Investigating reference genes for quantitative real-time PCR analysis across four chicken tissues

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Summary

Accurate normalisation of data is required to correct for different efficiencies and errors during the processing of samples in reverse transcription PCR analysis. The chicken is one of the main livestock species and its genome was one of the first reported and used in large scale transcriptomic analysis. Despite this, the chicken has not been investigated regarding the identification of reference genes suitable for the quantitative PCR analysis of growth and fattening genes. In this study, five candidate reference genes (*B2M*, *RPL32*, *SDHA*, *TBP* and *YWHAZ*) were evaluated to determine the most stable internal reference for quantitative PCR normalization in the two main commercial muscles (*pectoralis major* (breast) and *biceps femoris* (thigh)), liver and abdominal fat. Four statistical methods (geNorm, NormFinder, CV and BestKeeper) were used in the evaluation of the most suitable combination of reference genes. Additionally, a comprehensive ranking was established with the RefFinder tool. This analysis identified *YWHAZ* and *TBP* as the recommended combination for the analysis of *biceps femoris* and liver, *YWHAZ* and *RPL32* for *pectoralis major* and *RPL32* and *B2M* for abdominal fat and across-tissue studies. The final ranking for each tool changed slightly but overall the results, and most particularly the ability to discard the least robust candidates, were consistent between tools. The selection and number of reference genes was validated using *SCD*, a target gene related to fat metabolism. Overall, the results can be directly used to quantitate target gene expression in different tissues or in validation studies from larger transcriptomic experiments.

Keywords: gene expression; normalisation; endogenous control; expression stability; housekeeping gene; lipid metabolism

Introduction

Chicken production is spread worldwide and represents one of the main sources of dietary protein (in the form of meat and eggs) in the world. Chicken is an ideal model for examining animal growth trait development, which has increased spectacularly over the last 30 years. A 2001-strain broiler was estimated to have reached 1,815 g body weight at 32 days of age with a food conversion of 1.47, whereas a 1957-strain would not have reached that weight until 101 days of age with a food conversion of 4.42 (Havenstein et al., 2003). Unfortunately this growth rate is accompanied by increased body fat deposition, high mortality and high incidence of metabolic diseases and skeletal disorders (Julian, 2005). The genetic mechanisms of chicken growth traits have been studied using quantitative trait loci mapping through genome-scan and candidate gene approaches, genome-wide association studies (GWAS), comparative genomic strategies, microRNA and epigenomic analysis (reviewed in Xu et al. (2013)). Current trends of integration of genetics and functional genomics (in the form of analysis of global gene expression data using microarray or RNA-seq technology) will help characterising genes that play central roles in the processes leading to rapid growth. In this scenario, quantitative real-time PCR (qPCR) is the preferred independent method for the validation of global expression studies (VanGuilder et al., 2008).

Given its sensitivity, specificity, accuracy, large dynamic range of linear quantification, cost and its speed, qPCR has also become the more accepted standard for nucleic acid quantification. Nowadays this is a commonly available method in most molecular biology laboratories that can be used to detect even low abundant mRNAs and slight variation in gene expression levels. Nevertheless, the reliability of the final quantification result depends heavily on all elements in the workflow, such as the quality of the input template (RNA integrity and absence of inhibitors), reverse

transcription and qPCR efficiencies. To account for these and avoid bias, accuracy of qPCR relies on normalisation to an internal reference gene. Ideally, the expression of a reference gene should remain constant in all tissues analysed and under every experimental condition. Appropriate validation of reference genes in any new experimental system is therefore crucial. Moreover, it is today generally accepted that normalization to a single reference gene is clearly suboptimal for accurate data interpretation (Vandesompele et al., 2009). Currently, the use of multiple internal control genes is considered as an essential approach for an accurate normalization of data, which stresses the need to identify several candidate normalisation controls.

While the evaluation of expression stability of potential reference genes has been addressed on growth and fattening-related tissues for species such as pigs (McBryan et al., 2010), beef (Bonnet et al., 2013) and fish (Fuentes et al., 2013), reports in chicken have mostly concentrated on the immune cells (De Boever et al., 2008; Yue et al., 2010; Kuchipudi et al., 2012; Yang et al., 2013). Therefore, there is a general lack of information regarding suitable reference genes for qPCR analysis of target genes expressed in skeletal muscle, fat and liver. In the present study we have tested five commonly used reference genes for gene expression stability in the two most economically-important muscles in chicken (breast and thigh), liver and abdominal fat. Four algorithms have been used to assess the suitability of these control genes individually in each tissue and across tissues.

Materials and Methods

1.1 Animal material

Samples of *pectoralis major* (breast), *biceps femoris* (thigh), liver and abdominal fat were collected from 32 ISA Brown hens at 32 weeks of age. Animals were slaughter by

exsanguination and tissues collected within the following 15 minutes. Samples were immediately frozen in liquid nitrogen and then stored at -80ºC until analysis. All experimental procedures were approved by the Animal Ethics Committee from the Catalan Government (reference code: 7304 and 7305).

1.2 RNA isolation and retrotranscription

Liver and muscle samples (0.5 g approximately) were ground with mortar and pestle in liquid nitrogen and homogenized with a mechanical homogenizer (IKA Ultra-turrax T10, IKA-Werke GmbH) with a 5 mm rotor. RNA was isolated by the acid phenol method (Chomczynski and Sacchi, 1987) using the TRI Reagent (Sigma-Aldrich). RNA was quantified by a Nanodrop ND-1000 spectrophotometer and checked for integrity in formaldehyde-agarose gels.

Reverse transcription to cDNA was performed from 1.5 µg of DNase-treated (Turbo DNA-free, Ambion/LifeTechnologies) RNA with 50 U of Maxima H-Minus Reverse Transcriptase (Fermentas GmbH), in 1 x enzyme buffer, 0.5 mM dNTPs. 50 pmol of random hexamers, 50 pmol Oligo(dT) primer. The reactions were incubated at 25ºC for 5 min, 50ºC for 30 min and 85ºC for 10 min. Upon completion of the reactions, cDNA samples were diluted 1:30 with H_2O prior to expression analysis and stored at -40ºC.

1.3 Real-time Quantitative PCR (qPCR) analysis

Five potential reference genes were chosen for being frequently used as endogenous controls in expression studies in other livestock species (Erkens et al., 2006; McBryan et al., 2010; Damon et al., 2012), paying close attention to selecting genes that belong to different functional classes (Table 1): beta-2 microglobulin (*B2M*), ribosomal protein

L32 (*RPL32*), succinate dehydrogenase complex, subunit A, flavoprotein (Fp) (*SDHA*), TATA box binding protein (*TBP*) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (*YWHAZ*). For each gene, a set of primers was designed with Primer3Plus tool (www.bioinformatics.nl/primer3plus) using the qPCR default parameters (Table 1). Primer pairs were designed so as to fall in different exons, as inferred from chicken gene organization data available in Ensembl (www.ensembl.org, chicken genome assembly Galgal4), and to amplify a fragment of less than 150 bp (Table 1). Fleige and Pfaffl (2006) demonstrated that real-time qPCR based on short amplicons (in the range of 70-250 bp) is independent of RNA integrity and therefore give more accurate results than longer amplicons. The amplification reaction was performed in triplicate in a total volume of 8 µL containing 1x Maxima SYBR Green/ROX qPCR Master Mix (Fermentas), 200 nM of forward and reverse primers and 3 µL of 30-fold diluted cDNA as template. Amplification of the cDNA was achieved on an ABI 7500 Real Time PCR System (LifeTechnologies) following the manufacturer's conditions: an initial activation and denaturation step of 10 min at 95°C followed by 40 cycles consisting of 10 s at 95°C and 1 min at 60°C. Additionally, a dissociation curve protocol was run after every reaction in order to control the specificity of the amplified product.

Two different approaches were tested to determine the amplification efficiencies of the qPCR assay. Assay efficiency was evaluated with a serial 10-fold dilution of a pool of 12 cDNAs from the experiment (three from each tissue). These were used to generate standard curves for the five genes analysed. PCR efficiency (E) was calculated as follows:

$$E = (10^{(1/-S)} - 1)x100$$

where S is the slope from the standard curve (Hellemans et al., 2007).

In addition, individual efficiency of the assays was estimated with the statistical algorithm Real-time PCR Miner (Zhao and Fernald, 2005) using the raw fluorescence data as input.

1.4 Analysis of gene expression stability

Gene expression stability was evaluated with four different statistical algorithms: *BestKeeper* (Pfaffl et al., 2004), *geNorm* (Vandesompele et al., 2002), *NormFinder* (Andersen et al., 2004) and the comparative delta-Ct method (Silver et al., 2006). The four methods make use of the cycle threshold (Ct) values to determine the most stably expressed genes. *BestKeeper* analyses the inter-gene relationship, calculating the Pearson correlation coefficient (r), the probability and the sample integrity and the expression stability within each reference gene with an intrinsic variance of expression (Pfaffl et al., 2004). Data from the genes showing higher correlation values are combined to compute the geometric mean of Ct values (BestKeeper Index). Next, the Pearson's correlation coefficient between each candidate reference gene and the index (r_I) is calculated, which gives and estimation of the contribution of the gene to the BestKeeper Index. *GeNorm* determines the pairwise variation of a particular gene with all other control genes as the standard deviation of the logarithmically transformed expression ratios. A measure of internal control gene-stability (*M)* is defined by *GeNorm* as the average of the pairwise variation of one gene with all the other potential reference genes (Vandesompele et al., 2002). The lower the *M* value, the more stable the expression of that gene is. To select the best performing reference genes, the program recalculates the *M* stability measures after removal of the least stable gene and repeats the process until only the two most stable genes remain (Vandesompele et al., 2002). To test the minimum number of reference genes needed for adequate data normalization,

geNorm calculates a pairwise variation (*V*) between using n (number) and n+1 reference genes. Large *V* values indicate a significant effect of the additional gene on data normalization and endorse the need of including this gene among the controls. On the other hand, *NormFinder* (Andersen et al., 2004) is a model-based approach that enables estimation not only of the overall variation of the candidate normalization genes, but also of the variation between subgroups of the same sample set. *NormFinder* combines the intra- and intergroup variation to estimate, for each individual gene, a stability value (*Sv*), which represents a practical measure of the systematic error that will be introduced when using the investigated gene. Candidate reference genes can then be ranked according to the *Sv* value, where the lowest values correspond to the most stable genes. The *NormqPCR* and *ReadqPCR* bioconductor packages were used to compute the geNorm and Normfinder gene stability values in R. The comparative delta-Ct method compares Ct values from two candidate reference genes within each sample. The mean and SD from these data are computed. If the ΔCt value between the two genes remains constant when analysed in different samples, it means both genes are stably expressed (co-regulated) among those samples. If the ΔCt fluctuates, this indicates that one or both genes are variably expressed. The comparison is repeated for all gene-pairs and the mean of the SD is calculated (Silver et al., 2006). Finally, the Reffinder application (http://www.leonxie.com/referencegene.php) was used to assess the overall ranking for the five genes. Reffinder computes the rankings for each of the four methods above, assigns a weight to each gene according to its ranked position and calculates the geometric mean of the weights to produce an overall final ranking.

1.5 Validation of reference gene analysis

One gene of interest coding for the fatty acid desaturase stearoyl-coA desaturase (*SCD*) was used to validate the selected reference genes. Primers were designed using Primer3Plus software as described above (Table 1). The experimental procedure was the same as used in the selection of reference genes. The relative expression level of the target gene was calculated with different normalization factors based on the most stable gene, the most unstable gene or the geometric mean of the two most stable genes and the two most unstable genes.

Results

Data normalization using a set of reference genes is nowadays a current and crucial procedure when analysing the expression levels of target transcripts by qPCR in different tissues or under different conditions. In the present study, the transcript abundance of five potential reference genes was assessed in chicken tissues related to fat deposition and growth. A total of 128 cDNA samples (32 animals x 4 target tissues) were analysed.

2.1 Real-time qPCR experiment and PCR efficiency

Real-time qPCR was used to estimate the RNA transcription level of the five candidate reference genes in four adult tissues. Specificity of amplification and the absence of primer dimer formation were supported by the analysis of melting curves. The PCR efficiency of each primer pair was first calculated through the standard curve method in a pool RNA samples representing three biological replicates of each tissue. Results from the standard curve method were then compared with the efficiency value obtained through the algorithm RT-PCR Miner. In our hands, the efficiency of the qPCR assays did not differ substantially between the five genes. (Supplementary Table 1).

Efficiencies calculated by RT-PCR Miner were lower (range 81-88%) than those obtained with the standard curve method (range 97-108%). However, all genes gave comparable efficiencies within each method. The standard curve method is time consuming, requiring the production of repeatable and reliable standards (Pfaffl, 2001) and relies on the assumption that the PCR efficiency of each amplicon is constant in all samples, which rarely can be achieved in real experiments, strongly influencing Ct-based stability or quantification analyses. Therefore, RT-PCR Miner algorithm, which uses the single raw fluorescence data as an input (Zhao and Fernald, 2005), is a useful alternative to calculate the PCR efficiency for each primer pair in each tissue type.
The Ct values (number of cycles needed for the fluorescence to reach a specific threshold level of detection) were used directly in all analyses. The range of Ct values for each gene varied for each of the four tissues analysed (Supplementary Table 2). Abdominal fat and breast muscle gave more disperse Ct values than liver and thigh muscle. *RPL32* was the most abundant gene with a mean Ct in all tissues of 21.34, *B2M* and *SDHA* had intermediate expression levels (mean Ct 25.83 and 25.32, respectively) and *TBP* and *YWHAZ* were the least abundant (mean Ct 29.51 and 28.09, respectively).

2.2 Gene expression stability analysis

The gene expression stability of candidate reference genes was evaluated by using different approaches. Analyses using the RefFinder integration tool demonstrated an overall comprehensive ranking of reference genes integrated from four different algorithms. The reference genes calculated on the basis of different algorithms are presented in Table 2 and are ranked from the most stable to the least stable genes. Based on the rankings from each algorithm and its stability values, the geometric mean of the weights of individual genes was calculated by RefFinder for an overall final ranking for

each individual tissue. Also, records from all samples were pooled together to analyse gene expression stability across the whole sample set.

Overall, ranks obtained with the geNorm, Normfinder and Comparative Ct algorithms were more similar (particularly in pointing out the most stable genes) than rankings performed by Bestkeeper (Table 2). This is due to the fact that the first three algorithms use different measures of variation of Ct values while the Bestkeeper analysis is based on the correlation of Ct values between genes.

The optimal number of genes that are necessary for accurate normalization was determined for the whole set of samples with the geNorm algorithm (Figure 1). The pair-wise variation of two sequential normalization factors (Vn/n+1) shows that two reference genes are sufficient for the calculation of the normalization factor in all tissues analysed, since the V2/3 values were in the range of 0.012-0.026, which is below the cut off value of 0.15 suggested by the developers to include an additional reference gene (Vandesompele et al., 2009). The low Vn/n+1 value showed that the inclusion of additional reference genes had no significant effect on the normalisation of target genes and claims for the use the two most stable genes.

2.3. Validation of the selection and the number of candidate genes.

The conclusions from the analyses described above were applied to quantify the transcript level of a gene of interest. The expression of the fatty acid desaturase-encoding gene *SCD* was measured in the same 32 samples. The relative expression levels of this gene have been previously evaluated in 14 chicken tissues by Dridi et al. (2007).

For each tissue, normalisation of the expression levels was performed with different reference gene combinations corresponding to the one or two most stably and one or two most unstably expressed genes according to the RefFinder ranking (Table 2). The relative values obtained (Figure 2) agree with the expression of this gene reported in the literature where, in chickens, liver and fat express higher *SCD* levels than breast muscle (Dridi et al., 2007). Estimated expression in thigh was higher in the latter than with our data. Using only the most stable gene or the geometric mean of the two most stable to normalise the data did not change the relative levels between tissues (Figure 2). Even the geometric mean of the two most unstable genes was able to produce similar results. In contrast, using only the worst performing gene in each tissue changed the tissue expression profile altogether (Figure 2). In all tissues, there was a clear increase in the dispersion of data with the use of the most unstable reference gene.

Discussion

In order to select the most suitable reference for gene expression normalisation by real-time qPCR in chicken we analysed several tissues related to growth and fat deposition, including two muscles (*pectoralis major* and *biceps femoris*), liver and abdominal fat. The two muscles were selected as representative of quality cuts in the chicken (breast and thigh). Moreover, in chicken, *pectoralis major* is a particularly lean muscle, while *biceps femoris* is one of the fattest muscles (1.23% and 5.08% of intramuscular fat/ fresh meat, respectively; (Novello et al., 2009)). Therefore, they represent extremes in term of fat deposition events in muscle.

Case by case validation and the use of at least two validated reference genes involved in distinct cellular functions has been proposed by different studies, since no single gene can act as a universal reference. Therefore, five potential reference genes involved in

different biological roles, such as cytoskeleton structure (*B2M*), ribosomal complexes (*RPL32*), basal transcription (*TBP*), signal transduction (*YWHAZ*) and the citric acid cycle (*SDHA*), were assessed using several statistical approaches for the normalization of data.

The systematic validation of candidate genes demonstrated that none of them performed consistently well for all sample types and that the stability of the genes varied according to the tissue analysed (Table 2). Overall, from the different statistical algorithms used, geNorm, NormFinder and the comparative Ct method generated similar reference gene rankings, while BestKeepers gave slightly different results. It is now widely established that normalising against one single reference gene is clearly insufficient to account for all the small change in sample processing, particularly when comparing across tissues or treatments. Therefore, the use of two or more normalising genes is highly recommended. Is our case, the stepwise variation analysis performed with geNorm suggests that two reference genes are enough to normalise our data as including additional reference genes does not improve the variation coefficient and does not add a substantial contribution to the normalisation factor.

According to the comprehensive ranking provided by RefFinder, *YWHAZ* was the most stably expressed gene in the two muscles analysed and in liver. *TBP* was the second most stable gene in *biceps femoris* and liver and *RPL32* was so in *pectoralis major* (Table 2). The combination *B2M*/*RPL32* was the best option to normalise data from abdominal fat or across tissues. In many cases the difference between 2nd and 3rd position in the ranking was minimal. Therefore, other combinations including the 3rd classified gene are also possible. The most important outcome of reference gene validation studies is to be able to exclude bad performers, which would add dispersion to the data, as shown in our validation experiment were the expression of the *SCD* gene,

encoding for a fatty acid desaturase enzyme, was assessed with different combination of reference genes. Some of the variation in expression levels of the reference genes tested may be due to the role of the gene in specific tissues. A classical examples of this is the experiment performed by Barber et al. (2005). The authors tested the gene stability for the *GAPDH* gene in 72 human tissues or cell types and found up to 14-fold differences of expression between some of them. In our case, the role of *SDHA* in the oxidation of succinate, a substrate of the citric acid cycle, may account for the differences in expression levels between the tissues analysed. Also, the levels of the cytoskeleton protein *B2M* are likely to differ between tissues, according to the structure of the cells. Other factors affecting the stability of reference genes are disease and infection, developmental stage, stress and environmental factors such as diet or temperature (Kozera and Rapacz, 2013). Therefore, when samples differ in any of these factors, the candidate reference genes need to be validated first.

In conclusion, this study is the first attempt to identify reference genes in several chicken tissues related to growth and fat deposition. We conclude that *YWHAZ* and *TBP* are the most stable genes in *ms. biceps femoris* and liver, *YWHAZ* and *RPL32* in *ms. pectoralis major* and *B2M* and *RPL32* in abdominal fat and also in expression studies across tissues. These selected references genes were further validated in the transcriptional quantification of a target gene, known to be expressed preferentially in chicken liver and fat, and at lower levels, in muscle. These results should be a starting point to analyse the level of expression of genes related to growth and fat deposition in chicken or even be used in validation studies from large transcriptomic and genomic experiments.

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Table 1. Selection of candidate reference genes and validation gene, and sequence of primers used for the real-time quantitative PCR experiment

Gene Symbol	Gene Name	Main function	Primer	Sequence 5'→3'	Exon	Amplicon length
B2M	beta-2 microglobulin	Cytoskeletal protein involved in cell locomotion	Fw	GTGCTGGTGACCCTGGTG	E1	113 bp
			Rv	CAGTTGAGGACGTTCTTGGTG	E2	
RPL32	guanine nucleotide binding protein (G protein), ribosomal protein L32	Ribosomal protein that is a component of the 60S subunit	Fw	ATGGGAGCAACAAGAAGACG	E3	139 bp
			Rv	TTGGAAGACACGTTGTGAGC	E4	
SDHA	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	Involved in the oxidation of succinate, citric acid cycle	Fw	TCTGTCCATGGTGCTAATCG	E10	126 bp
			Rv	TGGTTTAATGGAGGGGACTG	E11	
TBP	TATA box binding protein	Basal transcription machinery. Coordinates initiation of transcription in core promoters	Fw	CCGGAATCATGGATCAGAAC	E2	85 bp
			Rv	GGAATTCCAGGAGTCATTGC	E3	
YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	Signal transduction	Fw	TTGCTGCTGGAGATGACAAG	E2	61 bp
			Rv	CTTCTTGATACGCCTGTTG	E3	
SCD	stearoyl-coA desaturase	Fatty acid desaturase, n-9	Fw	AGGCTGACAAAGTGGTGATG	E4	137 bp
			Rv	GATGGCTGGAATGAAGAAGC	E5	

Table 2. Integrated table of reference gene expression stability values as calculated by four different statistical methods.

	BestKeeper		geNorm		NormFinder		Comparative Ct		RefFinder	
	Gene	*Stab. Value*	*Gene*	*Stab. Value*	*Gene*	*Stab. Value*	*Gene*	*Average SD*	*Gene*	*Geomean*
biceps femoris	*TBP*	0.957	*TBP*	0.034	*TBP*	0.072	*TBP*	0.702	*TBP*	1.000
	YWHAZ	0.944	*YWHAZ*	0.034	*YWHAZ*	0.115	*RPL32*	0.770	*YWHAZ*	1.861
	RPL32	0.938	*B2M*	0.042	*RPL32*	0.121	*YWHAZ*	0.798	*RPL32*	2.711
	SDHA	0.927	*RPL32*	0.051	*B2M*	0.170	*B2M*	0.916	*B2M*	4.472
	B2M	0.868	*SDHA*	0.062	*SDHA*	0.199	*SDHA*	0.988	*SDHA*	3.557
pectoralis major	*SDHA*	0.990	*YWHAZ*	0.068	*YWHAZ*	0.377	*YWHAZ*	1.914	*YWHAZ*	1.316
	RPL32	0.977	*B2M*	0.068	*RPL32*	0.451	*RPL32*	2.027	*RPL32*	2.213
	YWHAZ	0.975	*SDHA*	0.084	*SDHA*	0.498	*B2M*	2.575	*SDHA*	2.340
	TBP	0.829	*RPL32*	0.108	*B2M*	1.147	*TBP*	2.752	*B2M*	2.783
	B2M	0.765	*TBP*	0.134	*TBP*	1.215	*SDHA*	5.846	*TBP*	4.229
Liver	*YWHAZ*	0.953	*YWHAZ*	0.027	*YWHAZ*	0.155	*YWHAZ*	0.679	*YWHAZ*	1.000
	RPL32	0.952	*TBP*	0.027	*TBP*	0.167	*TBP*	0.710	*TBP*	1.861
	TBP	0.943	*SDHA*	0.040	*RPL32*	0.247	*RPL32*	0.736	*RPL32*	2.449
	SDHA	0.924	*RPL32*	0.051	*SDHA*	0.258	*SDHA*	0.764	*SDHA*	3.722
	B2M	0.389	*B2M*	0.084	*B2M*	0.419	*B2M*	1.524	*B2M*	4.729
Abdominal Fat	*RPL32*	0.947	*TBP*	0.072	*YWHAZ*	0.296	*B2M*	1.374	*B2M*	1.565
	YWHAZ	0.943	*B2M*	0.072	*B2M*	0.342	*RPL32*	1.390	*RPL32*	1.861
	B2M	0.936	*RPL32*	0.082	*RPL32*	0.344	*TBP*	1.438	*YWHAZ*	2.213
	SDHA	0.933	*YWHAZ*	0.088	*TBP*	0.450	*YWHAZ*	1.523	*TBP*	2.783
	TBP	0.908	*SDHA*	0.101	*SDHA*	0.559	*SDHA*	1.929	*SDHA*	4.472

All tissues	*B2M*	1.630	*B2M*	0.070	*RPL32*	0.421	*RPL32*	2.190	*RPL32*	1.190
	RPL32	1.680	*YWHAZ*	0.070	*B2M*	0.458	*B2M*	2.360	*B2M*	1.410
	SDHA	2.080	*RPL32*	0.085	*YWHAZ*	0.490	*TBP*	2.560	*SDHA*	3.460
	YWHAZ	2.100	*SDHA*	0.117	*TBP*	0.744	*SDHA*	2.570	*TBP*	3.660
	TBP	2.210	*TBP*	0.121	*SDHA*	0.789	*YWHAZ*	3.500	*YWHAZ*	4.730

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Legends to Figures

Figure 1. Determination of the optimal number of reference genes for data normalization according to the geNorm software.

Pairwaise variation (Vn/n+1) analysis between the normalization factors NFn and NFn+1, carried out for all the samples (ALL) and in individual tissues (PM – *ms. pectoralis major*; BF – *ms. biceps femoris*; liver; and abdominal fat)

Figure 2. Use of *SCD* to validate the selection of reference genes. Relative expression levels of *SCD* in muscles *pectoralis major* (PM), *biceps femoris* (BF), liver and fat. Data were normalised against the most stable, the least stable or the geometric mean of the two most stable or the two least stable genes ranked by RefFinder in each tissue. Data are expressed as mean and standard error of relative expression units.

Figure 1

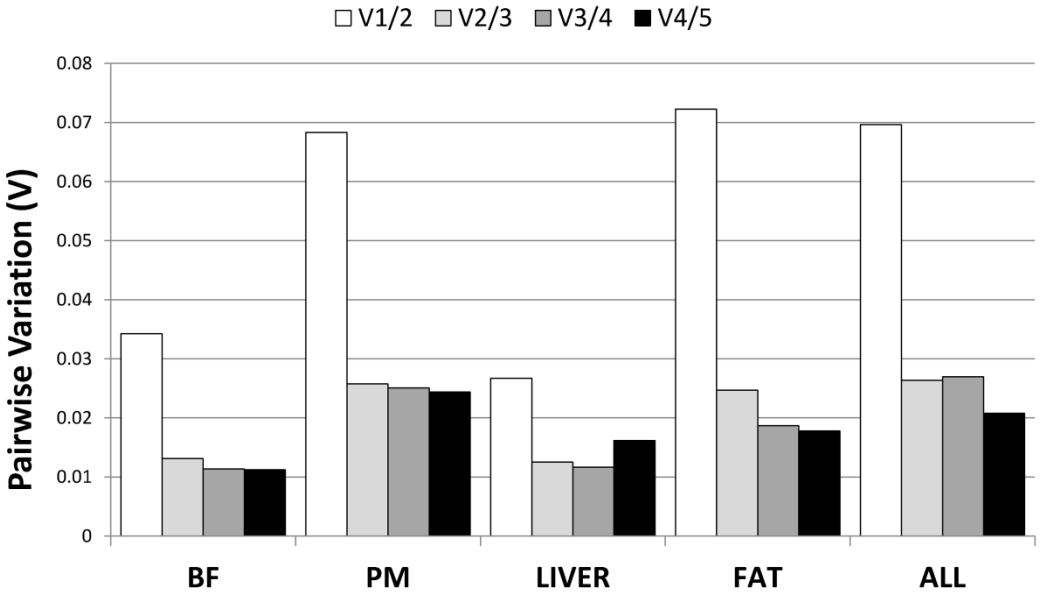
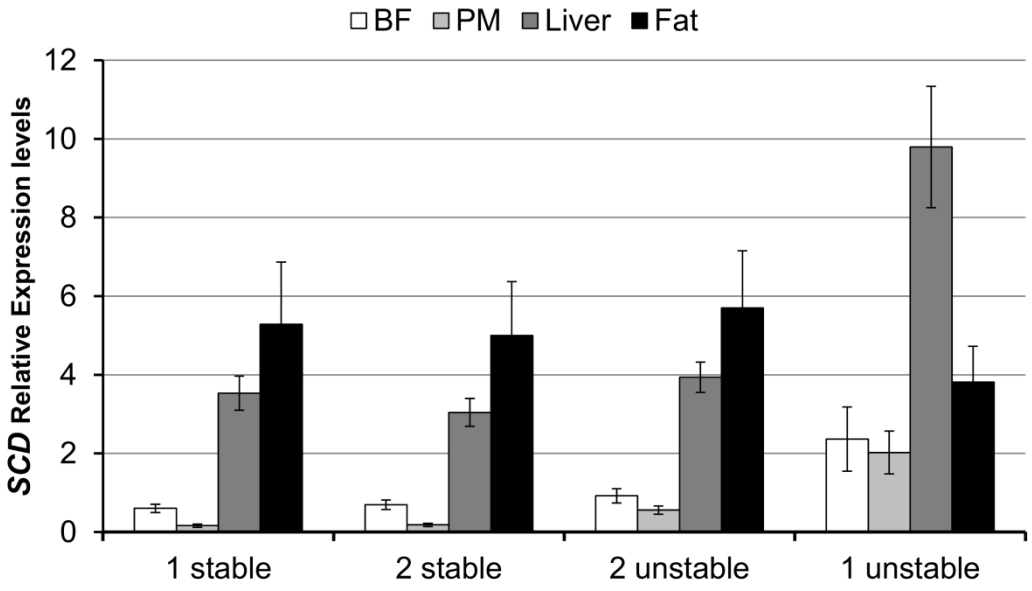

V1/2
V2/3
V3/4
V4/5
Pairwise Variation (V)
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
BF
PM
LIVER
FAT
ALL

Figure 2


BF
PM
Liver
Fat
SCD Relative Expression levels
12
10
8
6
4
2
0
1 stable
2 stable
2 unstable
1 unstable

Supplementary Table 1. Comparison of PCR efficiencies by the standard curve method and the RT-PCR Miner algorithm. For the standard curve method, quality parameters are indicated for each candidate reference gene and the PCR efficiency was calculated from the slope of the standard curve.

		Candidate reference gene				
Standard curve		***B2M***	***RPL32***	***SDHA***	***TBP***	***YWHAZ***
Slope		-3.153	-3.373	-3.265	-3.261	-3.137
R^2		0.996	0.998	0.998	0.990	0.996
PCR efficiency		107.59%	97.93%	102.45%	102.62%	108.36%
RT-PCR Miner		***B2M***	***RPL32***	***SDHA***	***TBP***	***YWHAZ***
PCR efficiency	*biceps femoris*	83.30%	85.10%	85.74%	84.58%	82.83%
	pectoralis major	84.19%	85.91%	85.03%	85.07%	82.09%
	Liver	82.90%	84.72%	86.55%	84.86%	82.21%
	Abdominal Fat	84.03%	85.41%	88.88%	86.79%	81.73%
	Average – all tissues	83.61%	85.29%	86.55%	85.33%	82.22%

Supplementary Table 2. Mean ± SD for the Ct values obtained for each gene and tissue in the real-time quantitative PCR experiment.

	Reference genes – Ct values				
	B2M	***RPL32***	***SDHA***	***TBP***	***YWHAZ***
pectoralis major	25.96±2.04	21.70±2.92	24.63±2.99	26.14±3.31	28.31±2.66
biceps femoris	26.47±1.09	21.86±1.40	24.10±1.73	30.92±1.27	28.78±1.48
Liver	26.04±1.01	22.17±1.53	26.75±1.46	30.30±1.26	28.50±1.24
Abdominal Fat	23.11±2.37	19.51±2.24	25.49±3.40	27.23±2.38	25.67±2.80
All tissues	25.83±2.16	21.34±2.33	25.32±2.59	29.51±2.69	28.09±2.54

Highlights

- Reference genes are necessary to validate expression data by quantitative PCR.
- We validated reference genes in chicken breast and thigh muscle, liver and fat.
- Gene stability studies rule out least stable genes (worse performers).
- Differences in the top ranking are not so critical for accurate expression analysis.