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

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Abstract

Insect juvenile hormone (JH) is well known to regulate post-embryonic development and reproduction in concert with ecdysteroids in a variety of insect species. In contrast, our knowledge on the role of JH in embryonic development is limited and inconsistent. Preceding studies indicate that JH biosynthesis or JH signaling genes are dispensable in holometabolous *Drosophila melanogaster* and *Bombyx mori*, while essential in hemimetabolous *Blattella germanica*. In the red flour beetle *Tribolium castaneum*, we performed functional analyses of key factors in JH signaling, i.e. the JH receptor *Methoprene-tolerant* (*Met*) and the early JH-response gene *Krüppel homolog 1* (*Kr-h1*) using parental RNA interference. Knockdown of *Met* resulted in a significant reduction in hatching rates and survival rates in the first and second larval instars. Meanwhile, knockdown of *Kr-h1* caused no significant effect on hatching or survival. The unhatched embryos under *Met* knockdown developed up to the late embryonic stage, but their body shape was flat and tubby compared with the controls. Attempts to suppress JH biosynthesis by parental RNA interference of JH biosynthetic enzymes were unsuccessful due to insufficient knockdown efficiency. These results suggested that *Met* but not *Kr-h1* is essential for the embryonic development of *T. castaneum*, although involvement of JH still remains to be examined. Taken together, the function of *Met* in embryonic development seems to be diverse among insect species.

Keywords

Juvenile hormone; Methoprene-tolerant; Embryonic development: *Tribolium castaneum*

1. Introduction

In post-embryonic development of insects, molting and metamorphosis are regulated by the molting hormone (ecdysone) and juvenile hormone (JH) (Riddiford, 1993). In general, JH is biosynthesized continuously by and released from the corpora allata (CA) in the juvenile stage. With abundant JH in the hemolymph, the molting hormone triggers larval-larval molts repeatedly. Once the larvae attain a certain body size, called the critical weight, JH biosynthesis ceases, and the molting hormone then causes metamorphic molts from larvae to pupae then to adults. Metamorphic molts proceed normally only with extremely low JH levels in the hemolymph: if an excessive dosage of chemicals with JH-like activity are applied when endogenous JH is negligible, metamorphic changes are prevented, resulting in developmental arrest and lethality. Thus, JH has an anti-metamorphic action suppressing ecdysone-induced metamorphic events in post-embryonic development.

In the last two decades, the molecular mechanism of JH signaling has been elucidated: Methoprene-tolerant (Met), a basic helix-loop-helix–Per-Arnt-Sim transcription factor, constitutes the JH receptor, with its partner Taiman (Li et al., 2011, Zhang et al., 2011). A liganded Met-Taiman complex induces the transcription of an early JH response gene *Krüppel homolog 1* (*Kr-h1*) (Jindra et al., 2013; Kayukawa et al., 2012; Li et al., 2014; Minakuchi et al., 2009). Subsequently, JH regulates the transcription of downstream key players such as the pupal specifier *broad* (Konopova and Jindra, 2008; Suzuki et al., 2008; Zhou and Riddiford 2002) and the adult specifier *E93* (Ureña et al., 2014), via direct binding of Kr-h1 to Kr-h1 binding sites in their upstream regulatory regions (Kayukawa et al., 2016, Kayukawa et al., 2017).

Meanwhile, our knowledge of the molecular mechanism of JH action in embryonic development is still limited, in contrast to the substantial progress on JH actions in post-embryonic development. Recent genetic studies revealed to some extent the role of JH in the embryonic and early larval stages in a few insect species. For example, in the fruit fly *Drosophila melanogaster*, genetically allatectomized larvae by expressing a cell death gene

ectopically in the CA survived the larval stage, but the defect became lethal around the time of head eversion (Riddiford et al., 2010). In addition, a double mutant of *Met* and its paralog *Germ cell-expressed* (*Gce*) resulted in lethality at the larva-pupa transition (Abdou et al., 2011). Thus, *D. melanogaster* can survive up to the end of the larval stage even with suppressed JH biosynthesis and signaling. In the silkworm *Bombyx mori*, a GAL4/UAS system-driven overexpression of a JH esterase gene that is involved in JH degradation resulted in precocious larval-pupal metamorphosis in the third larval instar without any detectable physiological change in embryonic development (Tan et al., 2005). In *B. mori*, JH-deficient *mod* mutants that lack the function of the JH-biosynthetic enzyme CYP15C1 survived to the embryonic stage and died at the third or fourth larval instar (Daimon et al., 2012). In addition, knockout of *JH acid O-methyltransferase* (*JHAMT*) encoding a critical enzyme for JH biosynthesis (Shinoda and Itoyama, 2003) resulted in a delay in embryonic development and reduced hatchability with a defect in shedding eggshells, but they could be rescued via removing eggshells with a knife in *B. mori* (Daimon et al., 2015). *Met1* mutants of *B. mori* also resulted in a slightly reduced hatching rate and delayed embryonic development, but most of them were able to hatch, and were subsequently arrested during molting from the second larval instar (L2) to the third larval instar (L3) (Daimon et al., 2015). These findings suggested that, although suppression of JH biosynthesis or JH signaling during embryonic stages caused minor defects, JH is not essential for the embryonic development of *B. mori*. The role of JH in embryonic development has also been studied in hemimetabolous insect species. In the cockroach *Blattella germanica*, parental RNA interference (RNAi) targeting *Kr-h1*, *Met*, or *JHAMT* resulted in arrest in mid and late embryogenesis, suggesting that JH signaling is essential for embryonic development in this species (Fernandez-Nicolas and Belles, 2017). Thus, the embryonic roles of JH seem to be diverse among insect species. Therefore, further investigation in various insect species will be needed in order to unravel JH function in embryonic development.

In this study, we examined the role of two transcription factor genes in JH signaling, *Met*

94 and *Kr-h1*, in the embryonic stage of the red flour beetle *Tribolium castaneum* (Coleoptera:
95 Tenebrionidae) to elucidate the embryonic role of JH in this species. Previously, we showed that
96 the transcript levels of JH biosynthetic enzymes *JHAMT* and *CYP15A1* increased in the middle
97 of the embryonic stage, suggesting that JH biosynthesis in CA started at this time point
98 (Minakuchi et al., 2015). To knockdown these genes, we utilized parental RNAi by injecting
99 double-stranded RNA (dsRNA) into adult females. A previous study showed that JH regulates
100 the transcription of *vitellogenins* (*Vgs*) in the fat body and oocyte maturation (Parthasarathy et
101 al., 2010b); therefore, we first monitored the transcript levels of *Vgs* in females after dsRNA
102 injection, and avoided using individuals with suppressed *Vg* transcripts after *Met* or *Kr-h1*
103 knockdown for further analysis. Knockdown of *Met* remarkably reduced hatching and survival
104 rates in the first and second larval instars, with defects in body shape. This study provided
105 evidence that *Met* plays important roles in embryonic development of *T. castaneum*.
106
107 **2. Materials and Methods**
108 **2.1. Insect rearing**
109 The wild-type strain of *T. castaneum* was provided by the National Agriculture and Food
110 Research Organization (Ibaraki, Japan). *T. castaneum* adults were fed on whole wheat flour in
111 the dark at 30 ± 1°C as described previously (Minakuchi et al., 2008).
112 **2.2. Examination of egg numbers and hatching rate**
113 Virgin adult females were mated with adult males. Three pairs of both sexes were kept in
114 a plastic container with 2.0 g of whole wheat flour, and the eggs were collected every 3–4 days.
115 The number of hatched larvae was counted 4–5 days after egg collection to calculate the
116 hatching rate as (number of hatched larvae)/(number of eggs laid).
117 **2.3. Parental RNAi of *Met* and *Kr-h1***
118 dsRNAs of *Met* and *Kr-h1* were synthesized as follows. Using the respective primer pairs

(Table S1) and Ex Taq HS DNA polymerase (TAKARA Bio), cDNA fragments with T7 promoter sequences on both ends were generated by RT-PCR and then purified using a QIAquick PCR Purification Kit (QIAGEN). The purified cDNA fragments were utilized as templates for dsRNA synthesis using a MEGAscript RNAi Kit (Ambion). As a negative control, a dsRNA fragment containing a partial *maltose binding protein E* (*malE*) sequence was also prepared as described previously (Yokoi et al., 2012).

Virgin female adults (14–28 days after adult emergence) were anesthetized using diethyl ether for 3–5 min and lined up on double-sided sticky tape. Then, 5 μg/μl dsRNA solution (approximately 0.20 μl for each insect) was injected into the abdominal hemocoel using a fine glass needle prepared using a needle puller (Narishige) and 3.5" Drummond glass capillary tubes. A group of dsRNA-treated females were allowed to mate with the same number of untreated males on the following day, and they were reared at 30 ± 1°C. Eggs were collected and counted every 3 days: 1–3 days after treatment (DAT) and 4–6 DAT. Four days after each egg collection, the number of hatched larvae was counted from the pooled egg population, and the hatching rate was calculated as above. Finally, the number of living larvae was counted 7 days after egg collection, and the survival rate of larvae was calculated as (number of living larvae)/(number of eggs laid).

To observe the morphology of embryos, adult females that had received *Met* or *malE* dsRNA were mated with untreated males on the following day, and the eggs were collected every 24 h. Eggs collected at 0–24 h, 24–48 h, 48–72 h, and 72–96 h after oviposition were rinsed with distilled water and then soaked in diluted breach containing 3% sodium hypochlorite for 2–3 min to remove the chorion. After rinsing with distilled water again, the eggs were mounted on a glass slide with Slide Seal for *in situ* PCR (Takara Bio) with phosphate-buffered saline, and the eggs were observed using an Olympus BX41 microscope.

To examine the morphological features of unhatched eggs in more detail, the egg shells of *Met*-knockdown embryos at 96–120 h after oviposition were carefully removed with fine forceps, and their morphology was observed using a microscope.

2.4. Quantitative RT-PCR analysis

To examine the efficiency of RNAi-mediated gene knockdown, total RNA was isolated using TRIzol reagent from either the whole body of individual adults or pooled eggs. First-strand cDNA was synthesized with a PrimeScript RT reagent with gDNA Eraser (Takara Bio).

To quantify the transcripts, qRT-PCR reactions were performed using SYBR Premix Ex Taq (Takara Bio) and Thermal Cycler Dice Real Time System TP800 (Takara Bio). The PCR conditions were 95°C for 30 sec followed by 45 cycles at 95°C for 5 sec and 60°C for 30 sec. The homogeneity of the amplicon was confirmed by melting curve analysis. Standard curves were prepared by serial dilution of plasmid DNAs containing the corresponding sequence of each target gene and utilized for quantitation. The amount of target cDNA of each sample was normalized to those of *T. castaneum ribosomal protein L32* (*rpL32*) in the same sample. Primer sequences were listed in Table S1.

2.5. Protein quantification and SDS-PAGE of egg extracts

Eggs within 6 h from oviposition laid by dsRNA-injected females were collected on 3 days after dsRNA treatment (3 DAT) and 6 days after dsRNA treatment (6 DAT). Ten eggs were pooled in a replicate, and the experiments were performed in triplicates. A pooled 10 eggs were homogenized in 20 µl of phosphate-buffered saline containing Protease Inhibitor Cocktail Set I (Fujifilm Wako), and the supernatant was collected after centrifugation (12,000 rpm for 10 min at 4°C). The extracted proteins were quantified with Protein Assay Reagent (Bio-Rad) with bovine serum albumin as the standard. In the meantime, the extracted proteins were mixed with

3x sample buffer (150 mM Tris-HCl of pH 6.8, containing 3% SDS, 9% 2-mercaptoethanol, 30% glycerol, and bromophenol blue), and denatured at 65°C for 2 min. Then, the denatured crude proteins (8 µl/lane; approximately 3 eggs equivalent/lane) were separated on a 10% SDS-polyacrylamide gel. After electrophoresis, proteins were visualized by with SimplyBlue Safe Stain (ThermoFisher Scientific) containing Coomassie G-250.

3. Results

3.1. Efficiency of RNAi-mediated gene knockdown of *Met* and *Kr-h1* and its effect on *Vg* transcription

We first determined the suitable range of age in days of adult females for parental RNAi experiments, using virgin adult females from A0 to A63. As shown in Fig. S1A, the number of eggs laid by females that were allowed to mate on A0 was low, but egg numbers then increased consistently by about 3- to 5-fold between A7 and A28, followed by a slight increase in A63 females. We also examined the hatching rate for each group of collected eggs and found that the hatching rate was almost consistent regardless of the age of mating, although moderate deviation among categories existed (Fig. S1B). In addition, the levels of *Vg1* and *Vg2* mRNA were determined in order to estimate the timing when females enter the vitellogenic phase. The levels of both *Vg1* and *Vg2* mRNA were relatively low until A4, they then increased and reached high and stable levels after A6 (Fig. S2). Based on these results, we decided to use adult females from A14 to A28 for parental RNAi experiments because the egg numbers, hatching rate and the levels of *Vg* mRNAs were reasonably stable.

Parental RNAi for *Met* and *Kr-h1* was performed on this basis using adult females from A14 to A28 to examine the role of JH signaling in embryonic stages. Firstly, the changes in transcript levels of *Met*, *Kr-h1*, and *Vg1* were investigated in dsRNA-treated adult females using

qRT-PCR. Upon *Met* dsRNA injection, the transcript level of *Met* was lower compared with *malE* dsRNA-treated control females, and the differences were statistically significant for those of 4 DAT and 11 DAT (Fig. 1A). In addition, *Kr-h1* dsRNA injection did not influence the transcript level of *Met*. Similarly, the transcript level of *Kr-h1* tended to decline after injecting *Kr-h1* dsRNA compared with *malE* dsRNA-injected controls (Fig. 1B). *Met* dsRNA treatment resulted in a reduction in *Kr-h1* transcript levels.

The transcript levels of *Vg* in females were also measured in knockdown conditions. The transcript levels decreased gradually from 4 DAT to 7 DAT with *Met* or *Kr-h1* knockdown and were significantly suppressed on 11 DAT in the case of *Kr-h1* knockdown compared with the controls (Fig. 1C). We inferred that *Vg* was still transcribed appreciably, along with Vg protein synthesis, at least until 7 DAT of *Met* or *Kr-h1* dsRNA, but began to decline toward 11 DAT. Therefore, we decided to use eggs that had been collected up to 7 DAT. To confirm that the yolk protein was not significantly reduced by *Met* knockdown, proteins in newly-laid eggs were quantified, and analyzed by SDS-PAGE (Fig. S3). Regarding the concentration of extracted protein, there was no statistically significant difference between controls and *Met* knockdown group (Fig. S3A). A previous study by Bai and Palli reported that two bands (~200 kDa and ~170 kDa) were detected by Western blotting of hemolymph and ovary extracts using a specific anti-Vg antibody, suggesting that these correspond to two subunits of Vg protein (Bai and Palli, 2016). Although we were not able to characterize the identity of each protein band in our SDS-PAGE analysis (Fig. S3B), the most intense band (~140 kDa) is likely to be the yolk protein vitellin, which has been processed form Vg. Importantly, the profiles of extracted proteins were comparable between controls and *Met* knockdown group (Fig. S3B).

3.2. Effects of parental RNAi of *Met* and *Kr-h1* on the progenies

The effects of knockdown of *Met* or *Kr-h1* were examined in eggs laid on 1–3 and 4–6 DAT. Both *Met* and *Kr-h1* knockdown reduced the number of eggs laid on 4–6 DAT at about 70% compared with the controls, though the reduction was statistically significant only in the *Met* knockdown (Fig. 2A).

Hatching rates were investigated by counting the number of larvae that hatched by 4 days after egg collection. The hatching rate was decreased slightly by *Met* knockdown at 4–6 DAT compared with *malE* dsRNA-injected controls with an observed hatching rate of 18 ± 5.5% (Mean ± S.D.) after *Met* dsRNA injection and 43 ± 14% after *malE* dsRNA injection (Fig. 2B). Importantly, almost all hatched larvae after *Met* knockdown died in a couple of days; the survival rate was 0.79 ± 1.6% at 1–3 DAT and 0.80 ± 0.92% at 4–6 DAT with *Met* dsRNA, and 19 ± 11% at 1–3 DAT and 34 ± 13% at 4–6 DAT with *malE* dsRNA (Fig. 2C). We should note here that the seemingly low survival rates of *malE* controls can be ascribed to similarly low hatching rate of the controls because we calculated the survival rate as (number of living larvae)/(number of eggs laid), not by (number of living larvae)/(number of hatched larvae). Meanwhile, in the case of *Kr-h1* knockdown, hatching and surviving rates were not significantly influenced (Figs. 2B and 2C).

Next, we tried to suppress JH biosynthesis by injecting dsRNA against the JH biosynthetic enzymes *CYP15A1* and *JHAMT*. Of note, parental RNAi of *CYP15A1* suppressed the amount of *CYP15A1* mRNA (Fig. S4A) in the eggs, but resulted in the elevation of *JHAMT* mRNA (Fig. S4B). Conversely, *JHAMT* parental RNAi resulted in a reduction in *JHAMT* mRNA (Fig. S4B) and elevation of *CYP15A1* mRNA levels in the resultant eggs (Fig. S4A). We further tried to suppress JH biosynthesis by knocking down both *CYP15A1* and *JHAMT*, but the results were still unsuccessful (Figs. S4C and S4D). Under the knockdown trials of *CYP15A1*, *JHAMT*, or both *CYP15A1* and *JHAMT*, the numbers of eggs, hatching and larvae survival rates

were not significantly suppressed in any cases (Figs. S5, S6, and S7).

3.3. Morphology of embryos and larvae with *Met* knockdown

As stated above, knockdown of *Met* caused severe lethality in the embryonic stage and following the first larval instar. We examined the morphology of the embryos in eggs after dechorionizing with sodium hypochlorite solution. In our hands, embryogenesis of *T. castaneum* at 30°C lasts for approximately 90 h from oviposition until hatching. At 25°C, embryonic events such as gastrulation (5 h after oviposition), germ band elongation (15–35 h), germ band retraction (45–95 h), and dorsal closure (105 h), are observed in 120-h development (Strobl et al., 2015). Based on this report, we estimated the approximate timing of each event at 30°C as follows: gastrulation (approximately 4 h after egg laying), germ band elongation (approx. 11–26 h), germ band retraction (approx. 34–71 h), and dorsal closure (approx. 79 h). As shown in Fig. 3 (left panels), embryos in *malE* dsRNA-treated groups seem to develop normally: formation of appendages progresses in 24–48 h and 48–72 h embryos, and dorsal closure is observed in 72–96 h embryos. Similar developmental events were visible in the *Met* knockdown group (Fig. 3, right panels), although a few arrested embryos were also found (Fig. 3G'). Thus, no clear differences between the *Met* knockdown group and *malE* dsRNA-treated controls were observed in their external morphology.

To examine the morphology in more detail, we removed the eggshells from unhatched eggs in *malE* dsRNA-injected controls, *Met* knockdown groups, and the groups without any parental RNAi treatment. As shown in Table 1, the majority of unhatched embryos in the controls without dsRNA treatment and *malE* dsRNA-treated controls were unfertilized (83% and 65%, respectively). In *Met* dsRNA-treated group, by contrast, embryos with malformed morphology (51%) and those with normal external morphology but had been arrested (36%)

were found. Morphological defects, such as flat and tubby, twist, or uncured, associated with *Met* knockdown were recognizable (Figs. 4C-4E) in comparison with the controls (Fig. 4B).

The morphology of a few hatched larvae after *Met* dsRNA treatment was also distinct from the controls. Most of the hatched larvae in the *Met* knockdown groups exhibited a shorter, flattened appearance, and they looked less mobile than the control ones (Fig. 5).

4. Discussion

In this study, we aimed to investigate the roles of *Met* and *Kr-h1* in embryonic development by adopting parental RNAi. The role of JH in reproduction in adult females has been well-established in several insect species: in the common bed bug *Cimex lectularius* and the linden bug *Pyrrhocoris apterus*, development of the ovary and the transcription of *Vg* genes were regulated by JH (Gujar and Palli, 2016, Smykal et al., 2014). Parthasarathy and colleagues reported that *Met* knockdown in *T. castaneum* pupae via dsRNA injection resulted in a reduction of *Vg* transcripts in adults (Parthasarathy et al., 2010b). These studies raised a possibility that *Met* knockdown in *T. castaneum* might suppress *Vg* transcription, which inhibits normal oogenesis. Therefore, we first examined the age when adult females were suitable for parental RNAi experiments, and we decided to use females from A14 to A28, when reproductive maturation seemed to be almost completed (Figs. S1 and S2). Although *Vg1* mRNA levels decreased after *Met* or *Kr-h1* dsRNA injection (Fig. 1), oogenesis and oviposition must proceed within a few days after dsRNA injection, using the Vg protein that had been synthesized and stored. We confirmed that neither the amount nor the profile of egg proteins was affected by parental RNAi of *Met* (Fig. S3). Using the same insect species, it has been reported that the expression of *Vg* was significantly suppressed by RNAi-mediated knockdown of either *Kr-h1* or *Met*, which resulted in lower egg numbers (Parthasarathy et al., 2010a, Parthasarathy et al.,

2010b). We think that the discrepancy between their study and ours may be due to a difference in experimental conditions for dsRNA treatment: Parthasarathy and colleagues performed dsRNA injection in 2 day-old pupae and assessed egg numbers from the fourth day until the eleventh day post-adult emergence, whereas we injected dsRNA into females from A14 to A28, in which enough Vg protein was likely to be stored. Thus, in our experimental conditions, we were able to analyze the role of *Met* and *Kr-h1* in embryonic development with parental RNAi tools with little effect on Vg protein synthesis and consequently on the number of eggs laid.

The hatching rate of eggs after *Met* knockdown was lower than the controls at 4–6 DAT (Fig. 2B), suggesting that *Met* is important in embryonic development in *T. castaneum*. We further verified the phenotype in more detail by observing the morphology of the embryos every 24 hours after oviposition. However, no remarkable defects were observed in morphology in the *Met* knockdown groups compared with *malE* dsRNA-treated controls throughout the whole embryonic stage (Fig. 3), indicating that most developmental events occured normally up to late embryonic stage. In *B. mori*, knockout of *JHAMT* resulted in decreased hatchability, but unhatched embryos could be rescued via removing the eggshells with a knife (Daimon et al., 2015). Referring to the *B. mori* study, we removed eggshells using forceps from the unhatched eggs of *T. castaneum* in *Met* knockdown groups and observed their morphology.

Firstly, we noticed that the eggshell-removed embryos as well as a few hatched larvae looked flat and tubby after *Met* knockdown compared with the controls (Figs. 4 and 5). This phenotype could be attributed to defects in the formation of the exoskeleton or muscles. Insect exoskeleton is composed of a wax layer, cuticular layer, and undermost epidermis. In *T. castaneum*, parental RNAi of genes involved in cuticular formation such as *Knickkopf*, *Retroactive*, and *chitin synthase-A* affected the layer structure of their progeny's cuticle and decreased hatchability (Chaudhari et al., 2015), which resembled the phenotypes we observed

311 with *Met* knockdown. In the cockroach *B. germanica*, parental RNAi of *Met* elevated the
312 expression of *Laccase 2*, a phenoloxidase involved in cuticular tanning, and resulted in arrest in
313 mid embryogenesis (Fernandez-Nicolas and Belles, 2017). We inferred that *Met* could be
314 involved in cuticular formation or its tanning of the first instar larvae of *T. castaneum*.

315 Secondly, we noticed that the first instar larvae in the *Met* knockdown group were less
316 mobile compared with the controls. This suggested that *Met* has roles in biological events such
317 as neurogenesis and myogenesis during embryonic development, and its knockdown might have
318 affected hatching behavior.

319 By contrast, knockdown of *Kr-h1*, which is located epistatically downstream of *Met* in JH
320 signaling, did not cause similar phenotypes to *Met* knockdown. Although we have not examined
321 the knockdown efficiency in the resulting embryos, this might be lower in the *Kr-h1* RNAi
322 groups than the *Met* RNAi groups. Alternatively, it is possible that *Met* regulates embryonic
323 development without going through *Kr-h1*.

324 To date, functional analyses of *Met* have been performed in several insect species. In *B.*
325 *mori*, most *Met1* mutants were viable throughout the embryonic stage and then arrested during
326 molting from L2 to L3 (Daimon et al., 2015). In *D. melanogaster*, a double mutant of *Met* and
327 its paralog *Gce* survived up to the end of the larval stage, but became lethal at the larva-pupa
328 transition (Abdou et al., 2011). In *B. germanica*, in contrast, knockdown of *Met* caused arrest in
329 mid and late embryogenesis (Fernandez-Nicolas and Belles, 2017). In unhatched oothecae from
330 *Met* dsRNA-treated females of *B. germanica*, 25% of the embryos were interrupted in early
331 embryogenesis, while 34% of the embryos were arrested in mid embryogenesis exhibiting
332 malformations such as unsealed dorsal closure and shorter appendages, and 41% were arrested
333 in late embryogenesis with relatively normal morphology but not hatching (Fernandez-Nicolas
334 and Belles, 2017). In this study, *Met* knockdown in *T. castaneum* resulted in malformed

335 morphology (51% of the unhatched embryos) and arrested embryos with normal external
336 morphology (36%). These results suggested that *Met* is essential for the embryonic development,
337 mainly in mid and late embryogenesis. Thus, the function of *Met* in embryonic development in *T.*
338 *castaneum* is somewhat similar to that in *B. germanica*. Nevertheless, the function of *Met* seems
339 to be quite diverse among insect species.

340 To determine whether or not the phenotype of *Met* knockdown is due to suppressed JH
341 signaling, parental RNAi of genes encoding JH biosynthetic enzymes, *JHAMT* and *CYP15A1*,
342 was performed. Unexpectedly, the knockdown of either *JHAMT* or *CYP15A1* resulted in
343 upregulation of the other gene (Fig. S4). In general, the transcription of genes encoding
344 biosynthetic enzymes is strictly regulated by a number of factors including the amount of its
345 substrates. In *T. castaneum*, JHAMT enables to methylate both farnesoic acid and
346 epoxyfarnesoic acid (JH acid) (Minakuchi et al., 2008) and CYP15A1 epoxydize both farnesoic
347 acid and methyl farnesoate (Minakuchi et al., 2015) Therefore, we hypothesized this
348 up-regulation might occur due to the accumulation of their substrates: knockdown of either
349 *JHAMT* or *CYP15A1* resulted in increased amounts of their common substrate, farnesoic acid,
350 which might in turn up-regulate the transcription of *JHAMT* and *CYP15A1* that catalyzes
351 farnesoic acid. Alternatively, the knockdown of *JHAMT* and *CYP15A1* might have successfully
352 inhibit the JH biosynthesis in some extent, and the declined JH titer might have enhance the
353 expression of JH biosynthesis genes including *JHAMT* and *CYP15A1* for compensation.

354 In any case, we were not convinced to decrease JH titer via RNAi-mediated knockdown
355 of *JHAMT* and *CYP15A1*. Future studies including knockout of these genes using genome
356 editing tools such as the CRISPR/Cas9 system may give us deeper insights into the function of
357 these genes during embryonic development.
358

5. Conclusion

In the present study, we demonstrated that knockdown of *Met* resulted in defects in embryonic development. *Met*-knockdown embryos developed up to late embryonic stage, but were arrested with morphological defects and impaired mobility. These results suggested that *Met* has an important role during embryonic development of *T. castaneum*. This study provides evidence that the function of *Met* in the embryonic development is diverse among insect species.

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Figure legends

Fig. 1 Relative mRNA amounts of *Met* (A), *Kr-h1* (B), and *Vg1* (C) in female adults treated with dsRNA

Total RNA was extracted from a whole body of dsRNA-treated female adults at 4, 7, and 11 days after dsRNA treatment. The relative expression levels of *Met*, *Kr-h1*, and *Vg1* were determined by qRT-PCR in comparison to those of *ribosomal protein L32* (*rpL32*). Mean ± S.D. of three biologically independent replicates are shown. *$p < 0.05$, and **$p < 0.01$ (Student's *t*-test), in comparison with the *malE* dsRNA-treated controls.

Fig. 2 Effects of *Met* and *Kr-h1* knockdown on egg numbers (A), hatching (B), and survival in the early larval stage (C).

dsRNA of *Met*, *Kr-h1*, and *malE* (control) were injected into 21-day-old virgin female adults. On the following day, dsRNA-treated females were mated with naive males of the same age. The eggs laid by the dsRNA-treated females were collected on 1–3 and 4–6 days after treatment (DAT) (A). Four days after egg collection, the hatching rates were examined (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 four replicates are shown. *$p < 0.05$ (Student's *t*-test), in comparison with the *malE* dsRNA-treated controls.

Fig. 3 Embryos with parental RNAi of *Met*.

The eggs laid by the females treated with *malE* dsRNA (control, A-D) and *Met* dsRNA (E-H and G') were dechorionized with sodium hypochlorite solution and the morphology of developing embryos was observed using a microscope (Olympus model BX41) every 24 hours after oviposition. Scale bars are 200 μm. Abbreviations in B-H: A, anterior; P, posterior.

Fig. 4 Hatched and unhatched larvae after parental RNAi of *Met*.

A larva that hatched normally (A) and an unhatched pharate first instar larva whose eggshell was manually removed (B), after injecting *malE* dsRNA maternally. (C-E) Unhatched embryos whose eggshells were manually removed after parental RNAi of *Met*.

Fig. 5 Morphological observation of offspring of first- to second-instar larvae from females treated with dsRNA of *malE* (A) and *Met* (B and C)

Scale bars are 300 μm.