Screening of Multiple Potential Control Genes for use in Caste and Body Region Comparisons Using RT-qPCR in *Coptotermes formosanus*

by

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ABSTRACT

Formosan subterranean termites, *Coptotermes formosanus*, are a significant worldwide pest. Molecular gene expression is an important tool for understanding the physiology of organisms. The recent advancement of molecular tools for *Coptotermes formosanus* is leading to the advancement of the understanding of termite physiology. One of the first steps in analyzing gene expression is the normalization to constant reference genes. Stable reference genes that have constant expression across multiple treatments are important for accurately comparing target genes' expression. The objective of this investigation was to analyze and validate a set of potential reference genes including *18S* rRNA; Glyceraldehyde 3-phosphate dehydrogenase (*Gadphd*); ribosomal protein L7 (*RPL*); *β-actin (BA1)*; *α-tubulin* (*Atube*); *α-actin* (*Aactin*); and elongation factor (*Elong*) as standards for analysis of transcriptional changes in the termite *Coptotermes formosanus*, across two phenotypic castes, body regions, and colonies. We also compared the expression of *hexamerin*-1 and 2 using stable and unstable reference genes to demonstrate the importance of consistent control genes. Our results demonstrate that *18S* and *RPL* can serve as reliable expression standards when comparing these different castes and body regions, and we show that *C. formosanus Hex-1* and *Hex-2* have expression patterns similar to that previously described in *R. flavipes*.

INTRODUCTION

Formosan subterranean termites (*Coptotermes formosanus*) are an extremely destructive urban pest and are expanding their range throughout

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the world. Following introduction of the species into the United States, they have successfully spread through the southern gulf coast states (Su & Scheffrahn 2000, Lax & Osbrink 2003). Research focused on the control of this species has advanced through the years but there is an increased need for species-specific control methods. The implementation of molecular biology resources has added to the knowledge of the basic biology of this serious pest. Understanding the molecular processes underlying termite growth, differentiation, and response to stress will lead to the identification of novel target sites for termite-specific control. Gene expression profiles will be beneficial in focusing the search for termite control targets. One established method for measuring gene expression is through reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR).

The RT-qPCR technique is an extremely sensitive method for nucleic acid detection, genotyping, snp analysis and the quantification of specific messenger RNA (mRNA) levels. Several factors, including quality of template RNA, choice of enzyme and other reagents, selection of control(s) (or reference) genes, and method of analysis can have a significant effect on results. Slight variations in the transcript abundances of chosen reference gene(s) thought to be temporally and/or spatially static can artificially amplify or lessen apparent changes in target transcript quantification. Application of insufficient reference standards can cause inaccurate normalization of experimental data leading to increased error or incorrect conclusions. Rather than using a single reference gene, the use of several internal reference standards can be applied to more accurately measure changes in gene expression (Vandersompele *et al*., 2002). Careful consideration must be taken in the choice of these standards for each system and experimental condition analyzed to ensure adequate normalization for accurate analyses of results.

In previous studies characterizing gene expression in termites, several different control genes were utilized (Scharf *et al*. 2003, 2005a, 2005b, Zhou *et al*. 2006a, 2006b, 2006c, 2007a, 2007b, 2008, Wheeler *et al*. 2010, Korb *et al*. 2009, Wiel *et al*. 2009, Hojo *et al*. 2009). However, it is unclear from these studies which control genes provide reliable high-quality standards for gene expression studies in *C. formosanus* termites. These types of analyses would benefit from a well characterized and validated set of control genes that provide a stable standard for analysis across various experimental conditions.

The objective of this investigation was to analyze and validate a set of potential reference genes including *18S* rRNA; Glyceraldehyde 3-phosphate dehydrogenase (*Gadphd*); ribosomal protein L7 (*RPL*); *β-actin (BA1)*; *α-tubulin* (*Atube*); *α-actin* (*Aactin*); and elongation factor (*Elong*) as standards for analysis of transcriptional changes in the termite *Coptotermes formosanus*, across two phenotypic castes, body regions, and colonies. In addition we incorporated one exogenous (Alien) RNA control into our study. A wide range of Ct (threshold cycle) variation was observed among this set of candidate control genes, and to test their utility we applied these control genes as standards for the analysis of two target genes *Hexamerin 1*(*Hex-1*) and *Hexamerin 2* (*Hex-2*). When we applied the most stable set of reference genes, we found that *Hex-1* and *Hex-2* expression in *C. formosanus* was similar to *Reticulitermes flavipes*, with an overall up-regulation in workers and increased expression in the carcass. Predictably, target gene comparison between variable and non-variable control genes resulted in statistically different results, underscoring the idea that careful selection of control genes is essential for accurate gene expression analysis.

MATERIALS AND METHODS

Termites

Formosan subterranean termites were collected from cardboard rolls placed in bucket traps at various locations in New Orleans City Park (New Orleans, LA.). The termites were removed from the collection material, separated, and placed into plastic containers (17.1 x 12.2 x 6.0cm Pioneer Plastics) containing slats of moist spruce (*Picea* sp.) wood. The containers were then placed into an incubator maintained at 25°C, 70 % relative humidity and constant darkness. Individuals collected from two independent colonies were used in the experiments. 16S mitochondrial rDNA sequencing was used to verify their identities as *Coptotermes formosanus* (Szalanski *et al*. 2003).

Dissection

A total of four sets of 25 workers and soldiers from a colony designated 1732 were dissected to obtain the tissue from each individual's head, gut and carcass for extraction of RNA. Termites were dissected using an Olympus SZX-12 dissecting microscope (Olympus America Inc., Center Valley,

PA). Each individual was decapitated and the entire gut removed with fine forceps by gently squeezing the hind end and pulling until the entire gut was withdrawn from the carcass. The gut was placed into a 1.5 ml eppendorf tube containing 100ul phosphate buffered saline (PBS). The remaining head and carcass were placed into separate 1.5 ml eppendorf tubes. This procedure was repeated 25 times to provide isolated head, gut, and carcass samples for each individual termite in separate tubes. The tubes were then placed into a -80°C freezer prior to extraction of RNA. This process was repeated to obtain 4 biological replicates of 25 heads, guts and carcasses from worker termites. The same procedures were followed to obtain 4 biological replicates of heads, guts, and carcasses from soldier termites as well. This was then repeated to obtain 4 biological replicates of 25 from workers and soldiers from a second colony designated 1314.

Extraction of RNA

Total RNA was isolated from each set of the 25 worker or soldier frozen body regions using the SV Total RNA Isolation kit (Promega Corp., Madison, WI) according to manufacturer's instructions. RNA was similarly extracted from the extirpated guts using the same kit. The extracted RNA was quantitated using a NanoDrop instrument (NanoDrop Technologies Inc., Wilmington, DE).

Design of primers

Gene targets and primers were chosen based upon homology to past termite control genes and control genes from other species (Hojo *et al.* 2009, Tarver *et al.* 2010. Zhou *et al.* 2006a,b,c; 2007a,b). Specific gene sequences were identified from an in-house EST library (D.Z. USDA-ARS-SRRC, unpublished results). Eight control and two target genes were chosen. Primers were designed using Beacon Designer 7.61 (Premier Biosoft, Palo Alto, CA) for Sybr qRT-PCR. *Hexamerin-1* and *-2* were chosen as test target genes based on previous research (Zhou *et al*. 2006a, 2007b). All primer sequences are listed in Table 1. Primers were purchased from Integrated DNA Technologies (IDT www.idtdna.com).

Reverse Transcription Quantitative Real-Time PCR

Reverse transcription reactions for cDNA synthesis were performed using a QuantiTect Reverse Transcription Kit (Qiagen Inc., Valencia, CA) and the

Table 1. Primer pair sequences designed for RT-qPCR.

	RT-qPCR Primer Pairs (5' - 3')	
Blastx Sequence Description	Forward	Reverse
Ribosomal protein L7 (*RPL*)	attgacaacagagttcgtaggc	cgttatgcaccagtaccttcc
18S ribosomal rRNA (*18S*)	cgagattgagcaataacaggtc	acgtaatcaacgcgagcttatg
β-Actin (*BA1*)	ctatgtcgccctggactt	cgttgccaatggtgatga
Glyceraldehyde 3-phosphate dehydrogenase (*GADPHD*)	ggtagttaatggtcacaagatt	gcagaagccttatcaatgg
α-tubulin (*Atube*)	cttaccacgagcagttgagt	cgaggatcacacttcaccat
α-actin (*Aactin*)	tcttggatgattacgagtgtga	tctcaatcctctcagcaattct
Elongation factor (*Elong*)	cttggatggtggaaaggagtaact	atcttggattggaaggcgaagt
Hexamerin 1 (*Hex-1*)	gaatctgctgctacccaagg	aatcccagtggcttgtcatc
Hexamerin 2 (*Hex-2*)	actctgctccgaaaggtgaa	tcgaaatcatgttcgctctg

Alien Reference RNA qRT-PCR Detection Kit (Agilent Technologies Inc., Santa Clara, CA). A 1 µl aliquot of the Alien RNA transcript ($3x10^{10}$ copies / µl) was diluted with 59 µl of RNAse free water to a final concentration of $5x10^{8}$ copies / µl. From the initial 1:60 dilution, four 10-fold serial dilutions were made to achieve a final concentration of $5x10^{4}$ copies / µl. For the initial reaction, 2 µl of 7X gDNA Wipeout Buffer (Qiagen Inc.) and a 2 µl aliquot of the $5x10^{4}$ concentration Alien RNA template were added. The amount of template RNA used from each sample was 130 ng and 240 ng for Colonies 1732 and 1314 respectively. Additional RNase free water was then added as needed until the final volume of each reaction was 14 µl. Samples were then incubated at 42°C for 2 minutes and immediately placed on ice. To each reaction, 1 µl of Quantiscript Reverse Transcriptase, 4 µl of 5X Quantiscript Reverse Transcriptase Buffer and 1 µl Reverse Transcriptase Primer Mix was added to bring the total volume of each sample to 20 µl. Samples were then incubated at 42°C for 15 minutes followed by a 3 minute incubation at 95°C to inactivate the Quantiscript Reverse Transcriptase. All above reagents were from Quiagen (Valencia, CA)

The qPCR reactions were performed in a Bio-Rad CFX96 real time PCR detection system (Bio-Rad Laboratories, Hercules, CA) using the fast protocol

with iTaq™ Fast SYBR® Green Supermix (Bio-Rad Laboratories). All primer sets were first run at a temperature gradient (53-63°C) to obtain the optimal annealing temperature. Optimal annealing temperature was determined to be 60°C. All samples (worker head, gut, carcass; soldier head, gut, carcass; Colony 1 and Colony 2) had four biological replicates per treatment (n=48). Three technical replicates were run for each biological sample. A dissociation curve was generated and used to validate that a single amplicon was present for each RT-qPCR reaction.

Data Analysis

Averaged biological C_t values of the reference genes were analyzed using BestKeeper software (Pfaffl *et al*., 2004) to obtain a ranking for control gene stability. Two reference genes (*18S*, *RPL*) and the Alien exogenous control gene were used to calculate the relative expression of the two target genes *Hex-1* and *Hex-2* according to the ABI Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR (Applied Biosystems, Foster City, CA) with the following modification: the geometric mean of the reference genes and Alien exogenous control gene was used to calculate the ΔC_t values for the individual target genes (Vandersompele *et al*., 2002). The ΔC_t values were normalized to the treatment that had the lowest overall expression to calculate the $\Delta\Delta C_t$. The values were then graphed using the SigmaPlot 11.0 software (Systat Software, Inc., San Jose, CA). Statistical differences were calculated by analysis of variance (ANOVA) and Student's t-test using JMP® 8.0 (SAS Institute Inc., Cary, NC, USA). Statistical differences were not calculated using the $\Delta\Delta C_t$ values because normalization using the $\Delta\Delta C_t$ leads to incorporation of values from one of the treatments and violates the ANOVA rules. To validate our findings and establish a set of stable control genes, the target gene *Hex-1* was analyzed using the two most consistent (*18S* and *RPL*) reference genes, and the least consistent (*Atube*) reference gene independently for normalization.

Results / Discussion

The RT-qPCR technique provides a precise method for characterizing changes in gene transcription. Inclusion of an appropriate normalization strategy is essential to the integrity of the data and its analysis. To establish a set of reliable control genes for *Coptotermes formosanus* we analyzed the expression pattern of several potential standards.

Table 2. RT-qPCR C_t values obtained from BestKeeper software

	RPL	*18S*	*BA1*	*Gadpdh*	*Atube*	*Alien*
n	48	48	48	48	48	48
geo Mean [C_t]	23.4	13.69	21.75	23.27	28.04	27.97
am Mean [C_t]	23.47	13.73	21.88	23.34	28.72	27.99
min [C_t]	21.3	11.17	17.93	20.77	19.27	26.45
max [C_t]	29.07	17.33	27.44	28.45	37.56	32.68
std dev [± C_t]	1.45	0.79	1.85	1.38	5.21	1
CV [% C_t]	6.19	5.77	8.47	5.9	18.13	3.59

Gene stability and ranking

We first compared the Ct values for each gene across all treatments to visualize the expression variability (Fig. 1). Data in Fig. 1 indicated that the *18S* and Alien controls provided the most consistent C_t values. Both the *RPL* and *Gadph* C_t values varied slightly more, while the *β-actin*, *α-actin*, *α-tubulin*, *Elong, Hexamerin-1,* and *Hexamerin-2* C_t values varied widely from the various body parts. C_t values were also entered into the BestKeeper analysis program and the results summarized in Table 2. This analysis provided a ranking of the expression of control genes based upon their standard deviation. The ranking based upon CV value was very similar with the Alien and *18S* genes providing the most stable reference standard. The *α-actin* and *Elong* were not included in the BestKeeper analysis because of the increased number of reactions for which no product was detected (*α-actin* 26/48 rx, *Elong* 41/48 rx). Undetectable reactions resulted in a lack of measurable product, which caused excessive variation in C_t value. However, none of the samples lacked RNA as evidenced by the expression levels of the other genes, indicating that transcript abundances of *α-actin* and *Elong* were below the limit of detection in these samples. The transcript abundances of valid reference genes should be present at sufficient levels to reliably generate detectable amplification products, and so *α-actin* and *Elong* may be invalid for use as reference genes due to their low expression levels.

Target gene analysis (single versus multiple gene normalization)

To validate a set of reference genes that can provide accurate normalization, we chose to normalize *Hex-1* and *Hex-2* expression profiles using *18S* and

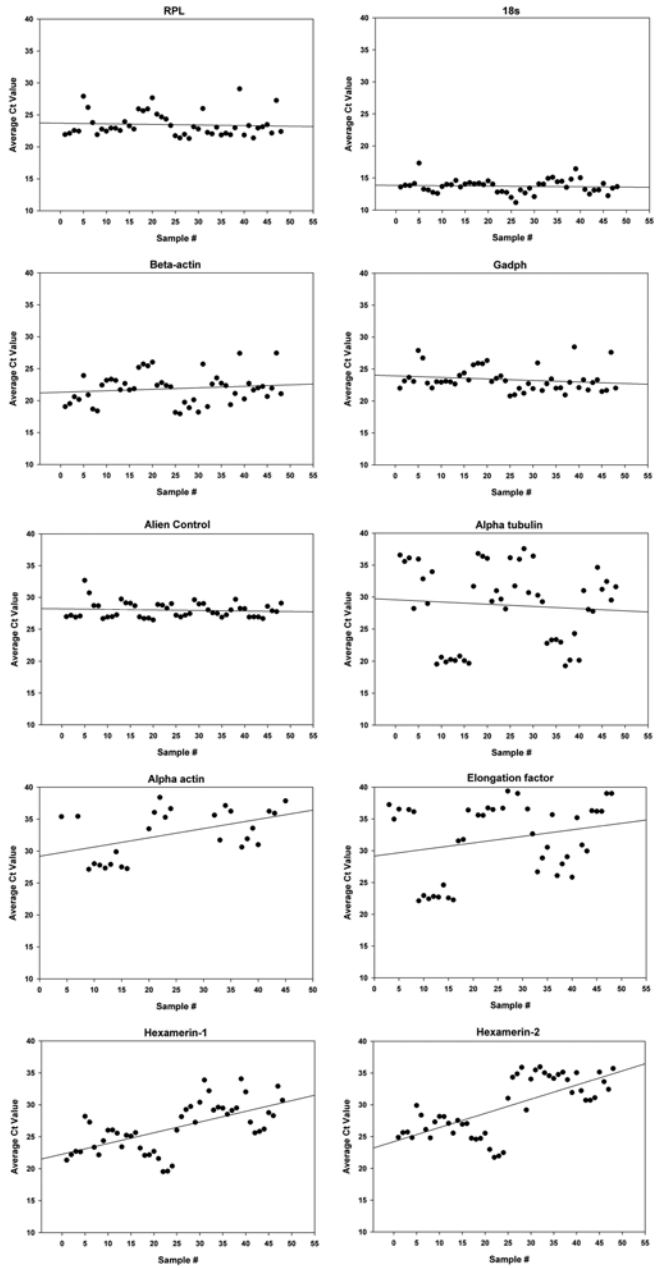

Fig. 1. Control and target gene Ct values across all treatments.

RPL, two internal control genes, and one exogenous control gene "Alien". The results with this set of three reference standards indicate that both *Hex-1* and *Hex-2* were more highly expressed in workers compared to soldiers (Fig. 2 A,B). *Hex-1* and *2* were also more expressed in the worker and soldier carcass compared to the head and gut. These findings are similar to results obtained with *R. flavipes* (Zhou *et al.* 2006c) that demonstrated up-regulation of *Hex-1* and *-2* in the termite fat body. Our results clearly demonstrate an up-regulation in the carcass which contained the fat body, therefore our results are consistent with the findings of Zhou *et al.* 2006c.

To demonstrate the potential pitfalls associated with application of a single non-validated reference gene for RT-qPCR normalization, we varied the application of our set of reference genes in the normalization of one target gene, *Hex-1*. The expression levels of *Hex-1* across soldier body regions were compared using individual control genes (*18S*, *RPL*, Alien, and *Atube*). Our findings indicated a similar expression pattern between two consistent control genes (*18S*, *RPL*), and demonstrated a higher *Hex-1* expression in the carcass compared to the head and gut (Fig. 3A and B). Even between these two "stable" control genes there is a significant difference in *Hex-1* expression in the gut. We find that with the application of *18S* alone as a reference, *Hex-1* expression in the gut is relatively higher compared to normalization with *RPL* alone (Fig. 3A and B).

Analysis of *Hex-1* expression using the most variable control gene (*Atube*) significantly alters the results. Applying *Atube* as a normalization control, *Hex-1* expression is lowest in the gut compared to the head and carcass (Fig. 3C). These results are dramatically different from the results using the two previous normalizers (*18S*, *RPL*) and clearly demonstrate that using only *Atube* as a control gene standard could result in misleading data analysis. This examination is consistent with similar types of analyses of multiple reference genes from other model systems and clearly supports the use of multiple standards (Vandesomepele *et al.* 2002). When performing gene expression analysis it is important for researchers to characterize and validate a set of control genes specific for the type of study being performed.

Relatively few studies have been conducted on termite gene expression using RT-qPCR. The majority of the past experiments have used only one gene as a reference, and in most cases this was the *β-actin gene* (Scharf *et al.*

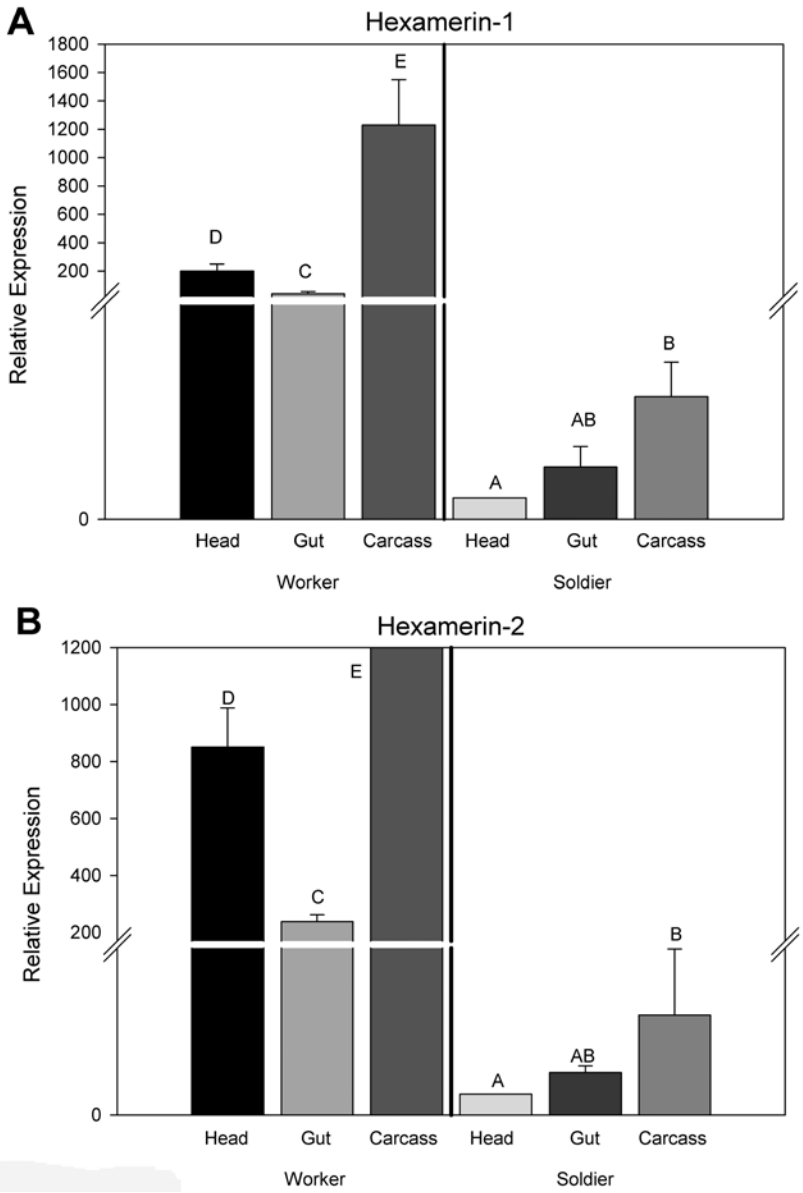

Fig. 2. Hexamerin 1 (A) and Hexamerin 2 (B) relative expression in worker and soldier termites in different body locations. *18S*, *RPL* and Alien were used as control genes with the "soldier head" being the control treatment. *Hex-2* relative expression in worker carcass (B) was too high to fit on to the graph (5663.5 ± 981 SE). Different letters represent significant differences between treatments ($P<0.05$).

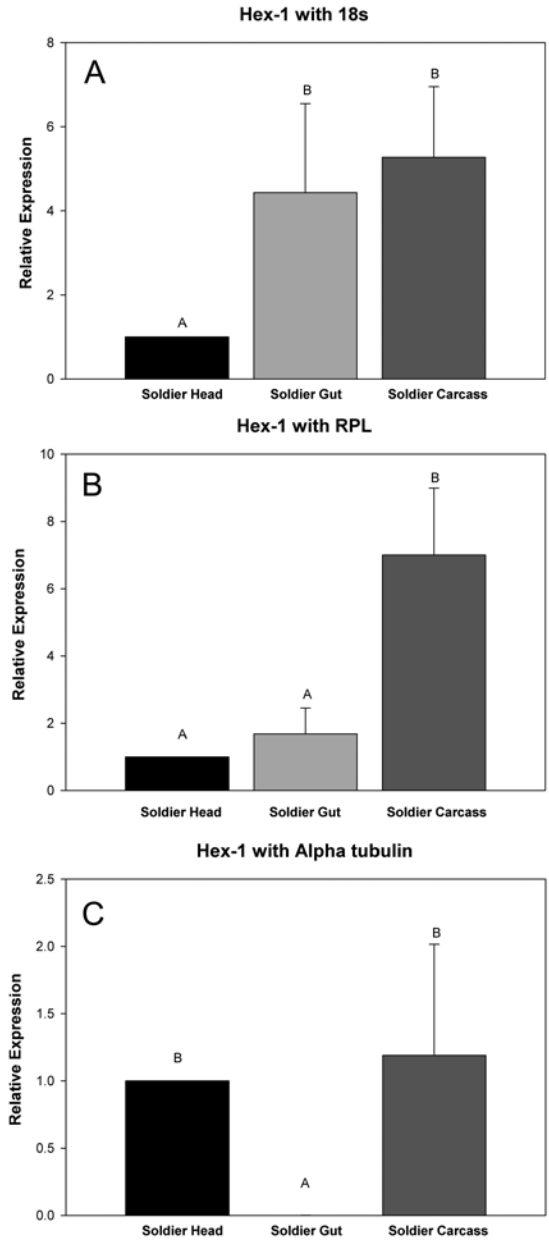

Fig. 3. Relative Hexamerin 1 expression in soldier termites after application of different reference standards (A) *18S*, (B) *RPL*, and (C) Atube. Soldier head was used as the normalizing treatment. Different letters represent significant differences ($P<0.05$).

2003, 2005a, 2005b, Wheeler *et al*. 2010). Zhou *et al*. (2006b) used only *NADH-dh* to compare different regions of the termite gut, while Korb *et al*. (2009) used only *18S* to compare gene expression in workers with different social situations. Weil *et al*. (2009) also used only *18S* and to compare gene expression in *Cryptotermes cynocephalus* and *Cryptotermes scundus* female neotenics.

A number of researchers utilized the BestKeeper software to compare three control genes. Zhou *et al*. (2006a; 2006b) used the BestKeeper software to compare *β-actin*, *HSP-70* and *NADH-dh* and determined that *β-actin* was the most stable when measuring *hexamerin* expression levels in worker termites. In another set of experiments Zhou *et al*. (2006b) used the BestKeeper software to compare *β-actin*, *HSP-70* and *NADH-dh* and determined that *NADH-dh* was the most stable when comparing cytochrome p450 expression levels in termite workers and *hexamerin* body location.

Weil *et al*. (2007) used the BestKeeper software to compare *18S*, *β-actin*, and *hexamerin* and determined that, similar to our findings, *18S* was the most stable when comparing castes in *C. secundus*. Hojo *et al*. (2009) also used the BestKeeper software and compared *18S*, *β-actin*, *Eelong*, and *NADH-dh* when measuring gene expression to compare soldier and minor workers in a higher termite. This study demonstrated that *β-actin* was the most stable and compared the stability of each potential control gene with each other. Zhou *et al*. (2008) again used the BestKeeper software to compare *β-actin*, *hsp-70* and *NADH-dh*, but this time normalized to all three control genes when documenting RNAi expression knockdown of *hexamerin* expression.

To date, the most extensive control gene identification experiment was performed in a study by Tarver *et al*. (2010) that analyzed 49 different genes across multiple treatments, days and colonies in *R flavipes*. This study compared the standard deviations of the C_t values across all genes and treatments and selected three genes with low variation to use as control genes (*Stero-1*, *LIM* and *Mev-1*).

Taken together, these different RT-qPCR gene expression studies indicate a need for control genes capable of performing as reference standards independent of termite colony, body-region, and experimental condition. Our results suggest that *18S* and *RPL* may serve as stable control genes when comparing the various samples that we investigated (caste, body location,

and colony) in our termite species, *C. formosanus*. Testing other treatments or species of termites may require a different set of control genes and a suite of control genes needs to be evaluated for variability. Predefined suites of control genes that can be applied across multiple species, time points, and treatments are highly desirable and would provide ease of use if it is possible to define them.

Finally, the use of the Alien Reference RNA (Stratagene) has a number of advantages. By "spiking" in an exogenous "alien RNA" before cDNA synthesis and then measuring Alien gene expression, a valid control is created to compensate for systematic error associated with pipetting and multiple reactions. Increased use of the Alien Reference RNA in future RT-qPCR studies will assist in the reduction of erroneous observations. Care must be taken not to rely solely on the Alien Reference RNA, but the addition of this reference with appropriate internal control genes should provide an additional reliable standard to more accurately quantify gene expression.

Conclusion

The RT-qPCR technique provides a precise method for characterizing gene transcription changes. Inclusion of an appropriate normalization strategy is essential to the accuracy of the data and its analysis. While it is labor and reagent intensive, the use of several well characterized reference genes ensures that small variations in target genes are more likely to be detected and this translates into more accurate results. Here we have characterized six potential control genes and applied the most stable (*18S*, *RPL*, Alien) to measure target gene expression across body region (head, gut, and carcass) and between castes (worker and soldier). Our results demonstrate that *18S* and *RPL* can serve as reliable expression standards when comparing these different castes and body regions, and we show that *C. formosanus Hex-1* and *Hex-2* have expression patterns similar to that previously described in *R.. flavipes*. Further use of these reference genes when comparing expression levels among various potential termite control treatments will allow more accurate assessment of effects on target gene expression.

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