



Methoprene and temperature effects on caste differentiation and protein composition in the Formosan subterranean termite, *Coptotermes formosanus*

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Abstract

The utilization of multiple castes is a shared feature of social insects. In termites, multiple extrinsic factors have been shown to impact caste differentiation; for example, increased temperature has been shown to increase soldier production. Also, application of exogenous methoprene has also been demonstrated to increase soldier production. The objective of this investigation was to examine and correlate the effects of temperature variation and methoprene treatments on termite caste differentiation, and identify the resulting changes in protein levels. Our results indicate that worker-to-soldier differentiation is modulated by temperature, where a greater number of soldiers developed at a higher rate at higher temperatures compared to lower temperatures. We analyzed total protein by sodium dodecyl sulfate polyacrylamide gel electrophoresis and N-terminal sequencing and found several changes. Specifically, four proteins affected by temperature change were identified: Hexamerin-1, Hexamerin-2, Endo-beta 1,4 glucanase, and myosin. These proteins were further examined for their response to temperature, assay length (time), and exposure to the juvenile hormone analog methoprene. Hexamerin-1 protein showed a temperature-and assay length-dependent effect, while Hexamerin-2, Endo-beta 1, 4 glucanase, and myosin protein levels were all affected by temperature, assay length, and exposure to methoprene. Our analysis allows the correlation of temperature, assay length, and presence of methoprene with specific changes in protein levels that occur during caste differentiation. These results can be directly applied to better understand the complex developmental factors that control termite differentiation and guide the use of juvenile hormone analogs to maximize efficiency of termite eradication in the field.

Keywords: hexamerin, LC/MS/MS, methoprene, phenotypic bioassay, social insect

Abbreviations: LC/MS/MS, liquid chromatography/mass spectrometry/mass spectrometry

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Introduction

Termites are significant and costly pests throughout the world; each year it is estimated that termites are responsible for billions of dollars in damage and control costs (Su 2002). One unique feature contributing to the success of these household pests is that they are one of the few organisms to have evolved eusocial behavior. Termite colonies are made up of tens of thousand of individuals, all working together. All social insects share three main characteristics: overlap of generations, cooperative care of young, and a caste system (Wilson 1971). Eusocial insects use multiple castes to perform different tasks within a colony. This task partitioning results in an increase in colony efficiency (Waibel et al. 2006).

Termite colonies are made up of three main behavioral/phenotypic castes: workers, soldiers, and reproductives (Wilson 1971). Other minor castes can be present (i.e., second and third form reproductives, presoldiers, alates). The differentiation of termites from one caste to another is dependent on a number of intrinsic and extrinsic factors (Lenz 1976; Henderson 1998; Mao et al. 2005; Scharf et al. 2007). Extrinsic factors may include ambient temperature, moisture, primer pheromones, etc. (Henderson 1998; Cabrera and Kamble 2001; Park and Raina 2004, 2005; Mao et al. 2005; Liu et al. 2005; Scharf et al. 2007; Dong et al. 2009).

Temperature has been shown to play a major role in the development and caste differentiation of termites (Fei and Henderson 2002; Liu et al. 2005; Scharf et al. 2007). Fei and Henderson (2002) investigated the effect of temperature on the formation of soldiers in *Coptotermes formosanus* Shiraki (Isoptera:

Rhinotermitidae) in groups starting with 100% workers. Their results show that soldier development was temperature-dependent, with a greater number of soldiers differentiating at higher temperatures. Liu et al. (2005) investigated the effects of both temperature and food quality on juvenile hormone (JH) titer and soldier formation. Workers fed on a high quality diet had a greater percentage of soldier differentiation and higher JH titers. They also showed that JH titer increased with temperature, showing that at higher temperatures there was a greater level of JH and a greater number of presoldiers formed. In termites, JH titer correlates with presoldier production, and higher levels of JH in workers leads to the formation of soldiers (Park and Raina 2004; Mao et al. 2004). Research done by Scharf et al. (2007) looked at the two major hemolymph proteins Hexamerin 1 (Hex-1) and Hexamerin 2 (Hex-2) in response to temperature and food consumption. They showed that temperature and feeding were correlated with Hex-1/Hex-2 protein levels and associated with presoldier/soldier development in *Reticulitermes flavipes*. The higher the temperature, the greater number of presoldiers/soldiers that were formed through Hex1/Hex2 proteins modulating JH binding (Zhou et al. 2006, 2007; Scharf et al. 2007). The number of soldiers and alates within a colony has also been shown to be temperature-dependent, with more soldiers and alates in the spring and summer months (Haverty 1981; Waller and La Fage 1988; Liu et al 2005).

The present research further investigates the influence of temperature on *C. formosanus* presoldier formation and protein abundance, and attempts to identify effects that can be correlated to temperature and time. This study

aims (1) to examine the extrinsic factor—temperature—on termite caste differentiation and protein abundance, (2) to correlate total protein abundance across temperature, assay length (time), and JH analog treatment, and (3) to identify *C. formosanus* genes/proteins that respond to changes in temperature, JH analog, and time. The findings presented here provide evidence that *C. formosanus* soldier differentiation is potentially controlled by temperature and multiple proteins temporally are affected by temperature and treatment with a JH analog.

Materials and Methods

Termites

Coptotermes formosanus termite colony fractions were collected from multiple locations within City Park, New Orleans, Louisiana, USA. In-ground traps consisted of irrigation boxes filled with spruce (*Picea* sp.) wood and cardboard rolls. In-ground traps were placed in 1999 and have since been regularly monitored. Collections were brought back to the laboratory, separated from the collection material, placed in plastic containers with moist spruce, and kept in an incubator at 25 °C. Colonies had been kept in the laboratory for at least two weeks, but not longer than three months before use in experiments. Five different colonies were used in these experiments. Colonies were identified as *C. formosanus* by 16S mitochondrial rDNA sequencing (Szalanski et al. 2003).

Phenotypic bioassays

Presoldier phenotypic bioassays were performed similarly to Scharf et al. (2003b) and Tarver et al. (2009), but with slight modification. Filter paper (42.5 mm) was treated either with methoprene (16 µg in acetone) (Sigma-Aldrich PS1040,

www.sigmaaldrich.com) or acetone. A concentration of 16 µg per assay replication of methoprene was determined by preliminary concentration responses (MRT and CF, unpublished data). Filter paper was allowed to air dry and then placed into plastic Petri dishes (50 mm × 9 mm). Filter paper was then moistened with 180 µl of de-ionized H₂O. Twenty worker termites were placed into each dish. Termites were carefully chosen to use only the worker caste (greater than third instar). Dishes were then placed into secondary containers containing moist paper towels to maintain humidity. Separate containers were then placed into five different incubators, each set at a different temperature (20, 23, 25, 28, 31 °C). Temperatures were chosen based on temperatures termites might encounter in the field. Temperature was also monitored by using a thermometer and checked regularly. Mortality and caste differentiation was monitored every five days for a total of 25 days. Water was added as needed. Experiments were replicated across five colonies; colony 1 had ten replicates per treatment, while colonies 2-5 had five replicates per treatment. Additional replicates for each treatment were added to colony 2 for protein analysis (three reps per day per treatment).

Protein analysis

The additional replicates from colony 2 were destructively sampled at multiple time points (0, 1, 5, 10, 15 days) for protein analysis. All worker termites from each treatment were collected and placed into 1.5 mL Eppendorf tubes and immediately frozen at -80 °C. Once all of the samples were collected from each time point, each sample was ground in 600 µL of 1% phosphate buffered saline (Sigma-Aldrich, P-4417) and centrifuged at 14,000 g for 10 min. The supernatant fraction was collected and saved. Total protein levels were

estimated using a Quick Start Bradford Protein Assay Kit (Bio-Rad Laboratories, www.bio-rad.com) using bovine serum albumin standards included in the kit.

An aliquot of 10 µg of total protein was loaded into each well of SDS-PAGE gels (Life Technologies, www.invitrogen.com) with 4-12 % gradient and run using an Invitrogen X-cell SureLock Mini-Cell system (Life Technologies). Each gel was run with in-gel bovine serum albumin standards at concentrations ranging from 0.125 to 0.50 (Scharf et al. 2007). The standards were included to allow in-gel comparisons using densitometry analysis of target proteins. Gels were stained using Invitrogen SimplyBlue SafeStain according to manufacturer protocol (Life Technologies). After staining, gels were scanned and protein bands of interest were compared to the in-gel standards for abundance (TotalLab Limited, www.totallab.com). For N-terminal sequencing analysis, SDS-PAGE gels were electroblotted to PVDF membrane (0.45 µm, Invitrogen) and stained with 0.1 % Ponceau S (in 5 % acetic acid). Bands of interest were excised for N-terminal sequencing (Baylor College of Medicine, www.bcm.edu).

Peptide analysis

Abundant protein bands were excised and digested using the In-Gel Tryptic Digestion Kit (Thermo Scientific, www.thermoscientific.com, No. 89871). The tryptic digests were analyzed via LC/MS/MS, using an Agilent 1260 LC system, an Agilent Chip-cube interface and an Agilent 6520 quadrupole time-of-flight tandem mass spectrometer (Agilent Technologies, www.agilent.com). Chromatographic separation was accomplished using a Chip consisting of a 40 nL enrichment column and a 43 mm analytical column packed with C18,

5 µm beads with 300Å pores. One µL aliquots of the sample were transferred to the enrichment column via the 1260 capillary pump operating at a flow of 4 µL/min. The 1260 nano pump was operated at a flow rate of 600 nL/min. An initial gradient (Solvent A - 100% H₂O, 0.1% formic acid; Solvent B - 90% acetonitrile (ACN), 10% H₂O, and 0.1% formic acid) of 97% Solvent A was changed to 30% Solvent A at 12 min, 0% at 13 min, 100% at 14 min, and 0% at 15 min. A post run time of 4 min was employed for column equilibration.

The mass spectrometry source was operated at 300 °C with 5 L/min N₂ flow and a fragmentor voltage of 175 V. N₂ was used as the collision gas with collision energy varied as a function of mass and charge using a slope of 3.7 V/100 Da and an offset of 2.5 V. Both quadrupole and TOF were operated in positive ion mode. Reference compounds of 322.048121 Da and 1221.990637 Da were continually leaked into the source for mass calibration. An initial mass spectrometry scan was performed from m/z 300 to 1600 and up to three multiply charged ions were selected for MS/MS analysis. Following the initial run, a second injection was made excluding ions previously targeted for MS/MS analysis.

Data files were transferred to an Agilent workstation equipped with Spectrum Mill software (Agilent Technologies). The raw MS/MS data files were extracted, sequenced, and searched against the TrEMBL database. Data was obtained from the TrEMBL Database Release 2010_11. Summed MS/MS search score is based upon a perfect score of 25 for an individual peptide, with values > 13 generally considered to be excellent fits.

Statistical analyses

Data from the phenotypic bioassays were analyzed using an ANOVA for the main effects of percent cumulative presoldier formation at day 25, colony, temperature, and treatment (JMP, www.jmp.com). Additional analyses of pairwise *t*-tests between methoprene and non-methoprene treatments were also performed. This method was repeated for percent mortality but the addition of the interaction of temperature*treatment was added to the effects test.

Data from protein densitometry was analyzed by ANOVA testing for the main effects of temperature, treatment, day, and the interactions between the effects (Table 1).

Results

Temperature dependent bioassays

Coptotermes formosanus soldier induction was monitored across multiple temperatures (20, 23, 25, 28, 31 °C) in the presence and absence of the juvenile hormone analog, methoprene. Results across five different colonies indicate an increase in presoldier formation with an increase in temperature

(ANOVA whole model - $df = 9,299$, $F = 18.7213$, $p < 0.01$; temperature - $df = 4$, $F = 20.8044$, $p < 0.01$; treatment - $df = 1$, $F = 31.814$, $p < 0.01$; colony - $df = 4$, $F = 13.3582$, $p < 0.01$) (Figure 1a). As expected, presoldier formation also increased with methoprene treatment (non-methoprene average 1.00 ± 0.15 , methoprene 5.00 ± 0.07). The addition of methoprene significantly increased presoldier formation across temperatures. There was a significant difference in presoldier formation with methoprene treatment at 28 and 31 °C compared to the non-methoprene treatment ($p < 0.05$) (Figure 1a). Presoldier differentiation was highest in the 28 °C methoprene treatment groups (16.5 ± 3.32). There was also an increase in presoldier differentiation in the non-methoprene treatments, with the greatest presoldier formation at the highest temperature (31 °C) (3.5 ± 0.87).

The overall mortality across all treatments and temperatures was (27.27 ± 1.93 SEM). The increase in temperature also raised the mortality rate as did the addition of methoprene in the higher temperatures (ANOVA whole model - $df = 13,299$, $F =$

Table 1. Whole model and effect tests for estimated protein abundance.

Hex-1						Beta					
Whole Model - Analysis of Variance						Whole Model - Analysis of Variance					
Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F	Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Model	39	3211057.8	82334.8	1.416	0.0973	Model	39	552180.3	14158.5	3.2941	< 0.0001
Error	77	4477259.7	58146.2			Error	74	318059.36	4298.1		
C. Total	116	7688317.6				C. Total	113	870239.66			
Effect Tests						Effect Tests					
Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F	Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Treatment	1	1	205762.78	3.5387	0.0637	Treatment	1	1	8126.59	1.8907	0.1733
Temp	4	4	676548.57	2.9088	0.0269	Temp	4	4	33088.83	1.9246	0.1152
Day	3	3	757399.49	4.3419	0.007	Day	3	3	447013.54	34.6675	< 0.0001
Treatment*Temp	4	4	336152.23	1.4453	0.2272	Treatment*Temp	4	4	10633.31	0.6185	0.6507
Treatment*Day	3	3	116119.84	0.6657	0.5757	Treatment*Day	3	3	18994.39	1.4731	0.2288
Temp*Day	12	12	835648.31	1.1976	0.3002	Temp*Day	12	12	20587.41	0.3992	0.9597
Treatment*Temp*Day	12	12	671615.72	0.9625	0.4914	Treatment*Temp*Day	12	12	20523.42	0.3979	0.9602
Hex-2						Myosin					
Whole Model - Analysis of Variance						Whole Model - Analysis of Variance					
Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F	Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Model	39	831427.8	21318.7	1.3578	0.1266	Model	27	188092	6966.37	1.9698	0.0139
Error	77	1208987.3	15701.1			Error	64	226338.09	3536.53		
C. Total	116	2040415.1				C. Total	91	414430.1			
Effect Tests						Effect Tests					
Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F	Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Treatment	1	1	197330.01	12.5679	0.0007	Treatment	1	1	1691.69	0.4783	0.4917
Temp	4	4	292004.04	4.6494	0.002	Temp	4	4	4367.34	0.3087	0.8711
Day	3	3	120405.42	2.5562	0.0613	Day	3	3	113875.91	10.7333	< 0.0001
Treatment*Temp	4	4	34274.98	0.5457	0.7026	Treatment*Temp	4	4	5167.43	0.3653	0.8325
Treatment*Day	3	3	27078.94	0.5749	0.6332	Treatment*Day	3	3	23894.05	2.2521	0.0908
Temp*Day	12	12	208747.9	1.1079	0.3662	Temp*Day	12	12	9437.72	0.2224	0.9967
Treatment*Temp*Day	12	12	33068.07	0.1755	0.999						

10.5408, $p < 0.01$; temperature - $df = 4$, $F = 25.3381$, $p < 0.01$; treatment - $df = 1$, $F = 6.4797$, $p < 0.01$; colony - $df = 4$, $F = 3.2575$, $p < 0.01$; treatment*temperature - $df = 4$, $F = 4.0421$, $p < 0.01$) (Figure 1b). As a result the increase in mortality reduced the number of presoldiers formed in the higher temperature treatments.

Temperature and methoprene effect on protein abundance

Side by side samples were destructively sampled at multiple time points (days 1, 5, and 10) and total extractable protein was analyzed by SDS-PAGE (Figure 2). Multiple protein bands were visualized and the most abundant proteins showing differing amounts with treatments or times were chosen for peptide sequencing. Four different protein bands were focused on for this research. Each

of the four bands was measured for differences in abundance using densitometry (Figure 2). N-terminal sequencing of two prominent protein bands revealed that one had the N-terminal amino acid sequence YPGTSPSQ and the other had DHHIEKKMAD. The first one matched one of our EST cDNA sequence, coding for Hexamarin-2 (#1424505), and the second closely matched the first 10 amino acids of *R. flavipes* mature Hexamarin-1 (AAU20851). The apparent molecular weights of these two proteins in SDS-PAGE (Figure 2) were about 85 kDa (Hex-2) and 80 kDa (Hex-1), respectively, which are similar to those of *R. flavipes*. Two other bands were excised and identified using tryptic digest followed by LC/MS/MS. Unknown protein band #1 (216 KD) was putatively identified as myosin (%12 coverage, MS/MS score - 379.03, accession

Table 2. Number of distinct peptides, MS/MS score, and sequence coverage on matching peptides from *Coptotermes formosanus* Bands 1 and 2.

A	1	MPKPAASQED	EDPTPYLFSV	LEQRRIQDSK	PYDSKKS C WV	PDEKEGYLLG	EIKATKGDIV	SVGLPGGEVK	DFKSEKVEKV	80
	81	NPKFKEKIED	MADMTVLNTP	CVLHNLQRQY	YHKLIYTKDF	KKDQLQVQNP	PKYEKAEDMS	NLTYLNDASV	LHNLQRQYYN	160
	161	KLIYTYSGLF	CVAINPYKRY	PVYTNRCAM	YRGKRRNEVP	PHIFAISDGA	YVDMLTNHVN	QSMILITGESG	AGKTENTKKV	240
	241	IAYFATVGAS	GKKDESQKNK	GSLEDQVVQT	NPVLEAFGNA	KTVRNDNSR	FGKFI R HF	PSGKLAGADI	ETYLLEKARV	320
	321	ISQOSLERSY	HIFYQIMSGA	VAGVKEICGL	TDNIYDYHIV	SQGKVTVPSI	DDSEEFHLTD	QAFDILGFTK	QEKEDVYKIT	400
	401	AAVMHMGGMK	FKQRGREQA	EQDGEEGGR	VSKLFGCDTA	ELYKNLLKPR	IKVGNFVVTQ	GRNVQVVTNS	IGALCKGVFD	480
	481	RLFKWLKVKC	NETLDTKQKR	QHFVGLDIA	GFEIFDYNFG	EQLCINFTNE	KLQFFFNHVM	FVLEQEEYTK	EGIHWFDFIDF	560
	561	GMDLLACIEL	TEKYNGFEQL	CINF T NEKLQ	QFFNHMFVFL	EQEEYKKEGI	EWAFIDFGMD	LLACIDLIEK	YNGFEQLCIN	640
	641	FTNEK L QQFF	NHHMFVLEQE	EYQREGIEWT	FIDFGMDLQL	CIDLIEKPMG	ILSILEEESM	FPKATDQTF	EKLTNTHLKG	720
	721	SAPFQKPKPP	KPGQQA A HF	IGHYAGVVAY	NITGWLEKNK	DPLND T VVDQ	FKKSQNKLLI	EIFADHPQS	GGGEQA K GGR	800
	801	GKKG G FATV	SSAYKEQLNS	LMTTLRSTQP	HFVRCIIPNE	MKQPGMVD A H	LVMHQLTCNG	VLEGI R CRK	GFPNRMVYPD	880
	881	FKMRYMILAP	AAMAAEKLPK	NAATK C LEAV	GLDQDLYRIG	NTKGI E DPK	CTKILV E STE	LADDQYRLGN	TKVFFRAGVL	960
	961	QOME E FRDER	LGKIMSWMQA	WARGYLSRRG	FKKLQEQRVA	LKVVRQNL R K	YLQRLTWPWY	KLWQKIKPLL	NVSR I EDEIA	1040
	1041	RLEEKAKKAE	ELHAAEVKVR	KELEVLNAKL	LAEKTALLDS	LSGEGQQLQD	FQERNAK L TA	QKNDLENQLR	DIQERLTQEE	1120
	1121	DARNQLFQQK	KKADQEISGL	KKDIEDLELS	VQKAEQDKAT	KDHQIRNLND	EIAHQ E DELIN	KLNKEKMQG	ESNQKTGEEL	1200
	1201	QA E EDKINHL	NKVKAKLEQT	LDELE S LER	EKKV R GDVEK	SKRKVEGD L K	LTQEAVSDLE	RNKKLE Q TI	QRKDKELSSI	1280
	1281	TAKLEDEQV	VGKHORQIKE	LQARIEELEE	EVEAERQARA	KA E QRADLA	RELEELGERL	EEAGGATSAQ	IELNKKREAE	1360
	1361	LSKLRRDLEE	ANIQHESTLA	NLRKKHND A V	AEMAEQVDQ	NKLKAKA E HD	RQ T CHNELNQ	TRTACDQLAR	DKAAQEKTAK	1440
	1441	QLQHTLNEVQ	SKLDET N RTL	NDFDASKK L Q	SIENS D LLR	LEEAESQV S Q	LSKIKISL T T	QLED T KRLAD	EESRERATLL	1520
	1521	GKFR N LEHDL	DNLREQVEEE	AEGKADLQ R K	LSKANAEAQV	WRSKYESD G V	ARSEEEEA K	RKLQARLAE A	ETTIESL N QK	1600
	1601	CIGLEKTKOR	LSTEVEDLQL	EVD R ANAIAN	AAEKKQKAFD	KIIGEWK L V	DDLAAELDAS	QKECRN Y STE	LFRLK G AYEE	1680
	1681	QEQLEAVRR	ENKNLADEVK	DLLDQIGEGC	RNIHEIEKAR	KRLEAEK D EL	QAAL E EA E EA	LEQ E ENKVL R	AQEL S QV R Q	1760
	1761	EIDRRIOEKE	EEFENTRKNH	QRALDSMQAS	LEAEAKGKAE	ALRMKK L EA	DINELEIALD	HANKANA E AQ	KNIKRYOQ Q L	1840
	1841	KDIQTAL E EE	QRARDAREQ	LGISERRANA	LQNEE S ERT	LLEQAD R GRR	QAEQELADAH	EQ L NEVSAQ N	ASISA A KRKL	1920
	1921	ESELQTLHSD	LDELLNEAKN	SEEKAKKAMV	DAARLADEL R	AEQDHAQ T QE	KLRKALEQ Q I	KELQVRLDEA	EANALKGG K	2000
	2001	AIQKLEQ R VR	ELENELDGEQ	RRHADAQKNL	RKSERRIKEL	SFQSEEDR K N	HERMQDLV D K	LQQKIK T YKR	QIEEA E EIAA	2080
	2081	LNLAKFRKAQ	QELEEAEERA	DLAEQAIS K F	RAKGRAGSVG	RGASPAPRAT	SVRPQFDGLA	FPPRFDLAP E	NEF	2153
B	1	MRVFFCLLSA	LALCQAAYDY	KTVLKNSLLF	YEAQRSGKLP	ADK K VTWRKD	SALNDK G QKG	EDLTGGYIDA	GDFV K FGFPM	80
	81	AYTVTVLAWG	LVDYESAYST	AGALDDGRKA	LKWGTYF L K	AHTAANE F YG	QVCGQDV D HA	YWRP E DM T M	SRPAYKID T S	160
	161	KPGSDLA A ET	AAALAAATAI	YKSAD S TYSN	NLITHAK O LF	DFANNY R GKY	SDSITDA K NF	YASGDY K DEL	VVA A AWLYRA	240
	241	TNDNTYLTKA	ESLYNEFGLG	SWNGAFNWDN	KISGVQVLLA	KLTSKQAY K D	KVQGV V DYLV	SSQK K TPKGL	VYIDQW G TLR	320
	321	HAANSALIAL	QAADLGINAA	SYRQYAKKQI	DYALDGGGRS	YVVGFGT N PP	VRPHRR S SSC	PDAPAA C DWN	TYNSAGP N AH	400
	401	VLTGALVGGP	DSNDSY T DSR	SDYISNEVAT	DYNAGFQSAV	AGLLKAGY				448

(A) Myosin. Distinct Peptides - 24, MS/MS Score Search - 379.03, % AA Coverage - 12, Database Accession # B4LUG2, Protein Name - Myosin heavy chain - *Drosophila melanogaster*. The matched peptides cover 12% (269/2153 AA's) of the protein.
 (B) Band-2-Endo-b-1,4-glucanase. Distinct Peptides - 5, MS/MS Score Search - 67.88, % AA Coverage - 15, Database Accession # Q9BH22, Protein Name - Endo-b-1,4-glucanase OS - *Coptotermes formosanus*. The matched peptides cover 15% (69/448 AA's) of the protein.

number B4LUG2) (Table 2). Unknown protein band #2 was identified as endo-beta 1,4 glucanase (Beta 1,4, 50 kDa, 15% coverage, MS/MS score - 67.88, accession number Q9BH22)(Table 2).

Using densitometry analysis relative to in-gel standards, Hex-1, Hex-2, Beta, and Myosin abundance were measured. Hex-1 protein abundance was affected by temperature and day, with an increase in protein abundance at the later days and greater temperature (Table 1). When the temperature and day effects were separated, it was shown that Hex-1 protein levels only increased in the 31 °C treatment at day 15 (Figure 3). These results indicate the Hex-1 protein increases at later time points and at higher temperature, the same treatments that resulted in greater presoldier formation.

Hexamerin-2 protein was shown to be affected by treatment (non-methoprene or methoprene) and temperature (Table 1). Hex-2 abundance levels increased with methoprene treatment and at the highest temperature (31 °C). When the temperature and treatment effects were separated Hex-2 levels increased in the methoprene treatments and increased only at the highest temperature (Figure 4).

Endo-beta-1,4 glucanase and myosin protein levels were affected by day (Table 1). When the day and treatment effects were separated Endo-beta-1,4 glucanase (beta-1,4) protein levels were highest at day 1 and were reduced through days 5 and 10, and increased significantly in day 15 non-methoprene treatments (Figure 5). When the day and treatment effects were separated for myosin protein levels, as with beta-1,4 they were the highest at day 1 and were reduced through days 5 and 10. But unlike beta-1,4, there was a significant increase in myosin protein levels

at day 15 in the presence of methoprene treatments (Figure 6).

Discussion

Termite caste differentiation is a complex process and the details of this biological process are still largely unknown. Because termites are cryptic and their colonies secluded, it makes it more difficult to observe their unique life style compared to other social insects (i.e., ants, bees, and wasps). Termite caste differentiation has been shown to be modulated by a multitude of extrinsic and intrinsic factors (Howard and Haverty 1981; Waller and La Fage 1988; Horiuchi et al. 2002; Fei and Henderson 2002; Liu et al. 2005; Park and Raina 2004; Mao et al. 2005; Scharf et al. 2007; Tarver et al. 2009, 2010).

The results presented here support previous findings that show that *C. formosanus* soldier production is modulated by temperature with a direct increase in presoldier formation as temperature increases. Presoldier percentage was found to be the greatest at 28 °C, and although there was an increase in presoldier formation at the highest temperature (31 °C), higher mortality may have had an impact at this higher temperature. No presoldiers formed at the lowest temperature (20 °C), while there were a few presoldiers in the methoprene treatment at 23 °C. These results suggest that soldier formation in colonies is less likely to occur at lower temperatures. This correlates with previous research that found an increase in soldier caste number in the spring season, suggesting a seasonal effect in caste regulation (Fei and Henderson 2002). These results also suggest that temperature factors should be considered when investigating other mediators of termite soldier formation.

The modulation of temperature has been shown to have an influence on caste differentiation and hexamerin protein levels in a related termite, *R. flavipes* (Scharf et al. 2007). Similar to the results shown here, an increase in temperature increased *R. flavipes* presoldier formation. Differences between the previous research (Scharf et al. 2007) and the research presented here are that additional temperatures were tested (five compared to two) and the Hex-1 and Hex-2 proteins were analyzed separately compared to being combined in the previous study.

By analyzing the Hex-2 and Hex-1 proteins separately we were able to determine that the Hex-2 is the protein most influenced by the JH analogue treatment, methoprene. Hex-2 only showed a significant temperature-dependent effect in the methoprene treatments, although there was a slight increase in the non-methoprene Hex-2 levels in relationship to temperature (Figure 3a). These results have a similar pattern to the presoldier formation bioassays, in which presoldier formation increased with temperature and methoprene. Hexamerin proteins have been suggested to directly bind/modulate JH levels in *R. flavipes*, thereby regulating caste differentiation (Zhou et al. 2006b, 2007; Scharf et al. 2007). Liu et al. (2005) measured JH levels in *C. formosanus* and found an increase in JHIII levels/presoldier formation with an increase in temperature.

Hexamerin-1 protein was shown to be affected by day and temperature, but there was an increase in protein abundance as assay day and temperature increased. Hex-1 in *R. flavipes* showed a unique hydrophobic tail, a putative JH binding site, and was shown to be potentially hemolymph-soluble (Zhou et al. 2006b). If *C. formosanus* Hex-1 has a similar

function, perhaps the lack of fluctuation with JH analog treatment could be due to an already abundant protein in the hemolymph or the use of JH analog instead of JHIII. Further experiments determining the potential binding of *C. formosanus* Hex-1 with JH or JH analog are needed.

The protein band that had homology to endo-beta-1,4 glucanase had an increase in abundance at day one with a decrease with time, but had an increase in abundance in the non-methoprene (control) treatment at day 15. This discrepancy between protein abundance at day 15 could be because a larger number of individuals were molting to soldiers in the methoprene treatments. Endoglucanases are digestive enzymes found in the termite digestive tract specifically, the endo-beta-1,4-glucanases are responsible for the cleavage of the internal beta-linked glycosidic bonds in glucose polymers (Zhou et al. 2010). This, coupled with the fact that termites molting between castes expel all contents from the gut before molting (Cleveland 1925; Raina et al. 2008), could explain the reduction in endo-beta-1,4 protein at the later time point. Additional research needs to be conducted to shed light on this hypothesis.

The final protein band that was analyzed had homology to myosin, a protein component of muscle. Myosin levels were high at the early time point and then reduced through days 5 and 10. At day 15 there was an increase in protein abundance in the methoprene treatments only. This increase correlates with the increase in presoldiers. Perhaps the increase in myosin protein abundance is in response/relationship to the increase in muscle needed for the soldier caste. Interestingly, previous research looking at gene expression in *R. flavipes* also showed an increase in myosin gene expression in relationship to

worker-to-soldier differentiation (Scharf et al. 2003; Tarver et al. 2010). Scharf et al. (2003) saw an increase in myosin soldier gene expression compared to worker gene expression. Results from Tarver et al. (2010) showed a significant increase in myosin gene expression in worker termites during the worker-to-soldier caste differentiation process. Together, these findings suggest that myosin plays an important role in termite soldier development. Future work combining gene expression, protein abundance, and soldier development in the same experiment needs to be carried out to functionally characterize this potentially important downstream gene.

Our gels demonstrate that other proteins are modulated by temperature and methoprene, but for these experiments we focused on Hex-2 and Hex-1 based on previous knowledge, and endo-beta-1,4 and myosin because of strong visible protein abundance changes and LC/MS/MS hits. Further research to identify other differently expressed proteins is currently ongoing.

One-dimensional SDS-PAGE separates proteins based on the mass/charge ratio. The drawback of using only 1D separation is that multiple proteins might have similar ratios; therefore, there might be multiple proteins within a single band. Absolute identification of the band #1 (endo-beta-1,4) and band #2 (myosin) may not be totally conclusive because of this reason. But relative size and strong mass spectral database matches gives us confidence of their identity. Further 2D and total proteomics analysis need to be conducted to verify their identity and to identify additional proteins responsible for pre-soldier formation.

These results together, along with past research (Scharf et al. 2007) in *C. formosanus* and *R. flavipes*, suggest that temperature plays an important role in caste differentiation, and that the Hex-2 protein could have the same role as in *R. flavipes* in JH modulation, which thereby has a direct impact on worker-to-soldier caste differentiation. Finally, the changes in abundance of the putative myosin protein correlate with worker-to-presoldier transformation and strongly suggest a need for the muscle protein in the soldier termite. Discovery of proteins responsible for or associated with caste differentiation will increase our understanding of termite differentiation and could provide new targets for development of termite specific control treatments.

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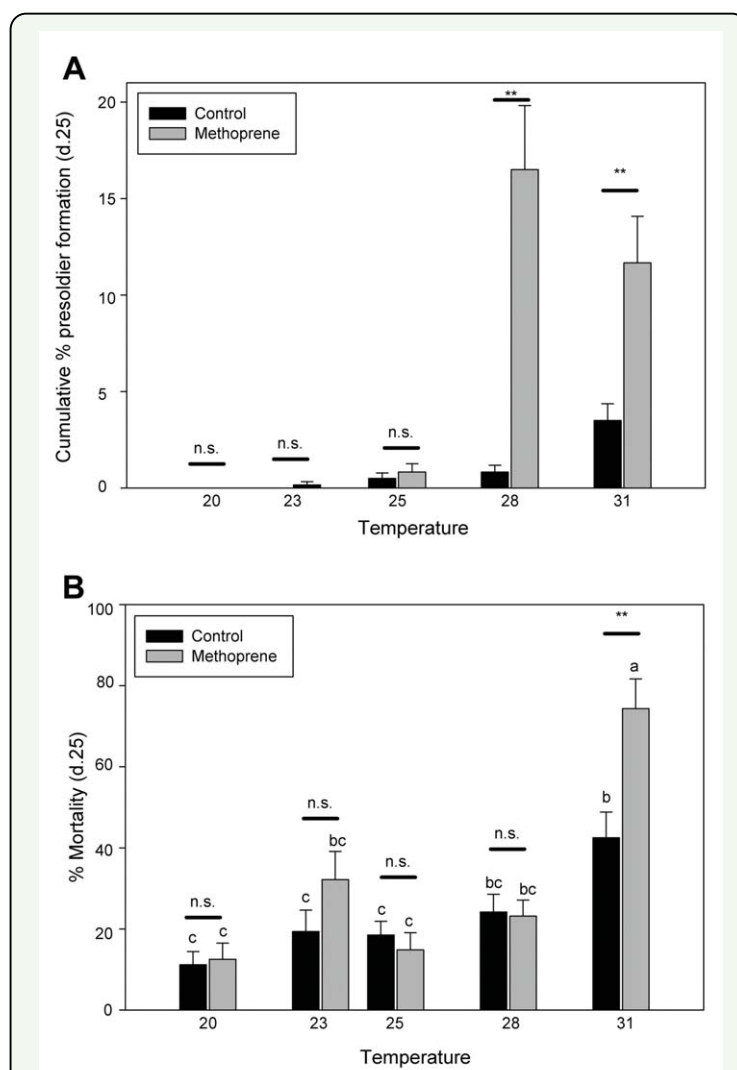


Figure 1. Impact of multiple temperatures on caste differentiation and mortality. Results of multiple temperatures 20-31 °C on (A) cumulative presoldier formation and (B) mortality. Asterisks (*) indicate a significant difference (t-test) with and without the JH-analog, methoprene. Different letters represent a significant difference ($p < 0.05$). N.S. indicates there was no difference between the two treatments. High quality figures are available online.

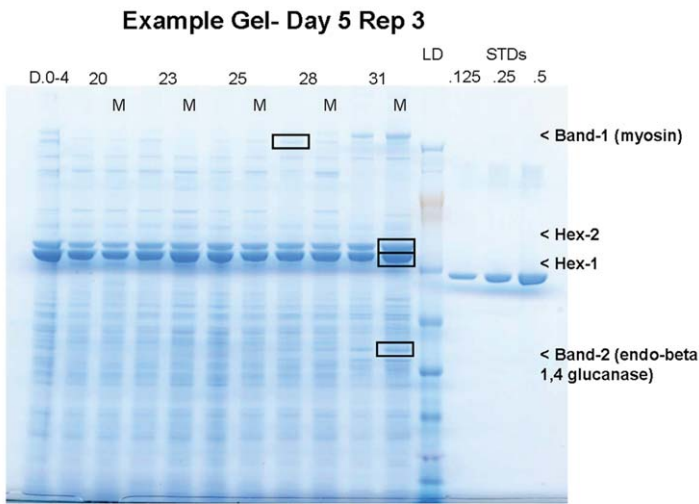


Figure 2. Representative SDS-PAGE gel. A typical gel image from the protein abundance experiment (Day 5, replicate-3). Each temperature with and without methoprene is shown. Molecular weight marker and three in-gel bovine serum albumin standards (0.125, 0.25, 0.5) were included for estimation of relative protein abundance. Hexamerin 1, 2, Band #1, and Band #2 are indicated. High quality figures are available online.

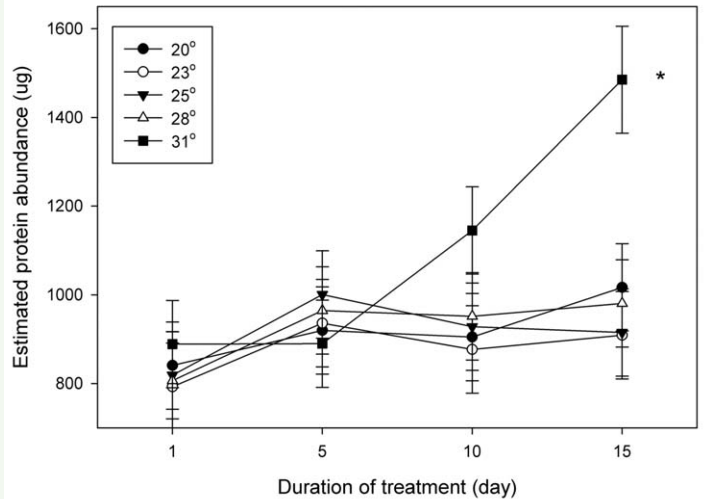


Figure 3. Hexamerin I protein abundance in relationship to temperature and day. Estimated Hexamerin I protein abundance of each temperature across days. Termites subjected to 15 days at 31 °C had significantly higher protein abundance than other temperatures ($p < 0.05$). High quality figures are available online.

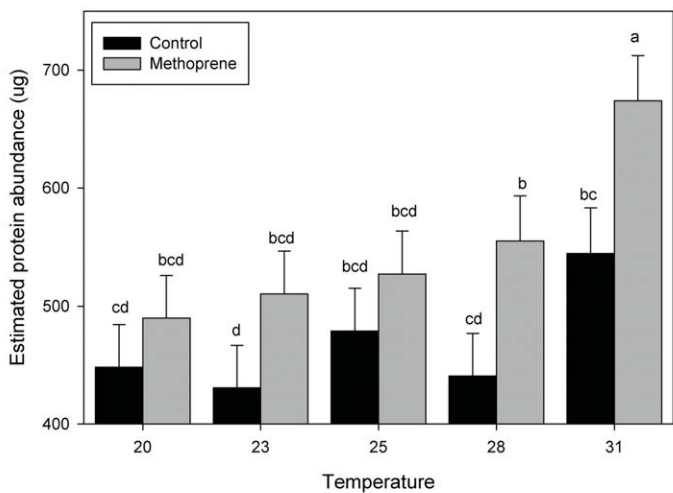


Figure 4. Hexamerin 2 protein abundance in relationship to temperature and treatment. Estimated Hexamerin 2 protein abundance for each temperature tested with and with out methoprene. Bars with different letters represent a significant difference ($p < 0.05$). High quality figures are available online.

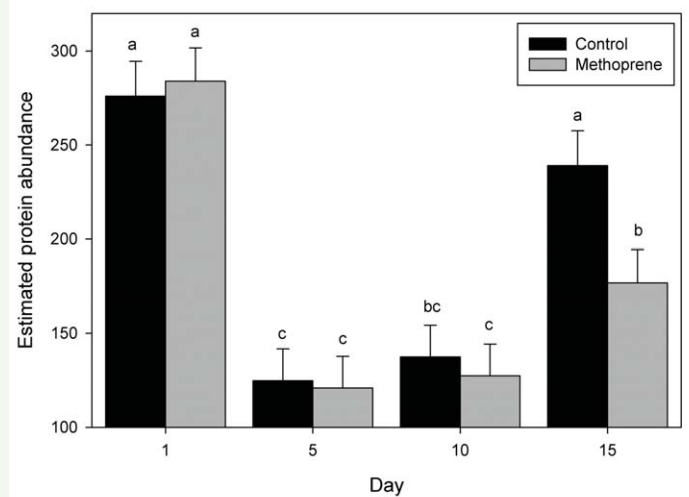


Figure 5. Band-2 (endo-beta 1,4 glucanase) protein abundance in relationship to day and treatment. Estimated Band-2 (endo-beta 1,4 glucanase) protein abundance by day with and without methoprene. Band-2 LC/MS/MS data was best fit to endo-beta 1,4, glucanase from *Coptotermes formosanus*. Bars with different letters represent a significant difference ($p < 0.05$). High quality figures are available online.

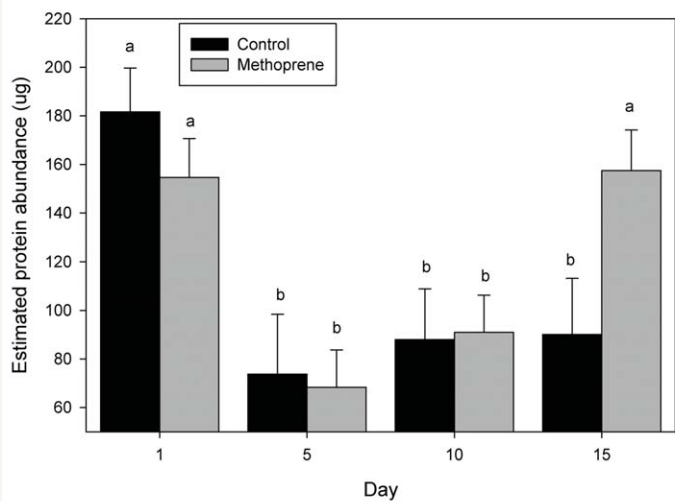


Figure 6. Band-I (myosin) protein abundance in relationship to day and treatment. Estimated Band-I (myosin) protein abundance by day with and without methoprene. Band-I LC/MS/MS data was best fit to myosin from *Drosophila melanogaster*. Bars with different letters represent a significant difference ($p < 0.05$). High quality figures are available online.