Identification of reference genes for studies of quantitative gene expression in male and female quail tissues

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ABSTRACT

In industrial poultry, quail production has gained increasing prominence over the years. It is known that the intensification of genetic studies has contributed greatly to this growth, through techniques, such as analysis of gene expression by PCR, for example. This study aimed to evaluate stability and recommend reference genes for quantitative real-time PCR in different tissues from male and female broiler quails. The stability of 10 housekeeping genes (GAPDH, RPL5, MRPS27, MRPS30, TFRC, HMBS, EEF1, LDHA, B2M, and UBC) by means of Bestkeeper, NormFinder, GeNorm softwares with ΔCq method. The tissues analyzed were: heart, thigh muscle, brain, and spleen, considering that they are tissues commonly used in nutrigenomic, immunological, and poultry performance research. As expected, the reference genes tested showed varying stability depending on the tissue evaluated. According to the present study, the most stable housekeeping genes were MRPS30, TFRC, and HMBS in heart; MRPS30, EEF1, and HMBS in thigh muscle; B2M, GAPDH, and UBC in brain; and EEF1, LDHA, and HMBS in spleen. Therefore, it is recommended to be used as reference genes for gene expression studies of male and female quails.

KEYWORDS

European quail; endogenous gene; expression of stability; RT-qPCR; expression

Introduction

For industrial poultry farming, quails rearing is an activity that has been emerging in the world. European quails (Coturnix coturnix coturnix) also known for meat production, began to develop commercially and gained space at expense of laying quails due to the first is considered an ease adaptation bird to breeding conditions, precocious sexual maturity, rapid growth, small feed consumption, great resistance to diseases, and also used as an animal model in the laboratory. However, it is established that success in productivity depends not only because optimization of the production system considering only on animal performance; other factors are also extremely important; like genetic, for example. Genetic studies are capable of providing intrinsic information revealing animal metabolism particularities, and these, when properly applied, can result in improvements in production system use and consequent development.

In the genetic context, gene expression analysis has been strengthened in the scientific community, with constant improvements in methods promoting better access to various platforms. Moreover, the epigenome comprising different mechanisms, e.g., DNA methylation, remodeling, histone tail modifications, chromatin microRNAs, and long non-coding RNAs, interact with environmental factors like nutrition, pathogens, and climate to influence the expression profile of genes and the emergence of specific phenotypes. Multi-level interactions between the genome, epigenome, and environmental factors might occur. Furthermore, numerous lines of evidence suggest the influence of epigenome variation on health and production. The expression of eukaryotic genes is temporarily and multidimensionally controlled. Only a relatively small set of the entire genome is expressed in each type of tissue, and the expression of genes depends on the stage of development. Therefore, gene expression in eukaryotes is specific to each tissue. Also, the number of gene products that are made in the same tissue as well as in other tissues that make up that product, regulates the expression of that gene. One of the basic activities in domestic animals is the study of...
genes and proteins related to economic traits and their study at the cellular or chromosomal level.\textsuperscript{11}

Among the methods of genetic analysis, quantitative real-time polymerase chain reaction (RT-qPCR) is recurrent and one of the most used in gene expression studies. Through this technique, the genome sequence of interest is amplified billions of times, for ease of analysis, and in conjunction with a fluorophore, the quantification of these copies is performed in real-time.\textsuperscript{13} The results obtained from RT-qPCR require normalization and this can be accomplished with internal control for, besides other functions, the correction of the results that may be distorted due to different initial amounts of nucleic acid.\textsuperscript{6,8,9}

The internal control analyses correspond to the study of a single or small set of genes, called reference genes, that have demonstrated significant expression on tissue and must remain invariable in every experimental condition, being their choice very influential and decisive in the results.\textsuperscript{10,11} The method commonly used to identify reliable reference genes is based on algorithms such as geNorm, NormFinder, BestKeeper and Delta Ct (ΔCt), assessing genes stability based on quantification cycle (Cq) variance values in each physiological or experimental condition.\textsuperscript{14} Thus, the choice of reference genes must be made carefully, based on previous tests that prove their effectiveness, since various physiological and experimental conditions affect the levels of expression.

Among the factors altering and interfering with these genes expression are the physiological state, type of tissue, experimental treatments, and both sexual and species differences.\textsuperscript{15–19} Some studies seek answers regarding animal genetic behavior in both sexes. For this purpose, is necessary to use the same set of reference genes to normalize the expression data from males and females samples, eliminating the sex effect.\textsuperscript{12} However, information about genes used as reference is still scarce for quails, reinforcing the importance of experiments that investigate their action, since these are considered based on the technique and, therefore, will influence subsequent genetic studies related to the current species.

Hence, the objective of this work was to assess 10-reference genes stability and, according to the results, recommend them for RT-qPCR in different tissues of male and female broiler quails.

Materials and methods

Animals

All procedures applied in this experiment were approved (process 09/2015) by the Animal Protection, Research, and Ethics Committee of the Federal University of Sergipe, Sergipe, Brazil. The tissues used in this study were: thigh muscle, brain, heart, and spleen, since they are commonly used in studies about animal productive performance and metabolic analyses of an investigative nature. The tissues were collected from six broiler quails (\textit{Coturnix coturnix}) at the age of 35 days, three males and three females (\(n = 6\)). These animals were obtained from a commercial production poultry farm in São Cristóvão—SE city, raised under standard management conditions with nutrition conducted in accordance with each growing phase requirements. At 35 days of age, the animals were slaughtered by cervical dislocation; and the organs and tissues were collected and stored in sterile tubes containing sterile RNA later solution (Ambion, RNA Carlsbad, CA, USA). The samples were stored at 4°C for 12 h and then at −20°C until total RNA extraction.

RNA extraction and cDNA synthesis

Total RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions, from 30 mg of each tissue. RNA concentration was determined by AstraGene UV/Vis spectrophotometer (AstraNet Inc., Bath, UK) using 2.5 μL of the extracted RNA. To eliminate possible sample contamination, they were subjected to treatment with DNase I Amplification Grade (Invitrogen, Carlsbad, CA, USA), then RNA integrity was evaluated using a 1% agarose gel stained with the gel red\textsuperscript{TM}, at 100 V for 30 min. After this verification, the cDNA was built from 1 μg of total RNA from each tissue using the GoScript Reverse Transcription System kit (Promega, Madison, WI, USA), following the manufacturer’s instructions. The resulting cDNA samples were stored at −20°C until PCR analysis was performed.

Selection of reference genes and RT-qPCR

Ten reference genes were chosen to assess stability during gene expression study in male and female broiler quails. These genes were selected based on literature,\textsuperscript{20} for \textit{Gallus gallus} species. The primers were designed using Primer Quest software provided by DNA Technologies, Inc (IDT, Coralville, IA, USA), and hereafter, the sequences were analyzed in Primer BLAST (http://blast.ncbi.nlm.nih.gov) to verify specificity; then were synthesized by Invitrogen Life
Expression stability of reference genes

The expression stability of the reference genes was analyzed using four methods: Bestkeeper,22 GeNorm,23 NormFinder,24 and ΔCt method,25 through RefFinder software, available at http://fulxie.0fees.us/?type=reference, which integrates the four algorithms to compare and classify genes stability. For GeNorm and NormFinder, the values used for calculation are those related to relative gene expression estimated from Cq values (min Cq—sample Cq). For Bestkeeper and ΔCq method, the values used for stability calculation were the Cq values introduced in the RefFinder. This tool analyzes each algorithm used separately and, also, provides a general classification of the best normalizer for experimental conditions tested, obtained by combining the results of the four algorithms. The results provided by RefFinder, considering gene expression stability for males and females broiler quails, can be displayed together or separately.

The BestKeeper algorithm provides values of standard deviation and coefficient of variation for the tested genes, where genes with a value of standard deviation higher than 1 are considered unstable and should be excluded from the analysis.22 Hence, a gene with the lowest standard deviation value is the first for stability classification. In GeNorm, classification is based on the value of average expression stability (M), which can reach the limit of 1.5 to be considered stable.23 Thus, the lower M value indicates a higher gene expression stability. NormFinder, on the other hand, shows results based on the variance between samples, where the lower value indicates less variation and, consequently, greater stability.24 The ΔCq method can be considered the most rigid testing each gene
stability by comparing its expression with all other genes, pair by pair. After obtaining a standard deviation for each comparison, this method obtains the average deviation for each gene. So, if ΔCq value between the pairs of genes remains constant for all tested samples, the smaller the deviation will be, and, consequently, the greater the stability of its expression.25

To obtain the final classification, RefFinder findings are based on rankings results of the more or less stable genes obtained by each method, designating an appropriate weight for an individual gene, and calculating the geometric mean of the weights. Consequently, average values of stability provided by each software can be obtained, favoring to analyze and perceive genes stability for different studied tissues.

Results

Amplification efficiency and reaction specificity

Considering regression coefficients ($R^2$) of the standard curve for the 10 reference genes, all were $>0.98$ and the amplification efficiencies were between 94 and 106%, revealing good linear correlation and relatively good specificity of the primers (Table 2).

Reference genes expression

High expression variability was observed between genes. In thigh muscle (Table 3), four genes were highly expressed (GAPDH, RPL5, TFRC, and EEF1) since they presented Cq values between 13 and 20. The other genes can be considered moderately expressed (Cq between 21 and 28).20 The MRPS30 (CV = 0.93%) and B2M (CV = 1.10%) genes showed low variance and dispersion; in contrast MRPS27 (CV = 3.50%) and LDHA (CV = 5.58%), genes showed high variance in this tissue. All genes were stable, according to literature,22 except LDHA (SD = 1.35).

Of eight genes evaluated on thigh muscle, three genes (GAPDH, TFRC, and EEF1) were highly expressed, with the lowest Cq values. The UBC gene was the least expressed (Cq $\geq 28$) among all others. LDHA, B2M, and UBC genes presented variation coefficients up to 2.5%, evidenced by low dispersion in broiler quails’ brain. Thus, considering standard deviation, only RPL5 ($SD = 1.28$) and HMBS ($SD = 1.01$) genes were shown to be unstable, and in this case can be observed that almost all evaluated genes showed moderate expression, with Cq values from 21 to 28. However, can be observed high expression variability in tissues. In this case of the brain, variability was quite heterogeneous where the gene with the least dispersion was the RPL5 (CV = 5.69%) with a high standard deviation ($SD = 1.18$); and in heart the EEF1 gene (CV = 18.91%) also having high standard deviation ($SD = 3.87$) (Table 3). The possibility of technical error for the EEF1 gene can be ruled out since the Cq values of the replicates were homogeneous within male/female tissues (Appendix).

In the spleen, seven genes were evaluated, and all of them showed moderate expression, except for GAPDH, which obtained Cq = 18.03, considered highly expressive. However, despite the expression, this gene was quite dispersed and unstable (CV = 9.06% and $SD = 1.64$) in the tissue (Table 3). Also, like the EEF1 gene evaluated in heart tissue, Cq values of replicates for the GAPDH gene were homogeneous within male/female, discarding any technical errors.

Expression stability by RefFinder

The general stability classification, assessed by tissue, obtained in the present research is shown in Table 4. According to this, the most stable genes in thigh muscle were MRPS30 and EEF1, followed by the HMBS gene; in brain, B2M, UBC, and GAPDH; in heart, MRPS30, TFRC, and HMBS, in that order. For spleen, the most stable genes identified were EEF1, LDHA, and HMBS.

Overall classification and sex effect

Sex effect was estimated through the $F$ test ($p < 0.05$), from the variability results generated in RefFinder for each reference gene on different tissues, and in male and female quails separately. Figures 1–4 show the overall classification of each gene expression variability, found in four tissues (thigh, brain, heart, and spleen) from male and female broiler quails, considering sex effect.

### Table 2. Characteristics of PCR amplification reactions for current studied genes.

| Gene symbol | PCR efficiency (%) | Slope | Regression coefficient ($R^2$) |
|-------------|--------------------|-------|-----------------------------|
| B2M         | 103.33             | -3.29 | 0.993                       |
| EEF1        | 102.75             | -3.25 | 0.990                       |
| GAPDH       | 94.75              | -3.44 | 0.995                       |
| HMBS        | 96.0%              | -3.41 | 0.995                       |
| LDHA        | 96.50%             | -3.40 | 0.996                       |
| MRPS27      | 101.67%            | -3.28 | 0.996                       |
| MRPS30      | 99.0%              | -3.35 | 0.997                       |
| RPL5        | 99.0%              | -3.34 | 0.997                       |
| TFRC        | 96.67%             | -3.40 | 0.998                       |
| UBC         | 106.0%             | -3.18 | 0.996                       |
Based on this classification, MRPS30, EEF1, and HMBS genes showed the most stable expression in thigh muscle; B2M, UBC, and GAPDH on brain; MRPS30 and TFRC followed by the HMBS on heart; and EEF1, LDHA, and HMBS on spleen of broiler quails.

**Discussion**

Can be perceived that the HMBS gene was one of the most stable in 75% of the evaluated tissues (thigh, heart, and spleen). Some studies evaluating HMBS gene stability, found this gene to be one of the most stable when considering several tissues in the same molecular analysis. Zhang,\(^{26}\) for example, evaluating the stability of eight reference genes in ten Boer goat tissues, found that the HMBS gene was the third most stable and, therefore, recommended to calibrate gene expression analyzes in goat tissues, deriving from this one, through RT-qPCR. In muscle tissue, HMBS gene was still found to be the most stable by Nascimento,\(^{20}\) in chickens’ Pectoralis major muscle. This is illustrated by the fact that the HMBS gene encodes hydroxymethylbilane synthase enzyme production. This enzyme is involved, also, in the production of a molecule called heme, considered vital for all organs of the body in both males and females. The use of the HMBS gene as an endogenous control for RT-qPCR analysis in various tissues of avian species has been reported and has shown positive results, due to its high expression and stability.\(^{27}\)

MRPS30 gene is a mitochondrial ribosomal protein encoded by nuclear genes and, with high activity in muscle tissues; hence, this protein synthesis is aided by this gene within the mitochondria. In addition, ribosomes where this gene is part of, consist of 75% of proteins for the composition of rRNA and, therefore, is abundant in cells and their expression can be considered continuous. For this reason, it is believed that such genes were shown to be stable in this study, as they are continually required in cells as part of processes essential to their maintenance.

### Table 3. Descriptive statistics and expression levels of reference genes in broiler quails at 35 days tested in four tissues (thigh, brain, heart, and spleen), obtained from the bestkeeper (n = 6) software.

| Gene symbol | Geo. Ave. (Cq) | Ari. Ave. (Cq) | Min. (Cq) | Max. (Cq) | SD (±Cq) | CV (% Cq) |
|-------------|----------------|----------------|-----------|-----------|----------|-----------|
| Thigh       |                |                |           |           |          |           |
| GAPDH       | 17.11          | 17.12          | 16.43     | 17.82     | 0.50     | 2.94      |
| RPL5        | 16.38          | 16.39          | 15.86     | 17.02     | 0.38     | 2.34      |
| MRPS27      | 28.39          | 28.41          | 27.28     | 29.56     | 0.99     | 3.50      |
| MRPS30      | 23.62          | 23.62          | 23.28     | 23.90     | 0.22     | 0.93      |
| TFRC        | 16.53          | 16.54          | 15.82     | 17.11     | 0.45     | 2.71      |
| HMBS        | 23.17          | 23.18          | 22.45     | 23.59     | 0.33     | 1.43      |
| EEF1        | 16.74          | 16.75          | 16.24     | 17.09     | 0.24     | 1.41      |
| LDHA        | 24.10          | 24.16          | 22.42     | 27.84     | 1.35     | 5.58      |
| B2M         | 26.27          | 26.27          | 25.76     | 26.70     | 0.29     | 1.10      |
| Brain       |                |                |           |           |          |           |
| GAPDH       | 16.00          | 16.01          | 15.47     | 16.62     | 0.44     | 2.73      |
| RPL5        | 22.42          | 22.46          | 20.28     | 24.64     | 1.28     | 5.69      |
| TFRC        | 16.68          | 16.71          | 15.62     | 17.80     | 0.79     | 4.71      |
| HMBS        | 27.62          | 27.64          | 26.27     | 29.13     | 1.01     | 3.64      |
| EEF1        | 18.04          | 18.05          | 17.11     | 18.66     | 0.52     | 2.90      |
| LDHA        | 24.00          | 24.01          | 22.88     | 24.84     | 0.61     | 2.61      |
| B2M         | 26.94          | 26.94          | 26.26     | 27.72     | 0.37     | 1.38      |
| UBC         | 29.27          | 29.27          | 28.70     | 29.86     | 0.38     | 1.30      |
| Heart       |                |                |           |           |          |           |
| GAPDH       | 19.21          | 19.22          | 18.58     | 19.89     | 0.52     | 2.70      |
| RPL5        | 26.16          | 26.18          | 24.19     | 27.57     | 0.95     | 3.62      |
| MRPS27      | 22.99          | 23.00          | 22.20     | 23.75     | 0.57     | 2.48      |
| MRPS30      | 25.46          | 25.46          | 25.17     | 25.73     | 0.17     | 0.67      |
| TFRC        | 26.31          | 26.31          | 26.14     | 26.50     | 0.13     | 0.49      |
| HMBS        | 27.71          | 27.72          | 27.31     | 28.20     | 0.38     | 1.38      |
| EEF1        | 20.09          | 20.46          | 16.47     | 24.50     | 3.87     | 18.91     |
| LDHA        | 25.72          | 25.73          | 24.69     | 26.55     | 0.53     | 2.05      |
| B2M         | 22.64          | 22.66          | 21.35     | 23.93     | 0.97     | 4.29      |
| Spleen      |                |                |           |           |          |           |
| GAPDH       | 18.03          | 18.11          | 15.90     | 20.22     | 1.64     | 9.06      |
| RPL5        | 26.78          | 26.79          | 25.31     | 28.09     | 0.70     | 2.62      |
| MRPS27      | 25.49          | 25.50          | 25.26     | 25.85     | 0.18     | 0.69      |
| MRPS30      | 25.24          | 25.28          | 23.79     | 26.89     | 1.35     | 5.33      |
| HMBS        | 27.08          | 27.08          | 26.53     | 27.73     | 0.41     | 1.52      |
| EEF1        | 24.86          | 24.87          | 24.29     | 25.62     | 0.39     | 1.58      |
| LDHA        | 25.63          | 25.64          | 24.99     | 26.35     | 0.47     | 1.85      |

Cq: quantification cycle; Geo. Ave.: Cq geometric mean; Ari. Ave.: Cq arithmetic average; Cq Min and Max: Cq minimum and maximum values; SD: standard deviation; CV: coefficient of variation.
Table 4. Stability values, algorithmic, and general classification for reference genes in four tissues, evaluated in male and female broiler quails.

| Gene symbol | BestKeeper | GeNorm | NormFinder | ∆Cq | Overall classification |
|-------------|------------|--------|------------|-----|------------------------|
| Thigh       |            |        |            |     |                        |
| GAPDH       | 0.503      | 0.481  | 1.978      | 0.850 | 6                      |
| RPL5        | 0.383      | 0.310 (2) | 0.433      | 0.810 | 5                      |
| MRPS27      | 0.993      | 0.727  | 1.389      | 1.500 | 8                      |
| MRPS30      | 0.220 (1)  | 0.228 (1) | 0.189 (2)  | 0.730 (1) | 1          |
| TFRC        | 0.449      | 0.531  | 0.402      | 0.890 | 7                      |
| HMBS        | 0.331      | 0.228 (1) | 0.298 (3)  | 0.780 (3) | 3          |
| EEF1        | 0.237 (2)  | 0.399 (3) | 0.136 (1)  | 0.750 (2) | 2          |
| LDHA        | 1.348      | 1.017  | 1.978      | 2.030 | 9                      |
| B2M         | 0.290 (3)  | 0.424  | 0.377      | 0.810 | 4                      |
| Brain       |            |        |            |     |                        |
| GAPDH       | 0.437 (3)  | 0.247 (1) | 0.648 (3)  | 1.040 (3) | 3          |
| RPL5        | 1.278      | 1.210  | 1.555      | 1.750 | 8                      |
| TFRC        | 0.787      | 0.366 (2) | 1.169      | 1.330 | 6                      |
| HMBS        | 1.005      | 1.031  | 1.218      | 1.410 | 7                      |
| EEF1        | 0.523      | 0.247 (1) | 0.799      | 1.100 | 4                      |
| LDHA        | 0.610      | 0.901  | 0.708      | 1.100 | 5                      |
| B2M         | 0.371 (1)  | 0.718 (3) | 0.240 (1)  | 0.970 (2) | 1          |
| UBC         | 0.381 (2)  | 0.822  | 0.370 (2)  | 0.970 (1) | 2          |
| Heart       |            |        |            |     |                        |
| GAPDH       | 0.518      | 0.492 (3) | 0.804      | 1.280 (3) | 4          |
| RPL5        | 0.948      | 0.949  | 1.037      | 1.770 | 8                      |
| MRPS27      | 0.570      | 0.548  | 0.918      | 1.360 | 6                      |
| MRPS30      | 0.170 (2)  | 0.258 (1) | 0.074 (1)  | 1.190 (1) | 1          |
| TFRC        | 0.130 (1)  | 0.258 (1) | 0.129 (2)  | 1.200 (2) | 2          |
| HMBS        | 0.383 (3)  | 0.423 (2) | 0.240 (3)  | 1.200 (2) | 3          |
| EEF1        | 3.870      | 1.729  | 4.425      | 4.460 | 9                      |
| LDHA        | 0.528      | 0.785  | 0.295      | 1.420 | 5                      |
| B2M         | 0.973      | 0.670  | 1.476      | 1.680 | 7                      |
| Spleen      |            |        |            |     |                        |
| GAPDH       | 1.640      | 1.019  | 1.873      | 1.965 | 6                      |
| RPL5        | 0.702      | 0.678  | 0.862      | 1.286 | 5                      |
| MRPS27      | 0.177 (1)  | 0.518 (3) | 0.270      | 1.011 | 4                      |
| MRPS30      | 1.348      | 1.324  | 2.001      | 2.085 | 7                      |
| HMBS        | 0.412 (3)  | 0.461 (2) | 0.237 (3)  | 0.959 (2) | 3          |
| EEF1        | 0.393 (2)  | 0.335 (1) | 0.168 (2)  | 0.958 (1) | 1          |
| LDHA        | 0.473      | 0.335 (1) | 0.168 (1)  | 1.001 (3) | 2          |

Classification of genes are the values into parenthesis.

Figure 1. Expression variability of reference genes evaluated in thigh muscle from broiler quails, within and between male and female individuals. a,bDifferent lowercase letters were significantly different ($p < 0.05$).
EEF1 gene encodes a protein responsible for the alpha-1 elongation factor, responsible for the enzymatic delivery of aminoacyl tRNAs to the ribosome during protein synthesis. This is also an FES-1 alpha subunit complex isoform, GTPase, and is involved with protein actin, in proteolysis.28,29 B2M gene, on the other hand, encodes a whey protein found in association with the largest heavy chain class I histocompatibility complex (MHC) on the cell surface of all nucleated cells. In brain tissue, ubiquitin (UBC) also acts, which is necessary for the proper creation, maintenance, and disassembly of protein subdomains within the neuron, being its function predominant throughout the life of this cell.30 Thus, all genes indicated as more stable in the current four tissues studied executing, on large scale, on cell survival and metabolism, with relatively little participation in only one of the sexes’ exclusive routes; for this reason, they

**Figure 2.** Expression variability of reference genes evaluated in brain from broiler quails, within and between male and female individuals. a,bDifferent lowercase letters were significantly different (p < 0.05).

**Figure 3.** Expression variability of reference genes evaluated in heart from broiler quails, within and between male and female individuals. a,bDifferent lowercase letters were significantly different (p < 0.05).
can be recommended for genic expression normalization in both male and female quails.

In the present study, the genes MRPS27 and LDHA in thigh muscle; RPL5 in brain, EEF1 in heart, and MRPS30 in spleen were considered unstable, and therefore, not recommended as a reference, when intended to analyze broiler quails gene expression in both sexes. However, due to their high coefficients of variation and deviation, the sex effect could be seen, enabling them to be recommended as reference genes when only one sex is being analyzed. In thigh muscle, MRPS27 protein, as the MRPS30, is encoded by nuclear genes with high activity in muscle tissues. However, besides its high expression, be stable and indispensable to mitochondrial cells; a difference in its production levels can be noted, due to differential protein metabolism between males and females, especially, in thigh muscle. This may explain to recommend this gene as an internal control for RT-qPCR analysis when intended to study muscle tissues of both male and female quails, which were analyzed separately.

For brain tissue, the present research indicates the GAPDH gene as a candidate for reference gene to standardize the expression of only from males, from females, or from males and females samples together. This gene has become one of the most used as a reference to standardize the expression of several samples in various animal species, due to the fact that Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is directly involved in metabolic and non-metabolic processes in the cell. This enzyme catalyzes the sixth step of glycolysis, such as glucose breakdown, activation of transcription, initiation of apoptosis, transport of ER vesicles to Golgi, and axonal transport rapid or axoplasmic; however, its expression varies greatly between individuals and in different stages of development. In avian species brain tissue, for example, GAPDH was used as endogenous control along with other genes, their expression in this tissue was stable and effective. In fact, in molecular studies seeking to evaluate the genic expression of sexually dimorphic genes related to gonadal differentiation in chickens’ brain tissue, the GAPDH gene is the one that showed the best results as a reference gene, according to the results obtained in the present study.

In the present research, the TFRC gene was the most stable and expressed in male quails heart. This gene encodes transferrin receptor Protein 1 responsible for cellular iron absorption. In healthy animals, male or female, both iron uptake and TFRC expression in heart tissue are stable, since this gene participates in cells’ oxidative phosphorylation. The deletion of this gene in cardiomyocytes is the origin of coronary problems and could motivate lethal cardiomyopathy.

In male and female quail spleen tissue, the EEF1 gene was the most stable when compared to the others. Genes expressed invariably in this tissue are related to immune response, independent of animal sex. In this case, when evaluating its genic expression in both sexes with normal health conditions, as accomplished in the current work, it is possible to recommend the use of the EEF1 gene as endogenous control of spleen analyzes involving, for example, T cells.
The present study was capable to identify stable genes than can be used in further genic expression studies, being necessary to evaluate both sexes and different quail tissues. The differences found between tissues showed variability, as an important factor to be analyzed, and must be considered in any experimental process. For some genes, the sex effect can be noticed suggesting, therefore, that every analysis can be performed to clarify differential expression found for contrasting tissues and sexes. Studies evaluating genetic behavior in animals without distinction of sex are important and should be subject to constant improvements. Due to research scarcity related to molecular genetics in quails, the present study serves as an informational basis to continue research on gene expression in this species. In addition, also can be a basis for investigations of the same family animals, such as chickens and partridges for example, and consequently, promotes improvements in the entire production chain.

Conclusion

Based on algorithms BestKeeper, GeNorm, NormFinder, and ΔCq; the genes MRPS30, EEF1, and HMBS in thigh muscle; B2M, UBC, and GAPDH in brain; MRPS30, TFRC, and HMBS in heart; and, EEF1, LDHA, and HMBS in spleen tissues; are the most stable and, therefore, can be recommended to be used as an endogenous control in further gene expression studies related to male and female broiler quails.

Author contributions

CSN, KRSS, APDV, and LTB: designed the experiment. FCBS, MSM, and IRSO: performed the experiment. FCBS, MSM, CSN, and LTB: analyzed and interpreted the data. MSM and LTB: wrote the paper. APDV, CSN, and KRSS: contributed reagents and critical revision of the manuscript for important intellectual content.

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Appendix

1. Values of Cq (quantification cycle) obtained from the amplification reaction in quantitative PCR in real time, for the reference gene GAPDH in different tissues of broiler quails.

| Replicate | Sex     | Thigh | Brain | Heart | Spleen |
|-----------|---------|-------|-------|-------|--------|
| 1         | Male    | 16.19 | 15.33 | 19.68 | 15.78  |
| 1         | Male    | 16.67 | 15.61 | 19.60 | 16.02  |
| 2         | Male    | 16.85 | 15.64 | 20.11 | 16.80  |
| 2         | Male    | 16.20 | 15.32 | 19.68 | 16.55  |
| 3         | Male    | 16.88 | 15.69 | 19.98 | 16.73  |
| 3         | Male    | 16.94 | 15.84 | 19.36 | 16.94  |
| 4         | Female  | 17.24 | 16.01 | 19.07 | 19.32  |
| 4         | Female  | 17.65 | 16.79 | 18.65 | 19.49  |
| 5         | Female  | 17.43 | 16.81 | 18.48 | 19.56  |
| 5         | Female  | 17.80 | 16.44 | 18.69 | 20.89  |
| 6         | Female  | 17.79 | 16.05 | 18.71 | 19.36  |
| 6         | Female  | 17.84 | 16.59 | 18.59 | 19.88  |

2. Values of Cq (quantification cycle) obtained from the amplification reaction in quantitative PCR in real time, for the reference gene RPL5 evaluated in different tissues of broiler quails.

| Replicate | Sex     | Thigh | Brain | Heart | Spleen |
|-----------|---------|-------|-------|-------|--------|
| 1         | Male    | 15.65 | 21.33 | 24.16 | 27.75  |
| 1         | Male    | 16.63 | 21.36 | 24.22 | 28.43  |
| 2         | Male    | 16.36 | 23.08 | 25.79 | 27.16  |
| 2         | Male    | 15.94 | 23.19 | 25.83 | 25.60  |
| 3         | Male    | 17.40 | 24.46 | 27.52 | 25.19  |
| 3         | Male    | 16.42 | 24.82 | 27.63 | 25.43  |
| 4         | Female  | 16.50 | 20.04 | 27.35 | 27.65  |
| 4         | Female  | 16.01 | 20.53 | 25.27 | 27.05  |
| 5         | Female  | 16.99 | 21.92 | 28.98 | 27.25  |
| 5         | Female  | 17.04 | 21.95 | 26.03 | 26.83  |
| 6         | Female  | 16.00 | 23.23 | 26.26 | 26.33  |
| 6         | Female  | 15.72 | 23.68 | 25.13 | 26.83  |

3. Values of Cq (quantification cycle) obtained from the amplification reaction in quantitative PCR in real time, for the reference gene MRPS27 evaluated in different tissues of broiler quails.

| Replicate | Sex     | Thigh | Brain | Heart | Spleen |
|-----------|---------|-------|-------|-------|--------|
| 1         | Male    | 29.62 | –     | 23.75 | 25.38  |
| 1         | Male    | 29.50 | –     | 23.58 | 25.44  |
| 2         | Male    | 29.06 | –     | 23.44 | 25.81  |
| 2         | Male    | 29.35 | –     | 23.12 | 25.89  |
| 3         | Male    | 29.22 | –     | 23.72 | 25.24  |
| 3         | Male    | 29.66 | –     | 23.78 | 25.61  |
| 4         | Female  | 27.45 | –     | 22.14 | 25.85  |
| 4         | Female  | 27.53 | –     | 22.25 | 25.48  |
| 5         | Female  | 27.94 | –     | 22.73 | 25.50  |
| 5         | Female  | 27.01 | –     | 22.86 | 25.20  |
| 6         | Female  | 27.26 | –     | 22.27 | 25.30  |
| 6         | Female  | 27.30 | –     | 22.28 | 25.21  |

–: tissue not used in gene evaluation.
4. Values of Cq (quantification cycle) obtained from the amplification reaction in quantitative PCR in real time, for the reference gene MRPS30 evaluated in different tissues of broiler quails.

| Replicate | Sex   | Thigh | Brain | Heart | Spleen |
|-----------|-------|-------|-------|-------|--------|
| 1         | Male  | 23.61 | –     | 25.58 | 26.04  |
| 1         | Male  | 23.84 | –     | 25.74 | 26.47  |
| 2         | Male  | 23.54 | –     | 25.55 | 26.90  |
| 2         | Male  | 23.06 | –     | 25.91 | 26.56  |
| 3         | Male  | 23.97 | –     | 25.48 | 26.86  |
| 3         | Male  | 23.82 | –     | 25.26 | 26.91  |
| 4         | Female| 23.75 | –     | 25.21 | 23.67  |
| 4         | Female| 23.68 | –     | 25.47 | 23.95  |
| 5         | Female| 23.72 | –     | 25.01 | 24.01  |
| 5         | Female| 23.85 | –     | 25.33 | 24.37  |
| 6         | Female| 23.31 | –     | 25.37 | 23.72  |
| 6         | Female| 23.25 | –     | 25.65 | 23.85  |

`: tissue not used in gene evaluation

5. Values of Cq (quantification cycle) obtained from the amplification reaction in quantitative PCR in real time, for the reference gene TFRC evaluated in different tissues of broiler quails.

| Replicate | Sex   | Thigh | Brain | Heart | Spleen |
|-----------|-------|-------|-------|-------|--------|
| 1         | Male  | 16.59 | 15.90 | 26.86 | –      |
| 1         | Male  | 16.78 | 15.33 | 25.97 | –      |
| 2         | Male  | 16.02 | 15.61 | 25.65 | –      |
| 2         | Male  | 15.80 | 15.64 | 26.63 | –      |
| 3         | Male  | 16.55 | 16.32 | 26.36 | –      |
| 3         | Male  | 16.73 | 16.69 | 25.94 | –      |
| 4         | Female| 15.94 | 17.84 | 26.40 | –      |
| 4         | Female| 15.70 | 17.01 | 26.42 | –      |
| 5         | Female| 17.94 | 17.79 | 26.50 | –      |
| 5         | Female| 16.17 | 17.81 | 26.01 | –      |
| 6         | Female| 17.18 | 17.44 | 26.99 | –      |
| 6         | Female| 17.04 | 17.05 | 26.00 | –      |

`: tissue not used in gene evaluation

6. Values of Cq (quantification cycle) obtained from the amplification reaction in quantitative PCR in real time, for the reference gene HMBS evaluated in different tissues of broiler quails.

| Replicate | Sex   | Thigh | Brain | Heart | Spleen |
|-----------|-------|-------|-------|-------|--------|
| 1         | Male  | 23.23 | 29.49 | 27.35 | 27.25  |
| 1         | Male  | 23.12 | 29.56 | 27.27 | 27.65  |
| 2         | Male  | 22.58 | 28.89 | 27.98 | 26.83  |
| 2         | Male  | 22.32 | 29.36 | 28.03 | 26.33  |
| 3         | Male  | 23.42 | 28.88 | 28.26 | 26.83  |
| 3         | Male  | 23.75 | 26.67 | 28.13 | 26.22  |
| 4         | Female| 23.58 | 26.41 | 27.75 | 27.61  |
| 4         | Female| 23.44 | 26.13 | 28.43 | 27.84  |
| 5         | Female| 23.12 | 26.94 | 27.16 | 27.54  |
| 5         | Female| 23.72 | 26.91 | 27.60 | 27.06  |
| 6         | Female| 22.78 | 26.87 | 27.19 | 26.97  |
| 6         | Female| 23.03 | 26.55 | 27.43 | 26.82  |

`: tissue not used in gene evaluation
7. Values of Cq (quantification cycle) obtained from the amplification reaction in quantitative PCR in real time, for the reference gene EEF1 evaluated in different tissues of broiler quails.

| Replicate | Sex | Thigh   | Brain   | Heart   | Spleen |
|-----------|-----|---------|---------|---------|--------|
| 1         | Male| 16.28   | 17.94   | 16.84   | 24.72  |
| 1         | Male| 16.19   | 17.17   | 16.53   | 23.85  |
| 2         | Male| 16.67   | 17.18   | 16.87   | 24.20  |
| 3         | Male| 16.85   | 17.04   | 16.07   | 24.80  |
| 3         | Male| 16.20   | 17.86   | 16.33   | 24.73  |
| 3         | Male| 16.88   | 17.97   | 16.90   | 24.66  |
| 4         | Female| 16.94 | 18.71   | 24.47   | 25.71  |
| 4         | Female| 17.24 | 18.39   | 24.53   | 25.52  |
| 4         | Female| 17.65 | 18.47   | 24.67   | 25.10  |
| 5         | Female| 16.43 | 18.56   | 23.95   | 25.47  |
| 6         | Female| 16.80 | 18.77   | 24.01   | 24.93  |
| 6         | Female| 16.79 | 18.54   | 24.37   | 24.64  |

8. Values of Cq (quantification cycle) obtained from the amplification reaction in quantitative PCR in real time, for the reference gene LDHA evaluated in different tissues of broiler quails.

| Replicate | Sex | Thigh   | Brain   | Heart   | Spleen |
|-----------|-----|---------|---------|---------|--------|
| 1         | Male| 22.32   | 24.62   | 25.22   | 25.48  |
| 1         | Male| 22.52   | 24.71   | 25.45   | 24.50  |
| 2         | Male| 23.08   | 24.77   | 24.94   | 25.20  |
| 2         | Male| 22.99   | 24.90   | 24.44   | 25.30  |
| 3         | Male| 23.66   | 24.51   | 25.58   | 25.45  |
| 3         | Male| 23.83   | 24.20   | 25.55   | 25.05  |
| 4         | Female| 24.79 | 23.94   | 26.61   | 26.86  |
| 4         | Female| 24.26 | 23.63   | 26.48   | 25.83  |
| 5         | Female| 23.27 | 23.49   | 26.21   | 26.00  |
| 5         | Female| 23.52 | 23.58   | 26.01   | 25.54  |
| 6         | Female| 29.62 | 22.71   | 26.37   | 25.46  |
| 6         | Female| 26.06 | 23.05   | 25.85   | 26.95  |

9. Values of Cq (quantification cycle) obtained from the amplification reaction in quantitative PCR in real time, for the B2M reference gene evaluated in different tissues of broiler quails.

| Replicate | Sex | Thigh   | Brain   | Heart   | Spleen |
|-----------|-----|---------|---------|---------|--------|
| 1         | Male| 26.10   | 27.75   | 23.41   | –      |
| 1         | Male| 25.99   | 27.68   | 23.13   | –      |
| 2         | Male| 26.16   | 26.72   | 23.94   | –      |
| 2         | Male| 26.62   | 26.85   | 23.91   | –      |
| 3         | Male| 25.66   | 27.31   | 23.87   | –      |
| 3         | Male| 25.86   | 27.25   | 23.55   | –      |
| 4         | Female| 26.85 | 26.04   | 22.47   | –      |
| 4         | Female| 26.55 | 26.47   | 22.23   | –      |
| 5         | Female| 26.62 | 26.90   | 21.12   | –      |
| 5         | Female| 26.57 | 26.56   | 21.58   | –      |
| 6         | Female| 25.98 | 26.86   | 21.32   | –      |
| 6         | Female| 26.3   | 26.91   | 21.42   | –      |

*: tissue not used in gene evaluation
10. Values of Cq (quantification cycle) obtained from the amplification reaction in quantitative PCR in real time, for the reference gene UBC evaluated in different tissues of broiler quails.

| Replicate | Sex   | Thigh | Brain | Heart | Spleen |
|-----------|-------|-------|-------|-------|--------|
| 1         | Male  | –     | 29.60 | –     | –      |
| 2         | Male  | –     | 29.68 | –     | –      |
| 3         | Male  | –     | 29.98 | –     | –      |
| 4         | Female| –     | 29.36 | –     | –      |
| 5         | Female| –     | 29.07 | –     | –      |
| 6         | Female| –     | 29.65 | –     | –      |
| 7         | Female| –     | 28.48 | –     | –      |
| 8         | Female| –     | 28.69 | –     | –      |
| 9         | Female| –     | 28.71 | –     | –      |
| 10        | Female| –     | 28.59 | –     | –      |
| 11        | Female| –     | 29.32 | –     | –      |

--: tissue not used in gene evaluation