Selection and validation of experimental condition-specific reference genes for qRT-PCR in *Metopolophium dirhodum* (Walker) (Hemiptera: Aphididae)

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*Metopolophium dirhodum* (Walker) (Hemiptera: Aphididae) is one of the most common aphid pests of winter cereals. To facilitate accurate gene expression analyses with qRT-PCR assays, the expression stability of candidate reference genes under specific experimental conditions must be verified before they can be used to normalize target gene expression levels. In this study, 10 candidate reference genes in *M. dirhodum* were analyzed by qRT-PCR under various experimental conditions. Their expression stability was evaluated with delta Ct, BestKeeper, geNorm, and NormFinder methods, and the final stability ranking was determined with RefFinder. The results indicate that the most appropriate sets of internal controls were SDHB and RPL8 across geographic population; RPL8, Actin, and GAPDH across developmental stage; SDHB and NADH across body part; RPL8 and Actin across wing dimorphism and temperature; RPL4 and EF1A across starvation stress; AK and RPL4 across insecticide treatments; RPL8 and NADH across antibiotic treatments; RPL8, RPL4, Actin, and NADH across all samples. The results of this study provide useful insights for establishing a standardized qRT-PCR procedure for *M. dirhodum* and may be relevant for identifying appropriate reference genes for molecular analyses of related insects.

The quantitative analysis of target gene expression is an essential part of most molecular studies. Quantitative real-time PCR (qRT-PCR) is a powerful tool for quantifying gene expression, combining improvements in both sensitivity and specificity with efficient techniques for signal detection. It is useful for the quantitative data analysis required for research related to molecular medicine, biotechnology, microbiology, and diagnostics and has become the preferred method for quantifying mRNA. Nevertheless, gene expression analyses are affected by many factors such as the quality of RNA samples, the efficiency of reverse transcription, and PCR efficiency. For accurate comparisons of expression levels, the expression data of the genes of interest are normalized against the expression data for a reference gene. Moreover, the reference gene compensates for the above-mentioned limitations. Because housekeeping genes are related to ubiquitous and basic cellular functions, they are considered to be constitutively expressed under diverse conditions. Housekeeping genes, including those encoding actin, glyceraldehyde-3-phosphate dehydrogenase, ribosomal protein, 18S ribosomal RNA, elongation factor 1α and heat shock proteins, have been extensively used as endogenous controls for normalizing real-time PCR data. However, several studies have indicated that the expression levels of the reference genes vary under diverse conditions. In fact, no single reference gene is appropriate for all experimental conditions. Therefore, evaluating and validating the stability of reference genes under different experimental conditions is critical.

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There have recently been several reports regarding reference genes for molecular research on insects, including bumblebee, Harmonia axyridis, Propylea japonica, Aphis craccivora Koch, Henosepilachna vigintioctomaculata, Chilo suppressalis, Galerusca daurica, Liriomyza trifolii, Coccinella septempunctata, Phenacoccus solenopsis, Lipaphis erysimi, Myzus persicae, Acyrthosiphon pisum, and Megoura viciea.3,14–27.

*Metopolophium dirhodum* (Walker) (Hemiptera: Aphididae) is one of the most major aphid pests affecting winter wheat and other cereals worldwide.28–31 Additionally, *M. dirhodum*, which was first detected in the 1970s, originated in the Holarctic and was subsequently introduced to South America and other regions.32,33 The *M. dirhodum* nymphs and adults damage cereals by directly feeding on plants, which may result in grain yield losses of 27–30%.34 Moreover, they damage crops by transmitting several viruses, especially the barley yellow dwarf virus.35 This aphid has most often been detected in semi-arid regions in South America, South Africa, Australia, and New Zealand, where it damages cereals, including wheat, barley, rye, and oat. A previous study revealed that *M. dirhodum* is the most abundant aphid species on cereals in the continental climate of central Europe.33 With the technical advances occurring in the post-genomic era, researchers may soon have additional options for studying *M. dirhodum* at the molecular level, which may contribute to the development of improved control measures. Thus, identifying suitable reference genes is important for analyzing the expression of functional genes at the molecular level, which may contribute to the development of improved control measures.

The objective of this study was to identify and evaluate a suite of experimental condition-specific reference genes to normalize target gene expression in *M. dirhodum*. Specifically, we analyzed the following 10 candidate genes: *Actin*, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), NADH dehydrogenase (NADH), arginine kinase (AK), succinate dehydrogenase B (SDHB), ribosomal protein L8 (RPL8), 18S ribosomal RNA (18S), elongation factor 1a (EF1A), ribosomal protein L4 (RPL4), and heat shock protein 68 (HSP68). The effects of the following factors on reference gene expression were evaluated: geographic population, developmental stage, body part, wing dimorphism, temperature, starvation stress, and exposure to an insecticide or antibiotic. The results indicate that the best reference genes for analyzing *M. dirhodum* gene expression vary among conditions.

**Results**

**Expression levels of candidate reference genes.** To evaluate the expression profiles of the selected candidate genes in all *M. dirhodum* sample sets, mRNA levels were measured for all genes. The gene expression levels varied considerably between Ct values of 12.70 (18S) and 30.88 (GAPDH) (Fig. 1). Of the 10 analyzed genes, the highest and lowest expression levels were detected for 18S (mean Ct value of 14.27) and GAPDH (mean Ct value of 28.90), respectively. The least variable expression among all samples was observed for *Actin* (mean Ct value ± SD of 26.79 ± 0.42) and *RPL8* (21.10 ± 0.35). In contrast, *HSP68* (24.82 ± 1.86) exhibited the most variable expression in all the tested samples.

**Analysis of gene expression stability.** The delta Ct method and the BestKeeper, NormFinder, and geNorm algorithms were used to assess the stability of the candidate reference gene expression levels. The rank order (most to least stable expression) was highly consistent among the four methods. Specifically, *SDHB*, *RPL4*, and *RPL8* were identified as the most stable genes, whereas *HSP68* and *GAPDH* were the least stable genes (Table 1). The RefFinder results for the geographic populations revealed a rank order (most to least stable expression) of *SDHB*, *RPL8*, *RPL4*, *NADH*, *AK*, 18S, *Actin*, EF1A, *GAPDH*, and *HSP68* (Fig. 2). On the basis of the GeNorm analysis, all pairwise variation values were below the 0.15 cut-off value, except for V5/6 (Fig. 3). Moreover, the RefFinder analysis indicated *SDHB* and *RPL8* are required for the normalization of target gene expression levels in different geographic populations.

![Figure 1](https://example.com/f1.png)

**Figure 1.** Candidate reference gene expression levels. Candidate reference gene expression levels in the whole *M. dirhodum* sample set are expressed in terms of the threshold cycle number (Ct value). Data are presented as whisker box plots. The box represents the 25th–75th percentiles, the median is indicated by a bar across the box, and the whiskers on each box represent the minimum and maximum values.
| Experimental conditions | Rank | Delta CT | Gene name Standard deviation | Gene name Standard deviation | Gene name Standard deviation | Gene name Stability value | Gene name Stability value |
|-------------------------|------|---------|-----------------------------|-----------------------------|-----------------------------|---------------------------|---------------------------|
|                         | 1    | 0.73    | SDHB                        | 0.04                        | RPL4                        | 0.292                     | SDHB/RPL8                 | 0.123                     |
|                         | 2    | 0.74    | RPL4                        | 0.12                        | SDHB                        | 0.311                     |                           |                           |
|                         | 3    | 0.78    | NADH                        | 0.13                        | RPL8                        | 0.439                     | NADH                      | 0.129                     |
|                         | 4    | 0.78    | RPL8                        | 0.20                        | NADH                        | 0.474                     | RPL4                      | 0.225                     |
|                         | 5    | 0.85    | AK                          | 0.29                        | 18S                         | 0.537                     | AK                        | 0.274                     |
|                         | 6    | 0.90    | 18S                         | 0.62                        | AK                          | 0.593                     | 18S                       | 0.507                     |
|                         | 7    | 0.96    | Actin                       | 0.68                        | Actin                       | 0.707                     | Actin                     | 0.640                     |
|                         | 8    | 1.06    | EF1A                        | 0.75                        | EF1A                        | 0.862                     | EF1A                      | 0.727                     |
|                         | 9    | 1.38    | GAPDH                       | 0.84                        | GAPDH                       | 1.310                     | GAPDH                     | 0.843                     |
|                         | 10   | 1.44    | HSP68                       | 1.08                        | HSP68                       | 1.366                     | HSP68                     | 0.962                     |
| Geographic populations  | 1    | 1.03    | GAPDH                       | 0.61                        | GAPDH                       | 0.149                     | Actin/RPL8                | 0.461                     |
|                         | 2    | 1.04    | RPL4                        | 0.64                        | Actin                       | 0.231                     |                           |                           |
|                         | 3    | 1.08    | RPL8                        | 0.85                        | RPL8                        | 0.410                     | GAPDH                     | 0.504                     |
|                         | 4    | 1.10    | NADH                        | 0.89                        | NADH                        | 0.458                     | NADH                      | 0.564                     |
|                         | 5    | 1.31    | RPL4                        | 0.94                        | RPL4                        | 0.934                     | RPL4                      | 0.653                     |
|                         | 6    | 1.55    | GAPDH                       | 0.96                        | AK                          | 1.199                     | EF1A                      | 0.840                     |
|                         | 7    | 1.55    | AK                          | 1.40                        | 18S                         | 1.266                     | 18S                       | 0.949                     |
|                         | 8    | 1.56    | EF1A                        | 1.50                        | SDHB                        | 1.503                     | SDHB                      | 1.123                     |
|                         | 9    | 1.75    | HSP68                       | 1.97                        | HSP68                       | 1.57                      | HSP68                     | 1.395                     |
|                         | 10   | 2.11    | HSP68                       | 2.92                        | HSP68                       | 2.18                      | HSP68                     | 1.059                     |
| Development stages      | 1    | 0.72    | GAPDH                       | 0.24                        | NADH                        | 0.043                     | NADH/SDHB                 | 0.085                     |
|                         | 2    | 0.74    | 18S                         | 0.28                        | SDHB                        | 0.043                     |                           |                           |
|                         | 3    | 0.77    | EF1A                        | 0.32                        | 18S                         | 0.168                     | Actin                     | 0.270                     |
|                         | 4    | 0.81    | SDHB                        | 0.36                        | Actin                       | 0.447                     | 18S                       | 0.314                     |
|                         | 5    | 0.88    | NADH                        | 0.42                        | EF1A                        | 0.568                     | AK                        | 0.377                     |
|                         | 6    | 0.95    | Actin                       | 0.50                        | GAPDH                       | 0.687                     | EF1A                      | 0.554                     |
|                         | 7    | 0.98    | RPL8                        | 0.61                        | AK                          | 0.711                     | GAPDH                     | 0.645                     |
|                         | 8    | 1.15    | AK                          | 0.66                        | RPL8                        | 1.056                     | RPL8                      | 0.757                     |
|                         | 9    | 1.25    | RPL4                        | 0.71                        | RPL4                        | 1.162                     | RPL4                      | 0.832                     |
|                         | 10   | 1.59    | HSP68                       | 1.23                        | HSP68                       | 1.557                     | HSP68                     | 0.983                     |
| Body parts              | 1    | 0.60    | Actin                       | 0.06                        | RPL8                        | 0.027                     | RPL8/EF1A                 | 0.053                     |
|                         | 2    | 0.60    | RPL4                        | 0.14                        | EF1A                        | 0.027                     |                           |                           |
|                         | 3    | 0.62    | HSP68                       | 0.19                        | RPL4                        | 0.039                     | RPL4                      | 0.087                     |
|                         | 4    | 0.64    | RPL8                        | 0.19                        | Actin                       | 0.094                     | Actin                     | 0.217                     |
|                         | 5    | 0.64    | HSP68                       | 0.23                        | HSP68                       | 0.332                     | HSP68                     | 0.309                     |
|                         | 6    | 0.67    | EF1A                        | 0.23                        | NADH                        | 0.406                     | NADH                      | 0.344                     |
|                         | 7    | 0.94    | SDHB                        | 0.51                        | SDHB                        | 0.889                     | SDHB                      | 0.458                     |
|                         | 8    | 1.01    | AK                          | 0.57                        | AK                          | 0.982                     | AK                        | 0.523                     |
|                         | 9    | 1.13    | GAPDH                       | 0.74                        | GAPDH                       | 1.035                     | GAPDH                     | 0.680                     |
|                         | 10   | 1.41    | 18S                         | 0.97                        | 18S                         | 1.401                     | 18S                       | 0.827                     |
| Wing dimorphism         | 1    | 0.72    | RPL8                        | 0.10                        | RPL8                        | 0.032                     | Actin/NADH                | 0.206                     |
|                         | 2    | 0.74    | RPL4                        | 0.16                        | RPL8                        | 0.064                     |                           |                           |
|                         | 3    | 0.75    | SDHB                        | 0.29                        | Actin                       | 0.141                     | RPL8                      | 0.280                     |
|                         | 4    | 0.78    | Actin                       | 0.30                        | EF1A                        | 0.266                     | RPL4                      | 0.310                     |
|                         | 5    | 0.81    | EF1A                        | 0.30                        | NADH                        | 0.347                     | EF1A                      | 0.341                     |
|                         | 6    | 0.83    | NADH                        | 0.32                        | SDHB                        | 0.397                     | SDHB                      | 0.386                     |
|                         | 7    | 0.94    | AK                          | 0.46                        | AK                          | 0.502                     | AK                        | 0.448                     |
|                         | 8    | 1.00    | GAPDH                       | 0.60                        | GAPDH                       | 0.626                     | GAPDH                     | 0.526                     |
|                         | 9    | 1.11    | 18S                         | 0.69                        | 18S                         | 0.915                     | 18S                       | 0.595                     |
|                         | 10   | 2.92    | HSP68                       | 2.18                        | HSP68                       | 2.885                     | HSP68                     | 1.059                     |
Developmental stage. The delta Ct and NormFinder analyses identified GAPDH and Actin as the most stable genes. In contrast, the most stable genes were RPL8 and RPL4 according to BestKeeper and Actin and RPL8 according to GeNorm. Regardless of the method, HSP68 was identified as the least stable gene (Table 1). According to the RefFinder results, the rank order (most to least stable expression) for the developmental stages was RPL8, Actin, GAPDH, RPL4, NADH, 18S, AK, SDHB, EF1A, and HSP68 (Fig. 2). The GeNorm analysis revealed that the values for V3/4 were less than the proposed 0.15 cut-off (Fig. 3). The RefFinder analysis indicated RPL8, Actin, and GAPDH are required for normalizing target gene expression levels for the different M. dirhodum developmental stages.

Body part. The gene expression stability rank order determined with BestKeeper differed from that obtained with the other three methods (Table 1). The BestKeeper algorithm identified GAPDH and 18S as the most stable genes. In contrast, the delta Ct method, NormFinder, and GeNorm identified NADH and SDHB as the most stable genes. All four analyses indicated RPL4 and HSP68 were the least stable genes. The RefFinder results for the different body parts revealed a rank order (most to least stable expression) of SDHB, NADH, 18S, Actin, GAPDH, EF1A, AK, RPL8, RPL4, and HSP68 (Fig. 2). On the basis of the GeNorm analysis, all pairwise variation values

### Table 1. Rank order of the M. dirhodum candidate reference genes under various experimental conditions.

| Experimental conditions | Rank | Gene name | Standard deviation | Gene name | Standard deviation | Gene name | Stability value | Gene name | Stability value |
|-------------------------|------|-----------|--------------------|-----------|--------------------|-----------|----------------|-----------|----------------|
| Starvation-stress       | 1    | RPL4      | 1.03               | 18S       | 0.06               | EF1A      | 0.026          | NADH/AK   | 0.050          |
|                         | 2    | EF1A      | 1.03               | Actin     | 0.33               | RPL4      | 0.026          |           |                |
|                         | 3    | RPL8      | 1.10               | GAPDH     | 0.46               | RPL8      | 0.484          | SDHB      | 0.175          |
|                         | 4    | AK        | 1.23               | RPL8      | 0.78               | AK        | 0.706          | RPL4      | 0.599          |
|                         | 5    | NADH      | 1.26               | EF1A      | 1.02               | NADH      | 0.771          | EF1A      | 0.687          |
|                         | 6    | GAPDH     | 1.31               | RPL4      | 1.05               | SDHB      | 1.034          | RPL8      | 0.790          |
|                         | 7    | SDHB      | 1.40               | AK        | 1.70               | GAPDH     | 1.066          | GAPDH     | 0.930          |
|                         | 8    | Actin     | 1.43               | NADH      | 1.74               | Actin     | 1.280          | Actin     | 1.017          |
|                         | 9    | 18S       | 1.77               | SDHB      | 1.89               | 18S       | 1.727          | 18S       | 1.125          |
|                         | 10   | HSP68     | 2.55               | HSP68     | 2.80               | HSP68     | 2.527          | HSP68     | 1.409          |
| Insecticide-stress      | 1    | RPL4      | 0.32               | HSP68     | 0.12               | AK        | 0.129          | Actin/AK   | 0.028          |
|                         | 2    | AK        | 0.32               | SDHB      | 0.22               | RPL4      | 0.135          |           |                |
|                         | 3    | Actin     | 0.33               | RPL8      | 0.22               | NADH      | 0.154          | RPL8      | 0.080          |
|                         | 4    | RPL8      | 0.33               | RPL4      | 0.23               | Actin     | 0.167          | RPL4      | 0.102          |
|                         | 5    | NADH      | 0.37               | Actin     | 0.29               | GAPDH     | 0.192          | HSP68     | 0.151          |
|                         | 6    | GAPDH     | 0.39               | NADH      | 0.29               | RPL8      | 0.208          | NADH      | 0.205          |
|                         | 7    | HSP68     | 0.44               | AK        | 0.31               | SDHB      | 0.384          | SDHB      | 0.245          |
|                         | 8    | SDHB      | 0.47               | GAPDH     | 0.50               | HSP68     | 0.388          | GAPDH     | 0.281          |
|                         | 9    | 18S       | 0.56               | 18S       | 0.69               | 18S       | 0.478          | 18S       | 0.347          |
|                         | 10   | EF1A      | 0.76               | EF1A      | 0.77               | EF1A      | 0.731          | EF1A      | 0.431          |
| Antibiotic-stress       | 1    | RPL8      | 0.54               | SDHB      | 0.03               | NADH      | 0.024          | GAPDH/18S | 0.013          |
|                         | 2    | RPL4      | 0.54               | Actin     | 0.15               | RPL8      | 0.086          |           |                |
|                         | 3    | AK        | 0.54               | NADH      | 0.18               | Actin     | 0.087          | AK        | 0.060          |
|                         | 4    | 18S       | 0.58               | RPL8      | 0.47               | RPL4      | 0.350          | RPL4      | 0.071          |
|                         | 5    | GAPDH     | 0.59               | RPL4      | 0.59               | SDHB      | 0.371          | EF1A      | 0.112          |
|                         | 6    | NADH      | 0.63               | AK        | 0.61               | AK        | 0.383          | RPL8      | 0.163          |
|                         | 7    | Actin     | 0.65               | 18S       | 0.67               | 18S       | 0.484          | NADH      | 0.297          |
|                         | 8    | EF1A      | 0.69               | GAPDH     | 0.68               | GAPDH     | 0.501          | Actin     | 0.370          |
|                         | 9    | SDHB      | 0.76               | EF1A      | 0.76               | EF1A      | 0.646          | SDHB      | 0.439          |
|                         | 10   | HSP68     | 1.99               | HSP68     | 0.95               | HSP68     | 1.987          | HSP68     | 0.749          |
| All above conditions    | 1    | RPL8      | 1.01               | Actin     | 0.54               | RPL8      | 0.401          | RPL8/RPL4 | 0.421          |
|                         | 2    | RPL4      | 1.03               | RPL8      | 0.54               | RPL4      | 0.497          |           |                |
|                         | 3    | NADH      | 1.09               | RPL4      | 0.82               | Actin     | 0.543          | EF1A      | 0.674          |
|                         | 4    | Actin     | 1.10               | 18S       | 0.96               | NADH      | 0.624          | NADH      | 0.747          |
|                         | 5    | EF1A      | 1.15               | SDHB      | 1.00               | SDHB      | 0.723          | GAPDH     | 0.786          |
|                         | 6    | GAPDH     | 1.16               | EF1A      | 1.01               | EF1A      | 0.724          | Actin     | 0.827          |
|                         | 7    | SDHB      | 1.17               | GAPDH     | 1.15               | GAPDH     | 0.752          | SDHB      | 0.868          |
|                         | 8    | AK        | 1.44               | NADH      | 1.16               | AK        | 1.159          | AK        | 0.955          |
|                         | 9    | 18S       | 1.51               | HSP68     | 1.46               | 18S       | 1.230          | 18S       | 1.061          |
|                         | 10   | HSP68     | 2.16               | AK        | 1.56               | HSP68     | 2.019          | HSP68     | 1.281          |
were below the 0.15 cut-off value, except for V9/10 (Fig. 3). The RefFinder analysis indicated SDHB and NADH are required for normalizing target gene expression levels in wing-dimorphic insects.

**Wing dimorphism.** The delta Ct and BestKeeper analyses identified Actin and RPL4 as the most stable genes, whereas both NormFinder and GeNorm identified RPL8 and EF1A as the most stable genes. All four analyses indicated that 18S, GAPDH, AK, and SDHB were the least stable genes (Table 1). The RefFinder data for the wing dimorphism revealed a rank order (most to least stable expression) of RPL8, Actin, RPL4, EF1A, HSP68, NADH, SDHB, AK, GAPDH, and 18S (Fig. 2). On the basis of the GeNorm analysis, all pairwise variation values were below the 0.15 cut-off value (Fig. 3). According to RefFinder, RPL8 and Actin are required for normalizing target gene expression levels in wing-dimorphic insects.

**Temperature-induced stress.** The delta Ct method identified Actin and RPL8 as the most stable genes. Both BestKeeper and NormFinder identified RPL8 and RPL4 as the most stable genes, whereas GeNorm identified Actin and NADH as the most stable genes. All four analyses indicated HSP68, 18S, GAPDH, and AK were the

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**Figure 2.** Stability of candidate reference gene expression levels in response to various treatments and conditions. In a RefFinder analysis, decreasing Geomean values correspond to increasing gene expression stability. The Geomean values for the following *M. dirhodum* samples are presented: adult samples from different geographic populations (Geographic population), samples for all developmental stages (Developmental stages), samples for different body parts of wingless adults (Body part), samples for winged and wingless adults (Wing dimorphism), adult samples exposed to different temperatures (Temperature-stress), fed and unfed adult samples (Starvation-stress), adult samples treated with different insecticides (Insecticide-stress), adult samples treated with antibiotic (Antibiotic-stress), and all samples for all treatments (All conditions). The candidate reference genes are as follows: Actin, Actin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NADH, NADH dehydrogenase; AK, arginine kinase; SDHB, succinate dehydrogenase B; RPL8, ribosomal protein L18; RPL4, ribosomal protein L4; HSP68, heat shock protein 68; 18S, 18S ribosomal RNA; and EF1A, elongation factor 1a.
least stable genes (Table 1). The RefFinder data for the different temperatures revealed a rank order (most to least stable expression) of RPL8, Actin, RPL4, NADH, EF1A, SDHB, AK, GAPDH, 18S, and HSP68 (Fig. 2). On the basis of the GeNorm analysis, all pairwise variation values were below the 0.15 cut-off value, except for V9/10 (Fig. 3). The RefFinder analysis indicated RPL8 and Actin are required for normalizing target gene expression levels in M. dirhodum exposed to different temperatures.

**Starvation-induced stress.** The delta Ct method and the NormFinder algorithm identified EF1A and RPL4 as the most stable genes and Actin, 18S, and HSP68 as the least stable genes (Table 1). However, BestKeeper identified 18S and Actin as the most stable genes and SDHB and HSP68 as the least stable genes (Table 1). The GeNorm algorithm identified NADH and AK as the most stable genes and Actin, 18S, and HSP68 as the least stable genes (Table 1). The RefFinder results for the starvation treatment revealed a rank order (most to least stable expression) of RPL4, EF1A, AK, NADH, RPL8, 18S, GAPDH, Actin, SDHB, and HSP68 (Fig. 2). The GeNorm analysis indicated that the pairwise variation values for V2/3 were less than the proposed 0.15 cut-off (Fig. 3). The RefFinder analysis indicated RPL4 and EF1A are required for normalizing target gene expression levels in starvation-stressed M. dirhodum.

**Insecticide-induced stress.** The delta Ct and NormFinder data revealed AK and RPL4 as the most stable genes, whereas the BestKeeper results identified HSP68 and SDHB as the most stable genes. In contrast, Actin and AK were the most stable genes according to GeNorm. All four analyses identified 18S and EF1A as the least stable genes (Table 1). The RefFinder data for the insecticide treatment revealed a rank order (most to least stable expression) of AK, RPL4, Actin, RPL8, HSP68, NADH, SDHB, GAPDH, 18S, and EF1A (Fig. 2). Based on the GeNorm analysis, all the pairwise variation values were below 0.15 cut-off value (Fig. 3). Thus, AK and RPL4 are required for normalizing target gene expression levels in insecticide-treated M. dirhodum.

**Antibiotic-induced stress.** The delta Ct method identified RPL8 and RPL4 as the most stable genes. The BestKeeper algorithm identified SDHB and Actin as the most stable genes, whereas NormFinder indicated NADH and RPL8 were the most stable genes. The GeNorm algorithm identified GAPDH and 18S as the most stable genes. All four analyses identified EF1A, SDHB, and HSP68 as the least stable genes (Table 1). The RefFinder data for the antibiotic treatment revealed a rank order (most to least stable expression) of RPL8, NADH, RPL4, 18S, GAPDH, AK, Actin, SDHB, EF1A, and HSP68 (Fig. 2). According to the GeNorm analysis, all pairwise variation values were less than the proposed 0.15 cut-off, except for V9/10 (Fig. 3). The RefFinder analysis suggested RPL8 and NADH are required for normalizing the target gene expression levels in antibiotic-treated M. dirhodum.

**Overall ranking of M. dirhodum candidate reference genes.** An examination of the candidate reference expression stability for all treatments and conditions with the four methods used in this study produced similar rank orders, with RPL4 and RPL8 identified as the most stable genes and AK, 18S, and HSP68 revealed as the least stable genes (Table 1). The RefFinder results for all treatments and conditions revealed a rank order (most to least stable expression) of RPL8, RPL4, Actin, NADH, EF1A, SDHB, GAPDH, 18S, AK, and HSP68 (Fig. 2). The GeNorm analysis indicated that the pairwise variation values for V4/5 were less than the proposed 0.15 cut-off (Fig. 3). Thus, an analysis of all treatments and conditions suggested that RPL8, RPL4, Actin, and NADH are suitable internal reference genes for normalizing target gene expression levels in M. dirhodum.
to various temperatures. However, we determined that SDHB was identified as the most stable gene following insecticide treatments (Fig. 2). In *H. armigera*, Actin was revealed as the least stable reference gene for analyses of developmental stages, temperature effects, and wing dimorphism (Fig. 2). Additionally, RPL4 was detected as the least stable gene in response to starvation and insecticide treatments, but was also almost the least stable gene during analyses of various *M. dirhodum* body parts (Fig. 2).

**Discussion**

There are several reports describing the application of qRT-PCR assays to clarify the gene expression levels associated with diverse biological processes. Reference genes used for molecular investigations can influence the accuracy of target gene expression levels. Therefore, a stable reference gene is an important prerequisite for gene expression investigations. Housekeeping genes, which are constitutively expressed to maintain basic cellular functions, have traditionally been used as internal reference controls. However, there is no universal reference gene that is stably expressed in all cell and tissue types under different experimental conditions. Therefore, every stable reference gene used to normalize gene expression data should be evaluated under each experimental condition.

In this study, qRT-PCR was used to evaluate the expression-level stability of 10 candidate reference genes in *M. dirhodum* across specific conditions. The best reference genes varied among conditions. Specifically, RPL8 (mean Ct value ± SD, 21.10 ± 0.35) and Actin (26.79 ± 0.42) had the least variable expression levels, whereas HSP68 (24.82 ± 1.86) produced the most variable expression levels among the examined candidate reference genes. Similarly, RPL8, RPL4, and Actin were the most stable reference genes, whereas HSP68 and 18S were the least stable reference genes under most conditions.

Ribosomal proteins (RPs), which are the principal components of ribosomes, are one of the most highly conserved proteins in all life forms. Earlier research proved that RP-encoding genes are among the most stably expressed reference genes, and have been widely used to normalize gene expression levels in insect molecular investigations during the past 10 years. For example, in *Brady sia odoriphaga*, RPS15 was the most stably expressed gene in response to various temperature treatments. However, another study indicated that the expression levels of RP-encoding genes may vary under some conditions. Moreover, RPS20 was detected as the least stably expressed gene for analyzing *Plutella xylostella* geographic populations as well as the effects of the temperature, photoperiod, and insecticides. Consistent with these earlier findings, we identified RPL8 as the most stable gene in *M. dirhodum* across various conditions (except for analyses of different body parts, starvation stress, and insecticide treatments). Additionally, RPL4 was detected as the most stable gene in response to starvation and insecticide treatments, but was also almost the least stable gene during analyses of various *M. dirhodum* body parts.

*Actin*, which encodes a major structural protein, is important for cell secretion, motility, cytoplasm flow, and cytoskeleton maintenance. Moreover, *Actin* is expressed at various levels in many cell types, and is considered the ideal reference gene for qRT-PCR, which may explain its frequent use. For example, it has been used to study the effects of diet on *B. odoriphaga* gene expression and for investigating *M. persicae* gene expression in different tissues and in response to the temperature, photoperiod, and wing dimorphism. However, in *Helicoverpa armigera*, Actin was revealed to be the least stable reference gene following temperature and photoperiod treatments. In our study, Actin was identified as one of the most stable reference genes for analyzing developmental stages, temperature effects, and wing dimorphism.

The GAPDH gene has been commonly used as a reference gene in the studies of gene expression. However, unstable GAPDH expression has been detected in *Tetranychus cinnabarinus* developmental stages, in the labial glands and fat bodies of *Bombus terrestris* and *Bombus lucorum*, and in various *Sogatella furcifera* body parts. In the current study, GAPDH was revealed as a stably expressed candidate reference gene for analyses of developmental stages. These results imply that the mechanism underlying the expression stability of endogenous reference genes is complex. Furthermore, the stability of potential reference genes in different biological samples should be tested prior to their use.

The protein encoded by EF1A affects translation by catalyzing the GTP-dependent binding of aminoacyl-tRNA to the acceptor site of the ribosome. The EF1A gene was recently used as a reference gene in multiple insect gene expression studies. Our results suggest that EF1A is an appropriate reference gene only for analyzing the effects of starvation stress on *M. dirhodum* gene expression.

The AK gene encodes the phosphagen kinase in invertebrates, and it has rarely been used as a reference gene. An earlier study revealed that AK is the most stably expressed gene in the *B. terrestris* labial gland and fat body. In this study, AK was identified as the most stable gene following insecticide treatments. In *A. pisum*, SDHB and NADH are reported as the most stable housekeeping genes in developmental stages and in response to various temperatures. However, we determined that SDHB and NADH are the most stable housekeeping genes only during examinations of different *M. dirhodum* body parts. These further suggest that reference gene expression stability is influenced by the experimental conditions.

The 18S rRNA gene is considered to be an ideal reference control because of its relatively stable expression levels. Accordingly, it has been applied in previous studies involving *Lucilia cuprina*, *Rhodnius prolixus*, and *Delphacodes kuscheli*. However, in this study, 18S was revealed as one of the least stable genes in almost all

| Conditions          | Reference gene | Conditions          | Reference gene |
|---------------------|----------------|---------------------|----------------|
| Population          | SDHB, RPL8     | Temperature         | RPL8, Actin    |
| Development stage   | RPL8, Actin, GAPDH | Starvation         | RPL4, EF1A    |
| Body part           | SDHB, NADH     | Insecticide         | AK, RPL4      |
| Wing dimorphism     | RPL8, Actin    | Antibiotic          | RPL8, NADH    |
| All conditions      | RPL8, RPL4, Actin, NADH | Sample          |                |

*Table 2.* Recommended reference genes for *M. dirhodum* under various experimental conditions.
sample sets, implying it is an inappropriate reference gene for *M. dirhodum* (Fig. 2). This observation is consistent with the results of previous studies that indicated 18S rRNA is not a stable reference gene in *Bactrocera dorsalis* and *Nilaparvata lugens* under specific experimental conditions. It is transcribed by a separate RNA polymerase, which may explain why rRNA is not a suitable reference control. Moreover, the utility of 18S for normalizing target gene expression levels in a qRT-PCR assay is limited by the potential imbalance between rRNA and mRNA fractions among samples.

The HSP68 gene, which belongs to the HSP70 family, encodes a highly conserved chaperone involved in protein assembly, folding, and transport as well as in antigen processing and presentation. The expression of genes encoding HSPs can be affected by high temperatures or other stresses (e.g., due to chemicals). In the current study, HSP68 was the least stable gene for all conditions (Fig. 2). In a previous study on *Coleomegilla maculata*, HSP70 was identified as the most stably expressed gene for sexes, but was the least stably expressed gene for analyses of different tissues, and dsRNA exposure.

It is becoming common for researchers to use multiple reference genes to normalize target gene expression levels in diverse studies because a single gene is usually insufficient for analyzing gene expression. An earlier investigation indicated that too many or too few reference genes may adversely affect the robustness of data normalizations. However, the simultaneous application of multiple reference genes in a given experiment may decrease the probability of biased normalizations. The optimal number of reference genes under specific experimental conditions can be determined with the geNorm algorithm, which calculates the pairwise variation $V_{n/n+1}$ based on the normalization factors $N_F$ and $N_{F_{n+1}}$ with $n \geq 2$. If $V_{n/n+1}$ is below 0.15, $n$ is the optimal number of reference genes. The results of this study indicate that the most appropriate number of reference genes varies under diverse experimental conditions (Fig. 3). This implies that the stability of reference genes must be evaluated before every qRT-PCR experiment.

**Conclusions**

To the best of our knowledge, this study is the first to evaluate and validate experimental condition-specific candidate reference genes for *M. dirhodum* gene expression analyses. We identified reference genes applicable for elucidating functional gene expression profiles. In this study, we examined 10 candidate reference genes under diverse conditions. Notably, the stability of candidate gene expression levels in *M. dirhodum* varies depending on the experimental conditions. Moreover, we identified internal reference genes suitable for normalizing and quantifying gene expression in *M. dirhodum* (Table 2). Our findings may be useful for establishing a more accurate and reliable method for normalizing *M. dirhodum* qRT-PCR data. They may also provide the basis for future investigations on RNA interference and gene transcription in *M. dirhodum* and other insect pests.

**Materials and methods**

**Insects.** Our original *M. dirhodum* colony was collected in Yinchuan (Ningxia), China (38° 48′ 54.78″ N, 106° 30′ 27.93″ E) in 2018. Other colonies were collected in Langfang (Hebei), China (39° 8′ 9.8″ N, 116° 10′ 4.05″ E) and Guiyang (Guizhou), China (26° 0′ 34.08″ N, 106° 35′ 4.35″ E) in 2018. The adult aphids were collected in wheat leaves of different plants of these geographic locations and were taken back to the lab to establish population. All the wheat aphid populations were reared on Luxuan 987 wheat seedlings in a thermostat chamber maintained at 20 ± 2 °C and 60% relative humidity, with a 16-h light:8-h dark cycle.

**Treatments.** Geographic population. Insects collected in Yinchuan (Ningxia), Langfang (Hebei), and Guiyang (Guizhou) in 2018 were examined to assess the effects of geography on gene expression. These three locations are separated by more than 1000 km. For each geographic population, three samples of 20 adults were selected, flash frozen in liquid nitrogen, and stored at −80 °C until total RNA extraction.

Developmental stage. Three *M. dirhodum* samples of about 30 first-instar nymphs, 30 second-instar nymphs, 20 third-instar nymphs, 20 fourth-instar nymphs, and 20 adults were collected, flash frozen in liquid nitrogen, and stored at −80 °C until total RNA extraction.

Body part. We used a dissection needle and a tweezer to separate the head, thorax, and abdomen from wingless *M. dirhodum* adults. These body parts as well as whole adult bodies were stored as described earlier.

Wing dimorphism. Three samples of 20 winged and wingless *M. dirhodum* adults were collected, flash frozen in liquid nitrogen, and stored at −80 °C until total RNA extraction.

Temperature-induced stress. Potted wheat seedlings infested with *M. dirhodum* were divided into five groups for a 24-h exposure to one of the following five temperatures: 4, 10, 15, 20, and 25 °C. For each temperature, three samples of 20 adults were collected, flash frozen in liquid nitrogen, and stored at −80 °C until total RNA extraction. None of the temperature treatments were lethal to the aphids.

Starvation-induced stress. Adult aphids were placed on moistened filter paper in a Petri dish (9 cm diameter) with no food for a 32-h incubation in a thermostatic chamber (20 ± 2 °C and 60% relative humidity, with a 16-h light:8-h dark cycle). The control (satiated) group comprised aphids able to feed on wheat seedlings in the same conditions. For the control and treatment groups, three samples of 20 adults were collected, flash frozen in liquid nitrogen, and stored at −80 °C until total RNA extraction. The mortality rate among the starved aphids was approximately 10%.
**Table 3. Functions, primer sequences, and amplicon characteristics of the candidate reference genes analyzed in this study.** *A* Amplicon length, *b* qRT-PCR efficiency (based on a standard curve). *c* Reproducibility of the qRT-PCR.

**Insecticide-induced stress.** The effects of insecticides on the stability of candidate reference genes were assessed in *M. dirhodum* subjected to one of the following three insecticide treatments: imidacloprid (9.87 mg/L), thiamethoxam (122.00 mg/L), and beta-cypermethrin (17.28 mg/L). These concentrations were selected because a bioassay indicated they are 30% to the mortality of the population (LC30) (Table S1). Aphids were treated with the insecticides via the leaf dip method. The 1% insecticide stock solutions prepared in acetone were serially diluted with water (containing 0.1% Tween-80) to produce five concentrations. Water (containing 0.1% Tween-80) was used as a control solution. Wheat leaves with *M. dirhodum* adults were fed a 30% sucrose solution containing 50 µg/mL rifampicin or an antibiotic-free sucrose solution (control) (25 aphids per feeder) for 48 h. For the control and treatment groups, three samples of 20 adults were collected, flash frozen in liquid nitrogen, and stored at −80 °C until total RNA extraction.

**Antibiotic-induced stress.** The *M. dirhodum* adults were fed a 30% sucrose solution containing 50 µg/mL rifampicin or an antibiotic-free sucrose solution (control) (25 aphids per feeder) for 48 h. For the control and treatment groups, three samples of 20 adults were collected, flash frozen in liquid nitrogen, and stored at −80 °C until total RNA extraction.

**Total RNA extraction and cDNA synthesis.** Total RNA was extracted with Trizol according to the protocol for the TRNZol Universal Reagent (Tiangen, Beijing, China). The ratio of the absorbance at 260 and 280 nm was 1.981–2.121, indicating the extracted RNA was pure. Next, 1 µg RNA was used as the template to synthesize first-strand cDNA with Oligo dT primers using the FastKing gDNA Dispelling RT SuperMix (Tiangen) following the manufacturer-recommended protocol. The synthesized cDNA was stored at −20 °C.

**Primer design and quantitative real-time PCR.** A qRT-PCR assay was completed with the Talent qPCR PreMix (SYBR Green; Tiangen) and the CFX Connect Real-Time system (Bio-Rad, Hercules, CA, USA). Details regarding the primers for *EF1A* and *18S* (Table 3) have been published by NCBI. Primers for the other target genes were designed based on our unpublished RNA sequencing data for *M. dirhodum*. The cDNA of each sample was prepared as a 50 ng/µL working solution. The qRT-PCR was completed in a 25-µL reaction volume comprising 12.5 µL 2 × Talent qPCR PreMix, 1 µL forward primer (100 µM), 1 µL reverse primer (100 µM), 1 µL cDNA working solution, and 9.5 µL RNase-Free ddH₂O. The PCR program was as follows: 95 °C for 5 min; 40 cycles of 95 °C for 30 s and 60 °C for 30 s. For each treatment, standard curves were produced based on a fivefold dilution series of cDNA as a template according to the linear regression model. The fixed threshold in this study

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**Table 3. Functions, primer sequences, and amplicon characteristics of the candidate reference genes analyzed in this study.** *A* Amplicon length, *b* qRT-PCR efficiency (based on a standard curve). *c* Reproducibility of the qRT-PCR.

| Gene symbol | Gene name | Gene ID | (Putative) Function | Primer sequences(5’-3’) | *R*² | *E* (%) | *t* (bp) |
|-------------|-----------|---------|---------------------|-------------------------|------|---------|---------|
| Actin       | Actin     | TR996[c1_g1 | Cytoskeletal structural protein | F:CCATGTACCCTGTATATTGC | 0.9984 | 1.106   |
| GAPDH       | Glyceraldehyde-3-phosphate dehydrogenase | TR3352[c0_g1 | Glycolytic enzyme | F:GGATTACCGAGCTAGGC | 0.9839 | 0.977   |
| NADH        | NADH dehydrogenase | TR12676[c0_g1 | Enzyme involved in redox reactions | F:CCAGCGACAAAGAGTTTA | 0.9973 | 0.941   |
| RPL8        | Ribosomal protein L18 | 18S | Structural constituent of ribosome | F:AGTACATAATTTCGACG | 0.9824 | 1.014   |
| SDHB        | Succinate dehydrogenase B | TR11034[c0_g1 | Structural constituent of ribosome | F:TCAGCCGAGATTCCG | 0.9998 | 0.888   |
| RPL4        | Ribosomal protein L4 | TR996[c0_g1 | Structural constituent of ribosome | F:GGCAGCACTGAGACACC | 0.9961 | 0.928   |
| HSP68       | Heat shock protein68 | TR7632[c0_g3 | Molecular chaperone | F:AAACGGCTCGGAGAC | 0.9983 | 0.955   |
| 18S         | 18S ribosomal RNA | KT204362.1 | Structural constituent of ribosome | F:CGATGATGACGACGTGTAGT | 0.999 | 0.904   |
| EF1A        | Elongation factor 1a | DQ005156.1 | Catalysis of GTP-dependent binding of aminoacyl-tRNA to the ribosome | F:GGGAACGGCTCTATTTGCC | 0.9989 | 0.924   |

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**Insecticide-induced stress.** The effects of insecticides on the stability of candidate reference genes were assessed in *M. dirhodum* subjected to one of the following three insecticide treatments: imidacloprid (9.87 mg/L), thiamethoxam (122.00 mg/L), and beta-cypermethrin (17.28 mg/L). These concentrations were selected because a bioassay indicated they are 30% to the mortality of the population (LC30) (Table S1). Aphids were treated with the insecticides via the leaf dip method. The 1% insecticide stock solutions prepared in acetone were serially diluted with water (containing 0.1% Tween-80) to produce five concentrations. Water (containing 0.1% Tween-80) was used as a control solution. Wheat leaves with *M. dirhodum* adults were immersed in the prepared solutions for 3–5 s and then placed on moistened filter paper in a Petri dish (9 cm diameter). The samples were incubated for 24 h at 20 ± 2 °C and 60% relative humidity, with a 16-h light:8-h dark cycle. For each concentration, the mortality rate based on three replicates of 30 aphids was calculated. Additionally, for the control and treatment groups, three samples of 20 adults were collected, flash frozen in liquid nitrogen, and stored at −80 °C until total RNA extraction.

**Antibiotic-induced stress.** The *M. dirhodum* adults were fed a 30% sucrose solution containing 50 µg/mL rifampicin or an antibiotic-free sucrose solution (control) (25 aphids per feeder) for 48 h. For the control and treatment groups, three samples of 20 adults were collected, flash frozen in liquid nitrogen, and stored at −80 °C until total RNA extraction.

**Total RNA extraction and cDNA synthesis.** Total RNA was extracted with Trizol according to the protocol for the TRNZol Universal Reagent (Tiangen, Beijing, China). The ratio of the absorbance at 260 and 280 nm was 1.981–2.121, indicating the extracted RNA was pure. Next, 1 µg RNA was used as the template to synthesize first-strand cDNA with Oligo dT primers using the FastKing gDNA Dispelling RT SuperMix (Tiangen) following the manufacturer-recommended protocol. The synthesized cDNA was stored at −20 °C.

**Primer design and quantitative real-time PCR.** A qRT-PCR assay was completed with the Talent qPCR PreMix (SYBR Green; Tiangen) and the CFX Connect Real-Time system (Bio-Rad, Hercules, CA, USA). Details regarding the primers for *EF1A* and *18S* (Table 3) have been published by NCBI. Primers for the other target genes were designed based on our unpublished RNA sequencing data for *M. dirhodum*. The cDNA of each sample was prepared as a 50 ng/µL working solution. The qRT-PCR was completed in a 25-µL reaction volume comprising 12.5 µL 2 × Talent qPCR PreMix, 1 µL forward primer (100 µM), 1 µL reverse primer (100 µM), 1 µL cDNA working solution, and 9.5 µL RNase-Free ddH₂O. The PCR program was as follows: 95 °C for 5 min; 40 cycles of 95 °C for 30 s and 60 °C for 30 s. For each treatment, standard curves were produced based on a fivefold dilution series of cDNA as a template according to the linear regression model. The fixed threshold in this study
was set to 500 to obtain all the threshold cycle (Ct) values of tested candidate reference genes. The qRT-PCR analyses were completed with three biological replicates and three technical replicates.

Data analysis. The stability of the 10 candidate reference housekeeping genes was evaluated with the geNorm19, NormFinder20, and BestKeeper21 algorithms and the comparative delta Ct method22. Finally, we compared and ranked the tested candidate reference genes with the web-based RefFinder analytical tool (https://www.heartcurve.com.au/for-researchers).

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Author contributions
X.L., P.G., J.J. and X.Z. conceived and designed the research. X.L., P.G., M.L. and B.W. conducted the experiments. X.L., H.G., C.W. and X.Z. analyzed the data. X.L. and X.Z. wrote the manuscript. Y.Z., X.L. and L.W. revised the manuscript. All authors have read and approved the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
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