RESEARCH ARTICLE

Soldier Morphogenesis in the Damp-Wood Termite Is Regulated by the Insulin Signaling Pathway

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ABSTRACT

Eusocial insects exhibit various morphological castes associated with the division of labor within a colony. Termite soldiers possess defensive traits including mandibles that are greatly exaggerated and enlarged, as compared to termite reproductives and workers. The enlarged mandibles of soldiers are known to result from dynamic morphogenesis during soldier differentiation that can be induced by juvenile hormone and its analogs. However, the detailed developmental mechanisms still remain unresolved. Because the insulin/insulin-like growth factor signaling (IIS) pathway has been shown to regulate the relative sizes of organs (i.e., allometry) in other insects, we examined the expression profiles of major IIS factors in the damp-wood termite *Hodotermopsis sjostedti*, during soldier differentiation. The relative expression patterns of orthologs for termite *InR* (*HsjInR*), *PKB/Akt* (*HsjPKB/Akt*), and *FOXO* (*HsjFOXO*) suggest that *HsjInR* and *HsjPKB/Akt* were up-regulated in the period of elongation of mandibles during soldier development. In situ hybridization showed that *HsjInR* was strongly expressed in the mandibular epithelial tissues, and RNA interference (RNAi) for *HsjInR* disrupted soldier-specific morphogenesis including mandibular elongation. These results suggest that signaling through the IIS pathway is required for soldier-specific morphogenesis. In addition, up-regulation of the IIS pathway in other body tissues occurred at earlier stages of development, indicating that there is tissue-specific IIS regulation. Because the IIS pathway is generally thought to act upstream of JH in insects, our results suggest the damp-wood termite may have evolved a novel feedback loop between JH and IIS that enables social interactions, rather than nutrition, to regulate caste determination. *J. Exp. Zool. (Mol. Dev. Evol.) 320B: 295–306, 2013.* © 2013 Wiley Periodicals, Inc.

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Insects show vast diversity in their morphology, and in extreme cases this can include exaggerated morphologies like horns or enlarged appendages (Emlen and Nijhout, 2000). Often, these extreme morphologies develop only when specific environmental cues exist, so that not all individuals produce the exaggerated traits [polyphenism, sensu West-Eberhard (2003)]. Castes in social insects are a classic and representative example, where highly differentiated developmental forms co-occur within colonies and exhibit cooperative social behavior (Nijhout, '99, 2003a; Miura, 2005).

Termites (order Isoptera), one of the major groups of social insects, possess three types of morphologically distinct individuals, that is castes, with specific morphologies that are linked to the division of labor within a colony (Weesner, '69; Wilson, '71). These castes include reproductives, workers, and soldiers. The soldier caste is distinctive in termites because of morphological and behavioral specialization for colony defense. In many species of termites, soldiers attack enemies (e.g., ants) with their weapon-like mandibles or frontal projections (Deligne et al., '81), which grow to exaggerated proportions.

In the damp-wood termite *Hodotermopsis sjostedti* (Isoptera, Termopsidae), seventh-instar larvae differentiate into soldiers (Miura et al., 2000, 2004; Fig. 1). These larvae are known as "pseudergates" and have worker roles but still maintain the potential to become reproductives (definition according to Korb and Hartfelder, 2008). This worker caste is hereafter referred to as "workers (W)." Soldier differentiation requires two subsequent molting events, the first leading to a "presoldier (PS)" stage, and the second leading to the mature soldier (S) (Miller, '69; Noirot, '69; Roisin, 2000).

In the course of soldier differentiation, termite body proportions are dramatically changed; the anterior portions in particular are modified to produce elongated or exaggerated mandibles (Deligne et al., '81; Koshikawa et al., 2002, 2003; Miura, 2005; Fig. 1). Prior to the molt into presoldiers, complicated morphogenetic processes are observed in the anteriormost body parts including the mandibles (Koshikawa et al., 2003), and several downstream genes are known to be up-regulated in these developing mandibles (Koshikawa et al., 2005). In the dynamic morphogenesis that occurs during soldier differentiation, genes related to this process are expected to have important roles in the formation of soldier-specific structures (Miura, 2001, 2005). These candidate genes can be predicted to include morphogenetic factors (Kojima, 2004; Moczek and Nagy, 2005) and tissue-specific downstream responses to endocrine regulators that control metamorphosis like juvenile hormone and ecdysteroids (Thummel, 2001). Here we focus on the insulin/insulin-like growth factor signaling (IIS) pathway because this pathway is known to regulate body size, organ size, and allometry in insects (Shingleton et al., 2005; Edgar, 2006; Emlen et al., 2006, 2012).

The IIS pathway affects metabolism (Unger et al., '78; Saltiel and Kahn, 2001), as well as numerous developmental processes

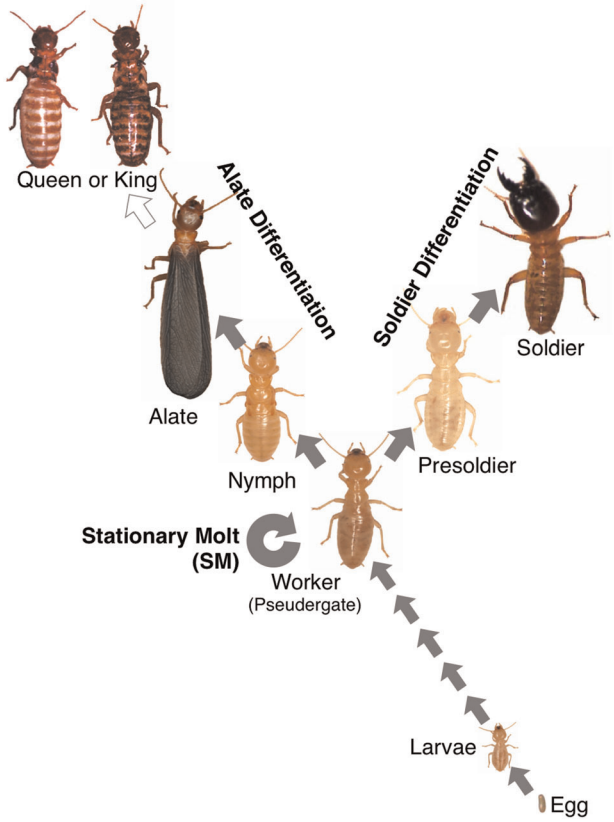

Figure 1. The caste developmental pathway of the damp-wood termite *Hodotermopsis sjostedti*, modified from Miura et al. (2004). The worker (pseudergate) is a termite in the seventh larval instar with the developmental potential to differentiate into an alate reproductive, a soldier or to maintain its undifferentiated worker caste state.

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(Juul et al., '94; Brüning et al., 2000; Renehan et al., 2006). During larval growth, this pathway is thought to integrate nutritional and hormonal signals to coordinate growth (Nijhout, 2003b; De Loof, 2008). When the ligands, that is, insulin and insulin-like peptides (ILPs), bind to InR (insulin receptor), the IIS cascade is activated (Wu and Brown, 2006). As a result, protein synthesis and cell growth are stimulated (Hara et al., '98; Ruvinsky and Meyuhas, 2006). In addition, the IIS pathway is known to regulate relative organ sizes in adult insects (Weinkove et al., '99; Puig et al., 2003; Shingleton et al., 2005, 2007), and this pathway was recently linked with caste differentiation in honeybees (Wheeler et al., 2006; Corona et al., 2007; de Azevedo and Hartfelder, 2008).

Based on these previous studies, we predicted that the IIS pathway would be involved in the allometric growth of soldier heads in termites. In this study using the damp-wood termite *Hodotermopsis sjostedti*, the expression profiles of termite orthologous genes to *InR* (insulin receptor), *PKB*/*Akt* (protein kinase B), and *FOXO* (forkhead box-containing protein, O), that is, *HsjInR*, *HsjPKB*/*Akt*, and *HsjFOXO*, were investigated during soldier differentiation induced by the application of juvenile hormone analog (JHA). In this pathway, *InR* and *PKB*/*Akt* work as positive regulators while *FOXO* regulates negatively (Puig et al., 2003; Wu and Brown, 2006). In order to examine the localization and functions of the IIS pathway, we also carried out in situ hybridization and functional analysis by RNAi for *HsjInR*.

MATERIALS AND METHODS

Insects

Colonies of *Hodotermopsis sjostedti* (*Hsj*) were sampled from rotten wood in evergreen forests on Yakushima Island, Kagoshima Prefecture, Japan, in May 2007 and May 2008. No specific permits were required for the described field studies. The sampling location is not privately owned or protected in any way and the field studies did not involve endangered or protected species. They were maintained in the laboratory as stock colonies at approximately 25°C under constant darkness.

Induction of Soldier Differentiation by JHA

Termite soldier differentiation is rare under natural and laboratory conditions and therefore in order to induce soldier formation we applied juvenile hormone (and/or its analogs) following previous studies (Ogino et al., '93; Cornette et al., 2006). Specifically, in this study we artificially induced soldier differentiation using the juvenile hormone analog pyriproxyfen as this has the most consistent and strongest induction in this species (Ogino et al., '93; Cornette et al., 2006).

Workers (pseudergates) were isolated in groups of 10 individuals in a Petri dish and fed with filter paper. Soldier differentiation was induced by the ingestion method of the juvenile hormone analogue (JHA), pyriproxyfen (Sigma–Aldrich, St. Louis, Missouri, USA; Ogino et al., '93), or the topical application of 5 µg pyriproxyfen diluted in 5 µL acetone per individual (Cornette et al., 2006). In both methods the presoldier molt is induced in 14 days or more after the onset of JHA application under laboratory conditions (25°C and constant darkness). This method is routine in termite research and produces normal presoldiers (and soldiers in a favorable condition) that are behaviorally and morphologically indistinguishable from soldiers developing naturally within colonies (Koshikawa et al., 2002).

To elucidate gene expression profiles during soldier differentiation, experimental stages were defined as follows: days post-JHA application—Day 1, Day 3, Day 7, and PS (presoldier, $n = 20$). In addition, the workers (W, $n = 20$), soldiers (S, $n = 20$), and workers before stationary molt (SM, $n = 20$), which were collected from stock colonies, were also included in order to compare the presoldier molt with other normal molts. The individuals sampled before SM were diagnosed based on their body color as previously described (Koshikawa et al., 2005).

Gene Cloning

Total RNA was extracted from whole bodies of workers (excluding the symbiont-containing gut by dissection) and subjected to RT-PCR to obtain cDNA fragments for orthologs of *InR*, *PKB*/*Akt*, and *FOXO*. Cloning primers for *HsjInR* were designed based on Kumar et al. (2008), while those for *HsjPKB*/*Akt* and *HsjFOXO* were newly designed.

After the removal of the symbiont-containing gut, total RNA was extracted from whole bodies of workers (W, 20 individuals) using the RNAgents total RNA extraction system (Promega, Madison, WI, USA), and reverse transcribed with random hexamer primers (Invitrogen, Carlsbad, CA, USA) and SuperScript III reverse transcriptase (Invitrogen). Using the reverse-transcribed cDNAs as templates, gene fragments of orthologs for *InR*, *PKB*/*Akt*, and *FOXO* were obtained by PCR amplification with specific degenerate primers, and with ExTaq polymerase mix (Takara, Shiga, Otsu, Japan). Primers for *HsjInR* were designed based on a previous study [forward: 5′-CTT YGG NAT GGT NTA YGA RGG-3′; reverse: 5′-CGT CAT NCC RAA RTC NCC RAT YTT-3′] (Roovers et al., '95), while conserved regions of cDNA sequences from *PKB*/*Akt* and *FOXO* genes of *Drosophila melanogaster*, *Tribolium castaneum*, *Aedes aegypti*, and *Apis mellifera* were used to design degenerate primers for *HsjPKB*/*Akt* [forward: 5′-AAR GAC GAA GTN GCN CAC AC-3′; reverse: 5′-CCC CAC CAA TCN ACA GCT CGT CC-3′] and *HsjFOXO* [forward: 5′-ACC GGA AAA TCN TCY TGG TGG ATG-3′; reverse: 5′-GAA GCT CGA GGN CKR AAR TCN GG-3′]. The PCR conditions were 94°C for 30 sec, 45–50°C for 30 sec and 72°C for 1 min for 35 cycles. The cDNA fragments were subcloned into pGEM-T vector (Promega), and transfected into *Escherichia coli* JM109. The nucleotide sequences of the cDNA fragments were determined with a Dye Terminator cycle sequencing kit and automatic sequencer model 3100 (Applied Biosystems, Carlsbad, CA, USA). Database searches for homologies were performed using BlastX at the NCBI server (http://blast.

ncbi.nlm.nih.gov/Blast.cgi). Molecular phylogenetic trees were constructed with orthologs from other animal species including insects by using the Neighbor-Joining method (Saitou and Nei, '87) with ClustalX software (http://www.clustal.org).

Real-Time Quantitative RT-PCR Analysis

Mandibles, heads (including mandibles), and bodies (thoraces and abdomens excluding guts) were dissected from all individuals, frozen in liquid nitrogen, and preserved at −80°C. Total RNA was extracted using the RNAgents total RNA extraction system (Promega), and total RNA was reverse transcribed with random hexamer primers (Invitrogen) and SuperScript III Reverse Transcriptase (Invitrogen).

Relative quantification of transcripts was performed using Power SYBR Green PCR Master Mix and the sequence detection system ABI PRISM 7000 (Applied Biosystems, Foster City, CA, USA). As an endogenous control of constitutive expression, the 18S rRNA gene (NCBI Accession Number: AF220567) was used, because previous studies showed that the transcript level of this gene was relatively constant and thus suitable as a reference gene (Ishikawa et al., 2010; Koshikawa et al., 2010). Primers for quantitative PCR were designed using Primer Express software (ver. 2.0.0, Applied Biosystems). Primer sequences of both targets and the endogenous control were as follows; *HsjInR* [103F: 5′-AAG GCC TTC AAC ACA CAC CAT-3′, 162R: 5′-TGG CTG GCC TTG TGA TAC C-3′], *HsjPKB/Akt* [34F: 5′-CAT CCC TTC CTT ATA TCG CTC AAG-3′, 108R: 5′-GTT CAC ATA CTC CAT GAC AAA GCA T-3′], *HsjFOXO* [53F: 5′-TGG AGA CCT CCA AGT TCG-3′, 107R: 5′-GCC TCC ACC TTC TTC TTG-3′], *Hsj18SrRNA* [1579F: 5′-CTT GCA ATT GTT CCC CAT GA-3′, 1646R: 5′-ACG TAA TCA ACG CGA GCT TAT G-3′]. Data acquisition and analysis were handled with ABI Prism 7000 SDS software ver. 1.2.3 (Applied Biosystems), with the relative standard curve method. For quantification accuracy, each experiment was done in technical triplicate, and statistical analyses were performed for the comparisons (one-way ANOVA and post hoc Tukey's multiple comparison tests, $P < 0.05$).

In Situ Hybridization

In order to examine the localization of *HsjInR*, in situ hybridization was carried out on JHA-induced workers at Day 7 and 14 postapplication. These stages were chosen based on the qPCR expression level of *HsjInR* from whole heads and mandibles.

Heads of the sample termites were fixed with 4% paraformaldehyde, and cryoprotected in sucrose solution. Then, the heads were embedded in O.C.T. compound (Sakura Finetek, Tokyo, Japan) and sliced to prepare 10 μm thick cryosections. The sections were hybridized with digoxigenin (DIG)-11-UTP-labeled antisense RNA probes (or sense RNA for control). The probes were prepared by in vitro transcription of the linearized *HsjInR*-ligated plasmids with the T7 promoter using the DIG RNA Labeling Kit (Roche Diagnostics, Basel, Switzerland).

The sections were treated sequentially with PBT (0.1% Tween 20; 10 min 2×), PBS (5 min), 4% PFA (10 min), PBS (5 min 3×), 1 μg/mL protenase K (10 min), 2 mg/mL Glycine in PBS (1 min), PBS (5 min), 4% PFA (10 min), PBS (5 min 3×) and 0.25% acetic anhydride in 0.1 M triethanolamine (10 min). After two washes in 4× saline sodium citrate (4× SSC) and 1 hr prehybridization at 42° C in a hybridization buffer [5× SSC, 50% formamide, 0.1% Tween 20, 5× Denhardt's solution (Nacalai tesque, Kyoto, Japan), 750 μg/mL Salmon sperm DNA (Roche Diagnostics, Mannheim, Germany)], sections were hybridized with the digoxigenin-labeled RNA probes at 42°C for approximately 48 hr. The probe concentration was approximately 0.5 μg/mL.

To visualize hybridization signals, sections were rinsed in buffer I (0.1 M Tris–HCl, pH 7.5, 0.15 mM NaCl) for 1 min, incubated in buffer II (1% blocking reagent in buffer I) for 30 min, and then applied anti DIG-AP conjugate (Roche Diagnostics) [500× dilution] in buffer II for 60 min. After washes with buffer I (15 min 2×) and buffer III (0.1 M Tris–HCl, pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$, 2 min), the hybridized probe was detected by alkaline phosphatase (AP) conjugated anti-DIG antibody using DIG nucleic acid detection kit (Roche Diagnostics). The NBT-BCIP solution of 100–200× dilution in buffer III was applied, and the NBT-BCIP reaction lasted for 12–24 hr at room temperature. To stop the reaction, sections were treated in buffer IV (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 10 min) and washed in distilled water (5 min). Finally, sections were air-dried, and then cover-slipped by using 70% glycerol in H_2O. The images were captured using a BX51 microscope (Olympus, Tokyo, Japan) connected to DP50 CCD camera (Olympus), and the images were digitized with the Viewfinder Lite version 1.0 program (Olympus).

RNA Interference

To clarify the IIS-signaling function in soldier morphogenesis in *H. sjostedti*, RNAi experiments were performed. RNAi knockdown during termite soldier differentiation has been previously established in *H. sjostedti* by injecting double stranded RNA (dsRNA) of target genes into the insect abdomen after JHA preparation, resulting in the repression of target gene expression (Koshikawa et al., 2010). Control treatments used standard GFP-dsRNA injection (Koshikawa et al., 2010). In this experiment, dsRNA was injected into JHA-treated workers at Day 3 post-JHA application. For the evaluation of RNAi phenotypes, principal component analyses of morphometric measurements were carried out.

To prepare the dsRNA for RNA interference, we performed PCR with M13 forward primer (5′-GTT TTC CCA GTC ACG TTG TA-3′) and *InR* gene degenerate primer, using InR plasmids as a template. The PCR conditions were 94°C for 30 sec, 45°C for 30 sec, and 72° C for 1 min for 35 cycles. PCR products were purified using the Wizard SV Gel and PCR clean-up system (Promega). RNA was prepared by in vitro transcription with the MEGAscript RNAi kit (Ambion, Austin, TX, USA). To prepare dsRNA, sense and

antisense RNA were mixed and heated at 75°C for 5 min, then left to cool at room temperature. After nuclease digestion to remove template DNA and single-stranded RNA, the product (540 bp) was diluted with PBS to a final concentration of 1 μg/μL.

JHA-treated workers at Day 3 after the onset of JHA application were collected and anaesthetized on ice. dsRNA were injected into the dorsal side between the fourth and fifth tergites, using glass capillaries attached to a micromanipulator (Narishige, Tokyo, Japan). Approximately 1 μg dsRNA ($n = 25$) or 1× PBS (controls, $n = 23$) was injected into each individual. Injected insects were placed in Petri dishes and maintained in an incubator at 25°C for 2–3 weeks. Only individuals that molted within the typical 2–3 weeks were used for the morphometric study. The RNAi control was dsRNA of green fluorescent protein (GFP) synthesized and injected as described above for IIS pathway genes.

Morphometric Study

To evaluate the effects of *dsInR* RNAi on termite caste differentiation, morphometric analyses were performed according to previous studies (Koshikawa et al., 2002, 2004). Principal component analyses (PCA) effectively describe the shape differences associated with alternative termite castes. We used the following 16 measurements: head length (HL); maximum head width (HW); head width at the base of the mandibles (HW2); labrum width (LW); postmentum length (PmL); postmentum width (PmW); right mandible length (ML); length between apical and first teeth of right mandible (AL); eye diameter (ED); pronotum length (PnL); pronotum width (PnW); mesonotum width (MsW); metanotum width (MtW); hind femur width (FW); hind femur length (FL); hind tibia length (TL). The measurements were performed using an image analysis system with a CCD camera, HIM-1N (HOGA, Kyoto, Japan). PCA was conducted using JMP (SAS Institute, Tokyo, Japan). A correlation matrix was used for extraction, which is equivalent to using standardized data. To evaluate the effects of RNAi treatments, Tukey's multiple comparison test ($P < 0.01$) was performed after ANOVA.

RESULTS

Identification of Insulin-Pathway Genes

Partial cDNA fragments of *InR*, *PKB*/*Akt*, and *FOXO* were obtained from *H. sjostedti* by degenerate PCR. By means of RT-PCR, cDNA fragments whose lengths were 387, 390, and 228 bp were obtained for orthologs of the damp-wood termite *InR*, *PKB*/*Akt*, and *FOXO*, respectively. All of these fragments encode amino acid sequences that have strong similarity to *InR*, *PKB*/*Akt*, and *FOXO* orthologs in *Tribolium castaneum* (percent identity 80.0%, 92.3%, and 73.7%, respectively). The damp-wood termite sequence information is deposited in the GenBank/EMBL/DDBJ database under the accession numbers; *HsjInR*: AB568260; *HsjPKB*/*Akt*: AB568261; *HsjFOXO*: AB568262. Molecular phylogenetic examination confirmed that the *Hsj* gene fragments are orthologous genes of *InR*, *PKB*/*Akt*, and *FOXO* (Fig. S1 and S2 in Supplementary Data).

Expression Profiles of IIS Genes in Mandibles and Other Body Parts

To evaluate expression profiles of the insulin-pathway genes in mandibles of different developmental stages of damp-wood termites, real-time quantitative PCR was performed for *HsjInR*, *HsjPKB*/*Akt*, and *HsjFOXO* (Fig. 2). Expression levels in untreated workers, JHA-induced individuals undergoing soldier differentiation (Day 7 and Day 14 post-JHA application, PS and S), and untreated workers prior to the stationary molt stage were compared. In mandibles, *HsjInR* and *HsjPKB*/*Akt* expression levels changed dramatically over the course of soldier differentiation (Fig. 2). In the course of soldier differentiation, at Day 14, which was just prior to the molt into presoldiers, the expression level of *HsjInR* in mandibles was three-fold the level found in workers (Fig. 2A). Although the expression levels dropped during the presoldier stage, they increased again in the mature soldiers, with mandibles of mature soldiers having fivefold higher transcript abundances than mandibles of workers. Even during the presoldier molt (i.e., Day 14), levels of *HsjInR* were elevated compared with those of animals at the stationary molt (SM in Fig. 2A).

Relative expression levels of *HsjPKB*/*Akt* showed even more dramatic changes (Fig. 2B). Relative expression of *HsjPKB/Akt* rose sharply when animals commenced solider development, peaked at Day 14 (fourfold, compared to the worker level), and remained higher than in stationary molt animals for the duration of soldier development. The level in soldiers was higher than in stationary molt animals, but not significantly different from levels in workers. In the case of *HsjFOXO*, in contrast, expression levels at Day 7 and 14 were not significantly different from levels in workers (Fig. 2C). However, *HsjFOXO* levels rose significantly higher in presoldiers and soldiers (peaking in presoldiers). Interestingly, *HsjFOXO* levels in stationary molt animals were extremely low.

To further characterize the localization of *HsjInR*, *HsjPKB*/*Akt*, and *HsjFOXO* gene expression in different developmental stages of termites, total RNAs were prepared separately from whole heads (including mandibles) and bodies (rather than mandibles), and expression levels of *HsjInR*, *HsjPKB*/*Akt*, and *HsjFOXO* were quantified by real-time quantitative PCR. In heads, *HsjInR* and *HsjPKB*/*Akt* expression levels were higher in soldiers than in workers, peaking at Day 7 after the onset of JHA treatment, and then becoming lower by Day 14 and in presoldiers (Fig. S3A, B in Supplementary Data; Isolated mandibles, in contrast with heads, peaked at Day 14). Both genes showed higher expression levels in all stages of soldier development, including presoldiers, compared with workers; the highest *HsjPKB*/*Akt* expression was found in soldiers, where it was about 10-fold higher than in workers. *HsjFOXO* was highly expressed in JHA-induced worker heads at Day 1 and Day 7 post-JHA application, but was present at low

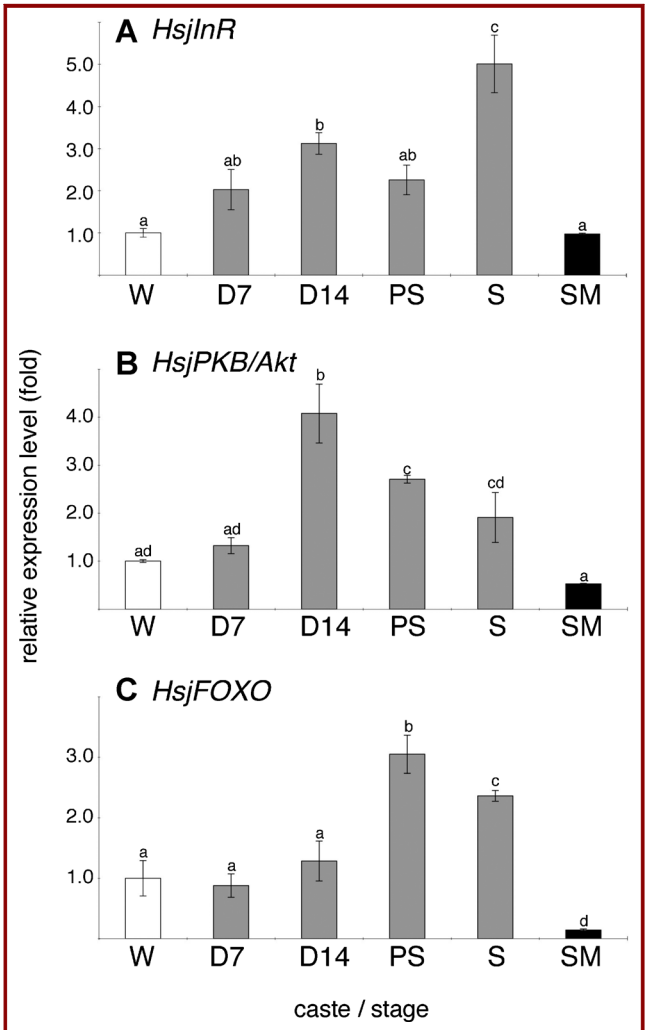

Figure 2. Expression profiles of IIS genes in mandibles during soldier differentiation, revealed by real-time quantitative PCR. An increase in IIS pathway activity is seen in developing soldiers relative to individuals undergoing a stationary molt. Relative expression levels of damp-wood termite developmental stages during soldier differentiation, that is, Day 7 (D7) and Day 14 (D14) post-JHA application, presoldier (PS), and soldier (S), were compared to the expression level of workers (W) and to workers prior to the stationary molt (SM). Significant variation by stage was found for all three genes (one-way ANOVA, $P < 0.05$). Bars denote mean $\pm$ SE for technical triplicates. Different letters on bars indicate significant differences (Tukey's multiple comparison, $P < 0.05$).

levels in the other stages, especially Day 14 individuals, presoldiers, and stationary molt animals (Fig. S3C in Supplementary Data).

In bodies, *HsjInR* showed an expression pattern similar to *HsjInR* in heads, except that it was relatively high in presoldiers and low in soldiers (Fig. S3D in Supplementary Data). *HsjPKB/Akt* consistently showed lower expression levels, with maximal amplitude about twofold to threefold (Fig. S3E in Supplementary Data). In contrast, the expression level of *HsjFOXO* rose at Day 1 and Day 3 post-JHA application in bodies, then dramatically dropped to the level of workers at Day 7 and Day 14 (Fig. S3F in Supplementary Data). The expression level in soldiers was relatively high (twofold higher than the worker level).

Localization of the Insulin Receptor Gene

To examine the localization of IIS activation in the heads of JHA-treated workers, in situ hybridization for *HsjInR* mRNA was carried out using DIG-labeled RNA probes. In individuals at Day 7, no specific signal was detected in mandibles (Fig. 3E), although overall hybridizations with the antisense probe were slightly more intense in the head than with the sense probe. At this stage, relatively strong signals were detected in the prothoracic glands, that is, molting glands (Fig. 3F). By Day 14 post-JHA application, the antisense probes for *HsjInR* produced intense hybridization signals in the epithelial cells of mandibles, in comparison with specimens with the sense probe (Fig. 3A–C). Other tissues including muscle also showed *HsjInR* expression but to a lower degree (Fig. 3D). Hybridization with *HsjInR* sense control probes produced no hybridization signals above background (Fig. 3B). Thus, localization of *HsjInR* within growing mandibles coincides with peak expression as revealed by quantitative real-time PCR, consistent with a role for IIS signaling in extreme growth of soldier mandibles.

Functional Analysis by InR RNAi

To clarify the IIS-signaling function in soldier morphogenesis in *H. sjostedti*, knock down of *HsjInR* by RNA interference (RNAi) was performed. RNAi treatments for *HsjInR* disrupted soldier-specific morphogenesis including mandibular elongation (Fig. 4A). In normal workers the size of the mandibles is relatively small, and they are hidden under the labrum, while mandibles in normal presoldiers (i.e., larvae induced to develop into soldiers by treatment with JHA) are elongated beyond the length of the labrum. Individuals injected with double-stranded RNA (dsRNA) of *HsjInR* at Day 3 after JHA treatment underwent presoldier molting, however, these termites possessed mandibles of reduced sizes. The mandibular length of some RNAi-treated presoldiers ($HsjInR^{RNAi}$ individuals) did not extend beyond the labrum, and resembled those of workers. The mandibular sizes of $HsjInR^{RNAi}$ individuals were relatively smaller than normal presoldiers (Fig. S4 in Supplementary Data) and significant differences between presoldiers and $HsjInR^{RNAi}$ individuals were detected in multiple body parts (i.e., head length, head width, labrum width, mandible length, pronotum width, pronotum length, mesonotum width, metanotum width; Tukey–Kramer, $P < 0.05$), suggesting that many body parts were affected by RNAi knockdown (Fig. S4 in Supplementary Data).

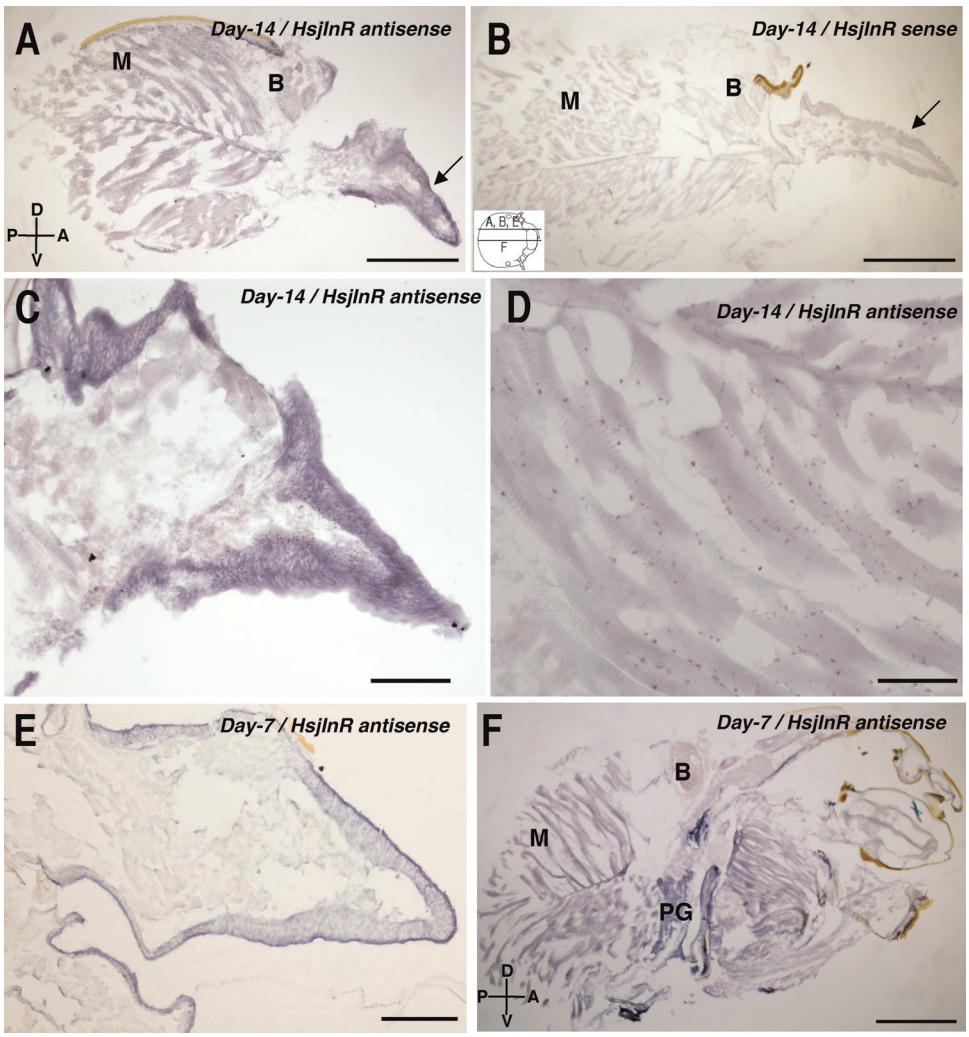

Figure 3. In situ hybridization of *HsjInR* mRNA in the heads of JHA-treated workers. InR expression is localized to the epidermis of developing mandibles in soldiers. Frozen sections were subjected to in situ hybridization with antisense (A, C, D, E, and F) and sense (B) DIG-labeled RNA probes. The probes were then detected with alkaline-phosphatase-conjugated anti-DIG antibody. (A–D) Day 14 individuals post-JHA application. Mandibular epidermis stained with antisense probes (arrows in A, and C). (C) Magnified image of mandibular tissues. (D) magnified image of muscles showing slight expression level of *HsjInR*. (E) Mandible and (F) head sections of Day 7 post-JHA application individuals stained with antisense *HsjInR* probes. Inset in (B) indicate the section planes shown in (A,B,E) and (F). Abbreviations: B = brain, M = muscle, PG = prothoracic gland (molt gland). A–P and D–V indicate anterior–posterior and dorsal-ventral axes, respectively. Bars = 500 μm (A, B, and F) and 200 μm (C, D, and E).

Among $HsjInR^{RNAi}$ individuals there were large morphological variations in which some individuals possessed small mandibles resembling workers, while others had larger mandibles resembling presoldiers. We assessed this variation using Principle Components morphometric analysis (PCA; Fig. 4B). The first principal component (PC1) accounted for 64.9% of the total variance and positively correlated with all measurements, while the second principal component (PC2) accounted for 8.9% of the total variance (Table S1 in Supplementary Data). PC2 was negatively correlated with HL, HW, PmL, PmW, ML, AL, PnW, FW, FL, and positively correlated with LW, ED, HW2, PnL, MnL, MenL, TL (Table S1 in Supplementary Data). The distinction between workers and presoldiers is well represented by PC1 so that PC1 is considered to reflect total body size as well as allometric differences. The variances for head and mandible sizes accounted by PC1 are relatively large (Table S1 in Supplementary Data).

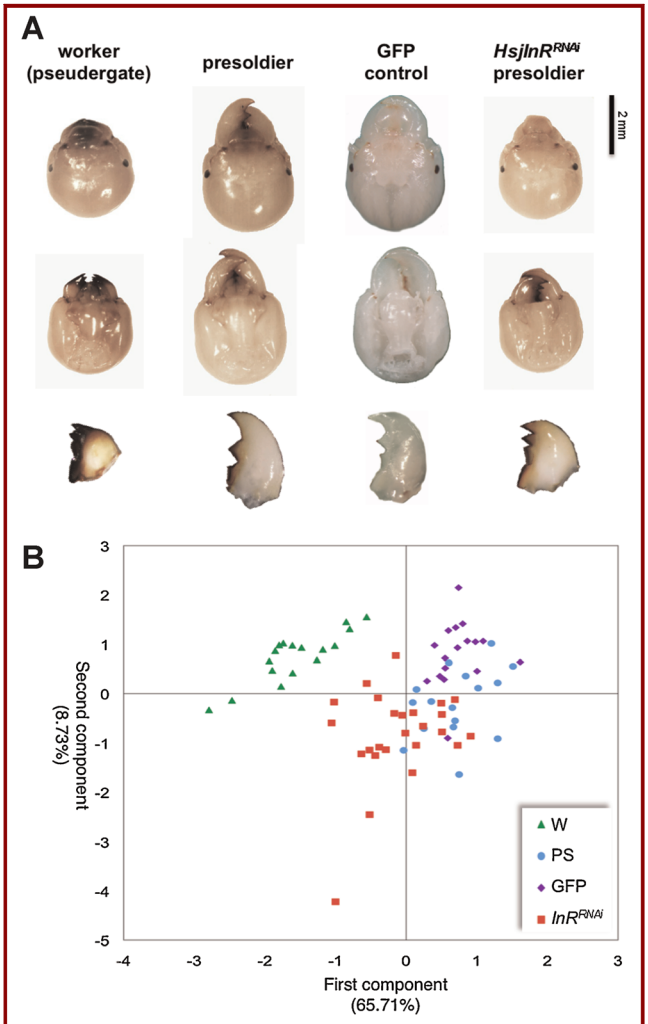

Figure 4. Confirmation of a functional role for IIS activity in the mandibular growth of termite soldiers, as measured by temporary knockdown of *HsjInR*. Effects of *HsjInR* suppression by RNAi on caste characteristics in *H. sjostedti*. (A–C) Dorsal (left) and ventral (center) view of termite heads, and right mandibles (right) are compared among four categories: untreated worker, JHA-induced presoldier, JHA-induced *GFP*-RNAi presoldier, JHA-induced *HsjInR*-RNAi presoldier (or *HsjInR*^{RNAi} individual). *HsjInR*^{RNAi} individuals have mandibles reduced in size relative to the labrum and compared to control presoldiers. Bars indicate 2 mm. (B) Data plots from principal component analysis (PCA), explained by the first and second principal component axes. The first principal component shows the difference among castes, reflecting the overall size and allometry. Note that about half of the *HsjInR*^{RNAi} individuals are located in between untreated workers and JHA-induced presoldiers, indicating a general shift toward the morphology of workers by knockdown of *HsjInR*.

About half of the *HsjInR*^{RNAi} individuals possessed intermediate morphologies resembling intercastes between workers and presoldiers, while the other half showed presoldier-like morphologies.

DISCUSSION

During soldier differentiation in the damp-wood termite, *H. sjostedti*, mandibles undergo rapid growth just prior to the molt from worker (pseudergate) to presoldier (Koshikawa et al., 2002, 2003). Since the IIS pathway is thought to regulate scaling relationships and relative amounts of trait growth (Shingleton et al., 2005), we predicted that IIS signaling would be involved with soldier-specific patterns of mandible growth in this termite species. We tested for a role for IIS signaling using a combination of expression and functional assays. Expression profiles of *HsjInR*, *HsjPKB/Akt*, and *HsjFOXO* indicated that, as predicted, the insulin-signaling pathway is activated in mandibular epithelia of putative presoldiers immediately prior to the molt from worker to presoldier (at Day 14; Figs. 2 and 3), and this localized activity is higher in presoldiers than in workers. Knockdown of *HsjInR* in putative presoldiers reduced the amount of mandible growth and altered head morphology in a direction that increased resemblance between treated animals and workers (Fig. 4B). Together these results suggest that caste-specific activation of the IIS pathway underlies exaggerated patterns of growth in termite mandibles during soldier differentiation.

The *HsjInR*^{RNAi} individuals showed morphological abnormalities especially in their heads (Fig. 4). In these *HsjInR*^{RNAi} individuals, head growth was repressed yielding the rounded shape typical of workers, while control presoldiers had rectangular heads. Generally, mandibular muscles involved in chewing, sucking, and biting constitute a large proportion of an insect head. During soldier differentiation the head capsule of presoldiers becomes elongated and rectangular, accommodating major changes in mandible function together with enlargement of the associated mandibular muscles (Koshikawa et al., 2002; Ishikawa et al., 2008). Thus, we speculate that the observed RNAi-induced changes to overall head shape reflect underlying changes to mandibular muscles and epidermis.

The enlargement of other body parts was also repressed in the *HsjInR*^{RNAi} individuals. The PCA showed that the *HsjInR*^{RNAi} individuals possessed intercaste-like morphological features between workers and presoldiers (Figs. 4B and S4 in Supplementary Data; Koshikawa et al., 2004). These results suggest that IIS activation is required for the normal formation of soldier morphologies specialized for defensive tasks. The *HsjInR*-RNAi effect on whole bodies (Fig. S4 in Supplementary Data) is consistent with the body size reduction observed in *Drosophila* by the knockdown of *DILP* or *DInR* genes (Brogiolo et al., 2001; Rulifson et al., 2002). In the honeybee *Apis mellifera*, queen bodies and ovaries were reduced by *amTOR*-RNAi, with these animals showing worker-like morphologies (Patel et al., 2007). As the TOR pathway and the IIS pathway are interconnected, it is not

surprising that inhibition of the TOR pathway shows phenotypes similar to IIS-suppressed individuals (Oldham et al., 2000; Zhang et al., 2000). Hence, the body size effects observed in the data presented here agree with demonstrated effects of interference of IIS (or the related TOR) activities in other insects.

The results of our study revealed differences in the expression dynamics of IIS-related genes among body parts (Figs. 2 and S3 in Supplementary Data). In addition to its effects on mandible growth, the IIS pathway may influence caste-specific growth of the nervous system, fat body, and endocrine system. IIS is known to regulate neuronal growth in both vertebrates (Robinson et al., '94) and insects (Song et al., 2003). In *H. sjostedti*, neural modifications associated with behavioral changes were suggested to occur during soldier differentiation (Ishikawa et al., 2008). In addition, the IIS pathway is also activated in the fat body, a key organ controlling insect nutrition, metabolism, and development (Colombani et al., 2003; Hwangbo et al., 2004). Fat body growth has been shown to occur within 3 days after JHA application (Cornette et al., 2007), and it is likely that the high expression of *HsjInR* in whole body extracts (Fig. S3 in Supplementary Data) is significantly related to fat-body growth.

The in situ results at Day 7 showed that the prothoracic glands (molting glands), exhibited strong staining of *HsjInR* mRNA (Fig. 3F). A histological study has shown that the prothoracic glands undergo growth at Day 7, prior to the presoldier molt (Cornette et al., 2008a). Recent evidence for a link between the IIS pathway and ecdysone action in determining body size (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005) suggests that IIS may accelerate soldier differentiation by triggering prothoracic gland activity. In *Drosophila*, IIS activity has been shown to affect JH synthesis (Tu et al., 2005). In addition, JH action coordinates life span under the control of insulin signaling (Tatar et al., 2001) and a link between JH and FOXO has been suggested (Puig and Mattila, 2011). Thus, IIS signaling is generally considered to act upstream of JH in insects.

In termite caste differentiation, it has long been known that JH plays a critical role (Nijhout and Wheeler, '82), and in *H. sjostedti*, JHA application induces growth of the corpora allata around Day 7 (Cornette et al., 2008a). Our results show that IIS signaling was increased by JHA application, suggesting that the IIS pathway may also act downstream of JH. Taken together, our results raise the possibility of a positive feedback loop between JH and IIS accelerating soldier differentiation. Of course, there is still a slight possibility that the observed physiological changes may be triggered by some undetected pharmacological effects of the artificial treatment of the high JHA dosage we used, but we have no evidence for this (Zera, 2007; Cornette et al., 2008b).

The IIS pathway is known to respond to nutritional conditions as seen in the case of other insects (Emlen et al., 2006). However, soldier differentiation in termites is triggered by social interactions, rather than nutrition (Noirot, '91; Tarver et al., 2010). In the honeybee, queen- or worker-destined larvae express differential levels of insulin signaling genes (Wheeler et al., 2006; de Azevedo and Hartfelder, 2008), but caste differentiation in the honeybee is deeply related to foods that contain royal jelly (Hartfelder and Emlen, 2005). Although there is still a possibility that nutritional conditions may affect caste fate, social interactions, probably via primer pheromones, play a key role in determining caste fate in termites (Henderson, '98). We suggest that a feedback loop connecting JH with IIS signaling may underlie the coupling of caste-specific patterns of growth (typically regulated primarily by nutrition) with novel social/pheromonal cues in these insects. Further studies will be required to reveal the link between the IIS activity and these critical social interactions.

Understanding molecular mechanisms of caste differentiation can help us understand the evolution of sociality (Robinson et al., 2005; Hunt et al., 2010), the evolution of exaggerated morphologies (Emlen and Nijhout, 2000), and the evolution of developmental plasticity (Nijhout, 2003a; West-Eberhard, 2003). Although this study shows that the IIS pathway contributes to the formation of soldier-specific characteristics, it is not yet known whether this pathway is also involved in the formation of other castes, for example in wing or ovarian development in the course of the differentiation of the winged reproductive (alate) termite caste. It is also unknown whether this mechanism is shared with other social insects. Considering that the function of insulin signaling is evolutionarily conserved across different species (Barbieri et al., 2003; Gonzalez et al., 2009), this pathway may provide a clue to understanding mechanisms of caste differentiation in general.

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LITERATURE CITED

Barbieri M, Bonafe M, Franceschi C, Paolisso G. 2003. Insulin/iIGF-I-signaling pathway: An evolutionarily conserved mechanism of longevity from yeast to humans. Am J Physiol Endocrinol Metab 285:E1064–E1071.

Brogiolo W, Stocker H, Ikeya T, et al. 2001. An evolutionarily conserved function of the *Drosophila* insulin receptor and insulin-like peptides in growth control. Curr Biol 11:213–221.

Brüning JC, Gautam D, Burks DJ, et al. 2000. Role of brain insulin receptor in control of body weight and reproduction. Science 289:2122–2125.

Caldwell PE, Walkiewicz M, Stern M. 2005. Ras activity in the drosophila prothoracic gland regulates body size and developmental rate via ecdysone release. Curr Biol 15:1785–1795.

Colombani J, Raisin S, Pantalacci S, et al. 2003. A nutrient sensor mechanism controls *Drosophila* growth. Cell 114:739–749.

Colombani J, Bianchini L, Layalle S, et al. 2005. Antagonistic actions of ecdysone and insulins determine final size in *Drosophila*. Science 310:667–670.

Cornette R, Koshikawa S, Hojo M, Matsumoto T, Miura T. 2006. Caste-specific cytochrome p450 in the damp-wood termite *Hodotermopsis sjostedti* (Isoptera, Termopsidae). Insect Mol Biol 15:235–244.

Cornette R, Koshikawa S, Miura T. 2008a. Histology of the hormone-producing glands in the damp-wood termite *Hodotermopsis sjostedti* (Isoptera, Termopsidae): A focus on soldier differentiation. Insectes Soc 55:407–416.

Cornette R, Gotoh H, Koshikawa S, Miura T. 2008b. Juvenile hormone titers and caste differentiation in the damp-wood termite *Hodotermopsis sjostedti* (Isoptera, Termopsidae). J Insect Physiol 54:922–930.

Cornette R, Matsumoto T, Miura T. 2007. Histological analysis of fat body development and molting events during soldier differentiation in the damp-wood termite, *Hodotermopsis sjostedti* (Isoptera, Termopsidae). Zool Sci 24:1066–1074.

Corona M, Velarde RA, Remolina S, et al. 2007. Vitellogenin, juvenile hormone, insulin signaling, and queen honey bee longevity. Proc Natl Acad Sci USA 104:7128–7133.

de Azevedo SV, Hartfelder K. 2008. The insulin signaling pathway in honey bee (*Apis mellifera*) caste development—Differential expression of insulin-like peptides and insulin receptors in queen and worker larvae. J Insect Physiol 54:1064–1071.

De Loof A. 2008. Ecdysteroids, juvenile hormone and insect neuropeptides: Recent successes and remaining major challenges. Gen Com Endocrinol 155:3–13.

Deligne J, Quennedey A, Blum M. 1981. The enemies and defence mechanisms of termites. In: Hermann H, editor. Social insects. London: Academic Press. p 1–76.

Edgar BA. 2006. How flies get their size: Genetics meets physiology. Nat Rev Genet 7:907–916.

Emlen DJ, Nijhout HF. 2000. The development and evolution of exaggerated morphologies in insects. Annu Rev Entomol 45:661–708.

Emlen DJ, Szafran Q, Corley LS, Dworkin I. 2006. Insulin signaling and limb-patterning: Candidate pathways for the origin and evolutionary diversification of beetle "horns." Heredity 97:179–191.

Emlen DJ, Warren I, Johns A, Dworkin I, Corley Lavine L. 2012. A mechanism of extreme growth and reliable signaling in sexually selected ornaments and weapons. Science 337:860–864.

Gonzalez JP, Farnes OC, Vasconcelos ATR, Perez AG. 2009. Conservation of key members in the course of the evolution of the insulin signaling pathway. Biosystems 95:7–16.

Hara K, Yonezawa K, Weng QP, et al. 1998. Amino acid sufficiency and mtor regulate p70 s6 kinase and eif-4e bp1 through a common effector mechanism. J Biol Chem 273:14484–14494.

Hartfelder K, Emlen D. 2005. Endocrine control of insect polyphenism. In: Gilbert LI, Iatrou K, Gill SS, editors. Comprehensive molecular insect science. Oxford: Elsevier. p 651–703.

Henderson G. 1998. Primer pheromones and possible soldier caste influence on the evolution of sociality in lower termites. In: Vander Meer RK, Breed MD, Winston ML, Espelie KE, editors. Pheromone communication in social insects: Ants, wasps, bees, and termites. Boulder, CO: Westview Press. p 314–330.

Hunt JH, Wolschin F, Henshaw MT, et al. 2010. Differential gene expression and protein abundance evince ontogenetic bias toward castes in a primitively eusocial wasp. PLoS ONE 5:e10674.

Hwangbo DS, Gershman B, Tu MP, Palmer M, Tatar M. 2004. Drosophila dFOXO controls lifespan and regulates insulin signalling in brain and fat body. Nature 429:562–566.

Ishikawa Y, Aonuma H, Miura T. 2008. Soldier-specific modification of the mandibular motor neurons in termites. PLoS ONE 3:e2617.

Ishikawa Y, Okada Y, Ishikawa A, et al. 2010. Gene expression changes during caste-specific neuronal development in the damp-wood termite *Hodotermopsis sjostedti*. BMC Genomics 11:314.

Juul A, Bang P, Hertel NT, et al. 1994. Serum insulin-like growth factor-I in 1030 healthy children, adolescents, and adults: Relation to age, sex, stage of puberty, testicular size, and body mass index. J Clin Endocrinol Metab 78:744–752.

Kojima T. 2004. The mechanism of *Drosophila* leg development along the proximodistal axis. Dev Growth Differ 46:115–129.

Korb J, Hartfelder K. 2008. Life history and development—A framework for understanding developmental plasticity in lower termites. Biol Rev Camb Philios Soc 83:295–313.

Koshikawa S, Cornette R, Hojo M, et al. 2005. Screening of genes expressed in developing mandibles during soldier differentiation in the termite *Hodotermopsis sjostedti*. FEBS Lett 579:1365–1370.

Koshikawa S, Cornette R, Matsumoto T, Miura T. 2010. The homolog of Ciboulot in the termite (*Hodotermopsis sjostedti*): A multimeric beta-thymosin involved in soldier-specific morphogenesis. BMC Dev Biol 10:63.

Koshikawa S, Matsumoto T, Miura T. 2002. Morphometric changes during soldier differentiation of the damp-wood termite *Hodotermopsis japonica* (Isoptera, Termopsidae). Insectes Sociaux 49:245–250.

Koshikawa S, Matsumoto T, Miura T. 2003. Mandibular morphogenesis during soldier differentiation in the damp-wood termite *Hodotermopsis sjoestedti* (Isoptera: Termopsidae). Naturwissenschaften 90:180–184.

Koshikawa S, Matsumoto T, Miura T. 2004. Soldier-like intercastes in the rotten-wood termite *Hodotermopsis sjostedti* (Isoptera: Termopsidae). Zool Sci 21:583–588.

Kumar S, Nei M, Dudley J, Tamura K. 2008. Mega: A biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief Bioinform 9:299–306.

Miller E. 1969. Caste differentiation in the lower termites. In: Krishna K, Weesner FM, editors. Biology of termites. New York: Academic Press. p 283–310.

Mirth C, Truman JW, Riddiford LM. 2005. The role of the prothoracic gland in determining critical weight for metamorphosis in *Drosophila melanogaster*. Curr Biol 15:1796–1807.

Miura T. 2001. Morphogenesis and gene expression in the soldier-caste differentiation of termites. Insectes Soc 48:216–223.

Miura T. 2005. Developmental regulation of caste-specific characters in social-insect polyphenism. Evol Dev 7:122–129.

Miura T, Hirono Y, Machida M, Kitade O, Matsumoto T. 2000. Caste developmental system of the Japanese damp-wood termite *Hodotermopsis japonica* (Isoptera: Termopsidae). Ecol Res 15:83–92.

Miura T, Koshikawa S, Machida M, Matsumoto T. 2004. Comparative studies on alate wing formation in two related species of rotten-wood termites: *Hodotermopsis sjostedti* and *Zootermopsis nevadensis* (Isoptera, Termopsidae). Insectes Sociaux 51:247–252.

Moczek AP, Nagy LM. 2005. Diverse developmental mechanisms contribute to different levels of diversity in horned beetles. Evol Dev 7:175–185.

Nijhout HF. 1999. Control mechanisms of polyphenic development in insects—In polyphenic development, environmental factors alter same aspects of development in an orderly and predictable way. Bioscience 49:181–192.

Nijhout HF. 2003a. Development and evolution of adaptive polyphenisms. Evol Dev 5:9–18.

Nijhout HF. 2003b. The control of growth. Development 130:5863–5867.

Nijhout HF, Wheeler DE. 1982. Juvenile-hormone and the physiological basis of insect polymorphisms. Q Rev Biol 57:109–133.

Noirot C. 1969. Formation of castes in the higher termites. In: Krishna K, Weesner FM, editors. Biology of termites. New York: Academic Press. p 311–350.

Noirot C. 1991. Caste differentiation in Isoptera—Basic features, role of pheromones. Ethol Ecol Evol Special issue 1:3–7.

Ogino K, Hirono Y, Matsumoto T, Ishikawa H. 1993. Juvenile-hormone analog, S-31183, causes a high-level induction of presoldier differentiation in the japanese damp-wood termite. Zool Sci 10:361–366.

Oldham S, Montagne J, Radimerski T, Thomas G, Hafen E. 2000. Genetic and biochemical characterization of dTOR, the *Drosophila* homolog of the target of rapamycin. Genes Dev 14:2689–2694.

Patel A, Fondrk MK, Kaftanoglu O, et al. 2007. The making of a queen: TOR pathway is a key player in diphenic caste development. PLoS ONE 2:e509.

Puig O, Mattila J. 2011. Understanding forkhead box class O function: Lessons from Drosophila melanogaster. Antioxid Redox Signal 14:635–647.

Puig O, Marr MT, Ruhf ML, Tjian R. 2003. Control of cell number by *Drosophila* FOXO: Downstream and feedback regulation of the insulin receptor pathway. Genes Dev 17:2006–2020.

Renehan AG, Frystyk J, Flyvbjerg A. 2006. Obesity and cancer risk: The role of the insulin-IGF axis. Trends Endocrinol Metab 17:328–336.

Robinson GE, Grozinger CM, Whitfield CW. 2005. Sociogenomics: Social life in molecular terms. Nat Rev Genet 6:257–270.

Robinson LJ, Leitner W, Draznin B, Heidenreich KA. 1994. Evidence that p21ras mediates the neurotrophic effects of insulin and insulin-like growth factor I in chick forebrain neurons. Endocrinology 135:2568–2573.

Roisin Y. 2000. Diversity and evolution of caste patterns. In: Abe T, Bignell D, Higashi M, editors. Termites: Evolution, sociality, symbiosis, ecology. Dordrecht: Kluwer academic publishers. p 95–119.

Roovers E, Vincent ME, et al. 1995. Characterization of a putative molluscan insulin-related peptide receptor. Gene 162:181–188.

Rulifson EJ, Kim SK, Nusse R. 2002. Ablation of insulin-producing neurons in flies: Growth and diabetic phenotypes. Science 296:1118–1120.

Ruvinsky I, Meyuhas O. 2006. Ribosomal protein S6 phosphorylation: From protein synthesis to cell size. Trends Biochem Sci 31:342–348.

Saitou N, Nei M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425.

Saltiel AR, Kahn CR. 2001. Insulin signalling and the regulation of glucose and lipid metabolism. Nature 414:799–806.

Shingleton AW, Das J, Vinicius L, Stern DL. 2005. The temporal requirements for insulin signaling during development in *Drosophila*. PLoS Biol 3:1607–1617.

Shingleton AW, Frankino WA, Flatt T, Nijhout HF, Emlen DJ. 2007. Size and shape: The developmental regulation of static allometry in insects. Bioessays 29:536–548.

Song JB, Wu LL, Chen Z, Kohanski RA, Pick L. 2003. Axons guided by insulin receptor in *Drosophila* visual system. Science 300:502–505.

Tarver MR, Zhou XG, Scharf ME. 2010. Socio-environmental and endocrine influences on developmental and caste-regulatory gene expression in the eusocial termite *Reticulitermes flavipes*. BMC Mol Biol 11:28.

Tatar M, Kopelman A, Epstein D, et al. 2001. A mutant *Drosophila* insulin receptor homolog that extends life-span and impairs neuroendocrine function. Science 292:107–110.

Thummel CS. 2001. Molecular mechanisms of developmental timing in *C. elegans* and *Drosophila*. Dev Cell 1:453–465.

Tu MP, Yin CM, Tatar M. 2005. Mutations in insulin signaling pathway alter juvenile hormone synthesis in *Drosophila melanogaster*. Gen Comp Endocrinol 142:347–356.

Unger RH, Dobbs RE, Orci L. 1978. Insulin, glucagon, and somatostatin secretion in the regulation of metabolism. Annu Rev Physiol 40:307–343.

Weesner FM. 1969. External anatomy. In: Krishna K, Weesner FM, editors. Biology of termites. London: Academic Press. p 19–48.

Weinkove D, Neufeld TP, Twardzik T, Waterfield MD, Leevers SJ. 1999. Regulation of imaginal disc cell size, cell number and organ size by *Drosophila* class I_A phosphoinositide 3-kinase and its adaptor. Curr Biol 9:1019–1029.

West-Eberhard MJ. 2003. Developmental plasticity and evolution. New York: Oxford University Press.

Wheeler DE, Buck N, Evans JD. 2006. Expression of insulin pathway genes during the period of caste determination in the honey bee, *Apis mellifera*. Insect Mol Biol 15:597–602.

Wilson EO. 1971. The insect societies. Cambridge: Belknap Press.

Wu Q, Brown MR. 2006. Signaling and function of insulin-like peptides in insects. Ann Rev Entomol 51:1–24.

Zera AJ. 2007. Endocrine analysis in evolutionary-developmental studies of insect polymorphism: Hormone manipulation versus direct measurement of hormonal regulators. Evol Dev 9:499–513.

Zhang HB, Stallock JP, Ng JC, Reinhard C, Neufeld TP. 2000. Regulation of cellular growth by the *Drosophila* target of rapamycin *dTOR*. Genes Dev 14:2712–2724.