Selection and Assessment of Reference Genes for Quantitative PCR Normalization in Migratory Locust *Locusta migratoria* (Orthoptera: Acrididae)

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**Abstract**

*Locusta migratoria* is a classic hemimetamorphosis insect and has caused widespread economic damage to crops as a migratory pest. Researches on the expression pattern of functional genes in *L. migratoria* have drawn focus in recent years, especially with the release of genome information. Real-time quantitative PCR is the most reproducible and sensitive approach for detecting transcript expression levels of target genes, but optimal internal standards are key factors for its accuracy and reliability. Therefore, it's necessary to provide a systematic stability assessment of internal control for well-performed tests of target gene expression profile. In this study, twelve candidate genes (*Ach, Act, Chl2, EF1α, RPL32, Hsp70, Tub, RP49, SDH, GAPDH, 18S*, and *His*) were analyzed with four statistical methods: the delta Ct approach, geNorm, Bestkeeper and NormFinder. The results from these analyses aimed to choose the best suitable reference gene across different experimental situations for gene profile study in *L. migratoria*. The result demonstrated that for different developmental stages, *EF1α, Hsp70 and RPL32* exhibited the most stable expression status for all samples; *EF1α* and *RPL32* were selected as the best reference genes for studies involving embryo and larval stages, while *SDH* and *RP49* were identified for adult stage. The best-ranked reference genes across different tissues are *RPL32, Hsp70* and *RP49*. For abiotic treatments, the most appropriate genes we identified were as follows: *Act* and *SDH* for larvae subjected to different insecticides; *RPL32* and *Ach* for larvae exposed to different temperature treatments; and *Act* and *Ach* for larvae suffering from starvation. The present report should facilitate future researches on gene expression in *L. migratoria* with accessibly optimal reference genes under different experimental contexts.

**Introduction**

In biological research, the fluorescence-based quantitative real-time reverse transcriptase PCR (qPCR) is the most reproducible and sensitive approach for gene expression analysis and has been widely used to measure and compare levels of gene transcription [1–3]. Although the technique of qPCR is usually described as the gold standard, the quality of results is influenced by several variables, including RNA stability, quantity, purity, reverse transcription efficiency and PCR efficiency [4,5]. To avoid bias, a number of strategies have been proposed for normalization in previous studies [6], including sampling similar tissue weight or volume to ensure similar sample size [6], targeting genomic DNA [7] and using an ‘artificial’ RNA molecule [4]. The strategy based on sample size may be straightforward, but it may not be biologically representative because that different samples may not contain the same cellular material [6]. The method based on genomic DNA is rarely used, as the copy number per cell may vary and DNA is usually eliminated during the RNA extraction procedures. The normalization approach using an artificial molecule remains an unvalidated theoretical ideal. Therefore, the most suitable method for mRNA quantification is to include internal standards, which are mainly housekeeping genes.

The transcription levels of these widely used housekeeping genes, including β-actin, 18S ribosomal RNA (18S rRNA), elongation factor 1-α (*EF1α*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) [8,9] had been assumed to have uniform levels of expression that are unaffected by experimental conditions since these genes are necessary in fundamental cellular processes [10]. These genes have been used as single internal standards for mRNA transcript levels can differ from actual expression even up to 20-fold if the normalization gene is regulated by the experimental conditions [19–21]. Therefore, it’s a key point to assure the expression of internal reference genes occur at a constant level. The endogenous control genes should be validated in different organism and for each specific experiment [10,22–27].
The migratory locust *Locusta migratoria* is the most common locust subspecies that is widely distributed in eastern and southern Asia including China, Cambodia, Indonesia, Japan and Thailand [20]. As a phytophagous insect, this pest feeds on gramineous and bulrush plants, and causes widespread economic damage to crops. Additionally, destructive outbreaks of locust is periodic [29]. In recent years, with the development of molecular technology and the release of the whole genome information of *L. migratoria*, qPCR has been widely used for study on phase changes [30] and gene expression in *L. migratoria* [31]. The systemic assessment of suitable internal control genes has been reported in several model insects such as *Bombyx mori* [32], *Apis mellifera* [33,34], and *Tribolium castaneum* [35]. For locusts, the initial several reference genes were validated for the brains of *Schistocerca gregaria* [36]. And then reference genes for locust were evaluated for *Chortoicetes terminifera* reared under different density treatments [37] and *L. migratoria* under hypobaric hypoxia stress [38]. There is no experimental data available on a systematic selection and assessment of reference genes in *L. migratoria* for gene profile analyses covering the commonly involved biotic and abiotic experimental contexts.

In this report, we analyzed the performance of twelve normalization genes (Ach, Act, Hsp70, 18S, EF1a, SDH, RPL32, His, Chit2, GAPDH, Tub, and RP49) for *L. migratoria* in a set of biotic factors (embryo stage, larvae stage, adult stage and 13 tissues) and under three abiotic stresses (insecticide, temperature and starvation). This work will provide benefits for identifying normalization genes in future gene expression studies in *L. migratoria*, saving time and expense in selecting reference genes.

**Materials and Methods**

**Ethics Statement**

For this study, there were no specific permits being required for the insect collected. The eggs of *L. migratoria* were originally collected from Cangzhou (38°13′12″N 116°59′24″E), Hebei Province, China. No endangered or protected species were involved in the field studies. The “List of Protected Animals in China” does not contain the migratory locust *Locusta migratoria* (Orthoptera: Acrididae) which are common insect.

**Insect Rearing**

The egg pods of *L. migratoria* were incubated in wet sand in an environmental chamber (Ningbo, China) at 30±1°C with a 14 h:10 h (L:D) photoperiod and 55% humidity. Grasshoppers were transferred to the laboratory after hatching and fed with fresh wheat seedlings, crop leaves and bran [30]. Pots filled with slightly moistened sterile sand were prepared for mature females to deposit their eggs. After oviposition, the egg pods were collected every day and incubated in an environmental chamber as described as above.

**Biotic factors**

**Embryo.** The eggs were collected from the fourth day after oviposion until the thirteenth day. Embryo was dissected from eggs in PBS solution (10 mmol Na2HPO4, 2 mmol/L KH2PO4, 157 mmol/L NaCl, 2.7 mmol/L KCl, pH 7.4) on ice.

**Larvae and adult.** Samples used in the studied comprised 10 first-instar nymphs, 8 second-instar nymphs, 5 third-instar nymphs, 3 fourth-instar nymphs, 2 fifth-instar nymphs, 1 male and female adults (collected at the first day and the tenth day after emergence) for each replication.

**Tissue.** Thirteen tissues and organs were obtained from adults using a dissection needle in ice-cold PBS solution [39]. Tissues included brain, antenna, wings, fore legs, middle legs, hind legs, Malpighian tube, ovary, testis, midgut, epidermis, hemolymph and fat body.

All the samples were kept in −80°C after snap frozen in liquid nitrogen for subsequent RNA extraction. We prepared three biological replications for every sample.

**Abiotic Stresses**

**Insecticide-induced stress.** Four insecticides, chlorpyrifos, cyhalothrin, acetamiprid, and chlorantraniliprole, were used in this study. These are commonly used insecticides in Orthoptera pest management programs. The method for insecticide bioassay was the leaf-dip bioassay. Crop discs (3 cm diameter) were dipped for 10 sec in distilled water solutions of formulated insecticide with 0.1% Triton X-100 and air-dried at 25°C for 3 h according to the leaf-dip bioassay [40] which was usually used for insecticide bioassay. The discs were then placed inside transparent plastic cups (6.5 cm×5.0 cm×5.5 cm) covered with clean gauze. For each replication, ten third-instar larvae that had been starved for 6 h were placed in the cup with four discs inside, and three replications were conducted. Five different concentrations were tested for each pesticide. For the controls, the crop discs were dipped in distilled water containing only 0.1% Triton X-100. Other treatments were the same as described above.

The larvae fed on treated crop discs were then reared under normal conditions. After 48 h, we checked for mortality. Mortality data from insecticide bioassays were analyzed for LG15 (sublethal dose) values and the SPSS program 17.0 was used to calculate their 95% confidence intervals based on probit analysis (Table S1). Third-instar nymphs were subjected to each insecticide with LG15 values derived from the toxicity test above. After 48 h rearing under routine conditions, five surviving nymphs were used for RNA extraction as one replication.

**Temperature-induced stress.** Third-instar larvae were transferred directly into thin glass test tubes (2 cm×8 cm) covered by gauze from their rearing conditions (30°C, 14L:12D) for incubation under series of temperatures (0°C, 15°C, 30°C, 36°C, 40°C) for 2 h [41]. For the 0°C incubation, the glass tubes were placed in an ice water mixture. For temperature treatments, five nymphs were used for RNA extraction as one replication.

**Starvation treatment.** Third-instar locust nymphs were placed in glass test tubes without food for 6 h and 12 h. Five nymphs were assembled for RNA extraction as one replication.

**Total RNA Extraction and cDNA Synthesis**

The RNasy Mini Kit (QIAGEN, Germany) was used to extract total RNA from treated samples following the manufacturer’s instructions. DNase I (RNase-Free DNase set, QIAGEN, Germany) was used to eliminate DNA contamination according to the recommended procedures. A spectrophotometer (NanoDrop-2000, Thermo Scientific) were used to measure the purity and concentration of total RNA for A260/A280 and A260/A230. The integrity of all RNA samples was then verified immediately via agarose gel electrophoresis. If an 18S band was clearly observed, the RNA samples were considered intact. It could not be identified for the band corresponding to 28S RNA because of the ‘hidden break’ present in insects [42]. M-MLV Reverse Transcriptase (Promega, USA) were used to synthesize complementary DNA (cDNA) from 2 μg of total RNA with Oligo(dT)18 primer.

**Candidate reference genes selection**

Twelve housekeeping genes were selected from previous studies in *L. migratoria* and the LocustDB (http://locusdb.genomics.org.cn/). These genes have been selected as reference genes for normalization factors in *L. migratoria* include EF1a (elongation...
factor 1 alpha), RPL32 (ribosomal protein L32), SDH (succinate dehydrogenase) [37], His (histone H3) [43], Hsp70 (heat shock protein) [44], Cal2 (probable chitinase 2), Ach (acetyl-CoA hydrolase), 18S (18S RNA) [30], RP49 (ribosomal protein 49), Tub (α-tubulin 1A), Act (β-actin), and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) [36]. The Primer Premier 5 software (http://www.PremierBiosoft.com/primerdesign/primerdesign.html) was used to design the primers. The parameters in Primer 5 were setting as follows: amplicon length 80–250 bp, melting temperature 58–62°C, primer lengths 15–28 bp, and GC content 40–60% (Table 1).

Quantitative Real time PCR analysis
An Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, USA) were used to perform Quantitative Real time PCR (qPCR) experiments in 96-wells reaction plates using SYBR Premix Ex Taq II (Takara, Japan). Each reaction was run in a 20 μL volume reaction [10 μL 2×SYBR Premix Ex Taq II (Tli RNaseH Plus), 0.4 μL ROX Reference Dye II, 6 μL nuclease-free water, 0.8 μL each primer and 2 μL diluted cDNA]. The reaction program was as follows: 95°C 30 s, followed by 40 cycles (95°C for 5 s, 55°C for 30 s and 72°C for 30 s). At the end of each PCR run, a dissociation protocol (melting curve analysis) was applied to all reactions. Each sample was prepared three technical and biological replicates. To estimate amplification efficiency and correlation coefficient (R²) of each primer pairs, a range of series dilution of cDNA (10-fold) was used to create the five-point standard curve. The equation \( E = [10^{-1/\text{slope}} - 1] \times 100\% \) was used to calculate the qPCR efficiency for each primer [45].

Validation of housekeeping gene selection
To assess the validity of selected internal control genes, the transcription level of the chiitin synthase 1 gene (CHS1) was estimated for different development stages. We compared the mRNA transcript level of CHS1 when using only one reference gene [the best (NF 1) and the worst gene (NF 12)] and two most stable reference genes (NF 1-2) recommended by RefFinder. Expression levels of a detoxification-related gene (GSTs1) were picked to evaluate the validity of selected reference genes in four tissues (midgut, Malpighian tube, fat body and spermatheca) and the third-instar larvae subjected to four insecticides. For the tissues, the expression profiles of the gene GSTs1 were estimated using one reference gene [the most (NF 1) and the least stable reference gene (NF 12)] and several stable reference genes (NF 1-2, NF 1-3, NF 1-3) together recommended by RefFinder. For the insecticide treatment, the method to evaluate the expression of GSTs1 was same as the gene CHS1 in different development stages. When normalizing using more than one internal reference gene, the geometric mean calculated from the cycle threshold values of the included housekeeping genes was used as the normalization factors (NF 1-n) and the algorithm \( \left( 2^{-\Delta \Delta Ct} \right) \) was used to calculate the transcription level of the interested gene. The effect of housekeeping gene selection and usage on the evaluation of interested gene expressions was assessed between those normalized by the least stable reference gene and the recommended combination of reference genes with the highest stability value. T-test was conducted for statistical analysis with software SPSS (ver. 17.0).

Statistical Analysis
The expression stability of twelve selected internal control genes was evaluated with the delta Ct methods, geNorm v. 3.5 [45], Bestkeeper [46] and NormFinder [47]. At the same time, RefFinder, a comprehensive tool (http://www.leonxie.com/referencgene.php), was adopted to assess and rank the selected housekeeping genes. This tool assigned an appropriate weight to an individual gene and calculated the geometric mean of their weights for the overall final ranking according to the results from each program. Raw Ct values were used for Bestkeeper and RefFinder. For the NormFinder and geNorm software programs, Ct values should be transformed to linear scale expression quantities.

Results
The Quality of Total RNA
In this study, the ratio A260/A280 of total RNA obtained from all samples ranged from 1.90 to 2.10 and A260/230 was above 1.90, indicating that all total RNA were adequately free from organic salts and protein contamination. The concentration of total RNA varied from 800 ng/μl to 2000 ng/μl, which was appropriate for synthesizing cDNA template.

PCR Amplification Efficiencies
For each set of the primer pairs, firstly, twelve candidate reference genes and two target genes were checked by normal PCR which produced a single amplicon with expected size. Then the dissociation curve derived from qPCR with single-peak confirmed the unique amplification and no primer dimer formation. Standard curve method was adopted to calculate the amplification efficiency of each primer pairs with cDNA isolated from third-instar nymphs in ten-fold serial dilution. The PCR efficiency of all the primer pairs ranked from 91.3% (Tub) to 108.7% (EF2a). The correlation coefficients R² ranged from 0.994 to 0.999 (Table 1).

Expression Profiles of Selected Reference Genes
The cycle threshold (Ct) values were adopted to compare the transcript abundance of the selected genes in different samples, assuming equal Ct on behalf of identical transcript amount, since an equal quantity of total RNA were performed in all qPCR reactions. The mean Ct values of the twelve reference genes varied from 11.96 to 25.35, with the lowest and highest Ct values obtained from 18S (Ct 8.45) and SDH (Ct 33.94) (Figure 1). 18S and EF2a showed the most abundant expression levels followed by Hsp70 (mean Ct 20.11), Act (mean Ct 20.47), RPL32 (mean Ct 20.81), Tub (mean Ct 21.11), RP49 (mean Ct 21.72), GAPDH (mean Ct 22.05) and His (mean Ct 22.97). The moderately abundant transcripts were the two target genes and remaining three reference genes, which had a Ct value of 23 or higher. Figure 1 also showed that the gene 18S displayed the lowest dispersion (6.3 cycles) followed by RPL32 (8.7 cycles). The gene His exhibited highest dispersion over all samples indicated by largest whiskers of the box.

Figure 2 revealed the distribution of relative expression level of the selected genes across different samples. For the biotic factors, RPL32 showed a more constant expression level among samples of different development stages than other candidate genes. The transcript level of SDH was also relatively constant in larvae and adult stages. The transcript levels of His and Cal2 were more stable in different tissues than other reference genes. For abiotic factors, the expression level of Ach was relatively constant in the third-instar larvae under temperature and starvation stress, while the transcript levels of SDH and Tub were more constant after insecticide treatment. These results revealed that there was not one reference gene suitable for all biological samples and experimental treatments.
Table 1. Details of twelve candidate reference genes used for real-time PCR.

| Symbol (AccessionNO.) | Gene name          | Description                                                                 | Primer sequence                | Size (bp) | Tm (°C) | E (%) | R²     |
|------------------------|--------------------|------------------------------------------------------------------------------|--------------------------------|-----------|---------|-------|--------|
| Ach (NP-651762)        | Acetyl-CoA hydrolase | Involved in acetate utilization in mitochondria and fatty acid metabolism    | F:CATGACAGGAGAAAACAGCAATAA     | 126       | 56.1    | 102.3 | 0.999  |
| Act (HQ388822)         | β-actin            | Cytoskeleton                                                                 | F:CGAAGCAGCAGTCAAAGAGGTA       | 156       | 60.2    | 94.2  | 0.999  |
| Hip70 (AY178988)       | Heat shock protein  | A member of 70-kDa heat shock protein                                         | F:CTGGTGTGTCATTACGGTAT          | 100       | 59.7    | 98.8  | 0.999  |
| I85 (AF370793)         | 18S rRNA           | Structural constituent of ribosome                                            | F:CGGTATACGTCAGTCCAAATG         | 121       | 60.1    | 102.4 | 0.999  |
| ERFs (HQ388819)        | Elongation factor 1 α | Translation elongation factor activity; GTPase activity; GTP binding         | F:AGCCCGAGAGATAAGGTTAAG        | 155       | 61.9    | 108.7 | 0.994  |
| SDH (HQ388823)         | Succinate dehydrogenase | Succinate dehydrogenase activity; Succinate-coA ligase activity; FAD binding;electron carrier activity | F:CCACTGAAACTGATCCAAGAGAG   | 98        | 60.2    | 91.5  | 0.997  |
| RPL32 (HQ388820)       | Ribosomal protein L32 | Structural constituent of ribosome                                            | F:ACTGGAAGCTTGATGATGCA         | 97        | 60.1    | 91.8  | 0.997  |
| His (AF370817)         | Histone H3         | A protein involved in the structure of chromatin in eukaryotic cells         | F:CACACGAGGAGGCGAGAAGA          | 250       | 64      | 102   | 0.997  |
| Chi2 (NP_477298)       | Probable chitinase 2 | To hydrolyze chitin                                                           | F:CTCTTATACCCTTGGTTC           | 258       | 60.1    | 94.9  | 0.996  |
| GAPDH (JF_915526)      | Glyceroldehyde-3-phosphate dehydrogenase | Oxidoreductase in glycolysis & Gluconeogenesis                                      | F:GTCGGATGAAAGGAGGGTGCAT      | 81        | 61.9    | 93.2  | 0.998  |
| Tub (JN676091)         | α-tubulin 1A       | Cytoskeletal structure protein                                                | F:TGACAGAGGCGCATCTATG          | 118       | 55.8    | 91.3  | 0.998  |
| RP49 (X00848)          | Ribosomal protein 49 | Translation                                                                  | F:CGCAGATGTCGTTGA              | 66        | 61.9    | 97.3  | 0.999  |
| CHS1 (AF221067.1)      | Chitin synthase 1   | A key enzymes involved in chitin biosynthesis                                | F:CTTGGAGGCAATTTGTGTTG         | 121       | 57.8    | 93.1  | 0.999  |
| GSTs1 (HM138136)       | Glutathione S-transferases | A dimeric enzymes involved in phase II detoxification of hydrophobic toxic compounds | F:CGGAAGATAGAATTTGGAACATG GCC | 181       | 60.1    | 96.9  | 0.998  |

Size: size of amplicon length; Tm: melt temperature; E: PCR efficiency; R²: coefficient of determination.

doi:10.1371/journal.pone.0098164.t001
Expression Stability of Selected Reference Genes

Biometric Factors. For embryo study, the overall ranking order of the best-suited reference genes generated by all the programs, except for Bestkeeper, were coherent, though the stability rankings fluctuated among separated analyses to some extent. Three methods (geNorm, NormFinder, and delta Ct methods) identified the top four ranked genes as RPL32, Hsp70, EF1α and Act for embryo, while Bestkeeper allocated RP49, RPL32, Tub and GAPDH as the four best-suited genes (Figure 3, Table 2). Interestingly, RPL32 was predicated stable by all software packages and EF1α by three programs. Our results displayed Cht2 as the least stable gene for embryo. RefFinder analysis showed the most stable genes were ranked as follows: Cht2 < His < 18S < Ach < GAPDH < SDH < Rp49 < Tub < Act < Hsp70 < RPL32 < EF1α (Table 2). According to the analysis of the pairwise variation, the V value (0.15) was below the default value (0.15) (Figure 4). This indicated that the addition of a third gene didn’t have great effect on normalization process. Therefore, two reference genes were appropriate to normalize gene expression.

For Larvae analysis, the rankings of the best-suited reference genes obtained by the delta Ct approach and geNorm were similar. Additionally, the top two ranked genes identified by geNorm were the same as those generated by NormFinder. The result from Bestkeeper was different from those generated by four other methods. Namely, the delta Ct method and other two programs ranked EF1α as the most stable gene, while GAPDH was ranked in the top position as Bestkeeper (Table 2). Cht2 appeared as the least stable gene for Larvae. RefFinder analysis showed the most stable genes were ranked as follows: Cht2 < Ach < 18S < Hsp70 < RP49 < Tub < Act < His < SDH < GAPDH < RPL32 < EF1α (Table 2). According to the analysis of the pairwise variation, the V value (0.15) was less than 0.15 (Figure 4). This indicated that the addition of a third gene didn’t have a great effect on normalization process. Therefore, two reference genes were appropriate to normalize gene expression.

For adult assessment, it was similar for the rankings of the best-suited reference genes got by the Delta Ct approach and Bestkeeper. They placed RP49, SDH, and RPL32 on the top three positions, although the rank order was slightly altered (Table 2). The top three genes identified by geNorm and NormFinder were largely different from the results generated by delta Ct method. Additionally, the most stable genes from geNorm also differed from those selected by NormFinder. NormFinder placed the gene SDH on the top position followed by Tub and Hsp70, while geNorm select RPL32 and RP49 as the most appropriate candidate genes with the lowest M value (0.054) (Table 2, Figure 3) and EF1α was placed on the third position with the M value (0.107). At the same time, His appeared as the least stable gene by four methods for adult. RefFinder analysis showed the most stable genes were ranked as follows: His < Cht2 < 18S < Ach < GAPDH < Act < EF1α < Hsp70 < Tub < RPL32 < RP49 < SDH (Table 2). According to the analysis of the pairwise variation, the V value (V_{2/3}) was below 0.15 (Figure 4). This indicates the best normalization factors for gene transcript analysis should contain at least two reference genes.

For tissue assay, the top ranked four genes exhibited by delta Ct method for different tissues were RPL32, Hsp70, RP49 and EF1α, which is similar to the results generated by NormFinder and geNorm (Table 2). Additionally, RPL32 was considered as the most appropriate gene by three these algorithms. However, Bestkeeper analysis identified 18S as the best one, followed by Cht2 and His. The gene Hsp70 was ranked as fourth. Likewise, the delta Ct method, geNorm and Normfinder ranked His as the least stable gene, but Bestkeeper selected Act as the least stable gene for all tissue samples. RefFinder analysis ranked the best suitable candidate reference genes as follows: Act < SDH < His < Ach < Cht2 < GAPDH < 18S < Tub < EF1α < Rp49 < Hsp70 < RPL32 (Table 2). According to the analysis of the pairwise variation, the V value (V_{2/3}) was less than the proposed value (0.15), but the V_{2/3} was below under the threshold value of 0.15. This indicates the best normalization factors for gene transcript analysis should contain at least five reference genes.

Abiotic stresses. For assay pretreated with different insecticide, it was similar for the top ranked three genes exhibited by the delta Ct approach and NormFinder, though the rank order was slightly altered. The delta Ct methods selected Act as the most suitable normalization factor for qPCR normalization followed by RPL32 and RP49 (Table 3). RPL32 was identified as the best endogenous control gene by NormFinder, while Act and RP49 were ranked in the second and third position, respectively. However, geNorm and Bestkeeper analysis generated different results. The top three genes identified by geNorm were SDH, Act and EF1α (Figure 3), but Hsp70 was selected as the most stable gene by Bestkeeper analysis followed by Tub and Act. RefFinder analysis showed the most suitable candidate genes were ranked as follows: Cht2 < His < 18S < Ach < GAPDH < EF1α < Tub < RP49 < RPL32 < Hsp70 < SDH < Act (Table 3). According to the analysis of the pairwise variation, the V value (V_{2/3}) was less than 0.15 (Figure 4). Thus, two reference genes were sufficient to normalize gene expression.

For survey after starvation, the top ranked four genes exhibited by NormFinder were Act, Ach, 18S and EF1α, which was the same as the results generated by Bestkeeper (Table 3). What’s more, the ranking orders of these four stable genes were also the same. Additionally, the best-suited reference genes generated by delta Ct analysis were also these four genes, though the stability rankings were slightly different. GeNorm analysis selected 18S and EF1α as the best suitable reference genes (Figure 3). The gene RPL32 was ranked in the third position followed by GAPDH. Interestingly, the candidate gene Cht2 was considered as the most unstable gene by all the algorithms. RefFinder analysis showed the most stable genes were ranked as follows: Cht2 < GAPDH < Tub < His < SDH < RP49 < RPL32 < Hsp70 < EF1α < 18S < Ach < Act (Table 3). The V value (V_{2/3}) was under the proposed value (0.15).
This indicated that the addition of a third gene didn’t have a great effect on normalization process. Therefore, two reference genes were appropriate to normalize gene expression.

For analysis of variant temperature, the most stable reference gene selected by the delta Ct methods was RPL32, which also the best gene identified by geNorm (Table 3). The delta Ct methods identified Tub as the second most stable gene followed by RP49, while geNorm identified RP49 and RPL32 both as the best suitable genes followed by Ach (Figure 3). According to the results of Normfinder analysis, the gene RPL32 was ranked in the third position. The gene SDH appeared as the most stable gene followed by Tub. The Bestkeeper analysis generated different results compared to the three other algorithms. Bestkeeper identified Hsp70 as the most suitable gene for normalizing gene expression and Ach was set in the second position followed by EF1a. All the statistical algorithms indicated Cht2 and His as the two least stable genes. RefFinder analysis showed the expression stability of genes was ranked as follows: His < Cht2 < 18S < Act < GAPDH < Hsp70 < Ach < SDH < EF1a < Tab < RP49 < RPL32 (Table 3). According to the analysis of the pairwise variation, the V2/3-value was under the threshold value (0.15) (Figure 4). Therefore, two reference genes were appropriate to normalize gene expression.

For the total investigated samples, the rankings of candidate reference gene stability obtained by three algorithms containing the delta Ct approach, Normfinder and geNorm were largely similar. These three methods indicated the same three most stable candidate genes (EF1a, Hsp70, RPL32), though the ranking order of these three reference genes were different. However, Bestkeeper generated different results: RPL32 and RP49 appeared as the two best normalization factors followed by 18S. Likewise, all the programs, except for Bestkeeper, selected Cht2 as the worst gene. His was selected as the most unstable reference gene by
Bestkeeper. RefFinder analysis showed the most stable genes were ranked as follows: His < Ch2 < SDH < GAPDH < 18S < Act < Ach < Tub < RP49 < EF1a < RPL32 < Hsp70 (Table S2).

Validation of reference gene selection

To assess the performance of selected reference genes, the transcript level of two target genes (CHS1, GSTs1), were assessed under various experimental conditions. For different development stages, using the best reference gene or the recommended two most stable references to normalize, CHS1 transcript level were higher in adult compared to larvae stages (Figure 5A). However, when the most unstable gene was used to normalize, no evident difference was detected. We also found that the expression level of CHS1 normalized against the combination of two best reference genes was very different from the least stable reference gene
Figure 4. Pairwise variation analysis for an accurate normalization. The pairwise variation analysis is performed by geNorm to determine the optimal number of internal control genes. Each pairwise variation value is compared with 0.15, below which the inclusion of an additional reference gene is not required. doi:10.1371/journal.pone.0098164.g004

(P<0.05) (Figure 5A). For qPCR data analysis in different tissues, if normalized using more than one reference gene (RPL32+Hsp, RPL32+Hsp+RP49, RPL32+Hsp+RP49+EF1a+Tu), the expression profile of GST1 in Malpighian tube and fat body was similar (Figure 5B) and higher compared to the other tissues. Using the single best reference gene as a normalization factor, similar results were found in the Malpighian tube and fat body. However, the transcript level of GST1 was decreased in Malpighian tube when normalized against the least stable reference gene (Hsp) (P<0.05) (Figure 5B). For insecticide treatment, when normalized against the combination of recommended reference genes (RPL32, Act), the expression of GST1 was increased by 1.07-fold for treatment of Cyhalothrin and 1.64 fold for Chlorpyrifos compared with the control insects, but was reduced for the other insecticides. Using the single best reference gene (RPL32) for normalization, similar expression levels were observed. However, notable differences were found when normalized against the least stable gene (Ghi2) (Figure 5C).

Discussion

As an accurate and sensitive method to quantify mRNA transcription levels, qPCR has played a very important role in molecular biology research. Normalization with endogenous reference genes is one very important factor which affects the correct measurement of gene transcript level changes. Researchers are required to prove that the candidate reference gene of choice is appropriate for data normalization of differentially expressed genes under a specific experimental condition. In this report, twelve candidate reference genes in L. migratoria were assessed for expression stability under the context of different development stages, tissues and special experiment treatments (insecticide, temperature and starvation).

The efficiencies of amplification of reference genes are directly related to the quality of results obtained from qPCR. Therefore, we calculated the efficiencies of each candidate gene prior to quantification. Twelve candidate genes were screened out with amplification efficiency above 91%, while genes with extreme efficiency value were excluded for further analysis.

The expression stability of selected internal control genes was evaluated with four statistical algorithms (the delta Ct approach, geNorm, Bestkeeper, and NormFinder). We assumed that a combination of different mathematical models enabled a better evaluation of the most reliable reference genes. However, there were discrepancies of best reference genes and the stability rankings among the different programs (Table 2, Table 3). This was caused by different mathematical models adopted by each program [47–49]. To solve this problem, RefFinder was adopted to comprehensively evaluate and rank reference genes. To evaluate reliable reference genes using different strategies, we need to be aware of their property. GeNorm software is one commonly used statistical method. The underlying principle of this method is that the transcript ratio of two best reference genes should be same in all test samples. This program ranks the reference gene via calculating M value (the average expression stability) for each gene. At the same time, it also revealed the optimal number of reference gene by analyzing the pairwise variation (V). A V score of less than 0.15 was ideal for valid normalization [46]. However, the disadvantage of geNorm was its sensitivity to co-regulation. It usually select genes with the highest degree of similarity in their transcript level [47].

Compared to geNorm, NormFinder was lack of sensitivity to co-regulation of the reference gene. When using the gene to normalize, NormFinder could supply an expression stability value for each gene that empowers the user to analyze the systematic error.

BestKeeper is another Excel-based tool that evaluates expression stability of reference genes based on two variations (SD and CV values). Genes with an SD value higher than 1 were unacceptable. It then determines the best reference gene according to the probability (p) value and the Pearson correlation coefficient (r) calculated from pair-wise correlation analysis of all pairs of selected reference genes [45]. Our results revealed that the rank of most suitable genes obtained from BestKeeper was slightly different from other statistical algorithms. As shown in Table 2, EF1a was ranked at the top position by three programs for embryo stage and ranked fifth by BestKeeper. A similar situation was observed with larvae and tissue samples. This might be caused by the statistical algorithms used by BestKeeper that combines highly correlated reference genes into an index. It usually represents the average of the best suitable reference genes. Therefore, BestKeeper may not be able to sensitively distinguish between stable and unstable reference genes [45,50,51].

The delta Ct approach is similar to that described by the report of Vandesompele for geNorm program, whereby ‘pairs of genes’ are compared [50]. This method uses delta Ct comparisons between genes to bypass the need to accurately quantify input RNA.

Consistent with the reports in Drosophila [51] and Spodoptera litura [52], this study revealed that it’s difficult to identify a universally appropriate reference genes for qPCR analyses, as all the selected reference genes exhibited notable variation of transcript levels under different experimental conditions. Furthermore, the best recommended reference genes were also different. According to RefFinder, which gave the overall final ranking based on the results from each program, EF1a was considered as the best reference gene in embryo and nymph stages, SDH was selected as the most appropriate gene in adult stage, Act was selected as the best gene in insecticide and starvation treatments, and RPL32 appeared to be the best normalization factor in different tissues and temperature treatments.

EF1a, one kind of protein that contributed to binding aminoclyl-transfer RNA to ribosomes during protein synthesis.
Table 2. Expression stability of the candidate reference genes under biotic conditions.

| Biotic | Reference | Delta Ct | Bestkeeper | Genorm | Normfinder | RefFinder |
|--------|-----------|----------|------------|--------|------------|-----------|
|        |           | StdDev   | Rank       | SD     | R          | Rank      |
|        | Gene      |          |            |        |            | M         | Rank      | SV     | Rank | Stability | Rank |
|        |           |          |            |        |            |           |           |        |       |           |      |
| Embryo | 18S       | 1.606    | 9          | 1.550  | 0.831      | 10        | 1.086     | 10     | 0.851  | 10     | 9.740    | 10   |
|        | Ach       | 1.335    | 8          | 1.449  | 0.969*     | 9         | 0.887     | 8      | 0.493  | 5      | 7.326    | 9    |
|        | Act       | 1.148    | 4          | 0.886  | 0.854      | 6         | 0.563     | 4      | 0.486  | 4      | 4.899    | 4    |
|        | Ch12      | 3.112    | 12         | 3.084  | 0.938*     | 12        | 1.521     | 12     | 2.168  | 12     | 12.000   | 12   |
|        | EF1α      | 1.048    | 1          | 0.848  | 0.961*     | 5         | 0.401     | 1      | 0.226  | 2      | 1.778    | 1    |
|        | GAPDH     | 1.327    | 7          | 0.841  | 0.794      | 4         | 0.739     | 6      | 0.649  | 8      | 6.055    | 8    |
|        | His       | 1.632    | 11         | 1.552  | 0.829      | 11        | 1.181     | 11     | 0.812  | 9      | 10.462   | 11   |
|        | Hsp70     | 1.103    | 2          | 1.000  | 0.932*     | 7         | 0.447     | 3      | 0.025  | 1      | 2.546    | 3    |
|        | RP49      | 1.625    | 10         | 0.652  | 0.508      | 1         | 0.983     | 9      | 0.991  | 11     | 5.609    | 6    |
|        | RPL32     | 1.118    | 3          | 0.777  | 0.911*     | 2         | 0.401     | 1      | 0.399  | 3      | 2.060    | 2    |
|        | SDH       | 1.245    | 5          | 1.054  | 0.927*     | 8         | 0.803     | 7      | 0.506  | 6      | 5.789    | 7    |
| Larvae | 18S       | 0.867    | 11         | 0.307  | –0.458     | 4         | 0.488     | 11     | 0.587  | 11     | 8.542    | 10   |
|        | Ach       | 0.748    | 10         | 0.561  | 0.800*     | 11        | 0.431     | 10     | 0.386  | 10     | 10.241   | 11   |
|        | Act       | 0.554    | 4          | 0.413  | 0.956*     | 9         | 0.337     | 7      | 0.069  | 3      | 5.244    | 6    |
|        | Ch12      | 2.097    | 12         | 2.044  | 0.842*     | 12        | 0.784     | 12     | 1.554  | 12     | 12.000   | 12   |
|        | EF1α      | 0.497    | 1          | 0.316  | 0.980*     | 5         | 0.116     | 1      | 0.040  | 1      | 2.115    | 1    |
|        | GAPDH     | 0.608    | 8          | 0.222  | 0.502      | 1         | 0.272     | 5      | 0.316  | 9      | 2.913    | 3    |
|        | His       | 0.519    | 3          | 0.362  | 0.968*     | 6         | 0.116     | 1      | 0.041  | 2      | 3.663    | 5    |
|        | Hsp70     | 0.606    | 7          | 0.414  | 0.847*     | 10        | 0.385     | 9      | 0.198  | 6      | 7.841    | 9    |
|        | RP49      | 0.641    | 9          | 0.387  | 0.549      | 7         | 0.362     | 8      | 0.243  | 7      | 7.707    | 8    |
| Adult  | 18S       | 0.983    | 10         | 0.481  | 0.215      | 8         | 0.550     | 10     | 0.524  | 9      | 9.212    | 10   |
|        | Ach       | 0.863    | 9          | 0.567  | 0.056      | 9         | 0.392     | 8      | 0.560  | 10     | 8.972    | 9    |
|        | Act       | 0.767    | 7          | 0.575  | 0.504      | 10        | 0.327     | 7      | 0.390  | 7      | 7.653    | 7    |
|        | Ch12      | 1.597    | 11         | 1.201  | 0.735      | 11        | 0.777     | 11     | 1.140  | 11     | 11.000   | 11   |
|        | EF1α      | 0.634    | 5          | 0.286  | 0.360      | 5         | 0.107     | 3      | 0.229  | 6      | 4.606    | 6    |
|        | GAPDH     | 0.817    | 8          | 0.430  | –0.012     | 7         | 0.438     | 9      | 0.468  | 8      | 7.969    | 8    |
|        | His       | 1.651    | 12         | 1.547  | 0.726      | 12        | 0.952     | 12     | 1.186  | 12     | 12.000   | 12   |
|        | Hsp70     | 0.631    | 4          | 0.384  | 0.722      | 6         | 0.210     | 5      | 0.114  | 3      | 4.356    | 5    |
|        | RP49      | 0.579    | 1          | 0.241  | 0.660      | 3         | 0.054     | 1      | 0.195  | 4      | 1.861    | 2    |
|        | RPL32     | 0.613    | 3          | 0.222  | 0.178      | 2         | 0.054     | 1      | 0.221  | 5      | 2.340    | 3    |
| Biotic | Reference | Delta Ct | Bestkeeper | Genom | Normfinder | RefFinder |
|--------|-----------|----------|------------|-------|------------|-----------|
|        |           | StdDev   | Rank       | SD    | R          | M         | Rank |
|        |           |          |            | R     | Rank       | SV        | Rank |
|        |           |          |            | Stability | Rank       | Stability | Rank |
|        |           |          |            |        |            |           |      |
| Condition | Gene |       |       |       |            |           |      |
| SDH     | 0.600 | 2      | 0.147 | 0.598 | 1          | 0.143     | 4     | 0.058 | 1 | 1.682 | 1 |
| Tub     | 0.652 | 6      | 0.267 | 0.551 | 4          | 0.273     | 6     | 0.109 | 2 | 4.120 | 4 |
| Tissue  | 18S      | 2.634    | 11 | 0.466 | 0.323 | 1 | 1.754 | 11 | 1.665 | 11 | 6.040 | 6 |
| Ach     | 1.885 | 7      | 2.757 | 0.929* | 8 | 0.988 | 7 | 1.011 | 7 | 7.238 | 9 |
| Act     | 2.020 | 9      | 3.098 | 0.954* | 12 | 1.201 | 9 | 1.080 | 9 | 9.671 | 12 |
| Cht     | 2.389 | 10     | 0.836 | 0.645 | 2 | 1.510 | 10 | 1.403 | 10 | 6.687 | 8 |
| EF1α    | 1.547 | 4      | 2.854 | 0.992* | 10 | 0.722 | 4 | 0.629 | 4 | 5.030 | 4 |
| GAPDH   | 1.697 | 6      | 2.777 | 0.959* | 9 | 0.818 | 5 | 0.814 | 6 | 6.344 | 7 |
| His     | 3.078 | 12     | 1.124 | 0.014 | 3 | 1.987 | 12 | 2.063 | 12 | 8.485 | 10 |
| Hsp70   | 1.497 | 2      | 2.405 | 0.974* | 4 | 0.554 | 1 | 0.494 | 3 | 2.213 | 2 |
| RP49    | 1.504 | 3      | 2.482 | 0.982* | 6 | 0.663 | 3 | 0.433 | 2 | 3.224 | 3 |
| RPL32   | 1.462 | 1      | 2.440 | 0.976* | 5 | 0.554 | 1 | 0.429 | 1 | 1.495 | 1 |
| SDH     | 1.922 | 8      | 3.003 | 0.950* | 11 | 1.089 | 8 | 1.050 | 8 | 8.663 | 11 |
| Tub     | 1.615 | 5      | 2.552 | 0.958* | 7 | 0.873 | 6 | 0.656 | 5 | 5.692 | 5 |

StdDev, standard deviation; SD, standard deviation; SV, stability value; R, Pearson correlation coefficient; *p<0.001.
doi:10.1371/journal.pone.0098164.t002
| Abiotic Condition | Reference Gene | Delta Ct StdDev | Rank | Bestkeeper SD R Rank | geNorm M Rank | Normfinder SV Rank | RefFinder Stability Rank |
|-------------------|----------------|-----------------|------|----------------------|--------------|--------------------|------------------------|
| Insecticide       | 18S            | 0.995           | 10   | 0.776 0.900* 10      | 0.443 10     | 0.554 10           | 10.000 10              |
|                   | Ach            | 0.679           | 9    | 0.394 0.446 8        | 0.291 9      | 0.321 9            | 8.739 9                |
|                   | Act            | 0.536           | 1    | 0.188 0.693 3        | 0.0852 1     | 0.120 2            | 1.565 1                |
|                   | Ch2            | 1.551           | 12   | 1.116 0.043 11       | 0.781 12     | 1.023 12           | 11.742 12              |
|                   | EF1α           | 0.603           | 5    | 0.249 0.511 5        | 0.121 3      | 0.226 7            | 5.439 7                |
|                   | GAPDH          | 0.626           | 8    | 0.381 0.784 7        | 0.201 5      | 0.214 6            | 7.200 8                |
|                   | His            | 1.296           | 11   | 1.146 0.843* 12      | 0.602 11     | 0.833 11           | 11.242 11              |
|                   | Hsp70          | 0.605           | 6    | 0.155 0.512 1        | 0.249 8      | 0.181 4            | 3.130 3                |
|                   | RPL32          | 0.574           | 3    | 0.371 0.835* 6       | 0.190 4      | 0.153 3            | 4.243 5                |
|                   | RPL32          | 0.567           | 2    | 0.395 0.911* 9       | 0.211 6      | 0.110 1            | 3.350 4                |
|                   | SDH            | 0.581           | 4    | 0.201 0.432 4        | 0.0852 1     | 0.211 5            | 2.991 2                |
| Starvation        | Tub            | 0.609           | 7    | 0.172 0.074 2        | 0.230 7      | 0.233 8            | 4.281 6                |
|                   | 18S            | 0.329           | 2    | 0.089 –0.197 3       | 0.001 1      | 0.085 3            | 2.711 3                |
|                   | Ach            | 0.333           | 3    | 0.051 –0.437 1       | 0.044 6      | 0.078 2            | 1.565 2                |
|                   | Act            | 0.328           | 1    | 0.053 –0.209 2       | 0.038 5      | 0.061 1            | 1.189 1                |
|                   | Ch2            | 0.751           | 12   | 0.576 –0.045 12      | 0.346 12     | 0.490 12           | 12.000 12              |
|                   | EF1α           | 0.368           | 4    | 0.156 0.668 4        | 0.001 1      | 0.119 4            | 4.000 4                |
|                   | GAPDH          | 0.523           | 11   | 0.374 0.614 11       | 0.017 4      | 0.295 11           | 10.462 11              |
|                   | His            | 0.502           | 9    | 0.269 0.287 9        | 0.195 10     | 0.264 9            | 9.240 9                |
|                   | Hsp70          | 0.422           | 5    | 0.254 0.114 8        | 0.113 8      | 0.221 7            | 6.117 5                |
|                   | RPL32          | 0.456           | 8    | 0.207 0.268 5        | 0.153 9      | 0.216 6            | 6.620 7                |
|                   | RPL32          | 0.435           | 6    | 0.231 0.741 7        | 0.012 3      | 0.211 5            | 6.192 6                |
|                   | SDH            | 0.443           | 7    | 0.216 0.382 6        | 0.077 7      | 0.233 8            | 6.701 8                |
| Temperature       | Tub            | 0.511           | 10   | 0.345 0.655 10       | 0.242 11     | 0.274 10           | 10.241 10              |
|                   | 18S            | 0.374           | 10   | 0.241 0.565 8        | 0.217 10     | 0.211 10           | 9.457 10               |
|                   | Ach            | 0.261           | 6    | 0.086 0.371 2        | 0.069 3      | 0.139 7            | 3.984 6                |
|                   | Act            | 0.308           | 9    | 0.256 0.743 10       | 0.161 8      | 0.145 8            | 8.712 9                |
|                   | Ch2            | 0.458           | 11   | 0.444 0.956* 11      | 0.268 11     | 0.294 11           | 11.00 11               |
|                   | EF1α           | 0.241           | 4    | 0.018 0.716 3        | 0.084 4      | 0.088 5            | 3.936 4                |
|                   | GAPDH          | 0.299           | 8    | 0.250 0.845* 9       | 0.188 9      | 0.126 6            | 7.896 8                |
|                   | His            | 0.561           | 12   | 0.513 0.967 12       | 0.324 12     | 0.398 12           | 12.00 12               |
|                   | Hsp70          | 0.298           | 7    | 0.049 –0.326* 1      | 0.114 6      | 0.181 9            | 4.409 7                |
|                   | RPL32          | 0.238           | 3    | 0.128 0.713 5        | 0.028 1      | 0.084 4            | 2.783 2                |
|                   | RPL32          | 0.235           | 1    | 0.127 0.755* 4       | 0.028 1      | 0.068 3            | 1.861 1                |
EF1α was placed on the top position for the stability ranking in embryo and larvae stage and ranked as the forth stable gene in tissues samples, starvation and temperature treatment. These results showed very similar correlations with the research in Drosophila across abiotic stress. EF1α was assumed to be the best gene in the brain of fifth-instar nymph of Schistocerca gregaria and Chortoicetes terminifera reared under different density treatments. Surprisingly, EF1α was not a good choice for adult according to the analysis of all programs except geNorm. This also highlighted the necessity for validation of the reference genes for different development stages.

Act plays a key role in cytoskeleton maintenance and cell motility. It is the most abundant protein in eukaryotic cells [54]. Although Act has been usually used as a normalization factor in molecular expression studies [55], several studies have shown that the expression of Act fluctuated with aging, growth [56,57], developmental stage and differentiation [58,59]. Our study also found Act displayed very low stability in different development stages and tissues. However, Act was still found to be the most reliable marker of internal control in the insecticide and starvation treatment.

RPL32 (ribosomal protein L32) is a ribosomal structural constituent. In this study, RPL32 was selected as the most appropriate reference gene in different tissues and temperature treatment. RPL32 also appeared as the second most suitable reference gene in embryo and nymphs stage. Our conclusions were in accordance with the research in Chortoicetes terminifera [37] and corpora allata of Drosophila punctata [60]. Additionally, the stability of RP49 was always behind RPL32 in our study, though they are both ribosomal proteins. Tub, a type of cytoskeletal structure protein, is another commonly used reference gene. In this study, Tub was identified as a moderately stable gene with stability rank around fifth in most samples except for temperature and starvation treatments. Tub appeared as the third most appropriate reference gene in third-instar nymphs subjected to temperature treatment and a variable gene under starvation stress.

To the best of our knowledge, Tub has been reported unsuitable to normalize gene expression in the brain of desert locust [36] and in virus-infected planthoppers [61].

SDH and GAPDH are two multifunctional enzymes involved in citrate cycle and metabolic pathways, respectively. Our results showed SDH was the best reference gene for adult stage and insecticide treatment. We also found that SDH and GAPDH were stable in larvae stage followed by EF1α and RPL32. For other experimental conditions, SDH and GAPDH were not good choices, especially for the abiotic stress. To our knowledge, the expression level of genes which participated in metabolic processes might fluctuate largely under heat stress [62], and GAPDH should be avoided to normalize gene expression in hypoxia experiments [63]. Hsp70, involved in translating one kind of 70 kDa heat shock protein [64], was chosen as the reference gene to assess the expression of AChE gene (acetylcholinesterase) after injection with dsRNA in L. migratoria manilensis [44]. In this study, Hsp70 was identified as the second most appropriate candidate gene in different tissues and third most stable in embryo stage and larvae treated with different insecticides. However, Hsp70 was detected to be unstable in larvae treated at different temperatures as it is sensitive to temperature. His, the housekeeping gene histone H3 which encodes histone protein [65], was rarely used as a normalization gene in insect. According to our study, His appeared as the second most suitable gene based on the assessment of NormFinder and geNorm in nymph stage. However, it was the most variable gene in other conditions.
The 18S ribosomal subunit was highly expressed in all samples with the lowest Ct values. The low Ct values reflect the large quantity of transcripts. mRNA only constitutes 5% of the total RNA, while rRNA corresponds to a large portion of the RNA. Therefore, it might not a good idea to choose 18S rRNA as the internal control factor. Interestingly, our study indicated that 18S was not stable enough based on the analysis of all the programs except for Bestkeeper. Many previous studies also showed that 18S was not a suitable reference gene [66–71]. Therefore, we did not recommend 18S rRNA to normalize gene expression in our experimental conditions.

Cht2 and Ach were used as two novel reference genes identified from the locust microarray data [38]. Ach is involved in fatty acid metabolism [72] and acetate utilization in mitochondria [73]. In this study, Ach was selected as the best gene in third-instar nymphs suffering starvation. However Cht2, which plays a role in hydrolyzing chitin, was considered as the worst gene in all samples in this study. The stability of Cht2 had also been found unreliable

Table 4. Preferable control genes in L. migratoria across different experimental conditions.

| Experimental conditions | Preferable reference genes |
|-------------------------|----------------------------|
| Biotic factors          | Embryo: EF1a, RPL32        |
|                         | Larvae: EF1a, RPL32        |
|                         | Adult: SDH, RP49           |
|                         | Tissue: RPL32, Hip70, RP49 |
| Abiotic factors         | Insecticide: Act, SDH      |
|                         | Starvation: Act, Ach       |
|                         | Temperature: RPL32, RP49   |

doi:10.1371/journal.pone.0098164.t004
for gene expression analysis in a previous study in locusts exposed to hypobaric hypoxia [30].

To accurately measure the expression levels of a target gene, normalization by multiple housekeeping genes is necessary. However, it is impractical to quantify more stable reference genes than necessary, especially when the amounts of template are limited. Vandesompele et al. [46] recommended to determine the ideal number of selected housekeeping genes by calculating the normalization factor (NF) with geNorm. If the pairwise variation \(V_{n/n+1}\) was below 0.15, it means adding n+1 gene has no obviously effect on normalization factors. Then, the geometric average of the top n candidate reference genes in the system would be the optimal normalization factor for the future experiment [46].

In our study, the \(V_{2/3}\) values were all below 0.15 for the development stages and abiotic stress, so two best reference genes are sufficient to analysis the expression of the gene of interest.

**Figure 5** showed that two most appropriate genes provided a more conservative estimation of target gene transcription compared to a single gene. Our results also demonstrated that the application of the least stable reference gene could result in false interpretation (**Figure 5A**). As for the different tissues, \(V_{2/6}\) was below the proposed 0.15 value, so the best number of selected reference genes should be five. However, it will require a large amount of resources using five control genes as a normalization factor, and our results demonstrated that the expression level and pattern of target gene GSTs1 in tissues were similar when normalized against three best reference genes and five most stable reference genes (**Figure 5B**). Therefore, we believe that using three best control genes is a valid normalization strategy for tissue samples.

As a proof of principle, our validation results were tested by evaluating the transcript of two target genes in different development stages, tissues and larvae subjected to insecticide treatment. CHSI plays an important role in chitin synthesis in insect cuticle [74]. The gene CHSI of *Locusta migratoria* is expressed consistently in every life stage and with the highest transcript amount in adults [75–77]. In this report, the expression level of *Loc*CHSI was highest in adult when normalized using the two best reference genes (**Figure 5A**), but not when using the least stable reference gene for normalization. Glutathione S-transferases (GSTs), a diverse family of dimeric enzymes, can eliminate toxicants from a cell [78–79]. Our results demonstrated that the highest transcript levels of GSTs1 mRNA were detected in fat body and Malpighian tubules when using the recommended set of reference genes for normalization (**Figure 5B**). This can be explained by the fact that the fat body of insects is the main metabolic detoxification center [76]. Our results were also in accordance with a previous report [77]. However, when only normalized by the least stable reference gene, the expression pattern of GSTs1 was very different. Similar results were observed when evaluating the expression level of CHSI in the third-instar larvae subjected to insecticides (**Figure 5C**). Therefore, using appropriate reference genes for normalization would be one of the key factors for accurate estimation of target gene expression, while unsuitable normalization factors might lead to deviated results and concealing of true outcome.

**Conclusions**

In conclusion, our study provides a comprehensive assessment for the suitable reference genes for qPCR in *L. migratoria* across all the development stages, tissues and three abiotic stress: EF1α and RPL32 were found to be reliable for embryo and larval stage; SDH and RP49 were optimal for adult stage; RPL32, Hsp70 and RP49 should be recommended for study in different tissues; Act and SDH would be appropriate for larvae treated with insecticide treatment; Act and Act should be used for larvae suffering starvation; and RPL32 and RP49 were selectable for larvae subjected to different temperature treatment (Table 4). Our data verified the caution that the expression stability of selected reference genes needed to be evaluated in different treated samples. This study will benefit future work on target gene expression in *L. migratoria*.

**Supporting Information**

**Table S1** The insecticide bioassay to the third-instar nymphs of *Locusta migratoria*. (DOC)

**Table S2** Expression stability of the candidate reference genes across all the samples. (DOCX)

**Acknowledgments**

We thank Yuanyuan Zheng for help with rearing the wheat seedlings for the nymphs.

**Author Contributions**

Conceived and designed the experiments: QPY QWZ XXL. Performed the experiments: QPY QWZ XXL. Analyzed the data: QPY ZL XXL. Contributed reagents/materials/analysis tools: QPY HJZ XYW. Wrote the paper: QPY XXL QWZ.

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