Selection and validation of reference genes for functional studies in the Calliphoridae family

Gisele Antoniazzi Cardoso¹,², Cleverson Carlos Matioli¹,², Ana Maria Lima de Azeredo-Espin¹,², Tatiana Teixeira Torres¹,³a

¹Centro de Biologia Molecular e Engenharia Genética (CBMEG), Universidade Estadual de Campinas (UNICAMP), Campinas, SP, Brazil
²Departamento de Genética, Evolução e Bioagentes, Universidade Estadual de Campinas (UNICAMP), Campinas, SP, Brazil
³Departamento de Genética e Biologia Evolutiva, Instituto de Ciências, Universidade de São Paulo (USP), São Paulo, SP, Brazil

Abstract

The genera Cochliomyia and Chrysomya contain both obligate and saprophagous flies, which allows the comparison of different feeding habits between closely related species. Among the different strategies for comparing these habits is the use of qPCR to investigate the expression levels of candidate genes involved in feeding behavior. To ensure an accurate measure of the levels of gene expression, it is necessary to normalize the amount of the target gene with the amount of a reference gene having a stable expression across the compared species. Since there is no universal gene that can be used as a reference in functional studies, candidate genes for qPCR data normalization were selected and validated in three Calliphoridae (Diptera) species, Cochliomyia hominivorax Coquerel, Cochliomyia macellaria Fabricius, and Chrysomya albiceps Wiedemann. The expression stability of six genes (Actin, Gapdh, Rp49, Rps17, α-tubulin, and GstD1) was evaluated among species within the same life stage and between life stages within each species. The expression levels of Actin, Gapdh, and Rp49 were the most stable among the selected genes. These genes can be used as reliable reference genes for functional studies in Calliphoridae using similar experimental settings.

Keywords: BestKeeper, Cochliomyia, Chrysomya, gene expression, geNorm, NormFinder

Correspondence:a ttorres@ib.usp.br

Received: 11 May 2012 Accepted: 13 August 2012 Published: 2 January 2014

Copyright: This is an open access paper. We use the Creative Commons Attribution 3.0 license that permits unrestricted use, provided that the paper is properly attributed.

ISSN: 1536-2442 | Vol. 14, Number 2

Cite this paper as:
Cardoso GA, Matioli CC, Azeredo-Espin AML, Torres TT. 2014. Selection and validation of reference genes for functional studies in the Calliphoridae family. Journal of Insect Science 14:2. Available online: http://www.insectscience.org/14.2
Introduction

The Calliphoridae family contains flies of economic, veterinary, and sanitary importance. Flies of this family feed on different sources, such as living tissues of a vertebrate host (obligate parasites) or decaying organic matter (saprophagous behavior). These infestations, known as myiasis, are caused by Calliphoridae species in the larval stage (Zumpt 1965). The evolutionary origins of parasitic behavior in Calliphoridae are unknown. However, given the evolutionary history of this family, it probably occurred in at least three independent events (Stevens and Wallman 2006). It has been proposed that this division between feeding behaviors reflects the progressive evolution of parasitism in Calliphoridae (Stevens 2003).

An interesting approach for investigating this biological question is to compare the expression of genes related to feeding behavior in closely related species, correlating their expression levels with the different feeding behaviors. Within Calliphoridae, the genera Cochliomyia and Chrysomya contain both obligate and saprophagous flies, which allows the comparison of the different feeding habits between closely related species. Due to its high sensitivity for the detection of PCR products with a fluorescence reporter, real-time qPCR has become the gold standard method for measuring mRNA levels (Wong and Medrano 2005; VanGuilder et al. 2008) and therefore an appropriate technique to compare expression levels between the different Calliphoridae species.

To ensure an accurate measure of the level of gene expression, it is compulsory to normalize the amount of the target gene with the amount of a reference gene. The normalization step is important to reduce experimental variability caused by factors such as the initial amount of total RNA, the integrity of the RNA, and the efficiency of the reverse transcriptase (Wong and Medrano 2005).

Since there is no universal gene that can be used as reference in functional studies, and as part of an effort to understand the evolution of parasitic behavior in Calliphoridae, 10 candidate genes were selected for qPCR data normalization. The expression stability was evaluated for six genes in three different species of the Calliphoridae (Diptera) family, Cochliomyia hominivorax Coquerel (parasite), Cochliomyia macellaria Fabricius (saprophagous), and Chrysomya albiceps Wiedemann (saprophagous). Here, a stable expression level among different Calliphoridae species and between two different life-stages within each species is provided. To our knowledge, this is the first cross-species validation study of reference genes for qPCR experiments in an evolutionary framework. A set of candidate genes (and primers to amplify them) that can be tested in different experimental settings is also provided. These are important resources for functional studies in the Calliphoridae family.

Materials and Methods

Fly collection and maintenance
Cochliomyia macellaria and C. albiceps were collected respectively in Campinas and Sorocaba, both in São Paulo State, Brazil. Adult flies were captured using a hand net and decaying meat or fish as bait. Cochliomyia hominivorax larvae were collected directly from wounds of infested animals in cattle breeding farms in Caiapônia, Goiás, Brazil. Larvae of the three species were reared at 30° C ± 5° C. Cochliomyia hominivorax larvae were maintained in a medium
consisting of fresh ground beef supplemented with blood and water (2:1). Cochliomyia macellaria and C. albiceps larvae were fed on rats donated by the São Leopoldo Mandic College in Campinas, São Paulo, Brazil. These rats were euthanized with a lethal dose of anesthetic (1 mL of 50% chloral hydrate) before they were received. Mature larvae of the three species were allowed to pupate in sawdust. Adults were maintained in cages (34 x 50 x 26 cm) at 25° C and fed a diet composed of dried milk, sugar, and yeast ferment.

**Reference gene selection and primer design**

Candidate reference genes were selected from previous insect studies. These candidates were chosen from functional studies using qPCR, in which they were used as reference genes, or from studies validating the stability of mRNA levels across different samples in which they showed a stable mRNA level. Actin was used as an endogenous control in a study comparing mRNA levels of genes involved in host specialization in Drosophila melanogaster, Drosophila sechellia, and Drosophila simulans (Dworkin and Jones 2009). The genes α-tub, Gapdh, GstD1, Rp49, RpL13A, and RpS18 were selected from a validation study in heads of the western honeybee, Apis mellifera, with a bacterial challenge (Scharlaken et al. 2008). Finally, the stability of α-tub, Gapdh, Rp49, and Ef1α100E in qPCR experiments was evaluated in brains of nymphs and adults of the locust Schistocerca gregaria (Van Hiel et al. 2009). Two additional housekeeping genes were selected, RpS17 and SdhA. The sequences of the candidate genes were recovered from the Flybase (www.flybase.org) and GenBank (www.ncbi.nlm.nih.gov/genbank) databases.

The sequences from the transcriptome of Co. hominivorax (Carvalho et al. 2010) were aligned against the sequences of D. melanogaster using the program tblastx (Altschul et al. 1990) to search for possible orthologs of the genes Actin, α-tub, Ef1α100E, Gapdh, GstD1, RpL13A, Rp49, RpS17, RpS18, and SdhA.

The mapped reads were used to perform a global alignment (Chenna et al. 2003) against the sequences of the 12 Drosophila species with whole genome sequences using the Clustal W program (Clark et al. 2007). The alignments were used to identify conserved regions, whereas primer pairs for each candidate gene were designed using Primer3 (Rozen and Skaletsky 2000).

**Amplification of the selected genes**

A PCR was performed using genomic DNA of five Calliphoridae (Diptera) species, C. albiceps, Chrysomya Megacephala Fabricius, Chrysomya putoria Wiedemann, Co. hominivorax, and Co. macellaria, to test the primers designed for the selected candidate genes.

PCR amplifications were performed in a GeneAmp PCR system 9700 (Applied Biosystems, Life Technologies, www.lifetechnologies.com) with a 20 µL final volume. All reactions contained MgCl₂ in a final concentration of 2.0 mM, 0.6 mM of primers, dNTPs in a final concentration of 200 µM, and 1 unit of Taq DNA polymerase (Fermentas, www.thermoscientificbio.com/fermentas) with 5–30 ng of DNA. After an initial denaturing step of 3 min at 94° C, 35 cycles were performed, each consisting of 50 sec at 94° C, 30 sec at 60° C, and 30 sec at 72° C. A final extension was performed at 72° C for 5 min.
RNA isolation and cDNA synthesis

The main motivation for this study was the investigation of feeding habits in Calliphoridae. The larval stages were initially chosen because the different feeding habits are exhibited during this stage. However, adult females are responsible for choosing the oviposition sites and, consequently, can play a major role in the evolution of feeding behavior (once the eggs hatch, the larvae have to feed on any resource the female has chosen). Hence, larvae and adult female samples were chosen for the evaluation of the reference genes.

Total RNA was extracted from adult females and 3rd instar larvae of the three species from two different generations. Three separate individuals of each generation were used, resulting in a total of six biological replicates. The Trizol reagent (Invitrogen, Life Technologies) was used according to the manufacturer’s protocol to extract total RNA. RNA integrity was confirmed through agarose gel electrophoresis.

All samples were treated with Turbo DNase (Ambion, Life Technologies) to avoid DNA contamination. The Turbo DNase inactivation was performed by heating the samples at 75°C for 10 min. To avoid RNA degradation when heated, EDTA was added to a final concentration of 2.5 mM. The RNA was quantified using the Qubit fluorometer (Invitrogen) with the Qubit RNA assay kit (Invitrogen) according to the manufacturer’s protocol.

PCRs were performed in a 20 µL final volume using 1 µL of each treated sample. Rp49 primer pairs were used in a final concentration of 0.4 mM and an annealing temperature of 60°C in the same conditions previously described to check if any of the samples were still contaminated with DNA after the Turbo DNase treatment.

The cDNA syntheses were performed using 0.4 µg of total RNA with the First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer’s protocol. After the cDNA synthesis, all samples were diluted 10 times for the qPCR assays.

mRNA levels quantification

qPCRs were performed in a 12.5 µL reaction volume following the manufacturer’s instructions for SYBR Green PCR Master Mix (Applied Biosystems). In each reaction, 1.5 µL of the cDNA sample were used and the primers were in a final concentration of 0.4 µM. The qPCRs were run in technical replicates to assess the intra-assay variation on an ABI 7500 PCR Real Time System (Applied Biosystems) using the following cycling conditions: 2 min at 50°C, 10 min at 95°C, and 40 cycles consisting of 15 min at 95°C and 60 sec at 60°C. To check for possible non-specific amplification and primer-dimer formation, after the 40 cycles, samples were submitted to a dissociation step consisting of an increase in temperature from 60°C to 95°C (increasing 1°C per minute for 35 min) to obtain the dissociation curve. PCR efficiencies were calculated using the equation $E = 10^{1/\text{slope}}$. To measure the expression stability of the selected genes, the ΔCt method (Vandesompele et al. 2002) was used to calculate the calibrated data. A control sample (a Co. hominivorax larva sample) with Rp49 primers was used in each qPCR run to account for inter-run variations. The inter-run variation in the Ct of the control sample was used to correct all raw Cts values. For each gene, 3-fold serial dilutions (six dilutions) of cDNA samples were used to construct a standard curve from Ct measures against the log of template quantity.
Gene stability analysis

The software geNorm (Vandesompele et al. 2002), NormFinder (Andersen et al. 2004), and BestKeeper (Pfaffl et al. 2004) were used to establish suitable reference genes for qPCR data normalization. These three programs have different statistical approaches that can be used to measure how stable a gene expression is between distinct conditions or among different species and developmental stages.

Results

Selection and amplification of the candidate genes

Ten candidate genes (Table 1) were selected from previous studies in insects: Apis mellifera (Scharlaken et al. 2008), Drosophila melanogaster (Dworkin and Jones 2009), and Schistocerca gregaria (Van Hiel et al. 2009). Using the sequence information from the Co. hominivorax transcriptome (Carvalho et al. 2010) it was possible to search for orthologs of these candidate genes and design specific primer pairs for Co. hominivorax (Table 1).

The primers were designed using the sequence information from Co. hominivorax. Consequently, all primer pairs were specific to this species. To show the utility of the designed primer pairs for a wider range of species, the selected genes were amplified from genomic DNA of five Calliphoridae species (C. albiceps, C. megacephala, C. putoria, Co. hominivorax, and Co. macellaria). Samples of C. megacephala and C. putoria were not used in qPCR, as colonies of these species were not maintained and it was not possible to obtain RNA.

The genes Actin, Eflα100E, Gapdh, GstD1, RpL13A, Rp49, RpS17, and RpS18 were amplified from genomic DNA in all five species. SdhA was amplified in only two species, Co. hominivorax and Co. macellaria, while α-tub was only amplified in Co. hominivorax.

Quantitative PCR

Gene expression analyses were performed in three species, C. albiceps, Co. hominivorax and Co. macellaria.

The PCR efficiency ranged from 88 to 97% (Table 2). The gene with the lowest efficiency was GstD1 (88%) in Co. hominivorax and Co. macellaria. The melting/dissociation curve showed that all qPCRs generated a single specific product. The coefficient of

| Symbol | Gene name       | Flybase ID   | Function                        | Accession no | Primer sequence (5' → 3') | Amplicon size (bp) |
|--------|-----------------|--------------|----------------------------------|--------------|---------------------------|--------------------|
| Actin  | Actin 5C        | FBgn0000042  | Cytokinesis                      | HQ910450     | F: GCCATGATGATTGCCATC    | 158                |
|        | a-tub (a-Tubulin at 84B) | FBgn0003884 | Protein Polymerization           | HQ910451     | F: GATTTGACCCCACTTTAAC  | 128                |
|        | Etfalpha100E    | FBgn0000557  | Translation                      | HQ910452     | F: ACAATCGCACTTGAGG     | 117                |
|        | Gapdh           | FBgn0001092  | Glycolysis                       | HQ910453     | F: GGATCAACGCTGGGAGG    | 128                |
|        | GstD1           | FBgn0001149  | Glutathione transferase activity | HQ910454     | F: AAAATCACTGCTGGACTC   | 150                |
|        | RpL13A          | FBgn0037351  | Translation                      | HQ910455     | F: CCAAGAATGGATCTTC     | 105                |
|        | Rp49            | FBgn0002626  | Translation                      | HQ910458     | F: GCCCAAGCCTTACATCC    | 169                |
|        | RpS17           | FBgn0005533  | Translation                      | HQ910456     | F: GCAGATGCGTGTGC       | 133                |
|        | RpS18           | FBgn0010441  | Translation                      | HQ910457     | F: ACCATGACTCAGTGGT      | 129                |
|        | SdhA            | FBgn0261439  | Electrons transport chain        | HQ910459     | F: AAGATGATGGAAATTG     | 133                |

1Genbank accession number of Cochliomyia hominivorax sequence used for primer design
2Primer sequences (F: forward; R: reverse)
determination, $R^2$, from standard curves generated for each gene ranged from 0.988 to 0.997, confirming that there were no inhibition contaminants present in the cDNA samples.

The expression of $Ef1\alpha100E$, $RpL13A$, $RpS18$, and, $SdhA$ was not detected in any of the three species, probably due to low mRNA levels. The amplification product for the gene $\alpha$-tub was only detected in $Co$. $hominivorax$, and $GstD1$ had low PCR efficiency in $C$. $albiceps$. Therefore, the suitable genes for comparing gene expression among the three different species were $Actin$, $Gapdh$, $Rp49$, and $RpS17$.

The Ct values (calibrated Cts, see Methods) in all samples ranged from 8.94 ($Actin$ in $Co$. $macellaria$ larvae) to 28.09 ($GstD1$ in $Co$. $macellaria$ larvae). The gene with the lowest variation in all dataset was $Rp49$, and the one with the highest variation was $GstD1$ (Figure 1a). Comparing the Cts within each life stage, $Rp49$ had the lowest while $RpS17$ the highest variation (Figure 1b). Among larval samples, the gene with the lowest variation was $Rp49$ and the gene with the highest variation was $RpS17$ (Figure 1b).

Cts between life-stages within species were also compared. In $C$. $albiceps$ and $Co$. $macellaria$, the gene with the lowest variation was $Rp49$ and the highest was $RpS17$ (Figure 1c). In $Co$. $hominivorax$, $Rp49$ had the lowest variation and $GstD1$ the highest (Figure 1c).

| Gene | Species | Slope | Efficiency | Stage | Average Ct | Average calibrated Ct |
|------|---------|-------|------------|-------|-------------|-----------------------|
| Actin | $C$. $albiceps$ | -3.46 | 95% | Adult female | 15.59 | 12.24 |
| Co. $hominivorax$ | -3.79 | 97% | Larva | 14.85 | 12.16 |
| Co. $macellaria$ | -3.45 | 95% | Adult female | 14.46 | 12.50 |
| $\alpha$-tub | $C$. $albiceps$ | -3.57 | 91% | Adult female | 20.03 | 19.58 |
| Gapdh | $C$. $albiceps$ | -3.79 | 91% | Larva | 15.70 | 13.30 |
| $GstD1$ | $C$. $albiceps$ | -3.61 | 89% | Larva | 16.82 | 14.76 |
| $Rps17$ | $C$. $albiceps$ | -3.6 | 90% | Larva | 16.14 | 14.19 |
| $Rp49$ | $C$. $albiceps$ | -3.66 | 88% | Larva | 23.97 | 23.59 |
| Co. $hominivorax$ | -3.65 | 88% | Larva | 23.81 | 23.37 |
| Co. $macellaria$ | -3.52 | 92% | Larva | 23.26 | 23.69 |
| $GstD1$ | $C$. $albiceps$ | -3.55 | 91% | Larva | 24.37 | 24.25 |
| $Rps17$ | $C$. $albiceps$ | -3.55 | 91% | Larva | 24.25 | 24.25 |
| $Rp49$ | $C$. $albiceps$ | -3.55 | 91% | Larva | 24.37 | 24.25 |

1 A control sample ($Cochliomyia$ $hominivorax$ larva with $Rp49$ primers) was used in each qPCR run to correct raw Cts for inter-run variation.

Table 3. Stability ranking of the reference genes by GeNorm, NormFinder and BestKeeper.

| Rank | $geNorm$ (M) | NormFinder (Stability value) | BestKeeper (BestKeeper index) |
|------|--------------|------------------------------|-------------------------------|
|     | Among species | Including $GstD1$ | Females | Within species | Among species | Including $GstD1$ | Females | Within species | Among species | Including $GstD1$ | Females | Within species | Among species | Including $GstD1$ | Females | Within species |
| 1 | $Actin$ (0.917) | $Rp49$ (0.755) | $Actin$ (0.109) | $Actin$ (0.461) | $Actin$ (0.172) | $Actin$ (0.690) | $Actin$ (0.189) | $Actin$ (0.526) | $Actin$ (0.192) | $Actin$ (0.690) | $Actin$ (0.189) | $Actin$ (0.526) | $Actin$ (0.192) | $Actin$ (0.690) | $Actin$ (0.189) | $Actin$ (0.526) |
| 2 | $Gapdh$ (0.947) | $Gapdh$ (0.755) | $Gapdh$ (0.109) | $Gapdh$ (0.461) | $Gapdh$ (0.172) | $Gapdh$ (0.690) | $Gapdh$ (0.189) | $Gapdh$ (0.526) | $Gapdh$ (0.192) | $Gapdh$ (0.690) | $Gapdh$ (0.189) | $Gapdh$ (0.526) | $Gapdh$ (0.192) | $Gapdh$ (0.690) | $Gapdh$ (0.189) | $Gapdh$ (0.526) |
| 3 | $Rps17$ (1.419) | $Rps17$ (1.383) | $Rps17$ (1.181) | $Rps17$ (0.903) | $Rps17$ (0.751) | $Rps17$ (0.861) | $Rps17$ (0.751) | $Rps17$ (0.861) | $Rps17$ (0.751) | $Rps17$ (0.861) | $Rps17$ (0.751) | $Rps17$ (0.861) | $Rps17$ (0.751) | $Rps17$ (0.861) | $Rps17$ (0.751) | $Rps17$ (0.861) |
| 4 | $GstD1$ (1.983) | $GstD1$ (1.383) | $GstD1$ (1.470) | $GstD1$ (1.600) | $GstD1$ (1.800) | $GstD1$ (1.900) | $GstD1$ (1.800) | $GstD1$ (1.900) | $GstD1$ (1.800) | $GstD1$ (1.900) | $GstD1$ (1.800) | $GstD1$ (1.900) | $GstD1$ (1.800) | $GstD1$ (1.900) | $GstD1$ (1.800) | $GstD1$ (1.900) |

1 Gene stability measure as calculated by $geNorm$.
2 Stability of gene expression among the three different species within each life stage.
3 Stability of gene expression in $Cochliomyia$ $hominivorax$ and $Cochliomyia$ $macellaria$ including $GstD1$.
4 Stability of gene expression between the two life stages within each species.
5 Stability value as calculated by NormFinder.
6 Stability index as calculated by BestKeeper.

$p < 0.05$
Genes in bold had a standard deviation lower than 1.
The Ct difference between gene-specific replicates was used to calculate the intra-run variation. The mean intra-run variation was 0.21. The variation was higher than 1 (higher than 2-fold) only in the C. albiceps sample amplified using primers for the Rp49 gene, for which the variation was 1.41.

Comparison among species within the same life stage
The expression stability of the candidate reference genes was evaluated among the different species within each life stage to select reference genes for qPCR experiments for the comparison of different species.

The suitable reference genes for normalization were ranked based on their expression stability (M) in a combined dataset of Co. hominivorax, Co. macellaria, and C. albiceps using geNorm (Table 3). Gapdh and Rp49 were the two most stable genes in samples of adult females.

By analyzing the gene expression stability using NormFinder in adult females, a similar result as geNorm was observed (Table 3). The best combination of two genes was Rp49 and Gapdh, with a stability value of 0.332. The software geNorm ranked RpS17 as the least stable gene, while NormFinder ranked Actin as the least stable. Using Bestkeeper, Gapdh was the candidate reference gene.
gene with the least variation, having an SD of 0.74, which represents a change in gene expression lower than 2-fold (SD smaller than 1 (Wong and Medrano 2005)). Gapdh was followed by Rp49, which had an SD of 0.78. The variation in the expression of the other candidate reference genes was greater than 2-fold. The pair-wise correlation between genes and the correlation between each gene and the BestKeeper index was also calculated (Table 3). The best correlation between the BestKeeper index and the candidate reference gene in females was obtained for Gapdh ($r = 0.805, p = 0.001$).

In larvae, geNorm ranked Gapdh and Actin (M = 0.917) as the best reference genes with higher stability values (Table 3). Based on the intra- and inter-group variation in NormFinder, Actin (0.461) and Gapdh (0.553) were identified as the most expression-stable, and RpS17 (0.868) as the least stable.

The analysis of BestKeeper results revealed that only Rp49 had an acceptable variation in gene expression (SD of 0.84), but it was ranked third according to its correlation with the BestKeeper index (Table 3). Even though the variation in their expression exceeded 2-fold, Actin and Gapdh had the highest correlation with the BestKeeper index. Hence, there was a consensus among the three different algorithms suggesting the same two genes with the highest expression stability among larval samples of different species.

The results for larvae were similar to those found for adult females. The suitable genes for normalization given by the three programs were Gapdh, Actin, and Rp49.

It was not possible to determine the expression levels of GstD1 in C. albiceps. Hence, the tests were repeated without the C. albiceps samples to validate the stability of this gene between Co. hominivorax and Co. macellaria (Table 3). Regardless of the algorithm, GstD1 was among the least stable in both larval and adult female samples. Only RpS17 had a performance worse than GstD1.

**Comparison between life stages within species**
The stability of the candidate reference genes between larvae and adult females within each species was also tested. Genes with the highest stability values between stages can be used as good reference genes in developmental studies aimed at comparing gene expression among different stages of Calliphoridae flies.

In C. albiceps, there was a consensus between NormFinder, geNorm, and BestKeeper results, allowing the identification of two good candidates, Gapdh and Rp49 (Table 3). In BestKeeper, both had a variation lower than 2-fold (SD of 0.42 for Rp49 and 0.67 for Gapdh). Actin was ranked second (Table 3) but it had a higher variation (SD of 1.08). RpS17 was the least expression-stable gene regardless of the algorithm employed.

In Co. hominivorax, Actin and Gapdh are the best choices based on the geNorm results (Table 3), while NormFinder ranked Gapdh and Rp49 as the most expression-stable genes. BestKeeper ranked the genes differently; GstD1 and Gapdh had the best correlation with the BestKeeper index (Table 3) but only Rp49 and Actin had an acceptable level of variation (SD of 1.01 and 1.05, respectively).
Finally, comparing different stages in *Co. macellaria*, the best reference genes according to geNorm and NormFinder results were *Actin* and *Gapdh* (Table 3). *Gapdh* and *Rp49* had the best correlations with BestKeeper index, but again, only *Rp49* had a variation in gene expression lower than 2-fold (SD of 0.75).

**Discussion**

Although qPCR is widely used, there is no consensus as to which gene or gene set should be used for data normalization, and the selection of genes that are expressed in the same levels across all samples and different conditions of a study is still one of the challenges of this technique (Bustin 2000; Bustin 2002). Differences in mRNA levels were observed in several housekeeping genes originally considered stable in their expression levels (Radonic et al. 2004; Bustin 2002). Therefore, using non-validated reference genes for normalization may result in an erroneous expression data interpretation. Thus, it is recommended to validate a set of candidate reference genes for each qPCR experiment (Bustin et al. 2010). The validation of reference genes is a difficult task, as the amount of the reference gene itself requires normalization for an accurate measure. An alternative is to use a combined set of candidate reference genes and their pair-wise variation in gene expression to evaluate the expression stability of each reference gene (Kubista et al. 2006; Nolan et al. 2006).

Recently, with the growing concern over an accurate normalization for qPCR data, there was an increase in the number of validation studies (Mallona et al. 2010). In insect research, there are only a limited number of efforts. Reference genes for qPCR were validated in *A. mellifera* (Lourenço et al. 2008; Scharlaken et al. 2008), ticks, *Rhipicephalus appendiculatus* and *Rhipicephalus microplus* (Nijhof et al. 2009), the locust *S. gregaria* (Van Hiel et al. 2009), and the psocid *Liposcelis bostrychophila* (Jiang et al., 2010). In Diptera, reference genes were validated in the fruit fly *Bactrocera dorsalis* (Shen et al. 2010) and in the blowfly *Lucilia cuprina* (Bagnall and Kotze 2010).

Here, the first effort of a cross-species validation of reference genes for evolutionary studies using qPCR is described. This cross-species approach presents some challenges for the selection of reference genes. First, it is necessary to design primers in conserved regions of orthologous genes. For non-model species, as in this study, conserved regions can be found by comparing divergent species. *Cochliomyia hominivorax* sequences were aligned to different species of *Drosophila*. However, not all primers designed recovered the desired product; *SdhA* was amplified only in two species, *Co. hominivorax* and *Co. macellaria*, α-tub was amplified only in *Co. hominivorax*, while primers for *GstD1* had a low efficiency of amplification in *C. albiceps*. A second challenge is the selection of genes with a stable expression across the different species. The divergence in the gene expression levels is correlated with the sequence divergence between species (Castillo-Davis et al. 2004; Nuzhdin et al. 2004; Lemos et al. 2005). For housekeeping genes there is, in general, a higher conservation in both regulatory and coding regions (Hurst et al. 2002; Farre et al. 2007). Hence, 10 housekeeping genes commonly used in previous insect studies (Scharlaken et al. 2008; Dworkin and Jones 2009; Van Hiel et al. 2009) were selected, and primer pairs for these genes were designed based on the sequence information from the *Co. homi-
inivorax transcriptome (Carvalho et al. 2010).

To evaluate the gene expression stability of the selected genes among different species, the most widely used programs were employed. Each program has its own statistical approach and each calculate a value for the expression stability of each gene. The Excel-based program geNorm (Vandesompele et al. 2002) uses the pair-wise variation of every reference gene as the standard deviation of the expression values and calculates a stability value. By using the geNorm algorithm Gapdh, Rp49, and Actin were identified as the most stable candidate genes in this experimental setting.

Similarly, BestKeeper (Pfaffl et al. 2004) calculates an index (BestKeeper index) using the geometric mean of each candidate reference gene. The pair-wise correlation is calculated with the BestKeeper index compared to each individual. The genes with best ranking among data in our study were Actin and Gapdh.

NormFinder (Andersen et al. 2004), however, estimates expression stabilities according to the intra- and inter-group expression variation, suggesting the most expression-stable genes. With NormFinder’s algorithm, Gapdh, Rp49, and Actin had the best stability values among the selected genes, both within and among the different species and developmental stages.

Several entomological studies demonstrated that Actin, Rp49, and Gapdh are stably expressed and, therefore, are good reference genes in experiments where normalization is required. Scharlaken et al. 2008 compared the expression stability of six reference genes, Actin, α-tub, Gapdh, Rpl13A, Rps18, and Ubq (UbiQuitin family member; ubq-1), between honeybees with and without a bacterial challenge. Actin and Gapdh were two of the best reference genes suggested by these authors. Rpl13A and Rps18 had higher expression stability values in the honeybee study but the expression of these genes was not detected in our samples.

In the locust S. gregaria (Van Hiel et al. 2009), the gene expression stability of nine candidate reference genes in the brain of nymphs and adults were compared. Actin, Ef1α100E, Gapdh, and Rp49 were selected in this study and were also identified as stable reference genes to be used for accurate normalization.

Nijhof et al. 2009 studied the stability of gene expression of nine candidate genes: Actb (beta actin), Btub (beta tubulin), Ef1α100E, Gapdh, GstD1, H3F3A (H3 histone family 3A), PPIA (Cyclophilin), Rpl4 (Risbosomal protein L4), and Tbp (TATA box binding protein) among life-stages of two species, R. appendiculatus and R. microplus. Gapdh was among the most expression-stable genes in the comparison using the combined data of both species. In line with the results of our study, GstD1 was the least expression-stable gene. However, Gapdh did not perform well in all validation studies. In the fruit fly B. dorsalis, the expression stability of 10 genes, including Actin, α-tub, Gapdh, and Ef1α100E, was analyzed in different tissues (Shen et al. 2010). Actin performed well as a reference gene, but Gapdh did not.

Recently, Bagnall and Koteze (2010) evaluated the stability of reference genes in different life stages of a closely related species in the Calliphoridae family, L. cuprina. Three of the 11 genes tested (Actin, Gapdh,
and \textit{GstD1}) overlapped with the genes selected in our study. One of the most expression-stable genes in our study, \textit{Gapdh}, was ranked by geNorm and NormFinder as the worst reference gene in the \textit{L. cuprina} study, with \textit{M} values ranging from 0.121 to 0.643 and NormFinder stability values ranging from 0.195 to 0.724 (Bagnall and Kotze 2010). The authors hypothesized that as \textit{Gapdh} is involved in metabolism, the individuals could have been in different energetic states during sampling. Combined, these results highlight the requirement of validation studies for each experimental setting. Although \textit{Gapdh} performed well in our study and some others, it was also among the least stable genes in some experimental settings.

Commonly used reference genes are involved in essential biological functions, such as cellular transport, translation, structural constitution, and metabolism, because they are thought to have stable expression. However, several studies demonstrated that such housekeeping genes could also vary according to experimental settings (Huitorel and Pantaloni 1985; Chang et al. 1998; Vandesompele et al. 2002; Axtner and Sommer 2009; Nijhof et al. 2009; Bagnall and Kotze 2010). This expression variation is probably a result of the involvement of these genes in additional cellular functions (Jain et al. 2006).

The genes \textit{RpS17} and \textit{GstD1} were the least stable genes in our study. \textit{RpS17} is involved in translation and encodes a protein related to lipid droplet formation (Cermelli et al. 2006). This lipid component is important for energy storage and utilization (Arrese and Soulages 2010). Furthermore, lipids play important roles in multiple metabolic functions during insect development (Arrese and Soulages 2010). The large variation of the expression of this gene may be related to individual differences in lipid storage between life-stages, but also may reflect different lipid requirements among the studied species. \textit{GstD1} encodes a glutathione-S-transferase involved in detoxification metabolism. This gene is regulated when an insect is exposed to a variety of organic compounds (Chahine and O'Donnell 2011). The compared species have different feeding preferences and were fed in different media. While we did not formally test the differences in gene expression of \textit{GstD1} among the different species, this was an interesting observation and further experiments should be performed to test the association of \textit{GstD1} with feeding habits.

Although several commonly used reference genes displayed a stable expression in many studies, reference genes must be validated for each experimental setting. In our study, the expression stability of six candidate reference genes was tested aiming at future functional studies in the Calliphoridae family. In our analyses, \textit{Actin}, \textit{Rp49}, and \textit{Gapdh} had a stable expression level among different Calliphoridae species and between two different life-stages within each species. Hence, they can be used as reliable reference genes for functional studies in Calliphoridae using similar experimental settings. These reference genes will be critical in studies involving behavior, development, and other biological processes in Calliphoridae species and will be an important resource for other validation studies in Calliphoridae and other insects.

\textbf{Acknowledgments}

We are very grateful to Maria Salete Couto for maintaining the screwworm colonies and
Rosângela A. Rodrigues for valuable technical assistance. This work was supported by grants to T. T. Torres from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, grant 2008/58106-0) and from the Brazilian National Council for Scientific and Technological Development (CNPq, 477335/2009-8). T. T. Torres received a research fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). G. A. Cardoso was supported by a fellowship from FAPESP (2009/13463-3).

Disclosure

Gisele Antoniazzi Cardoso carried out all experiments and data analysis. Cleverson Carlos Matioli helped with qPCR experiments. Ana Maria Lima de Azeredo-Espin contributed materials and analysis tools and helped in the project supervision. Tatiana Teixeira Torres conceived of the study, supervised the project, and coordinated all activities. Gisele Antoniazzi Cardoso and Tatiana Teixeira Torres designed the experiments and wrote the manuscript. All authors read and approved the final manuscript.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. *Journal of Molecular Biology* 215: 403–410.

Andersen CL, Jensen JL, Orntoft 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 Research* 64: 5245–5250.

Arrese EL, Soulages JL. 2010. Insect fat body: energy, metabolism, and regulation. *Annual Review of Entomology* 55: 207–225.

Axtner J, Sommer S. 2009. Validation of internal reference genes for quantitative real-time PCR in a non-model organism, the yellow-necked mouse, *Apodemus flavicollis*. *BMC Research Notes* 2: 264.

Bagnall NH, Kotze AC. 2010. Evaluation of reference genes for real-time PCR quantification of gene expression in the Australian sheep blowfly, *Lucilia cuprina*. *Medical Veterinary Entomology* 24: 176–181.

Bustin SA. 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *Journal of Molecular Endocrinology* 25: 169–193.

Bustin SA. 2002. Quantification of mRNA using real-time reverse transcription PCR: trends and problems. *Journal of Molecular Endocrinology* 29: 23–39.

Bustin SA, Beaulieu JF, Huggett J, Jaggi R, Kibenge FS, Olsvik PA, Penning LC, Toegel S. 2010. MIQE precis: Practical implementation of minimum standard guidelines for fluorescence-based quantitative real-time PCR experiments. *BMC Molecular Biology* 11: 74.

Carvalho RA, Azeredo-Espin AML, Torres TT. 2010. Deep sequencing of New World screw-worm transcripts to discover genes involved in insecticide resistance. *BMC Genomics* 11: 695.

Castillo-Davis CI, Hartl DL, Achaz G. 2004. cis-Regulatory and protein evolution in
orthologous and duplicate genes. *Genome Research* 14: 1530–1536.

Cermelli S, Guo Y, Gross SP, Welte MA. 2006. The lipid-droplet proteome reveals that droplets are a protein-storage depot. *Current Biology* 16: 1783–1795.

Chahine S, O’donnell MJ. 2011. Interactions between detoxification mechanisms and excretion in Malpighian tubules of *Drosophila melanogaster*. *Journal of Experimental Biology* 214: 462–468.

Chang TJ, Juan CC, Yin PH, Chi CW, Tsay HJ. 1998. Up-regulation of beta-actin, cyclophilin and GAPDH in N1S1 rat hepatoma. *Oncology Reports* 5: 469–471.

Chenna R, Sugawara H, Koike, T, Lopez R, Gibson TJ, Higgins DG, Thompson JD. 2003. Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Research* 31: 3497–3500.

Clark AG, Eisen MB, Smith DR, Bergman CM, Oliver B, et al. 2007. Evolution of genes and genomes on the *Drosophila* phylogeny. *Nature* 450: 203–218.

Dworkin I, Jones CD. 2009. Genetic changes accompanying the evolution of host specialization in *Drosophila sechellia*. *Genetics* 181: 721–736.

Dworin I, Jones CD. 2009. Genetic changes accompanying the evolution of host specialization in *Drosophila sechellia*. *Genetics* 181: 721–736.

Farre D, Bellora N, Mularoni L, Meseguer X, Alba MM. 2007. Housekeeping genes tend to show reduced upstream sequence conservation. *Genome Biology* 8: R140.

Huitorel P, Pantaloni D. 1985. Bundling of microtubules by glyceraldehyde-3-phosphate dehydrogenase and its modulation by ATP.

Jiang HB, Liu YH, Tang PA, Zhou AW, Wang JJ. 2010. Validation of endogenous reference genes for insecticide-induced and developmental expression profiling of *Liposcelis bостrychophila* Psocoptera: Liposcelididae. *Molecular Biology Reports* 37: 1019–1029.

Kubista M, Andrade JM, Bengtsson M, Forootan A, Jonak J, Lind K, Sindelka R, Sjöback R, Sjögren B, Strombom L, Stahlberg A, Zoric N. 2006. The real-time polymerase chain reaction. *Molecular Aspects of Medicine* 27: 95–125.

Lemos B, Bettencourt BR, Meiklejohn CD, Hartl DL. 2005. Evolution of proteins and gene expression levels are coupled in *Drosophila* and are independently associated with mRNA abundance, protein length, and number of protein-protein interactions. *Molecular Biology and Evolution* 22: 1345–1354.

Lourenço A, Mackert A, Cristiano A, Simões Z. 2008. Validation of reference genes for gene expression studies in the honey bee, *Apis mellifera*, by quantitative real-time RT-PCR. *Apidologia* 39: 372–385.
Mallona I, Lischewski S, Weiss J, Hause B, Egea-Cortines M. 2010. Validation of reference genes for quantitative real-time PCR during leaf and flower development in Petunia hybrida. BMC Plant Biology 10: 4.

Nijhof AM, Balk JA, Postigo M, Jongejan F. 2009. Selection of reference genes for quantitative RT-PCR studies in Rhipicephalus Boophilus microplus and Rhipicephalus appendiculatus ticks and determination of the expression profile of Bm86. BMC Molecular Biology 10: 112.

Nolan T, Hands RE, Bustin SA. 2006. Quantification of mRNA using real-time RT-PCR. Nature Protocols 1: 1559–1582.

Nuzhdin SV, Wayne ML, Harmon KL, Mcintyre LM. 2004. Common pattern of evolution of gene expression level and protein sequence in Drosophila. Molecular Biology and Evolution 21: 1308–1317.

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. Biotechnology Letters 26: 509–515.

Radonic A, Thulke S, Mackay IM, Landt O, Siegert W, Nitsche A. 2004. Guideline to reference gene selection for quantitative real-time PCR. Biochemical and Biophysical Research Communications 313: 856–862.

Rozen S, Skaletsky H. 2000. Primer3 on the WWW for general users and for biologist programmers. Methods in Molecular Biology 132: 365–386.

Scharlaken B, Graaf D, Goossens K, Brunain M, Peelman L, Jacobs F. 2008. Reference gene selection for insect expression studies using quantitative real-time PCR: The head of the honeybee, Apis mellifera, after a bacterial challenge. Journal of Insect Science 8:33. Available online: www.insectscience.org/8.33

Shen GM, Jiang HB, Wang XN, Wang JJ. 2010. Evaluation of endogenous references for gene expression profiling in different tissues of the oriental fruit fly Bactrocera dorsalis, Diptera: Tephritidae. BMC Molecular Biology 11: 76.

Stevens JR. 2003. The evolution of myiasis in blowflies Calliphoridae. International Journal of Parasitology 33: 1105–1113.

Stevens JR, Wallman JF. 2006. The evolution of myiasis in humans and other animals in the Old and New Worlds part I: phylogenetic analyses. Trends in Parasitology 22: 129–136.

Van Hiel MB, Van Wielendaele P, Temmerman L, Van Soest S, Vuerinckx K, Huybrechts R, Broeck JV, Simonet G. 2009. Identification and validation of housekeeping genes in brains of the desert locust Schistocerca gregaria under different developmental conditions. BMC Molecular Biology 10: 56.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3; RESEARCH0034.
Vanguarder HD, Vrana KE, Freeman WM. 2008. Twenty-five years of quantitative PCR for gene expression analysis. *Biotechniques* 44: 619–626.

Wong ML, Medrano JF. 2005. Real-time PCR for mRNA quantitation. *Biotechniques* 39: 75–85.

Zumpt F. 1965. *Myiasis in Man and Animals in the Old World*. Butterworths.