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ORIGINAL RESEARCH PAPER

Expressions of juvenile hormone biosynthetic genes during presoldier differentiation in the incipient colony of *Zootermopsis nevadensis* (Isoptera: Archotermopsidae)

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Abstract Soldier differentiations require high juvenile hormone (JH) titers in workers and two molts via a presoldier stage. However, it is still unclear when the endogenous JH titer increases in workers, because identifying an individual before it undergoes the presoldier molt is quite difficult in natural conditions. In an incipient colony of *Zootermopsis nevadensis* Hagen, the oldest 3rd instar larva (No. 1 larva) always differentiates into a presoldier and the second 3rd instar larva (No. 2 larva) molts into a 4th instar larva. Consequently, by focusing on the ontogeny of the No. 1 larva, the timing of the increase in the JH titer can be determined. Here, we compared the expression levels of JH biosynthetic genes in the heads of No. 1 and No. 2 larvae. Most genes involved in JH biosynthesis were found in a genome database, and two candidate *JH epoxidase* (*CYP15A1*) genes were identified. Expression levels of *JH acid methyltransferase* (*JHAMT*) and one candidate *CYP15A1*, probably involved in the final steps of the JH biosynthesis, were specifically high in No. 1 larvae at 2–3 and 4–5 days after the appearance, respectively. Expression patterns of these genes may be used for the prediction of endogenous JH titer changes during presoldier differentiation.

Keywords Social insects · Caste differentiation · Gene expression · *CYP15* · *JHAMT*

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Introduction

Insect societies are highly sophisticated because of the cooperation and division of labor among castes (Wilson 2000). Among sterile castes, which exhibit altruistic behaviors, the termite soldiers are a unique caste in terms of specific morphology and its role in nest defense (Deligne et al. 1981). All extant species, except for some lineages that have suffered a secondary loss, have a soldier caste (Lo and Eggeleton 2011). Consequently, it is strongly suggested that soldiers evolved just once in the course of eusocial termite evolution (Lo and Eggeleton 2011; Noirot and Pasteels 1987).

Caste differentiation in termites is an example of polyphenism; alternative phenotypes are generated through changes in the developmental process in response to environmental cues during the postembryonic period (Hartfelder and Emlen 2012; Miura 2005; Nijhout 2003; Roisin 2000; Watanabe et al. 2014). Generally, in insects, different phenotypes resulting from alterations in developmental processes are regulated by the endocrine system (Hartfelder and Emlen 2012; Nijhout 1999). For caste differentiation in termites, juvenile hormone (JH) plays a central role (Henderson 1998; Miura and Scharf 2011; Nijhout and Wheeler 1982). Soldiers differentiate from workers by undergoing two molts via a presoldier stage, which is associated with an increasing JH titer in workers (Cornette et al. 2008a). Previous studies reported that JH or JH analog (JHA) treatment of workers induced presoldier differentiation (e.g., Haward and Haverty 1979; Itano and Maekawa 2008; Maekawa et al. 2014; Ogino et al. 1993; Scharf et al. 2003; Toga et al. 2009; Tsuchiya et al. 2008). Based on these presoldier induction methods, soldier-specific morphogenesis and physiological/genetic changes after an increase in the JH titer in workers have been analyzed, and some important

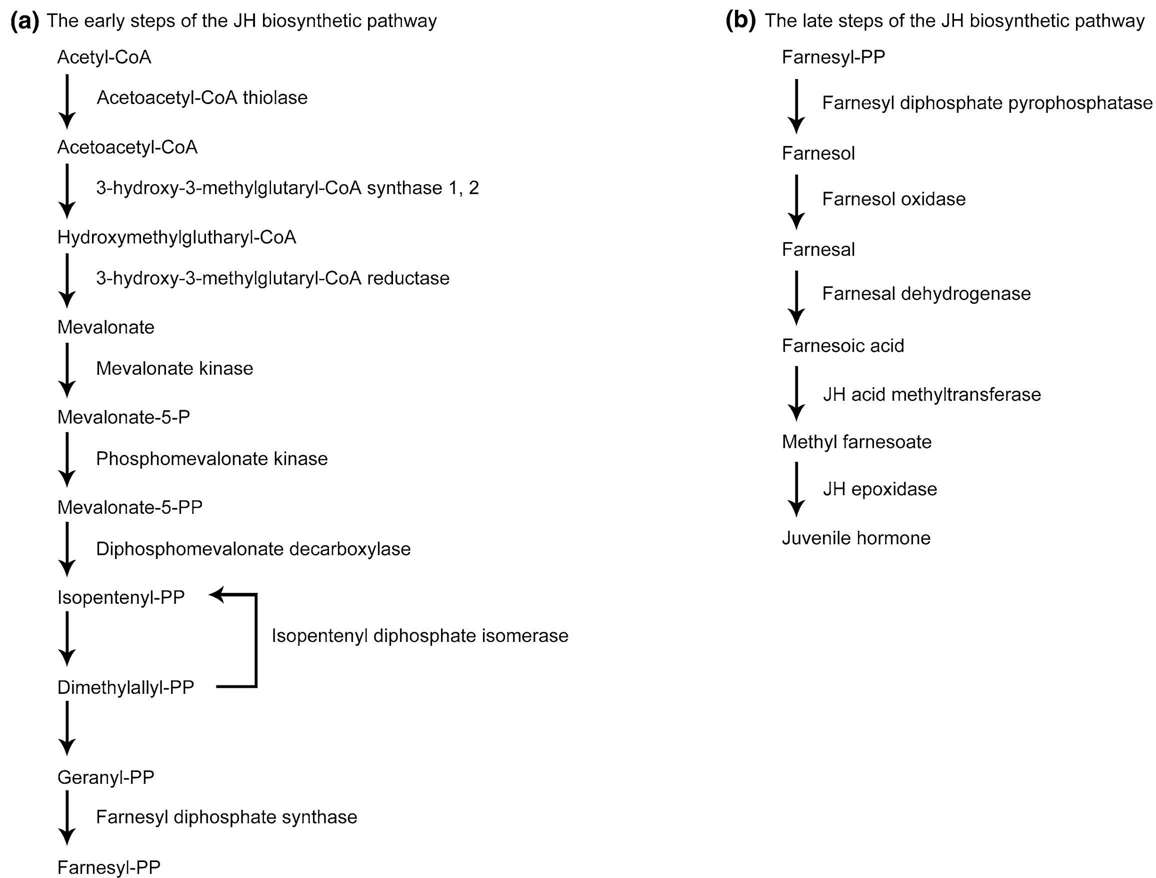

Fig. 1 Juvenile hormone (JH) biosynthetic pathway [early steps (**a**) and late steps (**b**)]. Each reaction is catalyzed by the enzyme shown next to the *arrow*

changes were identified (e.g., Cornette et al. 2008b, 2013; Koshikawa et al. 2005; Masuoka et al. 2013; Toga et al. 2012, 2013; Watanabe and Maekawa 2012). However, it is still unknown when the genes involved in JH biosynthesis are activated and/or the JH titer increases during soldier differentiation under natural conditions. This is because the soldier ratio in a colony is normally low compared with workers (usually below 10 %; Haverty 1977), and discriminating an individual worker that will differentiate into a presoldier and soldier is quite difficult under natural conditions.

In an incipient colony of the damp-wood termite *Zootermopsis nevadensis* Hagen (Isoptera: Archotermopsidae), which belongs to one of the basal lineages of termites, the oldest 3rd instar larva (No. 1 larva) always differentiates into a presoldier, and the 2nd oldest 3rd instar larva (No. 2 larva) molts into a 4th instar worker (Maekawa et al. 2012). When the No. 1 larva was artificially removed from the colony, the No. 2 larva differentiated into a presoldier, suggesting that presoldier differentiation was not determined genetically. This study was the first case to show that a particular individual differentiates into a presoldier before the molt. By focusing on the No. 1 larvae in incipient colonies of this species, the expression patterns of genes involved in JH biosynthesis can be elucidated during presoldier differentiation under natural conditions.

JH is synthesized in the corpora allata, and the biosynthetic pathway mainly comprises two enzymatic pathways: the early steps and late steps of the JH synthesis pathway (Bélles et al. 2005) (Fig. 1). The former is the broadly conserved mevalonate pathway, which includes eight enzymatic reactions. The latter is specific to insects and includes five enzymatic reactions. Based on the EST libraries derived from the corpora allata and corpora cardiaca of three insect species (cockroach *Diploptera punctata* Eschscholtz and mosquitoes *Aedes aegypti* L. and *Anopheles albimanus* Weidemann), JH biosynthetic genes were identified, and the expression of at least six genes was examined (Noriega et al. 2006). The expression of JH biosynthetic genes was also observed in the corpora allata and corpora cardiaca of the honeybee *Apis mellifera* L. (Bomtorin et al. 2014). Moreover, based on an EST library derived from multiple tissues of the silkworm moth *Bombyx mori* L., eight genes in the mevalonate pathway were identified, and specific expression in the corpora allata was elucidated (Kinjoh et al. 2007; Ueda et al.

2009). These studies indicated that the expression levels of JH biosynthetic genes influence endogenous changes in the JH titer. In particular, the JH titer may be strongly correlated with the expression levels of *JH acid methyltransferase* (*JHAMT*) and *JH epoxidase* (*CYP15A1*), both of which are involved in the final steps of JH biosynthesis (Fig. 1). For example, knockdown of *JHAMT* by RNA interference (RNAi) resulted in a lower level of JH synthesis in the corpora allata in the desert locust *Schistocerca gregaria* Forsskål (Marchal et al. 2011). A decrease in the JH titer in *A. mellifera* was also achieved by *JHAMT* RNAi (Bomtorin et al. 2014). CYP15A1, encoding JH epoxidase (Helvig et al. 2004), converts methyl farnesoate into JH III, and RNAi resulted in an increase in the level of methyl farnesoate in the corpora allata and a decrease in the JH titer in *S. gregaria* (Marchal et al. 2011). Moreover, knockdown of a *JH epoxidase* homolog, *CYP15F1*, by RNAi resulted in a decrease in presoldier differentiation from workers in the rhinotermitid termite *Reticulitermes flavipes* Kollar (Tarver et al. 2012). Consequently, changes in the endogenous JH titer may also be influenced by the expression levels of these genes in *Z. nevadensis*.

The genome sequence of *Z. nevadensis* was published, and genes involved in the JH biosynthetic pathways were predicted (Terrapon et al. 2014). In the present study, we first tried to quantify the JH titer in 3rd instar larvae using high-performance liquid chromatography-mass spectrometry (HPLC-MS) according to the previously described methods (e.g., Maekawa et al. 2010). Then, expression levels of JH biosynthetic genes were compared between the No. 1 and No. 2 larvae using real-time quantitative PCR (qPCR). Based on the results, we discuss the timing of changes in the endogenous JH titer during presoldier differentiation in *Z. nevadensis* in natural conditions.

Materials and methods

Incipient colony foundation

Mature colonies of *Z. nevadensis* were collected from Kawanishi-shi, Hyogo Prefecture, Japan, in April 2013. These colonies were maintained in constant darkness at room temperature in the laboratory. After alates (winged adults) emerged from the colonies, they were collected and separated by sex. To establish incipient colonies, male and female alates from different colonies were paired in 60-mm plastic dishes with crushed pieces of nest wood. These dishes were kept in constant darkness at 25 °C until the 3rd instar larvae (No. 1 and 2 larvae) appeared in each colony. The abdomens of the No. 1 and No. 2 larvae were marked with waterproof inks of different colors to discriminate each other (Maekawa et al. 2012).

JH titer quantification

Experimental details were reported previously (Maekawa et al. 2010; Saiki et al. 2015; Watanabe et al. 2011). Briefly, the 3rd instar larvae for JH extraction were sampled from a mature colony. Five and ten individuals per sample were collected, and each sample was prepared in triplicate. Based on the previous protocols, 30 ng of fenoxycarb (Wako, Osaka, Japan) was used as an internal standard, and JH extraction in the whole body was performed. The JH titer in each sample was quantified with an Agilent 1100 HPLC system with an autosampler (Agilent Technologies, Santa Clara, CA, USA). Mass spectral analysis was performed by electrospray ionization in the positive mode on a microTOF-HS (Bruker Daltonik GmbH, Germany).

Juvenile hormone biosynthetic genes identified from the *Z. nevadensis* genome database

Terrapon et al. (2014) reported gene sequences involved in the JH biosynthesis pathways, and the following 13 genes were obtained from the database (http://termitegenome.org/): acetoacetyl-CoA thiolase (*AcoAT*) (Gene ID: Znev_06783), 3-hydroxy-3-methylglutaryl-CoA synthase 1 and 2 (*HMGS1, 2*) (Znev_08283 and 14140), 3-hydroxy-3-methylglutaryl-CoA reductase (*HMGR*) (Znev_02974), mevalonate kinase (*MK*) (Znev_14029), phosphomevalonate kinase (*PK*) (Znev_12655), diphosphomevalonate decarboxylase (*DD*) (Znev_08133), isopentenyl diphosphate isomerase (*IPPI*) (Znev_09747), farnesyl pyrophosphate synthase (*FPS*) (Znev_02161), farnesal dehydrogenase 1 and 2 (*FD1, 2*) (Znev_01798 and 03358), JH acid methyltransferase (*JHAMT*) (Znev_12145) and JH epoxidase (*CYP15A1*) (Znev_14299).

Characterization of *JHAMT*

The sequence length of *JHAMT* in the genome database was extremely short (171 bp), and the conserved motif (SAM-binding motif, see results) could not be found. Thus, gene-specific PCR primers were newly designed, and subcloning and sequencing were performed.

cDNA preparation

Total RNA was extracted from the whole bodies of No. 1 larvae using ISOGEN (NipponGene, Tokyo, Japan). Five different individuals were used for RNA extraction. The extracted total RNA was purified with DNase treatment to remove contaminating DNA. RNA purity and quantity were measured using a NanoVue spectrophotometer (GE Healthcare BioSciences, Tokyo, Japan). The purified RNA was reverse-transcribed for cDNA synthesis using a

High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster, CA, USA).

Subcloning and sequencing

BLAST searches were performed against the *Z. nevadensis* genome database using *D. punctata JHAMT* (GenBank accession no. AHZ20738). For RT-PCR, a forward primer (5′-ATG CAC AAT GCG GAA CTC T–3′) and a reverse primer (5′-TTA CGT TTT TCT TGC GTA CGC–3′) were newly designed based on the obtained sequence using Primer3Plus (Untergasser et al. 2007). PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen, Tokyo, Japan) and subcloned into a pGEM easy T-vector (Promega, Madison, WI, USA). The inserted DNA sequence was amplified by PCR, and purified products were sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit and an automatic DNA Sequencer 3130 Genetic Analyzer (Applied Biosystems). Obtained sequences were subjected to BLAST database searches and compared with other published *JHAMT* sequences using MUSCLE in MEGA5 (Tamura et al. 2011).

Alignment and phylogenetic analysis of CYP15A1

According to Terrapon et al. (2014), there was only one *CYP15A1* gene in the *Z. nevadensis* genome (Znev_14299). However, BLAST database searches were performed using the published *CYP15A1* genes from other insect species [*R. flavipes* (Tarver et al. 2012; no. FJ792774), *D. punctata* (Helvig et al. 2004; no. AY509244) and *S. gregaria* (Marchal et al. 2011; no. HQ634703)] and found another similar gene sequence in *Z. nevadensis* (gene ID: Znev_06057). Multiple alignment was performed using these two amino acid sequences with seven published sequences derived from six insect species (including *CYP306A1* genes from fruit fly *Drosophila melanogaster* Meigen and from *Z. nevadensis*) using MUSCLE in MEGA5 (Tamura et al. 2011). For phylogenetic analysis, a maximum likelihood tree was constructed based on the WAG + Gamma distribution model using amino acid sequences (100 bootstrap replications).

Gene expression analyses using real-time quantitative PCR

Gene expression analysis between No. 1 and 2 larvae

No. 1 and 2 larvae were collected at three time points (0–1, 2–3 and 4–5 days after the appearance) in each colony. Eighteen different No. 1 and 2 larvae were sampled at each time point. Individuals were immersed immediately in liquid nitrogen and stored at −80 °C until use (three individuals in one tube). All individuals were separated into heads and other body parts, and to compare gene expressions between No. 1 and 2 larvae, head samples were used for cDNA preparation. Three different individuals were used for each cDNA sample, and in total six cDNA samples were prepared for each time point. In accordance with the procedure cited above, total RNA extraction, DNase treatment and cDNA synthesis were performed. For cDNA preparation, equal concentrations of total RNA (22 ng) were reverse-transcribed.

To perform real-time quantitative PCR, sequence-specific primers were designed against JH biosynthetic genes using Primer3Plus (Table 1). qPCR was performed using a Thunderbird qPCR mix (Toyobo, Osaka, Japan) using a MiniOpticon Real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The suitability of three reference genes [*EF-1α*, *β-actin* and *NADH-dh* from *Z. nevadensis* (GenBank accession no. AB915828, AB915826 and AB936819, respectively)] was evaluated using GeNorm (Vandesompele et al. 2002) and NormFinder software (Andersen et al. 2004). Because both softwares showed that the expression levels of *EF-1α* were the most stable, this gene was used as a reference gene. qPCR analysis was performed in biological sextuplicates (number of individuals examined = 3 × 6). Statistical analysis was carried out by two-way ANOVA and Tukey's test with Mac Statistical Analysis version 2.0 (Esumi, Tokyo, Japan).

Gene expression analysis in each body part of No. 1 larvae

For the gene expression analysis in each body part, heads and other body parts of No. 1 larvae were used for cDNA preparation. For cDNA preparation, equal concentrations of total RNA (360 ng) were reverse-transcribed. qPCR analysis was performed in biological sextuplicates (number of individuals examined = 3 × 6). In accordance with the method shown above, *JHAMT* and *CYP15A1* gene expression levels were quantified and compared between heads and other body parts.

Results

JH titer quantification using high-performance liquid chromatography-mass spectrometry

We performed JH titer quantification using 3rd instar larvae collected from a mature colony. Each sample consisted of 5 or 10 individuals, and measurement was replicated three times in each sample. Although the level of fenoxycarb could be quantified, the peak of JH III in the chromatograph

Table 1 Primer sequences used in this study

Gene name	Forward primer (5′–3′)	Reverse primer (5′–3′)	Annealing temperature (°C)
Acetoacetyl-CoA thiolase	ACGCAGGTTGACATTGAGG	GCCACCACATCCAGCTTATT	60
3-Hydroxy-3-Methylglutaryl-CoA synthase 1	GCGACATTGAAGGCGTAGAT	ACGACAGCGCATTGAAGAG	60
3-Hydroxy-3-Methylglutaryl-CoA synthase 2	CAAGCTGGTGCAGAAGTCAT	CCTCCAGCCTTACATTCTTGA	60
3-Hydroxy-3-Methylglutaryl-CoA reductase	CCACCATTACGCTCGACAC	AGCAACACAGCACCTCAAGA	60
Mevalonate kinase	TAACGCAGCTTGGAGAGTCA	TGCGATCTAGTGATGGATGTG	60
Phosphomevalonate kinase	GCACAGGCAAGATATGATTAAGTG	CCACAGCAGCTTGACAGAAG	60
Diphosphomevalonate decarboxylase	TGCTGCTGGTTATGCTTGTC	CAACGAACGAATCCTCCATAC	60
Isopentenyl-diphosphate σ-isomerase	GCGTGTTCCTGTTCAACTCA	CACCTCTGCCGCTATCTCAT	60
Farnesyl pyrophosphate synthetase	CACTGGTAAGATTGGCACTGAT	CACTGCTGCAACCTTCTCTG	60
Farnesal dehydrogenase 1	AGGCATTCATTCCACTCTTGA	GATCACTGCTGCTCGTAGAACTC	60
Farnesal dehydrogenase 2	GCACTGAATGCTATCACACGTT	CTTCCACAGTCATTGTTGCATT	60
JH methyltransferase	CGGTCAAGAAACGGATTGAC	AAGACACGGGATTCGAAGTG	60
JH epoxidase (Znev_14299)	TTGAGTCGGTCATCATGGAA	TCGAGTCCTTGAGTGCTCTG	60
JH epoxidase (Znev_06057)	AGACTTCTGGCAGGAACAGC	TCTGTTGGAATGCTTGGACA	60
Beta-actin	AGCGGGAAATCGTCCGTGAC	CAATGGTGATGACCTGCCCAT	60
NADH-dh	TTGGGTTGGGGGTATCAGC	CGGCAAGGAAGCAAATAAAG	60
EF1-alfa	GCATGCACTGTTGGCTTTTA	TTCCTCAAATCGGGTTTCAC	60

could not be measured, even in samples extracted from 10 individuals (data not shown).

Identification of JH biosynthetic genes and characterization of *JHAMT* and *CYP15A1*

Based on genomic information of *Z. nevadensis* (Terrapon et al. 2014), 13 JH biosynthetic genes were identified. Of these genes, the sequence length of *JHAMT* (gene ID: Znev_12145; Supplementary Table) was extremely short (171 bp) compared with those observed in other species [e.g., *S. gregaria*: 927 bp (no. HQ634702), *B. mori*: 837 bp (no. AB113578)], and there were no conserved motifs (e.g., SAM-binding motif) in this sequence. After subcloning and sequencing, we identified a 825-bp sequence, including the coding region predicted by Terrapon et al. (2014) with a SAM-binding motif (Fig. 2). The deduced amino acid sequence (275 aa) was subjected to BLASTP searches and found to have 64 and 44 % similarity with *JHAMT* homologs from *D. punctata* and *S. gregaria*, respectively. Thus, we concluded that this sequence is the *JHAMT* homolog from *Z. nevadensis*. The determined nucleotide and putative amino acid sequences are available in the DDBJ/EMBL/GenBank databases (accession no. LC049616).

Terrapon et al. (2014) proposed only one sequence as a gene encoding JH epoxidase (CYP15A1) (Znev_14299). We performed homology searches using CYP15A1 genes identified in *D. punctata*, *R. flavipes* and *S. gregaria* by BLASTX against a non-redundant database. As a result, we identified another sequence (Znev_06057) as well as the sequence reported previously (Znev_14299). Multiple alignments and molecular phylogenetic analysis showed that both of these sequences were included in the CYP15 family (Figs. 3, 4), although Znev_14299 lacks conserved P/G rich and Helix-C motifs, both of which contribute to protein conformation and the enzymatic function of CYP15 (Werck-Reichhart and Feyereisen 2000) (Fig. 3).

Expression levels of genes in the early steps of the juvenile hormone biosynthetic pathway

Expression analyses were performed by qPCR using head samples of No. 1 and 2 larvae collected at three time points (0–1, 2–3 and 4–5 days after the molting to the 3rd larval stage). The expression level of *AcoAT* was not significantly different between No. 1 and 2 larvae at each time point (two-way ANOVA followed by Tukey's test, $p < 0.05$), although interaction was detected between No. 1 and 2 larvae and time points ($df = 2$, $F = 5.599$, $p = 8.58E{-}03$) (Fig. 5a). Expression levels of *HMGR* and *HMGS1* were not significantly different in all cases (Fig. 5b, c). Statistical differences between No. 1 and 2 larvae were observed in *HMGS2* (No. 1/No. 2: $df = 1$, $F = 8.378$, $p = 7.02E{-}03$),

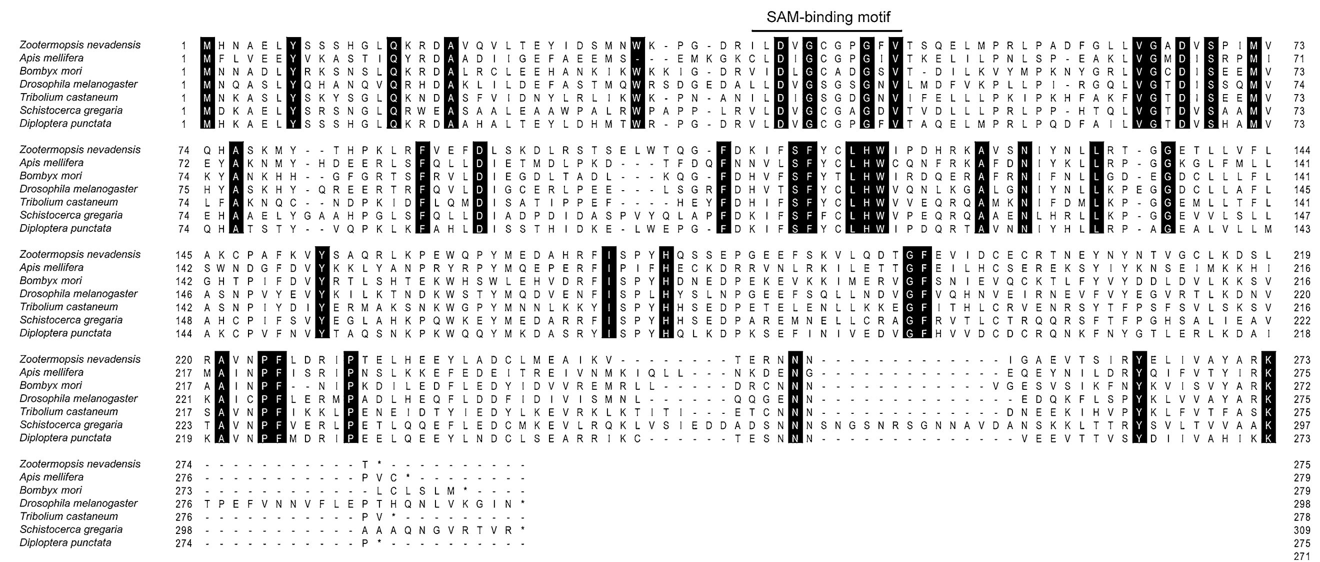

Fig. 2 Multiple alignment of JH acid methyltransferase (JHAMT) using the amino acid sequences of *Zootermopsis nevadensis* (obtained in this study, GenBank accession no. LC049616), *Apis mellifera* (XM_001119986), *Bombyx mori* (BAC98835), *Drosophila melanogaster* (BAC98836), *Tribolium castaneum* (AB360763), *Schistocerca gregaria* (ADV17350) and *Diploptera punctata* (AHZ20738). Amino acids common to each species are indicated by *white letters* with a *black background*. The SAM-binding motif is indicated with a *line above the sequences*

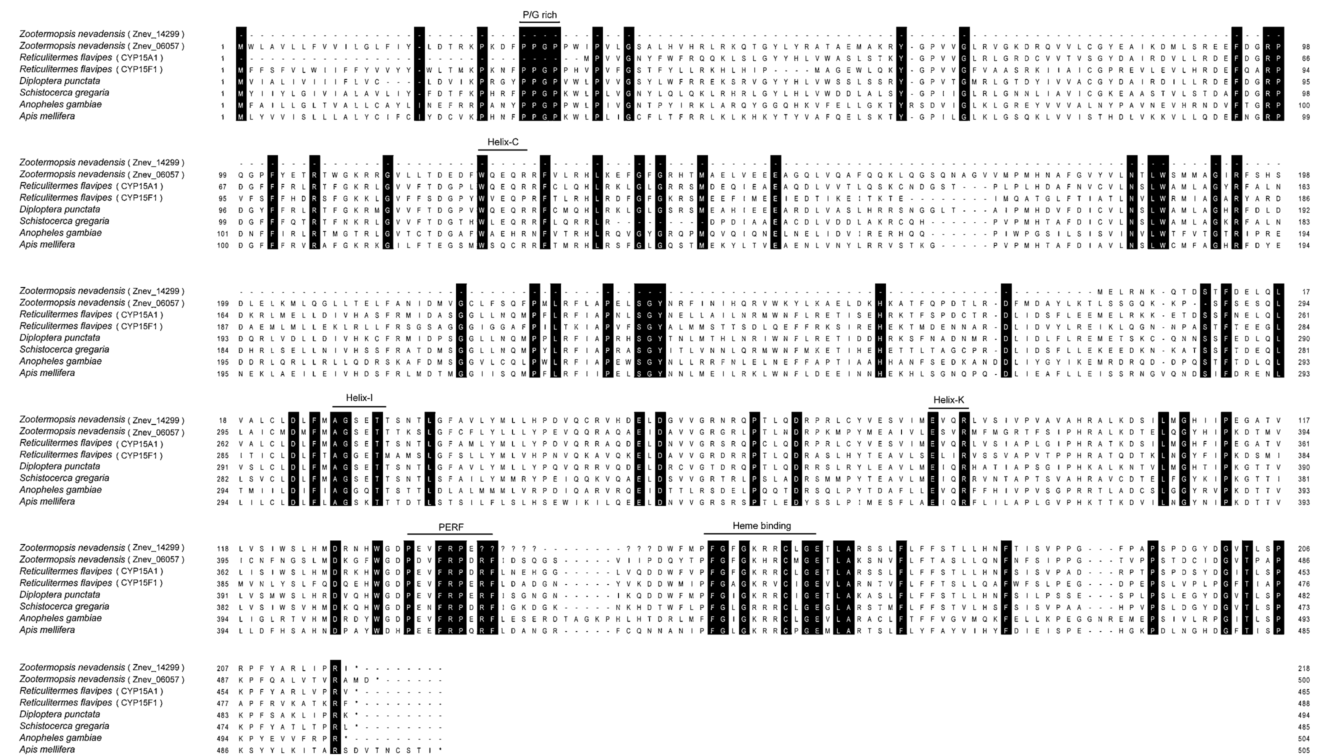

Fig. 3 Multiple alignment of JH epoxidase (CYP15A1) using amino acid sequences of *Zootermopsis nevadensis* (Znev_14299 and Znev_06057), *Apis mellifera* (GenBank accession no. XM_623572), *Schistocerca gregaria* (ADV17351), *Diploptera punctata* (AAS13464), *Anopheles gambiae* (XP_315675), *Reticulitermes flavipes* (ACN93795) and *R. flavipes* CYP15F1 (ACN93794). Amino acids common to each species are indicated by *white letters* with a *black background*. Six conserved motifs are shown with *lines above the sequences*

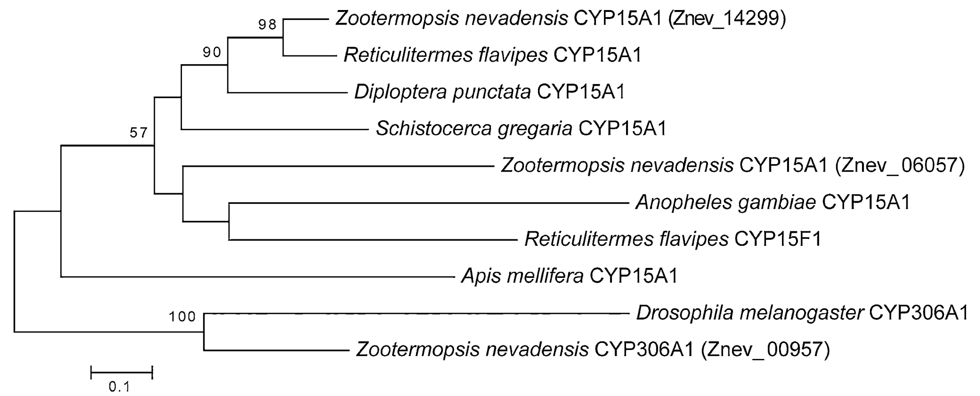

Fig. 4 Molecular phylogenetic tree of CYP15A1 using amino acid sequences. Two sequences of CYP306A1 involved in ecdysone biosynthesis were used as outgroups. The *numbers* above *each node* indicate the level of bootstrap probabilities more than 50 % (100 replicates)

Fig. 5 Expression levels of genes (average ± SD, $n = 6$) in the early steps of the JH biosynthetic pathway between No. 1 and 2 larvae at three time points after molting. **a** Acetoacetyl-CoA thiolase (*AcoAT*), **b** 3-hydroxy-3-methylglutaryl-CoA reductase (*HMGR*), **c** 3-hydroxy-3-methylglutaryl-CoA synthase 1 (*HMGS1*), **d** 3-hydroxy-3-methylglutaryl-CoA synthase 2 (*HMGS2*), **e** diphosphomevalonate decarboxylase (*DD*), **f** isopentenyl diphosphate isomerase (*IPPI*), **g** mevalonate kinase (*MK*) and **h** phosphomevalonate kinase (*PK*). Expression analyses were performed in the biological sextuplicates, and each data value was normalized to the expression level of *EF1α*. The *black* and *white columns* indicate No. 1 and 2 larvae, respectively. *Different letters above the bars* indicate a significant difference (two-way ANOVA followed by Tukey's test, $p < 0.05$)

but interaction was not detected between No. 1 and 2 larvae and time points (Fig. 5d). Significant differences of *DD* expression levels were found in all cases (No. 1/No. 2 larvae: $df = 1$, $F = 6.873$, $p = 0.0136$; days $df = 2$, $F = 14.004$, $p = 5.06\text{E}{-}05$; interaction: $df = 2$, $F = 11.296$, $p = 2.20\text{E}{-}04$). Expression levels of *DD* in No. 1 larvae

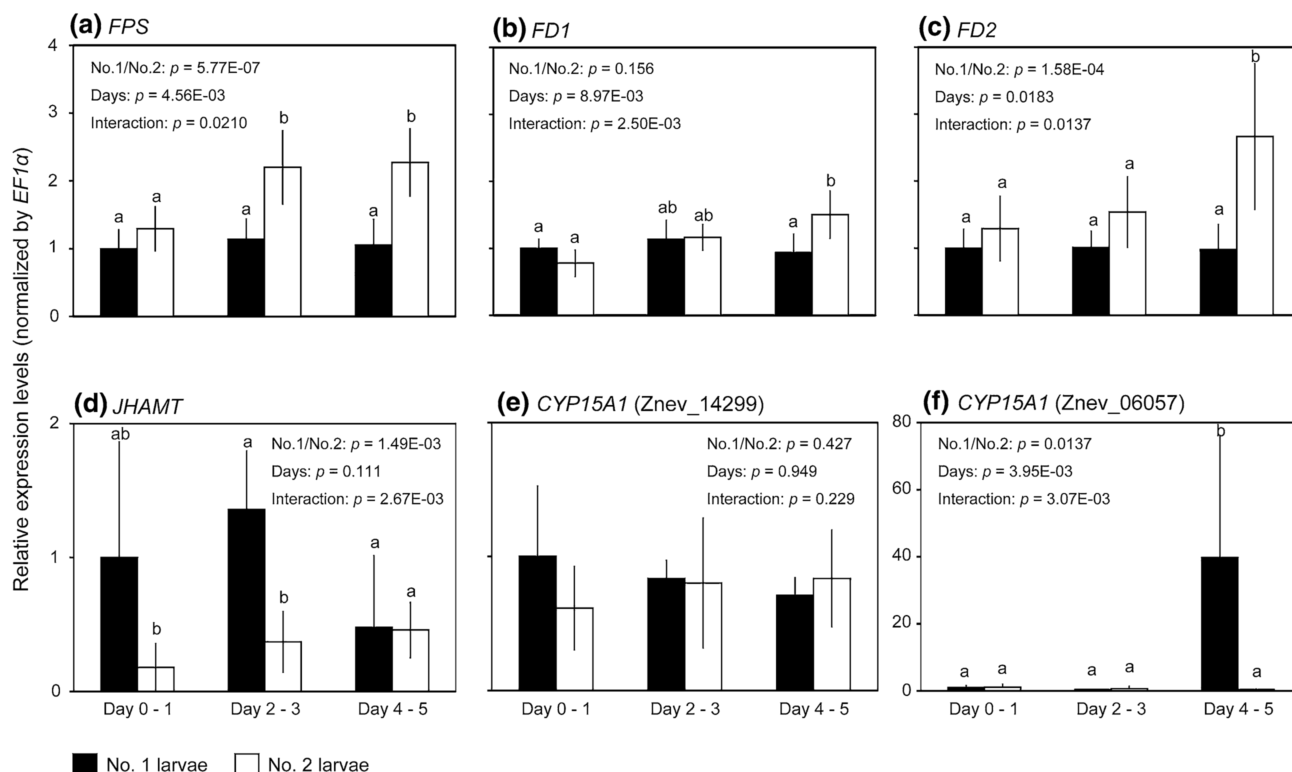

Fig. 6 Expression levels of genes (average ± SD, $n = 6$) in the late steps of the JH biosynthetic pathway between No. 1 and 2 larvae at three time points after molting. **a** Farnesyl pyrophosphate synthase (*FPS*), **b** farnesal dehydrogenase 1 (*FD1*), **c** farnesal dehydrogenase 2 (*FD2*), **d** JH acid methyltransferase (*JHAMT*), **e** JH epoxidase (*CYP15A1*) (Znev_14299) and **f** JH epoxidase (*CYP15A1*) (Znev_06057). Expression analyses were performed in the biological sextuplicates, and each data value was normalized to the expression level of *EF1α*. The *black* and *white columns* indicate No. 1 and 2 larvae, respectively. *Different letters above the bars* indicate a significant difference (two-way ANOVA followed by Tukey's test, $p < 0.05$)

2–3 days after molting were significantly higher than those of any other No. 1 larvae (two-way ANOVA followed by Tukey's test, $p < 0.05$) (Fig. 5e). Statistical differences were also observed in *IPPI* (No. 1/No. 2: $df = 1$, $F = 14.497$, $p = 6.46\text{E}{-}04$; interaction: $df = 2$, $F = 3.679$, $p = 0.0372$). Expression levels of *IPPI* in No. 2 larvae 4–5 days after molting were significantly higher than those of No. 1 larvae at the same time point (two-way ANOVA followed by Tukey's test, $p < 0.05$) (Fig. 5f). The expression level of *MK* was not significantly different in all cases (Fig. 5g). Statistical differences were observed in *PK* (No. 1/No. 2: $df = 1$, $F = 20.368$, $p = 9.18\text{E}{-}05$; days: $df = 2$, $F = 4.425$, $p = 0.0207$), but interaction was not detected between No. 1 and 2 larvae and time points (Fig. 5h).

Expression levels of genes in late steps of the juvenile hormone biosynthetic pathway

Two-way ANOVA was performed, and interaction was detected in all genes examined except for *CYP15A1* (Znev_14299) (*FPS*: $df = 2$, $F = 4.406$, $p = 2.10\text{E}{-}02$; *FD1*: $df = 2$, $F = 7.364$, $p = 2.50\text{E}{-}03$; *FD2*: $df = 2$, $F = 4.966$, $p = 0.014$; *JHAMT*: $df = 2$, $F = 7.269$, $p = 2.67\text{E}{-}03$; *CYP15A1* (Znev_06057): $df = 2$, $F = 7.060$, $p = 3.07\text{E}{-}03$) (Fig. 6). Statistical differences between No. 1 and 2 larvae were observed in four genes [*FPS*: $df = 1$, $F = 39.900$, $p = 5.77\text{E}{-}07$; *FD2*: $df = 1$, $F = 18.642$, $p = 1.58\text{E}{-}04$; *JHAMT*: $df = 1$, $F = 12.228$, $p = 1.49\text{E}{-}03$; *CYP15A1* (Znev_06057): $df = 1$, $F = 6.852$, $p = 0.014$]. Significant differences were also found among three time points in four genes [FPS: $df = 2$, $F = 6.487$, $p = 4.56\text{E}{-}03$; *FD1*: $df = 2$, $F = 5.539$, $p = 8.97\text{E}{-}03$; *FD2*: $df = 2$, $F = 4.585$, $p = 0.018$; *CYP15A1* (Znev_06057): $df = 2$, $F = 6.694$, $p = 3.95\text{E}{-}03$]. Expression levels of *FPS* in No. 2 larvae 2–3 and 4–5 days after molting were significantly higher than those of any other No. 1 larvae (Fig. 6a). Moreover, *FD1* and *FD2* expression levels in No. 2 larvae 4–5 days after molting were also significantly higher than those of No. 1 larvae (Fig. 6b, c). However, expression patterns of *JHAMT* and *CYP15A1* (Znev_06057) were completely different from those of other genes examined in the late steps. Namely, *JHAMT* expression levels in No. 1 larvae 2–3 days after molting were significantly higher than those of any

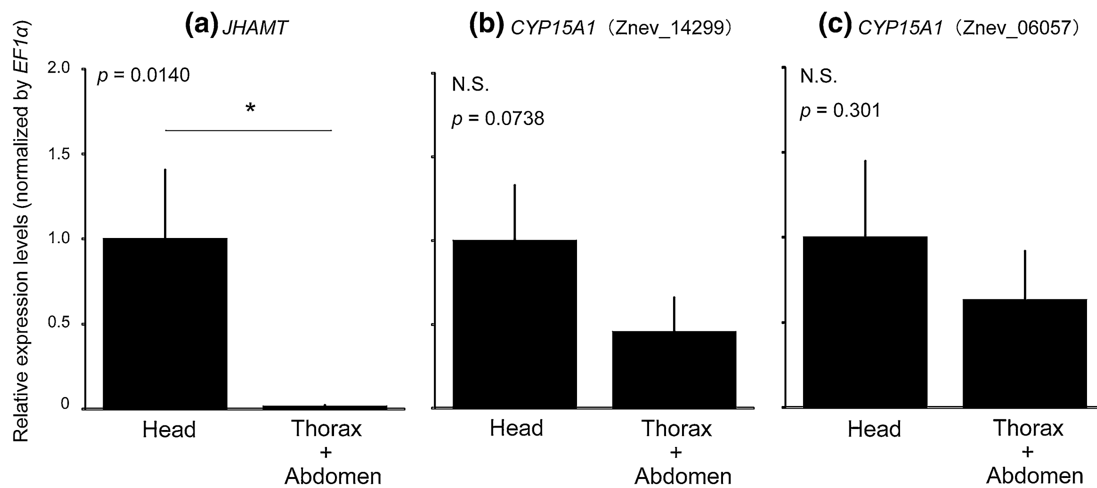

Fig. 7 Expression levels of genes (average ± SD, $n = 6$) in each body part of No. 1 larvae. **a** JH acid methyltransferase (*JHAMT*), **b** JH epoxidase (*CYP15A1*) (Znev_14299) and **c** JH epoxidase (*CYP15A1*) (Znev_06057). Expression analyses were performed in the biological sextuplicates, and each data value was normalized to the expression level of *EF1α*. An *asterisk over the bars* indicates a significant difference between groups (Welch's *t* test, $p < 0.05$)

other No. 2 larvae (Fig. 6d). *CYP15A1* (Znev_06057) expression levels in No. 1 larvae 4–5 days after molting were significantly higher and also the highest levels found among all larvae examined (two-way ANOVA followed by Tukey's test, $p < 0.05$) (Fig. 6f).

JHAMT and *CYP15A1* gene expression analyses between heads and other body parts

Expression levels of *JHAMT* in the heads of larvae were significantly higher than those in other body parts (predominantly the thorax and abdomen) of No. 1 larvae (Fig. 7a). Although expression levels of both *CYP15A1* genes in heads tended to be higher than those in other body parts, there were no significant differences between these levels (Fig. 7b, c) (Student's *t* test, $p = 0.074$ and $= 0.301$, respectively).

Discussion

JH quantification analyses

Because many termite species have been shown to have JH III [e.g., *Coptotermes formosanus* Shiraki (Park and Raina 2004), *Reticulitermes speratus* Kolbe (Maekawa et al. 2010) and *Hodotermopsis sjostedti* Holmgren (Cornette et al. 2008a)], *Z. nevadensis* is likely to have JH III. In accordance with previous studies on different termite species, JH quantification analyses using HPLC-MS were performed in 3rd instar larvae (5 or 10 individuals) sampled from mature colonies. Itano and Maekawa (2008) reported that the size of the corpora allata of 3rd instar larvae from a mature colony of *Z. nevadensis* was significantly larger than those from an incipient colony. Their results may indicate that the JH titers of those individuals would be higher. However, the present study fails to detect any significant levels of JH III. This failure may be attributed to the small amount of JH and/or high activity of JH-degrading enzymes during sample processing in *Z. nevadensis* larvae. The No. 1 larva is only a single individual in each incipient colony, and it is difficult to collect more individuals for JH quantification. Consequently, we focus on the expression patterns of JH biosynthetic genes.

Genes involved in juvenile hormone biosynthesis

Because we failed to detect measurable amounts of JH in the whole-body samples, we focused on genes involved in the JH biosynthesis in termites, of which homologs are shown to be expressed in the corpora allata of other insect species (e.g., *B. mori*, *Ae. aegypti*, *An. albimanus*, and *D. punctate*) (Kinjoh et al. 2007; Noriega et al. 2006). Of 13 genes examined, *JHAMT* is newly obtained by subcloning and sequencing. Because the obtained amino acid sequence is similar to those of other insects, and the conserved region (SAM-binding motif) is confirmed at the N-terminal site (Fig. 2), it may have enzymatic activity. *JHAMT* in *B. mori* was confirmed for the first time as the enzyme that converted JH acid to JH in the corpora allata (Shinoda and Itoyama 2003).

CYP15s belong to the cytochrome P450 gene family and are involved in JH biosynthesis (Feyereisen 2006). Multiple alignments of Znev_14299 and CYP15A1 genes previously described from other insects show that the

former lacks two conserved motifs (P/G rich and Helix-C) and has one incomplete motif (PERF). These motifs are important for protein conformation (Werck-Reichhart and Feyereisen 2000). Molecular phylogenetic analysis shows that Znev_14299 is most closely related to *R. flavipes CYP15A1*, which possesses an N-terminal site truncation and pseudogene defect conserved motif (Tarver et al. 2012). Based on these results, there is a possibility that Znev_14299 may not have sufficient function for JH biosynthesis in *Z. nevadensis*. Another sequence identified in this study (Znev_06067) is confirmed to have six conserved motifs. Phylogenetic analysis shows that Znev_06067 is more closely related to *R. flavipes CYP15F1* than to *R. flavipes CYP15A1*. *CYP15F1* was highly expressed at the head and gut of *R. flavipes* workers, and *CYP15F1* RNAi resulted in a decrease in the presoldier differentiation ratio (Tarver et al. 2012). Although further detailed analyses on these homologs (Znev_14299 and 06057) are required to determine whether they have different roles in JH biosynthesis, the expression levels of these two genes were compared between No. 1 and 2 larvae in this study.

Expression patterns of juvenile hormone biosynthetic genes

The present study shows that the expression of *DD*, *JHAMT* and *CYP15A1* (Znev_06057) is specifically upregulated during presoldier differentiation. In particular, *JHAMT* and *CYP15A1* are involved in the final steps of the JH biosynthetic pathway, and thus high expression levels of these genes may cause an increase in JH titer in No. 1 larvae. Expression levels of *JHAMT* in No. 1 larvae are significantly higher than those in No. 2 larvae 2–3 days after the appearance, suggesting that the JH biosynthesis in No. 1 larvae may be enhanced during this period. The expression level of the putative *CYP15A1* (Znev_06057) is remarkably high only in No. 1 larvae 4–5 days after the appearance. The JH titer in these individuals probably increases quickly during this period, and then gut purging (a release of the contents of the gut) occurs before the molt into a presoldier. *JHAMT* expression analysis of each body part in the No. 1 larva indicates that its level in heads (including the corpora allata) is extremely high (approximately 60 times higher) compared with other body parts. Although further detailed analyses of expression sites are needed, it is suggested that the JH titer in No. 1 larvae is strongly affected by *JHAMT* expression in the head. In *B. mori*, *JHAMT* is involved in the final step of JH biosynthesis, and *JH epoxidase* (*CYP15C1*) was constantly expressed before the final molt (Daimon et al. 2012). The present study shows that Znev_14299 is also constantly expressed during presoldier differentiation. On the other hand, the expression level of Znev_06057 is rapidly increased before the presoldier molt. In *D. punctata*, epoxidase was involved in the final step of JH biosynthesis (Helvig et al. 2004); thus, Znev_06057 may be the key gene to regulate the presoldier differentiation in natural conditions. Further gene function analyses of *JHAMT* and two epoxidases obtained in this study should be performed to conclude this hypothesis.

There are several genes (*IPPI*, *FPS*, *FD1* and *FD2*) with high expression levels until 5 days after the molt only in No. 2 larvae. The penultimate larva of the German cockroach *Blattella germanica* L. had consistently high JH titers during this stage (Treiblmayr et al. 2006). In the dry-wood termite *Cryptotermes secundus* Hill, JH titers of 3rd instar larvae were higher than those of nymphs (>4th instar) (Korb et al. 2009). In the incipient colony of *Z. nevadensis*, it took about 7–8 days for No. 1 larvae to differentiate into a presoldier, but No. 2 larvae molted into 4th instar workers in about 20 days (Maekawa et al. 2012). Thus, the JH titers of No. 2 larvae may increase gradually during the latter half of the 3rd larval stage. The fact that several genes (*IPPI*, *FPS*, *FD1* and *FD2*) express at high levels on day 4–5 may be related to this delayed increase in the JH titer in No. 2 larvae. To examine this possibility, the expression profiles of each gene during the entire 3rd instar larval period should be elucidated.

In the present study, the expression levels of *JHAMT* and *CYP15A1* (Znev_06057) at each point may be used for the prediction of endogenous JH titer changes in 3rd instar larvae of *Z. nevadensis*. The previous study of this species showed that JHA-induced presoldier differentiation from young instar larvae (3rd or 4th instars) was observed at least 10 days after the JHA treatment (Itano and Maekawa 2008). Although the expression patterns of *JHAMT* and *CYP15A1* (Znev_06057) are different from each other, both genes are highly expressed in the No. 1 larva within 5 days after the appearance (Fig. 6d, f), and several days later, a natural presoldier is differentiated (7.3 ± 0.84 days after the appearance; Maekawa et al. 2012). Consequently, the JH biosynthetic activity and endogenous JH titer levels may be rapidly increased much earlier than those after JHA treatment. In general, the JH titer is regulated not only by the JH biosynthetic activity in the corpora allata, but also by the action of other proteins involved in JH degradation and sequestration (Hartfelder 2000). Further research on JH-related genes should be performed to understand the physiological changes via the increased JH titer during presoldier differentiation in natural conditions.

Conclusion

This study clarified that *JHAMT* and *CYP15A1*, which are predicted to be involved in the final steps of the JH biosynthetic pathway, are highly expressed during presoldier

differentiation. No. 1 larvae of this species received proctodeal trophallactic food from reproductives significantly more often than No. 2 larvae (Maekawa et al. 2012). The frequent trophallactic behavior was especially observed 2–4 days after the appearance of No. 1 larvae. These periods are nearly equal to those when high expression levels of JH biosynthetic genes are observed in this study. The JH titer and/or JH biosynthetic gene expression is known to be affected by nutritional conditions in *M. sexta*, *Ae. aegypti*, *D. melanogaster* and *A. mellifera* (Bomtorin et al. 2014; Cymborowski et al. 1982; Noriega 2004; Tu et al. 2005). These results imply a crosstalk between nutritional signals and the JH biosynthetic pathway, and such interactions may also be important mechanisms to regulate presoldier differentiation in *Z. nevadensis*. Further gene expression and function analyses are to be performed to verify this hypothesis.

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