Article

Cyp15F1: A NOVEL CYTOCHROME P450 GENE LINKED TO JUVENILE HORMONE-DEPENDENT CASTE DIFFERENTION IN THE TERMITE *Reticulitermes flavipes*

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Termites are eusocial insects that jointly utilize juvenile hormone (JH), pheromones, and other semiochemicals to regulate caste differentiation and achieve caste homeostasis. Prior EST sequencing from the symbiont-free gut transcriptome of Reticulitermes flavipes unexpectedly revealed a number of unique cytochrome P450 (Cyp) transcripts, including fragments of a Cyp15 family gene (Cyp15F1) with homology to other insect Cyp15s that participate in JH biosynthesis. The present study investigated the role of Cyp15F1 in termite caste polyphenism and specifically tested the hypothesis that it plays a role in JH-dependent caste differentiation. After assembling the full-length Cyp15F1 cDNA sequence, we (i) determined its mRNA tissue expression profile, (ii) investigated mRNA expression changes in response to JH and the caste-regulatory primer pheromones γ-cadinene (CAD) and γ-cadinenal (ALD), and (iii) used RNA interference (RNAi) in combination with caste differentiation bioassays to investigate gene

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function at the phenotype level. Cyp15F1 has ubiquitous whole-body expression (including gut tissue); is rapidly and sustainably induced from 3 h to 48 h by JH, CAD, and ALD; and functions at least in part by facilitating JH-dependent soldier caste differentiation. These findings provide the second example of a termite caste regulatory gene identified through the use of RNAi, and significantly build upon our understanding of termite caste homeostatic mechanisms. These results also reinforce the concept of environmental caste determination in termites by revealing how primer pheromones, as socioenvironmental factors, can directly influence Cyp15 expression and caste differentiation. © *2012 Wiley Periodicals, Inc.*

Keywords: termite; cytochrome P450; Cyp15; juvenile hormone; caste differentiation; polyphenism; phenotypic plasticity; environmental caste determination

INTRODUCTION

Termites are eusocial insects that live in colonies and produce three primary castes (reproductives, soldiers, and workers) that function in reproduction, defense, and colony maintenance (Wilson, 1971). The worker caste of "lower" termites like *Reticulitermes flavipes* is a totipotent immature caste that retains the ability to differentiate into soldier and reproductive castes. Elevated juvenile hormone (JH) titers in *R. flavipes* workers induce soldier caste differentiation via the intermediate presoldier stage (Elliott and Stay, 2008). In *R. flavipes*, JH III is produced in specialized neuroendocrine tissue contained within the corpora allata (CA) (Yagi et al., 2005, 2008; Elliot and Stay, 2008; Elliott et al., 2009; Chan et al., 2011). Ectopic JH treatment also causes presoldier induction in *R. flavipes* workers (Scharf et al., 2005), presumably through a "primer pheromone" effect that stimulates endogenous JH production (reviewed by Miura and Scharf, 2010). Although there is no evidence that JH can transfer among individual termites as would a true primer pheromone, other transferable compounds have been identified from *R. flavipes* soldier head extracts (SHE) (Tarver et al., 2009, 2010, 2011). Two dominant terpene components of *R. flavipes* SHE have been identified: γ-cadinene (CAD) and γ-cadinenal (ALD). Both compounds are transferable from soldiers to workers in vivo, and when provided ectopically in conjunction with JH, CAD and ALD (respectively) enhance and inhibit presoldier differentiation (Tarver et al., 2011). These observations are consistent with the hypothesis that CAD and ALD are stimulatory and inhibitory primer pheromone components of *R. flavipes* SHE blends.

Cytochrome P450s (P450s) are a superfamily of oxidative, heme-containing, endoplasmic reticulum-bound enzymes that are universally present among insects (Feyereisen, 2005). Insect P450s play broad and physiologically relevant roles in the oxidation of endogenous substrates that include hormones and pheromones, and xenobiotic substrates like environmental toxins. Under the category of endocrine-active P450s, several forms have been identified that participate in the biosynthesis and metabolism of the sesquiterpenoid JH (Feyereisen, 2005). The only two P450 families known to play significant roles in JH biosynthesis and degradation are from families 15 (*Cyp15*) and 4 (*Cyp4*). In cockroaches and locusts, Cyp15A1 orthologs catalyze the final step in JH biosynthesis (Helvig et al., 2004; Marchal et al., 2011); and in the silkworm, *Bombyx mori*, the close homolog Cyp15C1 catalyzes the penultimate step in JH biosynthesis (Daimon et al. 2012).

Conversely, cockroach Cyp4C7 is involved in JH deactivation (Sutherland et al., 1998, 2000). Both *Cyp15A1* and *Cyp15C1*, as well as *Cyp4C7*, are highly expressed in the CA, located in the insect head. Previous investigations of *Cyp4* transcript diversity in *R. flavipes* revealed a close homolog of *Cyp4C7* in head tissue that is JH suppressible (*Cyp4C46*; Zhou et al., 2006a); however, to our knowledge no such information has been reported on *Cyp15s* of *R. flavipes* or any other termite species.

As eusocial organisms, termites engage extensively in trophallaxis and allogrooming behaviors that facilitate chemical signaling and communication (Wilson, 1971). In this context, the termite gut is considered to provide a physiological foundation that underlies termite sociality (e.g., Wheeler et al., 2010). In addition to revealing candidate JH esterases (Wheeler et al., 2010), prior EST sequencing from the symbiont-free gut transcriptome of *R. flavipes* revealed 16 unique cytochrome P450 transcripts, including the two *Cyp15* fragments *Cyp15F1* and *Cyp15A1* (Tartar et al., 2009). The present study specifically tested the hypothesis that *Cyp15F1* is linked to JH-dependent termite caste polyphenism. To test this hypothesis, we (i) assembled the full-length cDNA sequence of *Cyp15F1*, (ii) determined its baseline tissue expression profile, (iii) investigated its expression changes in response to JH and candidate primer pheromones (CAD and ALD), and (iv) used RNA interference (RNAi) to gain insights into *Cyp15F1* function at the molecular and organismal levels. In addition to revealing tissue, JH-induced and primer pheromone-induced expression profiles, findings show that *Cyp15F1* mediates JH signaling during JH-dependent soldier caste differentiation. These findings represent the second caste regulatory mechanism to be identified in *R. flavipes* through the use of RNAi and add appreciably to our understanding of mechanisms that control termite caste differentiation.

MATERIALS AND METHODS

Termites

For EST sequencing, gene expression, and RNAi studies, multiple *R. flavipes* colony fragments were collected from two locations near Gainesville, Florida, USA, separated by approximately 5 miles. For 5′ and 3′ RACE sequencing, termite colony fragments were collected in the vicinity of New Orleans, Louisiana and Poplarville, Mississippi USA. Termites were identified as *R. flavipes* by a combination of soldier morphology (Nutting, 1990) and *16S* mitochondrial-rRNA sequencing (Szalanski et al., 2003). Termites were held in the laboratory at least 2 months before use. At the time of testing, colonies were composed of workers, nymphs, larvae, soldiers and neotenic reproductives, and lacked primary reproductives. Colonies were maintained in darkness within sealed plastic boxes at 22°C. Termites were considered workers if they did not possess enlarged mandibles, wing buds, or distended abdomens.

Sequence Assembly and Analyses

Original sequence tags were obtained by Sanger sequencing from a host gut cDNA library as described in Tartar et al., (2009). *Cyp15F1* was represented by seven library clones (Genbank Accession Numbers FL640637, FL638893, FL640773, FL636262, FL635527, FL636088, and FL636256). To obtain additional sequence length and verify sequences, select library clones were picked and resequenced in both directions. Additionally, 5′ and 3′ RACE reactions were performed to extend sequences in the N-terminal and 3′ ORF

directions, respectively (Clontech SMART kit; Mountainview, CA). Sequence alignments were performed using the ClustalW algorithm in Megalign™ (Lasergene software package; Madison, WI). Sequence features (signal peptides, polyadenylation sites, and poly-A tails) were identified based on expected features of eukaryotic genes (Lewin, 1997). N-glycosylation sites, membrane anchor sequences, and signal peptides were predicted using online tools available at http://www.cbs.dtu.dk/services/.

Cadinene (CAD) and Cadineneal (ALD) Purification From SHE

SHE were prepared following established methods (Tarver et al., 2009, 2010, 2011). In brief, 150 heads were removed from soldiers of a single colony and homogenized in acetone using a Tenbroeck glass homogenizer. To remove particulate matter, the homogenate was fractionated by passing it through a glass Pasteur pipette filled with approximately 250 mg of silica gel (60–200 mesh) on top of a glass wool plug. The extract was eluted with 10 column volumes of acetone and then evaporated under N_2 to 400 μl. Samples were first analyzed by GC/MS (electron ionization, 70eV) to confirm the presence of predominant terpenoids, namely CAD and ALD (Nelson et al., 2001; Tarver et al., 2009), and then subsequently quantified using a 6890 gas chromatograph (Agilent; Santa Clara, CA) coupled to a flame ionization detector as described in full by Schmelz et al. (2001). High-purity CAD and ALD were isolated using preparative GC and verified by comparison to authentic standards as described previously (Tarver et al., 2009).

Bioassays

Two exposure methods were employed: (i) topical application and (ii) "dish" assays that provided for exposure via surface contact and feeding. Topical applications were made in 2 μL volumes with a PB-600-1 microapplicator equipped with a 50 μL syringe (Hamilton; Reno, NV). Treatments were applied in acetone at 0.32 μg per insect. Treatments included JH III (75% purity; Sigma; St. Louis, MO), purified CAD or ALD (see preceding section), or acetone in the case of controls. Topical assays were conducted in a time-course format in which whole termites were destructively sampled for RNA isolation at 3, 24, 48, and 120 h posttreatment. Prior to sampling, termites were held in groups of 15 in 5 cm plastic Petri dishes and on paper towel sandwiches (Georgia Pacific) moistened with 60 μl of deionized water, in complete darkness at 27°C. Upon sampling, replicate groups of termites were frozen together in 1.5 ml centrifuge tubes at −80°C. Four biological replicates were performed on a single colony.

Dish assays involved confinement of termites on paper towel sandwiches (Georgia Pacific) treated with acetone (controls), JH III in acetone, and/or dsRNA dissolved in water. Dish assays were used for both presoldier induction after RNAi, and for quantifying RNAi efficacy. JH III was applied to paired paper towel sandwiches on aluminum foil in a fume hood at 56 μg per sandwich in a volume of 50 μl acetone. After solvent evaporation, sandwiches were placed in 5 cm plastic Petri dishes and moistened with either deionized water or water containing 10 μg dsRNA. Preliminary studies testing 20 μg dsRNA per dish resulted in excessively high mortality for all treatments. Two dsRNAs were tested that included *Cyp15F1* and *GFP* (Green Fluorescent Protein) as a foreign control gene (further details provided below under *RNA interference*). For presoldier induction assays, fifteen worker termites were placed in each assay dish. Mortality and presoldier differentiation were scored every fifth day and deionized water was added as needed. The entire experiment was run in duplicate, both in the absence and presence of ectopic

Table 1. Primers Used for qRT-PCR and RNAi Studies. T7 Promoter Sequences in RNAi Template Primers Are Underlined

Gene	*Accession Number*	*Used for*	*Left primer (5′-3′)*	*Right primer (5′-3′)*
Cyp15F1	FJ792773	qRT-PCR	CGGCCTCAACATTCACAGAA	CTTCCCACAACTGCATCCAA
LIM	CB518301	qRT-PCR	GTCTTCAAGTGTGGCATGT	GTCCATCGTGAGACAACCAG
Beta actin	DQ206832	qRT-PCR	AGAGGGAAATCGTGCGTGAC	CAATAGTGATGACCTGGCCGT
Stero-1	FL639110	qRT-PCR	TTGGACTGTGGACCTTAAGAGG	CCCTTAGCAACGCAGACAAT
Cyp15F1	FJ792773	RNAi template amplification	TAATACGACTCACTATAGGG -TTTCTCTGATGGCCCGTACT	TAATACGACTCACTATAGGG -CCGGATTATTGCCTTGTAA
GFP	XXU50963	RNAi template amplification	TAATACGACTCACTATAGGG -GTGGTCCCAATTCTCGTGGA	TAATACGACTCACTATAGGG -AAGATCCCAAGCTAGAGCGG

JH III. Three and four biological replicates were performed, respectively, for presoldier induction and RNAi knockdown bioassays, each on a single colony. Whereas presoldier induction bioassays lasted 25 d, RNAi knockdown bioassays lasted only 48 h.

RNA Isolation and cDNA Synthesis

Depending on experiment, total RNA was isolated from either frozen tissue (gut, head, and carcass) or whole-body samples using the SV Total RNA Isolation System (Promega; Madison, WI) as outlined previously (Scharf et al., 2008). In-column DNase treatments were included in the RNA isolation protocol to eliminate contaminating genomic DNA. RNA quality and quantity were determined by agarose gel electrophoresis and spectrophotometry, respectively. Equal amounts of RNA were used in cDNA synthesis reactions. First-strand cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad; Hercules, CA) as outlined previously (Scharf et al., 2008).

Quantitative Real-Time PCR for Estimation of Gene Expression

Quantitative real-time PCR (qRT-PCR) was performed using an iCycler iQ real-time PCR detection system (Bio-Rad) using SYBR-green chemistry as outlined previously (Zhou et al., 2006a, b, c; Scharf et al., 2008). The target gene *Cyp15F1*, as well as three reference/control genes (*Stero-1, LIM, beta-actin*) were tested in all experiments using the PCR primers described in Table 1. Positions of qRT-PCR priming sites in the *Cyp15F1* cDNA sequence are shown in Supplementary Fig. S1. The most stable reference gene for normalization in each study was determined by comparing the standard deviation of CT values for respective genes across all treatments, tissues, and/or replicates using a modification of the BestKeeper analysis (Pfaffl et al., 2004) as described previously (Tarver et al., 2010). Relative expression levels were determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) with statistical analyses being performed on ΔCT (target gene minus reference gene CT) values. At least three biological replications were performed with parametric or nonparametric analysis as appropriate (see below). Unless indicated, reported relative expression values are $2^{-\Delta\Delta CT}$ values. All qPCR primer sets were verified to have similar amplification efficiencies using the template-standard dilution approach.

RNA Interference

RNA interference studies were performed using a previously developed dsRNA feeding bioassay (Zhou et al., 2008; Scharf et al., 2008). An approximately 500 bp fragment of the *Cyp15F1* cDNA (Supplementary Fig. S1) was PCR amplified using T7-appended primers (Table 1). The control gene for RNAi studies was a synthetic green fluorescent protein (GFP) construct (Zolotukhin et al., 1996; Genbank accession numbers XXU50963, U50963, AB078779). The synthetic GFP construct, its dsRNA template fragment, and corresponding T7-appended PCR primers are shown in Supplementary Fig. S2 and Table 1. Whole-body RNA isolation and cDNA synthesis were conducted as described above. Target and control dsRNAs were synthesized from T7-appended PCR products using the MEGAscript kit (Ambion; Austin, TX) as detailed previously (Zhou et al., 2006, 2008; Scharf et al., 2008).

RNAi impacts on *Cyp15F1* gene expression were investigated by qRT-PCR after 2 days of feeding on paper towel disks treated with (i) JH III at 56 μg per dish, (ii) 10 μg *Cyp15F1* dsRNA, or (iii) 10 μg *GFP* dsRNA. The dsRNA treatments were delivered in water and JH III in acetone. cDNA prepared from whole-body RNA isolations on pooled groups of individuals served as qRT-PCR templates. qRT-PCR was performed as described above using β-*actin* as the reference gene. RNAi "dish" assays were conducted as described above under *Bioassays*.

Statistical Analyses

When data could meet normality, variance, and other assumptions, standard one-way analysis of variance (ANOVA) was performed on whole models, with Tukey's HSD tests being used for mean separation only when model statements were significant. Otherwise, when ANOVA assumptions could not be met, nonparametric Kruskal–Wallis tests were used for whole-model analyses. In cases where Kruskal–Wallis analyses produced significant model statements, we then followed up with pairwise Mann–Whitney U-tests (A.K.A. Wilcoxon sum-rank tests) for comparisons between treatments and controls and among treatments.

RESULTS

Sequences and Sequence Analyses

Previously, over 5,000 ESTs were sequenced from a normalized *R. flavipes* gut cDNA library (Tartar et al., 2009). Two transcripts identified by Tartar et al. (2009) encoded predicted proteins with significant homology to Cyp15 proteins, i.e., *Cyp15F1* (FJ792773) and *Cyp15A1* (FJ792774). The *Cyp15F1* cDNA sequence was initially assembled from seven contiguous ESTs. To add length and build stronger contigs, library clones for both genes were resequenced in both directions and 5′ and 3′ RACE performed. *Cyp15A1* was encoded by only a single EST and appears to contain an intron at its 5′-end (Supplementary Fig. S3); thus, it apparently represents a processed pseudogene and was not considered further.

The *Cyp15F1* cDNA and translated amino acid sequences are shown in Fig. 1. The full-length *Cyp15F1* cDNA is 1,688 nucleotides long and contains a 1,461 bp open reading frame (ORF), 96 nucleotides of 5′ untranslated region (UTR) ahead of the ATG start codon, and a 3′ UTR of 128 nucleotides after the TGA termination codon. The 3′ UTR of *Cyp15F1* contains a polyadenylation signal "aataa" and a terminal poly-A tail that are

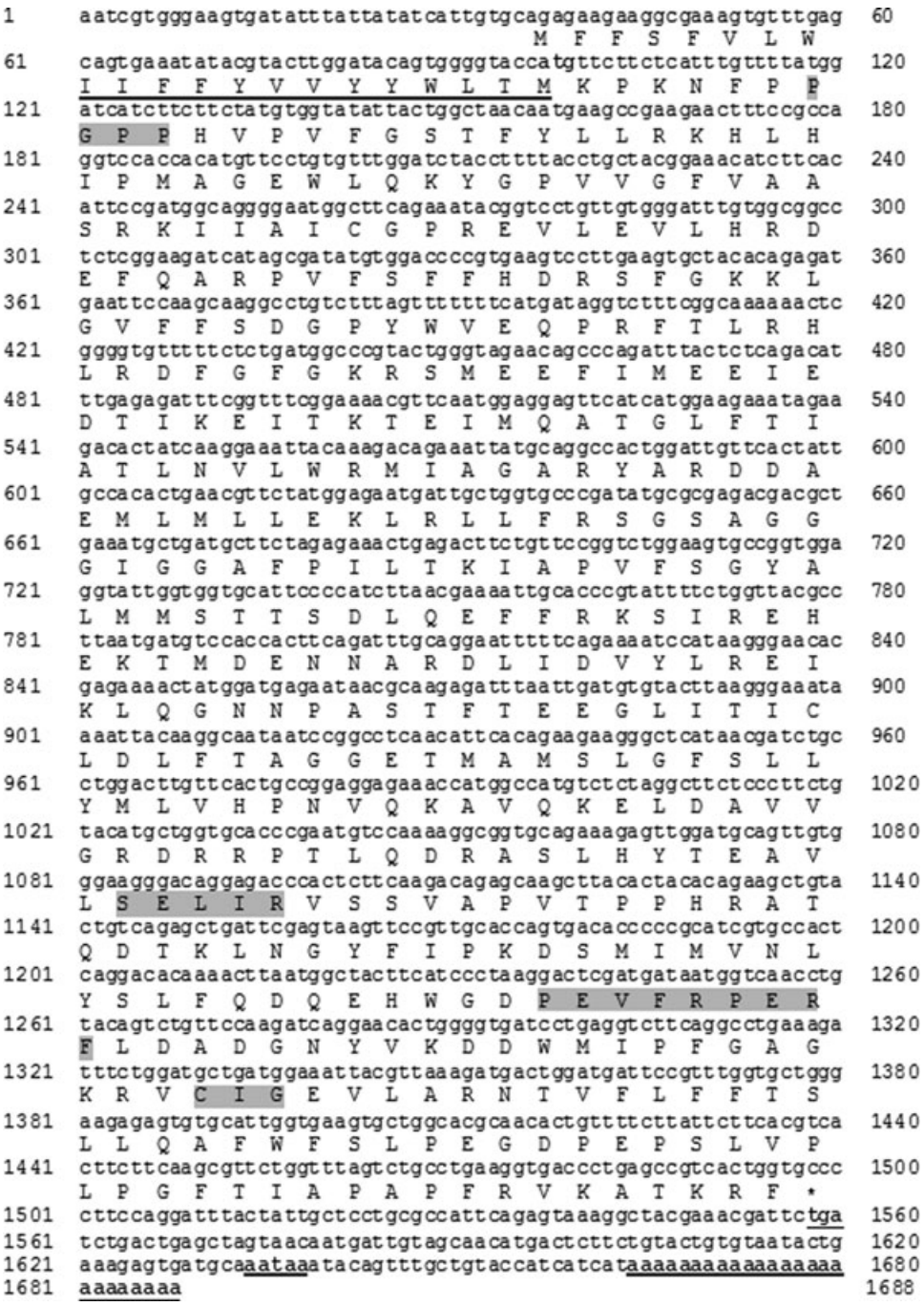

Figure 1. *Cyp15F1* cDNA sequence and amino acid translations. Important nucleotide features are underlined and shown in bold. A conserved N-terminal P450 membrane anchor sequence is underlined. The termination (stop) codon is denoted by an asterisk (*). Conserved P450 amino acid motifs are highlighted in gray. See the Results section for descriptions of sequence features.

Table 2. Cyp15F1 Transcript Tissue Distribution Profiles in R. Flavipes Colony Workers, as Determined by qRT-PCR. ANOVA Analysis Showed the Model To Be Highly Significant (df = 3,17 F = 5.7027, P = 0.0091). Data Points Within Rows With the Same Letter Are Not Significantly Different by Post Hoc Tukey's HSD Tests

	Cyp15F1 average relative expression (±standard error)		
qRT-PCR parameter	*Head*	*Gut*	*Carcass*
Δct^{a}	−0.06 (0.21)^{ab}	0.62 (0.22)^{a}	−0.61 (0.17)^{b}
$2^{-\Delta\Delta ct^{b}}$	2.00 (0.72)	1.00 (0.00)	2.20 (0.55)

[a] Δct = ct values normalized to the control gene *Stero-1* (Table 1). Statistical analyses were performed on these values.
[b] ΔΔct = ct values normalized to first the control gene *Stero-1* and then to the body region with the lowest level of expression.

highly consistent with numerous other *R. flavipes* cDNA sequences obtained to date. The translated Cyp15F1 protein sequence contains 464 amino acids and several identifiable motifs, including a 21-amino acid membrane anchor sequence MFFSFVLWIIFFYVVYYWLTM (Fig. 1). Other conserved peptide motifs in Cyp15F1 include the "PGPP hinge" involved in heme incorporation, the ETLR and PERF motifs "SELIR" and "PEVFRPERF" involved in secondary structure stabilization, and the heme-binding motif "CIG" with its heme-binding cysteine residue (Fig. 1; Feyereisen 2005). The Cyp15F1 protein has no predicted glycosylation sites and its predicted mass in kDa and pI are 55.6 and 7.19, respectively.

A translated amino acid alignment of *R. flavipes* Cyp15s with those of other hemimetabolous insects shows high degrees of conservation across lineages (Supplementary Fig. S4). The alignment includes (with protein accession numbers) *R. flavipes Cyp15F1* (ACN93794), the *R. flavipes Cyp15A1* pseudogene (ACN93795), *Diploptera punctata* Cyp15A1 (AAS13464), *Schistocerca gregaria* Cyp15A1 (ADV17351), and a partial *Blattella germanica* Cyp15A1 (CBL95272). Degrees of similarity among the five translated *Cyp15s* ranged from 41.5 to 72.5%. All sequence motifs as noted above are present across the translated alignment, with the exception of the "PGPP" hinge, which is lacking in the *R. flavipes* Cyp15A1 pseudogene and in the truncated in *B. germanica* Cyp15A1.

Baseline Expression and Tissue Localization

Baseline tissue expression profiles for *Cyp15F1* were determined in gut, head, and carcass tissues of colony workers using qRT-PCR (Table 2). Relative to gut tissue, *Cyp15F1* had 2.2- and 2.0-fold higher transcript abundance in carcass and head tissues. However, despite having lowest expression in the gut, preliminary qRT-PCR studies in colony workers indicated that *Cyp15F1* has 37-fold higher baseline expression in whole body than the apparent pseudogene *Cyp15A1* (not shown). In addition, *Cyp15F1* has now been sequenced repeatedly in several follow-up gut sequencing studies (unpublished results) and is thus considered as having whole-body expression in multiple tissues.

Temporal Impacts of JH and Purified Soldier Head Extract (SHE) Components on Cyp15F1 Gene Expression

Impacts of topically applied JH and purified SHE components on whole-body *Cyp15F1* expression are shown relative to solvent controls in Fig. 2. The SHE components CAD and

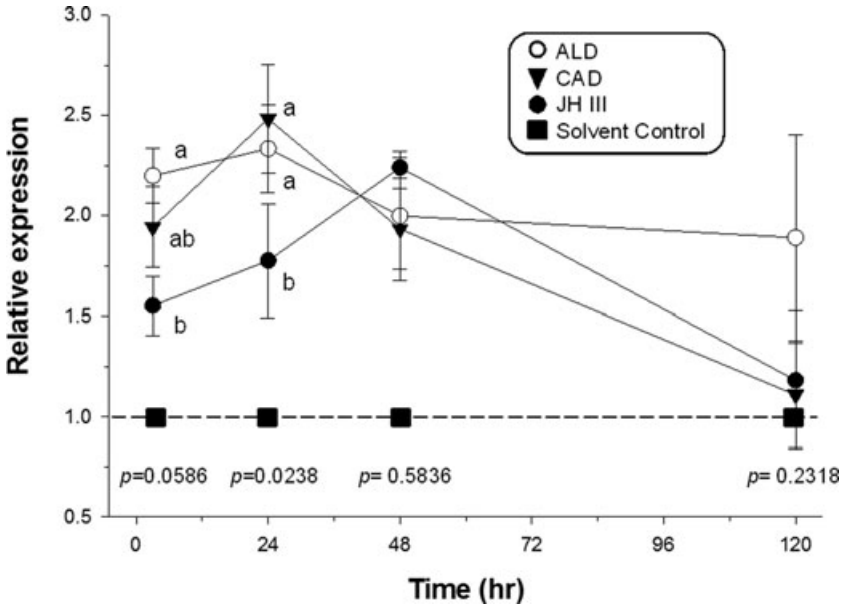

Figure 2. Time course of whole-body *Cyp15F1* transcript expression in *R. flavipes* workers in response to topical treatments of pure JH, cadinene (CAD), or cadineneal (ALD). Relative expression levels are $2^{-\Delta\Delta CT}$ values calculated relative to solvent controls, using *LIM* as the reference gene (see text and Table 1 for details). Statistical analyses were performed on ΔCT values using nonparametric Kruskal–Wallis, "ANOVA", and pairwise Mann–Whitney U-tests. The Kruskal–Wallis analysis for the whole *Cyp15F1* model was significant (df = 11; H = 22.608; $P = 0.020$) but for the reference gene *LIM*, it was not (df = 11; H = 10.793; $P = 0.461$). *P*-values shown across the bottom are the results of Kruskal–Wallis analyses within days for each treatment. Means within time points with different letters are significantly different based on pairwise analyses ($P < 0.05$). Error bars represent standard error of the mean from four biological replicates on a single colony.

ALD were obtained in highly pure form and their biological activity verified as described previously (Tarver et al., 2009, 2011). At the 3-h time point, *Cyp15F1* expression increased by 1.55- to 2.20-fold in response to treatment with the three test materials. At 24 h, CAD and ALD induced highest *Cyp15F1* expression levels overall (2.33- and 2.48-fold), followed by JH (1.77-fold). Peak induction of *Cyp15F1* expression by JH occurred at 48 h (2.24-fold); however, no differences among treatments were observable at 48 h and beyond. The reference gene *LIM* had stable expression across all treatments and time points (Kruskal–Wallis analysis: df = 11; H = 10.793; $P = 0.461$).

RNAi Knockdown Validations

RNAi studies were conducted using a previously developed feeding bioassay (Zhou et al., 2008). Knockdown validations were performed after a 48-h feeding period and results normalized to JH III treatments (Fig. 3). JH III was used as the reference treatment in this experiment because phenotypic bioassays also employed JH treatment in combination with RNAi (see following section). Also, *Cyp15F1* dsRNA and qRT-PCR primers were carefully designed so as to target different regions of the *Cyp15F1* cDNA sequence (Supplementary Figs. S1 and S2), and thus eliminate any possibility of amplifying aberrantly transcribed dsRNA. After 48 h, *Cyp15F1* transcript abundance was reduced by 27% in *Cyp15F1* dsRNA treatments relative to JH controls ($P = 0.0500$; Fig. 3). Alternatively, in

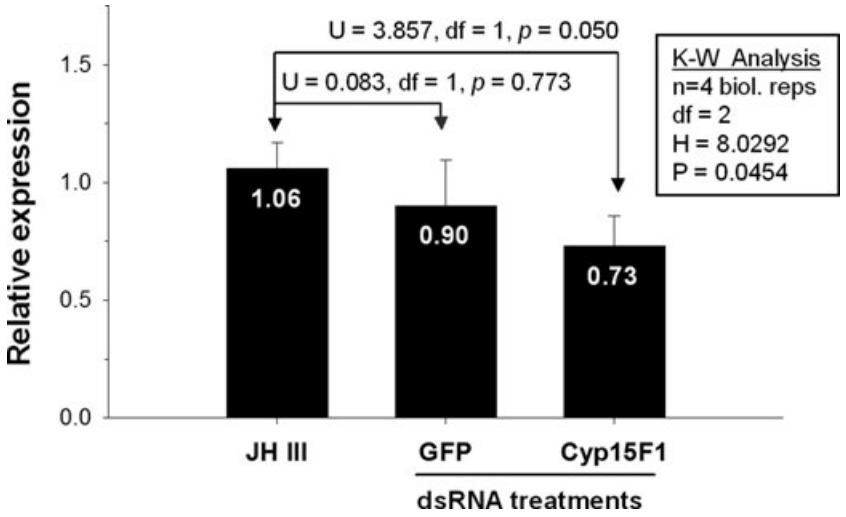

Figure 3. *Cyp15F1*dsRNA feeding impacts on *Cyp15F1* mRNA expression in *R. flavipes* workers. Bars represent relative expression ($2^{-\Delta\Delta CT}$) after 48 h ad lib feeding on filter papers treated with 56 μg JH III, 10 μg *GFP* dsRNA or 10 μg *Cyp15F1* dsRNA. Relative expression was normalized to the JH III treatment as described in the text and Table 1, using β-*actin* as the reference gene. Statistical analyses were performed on ΔCT values using nonparametric Kruskal–Wallis, "ANOVA," and pairwise Mann-Whitney U-tests. The Kruskal–Wallis analysis for the whole *Cyp15F1* model was significant ($P = 0.0454$; see graph), whereas for the reference gene β-*actin*, it was not (df = 2; H = 0.878; $P = 0.831$). *P* values shown with arrows indicate results of pairwise analyses between 48 h JH treatments and *GFP* or *Cyp15F1* dsRNA treatments. Error bars represent standard error of the mean from four biological replicates on a single colony.

GFP dsRNA treatments, *Cyp15F1* showed only a 10% reduction relative to JH controls ($P = 0.7730$). Transcript abundance of the control gene β-*actin* did not change across all dsRNA and control treatments (Kruskal–Wallis analysis: df = 2, H = 0.878, $P = 0.831$). These findings showing (i) comparatively little impact by *GFP* dsRNA on *Cyp15F1* transcript abundance, and (ii) uniform β-*actin* transcript abundance across all treatments provide reasonable evidence to support that *Cyp15F1* dsRNA effects were gene specific and not a generalized response to foreign dsRNA feeding.

Phenotypic Impacts Cyp15F1 RNAi on JH-Dependent Soldier Caste Differentiation

Phenotypic caste differentiation bioassays were used to compare the effects of two dsRNA feeding treatments (*Cyp15F1* and *GFP*) to no-dsRNA controls, both in the presence and absence of ectopic JH III (Fig. 4). The overall model for the entire experiment was highly significant (Kruskal–Wallis analysis: df = 5; H = 19.504; $P = 0.0015$). As expected (e.g., Scharf et al., 2005), a high proportion of presoldier differentiation (71.1%) occurred in JH positive controls that lacked dsRNA, and also as expected, no presoldier differentiation occurred in the absence of JH for any treatments. Most notably, presoldier formation in the *Cyp15F1* dsRNA + JH treatment was reduced to 15.6%, which is a 78.1% reduction relative to the no-dsRNA control treatment ($P = 0.0430$). The combination of *GFP* dsRNA + JH resulted in 80.0% presoldier differentiation, which was not different from the no-dsRNA control ($P = 0.4870$). There were no differences in mortality across all treatments and controls (Kruskal–Wallis analysis: df = 5; H = 5.923; $P = 0.3138$); average pooled mortality across all treatments was 14.2%.

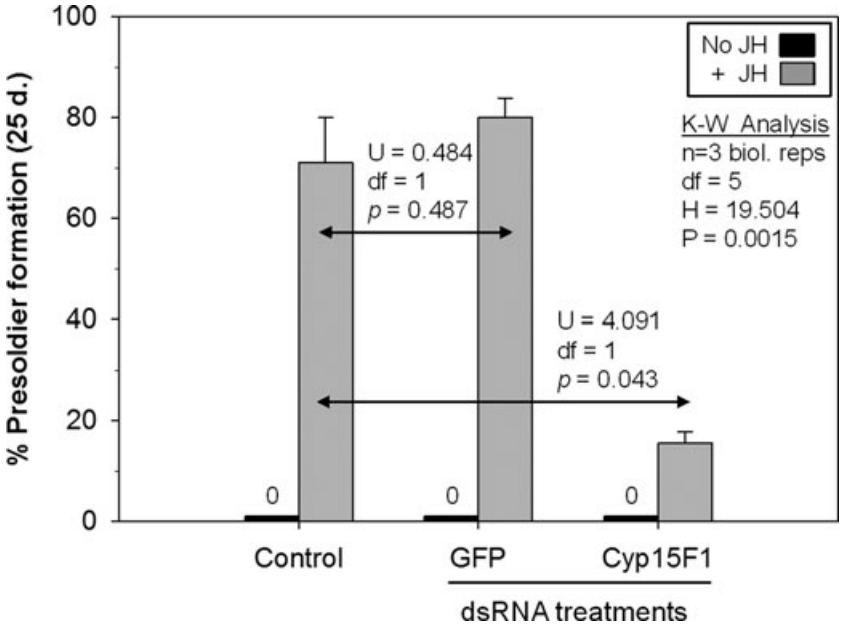

Figure 4. *Cyp15F1* RNAi significantly attenuates JH-dependent presoldier differentiation. The experiment was performed in parallel in the presence (gray bars) or absence (black bars) of 56 μg JH III. Bars represent cumulative percent presoldier differentiation through 25 days for three treatments that received no dsRNA (control), 10 μg *GFP* dsRNA, or 10 μg *Cyp15F1* dsRNA. Data were analyzed by Kruskal–Wallis "ANOVA" and pairwise Mann–Whitney U-tests. Kruskal–Wallis analysis results for the entire model are highly significant (see graph). Pairwise analyses (arrows) indicate a significant reduction in presoldier different for the *Cyp15F1*+JH treatment relative to the no-dsRNA control, but no significant effect by *GFP* dsRNA+JH ($P > 0.05$). Error bars represent standard error of the mean from three biological replicates on three separate colonies.

DISCUSSION

This research characterized a novel *Cyp15* gene from termites, *Cyp15F1*, initially discovered through EST sequencing of a normalized host gut cDNA library (Tartar et al., 2009). A follow-up study investigating expression changes of 49 candidate genes in response to socioenvironmental and endocrine treatments found that *Cyp15F1* expression is responsive to both JH and JH + crude soldier head extract (SHE) treatments; however, the experimental design of this prior study (Tarver et al., 2010) could not distinguish JH and SHE impacts, or impacts of individual SHE components. Thus, one goal of the study reported here was to discern impacts of JH and the individual SHE components CAD and ALD on *Cyp15F1* expression.

Cyp15s of closely related orthopteroid insects have established links to JH. In particular, members of the Cyp15A subfamily are expressed in neuroendocrine (CA) tissues where they catalyze the final step in JH biosynthesis (methyl farnesoate epoxidation). However, *Cyp15F1* is distinct from other Cyp15s and has thus been named as the first member of the Cyp15F sub-family (D.R. Nelson, personal communication). Because JH plays important roles in termite caste polyphenism (Miura and Scharf, 2010) and because of the similarity of *Cyp15F1* to other Cyp15s, the present study investigated *Cyp15F1* links to JH-dependent soldier caste differentiation in *R. flavipes*. In addition to investigating tissue expression and relative responses to JH and recently identified SHE primer pheromones

(Tarver et al., 2010, 2011), we used RNAi to specifically test the hypothesis that *Cyp15F1* is linked to JH-dependent caste polyphenism. Our findings lend strong support to this hypothesis by showing that (i) *Cyp15F1* responds directly to chemical signals that influence soldier caste differentiation (i.e., JH III and the soldier-derived primer pheromones CAD and ALD; Fig. 2), and (ii) reduction of *Cyp15F1* expression by RNAi significantly reduces JH-dependent soldier caste differentiation (Figs. 3 and 4).

Cyp15F1 is most closely related to a number of previously identified P450s from hemi and holometabolous insects, namely *Cyp15s* of the cockroach *D. punctata*, the locust *S. gregaria*, and the silkworm moth *B. mori*, all of which are expressed in CA tissues and catalyze methyl farnesoate epoxidation (Helvig et al., 2004; Marchal et al., 2011; Daimon et al., 2012). Through the same sequencing work that identified *Cyp15F1* (Tartar et al., 2009), a truncated *Cyp15A1* pseudogene was also identified (Supplementary Fig. S3). This apparent *Cyp15A1* pseudogene, which has only 0.027-fold expression levels relative to *Cyp15F1*, was not considered further in the current work. However, it is noteworthy that a truncated *Cyp15A1* homolog was also identified in the cockroach *B. germanica* (Maestro et al., 2010); and also several *Cyp4* pseudogenes were previously identified in *R. flavipes* (Zhou et al., 2006a). Further, a null mutant with a 68-bp deletion was identified in *B. mori Cyp15C1* that eliminates JH production (Daimon et al. 2012). Whether or not *Cyp15F1* and the apparent *Cyp15A1* pseudogene are functionally related, or if deleted *Cyp15A1* function is a unique feature of termite JH physiology are not clear at this time. Additional research will be required to investigate these possibilities.

Consistent with previous findings in orthopteroid insects relating to Cyp15 and JH biosynthesis, we found that *Cyp15F1* has expression in the head, which is the location of the CA and JH biosynthesis in *R. flavipes* (Yagi et al., 2005, 2008; Elliot and Stay 2008; Elliott et al., 2009; Chan et al., 2011). However, unlike *Cyp15A1* orthologs known to catalyze methyl farnesoate epoxidation, *Cyp15F1* is also expressed in the gut and other tissues throughout the body (e.g., carcass fat body; Table 2). Relative to solvent controls, *Cyp15F1* expression also responds rapidly (within 3 h) and in a sustained manner (through 48 h) to JH and primer pheromone treatment (Fig. 2). Finally, targeting *Cyp15F1* by RNAi reduces its transcript abundance after 2 days of feeding ($P = 0.0500$; Fig. 3) and reduces JH-dependent caste differentiation through 25 days ($P = 0.0430$; Fig. 4). Thus, these independent lines of evidence support the idea that *Cyp15F1* plays a role in regulating *R. flavipes* caste polyphenism by being responsive to primer pheromones and by participating in JH signaling.

This research builds on several previous studies of P450, JH, and primer pheromone signaling, and JH-related caste regulatory mechanisms in termites (Cornette et al., 2006; Zhou et al., 2006a, b, c, 2007; Scharf et al., 2007; Tarver et al., 2010, 2011). These previous studies have mostly considered molecular mechanisms underlying JH action and sequestration subsequent to its biosynthesis (reviewed by Miura and Scharf, 2010). Most notably, JH-binding/sequestering hexamerin proteins represented the first molecular caste regulatory mechanism to be characterized in termites (Fig. 5A). In brief, hexamerin proteins are JH inducible and capable of high-affinity JH sequestration, which results in attenuation of JH signaling, reduction of JH-dependent worker-to-presoldier differentiation, and maintenance of high worker caste proportions (reviewed by Miura and Scharf, 2010).

Cyp15F1 transcript expression levels responded positively to JH treatment, as well as to the purified SHE blend components CAD and ALD (Fig. 5B). Because *Cyp15F1* responds positively to JH and CAD, this supports the idea that *Cyp15F1* confers a mechanism by which primer pheromones and JH can directly influence caste differentiation. This conclusion is congruent with earlier findings in *R. flavipes* showing that CAD stimulates

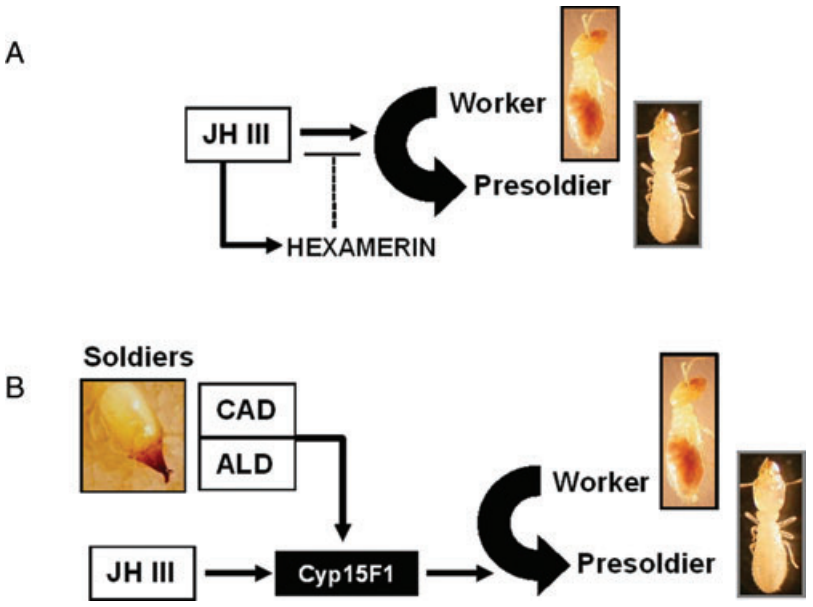

Figure 5. Synthesis models illustrating the relationships of prior and current results. (A) Prior results: *Hexamerins* respond positively to JH and inhibit its downstream impacts on presoldier differentiation. (B) Current results: JH and the soldier-derived terpene cadinene (CAD) both positively influence *Cyp15F1* expression. Because JH and CAD also influence presoldier differentiation (Tarver et al., 2011), this supports current RNAi results, suggesting *Cyp15F1*mediates JH and CAD signaling. However, because cadinene-aldehyde (ALD) induces *Cyp15F1* but inhibits presoldier differentiation (Tarver et al., 2011), this suggests as-yet unknown genes beyond *Cyp15F1* likely mediate ALD signaling.

presoldier differentiation and its levels increase in workers in the presence of soldiers (Tarver et al., 2011). However, the finding that ALD also induces *Cyp15F1* expression is paradoxical since (i) ALD significantly inhibits presoldier differentiation, (ii) live soldiers inhibit presoldier formation, and (iii) ALD levels increase substantially in workers held with soldiers (Tarver et al., 2011). These ALD results, therefore, suggest that ALD acts to inhibit soldier caste differentiation via an additional mechanism. Microarray studies to investigate global gene expression responses to JH and primer pheromones have recently been completed and results are forthcoming (MES, R. Sen and R. Raychoudhury, *in preparation*). Nonetheless, the current findings showing that soldier-derived primer pheromones can impact *Cyp15* expression and JH-dependent caste differentiation are significant and congruent with recent findings reported for other termite species; for example, live soldiers impact both JH titers and presoldier differentiation in *R. speratus* and *Coptotermes formosanus* workers (Mao et al., 2005; Park and Raina 2005; Watanabe et al., 2011).

The present findings, although expanding our understanding of caste homeostatic signaling mechanisms in termites, also underscore the long acknowledged complexity of these processes in termites and difficulty encountered in studying them (Lüscher, 1960, 1961). Together, suites of caste regulatory mechanisms that include diverse proteins like hexamerins and cytochrome P450s (and certainly others), acting in concert with JH prior to and subsequent to its biosynthesis, likely collaborate to maintain the finely balanced caste ratios that are observable in termite colonies and that have perplexed termite researchers for decades. As noted, recently completed microarray studies investigating

global gene expression responses to these treatments will lend significant resolution to this topic.

In conclusion, the findings presented here elucidate relevant new information on insect cytochrome P450, on the role of the environment in termite caste regulation, and on the use of RNAi to characterize termite socioregulatory mechanisms. First, this report focuses on *Cyp15F1*, the first member of the Cyp15F subfamily. *Cyp15F1* has ubiquitous whole body and gut expression, is responsive to JH and terpene primer pheromones, and mediates JH signaling as part of JH-dependent soldier caste differentiation. Thus, *Cyp15F1* functions in contrast to previously identified hexamerin proteins that serve a "*status quo*" function by inhibiting JH-dependent presoldier differentiation (Fig. 5A; Zhou et al., 2006a, b, 2007; Scharf et al., 2007). Second, although recent reports suggest a genetic basis to reproductive caste determination in termites (Hayashi et al., 2007; Matsuura et al., 2009; Vargo et al., 2012), the present work reinforces an equally substantial body of evidence indicating that environment (i.e., temperature) and primer pheromones (i.e., social environment) play significant roles in regulating soldier and neotenic reproductive differentiation (e.g., Goodisman and Crozier, 2003; Scharf et al., 2007; Korb et al., 2009; Matsuura et al., 2010; Tarver et al., 2009, 2010, 2011). In this regard, the current findings strengthen the view that soldier caste differentiation is a true environmentally dependent polyphenism (Nijhout, 1999, 2003; Scharf et al., 2007) by revealing how it can be regulated by primer pheromone influences on JH signaling mechanisms. Finally, the findings presented here reveal *Cyp15F1* as the second caste regulatory mechanism to be identified in *R. flavipes* through the use of RNAi. These findings build significantly on our understanding of the suite of mechanisms that underlie termite caste differentiation, and further illustrate how RNAi can be used to define gene function in social insects.

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