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Selection and Validation of Reference Genes for Quantitative Real-time PCR in the Mealworm Beetle, *Tenebrio molitor*L. (Coleoptera: Tenebrionidae)

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Abstract:*Tenebrio molitor is often referred to as mealworm; it is a good model organism due to its short life cycle and effortless collection. T. molitor was used as the model organism. Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) is a fast, dependable, and consistent technique evaluating gene amplification during different biological processes. The performance of five normalization genes (ACTB, RPS18, EF1α, ArgK and GADPH) were analysed for T. molitor in two biotic factors (larvae stage and adult stage). The stability of each candidate reference genes was evaluated by algorithms geNorm, NornFinder, BestKeeper, ΔCt method and RefFinder. As a result, GADPH has been found reliable for the larval stage; ArgK was optimal for the adult stage. This study is usefulness for forthcoming studies on target gene expression in T. molitor.*

Key Word:*RT-qPCR, reference genes,Cq,normalization,Tenebriomolitor*

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I. Introduction

Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) is a fast, dependable, and consistent technique evaluating gene amplification during different biological processes.[36]Even though RT-qPCR is seen as a significant innovation in PCR technology, there are restrictions, including changes in RNA isolation, reverse transcriptions, cDNA quantity, normalization, and PCR efficiency.[2,3, 12,30]In general, RT-qPCR requires normalization to the expression of the convenient set of reference genes. Although reference gene expression levels are ideally stable in a number of different conditions, mostly used reference genes vary from significantly between treatments.[4, 13, 14, 18, 29, 38,45]Obviously that gene transcript level of many candidate housekeeping genes is specific for different conditions. Therefore, there is no universal gene using for internal control for all experiments, and it strongly indicates that specific reference gene selection should be made for RT-qPCR analysis, even for the same species.[17]

The Tenebrionidae family is one of the largest in the insect taxonomy, containing about over 15,000 defined species.[39]*Tenebrio molitor*L.(Coleoptera: Tenebrionidae)is often referred to as mealworm; it is a good model organism due to its short life cycle and effortless collection.[6]Additionally, *T. molitor* larvae are commercially existing and easy to reproduce.[5]In many scientific studies, *T. molitor* was used as the model organism.[8, 19, 20]The mealworm beetle is a common secondary pest and scavenger of many stored grain products. *T. molitor* is also found in chickens and other birdhouses where feathers, food, and feces are mixed.[7]The mealworm beetle larvae are commonly used as favourited fresh pet food for birds, fish, reptiles, and others.[23]

In most studies, 5-10 candidate reference genes (recently more than 10 genes) were analysed to provide for gene expression level comparison and normalization. Furthermore, linear regression analysis did not display an important correlation between the numbers of housekeeping genes used in studies. [17]

In our study, we analysed the performance of five normalization genes (*ACTB, RPS18, EF1α, ArgK* and *GADPH*) for *T. molitor* in two biotic factors (larvae stage and adult stage). The aim of this study is to investigate the expression stability of various reference genes in model beetle in different life stages (larvae and adult) and to provide a selection of correct candidate reference genes for further molecular biology studies and analysis of gene expression in tenebrionids.

II. Material And Methods

Sample and ethics statement

For this research, there were no specific permits being required for the collected insect. *Tenebrio molitor* (Coleoptera: Tenebrionidae) was purchased from a commercial vender in Antalya/Turkey (Antalya Çekirge, https://www.antalyacekirge.net/).*Tenebrio molitor* larvae and adult were transferred to the laboratory after maintained in the RNA stabilization reagent RNAlater® (Qiagen, Cat. No:76106) and stored at -20°C for experimental analyses.

Total RNA extraction and cDNA synthesis

Total RNA was isolated using the GeneAll® Hybrid-R™ kit (GeneAll Biotechnology, Seoul, Korea)according to the manufacturer's instructions.The RNA integrity was confirmed by agarose gel electrophoresis and measure of the absorbance ratio of 260/280 nm using a MaestroNano micro-volume spectrophotometer (MaestroGen, Taiwan). GeneAll® HyperScript™ first strand synthesis kit (GeneAll Biotechnology, Seoul, Korea) was used for obtaining cDNA product with 1500 ng/µL of RNA.

Candidate reference genes selection

Five candidate housekeeping genes were selected from the literature. Specifications of each primer are provided in Table 1. PCR amplification efficiencies (E) and correlation coefficients (R^2) were checked to confirm the primers. Standard curves were set up using a 5-fold diluted cDNA series for each primer pair.

Quantitative Real-time PCR (RT-qPCR)

A StepOnePlusTM Real-Time PCR system (Applied Biosystems, USA) were used to performed quantitative real-time PCR (RT-qPCR) experiments in 96-well reaction plates using iGreen 2X qPCR Master Mix (Biomatik, Canada). All reactions were carried out in duplicate for each cDNA template with a final volume 20 µL [10 µL of 2X iGreen Master Mix, 0.7 µL of each specific primer (10 µM), 2 µL of the diluted first-strand cDNA template (1, 1/5, 1/25, 1/125 and 1/625) and 6.6 µL of nuclease-free water. The RT-qPCR program included and initial denaturation for 3 min at 95°C followed by 40 cycles of denaturation at 95°C for 10 s, annealing for 30 s at 58°C, and extension for 15 s at 72°C. For melting curve analysis, a dissociation step cycle (58°C for 1 min, and then 0.5°C for 10 s until 95°C) was added. At the end of each PCR reaction, a melting curve was generated to confirm a single peak and rule out the possibility of primer-dimers and non-specific product formation. PCR amplification efficiency (E) was calculated considering to the equation: $E= (10^{[-1/slope]}-1) \times 100$.

Data analysis

The stability of each candidate reference genes was evaluated by algorithms geNorm[36], NornFinder[1], BestKeeper[21] and ΔCt method.[28] Finally, we compared and ranked the tested candidate based on the comprehensive web-based analysis tool RefFinder (https://www.heartcure.com.au/reffinder/?type=reference). This tool assigned a convenient weight to each gene and calculates the geometric mean of their weights for the total final ranking in terms of results from each program.

Table 1. General information of five candidate housekeeping genes used for real-time qPCR.

Symbol	Gene name	Description	Primer sequence (5'→3')	Length (bp)	E (%)	R^2	Ref.
ACTB	B-actin	Cytoskeleton	F: TCCATCATGAAGTGCGATGT R: CCACATCTGTTGGAATGTCG	228	115.01	0,969	26
RPS18	Ribosomal protein S18	Ribonucleoprotein; Ribosomal protein; RNA-binding; rRNA-binding	F: TACACCTTTGATCGCTGTGAG R: GGCTCTGGTCATTCCAGATAAG	108	99.2	0,981	41
EF1α	Elongation factor 1α	Translation elongation factor activity; GTPase	F: ACCAGATTTGATGGCTTTGG R: CACCCAGAGGAGCTTCAGAC	194	109.05	0,964	25
ArgK	Arginine Kinase	Phosphotransferase activity	F: CTCGTGTGGTGCAACGAAGA R: GGTGGCTGAACGGGACTCT	130	101.4	0,972	10
GADPH	Glyceraldehyde -3-phosphate	Oxidoreductase in glycolysis & Gluconeogenesis	F: GCCAAGGTGATCCATGACAA R: GTCTTCTGAGTGGCAGTTGTAG	80	110.76	0,992	18

III. Results

The quality of total RNA

In this study, the A260 / A280 ratio of total RNA from all samples ranged from 1.90 to 2.12, and A260 / A230 is above 1.90, indicating that all total RNA does not contain organic salts and protein contamination. The total RNA concentration ranged from 1218ng/uL to 2000ng/uL, suitable for synthesizing the cDNA template.

Primer specificity and efficiency

All PCR amplification of each primers were confirmed by the presence of a single peak in melting curve analyses and specific band with expected size based on 1.5% agarose gel electrophoresis (data not shown). A primer efficiency value between 99.2% and 115.01% was displayed for all primers with a correlation coefficient (R^2) ranging between 0.96 and 0.99 and their specificity was verified with the BLAST program (Table 1).

Expression profiling of candidate reference housekeeping genes

The expression profiles of all RT-qPCR products for all primers and both experiments are shown in Figure 1. The Cq values of these five candidate reference genes (*ACTB*, *RPS18*, *EF1α*, *ArgK* and *GADPH*) under the two experimental conditions (larvae and adult) ranged between 22 and 35. For the larval and adult experiment, the most expressed reference gene were *ACTB* and *RPS18*, respectively, the least expressed reference gene was *ArgK* (Figure 1).

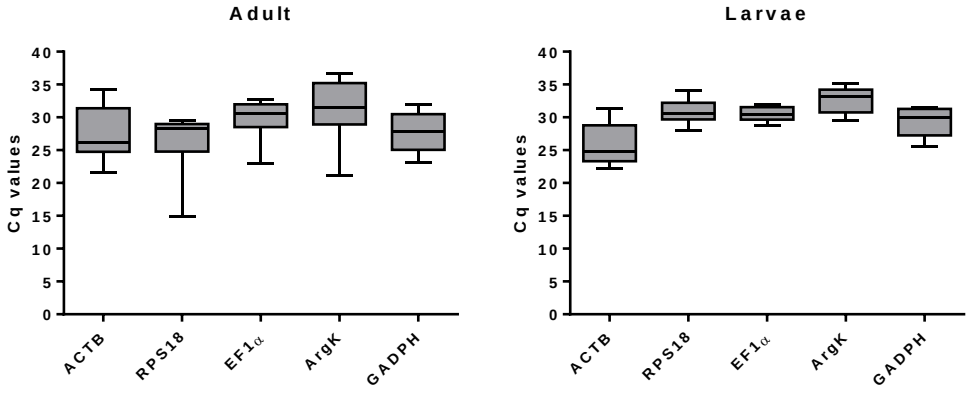

Figure 1. Expression profiles of the five candidate reference genes in adult and larvae of *Tenebrio molitor*. The dot indicates the maximum or minimum value of replicated samples, while whiskers indicate the standard error of the mean.

Expression stability of selected reference genes

Four different programs were used for the analysis of reference gene expression (geNorm, NormFinder, BestKeeper, and Δ-Ct method) to estimate the stability and ranking of five candidate reference housekeeping genes under two different experimental conditions. Additionally, five reference genes were compared and ranked by web-based analysis tool RefFinder. Figure 5 show the stability values of the remaining reference genes following stepwise exclusion analysis, which showed slightly different results in all algorithms.

Table 2. Expression stability of the candidate reference genes in case of life stages.

Biotic condition	Reference gene	Delta Ct		Bestkeeper			Genorm		Normfinder		RefFinder	
		StdDev	Rank	SD	R	Rank	M	Rank	SV	Rank	Stability	Rank
Larvae	*ACTB*	1.94	5	2.80	0.960*	5	1.528	4	1.751	5	5.00	5
	RPS18	1.38	2	1.48	0.939*	2	1.161	2	0.684	2	2.21	2
	EF1α	1.60	4	0.92	0.952*	1	1.254	3	1.264	4	2.83	4
	ArgK	1.41	3	1.80	0.912*	3	0.919	1	0.916	3	2.28	3
	GADPH	1.31	1	2.08	0.967*	4	0.919	1	0.539	1	1.41	1
Adult	*ACTB*	2.46	4	3.40	0.918*	4	1.494	1	1.825	4	2.83	4
	RPS18	3.24	5	4.32	0.940*	5	2.489	4	2.971	5	5.00	5
	EF1α	2.20	2	2.32	0.974*	1	1.914	2	1.003	2	1.86	2
	ArgK	2.18	1	3.20	0.978*	3	1.989	3	0.891	1	1.86	1
	GADPH	2.37	3	2.76	0.921*	2	1.494	1	1.724	3	2.06	3

StdDev, standard deviation; **SD**, standard deviation; **SV**, stability value; **r**, Pearson correlation coefficient; *****p≤0.001.

geNorm

The geNorm algorithm was used to calculate the stability of reference genes based on an "M" value. The lower the M value, the more stable the expression of the reference gene, and the M values exceeding the 1.5 shear value are not significantly stable between the samples.According to this program, *ArgK* and *GADPH* were the most stable genes with an *M* score of 0.919, and *ACTB* was the only gene slightly above the cut-off with an *M* score of 1.528 in the larval stage. On the other hand, while the most stable genes were *ACTB* and *GADPH* with an M score of 1.494, *RPS18* was the least stable gene with an *M* score of 2.489 in adults.

NormFinder

Candidate genes with the lowest stability values obtained with the NormFinder algorithm based on intra-group and inter-group expression variations are considered as the most stable reference genes. Results in larvae and adults, *GADPH* and *ArgK* were established as the most consistent gene (*SV*: 0.539; 0.891) and *ACTB* and *RPS18* were the least stable gene (*SV*: 1.751; 2.971), respectively.

BestKeeper

BestKeeper determines the most stably expressed genes based on the correlation coefficient with the Bestkeeper Index, the geometric mean of candidate reference gene Cq values. This algorithm calculates standard deviation (SD), lower values are considered more stable, and values exceeding the cut-off value (SD <1) are taken into account of unstable among all samples.This analysis indicates all housekeeping genes in both larvae and adults (except *EF1α* in larvae, *SD*, 0.92) exceeded the cut-off value.

ΔC_T method

This algorithm uses standard deviation means of Cq differences between each gene and the remainder of them in each situation. The standard deviation below 1 demonstrates the appropriate stability. None of current housekeeping genes were stable enough for comparison of larvae and adultsaccording to *ΔCT method***.** But**,** for all that, *GADPH* with a value of 1.31 was the closest to 1 and therefore the most stable gene in larvae.

RefFinder

RefFinder is an exhaustive algorithm that assembles all the software tools mentioned above to sort candidate reference genes according to their stability. The following rankings are listed in order of most-to-least stable reference genes. For the larval stage the comprehensive ranking was *GADPH, RPS18, ArgK, EF1α*and*ACTB*. The overall ranking for adult was *ArgK***,** *EF1α, GADPH, ACTB*and*RPS18* (Table 2).

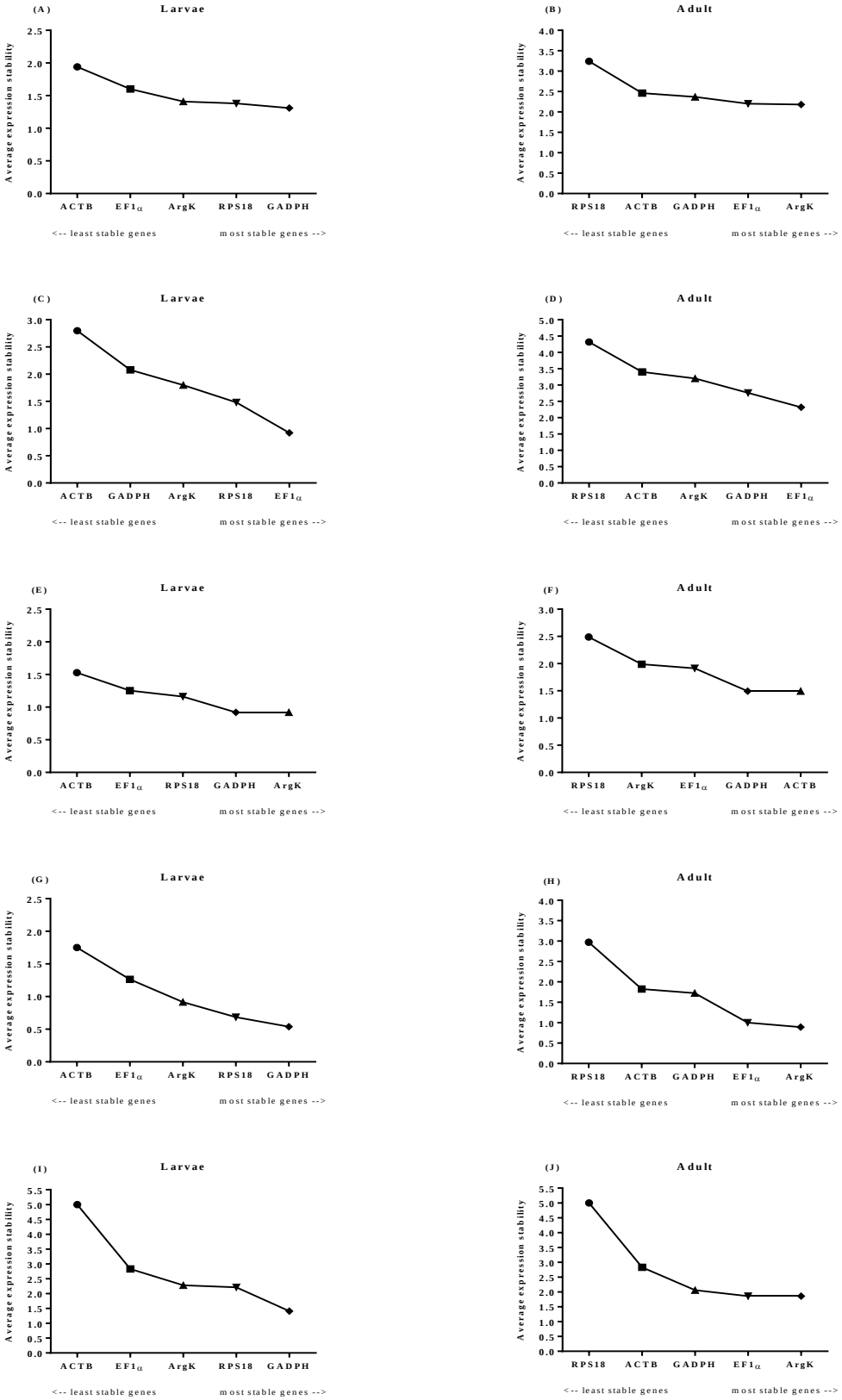

Figure 2. Gene expression stability and ranking of the five reference genes as calculated by ΔCt (A, B), Bestkeeper (C, D), Genorm (E, F), Normfinder (G, H), RefFinder (I, J). Mean expression stability was calculated following stepwise exclusion of the least stable gene across larvae and adult of *T. molitor*.

IV. Discussion

RT-PCR is controversial as the most properly used molecular method for the detection and quantification of nucleic acids. [34]At the same time, it is away from being the "gold standard" due to the lack of clearness, normalization, and technical/quality control.[4]Hellemans and Vandesompele[11] predicted that the average of the expression level differences of a gene interest after standardization with any of the two non-validated reference genes (indiscriminately selected) was between 3-6-fold between 10-25% of studies.This kind of discrepancy makes it impossible to infer a biologically appropriate conclusion. To arrest prejudices normalization, gradually scientists are beginning to admit the opinion of using multiple housekeeping genes to analyse the expression of gene.[9, 14, 15, 18, 42, 43, 45]However, each candidate gene should be assessed in terms of specific assay conditions for gene profiling to provide a constant level of expression. [34] Our results show that the most proper reference genes may be different in the larval and adult stages.

When we evaluated the results of five analyses (geNorm, BestKeeper, NormFinder, ΔCt method, and RefFinder) it produced different results in approximately all experiments. Therefore, our analysis did not produce a pervasive result for all algorithms and conditions. Experiments related to reference genes for RT-qPCR from other Coleoptera species in literature are consistent with our experiment results. [16, 18, 22, 24, 25, 26, 27, 31, 32, 33, 35, 37, 43, 44)

In summary, we tested five reference gene candidates and five statistical algorithms in two different experiments. The results obtained were used to produce the final ranking of all genes. RefFinder analysis tool showed that the most stable genes are listed as follows: *ACTB<EF1α<ArgK<RPS18<GADPH* for the larval stage; *RPS18<ACTB<GADPH<EF1α <ArgK* for adult stage.

V. Conclusions

Consequently, our study provides a comprehensive assessment of the reference genes convenient for RT-qPCR in larvae and adult stages of *T. molitor*. *GADPH* has been found reliable for the larval stage; *ArgK* was optimal for the adult stage (Table 2 and Figure 2). This study is usefulness for forthcoming studies on target gene expression in *T. molitor*.

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References

[1]. Andersen, C.L., Jensen, J.L., Orntoft, T.F. (2004). Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer research* 64:5245-5250.

[2]. Bustin, S.A., Benes, V, Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T. (2009). The MIQE guidelines: minimum information for publication of quantitative real- time PCR experiments. Clin Chem.;55(4):611- 622.

[3]. Bustin, S.A., Beaulieu, J., Huggett, J.,Jaggi, R.,Kibenge, F.S.B., Olsvik, A.P., Penning L.C., Toegel, S. (2010).MIQE précis: Practical implementation of minimum standard guidelines for fluorescence-based quantitative real-time PCR experiments. BMC Molecular Biol 11, 74.

[4]. Bustin, S. A. et al. (2013). The need for transparency and good practices in the qPCR literature. *Nat. Methods* 10, 1063–1067.

[5]. Castilla, A.M., Dauwe, T., Mora, I., Malone, J., Guitart, R. (2010). Nitrates and herbicides cause higher mortality than the traditional organic fertilizers on the Grain Beetle, *Tenebrio molitor*. *B Environ ContamTox* 84:101–105.

[6]. Costantino, R.F., Desharnais, R. A., Cushing, J. M., Dennis, B. (1997). Chaotic dynamics in an insect population. *Science* 275:389–391.

[7]. Cotton, R. T. (1956). Pests of Stored Grain and Grain Products Burgess Publishing Minneapolis, MN.

[8]. Frankel, G. (1951). Effect and distribution of vitamin B_T. *Arch BiochemBiophys* 34:457–467.

[9]. Fu, W. Xie, W., Zhang, Z., Wang, S., Wu, Q., Liu, Y., Zhou, X., Zhou, X., Zhang, Y. (2014). Exploring valid reference genes for quantitative real-time PCR analysis in Plutellaxylostella (Lepidoptera: Plutellidae). *Int. J. Biol. Sci*. 9: 792–802.

[10]. García-Reina, A., Rodríguez-García, M.J., Galián, J. (2018). Validation of reference genes for quantitative real-time PCR in tiger beetles across sexes, body parts, sexual maturity and immune challenge *Sci. Rep*. 8: 10743.

[11]. Hellemans, J., Vandesompele, J. (2014).Selection of reliable reference genes for RT-qPCR analysis. In Quantitative Real-Time PCR. *Springer New York*. 19–26.

[12]. Huggett, J., Dheda, K., Bustin, S., Zumla, A. (2005). Real-time RT-PCR normalisation; strategies and considerations. Genes Immun. 6: 279–284.

[13]. Kalushkov, P., Hodek, I. (2004). The effects of thirteen species of aphids on some life history parameters of the ladybird Coccinellaseptempunctata. *Biol. Control* 49, 21–32.

[14]. Li, R.,Xie, W., Wang, S., Wu, Q., Yang, N., Yang, X.,Pan, H., Zhou, X., Bai, L., Xu, B., Zhou, X., Zhang Y.(2013). Reference gene selection for qRT-PCR analysis in the sweetpotato whitefly, Bemisiatabaci (Hemiptera: Aleyrodidae). *PLOS ONE* 8: e53006.

[15]. Liang, P., Guo, Y. J., Zhou, X. G.,Gao, X. W. (2014). Expression profiling in Bemisiatabaci under insecticide treatment: indicating the necessity for custom reference gene selection. *PLOS ONE* 9: e87514.

[16]. Lord, J. C., Hartzer, K., Toutges, M., and Oppert, B. (2010). Evaluation of quantitative PCR reference genes for gene expression studies in Triboliumcastaneum after fungal challenge. *J. Microbiol. Meth*. 80: 219–221.

[17]. Lü, J., Yang, C., Zhang, Y., Pan, H. (2018). Selection of Reference Genes for the Normalization of RT-qPCR Data in Gene Expression Studies in Insects: A Systematic Review. Front. Physiol. 9:1560.

[18]. Pan, H. P., Yang, X. W., Siegfried, B. D., Zhou, X. (2015). A comprehensive selection of reference genes for RT-qPCR analysis in a predatory lady beetle, *Hippodamiaconvergens* (Coleoptera: Coccinellidae). *PLOS ONE* 10:e0125868.

[19]. Pedersen, S. A., Kristiansen, E., Hansen, B. H., Andersen, R. A., Zachariassen, K.E. (2006). Cold hardiness in relation to trace metal stress in the freeze-avoiding beetle *Tenebrio molitor*. *J Insect Physiol* 52:846–853.

[20]. Pedersen, S.A., Kristiansen, E., Andersen, R.A., Zachariassen, K. E. (2008). Cadmium is deposited in the gut content of larvae of the beetle *Tenebrio molitor* and involves a Cd-binding protein of the low cysteine type. *CompBiochemPhysiol Part C* 148:217–222.

[21]. Pfaffl, M.W., Tichopad, A., Prgomet, C., Neuvians, T.P. (2004). Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations. *Biotechnology letters* 26:509-515.

[22]. Rajarapu, S. P., Mamidala, P., Mittapalli, O. (2012). Validation of reference genes for gene expression studies in the emerald ash borer (Agrilusplanipennis). *Insect Sci.* 19: 41–46.

[23]. Rees, D. (2004). Insects of Stored Products Manson Publishing London 112–113.

[24]. Rodrigues, T. B., Dhandapani, R. K., Duan, J. J., Palli, S. R. (2017). RNA interference in the Asian longhorned beetle: identification of key RNAi genes and reference genes for RT-qPCR. *Sci. Rep.* 7:8913.

[25]. Rodrigues, T. B., Khajuria, C., Wang, H. C., Matz, N., Cardoso, D. C., Valicente, F. H., et al. (2013). Validation of reference housekeeping genes for gene expression studies in western corn rootworm (Diabroticavirgiferavirgifera). *PLOS ONE* 9:e109825.

[26]. Sang, W., He, L., Wang, X.P., Zhu-Salzman, K., Lei, C.L.(2015). Evaluation of reference genes for RT-qPCR in *Triboliumcastaneum* (Coleoptera: Tenebrionidae) under UVB stress. *Environ. Entomol*. 44:418–425.

[27]. Shi, X. Q.,Guo, W. C., Wan, P. J., Zhou, L. T., Ren, X. L., Ahmat, T., Fu,K.F.,Li, G.Q. (2013). Validation of referencegenesforexpressionanalysisbyquantitativereal-time PCR in Leptinotarsadecemlineata (say). *BMC Res. Notes*6:93.

[28]. Silver, N., Best, S., Jiang, J., Thein, S.L. (2006). Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. *BMC molecular biology* 7:33.

[29]. Sinha, D. K., Smith, C. M. (2014). Selection of reference genes for expression analysis in Diuraphisnoxia (Hemiptera: Aphididae) fed on resistant and susceptible wheat plants. *Sci. Rep*. 4:5059.

[30]. Strube C., Buschbaum S., Wolken S., Schnieder T. (2008). Evaluation of reference genes for quantitative real-time PCR to investigate protein disulfide isomerase transcription pattern in the bovine lungworm Dictyocaulusviviparus. *Gene* 425, 36–43.

[31]. Tan, Q. Q., Zhu, L., Li, Y., Liu, W., Ma, W. H., Lei, C. L., Wang, X.P. (2015). A de novo transcriptome and valid reference genes for quantitative real-time PCR in Colaphellusbowringi. *PLOS ONE* 10:e0118693.

[32]. Tan, Y., Zhou, X. R., Pang, B. P. (2017). Reference gene selection and evaluation for expression analysis using qRT-PCR in Galerucadaurica (Joannis). *B. Entomol. Res*. 107: 359–368.

[33]. Tang, P. A., Duan, J. Y., Wu, H. J., Ju, X. R., Yuan, M. L. (2017). Reference gene selection to determine differences in mitochondrial gene expressions in phosphine-susceptible and phosphine-resistant strains of Cryptolestesferrugineus, using qRT-PCR. *Sci. Rep*. 7:7047.

[34]. Thellin, O., Zorzi, W., Lakaye, B., Borman, B.D., Coumans, B., Hennen, G., Grisar, T., Igout, A., Heinen, E. (1999) Housekeeping genes as internal standards: use and limits. *J Biotechnol* 75:291–295.

[35]. Toutges, M. J., Hartzer, K., Lord, J., Oppert, B. (2010). Evaluation of reference genes for quantitative polymerase chain reaction across life cycle stages and tissue types of Triboliumcastaneum. *J. Agr. Food Chem.* 58:8948.

[36]. Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome biology* 3:RESEARCH0034.

[37]. Wang, Y., Wang, Z. K., Huang, Y., Liao, Y. F., Yin, Y. P. (2014). Identification of suitable reference genes for gene expression studies by qRTPCR in the blister beetle Mylabriscichorii. *J. Insect Sci.* 14:94.

[38]. Wang X. Y., Xiong M., Wang J. L., Lei C. L., Zhu F. (2015). Reference gene stability of a synanthropic fly, Chrysomyamegacephala. *Parasit. Vectors* 8:565.

[39]. Watt, J. C. (1974). A revised subfamily classification of Tenebrionidae (Coleoptera), *New Zealand Journal of Zoology*, 1:4, 381-452.

[40]. Xie, F., Xiao, P., Chen, D., Xu, L., Zhang, B. (2012). miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs. *Plant molecular biology* 80 (1), 75-84.

[41]. Yang, C. X., Pan H. P., Noland J. E., Zhang D. Y., Zhang Z. H., Liu Y., Xhou, X. (2015a). Selection of reference genes for RT-qPCR analysis in a predatory biological control agent, *Coleomegillamaculata* (Coleoptera: Coccinellidae). *Sci. Rep.* 5:18201.

[42]. Yang, C. X., Pan, H. P., Liu, Y., Zhou, X. G. (2014). Selection of reference genes for expression analysis using quantitative real-time PCR in the pea aphid, Acyrthosiphonpisum (Harris) (Hemiptera, Aphidiae). *PLOS ONE* 9: e110454.

[43]. Yang, C. X., Pan, H. P., Liu, Y., Zhou, X. G. (2015b). Stably expressed housekeeping genes across developmental stages in the two-spotted spider mite, Tetranychusurticae. *PLOS ONE* 10: e0120833.

[44]. Yang, C., Preisser, E. L., Zhang, H., Liu, Y., Dai, L., Pan, H., et al. (2016). Selection of reference genes for RT-qPCR analysis in Coccinellaseptempunctata to assess un-intended effects of RNAi transgenic plants. Front. *Plant Sci.* 7:e53006.

[45]. Zhu, X. Yuan, M., Shakeel, M., Zhang, Y., Wang, S., Wang, X., Zhan, S., Kang, T., Li, J.(2014). Selection and evaluation of reference genes for expression analysis using qRT-PCR in the beet armyworm Spodopteraexigua (Hübner) (Lepidoptera: Noctuidae). *PLOS ONE* 9: e84730.

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