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RESEARCH ARTICLE

DETERMINATION OF APPROPRIATE ENDOGENOUS REFERENCE GENES FOR RT-QPCR ANALYSIS IN SYRIAN (GOLDEN) HAMSTERS AND MONGOLIAN GERBILS

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ABSTRACT

Purpose: The use of hamsters and gerbils has increased significantly in a variety of fields, including biological rhythms, reproductive biology, immunology, oncology, and many others.
Material and Methods: The most stable genes in Syrian hamsters (*Mesocricetus auratus*) and Mongolian gerbils (*Meriones unguiculatus*) were assessed using 32 reference genes for normalization in RT-qPCR analysis. Adrenal, cerebral cortex, heart, hypothalamus, kidney, liver, lung and testis tissues were used to extract and purify RNAs. GeNorm was used to determine the gene expression stabilities of 14 candidate endogenous genes from each tissue that was compatible for both animals.
Results: Under our experimental conditions, we discovered that two endogenous genes are adequate for each tissue to perform RT-qPCR normalization. There were differences in the most stable genes between species and tissues.
Conclusion: We suggest that combinations of endogenous genes ought to be carefully chosen under various experimental circumstances.

Keywords: Hamsters, gerbils, reference genes, endogenous genes, qPCR

INTRODUCTION

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) has been used for measuring nucleic acids (1). A powerful method for quantifying nucleotide-based molecular studies is RT-qPCR (2). A common technique for data normalization in gene expression studies is the use of expressed reference genes as endogenous controls (3). The guidelines of “Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE)” indicate quantitative real-time PCR optimization methodologies (4).

RT-qPCR analyzes the comparison between reference genes and target genes to determine the level of relative gene expression (5). Gapdh, Actb, and 18S are frequently used universal targets for RT-qPCR, but their expression levels vary depending on the tissue. Additionally, a reference gene that is stable in one organism may be unstable in another, which may change the target gene's expression level (6). It is argued that the transcription level of universal reference genes may differ in organisms, tissues, or cell types (7,8). Algorithms such as GeNorm and NormFinder are commonly used for evaluating the stability of reference genes (9,10).

Syrian hamsters (*Mesocricetus auratus*) have advantageous features and are extensively used as animal models for studies in photoperiodism (11-14),

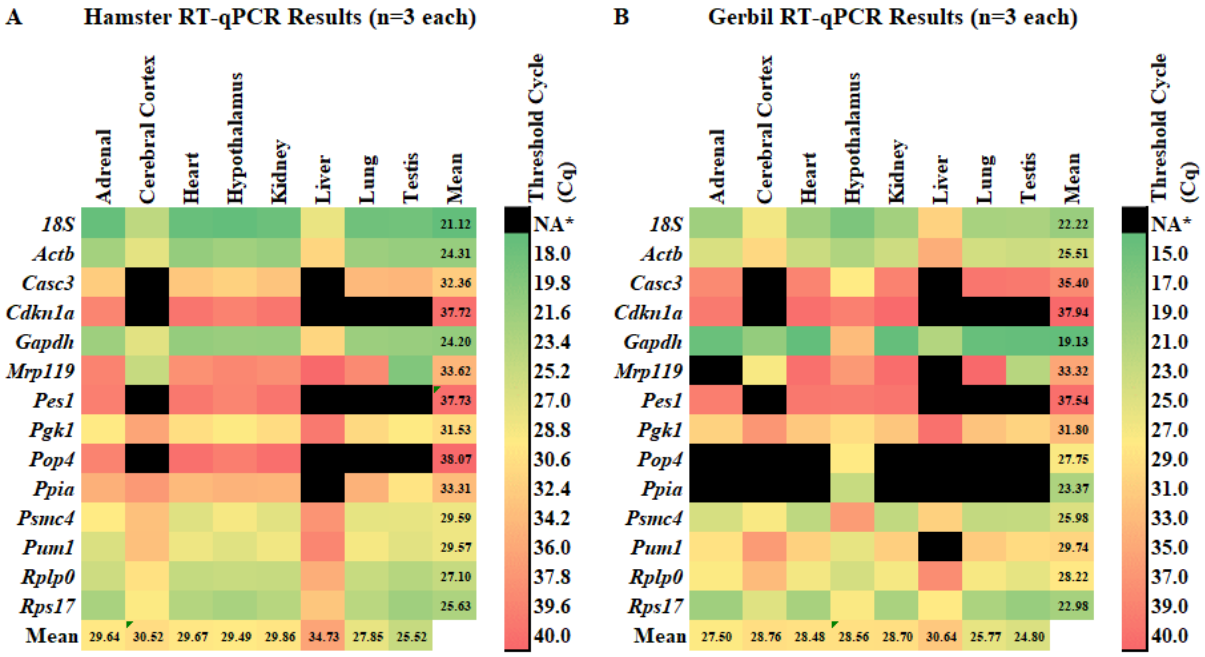

Figure 1. Heat map displays Cq values for hamster and gerbil tissues

reproduction (15,16), offspring development (17-20], infectious disease (21,22) and cancer (23). Another species used as an animal model for studies on reproduction (24,25), behavior (26-28), auditory (29,30), and inflammation (31,32) is the Mongolian gerbil (*Meriones unguiculatus*).

The limited availability of reagents for analyzing gene expression in hamsters and gerbils precludes the evaluation of transcriptional changes in physiological interactions in experimental studies of these species. In the present study, optimization of the TaqMan probe for 32 reference genes in hamster and gerbil hypothalamus was performed with RT-qPCR application. The stability rank of 14 reference genes in the adrenal, cerebral cortex, heart, hypothalamus, kidney, liver, lung, and testis was determined after analyzing RT-qPCR results with GeNorm. To properly use reference genes in future research, it was intended to identify an effective endogenous gene required for normalization in Syrian hamsters and Mongolian gerbils using RT-qPCR.

MATERIAL AND METHODS

Animals

The experiments were conducted following the ARRIVE guidelines (https://arriveguidelines.org) and guidelines of Canakkale Onsekiz Mart University and approved by the Ethical Council of Animal Research (Date: 13.04.2011, No: 2011/04-01). The study was conducted on two species (Syrian hamster and Mongolian gerbil). The 5 male adult Syrian hamsters used in the study weighed around 100 g and were 3-4 months old. Similarly, the 5 male adult Mongolian gerbils used in the study weighed around 90 g and were 3-4 months old. There were no problems with the animals' health during the 4-week study period, so no animals were removed or added to the study. Animals were reared from birth on long photoperiod 14 L (14 h light, 10 h dark; lights off at 20:00 h) in plastic cages (16 × 31 × 42 cm) with pine shaving bedding. The lighting system was provided by cool-white, fluorescent tubes (200 lux) controlled by automated and programmable timers. Temperature of 22 ± 2°C and relative humidity of 50 ± 5% in air-ventilated rooms were provided to animals. Animals had unlimited access to water and, unless stated otherwise in the experiments, were fed (Purina Rat Chow, catalog no. 5008: fat, 17 kcal%; carbohydrate, 56 kcal%; protein, 27 kcal%) ad libitum on a 14:10 light-dark cycle. Photoperiod was provided to hamsters and gerbils both before and during the 4-week experiment. Animals were decapitated at the end of the experiment, and tissue samples were collected. As an outcome measure stability of expression in reference genes were assessed.

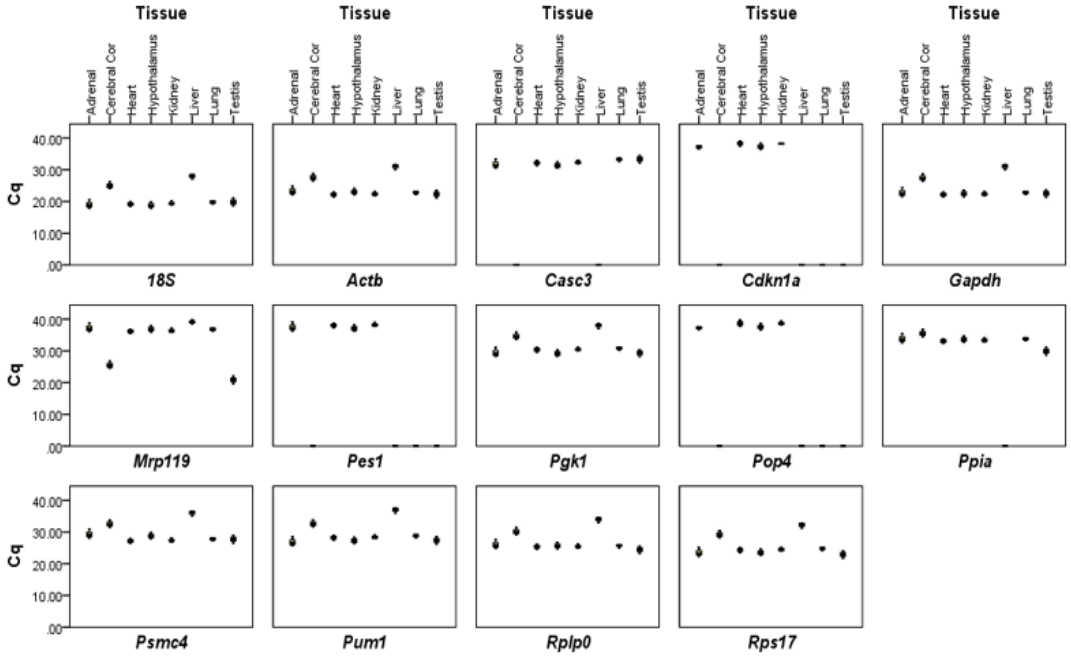

Figure 2. A boxplot chart for Cq values in hamster tissues

Tissue Collection

All animals were sacrificed by decapitation at noon (12:00 h). Immediately upon decapitation, the tissues of the adrenal cortex, cerebral cortex, heart, hypothalamus, kidney, liver, lung and testis were carefully removed without any deterioration. Tissues were immediately frozen in liquid nitrogen and stored at -80°C until analysis.

Isolation of RNA and Reverse Transcription

At the end of four weeks of ad libitum feeding, all RNAs from the adrenal cortex, cerebral cortex, heart, hypothalamus, kidney, liver, lung, and testis tissues were extracted and purified using RNA Mini Kit (PureLink) by adding DNase I (Invitrogen) in accordance with the instructions of the relevant kit. Pooling was used for each tissue and 3 randomly selected samples were studied. The samples were stored at -80ºC for a maximum of four weeks immediately after the measurement of their concentration, quality, and purity in the NanoDrop spectrophotometer (Thermo Fisher Scientific, MA, USA). In the analysis, samples with good concentration were used. Reverse transcription was performed according to the recommended procedure using the High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems™). In the first step, approximately 2 µg of total RNA was added to the 20 µl reaction mixture. cDNA synthesis was carried out as in the respective steps: 37°C for 60 min, 95°C for 5 min, and 4°C for 25 min.

Quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-qPCR)

RT-qPCR was performed on the StepOne instrument to measure quantitative mRNA (Applied Biosystems). Real Time PCR was performed in triplicate. In this study, there were two different approaches used. The first approach aims to understand 32 different gene probes as endogenous controls in the hypothalamus of hamsters and gerbils using a TaqMan Array Rat Endogenous Control Plate (Applied Biosystems). The second approach aims at understanding the potential of gene probes as endogenous controls in various tissues. For the second approach, gene probes are chosen based on how well they can hybridize with both animals.

To better understand their potential as endogenous genes in hamsters and gerbils, TaqMan probes for sixteen commonly used rat reference genes and sixteen human gene orthologs in rats were displayed. For each reaction, a 1:100 dilution of cDNA was applied. The reaction tube contained 5 µl of TaqMan probe master mix, 3.5 µl of RNase-free water, 1 µl of cDNA, and 0.5 µl of TaqMan probe. The reaction was carried out on a StepOne instrument (Applied Biosystems) in the following steps: 2 min. at 50°C, 10

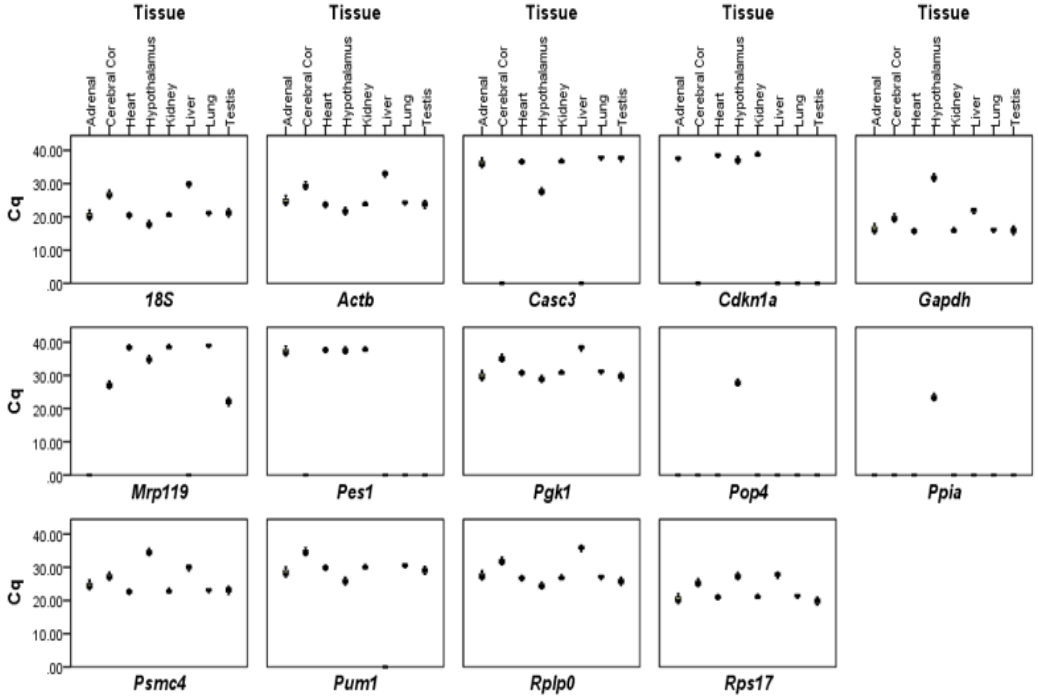

Figure 3. A boxplot chart for Cq values in gerbil tissues

min. at 95°C; 40 cycles of 15 sec. at 95°C, and 1 min. at 60°C.

Data Analysis

GeNorm (qbase+, Biogazelle) is used to determine the stability of gene expression in 14 of 32 reference genes. The mean RT-qPCR Cq value was used to determine the stability of mRNA gene expression in reference genes. GeNorm qbase+ determined the median reference stability measurement (M). The average GeNorm M value should be less than 1.5. V = 0.15 is the cutoff value for binary variation (10).

RESULTS

32 endogenous gene probes are listed in Supplementary Table 1. All gene probes were tested for their ability to be used as an endogenous control in hamsters and gerbils. While 14 of the gene probes produced a PCR product in both cases (*18S, Actb, Casc3, Cdkn1a, Gapdh, Mrpl19, Pes1, Pgk1, Pop4, Ppia, Psmc4, Pum1, Rplp0, Rps17*) 5 produced a product only in hamsters (*Gusb, Hmbs, Ppib, Rplp2, Tbp*) 5 produced a product only in gerbils (*Cdkn1b, Gadd45a, Hprt1, Rpl30, Tfrc*) and 8 produced no product at all (*Abl1, B2m, Eif2b1, Elf1, Mtatp6, Rpl37a, Ubc, Ywhaz*). Probes that produced PCR products in both species were used for further analyses in different tissues.

The heat map and boxplot charts were created for Cq values following the testing of 14 endogenous gene probes for male hamsters and gerbils on 8 different tissues (adrenal cortex, cerebral cortex, heart, hypothalamus, kidney, liver, lung and testis) using RT-qPCR (triplicate) (Figure 1, 2 and 3). All RT-qPCR reactions gave a single peak following melting curve analysis.

On male hamsters (Table 1) and male gerbils (Table 2), the average expression stability values (GeNorm M) of 14 endogenous genes are ranked from most stable (lowest GeNorm M) to least stable. To determine the optimal number of reference genes for normalization, pairwise variation (Vn/Vn+1) was used. As demonstrated, two control genes were sufficient to normalize eight tissues (Figure 4).

DISCUSSION

In this study, we evaluated the stability of gene expression in Syrian hamsters and Mongolian gerbils for 32 candidate reference genes. We demonstrated the optimal endogenous gene combination for use in 8 various tissues across two species. Additionally, the Mongolian gerbil was used as the first test subject to assess endogenous reference genes for normalization in RT-qPCR.

We evaluated mean Cq values in the hypothalamus of hamsters and gerbils. The most prevalent

Table 1. GeNorm was used to rank reference genes on hamster tissues from most stable to least stable

Ranking[a]	Adrenal	Cerebral Cortex	Heart	Hypothalamus	Kidney	Liver	Lung	Testis
1	***Psmc4***	***Rplp0***	***Pgk1***	***Pgk1***	***Casc3***	***Actb***	***Rplp0***	***Rplp0***
2	***Pgk1***	***Rps17***	***Psmc4***	***Psmc4***	***Pgk1***	***Rplp0***	***Pgk1***	***Rps17***
3	*Casc3*	*Actb*	*Pum1*	*Casc3*	*Ppia*	*Gapdh*	*Psmc4*	*Actb*
4	*Pum1*	*Gapdh*	*Rplp0*	*Pum1*	*Psmc4*	*Psmc4*	*Pum1*	*Gapdh*
5	*Rplp0*	*Psmc4*	*Casc3*	*Rplp0*	*Pum1*	*Pum1*	*Rps17*	*Psmc4*
6	*Ppia*	*Pum1*	*Ppia*	*Ppia*	*Rplp0*	*Rps17*	*Actb*	*Pum1*
7	*Mrp119*	*Pgk1*	*Pop4*	*Mrp119*	*Mrp119*	*Pgk1*	*Casc3*	*Pgk1*
8	*Pes1*	*18S*	*Rps17*	*Cdkn1a*	*Rps17*	*18S*	*Ppia*	*Ppia*
9	*Rps17*	*Mrp119*	*Mrp119*	*Pes1*	*Pes1*	*Mrp119*	*Gapdh*	*Mrp119*
10	*Actb*	*Ppia*	*Actb*	*Pop4*	*Pop4*	N[b]	*Mrp119*	*Casc3*
11	Gapdh	N[b]	*Cdkn1a*	*Rps17*	*Actb*	N[b]	*18S*	*18S*
12	*18S*	N[b]	*Gapdh*	*Actb*	*Gapdh*	N[b]	N[b]	N[b]
13	*Cdkn1a*	N[b]	*Pes1*	*Gapdh*	*18S*	N[b]	N[b]	N[b]
14	*Pop4*	N[b]	*18S*	*18S*	*Cdkn1a*	N[b]	N[b]	N[b]

[a]Depending on the results of pairwise variation, the top-ranked genes are displayed in bold [b]No available Cq data for analysis

Table 2. Ranking from most stable to least stable reference genes on gerbil tissues

Ranking[a]	Adrenal	Cerebral Cortex	Heart	Hypothalamus	Kidney	Liver	Lung	Testis
1	***Psmc4***	***Rplp0***	***Pgk1***	***Pgk1***	***Casc3***	***Actb***	***Rplp0***	***Rplp0***
2	***Pgk1***	***Rps17***	***Psmc4***	***Psmc4***	***Pgk1***	***Rplp0***	***Pgk1***	***Rps17***
3	*Casc3*	*Actb*	*Pum1*	*Casc3*	*Ppia*	*Gapdh*	*Psmc4*	*Actb*
4	*Pum1*	*Gapdh*	*Rplp0*	*Pum1*	*Psmc4*	*Psmc4*	*Pum1*	*Gapdh*
5	*Rplp0*	*Psmc4*	*Casc3*	*Rplp0*	*Pum1*	*Pum1*	*Rps17*	*Psmc4*
6	*Ppia*	*Pum1*	*Ppia*	*Ppia*	*Rplp0*	*Rps17*	*Actb*	*Pum1*
7	*Mrp119*	*Pgk1*	*Pop4*	*Mrp119*	*Mrp119*	*Pgk1*	*Casc3*	*Pgk1*
8	*Pes1*	*18S*	*Rps17*	*Cdkn1a*	*Rps17*	*18S*	*Ppia*	*Ppia*
9	*Rps17*	*Mrp119*	*Mrp119*	*Pes1*	*Pes1*	*Mrp119*	*Gapdh*	*Mrp119*
10	*Actb*	*Ppia*	*Actb*	*Pop4*	*Pop4*	N[b]	*Mrp119*	*Casc3*
11	Gapdh	N[b]	*Cdkn1a*	*Rps17*	*Actb*	N[b]	*18S*	*18S*
12	*18S*	N[b]	*Gapdh*	*Actb*	*Gapdh*	N[b]	N[b]	N[b]
13	*Cdkn1a*	N[b]	*Pes1*	*Gapdh*	*18S*	N[b]	N[b]	N[b]
14	*Pop4*	N[b]	*18S*	*18S*	*Cdkn1a*	N[b]	N[b]	N[b]

[a]Depending on the results of pairwise variation, the top-ranked genes are displayed in bold [b]No available Cq data for analysis

endogenous genes used for RT-qPCR normalization are 18S, Gadph, and Actb, which have higher expression levels than other endogenous genes in the hypothalamus of hamsters. However, only the expression of the 18S and Actb genes was noticeably higher in the hypothalamus of gerbils.

We obtained mean Cq values from the hypothalamus in the first phase of our research. The hypothalamus is thought to be the most important nervous system organizing region. All physiological systems in photoperiodic species, like hamsters and gerbils, are controlled by a circadian rhythm. The suprachiasmatic nucleus (SCN), also known as the biological clock and found in the hypothalamus, is the most significant neuron group in this rhythmic arrangement. Although rhythmic regulation in photoperiodic animals can be explained more clearly through the reproductive system, many physiological mechanisms of the organism (eating-drinking rhythm, hormonal rhythmic regulation, immunological responses, stress differences during the day, and so on) are rhythmically controlled. In photoperiodic animals, the critical photoperiod is crucial. For the Syrian hamster, this critical photoperiod is 12.5 hours, while for the gerbil, it is 10 hours (33-35). The hours above this critical photoperiod are important for normal reproduction and the proper operation of the animal's physiological systems. Nearly all of an

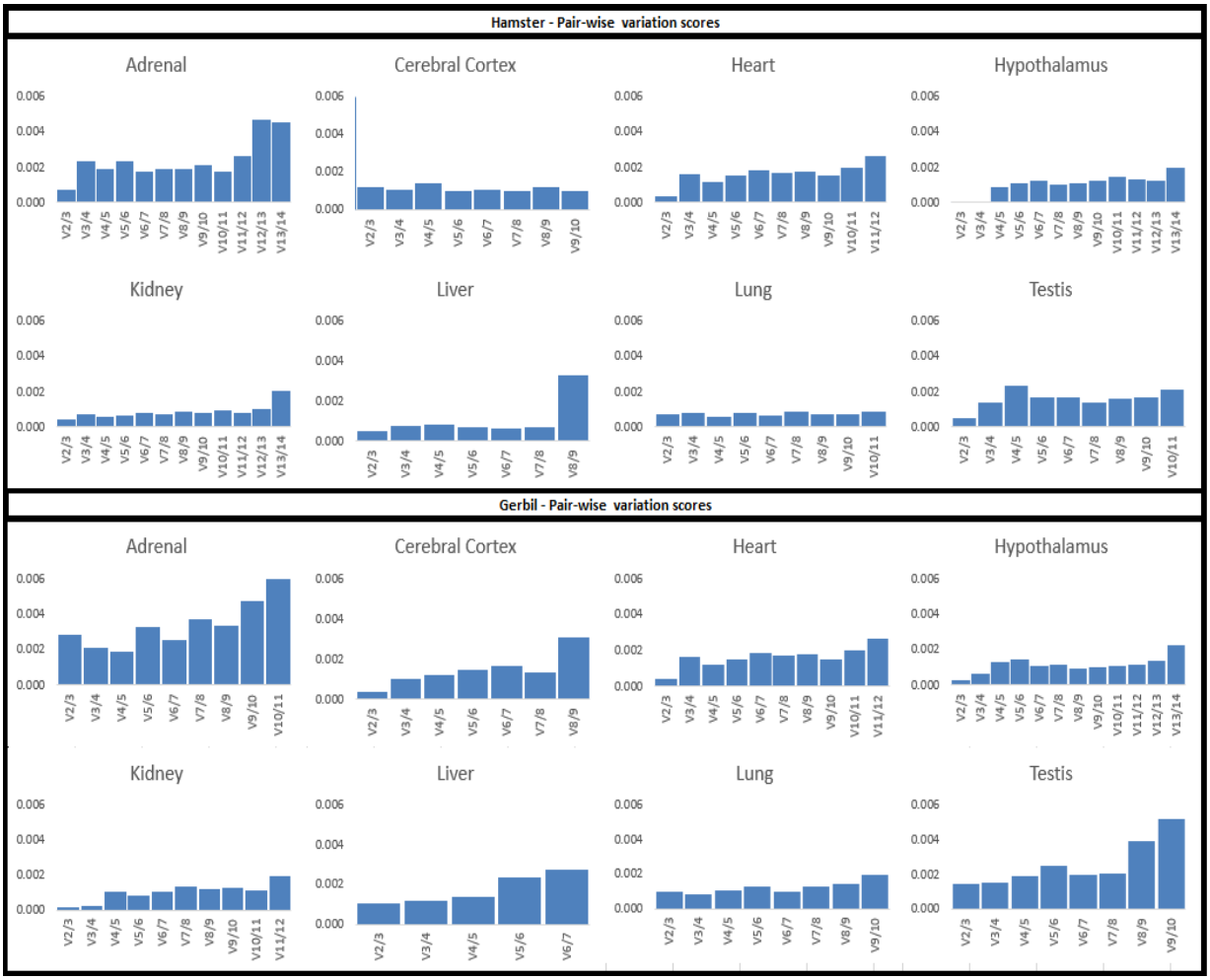

Figure 4. GeNorm V pair-wise variation scores for 8 different tissues were calculated to determine the ideal number of reference genes needed for normalization. (A) Vn/n+1 results for the hamster tissues. (B) Vn/n+1 results for the gerbil tissues. For stability, a GeNorm V threshold of less than 0.15 was chosen. The optimal number of control genes required for RT-qPCR normalization was determined to be two for all tissues in both species.

animal's physiological systems change (for example, adapting to winter conditions) and reproductive abilities decline during the hours below this critical photoperiod (36-43). The 14L photoperiod was chosen for our study because it would be the most suitable for both species. 32 endogenous genes were examined in hamster and gerbil species with distinct photoperiodic characteristics, focusing mainly on the hypothalamus.

We determined that *Psmc4/Pgk1, Rplp0/Rps17, Pgk1/Psmc4, Pgk1/Psmc4, Casc3/Pgk1, Actb/Rplp0, Rplp0/Pgk1 and Rplp0/Rps17* pairs in hamsters and *Rplp0/Pum1, 18S/Actb, Rplp0/Pum1, Pop4/Pgk1, Rplp0/Actb, Psmc4/18S, Actb/Rplp0,* and *Actb/Psmc4* pairs in gerbils, respectively, are the most stable reference genes for normalization in the following tissues: adrenal, cerebral cortex, heart, hypothalamus, kidney, liver, lung, and testis. Actb is the most commonly used reference gene for RT-qPCR normalization, but in our study, Actb was only the most stable gene in the livers of hamsters and the lungs and testis of gerbils. Furthermore, Actb was found to be the least stable in rats' hypothalamus and intestine (44). As a result, using Actb solely as a reference gene may lead to a misunderstanding of the expression levels of target genes in specific tissues. In the same tissues, stable reference genes may vary depending on the type of experiment (44). In addition, a recent study found that the gene expression of Actb varied among Syrian hamster tissues (21). In another study, different reference genes for aortic tissue in Syrian Hamsters were proposed (45). In a microarray analysis of Chinese Hamster ovary tissue, it was proposed species-specific reference genes (46). These findings

highlight the significance of selecting appropriate reference genes in future studies.

It was suggested that reference genes be used for normalization in RT-qPCR studies (10) but it is not feasible to analyze many endogenous reference genes in a study. According to our research, reference gene expression levels vary between species and tissues. According to our study's GeNorm analysis, pairwise variation results showed that two reference genes are adequate for RT-qPCR normalization in our experimental settings. It was strongly advised to choose appropriate reference genes in every experimental setting to accurately assess the target gene expression level in RT-qPCR analysis. For example, hamsters and gerbils are photoperiodic animals that are used in a variety of research studies. The limitation of the study was that only long photoperiod/normal conditions for these species were considered in the design of the study. In subsequent research, it would be appropriate to test the study under different conditions (food restriction, different ambient temperature, and different day cycles).Understanding the physiological mechanisms underlying the numerous species-specific diseases may be improved by identifying subtle variations in gene expression. Therefore, it is important to carefully choose the right reference genes for each unique experimental setting.

CONCLUSION

We compared two species for the first time in this study in terms of reference gene normalization for RT-qPCR analysis. Research revealed that the expression of reference genes may vary depending on the species and the tissue. Therefore, to identify differences that might arise because of sex differences, candidate reference genes in female Syrian hamsters and Mongolian gerbils should also be clarified. The findings pave the way for further research into the expression levels of various reference genes in various experimental settings (i.e. feeding types, and different photoperiodical conditions).

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Author contribution: All authors contributed to the study conception and design. Animal experiments were conducted by BO and BG. Data analysis was performed by TU, BO, and BG. TU and BO initially drafted the manuscript. BG reviewed and revised the manuscript. All authors read and approved the final manuscript.

Conflict of interests: The authors have no competing interests to declare that are relevant to the content of this article.

Ethical approval: The experimental procedures in this study were conducted following the guidelines of Canakkale Onsekiz Mart University and approved by the Ethical Council of Animal Research (Date: 13.04.2011, No: 2011/04-01).

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Supplementary File

Determination of Appropriate Endogenous Reference Genes for RT-qPCR Analysis in Syrian (Golden) Hamsters and Mongolian Gerbils

Supplementary Table 1. List of 32 TaqMan endogenous control gene probes

Gene Symbol	Gene Name	Assay ID
18S	Eukaryotic 18S rRNA	Hs99999901_s1
Abl1	ABL proto-oncogene 1, non-receptor tyrosine kinase	Rn01436238_g1
Actb	Actin, beta	Rn00667869_m1
B2m	Beta-2-microglobulin	Rn00560865_m1
Casc3	Cancer susceptibility candidate 3	Rn00595941_m1
Cdkn1a	Cyclin-dependent kinase Inhibitor 1A	Rn00589996_m1
Cdkn1b	Cyclin-dependent kinase Inhibitor 1B	Rn00582195_m1
Eif2b1	Eukaryotic translation eukaryotic translation initiation factor 2B, subunit 1 alpha	Rn00596951_m1
Elf1	E74-like factor 1	Rn00585356_m1
Gadd45a	Growth arrest and DNA-damage-include, alpha	Rn00577049_m1
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	Rn99999916_s1
Gusb	Glucuronidase, beta	Rn00566655_m1
Hmbs	Hydroxymethylbilane synthase	Rn00565886_m1
Hprt1	Hypoxanthine phosphoribosyltransferase 1	Rn01527840_m1
Mrpl19	Mitochondrial ribosomal protein L19	Rn01425270_m1
Mtatp6	Mitochondrially encoded ATP synthase 6	Rn03296710_s1
Pes1	Pescadillo homolog 1	Rn01443731_g1
Pgk1	Phosphoglycerate kinase	Rn00821429_g1
Pop4	Processing of precursor 4, ribonuclease P/MRP subunit (S. cerevisiae)	Rn02347225_m1
Ppia	Peptidylprolyl isomerase A (cyclophilin A)	Rn00690933_m1
Ppib	Peptidylpropyl isomerase B	Rn00574762_m1
Psmc4	Proteasome (prosome, Macropain) 26S subunit, ATPase 4	Rn00821605_g1
Pum1	Pumilio RNA binding family member 1	Rn00982780_m1
Rpl30	Ribosomal protein L30-like	Rn01504620_g1
Rpl37a	Ribosomal protein L37a	Rn02114291_s1
Rplp0	Ribosomal protein, large, P0	Rn00821065_g1
Rplp2	Ribosomal protein, large P2	Rn01479927_g1
Rps17	Ribosomal protein S17	Rn00820807_g1
Tbp	TATA box binding protein	Rn01455648_m1
Tfrc	Transferrin receptor	Rn01474695_m1
Ubc	Ubiquitin C	Rn01789812_g1
Ywhaz	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta	Rn00755072_m1