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ORIGINAL RESEARCH ARTICLE

Comparative analysis of reference genes in honey bees, *Apis cerana* and *Apis mellifera*

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ABSTRACT

Honey bees are vital pollinators in agriculture and important model insects. To understand the genetic and molecular aspects in their development, a reverse transcription quantitative polymerase chain (RT-qPCR) is used to investigate the target genes. However, it is essential to use the appropriate reference genes as endogenous controls for accurate normalization of target genes. To identify stable reference genes in two honey bee species, [*Apis mellifera* (*Am*) and *Apis cerana* (*Ac*)], we evaluated eight candidate reference genes including, *actin*, *atub*, *ef1α*, *gapdh*, *rpl13a*, *rpl32*, *rps18* and *tif*. Worker bees belonging to the two species were collected at each developmental day during the embryonic and postembryonic developmental stages. The *tyrosine hydroxylase* (*th*) gene was used as the target gene to validate the selected reference genes. Our results revealed that *rpl13a* was the most stable reference gene at all developmental stages of *Am* and *Ac*. In addition, gene combinations, including *Amrpl13a* & *Amrps18* & *Amactin*, *Amrpl13a* & *Amrpl32*, *Acrpl13a* & *Acrpl32*, *Acrpl13a* & *Acrpl32* & *Acef1α* followed by other combinations effectively normalized the expression of the target genes during the embryonic and postembryonic developmental stages of *Am* and *Ac*, respectively. Our findings provide a foundation for standardized RT-qPCR analysis to improve the accuracy of genes normalization during the different developmental stages of honey bees.

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RT-qPCR; reference genes; gene expression; *Apis mellifera*; *Apis cerana*

Introduction

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) has become the preferred method for the quantification of relative gene expression to understand the genetic and molecular mechanisms due to its high sensitivity, accuracy, specificity, rapidity and repeatability (Nonis et al., 2014; Park et al., 2008; Shakeel et al., 2018; Valasek & Repa, 2005).Stable reference genes are used as an internal control to normalize the RT-qPCR data (Feuer et al., 2015; Huggett et al., 2005; Pabinger et al., 2014). Evaluating reference genes is crucial to validate their stability to normalize the target gene expression for accurate RT-qPCR assays. This is because the stability of the reference genes differs among tissues, developmental stages, species, and responses to abiotic factors (Shakeel et al., 2018). For example, the appropriate reference genes for RT-qPCR assays in various insect species have been screened and validated (Deng et al., 2020; Freitas et al., 2019; Jeon et al., 2020; Niu et al., 2014).

Honey bees (*Apis mellifera*) play a vital role as plant pollinators in natural ecosystems (Hung et al., 2018; Klein et al., 2007). They are social insects with labor division and colony management, hence commonly used as model insects (Lee & Kim, 2017). Based on this, validation of their reference genes has been performed in the past. The expression of reference genes in *Apis mellifera* (*Am*) was initially examined in different tissues during the postembryonic development in larval and pupal stages after juvenile hormone exposure (Lourenço et al., 2008), and in head tissues after a bacterial challenge (Reim et al., 2013; Scharlaken et al., 2008). Reference genes have been validated in the *Am* head, thorax and abdomen during different seasons (Jeon et al., 2020; Moon et al., 2018a, 2018b), following infections with Israeli acute bee paralysis virus (IAPV) and chronic bee paralysis virus (CBPV) infections and under double-stranded RNA (dsRNA) treatment (Deng et al., 2020). In addition, the reference genes have been validated in *Apis cerana* (*Ac*) under the Chinese sacbrood virus (CSBV) (Deng et al., 2020). As a result, different reference genes have been identified under various conditions, indicating the variation in their expression under different conditions.

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Therefore, we evaluated the expression of eight common reference genes using five algorithms to identify the most suitable reference genes for RT-qPCR during the embryonic and postembryonic development of *Am* and *Ac*. These reference genes included *A. cerana actin-5C* (*Acactin*), *A. cerana tubulin alpha-1 chain like* (*Acatub*), *A. cerana elongation factor 1-alpha* (*Acef1α*), *A. cerana glyceraldehyde-3-phosphate dehydrogenase 2* (*Acgapdh*), *A. cerana 60S ribosomal protein L13a* (*Acrpl13a*), *A. cerana 60S ribosomal protein L32* (*Acrpl32*), *A. cerana 40S ribosomal protein S18* (*Acrps18*), *A. cerana transcription initiation factor TFIID subunit 10* (*Actif*), *A. mellifera actin related protein 1* (*Amactin*), *A. mellifera tubulin alpha-1 chain* (*Amatub*), *A. mellifera elongation factor 1-alpha F2* (*Amef1α*), *A. mellifera glyceraldehyde-3-phosphate dehydrogenase 2* (*Amgapdh*), *A. mellifera 60S ribosomal protein L13a* (*Amrpl13a*), *A. mellifera ribosomal protein L32* (*Amrpl32*), *A. mellifera 40S ribosomal protein S18* (*Amrps18*), *A. mellifera transcription initiation factor TFIID subunit 10* (*Amtif*). The expression of the target gene, *tyrosine hydroxylase* (*th*), which is involved in vital physiological processes in insects (Liu et al., 2010; Pendleton et al., 2002; Yang et al., 2018; Zhang et al., 2019), was investigated to validate the reference genes.

Materials and methods

Biological samples

The honey bee colonies (*Apis mellifera ligustica* and *Apis cerana cerana*) were obtained from the Honey bee Research Institute, Jiangxi Agricultural University, Nanchang, China (28.46°N, 115.49°E).

Worker bees (*Am*) were collected from three different colonies at each of the 21 developmental days at different stages, including eggs, larvae, prepupae, pupae and newly emerged bees to represent the embryonic and postembryonic development stages. In total, 30–40 eggs, 2–6 larvae and one prepupae, pupae and adult bee were pooled into each sample, with 8–9 samples collected each day. Similarly, *Ac* worker bees were collected from three different colonies during the 20 developmental days. Nine samples were collected each day, except for the 3rd developmental day, where seven samples were collected from two colonies. To control the age of the brood, the queen was restricted to lay eggs for eight hours in one empty comb, and the offspring (20–24 hours old) were collected as the samples at developmental day 1. Afterwards, samples were collected at intervals of 24 hours as the samples at developmental days 2 to 21 (*Am*) or 20 (*Ac*). The samples from developmental days 1 to 3 (egg stage) were used to evaluate the reference genes during the embryonic development, and days 4 to 21 (*Am*) or 20 (*Ac*) during the postembryonic development. The collected samples were immediately frozen in liquid nitrogen and stored at −70 °C for RNA extraction.

Total RNA extraction and cDNA synthesis

The total RNA was extracted using the TransZol Up Plus RNA Kit (TransGen Biotech, Beijing, China) following the manufacturer's protocol. The purity and quality of the total RNA were determined at 260/280 ratio using a DS-11 Spectrophotometer (DeNovix Inc, Wilmington, DE 19810, United States of America). For each sample, 1 μg of total RNA with 260/280 ratio ranging from 1.9 to 2.1 was used for cDNA synthesis using the Prime Script^{TM} RT reagent Kit with gDNA Eraser (Takara, Dalian, China) to eliminate genomic DNA contamination cDNA synthesis reactions following the manufacturer's instructions. All cDNA samples were stored at −20 °C until use.

Rt-qPCR assays

The specific primers amplifying the reference genes were designed per previous studies (Lourenço et al., 2008; Moon et al., 2018a; Scharlaken et al., 2008). In addition, the sequence information of *tif* and two target genes (*Acth* and *Amth*) were obtained from the NCBI (https://www.ncbi.nlm.nih.gov) database, and their primers were designed using the Primer3 program version 4.1.0 (Rozen & Skaletsky, 2000). To detect genomic DNA contamination, *rpl13a* and *rps18* primers were designed to span an intron, respectively. If genomic DNA contaminated the sample, besides the expected 191 bp, *rpl13a* would amplify 313 bp (*Am*) or 317 bp (*Ac*) fragment. *Rps18*, meanwhile, would amplify 443 bp (*Am*) or 451 bp (*Ac*) fragment and the expected 149 bp. These different amplification products would be distinguished based on the respective melting curves.

The RT-qPCR reactions were performed in a final volume of 20 μL containing 10 μL TB Green Premix Ex Taq II (2×) (Takara, Dalian, China), 0.4 μL ROX Reference Dye II (50×) (Takara, Dalian, China), 0.8 μL of 10 μM forward and reverse primers, 2 μL cDNA template, and 6 μL double-distilled H_2O for a total volume of 20 μL. The cDNA reaction was diluted at 1:10 with distilled water, and 2 μL distilled water was used in place of cDNA as the negative control. All reactions were performed in triplicate under the following cycling conditions: 50 °C for 2 min, 95 °C for 5 min (*Ac*) or 10 min (*Am*) followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Melting curves were generated to verify the specificity of the primers. Amplification was confirmed on gel electrophoresis, and the PCR products were cloned into the

Table 1. Primers of the candidate reference genes and target gene used for RT-qPCR.

Gene symbol	Gene name	Primer sequence (5′→3′)	Amplicon size (bp)	GenBank accession no.
Acactin *Amactin*	*Apis cerana actin-5C* *Apis mellifera actin related protein 1*	Forward: TGCCAACACTGTCCTTTCTG Reverse: AGAATTGACCCACCAATCCA	156	XM_017059067.2 NM_001185146.1
Acatub *Amatub*	*Apis cerana tubulin alpha-1 chain like* *Apis mellifera tubulin alpha-1 chain*	Forward: AGCATTCAGATTGCGCTTTT Reverse: GCAACGACGCTGTTATTGAA	141	XM_017061972.2 XM_396338.7
Acef1α *Amef1α*	*Apis cerana elongation factor 1-alpha* *Apis mellifera elongation factor 1-alpha F2*	Forward: GGAGATGCTGCCATCGTTAT Reverse: CAGCAGCGTCCTTGAAAGTT	154	XM_028669498.1 XM_006569892.2
Acgapdh *Amgapdh*	*Apis cerana glyceraldehyde-3-phosphate dehydrogenase 2* *Apis mellifera glyceraldehyde-3-phosphate dehydrogenase 2*	Forward: CACCTTCTGCAAAATTATGGCG Reverse: ACCTTTGCCAAGTCTAACTGTTAA	188	XM_017062468.2 XM_393605.7
Acrpl13a *Amrpl13a*	*Apis cerana 60S ribosomal protein L13a* *Apis mellifera 60S ribosomal protein L13a*	Forward: TGGCCATTTACTTGGTCGTT Reverse: GAGCACGGAAATGAAATGGT	191	XM_017065352.2 XM_623810.5
Acrpl32 *Amrpl32*	*Apis cerana 60S ribosomal protein L32* *Apis mellifera ribosomal protein L32*	Forward: AGTAAATTAAAGAGAAACTGGCGTAAA Reverse: TTAAAACTTCCAGTTCCTTGACATTAT	181	XM_017056470.2 XM_016914656.2
Acrps18 *Amrps18*	*Apis cerana 40S ribosomal protein S18* *Apis mellifera 40S ribosomal protein S18*	Forward: GATTCCCGATTGGTTTTTGA Reverse: CCCAATAATGACGCAAACCT	149	XM_017067400.2 XM_625101.6
Actif *Amtif*	*Apis cerana transcription initiation factor TFIID subunit 10* *Apis mellifera transcription initiation factor TFIID subunit 10*	Forward: TTGGTTTCATTAGCTGCACAA Reverse: ACTGCGGGAGTCAAATCTTC	149	XM_017054421.2 XM_006564672.3
Acth *Amth*	*Apis cerana tyrosine 3-monooxygenase* *Apis mellifera tyrosine hydroxylase*	Forward: GCTTGCGCGGAATATAGAAG Reverse: GGGCTCTTGATGTGACGAAT	221	XM_017059244.2 XM_006565075.3

pClone007 Versatile Simple Vector (TSINGKE, Beijing, China). The ligated products were transformed into TreliefTM 5α Chemically Competent Cells (TSINGKE, Beijing, China). The positive clones were sequenced using the ABI PRISM 3730XL analyzer with the M13 forward universal primer.

The threshold cycle (Ct) and amplification efficiency (E) were computed using qPCR package (Spiess & Ritz, 2010; Hornik, 2011).

Data analysis

The potential outliers were excluded using the Grubbs test (Grubbs, 1969). All statistical analyses were performed by the SPSS software (IBM SPSS Statistics, Rel. 22.0.0.0), where the quantitative data on genes were analyzed using a one-way ANOVA. The gene-expression stability of the candidate reference genes was evaluated based on five statistical algorithms, namely, BestKeeper version-1 (Pfaffl et al., 2004), delta-Ct method (Silver et al., 2006), geNorm^{PLUS} (qbasePLUS, version 3.2, Vandesompele et al., 2002), NormFinder version 0.953 (Andersen et al., 2004), and the online platform RefFinder (Xie et al., 2012).

BestKeeper determines the stability of reference genes based on their standard deviation (SD), coefficient of variation (CV), correlation (R^2) and P-value, where the candidate genes with lower SDs, CVs and P-value and relatively higher R^2 values are considered to be more stable (Pfaffl et al., 2004). Similarly, the delta-Ct method determines the stability of reference genes based on their average SD (Silver et al., 2006). GeNorm ranks the stability of the reference genes based on their M-value, which is the mean variation of a gene relative to all studied genes, where lower M-values are considered to be more stable expressions (Vandesompele et al., 2002). The geNorm M-value in geNorm^{PLUS} is slightly different as it denotes the average M-value after stepwise exclusion of the most unstable reference genes (the highest value); hence it is appropriate for evaluating the stability of a large set (ideally eight or more) of candidate reference genes. In addition, to determine the optimal number of reference genes needed for accurate RT-qPCR normalization, an average pairwise variation (V_n/V_{n+1}) between two consecutive normalization factors (n and n + 1) was calculated. As soon as the V_n/V_{n+1} value drops below the threshold (usually 0.15), the obtained n reference gene was enough to obtain accurate results (Vandesompele

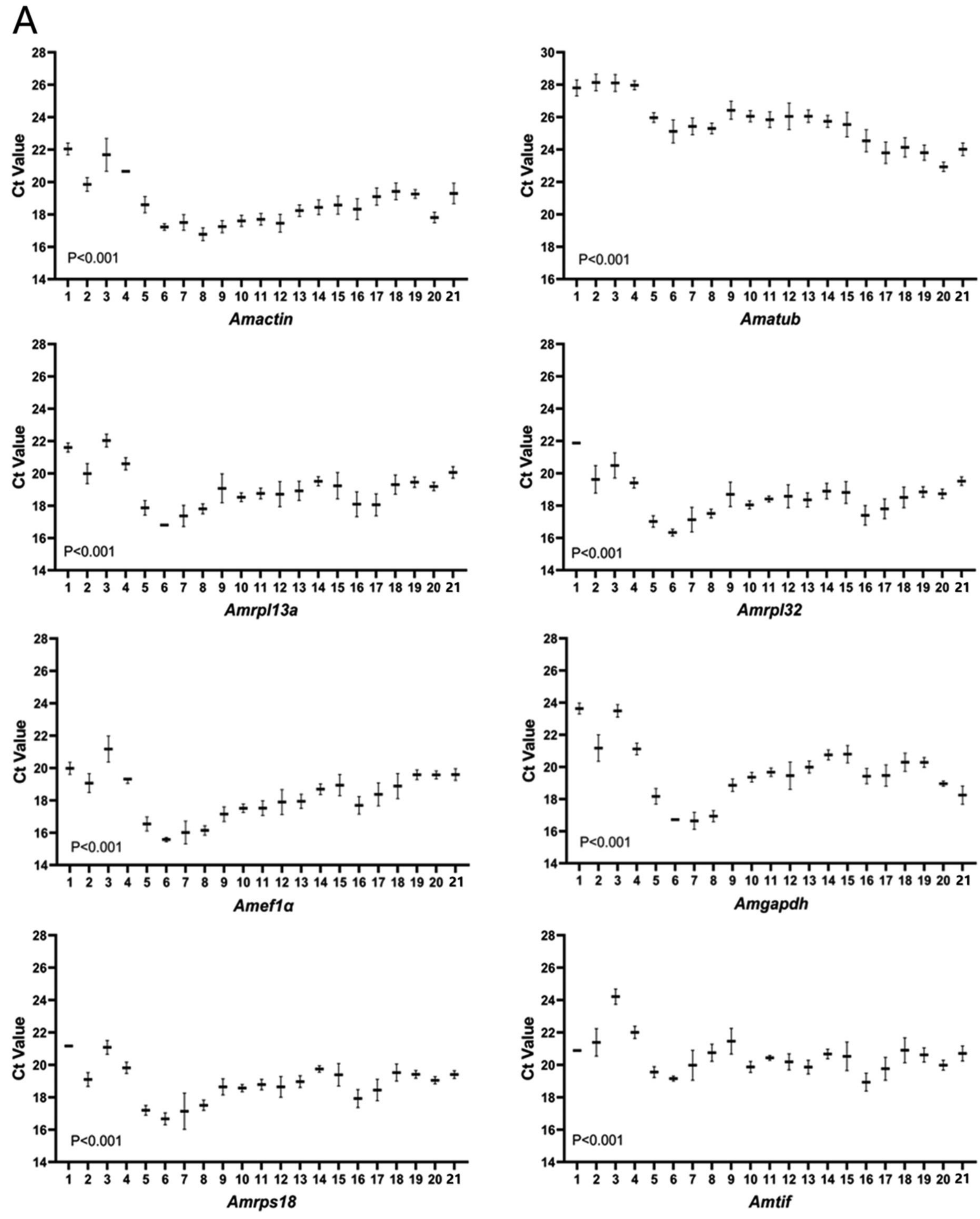

Figure 1. Threshold cycle (Ct) values of the eight reference genes during the embryonic and postembryonic developmental stages of *Am* (A) and *Ac* (B). P-value is given for each reference gene (One-Way ANOVA; Post-hoc: LSD). The numbers of the abscissa axis indicate the developmental days after egg-laying. Bars show the mean values, and error bars indicate the standard deviation of the mean.

et al., 2002). NormFinder calculates the stability of the candidate reference genes by estimating not only their overall variation but also the variation between sample subgroups of the sample set, where lower stability values demonstrate more stable genes (Andersen et al., 2004). Finally, RefFinder, a user-friendly web-based comprehensive tool, was used to evaluate and screen reference genes providing a comprehensive rank based on the geometric mean by integrating the above four computational algorithms results (Xie et al., 2012).

To validate the stability of the selected reference genes, the expression of *Amth* and *Acth* normalized with a single reference gene or multiple reference genes during the developmental stages of *Am* and *Ac* was statistically analyzed using the qbasePLUS software.

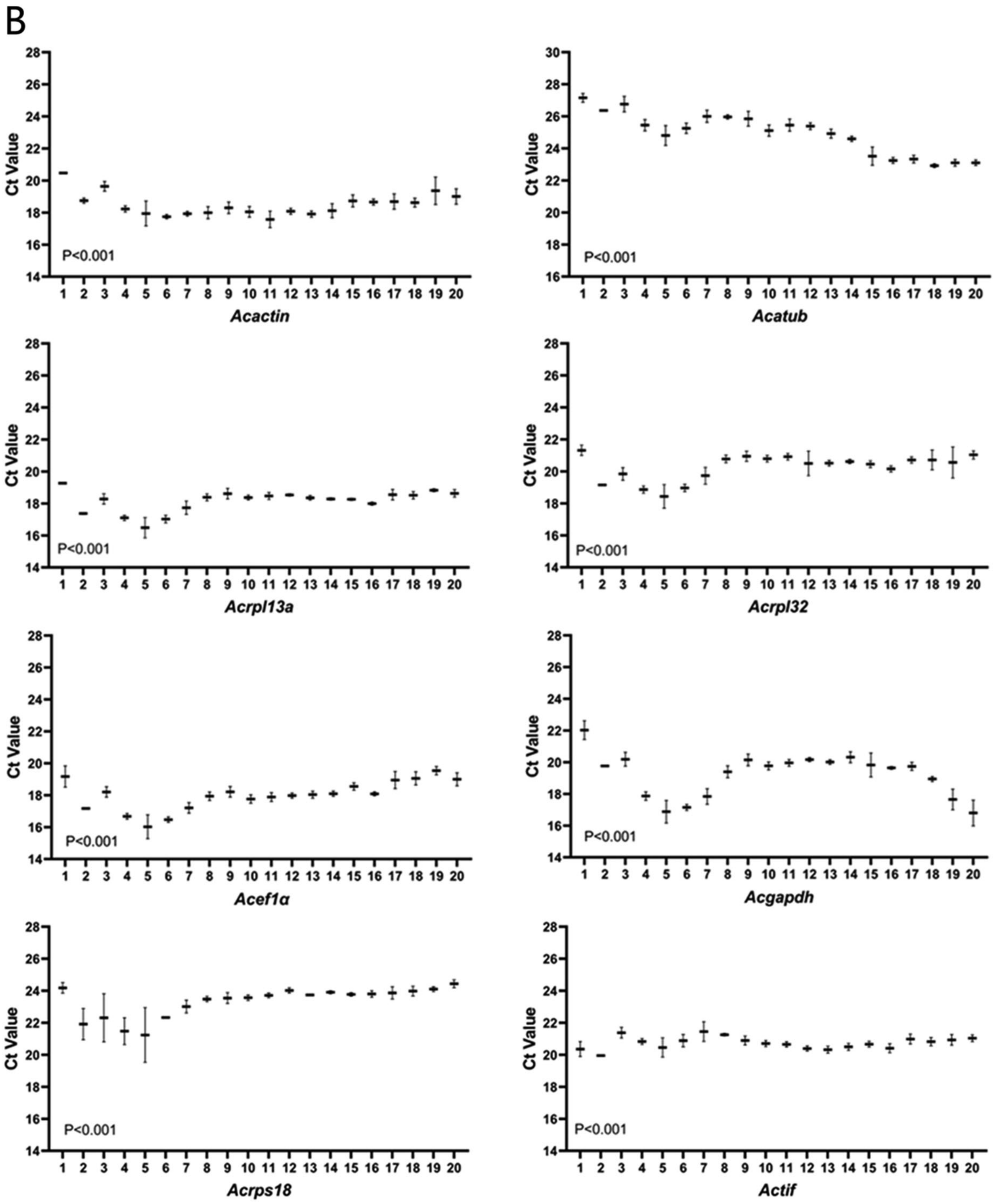

Figure 1. Continued.

Results

Primer optimization and amplification specificity

The primer sequences and characteristics of the candidate reference genes and target gene are summarized in Table 1. The amplification specificity and efficiency of the primers were investigated before the RT-qPCR assay was performed. A single band in 1% agarose gel and a single melting curve peak for each amplicon were observed. In addition, sequencing of the clones of the amplicons also revealed the specificity of the RT-qPCR products. The amplification efficiencies for each reference gene ranged from 1.87 to 1.93, and for the target genes (*Amth* and *Acth*) from 1.84 and 1.88 (Supplementary Table S1). The amplification efficiencies were calculated by fitting an exponential model, an objective method showing similar results to the standard curve method but having higher accuracy to avoid assumptions and subjective judgments (Zhao & Fernald, 2005).

Transcription profiles of reference genes during developmental stages of honey bee

RNA transcription levels of the eight candidate reference genes were compared at 21 and 20

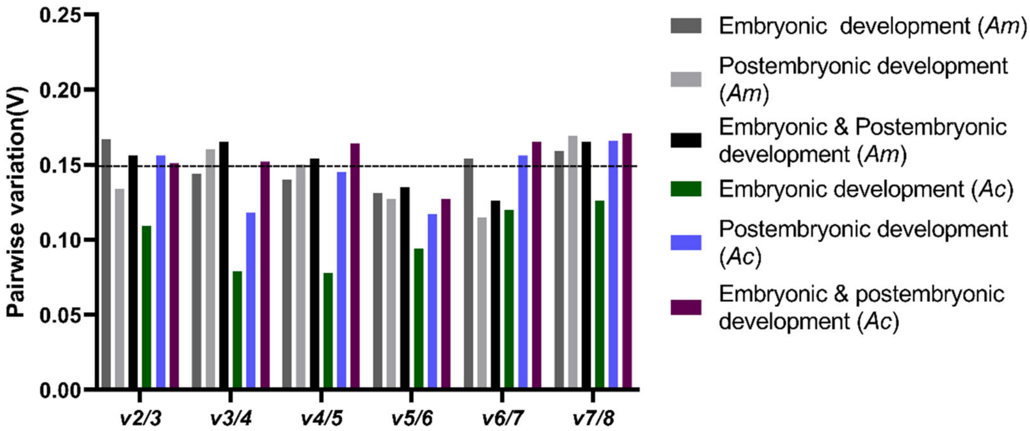

Figure 2. Pairwise variation calculated by geNorm to determine the optimal number of reference genes for the normalization of the target genes. The value of $V_n/V_{n+1} < 0.15$ means n is the optimal number of reference genes selected for RT-qPCR analysis.

developmental days of *Am* and *Ac*, respectively (Figure 1). The distribution of Ct values for all reference genes showed significant variations ($P < 0.001$), with the lowest mean values of *Amactin*, *Amef1α*, *Amgapdh*, *Amrpl13a*, *Amrps18* and *Amrpl32* observed at developmental day 5 to 8 of *Am*, and *Acef1α*, *Acrpl13a*, and *Acrpl32* at day 4 to 6 of *Ac*. In addition, *Amactin* and *Amgapdh* had significantly low Ct values at days 20 and 21, respectively, and *Acgapdh* on both days.

Stability of the candidate reference genes during developmental stages of honey bee

The expression stability of the candidate reference genes during the embryonic and postembryonic developmental stages in *Am* and *Ac*, analyzed using different algorithms, are summarized in Supplementary Table S2.

During the embryonic development of *Am*, *Amactin*, *Amrps18* and *Amrpl13a* were the most stable genes based on delta-Ct and geNorm with M-value below 1. The comprehensive result of RefFinder integrating those four algorithms was consistent with Delta-Ct and geNorm with M-value below 1 algorithms criterion for selecting accurate reference genes (Hellemans et al., 2007). With Normfinder, *Amrpl13a*, *Amrps18* and *Amgapdh* were identified as the most stable genes with SV value below 0.15, and *Amatub*, *Amef1α* and *Amrpl13a* based on the Bestkeeper with SD < 1. Although more stable candidate reference genes were identified based on these criteria, we were interested in the top 3. Thus, *Amrpl32*, *Amrpl13a* and *Amrps18* were the most stable genes based on geNorm, Normfinder and delta-Ct, and consistent with the comprehensive result of RefFinder during the postembryonic development were selected. Besides, the top three stable genes, during the whole development and during the postembryonic development of *Am*, were the same.

Similarly, the three most stable genes during the embryonic development of *Ac* were different based on the four algorithms, and the comprehensive ranking normalized by RefFinder were *Acrpl13a* > *Acactin* > *Acrpl32*. However, during the postembryonic development, *Acrpl13a*, *Acrpl32* and *Actif* were the most stable genes based on RefFinder, with *Acrpl13a* and *Acrpl32* as the top two stable genes in all algorithms except Bestkeeper. Besides, across all the developmental stages, *Acrpl13a*, *Acrpl32* and *Acef1α* were the most stable genes based on geNorm and delta-Ct, consistent with the result for RefFinder.

In addition, *Acatub* was the least stable gene in both *Am* and *Ac* based on all algorithms except BestKeeper, which showed *Acgapdh* as the least stable gene.

Subsequently, the pairwise variation (V_n/V_{n+1}) calculated by geNorm with 0.15 as the cutoff point to determine the optimal number of candidate reference genes for the normalization of the target gene is shown in Figure 2. In the present study, different numbers of reference genes were used to normalize the RT-qPCR data at different developmental stages of *Am* and *Ac*. The combination of *Amactin*, *Amrps18* and *Amrpl13a* during the embryonic development, *Amrpl32* and *Amrpl13a* during the postembryonic development, and *Amrpl13a*, *Amrpl32*, *Amrps18*, *Amef1α* and *Amactin* during the whole development of *Am*, were stable to normalize the expression of target genes at the respective stages. Likewise, the combination of *Acrpl13a* and *Acrpl32* during embryonic development, *Acrpl13a*, *Acrpl32* and *Acef1α*, and *Acrpl13a*, *Acrpl32*, *Acef1α*, *Acrps18* and *Acactin* during the whole development of *Ac* were recommended. Considering the high SD values of *Amactin* and *Amef1α* during the whole development of *Am* based on Bestkeeper, the combination of candidate reference genes at this stage was excluded in our next analysis.

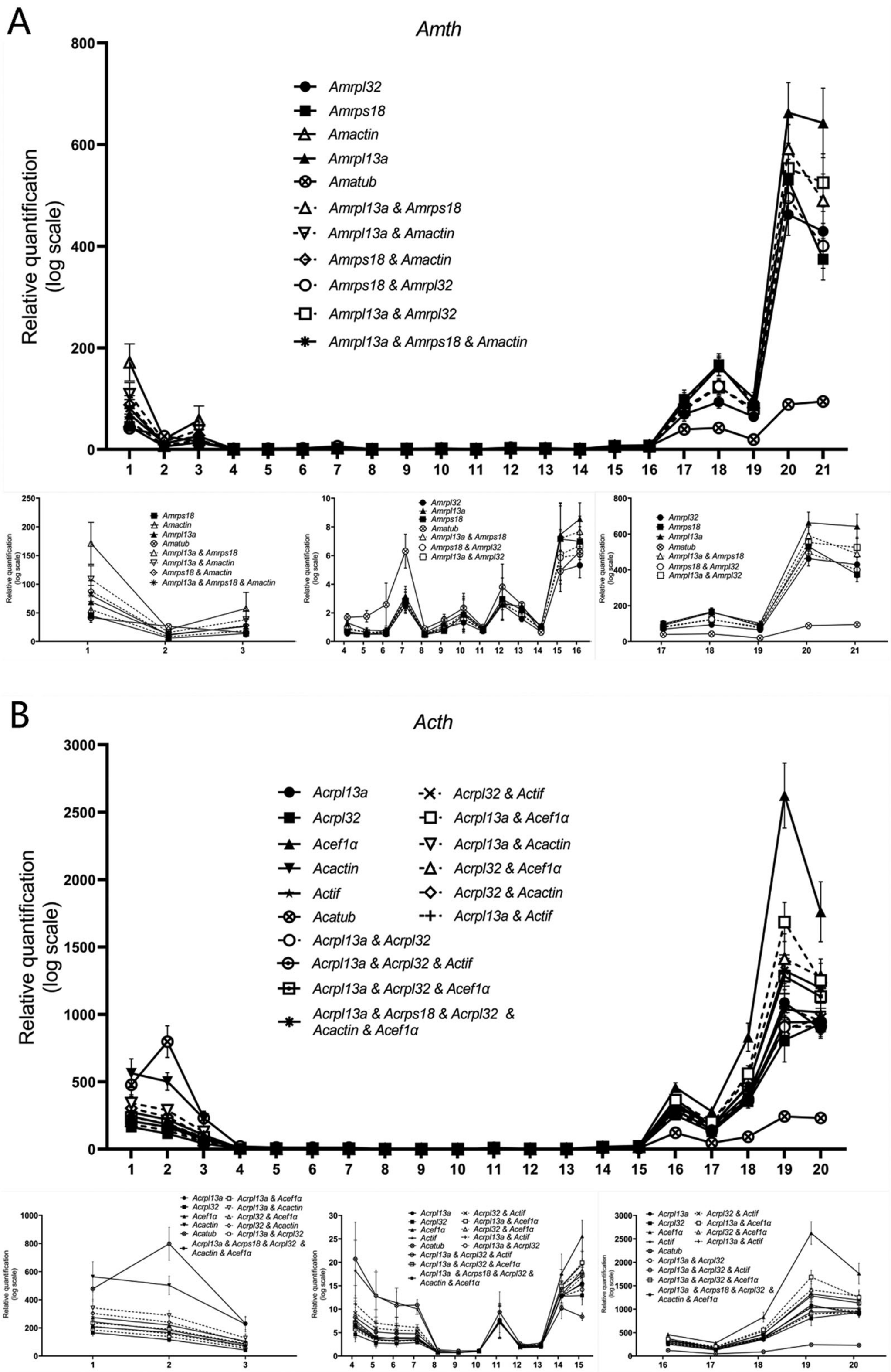

Figure 3. Relative quantification (log scale) of the target genes (*Amth*, and *Acth*) normalized with a single gene or a combination of two paired genes during the development of *Am* and *Ac*. (A) Expression levels of *Amth*, and (B) of *Acth*. The values in the abscissa axis indicate the developmental days after egg laying (days 1 to 3 represent the embryonic development, and days 4–21 for *Am* or 4–20 for *Ac* represent the postembryonic development). Bars show the mean values, and error bars indicate the standard error of the mean.

Validation of the selected reference genes

Considering the stability of the genes based on the different algorithms and the optimal number of candidate genes based on the V_n/V_{n+1} value, we compared the expression of the target genes (*Amth* and *Acth*) normalized with each of the three most stable reference genes and their combinations. Additionally, the optimal combination of reference genes based on the geNorm algorithm was analyzed as the benchmark. The expression profiles of the least stable genes (*Amatub* and *Acatub*) in each developmental day normalized with different combinations of the reference genes were also analyzed for comparison.

The expression profiles of *Amth* normalized with all the selected reference genes and their combinations during the embryonic development, and the postembryonic development of *Am*, was the same (Figure 3A).

However, the expression of *Amth* normalized with *Amactin* was significantly different from when normalized with *Amrpl13a* & *Amrps18* & *Amactin* at developmental day 1 and 2, and day 21 when normalized with *Amrps18* and *Amrpl13a & Amrpl32* ($P < 0.05$). In addition, normalization with *Amrps18* and a combination of *Amactin* and *Amrpl13a*, showed significant differences at developmental days 1 and 2 ($P < 0.05$), thus they were both excluded in the subsequent analysis. However, *Amrpl13a*, and the combinations of *Amrps18* and *Amrpl13a*, *Amactin* and *Amrps18* and *Amrpl13a* & *Amrps18* & *Amactin*, showed no significant difference compared to each other ($P > 0.05$) during the embryonic developmental stage. Likewise, *Amrpl13a* and *Amrpl32*, showed significant differences at developmental days 18, 19, 20 and 21 ($P < 0.05$), thus they were excluded in the subsequent analysis. Normalization with the other combinations, including *Amrpl13a* & *Amrps18*, *Amrpl13a* & *Amrpl32*, and the combination of *Amrps18* and *Amrpl32*, were the same during the postembryonic development of *Am*.

During the embryonic development of *Ac*, the expression profiles of *Acth* normalized with all combinations of selected reference genes except *Acactin* were the same (Figure 3B). During the postembryonic development, the expression profiles of *Acth* normalized with *Acrpl13a*, *Acrpl32*, *Acef1α*, and the combination of *Acrpl13a* and *Acef1α* were inconsistent with those of *Acrpl13a* & *Acrpl32* & *Acef1α* (Figure 3B).

Acth expression when normalized with the combination of *Acrpl13a* and *Acactin* was significantly different from *Acrpl13a* & *Acrpl32* at developmental days 1, 2, and 3 ($P < 0.05$). Normalizations with *Acrpl32* and the combination of *Acrpl32* and *Acactin*, were significantly different at developmental day 1 and 2 ($P < 0.05$), hence were excluded in the subsequent analysis. The remaining genes and their combinations including, *Acrpl13a*, *Acef1α*, *Acrpl13a* & *Acrpl32*, *Acrpl13a* & *Acef1α*, the combination of *Acrpl32* and *Acef1α*, and *Acrpl13a* & *Acrps18* & *Acrpl32* & *Acactin* & *Acef1α*, were the same during the embryonic development of *Ac* ($P > 0.05$). The expression of *Acth* normalized with *Actif*, *Acrpl13a* & *Acrpl32*, the combinations of *Acrpl13a* and *Actif*, *Acrpl32* and *Actif*, and *Acrpl13a*, *Acrpl32* and *Actif*, were significantly different from those normalized with *Acrpl13a* & *Acrpl32* & *Acef1α* during the postembryonic development, and hence excluded from the subsequent analysis ($P < 0.05$). However, *Acrpl32* & *Acef1α*, *Acrpl13a* & *Acrpl32* & *Acef1α* and *Acrpl13a* & *Acrps18* & *Acrpl32* & *Acactin* & *Acef1α*, were consistent with each other during the postembryonic development of *Ac*.

As expected, both the expression profile and comparison within groups for *Acth* (for *Ac*) or *Amth* (for *Am*) normalized with the least stable genes, *Acatub* or *Amatub*, were inconsistent with those normalized with the stable combinations.

Discussion

Appropriate reference genes as internal controls to normalize target gene expression are important for accurate RT-qPCR analysis. Therefore, it is necessary to validate multiple candidate reference genes before conducting RT-qPCR assays (Shakeel et al., 2018).

The selection of candidate reference genes during the postembryonic developmental stages has been performed at three larval and two pupal stages, which are critical stages in the postembryonic development of *Am* (Lourenço et al., 2008). In the present study, we re-evaluated suitable reference genes during the postembryonic development of *Am* at each developmental day with more candidate genes, and the embryonic and postembryonic development of *Ac*. To the best of our knowledge, this is the first time to evaluate reference genes during the development of *Ac*.

In general, single genes were not stable enough for RT-qPCR, where most reference genes selected were not stable when validated using a single reference gene as an internal control at the different developmental stages. Therefore, several combinations of reference genes were recommended for the accurate normalization of the target genes. Based on our findings, *Amrpl13a*, *Amrpl13a* & *Amrps18*, *Amrps18* & *Amactin*, *Amrpl13a* & *Amrps18* & *Amactin* during the embryonic development of *Am*, *Amrpl13a* & *Amrps18*, *Amrpl13a* & *Amrpl32*, and *Amrps18* & *Amrpl32* during the postembryonic development were the most stable for the normalization of target genes in *Am*. In *Ac*, *Acrpl13a*, *Acef1α*, *Acrpl13a* & *Acrpl32*, *Acrpl13a* & *Acef1α*, and *Acrpl32* & *Acef1α* during the embryonic development, and *Acrpl32* & *Acef1α*, and *Acrpl13a* & *Acrpl32* & *Acef1α* during the postembryonic development were stable enough for the normalization of the target genes. In addition, *Acrpl13a* & *Acrps18* & *Acrpl32* & *Acactin* & *Acef1α* was the most suitable combination for RT-qPCR assay during the whole development of *Ac*. It is worth noting that *rpl13a*, the first stable gene based on the comprehensive ranking, was stable enough as a single reference gene or part of the combinations during all the development stages, while *rpl32* was stable in combinations during all the development stages except during embryonic development of *Am*. *Rpl13a* was proposed as an endogenous control for transcript profiling studies of *Bemisia tabaci* (Collins et al., 2014), and was identified as an ideal reference gene, in different tissues of *Tribolium castaneum* during its development (Toutges et al., 2010), and under fungal challenge (Lord et al., 2010). Besides, *Rpl32* (formerly *rp49*) has shown relatively high stability in the brains, abdomens, during postembryonic development (Lourenço et al., 2008; Reim et al., 2013), under CSBV infection and dsRNA treatment (Deng et al., 2020) of honey bees playing different social roles. *Rpl32* was also highly stable during the development of three stingless bee species (Freitas et al., 2019). At the same time, *Rps18* was also commonly used as a reference gene in the honey bee (Deng et al., 2020; Moon et al., 2018a, 2018b; Scharlaken et al., 2008) and other bee species (Freitas et al., 2019) under different conditions. However, in the present study, *rps18* was stable in combination with other genes during *Am*'s embryonic and postembryonic development. Similarly, *ef1α* identified as a stable reference gene during the development of *Am* (Reim et al., 2013), was stable in combinations with other genes during the embryonic and postembryonic development of *Ac*. *Actin*, identified as a stable reference gene under bacterial and CBPV infection in *Am* (Deng et al., 2020), was also stable in combination with other genes during the embryonic development of *Am*. Therefore, these reference genes or their combinations can be used in RT-qPCR assays, and if the experimental condition changes, it is necessary to validate the expression stability of the suggested reference genes in each study as suggested by Kozera and Rapacz (2013).

Most candidate reference genes recorded the highest mRNA levels at developmental days 5 to 8, and 4 to 6 in *Am* and *Ac*, respectively. These days corresponded with the larval stages of the honey bee, implying that the physiological differences among the egg, larval and pupal stages influence the reference genes, increasing the difficulty to screen appropriate reference genes due to the high variance in the different developmental stages.

This study systematically analyzed eight commonly used reference genes for RT-qPCR assays using five algorithms during the different developmental stages of *Am* and *Ac*. The findings will contribute to research focusing on the different developmental stages in the honey bee.

Disclosure statement

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