RESEARCH ARTICLE

Assessment of *Aedes albopictus* reference genes for quantitative PCR at different stages of development

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Abstract

Members of the *Aedes* genus of mosquitoes are widely recognized as vectors of viral diseases. *Ae. albopictus* is its most invasive species, and are known to carry viruses such as Dengue, Chikungunya and Zika. Its emerging importance puts *Ae. albopictus* on the forefront of genetic interaction and evolution studies. However, a panel of suitable reference genes specific for this insect is as of now undescribed. Nine reference genes, namely ACT, *eEF1-γ*, *eIF2α*, PP2A, RPL32, RPS17, PGK1, ILK and STK were evaluated. Expression patterns of the candidate reference genes were observed in a total of seventeen sample types, separated by stage of development and age. Gene stability was inferred from obtained quantification data through three widely cited evaluation algorithms i.e. BestKeeper, geNorm, and NormFinder. No single gene showed a satisfactory degree of stability throughout all developmental stages. Therefore, we propose combinations of PGK and ILK for early embryos; RPL32 and RPS17 for late embryos, all four larval instars, and pupae samples; *eEF1-γ* with STK for adult males; *eEF1-γ* with RPS17 for non-blood fed females; and *eEF1-γ* with *elf2α* for both blood-fed females and cell culture. The results from this study should be able to provide a more informed selection of normalizing genes during qPCR in *Ae. albopictus*.

Introduction

*Aedes albopictus* (*Ae. albopictus*) is the most widely-travelled member of the *Aedes* genus [1]. Due to increased trade and ease of transcontinental travel [2,3], they are now found abundantly in not only the tropical Asian countries from which they originate, but also temperate Asia, Europe, the Americas, Australia, and Africa [4]. Though less notorious than *Aedes aegypti* (*Ae. Aegypti*) as a vector of arboviruses, *Ae. albopictus* is acknowledged as an efficient vector of at least 22 viral strains [5]. It also carries a multitude of cross-species infecting bacteria [6,7]. Throughout the course of history, this mosquito has been deemed responsible for a number of major outbreaks of diseases normally related to *Ae. aegypti*, such as Dengue [8,9] and Chikungunya [10]. *Ae. albopictus* is additionally susceptible to Zika, with high dissemination and transmission rates [11]. Nonetheless, it is typically presumed that where *Ae. aegypti*...
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algorithms of BestKeeper, geNorm, and NormFinder were used to analyse their stability and to rank the candidate genes in order of favourability for usage within any biological sample. The suitable reference gene(s) suggested through the outcomes of this study can be applied for normalization of qPCR data for whole organism *Ae. albopictus* tissue at multiple developmental stages as well as cell culture.

**Materials and methods**

**Rearing and sample collection**

Dried viable eggs of VCRU-lab strain *Ae. albopictus* were obtained from Vector Control Research Unit, Science University of Malaysia (USM). Roughly 500 were submerged in dechlorinated water at any one time. First instar larvae (1L) samples were collected immediately upon hatching. Hatchlings were maintained in relative humidity and natural light conditions at 28°C in plastic containers throughout the entire span of their aquatic lifecycle. Rearing water is changed every other day. Feed of approximately 1.0g mixture of crushed dog biscuits and pulverized chicken liver was administered daily. Second (2L), third (3L), and fourth (4L) instar individuals were collected successively. Pupal samples were a mixture of an equal number of individuals at first, second, and third day of pupation. Newly-eclosed adults were transferred to cages. They were maintained on 10% sucrose solution. Combined adult samples collected comprised of equal numbers of males and non-blood fed females aged 1 to 10 DAE. Adults of the same age range were also gender-separated in sampling. Food was removed from 5 to 7 DAE adults a full day before blood-feeding on lab-strain mice. Mosquitoes were returned to normal breeding conditions for females to lay eggs. Embryos were collected at three-hour intervals up to 12 hours; six-hour intervals from 12 to 24 hours; and 24-hour intervals from 24 to 72 hours after they were laid. C6/36 cells cultured in Gibco® L-15 media supplemented with 10% FBS, 1% Pen-Strep and 10% Tryptose Phosphate Broth (ThermoFisher Scientific, USA) were harvested at maximum confluency.

**RNA extraction and quality assurance**

This study adheres to the Minimum Information for Publication of Quantitative Real-Time PCR guidelines or MIQE. The amount of tissue collected per bioreplicate are as follows: ~500 eggs per embryonic sample; 50 individuals per first or second instar larval sample; 35 individuals per third larval sample; 20 individuals per fourth instar larvae or pupal sample; 20 individuals per adult sample; and 2ml of 4th day culture per cell sample. All samples were immediately stored -20°C in TRIZol® reagent (Invitrogen™, Ambion™, Life Technologies). Total RNA extraction was done within five days of collection with a protocol previously described for mosquito tissue samples. Culturing media was removed from c6/36 samples prior to RNA extraction as described by Abcam®. Extracts were quantified on the Quawell® Q3000 UV Spectrophotometer (Quawell Technology, Inc., California). The acceptable A260: A280 value was set between 1.75 and 2.05. All extracts used showed clear 18S banding and minimal smearing in 1.0% agarose gel, and were kept at -20°C for the duration of the experiment.

**Reference gene selection, primer design, and primer validation**

Genes were chosen if they fulfil either one of the following criteria: (a) potential as a good reference gene based on entomological literature (b) availability of an annotated sequence, or (c) at least 95% identical homology with annotated *A. aegypti* NCBI RefSeq. All primer pairs
were designed on the Primer3 software (bioinfo.ut.ee/primer3-0.4.0/). The Kalign nucleic acid alignment software (http://www.ebi.ac.uk/Tools/msa/kalign/) was used to specify regions spanning exon-exon boundaries. Restrictive parameters for primer selection were: melting temperatures between 59.0˚C and 61.0˚C, GC content between 40 and 60%, nucleotide length between 18 and 24, and amplicon length of between 150 to 225 bases. Other settings were kept at default. Singular amplicon product and mRNA specificity was confirmed in silico on the Sequence Manipulation Suite website (http://www.bioinformatics.org/sms2/). All genes, accession numbers, primer sequences and amplicon size used for this study is listed in Table 1.

Reverse transcription and qPCR

Reverse transcription was carried out with 1.5μg total RNA in 30μl reactions using the iScript Reverse Transcription Supermix (Bio-Rad Laboratories, California; cat. no. 1708840) according to manufacturer’s protocol. Pooled undiluted cDNA from all eleven developmental stages were serially diluted to the factor of 5 (1:1, 1:5, 1:25, 1:125, 1:625) for standard curve generation. All qPCR runs were performed on the BioRad CFX96 qPCR platform. Optimum qPCR reactions were in 10μl reactions of iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, California; cat. no. 1725120), ~10ng total cDNA, and 500nM each of forward and reverse primers. The standard protocol is initial denaturation at 95˚C for 2.30mins, followed by 40 cycles of denaturation at 95˚C for 15s, annealing at 59˚C for 15s and extension at 72˚C for 20s. A melting-curve analysis with a temperature range of between 65˚C and 95˚C immediately followed amplification. All samples were quantified in technical triplicates. Expression levels were recorded as cycle threshold (Ct). Efficiency values (E) were calculated according to the equation: E = (10^{(-1/slope)}) - 1) X 100.

Table 1. Specifications and amplification characteristics of candidate genes.

| Gene                                      | NCBI accession no. | Primer sequence       | Amplicon size (bp) | Ct range        | Std. Error | R2        | E%        |
|-------------------------------------------|--------------------|-----------------------|--------------------|-----------------|------------|-----------|-----------|
| RPS17 (40S Ribosomal Protein S17)         | AALF016139-RA      | FW 5′ GAACGACGACGACGAACTT | 192                | 14.25–22.80     | 1.944      | 0.998     | 97.2      |
|                                           |                    | RV 5′ GTCAAGAAGACGAGGATT |                   |                 |            |           |           |
| PP2A (Protein Phosphatase 2A)             | AALF009373-RA      | FW 5′ TGTTACGACCTGTTCTTGAGG | 160                | 20.67–29.15     | 1.804      | 0.995     | 105       |
|                                           |                    | RV 5′ CACGGATGAGGACAGGCTT |                   |                 |            |           |           |
| eEF1-γ (Eukaryotic Elongation Factor 1-Gamma) | AALF027751-RA      | FW 5′ GGAGAGTCCCCCAGCATTT | 159                | 23.84–29.89     | 1.412      | 0.977     | 117.1     |
|                                           |                    | RV 5′ CGCCAGCGCTTTGACCTT |                   |                 |            |           |           |
| RPL32 (60S Ribosomal Protein L32)         | AALF014668-RA      | FW 5′ TATGACAAGCTGGCCCCAA | 146                | 14.97–24.34     | 2.083      | 0.994     | 96.9      |
|                                           |                    | RV 5′ AGGACTCTTCTGGAACCGTG |                   |                 |            |           |           |
| PGK1 (Phosphoglycerate Kinase 1)          | AALF007981-RA      | FW 5′ TGGAAAAATGTCGATCTCACG | 179                | 18.75–29.67     | 2.268      | 0.972     | 103.1     |
|                                           |                    | RV 5′ GCCCTATGAGACTGTGCG |                   |                 |            |           |           |
| ILK (Integrin-Linked Kinase)              | AALF017749-RA      | FW 5′ CTTTAGCCCATTCACGTGTTG | 189                | 23.61–33.86     | 2.192      | 0.991     | 103.2     |
|                                           |                    | RV 5′ TGGCCACGTTCACATCC |                   |                 |            |           |           |
| STK (Serine-Threonine Kinase)             | AALF009209-RA      | FW 5′ TGCTATTAAAGGGATGCGCAACCTC | 166                | 21.84–29.20     | 1.647      | 0.993     | 95        |
|                                           |                    | RV 5′ ACCATGTACTCATCCACCAG |                   |                 |            |           |           |
| eIF2a (Eukaryotic Initiation Factor 2-Alp) | AALF024007-RA      | FW 5′ TGAAGTTCCACCCAGAGGACGAG | 196                | 21.75–30.66     | 2.034      | 0.982     | 104.8     |
|                                           |                    | RV 5′ GGTCGCCAGCAGCTGCTTCTC |                   |                 |            |           |           |
| ACT (Actin)                               | Tortosa et al., 2008 | FW 5′ GCAAACGTGGTATCTCTGAC | 135                | 18.98–28.70     | 2.940      | 0.99     | 98.9      |
|                                           |                    | RV 5′ GTGAGGAAACTGGTGGCT |                   |                 |            |           |           |

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were designed on the Primer3 software (bioinfo.ut.ee/primer3-0.4.0/). The Kalign nucleic acid alignment software (http://www.ebi.ac.uk/Tools/msa/kalign/) was used to specify for regions spanning exon-exon boundaries. Restrictive parameters for primer selection were: melting temperatures between 59.0˚C and 61.0˚C, GC content between 40 and 60%, nucleotide length between 18 and 24, and amplicon length of between 150 to 225 bases. Other settings were kept at default. Singular amplicon product and mRNA specificity was confirmed in silico on the Sequence Manipulation Suite website (http://www.bioinformatics.org/sms2/). All genes, accession numbers, primer sequences and amplicon size used for this study is listed in Table 1.
Data mining and selection of reference gene candidates with algorithms: geNorm, BestKeeper, and NormFinder

Publicly available evaluation tools i.e. BestKeeper [35,36], geNorm [17] and Normfinder [37] were utilized for selection of best candidate gene. The BestKeeper algorithm is an Excel program which generates a ranking through repeated pairwise correlation and regression analysis of a gene against all the other tested candidates. Raw data of Ct values (annotated as CP) and PCR efficiency of the primers were used to determine the correlation between each candidate gene and the index, expressed in the form of a coefficient of determination. For geNorm and NormFinder, raw data was converted into linear values relative to the lowest Ct recorded for each candidate gene. In geNorm, the stability of a gene is assessed through the consistency of its expression ratio across all samples. The software generates both a stability value i.e. M, and a pairwise variation value i.e. V. M represents the average variation in transcript levels of a gene in comparison to all other candidate genes, achieved through a repeated process of stepwise exclusion commencing from the least stable gene. Pairwise variation estimates the effect of including another gene [17] sequentially as per the established M-value rankings through the formula of $V_n/V_{n+1}$. A threshold of 0.15 is set; a V value below this would mean that an additional reference gene would not improve normalization. NormFinder is a mixed-effects model statistical analysis which estimates the stability value of a gene as a function of the approximate expression variation it would impose onto the target gene data during normalization [37]. The lower this value is, the less variation one would introduce to a normalization exercise should the candidate gene be used as a reference. It also estimates the variation between sample subgroups of the sample set. The BestKeeper vs. Pearson correlation coefficient value, geNorm M value, and NormFinder stability value are perceived as weightage. Geometric means i.e. central tendencies of these weightages for a candidate gene forms the basis for generation of a consensus ranking.

Results

Primer pair evaluation of candidate reference genes

The expression patterns of an upwards of sixteen housekeeping genes were initially observed from previously reported RNAseq data [38,39]. Seven genes including Ribosomal Protein L34 (RPL34), α-tubulin, β-tubulin, RNA Polymerase II (RNAPII), 18S, TATA-Box Binding Protein (TBP), and Ribosomal Protein S7 (RPS7) were eventually excluded due to any one or more of the following factors: (a) lack of introns or exon-exon boundaries enabling mRNA-specificity; (b) unsuitably low or high Ct values, which compromises sensitivity; and (c) poor primer design (see S1 File). Nine shortlisted candidates progressed through to the next stages of analysis. They can be divided into four functional classes: (i) ribosomal and protein-production genes: RPS17, RPL32, eEF1-γ, and eIF2α; (ii) metabolism-related gene: PP2A; (iii) signal-transduction genes: PGK1, ILK and STK; and (iv) structural integrity gene: ACT. A standard curve for each primer pair was generated with pooled cDNA serially diluted by a factor of 5. Primer quality is based on the efficiency (E) and linear regression coefficient ($R^2$) values as observed from the amplification of cDNA at very high to very low concentrations. All recorded acceptable E values between 95.0 and 117.1%. $R^2$ values range from 0.979 to 0.998 (Table 1). Amplification specificity was displayed through the production of a singular peak in melt-curve analysis, and confirmed on a 2% agarose gel (S1 Fig). PCR products were also sequenced and confirmed to have an alignment of at least 95% to the predicted gene region. All sequences are obtainable from GenBank’s BankIt depository with the accession numbers KY199533 to KY199541.
Expression levels and sample integrity

Expression levels were quantified and candidate gene variability in any developmental stage and cell culture are displayed as Box-Whisker plots in Fig 1. RPS17 recorded the lowest Ct value at 14.25, whereas the highest was by ILK with a reading of 33.86. The mean Ct values of candidates classifies the genes into two groups: (A) genes expressed at moderate levels, and (C) genes expressed at moderately-low levels. Group A genes RPS17 and RPL32 both recorded Ct means of 17.49. The remaining genes belong to Group B. Their mean Ct values are 24.52, 24.67, 25.83, 26.39, and 26.74 for PGK1, eIF2α, PP2A, eEF1-γ, STK, and ILK, respectively. Sample integrity is inferred from the intrinsic variation (InVar) score as generated by the BestKeeper algorithm. Removal of samples with scores in excess of ±3.0 is recommended [36]. Low InVar scores seen across the board signifies that triplicate variability was acceptable amongst the samples of each developmental stage.

BestKeeper analysis

BestKeeper estimates the standard deviation (SD) value of each candidate gene from raw Ct numbers. An SD>1 signifies that the variations in expression of a gene within a sample of
the same origin are high, and thus indicating its instability. Our data demonstrated that not all candidates were stable across all samples (see S1 Table). ILK and PGK1 frequently appeared to be unreliable. Both gave an SD above the acceptable threshold in 48 to 72 hour embryos, larval samples from second to the fourth instar stages, pupal samples, and blood-fed females. PGK1 were additionally unstable in 24 to 48 hour embryos and cell culture. STK displayed instability in embryos 24 to 72 hours in age as well as second instar larvae; PP2A in 48 to 72 hour embryos, second instar larvae, and blood-fed females; eEF1-γ in in third instar larvae and pupae; ACT in third instar larval and cell culture; and eIF2α in third and fourth instar larval samples, pupae, and blood-fed females. Both ribosomal-linked genes i.e. RPS17 and RPL32 were stable in all developmental time points. The SD of a gene factors into its position on a ranking based on the value given as BestKeeper vs Pearson correlation of coefficient. The closer this value is to 1, the greater the reliability of the gene. Here, those carrying SD values of above 1 are relegated to the bottom of the table regardless of its correlation of coefficient value. BestKeeper most recommends ACT for 24 to 48 hour embryos, second instar larva and blood-fed adult females; eEF1-γ for 18 to 24 hour embryos; eIF2α for each of the 18 to 24 hour and 48 to 72 hour embryonic stages along with cell culture samples; ILK for 0 to 3 hour, 3 to 6 hour, and 9 to 12 hour embryos; PGK1 for 6 to 9 hour embryos; PP2A from the fourth instar larval stage through male adulthood; RPL32 in third instar larva; and finally RPS17 during the first instar larval period as well as non-blood fed females. Rankings are shown in Table 2.

Table 2. Ranking of candidate genes based from BestKeeper.

| Rank | Developmental Stage |
|------|---------------------|
|      | 0-3h    | 3-6h  | 6-9h  | 9-12h | 12-18h | 18-24h | 24-48h | 48-72h | 1L |
| 1    | ILK     | ILK   | PGK1  | ILK   | eIF2α  | eEF1-γ | ACT    | eIF2α  | RPS17 |
| 2    | ACT     | PGK1  | RPS17 | STK   | pp2a   | ACT    | RPS17  | RPL32  | eEF1-γ |
| 3    | PP2A    | STK   | PP2A  | ACT   | PGK1   | PGK1   | ILK    | RPS17  | ILK   |
| 4    | PGK1    | PP2A  | RPL32 | eEF1-γ | ILK    | PP2A   | eEF1-γ | STK    |       |
| 5    | RPS17   | eEF1-γ| STK   | PGK1  | eEF1-γ | STK    | RPL32  | ACT    | RPL32 |
| 6    | eIF2α   | eIF2α| ILK   | eIF2α | STK    | eIF2α  | eEF1-γ | PGK1   | ACT   |
| 7    | eEF1-γ | ACT   | ACT   | pp2a  | ACT    | RPS17  | eIF2α  | ILK    | PGK1  |
| 8    | RPL32   | RPL32 | eEF1-γ| RPL32 | PP2A   | STK    | STK    | eIF2α  |       |
| 9    | STK     | RPS17 | eIF2α | RPS17 | RPS17  | RPL32  | PGK1   | PP2A   | PP2A  |

Genes highlighted red had an SD of over the recommended stability indicator of 1. BestKeeper values (not shown here; please refer S1 Table for more information) are based on the r-value, i.e. the BestKeeper vs Pearson coefficient of correlation.

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| Rank | 0-3h | 3-6h | 6-9h | 9-12h | 12-18h | 18-24h |
|------|------|------|------|-------|--------|--------|
| 1/2  | ILK/PP2A | 0.118 | RPL32/RPS17 | 0.093 | PGK1/RPS17 | 0.233 | RPL32/RPS17 | 0.146 | PGK1/PP2A | 0.168 | ACT/PGK1 | 0.228 |
| 3    | eEF1-γ | 0.160 | eIF2α | 0.123 | eEF1-γ | 0.301 | PP2A | 0.177 | STK | 0.212 | eIF2α | 0.246 |
| 4    | eIF2α | 0.189 | STK | 0.240 | ILK | 0.334 | eIF2α | 0.185 | eEF1-γ | 0.239 | eEF1-γ | 0.276 |
| 5    | ACT | 0.250 | PGK1 | 0.307 | eIF2α | 0.352 | ILK | 0.195 | ILK | 0.257 | RPS17 | 0.310 |
| 6    | RPS17 | 0.275 | ILK | 0.330 | PP2A | 0.371 | PGK1 | 0.228 | ACT | 0.280 | STK | 0.328 |
| 7    | RPL32 | 0.305 | eEF1-γ | 0.342 | RPL32 | 0.387 | STK | 0.256 | RPS17 | 0.305 | ILK | 0.343 |
| 8    | PGK1 | 0.323 | PP2A | 0.362 | STK | 0.403 | ACT | 0.384 | RPL32 | 0.329 | RPL32 | 0.364 |
| 9    | STK | 0.339 | ACT | 0.418 | ACT | 0.450 | eEF1-γ | 0.505 | eEF1-γ | 0.364 | PP2A | 0.382 |

**Table 3. Rankings of candidate genes by geNorm.**

| Rank | 24-48h | 48-72h | 1L | 2L | 3L | 4L |
|------|--------|--------|----|----|----|----|
| 1/2  | RPS17/ILK | 0.153 | RPL32/eIF2α | 0.354 | PGK1/ACT | 0.310 | eEF1-γ/ACT | 0.422 | RPL32/PP2A | 0.444 | eEF1-γ/RPS17 | 0.477 |
| 3    | ACT | 0.251 | RPS17 | 0.585 | RPL32 | 0.476 | eIF2α | 0.692 | STK | 0.723 | RPL32 | 0.678 |
| 4    | eEF1-γ | 0.398 | ACT | 0.670 | eEF1-γ | 0.729 | RPL32 | 0.955 | RPS17 | 0.944 | STK | 0.738 |
| 5    | RPL32 | 0.541 | eEF1-γ | 0.778 | RPS17 | 0.797 | RPS17 | 1.094 | eIF2α | 1.091 | ACT | 0.815 |
| 6    | eIF2α | 0.709 | PGK1 | 0.970 | ILK | 0.896 | PGK1 | 1.220 | PGK1 | 1.294 | PP2A | 0.900 |
| 7    | PP2A | 0.799 | ILK | 1.051 | STK | 0.955 | STK | 1.305 | ACT | 1.466 | eIF2α | 1.146 |
| 8    | STK | 0.935 | PP2A | 1.128 | eIF2α | 1.016 | PP2A | 1.437 | eEF1-γ | 1.565 | ILK | 1.436 |
| 9    | PGK1 | 1.050 | STK | 1.495 | PP2A | 1.106 | ILK | 1.631 | ILK | 1.766 | PGK1 | 1.646 |

Scores displayed are M values. The two top genes share the same value. A lower value denotes greater stability. M should not exceed 1.5.

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**geNorm analysis**

geNorm provides two assessment outcomes. The first is an average expression stability score as symbolized by M. The higher the M-value of a gene, the less stable it is perceived to be. This value should fall below 1.5. None of the candidate genes exceeded this in any developmental stage (Table 3). As considerations for a pairing of genes for normalization purposes is incorporated into the algorithm, two are viewed as equally stable or applicable for any single stage. An ILK and PP2A pairing is suggested for 0 to 3 hour embryos; RPL32 and RPS17 for the 3 to 6 hour and 9 to 12 hour embryonic stages as well as blood-fed females; PGK1 each with RPS17 for 6 to 9 hour embryos, with PP2A for the 12th to 18th hour, and with ACT for 18 to 24 hour embryos along with the first instar larval stage. RPS17 is to be paired with ILK for 24 to 48 hour embryonic samples; with eEF1-γ for fourth instar larvae and both sexes in non-blood fed adults; and with STK for pupal samples. Second instar larval samples is best normalized by an ACT and eEF1-γ pairing. For each of the remaining developmental time points i.e. 48 to 72 hour embryos, third instar larvae, and cell culture, RPL32 provides fairest equilibration when paired with eIF2α, PP2A, and eEF1-γ, respectively. Rankings are summarized in charts as provided by the software (Fig 2). The second outcome from geNorm estimates the effect of a gene...
Fig 2. Average stability values (M) of genes in individual developmental stages and cell culture. Lower values indicate better stability.

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addition event [17]. The proposed cut-off value, denoted as $V$, is 0.15. Our data shows that all samples of larval and pupal tissue, as well as those of 48 to 72 hour embryos and non-blood fed female adults, should require more than two candidate genes to be properly normalized (Fig 3).

**NormFinder analysis**

Similar to geNorm, the data utilized by this program is based on relative values. The algorithm produces a stability value for each gene when compared to others within the group. A lower value indicates greater stability. The program does not make suggestions for a cut-off value [37]. Rankings generated are summarized in Table 4. *eEF1-γ* is deemed the best reference gene for the 6 to 9 hour embryonic stage and both male and blood-fed female adults; *PGK1* for non-blood fed females, 9 to 12 hour and 12 to 18 hour embryos, and first instar larval samples; *PP2A* for 0 to 3 hour and 12 to 18 hour embryonic tissue as well as pupal samples; *RPL32* for 48 to 72 hour embryos, cell culture, and larval samples of the second through fourth instar; *RPS17* for 24 to 48 hour embryos, and finally *STK* for those aged between 3 to 6 hours. Despite the frequency at which *PGK1* is top-ranked, it is also the most commonly disrecommended candidate ($n = 4/17$). In the absence of group identifiers, it is presumed that the two genes with the lowest stability value within a sample set would provide the best combination for two-reference gene normalization strategies [40].

**Consensus list of reference genes**

Consensus rankings are obtained through geometrically averaging the weights assigned to a candidate gene (in the form of stability values from geNorm and NormFinder, and a function of $1 - ((BestKeeper vs. Pearson$ correlation coefficient value) from BestKeeper) by the three
Numbers depict stability; the lower the value, the greater the stability.

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programs. All genes are included regardless of BestKeeper SD values. Results are summarized in Table 5. The three top-ranked genes in the consensus list for any developmental stage are typically considered to be most reliable [37]. However, as these combinations vary greatly from one developmental stage to the next, reliability across sample types is also assessed on the basis of overall frequency at which a gene appears amongst the top-three. eEF1-γ and RPL32 are the most reliable, with a frequency of 0.157 and 0.137, respectively.

**Discussion**

The mosquito is intensively studied for a number of reasons: the central role of several of its species as propagators of disease-causing organisms notwithstanding, the insect’s wide-travelled nature and historically paralleled evolution alongside humans have also provided insights into adaptive organogenesis and development [41,42]. *Ae. albopictus* is especially interesting to study as it is both a potent carrier of arboviruses and the most invasive species of mosquito to have emerged in recent years [4,43]. As recognized pests, much of the research surrounding

### Table 5. NormFinder analysis and established rankings.

| Rank | PP2A | RPS17 | ILK | ACT | eEF1-γ | RPL32 | ILK | PP2A | RPS17 | ACT | eEF1-γ | RPL32 | ILK | PP2A |
|------|------|-------|-----|-----|--------|-------|-----|------|-------|-----|--------|-------|-----|------|
| 1    | 0.097| 0.119 | 0.130| 0.152| 0.159  | 0.198 | 0.219| 0.233| 0.237 | 0.240| 0.244  | 0.253 | 0.250| 0.259|
| 2    | 0.077| 0.112 | 0.217| 0.192| 0.209  | 0.225 | 0.278| 0.386| 0.374 | 0.397| 0.283  | 0.439 | 0.436| 0.272|
| 3    | 0.075| 0.215 | 0.217| 0.213| 0.220  | 0.223 | 0.275| 0.257| 0.274 | 0.274| 0.275  | 0.275 | 0.275| 0.275|
| 4    | 0.073| 0.215 | 0.217| 0.213| 0.220  | 0.223 | 0.275| 0.257| 0.274 | 0.274| 0.275  | 0.275 | 0.275| 0.275|
| 5    | 0.071| 0.215 | 0.217| 0.213| 0.220  | 0.223 | 0.275| 0.257| 0.274 | 0.274| 0.275  | 0.275 | 0.275| 0.275|
| 6    | 0.070| 0.215 | 0.217| 0.213| 0.220  | 0.223 | 0.275| 0.257| 0.274 | 0.274| 0.275  | 0.275 | 0.275| 0.275|
| 7    | 0.069| 0.215 | 0.217| 0.213| 0.220  | 0.223 | 0.275| 0.257| 0.274 | 0.274| 0.275  | 0.275 | 0.275| 0.275|
| 8    | 0.068| 0.215 | 0.217| 0.213| 0.220  | 0.223 | 0.275| 0.257| 0.274 | 0.274| 0.275  | 0.275 | 0.275| 0.275|
| 9    | 0.067| 0.215 | 0.217| 0.213| 0.220  | 0.223 | 0.275| 0.257| 0.274 | 0.274| 0.275  | 0.275 | 0.275| 0.275|

**Developmental stages**

- **0-3h**
- **3-6h**
- **6-9h**
- **9-12h**
- **12-18h**
- **18-24h**
- **24-48h**
- **48-72h**
- **1L**
- **2L**
- **3L**
- **4L**

**Numbers depict stability; the lower the value, the greater the stability.**
members of the Aedes genus is concerned with their control. Unlike *A. aegypti* however, *Ae. albopictus* remains largely susceptible to commonly used pesticides [44–47]. Regardless, comparative assays and subsequent investigations into how a single pesticide could affect these two genetically similar organisms vastly differently could be the key to understanding the genetics of mechanisms of resistance, and eventually offer a solution for overcoming the growing problem of pesticide resistance in *Ae. aegypti*.

These studies more often than not require qPCR. The method is hugely popular as it is robust, powerful, and fast. As it is widely utilized, many considerations for obtaining of assured data is also locked in place [28]. When these guidelines are followed, qPCR data is often viewed as highly reliable. Such a consideration is the normalization of expression levels through the application of reference genes [48,49]. These are typically chosen from a group classified as ‘reference genes’. However, current opinion has shied away from the assumption that only such genes need apply as references. In fact, it is highly likely that genes most suitable

| Developmental stage | 0-3h | 3-6h | 6-9h | 9-12h | 12-18h | 18-24h
|---------------------|------|------|------|-------|--------|--------
| **Rank**            | **ILK** | **PGK1** | **PGK1** | **ILK** | **PP2A** | **PGK1**
| 1                   | **ILK** | **PGK1** | **PGK1** | **ILK** | **PP2A** | **PGK1**
| 2                   | **PP2A** | **ILK** | **RPS17** | **STK** | **eEF2α** | **eEF1-γ**
| 3                   | **ACT** | **eEF2α** | **eEF1-γ** | **PGK1** | **PGK1** | **ACT**
| 4                   | **RPS17** | **STK** | **PP2A** | **RPS17** | **ILK** | **eEF2α**
| 5                   | **eEF1-γ** | **RPL32** | **ILK** | **PP2A** | **STK** | **ILK**
| 6                   | **eEF2α** | **eEF1-γ** | **eEF2α** | **eEF2α** | **ACT** | **STK**
| 7                   | **PGK1** | **PP2A** | **STK** | **RPL32** | **eEF1-γ** | **RPS17**
| 8                   | **RPL32** | **RPS17** | **RPL32** | **ACT** | **RPS17** | **PP2A**
| 9                   | **STK** | **ACT** | **ACT** | **eEF1-γ** | **RPL32** | **RPL32**
| 24-48h              | **ILK** | **eEF2α** | **PGK1** | **STK** | **PP2A** | **ACT**
| 48-72h              | **ILK** | **PGK1** | **ACT** | **RPL32** | **ACT** | **RPL32**
| 1L                  | **ILK** | **PGK1** | **PGK1** | **ACT** | **RPL32** | **PP2A**
| 2L                  | **STK** | **PP2A** | **eEF1-γ** | **TRP** | **ILK** | **STK**
| 3L                  | **PP2A** | **PGK1** | **ILK** | **STK** | **RPL32** | **PGK1**
| 4L                  | **ILK** | **eEF2α** | **STK** | **PP2A** | **ILK** | **STK**

Consensus rankings are based on the geometric means of weightages in the form of stability values from geNorm and NormFinder, and a function of 1-(BestKeeper vs. Pearson correlation coefficient value) from BestKeeper.

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as normalizers could be as particular as to be tissue-specific. For *Ae. albopictus*, a panel of reference genes that would satisfy each individual developmental stage is as of yet undescribed. As aforementioned, *ACT* [25] or *RPS7* [26] are often utilized, usually singularly and with little thought for the potential instability of these genes in respect to experimental variables. These practices are unadvisable; not only is the utilization of a single gene for normalization no longer accepted [50], but a misinformed selection of reference genes are known to lead to false positives or false negatives [15]. It is thus of utmost importance that this lack of information is promptly addressed. The challenge lies in the unannotated state of the *Ae. albopictus* transcriptome, which reduces confidence in the identity of genes of interest within the species. Nonetheless, through high-confidence sequence alignments in respect to the thoroughly annotated *A. aegypti* transcriptome, a group of candidate genes were chosen for validation within the context of this study. Ultimately, we hope that the findings here could act as a guide towards more careful selection of reference genes in qPCR assays involving *Ae. albopictus*.

A total of fifteen pre-determined stages of development in *Ae. albopictus* were sampled. A subgroup of female adults at 24-hours post-blood meal, a point where RNA production is doubled [51], as well as C6/36 cells are also included. Stability of nine candidate genes within each developmental stage is separately evaluated by the BestKeeper, geNorm, and Normfinder tools. The three gave largely differing results, though a level of congruence was seen amongst top three rankings in a majority of the stages considered. The gene most frequently observed within this group was *eEF1-γ*. It appeared eight times out of a possible fifty-one. The gene outperformed *RPL32* (n = 7/51), as well as *PP2A, ACT* and *PGK1* (each n = 6/51). In contrast, *STK* was most frequently found in the bottom-three rankings (n = 8/51), followed closely by *ILK* and *RPL32* (each n = 7/51). Given that *RPL32* was equally prominent in top three and bottom three rankings, and as inconsistencies in how most of the candidates were ranked across the consensus board are seen, it is more advisable to categorize performance on a tissue-type basis. As we are also wary of preferences in the scientific community to limit reference genes to two, the following suggestions will be composed of the pair with the best showing within each tissue-type grouping.

For embryo-derived samples up to 24h post-oviposition, *PGK1* and *ILK* is the most recommendable normalizer pairing. Both encode for kinases. Along with *STK*, they are chosen as candidates as previous validation studies have found members of this category of genes to be reliable references [52,53]. Kinases act as signal transducers. As information relay occurs around the clock, they are in constant demand, and this translates into the constitutive expression of their genes. However, the ‘reusable’ nature of these enzymatic proteins limits their numbers within the cell at any one time. This is hypothesized to assert a degree of expressional stability and subsequently, the applicability of this group of genes in normalization. Regardless, as most reference genes are regulated, their levels were shown to be affected by biological needs. It is thus important to note that both *PGK1* and *ILK* performed relatively poorly outside of the 0 to 24 hour embryo tissue-type.

Greatest reliability amongst candidate genes in embryos aged between 24 and 72 hours shifts towards ribosomal genes. This trend carries through into the larval and pupal stages as well. *RPL32* is prominent within the top-three rankings throughout these developmental periods. We therefore tentatively suggest the pairing of this gene along with *RPS17* for all tissues along this span of time. Ribosomes are the protein-generating machines of the cell system. Its two subunits, the 40S and 60S ribosomal proteins, are built from a total of over 80 components. Whereas *RPS17* is a component of the 40S subunit, *RPL32* is one of 60S. Both are common internal control genes and have displayed stability when utilized in cell culture [54–56], plants [57,58] and mammalian tissue [59,60]. In insects, *RPL32* [61] and *RPS17* [62] were shown to be reliable references regardless of tissue origin during bodily development. Cellular activity of
the evolving mosquito speeds up after the 24th hour within embryos. Our raw data shows expression of either gene to be high (Mean Ct value; \(RPL32 = 16.77, \ RPS17 = 16.61\)). When only the larval stages are considered, these values are further reduced, indicating even greater expression levels. This is the point of development whereby growth is most robust. Consequently, demand for proteins to facilitate the process is at an all-time high. Nonetheless, the kinetics of ribosomal activity is such that their components can dissociate and reassociate as needs dictate. The balancing act between availability, demand, and need here may altogether incur a stabilizing effect upon ribosomal genes and thus, their inherent usability as controls for these tissue types. This coincidentally explains why \(RPL32\) and \(RPS17\) are reasonably stable within rapidly growing cell culture as well. As C6/36 cells are also of larval origin, we are doubly confident with suggesting these two genes for normalization in context of \(Ae. \ albopictus\) cell culture.

Within adult tissues, another participant of protein synthesis emerged as the best reference gene. \textit{Eukaryotic Elongation Factor 1 Gamma} or \(eEF1-\gamma\) is a subunit of the protein \(eEF1\), which delivers aminoacylated-\(tRNA\)s to the ribosome during translation. However, the overall contribution of this highly versatile protein within the cell is much more widespread, potentially holding roles in nuclear export, proteolysis, and apoptosis, amongst others [63]. Our evaluation here marks \(eEF1-\gamma\) as superior to \(eIF2\alpha\) as a candidate reference gene. At the outset of this study, our expectations were such that these two would be comparable as like \(eEF1-\gamma\), \(eIF2\alpha\) is also a multifunctional player of protein synthesis [64]. It is unclear to us as to why the latter is the more volatile gene. Nonetheless, these two should provide proper normalization when utilized for expression within cell culture and blood-fed adult female tissues. For adult males and females, \(eEF1-\gamma\) is best paired with \(STK\) and \(RPS17\), respectively. We had also hypothesized an association between \(eEF1-\gamma\) and \(ACT\), as \(eEF1\) is known to effect actin polymerization and subsequent cytoskeletal formation [65,66]. True enough, the two were found closely placed in the consensus rankings in eight out of the seventeen sample subsets here, though unexpectedly this occurred mostly under circumstances where neither showed reliability as a normalizer.

An additional form of result supplied by geNorm is a pairwise variation (V) evaluation (Fig 3). The addition of a suitable reference gene during target gene normalization is expected to reduce the normalization factors (NF) value. However, geNorm dictates that if the stepwise inclusion event is valued at below 0.15, subsequent addition events will not positively affect normalization outcomes. The V values for our study show that in many cases, the combination of two genes is sufficient. Interestingly, the sample subsets which appear to benefit from stepwise inclusion all belong to the ‘rapid-growth’ group i.e. 48 to 72 hour embryos through to pupae. Where the inclusion of none of the genes evaluated would satisfy the 0.15 threshold value, it was proposed that three would be the ideal number to use during qPCR [67,68]. For this reason, in addition to the pair of genes suggested for usage on a tissue-type basis above, we recommend the addition of a third gene most top-ranked for an individual subset as seen Table 5. Nonetheless, this observation also alludes to there being a panel of reference genes more suitable for this period of development than the ones evaluated here.

In \textit{Aegypti}, we were able to recommend a two-gene combination of \(RPS17\) and \(ACT\) as a normalization strategy across all tissue-types on the basis of their inherent stability throughout development [62]. For \textit{Albopictus}, \(eEF1-\gamma\) and \(RPL32\) instead are the genes most commonly seen in top-three rankings within the consensus. Despite this, \(eEF1-\gamma\) is also seen as the least reliable gene in two stages i.e. 9 to 12h embryos and pupae. Similarly, \(RPL32\) is as equally ‘stable’ as it is ‘unstable’. Therefore, we cannot assuredly advise on a two-gene combination which would be suitable enough for general use regardless of tissue-type and experimental conditions.
The overall middling performance of ACT is another surprising outcome of the analysis, given the gene’s history as a ubiquitously used control in not only qPCR, but also proteomics. Its stability within the developing Ae. albopictus was however only on par to the equally middling PP2A. We had included the phosphatase in this study given its verified usability as a reference gene during development in plants [69–71]. In animal tissue, it is less commonly studied for this purpose. Our results suggest that although PP2A could be applied effectively as a reference for six tissue types, the lack of obvious trends to its stability renders the gene unpredictable. This emphasizes the need for a re-evaluation of suitability of genes as references on a species-by-species basis. Improvements to be made in the future include broadening the classes of genes evaluated as well as putting variation-contributing factors such as viral infection or environmental stresses under parallel consideration. In vastly researched species such as Drosophila, a large panel of reference genes has also been shown to benefit normalization as sample size and experimental complexity grows [72–74].

Conclusion
This is the first study of its kind in Ae. albopictus. Through the algorithms of BestKeeper, geNorm, and NormFinder, we recommend the implementation of two-gene combinations to provide satisfactory normalization for the described developmental stages and C6/36 cell culture on the basis of tissue-type. Based on consensus rankings, the proposed combinations are PGK and ILK for early embryos (0 to 24 hours post-oviposition); RPL32 and RPS17 for late embryos (24 to 72 hour post-oviposition) as well as all four larval instars and pupae samples; eEF1-γ with STK for adult males; eEF1-γ with RPS17 for non-blood fed females; and eEF1-γ with elf2α for both blood-fed females and cell culture. Application of an additional gene during the span of development from 48 to 72 hours post-oviposition embryos to the pupal stage comes highly recommended. These findings will benefit normalization practices in Ae. albopictus, and may additionally serve as a resource for screening reference genes in closely-related insects.

Supporting information
S1 Fig. PCR products in 2% agarose gel. In flanking lanes are 100bp ladder. (PDF)
S1 File. Expression level comparisons between candidate genes, and discarded primers. Table 1 is a summary of candidate gene expression levels and performance from previous publications; Table 2 is a list of primers eventually excluded from the study. (DOCX)
S1 Table. BestKeeper descriptive statistic analysis. A complete analysis of candidate genes performance by BestKeeper. (DOCX)

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References

1. Benedict MQ, Levine RS, Hawley WA, Lounibos LP (2007) Spread of the Tiger: Global Risk of Invasion by the Mosquito Aedes albopictus. Vector borne and zoonotic diseases (Larchmont, NY) 7: 76–85.
2. Reiter P, Sprenger D (1987) The used tire trade: a mechanism for the worldwide dispersal of container breeding mosquitoes. J Am Mosq Control Assoc 3: 494–501. PMID: 2904963
3. Craven RB, Eliason DA, Francy DB, Reiter P, Campos EG, et al. (1988) Importation of Aedes albopictus and other exotic mosquito species into the United States in used tires from Asia. J Am Mosq Control Assoc 4: 138–142. PMID: 2903907
4. Kraemer MU, Sinka ME, Duda KA, Mylne AQ, Shearer FM, et al. (2015) The global distribution of the arbovirus vectors Aedes aegypti and Ae. albopictus. Elife 4: e08347. https://doi.org/10.7554/eLife.08347 PMID: 26126267
5. Paupy C, Delatte H, Bagny L, Corbel V, Fontenille D (2009) Aedes albopictus, an arbovirus vector: from the darkness to the light. Microbes Infect 11.
6. Raquin V, Valiente Moro C, Saucreau Y, Tran F-H, Potier P, et al. (2015) Native Wolbachia from Aedes albopictus Blocks Chikungunya Virus Infection In Cellulo. PLoS ONE 10: e0125066. https://doi.org/10.1371/journal.pone.0125066 PMID: 25923352
7. Socolovschi C, Pagés F, Raoult D (2012) Rickettsia felis in Aedes albopictus Mosquitoes, Libreville, Gabon. Emerging Infectious Diseases 18: 1687–1689. https://doi.org/10.3201/eid1810.120178 PMID: 23017437
8. Kobayashi M, Nihei N, Kurihara T (2002) Analysis of northern distribution of Aedes albopictus (Diptera: Culicidae) in Japan by geographical information system. J Med Entomol 39: 4–11. PMID: 11931270
9. Simmons CP, Farrar JJ, Nguyen VV, Wills B (2012) Dengue. N Engl J Med 366: 1423–1432. https://doi.org/10.1056/NEJMra1110265 PMID: 22494122
10. Reiter P, Fontenille D, Paupy C (2006) Aedes albopictus as an epidemic vector of chikungunya virus: another emerging problem? The Lancet infectious diseases 6: 463–464. https://doi.org/10.1016/S1473-3099(06)70531-X PMID: 16870524
11. Wong P-SJ, Li M-zI, Chong C-S, Ng L-C, Tan C-H (2013) Aedes (Stegomyia) albopictus (Skuse): A Potential Vector of Zika Virus in Singapore. PLoS Negl Trop Dis 7: e2348. https://doi.org/10.1371/journal.pntd.0002348 PMID: 23938679
12. Gratz NG (2004) Critical review of the vector status of Aedes albopictus. Med Vet Entomol 18.
13. Tseltsarkin KA, Vanlindingham DL, McGee CE, Higgs S (2007) A Single Mutation in Chikungunya Virus Affects Vector Specificity and Epidemic Potential. PLoS Pathog 3: e201. https://doi.org/10.1371/journal.ppat.0030201 PMID: 18069894
14. Heid CA, Stevens J, Livak KJ, Williams PM (1996) Real time quantitative PCR. Genome Res 6: 986–994. PMID: 8908518
15. Bustin SA, Nolan T (2004) Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. J Biomol Tech 15: 155–166. PMID: 15331581
16. Yeung AT, Holloway BP, Adams PS, Shipley GL (2004) Evaluation of dual-labeled fluorescent DNA probe purity versus performance in real-time PCR. Biotechniques 36: 266–270, 272, 274–265. PMID: 14989091
17. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, et al. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3: research0034.0031-research0034.0011.

18. Bustin SA (2002) Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J Mol Endocrinol 29: 23–39. PMID: 12200227

19. Huggett J, Dheda K, Bustin S, Zumla A (2005) Real-time RT-PCR normalisation; strategies and considerations. Genes Immun 6: 279–284. https://doi.org/10.1038/sj.gene.6364190 PMID: 15815687

20. Fischer M, Skowron M, Berthold F (2005) Reliable transcript quantification by real-time transcriptionase-polymerase chain reaction in primary neuroblastoma using normalization to averaged expression levels of the control genes HPRT1 and SDHA. J Mol Diagn 7: 89–96. https://doi.org/10.1016/S1525-1578(10)60013-X PMID: 15681479

21. Goidin D, Mamessier A, Staquet MJ, Schmitt D, Berthier-Vergnes O (2001) Ribosomal 18S RNA prevails over glyceraldehyde-3-phosphate dehydrogenase and beta-actin genes as standard reference for comparative quantification of mRNA levels in invasive and noninvasive human melanoma cell subpopulations. Anal Biochem 295: 17–21. https://doi.org/10.1006/abio.2001.5171 PMID: 11476540

22. Nicot N, Hausman JF, Hoffmann L, Evers D (2005) Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress. J Exp Bot 56: 2907–2914. https://doi.org/10.1093/jxb/eri285 PMID: 16188960

23. Selvey S, Thompson EW, Matthaei K, Lea RA, Irving MG, et al. (2001) Beta-actin—an unsuitable internal control for RT-PCR. Mol Cell Probes 15: 307–311. https://doi.org/10.1006/mcpr.2001.0376 PMID: 11735303

24. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29: e45. PMID: 11328886

25. Tortosa P, Courtiol A, Moutaille S, Failloux AB, Weill M (2008) Chikungunya-Wolbachia interplay in Aedes albopictus. Insect Mol Biol 17: 677–684. https://doi.org/10.1111/j.1365-2583.2008.00842.x PMID: 19133077

26. Zhang M, Zheng X, Wu Y, Gan M, He A, et al. (2010) Quantitative analysis of replication and tropisms of Dengue virus type 2 in Aedes albopictus. Am J Trop Med Hyg 83: 700–707. https://doi.org/10.4269/ajtmh.2010.09-0193 PMID: 20810842

27. Bogaert L, Van Poucke M, De Baere C, Peelman L, Gasthuys F, et al. (2006) Selection of a set of reliable reference genes for quantitative real-time PCR in normal equine skin and in equine sarcoids. BMC Biotechnol 6: 24. https://doi.org/10.1186/1472-6750-6-24 PMID: 16643647

28. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, et al. (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55: 611–622. https://doi.org/10.1373/clinchem.2008.112797 PMID: 19246619

29. Hamalainen HK, Tubman JC, Vikman S, Kyrola T, Ylikoski E, et al. (2001) Identification and validation of endogenous reference genes for expression profiling of T helper cell differentiation by quantitative real-time RT-PCR. Anal Biochem 299: 63–70. https://doi.org/10.1006/abio.2001.5368 PMID: 11726185

30. Padmanabha H, Lord CC, Lounibos LP (2011) Temperature induces trade-offs between development and starvation resistance in Aedes aegypti (L.) larvae. Medical and Veterinary Entomology 25: 445–453. https://doi.org/10.1111/j.1365-2915.2011.00950.x PMID: 21410734

31. Roy JR, Hargraves PE, O’Connell SM (2009) Effect of selected marine and freshwater microalgae on development and survival of the mosquito Aedes aegypti. Aquatic Ecology 43: 987–997.

32. Tun-Lin W, Burkot TR, Kay BH (2000) Effects of temperature and larval diet on development rates and survival of the dengue vector Aedes aegypti in north Queensland, Australia. Medical and Veterinary Entomology 14: 31–37. PMID: 10759309

33. Bonizzoni M, Dunn WA, Campbell CL, Olson KE, Dimon MT, et al. (2011) RNA-seq analyses of blood-induced changes in gene expression in the mosquito vector species, Aedes aegypti. BMC Genomics 12: 1–13.

34. Zhou Y, Liu Y, Yan H, Li Y, Zhang H, et al. (2014) miR-281, an abundant midgut-specific miRNA of the vector mosquito Aedes albopictus enhances dengue virus replication. Parasites & Vectors 7: 1–11.

35. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT—PCR. Nucleic Acids Research 29: e45–e45. PMID: 11328886

36. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP (2004) Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper—Excel-based tool using pairwise correlations. Biotechnol Lett 26: 509–515. PMID: 15127793

37. Andersen CL, Jensen JL, Ortoft TF (2004) Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization,
applied to bladder and colon cancer data sets. Cancer Res 64: 5245–5250. https://doi.org/10.1158/0008-5472.CAN-04-0496 PMID: 15289330

38. Huang X, Poelchau MF, Armbruster PA (2015) Global Transcriptional Dynamics of Diapause Induction in Non-Blood-Fed and Blood-Fed Aedes albopictus. PLOS Neglected Tropical Diseases 9: e0003724. https://doi.org/10.1371/journal.pntd.0003724 PMID: 25897664

39. Poelchau MF, Reynolds JA, Elskic CG, Denlinger DL, Armbruster PA (2013) Deep sequencing reveals complex mechanisms of diapause preparation in the invasive mosquito, Aedes albopictus. Proc Biol Sci 280: 20130143. https://doi.org/10.1098/rspb.2013.0143 PMID: 23516243

40. Mehdi Khanlou K, Van Bockstaele E (2012) A critique of widely used normalization software tools and an alternative method to identify reliable reference genes in red clover (Trifolium pratense L.). Planta 236: 1381–1393. https://doi.org/10.1007/s00425-012-1682-2 PMID: 22718310

41. Ghaninia M, Larsson M, Hansson BS, Ignell R (2008) Natural odor ligands for olfactory receptor neurons of the female mosquito Aedes aegypti: use of gas chromatography-linked single sensillum recordings. J Exp Biol 211: 3020–3027. https://doi.org/10.1242/jeb.016360 PMID: 18775939

42. McBride CS, Baier F, Omondji AB, Spitzer SA, Lutomiah J, et al. (2014) Evolution of mosquito preference for humans linked to an odorant receptor. Nature 515: 222–227. https://doi.org/10.1038/ nature13964 PMID: 25391959

43. Honório NA, Silva WD, Leite PJ, Gonçalves JM, Lounibos LP, et al. (2003) Dispersal of Aedes aegypti and Aedes albopictus (Diptera: Culicidae) in an urban endemic dengue area in the State of Rio de Janeiro, Brazil. Memórias do Instituto Oswaldo Cruz 98: 191–198.

44. Ishak IH, Jaal Z, Hansson H, Wondji CS (2015) Contrasting patterns of insecticide resistance and knockdown resistance (kdr) in the dengue vectors Aedes aegypti and Aedes albopictus from Malaysia. Parasites & Vectors 8: 181.

45. Vontas J, Kioulas E, Pavlidis N, Morou E, della Torre A, et al. (2012) Insecticide resistance in the major dengue vectors Aedes albopictus and Aedes aegypti. Pesticide Biochemistry and Physiology 104: 126–131.

46. Kamganga B, Marcombe S, Chandra F, Ncourtoupen E, Nwane P, et al. (2011) Insecticide susceptibility of Aedes aegypti and Aedes albopictus in Central Africa. Parasit Vectors 4.

47. Marcombe S, Farajollahi A, Healy SP, Clark GG, Fonseca DM (2014) Insecticide Resistance Status of Aedes aegypti and Aedes albopictus in Central Africa. Parasit Vectors 4.

48. Kozera B, Rapacz M (2013) Reference genes in real-time PCR. Journal of Applied Genetics 54: 391–406. https://doi.org/10.1007/s13353-013-0173-x PMID: 24078518

49. Radonić A, Thulke S, Mackay IM, Landt O, Siegert W, et al. (2004) Guideline to reference gene selection for quantitative real-time PCR. Biochemical and Biophysical Research Communications 313: 866. https://doi.org/10.1006.bbrc.2003.0751 PMID: 14706621

50. Ferguson BS, Nam H, Hopkins RG, Morrison RF (2010) Impact of Reference Gene Selection for Target Gene Normalization on Experimental Outcome Using Real-Time qRT-PCR in Adipocytes. PLoS ONE 5: e15208. https://doi.org/10.1371/journal.pone.0015208 PMID: 21179435

51. Price DP, Nagarajan V, Churbanov A, Houde P, Milligan B, et al. (2011) The Fat Body Transcriptomes of the Yellow Fever Mosquito Aedes aegypti, Pre- and Post- Blood Meal. PLOS ONE 6: e22573. https://doi.org/10.1371/journal.pone.0022573 PMID: 21818341

52. Hildyard JC, Wells DJ (2014) Identification and Validation of Quantitative PCR Reference Genes Suitable for Normalizing Expression in Normal and Dystrophic Cell Culture Models of Myogenesis. PLOS Currents Muscular Dystrophy 1.

53. Riemer AB, Keskin DB, Reinherz EL (2012) Identification and validation of reference genes for expression studies in human keratinocyte cell lines treated with and without interferon-gamma—a method for qRT-PCR reference gene determination. Exp Dermatol 21: 625–629. https://doi.org/10.1111/j.1600-0625.2012.01537.x PMID: 22775998

54. Kriegova E, Baráczky-Kúzmán A, Zatloukal J, Mrazek F, et al. (2008) PSMB2 and RPL32 are suitable determinants to normalize gene expression profiles in bronchoalveolar cells. BMC Molecular Biology 9: 69. https://doi.org/10.1186/1471-2199-9-69 PMID: 18671841

55. Userek E, Baračzy-Kuzma A, Kuzma-Kozakiewicz M (2017) Validation of qPCR reference genes in lymphocytes from patients with amyotrophic lateral sclerosis. PLOS ONE 12: e0174317. https://doi.org/10.1371/journal.pone.0174317 PMID: 28328930

56. Pérez-Rico A, Crespo F, Sammartin ML, De Santiago A, Vega-Pla JL (2014) Determining ACTB, ATP5B and RPL32 as optimal reference genes for quantitative RT-PCR studies of cryopreserved stallion semen. Animal Reproduction Science 149: 204–211. https://doi.org/10.1016/j.anireprosci.2014.08.007 PMID: 25192831
57. Zhao X, Zhang X, Guo X, Li S, Han L, et al. (2016) Identification and Validation of Reference Genes for qRT-PCR Studies of Gene Expression in Dioscorea opposita. BioMed Research International 2016: 3089584. https://doi.org/10.1155/2016/3089584 PMID: 27381401

58. Zheng Y-T, Li H-B, Lu M-X, Du Y-Z (2014) Evaluation and Validation of Reference Genes for qRT-PCR Normalization in Frankliniella occidentalis (Thysanoptera:Thripidae). PLoS ONE 9: e111369. https://doi.org/10.1371/journal.pone.0111369 PMID: 25356721

59. Ahn K, Huh J-W, Park S-J, Kim D-S, Ha H-S, et al. (2008) Selection of internal reference genes for SYBR green qRT-PCR studies of rhesus monkey (Macaca mulatta) tissues. BMC Molecular Biology 9: 78. https://doi.org/10.1186/1471-2199-9-78 PMID: 18782457

60. Brattelid T, Winer LH, Levy FO, Liestøl K, Sejersen OM, et al. (2010) Reference gene alternatives to Gapdh in rodent and human heart failure gene expression studies. BMC Molecular Biology 11: 22. https://doi.org/10.1186/1471-2199-11-22 PMID: 20331858

61. Fu W, Xie W, Zhang Z, Wang S, Wu Q, et al. (2013) Exploring Valid Reference Genes for Quantitative Real-time PCR Analysis in Platulla xylostella (Lepidoptera: Plutellidae). Int J Biol Sci 9: 11.

62. Dzaki N, Ramli KN, Azlan A, Ishak IH, Azzam G (2017) Evaluation of reference genes at different developmental stages for quantitative real-time PCR in Aedes aegypti. Scientific Reports 7: 43618. https://doi.org/10.1038/srep43618 PMID: 28300076

63. Sasikumar AN, Perez WB, Kinzy TG (2012) The Many Roles of the Eukaryotic Elongation Factor 1 Complex. Wiley Interdisciplinary Reviews RNA 3: 543–555. https://doi.org/10.1002/wrna.1118 PMID: 22555874

64. Clemens MJ (2001) Initiation factor elf2 alpha phosphorylation in stress responses and apoptosis. Prog Mol Subcell Biol 27: 57–89. PMID: 11575161

65. Munshi R, Kandl KA, Carr-Schmid A, Whitacre JL, Adams AE, et al. (2001) Overexpression of translation elongation factor 1A affects the organization and function of the actin cytoskeleton in yeast. Genetics 157: 1425–1436. PMID: 11290701

66. Gross SR, Kinzy TG (2005) Translation elongation factor 1A is essential for regulation of the actin cytoskeleton and cell morphology. Nat Struct Mol Biol 12: 772–777. https://doi.org/10.1038/nsmb979 PMID: 16116436

67. Gu C, Chen S, Liu Z, Shan H, Luo H, et al. (2011) Reference Gene Selection for Quantitative Real-Time PCR in Chrysanthemum Subjected to Biotic and Abiotic Stress. Molecular Biotechnology 49: 192. https://doi.org/10.1007/s12033-011-9394-6 PMID: 21416201

68. Sun M, Lu M-X, Tang X-T, Du Y-Z (2015) Exploring Valid Reference Genes for Quantitative Real-Time PCR Analysis in Sesamia inferens (Lepidoptera: Noctuidae). PLOS ONE 10: e0115979. https://doi.org/10.1371/journal.pone.0115979 PMID: 25585250

69. Ray DL, Johnson JC (2014) Validation of reference genes for gene expression analysis in olive (Olea europaea) mesocarp tissue by quantitative real-time RT-PCR. BMC Research Notes 7: 304. https://doi.org/10.1186/1756-0500-7-304 PMID: 24884716

70. Sgambarra T, Pape J, Massiah A, Jackson S (2016) Selection of reference genes for diurnal and development mental time-course real-time PCR expression analyses in lettuce. 12: 21.

71. Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible W-R (2005) Genome-Wide Identification and Testing of Superior Reference Genes for Transcript Normalization in Arabidopsis. Plant Physiology 139: 5–17. https://doi.org/10.1104/pp.105.063743 PMID: 16166256

72. Chintapalli VR, Wang J, Dow JA (2007) Using FlyAtlas to identify better Drosophila melanogaster models of human disease. Nat Genet 39: 715–720. https://doi.org/10.1038/ng2049 PMID: 17534367

73. Zhai Y, Lin Q, Zhou X, Zhang X, Liu T, et al. (2014) Identification and Validation of Reference Genes for Quantitative Real-Time PCR in Drosophila suzukii (Diptera: Drosophilidae). PLoS ONE 9: e106800. https://doi.org/10.1371/journal.pone.0106800 PMID: 25198611

74. Ling D, Salvaterra PM (2011) Robust RT-qPCR Data Normalization: Validation and Selection of Internal Reference Genes during Post-Experimental Data Analysis. PLoS ONE 6: e17762. https://doi.org/10.1371/journal.pone.0017762 PMID: 21423626