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Regulation of polyphenic caste differentiation in the termite *Reticulitermes flavipes* by interaction of intrinsic and extrinsic factors

Michael E. Scharf*, Caitlin E. Buckspan†, Traci L. Grzymala and Xuguo Zhou
Entomology and Nematology Department, University of Florida, Gainesville, FL 32611-0620, USA
*Author for correspondence (e-mail: mescharf@ufl.edu)
†Present address: Department of Bioengineering, University of California – San Diego, La Jolla, CA 92093, USA

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Summary

Polyphenism is a key strategy used by solitary insects to adapt to changing environmental conditions and by eusocial insects for existing collaboratively in a social environment. In social insects, the morphogenetic juvenile hormone (JH) is often involved in directing the differentiation of polyphenic behavioral castes. The present study examines the effects of JH, environment and feeding on caste polyphenism in a eusocial insect, the termite *Reticulitermes flavipes* (Kollar). Our approach included a combination of model JH bioassays, SDS-PAGE and western blotting. Our findings revealed significant temperature-dependent effects on (1) JH-induced soldier caste differentiation, (2) abundance of soldier-inhibitory hexamerin proteins and (3) JH-sequestration by hexamerin proteins. Additionally, although it appears to be dependent on a complex interaction of factors, feeding apparently plays a significant upstream role in enhancing hexamerin accumulation under normal colony conditions. These findings offer important new information on termite eusocial polyphenism by providing the first mechanistic evidence linking an intrinsic caste regulatory factor (hexamerin proteins) to an upstream extrinsic factor (environment) and a downstream response (caste differentiation). These observations are consistent with the hypothesis that the hexamerins serve as an environmentally and nutritionally responsive switching mechanism that regulates termite caste polyphenism.

Key words: ecological-developmental biology, eco-devo, phenotypic plasticity, polyphenism, juvenile hormone, hexamerin.

Introduction

The ability of individuals to metamorphose into discrete phenotypes in response to changing environmental conditions is often critical to the perpetuation of a species. Phenotypic plasticity is the term used to describe such developmental changes; it can be divided into the two categories of 'reaction norms' and 'polyphenisms' (Nijhout, 1999; Nijhout, 2003; Evans and Wheeler, 2000). Reaction norms are gradual changes in phenotype that occur proportionally in response to incremental environmental changes. Polyphenisms, conversely, are alternative and discrete phenotypes that differentiate without intermediate forms. Because polyphenisms are environmentally dependent, they are inextricably linked to the ecology of a species (Gilbert, 2001). In insects, polyphenisms can arise in response to ecologically induced changes in hormone secretion or hormone sensitivity (Nijhout, 1999; Nijhout, 2003).

Castes of social insects such as termites represent an intricate system of phenotypic plasticity. Termite caste phenotypes are typically manifested as alternative phenotypes, or polyphenisms (Miura, 2004). Like all social insect colonies, termite colonies function because of the interconnected physiological and behavioral roles played by the different castes (Wilson, 1971). Thus, in termites, polyphenisms and sociality are inseparable. In termites, there are three distinct castes that include the soldier, worker and reproductive caste phenotypes (Noirot, 1985; Noirot, 1990). Reproductives produce offspring, soldiers defend the colony, and workers perform altruistic helping behaviors such as feeding, tunneling and brood tending. Workers are temporally arrested immature forms that retain the ability to differentiate to soldier or reproductive caste phenotypes (Myles and Nutting, 1988). As part of soldier caste differentiation, worker termites first pass through an intermediate 'presoldier' or 'white soldier' stage (Noirot, 1985; Noirot, 1990; Henderson, 1998). After ectopic juvenile hormone (JH) treatment of *R. flavipes* workers, presoldiers differentiate at about 10–16 days, with soldiers typically appearing from 25 to 30 days after treatment (Scharf et al., 2003; Scharf et al., 2005a). Soldier phenotypes differ from workers in that they possess a reduced digestive tract, an enlarged head with increased muscle mass and sclerotization, and greatly enlarged mandibles (Koshikawa et al., 2002). Presoldier and then soldier differentiation naturally peaks with rising temperatures in the spring of the year (Howard and Haverty, 1981; Waller and La Fage, 1988; Liu et al., 2005a) in response to rising titers of the morphogenetic insect hormone JH (Park and Raina, 2004; Mao et al., 2005). Thus, soldier caste differentiation appears to result from temperature and/or season-induced changes in hormone secretion.

A key component of polyphenic development is its regulation by hormone-responsive developmental switching mechanisms (Wheeler, 1986; Nijhout, 1999; Nijhout, 2003). In termites, a pair of hexamerin proteins is part of one such switching mechanism (Scharf et al., 2005a; Zhou et al., 2006a; Zhou et al., 2006b; Zhou et al., 2007). The hexamerins, which are both JH-inducible and capable of sequestering JH, play a key caste regulatory role by apparently modulating JH availability and limiting its influence over developmental gene networks. This mechanism is important in the context of sociality because it helps to ensure high worker caste proportions and maximal inclusive fitness of termite colonies. Insect hexamerins in general are well-known to participate in JH binding (Braun and Wyatt, 1996; Tawfik et al., 2006), as well as nutrient storage and nutritional signaling during immature instars (Burmester and Scheller, 1999). Compelling evidence implicates termite hexamerins in JH binding (e.g. Zhou et al., 2006b); however, responsiveness of termite hexamerins to environmental conditions and feeding/nutritional status are topics that remain uninvestigated.

The studies presented here were designed to test ecological-developmental effects of temperature and feeding on caste differentiation in the lower termite *Reticulitermes flavipes* (Kollar) and to investigate possible correlative relationships between select environmental and physiological parameters. The central hypothesis we tested was that environment and/or nutrition can influence caste-regulatory hexamerin abundance, which can in turn influence eusocial polyphenism. This hypothesis was tested by pursuing the following specific objectives: (1) to compare JH-induced phenotypic caste differentiation by worker termites at different temperatures, (2) to quantify survivorship, mass changes and feeding during caste differentiation; (3) to examine changes in expression/accumulation of caste regulatory hexamerin proteins and their JH-binding levels under the different temperature regimes and (4) to attempt to correlate statistically the assorted experimental response variables with main experimental effects and with each other. Our findings link both environment and nutrition to hexamerin-based caste regulation in termites and thus provide novel mechanistic evidence that advances our understanding of ecological-developmental factors that regulate social polyphenism.

Materials and methods

Experimental animals

Worker termites used in these experiments were from two different laboratory colonies collected near Gainesville, FL, USA. Colonies were maintained at a constant 22°C, with damp paper towels and pine wood 'shims' as harborage and food sources. Colonies were identified as *R. flavipes* by mitochondrial 16S rDNA sequence and soldier morphology. All caste phenotypes and developmental stages were readily observable in the colonies, including workers, soldiers, nymphs, supplementary reproductives, larvae and eggs. Sampled workers were from middle instars; they possessed sclerotized heads, rich symbiont communities and prominent fat bodies. Before assays, workers were also verified to possess no signs of wing buds and had pronotal widths wider than mesonotal widths. The first colony was assayed immediately after collection during the months of September and October. The second colony was collected one year later from a separate location and assayed during the months of September and November.

Bioassays

Model JH bioassays (Scharf et al., 2003; Scharf et al., 2005a; Zhou et al., 2006a; Zhou et al., 2007) were used to expose worker termites to the different experimental treatments. Four total experimental treatments were tested that compared treatments of either 150 μg JH III or acetone (untreated) controls at two temperatures (22 and 27°C). JH III was purchased from Sigma Chemical Co. (93% purity; St Louis, MO, USA) and diluted in analytical grade acetone (>99% purity; Sigma). Paired paper towel sandwiches were treated with either 300 μl of 0.5 mg ml^{-1} JH III in acetone, or 300 μl acetone for untreated controls. Rather than use a non-JH lipid or terpene as a control in these assays, acetone was used as a control because of broad-spectrum synergism of JH efficacy observed for a wide diversity of terpene compounds (M. R. Tarver and M.E.S., manuscript submitted for publication). After treatment, papers were allowed to dry for 30 min in a fume hood. While papers dried, worker termites were isolated from laboratory colonies using a vacuum apparatus, placed into 35 mm Petri dishes, and examined under a binocular viewing scope to identify and remove any non-worker individuals. Fifteen workers were added to each assay dish, the dishes were taped closed and then placed on wet paper towels in 30×15 cm plastic boxes with vented lids. The boxes were held in environmental chambers, which permitted precise temperature control. Assays ran for 15 days. Three replicated groups of 15 workers were tested per treatment per colony. For assays that examined feeding, body mass changes and hexamerin levels, nine replicate dishes were established per treatment for destructive sampling at assay days 5, 10 and 15. Feeding (mg/termite) was determined by comparing masses of paired paper sandwiches before and after confinement with termites, then by correcting for the number of live termites at the time of sampling. Papers were dried in a drying oven before weighing.

Protein isolation and protein assay

Whole termites were destructively sampled from colony 2 assays and used for protein isolation. Frozen groups of whole termites were homogenized in phosphate-buffered saline (PBS) (pH 7.6) on ice using a motorized Teflon^{TM}–glass homogenizer at low speed. The homogenate was centrifuged for 15 min at 4°C and 16 000 ***g***. The supernatant was carefully decanted and saved for protein quantification and polyacrylamide gel electrophoresis (PAGE) analysis. A commercial BCA (bicinchoninic acid) protein assay (Pierce BCA Assay; Rockford, IL, USA) was used for protein quantification against a standard curve of bovine serum albumin.

SDS-PAGE

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was conducted as described in several prior reports (e.g. Scharf et al., 2005a; Zhou et al., 2006a; Zhou et al., 2006b). PAGE resolving gels contained 8% acrylamide and 10% SDS. Stacking gels contained a lesser quantity of

acrylamide (4%) and the same amount of SDS. A discontinuous Tris-glycine buffering system was used, and protein sample buffer contained β-mercaptoethanol as a sulfhydryl reducing agent. 10 μg of protein was loaded per lane. Each gel was run with in-gel BSA protein standards at concentrations ranging from 0.3125 to 10.0 μg per lane. The BSA standards were used for densitometric quantification of hexamerin proteins directly on each gel. Molecular mass markers were Kaleidoscope™ broad-range markers (Bio-Rad, Hercules, CA, USA). After running, gels were stained for 12–16 h in a solution of water:methanol:acetic acid (50:40:10) + 0.5% Coomassie Blue R-250, then destained for ~2 h in several rinses of water:methanol:acetic acid (50:40:10). After photographing, gel images were analyzed densitometrically using Quantity-One™ software (Bio-Rad).

Western blotting

Anti-JH antiserum was obtained from Dr Walter Goodman (University of Wisconsin-Madison, USA). The antiserum was raised in rabbits to 10R JH III and has been shown to recognize JH from tobacco hornworm (Goodman et al., 1995; Cusson et al., 1997) and honeybee (Guidulgi et al., 2005) with high sensitivity by radioimmunoassay. The antibody is also useful for identification of JH that is covalently bound to denatured proteins (Zhou et al., 2006b). Western blotting was performed on proteins obtained and quantified as described above. After separation by SDS-PAGE (see above), proteins were transferred to nitrocellulose membranes (Bio-Rad). Prestained Kaleidoscope™ molecular mass markers (Bio-Rad) were used for western blotting and were visible prior to immunostaining. Membranes were blocked after protein transfer in 15 mg ml^{-1} non-fat dry milk in PBS. Dilutions were 1:500 for primary anti-JH antiserum. Secondary antiserum was goat-anti-rabbit AP-conjugate (Bio-Rad 170-6518), diluted 1:1000. Immunoreactive bands were visualized by incubation in a 10 ml solution prepared from BCIP-NBT (bromo-chloro-indolyl phosphate nitro-blue tetrazolium) Tablets (Sigma B-5655) dissolved in nanopure water.

Statistical analyses

Data obtained from bioassays (i.e. presoldier formation, survivorship and feeding) were analyzed with non-parametric Kruskal–Wallis tests using SAS software (Cary, NC, USA). Analysis of variance (ANOVA) testing of the main effects JH, temperature and time and their two- and three-way interactions on the dependent variables (with units) presoldier formation (%), feeding (mg) or hexamerin quantities (μg) were performed with SAS software using the PROC ANOVA procedure. Percentage presoldier formation data were arcsine transformed prior to conducting ANOVA. Linear regressions were performed with SAS software using the PROC REG procedure. Slopes from different replicates were compared statistically using non-parametric Kruskal–Wallis tests.

Results

Temperature dependence of soldier caste differentiation

Results for three independently replicated JH assays are shown in Fig. 1. Assays were run at 22 and 27°C, with and without ectopic JH. Irrespective of treatment, survivorship in all

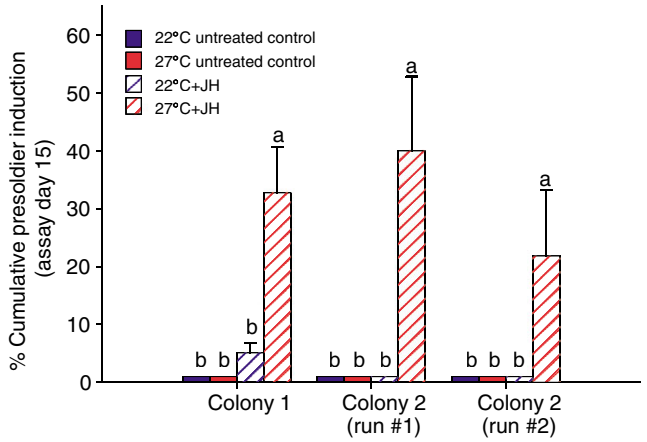

Fig. 1. Impacts of temperature and juvenile hormone (JH) on soldier caste differentiation. Results shown represent cumulative presoldier formation on day 15 of assays that were conducted under four different conditions of 22° and 27°C, with and without ectopic JH. Two colonies were tested at three different times over a 1-year period. Bars with the same letter within the colony are not significantly different by non-parametric Kruskal–Wallis tests ($P<0.05$, $N=3$).

treatments averaged >90%. By assay day 15, presoldier differentiation was significantly highest in all JH treatments at 27°C. Presoldier induction never occurred in the absence of JH and in only one replicate of the JH treatments at 22°C. In the one assay where presoldier induction did occur at 22°C (colony 1), it was below 5% and was not significantly different from zero. Induced presoldiers appeared morphologically normal and, to our knowledge, status quo worker-to-worker molts never occurred in any of the various treatments. These results establish that JH-induced presoldier differentiation is a temperature- and/or season-dependent phenomenon in *R. flavipes*.

Impacts of the temperature–JH interaction on survival, feeding and body mass

Survivorship, feeding and body mass were quantified on assay days 0, 5, 10 and 15. Survivorship under all JH and temperature conditions was greater than 90% and not different among all treatments ($P>0.05$; data not shown). Feeding on a per-termite basis progressed steadily over time through all treatments. No significant feeding differences were observed among treatments within each assay day ($P>0.05$) (Fig. 2A); however, an analysis of feeding rates over time showed that feeding rates are significantly greater in the absence of ectopic JH and are highest in 27°C treatments without ectopic JH (Fig. 2B). Body mass varied significantly among treatments and assay days ($P<0.05$; data not shown). The most pronounced mass changes occurred on assay days 10 and 15, where there were significant decreases with JH treatment. These body mass decreases are presumed to be associated with the presoldier molting process, which occurred for a proportion of individuals. In general terms, these results show significant but complex variation in both feeding and body mass in association with JH, temperature and presoldier differentiation. However, these results also indicate that feeding is enhanced in the absence of ectopic JH at higher temperatures.

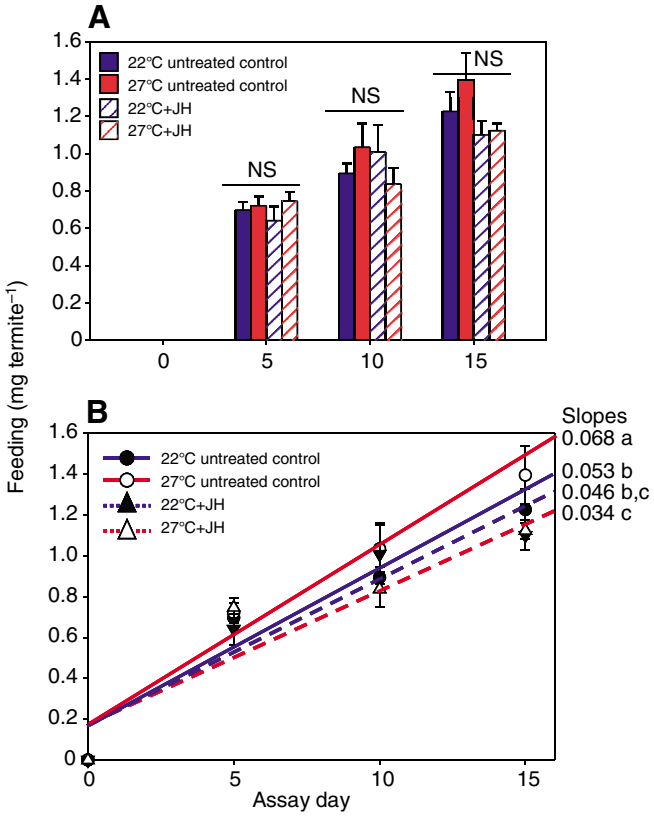

Fig. 2. Feeding over time as related to different combinations of juvenile hormone (JH) and temperature. (A) Mean (± s.d.) feeding for four treatments over time. Groups of bars within days are not significantly different (NS), as determined by non-parametric Kruskal–Wallis tests (P>0.05, N=3). (B) Linear regression results (mean ± s.d.) for feeding over time among different treatments. All regressions were significant (P<0.05, N=3). Slope values shown at the right with the same letter are not significantly different by linear regression analysis (GLM; P>0.05). Note that feeding rates were highest for the 27°C untreated controls, which have exposure to only endogenous JH.

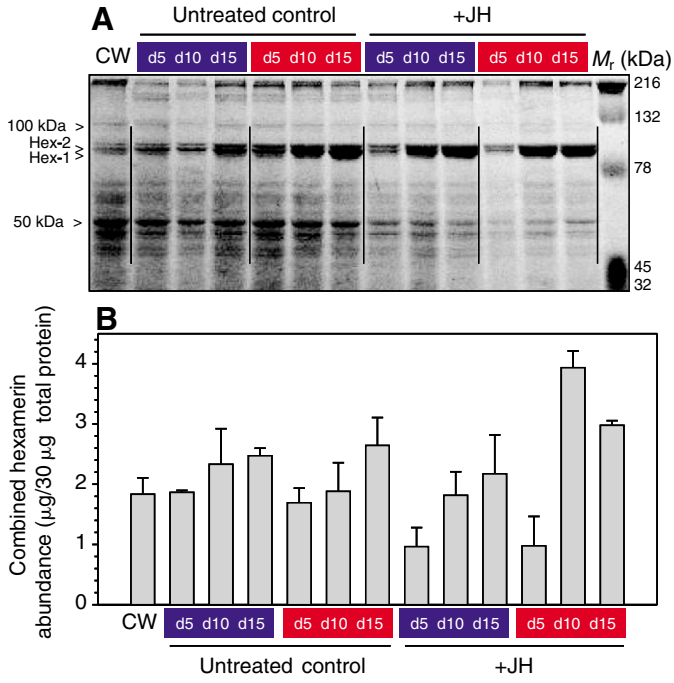

Fig. 3. Impacts of temperature and juvenile hormone (JH) on protein abundance. Blue and red boxes indicate 22° and 27°C treatments, respectively, across assay days (d) 5, 10 and 15. Colony workers (CW) were sampled directly from colonies and used to start assays on day 0. (A) Single replicate SDS-PAGE gel after Coomassie staining (10 µg protein per lane). Noteworthy proteins are indicated, which include unknown 100 and 50 kDa proteins as well as the caste regulatory Hex-1 and Hex-2 proteins that occur in the 80–85 kDa range. The lane labeled as M_r indicates molecular mass standards in kilodaltons (kDa). (B) Mean results for densitometric scans of three SDS-PAGE gels from three independent experimental replicates. Error bars represent s.e.m.

Impacts of temperature and JH on abundance of soldier-inhibitory hexamerin proteins

SDS-PAGE analyses were performed on termites that were destructively sampled at assay days 0, 5, 10 and 15. See Fig. 3A for a representative SDS-PAGE gel from six independent replicates that were performed. From previous research in *R. flavipes*, two soldier-inhibitory hexamerin proteins are readily identifiable in the vicinity of 80 kDa on 8% SDS-PAGE gels (Zhou et al., 2006a; Zhou et al., 2006b). For this doublet of protein bands, the upper band is Hex-2 and the lower band is Hex-1. Each replicate gel was run with an in-gel BSA protein standard curve. The BSA standards were used for densitometric quantification of hexamerin levels directly on each gel. Hexamerin densitometry results are summarized in Fig. 3B. Baseline hexamerin levels in workers sampled directly from the colony were ~2% of total protein. In acetone (untreated) controls, hexamerin levels did not change considerably. Changes were far more pronounced in JH treatments. In association with presoldier differentiation, hexamerin levels were most substantially increased with JH treatment at the higher temperature. At both temperatures, the hexamerins were suppressed by JH on assay day 5; however, these initial decreases were followed by >200% increases by day 15. These findings demonstrate that, like presoldier differentiation, hexamerin protein levels are significantly influenced by both temperature and JH (P<0.05; see statistical analyses below).

Verification of JH binding by hexamerin proteins

Established procedures (Zhou et al., 2006b) were used to assess JH-binding by hexamerin proteins from whole-body protein preparations on denatured western blots. A replicate blot from the same protein preparation shown above for SDS-PAGE is shown in Fig. 4. In agreement with previous findings (Zhou et al., 2006b), western blots indicate covalent JH binding by the Hex-1 protein; however, they also suggest JH binding two additional proteins that include a larger protein of ~100 kDa and a smaller protein of ~50 kDa. In agreement with established caste regulatory roles for the hexamerins (Zhou et al., 2006a; Zhou et al., 2006b; Zhou et al., 2007), western blots show weaker JH-binding for colony workers as well as non-JH-treated workers, but greater intensity in JH-treated workers. These results, which are the first examining JH binding by whole-body protein preparations, agree with earlier findings indicating covalent JH binding by the hemolymph-soluble Hex-

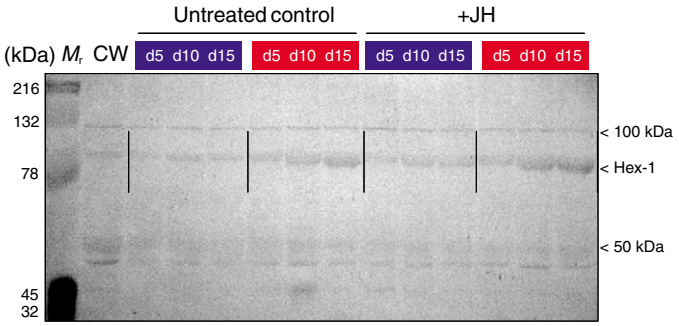

Fig. 4. Western blot showing impacts of temperature and juvenile hormone (JH) on JH-binding by various proteins. All colors and labeling correspond identically to that described for Fig. 3. Blots were probed with anti-JH antiserum, which directly enables identification of JH covalently bound to denatured proteins. Three key proteins were identified by the anti-JH, including the Hexamerin-1 (Hex-1) protein, an unknown 100 kDa protein and another unknown group of proteins in the 50 kDa range. Molecular mass markers were pre-stained and visible before immunodetection.

1 protein. These results also provide novel evidence suggesting JH binding by other ~100 and ~50 kDa proteins.

Statistical analyses of temporal and eco-physiological relationships

Results of three separate ANOVA runs are shown in Table 1. These analyses tested the effects of temperature, JH and time, as well as their two- and three-way interactions, on the dependent variables of presoldier formation, feeding and hexamerin abundance. The body mass ANOVA (not shown) is not significant and thus is not considered further. In the presoldier differentiation ANOVA, with the exception of the 'time' main effect, all main effects and interactions are significant. This confirms that a diversity of factors interact to influence presoldier differentiation. Alternatively, in the feeding ANOVA, only the 'time' effect contributes significantly to feeding variation across treatments. This finding is in agreement with the linear increase in feeding observed for all treatments across assay days (see Fig. 2A). For the hexamerin ANOVA, only the 'JH×temperature' interaction is significant. This result mostly reflects the observed increases in hexamerin levels with JH at the higher temperature of 27°C, but also the slightly increased hexamerin abundance in 27°C non-JH treatments.

Further comparisons of the relationships of the assay dependent variables feeding, body mass and hexamerin protein levels were made using linear regression analysis. These regressions compared feeding and body mass separately *versus* hexamerin abundance. The body mass *vs* hexamerin regression is very weak, with a low r^2 value of 0.068 (Fig. 5A). This finding is supported by ANOVA results indicating no significance for the body mass ANOVA model. The regression of feeding *vs* hexamerin abundance, however, is significant (P<0.05) and positively correlated (r^2=0.605) (Fig. 5B). Together, the ANOVA and regression results statistically support that feeding, JH and temperature all influence hexamerin protein levels. As discussed below, these factors can thus be considered as having impacts on regulating soldier caste differentiation in *R. flavipes*.

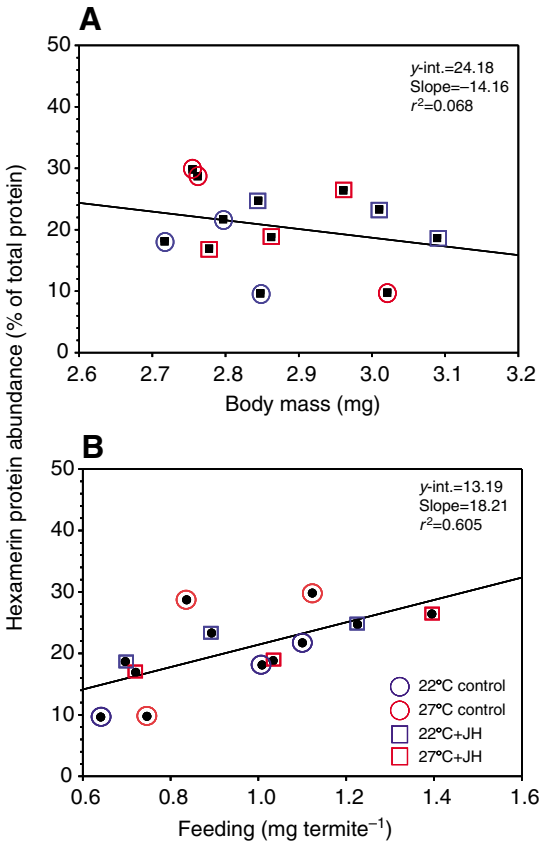

Fig. 5. Linear regression analysis of (A) body mass and (B) feeding *vs* combined hexamerin-1 and -2 protein abundance. The regression shown in A, which is not significant, indicates no relationship between body mass and hexamerin abundance. Alternatively, the regression shown in B of feeding *vs* hexamerin abundance is significant (P<0.05). Note also that the two highest feeding amounts recorded were in each of the untreated controls at assay day 15; these treatments had high hexamerin levels and no presoldier differentiation.

Discussion

Delineation of extrinsic and intrinsic regulatory factors

In a previous report (Zhou et al., 2007), we proposed a collection of extrinsic and intrinsic factors that, through apparent influences on caste regulatory hexamerin proteins and downstream JH-dependent gene networks (Fig. 6A), would be expected to have influences on caste differentiation. Included in this list of proposed extrinsic factors are temperature, season, moisture, food quality/quantity and colony. The list of proposed intrinsic factors includes nutritional status, JH titer, allatostatins, sex and instar. Some of these factors were considered in the present research, specifically temperature, seasonality and nutritional status (*via* feeding). Our findings not only link temperature and season to both hexamerin protein levels and soldier caste differentiation (Fig. 6B) but also link nutritional status to accumulation/stability of soldier-inhibitory hexamerin proteins (Fig. 6C).

With regard to extrinsic factors, it was observed that JH-dependent soldier caste differentiation in worker termites took place almost exclusively at the higher assay temperature of 27°C. From ANOVA analyses, it was confirmed that

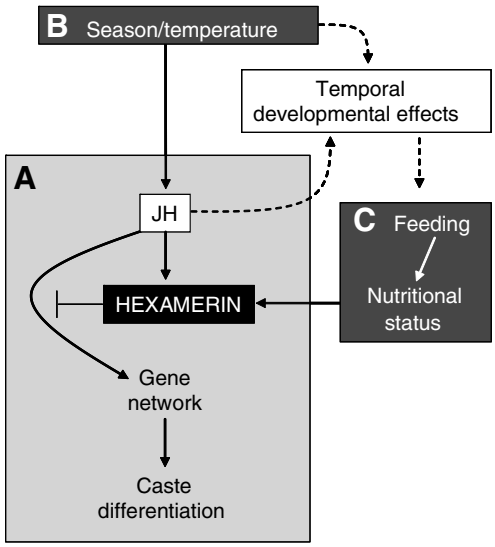

Fig. 6. Diagram integrating conclusions of previous studies on *R. flavipes* caste regulation with those of the present study. Solid arrows indicate known/observed relationships, and broken arrows represent putative or proposed relationships. (A) Relationship between juvenile hormone (JH), hexamerin and JH-responsive developmental gene networks [taken from Zhou et al. (Zhou et al., 2007)]. The two principal effects observed in the present study were those of temperature (B) and feeding/nutritional status (C). Significant temporal developmental effects were also observed; however, the potential influences of this remain unclear, as well as their effects on downstream factors such as feeding and nutritional status.

temperature significantly influences presoldier differentiation. However, while temperature has no direct effects on hexamerin protein levels, it interacts significantly with JH to influence hexamerin abundance. From these results it can be concluded that temperature must be either acting synergistically with JH or directly influencing its biosynthesis (Fei and Henderson, 2002; Liu et al., 2005b). Secondary temporal effects on feeding are also apparently occurring downstream as a result of JH-induced developmental changes (Fig. 6B). Although additional experiments testing a broad range of temperatures and a more diverse array of colonies will be imperative, these findings provide important evidence establishing that temperature and seasonality are significant ecological factors that impact caste differentiation in *R. flavipes*.

With respect to intrinsic factors, this study also confirmed that hexamerin protein levels correlate significantly and positively with nutritional status (as a direct result of cellulose feeding). Most notably, in assays where no ectopic JH was provided: (1) feeding levels were highest, (2) hexamerin titers were elevated and (3) no presoldier differentiation was observed (Fig. 5B). From previous studies that used RNA interference (RNAi) to silence hexamerin gene and protein expression in the absence of ectopic JH, presoldier differentiation was inducible to natural levels by constitutive JH titers alone (Zhou et al., 2006a). Our findings here indicate that feeding can confer a soldier-inhibitory effect through intrinsic elevation of nutritional reserves and induction of hexamerin titers (Fig. 6C). Thus, we conclude that feeding, especially in the absence of ectopic JH (i.e. under natural conditions), can indeed cause termite hexamerins to increase expression/accumulate in a manner consistent with their well-defined roles as nutrient storage proteins (Burmester and Scheller, 1999).

Despite identifying a connection between feeding and hexamerin levels, the specific causative factors that induce or repress feeding remain unclear. With respect to feeding inhibition, one plausible explanation supported by our findings is that feeding is suppressed in the latter temporal stages of JH-induced presoldier differentiation; for example, in synchronization with apolysis or other eclosion-related events. Although further detailed investigations will be required to better understand causative factors that positively impact feeding, the findings reported here provide the first direct evidence linking nutritional status (*via* feeding) to hexamerin levels in termites. This finding is noteworthy because it directly supports the idea that the hexamerins serve as a nutrition- and JH-dependent developmental switching mechanism (Zhou et al., 2006a; Zhou et al., 2006b; Zhou et al., 2007); a concept developed previously (Wheeler, 1986; Wheeler and Nijhout, 2003; Nijhout, 1999; Nijhout, 2003).

Table 1. *Results of ANOVA testing the bioassay effects of temperature, juvenile hormone (JH) and time, as well as their two- and three-way interactions on the dependent variables presoldier induction, feeding and hexamerin protein abundance*

	Presoldier (%)			Feeding (mg)			Hexamerin (μg)		
Source	d.f.	*F*	*P* value	d.f.	*F*	*P* value	d.f.	*F*	*P* value
Model	11	4.97	**0.0307**	11	6.14	**<0.0001**	11	2.49	**0.0297**
Temp.	1	4.51	**0.0443**	1	0.82	0.3755	1	1.12	0.2996
JH	1	4.51	**0.0443**	1	2.43	0.1319	1	1.71	0.2035
Time	2	3.18	0.0594	2	29.63	**<0.0001**	2	2.03	0.1537
Temp × JH	1	4.51	**0.0443**	1	1.37	0.2536	1	11.61	**0.0023**
Temp × Time	2	4.22	**0.0269**	2	0.77	0.4758	2	0.95	0.4017
JH × Time	2	4.22	**0.0269**	2	0.72	0.4965	2	2.76	0.0837
3-way	2	8.94	**0.0013**	2	0.35	0.7086	2	0.76	0.4774
Error	24			24			24		
Total	35			35			35		

Significant *P*-values are indicated in bold.

Soldier caste differentiation and regulation

Soldier caste proportions in *Reticulitermes* colonies from the southeastern USA are typically below 5% (Haverty, 1977). However, these proportions fluctuate throughout the year and usually peak above 5% during the spring season (Howard and Haverty, 1981). In another rhinotermitid termite, *Coptotermes formosanus*, soldier caste proportions show similar trends but peak at much higher proportions of 10–20% in the spring season (Haverty, 1977; Waller and La Fage, 1988; Henderson, 1998; Fei and Henderson, 2002).

To investigate seasonal influences on *C. formosanus* soldier proportions, Fei and Henderson examined the effects of ambient temperature and starting soldier proportion on worker feeding and soldier caste differentiation (Fei and Henderson, 2002). Of most importance, when starting with groups of 100% workers, Fei and Henderson determined that temperatures of 20°C resulted in no soldier production, while 25 and 33°C led to intermediate soldier production (~10% at 60 days), and 30°C led to maximal soldier production (~20% at 60 days). No correlation was identified between feeding and soldier differentiation, but consumption rates did increase with increasing temperatures. The findings of the present study on *R. flavipes*, showing no significant soldier differentiation at 22°C but significantly greater differentiation at 27°C, are strongly consistent with findings reported for *C. formosanus* by both Fei and Henderson (Fei and Henderson, 2002) and Waller and La Fage (Waller and La Fage, 1988). Together, these findings imply that soldier caste differentiation is a temperature- and/or season-dependent phenomenon among lower termites from the family Rhinotermitidae.

More recently, Liu et al. (Liu et al., 2005b) investigated the influence of temperature and food quality on JH titers and soldier production in *C. formosanus*. They found that workers fed a high-quality diet (pine wood) had higher JH titers and greater soldier production over 60 days than workers fed a lower quality diet (bleached filter paper). In a second experiment, Liu et al. subsequently found that JH titers remained unchanged and no soldiers were produced at 20°C, while JH titers and soldier production both increased significantly from 24 to 32°C. The results of the current study and Liu et al. (Liu et al., 2005b) are similar in that presoldier differentiation was observed exclusively at temperatures above 22°C. However, our findings for *R. flavipes* are paradoxical in that ectopic JH is required for presoldier induction (i.e. no presoldier or reproductive differentiation occurs in the absence of ectopic JH within our 25-day model assay system). As explained in a previous report (Zhou et al., 2007), we attribute this lack of differentiation to the hexamerin mechanism and its ability to attenuate the effects of normal, constitutive JH titers. However, recent evidence for *R. flavipes* suggests that ectopic JH has a primer pheromone-like effect that subsequently stimulates endogenous JH production (X.Z., unpublished). If this is occurring, our temperature assay results would be in complete agreement with those of Liu et al. (Liu et al., 2005b).

Termites are also important economic pests. From the perspective of termite control, our findings and those noted above for *C. formosanus* suggest that use of juvenoid insecticides, which mimic the effects of JH, may only be effective at elevated ambient temperatures. For example, in agreement with current findings, modest and variable presoldier induction results were observed when testing juvenoids on *Reticulitermes* termites at temperatures below 25°C (Scharf et al., 2003). Recent findings reported for both juvenoids and new 'juvenogen' insecticides (Hrdý et al., 2004) showed consistently high presoldier induction at 27°C (Hrdý et al., 2006). However, the juvenogens specifically have not been tested at lower temperatures, thus their true utility for year-round termite control in temperate climates remains unclear. The same can be said for all currently available juvenoid insecticides.

Hexamerin-based regulation

Over the past few years, an understanding of termite hexamerins has been gradually building. Prior to knowing their identity, hemolymph-soluble hexamerin protein forms were found to increase in abundance during JH-induced soldier caste differentiation (Scharf et al., 2005a). From nymph and presoldier array screens, expressed sequence tags representing the two hexamerins were identified and, subsequently, full-length cDNA sequences were obtained (Scharf et al., 2005b; Zhou et al., 2006b). The protein sequences deduced from the full-length cDNAs revealed that the Hex-1 protein has a unique hydrophobic tail with a putative JH-binding prenylation site and that the Hex-2 protein has a unique hydrophilic insertion with unknown function. That study also revealed receptor-like qualities for Hex-2 and the highly novel finding that the hemolymph-soluble Hex-1 protein is capable of covalent JH-binding (Zhou et al., 2006b).

With the assistance of RNAi, it was discovered subsequently that dual silencing of both hexamerins leads to significant increases in JH-dependent presoldier differentiation (Zhou et al., 2006a). This provided the first evidence that the hexamerins serve a 'status quo' presoldier-inhibitory function in workers, rather than an inductive function that facilitates presoldier differentiation. Later, using a combination of RNAi and gene expression profiling, it was discovered that effects of hexamerin silencing on downstream gene expression correlate significantly with JH-dependent changes in gene expression (Zhou et al., 2007). Collectively, this body of evidence lends support to the hypothesis that the hexamerins modulate JH availability and limit its effects on inducing worker differentiation to soldier caste phenotypes. The evolutionary significance of this regulation lies in its ability to sustain a high degree of colony fitness through maintenance of a sufficiently large work force.

In the current study, we tested the hypothesis that the hexamerins are an environmentally and nutritionally responsive switching mechanism. We found significant impacts by both environment and nutrition on hexamerin protein levels in older members of the colony work force. With respect to colony fitness, older workers are considered the most important members of the worker caste (Crosland et al., 1997). The present study also revealed that in older workers, the hexamerins are significantly impacted by several factors; this supports the contention that hexamerin abundance is under the control of an array of intrinsic and extrinsic factors. These findings lend some explanation to previous observations of a correlation between soldier caste proportions, JH titers, temperature and food quality (Fei and Henderson, 2002; Liu et al., 2005b). In a sociobiology context, this body of evidence supports the idea that termite

hexamerins serve interconnected proximate functions in hormonal and nutritional signaling. These proximate functions are clearly linked to maintenance of termite social structure and perhaps, ultimately, the evolution of present-day termite sociality.

In the current study, while we saw consistently strong increases on days 10 and 15, we also noted an initial decline in hexamerin levels on day 5 of JH assays at both temperatures. This decline suggests that, despite consistent increases in gene expression through this time frame (Scharf et al., 2005b; Zhou et al., 2006b; Zhou et al., 2007), hexamerin protein degradation may be occurring at the whole-body level in the initial stages of presoldier morphogenesis. Despite this initial decline, hexamerin levels increased substantially in later sampling times to levels consistent with previously observed levels in the hemolymph (Scharf et al., 2005b; Zhou et al., 2006b). This finding does not contradict our caste-regulatory hexamerin model, but it does suggest a possible modification for consideration; specifically, hexamerin degradation (and possible release of bound ligands) may be an integral enabling/inductive component of early presoldier morphogenesis.

Finally, in a broader context of insect development and metamorphosis, it was recently shown that in holometabolous insects such as *Manduca sexta*, JH functions in suppressing adult tissue differentiation by inhibiting intrinsic signaling independently of nutritional state (Truman et al., 2006). Our findings support that in termites, and possibly other hemimetabolous insects, the hexamerins are a bridging mechanism that brings together nutritional- and JH-signaling. In this context, it would be highly interesting to investigate how hexamerins, nutrition and development might be interconnected in other hemimetabolous insects. Cockroaches would seem to be an excellent starting point in such investigations (Nalepa, 1994; Holbrook and Schal, 2004). Investigating cockroach hexamerins through development and under different temperature and nutritional regimes would likely reveal important new information.

Conclusions

This study revealed several new and important concepts with respect to termite ecological-developmental biology and caste polyphenism. First, in agreement with previous findings for *C. formosanus*, temperature has a significant impact on JH-dependent presoldier differentiation in *R. flavipes*. This has important consequences to understanding both termite development and the mode of action for novel juvenoid termiticides. Second, we found that caste regulatory hexamerin proteins are not significantly impacted by JH alone, but rather they are significantly impacted by an interaction of JH and temperature. Third, as identified previously from hemolymph-soluble hexamerins (Zhou et al., 2006b), JH binding by one hexamerin protein (Hex-1) was verified from whole-body preparations. JH binding by other as yet unknown proteins was also identified in the same whole-body preparations. Further studies are currently in progress to better understand covalent JH binding by hexamerins, as well as the other potentially functionally related proteins.

Finally, feeding during soldier caste differentiation is significantly impacted by time but not by ectopic JH or temperature. However, feeding by older workers (particularly in the absence of ectopic JH) correlates significantly with hexamerin protein levels, supporting a link between nutritional signaling and hexamerin-based caste regulation in older status quo termite workers. This correlation specifically verifies in older termite workers [i.e. the true work force (Crosland et al., 1997)] that the hexamerins play a role in social regulation that is modulated by nutrition (feeding), endogenous factors (JH titers) and environment (temperature). In the broader context of polyphenism (Nijhout, 1999; Nijhout, 2003), these results provide novel evidence that establishes roles for the extrinsic and intrinsic factors of environment and nutritional status, respectively, in directing the differentiation of alternative caste phenotypes in termites.

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