Article

Validation and Evaluation of Reference Genes for Quantitative Real-Time PCR in *Macrobrachium Nipponense*

Yuning Hu [1] [ID]**, Hongtuo Fu** [1,2,]*****, Hui Qiao** [2]**, Shengming Sun** [2] [ID]**, Wenyi Zhang** [2]**, Shubo Jin** [2]**, Sufei Jiang** [2]**, Yongsheng Gong** [2]**, Yiwei Xiong** [2] **and Yan Wu** [2]

[1]   Wuxi Fishery College, Nanjing Agricultural University, Wuxi 214081, China; huxiaoao@163.com
[2]   Key Laboratory of Freshwater Fisheries and Germplasm Resources Utilization, Ministry of Agriculture, Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences, Wuxi 214081, China; qiaoh@ffrc.cn (H.Q.); sunsm@ffrc.cn (S.S.); zhangwy@ffrc.cn (W.Z.); jinsb@ffrc.cn (S.J.); jiangsf@ffrc.cn (S.J.); gongys@ffrc.cn (Y.G.); xiongyw@ffrc.cn (Y.X.); wuy@ffrc.cn (Y.W.)
*****   Correspondence: fuht@ffrc.cn; Tel: +86-510-8555-8835; Fax: +86-510-8555-3304

check for updates

Abstract: Quantitative real-time PCR (qPCR) is widely used in molecular biology, although the accuracy of the quantitative results is determined by the stability of the reference genes used. Recent studies have investigated suitable reference genes for some crustaceans under various conditions, but studies in *Macrobrachium nipponense* are currently lacking. In this study, we selected the following seven genes from among 35 commonly used housekeeping genes as candidate qPCR reference genes for temporal and spatial expression: *EIF* (*eukaryotic translation initiation factor 5A*), *18S* (*18S ribosomal RNA*), *EF-1α* (*elongation factor-1α*), *GAPDH* (*glyceraldehyde-3-phosphate dehydrogenase*), *TUB* (*α-tubulin*), *β-act* (*β-actin*), and *RPL18* (*Ribosomal protein L18*). The stability of each reference gene was evaluated by GeNorm, NormFinder, BestKeeper, and comparative ΔC_t methods, and was comprehensively ranked using RefFinder. *RPL18* was shown to be the most suitable reference gene for adult *M. nipponense* tissues, while *EIF* was the most stable in different ovarian and embryo stages and in white spot syndrome virus infection, and *β-act* was the most stable reference gene under hypoxia stress. The reliability of the rankings was confirmed by RNA interference experiments. To the best of our knowledge, this represents the first systematic analysis of reference genes for qPCR experiments in *M. nipponense*, and the results will provide invaluable information for future research in closely related crustaceans.

Keywords: *Macrobrachium nipponense*; reference gene; normalization; quantitative real-time PCR

1. Introduction

Quantitative real-time PCR (qPCR) is a widely used technique for investigating gene expression levels, with high accuracy and sensitivity, as well as a wide application. Real-time PCR can involve relative or absolute quantification methods, of which the relative quantification is simple and accurate [1,2]. Housekeeping genes (HKGs), as known as reference genes [3], are used to account for alignment errors (e.g., as a result of the differences in RNA concentration, efficiencies, and reverse transcription), thus allowing the expression levels of the target gene to be calculated relative to the housekeeping gene [4–6]. However, the appropriate reference genes need to be searched and used for specific experimental conditions, because there is no perfect reference gene that maintains a stable expression in all of the tissues and conditions [7,8]. Furthermore, the use of inappropriate reference genes may result in conflicting gene expression data for different tissues or situations [9,10].

Molecular research in crustacean has become popular in recent years, and numerous studies have examined the growth, development, reproduction, and sex differentiation of crustaceans [11]. qPCR has been widely used in these studies, and some research has been carried out to screen and identify suitable reference genes in crustaceans. One study showed that the commonly used reference gene *β-act* was not the most stable reference gene in *Penaeus stylirostris* infected with white spot syndrome virus (*WSSV*) [12]. In *Peneaus monodon*, the appropriate reference gene for the reproductive gene expression profile was identified [13]. Furthermore, the reference genes were screened before starting a quantitative study of heat-shock responses in *Palaemonetes varians* [14], while studies in *Macrobrachium rosenbergii* and *Macrobrachium olfersii* showed that reference genes were not universal, and the most appropriate reference gene depended on the specific conditions [15,16].

The oriental river prawn *Macrobrachium nipponense* is widely distributed in freshwater and low-salinity estuarine regions in China, and is of great market value. Although many molecular studies have investigated the reproduction, sexual control, stress, and nutrition in *M. nipponense* [17–19], most qPCR studies were performed using reference genes from other similar species without identification and verification, potentially leading to inaccurate results. It is therefore essential to screen for suitable specific reference genes in *M. nipponense* under different experimental conditions.

In the current study, we investigated several reference genes based on other model animals and crustaceans. After comparing them with the *M. nipponense* transcriptome data and gel electrophoresis detection, we identified seven candidate reference genes in the *M. nipponense* transcriptome library. We then measured the expression stability of those genes in different adult tissues, ovarian and embryo stages, under hypoxia stress, and in white spot syndrome virus (*WSSV*) infection, and analyzed the results using GeNorm [20], NormFinder [20], BestKeeper [21], and the comparative ΔC_t method [22], and ranked them using the web-based comprehensive tool RefFinder [23]. We also performed RNA interference (RNAi) experiments to verify the accuracy of the screened reference genes by detecting the expression of *SST* (*slow-tonic S2 tropomyosin*) gene before and after the RNAi, using difference reference genes for normalization. This study represents the first comprehensive systematic screening of reference genes for *M. nipponense* based on experiments involving temporal and spatial expression and stress. Furthermore, it also provides the first results of the reference gene screening and the verification of reference genes by RNAi experiments in crustaceans. The results of this study fill a gap in the *M. nipponense*-related research, thus increasing the accuracy and reliability of future research into the expression of target genes, and providing a useful reference for studies in other crustaceans.

2. Results

2.1. Selection of Target Internal Reference Genes

Based on previous research in model organisms (*Danio rerio, Mice, Bactrocera dorsalis*, and *Oryza sativa*) and crustaceans (*Procambarus clarkia, Macrobrachium rosenbergii*, and *Macrophthalmus japonicas*), we screened 35 candidate genes in the transcriptome libraries of *M. nipponense* [24–27]. Fifteen of the genes were ubiquitously expressed in different tissue libraries and passed the BLAST test. Further screening identified seven reference genes with effective specificity and amplification, *EIF* (*eukaryotic translation initiation factor 5A*), *18S* (*18S ribosomal RNA*), *EF-1α* (*elongation factor-1α*), *GAPDH* (*glyceraldehyde-3-phosphate dehydrogenase*), *TUB* (*α-tubulin*), *β-act* (*β-actin*), and *RPL18* (*Ribosomal protein L18*). The open reading frames of these sequences were verified using the primers in Table A1. The sequences of the candidate genes were submitted to NCBI GenBank (Table A1).

2.2. Primer Specificity and Amplification Efficiency for qPCR

The qPCR primer pairs of each reference gene are presented in Table 1. The specificity of each single PCR product was confirmed by 1.2% agarose gel electrophoresis (Figure 1), and was matched with their sizes. The amplification efficiencies of these primers ranged from 0.93–1.02 (Table 1) and the

standard curve for each gene from the cDNA dilutions displayed $R^2 > 0.99$ (Figure 2A); each primer produced a single melting peak (Figure 2B), reflecting their stability and specificity [3].

Table 1. Primer pairs for qPCR of candidate reference genes. EIF—eukaryotic translation initiation factor 5A; 18S—18S ribosomal RNA; EF-1α—elongation factor-1α; GAPDH—glyceraldehyde-3-phosphate dehydrogenase; TUB—α-tubulin; β-act—β-actin; RPL18—Ribosomal protein L18.

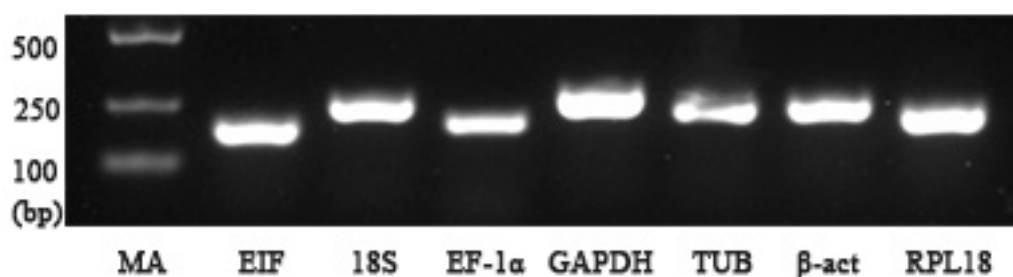| Gene	Primer Sequence (5′–3′) Forward/Reverse	Length (bp)	PCR Efficiency (%)	Correlation Coefficient (R^2)
EIF	CATGGATGTACCTGTGGTGAAAC CTGTCAGCAGAAGGTCCTCATTA	179	94.4	0.994
18S	GTTGGATGTTGCTGTTGAGAGAG CTGGGCATCATTCTCTGGGTAAA	250	96.8	0.997
EF-1α	CAAGGATCTGAAACGTGGCTTC GTACGTCTGTCGATCTTGGTCAG	198	93.7	0.999
GAPDH	GTCGGTAAGGTCATTCCAGAGC CGAAAGTTTTGCTGAGCTGGAT	274	95.5	0.991
TUB	AGGAATGGAAAATCAGGAAGCCC GTTTGTCGATCTGGAACCCTCT	224	97.0	0.996
β-act	CACGAGACCACCTACAATTCCA ATAGAGAAGCCAAGATAGAACCGC	226	99.4	0.991
RPL18	CTTTTTGTACCCACAGCTTGACC CACTTTTGATGTATTGGCCCGTC	202	102.7	0.997

Figure 1. Agarose gel electrophoresis of qPCR primers amplification of the candidate reference genes using hepatopancreas cDNA as a template. MA means DNA marker DL2000, which is in the left side and shows their expected sizes. *EIF—eukaryotic translation initiation factor 5A*; *18S—18S ribosomal RNA*; *EF-1α—elongation factor-1α*; *GAPDH—glyceraldehyde-3-phosphate dehydrogenase*; *TUB—α-tubulin*; *β-act—β-actin*; *RPL18—Ribosomal protein L18*.

2.3. Distribution of Cycle Threshold (Cq) Values

The detailed Cq values are presented in Table 2. The Cq values of these candidates' reference genes ranged from 17.75 (*β-act*) and 37.48 (*EF-1α*). A high Cq value represented a low expression level [3], indicating that in seven genes, *EIF*, *β-act*, and *RPL18* had the highest expression levels, and *EF-1α* and *18S* had the lowest levels.

The quantitative expression levels of each reference gene under various tissues and conditions, according to their Cq value, were represented by line charts and boxplots. The line charts showed that the reference genes remained stable within each tissue under different conditions, while some genes varied widely among different tissues, such as *EF-1α* in adult and embryonic tissue (Figure 3). The overall distribution and data dispersion [28] are illustrated by boxplots. These plots indicated that *EIF*, *RPL18*, *β-act*, and *TUB* showed small discrete fluctuations, while *EF-1α* and *GAPDH* varied widely between the adult tissues and embryo development stages (Figure 4).
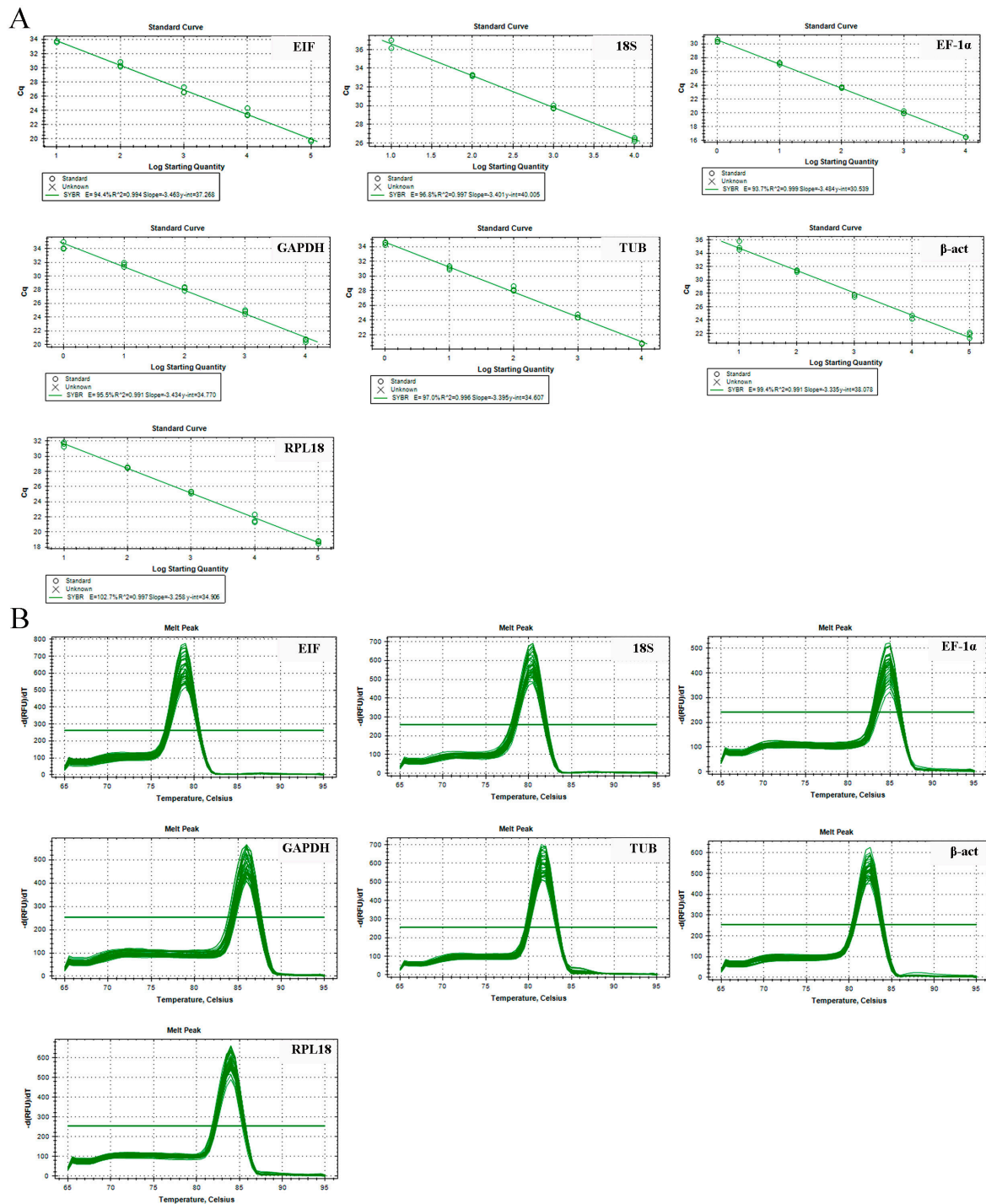
Figure 2. Dissolution and standard curve to verify the gene amplification efficiency and uniformity. (**A**) Dissolution curve of candidate reference genes. (**B**) Standard curve of candidate reference genes.

Table 2. Detailed distribution of cycle threshold (Cq) value information.

Experimental Conditions	Statistics	Reference Gene						
		EIF	*18S*	*EF-1α*	*GAPDH*	*TUB*	*β-act*	*RPL18*
Hypoxia stress	Group	5	5	5	5	5	5	5
	Mean	21.86	28.19	24.20	21.19	23.80	24.35	21.33
	Min Cq	20.64	26.08	22.50	20.02	23.13	23.01	19.03
	Max Cq	22.33	28.98	26.42	22.66	24.56	25.66	22.43
WSSV infection	Group	6	6	6	6	6	6	6
	Mean	24.82	32.02	34.89	26.78	30.44	26.47	24.40
	Min Cq	24.12	30.97	34.22	26.26	29.98	25.72	23.76
	Max Cq	25.30	33.28	36.04	27.44	31.34	27.64	24.96
Different adult tissues	Group	7	7	7	7	7	7	7
	Mean	21.65	27.81	25.05	22.55	25.86	20.82	21.83
	Min Cq	20.47	26.09	18.57	17.90	22.61	18.64	20.56
	Max Cq	23.59	30.26	32.62	26.74	29.17	23.36	23.88
Different ovarian stages	Group	5	5	5	5	5	5	5
	Mean	21.29	25.42	33.34	23.61	23.81	19.28	22.18
	Min Cq	20.24	23.77	31.56	20.71	21.06	17.75	20.59
	Max Cq	22.66	27.62	35.32	25.40	25.11	21.19	24.40
Different embryo stages	Group	7	7	7	7	7	7	7
	Mean	21.23	27.51	30.90	25.13	25.25	21.71	22.63
	Min Cq	18.58	25.37	21.12	21.25	22.53	18.26	18.95
	Max Cq	23.77	32.10	37.48	30.47	30.98	25.62	27.97

'Group' in this table represents how many states or tissues the genes are studied in. Each means five time points during hypoxia stress, six time points during *WSSV* infection, seven different organizations in adult tissues study, five different ovarian stages, and seven different embryo stage. *WSSV*—white spot syndrome virus.
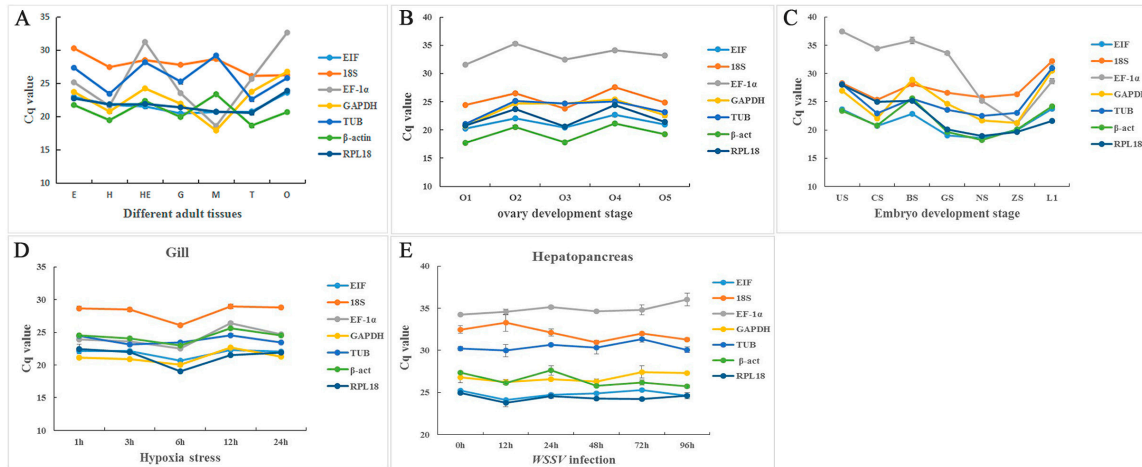

Figure 3. Variation in the reference genes expression using distribution of cycle threshold (Cq) values in line charts. (**A**) Different tissues in adult shrimps. (**B**) Five different ovary development stages, O1–5: Stage I, Stage II, Stage III, Stage IV, and Stage V of ovary. (**C**) Different embryo development stages, US—unfertilized egg stage; CS—cleavage stage; BS—blastula stage; GS—gastrul stage; NS—nauplius stage; ZS—zoea stage; L1—one day after larvae hatched. (**D**) Different times after hypoxia stress in gill. (**E**) Different times after infection in hepatopancreas.
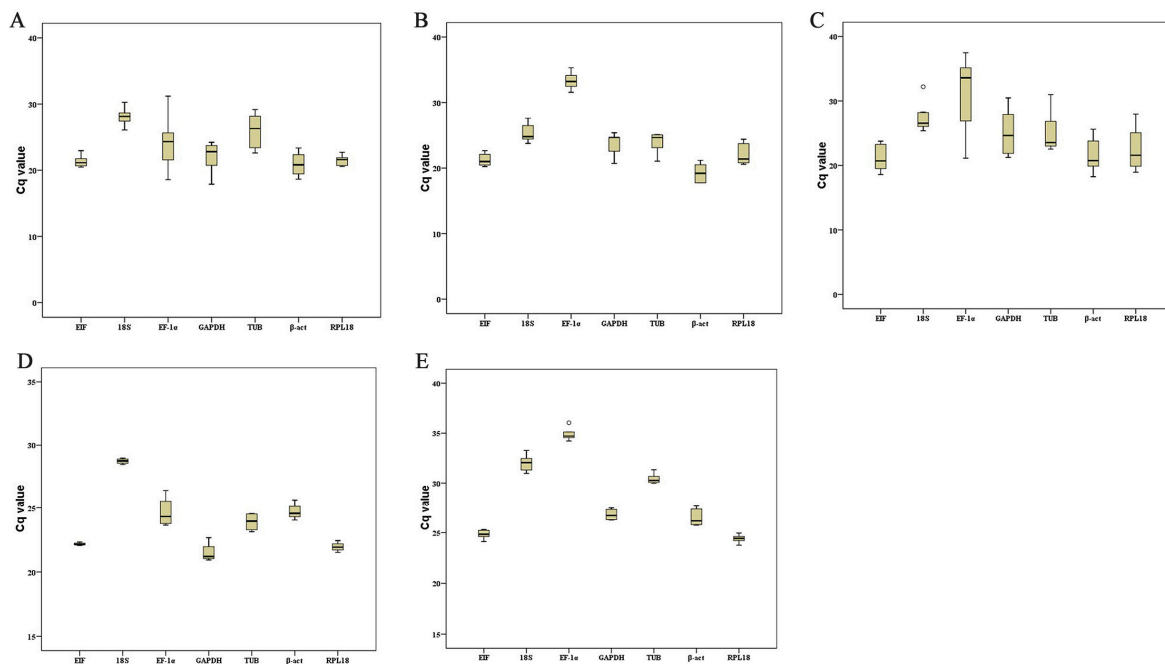
Figure 4. Variation in the reference genes expression using Cq values with boxplot. (**A**) Different tissues in adult shrimps. (**B**) Five different ovary development stages. (**C**) Different embryo development stages. (**D**) Different times after hypoxia stress in gill. (**E**) Different times after infection in hepatopancreas. Upper and lower whiskers mean maximum and minimum values, except outliers, and using a circle to mark the mild outliers and an asterisk to mark the extreme outliers. The upper and lower edges of the box represent the upper and lower quartiles, the middle black line is the median, and the whiskers represent the maximum and minimum values. Mild and extreme outliers are marked by circles and asterisks, respectively. The length of each graphic reflects its variation.

2.4. Stability Analysis

We analyzed the stabilities of the reference genes using four commonly used methods, comparative ΔC_t, BestKeeper, NormFinder, and GeNorm [20–23] (Tables A2 and A3). Comparative ΔC_t, BestKeeper, and NormFinder recommended RPL18 as the most stable reference gene in different adult tissues, while GeNorm identified that the combination of *EIF* and *RPL18* as the most stable genes. *TUB*, *GAPDH*, and *EF-1α* were the least stable genes according to all four methods. The comparative ΔC_t identified *β-act* as the most stable gene in the different ovarian stages, while BestKeeper and NormFinder considered *EIF* to be the best, and GeNorm considered the combination of *18S/RPL18* as the most stable target. *GAPDH* was the least stable gene according to all of these methods. Comparative ΔC_t and NormFinder considered *EIF* as the best reference gene in different embryonic stages, while BestKeeper picked *18S* and GeNorm chose the combination of *18S/TUB*. *GAPDH*, *RPL18*, and *EF-1α* were ranked the least stable. The comparative ΔC_t and NormFinder ranked *β-act* as the most stable reference gene in the *M. nipponense* gills under various durations of hypoxic stress, compared with *EIF* and *GAPDH/β-act*, according to GeNorm. *EF-1α* was not considered to be suitable in this situation. In the case of a *WSSV* infection, BestKeeper and NormFinder selected *RPL18*, comparatively ΔC_t picked *EIF*, and GeNorm selected *EIF/TUB* as the best reference genes, while all four ranked *β-act* and *18S* as the least stable.

The comprehensive scores for these methods were ranked using RefFinder to give a total ranking (Figure 5) [23]. The genes ranked from most- to least-stable in the adult *M. nipponense* tissues were *RPL18*, *EIF*, *18S*, *β-act*, *TUB*, *GAPDH*, and *EF-1α*. The equivalent ranking in the different ovarian stages was *EIF*, *β-act*, *RPL18*, *18S*, *EF-1α*, *TUB*, and *GAPDH*, while that in the different embryonic stages was *EIF*, *18S*, *β-act*, *TUB*, *GAPDH*, *RPL18*, and *EF-1α*. The most- to least-stable genes under hypoxic stress

were *β-act*, *GAPDH*, *EIF*, *18S*, *TUB*, *EF-1α*, and *RPL18*. *EIF* and *RPL18* were ranked highly under *WSSV* infection, followed by *TUB* and *GAPDH*, while *EF-1α*, *β-act*, and *18S* were not considered to be stable.
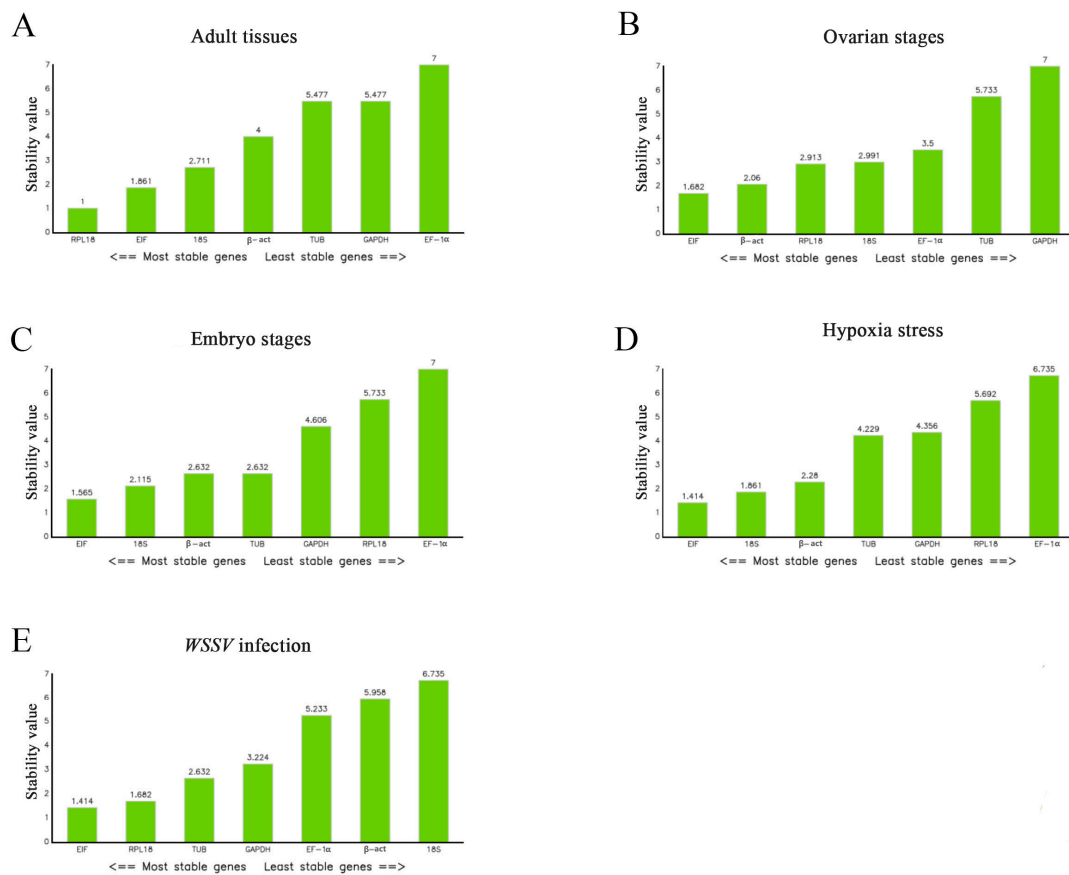

Figure 5. Comprehensive ranking of the reference genes under different conditions by RefFinder. (**A**) Different tissues in adult shrimps. (**B**) Five different ovary development stages. (**C**) Different embryo development stages. (**D**) Different times after hypoxia stress in gill. (**E**) Different times after infection in hepatopancreas. From left to right means the most suitable to the least suitable.

2.5. Validation of the Selected Reference Genes by RNAi

We confirmed the selected reference genes and verified their rankings based on qPCR by RNAi, which could reliably decrease the expression level of the target gene in *M. nipponense* [29,30]. The *SST* expression in the androgenic glands in *M. nipponense* was predicted to decrease by >90% over seven days after RNAi. qPCR was performed to measure the expression difference in the expression levels based on different reference genes. The Cq values show that the difference in the tissue expression was not significant before and after the experiment, except *EF-1α* and *GAPDH* (Table 3). The three most stable reference genes were *β-act*, *RPL18*, and *TUB* (Table 4, Figure 6), and the expression of *SST*, measured using different reference genes, were reduced to 1.35%, 1.20%, and 1.12%, respectively (Figure 7), which confirms the success of the interference experiment. *β-act* was chosen as the best reference gene, which indicated a decrease in expression of *SST* to 1.35%. *EF-1α* was considered the least stable gene, and indicated a decrease in *SST* to 14.65% (Figure 7).

Table 3. Detailed Cq value sorting of RNAi.

Experimental Conditions	Statistics	Reference Gene						
		EIF	*18S*	*EF-1α*	*GAPDH*	*TUB*	*β-act*	*RPL18*
RNAi (Androgenic glands)	Group	2	2	2	2	2	2	2
	Mean	21.29	27.62	24.51	22.14	23.46	19.11	20.58
	Min Cq	21.07	27.33	22.63	21.34	23.40	18.99	20.55
	Max Cq	21.51	27.91	26.38	23.14	23.51	19.23	20.62

'Group 2' in this table represents two states, before and after the RNAi experiment.

Table 4. Ranking of reference genes of RNAi by different methods.

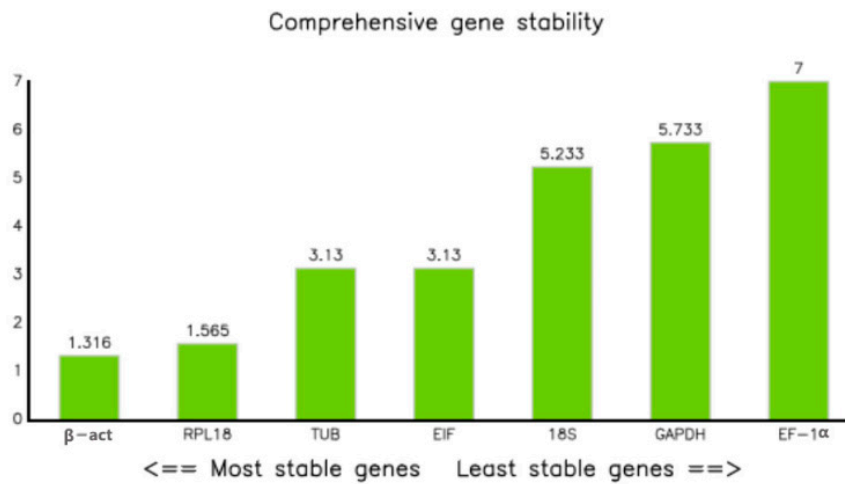| Experimental Conditions	Method	Rank						
		1	2	3	4	5	6	7
RNAi (Androgenic glands)	Comparative ΔC$_t$	*β-act*	*RPL18*	*EIF*	*TUB*	*18S*	*GAPDH*	*EF-1α*
	BestKeeper	*RPL18*	*TUB*	*β-act*	*EIF*	*18S*	*GAPDH*	*EF-1α*
	NormFinder	*β-act*	*EIF*	*RPL18*	*TUB*	*GAPDH*	*18S*	*EF-1α*
	GeNorm	*β-act/RPL18*		*TUB*	*EIF*	*18S*	*GAPDH*	*EF-1α*
	Recommended comprehensive ranking	*β-act*	*RPL18*	*TUB*	*EIF*	*18S*	*GAPDH*	*EF-1α*

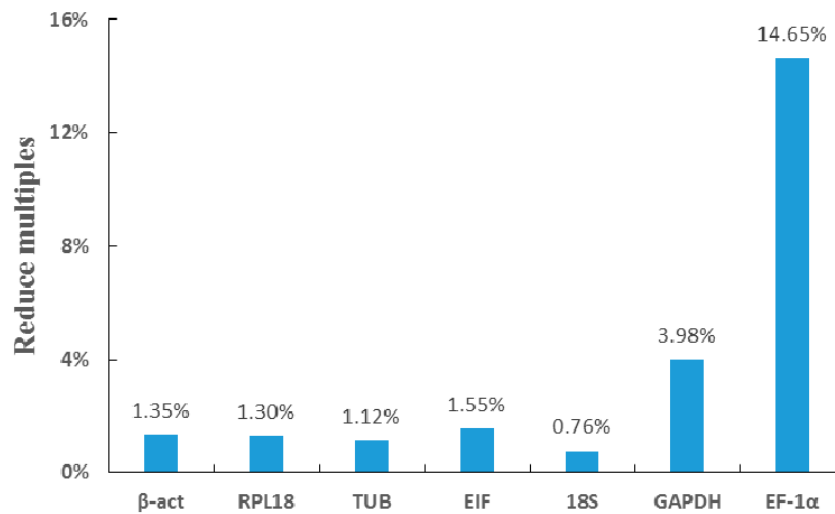**Figure 6.** Comprehensive ranking of reference genes of RNAi.

Figure 7. Using different genes as the reference gene for the reduce multiples of the *SST* gene expression, relative to the original level after interference.

3. Discussion

Molecular studies in crustaceans have indicated that many genes tend to change their expression levels under different situations or in different stages of development [29,31]. qPCR is a useful tool for analyzing gene expression, with good specificity, accuracy, efficiency, and reproducibility. However, numerous studies have shown that the reference genes, such as *GAPDH* and *β-act*, that are used to normalize the data in qPCR studies may not remain stable under all conditions [10,32,33].

In this study, we screened out seven reference genes (*EIF*, *β-act*, *RPL18*, *18S*, *EF-1α*, *TUB*, and *GAPDH*), and ranked and evaluated them in *M. nipponense* under different situations. We initially chose candidate reference genes based on the recommended gene studied in the model organisms and other crustaceans. It is interesting that, although there are evolutionary and organizational differences in the different species, reference genes like *alpha-1,2-mannosyltransferase* in *Saccharomyces cerevisiae* [34], *heterogeneous nuclear ribonucleoprotein 27C* and *TBC1 domain family member 22A* in *Oryza sativa* [35], *60S ribosomal* protein *L13a* in *Danio rerio* [36], *TATA-box binding* protein, and *eukaryotic translation elongation factor 2* in *Mus musculus* [32] can be found in the mRNA transcriptomes studied in our lab. The genes were further selected after following inspection with gel electrophoresis and sequencing. For example, the *TATA-binding protein* was eliminated because the PCR product was too weak in the gel electrophoresis and it was not widely present in all organizations. These results indicated that some genes considered as classical reference genes in some species may not be suitable in other species. Previous studies of crustaceans, especially shrimps, identified *EIF* and *18S* as the most stable reference genes in different tissues in *Procambarus clarkii* [9], while *GAPDH* and *β-act* were chosen in different male tissues in *Macrobrachium rosenbergii* [15], and *EF-1α* in the reproductive system in the *Peneaus monodon* [13] and the infected experiment in *Penaeus stylirostris* [12]. However, *EF-1α* was almost the least stable in all situations, including in *WSSV*-infected experiment in *M. nipponense*, suggesting that reference genes are not necessarily interchangeable in different shrimp, even under the same conditions. *RPL18* has rarely been used as a reference gene in crustaceans, although it was selected as a reference gene in different developmental stages and tissues in *Solenopsis invicta* [37], and in the different developmental stages in *Anastrepha obliqua* [38]. *RPL18* performed well in seven adult tissues in the current study, but was the least stable gene in the different embryo stages, suggesting that some reference genes only performed well in specific tissues or conditions. These results demonstrate the need to screen for specific suitable reference genes before conducting qPCR studies under new experimental conditions or means of stress. The Cq values and stability rankings of *GAPDH* and *EF-1α* both varied widely under different experimental conditions, and it is therefore not recommended to use them as reference genes in qPCR of *M. nipponense*. In contrast, *EIF* performed well in most of the stability rankings and should therefore be considered as a conventional reference gene for qPCR studies in *M. nipponense*.

An experiment with significant differences was designed and performed to further confirm the reliability of the screening results. Previous researchers in our lab have proved that RNAi is able to significantly reduce the expression of specific genes in *M. nipponense* [29,30]. The *SST* gene is specifically expressed in androgenic glands [39], and its expression was predicted to decrease by >90% by seven days after the injection. We analyzed this decrease using each of the seven candidate reference genes and showed that *β-act* and *RPL18* were the most stable reference genes, with similar ratings, followed by *TUB* and *EIF* tied for third, and then *18S*, *GAPDH*, and *EF-1α*. According to the qPCR data, the expression of *SST* in the androgenic glands dropped to 1.3%, based on the two most stable reference genes, compared with a drop to 14.6% compared with the least stable gene, *EF-1α*. This result was in accord with the stability rankings. A previous study identified *EF-1α* as the most suitable reference gene for measuring moderate and highly expressed genes in the infected *P. stylirostris*, while *GAPDH* was a better control for the lower expressed genes, corresponding to the expressions of *EF-1α* and *GAPDH* [12]. The current study showed that *18S*, with *EIF*, nearly showed (0.58 and 0.44) a difference in the mean Cq values, and a much slighter difference than *GAPDH* (1.80), but the rank score much lower (5.233 and 3.13) and the qPCR data shows its decline difference (0.76% and 1.5%), which

might indicate that, according to the expression level of the target gene, a medium high expression abundance of the reference gene was better. This suggests that some low-ranked genes may be suitable reference genes if the expression level of the target gene is very high or very low, depending on the Cq of the target gene. For example, *18S* may be suitable for target genes with low expression levels in adult tissues and under hypoxic stress, and *β-act* may be for highly expressed target genes in different ovarian stages in *M. nipponese* (Figures 3–5).

In this study, we screened seven potential reference genes' potential for *M. nipponense* from a large number of candidate genes, and analyzed their stability under various tissues and under different stresses using qPCR. *EIF* was stable in different situations, especially in *WSSV* infection and in different ovarian and embryo stages, while *RPL18* was the most stable reference gene in the adult tissues, and *β-act* was the best reference gene under hypoxia stress and RNAi. The five tools (GeNorm, NormFinder, BestKeeper, the comparative ΔC_t method, and RefFinder) used in this research for analyzing the stability rankings of the reference gene were widely used in crustaceans [12–16], but the reference genes have rarely been screened for conditions of hypoxia and RNAi. The current reference genes, identified under different conditions in *M. nipponense*, will thus provide a useful references for qPCR experiments in other crustaceans.

4. Materials and Methods

4.1. Selection of Reference Genes and Primer Design

The candidate gene selection was chosen from the reference genes that had been studied in other model animals and similar species. Then, their names were used to search the existing transcriptome libraries and the genes that were extensive in organizations were selected. The sequences were obtained from the transcriptome library. Nucleotide sequences were analyzed based on the nucleotide and protein databases using the BLASTX and BLASTN programs (http://www.ncbi.nlm.nih.gov/BLAST/). The protein prediction was performed using the open reading frame (ORF) finder (http://www.ncbi.nlm.nih.gov/gorf/). Multiple alignments of amino acid sequences' encoding were created using DNAMAN 6.0 [30].

All of the primers for the experiments were designed based on the open reading frame and using Primer-Blast tools in NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). A reaction without RNA templates was used as the negative control. The primers were designed to verify the ORFs of those sequences. After getting reliable ORFs, the primers for qPCR were designed considering the following parameters: primer size, 22–24 bp; product size, 150–300 bp; annealing temperature, 59–61 (°C) and a GC(composition) content of 40–60%. In order to get the most suitable primers, three attempts were taken to design and each time, with four pairs of primers design per gene.

4.2. Amplification Efficiency and Primers Specificity of Reference Genes

The specificity must be validated empirically with direct experimental evidence (electrophoresis gel, melting profile, DNA sequencing, amplicon size, and/or restriction enzyme digestion) [3]. The melting curve was measured to evaluate the specificity of the reference genes. The efficiency was based on the slope of a linear regression model and was calculated from the slope of a standard curve. The efficiencies (E) and correlation coefficients (R^2) were calculated for each reference gene [40]. The PCR efficiencies of the primer pairs were determined by qPCR, using a serial dilution of pooled hepatopancreas cDNA samples (×1, ×10, ×100, ×1000, and ×10000 dilutions).

4.3. Animals, Stress Experiments, and Tissue Collection

Our study does not involve endangered or protected species. This study was approved by the Institutional Animal Care and Use Ethics Committee of the Freshwater Fisheries Research Center, the Chinese Academy of Fishery Sciences (Wuxi, China, FFRC125, 26 August 2016). All of the prawns

were obtained from the Freshwater Fisheries Research Center, the Chinese Academy of Fishery Sciences, Wuxi, China (120°13′44″ E, 31°28′22″ N) [30].

In the spatiotemporal expression study, after one week, the laboratory culture, ovary, testis, gill, muscle, eyestalk, hepatopancreas, and heart were dissected out of the mature prawns (n = 3). The developmental stage of the embryo was classified into seven stages based on the criteria of Qiao et al. (2015) [41]. The ovarian cycle of the prawns was classified into five stages based on the previous results Gao et al., (2006) [42].

For the hypoxia stress experiment, every 30 shrimp in three tanks were maintained within the treatment tanks for 0, 3, 6, 12, and 24 h by nitrogen-filled manipulation in the hypoxic conditions (2.0 ± 0.2 mg O_2 L^{-1}). The hypoxic dissolved oxygen value was set on the basis of the previous observations of juvenile *M. nipponense* [43]. A gill of five time points of these shrimp was collected and all of the exposures were conducted in triplicate.

In the white spot syndrome virus (*WSSV*) infection, every 20 healthy shrimp in three tanks were bred for seven days in a laboratory condition, before infection. All of the experimental materials and methods are referenced from Zhao et al. (2017) [44]. The injection positions were between the third and fourth abdominal segments. Hepatopancreas was collected in triplicate at 0, 12, 24, 48, 72, and 96 h after post-inoculation (hpi) with *WSSV*.

4.4. RNA Extraction and cDNA Synthesis

All of the samples mentioned above were dissected out and frozen immediately in liquid nitrogen, and stored at -80 °C until processed. The total RNA was isolated from the different tissues of the prawns using RNAiso Plus Reagent (TaKaRa, Kusatsu, Japan), according to the manufacturer's protocols. At least three shrimp were analyzed for each type of tissue. For all of the RNA samples, the A260/A280 ratios were in the range of 1.9–2.1, and the RNA integrity was verified by 1.2% agarose gel electrophoresis and stored at 80 °C.

Approximately 1 μg of the total RNA from each tissue was reverse-transcribed by the iScript™ cDNA Synthesis Kit Perfect Real Time (BIO-RAD, Hercules, CA, USA), according to the manufacturerr's instructions. Synthesized cDNA were diluted to 2-fold and stored at -20 °C until use.

4.5. Quantitative Real-Time PCR (qPCR)

The qPCR amplification was performed in a total volume of 25 μL, which contained 1 μL cDNA (50 ng), 10 μL SsoFast™ EvaGreen Supermix (BIO-RAD, Hercules, CA, USA), 0.5 μL (10 μM) of the primers (Table 1), and 13 μL of nuclease-free water. The reaction mixture was initially incubated at 95 °C for 30 s to activate the Hot Start Taq DNA polymerase, followed by 40 cycles at 95 °C for 10 s and 60 °C for 10 s, and a melting cure analysis was performed at the end of the qPCR reaction at 65–95 °C (with increments of 0.5 °C) for 10 s. The differences of the expressions turned out to be significant ($p < 0.05$).

4.6. Methods for Analyzing Stability of Reference Genes

The expression stabilities of the candidate reference genes for the tissues and embryos were analyzed separately using RefFinder, an algorithm that integrates four widely used computational programmes for the analysis of the expression stability, namely GeNorm, NormFinder, BestKeeper, and the comparative ΔC_t method. Briefly, based on the rankings provided by individual programmes, RefFinder assigns an appropriate weight to each gene and calculates the geometric mean of the weights, in order to give an overall ranking [23].

4.7. Validation of Reference Genes by SST Gene RNA Interference

For the in vivo dsRNA injection experiment, 50 healthy mature male prawns (weights of 2.4 ± 0.6 g) were assigned to two groups. The experimental group (N = 25) was injected with *SST* dsRNA. Each prawn was injected with *SST* dsRNA through the pericardial cavity membrane of the

carapace at a dose of 4 μg/g.b.w. The control group (*N* = 25) was injected with diethy pyrocarbonate water at volumes equivalent to those applied to the experimental group (based on gram body weight). After the injection, 10 prawns from each group were randomly collected on the seventh day. The primer for the dsRNA of the *SST*, named *dsSST*, was designed using Snap Dragon tools (available online: http://www.flyrnai.org/cgi-bin/RNAi_find_primers.pl), and it is displayed in Table A1. The primers used for the qPCR of the *SST* was referred to in Jin et al. (2014) [39]. The purity and integrity of the double-stranded RNA (dsRNA) followed the instructions of the Transcript AidTMT7 High Yield Transcription kit (Fermentas, Waltham, USA).

Abbreviations

qPCR	quantitative real-time reverse transcription PCR
ORF	open reading frame
RNAi	RNA interference
M. nipponense	*Macrobrachium nipponense*

Appendix A

Table A1. Primers for validating open reading frames.

Abbreviation	Gene Name	Primer Sequence (5′–3′) Forward/Reverse	Genbank Accession No.
EIF	*eukaryotic translation initiation factor 5A*	GTTGTATGCAGTCGGCCATATTT TGTCCTGAAGGTGGTGATAATGA	MH540106
18S	*18S ribosomal RNA*	CTCCCCCTGAAGTGTATTATGGA GCGAATCTTTTTCAGTTGTTCCC	MH540107
EF-1α	*elongation factor 1-alpha*	CCTGGTATGGTTGTCACTTTTGC CTCCTTCTTCGACACCTCTTTGA	MH540108
GAPDH	*glyceraldehyde-3-phosphate dehydrogenase*	CCAAGGACATGAAGGTAGTCTCC TTCTGCATGTGCTTCAACAAGTC	MH540109
TUB	*α-tubulin*	GGTCTGGAATTCAGTCAAGTCG AGTGCATCTCAGTTCATGTTGG	MH540110
β-act	*β-actin*	CTCTTCTTCCCTGGAGAAGTCTTA ATCCACATCTGTTGGAAGGTAGA	MH540111
RPL18	*ribosomal protein L18*	ACTTTTTGTACCCACAGCTTGAC GTGAAGGGCGAATCTTTGTTGTT	MH540112
dsSST	*slow-tonic S2 tropomyosin*	TAATACGACTCACTATAGGGCGAG AGGTCTGAGGAACGAC TAATACGACTCACTATAGGGTAAG CTTCCTCACGCTGGTT	

Table A2. Ranking of reference genes under different conditions by different methods.

Experimental Conditions	Method	Rank						
		1	2	3	4	5	6	7
Different adult tissues	Comparative ΔC$_t$	*RPL18*	*EIF*	*β-act*	*18S*	*TUB*	*GAPDH*	*EF-1α*
	BestKeeper	*RPL18*	*EIF*	*18S*	*β-act*	*TUB*	*GAPDH*	*EF-1α*
	NormFinder	*RPL18*	*EIF*	*β-act*	*18S*	*GAPDH*	*TUB*	*EF-1α*
	GeNorm	*EIF/RPL18*		*18S*	*β-act*	*TUB*	*GAPDH*	*EF-1α*
	Recommended comprehensive ranking	*RPL18*	*EIF*	*18S*	*β-act*	*TUB*	*GAPDH*	*EF-1α*
Different ovarian stages	Comparative ΔC$_t$	*β-act*	*EIF*	*RPL18*	*18S*	*EF-1α*	*TUB*	*GAPDH*
	BestKeeper	*EIF*	*EF-1α*	*β-act*	*18S*	*TUB*	*RPL18*	*GAPDH*
	NormFinder	*EIF*	*β-act*	*EF-1α*	*RPL18*	*18S*	*TUB*	*GAPDH*
	GeNorm	*18S/RPL18*		*β-act*	*EIF*	*EF-1α*	*TUB*	*GAPDH*
	Recommended comprehensive ranking	*EIF*	*β-act*	*RPL18*	*18S*	*EF-1α*	*TUB*	*GAPDH*
Different embryo stages	Comparative ΔC$_t$	*EIF*	*β-act*	*TUB*	*18S*	*GAPDH*	*RPL18*	*EF-1α*
	BestKeeper	*18S*	*EIF*	*β-act*	*TUB*	*RPL18*	*GAPDH*	*EF-1α*
	NormFinder	*EIF*	*β-act*	*GAPDH*	*TUB*	*18S*	*RPL18*	*EF-1α*
	GeNorm	*18S/TUB*		*EIF*	*β-act*	*GAPDH*	*RPL18*	*EF-1α*
	Recommended comprehensive ranking	*EIF*	*18S*	*β-act*	*TUB*	*GAPDH*	*RPL18*	*EF-1α*
Hypoxia stress	Comparative ΔC$_t$	*β-act*	*GAPDH*	*EIF*	*18S*	*EF-1α*	*TUB*	*RPL18*
	BestKeeper	*EIF*	*TUB*	*GAPDH*	*β-act*	*18S*	*RPL18*	*EF-1α*
	NormFinder	*β-act*	*GAPDH*	*EIF*	*18S*	*TUB*	*EF-1α*	*RPL18*
	GeNorm	*GAPDH/β-act*		*EIF*	*18S*	*EF-1α*	*TUB*	*RPL18*
	Recommended comprehensive ranking	*β-act*	*GAPDH*	*EIF*	*18S*	*TUB*	*EF-1α*	*RPL18*
WSSV infection	Comparative ΔC$_t$	*EIF*	*RPL18*	*GAPDH*	*TUB*	*EF-1α*	*β-act*	*18S*
	BestKeeper	*RPL18*	*EIF*	*TUB*	*GAPDH*	*EF-1α*	*18S*	*β-act*
	NormFinder	*RPL18*	*EIF*	*GAPDH*	*TUB*	*β-act*	*EF-1α*	*18S*
	GeNorm	*EIF /TUB*		*GAPDH*	*RPL18*	*EF-1α*	*β-act*	*18S*
	Recommended comprehensive ranking	*EIF*	*RPL18*	*TUB*	*GAPDH*	*EF-1α*	*β-act*	*18S*

Table A3. Reference gene expression stability values based on several programs.

	Rank	ΔCt		BestKeeper		NormFinder		GeNorm	
		Genes	Average of Std dev	Genes	Std dev	Genes	Stability Index	Genes	Stability Value
Different adult tissues	1	*RPL18*	2.22	*RPL18*	0.83	*RPL18*	0.252	*EIF/RPL18*	0.392
	2	*EIF*	2.24	*EIF*	0.94	*EIF*	0.542		
	3	*β-act*	2.52	*18S*	1.10	*β-act*	1.767	*18S*	1.354
	4	*18S*	2.66	*β-act*	1.38	*18S*	1.934	*β-act*	1.55
	5	*TUB*	2.91	*TUB*	1.93	*GAPDH*	2.128	*TUB*	1.705
	6	*GAPDH*	3.07	*GAPDH*	2.16	*TUB*	2.369	*GAPDH*	2.192
	7	*EF-1α*	4.65	*EF-1α*	3.74	*EF-1α*	4.462	*EF-1α*	2.895
Different ovarian stages	1	*β-act*	0.85	*EIF*	0.87	*EIF*	0.439	*18S/RPL18*	0.319
	2	*EIF*	0.88	*EF-1α*	1.11	*β-act*	0.506		
	3	*RPL18*	0.91	*β-act*	1.25	*EF-1α*	0.609	*β-act*	0.388
	4	*18S*	0.95	*18S*	1.3	*RPL18*	0.637	*EIF*	0.513
	5	*EF-1α*	0.99	*TUB*	1.38	*18S*	0.749	*EF-1α*	0.653
	6	*TUB*	1.2	*RPL18*	1.5	*TUB*	1.032	*TUB*	0.892
	7	*GAPDH*	1.33	*GAPDH*	1.57	*GAPDH*	1.218	*GAPDH*	1.016
Different embryo stages	1	*EIF*	2.37	*18S*	1.89	*EIF*	0.642	*18S/TUB*	1.165
	2	*β-act*	2.47	*EIF*	1.73	*β-act*	0.786		
	3	*TUB*	2.74	*β-act*	5.07	*GAPDH*	1.775	*EIF*	1.44
	4	*18S*	2.77	*TUB*	3.11	*TUB*	1.906	*β-act*	1.578
	5	*GAPDH*	2.84	*RPL18*	2.55	*18S*	2.059	*GAPDH*	1.724
	6	*RPL18*	3.36	*GAPDH*	2.3	*RPL18*	2.295	*RPL18*	2.24
	7	*EF-1α*	5.35	*EF-1α*	2.92	*EF-1α*	5.137	*EF-1α*	3.129

Table A3. *Cont.*

	Rank	ΔCt		BestKeeper		NormFinder		GeNorm	
		Genes	Average of Std dev	Genes	Std dev	Genes	Stability Index	Genes	Stability Value
Hypoxia stress	1	*β-act*	0.6	*EIF*	0.49	*β-act*	0.092	*GAPDH/β-act*	0.185
	2	*GAPDH*	0.66	*TUB*	0.57	*GAPDH*	0.316		
	3	*EIF*	0.7	*GAPDH*	0.61	*EIF*	0.323	*EIF*	0.434
	4	*18S*	0.74	*β-act*	0.67	*18S*	0.45	*18S*	0.532
	5	*EF-1α*	0.95	*18S*	0.85	*TUB*	0.822	*EF-1α*	0.631
	6	*TUB*	0.97	*RPL18*	0.93	*EF-1α*	0.828	*TUB*	0.72
	7	*RPL18*	1.01	*EF-1α*	1.06	*RPL18*	0.912	*RPL18*	0.803
WSSV infection	1	*EIF*	0.66	*RPL18*	0.31	*RPL18*	0.297	*EIF/TUB*	0.397
	2	*RPL18*	0.67	*EIF*	0.32	*EIF*	0.334		
	3	*GAPDH*	0.69	*TUB*	0.38	*GAPDH*	0.391	*GAPDH*	0.454
	4	*TUB*	0.72	*GAPDH*	0.41	*TUB*	0.424	*RPL18*	0.489
	5	*EF-1α*	0.91	*EF-1α*	0.46	*β-act*	0.737	*EF-1α*	0.593
	6	*β-act*	0.92	*18S*	0.6	*EF-1α*	0.748	*β-act*	0.703
	7	*18S*	1.06	*β-act*	0.68	*18S*	0.929	*18S*	0.805

References

1. Bustin, S.A. Quantification of mRNA using real-time reverse transcription PCR (RTPCR): Trends and problems. *J. Mol. Endocrinol.* **2002**, *29*, 23–39. [CrossRef] [PubMed]

2. Bustin, S.A.; Nolan, T. Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. *J. Biomol. Tech.* **2004**, *15*, 155–166. [PubMed]

3. Bustin, S.A.; Benes, V.; Garson, J.A.; Hellemans, J.; Huggett, J.; Kubista, M.; Mueller, R.; Nolan, T.; Pfaffl, M.W.; Shipley, G.L.; et al. The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* **2009**, *55*, 611–622. [CrossRef] [PubMed]

4. Radonić, A.; Thulke, S.; Mackay, I.M.; Landt, O.; Siegert, W.; Nitsche, A. Guideline to reference gene selection for quantitative real-time PCR. *Biochem. Biophys. Res. Commun.* **2004**, *313*, 856–862. [CrossRef] [PubMed]

5. Thellin, O.; Zorzi, W.; Lakaye, B.; De Borman, B.; Coumans, B.; Hennen, G.; Grisar, T.; Igout, A.; Heinen, E. Housekeeping genes as internal standards: Use and limits. *J. Biotechnol.* **1999**, *75*, 291–295. [CrossRef]

6. Vandesompele, J.; De Preter, K.; Pattyn, F.; Poppe, B.; Van Roy, N.; De Paepe, A.; Speleman, F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **2002**, *3*, 1–12. [CrossRef]

7. Huggett, J.; Dheda, K.; Bustin, S.; Zumla, A. Real-time RT-PCR normalisation; strategies and considerations. *Genes Immun.* **2005**, *6*, 279–284. [CrossRef] [PubMed]

8. Zhang, S.; An, S.; Li, Z.; Wu, F.; Yang, Q.; Liu, Y.; Cao, J.; Zhang, H.; Zhang, Q.; Liu, X. Identification and validation of reference genes for normalization of gene expression analysis using qRT-PCR in *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Gene* **2015**, *555*, 393–402. [CrossRef] [PubMed]

9. Jiang, H.; Qian, Z.; Lu, W.; Ding, H.; Yu, H.; Wang, H.; Li, J. Identification and characterization of reference genes for normalizing expression data from red swamp crawfish *Procambarus clarkii. Int. J. Mol. Sci.* **2015**, *16*, 21591–21605. [CrossRef] [PubMed]

10. Wang, E.; Wang, K.; Chen, D.; Wang, J.; He, Y.; Long, B.; Yang, L.; Yang, Q.; Geng, Y.; Huang, X.; et al. Evaluation and selection of appropriate reference genes for real-time quantitative PCR analysis of gene expression in Nile tilapia (*Oreochromis niloticus*) during vaccination and infection. *Int. J. Mol. Sci.* **2015**, *16*, 9998–10015. [CrossRef] [PubMed]

11. Subramoniam, T. *Sexual Biology and Reproduction in Crustaceans*, 1st ed.; Academic Press: Amsterdam, The Netherlands, 2016.

12. Dhar, A.K.; Bowers, R.M.; Licon, K.S.; Veazey, G.; Read, B. Validation of reference genes for quantitative measurement of immune gene expression in shrimp. *Mol. Immunol.* **2009**, *46*, 1688–1695. [CrossRef] [PubMed]

13. Leelatanawit, R.; Klanchui, A.; Uawisetwathana, U.; Karoonuthaisiri, N. Validation of reference genes for real-time PCR of reproductive system in the black tiger shrimp. *PLoS ONE* **2012**, *7*, 1–10. [CrossRef] [PubMed]

14. Cottin, D.; Shillito, B.; Chertemps, T.; Thatje, S.; Léger, N.; Ravaux, J. Comparison of heat-shock responses between the hydrothermal vent shrimp *Rimicaris exoculata* and the related coastal shrimp *Palaemonetes varians*. *J. Exp. Marine Biol. Ecol.* **2010**, *393*, 9–16. [CrossRef]

15. Priyadarshi, H.; Das, R.; Kumar, A.P.; Babu-Gireesh, P.; Javed, H.; Krishna, G.; Marappan, M.; Chaudhari, A. Characterization and evaluation of selected house-keeping genes for quantitative RT-PCR in *Macrobrachium rosenbergii* morphotypes. *Fish. Technol.* **2015**, *52*, 177–183.

16. Jaramillo, M.L.; Ammar, D.; Quispe, R.L.; Guzman, F.; Margis, R.; Nazari, E.M.; Müller, Y.M. Identification and evaluation of reference genes for expression studies by RT-qPCR during embryonic development of the emerging model organism, *Macrobrachium olfersii*. *Gene* **2017**, *598*, 97–106. [CrossRef] [PubMed]

17. Jin, S.; Fu, H.; Sun, S.; Jiang, S.; Xiong, Y.; Gong, Y.; Qiao, H.; Zhang, W.; Wu, Y. Integrated analysis of microRNA and mRNA expression profiles during the sex-differentiation sensitive period in oriental river prawn, *Macrobrachium nipponense*. *Sci. Rep.* **2017**, *7*, 12011. [CrossRef] [PubMed]

18. Sun, S.; Xuan, F.; Fu, H.; Ge, X.; Zhu, J.; Qiao, H.; Jin, S.; Zhang, Y. Comparative proteomic study of the response to hypoxia in the muscle of Oriental River prawn (*Macrobrachium nipponense*). *J. Proteom.* **2016**, *138*, 115–123. [CrossRef] [PubMed]

19. Zhang, W.Y.; Jiang, S.F.; Xiong, Y.W.; Fu, H.T.; Qiao, H.; Sun, S.M.; Gong, Y.S.; Jin, S.B. Molecular cloning and expression analysis of *female sterile homeotic* gene (fsh) in the oriental river prawn *Macrobrachium nipponense*. *Genet. Mol. Res.* **2015**, *14*, 4318–4330. [CrossRef] [PubMed]

20. Andersen, C.L.; Jensen, J.L.; Ørntoft, T.F. 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 Res.* **2004**, *64*, 5245–5250. [CrossRef] [PubMed]

21. Pfaffl, M.W.; Tichopad, A.; Prgomet, C.; Neuvians, T.P. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper—Excel-based tool using pair-wise correlations. *Biotechnol. Lett.* **2004**, *26*, 509–515. [CrossRef] [PubMed]

22. Silver, N.; Best, S.; Jiang, J.; Thein, S.L. Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. *BMC Mol. Biol.* **2006**, *7*, 33. [CrossRef] [PubMed]

23. Xie, F.; Xiao, P.; Chen, D.; Xu, L.; Zhang, B. miRDeepFinder: A miRNA analysis tool for deep sequencing of plant small RNAs. *Plant Mol. Biol.* **2012**, *80*, 75–84. [CrossRef] [PubMed]

24. Qiao, H.; Fu, H.; Xiong, Y.; Jiang, S.; Zhang, W.; Sun, S.; Jin, S.; Gong, Y.; Wang, Y.; Shan, D.; et al. Molecular insights into reproduction regulation of female Oriental River prawns *Macrobrachium nipponense* through comparative transcriptomic analysis. *Sci. Rep.* **2017**, *7*, 12161. [CrossRef] [PubMed]

25. Gu, X.; Fu, H.; Sun, S.; Qiao, H.; Zhang, W.; Jiang, S.; Xiong, Y.; Jin, S.; Gong, Y.; Wu, Y. Dietary cholesterol-induced transcriptome differences in the intestine, hepatopancreas, and muscle of Oriental River prawn *Macrobrachium nipponense*. *Comp. Biochem. Physicol. Part D Genom. Proteom.* **2017**, *23*, 39–48. [CrossRef] [PubMed]

26. Qiao, H.; Fu, H.; Jin, S.; Wu, Y.; Jiang, S.; Gong, Y.; Xiong, Y. Constructing and random sequencing analysis of normalized cDNA library of testis tissue from oriental river prawn (*Macrobrachium nipponense*). *Comp. Biochem. Physicol. Part D Genom. Proteom.* **2012**, *7*, 268–276. [CrossRef] [PubMed]

27. Jin, S.; Fu, H.; Zhou, Q.; Sun, S.; Jiang, S.; Xiong, Y.; Gong, Y.; Qiao, H.; Zhang, W. Transcriptome analysis of androgenic gland for discovery of novel genes from the oriental river prawn, *Macrobrachium nipponense*, using Illumina Hiseq 2000. *PLoS ONE* **2013**, *8*, e76840. [CrossRef] [PubMed]

28. Krzywinski, M.; Altman, N. Points of significance: Visualizing samples with box plots. *Nat. Methods* **2014**, *11*, 119–120. [CrossRef] [PubMed]

29. Bai, H.; Qiao, H.; Li, F.; Fu, H.; Sun, S.; Zhang, W.; Jin, S.; Gong, Y.; Jiang, S.; Xiong, Y. Molecular characterization and developmental expression of vitellogenin in the oriental river prawn *Macrobrachium nipponense* and the effects of RNA interference and eyestalk ablation on ovarian maturation. *Gene* **2015**, *562*, 22–31. [CrossRef] [PubMed]

30. Li, F.; Qiao, H.; Fu, H.; Sun, S.; Zhang, W.; Jin, S.; Jiang, S.; Gong, Y.; Xiong, Y.; Wu, Y.; et al. Identification and characterization of opsin gene and its role in ovarian maturation in the oriental river prawn *Macrobrachium nipponense*. *Comp. Biochem. Phys. Part B Biochem. Mol. Biol.* **2018**, *218*, 1–12. [CrossRef] [PubMed]

31. Subramoniam, T. Mechanisms and control of vitellogenesis in crustaceans. *Fish. Sci.* **2011**, *77*, 1–21. [CrossRef]

32. Eissa, N.; Hussein, H.; Wang, H.; Rabbi, M.F.; Bernstein, N.; Ghia, J.-E. Stability of reference genes for messenger RNA quantification by real-time PCR in mouse dextran sodium sulfate experimental colitis. *PLoS ONE* **2016**, *11*, e0156289. [CrossRef] [PubMed]

33. Zhang, Y.; Han, X.; Chen, S.; Zheng, L.; He, X.; Liu, M.; Qiao, G.; Wang, Y.; Zhuo, R. Selection of suitable reference genes for quantitative real-time PCR gene expression analysis in *Salix matsudana* under different abiotic stresses. *Sci. Rep.* **2017**, *7*, 40290. [CrossRef] [PubMed]

34. Teste, M.A.; Duquenne, M.; François, J.M.; Parrou, J.L. Validation of reference genes for quantitative expression analysis by real-time RT-PCR in *Saccharomyces cerevisiae*. *BMC Mol. Biol.* **2009**, *10*, 99. [CrossRef] [PubMed]

35. Maksup, S.; Supaibulwatana, K.; Selvaraj, G. High-quality reference genes for quantifying the transcriptional responses of *Oryza sativa* L. (ssp. *indica* and *japonica*) to abiotic stress conditions. *Chin. Sci. Bull.* **2013**, *58*, 1919–1930.

36. Xu, H.; Li, C.; Zeng, Q.; Agrawal, I.; Zhu, X.; Gong, Z. Genome-wide identification of suitable zebrafish *Danio rerio* reference genes for normalization of gene expression data by RT-qPCR. *J. Fish Biol.* **2016**, *88*, 2095–2110. [CrossRef] [PubMed]

37. Cheng, D.; Zhang, Z.; He, X.; Liang, G. Validation of reference genes in *Solenopsis invicta* in different developmental stages, castes and tissues. *PLoS ONE* **2013**, *8*, e57718. [CrossRef] [PubMed]

38. Nakamura, A.M.; Chahad-Ehlers, S.; Lima, A.L.; Taniguti, C.H.; Sobrinho, I., Jr.; Torres, F.R.; de Brito, R.A. Reference genes for accessing differential expression among developmental stages and analysis of differential expression of OBP genes in *Anastrepha obliqua*. *Sci. Rep.* **2016**, *6*, 17480. [CrossRef] [PubMed]

39. Jin, S.; Jiang, S.; Xiong, Y.; Qiao, H.; Sun, S.; Zhang, W.; Li, F.; Gong, Y.; Fu, H. Molecular cloning of two tropomyosin family genes and expression analysis during development in oriental river prawn, *Macrobrachium nipponense*. *Gene* **2014**, *546*, 390–397. [CrossRef] [PubMed]

40. Kubista, M.; Andrade, J.M.; Bengtsson, M.; Forootan, A.; Jonák, J.; Lind, K.; Sindelka, R.; Sjöback, R.; Sjögreen, B.; Strömbom, L.; et al. The real-time polymerase chain reaction. *Mol. Asp. Med.* **2006**, *27*, 95–125. [CrossRef] [PubMed]

41. Qiao, H.; Xiong, Y.; Zhang, W.; Fu, H.; Jiang, S.; Sun, S.; Bai, H.; Jin, S.; Gong, Y. Characterization, expression, and function analysis of gonad-inhibiting hormone in Oriental River prawn, *Macrobrachium nipponense* and its induced expression by temperature. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* **2015**, *185*, 1–8. [CrossRef] [PubMed]

42. Gao, X.G.; Liu, H.; Xu, J.N.; Cai, S.L. Study on site of vitellogenin synthesis in the freshwater prawn *Macrobrachium nipponese*. *Biotechnol. Bull.* **2006**, 438–444. Available online: http://en.cnki.com.cn/Article_en/CJFDTotal-SWJT2006S1096.htm (accessed on 1 August 2018). (In Chinese)

43. Sun, S.M.; Xuan, F.J.; Ge, X.P.; Fu, H.T.; Zhu, J.; Zhang, S.Y. Identification of differentially expressed genes in hepatopancreas of oriental river prawn, *Macrobrachium nipponense* exposed to environmental hypoxia. *Gene* **2014**, *534*, 298–306. [CrossRef] [PubMed]

44. Zhao, C.; Fu, H.; Sun, S.; Qiao, H.; Zhang, W.; Jin, S.; Jiang, S.; Xiong, Y.; Gong, Y. Experimental inoculation of oriental river prawn *Macrobrachium nipponense* with white spot syndrome virus (*WSSV*). *Dis. Aquat. Organ.* **2017**, *126*, 125–134. [CrossRef] [PubMed]