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ARTICLE

Evaluation of Reference Genes for Quantitative Polymerase Chain Reaction across Life Cycle Stages and Tissue Types of *Tribolium castaneum*

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The genome of the genetic model for coleopteran insects, *Tribolium castaneum*, is now available for downstream applications. To facilitate gene expression studies in *T. castaneum*, genes were evaluated for suitability as normalizers in comparisons across tissues and/or developmental stages. In less diverse samples, such as comparisons within developmental stages or tissue only, normalizers for mRNA were more stable and consistent. Overall, the genes for ribosomal proteins rps6, rpl13a, rps3, and rps18 were the most stable normalizers for broad scale gene expression analysis in *T. castaneum*. However, their stability ranking was dependent upon the instrument as well as the analysis program. These data emphasize the need to optimize normalizers used in all real-time polymerase chain reaction experiments specifically for the experimental conditions and thermocycler and to carefully evaluate data generated by computational algorithms.

KEYWORDS: Normalization; gene expression; quantitative PCR; *Tribolium castaneum*

INTRODUCTION

Quantitative real-time polymerase chain reaction (qPCR) has rapidly become the method of choice for accurate quantification of gene expression, as well as validation of microarray analysis and other techniques that evaluate changes in gene expression. One of the primary challenges of qPCR analysis in any biological system is the availability of appropriate normalization genes. Using normalization genes that have variation in a biological system can lead to appreciable errors, up to 20-fold by some estimations (*1*). Normalizers have been described extensively for mammalian systems and are frequently applied to alternate systems without proper validation of their stability in that system. Many previously defined normalizers are not suitable for general use (*1*, *2*).

The red flour beetle, *Tribolium castaneum*, is a serious stored-products pest and coleopteran genetic model with a sequenced genome (*3*). The genomic sequence data provide the foundation for functional annotation and gene expression studies in *T. castaneum*. The rapid and biologically significant changes during insect development are often reflected by changes in gene expression. This characteristic lends to the challenges of establishing normalizers with which specific changes in gene expression can be evaluated. Furthermore, experimental conditions can be vastly different among gene expression studies in insects.

In gene expression studies of the *T. castaneum* larval gut, sequences from genes encoding the ribosomal protein rps6 and α-tubulin were used as normalizer genes to identify abundant transcripts (*4*). While these genes were satisfactory for gene expression studies within a single tissue (i.e., the larval gut), it was not evident if the same normalizers could be used for comparisons of tissues or developmental stages in *T. castaneum*. For example, it was determined that genes encoding ribosomal proteins rps3, rps18, and rpl13a were more appropriate for the evaluation of differential gene expression in fungus-challenged *T. castaneum* larvae (*5*).

To facilitate the growing need for stable reference genes in differential gene expression studies in different tissues or developmental stages of *T. castaneum*, we have evaluated nine potential normalizer genes across various life cycle stages and tissue types, using two independent qPCR instruments. The data demonstrate that different normalizers are more applicable to different experimental materials and that different normalizers were optimal with different instruments.

MATERIALS AND METHODS

Materials. The total RNA was extracted from untreated, pooled *T. castaneum* embryos, larvae, pupae, or adults, as well as tissues from head, gut, or whole carcass reared on 95% flour with 5% brewers yeast at 30 °C. Samples were flash frozen in liquid nitrogen, and the total RNA was extracted using the RNeasy Miniprep Kit (Qiagen, Valencia, CA) following the manufacturer's protocol, including one on-column and one additional in-solution DNase treatment. The total RNA was quantitated as described in the manufacturer's protocol (nanodrop 1000, Thermo Scientific, Wilmington, DE), and 500 ng was used as the template for cDNA synthesis using Superscript III First Strand Synthesis SuperMix for qRT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

Primers. qPCR primer sets were designed using Primer3 (*6*), and the amplicons (~200 bp) were validated against the *T. castaneum* genome (*5*). Nine reference genes were selected; the primer sequences and working concentrations are listed in **Table 1**. Primers were obtained from IDT Technologies (Coralville, IA).

qPCR. To minimize pipetting variation across instruments, 9 μL of the appropriate primer mix and 16 μL of the appropriate master mix

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Table 1. *T. castaneum* qPCR Primer Sets Used in This Study

symbol	description	Genbank accession	primer sequence (5′ to 3′) (forward primers are listed first)	final concn (μM)
β-actin	β-actin	XM_970977	TCCATCATGAAGTGCGATGT	900
			CCACATCTGTTGGAATGTCG	50
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	XM_969850	AATGGTCGACCTGACCGTAG	900
			AGTTTCGAGTAAGGGCCACA	900
rpl13a	ribosomal protein 13a	XM_969211	ACCATATGACCGCAGGAAAC	300
			GGTGAATGGAGCCACTTGTT	300
rps3	ribosomal protein S3	XM_965494	ACCTCGATACACCATAGCAAGC	300
			ACCGTCGTATTCGTGAATTGAC	900
rps6	ribosomal protein S6	XM_963302	AGATATATGGAAGCATCATGAAGC	50
			CGTCGTCTTCTTTGCTCAAATTG	300
rps18	ribosomal protein S18	XM_968539	CGAAGAGGTCGAGAAAATCG	900
			CGTGGTCTTGGTGTGTTGAC	900
E-cadherin	E-cadherin	XM_961215	AACGAGCCAAGGACAGCTAA	900
			TAGATTTGAGCGGTGGCTCT	300
syntaxin 1	syntaxin 1	XM_965112	GGCTTCATGGATGCATTTTT	300
			TTAAGCTTGGCACGGACTTT	900
syntaxin 6	syntaxin 6	XM_962400	CAGAGATCGTGATCGTACCG	900
			GGAATCACCGATAGCTTCCA	900

Table 2. Comparison of qPCR Normalizer Gene Primer Pair Stability, as Determined by geNORM Analysis (Stability Values), for Developmental Stages, Tissues, or Combined (All Samples)[a]

Gene Target					
all samples		developmental stages		tissues samples	
ABI	Stratagene	ABI	Stratagene	ABI	Stratagene
rps6/rpl13a (0.16)	rps6/rps3 (0.11)	rps18/rps3 (0.06)	rps6/rpl13a (0.08)	rps6/rpl13a (0.11)	rps18/rpl13a (0.08)
rps3 (0.19)	rps18 (0.19)	rpl13a (0.16)	rps3 (0.09)	rps3 (0.17)	syntaxin 6 (0.10)
rps18 (0.23)	rpl13a (0.21)	rps6 (0.18)	rps18 (0.17)	rps18 (0.23)	rps3 (0.12)
syntaxin 1 (0.32)	syntaxin 6 (0.28)	syntaxin 1 (0.25)	syntaxin 6 (0.22)	syntaxin 1 (0.30)	rps6 (0.13)
syntaxin 6 (0.46)	β-actin (0.49)	syntaxin 6 (0.40)	β-actin (0.45)	syntaxin 6 (0.42)	β-actin (0.34)
β-actin (0.60)	syntaxin 1 (0.92)	β-actin (0.53)	syntaxin 1 (0.63)	β-actin (0.53)	E-cadherin (0.91)
E-cadherin (1.42)	E-cadherin (1.63)	E-cadherin (1.49)	E-cadherin (1.48)	E-cadherin (0.98)	syntaxin 1 (1.32)

[a] Normalizers are listed in the order of most stable to least stable (top to bottom).

(containing 3 μL of a 1:50 dilution of cDNA/reaction) were used for qPCR reactions. Takara SYBR Premix Ex *Taq*II (Takara Bio, Madison, WI) reaction mix was used for optimization of concentrations and quantification according to the manufacturer's recommendations. All samples were divided equally and run on both the MX3000P (Stratagene, LaJolla, CA) and the StepOnePlus (Applied Biosystems Inc., Foster City, CA) instruments using the following cycling parameters: initial denaturation and enzyme activation at 95 °C for 30 s; 40 cycles of 95 °C for 5 s, 55 °C for 20 s, and 72 °C for 15 s with fluorescence collection at 80 °C for 10 s; cycling was followed by a standard melt curve. Three biological replicates from each of four developmental stages and three tissue types were evaluated using the raw reads from three technical replicates on each qPCR instrument.

Data Analysis. Two Microsoft Excel-based programs were used for reference gene comparisons: geNORM (*1*) (Version 3.5) and NormFinder (*7*) (Version 19). Samples were evaluated in three groups: (1) all samples, (2) developmental stages, and (3) tissue types. Data (less outliers) from each instrument were analyzed independently.

RESULTS AND DISCUSSION

For gene expression studies of comparisons across developmental stages and/or tissues of *T. castaneum*, we needed to identify stable and consistent normalizer genes. The GAPDH primer pair was expressed at undetectable levels under the experimental conditions and therefore was eliminated from the experimental analyses. Analysis of the eight remaining normalizers by geNORM indicated that overall the most stable primer pairs were consistently the ribosomal proteins (**Table 2**). However, their specific ranking was influenced by the instrument on which they were assayed as well as by the grouping within which they were analyzed. *T. castaneum* rps6 and rpl13a were the most common primer pair combination recommended by geNORM. While the top four primer pairs fluctuated in rankings between the separate analyses, they all were closely ranked, and any combination of the top four primer pairs is suitable for studies spanning developmental stages, tissue types, or even combinations of these two sample types (Supporting Information, Figure 1). The pairwise variation further supports this assumption, as two normalizer genes are consistently sufficient for qPCR normalization (pairwise variation < 0.15; Supporting Information, Figure 2).

The "Best gene" for normalization, as defined by NormFinder, varied between instruments and evaluation methods (**Table 3**). NormFinder defined rps6 as the "Best gene" for three of the six evaluations. The primary observation, however, is that the ranking of the normalizer genes was highly variable, allowing only two generalizations: (1) The ribosomal proteins were most frequently within the top three, and (2) the mRNA genes were most consistently in the bottom three, as ranked by NormFinder. There was no conclusive "Best gene" under our given experimental conditions. Evaluation of the defined "Best genes" with the NormFinder program is somewhat less intuitive than the geNORM program, as the predicted "best gene" was sometimes also the one that had the highest standard error (**Table 3**).

A good reference gene or normalizing gene is defined as a constitutive gene that is expressed at relatively constant levels and at a level that is similar to the gene(s) of interest. Given this definition, genes with a high standard error (standard deviation from the mean) would typically be excluded as normalizer

Table 3. Stability of Primer Pairs in qPCR of *T. castaneum* Developmental Stages, Tissues, or Combined (All Samples) as Defined by NormFinder Analysis[a]

Gene Target					
all samples		developmental stages		tissues samples	
ABI	Stratagene	ABI	Stratagene	ABI	Stratagene
rps6 (0.055/0.454)	rps6 (0.127/0.352)	rps18 (0.020/1.642)	syntaxin 6 (0.096/0.471)	rps6 (0.038/0.628)	β-actin (0.245/0.347)
rps18 (0.110/0.454)	β-actin (0.140/0.327)	rps3 (0.127/0.367)	rps18 (0.248/0.303)	rps18 (0.039/0.628)	rps6 (0.279/0.337)
rps3 (0.139/0.236)	rps18 (0.229/0.249)	B-actin (0.269/0.301)	rps6 (0.302/0.297)	rpl13a (0.079/0.345)	syntaxin 6 (0.321/0.332)
β-actin (0.183/0.212)	rps3 (0.284/0.233)	rps6 (0.284/0.301)	β-actin (0.302/0.297)	syntaxin 1 (0.111/0.280)	rps3 (0.402/0.338)
rpl13a (0.207/0.205)	syntaxin 6 (0.285/0.233)	rpl13a (0.339/0.303)	rpl13a (0.368/0.304)	β-actin (0.239/0.235)	rpl13a (0.405/0.345)
syntaxin 1 (0.432/0.210)	rpl13a (0.326/0.228)	syntaxin 6 (0.362/0.305)	rps3 (0.399/0.304)	rps3 (0.316/0.248)	rps18 (0.440/0.345)
syntaxin 6 (0.684/0.255)	syntaxin 1 (1.400/0.432)	syntaxin 1 (0.464/0.322)	syntaxin 1 (0.662/0.362)	syntaxin 6 (0.709/0.393)	E-cadherin (1.508/0.777)
E-cadherin (2.680/0.775)	E-cadherin (2.557/0.744)	E-cadherin (3.005/1.229)	E-cadherin (2.773/1.135)	E-cadherin (1.608/0.808)	syntaxin 1 (1.655/0.846)

[a] Normalizers are listed in the order of most stable to least stable (top to bottom) with stability values/standard error included in parentheses.

candidates. Although defined as "Best genes" by NormFinder, rps18, syntaxin 6, and β-actin had high standard errors by the programs' analyses, indicating that they are not likely the best candidates for normalization. However, the NormFinder program also has the ability to compare control and treatment groups through the assignment of group identifiers. Our analysis did not involve treatment groups and therefore may not have optimized the functionality of the program. Furthermore, while the NormFinder program requires only a minimum of three genes and three samples for analysis, it is recommended that optimal results will be obtained using eight or more biological eplicates, which was outside of the scope of our study.

E-cadherin is expressed in the gut of *T. castaneum* (*4*) and a closely related tenebrionid, *Tenebrio molitor* (*4*), and it has been used as a stable normalizer for analysis of gut-specific gene expression (unpublished data). E-cadherin was, however, the least stable of the normalizers that we evaluated for gene expression analysis across developmental stages or tissue types, regardless of the qPCR instrument. E-cadherin may have been a poor choice for a normalizer because the developmental stages within *T. castaneum* represent biologically different phenotypes with regard to feeding and therefore gut mRNA expression. Furthermore, analysis of the remaining mRNA normalizer genes, syntaxin 1, syntaxin 6, and β-actin, indicated that their use as normalizers under broad developmental or tissue type conditions also was generally not appropriate.

We have previously demonstrated that the commonly used housekeeping genes β-actin and ribosomal protein rps6 were inappropriate as reference genes in whole, fungus-challenged *T. castaneum* larvae (*5*). However, under the conditions defined in this study, rps6 was defined as one of the most stable normalizer genes. The discrepancy likely results from the previous normalizer analysis focusing on the stability of the primer pairs within a narrow range (one tissue type) while the current study evaluated primer pairs across developmental stages and tissue types. One would expect mRNA to be more constituently expressed within a single developmental stage or single tissue type than across the dynamic range of insect development stages and tissue types. This assumption was supported by the fact that when the analyses within this study focused on either tissue or stage, the mRNA primer sets were ranked as more stable (by *M* value), even though the raw data were unchanged. Furthermore, while the conditions were substantially different, the rankings of primer sets from fungus-challenged *T. castaneum* larvae (*5*), which was also assayed on the Stratagene MX3000P, were similar to those assayed on the Stratagene MX3000P in this study, with the rRNAs ranking in the top three and the mRNA demonstrating lower stability rankings.

Surprisingly, identical computational analyses of data from technical replicates from the different qPCR machines frequently defined different primers sets as optimal. Because the samples were prepared in one batch and divided, the same primer mix and master mix were used for technical replicates that were run on both instruments. In examination of reproducibility among technical replicates, the data obtained on the MX3000P had consistently lower standard errors, which may account for the decreased variability.

In summary, we have identified several stable reference genes for use in the analysis of gene expression across multiple life cycle stages and/or tissue types of *T. castaneum*. The most stable of these, the ribosomal protein genes, are transcribed by RNA polymerase I rather than RNA polymerase II, as are the mRNAs. This polymerase difference may introduce some error in the analysis of gene expression (*2*). However, de Jonge et al. (*8*) reported that 13 of the 15 top normalizer genes from a meta analysis of >13000 genes across human cell types and experimental conditions also were rRNAs. For broad spectrum gene analysis, such as among developmental stages and tissue types, the ribosomal proteins rps18, rps3, rps6, and rpl13a are well-suited for normalization of gene expression by qPCR in *T. castaneum*.

In conclusion, we have demonstrated that the ribosomal proteins rps3, rps6, rpl13a, and rps18 are appropriate for normalization of *T. castaneum* studies that require broad-spectrum gene expression analysis. Within a narrowed sample range (i.e., developmental stages or tissue types alone), the mRNA normalizers, β-actin, syntaxin 6, syntaxin 1, and E-cadherin were more stable. As the sample range is narrowed further (i.e., within a single developmental stage or tissue type), these mRNA genes may also represent excellent candidates as normalizers, particularly in studies of the larval gut.

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Supporting Information Available: Stability graphs and pairwise variation of primer sets for normalization of *T. castaneum* from geNorm analysis across development, tissue, and combined samples. This material is available free of charge via the Internet at http://pubs.acs.org.

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