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ORIGINAL ARTICLE

Identification of Suitable Reference Genes for Quantitative Gene Expression Analysis in Innervated and Denervated Adipose Tissue from Cafeteria Diet-Fed Rats

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Abstract Our previous studies show that cafeteria diet increases body adiposity, plasma insulin levels, and sympathetic activity to brown adipose tissue (BAT) and white adipose tissue (WAT) of Wistar rats, leading to rapid and progressive changes in the metabolic profile. The identification of suitable reference genes that are not affected by the experimental conditions is a critical step in accurate normalization of the reverse transcription quantitative real-time PCR (qRT-PCR), a commonly used assay to elucidate changes in the gene expression profile. In the present study, the effects of the cafeteria diet and sympathetic innervation on the gene expression of adrenoceptor beta 3 (*Adrb3*) from BAT and WAT were assessed using one of the most stable and one of the least stable genes as normalizers. Rats were fed the cafeteria diet and on the 17th day, interscapular BAT or retroperitoneal WAT was denervated and, 7 days after surgery, the contralateral innervated tissue was used as control. Ten reference genes were evaluated (*18S*, *B2m*, *Actb*, *CypA*, *Gapdh*, *Hprt1*, *Rpl32*, *Tbp*, *Ubc*, and *Ywhaz*) and ranked according to their stability using the following algorithms: geNorm, NormFinder, BestKeeper, and comparative delta threshold cycle (ΔC_t) method. According to the algorithms employed, the normalization of *Adrb3* expression by the least stable genes produced opposite results compared with the most stable genes and literature data. In cafeteria and control diet-fed rats, the three most stable genes were *Hprt1*, *Tbp*, and *Rpl32* for interscapular BAT and *Tbp*, *B2m*, and *Hprt1* for retroperitoneal WAT, while the least stable genes were *18S*, *Actb*, and *Gapdh* for both tissues.

Keywords Adipose tissue · Adrenoceptor beta 3 · Cafeteria diet · Expression gene · Sympathetic activity

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Abbreviations

18S	18S subunit ribosomal RNA
36B4	acidic ribosomal phosphoprotein P0
Actb	beta actin
Adrb3	adrenoceptor beta 3
B2m	beta-2 microglobulin
BAT	brown adipose tissue
ΔC_t	delta threshold cycle
CypA	cyclophilin A
CV	coefficient of variance
Gapdh	glyceraldehyde-3-phosphate dehydrogenase
Hprt1	hypoxanthine phosphoribosyltransferase

IBAT	interscapular brown adipose tissue
NE	norepinephrine
PCR	polymerase chain reaction
qRT-PCR	quantitative real-time PCR
Rpl	ribosomal proteins L
RWAT	retroperitoneal white adipose tissue
SD	standard deviation
Tbp	TATA box binding protein
Ubc	Ubiquitin C
UCP1	uncoupling protein-1
WAT	white adipose tissue
Ywhaz	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta

Introduction

Obesity is a significant and rapidly increasing global health issue (Wewege et al., 2017), affecting approximately 650 million adults worldwide (approximately 13% of the population) (World Health Organization, 2016). It is associated with several comorbidities, such as type 2 diabetes mellitus (American Diabetes, 2018), cardiovascular diseases (Parto and Lavie, 2017), cancer (Berger, 2014), dyslipidemia (Silva Figueiredo et al., 2017), nonalcoholic fatty liver disease (Ooi et al., 2017), osteoarthritis (Vina and Kwoh, 2017), and is characterized by increased adiposity (Galic et al., 2010). The increase of adipose mass is determined by the balance between lipogenesis and lipolysis (Coelho et al., 2013). Mammals have brown adipose tissue (BAT) and white adipose tissue (WAT), which present different morphology, distribution, gene expression, and function (Gomez-Hernandez et al., 2016). Due to its thermogenic function, BAT has emerged as a target for therapeutic strategies to reverse obesity (Bhatt et al., 2017; Loh et al., 2017). The BAT activity is inversely associated with body mass index, total adipose tissue content, and risk of type 2 diabetes (Lidell et al., 2014). The availability of free fatty acids for oxidation in mitochondria and the level of uncoupling protein-1 (UCP1) in mitochondrial membranes, both determinants of BAT thermogenesis, are regulated by the sympathetic nervous system (Morrison and Madden, 2014). In addition to regulating lipid stores, WAT functions as an endocrine organ acting to maintain the metabolic homeostasis (Harwood Jr., 2012). In recent years, considerable interest has been focused on the potential beneficial effects of inducing BAT features (browning) in nonclassic BAT locations, such as WAT (Rosell et al., 2014). The biochemical mechanisms involved in the regulation of lipogenesis, lipolysis, thermogenesis, and browning respond to marked changes in the gene expression profile, which are extensively studied in models of obesity.

Cafeteria diet increases the intake of calories, lipids, and carbohydrates, inducing an increase in the body adiposity (Chaves et al., 2006, 2008; Rothwell and Stock, 1982). The cafeteria diet also increases plasma insulin levels and the sympathetic stimulation of BAT and WAT (Chaves et al., 2006, 2008; Oliver et al., 2012), altering the expression of genes in the adipose tissue (Johnson et al., 2016; Oliver et al., 2012). In addition, the removal of sympathetic stimulation by a surgical denervation or antagonist administration also changes the expression of genes in WAT and BAT (Tang et al., 2015). Several tissues, including the adipose, have their gene expression changed by denervation (Dab et al., 2013; Ganta et al., 2006; Li et al., 2004; Raju et al., 2009; Veelken et al., 2008).

Reverse transcription quantitative real-time PCR (qRT-PCR) has been successfully used to evaluate the levels of messenger ribonucleic acid (mRNA) expression in each cell type (Rebouças et al., 2013). The accuracy of qRT-PCR relies on normalization to an internal control, often referred to as a reference gene (Silver et al., 2008). The best known reference genes in the literature are those encoding glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), beta actin (*Actb*), ribosomal proteins L (*Rpl*), ubiquitin, beta tubulin, 18S ribosomal protein, and phosphoglycerate kinase (Rebouças et al., 2013). Inappropriate selection of a gene as a normalizer may underestimate or overestimate the target gene expression, generating false results (Zhang et al., 2016), thus mathematical algorithms must be applied to evaluate the expression stability of reference genes. geNorm evaluates the *M* value, estimated from the arithmetic mean of pairwise variations of each gene (Vandesompele et al., 2002); NormFinder considers both intragroup and intergroup variations for normalization (Andersen et al., 2004); BestKeeper is based on the standard deviation (SD), coefficient of variance (CV), and coefficient of correlation (Pfaffl et al., 2004); and the delta threshold cycle (ΔC_t) method compares relative expression of "pairs of genes" within each sample (Silver et al., 2006). In the comprehensive ranking, the top classified genes are recommended as reference genes for data normalization in similar experimental systems (Zhang et al., 2016).

Given the increasing number of studies on obesity, especially those that evaluate the gene expression in adipose tissue, there are only a few studies evaluating the stability of reference genes in this tissue, furthermore, none of them employed the cafeteria diet or denervation (Nakao et al., 2017; Ragusa et al., 2017; Zhang et al., 2016). Thus, the present study aimed to identify the most stable gene(s) to use as reference gene(s). Subunit ribosomal RNA (*18S*); beta-2 microglobulin (*B2m*); Actb, cyclophilin A (*CypA*), *Gapdh*, hypoxanthine phosphoribosyltransferase (*Hprt1*), *Rpl32*, TATA box binding protein (*Tbp*), Ubiquitin C (*Ubc*) and tyrosine 3-monooxygenase/tryptophan 5-

monooxygenase activation protein, and zeta (*Ywhaz*) genes were tested in interscapular brown adipose tissue (IBAT) and retroperitoneal white adipose tissue (RWAT), both denervated and innervated, from cafeteria diet-fed or control diet-fed rats. In addition, the adrenoceptor beta 3 (*Adrb3*) gene expression was assessed using one of the most and one of the least stable genes as normalizers to demonstrate the importance of a careful selection of reference gene.

Materials and Methods

Animal Care and Study Design

In the present study, 28 male Wistar rats, initially weighing 65–75 g, were obtained from the Breeding Center of the Federal University of São João Del-Rei and kept in cages in an environmentally controlled room with a 12/12 h light/dark cycle at 23 ± 2 °C. The rats were divided into two groups: cafeteria diet or control diet. Rats fed a cafeteria diet for 24 days, which consisted of a chow diet (Nuvilab CR1, Nuvital, Brazil (22% protein, 55% carbohydrate and 4.5% lipid)) supplemented each day with four different lipid-rich palatable items selected from a list of 12 (Table 1). In addition, the water offered to these rats contained 20% sucrose. Control rats consumed the standard balanced diet only and water *ad libitum.* The energy intake of cafeteria diet-fed rats, which included the amount and composition of palatable items and the volume of water consumed, was approximately 40% higher than in controls (Chaves et al., 2006, 2008). In cafeteria diet-fed rats, protein contributed 15 ± 1%, carbohydrate 65 ± 1%, and lipid 20 ± 1% of the energy intake, compared with 25%, 63%, and 12%, respectively, in rats fed the control diet (Chaves et al., 2006, 2008). Surgical hemidenervation of IBAT or RWAT of each group was performed 7 days before using the animals in the experiments, thus the animals were divided into two subgroups. The rats weighed 210–230 g at the moment of the euthanasia, which was performed in the fed state between 8:00 and 10:00 AM. After euthanasia, the IBAT and RWAT were removed. Care and treatment of the rats received prior institutional approval by the Ethical Committee of the Federal University of São João del Rei (protocol 02/2012 and 31/2016).

Table 1 Composition (g/100 g) of palatable items present in cafeteria diet

	Carbohydrate	Lipid	Protein
Bacon	0	58.0	9.0
Caramel candy	72.7	9.1	18.2
Cashew nut	7.0	67.0	17.0
Cheese biscuit	65.0	17.5	7.5
Chocolate roll	50.0	33.3	10.0
Chocolate wafers	63.1	23.3	8.1
Cookies	63.4	23.3	3.3
Cornstarch biscuit	62.5	12.5	7.5
Nougat	80.0	7.5	10.0
Peanut candy	58.8	26.5	11.7
Potato chips	45.0	35.0	5.0
Toast	73.3	6.7	13.3

The water offered to cafeteria diet-fed rats contains 20% sucrose.

Unilateral IBAT and RWAT Sympathetic Denervation

Under ketamine:xylazine (70:7 mg/kg) anesthesia, five branches of the right intercostal nerve bundles that contain sympathetic fibers entering the right side of the IBAT were isolated and a section of approximately 5 mm was removed from these nerves, as described by Foster et al. (1982). For RWAT, three nerves were surgically denervated as described by Cantu and Goodman (1967). The three visible branches of the nerves entering the tissue were cut before and after their passage through the fat pad and the ~20 mm portions between the cuts were carefully removed. Seven days after the surgical hemidenervation, the norepinephrine (NE) concentration was measured using high-performance liquid chromatography, as described by Garofalo et al. (1996).

RNA Extraction and Reverse Transcription qRT-PCR

Total RNA, from innervated and denervated IBAT and RWAT, was isolated using the RNeasy Lipid Tissue Mini Kit (QIAGEN, Hilden, North Rhine-Westphalia, Germany), according to the manufacturer's protocol. The amount and purity of the RNA were determined by optical density ratios 260/280 and 260/230 (NanoVue Plus Spectrophotometer, GE Healthcare). Genomic DNA contamination was ruled out by running a polymerase chain reaction (PCR) on the RNA samples. Reverse transcription into complementary deoxyribonucleic acid (cDNA) was performed using 2 μg of total RNA with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. The qRT-PCR assays were carried out on a sequence detection system 7500 Fast Real-time PCR System (Applied Biosystems) using a SYBR Green PCR Master Mix and specific primers for *18S*, *B2m*, *Actb*, *CypA*, *Gapdh*, *Hprt1*, *Rpl32*, *Tbp*, *Ubc*, *Ywhaz*, and *Adrb3* genes (Table 2). The gene expression was quantified using the ΔC_t method (Livak and Schmittgen, 2001) by measuring the threshold cycle normalized to a reference gene and then expressed relative to the control groups.

Table 2 General information about the genes and primer sequences

Gene	Function	mRNA accession number	Primer sequence	Tm	PCR product
18S rRNA	Ribosome subunit	M11188.1	F: 5′-GTAACCCGTTGAACCCCATT-3′ R: 5′- CCATCCAATCGGTAGTAGCG-3′	54 °C	151 bp
Adrb3	Adrenergic signaling pathway	NM_013108.2	F: 5′-AACTCTGCCTTCAACCCGCTCAT-3′ R: 5′-TTCATGTGGGAAATGGACGCTCAC-3′	64 °C	202 bp
B2m	Antigen presentation	NM_012512.1	F: 5′-ATGGAGCTCTGAATCATCTGG-3′ R: 5′-AGAAGATGGTGTGCTCATTGC-3′	52 °C	155 bp
Actb	Cytoskeletal structural protein	NM_031144.3	F: 5′-CATGAAGATCAAGATCATTGCTCCT-3′ R: 5′-CTGCTTGCTGATCCACATCTG-3′	59 °C	109 bp
CypA	Cis-trans isomerization of peptide bonds	NM_017101.1	F: 5′-TATCTGCACTGCCAAGACTGAGTG-3′ R: 5′-CTTCTTGCTGGTCTTGCCATTCC-3′	57 °C	127 bp
Gapdh	Glycolytic enzyme	NM_017008.4	F: 5′-GCCAAAAGGGTCATCATCTC-3′ R: 5′-TACATTGGGGGTAGGAACAC-3′	56 °C	375 bp
Hprt1	Purine synthesis	NM_012583.2	F: 5′-CCCAGCGTCGTGATTAGTGATG-3′ R: 5′-TTCAGTCCTGTCCATAATCAGTCC-3′	57 °C	127 bp
Rpl32	Ribosome subunit	NM_013226.2	F: 5′-TCTGGTGAAGCCCAAGATCG-3′ R: 5′-CTCTGGGTTTCCGCCAGTT-3′	59 °C	101 bp
Tbp	Transcription factor	NM_001004198–1	F: 5′-CACCGTGAATCTTGGCTGTAAAC-3′ R: 5′-CGCAGTTGTTCGTGGCTCTC-3′	56 °C	124 bp
Ywhaz	Signal transduction	NM_013011.3	F: 5′-GATGAAGCCATTGCTGAACTTG-3′ R: 5′-GTCTCCTTGGGTATCCGATGTC-3′	57 °C	117 bp
Ubc	Remove damaged/unfolded proteins	NM_017314.1	F: 5′-CTCGTACCTTTCTCACCACAGT-3′ R: 5′-GACACCTCCCCATCAAACCC-3′	56 °C	74 bp

F, forward primer; PCR, polymerase chain reaction; R, reverse primer.

18S: 18S subunit ribosomal RNA; *B2m*: beta-2 microglobulin; *Actb*: beta actin; *CypA*: cyclophilin A; *Gapdh*: glyceraldeyde-3-phosphate dehydrogenase; *Hprt1*: hypoxanthine phosphoribosyltransferase; Rpl32: ribosomal protein L32; *Tbp*: TATA box binding protein; *Ubc*: ubiquitin C; and *Ywhaz*: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta.

Reference Gene Expression Stability Analysis

The statistical tools geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004), BestKeeper (Pfaffl et al., 2004), and the comparative ΔC_t method (Silver et al., 2006) were used to evaluate the stability of gene expression.

Statistical Analysis

The normality test was performed by the Shapiro–Wilk test and significant differences in mean values were measured by two-way ANOVA followed by Bonferroni's test. A p value <0.05 was adopted as the criterion of significance. Data were presented as the mean ± SEM.

Results

Norepinephrine Concentration after Denervation Surgery

First, the NE concentration in the adipose tissues was quantified to verify the efficiency of denervation. In IBAT, the NE concentration was reduced to approximately 3% in the denervated side compared with the control, contralateral innervated side (Fig. 1a). In contrast, in RWAT, the NE concentration was reduced to approximately 36% in the denervated side compared with the control, contralateral innervated side (Fig. 1b). In the intragroup comparison, the denervation did not affect IBAT and RWAT weight, but in the intergroup comparison, the cafeteria diet increased the weight of IBAT and RWAT, independent of innervation. The values found were 0.22 ± 0.01 (*CF*) versus 0.12 ± 0.01 (C) g in innervated IBAT [$p < 0.05$], 0.21 ± 0.01 (*CF*) versus 0.13 ± 0.01 (C) g in denervated IBAT [$p < 0.05$], 0.95 ± 0.06 (*CF*) versus 0.41 ± 0.01 (C) g in innervated RWAT [$p < 0.05$], and 0.97 ± 0.04 (*CF*) versus 0.48 ± 0.02 (C) g in denervated RWAT [$p < 0.05$]; all these values are presented for the cafeteria (*CF*) and control (C) groups, respectively.

RNA Purity, Primer Specificity, and Threshold Cycle of the Reference Genes

After demonstrating the efficiency of tissue denervation, RNA purity and primer specificity were tested. The RNA isolated from IBAT and RWAT tissues was pure and free of proteins and phenol. The $Abs_{260/280}$ ratio was 2.01 ± 0.01 (1.94–2.04) for IBAT and 2.05 ± 0.01 (2.01–2.14) for RWAT and the $Abs_{260/230}$ ratio was

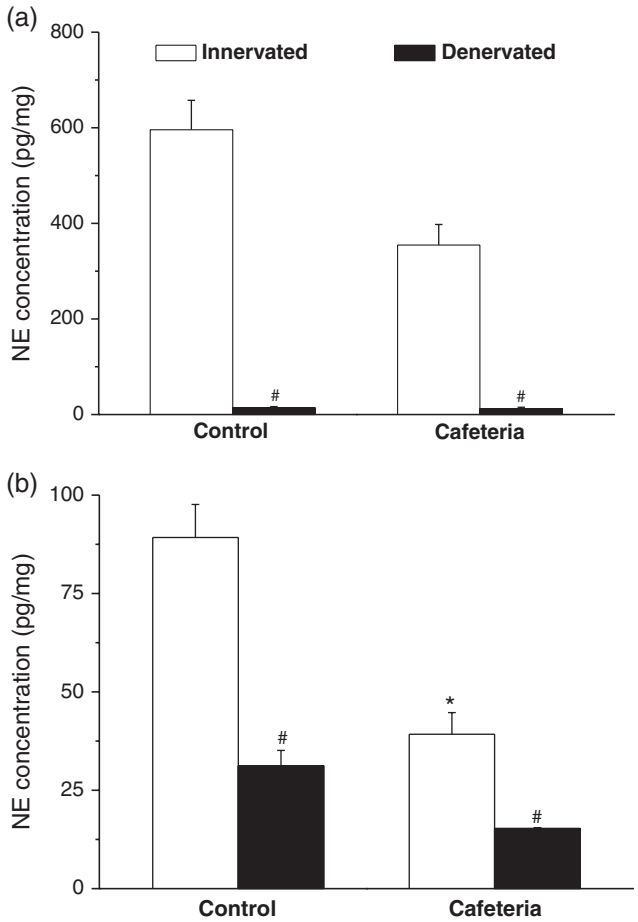

Fig. 1 Effect of cafeteria diet and denervation on norepinephrine (NE) concentration in the interscapular brown adipose tissue (IBAT) (a) and retroperitoneal white adipose tissue (RWAT) (b). Bars are means ± SEM of 4–6 rats. *$p < 0.05$ *versus* control. #$p < 0.05$ *versus* innervated

2.08 ± 0.04 (1.65–2.31) for IBAT and 2.02 ± 0.06 (1.60–2.33) for RWAT. The primer pairs for all candidate reference genes were specific, as evaluated by the analyses of the melting point using a corresponding cDNA sample (data not shown).

The threshold cycle (C_t) values of the reference genes, obtained from the real-time qRT-PCR analysis, were initially compared by their means among treatments. There were statistical differences for the following candidate reference genes: *Actb*, *CypA*, and *Gapdh* in IBAT and *18S*, *B2m*, *Actb*, *CypA*, *Gapdh*, *Hprt1*, *Tbp*, and *Ywhaz* in RWAT (Table 3).

Expression Stability of the Reference Genes

Four methods were used to evaluate the stability of gene expression in innervated and denervated IBAT and RWAT from cafeteria diet-fed or control diet-fed rats. The geNorm analysis (Fig. 2a, b) showed that the evaluated reference genes had an *M* value below 1.5, which is the maximum value for acceptable gene expression stability as defined by Vandesompele et al. (2002). The *Hprt1* and *Tbp* genes had the lowest *M* values in IBAT (0.507), followed by *Rpl32* (0.525), *Ywhaz* and *CypA* (0.538), *B2m* (0.553), *18S* (0.691), *Ubc* (0.711), *Actb* (0.815), and *Gapdh* (1.113) genes (Fig. 2a). The *Tbp* gene had the lowest *M* values in RWAT (0.505), followed by *B2m* (0.508), *Hprt1* (0.514), *Rpl32* (0.527), *Ywhaz* (0.569), *CypA* (0.577), *Ubc* (0.653), *Actb* (0.710), *Gapdh* (0.958), and *18S* (1.167) genes (Fig. 2b). These data indicate the rank of candidate reference genes from the most to the least stable in IBAT and RWAT.

According to the NormFinder analysis, the *Tbp* gene had the lowest stability value, indicating it as the most appropriate in IBAT (0.009). The *Tbp* gene was followed by *Hprt1* and *Ywhaz* (0.010), *Rpl32* (0.011), *CypA* (0.012), *B2m* (0.016), *Ubc* (0.018), *Actb* (0.022), *18S* (0.030), and *Gapdh* (0.034) genes (Fig. 3a). The *Rpl32* gene had the lowest *M* values in RWAT (0.011), followed by *Tbp* and *B2m* (0.012), *Hprt1* and *Ubc* (0.015), *Ywhaz* (0.017), *CypA* (0.018), *Gapdh* (0.032), *Actb* (0.033), and *18S* (0.065) genes (Fig. 3b).

According to the BestKeeper analysis, all the reference genes had an SD value lower than 1.0, which represents the cutoff value for acceptable gene expression stability, in IBAT and RWAT (Table 4). The ranking of reference genes by CV has a distinction from the ranking by the coefficient of correlation. Based on the CV results, the most stable gene to the least stable gene was ranked as follows: *Hprt1* > *Tbp* > *Rpl32* > *B2m* > *Ywhaz* > *CypA* > *Ubc* > *Actb* > *18S* > *Gapdh* for IBAT and *B2m* > *Tbp* and *Ubc* > *Hprt1* > *Rpl32* > *Ywhaz* > *CypA* > *Actb* > *Gapdh* > *18S* for RWAT (Table 4). However, when ordered by the coefficient of correlation, the results were as follows: *CypA* > *Ywhaz* > *Rpl32* > *Tbp* > *Hprt1* > *Actb* > *Gapdh* > *18S* > *B2m* > *Ubc* for IBAT and *Rpl32* > *Ywhaz* > *CypA* and *Tbp* > *Hprt1* > *Ubc* > *B2m* > *Gapdh* > *Actb* > *18S* for RWAT (Table 4).

The evaluation using the ΔC_t method does not have a cutoff value, but it allows the comparison of stability. According to the ΔC_t method analysis, the ranking was *Hprt1* > *Tbp*, *Rpl32* and *CypA* > *Ywhaz* > *B2m* > *18S* > *Ubc* > *Actb* > *Gapdh* for IBAT and *Hprt1* > *Tbp* > *B2m* > *Rpl32* > *Ywhaz* > *CypA* > *Ubc* > *Actb* > *Gapdh* > *18S* for RWAT (Table 5).

Thus based on the algorithms used, the three most stable genes were *Hprt1*, *Tbp*, and Rpl32 for IBAT and *Tbp*, *B2m*, and *Hprt1* for RWAT, while the least stable genes were *18S*, *Actb*, and *Gapdh* for both tissues.

Biological Application of Normalization of a Target Gene with Reference Genes

To identify how the stability of the reference genes affects the final results, the *Adrb3* mRNA was normalized by each one of the three most stable and by each one of the three least

Table 3 Effect of cafeteria diet and sympathetic denervation on the threshold cycle (C_t) of candidate reference genes in interscapular brown adipose tissue (IBAT) and retroperitoneal white adipose tissue (RWAT) from rats

IBAT	INN-CTRL	DEN-CTRL	INN-*CF*	DEN-*CF*	Cafeteria diet	Sympathetic denervation	Interaction
18S	09.76 ± 0.22	10.11 ± 0.23	10.08 ± 0.11	09.64 ± 0.11	NS	NS	NS
B2m	18.58 ± 0.12	18.44 ± 0.14	18.48 ± 0.08	18.13 ± 0.10	NS	NS	NS
Actb	18.84 ± 0.12	18.86 ± 0.19	18.35 ± 0.10	17.67 ± 0.39*	NS	NS	NS
CypA	19.77 ± 0.11	19.96 ± 0.20	19.11 ± 0.08*	19.68 ± 0.11$^{\#}$	NS	NS	NS
Gapdh	21.36 ± 0.27	22.09 ± 0.52	20.14 ± 0.22*	21.95 ± 0.20$^{\#}$	NS	NS	NS
Hprt1	20.76 ± 0.13	20.71 ± 0.14	20.64 ± 0.04	20.61 ± 0.09	NS	NS	NS
Rpl32	19.36 ± 0.18	19.36 ± 0.17	19.37 ± 0.12	18.99 ± 0.06	NS	NS	NS
Tbp	24.09 ± 0.12	24.17 ± 0.16	23.92 ± 0.05	24.18 ± 0.08	NS	NS	NS
Ubc	19.10 ± 0.24	18.94 ± 0.21	18.88 ± 0.20	18.84 ± 0.21	NS	NS	NS
Ywhaz	21.86 ± 0.14	21.92 ± 0.27	21.35 ± 0.06	21.64 ± 0.13	NS	NS	NS
RWAT	INN-CTRL	DEN-CTRL	INN-*CF*	DEN-*CF*	Cafeteria diet	Sympathetic denervation	Interaction
18 s	09.11 ± 0.11	08.69 ± 0.12	10.47 ± 0.27*	09.68 ± 0.10*$^{\#}$	$p < 0.05$	NS	NS
B2m	19.20 ± 0.11	19.7 ± 0.14$^{\#}$	19.17 ± 0.09	19.13 ± 0.10*	NS	NS	NS
Actb	19.34 ± 0.16	19.52 ± 0.16	18.46 ± 0.26*	18.54 ± 0.23*	$p < 0.05$	NS	NS
CypA	19.75 ± 0.16	20.68 ± 0.20$^{\#}$	19.49 ± 0.15	19.96 ± 0.10*$^{\#}$	NS	$p < 0.05$	NS
Gapdh	22.64 ± 0.32	23.95 ± 0.37$^{\#}$	22.85 ± 0.18	23.85 ± 0.31$^{\#}$	NS	$p < 0.05$	NS
Hprt1	20.47 ± 0.11	21.14 ± 0.16$^{\#}$	20.31 ± 0.14	20.50 ± 0.17*	NS	NS	NS
Rpl32	19.77 ± 0.19	20.03 ± 0.17	19.61 ± 0.14	19.65 ± 0.13	NS	NS	NS
Tbp	24.21 ± 0.16	24.90 ± 0.12$^{\#}$	23.92 ± 0.13	24.16 ± 0.14*	NS	NS	NS
Ubc	20.94 ± 0.18	21.33 ± 0.15	21.46 ± 0.18	21.53 ± 0.15	NS	NS	NS
Ywhaz	23.55 ± 0.15	24.12 ± 0.11	23.14 ± 0.18	23.27 ± 0.23*	NS	NS	NS

The data are represented as the mean ± SEM of rats ($n = 7$). **INN-CTRL**: innervated control; **DEN-CTR**: denervated control; **INN-*CF***: innervated cafeteria; **DEN-*CF***: denervated cafeteria; *18S*: 18S subunit ribosomal RNA; *B2m*: beta-2 microglobulin; *Actb*: beta actin; C_t: threshold cycle; *CypA*: cyclophilin A; *Gapdh*: glyceraldeyde-3-phosphate dehydrogenase; *Hprt1*: hypoxanthine phosphoribosyltransferase; IBAT: interscapular brown adipose tissue; *Rpl32*: ribosomal protein L32; RWAT: retroperitoneal white adipose tissue; *Tbp*: TATA box binding protein; *Ubc*: ubiquitin C; and *Ywhaz*: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta. *$p < 0.05$ versus control diet. $^{\#}p < 0.05$ versus innervated tissue.

stable reference genes in both adipose tissues. Similar results were obtained using each of the three most stable or least stable reference genes, thus only one most stable and one least stable gene were chosen to normalize the *Adrb3* gene. The cafeteria diet and sympathetic innervation did not change the expression of *Adrb3* mRNA in IBAT, when normalized by *Rpl32* mRNA (Fig. 4a). Conversely, independent of the diet, the denervation of IBAT from rats induced an increase of the *Adrb3* mRNA, when normalized by *Gapdh* mRNA (Fig. 4b). In the RWAT, sympathetic denervation from the control diet-fed rats induced a decrease of approximately 50% in *Adrb3* mRNA, when normalized by *B2m* (Fig. 4c), but when normalized by *Gapdh* mRNA, the cafeteria diet and sympathetic denervation did not change the expression of *Adrb3* mRNA in RWAT (Fig. 4d).

Discussion

The choice of an appropriate reference gene is fundamental to avoid an erroneous interpretation of the results (Iser et al., 2015). This process of normalization corrects variations caused, for example, by errors in sample quantification, real-time efficiency differences, or cDNA sample loading variation (Langnaese et al., 2008). Nevertheless, evidence has shown that the expression of individual reference genes does differ among samples under different experimental conditions (Yang et al., 2012). In the present study, we demonstrate that cafeteria diet or surgical denervation affects the C_t values of the *18S*, *Actb*, and *CypA* reference genes in RWAT (Table 3), thus making the evaluation of stability always necessary.

To the best of our knowledge, this is the first study to evaluate 10 candidates commonly used as reference genes for qRT-PCR in innervated and denervated IBAT and RWAT from cafeteria diet-fed or control diet-fed rats by the following algorithms: geNorm, NormFinder, BestKeeper, and comparative ΔC_t method. Initially, our efficiency of surgical sympathetic denervation agree with several studies that have demonstrated a similar reduction in IBAT and RWAT NE concentration without changes in tissue weight (Foster et al., 1982; Frasson et al., 2012;

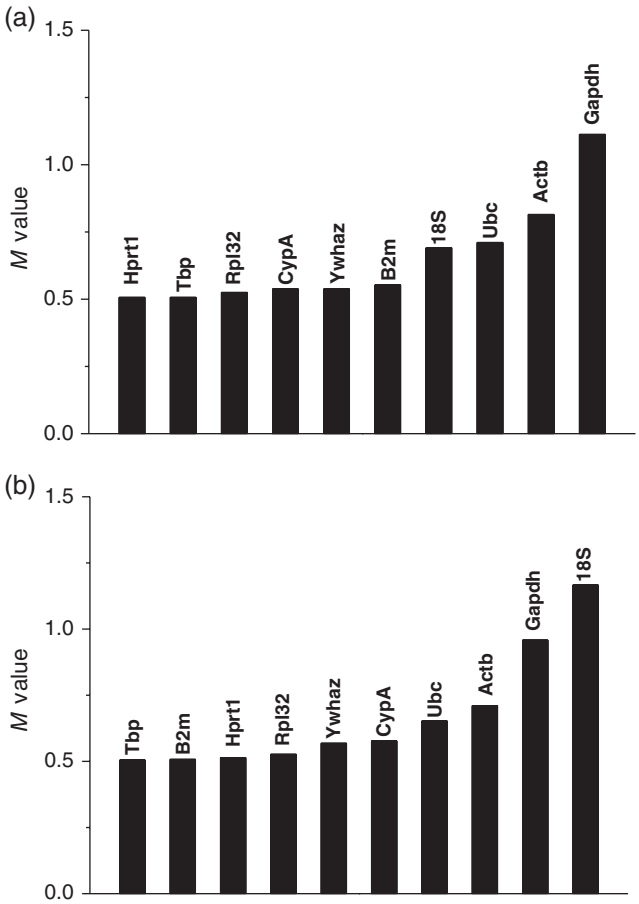

Fig. 2 Expression stability values and ranking of the 18S subunit ribosomal RNA (*18S*), beta-2 microglobulin (*B2m*), beta actin (*Actb*), cyclophilin A (*CypA*), glyceraldeyde-3-phosphate dehydrogenase (*Gapdh*), hypoxanthine phosphoribosyltransferase (*Hprt1*), ribosomal protein L32 (*Rpl32*), TATA box binding protein (*Tbp*), ubiquitin C (*Ubc*), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (*Ywhaz*) in the interscapular brown adipose tissue (IBAT—a) and retroperitoneal white adipose tissue (RWAT—b) from Wistar rats by geNorm ($n = 28$)

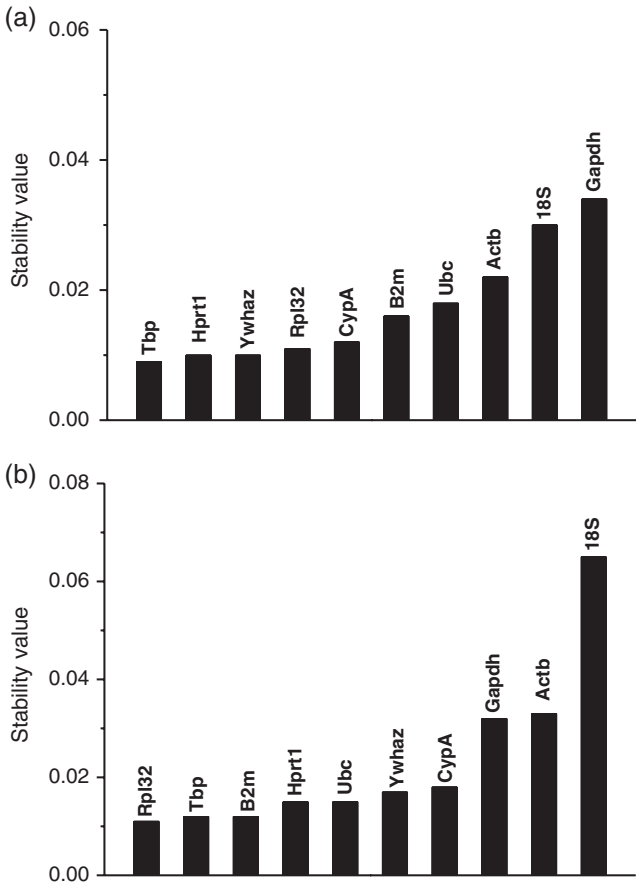

Fig. 3 Expression stability values and ranking of the 18S subunit ribosomal RNA (*18S*), beta-2 microglobulin (*B2m*), beta actin (*Actb*), cyclophilin A (*CypA*), glyceraldeyde-3-phosphate dehydrogenase (*Gapdh*), hypoxanthine phosphoribosyltransferase (*Hprt1*), ribosomal protein L32 (*Rpl32*), TATA box binding protein (*Tbp*), ubiquitin C (*Ubc*), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (*Ywhaz*) in the interscapular brown adipose tissue (IBAT—a) and retroperitoneal white adipose tissue (RWAT—b) from Wistar rats by NormFinder ($n = 28$)

Himms-Hagen et al., 1990; Kawashita et al., 2002). The absence of denervation effects in IBAT and RWAT weight demonstrates that the main stimulus for the increase in tissue weight in cafeteria diet-fed rats is hormonal. In a second part of the study, the gene expression stability was examined and the reference genes investigated presented acceptable gene expression stability in both IBAT and RWAT evaluated by geNorm, because the M value was below 1.5 (Vandesompele et al., 2002), and by BestKeeper analysis, because the SD was lower than 1.0 (Pfaffl et al., 2004). NormFinder and ΔC_t methods allow only the comparison of the gene expression stability between the candidate reference genes and the identification of the best one (Andersen et al., 2004; Silver et al., 2006).

According to all these algorithms, our results show that the three most stable genes were *Hprt1*, *Tbp*, and *Rpl32* for IBAT and *Tbp*, *B2m*, and *Hprt1* for RWAT, while the least stable genes were *18S*, *Actb*, and *Gapdh* for both tissues. The *Actb* and *Gapdh* genes are widely used in the literature as normalizers for the evaluation of target gene expression in WAT and BAT (Aguirre et al., 2016; Antony et al., 2017; Calderon-Dominguez et al., 2016; Chang and Kim, 2017; Li et al., 2015; Rodrigues et al., 2017; Tsubai et al., 2016; Yan et al., 2017), however, a few authors argue about the stability of the reference genes. A systematic review showed that the vast majority of the studies use a single gene as a normalizer, with *Actb* and/or *Gapdh* being commonly selected to evaluate the gene expression in vertebrates (Chapman and Waldenstrom, 2015). However, only a few studies (15%) have tested the expression stability of several potential reference genes before using them as normalizers (Chapman and Waldenstrom, 2015). Interestingly, among the studies specifically testing reference

Table 4 Expression stability of the candidate genes in interscapular brown adipose tissue (IBAT) and retroperitoneal white adipose tissue (RWAT) from rats (n = 28) evaluated by BestKeeper

IBAT	Geometric mean (C_t)	Arithmetic mean (C_t)	Minimum (C_t)	Maximum (C_t)	Standard deviation ($\pm C_t$)	Coefficient of variance ($\%C_t$)	Correlation coefficients (r)
18S	9.89	9.90	8.60	11.28	0.35	3.53	0.53
Actb	18.41	18.43	15.45	19.59	0.50	2.71	0.58
B2m	18.40	18.41	17.75	19.03	0.29	1.55	0.51
CypA	19.62	19.63	18.76	20.85	0.39	1.99	0.83
Gapdh	21.36	21.39	19.46	23.63	0.92	4.28	0.55
Hprt1	20.68	20.68	20.23	21.46	0.20	0.98	0.69
Rpl32	19.27	19.27	18.78	20.34	0.28	1.45	0.74
Tbp	24.09	24.09	23.64	24.87	0.24	0.99	0.73
Ubc	18.93	18.94	18.22	20.02	0.49	2.60	0.44
Ywhaz	21.69	21.69	20.56	23.06	0.38	1.75	0.80
RWAT	Geometric mean (C_t)	Arithmetic mean (C_t)	Minimum (C_t)	Maximum (C_t)	Standard deviation ($\pm C_t$)	Coefficient of variance ($\%C_t$)	Correlation coefficients (r)
18S	9.46	9.49	8.16	11.26	0.61	6.38	−0.10
Actb	18.95	18.97	17.27	20.10	0.54	2.83	0.55
B2m	19.29	19.30	18.68	20.35	0.29	1.49	0.71
CypA	19.96	19.97	18.93	21.50	0.44	2.19	0.75
Gapdh	23.30	23.32	21.41	25.40	0.75	3.24	0.63
Hprt1	20.60	20.61	19.80	21.84	0.38	1.82	0.74
Rpl32	19.76	19.76	18.93	20.62	0.36	1.84	0.80
Tbp	24.29	24.30	23.56	25.56	0.42	1.72	0.75
Ubc	21.31	21.31	20.19	22.32	0.37	1.72	0.73
Ywhaz	23.51	23.52	21.97	24.59	0.47	1.99	0.76

18S: 18S subunit ribosomal RNA; *B2m*: beta-2 microglobulin; *Actb*: beta actin; *CypA*: cyclophilin A; *Gapdh*: glyceraldeyde-3-phosphate dehydrogenase; *Hprt1*: hypoxanthine phosphoribosyltransferase; IBAT: interscapular brown adipose tissue; *Rpl32*: ribosomal protein L32; RWAT: retroperitoneal white adipose tissue; *Tbp*: TATA box binding protein; *Ubc*: ubiquitin C; and *Ywhaz*: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta.

gene stability, few found *Actb* or *Gapdh* to be optimal (Chapman and Waldenstrom, 2015). Furthermore, a few studies have evaluated the stability of reference genes in adipose tissue (Nakao et al., 2017; Ragusa et al., 2017; Zhang et al., 2016); however, none of them involved the cafeteria diet or adipose tissue denervation.

In inguinal beige adipose tissue and IBAT from obese and lean mice, the gene encoding the acidic ribosomal phosphoprotein P0 (*36B4*) was the best reference gene among *Actb*, *Gapdh*, *18S*, and *36B4*, while in epididymal WAT, *Gapdh* was the best (Zhang et al., 2016). More recently, it was demonstrated that the gene for *Tbp* was the most stable gene in adipose tissue from Zucker rats and *Gapdh* the least stable, both evaluated by geNorm, among the genes encoding *Gapdh*, *Tbp*, *Hprt1*, peptidylprolyl isomerase A, ribosomal protein L13A, and transferrin receptor (Ragusa et al., 2017). Although the two-way ANOVA identified that the *18S* was stably expressed throughout the day independent of feeding schedules in the liver and WAT, the peptidylprolyl isomerase A was the most stably expressed reference gene as evaluated by the RefFinder software (Nakao et al., 2017).

Slight differences in results by evaluation methods are expected, as programs rely on different mathematical approaches, for example the pairwise comparison approach, geNorm, tends to select those genes with the highest degree of similarity of expression profiles across the sample set (Nelissen et al., 2010). While NormFinder ranks the candidates with minimal estimated intragroup and intergroup variations, and, therefore, selects the genes taking into account the different experimental conditions used (Nelissen et al., 2010). These slight differences are also found in other studies of reference genes (Langnaese et al., 2008; Melgar-Rojas et al., 2015; Nelissen et al., 2010; Santos et al., 2016; Taki et al., 2014; Wan et al., 2010; Zhang et al., 2016).

To validate the results from the algorithms as suitable for normalization, we decided to evaluate the expression of the gene coding for *Adrb3*. It was demonstrated recently that the increased sympathetic activity to IBAT induced by 3-week cold exposure (at 4 °C) does not change the *Adrb3* expression (de Jong et al., 2017). Rats fed the cafeteria diet presented an increased norepinephrine turnover,

Table 5 Pair comparisons between reference candidate genes from interscapular brown adipose tissue (IBAT) and retroperitoneal white adipose tissue (RWAT) by the comparative ΔC_t method

Sample	Mean ΔC_t	SD	Mean SD	Stability ranking	Mean ΔC_t	SD	Mean SD	Stability ranking
			IBAT				RWAT	
18S versus *Actb*	8.53	0.76	0.66	5°	9.47	1.35	1.15	10°
18S versus *B2m*	8.50	0.54			9.80	1.04		
18S versus *CypA*	9.73	0.61			10.48	1.26		
18S versus *Gapdh*	11.49	1.29			13.83	1.34		
18S versus *Hprt1*	10.78	0.54			11.11	1.15		
18S versus *Rpl32*	9.37	0.47			10.27	1.04		
18S versus *Tbp*	33.99	0.60			14.81	1.18		
18S versus *Ubc*	9.04	0.58			11.82	0.77		
18S versus *Ywhaz*	11.79	0.63			14.03	1.22		
Actb versus *18S*	8.53	0.76	0.82	7°	9.47	1.35	0.70	8°
Actb versus *B2m*	0.02	0.70			0.33	0.56		
Actb versus *CypA*	1.19	0.77			1.00	0.59		
Actb versus *Gapdh*	2.95	1.34			4.35	1.10		
Actb versus *Hprt1*	2.25	0.73			1.64	0.53		
Actb versus *Rpl32*	0.84	0.62			0.79	0.53		
Actb versus *Tbp*	5.66	0.77			5.33	0.49		
Actb versus *Ubc*	0.51	0.96			2.34	0.83		
Actb versus *Ywhaz*	3.26	0.75			4.55	0.37		
B2m versus *18S*	8.50	0.54	0.54	4°	9.80	1.04	0.50	3°
B2m versus *Actb*	0.02	0.70			0.33	0.56		
B2m versus *CypA*	1.22	0.50			0.67	0.39		
B2m versus *Gapdh*	2.98	1.20			4.02	0.92		
B2m versus *Hprt1*	2.27	0.26			1.30	0.23		
B2m versus *Rpl32*	0.86	0.35			0.46	0.28		
B2m versus *Tbp*	5.68	0.32			4.99	0.22		
B2m versus *Ubc*	0.53	0.59			2.01	0.54		
B2m versus *Ywhaz*	3.28	0.46			4.22	0.36		
CypA versus *18S*	9.73	0.61	0.52	2°	10.48	1.2	0.56	6°
CypA versus *Actb*	1.19	0.77			1.00	0.59		
CypA versus *B2m*	1.22	0.50			0.67	0.39		
CypA versus *Gapdh*	1.75	0.83			3.34	0.75		
CypA versus *Hprt1*	1.05	0.38			0.63	0.31		
CypA versus *Rpl32*	0.35	0.46			0.20	0.41		
CypA versus *Tbp*	4.46	0.31			4.32	0.34		
CypA versus *Ubc*	0.68	0.60			1.34	0.59		
CypA versus *Ywhaz*	2.06	0.25			3.54	0.43		
Gapdh versus *18S*	11.49	1.29			13.83	1.34		
Gapdh versus *Actb*	2.95	1.34	1.12	8°	4.35	1.10	0.95	9°
Gapdh versus *B2m*	2.98	1.20			4.02	0.92		
Gapdh versus *CypA*	1.75	0.83			3.34	0.75		
Gapdh versus *Hprt1*	0.70	1.10			2.1	0.93		
Gapdh versus *Rpl32*	2.11	1.18			3.55	0.91		
Gapdh versus *Tbp*	2.70	1.00			0.97	0.90		
Gapdh versus *Ubc*	2.44	1.28			2.00	0.82		
Gapdh versus *Ywhaz*	0.30	0.86			0.20	0.95		
Hprt1 versus *18S*	10.78	0.54	0.48	1°	11.11	1.15	0.46	1°

Table 5 Continued

Sample	Mean ΔC_t	SD	Mean SD	Stability ranking	Mean ΔC_t	SD	Mean SD	Stability ranking
Hprt1 versus *Actb*	2.25	0.73			1.64	0.53		
Hprt1 versus *B2m*	2.27	0.26			1.30	0.23		
Hprt1 versus *CypA*	1.05	0.38			0.63	0.31		
Hprt1 versus *Gapdh*	0.70	1.10			2.71	0.93		
Hprt1 versus *Rpl32*	1.41	0.29			0.84	0.34		
Hprt1 versus *Tbp*	3.41	0.19			3.69	0.19		
Hprt1 versus *Ubc*	1.73	0.49			0.70	0.57		
Hprt1 versus *Ywhaz*	1.01	0.37			2.91	0.31		
Rpl32 versus *18S*	9.37	0.47	0.52	2°	10.27	1.04	0.52	4°
Rpl32 versus *Actb*	0.84	0.62			0.79	0.53		
Rpl32 versus *B2m*	0.86	0.35			0.46	0.28		
Rpl32 versus *CypA*	0.35	0.46			0.20	0.41		
Rpl32 versus *Gapdh*	2.11	1.18			3.55	0.91		
Rpl32 versus *Hprt1*	1.41	0.29			0.84	0.34		
Rpl32 versus *Tbp*	4.81	0.32			4.53	0.31		
Rpl32 versus *Ubc*	0.32	0.52			1.55	0.50		
Rpl32 versus *Ywhaz*	2.42	0.47			3.75	0.37		
Tbp versus *18S*	14.19	0.55	0.52	2°	14.81	1.18	0.49	2°
Tbp versus *Actb*	5.66	0.77			5.33	0.49		
Tbp versus *B2m*	5.68	0.32			4.99	0.22		
Tbp versus *CypA*	4.46	0.31			4.32	0.34		
Tbp versus *Gapdh*	2.70	1.00			0.97	0.90		
Tbp versus *Hprt1*	44.77	0.55			3.61	0.19		
Tbp versus *Rpl32*	4.81	0.32			4.53	0.31		
Tbp versus *Ubc*	5.14	0.55			2.98	0.61		
Tbp versus *Ywhaz*	2.39	0.34			0.77	0.22		
Ubc versus *18S*	9.04	0.58	0.69	6°	11.82	0.70	0.65	7°
Ubc versus *Actb*	0.51	0.96			2.34	0.83		
Ubc versus *B2m*	0.53	0.59			2.01	0.54		
Ubc versus *CypA*	0.68	0.60			1.34	0.59		
Ubc versus *Gapdh*	2.44	1.28			2.00	0.82		
Ubc versus *Hprt1*	1.73	0.49			0.70	0.57		
Ubc versus *Rpl32*	0.32	0.52			1.55	0.50		
Ubc versus *Tbp*	5.14	0.55			2.98	0.61		
Ubc versus *Ywhaz*	2.75	0.70			2.20	0.67		
Ywhaz versus *18S*	11.79	0.63	0.53	3°	14.03	1.22	0.54	5°
Ywhaz versus *Actb*	3.26	0.75			4.55	0.37		
Ywhaz versus *B2m*	3.28	0.46			4.22	0.36		
Ywhaz versus *CypA*	2.06	0.25			3.54	0.43		
Ywhaz versus *Gapdh*	0.30	0.86			0.20	0.95		
Ywhaz versus *Hprt1*	1.01	0.37			2.91	0.31		
Ywhaz versus *Rpl32*	2.42	0.47			3.75	0.37		
Ywhaz versus *Tbp*	2.39	0.34			0.77	0.22		
Ywhaz versus *Ubc*	2.75	0.70			2.20	0.67		

18S: 18S subunit ribosomal RNA; *B2m*: beta-2 microglobulin; *Actb*: beta actin; *CypA*: cyclophilin A; *Gapdh*: glyceraldeyde-3-phosphate dehydrogenase; *Hprt1*: hypoxanthine phosphoribosyltransferase; *Rpl32*: ribosomal protein L32; SD: standard deviation; *Tbp*: TATA box binding protein; *Ubc*: ubiquitin C; and *Ywhaz*: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta.

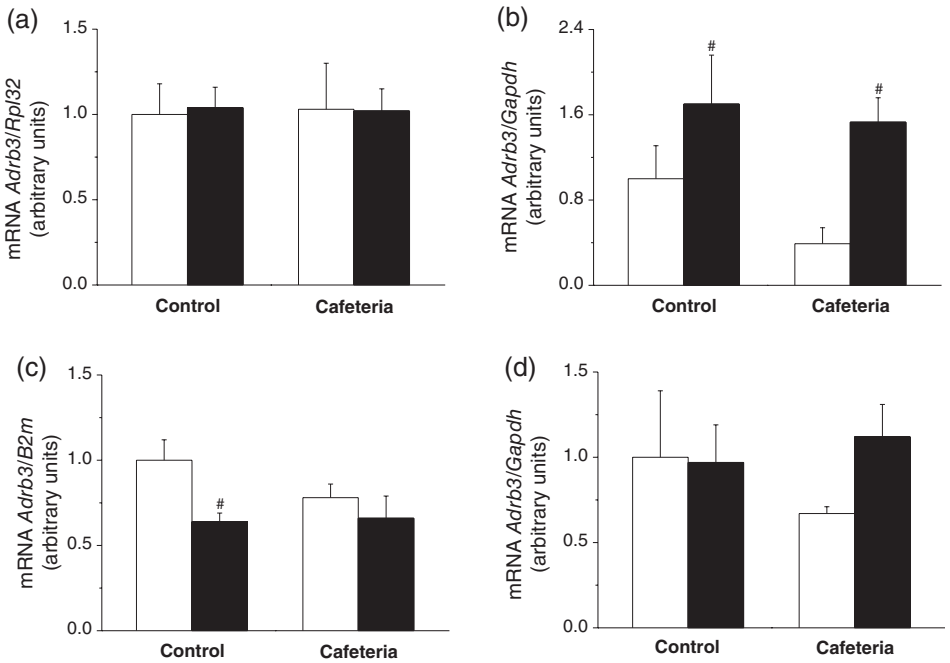

Fig. 4 Effect of denervation on the mRNA levels of *adrenoceptor beta 3* (*Adrb3*) normalized by ribosomal protein L32 (*Rpl32*—a) and glyceraldeyde-3-phosphate dehydrogenase (*Gapdh*—b) mRNA in the interscapular brown adipose tissue (IBAT) and by beta-2 microglobulin (*B2m*—c) and glyceraldeyde-3-phosphate dehydrogenase (*Gapdh*—d) retroperitoneal white adipose tissue (RWAT) from rats fed a control or cafeteria diet. Bars are means ± SEM of rats ($n = 7$). $^{*}p < 0.05$ versus control diet. $^{\#}p < 0.05$ versus innervated tissue

demonstrating an increase in sympathetic activity for this tissue (Chaves et al., 2008). Corroborating these findings, our results demonstrate that the expression of *Adrb3* was not changed by the elevated sympathetic stimulation induced by the cafeteria diet when the *Rpl32* gene was used for normalization (Fig. 4a). However, when *Gapdh* was used for normalization, a tendency for decreased expression was seen in rats fed with the cafeteria diet, although not statistically significant (Fig. 4b). In addition, the denervation procedure of both groups culminated in increased expression of *Adrb3* when normalized by *Gapdh* (Fig. 4b), but such changes did not occur when normalized by *Rpl32*, one of most stable genes (Fig. 4a). In WAT, a pioneering study using the RWAT sympathetic denervation to evaluate the *Adrb3* expression, by northern blotting, shows a decrease of the expression of this receptor in control diet-fed rats (Cousin et al., 1993). Similar results were obtained by normalization with one of most stable genes (*B2m*) (Fig. 4c). However, the *Adrb3* expression normalized by *Gapdh* was affected neither by the cafeteria diet nor denervation (Fig. 4d). In agreement with our results using the *B2m* gene, the cafeteria diet did not change the *Adrb3* expression in RWAT compared with the control rats (Llado et al., 2002). Conversely, when normalized with the *18S* and *Actb* genes, two other least stable genes, the *Adrb3* expression increased and decreased, respectively, in innervated RWAT (data not show), demonstrating the importance of a careful selection of reference genes to generate reliable results.

In summary, combining the various findings of the algorithms tested and literature data for *Adrb3* expression, *Hprt1*, *Tbp*, and *Rpl32* genes present as optimal reference genes to evaluate the effects of the cafeteria diet and sympathetic activity in IBAT and *Tbp*, *B2m*, and *Hprt1* genes for RWAT from rats. The *18S*, *Actb*, and *Gapdh* genes produced opposite results compared with the most stable ones regarding the *Adrb3* expression and, thus, should not be chosen under these experimental conditions. No statistical approach can cover all variables associated with gene expression studies, therefore, it is important to use more than one statistical approach. In addition, the test of more than one candidate reference gene increases the accuracy of the results and is strongly advised; for a good normalization, it is recommended to test at least three genes.

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Authors' Contribution G.H.M-O., H.M.S.V., J.A.A-F., I.C.K., H.C.F.O., and V.E.C. conceived and designed the study, interpreted the results, and drafted/edited the manuscript. G.H.M.-O., T.M.S., H.F.R., R.O.G.C, and M.A.R.G. collected data, conducted analyses, and revised the manuscript. All authors approved the content of this

manuscript. All authors contributed to the development, analysis, and drafting of this article.

Conflict of Interest The authors declare that they have no conflict of interest.

References

Aguirre, L., Milton-Laskibar, I., Hijona, E., Bujanda, L., Rimando, A. M., & Portillo, M. P. (2016) Effects of pterostilbene in brown adipose tissue from obese rats. *Journal of Physiology and Biochemistry*, **73**:457–464. https://doi.org/10.1007/s13105-017-0556-2

American Diabetes Association (2018) Obesity management for the treatment of type 2 diabetes: Standards of medical care in diabetes-2018. *Diabetes Care*, **41**:S65–S72. https://doi.org/10.2337/dc18-S007

Andersen, C. L., Jensen, J. L., & Orntoft, T. F. (2004) 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 Research*, **64**:5245–5250. https://doi.org/10.1158/0008-5472.CAN-04-0496

Antony, P. J., Gandhi, G. R., Stalin, A., Balakrishna, K., Toppo, E., Sivasankaran, K., … Al-Dhabi, N. A. (2017) Myoinositol ameliorates high-fat diet and streptozotocin-induced diabetes in rats through promoting insulin receptor signaling. *Biomedicine & Pharmacotherapy*, **88**:1098–1113. https://doi.org/10.1016/j.biopha.2017.01.170

Berger, N. A. (2014) Obesity and cancer pathogenesis. *Annals of the New York Academy of Sciences*, **1311**:57–76. https://doi.org/10.1111/nyas.12416

Bhatt, P. S., Dhillo, W. S., & Salem, V. (2017) Human brown adipose tissue-function and therapeutic potential in metabolic disease. *Current Opinion in Pharmacology*, **37**:1–9. https://doi.org/10.1016/j.coph.2017.07.004

Calderon-Dominguez, M., Sebastian, D., Fucho, R., Weber, M., Mir, J. F., Garcia-Casarrubios, E., … Herrero, L. (2016) Carnitine palmitoyltransferase 1 increases lipolysis, UCP1 protein expression and mitochondrial activity in Brown adipocytes. *PLoS One*, **11**:e0159399. https://doi.org/10.1371/journal.pone.0159399

Cantu, R. C., & Goodman, H. M. (1967) Effects of denervation and fasting on white adipose tissue. *The American Journal of Physiology*, **212**:207–212.

Chang, E., & Kim, Y. (2017) Vitamin D insufficiency exacerbates adipose tissue macrophage infiltration and decreases AMPK/SIRT1 activity in obese rats. *Nutrients*, **9**:1–15. https://doi.org/10.3390/nu9040338

Chapman, J. R., & Waldenstrom, J. (2015) With reference to reference genes: A systematic review of endogenous controls in gene expression studies. *PLoS One*, **10**:e0141853. https://doi.org/10.1371/journal.pone.0141853

Chaves, V. E., Frasson, D., Martins-Santos, M. E., Boschini, R. P., Garofalo, M. A., Festuccia, W. T., … Migliorini, R. H. (2006) Glyceroneogenesis is reduced and glucose uptake is increased in adipose tissue from cafeteria diet-fed rats independently of tissue sympathetic innervation. *The Journal of Nutrition*, **136**:2475–2480.

Chaves, V. E., Frasson, D., Martins-Santos, M. E., Navegantes, L. C., Galban, V. D., Garofalo, M. A., … Migliorini, R. H. (2008) Fatty acid synthesis and generation of glycerol-3-phosphate in brown adipose tissue from rats fed a cafeteria diet. *Canadian Journal of Physiology and Pharmacology*, **86**:416–423. https://doi.org/10.1139/y08-052

Coelho, M., Oliveira, T., & Fernandes, R. (2013) Biochemistry of adipose tissue: An endocrine organ. *Archives of Medical Science: AMS*, **9**:191–200. https://doi.org/10.5114/aoms.2013.33181

Cousin, B., Casteilla, L., Lafontan, M., Ambid, L., Langin, D., Berthault, M. F., & Penicaud, L. (1993) Local sympathetic denervation of white adipose tissue in rats induces preadipocyte proliferation without noticeable changes in metabolism. *Endocrinology*, **133**:2255–2262. https://doi.org/10.1210/endo.133.5.8404678

Dab, H., Hachani, R., Sakly, M., Bricca, G., & Kacem, K. (2013) Physiological regulation of pro-inflammatory cytokines expression in rat cardiovascular tissues by sympathetic nervous system and angiotensin II. *General Physiology and Biophysics*, **32**:569–575. https://doi.org/10.4149/gpb_2013054

de Jong, J. M. A., Wouters, R. T. F., Boulet, N., Cannon, B., Nedergaard, J., & Petrovic, N. (2017) The beta3-adrenergic receptor is dispensable for browning of adipose tissues. *American Journal of Physiology Endocrinology and Metabolism*, **312**:E508–E518. https://doi.org/10.1152/ajpendo.00437.2016

Figueiredo, P. S., Inada, A. C., Marcelino, G., Cardozo, C. M. L., Freitas, K. C., Guimaraes, R. C. A., … Hiane, P. A. (2017) Fatty acids consumption: The role metabolic aspects involved in obesity and its associated disorders. *Nutrients*, **9**:1–32. https://doi.org/10.3390/nu9101158

Foster, D. O., Depocas, F., & Zaror-Behrens, G. (1982) Unilaterality of the sympathetic innervation of each pad of rat interscapular brown adipose tissue. *Canadian Journal of Physiology and Pharmacology*, **60**:107–113.

Frasson, D., Boschini, R. P., Chaves, V. E., Santos, M. E., Paula Gomes, S., Valentim, R. R., … Kettelhut, I. C. (2012) The sympathetic nervous system regulates the three glycerol-3P generation pathways in white adipose tissue of fasted, diabetic and high-protein diet-fed rats. *Metabolism: Clinical and Experimental*, **61**:1473–1485. https://doi.org/10.1016/j.metabol.2012.03.014

Galic, S., Oakhill, J. S., & Steinberg, G. R. (2010) Adipose tissue as an endocrine organ. *Molecular and Cellular Endocrinology*, **316**:129–139. https://doi.org/10.1016/j.mce.2009.08.018

Ganta, C. K., Helwig, B. G., Blecha, F., Ganta, R. R., Cober, R., Parimi, S., … Kenney, M. J. (2006) Hypothermia-enhanced splenic cytokine gene expression is independent of the sympathetic nervous system. *American Journal of Physiology Regulatory, Integrative and Comparative Physiology*, **291**:R558–R565. https://doi.org/10.1152/ajpregu.00846.2005

Garofalo, M. A., Kettelhut, I. C., Roselino, J. E., & Migliorini, R. H. (1996) Effect of acute cold exposure on norepinephrine turnover rates in rat white adipose tissue. *Journal of the Autonomic Nervous System*, **60**:206–208.

Gomez-Hernandez, A., Beneit, N., Diaz-Castroverde, S., & Escribano, O. (2016) Differential role of adipose tissues in obesity and related metabolic and vascular complications. *International Journal of Endocrinology*, **2016**:1, 1216783–15. https://doi.org/10.1155/2016/1216783

Harwood Jr., H. J. (2012) The adipocyte as an endocrine organ in the regulation of metabolic homeostasis. *Neuropharmacology*, **63**:57–75. https://doi.org/10.1016/j.neuropharm.2011.12.010

Himms-Hagen, J., Cui, J., & Sigurdson, S. L. (1990) Sympathetic and sensory nerves in control of growth of brown adipose tissue: Effects of denervation and of capsaicin. *Neurochemistry International*, **17**:271–279.

Iser, I. C., de Campos, R. P., Bertoni, A. P. S., & Wink, M. R. (2015) Identification of valid endogenous control genes for determining gene expression in C6 glioma cell line treated with conditioned medium from adipose-derived stem cell. *Biomedicine & Pharmacotherapy*, **75**:75–82. https://doi.org/10.1016/j.biopha.2015.08.035

Johnson, A. R., Wilkerson, M. D., Sampey, B. P., Troester, M. A., Hayes, D. N., & Makowski, L. (2016) Cafeteria diet-induced obesity causes oxidative damage in white adipose. *Biochemical and*

Biophysical Research Communications, **473**:545–550. https://doi.org/10.1016/j.bbrc.2016.03.113

Kawashita, N. H., Moura, M. A., Brito, M. N., Brito, S. M., Garofalo, M. A., Kettelhut, I. C., & Migliorini, R. H. (2002) Relative importance of sympathetic outflow and insulin in the reactivation of brown adipose tissue lipogenesis in rats adapted to a high-protein diet. *Metabolism: Clinical and Experimental*, **51**: 343–349.

Langnaese, K., John, R., Schweizer, H., Ebmeyer, U., & Keilhoff, G. (2008) Selection of reference genes for quantitative real-time PCR in a rat asphyxial cardiac arrest model. *BMC Molecular Biology*, **9**: 53. https://doi.org/10.1186/1471-2199-9-53

Li, H., Zhang, J. B., Xu, C., Tang, Q. Q., Shen, W. X., Zhou, J. Z., … Wang, Y. P. (2015) Effects and mechanisms of auricular vagus nerve stimulation on high-fat-diet-induced obese rats. *Nutrition*, **31**: 1416–1422. https://doi.org/10.1016/j.nut.2015.05.007

Li, Z. S., Pham, T. D., Tamir, H., Chen, J. J., & Gershon, M. D. (2004) Enteric dopaminergic neurons: Definition, developmental lineage, and effects of extrinsic denervation. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, **24**: 1330–1339. https://doi.org/10.1523/JNEUROSCI.3982-03.2004

Lidell, M. E., Betz, M. J., & Enerback, S. (2014) Brown adipose tissue and its therapeutic potential. *Journal of Internal Medicine*, **276**: 364–377. https://doi.org/10.1111/joim.12255

Livak, K. J., & Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method. *Methods*, **25**:402–408. https://doi.org/10.1006/meth.2001.1262

Llado, I., Rodriguez-Cuenca, S., Pujol, E., Monjo, M., Estrany, M. E., Roca, P., & Palou, A. (2002) Gender effects on adrenergic receptor expression and lipolysis in white adipose tissue of rats. *Obesity Research*, **10**:296–305. https://doi.org/10.1038/oby.2002.41

Loh, R. K. C., Kingwell, B. A., & Carey, A. L. (2017) Human brown adipose tissue as a target for obesity management; beyond cold-induced thermogenesis. *Obesity Reviews: An Official Journal of the International Association for the Study of Obesity*, **18**:1227–1242. https://doi.org/10.1111/obr.12584

Melgar-Rojas, P., Alvarado, J. C., Fuentes-Santamaría, V., Gabaldón-Ull, M. C., & Juiz, J. M. (2015) Validation of reference genes for RT–qPCR analysis in noise–induced hearing loss: A study in Wistar rat. *PLoS One*, **10**:25. https://doi.org/10.1371/journal.pone.0138027

Morrison, S. F., & Madden, C. J. (2014) Central nervous system regulation of brown adipose tissue. *Comprehensive Physiology*, **4**: 1677–1713. https://doi.org/10.1002/cphy.c140013

Nakao, R., Okauchi, H., Hashimoto, C., Wada, N., & Oishi, K. (2017) Determination of reference genes that are independent of feeding rhythms for circadian studies of mouse metabolic tissues. *Molecular Genetics and Metabolism*, **121**:190–197. https://doi.org/10.1016/j.ymgme.2017.04.001

Nelissen, K., Smeets, K., Mulder, M., Jerome, J. A. H., & Ameloot, M. (2010) Selection of reference genes for gene expression studies in rat oligodendrocytes using quantitative real time PCR. *Journal of Neuroscience Methods*, **187**:78–83. https://doi.org/10.1016/j.jneumeth.2009.12.018

Oliver, P., Caimari, A., Diaz-Rua, R., & Palou, A. (2012) Diet-induced obesity affects expression of adiponutrin/PNPLA3 and adipose triglyceride lipase, two members of the same family. *International Journal of Obesity*, **36**:225–232. https://doi.org/10.1038/ijo.2011.92

Ooi, G. J., Mgaieth, S., Eslick, G. D., Burton, P. R., Kemp, W. W., Roberts, S. K., & Brown, W. A. (2017) Systematic review and meta-analysis: Non-invasive detection of non-alcoholic fatty liver disease related fibrosis in the obese. *Obesity Reviews: An Official Journal of the International Association for the Study of Obesity*, **19**:281–294. https://doi.org/10.1111/obr.12628

Parto, P., & Lavie, C. J. (2017) Obesity and cardiovascular diseases. *Current Problems in Cardiology*, **42**:376–394. https://doi.org/10.1016/j.cpcardiol.2017.04.004

Pfaffl, M. W., Tichopad, A., Prgomet, C., & Neuvians, T. P. (2004) Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper—Excel-based tool using pair-wise correlations. *Biotechnology Letters*, **26**: 509–515.

Ragusa, R., Cabiati, M., Guzzardi, M. A., D'Amico, A., Giannessi, D., Del Ry, S., & Caselli, C. (2017) Effects of obesity on IL-33/ST2 system in heart, adipose tissue and liver: Study in the experimental model of Zucker rats. *Experimental and Molecular Pathology*, **102**:354–359. https://doi.org/10.1016/j.yexmp.2017.03.002

Raju, B., Hultstrom, M., Haug, S. R., Ibrahim, S. O., & Heyeraas, K. J. (2009) Sympathectomy suppresses tumor growth and alters gene-expression profiles in rat tongue cancer. *European Journal of Oral Sciences*, **117**:351–361. https://doi.org/10.1111/j.1600-0722.2009.00646.x

Rebouças, E. L., Costa, J. J. N., Passos, M. J., Passos, J. R. S., Rvd, H., & Silva, J. R. V. (2013) Real time PCR and importance of housekeepings genes for normalization and quantification of mRNA expression in different tissues. *Brazilian Archives of Biology and Technology*, **56**:143–154.

Rodrigues, M. F. C., Ferreira, F. C., Silva-Magosso, N. S., Barbosa, M. R., Souza, M. V. C., Domingos, M. M., … Perez, S. E. A. (2017) Effects of resistance training and estrogen replacement on adipose tissue inflammation in ovariectomized rats. *Applied Physiology, Nutrition, and Metabolism*, **42**:605–612. https://doi.org/10.1139/apnm-2016-0443

Rosell, M., Kaforou, M., Frontini, A., Okolo, A., Chan, Y. W., Nikolopoulou, E., … Christian, M. (2014) Brown and white adipose tissues: Intrinsic differences in gene expression and response to cold exposure in mice. *American Journal of Physiology Endocrinology and Metabolism*, **306**:E945–E964. https://doi.org/10.1152/ajpendo.00473.2013

Rothwell, N. J., & Stock, M. J. (1982) Effects of feeding a palatable "cafeteria" diet on energy balance in young and adult lean (+/?) Zucker rats. *The British Journal of Nutrition*, **47**:461–471.

Santos, B. P., Diesel, L. F. C., Meirelles, L. S., Nardi, N. B., & Camassola, M. (2016) Identification of suitable reference genes for quantitative gene expression analysis in rat adipose stromal cells induced to trilineage differentiation. *Gene*, **594**:211–219. https://doi.org/10.1016/j.gene.2016.09.002

Silver, N., Best, S., Jiang, J., & Thein, S. L. (2006) Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. *BMC Molecular Biology*, **7**:33. https://doi.org/10.1186/1471-2199-7-33

Silver, N., Cotroneo, E., Proctor, G., Osailan, S., Paterson, K. L., & Carpenter, G. H. (2008) Selection of housekeeping genes for gene expression studies in the adult rat submandibular gland under normal, inflamed, atrophic and regenerative states. *BMC Molecular Biology*, **9**:64. https://doi.org/10.1186/1471-2199-9-64

Taki, F. A., Abdel-Rahman, A. A., & Zhang, B. (2014) A comprehensive approach to identify reliable reference gene candidates to investigate the link between alcoholism and endocrinology in Sprague-Dawley rats. *PLoS One*, **9**:e94311. https://doi.org/10.1371/journal.pone.0094311

Tang, L., Okamoto, S., Shiuchi, T., Toda, C., Takagi, K., Sato, T., … Minokoshi, Y. (2015) Sympathetic nerve activity maintains an anti-inflammatory state in adipose tissue in male mice by inhibiting TNF-alpha gene expression in macrophages. *Endocrinology*, **156**: 3680–3694. https://doi.org/10.1210/EN.2015-1096

Tsubai, T., Noda, Y., Ito, K., Nakao, M., Seino, Y., Oiso, Y., & Hamada, Y. (2016) Insulin elevates leptin secretion and mRNA

levels via cyclic AMP in 3T3-L1 adipocytes deprived of glucose. *Heliyon*, **2**:e00194. https://doi.org/10.1016/j.heliyon.2016.e00194

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., & Speleman, F. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*, **3**:1–12.

Veelken, R., Vogel, E. M., Hilgers, K., Amann, K., Hartner, A., Sass, G., … Tiegs, G. (2008) Autonomic renal denervation ameliorates experimental glomerulonephritis. *Journal of the American Society of Nephrology: JASN*, **19**:1371–1378. https://doi.org/10.1681/ASN.2007050552

Vina, E. R., & Kwoh, C. K. (2017) Epidemiology of osteoarthritis: Literature update. *Current Opinion in Rheumatology*, **30**:160–167. https://doi.org/10.1097/BOR.0000000000000479

Wan, G., Yang, K., Lim, Q. E., Zhou, L., He, B. P., Wong, H. K., & Too, H. (2010) Identification and validation of reference genes for expression studies in a rat model of neuropathic pain. *Biochemical and Biophysical Research Communications*, **400**:575:580–575:580. https://doi.org/10.1016/j.bbrc.2010.08.106

Wewege, M., van den Berg, R., Ward, R. E., & Keech, A. (2017) The effects of high-intensity interval training vs. moderate-intensity continuous training on body composition in overweight and obese adults: A systematic review and meta-analysis. *Obesity Reviews: An Official Journal of the International Association for the Study of Obesity*, **18**:635–646. https://doi.org/10.1111/obr.12532

World Health Organization. (2016) *Obesity and overweight.* Retrieved from http://www.who.int/mediacentre/factsheets/fs311/en/

Yan, K., Zhu, H., Xu, J., Pan, H., Li, N., Wang, L., … Gong, F. (2017) Lotus leaf aqueous extract reduces visceral fat mass and ameliorates insulin resistance in HFD-induced obese rats by regulating PPARgamma2 expression. *Frontiers in Pharmacology*, **8**:409. https://doi.org/10.3389/fphar.2017.00409

Yang, K. R., Allmeling, C., Liu, J., Lazaridis, A., & Vogt, P. M. (2012) Validation of differential gene expression in muscle engineered from rat groin adipose tissue by quantitative real-time PCR. *Biochemical and Biophysical Research Communications*, **421**:736–742.

Zhang, W. X., Fan, J., Ma, J., Rao, Y. S., Zhang, L., & Yan, Y. E. (2016) Selection of suitable reference genes for quantitative real-time PCR normalization in three types of rat adipose tissue. *International Journal of Molecular Sciences*, **17**:1–12. https://doi.org/10.3390/ijms17060968