A Crucial Caste Regulation Gene Detected by Comparing Termites and Sister Group Cockroaches

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ABSTRACT Sterile castes are a defining criterion of eusociality; investigating their evolutionary origins can critically advance theory. In termites, the soldier caste is regarded as the first acquired permanently sterile caste. Previous studies showed that juvenile hormone (JH) is the primary factor inducing soldier differentiation, and treatment of workers with artificial JH can generate presoldier differentiation. It follows that a shift from a typical hemimetabolous JH response might be required for soldier formation during the course of termite evolution within the cockroach clade. To address this possibility, analysis of the role of JH and its signaling pathway was performed in the termite *Zootermopsis nevadensis* and compared with the wood roach *Cryptocercus punctulatus*, a member of the sister group of termites. Treatment with a JH analog (JHA) induced a nymphal molt in *C. punctulatus*. RNA interference (RNAi) of JH receptor *Methoprene tolerant* (*Met*) was then performed, and it inhibited the presoldier molt in *Z. nevadensis* and the nymphal molt in *C. punctulatus*. Knockdown of *Met* in both species inhibited expression of 20-hydroxyecdysone (20E; the active form of ecdysone) synthesis genes. However, in *Z. nevadensis*, several 20E signaling genes were specifically inhibited by *Met* RNAi. Consequently, RNAi of these genes were performed in JHA-treated termite individuals. Knockdown of 20E signaling and nuclear receptor gene, *Hormone receptor 39* (*HR39/FTZ-F1β*) resulted in newly molted individuals with normal worker phenotypes. This is the first report of the JH–*Met* signaling feature in termites and *Cryptocercus*. JH-dependent molting activation is shared by both taxa and mediation between JH receptor and 20E signalings for soldier morphogenesis is specific to termites.

KEYWORDS 20-hydroxyecdysone; *Cryptocercus*; juvenile hormone; soldier differentiation; termites

THE complex eusocial society of one-piece termites (those using a single log as food and nest) consists of a reproductive caste (queen and king) and temporarily or permanently sterile castes (workers—also known as helpers, pseudergates, or alloparents—and soldiers, respectively). Termites are a monophyletic group within cockroaches (Lo *et al.* 2000; Inward *et al.* 2007; Bourguignon *et al.* 2017) and the soldier caste is regarded as the first acquired permanently

sterile caste (Nalepa 2011). The molecular basis of termite soldier evolution, however, is still far from fully understood. Increasing juvenile hormone (JH) titers triggers soldier differentiation in workers via an intermediate presoldier stage (Noirot 1985; Roisin 1996) which can be induced in many termite species by treating workers with JH or JH analogs (JHA) (Watanabe *et al.* 2014; Scharf 2015). This is in contrast to other insects in which JH maintains larval traits and has an inhibitory function on molting via suppression of prothoracicotropic hormone (PTTH) release (Gilbert 2012). It is also known that treatment with JHA can inhibit or delay 20-hydroxyecdysone (20E; the active form of ecdysone) synthesis and suppress expression of the 20E signaling genes (Berger *et al.* 1992; Zufelato *et al.* 2000; Aribi *et al.* 2006). In the German cockroach, *Blattella germanica*, JHA treatment of young instars inhibited 20E synthesis and resulted in developmental arrest in the nymphal stage (Hangartner and

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doi: <https://doi.org/10.1534/genetics.118.301038>

Manuscript received April 17, 2018; accepted for publication June 21, 2018; published Early Online June 22, 2018.

Supplemental material available at Figshare: <https://doi.org/10.25386/genetics.6564572>.

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Masner 1973; Masner *et al.* 1975). Furthermore, JH inhibits expression levels of the 20E-induced heat shock protein gene in *Drosophila melanogaster* (Berger *et al.* 1992), but in *D. melanogaster* and *Manduca sexta* JH activates expression level of the 20E-inducible nuclear receptor gene *E75* (Dubrovskaya *et al.* 2004). There is therefore a possibility that one or more unidentified JH signaling pathways related to the involvement of 20E in both molting (from worker to presoldier) and morphological modification (formation of weapons such as enlarged mandibles) were acquired during the course of termite evolution. To clarify this hypothesis, it is necessary to analyze the role of JH in nymphal development in additional cockroaches, particularly those of the sister group of termites, cockroaches in the family Cryptoceridae (wood roaches; *Cryptocercus* spp.).

Recently, the presence of JH signaling genes has been established in some model insect species (Jindra *et al.* 2015). In both hemimetabolous (without pupal stage, including termites and cockroaches) and holometabolous (with pupal stage) insects, a JH receptor, Methoprene tolerant (Met) and a steroid receptor coactivator (SRC; taiman; FISC) induce the expression of Krüppel homolog 1 (Kr-h1), which is necessary for JH to function in maintaining developmental status quo (Riddiford 2013; Jindra *et al.* 2015). *Met* and *Kr-h1* knockdown inhibited molts in the penultimate instar and induced precocious metamorphosis in *Tribolium castaneum* (Konopova and Jindra 2007; Minakuchi *et al.* 2009) and *B. germanica* (Lozano and Bellés 2011, 2014). On the other hand, although Met is generally involved in insect ovarian development, Kr-h1 function differed somewhat among species (Konopova *et al.* 2011; Song *et al.* 2014). Specifically, Kr-h1 was not required for ovarian development in the linden bug, *Pyrrhocoris apterus* (Smykal *et al.* 2014). In termites, a previous study demonstrated that RNA interference (RNAi) of *Met* suppressed soldier-specific morphogenesis in *Zootermopsis nevadensis* (Masuoka *et al.* 2015). Roles of other JH signaling genes, including *Kr-h1*, for termite soldier differentiation, however, have not been clarified. Moreover, in *Cryptocercus* cockroaches, no studies have focused on the function of JH signaling genes during molting.

To determine potential differences in the role of JH during molting in *C. punctulatus* and termites, JHA treatment of young nymphs was performed in *C. punctulatus*. To further clarify the function of JH signaling genes in these taxa, RNAi knockdown of *Met* and *Kr-h1* was conducted in both *Z. nevadensis* and *C. punctulatus*. Furthermore, expression and functional analysis of 20E signaling genes was performed during JHA-induced soldier differentiation. Based on the results, we discuss how the termite-specific JH pathway is related to soldier development, which involves notable morphological changes during the molting processes.

Materials and Methods

Insects

Seventh instars of *Z. nevadensis* were sampled from three mature colonies, which were collected at Hyogo Prefecture,

Japan, in May 2015 and 2016 and kept at ~25° in constant darkness until the following experiments were performed. Young instar nymphs [head width = 1.31–1.57 mm, class 1 (third or fourth instars); and head width = 1.91–2.12 mm, class 2 (probably fifth instars) (Nalepa 1984, 1990)] of *C. punctulatus* were collected at Mountain Lake Biological Station, Giles County, VA, in April 2015–2017. These individuals were kept at 15° in constant darkness until use.

JHA treatment

In *Z. nevadensis*, according to the methods of Saiki *et al.* (2014), filter paper was treated with 0 (for control) or 10 µg JHA (pyriproxyfen; Wako, Osaka, Japan) dissolved in 400 µl acetone and placed in a 90-mm petri dish with 10 individual seventh instars. In *C. punctulatus*, filter paper and 200 mg cellulose powder (Wako) was treated with 0 (for control) or 100 µg pyriproxyfen dissolved in 200 µl acetone and placed in a 60-mm petri dish with 10 class-1 or -2 nymphs. All petri dishes were kept in an incubator at 25° (*Z. nevadensis*) or 15° (*C. punctulatus*) in constant darkness for 30 days. Dishes were checked for dead and newly molted individuals every 24 hr. Molting rates in each species were compared between JHA and acetone control treatments. Fisher's exact test was performed using Mac Statistical Analysis version 2.0 (Esumi, Tokyo, Japan).

RNAi experiment

Each double-strand RNA (dsRNA) was generated by the partial complementary DNA (cDNA) sequences amplified by the gene-specific primers (Supplemental Material, Table S1) using T7 RNA polymerase with a MEGascript T7 Transcription Kit (Ambion, Austin, TX). As in previous studies (Masuoka *et al.* 2015, 2018; Masuoka and Maekawa 2016a,b), *GFP* was selected as a control gene, and dsRNA was generated using GFP vector pQBI-polII (Wako). Specific primers of the following genes of *Z. nevadensis* were designed from genome sequence data using Primer3Plus software (Untergasser *et al.* 2007): *ZnMet* (gene identifier Znev_09571; Terrapon *et al.* 2014), *ZnSRC* (Znev_05083), *ZnKr-h1* (Znev_04171), *ZnShr* (Znev_16529), *ZnSpo* (Znev_04417), *ZnEcR* (Znev_13925), *ZnE74* (Znev_00833), *ZnE75* (Znev_11406), *ZnHR3* (Znev_14707), and *ZnHR39* (Znev_00332). Specific primers of the following genes of *C. punctulatus* were designed from transcriptome sequence data (Hayashi *et al.* 2017; DNA Database of Japan Sequence Read Archive database accession number DRA004598) using Primer3Plus: *CpMet* (expressed sequence tag identifier Cp_TR6397) and *CpKr-h1* (Cp_TR7552). Each dsRNA [500 ng in 136 nl (*Z. nevadensis*); 4 µg in 272 nl (*C. punctulatus*)] was injected into the side of the thorax of individuals using a Nanoliter 2000 microinjector (World Precision Instruments, Sarasota, FL). Within 24 hr of the injection, all individuals were placed in a petri dish with a filter paper (and also cellulose powder for *C. punctulatus*) treated with pyriproxyfen or acetone, and the dish was kept in an incubator as in the previous section. Molting rate was compared between

Figure 1 Results of JHA (pyriproxyfen) and control (acetone) treatment in *C. punctulatus* and *Z. nevadensis* (inset). Molting rates indicate the ratio of nymphal (*C. punctulatus*) and presoldier (*Z. nevadensis*) molt. ** indicates a significant difference (Fisher's exact test). ** $P < 0.01$.

treatments, and Fisher's exact test was performed for the statistical analysis using statistical software R version 3.1.2 (Ihaka and Gentleman 1996). To evaluate the effects of *ZnMet* dsRNA injection timing, dsRNA was injected every 24 hr after JHA treatment (until 120 hr, day 0–5).

Gene expression analysis

Three individuals were collected 3 days after the dsRNA injection. Total RNA was extracted from the whole body of each individual using ISOGEN (NipponGene, Tokyo, Japan). The extracted RNA was purified with DNase treatment and used for cDNA synthesis using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Specific primers of 20E-related genes of *Z. nevadensis* and *C. punctulatus* (*Nvd*: Znev_04416 and Cp_TR25860; *Shr*: Znev_16529 and Cp_TR25505; *Spo*: Znev_04417 and Cp_TR54771; *Phm*: Znev_00957; *Dib*: Znev_08701 and Cp_TR16740; *Sad*: Znev_14659; *Shd*: Znev_02808; *EcR*: Cp_TR4152; *USP*: Znev_11534; *Br-C*: Znev_09723; *E63*: Znev_06687 and Cp_TR16589; *E74*: Znev_00833 and Cp_TR3685; *E75*: Cp_TR8108; *E93*: Znev_02008; *HR3*: Znev_14707 and Cp_TR38613; *HR4*: Znev_17691; *HR38*: Znev_16131; *HR39*: Znev_00332 and Cp_TR1259; *HR78*: Znev_03071; *HR96*: Znev_06284 and Cp_TR49824; *FTZ-F1*: Znev_18259) were newly designed as shown in the previous section (Table S1). JH signaling genes of *C. punctulatus* (*CpMet*: Cp_TR6397 and *CpKr-h1*: Cp_TR7552) were also

newly designed as shown in the previous section. Primers of JH signaling genes (*ZnMet*, *ZnSRC*, and *ZnKr-h1*) and 20E signaling genes of *Z. nevadensis* (*ZnEcR*, *ZnBr-C*, *ZnHR4*, and *ZnE75*) were previously described (Masuoka *et al.* 2015; Masuoka and Maekawa 2016a). The expression level of each gene was quantified using a THUNDERBIRD SYBR qPCR Mix (TOYOBO, Osaka, Japan) and MiniOpticon Real-Time System detection system (Bio-Rad, Hercules, CA). An endogenous control gene was selected from the following three genes, *EF1-α* (Zn: accession no. AB915828; Cp: accession no. AFK49795), *β-actin* (Zn: no. AB915826; Cp: Cp_TR19468), and *NADH-dh* (Zn: no. AB936819; Cp: Cp_TR49774), using GeNorm (Vandesompele *et al.* 2002) and NormFinder (Andersen *et al.* 2004). *EF1-α* was selected in all real-time quantitative PCR (qPCR) analyses performed in this study (Table S2). Real-time qPCR analysis was performed in biological triplicates. Statistical analysis was performed using Mann–Whitney's *U*-test for comparison between a target gene and *GFP* RNAi treatment using statistical software Mac Statistical Analysis version 2.0 (Esumi). For *Z. nevadensis*, prior to the use of ANOVA, we performed the Browne–Forsythe test on the variance equality using statistical software R version 3.1.2 (Ihaka and Gentleman 1996).

Data availability

The authors affirm that all data necessary for confirming the conclusion of the article are present within the article and

Figure 2 Phenotype of newly molted individual and molting rate after the dsRNA injection of JH signaling genes under JHA treatment in *Z. nevadensis*. The fraction on each column indicates number of molted individuals (numerator) and number of treated individuals (denominator). External morphologies of the molted individuals are shown in the top panels. These individuals were photographed 7 days after the molt. * indicates significant differences when compared to the control (GFP) (Fisher's exact test). * $P < 0.05$. n.s., not significant.

supplemental material. Supplemental material available at Figshare: <https://doi.org/10.25386/genetics.6564572>.

Results

JHA treatment in *C. punctulatus* and *Z. nevadensis*

In the class-1 nymphs (third or fourth instars) of *C. punctulatus*, the rates of nymphal molts within 30 days were significantly higher in the JHA-treated individuals than in the acetone controls (76.7 and 10.0%, respectively, $P < 0.01$; Figure 1). Additionally, JHA treatments in the class-2 nymphs (fifth instars) resulted in similar tendencies (JHA: 66.7%; acetone: 20%; $P < 0.01$; Figure 1). In *Z. nevadensis*, most JHA-treated individuals (85.0%) molted into presoldiers within 30 days, whereas no molted presoldiers were observed in the control treatment (Figure 1). These results are consistent with previous reports (Miura *et al.* 2003; Itano and Maekawa 2008; Saiki *et al.* 2014).

RNAi of JH signaling genes under the JHA treatment in *C. punctulatus* and *Z. nevadensis*

RNAi of JH signaling genes was performed in the JHA-treated individuals of *Z. nevadensis* and *C. punctulatus*. First, in *Z. nevadensis*, significant RNAi-knockdown effects were observed in *ZnMet*, *ZnSRC*, and *ZnKr-h1* compared to the GFP control (25.80, 52.62, and 39.00%, respectively; Figure S1). Knockdown of *ZnMet* strongly inhibited the presoldier molts and only 1 in 10 individuals molted into presoldier-like individuals with smaller head capsules and shorter mandibles

compared to the control (Figure 2). Knockdown of *ZnSRC* showed similar results and only 1 in 10 termites molted into a presoldier-like individual (Figure 2). However, *ZnKr-h1* RNAi did not have a significant effect on the molts, and 7 of 10 individuals molted into presoldiers with normal morphological characters (Figure 2). JHA-induced molting rates under *ZnMet* RNAi were significantly higher when dsRNA was injected 3–5 days after the JHA treatment (day 3–5) compared to those just before the treatment (day 0) (Figure S2). The former molted individuals had the enlarged mandibles of normal presoldiers (Figure S2).

In *C. punctulatus*, significant RNAi knockdown effects were observed in *CpMet* and *CpKr-h1* compared to the GFP control (41.99 and 51.31%, respectively; Figure S1). *CpMet* RNAi strongly inhibited the nymphal molts and only 1 in 10 individuals molted into the next instar. *CpKr-h1* RNAi, however, did not have a significant effect on the nymphal molts and 60% of individuals molted into a subsequent instar (Figure S3).

Expression of 20E synthesis and signaling genes under the *Met* RNAi

Changes in expression levels of 20E-related genes in the JHA-treated individuals were observed under the *Met* RNAi both in *Z. nevadensis* and *C. punctulatus*. In *Z. nevadensis*, *ZnMet* knockdown significantly inhibited the expression levels of two 20E synthesis genes (*ZnShr* and *ZnSpo*) and seven signaling genes including a 20E receptor gene (*ZnEcR*, *ZnE63*, *ZnE74*, *ZnE75*, *ZnHR3*, *ZnHR39*, and *ZnHR96*) (Figure 3). On

Figure 3 Expression levels (mean $\pm$ SE, biological triplicates) of 20E synthesis and signaling genes in 0–5 days after JHA treatment under *Met* RNAi in *Z. nevadensis*. Expression levels were normalized by *EF1- α* (*EF1a*) expression. Relative expression levels were calibrated using the mean expression level of individuals just before the JHA treatment (d0) as 1.0. The statistical results of two-way ANOVA are described in each box. The data are consistent with the use of parametric statistics by the Browne–Forsythe test [*ZnMet*: $P = 7.91\text{E-}01$ (GFP), $5.90\text{E-}01$ (*ZnMet* RNAi); *ZnNvd*: $P = 7.88\text{E-}01$ (GFP), $7.91\text{E-}01$ (*ZnMet* RNAi); *ZnShr*: $P = 5.37\text{E-}01$ (GFP), $5.89\text{E-}01$ (*ZnMet* RNAi); *ZnSpo*: $P = 7.77\text{E-}01$ (GFP), $4.93\text{E-}01$ (*ZnMet* RNAi); *ZnPhm*: $P = 4.43\text{E-}01$ (GFP), $2.89\text{E-}01$ (*ZnMet* RNAi); *ZnDib*: $P = 5.24\text{E-}01$ (GFP), $6.81\text{E-}01$ (*ZnMet* RNAi); *ZnSad*: $P = 7.50\text{E-}01$ (GFP), $7.52\text{E-}01$ (*ZnMet* RNAi); *ZnShd*: $P = 8.53\text{E-}01$ (GFP), $9.60\text{E-}01$ (*ZnMet* RNAi); *ZnEcR*: $P = 9.47\text{E-}01$ (GFP), $8.75\text{E-}01$ (*ZnMet* RNAi); *ZnUSP*: $P = 9.08\text{E-}01$ (GFP), $4.35\text{E-}01$ (*ZnMet* RNAi); *ZnBr-C*: $P = 5.31\text{E-}01$ (GFP), $2.30\text{E-}01$ (*ZnMet* RNAi); *ZnE63*: $P = 8.46\text{E-}01$ (GFP), $6.73\text{E-}01$ (*ZnMet* RNAi); *ZnE74*: $P = 8.57\text{E-}01$

the other hand, in *C. punctulatus*, expression levels of different 20E synthesis genes (*CpNvd* and *CpDib*) were decreased by *CpMet* RNAi treatment (Figure 4). Although expression of some 20E signaling genes (*CpE63*, *CpHR3*, and *CpHR96*) were negatively affected by the *CpMet* RNAi as shown in *Z. nevadensis*, expression levels of *CpEcR*, *CpE74*, *CpE75*, and *CpHR39* were not significantly decreased by the RNAi treatment (Figure 4).

RNAi of 20E synthesis and signaling genes during JHA-induced presoldier differentiation

RNAi of 20E synthesis (*ZnShr* and *ZnSpo*) and signaling genes (*ZnEcR*, *ZnHR3*, *ZnE74*, *ZnE75*, and *ZnHR39*) was performed during artificial presoldier differentiation (Figure 5). Expression levels of each of these genes except *HR3* were negatively affected by *Met* RNAi in *Z. nevadensis*, but not in *C. punctulatus*. Consequently, these expression changes might have crucial roles in presoldier-specific molting events. Expression levels of *HR3* were significantly decreased by *Met* RNAi in both species and thus *HR3* might have a similar role in their molting processes. The expression levels of *ZnSpo*, *ZnE75*, *ZnHR3*, and *ZnHR39* were also significantly repressed by *ZnSRC* RNAi treatment; however, *ZnKr-h1* RNAi did not affect expression levels of any gene examined (Figure S4). RNAi treatment of *ZnShr* and *ZnE74* did not affect JHA-induced presoldier differentiation, similar to those of *GFP* controls. *ZnSpo* and *ZnE75* RNAi significantly inhibited the molting process, but were nevertheless treated with JHA. Although knockdown of *ZnEcR* and *ZnHR3* did not affect the rate of gut-purged individuals (those that eliminate their gut contents before molt), all injected individuals failed to shed old cuticles (0% molting rate). Interestingly, *ZnHR39* RNAi did not inhibit the molting process, but the molted individuals had worker-like phenotypes with shorter mandibles and smaller head capsules.

Discussion

Termites and *Cryptocercus* have a similar JH-dependent molting system

Molting events were caused by the JHA treatments not only in *Z. nevadensis* (presoldier differentiation) but also in *C. punctulatus* (nymphal molts), suggesting that in these taxa JH has a role in activating the molting process. Generally, JH has an inhibitory role in molting via the repression of PTTH secretion and subsequent 20E synthesis (Gilbert 2012). In the German cockroach *B. germanica*, JHA treatment delays nymphal molt via inhibition of 20E synthesis (Hangartner and Masner 1973; Masner *et al.* 1975). In some lepidopteran

Figure 4 Expression levels (mean $\pm$ SE, biological triplicates) of 20E synthesis and signaling genes under *Met* RNAi in *C. punctulatus*. Expression levels were normalized by *EF1- α* (*EF1a*) expression. Relative expression levels were calibrated using the mean expression level of *GFP* dsRNA-injected individuals as 1.0. * denotes significant differences (Mann–Whitney’s *U*-test). * $P < 0.05$, ** $P < 0.01$. n.s., not significant.

species, however, JH can activate the prothoracic gland during pupation (Hiruma *et al.* 1978; Cymborowski and Stolarz 1979). Moreover, in the damp-wood termite *Hodotermopsis sjostedti*, JHA induced growth in the prothoracic gland of pseudergates (Cornette *et al.* 2008). Recent phylogenetic analyses strongly supported a monophyly of termites within the cockroach clade and sister group relationships between termites and *Cryptocercus* cockroaches (Bourguignon *et al.* 2017). Although further JH-treatment assays on some cockroach species are needed, there is a possibility that in both termites and *Cryptocercus* cockroaches, JH has a role in the activation of the molting process.

Role of JH signaling genes in termites and *Cryptocercus*

In both *Z. nevadensis* and *C. punctulatus*, knockdown of JH receptor, *Met*, inhibited the molting event instigated by JHA treatment of nonadult individuals. In addition, presoldier-specific morphogenesis (e.g., elongation of mandibles) was also inhibited by *Met* RNAi in *Z. nevadensis*. These phenotypic effects were similar to those when RNAi of the insulin receptor gene was performed in *H. sjostedti* (Hattori *et al.* 2013). Surprisingly, however, knockdown of the *Met* target gene, *Kr-h1*, had no influence on the JHA-induced molting rates in both termites and wood roaches or on morphogenetic changes in termites. These results suggest that the JHA-inducible process of molting (and also specific morphogenesis in termites) is activated via a JH receptor non-*Kr-h1* signaling pathway. During metamorphosis in holometabolous insects,

(*GFP*), 9.93E–01 (*ZnMet* RNAi); *ZnE75*: $P = 9.17E-01$ (*GFP*), 3.02E–01 (*ZnMet* RNAi); *ZnE93*: $P = 9.99E-01$ (*GFP*), 5.34E–01 (*ZnMet* RNAi); *ZnHR3*: $P = 2.61E-01$ (*GFP*), 9.48E–01 (*ZnMet* RNAi); *ZnHR4*: $P = 6.39E-01$ (*GFP*), 6.81E–01 (*ZnMet* RNAi); *ZnHR38*: $P = 6.44E-01$ (*GFP*), 3.78E–01 (*ZnMet* RNAi); *ZnHR39*: $P = 3.45E-01$ (*GFP*), 4.08E–01 (*ZnMet* RNAi); *ZnHR78*: $P = 7.95E-01$ (*GFP*), 9.23E–01 (*ZnMet* RNAi); *ZnHR96*: $P = 9.34E-01$ (*GFP*), 6.09E–01 (*ZnMet* RNAi); *ZnFTZ-F1*: $P = 9.88E-01$ (*GFP*), 6.62E–01 (*ZnMet* RNAi)] prior to the use of the ANOVA. Gene names with significant different expression levels between injected dsRNAs are shown in bold. * $P < 0.05$, ** $P < 0.01$, inter., interaction.

Figure 5 Phenotype of newly molted individual and molting and gut-purging rate after the dsRNA injection of 20E synthesis and signaling genes under JHA treatment in *Z. nevadensis*. The fraction on each column indicates number of molted or gut-purged individuals (numerator) and number of treated individuals (denominator). * indicates significant differences when compared to the control (GFP; Fisher's exact test). External morphologies of the molted individuals are shown in the top panels. These individuals were photographed 7 days after the molt. No molting individuals were obtained by *ZnEcR* and *ZnHR3* RNAi, but all gut-purged individuals died just before the molt because of a failure of the shedding of old cuticle, as shown in the top panel. * $P < 0.05$, ** $P < 0.01$. n.s., not significant.

JH acts to maintain developmental status quo in the larval stage via the *Kr-h1* pathway (Minakuchi *et al.* 2009). *Kr-h1* works as an important early transcription factor within the JH signaling pathway and is known to be involved in other JH-triggered phenomena such as ovarian development in *T. castaneum* and *Locusta migratoria* (Minakuchi *et al.* 2009; Konopova *et al.* 2011; Kayukawa *et al.* 2012). However, in the linden bug *P. apterus*, *Kr-h1* had little influence on ovarian development (Song *et al.* 2014). Further investigations are needed to determine whether there is a non-*Kr-h1* signaling pathway for the JH-inducible process of molting in termites and wood roaches, and in the specific morphogenesis found in termites.

Met regulates expression of 20E synthesis and signaling genes in both species

Met knockdown repressed expression levels of some 20E-related genes under JHA application both in *Z. nevadensis* and *C. punctulatus*. The expressions of the different 20E synthesis genes were inhibited by *Met* knockdown in *Z. nevadensis* (*ZnShr* and *ZnSpo*) and *C. punctulatus* (*CpNvd* and *CpDib*). There is a possibility that *Met* is involved in 20E synthesis activity via expression changes of different synthesis genes in the prothoracic glands of termites and *Cryptocercus* (Figure 6). A notable difference was also observed between the two species when the expression levels of 20E-related genes were

examined after *Met* RNAi. Expression levels of *ZnEcR*, *ZnE74*, *ZnE75*, and *ZnHR39* were significantly repressed after *ZnMet* RNAi in *Z. nevadensis* but no significant decreased levels were observed after *CpMet* RNAi in *C. punctulatus*. One possibility is that such differences in 20E-related gene expression changes via JH action may be related to soldier-specific morphogenesis in termites. RNAi-mediated function analysis was performed in this study to clarify this possibility.

The function of 20E-related genes in termites

Expression levels of both *ZnHR3* and *CpHR3* were significantly decreased by *Met* RNAi in the JHA-treated individuals. RNAi of *ZnHR3* resulted in the failure of ecdysis and all molting individuals died before the completion of ecdysis as shown in other insects [*T. castaneum* (Tan and Palli 2008a) and *L. migratoria* (Zhao *et al.* 2018)], including cockroaches [*B. germanica* (Cruz *et al.* 2007)]. These results suggest that an ecdysis-related function of *HR3* is conserved among insects and its expression occurs under JH signaling both in *Z. nevadensis* and *C. punctulatus*. To clarify the specific role of JH receptor signaling for 20E-related gene expression changes in termites, functional analyses of genes with different expression patterns after *ZnMet* and *CpMet* RNAi were performed. *ZnShr*- and *ZnE74*-knockdown treatments did not have any significant effects on presoldier differentiation and resulted in phenotypes similar to those found in the *GFP*

Figure 6 Hypothetical pathway of JH signaling in wood roaches and termites. A common pathway involved in 20E synthesis may control a downstream molting process via the JH receptor. In termites, soldier-specific morphogenesis may be regulated by a specific JH receptor pathway, probably involved in the 20E signaling genes including *HR39*.

control. These genes may not have an important role for the molting event accompanied with morphological changes. *ZnSpo* and *ZnE75* RNAi resulted in the inhibition of molting, although the individuals were treated with enough JHA to induce the presoldier molt. In *Bombyx mori*, *E75* was involved in the activation of expression of 20E synthesis genes including *Spo* (Li *et al.* 2016). In the early process of termite presoldier molting, *Spo* may have a critical role in 20E synthesis under JH signaling via *E75* expression (Figure 6). *ZnEcR* RNAi resulted in a failure of the shedding of old cuticle; although a newly formed cuticle was generated under the old cuticle, as shown in the presoldier-soldier molt in *Z. nevadensis* (Masuoka and Maekawa 2016a), the imaginal molt in *B. germanica* (Cruz *et al.* 2006), and the larval molt in *T. castaneum* (Tan and Palli 2008b). On the other hand, *ZnHR39* RNAi produced a unique effect and the newly molted worker-like individuals had no presoldier-specific morphogenesis. In holometabolous species, the orphan nuclear receptor gene *HR39* (*FTZ-F1β*) had multiple functions in metamorphosis including neuronal remodeling and muscle generation (Tan and Palli 2008a; Boulanger *et al.* 2011; Zirin *et al.* 2013). The present results strongly suggest that termite *HR39* is necessary for the drastic morphological changes that occur during soldier differentiation (Figure 6). Note that these changes in termites can be produced under the high levels of JH that result from artificial JHA treatment, whereas a metamorphosis in holometabolous insects is initiated by a reduction of larval JH titer. An important future topic will be to determine the differences in the JH–*HR39* regulatory mechanism between termites (soldier differentiation) and holometabola (metamorphosis).

Conclusion

In this study, a comparative analysis of the role of the JH signaling pathway during molting was done in termites

(*Z. nevadensis*) and sister group wood roaches (*C. punctulatus*). The results showed that JH-inducible molting via a receptor (Met) occurred in both termites (presoldier differentiation) and wood roaches (nymphal molt). Further, termite 20E signaling gene *HR39* is expressed under JH signaling via *Met* and has a crucial function in presoldier morphogenesis. The present study provides important insights into the proximate mechanisms of soldier evolution in termites. Namely, two crucial changes might be necessary for the evolution of termite soldiers: (1) the acquisition of a molting activation mechanism induced by high levels of JH (a feature shared by termites and wood roaches), and (2) a novel mediation between JH receptor and 20E signalings for specific morphogenesis (only in termites). Although some caution should be exercised when using the German cockroach *B. germanica* as a baseline for comparisons with termites, recent in-depth transcriptome analysis showed consistent expression patterns of 20E-related genes among *B. germanica* and termites (Harrison *et al.* 2018). Furthermore, we recently clarified that TGFβ signaling is involved in the mediation between JH and 20E pathways during soldier differentiation (Masuoka *et al.* 2018). These insights also support that a novel 20E signaling role might trigger a soldier evolution within the cockroach clade.

Acknowledgments

We thank the director and staff of Mountain Lake Biological Station for permission to collect *Cryptocercus punctulatus* on the grounds. Thanks are also due to Takumi Kayukawa and Tetsuro Shinoda for productive discussions. This study was supported in part by Grants-in-Aid for Japan Society for the Promotion of Science Fellows (nos. JP15J10817 and JP17J06352 to Y.M.) and Scientific Research (nos. JP25128705 and JP16K07511 to K.M.) from the Japan Society for the Promotion of Science.

Author contributions: Y.M. and K.M. designed experiments; Y.M., K.T., and C.A.N. collected samples and performed application analysis with the juvenile hormone analog; Y.M. performed molecular experiments and analyzed data; Y.M., C.A.N., and K.M. wrote the manuscript; and K.M. conceived of the study, designed the study, coordinated the study. All authors read and gave final approval for publication.

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Communicating editor: C. Peichel