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Reference gene selection for reverse transcription quantitative polymerase chain reaction in chicken hypothalamus under different feeding status

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Summary

This study was designed to investigate the stability of 10 candidate reference genes, namely *ACTB*, *B2M*, *GAPDH*, *HMBS*, *LBR*, *POLR2B*, *RN18S*, *RPS17*, *TBP*, and *YWHAZ* for the normalization of gene expression data obtained by quantitative real-time polymerase chain reaction (qPCR) in studies related to feed intake of chicken. Samples were isolated from hypothalamus under three different nutritional status (ad libitum, fasted for 24 hr, fasted for 24 hr then refed for 2 hr). Five different algorithms were applied for the analysis of reference gene stability: BestKeeper, geNorm, NormFinder, the comparative ΔCt method, and a novel approach using multivariate linear mixed-effects modelling for stable reference gene selection. *TBP* and *POLR2B* were identified as the two most suitable and *B2M* and *RN18S* as the two least stable reference genes for normalization. Despite our review, the current literature showing that *RN18S* is one of the most commonly used reference gene in chicken gene expression studies, its applicability for normalization should be evaluated before each qPCR experiment.

KEYWORDS
expression stability, fasting, internal controls, normalization, quantitative real-time polymerase chain reaction, refeeding

1 | INTRODUCTION

Quantitative real-time polymerase chain reaction (qPCR) was introduced by Higuchi, Dollinger, Walsh, and Griffith (1992) as an improvement over classical end-point PCR. Despite the appearance of transcriptomic tools, it is still a method of choice when someone wants to study the gene expressional changes of a limited number of targets. Its advantages over other techniques are sensitivity, real-time detection of reaction kinetics, speed of quantification (often less than an hour), high throughput and precise measurement of starting material.

Variation in gene expression data can originate from two distinguishable sources. One is true biological variation caused by genotype, tissue differences and individual different response to experimental conditions. The second one is related to technical factors. Experimenters introduce variance into qPCR results by the cumulative effect of differences in the starting material, extraction yield, RNA quantity and quality, reverse transcription and during assay setup. Efforts should be made to correct for technical variability to reliably measure the gene expression changes. Among these, ensuring similar amounts for RNA isolation, and transcribing similar amount of

RNA, using internal controls, called reference genes (formally called housekeeping genes) can help to reduce technical variance. The use of internal reference genes (internal controls) is a preferred way to normalize qPCR results, because their expression levels are affected by all sources of introduced variation during qPCR workflow, the same way as the expression of target genes (Huggett, Dheda, Bustin, & Zumla, 2005; Kozera & Rapacz, 2013). However, several studies concluded that no universal reference gene exist (Chervoneva et al., 2010; Nascimento et al., 2015; Olias, Adam, Meyer, Scharff, & Gruber, 2014). The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) recommends that the justification of number and choice of reference genes is essential before publication of quantitative RT-qPCR experiments to avoid misleading results (Bustin et al., 2009). There are already some examples for this type of publications in various farm animal species (Cappelli et al., 2008; Ji et al., 2013; Macabelli et al., 2014; Park et al., 2015; Zhu, Lin, Liao, & Wang, 2015). In the case of chicken, studies investigated the stability of reference genes during inflammation in circulating leukocytes (De Boever, Vangestel, De Backer, Croubels, & Sys, 2008) between *pectoralis major*, *biceps femoris*, liver and abdominal fat (Bagés, Estany, Tor, & Pena, 2015), between lymphoid organs (Borowska, Rothwell, Bailey, Watson, & Kaiser, 2016), fibroblasts infected with different viruses (Kuchipudi et al., 2012; Yang, Lei, Rodriguez-Palacios, Tang, & Yue, 2013; Yin et al., 2011; Yue, Lei, Yang, Li, & Tang, 2010) and in *pectoralis major* under the effect of different lysine supplementation and post-hatch periods (Nascimento et al., 2015).

In animal production, feed intake has a great significance, as it determines growth and performance of livestock, consequently the efficiency of production. Therefore, searching and studying feeding-related molecular markers has a great interest in poultry species as well. Ad libitum feeding, fasting, and refeeding of chicken are typically used to study not only the expression of neuropeptides involved in feed intake regulation (Sintubin et al., 2014) but also the carbohydrate metabolism (Coudert et al., 2015), fat metabolism (Honda et al., 2016), and muscle physiology as well (Nakashima & Ishida, 2015). Feed intake and feeding behaviour of animals are primarily regulated by the function of hypothalamus in the central nervous system. It receives signals from peripheral organs and other parts of the brain and integrates them. The result is the expressional differences in orexigenic and anorexigenic neuropeptides encoding genes affecting feed intake (Denbow & Cline, 2012). There are currently no studies investigating the reference gene expression stability in chicken hypothalamus under different feed intake.

The expression stability of 10 candidate reference genes was investigated in the current study using five different algorithms. The most commonly used NormFinder, geNorm, BestKeeper, and the comparative ΔCt method, and a recently published method by Chervoneva et al. (2010) were applied. Currently, there is no consensus on which is the most appropriate tool for stable reference gene selection (Vandesompele, Kubista, & Pfaffl, 2009). Results from each approach were compared and the most appropriate genes were picked. Samples were obtained from chicken's hypothalamus from different feeding status namely after ad libitum fed, after fasted for 24 hr and after fasted for 24 hr followed by refeeding for 2 hr.

2 | MATERIALS AND METHODS

2.1 | Literature review

A literature review was performed using Web of Science v5.22 bibliographic database (available at https://webofknowledge.com/) with keywords "chick* AND gene AND expression NOT fasting" between 2015 and 2016 (n = 100) and "chick* AND gene AND expression AND fasting" 2010 and 2016 to obtain adequate number of publications (n = 42). Only original articles investigating chicken samples and those which used qPCR for gene expression analysis were processed. Papers aimed directly for stable reference gene identification were excluded from analysis. Papers were evaluated according to the justification of choice and number of reference genes, and the computing method for reference gene expression stability measurement.

2.2 | Ethical approval

The experiment was approved by the local ethical boards of University of Debrecen, Hungary (registration number: DEMAB/12-7/2015).

2.3 | Birds and experimental conditions, sample collection

One day old broiler type chickens (Ross 308) were obtained from a commercial hatchery. Chickens were sexed using DNA extracted from wing feathers (Malagó, Franco, Matheucci, Medaglia, & Henrique-Silva, 2002) and a PCR-based method according to Li et al. (2012). Male chickens were selected and allocated into ground pens. The ground pens had wood shavings and were equipped with bowl feeders and Plasson drinkers. Temperature was maintained at 32°C at placement using electrical heaters, then gradually decreased by 1.5°C/week to ensure bird's requirement. Lighting regime was implemented in accordance with the Ross 308 management manual. At day 28 chickens were divided into three treatment groups: ad libitum fed, fasted for 24 hr, fasted for 24 hr then refed for 2 hr. Control male chickens were fed ad libitum with broiler grower diet (briefly: 20% crude protein with metabolizable energy of 13 MJ/kg). Water was freely available for all groups during the whole experiment. After the fasting period eightirds were slaughtered from both groups, while the fasted groups were refed with grower diet for 2 hr. After refeeding, eight birds were also slaughtered from the F24R2 group. All chickens were terminated by concussion as recommended in 40/2013. (II. 14.) Hungarian act about animal experiments. Following decapitation, whole hypothalamus samples were macrodissected within 10 min according to Griffin, Flouriot, Sharp, Greene, and Gannon (2001). Samples were snap chilled in liquid nitrogen and were stored refrigerated at −70°C.

2.4 | RNA isolation and reverse transcription

Whole hypothalamus samples were comminuted in liquid nitrogen using a mortar and pestle. 25 ± 2.5 mg grinded tissue were lysed in TRI Reagent solution (Thermo Fisher Scientific, Waltham, MA, USA)

with an Ultra-Turrax T10 at setting 5 for 1 min for efficient lysis and shredding of gDNA. Total RNA extracted using Direct-zol™ RNA MiniPrep (Zymo Research, Orange, CA, USA) using an on-column DNase I digestion step with 30 U enzyme for 15 min. Elution was conducted once with 50 µl DNase/RNase-Free water pre-heated to 70°C. Isolated RNA amounts and purity (260/280 and 260/230 nm ratios) were quantified in eluent using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). RNA integrity was checked with 1% agarose gel electrophoresis. Reverse transcription was conducted using 800 ng total RNA with qPCRBIO cDNA Synthesis Kit (PCR Biosystems, London, UK) in 20 µl reaction volume with oligo(dT)s and random hexamers using MMLV type reverse transcriptase. Reaction conditions were as follows: reverse transcription at 42°C for 30 min and RTase denaturation at 85°C for 10 min. Prior to qPCR, cDNA samples were diluted 10-fold, then stored in −20°C.

2.5 | Quantitative real-time PCR assays

Reference gene selection was based on our literature review (Figure 2) and publications (Nascimento et al., 2015; Olias et al., 2014). Intron spanning (expect for *RN18S*), cDNA specific primers were designed using Primer Express v3.0.1 software (Table 1) and in silico tested with NCBI primer BLAST (Ye et al., 2012) to avoid false priming to pseudogenes or any other unexpected targets. Primers were also compared against NCBI SNP database to avoid variations in priming sites. For efficient amplification under fast cycling conditions and to make assay independent from RNA integrity amplicons were designed <120 bp (Fleige & Pfaffl, 2006). Real-time PCR reactions were run in triplicates, same targets under same run using 384-well plates (4titude, Surrey, UK) on a LightCycler 480 Instrument II (Roche Life Science, Penzberg, Germany). Annealing temperatures were optimized using a PTC-200 thermal cycler (Bio-Rad, Hercules, CA, USA) between 58 and 69°C. Plates were centrifuged at 1500 *g* for 2 min before loading into qPCR machine. Thermal cycling conditions were the followings: initial denaturation at 95°C for 2 min, 40 cycles of denaturation at 95°C for 5 s and annealing/extension at 60°C for 30 s. Real-time PCR reactions (10 µl) contained 4 ng of cDNA template, 1X Xceed qPCR SG Hi-ROX mastermix (Institute of Applied Biotechnologies, Praha, Czech Republic), and 200 nM of each primer. Primers were purified with standard desalting, stocks diluted in molecular biology grade water and stored at −20°C (Integrated DNA Technologies, Leuven, Belgium). No template controls were included to each assay and reverse transcriptase negative control in the case of *RN18S* to check for DNA contamination. Primer specificities were tested using melt profile analysis after each run by gradually increasing temperature from 60 to 95°C and also with agarose gel electrophoresis.

2.6 | Data analysis

Raw fluorescent data were collected with LightCycler software 1.5.0 (Roche Life Science), converted using LightCycler 480 text file converter into input format for LinRegPCR (both available at http://www.hartfaalcentrum.nl/index.php?main=files&sub=LinRegPCR). Mean reaction efficiencies (*E*) for each primer pair were determined with linear regression analysis on individual amplification curves using LinRegPCR version 2016.0 (Ruijter, van Velden, & Ilgun, 2009), and also the quantification cycle values (Cq). Technical replicates with Cq that deviated of more than 1 cycle from the other two were excluded from averaging process. Efficiency corrected Cq values were calculated using the equation: $Cq_{eff.corr.} = Cq \cdot [\log(E)/\log(2)]$ as described by Kubista (2007) where *E* is the qPCR efficiency. Reaction efficiencies were also evaluated with a serial 10-fold dilutions of a pool containing all cDNA samples, then PCR efficiencies were calculated using the equation $E = 10^{(-1/S)}$ where *S* is the slope from the standard curve generated from plotting the log10 of dilution factors against Cq values. Five different approaches were applied to search for stable reference genes. Analysis by two Excel Visual Basic Application, geNorm (v3.5) and NormFinder (v0.953), the BestKeeper (v1) and the comparative ΔCt method were conducted on Microsoft Excel 2010 (v14.7163.5). The method described by Chervoneva et al. (2010) was performed on SAS Studio release 3.5 (SAS Institute, Cary, NC, USA). Correlation matrix between the results of the five algorithms was also calculated using SAS Studio, with non-parametric Spearman rank correlation.

2.7 | BestKeeper

BestKeeper input requires non-normalized Cq values (Pfaffl, Tichopad, Prgomet, & Neuvians, 2004). Analysis of expression stability of reference gene starts with descriptive statistic. The method computes the geometric mean, arithmetic mean, minimal, and maximal Cq value, standard deviation (*SD*), and coefficient of variance (CV). The first estimation of reference gene stability can be done by inspecting the calculated *SD* and CV values. Most stably expressed reference gene exhibits lowest variance and the least stable shows the highest variance. The authors suggest that any studied gene with *SD* lower than 1 can be considered stably expressed. BestKeeper also combines reference genes into an index (normalization factor) and then compares the correlation between this index and the reference gene to calculate the correlation coefficient (*r*) value. It does not imply that any calculated measures are more important than others, so all potential reference genes were ranked according to *SD*, CV, and *r* values separately and then calculate the geometric mean of the rankings to determine their final rank.

2.8 | geNorm

This approach uses relative scale as input (Vandesompele et al., 2002). For this, from each candidate reference gene with the lowest Cq value was subtracted with the Cq value for each sample, and the difference is exponentiated with the base 2 using the formula: $\Delta Cq = (\min Cq - Cq)$. The resulting relative quantities range between 0 and 1 and served for input for further calculations. During reference gene ranking, the stepwise elimination of least stable gene occurs. First, the pairwise variation for each reference gene with all other reference genes is calculated as the standard deviation of the logarithmic transformed expression ratios. This followed by the calculation of a reference gene stability value (*M* value) which is the average pairwise variation of a single reference

TABLE 1 Details of primers and amplicons

Gene symbol	Sequence accession number	Chromosome	Description	Encoded protein's function	Primers (5′–3′ sequence of forward and reverse)	Reaction efficiency (*E*)[a]	Amplicon length (bp)	Amplicon length on gDNA (bp)	Amplicon Tm (°C)[b]
ACTB	NM_205518.1	14	Beta cytoskeletal actin	Component of the cytoskeletal microfilaments.	F: AGATCACAGCCCTGGCACCTAG R: TTGCGCTCAGGTGGGGCAAT	1.899	61	416	80.9
B2M	XM_015279077.1	10	Beta-2-microglobulin	Component of the major histocompatibility complex class I.	F: ATCCCGAGTTCTGAGCTGTGC R: CCGTCATACCCAGAAGTGCGAT	1.855	115	1098	83.2
GAPDH	NM_204305.1	1	Glyceraldehyde-3-phosphate dehydrogenase	Takes part in glycolysis.	F: GCTGGCATTGCACTGAATGAC R: CACTCCTTGGATGCCATGT	1.780	113	470	82.3
HMBS	XM_417846.5	24	Hydroxymethylbilane synthase	The third enzyme of the heme biosynthetic pathway.	F: GCCTGAAGGAAACAATGCAGA R: GCTGTGATGCCAACATGCT	1.823	91	543	81.2
LBR	NM_205342.1	3	Lamin B receptor	Localized in the nuclear envelope inner membrane.	F: AAGGCACCTGAGGAAGACCTA R: AAACTGCCAATACGAGGGTTC	1.865	98	1032	80.7
POLR2B	NM_001006448.1	4	Polymerase (RNA) II (DNA directed) polypeptide B	Subunit of the DNA-dependent RNA polymerase II.	F: ACACGTATGAATGCCGGGGA R: AGGTGCAATGCTCATAGACATCA	1.902	110	1262	82.1
RN18S	AF173612.1	Unmapped	18S ribosomal RNA gene	Structural RNA of the small component of cytoplasmic ribosomes.	F: CTCTTTCTCGATTCCGTGGGT R: CATGCCAGAGTCTCGTTCGT	1.869	96	96 (single exon)	82.9
RPS17	NM_204217.1	10	Ribosomal protein S17	Ribosomal protein, component of small subunit.	F: TCCGGGGTATCTCCATTAAGCTG R: CATTTCCTTGGTGTCCGGGTC	1.896	119	1177	85.7
TBP	NM_205103.1	3	TATA box binding protein	Component of transcription factor IID.	F: ATCAAGCCAAGAATTGTTCTGC R: CTTCGTAGATTTCTGCTCGAACT	1.833	85	981	78.2
YWHAZ	NM_001031343.1	2	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta	Adapter protein in different general and specialized signalling pathways.	F: AGTCATACAAAGACAGCACGCTA R: GCTTCATCTCCTTGGGTATCCGA	1.872	85	416	79.6

[a]Determined with LinRegPCR (range between 1 and 2, 2 is equals to 100% efficiency).
[b]Determined with melt curve analysis.

gene with all other tested candidate reference genes. The lower of this value, the more stably expressed is a reference gene. As a cut-off value *M* was set to 0.5 as recommended for homogenous samples.

2.9 | NormFinder

This method described by Andersen, Jensen, and Ørntoft (2004) is a model-based approach that enables calculation not only the overall expression variation of the candidate reference genes, but also the variation between subgroups of the same sample set. It combines the intra- and intergroup variations to calculate a stability value (Sv) for each individual gene, which represents a practical measure of the systematic error that will be introduced when using the investigated gene. Candidate reference genes can be ranked according to the stability value, where the lowest values correspond to the most stable genes. This method requires a sample set minimally containing eight samples per group and 5–10 candidate reference genes are recommended. It requires relative quantities as input same as for geNorm.

2.10 | The comparative ΔCt method

This approach, described by Silver, Best, Jiang, and Thein (2006) uses relatively easy mathematical methodology for stable reference gene selection. First Cq values for each reference gene were normalized with a selected reference gene's Cq value, generating ΔCq values for each sample. The process is continued with the next selected reference gene, then with all the remaining, until ΔCqs are acquired for every combination. After this, *SD* values are calculated for every gene with each sample, followed by the averaging of mean *SD* values. Reference gene with lowest *SD* proposed to be the most stable, and the gene with the highest *SD* is the least stable.

2.11 | A model-based approach by Chervoneva et al. (2010)

This statistical model-based method requires gene expression levels before log transformation as input. The approach creates unstructured covariance matrix of all available candidate reference genes by estimations from a multivariate linear mixed-effects model. Robustness is achieved through bootstrapping (sampled with replacement) candidate reference genes data to obtain the bootstrap upper confidence limits for the variances of the normalizing factors (geometric means) for all possible gene subsets as well as the distribution of ranks of these variances. Then all possible subsets of reference genes are ranked from the smallest to the largest variance of the corresponding log geometric mean. This new approach identifies gene subset(s) with smaller variability of normalizing factors than current standard approaches when there is some non-trivial innate correlation among the candidate genes. Starting number for the bootstrap steps were $n = 1$, and desired number of $n = 1000$ as recommended by the authors. This approach was also used to select the optimal number of reference genes. The advantage of using more than one for normalization is that the variation of the geometric average of multiple genes is smaller than the variation of a single gene (Kozera & Rapacz, 2013; Vandesompele et al., 2002).

3 | RESULTS

3.1 | qPCR optimization

The annealing temperature at 60°C resulted a single strong band for each amplicon (between 61 and 119 bp) confirmed by agarose gel electrophoresis without detectable primer dimers or signs of non-specific amplification. Melt curve analysis further confirmed the specificity of primers with the same results (Figure 1). Amplification efficiencies (Table 1) were between 1.780 and 1.902 and considered as acceptable for each gene with coefficient of determination (R^2) higher than .995. Efficiencies derived from standard curve method were between 1.921 and 2.099 with R^2 higher than .990. Underestimation of qPCR efficiencies obtained by linear regression of the exponential phase were also reported by Robledo et al. (2014) and Bagés et al. (2015), therefore, we used efficiencies calculated with LinRegPCR because its results are not affected by the dilution steps involved in standard curve construction, which also dilutes potential inhibitors and might lead to efficiency overestimations ($E > 2$). No template controls were included in each assay and did not show any Cq value throughout the study nor the reverse transcriptase negative controls for *RN18S*.

3.2 | MIQE compliance of chicken gene expression studies

In the case of overall expression studies ($n = 100$) in chicken between 2015 and 2016, 12% of them stated that the used gene(s) is stable during the experimental conditions, but only three of those used methods like geNorm and NormFinder for evaluation. Their statement based mainly on results from earlier publications. In contrast, 88% of the papers do not address the question of reference gene stability (or do not wish to publish data). The most frequently used genes were *ACTB*, *GAPDH*, *RN18S*, with 37.2%, 35.5%, and 7.4% respectively (Figure 2a). 87% used one reference gene for normalization, 9% used two, 2% used three, 1% of them used five and 1% used six.

In the case of experiments related to fasting ($n = 42$), 19% of the publications stated that the used gene(s) is stable during the experimental conditions, but only one provided data of any software (geNorm) analysis (Serr, Suh, & Lee, 2011). In contrast, 81%-of the publications do not address the question of reference gene stability (or do not publish data). The most often used genes were also *ACTB*, *GAPDH*, and *RN18S* in this type of experiments, with 31.9%, 27.7% and 23.4% respectively (Figure 2b). 9.5% of publication used two reference genes for normalization, while 90.5% used one gene (Table S1).

3.3 | Descriptive statistic of the 10 candidate reference gene

The expression profiles of the 10 reference genes were evaluated as quantification cycles with qPCR in the chicken hypothalamus

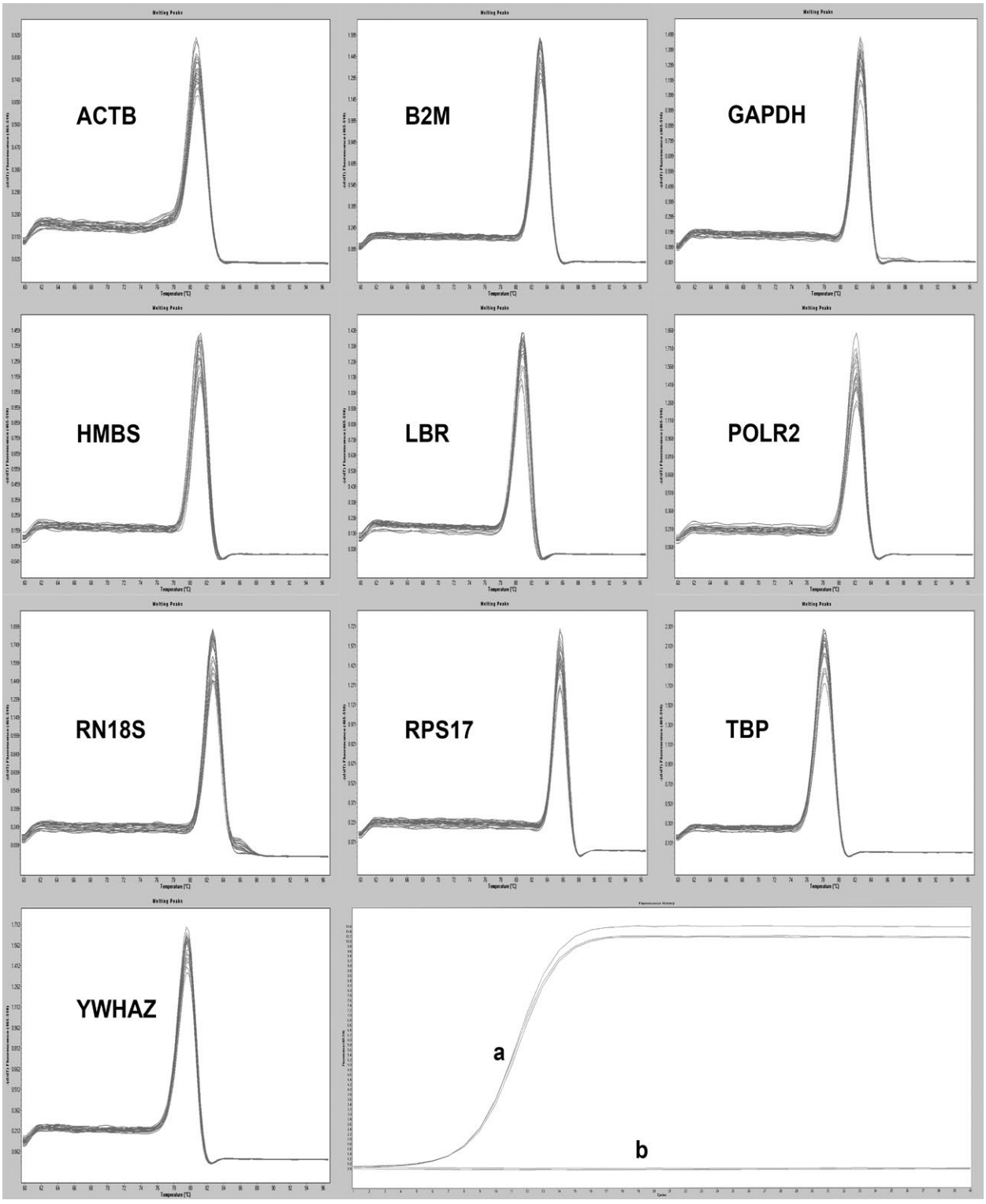

FIGURE 1 Specificity of qPCR amplification observed by melt curve analysis. The plots for 10 reference genes reveal unique melt peaks, suggesting specific reactions. *Y*-axis represents negative derivative of fluorescence over temperature (−d*F*/d*T*) and *X* represents temperature (°C). Amplification plot of a positive reaction (a), *RN18S* in this case (measured in triplicates) and RT^- for *RN18S* (b) show no amplification, indicating no gDNA amplification. *Y*-axis represents fluorescence and *X* represents PCR cycles (1–40)

(Figure 3) under ad libitum, fasted for 24 hr and fasted for 24 hr then refed for 2 hr conditions ($n = 8$ in each group). Under pooled conditions, Cq values ranged from 6.54 (*RN18S*) to 23.87 (*TBP*) in hypothalamus (Table 2). The wide range of Cq values suggested that these genes have different expression profiles, from the most abundant *RN18S* to the least abundant *TBP*. In the case of coefficient of variance *TBP* showed the narrowest variance (CV = 1.25%) and *RN18S* showed the highest one (CV = 6.62%).

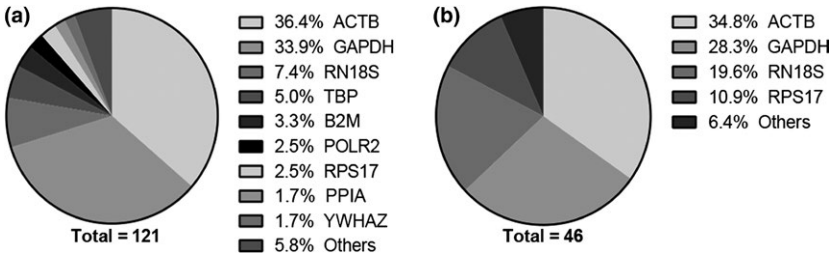

FIGURE 2 Percentage of different reference genes used in publications. It was found that *ACTB*, *GAPDH*, and *RN18S* are far the most frequently used ones in chicken relative gene expression studies overall (a) and in fasting experiments as well (b)

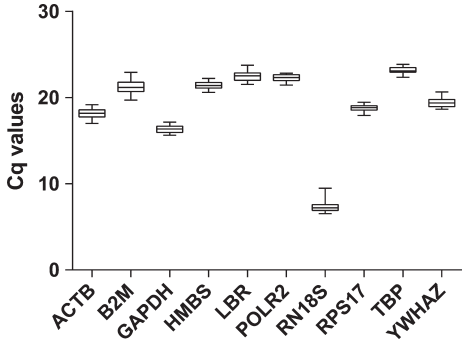

FIGURE 3 Mean quantification cycle (Cq) values for 10 candidate reference genes expressed in hypothalamus. Cq values of each reference gene from all samples (n = 24) are plotted in a box and whisker diagram. Boxes represent the interquartile interval (25–75%) with median value (50%), whiskers represent minimum and maximum values. *RN*18S showed very high expression with mean Cq below 10 as expected. Four genes (*ACTB*, *GAPDH*, *RPS*17, and *YWHAZ*) showed high expression with mean Cqs under 20. Five genes (*B2M*, *HMBS*, *LBR*, *POLR*2B, and *TBP*) showed moderate expression with mean Cqs above 20

3.4 | Gene expression stability of reference genes

Reference gene expression stability was analysed with five different algorithms and ranked them under pooled, ad libitum vs. fasted for 24 hr; fasted for 24 hr vs. fasted for 24 hr then refed for 2 hr; ad libitum vs. fasted for 24 hr then refed for 2 hr experimental conditions, ranking details are given in Table 3 and 4.

3.5 | Results under pooled conditions

Results by BestKeeper showed that under pooled conditions all genes had *SD* lower than 1. *B2M* showed the highest *SD* (0.66) and *TBP* the lowest (0.29). Highest correlation was observed with BestKeeper index in the case of *ACTB* (0.908) and the lowest of *POLR2B* (0.681). Ranking by geometric meaning of ranks of each indicator summarized in Table 3. According to geNorm analysis under pooled conditions, all genes reached the 0.5 cut-off criteria. Lowest stability was observed for *B2M* and *RN18S* with *M* value 0.423 and 0.476 respectively. Otherwise *TBP* and *RPS17* were considered as the most stably expressed genes. Analysis by the method described by Chervoneva et al. (2010) resulted with lowest mean rank of geometric mean variance for *TBP* (8.42) and the second lowest for *POLR2B* (36.32) and are considered as most stable. In the case of *B2M* and *RN18S*, both were identified as the two least stable genes. The optimal number of reference genes are suggested to be two, because the mean rank of geometric mean variance for *TBP* and *POLR2B* is lowest (2.95) when used in combination instead of using *TBP* only (8.42). Lowest stability value was observed for *POLR2B* (0.096) and *GAPDH* (0.103) when data were analysed with NormFinder, but in this case, *TBP* ranked only for third (0.114). *RN18S* (0.189) and *B2M* (0.220) were scored with the highest values, similarly to geNorm analysis, which means low expressional stability. With the comparative ΔCt method, *TBP* (0.39) and *RPS17* (0.40) were evaluated as most stable ones and similarly to the other four methods, *RN18S* (0.69) and *B2M* (0.65) proved to be the least stable reference gene candidates. Correlation analysis between the five algorithms was summarized in Table 5. A moderate, non-significant correlation was

TABLE 2 Descriptive statistics of ten candidate reference genes based on their quantification cycle (Cq) values under pooled experimental condition, obtained by BestKeeper

	ACTB	*B2M*	*GAPDH*	*HMBS*	*LBR*	*POLR2B*	*RN18S*	*RPS17*	*TBP*	*YWHAZ*
n	24	24	24	24	24	24	24	24	24	24
geo. Mean (Cq)	18.17	21.21	16.35	21.42	22.47	22.30	7.33	18.80	23.18	19.44
ar. Mean (Cq)	18.17	21.22	16.36	21.42	22.48	22.30	7.36	18.80	23.18	19.45
Min. (Cq)	17.00	19.71	15.65	20.61	21.55	21.47	6.54	17.95	22.36	18.66
Max. (Cq)	19.19	22.94	17.17	22.24	23.76	22.85	9.48	19.47	23.87	20.66
SD (±Cq)	.44	.66	.34	.34	.47	.33	.49	.30	.29	.47
CV (%Cq)	2.42	3.09	2.11	1.61	2.09	1.48	6.62	1.61	1.25	2.40
coeff. of corr. (*r*)	.908	.848	.737	.763	.880	.681	.741	.800	.825	.718

n, sample number; geo. Mean (Cq), geometric Cq mean; ar. Mean (Cq), arithmetic Cq mean; min. and max. (Cq), minimal and maximal Cq values; *SD* (±Cq), Cq standard deviation; CV (%Cq), coefficient of variance expressed as a percentage on the Cq level; coeff. of corr. (*r*), Pearson correlation coefficient with BestKeeper index.

TABLE 3 Summary of gene stability rankings (values in parentheses) of the five different methods under pooled conditions. Two of the most stable genes are shown in bold

Gene	Overall rank[a]	Best Keeper[b]	geNorm	Model-based[c]	NormFinder	ΔCt method
ACTB	6	3	7 (0.332)	8 (779.39)	6 (0.136)	6 (0.42)
B2M	9	8	9 (0.423)	9 (982.30)	10 (0.220)	9 (0.65)
GAPDH	5	7	5 (0.266)	6 (581.32)	2 (0.103)	5 (0.42)
HMBS	4	6	3 (0.231)	3 (189.67)	4 (0.116)	3 (0.40)
LBR	7	5	8 (0.362)	5 (542.01)	7 (0.138)	7 (0.47)
POLR2B	**2**	4	4 (0.239)	2 (36.32)	1 (0.096)	4 (0.42)
RN18S	10	10	10 (0.476)	10 (1023.00)	9 (0.189)	10 (0.69)
RPS17	3	2	1 (0.215)	4 (215.08)	5 (0.124)	2 (0.40)
TBP	**1**	1	1 (0.215)	1 (8.42)	3 (0.114)	1 (0.39)
YWHAZ	8	9	6 (0.301)	7 (725.94)	8 (0.164)	8 (0.49)

[a]Obtained by ranking the geometric average of the five rankings (as described by Chen, Pan, Xiao, Farwell, & Zhang, 2011).
[b]BestKeeper sub-results are summarized in Table 2.
[c]Method by Chervoneva et al. (2010).

TABLE 4 Ranking of reference gene expression stability in the hypothalamus in contrast to nutritional status. Two of the most stable genes are shown in bold

Gene	Pooled conditions	Ad libitum vs. fasted for 24 hr	Fasted for 24 hr vs. fasted for 24 hr then refed for 2 hr	Ad libitum vs. refed for 2 hr
ACTB	6	5	4	6
B2M	9	9	8	9
GAPDH	5	6	6	5
HMBS	4	4	3	**2**
LBR	7	7	4	7
POLR2B	**2**	3	**2**	4
RN18S	10	10	9	10
RPS17	3	**2**	5	3
TBP	**1**	**1**	**1**	**1**
YWHAZ	8	8	7	8

TABLE 5 Spearman correlation matrix between the five different methods. Correlation values are indicated between each approach and *p* values are given in parenthesis

Method	BestKeeper	geNorm	Model-based[a]	NormFinder	ΔCt method
BestKeeper	1	0.705 (0.023)	0.709 (0.022)	0.576 (0.082)	0.830 (0.003)
geNorm		1	0.863 (0.001)	0.748 (0.013)	0.960 (<0.001)
Model-based			1	0.794 (0.006)	0.891 (0.001)
NormFinder				1	0.794 (0.006)
ΔCt method					1

[a]Method by Chervoneva et al. (2010).

observed between ranks by NormFinder and BestKeeper indicating high discrepancy in scoring between the two methods. A very strong positive correlation (r = .964) was observed between the geNorm and ΔCt method indicating highest similarity in scoring. Strong positive correlation was observed (r > .7) in all the remaining comparisons.

When data from ad libitum vs. fasted for 24 hr conditions were analysed, it was found that *TBP* and *RPS17* were superior in stability in relation to others under these conditions, but *RN18S* and *B2M* were the least stable. When they were fasted for 24 hr vs. fasted for 24 hr then refed for 2 hr nutritional status were considered, *TBP* and *POLR2B* proved to be the most stable and *RN18S* and *B2M* the least stable genes. When investigating the ad libitum vs. fasted for 24 hr then refed for 2 hr the most stable genes were *TBP* and *HMBS*, but *B2M* and *RN18S* were proved again to be the most unreliable (breakdown can be found in Table S2). Overall stability (pooled conditions) of *TBP* and *POLR2B* was the highest, while *B2M* and *RN18S* ranked the worst.

3.6 | Optimal number of reference genes for normalization

The optimal number of reference genes for normalization was evaluated with the method by Chervoneva et al. (2010) with 1000 bootstrapped

TABLE 6 Ten different gene subsets with the lowest Mean rank of Var(GM) obtained with the method described by Chervoneva et al. (2010)

Set size	*ACTB*	*B2M*	*GAPDH*	*HMBS*	*LBR*	*POLR2B*	*RN18S*	*RPS17*	*TBP*	*YWHAZ*	Mean rank of Var(GM)[a]	Final rank
2	0[b]	0	0	0	0	1	0	0	1	0	2.95	1
3	0	0	0	1	0	1	0	0	1	0	7.98	2
1	0	0	0	0	0	0	0	0	1	0	8.42	3
3	0	0	0	0	0	1	0	1	1	0	12.33	4
3	0	0	0	0	1	1	0	0	1	0	14.78	5
4	0	0	0	0	1	1	0	1	1	0	15.42	6
4	0	0	0	1	1	1	0	0	1	0	15.84	7
4	0	0	0	1	0	1	0	1	1	0	17.53	8
2	0	0	0	1	0	0	0	0	1	0	18.34	9
5	0	0	0	1	1	1	0	1	1	0	18.64	10

[a]Mean rank of the geometric mean variation.
[b]1 Indicates that the corresponding gene is included in the subset and 0 if it is not included.

data sets using all Cq values (pooled conditions). Beside GeNorm and NormFinder, it can also select optimal number of reference genes, but with smaller empirical variability of normalizing factors. According to results with subset size ranged 1–5 (Table 6) the optimal subset of reference genes should contain two genes, *TBP* and *POLR2B* because their lowest value for Mean rank of Var(GM). Using the most stable (*TBP*) gene alone should not be optimal in this case because its mean rank of variance is higher (8.42), than it is used in combination with *POLR2B* (2.95).

4 | DISCUSSION

Fasting and refeeding experiments are widely used for understanding chicken feed intake regulation and carbohydrate, fat metabolism and muscle physiology as well. Quantitative real-time polymerase chain reaction is especially useful to study neuropeptides which are often expressed in small abundance, and commercial antibodies are not always available to study them. Even in the hand of a careful experimenter, qPCR results are affected by technical variations which can obscure real gene expressional changes. With the process called normalization, this technically related variation can be corrected. The use of reference genes is the preferred way for normalization as they can be used as internal controls. An ideal reference gene should be expressed at a stable level regardless of different experimental conditions or treatments. However, no universal reference gene exists because some variation is always observed therefore a careful selection of candidates is required for a reliable normalization to avoid misinterpretation of results. Bustin et al. (2009) published the MIQE guidelines to help improve the reliability and transparency of qPCR results and also made suggestions for reference gene selection. Literature review was performed to see how chicken gene expression studies comply with these guidelines in regard of reference genes. This review revealed that all the investigated publications (n = 142) used at least one reference gene for normalization of qPCR results. It can be concluded that, since the release of MIQE the research community already understood the need for normalization and the use of reference genes is favoured in chicken experiments over other alternatives (artificial molecules, gDNA, total RNA, etc.). Unfortunately, this compliance is only partial, because most of them do not question the reference gene stability; instead use mostly "classical" reference genes, assuming that their expression remains unaffected by all the experimental conditions. There are only a limited number of studies in which reference gene stability was investigated in chicken including these often used genes. Borowska et al. (2016) investigated six reference gene's stabilities including *GAPDH* and *ACTB* in chicken lymphoid tissues under stimulation of three different mitogens. They found that *GAPDH* and *TBP* were the two most stable while *ACTB* performed poorly when investigated with NormFinder and geNorm. De Boever et al. (2008) found that the use of *GAPDH* should be avoided to normalize the expression of pro-inflammatory cytokines in a lipopolysaccharide inflammation model in chickens while *ACTB* appeared to be a reliable reference gene. NormFinder and BestKeeper analysis revealed that *RN18S* is the most stable gene in virus-infected chicken lung cells while *ACTB* and *GAPDH* stability were highly affected (Kuchipudi et al., 2012). Studying the H5N1 level in chicken embryo fibroblasts *ACTB* was the most stable and performed better than *GAPDH* (Yue et al., 2010). In chicken embryo fibroblasts infected with Newcastle disease virus, *ACTB* was valuable as its expression remained stable in relation to others, while *RN18S* and *GAPDH* were considerably regulated during infection (Yin et al., 2011). Nascimento et al. (2015) investigated the stability of thirteen reference genes and found that *ACTB* and *GAPDH* showed moderate gene expression stability during different lysine supplementation while some less commonly used reference genes showed better overall stability, such as *HMBS* and *HPRT1*. On the basis of the aforementioned examples, it is clear that, the expression of the most commonly used reference genes is not stable under some experimental conditions and should not be used without experimental validation. We analysed the expression stability of 10 reference genes including the most commonly used *ACTB*, *GAPDH*, and *RN18S* with five different approaches under three different feeding status in chicken hypothalamus. Results showed that *RN18S* and *B2M* were consistently the most

variable reference genes in the hypothalamus under all feeding status, whereas *TBP* was scored among the most stable under all conditions. The optimal number of reference genes needed for qPCR analyses were determined by a method as described Chervoneva et al. (2010) and led the conclusion that the combination of *TBP* and *POLR2B* is adequate and favoured over other combinations for accurate normalization. *TBP* encodes the TATA-binding protein which is a transcriptional factor, part of the RNA polymerase II pre-initiation complex and required for basal transcription in eukaryotic cells (Boeger et al., 2005; Tora, 2002). *POLR2B* encodes the second largest subunit of RNA polymerase II, which is required for synthesizing messenger RNAs in eukaryotes. As the two gene products are essential for basic cell functions it seems that their expression regulation is independent of nutritional status in the chicken hypothalamus. The commonly used *ACTB* and *GAPDH* showed moderate expressional stability; meanwhile *RN18S* was the least stable among them and should not be used under these experimental conditions for normalization. Several problems associated with the use of *RN18S*, for instance it encodes the 18S rRNA ribosomal subunit and shows higher abundance than mRNAs (along with 28S rRNA, forms the most of total RNA). It is transcribed by RNA polymerase I, in contrast to RNA polymerase II which transcribes mRNAs, this allows independent regulation mechanism to take part (Radonić et al., 2004). In technical point of view, RN18S is also problematic, because for highly abundant targets it is necessary to use manual baseline settings for accurate Cq determination in older real-time thermal cyclers which makes the data analysis more complicated. Genes encoding ribosomal proteins often has enhanced stability over others when examined in the same tissue type (De Jonge et al., 2007). Indeed *RPS17* encoding ribosomal protein S17 ranked relatively good in hypothalamus in our experiment. However, Thorrez et al. (2008) found that they exhibit important tissue-dependent variation in mRNA expression and therefore they cannot be considered as universal reference genes. Correlating the scoring results, obtained from five different algorithm, strong relation was observed between the NormFinder and the recently described method by Chervoneva et al. (2010) as expected, since both methods use statistical linear mixed-effects modelling (MANOVA) for stable reference gene selection. Strong correlation was observed between the other ranking algorithms, except NormFinder and BestKeeper which can be explained by the very different scoring algorithm used by the two approaches. Quantitative polymerase chain reaction is still a method of choice for gene expression analysis, however, apart from a few examples it is difficult to find information for suitable reference genes for chicken studies. To the best of our knowledge, this is the first paper which identifies suitable reference genes in hypothalamus for chicken nutritional status related qPCR experiments.

5 | CONCLUSION

This study recommends the following: the geometric average of the most stable *TBP* and *POLR2B* genes are suggested to be used for normalization in chicken hypothalamus under different feeding status; despite their common appearance in literature, *ACTB*, *GAPDH* and *RN18S* are not the most stable reference genes and should not be used for accurate normalization of qPCR results from chicken hypothalamus samples related to feed intake regulation experiments. Other reference genes may exist beside the validated candidates, but their applicability should be evaluated with statistical approaches before any gene expression studies.

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