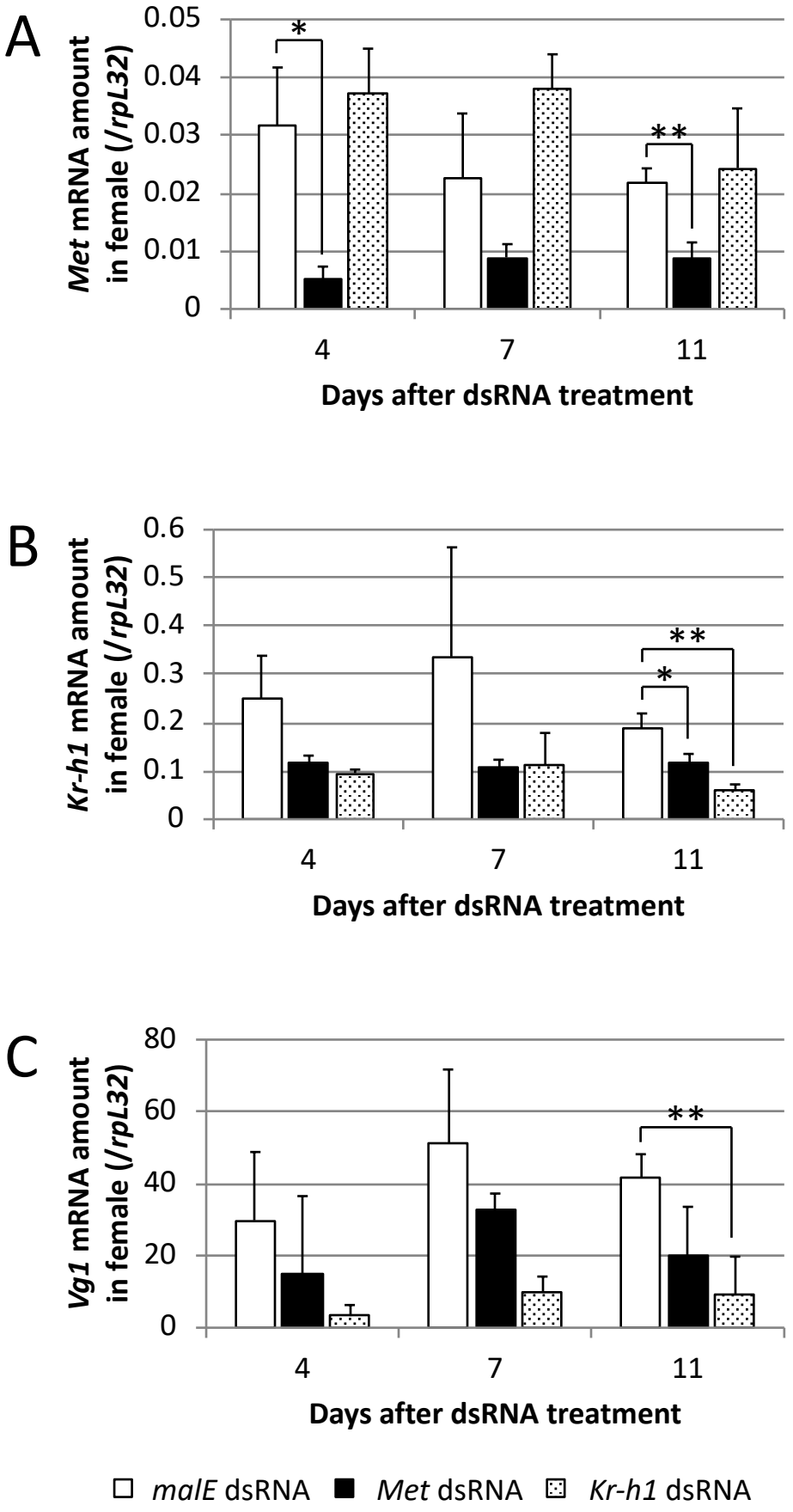
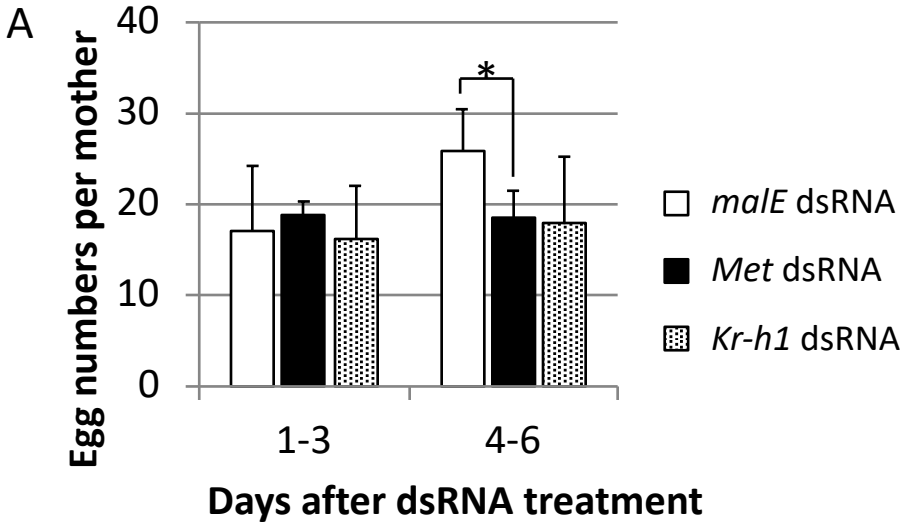
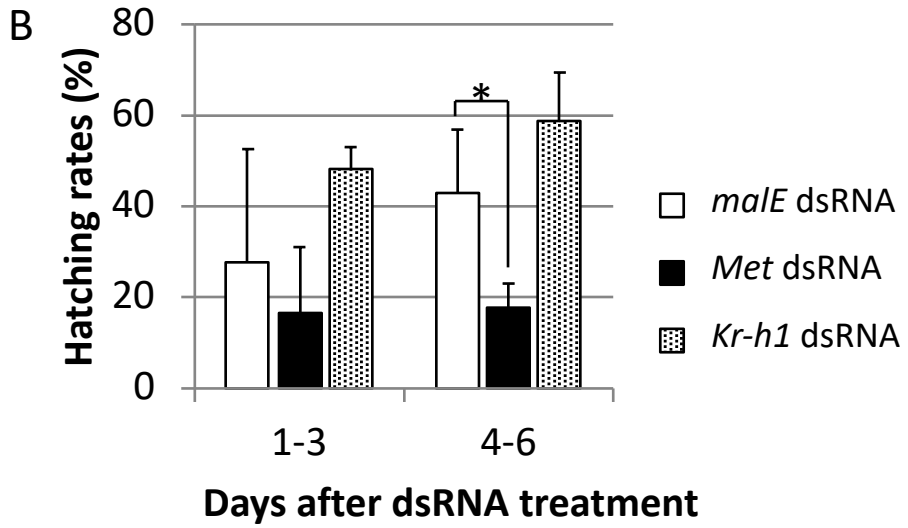

A
0.05
0.04
0.03
0.02
0.01
0
*
**
Met mRNA amount in female (/rpL32)
4
7
11
Days after dsRNA treatment
B
0.6
0.5
0.4
0.3
0.2
0.1
0
**
*
Kr-h1 mRNA amount in female (/rpL32)
4
7
11
Days after dsRNA treatment
C
80
60
40
20
0
**
Vg1 mRNA amount in female (/rpL32)
4
7
11
Days after dsRNA treatment
malE dsRNA
Met dsRNA
Kr-h1 dsRNA

Fig. 1


A
Egg numbers per mother
40
30
20
10
0
*
1-3
4-6
Days after dsRNA treatment
malE dsRNA
Met dsRNA
Kr-h1 dsRNA


B
Hatching rates (%)
80
60
40
20
0
*
1-3
4-6
Days after dsRNA treatment
malE dsRNA
Met dsRNA
Kr-h1 dsRNA

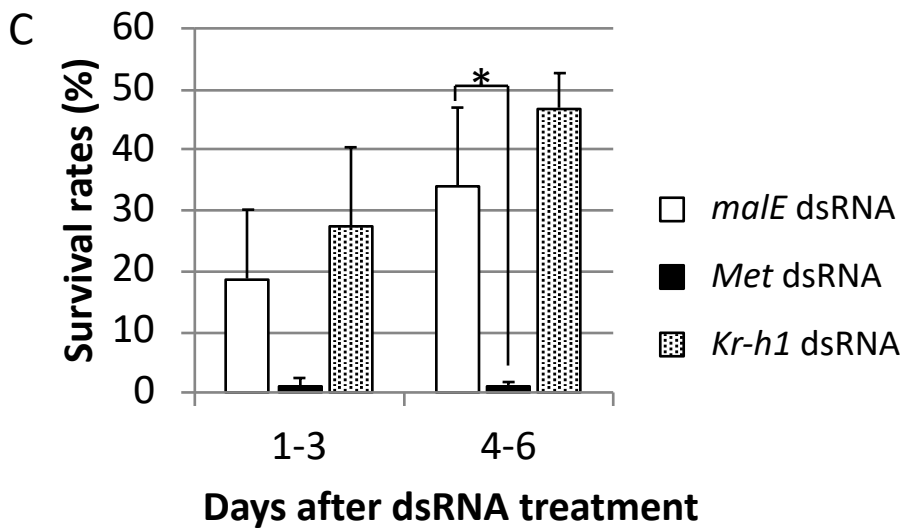

C
Survival rates (%)
60
50
40
30
20
10
0
*
1-3
4-6
Days after dsRNA treatment
malE dsRNA
Met dsRNA
Kr-h1 dsRNA

Fig. 2

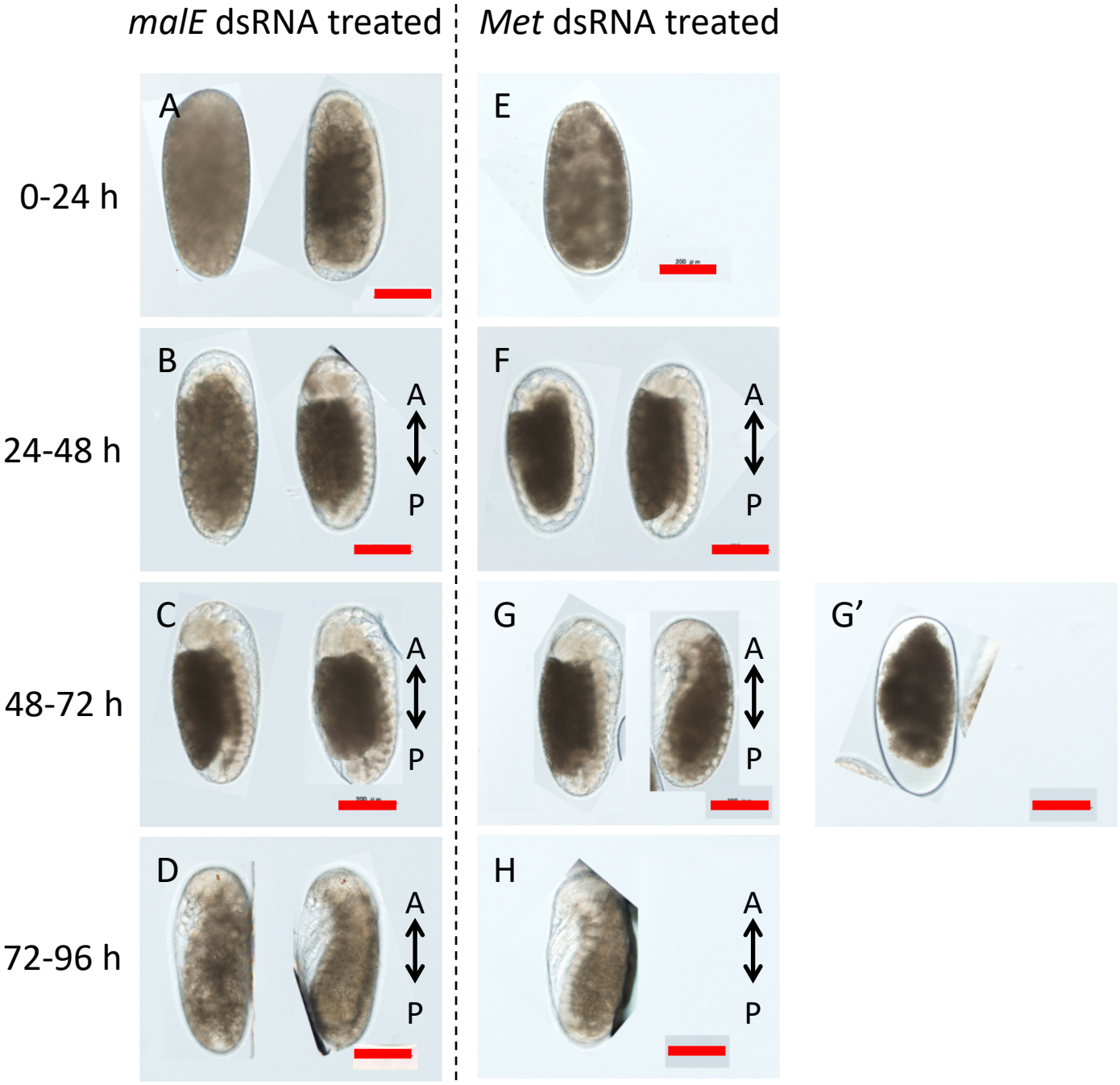

malE dsRNA treated
Met dsRNA treated
0-24 h
24-48 h
48-72 h
72-96 h
A
B
C
D
E
F
G
G'
H
A
P

Fig. 3

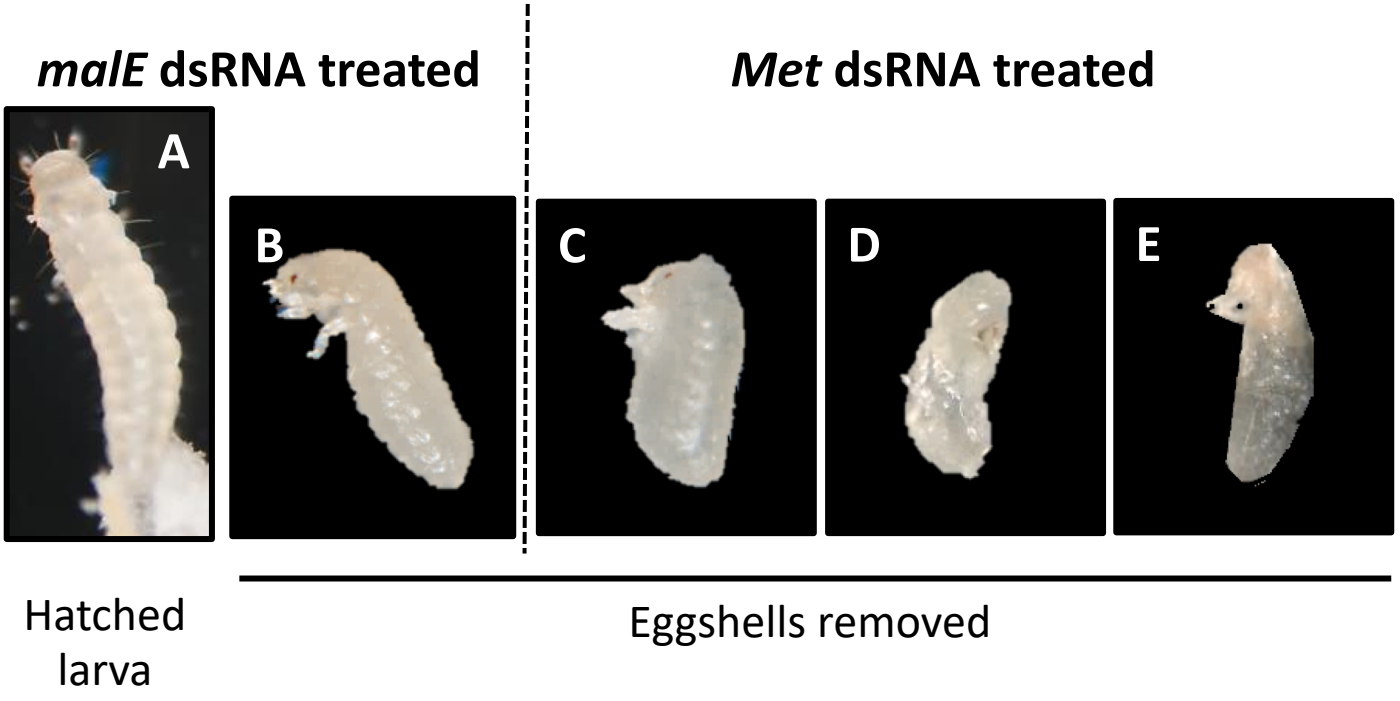

malE dsRNA treated
Met dsRNA treated
A
B
C
D
E
Hatched larva
Eggshells removed

Fig. 4

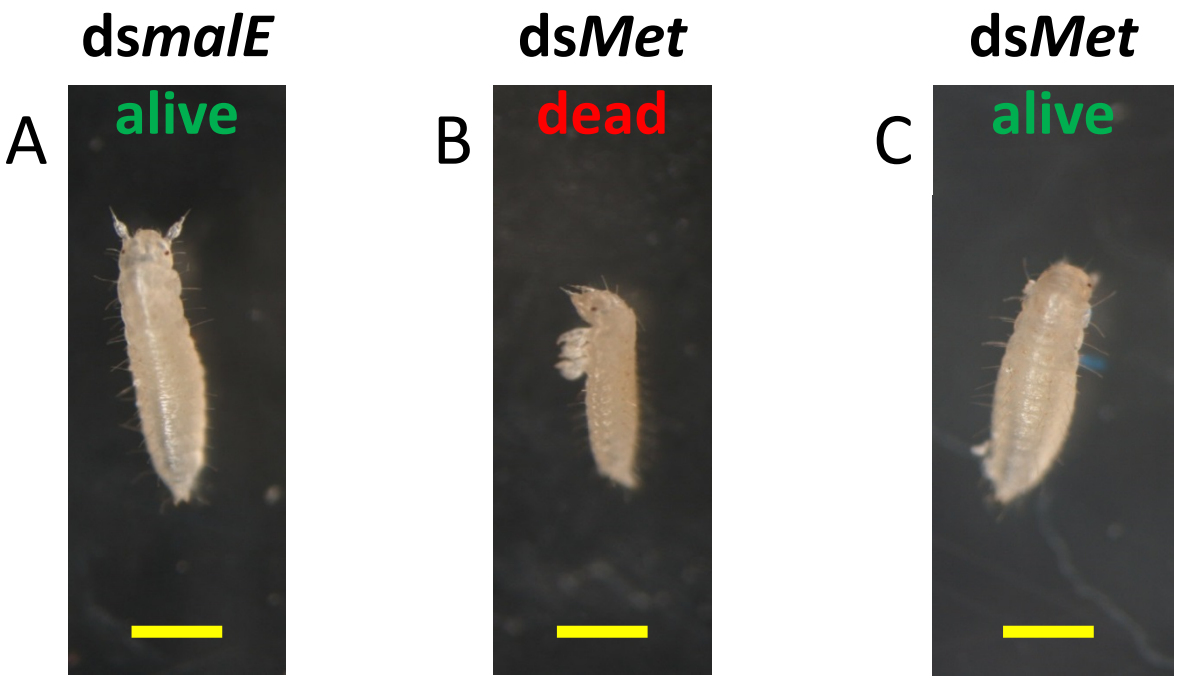


Fig. 5

Table 1 Phenotypes of unhatched embryos after parental dsRNA treatment

dsRNA	Observed time[a] (h after oviposition)	n	Unfertilized	Malformed morphology	Normal morphology (dead)	Normal morphology (alive)
No treatment	96-120	23	19 (83%)	0 (0%)	2 (8.7%)	2 (8.7%)
malE	90-96	16	9	0	4	3
	96-120	4	4	0	0	0
	(total)	**20**	**13 (65%)**	**0 (0%)**	**4 (20%)**	**3 (15%)**
Met	90-96	22	4	7	11	0
	96-120	17	1	13	3	0
	(total)	**39**	**5 (13%)**	**20 (51%)**	**14 (36%)**	**0 (0%)**

[a] Embryos that had not yet hatched were observed under the microscope after manually removing egg shells with forceps.

Ref: IP_2019_298

Title: Methoprene-tolerant is essential for embryonic development of the red flour beetle Tribolium castaneum

Journal: Journal of Insect Physiology

Author statement

Shouya Naruse: Conceptualization, Methodology, Investigation, Writing- Original Draft

Yumiko Washidu: Methodology, Investigation

Ken Miura: Writing- Review & Editing

Tetsuro Shinoda: Writing- Review & Editing

Chieka Minakuchi (corresponding author): Conceptualization, Writing- Review & Editing, Supervision, Funding acquisition

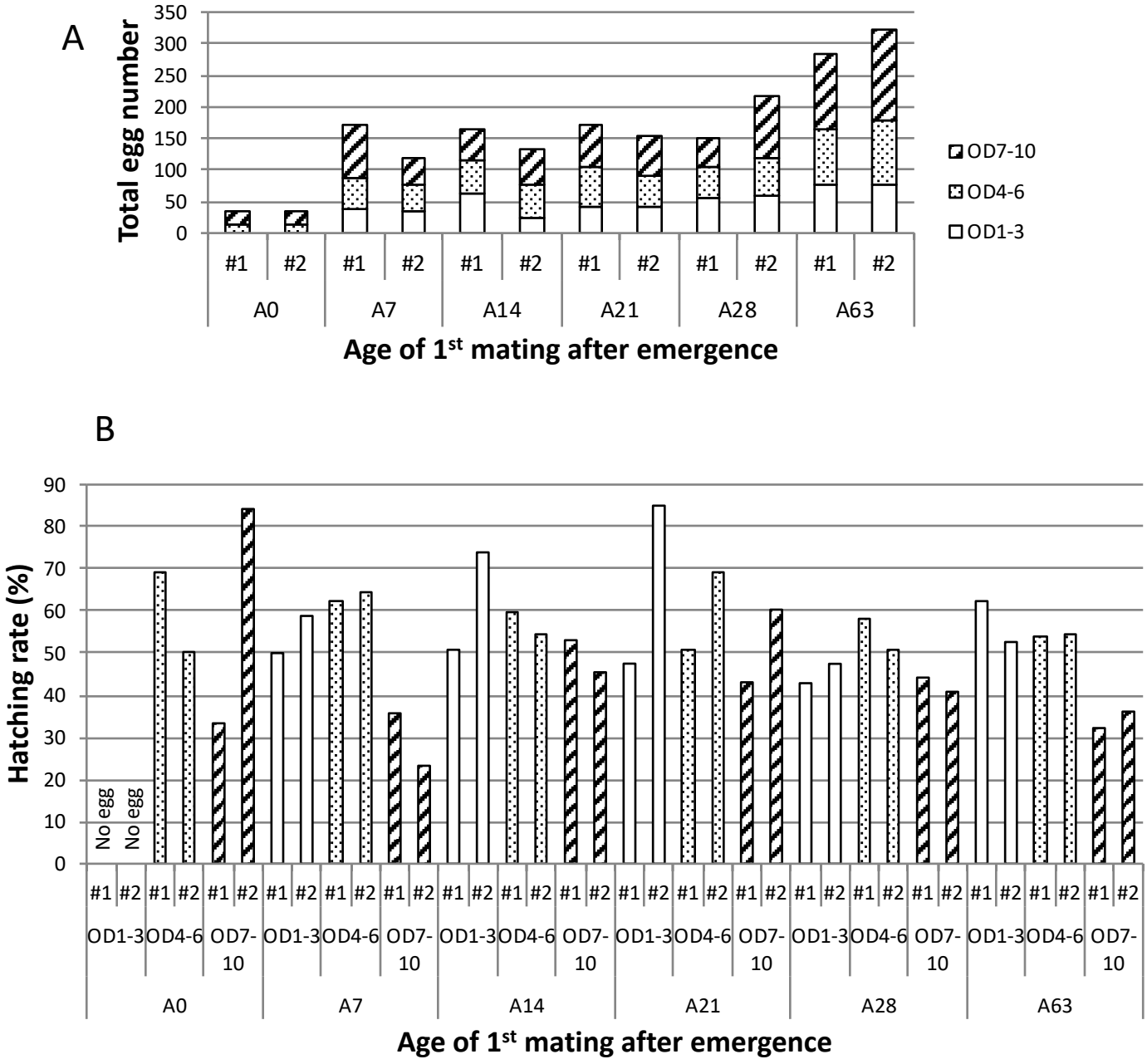


Fig. S1 Profiles of egg numbers (A) and hatching rates (B) throughout development of adult females.

(A) Three virgin females at 0, 7, 14, 21, 28, and 63 days after adult emergence (A0, A7, A14, A21, A28, and A63, respectively) were mated with 3 males, and the number of eggs was investigated every 3 or 4 days: on the first to third day of oviposition (OD1 to OD3), the fourth to sixth day of oviposition (OD4 to OD6), and the seventh to tenth day of oviposition (OD7 to OD10), according to the period after the day of mating. Day ages of the adult females when they were mated for egg collection are shown as A0, A7, A14, A21, A28, and A63, on the abscissa. Results from duplicate experiments are shown as #1 and #2.

(B) Hatching rates of the same series of eggs as Figure S1A investigated on four days after egg collection.

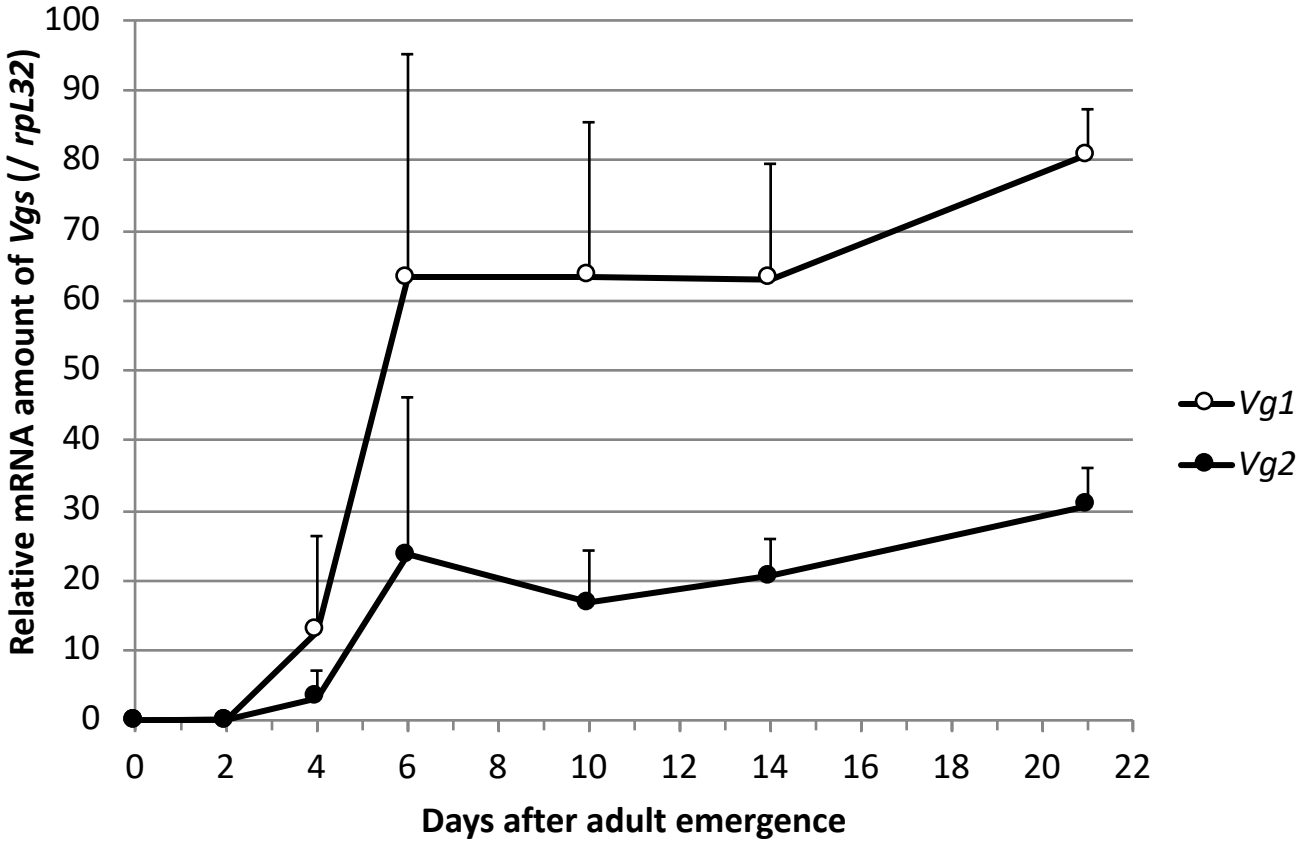


Fig. S2 Expression profiles of *Vg1* and *Vg2* in virgin female adults

Total RNA was extracted from the whole body of virgin female adults on 0, 2, 4, 6, 10, 14, and 21 days after adult emergence (A0, A2, A4, A6, A10, and A21, respectively). The relative expression levels of *Vgs* were determined by qRT-PCR in comparison with the transcript levels of *ribosomal protein L32* (*rpL32*). Mean ± S.D. of three independent replicates are shown.

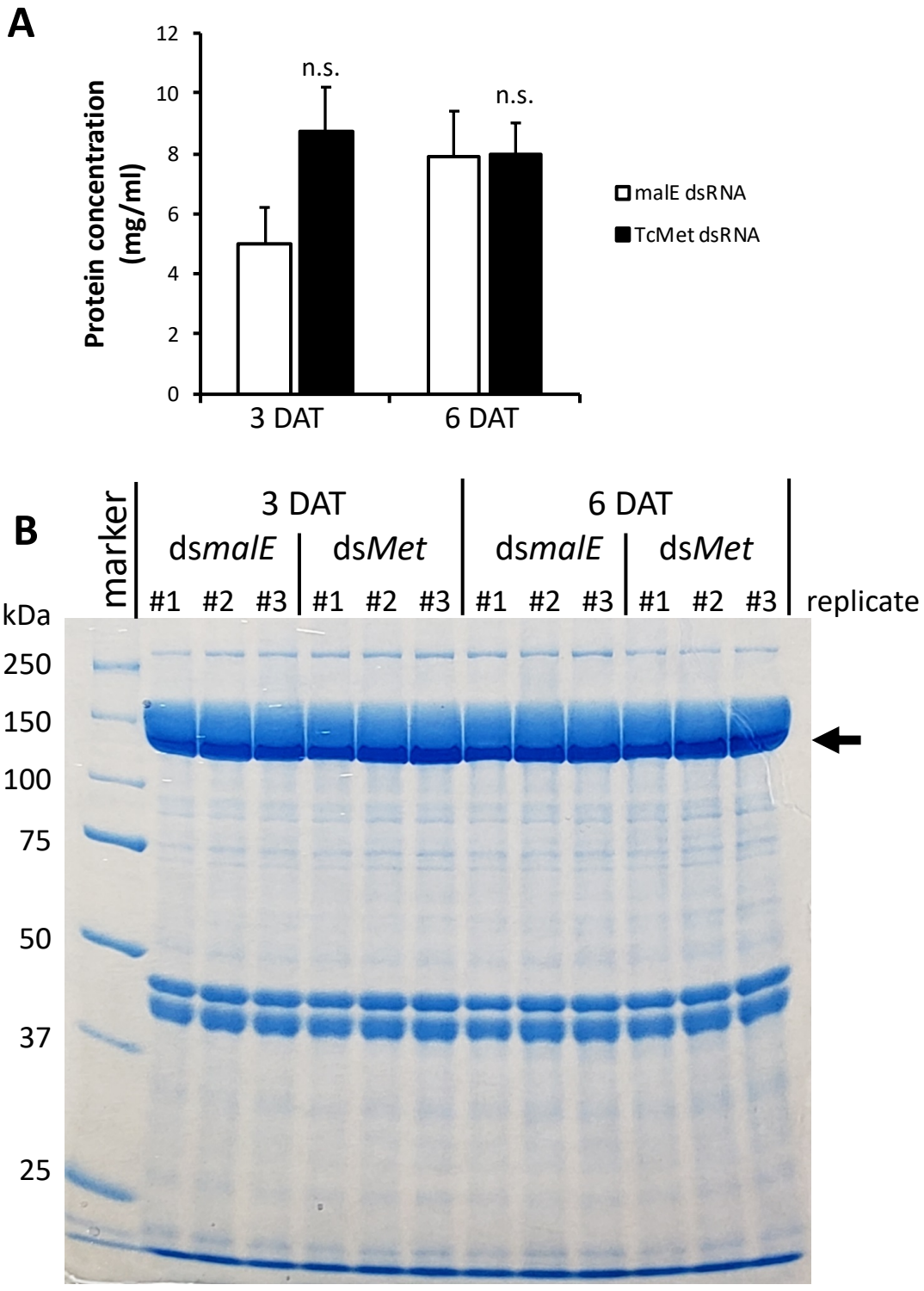


Fig. S3 Quantification (A) and SDS-PAGE (B) of proteins in the eggs laid by dsRNA-injected adult females.

Ten eggs (0—6 h from oviposition) laid by *malE* dsRNA- or *Met* dsRNA-injected females were homogenized in PBS supplemented with protease inhibitors, and centrifuged. (A) Protein concentration determined by Bradford method. n.s. indicates that there was not statistically significant difference (P>0.05, Student's t-test). (B) Protein extracts were separated on a 10% SDS-polyacrylamide gel, and visualized by Coomassie G-250. Putative Vitellin (~140 kDa) is indicated by an arrow. ds*malE*, *malE* dsRNA; ds*Met*, *Met* dsRNA; DAT, day after treatment of dsRNA to females. Numbers (#1, #2 and #3) represent replicates.

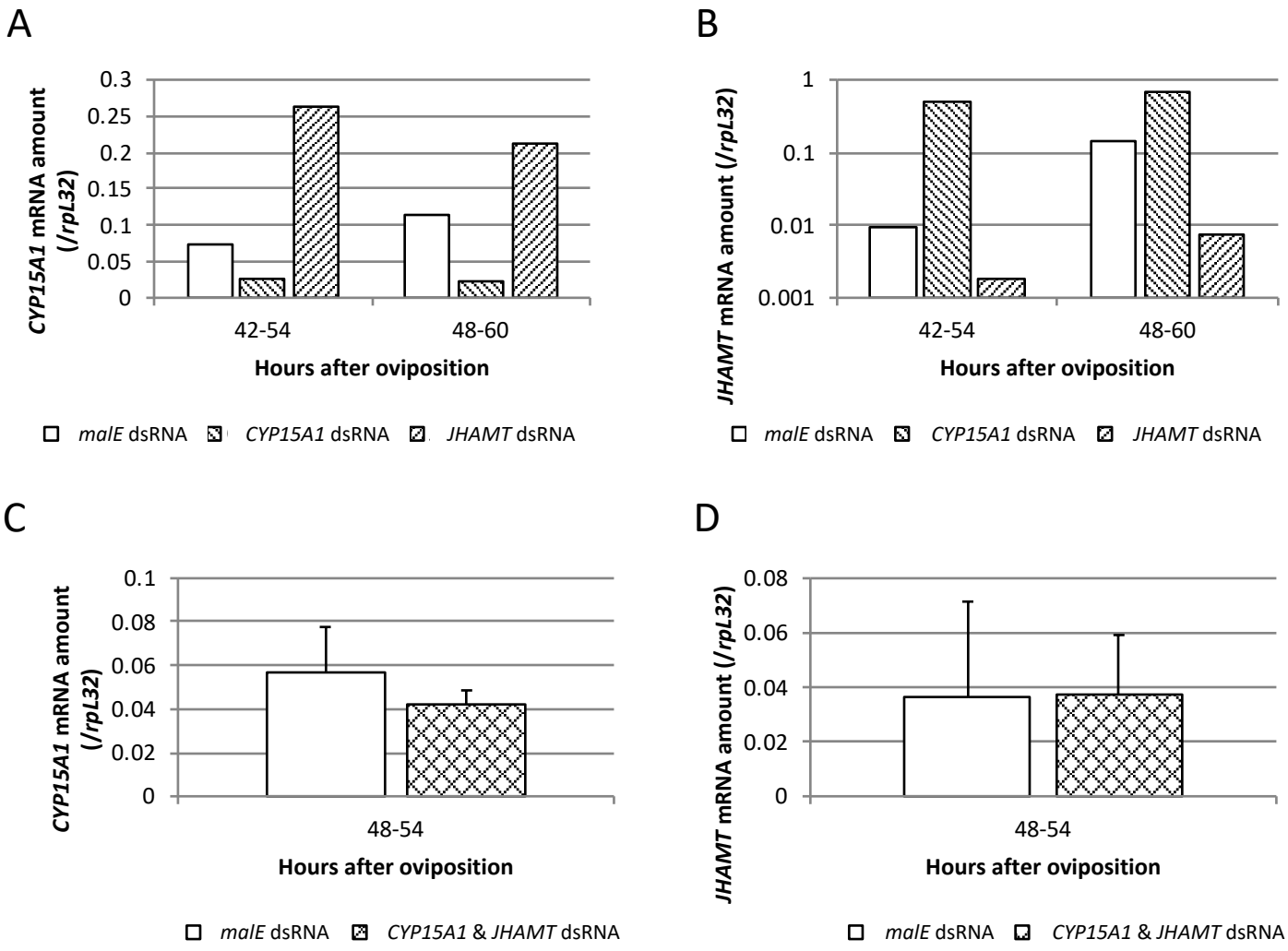


Fig. S4 Relative mRNA amounts of *CYP15A1* and *JHAMT* in eggs with single or double knockdown of *CYP15A1* and *JHAMT*

Single (A and B) or double knockdown (C and D) of *CYP15A1* and *JHAMT* were performed via dsRNA microinjection into virgin females. dsRNA of *malE* was applied as a control. The following day, the dsRNA-treated females were mated with the same-day-old males.

(A and B) The eggs laid by dsRNA-injected insects for 12 hours were collected on 4 and 8 days after dsRNA treatment (DAT). Total RNA was extracted from pools of 7 or 8 eggs [42–54 h after oviposition (HAO)] collected at 4 DAT and 10 eggs (48–60 HAO) collected at 8 DAT. The relative expression levels of *CYP15A1* and *JHAMT* were determined by qRT-PCR in comparison with the transcript levels of ribosomal protein (*rpL32*).

(C and D) The eggs laid by dsRNA-injected insects for 6 hours were collected on 4 and 5 DAT. Total RNA was extracted from pools of 10 eggs (48–54 HAO) collected at 4 and 5 DAT. The relative expression levels of *CYP15A1* and *JHAMT* were determined by qRT-PCR in comparison with the transcript levels of ribosomal protein (*rpL32*). Mean ± S.E. of two independent replicates are shown.

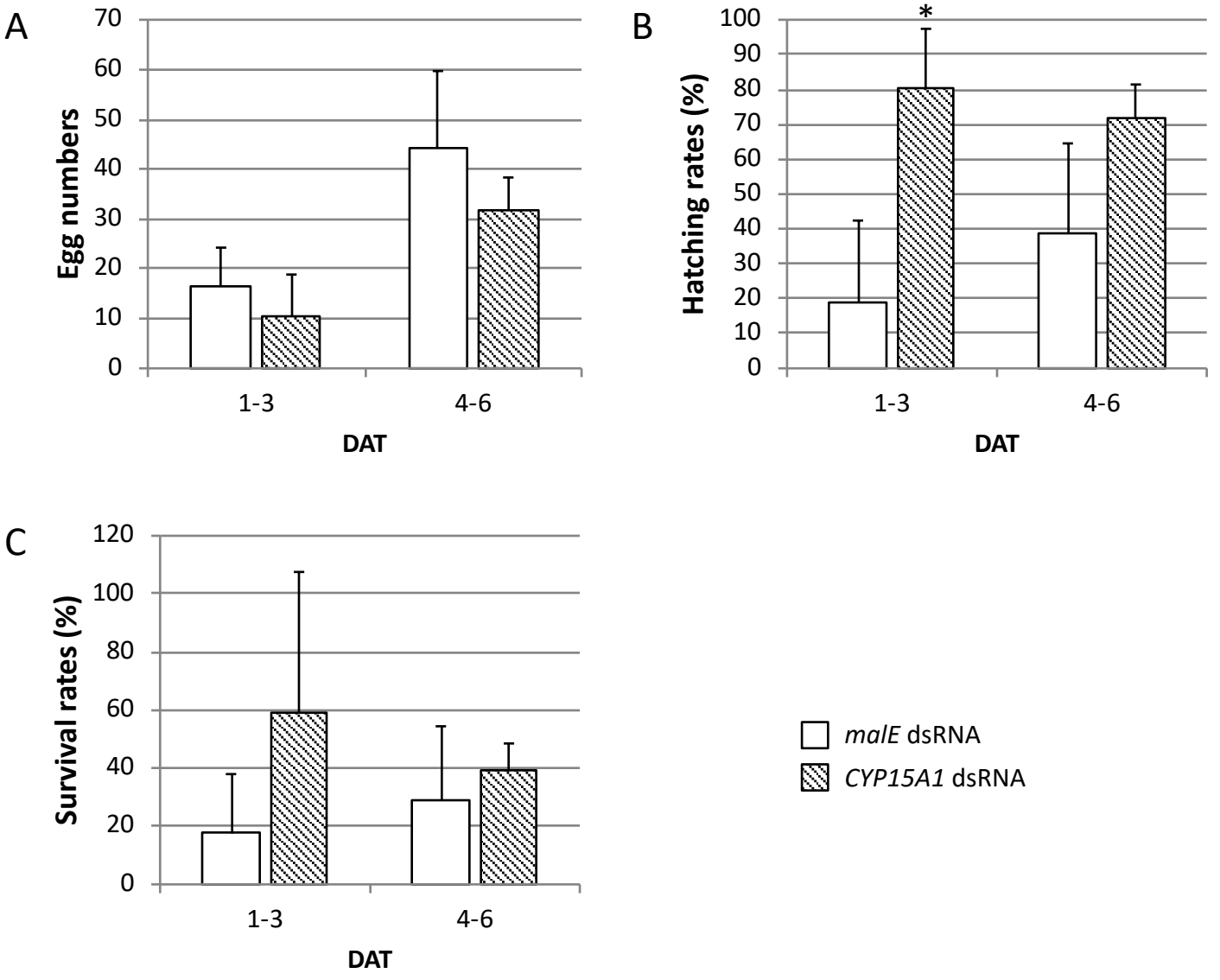


Fig. S5 Effects of single knockdown of *CYP15A1* on egg numbers (A), hatching (B) and survival in the early larval stage (C).

dsRNAs of *CYP15A1*, and *malE* (control) were injected into 22-day-old virgin female adults. The following day, the dsRNA-treated females were mated with the same-day-old males. The eggs laid by the RNAi-treated insects were collected on 1–3 and 4–6 days after dsRNA treatment (DAT) (A). Four days after egg collection, the hatching rates were investigated (B). A week after egg collection, the number of living larvae was counted and survival rates were calculated as (the number of living larvae)/(the number of eggs) (C). Mean ± S.D. of three replicates are shown. *$p < 0.05$ (Student's *t*-test), in comparison with the *malE* dsRNA-treated controls.

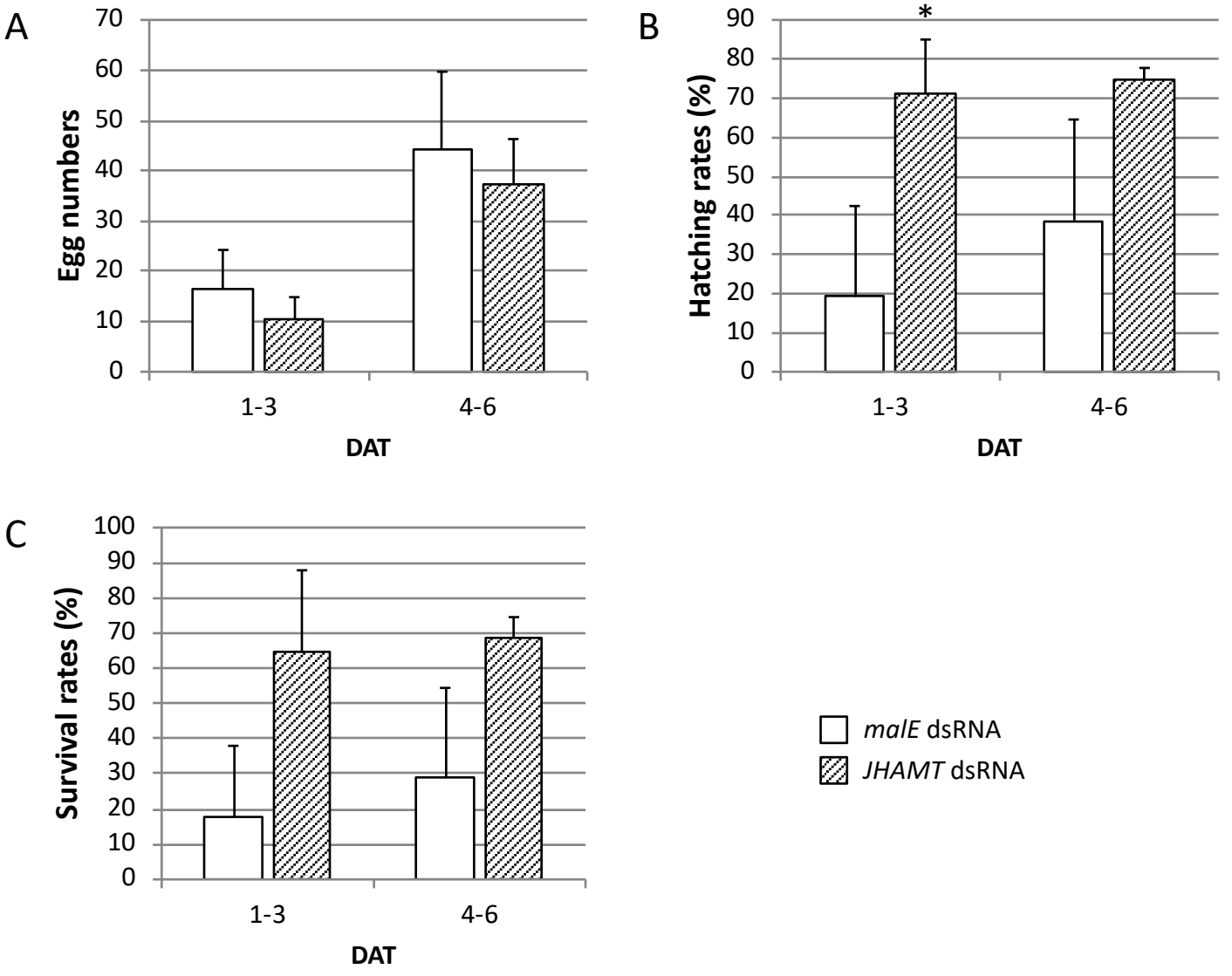


Fig. S6 Effects of single knockdown of *JHAMT* on egg numbers (A), hatching (B) and survival in the early larval stage (C).

dsRNAs of *JHAMT* and *malE* (control) were injected into 22-day-old virgin female adults. The following day, the dsRNA-treated females were mated with the same-day-old males. The eggs laid by the RNAi-treated insects were collected on 1–3 and 4–6 days after dsRNA treatment (DAT) (A). Four days after egg collection, the hatching rates were investigated (B). A week after egg collection, the number of living larvae was counted and survival rates were calculated as (the number of living larvae)/(the number of eggs) (C). Mean ± S.D. of three replicates are shown. *$p < 0.05$ (Student's *t*-test), in comparison with the *malE* dsRNA-treated controls.

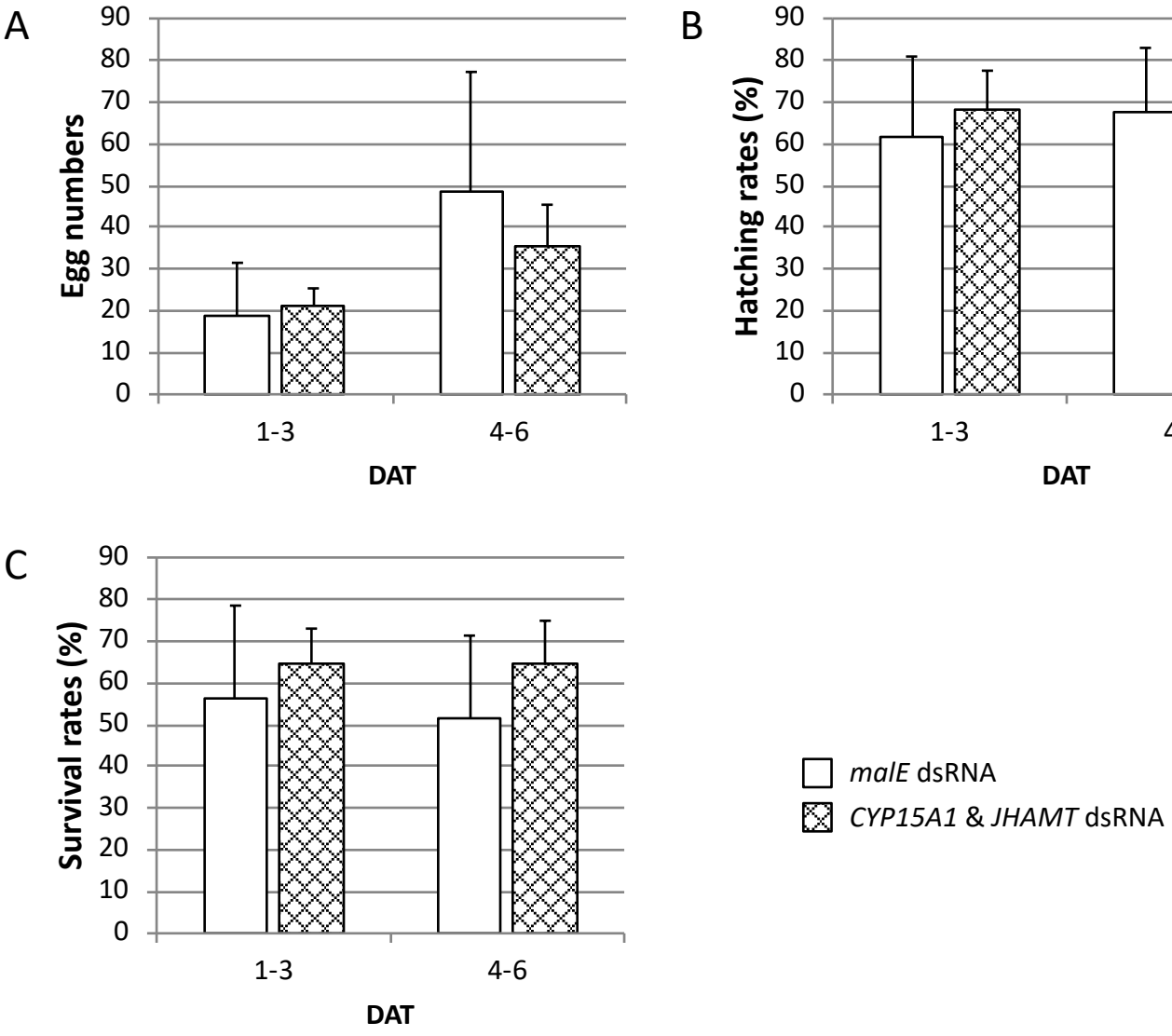


Fig. S7 Effects of double knockdown of *CYP15A1* and *JHAMT* on egg numbers (A), hatching (B) and survival in the early larval stage (C).

dsRNAs of *CYP15A1*, *JHAMT*, and *malE* (control) were injected into 20–24-day-old virgin female adults. The following day, the dsRNA-treated females were mated with the same-day-old males. The eggs laid by the RNAi-treated insects were collected and counted on 1–3 and 4–6 days after dsRNA treatment (DAT) (A). Four days after egg collection, the hatching rates were investigated (B). A week after egg collection, the number of living larvae was counted and survival rates were calculated as (the number of living larvae)/(the number of eggs) (C). Mean ± S.D. of three replicates are shown.

Table S1 Primers for qRT-PCR (A) and the synthesis of dsRNAs (B)

A	Primers	Sequences (5' – 3')
qRT-PCR	TcCYP15-QF1	ACCTGAGCGGTTTTTGAACAAGG
	TcCYP15-QR1	GCTAAAGATTCTCCCAAGCAGCG
	TcJHAMT-QF1	CATCTCGCCCTATCACCATTCG
	TcJHAMT-QR1	CCGCTGAAACCGATTTTGACAA
	TcMet-QF1	CATTGCAGGTTATATGACTGAGGAAGTGT
	TcMet-QR1	GAGTAAACGGTAACATGATGATCCTTTGCT
	TcKrh1-QF2	CCTGAGAAATTAGACTCCTTGGCAAAT
	TcKrh1-QR2	GGAGCAGCACCAGAGGGAAATTC
	TcrpL32-QF1	CAGGCACCAGTCTGACCGTTATG
	TcrpL32-QR1	GCTTCGTTTTGGCATTGGAGC
	TcVg1-QF1	CAACTCTGGCCGATCAGAAGA
	TcVg1-QR1	AGGGCTGTCGTATGCGTAAAC
	TcVg2-QF1	CCCACGTTGTTGACGCAATTG
	TcVg2-QR1	GTTTGTCGTGCTGGTTGTCTTT

B	Primers	Sequences (5' – 3')
RNAi	TcCYP15A_5_T7	TAATACGACTCACTATAGGGCCGTCAAGGAGGTGCTCACAAG
	TcCYP15A_3_T7	TAATACGACTCACTATAGGGTTTCGCCCAACAACTTCGTCC
	malE 5'T7	TAATACGACTCACTATAGGGTGATTGCTGCTGACGGGGGT
	malE 3'T7	TAATACGACTCACTATAGGGTTTCTGGGCGTTTTCCATAGTGG
	TcKr-h1_5'_T7_2	GGATCCTAATACGACTCACTATAGGTATTACACCGAAGACCCCTTGGC
	TcKr-h1_3'_T7_2	GGATCCTAATACGACTCACTATAGGCCCGAATGTTCAAATGCTCGTG
	TcMet_5'_T7	GGATCCTAATACGACTCACTATAGGGACGACCAGGGAACTGTTGAAAG
	TcMet_3'_T7	GGATCCTAATACGACTCACTATAGGCGACGGTTCGGTTTGTTGTGTTAC
	TcJHAMT_5'_T7	GGATCCTAATACGACTCACTATAGGNATGAACAAAGCCTCACTGTACTCAA
	TcJHAMT_3'_T7	GGATCCTAATACGACTCACTATAGGNCTCTGTTCCACCACCCAATGCAA