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Research paper

Identification and evaluation of reference genes for expression studies by RT-qPCR during embryonic development of the emerging model organism, *Macrobrachium olfersii*

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ABSTRACT

RT-qPCR is a sensitive and highly efficient technique that is widely used in gene expression analysis and to provide insight into the molecular mechanisms underlying embryonic development. The freshwater prawn, *Macrobrachium olfersii* is an emerging model organism, but, the stable reference genes of this species need to be identified and validated for RT-qPCR analysis. Thus, the aim of this study was to evaluate the expression stability of six genes (*β-act*, *GAPDH*, *EF-1α*, *RpL8*, *RpS6*, *AK*) in embryos and in adult tissues (cerebral ganglia, muscle and hepatopancreas) of *M. olfersii*. The expression stabilities of these genes were evaluated using geNorm, NormFinder, BestKeeper, ΔCt method and integrated tool RefFinder. In the general ranking, *RpL8* and *RpS6* were the most stable genes in embryos, while *RpS6* and *RpL8* were the most stable in a combined adult tissue analysis. Analysis of the adult tissues revealed that *β-act* and *AK* were the most stable genes in cerebral ganglia, *RpL8* and *AK* in muscle, and *RpS6* and *β-act* in hepatopancreas. *EF-1α* and *GAPDH* were the least stable genes and as normalizer genes in RT-qPCR affected expression of the *Distal-less* gene during *M. olfersii* development. This study provides suitable reference genes for RT-qPCR analysis and allows future studies of the gene expression in *M. olfersii* for understanding the molecular mechanisms of their development. To our knowledge, this is the first published study that identifies and evaluates reference genes for RT-qPCR analysis in *M. olfersii* and could be useful as basis for evaluations of reference genes in other prawns.

1. Introduction

During animal development, specific genetic programs and molecular mechanisms initiate various genetic events, such as morphogenesis and organogenesis, coordinated by cellular processes such as proliferation, fate determination, differentiation, apoptosis, migration, adhesion, and cell shape changes (Basson, 2012). The molecular mechanisms involved in these processes can be elucidated by gene expression analysis. RT-qPCR provides a highly sensitive, precise and reproducible technique for analyzing gene expression (Bustin, 2002; Bustin and Nolan, 2004; Kubista et al., 2006). However, one of the requirements for the relative expression analysis of target genes by RT-qPCR is the use of appropriate reference genes (Radonić et al., 2004). Reference genes typically constitute genes necessary to maintain basic cellular function and are stably expressed in biological samples from a particular study (Bustin, 2002; Radonić et al., 2004). The purpose of normalization with a reference gene is to remove sampling noise (such as differences in RNA concentration or efficiency of reverse transcription) in order to accurately estimate gene expression (Vandesompele et al., 2002; Bustin et al., 2009; Robledo et al., 2014).

Validation of reference genes in crustaceans is limited. In *Penaeus stylirostris*, four reference genes (*β-act*, *EF-1α*, *18S rRNA* and *GAPDH*)

Abbreviations: RT-qPCR, real-time reverse transcription quantitative polymerase chain reaction; cDNA, complementary DNA; MIQE, Minimum Information for Publication of Quantitative Real-Time PCR Experiments; β-act, β-actin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RpL8, ribosomal protein L8; RpS6, ribosomal protein S6; EF-1α, elongation factor-1α; AK, arginine kinase; M, muscle; HPT, hepatopancreas; CG, cerebral ganglia; Cq, quantification cycle; Ct, threshold cycle; E, efficiency; NFs, normalization factors; V, pairwise variation; SD, standard deviation.

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were evaluated for gene expression analysis associated with the immune system (Dhar et al., 2009). In *Penaeus monodon*, these same reference genes were validated for expression analysis in reproductive tissues (Leelatanawit et al., 2012). In *Rimicaris exoculata*, four reference genes (*β-act*, *GAPDH*, *18S rRNA* and *RpL8*) were evaluated to analyze the gene expression after exposure of the organism to heat stress (Cottin et al., 2010). In the *Macrobrachium* genus, five reference genes were evaluated in *Macrobrachium rosenbergii* (*GAPDH*, *RpL8*, *RpL18*, *β-act* and *EF-lα*) for gene expression in larvae, post-larvae and gills under saline stress conditions (Barman et al., 2012). Also, in *M. rosenbergii*, the expression stability of *EF-1α*, *GAPDH*, *β-act* and *18S rRNA* genes was evaluated for gene expression analysis in tissues (androgenic gland, gill, eyestalk, nerve cord and testis) of male morphotypes (Priyadarshi et al., 2015). Conversely, the reference gene validation studies for RT-qPCR analysis of the embryonic stages in *Macrobrachium* genus had not been conducted.

Within the decapods, the freshwater prawn, *Macrobrachium olfersii*, has wide distribution in the Americas (Holthuis, 1952) and is emerging as a potential model for development, developmental toxicity and environmental toxicology studies (Müller et al., 2003; Simões-Costa et al., 2005; Ribeiro and Mcnamara, 2009; Nazari et al., 2010; Barbieri et al., 2013; Rossi and Mantelatto, 2013; Zeni et al., 2015). The embryonic development of this species has been well-characterized both morphologically and chronologically (Mossolin and Bueno, 2002; Müller et al., 2003; Nazari et al., 2003; Simões-Costa et al., 2005). However, the molecular mechanisms of this species, including its development, particularly in the embryonic stages, remained unknown.

Previously, a transcriptome analysis of *M. olfersii* embryos (Jaramillo et al., 2016) provided candidate reference genes related to various metabolic processes. However, the expression stability of the reference genes in adult tissues and embryos of *M. olfersii* was not investigated. Six reference genes (*GAPDH*, *β-act*, *EF-1α*, *RpL8*, *RpS6* and *AK*) have been typically used for qPCR analysis, which may vary depending on the conditions. Therefore, the aim of this study was to determine the expression stability of these common reference genes, in embryos and adult tissues of *M. olfersii,* using RT-qPCR, tools of computational analysis (geNorm, NormFinder, BestKeeper and ΔCt method) and also the web-based comprehensive tool, RefFinder. Additionally, the reference genes were validated by analyzing the expression of the *Distal-less* (*Dll*) gene involved in appendage development, using either the candidate reference genes or their combinations, as normalizers. This study will allow the reliable and accurate quantification of the gene expression of *M. olfersii* by RT-qPCR and the use of this technique will provide insight into the molecular mechanisms involved in its embryonic development.

2. Materials and methods

2.1. Animals, embryos and tissue collection

Adults of *M. olfersii* were collected from Santa Catarina Island (Southern Brazil) (27°35′S, 48°35′W) and transported to the laboratory. The prawns were maintained in an aquarium (60 L) at 24 °C (±1), with constant aeration, and fed daily with balanced feed for aquatic organisms (Alcon Bottom Fish), at a density of 2 males: 6 females to facilitate breeding and obtain embryos. Eight embryonic stages were selected, from E3 (gastrulation) up to E10 (late morphogenesis/organogenesis), according to Simões-Costa et al. (2005). Also, cerebral ganglia, muscle and hepatopancreas tissues were dissected from adults and all tissues were frozen quickly in liquid nitrogen. Embryos and adult tissues were homogenized in Brazol (LGC Biotecnologia) for RNA extraction. The procedures for collecting and maintaining *M. olfersii* were approved by the National Environmental Agency (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis, number 15294-1/IBAMA/2008).

2.2. RNA extraction and cDNA synthesis

Extraction of RNA and qPCR experiments were performed according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guideline (Bustin et al., 2009) in order to ensure reliable results. Total RNA extraction of the samples (10–100 mg) was performed using Brazol, according to the manufacturer's instructions. To remove DNA, the total RNA of the samples was treated with DNAse I (1 U/μL, Thermo Scientific) at 37 °C for 30 min and at 65 °C for 10 min, according to the manufacturer's instructions. PCR of the *β-act* gene was performed using RNA samples to ensure no DNA contamination. Then, the samples (100 μL) were precipitated using 10 μL of sodium acetate (3 M, pH 5.2) with 110 μL of isopropanol, and centrifuged at 12,000 ×*g*, 4 °C, for 10 min. The pellets were washed with 75% ethanol, dried, and solubilized in diethylpyrocarbonate-treated water. The purity of the RNA was determined using a spectrophotometer (BIO-5000-BI, Kazuaki) and samples with absorbance ratios, 260/280 and 260/230, greater than 1.8 were selected for cDNA synthesis. RNA integrity was verified by electrophoresis on 1.5% agarose gel stained with GelRed (Biotium). For cDNA synthesis, 1 μg of total RNA from each sample (embryos and adult tissues) and GoScript™ Reverse Transcriptase (Promega) kit were used. The reverse transcription reaction consisted of 4 μL of GoScript™ 5× Reaction Buffer, GoScript™ Reverse Transcriptase (160 U), 0.5 mM of each dNTP, 0.5 μg of primer oligo $(dT)_{15}$, 2.5 mM $MgCl_2$, and ribonuclease inhibitor (20 U). The reactions were conducted at 25 °C for 5 min, 42 °C for 60 min, and 70 °C for 15 min.

2.3. DNA extraction and determination of exon–exon junctions

DNA was extracted from samples of *M. olfersii* embryos (E4) using Brazol, according to the manufacturers' instructions. To determine the location of the primers (in the same exon or in different exons of the same gene), PCR was performed on extracted DNA using the GoTaq® Green Master Mix kit (Promega). For each gene, PCR products with the same size, from both DNA and cDNA samples, indicate that the primers were designed in regions of the same exon. A PCR product of different size from DNA sample indicates that the primers were designed in different exons (exon–exon junctions). PCR was conducted at 94 °C for 5 min, followed by 35 cycles (94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min), and a final extension of 72 °C for 10 min. PCR products were loaded onto a 1.5% agarose gel for electrophoresis. After electrophoresis, the gel was stained with GelRed and photographed using the ChemiDoc MP system.

2.4. Selection of reference genes and primer design

Reference genes were identified via tBlastn against the sequences of the *M. olfersii* embryonic transcriptome (Jaramillo et al., 2016). Six candidate reference genes: β-actin (*β-act*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), elongation factor 1α (*EF-1α*), ribosomal protein L8 (*RpL8*), ribosomal protein S6 (*RpS6*) and arginine kinase (*AK*) were selected for evaluation. In addition, the *Dll* gene was chosen to validate the reference genes during embryonic development. Sequences of genes were deposited in the NCBI database under GenBank accession numbers indicated in Table 1. The primers for the genes were designed using either the program Oligo Explorer v1.5 (Gene Link) or Primer 3 Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The primers were designed considering the following parameters: a) primer size: 18–22 bp; b) product size: 50–200 bp; and c) GC% content of 40–60%. Primer–dimer and hairpin structures were analyzed using Oligo Analyzer v1.5 (Gene Link) and PRaTo web-tool (Nonis et al., 2011).

Table 1
Candidate reference genes and primer sequences designed for RT-qPCR amplification used in this study.

Gene symbol	Gene name	Gene function	Primers forward/reverse	Length (bp)	GenBank accession no.
β-act	β-actin	Cytoskeletal structural protein	5′-CACTTCCTCATGCCATCCTC-3′ 5′-GATGTCACGCACGATTTCTC-3′	128	KY027067
EF-1α	Elongation factor-1α	Protein biosynthesis	5′-TACTCCCCTGTGCTTGACTG-3′ 5′-TACTGCTGGAAGGTCTCAACG-3′	185	KY027069
RpS6	Ribosomal protein S6	Component of the 40S ribosomal subunit	5′-CTGTCTGTACTTGCCCTGATG-3′ 5′-GCGTGGAATGCTGTTGTCTG-3′	78	KY027064
RpL8	Ribosomal protein L8	Component of the 60S ribosomal subunit	5′-GTATTGTTGCTGGTGGAGGTC-3′ 5′-CTCTACAGGGTTCATCGCTAC-3′	125	KY027065
AK	Arginine kinase	Maintenance of ATP levels by the phosphorylation of the so called "phosphagens"	5′-TGGATTCTGGAGTTGGCATC-3′ 5′-CCTTAGGAGGGTGTTTGTCTTC-3′	126	KY027068
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase	Oxidoreductase in glycolysis and gluconeogenesis	5′-GTTGCCGTAAATGACCCCTTC-3′ 5′-CCTTCACCTCACCCTTGTAGAC-3′	94	KY027066
Dll	*Distal-less*	Development of appendage in arthropods	5′-CCGCACTATCTACTCTTCTC-3′ 5′-CCAGGCAGTTGTCCGTTC-3′	199	KY027070

2.5. Quantitative real-time PCR (qPCR)

qPCR reactions were performed using the GoTaq® qPCR Master Mix (Promega) and the equipment 7900HT Fast Real-Time PCR System (Applied Biosystems). Briefly, qPCR reactions consisted of 5 μL GoTaq® Master Mix 2×, 500 nM of each primer, 0.1 μL CXR Reference Dye, 1 μL of diluted cDNA (1/10), and nuclease-free water in a total volume of 10 μL. All cDNA samples ($n = 3$ whole egg mass of ovigerous females/embryonic stage or $n = 3$ adults/each tissue) were run in duplicate for each gene. The qPCR cycling conditions were as follows: denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, primer annealing and extension at 60 °C for 1 min. At the end of the cycles, the samples were subjected to a dissociation curve (95 °C for 15 s, 60 °C for 15 s, 95 °C for 15 s). The Cq value (i.e. number of cycles required to reach the quantification threshold) was determined from the same threshold fluorescence value for the six analyzed genes. The threshold was determined based on the individual backgrounds of the six genes and the highest value was then used for all six genes. Non-template controls for each gene were included on each PCR plate to confirm the absence of contamination.

2.6. Amplification efficiency and primers specificity of reference genes

The amplification efficiency for each candidate reference gene was tested by RT-qPCR using a series of dilutions (1/4, 1/8, 1/16, 1/32, and 1/64) from the sample cDNA pools. The efficiency was based on the slope of a linear regression model and was calculated from the slope of a standard curve, using the equation $E = 10^{-1/slope}$ (Rasmussen, 2001; Pfaffl, 2001, 2004). The efficiency (E) and correlation coefficient (R^2) were calculated for each reference gene. A range of 1.9–2.1 of the amplification efficiency and R^2 value of 0.99 was acceptable. The melting curve was used to evaluate the specificity of the primers. In addition, the RT-qPCR products were observed using electrophoresis on a 1.5% agarose gel and visualized with GelRed.

2.7. Determination of the stability of reference genes

The reference gene stability was determined using the comparative ΔCt method (Silver et al., 2006), geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004), BestKeeper (Pfaffl et al., 2004), and RefFinder (Xie et al., 2012). The latter web-based comprehensive tool integrates the other programs. In general, all computer programs use different algorithms based on the expression of reference genes that should be stable in the various embryonic developmental stages or adult tissues analyzed. The Cq values of reference genes obtained in the RT-qPCR experiments were used in the computer programs to determine their stability.

2.8. Impact of the normalization with reference genes

To determine the effect of the utilization of different reference genes as normalizers on the expression data of a gene of interest, was evaluated the *Dll* gene transcript levels during embryonic stages of *M. olfersii*. This gene was identified from sequences of the transcriptome of embryos *M. olfersii* (Jaramillo et al., 2016) and is important in development biology and evolution by being involved in appendage development in arthropods (Chen et al., 2016). The *Dll* gene expression level was normalized using the six candidate reference genes or their combinations. The expression level was determined using cDNA samples ($n = 3$ whole egg mass of ovigerous females/embryonic stage) and RT-qPCR. Specific primers were designed for gene *Dll*. The amplification efficiency of *Dll* gene was tested by RT-qPCR. RT-qPCR was performed using 0.3 μM primers, and annealing and extension at 62 °C for 1 min. The relative expression levels of *Dll* gene during embryonic stages were calculated by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.9. Statistical analysis

Data normality was verified using the Kolmogorov-Smirnov test in GraphPad Prism v5. The gene expression data was statistically analyzed by one-way ANOVA, using Statistica 10.0 for Windows. Pairwise comparison of means was performed with the Tukey test and a p-value < 0.05 was considered statistically significant.

3. Results

3.1. Determination of exon–exon junction, primer specificity and amplification efficiency

The expression stability of six candidate reference genes during embryonic stages and in adult tissues of *M. olfersii* was analyzed using primers designed for common reference genes used in the literature as normalizers in RT-qPCR analysis (Table 1). Ideally, primers are designed to span an exon-exon junction. However, in this study, due to the lack of sequenced *M. olfersii* genome, this was not considered feasible. Using DNA from *M. olfersii* embryos as a PCR template, it was determined that primers for each gene amplified within the same exon regions, with the exception of the *RpS6* gene primers that amplified in a different exon (Fig. 1a). Thus, eliminating DNA contamination in the total RNA of the samples, prior to cDNA synthesis, was important and necessary.

For each reference gene, the specificity of the primers was demonstrated by a single peak in the melting curve analysis (D'haene et al., 2010) and a single PCR product in gel electrophoresis (1.5% agarose gel) (Fig. 1b–d). The amplification efficiencies for the six reference genes ranged from 1.96 to 1.99 (embryonic stages) and from 1.93 to

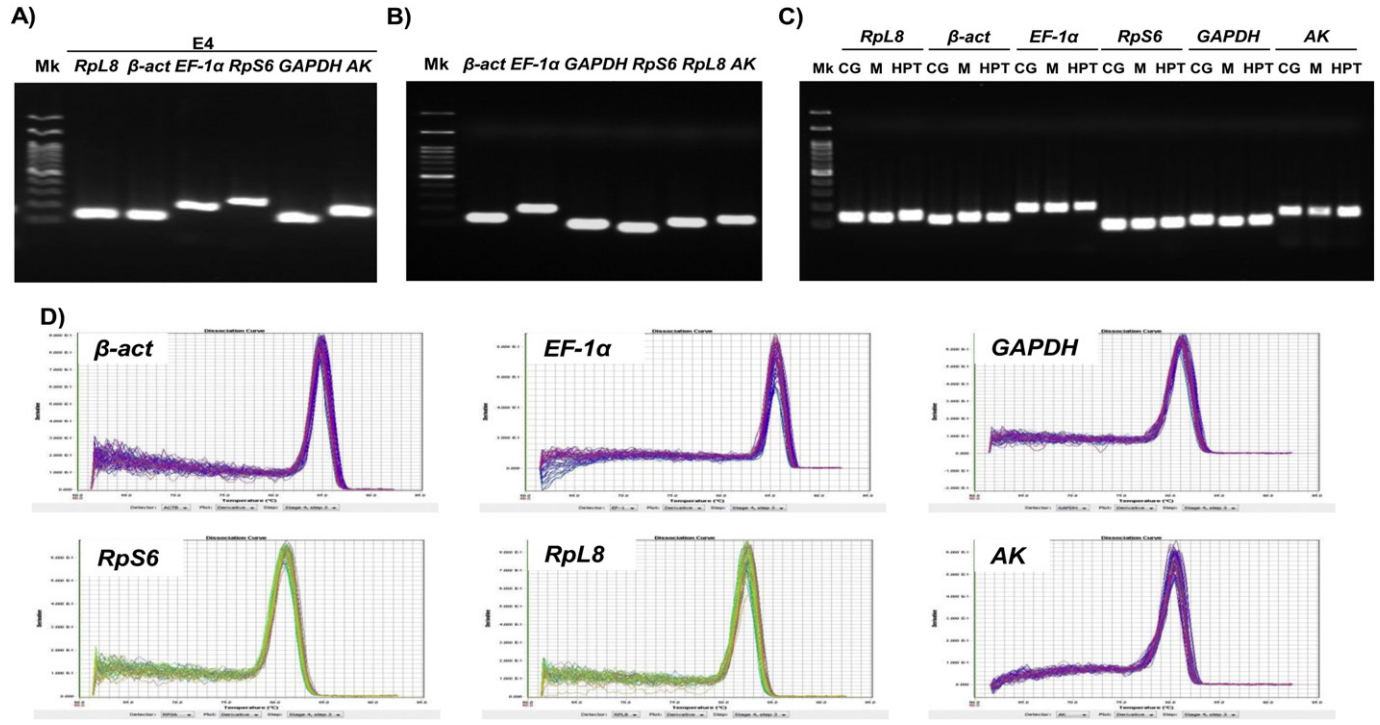

Fig. 1. Electrophoresis on 1.5% agarose gel showing amplification of reference genes. A) PCR from DNA sample of *M. olfersii* embryos at E4 stage. B) to D) Specificity of RT-qPCR amplicons showing amplification of a single product having the expected size and no dimer formation for each reference gene. B) In developmental stages. C) In adult tissues. D) Dissociation curves with single peaks generated from all amplicons. CG: cerebral ganglia; M: muscle; HPT: hepatopancreas, Mk: 100 bp DNA ladder.

1.97 (adult tissues). The standard curve for each gene from the cDNA dilutions displayed $R^2 > 0.99$ (Table 2).

3.2. Expression levels of reference genes in embryonic stages and tissues of *M. olfersii*

To gain an overview of the stability of the six reference genes involved in embryonic stages and in the adult tissues, the expression levels of the reference genes were evaluated by RT-qPCR using the Cq value of the samples. The descriptive statistics and the Kolmogorov–Smirnov test were used to evaluate the normality of the Cq values for each gene involved in embryonic stages and adult tissues of *M. olfersii* (Table 3). Considering the same threshold value for all the genes, the Cq values ranged from 15.26 (*β-act*) to 32.22 (*EF-1α*) for the embryonic stage samples (Table 3 and Fig. 2a) and 16.35 (*EF-1α*) to 29.79 (*EF-1α*) for the adult tissues (Table 3 and Fig. 2b).

In embryonic development stages (Fig. 2a), the expression of *RpL8*, *β-act* and *RpS6* genes were more abundant (lower median Cq values) than the *AK*, *GAPDH* and *EF-1α* genes (high median Cq values). In adult tissues (Fig. 2b), the expression of four genes (*β-act*, *RpL8*, *RpS6* and *GAPDH*) were more abundant than *AK* gene expression. Among all the genes, a greater variation of *EF-1α* gene expression and a smaller variation of *RpL8* and *RpS6* gene expressions were found in both groups (embryonic stages and adult tissues). The wide variation in expression of the six tested reference genes indicated that these genes did not have a constant expression level in the different samples of *M. olfersii*.

The inter- and intra-group variation of the gene expression levels in the eight developmental stages (E1–E8) of *M. olfersii* was analyzed to determine which variation had a greater influence on the Cq value during embryonic stages. The results demonstrated that the inter-group variation of Cq was comparable for the *RpL8*, *RpS6*, *AK*, and *β-act* genes. However, a high inter-group variation was found for the *EF-1α* and *GAPDH* genes. The highest Cq values (low expression levels) of the *EF-1α* gene were found in the initial stages (E3–E5) of development. Similarly, low expression levels were found for the *GAPDH* gene in the E3 stage (Fig. 3). Furthermore, when the Cq values of the genes were analyzed for each adult tissue, gene expression levels were found to be

Table 2
Reference genes and their parameters derived from RT-qPCR analysis to determine the amplification efficiency.

Gene	Developmental stages			Adult tissues		
	Slope	E	R^2	Slope	E	R^2
β-act	3.3908	1.9724	0.9977	3.3772	1.9774	0.9923
EF-1α	3.3755	1.9781	0.9985	3.4053	1.9663	0.9979
RpS6	3.3654	1.9822	0.9990	3.4456	1.9509	0.9996
RpL8	3.4176	1.9616	0.9986	3.4542	1.9476	0.9953
AK	3.3295	1.9968	0.9963	3.4880	1.9348	0.9997
GAPDH	3.3402	1.9924	0.9937	3.3425	1.9915	0.9958

E, efficiency; R^2, correlation coefficient.

Table 3
Descriptive statistics of the reference genes Cq values in the embryonic developmental stages and adult tissues of *M. olfersii*.

Developmental stages						
Gene	N	Mean	SD	Min Cq	Max Cq	KS-test p
β-act	24	16.44	0.56	15.26	17.50	0.076
EF-1α	24	23.44	4.35	17.70	32.22	0.167
RpS6	24	17.79	0.47	16.64	18.64	0.129
RpL8	24	16.36	0.25	15.91	16.77	0.142
AK	24	24.71	0.56	23.63	25.79	0.122
GAPDH	24	19.75	1.14	17.87	22.87	0.099
Adult tissues						
Gene	N	Mean	SD	Min Cq	Max Cq	KS-test p
β-act	9	21.05	2.35	17.99	23.65	0.191
EF-1α	9	22.35	5.45	16.35	29.79	0.205
RpS6	9	21.51	0.63	20.75	22.63	0.174
RpL8	9	20.44	0.69	19.76	21.93	0.143
AK	9	24.56	2.16	23.68	28.43	0.266
GAPDH	9	20.90	2.42	16.58	23.85	0.180

N, number of samples; SD, standard deviation; Min Cq, minimum Cq value; Max Cq, maximum Cq value; KS-test p, *p*-value of the Kolmogorov-Smirnov test.

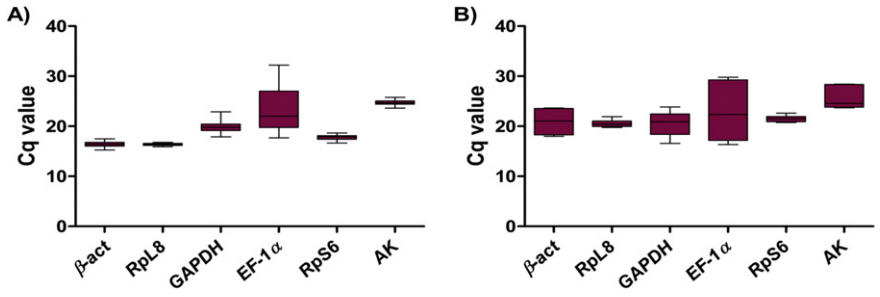

Fig. 2. Reference gene Cq value distributions. Boxplots of the Cq values in six different developmental stages (A) and three different adult tissues (B) for each of the six reference genes.

more similar for samples within the same tissue than between tissues (Fig. 3). The Cq values of the *EF-1α* gene were between 21.9 and 22.7 in the cerebral ganglia, 16.3–17.3 in muscle and 29.1–29.8 in the hepatopancreas. Similarly, the Cq values of *β-act*, *GAPDH* and *AK* genes were most similar in the same tissue. In general, the expression of reference genes analyzed in the hepatopancreas was lower than in the cerebral ganglia and muscle.

3.3. Expression stability of the candidate reference genes

The comparative ΔCt method was performed to estimate the variation in reference gene expression. According to the ΔCt method, the most stable genes were *AK* and *RpL8*, and the least stable genes were *GAPDH* and *EF-1α* (for embryonic stages). In adult tissues, *RpL8* and *RpS6* were the most stable genes, whereas the *AK* and *EF-1α* genes were least stable (Fig. 4a and Table 4).

Among the six genes, NormFinder analysis (Table 4) identified *AK* and *RpS6* as the most stable genes, while *GAPDH* and *EF-1α* were the least stable genes (for embryonic stages). In adult tissues, *RpS6* and *RpL8* were the most stable genes, whereas *AK* and *EF-1α* were the least stable genes. In embryonic stages, BestKeeper determined that the two most stable genes were *RpL8* and *RpS6* (Table 4). In adult tissues, the two genes most stable were *RpS6* and *RpL8*. The genes *GAPDH* and *EF-1α* were least stable in both embryonic stages and adult tissues (Table 4). According to the geNorm analysis, *RpL8* and *RpS6* were the most stable genes during embryonic stages, while the *GAPDH* and *EF-1α* genes were least stable (Fig. 4b and Table 4). In adult tissues, the *RpS6* and *RpL8* genes were the most stable, whereas the *AK* and *EF-1α* genes were least stable (Fig. 4b and Table 4).

Finally, the RefFinder analysis ranked the gene stability in embryonic stages as *RpL8* > *AK* > *RpS6* > *β-act* > *GAPDH* > *EF-1α*. In the adult tissues, gene stability decreased in the following order: *RpL8* > *RpS6* > *GAPDH* > *β-act* > *AK* > *EF-1α*. Based on the geometric mean ranking of each program, we devised a general gene stability ranking. In embryonic stages, the most stable reference genes were *RpL8* > *RpS6* and the least stable reference genes were *GAPDH* > *EF-1α* (Table 4). In the adult tissues, the most stable reference genes were *RpS6* > *RpL8* and the least stable reference genes were *AK* > *EF-1α*.

We also evaluated the reference gene stability on each specific tissue and based on the grouping of two different adult tissues using the RefFinder program. According to the comprehensive ranking of RefFinder, the *RpS6* (1.19) and *RpL8* (1.41) genes were most stable in cerebral ganglia and muscle. In cerebral ganglia and hepatopancreas, *RpS6* (1.32) and *AK* (2.21) were the most stable genes. In muscle and hepatopancreas, the *RpS6* (1.19) and *RpL8* (1.41) genes were the most stable. In cerebral ganglia, the genes *AK* (1.57) and *β-act* (1.68) were the most stable. In hepatopancreas, *RpS6* (1.73) and *β-act* (2.23) were the most stable genes. In muscle, *AK* (1.32) and *RpL8* (1.57) were the most stable genes. *GAPDH* and *EF-1α* were always ranked as the three least stable reference genes (Fig. 5).

3.4. Optimal number of reference genes for normalization

The greater the number of reference genes used for normalization gives the greater confidence in their gene expression level. Using the geNorm program, the optimum number of reference genes required for accurate normalization was calculated from the pairwise variation value (Vn/Vn + 1) to determine whether the addition of another reference gene (n + 1) was recommended (Vandesompele et al., 2002). In embryonic stages (Fig. 4b), three genes (*RpL8*, *RpS6*, *β-act*) were sufficient (V3/4) to normalize expression levels based on the recommended cut-off threshold of Vn/Vn + 1 = 0.15 (Vandesompele et al., 2002). Similarly, using the top four most stable ranked genes, NormFinder determined the *RpL8* and *RpS6* genes were the most suitable combination to normalize expression levels in embryonic stages. In the combined adult tissue analysis, *β-act* and *RpS6* were the most suitable combination according to NormFinder but were an unsuitable combination according to geNorm (value of M > 0.15) (Fig. 4b).

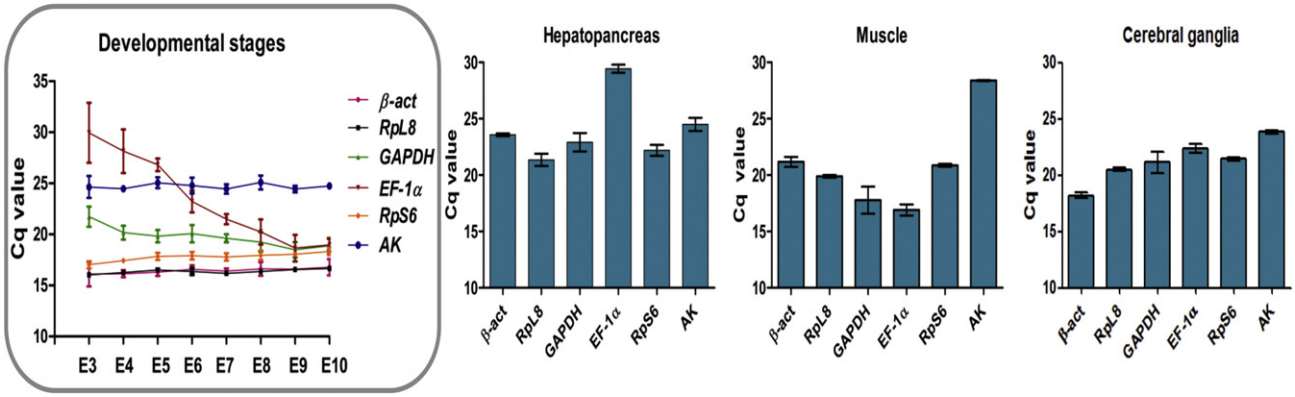

Fig. 3. Variation in the reference genes expression using the Cq values of each embryonic developmental stage and in each adult tissue of *M. olfersii* analyzed.

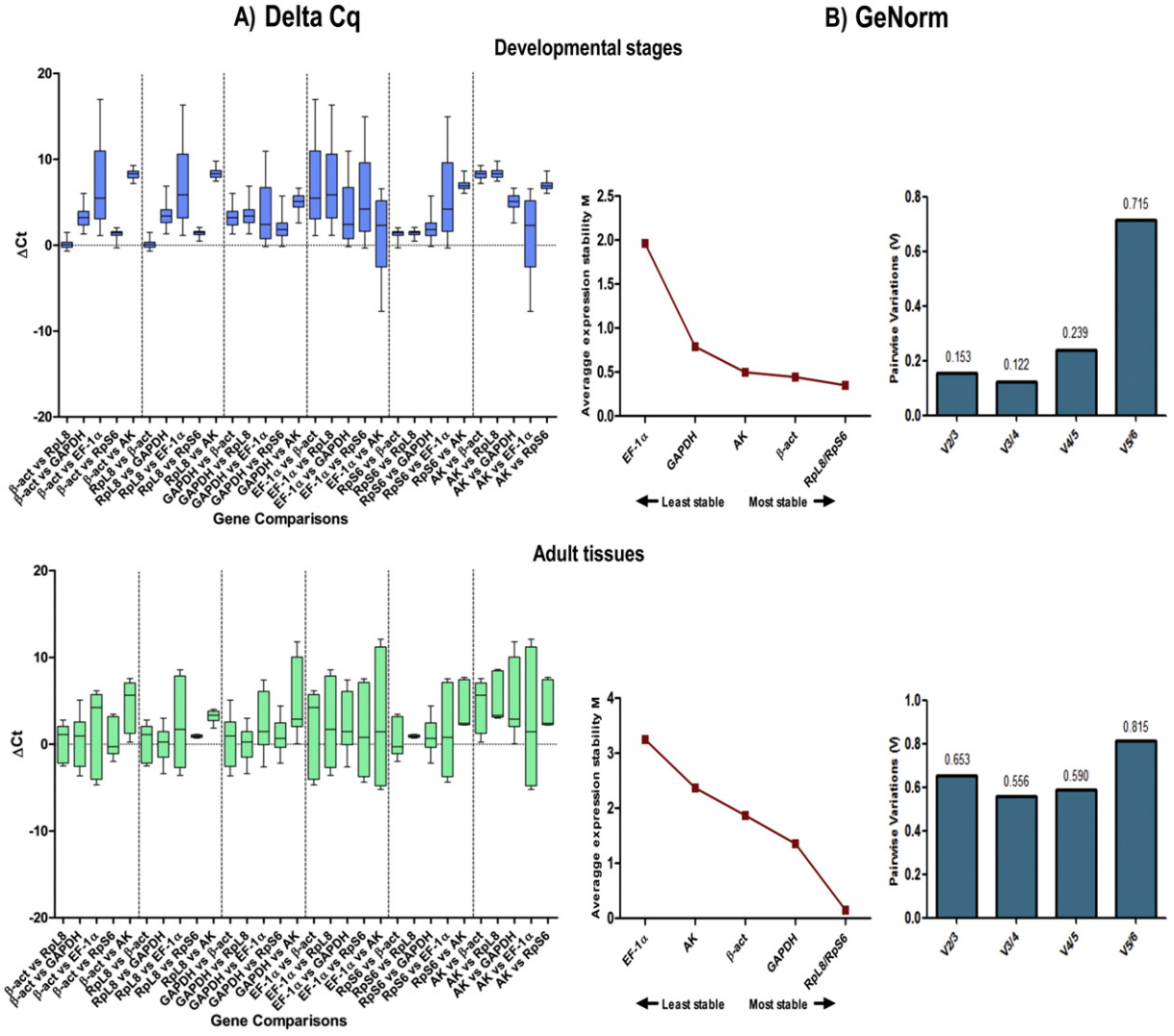

Fig. 4. ΔCt method and geNorm analysis of the reference gene selection. A) ΔCt variability in candidate reference genes is shown as medians (lines), 25th percentile to the 75th percentile (boxes) and ranges (whiskers) for samples of *M. olfersii*. Comparison of six reference genes in 24 developmental samples and nine adult tissue samples. B) Average expression stability value (M) is the mean pair-wise variation between an individual gene and all other tested genes, calculated by geNorm. Pairwise variation (V) between the normalization factors (NFn and NFn + 1) was used to determine the optimal number of reference genes.

3.5. Impact of reference genes on RT-qPCR data analysis

The selection of a reference gene for normalization of RT-qPCR can have a distinct influence on the expression profile of target genes (Suzuki et al., 2000; Jiang et al., 2015). Therefore, to evaluate the impact of reference genes on the RT-qPCR data, the *Dll* gene expression was normalized with each, or a combination, of the reference genes analyzed (Fig. 6). Additionally, the E10 stage was used as a second

Table 4
Reference gene expression stability values in embryonic developmental stages and adult tissues of *M. olfersii* based on several programs.

	Rank	NormFinder		BestKeeper		geNorm		ΔCt		General ranking
		Genes	Stability index	Genes	SD	Genes	M value	Genes	Average SD	Genes
Developmental stages	1	*AK*	0.255	*RpL8*	0.210	*RpL8/RpS6*	0.350	*AK*	1.404	*RpL8*
	2	*RpS6*	0.263	*RpS6*	0.377			*RpL8*	1.438	*RpS6*
	3	*β-act*	0.280	*AK*	0.427	*β-act*	0.443	*β-act*	1.460	*AK*
	4	*RpL8*	0.310	*β-act*	0.430	*AK*	0.497	*RpS6*	1.474	*β-act*
	5	*GAPDH*	0.580	*GAPDH*	0.861	*GAPDH*	0.861	*GAPDH*	1.690	*GAPDH*
	6	*EF-1α*	2.969	*EF-1α*	3.716	*Ef-1α*	1.961	*EF-1α*	4.309	*EF-1α*
Adult tissues	1	*RpS6*	0.699	*RpS6*	0.473	*RpL8/RpLS6*	0.147	*RpL8*	2.332	*RpS6*
	2	*RpL8*	0.706	*RpL8*	0.527			*RpS6*	2.348	*RpL8*
	3	*β-act*	1.052	*AK*	1.881	*GAPDH*	1.356	*GAPDH*	2.932	*GAPDH*
	4	*GAPDH*	1.329	*β-act*	1.897	*β-act*	1.869	*β-act*	2.942	*β-act*
	5	*AK*	1.945	*GAPDH*	1.947	*AK*	2.369	*AK*	3.931	*AK*
	6	*EF-1α*	2.380	*EF-1α*	4.363	*EF-1α*	3.249	*EF-1α*	5.009	*EF-1α*

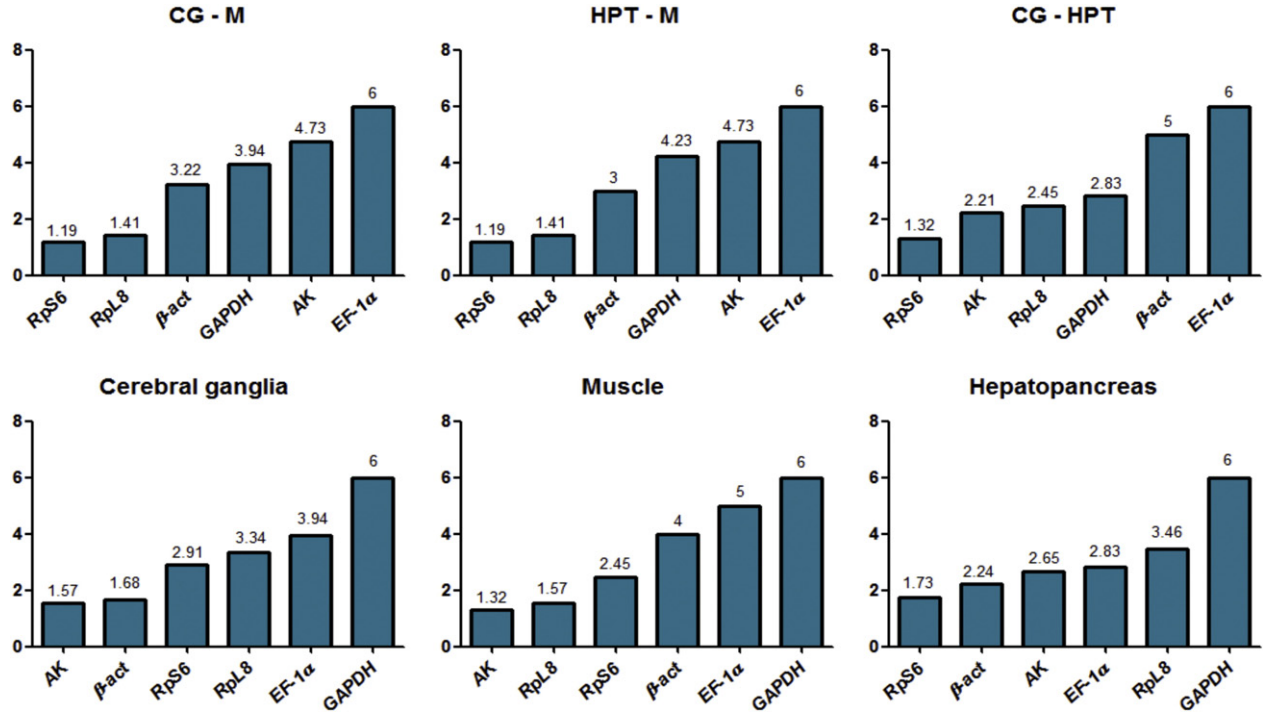

Fig. 5. Comprehensive expression stability of candidate reference genes calculated by RefFinder on each specific tissue and based on the grouping of two different adult tissues of *M. olfersii*. Reference genes were plotted from most stable (left) to least stable (right). CG-M: cerebral ganglia and muscle, HPT-M: hepatopancreas and muscle, CG-HPT: cerebral ganglia and hepatopancreas.

normalizer of the gene expression during embryonic development. The amplification efficiency for *Dll* gene was 1.95. Samples from various stages of development (E3, E5, E7 and E10) were used in this experiment. Generally, the use of multiple reference genes provides more accurate data than the use of a single reference gene. The *Dll* gene expression level was similar when either the most stable reference gene (*RpL8* or *RpS6*) or the geometric mean of reference gene combinations: (*RpL8* and *RpS6*), (*RpL8*, *RpS6* and *β-act*), (*RpL8*, *RpS6* and *AK*) was used as the normalizer. *Dll* gene expression tended to decrease from stage E3 up to E7 and increase at stage E10. Normalization of the *Dll* gene expression with the third (*AK*) or fourth (*β-act*) ranked gene was similar to the expression obtained when either of the reference genes, *RpL8* or *RpS6*, were used. The expression levels were considerably diminished from stages E3 up to E10 when the *GAPDH* and *EF-1α* genes were used.

4. Discussion

Analysis of gene expression is a powerful tool for understanding the molecular mechanisms during embryonic stages and the biological processes of the adult tissues. This approach could be useful for *M. olfersii*, considering the available sequences in a transcriptome analysis (Jaramillo et al., 2016) and the potential of this species to become a model for developmental, toxicological and environmental studies. One of the methods commonly used to analyze gene expression is the RT-qPCR (Valasek and Repa, 2005). However, suitable reference genes,

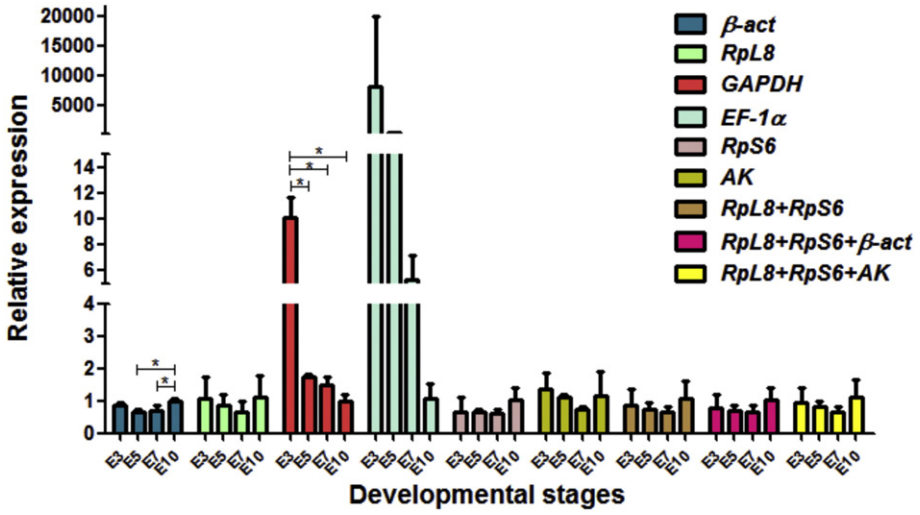

Fig. 6. Relative expression of the *Dll* gene in the embryonic development of *M. olfersii*, using the reference genes or their best combinations. Variability of *Dll gene* expression is shown for each tested reference gene used as a normalizer. Bars indicate mean ± standard deviation ($n = 3$ whole egg mass of ovigerous females/embryonic stage). (*) Represents significant difference at $p < 0.05$ among embryonic stages.

a prerequisite for to obtain reliable and accurate data in RT-qPCR, had not been identified and validated in *M. olfersii*. Evaluation and validation of reference genes in crustaceans are limited and typically, the *EF-1α*, *GAPDH*, *18S rRNA*, *RpL8*, *RpL18* and *β-act* genes are used (Dhar et al., 2009; Cottin et al., 2010). Most expression studies in freshwater prawns, particularly in *Macrobrachium* genus, have used a single reference gene without evaluating the expression stability of other genes (Feng et al., 2007; Zhang et al., 2010, 2011, 2014; Arockiaraj et al., 2013; Bai et al., 2015). In this study, we evaluated the stability of six candidate reference genes (*β-act*, *GAPDH*, *EF-1α*, *RpL8*, *RpS6* and *AK*) in three adult tissues and during embryonic developmental stages using the geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004), BestKeeper (Pfaffl et al., 2004), ΔCt method (Silver et al., 2006) and RefFinder programs.

The three most stable genes (*RpL8*, *RpS6* and *AK*/*β-act*) in embryonic stages were ranked differently, depending on the program used to analyze the data (geNorm, NormFinder, BestKeeper, ΔCt method and RefFinder). In contrast, for adult tissues, the three most stable reference genes were *RpL8*, *RpS6* and *β-act*/*AK*/*GAPDH*. The differences in the resultant gene rankings could be associated with the particular algorithms used by each program. The comprehensive RefFinder ranking, which integrates the four programs (ΔCt method, NormFinder, BestKeeper and geNorm), indicated that *RpL8* was the most stable gene in embryonic stages and adult tissues of *M. olfersii*. However, based on the geometric mean ranking of each program, we devised a general ranking stability and found that the *RpL8* and *RpS6* genes were most stable in embryonic stages and adult tissues, respectively. These ranking differences may be primarily associated with the results obtained in RefFinder, which does not include the identification of the group (i.e., different tissues) in contrast to NormFinder. In instances where the group identifications (different tissues) were not considered, the same general ranking results were obtained. The results of the *RpL8* gene stability is consistent with previous reports describing stable *RpL8* expression in gills of *Portunus trituberculatus* (Xu and Liu, 2011), in the larvae, post-larvae, and gills of *M. rosenbergii* (Barman et al., 2012), and in tissues from the abdomen of *R. exoculata* and *Palaemonetes varians* (Cottin et al., 2010). In another study, the *RpS6* gene expression was a more stable normalizer for broad-scale gene expression analysis in *Tribolium castaneum* (Toutges et al., 2010). These results demonstrate why various genes for ribosomal proteins have been validated as normalizer genes for RT-qPCR in many organisms (Lu et al., 2013). In this study, *AK* was ranked as the third most stable gene in developing embryos and fifth most stable in adult tissues. This gene has been used to study arthropods and was stable in the labial gland and fat body samples of *Bombus terrestris* (Horňáková et al., 2010). Additionally, *AK* was the second most stable gene in tissues from third-instar larvae of *Spodoptera litura* treated with various insecticides (Lu et al., 2013).

GAPDH and *EF-1α* were the least stable genes during embryonic stages of *M. olfersii*. We considered that this is due to the low expression of these two genes in early developmental stages. Several studies in other arthropods have demonstrated low *GAPDH* expression stability in certain life stages of *Tetranychus cinnabarinus* (Sun et al., 2010) and in the labial gland and fat tissues of *B. terrestris* and *Bombus lucorum* (Horňáková et al., 2010). However, the *EF-1α* gene was considered the most stable gene in the reproductive system of *P. monodon* (Leelatanawit et al., 2012) and second most stable in immune gene expression of *Penaeus stylirostris* (Dhar et al., 2009). Furthermore, *GAPDH* has been used as a reference gene for low gene expressions in shrimps (Dhar et al., 2009). In *M. rosenbergii, EF-1a* was the most stable gene for expression analysis in the androgenic gland, testis, nerve cord and gills, while *GAPDH* was the most suitable reference gene in eyestalk (Priyadarshi et al., 2015). Additionally, *EF-1a* was found to be the most stable gene in larval developmental stages and in temperature-stressed larvae, while it was the second most stable gene in larvae fed various foods (Lu et al., 2013). In this study, the *EF-1α* gene was the least stable of the reference genes evaluated in embryonic stages and adult tissues of *M. olfersii*. Similar results were found when evaluating reference gene stability in the development and tissues of *Procambarus clarkii* (Jiang et al., 2015). Thus, the use of *EF-1α* and *GAPDH* as reference genes should be avoided in future RT-qPCR experiments focused on the embryonic development of *M. olfersii*.

Studies with various organisms have demonstrated that the expression of reference genes varies, in part, because the proteins they encode could be involved in basal metabolism and other biological functions (Schmittgen and Zakrajsek, 2000; Thornton et al., 2003, Zhang et al., 2013). In this study, we evaluated each tissue independently and the combination of two tissues of *M. olfersii* to determine the effects on the combined ranking analysis. Regardless of the different tissue combinations, the *RpS6* gene was always the most stable. Furthermore, when each tissue was assessed independently, the two most stable genes were always among the *AK*, *β-act*, *RpL8* and *RpS6* genes, except the *AK* gene in hepatopancreas. The *EF-1α* gene was always ranked among the three least stable reference genes. These results confirmed that reference gene expression varies in different tissues of *M. olfersii*. Also, the expression of these reference genes could vary in *M. olfersii* tissues other than those analyzed in this study and this should be validated in subsequent studies. Indeed, these current results showed that reference gene expression stability was affected by differences in gene expression in the tissue, indicating the importance of validating reference genes in each tissue analyzed.

Previous studies have demonstrated the variation in reference gene expression under different experimental conditions, therefore there is no universal reference gene and more commonly, multiple reference genes are used (Bustin, 2002; Dheda et al., 2005; Zhu et al., 2005; Bogaert et al., 2006; Nestorov et al., 2013; Reddy et al., 2015). Many studies have shown that the use of multiple reference genes provides greater accuracy in RT-qPCR analysis (Vandesompele et al., 2002; Gutierrez et al., 2008; Derveaux et al., 2010; Le et al., 2012). In this context, the impact of the normalization with different reference genes or gene combinations was evaluated in embryonic stages of *M. olfersii* using the *Dll* expression level. Normalization with the most stable genes (*RpL8* or *RpS6*) showed a decrease in *Dll* gene expression up to stage E7. A similar profile was obtained using the geometric mean of the reference genes suggested by NormFinder (*RpL8* and *RpS6*) and geNorm (*RpL8*, *RpS6* and *β-act*) programs, and the three genes most stable in general ranking (*RpL8*, *RpS6* and *AK*). We found a similar *Dll* expression profile, using the third and fourth most stable genes (*AK* and *β-act*, respectively). However, we considered that the more precise expression profile was acquired using the *RpL8* or *RpS6* genes or their combination. Indeed, when we used the least stable genes, *GAPDH* and *EF-1α*, there was a substantial decrease in the *Dll* expression profile. Therefore, the selection of reference genes impacted the expression levels of target genes, and our results indicated that *EF-1α* and *GAPDH* were not suitable reference gene candidates for developmental studies of *M. olfersii*. Similar impacts of reference genes have been demonstrated in *P. clarkii* (Jiang et al., 2015).

In summary, our results suggested that *RpL8* and *RpS6* were the most stable genes during *M. olfersii* embryonic stages and *RpS6* and *RpL8* were the most stable genes in the adult tissues. Thus, *RpL8* and *RpS6*, or geometric mean of two, could be considered as optimal reference genes for differential qPCR analysis in *M. olfersii*. Additionally, we demonstrated that the reference genes, *EF-1α* and *GADPH*, impacted the expression level of a target gene in *M. olfersii* embryonic development, and, therefore, was unsuitable for RT-qPCR normalization. The geometric mean of three genes (*RpL8*, *RpS6* and *AK* or *RpL8*, *RpS6* and *β-act*) also provided a robust approach to normalizing gene expression in *M. olfersii* development. To the best of our knowledge, this research is the first published study to identify and compare expression stability of candidate reference genes for RT-qPCR analysis in *M. olfersii*. The most stable reference genes identified will aid future RT-qPCR studies of *M. olfersii* gene expression and contribute to understanding the molecular mechanisms of its development. Furthermore, considering the similarity of the

embryonic development of other species that have superficial cleavage and embryonized nauplius, this study may be useful as a basis for future evaluations of reference genes in other prawns.

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