Evaluation of the expression stability of reference genes in *Apis mellifera* under pyrethroid treatment

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Honeybees (*Apis mellifera* L.), which unquestionably play an economically important role in pollination and agricultural production, are at risk of decline. To study changes in gene expression in insects upon exposure to pesticides or other external stimuli, appropriate reference genes are required for data normalization. Since there is no such gene that is absolutely invariable under all experimental conditions, the aim of this study was to identify the most stable targets suitable for subsequent normalization in quantitative experiments based on real-time polymerase chain reaction in honeybee research. Here, we evaluated the expression of fifteen candidate housekeeping genes from three breeding lines of honeybees treated with pyrethroids to identify the most stable genes. The tested insects were exposed to deltamethrin or lambda-cyhalothrin, and then, changes in the accumulation of selected transcripts were assessed, followed by statistical analyses. We concluded that *AmRPL32*, *AmACT* and *AmRPL13a* were the commonly recorded most stable genes in honeybees treated with the selected pyrethroids.

The importance of honeybees (*Apis mellifera* L.) as pollinators is unquestionable. However, since intensive crop production is currently strictly dependent on pesticide use, honeybees are exposed to agrochemicals during pollination, and these chemicals seem to impact the insects.

Pyrethroids are an important group of insecticides used for insect pest management. They are the most frequently used crop protection products for crops pollinated by honeybees, and their residues are most often found in these insects. Pyrethroids target the nervous system of the treated individuals; their active compound binds to voltage-gated sodium channels (VGSCs) in neurons and alters the function of these channels by maintaining the channel opening on axonal membranes. As a result, the neuron membranes cannot repolarize, leaving the axonal membrane permanently depolarized, thereby paralysing the organism. By performing gene expression analyses, it is possible to understand how honeybees react to pyrethroids at the molecular level. As a result, the data obtained are then relevant for sustainability programmes. For this purpose, several methods can be used, such as next-generation sequencing (RNAseq), microarrays, northern hybridization or quantitative reverse transcription polymerase chain reaction (RT-qPCR). Because of its versatility, low cost and high detection rate, RT-qPCR is now not only a very important tool for a majority of gene expression studies but also a standard for validating results derived from RNAseq or microarray analyses in which differentially expressed genes are studied. However, similar to other RNA-based quantitative techniques, RT-qPCR experiments need to be carefully designed and performed. The experimental design is based on, among other factors, the selection of a good internal control, that is, a gene that exhibits stable expression under the experimental conditions being tested. Only by using this approach is it possible to accurately estimate the accumulation of target mRNA molecules. However, it should be noted that there is no universal gene that might be used for RT-qPCR normalization under every experimental condition. The expression of housekeeping genes (HKGs) can be influenced by many factors; therefore, validation of their stability should always be performed before quantitation of mRNA targets. The need of careful selection of reference genes for gene expression studies in insects was widely reviewed previously.
In this study, we analysed the stability of the expression of fifteen genes, used previously in selection of reference genes in insects\(^1\), involved in basic metabolism of the honeybees, namely, actin (AmACT), α-tubulin (AmTUB), glutathione-S-transferase (AmGST1), glyceraldehyde-3-phosphate dehydrogenase (AmGAPDH), porphobilinogen deaminase (AmHMBS), ribosomal protein L32 (AmRPL32), 60S ribosomal protein L13a (AmRPL13a), 40S ribosomal protein S18 (AmRP18S), succinate dehydrogenase (AmSDHA), TATA-box-binding protein (AmTBP), elongation factor 1-alpha (AmEF1a), arginine kinase (AmARGK), chitin synthase 6 (AmCHS6), dorsal (AmDORS), and 18S ribosomal RNA (Am18S), in A. mellifera L. exposed to two types of pyrethroids: deltamethrin and lambda-cyhalothrin. The aim of the study was to identify the HKGs stably expressed in honeybees. We performed experiments to determine the best reference genes (a) for all the experimental conditions tested (namely, for all the breeding lines under treatment with pyrethroids), (b) for a particular breeding line individually, and (c) by focusing on two different active pyrethroid compounds. By utilizing RT-qPCR and four statistical algorithms, we concluded that regardless of the conditions tested, the genes AmRPL32, AmACT or AmRPL13a were commonly found among the most stable genes in honeybees treated with the mentioned pyrethroids. Moreover, by performing pairwise variation analysis (\(V_{\text{n/n+1}}\)), we determined that two of the identified reference genes would be sufficient for accurate normalization of RT-qPCR experimental results. Finally, we validated the results and used the selected reference genes to measure the expression of two cytochrome P450 monooxygenase (AmCYP450) genes described previously to be influenced in honeybees treated with insecticides\(^13,14\), and therefore the expression of AmCYP6AQ1\(^17\) and AmCYP305a1\(^17\) assayed in the research.

**Results**

**Determination of the specificity of the designed primers.** In this study, we evaluated fifteen candidate genes from honeybees to check their stability in insects exposed to pyrethroid treatment (Table 1). The main goal of the research was to identify the most stable genes that could be used as internal controls in experiments based on RT-qPCR to determine or verify differentially expressed genes in honeybees treated with pyrethroids.

First, from the GenBank database, we retrieved cDNA sequences of A. mellifera L. encoding these genes and used the data as input in Primer3 for designing the best primer pairs. By doing so, fifteen primer pairs that matched the implemented parameters were chosen, with the resulting amplicon lengths between 100 and 250 bp and the annealing temperature of the primers set at approximately 60 °C. Then, by performing a pilot experiment (end-point RT-PCR), we tested the designed primers for their specificity. The products of the RT-PCR were resolved on an agarose gel, and after staining, a single DNA band was detected for each tested primer pair (Fig. 1). No amplification products were detected in the no-template control reactions. Moreover, the results from Sanger sequencing of the cloned amplicons verified the sequence specificity of the primers used.

Next, we examined the expression rates of the tested transcripts: after each RT-qPCR, all the \(C_T\) output data were grouped in a table, and a combined box plot was prepared. All of the fifteen tested genes were amplified by RT-qPCR. The highest expression rate was observed for Am18S rRNA, with a \(C_T\) value of approximately 5.61. However, we omitted Am18S rRNA in further analyses because of the extremely high accumulation of rRNA in the analysed insects. The \(C_T\) values of all other candidate genes ranged between 14.33 and 25.62, which were the values for AmEF1a and AmGST, respectively (Fig. 2). Importantly, the expression levels of the analysed genes were similar among the three tested breeding lines (see Supplementary Fig. S1). Next, analysis of melting curves generated during the melting stage of RT-qPCR verified the presence of a single amplification product in each reaction: the generated melting curves were sharp and symmetric (Fig. 3), indicating reaction specificity. The melting temperature of the amplicons ranged from 75.46 to 84.33 °C, which were the values for AmHMBS and Am18S, respectively.

**Stability analysis of candidate reference genes.** In the present study, we focused on fourteen candidate HKGs of A. mellifera L. treated with two pyrethroids: deltamethrin and lambda-cyhalothrin. Our general goal was to identify the most stable reference genes in honeybees (a) regardless of breeding line and chemical compounds used for treatment and (b) individually for three breeding lines, and (c) for the two pyrethroids used for treating the insects. To achieve this goal, we used the following statistical tools: geNorm, BestKeeper, NormFinder and ΔCT. The comprehensive analysis was performed using RefFinder (https://www.heartbeat.com.au/reffinder).

*Search for the most stable reference genes for all breeding lines exposed to pyrethroids.* geNorm software, installed as a Bioconductor “NormqPCR” package in R software\(^16\), was used as the first program to identify the stability of the predicted HKGs, and the resulting values were used to order the HKGs from the most stable (the lowest \(M\) value) to the least stable (the highest \(M\) value). The AmHMBS gene and the AmSDHA gene had the highest expression stability values (0.0597 and 0.0604 M, respectively), followed by AmTub, AmTBP, and AmRPL32. The five least stable genes were AmRPL13a, AmGST, AmRPL18S, AmGAPDH and AmEF1a (Table 2).

Next, three additional methods were used to calculate the most stable reference genes within the pool of all tested samples. NormFinder analysis classified the AmRPL32, AmHMBS, AmCHS6, AmTub and AmAct genes as the most stable genes, whereas the AmEF1a, AmGST, AmTBP, AmGAPDH and AmDORS genes were indicated as being the least stable. The ΔCT analysis showed that AmRPL32, AmHMBS, AmTub, AmCHS6 and AmAct had the highest expression stability in comparison to AmEF1a, AmGST, AmTBP, AmGAPDH and AmDORS, which exhibited unstable expression. Then, based on the standard deviation (SD) of the \(C_T\) measurements, the stability values for the expression of fourteen candidate reference genes were calculated using the BestKeeper program, which showed slight differences compared to previous algorithms. The AmRPL32, AmSDHA, AmCHS6, AmHMBS, and AmRPL13a genes were identified as the most stable genes, and the AmTBP, AmEF1a, AmDORS, AmRP18S and AmGST genes were identified as the least stable genes.
Finally, to prepare a general ranking of most stable/unstable genes, a comprehensive analysis was performed with the support of RefFinder. According to the recommended comprehensive ranking, the **AmRPL32, AmAct, AmHMBS, AmTub, and AmCHS6** genes were identified as the five most stable genes, and the **AmRP18S, AmDORS, AmTBP, AmGAPDH, and AmEF1α** genes were identified as the five least stable genes.

**Analysis of HKG stability among the three breeding lines.** Next, the mentioned calculating methods were implemented to check the most stable HKGs in honeybees with regard to their breeding origin (breeding line) separately.

For the Kortówka line (Table 3), the five most stable genes were as follows: **AmGST, AmRPL32, AmTub, AmARGK** and **AmAct** (according to geNorm); **AmAct, AmARGK, AmRPL13a, AmCHS6** and **AmSDHA** (according to NormFinder); **AmGST, AmARGK, AmRPL32, AmTub** and **AmAct** (according to BestKeeper); **AmAct, AmARGK, AmRPL13a, AmCHS6** and **AmSDHA** (according to the ΔCT method).

For the Kortówka breeding line, the RefFinder method ordered the most stable genes as follows: **AmAct, AmARGK, AmRPL13a, AmGST** and **AmGAPDH**.

| Target name | Sequence of used oligonucleotides 5’→3’ | Forward primers | Reverse primers | Accession number | Estimated length in RT-PCR [bp] | Calculated Tm after RT-qPCR [°C] |
|-------------|------------------------------------------|----------------|----------------|-----------------|-----------------------------|-------------------------------|
| Actin       | AmAct_F TGGCAACAACCTTGCTCTTTCTG         | AmAct_R AGAAATGAGCACCACCA             | ABO23025.1      | 156             | 79.89                      |
| Tubulin alpha-1 | AmTub_F AATCGGCAAAAGAATTGTG          | AmTub_R TACCAACACGAAGTG                | XM_396338.6     | 107             | 78.82                      |
| Glutathione-S-transferase 1 | AmGST_F ACGTGCTCCGTGTGCTTTCTG         | AmGST_R CCGCTCACAAATTGACCT            | AY620822.2      | 174             | 83.43                      |
| Glyceraldehyde-3-phosphate dehydrogenase 2 | AmGAPDH_F TGGCTCAGGTTGTTGCTGAAT      | AmGAPDH_R CAGCTCCAGCTTTTGCTCAT         | XM_393605.6     | 197             | 75.91                      |
| Porphobilinogen deaminase | AmHMBS_F AAAAGCGGATGTTGCTCTGAA    | AmHMBS_R AATCAGACGGCGCACTTTC          | XM_624258.5     | 197             | 75.46                      |
| Ribosomal protein L32 | AmRPL32_F TGGTCTGCTAATCTGGTTG          | AmRPL32_R CGTAAACCTTGCTGACT           | NM_001011587.1  | 104             | 77.81                      |
| Ribosomal protein L13a | AmRPL13a_F TGGCCATTACCTTGGTT          | AmRPL13a_R AGACGGAGAAATGAGTA          | XM_623810.5     | 191             | 77.51                      |
| 40S ribosomal protein S18 | AmRP18S_F GATTCCGAGTTGTTTTGTA       | AmRP18S_R CCCAAATGACGCAACCTTT         | XM_625101.5     | 149             | 76.79                      |
| Succinate dehydrogenase [ubiquinone] flavoprotein subunit | AmSDHA_F GGCAAAAGCTGCAAATCATCTC   | AmSDHA_R AAAGTGGCAGTAATCTCGTCT         | XM_623062.5     | 109             | 79.15                      |
| TATA-box-binding protein | AmTBP_F TGATGCGGAACCCACAAA             | AmTBP_R AAGCGGTTGCTAGTGTGCT            | XM_623085.5     | 189             | 78.67                      |
| Elongation factor 1-alpha F2 | AmEF1a_F TGATGTCCTGCTGGACAGAGAGAAGAATC   | AmEF1a_R GAAATGCGCTCTGGTCTCTG          | NM_0010115603.1 | 192             | 82.57                      |
| Arginine kinase | AmARGK_F GTGCAACATCAAGCTGCTAA       | AmARGK_R GATTCATCGTGCATCTGGCT         | XM_006569890.3  | 114             | 78.27                      |
| Chitin synthase 6 | AmCHS6_F GGAGCACATGATTGGTTTCTG       | AmCHS6_R CGATCGTTTCCTCTGACCTGTA       | XM_001123000.3  | 150             | 78.55                      |
| 18S ribosomal RNA | Am18S_F CGGACCGAGATGTAGCGGAAATTA     | Am18S_R TCCGTCCTGGAAGGGAATAA          | AY703484.1      | 170             | 84.22                      |
| Dorsal (transcription factor) | AmDorsal_F TGCCATGGTCTACGAGGCA      | AmDorsal_R AACAGTGGCTCTACGTCTGCT       | NM_001011577    | 153             | 79.59                      |
| Cytochrome P450 (CYP6AQ1) | AmCYP450_R TGCACTGGTATGCGCAGTGGCACTAG | AmCYP450_R AAGAGTGAACCCAGCGGGA         | NM_001205062    | 192             | 78.72                      |
| Cytochrome P450 305a1 (CYP305a1) | AmCYP450_305a1_F TGTCATTTCGTTCTG      | AmCYP450_305a1_R TTGCTTTCGTTCTCCA       | XM_623618.6     | 156             | 77.24                      |

Table 1. List of primers used in the study.
For the Alpejka line (Table 4), the most stable genes were AmTBP, AmDORS, RPL32, AmHMBS, and AmRP18S (according to geNorm); AmDORS, AmRP18S, AmEF1a, AMHMBS and AmRP13a (according to NormFinder); AmDORS, AmRP18S, AmRPL32, AmGST and AmRP13a (according to BestKeeper); and AmDORS, AmRP18S, AmEF1a, AmHMBS and AmRP13a (according to the ΔCT method).

On the basis of the abovementioned results, the RefFinder analysis identified AmDORS, AmRP18S, AmRPL13a, AmGAPDH, and AmEF1a as the most stable genes for the Alpejka breeding line.

For the Nieska line (Table 5), the most stable genes were AmTBP, AmDORS, AmRPL32, AmHMBS, AmRP18S (according to geNorm); AmARGK, AmTub, RPL13a, AmCHS6 and AmTBP (according to NormFinder); AmRPL32, AmRP18S, AmRPL13a, AmAct and AmGST (according to BestKeeper); and AmARGK, AmTub, AmRPL13a, AmRP18S and AmRPL32 (according to the ΔCT method).

Thus, the following genes were selected as the most stable genes according RefFinder calculations for the Nieska line: AmARGK, AmRPL32, AmRP18S, AmRPL13a, and AmTub (Table 5).

Analysis of the HKG stability with regard to the active substance of pyrethroid insecticide. Analysis of the influence of each insecticide used for insect treatment on HKG stability was also performed (Table 6). All calcula-
Figure 3. Melting plots generated after RT-qPCR. The fifteen primer pairs were evaluated by quantitative RT-PCR. The melting temperature is indicated on each plot.

Table 2. Stability ranking of fourteen candidate reference genes in *Apis mellifera* L. Carnolian honeybees under pyrethroid treatment. The calculations were performed by geNorm, NormFinder, BestKeeper, ΔCT and RefFinder.
Table 3. Stability ranking of fourteen candidate reference genes in the *Apis mellifera* L. Kortówka breeding line under pyrethroid treatment. The calculations were performed by geNorm, NormFinder, BestKeeper, ΔCT and RefFinder.

| Analy ses | Program | Genes stability | Most stable | Less stable |
|-----------|---------|-----------------|-------------|-------------|
| geNorm    | AmGST   | AmRP  | AmTub | AmAR   | AmAct | AmCHS6 | AmSDHA | AmHMBS | AmRBP  | AmL13a | AmARGK | AmORS | AmTB  | AmEF  | AmRPL  | AmTBP |
|           |         | L32   |       | 6      |       | S6     |        |        | L32    | 1a     |       | S6     | 6     |       | 1a     | S6    |
|           |         | 0.042 | 0.048 | 0.050  | 0.053 | 0.056  | 0.058  | 0.060  | 0.062  | 0.064  | 0.067  | 0.069  | 0.074 | 0.078 |
| NormFinder| AmAct   | AmAR  | AmRPL | AmC   | AmSD | AmMBS | AmGAPDH | AmM5 | AmH    | AmTu   | AmRPL | AmEF  | AmD   | AmRBP | AmEF  | AmTBP |
|           | GR      | L32a  |       | HS6   |      |       |        |       |        |        |       |       |       |       |       |       |
|           | 0.385   | 0.401 | 0.4677 | 0.486 | 0.4921 | 0.547 | 0.641 | 0.657 | 0.6921 | 0.695 | 0.826 | 0.893 | 0.9399 | 1.049 |
|           | 59      | 1      | 62    | 6     | 1     | 6     | 6      | 6      | 6      | 8      | 4      | 6      | 8      | 1      | 3      |
| BestKe eper Kortówka line | AmAct | AmAR | AmRPL | AmTub | AmMBS | AmM | AmCHS6 | AmSDHA | AmHMBS | AmRBP  | AmL13a | AmEF  | AmD   | AmRBP | AmEF  | AmTBP |
|           | GR      | L32  |       |       |       |       |        |        |        | L32    | 1a     |       | S6    | 6     |       | 1a     | S6    |
|           | 0.334   | 0.375 | 0.508 | 0.537 | 0.545 | 0.699 | 0.709  | 0.764 | 0.794  | 0.908  | 0.947  | 0.971 | 1.005 |
|           | 4       | 7      | 6     | 6     | 7      | 6     | 6      | 6      | 6      | 6      | 6      | 6      | 6      |
| ΔCT       | AmAct   | AmAR | AmRPL | AmC   | AmSD | AmMBS | AmGAPDH | AmM5  | AmH    | AmTu   | AmRPL | AmEF  | AmD   | AmRBP | AmEF  | AmTBP |
|           | GR      | L32a |       | HS6   |      |       |        |        |        | L32    | 1a     |       | S6    | 6     |       | 1a     | S6    |
|           | 0.786   | 0.790 | 0.814 | 0.832 | 0.833 | 0.853 | 0.917  | 0.942 | 0.947  | 0.955  | 1.025  | 1.099  | 1.120  | 1.200  |
|           | 6       | 2      | 6     | 6     | 2      | 6     | 6      | 6      | 6      | 6      | 6      | 6      | 6      | 6      |

Determination of the minimum number of reference genes necessary for normalization. Pairwise variation analysis (V_{n/n+1}) performed using the geNorm method\(^{15}\) indicated that the expression of the target gene in each considered experimental variant needs to be normalized using two selected reference genes. This was indicated by pairwise variation (V) with the threshold value set at 0.15\(^{15}\). In all tested experimental variants, the V_{n/n} value was lower than 0.15 (Fig. 4).

Validation of reference genes. To validate the obtained results (the indicated stable HKGs for each experimental condition), we performed an analysis of the expression of two cytochrome P450 monooxygenase (*AmCYP450*) genes in honeybees exposed to pyrethroid treatment. CYP450s are known to be involved in xenobiotic detoxification in insects\(^{14}\). Importantly, it was described previously that the expression of *AmCYP6AQ1*\(^{15}\) and *AmCYP305a1*\(^{13}\) was influenced by insecticides. Validation experiments aimed at normalization...
of the expression of both AmCYP450 genes were performed in the following contexts: first, for the entire set of tested Carnolian honeybees exposed to pyrethroid treatment; second, for testing the effect of pyrethroid treatment on the expression of AmCYP450 in each breeding line separately; and finally, for analysing the expression of AmCYP450 separately in deltamethrin- or lambda-cyhalothrin-treated insects.

As indicated earlier in the manuscript, using two reference genes is sufficient for accurate normalization of genes in pyrethroid-treated insects. For normalization of AmCYP450 expression in Carnolian honeybees, the two following HKGs were used: AmRPL32 and AmRPGT.

The results showed (Fig. 5) that expression of the AmCYP6AQ1 gene increased slightly in honeybees treated with deltamethrin (1 h and 24 h after treatment) and lambda-cyhalothrin (24 h after treatment) with a 1.35-fold change, 1.28-fold change and 1.47-fold change (all with p < 0.01), respectively. On the other hand, the expression of the AmCYP305a1 gene increased slightly in honeybees treated with deltamethrin. These data also showed that each breeding line of tested honeybees treated with pyrethroids, we observed that the expression of AmCYP6AQ1 and AmCYP305a1i in insects belonging to the Alpejka breeding line changed slightly. On the other hand, in the Kortówka breeding line, the level of expression of the AmCYP6AQ1 gene increased 1.40-fold 1 h post treatment and 24 h after pyrethroid exposure (1.40-fold

| Analyses   | Progamm | Genes stability | Most stable | Less stable |
|-----------|---------|----------------|-------------|-------------|
| geNorm    | AmTB   | 0.042          | AmRDP5     | AmRPI32    |
|           | AmD    | 0.044          | AmRPI85    | AmRPI85    |
|           | AmGST  | 0.048          | AmRPI85    | AmRPI85    |
|           | AmTu   | 0.051          | AmRPI13a   | AmRPI13a   |
|           | AmEF1  | 0.053          | AmRPI13a   | AmRPI13a   |
|           | AmEF2  | 0.054          | AmFE1a     | AmFE1a     |
|           | AmEF3  | 0.055          | AmELa      | AmELa      |
|           | AmEF4  | 0.057          | AmELa      | AmELa      |
|           | AmEF5  | 0.058          | AmELa      | AmELa      |
|           | AmEF6  | 0.059          | AmELa      | AmELa      |
|           | AmEF7  | 0.061          | AmELa      | AmELa      |
|           | AmEF8  | 0.064          | AmELa      | AmELa      |

**Table 4.** Stability ranking of fourteen candidate reference genes in the *Apis mellifera* L. Alpejka breeding line bred under pyrethroid treatment. The calculations were performed by geNorm, NormFinder, BestKeeper, ΔCT and RefFinder.
change and 1.26-fold change, respectively, \( p < 0.01 \) was indicated. Accordingly, in lambda-cyhalothrin-treated insects, AmCYP6AQ1 showed a modest, statistically significant increase in expression level 24 h after pyrethroid treatment (1.34-fold change, with \( p < 0.01 \)). Similarly, the expression level of the AmCYP305a1 gene was somewhat stable over time, reaching a 1.35-fold change (with \( p < 0.05 \)) in bees 1 h after treatment with lambda-cyhalothrin (Fig. 7).

Additionally, changes in AmCYP450s expression levels normalized against two unstable HKGs were also analysed (see Supplementary Figs. S2, S3 and S4). The use of inappropriate normalizers in differential gene expression analysis resulted in increased statistical significance at the expense of an increased error range and changes in the expression levels of target genes in individual research models (e.g., for the Nieska line, the AmCYP305a1 gene expression level 24 h after pyrethroid treatment was almost 40 times higher than that obtained if the least stable genes were selected (see Supplementary Fig. S3). Moreover, the use of the highly unstable HKGs for validation gives different, highly discrepant results, as in the case of the Kortówka line, where changes in the expression levels of target genes in individual research models (e.g., for the Nieska line, the AmCYP450s expression analysis resulted in increased statistical significance at the expense of an increased error range and changes in the expression levels of target genes in individual research models (e.g., for the Nieska line, the AmCYP305a1 gene expression level 24 h after pyrethroid treatment was almost 40 times higher than that obtained if the least stable genes were selected (see Supplementary Fig. S3)).

**Discussion**

To minimize both biological and experimental errors in quantitative analyses performed by means of real-time qPCR, it is important to choose the most stable reference genes for normalization of RNA input. However, this requires an individualized research approach for each analysed parameter. One such parameter is the fitness of insects, which has been extensively discussed.

In this study, we investigated the expression stability of 14 candidate reference genes of *A. mellifera* L., belonging to Carnolian honeybees, exposed to pyrethroids. The selected subspecies of the honeybee was treated with two insecticides: deltamethrin and lambda-cyhalothrin. It should be remembered that honeybees of various genetic background (like the three breeding lines described in the study: Alpejka, Nieska and Kortówka) might react differently at the level of insecticide sensitivity, what can expressed at the molecular level.

Carnolian honeybees are highly adapted to nectar and climatic flow both in Poland and worldwide. Analysis of all the obtained data described in this study on Carnolian honeybees under pyrethroid treatments indicated

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**Table 5.** Stability ranking of fourteen candidate reference genes in the *Apis mellifera* L. Nieska breeding line under pyrethroid treatment. The calculations were performed by geNorm, NormFinder, BestKeeper, ΔCT and RefFinder.
The calculations were performed by geNorm, NormFinder, BestKeeper, ΔCT and RefFinder.

| Analyses  | Progra m | Gene stability |
|-----------|----------|----------------|
|           |          | Most stable     | Less stable |
| Lambda - | Cyhalot rin |  |  |
| geNorm m | Genes stability (by M Value) | 0.056 | 0.056 | 0.059 | 0.068 | 0.072 | 0.073 | 0.082 | 0.088 | 0.089 | 0.089 | 0.103 | 0.112 | 0.115 |
|          |          | 0.056 | 0.056 | 0.059 | 0.068 | 0.072 | 0.073 | 0.082 | 0.088 | 0.089 | 0.089 | 0.103 | 0.112 | 0.115 |
| NormFind er | Genes stability (by SD Value) | 0.574 | 0.610 | 0.646 | 0.647 | 0.656 | 0.658 | 0.735 | 0.774 | 0.904 | 0.907 | 0.988 | 0.992 | 1.001 | 1.013 |
|          |          | 0.574 | 0.610 | 0.646 | 0.647 | 0.656 | 0.658 | 0.735 | 0.774 | 0.904 | 0.907 | 0.988 | 0.992 | 1.001 | 1.013 |
| BestKe eper | Genes stability (by SD Value) | 0.467 | 0.53 | 0.591 | 0.661 | 0.661 | 0.745 | 0.764 | 0.817 | 0.83 | 0.84 | 1.003 | 1.025 | 1.056 | 1.07 |
|          |          | 0.467 | 0.53 | 0.591 | 0.661 | 0.661 | 0.745 | 0.764 | 0.817 | 0.83 | 0.84 | 1.003 | 1.025 | 1.056 | 1.07 |
| ΔCT | Genes stability (by Mean SD Value) | 0.996 | 1.012 | 1.028 | 1.029 | 1.033 | 1.036 | 1.080 | 1.094 | 1.188 | 1.192 | 1.238 | 1.251 | 1.253 | 1.258 |
|          |          | 0.996 | 1.012 | 1.028 | 1.029 | 1.033 | 1.036 | 1.080 | 1.094 | 1.188 | 1.192 | 1.238 | 1.251 | 1.253 | 1.258 |
| RefFin ders | Genes stability (by SD Value) | 0.055 | 0.055 | 0.064 | 0.069 | 0.073 | 0.076 | 0.077 | 0.082 | 0.086 | 0.088 | 0.094 | 0.097 | 0.11 | 0.117 |
|          |          | 0.055 | 0.055 | 0.064 | 0.069 | 0.073 | 0.076 | 0.077 | 0.082 | 0.086 | 0.088 | 0.094 | 0.097 | 0.11 | 0.117 |
| Lambda - | Cyhalot rin |  |  |
| geNorm m | Genes stability (by M Value) | 0.655 | 0.655 | 0.660 | 0.671 | 0.685 | 0.704 | 0.705 | 0.793 | 0.814 | 0.822 | 0.944 | 0.965 | 1.001 | 1.092 |
|          |          | 0.655 | 0.655 | 0.660 | 0.671 | 0.685 | 0.704 | 0.705 | 0.793 | 0.814 | 0.822 | 0.944 | 0.965 | 1.001 | 1.092 |
| NormFind er | Genes stability (by SD Value) | 0.524 | 0.545 | 0.661 | 0.713 | 0.755 | 0.807 | 0.914 | 0.93 | 0.995 | 1.036 | 1.073 | 1.092 | 1.212 | 1.274 |
|          |          | 0.524 | 0.545 | 0.661 | 0.713 | 0.755 | 0.807 | 0.914 | 0.93 | 0.995 | 1.036 | 1.073 | 1.092 | 1.212 | 1.274 |
| BestKe eper | Genes stability (by SD Value) | 1.005 | 1.032 | 1.037 | 1.044 | 1.046 | 1.060 | 1.064 | 1.107 | 1.119 | 1.128 | 1.206 | 1.229 | 1.243 | 1.319 |
|          |          | 1.005 | 1.032 | 1.037 | 1.044 | 1.046 | 1.060 | 1.064 | 1.107 | 1.119 | 1.128 | 1.206 | 1.229 | 1.243 | 1.319 |

Table 6. Stability ranking of fourteen candidate reference genes in *Apis mellifera* L. under pyrethroid treatment. The calculations were performed by geNorm, NormFinder, BestKeeper, ΔCT and RefFinder.
Figure 4. Optimal number of reference genes for various conditions. The geNorm algorithm was used to determine the pairwise variation (V) between the reference genes for treatments with pyrethroids together (Carnolian honeybees) or separately (deltamethrin or lambda-cyhalothrin). The effect of pyrethroid treatments on three breeding lines was also indicated (Kortówka, Alpejka and Nieska). The threshold for adequate normalization was V ≤ 0.15.

Figure 5. Expression of the two AmCYP450 genes AmCYP6AQ1 and AmCYP305a1 in Apis mellifera L. treated with either deltamethrin (A) or lambda-cyhalothrin (B) normalized against the indicated reference genes (AmRPL32 and AmHMBS). Blue bars: 1 h post treatment, orange bars: 24 h post treatment. Error bars represent the standard deviation. The Mann–Whitney U-test was used. **p < 0.01, *p < 0.05.
Figure 6. Expression of the two AmCYP450 genes (AmCYP6AQ1 and AmCYP305a1) in three breeding lines of Apis mellifera L., namely, Alpejka (A), Kortówka (B) and Nieska (C), treated with either deltamethrin or lambda-cyhalothrin, normalized against the indicated reference genes: (A) AmDORS and AmRP18S; (B) AmAct and AmARGK; (C) AmRPL32 and AmRPL13a). Blue bars—1 h post treatment, orange bars—24 h post treatment. Error bars represent the standard deviation. Error bars represent the standard deviation. The Mann–Whitney U-test was used. **p < 0.01, *p < 0.05.
5 stably expressed genes: AmHMBS (responsible for haem synthesis and porphyrin metabolism), AmCHS6 (responsible for synthesis of chitin), AmRPL32 (ribosomal protein gene), AmAct (encoding cytoskeletal structural proteins), and AmTub (encoding cytoskeletal structural proteins).

Analysis of the expression stability of selected candidate reference genes with respect to individual breeding lines distinguished a common high-scoring gene, AmRPL13a, in terms of stability for all the tested lines. On the other hand, AmDORS, AmAct and AmTub were selected as the most stable genes in the Alpejka, Kortówka and Nieska breeding lines, respectively. Similarly common most stable genes were also observed between the Kortówka and Nieska lines (the AmARGK gene) and between the Alpejka and Nieska lines (the AmRP18S gene). The expression level of the AmARGK gene does not change after carbon dioxide narcosis in honeybee workers28; however, it should be noted that the amount of ARGK protein in the antennae can vary between bee families29.

The ribosomal genes (from the functional rRNA-coding regions) are structurally conserved and homogeneous throughout the nuclear and mitochondrial genomes in honeybees30 and are often used as reference genes for differential expression studies31–33. In research on the effects of imidacloprid treatment on honeybees, ribosomal genes have been shown to be upregulated34, which means that they should be approached with caution as potential reference genes. The analyses also show the variable levels of expression of target genes relative to the AmDORS gene described in the literature34. Depending on the breeding line tested, the expression stability results for individual genes were classified slightly differently (Tables 3, 4, 5); therefore, in experiments, both the population and the breeding line should be determined with full accuracy to avoid statistical errors in research.

The stability ranking of HKGs in honeybees under pyrethroid treatment, when the active compounds (deltamethrin or lambda-cyhalothrin) were considered separately, showed three common most stable reference genes: AmRPL32, AmTub and AmHMBS. These results were confirmed with the data previously obtained when active substances were analysed together; however, it should again be noted that the genes were placed at different positions in the ranking order (after deltamethrin treatment: AmRPL32, AmCHS6, AmTub, AmARGK and AmHMBS; after lambda-cyhalothrin treatment: AmRPL32, AmAct, AmRPL13a, AmHMBS and AmTub). Such differences may occur due to differences in the sample sizes analysed individually. For the entire set of Carnolian honeybees, all data obtained in the experiments were taken into account. In turn, for the analysis of bees after treatment with deltamethrin or lambda-cyhalothrin, data obtained for a specific pyrethroid active substance
treatment/exposure were taken (limiting the sample size from 108 bees to 72 individuals). This is why the selection of the sample is such an important aspect of research related to differential gene expression.

The validation of the indicated most stable reference genes showed that the selection of inappropriate normalizers, the expression of which is not stable under the conditions being tested, can significantly affect the final results of the analysis of the target gene of interest. The values may vary by up to 40 times, as was observed for the expression level of the AmCYP6AQ1 gene in the Nieska line exposed to lambda-cyhalothrin (24 h after treatment), when we compared the results obtained by using the most stable genes and least stable genes for normalization (see Supplementary Fig. S3). The statistical significance and direction of changes in the level of expression between two time points were also divergent after the selection of relatively less stable reference genes for analysis (as was the case for the Nieska breeding line, as stated earlier) (see Supplementary Figs. S2, S3, S4).

Therefore, optimization of testing data by using various statistical programs is very important when studying changes in the expression of target genes relative to that of a reference gene. It should also be noted that the selection of HKGs may differ if the research model assumes testing on populations, not on specific breeding lines, as presented in Tables 2, 3, 4, 5. Previous work also showed that the differences in the expression stability results for reference genes may be due to the season in which the study was conducted and the stage of maturation of the tested individuals. The expression levels of the AmCYP450 genes validated against the selected HKGs confirmed some behavioural observations for the developmental lines tested. Namely, slight changes in the expression of the AmCYP6AQ1 and AmCYP305a1 genes were observed for the Alpejka line (Fig. 6), in which individuals showed the highest liveliness among the three breeding lines during the experiment. Accordingly, the most considerable increase in the expression of these genes was demonstrated for the Nieska line (Fig. 6), the individuals of which tended to gather in groups and exhibited low activity (data not shown).

To summarize, regardless of the experimental conditions or tested breeding line of the examined insects, the above studies indicated the three following HKGs as reference genes to be considered, as they were classified by each analysis as being the most stable genes: AmRPL32, AmAct and AmRPL13a.

Methods
Insects used in the study. In this study, three breeding lines of A. mellifera L. were used, namely, Kortówka, Alpejka and Nieska, all belonging to Carniolan honeybees. The insects were taken from original hives by a bee-keeper and were individually treated with a 1 µl dose of one of the following pyrethroids: deltamethrin (0.75 ml/L (4.8%)) or lambda-cyhalothrin (0.75 ml/L (4.81%)). Non-treated insects were used as a control. Then, the treated bees were gathered (6–15 insects, whereas for RNA isolation 6 insects were taken) in bee cages and collected 1 h and 24 h after treatment. During this time, the insects were kept at room temperature on a laboratory bench. Then, the insects were immediately frozen in liquid nitrogen and stored at −80 °C.

RNA isolation and cDNA synthesis. Single insects (from six biological replicates) were pulverized in liquid nitrogen using a mortar and pestle and were subsequently stored at −80 °C for further analyses. Next, up to 100 mg of pulverized material was taken for total RNA extraction. RNA isolation was performed using 1 ml of TriReagent Solution (Invitrogen) followed by RNA precipitation with propanol. The resulting RNA pellet was washed with 70% ethanol, air-dried and resuspended in nuclease-free water. The concentration of the RNA, as well as its purity (the 260/230 and 260/280 values) were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific), whereas the quality of the RNA was assessed by means of gel electrophoresis. Contaminant genomic DNA in the RNA samples was removed using dsDNAse enzyme (Thermo Scientific). Next, cDNA synthesis was performed using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific) and 3 µg of total RNA. The resulting cDNA was finally diluted 3 times with water (50 ng/µl).

Primer selection and real-time quantitative PCR. The primers used in this study were designed using Primer3 online software. The coding sequences of target transcripts were retrieved from GenBank and further analysed with Primer3 software to indicate the best pairs for RT-qPCR (Table 1). The selected primers were tested for their specificity: initially, all the tested sequences were verified by BLAST, and next, the primers were used in subsequent end-point RT-PCR to check the estimated size of the resulting amplicons. The RT-PCRs were performed in 20 µl reactions containing 1 × reaction master mix (DreamTaq PCR Master Mix, Thermo Scientific), 0.5 µM forward primer, 0.5 µM reverse primer and 1 µl of cDNA. The reaction was incubated for 3 min at 95 °C, followed by 35 cycles of 95 °C for 20 s, 60 °C for 30 s, and 72 °C for 30 s. After incubation at 72 °C for an additional 10 min, the reactions were resolved on a 1% agarose gel, and the PCR products were gel-purified and subsequently cloned into Escherichia coli DH10B using the CloneJET PCR Cloning Kit (Thermo Scientific). The recombinant plasmids were isolated from transformed bacteria, and the inserted cDNAs were sequenced by Genomed (Warsaw, Poland).

RT-qPCR was performed as follows: the 10 µl reaction mixture contained 1 × master mix (iTaql Universal SYBR Green Supermix, Bio-Rad), 0.5 µM forward primer, 0.5 µM reverse primer and 1 µl of cDNA. The reaction was incubated for 3 min at 95 °C, followed by 40 cycles of 20 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. After the last cycle, a melting curve was generated by increasing the temperature from 60 °C to 95 °C. RT-qPCR was performed in three technical replicates using the real-time PCR system (QuantStudio5, Thermo Scientific).

Statistical analysis of HKGS and validation. Selection of the best reference genes was performed using previously described calculation algorithms, namely, geNorm, BestKeeper, NormFinder and the ΔCT method. The detailed description of the methods was indicated in Supplementary File.
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Author contributions
A.O.S. and P.W. designed the experiment; P.W. carried out all molecular biological procedures; PF performed all statistical analyses; PW, PF, and AOS analysed and interpreted the obtained results; and AOS, PF and PW wrote the manuscript.

Competing interests
The authors declare no competing interests.

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