

RESEARCH ARTICLE

# Identification of endogenous normalizing genes for expression studies in inguinal ring tissue for scrotal hernias in pigs

William Raphael Lorenzetti<sup>1,2</sup>, Adriana Mercia Guaratini Ibelli<sup>2,3</sup>, Jane de Oliveira Peixoto<sup>2</sup>, Marcos Antonio Zanella Mores<sup>2</sup>, Igor Ricardo Savoldi<sup>3</sup>, Kamilla Bleil do Carmo<sup>3</sup>, Haniel Cedraz de Oliveira<sup>4</sup>, Mônica Corrêa Ledur<sup>1,2\*</sup>

**1** Programa de Pós-graduação em Zootecnia, Centro de Educação Superior do Oeste (CEO), Universidade do Estado de Santa Catarina, UDESC, Chapecó, Santa Catarina, Brazil, **2** Embrapa Suínos e Aves, Concórdia, Santa Catarina, Brazil, **3** Universidade do Contestado, Concórdia, Santa Catarina, Brazil, **4** Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil

\* [monica.ledur@embrapa.br](mailto:monica.ledur@embrapa.br)



**OPEN ACCESS**

**Citation:** Lorenzetti WR, Ibelli AMG, Peixoto JdO, Mores MAZ, Savoldi IR, Carmo KBd, et al. (2018) Identification of endogenous normalizing genes for expression studies in inguinal ring tissue for scrotal hernias in pigs. PLoS ONE 13(9): e0204348. <https://doi.org/10.1371/journal.pone.0204348>

**Editor:** Ramona Natacha Pena i Subirà, Universitat de Lleida, SPAIN

**Received:** April 18, 2018

**Accepted:** September 6, 2018

**Published:** September 20, 2018

**Copyright:** © 2018 Lorenzetti et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Data Availability Statement:** All relevant data are within the paper and its Supporting Information files.

**Funding:** This study was supported by project #476146/2013-5 from the National Council of Scientific and Technological Development (CNPq) to MCL in which she is a CNPq fellow. WR Lorenzetti was sponsored by a PROMOP/Udesc scholarship. IR Savoldi and KB do Carmo are recipients of a PIBIC/CNPq scholarship at Embrapa Swine and Poultry National Research Center. The

## Abstract

The use of reference genes is required for relative quantification in gene expression analysis and since the stability of these genes could be variable depending on the experimental design, it has become indispensable to test the reliability of endogenous genes. Therefore, this study evaluated 10 reference candidate genes in two different experimental conditions in order to obtain stable genes to be used as reference in expression studies related to scrotal hernias in pigs. Two independent experiments were performed: one with 30 days-old MS115 pigs and the other with 60 days-old Landrace pigs. The inguinal ring/canal was collected, frozen and further submitted to real-time PCR analysis (qPCR). For the reference genes stability evaluation, four tools were used: GeNorm in the SLqPCR, BestKeeper, NormFinder and Comparative CT. A general ranking was generated using the BruteAggreg function of R environment. In this study, the *RPL19* was one of the most reliable endogenous genes for both experiments. The breed/age effects influenced the expression stability of candidate reference genes evaluated in the inguinal ring of pigs. Therefore, this study reinforces the importance of evaluating the stability of several endogenous genes previous their use, since a consensual set of reference genes is not easily obtained. Here, two sets of genes are recommended: *RPL19*, *RPL32* and *H3F3A* for 30-days MS115 and *PPIA* and *RPL19* for the 60 days-old Landrace pigs. This is the first study using the inguinal ring tissue and the results can be useful as an indicative for other studies working with gene expression in this tissue.

## Introduction

The real time PCR (qPCR) is one of the main approaches used for gene expression studies, being highly sensitive [1]. However, many factors related to this technique, since the quality of biological material up to the laboratorial procedures, might compromise the reliability of the

fundamental role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing interests:** The authors have declared that no competing interests exist.

qPCR results [2]. Furthermore, qPCR is a powerful technique to validate differentially expressed genes from global expression approaches, such as microarrays and, more recently, RNA-Seq [3]. Therefore, it is essential to standardize the methodologies to be used and, specifically considering gene expression studies using qPCR, the correct choice and use of reference genes, also known as endogenous genes, avoid mistaken results. The use of stable endogenous reference genes ensures that any variation in input RNA levels between samples is normalized, avoiding errors in the quantification [2]. Thus, knowing the behavior of these genes in each experimental design is crucial to obtain reliable results [1,4].

To be considered a valid reference gene its expression must not be variable between different experimental conditions, tissues or physiological state of the tissue or organism [2]. In relative gene expression analyses, the use of reference genes is required to normalize and obtain the fold-change, through mathematical algorithms, such as those described previously by Pfaffl (2001), Livak & Schmittgen (2001) and Schmittgen & Livak (2008) [5–7]. Some of the most well-known reference genes are *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase), *PGK* (phosphoglycerate kinase), *UBQ* (ubiquitin), *RPL19* (ribosomal protein L19), *18S rRNA* (ribosomal RNA 18S),  $\beta$ -actin and  $\beta$ -tubulin [1] and they have been used in several studies, in many species, including pigs. However, the stability of the reference genes can be altered depending on the tissue, age, treatment and other conditions, which makes indispensable to test the stability of several genes before using those as reference [8–10]. Several studies searching for reliable endogenous genes in pigs have been reported [11–14] with different breeds, tissues and conditions. However, studies aiming to verify stable reference genes in the inguinal ring for scrotal hernia studies have not been reported to date.

The scrotal hernia is a malformation whereby intestinal loops traverse the abnormally open inguinal ring [15]. Although there are indications about the involvement of genetic components in the occurrence of this anomaly in humans [16,17] and other species [16], including the pig [18,19], the genes affecting this condition remain unknown. Therefore, expression studies are required to clarify the genetic mechanism involved in this malformation. Most of the expression studies searching for reference genes are based on muscular tissues with better characterized anatomy, such as *longissimus dorsi* [10,20–23] or even with a broader set of tissues [9,12,24]. No gene expression study is available in livestock using inguinal ring tissue, which is composed by connective and muscular tissues, and it is the site of occurrence of the scrotal hernia. Thus, knowing reliable reference genes for the inguinal canal is essential to obtain accurate gene expression assays with this tissue. Therefore, to obtain stable genes to be used as reference in expression studies related to scrotal hernias in pigs, 10 endogenous candidate genes were evaluated in the present study in two different experimental conditions.

## Materials and methods

### Animals and sample collection

This study was performed with the approval of the Embrapa Swine and Poultry Ethical Committee of Animal Use (CEUA) under the protocol number 011/2014. Two experiments were carried out to detect the best reference genes in two different ages: at 30 and 60 days of age. The details of each experiment are presented below:

**Experiment 1 (E1).** Animals were raised at the Embrapa Swine and Poultry National Research Center farm until 30 days of age. A total of 18 entire male pigs of the MS115 synthetic line were used. The animals were grouped in normal ( $n = 9$ , absent from malformations and coming from litters with no history of hernias) and affected ( $n = 9$ , from litters with the presence of more than one animal with scrotal hernia).

**Experiment 2 (E2).** Eight Landrace pigs with 60 days of age from the same nucleus farm, located in Santa Catarina State, Brazil, were used in this study. These animals were transported from the farm to the necropsy room at the Embrapa Swine and Poultry. As in the experiment 1, the animals were grouped in normal ( $n = 4$ ) and affected ( $n = 4$ ) with scrotal hernia.

For both experiments, animals were not related, i.e., they were chosen from different families in a case and control design, with cases and controls being from the same contemporary group. The euthanasia was performed by electrocution for 10 seconds, followed by immediate exsanguination, according to the practices recommended by the Ethics Committee. The necropsy was performed for the evaluation of possible problems and additional characteristics that could interfere in the accuracy of the data, as well as for the correct characterization of the hernia phenotype. Tissue samples from the inguinal ring/canal of normal and scrotal hernia-affected groups were collected and immediately frozen in liquid nitrogen and then stored at  $-80^{\circ}\text{C}$  for subsequent RNA extraction. After necropsy and tissue collection, the piglets' carcasses were destined for composting.

### RNA extraction

Tissue RNA extraction was performed according to the Trizol Reagent (Invitrogen, Carlsbad, CA) protocol. Samples containing about 100 mg of tissue were initially macerated in liquid nitrogen with mortar and pestle, properly treated for this procedure. After maceration, the generated contents were placed into 1.5 mL microtube containing 1 mL of the Trizol reagent, vortexed and then incubated for 5 minutes at room temperature ( $25^{\circ}\text{C}$ ). Next, 200  $\mu\text{L}$  of chloroform was added to the tube, shaken vigorously with the hands for 15 seconds, and finally incubated at room temperature for 5 minutes. After incubation, centrifugation was performed at 11,000 rpm (rotations per min) at  $4^{\circ}\text{C}$  for 15 minutes. Thereafter, the aqueous phase was removed into a clean polypropylene tube and 500  $\mu\text{L}$  of isopropanol was added. The tube was stirred and subsequently incubated for 10 minutes at room temperature. After 10 minutes, the tubes containing the sample were centrifuged for 10 minutes at 10,000 rpm at  $4^{\circ}\text{C}$ . The supernatant was discarded and the pellet washed with 1 mL of 75% ethanol and homogenized in vortex. This was centrifuged at 9,000 rpm for 5 minutes at  $4^{\circ}\text{C}$ . The supernatant was discarded and the pellet dried for 15 minutes at room temperature, resuspended in DEPC water and heated at  $55^{\circ}\text{C}$  for 10 minutes. The quality and quantity of the total RNA were evaluated by spectrophotometer (Biodrop, UK) and also in 1% agarose gel. Finally, the total RNA extracted was conserved in ultrafreezer— $80^{\circ}\text{C}$ .

### Complementary DNA (cDNA) synthesis

For the synthesis of complementary DNA (cDNA), the SuperScript III First-Strand Synthesis Supermix Kit (Invitrogen, USA) was used. For each 3  $\mu\text{g}$  of total RNA, 1  $\mu\text{L}$  of Annealing buffer, 1  $\mu\text{L}$  of oligo dT 0.5  $\mu\text{g} / \mu\text{L}$  and water until the volume was completed in 10  $\mu\text{L}$  were added, incubated at  $65^{\circ}\text{C}$  for 5 minutes and then cooled in ice for 1 minute. Then, 10  $\mu\text{L}$  of 2X First-Strand reaction mix and 2  $\mu\text{L}$  of SuperscriptIII/RNaseOUT enzyme mix (Invitrogen, USA) were added to the mixture, being incubated for 50 minutes at  $50^{\circ}\text{C}$  and subsequently inactivated for 5 minutes at  $85^{\circ}\text{C}$ , and then stored at  $-20^{\circ}\text{C}$ .

### Relative quantification using qPCR

The relative quantification of each putative reference gene was performed by qPCR. The expression pattern of the following genes was evaluated: hydroxymethylbilane synthase (*HMBS*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*), succinate dehydrogenase complex flavoprotein subunit A (*SDHA*), topoisomerase

(DNA) II beta (*TOP2B*), ribosomal protein L13A (*RPL13A*), H3 histone, family 3A (*H3F3A*), eukaryotic translation elongation factor 1 alpha 1 (*EEF1A1*), ribosomal protein L32 (*RPL32*), ribosomal protein L19 (*RPL19*) and peptidyl prolyl cis-trans isomerase A (*PPIA*). The sequences and annotations for these 10 genes were obtained in the swine genome (*Sus scrofa*, v. 10.2) available in GeneBank (<https://www.ncbi.nlm.nih.gov/genbank/>) and Ensembl 86 (<http://www.ensembl.org/index.html>). Primers were designed in exon-exon junction regions, in order to avoid the genomic DNA amplification, using the Primer-Blast program [25] and are shown in Table 1. The qPCR reactions were carried out in duplicate in 15 µL final volume containing 1X of Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Fisher Scientific, USA), 0.05 to 0.13 µM of each primer and ~20 ng of cDNA. Reactions were performed in the Quantstudio 6 equipment (Thermo Fisher Scientific, USA) using SYBR Green as fluorescence dye with the following cycling condition: 95° for 10 min, 40 cycles of 15 seconds at 95°C and 30 seconds 60°C. In addition, the melting curve stage of 70°C to 95°C at 0.1°C/s for all genes studied were included to verify the primers specificity. The maximum allowed difference in Ct values between technical replicates was 0.3 Ct.

### Reference gene stability evaluation

A total of four algorithms widely used to identify the most stable expressed genes: the geNorm [26], NormFinder [27], BestKeeper [28] and Comparative ΔCt [29] were used to evaluate the reference candidate genes in the present study. The geNorm is a robust software that calculates

**Table 1. Primers for the 10 reference candidate genes for the qPCR analysis in the inguinal ring of pigs.**

Gene	Function	Primer Sequences (5'– 3')	Ensembl ID
<b>HMBS</b> hydroxymethylbilane synthase	Third enzyme of the biosynthetic pathway of the Heme group	F: AGGATGGGCAACTCTACCTGA R: ATGGATGGTGGCTGCATAG	ENSSSCG00000015108
<b>RPL19</b> ribosomal protein L19	Ribosomal protein 60S subunit component, L19E family	F: ACCGCCACATGTATCACAGTC R: TGTGCTCCATGAGAATCCGC	ENSSSCG00000017509
<b>RPL32</b> ribosomal protein 32	Ribosomal protein 60S subunit component, L32E family	F: CAAAATTAAGCGGAAGTGGCGG R: GCACATTAGCAGCACTTCAAGC	ENSSSCG00000027637
<b>EEF1A1</b> eukaryotic translation elongation factor 1 alpha 1	Enzymatic delivery of aminoacyl tRNAs to the ribosome.	F: CCGCCAGGACACAGGT R: TTCCCATCTCCGAGCCT	ENSSSCG00000004489
<b>H3F3A</b> H3 histone, family 3A	3rd component of nuclear histones	F: CTTTGCAGGAGGCAAGTGAG R: TGGCATGGATAGCACACAGG	ENSSSCG00000023971
<b>RPL13A</b> ribosomal protein 13A	Ribosomal protein 60S subunit component, L13A family	F: CCAAGCAGGTACTTCTGGGC R: GGCAGCATGCCTCGCA	ENSSSCG00000003166 ENSSSCG00000003167
<b>TOP2B</b> topoisomerase (DNA) II beta	DNA transcription and replication	F: AGAAGAGCTGCTGCTGAAAGG R: TCCCCGTCATTTGTCACAGG	ENSSSCG00000011213
<b>SDHA</b> succinate dehydrogenase complex flavoprotein subunit A	Encodes a major catalytic subunit of succinate-ubiquinone oxidoreductase, in the mitochondrial respiratory chain	F: TTGTACGGAAGGTCTCTGCG R: GATGACTCCACGACACTCCC	ENSSSCG00000020686
<b>YWHAZ</b> tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta	Regulation of signal transduction pathways through binding phosphoserine proteins	F: ATCAGATGGGTCTGGCCCT R: GGTATCCGATGTCCACAATGTC	ENSSSCG00000006062
<b>PPIA</b> peptidyl-prolyl cis-trans isomerase A	Accelerate the folding of proteins	F: GCGTCTCCTTCGAGCTGTTT R: ACTTGCCACCAGTGCCATTA	ENSSSCG00000016737

F: forward; R: reverse.

<https://doi.org/10.1371/journal.pone.0204348.t001>

an internal control gene-stability measurement (M) for each combination of two control genes tested, obtaining a transformed expression ratio and then, calculates a standard deviation of these pairwise gene combinations. The two most stable genes are determined based on the lowest M value, and values lower than 1.5 indicate stable genes [26]. The M values from geNorm were obtained using the SLqPCR package on R (<http://bioconductor.org/packages/release/bioc/html/SLqPCR.html>).

The NormFinder is a visual basic application for Microsoft Excel that calculates a stability value (S) based on intra and intergroup variation of genes tested, taking into account their co-regulation, ranking the genes according to their expression stability and similarity. The smallest S values indicate the best or the most stable genes to be used as normalizers [27]. In the NormFinder, the data used was transformed in log<sub>2</sub>, as suggested by the developer [27].

The BestKeeper is also an Excel-based tool for scoring the genes using an index (power of the gene) composed by the values of Ct, fold-change, standard deviation (SD) and coefficient of variation (CV) [28]. According to Pfaffl et al. (2004) [28], the most consistent genes will present values of SD of Cts lower than 1 and SD of X-Fold lower than 2. The authors also suggest not using genes with SD of Cts above 1.5 [28].

The Comparative  $\Delta$ Ct [29] uses a basic  $\Delta$ Ct approach to compare the relative expression of pairs of genes, creating a stability rank based on the  $\Delta$ Ct and average standard deviations. The genes with the lowest average SD and with constant  $\Delta$ Ct values are considered to be the most stable [29].

In addition, once all of the stability values for all tools were obtained, the BruteAggreg function, a weighted rank aggregation tool from the RankAggreg package [30] of R environment [31], that calculates a Spearman distance based on Monte Carlo algorithm, was used to determine a general ranking of the most stable genes for each experiment and analyzed tools (SLqPCR, NormFinder, BestKeeper and Comparative Ct). The BruteAggreg function was used twice for each experiment. This had to be done because the geNorm software ranks the two best genes at the same time. Then, these genes were both put in the 1<sup>st</sup> and 2<sup>nd</sup> positions for each experiment in BruteAggreg to improve the prediction of the best endogenous control gene.

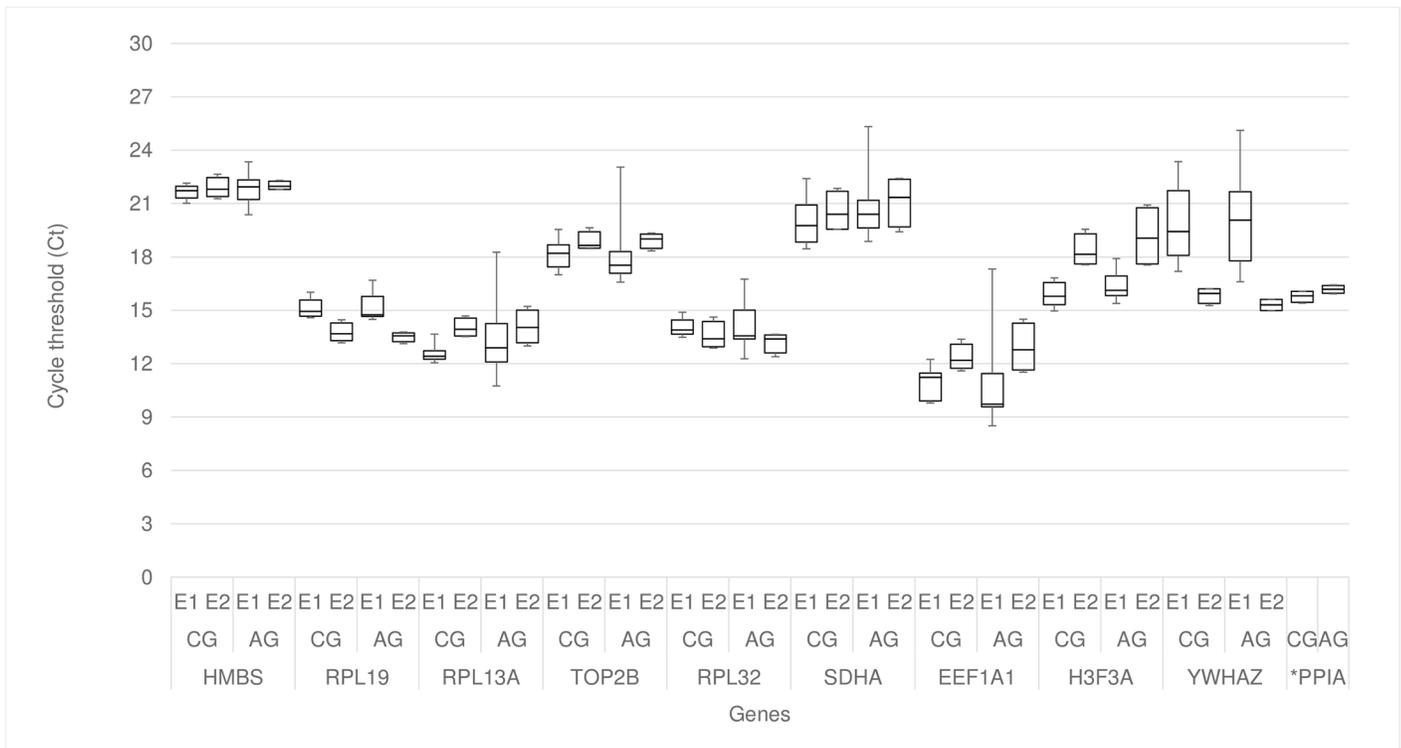
## Results

The total RNA average concentration was 1,033.19 ng/ $\mu$ L for the normal and 1,052.66 ng/ $\mu$ L for the affected group in the Experiment 1, and 918.55 ng/ $\mu$ L for the normal and 995.03 ng/ $\mu$ L for the affected group, in the Experiment 2. Regarding the RNA quality, the average A260/280 ratio was  $1.90 \pm 0.04$  and  $2.06 \pm 0.01$  for the unaffected pig samples and  $1.92 \pm 0.05$  and  $2.07 \pm 0.02$  for herniated pig samples in the E1 and E2, respectively, evidencing a good quality of the RNA samples to be used in the further analysis.

The mean Ct values ( $\pm$  SD) of the reference candidate genes ranged from approximately 10.5 to 22 (Fig 1, Table 2) according to each experiment. The *PPIA* gene was removed from the Experiment 1 analysis since there was no amplification for some of the samples, differing from the Experiment 2, where all samples amplified for this gene, with average Ct mean of  $15.98 \pm 0.34$  and the smallest standard deviation.

Most of the genes started the amplification between cycles 10 to 20 cycles (Fig 1), indicating high levels of expression. Also, it was possible to identify a higher dispersion of the Cts for the *YWHAZ* gene in E1 compared to E2. According to the melting curve analysis, all genes presented a specific amplification (Fig 2).

Regarding the experiment 1, it was possible to observe a similar expression profile among *RPL19*, *RPL32*, *H3F3A* and *HMBS* genes obtained with the several evaluated tools (Table 3).



**Fig 1. Cycle threshold (Ct) variation in normal and hernia-affected pigs in the two experiments.** CG: control group; AG: affected group; 1 –experiment 1 and 2 – experiment 2. \*PPIA: just the information about the experiment 2 was plotted, since there was no amplification for some samples in the experiment 1.

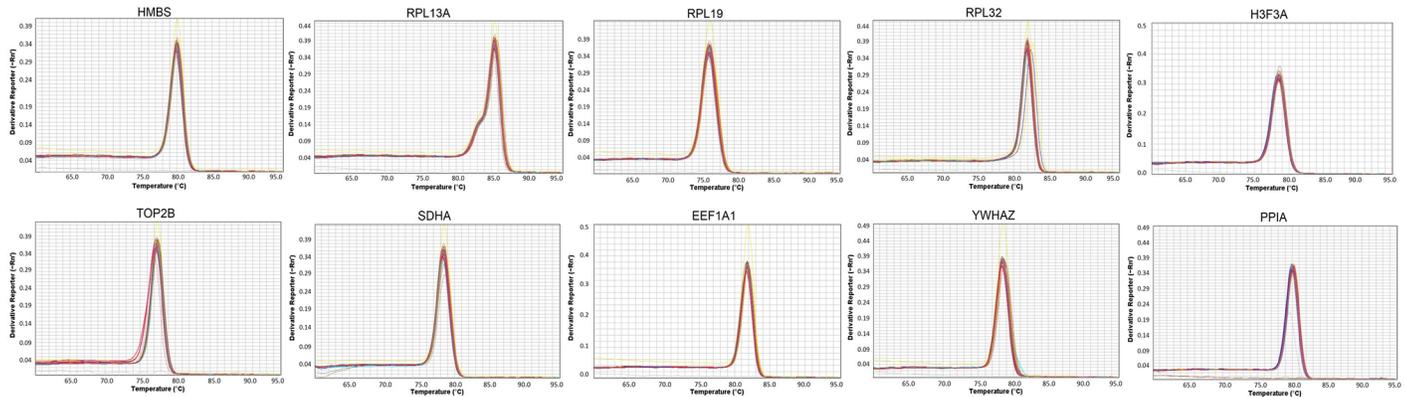
<https://doi.org/10.1371/journal.pone.0204348.g001>

These were the first four genes ranked with the BestKeeper (S1 Table) and with the geNorm (Fig 3A) tools and also presented the smallest dispersion of Ct values (Fig 1). The geNorm classified the *RPL19* / *H3F3A* and *HMBS* genes with the lowest M values:  $M = 0.620$  and  $M = 0.659$ , respectively (Fig 3A). The NormFinder program included the *TOP2B* among the most stable genes, while BestKeeper, geNorm and DeltaCt ranked those genes in the last five positions, showing a reduced stability (Table 3). Another important observation was that the *RPL13A*, *SDHA*, *EEF1A1* and *YWHAZ* genes demonstrated the lowest stability values

**Table 2. Average Ct means for the 10 reference candidate genes by group in each experiment.**

Gene	Ct Mean ± SD					
	Experiment 1			Experiment 2		
	Normal	Affected	Average	Normal	Affected	Average
HMBS	21.64 ± 0.39	21.82 ± 0.86	21.73 ± 0.66	21.89 ± 0.57	22.01 ± 0.25	21.95 ± 0.41
RPL19	15.12 ± 0.50	15.15 ± 0.80	15.14 ± 0.65	13.75 ± 0.53	13.51 ± 0.27	13.63 ± 0.41
RPL13A	12.56 ± 0.47	13.45 ± 2.14	13.00 ± 1.57	14.02 ± 0.53	14.07 ± 0.95	14.05 ± 0.71
TOP2B	18.15 ± 0.81	18.11 ± 1.95	18.13 ± 1.45	18.85 ± 0.54	18.93 ± 0.43	18.89 ± 0.45
RPL32	14.05 ± 0.47	14.12 ± 1.31	14.09 ± 0.96	13.57 ± 0.76	13.20 ± 0.57	13.39 ± 0.65
SDHA	19.93 ± 1.32	20.80 ± 1.90	20.37 ± 1.45	20.55 ± 1.16	21.13 ± 1.43	20.84 ± 1.24
EEF1A1	10.86 ± 0.88	10.89 ± 2.62	10.88 ± 1.89	12.34 ± 0.75	12.90 ± 1.39	12.62 ± 1.08
H3F3A	15.90 ± 0.65	16.34 ± 0.83	16.12 ± 0.76	18.35 ± 0.91	19.15 ± 1.72	18.75 ± 1.34
YWHAZ	19.90 ± 2.09	19.92 ± 2.65	19.91 ± 2.32	15.85 ± 0.43	15.30 ± 0.36	15.58 ± 0.47
PPIA	-	-	-	15.77 ± 0.33	16.18 ± 0.23	15.98 ± 0.34

<https://doi.org/10.1371/journal.pone.0204348.t002>



**Fig 2. Melting curve analyses of the 10 reference candidate genes evaluated in this study.**

<https://doi.org/10.1371/journal.pone.0204348.g002>

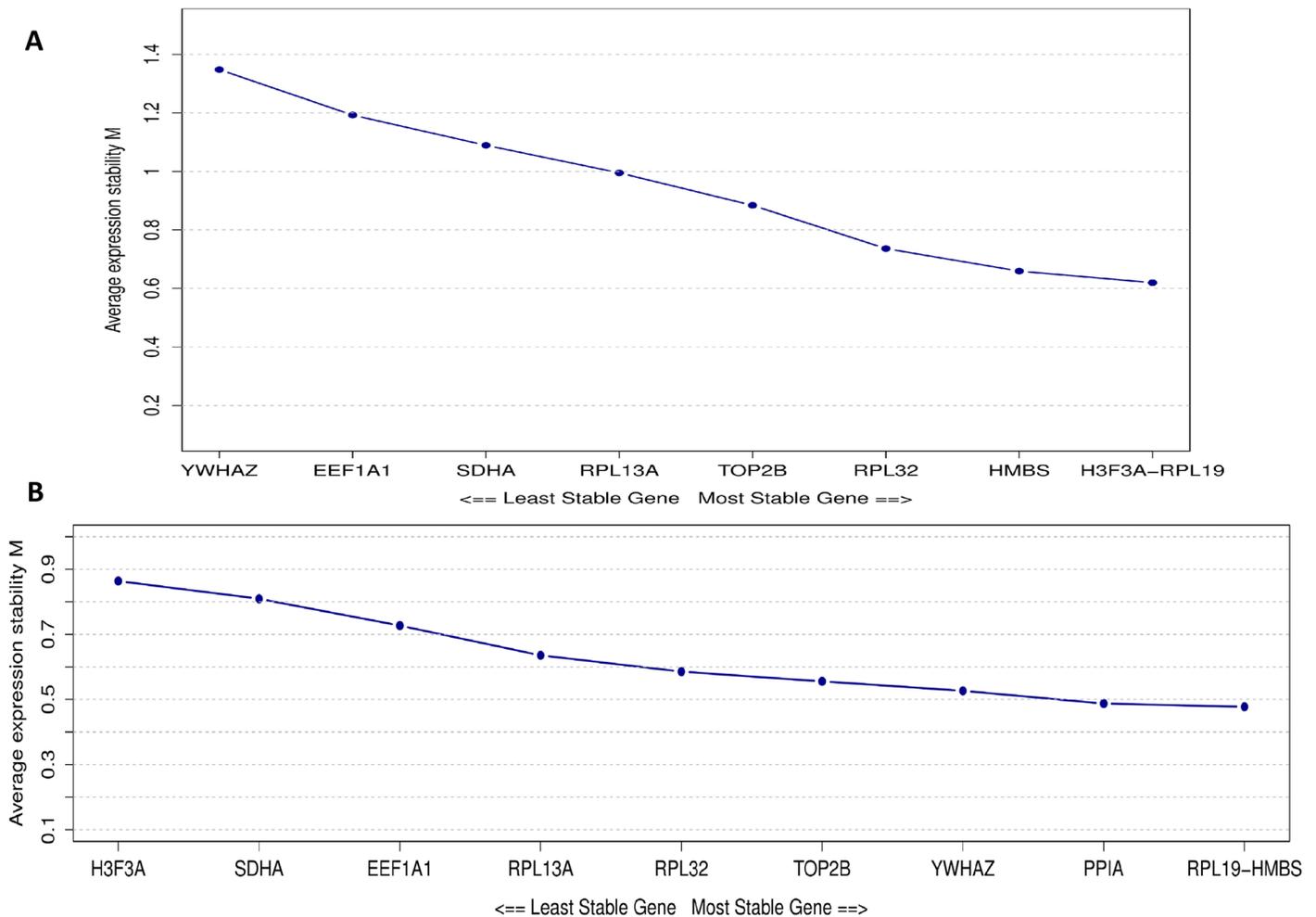
according to the BestKeeper, geNorm and NormFinder tools (Table 3). However, when the  $\Delta C_t$  comparative approach was evaluated, the *YHWAZ* gene was scored as the most stable, differing from the other three tools previously mentioned (Table 3).

When the analysis was performed in the experiment 2, several differences among the most suitable genes were found in comparison to experiment 1. Also, it is interesting to note that each algorithm/tool indicated one different gene as most stable (Table 3). Using geNorm, the *RPL19/HMBS* and *PPIA* genes presented the lowest M value, of 0.478 and 0.492, respectively, which suggest that those genes should be used as reference in E2 (Table 3, Fig 3B). The best genes according to BestKeeper were *HMBS*, *TOP2B* and *YHWAZ* (Table 3), respectively, while the *RPL13A*, *RPL32* and *PPIA* genes were listed by NormFinder. Furthermore, for the  $\Delta C_t$  method, the *PPIA*, *YHWAZ* and *RPL13A* were the top three reliable genes (Table 3). The variation in the stability could occur by differences in the biological expression levels and also due to technical issues. Although all the evaluated primers were specific, a small “shoulder” in the *RPL13A* melting curve (Fig 2) was observed, possibly indicating the amplification of more than one *RPL13A* isoform. This could influence the observed Ct levels, however, since all the samples had the same shoulder and the quantification is relative, probably this shoulder has slightly or no influence on the results.

**Table 3. Gene classification values and ranking (in parenthesis) according to the four algorithms analyzed and the general rank generated by the BruteAgreeg for experiments 1 (E1) and 2 (E2).** 1<sup>st</sup> and 2<sup>nd</sup> are the rank after running the BruteAgreeg twice.

Gene	BestKeeper Power of the gene		DeltaCt Mean StdDev		NormFinder S-value		geNorm M-value		BruteAgreeg			
	E1	E2	E1	E2	E1	E2	E1	E2	E1		E2	
									1 <sup>st</sup>	2 <sup>nd</sup>	1 <sup>st</sup>	2 <sup>nd</sup>
HMBS	1.334 (1)	0.000 (1)	1.202 (4)	0.811 (5)	0.542 (4)	0.205 (4)	0.659 (3)	0.478 (2)	3	3	3	4
RPL19	1.386 (2)	1.242 (4)	1.145 (2)	0.796 (4)	0.477 (2)	0.220 (6)	0.620 (2)	0.478 (1)	1	1	2	2
H3F3A	1.393 (3)	5.416 (10)	1.214 (5)	1.081 (10)	0.551 (5)	0.270 (9)	0.620 (1)	1.097 (10)	4	2	10	10
RPL32	1.632 (4)	1.549 (6)	1.157 (3)	0.871 (6)	0.434 (1)	0.192 (3)	0.736 (4)	0.646 (6)	2	4	6	6
TOP2B	2.244 (5)	0.963 (2)	1.259 (6)	0.909 (8)	0.498 (3)	0.321(10)	0.884 (5)	0.599 (5)	5	5	7	7
RPL13A	2.325 (6)	2.485 (7)	1.338 (7)	0.724 (3)	0.633 (6)	0.118 (1)	0.995 (6)	0.761 (7)	6	6	5	5
SDHA	2.351 (7)	4.652 (9)	1.376 (8)	1.023 (9)	0.660 (7)	0.264 (8)	1.089 (7)	1.081 (9)	7	7	9	9
EEF1A1	2.742 (8)	4.002 (8)	1.584 (9)	0.897 (7)	0.900 (8)	0.217 (5)	1.192 (8)	1.001 (8)	9	9	8	8
YHWAZ	3.204 (9)	1.225 (3)	1.141 (1)	0.715 (2)	1.175 (9)	0.238 (7)	1.348 (9)	0.566 (4)	8	8	4	3
PPIA	-	1.422 (5)	-	0.701 (1)	-	0.218 (2)	-	0.492 (3)	-	-	1	1

<https://doi.org/10.1371/journal.pone.0204348.t003>

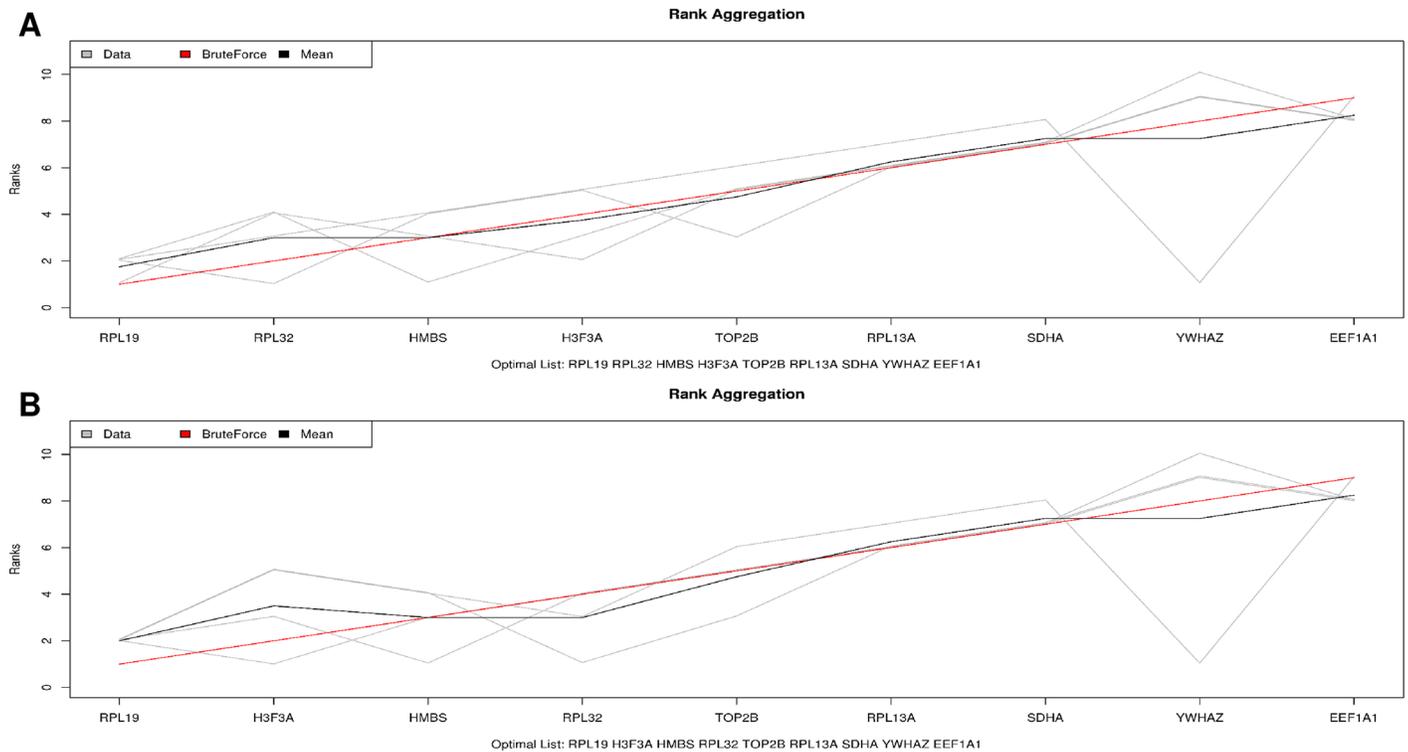


**Fig 3. Ranking of reference candidate genes based on the average expression stability using the geNorm software.** A: results obtained in the Experiment 1. B: results obtained in the Experiment 2.

<https://doi.org/10.1371/journal.pone.0204348.g003>

A great variation on the rank of the best reference genes were observed, depending on the evaluated tool. Therefore, a general rank considering those four tools was performed using the BruteAggreg function. For the experiment 1, *RPL19* and *RPL32*, and *RPL19* and *H3F3A* were pointed out as the first and second most stable genes, followed by *HMBS*, after performing the BruteAggreg function twice (Fig 4A and 4B, respectively). The results from the BruteAggreg function are similar to those obtained with the geNorm evaluation, including for the genes *EEF1A1* and *YWHAZ*, which were the worst genes evaluated (Fig 4). For the experiment 2, the *PPIA* and *RPL19* were scored as the best genes in both BruteAggreg analyses, while the *H3F3A* and *SDHA* were the most variable genes (Fig 5A and 5B, respectively).

The *RPL19* was ranked as one of the less variable genes, showing a similar classification (Table 3) for both experiments, which were run independently. Also, the *HMBS* gene was classified as the 3<sup>rd</sup> most stable in the general rank for E1 and the 3<sup>rd</sup> and 4<sup>th</sup> for E2 (Figs 4 and 5). However, despite of these similarities, there were two important differences in the general score: one related to the *H3F3A* gene, that was the 2<sup>nd</sup>/4<sup>th</sup> most stable gene in experiment 1, but was the worst gene evaluated in the experiment 2, and the *PPIA* gene, which was the best



**Fig 4. Suitable genes ranked by the BruteAgreeg tool in the two simulations for Experiment 1.** A: simulation 1, genes *RPL19*, *RPL32* and *HMBS*; B: simulation 2, genes *RPL19*, *H3F3A* and *HMBS* (Table 3).

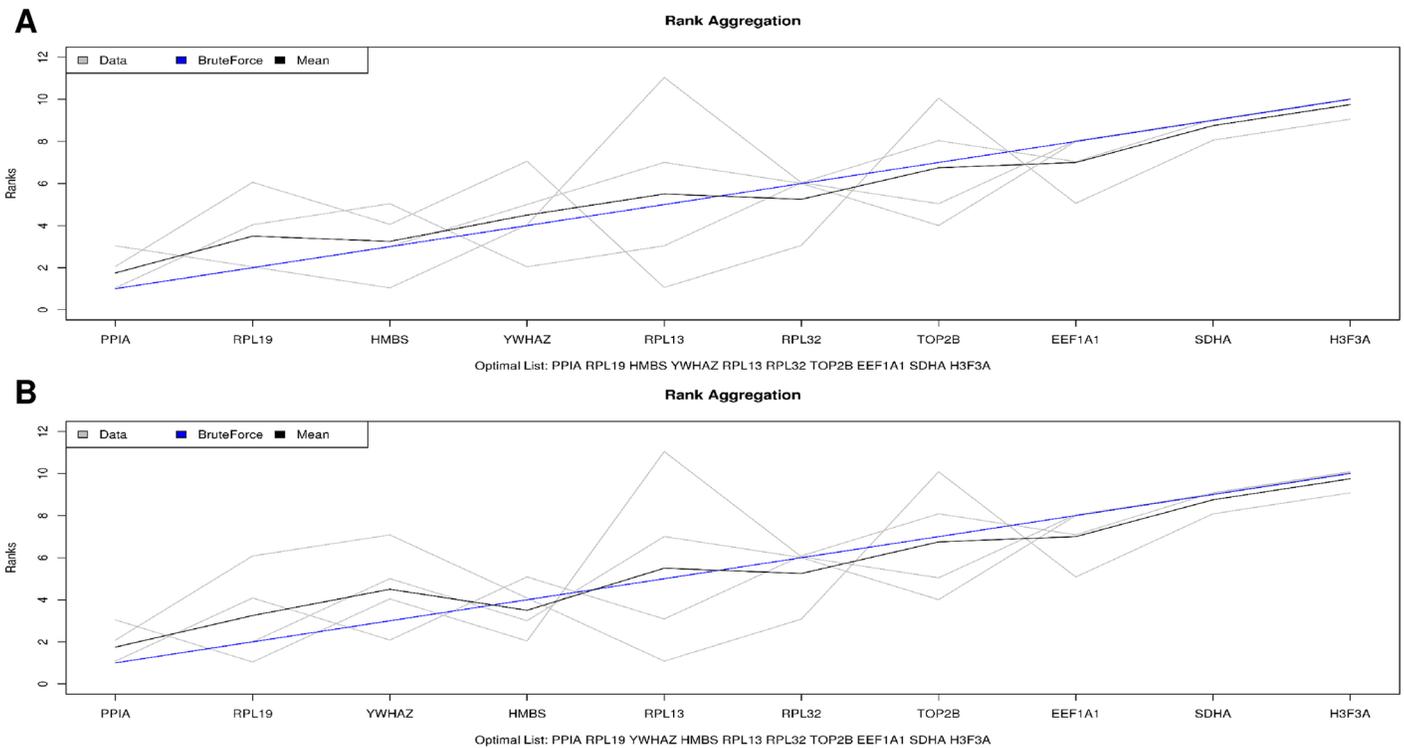
<https://doi.org/10.1371/journal.pone.0204348.g004>

gene to be considered as reference in the experiment 2, while several samples did not amplify in the experiment 1.

## Discussion

The studies using gene expression methodologies have been increasing and the use of qPCR for mRNA quantification might be highlighted [32]. Although the qPCR analysis is widely disseminated, some concerns are always important to improve the quality of the laboratory analyses. One of them is related to the RNA amount and integrity, which helps in achieving high accuracy, sensitivity and reproducibility of the further analysis. In this study, the total RNA with good quality according to the usually recommended [32,33] and an amount necessary for all the expression analysis in just one batch was obtained.

The evaluation of a large set of reference candidate genes is essential to obtain reliable data in qPCR studies [28,34]. For this purpose, 10 putative reference genes were evaluated according to their expression stability and consistency with four different specific tools: geNorm, NormFinder, BestKeeper and  $\Delta$ Ct method (Table 3), which are widely used in similar studies. In the last years, several studies have been published discovering many candidate genes that might be used as internal control [28,34]. However, the search for the best reference gene is not trivial, since there are many approaches available and no standard methodology is established. In addition, some of the algorithms include a threshold stability value, while others do not and these values are helpful to verify if the top genes are indeed stable. Therefore, the use of those tools jointly provides more information on the variability of gene expression, which improved our decision on choosing the most reliable reference genes among the studied



**Fig 5. Suitable genes ranked by the BruteAgreeg tool in the two simulations for Experiment 2.** A: Simulation 1, genes *PPIA*, *RPL19* and *HMBS*; B) Simulation 2, genes *PPIA*, *RPL19* and *YWHAZ* (Table 3).

<https://doi.org/10.1371/journal.pone.0204348.g005>

candidates. Moreover, each experiment/condition requires a specific search for genes with non-variable expression patterns to be used as control [34].

In this study, two independent experiments were carried out in pigs from two different lines and ages. In general, it was possible to observe a discordance of the best normalizer genes chosen among the four methodologies in both experiments for the inguinal ring tissue (Table 3). These results reinforce the need for checking a certain number of reference candidate genes before initiating a gene expression analysis, in order to have an appropriate normalization of the transcript level [35].

In pigs, there are some studies validating internal control genes in multiple tissues, such as backfat, muscle, heart, adipose, skin, liver, pancreas, lung, within others [10,20,24] and also, in various developmental stages [22]. Although several tissues have already been evaluated, no information about expression profile of the inguinal ring has been reported to date, especially considering the presence or absence of scrotal hernia phenotype.

In our study, 10 reference candidate genes were tested. However, some issues to select the best genes were encountered, since the most stable reference genes varied when each tool was evaluated separately. For example, while the *HMBS* was the best ranked in the BestKeeper in both experiments, it was the 2<sup>nd</sup> and 3<sup>rd</sup> in the geNorm (Fig 3), the 4<sup>th</sup> and 5<sup>th</sup> for Delta Ct, and 4<sup>th</sup> in the NormFinder, for E1 and E2, respectively. Other studies, such as the one reported by Perez, Tupac-Yupanqui & Dunner (2008) [36], also found a divergent pattern among the tools evaluated for internal control genes in bovine muscle tissues. Obviously, this variation could happen, since the algorithms and data transformation of those tools are different and were developed to address different types of experiments. While the geNorm uses an arithmetic mean of all pairwise variation to obtain the M value and do not account for co-expression of

the reference candidate genes, ranking the 2 most stable reference genes [26], the NormFinder uses a model-based approach generating the S value, having low sensitivity to the co-regulation of the genes [27]. In addition, Bestkeeper allows the visualization of raw and transformed data and generates a combined index of the best genes, being sensitive to those with large differences in their Ct levels [28]. While a similar amount of mRNA are required to the use of the previous tools, the comparative DeltaCt bypasses this requisite, being advantageous for experiments with limited amount of input RNA [29]. Therefore, when such a great variation is observed on the genes ranked by different tools, there is no recommendation of the best method to select the genes, as well as there is no standard score or threshold indicating good or bad stability. Mosley et al. (2017) [37], after analyzing 5 tools (BestKeeper, geNorm, NormFinder, DeltaCt and RefFinder), concluded that the geNorm seems to be the best tool for choosing the most reliable genes.

Some studies have generated a rank when several approaches are used as an alternative to choose the best normalizer genes [38–40]. Thus, the validation with another tool is essential to improve the quality of the genes to be chosen [41]. Therefore, a general ranking obtained with the BruteAggreg function pointed out that for the E1, *RPL19*, *H3F3A/RPL32* and *HMBS* (Fig 4) were the most stable genes, while for the E2 the most stable genes were *PPIA*, *RPL19* and *HMBS/YWHAZ* (Fig 5, Table 3). Regarding gene stability, the top genes in the general rank were also considered stable genes in most of the tools separately, since they had values within the parameters suggested by each tool, i.e.  $S < 0.5$ ,  $M < 1.5$  and  $[\pm Ct] SD < 1.5$  (Table 2, S1 Table). Also, a similar pattern was observed when the least stable genes were ranked, i.e., the *YWHAZ* in E1, which was considered unstable in the NormFinder ( $S = 1.175$ ) and Bestkeeper ( $[\pm Ct] SD = 1.87$ , S1 Table) tools and the most variable in the geNorm ( $M = 1.348$ ). Therefore, the use of several tools to choose the normalizer gene(s) allowed us to verify the variation on the expression of those reference candidate genes in a widely way. Even though BruteAggreg provides a general rank of the genes, this does not mean that all genes are stable or vice-versa. In this context, there is the need to evaluate the output from different tools according to their stability values to consider if the genes are indeed stable or not. The *H3F3A* and *HMBS* have been previously described as reference genes in swine tissues, where the *H3F3A* was the most stable and the *HMBS* was regulated in some of the evaluated tissues [23,24]. Few studies have been performed using the *RPL19* as reference gene in pigs [42], but it has been considered as a good internal reference gene in other livestock species [43–46].

Regarding the best endogenous genes for the inguinal ring tissue, the *RPL19* showed the highest uniformity in its expression within the tools and experiments (Table 3, Table 2). Ribosomal proteins have been suggested as good reference genes in many studies [47], because of their function on ribosome production [48]. Schulze et al. (2017) [45] and Lenart, Kogut & Salinska (2017) [46] also found stable expression of this gene on sheep bone cells and in chick brain, respectively. In pigs, *RPL19* was recommended as endogenous gene in studies using peripheral blood mononuclear and dendritic cells [42]. The *RPL19* amplified in early Cts (before 20) and had small coefficient of variation in each experiment (Fig 1, Table 2), which can indicate that this gene would be a good normalizer. The *RPL32*, *H3F3A* and *RPS18* (ribosomal protein 18S), involved in the development of cellular machinery, have also been chosen as endogenous gene for multiple tissues and swine breeds [14,23]. In addition, Zhang et al. (2012) [23], testing six endogenous genes in the *longissimus dorsi* of pigs, found differences on the best genes according to the breeds studied, where *RPL32 / RPS18* were the most stable in the Landrace and *H3F3A / RPS18* in the Toncheng breed. In our study, both *RPL32* and *H3F3A* genes were the 2<sup>nd</sup> most stable genes in the E1 (Table 3, Fig 4), endorsing the results obtained by Zhang et al. (2012) [23]. On the other hand, for the E2, the *RPL32* and *H3F3A* were not considered as stable genes.

The *PPIA* gene, that is involved in protein folding [49], has also been recommended for being used as endogenous control in several tissues, species and ages [9,14,21]. In our study, the *PPIA* was ranked as the most invariable gene in the E2, where Landrace pigs with 60 days of age were evaluated, being one of the most indicated as endogenous gene from the geNorm, Norm-Finder and Comparative Ct tools (Table 3, Fig 3). A similar pattern was observed when several tissues of Berkshire, Duroc, Landrace and Yorkshire pigs were evaluated [14], suggesting that *PPIA* is a reliable gene for expression studies in adult pigs. However, in our study, a variation in the *PPIA* expression between the two experiments was observed (Table 3). Although the *PPIA* was the best normalizer gene in the E2, for E1, in which the samples were obtained from 30 days-old MS115 pigs, the expression of this gene was impossible to be analyzed, since many samples did not amplify. This might be due to the different ages and breeds used in each experiment. Uddin et al. (2011) [9] observed that distinct genes should be used as reference gene depending on the pig's age. In addition, stability differences can also occur among the tissues analyzed [12]. Here, samples of the inguinal ring were collected and, albeit a unique technician had collected all samples in a specific anatomical region, it could be possible that the tissues were slightly unequal among samples and experiments, since this tissue is highly complex to collect. Therefore, studies evaluating the distinction among breeds, phenotypes and age of the animals are essential [9] to the better characterize the expression profile of the tissues.

The *HMBS* gene had a good general ranking and in most of the other tools for both experiments (Table 3). This gene has been used as endogenous in many species, pig lines, tissues and ages [24,38,50,51]. However, the regulation of this gene depends on the muscle tissue, sex, age and experimental conditions [38,50].

The *H3F3A* and *YWHAZ* were the most variable genes between both experiments. For instance, *H3F3A* was considered reliable in the experiment 1, with 30 days-old MS115, while it was the least reliable in the experiment 2, with 60 days-old Landrace pigs. The same pattern was observed with the *YWHAZ*, which in this case was stable with 60 days-old Landrace samples and variable with the 30 days-old MS115, reinforcing the statement that there are no general reference genes that might be used in all situations. The *SDHA*, *TOP2B*, *EEF1A1* and *YWHAZ* genes were highly variable regarding the general score in both experiments, possibly because of the late Ct and its variation between and within groups. Furthermore, the variability presented by these genes could be possibly due to the non-homogeneity of the tissue used in this study.

Although more than two genes should be used as reference in gene expression studies [33], the average number of genes used is only 1.2, which means, below the recommendation [33,34]. Moreover, it is usual studies with relatively common genes such as GAPDH,  $\beta$ -actin and 18S RNA, without testing for stability. Given the complexity of the experimental designs and tissues to be evaluated, a broad panel of genes and tools should be used to search for the best reference genes [34]. It is also important to note that when candidate reference genes are being evaluated, the most or least stable genes chosen are based only in that experiment, and not necessarily will happen in other conditions. Furthermore, the most stable genes found in one experiment does not mean that only those genes are stable, reinforcing the need of always testing several candidate reference genes. The use of more than three genes is indicated to reduce the selection of false endogenous genes that may impact on the reliability of the results [52]. One example could be observed in our study, where the same tissue was collected from animals of two different lines and ages and, despite of being from the same species, two sets of genes should be used as reference: the *RPL19*, *RPL32* and *H3F3A* for 30-days MS115 (E1) and *PPIA* and *RPL19* for the 60 days-old Landrace pigs (E2).

In this study, even though there was a confounding between age and breed effects, the experimental conditions influenced the stability of the evaluated genes. Therefore, further

studies are recommended to clarify the isolated contribution of age and breed to variations on the genes' expression profile in the inguinal ring tissue of pigs. The effect of breed is expected to influence scrotal hernia congenital anomaly. Vogt & Ellersieck (1990) [18] found significant differences in frequency of this defect among Duroc, Landrace and Yorkshire male lines. Sevilano et al. (2015) [19] observed a slightly higher incidence of scrotal hernia in Large White (0.42%) compared to Landrace breeds (0.34%). In addition, these authors mapped distinct genomic regions associated to scrotal hernia between Landrace e Large White pigs. Probably, intrinsic conformation and anatomical differences of each breed could affect the inguinal ring tissue composition causing variation in the expression profile of the endogenous candidate genes. Regarding the age effect, since hernias are related to development, usually resulting from failed obliteration of the *processus vaginalis* after descent of the testis, it is expected that the age would be important to this malformation. As evidence, most scrotal hernias are diagnosed at the time of castration, an early phase in the pig's life [53]. Therefore, the age effect should be evaluated independently in different ages, especially in early stages of life.

## Conclusions

The breed/age effects influenced the expression stability of candidate reference genes evaluated in the inguinal ring of pigs. A consensual set of reference genes was not obtained for the two experimental conditions, evidencing the importance of evaluating the stability of several endogenous genes previous their use. The *RPL19* was one of the most reliable endogenous genes for both experiments. Therefore, two set of genes are recommended for the inguinal ring tissue: *RPL19*, *RPL32* and *H3F3A* for 30-days MS115 and *PPIA* and *RPL19* for the 60 days-old Landrace pigs. This is the first study using the inguinal ring tissue and the results can be useful as an indicative for other studies working with gene expression in this tissue.

## Supporting information

**S1 Table. Results from the Bestkeeper tool.**  
(XLSX)

## Acknowledgments

This study was supported by project #476146/2013-5 from the National Council of Scientific and Technological Development (CNPq) to MCL in which she is a CNPq fellow. WR Lorenzetti was sponsored by a PROMOP/Udesc scholarship. IR Savoldi and KB do Carmo are recipients of a PIBIC/CNPq scholarship at Embrapa Swine and Poultry National Research Center. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## Author Contributions

**Conceptualization:** Adriana Mercia Guaratini Ibelli, Jane de Oliveira Peixoto, Mônica Corrêa Ledur.

**Data curation:** William Raphael Lorenzetti, Adriana Mercia Guaratini Ibelli, Jane de Oliveira Peixoto, Haniel Cedraz de Oliveira.

**Formal analysis:** William Raphael Lorenzetti, Adriana Mercia Guaratini Ibelli, Jane de Oliveira Peixoto.

**Funding acquisition:** Mônica Corrêa Ledur.

**Investigation:** William Raphael Lorenzetti, Adriana Mercia Guaratini Ibelli, Marcos Antonio Zanella Mores, Igor Ricardo Savoldi, Kamilla Bleil do Carmo, Mônica Corrêa Ledur.

**Methodology:** William Raphael Lorenzetti, Adriana Mercia Guaratini Ibelli, Jane de Oliveira Peixoto, Marcos Antonio Zanella Mores, Igor Ricardo Savoldi, Kamilla Bleil do Carmo, Haniel Cedraz de Oliveira, Mônica Corrêa Ledur.

**Supervision:** Mônica Corrêa Ledur.

**Visualization:** William Raphael Lorenzetti, Adriana Mercia Guaratini Ibelli.

**Writing – original draft:** William Raphael Lorenzetti, Adriana Mercia Guaratini Ibelli, Jane de Oliveira Peixoto, Igor Ricardo Savoldi, Mônica Corrêa Ledur.

**Writing – review & editing:** William Raphael Lorenzetti, Adriana Mercia Guaratini Ibelli, Jane de Oliveira Peixoto, Marcos Antonio Zanella Mores, Kamilla Bleil do Carmo, Haniel Cedraz de Oliveira, Mônica Corrêa Ledur.

## References

1. de L Rebouças E, do N Costa JJ, Passos MJ, de S Passos JR, van den Hurk R, Silva JRV. Real time PCR and importance of housekeeping genes for normalization and quantification of mRNA expression in different tissues. *Brazilian Arch Biol Technol.* 2013; 56: 143–154. <https://doi.org/10.1590/S1516-89132013000100019>
2. Kozera B, Rapacz M. Reference genes in real-time PCR. *J Appl Genet.* 2013; 54: 391–406. <https://doi.org/10.1007/s13353-013-0173-x> PMID: 24078518
3. Fang Z, Cui X. Design and validation issues in RNA-seq experiments. *Brief Bioinform.* 2011; 12: 280–287. <https://doi.org/10.1093/bib/bbr004> PMID: 21498551
4. Dheda K, Huggett JF, Bustin SA, Johnson MA, Rook G, Zumla A. Validation of housekeeping genes for normalizing RNA expression in real-time PCR. *Biotechniques.* 2004; 37: 112–119. <http://dx.doi.org/10.1016/j.ymeth.2010.01.003> PMID: 15283208
5. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 2001; 29: 2002–2007. <https://doi.org/10.1093/nar/29.9.e45>
6. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2<sup>-ΔΔCT</sup> Method. *Methods.* 2001; 25: 402–408. <https://doi.org/10.1006/meth.2001.1262> PMID: 11846609
7. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. *Nat Protoc.* 2008; 3: 1101–1108. <https://doi.org/10.1038/nprot.2008.73> PMID: 18546601
8. Wang Q, Ishikawa T, Michiue T, Zhu B-L, Guan D-W, Maeda H. Stability of endogenous reference genes in postmortem human brains for normalization of quantitative real-time PCR data: comprehensive evaluation using geNorm, NormFinder, and BestKeeper. *Int J Legal Med.* 2012; 126: 943–952. <https://doi.org/10.1007/s00414-012-0774-7> PMID: 23010907
9. Uddin M, Cinar M, Tesfaye D, Looft C, Tholen E, Schellander K. Age-related changes in relative expression stability of commonly used housekeeping genes in selected porcine tissues. *BMC Res Notes.* 2011; 4: 441. <https://doi.org/10.1186/1756-0500-4-441> PMID: 22023805
10. Wang Y, Zhao Y, Li J, Liu H, Ernst CW, Liu X, et al. Evaluation of housekeeping genes for normalizing real-time quantitative PCR assays in pig skeletal muscle at multiple developmental stages. *Gene.* 2015; 565: 235–241. <https://doi.org/10.1016/j.gene.2015.04.016> PMID: 25865298
11. Cinar MU, Islam MA, Uddin MJ, Tholen E, Tesfaye D, Looft C, et al. Evaluation of suitable reference genes for gene expression studies in porcine alveolar macrophages in response to LPS and LTA. *BMC Res Notes.* 2012; 5: 107. <https://doi.org/10.1186/1756-0500-5-107> PMID: 22340302
12. Gu YR, Li MZ, Zhang K, Chen L, Jiang AA, Wang JY, et al. Evaluation of endogenous control genes for gene expression studies across multiple tissues and in the specific sets of fat- and muscle-type samples of the pig. *J Anim Breed Genet.* 2011; 128: 319–325. <https://doi.org/10.1111/j.1439-0388.2011.00920.x> PMID: 21749479
13. Li Q, Domig KJ, Eittle T, Windisch W, Mair C, Schedle K. Evaluation of Potential Reference Genes for Relative Quantification by RT-qPCR in Different Porcine Tissues Derived from Feeding Studies. *Int J Mol Sci.* 2011; 12: 1727–1734. <https://doi.org/10.3390/ijms12031727> PMID: 21673918

14. Park S-J, Kwon SG, Hwang JH, Park DH, Kim TW, Kim CW. Selection of appropriate reference genes for RT-qPCR analysis in Berkshire, Duroc, Landrace, and Yorkshire pigs. *Gene*. 2015; 558: 152–158. <https://doi.org/10.1016/j.gene.2014.12.052> PMID: 25550045
15. Grindflek E, Moe M, Taubert H, Simianer H, Lien S, Moen T. Genome-wide linkage analysis of inguinal hernia in pigs using affected sib pairs. *BMC Genet*. 2006; 7: 25. <https://doi.org/10.1186/1471-2156-7-25> PMID: 16672048
16. Carman GM. Hernia and Its Heredity. *Iowa State Univ Vet*. 1952; 14. [http://lib.dr.iastate.edu/iowastate\\_veterinarian/vol14/iss3/3](http://lib.dr.iastate.edu/iowastate_veterinarian/vol14/iss3/3)
17. Burcharth J, Pommersgaard HC, Rosenberg J. The inheritance of groin hernia: a systematic review. *Hernia*. 2013; 17: 183–189. <https://doi.org/10.1007/s10029-013-1060-4> PMID: 23423330
18. Vogt DW, Ellersieck MR. Heritability of susceptibility to scrotal herniation in swine. *Am J Vet Res*. 1990; 51: 1501–1503. PMID: 2396801
19. Sevillano CA, Lopes MS, Harlizius B, Hanenberg E, Knol EF, Bastiaansen J. Genome-wide association study using deregressed breeding values for cryptorchidism and scrotal/inguinal hernia in two pig lines. *Genet Sel Evol*. 2015; 47: 18. <https://doi.org/10.1186/s12711-015-0096-6> PMID: 25886970
20. Erkens T, Van Poucke M, Vandesompele J, Goossens K, Van Zeveren A, Peelman LJ. Development of a new set of reference genes for normalization of real-time RT-PCR data of porcine backfat and longissimus dorsi muscle, and evaluation with PPARGC1A. *BMC Biotechnol*. 2006; 6: 41. <https://doi.org/10.1186/1472-6750-6-41> PMID: 17026777
21. Feng X, Xiong Y, Qian H, Lei M, Xu D, Ren Z. Selection of reference genes for gene expression studies in porcine skeletal muscle using SYBR green qPCR. *J Biotechnol*. 2010; 150: 288–293. <https://doi.org/10.1016/j.jbiotec.2010.09.949> PMID: 20887758
22. Niu G, Yang Y, Zhang Y, Hua C, Wang Z, Tang Z, et al. Identifying suitable reference genes for gene expression analysis in developing skeletal muscle in pigs. *PeerJ*. 2016; 4: e2428. <https://doi.org/10.7717/peerj.2428> PMID: 27994956
23. Zhang J, Tang Z, Wang N, Long L, Li K. Evaluating a Set of Reference Genes for Expression Normalization in Multiple Tissues and Skeletal Muscle at Different Development Stages in Pigs Using Quantitative Real-Time Polymerase Chain Reaction. *DNA Cell Biol*. 2012; 31: 106–113. <https://doi.org/10.1089/dna.2011.1249> PMID: 21631284
24. Nygard A-B, Jørgensen CB, Cirera S, Fredholm M. Selection of reference genes for gene expression studies in pig tissues using SYBR green qPCR. *BMC Mol Biol*. 2007; 8: 67. <https://doi.org/10.1186/1471-2199-8-67> PMID: 17697375
25. Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics*. 2012; 13: 134. <https://doi.org/10.1186/1471-2105-13-134> PMID: 22708584
26. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol*. 2002; 3: research0034.1–0034.11. <https://doi.org/10.1186/gb-2002-3-7-research0034>
27. Andersen CL, Jensen JL, Ørntoft TF. Normalization of Real-Time Quantitative Reverse Transcription-PCR Data: A Model-Based Variance Estimation Approach to Identify Genes Suited for Normalization, Applied to Bladder and Colon Cancer Data Sets. *Cancer Res*. 2004; 64: 5245–5250. <https://doi.org/10.1158/0008-5472.CAN-04-0496> PMID: 15289330
28. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper—Excel-based tool using pair-wise correlations. *Biotechnol Lett*. 2004; 26: 509–515. <https://doi.org/10.1023/B:BILE.0000019559.84305.47> PMID: 15127793
29. Silver N, Best S, Jiang J, Thein SL. Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. *BMC Mol Biol*. 2006; 7: 33. <https://doi.org/10.1186/1471-2199-7-33> PMID: 17026756
30. Pihur V, Datta S, Datta S. Weighted rank aggregation of cluster validation measures: a Monte Carlo cross-entropy approach. *Bioinformatics*. 2007; 23: 1607–1615. <https://doi.org/10.1093/bioinformatics/btm158> PMID: 17483500
31. R Core Team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2013.
32. Bustin SA, Wittwer CT. MIQE: A Step Toward More Robust and Reproducible Quantitative PCR. *Clin Chem*. 2017; 63: 1537–1538. <https://doi.org/10.1373/clinchem.2016.268953> PMID: 28606913

33. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clin Chem*. 2009; 55: 611–622. <https://doi.org/10.1373/clinchem.2008.112797> PMID: 19246619
34. Chapman JR, Waldenström J. With Reference to Reference Genes: A Systematic Review of Endogenous Controls in Gene Expression Studies. *PLoS One*. 2015; 10: e0141853. <https://doi.org/10.1371/journal.pone.0141853> PMID: 26555275
35. Liu J, Huang S, Niu X, Chen D, Chen Q, Tian L, et al. Genome-wide identification and validation of new reference genes for transcript normalization in developmental and post-harvested fruits of *Actinidia chinensis*. *Gene*. 2018; 645: 1–6. <https://doi.org/10.1016/j.gene.2017.12.012> PMID: 29242074
36. Perez R, Tupac-Yupanqui I, Dunner S. Evaluation of suitable reference genes for gene expression studies in bovine muscular tissue. *BMC Mol Biol*. 2008; 9: 79. <https://doi.org/10.1186/1471-2199-9-79> PMID: 18786244
37. Mosley Y-YC, HogenEsch H. Selection of a suitable reference gene for quantitative gene expression in mouse lymph nodes after vaccination. *BMC Res Notes*. BioMed Central; 2017; 10: 689. <https://doi.org/10.1186/s13104-017-3005-y> PMID: 29208024
38. Cedraz de Oliveira H, Pinto Garcia AA, Gonzaga Gromboni JG, Vasconcelos Farias Filho R, Souza do Nascimento C, Arias Wenceslau A. Influence of heat stress, sex and genetic groups on reference genes stability in muscle tissue of chicken. *PLoS One*. 2017; 12: e0176402. <https://doi.org/10.1371/journal.pone.0176402> PMID: 28459824
39. Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet*. 2009; 10: 57–63. <https://doi.org/10.1038/nrg2484> PMID: 19015660
40. Xie F, Xiao P, Chen D, Xu L, Zhang B. miRDeepFinder: A miRNA analysis tool for deep sequencing of plant small RNAs. *Plant Mol Biol*. 2012; 80: 75–84. <https://doi.org/10.1007/s11103-012-9885-2> PMID: 22290409
41. De Spiegelaere W, Dern-Wieloch J, Weigel R, Schumacher V, Schorle H, Nettersheim D, et al. Reference Gene Validation for RT-qPCR, a Note on Different Available Software Packages. *PLoS One*. 2015; 10: e0122515. <https://doi.org/10.1371/journal.pone.0122515> PMID: 25825906
42. Facci MR, Auray G, Meurens F, Buchanan R, van Kessel J, Gerdtz V. Stability of expression of reference genes in porcine peripheral blood mononuclear and dendritic cells. *Vet Immunol Immunopathol*. 2011; 141: 11–15. <https://doi.org/10.1016/j.vetimm.2011.01.005> PMID: 21354629
43. Ibelli AMG, Nakata LC, Andréo R, Coutinho LL, Oliveira MCS, Amarante AFT, et al. mRNA profile of Nellore calves after primary infection with *Haemonchus placei*. *Vet Parasitol*. 2011; 176: 195–200. <https://doi.org/10.1016/j.vetpar.2010.11.013> PMID: 21144661
44. Zaros LG, Coutinho LL, Sider LH, de Medeiros HR, das Neves MRM, Benvenuti CL, et al. Evaluation of reference genes for real-time PCR studies of Brazilian Somalis sheep infected by gastrointestinal nematodes. *Genet Mol Biol*. 2010; 33: 486–490. <https://doi.org/10.1590/S1415-47572010000300018> PMID: 21637421
45. Schulze F, Malhan D, El Khassawna T, Heiss C, Seckinger A, Hose D, et al. A tissue-based approach to selection of reference genes for quantitative real-time PCR in a sheep osteoporosis model. *BMC Genomics*. BMC Genomics; 2017; 18: 975. <https://doi.org/10.1186/s12864-017-4356-4> PMID: 29258442
46. Lenart J, Kogut K, Salinska E. Lateralization of housekeeping genes in the brain of one-day old chicks. *Gene Expr Patterns*. 2017; 25–26: 85–91. <https://doi.org/10.1016/j.gexp.2017.06.006> PMID: 28625896
47. Zhou L, Lim Q, Wan G, Too H. Normalization with genes encoding ribosomal proteins but not GAPDH provides an accurate quantification of gene expressions in neuronal differentiation of PC12 cells. *BMC Genomics*. 2010; 11: 75. <https://doi.org/10.1186/1471-2164-11-75> PMID: 20113474
48. Robledo S, Idol RA, Crimmins DL, Ladenson JH, Mason PJ, Bessler M. The role of human ribosomal proteins in the maturation of rRNA and ribosome production. *RNA*. 2008; 14: 1918–1929. <https://doi.org/10.1261/rna.1132008> PMID: 18697920
49. Gong Z-K, Wang S-J, Huang Y-Q, Zhao R-Q, Zhu Q-F, Lin W-Z. Identification and validation of suitable reference genes for RT-qPCR analysis in mouse testis development. *Mol Genet Genomics*. 2014; 289: 1157–1169. <https://doi.org/10.1007/s00438-014-0877-6> PMID: 24952483
50. Nascimento CS, Barbosa LT, Brito C, Fernandes RPM, Mann RS, Pinto APG, et al. Identification of Suitable Reference Genes for Real Time Quantitative Polymerase Chain Reaction Assays on Pectoralis major Muscle in Chicken (*Gallus gallus*). *PLoS One*. 2015; 10: e0127935. <https://doi.org/10.1371/journal.pone.0127935> PMID: 26020643
51. Paludo E, Ibelli AMG, Peixoto JO, Tavernari FC, Lima-Rosa CA V., Pandolfi JRC, et al. The involvement of RUNX2 and SPARC genes in the bacterial chondronecrosis with osteomyelitis in broilers. *Animal*. 2017; 11: 1063–1070. <https://doi.org/10.1017/S1751731116002433> PMID: 27881195

52. Jacob F, Guertler R, Naim S, Nixdorf S, Fedier A, Hacker NF, et al. Careful Selection of Reference Genes Is Required for Reliable Performance of RT-qPCR in Human Normal and Cancer Cell Lines. *PLoS One*. 2013; 8: e59180. <https://doi.org/10.1371/journal.pone.0059180> PMID: 23554992
53. Mattsson P. Prevalence of congenital defects in Swedish Hampshire, Landrace and Yorkshire pig breeds and opinions on their prevalence in Swedish commercial herds. M.Sc. Thesis, Swedish University of Agricultural Sciences. 2011. [https://stud.epsilon.slu.se/2390/1/mattsson\\_p\\_110330.pdf](https://stud.epsilon.slu.se/2390/1/mattsson_p_110330.pdf)