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Reference Gene Selection in Adipose and Muscle Tissues of Fat-tailed Lori-Bakhtiari Lambs

Alireza Aziziyan[1], Mostafa Sadeghi[*1], Mahdi Ganjkhanlou[1], Hossein Zakariapour Bahnamiri[1]

1. Department of Animal Sciences, College of Agriculture and Natural Rsources, University of Tehran, Karaj, Iran

Abstract

BACKGROUND: Fat-tailed sheep breeds have a unique ability to tolerate periods of negative energy balance due to seasonal changes in feed availability. This ability is attributed to presence of fat-tail as a body energy reserve, however the exact underlying mechanisms controlling the response of adipose tissue depots to variations in energy balance in fat-tailed breeds are not well understood.

OBJECTIVES: As definition of a set of stable reference gene is an absolute prerequisite of any gene expression study, therefore the current research was conducted to define the most stable reference genes in adipose tissue depots and muscle of fat-tailed Lori-Bakhtiari lambs during periods of negative and positive energy balances.

METHODS: Eighteen fat-tailed Lori-Bakhtiari male lambs were divided into 3 groups according to their body-weight. The experiment was consisted of an adaptation period (2 weeks), negative energy balance period (3 weeks), followed by positive energy balance period (3 weeks). The 3 groups of lambs were randomly selected and slaughtered at the beginning and end of negative energy balance and at the end of positive energy balance to collect samples of muscle and adipose tissue depots.

RESULTS: The stability of the reference genes differed among different tissues and also between various depots of adipose tissue. Average of ranking by different software programs showed that glyceraldehyde 3-phosphate dehydrogenase (GAPDH), B-actin and peptidylprolyl isomerase A (PPIA) were the 3 most stable reference genes in mesenteric adipose tissue, whereas in fat-tail adipose tissue, PPIA, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ) and RNA polymerase II subunit A (POLR2A) were considered as genes with least expression variability during periods of negative and positive energy balance. B-actin, YWHAZ and phosphoglycerate kinase 1 (PGK1) were defined as the most stable reference genes in longissimus dorsi muscle tissue of Lori-Bakhtiari lambs.

CONCLUSIONS: The results of the current study demonstrate that the stability of the reference genes varied between mesenteric and fat-tail adipose tissues and the level of energy balance affects the stability of the reference genes. In addition, ranking of the reference genes differs among different software programs possibly due to different mathematical algorithms used by different programs.

KEYWORDS: Adipose tissue, Energy balance, Fat-tailed sheep, Longissimus dorsi muscle, Reference gene.

Correspondence
Mostafa Sadeghi, Department of Animal Sciences, College of Agriculture and Natural Rsources, University of Tehran, Karaj, Iran, Tel: +98 (026) 32246752, Fax: +98 (026) 32246752, Email: sadeghimos@ut.ac.ir
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Introduction

There are 27 breeds of sheep in Iran, 26 of which are fat-tailed breeds. As Iran is located in arid and semi-arid regions of the world and experiences periods of feed abundance and scarcity during the year, fat-tail adipose tissue as an energy reserve developed in native sheep breeds of Iran as an evolutionary adaptation that serves as a source of energy to increase survival during the shortage in pasture and of feed scarcity. In fat-tailed breeds, deposition of fat in tail region during feed abundant season can keep the animal alive during periods of scarcity without considerable elevation of plasma non-esterified fatty acid (NEFA) concentration. To our knowledge, there is no study investigating the biological pathways such as gene expression of regulators and enzymes involved in adipose tissues metabolism of fat-tailed sheep breeds during periods of negative and positive energy balance. Evaluating the effect of negative and positive energy balances on gene expression might reveal the pathways involved in adipose tissue metabolism, as quantitative real time PCR (RT-qPCR) can produce data with high sensitivity and reproducibility. As stability of the reference genes varies according to type of tissue and physiological stage (Svingen et al., 2015; Kaur *et al*., 2018), defining a suitable set of reference genes is an absolute prerequisite for any RT-qPCR analysis, since a stable reference gene in various physiological and environmental conditions as an internal standard can accurately depict changes in expression of target genes. Moreover, several software programs have been developed to rank the reference candidate genes from the most stable to the least stable one. These software programs have been shown to rank the reference genes differently (Najafpanah *et al*., 2013) due to acquisition of different algorithms (Kim *et al*., 2011). Hence, the objective of the current study was to evaluate the stability of 8 commonly used candidate reference genes including Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Peptidylprolyl isomerase A (PPIA), Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, Zeta polypeptide (YWHAZ), B-actin, Glucose-6-phosphate dehydrogenase (G6PDH), RNA polymerase II subunit A (POLR2A), Phosphoglycerate kinase 1 (PGK1) and Beta-2-microglobulin (B2M) in adipose and muscle tissues of fat-tailed Lori-Bakhtiari male lambs under periods of negative and positive energy balance by 3 software programs including BestKeeper, NormFinder and geNorm and also consensus ranking of these software programs.

Materials and Methods

Ethics statement

The experiment was done according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the Research Station of Department of Animal Science, University of Tehran, Iran. The protocols were approved by the Animal Care and Use Committee of the University of Tehran Institutional Animal Care and Use Committee.

Animal, housing and sampling

The experiment was carried out at the Natural Resources & Agricultural Research Farm of Tehran University, Karaj, Iran. Eighteen Lori-Bakhtiari male lambs with average body-weight of 45.10 ± 3.50 and age

of 5-6 months were divided into 3 groups of 6 lambs in each treatment according to their body-weight. Lambs were placed in individual pens. The experiment began after two weeks of an adaptation to pen and lasted for about 42 days. All lambs were fed a balanced total mixed ration (TMR) formulated by Cornell net carbohydrate and protein system (CNCPS) software program 1.5 fold of their maintenance requirement during adaptation period. The diet was consisted of concentrate (44 %) and forage (56 %; alfalfa hay and wheat straw; Table S1). The amount of feed was adjusted weekly according to lambs body-weight change during the whole experiment. The lambs were fed twice daily at 8:00 and 17:00 (equal amount) and had free access to water. At the end of adaptation period, the first group (6 lambs) was randomly selected and weighted after 16 h depriving from feed and slaughtered to collect samples of adipose tissues and longissimus dorsi muscle. The remained 2 groups were fed 90, 80 and 70 % of their maintenance requirement in weeks 1, 2 and 3 of the experiment respectively. At the end of week 3, the second group was randomly selected and slaughtered to collect samples and the remained group (group 3) was fed ad-libitum until the end of experiment (day 42) and then was slaughtered to collect samples. All samples were immediately frozen in liquid nitrogen, transferred to the laboratory and kept at -80 °C until analysis. Lambs were weighed and bled weekly for calculation of changes in body-weight and plasma NEFA concentration.

Total RNA extraction, clean-up, and cDNA synthesis

Total RNA was extracted according to the method of Chomczynski and Sacchi (2006) using YTzol reagent (Yekta Tajhiz Azma Co., Tehran, Iran) and treated with RNase-free DNase I in order to remove the remnant genomic DNA from the samples (TaKaRa, Shuzo, Kyoto, Japan). The RNA abundance was estimated by nanodrop spectrophotometry at 260 nm, and the purity was checked by determining the absorption ratio at 260/280 nm. The quality of the extracted RNA was assessed by electrophoresis at 1% agarose-gel that contained ethidium bromide. The first-strand complementary DNA (cDNA) was synthesized from 100 ng of total RNA by cDNA synthesis kit (M- MuLV Reverse Transcriptase, Cinaclon Co, Tehran, Iran, CAT No; PR911658), an oligo (dT) primer and random hexamers according to manufacturer's instructions. The process of cDNA synthesis was initiated by annealing of the primers at 37 ºC for 1 min followed by cDNA synthesis at 42 ºC for 60 min and terminated by inactivation of the reverse transcriptase enzyme at 85 ºC for 5 min. The synthesized cDNA was kept at -20 ºC to be used later.

Primer design

The nucleotide sequence of 8 candidate reference genes belonging to the sheep (*Ovis aries*) was obtained from public databases (GenBank, National Center for Biotechnology Information). Primer pairs were designed according to these sequences (optimal T_m at 61 °C and GC between 45-50%) using primer3Plus (Untergasser *et al*., 2007) online software programs and the suitability of primers was evaluated by OligoAnalyzer 3.1 (http://eu.idtdna.com/analyzer/applications/oligoanalyzer/) and OligoCalc (Kibbe, 2007). The specificity of designed primers was examined through PrimerBLAST software of NCBI database (Ye *et al*., 2012). The sequence and some other characteristics of designed primers are presented in Table 1.

Table 1. The sequence and characteristics of primers used for evaluation of expression of reference genes

	Accession number	Forward and reverse sequence	Fragment length (bp)	Annealing temperature (º C)
GAPDH	NM_001190390.1	ACGCTCCCATGTTTGTGATG	146	58.83
		CATAAGTCCCTCCACGATGC		58.13
PPIA	NM_001308578.1	TTGCAGACAAAGTCCCGAAG	121	58.41
		CCACCCTGGCACATAAATCC		58.60
YWHAZ	NM_001267887.1	GTTCTTGATCCCAAACGCTTC	119	57.80
		CCACAATCCCTTTCTTGTCATC		57.29
B-actin	NM_001009784.1	TGGCACCACACCTTCTACAAC	105	60.48
		GGTCATCTTCTCACGGTTGG		58.27
G6PDH	NM_001093780.1	CAAGCTGGAGGAGTTCTTTGC	131	59.46
		GGTAGAAGAGGCGGTTGGTC		60.11
POLR2A	XM_004013289.3	GGATCAGGAGTGGGTGAATG	110	57.66
		TCCGGTCAGTCATGTGCTTC		60.04
PGK1	NM_001142516.1	TAAGGTGCTCAACAACATGGAG	203	58.59
		CCATCCAGCCAACAGGTATG		58.32
B2M	NM_001009284.2	CAGCGTATTCCAGAGGTCCAG	199	60.20
		CAGCGTGGGACAGAAGGTAG		60.11

Real-time RT-PCR

The real-time quantitative PCR was performed using SYBR Green I technology on an iQ5 System (BioRad, USA). The reactions consisted of 10 µL SYBR Green PCR Master Mix (SYBR biopars, GUASNR, Iran), 10 pmol (1 µL) of each specific forward and reverse primer, 3 µL of cDNA, and 5 µL nuclease free water, for a final volume of 20 µL. Real-time quantitative PCR was performed for samples with 6 biological replicates. The PCR temperature cycling program was an initial denaturation at 95 ºC for 15 min followed by 40 cycles at 95 ºC (denaturation, 15 sec), 62 ºC (annealing, 30 sec), and 72 ºC (elongation, 30 sec), followed by a final extension at 72 ºC for 5 min. The amplified DNA was incubated at 4 ºC, and 5.5 µL of PCR amplified product was purified using horizontal electrophoresis in a 2% agarose gel and visualized by ethidium bromide to confirm the specificity of amplified fragments. The efficiency of RT-PCR was assessed for each gene based on the slope of a linear regression model. The bulk of each cDNA sample was used as a PCR template to produce a graph of the cycle threshold (Ct) in a range of 10-fold dilution series. The corresponding RT-PCR efficiencies were calculated based on the slope of the standard curve using the following equation: (E=10 -1/slope-1) (Radonić *et al*., 2004). A melt-curve analysis was conducted for each amplification between 55-95 ºC to ascertain that non-specific products were not amplified. Three software programs of NormFinder, geNorm and BestKeeper were used to rank the candidate reference genes according to their stability. The arithmetic mean of the reference genes ranks by 3 software programs was calculated as consensus ranking.

Statistical analysis

Data were analyzed by GLM procedure of SAS software (SAS 2002) to evaluate the difference in Ct value of the candidate reference genes and SAS MIXED procedure was used to analyze bodyweight and plasma NEFA concentration during periods of negative and positive energy balance. The difference between treatments was considered to be significant if $P<0.05$.

Results

Induction of Negative energy balance

As it is shown in Figure 1, feeding 90, 80 and 70 % of maintenance requirement respectively in weeks 1, 2 and 3 of negative energy balance period significantly reduced body-weight and increased plasma NEFA concentration, hence successfully induced negative energy balance in Lori-Bakhtiari lambs. The lambs experienced the most severe negative energy balance and body-weight loss during the third week of the restricted feeding and by elimination of the restricted feeding, started to gain weight and plasma NEFA concentration was returned (decreased) to the basal level.

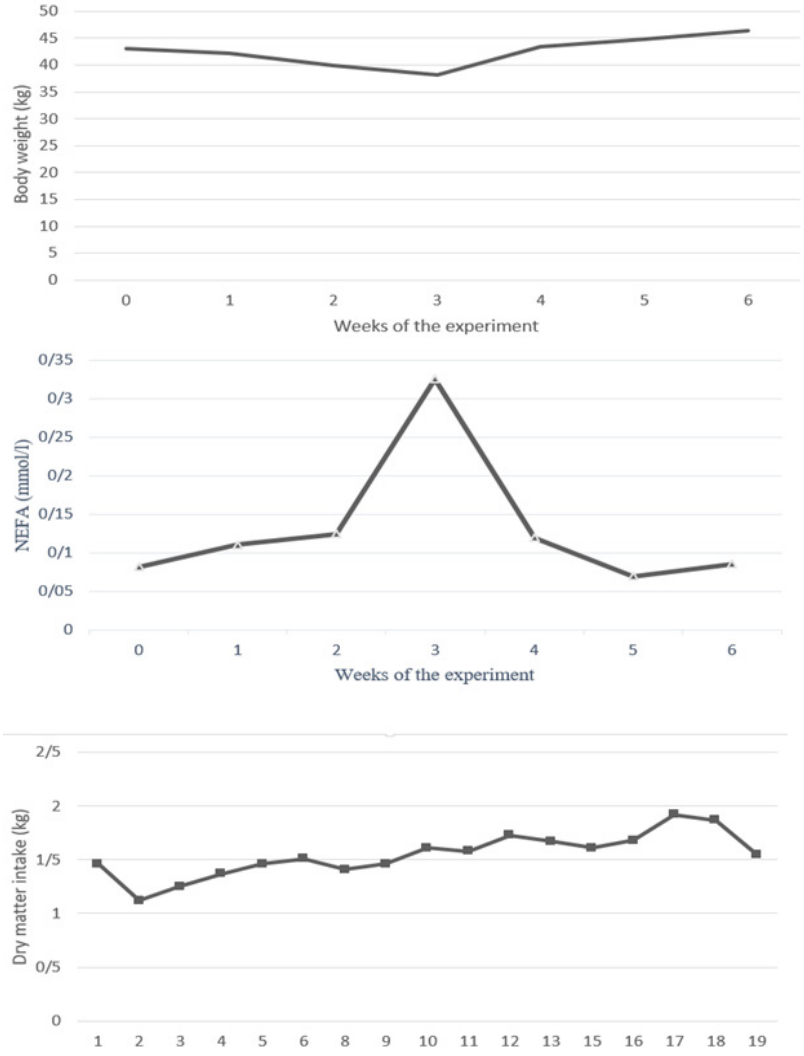

Figure 1. The bodyweight and plasma NEFA concentration changes during negative and positive energy balances

Reference genes stability

The mean Ct value with standard deviation and also Ct distribution of candidate reference genes are presented in Figures 2 to 7. In mesenteric adipose tissue, lowest and highest Ct values were observed with B-actin and PGK1 respectively (Figure 2). The range of the Ct value distribution was parallel to the standard deviation of the reference genes as genes with the lowest standard deviation showed Ct values distributed in a narrower range (Figure 3). In fat-tail adipose tissue, the lowest Ct value was observed with B2M and PPIA, whereas POLR2A and YWHAZ showed the highest Ct value (Figure 4). The Ct value of G6PDH showed a narrower distribution range compared to other candidate reference genes in fat-tail adipose tissue (Figure 5). In longissimus dorsi muscle, G6PDH showed the lowest standard deviation (Figure 6) and also had the narrowest distribution (Figure 7). There was no significant change in Ct value of the reference genes in mesenteric adipose tissue (Figure 8), except for B-actin and G6PDH which increased as the experiment progressed (from 19.32 and 21.65 at the beginning of the experiment to 21.62 and 24.19 at the end of the experiment respectively for B-actin and G6PDH). Induction of negative energy balance increased the Ct value of all candidate reference genes and shifting to positive energy balance reduced their Ct value, however the difference was significant only for G6PDH ($P<0.02$) and PGK1 ($P<0.05$). The negative energy balance caused a significant enhancement in Ct value of GAPDH, B-actin, B2M and PGK1 followed by a reduction in response to positive energy balance, whereas the Ct value of G6PDH was reduced as a consequence of negative energy balance ($P < 0.05$), however, the difference was not significant ($P > 0.11$).

G6PDH was the most stable gene in the mesenteric adipose tissue defined by NormFinder and geNorm software programs, whereas by BESTKEEPER software, it ranked 3 and B-actin was defined as the most stable gene (Table 2). Gene expression of POLR2A, PGK1 and B2M showed the least stability in mesenteric adipose tissue calculated by NormFinder and geNorm software programs and POLR2A was replaced by YWHAZ when BestKeeper was used. Arithmetic mean of the ranking by 3 software programs showed that GAPDH, B-actin and PPIA were the most stable and POLR2A, PGK1 and B2M were the least stable genes in mesenteric adipose tissue during negative and positive energy balances.

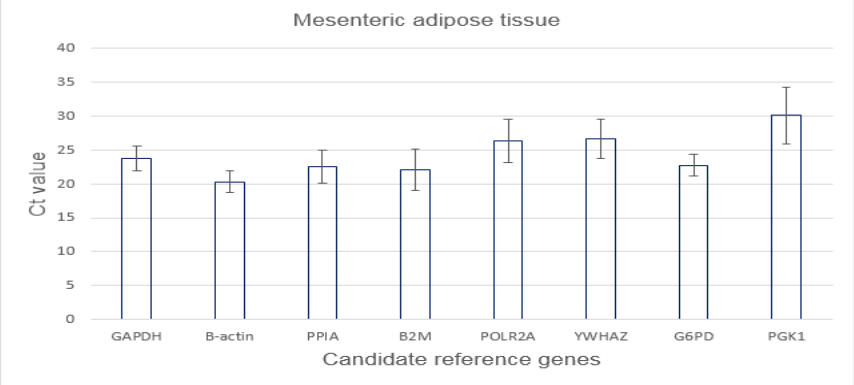

Figure 2. The Ct value with standard deviation of mesenteric adipose tissue

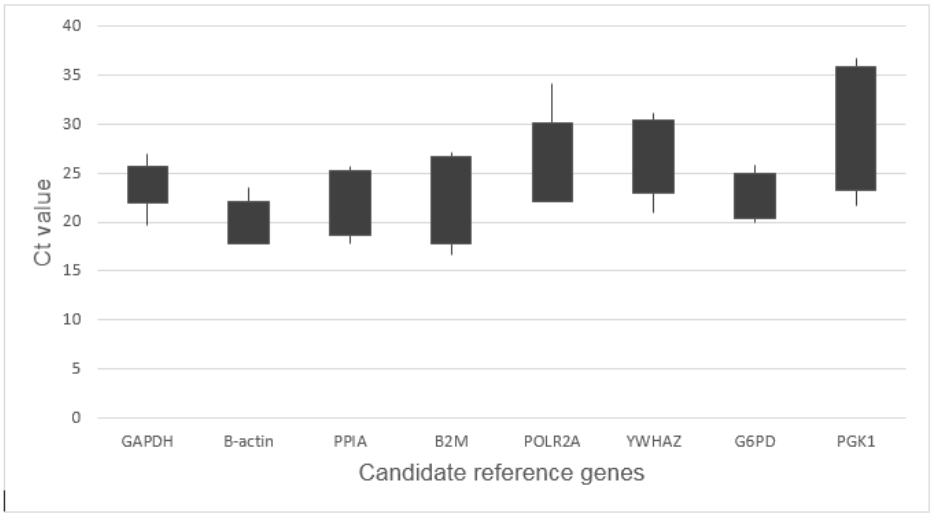

Figure 3. The distribution of Ct value of mesenteric adipose tissue

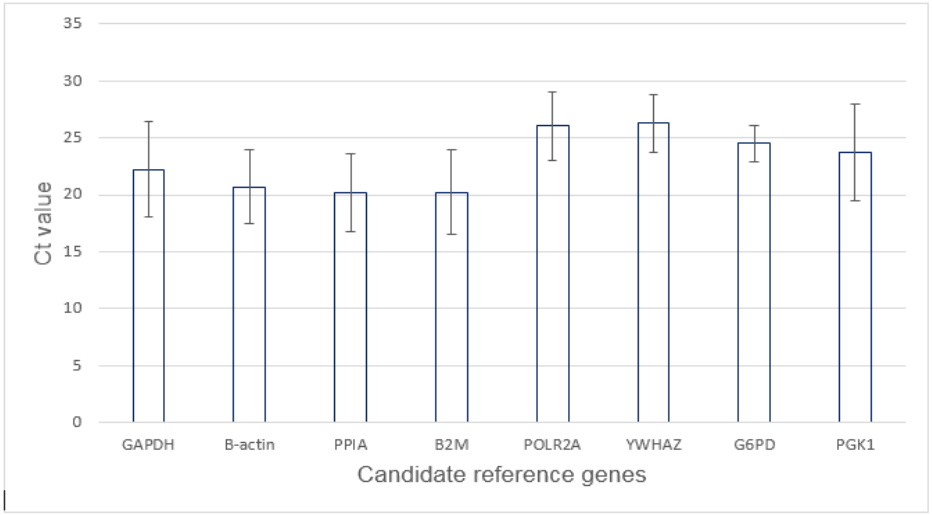

Figure 4. The Ct value with standard deviation of fat-tail adipose tissue

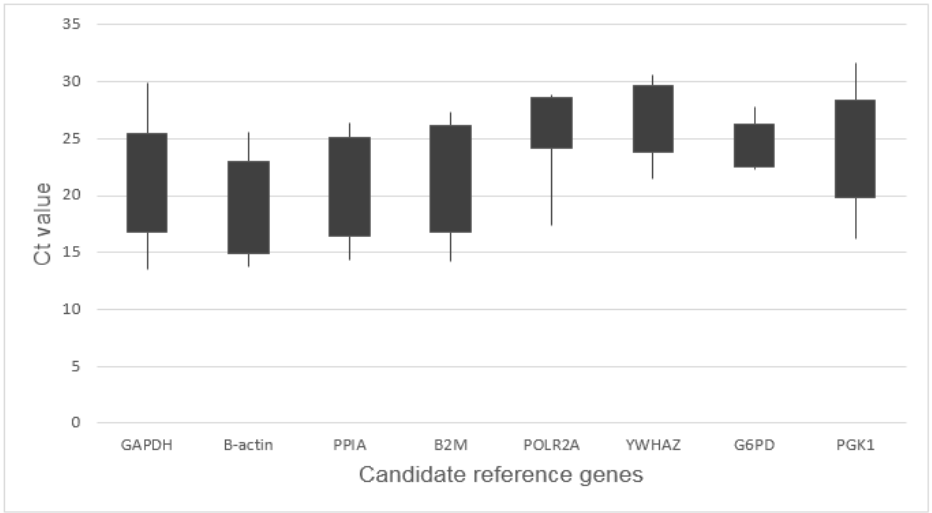

Figure 5. The distribution of Ct value of fat-tail adipose tissue

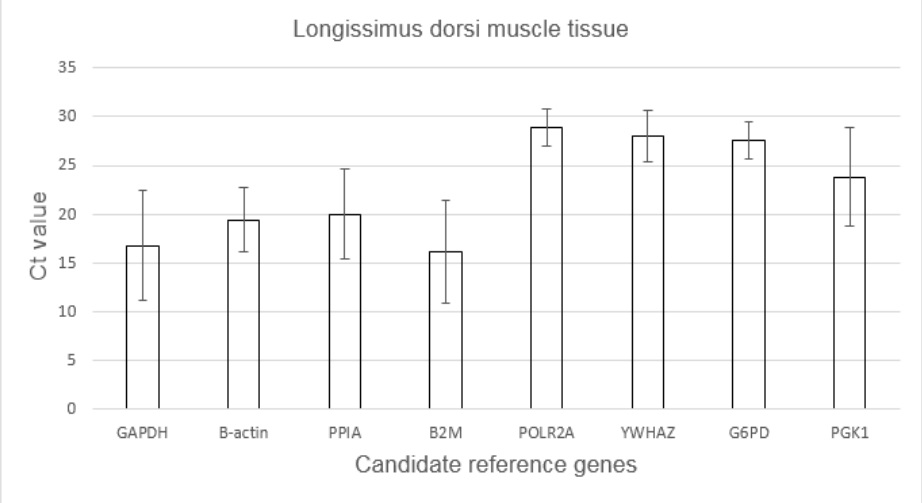

Figure 6. The Ct value with standard deviation of longissimus dorsi muscle tissue

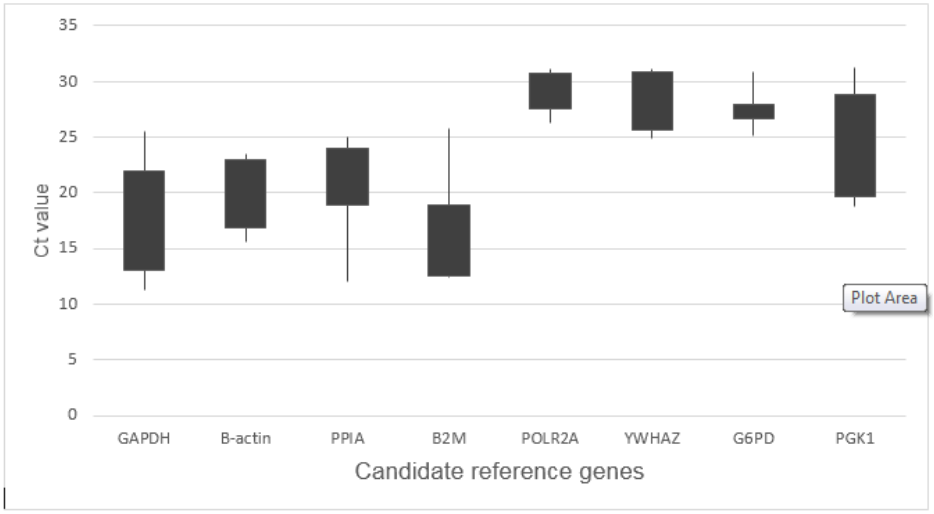

Figure 7. The distribution of Ct value of longissimus dorsi muscle tissue

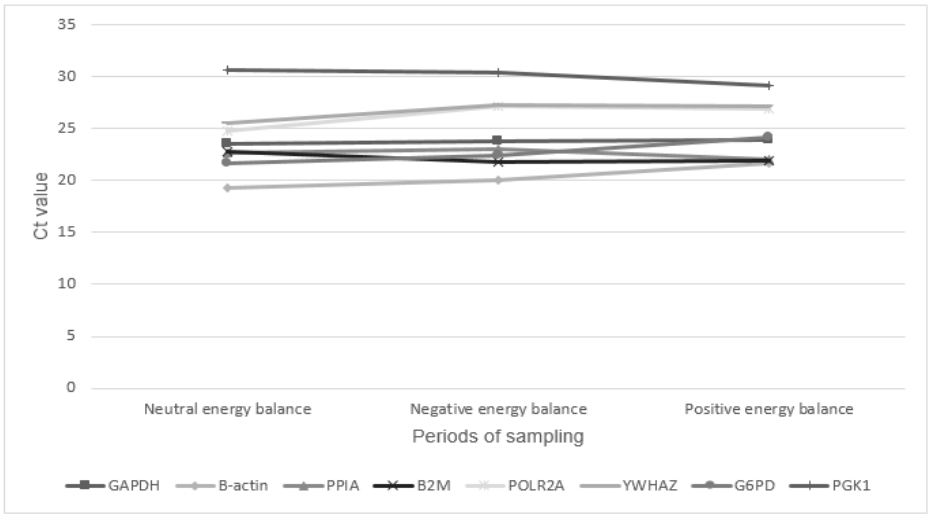

Figure 8. The Ct value of reference genes in mesenteric adipose tissue in different energy balances

Table 2. The candidate genes ranked by different software programs and the consensus ranking in mesenteric depot

Rank of stability	Best Keeper	NormFinder	geNorm	Consensus ranking
1	B-actin	GAPDH	GAPDH	GAPDH
2	G6PDH	PPIA	B-actin	B-actin
3	GAPDH	YWHAZ	PPIA	PPIA
4	PPIA	B-actin	YWHAZ	G6PDH
5	POLR2A	G6PDH	G6PDH	YWHAZ
6	YWHAZ	POLR2A	POLR2A	POLR2A
7	B2M	PGK1	PGK1	PGK1
8	PGK1	B2M	B2M	B2M

Ranking of 8 candidate reference genes in fat-tail adipose tissue by NormFinder and geNorm software programs was quite similar except for B-actin and B2M which were exchanged between ranks of 5 and 8 (Table 3). PPIA, PGK1 and YWHAZ were the most stable reference genes defined by NormFinder and geNorm, whereas Best Keeper calculated G6PDH, YWHAZ and POLR2A as genes with least variability with YWHAZ as the only similarity among 3 software programs. PGK1 was defined as the second stable reference gene by NormFinder and geNorm software programs, whereas it showed the least stability calculated by BestKeeper software program. Average of the ranking by 3 software programs showed that PPIA, YWHAZ and POLR2A were the most and B-actin, GAPDH and B2M were the least stable genes in fat-tail adipose tissue.

Table 3. The candidate genes ranked by different software programs and the consensus ranking in fat-tail depot

Rank of stability	Best Keeper	NormFinder	geNorm	Consensus ranking
1	G6PDH	PPIA	PPIA	PPIA
2	YWHAZ	PGK1	PGK1	YWHAZ
3	POLR2A	YWHAZ	YWHAZ	POLR2A
4	B-actin	POLR2A	POLR2A	PGK1
5	PPIA	B-actin	B2M	G6PDH
6	B2M	GAPDH	GAPDH	B-actin
7	GAPDH	G6PDH	G6PDH	GAPDH
8	PGK1	B2M	B-actin	B2M

When mesenteric and fat-tail adipose tissues were considered together, GAPDH, PPIA and YWHAZ were considered as the most stable genes during negative and positive energy balances by NormFinder and geNorm software programs, while by using Best Keeper, G6PDH, B-actin and POLR2A were defined as genes with least variability (Table 4). PPIA which was defined as the second stable gene by NormFinder and geNorm software programs, was considered as a gene with low stability (ranked sixth) by BestKeeper software program. In addition, G6PDH which was

considered as the best reference gene with least variability by BestKeeper, was among genes with the lowest stability defined by other software programs.

Table 4. . The candidate genes ranked by different software programs and the consensus ranking in adipose tissue

Rank of stability	Best Keeper	NormFinder	geNorm	Consensus ranking
1	G6PDH	GAPDH	GAPDH	GAPDH
2	B-actin	PPIA	PPIA	PPIA
3	POLR2A	YWHAZ	YWHAZ	YWHAZ
4	YWHAZ	POLR2A	POLR2A	POLR2A
5	GAPDH	B-actin	B-actin	B-actin
6	PPIA	B2M	B2M	G6PDH
7	B2M	G6PDH	G6PDH	B2M
8	PGK1	PGK1	PGK1	PGK1

For longissimus dorsi muscle, G6PDH, POLR2A and YWHAZ were defined as the most stable genes by BestKeeper software program, while by NormFinder and geNorm software programs, B-actin, PGK1 and YWHAZ were defined as the most stable reference genes with YWHAZ as the only similarity (Table 5). G6PDH was considered as the most stable reference gene in muscle tissue by BestKeeper program, whereas by NormFinder and geNorm, it was considered as a gene with low stability. In addition, GAPDH was considered as a gene with low stability by all 3 software programs. Consensus ranking of all software programs defined B-actin, YWHAZ and PGK1 as the 3 most and B2M, G6PDH and GAPDH as the 3 least stable reference genes in muscle tissue during periods of negative and positive energy balance.

Table 5. The candidate genes ranked by different software programs and the consensus ranking in skeletal muscle

Ranking of stability	Best Keeper	NormFinder	geNorm	Consensus ranking
1	G6PDH	B-actin	B-actin	B-actin
2	POLR2A	PGK1	PGK1	YWHAZ
3	YWHAZ	YWHAZ	YWHAZ	PGK1
4	B-actin	PPIA	POLR2A	POLR2A
5	PPIA	B2M	B2M	PPIA
6	B2M	POLR2A	PPIA	B2M
7	PGK1	GAPDH	G6PDH	G6PDH
8	GAPDH	G6PDH	GAPDH	GAPDH

The ranking of the candidate reference genes by different software programs and also the consensus ranking were affected by energy balance (Table 6). Consensus ranking of the software programs showed that in mesenteric adipose tissue, GAPDH

was among the 3 most stable reference genes in all periods of different energy balance, whereas B-actin was not considered as a stable reference gene in negative energy balance. In fat-tail adipose tissue, the stability of reference genes was considerably affected by energy balance, however, B-actin and PGK1 were among the 3 most stable genes in both negative and subsequent positive energy balances. When mesenteric and fat-tail adipose tissues were considered together, GAPDH was among the 3 most stable genes in all periods and then B-actin and PPIA were the most frequent genes selected as the 3 most stable reference genes.

Table 6. Three most stable reference genes in various tissues during neutral, negative and positive energy balances.

	BestKeeper	NormFinder	geNorm	Concensus ranking
Mesenteric adipose tissue				
Beginning (Neutral energy balance)	G6PDH	GAPDH	GAPDH	B-actin
	B-actin	B-actin	B-actin	GAPDH
	GAPDH	PPIA	PPIA	PPIA
Middle (Negative energy balance)	GAPDH	GAPDH	GAPDH	GAPDH
	G6PDH	G6PDH	G6PDH	G6PDH
	B-actin	PPIA	PPIA	PPIA
End (Positive energy balance)	POLR2A	GAPDH	GAPDH	GAPDH
	G6PDH	PPIA	POLR2A	POLR2A
	B-actin	YWHAZ	B-actin	B-actin
Fat-tail adipose tissue				
Beginning (Neutral energy balance)	G6PDH	PPIA	PPIA	PPIA
	POLR2A	YWHAZ	YWHAZ	POLR2A
	PPIA	POLR2A	POLR2A	YWHAZ
Middle (Negative energy balance)	G6PDH	B-actin	B-actin	B-actin
	B-actin	G6PDH	G6PDH	G6PDH
	POLR2A	GAPDH	GAPDH	PGK1
End (Positive energy balance)	G6PDH	B-actin	PGK1	B-actin
	YWHAZ	PGK1	B-actin	YWHAZ
	B-actin	YWHAZ	PPIA	PGK1
All adipose tissue				
Beginning (Neutral energy balance)	G6PDH	PPIA	PPIA	PPIA
	B-actin	B2M	B2M	GAPDH
	POLR2A	GAPDH	GAPDH	B2M
Middle (Negative energy balance)	GAPDH	GAPDH	GAPDH	GAPDH
	B-actin	PPIA	B-actin	B-actin
	PPIA	YWHAZ	YWHAZ	PPIA

	BestKeeper	NormFinder	geNorm	Concensus ranking
End (Positive energy balance)	G6PDH	B-actin	B-actin	B-actin
	B-actin	GAPDH	POLR2A	POLR2A
	YWHAZ	PPIA	PPIA	GAPDH
Longissimus dorsi muscle				
Beginning (Neutral energy balance)	G6PDH	B-actin	B-actin	B2M
	B2M	B2M	B2M	B-actin
	YWHAZ	GAPDH	PGK1	GAPDH
Middle (Negative energy balance)	B-actin	B-actin	B-actin	B-actin
	PPIA	PPIA	PPIA	PPIA
	POLR2A	PGK1	PGK1	POLR2A
End (Positive energy balance)	POLR2A	G6PDH	G6PDH	G6PDH
	PPIA	B2M	PGK1	B2M
	B2M	PGK1	B-actin	PGK1

Discussion

To date, there is no study investigating the underlying mechanisms controlling adipose tissue metabolism including gene expression of regulators and enzymes in fat-tailed sheep breeds profoundly. RT-PCR/quantitative PCR is a sensitive and reliable analysis for investigation of biological pathways involved in tissue metabolism (Fan *et al*., 2013) which needs some stable reference genes as an internal normalization factor to depict changes in target genes expression. Researchers choose the reference genes from other carried out researches even in closed species which does not seem suitable as there is not any reference gene to be stable in all environmental and physiological conditions and also nutritional treatments. Negative energy balance is the consequence of increased demands, reduced intake or both which force the body to use its energy reserve to continue the vital metabolic pathways. Enhanced release of free fatty acids as a consequence of stimulated lipolysis in response to negative energy balance leads to increased plasma concentration of NEFA. In the current study, plasma NEFA concentration increased more than 4 fold at the end of week 3 compared to the beginning of the experiment which demonstrates that feed restriction was influential to induce negative energy balance.

The rankings of the candidate reference genes by NormFinder and geNorm software programs were similar in all studied tissues with some negligible differences, whereas ranking by BestKeeper software program was totally different from those of NormFinder and geNorm. For example, G6PDH was defined as the most stable reference gene in fat-tail and muscle tissues by BestKeeper software program, whereas by NormFinder and geNorm software programs it was considered as the least stable reference gene. The difference in ranking of the reference genes by different software programs is in agreement with Najafpanah *et al*. (2013) and Kaur *et*

al. (2018) who reported different ranking of candidate reference genes by NormFinder and geNorm software programs compared to BestKeeper in different tissues. The geNorm program ranks the candidate reference genes according to mean pairwise variation in a special candidate gene compared to all other candidate reference genes and represents it as M value and subsequently by stepwise elimination of gene with highest M value (Vandesompele *et al*., 2007). NormFinder uses an algorithm rooted in mathematical model of gene expression and a solid statistical framework to estimates both variation of inter and intra-group and provide a stability value for each candidate reference gene (Mallona *et al*., 2010), whereas BestKeeper determines the optimal reference genes by repeated pairwise correlation analysis (Pfaffl 2001). These differences in mathematical algorithms used by various software programs can explain the observed difference in ranking of the reference genes by different software programs.

Consensus ranking which is the average of candidate reference genes rank calculated by 3 software programs, showed that the 3 most stable genes defined for adipose tissues (GAPDH, PPIA and YWHAZ) are different from those defined for longissimus dorsi muscle (B-actin, YWHAZ and PGK1), except for YWHAZ which was among the 3 most stable reference genes in both adipose and muscle tissues. In the studies of Najafpanah *et al*. (2013) and Bonnet *et al*. (2013), a significant difference in stability of candidate reference genes among various tissues was reported in caprine and bovine respectively. Moreover, in mesenteric adipose tissue, GAPDH, B-actin and PPIA was the first 3 most stable reference genes defined by consensus ranking of 3 software programs, whereas in fat-tail adipose tissue, PPIA, YWHAZ and POLR2A were the most stable defined reference genes. In the study reported by Zhang *et al*. (2016), adipose tissues from different depots in rat had different most stable reference gene defined by software programs of NormFinder, geNorm and BestKeeper. The results of current study demonstrate that the stability of reference genes not only varies among different tissues, but also various depots of a special tissue such as adipose tissue can be influential on reference gene stability. Moreover, as it was shown in Fig. 3, Ct value of some reference genes including GAPDH, B-actin and PGK1 in longissimus dorsi muscle, PGK1 and G6PDH in fat-tail adipose tissue and B-actin and G6PDH in mesenteric adipose tissue were affected by induction of negative energy balance. These variation in Ct of reference genes can explain the difference in selection of different 3 most stable genes in different periods of energy balance. Gene expression of candidate reference genes was affected by physiological stage of dairy cows (Macabelli *et al*., 2014; Jatav *et al*., 2016). The results indicate that the stability of a reference gene can be affected by physiological status of the animal.

The results of the current study demonstrate that the stability of the reference genes varied between mesenteric and fat-tail adipose tissues and the level of energy balance affects the stability of the reference genes. Therefore, the ideal way for normalization of data related to RT-PCR/quantitative PCR is to define reference genes separately for different tissues and

various depots of tissues such as adipose tissue in every special experimental and environmental condition as the stability of the reference genes varies considerably in various environmental conditions. In addition, ranking of the reference genes differs among different software programs possibly due to different mathematical algorithms used by different programs, hence considering consensus ranking of all software programs would be more logical as it can consider all influential factors used by different software programs.

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Conflict of interest

The authors declared that there is no conflict of interest.

References

Bonnet M., Bernard L., Bes S. & Leroux C. (2013) Selection of reference genes for quantitative real-time PCR normalisation in adipose tissue, muscle, liver and mammary gland from ruminants. *anim*, 7, 1344-53. https:// doi.org/10.1017/S1751731113000475. PMID:23552195

Chomczynski P. & Sacchi N. (2006) The single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction: twenty-something years on. *Nat. Protoc*, 1, 581-5. https:// doi.org/10.1038/nprot.2006.83. PMID:17406285.

Fan C., Ma J., Guo Q., Li X., Wang H. & Lu M. (2013) Selection of reference genes for quantitative real-time PCR in bamboo (*Phyllostachys edulis*). PLOS ONE, 8, e56573. https:// doi.org/10.1371/journal.pone.0056573. PMID:23437174.

Jatav P., Sodhi M., Sharma A., Mann S., Kishore A., Shandilya U.K., Mohanty A.K., Kataria R.S., Yadav P. & Verma P. (2016) Identification of internal control genes in milk-derived mammary epithelial cells during lactation cycle of I ndian zebu cow. J Anim Sci, 87, 344-53. https://doi.org/10.1111/asj.12384. PMID:26762603.

Kaur R., Sodhi M., Sharma A., Sharma V.L., Verma P., Swami S.K., Kumari P. & Mukesh M. (2018) Selection of suitable reference genes for normalization of quantitative RT-PCR (RT-qPCR) expression data across twelve tissues of riverine buffaloes (*Bubalus bubalis*). PLOS ONE, 13, e0191558. https://doi.org/10.1371/journal.pone.0191558. PMID: 29509770.

Kibbe W.A. (2007) OligoCalc: an online oligonucleotide properties calculator. Nucleic Acids Res, 35, W43-W6. https:// doi.org/10.1093/nar/gkm234. PMID:17452344.

Kim I., Yang D., Tang X. & Carroll J.L. (2011) Reference gene validation for qPCR in rat carotid body during postnatal development. BMC Res Notes, 4, 1. https:// doi.org/10.1186/1756-0500-4-440. PMID:22023793.

Macabelli C.H., Ferreira R.M., Gimenes L.U., de Carvalho N.A.T., Soares J.G., Ayres H., Ferraz M.L., Watanabe Y.F., Watanabe O.Y. & Sangalli J.R. (2014) Reference gene selection for gene expression analysis of oocytes collected from dairy cattle and buffaloes during winter and summer. PLOS ONE, 9, e93287. https://doi.org/10.1371/journal.pone. PMID:24676354.

Mallona I., Lischewski S., Weiss J., Hause B. & Egea-Cortines M. (2010) Validation of reference genes for quantitative real-time PCR during leaf and flower development in *Petunia hybrida*. BMC Plant Biol, 10, 4. https:// doi.org/10.1186/1471-2229-10-4. PMID: 20056000.

Najafpanah M.J., Sadeghi M. & Bakhtiarizadeh M.R. (2013) Reference genes selection for quantitative real-time PCR using RankAggreg method in different tissues of Capra *hircus*. PLOS ONE, 8, e83041. https:// doi.org/10.1371/journal.pone.0083041. PMID:24358246.

Pfaffl M.W. (2001) A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res, 29, e45-e. https:// doi.org/10.1093/nar/29.9.e45. PMID:11328886.

Radonić A., Thulke S., Mackay I.M., Landt O., Siegert W. & Nitsche A. (2004) Guideline to reference gene selection for quantitative real-time PCR. Biochem Biophys Res Commun, 313, 856-62. https:// doi.org/10.1016/j.bbrc.2003.11.177. PMID:14706621.

SAS (2002) STAT user's guide: statistics. Cary, NC: Statistical Analysis System Institute Inc.

Svingen T., Letting H., Hadrup N., Hass U. & Vinggaard A.M. (2015) Selection of reference genes for quantitative RT-PCR (RT-qPCR) analysis of rat tissues under physiological and toxicological conditions. Peer J, 3, e855. https:// doi.org/10.7717/peerj.855. PMID: 25825680.

Untergasser A., Nijveen H., Rao X., Bisseling T., Geurts R. & Leunissen J.A. (2007) Primer3Plus, an enhanced web interface to Primer3. Nucleic Acids Res, 35, W71-W4. https:// doi.org/10.1093/nar/gkm306. PMID:17485472.

Vandesompele J., De Preter K., Pattyn F., Poppe B., Van Roy N., De Paepe A. & Speleman F. (2007) GeNorm software manual. Last updated on March 13.

Ye J., Coulouris G., Zaretskaya I., Cutcutache I., Rozen S. & Madden T.L. (2012) Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinform, 13, 134. https:// doi.org/10.1186/1471-2105-13-134. PMID:22708584.

Zhang W.-X., Fan J., Ma J., Rao Y.-S., Zhang L. & Yan Y.-E. (2016) Selection of suitable reference genes for quantitative Real-Time PCR normalization in three types of rat adipose tissue. Int J Mol Sci, 17, 968. https:// doi.org/10.3390/ijms17060968. PMID: 27338366.

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انتخاب ژن رفرنس در بافت‌های چربی و ماهیچه بره‌های دنبه‌دار لری‌بختیاری

علیرضا عزیزیان[۱]، مصطفی صادقی*[۱]، مهدی گنج‌خانلو[۱]، حسین ذکریاپور بهنمیری[۱]

[۱] گروه علوم دامی، پردیس کشاورزی و منابع طبیعی کرج، دانشگاه تهران، کرج، ایران

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چکیده

زمینه مطالعه: گوسفندان دنبه دار توانایی منحصر به فردی برای تحمل دوره‌های با تعادل منفی انرژی دارند. این توانایی به وجود بافت چربی دنبه به عنوان یک ذخیره انرژی بدنی ربط داده شد هرچند مکانیسم‌های کنترل کننده پاسخ بافت‌های چربی به نوسانات تعادل انرژی به خوبی مشخص نشده است.

هدف: با توجه به این امر که مشخص کردن ژن رفرنس پایدار یک پیش نیاز برای هرگونه مطالعه بیان ژنی است، مطالعه حاضر بر آن شد تا ژن‌های رفرنس با بیشترین میزان پایداری در بافت‌های چربی و ماهیچه بره های دنبه‌دار لری بختیاری در طول دوره‌های با تعادل منفی و مثبت انرژی را مشخص کند.

روش کار: در این آزمایش ۱۸ بره دنبه دار نر لری-بختیاری بر اســاس وزن بدن به ســه گروه تقسیم شدند. آزمایش شامل دوره‌های عادت‌پذیری، تعــادل منفــی و مثبت انرژی به ترتیب در ۲، ۳ و ۳ هفته بود. ۳ گروه از بره ها به ترتیب در انتهای دوره عادت پذیری، انتهای دوره با تعادل منفی انرژی و انتهای دوره با تعادل مثبت انرژی کشتار شدند و نمونههای بافت مختلف چربی و ماهیچه گرفته شد.

نتایــج: پایداری ژن‌های رفرنس در بین بافت های مختلف و همچنین بین مکان‌های مختلف بافت چربی متفاوت بود. میانگین رتبه‌بندی توسط نرم افزارهای مختلف نشان داد که B-actin، glyceraldehyde 3-phosphate dehydrogenase (GAPDH) و peptidylprolyl isomerase A (PPIA) جزء سه ژن با بیشترین پایداری در بافت چربی چادرینهای بودند در حالی که در بافت چربی دنبه ژن‌های PPIA، Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ) و RNA polymerase II subunit A (POLR2A) جزء ژن‌های با کمترین نوسان در بیان در طی دوره‌های با تعادل منفی و مثبت انرژی بودند. ژن‌های B-actin، YWHAZ و phosphoglycerate kinase 1 (PGK1) جزء ژن‌های با بیشترین ثبات در بیان در بافت عضله چشمی بره‌های لری بختیاری در تعادل‌های مختلف انرژی بودند.

نتیجه گیری نهایی: نتایج مطالعه حاضر نشان داد که ثبات بیان ژن‌های رفرنس در بین بافت‌های چربی چادرینه و دنبه متفاوت است و سطح تعادل انرژی باعث اثر بر میزان بیان آنها می‌شود. به علاوه، رتبه‌بندی ژن‌های رفرنس توسط نرم‌افزارهای مختلف متفاوت می‌باشد که این امر می‌تواند به دلیل الگوریتم‌های ریاضی متفاوت بکار گرفته شده توسط نرم‌افزارهای مختلف باشد.

واژه‌های کلیدی:

بافت چربی، تعادل انرژی، گوسفند دنبه دار، عظه چشمی، ژن رفرنس.

نویسندهٔ مسئول: مصطفی صادقی، گروه علوم دامی، پردیس کشاورزی و منابع طبیعی کرج، دانشگاه تهران، کرج، ایران. ایمیل: *sadeghimos@ut.ac.ir*