
B
(10S)-JH III
C
(10R)-JH III
D
TcCYP15+MF
E
racemic JH III acid
F
(10S)-JH III acid
G
(10R)-JH III acid
H
TcCYP15+FA
UV 219 nm (mV)
Time (min)

Figure 8

dsRNA *malE* *CYP15A1* *JHAMT* *CYP15A1+JHAMT*_mild



*CYP15A1+JHAMT*_severe *CYP15A1+JHAMT*_severe

Figure 9

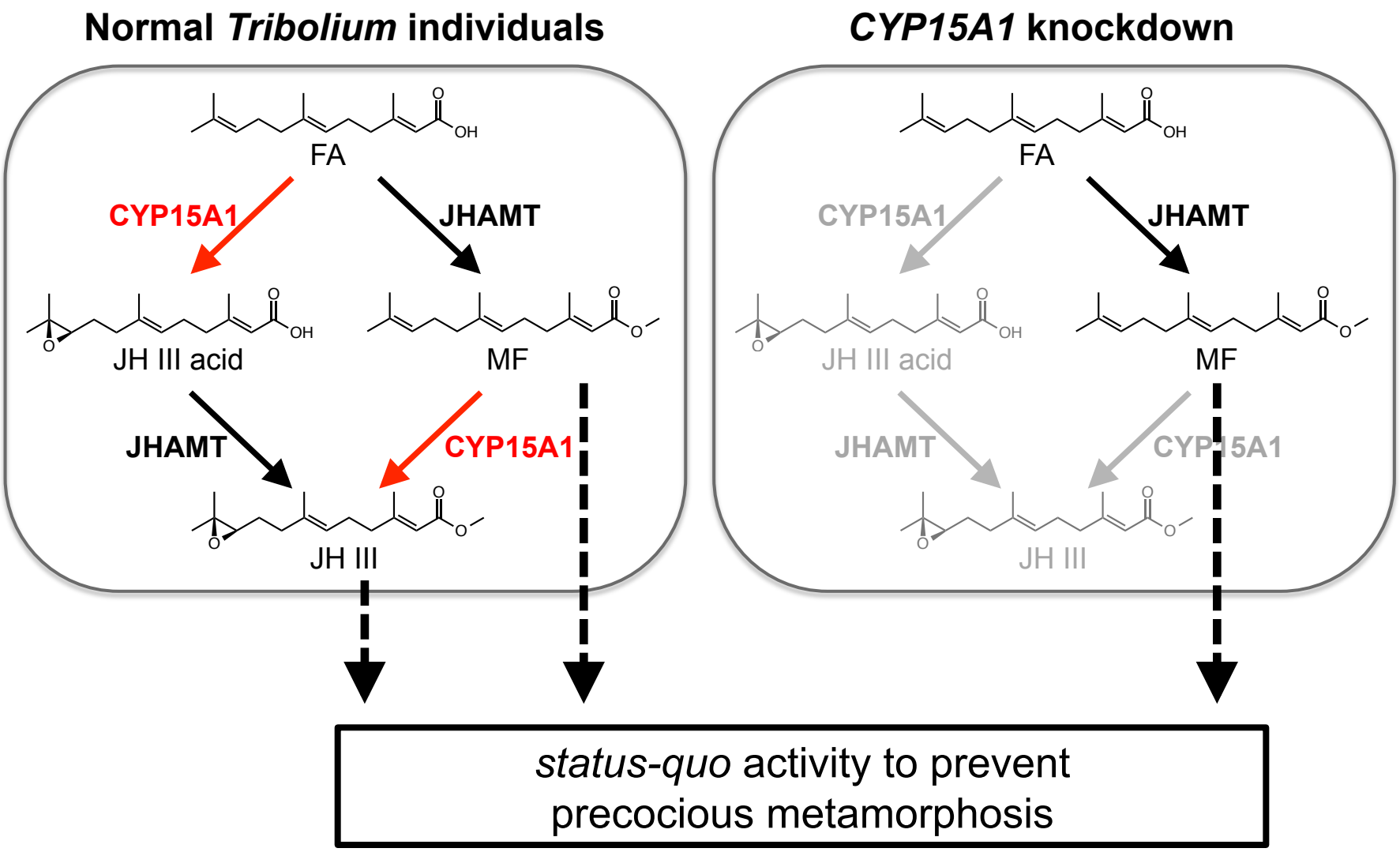

Normal *Tribolium* individuals
CYP15A1 knockdown
FA
CYP15A1
JHAMT
JH III acid
MF
JHAMT
CYP15A1
JH III
FA
CYP15A1
JHAMT
JH III acid
MF
JHAMT
CYP15A1
JH III
status-quo activity to prevent precocious metamorphosis

Table 1 Phenotype of *T. castaneum* larvae injected with 5 µg/µl dsRNA.

			Pupation stage				
injection stage	dsRNA	N	4th	5th	6th	7th	8th
Day1_4th							
	malE	16	0	0	0	14	2
	TcCYP15A1	24	0	0	3	21	0
Day0_5th							
	no injection	47	–	0	0	12	35
	malE	22	–	0	0	2	20
	TcCYP15A1	24	–	0	0	9	15
	TcJHAMT	23	–	0	14	9	0

Table 2 Phenotype of *T. castaneum* larvae injected with 5 µg/µl dsRNA on Day 0 of the 8th larval instar.

		Lethal stage			Normal adults, pupation stage	
dsRNA	N	L8-pupa ecdysis	pupa	adult eclosion	8th	9th
no injection	24	0	0	0	24	0
malE	37	0	0	0	37	0
TcCYP15A1	39	0	0	0	32	7
TcJHAMT	32	0	0	0	30	2
TcCYP15A1+TcJHAMT	39	3	9	18	8	1

Figure S1

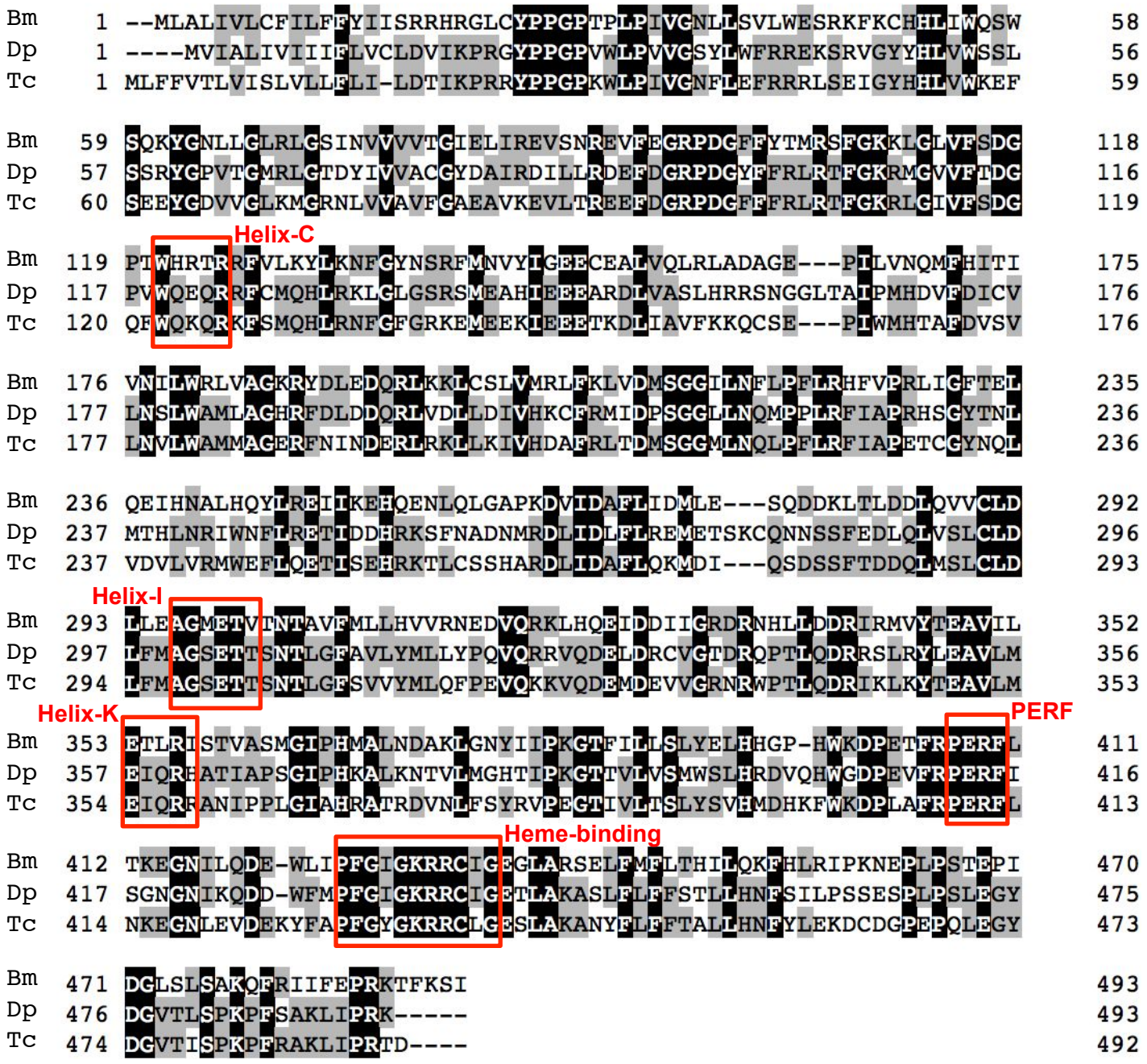


Figure S2

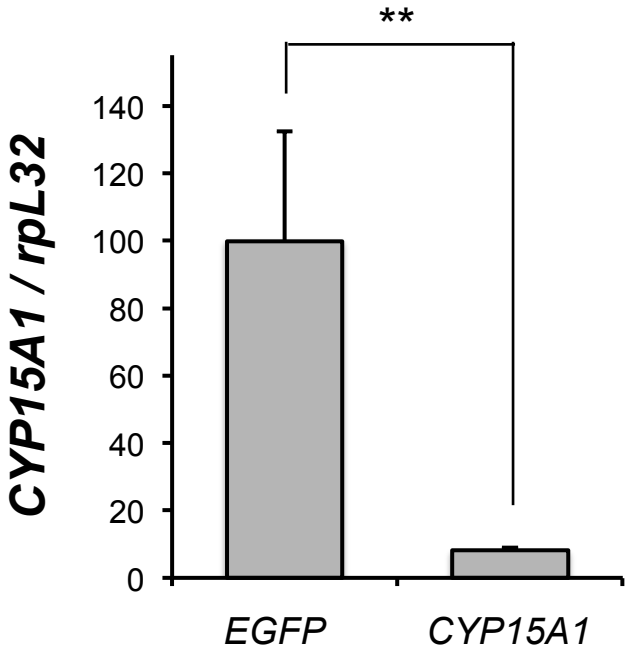


Table S1 Oligonucleotide primers used for RT-PCR.

Primers	Sequences (5' – 3')
RT-PCR	
TcCYP15-F1	ATGCTCTTCTTCGTAACTTTAG
TcCYP15-R1	TTAATCAGTCCGTGGGATGAGC
dsRNA synthesis	
TcCYP15_5	CCGTCAAGGAGGTGCTCACAAG
TcCYP15_5T7	TAATACGACTCACTATAGGGCCGTCAAGGAGGTGCTCACAAG
TcCYP15_3	TTTCGCCCAACAACTTCGTCC
TcCYP15_3T7	TAATACGACTCACTATAGGGTTTCGCCCAACAACTTCGTCC
quantitative RT-PCR	
TcCYP15-QF1	ACCTGAGCGGTTTTTGAACAAGG
TcCYP15-QR1	GCTAAAGATTCTCCCAAGCAGCG
ORF amplification for subcloning into pIZT/V5-His vector	
TcCYP15-F2	ACCGAGCTCGACATGCTCTTCTTCGTAACTTTAGTG
TcCYP15-R2	CGAGCGGCCGCCTTAATCAGTCCGTGGGATGAGCTT

Supplementary Table 2 Oligonucleotide primers used for quantitative RT-PCR.

Primers	Sequences (5' - 3')	size of amplicon (bp)	reference
TcCYP15-QF1	ACCTGAGCGGTTTTTGAACAAGG	102	this study
TcCYP15-QR1	GCTAAAGATTCTCCCAAGCAGCG		
TcJHAMT-QF1	CATCTCGCCCTATCACCATTCG	149	Minakuchi et al., 2008
TcJHAMT-QR1	CCGCTGAAACCGATTTTGACAA		
TcMet-QF1	CATTGCAGGTTATATGACTGAGGAAGTGT	145	Minakuchi et al., 2009
TcMet-QR1	GAGTAAACGGTAACATGATGATCCTTTGCT		
TcKrh1-QF2	CCTGAGAAATTAGACTCCTTGGCAAAT	168	Minakuchi et al., 2009
TcKrh1-QR2	GGAGCAGCACCAGAGGGAAATTC		
TcrpL32-QF1	CAGGCACCAGTCTGACCGTTATG	140	Minakuchi et al., 2008
TcrpL32-QR1	GCTTCGTTTTGGCATTGGAGC		

ITEM TO CHECK	IMPORTANCE	CHECKLIST
EXPERIMENTAL DESIGN		
Definition of experimental and control groups	**E**	Temporal and spatial expression pattern was analyzed.
Number within each group	**E**	Several individuals were pooled in each sample (please see Materials and Methods).
Assay carried out by core lab or investigator's lab?	D	Sample preparation and quantitative RT-PCR were carried out in NIAS (Figs. 1 and 3) and Nagoya University (Fig. 2).
Acknowledgement of authors' contributions	D	C. M. and T. S. designed the experiments; C. M., F. I. and Y. W. performed experiments and analyzed the data.
SAMPLE		
Description	**E**	The red flour beetle, *Tribolium castaneum*
Volume/mass of sample processed	D	Several individuals were combined for RNA isolation of the embryos and the first, second, and third larval instars. RNA was isolated from individuals for the sixth and seventh larval instars, pupal and adult stages.
Microdissection or macrodissection	**E**	Whole body was used for RNA extraction (Fig. 1). Whole embryo was used for RNA extraction (Fig. 2). Larvae were cut in half between thoracic segments T2 and T3 (Fig. 3A).
Processing procedure	**E**	Basically, insects or embryos were homogenized in homogenizing reagent for RNA isolation (i.e. TRIzol or RNeasy Plus mini kit), without freezing.
If frozen - how and how quickly?	**E**	Insects were homogenized without freezing.
If fixed - with what, how quickly?	**E**	Insects were homogenized in homogenizing reagent for RNA isolation (i.e. TRIzol or RNeasy Plus mini kit), without freezing.
Sample storage conditions and duration (especially for FFPE samples)	**E**	Homogenized samples in homogenizing reagent for RNA isolation were stored at -80º C until RNA extraction.
NUCLEIC ACID EXTRACTION		
Procedure and/or instrumentation	**E**	TRIzol reagent (Fig. 2) or RNeasy Plus mini kit (Figs. 1 and 3) were used for RNA extraction.
Name of kit and details of any modifications	**E**	TRIzol reagent (Fig. 2) or RNeasy Plus mini kit (Figs. 1 and 3). RNA extraction was performed according to the manufacturer's instruction.
Source of additional reagents used	D	Chloroform, isopropanol and ethanol for molecular biology were used for RNA isolation using TRIzol.
Details of DNase or RNAse treatment	**E**	When using RNeasy Plus mini kit, gDNA Eliminator Column was used according to the instruction. When using TRIzol reagent, total RNA was treated with DNase I in order to eliminate gDNA before reverse transcription.
Contamination assessment (DNA or RNA)	**E**	In subsequent quantitative RT-PCR, no reverse transcription control was prepared to access the amount of amplicon generated from contaminated genomic DNA.
Nucleic acid quantification	**E**	Total RNA was quantified by measuring the absorbance at 260 nm.
Instrument and method	**E**	Nano-drop Spectrophotometer, Hitachi double-beam uv-vis Spectrophotometer, or SimpliNano Spectrophotometer (GE Healthcare)
Purity (A260/A280)	D	A260/A280 was calculated to confirm that the purity of total RNA is high enough.
Yield	D	RNA concentration was calculated from A260.
RNA integrity method/instrument	**E**	Not examined
RIN/RQI or Cq of 3' and 5' transcripts	**E**	Not examined
Electrophoresis traces	D	Not performed
Inhibition testing (Cq dilutions, spike or other)	**E**	Not performed
REVERSE TRANSCRIPTION		
Complete reaction conditions	**E**	In experiments shown in Figs. 1 and 3A, cDNAs were synthesized with an oligo(dT)18 primer and M-MLV reverse transcriptase (Clontech Laboratories). In experiments shown in Fig. 2, cDNAs were synthesized with an oligo dT primer and PrimeScript II reverse transcriptase (Takara Bio).
Amount of RNA and reaction volume	**E**	In experiments shown in Figs. 1 and 3A, 300-400 ng total RNA was reverse-transcribed in 20 µl reaction mixture. In experiments shown in Fig. 2, 8 µl total RNA (generally including 400 ng or less RNA) was reverse-transcribed in 20 µl reaction mixture.
Priming oligonucleotide (if using GSP) and concentration	**E**	Not applicable
Reverse transcriptase and concentration	**E**	In experiments shown in Figs. 1 and 3A, cDNAs were synthesized with an oligo(dT)18 primer and M-MLV reverse transcriptase (Clontech Laboratories). In experiments shown in Fig. 2, cDNAs were synthesized with an oligo dT primer and PrimeScript II reverse transcriptase (Takara Bio). The concentration of RTase followed the manufacturer's instructions.
Temperature and time	**E**	Reverse transcription was performed at 42ºC for 1 hr.
Manufacturer of reagents and catalogue numbers	D	M-MLV reverse transcriptase (Clontech Laboratories) and PrimeScript II reverse transcriptase (Takara Bio).
Cqs with and without RT	D*	In some samples, Cqs of the control samples without RT were examined in qRT-PCR to access the amount of genomic DNA.
Storage conditions of cDNA	D	cDNAs were stored at -25ºC.
qPCR TARGET INFORMATION		
If multiplex, efficiency and LOD of each assay.	**E**	Not applicable
Sequence accession number	**E**	TcCYP15A1 (AB987827); TcJHAMT (AB360763); TcMet (AB360765); TcKr-h1 (AB360764); TcrpL32 (i.e. Tcrp49, XM_964471)
Location of amplicon	D	Not described
Amplicon length	**E**	Amplicon length and the primer sequences were listed in an additional table.
In silico specificity screen (BLAST, etc)	**E**	Not performed
Pseudogenes, retropseudogenes or other homologs?	D	Not applicable
Sequence alignment	D	Not applicable
Secondary structure analysis of amplicon	D	Not performed
Location of each primer by exon or intron (if applicable)	**E**	Not applicable
What splice variants are targeted?	**E**	Not applicable
qPCR OLIGONUCLEOTIDES		
Primer sequences	**E**	Primer sequences were listed in Supplementary Tables of this paper and in our previous studies (Minakuchi et al., FEBS J 2008; Minakuchi et al., Dev Biol 2009). Amplicon length and the primer sequences were listed in an additional table.
RTPrimerDB Identification Number	D	Not applicable
Probe sequences	D**	Not applicable
Location and identity of any modifications	**E**	Not applicable
Manufacturer of oligonucleotides	D	Operon Biotechnologies, and Life Technologies Japan
Purification method	D	Desalted

qPCR PROTOCOL		
Complete reaction conditions	**E**	In experiments shown in Figs. 1 and 3A, qPCR was performed using LightCycler 2.0 (Roche Diagnostics). Quantitative RT-PCR was carried out in a 20 µl reaction volume containing SYBR Premix Ex Taq (Takara Bio), 0.2 µM of each primer and 2–3µl of template cDNAs or standard plasmids. PCR conditions were 95 °C for 10 s, followed by 45 cycles at 95°C for 5 s and 60°C for 20 s. After PCR, the absence of unwanted byproducts was confirmed by melting curve analysis. For standards, serial dilutions of a plasmid containing the ORF of each gene were used. TcrpL32 (synonymous with rp49) was used as a reference gene. In experiments shown in Fig. 2, qPCR was performed using Thermal Cycler Dice Real Time System (model TP800, Takara Bio). Quantitative RT-PCR was carried out in a 12 µl reaction volume containing SYBR Premix Ex Taq (Takara Bio), 0.2 µM of each primer and 1 µl of template cDNAs or standard plasmids. PCR conditions were 95 °C for 30 s, followed by 45 cycles at 95°C for 5 s and 60°C for 30 s. After PCR, the absence of unwanted byproducts was confirmed by melting curve analysis. For standards, serial dilutions of a plasmid containing the ORF of each gene were used. TcrpL32 was used as a reference gene.
Reaction volume and amount of cDNA/DNA	**E**	In experiments shown in Figs. 1 and 3A, qPCR was carried out in a 20 µl reaction volume containing SYBR Premix Ex Taq (Takara Bio), 0.2 µM of each primer and 2–3µl of template cDNAs or standard plasmids. In experiments shown in Fig. 2, qPCR was performed in a 12 µl reaction volume containing SYBR Premix Ex Taq (Takara Bio), 0.2 µM of each primer and 1 µl of template cDNAs or standard plasmids.
Primer, (probe), Mg++ and dNTP concentrations	**E**	Primer concentration was 0.2 µM. Mg2+ and dNTP were already included in 2x SYBR Premix Ex Taq.
Polymerase identity and concentration	**E**	Ex Taq HS DNA polymerase
Buffer/kit identity and manufacturer	**E**	SYBR Premix Ex Taq (Takara Bio)
Exact chemical constitution of the buffer	D	TaKaRa Ex Taq HS, dNTP Mixture, Mg2+, SYBR Green I
Additives (SYBR Green I, DMSO, etc.)	**E**	SYBR Green I was already included in 2x SYBR Premix Ex Taq.
Manufacturer of plates/tubes and catalog number	D	In experiments shown in Figs. 1 and 3A, LightCycler capillaries (Roche Diagnostics) were used. In experiments shown in Fig. 2, Hi-8 tubes with flat caps (Takara Bio) were used.
Complete thermocycling parameters	**E**	In experiments shown in Figs. 1 and 3A, PCR conditions were 95 °C for 10 s, followed by 45 cycles at 95°C for 5 s and 60°C for 20 s. In experiments shown in Fig. 2, PCR conditions were 95 °C for 30 s, followed by 45 cycles at 95°C for 5 s and 60°C for 30 s. In both cases, the absence of unwanted byproducts was confirmed by melting curve analysis.
Reaction setup (manual/robotic)	D	Manual
Manufacturer of qPCR instrument	**E**	In experiments shown in Figs. 1 and 3A, LightCycler 2.0 (Roche Diagnostics) was used. In experiments shown in Fig. 2, Thermal Cycler Dice Real Time System (model TP800, Takara Bio) was used.
qPCR VALIDATION		
Evidence of optimisation (from gradients)	D	Not applicable
Specificity (gel, sequence, melt, or digest)	**E**	Melting curve analysis was performed to confirm the absence of unwanted byproducts.
For SYBR Green I, Cq of the NTC	**E**	The difference in Cq between NTC controls and cDNA samples was large.
Standard curves with slope and y-intercept	**E**	Not applicable
PCR efficiency calculated from slope	**E**	PCR efficiency was 83-99%.
Confidence interval for PCR efficiency or standard error	D	Not applicable
r2 of standard curve	**E**	r2 of standard curve was >0.995.
Linear dynamic range	**E**	Not performed
Cq variation at lower limit	**E**	Not performed
Confidence intervals throughout range	D	Not performed
Evidence for limit of detection	**E**	Not performed
If multiplex, efficiency and LOD of each assay.	**E**	Not applicable
DATA ANALYSIS		
qPCR analysis program (source, version)	**E**	LightCycler Software (Roche Diagnostics) and Thermal Cycler Dice Real Time System Software (Takara Bio)
Cq method determination	**E**	Second derivative maximum method
Outlier identification and disposition	**E**	Not performed
Results of NTCs	**E**	The difference in Cq between NTC controls and cDNA samples was large.
Justification of number and choice of reference genes	**E**	The transcript levels of the target genes were normalized to the amount of rpL32 (Rp49) mRNA in the same sample.
Description of normalisation method	**E**	The transcript levels of the target genes were normalized to the amount of rpL32 (Rp49) mRNA in the same sample.
Number and concordance of biological replicates	D	In Figs. 1B and 2, at least three biological replicates were examined, and the average and SD were calculated.
Number and stage (RT or qPCR) of technical replicates	**E**	One techincal replicate was carried out.
Repeatability (intra-assay variation)	E	Not examined
Reproducibility (inter-assay variation, %CV)	D	Not examined
Power analysis	D	Not performed
Statistical methods for result significance	**E**	Not applicable
Software (source, version)	E	LightCycler Software and Thermal Cycler Dice Real Time System Software (ver. 3.0)
Cq or raw data submission using RDML	**D**	Not performed

MIQE checklist for authors, reviewers and editors. All essential information (E) must be submitted with the manuscript. Desirable information (D) should be submitted if available. If using primers obtained from RTPrimerDB, information on qPCR target, oligonucleotides, protocols and validation is available from that source.

*: Assessing the absence of DNA using a no RT assay is essential when first extracting RNA. Once the sample has been validated as RDNA-free, inclusion of a no-RT control is desirable, but no longer essential.

**: Disclosure of the probe sequence is highly desirable and strongly encouraged. However, since not all commercial pre-designed assay vendors provide this information, it cannot be an essential requirement. Use of such assays is advised against.